1

CANADIAN THESES ON MICROFICHE

I.S.B.N.

THESES CANADIENNES SUR MICROFICHE

National Library of Canada
Collections Development Branch

Canadian Theses on
Microfiche Service

Ottawa, Canada
K1A 0N4

Bibliothèque nationale du Canada
Direction du développement des collections

Service des thèses canadiennes
sur microfiche

NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us a poor photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30. Please read the authorization forms which accompany this thesis.

THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de mauvaise qualité.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30. Veuillez prendre connaissance des formulés d'autorisation qui accompagnent cette thèse.

LA THÈSE A ÉTÉ
MICROFILMÉE TELLE QUE
NOUS L'AVONS REÇUE

National Library
of CanadaBibliothèque nationale
du Canada

Canadian Theses Division · Division des thèses canadiennes

Ottawa, Canada
K1A 0N4

60026

PERMISSION TO MICROFILM — AUTORISATION DE MICROFILMER

• Please print or type — Écrire en lettres moulées ou dactylographier

Full Name of Author — Nom complet de l'auteur

ELIA, Andrew James

Date of Birth — Date de naissance

July 10, 1955

Country of Birth — Lieu de naissance

Canada

Permanent Address — Résidence fixe

1319 Prince of Wales Drive
Ottawa, Ontario. K2C 1N2

Title of Thesis — Titre de la thèse.

The long-term effects of DDT and analogs on the central nervous
system of the cockroach, Periplaneta americana (L.)

University — Université

Carleton

Degree for which thesis was presented — Grade pour lequel cette thèse fut présentée

M.Sc.

Year this degree conferred — Année d'obtention de ce grade

1982

Name of Supervisor — Nom du directeur de thèse

Dr. D.R. Gardner

Permission is hereby granted to the NATIONAL LIBRARY OF
CANADA to microfilm this thesis and to lend or sell copies of
the film.The author reserves other publication rights, and neither the
thesis nor extensive extracts from it may be printed or other-
wise reproduced without the author's written permission.L'autorisation est, par la présente, accordée à la BIBLIOTHÈ-
QUE NATIONALE DU CANADA de microfilmer cette thèse et de
prêter ou de vendre des exemplaires du film.L'auteur se réserve les autres droits de publication; ni la thèse
ni de longs extraits de celle-ci ne doivent être imprimés ou
autrement reproduits sans l'autorisation écrite de l'auteur.

Date

March 29/1982

Signature

THE LONG-TERM EFFECTS
OF DDT AND FOUR OF ITS ANALOGS ON THE
CENTRAL NERVOUS SYSTEM
OF THE COCKROACH, Periplaneta americana (L.)

by

ANDREW JAMES ELIA, B.Sc.

A thesis submitted to the Faculty of
Graduate Studies and Research in partial fulfilment
of the requirements for the degree of
Master of Science

Department of Biology
Carleton University
Ottawa, Ontario
CANADA

JANUARY 29, 1982.

The undersigned hereby recommend to the Faculty of
Graduate Studies and Research acceptance of this
thesis, submitted by Andrew James Elia, B.Sc., in
partial fulfilment of the requirements for the degree
of Master of Science.

Chairman, Department of Biology

Supervisor

Carleton University

February 11, 1982.

ABSTRACT

The effects of p,p'-DDT and four of its analogs on activity in the CNS of the cockroach, Periplaneta americana (L.), were investigated using extracellular recording techniques. Cockroaches were injected intra-abdominally with organochlorine (O.C.) compounds at LD50 96 H doses. Extracellular recordings were made from the central nervous system (CNS) at 1 h, 24 h, or 3-weeks post-injection.

Experiments indicated that p,p'-DDT, methoxychlor, and p,p'-DDD induced behavioural signs (tremors, jitters, hyperexcitability) and CNS symptoms (repetitive firing) of poisoning at 1 h post-injection. At 24 h post-injection, behavioural signs of poisoning disappeared, though repetitive firing could be readily elicited in the CNS. Furthermore, cockroaches treated with p,p'-DDT or o,p'-DDT were behaviourally normal at 3-weeks post-injection but still displayed a significant occurrence of repetitive firing in their CNS's.

A mechanism is proposed to explain how the cockroach might recover from a sublethal dose of O.C. compound but still display repetitive firing in its CNS. The "cause and effect" relationship between repetitive firing in the CNS and mortality (and signs of poisoning) is therefore questioned.

An investigation into a possible site of action of DDT (neuro-plasma membrane or CNS mitochondria) was also pursued. The amount of DDT determined to be associated with the mitochondrial fraction *in vivo*, was calculated to be insufficient to cause a significant degree of inhibition of the Mg-ATPase and mitochondrial function.

TABLE OF CONTENTS

Acknowledgements.....	1
List of Abbreviations.....	ii
List of Figures.....	v
List of Tables.....	viii
List of Appendices.....	ix

Introduction

1. General Organization of the Cockroach Nervous System.....	1
2. The DDT Controversy - Current Hypotheses on the Mode of Action of DDT	
(a) General.....	9
(b) Direct Effects on the Nervous System..	11
(c) Interaction of DDT with Fractioned Components of Nerve Membrane.....	23
(d) Molecular Aspects of DDT Action.....	26
(e) Possible Involvement of an Endogenous Toxic Substance.....	39
(f) Effects on the Adenosine Triphosphatase (ATPase) Enzymes.....	42
3. Metabolism of DDT, <u>in vivo</u>	49
4. Experimental Objectives of this Study.....	51

Materials and Methods

1. The Experimental Animal The Cockroach, <u>Periplaneta americana</u> (L.).....	54
--	----

(b) The Cockroach Colony.....	54
2. Whole Animal Toxicity	
(a) Injection Procedure.....	57
(b) Doses of DDT and Analogs.....	59
(c) LD50 96 h Determination for o,p'-DDT..	61
3. Organochlorine Compounds.....	61
4. Neurophysiological Methods	
(a) The Isolated Nerve Preparation.....	63
(b) Preparation of the Ventral Nerve Cord (VNC) for Stimulation and Recording...	64
(c) The Apparatus: Stimulating and Recording from the VNC.....	65
5. Saline Solution and Fractionation Medium	
(a) Normal Saline.....	70
(b) Fractionation Medium.....	71
6. Thin Layer Chromatography (TLC) of Organochlorine Compounds.....	71
7. Cockroach Central Nervous System (CNS) Fractionation Experiments	
(a) Localization of 14C-DDT in the CNS of the Cockroach, <u>Periplaneta americana</u> (L.).....	74
(b) Fractionation Procedure.....	74
(c) Electron Microscopic (E.M.) Identification of Fractioned Pellets.....	78
(d) Experiments Employing 14C-p,p'-DDT....	80

8. Protein Determination

- (a) The Lowry Method.....85
- (b) Coomassie Brilliant Blue G-250
Method.....88

Results

1. Mortality Studies

- (a) Signs and Symptoms of DDT Poisoning
in the Cockroach.....92
- (b) Cumulative Data on Mortality
Following the LD50 96 h Dose.....94
- (c) Toxicity of o,p'-DDT.....96

2. Activity in the Cockroach Ventral Nerve Cord

I Classifying Repetitive Firing

- (a) Pseudo Repetitive Firing and other
Spontaneous Activities.....99
- (b) Common Characteristics of Repetitive
Firing.....104

II Efficacy of Organochlorine Compounds

to Elicit Behavioural and
Neurophysiological Symptoms of Poisoning

- (a) 24 h Preparations: Behavioural
Abnormalities.....108
- (b) Activity in the VNC 1 h
Post-Injection.....110
- (c) Activity in the VNC 24 h
Post-Injection.....111
- (d) p,p'-DDD Injected Cockroaches

Near Prostration.....	119
(e) Activity in the VNC 48 and 96 h	
Post-Injection.....	119
(f) Preliminary Results from Severed CNS	
Experiments (T3-A1).....	124
(g) 3-Week Experiments:	
i Behavioural Abnormalities.....	124
ii Activity in the VNC 3-Weeks	
Post-Injection.....	125
iii Characteristics of Repetitive	
Firing at 3-Weeks.....	129
3. The Activity Quotient (A.Q.)	
(a) 24 h Experiments.....	135
(b) 3-Week Experiments.....	137
4. Organochlorine Chromatography.....	139
5. Cockroach CNS Fractionation Experiments	
(a) Morphological Identification of	
Fractioned Pellets.....	143
(b) Association of 14C-DDT with the	
CNS, <u>in vivo</u>	143
(c) Affinity of the Fractions	
for 14C-DDT, <u>in vitro</u>	147
(d) Contamination of the	
Mitochondrial Fraction.....	150
(e) Estimate of DDT Contained in	
each Fraction.....	150
6. Protein Determination of the Three Isolated	

CNS Fractions

(a) Lowry Protein Determination.....153

(b) Coomassie Brilliant Blue G-250

Protein Determination.....153

Discussion.....157

1. Rationale and Treatment with O.C. Compounds..157

2. o,p'-DDT159

3. Repetitive Firing in Controls.....163

4. Repetitive Firing in O.C. Treated VNCs.....167

5. Possible Correlations

with Endotoxin Research.....177

6. The ATPase Question.....179

7. Localization of DDT in the CNS, in vivo.....180

General Conclusions.....186

References.....188

Appendices.....198

For Sue

ACKNOWLEDGEMENTS

I wish to express my gratitude to Dr. David R. Gardner for his assistance during my research endeavours at Carleton. His ready willingness to discuss various aspects of my research have been invaluable. His critical comments in the latter stages of preparation of this thesis have provided me with a new understanding for the English language.

My two advisors, Drs. S. L. Jacobson and J. Sinclair, also deserve a note of thanks for their generous contributions of equipment, guidance, and kindness. Also, Mr. Borys L. Brezden's ability to inject both humour and science into my daily life at Carleton was greatly appreciated.

Mr. Clifford Wilson's technical assistance and charming wit were readily dispensed when, and sometimes when not, asked for. I am grateful for both. Also, the assistance of Drs. G. Setterfield and P. Lee are gratefully acknowledged.

I must also thank Al Barlow for his tolerance and steady nerves.

The final, deepest note of appreciation must go to my parents. It has been their constant encouragement and understanding that has made it possible for me to complete this, and previous, scholastic endeavours.

LIST OF ABBREVIATIONS

- A.Q.: Activity Quotient; a measure of the effectiveness of a compound to induce repetitive firing in the CNS
- ATPase: Ion-sensitive adenosine triphosphatase
- °C: Temperature measured in degrees centigrade
- CAP: Compound Action Potential
- ¹⁴C-DDT: The isotopically labelled form of the DDT molecule
- cm: centimeter
- CNS: Central Nervous System
- DDD: a toxic analog of DDT (see Table 1, Pg. 58, or Pg. 63)
- DDE: a non-toxic breakdown product of DDT (see Table 1 or Pg. 63)
- dpm: Disintegrations per minute -- 1 Curie is equal to about 2.22×10^{-9} dpm
- dpm/mg: dpm per milligram (1×10^{-3} g)
- ED50: dose of a compound required to elicit a response in 50% of treated preparations
- g: grams
- xg: the gravitational force exerted upon an object
- h: hours
- Hz: Hertz -- unit of frequency defined as one cycle per second
- LD50 96 h: Lethal dose for 50% of a treated population in 96 h

M: Molar (moles per litre of solution)

mCi/mg: millicuries (1×10^{-3} Curies) per milligram
of sample

MeO-DDT: Methoxychlor - the -OCH₃ para-substituted
analog of DDT (see Table 1 or Pg. 63)

mg: milligram (1×10^{-3} g)

min: minutes

ml: millilitre (1×10^{-3} litres)

mm: millimeter (1×10^{-3} meters)

mM: millimolar (1×10^{-3} moles/litre)

ms: millisecond (1×10^{-3} seconds)

mV: millivolt (1×10^{-3} volts)

nM: nanomolar (1×10^{-9} moles/litre)

O.C.: organochlorine

ppm: concentration expressed as parts per million

R-DDD: the migration of O.C. compounds on TLC relative
to the mobility of p,p'-DDD (as defined by
Sunshine (1972) - CRC Handbook of Chromatography)

R_f: the distance migrated by a test compound relative
to the distance migrated by the mobile phase (i.e.,
solvent front)

sec: seconds

TBM: Trout brain mitochondria

TEA: tetraethylammonium - quaternary ammonium compound
that selectively blocks the potassium conductance
channel in neurons and muscle fibres

TTX: tetrodotoxin - a toxin which specifically blocks

the sodium conductance channel in neurons and
muscle fibres

ug: microgram (1×10^{-6} g)

ul: microlitre (1×10^{-6} litre)

umols: micromoles (1×10^{-6} moles)

UV: Ultraviolet

VNC: Ventral Nerve Cord

x-DDT: variously substituted p,p'- positions in the DDT molecule; e.g., NO₂-DDT - NO₂ replaces the Cl in the para- positions on the phenyl rings (similarly with NH₂, OH-, OCH₃-) (see Table 1 for DDT structure)

LIST OF FIGURES

Fig. No.	Title	Page
1(a)	Diagramatic Representation of the Cockroach CNS...	2
1(b)	Electron Micrograph of Cross-Section Through Cockroach VNC Connective (Abdominal Segment).....	4
2	Diagramatic Representation of the Giant Fibre System of the Cockroach, <u>Periplaneta americana</u> (L.).....	7
3	Alteration of the Action Potential Following Incubation with p,p'-DDT.....	14
4	Depolarizing After-Potential in the Cockroach CNS.....	16
5	Diagram of Cockroach Colony.....	56
6	Route of Injection into Cockroach.....	59
7	Block Diagram of the Stimulating and Recording Apparatus.....	67
8	Photograph of Faraday Cage and Electrodes.....	68
9	Dosage-Mortality Curve for o,p'-DDT - Probit Analysis.....	97
10	Normal Activity and Activity in the Cockroach Nervous System not Associated with O.C. Poisoning.....	101
11	Some Characteristics of Repetitive Firing.....	106
12	Activity Records for 24 h p,p'-DDD (near-prostrate) and	

	1 h MeO-DDT Injected Cockroaches.....	109
13	Graphical Representation of Percent of Preparations Displaying Repetitive Firing vs. Compound Injected - 24 h Preparations.....	112
14	Activity Records for 24 h p,p'-DDT, MeO-DDT, and p,p'-DDD Injected Cockroaches.....	114
15	Repetitive Firing in the VNC of a Cockroach Injected 24 h Previously with p,p'-DDT.....	115
16	Repetitive Firing in the VNC of a Cockroach Injected 24 h Previously with MeO-DDT.....	117
17	Repetitive Firing in the VNC of a Cockroach Injected 24 h Previously with p,p'-DDD.....	118
18	Activity Records for 48 and 96 h o,p'-DDT Injected Cockroaches.....	121
19	Repetitive Firing in the VNC of a Cockroach Injected 48 h Previously with o,p'-DDT.....	122
20	Repetitive Firing in the VNC of a Cockroach Injected 96 h Previously with o,p'-DDT.....	123
21	Graphical Representation of Percent of Preparations Displaying Repetitive Firing vs. Compound Injected for 3-Week Preparations.....	127
22	Activity Records for 3-Week Saline, Acetone,	

	p,p'-DDT, and o,p'-DDT Injected Cockroaches.....	128
23	Activity Records for 3-Week MeO-DDT, p,p'-DDD, and p,p'-DDE Injected Cockroaches.....	130
24	Repetitive Firing in the VNC of a Cockroach Injected 3-Weeks Previously with p,p'-DDT.....	131
25	Repetitive Firing in the VNC of a Cockroach Injected 3-Weeks Previously with p,p'-DDT.....	133
26	Graphical Representation of Activity Quotient vs. Compound Injected for 24 h Preparations.....	136
27	Graphical Representation of Activity Quotient vs. Compound Injected for 3-Week Preparations...	138
28	TLC-Radiochromatogram of O.C. Compounds.....	140
29	Electron Micrograph of Mitochondrial Fraction...	144
30	Electron Micrograph of Microsomal Fraction.....	145
31	Electron Micrograph of Membranal Fraction.....	146
32	Percent of Pellet Dry Weight as Protein for the Various CNS Fractions.....	155

Note: All chart records of repetitive firing
are unretouched photographs of original
records made on the Siemen's chart recorder.

LIST OF TABLES

No.	Title	Page
1	The Structural Formulae of p,p'-DDT and the DDT Analogs.....	58
2	Doses of Organochlorine Injected.....	60
3	Doses of o,p'-DDT Injected for LD50 96 h Determination.....	62
4	Cumulative Mortality Data Following the Putative LD50 96 h Doses of Organochlorine Compounds.....	95
5	Doses of o,p'-DDT Employed to Determine Percent Mortality at 96 h and 3-Weeks.....	98
6	Rf and R-DDD Values.....	141
7	Radioactivity Associated with Different Fractions from the CNS.....	148
8	Affinity of the CNS Fractions for 14C-DDT, in vitro (14C-DDT Injected into the homogenate).149	149
9	Contamination of the Mitochondrial Pellet with 14C-DDT During the Isolation Procedure.....	151
10	Estimates of Total Protein of CNS Fractions.....	154

LIST OF APPENDICES

Letter	Title	Page
A	Modified Procedure of Chappel and Hansford (1971) for Isolation of Membranal, Mitochondrial, and Microsomal Fractions from the CNS.....	201
B	Procedures Employed to Prepare Tissue from the CNS for Embedding in Epon-Araldite.....	202
C	Protein Extraction Procedure Employed for the Lowry and Coomassie Brilliant Blue G-250 Protein Determinations.....	203
D	Protein Determination by the Method of Lowry et al. (1951).....	204
E	Sample Standard Curve for Protein Determination by the Method of Lowry et al. (1951).....	205
F	Protein Determination by the Method of Bradford (1976) (Coomassie Brilliant Blue G-250) and Sample Standard Curve.	206
G	Sample Calculations for ¹⁴ C-DDT Experiments.....	207
H	Discussion of Results for Protein Determinations.....	208

INTRODUCTION

1. General Organization of the Cockroach Central Nervous System (CNS)

The most characteristic feature of the cockroach CNS is the decentralization of function and response. The ganglionic mass which resides in the head is referred to as the "brain" mainly because of its position in relation to the other ganglia. It consists of three fused ganglia (protocerebrum, deutocerebrum, and tritocerebrum) which occupy three distinct anatomical regions in the brain. The functions of each region have been delineated (see Willey (1961) for a comprehensive account of the brain of Periplaneta americana) (Cornwell, 1968). Nerves that originate in the brain supply the suboesophageal and frontal ganglia. Nerves originating in these two ganglia innervate the endocrine structures associated with the brain (corpora cardiacum and corpora allatum) and supply the nerves to innervate the first thoracic ganglion (suboesophageal ganglion).

The ventral nerve cord is comprised of two discrete bundles of axons side by side and joined by an acellular sheath composed of collagenous-type fibrils. The ventral nerve cord connects the brain with three thoracic and six abdominal ganglia (Fig. 1(a)). Although the head ganglia's main function is to receive and interpret input

Figure 1(a): Diagrammatic Representation of the Central Nervous System (CNS) of the Cockroach, *Periplaneta americana* (L.) The pro-, meso-, and meta- thoracic ganglia (T1, T2, T3, respectively) are shown with associated nerve roots. The abdominal ganglia (except A6) are shown without associated nerve roots. The connectives between ganglia are designated by the associated ganglia (e.g., T3-A1; A1-A2; etc.) In the head: B, brain; SG, subesophageal ganglion.

from sensory organs located on the external surface of the head, it also receives afferent input from other sensory structures located on the legs and abdomen (Lane, 1974; Horn, 1976). It is believed that the brain acts as the chief association centre as well as the seat of long-term organized behaviour patterns (Bullock and Horridge, 1965; Lane, 1974). However, a decapitated cockroach will continue to respond to external (mechanical) stimuli for periods often exceeding 24 hours (personal observation), thus illustrating the relative autonomy of the various segments of the Central Nervous System (CNS).

Arising from the ventral nerve cord ganglia are tracks of nerve cell processes, or axons, which serve to interconnect ganglia and transmit nerve impulses (both afferently and efferently). The axons normally lie closely apposed to one another within a bundle. Depending on fibre type and position in the interganglionic connective, the axons are isolated from one another by varying numbers of glial cells (Chapman, 1971). Each side of the ventral nerve cord is surrounded by an acellular sheath (neural lamella) and an internal cellular perineurial layer separating axons and cell bodies from the neural lamella (Fig. 1(b)). Both layers extend the entire length of the ventral nerve cord (Lane, 1974) with the perineurial layer of the connectives noticeably thinner than that surrounding the ganglia (Huddart, 1971).

Figure 1(b): A cross-section through one side of the abdominal connective reveals the different morphology of the neural lamella (NL) and perineurium (P). The neural lamella is composed mostly of collagen-like fibrils running in different directions. Clumps of other fibrils cut in cross-section are also evident (CL). Also embedded in the neural lamella is the occasional tracheole cell (T). The lamella ensheaths the perineurium. Perineurial cell processes can be seen to interdigitate one another (I). Note the dense population of mitochondria (M) in the perineurial layer. Various sizes of axons (A) can be seen beneath the perineurial layer. These axons may or may not be ensheathed by glial cells (G) (all those in this photograph are ensheathed). Above left can be seen a collateral branch which emanates from an adjacent ganglion. A nucleus (N) with condensed chromatin (C) is evident. 7600 X

These two structures serve as a support and a diffusion barrier within the cockroach (Treherne, 1970; Pichon et al., 1971; Lane, 1974; Treherne, 1974).

The neural lamella is composed of an outer layer and an inner less dense region, which normally contains the tracheoles. Collagen-like fibrils run at right angles and parallel to the longitudinal axis of the nerve cord and are the main component of the neural lamella (Lane, 1974). The perineurium functions as a storage depot and transfer site of nutrients and metabolites. It is also believed to be the equivalent, in many respects, of the mammalian blood-brain barrier (Lane, 1974; Treherne, 1974).

As mentioned above, the sheath which the glial cells form around an axon also varies considerably. An axon may have a single glial cell surrounding it, it may be wrapped by several folds of the same glial cell, or it may be enclosed by overlapping cells (Chapman, 1971; Lane, 1974). Also, small axons may be naked, several axons may share the same glial sheath, or each axon may have its own cellular sheath (Lane, 1974). It is believed that glial cells can act as an insulator between cells, although this function does not appear to be relevant to naked, grouped axons (Horn, 1976; Chapman, 1971). The glial lacunar system (extracellular spaces between glial cells) is thought to be concerned with nutrition and would supply the perikarya, (nerve cell bodies) and axons with vital

nutrients (Lane, 1974; Treherne, 1974).

The axons arise from the nerve cell body within a ganglion or in a sense organ of the periphery. They serve to interconnect the ganglia of the CNS and relay messages (in the form of nerve impulses) to and from the various sensory and motor structures. The nerve impulse then enters the neuropile (central portion of the ganglion containing the axonal tracks and devoid of cell bodies) where it will be transmitted afferently or efferently. The axons in the neuropile are morphologically distinct from their cell bodies by containing only mitochondrial organelles, neurotubules (the microtubules of nervous tissue) that run parallel to the plasma membrane of the axon, and axoplasm.

Both sides of the ventral nerve cord of Periplaneta americana contain the axons of seven to eight giant interneurons (4 to 5 ventral and 3 dorsal giant fibres, 20 to 60 um in diameter) (Spira et al., 1969b; Parnas and Dagan, 1971; Westin et al., 1977; Ritzmann and Camhi, 1978), each bilaterally paired with an axon in the contralateral bundle. Their cell bodies are located in the terminal (sixth) abdominal ganglion. From there, the axons are continuous for the entire length of the Ventral Nerve Cord (VNC) (A6 to SG, Fig. 2) (Parnas et al., 1969; Spira et al., 1969(a,b)). Their form remains constant through the entire abdominal section of the nerve cord.

A6 A5 A4 A3 A2 A1 T3 T2 T1 SG

DORSAL

VENTRAL

GIANT AXONS

Figure 2: Representation of the Continuous Giant Fibre System in the Cockroach, *Periplaneta americana* (L.) (not drawn to scale) A1-A6, abdominal ganglia; T1-T3, thoracic ganglia; SG, suboesophageal ganglia; M, outputs to motor system of the legs; S, possible sensory input and synaptic modulation (after Parnas et al.(1969) and Castel et al.(1976)).

However, axonal tapering and synaptic input in the three thoracic ganglia modulate the action potential as it passes through each of these ganglia (Parnas et al., 1969; Spira et al., 1969(a,b); Castel et al., 1976; Parnas et al., 1976; Spira et al., 1979). These characteristics of the giant fibres led early investigators to believe that they mediated leg movements during the escape reflex (Roeder, 1948). Some doubt concerning this possible function was raised by Dagan and Parnas (1970).

Stimulation of the giant axons at frequencies up to 200 Hz did not evoke a response in any of the motor neurons that were recorded from simultaneously. However, more recent evidence (Westin et al., 1977; Ritzmann and Camhi, 1978; Delcomyn and Daley, 1979) has shown that the giant interneurons are involved with mediating sensory input and motor activity. High frequency input (greater than 200 Hz) from the abdominal giant interneurons evoked action potentials in motor neurons (Ritzmann and Camhi, 1978). Previously, (Westin et al., 1977; Fourtner and Drewes, 1977) it was demonstrated that wind puffs to the abdominal cerci or electrical stimulation of the cercal nerves evoked trains of action potentials of very high frequency (200 to 400 Hz) and short duration (10 to 40 ms) in the giant interneurons. Furthermore, decapitation did not suppress, but occasionally enhanced, giant axon to motor nerve responses (Ritzmann and Camhi, 1978) and increased general activity in the CNS (Horn, 1976). Modulation of

spike frequency by regions of special axonal geometry (e.g., regions that contain branch-points or points of sudden change in axon diameter) and by synaptic inputs was shown to occur in the metathoracic (third) ganglion (Parnas et al., 1976; Spira et al., 1976). Alteration of the normal action potential and partial or complete conduction block in the metathoracic ganglion was demonstrated using various frequencies and durations of electrical stimulation between the second and third thoracic ganglia (T2-T3) and between the fifth and sixth abdominal ganglia (A5-A6). The possible significance of these findings in relation to poisoning by DDT and its effects on the nervous system will be presented in the Discussion (Section 5).

2. THE DDT CONTROVERSY - Current Hypotheses on the Mode of Action of DDT

(a) General

The problem of understanding the mode of DDT action has been complicated by the great diversity of experimental findings. The exact mechanism which is ultimately responsible for the death of the insect has remained elusive despite considerable effort spent attempting to resolve this problem. In vitro, experiments have shown that DDT applied directly to an isolated nerve preparation causes a destabilization of the nerve membrane (Narahashi and Yamasaki, 1960(a,b); Uchida et al., 1974;

Wu et al., 1975). In vivo, injection or topical application of DDT to cockroaches was found to induce the same destabilizing characteristics in the subsequently isolated abdominal nerve cord (Gardner and Vincent, 1978) or in vivo "wired" free-walking cockroach preparation (Gammon, 1977; 1978).

A theory which has not received widespread support, although it has remained prevalent in the literature, is the possible production of an endogenous toxic factor (Sternberg and Kearns, 1952; Shankland and Kearns, 1959; Milburn et al., 1960; Maddrell and Reynolds, 1972; Tashiro et al., 1972). The production and/or release of this toxic factor may be stimulated by the application of DDT to the insect or by some other form of severe physical stress.

Complicating matters further, experiments implicating the sodium-potassium-dependent adenosine triphosphatase (Na-K-ATPase) and the magnesium-dependent adenosine triphosphatase (Mg-ATPase or oligomycin-sensitive ATPase) enzyme systems as the targets for the DDT molecule have been published (Matsumura and Narahashi, 1971; Cutkomp et al., 1971; Doherty and Matsumura, 1975; Cheng and Cutkomp, 1975; 1977).

More recently, another ATPase enzyme, the calcium-dependent ATPase (Ca-ATPase), has been implicated as a possible target of DDT. This Ca-ATPase has been

isolated from the brain of sensitive and resistant cockroaches (Blattella germanica (L.)) and from peripheral nerves and tail muscle of lobster (Homarus americanus) (Matsumura and Ghiasuddin, 1978; Ghiasuddin and Matsumura, 1979, 1981; Ghiasuddin et al., 1981).

(b) Direct Effects on the Nervous System

1/ The Negative Temperature Coefficient of DDT Poisoning

Excitability, tremors, and loss of coordinated activity have been associated with DDT and its effects on the nervous system since the earliest investigations (Yeager and Munson, 1945; Roeder and Weiant, 1945, 1948; Welsh and Gordon, 1947; Lalonde and Brown, 1954). At that time (Roeder and Weiant, 1948) there was a distinction made between DDT's effects on the CNS and on the peripheral sensory neurons. The difference in sensitivities between central and peripheral nerves was later apparently substantiated by Eaton and Sternberg (1964, 1967). The frequency of appearance of trains and the number of impulses in each train in the peripheral nervous system (PNS) increased with increasing temperature (positive temperature coefficient). This was apparently contrary to the effect on the CNS which displayed an increase in impulse trains and poisoning symptoms at lower temperatures (Yamasaki and Ishii, 1953, 1954; Eaton and Sternberg, 1964), i.e., a negative temperature

coefficient. Raising the temperature of a repetitively firing ventral nerve cord prevented the repetitive response (Eaton and Sternberg; 1967). This effect on the CNS (the negative temperature coefficient) paralleled the symptoms of poisoning and toxicity displayed in the whole animal. Thus, it was concluded that the CNS was responsible for the overall negative temperature coefficient of DDT-toxicity (Eaton and Sternberg; 1964, 1967).

