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Sites of Action
for Neurotoxic Pesticides

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Foreword

Preface

APPROXIMATELY 200 ACTIVE INGREDIENTS have been commercialized as insecticides since World War II. This number might seem to be ample to solve any imaginable problem posed by insects and other arthropods in agriculture and public health. However, this conclusion is far from the truth. Evolving pest resistance, regulatory actions based on concerns about environmental and human health, enhanced degradation in soils, and the development of new agricultural technologies continually erode the number of chemicals available for pest control. Increasingly, we remain but one step ahead of important pests in the struggle for dominance. This situation is particularly true with insect pests, among which resistance is commonplace and the discovery of selective toxicants is made difficult by numerous biochemical similarities to vertebrates.

Meanwhile, the odds against the discovery of new pesticides that satisfy stringent requirements of cost-effectiveness and safety continue to rise. Whereas in the 1950s the frequency of success in discovering marketable pesticides was about 1 in 2000 compounds screened, this success ratio declined to less than 1 in 20,000 by the mid-1980s. One hope for improving these uneconomical odds lies in finding ways to apply our increasing knowledge of pest physiology and biochemistry and of comparative toxicology in vertebrates and invertebrates. This knowledge can help to increase screening efficiency, to optimize and evaluate leads, and to define new target sites for insecticide action that may lead to the design of novel toxicants.

The symposium on which this volume is based was organized to review and evaluate the state of knowledge of how insecticides interact with important target sites and to generate ideas about new sites. Attention was focused on the nervous system as the major locus of action of current, and assuredly, many future insecticides.

Although pesticide innovation was our primary objective in arranging the symposium, knowledge of the neurotoxic actions of pesticides has other important applications. It is critical to understanding the toxicological consequences and hazards of exposure to nontarget organisms. Also, if we view insecticides as potent and specific biological probes, they tell us much regarding normal nervous system functions and processes. Knowledge of modes and sites of action is one essential component of attempts to develop resistance management programs for insecticides. We therefore feel

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confident that the contents of this book will be of interest to mammalian and environmental toxicologists, neurobiologists, and entomologists, as well as to a more specific audience concerned with pesticide chemistry, biochemistry, and physiology.

Like any volume based on a symposium, this book presents a snapshot of its field at one moment in a long sequence of growth and development. In 1970, few of the topics covered here could have been predicted. The emphasis would have been strongly on the cholinergic aspects of the nervous system and particularly on cholinesterase inhibitors. This area is now relatively neglected. However, a renewed interest in acetylcholine receptors is evident, and some of this work is reported here. In the past 10 years, the focus has shifted to noncholinergic sites in the nervous system under the impetus of the discovery of the importance of the γ-aminobutyric acid (GABA) receptor-ion channel complex as a site of action for insecticides and related pesticides. This inhibitory system is now known to be the target site for cyclodiene insecticides and for the recently discovered avermectins. In turn, these observations have stimulated the study of other compounds, such as bicyclophosphate esters and related cage structures, which are thought to block the GABA-gated chloride channel.

Another event that has helped to focus attention on noncholinergic systems as possible targets for insecticide action in recent years is the discovery that formamidine pesticides are powerful agonists at octopamine receptors in insects. Further developments of this discovery are described. Initial attempts to develop compounds that would affect receptors for glutamate as the excitatory transmitter at many insect skeletal muscles were disappointing. However, recent successes are reviewed here as the target has shifted from the highly specific receptor site to what may be a more tractable target in the form of the associated ion channel. Finally, a topic that has burgeoned in significance since 1970 is that of the sodium channel as the major target for the synthetic pyrethroids. Much work continues in this area, and recent advances in understanding this channel as a target site are the topics of several chapters.

A panel discussion was convened at the end of the symposium to summarize impressions and highlight problems and approaches to the integration of basic knowledge of target sites and responses into a more rational approach to pesticide discovery. The results of this discussion are presented here, and we feel that they will be an illuminating record of the current state of the art.

In closing, we cannot help but wonder as we head for the 21st century what advances the next 15 years will bring in discovering, defining, and exploiting specific sites for insecticide action. The future of this area is as unpredictable as ever, but it is reasonable to hope that we shall see new natural products as insecticide models, new sites of action defined, such as

those for neuropeptides, and an improved capacity to conceive and design
selective agents to affect these sites.

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Chapter 1

Actions of GABA Agonists and Antagonists on Invertebrate Nerves and Muscles

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Deltamethrin did not affect the GABA system of the crayfish stretch receptor organ, but caused severe disruption at pM concentrations by acting on the voltage dependent sodium channels. Avermectin potentiated GABA actions on crayfish dactyl abductor muscle with a threshold of between 5 and 50 nM. Picrotoxin reversed the effect. Avermectin at 0.1 - 1 μM caused irreversible changes in muscle properties. Actions of suspected GABAergic drugs can be tested on various invertebrate preparations with crayfish, not insect, being the preferred test animal. Conclusions from these studies are limited by the type of GABA response a given preparation has to offer.

Styles change - thirty years ago, studying cholinesterase inhibition was all the rage and the GABA system had just been described. Today relatively little research attention is paid to cholinesterase inhibition and the search is on to find selective insecticides that lack side effects on nontarget species or on the environment.

Pyrethroid insecticides are also of interest currently, probably because they are selective and are fairly safe to mammals, but mostly because they are a few orders of magnitude more potent than previous categories of insecticides and are, therefore, marketable despite their higher price.

The history of the development of pyrethroids carries some important lessons and leads to a point to be made. The pyrethroids as templates or lead compounds languished for years as most prominent scientists and research directors recommended against work on them. This is an unfortunate fact of life for us: there is a certain lethargy about looking at entirely new chemical or biological systems in the search for new leads to insecticides. This is my first point.

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The approaches taken to find new insecticides include a tendency not to consider sites of action that are universal among animals. This bias against universal sites has been expressed more than once, and it is somewhat naive - my second point. There are too many examples of highly selective compounds even among cholinesterase inhibitors that would argue otherwise. To find selective and acceptable candidates for development as insecticides, one needs to find activity first, preferably potent activity and design selectivity into the final chemical structure.

In the search for novel insecticides, the so-called GABA system (synapses employing gamma-aminobutyric acid as a putative neuro-transmitter) was known for many years as a possible lesion site. Starting from a physiological lesion and working backwards to find insect control products has been rare. It is more common to find and then develop natural products with insecticidal activity or to find active chemicals fortuitously from random screens.

Some older compounds such as toxaphene, lindane and cyclodiene insecticides have been around for some time without their mode of action being known. My third observation is that, almost as a tradition, some insecticidal activity has been found and then later a physiological lesion has been discovered. The initial acaricidal activity of chlordimeform, the nematicidal activity of avermectin, or the diptericidal activity of cyromazine are good examples.

When possible sites of lesion are considered, the synapse is always a favorite. Many examples come to mind of compounds with some activity as synaptic poisons in insects. Organophosphorus and carbamate insecticides, and nicotine are the most obvious examples of cholinergic poisons; however, relatively few synapses beyond the cholinergic synapses have been described in insects in any detail. During the last Congress of Entomology in August 1984, Dr. M. O'Shea startled an audience by predicting that perhaps 200 neurotrans-mitters were yet to be found in insects. Nevertheless there are few drugs or natural toxicants upon which to base a rational search for new insecticides.

The biggest exception to this, of course, is the GABA system which is associated with a variety of active drugs. Besides the older picrotoxinin (I) and bicuculline (II), there are barbiturates (III), nipecotic acid (IV), muscimol (V), benzodiazepines (VI), baclofen (VIII), and avermectins to guide the search for active leads. The recent suggestion of similarities in structure and mode of action of lindane, cyclodienes and picrotoxinin analogs (1) throws open even more possibilities.

Besides the wide variety of possible GABA drugs to choose from for lead insecticides, there is active work leading to the isolation and characterization of the GABA receptor. Experience suggests that such synaptic receptors might be similar across the phyla. GABA drugs indeed tend to have a certain universality, but with some important exceptions.

Table I shows a classification of two types of vertebrate GABA receptors; a muscimol type (GABA/A) and baclofen type (GABA/B). A third column was added to the listing that is based on known invertebrate activities of GABA agents and is in keeping with the subject of the symposium.
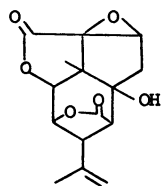
I
picrotoxinin

II
Bicuculline

Et   CH—nPr
        CH$_3$
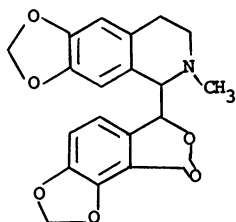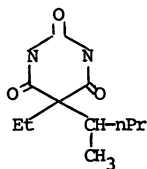
III
pentobarbital

IV
nipecotic acid

H$_2$NCH$_2$ OH

V
muscimol

CH$_3$

Cl

VI
diazepine
(diazepam)

Cl

H$_2$NCH$_2$CHCH$_2$COOH
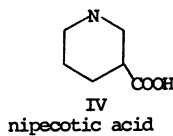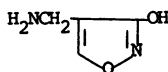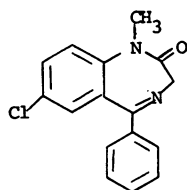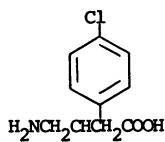
VII
baclofen

Table I. Classification of vertebrate GABA-receptors

	Structure	GABA/A	GABA/B	Lead potential
Agonists				
muscimol	V	potent	weak	good
3-APS	-	potent	inactive	good
baclofen	VII	inactive	potent	poor
Antagonists				
bicuculline	II	potent	inactive	moderate
picrotoxinin	I	potent	inactive	good
Potentiators				
benzodiazepines	VI	potent	-	poor
pentobarbitone	III	moderate	-	poor
nipecotic acid	IV	weak	-	poor
Other				
bicyclophosphate	-	potent	?	good
avermectin	-	potent	?	good

Taken from (2) and (3)

There are some similarities between the actions of GABA drugs on vertebrates and actions on invertebrates and some differences. The similarities are strong enough to postulate a good deal of receptor similarity; however, the differences tend to suggest that caution is in order in deciding which compounds might be convulsants in invertebrates, or otherwise good leads.

GABA binding in crayfish muscle shows similar characteristics to those of housefly and locust preparations. Isoguvacine, imidazole acetic acid and 3-aminopropanesulphonate potently inhibit muscimol binding to housefly head preparations with LC_{50}s of 100, 300, and 600 nM, respectively. Bicuculline is much less active here than on the mammalian GABA receptor. An important difference between insect and mammal involves pentobarbital which enhances muscimol binding in mammals but is inactive in similar insect preparations (4). This is point four: there are important differences in the actions of GABA drugs between insects or mammals.

Aside from the responses of GABA agents there are also some misleading facts. For example, on first inspection, picrotoxinin has little or no convulsive actions on insects. The topical toxicity on houseflies is greater than 50 μg/female. By synergizing with piperonyl butoxide the LD_{50} is reduced to 18 μg/female, which is about 300 times less toxic than the carbamate insecticide carbofuran (LD_{50} = 50 ng/female).

When comparing concentrations necessary to produce convulsions in 30 min by perfusing compounds onto the exposed thoracic ganglion of adult housefly, picrotoxin required more than 10 μM and carbofuran required 1 μM. When assaying the same materials on the desheathed ganglion, less than 100 nM picrotoxinin and more than 100

nM carbofuran was required to produce the convulsions (5) making picrotoxinin more intrinsically active than carbofuran. Evidently, a considerable barrier exists to keep picrotoxinin from penetrating into the central nervous system of insects and this is the fifth point to be made: Structure-activity studies are only as good as the activity that is measured.

The GABA system is studied physiologically and biochemically, depending on whether the tissue is left intact, or disrupted. Both approaches have their advantages and one tends to keep the other honest. A recent report by Dr. M. Eldefrawi that elements of GABA-like activity were demonstrable from the Torpedo electoplax preparation (TBPS binding of electroplax tissue extracts was inhibited reversibly by picrotoxin in a dose-dependent manner) which has no GABA system physiologically is an excellent example that caution is needed in interpreting binding studies.

The greatest drawback to physiological studies resides in the lack of a variety of preparations and the difficulty of finding preparations in the central nervous system. Usually results from studies on peripheral GABA sites are extrapolated to the central nervous system. This also should be done with caution.

With these thoughts in mind, we examined the effects of various drugs on GABA-mediated synapses in the crayfish. While there are a number of GABAergic neuromuscular junction preparations in invertebrates which are accessible for physiological research, the crayfish stretch receptor is one of very few invertebrate preparations which can be used for studies of synapses between nerves. The anatomy and physiology of these receptor organs from the abdomen of the crayfish has been extensively studied (6).

The receptor organ consists of two muscle fibers, each associated with a sensory cell (Fig. 1). The sensory cells send impulses to the central nervous system when the receptor muscles are stretched. Both of the sensory cells are innervated by a single inhibitory fiber from the CNS, which synapses with the dendrites and the cell soma and is thought to release GABA as the neurotransmitter.

The somata of the sensory neurons are large enough to allow impalement with two electrodes. Current passed through one electrode (Fig. 2, inset I) causes a voltage change at a second electrode (Fig. 2, inset E), that is related to the conductance of the neuron membrane. Drugs such as GABA or tetrodotoxin (TTX), which alter membrane conductance by opening chemically activated channels or blocking voltage dependent sodium channels respectively, cause an alteration in this voltage response. Stretch-induced nervous impulses can also be recorded from the axons of the receptor neurons (Fig. 3). Control recordings show that these axon spikes correlate with potentials recorded within the cell soma.

Effects of GABA and GABA antagonists on the stretch receptor organ can be compared with their effects on the neuromuscular junction. The dactyl abductor muscle of the crayfish is thought to be innervated by an inhibitory nerve which releases GABA as the neurotransmitter (7). Two electrodes can be inserted into a single muscle fiber, and conductance measurements can be made as described for the stretch receptor neuron. In both preparations, dose response curves can be constructed by applying a series of concentrations of GABA, then comparing these with curves produced after the addition of putative GABA antagonists.
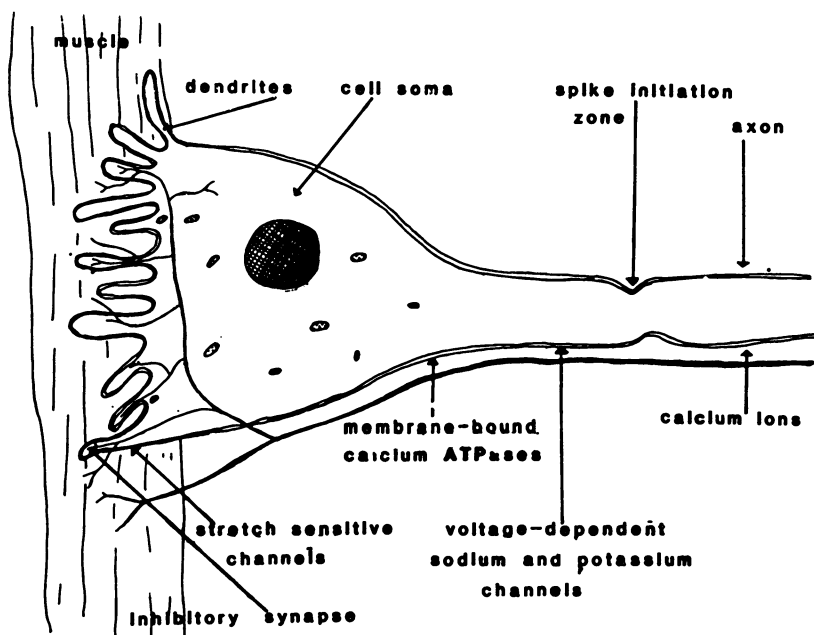
Figure 1. Anatomy of the stretch receptor organ (SRO) of the crayfish *Procambarus clarkii*.

Figure 2. Dose response curve of conductance changes elicited by GABA alone (x) or with 10 µM picrotoxin (o). (Reproduced with permission from ref. 11. Copyright 1987 Academic Press.)
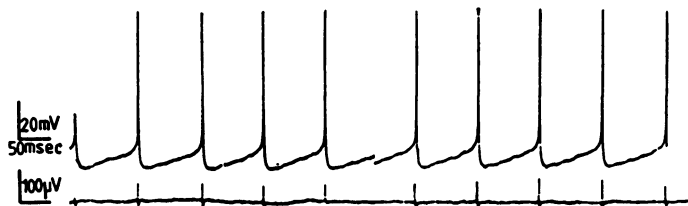
Figure 3. Control recording from the soma of a stretch receptor organ neuron (upper trace) and nervous impulses recorded from the axon of the SRO (lower trace).

Picrotoxin at 10 μM reduced the GABA response in the stretch receptor neuron (Fig. 2) and the dactyl abductor muscle. The muscle response was also slightly reduced by 0.5 mM bicuculline (Fig. 4), but this had no effect on the stretch receptor. No change in the neuron or muscle response to GABA was seen after treatment with the steroid R5135 (mM), ethyl bicyclophosphate (mM) or pentobarbitone (mM).

The action of deltamethrin on the GABA response was more difficult to determine, because low concentrations of deltamethrin caused depolarization and rapid firing of action potentials, accompanied by a decrease in membrane resistance. Deltamethrin at 0.1 nM caused a massive increase in firing frequency, and a depolarization of 30mV, after perfusion for ten minutes. Application of TTX, which specifically blocks voltage dependent sodium channels, abolished the action potentials, and also reversed the pyrethroid-induced depolarization and decrease in membrane resistance.

In the presence of TTX, the response of the preparation to GABA was not altered even after perfusion with deltamethrin for sixty minutes. Figure 5 shows dose response curves to GABA obtained before and after perfusion of 10 mM deltamethrin. This concen-tration of deltamethrin is 1,000 times greater than the threshold concentration for an effect on the sodium channels, indicating that the sodium channel effect is much more important to the poisoning process than any effect on the GABA system.

The effect of pyrethroids on the stretch receptor neuron is described in more detail by Chalmers and Osborne (8). These results agree with earlier studies on the insect neuromuscular junction in which the actions of pyrethroids were interpreted entirely on the basis of their actions on the sodium channels of the presynaptic axons (9).

The two crayfish preparations were also used to investigate the effects of avermectin on GABA-mediated synapses. In both preparations, concentrations of avermectin above 10 nM caused an increase in conductance which was reversed by the addition of picrotoxinin. If the concentration of avermectin was increased above 0.1 μM, the conductance increase could no longer be reversed, even by addition of 0.1 mM picrotoxinin.

One additional effect of avermectin was observed in some muscle preparations, but never in the stretch receptor preparations. Concentrations of avermectin between 5 and 50 nM, which had no obvious effect on the membrane resistance, appeared to potentiate the effects of GABA. In these preparations, the application of GABA caused an apparently normal conductance increase, but the conductance remained high even after the GABA was washed away (the recovery from GABA treatment is normally very rapid). This conductance increase was reversed by picrotoxinin.

These experiments show that, even in a single animal, there may be GABA receptors with different properties. It is not surprising, therefore, that the pharmacology of the GABA receptor appears to differ in different invertebrates. The number of preparations suitable for physiological investigation of the GABA response is limited, but physiological experiments must continue alongside biochemical studies if we hope to obtain a clear understanding of
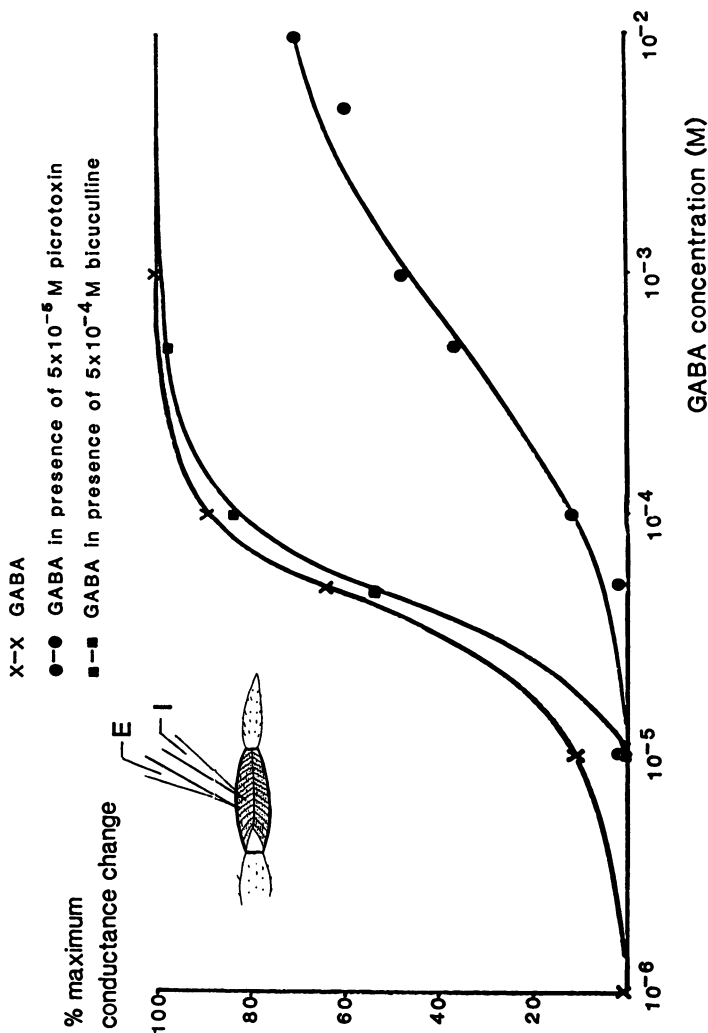
Figure 4. Dose response curve of conductance changes elicited by GABA alone (x) or with picrotoxin and bicuculline on the dactyl abductor muscle preparation.
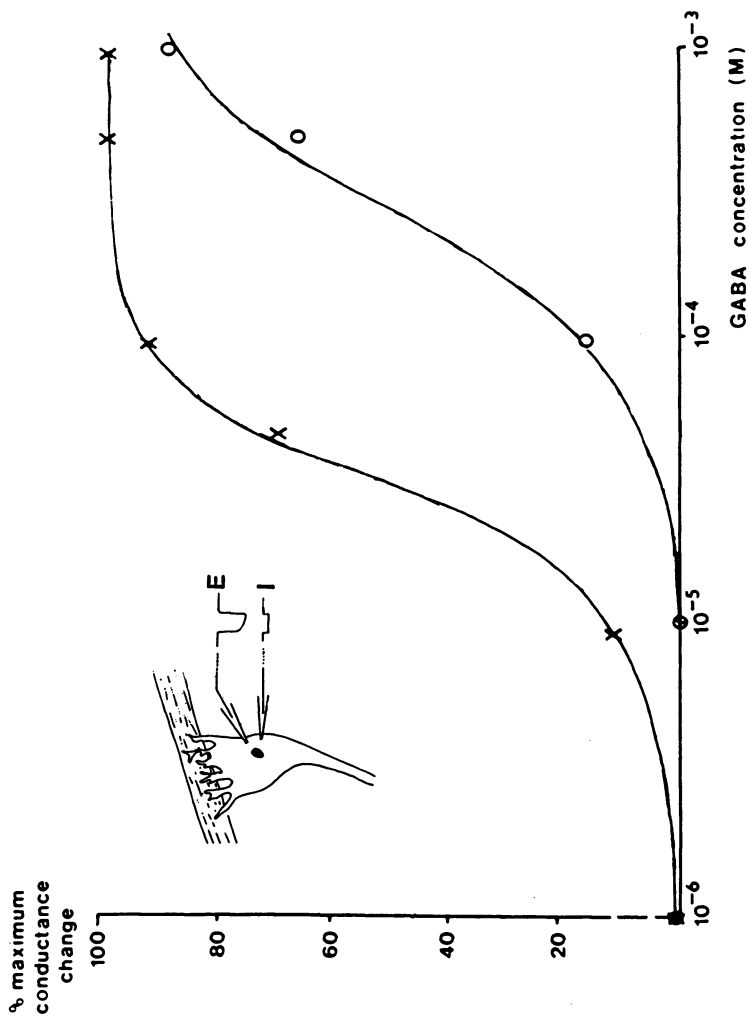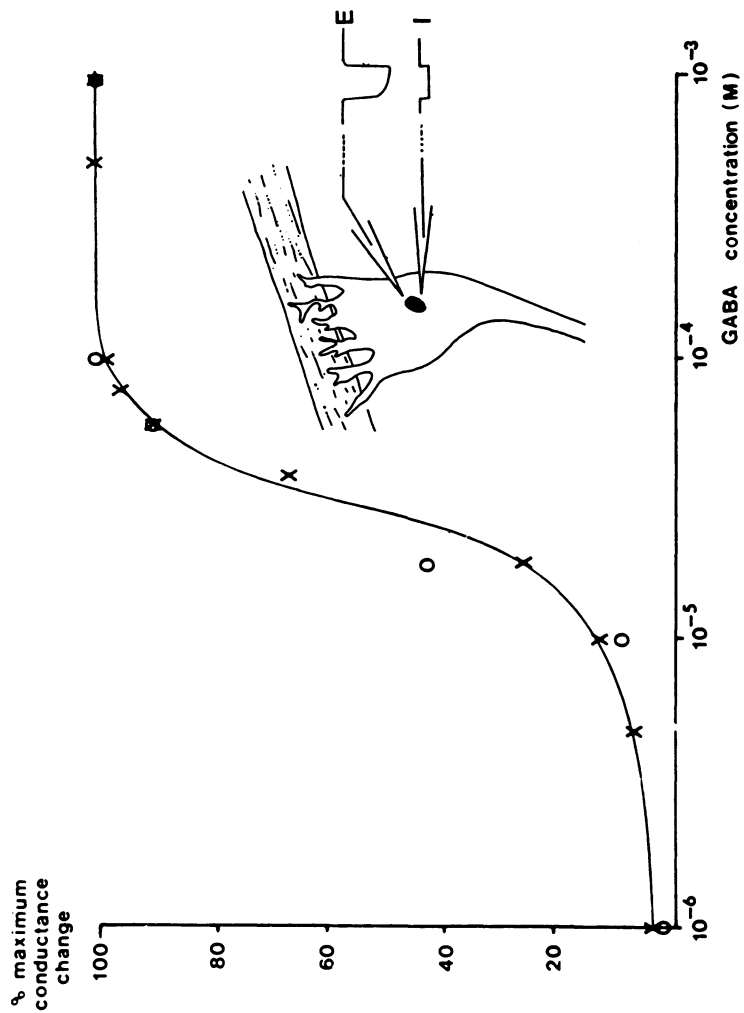
Figure 5. Dose response curve of conductance changes elicited by GABA alone (x) or with 10 nM deltamethrin (o). (Reproduced with permission from ref. 11. Copyright 1987 Academic Press.)

the role played by different elements of the GABA receptor complex. This understanding is vital if this type of study is to lead us to the rational design of chemicals that interfere with the GABA synapse.

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Chapter 2

GABA Receptors of the Insect Central Nervous System

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The central nervous system of the cockroach Periplaneta americana contains saturable specific binding sites for [^{3}H]GABA, [^{3}H]flunitrazepam and [^{35}S]TBPS. The [^{3}H]GABA binding site exhibits a pharmacological profile distinct from that reported for mammalian GABA$_A$ and GABA$_B$ receptors. Muscimol and GABA were the most effective inhibitors of binding whereas isoguvacine and 3-aminopropane sulphonic acid were less effective. Bicuculline methiodide and baclofen did not inhibit binding. The pharmacological profile of the [^{3}H]flunitrazepam binding site showed some similarities with the vertebrate peripheral benzodiazepine binding site, with Ro 5-4864 being a more effective inhibitor than clonazepam. Voltage-clamp and current-clamp experiments on an identified motorneurone confirmed the presence of a class of insect GABA receptor-chloride channels complexes with a different pharmacology and anion selectivity to their vertebrate counterparts.

Gamma-aminobutyric acid (GABA) is a widely distributed amino acid in the nervous tissue of both vertebrate and invertebrate organisms, where it functions predominantly as an inhibitory neurotransmitter (1,2). Receptor sites for GABA have been extensively studied in vertebrate central nervous systems (CNS), where there are at least

two distinct classes (3). GABA$_A$ receptors are blocked by bicuculline and activated by a number of GABA agonists including isoguvacine, whereas GABA$_B$ receptors are insensitive to bicuculline and can be activated by the agonist baclofen.

There is considerable evidence that GABA is an important neurotransmitter in the CNS of insects, although the details of insect GABA receptor pharmacology are only just beginning to emerge (4-9). We have used the CNS of the cockroach Periplaneta americana to investigate these receptors using both biochemical and physiological techniques.

Experimental Approaches

For a radioligand binding site to be considered as a neurotransmitter receptor site it is vital to establish a functional role for the putative receptor. Thus a preparation which permits physiological and biochemical experiments to be performed on the same tissue is particularly suitable for the study of neurotransmitter receptor interactions. Despite the paucity of tissue available and the problems of transferring technology developed for vertebrate tissues, insect central ganglia allow such a multidisciplinary experimental approach. Particularly significant advances have been made in electrophysiological studies on identified neurones. Thus the use of single identifiable cells, which are recognizable from preparation to preparation, allows detailed pharmacological investigations of the actions of various putative and known neurotransmitters; this has many advantages over more complex preparations. Insect neurones of all major classes (motorneurones, sensory neurones, interneurones and modulator cells) are accessible to microelectrode impalement as a result of their size and location.

Characterization of Putative Insect CNS GABA receptors by Radioligand Binding Studies

In order to examine radioligand binding to insect CNS GABA receptors, we used a crude membrane preparation from 40-60 thoracic and abdominal nerve cords (100-200 mg tissue w/w). A 40,000xg pellet was prepared in sodium-free buffer to minimize possible binding to uptake sites, and was then washed in excess buffer to remove endogenous GABA. The reaction was started by the addition of [^{3}H]GABA (100nM unless otherwise stated) and was terminated by centrifugation in an Eppendorf microfuge for 4 min. Non-specific binding to this membrane pellet was determined in the presence of 10^{-4}M muscimol. Using [^{3}H]GABA as a receptor probe, we demonstrated a

specific component of saturable binding to membranes
from cockroach nerve cords. Scatchard plots yielded
an estimate of the K_d (3.84 x 10^{-7}M) and B_{max}
(1.42pmol mg^{-1} protein). The Hill coefficient was
close to unity, suggesting non-cooperative binding to
a single class of receptor sites.

To examine the pharmacological profile of these
insect CNS GABA receptor binding sites, the
displacement of [^{3}H]GABA binding by a variety of
GABAergic ligands was explored (see Table I). The
most potent displacers were GABA and muscimol, whereas
the vertebrate $GABA_A$ agonists isoguvacine,
thiomuscimol and 3-aminopropane sulphonic acid were
less effective. Piperidine-4-sulphonic acid, a good
inhibitor of vertebrate [^{3}H]GABA binding, was
ineffective, as were both bicuculline methiodide (a
$GABA_A$ antagonist) and baclofen (a $GABA_B$ agonist) at
concentrations of 10^{-4}M and 10^{-3}M respectively. Thus
the pharmacological profile of this insect CNS GABA
binding site differs from that of the vertebrate $GABA_A$
receptor. Further studies would be necessary to
determine if the insect [^{3}H]GABA binding site has a
pharmacological profile closer to that of the
vertebrate $GABA_B$ receptor, but the lack of effect of
baclofen, a potent $GABA_B$ agonist, suggests that this
is unlikely. Nipecotic acid, a vertebrate GABA uptake
inhibitor, and β-alanine, a relatively specific glial
uptake inhibitor, failed to inhibit [^{3}H]GABA binding
to cockroach membranes at 1.0 x 10^{-4}M. However, these
two compounds were effective in displacing [^{3}H]GABA
binding in the presence of sodium. The presence of
sodium itself resulted in a 7-8 fold enhancement of
specific binding. This points to sodium-dependent
GABA binding to uptake sites, or sodium-dependent
uptake into membrane vesicles.

Examination of the rates of association and
dissociation of [^{3}H]GABA binding assumed the simple
kinetic model:

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

(where L = ligand and R = receptor). The dissociation
rate determined from the half life of the binding
complex (k_{-1} = ln2/$t_{0.5}$) was calculated to be 0.116
min^{-1}. For determination of the association rate
constant, the initial rate portion of the curve was used
and k_{app} was determined from the slope of the plot using
the equation k_{app} = k_{+1} [S] + k_{-1}. A value for k_{app} of
0.125 was obtained and thus k_1 = 3.16 x 10^{-4} M^{-1} min^{-1}.
The dissociation constant calculated from these kinetic
studies (K_d = k_{-1}/k_{+1}) was 365nM, similar to the value
obtained from equilibrium studies (data from ref.5).

Table I: Comparison of cockroach and vertebrate IC_{50} values (concentrations that inhibit 50% of specific [^{3}H]GABA binding)

Ligand	IC_{50} (M)		
	Cockroach CNS	Vertebrate CNS	
		$GABA_A$	$GABA_B$
GABA	1.3×10^{-7}	1.2×10^{-7}	8.0×10^{-6}
Muscimol	7.1×10^{-7}	2.0×10^{-8}	5.4×10^{-6}
Thiomuscimol	3.0×10^{-5}	1.2×10^{-7}	n.t.
THIP	3.9×10^{-4}	1.1×10^{-7}	i.a.
3-Aminopropane sulphonic acid	3.9×10^{-4}	1.1×10^{-7}	1.0×10^{-5}
Isoguvacine	4.5×10^{-4}	1.9×10^{-7}	i.a.
Bicuculline methiodide	i.a.	2.7×10^{-5}	i.a.
Baclofen	i.a.	i.a.	1.3×10^{-7}
Piperidine-4- sulphonic acid	i.a.	3.0×10^{-8}	i.a.
Nipecotic acid	i.a.	i.a.	i.a.
Picrotoxin	i.a.	i.a.	i.a.

Data as in ref.6. Abbreviations: i.a. = inactive; n.t. = not tested.

Autoradiographical Localization of [^{3}H]GABA Binding Sites

The localization of [^{3}H]GABA binding sites was examined in frozen sections of the sixth abdominal ganglion of the cockroach under sodium-free conditions. Controls were treated with 10^{-4} M muscimol. Dark-field autoradiographs showed binding in the neuropile (synaptic region) and some binding to the outer neural lamella, though binding was difficult to detect over the peripheral cell bodies or glia (6). These findings are consistent with a functional role for GABA in insect CNS synaptic transmission.

Benzodiazepine Binding Sites in Insect CNS

A wide variety of vertebrate species have been shown to possess CNS benzodiazepine binding sites and a peripheral benzodiazepine binding site has been described (10). The vertebrate neuronal benzodiazepine receptors are thought to be intimately associated with GABA receptors, whereas the peripheral binding sites, initially discovered in kidney and

liver, appear to be associated with calcium channels. Early reports suggested a lack of benzodiazepine receptors in invertebrates (11), but more recent studies using radiolabelled probes such as [3H]flunitrazepam have demonstrated the presence of benzodiazepine binding sites in the insect CNS (4,5,12). We have examined the binding of [3H]flunitrazepam to a membrane preparation of cockroach nerve cords. A saturable component of specific binding has been demonstrated and K_d (3.83 x 10^{-7}M) and B_{max} (5.5pmol mg^{-1} protein) were obtained from Scatchard plots. These cockroach benzodiazepine binding sites appear to be closely associated with GABA receptor sites; GABA, over a narrow concentration range, was found to enhance the binding of [3H]flunitrazepam with a maximum effect at 10^{-7}M GABA. A similar result has been demonstrated for locust (12), suggesting that at least some of these insect benzodiazepine binding sites are coupled to GABA receptors.

     The pharmacological profile of these [3H]flunitrazepam binding sites is shown in Table II. Ro 5-4864, a vertebrate peripheral benzodiazepine ligand, was the most effective in displacing specific [3H]flunitrazepam binding, whereas clonazepam and Ro 15-1788, which are specific neuronal benzodiazepine ligands in vertebrates, were much less effective. These results therefore suggest that the cockroach benzodiazepine binding sites show more similarities with peripheral rather than neuronal vertebrate benzodiazepine binding sites. The pharmacological profile is similar to that seen for the housefly thorax [3H]flunitrazepam binding sites where flunitrazepam, diazepam and Ro 5-4864 are the most potent inhibitors of binding and clonazepam is least effective (4).

Table II: IC_{50} values for [3H]flunitrazepam binding in cockroach CNS

Ligand	IC_{50} (10^{-6}M)
Ro 5-4864	0.6
Diazepam	1.0
Flunitrazepam	1.6
Clonazepam	25.0
Ro 15-1788	100.0

Data from ref. 5.

Binding Sites for the GABA-activated Chloride Ion Channel in the Insect CNS

[^{35}S]TBPS (t-butylbicyclophosphorothionate) binds to picrotoxin sites which are allosterically coupled to both the GABA receptors and chloride ion channels in the GABA receptor complex (13). This ligand was found to bind with high affinity to a cockroach nerve cord membrane preparation. Scatchard plots revealed a K_d of 1.85 x 10^{-8}M and a B_{max} of 177fmol mg^{-1} protein. Micromolar concentrations of GABA and avermectin (AVM) were found to partially inhibit [^{35}S]TBPS binding. These results suggest that the TBPS binding site in insects, as in vertebrates, is closely associated with the AVM and GABA binding sites.
    Thus the radioligand binding data suggest that in the CNS of the cockroach, there is a GABA receptor/ion channel complex which is in some ways comparable to the vertebrate GABA receptor/ion channel complex. The detailed characteristics of the individual binding sites appear to differ between insects and vertebrates but the interactions between some of these insect binding sites show some similarities to those of the corresponding sites in vertebrates.

Electrophysiological Characterization of Insect CNS GABA receptors

Using the cell body of an identified neurone, the fast coxal depressor motorneurone (D_f) of the cockroach *Periplaneta americana*, the pharmacological profile of the GABA receptor and ionic events following GABA receptor activation were investigated with current-clamp and voltage-clamp techniques. The cell body of $D_{f.}$ was visually located in the isolated, desheathed, metathoracic ganglia of the cockroach. The ganglion was mounted in a perspex experimental chamber (0.5ml volume), perfused with saline (2ml min^{-1}, 18-20°C) and the neuronal cell body was impaled with two microelectrodes. Drugs were applied in the perfusing saline or by ionophoresis. Intracellular ion substitutions were achieved by changing the electrolyte content of the intracellular recording electrodes. During extracellular ion substitutions osmotic pressure was controlled by altering the concentration of sucrose in the saline (14). Changes in intracellular chloride concentration were monitored with intracellular recording electrodes containing the chloride sensitive resin Corning 47715.

Ionic Basis of the GABA Response

Under current-clamp conditions, activation of GABA receptors produced an increase in conductance and hyperpolarization of the membrane potential. Under voltage-clamp conditions this was seen as an outward current or influx of negative charge. The reversal potential for the GABA-induced hyperpolarizations was -77 ± 2 mV (mean $\pm$s.e.m., n=22).

The reversal potential for GABA was highly sensitive to changes in external chloride concentration, weakly affected by changes in external potassium concentration, and was independent of changes in either sodium or calcium concentration. When isethionate was exchanged for chloride in the perfusing solutions, the reversal potential for GABA moved to less negative values and the hyperpolarizing responses became depolarizing. When bromide or iodide were exchanged for external chloride there was no change in the reversal potential for GABA. The use of intracellular ion-sensitive microelectrodes indicated that an influx of chloride ions rather than potassium ions mediated the GABA response. Increasing potassium in the bathing solution caused the neurone to depolarize and produced a positive shift in the GABA reversal potential of smaller amplitude than would be predicted for a pure potassium event. Additionally, replacement of potassium with caesium caused no change in the GABA reversal potential. Removal of either sodium or calcium from the bathing solutions was without effect on the reversal potential for GABA.

Intracellular injections of acetate, citrate, sulphate, fluoride or ammonium ions caused no change in the reversal potential for GABA. However, intracellular injection of chloride, bromide, chlorate, bromate, or methyl sulphate caused the reversal potential for GABA to move in a positive direction. The data are summarized in tables III and IV.

Evidence for chloride accumulation and extrusion mechanisms were examined with putative inhibitors. Intracellular injection of ammonium ions, bathing the preparation in 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulphonic acid (SITS), 4,4'-diisothiocyanato-stilbene-2,2'-disulphonic acid (DIDS), acetazolamide, furosemide, NH^{4+}, Zn^{2+} or Cu^{2+} were without effect on the reversal potential for GABA. The data obtained so far suggest that chloride-regulating mechanisms present in the cockroach are not sensitive to the inhibitors which block other invertebrate (15,16) or vertebrate (17) chloride pumps.

Table III. Effects of extracellular ion changes on GABA reversal potential

External Ion Changed	Effect on Reversal Potential
Replace chloride with: Isethionate	Moves positive[a]
Methyl sulphate	No change
Bromide	No change
Iodide	No change
Replace sodium with N-methyl glucamine	No change
Replace calcium with magnesium	No change
Replace potassium with caesium	No change
Increase potassium	Moves slightly positive

[a] Movement in a positive direction indicates the reversal potential moved to a value closer to 0mV than the control value.

Table IV. Effects of various intracellular anion substitutions on the reversal potential for neuronal GABA response

Anion	Radius[a]	Insect[b]	Snail	Cortex
Bromide	0.93	+	+	+
Iodide	0.96	+	+	+
Chloride	0.96	+	+	+
Chlorate	1.13	+	+	+
Bromate	1.32	+	+	0
Fluoride	1.33	0	0	0
Methylsulphate	1.39	+	0	0
Acetate	1.83	0	0	0
Sulphate	1.84	0	0	0
Citrate	3.08	0	0	0

[a] The anionic radius given is the value relative to the hydrated potassium ion. [b] The direction in which the response moves is indicated by + for movement in positive direction (i.e. closer to 0mV than the control value), 0 for no change. The data for snail neurones and cortical neurones is derived from refs 18 and 19.

GABA receptor pharmacology

The cockroach motorneurone (D_f) was sensitive to GABA applied either in the bath or by ionophoresis. Prolonged application of GABA resulted in desensitization. Brief ionophoretic pulses of increasing quantities of GABA caused progressively larger

responses; Hill coefficients determined from such data were greater than 2 suggesting that a minimum of two molecules of GABA were required to bind to the receptor to open the chloride channel.

Clear differences between vertebrate GABA receptors and insect neuronal GABA receptors were established (see Table V). On the GABA receptor of motorneurone D_f isoguvacine ($EC_{50}=2X10^{-5}M$) was more active than muscimol ($EC_{50}=8X10^{-5}M$), GABA ($EC_{50}=1X10^{-3}M$) and 3-aminopropane sulphonic acid ($EC_{50}=1X10^{-3}M$). This is a different rank order of potency to that found in vertebrates ([20]) for the $GABA_A$ receptor. The $GABA_B$ receptor agonist baclofen was inactive on the neurone D_f. The role of GABA uptake in the GABA response was not an important factor in these experiments since GABA responses were only weakly potentiated by the uptake blocker ([21]) nipecotic acid ($10^{-3}M$).

Of the potent vertebrate $GABA_A$ receptor antagonists bicuculline ([20]), pitrazepin ([22]), RU5135 ([23]) and picrotoxin, only picrotoxin produced a potent reversible blockade of the GABA response on motorneurone D_f. The block of the GABA response by picrotoxin ([24]) at concentrations below $10^{-7}M$ was weakly voltage-dependent, but at higher concentrations no significant voltage dependence was observed. Picrotoxinin and picrotin, the contituents of picrotoxin, were tested separately and both were found to be active. Bicuculline hydrochloride and bicuculline methiodide were both ineffective at $10^{-4}M$ on the GABA response at resting membrane potential. However, at hyperpolarized levels partial block of GABA currents was occasionally observed. Pitrazepin ($10^{-4}M$) caused a partial block of GABA currents. This block was voltage-independent. The steroid derivative RU5135 had very little effect on the GABA response at doses as high as $10^{-4}M$. In contrast to the potent competitive blockade of vertebrate $GABA_A$ receptors by bicuculline, pitrazepin and RU5135 ([20,22,23]), none of the weak GABA antagonism caused by these drugs on motorneurone D_f was competitive.

The response to GABA could be potentiated by the benzodiazepine flunitrazepam ($10^{-6}M$), suggesting that the insect GABA receptor may have a functional benzodiazepine site on the GABA receptor complex.

Conclusion

In the CNS of the cockroach Periplaneta americana, we have demonstrated the presence of GABA receptor-activated chloride ion channels. Both the radiolabelled ligand binding and the electrophysiological studies have shown that the pharmacology of these insect CNS GABA receptors differs from that of the GABA receptors

Table V. Pharmacology of GABA agonists and antagonists
on $GABA_A$, $GABA_B$ and insect neuronal GABA receptors

Drug	$GABA_A$	$GABA_B$	Insect neurone
Part (i)			
Antagonists:			
Bicuculline	Competitive	Not active	Not active
Pitrazepin	Competitive	Not active	Weak, Non-Competitive
RU5135	Competitive	Not active	Not active
Picrotoxin	Non-Competitive	Not active	Non-Competitive
Part (ii)			
Agonist potency:	Muscimol⟩ Isoguvacine⟩ 3-APS⟩ GABA	Baclofen⟩ GABA≫ Muscimol	Isoguvacine ⟩ Muscimol= GABA⟩ 3-APS

identified and characterized in vertebrate CNS, and it
is apparent from the permeability studies that the
anion selectivity of the GABA-activated chloride
channel also differs between insects and vertebrates.
At least some of the insect CNS GABA receptors appear
to be linked to benzodiazepine binding sites, although
these binding sites have different pharmacological
characteristics from those in vertebrate CNS. Further
understanding of these insect receptors could lead to
a rational basis for insecticide design and may help
to clarify the molecular basis of insecticide
resistance.

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Chapter 3

Responses to GABA and Other Neurotransmitters in Insect Central Neuronal Somata In Vitro

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Using conventional and whole cell patch techniques, we have current- and voltage-clamped the somata in cultures of dissociated embryonic cockroach brain neurons and preparations of isolated thoracic neurons from locusts to examine their responses to GABA and other putative neurotransmitters and neuropeptides. These neurons respond to the micro-application of GABA with a transient membrane conductance increase. The effect is blocked by picrotoxin but unaffected by bicuculline up to 10^{-4} M. Using the whole cell patch method, we have changed the $[Cl^-]_i$ of the cockroach neurons, thereby shifting the Cl^- equilibrium potential to demonstrate that the GABA response results from the activation of a Cl^- current. Application of flunitrazepam or Na-pento-barbital to the locust neurons increases the GABA-evoked current by up to 70%. However, application of diagnostic agonists and antagonists indicates that these locust somal GABA receptors differ from both the $GABA_A$ and $GABA_B$ receptors of the vertebrates. Octopamine, serotonin and FMRFamide all evoke consistent changes in membrane potential and resistance in varying proportions of the cultured cockroach brain neurons.

The pharmacology of insect central nervous system transmitter receptors and their associated modulatory sites and ion channels is less thoroughly known than that of the peripheral neuromuscular systems. This is in part due to the relative inaccessibility of the central

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neurons and especially their synapses. In recent years, two preparations have been developed which greatly reduce this accessibility problem. Usherwood and his co-workers described a technique for isolating neuronal somata from the thoracic ganglia of the locust Schistocerca gregaria and, using a method based on the earlier work of Levi-Montalcini (1), they were able to culture isolated neuronal somata from locusts and cockroaches (2,3). At about the same time, Beadle and his co-workers developed a similar preparation to show that neurons from the brains of embryonic Periplaneta americana can be maintained in primary culture for many weeks and under these conditions produce multiple inter-connecting neurites (4,5). In addition, the cultured neurons are free of glia and thus well-suited to patch-clamping. Both laboratories have found that the neuronal somata in these preparations are endowed with a considerable range of receptor types. In this paper, we will describe our recent observations on the responses of the cultured embryonic cockroach brain neurons and freshly dissociated locust thoracic neurons to neuroactive amines and neuro-peptides, and particularly the response to γ-aminobutyric acid (GABA), its ionic basis and its modulation.

Methods

To prepare the locust neuronal somata, the desheathed thoracic ganglia of Schistocerca gregaria or Locusta migratoria were dissociated mechanically, without enzyme treatment, by repeated passage through the tip of a Pasteur pipette (2). Tissue dissection, dissociation and intracellular recording were carried out in a saline with the following composition: NaCl, 180 mM; $MgCl_2$, 15 mM; $CaCl_2$, 2 mM; KCl, 10 mM; HEPES, 10 mM; pH 6.8. The dissociated cells were plated in a few drops of saline onto 50 mm "Falcon tight-seal" petri dishes and left for 10 min - 4 hr to adhere to the plastic surface of the dish. The resulting monolayer of neuronal somata was viable for recording for up to 12 hr. Neurons from younger animals were more stable during intracellular recording. The cells ranged between 10 and 100 μm in diameter and often bore axon stumps with apparent regions of regenerated membrane at their tips. Only the larger neurons (30-100μm) were selected for impalement. Conventional recording and single electrode voltage-clamping methods were used.

The preparation of the embryonic cockroach brain neuronal cultures is a complicated procedure which has been described in detail by Beadle and Lees (6). In brief, the brains were dissected from 23-26 day old embryos of Periplaneta americana and broken up by passage through Pasteur pipettes of decreasing diameter. The cultures were initiated in a medium consisting of 5 parts Schneider's Drosophila medium and 4 parts Eagle's Basal medium, and after 7 days' growth were transferred to a

medium consisting of equal parts of Leibovitz's L-15 medium and Yunker's modified Grace's medium. The cells were grown at 29°C in 50 mm "Falcon tight seal" petri dishes using a modification of the hanging drop method.

To carry out electrophysiological recordings on the cultured neurons, the growth medium was removed and replaced by a recording saline with the following composition: NaCl, 200 mM; $CaCl_2$, 9 mM; KCl, 3.1 mM; HEPES, 5 mM; pH 7.2. For conventional recording and voltage clamping, the recording electrode was filled with 1 M K-acetate.

FMRFamide and substance P were obtained from Cambridge Research Biochemicals Ltd. and proctolin from Vega Biochemicals Inc. Pitrazepin was kindly provided by Sandoz Ltd. Flunitrazepam and baclofen are CIBA-GEIGY analytical standards and all other test compounds were obtained from Sigma.

Results and Discussion

Cultured cockroach neurons. Several biogenic amines, neuropeptides and other putative neurotransmitters were applied by pressure ejection from micropipettes onto the surface of individual neuronal somata under current clamp. The results are illustrated in Fig. 1 and have been reported briefly elsewhere (7). All of the cells tested (N>50) responded to acetylcholine with a voltage-dependent depolarization and a decrease in membrane resistance, as described in detail by Lees et al. (8). In addition, 60% of the cells were sensitive to GABA, responding with a decrease in membrane resistance and a concurrent hyperpolarization, except in somata with very negative resting potentials (more negative than -55 to -65 mV) where the response was a depolarization (Fig. 1a). The GABA response will be considered in greater detail below. Octopamine evoked hyper-polarizations accompanied by membrane resistance increases in 2 of 9 neurons tested. It slowed the rate of spontaneous spiking where this occurred (Fig. 1b) and the evoked hyperpolarization was larger at more negative membrane potentials (Fig. 1c). Serotonin (5-hydroxy-tryptamine) evoked depolarizations accompanied by decreased membrane resistance (Fig. 1d), with the evoked potentials increasing in magnitude with hyperpolarization of the membrane potential. Repeated pulses of serotonin resulted in a response of decreasing magnitude (Fig. 1e) suggesting receptor desensitization. Glutamate evoked responses in up to 30% of the neurons in some cultures. The majority of the responses were depolarizations accompanied by decreases in membrane resistance but some cells were hyperpolarized (Horseman et al., unpublished data). Histamine (10^{-4} M, n=13) produced no unequivocal responses and neither did proctolin (10^{-5} M, n=6, pulses of up to 2 sec duration) or substance P (10^{-5} M, n=9). However, the molluscan cardioexcitatory
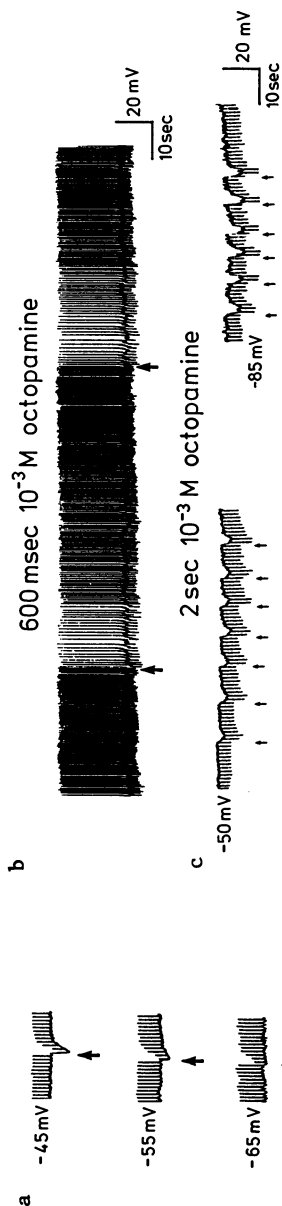
Figure 1. Responses of current-clamped, cultured, embryonic cockroach brain neurons to putative neurotransmitters and a neuropeptide. The test compounds were applied by pressure ejection from glass micropipettes onto the soma of the impaled neuron. The membrane resistance was monitored by repeated injection of constant-amplitude current pulses.
a) The response of a neuron (26 days in culture) to 100 msec pulses of 10^{-3} M GABA consisted of a change in membrane potential which reversed direction at -65 mV and was accompanied by a decrease in membrane resistance.
b) Spontaneous firing of action potentials in a neuron (22 days in culture) was partially inhibited by 600 msec pulses of 10^{-3} M octopamine.
c) 2 sec pulses of 10^{-3} M octopamine hyperpolarized a neuron (20 days in culture) in a voltage-dependent manner and accompanied by an increase in membrane resistance.
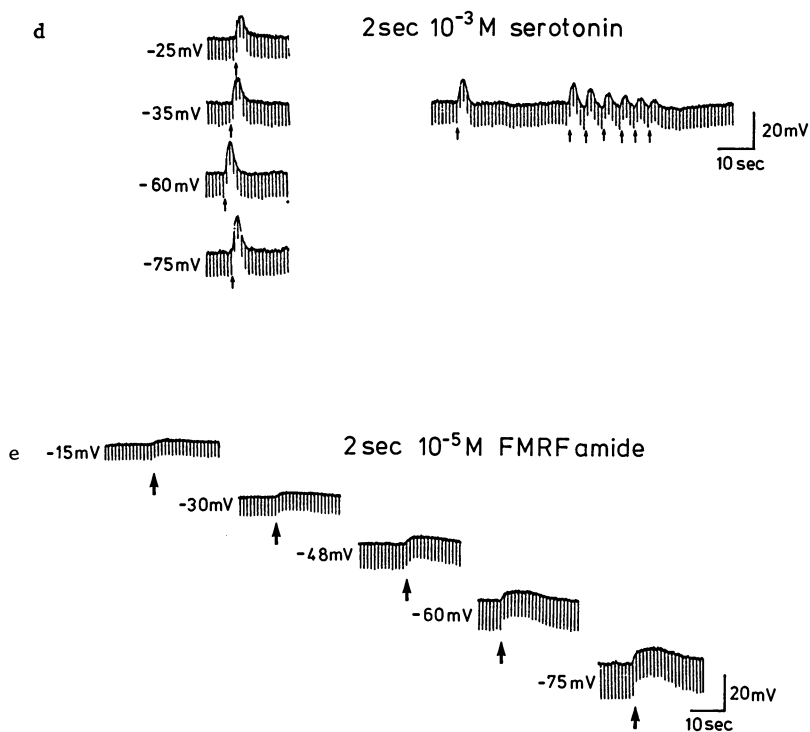
Figure 1.--Continued.

d) 2 sec pulses of 10^{-3} M serotonin depolarized a neuron (21 days in culture) in a voltage-dependent manner and accompanied by a decrease in membrane resistance. Repeated applications to the same cell resulted in partial desensitization.
e) 2 sec pulses of 10^{-5} M FMRFamide evoked comparatively long-lasting, voltage-dependent depolarizations and reduced the membrane resistance.

neuropeptide, FMRFamide, evoked clear responses in 4 of 20 neurons. The response consisted of a depolarization of long duration accompanied by a decrease in membrane resistance (Fig. 1e). The FMRFamide-evoked depolarization increased in amplitude with hyperpolarization of the cell membrane.

Insect GABA receptors have been implicated as target sites for a number of insecticides including the cyclodienes (9), the α-cyano-pyrethroids (at high concentrations) (10,11) and the avermectins (12). We therefore decided to study the GABA response of the insect central neuronal somata more closely. Current clamp recordings showed that the GABA-evoked potential reversed direction at about -65 mV, being a hyperpolarization at potentials positive to -65 mV and a depolarization at more negative potentials (Fig. 1a). Since the removal of Cl^- ions from the bathing medium blocks the somal GABA response of locust DUM neurons (13) and Cl^- ions mediate the vertebrate $GABA_A$ response (reviewed by Simmonds (14)), we tested for this possibility in the cockroach brain neurons. To do this, we changed the Cl^- equilibrium potential, E_{Cl}, in a controlled manner by altering the ratio of Cl^- ion concentrations across the neuronal membrane. The value of E_{Cl} was calculated from the transmembrane $[Cl^-]$ ratio by means of the Nernst equation. We then checked for a corresponding shift in the reversal potential, E_R, of the GABA-evoked current. This is often carried out by modifying $[Cl^-]_o$, the Cl^- concentration of the bathing saline. However, to control both $[Cl^-]_o$ and the intracellular Cl^- concentration, $[Cl^-]_i$, we chose instead to change $[Cl^-]_i$ by means of the whole cell patch clamp method of intracellular perfusion (15). Using an intra-electrode $[Cl^-]$ of 114 mM and $[Cl^-]_o$ of 221 mM, the E_R of the GABA-evoked current was shifted to -16 mV (Fig. 2) which is equal to the value predicted for E_{Cl} by the Nernst equation. From these experiments, we conclude that the GABA response of the cultured embryonic cockroach brain neurons is mediated by Cl^- ions, as in the vertebrate $GABA_A$ system, and that rapid equilibration occurs between the intra-electrode and intracellular $[Cl^-]$ when the whole cell patch clamp method is employed.

Dissociated locust neurons. To study the pharmacology of the receptors and channels involved in the central somal GABA responses, we utilized the less laborious and more robust isolated locust thoracic neuronal somata preparation. Usherwood and his colleagues found that freshly isolated adult as well as cultured nymphal neurons from Schistocerca responded to GABA usually with a hyperpolarization (2,3) and Goodman and Spitzer reported that the GABA response of the thoracic DUM neuronal somata of Schistocerca nitans reverses at -70 mV (13). We routinely voltage-clamp the isolated thoracic
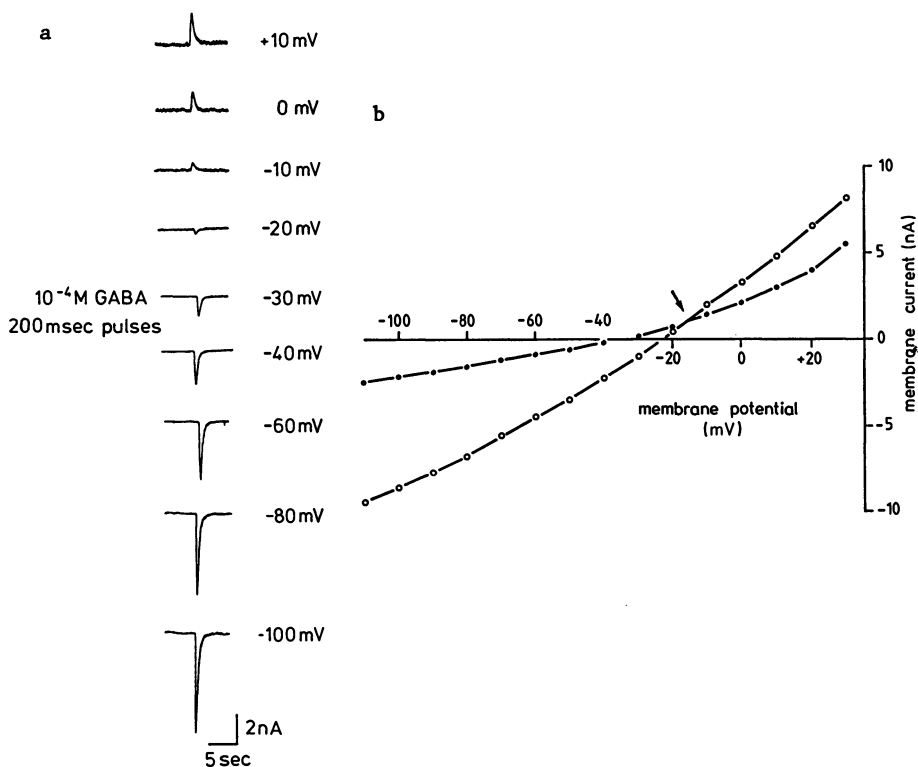
Figure 2. Currents recorded from a cultured, embryonic cockroach brain neuron intracellularly perfused and voltage-clamped using the whole cell patch method. The intra-electrode [Cl^-] was 114 mV and [Cl^-]$_o$ was 221 mM. For this ratio of [Cl^-] across the cell membrane, the Nernst equation predicts a value of -16 mV for the Cl^- equilibrium potential (E_{Cl}).
a) Records of the current evoked by 200 msec pressure microapplications of 10^{-4} M GABA.
b) Current-voltage (I-V) curves plotting the resting (closed circles) and evoked (open circles) currents shown in a). The resting current is the net membrane current flowing in the absence of GABA, so the point of intersection of the two curves (arrow) indicates the reversal potential of the current evoked by the GABA pulses. It coincides with the predicted E_{Cl} indicating that the GABA-evoked current is a Cl^- current.

neurons of Locusta and Schistocerca and, of the more than
300 neurons tested, all have shown a response to GABA or
its analog muscimol, both of which act on the same
receptor population (16). This response is usually
monophasic, but in a small number of Locusta neurons a
biphasic response has been recorded (16-18).

The monophasic response of the locust neurons is
very similar to that of the cultured cockroach neurons in
its fast time course and E_R (Fig. 3). However, the
evoked current recorded in the locust neurons is always
outwardly rectifying (Fig. 3) whereas this appears to be
not always the case in the cockroach neurons (Fig. 2).

In some Locusta neurons, GABA and muscimol evoked a
biphasic response. In the current-clamped neurons showing
this response, the predominant phase was a large and
slowly decaying depolarization which could be elicited by
a comparatively brief pulse of agonist (Fig. 4a). The
amplitude of this depolarization was not linearly related
to the observed concurrent conductance increases. Under
voltage-clamp conditions, at resting membrane potentials
(mostly -40 to -45 mV), the underlying current consisted
of a fast outward current component (indistinguishable
from the fast hyperpolarizing current described above),
together with an inward current component which had
slower onset kinetics and lasted up to 30 sec in response
to a 500 msec pulse of muscimol (Fig. 4b). The faster
outward current reversed direction at a membrane
potential of about -55 mV and was an inward current at
more negative potentials, but the slow current was inward
over the whole range of holding potentials tested (0 to
-80 mV) and increased with hyperpolarization (Fig. 4c).

Picrotoxin is well-known as a specific blocker of
Cl^- channels, in particular those associated with the
$GABA_A$ receptor in the vertebrate brain, and Cl^--
mediated responses in arthropods (14), including insect
neuron somal GABA responses (13). Bath application of
picrotoxin at concentrations of 10^{-6} M or higher
completely abolished the hyperpolarizing responses evoked
by 10^{-4} M GABA pulses applied for periods of up to 10
sec. This effect was only partially reversible even
following prolonged superfusion with control saline. At
10^{-7} M, picrotoxin reduced the amplitude of the
response to GABA or muscimol by 50-80%. The voltage-
dependence of the blockade was examined using 10^{-7} M
picrotoxin. In all stable recordings (n=11), the
percentage reduction in response was similar at all
potentials tested (Fig. 5a). It was possible to test the
action of picrotoxin on two of the Locusta neurons in
which the biphasic GABA- and muscimol-evoked response was
observed. In both cells, 10^{-7} M picrotoxin reduced the
amplitude of both the inward and the outward components
(Fig. 5b). At 10^{-6} M, picrotoxin completely blocked
both components of the response. These observations
suggest that picrotoxin binds or has part of its action
elsewhere than at the Cl^- channels in these neurons

a

$- 40$ mV

$- 50$ mV

10^{-4}M GABA
1 sec pulses

$- 60$ mV

$- 70$ mV

$- 80$ mV

$- 90$ mV

1nA
30 sec
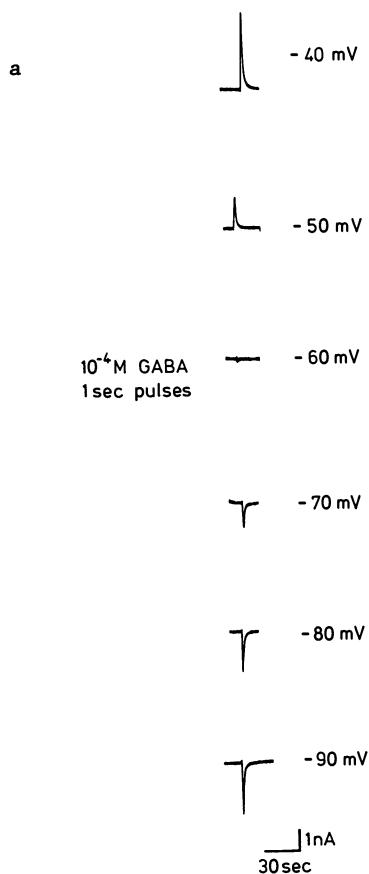
Figure 3. Currents recorded from isolated locust
thoracic neuronal somata voltage-clamped by a
conventional single-electrode method.
a) Application of 1 sec pressure pulses of 10^{-4} M
GABA to a neuron clamped at a series of different
holding potentials shows the direction and voltage-
dependence of the current underlying the change in
membrane potential seen in all cells in response to
GABA or muscimol.

b
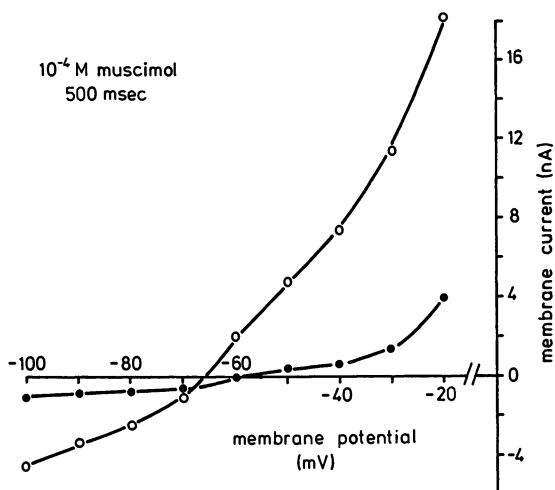
Figure 3.--Continued.
b) The current-voltage (I-V) curve of this voltage-
clamped neuronal soma shows the voltage-dependence of
the outward rectification of the resting cell (filled
circles) and of the current evoked by 500 msec
pressure pulses of 10^{-4} M muscimol (open circles).
The point of intersection of the two curves indicates
the reversal potential (-65 to -70 mV) of the
muscimol-evoked current (not significantly different
from the GABA-evoked current).
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1987, Elsevier Press.
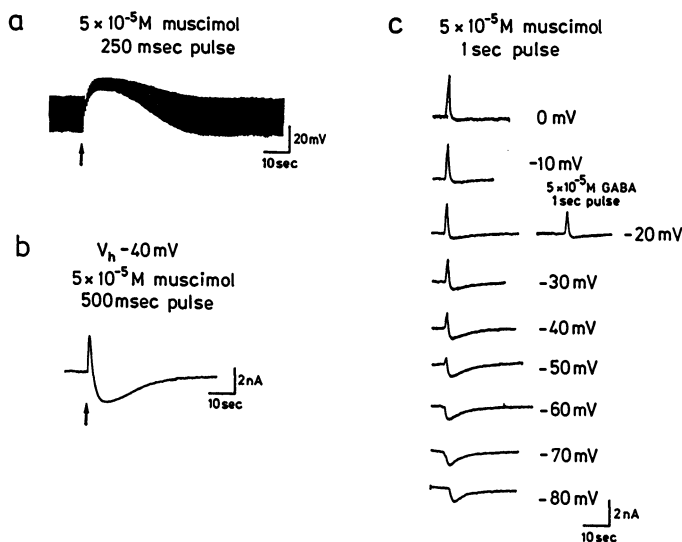
Figure 4. Muscimol and GABA evoked a biphasic response in some <u>Locusta</u> neuronal somata.
a) Pressure application of 5 x 10^{-5} M muscimol for 250 msec induced a brief hyperpolarization (partially obscured by the test current pulses in this trace) followed by a predominant, prolonged depolarization in a current-clamped neuron with normal resting potential (about -45 mV). Repetitive, constant current pulses throughout the experiment gave a measure of the membrane conductance.
b) Muscimol (5 x 10^{-5} M, 500 msec pressure pulse) applied to a <u>Locusta</u> neuron clamped at -40 mV evoked a two-component current paralleling the muscimol-evoked potential change in the current clamped neuron in a). A fast outward current was followed by a prolonged inward current.
c) The voltage-dependence of the two muscimol-evoked current components is shown for a <u>Locusta</u> neuron clamped at holding potentials from 0 mV to -80 mV. The fast component reversed sign at close to -60 mV, while the slow component remained inward and increased in amplitude with hyperpolarization over the range tested. The inset shows a similar response in the same neuron to an equivalent pulse of GABA, indicating that the biphasic response is not agonist specific. Reproduced with permission from Ref. 16. Copyright 1987, Elsevier Press.

a

membrane potential
(mV)

-90   -80   -70

-60   -50   -40   -30   -20

10 mins 10^{-7} M
picrotoxin

control

evoked current (nA)

20

15

10

5

0

-5

-10

b

control V_h -40 mV 10^{-7} M picrotoxin

-20 mV

-40 mV

10^{-7} M picrotoxin   -80 mV   2nA

10 sec
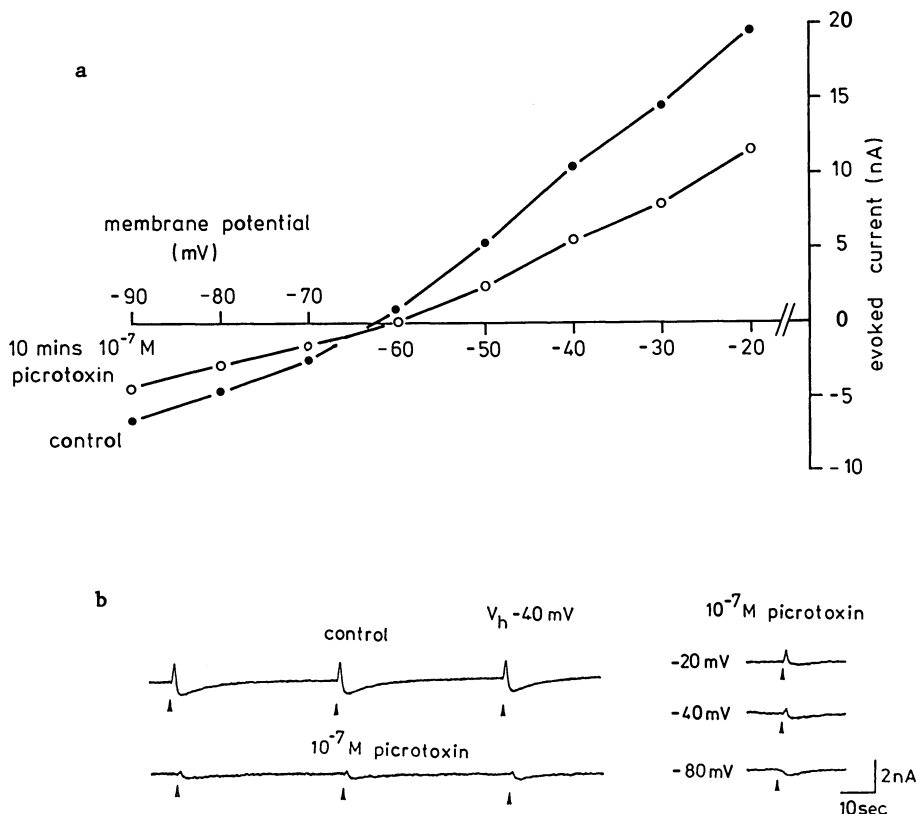
Figure 5.   Picrotoxin blocked the GABA- and muscimol-
evoked responses in the locust thoracic neuronal
somata.
a) The amplitudes of the current evoked by 500 msec
pressure pulses of 10^{-4} M muscimol are plotted
against the holding potential of the clamped neuron in
normal saline (filled circles) and during superfusion
with 10^{-7} M picrotoxin (open circles) to show the
degree of blockade at different membrane potentials.
b) Picrotoxin at 10^{-7} M (as shown) or 10^{-6} M
inhibited both components of the biphasic response
generated by 1 sec pulses of 5×10^{-5} M muscimol in
this neuron clamped at membrane potentials between
-20 mV and -80 mV.
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since the slow current component cannot be accounted for by a change in Cl$^-$ conductance, gCl$^-$.

To determine whether the locust somal GABA receptor fits the vertebrate GABA$_A$/GABA$_B$ classification, we tested a selection of diagnostic agonists and antagonists. The blocking action of picrotoxin is characteristic of the GABA$_A$ receptor-channel complex and muscimol is a potent GABA$_A$ agonist which mimics the action of GABA on the locust neuronal somata (13,16). Baclofen, in contrast, is a GABA$_B$ agonist. It was without significant effect when applied to the locust somata at concentrations of up to 10^{-4} M (n=20). In terms of these well-characterized agonists, the locust somal GABA receptor appears to be Type A. However, this conclusion is contradicted by the observations made using antagonists. Bicuculline base, its water-soluble methiodide salt and pitrazepin (a competitive GABA$_A$ antagonist recently reported to be an order of magnitude more potent than bicuculline at the GABA$_A$ receptor in vertebrates (19)), were freshly prepared and tested by bath application at concentrations up to 10^{-4} M. The current evoked by GABA (10^{-5} to 10^{-4} M) was completely unaffected by all three compounds over a range of holding potentials from -20 to -100 mV. From these experiments, we conclude that the locust somal GABA receptor does not fit the vertebrate GABA$_A$/GABA$_B$ classification. This feature appears to be common to other invertebrate GABA receptors (14) and similar conclusions have been drawn from binding studies on the cockroach nervous system (20).

There is a second major distinction between the GABA$_A$ and GABA$_B$ receptors. Modulators are substances that alter the responsiveness of neurons to excitation or inhibition (21). The GABA$_A$ receptor is part of a complex which includes binding sites for benzodiazepines and barbiturates which modulate the response of the complex to GABA and other agonists. GABA$_B$ responses appear not to be modulated by these compounds. We were interested to know whether these modulatory sites occur in conjunction with the insect somal GABA receptors both as part of the general pharmacological characterization of the receptor and also because such sites, if they occur, could provide additional targets for insecticide design.

Flunitrazepam is a sedative benzodiazepine which has its clinical effect by enhancing the inhibitory action of GABA when it binds to its receptors in the vertebrate CNS. Applying this compound (10^{-6} to 10^{-5} M) prior to and concurrently with the application of the agonist reversibly enhanced the amplitude of the GABA- or muscimol-evoked current in somata voltage-clamped at -40mV (Fig. 6a,b). The rise rate of the response was increased but the duration was not significantly altered (Fig. 6a). At these concentrations, flunitrazepam did not affect the resting membrane potential or the membrane
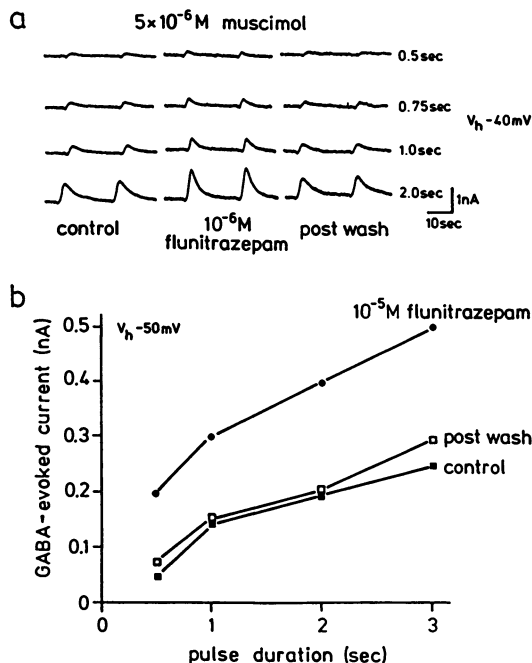
Figure 6. Flunitrazepam increased the amplitude but not the duration of the GABA- and muscimol-evoked current in locust thoracic neuronal somata.
a) Muscimol pulses were applied by pressure ejection for the durations indicated to a neuron clamped at -40 mV. During continuous microapplication of 10^{-6} M flunitrazepam, the amplitude of the muscimol-evoked current was increased. The effect of flunitrazepam was rapidly reversed by continuation of the normal perfusion of the experimental chamber.
b) The amplitude of the GABA-evoked current recorded in a neuron, clamped at -50 mV, before (filled squares), during (filled circles) and after (open squares) treatment with 10^{-5} M flunitrazepam is plotted against the GABA pulse duration to show the reversible enhancement of the evoked current amplitude by flunitrazepam.
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conductance. The response enhancement was seen in all
cells tested but to varying degrees. The maximum response
enhancement was 60-70%. Recent binding studies have
indicated the presence of benzodiazepine binding sites in
the thoracic muscles of insects as well as in the insect
CNS (22-24). Our electrophysiological results provide
evidence that at least some of these binding sites may be
functionally associated with GABA receptors and Cl$^-$
channels in a similar way to those which form part of the
vertebrate GABA$_A$ complex.

In the vertebrate brain, the GABA$_A$ receptor-
channel complex has a fourth element, a barbiturate
modulatory site. Anaesthetic barbiturates such as Na-
pentobarbital enhance and prolong the inhibitory effects
of GABA in the vertebrate brain (e.g. 25). We therefore
examined the effects of Na-pentobarbital on the GABA-
evoked Cl$^-$ current in the locust neuronal somata. When
applied by microperfusion at 10^{-4} M, this compound
reversibly enhanced both the amplitude and the duration
of the current evoked by GABA or by muscimol (Fig. 7a,b).
In common with the modulatory effect of flunitrazepam,
the action of Na-pentobarbital was seen in all cells
tested, but the magnitude of the enhancement was
variable. The maximum enhancement observed was about 70%.
For 10^{-4} M Na-pentobarbital, the interpretation of the
results was occasionally complicated by the observation
of direct effects on the resting membrane potential and
conductance (16). During the application of Na-
pentobarbital, depolarization, accompanied by a decrease
in the input resistance, was seen in some cells. From
these observations, we conclude that the locust somal
GABA receptor complex, like the vertebrate GABA$_A$
receptor, has a binding site for barbiturates and that
the anaesthetic barbiturates enhance the GABA response in
a way similar to their mode of action in the vertebrates.

Conclusions

Both the long-term primary cultures of neurons from the
brains of embryonic cockroaches and the freshly
dissociated neuronal somata from the thoracic ganglia of
locusts can readily be voltage-clamped for many hours so
that the resting current as well as the voltage-activated
and agonist-evoked currents can be recorded (8, 16-18).
For the study of receptor pharmacology, these
preparations provide relatively simple systems in
comparison with intact ganglia, stable in vitro and
devoid of diffusion barriers. Up until now, we have found
the pharmacology of particular receptor types to be
uniform from one individual soma to another, so that it
has not proved necessary to attempt the dissection of
somata from identified neurons. This picture may change
as we accumulate more detailed pharmacological
information. It is also important to note that at least
some of the insect somal receptor types are likely to

a

10^{-4}M GABA
400 msec pulses
V_h -40 mV

control

b

5×10^{-5}M
pentobarbital

post-wash

2·5nA
1sec
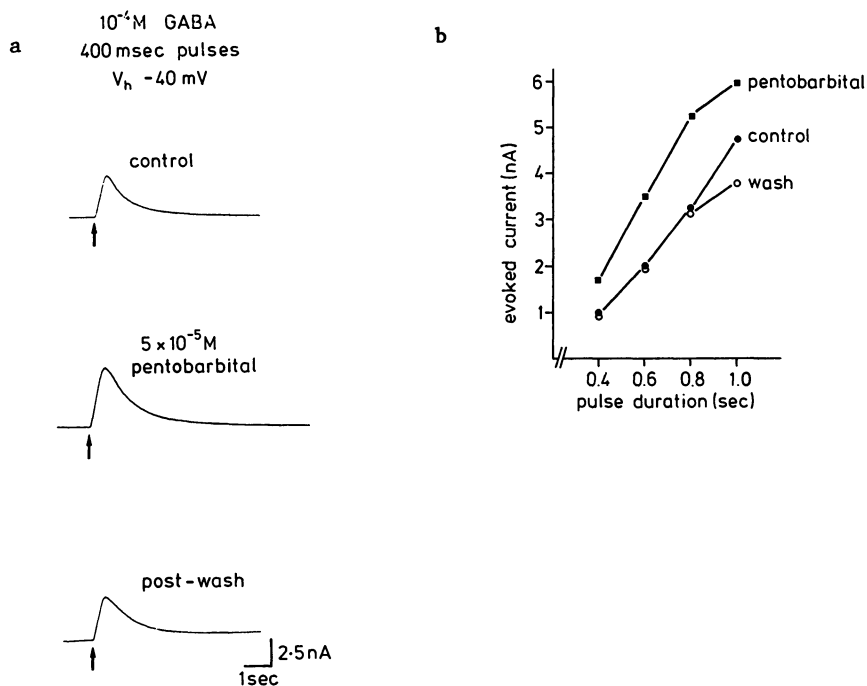
Figure 7. Na-pentobarbital increased the amplitude and duration of the GABA- or muscimol-evoked current in the locust thoracic neuronal somata.
a) Application of 400 msec pressure pulses of 10^{-4} M GABA to a neuron clamped at -40 mV resulted in an outward current which was increased in both amplitude and duration by exposure of the neuron to 5×10^{-5} M Na-pentobarbital. The Na-pentobarbital effect was easily reversed by a few minutes superfusion with normal saline.
b) The amplitude of the muscimol-evoked current was linearly dependent on the duration of the application pulse. Na-pentobarbital increased the amplitude of this current, as for the GABA-evoked current, over the range of pulse durations tested. The current was increased in amplitude by similar proportions for all pulses except the longest pulse (1 sec) when the enhancement was proportionately less.
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differ from the insect central synaptic receptors for the same neurotransmitters. Nevertheless, from a practical point of view, if the somal receptors are present in vivo as they are in vitro, they might in any case provide targets for insecticides.

The pharmacology of the locust somal GABA receptors is summarized in Table I. In common with other invertebrate GABA receptors, these receptors differ in pharmacology from those of the vertebrates. At first sight, this might appear to present an opportunity for specificity in designing insecticides. However, more data are required for anything other than cautious optimism, since the agonists active on the insect somata are also active at the vertebrate central or peripheral receptors. It is only the agonist/antagonist combinations that are different among the three receptor types (GABA$_A$, GABA$_B$, insect soma) and so far there are no compounds known to be uniquely active as agonists or antagonists at the insect GABA receptor.

Table I. Pharmacology of GABA receptor-channel subtypes

	Vertebrate		Insect
	GABA$_A$	GABA$_B$	soma
Agonists			
muscimol	potent	very weak	potent
baclofen	inactive	potent	inactive
Antagonists			
picrotoxin	potent	inactive	potent
bicuculline	potent	inactive	inactive
pitrazepin	potent	inactive	inactive
Modulators (potentiators)			
benzodiazepine	potent	?	potent
barbiturate	potent	?	potent
Mechanism			
gCl$^-$	increase	no link	increase
gK$^-$	no link	increase	no link

Some years ago, Nielson (26) carried out a survey of the benzodiazepine binding sites in the neural tissue of some vertebrates and invertebrates, including locusts, and the results suggested that benzodiazepine receptors do not occur in the invertebrates but arose with the evolution of the bony fishes. Binding studies (22-24) together with our electrophysiological data indicate the presence of benzodiazepine receptors functionally associated with at least some insect central GABA receptors.

Additionally, we have presented evidence that there is a barbiturate receptor which most likely forms part of the GABA receptor-Cl$^-$ channel complex as in the vertebrate GABA$_A$ system. We conclude that the insect thoracic somal GABA receptor is part of a complex bearing strong similarities to the vertebrate GABA$_A$ receptor complex but exhibiting a distinctive agonist/antagonist profile.

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Chapter 4

GABA-Related Systems as Targets for Insecticides

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Gamma-aminobutyric acid (GABA) is an inhibitory
transmitter substance in the central nervous system of
both insects and higher animals. However, there are
subtle differences in the properties of the GABA-
receptor complex between these two groups of animals.
The picrotoxinin receptor of the cockroach head differs
in several respects from that of the rat brain in its
response to cyclodiene-type as well as pyrethroid in-
secticides. The molecular topography of the cockroach
picrotoxinin receptor has been studied by synthesizing
various picrotoxinin analogs. There appears to be a
minimum requirement for an active ligand to possess at
least two of three critical moieties comprised of two
electronegative and one steric bulkiness (hydropho-
bicity) centers. In the case of the chloride channel
the most conspicuous species difference was noted when
the action of avermectin B_{1a} was studied. Mammalian
systems respond by increases in GABA release, benzodi-
azepine binding, GABA-binding and chloride perme-
ability. In the case of the cockroach this pesticide
causes a profound change only in the putative chloride
channel.

Dieldrin (1), γ-BHC (2) and picrotoxinin (3,4) have been shown to
influence the presynaptic events on the American cockroach
(Periplaneta americana) central nervous system (CNS) and thereby to
stimulate excitatory neurotransmitter release. As to the cause for
such stimulation, we have proposed that these agents specifically
interact with the putative picrotoxinin receptor closely associated
with the chloride ionophore in the γ-aminobutyric acid-chloride iono-
phore complex (designated as the GABA receptor system in this paper)
at the presynaptic region, and that such interaction causes inhibi-
tion of chloride ion uptake. This uptake is regulated by GABA to
modulate the presynaptic membrane potential (4-8).

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Furthermore, we have reported that cyclodiene- and
γ-BHC-resistant German cockroach strains are also resistant to picro-
toxinin, a naturally occurring neuroexcitant and GABA antagonist
(6,7,9). Moreover, the components of the CNS from the cyclodiene-
resistant German cockroaches (Blattella germamica) have been found to
have lower specific binding capacities toward picrotoxinin than do
those of their susceptible counterparts (7). It has also been shown
that cyclodienes, γ-BHC and some of the known GABA antagonists bind
competitively with the picrotoxinin receptor in the CNS of the
American cockroach (4). Other agents such as toxaphene and bicyclo-
phosphates also bind competitively with the picrotoxinin receptor in
the CNS of the American cockroach (4). In agreement with these
observations Lawrence and Casida (10), and Abalis et al. (11), have
found that there is an excellent correlation between the inhibitory
potencies of various cyclodiene-type insecticides on ^{35}S-TBPS binding
to mammalian brain components and their insecticidal activities.

An important message emerging from such a series of studies is
that the GABA system is an important and integral component of the
insect CNS as shown by the supreme effectiveness of these insecti-
cides in eliciting violent CNS excitation symptoms in many insect
species. Despite its importance, not much is known about the proper-
ties of the GABA system in the insect CNS. In this paper, therefore,
we have made an effort to describe the general biochemical properties
of two of the major components of the insect's GABA receptor system;
the picrotoxinin receptor and the chloride channel. Whenever pos-
sible we have compared their properties to those already reported in
the mammalian CNS.

All methodologies and materials used to obtain the data pre-
sented in this paper have been published (4,6-9).

Picrotoxinin Receptor (PTX-R)

Both mammalian and insect picrotoxinin receptors have recently been
reported to respond to a variety of cyclodiene-type insecticides. By
using ^3H-dihydropicrotoxinin to identify GABA receptors, Matsumura and
Ghiasuddin (6) have shown that the I_{50} of heptachlor epoxide against
the rat brain picrotoxinin receptor is in the order of 2 μM
(Figure 1). When the same insecticide was tested against the ^3H-
dihydropicrotoxinin binding in cockroach nerves, the corresponding
value was in the order of $2 \times 10^{-7}M$ (for the brain PTX-R) to $5 \times 10^{-7}M$
(for the PTX-R from the nerve cord) (Figure 2), indicating that the
cockroach nerve PTX-R is more sensitive to heptachlor epoxide than
that from the rat brain.

The Scatchard plot analysis of ^3H-DHPTX binding (Figure 3) in-
dicates that the dissociation constant, K_d, is in the order of
$5.8 \times 10^{-7}M$ using a brain membrane preparation from the CSMA strain of
the German cockroach. The corresponding data from the cyclodiene-
resistant strain (Lpp) are also shown in this figure for comparison.
The data indicate that the PTX-R in the resistant nervous system has
only 1/10 as high an affinity as that from the susceptible strain
toward dieldrin. Also, the number of receptors was reduced in the
preparation from the resistant insects.

In the next series of experiments the relative sensitivity of
the PTX-R in the American cockroach head to various insecticides and
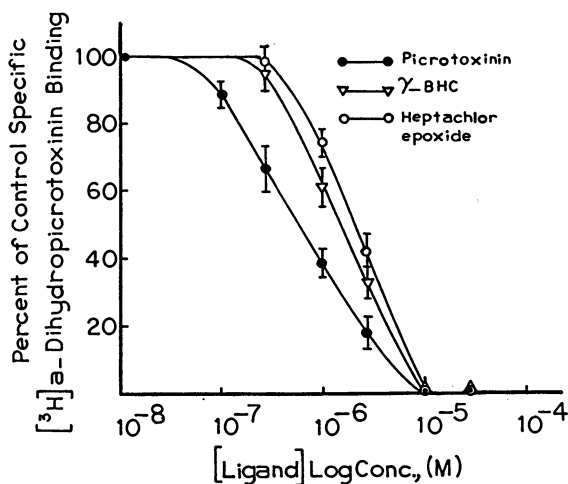
Figure 1. Binding of [^{3}H]α-dihydropicrotoxinin to rat brain
   synaptosomes and its displacement by picrotoxinin, γ-BHC and
   heptachlor epoxide. Reproduced with permission from Ref. (6)
   (Copyright, 1983 Marcel Dekker).
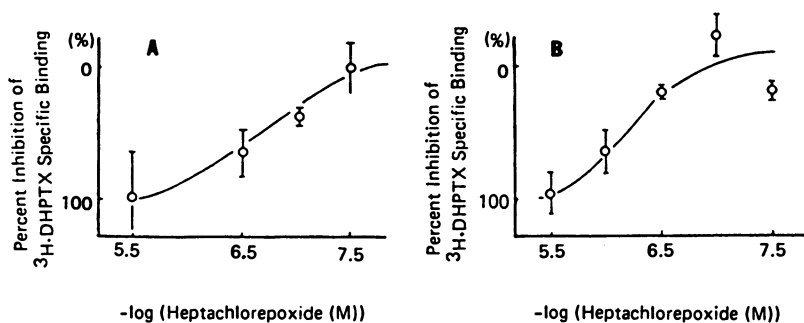
Figure 2. Inhibition of [^{3}H]α-dihydropicrotoxinin (^{3}H-DHPTX)
   binding to nerve components of P. americana by heptachlor
   epoxide. A) Brain. B) Nerve cord. Reproduced with permission
   from Ref. (44) (Copyright, 1985 Academic Press)

neuroactive agents was examined (Table I). The results clearly
indicate that the cockroach PTX-R is sensitive to a variety of cyclo-
diene insecticides, but not to any other types of insecticides
tested. Gamma-BHC is an exception, but it has long been recognized
that this insecticide acts in a very similar manner to cyclodienes
and as such it is not surprising to find that it mimics cyclodienes
in this respect. Also it is important to note that toxaphene acts as
a cyclodiene-type insecticide as far as its insecticidal mechanisms
are concerned.

The differences in potency among BHC isomers are of great inter-
est. In the case of the American cockroach system the gamma-isomer
is clearly the most potent isomer followed by delta, alpha and beta.
The relationship in the rat brain system is similar to the cockroach
brain (Table II), but in the rat the potency of the delta isomer is
almost as high as that of the gamma-isomer. Since the gamma isomer
is the only insecticidal isomer, the above data have been interpreted
to mean that the receptor binding potency of the delta isomer is
probably related to its strong blocking (depressant instead of
excitant) action shown to occur in vivo in the mammalian CNS.

Another conspicuous feature of the cockroach PTX-R is its insen-
sitivity to pyrethroids. Earlier Lawrence and Casida (12) reported
that in rat brain, pyrethroids, particularly type II pyrethroids,
were strong competitors of the binding of ^{35}S-TBPS to its receptor.
Since bicyclophosphates such as TBPS have been shown to interact with
the mammalian PTX-R (13-15) the above event was interpreted as show-
ing a direct action of pyrethroids on the PTX-R. Since the radio-
ligand used in this study is different from that employed by us, a
question may be raised whether such a discrepancy is due to the
difference in the source of the PTX-R or the ligands. More recent
information (Palmer and Casida, this volume) indicates that the
housefly PTX-R is also refractory to pyrethroids as judged by the
^{35}S-TBPS binding test. This suggests that the difference is probably
due to the biological origin of the PTX-R itself.

Structural Requirements of Chemicals Interacting with Insect PTX-R

The structure-insecticidal activity relationships of picrotoxinin
analogs and related compounds have been studied by several research-
ers (3,16), who noted that the bridged bicyclic lactone skelton and
the trans-isopropenyl or isopropyl group are essential for insecti-
cidal activity. Structure-activity relationships of cyclodiene
insecticides and BHC have also been thoroughly discussed (17-19).
However, these discussions were published before the nature of the
biological target site(s) for cyclodienes was known.

In view of the recent discovery of the similarities of action
between picrotoxinin and cyclodiene-type insecticides as described
above (3,8), it appears worthwhile to reexamine the structural
requirement of picrotoxinin-type convulsants for interaction with the
specific picrotoxinin binding site. Another objective of this study
is to obtain supporting evidence for the role of the picrotoxinin
receptor in the mode of action of cyclodiene-type insecticides by
synthesizing compounds that structurally bridge the gap between
cyclodiene compounds and picrotoxinin.

Table I.   Effect of cyclodienes and other agents on [^{3}H]α-
                dihydropicrotoxinin binding[a]

Agents[b]	% Inhibition of ^{3}H-dihydropicrotoxinin (DHPTX) specific binding[c]
Dihydropicrotoxinin	100%
Aldrin	36.6 ± 0.6
Dieldrin	58.5 ± 2.3
Photodieldrin	79.0 ± 4.3
Heptachlor	36.6 ± 18.6
Heptachlor epoxide	57.3 ± 31.3
Isodrin	35.6 ± 3.8
Endrin	62.3 ± 12.4
γ-Chlordane	56.2 ± 2.0
Oxychlordane	77.8 ± 6.2
Hexachlorocyclopentadiene	0.3 ± 2.2
Mirex	14.7 ± 15.7
Kepone	135.5 ± 18.4
Toxaphene	72.3 ± 28.1
DDT	5.4 ± 3.6
alpha-BHC	21.4 ± 18.5
beta-BHC	15.3 ± 15.4
gamma-BHC	91.5 ± 20.0
delta-BHC	55.0 ± 16.0
Parathion	4.1 ± 26.3
Allethrin	13.2 ± 6.7
Cypermethrin	7.7 ± 3.1
Decamethrin	-0.1 ± 1.0
Fenvarelate	-0.4 ± 5.8
Chlordimeform	7.8 ± 4.7
Pentobarbital	21.1 ± 8.1
Benzodiazepam	45.1 ± 10.8
SQ-65396	21.5 ± 5.7
SQ-20009	-8.3 ± 4.2
Tetramethylenedisulfotetramine	27.1 ± 13.0
t-Butyl bicyclic phosphate	46.6 ± 2.9

[a]^{3}Hα-dihydropicrotoxinin: 11.1 nM. Five American cockroach heads
 were used for one experiment (one agent), which involves three
 determinations.
[b]Dihydropicrotoxinin: 100 μM, others: 10 μM.
[c]Data are expressed as means ± SE of two or three experiments, each
 experiment involving three determinations (4).

Figure 3. Scatchard plot analysis of [^{3}H]-DHPTX binding to the brain membrane preparations from two German cockroach strains: Dieldrin-susceptible CSMA (●) and -resistant LPP (○) strains. Bmax (receptor number (mole/mg protein) was: CSMA, 9.0×10^{-13}; LPP, 7.1×10^{-14}; Kd (dissociation constant (M)) was: CSMA, 5.84×10^{-7}; LPP, 6.45×10^{-8}. Data are expressed as means of two independent experiments, each experiment involving three determinations. Reproduced with permission from Ref. (44) (Copyright, 1985 Academic Press).

Table II. Effect of BHC isomers on [^{3}H]-dihydropicrotoxinin[a] binding to the rat brain picrotoinin receptor

Isomers[b]	Specific binding[c] (dpm/mg protein)	(% of picrotoxinin value)
Picrotoxinin	420 ± 37	(100.00)
α-BHC	1 ± 84	(0.22)
β-BHC	53 ± 77	(12.79)
γ-BHC	299 ± 46	(71.18)
δ-BHC	259 ± 81	(61.47)

[a]Concentration of ^{3}H-dihydropicrotoxinin was 1.65×10^{-8} M.
[b]All at 10^{-5} M.
[c]Total binding was 14052 dpm/mg protein.

Chemistry. On the basis of the discussion by Matsumura and
Ghiasuddin (6), several bridged bicyclic compounds were synthesized
(Table III). Cycloaddition of allyl cations to isopropylidenecyclo-
pentadiene yielded three epimers of 2,4-dimethyl-8-
isopropylidenebicyclo[3.2.1]oct-6-en-3-one, i.e., the equatorial cis-
2,4-dimethyl [1], axial cis-2,4-dimethyl [8], and trans-2,4-dimethyl
[12] analogs, and two epimers of the 2,4-dibromo analog [7 and 11].
Several epoxy and hydroxy analogs were prepared by epoxidation of
double bonds and reduction of carbonyl groups, respectively.
     The Diels-Alder reaction was utilized to construct bicyclo
[2.2.1]heptane or bicyclo[2.2.1]heptene structures. The reaction of
isopropylidenecyclopentadiene with maleic anhydride produced the endo
and exo configurational isomers of 8-isopropylidenebicyclo[2.2.1]
hept-2-ene-5,6-dicarboxylic anhydride. Similar reactions were ap-
plied to unsubstituted and 1-(methoxycarbonyl)cyclopentadienes to
give the corresponding anhydrides. The anhydrides were reduced to
alcohols, which were then allowed to react with thionyl chloride or
tosyl chloride to give cyclic sulfites or tosylates. Reaction of the
tosylates with lithium chloride gave chlorinated compounds. Hydra-
tion of the double bonds of the chlorinated compounds was accom-
plished by hydroboration-oxidation. Diol 31 thus obtained was
converted to 5,6-bis(chloromethyl)-7-isopropylidene-bicyclo[2.2.1]
heptan-2-one [33] by chromium trioxide oxidation of the secondary
hydroxyl group followed by dehydration at the C-7 substituent.

Insecticidal Activity. Generally, the insecticidal activity of these
synthesized compounds was not high (Tables III, IV and V). However,
they were active enough to serve as a tool for toxicological study.
Generally speaking, active compounds (e.g. 1 and 15) caused convul-
sions, and some of them (e.g. 33) caused incoordinated walking in
the German cockroach. The onset of signs of poisoning was rapid, as
compared with those caused by cyclodiene insecticides such as diel-
drin. Table III shows the insecticidal activities of 2,4-dimethyl-8-
isopropylidenebicyclo[3.2.1]oct-6-en-3-one and related compounds by
injection. The equatorial cis-2,4-dimethyl epimer 1 was most active
among the three epimers of 2,4-dimethyl-8-isopropylidenebicyclo-
[3.2.1]oct-6-en-3-one. The trans isomer [12] was about 10 times less
active than the equatorial cis isomer. The epoxide derivatives [4
and 6] had almost the same activity as the parent compound. Reduc-
tion of the carbonyl group [2 and 3] lowered the activity. The 2,4-
dibromo derivatives [7] and [11] were inactive.
     Table IV lists the insecticidal activity of cyclic sulfites. In
this case, the exo cyclic sulfites of 5,6-bis(hydroxymethyl)-7-
isopropylidenebicyclo[2.2.1]hept-2-ene [20] was active whereas the
corresponding endo isomer [18] was inactive. Monoepoxidation [21 and
22] and diepoxidation [23] of the exo cyclic sulfite resulted in a
slight increase in activity. Compound 15 was the most active among
this series of compounds. The LD_{50} value of this compound was
estimated to be about 1 μg/fly when topically applied to houseflies
without any synergists (data not shown). The C-7 isopropylidene
group proved to be unnecessary for high activity by comparison with
compound 18. The activity decreased after epoxidation [16] or hydro-
genation [17] of the endocyclic double bond. The cyclodiene insecti-
cide endosulfan was about 10 times as active as [15].

Table III.   Insecticidal Activity of Bicyclo[3.2.1]oct-6-en-3-one Analogues

compd no.	X	Y	Z	R	dose, μg/roach	mortality
2,4-Diequatorial Cis Isomers						
1	HC—CH	C=C(CH₃)₂	C=O	CH₃	10 / 3	6/11 / 3/10
2	HC—CH	C(CH₃)₂	CH(OH) (eq)	CH₃	10	0/10
3	HC—CH	C(CH₃)₂	CH(OH) (ax)	CH₃	10	0/10
4	HC—CH	C—C(CH₃)₂	C=O	CH₃	10 / 3	8/10 / 2/10
5	HC—CH	C(CH₃)₂	CH(OH)	CH₃	10	1/10
6	HC—CH (epoxide)	C—C(CH₃)₂	C=O	CH₃	10 / 3	7/10 / 0/10
7	HC—CH	C=C(CH₃)₂	C=O	Br	10	0/10
2,4-Diaxial Cis Isomers						
8	HC—CH	C=C(CH₃)₂	C=O	CH₃	10	1/10
9	HC—CH	C—C(CH₃)₂	C=O	CH₃	100 / 10	6/10 / 0/10
10	HC—CH (epoxide)	C—C(CH₃)₂	C=O	CH₃	10	0/10
11	HC—CH	C=C(CH₃)₂	C=O	Br	10	0/10
2,4-Trans Isomers						
12	HC—CH	C=C(CH₃)₂	C=O	CH₃	10	0/10
13	HC—CH	C—C(CH₃)₂	C=O	CH₃	100 / 10	9/10 / 0/10
14	HC—CH (epoxide)	C—C(CH₃)₂	C=O	CH₃	10	1/10

Table IV. Insecticidal Activity of the Cyclic Sulfites of 5,6-Bis(hydroxymethyl)bicyclo[2.2.1]hept-2-ene Analogues

compd no.	X	Y	R		dose, μg/roach	mortality
Endo Cyclic Sulfites						
15	HC—CH	CH_2	H		10	10/10
					3	9/10
					1	3/10
					0.3	0/10
16	(HC—CH epoxide)	CH_2	H		10	10/10
					3	4/10
					1	0/10
17	$H_3C CH_2$	CH_2	H		10	8/10
					3	0/10
18	HC—CH	$C=C(CH_3)_2$	H		10	0/10
19	HC—CH	$C C(ClCH_3)_2$	H	I	30	1/10
					10	0/10
					30	6/10
					10	0/10
	ClC—CCl	CCl_2 (endosulfan)	Cl	II'	0.3	10/10
					0.1	8/10
					0.3	8/10
					0.1	0/10
Exo Cyclic Sulfites						
20	HC—CH	$C=C(CH_3)_2$	H		10	5/10
					3	1/10
21	(HC—CH epoxide)	$C=C(CH_3)_2$	H		10	8/10
					3	0/10
22	HC—CH	(ClCH₃)₂	H		10	9/10
					3	1/10
23	(HC—CH epoxide)	$C C(ClCH_3)_2$	H		10	10/10
					3	0/10
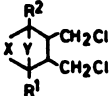
Another series of compounds are 5,6-bis(chloromethyl)bicyclo
[2.2.1]hept-2-ene and related compounds (Table V). Compound 24 was
active. The activity was slightly raised by the introduction of a
chloromethyl group at the bridgehead [25]. Ketone [27] prepared by
hydroboration-oxidation of [24] was active. Introduction of a chlor-
omethyl group at the bridgehead of [27] resulted in a decrease in
activity [28]. Compound 30 was inactive, indicating that the isopro-
pylidene group at the 7-position is not desirable for insecticidal
activity. Ketone 33, which has an isopropenyl group at the 7-
position, had some activity.

Inhibition of Specific [^{3}H]-α-Dihydropicrotoxinin Binding. Specific
binding of [^{3}H]-α-dihydropicrotoxinin was inhibited by some bridged
bicyclic compounds (Table VI). Compound 33 was a potent inhibitor,
which was comparable to endosulfan, although the insecticidal
activity of the former compound was somewhat lower than that of the
latter. Cyclic sulfite [15] was also active in displacing [^{3}H]-
α-dihydropicrotoxinin. The non-insecticidal analogue, 10, was not
very active in this regard, nor is the insecticidal equatorial cis
isomer 6. A simple lactone and norcamphor were also weak inhibitors.
Cyclohexane had almost no activity. Judging by the fact that the
degree of resistance shown by LPP to the lactone compound and to com-
pound 6 was very modest, it appears that the extent of interaction of
these compounds with the picrotoxinin receptor of the cockroach is
less significant than that of [15] and [33].

Discussion on Molecular Topography of Insect PTX-R

The molecular topography of cyclodiene insecticides was extensively
studied by Soloway (17), who noted the presence of two electronega-
tive centers in each active cyclodiene or gamma-BHC. We provide a
modified interpretation of the molecular topography of picrotoxinin-
type convulsants in this paper. The similarity between the two ap-
proaches is that in the case of cyclodiene compounds such as aldrin,
dieldrin and heptachlor epoxide two electronegative centers are pro-
vided by the olefinic chlorines of the hexachloronorbornene nucleus
and the double bond or oxygen atom of the second ring system (A and B
in Figure 4). The differences are that we propose that there is one
additional hydrophobic center that provides steric bulkiness (C in
Figure 4) and that to act on the receptor the compound must possess
at least two of these three characteristics. γ-BHC has three equa-
torial chlorines and two axial chlorines as the electronegative cen-
ter. However, the isopropenyl group of picrotoxinin, the center
axial chlorine of γ-BHC, and one chlorine of the dichloromethylene
bridge of cyclodiene insecticides appear to work as steric bulkiness
or   hydrophobic centers rather than as electronegative centers.
Another type of convulsant, the bicyclic phosphates (13-15), which
also act at the picrotoxinin binding site in cockroaches (4), has a
phosphoryl moiety as the single electronegative center. The bulki-
ness or hydrophobicity of the bridgehead substituent plays an impor-
tant role as the second center for high affinity to the binding site
(20). Another example is a picrotoxinin-like convulsant, anisatin,
which is isolated from the seed of a toxic plant. This compound does
not seem to have the second electronegative center (Figure 4).

Table V.   Insecticidal Activity of 5,6-Bis(chloromethyl)
bicyclo[2.2.1]hept-2-ene Analogues

compd no.	X	Y	R¹	R²	dose, µg/roach	mortality
		endo-Bis(chloromethyl) Analogues				
24	HC=CH	CH₂	H	H	10 / 3	6/10 / 1/10
25	HC=CH	CH₂	CH₂Cl	H	10 / 3	10/10 / 0/10
26	CH₂CH(OH)	CH₂	H	H	10	0/10
27	CH₂CO	CH₂	H	H	10 / 3	5/10 / 0/10
28	CH₂CO	CH₂	CH₂Cl	H	10	2/10
29	CH₂COO	CH₂	H	H	10	2/10
30	HC=CH	>C=C(CH₃)₂	H	H	10	0/10
31	CH₂CH(OH)	>CHC(CH₃)₂(OH)	H	H	10	0/10
32	CH₂CO	>CHC(CH₃)₂(OH)	H	H	10	0/10
33	CH₂CO	>CHC(CH₃)=CH₂	H	H	10 / 3	5/10 / 0/10
	ClC=CCl	>CCl₂ (alodan)	Cl	Cl	0.1 / 0.03	5/10 / 0/10
		exo-Bis(chloromethyl) Analogues				
34	HC=CH	>C=C(CH₃)₂	H	H	10	0/10
35	(epoxide HC—CH)	>C=C(CH₃)₂	H	H	10	0/10
36	CH₂CH(OH)	>C=C(CH₃)₂	H	H	10	0/10
37	CH₂CO	>C=C(CH₃)₂	H	H	10	0/10

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Society.

Table VI. Inhibition of specific [³H]-α-Dihydropicrotoxinin Binding
to American cockroach head components by Bridged Bicyclic and
Related Compounds

compd	concn, µM	inhibn,[a] %
cyclohexane	30	10 ± 19
β,β,γ,γ-tetramethyl-γ-butyrolactone	10 / 30	20 ± 8 / 17 ± 3
norcamphor	10 / 30	23 ± 2 / 22 ± 10
6	10 / 30	38 ± 21 / 16 ± 9
10	10 / 30	21 ± 12 / 27 ± 30
15	10 / 30	28 ± 8 / 58 ± 18
33	10 / 30	60 ± 21 / 102 ± 33
endosulfan (α)	10	63 ± 3
alodan	10	78 ± 18

[a] Each value is the mean ± SD of three or four experiments, each
performed in triplicate.

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Society.

Picrotoxinin   Heptachlor epoxide   T-BHC

Anisatin   Bicyclic phosphate   33

1   15   25

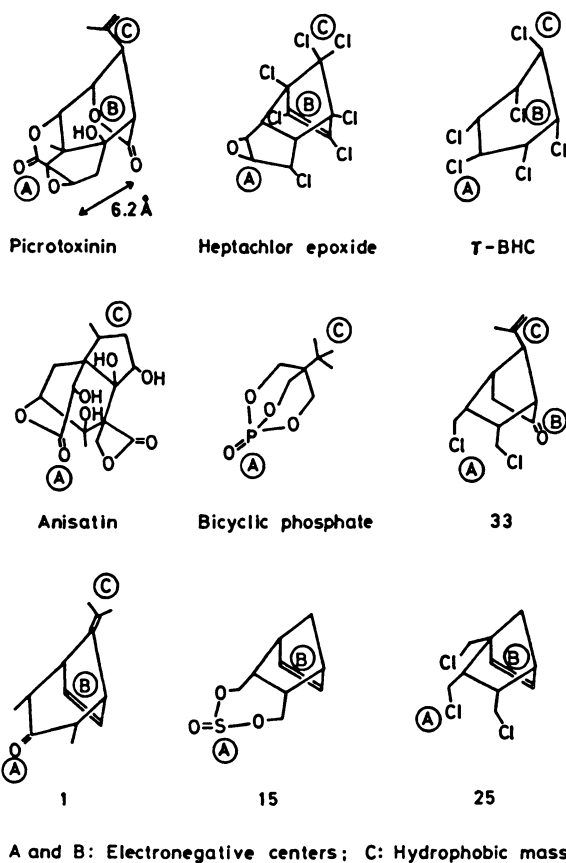A and B: Electronegative centers; C: Hydrophobic mass.

Figure 4. Electronegative and steric bulkiness sites of compounds acting on picrotoxinin receptors. Reproduced from Ref. (57) (Copyright, 1986 Amer. Chem. Society).

The insecticidal compounds synthesized for the present study satisfy the structural requirement as described above although not completely. They inhibited $[^3H]$-α-dihydropicrotoxinin binding and were less toxic to the cyclodiene-resistant strain of the German cockroach than the susceptible strain, indicating that they act at the picrotoxinin site. In the case of bicyclo[3.2.1]oct-6-en-3-one analogs, all insecticidal (below 10 μg) compounds have equatorial methyl groups at the 2- and 4-positions. The equatorial cis 2,4-dimethyl isomers are in the chair conformation whereas axial cis isomers are in the boat form, in which 1,3-diaxial nonbonded interactions are minimized (A of Figure 5). On the other hand, trans isomers are supposed to interconvert between the chair and boat conformations. The chair conformation and substituents in the equatorial position seem to be favorable for the interaction with the picrotoxinin binding site. This is also the case for picrotoxinin, γ-BHC, and cyclodiene insecticides that have equatorial or endo substituents at the corresponding positions. The carbonyl oxygen of boat conformers possibly cannot constitute the electronegative center with the double bond or epoxy group at the 6- and 7-positions, whereas that of chair conformers can. This may be tested by ligand-receptor binding assay. However, there was no great difference in specific $[^3H]$-α-dihydropicrotoxinin binding inhibitory activity between compounds 6 and 10. The binding assay seems to be less sensitive in estimating the biological activities of compounds than bioassay with the German cockroach. Although the binding inhibitory activity of [6] was not so high, the cross-resistance shown by cyclodiene-resistant roaches clearly indicated that this compound acted on the picrotoxinin receptor. On the other hand, epoxidation of the double bonds of compound 1 did not improve the insecticidal activity. This finding indicates that compound 1 with double bonds is possibly converted to analogs with epoxy groups [4 and 6] in the insect body to exert an activity similar to the latter compounds, or that both the epoxy group and double bond work almost equally as electronegative centers in the 6- and 7-positions, i.e., B of [1] in Figure 4. As to the 8-position (C of [1] in Figure 4), neither the epoxy group nor the isopropylidene group may be adequate for interaction with the binding site.

Compound 15 was the most highly insecticidal analog. This compound can be regarded as a dechlorinated analog of endosulfan. The difference in insecticidal activity between the two compounds was only 10-fold, which could be explained by expected metabolic differences. The chlorine atoms of cyclodiene insecticides are not necessarily essential for insecticidal activity. Soloway (17) observed a very high toxicity for the 1,4,10,10-tetrachloro analogue of aldrin, indicating that the double bond of the hexachloronorbornene nucleus can replace the olefinic chlorines as a part of the electronegative center. It is also worth noting the difference in activity between compounds 15 and 18 (B of Figure 5). The difference in structure is just an isopropylidene group at the 7-position. The isopropylidene group, unlike the isopropenyl moiety of picrotoxinin, has a projection angle away from the electronegative center A. This makes the distance between A and C too great for 18, indicating that the isopropylidene group is unfavorable for the interaction with the picrotoxinin binding site. The fact that compound 20 is active despite having the unfavorable exo configuration supports the above
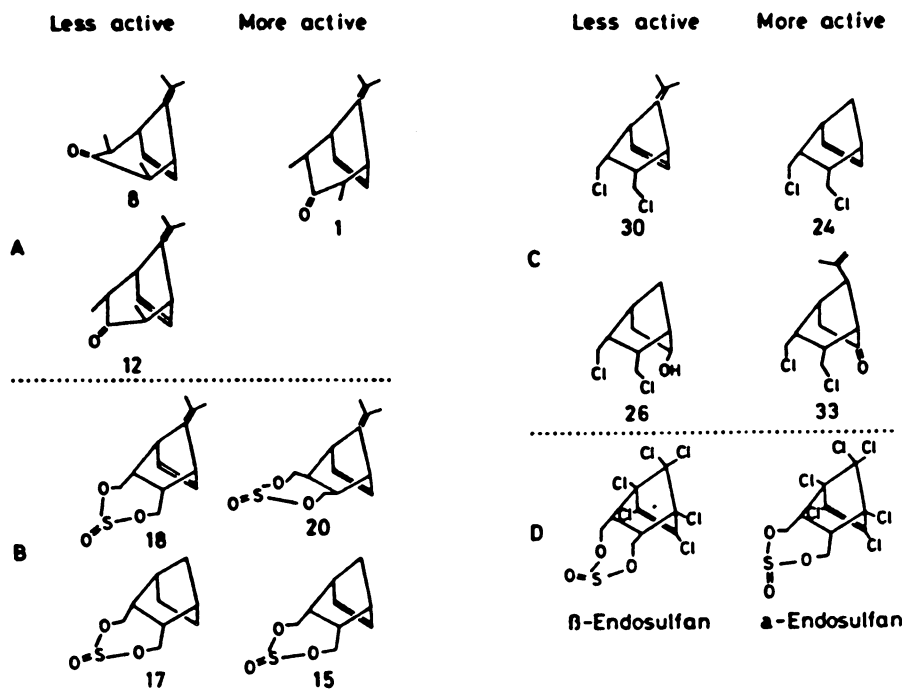
Figure 5. Structure-activity relationships of bridged bicyclic compounds. Reproduced from Ref. (57) (Copyright, 1986 Amer. Chem. Society).

conclusion (B of Figure 5). A similar argument may be made for
compound 24, for [33] versus [30], and for α- versus β-endosulfan (C
and D of Figure 5). The activity of the epoxidized and hydrogenated
analogs [16 and 17] of compound 15 decreased in that order due to
reduced electronegativity. This may indicate the significance of the
electronegativity of the B site of [15] (Figure 4), which, with the
cyclic sulfite moiety A, provides two electronegative centers.

Several endo-5,6-bis(chloromethyl)bicyclo[2.2.1]heptenes and
heptanones had insecticidal activity. The lactone moiety of picro-
toxinin is a metabolically vulnerable point, and the insecticidal
activity may increase by replacement of the lactone by a carbonyl
group. This is the reason why some ketone analogs were synthesized.
Compound 33 has an isopropenyl group like picrotoxinin and two endo
chloromethyl groups like the cyclodiene insecticide alodan. In this
case, two chloromethyl groups and the carbonyl group probably con-
stitute two electronegative centers (Figure 4). The isopropenyl
group may play an important role in interacting with the critical
binding site through its steric bulkiness. This compound was intrin-
sically the most active, although the insecticidal activity was
weaker than expected. The inhibitory activity of specific [3H]-
α-dihydropicrotoxinin binding was comparable to that of the cyclo-
diene insecticides, indicating that compound 33 to a great extent
satisfies structural requirement as a ligand for the picrotoxinin
site. However, a high affinity of a compound for the site of action
does not necessarily mean that it will have high insecticidal activ-
ity. The hydrophobicity of the whole molecule and its metabolic vul-
nerability should also be optimized to obtain highly insecticidal
compounds.

In contrast to the above groups of compounds, there is some
evidence to show that γ-butyrolactones do not act on the picrotoxinin
receptor, though they have a partial structure of picrotoxinin. Eto
(20) described the insecticidal activity of β-isopropyl-γ-
butyrolactone. Klunk et al. (21) presented a hypothesis that alkyl-
γ-butyrolactones act at the same site as picrotoxinin, based on
structural and pharmacological studies. If simple alkyl-γ-
butyrolactones act at the picrotoxinin binding site, cyclodiene-
resistant German cockroaches must show cross-resistance to the
compounds and specific [3H]-α-dihydropicrotoxinin binding must be in-
hibited by them. Table VI shows the results of ligand-receptor bind-
ing assays of β,β,γ,γ-tetramethyl-γ-butyrolactone. The lactone
interacted with the picrotoxinin receptor to some extent in vitro,
but, judging from its very low resistance ratio, the interaction is
not very significant. These findings suggest that other site(s) of
action than the picrotoxinin binding site exist for these compounds.

In conclusion, the picrotoxinin binding site can accept a
variety of compounds such as cyclodiene insecticides, γ-BHC,
toxaphene, bicyclic phosphates, and picrotoxinin (4). While the mode
of interaction seems to be somewhat flexible, there appears to be a
minimum requirement for active compounds to possess at least two of
the three essential sites, i.e. two electronegative and one steric
bulkiness (or hydrophobicity) sites within a definite range of dis-
tance. Bridged bicyclic structures are suited for the preparation of
compounds which satisfy such structural requirements.

Mechanism of Action of Avermectins: Studies on the Chloride Channel in the American Cockroach

Avermectins (22-24) and milbemycins (25,26) are macrocyclic lactones which are produced by certain streptomycetes. They are reported to have high insecticidal and anthelmintic activities. The former compounds are oleandosyloxy derivatives of the latter compounds (27). Using avermectin B_{1a}, one of the most potent components of the avermectin complex, extensive mode-of-action studies have been conducted by several research groups. When applied to the extensor muscles of lobster walking legs, avermectin B_{1a} blocked neuromuscular transmission, which was associated with a reduction of the membrane resistance of the muscle fibers (28). This reduction of membrane resistance is proposed to be closely related to γ-aminobutyric acid (GABA)-mediated chloride conductance changes (28). Kass et al. (29) reported that avermectin B_{1a} rapidly paralyzed nematodes without causing hypercontraction or flaccid paralysis. They also found that this drug blocks neurotransmission in the ventral nerve cord and the transmission between motoneurons and muscle.

GABA, an inhibitory neurotransmitter in the vertebrate brain and the invertebrate muscle, exerts its effect through increased post-synaptic membrane permeability to chloride ion (30-33). Its effect is initiated by binding to its receptor in the GABA system, i.e., GABA, benzodiazepine, picrotoxinin receptors, and Cl^- channel complex. There is biochemical evidence that avermectin B_{1a} potentiates GABA action in the mammalian brain in several ways. GABA release from brain synaptosomes is specifically stimulated by avermectin (34), and ligand binding to the GABA and benzodiazepine sites in mammalian brain is also stimulated (35-41).

Compared with the accumulated biochemical knowledge on GABA, benzodiazepine, and picrotoxinin receptors, and the Cl^- channel complex in mammalian brains (see Ref. 41), the available information on insects and nematodes is quite limited.

Difficulties in measuring ligand binding to GABA receptors in insects (42) could be one of the reasons for such a scarcity of information. The only results available are a few studies on picrotoxinin receptors and Cl^- channels in the central nervous systems and muscles of the American and German cockroaches (4,5,7), and on putative benzodiazepine receptors in housefly thoracic muscles (42). According to these reports there are several differences in drug specificities between the receptors of mammals and insects (4,42). One of the most convincing species differences in the susceptibility of the GABA system to neuroactive agents is that avermectins are more toxic to insects and nematodes than to mammals (43).

Avermectins and the related milbemycins may be a very useful biochemical probe for understanding the function and mechanism of nervous systems in invertebrates and vertebrates.

In the current study, we have made an attempt to characterize the mechanism of action of avermectin B_{1a} in the cockroach muscle and central nervous system. We report finding that avermectin opens the Cl^- channel apparently independent of actions on GABA, benzodiazepine, and picrotoxinin receptors. The methods used for this part of the study have been published elsewhere (44).

Effect of Avermectin B1a on the Flex-Contraction of the Perfused Cockroach Leg

Male American cockroaches were prepared as described earlier (44).
Under the normal test conditions, the leg perfused with Ringer solu-
tion (0.5 ml/min.) responded to electric stimuli (2-10 V, 2 msec
duration, every 5 sec.) by rhythmically contracting as shown in
Figure 6. When the perfusate was changed to avermectin-containing
Ringer solution (10^{-5} M) the leg continued to respond normally to the
stimuli for about 15 min. Thereafter, it started to show signs of
failing to respond. By 30 min, the leg did not show any response to
electric stimuli, even when their intensity was increased to 15 V.
On the other hand, the force of contraction did not change until the
last response.
        The results agree with the data obtained by Kass et al. (29),
who observed that avermectin paralyzes nematodes without causing
hypercontraction or flaccid paralysis.

$^{36}Cl^-$ Influx Studies. Among the leg muscles used in this study, the
coxal fibers of muscles 177d and 177e are known to be innervated by
three inhibitory axons and a slow excitatory axon (45). Muscles 182c
and 182d are also known to receive three inhibitory axons (46,47).
        From preliminary experiments using radioactive $^{36}Cl^-$, it was
found that avermectin B1a significantly enhanced the $^{36}Cl^-$ influx
into the muscle as compared to that for the control muscle. More-
over, at least a few minutes of preincubation of the muscles in
saline containing avermectin is sufficient for the maximum enhance-
ment of the $^{36}Cl^-$ influx into the muscles. Without preincubation of
the muscles in the presence of avermectin little increase of $^{36}Cl^-$
influx was observed. The muscles preincubated for over 10 min with
avermectin took up significantly higher amounts of $^{36}Cl^-$. Interest-
ingly, the increase in $^{36}Cl^-$ influx of the muscle preincubated for 20
min was almost as high as in the ones preincubated for 3, 6, and 10
min.
        The enhancement of $^{36}Cl^-$ influx induced by avermectin was found
to be concentration-dependent (Table VII). The ion flux increased
slightly at 10^{-8} M avermectin. At 10^{-7} M, avermectin action is max-
imal. GABA is believed to cause an increase in Cl^- permeability of
the postsynaptic muscle cell membrane in invertebrates (5,30,31).
Desensitization of GABA action has been reported in some crustacean
muscles (48) and the mammalian central nervous system (23), but not
in other systems. Therefore, the action of avermectin was compared
to that of GABA (48,49). As shown in Table VII, avermectin (10^{-7} M)
was found to be more potent than GABA in increasing $^{36}Cl^-$ uptake. In
combination with avermectin, GABA did not increase or decrease the
$^{36}Cl^-$ influx induced by avermectin, suggesting that they have some
common site of action. Milbemycin, an analog of avermectin (25),
activated $^{36}Cl^-$ influx at 10^{-7} M to the same extent as avermectin.
        Picrotoxin, a blocker of the Cl^- channel, has been reported to
antagonize avermectin's action in various preparations (28,29,43,
49). Bicuculline, another GABA antagonist, blocked the action of
avermectin on lobster stretcher muscles (49). These two GABA antago-
nists are also known to inhibit $^{36}Cl^-$ uptake induced by GABA (4,50).
Picrotoxinin (10^{-4} M) blocked 50% of the $^{36}Cl^-$ influx enhanced by
avermectin (10^{-7} M) (Table VII). Bicuculline methiodide (10^{-4} M) did
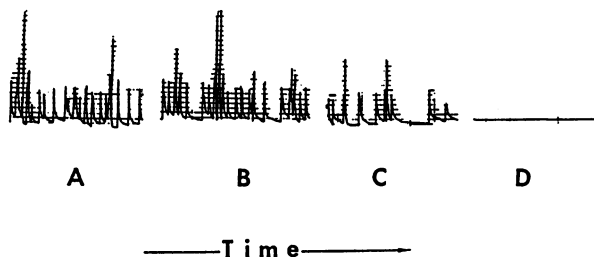
Figure 6. The effect of avermectin on muscle contractions of the
perfused cockroach hind leg (stimulated by 5-V square impulses).
The most frequently appearing (medium height) peaks are the ones
caused by contraction of the hind leg. The higher peaks are due
to compound contractions involving additional muscles. (A) Con-
trol preparation perfused with Ringer solution. (B) A saline
solution containing avermectin (10^{-5} M) was injected into the
hind leg. Recording started at 1 min after injection. (C) 15
min from the time of injection of avermectin. (D) Recording
after 30 min. Reproduced with permission from Ref. (44) (Copy-
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not reverse the action of avermectin Bla. Changing the order of preincubation (i.e., first antagonist and then avermectin plus antagonist) did not affect the inhibition pattern (data not shown).

The Effect of Na^+, K^+, and Cl^- on the Action of Avermectin

To examine the effect of the counter cation of Cl^- (i.e., Na^+ and K^+) on the $^{36}Cl^-$ influx induced by avermectin, all Na^+ except a part of the sodium phosphate (2 mM) was replaced by cholinium cations. The results (Table VIII) indicate that a similar $^{36}Cl^-$ influx enhancement by avermectin was observed, although the extent of enhancement was slightly less than that observed in the normal saline.

The resting membrane potential of the muscle and nerve is known to be close to the Cl^- equilibrium potential. The potassium equilibrium potential is also close to the resting membrane potential. When the muscles are exposed to the K^+ free solution, the resting membrane potential is known to hyperpolarize below the chloride ion equilibrium potential (28). When the avermectin-treated muscles were exposed to the K^+-free saline solution containing $^{36}Cl^-$, the $^{36}Cl^-$ influx was suppressed to 60% of the control.

Neurophysiologically, muscles of vertebrates have different characteristics from those of invertebrates. The latter muscles are innervated by excitatory and inhibitory motor neurons to form neuromuscular junctions which are believed to be operated by the excitatory transmitter, glutamate, and the inhibitory transmitter, GABA, respectively. On the other hand, vertebrate skeletal muscles have only excitatory innervation in which acetylcholine is a transmitter. To study the effect of avermectin on vertebrate muscles, those from frog hind legs were used. As expected, no difference was observed in $^{36}Cl^-$ influx between control and avermectin treated leg muscles (data not shown).

Effects of Avermectin on Muscimol, Benzodiazepam and Picrotoxinin Binding

From the above experiments it was evident that avermectin increases the muscle membrane conductance to Cl^- ions by opening the chloride channel. However, it is still unknown whether avermectin opens the chloride channel directly or indirectly by modulating other components of GABA (34,35) and benzodiazepine binding (34,35). Another possibility is the stimulation of GABA release by avermectin as in the case of rat brain synaptosomes (36).

By employing a Na^+-free assay solution and thoroughly disrupting and washing muscle and brain membrane preparations of the American cockroach, reproducible and specific [^{3}H]muscimol binding data were obtained (Table IX). This specific muscimol binding was completely inhibited by bicuculline methiodide (10^{-5} M).

As shown in Table IX, avermectin (10^{-7}M) failed to increase [^{3}H]muscimol binding. At 10^{-5} M, a marginal inhibition of binding was seen. For comparison, the same experiment was repeated using a rat brain membrane preparation. In this case, avermectin at 10^{-5} M clearly stimulated [^{3}H]muscimol binding to rat brain membrane as also reported for [^{3}H]GABA binding (34,35).

Table VII. Effect of Various Agents and Treatments the Level
of $^{36}Cl^-$ Uptake by the Leg Muscles of the American
Cockroach

Compounds	n	$^{36}Cl^-$ influx (%)
Experiment A		
Control	4	100.00 ± 4.39
Avermectin[a] (10^{-5} M)	4	115.71 ± 5.60
Avermectin (10^{-6} M)	4	116.37 ± 6.89
Avermectin (10^{-7} M)	4	115.28 ± 5.18
Avermectin (10^{-8} M)	4	107.44 ± 6.13
Avermectin (10^{-9} M)	4	101.36 ± 4.75
Experiment B		
Control	8	100.00 ± 4.32
Avermectin (10^{-7} M)	8	115.87 ± 7.95
Milbemycin (10^{-7} M)	8	116.63 ± 4.99
GABA (10^{-3} M)[a]	8	104.18 ± 5.92
Avermectin (10^{-7} M)		
+ GABA (10^{-3} M)	8	116.30 ± 8.22
Experiment C		
Control	8	100.00 ± 4.32
Avermectin (10^{-7} M)	8	115.87 ± 7.95
Avermectin (10^{-7} M) + picrotoxinin (10^{-4} M)	8	108.12 ± 6.72
Avermectin (10^{-7} M) + bicuculline methiodide (10^{-4} M)	8	113.08 ± 4.73

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Table VIII. Stimulation of $^{36}Cl^-$ Uptake by the Cockroach Muscles
with Avermectin in Na^+-Free Saline Solution

Compounds	n	$^{36}Cl^-$ influx (%)
Na^+-free saline	4	100.00 ± 4.83
+ Avermectin (10^{-7} M)	4	111.90 ± 5.88

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Table IX. Effect of avermectin on ^{3}H-Muscimol, ^{3}H-Benzodiazepam and ^{3}H-Dihydropicrotoxinin specific binding

	dpm/mg Protein		
	American Cockroach		Rat
	Head	Muscle	Brain
1. ^{3}H-Muscimol[a]			
Control	5813 ± 397	4607 ± 169	12730 ± 278
+ GABA (10^{-4} M)	2688 ± 61	3293 ± 81	2125 ± 40
+ Avermectin (10^{-5} M)	5486 ± 330	4470 ± 115	15431 ± 154
+ Avermectin (10^{-7} M)	5792 ± 232	4586 ± 145	12123 ± 42
2. ^{3}H-Benzodiazepam[b]			
Control	59008 ± 262	45941 ± 334	63412 ± 2065
+ Benzodiazepam (10^{-4} M)	49801 ± 443	44521 ± 535	35866 ± 717
+ Avermectin (10^{-5} M)	57724 ± 310	45646 ± 333	74054 ± 1461
+ Avermectin (10^{-7} M)	57842 ± 841	45667 ± 282	69315 ± 1079
3. ^{3}H-Dihydropicrotoxinin[c]			
Control	29038 ± 200	20403 ± 84	
+ Dihydropicrotoxinin (10^{-4} M)	27044 ± 260	19659 ± 186	
+ Avermectin (10^{-5} M)	28650 ± 233	20392 ± 442	
+ Avermectin (10^{-7} M)	29128 ± 506	20434 ± 799	

The concentration of these radioligands is as follows:
a) ^{3}H-Muscimol (7.8 x 10^{-9} M),   b) ^{3}H-Benzodiazepam (2.43 x 10^{-9} M),   c) ^{3}H-Dihydropicrotoxinin (22.2 x 10^{-9} M)

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Electrophysiological and pharmacological studies in mammalian species have suggested a role for GABA in mediating the effects of benzodiazepines (42). This suggestion is supported by the discovery of the enhancement of benzodiazepine-receptor binding by GABA and its analogs. Avermectin is also known to cause a concentration-dependent increase in benzodiazepine receptor binding in synaptic membranes in a solubilized receptor fraction by changing the affinity and also the receptor number (37,39,40,52).

Using membrane preparations of the brain and the muscle of the American cockroach, [^{3}H]benzodiazepam binding was studied in the presence of avermectin. At 10^{-5} and 10^{-7} M, there was no activation of [^{3}H]benzodiazepam binding. By contrast, in the case of the rat brain preparation, avermectin increased [^{3}H]benzodiazepam binding as previously shown by other workers (see above).

The picrotoxinin receptor, though it is an integral part of the GABA complex, is known to be a receptor independent from the GABA and benzodiazepine receptors in mammalian brains (52).

Using the [^{3}H]dihydropicrotoxinin binding test (52), the effect of avermectin on the picrotoxinin receptor was examined. No significant effect of avermectin on [^{3}H]dihydropicrotoxinin binding to the membranes of the brain and the muscle of the American cockroach was found. At higher concentrations of avermectin (10^{-5} M) a slight inhibition was observed.

Effect on the Central Nervous System. Perfusion of the central nerve cord with 10^{-7} M avermectin eliminated the nerve excitation induced by γ-BHC (10^{-6} M) (44). Within 15–20 min after perfusion of avermectin the nerve became completely calm. Under the experimental conditions a few random single spikes per minute were observed but only in the control nerves. Avermectin completely eliminated such background signals. Shortly before transmission blockage occurred, the nerve treated with γ-BHC and avermectin showed severe but transient excitation. Recently Mellin et al. (49) observed an initial enhancement by avermectin of the facilitation response of excitatory postsynaptic potentials to a train of stimuli in the stretcher muscle of the lobster. Nerve excitation by DDT (10^{-5} M) was also found to be eliminated by avermectin (10^{-7} M).

Discussion on the Properties of the Cockroach "Chloride Channel"

It must be stated at the beginning that the term "chloride channel opening" instead of "ionophore action" has been used in this paper to describe the overall process of increased chloride ion permeability through the plasma membranes of muscle and nerves. Such usage does not necessarily imply that the presence of specific chloride channels has been proven in insect excitable cells. Other terms such as chloride ionophore, membrane permeability, or conductance changes could also be used. In the absence of solid evidence to preferentially support any of the terms, and in view of the antagonistic action of picrotoxinin on this process, the term chloride channel has been adopted with the above reservations. A recent report (D. Soderlund, personal communication, 1986) indicates that in the rat brain postsynaptic vesicles avermectin blocks Cl$^-$ passage, indicating that

avermectin is not Cl⁻ ionophore, and that it can fix the "channel" either at an open state or a closed state. Such a finding supports the idea of the existence of Cl⁻ channels. However, more evidence would be needed to unequivocally prove their existence.

The most important finding of this investigation is that avermectin causes its insecticidal activity by directly opening chloride channels in the membrane of the leg muscles in the American cockroach. This action of avermectin is not mediated through GABA or benzodiazepine receptors in the cockroach muscles. The action of avermectin itself otherwise appears to be similar to that of GABA in fundamental aspects, although avermectin was found to be more potent than GABA. The observation that avermectin did not interact with the picrotoxinin receptor of the CNS of the American cockroach, which is known to be closely associated with Cl⁻ channel (31,43), is also significant.

It is well known that the GABA receptor regulates the opening of the Cl⁻ channel in crustacean muscles and the mammalian CNS (31,51, 53). Benzodiazepines are one group of chemicals which can facilitate the action of GABA (54,55). The modulation of GABA and benzodiazepine receptors of the mammalian CNS by avermectins has been reported by several researchers (34,35,37,39,40). In the case of the mammalian CNS, avermectin itself does not directly compete against GABA at its binding site (34). Instead it appears to enhance the specific binding of [³H]GABA by increasing the apparent number of GABA binding sites without significantly changing their apparent dissociation constant (35). If this enhancement of GABA binding could couple with the stimulation of GABA release by avermectin as reported by Pong and Wang (34), a potent avermectin-evoked GABA action would be expected. However, at least in the American cockroach systems, such an action is unlikely, since avermectin did not activate muscimol binding at all. Na⁺-dependent GABA uptake is a very important step in the elimination of GABA from the receptor area. It is more critical than the enzymatic degradation of GABA. Nipecotic acid is known to be an inhibitor of GABA uptake (56). However, nipecotic acid did not show any synergistic activity on avermectin's toxicity to the German cockroach (LT_{50} by film contact method, 100 mg/500-ml jar was 680 min with avermectin alone and in combination with nipecotic acid (100 μg/roach), it was 660 min). The data suggest that an increased GABA release by avermectin might not be involved in its toxicity in this species. This conclusion is supported by another observation that the muscles of the American cockroach preincubated with avermectin for 20 min still open their chloride channels as much as the ones incubated for shorter times (i.e., 3, 6, and 10 min). Such a prolonged GABA release from the presynaptic area is highly unlikely. The observation by Fritz et al. (28) that avermectin's effects on the lobster muscle could not be reversed by washing also supports the above conclusion that these effects in invertebrate systems may not be directly related to GABA itself, because free GABA would be eliminated by extensive washing. Similarly, the possibility of the involvement of benzodiazepine receptors in the action of avermectin on the American cockroach could be excluded since little effect on the benzodiazepine receptor by avermectin was found.

The loss of contraction in the leg of the American cockroach by avermectin was studied by the experiment using the perfused cockroach leg preparation (50). The observation that the leg contracted at the

same strength until losing the contraction itself suggests that
avermectin blocks stimulus transmission from the central nervous
system to muscles, but does not affect their tension. This sugges-
tion is in agreement with the earlier electrophysiological observa-
tions by Fritz et al. (28) and Mellin et al. (49) that avermectin
blocked synaptic transmission in the lobster stretcher and opener
muscles. They observed a rapid loss of inhibitory postsynaptic
potentials with a slow decline in the amplitude of excitatory post-
synaptic potentials, accompanied by a shortening of the excitatory
postsynaptic potential duration and a decrease of the input resis-
tance in the muscle fiber. The site of action was proposed to be
postsynaptic rather than presynaptic on the basis of electrophysio-
logical observations (28). Kass et al. (29) also found electro-
physiologically that avermectin blocks transmission between
inhibitory motoneurons and muscles of a nematode species, in addition
to the blockage of transmission between interneurons and excitatory
motoneurons in the ventral nerve cord.

As for the meaning of the action of avermectins in K^+-free
saline, Fritz et al. (28) observed that when avermectin was applied
in the standard lobster Ringer solution, the muscle membrane hyper-
polarized by up to 5 mV within a few minutes. The addition of
avermectin to muscles in K^+-free solution, where the resting poten-
tial is more negative than the Cl^- equilibrium potential, caused a
depolarization of the membrane by several millivolts. The suppres-
sion of $^{36}Cl^-$ influx of the avermectin-treated muscles in the K^+-free
saline solution may be explained by the electrophysiological observa-
tion by Fritz et al. (28) that, in K^+-free solution, efflux of intra-
cellular Cl^- in avermectin-treated muscles increases for a while due
to the depolarization. The evidence suggests that avermectin selec-
tively increases Cl^- ion permeability across the muscle membrane.

Recent studies from our research group have demonstrated the
presence of GABA and picrotoxinin receptors and the Cl^- channel com-
plex in the central nervous system of American and German cockroaches
(4,7). In the current study, avermectin was found to increase chlo-
ride uptake in the leg muscle cells of the American cockroach.
Avermectin may act as a presynaptic inhibitor by opening Cl^- channels
of the GABA synapse so that the presynaptic membrane is not able to
release an excitatory transmitter, or as a postsynaptic inhibitor by
opening the chloride channel in the postsynaptic region.

The elimination of γ-BHC symptoms by avermectin may be explained
as follows. The primary action site of γ-BHC is likely to involve
the picrotoxinin receptor (5). Therefore, one could assume that γ-
BHC, at least in part, acts in an identical manner to picrotoxinin
which has already been shown to antagonize the action of avermectin.
Kass et al. (29) reported two types of avermectin action on the
nervous system of the nematode, based on the inhibition by picro-
toxinin. One is the avermectin-induced blockage of interneuron-
excitatory motorneuron transmission, which can be reversed by
picrotoxinin. The other is the avermectin-induced blockage of the
transmission between inhibitory motoneurons and muscle, which cannot
be reversed by picrotoxinin.

The excitatory action of γ-BHC was recorded on a central nerve
preparation. Therefore, the current observation agrees well with
that made by Kass et al. (29). On the other hand, the antagonistic

action of avermectin on DDT-induced excitation, probably produced by actions on the axonal Na channel, suggests that avermectin, at high concentrations, can block any type of nerve excitation in addition to the picrotoxinin receptor-mediated process.

In summary, avermectin has been found to interact directly with the chloride channel in the muscle and the central nervous system of the American cockroach. This action of avermectin appears to occur independently from GABA, benzodiazepine, and picrotoxinin receptors in this species.

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Chapter 5

Bicycloorthocarboxylates

Potent Insecticides Acting at the GABA-Regulated Chloride Ionophore

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Bicycloorthocarboxylates or 2,6,7-trioxabicyclo-[2.2.2]octanes of the type $R-C(CH_2O)_3C-R'$ (R and R' are the 4- and 1-substituents, respectively) are readily prepared by several methods, the most versatile of which involves rearrangement of an acylated hydroxymethyloxetane with boron trifluoride etherate. They are potent insecticides when R is propyl, butyl, cyclopentyl or cyclohexyl and R' is cyclohexyl, cycloheptyl or a suitably-substituted phenyl group. Piperonyl butoxide synergizes many of the active compounds as topical toxicants for adult houseflies and American cockroaches. Suitable combinations of 1- and 4-substituents confer selective toxicity to houseflies versus mice. The bicycloorthocarboxylates act in mammalian brain membranes at the GABA-regulated chloride ionophore as $GABA_A$ receptor antagonists based on studies on the inhibition of ^{36}chloride uptake and displacement of the radioligand $[^3H]\underline{t}$-butylbicycloorthobenzoate from its specific binding site. They antagonize GABA-mediated relaxation of a functional insect nerve-muscle synapse. 1,4-Disubstituted-bicycloorthocarboxylates are a new class of potent $GABA_A$ receptor antagonists.

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system of insects and mammals and at the neuromuscular junction of insects (1). The GABA-regulated chloride ionophore or $GABA_A$ receptor component of the GABAergic system is sensitive to antagonism by polychlorocycloalkane insecticides (e.g., lindane, dieldrin, toxaphene) (2-6), picrotoxinin (PTX) analogs (7, 8) and bicyclophosphorus esters (9-11). The bicyclophosphorus esters such as t-butylbicyclophosphorothionate (TBPS) have little or no insecticidal activity except on injection into insects pretreated with the oxidase inhibitor piperonyl butoxide (PB), and they are highly toxic to mammals (12, 13). The related bicycloorthocarboxylates are similar to the bicyclophosphorus esters in their mode of action in mammals (9, 11, 14) and offer greater opportunity

for structural modification in the 1- and 4-positions for optimizing insecticidal potency, penetration and selective toxicity (13). This review considers the structure-activity relationships and mode of action of 1,4-disubstituted-bicycloorthocarboxylates.

R_1, R_4 = alkyl, aryl
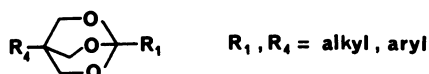
Synthesis

Bicycloorthocarboxylates are obtained by transesterification of the appropriate 2-hydroxymethyl-1,3-propanediol (triol) with a trialkylorthoester (9, 15) when acid-sensitive functionalities are not present.
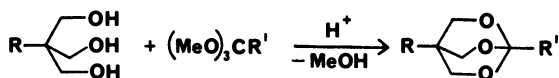
The triol undergoes direct esterification with carboxylic acids but only when they have electronegative acyl substituents (16).
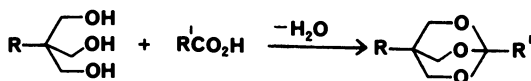
The most versatile method involves rearrangement of an acylated hydroxymethyloxetane catalyzed by boron trifluoride etherate (13, 17). The triols are readily converted to the required hydroxy-methyloxetanes via pyrolysis of the carbonate esters (18) and then to the acylated hydroxymethyloxetanes with acid chlorides.
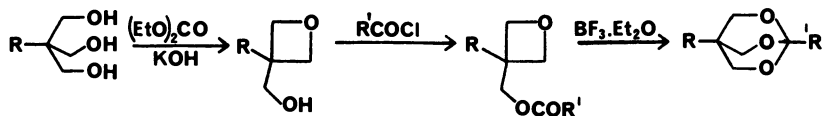
The radioligand [3H]t-butylbicycloorthobenzoate, [3H]TBOB, is prepared at high specific activity from the 4-Cl and 3,4-Cl$_2$ analogs of TBOB by reductive dechlorination with tritium gas (19, 20).
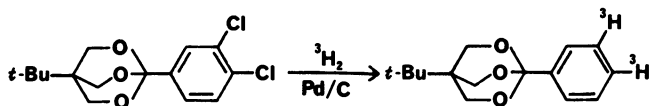
[^{35}S]TBPS, with similar properties to [^{3}H]TBOB as a radioligand, is synthesized from the corresponding phosphite by addition of ^{35}sulfur at high specific activity (<u>11</u>).
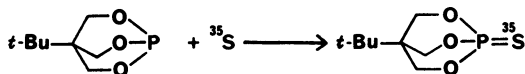
The abbreviations used for chemical substituents are as follows: Me-methyl, Et-ethyl, Pr-propyl, Bu-butyl, Pen-pentyl, Hex-hexyl, Hept-heptyl, Ph-phenyl, <u>n</u>-normal, <u>i</u>-iso, <u>sec</u>-secondary, <u>t</u>-tertiary, and <u>c</u>-cyclo.

Insecticidal Activity

Although bicycloorthocarboxylates were first prepared more than 30 years ago (<u>15</u>, <u>21</u>), they were only recently recognized as insecticides (<u>13</u>). The following discussion considers the effects of the 1- and 4-substituents on insecticidal activity based on houseflies treated topically with PB (250 μg/g) and then with the candidate insecticide (<u>13</u>). Suitable 4-substituents appear to be <u>n</u>-Pr, <u>i</u>-Pr, <u>n</u>-Bu, <u>sec</u>-Bu, <u>t</u>-Bu, <u>c</u>-Pen, <u>c</u>-Hex and Ph with <u>t</u>-Bu and <u>c</u>-Hex at or near the optimum (Table I). Orthobenzoates are most active with the 3-Cl, 4-halo, 3,4-Cl$_2$, 4-CN and 4-NO$_2$ substituents (Table II). Suitable non-aromatic 1-substituents for high activity are <u>c</u>-Hex and <u>c</u>-Hept; two of the most effective compounds combine the 4-<u>c</u>-Hex substituent with 1-<u>c</u>-Hex or 1-<u>c</u>-Hept (Table III). Additional 1-substituents conferring moderate activity are 2-bicyclo[2.2.1]heptyl, cyclohex-3-enyl, 4-azidophenyl and penta-fluorophenyl (Tables II and III). A few orthocarboxylates show high housefly toxicity without PB, <u>e</u>.<u>g</u>., the bicycloorthobenzoates with the 4-<u>t</u>-Bu group and 4-Br, 4-CN or 3,4-Cl$_2$ aromatic substituents and with the 4-<u>c</u>-Hex group and the 4-Br aromatic substituent or the 1-<u>c</u>-Hex substituent (Tables II and III). Some of the orthocarboxylates are almost equitoxic to houseflies when applied topically or injected, indicating that they are able to penetrate the insect integument very effectively (<u>13</u>). A few of the compounds exhibit high toxicity in combination with PB to American cockroaches (Table I) although toxicity to cockroaches does not always parallel that to houseflies (<u>13</u>). The bicycloorthocarboxylates exhibit a positive temperature coefficient, <u>i</u>.<u>e</u>. representative compounds are 3-6 fold more toxic at 25°C than at 11°C (Table IV). At the higher temperature in these tests they compare favorably in insecticidal activity with dieldrin, parathion, DDT and bioallethrin.

Toxicity to Mammals and Selective Toxicity

The bicycloorthocarboxylates and bicyclophosphorus esters share the same structural requirements for the 4-substituent based on their mouse toxicity, <u>i</u>.<u>e</u>. <u>t</u>-Bu > <u>i</u>-Pr > <u>n</u>-Pr > Et (Table V). Interestingly, the bicycloorthocarboxylates exhibit a discontinuous structure/activity relationship for substituents in the 1-position

Table I. Effect of 4-Substituent on the Topical Toxicity to Houseflies and American Cockroaches of 1-(4-Chlorophenyl)-bicycloorthocarboxylates

| | LD_{50}, µg/g | | |
| | Housefly | | Cockroach |
4-Substit.[a]	with PB	alone	with PB
Et	105	(>500)	-
n-Pr	2.5	(23)	2
i-Pr	8.3	(140)	-
n-Bu	3.5	(17)	3
sec-Bu	2.7	(58)	-
t-Bu	1.5	(10)	1
c-Pen	2.0	(21)	2
c-Hex	0.53	(10)	1
Ph	2.5	(41)	7

[a]4-Substituents that do not confer insecticidal activity alone or with PB, i.e. LD_{50} >500 µg/g, are 4-Me-Ph and NO_2.
SOURCE: Data are from ref. 13.

Table II. Effect of Substitution on 1-Phenyl Group on the Topical Toxicity to Houseflies of 4-Alkyl-bicycloortho-carboxylates

| | LD_{50}, µg/g, with PB (and alone) | | | |
1-Phenyl substit.[a]	4-t-Bu		4-c-Hex	
H	23	(>500)	13	(>500)
2-F	30	(>500)	-	-
2-Cl	105	(>500)	-	-
3-Cl	6.3	(375)	-	-
4-F	5.5	(>500	1.9	(>500)
4-Cl	1.5	(10)	0.53	(10)
4-Br	0.83	(3.5)	0.25	(6.5)
4-CF_3	53	(>500)	-	-
4-NO_2	5.0	(>500)	-	-
4-CN	0.23	(4.8)	0.65	(115)
4-N_3	13	(160)	-	-
3,4-Cl_2	0.88	(4.3)	2.5	(30)
F_5	18	(240)	-	-

[a]1-Phenyl substituents in the 4-n-Pr or 4-i-Pr series that do not confer appreciable insecticidal activity alone or with PB are: 4-Me, 4-MeO, 3,4-OCH_2O, 3-PhO, 4-MeS, 4-$MeSO_2$ and 3-NO_2-4-Cl.
SOURCE: Data are from ref. 13.

Table III. Effect of 1-Substituent on the Topical Toxicity to Houseflies of 4-Isopropyl-, 4-$\underline{t}$-Butyl- and 4-Cyclohexyl-bicyclo-orthocarboxylates

1-Substit.[a]	LD$_{50}$, µg/g, with PB (and alone)					
	4-$\underline{i}$-Pr		4-$\underline{t}$-Bu		4-$\underline{c}$-Hex	
$\underline{n}$-Pr	–	–	425	(>500)	–	–
$\underline{n}$-Bu	–	–	55	(450)	–	–
$\underline{n}$-Pen	–	–	33	(365)	–	–
$\underline{n}$-Hex	160	(>500)	–	–	–	–
$\underline{c}$-Hex	14	(>500)	3.5	(165)	0.63	(8.5)
$\underline{c}$-Hept	8.5	(300)	2.0	(44)	2.0	(13)
Ethynyl	325	(>500)	90	(175)	–	–
Benzyl	–	–	210	(>500)	–	–

[a]1-Substituents in the 4-$\underline{i}$-Pr or 4-$\underline{t}$-Bu series that do not confer insecticidal activity alone or with PB, $\underline{i}$.$\underline{e}$. LD$_{50}$ ≥500 µg/g, are H, Me, Et, $\underline{i}$-Pr, $\underline{sec}$-Bu, $\underline{neo}$-Pen, $\underline{c}$-Pr, $\underline{c}$-Bu, $\underline{c}$-Pen, vinyl, 1-BrEt and 1,2-Br$_2$Et. Other active compounds (LD$_{50}$ alone and with PB) in the 4-$\underline{n}$-Pr series are 1-(2-bicyclo[2.2.1]heptyl) 10 (160) and 1-(cyclohex-3-enyl) 19 (110).
SOURCE: Data are from ref. 13.

Table IV. Effect of Temperature on the Topical Toxicity to Houseflies of Three 1,4-Disubstituted-bicycloorthocarboxylates and Four Insecticides of Other Types

Insecticide	LD$_{50}$, µg/g, with PB (and alone)		
	25°C		11°C
Compounds with Positive Temperature Coefficients			
$\underline{c}$-Hex-C(CH$_2$O)$_3$C-Ph-Br-4	0.25	(6.5)	1.3
$\underline{t}$-Bu-C(CH$_2$O)$_3$C-Ph-CN-4	0.23	(4.8)	1.3
$\underline{t}$-Bu-C(CH$_2$O)$_3$C-$\underline{c}$-Hex	3.5	(165)	9.5
dieldrin	0.83	(0.65)	14
parathion	0.43	(1.3)	1.0
Compounds with Negative Temperature Coefficients			
DDT	12	(14)	4.8
S-bioallethrin	0.32	(14)	0.04

SOURCE: Data are from ref. 13.

with optimal toxicity conferred by small (H and Me) or large (Ph, n-Pr and n-Bu) groups rather than substituents of intermediate size (i.e., Et and i-Pr) (Table V). This finding was rationalized as indicating that there are two similar but distinct receptors, one with a conformation to accept small substituents and the other large substituents (14). A more extensive study (22) established the high mammalian toxicity of 4-t-butylbicycloorthocarboxylates with selected 1-substituents, e.g. [mouse intraperitoneal (ip) LD_{50}, mg/kg], 1-ethynyl (0.43), 1-(2- or 4-F-Ph) and 1-(3,4-Cl_2-Ph) (0.7-0.9) and particularly the 1-(4-CN-Ph) analog (0.06). The mouse ip LD_{50} is also below 1 mg/kg for the 4-c-Hex-1-(4-CN-Ph) analog.

4-t-Bu-bicyclophosphate is highly toxic to mice but essentially inactive to houseflies (Table VI). Some bicycloorthocarboxylates are selectively toxic to mice and others to PB-treated houseflies. The 4-c-Hex substituent is favorable relative to mammalian toxicity.

Metabolism

Bicycloorthocarboxylates are detoxified in houseflies and cockroaches primarily by oxidative processes judged by the high degree of synergism by PB (13). Dependent on structure, there is enormous variation in the synergistic ratio on topical treatment of houseflies, i.e. 2-260 fold. In mice, only a few bicycloortho-carboxylates show a PB synergism factor of >2, the greatest ratio being 10 for the 4-c-Hex-1-(4-Cl-Ph) analog (22). A coupled [^{35}S]TBPS binding site-microsomal oxidase assay showed distinct detoxification of this compound and its 4-bromophenyl analog but not of the 4-t-Bu-1-(4-Cl-Ph) compound (Table VII). TBOB is metabolized in microsomal oxidase systems of mouse liver and houseflies by hydroxylation at an O-methylene substituent and probably also at a methyl group and the phenyl moiety (20).

Mode of Action

The convulsions and poisoning signs of the bicycloortho-carboxylates on ip administration to mice are similar to those of PTX and the bicyclophosphorus esters (9), and in each case the toxicity is decreased on pretreatment with phenobarbital and diazepam (Table VIII) (22, 23). A representative bicycloortho-carboxylate antagonizes GABA-mediated relaxation of a functional insect nerve-muscle synapse in a manner similar to that caused by PTX (Figure 1). Inhibitory potencies of various bicycloortho-carboxylates and bicyclophosphorus esters for GABA-stimulated ^{36}chloride uptake in membrane vesicles from rat cerebral cortex are consistent with those obtained for [^{35}S]TBPS binding in human brain preparations (24). These findings indicate that the bicyclo-orthocarboxylates are GABA antagonists through blockade of the chloride ionophore.

TBOB is a potent GABA$_A$ receptor complex antagonist (22) and [^{3}H]TBOB is a sensitive probe for a chloride ionophore-associated binding site within the GABA receptor-ionophore complex (19). GABA and muscimol are allosteric inhibitors of [^{3}H]TBOB binding to rat brain

Table V. Effects of 1- and 4-Substituents on the Intraperitoneal Toxicity to Mice of Bicycloorthocarboxylates

1-Substit.	LD_{50}, mg/kg[a]			
	4-Et	4-n-Pr	4-i-Pr	4-t-Bu
H	95	23	12	6.5
Me	>500	85	42	6.0
Et	500	>500	>500	80
i-Pr	350	>500	>500	>500
n-Pr	420	29	29	12
n-Bu	22	11	7.3	2.5
Ph	9.2	7.5	5.8	1.3

[a]LD_{50} values (mg/kg) for the analogous bicyclophosphite, bicyclophosphate, and bicyclophosphorothionate are: in the 4-Et series 1.0-1.1; in the 4-n-Pr series 0.39-0.79; in the 4-i-Pr series 0.18-0.26; in the 4-t-Bu series 0.036-0.053.

SOURCES: Data are from refs. 9 and 14.

Table VI. Effects of 1- and 4-Substituents on the Selective Toxicity of Bicycloorthocarboxylates

Substituents[a]		LD_{50}, mg/kg		
R_4	R_1	Housefly topical with PB	Mouse ip	LD_{50} ratio, mouse/housefly
t-Bu	Me	>500	6	<0.01
t-Bu	Ph	23	1.3	0.06
t-Bu	Ph-CN-4	0.23	0.06	0.26
c-Hex	c-Hex	0.63	42	67
c-Hex	Ph-Cl-4	0.53	52	98

[a]Analogous LD_{50} data (mg/kg) for the 4-t-Bu-bicyclophosphate are >500 for housefly and 0.036 for mouse giving an LD_{50} ratio of <0.00007.

SOURCES: Data are from refs. 13 and 22.

Table VII. Effect of Mouse Microsomal Oxidases on Potency of
Three Bicycloorthocarboxylates as Inhibitors of Specific [^{35}S]TBPS
Binding to Human Brain Membranes

		Inhibition, %[a]	
Compound	nM	Control	Oxidase
t-Bu-C(CH$_2$O)$_3$C-Ph-Cl-4	7	29	34
c-Hex-C(CH$_2$O)$_3$C-Ph-Cl-4	13	50	11
c-Hex-C(CH$_2$O)$_3$C-Ph-Br-4	19	41	7

[a]The human brain receptor was incubated with mouse liver
microsomes alone (control) or with NADPH (oxidase) as detailed in
Ref. 22.
SOURCE: Data are from ref. 22.

Table VIII. Protective Effects of Phenobarbital and Diazepam on
the Toxicity of Three Bicycloorthocarboxylates to Mice

		Protective factor[a]	
Compound	LD$_{50}$ mg/kg	Phenobarbital	Diazepam
t-Bu-C(CH$_2$O)$_3$C-Ph-Br-4	1.2	1.9	1.8
c-Hex-C(CH$_2$O)$_3$C-Ph-Br-4	4.8	9.0	5.8
t-Bu-C(CH$_2$O)$_3$C-C≡CH	0.43	3.4	3.1

[a]Increase in LD$_{50}$ on pretreatment with sodium phenobarbital
at 100 mg/kg or diazepam at 10 mg/kg administered ip 5 and 15
min, respectively, before the convulsant.
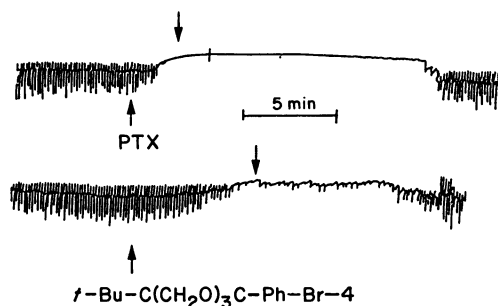SOURCE: Data are from ref. 22.

Figure 1. Similar actions of PTX and a bicycloorthocarboxylate
at 5 μM (↑) as antagonists of GABA-mediated relaxation events
in the cockroach coxal depressor muscle. Transient relaxation
events in the muscle, shown as downward deflections, are caused
by brief trains of 8-Hz stimuli applied every 10 s to GABA-ergic
inhibitory motorneurons. A saline wash (↓) permits recovery.
Data are from ref. 22.

P_2 membranes and a variety of cage convulsants competitively displace this radioligand (Table IX). The GABA-regulated chloride ionophore binding sites for [^{3}H]TBOB are localized in the frontal cortex, islands of Calleja, ventral pallidum and lateral amygdaloid nucleus based on *in vitro* quantitative autoradiography of rat brain sections (25) (Figure 2) and in the cerebral cortex, cerebellum and dorsal hippocampus based on filtration assays of washed P_2 membranes (19). [^{3}H]TBOB acts at the same binding site as [^{35}S]TBPS in rat and human brain tissues (19, 22). This indicates that bicycloortho-esters with small and large substituents in the l-position probably act at the same binding site (19) rather than two distinct binding sites suggested from the discontinuous structure/activity relation-ships (14).

The potency of the bicycloorthocarboxylates in decreasing [^{3}H]TBOB and [^{35}S]TBPS binding is strongly influenced by the nature of the substituents at both the 1 and 4 positions (19, 22). The optimal 4-substituents are C_4 branched chain alkyl or C_5 or C_6 cycloalkyl groups, e.g. t-Bu, sec-Bu and c-Hex. Apparently the size and steric features of these group(s) confer appropriate hydrophobic inter-action of the 4-substituent at one region of the binding site. The l-substituent is optimally a phenyl moiety with a cyano or one or more halogen group(s). Other substituted phenyls and alkyl and cycloalkyl groups in the 1 position are less effective. The potency of TBOB, which is similar to that of TBPO and TBPS, is increased 5-10 fold on introducing 4-CN, 4-Br, 4-Cl, 3,4-Cl_2 and F_5 substituents in the phenyl group. These bicycloorthocarboxylates are the most potent known GABA$_A$ receptor antagonists based on their inhibitory activity in the TBPS binding assay.

Relationships between potency at the TBPS binding site and toxicity indicate that the bicycloorthobenzoates with varying substituents in the 4 position and on the aryl groups may act somewhat differently than the bicyclophosphates with varying 4-substituents, giving different lines with moderate (r=0.62) and high (r=0.89) correlation coefficients, respectively (Figure 3). Most 1,4-dialkylbicyclo-orthocarboxylates fall between the lines for the bicycloortho-benzoates and the bicyclophosphorus esters, with a much higher variation (r=0.33), suggesting that they overlap the other two groups possibly due in part to more variable target site inter-actions. In addition although TBOB and TBPS bind at the same hydrophobic site of the chloride channel there are two important differences in their interactions with this site, i.e. [^{35}S]TBPS binding is dependent on temperature and the modulation of its binding by GABA is dependent on salt concentration, whereas [^{3}H]TBOB binding is independent of both temperature and the effect of varying salt concentration on GABA modulation (19). These findings suggest that there are substituent-dependent differences in the degree to which inhibition of [^{35}S]TBPS binding is related to a block in GABA-mediated neurotransmission.

Conclusion

There is a continuing need for new insecticides acting by

Table IX. Potencies of Cage Convulsants, Muscimol and GABA as Inhibitors of [^{3}H]TBOB binding to rat brain membranes

Compound	IC$_{50}$, nM
t-Bu-C(CH$_2$O)$_3$C-PhCl-4	20
t-Bu-C(CH$_2$O)$_3$P=S (TBPS)	39
12-Ketoendrin	52
t-Bu-C(CH$_2$O)$_3$C-Ph (TBOB)	61
t-Bu-C(CH$_2$O)$_3$C-Bu-n	89
Picrotoxin	843
Muscimol	3560
GABA	12200

SOURCE: Data are from ref. 19.
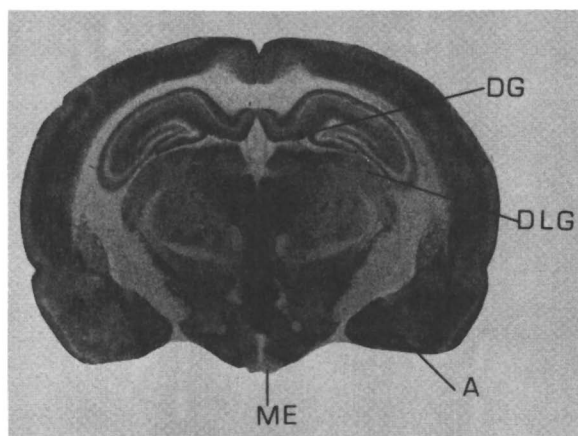
Figure 2. Autoradiographic localization of [^{3}H]TBOB binding sites in rat brain. Autoradiographic grains appear black against a white background. Autoradiogram was generated as described in Ref. 25. Abbreviations: DG - dentate gyrus, DLG - dorsolateral geniculate, A - amygdala, ME - median eminence
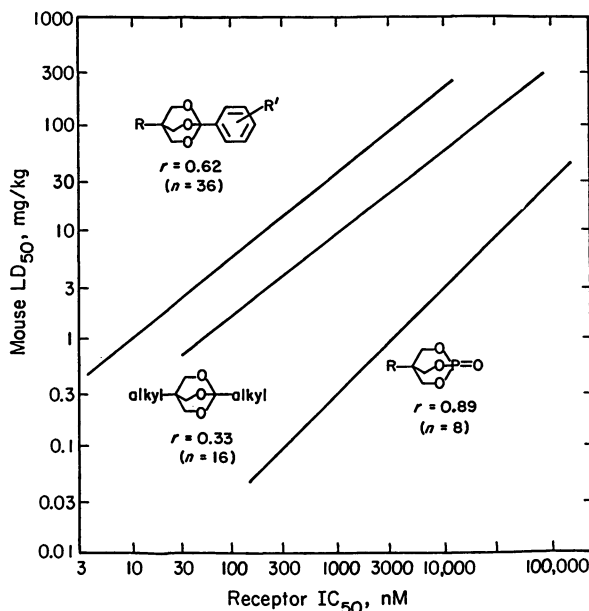
Figure 3. Relation of potency in inhibiting [^{35}S]TBPS binding to human brain membranes and ip toxicity to mice of three types of trioxabicyclooctanes with correlation coefficients and numbers of examples for each type of compound. Data are from. ref. 22.

mechanisms different than those of the major commercial compounds, which are cholinesterase inhibitors or disrupt sodium channels. Optimization of a new class of insecticides may be more easily achieved with compounds having a known mode of action for which there is a rapid and convenient *in vitro* enzyme or receptor inhibition assay. Bicycloorthocarboxylates act at the GABA-regulated chloride ionophore as GABA$_A$ receptor antagonists. They have the potential for exceptional potency at this receptor in insect and mammalian systems. The first steps have been taken in optimizing the structure of the bicycloorthocarboxylates for increasing insecticidal activity and reducing mammalian toxicity. The 1,4-disubstituted-bicycloorthocarboxylates include the most potent known GABA$_A$ receptor antagonists and some of the most insecticidal compounds. They are ideal probes in establishing selectivity mechanisms between the insect and mammalian GABAergic systems.

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Chapter 6

Bicyclic Phosphorus Esters

Insecticidal Properties and Binding Sites in the Housefly

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2,6,7-Trioxa-1-phosphabicyclo[2.2.2]octane GABA antagonists [$RC(CH_2O)_3X$; X = P, P=O, P=S] with appropriate substituents exerted toxic actions on houseflies even by topical application when synergized with piperonyl butoxide. Structure-insecticidal activity relationship studies suggested the presence of a critical binding site for the bicyclic phosphorus esters (BPEs) in the nervous system. High-affinity binding site(s) for BPEs in the fly head were demonstrated and characterized by radioreceptor assays using a tritiated 4-n-propyl oxon analog (R = n-C_3H_7, X = P=O) as a ligand. The binding properties of the site differed in many respects from those already characterized with the ^{35}S-labeled 4-t-butyl thiono analog (R = t-C_4H_9, X = P=S) mainly in rat brain membranes. The binding site could not be related to the GABA-mediated neurotransmission system in the housefly.