This apparent difference in response of the central and peripheral nervous system to DDT may be a result of the differences in parameters measured in the two groups of studies (Narahashi; 1979). Yamasaki and Ishii (1953, 1954) determined the potency of DDT as a function of concentration. Lowering the temperature greatly enhanced the appearance of repetitive firing trains and symptoms of poisoning, i.e., an increased potency at lower temperatures. The dose for a LD50 was also much less at lower temperatures. Eaton and Sternberg (1964, 1967) measured the frequency of appearance of trains and the number of impulses in each train. These parameters relate to the efficacy, not the potency. They (frequency of appearance of trains and the number of impulses in each train) were not directly related to the potency (and ultimate toxicity) of DDT. Evidence supporting this was the positive temperature coefficient of these two efficacious parameters, (compared with the negative

temperature coefficient of mortality), and the reversal of signs and symptoms of poisoning when a CNS from a treated cockroach or a near prostrate cockroach was raised in temperature (Eaton and Sternberg, 1964, 1967; Gammon, 1978). Therefore, the controversy with respect to the temperature coefficient of DDT action may be accounted for by the different parameters measured by the two groups (Narahashi; 1971, 1979).

A more definitive study on the negative temperature coefficient of DDT poisoning was conducted by Gammon (1978). In an elegant series of experiments, employing free-walking cockroaches with electrodes implanted at various sites along their central nervous systems, he showed that the effects of DDT on the CNS became more marked at lower temperatures. All cockroaches received a LD95 120 h dose of DDT. (However, the LD95 dose at 16.5°C was much lower (5.2 ug/cockroach) than that at 32°C (27.6 ug/cockroach).) Maximum duration of abdominal repetitive discharges following a single electrical stimulus was significantly longer at lower (16.5°C) than at higher (25°C and 32°C) temperatures. Several other important characteristics of DDT poisoning were revealed. Each experimental (poisoned) animal tested exhibited repetitive discharges in their ventral nerve cords before overt behavioural signs of poisoning were elicited. In prostrate cockroaches it was noted that the nervous system

would waver between block and recovery, before becoming irreversibly blocked. This oscillatory period of nervous activity lasted 2 to 3 hours.

Moving a poisoned cockroach from 16.5°C to 25°C or 32°C would abolish signs and symptoms of poisoning. These signs and symptoms would return if the cockroach was returned to the cooler environment (Gammon, 1978). Thus the negative temperature coefficient in relation to toxicity and effects on the CNS has been well demonstrated.

2/ Alteration of the Action Potential by DDT

The effects of DDT on nerve membrane characteristics were most notably studied by Narahashi, Yamasaki, and Haas. Using intracellular glass capillary microelectrodes, Narahashi and Yamasaki (1957, 1960) first described the marked increase and prolongation of the depolarizing after-potential in cockroach giant axons (Fig. 3).

Figure 3: Alteration of the Action Potential following incubation with DDT (from Narahashi and Yamasaki (1960(a))).

These early experiments, combined with later work (Narahashi and Haas, 1967; 1968) using the sucrose-gap voltage-clamp technique on single lobster axons provided valuable information regarding the alterations in conduction by the nerve when exposed to DDT.

A latent period of between 10 and 20 min was experienced before the axons showed any sign of effects due to the applied DDT. As time progressed the first signs of poisoning appeared as a depolarizing after-potential. With a slight increase in depolarizing after-potential, a single input stimulus eliciting an action potential would give rise to multiple afterdischarges (Fig. 4). As the depolarizing after-potential amplitude becomes larger (ca. 30 mV), a point is reached at which the repetitive afterdischarges cease. The time to reach this point will depend upon the dose of DDT applied and type of nerve fibre (e.g., for cockroach giant axons treated with 10^{-4} M p,p'-DDT, 90 to 120 min). The relationship between size of the depolarizing after-potential and repetitive discharges is specific for each type of neuron (Narahashi and Yamasaki, 1960(a-c); Narahashi, 1980). Whether or not a neuron fires repetitively will depend upon the inherent relationships amongst ionic conductance mechanisms, the membrane potential, the amplitude and time course of the depolarizing after-potential, and on environmental factors such as the ionic composition of the bathing medium and

temperature (Narahashi, 1980). van den Bercken (1972), found that in myelinated nerve fibres of the frog an increase in the size of the depolarizing after-potential usually corresponded to an increase in the number of repetitive action potentials and a decrease in the interval between action potentials within a repetitive firing sequence. However, a large depolarizing after-potential (20 to 30 mV) was not essential for the generation of the repetitive response, 5 to 10 mV was apparently sufficient (Narahashi and Haas, 1968). After the last spike of the repetitive discharge and decline of the depolarizing after-potential, the membrane potential returned to its normal resting value (Narahashi and Yamasaki, 1960(a,b); van den Bercken, 1972; Pichon, 1976).

Figure 4: Repetitive after discharge superimposed upon a depolarizing after potential (from Narahashi and Yamasaki (1960a).

Voltage clamp experiments using the sucrose-gap technique were employed to study the effects of DDT on membrane currents in lobster axons. Two major

alterations, in both peak transient (normally an inward current created by movement of sodium ions across the membrane) and steady state (normally an outward current created by movement of potassium ions across the membrane) currents, were detectable from a comparison of the families of membrane currents for normal and DDT-poisoned axons. The differences noted between current-clamp curves from normal and DDT treated axons were: 1/ the much slower decline of the peak transient current (inward) and the residual inward steady state current; and 2/ the steady state current (outward) was much reduced in size. The inward steady state current upon small voltage-clamp depolarizations was apparently accounted for by the slow (delayed) turn-off of the peak-transient current (Narahashi and Haas, 1968). The time course of the decline of peak transient current is markedly prolonged into at least two phases, as opposed to the single-slope exponential decline experienced with untreated, normal axons (Hille, 1968; Narahashi and Haas, 1968; Narahashi, 1979). This steady state (inward) current could be calculated by subtraction of the membrane current of axons treated with DDT and TTX from the membrane current of the axon treated with DDT alone.

The slow turn-off of the peak transient current initially obscured conductance changes of the steady state current. However, if the DDT treated axon was also exposed to TTX then only the K⁺ steady state conductance

could be seen. (TTX selectively blocks the sodium channels from outside the nerve membrane. This implicated sodium and sodium channels as the source of the steady state inward current after small depolarizations (Moore et al., 1967).) Also, the maximum steady state conductance of a DDT-treated axon was found to be only 37% the normal value (Narahashi and Haas, 1968). The time required before maximum steady state current was reached for DDT-treated axons was increased between 1.4 and 1.6 fold, depending on the membrane potential.

3/ DDT in Relation to Selected Pharmacological Agents

Narahashi and Haas (1968) hypothesized that DDT exerted its effects by physically entering the nerve membrane (DDT being extremely lipid soluble). By dissolving in the lipid phase of the membrane, the DDT molecule could interact directly with the lipoprotein molecular structure and possibly have access to the inner part of the nerve membrane. It might then influence the molecular structure controlling the gating mechanism for shutting off the peak transient current (sodium inactivation mechanism). (Opening and closing rates for sodium channels are relatively insensitive to the ionic composition with respect to monovalent cations in the bathing medium, implying that gating is a structural property of the channel rather than a result of the movement or accumulation of particular ions around the

channel (Hille, 1972).)

As mentioned earlier, the turning on process and the rise to peak transient current for an action potential in an axon poisoned with DDT is not significantly altered. However, the peak transient current is severely impaired by TTX at concentrations as low as 90 nM (Narahashi, 1965; Moore et al., 1967; Pichon, 1969-cited in Pichon, 1974). TTX is not lipid soluble and interacts with the nerve membrane on its external surface. Its blocking action is very specific for peak transient current, not ionic species (i.e., lithium substituted for sodium would also be blocked (Hille, 1972)), without interfering with activation and inactivation mechanisms (Pichon, 1971; 1974; 1976).

Two compounds, N-bromoacetoamide and pronase, destroy sodium inactivation when perfused inside squid axons (Armstrong et al., 1973; Oxford et al., 1978; Meves, 1978). The inhibition of the sodium inactivation was very specific and did not alter sodium and potassium activations. This observation was important because it indicated that a protein which was accessible from the inner side of the membrane was an essential part of the inactivation mechanism. Thus, it is likely that DDT must at least enter the nerve membrane in order to exert its proposed effect on the inactivation protein.

It has also been shown that DDT exerts an effect on the steady state potassium (outward) current. Studies performed on squid giant axons using tetraethylammonium chloride (TEA) have indicated that a gating mechanism for the increase in steady state current was located on the internal surface of the nerve membrane (luminal side) (Tasaki and Hagiwara, 1957; Armstrong and Binstock, 1965; Adelman and Senft, 1966). However, other studies indicated that TEA applied externally (ca. 25 mM) in squid and cockroach axons and frog nerves also selectively blocked potassium channels (Hille, 1967; Pichon, 1974; 1976), possibly by TEA passing through sodium channels or via an alternate membrane mechanism (Pichon, 1971; 1976). The decrease in potassium conductance of an axon without significant alteration of the kinetics involved is a characteristic of poisoning with both DDT and TEA. The time course for the increase in potassium conductance was not significantly altered by TEA (Pichon, 1974; 1976), implying that the "channels" are blocked and the kinetics of the increase in potassium conductance are unaffected. Similarly, only the magnitude of potassium conductance was affected by DDT treatment.

The outline above indicates that alteration of the action potential by DDT was accounted for by a delay in the onset of sodium inactivation and a decrease in potassium conductance. Research on myelinated nerve

membrane from the frog has revealed that there is another possibility regarding the mechanism of action potential alteration by DDT.

An alternative hypothesis was presented by van den Bercken and Vijverberg (1980). They suggest that in the nodal membrane from frog myelinated nerve, it is not the sodium-inactivation process that is altered (h-gates) but that the activation process (m-gates) does not close when the membrane potential is repolarizing following an action potential. Normally, at the resting membrane potential most of the m-gates are hypothesized to be in the closed configuration while most of the h-gates are open (Hodgkin and Huxley, 1952). As the membrane is depolarized the m-gates open and the h-gates begin to close, thus allowing sodium ions to flow through the open channel. But, as the membrane becomes more depolarized, the h-gates (inactivation) begin to close. (The closing of the h-gates occurs at a much slower rate than the opening of the m-gates.) Membrane potential then begins to repolarize as more and more h-gates close, blocking further entry of sodium ions. Repolarization of the membrane leads to the reactivation state (i.e., h-gates reopen and m-gates close). However, according to van den Bercken, in the DDT-poisoned nerve, all proceeds normally except the final closing of the m-gates upon repolarization. The DDT molecule(s) prevent some of the m-gates from closing. Thus, when the h-gates reopen upon

repolarization sodium ions are free to rush through the DDT-affected m-gates. This sudden influx of sodium is presumed to induce a further action potential, which multiplies to form the characteristic DDT spike-train. Thus, the delayed closing of the m-gates is hypothesized as being responsible for the repetitive firing response in frog myelinated nerves poisoned by DDT. Opening and closing of the h-gate and opening of the m-gate are little affected by DDT, according to van den Bercken's hypothesis.

As the lipophilic nature of the DDT molecule facilitates its interaction with membrane components, it is likely that the molecule actually dissolves in the lipid phase of the membrane and interacts there by exerting an intermolecular force within the lipoprotein matrix (Holan, 1969; Pichon, 1974). The force exerted must act in such a way as to alter sodium channel characteristics (inactivation) and potassium conductance, simultaneously, according to the Narahashi hypothesis of DDT action. From evidence gained regarding channel characteristics employing DDT, TTX, TEA, pronase, and N-bromoacetamide it is hypothesized that the phenomena of sodium activation and inactivation are independent processes and that sodium channels are distinct from potassium channels (Armstrong et al., 1973; Pichon, 1976; Oxford et al., 1978; Meves, 1978). However, the details of DDT's interaction with these nerve membrane processes

remains an unresolved problem.

(c) Interaction of DDT with Fractioned Components of the CNS

The following experiments are illustrative of some of the problems and traps associated with extrapolating in vitro evidence to an in vivo system. However, they also illustrate and support the hypothesis that DDT is capable of interacting with membrane lipoprotein complexes.

The actual molecules in the membrane with which DDT interacts remain unknown. Several research groups have employed ¹⁴C-DDT to trace the movement of DDT on and into nerve membrane components (Camejo et al., 1969; Barnola et al., 1971; O'Brien, 1975)). They used a fractionation procedure which isolated two particular membrane fractions from the nerves of squid. The two isolated fractions were: 1/ the axolemma or axon excitable membrane (Membrane Fraction 1); and 2/ plasma membrane of the periaxonal cells, including Schwann cells (Membrane Fraction 2). A soluble cytoplasmic fraction was also obtained during the membrane isolation procedure. When experiments were performed this fraction was found to contain an insignificant amount of radioactivity compared to the other fractions. This indicated to them that DDT associated mainly with cellular membranes.

Results showed that nerves incubated with ¹⁴C-DDT prior to isolation of the membrane fractions picked-up a

significant amount of ^{14}C -DDT. Subsequent isolation of Membrane Fraction 1 and 2 revealed that there was little, if any, difference in radioactivity associated with the two fractions (Barnola et al., 1971). They found 0.4 nmol DDT/mg membrane associated with Membrane Fraction 1 and 0.5 nmol DDT/mg membrane associated with Membrane Fraction 2. This indicated that the two Membrane Fractions apparently had very similar affinities for the ^{14}C -DDT. But, they do not discuss the possibility that this result may also indicate that the two fractions could not be separated without equal partition of DDT between them.

However, further experiments entailed ^{14}C -DDT being injected below clean films (monolayers) of sonicated Membrane Fractions 1 and 2 (Barnola et al., 1971). (The surface pressure used (17 dynes/cm) was found previously to enhance ^{14}C -DDT's association with lipid monolayers.) They calculated that a greater amount of ^{14}C -DDT associated with Membrane Fraction 2 films (0.36 nmol DDT/cm film area) than with those of Membrane Fraction 1 (0.29 nmol DDT/cm film area). It was then postulated that the greater protein content of Membrane Fraction 2 (48.3% of total dry weight) was responsible for the greater affinity of that fraction for ^{14}C -DDT (Membrane Fraction 1 Protein = 29.5% of total dry weight). (The percent of dry weight not due to protein was lipid.) That DDT would associate with the fraction containing a greater amount of

protein may seem peculiar considering DDT's lipophilic nature. However, much of the protein associated with the membrane is hydrophobic, which would increase the possibility of DDT binding. Also, the higher cholesterol content of Membrane Fraction 1 (19% of total lipid) compared to Membrane Fraction 2 (13% of total lipid) may have contributed to the relative lack of affinity Membrane Fraction 1 had for DDT. (The apparent difference in affinity for DDT between monolayers of Membrane Fraction 1 and 2 supports the suggestion above, i.e., that the two fractions could not be separated from a pretreated CNS (14C-DDT) without cross-contamination). O'Brien (1975) indicated that surface tension of cholesterol monolayers was completely unaffected by DDT. Furthermore, he showed that there was little molecular interaction between DDT and cholesterol until the monolayer film was compressed. This suggests that the higher cholesterol content of Membrane Fraction 1 may contribute to the net forces excluding DDT from the monolayer. The higher concentration of cholesterol would also increase both rigidity and stability of the monolayer (Gregoriadis and Davis, 1979; Kirby et al., 1980) which may also contribute to the final net reduction of DDT binding. Other results (Hilton and O'Brien, 1973) showed that monolayers made from extracts (lipid and protein) of electroplax membrane of the electric skate (which is rich in neural membranes) complexed with the DDT molecule to the same extent as artificial lecithin monolayers.

Given the lipophilic nature of DDT and the evidence presented above, it may be inferred that a DDT-membrane

complex would be influenced by both the lipid molecules and hydrophobic portion of the protein constituents in the membrane, as postulated by the fluid mosaic model of membrane structure (Singer and Nicolson, 1972). The interaction between the two components (lipid and protein) are thus inseparable and mutually dependent. However, O'Brien (1975) suggested that the monolayer represents a special situation. In the monolayer, DDT is free to make complexes with the hydrophobic component of phospholipids which are exposed to air. In a bilayer, by contrast, the DDT molecule would associate with the inside (hydrophobic) surface between the two monolayers of lipid. Consequently, the effects of DDT on lipid-protein monolayers must be interpreted and extrapolated with the utmost of caution. Any apparent relation to an in vivo system may be fortuitous. However, the experiments did indicate that DDT would associate with a proteinaceous medium and that a high lipid content is not the only criterion for increased DDT association.

(d) Molecular Aspects of DDT Action

Several hypotheses have been advanced regarding the actual molecular mechanisms involved in DDT poisoning (Matsumura and O'Brien, 1966; Holan, 1969; Metcalf et al., 1971; Fahmy et al., 1973; Coats et al., 1977; 1979). These studies have dealt mainly with DDT's structure-activity relationships based on the use of

molecular models. Attempts have been made to correlate the size and shape of the molecular model with interaction at a hypothetical DDT receptor site.

In 1956, Mullins proposed that DDT entered a membrane interspace (i.e., the channel he theorized was responsible for movement of sodium and potassium ions during an action potential) in a single, specific orientation (Mullins, 1956). Up to 20 molecules of DDT could fit across the membrane in this interspace. Positioning of DDT in these channels was hypothesized to cause an enlargement of adjacent interspaces and induce leakage of sodium through the axonal membrane. However, his model for the interaction of the DDT molecule in the membrane was purely theoretical and does not satisfy the present electrophysiological evidence or the pharmacological data (which confers support on the two channel hypothesis previously described). (For example, the leakage of sodium across the membrane postulated to account for repetitive discharges (by Holan, 1969) would also induce a change in resting membrane potential. However, this has been shown not to be the case (Narahashi and Yamasaki, 1960(a,b); Pichon, 1976).) Also, Mullins' hypothesis is based upon interaction of DDT with a single channel which was predicted to allow both sodium and potassium to flow through it during an action potential.

However, evidence presented for DDT and TTX may be used to support the dual-channel hypothesis for ionic

conduction (i.e., that sodium and potassium ions cross the axonal membrane via structurally distinct channels). The evidence indicates that the inward steady state current (carried by sodium ions) induced by poisoning of the axon with DDT flows simultaneously with the outward steady state current (carried by potassium ions). This is in direct contrast to the single-channel hypothesis proposed by Mullins (1956). Also, controlling mechanisms for peak transient current (activation and inactivation) have been postulated to exist at two independently operative sites (Moore et al., 1967; Armstrong et al., 1973; Oxford et al., 1978; Meves, 1978).

Holan (1969) revealed that there were certain toxic DDT analogs and cyclopropanes whose actions could not be accounted for by Mullins' steric receptor model. (Holan employed cyclopropane-DDT analogs for ease of synthesis and increased stability over some of their DDT-counterparts. Symptoms of poisoning by cyclopropane analogs were apparently indistinguishable from their DDT counterparts.) His model was a modified version of the model created by Mullins (1956). It related the toxicity (activity) to two basic structural features of the molecule: 1/ the nature of substituents and their π -electron density found on the phenyl rings; and 2/ the size of the cyclopropane ring.

Cyclopropane

Cyclopropane-DDT Analog

The DDT molecule was hypothesized to associate with the lipid-protein nerve membrane interface. The phenyl portion of the molecule would interact with the protein layer while the cyclopropane moiety would act as an apex and fit into the channel of the pore in the lipid part of the membrane forming a "molecular wedge" (Holan, 1969). The channel would thus be locked open, allowing more sodium ions to flow across the membrane. The apparent paradox created (i.e., the cyclopropane moiety both plugging the channel and causing an increase in the sodium permeability) was resolved by hypothesizing that 'the phospholipoprotein found in biological membranes forms a "spring" which when expanded (by the DDT molecule) is selective for sodium ions' (Holan, 1969). The complexing of the DDT analog with protein on the nerve membrane was suggested because of evidence presented by Matsumura and O'Brien (1966). They claimed that neither active cyclopropanes or DDT would form complexes with pure lipid nerve components. This suggested that a charge-transfer

complex was formed with the protein component of the nerve membrane. However, the validity of the charge-transfer complex hypothesis (O'Brien and Matsumura, 1964; Matsumura and O'Brien, 1966) has since been questioned (Hatanaka et al., 1967; Wilson et al., 1971), although it is likely that both lipid and protein act in concert to bind the DDT molecule.

Fahmy et al. (1973) studied the effects of silicon-containing DDT analogs using a multiple regression analysis to compare the effects of various substituents on toxicity. They envisaged the receptor site as a cavity or pouch with a limited amount of flexibility (i.e., not rigid). The most effective analogs would fill this receptor site as closely as possible with little or no distortion of the receptor molecule(s). Maximum interaction without torsional strain would result in the greatest possible activity (i.e., toxicity of the compound). Reduced interaction would result in reduced activity. Overall size of the molecule (and not whether an analog's constituents were symmetrical) was the important factor in ultimately determining toxicity (Fahmy et al., 1973).

Although Fahmy et al. (1973) lacked sufficient evidence to substantiate their steric fit hypothesis, later evidence supported their contention (Coats et al., 1977). Coats et al. employed effective (i.e., toxic) DDT analogs

with altered aliphatic moieties (isobutane and chloropropane substituted), and studied the toxicity of these analogs to various insects in relation to the van der Waals volume of the various constituents (i.e., the probable volume occupied by a molecule in space, determined by the density of electron charge surrounding a specific nucleus). Analysis revealed that a sharply defined minimum volume was required and a progressive loss of toxicity paralleled an increase in the size of substituents (Coats et al., 1977).

Steric interaction between the aliphatic moiety and the phenyl rings were shown to be ultimately responsible for the insecticidal activity of a particular analog (Coats et al., 1977; 1979). If aromatic substituents were kept constant, altering the aliphatic moiety would increase or decrease the toxicity of the molecule, depending upon the final overall volume occupied.

Thus, the evidence suggests that the hypothetical DDT receptor site of the nerve membrane is specific for the DDT molecule for several reasons: 1/ the overall "volume" of the DDT molecule closely fits the available space at the receptor site; 2/ the interactions between aliphatic and aromatic substituents are optimum; and 3/ although symmetry of the substituents on the phenyl rings is not essential for toxicity, steric interaction between phenyl substituents may alter insecticidal activity. These

requirements for activity are consistent with the previously mentioned evidence regarding DDT's possible interaction with a membrane protein to alter sodium inactivation.

The above hypotheses are based upon the relation between the insecticidal activity (potency) of a compound, determined by topical application or injection, and the interaction of the molecule with a proposed receptor site in the membrane. However, topical application of DDT or analog would be followed by a series of reactions involving cuticular penetration, metabolism, circulation within the body and deposition in various tissues, and penetration to the site(s) of action (O'Brien, 1967; Narahashi, 1971). Insecticide toxicity studies employing isolated sections of a nervous system largely circumvent any of these other interactions which could occur if the insecticide had been applied topically. These studies have attempted to elucidate the structure-activity relationships of DDT and analogs with direct action on the nerve membrane (Uchida et al., 1974; Wu et al., 1975; Gardner and Vincent, 1978; Salvisberg et al., 1980; Brown et al., 1981) using the ability of a particular compound to disrupt an isolated nervous system by inducing a depolarizing after-potential and/or repetitive discharges as a measure of neurotoxicity. However, only with caution may results obtained from in vitro neurotoxicity studies

be extrapolated to the whole animal.

Uchida et al. (1974) compared the minimum effective dose to induce repetitive firing in the isolated ventral nerve cord of cockroaches to the minimum effective dose (injected intra-abdominally) required to induce convulsive behaviour in cockroaches. From their analysis, they reported that an increase in convulsive activity was directly proportional to the neuroexcitation induced in the nervous system by that compound. However, no attempt was made to actually correlate the convulsive behaviour displayed by a particular cockroach with the simultaneous or subsequent activity in the CNS of that same cockroach. Without this analysis, the direct relationship described may have been fortuitous. Confidence in his data relating neurotoxicity, behaviour, and mortality is further reduced by their hypothesis that a direct relationship existed between neurotoxicity and convulsive behaviour in cockroaches and the mortality in Azuki bean weevils (Uchida et al., 1974).

A further attempt to relate neurotoxicity of cockroach nerve (crural) and insecticidal activity in unrelated species (house fly and mosquito larva) was made by Brown et al. (1981). The doses of various compounds which induced trains of impulses in 50% of treated crural nerves within 30 min of application (ED50) were compared to LD50 values reported for various species of flies and

mosquito larvae. Linear regression analyses were performed on the graphical representations of neurotoxicity versus LD50 for two groups of compounds (ethoxychlor analogs with altered aliphatic moieties and p,p'-substituted diaryl trichloroethanes). The regression analysis performed by Brown et al. (1981) attempted to determine whether a correlation existed between threshold toxicity to crural nerve and topical toxicity of ethoxychlor analogs to susceptible houseflies. (They reported that the regressions were significant, although the r-values were only 0.756 and 0.617, respectively, for the two groups of compounds.) However, although the significance of the relationship appears to be dubious, a graph illustrating the regression between threshold toxicity and size of the aliphatic portion of ethoxychlor analogs was more convincing. It appeared to illustrate that there was an optimum aliphatic volume for neurotoxicity (Brown et al., 1981). As the aliphatic moiety of a series of analog molecules became smaller or larger, their ability to induce trains of impulses in the crural nerve became progressively less. Also, molecules with greater aliphatic conformational freedom displayed greater neurotoxicity. (This analysis compared neurotoxicity to molecular structure, without the intervention of a comparison with LD50 values for an unrelated species.) They also examined the effects of altering the p,p'-substituents of the phenyl rings.

Their results appeared to indicate that overall, ED50 values did not correlate well with lethality, although they claimed a correlation did exist between the analogs tested. Certainly the curve (ED50s vs. insecticidal toxicity) does exist but it should be noted that the correlation coefficient values were low (see above). On the other hand, the structure-activity (neurotoxicity) relationship elaborated was very similar to the structure-activity relationships and hypothetical receptor site previously elucidated (Fahmy et al., 1973; Coats et al., 1977).

Other reports of structure-activity relationships have been more conservative in their generalizations and hypotheses (Wu et al., 1975; Takeno et al., 1977; Gardner and Vincent, 1978; Salvisberg et al., 1980; Adams and Miller, 1980). Characterization of the response elicited by a nerve preparation in response to DDT or analog treatment were examined. The data was not extended to attempt to elucidate the relation between neurophysiological symptoms of poisoning, behaviour, and mortality (except for Adams and Miller, 1980). They merely documented the effects of various compounds on the isolated nervous system. Adams and Miller (1980), compared pyrethroid and DDT-type insecticides and their abilities to induce behavioural and neural symptoms of poisoning. They employed the housefly for both

neurophysiological and behavioural experiments and questioned whether or not in vivo physiological measurements could be correlated with the differences observed in symptomology. The specific physiological parameter measured was the repetitive discharging and motor backfiring in flight motor units of intact flies. (Motor backfiring was described as follows: when a single orthodromic nerve spike, upon reaching the motor terminals, triggers a discharge which passes postsynaptically and also "backfires" into the main part of the axon (Adams and Miller, 1979).) They concluded that the motor backfiring was probably not directly related to the onset of poisoning symptoms even though their time courses of onset were parallel. Some of the pyrethroid analogs which were highly toxic did not induce motor backfiring. Symptoms of poisoning and toxicity followed a negative temperature coefficient relation while motor backfiring disappeared at lower temperatures. This evidence indicated to them that backfiring was probably a side effect of the poisoning process and not related to the symptoms of poisoning and toxicity (Adams and Miller, 1980).

The lack of correlation between neurophysiological activity and symptoms of poisoning illustrates the large degree of care which must be exercised when interpreting results. Other results which further illustrate this point are the DDT analogs which have displayed

neurotoxicity following in vitro application to nerve preparations but have failed to be classified as active insecticides (e.g., p,p'-NO₂-DDT, p,p'-NH₂-DDT, and p,p'-OH-DDT: Metcalf and Fukuto, 1968; p,p'-HO-DDT, p,p'-NO₂-DDT: Wu et al., 1975), presumably because of some physical or metabolic barrier which prevents these compounds reaching their site of action.

Several points regarding the structure-activity relationships concerning the toxicity of DDT and analogs may be summarized:

1/ There is a specific range of steric forms for DDT and analogs that can be accommodated by a hypothetical nerve receptor site and be classified as insecticidal.

2/ There is an optimum steric volume which the DDT or analog molecule must conform to in order to exhibit insecticidal and/or neurotoxicity.

3/ Substituents located both on the phenyl rings and the benzylic (alpha) carbon are important in determining toxicity of the analog. Interactions between the two groups must also be considered.

4/ The substituents and conformational flexibility of the aliphatic moiety are important determinants of neurotoxicity, in vitro. A greater flexibility of the aliphatic moiety allowed the molecule to better "fit" into the receptor site.

5/ Because of the complex series of interactions

between the applied insecticide and whole animal, in vivo,
caution must be exercised when attempting to extrapolate
data from in vitro to in vivo.