Bicyclic phosphorus esters (BPEs) with relevant substituents, the general structure of which is shown in Figure 1, are highly toxic to mammals (1). This group of compounds is different from usual organophosphorus insecticides and is unique in that they interfere with the neurophysiological action of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) in the mammalian central nervous system (2). The bicyclic phosphorus esters and a toxicant of plant origin, picrotoxinin, probably share a site of action, which is related to chloride ion channels in synapses mediated by GABA (3,4). A considerable amount of data that characterize this site in the rat brain have accumulated through ligand-receptor binding studies using two ligands, [^3H]α-dihydropicrotoxinin ([^3H]DHP)(5) and ^{35}S-labeled 4-t-butyl bicyclic phosphorothionate ([^{35}S]TBPS)(4), in addition to our initial attempt with [^3H]4-n-propyl bicyclic phosphate ([^3H]Pr-BP, Figure 1)(6).

Bicyclic phosphorus esters are also toxic to invertebrates although invertebrates are less sensitive than vertebrates (7,8). With invertebrates they have been shown to possess GABA antagonistic

action (9,10). However, there are not sufficient data to character-
ize the invertebrate site of action, especially at the molecular
level. Thus we examined the insecticidal activity of BPEs, mainly
bicyclic phosphates (BPs), against the housefly, *Musca domestica* L.
and characterized their binding site(s) in the housefly using [^{3}H]-
Pr-BP.

Insecticidal Activity against Houseflies

Table I lists the LD$_{50}$ values of selected BPEs determined by four
different methods for the housefly. The value for the well-known
GABA antagonist picrotoxinin is also included for comparison. Bicy-
clic phosphates were not very insecticidal as judged from their topi-
cal 24-h LD$_{50}$ values without any synergists. However, the esters ex-
erted greater toxic action by injection or pretreatment with the mix-
ed-function oxidase inhibitor piperonyl butoxide (pb). The poisoning
symptoms were convulsions and paralysis, both of which are typical of
neurotoxins. 4-*t*-Butyl BP (compound 1) was outstandingly toxic to
mammals (mouse ip LD$_{50}$, 0.053 mg/kg)(11) but not so active against
the housefly (8). There was no essential difference in insecticidal
activity between Pr-BP (compound 2) and 4-*t*-butyl BP by injection.
When judged by the ratio of mouse ip LD50/housefly-injection LD$_{50}$
(*rs*), Pr-BP (*rs* = 2.2) appears to have a higher specificity in action
on houseflies than 4-*t*-butyl BP (*rs* = 0.43). This might indicate
that the former compound has a high affinity for a target site in
houseflies and a low affinity for that in mice. In this respect,
Pr-BP may be a suitable analog for characterization of a housefly-
specific site. Introduction of a methyl group into the 3 position of
Pr-BP resulted in an increase in insecticidal activity (*i.e.*, com-
pound 3) while it had the opposite effect on mammals. As for the 4-
n-propyl phosphorothionate (compound 5) and phosphite (compound 6)
analogs, a topical application method was effective even without pb.
The high activity of the 4-*n*-propyl thionate ester has been reported
to be related to its low internal lethal level, compared with the
phosphate (12).

Table I. Insecticidal Activity of Bicyclic Phosphorus Esters
against Houseflies

				LD$_{50}$ (μg/g)[a]		Synergistic ratio[a]
No.	R^1	R^2	X	Alone	With pb	
				Injection		
1	*t*-C$_4$H$_9$	H	P=O	35	6.2	5.6
2	*n*-C$_3$H$_7$	H	P=O	22	8.5	2.6
3	*n*-C$_3$H$_7$	CH$_3$	P=O	NT[b]	5.1	–
4	Picrotoxinin			NT	5.3	–
				Topical application		
2	*n*-C$_3$H$_7$	H	P=O	3300	11	300
5	*n*-C$_3$H$_7$	H	P=S	14	3.1	4.5
6	*n*-C$_3$H$_7$	H	P	23	11	2.1

a) Calculated using data from Ref. 12.
b) Not tested.

We have determined LD_{50} values of 28 BP analogs for the housefly by injection after pretreatment with pb. The most toxic compound was 3-methyl-4-*n*-propyl BP (compound 3). Their toxicity was greatly dependent on the nature of the bridgehead substituent. The following equation has been derived by multiple regression analysis of some of those synergized toxicity data for a series of 4-substituted BPs (8):

$$\log(1/LD_{50}) = 5.193(\pm 0.759)\pi - 1.356(\pm 0.207)\pi^2 - 0.316(\pm 0.141)E_s^c$$
$$+ 1.711(\pm 0.370)\sigma^* + 4.049$$
$$n = 12, \ r = 0.992, \ s = 0.108, \ F = 112.86$$

In this equation, π is the hydrophobic parameter, E_s^c the Hancock steric parameter, and σ^* the electronic constant for the bridgehead substituent. Median lethal dose is expressed as mol/fly. The bicyclic phosphates have been shown to be mainly degraded by microsomal mixed-function oxidases in the housefly (12). Thus, the toxicity determined by injection after pb pretreatment is regarded as a good measure of the intrinsic toxicity of BPs, and it is probable that this equation reflects significant interactions between the bridgehead substituent of BPs and its site of action, *i.e.*, a type of receptor. Little is known about the hypothetical BP receptor in the insect while a considerable amount of information has been obtained on a mammalian site. Three-dimensional receptor models have been proposed based on structure-activity relationships for BPs and related compounds (13,14). It is now important to substantiate the existence of the putative receptor in insects for the elucidation of the detailed mode of insecticidal action of BPEs.

Internal Distribution of [3H]Pr-BP Applied on Houseflies

First, distribution of [3H]Pr-BP in the fly body was examined when topically applied after pretreatment with pb (Figure 2). Since metabolism of Pr-BP is inhibited by the synergist to a great extent, most of radioactivity can be regarded as that of Pr-BP, especially at an early stage of intoxication. The internal Pr-BP level increased in the thorax and abdomen for 5 h. The head contained about 8% of the total amount of internal Pr-BP at 5 h. It is impossible to determine the site of action from these data but the brain cannot be excluded as a site of action.

Binding of [3H]Pr-BP to the P2 Fraction from Housefly Heads

Binding Characteristics. For ligand-receptor binding assays, we used an EDTA/Tris-citrate (1 mM, pH 7.4)-dialyzed mitochondrial fraction of housefly heads, the so-called P2 fraction, which was prepared by a modification of Squires's method (4). The final pellet was frozen at -25°C and then suspended in 50 mM Tris-citrate buffer (pH 7.4 at 3°C) for assays. 4-*n*-Propyl[2,3-3H]-2,6,7-trioxa-1-phosphabicyclo[2.2.2]-octane 1-oxide ([3H]Pr-BP, 41 Ci/mmol) was used as a ligand for the reason described above. Radioreceptor assays were performed by filtration techniques with glass fiber filters. Specific [3H]Pr-BP binding was taken as that displaced by excess unlabeled Pr-BP(10 μM). Specific [3H]Pr-BP binding was linear up to 1.0 mg of membrane protein per 1 ml. Specific binding was 89% of total binding under
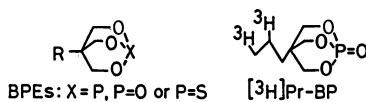
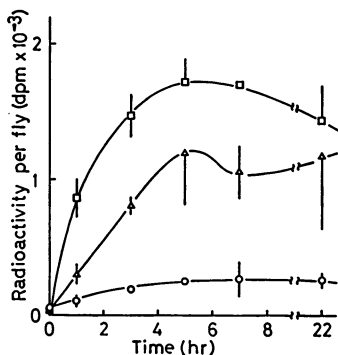Figure 1. Structures of bicyclic phosphorus esters and [3H]-propyl bicyclic phosphate.

Figure 2. Distribution of radioactivity in the fly body. An acetone solution containing [3H]Pr-BP (*ca.* 0.33 μg = *ca.* 11,000 dpm; sp. act., 2.83 mCi/mmol) was topically applied on the dorsal portion of the thorax of 3 to 5-day-old female houseflies 1 h after pretreatment with 10 μg of piperonyl butoxide. The flies were held at 25°C, rinsed twice with acetone, ground in a mortar containing aqueous acetone, and centrifuged at 5,000 x g for 5 min. The supernatant was analyzed for radioactivity in the head (O), thorax (□), and abdomen (Δ). Each point represents the mean ± SD of three experiments.

standard conditions: [³H]Pr-BP, 1.7 nM; protein, 1 mg; volume, 1 ml; reaction for 1 h in 50 mM Tris-citrate buffer (pH 7.4) on ice. Figure 3 shows the temperature dependence of specific binding. Specific Pr-BP binding showed a marked negative temperature coefficient which was also observed in the insecticidal activity of Pr-BP. The optimal temperature was 0°C, in contrast to about 20°C for TBPS binding to housefly thorax/abdomen membranes as well as BPE binding to the rat brain (4,6,15). The specific binding capacity of housefly head membranes was stable on heating at 30°C for 1 h but it fell at higher temperature. When kept frozen at -25°C for three months, 54% of the initial capacity was retained. This stability against freezing was also in contrast to a recent observation on a TBPS binding site in the housefly thorax and abdomen whose binding capacity was lost by storage overnight at -20°C (15,16). The interaction between Pr-BP and its specific binding site had a broad pH optimum (pH 5-9), suggesting the lack of significant electronic interaction between Pr-BP and its binding site. The binding activity-pH curve gradually declined at basic and acidic pH.

Under standard conditions, specific binding reached about 80% of its maximal value after 30 min of incubation (Figure 4A). A further 20% increase in binding was observed from that time on. It is likely that Pr-BP does not bind to a single population of sites. Half of the specifically bound Pr-BP dissociated within 3 min after addition of excess unlabeled Pr-BP (Figure 4B). Dissociation was found to proceed in a biphasic manner from pseudo-first-order plots.

Figure 5A shows plots of total, specific and nonspecific binding of [³H]Pr-BP *versus* the concentration of [³H]Pr-BP added in the incubation medium. Specific binding was saturable and nonspecific binding increased linearly. Half maximal binding was obtained with about 4 nM [³H]Pr-BP. The affinity of Pr-BP for the housefly-specific sites is much higher than that of TBPS (K_d = 0.17 µM (15,16)) as well as that of Pr-BP to rat brain synaptic sites (K_d = 30 µM (6)). The apparent number of binding sites at saturation was estimated to be approximately 30 fmol/mg protein. The Scatchard analysis of these data did not give a simple straight line, indicating the presence of two or more populations of binding sites (Figure 5B). The slope of the Hill plot was, however, linear with a coefficient of 0.94, suggesting neither positive nor negative cooperativity among the binding sites.

Effect of Chemicals on Specific Binding. The effects of inorganic salts on specific Pr-BP binding were examined, since binding of BPEs to rat brain membranes required the presence of certain anions such as bromide and chloride in the incubation medium (4,6). Sodium chloride had only a slight inhibitory effect on the specific binding and sodium thiocyanate was the most inhibitory salt (Table II). On the other hand, potassium fluoride and sodium sulfate enhanced specific Pr-BP binding. The bicyclic phosphorus esters are thought to block Cl⁻ channels either directly or allosterically (17). In this connection, it is interesting to note that the effects of inorganic salts are related to the ability of the anions to pass through Cl⁻ channels (18). Permeable anions (Br⁻, Cl⁻ and SCN⁻) had inhibitory effects and impermeable anions (F⁻ and SO_4^{2-}) had the opposite effect. It remains to be elucidated whether the present Pr-BP binding site is indeed associated with Cl⁻ channels, because the decrease in binding
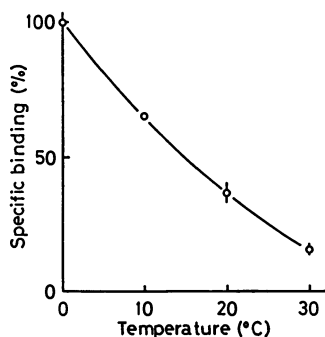
Figure 3. Effect of incubation temperature on specific binding of [3H]Pr-BP. Membranes (1 mg protein) were incubated with 1.7 nM [3H]Pr-BP in 1 ml of 50 mM Tris-citrate buffer (pH 7.4 at 3°C) for 1 h at various temperatures. After termination of the reaction, filters were rinsed with the buffer maintained at the incubation temperature. Each point represents the mean ± SD of three experiments. Reproduced with permission from Ref. 39. Copyright 1986, Academic Press Inc.
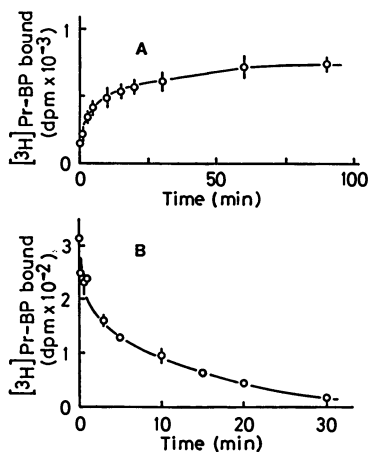

Figure 4. Association of [3H]Pr-BP with specific sites in housefly head membranes and dissociation of specifically bound [3H]Pr-BP. For association (A), incubation of membranes (1 mg protein) with 1.7 nM [3H]Pr-BP in 1 ml of 50 mM Tris-citrate buffer (pH 7.4) at 0°C was terminated by filtration at various time intervals. For dissociation (B), membranes (1 mg protein/ tube) were incubated with 1.5 nM [3H]Pr-BP in 50 mM Tris-citrate buffer (pH 7.4) for 1 h at 0°C, diluted 1.1-fold with the buffer containing excess unlabeled Pr-BP, and the mixture was incubated for the indicated time at 0°C prior to filtration. Reproduced with permission from Ref. 39. Copyright 1986, Academic Press Inc.
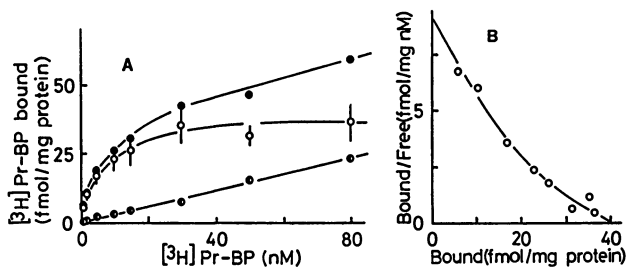
Figure 5. Binding of [3H]Pr-BP to housefly head membranes at increasing ligand concentrations. Membranes (1 mg protein) were incubated with various amounts of [3H]Pr-BP in 1 ml of 50 mM Tris-citrate buffer (pH 7.4) for 1 h at 0°C. Total (●), specific (○), and nonspecific (◑) binding are defined in the text. Panel B shows the Scatchard plot of the specific binding data. Reproduced with permission from Ref. 39. Copyright 1986, Academic Press Inc.

Table II. Effects of Salts on Specific Binding of [^{3}H]Pr–BP

Salt	Specific binding (dpm)[a]	% of control
None	529 ± 19	100
NaBr	*325 ± 46	61
KBr	*352 ± 6	66
NaCl	498 ± 14	94
NaSCN	*182 ± 33	34
KF	*697 ± 31	132
Na$_2$SO$_4$	*726 ± 31	137

a) Specific [^{3}H]Pr–BP binding was measured in 50 mM Tris-
    citrate buffer (pH 7.4) containing 200 mM salt under the
    standard conditions. *Significantly different from
    control at $P < 0.01$ (Student's t-test). The values are
    the means ± SD of three experiments.

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by Br$^-$ and SCN$^-$ may be due to their chaotropic action that causes
membrane disruption and solubilization of membrane protein (19).
       Table III compares the specific [^{3}H]Pr–BP displacing and insec-
ticidal activities of bridged bicyclic and related compounds. Six
BPEs with insecticidal activity inhibited [^{3}H]Pr–BP binding at IC$_{50}$
values in the nanomolar range. Compounds with LD$_{50}$ values below
about 10 µg/g (Ib, Ic, If and Ih) had IC$_{50}$ values of less than 40 nM
except the phosphite analog (Ig). The phosphite analog had a rela-
tively low binding inhibitory activity while it was insecticidal in
a range similar to other toxic phosphorus analogs. Phosphorus com-
pounds with LD$_{50}$ values in the range of 100–1000 µg/g (Ia and Id)
gave IC$_{50}$ values in the order of 100 nM. Propyl bicyclic phosphate
(Ib) was the most potent compound in displacing specifically bound
[^{3}H]Pr–BP, although its activity may be overestimated because in this
case the displacer is the same compound as the radioligand. The
binding inhibitory activity of methyl BP (Ia) appeared to be higher
than that expected from its LD$_{50}$ value. It is likely that Ia hardly
reaches the binding site in the fly body due to its relatively high
hydrophilicity although its intrinsic activity is high. Amino BP(Ie)
was almost nontoxic and had very low binding inhibitory activity.
       Bicyclic orthocarboxylates are compounds of a group with a
structure analogous to BPEs. The structural difference lies in the
replacement of the phosphorus moiety of BPEs by a carbon moiety.
They have been shown to inhibit specific [^{35}S]TBPS binding to a rat
brain membrane site (4), while t-butyl orthobenzoate was recently
reported to be weakly active or inactive at a housefly TBPS site
(15). Orthocarboxylates Ii and Ij possessed insecticidal activity
comparable to that of BPEs (Table III, also cf. 20,21). Neverthe-
less, these compounds were inactive in displacing specifically bound
[^{3}H]Pr–BP at 10 µM. It is striking that the Pr–BP binding site rec-
ognizes the difference in the bridgehead moieties (1 and 4 positions)
so selectively. Neither a hydrolyzed product (II) of Pr–BP nor
fenitroxon (III), the oxon analog of the insecticide fenitrothion,
was active.
       It was well established that BPEs act as GABA antagonists (2,4,
9). The binding of [^{35}S]TBPS to rat brain membranes was inhibited by
GABA agonists and antagonists (4). Recently, GABA was reported to
enhance TBPS binding to membranes from the housefly thorax and

Table III. Inhibition (as IC$_{50}$) of Specific [^{3}H]Pr-BP Binding to Housefly Head Membranes by Organophosphorus Esters and Related Compounds and their Insecticidal Activity (as LD$_{50}$)

Compound	IC$_{50}$ (nM)[a]	LD$_{50}$ (µg/g)[b]
Ia	203.2 ± 19.1	1300[c]
Ib	6.1 ± 1.3	8.5[c]
Ic	34.6 ± 3.0	6.2[c]
Id	215.9 ± 17.5	100[c]
Ie	79500 ± 11900	>2000(18.2)[c],[d]
If	20.6 ± 0.5	5.1[c]
Ig	1005.0 ± 237.6	11[e]
Ih	38.7 ± 8.9	3.1[e]
Ii	>10000	1400[e]
Ij	>10000	36[e]
II	>10000	>2000(2)[c],[d]
III	>10000	–

a) Concentration causing 50% inhibition of specific [^{3}H]Pr-BP binding under the standard assay conditions. Most values are expressed as the means ± SD of 3-4 experiments. Taken from Ref. 39.

b) Median lethal dose against houseflies. Calculated using data from Ref. 39.

c) Determined (24 h) by injection into the dorsal portion of the thorax 1 h after topical treatment with piperonyl butoxide (10 µg/fly).

d) Mortalities at 40 µg/fly are given in parentheses.

e) Determined (24 h) by topical application on the dorsal portion of the thorax 1 h after treatment with piperonyl butoxide (10 µg/ fly).

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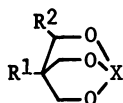
Ia: R^1 = CH$_3$, R^2 = H, X = P=O   If: R^1 = n-C$_3$H$_7$, R^2 = CH$_3$, X = P=O
Ib: R^1 = n-C$_3$H$_7$, R^2 = H, X = P=O   Ig: R^1 = n-C$_3$H$_7$, R^2 = H, X = P
Ic: R^1 = t-C$_4$H$_9$, R^2 = H, X = P=O   Ih: R^1 = n-C$_3$H$_7$, R^2 = H, X P=S
Id: R^1 = CH$_2$Br, R^2 = H, X = P=O   Ii: R^1 = n-C$_3$H$_7$, R^2 = H, X = CH
Ie: R^1 = NH$_2$, R^2 = H, X = P=O   Ij: R^1 = n-C$_3$H$_7$, R^2 = H, X = CC$_6$H$_5$
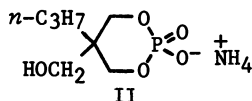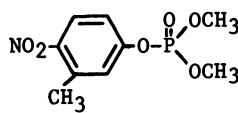
abdomen (15). These findings are believed to indicate coupling between the TBPS binding site and the GABA$_A$ receptor. Significantly, a mutual inhibitory relation was found to hold between the noncompetitive GABA antagonists picrotoxinin and BPEs in binding to specific sites, and there are several lines of evidence that they share a common site of action (3,4,22). In the present study, however, GABA, muscimol, and bicuculline methiodide, as well as picrotoxinin proved to have little effect on specific [^{3}H]Pr-BP binding even at relatively high concentrations. The lack of inhibitory activities of

muscimol and picrotoxinin was confirmed by using a similar membrane
preparation from the thorax and two other head membrane preparations
obtained by modifications of the methods of Abalis *et al.* (23) and
Kadous *et al.* (24) without freezing prior to the binding assays. The
addition of NaCl to the incubation medium and the preincubation of
head membranes with the two compounds were not effective in inhibit-
ing Pr-BP binding. These findings indicate that muscimol and
picrotoxinin do not compete for the present Pr-BP binding site.
        It has been clearly shown that cyclodiene-type insecticides act
on the TBPS site (15,16,25) and the cockroach picrotoxinin receptor
(22,24) which is presumed to be the site of action for BPEs, but
neither dieldrin nor γ-BHC at 10 μM affected Pr-BP binding. Other
GABA receptor-related and Cl⁻ channel-related compounds described
below, some of which have been reported as TBPS binding modulators
(4,26-31), were inactive in inhibiting [^{3}H]Pr-BP binding to the
housefly head membranes: deltamethrin (10 μM), allethrin (10 μM),
suriclone (0.1 μM), avermectin B$_1$ (1 μM), clonazepam (10 μM),
etomidate chloride (10 μM), pitrazepin (5 μM), phenobarbital sodium
(10 μM), Ro 5-4864 (10 μM), Ro 15-1788 (5 μM), RU 5135 (0.5 μM), and
DIDS (10 μM).

Binding of GABA Site Ligands

It is known that the mammalian GABA system is composed of at least
three postsynaptic components, *i.e.*, GABA receptors, benzodiazepine
recognition sites and chloride ion channels, which are functionally
coupled to each other (32). There are several reports on specific
binding of ligands to each site in insects (15,22-24,33-37), although
the existence of the same system as that of mammals is still contro-
versial. The present *in vitro* assay conditions might not be suitable
for the binding of other GABA site ligands so that the coupling of
the putative GABA receptor/ion channel/benzodiazepine site complex in
the housefly is prevented. It is necessary to take into account the
possibility that this resulted in the lack of effect of GABA site
ligands on [^{3}H]Pr-BP binding.
        In order to make this point clear, we attempted to detect spe-
cific binding of [^{3}H]DHP, [^{3}H]muscimol and [^{3}H]diazepam to EDTA-
treated membranes under the same conditions as that for [^{3}H]Pr-BP
binding. Binding should decrease on addition of excess unlabeled
ligands if a specific site existed. The binding of [^{3}H]muscimol was
reduced on addition of excess muscimol and GABA. Bicuculline
methiodide was less potent in displacing [^{3}H]muscimol (Table IV).
The saturation isotherm of specific binding of [^{3}H]muscimol to EDTA-
treated membranes is shown in Figure 6A. The Scatchard plots gave a
K_d value of about 12 nM and a B_{max} value of about 30 fmol/mg protein
(Figure 6B). The GABA receptor, at least, appears to be present in
EDTA-treated housefly head membranes although it is not fully charac-
terized in these studies. This finding suggests that BPEs act on
site(s) apart from the GABA system or that the binding site for BPEs
was decoupled from the GABA receptor during preparation of the P$_2$
fraction. Specific [^{3}H]DHP and [^{3}H]diazepam binding failed to occur.
Taken together with the lack of DHP inhibition of specific [^{3}H]Pr-BP
binding, picrotoxinin appears to act on other site(s) than the Pr-BP
site in the housefly. Sites for benzodiazepines as well as those for
picrotoxinin do not exist or are inactivated in the present housefly
head extracts.

Table IV. Effect of Unlabeled Ligands on Binding of
[³H]α-Dihydropicrotoxinin, [³H]Diazepam and [³H]Muscimol
to EDTA-Treated Membranes from Housefly Heads

Radioligand	Unlabeled ligand[d]	Reduction in binding on addition[f] of unlabeled ligand (% of total)
[³H]DHP[a]	DHP	0.4 ± 0.7 (n = 3)
	Pr-BP	-0.2 ± 1.5 (n = 3)
[³H]Diazepam[b]	Diazepam	0.7 ± 4.6 (n = 3)
[³H]Muscimol[c]	Muscimol	*43.7 ± 16.2 (n = 6)
	GABA	*33.0 ± 12.0 (n = 3)
	BM[e]	**4.8 ± 2.8 (n = 3)

a) α-Dihydropicrotoxinin is abbreviated as DHP. Membranes were al-
lowed to react with 1.8 nM or 8.0 nM [³H]DHP (sp. act., 59.5 Ci/
mmol) at 0°C for 30 min in the absence and the presence of unla-
beled ligands and then centrifuged at 15,000 x g for 30 min.
The pellet was analyzed for bound radioactivity. Other condi-
tions were the same as those for [³H]Pr-BP binding.
b) The concentration of [³H]diazepam (sp. act., 90.0 Ci/mmol) was
1.4 nM. Other conditions were the same as those for [³H]Pr-BP
binding.
c) The concentration of [³H]muscimol (sp. act., 12.2 Ci/mmol) was
9.8 nM. Other conditions were the same as those for [³H]Pr-BP
binding.
d) The concentrations of unlabeled ligands were 10 μM except for
DHP (100 μM).
e) (–)Bicuculline methiodide is abbreviated as BM.
f) Each value represents the mean ± SD of indicated number of ex-
periments. *Statistically significant reduction at $P < 0.01$
(Student's t-test). **Statistically significant reduction at $P
< 0.05$.

GABA Content and Density of Pr-BP Binding Sites

Finally, attempts were made to compare the distribution of specific
Pr-BP binding sites with the GABA content in several parts of the
housefly body (Table V). Both Pr-BP binding sites and GABA were most
abundant in the thorax (total mol). The abdomen which contained the
lowest level of GABA displayed the lowest Pr-BP binding capacity. Of
course this observation is necessary but not sufficient evidence for
an association of Pr-BP binding sites with the GABA receptor.

Concluding Remarks

In the present study, high-affinity binding site(s) for [³H]Pr-BP
were demonstrated in the housefly head extracts. Specific [³H]Pr-BP
binding was selectively inhibited by insecticidal BPEs but not other
GABA site ligands. The Pr-BP binding site was different from rat
brain-site(s) already characterized with [³H]Pr-BP or [³⁵S]TBPS in
many respects. There is both electrophysiological and biochemical
evidence that BPEs act as GABA antagonists. Especially, [³⁵S]TBPS
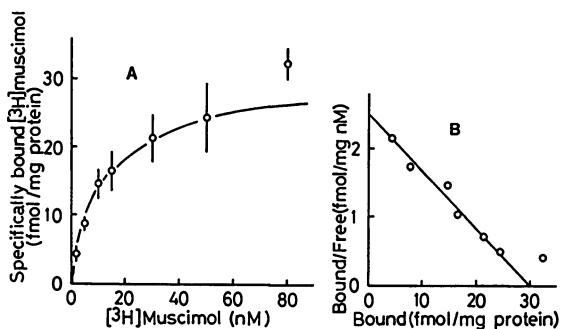has been established as a suitable probe for GABA$_A$ receptor-coupled

Figure 6. Specific binding of [^{3}H]muscimol to EDTA-treated membranes from housefly heads. Binding assays were performed in the same manner as [^{3}H]Pr-BP binding. Each point represents the mean ± SD of three experiments.

Table V. GABA Content and [^{3}H]Pr-BP Binding Activities
in Housefly Head, Thorax and Abdomen

| Tissue | GABA content[a] | | [^{3}H]Pr-BP binding activity[b] | | |
	Total nmol	nmol/ g wet tissue	fmol/ mg protein	Total fmol	fmol/ g wet tissue
Head	0.98 (±0.07)	757.4 (±64.1)	16.5 (±12.5)	0.6 (±0.4)	387.8 (±59.3)
Thorax	1.98 (±0.18)	268.9 (±30.9)	56.9 (±32.1)	3.7 (±2.0)	556.9 (±300.0)
Abdomen	0.59 (±0.10)	118.4 (±13.1)	4.2 (±1.7)	0.2 (±0.1)	44.6 (±8.9)

a) Assayed according to the method of Graham and Aprison (40).
b) Specifically bound [^{3}H]Pr-BP was determined by incubating mem-
branes with 1.7 nM [^{3}H]Pr-BP in 1 ml of 50 mM Tris-citrate
buffer (pH 7.4) for 1 h at 0°C. Data taken from Ref. 39.

chloride ion channels (38). However, the bicyclic phosphorus esters
may act on site(s) apart from the GABA-mediated neurotransmission
system as well in the housefly. This must be confirmed by more
binding assays under various conditions. In this respect, it is
interesting that TBPS has been recently reported to act on a site
independent of GABA receptors in *Torpedo* electric organs (26).

Abbreviations Used

GABA, γ-aminobutyric acid; BPE, bicyclic phosphorus ester; DHP,
α-dihydropicrotoxinin; TBPS, 4-*t*-butyl-2,6,7-trioxa-1-phosphabicyclo-
[2.2.2]octane 1-sulfide; Pr-BP, 4-*n*-propyl-2,6,7-trioxa-1-phospha-
bicyclo[2.2.2]octane 1-oxide; BP, bicyclic phosphate; pb, piperonyl
butoxide; EDTA, ethylenediaminetetraacetic acid disodium salt; BM,
bicuculline methiodide

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Chapter 7

Neurotoxic Insecticides as Antagonists of the GABA$_A$ Receptor Function

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Eight cyclodiene insecticides or insecticide metabolites
inhibited γ-aminobutyric acid (GABA)-dependent chloride
uptake by mouse brain synaptic vesicles with potencies
closely related to their acute toxicities. Lindane and
deltamethrin also inhibited GABA-stimulated chloride
flux, but low potency (lindane) and incomplete
stereospecificity (deltamethrin) suggest that the GABA
receptor-ionophore complex is not the principal site of
action for these compounds. Abamectin, which has been
shown to activate chloride channels, failed to stimulate
chloride uptake but was a very potent inhibitor of GABA-
dependent chloride channel activation. These studies
illustrate the value of this assay for defining the
effects of insecticides on GABA receptor function.

The γ-aminobutyric acid (GABA) receptor-chloride ionophore complex
mediates inhibitory synaptic neurotransmission in both vertebrate and
invertebrate nervous systems. This complex has been implicated as a
site of neurotoxic action for several groups of insecticides, includ-
ing cyclodienes and lindane (1-5), pyrethroids containing the α-
cyano-3-phenoxybenzyl moiety (6,7), the avermectins (8-11), and a new
series of bicyclic orthobenzoate esters related to the cage convul-
sant t-butylbicyclophosphorothionate (TBPS) (12,13). Most of the
evidence for insecticide effects on this target has been obtained
from physiological studies at peripheral synapses in invertebrates
(7,8,11) or from radioligand binding studies using subcellular frac-
tions of mammalian brain or insect ventral nerve cord homogenates (2-
6,9,10,13). Physiological methods are able to provide evidence for
insecticide-dependent modifications of GABA receptor-ionophore func-
tion, but application of these methods to identified GABAergic
synapses in central nervous system (CNS) preparations is technically
difficult. Radioligand displacement studies provide evidence for
binding interactions between insecticides and this target in the CNS,
but they are unable to demonstrate the consequences of binding in
terms of altered receptor function. Thus, additional methods to
define the consequences of insecticide-GABA receptor interactions in
the CNS are clearly needed.

Recently, Harris and Allan (14) described a method for studying the coupling of GABA receptor binding sites to their associated chloride ionophore by measuring GABA-stimulated $^{36}Cl^-$ uptake into synaptic vesicles prepared from mouse brain. Preliminary studies in this laboratory (15) and elsewhere (16) demonstrated the utility of this system to define the effects of insecticides on GABA receptor-ionophore function. This paper summarizes further use of these methods to define the effects of cyclodienes, lindane, pyrethroids, and avermectins at the GABA receptor-ionophore complex and to correlate in vitro effects with the neurotoxic actions of these compounds in vivo.

Methods

Chemicals. Abamectin (a mixture of 80% avermectin B_{1a} and 20% avermectin B_{1b}) was provided by Dr. J. Mollet, Merck Sharp & Dohme Research Laboratories, Three Bridges, NJ. The sources of other compounds used in these assays are given elsewhere (15,17).

Chloride flux assay. Synaptic vesicles were prepared from brains of male ICR mice (20-30 g; Blue Spruce Farms, Altamont, NY) (15). Assays of $^{36}Cl^-$ uptake involved preincubation (10-20 min) of vesicles with carrier solvent (ethanol or acetone, 0.5-1 µl) or insecticide, followed by incubation (4 sec) with $^{36}Cl^-$ with or without GABA or added insecticides and isolation of labelled vesicles by rapid vacuum filtration. Detailed descriptions of the assay are published elsewhere (15,17). Abamectin stock solutions were prepared in absolute ethanol in silanized glass vials. Abamectin in ethanol (1 µl) was added to give concentrations of 3 nM-3 µM in a final volume of 200 µl of vesicle suspension during preincubation or 3 µM in a final volume of 200 µl of $^{36}Cl^-$ uptake medium.

Determination of signs of intoxication. Muscimol in physiological saline and abamectin in triethyleneglycol dimethyl ether were administered to mice by intraperitoneal (IP) injection (50 µl of vehicle) or intracerebral injection as described by Lawrence and Casida (18; 1-3 µl of vehicle). Animals were observed after dosing for the appearance of excitatory or depressant signs of intoxication.

Effects of GABA Agonists and Antagonists

Harris and Allan (14) showed a concentration-dependent stimulation of chloride uptake by GABA in mouse brain preparations and the appropriate stimulation or inhibition of this uptake by drugs and toxins known to act at the $GABA_A$ receptor. We confirmed a significant and highly reproducible stimulation of chloride uptake by 100 µM GABA (15) and a similar effect of 100 µM muscimol, a GABA agonist (19). GABA-stimulated chloride flux was inhibited by picrotoxinin (I_{50}= 11.2 µM; 15) and TBPS (I_{50}= 1.3 µM; 17), which are established physiological antagonists of GABA-gated chloride conductance. These findings demonstrate that the chloride flux assay is suitable for investigating the inhibitory $GABA_A$ receptor and its associated chloride ionophore as a functional unit.

Effects of Polychlorocycloalkane Insecticides

Cyclodiene insecticides or their neurotoxic metabolites have been found to be potent inhibitors of [^{3}H]dihydropicrotoxinin (DHPTX) or [^{35}S]TBPS binding to membranes from mammalian and insect CNS preparations (2-5). We evaluated eight cyclodiene insecticides or insecticide metabolites as inhibitors of GABA-dependent chloride flux in mouse brain vesicular preparations (17). 12-Ketoendrin, the most toxic compound in this series, was also the most potent inhibitor (I_{50} = 0.86 μM; Figure 1). Inhibitory potency decreased for other compounds in the following order: isobenzan; endrin; dieldrin; heptachlor epoxide; aldrin; heptachlor (17; see Figure 1). Aldrin trans-diol, the least effective compound in the series, produced approximately 25% inhibition at 100 μM. We found a significant linear correlation between potency in the chloride flux assay and acute oral toxicity for the seven most potent cyclodienes (Figure 2A). For these seven compounds, potency in the chloride flux assay was also highly correlated with potency in the inhibition of [^{35}S]TBPS binding to rat brain membranes (Figure 3). The tenfold lower sensitivity of the chloride uptake assay may reflect the effects of endogenous GABA on the cyclodiene-chloride ionophore interaction during preincubation. Unlike the vesicles used for chloride uptake, the membranes used in binding studies are extensively washed to remove endogenous GABA (4-6,20), which antagonizes [^{35}S]TBPS binding (20). These results show that the interaction of cyclodiene insecticides with the TBPS binding site on the GABA receptor-ionophore complex is directly correlated with inhibition of chloride ionophore activation, which in turn appears to be a critical event in determining acute toxicity in this series. Thus, chloride flux studies confirm and strengthen the hypothesis that the GABA receptor-ionophore complex is the principal site of action of cyclodiene insecticides.

Recent studies have grouped lindane with the cyclodienes on the basis of its potent and stereospecific inhibition of [^{3}H]DHPTX (2,3) and [^{35}S]TBPS (4,5) binding. However, the quantitative correlations between potency in the chloride flux assay, potency in the [^{35}S]TBPS binding assay, and acute toxicity that were observed for the cyclodienes do not extend to lindane. Lindane was a very weak inhibitor of chloride uptake (I_{50} = 1 mM; 17). Inclusion of the data point for lindane in the plot correlating potency in the chloride uptake assay with acute toxicity (Figure 2B) clearly shows that lindane cannot be grouped with the cyclodienes in relating in vitro potency with intoxication. The failure of lindane to fit this correlation may simply reflect the divergent pharmacokinetic behavior of hexachlorocyclohexane isomers and cyclodienes. However, lindane also does not fit the strong correlation between the two in vitro potency indices observed for the cyclodienes (Figure 3). In this relationship, lindane is 50-fold less potent as an inhibitor of chloride uptake than would be predicted from its potency as an inhibitor of [^{35}S]TBPS binding. These data suggest that lindane, unlike the cyclodienes, can bind stereospecifically with high affinity to the TBPS recognition site without significantly altering chloride ionophore function. We conclude from these studies that sites other
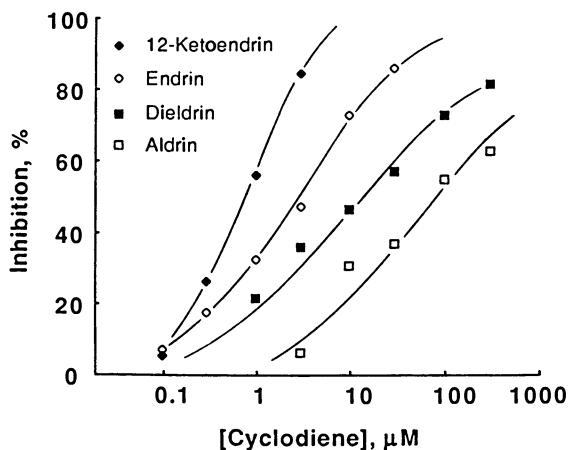
Figure 1. Concentration-dependent inhibition of GABA-stimulated chloride flux in mouse brain vesicles by four cyclodienes. Redrawn from data in Ref. 15 (endrin, dieldrin) and Ref. 17 (12-ketoendrin, aldrin).
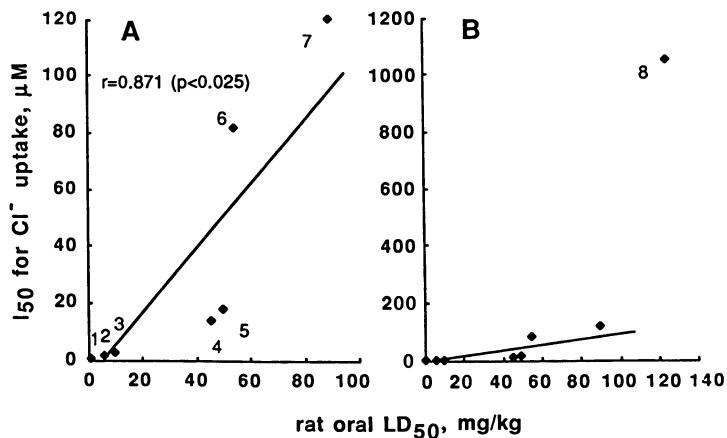

Figure 2. (A) Correlation by least squares linear regression of inhibitory potency in chloride flux assays with acute oral toxicity for 12-ketoendrin (1), isobenzan (2), endrin (3), dieldrin (4), heptachlor epoxide (5), aldrin (6), and heptachlor (7). Redrawn from Ref. 17. (B) Relationship of the regression of Figure 2A to the data point for lindane (8).

than the GABA receptor-ionophore complex may be involved in lindane neurotoxicity.

The polychlorinated cage compounds mirex and chlordecone were very poor inhibitors of GABA-dependent chloride uptake, producing little or no inhibition at 100 μM (17). These compounds also failed to inhibit [^{35}S]TBPS binding at concentrations up to 10 μM (4). Thus, despite the potent inhibition of [^{3}H]DHPTX binding in cockroach CNS preparation by chlordecone (3), it appears that these compounds act at sites other than the GABA receptor-ionophore complex in mammals.

Effects of Pyrethroid Insecticides

Electrophysiological studies have identified the voltage-dependent sodium channel of nerve membranes as the principal site of pyrethroid action (21). However, pyrethroids that contain the α-cyano-3-phenoxybenzyl moiety and produce the CS or "Type II" poisoning syndrome in mammals (22) inhibit the binding of [^{35}S]TBPS (6) or the convulsant benzodiazepine [^{3}H]Ro5-4864 (23) to rat brain membranes, thus implicating the GABA receptor-ionophore complex as a site of action for these compounds. We explored the effects of pyrethroids on GABA receptor-ionophore function in the chloride flux assay using deltamethrin, its nontoxic enantiomer, and NRDC 157 (3-phenoxybenzyl [1R,cis]-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate) as test compounds.

Preincubation of membranes with deltamethrin, which gives the CS poisoning syndrome, produced concentration-dependent inhibition of GABA-dependent chloride flux (Figure 4). Inhibition was not complete but reached a maximum of slightly more than 50% at high concentrations. NRDC 157, the non-cyano analog of deltamethrin that produces the T or "Type I" poisoning syndrome (22), was not inhibitory in this assay (17). The enantiomer of deltamethrin, which is more than 500-fold less toxic than deltamethrin by intracerebral administration to mice (24), was also inhibitory and approximately ten-fold less potent than deltamethrin (Figure 4). Thus, the actions of pyrethroids stereoisomers in this system fail to exhibit the profound stereospecificity observed in toxicity determinations and also do not confirm the extent of stereospecificity claimed for the inhibition of [^{35}S]TBPS binding by deltamethrin and related compounds (6). Because stereospecificity in pyrethroid intoxication appears to result from the chirality of the pyrethroid receptor site (18,22), pharmacological effects lacking this stereospecificity, such as those shown in Figure 4, are probably not critically involved in intoxication. Our findings therefore tend to rule out the GABA receptor-ionophore complex as a toxicologically relevant site of pyrethroid action.

Effects of Abamectin

The avermectins have been shown to increase chloride conductance in invertebrate electrophysiological preparations (8,11,25) and modulate the binding of several GABA receptor-ionophore ligands (9,10), but the molecular mechanisms underlying their neurotoxicity remain poorly defined. Recently, Abalis et al. (16) reported an avermectin B$_{1a}$-dependent stimulation of chloride uptake in rat brain vesicles that
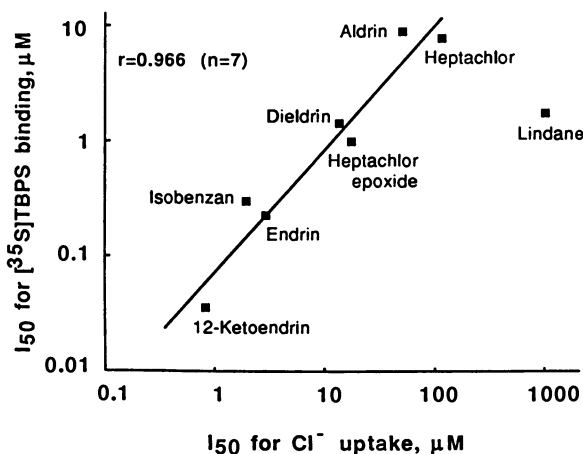
Figure 3.   Correlation by least squares linear regression of inhibitory potencies in [^{35}S]TBPS binding assays (4) with inhibitory potencies in chloride flux assays (15, 17) for seven cyclodienes. The data point for lindane is also shown but not included in the calculated regression. Redrawn from Ref. 17.
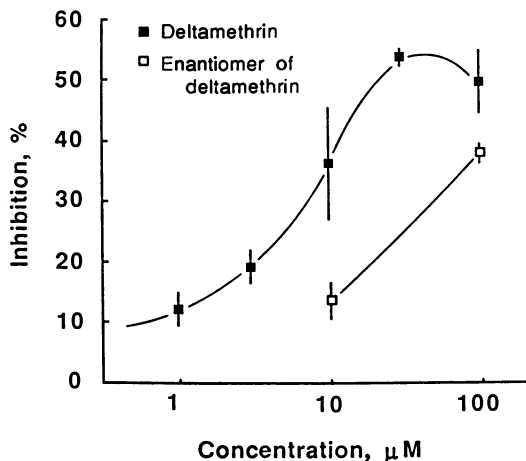

Figure 4.   Concentration–dependent inhibition of GABA–stimulated chloride flux in mouse brain vesicles by deltamethrin and its nontoxic enantiomer. Redrawn from Ref. 17.

was sensitive to bicuculline inhibition, thus suggesting a GABA-mimetic action for this compound.

We explored the action of abamectin in the mouse brain chloride flux assay under several conditions (Figure 5). Abamectin at 3 μM in either the preincubation medium or the $^{36}Cl^-$ uptake medium did not stimulate chloride uptake above background levels, whereas GABA at 100 μM produced a typical stimulation of uptake, thereby confirming the viability of the tissue preparation. Moreover, abamectin at 3 μM in the preincubation medium completely inhibited GABA-dependent stimulation. This antagonistic effect of abamectin clearly differs from its GABA-mimetic activity in similar experiments with rat brain (16). In further experiments, we characterized the effect of abamectin concentration on this inhibitory effect (Figure 6). Inhibition of GABA-dependent uptake was barely detectable at 10 nM and extensive at 1 μM. The I_{50} for abamectin in this assay is approximately 100 nM, making this compound almost 9-fold more potent as an inhibitor than 12-ketoendrin, the most potent cyclodiene (Figure 1).

We performed preliminary studies of the signs of abamectin intoxication in mice to determine whether antagonism of GABA-mediated inhibitory transmission in vivo might produce neuroexcitation (19). Abamectin administered either IC or IP at just-lethal doses produced an initial period of hyperexcitation, hypersensitivity to physical and auditory stimuli, increased locomotor activity, and tremor (IP only). The duration of this period varied with both route of administration and dose: excitatory signs were observed for less than an hour at just-lethal IC doses and intermittently for up to 6 hours at just-lethal IP doses, but these periods were shortened or completely absent at lethal overdoses by both routes of administration. These preliminary findings agree with our in vitro studies and suggest that abamectin may produce both neuroexcitatory effects, perhaps by antagonism of GABA-mediated inhibition, and depressant effects, perhaps by activation of chloride channels. The latter effect is not observed under our in vitro assay conditions but has been documented in invertebrate nerve preparations (11).

Conclusions

Our studies show that chloride flux assays using synaptic vesicle preparations from CNS homogenates provide unique and valuable information on the alteration of normal GABA receptor-chloride ionophore function by neurotoxic insecticides. Whereas radioligand binding assays define molecular interactions between toxicants and target sites, functional assays provide information on the consequences of binding interactions. The divergent behavior of cyclodienes and lindane in the binding and chloride flux assays illustrates both the different types of information obtained in each assay and the value of the latter system in describing toxicant effects on target site function. Using the chloride flux assay as an index of altered function, we have provided strong evidence that the neurotoxic effects of cyclodienes result from inhibition of GABA-gated chloride conductance at inhibitory synapses. We have also shown that lindane and deltamethrin produce qualitatively similar effects in vitro, but that these effects are poorly correlated with intoxication events in vivo. Finally, we have documented an unanticipated antagonistic effect of abamectin on GABA-dependent chloride uptake.
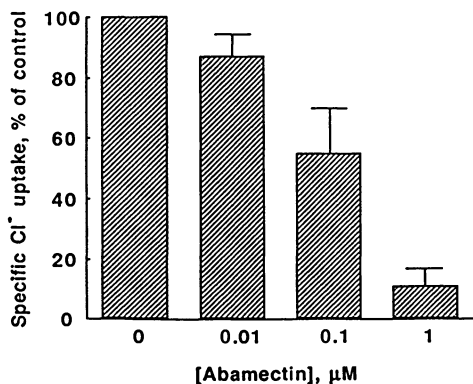
Figure 5. Effects of abamectin (AM) at 3 µM in either the preincubation or ^{36}Cl$^-$ uptake medium on basal chloride uptake and at 3 µM in the preincubation medium on uptake stimulated by GABA (100 µM). Ethanol (EtOH; 1 µl) was the control for AM addition to the preincubation medium. Results are means ± standard errors of total chloride uptake per mg protein from three experiments using different vesicle preparations (26).
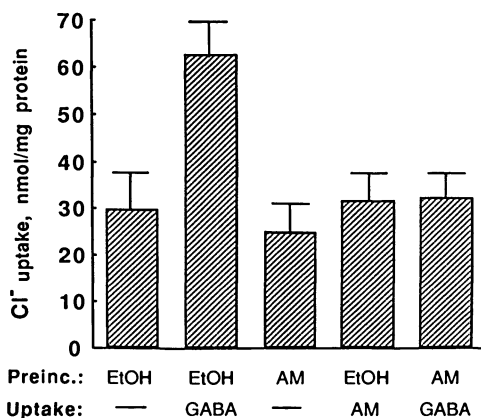

Figure 6. Effect of preincubation with different concentrations of abamectin on subsequent chloride flux stimulated by 100 µM GABA. Results are means ± standard errors of normalized specific chloride uptake in three experiments using different vesicle preparations (26).

    To our knowledge, the successful use of this synaptic vesicular assay is limited to mammalian CNS preparations (14–17). We have attempted to transfer the methodology to study GABA receptor-ionophore function in subcellular fractions prepared from cockroach (*Periplaneta americana*) thoracic nerve cords. These studies so far have provided some evidence for GABA-dependent chloride uptake, but the magnitude of this effect is small and highly variable from preparation to preparation (26). It is likely that the limiting constraint in this effort is the isolation of stable, viable postsynaptic vesicles from insect CNS homogenates. The use of novel methods to prepare functional synaptosomes from insect CNS tissue after many years of failure (27,28) suggests that new methods for isolation of mixed presynaptic and postsynaptic vesicles can be identified that are appropriate to the neuronal architecture of insect ganglia, the size distribution of insect postsynaptic terminals, and the susceptibility of the resulting vesicles to physical and osmotic insult. Recent studies indicating that insect CNS GABA receptors differ in their pharmacological profile from both types of mammalian GABA receptor (29) should provide further stimulus for the extension for these methods to insect tissue.

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Chapter 8

Interactions of Insecticides
with GABA-Operated and
Voltage-Dependent Chloride Channels

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GABA$_A$ receptors of mammalian and insect brains are
identified by binding of radioactive ligands and GABA-
induced $^{36}Cl^-$ fluxes.   Cyclodienes and lindane inhibit
stereospecifically and competitively the binding of
[^{35}S]t-butylbicyclophosphorothionate ([^{35}S]TBPS) to the
Cl$^-$ channel component of the GABA$_A$ receptor in rat brain.
Endrin and endosulfan I are the most potent inhibitors at
nM concentrations, followed by endosulfan II and
heptachlor epoxide, then dieldrin, lindane, heptachlor and
aldrin. There is good correlation between their potencies
as inhibitors of [^{35}S]TBPS binding and the GABA-
stimulated, bicuculline-inhibited $^{36}Cl^-$ influx into rat
brain microsacs.   This suggests that they act as
noncompetitive blockers of the GABA$_A$ receptor. They also
inhibit with a different order of potency [^{35}S]TBPS
binding to the voltage-dependent chloride channel in
Torpedo electric organ, which has no GABA or glycine
receptors, with lindane being much more potent than
endrin.   α-Cyanophenoxybenzyl (type II) pyrethroids are
generally more potent than type I pyrethroids in
inhibiting the GABA-induced $^{36}Cl^-$ flux. Avermectin B$_{1a}$
(AVM) potentiates binding of [^3H]flunitrazepam, inhibits
binding of [^3H]muscimol and [^{35}S]TBPS to rat brain
membranes, and induces bicuculline-inhibited $^{36}Cl^-$ influx
into brain microsacs. AVM also induces another $^{36}Cl^-$ flux
that is not affected by GABAergic drugs. It is suggested
that both the GABA$_A$ receptor and voltage-dependent
chloride channel bind TBPS, cyclodienes, lindane and AVM
with different affinities.   Insect GABA$_A$ receptors differ
from those of mammalian brain in having a lower affinity
for bicuculline and a higher affinity for AVM as shown by
[^3H]muscimol binding to honey bee brain.   The house fly
muscle receptor also has a lower affinity for clonazepam
and a higher one for Ro5-4864 than the rat brain receptor.
Such differences can be utilized to develop selective
insecticides.

ɣ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in vertebrate and invertebrate brains as well as crustacean and insect skeletal muscles. GABA is synthesized in GABAergic neurons by enzymatic decarboxylation of glutamic acid, is stored within vesicles in GABAergic nerve terminals, is released by a Ca^{2+}-dependent process, interacts with GABA receptors, and is reaccumulated in the nerve terminal by an active transport process (see Enna (1)). GABA synapses, which are abundant in mammalian brains, are divided into two types based on the pharmacology of their receptors. GABA_A receptors are predominently postsynaptic, are activated by muscimol but not baclofen, and are sensitive to picrotoxin and bicuculline. GABA_B receptors are localized presynaptically, are activated by baclofen but not muscimol, and are insensitive to picrotoxin and bicuculline. The activation of GABA receptors results in sedation and a decrease in muscle tone and motor activity, while their inhibition leads to excitation and generalized seizures.

It is generally held that the GABA_A receptor (designated GABA receptor from here on) is a glycoprotein which traverses postsynaptic membranes and functions as a chemically gated chloride channel (2,3). GABA receptors of the mammalian brain carry several binding sites: the recognition site for GABA and the competitive inhibitor bicuculline, the site that binds the tranquilizing benzodiazepines (e.g., valium), and sites that are more closely associated with the chloride channel moiety of the receptor, which bind the depressant barbiturates and convulsants (e.g., picrotoxin and t-butylbicyclophosphorothionate (TBPS) (4,5) (see Figure 1)). It is important to know that all these sites are allosterically coupled so that binding of one drug affects binding of another and that binding to any of the sites may inhibit the receptor's function. We are using [3H]-muscimol, [3H]flunitrazepam and [35S]TBPS to label the GABA recognition site, the benzodiazepine-tranquilizer site and the picrotoxin-convulsant site, respectively, utilizing binding assays that are measured by filtration on Whatman GF/B filters (6-8). As shown in Table I, GABA affects the binding of all three ligands differently. We are also studying GABA receptor function by means of its GABA-induced, bicuculline-sensitive $^{36}Cl^-$ influx into membrane microsacs (9).

Table I. Binding of [3H]Flunitrazepam, [3H]Muscimol and [35S]TBPS to Rat Brain Membranes in the Presence and Absence of Specific Drugs

Radioactive ligand ± drug	Specific binding (pmol/g tissue)
[3H]Flunitrazepam (1 nM)	6.3 ± 0.2
[3H]Flunitrazepam (1 nM) + 10 μM GABA	12.3 ± 0.3
[3H]Muscimol (4 nM)	6.7 ± 0.3
[3H]Muscimol (4 nM) + 20 μM GABA[a]	1.1 ± 0.06
[35S]TBPS (2 nM)	1.6 ± 0.14
[35S]TBPS (2 nM) + 10 μM GABA	0.1 ± 0.08

[a]Final solution contained 1% ethanol.
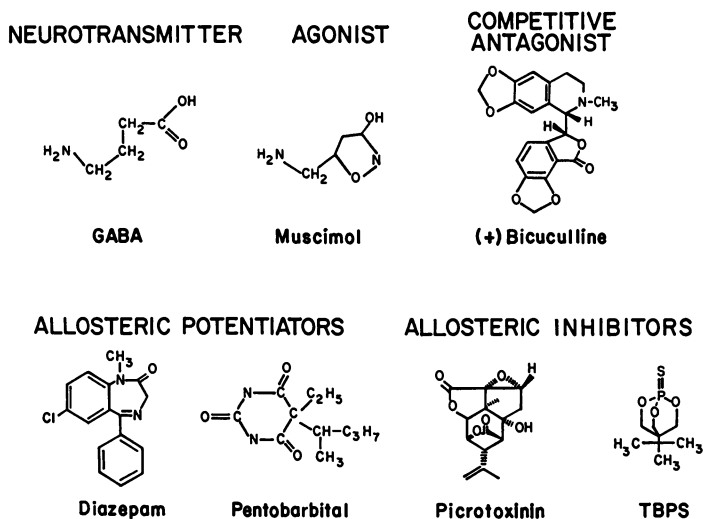
SOURCE: Data are from ref. 6.

Figure 1. Chemical structures of drugs that interact with the GABA_A receptor.

Action of Cyclodienes and Lindane on GABA Receptor

Cyclodienes and lindane have no effect on the binding of [3H]muscimol or [3H]flunitrazepam to rat brain membranes, but are potent inhibitors of [35S]TBPS binding (6) and the GABA-induced $^{36}Cl^-$ influx into brain microsacs (10). Similar actions have been reported on both [35S]TBPS binding (11,12) and the GABA-induced flux (13). The action is stereospecific and correlates for the most part with their mammalian toxicities (Table II).

Table II. Effect of Insecticides
on [35S]TBPS Binding to, and GABA-induced $^{36}Cl^-$ Influx into,
Rat Brain Microsacs

Insecticide (1 μM)	% of control $^{36}Cl^-$-influx[a]	[35S]TBPS binding IC$_{50}$ (nM)[b]	Rat toxicity (oral LD$_{50}$, mg/kg)[c]
Control, 100 μM GABA and no insecticide	100	---	---
C clodienes			
Endrin	18	3	10
Dieldrin	32	100	46
Endosulfan I	0	3	18
Endosulfan II	30	60	240
Heptachlor	55	400	90
Heptachlor epoxide	36	70	40
Hexachlorocyclohexanes (BHC)			
γ-BHC (lindane)	54	150	125
β-BHC	93	>10,000	6,000
Pyrethroids			
Allethrin	83	---	680
Fluvalinate	56	---	---
1R, cis, αS-Cypermethrin	28	---	---
1R, trans, αS-Cypermethrin	64	---	---

[a]Each insecticide was added in 20-μl aqueous aliquots to 180 μl of rat brain membrane for 10 min at 37°C before addition of the buffer containing 100 μM GABA and 0.2 μCi of $^{36}Cl^-$ then filtration. Each value is the mean of three experiments.
[b]The concentration of insecticide that inhibits 50% of the binding of 2 nM [35S]TBPS to rat brain membranes.
[c]Rat toxicity values were obtained from EPA publication-600/2-81-011, "Analytic Reference Standards and Supplemental Data for Pesticides and Other Organic Compounds". The rat oral LD$_{50}$ for the racemic mixture of cypermethrin is 251 mg/kg. The mouse intracerebral LD$_{50}$ values are 0.6 μg/g brain weight for the 1R, cis, αS and 1.6 for the 1R, trans, α-S isomers (15).
SOURCE: Data are from ref. 14.

Endosulfan I, which is more toxic than endosulfan II, is also more potent in displacing [^{35}S]TBPS binding (Figure 2) and in inhibiting the GABA-induced ^{36}Cl$^-$ influx (Figure 3). The same is found for endrin and dieldrin (Figures 2 and 3). It should be noted that toxicity is a reflection of not only drug-receptor interactions but also drug absorption, distribution, metabolism and elimination.

Endrin and endosulfan I are the most potent inhibitors of binding of [^{35}S]TBPS to GABA receptors (IC$_{50}$ of 3 nM), followed by endosulfan II, heptachlor epoxide, dieldrin, lindane, heptachlor and aldrin. The β- isomer of hexachlorocyclohexane has no effect on GABA receptors at concentrations at which the ɣ - isomer causes total inhibition (Figure 4).

These insecticides also inhibit the GABA-induced ^{36}Cl$^-$ influx in the following order of decreasing potency: endrin = endosulfan I > heptachlor epoxide > dieldrin > endosulfan II > lindane = heptachlor (10). It is clear that heptachlor epoxide, which is more toxic than the parent compound heptachlor, is also a more potent inhibitor of the GABA-induced ^{36}Cl$^-$ influx and binding of [^{35}S]TBPS to rat brain (Figure 3). It should be pointed out that the higher concentrations of these insecticides that are needed to inhibit ^{36}Cl$^-$ flux than [^{35}S]TBPS binding result from the need to maximize ^{36}Cl$^-$ flux in the assay by using 100 ␮M GABA. This requires much higher concentrations of insecticide to inhibit ^{36}Cl$^-$ flux than if lower GABA concentrations are used. Cyclodienes apparently bind to the convulsant site of the GABA receptor that binds picrotoxinin, as suggested by the competitive displacement of [^{35}S]TBPS binding by endrin (Figure 5). The high affinity that GABA receptors have for cyclodienes and their stereospecificity suggest that these receptors are likely to be primary targets for the toxic action of cyclodienes.

Action of Cyclodienes and Lindane on a Voltage-dependent Chloride Channel

[^{35}S]TBPS also binds to a voltage-dependent chloride channel found in abundance in the electric organ of the electric ray *(Torpedo* sp.) (Figure 6). This tissue contains only acetylcholine receptors but no GABA or glycine receptors. Cyclodiene insecticides and lindane also inhibit this binding, but lindane is much more potent than the cyclodienes (Table III) (16). It is interesting to note that the GABAergic drugs that bind to the GABA or the benzodiazepine sites have no effect on [^{35}S]TBPS binding to the voltage-dependent chloride channel, while drugs that bind to, or are near, the ionic channel moiety of the receptor (e.g., picrotoxinin and pentobarbital) are active, though less so than the insecticides (16).

It is reasonable to assume that binding of [^{35}S]TBPS to brain membranes encompasses binding to both the GABA-operated and the voltage-dependent chloride channels. The more toxic stereoisomers of several cyclodiene pairs are more potent in displacing [^{35}S]TBPS from the voltage-dependent Cl$^-$ channel of *Torpedo*. For example, at 1 ␮M, dieldrin and endrin inhibit 40 and 100%, respectively, and endosulfan I and II inhibit 42 and 72%, respectively. In addition, the more toxic heptachlor epoxide is more potent than heptachlor. These results suggest that if present in higher concentrations, cyclodienes will affect voltage-dependent chloride channels as well, but at lower concentrations their effect may be restricted to the GABA-operated chloride channels. The situation is reversed for lindane, which has
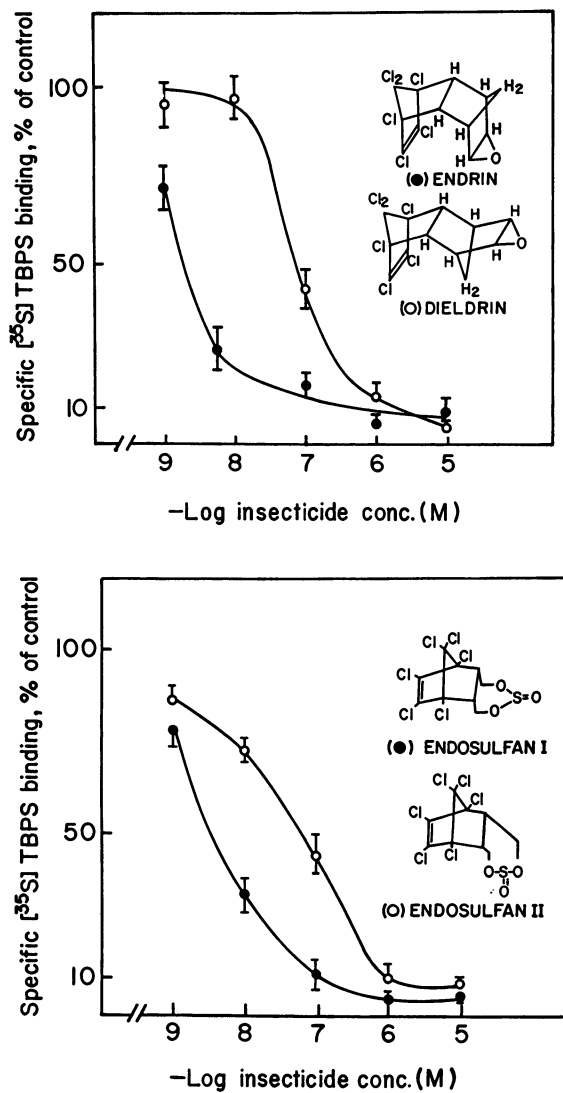
Figure 2. Inhibition by endrin, dieldrin, endosulfan I and II of specific 2 nM
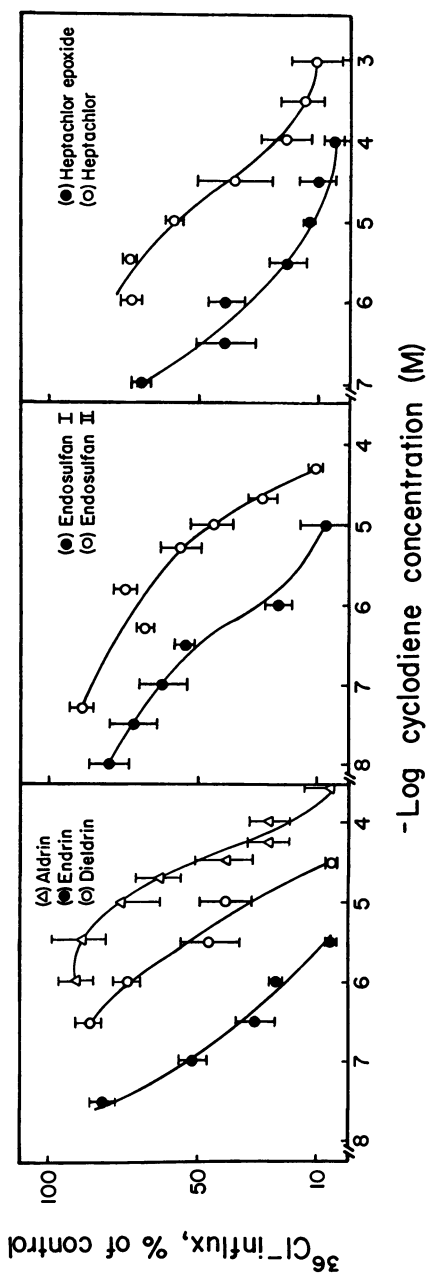(Reproduced with permission from ref. 6. Copyright 1985 Academic.)

Figure 3. Inhibition of 100 μM GABA-induced $^{36}Cl^-$ influx into rat brain microsacs by cyclodiene insecticides. (Reproduced with permission from ref. 10. In press, Academic.)
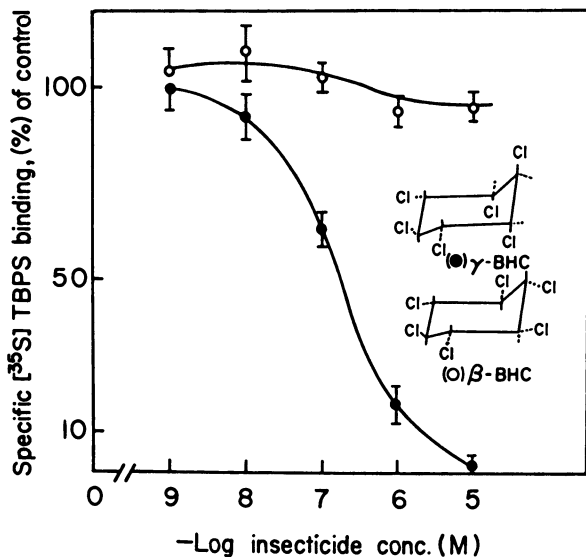
Figure 4.   Inhibition by hexachlorocyclohexane isomers of specific 2 nM [^{35}S]TBPS binding to rat brain membranes.
(Reproduced with permission from ref. 6. Copyright 1985 Academic.)
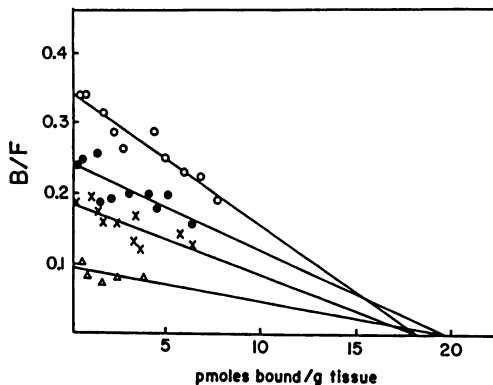
Figure 5.   Scatchard plot of the binding of [^{35}S]TBPS to the GABA receptor of rat brain in the absence (o) and presence of 1 nM (•), 3 nM (x), and 10 nM (Δ) endrin.   B, amount bound in pmol/g tissue; F, free [^{35}S]TBPS concentration.
(Reproduced with permission from ref. 6. Copyright 1985 Academic.)
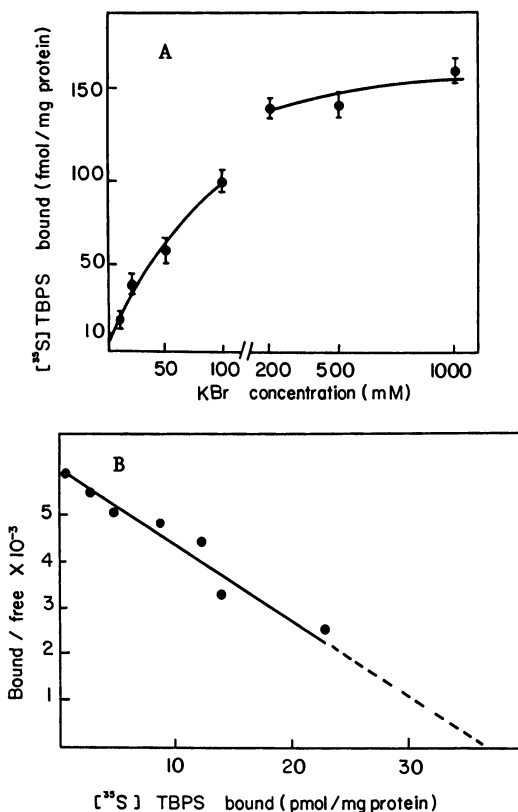
Figure 6.   Binding of [^{35}S]TBPS to *Torpedo* electric organ membranes.
A.   Effect of KBr concentration on 3 nM [^{35}S]TBPS binding.   Symbols
and bars represent means + S.D. of triplicate experiments.   B.
Scatchard plot of [^{35}S]TBPS binding in presence of 200 mM KBr at
21°C.   Nonspecific binding was that observed in the presence of 4.5
μM unlabeled TBPS.   The points are the means of three experiments.
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Press.)

a 10-fold lower affinity for the GABA receptor's chloride channel,
and thus would preferentially inhibit the voltage-dependent chloride
transport at lower concentration, and also would inhibit the GABA-
operated chloride channel at higher concentrations.

Table III. Inhibition of [^{35}S]TBPS Binding to the Putative Voltage-
Dependent·Chloride Channel in *Torpedo* Electric Organ and the GABA
Receptor Chloride Channel in Rat Brain

Drug	K_i (μM)	
	Torpedo electroplax	Rat brain
TBPS	1.37	0.05
Picrotoxinin	100.00	0.20
Pentobarbital	110.00	58.00
Endrin	0.75	0.03
Lindane	0.04	0.15

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Action of Pyrethroids on GABA Receptors

Since diazepam delayed the onset of toxicity symptoms produced by
type II pyrethroids, it was suggested that they inhibited GABA
receptors (17).   Later, it was found that type II pyrethroids were
more potent than type I in inhibiting [^{35}S]TBPS binding to rat brain
GABA receptors (18).   We also find that pyrethroids inhibit GABA-
induced ^{36}Cl$^-$ flux with the α-cyano-containing type II fluvalinate
being more potent than the type I allethrin (Table II).   The higher
potency of the 1R,cis,αS isomer than the 1R,trans,αS isomer of
cypermethrin in inhibiting the GABA-induced ^{36}Cl$^-$ influx suggests
that their effects are stereospecific and related to their mammalian
toxicities.   Based on the characteristics of inhibition of [^{35}S]TBPS
binding to the GABA receptor, the action of pyrethroids is suggested
to be allosteric at a site different from the cyclodiene binding
site.   The data suggest that though the voltage-dependent sodium
channel may be the primary target for pyrethroid action, the GABA
receptors may be a secondary target.   The high affinity that the GABA
receptor has for type II pyrethroids and the close relationship of
their effects to their mammalian toxicities, suggests that this site
may in fact be an important site of action for the type II
pyrethroids, although not the type I pyrethroids.   However, the
higher potency of type II pyrethroids on the voltage-dependent sodium
channel, and the disruption of conduction that results from
inhibition of inactivation of less than 0.1% of sodium channels in an
axon (19), suggests that the sodium channel may be the primary target
for type I and type II pyrethroids.

Action of Avermectin on GABA Receptor and Chloride Channel

Avermectin B$_{1a}$ (AVM) acts on both GABA-operated and voltage-dependent
chloride channels, but its action on both is quite different from
those of cyclodienes, lindane or pyrethroids.   In rat brain
preparations, AVM induces ^{36}Cl$^-$ influx which is inhibited by the

competitive antagonist bicuculline. The AVM–induced influx is much less than that induced by GABA (Figure 7).

AVM acts like GABA in potentiating binding of [^{3}H]flunitrazepam and inhibiting binding of [^{3}H]muscimol (Figure 8) and [^{35}S]TBPS to rat brain membranes, and induces bicuculline–sensitive ^{36}Cl$^-$ influx, though to a lower degree than does GABA. This suggests that AVM binds to the GABA recognition site and acts as a partial agonist (8). AVM also binds to the GABA recognition site of insect GABA receptors (Table IV). Because of the complexity of the chemical structure of AVM (a macrocyclic lactone), it is likely that only a very small part of the molecule binds to the GABA receptor. Inhibition of [^{35}S]TBPS binding by AVM could result from the allosteric effect of occupation of the GABA site, or from direct occupation of the TBPS site, thus acting as a hyperpolarizing blocker.

Table IV. The Effects of GABAergic Drugs on the High Affinity Binding of [^{3}H]Muscimol to Honey Bee Brain Membranes in Comparison with Their Reported Effects on [^{3}H]Muscimol Binding to Rat Brain' Membranes

	Apparent IC$_{50}$ (μM)	
Drugs	Honey bee brain[a]	Rat brain[b]
Muscimol	0.006	0.01
AVM	0.003	---
GABA	0.042	0.038
(+)Bicuculline	>1000	6.2

[a]Data presently obtained using 5 nM [^{3}H]muscimol. Thus, apparent IC$_{50}$ values, calculated from log dose–response curves, are based on inhibition of binding that represented ~75% high affinity and 25% low affinity binding. The actual IC$_{50}$ values are significantly lower.
[b]Using 8.4 nM [^{3}H]muscimol (20).
SOURCE: Data are from ref. 7.

AVM also induces another ^{36}Cl$^-$ transport in rat brain neurosynaptosomes that is insensitive to bicuculline but is inhibited by the specific chloride channel inhibitor 4,4–dithiocyano-2,2'-stilbenedisulfonic acid (DIDS) (Figure 9). This ^{36}Cl$^-$ flux is sensitive to GABAergic drugs that affect the channel function (e.g., picrotoxinin, TBPS and pentobarbital). It suggests that AVM also activates chloride channels and agrees with the activation by AVM of the GABA–operated and voltage–dependent chloride channels in locust muscle (21).

Comparison of Insect and Vertebrate GABA Receptors

It is important to note that there are differences in drug specificities of GABA receptors of insects and mammals. Examples are the insensitivity to bicuculline of [^{3}H]muscimol binding to honey bee brain, and the very high potency of AVM (7) (Table IV), compared to rat brain binding. Bicuculline is also ineffective in inhibiting GABA–induced ^{36}Cl$^-$ influx into membranes from cockroach nerve cord
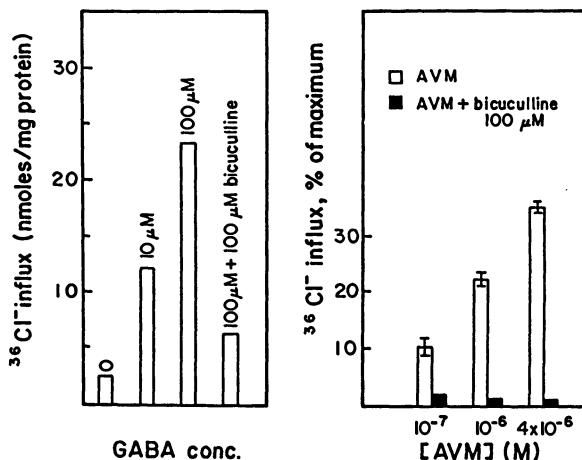
Figure 7.   Influx of ^{36}Cl$^-$ into rat brain microsacs, which is activated by GABA (left panel) or AVM (right panel), and is inhibited by bicuculline.   Maximum ^{36}Cl$^-$ influx is that obtained with 100 μM GABA.   Each bar represents the S.D. of three experiments. ^{36}Cl$^-$ influx into microsacs in absence of drugs was 808 dpm, in presence of 100 μM GABA, 1795 dpm, and in presence of 1 μM AVM, 1104 dpm.   Thus, GABA- and AVM- induced ^{36}Cl$^-$ influxes were 987 dpm and 296 dpm, respectively.

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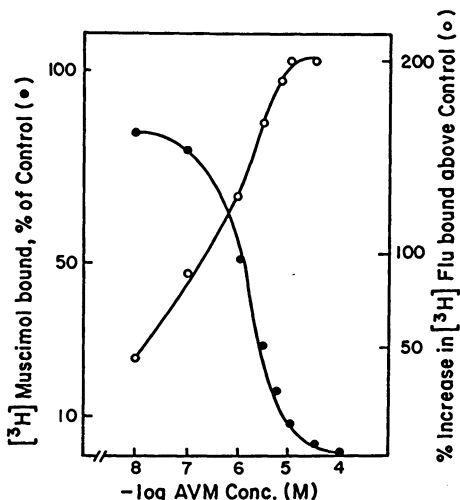
Figure 8.   The dose-dependent effect of AVM on the binding of 4 nM [^{3}H]muscimol ($\bullet$) and 2 nM [^{3}H]flunitrazepam ([^{3}H]Flu) (o) to rat brain membranes.   Control binding of [^{3}H]muscimol and [^{35}S]TBPS in the absence of AVM was 5.6 pmol/g tissue and 1.3 pmol/g tissue, respectively, while in [^{3}H]flunitrazepam binding, the control was 5.8 pmol/g tissue.

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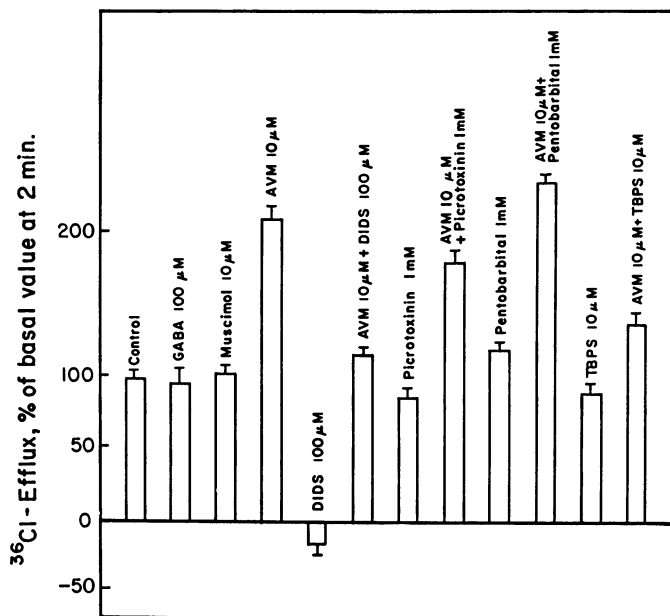
Figure 9. The effect of AVM and GABAergic drugs on $^{36}Cl^-$ efflux from rat brain microsacs. Each bar represents the S.D. of three experiments. The membranes were incubated with 10 μCi $^{36}Cl^-$ in 3 ml for 60 min at 4°C, then 100 μl aliquots were incubated for 2 min at 21°C in 5 ml of buffer containing AVM, DIDS or both simultaneously. The ordinate is the difference in $^{36}Cl^-$ count inside the microsacs between 2 min and 0 time in the presence of drug divided by the values in the absence of any drug (i.e., basal $^{36}Cl^-$ efflux) and calculated as a %. A negative value is obtained when the count at 2 min (e.g., 8171 ± 421) in presence of 0.2 mM DIDS is above that in absence of drugs at 0 time.

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consistent with its ineffectiveness on identified neurons in the same cord measured electrophysiologically (Wafford et al. (22)).

GABA receptors identified in house fly thoracic muscle membranes by [3H]flunitrazepam binding also show differences from rat brain GABA receptors (23). The insect muscle receptors have higher affinity than the rat brain receptor for the convulsant benzodiazepine Ro5-4864 and lower affinity for clonazepam in inhibiting [3H]flunitrazepam binding (Table V). This makes them more like the peripheral-type benzodiazepine receptors in mammalian tissues, which are not linked to GABA-regulated anion channels and are suggested to be calcium channels (24). Such differences in drug specificities might be exploited to develop insect-specific and environmentally safer insecticides.

Table V. Comparison of Benzodiazepine Binding Sites
in Different Species and Tissues

Compound	K_i (nM)	
	House fly thorax[a]	Mammalian brain
Flunitrazepam	290 + 35	2.72[b]
Diazepam	488 + 60	27.4[b]
Clonazepam	146,000 + 3,522	1.13[b]
Ro5-4864	680 + 75	100,000[c]

[a]Each value is the mean of three separate experiments, performed in triplicate, + standard deviation.
[b]Data on [3H]flunitrazepam binding to human cerebral cortex membranes from Speth et al. (25).
[c]Data calculated from IC50 values of [3H]diazepam binding to rat tissues from Braestrup and Squires (26).
SOURCE: Data are from ref. 23.

The data presented above show clearly that GABA receptors of mammalian brain are targets for the toxic action of cyclodiene, ɣBHC and pyrethroids. Although, the binding and flux assays, used so sucessfully to study interactions of these insecticides with mammalian brain, have had limited success when applied to insect tissues, there is ample evidence to suggest that GABA receptors of insects are targets for AVM (7, 21) cyclodienes and pyrethroids (17, 22).

Conclusions

In summary, our data suggest that GABA receptors in mammalian and insect brains are targets for insecticides, which are either inhibitors or activators of receptor function. The GABA receptor may be a primary target for cyclodienes and possibly AVM, but a secondary target for lindane and pyrethroids. On the other hand, the voltage-dependent chloride channel may be a primary target for lindane and possibly AVM. There are differences in the drug specificities of the GABA receptors of insects and vertebrates, which can be a basis for development of selective insecticides. Both the GABA-operated and the voltage-dependent chloride channels are molecular targets for the

action and neurotoxicity of insecticides with different chemical structures, and potential targets for many more to be developed.

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Chapter 9

GABA Transaminase and Glutamic Acid Decarboxylase as Targets for Insecticides

Biochemical and Toxicological Assessment

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A series of inhibitors of GABA transaminase (GABA-T)
and glutamic acid decarboxylase (GAD) was synthe-
sized. Analogs of aminooxyacetate were tested
against bacterial GABA-T. Examples of Kd values
were: aminooxy-butyrate (AOB) 0.1 μM, AOB ethyl
ester 1.5 μM, aminooxyacetate 5.5 μM and gabaculine
21 μM. Toxicity to the American cockroach (by
injection) did not correlate with GABA-T inhibition,
LD50's being 350, 25, 750 and >1000 μg/g,
respectively. When [^{14}C]-GABA was injected into
cockroaches pretreated with gabaculine, AOB ethyl
ester or saline, the evolution of $^{14}CO_2$ was virtually
eliminated (gabaculine) or partially reduced (AOB
ethyl ester) compared with the control. However,
since none of the insects died, GABA-T can be ruled
out as a lethal target. Indeed, GABA-T inhibition *in
vivo* using gabaculine abolished the symptoms caused
by AOB ethyl ester but did not affect symptoms caused
by the non GABA-ergic insecticide chlordimeform
indicating a specific effect of AOB ethyl ester on
GABA/glutamate systems. Many compounds also
inhibited bacterial and insect GAD, *in vitro*.
However, cockroaches dosed with AOB ethyl ester *in
vivo* showed 70-80% inhibition of flight muscle GAD,
irrespective of symptoms. This suggests that GAD may
not be a very promising target site for insecticides
either.