(e) Possible Involvement of an Endogenous Toxic Substance

Sternberg and Kearns (1952) postulated that there was an endogenous toxic factor released into the haemolymph of DDT-poisoned cockroaches. Haemolymph from cockroaches poisoned by DDT was removed, quick frozen, thawed and centrifuged before small quantities (up to 5 ul) were injected into houseflies. The haemolymph from treated cockroaches was toxic to both DDT-sensitive and resistant flies. These injected flies displayed poisoning symptoms, including a negative temperature coefficient, and death characteristic of DDT poisoning. The level of DDT in the haemolymph was quantitatively determined to be from 0.5 to 15 ppm (Sternberg and Kearns, 1952), although the toxicity of the haemolymph did not correlate with the concentration of DDT. But, credibility would have been jeopardized had they not quantitatively removed the traces of DDT from further haemolymph samples. Extraction with ether or benzene effectively removed traces of DDT previously detected (to a level < 0.05 ug). The extraction procedure did not alter the potency of the haemolymph and its effectiveness to induce signs and symptoms of poisoning.

Further evidence (Shankland and Kearns, 1959; Sternberg et al., 1959; Sternberg, 1960, 1963; Hawkins and Sternberg, 1964; Flattum and Sternberg, 1970) supported the existence of an endogenous toxic factor in the haemolymph of cockroaches poisoned or subjected to severe

physical stress. This endogenous toxic factor (endotoxin) was neuroactive and produced symptoms of poisoning in the isolated ventral nerve cord (cockroach). The symptoms elicited were very similar to those observed in DDT-poisoned preparations. Nerve cords treated with the endotoxin displayed an increase in spontaneous activity and symptoms of hyperexcitability. If only the sixth abdominal ganglion was treated, followed by stimulation of a cercal nerve, recurrent trains of impulses would be elicited in the abdominal connectives. Hyperexcitation was normally followed by synaptic blockage in the abdominal ganglia (Shankland and Kearns, 1959). This neuroactive substance increased in concentration in the haemolymph of poisoned cockroaches as poisoning symptoms progressed. Thus, the haemolymph/endotoxin isolated from cockroaches displaying only minor signs of poisoning was not as effective in producing hyperactivity in an isolated nervous system. However, Gammon (1978) found that cockroaches poisoned with DDT elicited repetitive discharges in their nervous systems before any overt signs of poisoning were displayed. Haemolymph/endotoxin isolated from cockroaches poisoned but not displaying signs of poisoning was not effective in inducing hyperexcitation in an isolated nervous system (Shankland and Kearns, 1959). Thus, the endotoxin apparently cannot account for the symptoms of poisoning in the nervous system before overt signs become evident, or the

concentration of endotoxin in the haemolymph of cockroaches showing symptoms of nervous system disruption but not overt behavioural signs, may be too low to be detected (using their bioassay technique).

Further studies delineating the chemical nature of the endotoxin and studies charting its appearance and concentration in the haemolymph from time of poisoning are required before a reasonable correlation between endotoxin and neurotoxicity/lethality is established.

As mentioned above, the chemical nature and origin of the endotoxin have not been elucidated. An unsubstantiated hypothesis (Tashiro et al., 1972) implicated L-leucine as the neuroactive factor. A more convincing hypothesis proposed that the corpora cardiaca were responsible for the production and secretion of a neuroactive factor which induced hyperexcitability in the nervous system (Hodgson and Gelding, 1959; Milburn et al., 1960; Brown, 1965; Natalizi et al., 1970). However, evidence regarding the neuroactive characteristics of this factor suggested that Sternberg's endotoxin associated with DDT poisoning was not the same as that isolated from the corpora cardiaca.

The direct action of insecticides on neurosecretory neurons has also been investigated. It has been found that insecticides can induce the release of neurohormones from neurosecretory cells (Granett and Leeling, 1972;

Maddrell and Reynolds, 1972; Orchard and Osborne, 1979). These neurohormones could arise from many different cells during the intoxication process. As yet no attempts have been made to correlate one of these neurohormones to the DDT endotoxin of Sternberg and Kearns (1952).

(f) Effects on the ATPase Enzymes

1. Mg-Dependent and Na-K-Dependent ATPase

There is a hypothesis for the mode of DDT action which implicates the mitochondrial magnesium-dependent adenosine triphosphatase enzyme (Mg-ATPase - also known as the oligomycin-sensitive Mg-ATPase). This particular ATPase system normally catalyzes the terminal step in oxidative phosphorylation (Penefsky, 1974). It is quite distinct from the sodium-potassium-dependent ATPase system that is found in the excitable membranes of the nervous system and is responsible for maintaining ionic gradients of sodium and potassium across those membranes.

Although early speculation supported the latter ATPase system as a potential target for DDT (Matsumura and Patil, 1969), later work indicated that the sodium pump Na-K-ATPase was not likely to be the target (Matsumura, 1970; Matsumura and Narahashi, 1970; Cutkomp et al., 1971). This conclusion was mainly the result of a study exploring the possible correlations between electrophysiological changes in the nerve membrane and

various pharmacological agents. They found that ouabain (a specific inhibitor of Na-K-ATPase) induced symptoms of poisoning entirely different than those found to occur with DDT. Furthermore, if the Na-K-ATPase associated with the membrane Na-K pump was the target for the DDT molecule, then substantial changes would have been realized in the resting membrane potential (as was seen with ouabain). Such was not the case (Narahashi and Yamasaki, 1960(a,b); Narahashi and Haas, 1968; Gardner and Bailey, 1975). The Na-K ATPase as possible target site for DDT, in vivo, was not pursued any further. Other results (Cutkomp et al., 1971; Desai et al., 1974; Desai et al., 1975; Cutkomp et al., 1976) indicated that the Mg-ATPase was more sensitive than the Na-K ATPase to DDT poisoning. Mg-ATPase of muscle, kidney, and liver tissue were the most sensitive to DDT; brain Mg-ATPase was less sensitive. Therefore, extrapolation of data between different tissue types is difficult. However, muscle Mg-ATPase has normally been utilized when performing various DDT inhibition studies because of its greater abundance there and its inherently greater sensitivity. But, muscle has been shown to be virtually unaffected by DDT; the primary site of action of DDT appears to be the nervous system (Matsumura and Narahashi, 1971; Cutkomp et al., 1971; Gammon, 1978).

Cutkomp et al. (1971) claimed to find that the

Mg-ATPase of fish brain mitochondria were sensitive to DDT. They further speculated that it was a disruption of oxidative phosphorylation in the mitochondria that was ultimately responsible for the altered electrical activity in the nerve. (It should be noted that the outcome of oxidative phosphorylation is the production of ATP. However, they examined the Mg-ATPase as an enzyme (in vitro) which catalyzed the cleavage of ATP, contrary to it's in vivo function!) However, the lack of resting membrane potential change (Narahashi and Yamasaki, 1960(a,b); Pichon, 1976) is evidence that the Na-K pump, which is driven by the metabolic energy produced in oxidative phosphorylation (i.e., ATP), is operative and not starved for a supply of ATP. If the inhibition of the Mg-ATPase of mitochondria (in vivo) was affecting the supply of ATP for the Na-K pump, then a 6 to 8 mV depolarization of the resting membrane potential would be expected eventually (Gardner and Bailey, 1975). Furthermore, the permeability changes which occur and conduction of the action potential are processes which are independent of metabolic energy (Kuffler and Nicholls, 1976; Narahashi, 1980). Evidence supporting this can be derived from experiments with axons poisoned with potent metabolic inhibitors, such as 2,4-DNP, cyanide, and azide (Hodgkin and Keynes, 1955; Narahashi, 1980). Therefore, the contention that the disruption of the Mg-ATPase and oxidative phosphorylation process is responsible for the

alteration of nervous activity and ultimately mortality cannot be supported.

Still attempting to relate the Mg-ATPase to DDT toxicity, several reports (Doherty and Matsumura, 1975; Cheng and Cutkomp, 1975; 1977) showed that their oligomycin-sensitive Mg-ATPase was more sensitive to DDT, DDD, methoxychlor, and DDE at cool temperatures than at warmer temperatures, thus implicating a negative temperature coefficient of inhibition (in vitro). Although this negative temperature coefficient parallels the temperature dependence of toxicity to DDT in vivo, they found that oxidative phosphorylation in their mitochondrial preparation displayed a greater sensitivity to DDT at higher temperatures, i.e., a positive temperature coefficient!

Therefore, the supposition of Cheng and Cutkomp (1977), that the negative temperature coefficient of the isolated oligomycin-sensitive-Mg-ATPase parallels the in vivo toxicity of DDT, could be merely fortuitous.

2. Ca-Dependent ATPase

Two types of Ca-ATPase have been isolated and identified (Matsumura and Ghasuddin, 1979). An inner membrane Ca-ATPase is reportedly responsible for actively expelling calcium ions from the axoplasm, maintaining a low concentration inside (ca. 0.5 μM) (Kuffler and

Nicholls, 1976; Matsumura and Ghiasuddin, 1979). The ecto-Ca-ATPase is believed to be situated on the external surface of the axonal membrane. It has been hypothesized to be responsible for the maintenance of a high calcium concentration (10^{-2} to 10^{-3} M) on the surface of the membrane (Trams and Lauter, 1977; Matsumura and Ghiasuddin, 1979). Presumably, if the concentration of calcium decreases below the optimum concentration then the ecto-Ca-ATPase would actively (i.e., employing metabolic energy in the form of ATP) sequester calcium from the bathing medium. If it was above the optimum, then the ecto-Ca-ATPase would cease to function or actively expel calcium ions from the membrane's surface.

This ecto-Ca-ATPase has been implicated as a possible site of action of DDT (Matsumura and Ghiasuddin, 1979; Ghiasuddin and Matsumura, 1979; Ghiasuddin et al., 1981). Of all the ATPases examined, this appears to be the only one which has a theoretical basis which may allow it to contribute to the general DDT-poisoning syndrome. They propose that DDT inhibits the ecto-Ca-ATPase associated with the axonic membrane. This inhibition would supposedly allow the external membrane calcium to fall below optimum concentrations and thus cause the membrane to become unstable. The instability would lead to spontaneous oscillations of the membrane potential or repetitive activity (Frankenhauser and Hodgkin, 1957).

The ecto-Ca-ATPase has been isolated from the

peripheral nerves of the lobster (Matsumura and Ghiasuddin, 1979; Ghiasuddin and Matsumura, in press), lobster muscle (from sarcoplasmic reticulum) (Ghiasuddin and Matsumura, in press), and from the brains of cockroaches (Ghiasuddin et al., 1981). Preparations from peripheral lobster nerve and cockroach brain displayed identical enzymatic characteristics whereas that from sarcoplasmic reticulum of muscle was much less sensitive to DDT inhibition. (This was contrary to the muscle Mg-ATPase which displayed a higher sensitivity than nerve Mg-ATPase.) The concentration of DDT required to inhibit 50% of the Ca-ATPase activity was 10 to 10,000 times lower than that reported for other ATPases against DDT (Matsumura and Patil, 1969; Cutkomp et al., 1971; Desai et al., 1974; Cheng and Cutkomp, 1976). 50% inhibition was obtained with 10^{-9} M p,p'-DDT. The sensitivity at this dose is comparable to the concentration of tetrodotoxin employed to block sodium channels (Matsumura and Ghiasuddin, 1979), illustrating the apparent specificity and vulnerability of the ecto-Ca-ATPase to inhibition by DDT.

Employing axonal membrane fragments containing the ecto-Ca-ATPase and the radioactive isotope ^{45}Ca , it was also shown that DDT inhibited calcium binding to the membrane, in vitro. ATP was required in the reaction medium for the operation of the Ca-ATPase. This supported their hypothesis that DDT may decrease calcium binding to

the nerve membrane by inhibiting the ecto-Ca-ATPase (Matsumura and Ghiasuddin, 1979).

The negative temperature coefficient of DDT toxicity is an important factor when considering possible sites of action of DDT. One of the major shortcomings of the Mg-ATPase hypothesis was its lack of correlation with the negative temperature coefficient. However, the inhibition of the ecto-Ca-ATPase by DDT did follow a negative temperature coefficient (Matsumura and Ghiasuddin, 1979).

Ecto-Ca-ATPases isolated from two resistant strains of cockroaches (VPIDLS and VT - Ghiasuddin et al., 1981) were found to be relatively insensitive to DDT when compared to the susceptible one. The enzymatic characteristics of the ecto-Ca-ATPase from susceptible and resistant cockroaches were otherwise identical, differing only in their sensitivity to DDT.

The ecto-Ca-ATPase has potential to actively contribute to the general poisoning syndrome. Its response to DDT poisoning, in vitro and in vivo, appear to parallel some of the symptoms of poisoning displayed by an intoxicated insect. A critical analysis must be performed to determine whether or not the binding of calcium, in vivo, could be altered. The relationship between inhibition of the ecto-Ca-ATPase and neurophysiological symptoms of poisoning have not been elucidated experimentally. However, it is the only ATPase system

which deserves further consideration as a possible factor in the process of nerve poisoning, and ultimately, mortality, caused by DDT.

3. METABOLISM OF DDT, in vivo

An insecticide exerts its effect on a target organism by producing abnormalities in essential biophysical (and/or biochemical) processes. The extent of disruption will determine the potency of a particular compound (Metcalf, 1967). However, at least several factors contribute to the susceptibility of a particular organism. These factors may be significant enough to render a potentially lethal compound relatively innocuous to a non-target organism or lethal to a susceptible organism (i.e., species selectivity). Physiological selectivity (Metcalf, 1967; Winteringham, 1969) can be achieved in three principal ways: 1/ by altering the insecticide such that its ability to be absorbed by the cuticle of insects far exceeds its ability to penetrate the epidermis of non-target organisms; 2/ rapid metabolic detoxification of the insecticide in the non-target organism; and 3/ a differential sensitivity at the site of action in the organism, in vivo (i.e., a very low sensitivity of the site of action in the non-target organism).

The latter two factors are chiefly responsible for the resistance or lack of resistance an insect species may

display. Resistant strains of insects normally arise from selection of a naturally occurring mutant within a population. One manner in which a mutant may increase its resistance to a pesticide is by producing an enzyme which metabolizes the pesticide to a non-toxic form at a much higher rate than normal (Metcalf, 1967). The best known metabolite of DDT in insects is DDE (Metcalf, 1967; O'Brien, 1967; Esaac and Matsumura, 1980). The rate of formation of DDE from DDT is likely to be the major cause of resistance in many species. The enzyme responsible, DDT-dehydrochlorinase, is usually specific for p,p'-DDT. However, some insects contain a DDT-dehydrochlorinase which also metabolizes MeO-DDT and p,p'-DDD, but does not act on o,p'-DDT (O'Brien, 1967). This illustrates that DDT-dehydrochlorinase may differ between species. It was reported (Esaac and Matsumara, 1980) that stable flies treated with ^{14}C -DDT produced DDE as the sole in vivo metabolite. Further inter-species variation is illustrated by the report that Grain weevils fed DDT-treated wheat apparently metabolized DDT mainly to DDD, with only partial conversion to DDE and other products (Rowlands and Lloyd, 1969).

It has also been suggested (Patil et al., 1980) that the ATPase enzyme system may be altered in resistant insects. The study indicated that the Mg-ATPase of sensitive and resistant strains were equally sensitive to

inhibition by DDT while the Na-K-ATPase of resistant strains displayed a marked tolerance to inhibition by DDT. However, this ATPase has previously been disregarded as a possible site of action for DDT (Matsumura, 1970; Matsumura and Narahashi, 1970; Cutkomp et al., 1971).

The conferring of resistance to DDT by alteration of the site of action remains largely unsubstantiated, although evidently a possibility that should be considered. It seems as though a simpler, more rapid method for resistance has already been 'implimented' by many insect species, namely, the enzymatic detoxification of DDT to DDE or other analog, depending upon the particular insect species involved.

4. EXPERIMENTAL OBJECTIVES OF THIS STUDY

Although DDT's effects on the isolated nervous system have been well established, there is a paucity of information regarding the effects of DDT on the in vivo treated nervous system (Gammon (1978) being the notable exception). The methods employed in the experiments presented below attempted to bridge the gap which exists between in vitro and in vivo results.

Gardner and Vincent (1978) first reported the possible long-term (3-week) effects of DDT on the cockroach CNS. Cockroaches which behaved in an entirely normal fashion, (3-weeks following a LD50 96 h dose of

p,p'-DDT or MeO-DDT) still displayed symptoms of poisoning (repetitive firing) in their CNS's. That report (Gardner and Vincent, 1978) was the initial stimulus for the work presented in this thesis, which attempts to resolve two main issues concerning the mode of DDT action:

1/ to resolve discrepancies associated with the cause and effect hypothesis for the mode of action of DDT (the hypothesis infers that DDT induced repetitive firing in the CNS is responsible for the mortality of the insect), and

2/ to obtain some evidence regarding the localization of DDT in the CNS.

The investigation of these two problems took the following directions:

1(a) Those analogs of DDT which are toxic and induce behaviour consistent with DDT poisoning would be expected to also elicit neurological symptoms of poisoning. Investigating the activity induced by electrical stimuli in ventral nerve cord from cockroaches poisoned with a LD50 96 h dose of DDT or analog was pursued to verify or refute this supposition.

(b) Because of the loss of behavioural signs of poisoning (usually approximately 24 h post-injection for DDT), the persistent nature of the DDT molecule, and the slow elimination of DDT from the susceptible cockroach, long-term (3-week) studies were of particular interest.

Evaluation of activity in the ventral nerve cord and its possible correlation with behaviour observed was pursued to further evaluate the validity of the cause and effect hypothesis. For the cause and effect hypothesis to be supported, the CNS of a cockroach surviving 3 weeks following a LD50 dose should respond in a normal fashion (as defined by control experiments) to a series of standard test electrical stimuli. To be refuted, the CNS must display symptoms of instability while the whole cockroach shows no sign of poisoning.

2. The site of action of DDT was investigated by performing several CNS fractionation experiments. They involved isolating a gross membranal fraction, mitochondrial fraction, and microsomal fraction from the cockroach CNS. Employing ^{14}C -DDT, the affinity of the various fractions for DDT, in vivo and in vitro, were determined. It was hypothesized that, if the Mg-ATPase hypothesis of DDT action was to be supported, sufficient DDT must reach the mitochondria to cause a significant inhibition of the Mg-ATPase.

MATERIALS AND METHODS

1. EXPERIMENTAL ANIMALS

(a) The Cockroach, Periplaneta americana (L.)

The experimental animal employed in all experiments was the American Cockroach, Periplaneta americana, (L.). The original parental stock of cockroaches was obtained from Carolina Biological Supply Co., Burlington, North Carolina. None were used in experimentation because of the possibility that some of them may have previously been exposed to an insecticide. Instead, egg cases deposited by females were collected and then isolated in a specially prepared tank where the temperature and relative humidity were maintained between 26 to 28°C and 60 to 80%, respectively.

(b) The Cockroach Colony

Housing units for the nymphs were comprised of cylindrical tubes up to 30 cm long and 2 to 4 cm in diameter. Several pieces of corrugated cardboard were also used (rolled or flat) to accomodate the first instar nymphs. This arrangement allowed cockroaches of various sizes to hide from each other and sun light. As adults and large nymphs began to appear, they were removed from the controlled environment of the hatchery and transferred to larger housing aquaria (150 litres - 40 x 30 x 60 cm), where humidity was not controlled and temperature was maintained between 23 to 26°C. A humid environment was

not essential for the well being of adult cockroaches. In fact, a lower humidity discourages pests such as lice and mites from infesting and destroying the colony (Cornwell, 1976). Specially cut, inverted rectangular boxes (14 x 14 x 30 cm.) were used as housing units in these tanks. (The boxes served to increase surface area within the tank (Fig. 5).)

The cockroach aquaria were placed in galvanized steel pans (8 cm high and 10 cm greater in length and width than the surrounded tank) which were filled with a strongly alkaline soap solution. This moat prevented mites and lice from crawling into the colony. An additional benefit of the moat was the further degree of protection provided against cockroaches escaping. Food (Ogilvie's rolled oats (Ogilvie Mills Ltd. Montreal) : debittered Brewer's Yeast, (4:1)) and water were provided, ad libitum. Food was contained in 30 cm diameter aluminum pie plates (two per tank) while water was supplied by an inverted bottle on glass wool. The colonies set up in this fashion were virtually self sustaining and able to provide the mature male cockroaches required for experimentation and egg cases for development of new colonies.

Only male cockroaches were utilized in the experimentation.

Experimental subjects were caught at random by hand. Cockroaches were then treated and housed individually in

Figure 5: Diagram of Cockroach Colony

Inverted boxes (B) increased the internal surface area of the aquarium and provided a dark hiding places for the cockroaches. Food in aluminum pie plates (F) and water-saturated glass wool (W, the type used in aquarium filters) were provided, ad lib. Vaseline, V, prevented the cockroaches from reaching the wire-mesh top. The moat, M, containing a strong alkaline solution, prevented mites and other creatures from crawling into the colony. T, wire-mesh top, not shown. Dimensions of the tank: length x width x height: 60 x 30 x 40 cm

one litre flasks with food and water provided, ad lib., until used. At no time were the cockroaches exposed to any anaesthetics (such as carbon dioxide). When surgery to remove the ventral nerve cord was required, the cockroaches were caught by hand, securely held and rapidly decapitated. Cockroaches did not normally struggle if held without undue pressure in one's hand.

2. WHOLE ANIMAL TOXICITY

(a) Injection Procedure

Each male cockroach to be injected was weighed in a pre-tared petri plate (15 x 50 mm). The dose, calculated according to his weight and the particular analog to be used, was then prepared in acetone (Anachemia Chemicals Ltd., Reagent UV Spectrophotometric Grade). Doses to be injected were freshly made for each experiment from stock solutions of organochlorine (O.C.) compounds (Table 1). O.C. stock solutions were kept in tightly sealed vials. The volume contained in each stock vial was marked and the weight recorded to ensure that a concentrating effect did not occur due to loss of solvent by evaporation.

The dose was injected in $1.0 + 0.05$ ul of acetone between the third and fourth abdominal sternite. This amount of acetone or saline alone proved to be non-toxic to the cockroaches over a three week period. The microsyringe (Hamilton Co., Model 701N 10 ul) was inserted so that the tip was directed toward the dorsal surface and

Table 1: The Structural Formulae of p,p'-DDT and the DDT Analogs.

DDT Analogs	V	W	Y	Z
p,p'-DDT	HCCC1 ₃	H	Cl	Cl
p,p'-OCH ₃ -DDT (methoxychlor)	HCCC1 ₃	H	OCH ₃	OCH ₃
p,p'-DDD	HCCHCl ₂	H	Cl	Cl
o,p'-DDT	HCCC1 ₃	Cl	H	Cl
p,p'-DDE	CCC1 ₂	H	Cl	Cl
14C-DDT	HCCC1 ₃ 14	H	Cl	Cl

approximately 1 cm from the sternite joint penetration site (Fig. 6). A different microsyringe was designated for each compound used, including saline and acetone. This minimized the chances of contamination occurring between the compounds. A cockroach held firmly between the fingers did not usually struggle or attempt to escape during the injection procedure. Injected cockroaches were then housed singly in one-litre flasks until used. Food and water were provided, ad lib.

Figure 6: Injection Route into Abdomen of Cockroach

(b) Doses of DDT and Analogs

Initially, the amount of O.C. delivered, in vivo, to a cockroach for (approximately) an LD₅₀ 96 hour dose was based upon previous authors' results (Uchida et al., 1974; Gardner and Vincent, 1978). Some changes to these were made for the present study. The doses injected for the O.C. compound used are presented in Table 2. o,p'-DDT was found to be toxic to male cockroaches when doses approximately 35 times greater than that used for p,p'-DDT

Table 2: Doses of Organochlorine Injected

O.C. Compound	Dose Delivered, umole/g cockroach
p,p'-DDT	0.017 $\pm$ 0.001
MeO-DDT	0.029 $\pm$ 0.001
p,p'-DDD	0.150 $\pm$ 0.007
p,p'-DDD	0.190 $\pm$ 0.009
o,p'-DDT ¹	0.50-0.85 $\pm$ 0.03
p,p'-DDE	0.80-1.60 $\pm$ 0.05

¹see Table 3

were injected. Therefore, an LD50 96 hour dose was determined for o,p'-DDT.

(c) LD50 96 Hour Determination for o,p'-DDT

Groups of male cockroaches were injected, following the procedure outlined above, with doses of o,p'-DDT ranging from 0.50 to 0.85 umole/gram (Table 3). Mortality counts were taken daily and on the fourth day (96 hours post-injection), the lethal dose 96 hours was calculated. Although failure to exhibit motion has previously been defined as the criterion for death (Cochran, 1955; Gardner and Vincent, 1978), this was found to be inappropriate on several occasions. In these cases, cockroaches that were apparently "dead", recovered from their apparent state only to walk about again. (However, it was unusual for any of these cockroaches to survive for more than 24 hours following such a display.) For this reason, mortality counts were taken with extreme care and rigorous documentation. Roaches were observed several times during the fourth day post-injection in order to accurately assess their condition.

3. ORGANOCHLORINE COMPOUNDS

Five O.C. compounds were used during this investigation.

The following were Aldrich Analytical Grades (99+); (Aldrich Chemical Company, Milwaukee, USA):

p,p'-DDT (1,1,1-trichloro-2,2-bis(p-chloro-

Table 3: Doses of o,p'-DDT Injected for
LD50 96 h Determination

Dose Delivered, umole/g cockroach	Number of Cockroaches Injected, n
0.50 ± 0.02	14
0.60 ± 0.03	30
0.65 ± 0.03	30
0.70 ± 0.03	10
0.85 ± 0.04	10

phenyl)ethane; o,p'-DDT (1,1,1-trichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl)ethane); p,p'-DDD (1,1-dichloro-2,2-bis(p-chlorophenyl)ethane); and p,p'-DDE (1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene). p,p'-methoxychlor (MeO-DDT) (1,1,1-trichloro-2,2-bis(p-methoxyphenyl)ethane) was Sigma Analytical Grade (99+%; Sigma Chemical Company, St. Louis). Structural formulae are presented in Table 1.

4. NEUROPHYSIOLOGICAL METHODS

All neurophysiological experiments were prepared for and conducted following standardized procedures. The only variable involved when considering a particular experiment in a group was the time between injection and recording from the ventral nerve cord (VNC). (A group was defined as a series of experiments in which the injected compound, dose, and time between injection and isolation were the same for each experiment within the group.)

Various lengths of time were allowed for incubation of any injected compound, in vivo. Standard time periods were set at 1 hour, 24 hours, 48 hours, 96 hours, and 3 weeks. Only o,p'-DDT injected cockroaches had their nerve cords tested at 48 and 96 hours. All compounds were tested at 1 hour, 24 hours and 3 weeks.

(a) The Isolated Nerve Preparation

Preparation of the cockroaches and O.C. compounds, or control compounds, for injection followed the procedures outlined above. Following injection and a predetermined time period, the cockroach was removed from the housing flask, quickly decapitated and de-limbed; the nerve cord was removed (three thoracic and six abdominal ganglia included) through the ventral surface of the animal. Great care was taken not to disturb the internal organs surrounding the VNC, especially the fat body as tissue disruption was considered to be a possible source of O.C. contamination. (Preliminary radioactive tracer experiments showed that the fat body acted as a sink for ^{14}C -DDT which could then readily contaminate other organs.) The total time required from decapitation to isolation of the nerve cord for electrical recording ranged from seven to eleven minutes. During the entire isolation procedure, the cockroach and nerve cord were perfused with normal physiological saline (Uchida et al., 1974 - see below).

(b) Preparation of the VNC for Stimulation and Recording

The isolated nerve cord was mounted in a 15x50 mm disposable wax-bottomed (Parawax, Esso Research) petri dish and bathed in normal physiological saline. (A layer of black mineral wax was poured and allowed to set before the layer of Parawax was applied. The black wax provided a dark background which facilitated viewing of the

pale-white coloured nerve cord.) The preparation was viewed through an Olympus Binocular Microscope with illumination supplied by a fibre optics light source (Model 11-80, American Optical Corporation). The abdominal connectives joining the third thoracic to the first abdominal ganglion (T3 to A1) and the fifth abdominal to the sixth abdominal ganglion (A5 to A6) (Fig. 1(a)) were crushed with forceps. This effectively provided an isolated abdominal nerve cord preparation. Without this, extraneous nerve activity from the thoracic ganglia and especially the sixth abdominal ganglion were found to intermittently provide input into the abdominal chain, which made interpretation of the results difficult. Isolating the abdominal chain in this fashion assured that any electrical output recorded was the result of the experimental input stimulus or had arisen spontaneously from within the abdominal chain (excluding the sixth abdominal ganglion). Collaterals extending from abdominal ganglia were carefully cut short with very fine scissors to facilitate handling of the VNC with the stimulating and recording electrodes.

(c) The Apparatus: Stimulating and Recording from the VNC

Methods and equipment employed in the excitation, monitoring, recording, and transcribing of nerve activity were standardized and used in all experiments investigating activity in the abdominal nerve cord. A

block diagram of the recording apparatus is presented in Figure 7.

Extracellular recording and stimulating of the preparation was performed within a copper-screened (Faraday) cage (Figure 8). Stimulating and recording was accomplished with two pairs of hooked tungsten wires (0.006 inches diameter). The tungsten wires were mounted in a miniature manipulating device which allowed variable separation of the two pairs. To further facilitate positioning, the third electrode (first recording electrode) was capable of independent movement. Stimulating electrodes were placed between the 5th and 6th abdominal ganglia. Recording electrodes were placed between the 1st and 2nd abdominal ganglia.

Stimulation of the preparation was achieved with a standard square wave of 0.2 millisecond duration and variable voltage (up to twice threshold value), from a SD48 stimulator (Grass Medical Instruments Co., Quincy, Mass.). Before reaching the preparation, the stimulus pulse was passed through a Stimulus Isolation Unit (Model SIU-5, Grass Medical Instruments Co.). The SIU-5, used in direct coupling mode, effectively isolated the stimulus from ground and in so doing substantially reduced the size of the stimulus artifact (Fig. 7).

A P511-J AC Preamplifier (Grass Medical Instruments Co.) with high impedance probe (HIP-5, Grass Medical

Figure 7: Block Diagram of Stimulating and Recording Apparatus

A and I within

CO enclosure: Vertical amplifiers of the
oscilloscope

CH1 and CH2: Recording channels of FM tape
recorder

CO: Cathode ray oscilloscope

CT: Cathode ray tube

FM: Stereo (FM) tape recorder

H: High impedance input probe

P: AC Pre-amplifier (P-511J)

R: Recording electrodes (independent
adjustment)

s: Loudspeaker with adjacent amplifier

S: Stimulating electrodes

SD-48: Electrical stimulator

SIU: Stimulus isolation unit

T: Stimulator trigger output

TP: Temperature monitor unit with attached
probe

Figure 8: Photograph of Faraday Cage and Electrodes

Bath: Preparation mounting chamber and saline bath

E: Stimulating and recording electrodes

EH: Electrode holder and manipulating device

F: Fibre optics light source

I: Saline inflow line

M: Microscope

S: Saline outflow line

SC: Saline cooling Column

T: Temperature Monitor

TP: Temperature probe

U: Micromanipulator

Z: High impedance input probe

Instruments Co.) was calibrated using internal circuitry and checked with an external, independently operating calibrated voltage supply (Medistor Calibration Unit). During the experiment, activity in the nerve cord was viewed simultaneously on both a Tektronix 502A and 564B (storage) Oscilloscope (Tektronix Corp., Oregon). It was then circuited through a switching device to a FM Tape Recorder (Model PI 6104, Precision Instruments, Palo Alto, California) where the nerve cord activity was recorded on magnetic tape (Ampex Instrumentation Tape). During the experiment, all data recorded on magnetic tape were simultaneously monitored with the original output trace from the AC-preamplifier. Permanent records of data were made from the magnetic tape using a Tektronix Camera (Tektronix Oscilloscope Camera C-12) with Polaroid Film (Polaroid Type 47, High Speed Land Film, Polaroid Corporation, Cambridge, Massachusetts) and a Siemens Oscillomink High Speed Chart Recorder (Siemens, Germany).

Experiments were conducted at room temperature, which ranged between 22 to 26°C. It was monitored in all experiments using a Telethermometer with Micro Probe (Model 43TD, Yellow Springs Instruments, Yellow Springs), and a Coleman 165 chart recorder (Hitachi, Japan). The thermo-probe was attached to the electrode holding/manipulating device and extended into the saline bath adjacent to the nerve preparation. A 10 to 15 minute period was normally allowed, following isolation, before

recording. This time was usually sufficient for the preparation to recover its normal low level of spontaneous activity and normal action potential (AP). Each nerve preparation was then followed for a period of 4 to 5 hours. Spontaneous and stimulated activity was monitored at 10 min (with intermittent 15 min) intervals. Each recording interval was assessed with respect to the type of activity observed and placed into one of three categories. Thus, the activity could be quantified. The three categories were: 1/ normal compound action potential (with or without spontaneous [non-repetitive firing] activity); 2/ repetitive firing elicited consistently during the recording interval; and 3/ no compound action potential or repetitive firing elicited (i.e., conduction block). Thus, with information from each recording interval, the data was assembled in the form of activity records (for example, see Figure 12).

5. SALINE SOLUTION AND FRACTIONATION MEDIUM

The normal cockroach physiological saline employed was that outlined by Uchida et al. (1974). Its composition is presented below:

Salt	Concentration (mM)
NaCl	210
KCl	2.9
CaCl ₂	1.8
NaH ₂ PO ₄	0.2
Na ₂ HPO ₄	1.8

The pH of the saline was adjusted to 7.2 with NaH₂PO₄.

The osmotically balanced solution (Medium 1) employed during the cockroach CNS fractionation experiments was the same as that used by Chappel and Hansford (1972). Its composition is presented below:

Solute	Concentration (mM)
Sucrose	250
EDTA*	1.0
Tris-HCl	10

*EDTA = Ethylenediamine tetra-acetic acid

The pH of Medium 1 was then adjusted to 7.2 with concentrated HCl.

6. Thin Layer Chromatography (TLC) of Organochlorine Compounds

¹⁴C-p,p'-DDT was used in several experiments. The labelled p,p'-DDT (Specific Activity = 0.0294 mCi/mg) was prepared by the method of Fields et al. (1950) by

Amersham-Searle Chemical Co. (Oakville, Ontario.) The preparative technique employed, labelled the same, single carbon atom per molecule DDT. The ^{14}C -DDT batch (#30) was analyzed by Amersham-Searle Corporation and was reported as being greater than 99% pure. Thin layer chromatography (TLC), using the method of Morley and Chiba (1964) and the Chemical Rubber Company (CRC) Handbook of Chromatography (Sunshine, 1972), was performed on the ^{14}C -p,p'-DDT sample, p,p'-DDT and other analogs listed above. This was done in order to verify the product information supplied by Amersham-Searle, Aldrich, and Sigma Chemical Companies concerning the identity and purity of the O.C. compounds. n-hexane (Pesticide Quality, Matheson Coleman and Bell Manufacturing Chemists, Norwood, Ohio) was used as the mobile phase. Four microlitres of each O.C. compound (0.01 M) were spotted on the TLC plates (Alumina G (250 microns), Analtech, Inc., Newark, Delaware) 2.5 cm from the bottom edge, with a 10 μl Hamilton microsyringe (Hamilton Company, Brockville, Ontario) and 9 cm piece of PE20 intramedic tubing (Clay-Adams). (Note: only 2.0 μl of a concentrated ^{14}C -DDT sample were spotted.) Spot diameter was kept to less than 0.8 cm. After the solvent front had moved a premarked distance (15 cm), the plates were dried and sprayed with a chromogenic reagent (1.7 g silver nitrate in 10 ml distilled water and 5 ml ammonium hydroxide (spectral grade, specific gravity 0.90); diluted to 200 ml with acetone). The plates were then exposed to

2

short wavelength UV radiation (wavelength = 285 nm) at a distance of 15 cm for 20 minutes. Dark spots appeared indicating the location of the particular O.C. compound. Distances moved were measured and R_f and R-DDD values calculated according to the procedure described in the CRC Handbook. To calculate R-DDD values, the distance an O.C. compound ran was divided by the distance run by p,p'-DDD multiplied by 100. The ratios calculated were related to standard values presented in the CRC Handbook for the particular compound, system, and TLC plate used.

7. COCKROACH CNS FRACTIONATION EXPERIMENTS

(a) Localization of ^{14}C -DDT in the CNS of the Cockroach, Periplaneta americana, (L.)

In an effort to investigate one of the putative sites of action of DDT, experiments involving ^{14}C -DDT and the cockroach CNS were performed. These experiments involved isolating various fractions from the CNS, and following the distribution of ^{14}C -DDT throughout the various cellular fractions.

(b) Fractionation Procedure: Isolation of Gross Membranal, Mitochondrial and Microsomal Fractions

The experiments performed required that at least thirty VNCs be isolated for each experiment. Pooling of nerve cords was essential in order to obtain sufficient tissue for analyses. Following the isolation of the nerve cords, all procedures were done on ice or at 0°C . All glassware and solutions were chilled on ice before use. Tissue samples not being centrifuged were kept on ice until processed.

Adult males were used exclusively. Nerve cords were dissected from cockroaches under a constant flow perfusion of cold normal physiological saline at pH 7.2. Perfusion with saline at 2°C allowed the preparation to be maintained at a temperature of 4 to 6°C during the entire dissection procedure. Following dissection, isolated VNCs were kept on ice in normal saline until the predetermined

number of cords required had been pooled.

Once isolated, the tissue was blotted on Whatman #1 filter paper and the fresh weight determined as quickly as possible (Mettler Model H10T, Mettler, Germany).

(Experiments were performed to determine: 1/ the length of time required to weigh a vial, time zero set at removal of the vial from the drying plate; and 2/ the gain in weight over time of the sample once removed from the drying plate. Total time required to weigh a sample was 20 to 30 sec. The weight of the vial and sample did not begin to change (increase) until approximately 60 sec after removal from the drying plate.) Nerve cords were then transferred to ice cold Medium 1 (see Materials and Methods, Section 4) and chopped with a razor blade. The tissue was homogenized by hand in a Pyrex ground glass homogenizer (# 7725, Corning Glass Works), in Medium 1 employing 15 twisting up and down strokes. Separation of membrane, mitochondrial, and microsomal fractions by differential centrifugation followed the method of Chappel and Hansford (1972) with minor alterations. A detailed outline of the procedure is given in Appendix A. The procedure was reported to allow the separation of very pure mitochondrial and microsomal fractions (Chappel and Hansford, 1972). The membrane fraction contained the occasional mitochondrion which was trapped and not separated during the homogenization or differential centrifugation process. (However, the appearance of

mitochondria in microscopic sections of the membrane fraction was infrequent and was not considered to be significant when compared to the amount of other tissue present. The main object of the exercise was to obtain as pure a mitochondrial fraction as possible. This was accomplished (see Results, Section 5).)

Separation of membrane and mitochondrial fractions required rapid acceleration of the centrifuge rotor (IEC, Model B-20; International Equipment Co., Needham Hts., Mass.) to 2000 xg for 25 seconds (total time at 2000 xg). The supernatant was then carefully pipetted into another centrifuge tube and the crude membrane pellet resuspended. The supernatant from this first spin contained the mitochondrial and microsomal fractions to be isolated. Both samples were respun at 2000 xg. Membrane supernatant from this spin was carefully pipetted and discarded. The original membrane supernatant (containing mitochondria and microsomes) was pipetted to a clean, chilled centrifuge tube. Three washes of the membrane pellet with isolation Medium 1 and three washes with twice distilled water preceded isolation of the pellet in twice distilled water for drying at 70°C. The distilled water washings were saved for further analysis. (Pellets were washed in distilled water to remove any low molecular weight molecules that would have contributed to the final dry weight of the pellet.)

The original membrane supernatant was taken as rapidly as possible to 20,000 xg (14,500 RPM) where it was spun for 25 seconds. It was then rapidly braked to a stop. Mitochondria were pelleted and the resulting supernatant contained the microsomal fraction. After careful removal of the supernatant, the mitochondrial pellet was resuspended in fresh, ice cold Medium 1 and was respun at 20,000 xg for 25 seconds (following the same procedure as outlined above). This supernatant was then discarded and the pellet was washed with Medium 1 and twice distilled water. The final mitochondrial pellet was then removed for drying at 70°C.

The initial mitochondrial supernatant was then spun at 20,000 xg for 30 min. The resultant microsomal pellet was then washed and collected following the routine used for the membrane and mitochondrial pellets except that the microsomal supernatant was saved for analysis as it was and not respun.

The method of differential centrifugation and fractionation of the cockroach CNS employed here was similar to the method employed by Cheng and Cutkomp (1977). They used a sucrose solution of 0.32 M and centrifuged at a lower force (13,000 xg) for a longer period of time (20 minutes). This method may have been adequate. However, Cheng and Cutkomp (1975, 1977) provided no morphological evidence that the fractions they

isolated were what they supposed them to be. (Koch (1969), using the same method, did present several micrographs of the B fraction, the one reported to have been isolated by Cheng and Cutkomp.) Identification and morphological dissection of the fractioned pellets obtained following the method adapted from Chappel and Hansford (1972) indicated that mitochondrial and microsomal fractions were very pure and the membranal fraction was quite acceptable (mostly composed of plasma membrane (neurolemma) and membrane fragments - see Results, Section 5).

(c) Electron Microscopic (E.M.) Identification of Fractioned Pellets

Ten cockroach CNSs were isolated and fractioned following the procedures outlined above. However, pellets were not washed with distilled water. Instead, the sucrose medium was pipetted off and the pellets submerged in a 1.6% glutaraldehyde solution for a period of one hour. It was then replaced with 4.0% glutaraldehyde for 2.0 h. Following glutaraldehyde fixation, the tissues were washed in 0.2 M phosphate buffer and then post-fixed in 1% osmium tetroxide for 1.0 h. They were then washed again and dehydrated in a typical alcohol series followed by propylene oxide (undiluted), propylene oxide:epon-araldite (3:1), propylene oxide:epon-araldite

(1:1), propylene oxide:epon-araldite (1:3), and epon-araldite (undiluted). After 24 h the epon-araldite (undiluted) was replaced with fresh stock for final embedding in flat moulds. The epon-araldite was activated prior to use with DMP-30 (J.B. EM Services, Montreal). A detailed outline of the steps involved from fixation to embedding is presented in Appendix B.

Two separate experiments were performed to determine the purity and consistency of the fractionation procedure. Ten CNSs were isolated for each experiment and processed, strictly following the procedures outlined in Appendix A.

From the pelleted tissue a total of 24 blocks were prepared with membrane fraction tissue, 16 blocks with mitochondrial fraction tissue, and 9 blocks with microsomal fraction tissue. All blocks containing mitochondrial tissue were sectioned. Approximately half the membrane and half the microsomal blocks were selected at random and sectioned. Ultra-thin sections (grey; silver; silver-gold : 60 nm; 70 to 90 nm; 90 to 100 nm; respectively) were cut using freshly broken glass knives (LKB Knife Maker, Model 7800A; LKB Produkter, Stockholm, Sweden) on a Porter-Blum Ultra-microtome (Model MT-1, Ivan Sorvall, Inc.; Norwalk, Connecticut) or Reichert Automatic Ultramicrotome (Type 700121, Vienna, Austria). Thin sections were picked-up on copper grids coated with formvar (0.2-0.4% formvar in dichloroethane) and carbon.

The formvar coating provided support for sections while the carbon (applied using a Vacuum Evaporator, Model VE404, Mikros Inc., Portland, Oregon) aided in dissipating heat generated by the electron beam during viewing.

After drying, the grids were stained in 25% methanolic uranyl acetate (10 to 20 min) followed by counter staining with lead citrate (Reynolds Procedure - 5 to 10 min). Both stains were filtered through Whatman #1 filter paper before use. Grids were then viewed and photographed using a Siemens Elmiskop 1a electron microscope (Siemens, Germany) and Kodak Electron Image film (Type 4463).

(d) Experiments Employing ^{14}C -p,p'-DDT

Once it was verified that the fractionation procedure provided pure preparations of mitochondria, microsomes, and membrane fragments, experiments employing ^{14}C -DDT were performed. (The long half life (5720 years) and slow metabolic degradation of p,p'-DDT in vivo means that it can be safely assumed the major portion of radioactivity detected was associated with a DDT molecule and that it was due to the disintegration of the carbon-14 atom associated with that molecule.)

Three experiments were designed to provide data and supportive evidence on the localization of ^{14}C -DDT in the cockroach CNS. The first experiment was conducted to

determine whether or not the injected ^{14}C -DDT would associate more with one fraction than another.

Cockroaches were injected with one microlitre of ^{14}C -DDT solution in acetone (Specific Activity = 0.0294 mCi/mg; Amersham-Searle Chemical Co., Oakville, Ontario). The cockroaches were then left for 60 min in one-litre flasks. The CNSs were then isolated and prepared following the procedures outlined previously for fractionation experiments. Pellets were transferred to glass scintillation vials (which had been drying for 96 hours at 70°C) and dried at 70°C for at least 60 hours or until the pellet weight stabilized. (The length of time required to dry the pellets depended upon the initial amount of twice distilled water required in transferring the pellet from the Corex centrifuge tubes.) The dry weight of each pellet was then determined on the Mettler H10T balance to within 0.1 mg. Each pellet was weighed five times over a period of at least two hours. A stable pellet weight was one in which the values for a single pellet did not deviate more than ± 0.1 mg. Once the dry weight was determined the pellet was processed for liquid scintillation counting. A series of empty vials were filled with twice-distilled water to determine if the dried down water would alter the weight determination of the pellets. (It was found that the twice-distilled water did not contribute any weight to the dried contents of the vial.)

The second experiment was performed to determine the in vitro affinity of the various fractions for ^{14}C -DDT. It involved isolating and preparing thirty untreated (cold) CNSs for homogenization. Immediately following the transfer of the 30 chopped cold CNSs to the homogenizer, 30 μl of ^{14}C -DDT was injected into the homogenization medium. The experiment proceeded with subsequent isolation of the various pellets. Pellets were dried, weighed, and processed for liquid scintillation counting. The radioactivity associated with each pellet was then determined.

The third experiment was performed to determine the possible degree of 'pick-up' of ^{14}C -DDT by the mitochondrial fraction during the homogenization procedure.

Sixty CNSs were isolated, 30 from cockroaches treated one hour previously with ^{14}C -DDT (hot CNSs) and 30 from untreated cockroaches. Dissections were alternated between treated and untreated cockroaches. (Two separate sets of surgical instruments and dissection chambers were used to avoid any chance of contamination.) Hot and cold pellets were isolated from all fractions. The hot membrane fraction, microsomal fraction, and microsomal supernatant were then pipetted back into the homogenizer. The cold mitochondrial pellet was then pipetted into the same homogenizer. Re-homogenization and re-fractionation

was performed on these pellets and supernatant. Membrane, mitochondrial, and microsomal pellets were dried, weighed, and prepared for liquid scintillation counting. The radioactivity associated with each pellet was determined.

Samples were rehydrated with 4 to 6 drops of twice-distilled water in their scintillation vials. They were then solubilized with 1 to 3 ml of NCS tissue solubilizer (NCS, Amersham-Searle Chemical Co., Oakville) at 50°C, with occasional gentle mixing, until all tissue was in solution (24-48 h). Ten millilitres of the toluene based scintillation cocktail (1 litre toluene, 6 g PPO, 2 mg POPOP; Fisher Scientific Co.) was added to each cooled sample. Each sample was mixed vigorously on a vortex mixer to ensure complete solution of the sample. Occasionally, a further aliquot of 0.5 ml of NCS had to be added to a vial which resisted total solution, instead forming two distinctly separate phases. This additional 0.5 ml NCS was usually sufficient to ensure a uniform solution.

The pH of each vial was checked and adjusted to pH 7.0 with Glacial Acetic Acid (Baker Chemical Co.). pH adjustment was important because the NCS made the samples extremely alkaline (ca. pH 11+) which would have induced a significant error in radioactivity detected due to chemiluminescence. The vials were then wiped clean with kleenex and methanol to remove any finger prints or other

substances which might have affected counting efficiency. The vials were kept in the dark for a minimum of two hours before beginning the counting procedure. Counting was done on a Packard Tri-Carb Liquid Scintillation Spectrophotometer (Model 574, Packard Instrument Co., Inc.) at 0 to 4 C. Window settings were set at 50-1000 for all three channels. Gain controls were set at 14%, 17%, and 2%. 14% gain and the described window setting were determined to be the optimum settings. The efficiency of this Packard TriCarb for carbon-14 using a set of known standards had been determined to be 81%.

Vials were counted overnight to enable uninterrupted counting and a ten minute count per vial with 3 to 5 complete counting cycles. When complete, each cycle was compared with the others for consistency. The counts from the channel counting with 14% gain were then tabulated and the mean counts per minute calculated. The disintegrations per minute (dpm) employing the factor of 81% were then calculated. This procedure was followed for each vial. Vials were used once and disposed of following precautions necessary when handling radioactive material.

8. PROTEIN DETERMINATION

Both the Lowry procedure (Lowry et al., 1951) and Coomassie Brilliant Blue G-250 method (Bradford, 1976) of protein determination were employed to quantitatively

determine the protein content of the three fractionated pellets. Prior to protein determination, using either method, the protein was extracted from the tissue by the following procedure (see also Appendix C).

One millilitre of 10% (w/v) trichloroacetic acid (TCA) was added to the 0.5 ml volume of fractionated tissue homogenate in twice-distilled water. After vigorous mixing, the homogenate-TCA mixture was boiled (100°C) in a water bath for 15 minutes. The samples were then centrifuged for 10 min at 700 xg (2000 RPM IEC Centrifuge) followed by very careful removal of the supernatant. The pellet was then resuspended in 1.0 ml of 10% TCA and stored overnight (15 to 18 hours) at 0°C . The sample was then removed from the cooler, spun 10 min at 700 xg, supernatant removed, and the final pellet dissolved in 3 to 10 ml of 0.10 N or 0.50 N NaOH. Dissolution of the protein sample was greatly facilitated by sonication at 55°C . (Dissolution of the protein sample in 0.10 N NaOH was used as a compromise between the Lowry and Coomassie Blue procedures. The compromise proved to be satisfactory as long as the standard protein samples Bovine Serum Albumin (BSA, essentially fatty acid free, prepared from fraction V, Sigma Chemical Co., St. Louis) were also dissolved in 0.10 N NaOH.)

(a) The Lowry Method of Protein Determination

The reagents used in the Lowry Assay were prepared

according to Lowry et al., (1951):

Reagent A : 2% Na_2CO_3 in distilled water

Reagent B : 0.5% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 1% Sodium Tartrate

Reagent D : Reagents A and B freshly mixed,
50.0 ml A to 1.0 ml B

Reagent E : 1 N phenol reagent prepared from
2 N phenol reagent (Folin-
Ciocaltean) by dilution with
distilled water

Reagent D was used for protein samples dissolved in base (NaOH); Reagent C was not used in these experiments as the pH was already alkaline.

The procedure followed for protein determination (Lowry Method, Appendix D; Lowry et al., 1951) was carried out at room temperature in clean glass test tubes. To duplicate 1.0 ml volumes of protein sample in 0.10 N NaOH, 5.0 ml of Reagent D was added, followed immediately by vigorous mixing. After 10 min of standing, 0.5 ml of freshly made Reagent E was added to each tube, again followed by vigorous mixing. These tubes were then allowed to stand 35 min for maximal colour development. The absorbances were then read at 560 nm (Bausch and Lomb, Spectronic 20, Spectrophotometer) against a reagent blank prepared in the same fashion as the protein sample (except 1.0 ml of 0.10 N NaOH was substituted for the 1.0 ml of protein solution).

A new standard curve was prepared for each day's analysis. The protein used as standard was Bovine Serum Albumin (BSA). From a stock solution of 250 ug BSA/ml 0.10 N NaOH, seven duplicate standards were prepared (12.5, 25, 50, 100, 150, 200, and 250 ug). These seven BSA protein samples were treated in the same fashion as the CNS tissue samples. The protein content of the tissue samples were then read directly from the standard curve constructed (Appendix E). (This method of reading unknown protein sample content directly from the standard curve was preferred over calculating a conversion factor from the slope of the curve because the standard curve was found to be linear with two distinct slopes. The two ranges of linearity were from 0 to 50 ug and 50 to 250 ug protein content. This trend was very consistent and occurred with each standard curve (see Appendix E), despite new stock solution.) Total protein was then determined by multiplying the protein content of the sample tested by the dilution factor.

Tissue samples were normally diluted in such a fashion as to enable reading absorbances in the lower range of the scale. This, combined with reading protein content directly from the standard curve, was found to enhance the accuracy of determining protein content since the standard curve tended to level off at high optical densities (Lowry et al., 1951). Dilution factors were adjusted according to the protein content of the samples as estimated in

trial runs.

(b) Coomassie Brilliant Blue G-250 Protein Determination

Coomassie Brilliant Blue G-250 (Coomassie Blue) has been used to stain for proteins in polyacrylamide gels following electrophoresis (Reisner et al., 1975 - cited in Sedmak and Grossberg, 1977). However, it was not until Bradford (1976) and later Sedmak and Grossberg (1977) that a method was elaborated that enabled the use of Coomassie Blue as a quantitative assay in protein determination. The method employed was very simple and economical in both time and expense with respect to reagents. Only a single colourmetric reagent is required to be added to a protein sample (unlike the Lowry reagents, the Coomassie Blue reagent is stable at room temperature, and has a very long shelf life). Total colour development is normally complete and stable within 10 minutes.

Much smaller volumes of protein sample (0.1 ml) are required. This is suitable since the real strength of this assay procedure is the determination in samples containing less than 50-100 ug protein.

Protein was extracted as described for the Lowry Method. Normally, the protein sample and standards would be dissolved in 0.15 M NaCl (Bradford, 1976). However, to remain compatible with the Lowry Assay procedure, protein samples were dissolved in 0.10 N NaOH. Standard curves prepared using BSA as the protein standard dissolved in

0.10 N NaOH and 0.15 M NaCl indicated that the range of linearity and sensitivity of the assay procedure remained intact regardless of which solvent was used. Thus, the use of 0.10 N NaOH to dissolve protein samples allowed both protein determination procedures to be performed on the same stock protein sample.

The protein reagent required in the Coomassie Blue assay was prepared according to Bradford (1976). Coomassie Blue (100 mg-Sigma Chemical Co.) was dissolved in 50 ml of 95% ethanol. To this solution, 100 ml of 85% (w/v) phosphoric acid was added. This solution was then diluted to a volume of 1.0 litre with distilled water. Undissolved matter was removed by qualitative filtration of the reagent through Whatman #1 filter paper. Final concentrations in the protein reagent were: 0.01% (w/v) Coomassie Blue; 4.7% (w/v) ethanol; and 8.5% (w/v) phosphoric acid.

The procedure followed for the protein determination was essentially the method outlined by Bradford (1976) with the following exceptions: 1/ as previously noted, protein standards and samples were dissolved in 0.10 N NaOH; and 2/ following addition of the protein reagent, three minutes was allowed for colour development (Appendix F). Protein determination was conducted at room temperature (in clean glass test tubes) and normally just before or after the Lowry protein determination had been performed on the same protein samples.

Using preliminary trials, the approximate protein in each sample was determined. If the protein contained in 0.1 ml of protein sample exceeded 70 ug protein, then only a fraction of the 0.1 ml protein sample was pipetted into the test tube to enable protein determination within the range 10 to 70 ug. The volume in the test tube was then adjusted to 0.1 ml with 0.10 N NaOH. (This procedure was required only for membrane protein samples.) Five millilitres of the protein reagent was then rapidly added to each sample and the contents mixed thoroughly by vortexing. The absorbance at 595 nm was measured three minutes after vortexing the protein-reagent mixture. Duplicate samples of each protein were run simultaneously to ensure consistency. The glass cuvettes and spectrophotometer were the same as those in the Lowry protein determination.

A new standard curve was prepared for each day's protein determinations. From a stock solution of 200 ug BSA/0.1 ml (the same BSA as used in the Lowry Method) in 0.10 N NaOH, six duplicate standards were prepared (12.5 ug, 25 ug, 50 ug, 100 ug, 150 ug, 200 ug). Protein was determined as outlined above and the standard curve plotted (see Appendix F for sample standard curve). Values of protein from tissue samples were read directly from the standard curve. Total protein for each sample was then determined by multiplying by the appropriate dilution factor.

For both the Coomassie Blue and Lowry protein determination procedures, if the samples in a duplicate standard protein pair differed by more than 2%, then all new standards were prepared for that curve.

RESULTS

1. MORTALITY STUDIES

(a) Signs and Symptoms of DDT Poisoning in the Cockroach

Injection of 1 ul physiological saline or acetone did not cause any signs of poisoning. All these cockroaches survived at least three weeks following the injection while being maintained under identical conditions as O.C. treated cockroaches.

Following injection of a LD50 96 h dose of DDT there was normally a delay of 10 to 15 minutes before any signs of poisoning became evident. The first symptoms of poisoning were usually minor, intermittent tremoring of the legs, with an increase in the severity and intensity of the tremor as poisoning progressed. Following the initial symptoms the cockroach became hyperexcitable, i.e., its response to a minor abrupt stimulus (such as tapping the containing flask on the bench top) was grossly exaggerated. The exaggeration of the startle response rapidly increased in severity to a peak level. This symptom of poisoning normally outlasted the spontaneous tremor and jitter also seen. Hyperexcited activity also arose spontaneously or could be elicited by directing wind puffs toward the posterior of the abdomen.

Jitters usually ensued and occurred simultaneously with the tremorous behaviour. The jitters appeared as

uncoordinated jerky movements of individual appendages. A severe jitter episode could develop into uncoordinated convulsive movements in the later stages of lethal poisoning.

Within the first 24 h visible signs of poisoning in some cockroaches subsided and became almost undetectable. In others, signs of poisoning persisted and advanced to further stages: intense jitters with intermittent spontaneous hyperexcited and convulsive behaviour intensified. The cockroach then became unable to maintain an upright stance and remained inverted, struggling on its dorsal surface. Jittering and intense movements became reduced as the cockroach entered the prostrate (exhausted) stage of poisoning. Intermittent tremor of varying degrees of severity became the predominant form of movement. Frequency of tremorous activity decreased to the point at which no movement could be detected. At this time the cockroach may be considered dead, although caution must be exercised because, as noted previously, several cockroaches were observed to subsequently revive from this apparent state to walk about again. All 'dead' cockroaches were retained and observed for at least 12 h after being pronounced dead. At this time the final mortality count was completed.

As defined by the dose (LD50 96 h), approximately 50% of cockroaches die within 96 h of being treated. The other 50% apparently recover and begin to behave

'normally' again within 24 to 48 h. However, of those cockroaches in which visible signs of poisoning disappeared, neurological symptoms persisted well past the 24 and 96 h times post-injection, as will be outlined below. Once past 96 h post-injection most cockroaches survived for at least three weeks without any sign of the return of poisoning symptoms.