The major inhibitory neurotransmitter in invertebrates is
γ-aminobutyric acid (GABA). Inhibitory neurones are found both in
the CNS and at the neuromuscular junction in invertebrates (1),
whereas they are not found at the neuromuscular junction in mammals.
The widespread distribution of GABA-ergic neurones in insects is
probably partly responsible for the selective toxicity towards
insects of certain commercial insecticides, e.g., cyclodienes (2-4),
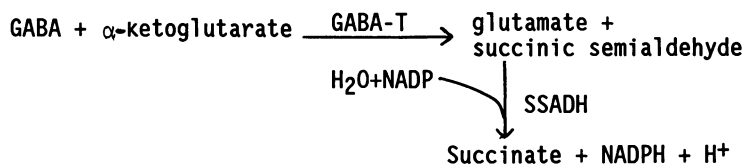avermectin (5), and perhaps some pyrethroids (6).

In mammals, the enzymes GABA-transaminase (GABA-T) and glutamic acid decarboxylase (GAD) are largely responsible for the degradation and synthesis of GABA, respectively. Furthermore, both of these enzymes have been identified in invertebrate species (7,8). Initially, the inhibition of GABA-T was addressed as a possible target site for insecticides using as a model the selective, irreversible, suicide substrate (mechanism-based inhibitor) gabaculine. This naturally occurring compound (9), inhibits GABA-T by virtue of the formation of a stable, covalent bond to the coenzyme pyridoxal phosphate, but only after first undergoing an enzyme-induced aromatization of the cyclohexadiene (10). In the present study a kinetic analysis has been used which was based on the one previously developed for studying irreversible inhibitors of acetylcholine esterase (11). This involved calculating the constants which govern the affinity of the inhibitor for the enzyme (Kd) and the rate of enzyme inactivation (kcat). Likewise, a series of analogs of aminooxyacetate, an irreversible inhibitor which does not have a suicide mechanism but instead is a general carbonyl trapping reagent, was made and tested against GABA-T using the same analysis.

A complementary study of the toxicology and symptomology of these compounds in the cockroach *in vivo* led to the suggestion that some of them might be inhibiting GAD. This could lead not only to reduced GABA levels, but also to enhanced concentrations of glutamate, the putative excitatory transmitter at the arthropod neuromuscular junction (12). An analysis of the inhibition of GAD from cockroach flight muscles was, therefore, conducted.

Inhibition of GABA-T, *In Vitro*

Gabase, a commercial (Boehringer-Mannheim Biochemicals) preparation from *Pseudomonas fluorescens* which contains GABA-T and succinic semialdehyde dehydrogenase (SSADH), was used as a source of GABA-T. The activity of GABA-T was measured indirectly in a coupled enzyme assay (13) by measuring spectrophotometrically the rate of formation of NADPH:

$$\text{GABA} + \alpha\text{-ketoglutarate} \xrightarrow{\text{GABA-T}} \text{glutamate} + \text{succinic semialdehyde}$$

$$\text{H}_2\text{O} + \text{NADP} \searrow \Big|_{\text{SSADH}}$$

$$\text{Succinate} + \text{NADPH} + \text{H}^+$$

The formation of NADPH was monitored by measuring its absorbance at 340nm. In the presence of an excess of SSADH, this absorbance is proportional to the activity of GABA-T. The additional SSADH used in the enzyme reaction was prepared by adding Gabase which had been treated with gabaculine to inactivate the GABA-T and then dialyzed to remove any excess gabaculine.

The Km of the enzyme for GABA was 10^{-3}M at 25°C. The enzyme was generally freshly prepared although it could be stored at -20°C dissolved in 0.1M Hepes buffer (pH 7.1) containing 5 mM dithiothreitol (DTT) for up to 2 weeks without appreciable loss of activity. Gabaculine and other inhibitors were usually prepared at

10^{-2}M in H_2O and diluted appropriately. The assay was adapted to an automated clinical analyzer, the Chemetrics Analyzer II (Worthington Diagnostics). Absorbances (340nm) were recorded at 5 sec. intervals and printed out for 1-2 min.
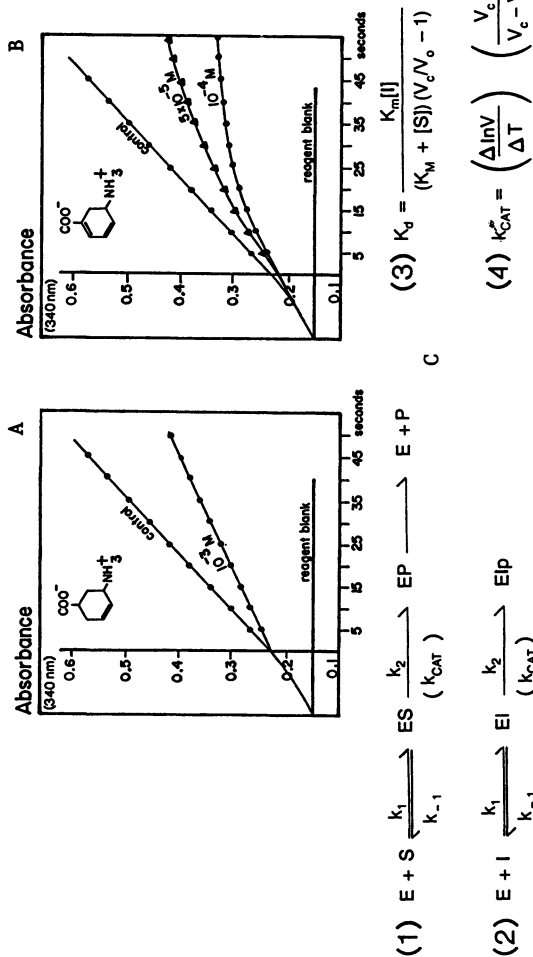
The rate of absorbance change for the uninhibited enzyme was linear with time (Figures 1A and B). A lower, but constant, rate was observed with trans-3-amino-cyclohexene carboxylate, which is characteristic of a reversible, competitive inhibitor (Figure 1A). In contrast, gabaculine, which is known to act as an irreversible, competitive inhibitor (10), progressively decreased the rate of enzyme activity, indicating a permanent inactivation of enzyme molecules (Figure 1B). When the log of the rate of enzyme activity was plotted against time, it decreased linearly in the presence of gabaculine (Figure 2). The dissociation constant (K_d) and the catalytic constant (kcat) were calculated with a computer program using the equations given in Figure 1C (11).

The inhibition data for the aminooxy acids was treated similarly (Table I). For the series of aminooxy acids ranging from the acetate to the valerate, and for gabaculine, there was little change in kcat (range from 0.11 to 0.18 sec^{-1}) whereas there was a 210-fold range of values of K_d (0.1 to 21 μM). Therefore, K_d was used as the basis of comparison to describe the derivatives (Table I). Inhibition by the aminooxy acids increased as the carbon chain lengthened from 1 to 4 (acetate to the butyrate) but then decreased as the length increased to 5 with the valerate. This is perhaps to be expected since the butyrate most closely resembles GABA, the substrate.

Several derivatives of the aminooxy acids also inhibited GABA-T (Table I). Once again, those compounds remaining closest to the GABA skeleton were most potent, as judged from the K_d values. Esters of aminooxybutyrate decreased in inhibitory potency as the chain length increased from one to eight (compounds 5-8, Table I), moving away from the GABA backbone. Isogabaculine (compound 14, Table I) was also active as a GABA-T inhibitor, being approximately three times as potent as gabaculine. The hydroxylamine derivatives of aminobutyrate (compounds 11 and 12, Table I) were both active as GABA-T inhibitors, but with very little toxicity, presumably also through reacting with pyridoxal phosphate.

Assessment of the Toxicological Relevance of GABA-T Inhibition in the Cockroach

Compounds were dissolved in sodium phosphate buffer and injected into the thorax of the American cockroach. This allowed for rapid access to the leg muscles while avoiding much of the potential metabolic degradation in the abdomen. Compounds with the highest affinity for GABA-T appeared to be the most toxic. However, the specific GABA-T inhibitor gabaculine caused no toxic effects even at 1000 μg/g. The aminooxy compounds caused prolonged convulsions prior to prostration which, superficially, would not be anticipated if GABA-T were the only enzyme inhibited since this should cause an elevation of GABA levels. An *in vivo* experiment was designed to determine definitively the importance of GABA-T inhibition as a lethal lesion in insects.

$$(1)\quad E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow[(k_{CAT})]{k_2} EP \longrightarrow E + P$$

$$(2)\quad E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow[(k_{CAT})]{k_2} EIp$$

Where: E = Enzyme
I = Inhibitor
EI = Reversible Enzyme-Inhibitor Complex
EIp = Irreversible Enzyme-Inhibitor Complex
k_1 = Association Rate Constant
k_{-1} = Dissociation Rate Constant
$k_2 = k_{CAT}$ = Catalytic Rate Constant
$k_{-1}/k_1 = K_d$ = Dissociation Constant for Inhibitor.

$$(3)\quad K_d = \frac{K_m[I]}{(K_M + [S])(V_c/V_o - 1)}$$

$$(4)\quad k_{CAT} = \left(\frac{\Delta \ln V}{\Delta T}\right)\left(\frac{V_c}{V_c - V_o}\right)$$

Where: K_M = Michaelis Constant
$[I]$ = Inhibitor Concentration
$[S]$ = Substrate Concentration
V_o = Inhibited Zero-Time Velocity
V_c = Control Zero-Time Velocity
$\Delta \ln V$ = Natural Log of V_1/V_2
ΔT = Time Interval Between V_1 and V_2

Figure 1: The effects of a reversible (A) and an irreversible (B) inhibitor on GABA-T activity plotted against time since mixing. C: The equations used to calculate the catalytic rate constant (kcat) and the dissociation constant for the inhibitor (Kd). Reactions in A and B were initiated 16-18 seconds before the indicated time zero.
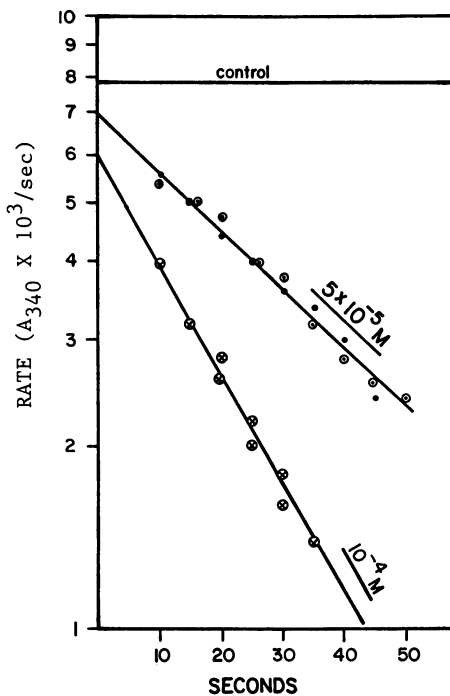
Figure 2:   Semi-log plot of the change in GABA-T activity in the presence of gabaculine, at $5X10^{-5}$M (two experiments) and 10^{-4}M with time, showing first order kinetics.

Table I

Summary of the Effects of GABA-T Inhibitors and Related Compounds *In Vitro* and *In Vivo*

| | E coli GABA-T Inhibition | | P. americana | |
	K_d, μM	k_{cat}, sec^{-1}	Approx. LD_{50} $\mu g/g$	Comments
Aminooxy Acids				
1. Acetate	5.5	0.18	750	prostration at 2-4 h, after tremors.
2. Propionate	0.4	0.16	750	prostration at 3-24 h.
3. Butyrate	0.1	0.12	350	tremors 30 min - 2 h then prostration.
4. Valerate	1.0	0.11	400	prostration $\leq$ 1 h.
Aminooxy Butyrate Derivatives				
A. Acid				
5. Methyl ester	0.3		50	tremors & prostration 2-4 h.
6. Ethyl ester	1.5		25	tremors 40 min - 3 h then prostration.
7. Hexyl ester	20		225[A]	tremors > 1 h; prostration 7-24 h; $LD_{50} \simeq$ 75 in thorax.
8. Octyl ester	36		>500[A]	Abnormal posture, some tremors.
B. Amine[B]				
9. Boc	30		100	prostrate at 200 in < 7 min (n = 3).
10. Boc, Me ester	—		>500[A]	some uncoordination.
11. Hydroxylamine	17		$\geq$ 500	some tremors & temporary prostration at highest dose.
12. Hydroxylamine, Et ester	300		>1000	little effect.
Suicide Substrates				
13. Gabaculine	21	0.16	>1000	no symptoms.
14. Isogabaculine	6.9	0.11	—	
Related Compounds				
15. GABA	—		>1000	1-2 min slight tremor.
16. Muscimol	—		40	slight tremors 1st few min, prostration in 30-90 min.
17. Bicuculline	—		150[A]	tremors
18. Glutamate	—		400	immediate tremors then prostration and recovery after 10 min.

[A] — Abdominal injection, DMSO solvent.

[B] — t-butyloxy carbonyl

Metabolism of [^{14}C(U)] GABA in the Cockroach

The following experiment was carried out to determine to what extent
GABA-T can be inhibited *in vivo* after administration of inhibitors
shown to be active *in vitro*. The rationale for such an approach
assumes that GABA-T is, quantitatively, the most important enzyme in
GABA metabolism. Thus, in the following scheme, administration of a
GABA-T inhibitor will prevent the oxidation of GABA to CO_2.

```
         GABA-T                                    KREBS
GABA --------->  SUCCINIC   ---->  SUCCINIC ----> TCA   ----> CO2
                 SEMIALDEHYDE       ACID          CYCLE
```
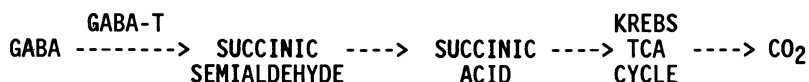
Gabaculine was injected at 1000 µg/g ($\approx$35 mM hemolymph concen-
tration); ethyl 4-aminooxybutyrate at 12.5 µg/g, half the LD_{50} ($\approx$0.4
mM hemolymph concentration). Cockroaches were then injected with
[^{14}C(U)]GABA (79,600 dpm, abdominal route) and placed in closed
flasks containing 0.2 ml N KOH as a CO_2 trap. At 2, 5, and 20.5 h
the traps were removed for measurement of $^{14}CO_2$.

Gabaculine inhibited GABA oxidation by 98% within two hours
(Figure 3). The nonspecific inhibitor, ethyl aminooxybutyrate also
inhibited GABA oxidation *in vivo* ($\approx$50% at 2 h). Since neither
compound caused symptoms or mortality at these dosages, it appears
that GABA-T is not important as a target site for insecticides. An
alternative explanation for the toxicity of the aminooxy compounds
is the inhibition of GAD, another enzyme using pyridoxal phosphate
as a cofactor.

Inhibition of GAD *In Vitro*

Studies of GAD inhibition used enzyme from *E. coli* (Sigma) or
American cockroach flight muscle. GAD was incubated in 20 mM
potassium phosphate buffer in scintillation vials at pH 5.0
(bacterial) or pH 7.0 (insect). Inhibitors were added in buffer or
DMSO, final concentration 2%. Reactions were initiated by adding
0.5 µCi of [^{14}C(U)] glutamate (specific activity 293 mCi/mmol.) or
0.1 µCi [1-^{14}C] glutamate (specific activity 50 mCi/ mmol.). A
center well contained 1M hyamine hydroxide in methanol to trap the
$^{14}CO_2$ which was quantified by scintillation counting. Reactions
were run for 45 min at 25°C.

GAD was partially purified from cockroach flight muscle. All
steps were carried out at 0-4°C. Muscle was dissected from male
cockroaches and held in 250 mM sucrose, 1 mM EDTA and 1 mM DTT (SED)
until enough tissue was accumulated. The SED solution was replaced
with 10 volumes of fresh medium and the tissue was homogenized with
a Dounce homogenizer. The homogenate was filtered through 4 layers
of cheesecloth and centrifuged at 15,000X g for 20 min. The result-
ing pellet was resuspended in the original volume of SED and the
homogenization and centrifugation steps repeated. The pellet was
stored at -80°C until used. The pellet was thawed, homogenized and
centrifuged at 70,000X g (25 min). The pellet was resuspended in
cold water and stirred for 30 min. After centrifugation at 100,000X
g, the supernatant was dialyzed against 20 mM potassium phosphate
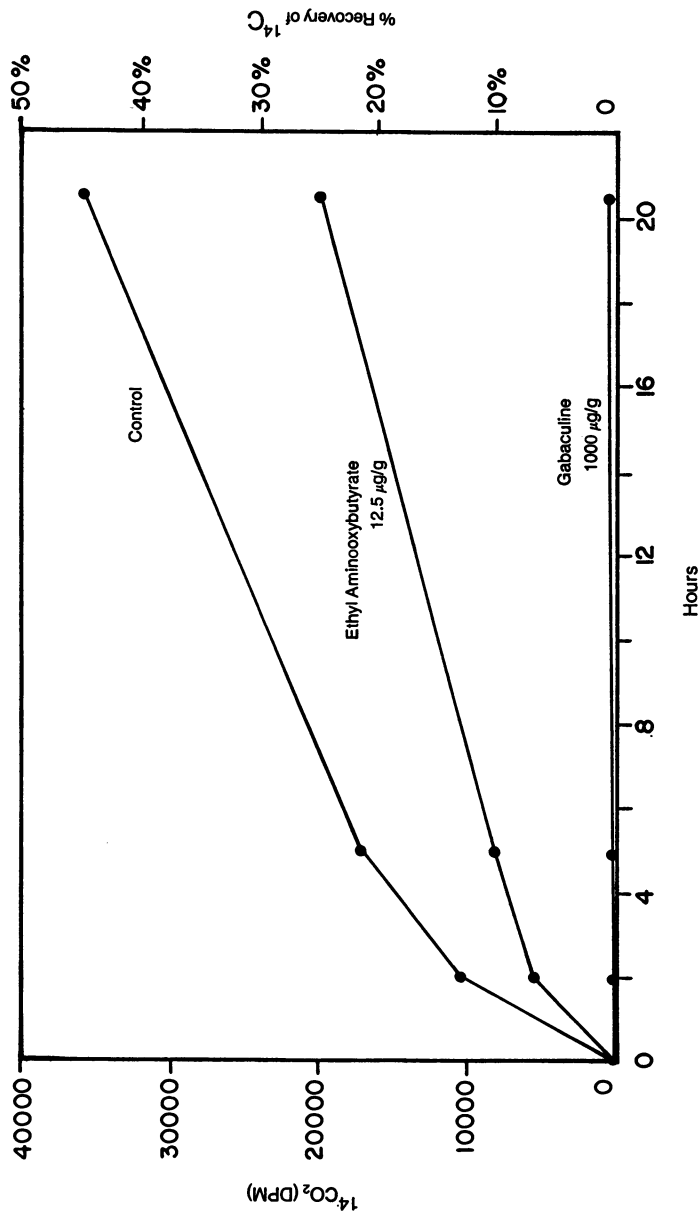buffer (pH 7.0) containing 1 mM DTT for 3 hours and then used for

Figure 3: Cockroaches were dosed with gabaculine, ethyl 4-aminooxybutyrate and saline (control). They were then given [^{14}C (U)]GABA and the $^{14}CO_2$ liberated was measured at intervals.

the GAD assay. This protocol was based on methods used for the preparation of GAD from mouse brain (14) and effected a 20- to 30-fold purification of the crude enzyme; it not only removed other enzymes which might have metabolized glutamate, but also removed endogenous inhibitors, such as GABA.

The inhibitory potencies of the compounds, along with those of several GAD inhibitors from the literature, are summarized in Table II. Reasonable agreement was found between the bacterial and insect enzymes. Toxic compounds were good inhibitors of GAD, in general, although aminooxy acids 1 and 2 (Table II) were potent inhibitors without being very toxic. A previous observation that insect GAD is not inhibited by aminooxyacetate (15) may be explained by the use of different enzyme preparations in the two studies. In the present work it was found that GAD in a crude muscle homogenate was not consistently inhibited by aminooxyacetate. It was this observation that led to the above purification protocol.

GABA-T inhibition does not seem to be detrimental to insects, but elevated GABA levels may have an anticonvulsant effect towards convulsant insecticides which act on GABA/glutamate systems. The following experiment was, therefore, devised.

Protective Effect of Gabaculine *In Vivo* Against Ethyl Aminooxybutyrate (Compound 6)

An attempt was made to study the possible anticonvulsant effects of gabaculine against symptoms caused by ethyl aminooxybutyrate. This compound inhibits both GABA-T and GAD, so that symptoms could be caused by inhibition of GAD, whereas gabaculine is a specific suicide substrate of GABA-T. Cockroaches were injected intrathoracically with gabaculine at 1000 µg/g and 2.5 hours later, were injected similarly with 50 µg/g (2X LD_{50}) of compound 6 (Tables I and II). At no stage during the next 24 hours were any symptoms observed. However, a control group of five insects, which received compound 6 alone, began to show tremoring between 50 and 120 min. after dosing. By 4 hours, 80% were prostrate. These results clearly showed that GABA-T inhibition can block the neurotoxic effects of compound 6. It is, therefore, possible that a compound which inhibits both enzymes may elicit no symptoms because the two effects may cancel one another out, since the elevation of GABA levels caused by GABA-T inhibition would tend to compensate for the reduction in GABA levels (and increase in glutamate levels) caused by GAD inhibition. An indication that this effect is specific was obtained by repeating the experiment using chlordimeform, with and without pretreatment with gabaculine. This insecticide acts as an octopamine agonist as well as on other systems at higher doses, and so it does not specifically interfere with GABA systems (16,17). In this case, there was no difference in onset of symptoms whether or not the insects had been treated with gabaculine. At 500 µg/g (2X LD_{50}), injected into the abdomen, chlordimeform caused uncoordination within 15 to 30 min of dosing followed by prostration between 5 and 24h after injection. This experiment suggests that only insecticides acting either by potentiating glutamate effects or reducing the effects of GABA will be antagonized by a specific GABA-T inhibitor.

Table II

Inhibition of Glutamic Acid Decarboxylase (GAD)

Inhibitor	E. Coli GAD IC_{50} (μM)	Cockroach GAD,IC_{50} (μM)	LD_{50}
Aminooxy Acids			
1. Acetate	—	< 10f	c
2. Propionate	—	< 10f	c
3. Butyrate	10-100	< 10f	b
4. Valerate	—	< 10f	b
Aminooxy Butyrate Derivatives			
A. Acid			
5. Methyl ester	10-100	< 10f	a
6. Ethyl ester	10-100	1-10	a
7. Hexyl ester	—	≈100	b
8. Octyl ester	—	≈100	c
B. Amine			
9. Boc	10-100	10-100	a
10. Boc, Me ester	—	—	c
11. Hydroxylamine	≈1000	—	c
12. Hydroxylamine, Ethyl ester	>1000	— ·	c
Suicide Substrate			
13. 4-Amino-Hex-5-ynoic acid	—	100-1000	c
Other, non-specific GAD Inhibitors			
14. MDTC [d]	>1000	> 1000	c
15. Isonicotinic acid hydrazide	>1000	> 1000	c
16. Allyl Glycine [e]	—	> 1000	c

LD_{50} = a. <100 μg/g
  b. 100-400 μg/g
  c. >400 μg/g
  d. methyl dithio carbazinate. see Ref. (18)
  e. see Ref. (19)
  f. for compounds 1-5, 10 μm caused 100% GAD Inhibition

Inhibition of GAD *In Vivo*

An experiment was designed to assess directly the importance of GAD
inhibition in generating the symptoms displayed by the relatively
toxic ethyl aminooxybutyrate (compound 6, Tables I and II). Cock-
roaches were injected with 25 µg/g (LD_{50}) or 500 µg/g of this com-
pound and were sacrificed 2 hours later. The GAD activity in the
flight muscles was assayed in exactly the same manner as for the *in
vitro* assays. Several untreated insects served as the control. The
enzyme assays were run in triplicate at two different protein
concentrations (28 and 56 µg protein per assay) and the amount of
$^{14}CO_2$ generated in the controls (17 pmol/min/mg protein) was very
similar to that obtained in the control preparation used for the
earlier *in vitro* experiments. The treated insects showed reduced
GAD activity at each dose. At 25 µg/g, 68% and 78% inhibition were
observed at the two protein concentrations, whereas at 500 µg/g, 70%
and 77% inhibition were observed. These very similar degrees of GAD
inhibition were thus obtained from cockroaches which had been
treated with widely differing doses causing different toxic
manifestations: the insects at 25 µg/g were either unaffected or
else were showing only mild tremoring, but those at 500 µg/g were
prostrate with little leg movement at the time of sacrifice. The
similarity of GAD inhibition combined with the large difference in
symptomology suggests that GAD inhibition by this compound probably
plays only a minor role in causing toxicity.

Reactivity of Aminooxy Compounds with Pyridoxal Phosphate

Pyridoxal phosphate (PLP) in aqueous solution has an absorbance
maximum at 380 nm, with a smaller peak at 330 nm (Figure 4). After
0.1 mM PLP was reacted with 1 mM of the aminooxy acids in 100 mM Na
Bicine buffer, pH 7.5, for 16 h at 4°C (1-4 in Tables I and II), the
peak at 380 nm was abolished and instead a new peak at about 335 nm
appeared (Figure 4). A similar spectral shift is observed during
the reaction occurring between hydroxylamine and pyridoxal phosphate
where the reaction product formed is an oxime. No reaction with PLP
was observed with either of the BOC(*t*-butyloxy carbonyl) compounds
(compounds 9 and 10, Table I). It is perhaps worth noting that the
inhibition of GABA-T by aminooxy acids *in vitro* could be abolished
by adding excess PLP to the incubation mixture. Furthermore,
compounds 1-4 (Tables I and II) were very active against alanine
aminotransferase, (from pig heart, Boehringer-Mannheim Biochemicals)
another PLP-dependent enzyme, having Kd values in the range of 40 to
240nM.

Discussion

The original purpose of this study was to address the issue of
whether GABA-T was a realistic target site for insecticides. A
range of inhibitors was synthesized and tested *in vitro* and *in vivo*.
One interesting finding from the *in vitro* experiments was that
aminooxybutyrate was over 50 times more potent than aminooxyacetate,
which is a "standard" inhibitor in the literature (17). The data
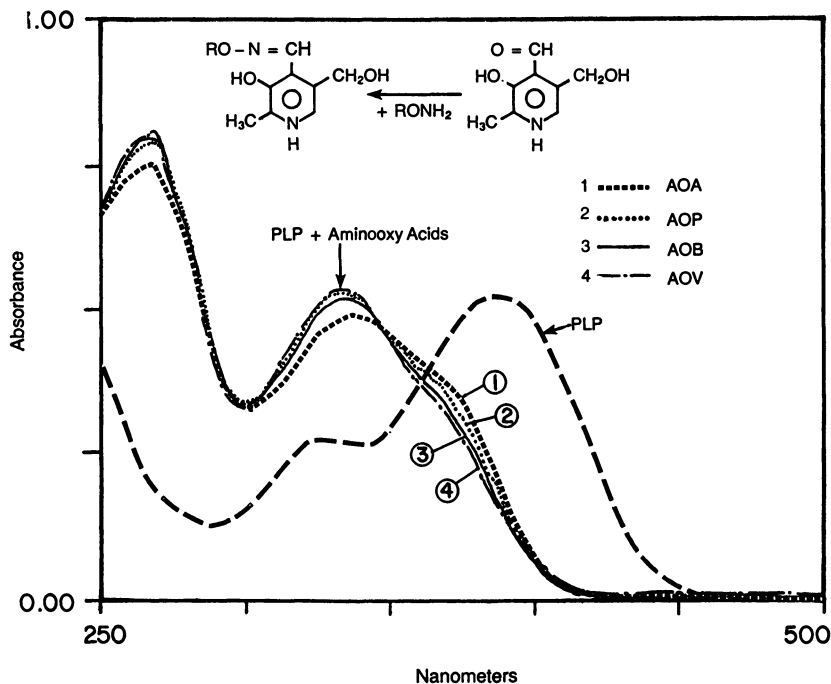
Figure 4:   Absorbance spectrum for pyridoxal phosphate, alone and in the presence of aminooxy acetate(1), propionate(2), butyrate(3) and valerate(4).

presented suggest that the butyrate may fit the active site of GABA-T better because its skeleton most resembles the substrate GABA.

In vivo, none of the GABA-T inhibitors showed any significant insecticidal activity in conventional screens against pest species. In the cockroach, however, activity was observed when certain compounds were injected into the thorax. The toxicity correlated only slightly with inhibitory potency against GABA-T, but a variety of pharmacokinetic factors could have been responsible for the limited correlation. However, the experiment involving injection of [14C]GABA into cockroaches that had been pretreated with either gabaculine or ethyl aminooxybutyrate, in which no symptoms were observed although 14CO2 evolution was inhibited, compared with control, showed clearly that inhibition of GABA-T does not represent a feasible mechanism of action for an insecticide. Indeed, the protection against ethyl aminooxybutryate toxicity afforded by gabaculine strongly suggests that GABA-T inhibition will tend to counteract the effects of certain types of toxicant.

In an attempt to explain the symptomology and toxicity of the aminooxy compounds, the inhibition of GAD was also studied in the cockroach. There was some correlation between inhibitory potency *in vitro* and toxicity, as was the case with GABA-T inhibition. Both toxic and non-toxic doses of ethyl aminooxy butyrate caused the same degree of suppression of *in vivo* GAD activity, suggesting that GAD

may not be a good target for insecticidal action either. The
possibility that there are two isozymes of GAD (15) with different
susceptibilities to inhibition cannot be excluded. For example, it
is possible that GAD activity in the CNS, rather than in the flight
muscles, is better correlated with symptomology and toxicology. A
definitive answer to the question of whether GAD represents a
potential target site for insecticides must await the synthesis of a
specific GAD inhibitor, analogous to gabaculine for GABA-T.
     The biochemical basis for the toxic effects of some of these
compounds remains speculative. GAD inhibition may account for some
of the symptoms, but it is possible that the aminooxy compounds are
interfering with a neuroreceptor, perhaps for GABA or glutamate. One
compound, the BOC amine of aminooxybutyrate, caused rapid
prostration without tremoring, and although it caused some
inhibition of both GAD and GABA-T, it is not able to form a Schiff's
base with PLP. It must have a different mechanism of action,
therefore, from the other aminooxy compounds, perhaps instead acting
as a glutamate receptor antagonist or GABA agonist.

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Chapter 10

Octopamine in Insects

Control of Hemolymph Lipid and Visceral Muscle in Locusts

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The distribution, physiological role, mode of action
and pharmacology of octopamine
(1-(p-hydroxyphenyl)-2-aminoethanol) in insects is
reviewed. Octopamine is found throughout the ventral
nerve cord, being concentrated within the ganglia.
Identified octopaminergic neurons belong to the
unpaired median class. Peripherally, octopamine is
associated with a broad range of tissues, including
muscular, reproductive and lantern tissue. Octopamine
functions as a neurotransmitter, neurohormone and
neuromodulator in insects, and octopamine has been
proposed as the mediator of a sympathomimetic response.
Two examples of the multifunctional properties of
octopamine in locusts are highlighted; namely the
control of haemolymph lipid and oviduct visceral
muscle. Adenosine 3',5'-monophosphate (cylcic AMP) is
believed to act as the cellular mediator for many of
the physiological actions of octopamine, although at
least one other mechanism of action exists.
Pharmacologically, octopamine receptors exhibit a
specificity for monophenolic amines with a single
hydroxyl group on the aromatic ring. They are
susceptible to vertebrate α-adrenergic receptor
antagonists, although the overall pharmacological
properties of the octopamine receptors do not conform
to any one recognised vertebrate receptor.

Octopamine, (1-(p-hydroxyphenyl)-2-aminoethanol), derives its name
from the octopus in whose salivary glands it was first discovered
(1). As a biogenic amine, octopamine remained in relative obscurity
until reports of its presence in the nervous tissue of a variety of
invertebrates began to be published (2,3). These studies were made
possible by the development of a sensitive and specific
radioenzymatic assay (4-6). By means of this assay octopamine has
now been found to be present in the nervous tissue of all
invertebrates examined and, of vital importance, found within

individual identifiable neurons (7-10). Physiological studies have
now indicated that octopamine performs many roles in invertebrates,
and certainly within insects is multifunctional, demonstrating
properties of a neurotransmitter, neurohormone and neuromodulator
(for definitions see 11, 12). The scientific importance of
octopamine may be attested to by the number of reviews it has
prompted in such a short time (11-14). The present review will build
upon the earlier ones and examine the distribution, physiological
role, mode of action and pharmacology of octopamine in insects. In
particular the review will highlight two examples in locusts in which
octopamine demonstrates its multifunctional properties, namely the
regulation of haemolymph lipid levels and the regulation of visceral
muscle.

Distribution

Octopamine is structurally very similar to norepinephrine being
different only in lacking the 3-hydroxyl group on the aromatic ring
(Figure 1). It may therefore be considered the monophenolic analogue
of norepinephrine. In the vertebrate nervous system octopamine is
synthesized by a decarboxylation of tyrosine to tyramine, and then by
a subsequent β-hydroxylation of tyramine (15). Whilst the enzymes
have not been purified and characterized in insects a similar pathway
appears to occur, since radiolabelled tyrosine and tyramine may be
metabolized to octopamine by insect nervous tissue (3,16-17).
Further metabolism of octopamine by phenylethanolamine-N-methyl
transferase (PNMT) may produce synephrine (Figure 1). Although
synephrine does not occur naturally in invertebrates, it lays the
foundation for the radioenzymatic assay for octopamine in which
endogenous octopamine in tissue extracts is converted to
radiolabelled synephrine using PNMT and $[^3H]$-S-adenosylmethionine as
the methyl donor. The amount of $[^3H]$-synephrine formed is measured
following suitable solvent extraction. The naturally occurring
isomer of octopamine in insect haemolymph and nervous tissue is
D(-)octopamine (18,19). By necessity (since D(-)octopamine is not
commercially available) DL-octopamine is used as a standard in these
assays which results in a higher estimation of naturally occurring
octopamine. A correction factor is rarely applied.
      Measurements of octopamine in insects have almost exclusively
been made using the radioenzymatic assay. However, recent advances
have now resulted in the availability of high performance liquid
chromatography coupled with electrochemical detection for the
measurements of catecholamines, monoamines and their metabolites
(20,21). This technique affords the advantage of estimating several
compounds simultaneously in a single sample. One may anticipate an
increase in the use of this technique in the near future.
      The typical distribution of octopamine in the central nervous
system of insects is depicted in Figure 2. Octopamine is found
throughout the ventral nerve cord, being concentrated within the
ganglia. The cerebral ganglion and optic lobe contain particularly
high concentrations. Insect neurohaemal organs (the corpora cardiaca
and perisympathetic organs) also contain octopamine where it may be
released into the haemolymph as a neurohormone and/or control the
release of peptidergic hormones (see later). Peripherally,
octopamine is associated with a broad range of tissues, including
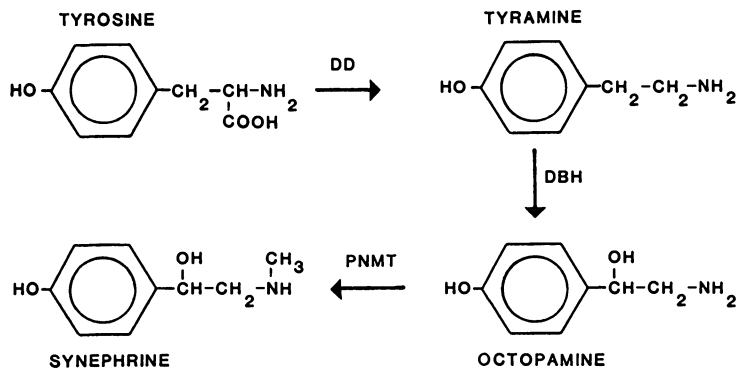
Figure 1. Pathway for the production of phenolamine from tyrosine. The reactions are catalyzed by the following enzymes: DD, DOPA decarboxylase; DBH, dopamine β-hydroxylase; PNMT, phenylethanolamine-N-methyltransferase. Data from reference 12.

STRUCTURE	OCTOPAMINE (pmol/structure)
optic lobe (per pair)	17.2
cerebral ganglion	11.6
corpora cardiaca (per pair)	0.5
corpora allata (per pair)	0.2
suboesophageal ganglion	4.7
prothoracic ganglion	5.4
pro-meso connectives (per pair)	0.7
mesothoracic ganglion	6.2
metathoracic ganglion	4.3
perisympathetic organ	0.05
abdominal ganglion	1.0
terminal abdominal ganglion	2.7

Figure 2. Distribution of octopamine throughout selected parts of the central nervous system of Schistocerca americana gregaria. Data from reference 22.

both skeletal and visceral muscle (heart, spermiduct and oviduct), salivary glands, malpighian tubules, fat body, eggs, haemolymph and light organ of fireflies (see 12).

At the present time there is no specific histochemical technique available for the localization of octopamine. Octopaminergic cells do not fluoresce with the Falck-Hillarp or glyoxylic acid histofluorescent techniques for catecholamines and indolalkylamines. This may be used to some advantage, however, since octopaminergic neurons, like other aminergic neurons of invertebrates, stain specifically with the dye neutral red. Thus any cell which stains with neutral red but does not fluoresce with the above techniques may be tentatively identified as octopaminergic. Caution must be exercised however since neutral red may not be selective for aminergic neurons (see 12) and so it is necessary to supplement the information with biochemical analysis. Such has been done for a variety of systems. For example, the dorsal surface of a number of insect thoracic and abdominal ganglia contain a group of highly specialized cells which are unpaired and have bifurcating axons which project symmetrically into left and right peripheral nerve roots of the ganglion (see 23). Accordingly they are referred to as dorsal unpaired median (DUM) neurons (24). More recently neurons with the same appearance were found on the ventral surface of the VIIth abdominal ganglion of locusts and referred to as ventral unpaired median (VUM) neurons (10). Both DUM and VUM neurons stain with neutral red but show no fluorescence with Falck-Hillarp or glyoxylic acid staining methods. **Table I** illustrates the octopaminergic nature of some of these neurons. These studies have led to the belief that all DUM cells are octopaminergic. In addition, golgi cells of the calyx of the mushroom bodies of cockroach, bee, locust and cricket brains have also been shown to contain octopamine (26,27).

A knowledge of the distribution of octopamine throughout the nervous system and in particular the identification of octopamine-containing cells has led to rapid advances in our understanding of the role of octopamine in insects.

Physiological role

Much evidence has accumulated that octopamine functions as a neurotransmitter, neurohormone and neuromodulator in insects (for reviews see 11, 12). The physiological events influenced by octopamine are those that might be expected to be associated with a generalized sympathetic response and indeed, octopamine has been proposed as the mediator of a sympathomimetic response in insects. It is the insect equivalent of epinephrine and norepinephrine. Certainly much of the interest in octopamine has arisen because it appears to combine many of the roles of these two vertebrate substances and therefore acts in a multifunctional fashion.

Table II illustrates some of the systems in which there appears to be evidence for physiological control by octopamine. A most noticeable feature of these examples is the broad range of actions which octopamine possesses.

Dual role for octopamine in the control of haemolymph lipid during flight in locusts. In locusts, octopamine performs a dual role in the control of haemolymph lipid during flight (Figure 3). It behaves

Table I. Octopamine content of isolated cells

Insect	Cell	Octopamine/soma (pmol)	Reference
Schistocerca americana	DUMETi	0.099 ± 0.023	7
Schistocerca nitens	DUM(metathorax)	0.14 ± 0.02	25
Locusta migratoria	DUMOV1	0.35 ± 0.14	9
	DUMOV2	0.33 ± 0.09	9
	VUM	0.39 ± 0.05	10
Photuris versicolor	DUM(lantern)	0.03 ± 0.006	8
Periplaneta americana	DUM(metathorax)	0.085 ± 0.014	26
	DUM(abdomen)	0.140 ± 0.038	26

DUM(abdomen), unidentified dorsal unpaired median neurons in abdominal ganglion
DUM(metathorax), unidentified dorsal unpaired median neurons in metathoracic ganglion
DUM(lantern), dorsal unpaired median neurons innervating the lantern
DUMETi, dorsal unpaired median neurons innervating the extensor tibiae muscle
DUMOV, dorsal unpaired median neurons innervating the oviducts
VUM, unidentified ventral unpaired median neurons of VIIth abdominal ganglion

Table II. Physiological roles for octopamine in insects

	Insect	Target tissue	Role for octopamine	Source of octopamine	Reference
Neurotransmitter	Photuris	Lantern	Control of light production	DUM	8,28,29
	Locusta	Glandular lobe	Release of adipokinetic hormone	Brain	30-32
	Periplaneta	Corpus cardiacum	Release of hyper-trehalosemic hormone	Brain	33
Neurohormone	Locusta	Fat body	Release of lipid	Neurohaemal organs?	34,35
	Periplaneta	Fat body	Stimulates glycogenolysis	Neurohaemal organs?	36,37
	Periplaneta	Haemocytes	Unknown	Neurohaemal organs?	38,39
	Periplaneta	Nerve cord	Stimulates glycogen phosphorylase and glyco-genolysis	Neurohaemal organs?	40
Neuromodulator – peripheral	Schistocerca	Extensor – tibiae muscle	Inhibits myo-genic contractions Modulates neuro-muscular transmission	DUMETi	7,41,42
	Locusta	Oviduct visceral muscle	Inhibits myo-genic contractions Modulates neuro-muscular transmission	DUMOV	9

Table II.--Continued

	Insect	Target tissue	Role for octopamine	Source of octopamine	Reference
	Manduca	Dorsal longi-tudinal flight muscles	Modulates neuro-muscular trans-mission	Unknown	43
	Hemideina	Extensor-tibiae muscle	Modulates neuro-muscular trans-mission	DUMETi	44,45
			Induces catch-like tension		
Neuromodulator – central	Schistocerca	Central motor neurons	Dishabituation and sensitisa-tion of excita-tory synapses	DUM?	46
			'Orchestration hypothesis' generation of specific behaviours	DUM?	47
	Manduca	Central neurons (flight pattern generator)	Alters level of excitation and effectiveness of synaptic trans-mission	Unknown	48
	Corydalus	Central neurons	Increases frequency of ventilatory rhythm	Unknown	49
	Phormia	Central nervous system	Stimulates feeding behaviour	Unknown	50
	Apis	Brain	Enhances light-evoked poten-tials in mush-room bodies	Golgi cells in mushroom bodies?	51

DUM, dorsal unpaired median neuron; DUMETi, dorsal unpaired median neuron innervating the extensor tibiae muscle; DUMOV, dorsal unpaired median neuron innervating oviducts
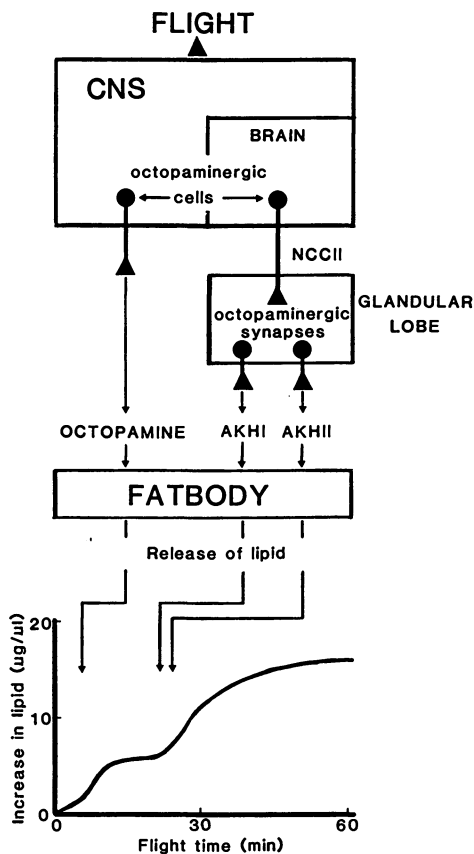
Figure 3. Model depicting the involvement of octopamine in the control of haemolymph lipid levels during flight in <u>Locusta migratoria</u>. Explanation in text. Redrawn with modifications from reference <u>52</u>.

as a neurotransmitter controlling the release of peptidergic
adipokinetic hormones as well as a neurohormone with direct action
upon the fat body (see 52). The glandular lobe of the corpus
cardiacum of locusts is composed of neurosecretory cells which
possess short axon-like processes (53). These neurosecretory cells
are the source of two peptidergic adipokinetic hormones (AKHI and
AKHII), both of which are released in response to flight (54). These
hormones mediate a mobilization of lipid from the fat body, thereby
providing an energy source for sustained flight. Electrical
stimulation (in vitro) of the nervi corporis cardiaci II (NCCII),
which project into the glandular lobe from the brain, results in the
release of both AKHs (30,55). The neurally-evoked release of AKHs
involves synapses between axons of NCCII and the neurosecretory
cells. These synapses are characterized by the presence of large
electron dense granules (100 nm diameter) and numerous small clear
vesicles (56). These ultrastructural features are similar to those
of identified octopaminergic neurons (57,58) and this, coupled to the
demonstration of the presence of octopamine within the glandular lobe
(30, 59,60) and within NCCII (60) suggests that octopamine may be the
natural transmitter mediating the release of AKHs. Physiological
evidence has now accumulated to indicate that this may be true.
Thus, the neurally-evoked release of AKHs is abolished after
reserpine treatment and by treatment with α-adrenergic receptor
antagonists (30). Exogenous application of the natural transmitter
to the in vitro glandular lobe should mimic the effect of neural
stimulation, and indeed, low concentrations of octopamine (10^{-7}M),
but not of other naturally occurring amines, induce the release of
significant amounts of hormone (32). This response is again blocked
by α-antagonists (30). It seems clear therefore that octopamine is
the transmitter mediating the release of peptidergic AKHs in
locusts.
     The possibility that octopamine may be a neurohormone arose with
the demonstration of its fluctuating presence in the haemolymph of
insects. It is released into insect haemolymph at times of stress
(20,34,61,62) where it appears to mediate a mobilization of lipid and
trehalose (35, 37). In both locusts and cockroaches octopamine has
been shown to have a direct action upon the fat body (35-37). These
effects suggest that octopamine can act as a 'sympathetic'
circulating hormone. Of particular importance to the present review
is the fact that in both Schistocerca and Locusta the titres of
haemolymph octopamine rise during the initial minutes of flight (18,
52). The increase in Locusta is above that induced by stress alone
(52). Since octopamine acts directly upon locust fat body to release
lipid (35) it seems clear that octopamine must be used as an
adipokinetic hormone during the initial minutes of flight. Careful
examination of the increase in haemolymph lipid concentration during
flight in Locusta reveals a biphasic pattern (54). There is an
initial elevation within the first 10 min of flight, and a second
larger elevation which begins after approximately 20 min.
Examination of the titres of the three adipokinetic hormones,
octopamine, AKHI and AKHII (see 52) reveals a model as shown in
Figure 3. Flight induces an elevation in haemolymph octopamine which
acts on the fat body to result in the initial elevation in haemolymph
lipid. After some minutes, the release of the peptidergic AKHs is
stimulated by octopaminergic synapses within the glandular lobe.

These peptidergic hormones then result in the sustained output of
lipid from the fat body. Octopamine thus serves a role as a
neurotransmitter controlling release of peptidergic AKHs and a
neurohormone acting directly upon the fat body during flight in
locusts. It is now apparent that octopamine may also mediate the
release of other peptides from the corpus cardicaum (63–65).

Neuromodulation of visceral muscle. The most extensive studies of
identified octopaminergic neurons have been made using DUMETi (dorsal
unpaired median neuron innervating the extensor tibiae muscle), and
it is now well established that octopamine acts as a modulator of
neuromuscular transmission and muscle contraction in insect skeletal
muscle (see 12 for complete review). Recently, a system has been
described which illustrates that insect visceral muscle is also under
the modulatory influence of octopaminergic neurons. In Locusta, two
dorsal unpaired median neurons (DUMOV1,2) lying in the VIIth
abdominal ganglion (Figure 4) project axons to the visceral muscles
of the oviduct (66). These cells possess morphological and
physiological characteristics in common with DUMETi and the firefly
DUM neurons, and like these neurons, stain selectively with neutral
red dye. Radioenzymatic assays reveal that DUMOV1 and 2 are
octopaminergic (9), with octopamine present in the cell bodies as
well as in the oviducal nerve and innervated regions of the oviducal
muscle (Figure 4). Intrasomatic stimulation of DUMOV results in a
calcium-dependent release of octopamine, as does stimulation of DUMOV
axons (67). This represents only the second demonstration of the
release of octopamine from identified neurons (68), although a recent
study has demonstrated calcium-dependent, high potassium-induced
release of octopamine from firefly lanterns (69) which clearly
represents release from DUM terminals. Physiologically, octopamine
reduces the amplitude of neurally-evoked contractions of the oviduct,
with a threshold lying between 5×10^{-10}M and 7×10^{-9}M, and
half-maximum effect at about 5×10^{-7}M (9). It also results in a
relaxation of basal tonus and inhibition of myogenic contractions
(70). Recent studies (71) indicate that octopamine reduces the
amplitude of excitatory junction potentials and hyperpolarizes the
muscle membrane potential. Thus, octopamine is modulating
contractions of this visceral muscle. Octopamine has previously been
shown to potentiate neuromuscular transmission in a number of
arthropod preparations and in some systems alter the myogenic
activity (see 9). In locust oviducts, octopamine inhibits myogenic
contractions and contractions induced by both glutamate and proctolin
(70) indicating that octopamine receptors occur post-synaptically in
this system. This is similar to the situation proposed for some
lobster (72) and locust neuromuscular preparations although in other
preparations octopamine has been shown to have a presynaptic action
(see 9,12). Whether or not the reduction in amplitude of excitatory
junction potentials and neurally-evoked contractions in locust
oviducal muscle are produced by pre-synaptic receptors remains to be
seen.

Central actions of octopamine. There is no doubt that, at the
present time, more detail is known about the peripheral effects of
octopamine than its central effects. This is almost certainly due to
the amenability of peripheral preparations to experimentation. Table

OCTOPAMINE CONTENT

0.63 ± 0.14 nmol/g wet wt

0.34 ± 0.08 pmol/cell body
$(1.89 \times 10^{-3} M)$

1.62 ± 0.3 pmol/cm nerve

13.7±4.7 nmol/g wet wt

lateral oviduct

common oviduct
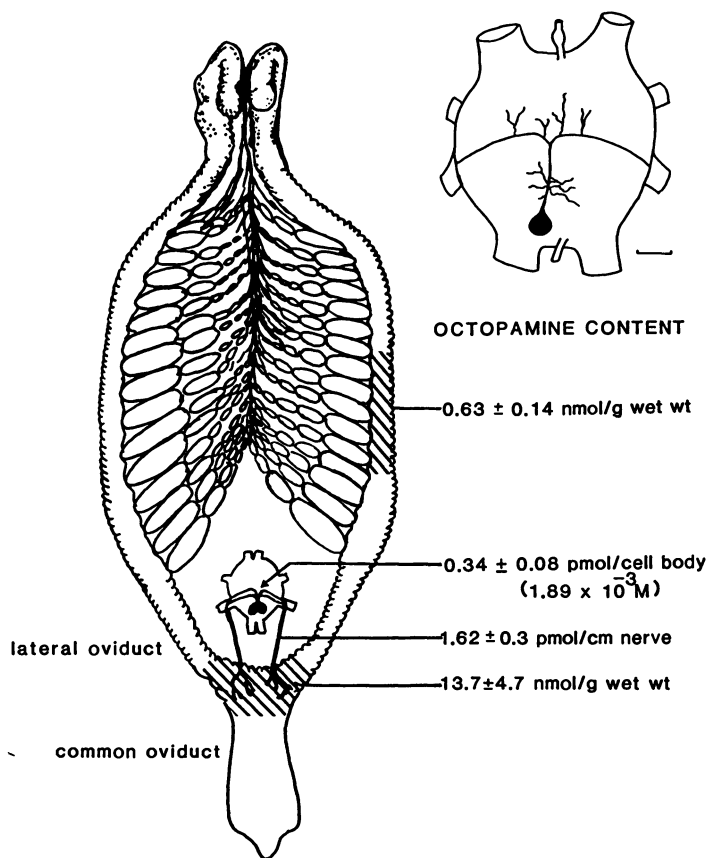
Figure 4. Octopamine content of various tissues associated with
the oviducts of <u>Locusta</u> <u>migratoria</u>. Shaded areas of oviducts
illustrate the approximate sites which were assayed. Inset upper
right depicts a camera lucida drawing of one of the dorsal
unpaired median neurons (DUMOV) injected with Lucifer yellow.
Scale bar: 50 µm. Data from reference 9.

II illustrates some of the possible targets for octopamine within the central nervous system, and indicates that octopamine plays an important physiological role as a transmitter or modulator in this area. In addition, injections of octopamine or its mimics into the haemolymph or ganglia, induces behavioural responses. Honeybees show enhanced responsiveness to light and olfactory stimuli after injection of octopamine into the brain (51,73). Blowflies develop a decreased threshold for the intake of sucrose solutions and also consume more sucrose after the injection of octopamine or related compounds (50), while cockroaches exhibit anorexia (74).

Iontophoretic studies (47), in which octopamine has been injected into localized areas of a ganglion, indicate that the primary role of octopamine may well be to generate specific behaviours. These studies have led to the "orchestration hypothesis" (47). In this hypothesis octopamine is released from modulator (M) neurons. It is suggested that a behaviour cannot occur, or is unlikely to occur, without the release of octopamine from the appropriate M neurons, along with neural circuit activation for the particular behaviour. While the general release of octopamine may increase the probability of any behaviour occurring, the behaviour which actually occurs will depend upon which group of M neurons is most strongly active at that time. Thus, octopamine would play a pivotal role in co-ordinating behaviours at the appropriate times.

In view of the ability of the formamidines and imidazolines to upset the behaviour of certain pest species, it seems likely that the central nervous system may well be an important site of action for such compounds, and highlights the need for further, more detailed studies, upon the central actions of octopamine.

Mode of action

Evidence has accumulated over recent years for the presence of octopamine-sensitive adenylate cyclase in invertebrate tissues, in particular those of insects (for references see 75). The elevated levels of adenosine 3',5'-monophosphate (cyclic AMP) induced by octopamine are believed to act as the cellular mediators for the physiological actions of octopamine. Thus, octopamine has been shown to either activate adenylate cyclase or elevate cyclic AMP in homogenates of thoracic ganglia of cockroach (76), brains of various insects (77-79), locust flight muscle (80), locust extensor-tibiae muscle (75), locust oviduct (81), locust glandular lobe (31), firefly lantern (82), locust and cockroach fat body (35,79,83) and haemocytes of the forest tent caterpillar (38,39). The properties of the octopamine-sensitive adenylate cyclase in insects are very similar to those of catecholamine-sensitive adenylate cyclase in vertebrate tissue (see 84). Furthermore, stimulation of this enzyme by various agonists and antagonists correlates well with the rank order of potency of these same agents in causing the physiological effects of octopamine (see later). This in itself, of course, is not sufficient to distinguish cause and effect and until recently little was known about the physiological significance of octopamine-sensitive adenylate cyclase in invertebrates. This question has recently been addressed in three tissues known to receive innervation from identified octopaminergic neurons. Nathanson (85) found that injection of cholera toxin into fireflies caused a delayed,

non–hormone–dependent activation of adenylate cyclase, an elevation
in cyclic AMP, and a persistent glow of the firefly light organ.
This, coupled to the fact that phosphodiesterase inhibitors can cause
glowing and potentiate the effects of octopamine (see 85), indicates
that the initiation of the normal adult firefly flash is mediated
through an elevation of cyclic AMP levels secondarily to the
activation of an octopamine–sensitive adenylate cyclase.

      With the discovery of identified octopaminergic neurons in
insects it has now been possible to extend the observations of
effects of octopamine upon broken cell preparations (77,78,82) and
intact tissue (31,35,83) and to include direct activation via
neurons. Recently, in a pioneering series of experiments Evans (75)
demonstrated the ability of an identified octopaminergic neuron
(DUMETi) to increase the levels of cyclic AMP in the extensor–tibiae
muscle of locusts. Furthermore, it was shown that elevation of
cyclic AMP by means of the phosphodiesterase inhibitor isobutyl
methylxanthine (IBMX), and forskolin (a direct activator of the
catalytic sub–unit of adenylate cyclase) mimicked some of the
physiological effects of stimulating DUMETi and of octopamine. Thus
for the first time, neurally–mediated and octopamine–mediated changes
in cyclic AMP have been related to their corresponding physiological
effects.

      A similar series of experiments has now been performed in insect
visceral muscle (81). Octopamine elevates cyclic AMP content of the
lateral oviduct of locusts. This effect is dose–dependent in the
presence of IBMX, with a threshold dose at about 10^{-8}M and
half–maximal stimulation at 7×10^{-7}M. Forskolin also elevates cyclic
AMP content and IBMX potentiates the action of forskolin.
Stimulation of the two identified octopaminergic neurons which
project to the lateral oviducts also results in an elevation in
cyclic AMP. The major physiological effects of octopamine (reduction
in amplitude of neurally–evoked contractions and inhibition of
myogenic contractions) are induced by agents which artificially
elevate cyclic AMP levels (IBMX, forskolin, dibutyryl cylic AMP).
The evidence strongly suggests that, as with skeletal muscle, the
octopaminergic control of this insect visceral muscle is mediated at
least in part by cyclic AMP.

      An octopamine–sensitive adenylate cyclase is not the only means
by which octopamine may exert its effects. Whilst DUMETi and
octopamine modulate the amplitude and rate of relaxation of twitch
tension in the extensor tibiae muscle of locusts via cyclic AMP,
their effects upon the myogenic rhythm are mediated via a different
route (86). Thus, elevated cyclic AMP does not induce a decrease in
the frequency of myogenic rhythm as does octopamine, and the effects
of octopamine are not potentiated by IBMX. In fact the mode of
action of octopamine in reducing the frequency of myogenic
contractions remains unknown.

Pharmacology

Extensive pharmacological investigations of octopamine receptors have
been reported for locust skeletal muscle (87), firefly lantern
(82,88,89), tobacco hornworm central nervous system (88,89),
cockroach brain (77), fruit fly heads (90), locust visceral muscle
(91), locust glandular lobe (92) and cockroach haemocytes (39).

Comparing the effectiveness of compounds upon these preparations,
however, can be very frustrating. The compounds have been tested on
the physiological response, adenylate cyclase activity, cyclic AMP
accumulation and receptor binding, and these varied methods give
different results. A major problem of course is that there are
clearly different types of octopamine receptors. For example, Evans
(87) distinquished three different classes of octopamine receptor
mediating physiological changes in the extensor tibiae muscle of
locusts. These are referred to as octopamine-1, octopamine-2A and
octopamine-2B receptors on the basis of sensitivity to various
agonists and antagonists. Subsequent work revealed that the
octopamine-2 receptors appeared to be linked to adenylate cyclase
whereas octopamine-1 receptors were not (75,86). If these
populations of receptors (and possibly others?) exist in
preparations, then the biochemical analysis may not reflect the
response measured physiologically (and vice – versa). For example,
 the α-adrenergic agonist tolazoline is a full agonist of octopamine
1, 2A and 2B receptors in the extensor-tibiae muscle assay (87).
However, when studying cyclic AMP accumulation in the same
preparation, tolazoline is only a weak partial agonist (75).
Similarly, tolazoline is a weak agonist of the octopamine receptor
linked to adenylate cyclase in firefly lantern (93) and tobacco
hornworm central nervous system (88) and has no agonistic activity at
$10^{-5}M$ on cockroach haemocyte adenylate cyclase (39) or locust oviduct
cyclic AMP accumulation (91). With these problems in mind, what can
be said about octopamine receptors? Certainly octopamine receptors
exhibit a specificity for monophenolic amines with a single hydroxyl
group on the aromatic ring. The N-methylated analogue of octopamine,
synephrine, is a potent agonist of octopamine receptors, although the
relative potencies of octopamine and synephrine vary between
preparations. Thus, in the locust extensor-tibiae muscle (7,42) and
firefly light organ (94) synephrine is more potent than octopamine
when the physiological response is examined, whereas in locust
oviduct muscle they are equipotent (9). In locust oviduct this
equipotency is reflected in their abilities to elevate cyclic AMP
(91), whereas in studies of adenylate cyclase activity in cockroach
brain (77) and cockroach haemocytes (39), octopamine is more potent
than synephrine.
    One of the general features of octopamine receptors appears to
be their susceptibility to vertebrate α-adrenergic receptor
antagonists, and their insensitivity to β-adrenergic antagonists.
Thus antagonists such as phentolamine are competitive inhibitors of
octopamine whereas agents such as propranolol are not. Similarly,
vertebrate α-adrenergic agonists such as clonidine and tolazoline
have been shown to be agonists of certain octopamine receptors (87).
These results have led to the general view that octopamine receptors
are similar to vertebrate α-adrenergic receptors. It is important
to emphasize, however, that the overall pharmacological properties of
the octopamine receptor do not conform to any one recognized
vertebrate receptor. Thus, while being sensitive to α-antagonists,
the octopamine receptor is also antagonized by vertebrate
serotoninergic and histaminergic antagonist such as cyproheptadine,
gramine and mianserin (39,77,82,87,91). Furthermore vertebrate α-
 receptors are not typically coupled to adenylate cyclase, whereas
octopamine receptors are (see earlier).

Interest in octopamine receptors has grown with the discovery that a novel group of pesticides, the formamidines, are octopamine agonists (95). Thus chlordimeform (CDM) or demethylchlordimeform (DCDM) are able to activate octopamine receptors in firefly lantern (95,96), tobacco hornworm central nervous system (88), locust skeletal muscle (97), locust oviduct muscle (91), locust corpora cardiaca (98), locust fat body (99), and cockroach haemocytes (39). In some preparations CDM itself is active (97) whereas in others it appears to require metabolic conversion to DCDM (95,96). In terms of octopamine-sensitive adenylate cyclase (96) and octopamine-mediated elevations in cyclic AMP (39,91,100), DCDM is a potent though partial agonist of octopamine receptors, being anywhere from 80%-53% as effective as octopamine. However in tobacco hornworm central nervous system, DCDM is a complete agonist (88). The pesticidal properties of the formamidines appear to be mediated through cyclic AMP increases (see 88,89,93) and, it is believed, via octopamine receptors. Formamidines induce behavioural changes in insects (eg. feeding, mating, dispersion), with the insect dying as a result of abnormal behaviour rather than from any direct lethal actions of the pesticide (see 88). More recently (88,89,93) a different class of potent octopamine agonists has been defined and characterized, the substituted phenyliminoimidazolidines (PIIs). Some of these have potencies exceeding those of any previously described agonists of octopamine-sensitive adenylate cyclase, whereas others are antagonists. In a similar fashion to the formamidines, the PIIs also cause disruption of motor and feeding behaviour in tobacco hornworms, leading to insect death.

Concluding remarks

Octopamine is one of the most abundant biogenic amines found in the insect nervous system. Its ubiquitous presence in nervous systems coupled to its broad range of actions has led to a rapid accumulation of knowledge about this amine, which is considered to be the insect equivalent of epinephrine and norepinephrine. Octopamine has both central and peripheral actions, where it serves as a neurotransmitter, neurohormone or neuromodulator. It thus manages to provide flexibility in the speed of delivery, length and privacy of messages it sends to key physiological processes. Its messages are made even more flexible by the possession of more than one mode of action. Whilst much interest has focussed on octopamine-sensitive adenylate cyclase, there appears to be at least one other action not associated with adenylate cyclase. As future studies extend our knowledge of mechanisms controlled by identified octopamine neurons, so our knowledge of the mode of action of octopamine should grow. Octopamine receptors show some pharmacological similarities to vertebrate α-adrenergic receptors, and recently a variety of potent agonists have been characterized (the formamidines and PIIs). The overall importance of octopamine in insects is emphasised by the destructive influence of the formamidines and PIIs. Thus octopamine 'orchestrates' appropriate behaviors and "prepares" the insect for stressful conditions. It mediates a "flight or fight" response. Disturbing octopamines' homeostasis results in behaviors occurring at inappropriate times. Clearly a complete understanding of octopamine in insects may well lead to the development of

selective pesticidal agents with decreased toxicity for vertebrates.

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Chapter 11

Phenylethanolamine Receptors as Selective Targets for Pesticide Action

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Exogenously applied octopaminergic agonists can disrupt
insect behavior and interfere with feeding. Therefore,
the potential exists for the development of new, potent
octopamine analogs which would have pesticidal toxicity
for insects. Because membrane receptors for octopamine
appear to be present primarily in invertebrates, such
analogs would have reduced toxicity for mammals and
other vertebrates. Recently developed synthetic octop-
amine analogs, such as the substituted phenyliminoimid-
azolidines, should help to clarify the characteristics
of octopamine receptor subtypes, some of which are
associated with the activation of adenylate cyclase.
Furthermore, certain derivatives of these ligands can
be used to irreversibly label octopamine receptors,
thereby providing a mechanism for characterizing the
biochemical properties of insect phenylethanolamine
receptors.

Substantial biochemical and physiological evidence indicates that
octopamine is a major aminergic neurotransmitter in invertebrates,
having both neurohumoral and neurotransmitter roles (1-3). Studies
from this and other laboratories indicate that octopamine may be a
selective neurotransmitter, having a physiological role in inverte-
brates but not vertebrates. As will be described below, this selec-
tivity should allow the development of potent octopamine analogs
which have selective toxicity for insects but not for mammals and
other vertebrates. To help achieve such a goal, what is needed is a
greater understanding of the pharmacology of octopamine receptors in
insects and a better knowledge of how these receptors may vary among
different insect species.   Recently, we have characterized a new
series of reversible and irreversible octopamine receptor ligands.
As described in preliminary studies below, these ligands, may help
us to begin to understand the molecular pharmacology of octopamine
receptors and, ultimately, to develop compounds which will be useful
as pesticides with very low toxicity for mammals.

Background

Octopamine (p-hydroxyphenylethanolamine) was first discovered in the
posterior salivary gland of the octopus over 30 years ago by
Erspamer and Boretti (4).   Although similar to norepinephrine in
structure, octopamine was soon found to have very little activity as
a sympathomimetic when injected into mammals (5).   In addition,
compared with norepinephrine, octopamine was found to be present in
very low concentrations in vertebrate tissues (6). Because of these
facts, relatively little attention was paid to octopamine until the
early 1970's, when Molinoff and Axelrod (7) developed a sensitive
radioenzymatic assay for the compound and reported that octopamine
was present in much higher concentrations in invertebrates, particu-
larly in invertebrate nerve tissue.
    In 1973, we reported the first identification of an octopamine
receptor (8,9).   Because this receptor was present in highest con-
centrations in insect nerve cord, we postulated that octopamine
might function as a neurotransmitter.   Furthermore, because these
receptors were undetectable in mammalian tissues, we also postulated
that the neurotransmitter function of octopamine might be restricted
to invertebrates (10,11).   At about the same time, Kravitz and co-
workers independently reported the presence of octopamine-containing
neurons in crustacea (12), and, somewhat later, Hoyle (13) reported
the presence of large octopaminergic neurons in insect ganglia.
Subsequent work by a number of investigators has firmly established
the role of octopamine as a neurohormone and neurotransmitter in
insects.
    Our original search for the octopamine receptor had been
triggered by a report of Robertson and Steele (14) that octopamine
could activate glycogen phosphorylase in insects.   In a fashion
somewhat analogous to the action of norepinephrine in mammalian
liver, we found that, in insects, octopamine can stimulate the
production of cyclic AMP through activation of a specific octop-
amine-sensitive adenylate cyclase.   The activity of this enzyme
could be assayed in vitro using membrane fractions from cells.
Therefore, by measuring the degree of activation of octopamine-
sensitive adenylate cyclase by various agonists and antagonists, it
was possible to learn something of the pharmacology of octopamine
receptors (11,15). However, because several other hormone receptors
(e.g., those for dopamine, serotonin, and proctolin) are also
positively coupled to cyclic AMP production in these tissues, it was
difficult (due to the confounding effects of these other receptors)
to completely define the pharmacological characteristics of octop-
amine-sensitive adenylate cyclase.
    In 1979, we discovered that the light organ of the firefly has
a virtually pure population of octopamine receptors, with no
evidence of adenylate cyclases activated by other hormones (16).
Indeed, the octopamine-sensitive adenylate cyclase in this tissue is
more active than any other hormone-activated adenylate cyclase yet
reported in the animal kingdom.   This allowed us to carry out the
first detailed pharmacological characterization of an octopamine-
sensitive adenylate cyclase in the absence of other amine receptors
(16-18).   It also allowed us, more recently, to characterize a new
chemical class of octopamine receptor agonists, the phenylimino-
imidazolidines (see below) (19-21).

Through use of the virtually pure octopaminergic firefly system, we have now acquired some idea of the structure-activity requirements for activation of this octopamine-sensitive adenylate cyclase. Furthermore, by quantitating the octopamine agonist potencies of a large series of chemically-related derivatives, we are now in an excellent position to characterize octopamine receptors in other, less homogenous tissues as well as in other insect species.

Toxicity of Octopamine Agonists

Because of our earlier observations that octopamine might be a neurotransmitter in insects but not vertebrates, several years ago we carried out an experiment to determine whether large doses of exogenous octopamine might exert detrimental behavioral effects in insects, analogous to what might happen if a vertebrate received an overdose of adrenalin or amphetamine. As a control, we decided to compare the activity of octopamine (chemically para-octopamine) with a positional isomer, meta-octopamine, which we found had little activity on insect adenylate cyclase (17).

Figure 1 shows the relative effect of the two compounds, when sprayed on tomato leaves, to alter the feeding of first instar Manduca sexta larvae. Consistent with their relative potency on octopamine-sensitive adenylate cyclase, octopamine was much more potent than m-octopamine in inhibiting feeding of Manduca. Animals receiving octopamine showed hyperactivity, tremors, and leaf "walk-off" behavior. Unfortunately, because octopamine is rather easily degraded, high concentrations were required in order to exert a leaf protective effect. Nonetheless, this experiment demonstrated to us that potent octopamine agonists can be detrimental to insect behavior. Furthermore, because octopamine has a relatively low degree of toxicity in mammals and because octopamine receptors are present primarily in invertebrates, such agonists should have some degree of insect selectivity.

In 1980, Hollingworth and Murdock (22) reported that application of the formamidine pesticide, chlordimeform (CDM), to firefly tails resulted in light emission (see also, Ref. 23). Because of previous evidence that the firefly light organ contains octopamine-sensitive adenylate cyclase, these workers postulated that the formamidines were exerting their pesticidal actions by affecting octopamine receptors. In subsequent biochemical work by Hollingworth and by our own lab (24), it was found that the formamidines were, indeed, potent agonists of light organ adenylate cyclase and that they interacted directly with octopamine receptors. In further work, we showed that the active form of CDM at the receptor was the N-demethyl metabolite, DCDM, and not the primary compound itself. Since the formamidine pesticides exert behavioral effects similar to those observed with octopamine, these findings provided additional support for the hypothesis that potent octopamine agonists might be useful as selective pesticides.

One problem with the formamidines as pesticides is that their range of activity is limited to a relatively few insect species (25). This species selectivity appears to be directly related to the ability of the compounds to activate adenylate cyclase activity in different insects. For example, as shown in Table I, we have
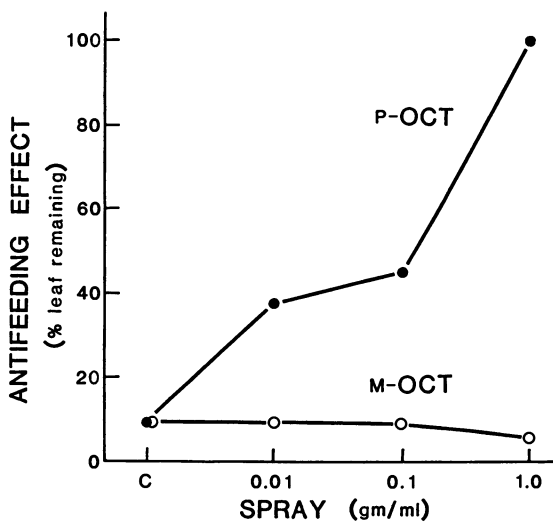
Figure 1. Antifeeding effects of para-octopamine versus meta-octopamine (a positional isomer with little activity as an agonist of insect adenylate cyclase), when sprayed on tomato leaves which were then fed to first instar Manduca sexta larvae.

observed that didemethyl-CDM is a full agonist, 20-fold more potent
than octopamine in <u>Manduca</u>, but only a very weak and partial agonist
in the cockroach. Consistent with this <u>in vitro</u> activity, the
formamidines inhibit feeding in the former species but not in the
latter. Hollingworth and Johnstone (<u>26</u>) have reported that CDM and
monodemethyl-CDM are also more potent in <u>Manduca</u>. We have obtained
similar correlations between <u>in vivo</u> toxicity and <u>in vitro</u> adenylate
cyclase activity with some of the newly characterized octopamine
agonists described below.

TABLE I. Effects of Phenyliminoimidazolidines (NC-5 and NC-7),
Octopamine (OCT), and a Formamidine (DDCDM) on Activating
Adenylate Cyclase in Three Insect Species

Compound	Firefly Light Organ		Cockroach Nerve Cord		Manduca Nerve Cord	
	V_{max}	K_a ratio	V_{max}	K_a ratio	V_{max}	K_a ratio
NC-5	97 $\pm$ 5	19	91 $\pm$ 3	11	96 $\pm$ 4	8
NC-7	68 $\pm$ 10	10	45 $\pm$ 5	12	100 $\pm$ 3	16
OCT	100 $\pm$ 5	1	100 $\pm$ 5	1	100 $\pm$ 5	1
DDCDM	68 $\pm$ 5	4	12 $\pm$ 1	3	97 $\pm$ 3	20

Activity is expressed relative to octopamine (V_{max} = 100%; K_a Ratio
= 1) (Reproduced with permission from Ref. 21. Copyright 1985, Am.
Soc. Pharmacol. Exptl. Ther.)

These results further support the hypothesis that potent synthetic
agonists of octopamine-sensitive adenylate cyclase should be useful
as selective pesticides. The results also suggest that if compounds
could be developed which were potent against the octopamine-sensi-
tive adenylate cyclases present in a wide variety of insect species,
they should, similarly, have a wide spectrum of insecticidal
activity. However, in order to develop such compounds, much more
needs to be known about: a) the molecular pharmacology of octopamine
receptors and b) the interspecies differences which seem to exist
in the characteristics of such receptors. In other words, we need
to know if there exist single or multiple octopamine receptor
subtypes which interact with adenylate cyclase, and if multiple, how
these subtypes vary among different species.
    It is now clear, from the studies of Evans and others (<u>27,28</u>),
that there exist high affinity octopamine receptors not associated
with adenylate cyclase. It is as yet unknown whether such receptors
are involved in the behavioral abnormalities and pesticidal effects
seen with octopamine agonists. On the other hand, we have evidence
that octopamine receptors linked to adenylate cyclase activation
definitely are associated with behavioral abnormalities and insect
toxicity. Thus, in a series of experiments (<u>19,21,29,30</u>), we have
found, for example, that: 1) agents which inhibit the breakdown of
cyclic AMP augment the antifeeding effects of octopamine agonists;
2) direct activators of adenylate cyclase, such as forskolin, mimic
the effects of octopamine agonists; and 3) cell permeable analogs of
cyclic AMP also exert pesticidal activity.

Phenyliminoimidazolidine Octopamine Agonists

We have obtained biochemical structure-activity data on a large number of compounds in a newly defined class of octopamine agonists, the phenyliminoimidazolidines (PII's) (20,21). These compounds interact with octopamine receptors which are distinct from mammalian adrenergic (including alpha-1, alpha-2, beta-1, beta-2), dopaminergic, $5HT_1$, and $5-HT_2$ receptors (21). Furthermore, relevant to understanding interspecies differences, we have in preliminary experiments found certain derivatives which show some selectivity for activating adenylate cyclase in different species. For example, the compound NC-7 shows preferential selectivity for octopamine-sensitive adenylate cyclase in Manduca as compared with firefly or cockroach (see Table I). One the other hand, NC-5 shows the converse. NC-5 also displays activity in species, such as the cockroach, for which the formamidines are largely inactive (Table I). In future studies, these derivatives, along with other agonists and antagonists, should help to pharmacologically define the octopamine-sensitive adenylate cyclases present in certain target insect species and tissues.

Labeling Octopamine Receptors

To be sure that species differences seen in the activation of octopamine sensitive adenylate cyclase are due to real differences in receptor binding (as opposed to differential metabolism of agonists or differential access to the receptor), it is also useful to make direct measurements of receptor binding. In order to carry out binding experiments, one needs to have a radiolabeled ligand whose binding can be quantitated and which interacts only (or primarily) with those octopamine receptors which are involved in mediating the detrimental behavioral alterations seen in species exposed to octopamine agonists.

One possibility would be to use labeled octopamine, itself, and to perform ligand binding studies of the type which are frequently used in mammalian neurotransmitter receptor research. The problem with this approach is that octopamine binds, to a large extent, to octopamine receptors other than those associated with adenylate cyclase. This is shown by the fact that the binding affinity reported for ^{3}H-octopamine in insect nerve tissue is in the low nanomolar range (31), whereas the binding affinity of octopamine for activating adenylate cyclase has been reported by many investigators to be in the low micromolar range (1000-fold higher) (2). Indeed, with current techniques, it is almost impossible to use any sort of reversible ligand to measure a receptor with an affinity in the micromolar range. This is because the off-time for such a ligand is in the range of 0.1 seconds, so fast that most of the label is gone by the time the tissue is treated (by filtration or centrifugation) to separate free from bound label.

In order to circumvent these difficulties, we have tried to develop a ligand of the PII-type which will bind irreversibly to the octopamine receptor(s) associated with activation of adenylate cyclase. Since binding is permanent one can then easily distinguish between free and bound ligand. In addition, such a ligand should make it possible to permanently label octopamine receptors, which

will allow the biochemical characterization of receptor structure. Recently, we have demonstrated the feasibility of this approach by using an azido-labeled, photoaffinity ligand developed from the PII's. Ligands of this type have the property of binding to receptors reversibly in the dark. In the presence of intense short-wave UV light, however, the ligands photolyze and bind covalently to proteins. Because of this, ligand reversibly bound to the receptor at the time of photolysis becomes permently bound to a site on or very close to the receptor protein. This irreversible binding occurs even if the affinity of the ligand is in the micromolar range. If the ligand is tritiated, then the amount of binding can be measured. Furthermore, the receptor protein(s) can be solubilized and isolated on the polyacrylamide gels. In order to show specific receptor labeling, one must demonstrate that the irreversible labeling can be prevented or substantially reduced with a reversible agonist or antagonist.

    In preliminary studies, using an azido-labeled PII, we have been able, in the absence of UV light, to demonstrate reversible activation of firefly octopamine-sensitive adenylate cyclase, with a K_a for the azido compound similar to that for octopamine. In the presence of UV light, a portion of the label binds irreversibly, and preliminary evidence indicates that about 40-50% of the labeling occurs specifically at octopamine receptor sites. To carry out these latter experiments, firefly light organ membranes were labeled with the photoaffinity agonist, in the absence or presence of unlabeled octopamine. Membrane proteins were than solubilized and separated on SDS-polyacrylamide gels, the gels dried, and exposed to X-ray film. Densitometry tracings of labeled proteins on the resultant autoradiogram were made and the peaks quantitated. We found that six proteins ranging in the MW from 17,000 to 90,000 were labeled by the azido agonist. However, for only two of these proteins (MW 65,000 and MW 90,000) was the labeling specific. This is shown by the fact that <u>unlabeled</u> octopamine was able to reduce binding only for these proteins and not for the others. These two proteins, therefore, are presumptive octopamine receptors. Although these could represent hydrolysis products of a larger protein, other data we have suggests the light organ may, in fact, contain two cyclic nucleotide-linked octopamine receptors. As yet, we do not know whether non-cyclic nucleotide-linked octopamine receptors (so-called, octopamine-1 receptors) might also be labeled by this azido analog. If so, use of the photoaffinity technique, together with the reversible PII's described above, should make it possible to carry out interspecies characterization of those octopamine receptor subtypes linked to adenylate cyclase as well as those not linked to this intracellular second messenger.

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Chapter 12

Effects of Octopaminergic Agents on the Sex Pheromone Mediated Behavior of Adult Lepidoptera

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When injected into adult male cabbage looper moths, lower doses of octopamine (30 and 100 µg/g) heightened male sensitivity to pheromone, as evidenced by a significant shift in the dosage-response curve. Higher doses (300 and 1000 µg/g), in addition to affecting male sensitivity, significantly disrupted the ability of males to execute the upwind oriented flight response. A series of agonists mimicked the effects of octopamine, while a series of antagonists decreased the responsiveness of males when injected alone, and blocked the effect of exogenous octopamine. The phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine also significantly potentiated the effect of octopamine on male sensitivity to pheromone. The agonists and antagonists displayed a wide range of potencies, with the rank order of both similar to that found for the octopamine-2 receptors in the locust extensor tibiae muscle. The results support the proposed role of octopamine as an endogenous neuromodulator of moth olfactory perception.

The biogenic amine octopamine (OA) is widely recognized as an important neuroregulator in many invertebrates. It has been proposed as a neurotransmitter in the firefly light organ, and as a neuromodulator of muscle action and a neurohormone in the locust ($\underline{1},\underline{2}$). Octopamine also appears to act on central nervous system (CNS) sites affecting coordinated motor actions ($\underline{3},\underline{4}$), and has been shown to modulate CNS thresholds for feeding in blowflies ($\underline{5}$), and olfaction in honeybees ($\underline{6}$).

We have recently demonstrated that OA, along with 5-hydroxytryptamine (5-HT), can significantly alter behavioral thresholds controlling the response of male moths to sex pheromone ($\underline{7}$). Mating success in these insects is dependent on male perception of a specific female-released multicomponent pheromone. Behavioral studies have shown that males possess a high degree of specificity with respect to the qualitatitive and quantitative properties of the chemical signal, with maximal response occurring to a narrow range of blend-concentration combinations around the female-released signal.

This specificity for the female released blend and ratio of components is controlled by two prominent threshold effects on male flight behavior, one affecting plume orientation and the other sustained upwind flight (8,9).

Optimal flight behavior, however, is also dependent on appropriate environmental conditions. Mating typically occurs during a period of the diel cycle that is characteristic for a given species and is governed by a circadian rhythm that also controls general locomotor activity (10). Photoperiod and temperature cues are important in regulating both the timing of the response to pheromone, and the sensitivity of males to the qualitative and quantitative properties of the signal. For example, in the nocturnally active cabbage looper moth (CL), Trichoplusia ni Hubner, random flight activity and response to pheromone peak during the mid to latter part of the scotophase, with maximal response levels dependent not only on the ´lights off´ cue, but also the subsequent scotophase light intensity and temperature.

The observed relationship between exogenous cues and their modulatory effect on neural pathways controlling flight behavior and sensitivity to sex pheromone provided the rationale for investigating the possible regulatory role of OA and other biogenic amines. This paper will review our initial findings on the effects of OA and then present new information on the ability of selected agonists and antagonists to mimic the action of OA on moth sensitivity and response to pheromone. Preliminary data on the effects of a phosphodiesterase inhibitor will also be presented.

Methods

Insects. Cabbage looper moths were obtained from a laboratory colony reared on semisynthetic medium, on a 16:8 L:D photoperiod, at 25–27° C. Adult males were separated from females and segregated daily by age (11).

Chemicals. The proportions of synthetic chemicals in the 6-component pheromone were as follows: 12:OAc (6.8), Z5-12:OAc (7.6), Z7-12:OAc (100), 11-12:OAc (2.3), Z7-14:OAc (0.9), and Z9-14:OAc (0.6) (11). A solution of this blend (in Skelly B) was checked by capillary GLC (45-m Carbowax 20M column) and from a dilution series 100 ul amounts were placed in polyethylene cap sources to achieve the desired source dosages (11).

Solutions of drugs were prepared in 145 mM aqueous NaCl, from which a dose series was prepared. dl-Octopamine, dl-synephrine, dopamine, tolazoline, clonidine, yohimbine, cyproheptadine, gramine, chlorpromazine, promethazine, propranolol, metoclopramide, and 3-isobutyl-1-methylxanthine were obtained from Sigma Chemical Co.; naphazoline from Aldrich Chemical Co.; chlordimeform and phentolamine from Ciba Geigy; mianserin from Research Biochemicals Inc.; and lofexidine, XAMI, and tramazoline were gifts from Dr. R. Hollingworth, Purdue Univ.

The doses of all compounds are expressed as an amount per unit fresh weight of moth (µg/g). The average weight of male CL moths was 0.096 ± 0.011 g (N = 100, selected randomly over the series of tests).

<u>Assay Methodology.</u> Details of the flight tunnel and procedures for handling and testing males can be found in (<u>11</u>). Briefly, for all tests the compounds were injected (as a dose series in 1 µl saline) into four day-old male CL moths 1 hour prior to the initiation of scotophase (16:8 L:D). Male response to pheromone was observed in the sustained-flight tunnel, five to six hours after injection, during the period of the scotophase when peak response to pheromone occurs. Flight tunnel conditions were 0.3 lux light intensity, 23 $^\circ$C, 50 cm/sec airspeed. Males were scored for three key behaviors in the flight sequence: taking flight, upwind flight over a 1.5 m distance, and source contact (<u>11</u>).

Effect of Octopamine on Pheromone Response

When injected into male CL moths OA induced two distinct, dose-related effects on male response to pheromone (<u>7</u>, see Figure 1). Over the range of 10 to 100 µg/g OA a significant shift occurred in the pheromone dosage-response curve, with OA-injected males exhibiting peak response to 0.01 mg pheromone rather than 1 mg as in the controls. This change in male sensitivity, and thus the behavioral thresholds controlling male response, was not associated with any observed change in the ability of males to execute upwind oriented flight behavior.

In contrast, higher doses of OA (300 and 1000 µg/g) affected male sensitivity to the chemical signal as well as their ability to execute the upwind oriented flight response. At 300 µg/g males were unable to sustain upwind oriented flight, once having initiated the response. These males exhibited excessively wide casting patterns across the tunnel within the first 30 cm of upwind flight, eventually falling to the floor of the tunnel and, while on their backs, exhibiting rapid circular motions. At 1000 µg/g OA males became activated by the pheromone, but were unable to take flight, instead falling to the floor of the tunnel and exhibiting a paralysis characterized by the insect assuming a rigid position with the wings extended vertically over the head and the abdomen bent under the thorax.

Effects of Agonists on Male Sensitivity to Pheromone

Our agonist studies were designed to further document the effect of lower doses of OA on male sensitivity to pheromone (as shown in Figure 1), and to provide a pharmacological profile that could be compared with data from other published studies on OA receptors (<u>12-14</u>). The compounds included 1) the phenylethylamines: synephrine and dopamine, 2) the formamidine: chlordimeform, and 3) the substituted imidazolines: naphazoline (2-[1-naphthylmethyl]imidazoline), lofexidine (2-[1-(2,6-dichlorophenoxy)ethyl]-2-imidazoline), XAMI (2,3-xylylaminomethyl-2-imidazoline), clonidine (2-(2,6-dichloroanilino)-2-imidazoline), tolazoline (2-benzyl-2-imidazoline), and tramazoline (2-[5,6,7,8-tetrahydro-1-naphthyl]amino-2-imidazoline).

We first determined the ability of selected agonist compounds to mimic the effect of OA on male sensitivity to the pheromone by treating males with a dose series of each compound and then testing them with the 0.01 mg dosage of pheromone, which under control conditions elicits a low level of response (Figure 2). It is important to note
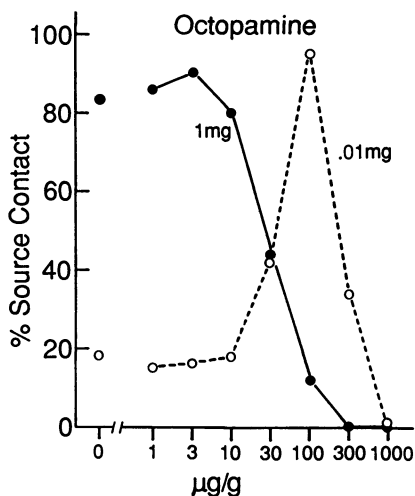
Figure 1. Percentage of male CL flying upwind (1.5m) and making contact with a 1 or 0.01 mg pheromone source 4-5 hours after injection with OA (μg/g). N = 85 for each dose of OA and dosage of pheromone. Control values are indicated by the 0 dose points.
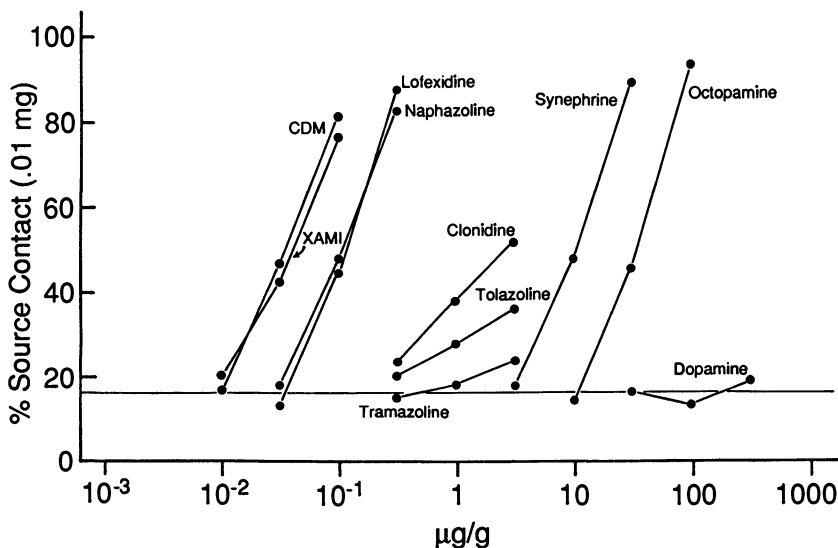
Figure 2. Percentage of male CL making source contact (0.01 mg pheromone) 4-5 hours after injection of OA and agonist compounds (μg/g). The curves for each compound represent the range of doses over which an effect was observed only on male sensitivity to pheromone, without a significant effect on male flight perfor- mance. N = 80 for each dose. The solid line indicates control response level (16%).

that the curves in Figure 2 show the range of doses for each agonist at which male sensitivity, and not upwind oriented flight, was affected. At higher doses of each compound increasing numbers of moths were affected during the upwind flight response, resulting in lower percentages of source contacts. These response levels have been excluded from figure 2 for purposes of clarity and to focus specifically on the effect on sensitivity.

The agonists were found to vary considerably in their potency, with peak response in chlordimeform and XAMI treated insects occurring at 1 µg/g vs 100 µg/g for OA (Figure 2). The dose-response curves also show that males treated with chlordimeform, XAMI, lofexidine, naphazoline, and synephrine exhibited levels of response to 0.01 mg pheromone that were equal to those of OA-treated (100 µg/g) males, but that clonidine, tolazoline, and tramazoline were much less effective, and that dopamine was without effect on male sensitivity.

A more detailed examination of the flight response of clonidine- and tolazoline-treated males suggested that the low response levels observed with 0.01 mg of pheromone were possibly due to the dosage of pheromone used, rather than to the efficacy of the compounds. This was suggested by the fact that tolazoline-treated males (3 µg/g) exhibited a significant level of arrestment of upwind flight (characteristic of a flight pattern exhibited by untreated insects to a high dosage of pheromone), whereas clonidine treated males (3 µg/g) exhibited low levels of plume orientation and initiation of upwind flight (characteristic of control responses to lower than optimal dosages of pheromone).

To examine this further, and to obtain a more complete profile of the efficacy of the agonists, male response was next observed to a dosage series of pheromone after treatment with the dose of agonist from figure 2 that gave peak effect on male sensitivity to 0.01 mg pheromone. These data are presented in Figure 3, and show that there were considerable differences in the potency and efficacy of the compounds. For example, whereas the potencies of chlordimeform and XAMI with 0.01 mg pheromone were higher than those of naphazoline or tolazoline (0.1 vs 0.3 and 3 µg/g respectively), naphazoline- and tolazoline-treated males exhibited peak response to a much lower dosage of pheromone (0.0001 vs 0.01 mg). It is unclear whether the differences in potency and efficacy of the compounds reflect differences in affinity of the agonists for target sites or differences in penetrability of the compounds across CNS barriers. In either case the efficacy of several of the agonists is striking given that male sensitivity was measured five hours or more after injection of the compounds.

Effects of Agonists on Pheromone-Mediated Flight Behavior

As noted above the agonists also induced, at doses higher than those shown in Figure 2, two significant effects on moth flight behavior. The first was a disruption of oriented flight, leading to uncontrolled locomotory behavior on the tunnel floor. The second was evidenced as a complete inability to take flight. These effects on flight behavior were observed with all of the agonist compounds, including the phenylethylamine dopamine.

Effects of Antagonists on Male Sensitivity to Pheromone

We also examined the effects of selected antagonists on male sensitivity to pheromone. These included the alpha adrenergic blockers phentolamine, yohimbine, and metoclopramide; the (generally considered) 5-HT antagonists cyproheptadine, mianserin, and gramine; the phenothiazines chlorpromazine and promethazine, generally classed as dopamine antagonists; and the beta adrenergic blocker propranolol. As with the agonists, these compounds were selected on the basis of other OA systems that have been studied (12-14).

The potency of the antagonists was first measured by injecting them alone. As with the agonists, the results showed a broad range of effectiveness in the ability of these compounds to decrease male sensitivity to the pheromone (Figure 4). It is important to note that while the antagonist effect is expressed as a decrease in source contacts, the effect included all phases of the behavioral response, beginning with activation. Further, the effect of the antagonists was to raise the threshold for response to pheromone, and not to disrupt flight motor function. As in the case with lower doses of agonists, over the range of antagonist doses shown, there was no effect on the ability of males to fly when released inside the room housing the flight tunnel after being disturbed from a quiescent state.

In the second series of tests the ability of several of the antagonists to block the effect of OA on male sensitivity was assessed. Data in Figure 5 show that mianserin, chlorpromazine, and yohimbine reversed the effect of OA on male sensitivity to the 1 mg dosage of pheromone, with chlorpromazine and yohimbine exhibiting greater potency than when presented alone (Figure 4).

Effects of a Phosphodiesterase Inhibitor on Moth Sensitivity

One of the proposed mechanisms by which OA exerts its regulatory actions is via an adenylate cyclase (12-18). In a preliminary test male CL were treated with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX), alone and with OA. The dose of OA (10 µg/g) was one that induced little or no effect on male sensitivity (see Figure 1). Results showed that when treated with IBMX alone (10 µg/g) male response to a low dosage of pheromone (0.01 mg) was not significantly enhanced over that observed with controls, or OA alone (20 vs. 15% source contacts respectively). However, when treated with 10 µg/g OA and 10 µg/g IBMX, 78% of the males successfully reached the source. IBMX clearly potentiated the effect of a low dose of OA on male sensitivity to pheromone.

Conclusions

The results of the present study continue to support our hypothesis that OA is an endogenous modulator of male response to sex pheromone. Over a series of doses OA induced two distinct effects on male behavior. The first, at lower doses, was on male sensitivity, and was evidenced by a significant shift in the pheromone dosage-response curve. The second, at higher doses, was on flight motor behavior, and was evidenced by a disruption of the oriented flight response and eventually a total inhibition of flight. The effects on male sensitivity were specific for OA, and not for other biogenic amines such
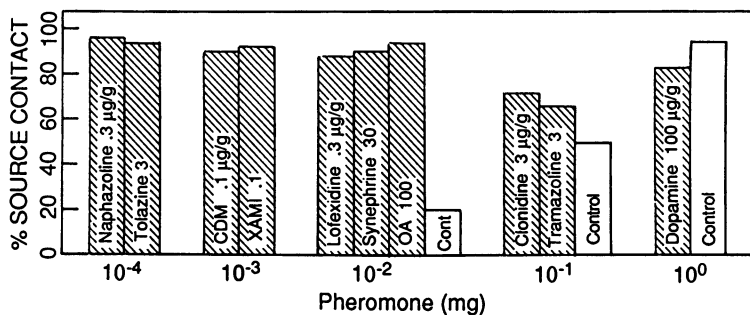
Figure 3. Percentage of male CL reaching source (mg pheromone) 4-5 hours after injection of indicated dose of OA or agonist compound (μg/g, from figure 2). These doses represent ones at which the maximal effect on male sensitivity was observed, without a significant effect on male flight performance. The percentage response for each agonist is indicated at the dosage of pheromone eliciting peak levels of response over the dosage series tested. N = 50 for each dose of agonist and dosage of pheromone.
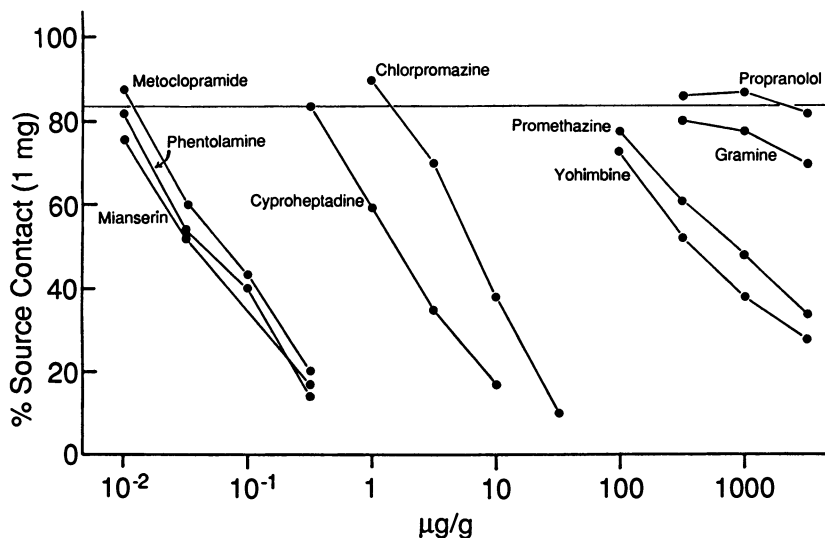
Figure 4. Percentage of male CL reaching source (1 mg pheromone) 4-5 hours after injection of antagonists. N = 80 for each dose of antagonist. Solid line represents control response (84%).
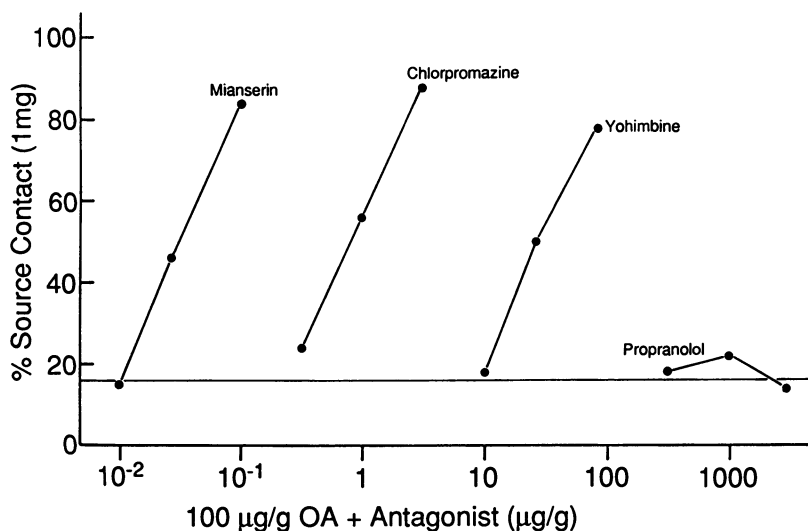
Figure 5. Percentage of male CL reaching source (1 mg pheromone) 4-5 hours after injection of 0.1 µg/g OA plus the dose of antagonist indicated. N = 40 for each dose of antagonist. Solid line represents control response (16%).

as 5-HT (7), or dopamine (present study). The hypothesis is also
supported by the actions of selected agonists and antagonists that
were tested, all of which have been shown in previous studies to
interact at receptor sites associated with OA-induced actions (12-
14).

   With respect to the effect on male sensitivity, we propose that
OA was mimicking an endogenous effect of this amine on neural path-
ways involved in the qualitative and quantitative assesment of the
pheromone, and not on motor units involved in the flight response. It
should be stressed that lower doses of all agonists, as well as all
doses of antagonists, did not affect the ability of males to execute
the oriented upwind flight response, or to fly in a normal manner if
mechanically disturbed. Rather the effect appeared to be on the
thresholds controlling male sensitivity to the chemical signal. We
have shown in previous studies that optimal male response to the
pheromone is dependent on the appropriate blend and ratio of com-
ponents (8,9), as well as the appropriate environmental conditions
(10). The response is thus a product of CNS integration of informa-
tion from several sensory modalities, as well as the influence of a
circadian clock. This suggests a number of possible sites of action,
including pathways at the peripheral and deutocerebral (19) levels,
or in higher brain centers involved in regulating the general
locomotor rhythm. At the present time electroantennogram studies are
being conducted to evaluate the impact of selected compounds on
peripheral pathways.

   The most surprising aspect of the present study was the duration
of effect on male behavior. Males were tested in the flight tunnel 4
to 5 hours after injection of the chemicals, a period one might
predict would be long enough for all of the compound to be metabo-
lized or excreted. Whereas we cannot at the present time provide a
definitive explanation for the duration of these effects, one feature
of this system that might be involved in the modulatory action of OA
deserves discussion. This concerns the fact that male locomotor
activity, and response to pheromone, are dependent on the transition
to appropriate scotophase conditions (0.3 to 1 lux). In the absence
of this photoperiodic cue injection of OA does not enhance male
sensitivity or response to pheromone (preliminary data from our
laboratory). This suggests that the modulatory action of OA is linked
to physiological changes that occur with the transition from
photophase to scotophase conditions, and that the magnitude of the
effect might be dependent on the amount of OA released during a
critical period at the beginning of the scotophase, close to the time
at which injection occurred in the present study. The duration of the
effect would then be related to a continued release of the compound
over the scotophase period. While this hypothesis is highly specula-
tive it can be tested. Future studies in our laboratory will address
in detail the relationship between the timing of treatment, the
initiation of scotophase, and the subsequent effect of OA on male
behavior, as well as the relationship between the effect of OA and
the light intensity during the scotophase period. Evidence also
exists from our previous study that 5-HT might be involved in the
regulation of spontaneous flight activity that also occurs with the
initiation of scotophase, and we will address several questions relat-
ing to the possible interaction of OA and 5-HT (7).

While the effects on male sensitivity were somewhat surprising considering the complexity of the behavioral response, and the uncertainity of where the compounds might be acting within the insect, the effect of higher doses of agonist compounds on flight motor behavior was one that we did expect. Results of studies with Manduca sexta (4), as well as the locust Shistocerca americana (3), suggest that modulation of motor axon activity by OA may be an important regulatory component of the flight generator in the thoracic ganglia. It should also be noted that higher doses of agonist compounds, as well as the antagonists, affected levels of spontaneous motor activity exhibited by males during the scotophase period. With the agonists this was evidenced as an increase in spontaneous flight activity, while the antagonists decreased this response. We are at the present time investigating in greater detail the effects of these compounds on spontaneous flight activity, and this will be reported elsewhere.

Our study further suggests that the action of OA on neural centers associated with male detection of and response to the pheromone may be via an adenylate cyclase. This mechanism of action has been proposed for one type of OA receptor in the locust extensor muscle, as well as in the firefly light organ. Extensive studies with this latter system have demonstrated that many of the agonist compounds in the present study interact with an OA-sensitive adenylate cyclase from this tissue (13,14). In addition, OA-sensitive adenylate cyclase has been isolated and studied from the CNS in several insects, including the moth Manduca sexta (see 13).

A comparison of our results with those of other studies suggests that the OA receptors involved in the heightened sensitivity to pheromone and control of flight behavior are similar to those characterized by Evans (12) as octopamine-2 receptors, affecting twitch tension and relaxation in the locust extensor tibiae muscle. This claim is made on the basis of a ranking of the agonists and antagonists with respect to efficacy in enhancing male sensitivity to pheromone (tolazoline = naphazoline > chlordimeform = XAMI > OA = lofexidine = synephrine > clonidine = tramazoline), or in their ability to lower male sensitivity (phentolamine = mianserin = metoclopramide > cyproheptadine > chlorpromazine > yohimbine > promethazine > propranolol = gramine). These profiles differ from the octopamine-1 receptors affecting the rhythm of the myogenic bundle in the locust extensor muscle, in that clonidine and tramazoline are strong agonists in that system, while yohimbine and promethazine are strong antagonists. Our profiles also differ from similar types of rankings for the OA activation of adenylate cyclase in the firefly lantern and tobacco hornworm CNS, in that chlorpromazine was a much better blocker in these systems than phentolamine and metoclopramide, and tramazoline a better agonist than tolazoline (see 13 for a discussion of these profiles). In agreement with all other OA systems that have been studied, however, is the observation that alpha adrenergic blockers were much more effective than beta blockers.

The rank order of effectiveness for the substituted imidazolines was of particular interest because it indicates a low degree of correlation with respect to structure and activity. This result is in agreement with other published studies demonstrating that more structural variations are possible in the imidazolines than in the case of the formamidines, even though many of the substituted imidazolines

elicit formamidine-like effects on the locomotor behavior of insects
([13],[14]). In the present study we tested only one formamidine com-
pound, chlordimeform, and it was found to exhibit a high degree of
potency, although not the highest efficacy among the agonist com-
pounds. The formamidines are of great interest because of their
potent sublethal effects on insect locomotor behavior, and because a
substantial number of studies have provided evidence that at least
one of the actions of the formamidines is to interact at octopaminer-
gic receptor sites ([20-22]). Our present study supports the findings
of an earlier investigation in which we observed both heightened
sensitivity to sex pheromone in chlordimeform-treated moths, but also
a significant increase in spontaneous activity and hyperreflexic
response to a visual stimulus ([23]). This latter behavior and the role
that OA plays in its expression is at present being investigated in
our laboratory using a more complete list of formamidines and sub-
stituted imidazolines.

Finally, the present studies support the hypothesis that valu-
able pharmacological data can be obtained from an *in vivo* assay
involving the behavior of individual organisms. This was possible
because of the sensitivity of the assay and the detailed knowledge we
have of the biology of the insects involved. Under optimal conditions
80 to 100 males can be tested in a 2 hour period, and with 3 test
periods per day a study of the present magnitude (>7400 insects)
could be done in a period of two months. The complexity of the be-
havioral sequence, its biological relevance, and the wealth of
information that can be obtained suggest that our flight tunnel assay
can provide an excellent research tool for investigations on a
variety of pharmacological questions, as well as a screening method
for new, behaviorally-active compounds that might have application in
pest management situations.

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Chapter 13

Effects of Formamidines
on Acarine Dispersal and Reproduction

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Formamidine effects on twospotted spider mites, Tet-
ranychus urticae, included elicitation of walk-off
and spin-down dispersal and reduction of fecundity
and egg hatch. Formamidine-induced walk-off and
spin-down were not correlated, but the former was
significantly associated with formamidine-induced
lethality. A moderately strong correlation existed
among the activities of formamidines in reducing
fecundity, egg hatch, and reproductive potential,
whereas only weak associations were found between
these parameters and formamidine-induced lethality.
These results suggest that the mechanism(s) in-
volved in formamidine-induced lethality and walk-
off in mites may be similar, but other mechanisms
possibly are involved in formamidine-induced spin-
down and reduction of fecundity and egg hatch.
Based on analogy with studies of formamidine action
in other organisms, it is suggested that interfer-
ence with biogenic amine regulatory mechanisms may
be involved in some of these effects on mites. In
this connection, chlordimeform treatment of bulb
mites, Rhizoglyphus echinopus, altered whole body
levels of dopamine and 3,4-dihydroxyphenylalanine,
and chlordimeform, its formamidine and formanilide
metabolites, and other formamidines and formanilides
inhibited the degradation of 2-phenylethylamine by
bulb mite homogenates.

The formamidine acaricides and insecticides, chlordimeform and ami-
traz, are lethal to all life stages of acarines, but their activity
to insects is considerably more restricted (1). Using the southern
cattle tick, Boophilus microplus, and the twospotted spider mite,
Tetranychus urticae, the structural requirements of formamidines
for lethality have been elucidated (2-5), and the structure (lethal
moiety) for maximum activity is shown in Figure 1. Briefly, the
ring must contain two substituents, and they must be located at
positions two and four; maximum activity was observed when position

two was methyl and R was halogen, preferably chlorine, or methyl.
At the amino nitrogen, there should be one lower alkyl group,
preferably methyl, whereas the other substituent at R' could be
hydrogen, lower alkyl, methylthiomethyl, thiophenyl, or other more
complex moieties (2-5). Chlordimeform and amitraz also have toxic
actions in acarines other than lethality (1). Depending upon dose,
they can elicit dispersal behavior, disrupt feeding, and interfere
with reproduction (5-17). Recently our interest in formamidines
has been directed toward their activity as elicitors of dispersal
behavior and inhibitors of reproduction in mites. Previous inves-
tigations of formamidine effects on mite dispersal (8,9,17) and
reproduction (12) have used chlordimeform and related formamidines
that possessed the lethal moiety. The direct lethal action of
formamidines might not be inextricably linked with their effects
on mite dispersal and reproduction. If so, evaluation of the ef-
fects of the many nonlethal formamidines on these parameters might
reveal compounds with practical applicability in pest management
programs. Also, such studies could provide valuable insight into
some of the interesting mechanistic questions associated with this
class of compounds. This paper summarizes the results of these
studies of the effects of formamidines on twospotted spider mite
dispersal behavior (18) and reproduction (19,20). Also included
are the results of investigations of bulb mite, Rhizoglyphus ech-
inopus, biogenic amines and regulatory mechanisms (21,22), along
with associated effects of formamidines (23).

Formamidines and Mite Dispersal Behavior

To examine the activity of formamidines on spider mite dispersal
behavior, young adult females were placed on a bean leaflet prep-
aration and dipped for ten seconds in an emulsion of the test
compound (18). As a result of dose range experiments with chlor-
dimeform, we selected 62.5 ppm and 500 ppm as low and high discrim-
inating concentrations, respectively. In some cases, EC_{50} values
were determined. The treated leaflets were allowed to drip to
dryness and were suspended on a rack in an environmental chamber
operated at 24°C and 50 ± 5% relative humidity under continuous
light. Two dispersal parameters, % spin-down and % walk-off, were
measured, and a third, total dispersal, was calculated by summing
the values for walk-off and spin-down. Spin-down, or the number
of mites suspending themselves from the lower leaf surface by a
silken thread, was assessed at 15-minute intervals for eight hours
and at 24 hours posttreatment. Quantification of walk-off, or the
number of mites trapped in a Tanglefoot rim at the leaflet rachis,
was determined at 24 hours (18).
    Sixty-seven compounds, including two formanilides and two
anilines, were examined (18). Twenty-eight formamidines elicited
dispersal behavior significantly greater than controls; 39 com-
pounds, including the two formanilides and two anilines, were
inactive. Fifteen formamidines elicited both walk-off and spin-
down, whereas three induced walk-off only and ten spin-down only
(18).
    The effects of 15 formamidines on twospotted spider mite dis-
persal behavior are given in Table I. Compounds are ranked in
order of their decreasing activity as elicitors of total dispersal

Table I. Effects of Formamidines on Twospotted Spider Mite
Dispersal Behavior[a]

Compound Number	Structure	Dispersal					
		Total		Walk-off		Spin-down	
		Rank	%	Rank	%	Rank	%
102	Me-2,4-Ph-N=CH-NMeSPh	1	75	1	69	12	6
105	(Me-2,4-Ph-N=CH-NMe)$_2$S	2	77	3	45	1	32
87	Cl-4,Me-2-Ph-N=CH-NHMe	3	79	2	71	16	8
101	Cl-4,Me-2-Ph-N=CH-NMeSPh	4	76	4	54	5	22
94	Me-2,4-Ph-N=CH-NHMe	5	79	5	54	3	25
52	Cl-4,Me-2-Ph-N-CH-NMe$_2$	6	65	6	50	11	15
104	(Me-2,4-Ph-N=CH)$_2$NMe	7	47	8	29	4	18
50	Cl-3,Me-2-Ph-N=CH-NMe$_2$	8	63	7	57	25	6
49	Cl-2,Me-6-Ph-N=CH-NMe$_2$	9	37	10	28	14	9
48	Cl-2,Me-4-Ph-N=CH-NMe$_2$	10	36	11	25	15	11
95	Cl-4,Me-2-Ph-N=CH-cyclohexylidene	11	31	16	11	9	20
37	Me-2,4-Ph-N=CH-NMe$_2$	12	36	9	32	29	4
28	Cl-2,4-Ph-N=CH-NMe$_2$	13	30	13	17	13	13
90	Cl-4,Me-2-Ph-N=CH-NHBu-n	14	28	23	4	6	24
91	Cl-4,Me-2-Ph-N=CH-NHBu-i	15	24	31	2	2	22

[a]Data from Ref. 18. Compounds are listed in decreasing order
according to total dispersal activity. Ranking of compounds
was accomplished using two-way ANOVA and Duncan's multiple
range test ($P<=0.05$) for transformed (arcsin) main effect means
(500 and 62.5 ppm). Data for % dispersal in each category are
for 500 ppm treatment only. Pooled control values were 2% for
walk-off and 2% for spin-down. Compounds numbered after Chang
and Knowles (3); 52 = chlordimeform, 104 = amitraz. Abbrevia-
tions: Me = methyl, Et = ethyl, Bu = butyl, and Ph = phenyl.

which includes both walk-off and spin-down. It should be empha-
sized that the rank ordering takes into account formamidine effects
at 62.5 ppm and 500 ppm, whereas the % dispersal values are from
500 ppm only. The most active elicitors of total dispersal and
walk-off were formamidines 102 (U-42564), 105 (U-44193), 87
(demethylchlordimeform), 101 (U-42558), 94 (BTS-27271), and 52
(chlordimeform). EC$_{50}$ values (ppm) for total dispersal and walk-
off were 23 and 46 for 102, 35 and 172 for 105, 70 and 112 for 87,
109 and 193 for 101, 128 and 232 for 94, and 296 and >500 for 52
(chlordimeform). EC$_{50}$ values for spin-down were >500 ppm in all
cases. These 15 formamidines all had the aryl moiety substituted
with 2,4-dimethyl or 4-chloro-2-methyl, with the exceptions of 50,
49, 48, and 28, which were substituted with 3-chloro-2-methyl, 2-
chloro-6-methyl, 2-chloro-4-methyl, and 2,4-dichloro, respectivly
(18).
    The most active compounds for elicitation of spin-down be-
havior in decreasing order of activity, were 105, 91, 94, 104
(amitraz), 101, 90, 84, 92, 95, 96, and 52 (chlordimeform).
Compound 96 is similar to 95 except that the nitrogen-containing
ring is piperidino. Moreover, formamidines 105, 94, 104

(amitraz), 101, 95, and 52 (chlordimeform) educed both spin-down
and walk-off, while 91, 90, 84, 92, and 96 elicited spin-down
only. Time courses for ten of these compounds are given in
Figure 2. Spin-down began during the initial hour after treat-
ment and was completed by seven hours; it only exceeded 25% at
500 ppm in the case of formamidine 105. The nature of the sub-
stituents at the amino nitrogen was important for those formam-
idines that elicited spin-down only, with the monobutyl compounds,
except for the tertiary isomer (18), being especially active.
    In a study of arylthioformamidine-mediated dispersal in the
twospotted spider mite (8) and the carmine spider mite, Tetrany-
chus cinnabarinus (9), it was concluded that walk-off was a func-
tion of the formamidine portion of the molecule, whereas spin-down
was a function of the thiophenyl moiety. The present study (18)
corroborates this conclusion.
    A dispersal and mortality correlation matrix for formamidines
and spider mites is given in Table II. Total dispersal was cor-
related with walk-off and spin-down, but the former clearly was
the major component. Total dispersal also was correlated with
mortality. However, spin-down was not correlated with walk-off
or with mortality. Walk-off was correlated with mortality (18).

Table II. Correlation Matrix for the Effects of Formamidines on
Twospotted Spider Mite Dispersal and Mortality at 72 Hours[a]

	Spin-down	Walk-off	Mortality[b]
Total Dispersal	0.61 P<0.0001	0.92 P<0.0001	0.58 P<0.0001
Spin-down		-0.28 P>0.05	-0.24 P>0.05
Walk-off			-0.62 P<0.0001

[a]Reproduced with permission from Ref. 18. Copyright 1984, Entomo-
logical Society of America. Correlation coefficients calculated
from transformed (arcsin) dispersal and mortality data at 500
ppm.

[b]Calculated from data in Ref. 3.

Formamidines and Mite Reproduction

The procedures for examining the effects of formamidines on two-
spotted spider mite reproduction were similar to those for dis-
persal mentioned above with the following exception. After the
treated leaflets had dripped dry, the surviving mites were
transferred to untreated leaf discs. This initial transfer was
made to minimize potential residual activity and to ensure that
formamidine effects on reproduction were due to the treatment
applied to the adult female mites. Under these conditions, there
should be little, if any, direct ovicidal action or mortality of
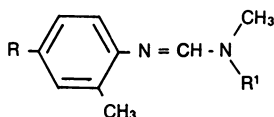
Figure 1. Essential moiety for lethal activity of formamidines in acarines. Reproduced with permission from Ref. 1. Copyright 1982, Academic Press.
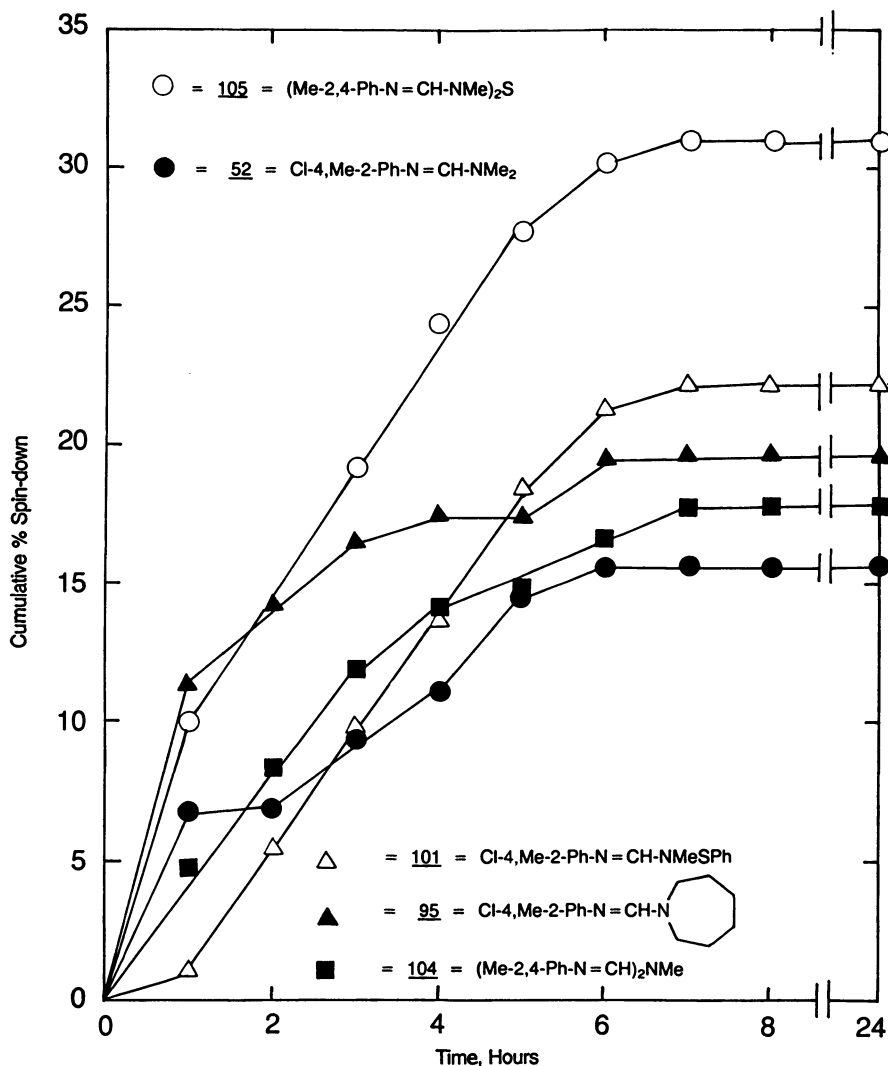
Figure 2. Cumulative percentage spin-down by twospotted spider mites exposed to formamidines at a concentration of 500 ppm. (Reproduced with permission from ref. 18. Copyright 1984 Entomological Society of America.) Continued on next page.
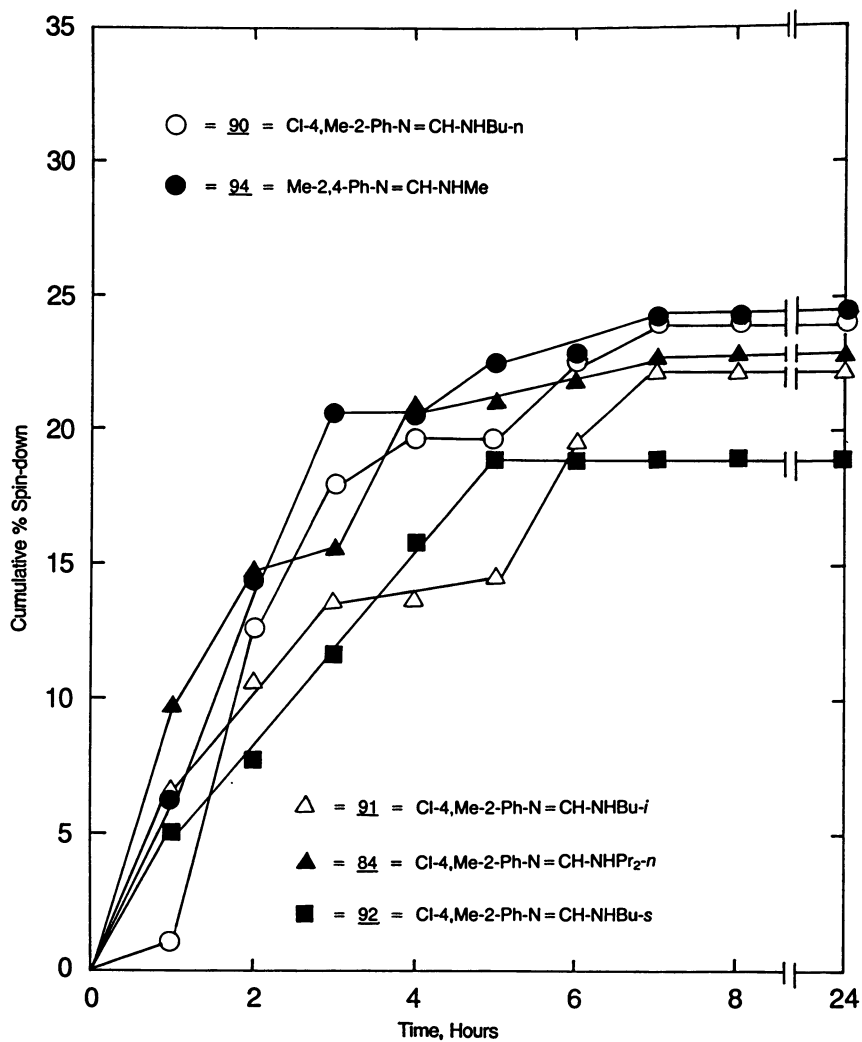
Figure 2.--Continued. Cumulative percentage spin-down by twospotted spider mites exposed to formamidines at a concentration of 500 ppm. (Reproduced with permission from ref. 18. Copyright 1984 Entomological Society of America.)

any newly hatched larvae as a result of their contact with any
residual deposit. Similar transfers were made at 24 and 48 hours
posttreatment. These tests were conducted in an environmental
chamber at 22-23°C, 50-65% relative humidity, and 16:8 hour photo-
period. The total number of eggs and the number of unhatched eggs
were recorded at 24, 48, and 72 hours after treatment (19).

In these studies, the activity of 105 formamidines, two form-
anilides, and two anilines on mite fecundity and egg hatch was
investigated. With regard to fecundity, 82 compounds yielded a
significant effect. Sixty-three compounds reduced fecundity at
one or both concentrations; 54 of these remained effective in re-
ducing egg production for as long as or by 72 hours posttreatment.
Eight compounds increased fecundity, while one formamidine was
stimulatory at the low concentration and inhibitory at the high
concentration (19).

Tables III, IV, and V present in decreasing order the ten
most active formamidines affecting fecundity, egg hatch, and re-
productive potential, respectively. The three most effective
compounds were formamidines 102, 105, and 101 in each case. Form-
amidine 17 was moderately active against fecundity and reproduc-
tive potential but did not affect egg hatch (19).

Table III. Ranking of Formamidines in Order of Their Decreasing
Potencies as Inhibitors of Twospotted Spider Mite Egg
Production at Seventy-two Hours Posttreatment[a]

Compound Number[a]	Structure	Inhibition of Egg Production, %
102	Me-2,4-Ph-N=CH-NMeSPh	97.2
105	(Me-2,4-Ph-N=CH-NMe)$_2$S	92.9
101	Cl-4,Me-2-Ph-N=CH-NMeSPh	85.6
17	n-Bu-4-Ph-N=CH-NMe$_2$	56.5
50	Cl-3,Me-2-Ph-N=CH-NMe$_2$	38.7
71	Me-2,4,6-Ph-N=CH-NMe$_2$	36.0
95	Cl-4,Me-2-Ph-N=CH-cyclohexylidene	34.2
100	Cl-4,Me-2-Ph-N=CH-NMeCH$_2$SMe	32.1
37	Me-2,4-Ph-N=CH-NMe$_2$	31.6
98	Cl-4,Me-2-Ph-N=CH-methylmorpholino	31.6

[a]From Ref. 19. Ranking was accomplished for 500 ppm treatment
data.

A correlation matrix for the effects of formamidines on two-
spotted spider mite reproduction and mortality is shown in Table
VI. Highly significant relationships existed among the effects
of formamidines on fecundity, egg hatch, and reproductive poten-
tial, whereas only weak associations were found between these
parameters and formamidine-induced mortality (19).

The weak activity of 52 (chlordimeform) and 104 (amitraz) as
reproduction inhibitors in individual mites was surprising. Of
the 109 compounds examined, the rank order for inhibition of re-
productive potential at 500 ppm at 72 hours was 50 for formamidine
52 (chlordimeform); it yielded 11.7%, 5.6% and 16.6% reduction of

fecundity, egg hatch, and reproductive potential, respectively. The rank order for inhibition of reproductive potential was >64 or nonsignificant for formamidine 104 (amitraz); it yielded 10.0%, 1.4%, and 12.3% reduction of fecundity, egg hatch, and reproductive potential, respectively.

Table IV.   Ranking of Formamidines in Order of Their Decreasing
    Potencies as Inhibitors of Twospotted Spider Mite Egg Hatch
                at Seventy-two Hours Posttreatment[a]

Compound Number	Structure	Inhibition of Egg Hatch, %
102	Me-2,4-Ph-N=CH-NMeSPh	82.6
101	Cl-4,Me-2-Ph-N=CH-NMeSPh	65.3
105	$(Me-2,4-Ph-N=CH-NMe)_2S$	45.8
18	$MeO-2-Ph-N=CH-NMe_2$	13.5
44	$MeO-3,4-Ph-N=CH-NMe_2$	13.2
43	$MeO-2,5-Ph-N=CH-NMe_2$	12.8
22	$NO_2-3-Ph-N=CH-NMe_2$	12.3
16	$Et-4-Ph-N=CH-NMe_2$	12.3
2	$F-2-Ph-N=CH-NMe_2$	11.2
60	$MeO-4,Me-2-Ph-N=CH-NMe_2$	10.0

[a]From Ref. 19.   Ranking was accomplished for 500 ppm treatment data.

      To explore this further, a demographic study was conducted with chlordimeform, amitraz, and compound 17, which was the most active mite reproduction inhibitor among the nonlethal formamidines (20).   Each of the three formamidines caused a reduction in survivorship and fecundity which resulted in a significant decrease in the intrinsic rate of increase of the mite population. In these experiments, amitraz was most potent, chlordimeform was intermediate, and compound 17 was least potent (20).   This apparent disparity between the strong activity of chlordimeform and amitraz on mite reproduction at the population level and their weak activity at the individual level can be explained as follows. The effects of amitraz on population growth were due mainly to its lethal activity to the various life stages, and the effects of chlordimeform were due to its lethality to life stages as well as its reduction of fecundity.   The activity of compound 17 resulted only from its reduction of fecundity (20).

Causative Agent(s) of Formamidine Actions in Mites

Since the demonstration by Knowles and co-workers (5,24-26) that the lethal action of chlordimeform in southern cattle tick larvae was due to its N-demethyl metabolite which was formed by oxidative N-demethylation, there has been considerable interest relative to the actual compound(s) responsible for the lethal and sublethal actions of formamidines in mites.   With regard to lethality, chlordimeform and demethylchlordimeform were approximately equitoxic to the twospotted spider mite (3), the Kanzawa

Table V.   Ranking of Formamidines in Order of Their Decreasing
Potencies as Inhibitors of Twospotted Spider Mite Reproductive
Potential at Seventy-two Hours Posttreatment[a]

Compound Number	Structure	Inhibition of Reproductive Potential, %
102	Me-2,4-Ph-N=CH-NMeSPh	99.5
105	(Me-2,4-Ph-N=CH-NMe)$_2$S	96.2
101	Cl-4,Me-2-Ph-N=CH-NMeSPh	95.0
17	n-Bu-4-Ph-N=CH-NMe$_2$	57.2
50	Cl-3,Me-2-Ph-N=CH-NMe$_2$	41.1
95	Cl-4,Me-2-Ph-N=CH-cyclohexylidene	38.6
71	Me-2,4,6-Ph-N=CH-NMe$_2$	38.1
100	Cl-4,Me-2-Ph-N=CH-NMeCH$_2$SMe	36.1
37	Me-2,4-Ph-N=CH-NMe$_2$	33.3
98	Cl-4,Me-2-Ph-N=CH-methylmorpholino	32.6

[a]From Ref. 19. Ranking was accomplished for 500 ppm treatment
data. Inhibition of reproductive potential = 1 -[(mean number
of eggs in treatment/mean number of eggs in control) x (mean
% hatch in treatment/mean % hatch in control)].

spider mite, Tetranychus kanzawai (27), the cheese mite, Tyropha-
gus putrescentiae (27), and Macrocheles muscadomesticae, a mite
predator of house fly, Musca domestica, eggs (27). Twospotted
spider mites have been shown to degrade chlordimeform to demethyl-
chlordimeform, N'-(4-chloro-o-tolyl)formamidine (didemethylchlor-
dimeform), 4-chloro-2-methylformanilide, and 4-chloro-2-methyl-
aniline (3). Didemethylchlordimeform also was lethal to two-
spotted spider mites, but it was not as toxic as chlordimeform
and demethylchlordimeform; the formanilide and aniline metabolites
were not lethal to mites (3).
      Twospotted spider mites also have been shown to degrade ami-
traz and U-44193 to N'-(2,4-dimethylphenyl)-N-methylformamidine
(BTS-27271), 2,4-dimethylformanilide, and 2,4-dimethylaniline
(28). Amitraz was twice as toxic as BTS-27271 to twospotted
spider mites (3). It is clear that chlordimeform, amitraz, and
U-44193 were each converted in mites to a formamidine metabolite
that possesses lethal action. Moreover, it is clear that in the
other 2,4-dimethylphenyl- and 4-chloro-2-methylphenylformamidines
those compounds capable of forming N'-aryl-N-monomethylformami-
dines were lethal (3). However, in the case of N'-(4-chloro-o-
tolyl)-N,N-diethylformamidine and its N-monoethyl analog, both
compounds were lethal to twospotted spider mites, but the N,N-
diethyl derivative was three-fold more toxic than the N-monoethyl
derivative (3). With the N,N-di-iso-propyl and N-mono-iso-propyl
compounds, the former was not lethal to spider mites, whereas the
latter was quite toxic (3). At this time, there are no unequivo-
cal data to show that metabolic conversion of formamidines to
N-monoalkylformamidines is a prerequisite for killing action in
mites. However, such conversion clearly would contribute to over-
all toxicity and may be required in some instances.

Table VI. Correlation Matrix for the Effects of Formamidines on Twospotted Spider Mite Reproduction and Mortality at 72 Hours[a]

	Fecundity	Egg Hatch	Mortality[b]
Reproductive Potential	0.98 $P \leq 0.0001$	0.70 $P < 0.0001$	0.32 $P < 0.001$
Fecundity		0.73 $P < 0.0001$	0.35 $P < 0.0079$
Egg Hatch			0.27 $P \leq 0.041$

[a] Reproduced with permission from Ref. 19. Copyright 1986, Entomological Society of America. Concentration of test compounds was 500 ppm.

[b] Calculated from data of Chang and Knowles (3).

In the case of dispersal, chlordimeform, amitraz, and U-44193 and their metabolites with the formamidine moiety were active, whereas their respective formanilide and aniline metabolites were inactive (Table I). It is apparent that conversion of chlordimeform and amitraz to demethylchlordimeform and BTS-27271, respectively, would result in the formation of metabolites that are more active than the parent formamidines as elicitors of dispersal behavior in mites, but whether this conversion is required for activity remains to be demonstrated. U-44193 was appreciably more active in eliciting dispersal behavior than was BTS-27271 as the EC_{50} values listed above and the data in Table I indicate. This may be related at least in part to the fact that U-44193 is a labile molecule and that conversion of U-44193 to BTS-27271 yields two molecules of the latter per molecule of the former (see below). Since some parent formamidines that apparently do not degrade to formamidine metabolites (e.g., formamidines 95 and 96) also elicited dispersal behavior (18), dispersal activity must be due at least in part to the parent compound and not exclusively to a metabolite.

With regard to reproduction, demethylchlordimeform was more active than chlordimeform in decreasing fecundity and egg hatch in spider mites; 4-chloro-2-methylaniline also possessed some activity against egg hatch only, while the formanilide was inactive (19). Thus it seemed that with chlordimeform the demethyl metabolite played a major role in its effects on spider mite reproduction. However, the results with amitraz and U-44193 were more difficult to interpret. Their common metabolites, BTS-27271, 2,4-dimethylformanilide, and 2,4-dimethylaniline all significantly decreased fecundity and egg hatch, yet U-44193 was one of the most active inhibitors of these processes in mites, and amitraz had no significant effect. Examination of metabolism data (28) revealed that in twospotted spider mites exposed to U-44193 or amitraz, levels of parent compounds were low (<1.0%), but levels of BTS-27271 were several fold higher in mites exposed to

U-44193 than in those exposed to amitraz (28). Further, there was
evidence to indicate that BTS-27271 once formed would be degraded
slowly (28). The fact that cleavage of one molecule of U-44193
yielded two molecules of BTS-27271, while cleavage of one molecule
of amitraz yielded only one molecule of this compound may partly
explain their differential effects on spider mite reproduction.
Thus it appeared that with U-44193 the formamidine metabolite BTS-
27271 was present in high levels and played a major role in the
effects of this bis-formamidine on reproduction. With amitraz it
appeared that BTS-27271 did not achieve levels high enough to in-
hibit reproduction. It follows that the effects of chlordimeform,
amitraz, and U-44193 on mite reproduction were due mainly to
metabolites, especially the respective N'-aryl-N-monomethylform-
amidines. However, it should not be assumed that this was the
case with the other active compounds, especially the nonlethal
formamidines which cannot be degraded to either of these two N'-
aryl-N-monomethylformamidines. In this instance, the active agent
must be the parent compound and/or some other metabolite(s).

Mechanisms Associated with Formamidine Actions in Mites

Indirect evidence for a neurotoxic action of formamidines in mites
was provided when Lund et al. (29), using some of the bioassay
data of Chang and Knowles (3), demonstrated that a strong cor-
relation existed between the lethal action of some compounds to
twospotted spider mites and their ability to cause neuronal excit-
ation in tobacco hornworm, Manduca sexta, fourth abdominal ganglia.
The most potent formamidines possessed the lethal moiety, a fea-
ture later shown to be required for stimulation of octopamine rec-
eptors by formamidines in insects (30). Using a similar approach,
it was shown that a relationship existed between lethality to two-
spotted spider mites of a small series of N'-aryl-N,N-dimethyl-
formamidines (3) and the stimulation of cyclic AMP synthesis in
firefly, Photinus pyralis, lantern preparations by their corres-
ponding N'-aryl-N-methylformamidines (31). Since the firefly
lantern adenylate cyclase is sensitive to octopamine, these
results suggest that interference with octopaminergic transmis-
sion may be involved in some of the actions of formamidines in
mites. For example, the fact that formamidine-induced lethality
and walk-off dispersal in mites were strongly correlated impli-
cates a similar mechanism(s) in these events, probably involving
perturbation of octopamine-mediated transmission. However, this
association of formamidine interaction with octopamine receptors
in mites is suggested with some caution because the presence of
this amine has yet to be demonstrated in these organisms and evi-
dence is accumulating that formamidines also can interact with
other biogenic amine receptors in ticks (32), snails (33), and
insects (34) as well as in mammals (35). The lack of a signifi-
cant relationship between formamidine-induced lethality and spin-
down dispersal suggests that a different, but not necessarily
unrelated, mechanism may be involved with this type of behavior.
Interestingly, spin-down dispersal was elicited in the carmine
spider mite by several formamidines and nonformamidines including
cyhexatin and dienochlor (17).

In terms of understanding the formamidine effects on mite re-
production, the picture is even less clear than that of dispersal
behavior. The lack of significant relationships between formam-
idine-induced lethality and effects on fecundity and egg hatch
suggest that other types of receptors and/or mechanisms are in-
volved in these actions. Interestingly, chlordimeform and amitraz
have been shown to inhibit synthesis of prostaglandin E_2 from
arachidonic acid by bovine seminal vesicle microsomes (36), and
prostaglandins have been shown to stimulate oviposition in some
insects (37). It might be instructive to investigate the activity
of formamidines and their metabolites as inhibitors of prostag-
landin synthesis in mites.

Direct evidence for the mechanisms of these toxic actions of
formamidines in mites is lacking. This is primarily a consequence
of the limited knowledge of mite neurochemistry and physiology.
In an attempt to overcome these deficiencies, we have conducted
some studies with the bulb mite (21-23). As compared to the two-
spotted spider mite, it is larger and easier to rear. Initially
we have turned our attention to biogenic amines and their regu-
latory mechanisms. Using HPLC with electrochemical detection,
high levels of 3,4-dihydroxyphenylalanine (DOPA) and dopamine (21)
were found in female bulb mites and their eggs (Table VII).
Norepinephrine, 3,4-dihydroxyphenylacetic acid, N-acetyldopamine,
5-hydroxytryptamine, 5-hydroxytryptophan, 5-hydroxyindoleacetic
acid, 5-hydroxytryptaphol, or N-acetyl-5-hydroxytryptamine were
not detected, and results with epinephrine and deoxyepinephrine
thus far have been inconclusive (21). Work with octopamine and
2-phenylethylamine (PEA) is in progress.

Table VII.   Levels of Dopamine and Dihydroxyphenylalanine (DOPA)
         in Females, Males, and Eggs of the Bulb Mite[a]

Mite	Level (ng/g)[b]	
	Dopamine	DOPA
Females	390(71)	1283(134)
Males	95(19)	1126(123)
Eggs	1187(142)	2594(112)

[a]From Ref. 21. Levels are means (n=3) with standard deviations in
   parentheses.

Table VIII gives the effects on levels of dopamine and its
precursor DOPA after exposure of bulb mites in vials precoated
with chlordimeform. Chlordimeform resulted in an initial increase
in levels of dopamine and DOPA; after 24 hours, DOPA levels still
were increased, but there was a significant decrease in levels of
dopamine (23). In other studies (22), it was shown that whole
homogenates of bulb mites rapidly metabolized PEA, but were appre-
ciably less active against tryptamine, 5-hydroxytryptamine, and
dopamine. Moreover, no deamination of octopamine was detected
(Table IX). PEA metabolism by bulb mite homogenates was inhibited

Table VIII. Effect of Chlordimeform on Levels of Dopamine and
Dihydroxyphenylalanine (DOPA) in the Adult Female Bulb Mite[a]

Treatment and	% of Control	
Time	Dopamine	DOPA
Chlordimeform, 1 Hr	116*	126**
Chlordimeform, 24 Hr	67**	331***

[a]From Ref. 23. Mites were exposed in vials precoated with an
acetone solution of chlordimeform at a concentration of
1 mg/ml. *** = P<0.001, ** = 0.01>P>0.001, * = 0.05>P>0.01
(Student's t test).

by chlordimeform and its formamidine (Figure 3) and formanilide
(Figure 4) metabolites, as well as other formamidines and form-
anilides (Figure 4) (23). PEA degradation also was inhibited by
two irreversible monoamine oxidase (MAO) inhibitors, pargyline
and tranylcypromine, and a reversible MAO inhibitor, harmaline
(22). The fact that PEA, but not 5-hydroxytryptamine, was rapidly
metabolized and that PEA degradation was almost completely blocked
by classical MAO inhibitors strongly suggested the presence in
bulb mites of a Type B MAO (22). This apparent involvement of MAO
in the degradation of some biogenic amines by the bulb mite and by
the cheese mite (38) is interesting since N-acetylation and not
oxidative deamination probably is the major pathway for biogenic
amine inactivation in insects (39-42).
     These in vitro and in vivo investigations indicate that
chlordimeform as well as other formamidines and formanilides
can interfere with biogenic amine regulatory mechanisms in bulb
mites. Whether these actions are related to any of the form-
amidine effects on mite dispersal and reproduction remains to
be demonstrated.

Table IX. Oxidative Deamination of Biogenic Amines
by Bulb Mite Homogenates[a]

Amine	pmoles/hr/mg (wet weight)
2-Phenylethylamine	12.75(2.2)
Tryptamine	1.52(0.1)
5-Hydroxytryptamine	0.24(0.1)
Dopamine	0.12(<0.1)
Octopamine	<0.01

[a]Reproduced with permission from Ref. 22. Copyright 1985,
Elsevier Science. Homogenate and substrate concentrations
were 15 mg/ml and 1 µM, respectively. Data are means (n=3)
with standard deviations in parentheses.
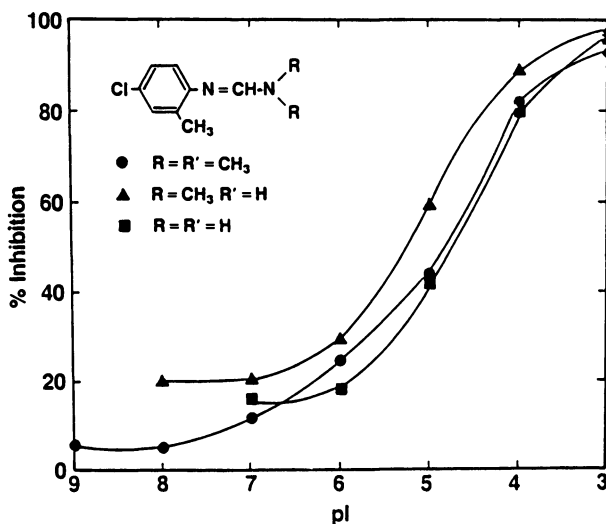
Figure 3. Inhibition of 2-phenylethylamine degradation in bulb mite homogenates by chlordimeform and its formamidine metabolites. Homogenate and substrate concentrations were 15 mg/ml and 1×10^{-6} M, respectively. Points are means (n=3). Redrawn with permission from Ref. 23. Copyright 1985, Society of Chemical Industry.
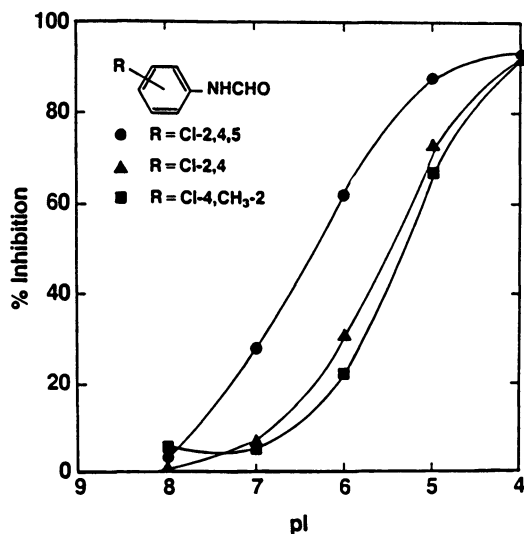
Figure 4. Inhibition of 2-phenylethylamine degradation in bulb mite homogenates by the formanilide metabolite of chlordimeform ($R=Cl-4,CH_3-2$) and other formanilides. Homogenate and substrate concentrations were 15 mg/ml and 1×10^{-6} M, respectively. Points are means (n=3). Redrawn with permission from Ref. 23. Copyright 1985, Society of Chemical Industry.

Summary

The studies discussed herein show that formamidines without the conventional lethal moiety can elicit dispersal behavior and can interfere with reproduction in twospotted spider mites. Walk-off dispersal elicited by formamidines was correlated with formamidine-induced mortality. Since these structure-activity relationships of formamidines are similar to those for pertubation of octopaminergic transmission in insects, a similar mechanism could be involved in these actions in mites. Since structure-activity relationships for formamidine effects on spin-down and reproduction were not correlated with those for lethality, other mechanisms, which may or may not involve octopamine or other biogenic amines, are probably associated with these actions.

Acknowledgments

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Chapter 14

Actions of Drugs and Pesticides on Components of Octopaminergic Neurotransmission

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The stimulation of the octopaminergic nervous system of invertebrates is a proven strategy for the control of important pest species. This has been achieved in the past by the use of octopamine receptor agonists such as formamidine and imidazoline derivatives. However, other potential strategies to achieve this end include the inhibition of cyclic nucleotide phosphodiesterase, inhibition of the neural reuptake of octopamine, and inhibition of octopamine N-acetyltransferase. Using the American cockroach nervous system, formamidines were found to inhibit both the uptake and acetylation of octopamine, but not with a potency comparable to their effect on octopamine receptors. The tricyclic antidepressant, desipramine, and the benzylamine, xylamine, were the most active inhibitors of these octopamine removal systems. The pharmacological profiles for uptake and N-acetylation appear to be quite similar, but differ from that of the adenylate cyclase-linked octopamine receptor.

Over the last decade it has become increasingly clear that octopamine (OA) plays a number of discrete and vital roles in the physiology of insects through its actions as a neuromodulator, neurohormone and neurotransmitter (1,2,3). Much is known regarding its importance as a peripheral neuroeffector (4), but its functions in setting the degree of excitability in parts of the central nervous system and as a key elicitor and coordinator of specific behaviors (5,6,7), though less well understood, may be even more significant for exploitation in insect control.

Formamidines (such as chlordimeform (Figure 1) and amitraz) and imidazolines (such as naphazoline and XAMI; Figure 1) cause a series of behavioral and lethal effects in insects and acarines that may be ascribed entirely or in part to the actions of these compounds and their common metabolites as powerful agonists at octopamine receptors

Octopamine (OA)

Chlordimeform (CDM)

N-Acetyloctopamine (NAOA)

Naphazoline

XAMI

Xylamine

Desipramine
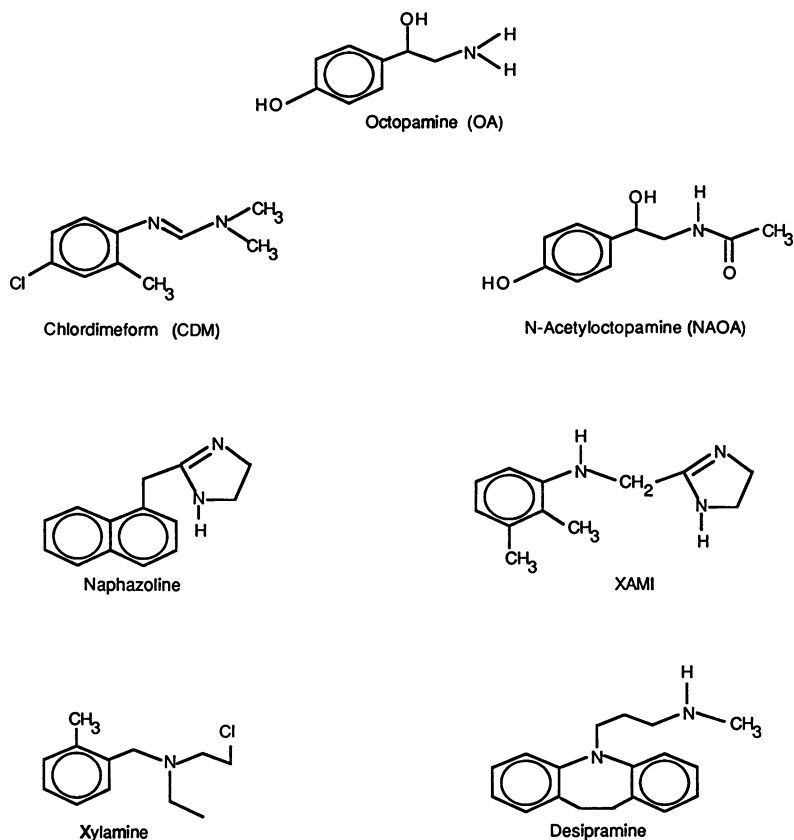
Figure 1. The structures of some compounds which affect octopaminergic systems.

(8,9). This validates the stimulation of octopaminergic systems as
an effective strategy for insecticidal/acaricidal action. However,
there are several approaches to achieving this end other than by a
direct stimulatory effect on the octopamine receptor.
      The action of octopamine is probably terminated by
carrier-mediated reuptake systems as with other biogenic amines (10).
Blocking uptake could result in an increased level of octopaminergic
activation. It appears that a major catabolic route for octopamine
in insects is through N-acetylation, a situation not paralleled in
vertebrates (1,3). Inhibitors of this N-acetyltransferase activity
could also enhance octopaminergic action. In the majority of cases
so far studied, the stimulation of octopamine receptors results in an
enhanced activity of adenylate cyclase and elevated cyclic AMP (cAMP)
levels in the target tissue (1,3,11). Cyclic nucleotide
phosphodiesterase activity is an important component regulating the
intracellular level of cAMP and inhibition of this enzyme also leads
to elevated cAMP levels. Phosphodiesterase inhibitors such as
methylxanthines cause behavioral effects similar to those induced by
octopaminergic agonists and synergize the activity of these compounds
in insects (8,12). These alternatives to the direct stimulation of
OA receptors deserve further evaluation as prospects for insecticidal
and insectistatic actions. Additionally, although it is clear that
formamidines and imidazolines have potent direct effects on the
receptor itself, it is also possible that the octopaminergic
stimulation could be augmented by effects on other components of
octopamine-activated neurotransmission. Entities such as the uptake
carrier and the active site of octopamine N-acetyltransferase
presumably have binding sites for OA and may, like the receptor, be
able to bind these synthetic analogs of OA. Using quantum mechanical
calculations Tosi et al. (13) have shown that there are strong
conformational similarities between formamidine insecticides and
(R)-octopamine. Little has been done so far to evaluate the
contribution that these alternative actions may make to the overall
effects of formamidines and imidazolines on invertebrates.
Preliminary results from studies in these areas are described below.

Materials and Methods

Animals. Adult male cockroaches (Periplaneta americana) were reared
at 24°C and fed rat chow and water ad lib. For 24 hours prior to
dissection they were isolated in individual containers. Fireflies
(Photinus pyralis) were caught locally and either used immediately,
or the whole insects were frozen at -80°C until use. Tobacco
hornworms (Manduca sexta) were reared on an artificial diet and
utilized in the 3rd or 4th day of the final larval instar.

Chemicals. Ring-labelled [^{3}H]-octopamine (44 Ci/mmol) and
[2,8-^{3}H]adenosine 3',5'-cyclic phosphate (41 Ci/mmol) were purchased
from Amersham Corp., Arlington Hts., IL. Xylamine was synthesized by
the method of Ransom et al. (14). Chlordimeform (CDM),
N-demethylchlordimeform (DCDM), and N,N-bisdemethylchlordimeform
(DDCDM) were synthesized as described by Hollingworth (15).
2-(2,3-xylylaminomethyl)-2-imidazoline (XAMI) was synthesized from
2-chloromethyl-2-imidazoline (16). N-Acetyloctopamine was

synthesized using the method of Anderson (17). All other compounds
were obtained from Sigma Chemical Co., St. Louis, MO.

Biochemical Assays. Phosphodiesterase activity was assayed in
homogenates of the cockroach central nervous system using a
modification of the anion exchange column method described by
Thompson et al. (18) with [3H]-cAMP as the substrate. Cockroach
nerve cords (10-20 mg tissue/ml) were homogenized in ice-cold Tris
buffer (62.5 mM, pH 7.7 (25°C) containing 6.3 mM MgSO4 and 2.5 mM
mercaptoethanol). The incubation mixture consisted of 140 ul buffer,
20 ul tissue homogenate, 20 ul of inhibitor or buffer, and 20 ul of
cAMP solution (1 mM, 2.4 uCi/ml). This mixture was incubated at 30°C
for 20 min, the reaction was stopped by boiling, and after cooling,
100 ul cobra venom solution (1 mg/ml) was added. This was incubated
at 30°C for 30 min, then 1 ml methanol was added. The mixture was
loaded onto an anion exchange column (5.0 x 0.5 cm, Dowex-1, 200-400
mesh, pH 5.0 in methanol) and eluted with 1 ml methanol. The column
was drained and the eluent was counted in a liquid scintillation
counter (LSC).
     Adenylate cyclase activity was measured essentially as described
by Nathanson and Greengard (19) except that a mixture of ATP (0.75
mM) and GTP (0.15 mM) was used to initiate the reaction. The amount
of cAMP in the reaction mixture was determined with an assay kit from
Amersham Corp. based on the method of Tovey et al. (20). Tissue
homogenates were made from firefly light organs, and the ventral
nerve cords of adult Periplaneta and larval Manduca.
     The uptake of octopamine into the ventral nerve cord of P.
americana (21) and octopamine N-acetyltransferase activity (22) were
also assayed on the abdominal portion of the ventral nerve cord from
cold-anesthetized cockroaches. For the uptake experiments, the nerve
cord was blotted, weighed, then pre-incubated in normal or Na-free
saline (augmented with Tris) for 10 min. The nerve cord was then
incubated in OA-containing saline (2 uM, 4 uCi/ml) plus compound (100
uM) for 10 min at 28°C. The nerve cords were rinsed briefly in ice
cold saline, digested, then counted by LSC. Na-dependent uptake was
calculated as: total uptake (in the presence of Na) minus
Na-independent uptake (in the absence of Na). For the NAT assays,
the nerve cords were homogenzied (5 cords/ml) in ice-cold phosphate
buffer (50 mM, pH 7.0, containing 0.65 mM dithiothreitol). The
homogenate was centrifuged at 17,000xg for 5 min and the supernatant
was stored at -80°C for up to two weeks. The nerve cord homogenates
were assayed for NAT activity by incubating with equal amounts (25
ul) of 1mM acetyl CoA, [3H]-octopamine (40 uM, 16 uCi/ml) and
phosphate buffer (pH 7.0) or compound (1 mM). After incubation at
30°C for 30 min, a 10 ul sample was spotted on a methanol-prewashed
silica gel TLC plate together with OA and NAOA standards. The plate
was developed in n-butanol:acetic acid:water (4:1:5), then dried
thoroughly. The resulting separation was visualized with iodine, the
spots were scraped, the compounds eluted with water, and the amounts
of OA and NAOA were determined by LSC. The percent OA converted to
NAOA was calculated as: 100 x (cpm NAOA)/ (cpm NAOA plus cpm OA).

Results and Discussion

Phosphodiesterase Inhibition. The formamidine, chlordimeform (CDM), its two active N-demethylated metabolites (DCDM and DDCDM), and a representative imidazoline, XAMI, were studied as inhibitors of the cAMP phosphodiesterase of the cockroach CNS (Table I).

TABLE I.   Inhibition of cAMP Phosphodiesterase from Cockroach CNS

Inhibitor	% Inhibition[a] (mean ± S.D.)	IC50 (mM)
Theophylline	57 ± 4	0.56
Papaverine	66 ± 7	0.51
IBMX	89 ± 2	0.11
Chlordimeform (CDM)	(+) 7 ± 5	--
DCDM	(+) 3 ± 6	--
DDCDM	14 ± 4	--
XAMI	(+) 6 ± 26	--

[a](+) indicates stimulation rather than inhibition
Final concentration, 1 mM

At a screening concentration of 1.0 mM, none of these compounds gave noteworthy inhibition. The known phosphodiesterase inhibitors theophylline, isobutyl-3-methylxanthine (IBMX), and papaverine caused considerable inhibition at 1 mM and had IC50 values between 0.1 and 1.0 mM. It therefore seems unlikely that the excitatory effects caused by these octopaminergic agonists in invertebrates can be related to inhibition of cAMP phosphodiesterase activity.

Cyclic AMP Synthesis. The activity of adenylate cyclase from several insect sources was measured in the presence of OA and other octopaminergic agonists (Table II). The results are corrected for basal (unstimulated) adenylate cyclase activity and expressed as the Ka (concentration to cause a half-maximal stimulation of cAMP synthesis) and Vmax (maximum stimulation observed, related to the maximum stimulation observed with octopamine). The characteristics of the three preparations were similar in regard to their Ka values for octopamine but differed widely with the synthetic agonists, DCDM and XAMI. In each case the Ka values for these compounds were lower than those for OA, sometimes by a considerable margin (e.g. DCDM with Manduca and XAMI with Periplaneta). On the other hand these synthetic agents were only partial agonists except in the case of DCDM with Manduca where the maximum level of stimulation was indistinguishable from that seen with OA. Somewhat similar results showing enhanced but varying potencies of synthetic agonists for octopamine receptors in insects have been reported previously (9,23). Since a full physiological response may be achieved in vivo with only partial (10-20% of maximum) stimulation of tissue cAMP levels, the potential activity of such synthetic agonists in stimulating some insect octopaminergic systems may approach the low nanomolar range, but this will vary considerably between species.

TABLE II.   Effects of Drugs on cAMP Synthesis in Several Insect
Tissues

	Octopamine		DCDM		XAMI	
	Ka (nM)	Vmax (%OA)	Ka (nM)	Vmax (%OA)	Ka (nM)	Vmax (%OA)
Firefly Lantern	2500	100	470	85	280	85
Cockroach nerve cord	2000	100	600	57	7	76
Hornworm nerve cord	3000	100	33	100	150	74

Effects of Drugs on the Uptake of Octopamine.   Several drugs were
tested for their effects on both components (Na-dependent and
Na-independent) of uptake. The final concentration of
[^{3}H]-octopamine (2 uM) was chosen so that about 50% of the uptake was
Na-dependent and 50% was Na-independent. Based on the study of Evans
(21), the former fraction, which is readily saturable and shows a
high affinity for OA, probably relates to one or more specific uptake
systems, while the Na-independent fraction is not readily saturable,
shows a low affinity for OA and may relate to its absorption onto the
perineurium. Drugs were assayed on both fractions at 0.1 mM. No
significant (>20%) inhibition of the Na-independent fraction was seen
with any compound. However, several of these agents proved to be
effective inhibitors of Na-dependent uptake (Table III).

TABLE III.   Inhibition of Na-Dependent Octopamine Uptake and
N-Acetyltransferase in the Cockroach CNS

Inhibitor	% Uptake Inhibition[a] (mean + S.D.)	% NAT Inhibition[b] (mean + S.D.)
Xylamine	61 + 6	93 + 2
Desipramine	86 + 11	65 + 3
Chlordimeform	(+)3 + 7	16 + 5
DCDM	17 + 9	28 + 4
DDCDM	33 + 9	52 + 6
Synephrine	100 + 8	88 + 2
NAOA	18 + 9	1 + 6
Naphazoline	28 + 7	(+)2 + 5
XAMI	20 + 11	(+)7 + 5

[a]Final concentration, 0.1 mM
[b]Final concentration, 0.25 mM

While chlordimeform itself did not block uptake at this
concentration, DCDM and, particularly, DDCDM did cause inhibition.
It is worth noting that the unmethylated formamidine, DDCDM, most
closely approximates the structure of OA in this series. These

results can be compared to those reported recently by Scott and Knowles (24) who concluded that CDM and DCDM did not inhibit the uptake of labelled OA into the cockroach nerve cord. However, it should be noted that these workers did not demonstrate any Na-dependent uptake in their preparation.

Synephrine, the N-methylated analog of OA, completely blocked uptake at this concentration. The two imidazolines, naphazoline and XAMI, were somewhat effective as uptake blockers (20-30% inhibition), comparable to DCDM and DDCDM. The tricyclic antidepressant desipramine, a known amine uptake blocker in vertebrates, showed good potency in the cockroach system, as originally described by Evans (21). Finally, the N-chloroethyl benzylamine derivative, xylamine, has been described as a potent, specific and irreversible inhibitor of norepinephrine uptake in mammals (25). It was an active inhibitor of uptake in the cockroach preparation.

Effect of Drugs on the N-Acetyltransferase Activity of the Cockroach Ventral Nerve Cord. In order to determine the metabolic fate of octopamine taken into the cockroach nerve cord, nerve cords were incubated with high specific activity [3H]-octopamine according to the methods used to determine uptake, homogenized and the supernatant spotted on a TLC plate. After development in butanol:acetic acid:water (4:1:5), the plates were subjected to electrophoresis at pH 2.05 (formic acid, 0.47 M; acetic acid, 1.40 M) in the second dimension. A typical autoradiograph is shown in Figure 2. Virtually all of the radioactivity was located in the peaks that cochromatographed with octopamine (47.5%) and N-acetyloctopamine (50.8%). Two other minor unknown metabolites (1 and 2) were also detected. It is noteworthy that p-hydroxymandelic acid (p-OHMA), the potential product of monoamine oxidase (MAO) activity, was not detected. This agrees with the now generally accepted idea that MAO is not an important means of octopamine metabolism in those insects so far studied and that N-acetylation is the major metabolic fate for biogenic amines in the insects CNS (1,22,26-28). The rapidity of this reaction in the nerve cord is clear from the observation that even after a brief exposure to labelled OA, greater than 50% was converted to the N-acetyl analog.

As inhibitors of N-acetyltransferase activity, the formamidines showed a pattern similar to their effect on OA uptake i.e. CDM was the least active member of the series with DCDM and DDCDM increasingly more active. At 0.25 mM, DDCDM caused about 50% inhibition of NAT activity. Little previous work has been published on N-acetyl transferase inhibitors in insects. However, the inhibition by CDM of NAT acting on tryptamine was reported by Allais et al. (29). They showed that 1.2 mg of CDM applied topically to locusts reduced subsequent NAT activity in the brain in vitro by approximately 35%.

Synephrine, as a potential substrate for NAT, showed considerable inhibition, but naphazoline and XAMI were inactive. Desipramine proved to be quite effective as an inhibitor of NAT, but the most active compound in this group was xylamine. More detailed study of the inhibition by this compound gave an IC50 of less than 1 uM (without preincubation with the enzyme before addition of the substrate) and showed that the inhibition was progressive and

irreversible. Probably the mechanism of inhibition involves
alkylation through a reactive aziridinium product (14).

Significance of N-Acetyloctopamine as a Metabolite of Octopamine.
Since N-acetyloctopamine (NAOA) is the major product of OA metabolism
and is rapidly produced after uptake of OA, we thought that
N-acetylation might represent an inactivation process.
N-Acetyloctopamine was already found to be inactive compared to OA on
crayfish heart and skeletal muscle (30). To shed further light on
this question we examined the ability of NAOA to stimulate adenylate
cyclase activity in homogenates from the isolated adult firefly light
organ. As shown in Figure 3, OA stimulated this preparation with a
concentration for half maximal stimulation of about 2 uM.
N-Acetyloctopamine on the other hand caused virtually no increase in
cAMP synthesis over the very low basal level. A slight but
reproducible stimulation was observed at about 1 mM, but this could
have been due to small amounts of the precursor OA being present as
an impurity. In any case, in this well-studied octopaminergic
system, NAOA is at best a very weak agonist. Further study showed
that it did not act as an antagonist of octopamine-stimulated
adenylate cyclase either. The data presented in Table III indicate
that NAOA has little or no effect on either Na-dependent OA uptake or
on NAT. Thus it appears to have no significant interaction with
octopaminergic systems. Tentatively then, one can conclude that
N-acetylation represents a mechanism of inactivation for OA.

Relationship of Uptake, N-Acetyltransferase and the OA Receptor. As
pointed out initially, all three of these biochemical entities have
recognition sites for octopamine. It is interesting therefore to
compare their responses to the same series of drugs. There are
distinct differences in the properties of the octopamine-sensitive
adenylate cyclase compared to the other two activities e.g.
naphazoline and XAMI (Table II) are very active in stimulating cAMP
synthesis, but do not interact strongly with the uptake system or
NAT. Also the effect of the formamidines, DCDM and DDCDM, which
stimulate adenylate cyclase at micromolar concentrations, is much
more potent in the case of the adenylate cyclase than with uptake or
NAT. On the other hand, xylamine is active against both uptake and
NAT, but does not interact with comparable potency with the adenylate
cyclase system of the firefly light organ (Hollingworth,
unpublished).
        There is an intriguing degree of correlation between the
responses of uptake and NAT to the limited series of drugs studied,
as shown in Table III (r = 0.81, p < 0.01). Although there is no
reason a priori why these two systems should have similar
pharmacology, it may be that N-acetylation in some way limits or is
involved in the uptake process into the nerve cord, particularly in
view of the efficiency with which N-acetylation occurs. This
possibility is being studied further.
        These results indicate that the N-demethylation products of
chlordimeform have inhibitory effects on both the reuptake of OA into
the cockroach nerve cord and on its subsequent N-acetylation to the
putatively inactive NAOA. In both cases the tendency of these
results would be to increase the activity of endogenous octopamine.
Whether these effects occur with a potency that would make them
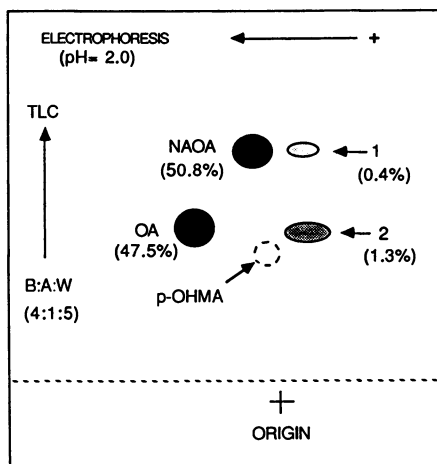
Figure 2.   Illustration of the metabolism of OA by isolated cockroach nerve cords. Nerve cords were incubated according to the methods for measuring OA uptake, then homogenized (3 nerve cords/300 ul), and 30 ul of the homogenate was spotted on the TLC plate for two dimensional analysis. Autoradiographs were made and the spots were then scraped and counted. Values shown are the mean of two determinations. The identities of compounds 1 and 2 were not determined.
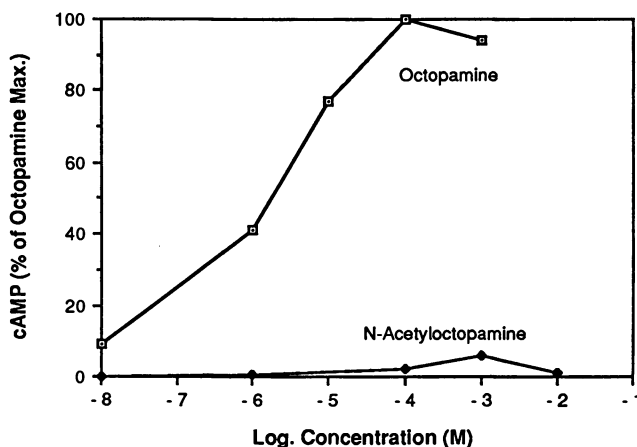
Figure 3.   Stimulation of adenylate cyclase from the firefly lantern by either octopamine or N-acetyloctopamine.

significant contributors to the overall octopaminergic action of the
formamidines is not clear. However, since DCDM and DDCDM stimulate
adenylate cyclase activity in cockroach nerve cord homogenates at
concentrations well below 1 uM in vitro (Table II, 9, 31) while the
effects on uptake and NAT are most evident at millimolar
concentrations, it is likely that these other actions are not the
major cause of octopaminergic stimulation in vivo. The same
arguments hold with the imidazoline, XAMI.
        The observation that both xylamine and desipramine are potent
inhibitors of octopamine uptake and acetylation suggests that they,
and their relatives, represent potential tools for the assessment of
the suitability of these systems as a target for the development of
novel insect control agents. Preliminary studies with xylamine
injected into adult American cockroaches and adult tobacco budworms
suggests that the activity is depressive rather than stimulatory.
However, more detailed studies of the physiological and behavioral
actions of these compounds are warranted.