(b) Cumulative Data on Mortality Following the LD50 96 h Dose

Mortality counts were taken and recorded for each experiment and O.C. compound for the first seven consecutive days following injection and again at three weeks (depending upon the experiment). At the completion of study on each compound, the cockroach mortality at 96 h was calculated.

From Table 4 it can be seen that injection of physiological saline, acetone, and p,p'-DDE apparently did not alter the survival of the cockroaches.

The final calculated 96 h mortality for p,p'-DDT treated cockroaches was 42% (Table 4). Thus, it is evident that the injected dose of 0.017 μ moles p,p'-DDT/g (LD42 96 h) was less than that required to attain a proposed 50% mortality rate in 96 h.

The doses of MeO-DDT and p,p'-DDD employed (Table 2) resulted in levels of 50% mortality in 96 h (LD50 96 h) (Table 4). Because of its peculiarities, o,p'-DDT was

Table 4: Cumulative Mortality Data Following the Putative LD50 96 h Doses of Organochlorine Compounds¹

Compound	Dose of Compound Injected ² , umole/g	Total Number of Cockroaches Injected, n	%Mortality in 96 h
Normal Saline , and Acetone		24	0
p,p'-DDT	0.017	57	42
MeO-DDT	0.029	18	50
p,p'-DDD	0.15	30	47
p,p'-DDD	0.19	47	62
p,p'-DDE	0.8-1.6	16	0

¹see Table 5 for o,p'-DDT Mortality Data

²Total volume injected = 1.0 ul

considered separately (see below).

(c) Toxicity of o,p'-DDT

It was determined that at a dose of 30 to 40 times that used for p,p'-DDT (0.5 to 0.68 umoles/g cockroach) o,p'-DDT was toxic to cockroaches. The LD50 96 h dose was estimated to be between 0.50 and 0.85 umoles/g of cockroach. A series of doses in this range were tested (Table 5). The dosage-mortality curve (Fig. 9) was fitted according to the statistical procedure of Finney (Probit Analysis) as outlined by Woolf (1968). From this, the LD50 96 h dose was determined to be 0.65 umole/g cockroach (Fig. 9). Examination of the slope of the curve (Fig. 9) indicates that it describes a very narrow range of doses over which injected o,p'-DDT has intense effects on mortality within 96 h. An increase of 0.05 umole/g in the dose of o,p'-DDT increased mortality by approximately 20%. A similar increase in the dose of p,p'-DDD (Table 4) provided less than 15% increase in mortality.

Cockroaches treated with o,p'-DDT responded to the injection in a manner that was characteristic only of o,p'-DDT treated cockroaches. Signs of poisoning did not present themselves until 48 to 72 h post-injection. When signs and symptoms did appear, they were much less pronounced and never became as severe as in cockroaches injected with p,p'-DDT.

Although 50% of injected cockroaches died within 96 h

Figure 9: Dosage Mortality Curve for o,p'-DDT -
Probit Analysis
Probit values were calculated as outlined by
Woolf (1968).

Table 5: Doses of o,p'-DDT Employed to Determine %Mortality at 96 h and 3-weeks

Dose Injected, umoles/g cockroach	Total Number of Cockroaches Injected	%Mortality in 96 h	%Mortality in 3-weeks
0.50	14	14	79
0.60	30	47	83
0.65	30	50	90
0.70	10	70	90
0.85	10	80	100

at a dose of 0.60 to 0.65 umole/g, it was observed that only 10 to 17% of the total number injected survived a three week period (Table 5). This further indicated that there is a delay characteristic of poisoning and mortality induced by o,p'-DDT.

2. ACTIVITY IN THE COCKROACH VENTRAL NERVE CORD

I Classifying Repetitive Firing (R.F.)

Classically, the nervous system has been cited as the target organ on which the DDT molecule exerts its poisoning effects. The destabilizing effects on the nervous system have been well documented using both intracellular and extracellular recording techniques. Using intracellular recording and voltage-clamp techniques, the effects of DDT on ionic conductance changes in the axonal membrane have been elucidated (Narahashi and Haas, 1968; van den Bercken, 1980). Extracellular recording (the same as that utilized in the present research) allows the simultaneous monitoring of many axons within the VNC without the intricacies involved with intracellular recording (Pearson et al, 1970). In this way, a more general status of the nervous system is described.

(a) Pseudo Repetitive Firing and other Spontaneous Activities not Associated with O.C. Poisoning

Because of the complexity of the numerous responses

recorded from the VNC, a set of guidelines were established which allowed the recognition and distinction of extraneous activity. The identification of repetitive firing in a consistent and unambiguous fashion was then possible. The guidelines are described below.

1/ A compound action potential stimulated at A5-A6 and recorded at A1-A2 usually appeared as a very rapid, large, single spike of amplitude ranging from 5 to 25 mV (Fig. 10(a)). However, various types of spontaneous activity were occasionally recorded simultaneously with this compound action potential. A repetitive type of firing activity was recognized as not being associated with poisoning by DDT or analog. It was essential that the pattern of this activity be established and not considered part of the general poisoning syndrome. The most characteristic element of this spontaneous activity was its spike frequency, which ranged from 40 to around 60 Hz (Fig. 10(b)). Frequencies less than 40 Hz and as high as 70 Hz were infrequently noted. An electrical stimulus was not essential for this activity to be elicited. (A repetitive firing discharge associated with O.C. poisoning usually required an electrical stimulus to initiate the discharge.) The duration of the spontaneous discharge was so variable that it almost precluded definition, ranging from milliseconds to seconds in length. The spike characteristics associated with the termination of a spontaneous discharge were also very inconsistent. This

Figure 10: Normal Activity and Activity in the Cockroach
CNS not Associated with O.C. Poisoning

(a) Normal compound action potential.
Stimulated to fire at a rate of approximately
7 Hz (scale bar: 0.4 mV, 50 ms).

(b) Irregular, low frequency, spontaneous
activity. Not repetitive firing (scale bar:
1.0 mV, 50 ms).

(c) Random, low amplitude spontaneous
activity, Not repetitive firing (scale bar:
0.2 mV, 50 ms).

(d) Post-CAP-Activity -- Note the irregular
amplitudes and random nature of the firing
pattern. Not repetitive firing (scale bar:
0.2 mV, 50 ms).

7

c

d

Figure 11: Some Characteristics of Repetitive Firing

(a) Repetitive firing with virtually no taper of frequency in the terminal portion of the train (scale bar: 2 mV, 50ms).

(b) Note the pronounced taper of frequency in the terminal portion of the train (scale bar: 2 mV, 50 ms).

(c) Note that the size of the compound action potential is considerably larger than the spikes within the repetitive firing train. The uniform amplitude of the spikes within a train is evident (also see (a) and (b) this figure) (scale bar: 2 mV, 25 ms).

inconsistency was a characteristic that could be used to help identify this type of activity. Rather than an abrupt end to the discharge, it commonly tapered off in an irregular fashion. The frequency of spikes became erratic and sometimes persisted in this way for various lengths of time. This activity occasionally arose despite isolation of the abdominal segment of the VNC by crushing connectives between A1-T3 and A5-A6 (see Introduction, Fig. 1(a)). Usually, spontaneous activity arises within the sixth abdominal ganglion, thoracic ganglia, or head.

2/ An irregular type of spontaneous activity was identified in both control and treated preparations. The firing frequency of these action potentials was entirely random, without any form or order (Fig. 10(c)). This element in itself was sufficient to identify and subsequently used to disqualify it as possible repetitive firing.

3/ Another type of activity which was occasionally found to occur with both control and treated preparations was a low, irregular amplitude, random array of spike activity following a stimulated action potential (Fig. 10(d)). The term "post-CAP-activity" (CAP, compound action potential) is used to describe this type of noise following the stimulated action potential. However, the post-CAP-activity had to be carefully distinguished from a repetitive firing discharge involving several axons firing

simultaneously following an electrical stimulus (see below). Post-CAP-activity had no apparent coherent structuring or order of spiking, even within the final few milliseconds of the discharge. Again, the final segment of the activity discharge was important in differentiating between random, insignificant activity and repetitive firing.

4/ Some acetone and saline control preparations at 1h, 24 h, and 3-weeks displayed repetitive firing in their isolated VNCs. There was no significant difference in the occurrence of repetitive firing in these controls at any of these time periods (Student's t-test, $P=0.05$). For a representative activity record for acetone and saline controls see Figure 22(a,b), which demonstrates that repetitive firing could be associated with an apparently normal nervous system and cockroach. There were no behavioural symptoms of poisoning or signs of severe nervous system disruption in any of these cockroaches. As will be presented, nervous system disruption did not necessarily correlate directly with behaviour displayed. The occurrence of repetitive firing in controls was low enough that it allowed distinction from repetitive firing in VNCs of cockroaches treated with O.C. compounds. The distinction (or lack of) was verified for each O.C. compound at the standard incubation times (1 h, 24 h, 3-weeks) by employing a statistical procedure (Student's

t-test). It could thus be evaluated whether or not the occurrence of repetitive firing in treated cockroaches was significantly different from controls. The comparison of the occurrence of repetitive firing in O.C. treated preparations with control treated preparations is presented below.

(b) Common Characteristics of Repetitive Firing

To be considered as repetitive firing the initial spiking frequency within a discharge had to be greater than or equal to 80 Hz. Although 80 Hz represents the lower limit, initial spike frequencies between 125 and 250 Hz were most commonly observed. No upper limit was assigned. (The lower limit was not an arbitrary choice. It (80 Hz) represented a frequency of firing greater than the maximum spontaneous activity occasionally displayed (see Results Section 2.I(a)1).) It was essential that the spikes of a discharge be distinct from other possible activity associated with the stimulated compound action potential. As mentioned previously, post-CAP-activity could be associated with either control or treated preparations and may partially obscure the initial phases of a discharge. This normally did not present a problem because the duration of the repetitive firing discharge usually outlasted the post-CAP-activity. Furthermore, post-CAP-activity was not commonly associated with a repetitive firing discharge.

Duration of repetitive firing discharges were quite variable. They ranged from 10 ms to several seconds in length. However, the duration, per se, was not as significant as the related features of the discharge, such as the characteristics of the spiking frequency. The importance of the initial spike frequency has already been mentioned. The termination of the repetitive firing discharge was of equal importance in identifying and characterizing repetitive firing. There were two distinct ways in which a repetitive firing discharge ended: 1/ it could terminate abruptly with little or no change in spiking frequency (Fig. 11(a)); or 2/ it could terminate with a pronounced tapering in the frequency of spiking (Fig. 11(b)). The second alternative must be qualified: the decline in frequency of firing followed a regular pattern with increasing time between spikes as the discharge progresses (i.e., a decrease in firing frequency), and continued to the terminal spikes of the discharge.

Repetitive firing spike amplitude (mV) was not a critical factor in distinguishing repetitive firing. However, several consistencies were observed. One was the difference in amplitude between the initial stimulated compound action potential and the following spikes of a discharge. The initial compound action potential was always larger than the following spikes (Fig. 11(a-c)).

This implies that all axons within the nerve cord do not fire repetitively. (When more than one axon repetitively fired, they did not normally summate, in contrast to the initial compound action potential.) Another feature was the consistency of amplitude of spikes within a single discharge. Although exceptions were found, this was the more usual occurrence.

II. EFFICACY OF O.C. COMPOUNDS TO ELICIT BEHAVIOURAL AND NEUROPHYSIOLOGICAL SYMPTOMS OF POISONING

(a) 24 h Preparations: Behavioural Abnormalities

Cockroaches injected with a LD₅₀ 96 h dose of p,p'-DDT, MeO-DDT, and p,p'-DDD elicited behavioural abnormalities consistent with poisoning (tremors, jitters, and hyperexcitability) within one hour of being injected. Signs and symptoms worsened in some and subsided in others (as previously described). At approximately 24 h post-injection they were near-prostrate, prostrate, or behaviourally almost recovered. VNCs that were removed from near-prostrate or prostrate cockroaches were found to rarely exhibit repetitive firing in response to single or multiple electrical stimuli up to twice the threshold voltage required to produce a compound action potential. These nerve cords also had poor survivability, in vitro, i.e., they usually remained functional and producing at least a small compound action potential for only 1 to 2 h instead of the minimum 4.5 h recorded for all other preparations, as shown in the activity record for p,p'-DDD at 24 h (Figure 12(a)).

Cockroaches that had apparently recovered from poisoning symptoms in 24 h would respond only occasionally to a mechanical stimulus in a hyperexcited manner or in a manner indistinguishable from a control response. The occasional hyperexcited response elicited by some

Figure 12: Activity Records for 24 h p,p'-DDD (near-prostrate) and 1 h MeO-DDT Injected Cockroaches

Each vertical bar represents a recording interval in which repetitive firing was consistently displayed. Each dot represents an interval in which no repetitive firing was observed. Crosses represent an occasion when an action potential could not be elicited. Total recording time = 4.5 h

(a) The records were obtained from VNCs which had been removed from cockroaches injected 24 h previously with a LD50 96 h dose of p,p'-DDD. All cockroaches were near-prostrate i.e., unable to right themselves or remain upright, severe intermittent tremors, and jitters. Also note the low incidence of repetitive firing and short survival time of 3 of the 4 preparations.

(b) Cockroaches were poisoned with MeO-DDT (LD50 96 h) one hour before removal of their CNS for recording. Note the consistent occurrence of repetitive firing and lack of normal activity.

0 60 120 180 240 MIN

cockroaches was intermittent and not as strong or pronounced as seen in cockroaches immediately following poisoning. (This is a very important observation when compared with the 24 h VNC activity data presented below.)

Cockroaches injected with o,p'-DDT or p,p'-DDE did not show any visible sign of poisoning immediately following, or at 24 h post-injection. Similar results were obtained with saline and acetone injected cockroaches.

(b) Activity in the VNC of Cockroaches Injected 1 h Previously with MeO-DDT (LD50 96 h)

Cockroaches were injected with MeO-DDT (LD50 96 h). Following an incubation period of one hour, in vivo, the VNC was removed and its activity recorded. The five cockroaches of this group presented here were displaying overt signs of poisoning (tremors, jitters, and hyperexcitability). The activity recorded from their VNCs apparently paralleled their behaviourally disrupted state. When a threshold or greater electrical stimulus was applied to the VNC a repetitive firing discharge was elicited (Fig. 12(b)). (Thus, each recording interval contained many instances of repetitive firing.) The occurrence of repetitive firing during a recording interval is represented by Figure 12(b). The figure does not provide data on the number of instances of repetitive firing within a recording interval. Rather, it is

designed to provide information regarding the consistency of occurrence of repetitive firing over the total in vitro recording time period (4.5 h).

Very similar results were obtained with both p,p'-DDT and p,p'-DDD. Cockroaches injected with p,p'-DDE, o,p'-DDT, or control compound did not display a significant level of repetitive firing 1 h post-injection.

(c) Activity in the VNC 24 h After Treatment

p,p'-DDT, MeO-DDT, and p,p'-DDD induced a significant occurrence of repetitive firing in the VNC of cockroaches injected 24 h previously (Student's t-test, $P < 0.005$), while o,p'-DDT and p,p'-DDE did not induce a significant occurrence of repetitive firing in the VNC of cockroaches injected 24 h previously (Student's t-test, $P = 0.05$) (Fig. 13). The significance of the occurrence of repetitive firing in O.C. treated cockroaches was determined by comparing repetitive firing in treated cockroaches with the occurrence of repetitive firing in saline and acetone injected (control) cockroaches. As there was no significant difference between levels of repetitive firing in saline and acetone injected cockroaches (Student's t-test, $P = 0.05$), these results were pooled and then tested against results for O.C. compounds.

1/ p,p'-DDT:

VNCs isolated from cockroaches which were injected 24h previously with p,p'-DDT elicited a repetitive discharge

Figure 13: Graphical Representation of Percent of Preparations Displaying Repetitive Firing vs. Compound Injected - for 24 h Preparations

All compounds except saline, acetone, and p,p'-DDE were injected at a LD50 96 h dose. Note that at 24 h, o,p'-DDT (and p,p'-DDE) injected cockroaches did not display a significantly greater occurrence of repetitive firing than controls (Student's t-test, $P=0.05$).

virtually every time an electrical stimulus of threshold or greater strength was applied to the nerve cord. This can be seen in Fig. 14(a). This persisted for more than 4.5 h, in vitro, without significant alteration in threshold stimulus. The small variation in amplitude of the compound action potential and of the spikes within a discharge recorded during different recording intervals was not considered to be significant. It was probably due to a slightly different positioning of the electrodes on the nerve and/or the extent of saline meniscus between nerve and electrodes. Average compound action potential amplitude did not change over the 4.5 h recording period. The pattern of repetitive firing assumed by a particular VNC was not consistent and could vary considerably. For instance, the DDT-treated example shown in Fig. 15(a) displayed repetitive firing of very high frequency (280 Hz) but of short duration (80-100 ms) at 47 min after isolation. At 1 h 32 min post-isolation this VNC was repetitively firing at the same initial frequency (280 Hz) but the discharges were persisting for durations ranging from 650 to 850 ms (Fig. 15(b)). Later recordings (at 2 h 32 min) from the same VNC indicated repetitive firing of frequency ranging from 140 to 150 Hz and durations similar to those obtained at 1 h 32 min. A consistent feature of the repetitive firing in the example shown in Fig. 15(a) was the incidence of more than one axon firing during the discharge independently of each other. The second axon

Figure 14: Activity Records for 24 h p,p'-DDT, MeO-DDT, and p,p'-DDD Injected Cockroaches (vertical bar: occurrence of repetitive firing during recording interval. Dots: normal activity, no repetitive firing)

(a) Activity in the VNCs of cockroaches injected 24 h previously with a LD50 96 h dose of p,p'-DDT. Note the more frequent occurrence of repetitive firing in (a) as compared with (b) or (c).

(b) Activity in the VNCs of cockroaches injected 24 h previously with a LD50 96 h dose of MeO-DDT. Note the frequent occurrence of repetitive firing interspersed with normal activity.

(c) Activity in the VNCs of cockroaches injected 24 h previously with a LD50 96 h dose of p,p'-DDD.

0 60 120 180 240 MIN

Figure 15: Repetitive Firing in the VNC of a Cockroach
Injected 24 h Previously with a LD50 96 h Dose
of p,p'-DDT

(a) Short duration, high frequency repetitive firing (scale bar: 0.2 mV, 50 ms).

(b) Long duration, high frequency repetitive firing. Note: the cockroach this VNC was removed from was not displaying any signs of poisoning at the time of decapitation (scale bar: 1 mV, 50 ms).

a

b

was firing at an initial frequency of 160 to 180 Hz with discharge durations of approximately 300 ms.

2/ MeO-DDT and p,p'-DDD:

Compared with p,p'-DDT data, the activity records for MeO-DDT and p,p'-DDD at 24 h are not as intensely populated with sequences of repetitive firing (Fig. 14(b) and 14(c), respectively). This reduced occurrence of repetitive firing, when compared with p,p'-DDT at 24 h and MeO-DDT at 1 h post-injection, possibly reflects the less persistent nature of MeO-DDT and p,p'-DDD.

The characteristics of the repetitive firing discharge in VNCs from cockroaches treated with MeO-DDT and p,p'-DDD were very similar to that seen with p,p'-DDT. Discharges from two axons were readily elicited from both MeO-DDT and p,p'-DDD treated VNCs (Fig. 16(a) and 17(a), respectively). In fact, an example was recorded containing discharges from at least three axons (Fig. 16(b)). However, the distinctive characteristics for a repetitive firing discharge (see Results, Section 2.I(b)) became more evident when only a single giant axon was involved, as was commonly seen with all three O.C. compounds which induced repetitive firing at 24 h post-injection (Fig. 17(b)). It was not possible to distinguish between O.C. compounds by the form of the repetitive firing discharge.

Figure 16: Repetitive Firing in the VNC of a Cockroach
Injected 24 h Previously with a LD50 96 h dose
of MeO-DDT

(a) At least two axons firing repetitively,
independent of one another.

(b) At least three axons firing repetitively,
independent of one another. Note the almost
indiscernable separation of spikes when all
three axons are firing (during the first
50 to 100 ms after stimulation).
(scale bar for (a) and (b): 1 mV, 50 ms)

a

b

Figure 17: Repetitive firing in the VNC of a Cockroach
Injected 24 h Previously with a LD50 96 h Dose
of p,p'-DDD

(a) Incidence of two axons firing in a
repetitive fashion, independent of one another.

(b) A single train of repetitive firing from
the same VNC as in (a). Note the consistency
of spike amplitude, frequency, and the abrupt
way in which the train terminates (scale bar
for (a) and (b): 2 mV, 50 ms).

a

b

(d) 24 h p,p'-DDD Near-Prostrate Cockroaches

The group of four cockroaches described here had been injected 24 h previously with a LD50 96 h dose of p,p'-DDD and were now all near prostration. They were displaying symptoms consistent with severe O.C. poisoning: tremors, intense intermittent jitters, and hyperexcitability coupled with difficulty in maintaining an upright position. Recordings from the VNCs were obtained following the standard procedures outlined above (Materials and Methods, Section 4(a,b)). In spite of the precautions employed, it was evident that the longevity of the VNCs, in vitro, had been reduced, presumably due to their advanced state of poisoning. Furthermore, there was a lack of repetitive firing in these obviously poisoned cockroaches (Fig. 12(a)) although some alternations between conduction and conduction-block were also noted. These same observations were also noted in VNCs taken from near-prostrated cockroaches after p,p'-DDT and o,p'-DDT treatment. Gammon (1978) also observed an alternation between conduction and conduction-block in severely poisoned (p,p'-DDT, LD95 120 h) cockroaches.

(e) Activity in VNCs from Cockroaches Treated 48 and 96 h Previously

The effect of the O.C. compound o,p'-DDT on the VNC of cockroaches injected 48 and 96 h previously was investigated because of a suspected delayed neurotoxicity.

Results of recordings taken, following 48 h incubation in vivo, indicated that the VNCs of o,p'-DDT poisoned cockroaches were now repetitively firing at a level significantly greater than that found in controls and o,p'-DDT treated cockroaches at 24 h (Student's t-test, $P < 0.005$ - Figure 18(a,b)). 6 out of 9 cockroaches tested displayed reproducible repetitive firing in their VNCs. The trend was continued at 96 h when 5 out of 7 VNCs tested were found to elicit a significant level of repetitive firing (Student's t-test, $P < 0.005$ - Figure 18(c)). There was no significant difference between the occurrence of repetitive firing in 48 and 96 h cockroaches (Student's t-test, $P = 0.05$).

The characteristic form of the repetitive firing discharge was not altered in either 48 h (Fig. 19) or the 96 h (Fig. 20) treated cockroaches. Examples of repetitive firing from recordings made for 48 h experiments showed that the discharges may involve one (Fig. 19(a)) or more (Fig. 19(b)) axons in the form typically seen with other efficacious O.C. compounds. Repetitive firing discharges from 96 h experiments are presented for comparison in a similar fashion (Fig. 20(a,b)).

Some very low amplitude repetitive firing was also detected in 48 h o,p'-DDT treated cockroaches (Fig. 19(c)).

Figure 18: Activity Records for: (a) 24 h, (b) 48 h,
and 96 h o,p'-DDT Injected Cockroaches
(vertical bars: occurrence of repetitive
firing during that recording interval. Dot:
normal activity, no repetitive firing
recorded)

0 60 120 180 240 MIN

Figure 19: Repetitive Firing in the VNC of a Cockroach
Injected 48 h Previously with a LD50 96 h Dose
of o,p'-DDT

- (a) Single train repetitive firing.
- (b) Dual train repetitive firing.
- (scale bar for (a) and (b): 2 mV, 50 ms)
- (c) Low amplitude (0.5 mV) repetitive firing
- (scale bar: 0.5 mV, 50 ms).

Figure 20: Repetitive Firing in the VNC of a Cockroach
Injected 96 h Previously with a LD50 96 h Dose
of o,p'-DDT

(a) Note the taper in frequency of spikes
towards the end of the discharge.

(b) Careful observation reveals that the
discharge is not random but in fact represents
two axons firing simultaneously, independent of
one another. Note the slight difference in
amplitude and spike frequency. The spikes of
one train are marked with arrows.
(scale bar for (a) and (b): 2 mV, 50 ms)

a

b

(f) Preliminary Results from Severed CNS Experiments
(T3-A1).

Five cockroaches which had their VNC's severed at T3-A1 24 h prior to treatment responded in the typical fashion to a LD50 96 h dose of DDT injected intra-abdominally. Tremors and symptoms of hyperexcitability were quite evident in all five cockroaches within 30 min of injection. Subsequent removal of the abdominal nerve cord (during the display of poisoning, ca. 1 to 2 h post-injection) followed by electrical stimulation and recording, revealed that the axons fired repetitively at high frequency in response to a single threshold (or greater) electrical stimulus. The nerve response elicited was identical to responses recorded from intact (i.e., unsevered) VNCs of cockroaches injected 1 and 24 h previously.

(g) 3-Week Experiments

The experiments described below pertain to the behavioural and neurophysiological symptoms of poisoning in cockroaches treated 3-weeks previously with an O.C. (LD50 96 h - except p,p'-DDE; see Materials and Methods, Section 2(b)) or control compound.

1/ Behavioural Abnormalities

None of the cockroaches injected 3-weeks previously with O.C. (LD50 96 h), physiological saline, or acetone displayed any sign of poisoning. Startle responses were

entirely normal. Startle stimuli and behaviour observed included: response to wind puffs directed toward the caudal end of the abdomen (cerci) - this did not cause an abnormal response, or a response different from control injected cockroaches. Tapping the containing flask on the work bench did not produce an abnormal response either. Further, no evidence of tremoring or other symptoms of hyperexcitability were noted. These behavioural responses were not surprising given that near normal responses had been obtained since about 24 h after injection with O.C. compounds. This was the case, with the exception of o,p'-DDT, where the behavioural responses returned to normal after about 96 h post-injection (having appeared only at 48 h). The onset of symptoms by a population of o,p'-DDT treated cockroaches occurred over a period of approximately seven days. Thus, there was a great deal of variation as to when a particular cockroach from an o,p'-DDT group would begin to show overt signs of poisoning. This was dissimilar to other efficacious O.C. compounds which induced a treated population to display symptoms of poisoning almost synchronously (i.e., within 1 h).

2/ Activity in the VNC 3-Weeks After Treatment

Of the five O.C. compounds tested, only p,p'-DDT and o,p'-DDT were shown to induce repetitive firing in the VNCs of cockroaches treated 3-weeks previously with an

LD50 96 h dose. The levels of repetitive firing displayed in VNCs of cockroaches injected with either of these two compounds were significantly different from control levels of repetitive firing (Student's t-test, $P < 0.005$). 73% of p,p'-DDT injected cockroaches that survived 3-weeks ($n=22$) and 92% of o,p'-DDT injected cockroaches that survived 3-weeks ($n=12$) displayed repetitive firing in their VNCs (Fig. 21). Levels of repetitive firing noted in cockroaches treated 3-weeks previously with MeO-DDT, p,p'-DDD, and p,p'-DDE were not significantly different from pooled control levels of repetitive firing (Student's t-test, $P=0.05$ - Fig. 21). (Levels of repetitive firing in saline and acetone injected cockroaches were not significantly different (Student's t-test, $P=0.05$) and therefore, these results were pooled.)

The frequency of occurrence of repetitive firing in response to an electrical stimulus in cockroach VNCs 3-weeks after treatment with p,p'-DDT and o,p'-DDT was much less than that noted in 24 and 96 h preparations that displayed a significant level of repetitive firing (Fig. 22(a,b) compared with Fig. 14(a) and 18(c)). However, even though the frequency of occurrence apparently declined over the 3-week incubation period, the difference from control levels of repetitive firing was still apparent (Fig. 21; Fig. 22(a,b)).

Some representative examples of activity records for

Figure 21: Graphical Representation of Percent of Preparations Displaying Repetitive Firing vs. Compound Injected - for 3-Week Preparations

All compounds except saline, acetone, and p,p'-DDE were injected with a LD50 96 h dose. Only the VNCs from cockroaches injected with p,p'-DDT and o,p'-DDT displayed a level of repetitive firing significantly different from controls (Student's t-test, $P < 0.005$).

Figure 22: Activity Records for 3-Week Preparations
(vertical bars: occurrence of repetitive
firing during that recording interval. Dot:
normal activity, no repetitive firing.)

- (a) saline control
- (b) acetone control
- (c) p,p'-DDT
- (d) o,p'-DDT

MeO-DDT, p,p'-DDD, and p,p'-DDE are presented for comparison. They were not significantly different from control records (Student's t-test, $P=0.05$ - Fig. 23(a-c)).

3/. Characteristics of Repetitive Firing at 3-Weeks p,p'-DDT:

The characteristic form of repetitive firing as defined (Results, Section 2.I(b)) was also found in VNCs of cockroaches treated 3-weeks previously with p,p'-DDT or o,p'-DDT. However, the repetitive firing discharge was not as readily elicited in 3-week preparations as in 24 or 96 h preparations. Despite this it was reproducible on a level that distinguished it from any of the other compounds tested, as shown by the activity records (compare Fig. 22(c,d) with Fig. 23(a-c)) and statistical analysis (Student's t-test, $P<0.005$).

As with 24 h preparations, there were notable variations within the parameters defining repetitive firing. For example, VNCs from cockroaches treated with p,p'-DDT 3-weeks previously displayed repetitive firing of high initial frequency (>220 Hz) with a short duration (50 to 100 ms) (Fig. 24(a)). The low amplitude noise which followed the compound action potential was readily distinguished from, and not considered to be related to, the repetitive firing discharge. (This type of activity was not unusual as it was observed in both O.C. treated and control preparations.)