Acknowledgments

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Chapter 15

N-Acetylation of Octopamine

A Potential Target for Insecticide Development

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High performance liquid chromatography with coulometric electrochemical detection was used to separate and quantify octopamine and N-acetyl octopamine in haemolymph and incubated tissues of the American cockroach, Periplaneta americana. Injected octopamine is rapidly removed from the insect haemocoel and this removal of octopamine occurs concomitantly with elevated haemolymph levels of N-acetyl octopamine. Studies with isolated tissues indicate that the gut, especially the Malpighian tubules, contribute substantially to the removal of octopamine by N-acetylation. The inclusion of the formamidine pesticide, N-demethylchlordimeform in incubations containing octopamine and Malpighian tubules inhibits the N-acetylation of octopamine by the incubated tissue.

The central role of octopamine (OA) in mediating a number of important physiological processes in insects (1,2) suggests that this compound may be a target against which specific insecticides could be developed. The physiological actions of OA appear to be mediated through interaction of the monoamine with receptors that have a high binding affinity for OA and which are located on the outer cell membrane of OA-sensitive cells. Much of the research effort directed towards exploitation of OA as a target has concentrated on the development of compounds which compete with OA for binding sites on the OA-sensitive receptors. This approach has been encouraged by the formamidine group of insecticides which exert at least part of their insecticidal action by acting as partial agonists of OA-sensitive adenylate cyclase (3,4) and by the phenylimidazolines which also show high biological activity in insects and acarines through agonistic activity on OA-sensitive receptors (5).

In contrast to the considerable research effort devoted towards the development of compounds that perturb OA-receptor interactions, few studies have examined the nature of OA-degradation or the possibility of developing insecticidal compounds that

interfere with this process. This situation is particularly surprising in light of the importance of acetylcholinesterase as a target for attacking the cholinergic system of insects.

Studies on monoamine inactivation mechanisms in insects have yielded equivocal results with N-acetylation (6-9), oxidative deamination (10,11) and O-sulphate or β-alanyl conjugation (12,13) proposed as possible routes of monoamine catabolism; however, the weight of evidence favours N-acetylation as the principal mechanism of monoamine breakdown. Sodium-sensitive and sodium-insensitive uptake mechanisms for OA have also been described in the cockroach central nervous system (14).

The present study was undertaken with the objective of developing a simple procedure using high performance liquid chromatography with electrochemical detection (HPLC/ElCD) to monitor the capacity of various insect tissues to effect N-acetylation of OA. The resulting protocol has been used to identify the Malpighian tubules as a major site of N-acetylation of OA and to demonstrate the potential for perturbing this process by chemical agents.

Materials and Methods

Reagents. DL-p-Octopamine hydrochloride, trehalose dihydrate and ethylenediaminetetraacetic acid (disodium salt) (EDTA) were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO.). Phosphoric acid and HPLC-solvents were obtained from Caledon Laboratories Ltd. (Georgetown, Ontario) and 1-octanesulphonate (sodium salt) (SOSA) from Regis Chemical Co. (Morton Grove, IL.). All other chemicals were Baker Analytical grade from Canlab (Toronto, Ontario). Water used for the preparation of all solutions was deionized, distilled and redistilled over potassium permanganate. N-acetyl p-octopamine (N-acetyl OA) was generously provided as a gift from Dr. J.S. Kennedy (NIMH, Rockville, MD.) and the formamidine pesticides chlordimeform (CDM) and N-demethylchlordimeform (DCDM) were donated by American Cyanamid (Princeton, N.J.).

Insects. Adult male cockroaches were taken at 1-3 months after the final moult from a colony of Periplaneta americana maintained under standard conditions in this laboratory (15). Insects were removed from the colony and held in individual petri dishes for 12-16 hours prior to experimentation.

The rate of removal of p-octopamine and N-acetyl OA was determined by injecting 5 μl of a solution containing 26 nmoles of the compound in cockroach physiological saline (16) (CPS) into the insect haemocoel and withdrawing 5 μl aliquots of haemolymph at various times after the initial injection. Procedures for injection and haemolymph collection have been described previously (17). Each haemolymph sample was transferred immediately to a 1.5 ml polypropylene tube containing 370 μl 0.3 mM EDTA in 0.1 M perchloric acid and vortexed. Following centrifugation at 40,000 g for 30 min, a 5 μl aliquot of the supernatant was injected directly onto the HPLC column for analysis.

Whole gut and/or Malpighian tubules were dissected from insects under ice cold CPS and pre-incubated for 10 min at 25°C in CPS containing glucose and trehalose (18) (GTR). The whole gut preparation was ligated at the anterior end of the oesophagus and

the posterior end of the rectum. The preparation was then transferred to 500 µl GTR containing 5×10^{-5}M OA or 5×10^{-5}M OA and an equimolar concentration of CDM or DCDM. Following incubation, 100 µl of the incubation medium was transferred to a 1.5 ml polypropylene tube containing 0.3 mM EDTA in 700 µl 0.1M perchloric acid and a 5 µl aliquot used for chromatographic analysis.

Chromatography. Chromatographic separation of OA and N-acetyl OA was achieved on a 5cm x 4.6mm ID column packed with 3 micron, end-capped octadecyl Spherisorb in series with a 15cm x 4.6mm ID column packed with 5 micron, end-capped octadecyl Spherisorb (both columns were obtained from Regis Chemical Co., Morton Grove, IL). Samples were injected onto the HPLC column using a WISP 710A autosampler (Waters, Mississauga, Ontario) that was modified to accommodate pure teflon seals in the injector housing. The mobile phase was delivered using a Spectra-Physics Model 740B pump (Spectra-Physics, San Jose, CA.) equipped with two pulse dampeners (Waters, Mississauga, Ontario). The mobile phase contained 0.1M NaH_2PO_4, 1.0 µM EDTA, 0.3 mM SOSA, 10% methanol and 0.2% tetrahydrofuran. The final pH was adjusted to 3.6 with 85% phosphoric acid. The mobile phase was continually degassed with a stream of helium and pumped at a flow rate of 1 ml/min.

    Eluted peaks were detected by electrochemical oxidation using the ESA 5100A coulometric detector equipped with an ESA 5010 dual electrode detector cell and a guard cell (ESA, Bedford, MA). The guard cell was placed between the pump and injector (19) and set at a potential of 0.75V. The first electrode of the analytical cell was set at a potential of 0.5V and the second electrode at which OA and N-acetyl OA are oxidized was set at 0.7V.

Results and Discussion

Chromatography. A rapid, convenient procedure for estimation of OA by HPLC/E1CD has been described (19). Modification of this method enables simultaneous estimation of OA and N-acetyl OA as indicated in the chromatogram illustrated in Figure 1. Confirmation of the identity of N-acetyl OA was achieved by comparing the current generated by oxidation of standard N-acetyl OA with that resulting from the putative compound produced by a whole gut preparation. The results are indicated in Table I and demonstrate complete duplication of oxidation characteristics for the two samples at various electrode potentials. These data, together with the identical retention times of the standard and biological compound, validate the separation and identification of N-acetyl OA by the HPLC/E1CD procedure employed in the present study.

Removal of haemolymph OA. OA levels in the haemolymph of P. americana increase rapidly following excitation and/or the initiation of exercise and are rapidly restored to normal levels when the activity ceases (17). The possible contribution of N-acetylation to OA removal from haemolymph was investigated by injecting a 5 µl solution containing 26 nmoles OA into the haemocoel of adult male cockroaches and monitoring the rates of OA removal and N-acetyl OA appearance. The results indicate rapid removal of OA and appearance of N-acetyl OA in the haemolymph
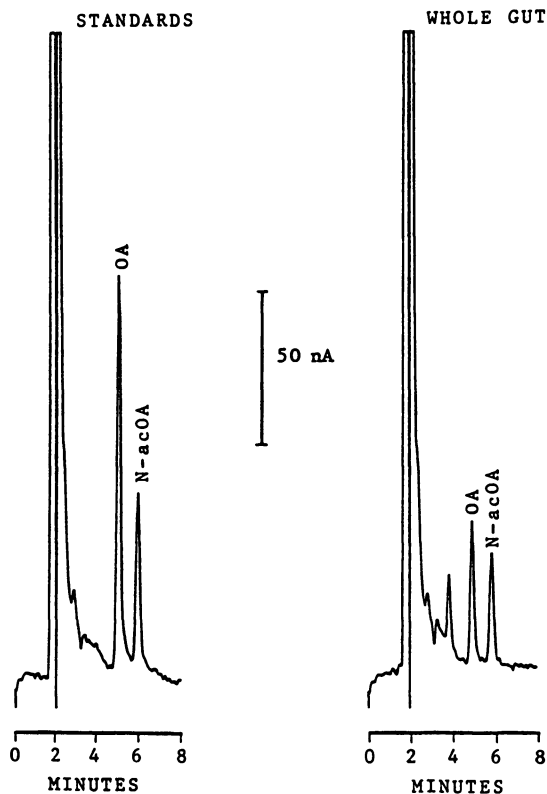
Figure 1. Chromatograms of p-octopamine (OA) and N-acetyl p-octopamine (N-acOA) obtained from standards (2ng OA and 1ng N-acOA) and a whole gut preparation incubated for 20 minutes in medium containing 5x10^{-5}M OA. (Chromatographic conditions as described in text.)

Table I. Comparison of the Current Generated
by the Oxidation of Standard N-Acetyl Octopamine
and Biologically Produced N-Acetyl Octopamine
(from whole gut preparation of Periplaneta americana)
at Different Detector Potentials

Potential (V)	Current Ratio	
	Standard	Biological
0.85	1.00	1.00
0.80	0.9635 ± 0.0050	0.9629 ± 0.0064
0.75	0.8608 ± 0.0074	0.8604 ± 0.0099
0.70	0.7519 ± 0.0034	0.7456 ± 0.0038
0.65	0.6885 ± 0.0007	0.6925 ± 0.0048
0.60	0.6412 ± 0.0064	0.6281 ± 0.0077
0.55	0.4116 ± 0.0041	0.4123 ± 0.0054
0.50	0.0918 ± 0.0011	0.0931 ± 0.0028
0.45	0	0

Values indicate mean ± SEM for 3 determinations.

(Table II). The levels of N-acetyl OA are maximal at about 15 min
following the injection of OA and decline thereafter, whereas the
most rapid drop in OA concentration occurs during the first five
minutes following injection. The initial rapid removal of OA is
consistent with previous studies on the restoration of normal
levels following flight-induced elevation of haemolymph OA (20,21).

Table II. Fate of Injected Octopamine in Haemolymph of the
American Cockroach Periplaneta americana

Time after Injection	Concentration (μM)	
	Octopamine	N-acetyloctopamine
1 min	157.5 ± 26.9 (14)	Not Detected
5	37.7 ± 8.3 (12)	25.5 ± 6.3 (5)
10	14.4 ± 3.1 (10)	35.6 ± 3.3 (5)
15	4.7 ± 1.0 (8)	39.4 ± 5.2 (5)
20	1.8 ± 0.8 (8)	36.2 ± 2.9 (5)
30	0.2 ± 0.2 (10)	17.3 ± 2.5 (5)
60	Not Determined	18.6 ± 0.9 (5)

Amount of p-octopamine injected was 26 nmoles in 5 μl CPS
Values indicate mean ± SEM for number of determinations indicated
  in parentheses

Only about 25% of the OA that is removed from the haemolymph is
subsequently detected in this tissue as N-acetyl OA. A number of
insect tissues possess N-acetyl transferase activity and have been
shown to retain N-acetylated derivatives of other monoamines (22),
therefore, it can be assumed that much of the N-acetyl OA formed
following OA-injection is not released into the haemolymph.
Indeed, preliminary studies indicate that the gut plays a major

role in removal of haemolymph OA (23); therefore, it is likely that large amounts of N-acetyl OA accumulate in the lumen of this organ. The results presented in Table II suggest also that N-acetyl OA is removed from haemolymph following release of the compound from tissues with N-acetyltransferase activity.

This possibility was confirmed by injecting 5 μl N-acetyl OA (26 nmoles) into the cockroach haemocoel and monitoring levels of the derivative at various times following injection. If it is assumed that the haemolymph volume is 175 μl (24), the concentration of N-acetyl OA immediately following injection is 148.57 μM and, as indicated by the results in Table III, this value is reduced by 44% within 30 min. N-Acetyl OA is inactive against neuromuscular preparations from decapod crustaceans (25) and studies from this laboratory demonstrate that the derivative has no effect on adenylate cyclase activity in the insect nervous system (26). The relatively low rate of removal of N-acetyl OA compared with that of OA (see Table II) suppports the contention that the N-acetylation pathway produces a neurally non-active product.

Table III. Rate of Removal of N-Acetyl Octopamine
from Haemolymph of *Periplaneta americana*

Time After Injection (min)	Concentration of N-acetyl OA (μM)
10	130.0 ± 10.0
20	95.2 ± 4.5
30	85.6 ± 4.5

Amount of N-acetyl p-octopamine injected was 26 nmoles.
Values indicate mean ± SEM for 5 determinations.

Role of Malpighian tubules. The ability of locust Malpighian tubules to remove OA from medium *in vitro* has been demonstrated previously (20) although the fate of the OA was not determined. Studies from this laboratory have indicated that the Malpighian tubules of P. americana contribute substantially to the catabolism of haemolymph OA (23). This observation was confirmed by incubating freshly dissected Malpighian tubules in medium containing OA and monitoring the rate of OA removal. The results clearly indicate that Malpighian tubules have considerable capacity for removing OA from the incubation medium (Table IV). Analysis of the Malpighian tubule preparation for N-acetyl OA reveals that all of the OA that has been removed from the incubation medium can be accounted for by the newly formed N-acetyl OA (Table IV).

Under the experimental conditions, the capacity of the Malpighian tubules to remove OA may be calculated as 261 pmoles/min, which is compatible with a calculated rate of production of N-acetyl OA of 250 pmoles/min. The OA level of haemolymph following flight-induced excitation rises to 65 nM (17) and, again assuming a haemolymph volume of 175 μl this amounts to 11.38 pmoles/insect. These calculations indicate that the capacity of Malpighian tubules

to remove haemolymph OA greatly exceeds that required to effect
rapid removal of physiological levels of OA. Furthermore, the

Table IV. Rate of Removal of p-Octopamine and Production
of N-Acetyl p-Octopamine by Incubated Malpighian Tubules
of Periplaneta americana

	Amount of Amine	
Time (Minutes)	p-Octopamine (nmoles Removed)	N-acetyl p-Octopamine (nmoles Produced)
5	1.5 ± 0.4	1.4 ± 0.1
10	2.7 ± 0.3	3.0 ± 0.5
15	4.5 ± 0.7	3.8 ± 0.5
20	5.0 ± 0.7	5.0 ± 0.5
Rate	0.26 nmoles/min	0.25 nmoles/min.

Values indicate mean ± SEM for 5 determinations

calculations suggest that the absolute amount of OA that is
released into the haemolymph in response to excitation is
considerably greater than has previously been assumed; therefore,
perturbation of the OA removal mechanism may be expected to have
deleterious consequences. This suggestion is supported by the
report that a positive correlation exists between the extent of
toxication of Locusta migratoria and inhibition of N-acetylation of
tryptamine in the brain (27). The results of the present study are
consistent with previous reports of N-acetyl transferase activity
in the Malpighian tubules of Locusta migratoria (6) and Ostrinia
nubilalis (7) and serve to demonstrate that this enzyme represents
the major catabolic step for degradation of circulating levels of
OA.

Perturbation of OA removal by formamidines. The potential for
interfering with N-acetylation was tested by determining the effect
of formamidine insecticides on the ability of Malpighian tubules to
remove OA from incubation medium. The results of these experiments
are presented in Table V and demonstrate that DCDM, but not CDM,
reduces the amount of N-acetyl OA produced by the Malpighian tubule
preparation. Studies on N-acetylation of dopamine in cerebral
ganglia of Periplaneta americana in vivo failed to demonstrate any
formamidine-mediated inhibition of N-acetyl transferase activity
(28). The results of the present study clearly demonstrate that
DCDM inhibits the formation of N-acetyl OA from OA by Malpighian
tubules. Evans et al. (7) demonstrated that N-acetyl transferase
from larval carcass of the European Corn Borer has a greater
preference for β-hydroxylated amines than unsubstituted analogues
but this distinction was not observed with an enzyme preparation
derived from brain tissue. Thus the results of the present study
support the proposal that the enzyme associated with nervous
tissues may differ from that of peripheral tissues.
    The results do not indicate if DCDM is acting at the level of
the N-acetyl transferase or is interfering with an OA-uptake

mechanism similar to that which has been described in the central
nervous system of cockroaches (14). Irrespective of the site of
action of DCDM, the results demonstrate that, in addition to
demonstrated effects on OA-sensitive receptors (4) and dopamine-
and serotonin-sensitive receptors (29), some formamidines also
perturb the process by which OA is degraded by N-acetylation. The
contribution of this effect to the cumulative insecticidal activity
of formamidine insecticides awaits further elucidation.

Table V. Effect of Chlordimeform and N-Demethylchlordimeform
          on N-Acetylation of p-Octopamine by Incubated
          Malpighian Tubules of Periplaneta americana

Treatment	N-acetyl p-Octopamine Formed (nmoles/20 min)	% Inhibition
Control	4.9 ± 0.2 (7)	–
Chlordimeform	4.5 ± 0.1 (4)	n.s
N-Demethylchlordimeform	3.0 ± 0.5 (4)*	39.0

Values indicate mean ± SEM for number of determinations shown in
parentheses.
* $p < 0.05$ using model 1 ANOVA
n.s. not significant

Conclusions

The present study provides a convenient protocol for monitoring the
ability of Malpighian tubules and other tissues to remove OA from
incubation medium and convert it to N-acetyl OA. The results
obtained with DCDM also indicate the potential to interfere with
the process. Studies are currently in progress to determine the
capacity of other tissues to effect N-acetylation of OA and to
screen a spectrum of potential blockers of the effect.

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Chapter 16

Sodium Channels

Primary Targets for Insecticide Action?

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Three series of experiments have been performed on the cockroach, Periplaneta americana, to examine to what extent the modifications of the sodium channels of the nerve membrane induced by pyrethroids are sufficient to account for their insecticidal properties. Topical applications of lethal doses of deltamethrin modify nerve activity in a way which is compatible with the observed changes in behaviour of the dosed animal. These changes in activity are associated with a slow membrane depolarization preceded, for most molecules, by a marked increase in the depolarizing afterpotential. Both effects are linked with a time dependent modification of the properties of a small proportion of the sodium channels. Computer reconstruction of nerve membrane behaviour indicates that this modification is sufficient to account both qualitatively and quantitatively for the pyrethroid-induced changes in nerve activity.

Although it is generally accepted that most insecticides are nerve poisons, a direct correlation between their mode of action on the nervous system and their efficacy in the field is often lacking. Furthermore, the precise mechanism by which the insecticide (or its metabolites) modifies nerve activity is not fully understood. Thus, whereas the mode of action of pyrethroid insecticides have been studied in a variety of preparations (1–6) and consistently been found to be the induction of a voltage and time dependent increase in sodium conductance, the in vitro efficacy of some of these molecules was often found to be significantly different from their in vivo effect. This was particularly true in the case of deltamethrin. It has been suggested (7) that such a discrepancy could arise from a larger effect on the peripheral sensory receptors of from a direct effect of the molecule on the GABA receptor/chloride ion channel complex (8).
    The experiments summarized in the present paper have been

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designed in an attempt to correlate the insecticidal effects of pyrethroid insecticides with their effects on the sodium channels which are considered to be their main target site. Three levels have been considered, corresponding to increasing sophistication of the experimental set-up: (1) in vivo effect of topical application of known doses of insecticide. (2) in vitro microelectrode recording of the modifications of the resting and action potentials induced by bath application of known concentrations of insecticides. (3) Quantitative analysis on isolated axons of the modifications of the ionic conductances induced by various insecticide molecules. The results of this last set of experiments were then used to reconstruct nerve membrane behaviour according to a technique described earlier (9).

Effects of topical application of deltamethrin on cockroach behaviour

Experiments were performed at room temperature on adult male American cockroaches. Doses of formulated deltamethrin (RU-22974) ranging from 50 pg to 500 ng were applied to the first 2 abdominal tergites and the behaviour of the dosed insects followed during a 5 day period. The main findings are summarized in Table I where it

Table I.   Response of American cockroaches to 3 doses of deltamethrin

Time after dosing	Dose		
	5 ng	50ng	500 ng
1-15min.	Excitation	Excitation	Excitation
15-60 min.	Incoordination	Prostration	Paralysis
1-4 hours	Prostration	Partial Paralysis	Paralysis
4 hours-5 days	Death/Recovery	Death	Death

can be seen that, for all concentrations, the first effect of the insecticide is an excitation. This phase is followed by a phase of incoordination leading, for the larger doses, to prostration, then paralysis. Death occured when the applied dose was larger than 5ng, a dose corresponding to the LD50 for our batch of cockroaches.

Effects of topical application of deltamethrin on nerve activity

As expected from the data of Table I, 2 hours after topical application of 50 ng deltamethrin, nerve activity recorded in situ from the abdominal nerve cord was very much reduced (10). To determine the origin of this reduction, these experiments were repeated with a slightly modified technique enabling more consistent recordings of nerve activity for extended periods of time. The animals were treated as before but the nerve cord was

removed from the animal a given time after the application. The
recordings were made in vitro in a moist chamber which allowed
stable recordings of activity from the cercal nerves and the
abdominal nerve cord as well as electrical stimulation of the
cercal nerves, i.e. distal to the synapses between the cercal
nerves fibres and the giant axons of the abdominal nerve cord.

In the experiments illustrated in Fig.1, two insects were
treated with 500 ng formulated deltamethrin 1 hour (c and d) and 4
hours (e and f) prior to the dissection. Under these conditions,
the insects were completely paralyzed (see Table I). The
postsynaptic spike corresponding to suprathreshold stimulation of
the cercal nerve decreased progressively during intoxication and
disappeared after 8 hours (not shown). This decrease was associated
with a lengthening of the synaptic and conduction delay (the
interval between the sharp deflection in the recording and the
multiphasic postsynaptic response) and a reduction in the amplitude
of the presynaptic spike recorded directly on the cercal nerve (c
and e). This result shows unequivocally that, under our
experimental conditions, the primary target of the insectide
molecule (or its metabolites) is the conduction processes and not
the synapse.

The question as to whether this effect is due to a direct
action of the molecule on the nerve membrane or to an indirect
effect remained to be answered. The following experiments, in
which the nerve was perfused with solutions containing various
concentrations of the insecticide molecules, suggest that there is
a direct effect on the nerve membrane.

Direct effects of pyrethroid insecticides on giant axons in the
abdominal nerve cord (microelectrode recordings).

When applied directly onto the abdominal nerve cord of
Periplaneta, micromolar concentrations of pyrethroid molecules such
as allethrin (12-13), S-bioallethrin, biotetramethrin,
biopermethrin, bioresmethrin, cismethrin and 'kadethrin' (RU-15525)
(2) have been found to induce prolonged depolarizing after-
potentials. Under those conditions, high frequency stimulation of
the nerve cord resulted in a summation of these after-potentials as
illustrated in Fig. 2 for kadethrin. Larger concentrations of
several of these compounds were also found to depolarize the
resting membrane. This slow depolarization, which was also seen
for micromolar concentrations of deltamethrin (1), led to a block
of conduction, probably through an increased inactivation of the
sodium channels (see later). This is most probably due to an
increased resting sodium conductance since it disappeared following
removal of sodium ions from the external medium or addition of
tetrodotoxin (1μM), a well known inhibitor of the sodium
conductance in insect axons (14). The nature of the prolonged
increase in the depolarizing after-potential remained however to be
elucidated. This has been done for several pyrethroid molecules in
isolated giant axons under voltage-clamp conditions.

CERCAL NERVE   ABDOMINAL CONNECTIVES

A

untreated

B

C

1 h after
treatment

D

E

4 h after
treatment

F

2 mV

5 ms
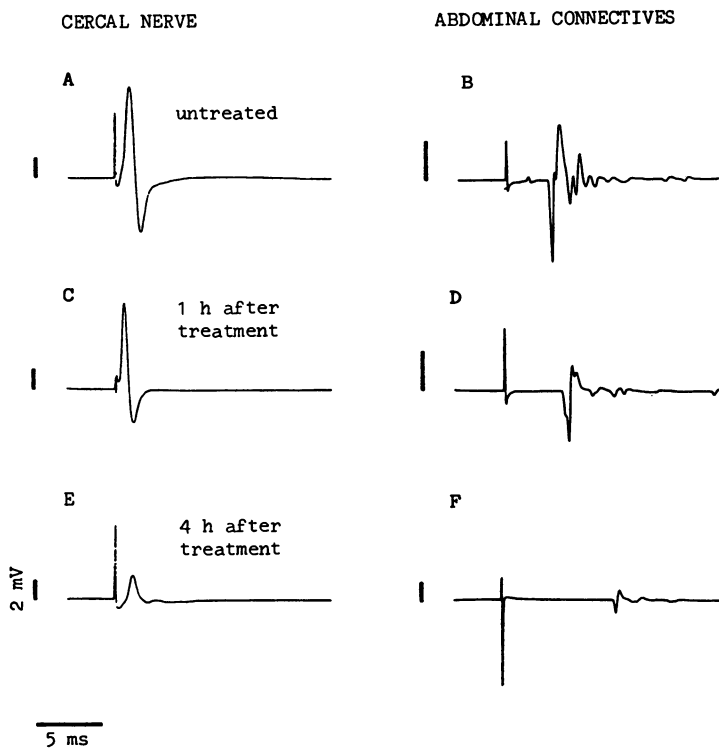
Fig.1: <u>In vitro</u> recordings of the effects of topical application
of 500 ng formulated deltamethrin on pre- and postsynaptic
activity in the abdominal nerve cord of the cockroach,
<u>Periplaneta americana</u>.

A

"Kadethrin" 0.14 uM

1 Hz

B

10 Hz

100 Hz

C

2 mV

0.5 s
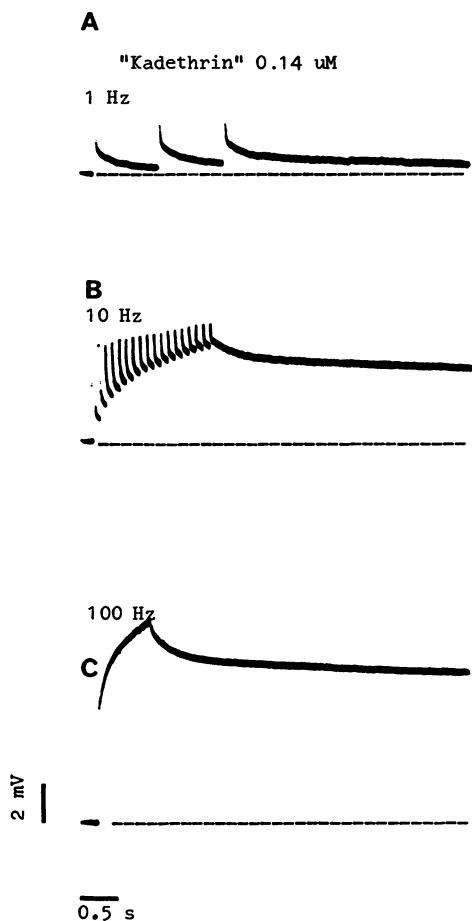
Fig.2: Effects of repetitive stimulation on membrane potential
in a cockroach giant axon treated with 0.14 μM kadethrin. The
horizontal broken lines designate the resting membrane
potential.

Effects of pyrethroid molecules on isolated axons (current-clamp experiments).

Giant axons were isolated from the abdominal nerve cord of Periplaneta and studied using external electrodes with the "oil-gap" technique (15-16). The response of these axons to external application of various pyrethroid molecules was found to be essentially similar to that observed with intracellular microelectrodes: development of prolonged depolarizing after-potentials and slow membrane depolarization (1-2).

The origin of these modifications has been studied using the voltage-clamp technique.

Effects of pyrethroid molecules on isolated axons (voltage-clamp experiments).

The method used to voltage-clamp the axons was similar to that described earlier (16,17) with the exception that most experiments were carried-out under computer control and the results digitized and stored on digital discs or cartridges for subsequent off-line analysis.

Pyrethroids were found to selectively increase the sodium conductance. The time- and voltage-dependency of this increase has been analysed quantitatively for two molecules: S-bioallethrin (9) and methanotetramethrin (3,4,5,6-tetrahydrophthalimidomethyl (1RS)-cis-3 [(RS)-2,2-dimethylcyclopropyl]-2,2 dimethylcyclopropane-carboxylate also referred to as Pyrethroid I). For small concentrations (up to 5 μM), the most obvious effect is the development of a low voltage-sensitive sodium conductance which turns off very slowly at the end of the depolarizing voltage pulses, producing large tails of inward current (Fig.3).

The time- and voltage-dependencies of this tail have been studied quantitatively during the development of the action of the insecticide. It was found that, during the first minutes of action, the voltage-sensitivity of the tail current followed quite closely that of the unmodified (fast) sodium conductance, although the activation curve was usually shifted to the right towards more positive potentials (11) (Fig.3C). Later, the voltage-sensitivity of the pyrethroid-induced current was found to decrease as indicated by the decrease in the slope of the activation curve. This decrease corresponded to the development of a small resting current (Fig. 3C, Fig. 4A). The difference between the normal sodium activation curve (dotted line) and the slow insecticide-induced sodium activation curve (broken line) is obvious 20 min. after application of 5 μm S-bioallethrin (Fig. 4A). This figure also illustrates the slow turning on of the insecticide-induced sodium conductance (Fig.4B) (from 9).

The extent to which these modifications can account for the observed changes in nerve activity has been evaluated through a computer reconstruction of nerve membrane behaviour.

A
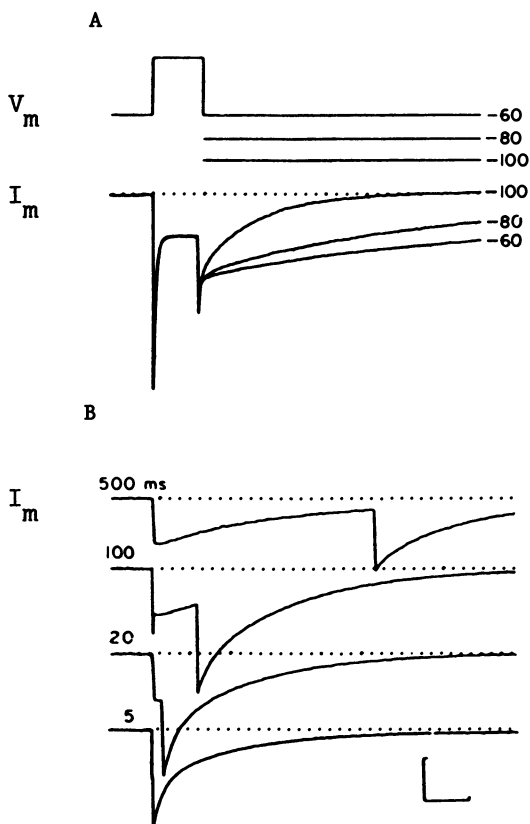
Fig.3: Effects of methanotetrametrin (pyrethroid I) on the
sodium current and conductance in isolated cockroach axons. The
potassium current was blocked using 0.2 mM 3,4-diaminopyridine
in the external solution.
A: Time course of the ionic currents for a square membrane de-
polarization to -10 mV followed by a repolarization to various
potential   levels   as   indicated.   Vertical   scale: 0.1 µA;
horizontal scale: 5 ms.
B: Time course of the ionic currents for square membrane de-
polarizations to -10 mV of varying duration as shown on each
tracing. Vertical scale: 0.1 µA; horizontal scale; 100 ms.
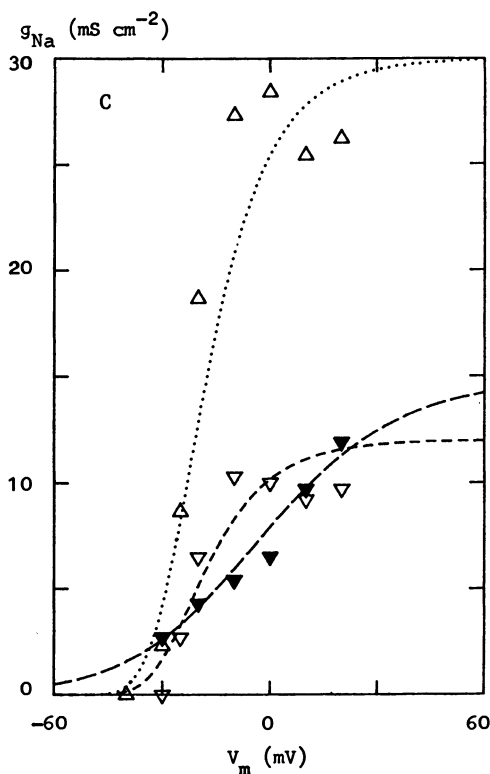
Fig.3: Continued.
C: Conductance-potential relationship for the peak (△) and
tail currents 10 min. (▽) and 22 min. (▼) after application of
the insecticide. The peak conductance was tentatively fitted
with a normal sodium activation curve shifted by 10 mV towards
more positive membrane potentials. After 10 min., the tail
current could still be fitted with a similar curve whereas,
after 22 min., the voltage-dependency was reduced by one half
and a resting sodium conductance appeared. Modified from (11).
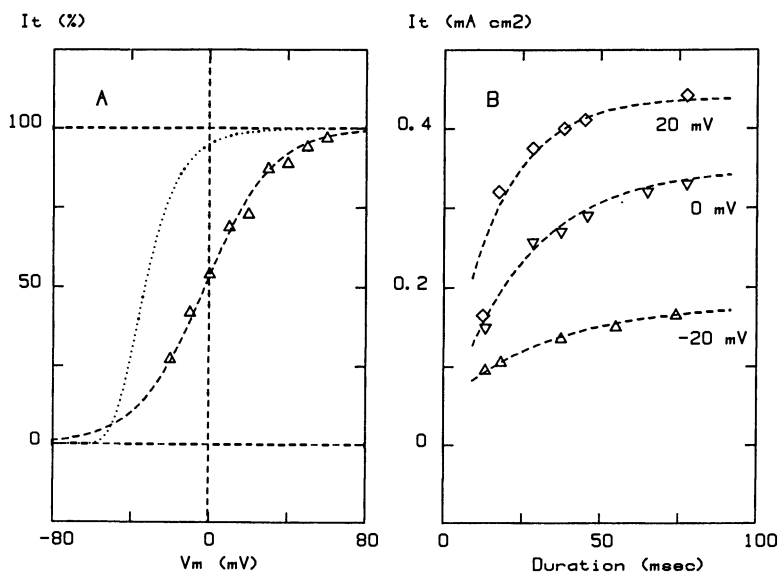
Fig.4: Voltage (A) and time (B) dependency of the tail current following a 20 min treatment of the axon with 5µM S-bioallethrin. Here again, the voltage sensitivity (A) of the tail conductance could no longer be fitted with the normal sodium activation curve (dotted line) but exhibited a reduced voltage sensitivity (interrupted line). In B, the experimental data could be fitted with a combination of a step increase and a slow exponential increase consistent with the model presented in the text. Modified from (10).

Computer reconstruction of nerve membrane behaviour in the presence of pyrethroids

The method used to reconstruct nerve behaviour derives directly from that originally proposed by Hodgkin and Huxley (18) for squid axons. The parameters used in these equations have been taken from earlier experiments on isolated cockroach axons (19-20). To reproduce the effects of the insecticide, a set of new equations, the "p" equations, have been derived from the above voltage-clamp experiments. The steady-state values of p as well as the values of the time constants used in the reconstructions are illustrated in Fig.5. It is assumed that activation of the slow channels is a first order process and that these channels do not inactivate.

The principle of the reconstruction is that, under space-clamp conditions, the observed changes in membrane potential represent charging and discharging of the membrane capacitance by the sum of all transmembrane currents according to the following equation :

$$C_m \frac{dV}{dt} = I_{Na} + I_K + I_I \qquad (1)$$

Typical applications of this equation are shown in Fig. 6. In A and B, the progressive poisoning of the nerve membrane was mimicked by assuming that, following the time of application of the insecticide (lowermost recording), the number of modified channels increased exponentially (A represents the proportion of modified channels, 1-A being the number of unmodified channels). Fig. 6A represents the early phase of poisoning and fig. 6B the late phase (characterized by a reduced voltage sensitivity of the insecticide modified sodium channels).

During the early phase of poisoning, the main effect of the insecticide is to induce a depolarizing after-potential of long duration, membrane depolarization (which was calculated for each concentration) being restricted to a few mV. As in the actual experiments, the size and duration of the afterpotential increased with insecticide concentration.

During the late phase of poisoning, the resting sodium conductance (predicted from the change in voltage sensitivity of the slow sodium channels, see Fig. 4C and 5A) is such that the membrane depolarizes significantly, resulting in a reduction in the spike and the depolarizing after-potential.

Fig. 6C shows that, when the sodium inactivation curve is shifted by 10 mV towards more positive potentials to account for the properties of some regions of the axons such as the site of initiation of the nerve impulse, the equations predict that the same small insecticide concentration that would normally only increase the afterpotential (lower trace) induces repetitive activity (upper trace).

Steady-state values

Time constant (ms)
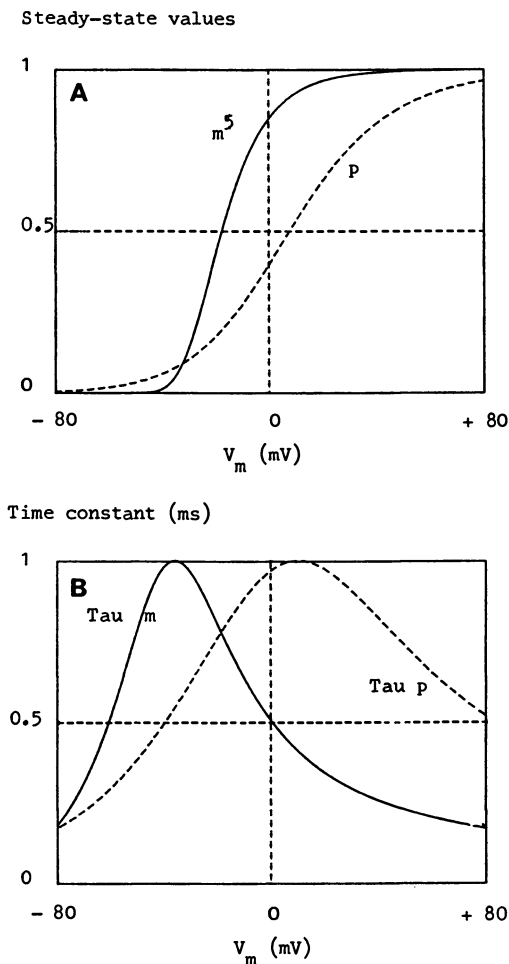
Fig.5: A: Voltage-sentitivity of m5 (the steady-state value of
the sodium activation parameter raised to the fifth power to
describe the sodium current in insect nerves , see 19 and 20),
and of the insecticide induced sodium conductance (p) . B:
voltage-sensitivity of the corresponding time constants of
activation (tau m and tau p). Tau p is larger that tau m by a
factor   of 10 to 1000, depending on the nature of the
insecticide, its concentration and time. The values used for
the computations illustrated in this paper were derived from
experiments on pyrethroid I.

V_m (mV)   Exponential increase in A to 0.048

+ 60
A
         Tau p x 200
    Normal voltage dependency

- 90
         40 ms

Exponential increase in A to 0.048

+ 60
B
         Tau p x 200
    Voltage dependency/2

- 90
         40 ms

A = 0.039 — Normal voltage dependency

+ 60
C
         Tau p x 200

- 62

- 90
         40 ms
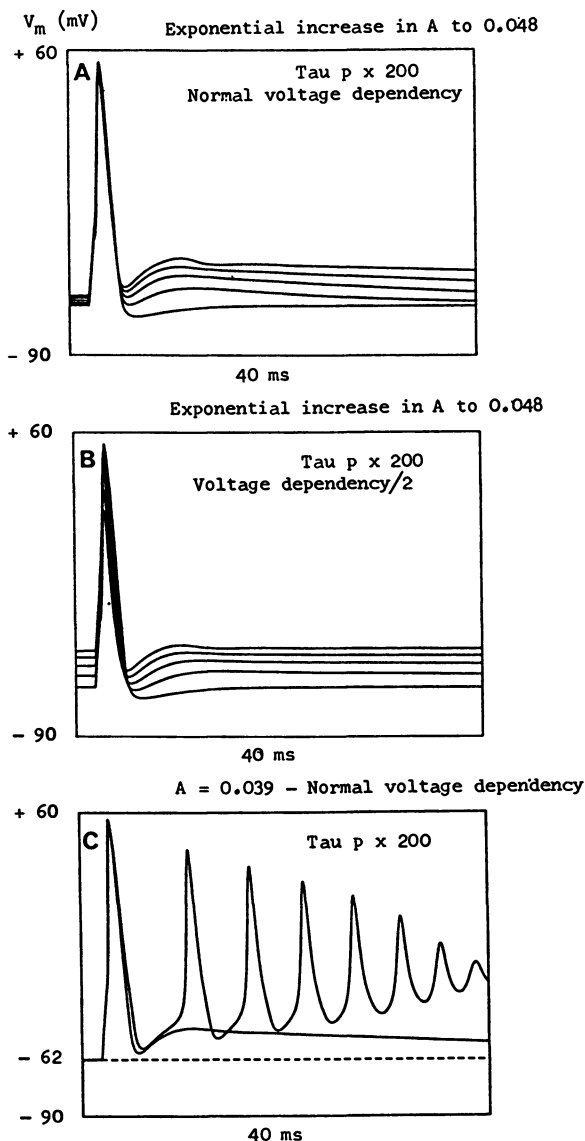
Fig.6: Computer reconstruction of nerve membrane activity based on the existence in the membrane of a small proportion (A) of modified sodium channels. In panels A and B, this proportion was varied from zero to 0.048 (i.e. 4.8 % of modified channels) to reproduce the membrane potential changes observed in strongly poisoned insects. In panel C, repetitive firing was obtained by shifting the sodium inactivation curve towards more positive potentials. Tau p x 200 indicates that, for these examples, the time constant of activation of the modified channels was 100 times slower than that of the normal channels.

Conclusion

A first conclusion of our work is that a simple modification of the time and voltage dependency of a small proportion (less than 5 per cent in the computed examples) of the sodium channels of the nerve membrane is sufficient to account for the insecticidal activity of pyrethroid insecticides. This modification leads to a lengthening of the spikes, repetitive firing and axonal depolarization.

These primary effects are likely to be amplified at different levels (sensory receptors, synapses) through various mechanisms such as potassium accumulation in the vicinity of the nerve membrane, sodium accumulation inside the axons and abnormal release of neurotransmitters, neuromodulators or neurohormones.

The experiments described in the first part of this paper indicate that, under our experimental conditions, the synapse is unlikely to be the primary target of deltamethrin or its metabolites. This does not mean that other mechanisms must be ignored since they may be used as a starting point in the search for new insecticides.

The extent to which our conclusions can be extended to other known families of insecticides such as DDT or lindane remains to be investigated.

As far as the design of new insecticides is concerned, it appears that sodium channels play such a key role in nerve activity that they are and will remain one of the best target sites. Sodium channels have recently been isolated and purified, their molecular sequence is known and their pharmacological properties are being studied with powerful biochemical and biophysical techniques. This increase in our knowledge of the functional properties of the channel is likely to result in the near future in the design of new, more powerful and more selective insecticides.

Acknowledgments

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Chapter 17

Neuronal Target Sites of Insecticides

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Pyrethroids have been shown to act on several target
sites in the nervous system including the sodium
channel, calcium channel and GABA receptor-channel
complex. This chapter gives highlights of our
recent studies of the mechanisms of action of
pyrethroids, DDT and lindane. The sodium channel is
the major, if not the sole, target site of type I
and type II pyrethroids and DDT, and changes in
nerve activity as manifested by repetitive
discharges, membrane depolarization, conduction
block and synaptic facilitation can be explained on
the basis of modification of the sodium channel
gating kinetics including prolonged openings. Some
effects of type I pyrethroids on the calcium channel
are noted, but the toxicological significance
remains to be seen. The GABA receptor-channel complex,
which was claimed by some investigators to be
suppressed by type II pyrethroids, has recently been
shown to be totally unaffected by deltamethrin while
the sodium channel undergoes drastic modification.
Lindane effectively blocks one of the components of
the GABA-activated chloride channel current, and
this action appears to be responsible for synaptic
hyperactivity.

Whereas the mechanism of action of insecticides has been
studied for many years since the development of synthetic
insecticides such as DDT, lindane and parathion during and after
World War II, it was not until around the mid-1960's that their
actions on the nervous system were understood at the cellular and
membrane level. Since these neuroactive insecticides are known
to alter membrane excitation which takes place with a time course
of milliseconds, the study of their mechanisms of action can best
be performed with the aid of advanced electrophysiological
techniques such as voltage clamp which allows us to measure the
ionic permeabilities of excitable membranes. Studies along this
line unveiled a variety of important features concerning the

interactions of insecticides with ion channels and receptors in
the nervous system. The present chapter gives a brief summary of
the historic development of this field and highlights of recent
discoveries made in our laboratory.

Pyrethroids

Certain pyrethroids such as pyrethrins, allethrin and
tetramethrin stimulate and then paralyze insects. Various nerves
were stimulated to produce repetitive discharges either
spontaneously or in response to a single stimulus (1-5). The
depolarizing after-potential was elevated by the pyrethroids and
reached the threshold for repetitive after-discharges (6,7). At
high concentrations of pyrethroids the membrane was gradually
depolarized and impulse conduction was eventually blocked (6,7).
Repetitive responses in the postsynaptic element in the
pyrethroid-poisoned preparations were induced at the presynaptic
nerve terminals (8-10). Thus the nerve membrane appears to be
the major target site of pyrethroids.
    Earlier voltage clamp experiments clearly demonstrated that
prolongation of sodium current and partial inhibition of
potassium current were responsible for the increase in
depolarizing after-potential caused by the pyrethroid allethrin
(11-13). The peak amplitude of sodium current was suppressed
resulting in conduction block. Despite these multiple actions,
the prolongation of sodium current was produced at low
concentrations of the pyrethroid, and was directly responsible
for the elevation of the depolarizing after-potential which
triggered repetitive discharges, the major cause of the symptoms
of poisoning in animals (14,15).
    Pyrethroids may conveniently be classified into two groups
based on the chemical structure and toxic action (16-18). Type I
pyrethroids do not possess an alpha-cyano group and include many
conventional ones such as allethrin, tetramethrin, phenothrin and
permethrin. Type II pyrethroids possess a cyano group at the α
position and include cyphenothrin, cypermethrin, deltamethrin and
fenvalerate.

Effects of Pyrethroids on Resting and Action Potentials

The effects of type I pyrethroids such as tetramethrin and
allethrin on the giant axons of the crayfish and squid were
characterized by an increase and prolongation of the depolarizing
after-potential which, at the threshold for action potential
generation, led to repetitive after-discharges. This occurred at
low concentrations in the nanomolar range, without much change in
the resting and action potential amplitude (14). Type II
pyrethroids such as fenvalerate and cyphenothrin caused a
membrane depolarization and a decrease in action potential
amplitude. No large change in the depolarizing after-potential
occurred, and no repetitive after-discharges were induced (17).

Effects of Type I Pyrethroids on Sodium Channels

In normal crayfish and squid giant axons perfused internally with

potassium-free cesium solutions, the membrane ionic current associated with a step depolarization (e.g. to -20 mV) from a holding potential (e.g. -100 mV) under voltage clamp conditions was composed of a peak transient inward current (I_p) which was followed by a small slow current (I_s). The tail current (I_{tail}) associated with a step repolarization decayed quickly. When perfused internally with (+)-trans tetramethrin (20 μM), the peak current was not changed but the slow current was greatly increased in amplitude. The tail current was also markedly increased in amplitude (Figure 1). Both the slow current and tail current decayed very slowly (14,15).

Kinetic analyses of the peak sodium current have revealed that the time constants of activation (τ_m), inactivation (τ_h), and the initial phase of the tail current (τ_{tail}) are not affected by tetramethrin (14,15). However, in the presence of tetramethrin the slow component of tail current and the slow steady-state inactivation curve were both shifted in the hyperpolarizing direction. The amplitude of the slow tail current was dose-dependent, whereas its time constant was not. These observations led to the suggestion that tetramethrin modifies a population of sodium channels to give rise to slow activation and inactivation kinetics. The normal peak transient current in tetramethrin simply represents the activity of the unmodified channels.

Calculations show that only a very small fraction of sodium channels, less than 1%, needs to be modified by tetramethrin in order to elevate the depolarizing after-potential to the level of threshold for repetitive after-discharges (19). This represents a unique amplification of the toxicological effect from channel modulation to the symptoms of poisoning in animals, and accounts in part for the high potency of the pyrethroids.

Development of the slow tail current during a depolarizing pulse was taken as a measure of the rate at which the sodium channels are modified. It had a fast and a slow phase, and the latter disappeared after removal of sodium channel inactivation with pronase. Based on these and other results, a kinetic scheme was developed (Figure 2). Tetramethrin modifies the sodium channel in both closed and open states, and the modified channel opens and is inactivated much more slowly than the normal channel (15). However, we have very recently shown that the apparent inactivation of the modified sodium channel is a result of depletion of sodium ions in the periaxonal space (20). Figure 2 incorporates the revised version of the kinetic scheme.

Effects of Type II Pyrethroids on Sodium Channels

Type II pyrethroids also modify the sodium channel kinetics (20-24). In a squid axon internally perfused with 10 μM deltamethrin a step depolarization from a holding membrane potential of -80 mV to -20 mV produced a peak transient sodium current which was followed by a slow current (Figure 3). With a prolonged, 510 msec depolarization the slow component of sodium current was hardly inactivated. The tail current associated with step repolarization of the membrane decayed very slowly with a dual time constant of 33 msec and 1074 msec. Like the peak
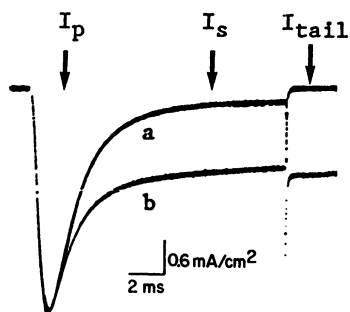
Figure 1. Membrane sodium currents in a squid giant axon before (a) and during (b) internal perfusion with 1 μM (+)-trans allethrin. Sodium current associated with a step depolarization from -100 mV to -20 mV was recorded after cesium and tetramethylammonium had been substituted for internal K^+ and external K^+, respectively, to eliminate the potassium current. In the control record (a), the peak sodium current (I_p) is followed by a small slow sodium current (I_s) during a depolarizing pulse, and the tail current (I_{tail}) associated with step repolarization decays quickly. In the presence of allethrin (b), I_p remains unchanged while I_s is greatly increased in amplitude. I_{tail} is also increased in amplitude and decays very slowly.
(Reproduced with permission from ref. 73. Copyright 1980 Society of Chemical Industry.)
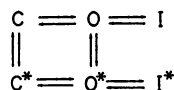
Figure 2. Kinetic model for the action of tetramethrin on the sodium channel. The normal closed channel (C) opens upon membrane depolarization to produce O, which is inactivated to become I during a prolonged depolarization. Tetramethrin binds to both closed and open channels yielding the modified closed (C^*) and open (O^*) channels, respectively. The modified open channel becomes inactivated very slowly during a prolonged depolarization to yield I^*.
(Reproduced with permission from ref. 74. Copyright 1982 ANKHO International.)
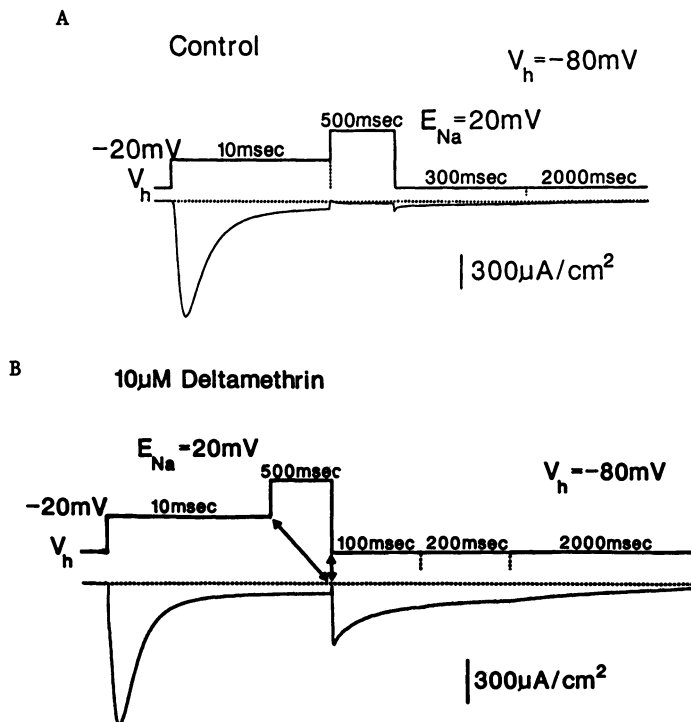
Figure 3. Sodium currents recorded from the squid giant axons
before (A) and after (B) internal application of 10 μM
deltamethrin.   External and internal sodium concentrations were
111 mM and 50 mM, respectively. A, a depolarizing pulse from
the holding potential (V_h) of -80 mV to -20 mV elicited the
normal transient inward sodium current which decayed within 10
msec. Depolarization to a second depolarizing pulse (500 msec)
to the sodium reversal potential (E_{Na} = +20 mV) yielded a
negligible current. Repolarization to the holding potential
(-80 mV) produced a very small inward sodium tail current. B,
the same pulse protocol as that for A but in the presence of
deltamethrin in another axon. Note a large and prolonged tail
current upon repolarization from +20 mV to -80 mV.
(Reproduced with permission from ref. 21. Copyright 1987
Academic Press.)

transient current, the slow and tail currents were both blocked by 1 μM tetrodotoxin (TTX) indicating that they flowed through the sodium channel. These and other observations are compatible with the kinetic model originally developed for type I pyrethroids (Figure 2). Fenvalerate, another type II pyrethroid prolongs the sodium current in a manner very similar to that of deltamethrin. In the presence of fenvalerate, the sodium channel opens normally and is modified by the pyrethroid. Fenvalerate also binds to the sodium channel at its closed state causing a modified closed channel. The latter opens slowly upon depolarization to generate a slow current. The voltage dependence of activation of closed modified channels was shifted 20-30 mV in the direction of hyperpolarization. This, together with the absence of inactivation, causes a depolarization. It appears that type I and type II pyrethroids act on the nerve membrane sodium channel in a qualitatively similar manner. However, the opening and closing kinetics of the modified channel are much slower for type II than for type I pyrethroids.

Effects of Pyrethroids on Gating Currents

Gating current, which is generated by channels as they change their conformation in response to changes in membrane potential, is an important tool for the study of sodium channels, because it can give more direct information about the conformational changes of channels than can ionic current. The prolonged sodium current in pyrethroid-poisoned axons shows that the conducting pore of the channel is held open, but it is not known whether the gating charges themselves are held in the open position. When sodium channels of crayfish axons were put into the modified open state by repetitive stimulation in the presence of fenvalerate, the intermediate component of the ON gating current ($\tau = 150$ μs at +20 mV), which corresponds to sodium channel activation, and the fast component of the OFF gating current ($\tau = 50$ μs at -160 mV), which corresponds to sodium channel deactivation, where inhibited in parallel with activation and deactivation of the sodium current. There was a slow component of ON gating current with the same time constant as sodium inactivation ($\tau = 600$ μs at +20 mV). This component and sodium inactivation were both abolished by fenvalerate, suggesting that this gating current component is associated with sodium inactivation. The fast component of the ON gating current ($\tau = 45$ μs at +20 mV) was not affected by fenvalerate, suggesting that it is not associated with sodium channel gating. It was concluded that when sodium channels are in the fenvalerate-modified open state, their activation and inactivation gating charges are immobilized. It appears that the activation gating charges are stuck in the open configuration while the inactivation gating charge is stuck in the resting or open configuration.

Effects of Pyrethroids on Single Sodium Channels

The voltage clamp experiments outlined above permit recording of opening and closing of many sodium channels contained in a large nerve membrane area. A question arises as to how individual

sodium channels behave in response to depolarizing stimulation
and how they are modified by pyrethroids. Neher and Sakmann (25)
developed a technique whereby the opening and closing of
individual ion channels could be recorded from a limited area of
the membrane by means of a glass capillary electrode. The
technique, called patch clamp, was later improved so that the
activity of individual channels could be distinguished more
clearly (26). This method, referred to as gigaohm seal patch
clamp, was combined with a technique by which a small patch was
pulled off the cell membrane and attached to the tip of the
recording capillary electrode (27).

We started working on patch clamp techniques as applied to
cultured neuroblastoma cells (N1E-115 line) in 1980, and have
since then studied the interaction of pyrethroids with single
sodium channels (28-32). Prior to application of tetramethrin,
individual sodium channels opened for a short period of time
during a depolarizing step as seen in the records as inward-going
current pulses (downward deflections) (Figure 4A). After
exposure to tetramethrin, individual sodium channels opened for a
much longer period of time (Figure 4B; note change in time scale
compared to 4A). The currents before and after application of
tetramethrin were both blocked by TTX, indicating that we were
observing the sodium channels. The amplitude histograms reveal
no change in the distribution of single channel currents
following application of tetramethrin (Figures 4C and 4D). The
open times (lifetimes) of single channels follow the Poisson
distribution with a time constant of 1.7 msec in the control
(Figure 4E). After exposure to tetramethrin, however, the open
time distribution is expressed by two exponential functions: One
has a time constant of 1.8 msec (inset) which is similar to that
of the control, and the other has a constant of 16.6 msec, much
longer than the control (Figure 4F). Thus the open time
distribution shows clearly that in the tetramethrin-poisoned
membrane there are two populations of sodium channels, one having
the normal characteristics and the other having modified
characteristics. The former represents the sodium channels not
bound by tetramethrin, and the latter those bound by
tetramethrin. This indicates that individual sodium channels are
modified by tetramethrin in an all-or-none manner.

The effects of fenvalerate and deltamethrin on single sodium
channels were also studied using the patch clamp method. The
fenvalerate-modified channel, once opened, remained open
throughout the entire depolarizing pulse (200 msec) and long
after repolarization (30). The mean open time was about 1 sec at
-80 mV and exhibited a voltage dependence similar to that of
macroscopic tail currents. This type of modified channel
activity, designated mode I, can account for the prolongation of
sodium current during and after a depolarizing pulse under
macroscopic voltage clamp conditions. Occasionally, the channel
exhibited spontaneous openings at the holding potential. These
openings had a mean open time of about 50 msec at -80 mV and
occurred in clusters lasting about 10 sec. This type of
activity, designated mode II, may explain the increase in holding
current seen in macroscopic studies. The two modes of activity
do not occur simultaneously. The opening and closing behavior of

the modified channel is compatible with the notion that
fenvalerate and deltamethrin stabilize a variety of channel
states by reducing the transition rates between them (29).
Similar conclusions were obtained by measurement of sodium
channel gating current as described above.

Sites of Action of Pyrethroids on Sodium Channels

Two questions were raised regarding the site of action of
pyrethroids on the sodium channels, i.e. 1) whether various
isomers of a pyrethroid act on the same site, and 2) whether
pyrethroids act on the same site as that of other chemical agents
known to interact with the sodium channel. The results to be
described below are compatible with the notion that the sites of
pyrethroid action are not located inside the sodium channel.
This led to the hypothesis that the properties of the open sodium
channels are not changed by pyrethroids.

Large differences in potency have been found among geometric
and optic isomers of pyrethroids. This provides us with an
excellent opportunity to study the stereospecificity of
pyrethroid binding sites (19). Four isomers of tetramethrin were
used and the amplitude of the tail current associated with step
repolarization was taken as a measure of activity. Whereas the
(+)-trans (1R-3-trans) and (+)-cis (1R-3-cis) forms of
tetramethrin were effective in increasing the tail current
amplitude at concentrations as low as 3×10^{-7}M and 5×10^{-6}M,
respectively, the (-)-trans (1S-3-trans) and (-)-cis (1S-3-cis)
forms were ineffective even at 3×10^{-4}M, the highest
concentration used. When pretreated with either (-)-trans or
(-)-cis tetramethrin, the effects of the subsequently applied
(+)-trans or (+)-cis tetramethrin were diminished greatly.
(-)-trans tetramethrin decreased the effect of (+)-trans
tetramethrin largely in a non-competitive manner. Similar
non-competitive antagonism was observed in the combinations of
(-)-trans and (+)-cis forms, and (-)-cis and (+)-trans forms.
However, (-)-cis tetramethrin antagonized the action of (+)-cis
tetramethrin in a competitive manner. Tetrodotoxin antagonized
(+)-trans tetramethrin in a non-competitive manner.

All of these results can be interpreted by a scheme
illustrated in Figure 5. There are a trans site and a cis site
in the sodium channel to which the (+)-trans form and (+)-cis
form bind respectively with a high affinity causing modification
of the sodium channel. The (-)-cis form binds to the cis site
with a high affinity thereby antagonizing the effect of (+)-cis
form in a competitive manner. However, the (-)-trans form binds
to the trans site only with a low affinity; its major binding
occurs at a negative allosteric site, modification of which
causes an inhibitory effect on the trans and cis sites. The
negative allosteric site is also bound by the (-)-cis compound
but only with a low affinity. Tetrodotoxin binds to another site
causing a non-competitive antagonism against the active (+)
forms.

Since certain sodium channel agents have been shown to bind
to a site inside the channel, they can be used as a useful probe
to determine the site of action of pyrethroids in the sodium
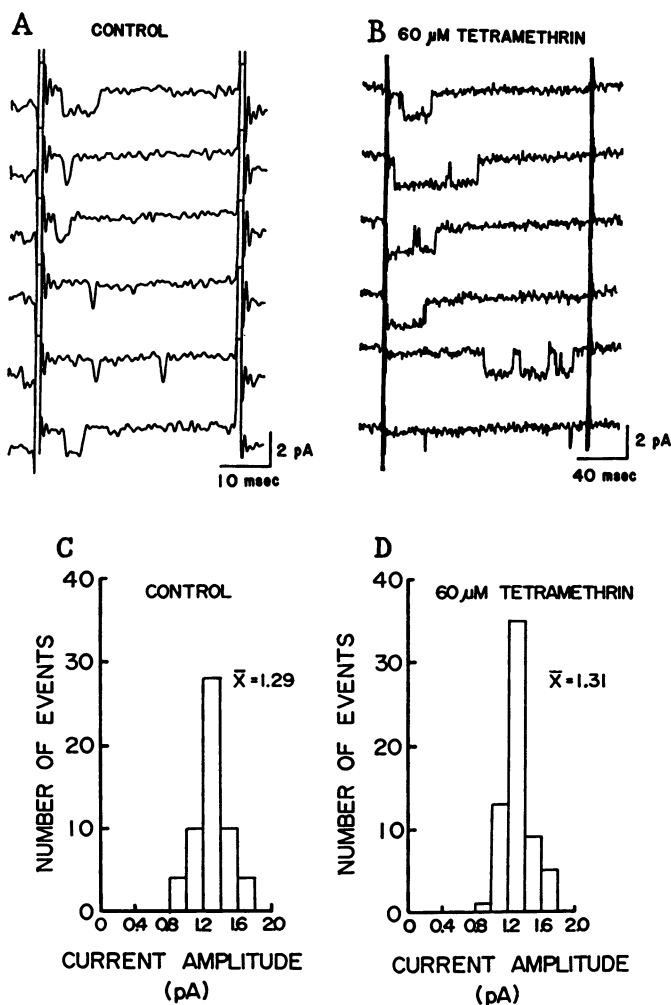
Figure 4. Effects of 60 μM (+)-trans tetramethrin on single
sodium channels in an inside-out membrane patch excised from a
neuroblastoma cell (N1E-115 line). A, sample records of sodium
channel currents (inward deflections) associated with step
depolarizations from -90 mV to -50 mV. B, as in A, but after
application of tetramethrin to the internal surface of the
membrane. C, current amplitude histogram in the control. D,
as in C, but after application of tetramethrin. (Reproduced
with permission from ref. 31. Copyright 1983 Elsevier.)

E

F
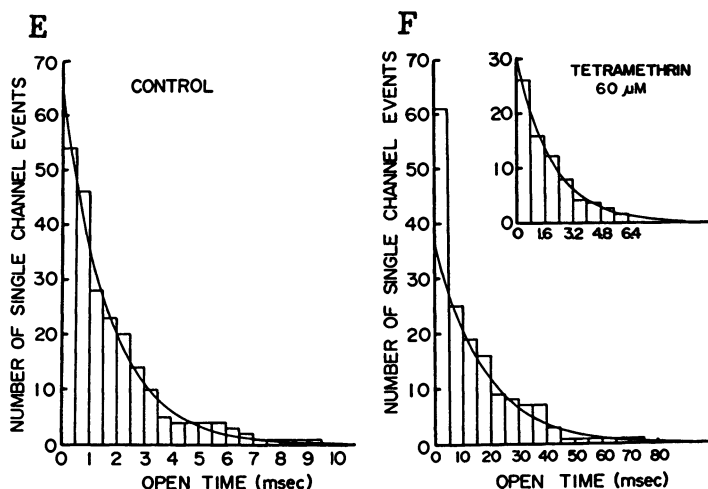
Figure 4.--Continued. E, channel open time distribution in the control. F, as in E, but after application of tetramethrin. Inset shows the distribution of short open times.
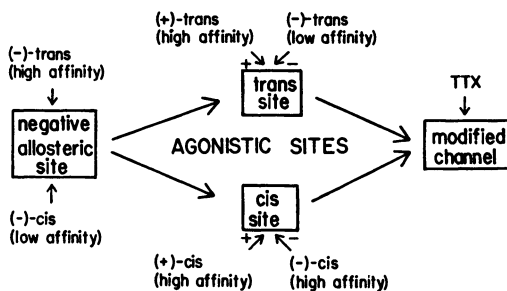
Figure 5. Hypothetical model for the interactions of tetramethrin with the sodium channel.
(Reproduced with permission from ref. 19. Copyright 1982 Intox Press.)

channel. Batrachotoxin (BTX), a sodium channel modulator, has been demonstrated to bind to the "inactivation receptor" site (33). BTX was first perfused internally through the squid giant axon, and repetitive stimulations were applied until the typical BTX modification of the sodium channel occurred. This was illustrated by the development of non-inactivating sodium currents during step depolarization and by the appearance of these modified currents at large negative membrane potentials. No drastic change occurred in tail sodium current. When tetramethrin was added to the BTX-treated axon, a large and prolonged tail current characteristic of the tetramethrin modified sodium channel developed. Thus tetramethrin binds to a site different from the binding site of BTX which is located inside of the channel. This result is compatible with the hypothesis that the pyrethroid molecules bind to the channel gating machinery via the membrane lipid phase thereby altering the kinetics of channel gating.

Whereas the pyrethroids modify the gating kinetics of sodium channels drastically, it remains to be seen whether they modify the properties of the sodium channel when it is in the open configuration. If the pyrethroid molecules bind to the membrane lipid phase in the vicinity of the channel thereby affecting the gating kinetics, the properties of the open channel may not be altered. Experiments were performed with the internally perfused, voltage clamped squid giant axon to examine the open sodium channel properties (34,35). The permeability ratio $P_{Na}:P_{Li}:P_{NH4}:P_{guanidine}:P_{formamidine}$ in the presence of 300 mM Na or test cations in the external solution was 1:1.13:0.27:0.34:0.23 in control and 1:0.93:0.29:0.21:0.21 in 50 μM tetramethrin. In the presence of 600 mM Na$^+$ or test cations, the ratio was 1:1.19:0.21:0.28:0.20 in control, and 1:1.18:0.29:0.29:0.25 in 50 μM tetramethrin. Thus there was no difference between normal and tetramethrin modified channels in their selective permeability to cations. The instantaneous current-voltage (I-V) curves for channels were also examined. In Na$^+$ solution, the instantaneous I-V curve showed a U-shape, reflecting the voltage-dependent block of sodium channels by external calcium ions at hyperpolarizing potentials. The instantaneous I-V curve for the tetramethrin-modified channels in various solutions was practically identical to those for normal channels (Figure 6).

Patch clamp single channel recording experiments with neuroblastoma (N1E-115) cells provided additional support to the above hypothesis (32). The single sodium channel conductance of neuroblastoma cells was reduced by increasing the external calcium concentration from 0.18 to 9.0 mM. The dissociation constant for calcium block was estimated to be 32.4 $\pm$ 1.05 mM. The block was intensified by hyperpolarization. The voltage dependence of block indicates that calcium ions bind to sodium channels at a site located 37 $\pm$ 2% of the electrical distance from the outside. Sodium ions also blocked sodium channels in a voltage-dependent manner, with dissociation constants of 185 and 204 mM at -50 and 0 mV, respectively. A model consisting of a one-ion pore with four barriers and three wells can account for the observations that deviate from the independence principle,
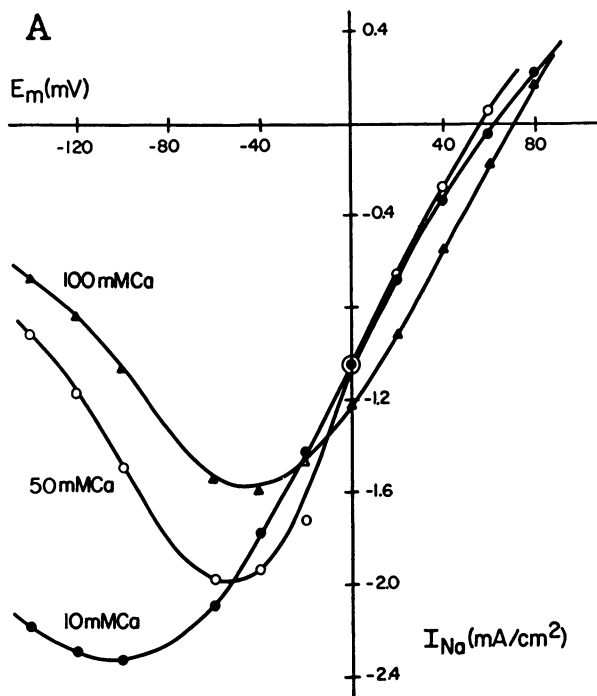
Figure 6. Instantaneous current–voltage relationships in 10, 50, and 100 mM Ca^{2+} solutions in a normal axon (A). The control and tetramethrin data were obtained from different axons. (Reproduced with permission from ref. 35. Copyright 1986 Elsevier.)
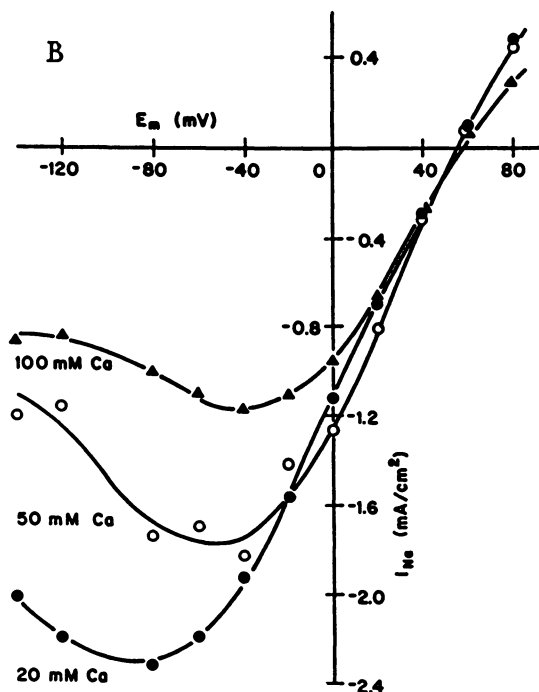
Figure 6.--Continued. Instantaneous current-voltage relationships in 10, 50, and 100 mM Ca^{2+} solutions in 20, 50, and 100 mM Ca^{2+} solutions in an axon internally perfused with 50 μM tetramethrin (B). The control and tetramethrin data were obtained from different axons. (Reproduced with permission from ref. 35. Copyright 1986 Elsevier.)

namely, the saturation of current, block by calcium ions, and
rectification in current-voltage relationship. Tetramethrin at a
concentration of 50 μM had no effect on any of these parameters
examined. Thus it can be concluded that despite the drastic
prolongation of open time no modification is brought about by
tetramethrin in the channel properties that control ion
permeation. These results are compatible with our hypothesis
that the pyrethroid molecules do not bind to the inside of the
sodium channel but bind to the membrane lipid phase thereby
modifying the gating kinetics.

Structure-Activity Relationship of Pyrethroids and DDT

Intoxication with DDT results in ataxia, loss of coordination,
convulsions, and hyperexcitation of insects and mammals. Various
regions of the nervous system were stimulated to discharge
repetitively either in response to a single stimulus or
spontaneously. These included sensory cells, synapses, and nerve
fibers (2,3,36,37). Repetitive after-discharges in nerve fibers
were due to an increase in the depolarizing (negative)
after-potential by DDT (38,39). Repetitive responses in
neuromuscular junctions have been shown to originate in
presynaptic nerve terminals (40). It appears that DDT and
pyrethroids exert similar effects on the nerve membrane sodium
channel (41). Detailed analyses as described below clearly show
that this is actually the case.

Comparison of Action of Pyrethroids and DDT at the Sodium Channel Level

Any structure-activity study must carefully define "activity" so
that only those pyrethroids with the same type of activity are
compared with respect to potency. The effects of a wide range of
pyrethroids and DDT analogs on the membrane potential and
membrane sodium currents were studied in crayfish, lobster and
squid giant axons (17,42,43). DDT, plifenate (2, 2,
2-trichloro-1-(3, 4-dichlorophenyl)-ethanol acetate), and EDO
(2,2-bis(p-ethoxyphenyl)-3, 3-dimethyloxetane) produced
depolarizing after-potentials which developed quickly and decayed
quickly, and had little effect on the resting potential.
Tetramethrin, allethrin, resmethrin and proparthrin produced
depolarizing after-potentials which were more clearly detectable
during multiple after-potentials which developed more slowly but
lasted longer. The phenoxybenzyl compounds NRDC 157, permethrin
and phenothrin produced very persistent depolarizing
after-potentials and partially depolarized the resting membrane.
Finally, the α-substituted compounds cyphenothrin, deltamethrin
and fenvalerate produced very small depolarizing after-potentials
which were more clearly detectable during multiple stimulation.
The resulting depolarization was so persistent that it caused a
change in the resting potential. Eventually the depolarization
became so large that further conduction was blocked. No
repetitive firing was observed with these compounds in giant
axons.
     The depolarizing after-potentials, the use-dependent

depolarization and the membrane depolarization caused by various
pyrethroids and DDT analogs could all be explained by a prolonged
increase in sodium conductance. The α-cyano pyrethroids
fenvalerate, cyphenothrin and deltamethrin were slow to modify
channels (type II effect). A relatively small fraction of the
sodium channels was retained in the modified open state with each
depolarization, but the lifetime of the channels in the modified
open state was very long. Thus, axons poisoned with these
compounds were depolarized until the action potential was blocked.
In the course of slow depolarization, some neurons fire
repetitively, especially those in the sensory nervous system
(44,45). Repetitive discharges from sensory neurons appear to be
the cause of tingling sensation of the skin when exposed to type
II pyrethroids. Membrane depolarization at nerve terminals caused
by type II pyrethroids would cause an increase in transmitter
release which in turn disturbs synaptic transmission (46,47).
     By comparison, DDT and plifenate modified sodium channels
rapidly but the lifetime of the channels in the modified open
state was much shorter (10-20 msec) (type I effect). Thus, axons
poisoned with these compounds showed long trains of potentials
with very little effect on the resting potential at low
concentrations. These two groups of compounds apparently
represent two extreme cases of a continuous variation in the
rates at which sodium channels reach the modified open state and
return to the resting state. Most of the other compounds tested
were intermediate and showed repetitive activity as well as some
decrease in the resting potential (Table I). Since the kinetics

Table I. Time Constants (msec) of Tail Currents Associated with
          Step Repolarizations of the Membrane to the Levels
          Indicated in Crayfish Giant Axons Treated with Various
          Compounds

Compound	Conc. (M)	Tail Current Time Constant (msec) at		
		-160 mV	-120 mV	-100 mV
DDT	1×10^{-4}	3.0	6.1	9.5
Plifenate	3×10^{-6}	9.4	14.8	17.0
EDO	1×10^{-4}	16	44	86
Tetramethrin	2×10^{-5}	30	225	620
Phenothrin	3×10^{-5}	200	750	1340
GH401	1×10^{-4}	700	1450	2220
Cyphenothrin	1×10^{-6}	(min)	(min)	(∞)
Fenvalerate	1×10^{-6}	(min)	(min)	(∞)
Deltamethrin	1×10^{-6}	(min)	(min)	(∞)

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1983 Academic Press.

of sodium channel modification vary continuously with the type of insecticide, structure-activity studies must attempt to measure the interactions between the insecticides and sodium channels. The most precise measure of this interaction requires the voltage clamp technique, but a qualitative indication can be obtained using conventional intracellular potential recording.

Effects of Pyrethroids on Calcium Channels

In view of the very critical role calcium channels play in a variety of nerve and muscle function, possible effects of various insecticides on calcium channels are too important to be overlooked. It was indeed shown that neurosecretory cells of the stick insect generate action potentials by inward calcium currents (48), and that permethrin increases the electrical activity of these cells at a concentration as low as 5 x 10^{-11}M (49). Although direct demonstration still remains to be seen, it is possible that pyrethroids act on calcium channels to exert their toxic effects (48,50).

We have launched an extensive study of calcium channels in connection with insecticidal action. First, we had to characterize the normal physiological properties of calcium channels in neuroblastoma cells as they were largely unknown. Whole cell patch clamp technique as combined with internal perfusion of neuroblastoma cells (N1E-115) (51) has proved highly successful as described below.

Patch clamp techniques as applied to the whole cell calcium channels led to the discovery of two types of calcium channels in neuroblastoma cells (52-56). These two types exhibited different physiological and pharmacological properties. Barium (50 mM) was used in the external solution as the carrier of current through calcium channels. Step depolarizations from a holding potential of -80 mV to potentials more positive than -50 mV evoked transient inward Ba^{2+} currents which reached a maximum amplitude at -20 mV (type I calcium channel). A second component of the inward current appeared around -20 mV and reached its maximum at 0 to +10 mV. This component was not inactivated during prolonged depolarizing steps lasting more than 200 msec (type II calcium channel). When the holding potential was changed to -50 mV, step depolarizations failed to evoke the fast, transient component due to inactivation. However, they induced the slow, non-inactivating component in the isolated form. Both components of the inward current was abolished by 1 mM La^{3+}, indicating that the non-inactivating component of the current also flowed through calcium channels. Dibutyryl cyclic AMP (1 mM) caused an increase in the amplitude of the non-inactivating component by 30-50%, but failed to alter the transient component significantly. The two types of channels also differed in their ionic selectivity as estimated from the peak current amplitude; $Ba^{2+}:Sr^{2+}:Ca^{2+}$ = 1.0:1.0:0.6 for type I channel, and $Ba^{2+}:Sr^{2+}:Ca^{2+}$ = 1.0:0.7:0.3 for type II channel. Replacement of Ba^{2+} with Ca^{2+} caused a positive voltage shift in the I-V relationship for type II, but not for type I channels. La^{3+}, Cd^{2+}, Ni^{2+} and Co^{2+} blocked both types of calcium channels in a dose-dependent manner with one-to-one stoichiometry. For the type I channel, the sequence

of blocking potency as estimated from the apparent K_d (μM) was
La^{3+} (1.5) >> Ni^{2+} (47) > Cd^{2+} (160) = Co^{2+} (160). For the type
II channel, the sequence was La^{3+} (0.9) > Cd^{2+} (7.0) >> Ni^{2+}
(280) > Co^{2+} (560). These results strongly suggest that the two
types of calcium channels found in neuroblastoma cells represent
different entities.

Tetramethrin (50 μM) caused a progressive block of type I and
type II calcium channel currents over a 10-15 min period (56).
When a steady state was achieved, type I channel current was
blocked by 75%, while type II current was blocked only by 30%.
The tetramethrin block of both channel types was time-dependent,
being enhanced during a 400 msec depolarizing pulse. The
time-dependent component of block was easily reversible after
washing with drug-free solution, while the time-independent
component or resting block persisted for at least 40 min after
washing. Deltamethrin and fenvalerate (10 μM) had no effect on
either type of calcium channel currents during 30 min of
exposure. The results indicate that type I pyrethroids are
calcium channel blockers as well as sodium channel modulators.
The two components of calcium channel block, a time-dependent
reversible block and a time-independent irreversible block,
suggest two separate sites of action of tetramethrin in calcium
channels. However, type II pyrethroids modify sodium channels
only. This difference in action between type I pyrethroids and
α-cyano type II pyrethroids may be partially responsible for the
different symptoms of poisoning in animals.

Neuroreceptors as Target Sites of Pyrethroid and DDT-like Insecticides

It has recently been shown that the active isomers of type II
pyrethroids bind to a site closely associated with the GABA
receptor-ionophore complex (57). Binding of sulfur-35-labeled
t-butylbicyclophosphorothionate (TBPS), a ligand for the
picrotoxinin binding site, was inhibited by type II pyrethroids,
but not by type I pyrethroids. Measurements of input resistance
of crayfish muscle fibers also supported the antagonism between
type II pyrethroids and GABA receptor-ionophore complex (58).
Further support to the hypothesis was provided by the observation
that diazepam protected cockroaches against the action of type II
pyrethroids (59). However, the potency of deltamethrin, a type II
pyrethroid, was 100 times lower on GABA receptors than on sodium
channels (60). It has also been suggested that type II
pyrethroids may interact with the brain binding sites for
dihydropicrotoxinin (61) or kainic acid (62).

Our recent study clearly excludes the possibility that the
GABA receptor-channel complex is a major target site of
deltamethrin, a type II pyrethroid (63). Patch clamp experiments
were performed with the primary cultured neurons isolated from
the rat dorsal root ganglion. These neurons are endowed with
GABA receptor-channels. Both GABA-induced inward chloride current
and voltage-activated sodium channel current were recorded from
the same cell. The GABA-induced chloride current was unaffected
by application of 10 μM deltamethrin, while the sodium current
was greatly prolonged indicating drastic modification of the

channel gating kinetics. Thus, deltamethrin does not affect the mammalian GABA receptor-channel complex. It remains to be seen whether the same conclusion is applied to the insect GABA receptor-channel complex.

Nicotinic acetylcholine (ACh) receptors from Torpedo electric organ were also affected by pyrethroids (64-66). The binding of ^{3}H-perhydrohistrionicotoxin, but not of ^{3}H-ACh, was inhibited by type I pyrethroids. Type II pyrethroids were less potent. The inhibitory effect had a negative temperature coefficient. The receptor-regulated calcium flux was also inhibited. Thus the channels associated with nicotinic ACh receptors may be another site of action of pyrethroids. However, electrophysiological experiments did not support this idea. The amplitude of the end-plate potentials in frog skeletal muscles was unaffected by type I pyrethroids (8,10). This controversy remains to be solved.

EDO and Glutamate Receptors

The effects of EDO, a biodegradable DDT analog, on glutamate receptor-channels were examined using crayfish neuromuscular junctions as a model (40). EDO at a concentration of 40 nM greatly augmented the excitatory junctional potentials (EJPs). Repetitive EJPs were evoked by a single nerve stimulus. However, focal recording by means of an extracellular microelectrode placed in the immediate vicinity of the junction revealed that each excitatory junctional current (EJC) was preceded by a nerve terminal action current (Figure 7). Thus, in the presence of EDO, the nerve generated repetitive discharges in response to a single stimulus, thereby augmenting the EJPs. Junctional depolarization evoked by direct iontophoretic application of L-glutamate was not affected by EDO at all (Figure 8). Thus it was concluded that EDO had no effect on the glutamate receptor-channel complex.

Pyrethroids and Glutamate Receptors

The effects of type I and type II pyrethroids on the evoked potential were studied in vitro using guinea pig olfactory cortex slices (67). A pair of stimuli, 50 msec apart, was applied at a frequency of 0.4 Hz, and the field potentials were recorded by means of a glass capillary microelectrode filled with 0.9% NaCl. In normal preparations, the amplitude of the second evoked potential was slightly larger than that of the first by approximately 15%. After application of 10 μM deltamethrin (R,S-isomer), fenvalerate (S,S-isomer), or tetramethrin ((+)-cis-isomer), the second evoked potential became smaller than the first by 10-25%. The first response was not markedly changed in amplitude, but was slightly prolonged in duration. The change in the second response was abolished by a decrease in calcium concentration from 2 to 1 mM. These results are compatible with the notion that the decrease in the second response by pyrethroids is due to suppression of part of the mechanisms responsible for transmitter release. The glutamate receptor-channel complex in this preparation is not affected by pyrethroids.