Figure 23: Activity Records for 3-Week Preparations
(vertical bars: occurrence of repetitive
firing during that recording interval. Dot:
normal activity, no repetitive firing.)

- (a) MeO-DDT
- (b) p,p'-DDD
- (c) p,p'-DDE

Figure 24: Repetitive Firing in the VNC of a Cockroach
Injected 3-Weeks Previously with p,p'-DDT

(a) High frequency, short duration. (scale bar:
0.2 mV, 25 ms)

(b) Medium-high frequency, long duration.
(scale bar: 0.5 mV, 50 ms).

Repetitive firing discharges of lower initial frequency (ca. 180 Hz) and longer duration (ca. 600 ms) were also observed (Fig. 24(b)). There was no consistent pattern concerning the initial spike frequency and duration of discharge. Discharges of lower initial frequency (100 to 150 Hz) and short duration (50 to 100 ms) were also common, as was the reverse situation, i.e., a high initial frequency (220 to 280 Hz) and long duration of discharge (500 to 800 ms - Fig. 25(a)).

o,p'-DDT:

A consistent feature of repetitive firing elicited from the VNCs of cockroaches previously poisoned with *o,p'*-DDT was the exaggerated decline (taper) in firing frequency as the discharge progressed (Fig. 25(b)). This characteristic was pronounced in 3-week preparations and those isolated at 48 and 96 h (Fig. 19 and 20). (Usually only a slight decline, if any, in firing frequency was observed with control and treated preparations, as the discharge progressed to termination.) However, the abrupt termination of the discharge remained as described in previous examples. Other aspects of the repetitive firing discharge associated with *o,p'*-DDT treated cockroaches were consistent with the repetitive firing examples outlined above.

Saline and Acetone:

Some saline and acetone control isolated VNC

Figure 25: High Frequency, Long Duration Repetitive Firing in the VNC of a Cockroach Injected 3-Weeks Previously

(a) p,p'-DDT injected (scale bar:0.5 mV, 50 ms).

(b) o,p'-DDT injected. Note the taper in firing frequency in the terminal portion of the spike train, compared to (a) (scale bar: 1 mV, 50 ms).

a

b

preparations at 1 h, 24 h, and 3-weeks displayed repetitive firing (Fig. 22(a,b)). These activity records demonstrate that repetitive firing could be associated with an otherwise apparently normal nervous system and cockroach. However, the occurrence of repetitive firing in controls was low enough for a distinction to be made from repetitive firing in VNCs of cockroaches treated with O.C. compounds, when required. There were no behavioural symptoms of poisoning or severe nervous system disruption in any of these cockroaches. As will be discussed later, neurological disruption did not necessarily correlate directly with behaviour.

3. THE ACTIVITY QUOTIENT (A.Q.)

A quantitative method to compare the ability of DDT and its analogs to produce repetitive firing in the VNCs of poisoned cockroaches was devised. The data were quantified by relating the total number of observation intervals over a specific period of time, the total number of recording intervals in which repetitive firing was reproducibly elicited, and the total number of preparations employed in the experiment. The Activity Quotient delineated better the degree with which a particular preparation was repetitively firing. The Activity Quotient is given by the following formula:

$$A.Q. = \frac{\sum_{i=1}^n \frac{\text{No. of +ve responses}}{\text{total No. of observations}}}{n}$$

where n = the total number of preparations employed.

Data to calculate the A.Q. were deduced from activity records for each cockroach VNC. An Activity Quotient of 1.0 would represent a compound which reproducibly induced repetitive firing during every observation interval.

(a) 24 h Experiments

A.Q. values indicated that there were significant differences in the occurrence of repetitive firing detected in controls and certain O.C. treated cockroaches (p,p'-DDT, MeO-DDT, p,p'-DDD) 24 h following injection (Student's t-test, $P < 0.005$). It also showed that there were no significant differences between controls and p,p'-DDE or o,p'-DDT treated cockroaches at 24 h (Student's t-test, $P = 0.05$).

It can be seen (Fig. 26) that, despite the fact that all cockroaches (100%) treated for 24 h with p,p'-DDT, MeO-DDT, and p,p'-DDD displayed many periods of reproducible repetitive firing (Fig. 13), p,p'-DDT consistently provided more such displays (compare also Fig. 14). Furthermore, the very inconsistent nature of the repetitive type activity displayed by controls became

Figure 26: Graphical Representation of Activity Quotient vs. Compound Injected for 24 h Preparations and 48 and 96 h o,p'-DDT Preparations

The incidence of repetitive firing in 24 h o,p'-DDT injected cockroaches was not significantly different from control cockroaches (Student's t-test, $P=0.05$). However, at 48 and 96 h, they were significantly different (Student's t-test, $P<0.005$). (error bars = standard error of the mean)

$$A.Q. = \frac{\sum_{i=1}^n \text{No. of +ve responses}}{\text{total No. of observations}}$$

where n = the total number of preparations employed

much more evident (Fig. 26).

The insignificant difference between controls and 24 h o,p'-DDT treated preparations and the significant difference between controls and 48 and 96 h o,p'-DDT treated preparations was also distinct (Student's t-test, $P=0.05$ and $P<0.005$, respectively). Levels of repetitive firing in 48 and 96 h o,p'-DDT treated cockroaches were comparable and not significantly different from levels of repetitive firing in 24 h p,p'-DDD and MeO-DDT treated cockroaches (Student's t-test, $P=0.05$; 48 h o,p'-DDT and 24 h MeO-DDT showed a slight significant difference at $P=0.05$, but not at $P=0.02$).

(b) 3-Week Experiments

The A.Q. bar graph provided a profile of the occurrence of repetitive firing in the nervous systems of treated and control cockroaches (Fig. 27). Both p,p'-DDT and o,p'-DDT displayed levels of repetitive firing significantly different from all other treated and control preparation (Student's t-test, $P<0.005$). The variable occurrence of repetitive firing indicated by the activity records (Fig. 22(c,d)) was reflected in the lower value for the A.Q.. The VNCs of other O.C. treated cockroaches did not display a level of repetitive firing significantly different from controls (Student's t-test, $P=0.05$).

It must be noted that the VNCs from p,p'-DDE injected cockroaches displayed the least percentage drop in

Figure 27: Graphical Representation of Activity Quotient vs. Compound Injected for 3-Week Preparations

Note that only p,p'-DDT and o,p'-DDT injected cockroaches displayed occurrence of repetitive firing significantly different from controls (Student's t-test, $P < 0.005$). (error bars = standard error of the mean)

occurrence of repetitive firing (except for controls which decreased by only 23%) when comparing 24 h and 3-week Activity Quotients (Fig. 26 and 27). *p,p'*-DDE treated preparations retained 68% of the level of repetitive firing recorded at 24 h. *p,p'*-DDT retained 24% followed by MeO-DDT (10%) and *p,p'*-DDD (8%). *o,p'*-DDT was not comparable with the other compounds because of its delayed neurotoxic effects. However, comparing 96 h and 3-week Activity Quotients, *o,p'*-DDT treated cockroaches retained 76% of the level of repetitive firing recorded at 96 h.

4. ORGANOCHLORINE CHROMATOGRAPHY

The five authentic (Aldrich spectra grade, 99%+; see Materials and Methods, Section 2(b)) organochlorine compounds were spotted on an Alumina G TLC plate beside a sample of ¹⁴C-DDT. Although the chromatography served as a check of the qualitative nature of the O.C. samples, it was mainly employed to verify and determine the chemical characteristics of the ¹⁴C-DDT sample.

There were no indications of any pesticides other than those spotted for each organochlorine compound (cold) sample. Migration points were quite distinct and covered an area of approximately one square centimeter. Movement of the O.C. compounds on the thin layer chromatographic plate relative to the origin is presented in Figure 28. *R_f* and *R*-DDD values were tabulated (Table 6) and compared

Figure 28: TLC-Radiochromatogram of O.C. Compounds

Positions the O.C. compounds migrated to were plotted according to the distances migrated from the origin (right margin). The associated R-DDD values were also plotted (left margin) for comparison with similar values from the CRC Handbook of Chromatography (Sunshine, 1972). The radioactivity associated with each square centimeter above and below the point at the origin where the sample of ^{14}C -DDT was spotted were determined (histogram, using the right margin as the baseline and top margin for graduation). Note the high intensity of radioactivity associated with the position identified as p,p'-DDT.

Table 6 : Rf and R-DDD Values

O.C. Compound	Rf. (x100) (Calculated) ¹	R-DDD (x100) (Calculated)	R-DDD (x100) (CRC) ²
p,p'-DDD	39	100	100
MeO-DDT	11	29	27
p,p'-DDT	66	168	160
p,p'-DDE	87	209	200
o,p'-DDT	78	198	190
14C-DDT	67	170	—

¹From TLC data

²From the CRC Handbook of Chromatography

with previously published data (CRC Handbook, 1972).

R-DDD values calculated were judged to be not significantly different from the published figures. Small differences noted were consistent with each compound and did not obscure the separation and subsequent identification of any compound.

94% of the radioactivity associated with the ^{14}C -DDT sample spotted was located over a position on the plate characteristic of the authentic p,p' -DDT sample. The remaining 6% of radioactivity spotted was found at positions other than the square centimeter associated with the ^{14}C - p,p' -DDT position. Radioactivity that was possibly associated with o,p' -DDT and p,p' -DDE totalled approximately 1%. Although the concentrated ^{14}C -DDT sample used facilitated detection and increased the signal to noise ratio, it may have been responsible for some streaking of the sample. This may at least partially account for the radioactivity detected at the positions for o,p' -DDT and p,p' -DDE had they been present in the radioactive sample. (The 1 μl of ^{14}C -DDT normally injected into a cockroach contained approximately 125,000 dpm. This represents 5.39×10^{-9} moles of p,p' -DDT and a total of approximately 4.32×10^{-11} moles of o,p' -DDT and p,p' -DDE (1% of the injected sample).)

5. COCKROACH CNS FRACTIONATION EXPERIMENTS

(a) Morphological Identification of Fractionated Pellets

The procedure employed to isolate the three fractions from the CNS by differential centrifugation provided very pure mitochondrial and microsomal preparations (Fig. 29 and 30, respectively). There was virtually no cross contamination in these two fractions by material from any of the other fractions. From morphological (E.M.) examination of sections from the membrane fraction, it was found to be inhomogeneous (fractions defined - see Materials and Methods, Section 6) containing pieces of perineurium with neural lamella and collagenous material, cell nuclei, glial cells (whole and broken), and nerve membrane fragments (Fig. 31). Mitochondria were occasionally found bound to fragments of neural lamella and perineurium. However, intact pieces of neural lamella and perineurium were not commonly found and sighting of mitochondria was even less common. Free mitochondria in the membrane fraction were not observed. Considering the mass of the membrane fraction compared to the few mitochondria sighted, it was judged that they would not contribute significantly in any way to the membrane fraction.

(b) Association of ¹⁴C-DDT with the CNS, in vivo

An experiment was designed to determine which fraction of the CNS the ¹⁴C-DDT would be associated with after being injected and allowed a 1 h incubation period.

Figure 29: Morphology of the Mitochondrial Fraction

A typical cross-section through the mitochondrial fraction pellet. The presence of mitochondria (M) with intact membrane and cristae are evident. Note the mitochondria which seem to be in the process of breaking-up and releasing their contents into the medium (MR). Also present are mitochondrial ghosts, those devoid of any internal structure or content (MG). Cristae material (C) that has been released is also present.

43,000 X

Figure 30: Morphology of the Microsomal Fraction

Typical section through the microsomal fraction pellet. No mitochondria were visible in any section made. Rounded-up fragments of membrane structures were commonly seen (RM). Note the lack of internal structure in the vesicle-like structures. Some mitochondrial ghosts may also be present (MG). Note the separation of the inner and outer membrane in some vesicles (arrow heads).

41,000 X

Figure 31: Morphology of the Membrane Fraction

Cross-section through a membrane fraction pellet. Note the general inhomogeneous appearance of the material in the section. Most large membrane structures have been damaged, probably during the homogenization process. A large nucleus (Nu) with a perforation in the nuclear membrane (NP) is visible. Also present are remnants of glial cells (G) and a neurosecretory cell (NC) with several neurosecretory granules (NS). Strands of collagenous material from the neural lamella are also present (C). Spherical structures with darkened border (Ar) may be remnants of axons with some associated glial cell. Axonal fragments without glial cells (A) are also seen. A single, identified mitochondrion (M) is also evident. 30,000 X

3 3
OF / DE

It was found that the membrane fraction was normally 1.5 to 2.6 times as radioactively labelled as the mitochondrial fraction (Table 7, n=4 experiments - 30 cockroaches per experiment). The microsomal fraction was labelled even less than the mitochondrial fraction. The values of radioactivity were represented as disintegrations per minute per milligram dry weight (dpm/mg dry weight) and corrected for background (Table 7).

(c) Affinity of the Fractions for ^{14}C -DDT, in vitro

The lipophilicity of DDT suggested that DDT would be rapidly taken-up by a membranous structure rather than be left unassociated in the aqueous solution of the homogenizer. Therefore, the extent to which each fraction picked-up, ^{14}C -DDT during the homogenization and fractionation procedure was investigated by injecting a quantity of ^{14}C -DDT into the homogenizer containing 30 cockroach CNSs not previously exposed to ^{14}C -DDT. The standard procedure of homogenization and isolation of the various pellets was then followed. The experiment indicated that there was no significant difference in amounts of radioactivity associated with isolated membranal and mitochondrial fractions when ^{14}C -DDT was applied, in homogeno, (Table 8). However, the microsomal fraction contained significantly less radioactivity (ca. 15,000 dpm/mg dry weight vs. ca. 46,500 dpm/mg dry

Table 7: Radioactivity¹ Associated with Different Membrane Fractions from the CNS Injected in vivo with ¹⁴C-DDT 1 h Prior to Isolation

Fractioned Pellet	Experiment Number				Mean Total dpm	Mean dpm/mg
	1	2	3	4		
MEMBRANE						
Dry weight, mg	15.8	12.8	19.2	12.7		
Total dpm	1383	455	791	752	845	
dpm per mg	87	36	42	59		56
MITOCHONDRIA						
Dry weight, mg	3.1	4.0	2.9	2.6		
Total dpm	106	65	83	71	81	
dpm per mg	34	16	28	27		26
MICROSOME						
Dry weight, mg	4.0	5.0	4.3	3.1		
Total dpm	(324) ²	46	71	42	53	
dpm per mg	(81) ²	10	16	13		13
Membr. (dpm/mg)	2.6	2.2	1.5	2.2		
Mitoch. (dpm/mg)						

¹Corrected for background

²Contaminated during isolation, (not included in "mean total dpm (per mg)")

Table 8: Affinity of the CNS Fractions for ^{14}C -DDT, in vitro
(^{14}C -DDT injected into the homogenate)

Pellet Type	Dry Weight, mg	Radioactivity, dpm/mg
Membrane	15.5	47 788
Mitochondria	1.7	45 136
Microsome	2.88	15 648

weight). Thus, a difference in binding affinity was demonstrated between the microsomal fraction and the other two fractions.

Recovery of the radioactivity associated with the three fractioned pellets was approximately 27%. The remainder of radioactivity was lost with the lipid associated with the final supernatant, during sucrose washing procedures, and to DDT that was left associated with the glassware.

(d) Contamination of the Mitochondrial Fraction During the Isolation Procedure

The final experiment: a 'cold' mitochondrial fraction was placed in the homogenizer containing all the tissue from 30 'hot' CNSs, EXCEPT the 'hot' mitochondrial fraction. During rehomogenization and refractionation, the originally cold mitochondrial fraction 'picked-up' ^{14}C -DDT totalling 82% of the label found associated with the original hot mitochondrial fraction (Table 9).

(e) Estimate of DDT Contained in each Fraction, in vivo

The Specific Activity of the ^{14}C -DDT employed was 0.0294 mCi/mg. It was then determined that the total number of moles of p,p'-DDT injected into each cockroach (for ^{14}C -DDT fractionation experiments) averaged 0.0054 umoles (range = 0.0043 to 0.0065 umoles - see Appendix G for calculation procedure).

Following the 1 h incubation period for the ^{14}C -DDT

Table 9: Contamination of the Mitochondrial Pellet with ^{14}C -DDT During the Isolation Procedure

Pellet Type	Dry Weight, mg	Associated Radioactivity ² , dpm/mg
Membrane		
Group 1 (hot) ¹	8.3	(1052) ³
Group 2 (cold)	11.2	—
Mitochondria		
Group 1 (hot)	4.5	85
Group 2 (cold) ¹	2.6	(69) ³
Microsome ⁴		
Group 1 (hot) ¹	4.4	(19) ³
Group 2 (cold)	4.3	—

¹ Pellets that were rehomogenized and refractioned together

² Corrected for background

³ Activity after rehomogenization and refractionation in the Group 1 (hot) homogenizer

⁴ Special Note: The total radioactivity associated with the microsomal supernatant (Group 1 - Hot) was 757 dpm. It can also be argued that comparison of this data with those of Jackson and Gardner (1978) should use the total amount of DDT present in the supernatant as the pool to which the mitochondria are exposed. The microsomal supernatant in the homogenizer contained ca. 3.0×10^{-11} moles DDT (4.5×10^{-11} moles DDT/mg mitochondrial protein). Jackson and Gardner required 1.4×10^{-5} moles DDT/mg TBM protein to achieve 50% inhibition of Mg-ATPase activity. Thus, even using this method of calculation, it appears that there is insufficient DDT available to cause 50% inhibition of the mitochondrial ATPase. (This probably represents the "worst case" as the supernatant also contains emulsified fat which can be expected to reduce the DDT effectively available to the mitochondria.)

injected, in vivo, the CNSs were removed, the fractions isolated, and associated radioactivity determined by the standard procedures (see Materials and Methods, Section 7(e) and Appendix A). Then, from the Specific Activity and the data presented (Table 7) it was calculated that the membranal and mitochondrial fractions (of 30 CNSs) contained approximately 3.64×10^{-11} moles and 3.50×10^{-12} moles of DDT, respectively. The microsomal fraction contained less (2.29×10^{-12} moles). Sample calculations are presented in Appendix G.

It must be recalled that up to about 80% of the radioactivity associated with the mitochondrial fraction could have been 'picked-up' during the isolation procedure (Results Section 5(d)). This can also be inferred for the membranal fraction because of the demonstrated equal affinities both fractions had for ^{14}C -DDT (Results, Section 5(c)). Therefore, if one assumes all of the DDT becomes available to reassociate, then the values for DDT contained in the outlined fractions were liberal estimates and probably reflect artificial maxima. If corrected for contamination (or the association), the values of DDT contained in the membranal and mitochondrial fractions would be 20% of the values presented above (membranal: mitochondrial; 6.55×10^{-12} moles: 6.30×10^{-13} moles). This would represent the most conservative estimate of DDT contained in the fraction, in vivo.

6. PROTEIN DETERMINATION OF THE THREE ISOLATED CNS FRACTIONS

(a) Lowry Protein Determination

Three groups of 30 male cockroaches were isolated from the colony. The CNSs of each group were individually fractionated following the same procedure as that used in all fractionation experiments outlined above. After dry weight determination the protein content of each pellet was determined according to the procedure of Lowry et al.(1951). From the three sets of data (Table 10) it was determined that the gross membrane fraction contained the greatest amount of protein by weight (22%) followed by the mitochondrial (19%) and microsomal (12%) fractions. However, there is not a significant difference between the amount of protein in the membrane and mitochondrial fractions (Student's t-test, $P=0.05$ - Fig. 32).

(b) Coomassie Brilliant Blue G-250 Protein Determination

Employing tissue samples from that processed for the Lowry protein determination (Results Section 5(a)), the protein content of the various fractions were determined following the procedure of Bradford (1976 - Coomassie Brilliant Blue G-250). Contrary to evidence presented for the Lowry protein determination, the Coomassie Blue method indicated that the mitochondrial fraction contained the greatest proportion of protein by weight (20%) followed by the microsomal (14%) and membrane (14%) fractions (Table

Table 10: Estimates of Total Protein of CNS Fractions¹

Method of Protein Determination and Pellet Type	Total Protein, ug	Dry Weight, mg	Percent of Total Dry Weight as Protein	Standard Deviation, s	Estimated Standard Error of the Mean, $s_{\bar{x}}$ (2)
Lowry Method ³					
Membrane	3673	16.6	22.2	3.15	1.82
Mitochondria	737	3.8	19.1	2.68	1.55
Microsome	563	4.5	12.4	1.82	1.05
Coomassie Blue Method ⁴					
Membrane	2293	16.6	14.0	1.84	1.06
Mitochondria	767	3.8	20.0	1.40	0.81
Microsome	640	4.5	14.2	2.53	1.46

¹Mean of three experiments⁽²⁾Estimated standard error of the mean, $s_{\bar{x}} = \frac{s}{\sqrt{n}}$ ³Lowry et al., 1951⁴Bradford, 1976

**Figure 32: Percent of Pellet Dry Weight as Protein
of the Membranal, Mitochondrial, and Microsomal
Fractions of the Cockroach CNS**

Protein content was determined by the Methods
of Lowry et al.(1951) and Bradford (1976). See
text for explanation.

**% OF TOTAL PELLET DRY WEIGHT AS PROTEIN VS. FRACTIONED
PELLET TYPE AND METHOD OF PROTEIN DETERMINATION**

10). Using this data, there is a significant degree of difference between membrane and mitochondrial fractions (Student's t-test, $P < 0.005$ - Fig. 32).

The protein content of the mitochondrial fraction determined by the Lowry method was not significantly different from the level determined by the Coomassie Blue method. Similarly, this was found to be the case comparing microsomal fraction protein determinations. However, the great difference between membrane protein as determined by Lowry and Coomassie Blue was apparently significant. The almost 10% greater estimation of membrane protein using the Lowry assay was a consistent feature in all protein determinations. This may be accounted for by the differing specificities of the two protein assays (see Appendix H).

DISCUSSION

The main purpose of this research project was to determine whether or not a sublethal dose of DDT or analog would induce symptoms of poisoning in the CNS of the cockroach and whether these symptoms of poisoning would correlate with behavioural changes.

1. RATIONALE AND TREATMENT WITH O.C. COMPOUNDS

An insecticide is often picked-up by an insect through its cuticle. Once into the euticular layer it is transferred to the haemolymph where it will be distributed to the target site(s). Depending upon the insecticide, an activation and/or detoxification step may precede the action of the insecticide on the target (nerve) (Narahashi, 1979). Furthermore, it is likely that the insecticide acts at a number of sites throughout the insect, the sum total of which determine the potency and resultant mortality. By treating an isolated nerve with insecticide (in vitro), the factors involved in poisoning, in vivo, do not contribute to the toxic syndrome. Therefore, research conducted regarding activity in the VNC was performed on VNCs which had been removed from cockroaches treated in vivo with an O.C. or control compound.

All experiments examining the effects of DDT or analog on the nervous system employed cockroaches that had

been injected with a LD50 96 h dose. Injection was preferred to topical application for several reasons: 1/ the quantity of DDT entering the body of the cockroach could be strictly controlled; 2/ there would be little delay time between injection and response; and 3/ the dose could be delivered in an acute fashion.

It was intended that all the analogs tested (except p,p'-DDE) be injected at an equitoxic dose (LD50 96h). This was confirmed for p,p'-DDD, MeO-DDT, and o,p'-DDT, but, it was determined that p,p'-DDT was injected at a dose equal to a LD42 96 h (n=57). Even at this dose, the effects on the nervous system were significantly different (Student's t-test, $P < 0.005$) from control experiments (Fig. 26 and 27). If the dose injected had been greater, i.e., closer to a LD50 96 h value, the effects on the nervous system may have been even more pronounced, (i.e., one would expect a larger percentage of cockroaches displaying repetitive firing and a larger Activity Quotient at 3-weeks).

Cockroaches were injected with p,p'-DDE at approximately 70 times the dose used for p,p'-DDT. The concentration of p,p'-DDE used was at its limit of solubility in acetone (at 25°C, ca. 1.6 M). However, despite the high concentration injected, the occurrence of repetitive firing in the nerve cords of p,p'-DDE injected cockroaches was not significantly different from controls

(Student's t-test, $P=0.05$).

2. o,p'-DDT

o,p'-DDT was previously reported as being non-toxic to cockroaches (Gardner and Vincent, 1978), although it did induce repetitive firing in an isolated ventral nerve cord (Gardner and Vincent, 1978; confirmed by Elia). The high dose required for a LD50 96 h in the present experiments (0.60 to 0.65 umole/g cockroach) is suggestive of either an imprecise action at the target site, a metabolic conversion (activation/detoxification) in vivo, or a delay in reaching the target site (O'Brien, 1967). In vitro, a minimum concentration of 5×10^{-4} M was required to induce repetitive firing in a nerve cord (Gardner and Vincent, 1978). This dose was 625 times greater than the minimum dose required for p,p'-DDT (8.0×10^{-7} M) to induce repetitive firing in the VNC, in vitro. This considerable difference, however, only pertains to the in vitro system. In vivo, the dose of o,p'-DDT (0.65 umol/g cockroach) to obtain a LD50 96 h level of mortality was only about 35 times greater than the dose required for p,p'-DDT (0.017 umol/g cockroach). Thus, it appears as though an in vivo mechanism potentiates the effect of o,p'-DDT on the nervous system which is not realized in vitro. (This also substantiates the contention that purely in vitro studies of insecticides on the CNS must

not be taken out of context and points to the dangers of extrapolating such in vitro data to the in vivo situation.) Comparing the minimum effective dose to induce repetitive firing in vitro, for MeO-DDT and p,p'-DDD, with that required for p,p'-DDT reveals that there is not as great a difference in dose as was found with o,p'-DDT. In vitro, MeO-DDT and p,p'-DDD required only 1.3 and 30 times the dose of p,p'-DDT, respectively (Gardner and Vincent, 1978). In vivo, the difference in dose required for a LD50 96 h comparing MeO-DDT and p,p'-DDD with p,p'-DDT was similar (1.7 and 8.8 times, respectively) as that found for the in vitro dose. However, further study is required to clarify the significance of these points.

By injecting o,p'-DDT into whole animals in these experiments, the possibility that the cuticle acted as a diffusion barrier was eliminated. (The cuticle may still absorb some of the DDT analog injected, but this is not a primary sink or target (O'Brien, 1967).) The possibility of conversion of o,p'-DDT to p,p'-DDT (or another toxic analog) was considered and pursued because of the steady increase in mortality after 48 h incubation of o,p'-DDT, in vivo. Although no literature could be found to support such a possible conversion in insects, several papers reported that o,p'-DDT was converted to p,p'-DDT in rats and pigeons (Klein et al., 1964; Klein et al., 1965;

French and Jefferies, 1969). However, since then, evidence has emerged refuting the possible conversion of o,p'-DDT to p,p'-DDT in rats, sheep, chicken, and quail (Bitman et al., 1971; Cranmer, 1972). Evidence was presented which indicated that samples of o,p'-DDT used by earlier authors contained p,p'-DDT as an impurity. The p,p'-DDT impurity rapidly accumulated in fat tissues while o,p'-DDT was rapidly eliminated by conversion to hydroxy and methoxy metabolites and excreted in the faeces (Bitman et al., 1971; Cranmer, 1972).

However, despite its rapid in vivo metabolism (in homeotherms), o,p'-DDT at high enough doses is toxic to both cockroaches and homeotherms (Dale et al., 1966; Okey and Page, 1974). A dose of p,p'-DDT large enough to induce symptoms of poisoning in a rat was correlated with the amount of p,p'-DDT found in the brain (Dale et al., 1966). The same dose of o,p'-DDT did not induce poisoning symptoms although an equivalent amount of o,p'-DDT was found in the brain. It required a 6 to 9 times greater concentration of o,p'-DDT in the brain compared with p,p'-DDT before symptoms of poisoning occurred (Dale et al., 1966). An assay was performed and revealed that there had been an insignificant (<4.0 ppm, minimum detection value) conversion in the brain of o,p'-DDT to o,p'-DDE (an inactive analog - o,p'-DDT was present at levels of 1610 to 2843 ppm). Although this evidence pertains to rats, some parallels exist with the evidence

presented for o,p'-DDT toxicity in cockroaches. Most notable was the much greater dose of o,p'-DDT required to induce symptoms of poisoning. That levels of o,p'-DDT and p,p'-DDT were the same in the brains of rats injected with an equal concentration of each suggests that the permeability of the CNS in the rat was not a factor in determining toxicity. No parallel evidence concerning the permeability and accumulation of o,p'-DDT in the CNS presently exists for the cockroach (or any other insect). Experiments could be performed to clarify whether or not o,p'-DDT accumulates in the cockroach CNS at the same rate and to the same concentration as p,p'-DDT. Ultimately, this may help to elucidate the molecular nature of the target site within the membrane which is attacked by p,p'-DDT. Furthermore, the low toxicity of o,p'-DDT supports the contention that the receptor site for p,p'-DDT in the membrane is stereo-specific (Holán, 1969; Fahmy et al., 1973; Coats et al., 1977; 1979) (also see Introduction, Section 2(d)).

Another possible explanation for the higher dose of o,p'-DDT required for 50% mortality is a delay in reaching the target site. The slow development of poisoning symptoms (beginning at approximately 48 h post-injection) and the high mortality over the 3-week period following injection, lend support to the supposition that o,p'-DDT may only slowly accumulate at the target site.

Eventually, enough would accumulate and exert an effect. The amount injected, and thus contained in a susceptible cockroach, would not change significantly because they do not rapidly detoxify DDT analogs (Metcalf and Fukuto, 1968). This is evident from the 3-week mortality counts and neurophysiological evidence that indicated the VNC of cockroaches treated 3-weeks previously with o,p'-DDT displayed a significant level of repetitive firing (Student's t-test, $P < 0.005$). The slight difference in partition coefficients between o,p'-DDT and p,p'-DDT does not seem a likely explanation for the difference in toxicity observed.

Other possibilities exist for the mode of action of o,p'-DDT, although all remain unsubstantiated. Of those listed above, metabolic conversion seems unlikely (but not disproven in the cockroach). Target site specificity and delay in reaching the target site probably combine to determine the degree to which o,p'-DDT is toxic.

3. REPETITIVE FIRING IN CONTROLS

Various types of spontaneous activity were normally associated with the cockroach ventral nerve cord preparation. The only one which required special attention was the type illustrated in Figure 10(b). Careful determination of initial and final spiking

frequencies was essential. Characterization of the terminal portion of the spike-train also contributed to the identification. If during the latter portion of a discharge the frequency of firing became random, and the initial spike frequency was low (40-100 Hz), then the discharge would not be considered repetitive firing. Criteria employed usually allowed distinction between classical repetitive firing (O.C. induced) and normal repetitive-type activity in the CNS. It was perhaps fortuitous that the terminal portion of the non-organochlorine induced repetitive-type activity could usually be characterized and thus distinguished from repetitive firing induced by an organochlorine compound. On the other hand, it supports the supposed specificity of DDT action on the nervous system, i.e., trains of spikes induced by DDT are usually distinguishable from endogenous activity.

However, that the nervous system from an untreated cockroach should repetitively fire indicates that the event itself is not particularly unusual. The uncontrolled, high frequency of firing and occurrence of discharges are the factors which distinguish a normal CNS from a poisoned one (Figure 26 and 27).

An irregular type of low amplitude, spontaneous activity was noted occasionally (Fig. 10(c)). It was present in both control and O.C. treated cockroach CNSs.

Its characteristics were entirely different from any type of repetitive firing activity. This spontaneous activity could be minimized by careful handling of the nerve preparation during excision from the cockroach and while being placed on the stimulating and recording electrodes. Some preparations appeared to be more sensitive than others to the procedure of being raised clear of saline in order to stimulate and record. These preparations would display an increased amount of random spontaneous activity for 30 to 60 sec before decreasing to a normal background level. However, the low firing frequency and random nature of this spontaneous activity readily distinguished it from a repetitive discharge.

The random frequency and amplitude of the spikes comprising the post-CAP-activity served to distinguish it from an incidence of high frequency repetitive firing (Fig. 10(d)). The various frequencies and amplitudes implied that a number of axons were firing independently of one another (after the main compound action potential). When a faster time base was used, it became evident that the spikes within the group did not form any regular firing patterns, unless a repetitive firing discharge happened to follow the post-CAP-activity. Only then could a regular firing pattern be distinguished (Fig. 20(b)).

Qualitatively, post-CAP-activity was seen to occur more frequently in O.C. treated preparations than in controls. It could have been a symptom of poisoning, although its frequency of occurrence was nowhere near that observed for repetitive firing. Furthermore, its very inconsistent nature (i.e., the randomness of amplitude, spike frequency, and relative infrequent occurrence) made it very difficult to characterize and compare with a similar discharge which occurred in another preparation. For these reasons, an attempt to correlate post-CAP-activity with O.C. poisoning was not attempted, although a record of incidence was made.

It is evident from Figures 26 and 27 that some control preparations at 24 h and 3-weeks displayed signs of instability by repetitively firing in response to a single electrical stimulus. The form of these repetitive responses were indistinguishable from repetitive firing induced by the efficacious O.C. compounds. However, the single difference between repetitive firing in control and O.C. treated cockroaches was the frequency of occurrence in that particular preparation and in the entire group (Fig. 22(a,b compared with c,d)). It is apparent that all control preparations do not repetitively fire. Those that do, usually do so with a very low frequency of occurrence. An average of about 10% (n=38) of control cockroaches at 24 h and 3-weeks (Figure 13 and 21, respectively)

displayed some sign of repetitive firing. This, in addition to the very low activity quotient (Fig. 26 and 27) indicated several things: 1/ that the level of instability displayed by some control preparations was insufficient to induce behavioural abnormalities; 2/ an unpoisoned CNS was capable of producing repetitive firing in response to a single electrical stimulus. This implies that the 'facility' to create and cope with high frequency spike trains existed before the application of an O.C. compound; and 3/ due to the low incidence of occurrence of repetitive firing in controls it (repetitive firing) may be used as a criterion to help evaluate the extent to which a nervous system has been poisoned.

4. REPETITIVE FIRING IN O.C. TREATED VNCs

As indicated above, preparations from control cockroaches at 24 h and 3-weeks that displayed repetitive firing did not elicit any behavioural abnormalities. A similar finding was discovered in O.C. treated cockroaches. The failure of MeO-DDT or p,p'-DDD treated preparations to display repetitive firing activity after 3-weeks is presumably a reflection of the rapid degradation of these analogs to non-toxic metabolites (O'Brien, 1967; Gardner, 1973). At 3-weeks, most of the CNSs from p,p'-DDT (73%, n=22) and o,p'-DDT (92%, n=12) treated cockroaches displayed repetitive firing. The

occurrence of repetitive firing in these cockroaches was significantly greater than that found in control preparations. However, despite the significantly greater occurrence of repetitive firing in these cockroaches, none displayed any sign of abnormal behaviour prior to decapitation. At 24 h, all CNSs from cockroaches treated with p,p'-DDT, MeO-DDT, and p,p'-DDD displayed repetitive firing in a very consistent manner (Fig. 14(a-c)). Despite evidence of a severely disrupted CNS, behavioural signs of poisoning in these cockroaches had either disappeared or were present in a much reduced form. Cockroaches in which behavioural signs had persisted displayed an adaptive response to the startle stimulus. That is, initially, hyperactivity in the cockroach could be elicited by a sudden tapping of the flask containing the cockroach. However, with several further startle (tapping) stimuli, the cockroach did not usually continue to respond in a hyperexcited fashion. Before discussing the possible relevance and mechanism of the nervous system/behaviour uncoupling, two other points should be noted. Recordings made from VNCs of cockroaches in early stages of poisoning (30 min to several hours) indicated that severe disruption (i.e., extensive repetitive firing) in the CNS at this time paralleled changes in behaviour. However, the VNC of cockroaches which were near-prostrate or prostrate did not usually display repetitive firing at a level significantly different from controls, though, the

compound action potential during each recording was attenuated and was intermittently blocked in the abdominal segment of the VNC altogether. (This oscillatory phenomenon between block and recovery was also noted by Gammon (1978) in the CNS of DDT-poisoned cockroaches.)

Therefore, the progressive poisoning in the cockroach following a LD₅₀ 96 h dose of DDT appears to follow a particular pattern. This pattern is based upon behavioural and neurophysiological correlates and is consistent among cockroaches injected with MeO-DDT, p,p'-DDD, and p,p'-DDT. The first step following injection of one of the effective O.C. compounds is a delay of approximately 15 to 30 min before any visible sign of poisoning becomes apparent. (Gammon (1978) presented evidence that indicated neurophysiological symptoms of poisoning arose prior to behavioural signs: recording from a "wired", free-walking cockroach was required to expose this feature of poisoning.) Nerve cords which were removed immediately following initial signs of poisoning displayed extensive repetitive firing (i.e., CNS disruption paralleled behavioural signs). As time progressed, behavioural signs of poisoning continued to parallel neurophysiological disruption in cockroaches that became prostrate and subsequently died in 24 to 48 h (repetitive firing followed by nerve block and death). However, approximately 50% of treated cockroaches apparently recovered from poisoning symptoms (as defined

by the LD50 dose). In these cockroaches, behavioural signs of poisoning disappeared while neurophysiological signs (extensive repetitive firing) persisted. This was the first sign of CNS/behaviour uncoupling. The activity in these VNCs did not usually degenerate to intermittent block and recovery, (ultimately leading to complete block with no recovery). However, the VNC is actually a conglomeration of neurons, and since the axon of each neuron contributes to the final compound action potential, it is possible that some neurons were blocked while the majority (which were still firing) formed the compound action potential recorded. The slight decrease in size of the compound action potential that would result may not be noticed because of the normal variation in amplitude that occurred. Also, if small diameter axons were blocked, the change in size of the compound action potential would be less than if a large diameter axon were blocked (Pearson et al., 1970).

The following question still remains: why did the instability (extensive repetitive firing) which existed in the VNC at 24 h and 3-weeks not parallel behaviour exhibited by these cockroaches?

It was first proposed by Roeder (1948) that the abdominal giant fibres ascend the VNC and excite leg motorneurons. However, Dagan and Parnas (1970) provided

evidence that apparently contradicted Roeder's earlier assumption. Intracellular stimulation of individual abdominal giant axons up to a frequency of 200 Hz (for 1 sec) did not cause excitation of the motor nerve and consequently did not induce leg movements. But, extracellular stimulation of the whole VNC at A5-A6 did elicit leg movements. They concluded that the giant axons of the abdomen were not responsible for conduction of the nerve impulse mediating the escape reflex. However, more recent evidence (Camhi, 1976; Westin et al., 1977; Ritzmann and Camhi, 1978) indicates that with high enough frequencies of firing (>200 Hz) the abdominal giant axons will excite leg motoneurons. Mechanical stimuli (wind puffs) directed toward the cerci are known to excite a minimum of eight giant interneurons in the VNC (Westin et al., 1977), followed by excitation of the leg motoneurons.

With respect to cockroaches treated 24 h previously with p,p'-DDT, MeO-DDT, and p,p'-DDD, their lack of hyperexcited response to a startle stimulus apparently did not correlate with symptoms of poisoning in the VNC. When startled, these poisoned cockroaches responded in a fashion indistinguishable from controls. In controls, the response to a startle stimulus was recorded in the VNC (giant axons) as a brief volley of spikes, normally at a frequency of greater than 200 Hz (Camhi, 1975; Westin et

al., 1977; Ritzmann and Camhi, 1978). The outcome of this giant axon activity was excitation of motorneurons and subsequently an escape manoeuvre.

In the poisoned cockroach at 24 h a single action potential in the VNC usually induced a train of spikes of frequency greater than 200 Hz. Therefore, the normal volley of impulses elicited in response to a startle stimulus would be compounded many times over in the poisoned cockroach. The compounded firing in the giant axons (and likely in some of the smaller ascending fibres) is hypothesized to induce the exaggerated startle response during initial stages of poisoning. However, the validity of this hypothesis becomes questionable when considering 24 h and 3-week data. From the evidence presented, it is suggested that the instability (repetitive firing) in the VNC which may have previously been responsible for behavioural signs of poisoning, does not exert that effect beyond approximately 24 h post-injection of a LD50 96 h dose (of toxic compounds listed above). Cockroaches at about 24 h post-injection began to show an apparently normal behavioural response to a startle stimulus. The cerci mainly respond to currents of air passing by them (Westin et al., 1977). If the cerci become unresponsive and/or the abdominal ganglionic chain becomes 'disconnected' from the thoracic ganglia, it is possible that the wind puffs and tapping of the containing flask would activate sensory receptors on the thorax and head.

This should induce these sensory organs to acknowledge receipt of the external mechanical stimulus by creating a generator-, and subsequently, action-potential(s). These action potentials enter the CNS and normally would be transmitted to the effector muscles for the startle reflex. Although direct evidence of repetitive firing in the thoracic connectives induced by DDT and analogs was not obtained, it seems unlikely that nervous system disruption would occur only in the abdominal segments of the VNC. Furthermore, cockroaches which had their VNCs severed at T3-A1 24 h prior to treatment, responded in the typical fashion to a LD50 96 h dose of DDT injected intra-abdominally. From this it may be concluded: 1/ the apparent spread of DDT from the abdominal cavity to the thorax is rapid and does not require an intact connection between abdominal and thoracic segments of the VNC; and 2/ development of overt signs of poisoning (tremors, jitters, hyperexcitability) do not require input from the giant interneurons (or any other neuron) from the abdominal VNC. Obviously then, input from the abdominal VNC alone was not responsible for behavioural signs of poisoning. Possibly, a neural mechanism which enables the cockroach to tolerate the extremely unstable nervous system at 24 h post-injection also resides in the thoracic ganglia.

The hypothesis that a neural mechanism may be responsible for preventing invasion of multiple discharges

into motor neurons is supported by evidence regarding modulation of spike frequency by regions of special axonal geometry in the thoracic ganglia (see Introduction, Section 1) (Castel et al., 1976; Parnas et al., 1976; Spira et al., 1976). It was found that descending and ascending impulses passing through the metathoracic ganglion (T3) could be altered. Stimulating at a frequency of 33 Hz for 120 sec at T2-T3 induced complete block of the invading spike recorded (intracellularly) from the posterior base of T3. Single spikes could be recorded again within 5 sec after cessation of the stimulation (33 Hz). However, if the axon was again stimulated at this frequency within 20 min of a previous block, then conduction block redeveloped after only several impulses. The same sequence of events transpired if the axon was stimulated caudally (A4-A5) and recorded from at the anterior base of T3. At a higher frequency of stimulation (50 Hz), the invasion of descending and ascending impulses were blocked much more rapidly (25 sec and 40 sec, respectively) (Spira et al., 1976). Furthermore, conduction block of impulses descending through T3 could be blocked by stimulation of the connective A4-A5 (ascending impulses) at a frequency of 100 Hz. (Similar conduction blocks could be obtained by stimulation of single giant axons if the stimulation was applied for 6 to 10 times longer than that used when stimulating the entire VNC (Spira et al., 1976).)

Therefore, a conduction block in the metathoracic ganglion which prevents invasion of further input from the abdominal segment of the VNC, seems to be a distinct possibility. Certainly frequencies of repetitive firing recorded were high enough to induce such a block, as compared with results from Spira et al. (1976).

Regions of similar axonal geometry and synaptic input in the meso- and prothoracic ganglia have been hypothesized to exist with the same operational characteristics as the metathoracic ganglion. Ultrastructural and electrophysiological analysis of regions of axonal tapering and synaptic input in the meso- and prothoracic ganglia support this hypothesis (Parnas et al., 1969; Spira et al., 1969(a,b); Castel et al., 1976).

Major neuronal pathways invading the thoracic ganglia may be inhibited (blocked) by the method described above. However, although the effect of DDT on motor-axons appears to be similar to that on the CNS (i.e., a repetitive discharge in response to a single input stimulus, either electrical or endogenous) (Roeder and Weiant, 1948; etc.) a leg (coxa to tarsus, inclusive) isolated from a cockroach displaying signs of DDT poisoning does not continuously jitter or tremor in its isolated state. This suggests that a high level of spontaneous excitation in the motor-axon does not exist. Furthermore, a viable connection between motor-neurons and excitor neurons

and/or a thoracic ganglion presumably must exist in order for the in situ leg to display the signs of poisoning observed. During early stages of poisoning, therefore, this connection must exist. However, as the blockage to invading spikes to the thorax increases, one would expect to see a decline in poisoning symptoms displayed. This is what happens, although it is impossible to distinguish (using the results presented here) whether a tremor or jitter in a leg was the result of a spontaneous discharge in the motoneuron or a discharge initiated from the CNS. This hypothesis however, does require the assumption that the giant axons do not directly control neural circuits responsible for such normal behaviour patterns as walking (Parnas and Dagan, 1971). The transition between central and peripheral excitation in DDT poisoning is not clear and needs to be elucidated. Recording simultaneously at various locations in the CNS and from motoneurons of DDT-poisoned cockroaches may help to answer this question.

The possibility of hyperexcitation of the common inhibitory leg motor neuron also exists. It has been shown (Fourtner and Drews, 1977) that this inhibitory neuron may be excited by tactile stimulation of the cercus or electrical stimulation of the cercal nerve. The primary function of the common inhibitory motor neuron appears to be related to coordination during walking or running (Pearson, 1973). This is accomplished by

increasing the rate of relaxation in tonic muscle fibres. The inhibitory motoneuron has also been shown to fire during the very initial stages of the startle response, thus acting to decrease and limit tension in tonic muscles (extensors) of the limbs (Fourtner and Drews, 1977).

Thus, normal muscle contraction and leg movements are not grossly inhibited by the common inhibitory motor neuron.

Therefore, during early stages of poisoning, increased activity in this neuron may contribute to the general poisoning syndrome by releasing normal tone in the extensors, causing movements to appear jittery. Whether or not the inhibitory motor neuron is blocked during later stages of poisoning would determine its subsequent effect on poisoning symptoms displayed.

However, the question remains: is the electrically stimulated activity in the nervous system actually representative of naturally arising repetitive firing? Evidence by Gammon (1978) indicated that spontaneous repetitive firing does occur in the CNS of his "wired" free-walking cockroach preparation. Thus, it is evident that repetitive firing induced by an electrical stimulus, in vitro, is not an artifact of isolation and does occur in the intact animal.

5. POSSIBLE CORRELATIONS WITH ENDOTOXIN RESEARCH

The possibility of release of an endogenous toxic

substance (endotoxin or "stress-toxin") into the haemolymph as a result of DDT poisoning was previously described (see Introduction, Section 2(e)). Although the present investigation did not explore this possibility, it is interesting to compare data from endotoxin research with neurophysiological results reported here.

The appearance of the endotoxin in the haemolymph was reported as being approximately simultaneous with the onset of poisoning symptoms (Sternburg and Kearns, 1952; Shankland and Kearns, 1959). Endotoxin injected into an untreated cockroach induced that cockroach to become hyperexcitable and display symptoms of poisoning similar to those seen in DDT poisoned cockroaches. More recent evidence (Gammon, 1977) showed that there was a considerable amount of spontaneous activity in the CNS of cockroaches for several hours following the surgical procedure employed to implant electrodes at various sites along the CNS. This increased spontaneous activity was attributed to build-up of "stress-toxins" in the haemolymph (Gammon, 1977). If this was the case, then it is feasible that the hyperactivity in the nervous system induced by DDT may cause release (and/or production) of such a stress-toxin. This stress-toxin would then circulate in the haemolymph and subsequently induce a high level of spontaneous activity in the nervous system. The gradual disappearance of the stress-toxin or the habituation of the response to it has to be postulated

to coincide with the observed remission of behavioural poisoning symptoms and consequently a decrease in spontaneous activity in the motorneurons.

Although the relation between DDT and production and/or release of a stress-toxin remains largely unsubstantiated, it should not be ignored as a possible mechanism contributing to the intoxication of the cockroach by DDT.

6. THE ATPase QUESTION

Evidence presented in the Introduction (Section 2(f)) showed that inhibition of the Na-K-ATPase and/or the Mg-ATPase could not be responsible for the signs and symptoms of poisoning elicited by a cockroach following a dose of DDT. Inhibition of the Na-K-ATPase followed a positive temperature of DDT poisoning. This is contrary to the toxic effects on the insect, which follow a negative temperature coefficient of toxicity. Also, symptoms of poisoning by cardiac glycosides such as ouabain (which inhibits the Na-K-ATPase) do not parallel the effects of DDT on the nervous system.

Also, the complete process of oxidative phosphorylation (in which the Mg-ATPase catalyses the final stage, (the phosphorylation of ADP to ATP)) showed a positive temperature coefficient of inhibition while the isolated Mg-ATPase displayed a negative temperature

coefficient of inhibition (Cheng and Cutkomp, 1977). Furthermore, it seems unlikely that inhibition of the Mg-ATPase could disrupt the nervous system in such a radical manner considering mitochondrial function does not directly affect the mechanism of the action potential (Gardner and Bailey, 1975; Brooks, 1980). Also, from the available evidence presented in the Results (Section 5), it seems unlikely that sufficient DDT reaches the mitochondria to have the inhibitory effect on Mg-ATPase, as claimed by Cheng and Cutkomp (1977).

The inhibition of ecto-Ca-ATPase located on the external surface of the axonal membrane by DDT has also been considered as a possible target for the DDT molecule (Matsumura and Ghasuddin, 1979; Ghasuddin and Matsumura, 1979; Ghasuddin et al., 1981). Inhibition of the ecto-Ca-ATPase would presumably result in the destabilization of the membrane in a fashion similar to that induced by DDT (Matsumura and Ghasuddin, 1979). However, the link between biochemical and electrophysiological evidences have not been sufficiently elucidated to allow any conclusions to be drawn.

7. LOCALIZATION OF DDT IN THE CNS, in vivo

Fractionation of the CNS of cockroaches treated with a dose of ^{14}C -DDT was pursued in an attempt to determine

whether there was a difference in the affinities of the fractions for ^{14}C -DDT, in vivo. Because of the relatively short incubation time (1 h) and the slow metabolism of DDT (in vivo), it was assumed that the majority of the ^{14}C -label would remain associated with the DDT molecules. (It was also shown by TLC that less than 1% of radioactivity could possibly be associated with any other analog.) The data obtained concerning the amount of DDT associated with each fraction was for use in comparison with the amount of DDT required to inhibit the Mg-ATPase. Unfortunately, most publications dealing with the inhibitory effects of DDT on the ATPase enzyme (Cutkomp et al., 1971; Doherty and Matsumura, 1975; Cutkomp et al., 1976; Cheng and Cutkomp, 1977) do not specify the amount of DDT employed per mg protein. Their use of concentrations of DDT in the reaction mixture and units of $\mu\text{moles Pi}$ (inorganic phosphate) produced/mg protein/hour (the unit denoting ATPase activity) without alluding to the actual amount of protein, precluded any attempt at comparison with results presented here concerning the amount of DDT per fraction.

Jackson and Gardner (1978(a)) were an exception. Unfortunately, they employed trout brain mitochondria in their experiments. However, until data using cockroach CNS becomes available, a cautious comparison between trout brain and cockroach CNS must suffice. By specifying the number of moles of DDT employed per mg trout brain

mitochondrial (TBM) protein, it was determined that 1.4×10^{-5} moles DDT/mg TBM protein was required for approximately 50% inhibition of Mg-ATPase. Using the mean total protein (Lowry Method of analysis) of the mitochondrial fraction (7.37×10^{-1} mg - see Table 9) and the total amount of DDT calculated to be associated with the mitochondrial fraction (3.50×10^{-12} moles - see Appendix G), then 4.75×10^{-12} moles DDT/mg protein were associated with the isolated mitochondrial fraction. Thus, Jackson and Gardner (1978(a)) required about $3 \times 10^{+6}$ times more DDT to inhibit 50% of the TBM ATPase than found associated with the mitochondrial fraction in vivo. According to these results, the amount of DDT associated with the mitochondrial fraction (in vivo), would be insufficient to cause inhibition. In addition, the value of 4.75×10^{-12} moles DDT/mg protein associated with the mitochondrial fraction was a liberal estimate as the mitochondrial fraction may have picked-up about 80% of its associated ¹⁴C-DDT during the homogenization and fractionation procedures (see Results, Section 5(d)). Therefore, the actual amount of DDT in the mitochondrial fraction could be considerably less (ca. 6×10^{-13} moles). This tends to reinforce the hypothesis that insufficient DDT reaches the mitochondria of the CNS to cause a significant degree of inhibition.* Then implicitly, this further substantiates the argument that the overt signs of poisoning displayed by the cockroaches injected with ¹⁴C-DDT were not due to the inhibition of the Mg-ATPase.

* see Pg. 151

GENERAL CONCLUSIONS

Signs of poisoning in cockroaches treated with p,p'-DDT, MeO-DDT, and p,p'-DDD (LD50 96 h) begin between 15 and 30 min post-injection and persist until approximately 24 h post-injection. During this period, repetitive firing in the abdominal nerve cord was readily elicited. However, cockroaches that had apparently recovered from signs of poisoning at 24 h still displayed symptoms of poisoning in their isolated nervous systems (i.e., repetitive firing). Furthermore, this symptom of poisoning persisted at least 3-weeks in cockroaches treated with p,p'-DDT and o,p'-DDT.

Cockroaches treated with o,p'-DDT required an incubation period of approximately 48 h before overt signs of poisoning were displayed. After this 48 h incubation period, activity in the VNC was similar to that found in VNCs from cockroaches treated with p,p'-DDT, MeO-DDT, and p,p'-DDD. Target site specificity and delay in reaching the target site in the CNS are hypothesized to determine the degree to which o,p'-DDT is toxic.

Although repetitive firing was occasionally elicited in control VNC preparations, its occurrence was significantly less than the occurrence of repetitive firing induced by O.C. compounds at 24 h (p,p'-DDT,

MeO-DDT, p,p'-DDD); 48 h (o,p'-DDT); and 3-weeks (p,p'-DDT, o,p'-DDT).

Initially, the development of behavioural signs of poisoning may result from DDT's interaction with the CNS. But, CNS disruption persists well beyond the stage of behavioural abnormalities. Thus, it is suggested that action potential modulation in the thoracic ganglia may be responsible for the suppression of behavioural signs of poisoning. However, an alternate hypothesis suggesting that the DDT induces the release and/or production of an endogenous toxic substance should not be ignored. A form of habituation by the cockroach may then be hypothesized to account for the disappearance of signs of poisoning while CNS disruption persists. Ultimately, a combination of factors, which may include the above hypotheses, conspire to kill the animal.

Experiments indicated that the amount of DDT that reaches the mitochondrial fraction of the CNS in vivo, was insufficient to induce a significant level of inhibition of Mg-ATPase, making this an unlikely site of action of DDT, in vivo.

REFERENCES

- Adams, M. E. & Miller, T. A. (1979). Site of action of pyrethroids: Repetitive "backfiring" in flight motor units of house fly. *Pestic. Biochem. Physiol.* 11: 218-231.
- Adams, M. E. & Miller, T. A. (1980). Neural and behavioral correlates of pyrethroid and DDT-type poisoning in the house fly, Musca domestica (L.). *Pestic. Biochem. Physiol.* 13: 137-147.
- Adelman, W. J. Jr. & Senft, J. P. (1966). Voltage clamp studies on the effect of internal cesium ion on sodium and potassium currents in the squid giant axons. *J. Gen. Physiol.* 50: 279-293.
- Armstrong, C. M., Benzanilla, F. & Rojas, E. (1973). Destruction of sodium conductance in squid axons perfused with pronase. *J. Gen. Physiol.* 62: 375-391.
- Armstrong, C. M. & Binstock, L. (1965). Anomalous rectification in the squid giant axon injected with tetraethylammonium chloride. *J. Gen. Physiol.* 48: 859-872.
- Barnola, F. V., Camejo, G. & Villegas, R. (1971). Ionic channels and nerve membrane lipoproteins: DDT-nerve membrane interaction. *Intern. J. Neuroscience* 1: 309-316.
- Bitman, J., Cecil, H. C. & Fries, G. F. (1971). Nonconversion of o.p'-DDT in rats, sheep, chickens, and quail. *Science* 174: 64-66.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochem.* 72: 248-254.
- Brooks, G. T. (1980). Biochemical targets and insecticide action. pg.41-56. In: *Insect Neurobiology and Pesticide Action (Neurotox. 79)*. Narahashi, T. (editor). Society of Chemical Industry, (Publishers), London.
- Brown, B. E. (1965). Pharmacologically active constituents of the cockroach corpus cardiacum: Resolution and some characteristics. *Gen. Comp. Endocrinology.* 5: 387-401.
- Brown, D. D., Metcalf, R. L., Sternburg, J. G. & Coats, J. R. (1981). Structure-activity relationships of DDT-type Analogs based on in vivo toxicity to the sensory nerves of the cockroach Periplaneta americana (L.). *Pestic. Biochem. Physiol.* 15: 43-57.

Bullock, T. H. & Horridge, G. A. (1965). In: Structures and Function in the Nervous Systems of Invertebrates. Freeman & Co., London (England).

Camejo, G., Villegas, G. M., Barnola, F. V. & Villegas, R. (1969). Characterization of two different membrane fractions isolated from the first stellar nerves of the squid Dosidicus gigas. Biochim. Biophys. Acta 193: 247-259.

Camhi, J. M. (1976). Non-rhythmic sensory inputs: Influence on locomotory outputs in arthropods. In: Neural Control of Locomotion. Herman, R. M., Grillner, S., Stein, P. S. G. & Stuart, D. G., (editors). Plenum Press, London & New York. pg. 516-586.

Castel, M., Spira, M. E., Parnas, I. & Yarom, Y. (1976). Ultrastructure of region of low safety factor in inhomogenous giant axon of the cockroach. J. Neurophysiol. 39(4): 900-908.

Chapman, R. F. (1971). THE INSECTS - Structure and Function, Section E. American Elsevier Publishing Co., Inc. New York.

Chappell, J. B. & Hansford, R. G. (1972). Preparation of mitochondria from animal tissues and yeasts. In: Subcellular Components: Preparation and Fractionation. Birnie, G. D. (editor). University Park Press, Baltimore. pg. 77-91.

Cheng, E. Y. & Cutkomp, L. K. (1975). The ATPase system in American cockroach muscle and nerve cord. Insect Biochem. 5: 421-427.

Cheng, E. Y. & Cutkomp, L. K. (1975). The ATPase system in American cockroach muscle and nerve cord. Insect Biochem. 5: 421-427.

Cheng, E. Y. & Cutkomp, L. K. (1977). The sensitivity of mitochondrial Mg -ATPase to DDT and analogs at different temperatures. Pestic. Biochem. Physiol. 7: 360-366.

Coats, J. R., Metcalf R. L. & Kapoor, I. P. (1977). Effective DDT analogues with altered aliphatic moieties, isobutanes and chloropropanes. J. Agric. Food Chem. No. 4, 859-868.