2×10^{-6}M EDO

SA

0.2 mV

20 msec
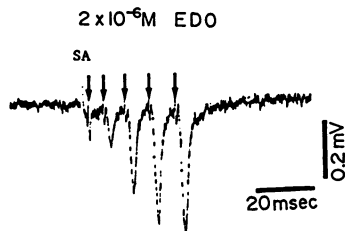
Figure 7. A focal recording of multiple EPPs induced by a single stimulus of the excitatory nerve in the presence of 2 x 10^{-6}M EDO. The initial upward deflection is the stimulus artifact (SA). The small deflections indicated by the arrows prior to each EPP are nerve terminal potentials. Note the failure of the first nerve terminal potential to produce an EPP.
(Reproduced with permission from ref. 40. Copyright 1979 Intox Press.)

A. CONTROL

B. 2×10^{-6}M EDO
30 min

5 mV

200 msec
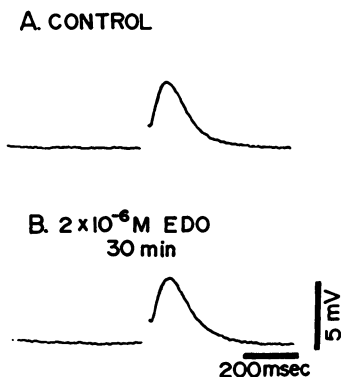
Figure 8. Effect of 2 x 10^{-6}M EDO on the iontophoretically induced glutamate potential. A, glutamate potential in van Harreveld's saline. B, glutamate potential after 30 min of exposure to EDO. The potential in B was obtained at a time when spontaneous nerve activity had occurred in the nerve. Both records are from the same neuromuscular junction.
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Lindane on Acetylcholine and GABA Receptors

It has been well established that synapses are the major site of action of lindane. Synaptic transmission is greatly facilitated by the action of lindane in both insects and vertebrates (68-71). However, little is known about the mechanism whereby synaptic transmission is facilitated by lindane.

An example of the lindane-induced synaptic facilitation is illustrated in Figure 9 (71). Synaptic transmission across the sixth (last) abdominal ganglion of the cockroach was studied using extracellular electrodes. The cercal nerve was stimulated and the postsynaptic response was recorded from the abdominal nerve cord. Prior to lindane application, a single presynaptic stimulus induced a postsynaptic response which was composed of the initial large spikes followed by after-discharges of small amplitudes (Figure 9A). After application of 10 μM lindane, the postsynaptic after-discharges were greatly augmented and prolonged (Figures 9B and 9C) and eventually a single presynaptic stimulus evoked bursts of postsynaptic responses (Figure 9D).

More detailed analyses of the effects of lindane on synaptic transmission were made using the frog neuromuscular junction (72). Lindane (100 μM) greatly increased the frequency of spontaneous miniature end-plate potentials (MEPPs) while decreasing their amplitudes. End-plate depolarization evoked by iontophoretic application of ACh was also decreased by lindane. The quantal content of end-plate potentials was increased to 180% of control by lindane. When this factor was taken into consideration, the degree of end-plate block by lindane was estimated to be 30%. No detectable effect of lindane on transmitter release could be shown in normal Ringer's solution. However, when the mean quantal content was small (e.g. in low Ca^{2+}-high Mg^{2+}), transmitter release was greatly enhanced. Thus lindane has two effects; one is to suppress the end-plate response to ACh, and the other is an effect consistent with a small increase in intracellular free Ca^{2+}. The calcium channels of neuroblastoma cells, both inactivating (type I) and non-inactivating (type II), were not affected by lindane (72).

Our recent study using the primary cultured neurons isolated from the rat dorsal root ganglion has thrown light on the mechanism of action of lindane (63). Bath application of GABA induced two components of inward chloride current. One was a transient component which was desensitized with time, and the other was a non-desensitizing component. The desensitizing component of chloride current was completely blocked by 10 μM lindane, while the non-desensitizing component remained unaffected. Thus lindane blocks one of the GABA-activated receptor-channel complexes leading to hyperexcitation. This accounts for synaptic facilitation observed earlier with the lindane-treated cockroach ganglion and vertebrate preparations.

Conclusion

The results of experiments so far obtained in our laboratory and
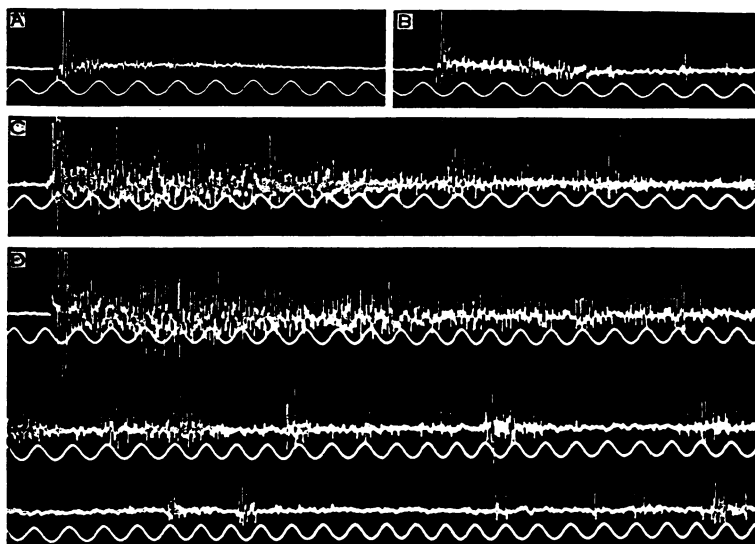
Figure 9. After-discharges of the giant fibers induced by a
single supramaximal stimulus to the cercal (presynaptic) nerve
of the American cockroach before and after treatment with 1 x
10^{-5}M lindane. 17°C, time marker 50 c.p.s. A, before
treatment; very short after-discharge (100 msec). B, 1 hour
and 25 minutes after treatment; slight after-discharge (380
msec). C, 4 hours and 10 minutes after treatment; prolonged
after-discharge (940 msec). D, 6 hours and 10 minutes after
treatment. The three lines are continuous. Very prolonged
after-discharge (2380 msec).
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elsewhere clearly indicate that the nerve membrane sodium channel
is the major target site of type I and type II pyrethroids and
DDT. The gating kinetics of single sodium channels are modified
by pyrethroids and DDT resulting in marked prolongation of
channel open time. This change causes a prolonged sodium current
to flow across the nerve membrane which in turn increases and
prolongs the depolarizing after-potential in the presence of type
I pyrethroids and DDT, or depolarizes the membrane in the
presence of type II pyrethroids. Thus repetitive discharges are
evoked in nerve fibers (type I pyrethroids and DDT) or in sensory
neurons (type II pyrethroids and DDT). In either case, the end
product is represented by synaptic disturbances of various types
which explain the symptoms of poisoning in animals. In certain
neurons, pyrethroids have been shown to affect calcium channels,
but the exact role of the channel in poisoning remains to be
seen. Our recent study has shown that the GABA receptor-channel
complex, which was claimed by some investigators to be a target
of type II pyrethroids, is not affected at all by deltamethrin
while the sodium current is greatly prolonged. Thus the GABA
receptor-channel complex is unlikely to be the major target site
of type II pyrethroids. Lindane has recently been shown to
effectively block one of the components of the GABA-induced
chloride channel current, and this action appears to be
responsible for synaptic hyperexcitation.

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Chapter 18

Enhancement of Veratridine-Dependent Sodium Channel Activation by Pyrethroids and DDT Analogs

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Pyrethroid insecticides (deltamethrin, NRDC 157, cismethrin), DDT analogs (p,p´-DDT, o,p´-DDT, methoxychlor, EDO), and a DDT-pyrethroid hybrid compound (GH401) enhanced veratridine-dependent sodium uptake by mouse brain synaptosomes. The effectiveness of these compounds in the sodium uptake assay was in good agreement with their acute mammalian toxicities. p,p´-DDT also enhanced veratridine-dependent sodium uptake by fish brain synaptosomes. These findings demonstrate the utility of ion flux assays to study interactions of insecticides with sodium channels in the central nervous system and to explore species differences in insecticide target site sensitivity.

Abundant neurophysiological evidence implicates the voltage-dependent sodium channel as an important target site for DDT and pyrethroids. These compounds cause repetitive firing and depolarization in insect motor nerve terminals (1,2), vertebrate sensory neurons (3), and arthropod giant axons (4). Voltage clamp studies have shown that all these effects of pyrethroids and DDT analogs can be ascribed to a prolongation of sodium channel currents (4).

   Electrophysiological studies are well suited for measuring the effects of pyrethroids on sodium channel kinetics, but they are less useful for studying the pharmacology of pyrethroid-receptor interactions. Moreover, these techniques are difficult to employ in studies of insecticide effects on neurons in the central nervous systems of mammals and insects. Radiotracer ion flux measurements have emerged as a useful alternative method for studying the action of pyrethroids on the sodium channel. Investigations using neuroblastoma cells (5) and mouse brain synaptosomes (6) showed that pyrethroids had no significant effect on $^{22}Na^{+}$ uptake when applied alone but enhanced the specific stimulation of sodium uptake caused by established sodium channel activators such as veratridine.

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In this paper, we report that DDT and its analogs, as well as pyrethroids, enhance veratridine-dependent sodium uptake in mouse brain synaptosomes. We also demonstrate the extension of these methods to study insecticide effects on sodium channel function in rainbow trout.

Methods

Chemicals. p,p´-DDT was purchased from Chem Service, West Chester, PA, and methoxychlor was purchased from Aldrich Chemical Co., Milwaukee, WI. DDE and o,p´-DDT were obtained from Ciba-Geigy Corp., Greensboro, NC. Deltamethrin was a gift from J. Martel, Roussel-Uclaf, Romainville, France, and cismethrin was a gift from M. Elliott, Rothamsted Experimental Station, Harpenden, England. GH401 (3-phenoxybenzyl [S]-2,2-dichloro-1-(4-ethoxyphenyl)-cyclopropanecarboxylate) and EDO (2,2-bis-(4-ethoxyphenyl)-3,3-dimethyloxetane; GH149) were gifts from G. Holan, CSIRO, Melbourne, Australia. The sources of other chemicals used in the sodium uptake assay are published elsewhere (7).

Preparation of synaptosomes. Synaptosomes were prepared from the brains of male mice (20-30 g; Blue Spruce Farms, Altamont, NY) either by a modification of the method of Hajos (8) or by the method of Dodd et al. (9). Both preparations gave qualitatively similar results, but the magnitude of all sodium fluxes per mg of synaptosomal protein was much greater with the latter preparation. A preparation enriched in synaptosomes was prepared from the brains of juvenile rainbow trout (Salmo gairdneri; obtained from the New York State Fish Hatchery, Bath, NY) by homogenization in 14 volumes of 0.7 M sucrose, and centrifugation first at 2000 g for 10 min and then at 31000 g for 30 min. The pellet from the second centrifugation was resuspended in sodium-free buffer identical to that used in previous studies (7) except that it also contained 370 mM sucrose.

Sodium uptake assay. Assays using mouse brain synaptosomes were performed as described previously (6,7), except that insecticides were introduced to resuspended synaptosomes in 0.2-0.4 µl of ethanol rather than as a residue in the incubation tube. This amount of ethanol improved the delivery of insecticides, thereby increasing the reproducibility of the assay, and had no measurable effect on veratridine-dependent sodium channel activation. These methods were also used for assays with fish brain membranes, except that all buffers were augmented with sucrose to give osmolarities equivalent to the 0.7 M sucrose used for membrane isolation.

Intracerebral toxicity determinations. EDO and GH401 were administered to mice by intracerebral injection in 3 µl of triethylene glycol dimethyl ether under light ether anaesthesia as described previously (6). Control animals received vehicle only and were apparently normal following recovery from anaesthesia.

Effects of Pyrethroids on Mouse Brain Sodium Channels

Our initial studies in this system employed deltamethrin at a high concentration (10 µM) to characterize the effects of pyrethroids on

synaptosomal sodium uptake. Deltamethrin alone had no effect on
basal levels of synaptosomal sodium uptake, but it markedly enhanced
the stimulation of sodium uptake caused by the alkaloid veratridine,
a sodium channel activator (6; Table I). Both the veratridine
stimulation and the enhancement of this effect by deltamethrin were
completely blocked by tetrodotoxin (Table I), thus confirming that
the observed sodium fluxes reflect a specific effect on the voltage-
dependent sodium channel. The effect of deltamethrin on the potency
curve for veratridine-dependent sodium channel activation is shown in
Figure 1. Deltamethrin increased the amount of uptake observed at
any given veratridine concentration, but it had no significant effect
on the potency of veratridine. This finding is consistent with the
effects of deltamethrin on veratridine-dependent sodium channel
activation in cultured neuroblastoma cells (5). Maximum enhancement
of sodium channel activation was observed at 50 µM veratridine
(Figure 1); this concentration was used as the standard activator
concentration in all subsequent experiments.

Table I. Effect of Deltamethrin on Basal and Veratridine-Stimulated
          Sodium Uptake by Mouse Brain Synaptosomes[a]

Preincubation conditions	Uptake, nmol/mg protein[b]
Control	1.40±0.18
Deltamethrin (10 µM)	1.35±0.12
Veratridine (50 µM)	1.90±0.17[c]
Deltamethrin + veratridine	2.36±0.15[d]
Deltamethrin + veratridine + tetrodotoxin (5 µM)	1.26±0.09

[a]Data from Ref. 6.

[b]Means ± standard errors of 4-7 determinations; 15 sec incubations.

[c]Differs significantly from control uptake (p<0.025; unpaired t-test,
8 d.f.).

[d]Differs significantly from veratridine-stimulated uptake (p<0.025;
unpaired t-test, 6 d.f.).

     We explored the potency of pyrethroid action in this system
using deltamethrin, NRDC 157 (3-phenoxybenzyl [1R,cis]-3-(2,2-
dibromovinyl)-2,2-dimethylcyclopropanecarboxylate; the non-cyano
analog of deltamethrin), and their nontoxic enantiomers as test
compounds (6; Table II). Deltamethrin produced half-maximal enhance-
ment of veratridine-dependent activation at 25 nM, whereas NRDC 157
was approximately tenfold less potent and the nontoxic enantiomers
were inactive. These findings demonstrate that the effect of
pyrethroids on mouse brain sodium channels is both potent and
stereospecific for toxic isomers. The relative potencies of del-
tamethrin and NRDC 157 in this assay also agree well with their
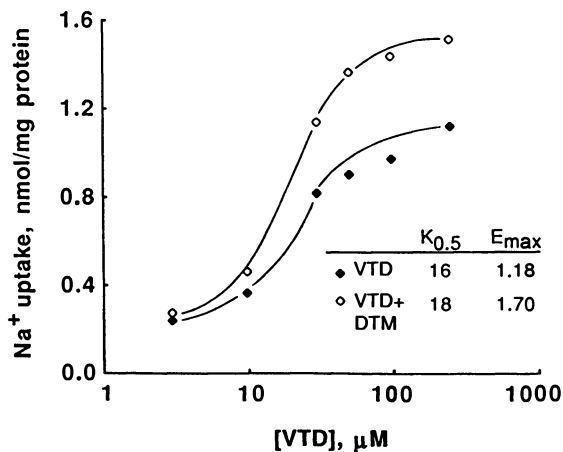
Figure 1. Effects of veratridine (VTD) concentration on veratridine-stimulated sodium uptake by mouse brain synaptosomes in the presence and absence of deltamethrin (DTM; 10 μM). Data points are means of two replicate experiments using different membrane preparations corrected for veratridine-independent sodium uptake. Tabulated values are: $K_{0.5}$, concentration giving half-maximal uptake in μM; E_{max}, maximum uptake at saturating concentrations in nmol/mg protein. Data are from ref. 10.

relative acute toxicities measured by intracerebral injection (Table II).

Table II. Effects on Sodium Channel Activation and Acute Intracerebral Toxicity of Deltamethrin, NRDC 157, and Their Enantiomers in Mice[a]

Compound	$K_{0.5}$, nM[b]	LD_{50}, µg/kg
NRDC 157	220	190
Enantiomer of NRDC 157	>10,000	>10,500
Deltamethrin	25	19
Enantiomer of deltamethrin	>10,000	>10,500

[a]Data from Ref. 6.

[b]Concentration giving half-maximal enhancement of veratridine-stimulated sodium uptake.

Current efforts in these studies involve a detailed characterization of pyrethroid-activator interactions and the examination of a broader range of pyrethroids and other compounds as enhancers of activation. Recently, we have found that cismethrin is one of the most effective pyrethroids in this system, producing enhancement at high concentrations equal to or greater than that observed with deltamethrin (Figure 2). The action of cismethrin in the mouse brain system is quite different from its action on neuroblastoma cell sodium channels, where it has no effect on veratridine-dependent activation but is able to antagonize the enhancement produced by other pyrethroids (5).

Effects of DDT Analogs on Mouse Brain Sodium Channels

The similar actions of pyrethroids and DDT analogs on sodium channels in electrophysiological preparations (4) prompted us to evaluate these compounds in the synaptosomal sodium flux assay. Initial experiments using p,p′-DDT at a high concentration (100 µM) showed an enhancement of veratridine-dependent sodium uptake that was similar to that observed with high concentrations of pyrethroids (Figure 2). Enhancement of sodium uptake by p,p′-DDT was concentration-dependent (Figure 3), but the low solubility of p,p′-DDT prevented completion of the curve using concentrations higher that 100 µM. Nevertheless, the available data suggest that the $K_{0.5}$ for this effect is approximately 30 µM. From these data, it is clear that p,p′-DDT is much less potent in this system than deltamethrin and NRDC 157 (Table II). However, p,p′-DDT produces low but significant levels of enhanced activation at concentrations as low as 0.1 µM. Since altered nerve function can result from modification of only a small percentage of total sodium channels (11), p,p′-DDT may be more potent in intact nerves than its $K_{0.5}$ value would indicate.
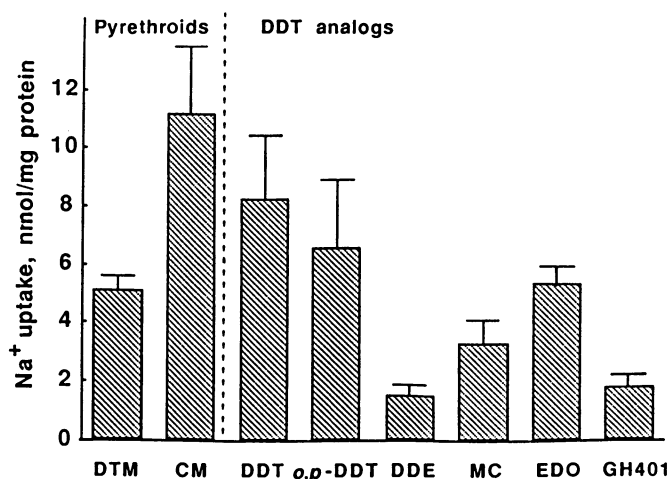
Figure 2. Enhancement of veratridine-stimulated sodium uptake in mouse brain synaptosomes by pyrethroids and DDT analogs. Each value is the mean of three determinations using different membrane preparations corrected for veratridine-stimulated uptake in the absence of insecticide; bars show standard errors. Compound abbreviations: DTM (deltamethrin); CM (cismethrin); MC (methoxychlor). Concentrations were 100 μM for all compounds except DTM (10 μM).
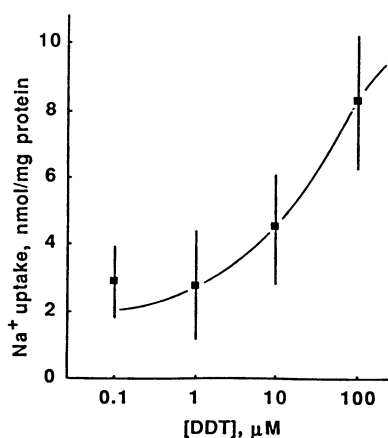
Figure 3. Effect of DDT concentration on the enhancement of veratridine-stimulated sodium uptake by mouse brain synaptosomes. Data points are means of three determinations using different membrane preparations corrected for veratridine-stimulated uptake in the absence of DDT; bars show standard errors.

We have also surveyed the effects of a limited group of DDT analogs on veratridine-dependent sodium uptake (Figure 2). The o,p'isomer of DDT was almost as active as p,p'-DDT in this assay, but the dehydrochlorinated metabolite DDE was much less effective. The relative effectiveness of p,p'-DDT, o,p'-DDT, and DDE as enhancers of veratridine-dependent sodium uptake is generally consistent with their acute oral toxicities to mammals (12). Methoxychlor, a DDT analog with much lower mammalian toxicity (12), also enhanced veratridine-dependent sodium uptake but was somewhat less effective than DDT. This finding provides further evidence that the low mammalian toxicity of methoxychlor results from its rapid biodegradation rather than from its lack of intrinsic neurotoxicity. EDO, an oxetane analog of DDT with very low mammalian toxicity (13), approached the activity of p,p'-DDT in this assay (Figure 2). GH401, which was designed as a hybrid molecule incorporating structural elements of both DDT and pyrethroids (14) and which is relatively nontoxic to mammals (13,14), was weakly active in this system. A recent study showed that EDO and GH401, unlike pyrethroids, were unable to enhance sea anemone toxin-stimulated sodium uptake in cultured neuroblastoma cells (13). These authors proposed that the mammalian (neuroblastoma) sodium channel was intrinsically insensitive to EDO and GH401, thus explaining their low acute toxicities to mammals.

Since our sodium uptake experiments suggested that EDO might exhibit DDT-like effects in vivo, we undertook a preliminary survey to estimate the intrinsic neurotoxicity of EDO and GH401 following intracerebral administration to mice. Of the four animals treated with EDO at 1 mg/kg, two exhibited a rapid onset of whole body tremor, followed by convulsions and death. The remaining two animals exhibited less severe tremor for approximately 30 min. after treatment and then recovered. These preliminary findings suggest that EDO produces the T or Type I syndrome of intoxication in mammals with a potency somewhat lower than neurotoxic pyrethroids that produce the T syndrome (15). In contrast, mice treated with GH401 at 3 mg/kg (n=4) were indistinguishable from vehicle-treated controls, suggesting that this compound has little or no neurotoxic activity in vivo. These findings, though preliminary in nature, indicate a good correlation for EDO and GH401 between activity in sodium uptake assays and acute neurotoxicity. These results suggest that the ineffectiveness of EDO on sodium channels in neuroblastoma cells (13) reflects the unique and atypical selectivity of neuroblastoma cell sodium channels. The low mammalian toxicity observed for EDO when administered by other routes probably reflects the rapid metabolic detoxication of this compound. In contrast, the low toxicity of GH401 appears to involve reduced sensitivity of the target site in mammals, a phenomenon that has been noted previously for several insecticidal pyrethroids (15).

Effects of DDT on Fish Brain Sodium Channels

The high acute toxicity of DDT analogs and most pyrethroids to fish is one of the most significant hazards associated with the widespread use of these compounds. The high toxicity of pyrethroids to fish appears to result in part from high intrinsic sensitivity of the CNS to these compounds (16,17). Fish are also highly sensitive to some

insecticidal pyrethroids, such as trans-permethrin, that are com-
pletely nontoxic to mammals (16). These data suggest that
significant species differences exist between sodium channels from
fish and mammals. To explore these differences, we have adapted the
methods for studying synaptosomal sodium uptake to examine the ef-
fects of insecticides on sodium channels in fish brain synaptosomes.

Our initial studies used veratridine and p,p´-DDT to explore
sodium channel pharmacology in the fish brain system. Veratridine
(50 µM) produced a small but reproducible and statistically sig-
nificant ($p < 0.005$; paired t-test, 3 d.f.) stimulation of sodium
uptake, and this effect was completely blocked by 5 µM tetrodotoxin
(Figure 4). Inclusion of p,p´-DDT (100 µM) produced a statistically
significant ($p < 0.01$; paired t-test, 3 d.f.) enhancement of
veratridine-dependent sodium uptake in a manner identical to that
previously observed in mouse brain preparations (Figure 4). Both
veratridine-dependent stimulation and the enhancing effect of p,p´-
DDT were completely inhibited by coincubation with tetrodotoxin, a
highly specific blocker of the sodium channel (Figure 4). These
results demonstrate a sodium channel-specific component of sodium
uptake by trout brain vesicles and also show that the radiosodium
uptake assay can be used to explore the interactions between insec-
ticides and fish CNS sodium channels.

We have also defined both the effect of veratridine concentra-
tion on sodium channel activation in this system and the impact of
p,p´-DDT on the concentration-effect curve for veratridine-dependent
activation (Figure 5). Half-maximal activation by veratridine oc-
cured at approximately 50 µM, a value very close to that found for
the action of this compound in mouse brain synaptosomes (7). p,p´-
DDT significantly enhanced veratridine-dependent uptake at all
veratridine concentrations above 10 µM, and maximum sodium uptake in
the presence of both compounds was achieved at 50 µM, which is the
approximate $K_{0.5}$ for veratridine alone. These findings suggest that
p,p´-DDT not only enhances veratridine-dependent activation but also
increases the potency of veratridine. This result contrasts with the
interaction of deltamethrin and veratridine in mouse brain synap-
tosomes (Figure 1) where the insecticide enhanced activation but did
not affect the potency of the activator. The only previous report of
insecticide-dependent potency shifts in sodium channel activation is
in neuroblastoma cells, where pyrethroids increase the potency of
batrachotoxin and dihydrograyanotoxin II as sodium channel ac-
tivators, but do not alter the potency of veratridine (5). These
experiments provide the basis for further studies to compare directly
the selectivity and sensitivity of sodium channels from mammalian and
fish brain in their interactions with insecticides.

Conclusions

Radiosodium uptake studies offer a new technical approach to the
description of the actions of insecticides with sodium channels.
This method is particularly well suited to the study of CNS sodium
channels, which pose serious technical difficulties for the applica-
tion of intracellular recording and the current-voltage manipulations
required to study normal and modified channel kinetics. Our studies
to date have described an enhancement of sodium channel activation in
this system by DDT analogs and pyrethroids that is consistent with
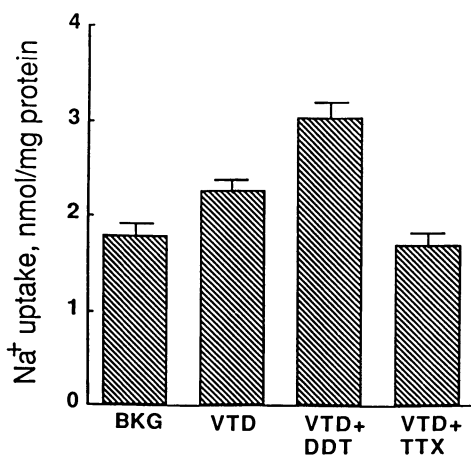
Figure 4. Sodium uptake by fish brain synaptosomes: BKG, background uptake; VTD, uptake stimulated by 50 µM veratridine; VTD+DDT, enhancement of veratridine-stimulated uptake by 100 µM DDT; VTD+TTX, inhibition of veratridine-stimulated uptake by 5 µM tetrodotoxin. Values are means of three determinations using different membrane preparations; bars show standard errors.
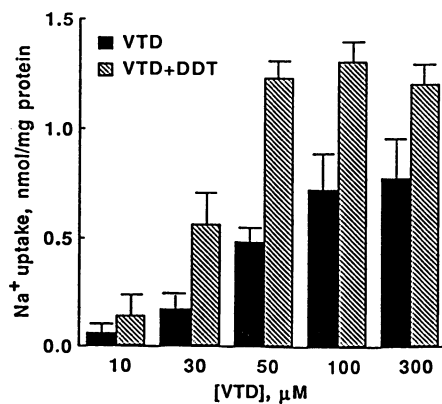
Figure 5. Effect of veratridine (VTD) concentration on veratridine-stimulated sodium uptake in fish brain synaptosomes in the presence and absence of DDT (100 µM). Values are means of three determinations using different membrane preparations; bars show standard errors.

the known actions of these compounds on axonal sodium channels.
Moreover, the results obtained to date are in good agreement with the
acute neurotoxicity in mammals of the compounds we have examined.

The use of cloned mouse neuroblastoma cells as a model system
for in vitro studies of sodium channel function has become increas-
ingly popular over the past decade. Comparison of published
information on toxin and insecticide interactions with neuroblastoma
cell sodium channels (5,13) with our data (6,7) shows large qualita-
tive differences between native CNS channels and those in cultured
cells. We previously documented the quantitative differences between
brain and neuroblastoma channels in the relative efficacy of alkaloid
sodium channel activators (7). A further difference is seen in the
effect of polypeptide neurotoxins, which do not activate rat brain
synaptosomal sodium channels (18) but produce marked activation in
neuroblastoma cells (5). There are also important differences be-
tween these two systems in the action of insecticides. Non-cyano
pyrethroids such as NRDC 157 and cismethrin are effective enhancers
of veratridine-dependent activation of brain sodium channels, but
cismethrin and other neurotoxic non-cyano pyrethroids were ineffec-
tive in combination with veratridine in neuroblastoma cells (5).
Similarly, EDO enhanced veratridine-dependent activation in our
studies, but this compound in neuroblastoma cells was ineffective in
combination with a polypeptide activator (13), a method that is
effective with a broader range of pyrethroids than the insecticide-
veratridine combination in that system (5). We conclude that sodium
channels in neuroblastoma differ significantly in their properties
from mammalian CNS sodium channels. Thus, data from insecticide
effects on neuroblastoma cells are of limited value in defining or
predicting toxicologically relevant target site interactions.

Our preliminary results with fish brain preparations suggest
that ion flux techniques may be valuable in studies of target site
differences between species. We have demonstrated veratridine-
stimulated, tetrodotoxin-sensitive sodium uptake in a vesicular
preparation from fish brain, thus confirming the presence of func-
tional sodium channels in this preparation. Our results with p,p'-
DDT in this system also agree well with the action of DDT analogs and
pyrethroids in mouse brain assays. Further studies wih both prepara-
tions should allow the exploration of target site differences between
mammals and fish that have been inferred from whole animal toxicity
studies.

Finally, it is possible that these techniques can be extended
successfully to insect CNS preparations. Methods now exist to
prepare functional insect synaptosomes from insect ganglia (19), and
these preparations have been used to demonstrate veratridine-
dependent neurotransmitter release and enhancement of this release by
deltamethrin in a superfusion assay (20). Further refinement of
these methods should allow direct measurement of sodium channel-
mediated sodium fluxes in insect CNS preparations, thus allowing the
investigation of target site differences not only between mammals and
insects but also between susceptible and resistant insect strains.

Acknowledgments

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Chapter 19

Insect Synaptosomes in Superfusion

A Technique To Investigate the Actions of Ion Channel Directed Neurotoxicants by Monitoring Their Effects on Transmitter Release

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A study of the interaction of selected neurotoxicants with ion channels in the synaptosomal membrane as measured by a superfusion technique is described. The alkaloid neurotoxin veratridine (10^{-4}M) stimulated release of radiolabel from [^{3}H]-choline loaded synaptosomes in superfusion. Hemicholinium-3 (10^{-4}M) was inactive. Veratridine-induced release, although unaffected by picrotoxinin (10^{-4}M) was strongly inhibited by tetrodotoxin (IC_{50}:5×10^{-10}M). Deltamethrin produced a potentiation of veratridine-stimulated transmitter release, this effect being more pronounced upon preincubation with the pyrethroid. Ivermectin and certain milbemycins induce release of label from insect synaptosomes. Picrotoxinin, dieldrin and γ-hexachloro-cyclohexane are potent inhibitors of ivermectin-dependent release whereas t-butylbicyclophosphorothionate (10^{-6}M) and tetrodotoxin (10^{-6}M) have no antagonist activity. These observations support the concept that insect central nerve terminals contain functional sodium and chloride channels.

Certain neurotoxicants act by directly influencing the electrical activity of the insect nerve membrane through a specific action at ion-selective channels. These phenomena can be studied using many of the neurobiochemical techniques that have successfully been applied to vertebrate nervous systems. In this study we use the technique of insect synaptosomes in superfusion to examine the interaction of a range of insecticidal agents with ion channels in the nerve terminal by monitoring effects on transmitter release.

Methods

Head and thoracic ganglia from eighty cockroaches (P. americana) were homogenized in ice-cold 0.25M sucrose (buffered to pH 7.2 with Tris.HCl) and fractionated as far as the crude synaptosomal

(P_2) stage according to procedures published for locust nervous tissue ([1]). In a previous structural analysis of cockroach P_2 fraction we had confirmed the presence of considerable numbers of intact synaptosomal profiles which retained the characteristic morphology of terminals within the intact cockroach nervous system ([2]). Contaminating fragments (mitochondria, large empty vesicles, elongated membranous profiles and other cellular debris) are also present. The P_2 fraction was gently resuspended in buffered sucrose (0.2ml) and then slowly introduced into oxygenated insect saline (1.8ml) of the following composition: NaCl 12.5g/l; KCl 0.23g/l; $CaCl_2 . 6H_2O$ 0.45g/l; $Na_2HPO_4 . 12H_2O$ 0.85g/l; $NaH_2PO_4 . 2H_2O$ 0.03g/l; $MgCl_2 . 6H_2O$ 1.3g/l; glucose 3g/l. Synaptosomes suspended in saline were incubated with methyl[^{3}H]-choline for 15 minutes at 30°C and distributed between twenty superfusion units containing Whatman GF/B filters. [^{3}H]-Choline loaded synaptosomes were super-fused with oxygenated saline to wash out most of the extrasynapto-somal radioactivity. Saline (20ml) was then added which contained the neurotoxicant(s). Superfusion rates were maintained at 0.88ml/minute using a peristaltic pump. Depending on the experiment, 13 - 20 fractions were collected. When the effect of pre-incubation with deltamethrin was examined, synaptosomes were exposed to the pyrethroid in saline 34 minutes prior to a challenge with veratridine plus pyrethroid. Lipophilic neurotoxicants were added to the superfusion saline dissolved in acetone (final carrier concentration 0.1%). Radioactivity in superfusate samples was determined using liquid scintillation counting.

Synaptosomes isolated from the central nervous system of the cockroach and maintained on filter platforms, as outlined, show no deterioration in response to the neurotoxicants examined for at least one hour after the start of superfusion. Variability within an experiment was less than 8%, and there was good agreement between experiments carried out on different days.

Results and Discussion

Isolated nerve endings (synaptosomes) in superfusion represent an ideal preparation for the study of neurotoxicant action. Indeed the use of such an approach to examine the interaction of pharmaco-logical agents with ion channels in vertebrate synaptosomal preparations is well established ([3]), ([4]). It is only relatively recently that fractionation procedures for invertebrate synaptosomes have been optimized and superfusion techniques applied to the study of drug and toxin action in insects ([1]), ([5]). In the present communication we have attempted to clarify the mode of action of certain insecticidal neurotoxicants by determining their effects on transmitter release in the presence and absence of channel specific modulators.

The effects of veratridine on superfused synaptosomes preloaded with[^{3}H]-choline are summarized in Figures 1 and 2. Upon exposure of the synaptosomal bed to the alkaloid there follows a rapid increase in the rate of release of label which peaks after ten minutes then declines rapidly. Veratridine is of moderate potency, producing threshold effects at 10^{-6}M and a maximal response at 2.5×10^{-4}M, the latter concentration approaching the
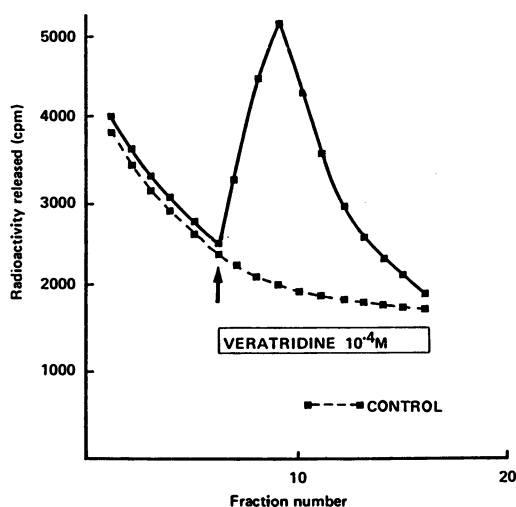
Figure 1. The effect of veratridine (added at arrow) on release of label from superfused insect synaptosomes loaded with [³H]-choline.
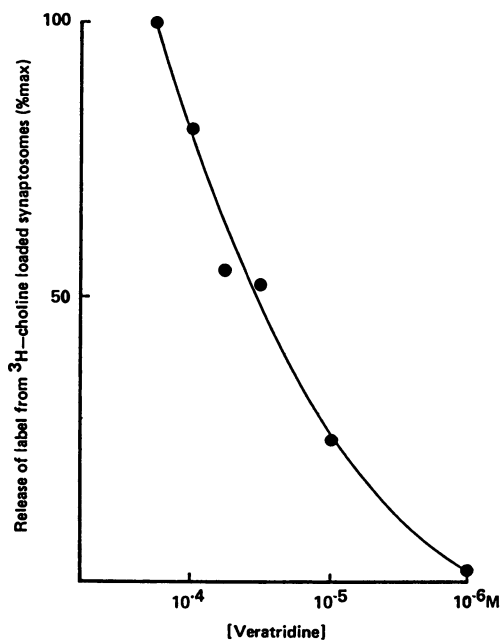
Figure 2. The relationship between veratridine concentration and release of neurotransmitter. Reproduced with permission Ref. 2. Copyright 1985, Society of Chemical Industry.

limit of its solubility in saline. The veratridine-stimulated
efflux of label from the synaptosomal preparation could arise from
its classical neurotransmitter-releasing action through sodium
channel activation, as described for mammalian nerve terminals (4).
Alternatively it could act by inhibiting reuptake into the terminals
of any[^{3}H]-choline that might diffuse into the extraterminal
spaces of the synaptosomal bed. Clearly an important requirement
in any release study is that reuptake of released radiolabelled
material is prevented. If this is not achieved, inhibition of
reuptake may be misinterpreted as a releasing effect. Evidence
from vertebrate superfusion experiments demonstrates that reuptake
of released neurotransmitters can be overcome if synaptosomes are
superfused as a thin layer at appropriate flow rates (6). In the
present study inhibition of reuptake of 'diffusible' choline can be
discounted because hemicholinium-3, which has been shown to be an
effective inhibitor of [^{3}H]-choline accumulation in locust central
nerve terminals (7), failed to stimulate release of label at 10^{-4}M
(Figure 3). Furthermore, the results show clearly that tetrodo-
toxin, a selective blocker of sodium channels (8), is a very potent
inhibitor of veratridine-induced release (Figure 4), with 90%
suppression occurring at an inhibitor concentration of 10^{-7}M. The
highly specific nature of these interactions are further emphasized
by the observation that picrotoxinin which acts by blocking chloride
channels (9) fails to influence veratridine-dependent release, even
at high concentrations (Figure 5). In mammalian synaptosomal
preparations low concentrations of pyrethroids have been shown to
stimulate, in a tetrodotoxin-sensitive fashion, both sodium entry
(10) and release of the neurotransmitter GABA (11). In view of
these experiments and the strong electrophysiological evidence that
exists for the occurrence of nerve terminal depolarization in
insects poisoned with pyrethroids (12), the action of deltamethrin
was investigated using the superfusion technique. Insect
synaptosomes must be exposed to deltamethrin at high concentrations
(10^{-5}M) to observe immediate effects or for much longer periods at
lower concentrations (10^{-6}M to 10^{-8}M) to observe significant changes
in the rate of transmitter release in this system. In both cases
the stimulations observed are a great deal weaker (<10%) than those
observed with veratridine at 10^{-4}M. Veratridine's action however,
is potentiated by deltamethrin with the effect being more marked
upon pre-incubation with the pyrethroid (Figure 6). Such an inter-
action which has also been reported in mammalian systems (10), (13),
suggests that deltamethrin exerts its depolarizing action in
synaptosomes by combining with an open form of the sodium channel.
In view of the sensitivity of central nerve terminals to this
agonist combination, these sites should be considered potential
targets for pyrethroids in insects.

Avermectin B$_{1a}$ has been shown to stimulate chloride ion uptake
into cockroach muscle (14) and block transmission at the arthropod
neuromuscular junction by increasing GABA-mediated chloride ion
permeability (15), (16). To extend our understanding of ion channel
types present in the surface membrane of the synaptosome we
examined the effects of ivermectin (22,23-dihydroavermectin B$_1$)
and some closely related milbemycins. Both ivermectin and the
milbemycins examined, with the exception of milbemycin α9, were
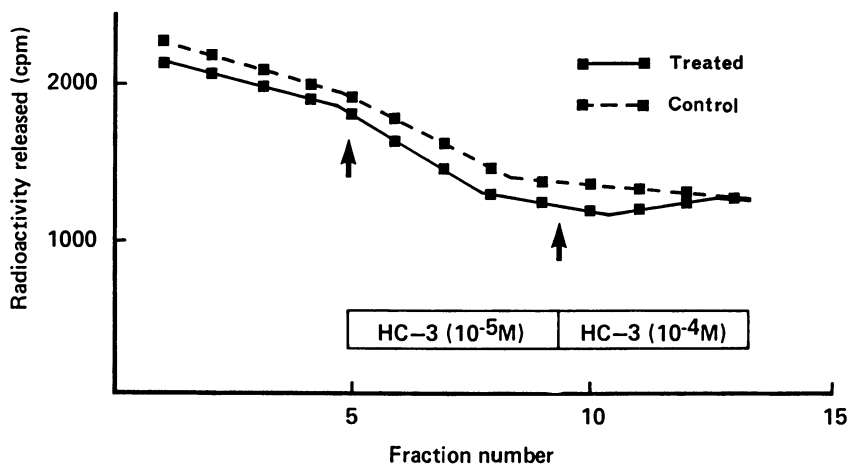
Figure 3. The failure of hemicholinium-3 (added at arrow) to stimulate efflux of label from [^{3}H]-choline-loaded synaptosomes in superfusion.
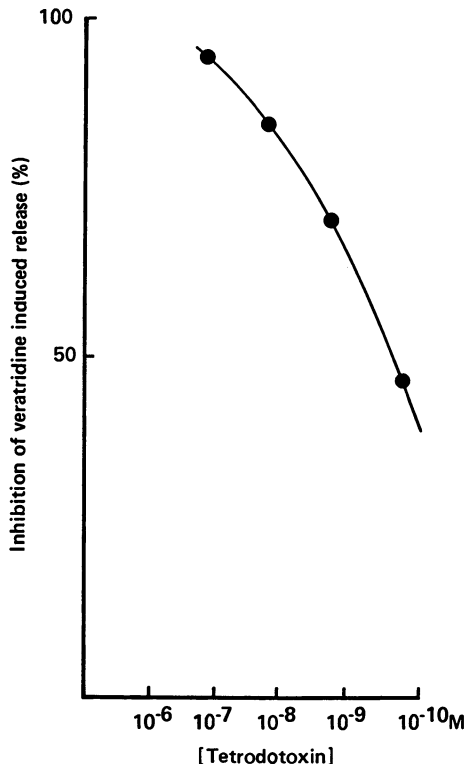
Figure 4. The inhibition of veratridine-dependent transmitter release by tetrodotoxin. Reproduced with permission Ref. 2. Copyright 1985, Society of Chemical Industry.
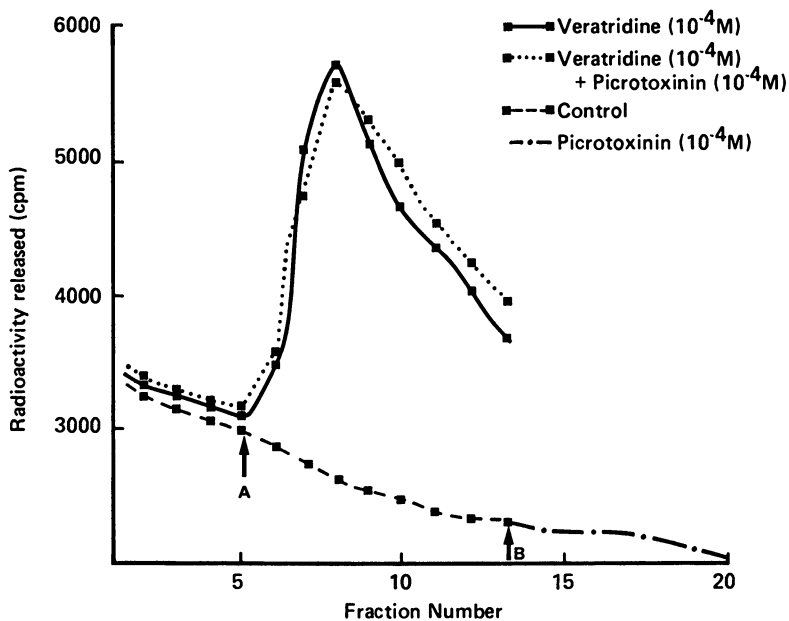
Figure 5. The lack of effect of picrotoxinin on veratridine-dependent release. (Veratridine with or without picrotoxinin added at A; picrotoxinin added at B).
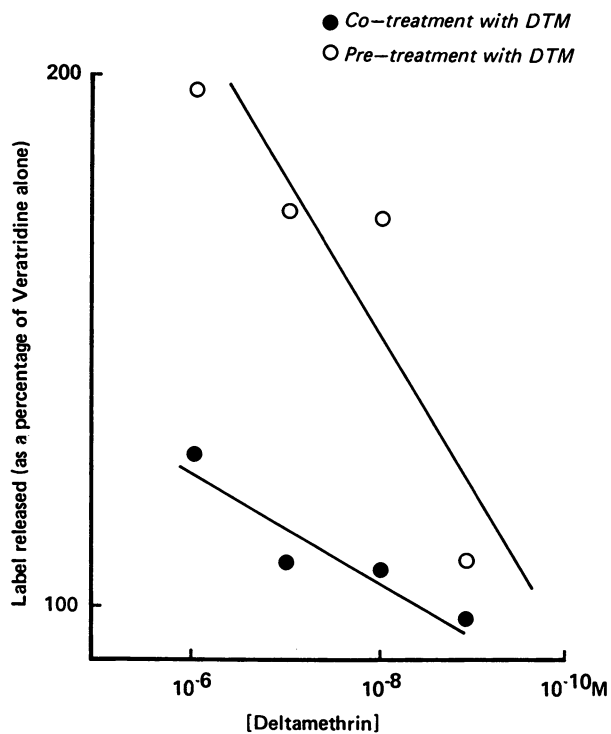
Figure 6.   The potentiation of veratridine-induced release by deltamethrin (DTM):- a comparison of preincubation with co-treatment (veratridine at 10^{-4}M throughout: the period of pre-incubation with DTM was 34 minutes).

found to stimulate the release of radioactivity from insect
synaptosomes (Figure 7). In this respect ivermectin was
demonstrably the most potent of the macrolactone analogues producing
greater than 50% of the maximum response at 10^{-8}M. Ivermectin's
stimulatory action could be inhibited by picrotoxinin, dieldrin
and γ-hexachlorocyclohexane (Figure 8). In view of the chloride
channel blocking actions reported for these classes of antagonist
(9), (17), it is likely that ivermectin-induced release of neuro-
transmitter involves an opening of membrane channels that are
selective for chloride ions. t-Butylbicyclophosphorothionate (TBPS)
a potent mammalian convulsant (18), reported to bind with high
affinity to the picrotoxinin recognition site in rat brain synaptic
membranes (19), was unable at 10^{-6}M to influence ivermectin-induced
release (Figure 9). This result raises the possibility of a
difference between mammalian and insect chloride channels in terms
of their responses to bicyclophosphorothionates. As expected,
tetrodotoxin (10^{-6}M) has no inhibitory effect on the ivermectin
response (Figure 10). Ivermectin-dependent release is reduced
slightly by the removal of ionic calcium from the superfusing saline
(Figure 11) indicating that opening of voltage-sensitive calcium
channels may play a part in this phenomenon.
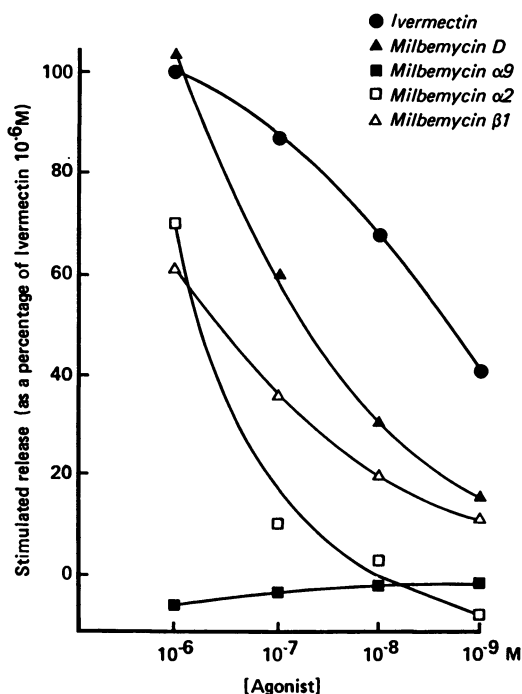
Figure 7. Ivermectin- and milbemycin-stimulated release of
neurotransmitter from cockroach central nerve terminals in
superfusion.
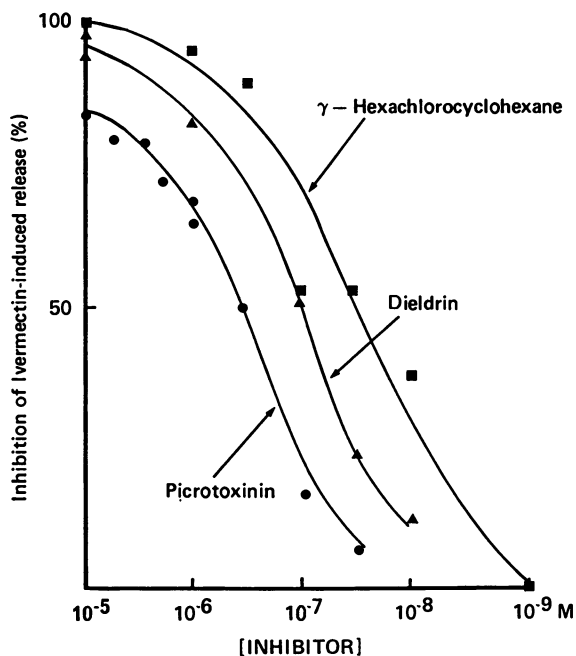
Figure 8. The inhibition of ivermectin-dependent release of label from synaptosomes by selected chloride channel antagonists.
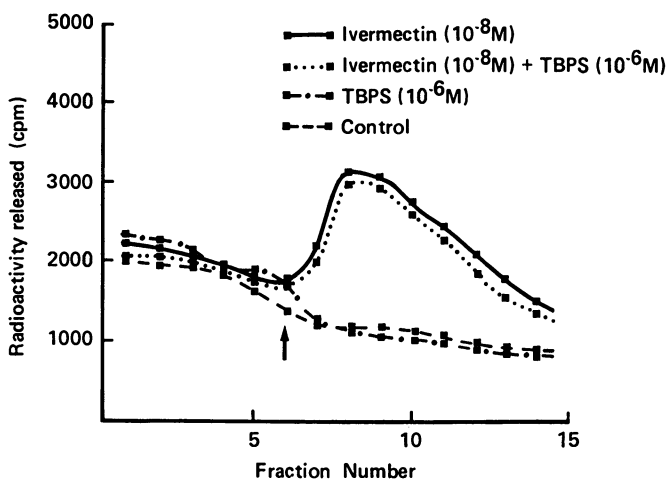
Figure 9. The lack of effect of TBPS on ivermectin-induced release of transmitter from cockroach synaptosomes. (Compounds added at arrow).
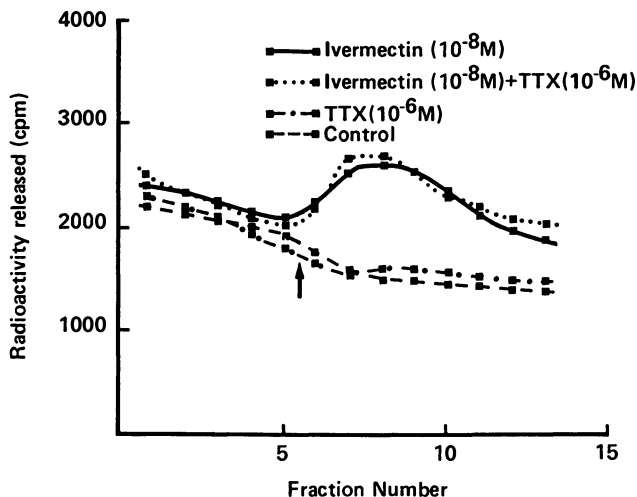
Figure 10. The lack of effect of tetrodotoxin (TTX) on ivermectin-induced release of transmitter from cockroach synaptosomes. (Compounds added at arrow).
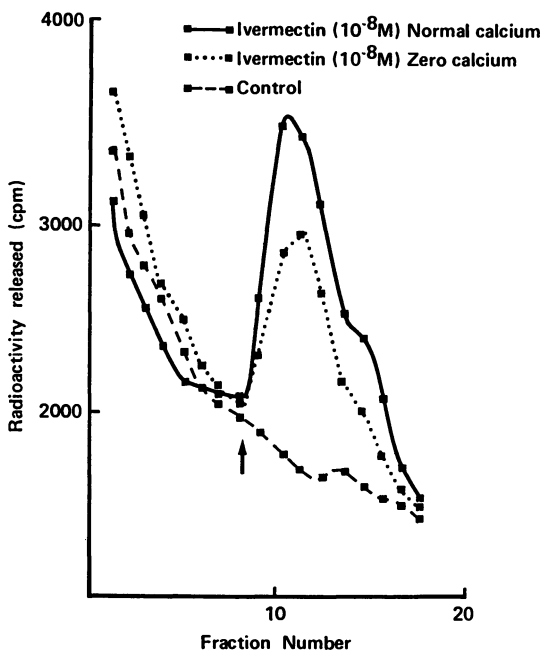
Figure 11. The effect of removing calcium from the superfusion saline on ability of synaptosomes to respond to ivermectin. (Ivermectin added at arrow; EGTA added to zero calcium saline).

In addition to clarifying the mechanism of action of selected insecticidal agents this study provides clear evidence for the existence of sodium, chloride and possibly calcium channels in the surface membrane of insect central nerve terminals. The observed release of neurotransmitter in the presence of ivermectin was unexpected but may indicate that insect cholinergic nerve terminals contain high levels of chloride in the resting state. Under these circumstances, a challenge with a chloride channel activator would produce an outward passage of negatively charged ions which may be sufficient to depolarize the terminal and activate the release mechanism. A similar phenomenon has been reported in dendritic regions of spinal cord neurones where iontophoretic application of GABA produces depolarizing responses (20). The ionic basis of ivermectin's stimulatory action on release of neurotransmitter from insect synaptosomes is currently under investigation.

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Chapter 20

Methyllycaconitine

A Potent Natural Insecticide Active on the Cholinergic Receptor

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A chloroform extract from the seeds of Delphinium plants was shown to be toxic to a number of insect species, and to have a very high affinity for the nicotinic acetylcholine receptor of the housefly. The major active fraction of the extract was an alkaloid, methyllycaconitine (MLA). A pure sample of MLA.citrate showed similar insecticidal activity to the crude extract, and had a similarly high affinity for the acetylcholine receptor. Previous experiments on a vertebrate preparation had indicated an action of MLA on the nicotinic acetylcholine receptor, but the concentrations used were many times greater than those affecting the housefly receptor. There may therefore be significant differences between the acetylcholine receptors of insects and vertebrates, and MLA could be a very useful tool for studying these differences.

A large number of commercially important insecticides act on the nervous system of the insect. By investigating certain areas of this system in detail, it may be possible to find new classes of chemicals which can disrupt the normal nervous activity, and this may lead to the development of new insecticides. It is important to look not only at chemicals which kill the insect, but also at those groups of chemicals which can disrupt the insect's behavior sufficiently to prevent its survival in the field.

One target of interest in the search for new insecticides is the nicotinic acetylcholine receptor. Chemicals which act at this site are known to kill insects, and two are used as insecticides. These are nicotine, which has been used for over 200 years, mainly against aphids and thrips, and Padan, an insecticide used against rice pests, which was derived from a toxin produced by marine worms of the genus Nereis (1). The fact that one of these insecticides was derived from a plant and one from an animal underlines the

importance of investigating natural products as potential sources
of new insecticides.

Studies on the Nicotinic Acetylcholine Receptor

One way of investigating the effect of chemicals at the nicotinic
acetylcholine receptor is to isolate the receptor and measure the
ability of chemicals to bind to it. In the experiments described
here, housefly heads were used as a source of nervous tissue, and
a crude membrane preparation was obtained using a method based on
that described by Jones et al. ([2]). The snake toxin, α-bungarotoxin
(α-BGTx), is known to bind to this receptor with a very high affin-
ity (KD=1.1 +/- 0.1nM.([3]))
      Chemicals of interest are incubated with the housefly head
receptor preparation, then the radioligand [3H]- propionyl-$_\alpha$-bun-
garotoxin (3Hα-BGTx) is added. If the chemical has bound to the
receptor, this will reduce the number of binding sites available
to the 3Hα-BGTx. The percentage reduction of binding of 3Hα-BGTx
can be measured, and the Kinh of the test chemical can be calculated.
Kinh for these experiments was estimated using the Cheng-Prusoff
relation ([4]) after a logit transformation of the dose-inhibition
data. This allows the quantitative evaluation of the inhibitory
potency of test chemicals. Those chemicals showing inhibitory activ-
ity can be further investigated using physiological tests to show
whether they are acting as agonists or antagonists of acetylcholine.
Their toxicity to a range of insect species can also be investigated.
      The assay described above has been used to investigate the
actions of extracts from several species of plants, some of which
are known to be insecticidal. The insecticidal properties of ex-
tracts from Delphinium seeds were first reported by Pliny the Elder,
who described their use as a treatment for "vermin in the head and
other parts of the body" ([5]). More recently, it has been reported
that extracts of Delphinium were used extensively in Russia as an
antiparasitic, being effective against lice and bed-bugs ([6]). Some
testing of the insecticidal properties of Delphinium brownii alka-
loids was conducted by the Canada Dept. of Agriculture ([7,8]), but
other than confirming that the alkaloidal fraction possessed insecti-
cidal properties, no further chemical analysis or biological mode
of action work was performed. A recent study of extracts from
Delphinium geyeri also revealed insecticidal activity of certain
alkaloids, and some of these were isolated and characterized ([9]).

Effects of Delphinium Extracts on the Nicotinic Acetylcholine Receptor

The effects of seeds of Delphinium hybrid, cv."Pacific Giant, King
Arthur" were investigated in the housefly nicotinic receptor assay,
and their toxicity to insects was investigated. Chloroform extracts
of the Delphinium seeds were assayed for activity against a number
of species of insects and mites, and the results of these tests
are summarized in Table I. The extract caused mortality in a number
of different species, and protected leaves from feeding damage.
      The chlorform extract of the Delphinium seeds displayed a very
potent inhibition of 3H α-BGTx binding in the nicotinic receptor

assay and was much more potent than nicotine, which was used as
a standard. In order to identify the active component, the extract
was first divided into an alkaloidal and a non-alkaloidal fraction.
Tests on the nicotinic receptor and on several insect species in-
dicated that both the nicotinic effects and the insecticidal activ-
ity were localized in the alkaloidal fraction.

        The alkaloidal fraction was then further separated using pre-
parative silica gel thin layer chromatography (TLC). The material
was separated on Silica Gel 60 (0.25 mm, Merck) using cyclohexane:
chloroform:diethylamine (5:4:1), and six discrete fractions were
identified. The major alkaloid had an Rf of 0.43, and this fraction
was by far the most active in the nicotinic receptor assay. This
active fraction was then further characterized by mass spectrometry,
proton and C13 NMR spectroscopy, and was identified as methylly-
caconitine (MLA,Fig.1). It was found to have a Kinh value for dis-
placing $3H\alpha$-BGTX of less than 0.5 nM (Fig.2).

        This MLA fraction was also tested for insecticidal activity
against <u>Spodoptera</u> <u>eridania</u> and <u>Musca</u> <u>domestica</u>, and was toxic to
both. A previous study by Aiyar et al. had shown that MLA was the
alkaloid responsible for causing the death of cattle feeding on
<u>Delphinium</u> <u>brownii</u> in Western Canada (<u>10</u>). They also demonstrated
that MLA had a neuromuscular blocking action in a rat phrenic nerve-
diaphragm preparation, which was probably due to an effect on the
nicotinic cholinergic receptor. The EC50 for this effect was
2.3 µM.

        A pure sample of MLA.citrate was obtained, and its affinity
for the housefly nicotinic acetylcholine receptor was found to be
identical to that of the most active fraction isolated by TLC.
The Kinh value of MLA.citrate at the insect receptor was 0.25 +/-
0.05 nM. This shows that the activity at the insect receptor is
far greater than that reported for rat muscle. The rat data was
obtained from a physiological experiment, not a binding study, so
it is not possible to compare it directly with these insect experi-
ments, but the great difference between the effective concentration
at rat muscle and the Kinh for the insect receptor may indicate
that the rat and insect receptor differ pharmacologically. This
idea is supported by experiments on other alkaloids. Aconitine,
which is more active than MLA at the rat muscle, was considerably
less active at the insect receptor, having a Kinh of 2.7 +/-
0.08 mM. Lycoctonine, which differs from MLA in the absence of the
aromatic ester group (Fig.1), also inhibited $3H\alpha$-BGTx binding in
the insect cholinergic receptor assay, but was much less potent
(Kinh 0.38 +/- 0.06 µM). It was not toxic to <u>Spodoptera</u> <u>eridania</u>
larvae at concentrations at which MLA caused to kill. The order of
potency at the insect receptor was therefore MLA>lycoctonine>aconi-
tine, while that in the rat phrenic diaphragm preparation was aconi-
tine>MLA>lycoctonine (<u>10</u>).

Insecticidal Activity

The activity of MLA.citrate against insects and mites was tested
and compared with the activity of the Delphinium seed extract
(Table 1). The properties were found to be very similar, the main
difference being that the MLA.citrate was not active against <u>Tetra-</u>
<u>nychus</u> <u>urticae</u> or <u>Anopheles</u> <u>quadrimaculatus</u>. It is thought that

Table I.   Insecticidal and acaricidal activity of <u>Delphinium</u>
extracts compared with MLA

		Chloro-form extract	Alkaloid fraction	MLA. citrate
<u>Spodoptera eridania</u>	Larvae	+	+	+
<u>Aphis fabae</u>		−	N.D.	−
<u>Tetranychus urticae</u>		+	−	−
<u>Diabrotica undecimpunctata howardi</u>	Larvae	−	N.D.	−
<u>Anopheles quadrimaculatus</u>	Adult	+	N.D.	−
	Larvae	N.D.	−	−
	Eggs	+	−	−
<u>Empoasca abrupta</u>		+	+	+
<u>Heliothis virescens</u>	Larvae	+	+	+
	Eggs	+	+	+
<u>Musca domestica</u>		+	N.D.	+

(+) active, (−) inactive,(N.D.) not determined. Activity on eggs
indicates a contact ovicidal activity, reducing viable egghatch.
Active denotes significant (50% +) mortality at a screening rate
of 1000 ppm (<u>Spodoptera</u>, <u>Heliothis</u> larvae, <u>Musca</u>), 300 ppm
(<u>Heliothis</u> eggs) or 100 ppm (<u>Empoasca</u>). Copyright 1986 American
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R = H Lycoctonine

Aconitine

R =   Methyllycaconitine
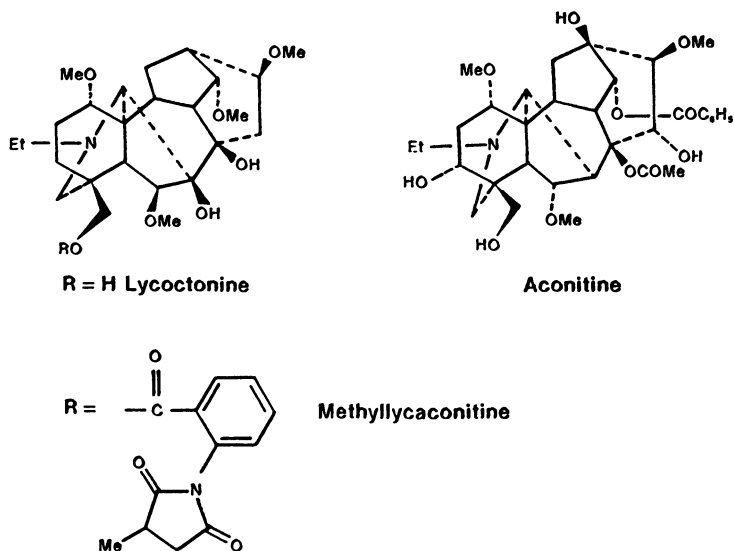
Figure 1. Structures of aconite alkaloids investigated.
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these organisms are killed by the saponins in the seed extract, and this would explain why they were unaffected by the MLA. The similar spectrum of activity of the seed extract and the MLA.citrate implies that MLA is the main insecticidal agent in the <u>Delphinium</u> plant. It seems likely that it is the very potent action of MLA at the nicotinic cholinergic receptor that causes the toxicity.

The symptoms caused by MLA in <u>Spodoptera</u> larvae are consistent with the hypothesis that it is acting on the nervous system. When the larvae were fed on treated bean leaves, observed mortality was rapid, with significant numbers of larvae being killed within 24 hours at concentrations as low as 100 ppm. MLA gave significant protection against feeding damage at levels of 300 ppm and above. Figure 3 illustrates the protection afforded at twenty-four hours. After 72 hours, feeding damage in the treated leaves was approximately 5%, whereas controls showed over 95% feeding damage. An approximate LC50 for MLA.citrate was calculated by dipping lima beans in different concentrations of MLA in 2:1 acetone:water, and exposing groups of ten larvae to the treated leaves. This experiment gave an LC50 value of 308 +/- 48 ppm. The LC50 for nicotine in the same assay was much greater than 1000 ppm.

Physiological Experiments

The binding studies on the housefly head preparation indicate that MLA binds to the nicotinic acetylcholine receptor with a very high affinity, but they do not show whether it acts as an agonist or an antagonist of the natural neurotransmitter. A useful preparation for examining the physiological effects of drugs at cholinergic synapses is the cercal giant synapse of the cockroach. Sensory nerves from the cerci form synapses in the sixth abdominal ganglion with the giant fibres in the ventral nerve cord, and these synapses are thought to be cholinergic (11). The effects of bungarotoxin and nicotine on this preparation have previously been investigated, using mannitol gap or oil gap techniques (12).

Initial experiments on MLA were carried out using the mannitol gap technique described by Callec et al. (12). However, comparable results could be obtained using a much simpler preparation, in which the sixth abdominal ganglion and cercal nerves were placed in a saline filled chamber, and the ventral nerve cord was draped over a wax partition into a separate chamber (Fig.4). The saline contained 210 mM NaCl, 3.1 mM KCl, 5.4 mM $CaCl_2$, 0.1 mM NaH_2PO_4, 2.0 mM $NaHCO_3$ and 5 mM $MgCl_2$. The nerve was embedded in vaseline as it passed over the partition. One cercal nerve was picked up in a suction electrode, and stimulated at 10 sec intervals. The resulting excitatory post-synaptic potential (epsp) was recorded using a differential amplifier. Stable recordings could be obtained for over two hours.

The effects of nicotine, MLA.citrate and α-BGTx on the electrical activity of this preparation were investigated. As α-BGTx is not water soluble, it was first dissolved in DMSO, then added to saline to give a final concentration of 1% DMSO. 1% DMSO was therefore included in all salines, to abolish any differences in response which might be due to solvent effects.
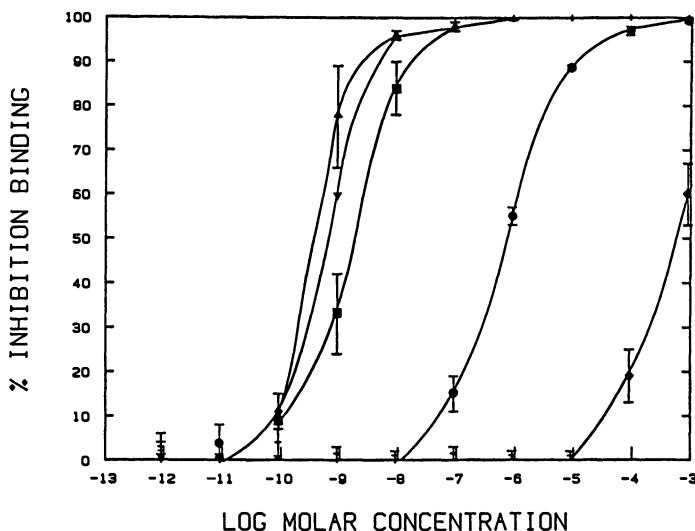
Figure 2. Inhibitory effects of test compounds on binding of $^3H\alpha$-BGTx to *Musca domestica* head homogenates. Test compounds were preincubated for 20 min with the tissue homogenate at 22–24 °C in pH 7.4 sodium phosphate buffer containing 1 mg/mL bovine serum albumin and 9% DMSO. The binding reaction was carried out for 30 min with 5 nM $^3H\alpha$-BGTx and terminated by filtration on Whatman GF/C filters. Data shown are percentage inhibition of binding ± SD. Δ, MLA.citrate; ∇, MLA purified from *Delphinium* seed; □, α-BGTx; ○, lycoctonine; ♦, aconitine. (Reproduced with permission from American Cyanamid Co. Copyright 1986.)
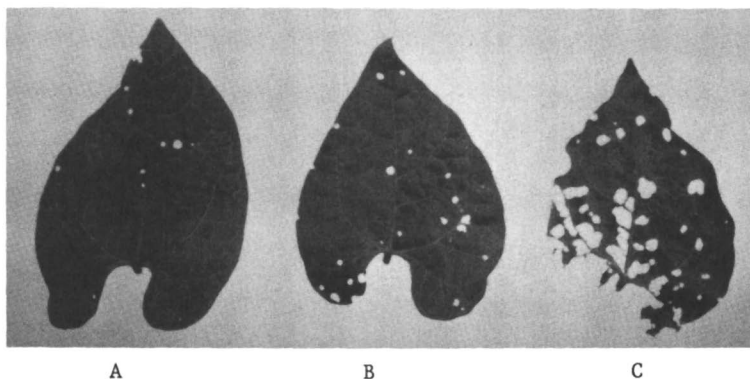
Figure 3. The insecticidal effect of methyllycaconitine against *Spodoptera eridania*. Lima bean leaves were dipped in 2:1 acetone:water solutions containing 1000 ppm MLA.citrate (A), 300 ppm MLA.citrate (B), acetone:water control (C). Leaves were exposed to 5 third instar *Spodoptera eridania* larvae for 24 h at an ambient temperature of 27 °C. (Reproduced with permission from American Cyanamid Co. Copyright 1986.)

The effect of nicotine (1 μM) on this preparation was to cause
a massive increase in spontaneous activity, which tended to obscure
the synaptic response. If the concentration was increased to 10 μM,
all synaptic activity was rapidly blocked. Both MLA and α-BGTx
caused a gradual block of synaptic response. Concentrations of MLA
above 10 μM also caused a slight increase in spontaneous activity
in approximately 50% of preparations tested, but neither MLA nor
BGTx ever caused the violent bursts of activity observed in response
to nicotine.

α-BGTx had no effect in preparations with an intact sheath
around the ganglion. Even in desheathed preparations, it took over
thirty minutes for α-BGTx to have an effect. 100 nM α-BGTx caused
synaptic block in 52 +/- 4.9 min, but increasing the concentration
one-hundred fold did not significantly reduce the time to block.
MLA was also inactive in preparations with an intact ganglionic
sheath, but its effects in desheathed preparations were more dose
dependent (Fig.5). The lowest concentration ever to cause block
within one hour was 10 nM for α-BGTx and 100 nM for MLA. The simi-
larity of action of MLA and BGTx indicates that MLA may be acting
as an antagonist.

The concentration of MLA to cause a physiological effect in
the cockroach was significantly higher than that inhibiting α-BGTx
binding in the housefly head preparation. This difference was inves-
tigated further by measuring the binding of MLA to cockroach nerve
cord. Preliminary results indicate that MLA is approximately 100
times less active at the cockroach receptor than at the housefly
head receptor. Part of this reduction might be due to a difference
in the techniques used, as the IC50 for nicotine was also slightly
less for the cockroach than for the housefly, but it might be an
indication that there are different sub-classes of nicotinic recep-
tor in different insects.

Significance

The high affinity of MLA for the insect nicotinic acetylcholine
receptor suggests that this chemical may have evolved within the
plant as an insecticide, giving protection against a wide range
of insect species. The spectrum of insecticidal activity of MLA
is wider than that of nicotine, which, unlike MLA, has little effect
on Lepidoptera. Nicotine was also approximately 10,000 times less
active than MLA as an inhibitor of 3H α-BGTx binding to housefly
head homogenates, and this difference in potency is reflected in
the differences in insecticidal action.

The concentrations of MLA necessary to displace α-BGTx binding
to the nicotinic acetylcholine receptor are many times less than
the concentrations having a physiological effect in a vertebrate
nerve-muscle preparation. Although binding experiments cannot be
directly compared with physiological experiments, this apparent
difference in response may indicate a difference in the pharmacology
of the vertebrate and the invertebrate nicotinic acetylcholine
receptor.
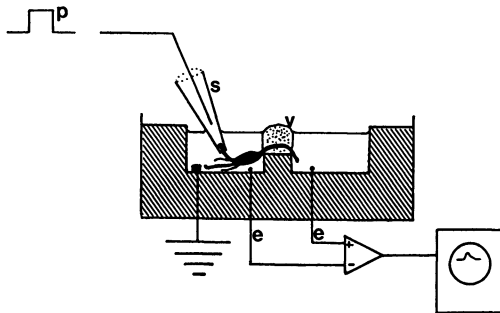
Figure 4. Experimental chamber for recording from the 6th abdominal ganglion of the cockroach. The ganglion (black) is placed in a wax chamber (shaded) and the nerve cord is embedded in vaseline, thus isolating the cut end of the nerve cord from the ganglion. A stimulus pulse (p) was applied to the cercal nerve via a suction electrode (s). Recordings from the nerve cord were made via silver wire electrodes (e), and chemicals were added to the left side of the saline-filled chamber. Copyright 1986 American Cyanamid Co. Reprinted with permission.
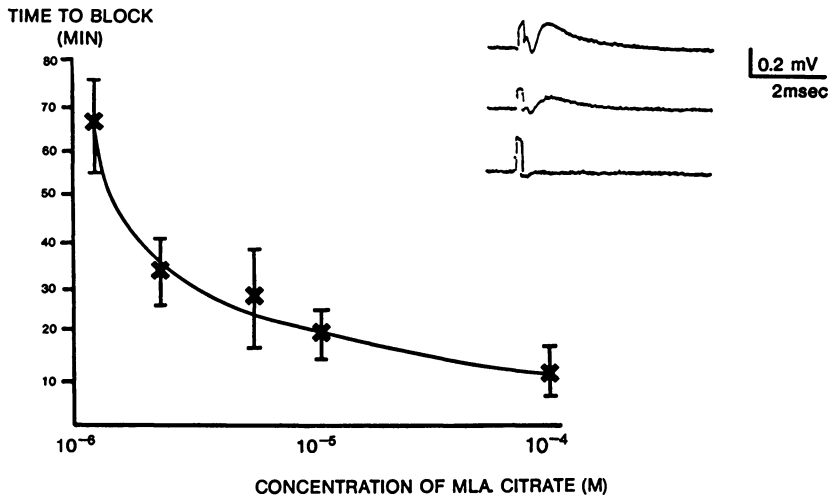
Figure 5. Time to block of the evoked epsp in cockroach nerve cord after application of MLA.citrate. Only those concentrations giving 100% response within 90 min are plotted.(mean +/- SD, n=5) Inset: Epsp recorded before treatment (upper trace), 40 min after addition of 200 nM MLA.citrate (middle trace) and 80 min after MLA.citrate (lower trace). Copyright 1986 American Cyanamid Co. Reprinted with permission.

The affinity of MLA for the nicotinic receptor in the cockroach nerve cord also appeared to be rather less than that for the housefly head receptor, suggesting that there might be different sub-classes of insect receptor. This theory was proposed recently by Battersby and Hall (13), who showed that lobeline, another plant-derived cholinergic ligand, had a higher affinity for the cockroach receptor than for the housefly head receptor.

The experiments described here suggest that MLA could be a very powerful tool for investigating the comparative pharmacology of different insect receptors, and for studying the differences between insect and vertebrate receptors. It also appears likely that chemicals acting in a similar way to MLA could be powerful insecticides, and the differential affinity for the insect receptor might confer low mammalian toxicity.

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Chapter 21

Anthelmintics Affecting GABA
and Acetylcholine Receptors
on Ascaris Muscle Bags

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We have shown that the anthelmintics; pyrantel,
morantel and levamisole, activate acetylcholine
(Ach) receptors on the muscle cell membranes of
Ascaris suum and also that the avermectin;
22,23-dihydroavermectin B1a (DHAVM) blocks the
function of muscle cell gamma-aminobutyric acid
(GABA) receptors. In experiments using
micro-iontophoresis, the cholinergic anthelmintics
were found to act directly on the muscle bag
membrane, evoking a depolarizing response.
Current-voltage relationships for Ach, pyrantel,
morantel, and levamisole have similar reversal
potentials (approx. +10 mV), suggesting that each
activates the same cation channel. DHAVM blocks
GABA-induced responses recorded from the muscle bag
membrane. The blockade is voltage-dependent
increasing with membrane depolarization, suggesting
that DHAVM interacts with the ion-conducting site
of the GABA receptor/chloride channel.

Modern pharmaceutical research has led to the discovery of numerous
drugs that provide highly effective control of helminth parasites.
These anthelmintic agents which are comprised of several chemical
classes of compounds include many that are neuroactive. For
example, the avermectins (1-3), piperazine (4), imidothiazoles
(5-7), pyrimidines (7-8) and organophosphates (9) interact with
target sites in the central nervous system and/or neuromuscular
systems of parasites. Other anthelmintics, such as the
benzimidazoles, salicylamides and substituted phenols act on
biochemical targets to disrupt energy production in helminth
parasites (10-14).
In this paper we will review recent studies in our laboratory
on the mode of action of neuroactive anthelmintics. We have
concentrated on investigation of the following cholinergic
anthelmintics; the pyrimidines, pyrantel and morantel, and the
imidothiazole, levamisole (Figure 1). Our findings from these

studies will be described (published in detail elsewhere (7)) together with those obtained in studies on the effects of 22,23-dihydroavermectin B1a (DHAVM) on GABA receptor function.

All experiments were performed using neuromuscular preparations from the intestinal parasite Ascaris suum. This pig parasite has large muscle cells which facilitate intracellular recording. The cells are composed of three parts: the spindle, bag and arm (Figure 2). The spindle is the contractile region of the muscle cell. The bag is spherical, about 200 um in diameter, and contains the cell nucleus. The arm is a thin process which reaches transversely from the bag to one of the longitudinally running nerve cords. The arms come together to form a syncitium over the nerve cord and are electrically coupled in this region (15). Neuromuscular synapses with both excitatory and inhibitory motorneurones are present in the syncitium (see reviews 16-18). In addition to the receptors on the post-synaptic membranes, non-synaptic acetylcholine (Ach) and gamma-aminobutyric acid (GABA) receptors are present on the bag membrane (4, 19, 20). These receptors are directly accessible for quantitative pharmacological investigations. Compounds can be applied to the bag membrane in the bathing medium by local perfusion or iontophoretically from micropipettes placed within 50 um of the membrane.

Action of Morantel, Pyrantel and Levamisole on the Function of Muscle Ach Receptors

Exposure of nematode parasites to morantel, pyrantel and levamisole causes spastic paralysis (21) consistent with the activation of excitatory Ach receptors. In experiments on A. suum, Aubry et al. (8) showed that muscle contractions evoked by pyrantel are sensitive to block by the cholingeric antagonist, d-tubocurarine. In our experiments we used current or voltage clamped muscle bag preparations to investigate directly the mechanism of the paralyzing action of morantel, pyrantel and levamisole.

In an initial series of experiments, changes in membrane input conductance in response to the microperfusion of Ach, levamisole, pyrantel and morantel to the muscle bag membrane were measured under current clamp. Dose-conductance relationships were generated by the sequential application of increasing drug concentrations (no washing between applications) within the range 10 nM to 10 mM (Figure 3a). The Ach-induced conductance increase was dose-dependent and the log dose-conductance relationship was sigmoid (Figure 4a). The concentration of Ach required to produce a half-maximal conductance increase (EC50) was 110 uM. Dose-dependent desensitization to Ach was not observed in any experiments performed at the resting potential. The effects of Ach on both input conductance and membrane potential were readily reversed on washing. The levamisole-induced conductance increase was also dose-dependent, with a sigmoid dose-conductance relationship. The EC50 for levamisole was 20 uM. The maximal increase in input conductance obtained with levamisole and Ach were similar, being 1.29 $\pm$ 0.2 uS (mean $\pm$ s.e.) and 1.32 $\pm$ 0.24 uS, respectively. The time course of the levamisole-induced response was prolonged when compared with responses induced by similar concentrations of Ach.

IMIDOTHIAZOLE AND PYRIMIDINE ANTHELMINTICS

IMIDOTHIAZOLE : Levamisole
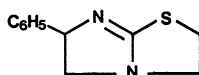
PYRIMIDINES :   Morantel   Pyrantel
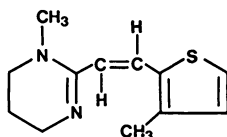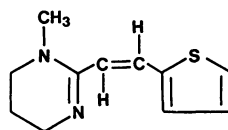
Figure 1. Chemical structures of the cholinergic anthelmintics levamisole, morantel and pyrantel.
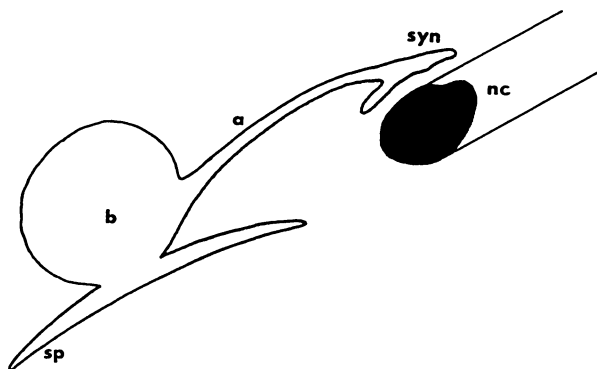
Figure 2. Diagram of the <u>Ascaris</u> muscle cell illustrating its anatomical features: b, bag; a, arm; syn, syncitium; sp, spindle; nc, nerve cord.
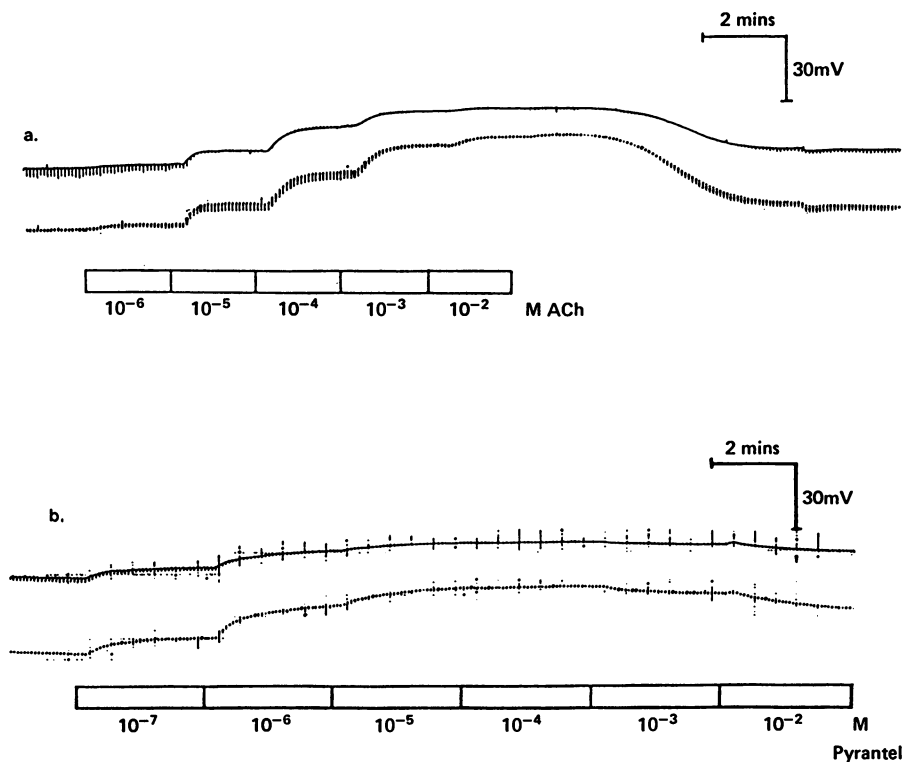
Figure 3. Changes in the membrane potential and input conductance of muscle bags during the sequential application of increasing concentrations of a) acetylcholine (Ach) and b) pyrantel. The input conductance was determined from the amplitude of the hyperpolarizations resulting from 400 ms pulses of current (50 nA) passed across the bag membrane. Ach and pyrantel were applied by microfusion (concentrations shown) to different muscle bags. Note the decrease in input conductance and repolarization in the presence of 1 and 10 mM pyrantel. (Adapted with permission from Ref. 7. Copyright 1985, Society of Chemical Industry).
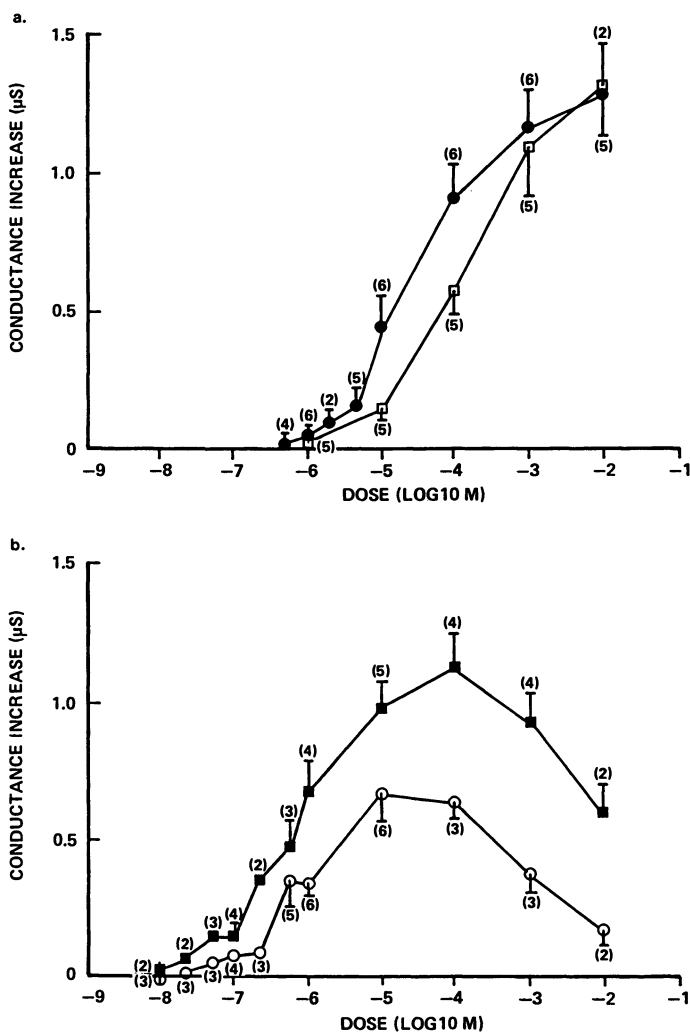
Figure 4. Dose-conductance relationship for a) acetylcholine (□) and levamisole (●), and b) morantel (O) and pyrantel (■). Each point is the mean of results from at least two cells: Vertical lines indicate the standard error of the mean. The number of cells contributing to each point is shown in parenthesis above or below the error bar. (Reproduced with permission from Ref. 7. Copyright 1985, Society of Chemical Industry).

Microperfusion of both morantel and pyrantel, within the concentration range 10 nM to about 100 uM, caused dose-dependent increases in both input conductance and membrane depolarization. Further increases in concentration, up to 10 mM, resulted in a decrease in input conductance and membrane repolarization (Figure 3b). As a consequence, the dose-conductance relationships for these compounds were bell-shaped (Figure 4b). We feel that the most likely interpretaion of the bell-shaped dose-conductance relationships is that both morantel and pyrantel act as antagonists at the Ach-receptor ion-channel complex when applied in high concentrations. However, it is also possible that the decrease in input conductance at high doses could result from dose-dependent desensitization, although desensitization was not observed during prolonged applications of morantel or pyrantel at concentrations up to 100 uM.

To further investigate the effects of the anthelmintics on muscle bag Ach receptors, membrane currents were recorded under voltage clamp. Figure 5a shows the current-voltage relationship obtained by microperfusion of 100 uM Ach. The relationship between Ach peak current amplitude and membrane potential was linear over the range -30 mV to +10 mV. The reversal potential had a value close to +10 mV. For membrane potentials more negative than -30 mV (range -40 to -80 mV) the current-voltage relationship was non-linear. We found that the non-linear component was both dose-dependent, increasing with Ach concentration, and voltage-dependent, suggesting that it is caused either by agonist-induced cation channel block or by voltage-dependent desensitization.

The current-voltage relationships for membrane currents induced by microperfusion of Ach (100 um), levamisole (100 um), morantel (10 um) and pyrantel (10 uM) to the same muscle bag are shown in Figure 5b. The current-voltage relationships for the anthelmintics resemble that obtained with Ach. Each possesses a linear component at membrane potentials between -30 mV and 0 mV, and a non-linear component at potentials more hyperpolarized than -30 mV. The reversal potential for each compound was close to +10 mV suggesting that each activate the same cation channel in the muscle bag membrane. Since the experiments were performed in calcium-free saline it seems likely that the membrane currents were carried mainly by sodium ions (and possibly potassium ions).

In an additional series of experiments, double-barrelled iontophoresis was used to investigate the interaction of Ach and pyrantel at the Ach receptor. Figure 6a compares the inward membrane currents, recorded at a holding potential of -30 mV, in response to these two ligands applied at the same iontophoretic dose. Pyrantel evoked a larger peak inward current than Ach, indicating that it is a more potent agonist under these conditions. The pyrantel-induced current was also prolonged in comparison to the Ach response (even when the iontophoretic dose was adjusted to produce the same peak inward current; not shown). When pyrantel was applied immediately prior to an Ach application (Figure 6b) it caused a reversible block of the Ach-induced current, suggesting that the two ligands were competing for the same receptor site. In the same experiment, two iontophoretic pulses of pyrantel, applied in succession, roughly doubled the peak inward current indicating

(a)

MEMBRANE POTENTIAL (mV)

−80 −70 −60 −50 −40 −30 −20 −10   0   10

INWARD CURRENT (nA)

−10
(3)
−20
(3)
−30
(6)
−40
−50
(6)
−60
−70
(6)
−80

(b)

MEMBRANE POTENTIAL (mV)

−80 −70 −60 −50 −40 −30 −20 −10   0   10

INWARD CURRENT (nA)

−5
−10
−15
−20
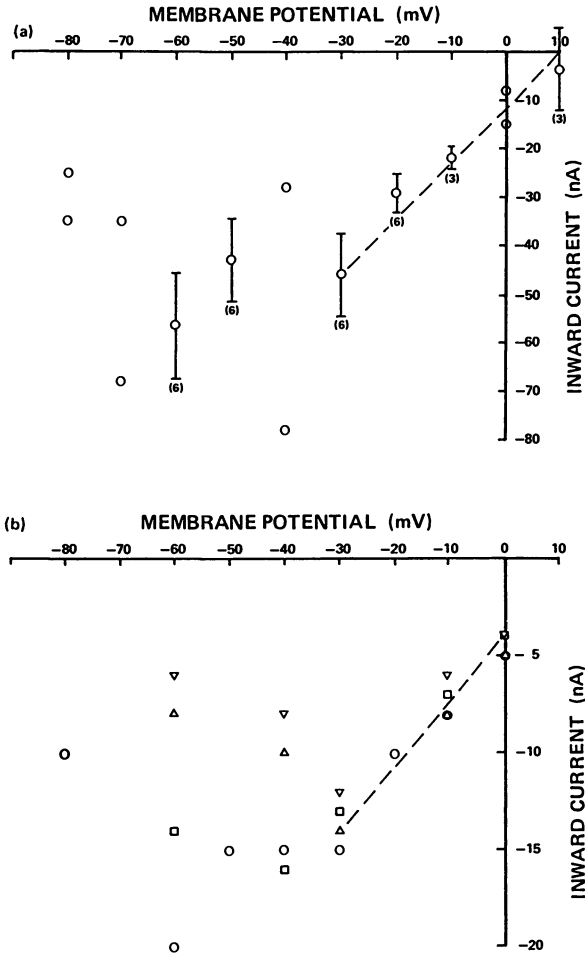
Figure 5. a) Current-voltage relationship for membrane currents
evoked by microperfusion of acetylcholne (100 uM; 1 min). The data
are pooled from nine different muscle cells. Means are plotted with
the number of cells contributing to each point given in parenthesis:
Vertical bars indicate the standard error of the mean. The dashed
line was fitted by eye to the linear component of the
current-voltage relationship (−30 mV to 0 mV), giving a reversal
potential of about +10 mV. b) Current-voltage relationship for; (O)
acetylcholine (100 uM), (□) levamisole (100 uM), (Δ) morantel (10
uM) and (▽) pyrantel (10 uM). The dashed line, which was fitted by
eye to all datum points between −30 and 0 mV, extrapolates to +12
mV. (Adapted with permission from Ref. 7. Copyright 1985, Society
of Chemical Industry).
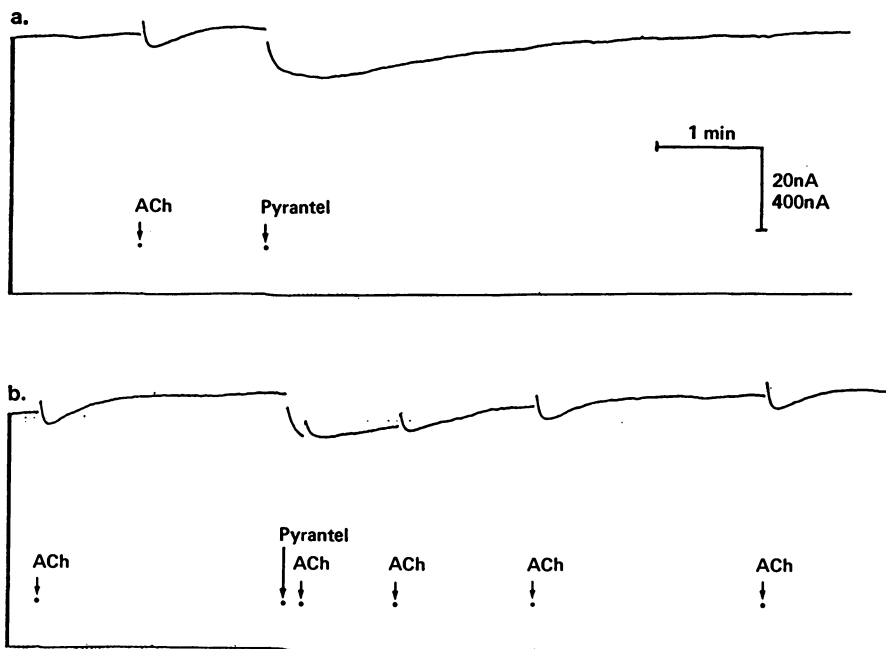
Figure 6.   Iontophoretic application of acetylcholine (Ach) and
pyrantel: (a) Comparison of inward currents (upper trace) generated
by the iontophoresis of Ach and pyrantel from a double-barrelled
micropipette, b) Interaction of Ach and pyrantel showing reversible
block of Ach currents following pyrantel application. The
micropipette was placed within a distance of 50 um from the bag
membrane. Ejection currents of 250 nA for 1s were used. Vertical
calibration: 20 nA, membrane current. 400 nA, iontophoretic
current (lower traces). (Adapted with permission from Ref. 7.
Copyright 1985, Society of Chemical Industry).

that the pyrantel-induced Ach current block was not due to a maximum
current limit being reached.
    The experiments described show that morantel, pyrantel and
levamisole occupy Ach receptors on A. suum muscle bags. At the
normal resting potential (approx. -30 mV) levamisole acted as an
agonist at all concentrations tested. Morantel and pyrantel also
acted as agonists, but only when applied in low concentrations. On
increasing the dose of these pyrimidines, antagonistic effects were
found. Increases in input conductance and membrane currents evoked
by morantel and pyrantel had prolonged time courses when compared to
responses induced by similar concentrations of Ach. Brief
applications (1-2 min) of either pyrimidine resulted in conductance
increases which persisted for up to 20 minutes whilst washing in
drug-free saline. These prolonged increases, which result in
membrane depolarization, cause maintained muscle contraction. The
electrophysiological results are consistent with the effects
observed when intact nematodes are exposed to morantel or pyrantel.
Under these conditions the worms exhibit spastic paralysis.
    Previous studies to characterize the pharmacology of Ach
receptors on A. suum muscle show that they possess a nicotinic
profile (22, 23). Of all the agents reported to have agonist
activity at these receptors, our results show that morantel and
pyrantel are the most potent agonists described to date. We have
also shown that levamisole is an agonist at the muscle Ach receptor
of A. suum, but is much less potent than the pyrimidines.
Levamisole causes rapid muscle contractions that lead to spastic
paralysis of nematodes. It has been suggested that these
contractions are caused by levamisole acting as a nerve ganglion
stimulant (6, 24). The results of our study show that levamisole
also acts directly on Ach receptors on the muscle cells of A. suum.
Both the increases in input conductance and the depolarization of
muscle cells were recorded from preparations bathed in a
calcium-free, high magnesium saline: under these conditions
transmission at chemical synapses is completely blocked. We have
also obtained further evidence for the direct action of levamisole
on the muscle cell membrane by recording depolarizing responses
evoked by the iontophoretic delivery of levamisole to the muscle bag
membrane.

The Effects of 22,23-Dihydroavermectin B1a on the Function of Muscle
GABA Receptors

The avermectins (AVM) are a family of naturally occurring macrolides
which are produced by Streptomyces avermitilis and possess potent
broad-spectrum anthelmintic, acaricidal and insecticidal activity
(25-27). One of the major components of the avermectin complex is
AVM B1a. This has been shown to interfere with neurotransmission at
excitatory neuronal (nerve-nerve) synapses in the ventral nerve cord
of A. suum where the mode of action seems to involve a GABA-ergic
mechanism (1, 3). AVM B1a also abolishes the hyperpolarizing
potentials recorded from muscle bags of A. suum in response to
stimulation of inhibitory motorneurones (1, 3). Kass and co-workers
(2, 28) suggest that the effect on inhibitory neuromuscular
transmission results from blockade of GABA receptors present on the
postsynaptic muscle membrane. As the postsynaptic receptors are

inaccessible to direct electrophysiological study, we have
investigated the effects of AVM on muscle GABA receptor function
using the non-synaptic GABA receptors on the muscle bag membrane
(19, 20).

In our study the AVM tested was 22,23-dihydroavermectin Bla
(DHAVM) which is a synthetic derivative of AVM Bla and the major
component of Ivermectin. DHAVM is about 70% less active than AVM
Bla in studies on parasite toxicity and avermectin-induced GABA
release from synaptosomes (29). Figure 7 shows the blocking effect
of a 30 min bath application of DHAVM (10 ug/ml) on the amplitude of
iontophoretically-evoked GABA hyperpolarizations recorded from a
muscle bag at its resting potential (approx. -30 mV). Washing in
drug-free saline for 25 min failed to restore the amplitude of the
GABA-induced responses, showing that the block was virtually
irreversible. Brief (2 min) applications of DHAVM at concentrations
of 0.01 ug/ml and above also blocked GABA hyperpolarizations. This
is illustrated in Figure 8 which shows typical recordings obtained
when GABA and DHAVM were microperfused onto current clamped muscle
bags. The GABA response was reversibly reduced when DHAVM (0.01 and
0.1 ug/ml) was applied either during or immediately prior to a
GABA-induced conductance change. A higher concentration of DHAVM
(1.0 ug/ml) caused an irreversible reduction in GABA response
amplitude. The resting potential and input conductance of the
muscle bag were unaffected by DHAVM up to 10 ug/ml.

To further investigate the mode of action of avermectins
against Ascaris muscle GABA receptors we studied the membrane
potential dependence of the blocking action of DHAVM. Exposure of
the muscle bag membrane to DHAVM (0.1 ug/ml) resulted in partial
suppression of the GABA-induced current amplitude at membrane
potentials within the range -20 mV to -90 mV (Figure 9a). The
degree of block was voltage-dependent, increasing with membrane
depolarization (Figure 9b).

Our studies show that DHAVM has no effect on the resting input
conductance or resting potential of the bag membrane of A. suum
muscle cells. This is in contrast with the effects of AVMs on other
invertebrate muscle preparations. In the opener and stretcher
muscles of lobster, AVM Bla causes an increase in the conductance of
the muscle membrane to chloride ions which is thought to result from
the activation of GABA receptor/chloride ion channels (31).
Similarly, the chloride conductance of locust extensor tibiae muscle
fibers is increased by exposure to DHAVM (30).

We have found that DHAVM antagonizes non-synaptic GABA
responses on A. suum muscle bags and that the ability of DHAVM to
block GABA-induced currents is voltage-dependent. The simplest
interpretation of this result is that DHAVM interacts with the
ion-conducting site of the GABA receptor/chloride channel.
Non-competitive inhibition of GABA responses by DHAVM has been
observed in the locust extensor tibiae preparation (30), and this
has also been interpreted as showing that DHAVM can interact with a
site that is distinct from the GABA recognition site (possibly, the
chloride ion channel).

There are close similarities between the effects of DHAVM on
the hyperpolarizing potentials evoked by activation of non-synaptic
GABA receptors on the bag membrane of A. suum and the effects of AVM
Bla on the hyperpolarizing potentials of muscle cells in response to
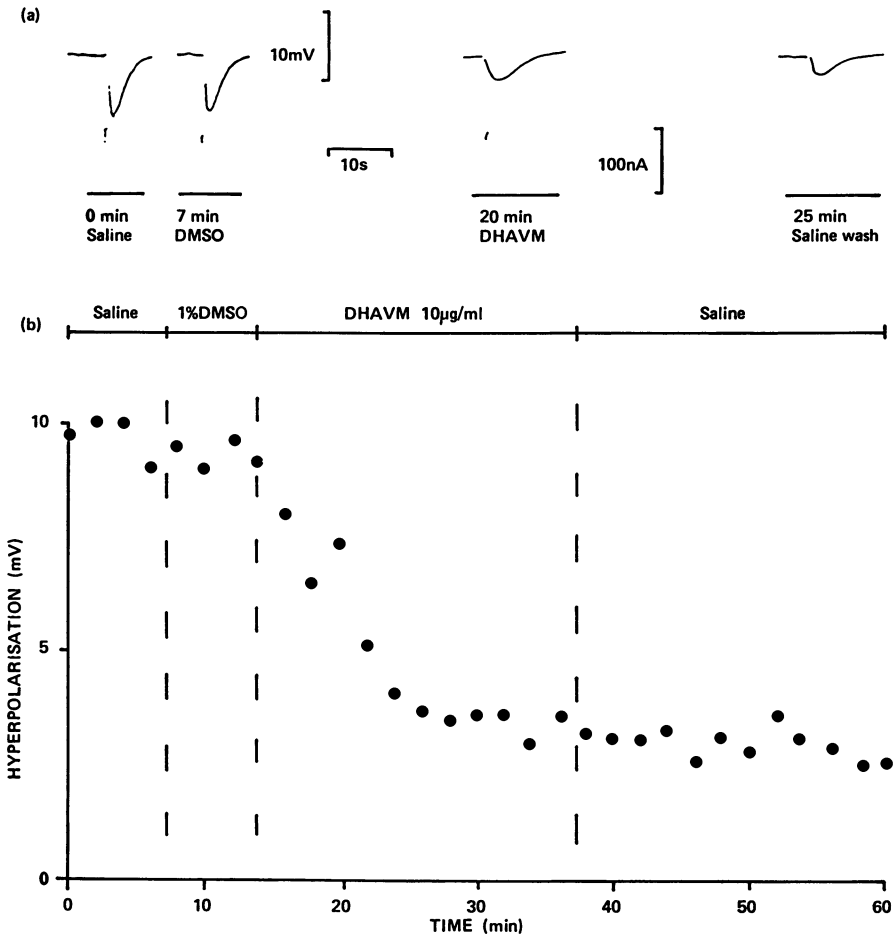
Figure 7. Effect of prolonged (25 min) bath application of
22,23-dihydroavermectin B1a (DHAVM, 10 ug/ml) on the amplitude of
iontophoretically evoked GABA hyperpolarizations. a) Examples of
GABA responses recorded before and after DHAVM application. b) Plot
of GABA response amplitude versus time. DHAVM was first dissolved
in dimethylsulfoxide (DMSO) and then diluted with saline; the final
DMSO concentration in saline of 1% had no detectable effect in
control experiments.
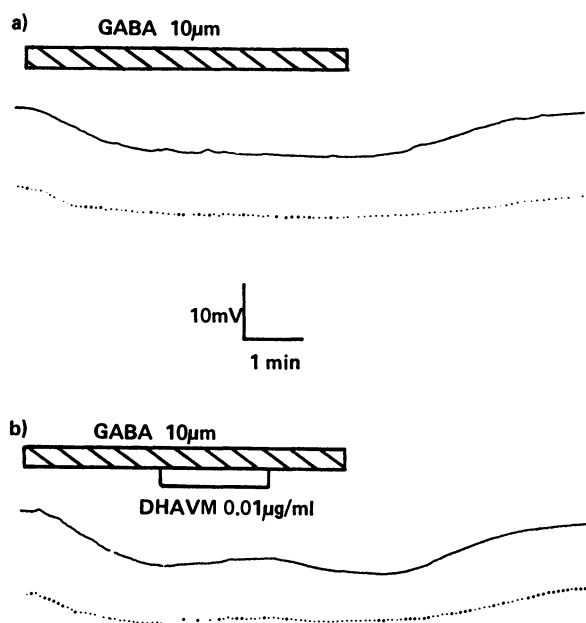
Figure 8. Effect of brief (2 min) application of
22,23-dihydroavermectin B1a (DHAVM, 0.01 ug/ml) on the conductance
increase evoked by GABA. a) Control response evoked by GABA (10
uM), b) co-application of GABA and DHAVM. Both GABA and DHAVM were
applied by microperfusion.
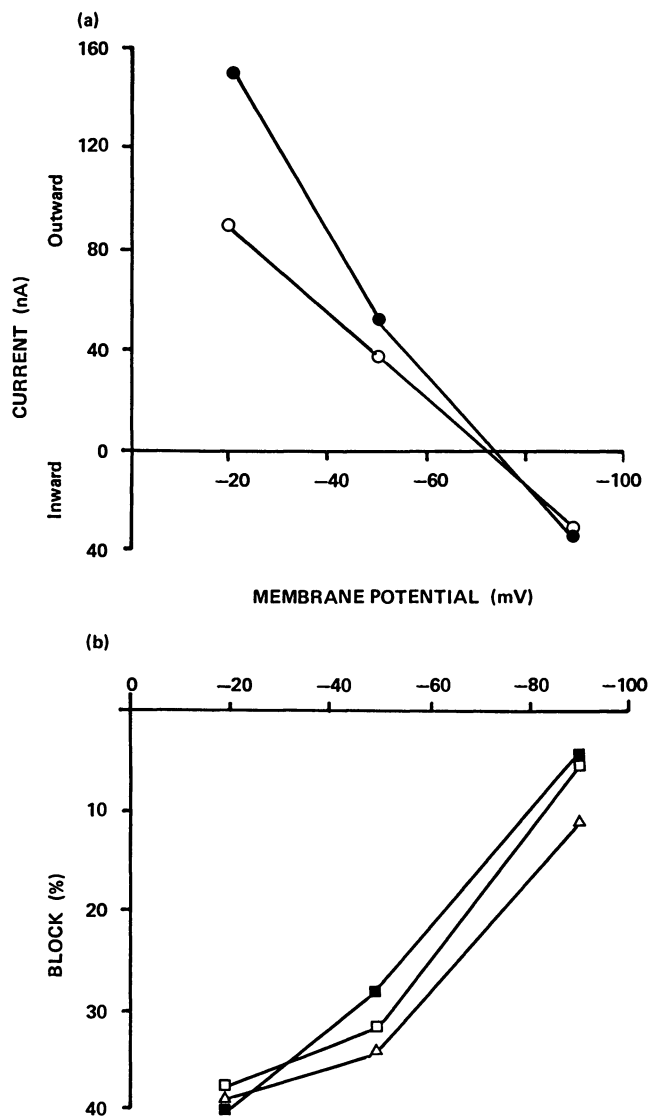
Figure 9. a) Current-voltage relationship for GABA currents recorded in the presence (O) and absence (●) of 0.1 ug/ml 22,23-dihydroavermectin B1a (DHAVM). The DHAVM was applied for 30 min. Both GABA and DHAVM were applied by microperfusion. b) The percentage inhibition of the peak GABA-induced current is plotted as a function of membrane potential for 3 different cells (■,□,△) each exposed to 0.1 ug/ml DHAVM.

stimulation of the ventral inhibitory (VI) motorneurone (1-3). In both cases, exposure of the muscle cells to micromolar doses of AVM produces irreversible abolition of muscle hyperpolarization. These similarities, together with the proposition that GABA is the putative neurotransmitter at inhibitory nerve-muscle synapses in A. suum (32), suggest that the AVM-induced blockade of inhibitory nerve-muscle synaptic transmission might result from antagonism, possibly channel blockade, at the post-synaptic GABA receptor/chloride ion channels.

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Chapter 22

Noncompetitive Antagonism
of Glutamate Receptors

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Recent discoveries of potent non-competitive antagon-
ists of glutamate receptors present on excitable cells
of vertebrates and invertebrates offer opportunities
to the chemical industry for rational development of
novel therapeutics and pesticides. The interactions
of a number of non-competitive antagonists of locust
muscle glutamate receptors with the channels gated by
these membrane proteins are described and discussed
qualitatively and quantitatively in terms of channel
blocking kinetics.

It is generally accepted that acidic amino acids probably function
as transmitters at synaptic sites in the central and peripheral
nervous systems of many animals including man. L-Glutamic acid is
the most studied of these compounds, both neuropharmacologically and
neurochemically, and much current research, both academic and
industrial, is directed towards the discovery of antagonists of this
amino acid. Given the possible importance of L-glutamate in central
nervous function in mammals it is hardly surprising that studies of
its receptors are considered a prerequisite for understanding the
functional properties of the mammalian central nervous system (CNS)
and for the rational development of therapeutic compounds (1).
    Pharmacological studies of mammalian (and other vertebrate)
glutamate receptors using electrophysiological techniques have led
to the currently-held view that they exist in the CNS as three
distinct populations based upon the most selective agonists, N-
methyl-D-aspartate (NMDA), L-quisqualate and L-kainate (2-3). An
additional class of glutamate receptors described by some authors is
highly sensitive to L-amino-4-phosphonobutyrate (L-APB) (4). The
results of biochemical studies of CNS glutamate receptors lend some
support to this overall classification, although there are signifi-
cant differences between the electrophysiological and biochemical
data (1). In a recent publication Fagg et al. (1) have focused
attention on possible methodological discrepancies between elec-
trophysiological and biochemical (ligand binding) studies to deter-
mine whether these might account for the differences between the two

sets of data. Although unsuccessful in this respect they have obtained evidence for Cl^-- and Ca^{2+} dependent glutamate binding sites in murine brain membrane fractions closely resembling those of the L-APB and NMDA receptor populations identified using electrophysiological techniques (1). Unfortunately both biochemical and electrophysiological studies of CNS glutamate receptors are handicapped by the lack of a wide range of radiolabelled ligands, particularly of specific antagonists with low unbinding rates.

The physiological functions ascribed to each of the receptor populations are not entirely clear as yet and there is some uncertainty about which endogenous ligand interacts with each population. L-glutamate seems to have high affinity for all three (or four) receptor populations, while L-aspartate and quinolate (pyridine 2,3-dicarboxylic acid) act preferentially on the NMDA receptor population. Somewhat surprisingly, single channel studies show that putative NMDA receptors in mouse CNS are blocked in their open channel conformation by Mg^{2+} at concentrations similar to those found in vivo (5). The negative slope conductance caused by this voltage-dependent block may confer regenerative properties to cells with MNDA receptors. Depolarization of such cells, for example, through the activation by L-glutamate of quisqualate-type postjunctional receptors, would reduce open channel block of the MNDA receptors.

The search for antagonists of mammalian CNS glutamate receptors has mainly involved structural modifications of glutamate and aspartate molecules (6-7). Most of these compounds act non-specifically on two or more of the glutamate receptor populations. However, highly selective antagonists of the NMDA receptor have been discovered recently using this approach (6-7). These are the ω-phosphono-α-amino-carboxylic acids, 2-amino-5-phosphonovaleric acid and 2-amino-7-phosphonoheptanoic acid. It remains to be established unequivocally whether these are competitive antagonists. The regenerative nature of MNDA responses, which some authorities consider is likely to account in part for excitotoxicity in the vertebrate CNS, may be a central factor linking certain disorders such as epileptic seizure activity, ischemic damage, neurodegenerative disease and neurodepressive phenomena. The NMDA receptor antagonists either prevent or suppress epileptic activity in many animal seizure models and exhibit strong anti-convulsant properties when injected intracerebrally (8). According to Schwarcz et al. (9) they block ibotenate and NMDA responses in rat hippocampus but not those to kainate. This has lead to the claim that antagonism of NMDA receptors in mammalian CNS may protect against ischemic damage to the brain (10) and to suggestions that NMDA antagonists might be used for treatment of specific neurodepressive disorders such as Huntington's disease (8). Implicit in these proposals is the philosophy of rational drug design based upon a knowledge of mammalian neurobiology.

Despite the fact that excitable tissues are the targets for most pesticides and that the nervous systems of pest insects share many basic properties in common with those of vertebrate animals a philosophy of rational insecticide design has not, hitherto , been widely welcomed in the pesticide industry, although recent signs suggest that it may now be gaining ground. The reasons for this reluctance are well understood within this industry, as is the

growing awareness of the possible virtues of a rational approach to
pesticide discovery to set alongside more traditional approaches.
My reasons for drawing attention to current developments in glutamate
receptor research on central nervous systems of vertebrates in
general and those of mammals in particular are twofold: first, to
demonstrate the value of the rational approach, even when it is
applied to biological systems which have raised considerable
technical and conceptual difficulties for electrophysiologists and
biochemists alike; second, to identify those properties of
vertebrate central nervous system function of which the pesticide
biologist should be aware when attempting rationally to develop
compounds targeted at glutamate receptors of pest invertebrates.

Amino Acid Synapses In Insects

Transmission at most, if not all, excitatory nerve-muscle junctions
on insect skeletal muscle and perhaps at excitatory junctions on
visceral muscles in these animals is glutamatergic. The discovery
of the involvement of amino acids in nerve-muscle transmission in
insects was made by Usherwood & Grundfest (11-12) in the early 1960s
when they showed that γ-aminobutyric acid was the transmitter at
inhibitory nerve-muscle junctions on locust leg muscle (metathoracic
extensor tibiae). This was soon confirmed by Kerkut et al. (13) who
studied the inhibitory influence of this amino acid on transmission
at cockroach nerve-muscle junctions. It was at this time that
Usherwood & Grundfest (12) and Kerkut et al. (13) presented the
first evidence that glutamic acid might be the excitatory counter-
part of γ-aminobutyric acid in insect nerve-muscle systems. Since
that time a considerable body of electrophysiological information
has been accumulated which supports this contention. The most
compelling pieces of evidence for L-glutamate being the excitatory
neuromuscular transmitter in insects have derived from: the demonst-
ration of depolarizations to this amino acid at excitatory, but not
inhibitory nerve-muscle junctions (14-15); the demonstration of
glutamate supersensitivity following denervation of locust muscle
(16); the demonstration of extrajunctional receptors for L-glutamate
on innervated locust muscle fibres (17-18); the demonstration,
through noise analysis, of the equivalence of the unit conductance
of noise generated by exogenous L-glutamate with that generated by
the excitatory transmitter at locust excitatory nerve-muscle
junctions (19); and the recording of single channels from locust
muscle fibres with pipettes containing L-glutamic acid and the
qualitative correspondence of the properties of these channels to
those observed at excitatory nerve-muscle junctions using noise
analysis (20). The physiological and pharmacological properties of
both junctional and extrajunctional receptors on locust muscle are
now quite well established and much progress has already been made
in understanding the gating mechanisms underlying channel opening
and closing transitions which occur during the interaction of
L-glutamate and agonists with binding sites on the glutamate
receptor protein (21-25). (See Piek (26) for an excellent summary of
insect nerve-muscle pharmacology.) Despite this progress and the
effort that has been made to study insect muscle glutamate receptors
the discovery of potent competitive antagonists of insect glutamate
receptors has eluded us.

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This failure to discover potent competitive antagonists of insect glutamate receptors perhaps accounts in part for the reluctance of much of the pesticide industry, hitherto, to consider the excitatory nerve-muscle junctions of insects as suitable target sites for insect pest control. The major objective of this review is to identify an alternative approach involving non-competitive rather than competitive antagonists of glutamate receptors as leads for pesticide discovery. In this context compounds which interact with transmitter receptors to block the channels that they gate make a useful starting point to such an approach.

For the rest of this chapter I will summarize work undertaken mainly in my laboratory on blockers of the glutamate receptor channel of locust leg muscle and give some indication of the variety of mechanisms which may contribute to the general phenomenon of channel block. However, before embarking on this exercise it is necessary to give a brief account of our current knowledge of the properties, including the kinetics of gating, of the glutamate receptor channel which have been recently derived from single channel studies of glutamate D-receptors in locust extrajunctional muscle membrane.

Glutamate Receptor Channels of Locust Leg Muscle

Like most other membrane channels the glutamate receptor channel of locust muscle can be described biophysically in terms of its gating properties, ion selectivity and conductance. The gating properties have been thoroughly studied using single channel analysis and will be summarized briefly below. The ion selectivity is a function of the highest energy barrier that ions must cross to pass through the channel (27). Ion substitution experiments undertaken using the patch clamp, single channel approach have indicated that the glutamate receptor channel is highly permeable to the monovalent cations Li^+, Na^+, K^+, Rb^+, Cs^+ and NH_4^+, slightly permeable to Ca^{2+} and Mg^{2+}, and impermeable or at best poorly permeable to tetraethylammonium, guanidinium and choline (28). Since both excitatory postjunctional receptors and extrajunctional glutamate D-receptors on locust leg muscle have reversal potentials close to 0mV it is reasonable to assume that during transmission at the glutamatergic synapses on this muscle Na^+ and K^+ are the permeant ions, with Ca^{2+} also possibly making a minor contribution to the postsynaptic current (29-31). However, at high concentrations (10mM) Ca^{2+} blocks the open channel (32-33). The conductance of the glutamate receptor channel which is normally ca. 150pS depends upon the channel filter, which determines ion selectivity and probably resides closer to its inner orifice than to its outer orifice, and how tightly ions bind to sites within the open channel (34).

Most membrane channels can be blocked when they are open by drugs of diverse chemical structure but usually possessing an appropriate charge. This has led many investigators to conclude that these drugs interact strongly with sites within the channel possibly close to the channel filter. As a result they dwell within the open channel for sufficient time to block the movement through it of those permeant ions which normally carry the channel current. However, this explanation is probably an oversimplification, since just as there is a variety of compounds (some of which are not

charge carriers) which can block membrane channels there is also a
variety of ways in which this block may come about. Possible
schemes for channel block involve both open and closed channel
states and include hydrophobic compounds which may influence the
channel after entering the membrane lipid. According to Ketterer et
al. (35) such hydrophobic compounds would concentrate near the inner
and outer faces of the membrane, so that one might anticipate their
receptor binding sites to be distal to the channel gate (36).
    The pharmacology of channel block is perhaps best understood in
terms of the nicotinic acetylcholine receptor. It might be assumed
that because of the diverse structure of compounds which interact
with this channel, binding to the channel blocking site(s) is
relatively non-specific compared to the binding of acetylcholine to
the acetylcholine recognition site on the receptor. However, there
may be many binding sites within the channel, each of which may
possibly exhibit a high pharmacological specificity.

Glutamate Receptor Channel Kinetics. From records of single channel
data recorded from locust extrajunctional membrane using patch
pipettes filled with saline containing different concentrations
(10^{-6}–10^{-2}M) of L-glutamate it is possible, under carefully control-
led conditions, to obtain a major insight into the gating kinetics
of the glutamate receptor channel. For example, from the analysis
of open and closed time distributions it is possible to draw certain
inferences about the complexity of the underlying gating mechanisms.
Such an approach led Kerry et al. (25) to conclude that with 10^{-4}M
L-glutamate in the patch pipette the open channel exhibits a minimum
of 3 kinetic states and the closed channel a minimum of 4 (Table
1). By subjecting the single channel data to auto-correlation
function analysis it was also possible to demonstrate that dwell
times (open and closed) are correlated in such a manner which indi-
cates that there is more than one pathway connecting the open and
closed states of the receptor channel. On the basis of these
results Kerry et al. (25; to be published) proposed a tentative
model for the glutamate receptor channel comprising five open and
five closed states corresponding to different numbers (one to four)
of glutamate receptor molecules bound to the glutamate receptor,
with one open and one closed state unliganded. This model
corresponds with the known oligomeric nature of proteins in general
(37) and is based upon the allosteric model (38) for regulation of
enzyme activity (39). Much more needs to be done to test the
validity of this model, although the results of studies of the
concentration dependencies of the channel kinetics of the glutamate
receptor (40) suggest that it is not unreasonable.
    One useful consequence of investing so much time in studying
the kinetics of this system is that it is now possible to gain some
quantitative insight into the influence of non-competitive antagon-
ists on the kinetics of glutamate receptor channel gating, although
at present, the complexity of the gating kinetics currently imposes
some limitations on the progress that can be made in this direction.

(1) +-Tubocurarine - Open Channel Blocker With Low Unblocking Rate

Although (+)-tubocurarine ((+)-TC) has been classically regarded as
a competitive antagonist of nicotinic cholinergic synapses (41)

recent studies of this compound, including some made on insect nerve-muscle preparations, have indicated two additional properties, open channel block (42-43) and agonist-like channel gating (44). Yamamoto & Washio (45-46) showed that (+)-TC blocks transmission postsynaptically at nerve-muscle junctions on Tenebrio molitor muscle and Cull-Candy & Miledi (47) described possible open channel block of postjunctional glutamate receptors of locust leg muscle by this compound. The proposal of open channel block by (+)-TC has received support from recent work of Kerry et al. (48-49) who have gained insight into the kinetics of the blocking mechanism by studying the effects of (+)-TC on the single glutamate receptor channel of locust extrajunctional muscle membrane. They found that the glutamate concentration-dependent increase in the probability (p_o) of the channel being open (22-23, 50-51), which is normally a property of this receptor protein channel, does not occur in the presence of (+)-TC (5 x 10^{-4}M). (+)-TC causes a (+)-TC concentration-dependent (studied over the (+)-TC concentration range 5 x 10^{-6} to 5 x 10^{-4}M) decrease in mean channel open time and in p_o. Correlations between successive channel openings and closings which are characteristic of the kinetics of the channel in the absence of (+)-TC are weakened when this compound is present in the patch pipette along with L-glutamate. All of these observations are consistent with the notion that (+)-TC blocks the cation-selective channel gated by the glutamate receptor.

A simple sequential model for open channel block of the general type first proposed by Adams (52) provides a good first fit to the (+)-TC data;

$$\text{A} + \text{R}_c \rightleftharpoons \text{AR}_c \rightleftharpoons \text{AR}_o \overset{B}{\rightleftharpoons} \text{AR}_o\text{B} \quad .. \quad .. \quad .. \quad \text{(equ. 1)}$$
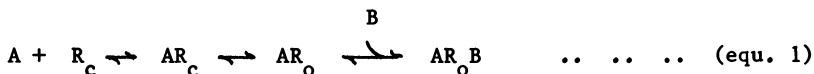
where R and R are the open and closed receptor channel conformers, A is the agonist (e.g. L-glutamate) and B the blocking agent (i.e. (+)-TC). From the relationship between (+)-TC concentration and p_o it is estimated that the dissociation constant for open channel block of locust extrajunctional glutamate receptors is 1.75μM (at a membrane potential (Vm) of -100mV). The single channel data may also be used to estimate the rates of association and dissociation of (+)-TC with the glutamate receptor channel. The rate of association is estimated to be 9.44 x $10^3 \text{ms}^{-1}\text{M}^{-1}$ (Vm -100mV). The rate of dissociation is estimated to be 1.53 x 10^{-2}ms^{-1}, which gives a mean channel block time of 65.4ms.

In these studies of Kerry et al. (48-49) there was no evidence of voltage dependence of block, although one might have anticipated from the net positive charge carried by the (+)-TC molecule that its binding to the glutamate receptor channel would be enhanced with membrane hyperpolarization, since the Adams' (52) open channel block scheme envisages that the blocking molecule enters the cation selective channel of the receptor protein. However, the studies of Kerry et al. (48-49) were restricted to the membrane potential range of -70mV to -100mV which may have been insufficient to disclose any voltage dependence for the action of (+)-TC.

It is important to remember that the scheme for channel block referred to in equ. 1 is undoubtedly an oversimplification. This model treats channel gating by agonist as a pseudo-unimolecular

process, yet recent studies of the gating of the locust muscle glutamate receptor channel by Ashford et al. (50) and Kerry et al. (25) have demonstrated that the gating mechanism for this receptor is more complex than this (Table I). (However, it is assumed that, in principle, (+)-TC can bind to each of the open channel states of the glutamate receptor channel.) The fact that the correlations between successive channel openings and closings are reduced by (+)-TC is consistent with block of a non-linear, multistate channel in its open states by a blocking agent which has a relatively low unblocking rate.

How then do these single channel data on the action of (+)-TC relate to the effects of this compound on the whole nerve-muscle preparation? If (+)-TC is perfused over the locust retractor unguis nerve-muscle preparation (15, 53) at concentrations > 10 ^{5}M there is a reduction in the amplitude of the neurally-evoked twitch of the retractor unguis muscle. The magnitude of this reduction in twitch amplitude is directly proportional to the frequency of stimulation of the muscle (49). These observations are entirely consistent with the notion the (+)-TC is an open channel blocker with a relatively low unblocking rate.

Chlorisondamine - An Open And Closed Channel Blocker With Complex Blocking Kinetics

Shinozaki & Ishida (54) and Lingle et al. (55) have shown that chlorisondamine blocks the open channel gated by postjunctional glutamate receptors of crustacean (crab, lobster and crayfish) muscle in a voltage-dependent fashion. In addition Lingle (56) has suggested that this compound can induce a stable, closed-but-blocked state of the acetylcholine receptor-gated, cation-selective channel of crustacean muscle. This compound also blocks the open channel gated by the glutamate D-receptor of locust leg muscle extra-junctional membrane (57-58). The block of this glutamate receptor channel is voltage sensitive, but somewhat surprisingly the voltage sensitivity is in the opposite sense to that expected of a molecule carrying a net positive charge. Ashford et al. (57-58) also found that chlorisondamine blocks the closed channel of the unliganded (i.e. no glutamate bound) glutamate receptor and that this may be more significant than open channel block in terms of the non-competitive antagonism exerted by chlorisondamine in this system. However, reversal of closed channel block is enhanced in the presence of agonist (e.g. L-glutamate) which suggests that the affinity of chlorisondamine for its binding site on the receptor channel complex is lowered during agonist binding to the glutamate receptor.

When chlorisondamine (>5 x 10^{-5}M) was perfused over the locust isolated retractor unguis nerve-muscle preparation the neurally-evoked twitch contraction of the retractor unguis muscle was reduced in amplitude; with 5 x 10^{-4}M chlorisondamine the twitch was almost abolished. Part of this reduction in twitch amplitude was independent of the recent stimulation history of the retractor unguis muscle, unlike the remainder which was clearly dependent upon the stimulation frequency. Although it is not possible with this type of preparation to gain much insight into the site and mode of action of compounds such as chlorisondamine the results of these

preliminary studies suggested that chlorisondamine may be an antagonist of the glutamate receptor found postjunctionally on the retractor unguis muscle. Support for this notion came from studies of its effects on the response (glutamate potential) of the excitatory postjunctional membrane of locust extensor tibiae muscle to glutamate ionophoresis. In the presence of $\geq$ 2 x 10^{-5}M chlorisondamine the slope of the relationship between ionophoretic dose of glutamate and amplitude of the resultant glutamate potential was reduced, a result which suggests non-competitive antagonism of the glutamate receptor channel complex. However, the ionophoretic studies failed to indicate whether this was at the level of the open or the closed receptor channel. When chlorisondamine was tested on the excitatory post-synaptic current (EPSC) recorded from voltage clamped extensor tibiae muscle fibres it became clear that it was causing both open and closed channel block. A reduction in amplitude of the EPSC and spontaneous miniature EPSC seen when the muscle was exposed to 5 x 10^{-5}M chlorisondamine suggested the occurrence of closed channel block. However, a comcomitant decrease in the EPSC rise time (20-80% peak EPSC amplitude) and the appearance of biphasically-decaying EPSC's and prolonged EPSC decays were indicative of open channel block. Somewhat surprisingly these changes were most marked when the muscle fibre was held at low membrane potentials and became progressively less marked as the membrane potential was increased (hyperpolarization). This is the opposite result to that expected for a positively charged drug blocking a cation-selective receptor channel.

Ashford et al. (57,58) suggested that during hyperpolarization chlorisondamine may be 'forced' through the open channel of the glutamate receptor into the muscle fibre by the increased potential difference across the muscle fibre membrane. This could account for the return of the amplitude and time course of the synaptic current towards values obtained in the absence of drug. Hyperpolarization could also conceivably enhance the conversion of the closed-but-blocked channel to the open-but-blocked state for which the chlorisondamine molecule seemingly has lower affinity ;

$$A \;+\; R_c \;\longrightarrow\; AR_c \;\longleftarrow\; AR_o$$
$$\left\downarrow B \qquad\qquad \downarrow \qquad\qquad \downarrow (v) \; B \quad .. \quad .. \quad .. \quad .. \quad (2)\right.$$
$$A \;+\; R_c B \;\longrightarrow\; AR_c B \;\xleftarrow{(V)}\; AR_o B$$
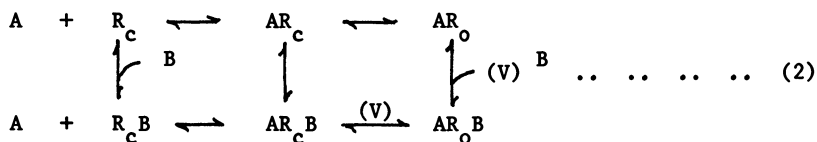
Adams (36) proposed a somewhat similar model to account for the complex block of the acetylcholine receptor channel at frog motor end-plate by procaine.

The conclusion, drawn from studies of the glutamate potential and EPSC of locust muscle that chlorisondamine blocks both the open and closed channel of the postjunctional glutamate receptor received substantial support when the action of the drug on the single extrajunctional glutamate D-receptor channel was investigated. With patch pipettes containing 5 x 10^{-5}M chlorisondamine in addition to 10^{-4}M L-glutamate channel openings appeared as bursts of short pulses, suggestive of open channel block with high blocking and unblocking rates (58). Table I shows that there are major differences in the channel dwell time (open and closed)

distributions when recordings with 10^{-4}M L-glutamate alone are
compared to those obtained with patch pipettes containing glutamate
plus chlorisondamine. With chlorisondamine plus glutamate in the
patch pipette the open time distribution contained 2 rather than 3
components, due to the loss of long openings and was also charac-
terized by a high proportion of brief openings compared with the
control distribution (i.e. L-glutamate alone) (Table 1).   50μM
chlorisondamine also reduced p_o, from about 6% to less than 1%, a
change which probably cannot be accounted for in terms of open
channel block with rapid blocking and unblocking kinetics (Equ. 1).

Table I. Rate constants (ms^{-1}) for components of open and closed time distri-
          butions of single channel data recorded from extrajunctional glutamate
          D-receptors of locust muscle with patch pipette containing 10^{-4}M
          L-glutamate alone (control) or with L-glutamate plus channel blocker

Control		2 x 10^{-4}M (+)-Tubocurarine		5 x 10^{-5}M Chlorisondamine		10^{-10}M Argiotoxin (636)	
Open States							
2.53	(44)	10.81	(5)	5.33	(79)	4.27	(27)
0.689	(52)	2.42	(90)	2.62	(21)	0.538	(58)
0.294	(4)	0.92	(5)	-		0.134	(15)
Closed states							
2.48	(22)	2.20	(10)	1.06	(57)	2.33	(20)
0.135	(37)	0.169	(17)	0.032	(31)	0.189	(46)
0.0499	(35)	0.0202	(56)	0.01	(32)	0.087	(31)
0.0106	(6)	0.0053	(17)			< 10^{-5}	

The rate constants were derived from multi-exponential fits to open and closed
time probability density functions (25). The number of open and closed states
represents the number of exponential components required to fit each of the
probability density functions, e.g. fitting the open and closed time distribu-
tions for the control data (10^{-4}M L-glutamate alone) required 3 and 4 components
respectively.   Numbers in brackets are percentages of channel populations
occupying given open or closed states.

According to the sequential blocking model (Equ. 1) of Adams (52)
direct transition from the blocked-but-open channel to the closed
channel is not allowed. As a result one would predict that the sum
of all the short open periods observed in an open burst (see above)
during chlorisondamine application would, on the average, be equal
to the mean open time of the unblocked channel (25). It follows,
therefore, that with low channel opening rates, such as those seen
with 10^{-4}M L-glutamate (i.e. ca. $50s^{-1}$), p_o should not be markedly
affected by the presence of chlorisondamine in the patch pipette,
yet it falls dramatically. It seems likely, therefore, that this
drug blocks the closed as well as the open glutamate receptor

channel, a conclusion which is in accord with that derived from the macrosystem studies referred to above. However, without further experiments one cannot completely exclude some contribution from competitive antagonism by chlorisondamine at the glutamate binding site(s) on the receptor.

If each of the bursts of channel openings seen with chlorisondamine represents a single channel opening interrupted by high-frequency blocking (and unblocking) transients, such as might be expected from open channel block with rapid blocking and unblocking kinetics, it follows that an increase in the frequency of blocking events might be expected to follow both an increase in drug concentration and hyperpolarization of the locust muscle fibre because of the higher blocking rate expected under these conditions. However, changes in drug concentration would not be expected to influence the open channel unblocking rate, although this parameter might be expected to decrease with hyperpolarization (36). Nevertheless, when the chlorisondamine concentration was increased, and particularly at relatively high membrane potentials, the conductance (normally ca.150pS) of the open glutamate receptor channel apparently was lowered. One explanation is that channel block (and unblock) under these conditions was so rapid that the conductance of the open, unblocked channel could no longer be fully resolved given the limited frequency response characteristics of the recording apparatus. It seems likely that both the blocking and unblocking rates are increased. Further single channel studies of the effects of chlorisondamine on the glutamate receptor channel are necessary to clarify some of the issues raised by these preliminary investigations. The introduction of giga-ohm seal recording techniques for studying locust muscle glutamate receptors will considerably facilitate such studies.

Argiotoxin (636) – Open Channel Blocker With Very Low Unblocking Rate

Venoms for spiders of the Araneid family (the orb-web weavers) have recently been shown to contain highly potent blockers of the post-junctional glutamate receptor channel of locust muscle (59-62) and to interact with glutamate receptors found on a variety of crustacean and vertebrate excitable cells (63-68). It remains to be established unequivocally whether these toxins bind specifically to glutamate receptor channels, although the literature on open channel blockers suggests that this is unlikely to be the case. Recent studies in my laboratory have concentrated on the effects of argiotoxin (636) on the extrajunctional glutamate D-receptor channel of locust leg muscle. Argiotoxin (636) is a highly modified peptide of low molecular weight (636 daltons) (62). In single channel studies with this toxin it has been applied either via the patch pipette or via the bathing medium of the nerve-muscle preparation.

Channel openings were never seen when patch pipettes containing 10^{-4}M L-glutamate and concentrations of argiotoxin (636) of 10^{-9}M or greater were used. In the absence of toxin the glutamate receptor channel would be expected to have an open channel probability of about 5% for 10^{-4}M L-glutamate (22-23, 25). However, channels of duration < 100μs were not readily resolved, so one cannot assume from this observation that no channel openings occurred. If

argiotoxin (636) is an open channel blocker, then its blocking rate at 10^{-9}M may be sufficiently high to limit channel open events to < 100μs duration. An alternative explanation for the apparent absence of channels with 10^{-9}M argiotoxin (636) is that this toxin exhibits properties in addition to that of open channel block. A few brief openings were seen with 5 x 10^{-10}M argiotoxin (636) during recording periods extending over many minutes but it was necessary to lower the toxin concentration to about 10^{-10}M before the frequency of channel openings was > 0.015. Analysis of channel dwell time distributions for 10^{-10}M argiotoxin (636) indicated the presence of longer duration openings than for control distributions (Table I) which suggests that the toxin may increase the affinity of the glutamate receptor for L-glutamate in addition to blocking the channel gated by this receptor. However, there are other equally plausible explanations for the appearance of these long channel open times. For example, the kinetics of the glutamate receptor channel are complex (25) and may have led coincidentally to the results described in Table 1, which were from a single experiment. More extensive studies are in progress to determine whether the mean open time of the channel is increased by argiotoxin (636). The closed time distribution obtained by measuring closed times present within the bursts of channel openings was not greatly different from the overall control distribution of channel closed times. However, distributions of all closed times were extended in the direction of long closings by the prolonged interburst intervals which characterized the channel activity when argiotoxin (636) was present in the patch pipette. There was no evidence in either the raw data or the dwell time distributions for brief closing transients during channel openings, which one would have expected if argiotoxin (636) were rapidly blocking and unblocking the open channel. Compared with (+)-TC, argiotoxin (636) apparently has a very low unblocking rate since the mean channel block time was about 13s compared with about 65ms for (+)-TC. To check whether some of the changes in channel kinetics caused by argiotoxin (636) were due to competitive antagonism or to closed channel block or to both of these phenomena the concentration of L-glutamate in the patch pipette was raised to 10^{-3}M (channel open probability normally about 70% (22-23, 40)) whilst keeping the concentration of argiotoxin (636) constant at 10^{-10}M. This apparently completely eliminated channel openings (although very brief openings may well have been missed), a result which is not in accord with closed channel block or competitive antagonism.

But can this result be explained solely on the basis of open channel block? Undoubtedly the high p_o at 10^{-3}M L-glutamate must considerably enhance the probability of open channel block and given the low unblocking rate of argiotoxin (636), this would reduce the frequency of channel openings. However, an alternative explanation for the effects are argiotoxin (636) on the glutamate D-receptor channel of locust leg muscle is that it induces densitization or refractoriness of the receptor channel complex. Although this is an unlikely explanation since the single channel studies were undertaken after pretreatment of the muscle with concanavalin A, which blocks desensitization in this system, it will receive further consideration as our patch clamp experiments with argiotoxin (636) progress.

When argiotoxin (636) was applied in the medium bathing the locust nerve-muscle preparation quantitatively different results were obtained. In these experiments the patch pipette contained 10^{-3}M L-glutamate, but no toxin. It was necessary to reduce the toxin concentration to 10^{-11}M before any channels were seen under these conditions, even then the channel openings did not persist. With prolonged (e.g. 60 min) bath application it was still possible to demonstrate an effect of the toxin on channel kinetics with concentrations of argiotoxin (636) as low as 10^{-14}M. These results raise some interesting questions. For example, how does the toxin reach a glutamate receptor channel which is effectively isolated from the bathing medium by the tip of a patch pipette, and what is the basis for the time-dependent effect on channel kinetics seen with low concentrations of bath-applied argiotoxin (636)? One obvious explanation for both of these observations is that the toxin can block the glutamate receptor channel via the membrane lipid phase. Studies currently in progress in my laboratory are intended to test the validity of this proposal.

The data obtained so far suggest that argiotoxin (636) has no effect on channel conductance.

δ-Philanthotoxin - Open Channel Blocker with Complex Unblocking Kinetics

One component (δ-philanthotoxin) of venom obtained from the wasp Philanthus triangulum blocks transmission postsynaptically at glutamatergic synapses on locust muscle (69-73). This toxin depresses both the post-junctional current which follows stimulation of an excitatory motoneurone and the postjunctional responses to glutamate ionophoresis in a manner suggestive of open channel block. Studies of the effects of δ-philanthotoxin on the kinetics of channel gating of the extrajunctional glutamate D-receptor have disclosed major modifications of the channel open-closed kinetics in this system. The mean channel closed time was greatly increased when δ-philanthotoxin was included in the patch pipette along with L-glutamate, with channel openings occurring in bursts separated by long closed intervals. The mean channel closed times within the bursts of channel openings were not significantly different from control mean closed times recorded in the absence of toxin. These observations, and the fact that the frequency of channel openings was reduced by the toxin, could be accounted for by either competitive or non-competitive (closed or open channel block) antagonism of the glutamate receptor channel. However, the mean channel open time was also reduced by the toxin, a result which clearly indicates an influence on the open channel state. Unfortunately, the limited resolution of the recording system employed in these early single channel studies and the very limited availability of δ-philanthotoxin precluded investigations of the effects of membrane potential on the antagonism of the glutamate receptor channel by this toxin. However, the shortening of the decay of the excitatory postsynaptic current (EPSC), which was observed when the locust nerve-muscle preparation was bathed in saline containing δ-philanthotoxin, was not influenced by muscle membrane potential. It seems reasonable to assume, therefore, that the open channel block of this toxin is not voltage-dependent. δ-Philanthotoxin had

no effect on the open channel conductance. Although the single
channel data obtained in these studies were filtered at 1kHz, which
reduced the resolution of brief channel openings and closings, it
was possible to estimate the channel unblocking rate by comparing
the closed time distributions obtained in the presence and absence
of toxin. An estimate for the unblocking rate of $1.0 - 1.5s^{-1}$ was
obtained in this manner. Since the molecular weight of δ-philantho-
toxin was not known and highly pure toxin was not available at the
time these single channel studies were undertaken, it was not
possible from the single channel data to estimate the blocking rate
for this compound.

    There are close similarities between argiotoxin (636) and
δ-philanthotoxin in terms of their interactions with the glutamate
receptors (junctional and extrajunctional D-receptors) of locust
muscle. Both appear to be potent open channel blockers with very
low unblocking rates and both seem to enter a compartment of the
muscle (probably the membrane lipid) from which they discharge only
slowly during washout. (It remains to be established whether these
compounds possess chemical properties in common which account for
their similar channel blocking properties.) However, studies of the
effect of δ-philanthotoxin on the glutamate potential arising from
ionophoresis of L-glutamate to a superficial nerve-muscle junction
on locust extensor tibiae muscle suggest that recovery from channel
block by this toxin is more complex than recovery from channel block
by argiotoxin (636). The presence of agonist increases the rate of
recovery from the former but reduces the rate of recovery from the
latter. Clark et al. (73) showed that recovery of the amplitude of
the glutamate potential is rapidly reversible, provided that L-glu-
tamate is applied throughout the period of δ-philanthotoxin washout
(the toxin having been applied to the nerve-muscle preparation in
the superfusing saline). However, recovery is significantly slowed
if the application of L-glutamate during washout is interrupted. In
other words the open channel block of locust muscle glutamate
receptors by δ-philanthotoxin is more rapidly reversed when agonist
binds to the glutamate receptor. This behaviour is qualitatively
similar to chlorisondamine interaction with acetylcholine receptor
channels of the spiny lobster gastric mill muscle (56). For this
system Lingle (56) proposed a sequential scheme in which, following
the binding of chlorisondamine to the open ion channel, the channel
may undergo a transition to a closed-but-blocked state that requires
reactivation by agonist to become unblocked. The following scheme
describes qualitatively how this is envisaged for δ-philanthotoxin:

$$A + R_c \rightleftharpoons AR_c \rightleftharpoons AR_o \overset{B}{\rightleftharpoons} AR_oB \quad .. \quad .. \quad .. \quad ... \quad (3)$$

$$\updownarrow$$

$$R_c B$$

$$+$$

$$A$$

Where $R_c B$ is the closed-but-blocked receptor channel complex. If
the interaction of A with $R_c B$ is rapid compared with unblocking of

the open channel then it is unlikely that Clark <u>et al</u>. (<u>73</u>) would have observed the R$_c$B state in their single channel studies.

Conclusions

This review has summarized much of the recent work on non-competitive antagonism of locust muscle glutamate receptors and has concentrated on single channel studies. It illustrates the variety of compounds which interact non-competitively with the glutamate receptor channel and highlights the different ways in which these compounds exert their antagonistic actions. Undoubtedly there are many other compounds which are channel blockers of the glutamate receptor. Indeed some of these, such as the aminoglycoside antibiotics (e.g. streptomycin) (<u>21, 26</u>) and esters of tryptophan (Usherwood, unpublished data) have already been tested on the locust nerve-muscle system. Those compounds with high affinity for binding sites on the glutamate receptor channel protein clearly deserve most attention initially if one is seeking leads for the chemical industry. The toxins of certain spiders and other venomous arthropods would seem to offer excellent opportunities in this respect.

In writing this review I had in mind the need to demonstrate how the rational approach to pesticide discovery could be facilitated by recent discoveries in basic research. Although I am unable at this time to offer chemical structures for industrial exploitation I am confident that there is much to be gained commercially by considering non-competitive antagonists of glutamate receptors as lead structures.

Acknowledgments

Addendum

Since this manuscript was prepared Volkova <u>et al</u>. (*) have presented a structure for a 636 molecular weight toxin isolated from the venom of <u>Argiope lobata</u>. Also Aramaki <u>et al</u>. (**) have presented somewhat similar structures for low molecular weight toxins present in the venoms of <u>Nephila clavata</u> and <u>Nephila maculata</u>. These compounds share in common the presence of amino acids, a phenyl group (<u>62</u>) and a polyamine.

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Chapter 23

Panel Discussion

Role of Biochemical, Physiological, and Neurochemical Research in the Insecticide Discovery Process

Chairman: A. E. Lund[1]
Panel members: D. W. Gammon[2], S. M. Sieburth[2], M. A. Brown[3], M. E. Schroeder[4], K. R. Jennings[5]

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A. E. Lund: I want to provide a context for our discussion on the role of biochemical, physiological, and neurochemical research in this process. Figure 1 shows a schematic view of insecticide discovery that highlights some key steps taken by most agricultural chemical companies. Probably no company fully integrates all the functions shown here, and many undoubtedly have chosen not to develop one or more steps in the process as I have drawn it.

Traditionally, the discovery process begins when a chemist synthesizes a new compound. The chemist may choose to make compounds based on an understanding of vertebrate pharmacology, biologically active natural products, or proven insecticidal chemistry; or he may choose to make completely novel structures for which no biological information is available. In any case the compounds are evaluated for toxic effects in insect species which usually represent desirable insecticide markets. The objective of this initial testing is to eliminate inactive compounds from further tests, and to roughly define the species spectrum of activity. Active compounds continue to higher level testing where attributes deemed important to successful product marketing are measured and compared with standards. The most attractive compounds are tested in initial field trials from which product development candidates are selected -- again based on a knowledge of the relevant markets.

This sequence between chemical synthesis and product development is, and will surely continue to be, the heart of the insecticide discovery process. The compelling reason for this is the simple fact that the experimental proof that a new compound possesses those attributes which will provide the required value to some customer can only be obtained from tests for those attributes on target insect species under realistic conditions. It seems unlikely to me that biochemical or physiological research will have a significant impact on this part of the product discovery process in the foreseeable future. A more plausible role for this research
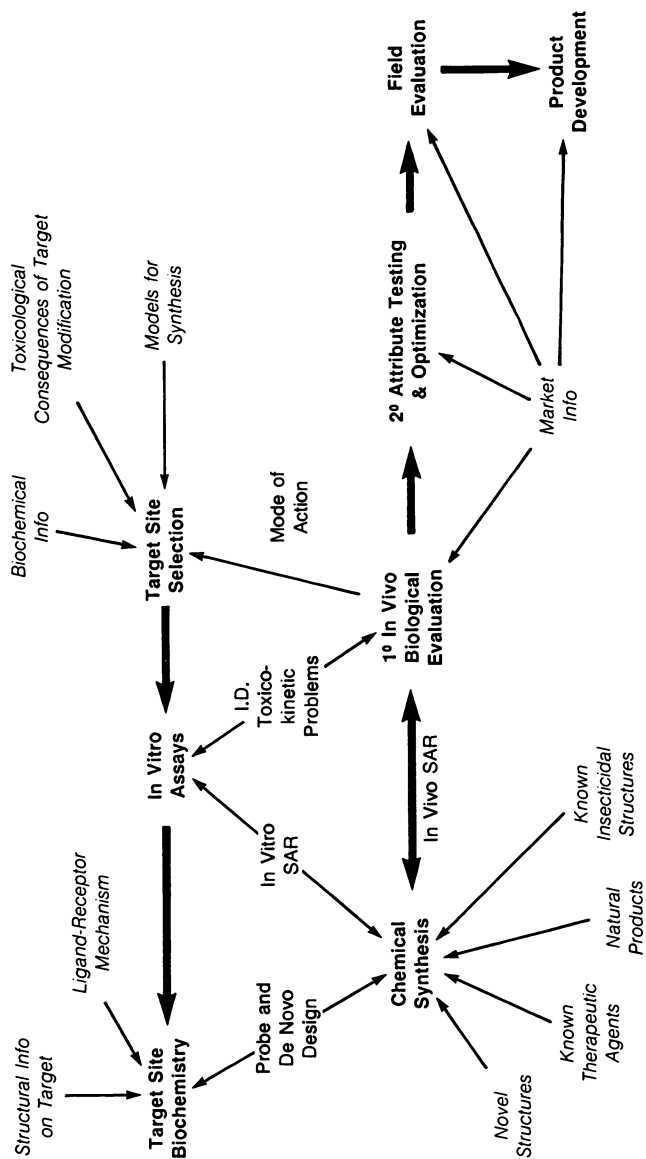
Figure 1. An insecticide discovery process.

seems to be the facilitation of the discovery of new early leads
through some knowledge of biochemical target sites within the pest
insect.

Target site research logically begins with the selection of an
appropriate target site. A likely source of clues to new target
sites comes from studying the mode of action of an insecticidal
compound discovered in the in vivo evaluations, but targets can also
be selected based on a thorough knowledge of physiology. There are
many factors which one might consider in the selection of a target,
but I have included a few that I think are particularly important.
Some knowledge of the toxicological consequences of target site
modification is crucial. If inhibiting a particular receptor or
enzyme by 5% results in a desirable toxic effect, that target is
more sensitive to manipulation than one that requires 95% inhibition
before in vivo activity is observed. At least some rudimentary
information on the biochemistry of the target site is helpful, since
the development of efficient binding and functional assay systems is
certainly facilitated by background information. The availability
of chemical models provides the clues on which to base initial
synthesis. Thus known toxins or drugs represent starting points for
the chemist and tools for the testing of new assays by the
biologist.

Once a biochemical target has been selected, in vitro assays
can be developed which measure both the binding of ligands and the
functional integrity of the target. These assays provide activity
information at the target site without the complicating influences
of penetration, metabolism, and excretion. Of course, comparing the
in vitro and in vivo results is the first step in identifying and
solving these toxicokinetic problems. The in vitro assays also
provide the foundation for the study of target site biochemistry. I
think the key issue here is the need to collect biochemical
information about the target site that is useful to the synthesis
chemist. For example, the molecular weight, number of subunits, or
even amino acid sequence of a receptor is not particularly helpful,
while the location and identity of functional groups adjacent to a
particular toxin binding site and the nature of the chemical
interaction between the ligand and receptor which results in
receptor activation give clues to the design of potential
insecticides. Thus the interaction between the chemist and the
target site biochemist tends to focus on the design of various
chemical probes to gain this information.

I will conclude this introduction by stating the obvious. It
seems clear to me that biochemical, physiological, and neurochemical
research has much to contribute to the product discovery process,
but the success of this approach is crucially dependent on the close
interdisciplinary interactions that I have indicated in the figure.

T. Narahashi: Could you give us some examples of insecticide
development based on this scheme?
M. E. Schroeder: I don't think there are any commercial
insecticides whose discovery or development was significantly
impacted by in vitro testing. All that I know about were discovered
through the traditional screening process. However, as you know, it
is becoming more and more difficult to discover new classes of
insecticides. So I think that in vitro testing and input from
biochemists that provide models for synthesis based on biochemical

and physiological information will play an increasingly important role in the product discovery process in the future.

D. W. Gammon: One example where in vitro testing has had an impact is in the case of the bicycloorthocarboxylate esters which were designed to attack GABA receptors based on the known mode of action of the bicyclophosphates. An in vitro assay was used to optimize the potency of these compounds at a receptor site in a process that was parallel to the in vivo testing.

M. A. Brown: Even in this case, however, the original observation was the in vivo toxicity of these compounds. In this sense, the bicycloorthocarboxylates are more of an example of in vitro optimization of an existing in vivo lead.

R. M. Hollingworth: If you want an example, it is difficult to pick one from the agricultural area, but it is my understanding that one of the most important recent drugs in the U.S., Tagamet, was discovered through a receptor screening process. Also, another major recent drug, the antihypertensive, captopril, was designed specifically to inhibit the angiotensin converting enzyme based on a knowledge of the substrate specificity and mechanism of this enzyme. So we do not have to look too far afield to find commercial success stories that came through an in vitro approach.

M. E. Eldefrawi: Did we hear in this symposium about a possible product? I am referring to Dr. Usherwood's work on toxins from spider venoms. Does he think that this might be an example of a possible product starting from in vitro assays?

P.N.R. Usherwood: It is possible that a product may arise from this research, but I think it is a long way off yet. However, we are in danger of missing the point about the role of in vitro assays in pesticide research and discovery. One talks of random screening having been the way forward in the past, and that there is no evidence, past or present, that in vitro screening has any real value in the discovery of insecticides. However, even with so-called random screening rational decisions are made about which compounds should be tested. Biologists working with in vitro preparations are now injecting additional ideas into such screening programmes. Hopefully, in due course, this added input will make its present felt. I don't see this as a different process or as a new way of proceeding towards product discovery. I see it simply as a contemporary development of traditional procedures.

R. M. Hollingworth: I think we spend too much time trying to justify the top line of what you have in Figure 1. I think it should be self-evident that this is another level or type of knowledge that feeds into the product discovery process. It's obvious that we must do this. I would like to see us talk more about how we can best integrate and use this approach to enhance insecticide discovery.

K. R. Jennings: One type of contribution can be to aid in the pre-selection of chemistry. We have recently introduced the amidinohydrazones which were developed by American Cyanamid for

cockroach and fire ant control. The original lead came from a drug
synthesis program. Frequently we see that compounds made for
another program show activity against insects; so a knowledge of
other areas such as vertebrate pharmacology can positively impact
product discovery.

F. Matsumura: There are certainly other such examples. One that
comes to mind is the carbamate insecticides which came originally
from a natural product model. Although I think it is true that
every 10 or 20 years someone will discover a completely new
structure with a new mode of action such as Merck has done with the
avermectins, there may come a time when we run out of really good
models for synthesis. Then we may want to come back to some of the
older but proven targets such as GABA receptors for the design of
new insecticides.

M. E. Eldefrawi: I would like to direct a related question to Scott
Sieberth as the chemist on the panel. You have heard about the
action of cyclodienes on GABA receptors from several groups at this
symposium. Would you consider investigating the possibility of
making a non-chlorinated, easily biodegradable "cyclodiene"? Would
that be a viable kind of product?

S. M. Sieburth: Sure. Anything that has demonstrated appropriate
intrinsic activity is fair game for further work. Once you know
that cyclodienes interfere with GABA receptors, it's a logical step
to investigate other compounds which interfere with this target with
some expectation of getting similar types of activity. The
bicycloorthocarboxylates are an example. On the other hand, one
might worry about the likelihood of cross-resistance occurring since
resistance to cyclodienes is documented.

D. M. Soderlund: I agree that further work on established targets
is worthwhile. The fact that we have a group of compounds which act
on a given target is a conceptual lead of considerable value as
opposed to a target that has hypothesized but not proven
toxicological significance. Now, considering the potential negative
aspects of preselected resistance to, say, pyrethroids diminishing
the value· of the sodium channel as a target, we should keep in mind
that Jeff Bloomquist working in Tom Miller's lab, and now others,
have shown that the dreaded kdr target site resistance to
pyrethroids does not modify the sodium channel sensitivity to all
toxins acting there. So there is a great deal to be learned about
overcoming broad target site resistance by understanding existing
target site interactions. I think this emphasizes the idea that
just because you have a group of compounds which act in some
ill-defined way on a target, it does not mean that one cannot find
other chemical means to attack the same target successfully.

F. Matsumura: An important contribution of this more basic research
is identifying areas on which we can focus our synthesis effort. We
know that the sulfonylurea herbicides inhibit an enzyme that is
important in the synthesis of amino acids in plants but not mammals.
Now it is turning out that several new classes of herbicides attack
plants at the same site. Another example are the new sterol

inhibitor fungicides. We know now that inhibition of ergosterol
synthesis is very fungicidal. Other enzymes in this pathway may be
important targets for further synthesis. By identification of these
critical areas of biochemistry, we can focus synthesis effort in
what may be more fruitful areas.

Unknown: Another use of this sort of system is in the
identification of compounds from natural sources where there is
often such a small quantity of compound available that you would
never see activity in more traditional whole animal or insect
assays. The test system doesn't have to be a single receptor or
enzyme. The kind of thing I have in mind is that we would never
have discovered one of our antibiotics if we had not had a bacterial
cell wall assay. The fermentation mixture itself has no
antibacterial activity.

A. E. Lund: That is something that is not even on this chart (Fig.
1), and perhaps should be. It is certainly being used by companies
today. Screening fermentation broths or crude extracts from plants
to discover new agricultural chemicals can be done using traditional
kinds of in vivo assays or one can screen for specific in vitro
activities. It is, of course, crucial in the latter case that one
carefully identify the activity of interest. Once this is
accomplished, one can go about designing an assay to detect the
activity in very small samples.

K. R. Jennings: A further point on natural products concerns the
purification and identification of the active component. It is
advantageous if not necessary in most cases to have a rapid in vitro
assay that can give results on active fractions in a matter of hours
using very small quantities of compound. Otherwise, of course, you
loose too much of your sample at each bioassay. So a good in vitro
assay is certainly important for successful purification and
structural identification.
    This is what we did at the American Cyanamid Research Center in
Princeton, when we purified methyllycaconitine (an insecticidal
plant alkaloid active on insect nicotinic receptors) from Delphinium
plants. As reported by Dr. Chalmers in the Tuesday afternoon
session, we were able to employ the in vitro cholinergic receptor
binding assay to follow the active material present in small
quantities on TLC plates. Results were available in a matter of
hours. This type of strategy could expedite purifications of active
materials from other natural sources. Also, knowing the mode of
action of the insecticidal component at an early stage facilitated
our decision to purify the active material.

A. T. Eldefrawi: I would like to bring attention to something else
that in vitro binding assays can provide, and that is a measure of
target selectivity. If we do the same kinds of assays with both
insect and mammalian preparations we can learn what kind of
structures within a large group of drugs we should study. Let me
give you an example. If you look at the nicotinic acetylcholine
receptor, you may find 30-40 drugs which have similar potency in
vertebrates and invertebrates. However, there may be one or two to

which the insect target is much more sensitive. This gives us a
clue to zero in on for further investigation. This comparative <u>in
vitro</u> approach can be useful in detecting selective compounds for
development as insecticides.

M. E. Schroeder: Another related area where basic research may have
an impact on product discovery is in the support of optimization
once lead compounds are identified. In this case the <u>in vitro</u> assay
can tell the chemist if the modifications that he is making are
increasing or decreasing the activity at the target site. I think
most chemists would like to know whether changes in insecticidal
activity of a series of compounds are due to changes in penetration
or metabolism or due to changes in the inherent activity.

S. M. Sieburth: I agree that <u>in vitro</u> assays should be a component
of any optimization scheme. It is also helpful to have a
side-by-side comparison of vertebrate and invertebrate assays to
give some clue as to target site selectivity even though we
recognize that multiple factors will ultimately determine the whole
animal selectivity.

M. A. Brown: I think one of the key problems that we face in trying
to discover a new insecticide and that pharmacologists probably face
to a lesser extent is the problem of selectivity. After compounds
have been shown to be active at a particular target site, the key
problem frequently is not to increase potency but to find proper
selectivity. I think it is fair to say that most
commercially-available insecticides owe there selectivity to
selective metabolism. This is a feature of compounds that can only
be identified, reliably, in whole animal assays.

A. E. Lund: One part of me likes very much the idea of using <u>in
vitro</u> assays to hint at selectivity between insects and mammals.
However the problem that frequently arises if one tries to use such
assays to guide synthesis is a marked discomfort in making synthesis
decisions based on these results. If one limits synthesis to an
area of chemistry with target site selectivity, one is haunted by
the concern that the most selective compound <u>in vivo</u> is one that is
less selective on the target site because it possesses some
toxicokinetic vulnerability in mammals. On the other hand,
compounds which are inactive on the mammalian target may ultimately
be less safe because of a deleterious action on some totally
unrelated system in the mammal. So the discrepancies between
selectivity measured <u>in vivo</u> and <u>in vitro</u> cloud our ability to make
synthesis decisions with much confidence. Perhaps if one
continually compares <u>in vivo</u> and <u>in vitro</u> results with a group of
compounds, you can minimize these errors.

K. R. Jennings: I think this is an important point with a corollary
that concerns target selection. It is not necessarily true that
target sites common to insects and mammals will results in
non-selective agents. Therefore it is unwise to rule out all
targets which have been evolutionarily conserved. Again the example
I will give is our amidinohydrazones which Dr. Hollingshaus working
in our group has shown to inhibit electron transport in

mitochondria. This is a highly conserved pathway across all living
organisms. So if you were looking at this process a priori, one
might rule it out as too conserved to allow for selectivity and yet
these compounds show very good selectivity. So I think that while
it is important to look at pharmacological differences between
insects and mammals, we should keep our eyes open to the fact that
there are other factors involved in determining selectivity.

R. Neumann: It seems to me somehow naive to talk about the insect
or the mammalian receptor. I think the movement of companies away
from screening houseflies to screening market-relevant insects
indicates a widespread belief that target sites and detoxification
mechanisms differ widely between insect species. Therefore it is
very difficult to relate conclusions about receptor systems in
cockroaches, locusts or some other model system to the economic
"real world".

Unknown: When you assay compounds that are active in some in vitro
system and they are inactive in vivo, how often do you assay those
insects to determine if the enzyme or receptor was, in fact,
affected in the whole animal?

M. E. Schroeder: The answer is very rarely. In order to justify
the major research effort required to go after a biochemical target,
one needs to determine the toxicological consequence of inhibiting
that target. It is one thing to inhibit a GABA-chloride channel
complex in vitro and another to correlate that inhibition with in
vivo toxicity. I think that bridge must be made before you can gain
real confidence in your target.

D. W. Gammon: This has been done though. The example of GABA
transaminase I reported on yesterday is a case where we could gain
some insight into the relative sensitivity of this enzyme as an
insecticide target, and allow a decision to be made on future
synthesis. People have, of course, been doing these experiments for
a long time trying to measure enzyme activities in vitro and in
vivo. It is often the only way to determine if a given target site
is important. In the case of receptor binding, one can use
radiolabeled ligands to do similar experiments. I believe Jeff
Lawrence is in the audience; perhaps he would comment.

L. J. Lawrence: We tried for a while to demonstrate involvement of
GABA receptors in deltamethrin poisoning in rats by dosing the rats
in vivo, removing the brain and assaying for the number of
TBPS-binding sites still available. We could never demonstrate
complete inhibition of these sites or inhibition to the extent that
one would have expected from the in vitro studies. We could show
some inhibition, but its extent was too variable for detailed
structure-activity studies. I think that making the step from the
in vitro to in vivo situation is difficult due to various
toxicokinetic complications.

D. M. Soderlund: There is one very simple and important barrier to
the success of these experiments. Unless the target site is
covalently modified, you will never be able to show a clear 1:1

correlation between what happens during equilibration of some dose
at a target site in vivo and subsequent isolation and preparation of
some in vitro system. If you have a reversible insecticide-target
interaction, the minute you begin preparing the tissue you start
altering the distribution of the toxicant by destroying the
equilibrium with the receptor site.

Unknown: Most of our commercial insecticides today are nerve
poisons with relatively quick kill. I think that if you are
familiar with some of the insect neurobiology conferences which have
occurred over the past five years or so, there is much more
attention being given to insect neurohormones and neuromodulators
— things which may not act like neurotransmitters but have longer
term effects on insect behavior and reproduction. I think these are
areas which can be exploited to the detriment of the insect even
though you do not see dead insects in 24 hours. You may effect
sufficient crop protection to realize a yield increase at the end of
your growing season. This has a much larger impact on how we look
for activity of compounds, because most of our screens look for
mortality in 24 or at most 72 hours. I think the biologists and
biochemists have to give feedback to the people doing the screens so
that they can structure their evaluation program to pick up
activities that are interfering with modulatory sites that may give
the same crop protection as an organophosphate or pyrethroid.

R. M. Hollingworth: That is a very important point. We seriously
underestimate the impact on insect populations of compounds which
have deleterious effects on every life stage of the insect but are
not spectacular on any one of them. Current screens are not going
to pick those up with any great fidelity. I was very interested to
see Dr. Knowles' life table approach to the action of formamidines
which impact the insect at every point from the egg, through the
larva, to the adults and their reproduction. Even a relatively
small effect at each life stage can add up to excellent economic
control of the overall population, particularly over more than one
generation. Accepting this approach and developing appropriate
screens is a degree of sophistication that we have not yet attained,
but I hope we will be able to use it in the future.

R. Neumann: Shouldn't we discuss de novo design a little? I
suspect that most if not all chemicals that have been synthesized
have been properly evaluated in vivo. Most of the basic research
that is done is to just prove why compounds are active. What I
think industry hopes to get out of basic research is ideas on how to
design new chemicals de novo. I wonder if anyone would like to
comment on how the research that we have been hearing about the last
two and one-half days could contribute to de novo design. I must
say that I am still somewhat surprised that new compounds have not
been designed to attack targets that are so well known. There are
at least a half a dozen compounds that interfere with the sodium
channel, but still we are completely unable to design anything de
novo that can act on that target. Even in such a well known and
researched system, our knowledge that can be exploited for de novo
synthesis seems to be very limited.

R. M. Hollingworth: This is a very promising area. In fact, progress is being made. One area of great current promise is the design of compounds to inhibit photosystem II in plants. The plastaquinone binding site has been defined by biochemical genetic and crystallographic techniques. The amino acid sequence and three dimensional structure are known, and this information now is being used to synthesize photosynthesis inhibitors which are active and novel. I believe it will not be many more years, perhaps, until we are at this point with the acetylcholine receptor. We already have a good deal of information from Torpedo electroplax on the molecular nature of the receptor. There are detailed models of the active site. There is some relevant information available on the associated ion channel as a second target area on the receptor complex. I think we have to keep in mind, though, that you have to have this very exact information in order to design compounds, and it just isn't there in most cases. But let's be optimistic and hopeful about it for the future. Again, I think we will suffer if we insist on precedents and on seeing proofs of probable success before the fruit on this particular tree has ripened.

A. E. Lund: I agree with you completely that there is new information accumulating all the time that is getting us closer to de novo design. Certainly the growing application of molecular genetic techniques to neurobiology promises to provide much of the needed information. I think, however, there is something else one can do along these lines that is a little more conventional and that puts us closer to design without waiting for molecular biologists and x-ray crystallographers to hand us the total picture. It should be possible to study the chemistry of the interaction between small organic molecules and a protein target that can reveal certain physico-chemical characteristics of the target. We might then design molecules with the appropriate complementary characteristics. The tools needed for this research include two reliable assays -- one to measure the binding of a ligand to a specific site on the protein, and a second to measure the functional consequence of that interaction. One also needs to construct a series of chemical probes with group specific reagents or affinity labels at various locations around the known ligand. One then can begin to map out certain features of the topography of the binding site. With the aid of computer modeling to help the chemist visualize new possibilities, this new information allows the development of structure-activity hypotheses that can be tested using a conventional SAR approach. This process can result in a parallel development of in vitro active compounds and a structure-topography which drifts more and more into focus. The ultimate aim of this approach, as I see it, is to step away from known chemistry and to design a molecule that has the characteristics (overall shape, lipophilicity, electronic configuration, etc.) needed to fit the hypothesized site but actually uses different chemistry. Perhaps I shouldn't call this process de novo design, since it is really an extension of a conventional structure-activity approach, but chemical design based on an important input of structural information on the target site is certainly involved.

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