Coats, J. R., Metcalf, R. L., Kapoor, I. P., Chio, L. & Boyle, P. A. (1979). Physical-chemical and biological degradation studies on DDT analogues with altered aliphatic moieties. J. Agric. Food Chem. 27(5): 1016-1022.

Cochran, D. G. (1955). Differential susceptibility of the sexes and developmental stages of the American cockroach to several insecticides. *J. Econ. Entomol.* 48(2):

Cornwell, P. B. (1968). *The Cockroach*. Vol. I, Hutchinson & Co., (Publishers) Ltd. London.

Cornwell, P. B. (1976). *The Cockroach*. Volume 2. Associated Business Programmes (Publishers). London, England.

Cranmer, M. F. (1972). Absence of conversion of o,p'-DDT to p,p'-DDT in the rat. *Bull. Environ. Contam. Toxicol.* 7: 121-124.

Cutkomp, L. K., Desai, D., Cheng, E. Y., Veal, E. V. & Koch, R. B. (1976). The *in vivo* sensitivity of ATPases to DDT, DDE, and physical immobilization in American cockroaches. *Pestic. Biochem. Physiol.* 6: 203-208.

Cutkomp, L. K., Yap, H. H., Cheng, E. Y. & Koch, R. B. (1971). ATPase activity in fish tissue homogenates and inhibitory effects of DDT and related compounds. *Chem.-Biol. Interactions.* 3: 439-447.

Cutkomp, L. K., Yap, H. H., Veal E. V. & Koch, R. B. (1971). Inhibition of oligomycin-sensitive [mitochondrial] Mg -ATPase by DDT and selected analogs in fish and insect tissue. *Life Sci.* 10: 1201-1209.

Dagan, D. & Parnas, I. (1970). Giant fibre and small fibre pathways involved in the evasive response of the cockroach, Periplaneta americana. *J. Exp. Biol.* 52: 313-324.

Dale, W. E., Copeland, M. F., Pearce, G. W. & Miles, J. W. (1966). Concentration of o,p'-DDT in rat brain at various intervals after dosing. *Archives Internat. de Pharmacodynamie et de Therapie.* 162: 40-43.

Delcomyn, F. & Daley, D. L. (1979). Central excitation of cockroach giant interneurons during walking. *J. Comp. Physiol.* 130(1): 39-48.

Desai, D., Cutkomp, L. K. & Koch, R. B. (1974). A comparison of DDT and its biodegradable analogues tested on ATPase enzymes in cockroaches. *Pestic. Biochem. Physiol.* 4: 232.

Desai, D., Jarvinen, L. K. C., Jarvinen, R. R. K. & Jarvinen, A. (1975). DDT: effect of continuous exposure on ATPase activity in fish, Pimephales promelas. *Arch. Environm. Contam. Toxicol.* 3: 132.

Doherty, J.D. & Matsumura, F. (1975). DDT effects on certain ATP related systems in the peripheral nervous system of the lobster Homarus americanus. Pestic. Biochem. Physiol. 5: 242-252.

Eaton, J. L. & Sternburg, J. (1964). Temperature and the action of DDT on the nervous system of Periplaneta americana (L). J. Insect Physiol. 10: 471-485.

Eaton, J. L. & Sternburg, J. (1967). Temperature effects on nerve activity in DDT-treated American cockroach. J. Econ. Entomol. 60: 1358-1364.

Eldefrawi, M. E. & Eldefrawi, A. T. (1973). Purification and molecular properties of the acetylcholine receptor from torpedo electroplax. Archives of Biochem. Biophys. 159: 362-373.

Esaac, E. G. & Matsumura, F. (1980). Metabolism of insecticides by reductive systems. Pharmac. Ther. 9: 1-26.

Fahmy, M. A. H., Fukuto, T. R., Metcalf, R. L. & Holmstead, R. L. (1973). Structure-activity correlations in DDT analogs. Agricul. Food Chem. 21(4): 585.

Fields, M., Gibbs, J. & Walz, D. E. (1950). The synthesis of 1,1,1-trichloro-2,2-bis-(p-chlorophenyl)ethane. Science 112:

Flattum, R. F. & Sternburg, J. (1970). Release of a synaptically active material by nicotine in the central nervous system of the American cockroach. J. Econ. Ent. 63: 67-70.

Fourtner, C. R. & Drewes, C. D. (1977). Excitation of the common inhibitory motor neuron: A possible role in the startle reflex of the cockroach, Periplaneta americana. J. Neurobiology 8(5): 477-489.

Frankenhaeuser, B. & Hodgkin, A. L. (1957). The action of calcium on the electrical properties of squid axons. J. Physiol. 137: 218-244.

French, M. C. & Jefferies, D. J. (1969). Science 165: 914.

Fries, G. R., Marrow, G. S. & Gordon, C. H. (1969). Metabolism of o,p'-DDT by rumen microorganisms. J. Agric. Food Chem. 17: 860-862.

Gammon, D. W. (1977). Nervous effects of toxins on an intact insect: A method. Pestic. Biochem. Physiol. 7: 1-9.

Gammon, D. W. (1978). Effects of DDT on the cockroach nervous system at three temperatures. *Pestic. Sci.* 9: 95-104.

Gammon, D. W. (1980). Pyrethroid resistance in a strain of Spodoptera littoralis is correlated with decreased sensitivity of the CNS in vitro. *Pestic. Biochem. Physiol.* 13: 53-62.

Gammon, D. W. & Holden, J. S. (1980). A neural basis for pyrethroid resistance in larvae of Spodoptera littoralis. In: *Insect Neurobiology and Pesticide Action*. (Neurotox. 79). Narahashi, T. (editor). Society of Chemical Industry, (Publisher), London.

Gardner, D. R. & Bailey, J. R. (1975). Methoxychlor: Its effects on environmental quality. Associate Committee on Scientific Criteria for Environmental Quality, National Research Council. Publication No. NRCC 14102 of the Environmental Secretariat, Ottawa.

Gardner, D. R. & Vincent, R. S. (1978). Insecticidal and neuroexcitant actions of DDT analogs on the cockroach, Periplaneta americana. *Bull. Environm. Contam. Toxicol.* 20: 294-302.

Ghiasuddin, S. M., Kadous, A. A. & Matsumura, F. (1981). Reduced sensitivity of a Ca-ATPase in the DDT-resistant strains of the German cockroach. *Comp. Biochem. Physiol.* 68C: 15-20.

Ghiasuddin, S.M. & Matsumura, F. (1979). DDT inhibition of Ca-ATPase of the peripheral nerves of the American lobster. *Pestic. Biochem. Physiol.* 10: 151-161.

Ghiasuddin, S. M. & Matsumura F. (1981). DDT inhibition of Ca-Mg ATPase from peripheral nerves and muscles of lobster, Homarus americanus. *Life Sciences* (in press).

Granett, J. & Leeling, N.C. (1972). A hyperglycaemic agent in the serum of DDT prostrate American cockroaches, (Periplaneta americana). *Ann. Entomol. Soc. Amer.* 65: 229.

Gregoriadis, G. & Davis, C. (1979). Stability of liposomes in vivo and in vitro is promoted by their cholesterol content and the presence of blood cells. *Biochem. Biophys. Res. Comm.* 89: 1287-1293.

Hatanaka, A., Hilton, B. D. & O'Brien, R. D. (1967). The apparent binding of DDT to tissue components. *J. Agr. Food Chem.* 15: 854-857.

Hawkins, W. B. & Sternburg, J. (1964). Some chemical characteristics of a DDT-induced neuroactive substance from cockroaches and crayfish. *J. Economic Ent.* 57(2):

Hille, B. (1972). The permeability of the sodium channel to metal cations in myelinated nerve. *J. Physiol.* 59: 637-658.

Hille, B. (1967). The selection inhibition of the delayed potassium currents in nerve by tetraethylammonium ion. *J. Gen. Physiol.* 50: 1287.

Hille, B. (1968). Pharmacological modification of the sodium channels of frog nerve. *J. Gen. Physiol.* 51: 199-219.

Hilton, B. D. & O'Brien, R. D. (1973). The effects of DDT and its analogs upon lecithin and other monolayers. *Pestic. Biochem. Physiol.* 3: 206.

Hodgkin, A. L. & Huxley, A. F. (1952). A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol.* 117: 500-544.

Hodgkin, A. L. & Keynes, R. D. (1955). Active transport of cations in giant axons from Sepia and Loligo. *J. Physiol.* 128: 28-60.

Hodgson, E. S. & Geldiay, S. (1959). Experimentally induced release of neurosecretory materials from roach corpora cardiaca. *Biol. Bull.* 117: 275-283.

Holan, G. (1969). New halocyclopropane insecticides and the mode of action of DDT. *Nature* 221: 1025-1029.

Horn, D. J. (1976). *Biology of Insects*. W. B. Saunders Co. (Publishers). Toronto. pg. 194-199.

Huddart, H. (1971). Ultrastructure of the prothoracic ganglion and connectives of the stick insect in relation to function. *J. Insect Physiol.* 17: 1451-1469.

Jackson, D. A. & Gardner, D. R. (1978). Effects of DDT on electrical properties of lecithin-decane bilayer membranes. *Pestic. Biochem. Physiol.* 8: 129-136.

Jackson, D. A. & Gardner, D. R. (1978a). In vitro effects of DDT analogs on trout brain Mg -ATPases. I. Specificity and physiological significance. *Pestic. Biochem. Physiol.* 8: 113-122.

Kirby, C., Clarke, J. & Gregoriadis, G. (1980). Effect of the cholesterol content of small unilamellar liposomes on

their stability in vivo and in vitro. Biochem. J. 186: 591-598.

Klein, A. K., Lang, E. P., Datta, P. R. & Mendel, J. L. (1965). J. Amer. Chem. Soc. 87: 2520.

Klein, A. K., Lang, E. P., Datta, P. R., Watts, J. O. & Chen, J. T. (1964). J. Ass. Offic. Anal. Chem. 47: 1129.

Koch, R. B. & Norring, N. L. (1969). Fractionation of olfactory tissue homogenates. Isolation of a concentrated plasma membrane fraction. J. Neurochem. 15: 145-157.

Kuffler, S. W. & Nicholls, J. C. (1976). From Neuron to Brain. Sinauer Associates, Inc. Publishers; Sunderland, Massachusetts.

Lalonde, D. I. V. & Brown, A. W. A. (1954). The effect of insecticides on the action potentials of insect nerve. Can. J. Zool. 32: 74-81.

Lane, N. J. (1974). The organization of insect nervous system.

In: Insect Neurobiology. J. E. Treherne (editor). North Holland Publishing Co. Amsterdam, Oxford. pg. 1-72.

Lowry, O. H., Rosebrough, N. J., Lewis Farr, A. & Randall, R. J. (1951). Protein measurement with the Folin Phenol Reagent. J. Biol. Chem. 193: 265-275.

Lehninger, A. L. (1975). Biochemistry. Worth Publishers, Inc., New York.

Maddrell, S. H. P & Reynolds, S. E. (1972). Release of hormones in insects after poisoning and in insecticides. Nature (London), 236: 404.

Matsumura, F. (1970). DDT action and adenosine triphosphate-related systems. Science 169: 1343.

Matsumura, F. & Ghiasuddin, S.M. (1978). Highly sensitive Ca-ATPase in axonic membranes of American lobster. Abs. Amer. Chem. Soc. 174: 17.

Matsumura, F. & Ghiasuddin, S. M. (1979). Characteristics of DDT-sensitive Ca-ATPase in the axonic membrane. In: Neurotoxicology of Insecticides and Pheromones. Narahashi, T. (editor). Plenum Press, New York.

Matsumura, F. & O'Brien, R. D. (1966). Interactions of DDT with components of American cockroach nerve. J. Agr. Food Chem. 14(1): 39-43.

Matsumura, F. & Patil, K. C. (1969). Adenosine

triphosphatase sensitive to DDT in synapses of rat brain. Science 166: 121.

Matsumura, F. & Narahashi, T. (1971). ATPase inhibition & electrophysiological change caused by DDT and related neuroactive agents in lobster nerve. Biochem. Pharmacol. 20: 823-837.

Metcalf, R. L. (1967). Metabolism and fate of pesticides in plants and animals. In: Scientific Aspects of Pest Control.

Metcalf, R. L. & Fukuto, T. R. (1968). The comparative toxicity of DDT and analogs to susceptible and resistant houseflies and mosquitoes. Bull. W. H. O. 38: 633-647.

Metcalf, R. L., Kapoor, I. P. & Hirwe, A. S. (1971). Development of biodegradable analogues of DDT. Bull. W. H. O. 44: 363-367.

Meves, H. (1978). Inactivation of the sodium permeability in squid giant nerve fibres. Prog. Biophys. Molec. Biol. 33: 207-230.

Milburn, N., Weiant, E. A. & Roeder, K. D. (1960). The release of efferent nerve activity in the roach, Periplaneta americana. Biol. Bull. 118: 111.

Moore, J. W., Blaustein, M. P., Anderson, M. C. & Narahashi, T. (1976). Basis of tetrodotoxin's selectivity in blockage of squid axons. J. Gen. Physiol. 50: 1401-1411.

Morley, H. V. & Chiba, M. (1964). Thin-layer chromatography for chlorinated pesticide residue analysis without cleanup. J. Assoc. Official Agric. Chemists. 47: 307-310.

Mullins, L. J. (1956). The structure of nerve cell membranes. In: Molecular Structure and Functional Activity of Nerve Cells. Grenell, R. G. & Mullins, L. J. (editors). American Institute for Biological Sciences Publisher, Washington.

Narahashi, T. (1965). The Physiology of Insect Axons In: The Physiology of the Insect CNS. Treherne, J. E. and Beament, J. W. L. (editors), Academic Press, London & New York. pg.1-22.

Narahashi, T. (1971). Effects of insecticides on excitable tissue. In: Advances in Insect Physiology. Beament, J. W. L., Treherne, J. E. and Wigglesworth, N. B. (editors), 8: 1-93. Academic Press. N.Y.

- Narahashi, T. (1979). Nervous system as a target site of pheromones and insecticides. In: Neurotoxicology of Insecticides and Pheromones. T. Narahashi (editor). Plenum, Press. New York. London. pg. 1-4.
- Narahashi, T. & Haas, H. G. (1967). DDT: Interaction with nerve membrane conductance changes. Science 157: 1438.
- Narahashi, T. & Haas, H. G. (1968). Interaction of DDT with the components of lobster nerve membrane conductance. J. Gen. Physiol. 51(2): 177-198.
- Narahashi, T. & Yamasaki, T. (1960a). Behaviors of membrane potential in the cockroach giant axons poisoned by DDT. J. Cell. Comp. Physiol. 55: 131-142.
- Narahashi, T. & Yamasaki, T. (1960b). Mechanism of after-potential production in the giant axons of the cockroach. J. Physiol. 151: 75-88.
- Narahashi, T. & Yamasaki, T. (1960c). Mechanism of increase in negative afterpotential by DDT in the giant axons of the cockroach. J. Physiol. 152: 122-140.
- Natalizi, G. M., Pansa, M. C., D'Ajello, V., Casaglia, O., Bettini, S. & Frontali, N. (1970). Physiologically active factors from corpora cardiaca of Periplaneta americana. J. Insect Physiol. 16: 1827-1836.
- O'Brien, R. D. (1967). Insecticides: Action and Metabolism. Academic Press, New York.
- O'Brien, R. D. (1975). Nonenzymatic effects of pesticides on membranes. In: Environmental Dynamics of Pesticides. Hague, R. & Freed, V. H. (editors). Plenum Press, New York.
- O'Brien, R. D. & Matsumura, F. (1964). DDT: A new hypothesis of its mode of action. Science 146: 657-658.
- Orchard, I. & Osborne, M. P. (1979). The action of insecticides on neurosecretory neurons in the stick insect, Carausius morosus. Pestic. Biochem. Physiol. 12: 197-202.
- Oxford, G. S., Wu, C. H. & Narahashi, T. (1978). Removal of sodium channel inactivation in squid giant axons by n-bromoacetamide. J. Gen. Physiol. 71: 227-247.
- Parnas, I. & Dagan, D. (1971). Functional organization of giant axons in the CNS of insects: New aspects. Adv. Insect Physiol. 9: 95-143.
- Parnas, I., Hochstein, S. & Parnas, H. (1976).

Theoretical analysis of parameters leading to frequency modulation along an inhomogeneous axon. J. Neurophys. 39(4): 909-923.

Parnas, I., Spira, M. E., Werman, R. & Bergmann, F. (1969). Non-homogenous conduction in giant axons of the nerve cord of Periplaneta americana. J. Exp. Biol. 50: 635-649.

Patil, T. N., Abd-El-Fattah, A. S. A., Plapp, F. W. & Koch, R. B. (1980). ATPase and dehydrogenase activities from house flies susceptible and resistant to organochlorine insecticides. Pestic. Biochem. Physiol 13: 5-12.

Patil, T. N. & Koch, R. (1979). Differential inhibition responses caused by DDT on oligomycin-sensitive Mg -ATPase activity: Nature of the requirements for DDT sensitivity. Pestic. Biochem. Physiol. 12: 205-215.

Patil, T. N., Telford, J. N., Plapp, F. W. & Koch, R. B. (1979). Isolation, characterization, and properties of factor F1 from mitochondrial preparations of housefly. Pestic. Biochem. Physiol 12: 95-104.

Pearson, K. G. (1973). Function of peripheral inhibitory axons in insects. Amer. Zool. 13: 321-330.

Pearson, K. G., Stein, R. B. & Malhotra, S. K. (1970). Properties of action potentials from insect motor nerve fibres. J. Exp. Biol. 53: 289-316.

Penefsky, A. S. (1974). Mitochondrial and chloroplast ATPases. In: The Enzymes. Boyer, P. D. (editor). Vol. X, pg.375. Academic Press, New York. Conductance of the sodium channel in myelinated nerve fibres with modified sodium inactivation. J. Physiol. 262: 729-742.

Pichon, Y. (1971). Mode of action of tetraethylammonium (TEA) ions on insect nerve membrane. Proc. Int. Union Physiol. Sci. 9: 452.

Pichon, Y. (1974). Axonal conduction in insects. In: Insect Neurobiology. Treherne, J. E. (editor). American Elsevier Publishing Co., Inc. New York.

Pichon, Y. (1976). Pharmacological properties of the ionic channels in insect axons. In: Perspectives in Experimental Biology, Vol.1. P. Spencer Davies (editor). Pergamon Press, Oxford, New York, Toronto.

Pichon, Y., Moreton, R. B. & Treherne, J. E. (1971). A quantitative study of the ionic basis of extraneuronal potential changes in the central nervous system of the

cockroach (Periplaneta americana (L.)). J. Exp. Biol. 54: 757-777.

Ritzmann, R. E. & Gamhi, J. M. (1978). Excitation of leg motor neurons by giant interneurons in the Cockroach. J. Comp. Physiol. 125: 305-316.

Roeder, K. (1948). Organization of the ascending giant fibre system in the cockroach (Periplaneta americana). J. Exp. Zool. 108: 243-261.

Roeder, K. D. & Weiant, E. A. (1948). The effect of DDT on sensory and motor structures in the cockroach leg. J. Cell. Comp. Physiol. 36: 175-186.

Rowlands, D. G. & Lloyd, C. J. (1969). DDT metabolism in susceptible and pyrethrin-resistant Sitophilus granarius (L.). J. Stored Prod. Res. 5: 413-415.

Salvisberg, W., Becker, G. & Voss, G. (1980). Insecticides and electrophysiological symptoms: A comparative study on a tarsal motor nerve of Locusta migratoria. Pestic. Biochem. Physiol. 11: 135-158.

Sedmak, J. J. & Grossberg, S. E. (1977). A rapid, sensitive, and versatile assay for protein using Coomassie Brilliant Blue G250. Analytical Biochem. 79: 544-552.

Shankland, D. L. & Kearns, C. W. (1959). Characteristics of blood toxins in DDT-poisoned cockroaches. Annal. Entomological Society America. 52: 386-394.

Singer, S. J. & Nicolson, G. L. (1972). The fluid mosaic model of the structure of cell membranes. Science 175: 720-731.

Spira, M. E., Parnas, I. & Bergmann, F. (1969a). Histological and electrophysiological studies on the giant axons of the cockroach Periplaneta americana. J. Exp. Biol. 50: 629-634.

Spira, M. E., Parnas, I. & Bergmann, F. (1969b). Organization of giant axons of the cockroach Periplaneta americana. J. Exp. Biol. 50: 615-627.

Spira, M. E., Yarom, Y. & Parnas, I. (1976). Modulation of spike frequency by regions of special axonal geometry and by synaptic inputs. J. Neurophys. 39(4): 882-889.

Sternburg, J. (1963). Autointoxication and some stress phenomena. Ann. Rev. Entomol. 8: 19.

Sternburg, J., Chang, S. C. & Kearns, C. W. (1959). The release of a neuroactive agent by the American cockroach

after exposure to DDT or electrical stimulation. J. Econ. Entomol. 52(6):

Sternburg, J. & Kearns, C. W. (1952). The presence of toxins other than DDT in the blood of DDT-poisoned roaches. Science 116: 144-147.

Takeno, K., Nishimura, K., Parmentier, J. & Narahashi, T. (1977). Insecticide screening with isolated nerve preparations for structure-activity relationships. Pestic. Biochem. Physiol. 7: 486-499.

Tasaki, I. & Hagiwara, S. (1957). Demonstration of two stable potential states in the squid giant axon under tetraethylammonium chloride. J. Gen. Physiol. 40: 859-885.

Tashiro, S., Taniguchi, E. & Eto, M. (1972). L-leucine: A neuroactive substance in insects. Science 175: 448-449.

Treherne, J. E. (1974). The environment and function of insect nerve cells. In: Insect Neurobiology. Treherne, J. E. (editor). American Elsevier Publishing Co., Inc. New York.

Trams, E. G. & Lauter, C. J. (1977). A comparative study of brain Ca-ATPases. Comp. Biochem. Physiol. 59B: 191-194.

Uchida, M., Naka, H., Irie, Y., Fujita, T. & Nakajima, M. (1974). Insecticidal and neuroexciting actions of DDT analogs. Pestic. Biochem. Physiol. 4: 451-456.

van den Bercken, J. (1972). The effect of DDT and dieldrin on myelinated nerve fibres. European J. Pharmacology 20: 205-214.

van den Bercken, J. & Vijverberg, H. P. M. (1980a). Effects of insecticides on the sensory nervous system of Xenopus. pg. 391-397. In: Insect Neurobiology and Pesticide Action (Neurotox. 79). Narahashi, T. (editor). Society of Chemical Industry, (Publishers), London.

van den Bercken, J. & Vijverberg, H. P. M. (1980b). Voltage clamp studies on the effects of allethrin and DDT on the sodium channels in frog myelinated nerve membrane. pg. 79-85. In: Insect Neurobiology and Pesticide Action. Society of Chemical Industry (Publishers), London.

Welsh, J. H. & Gordon, H. T. (1947). The mode of action of certain insecticides on the arthropod axon. J. Cell. Comp. Physiol. 30: 147-172.

Westin, J., Langberg, J. J. & Camhi, J. M. (1977).

Responses of giant interneurons of the cockroach Periplaneta americana to wind puffs of different directions and velocities. J. Comp. Physiol. 121: 307-324.

Willey, R. B. (1961). The morphology of the stomodtal nervous system in Periplaneta americana (L.) and other Blattaria. J. Morph., 108(2): 219-261.

Wilson, W. E., Fishbein, L. & Clements, S. T. (1971). DDT: Participation in ultraviolet-detectable, charge-transfer complexation. Science 171: 180-182.

Winteringham, F. P. W. (1969). Mechanisms of selective insecticidal action. Ann. Review Entomology 14: 409-442.

Wu, C. H., van den Bercken, J. & Narahashi, T. (1975). The structure-activity relationship of DDT analogs in crayfish axons. Pestic. Biochem. Physiol. 5: 142-149.

Yamasaki, T. & Ishii, T. * (1953). Studies on the mechanism of action of insecticides (VI) DDT symptoms in the cockroach, with special reference to the effects of temperature. English Translation (1957) In: Japanese Contributions to the Study of the Insecticide-Resistance Problem. 130-139 W. H. O.

Yamasaki, T. & Ishii, T. (1954). Studies on the mechanism of action of insecticides (VIII). Effects of temperature on the nerve susceptibility to DDT in the cockroach. English Translation (1957) In: Japanese Contributions to the Study of Insecticide-Resistance Problem. W. H. O.

Yeager, J. F. & Munson, S. C. (1945). Physiological evidence of a site of action of DDT in an insect. Science 102: 305-307.

Younis, H. M., Telford, J. N. & Koch, R. B. (1978). Adenosine triphosphatase from cockroach coxal muscle mitochondria. Isolation, properties, and response to DDT. Pestic. Biochem. Physiol. 8: 271-277.

* Former name of T. Narahashi.

Appendix A: Modified Procedure of Chappel and Hansford (1971) for isolation of Membranal and Microsomal Fractions from the CNS¹.

¹ All procedures carried out on ice or at 0°C

Appendix B: Procedures Employed to Prepare Tissue From the CNS
for Embedding in Epon-Araldite

1. Abdominal Cavity of Cockroach opened and perfused with 1.6% Glutaraldehyde - followed immediately by the thorax
2. CNS removed (7-10 min) and cut into pieces less than or equal to 1 mm³, in 1.6% Glutaraldehyde - remain in 1.6% Glutaraldehyde for 1.0 hour
3. Transfer samples to 3.0% Glutaraldehyde - 2.0 hours
4. Wash in sodium phosphate buffer (0.2 M)²
5. Post fix in 1.0% Osmium Tetroxide - 1.0 hour
6. Wash in sodium phosphate buffer - series of 5 washes over 25 minutes
7. Into 50% Ethanol - 30 minutes
8. Into 70% Ethanol - 30 minutes
9. Into 95% Ethanol - 2 changes, 20 minutes each
10. Into absolute Ethanol - 2 changes, 20 minutes each
11. From absolute Ethanol into Propylene Oxide - 2 changes, 15 minutes each
12. Propylene oxide: Resin³ = 3:1, 1.0 hour
13. Propylene oxide: Resin = 1:1, 1.0 hour
14. Propylene oxide: Resin = 1:3, minimum 15 hours
15. Into Pure Resin, 24 hours
16. Replace with fresh Resin, vacuum ca. 20 minutes
17. Place and orient in flat molds - Bake 24-48 hours at 78°C

...Appendix B continues

Continued Appendix B:

²Stock solutions of: 0.2 M NaH_2PO_4 (sol. A) & 0.2 M Na_2HPO_4 (sol. B), buffer solution = 28 ml sol. A 72 ml sol. B, to pH 7.2

³All resin contained activator, DMP-30, 1.5 Drops/ml resin

¹References: Lane, N.J. (1974). The Organization of Insect Nervous Systems, In: Insect Neurobiology, ed. J.E. Treherne, American Elsevier Publ. Co. Inc., New York.

Ruthman, A. (1970). Methods in Cell Research. G. Bell & Sons Ltd., (Publ.) London.

Appendix C: Protein Extraction Procedure Employed for the Lowry
and Coomassie Brilliant Blue G-250 Protein
Determinations

1. 1.0 ml 10% (w/v) TCA added to each 0.5 ml fractioned tissue homogenate in twice distilled water
2. Mix vigorously
3. Boil in water bath (100°C) for 15 minutes
4. Centrifuge samples at 700 xg - 10 minutes
5. Careful removal of supernatant, discard
- Pellet resuspended in 1.0 ml 10% TCA,
stored 15-18 hours @ 0°C
6. Centrifuge at 700 xg - 10 minutes
7. Careful removal of supernatant, discard
- Final pellet dissolved in 0.10 N or 0.50 N NaOH
8. Sonicate samples in Branson water bath sonicator at 55°C , 10-15 minutes or until total solution of pelleted material achieved

Appendix D: Protein Determination by the Method of Lowry et al., 1951.

1. To duplicate 1.0 ml samples of protein in 0.10 N NaOH, add 5.0 ml Reagent D¹, followed immediately by vigorous mixing.
2. Let stand ten minutes.
3. Add 0.5 ml freshly made Reagent E to each sample with immediate vigorous mixing.
4. Let stand 35 minutes for optimal colour development.
5. Read absorbance at 560 nm against reagent blank.²

²Preparation of reagent blank (simultaneous with protein samples):

Add 5.0 ml Reagent D to 1.0 ml 0.10 N NaOH

↓ 10 minutes

Add 0.5 ml freshly made reagent E

↓ 35 minutes

Adjust absorbance at 560 nm to zero

↓

Reset zero following each sample reading

¹Reagents required outlined in Materials and Methods, Section 7.

Appendix E: Sample Standard curve for Protein Determination by the
Method of Lowry et al., 1951.

Appendix F: Protein Determination by the Method of Bradford, 1976
(Coomassie Brilliant Blue G-250) and Sample Standard
Curve.

To 0.1 ml protein sample in 0.1 N NaOH¹ (10-150 ug),
add 5.0 ml of Bradford protein reagent¹

mix immediately by vortexing

↓
let stand 3 minutes

↓
read absorbance at 595 nm against reagent blank²

¹As outlined in Materials & Methods, Section 7(b).

²Reagent blank prepared the same as a protein sample except
0.1 ml of 0.1 N NaOH was substituted in place of the protein.

Appendix G: Sample Calculations for ^{14}C -DDT Experiments

Experiments presented employed ^{14}C -DDT of Specific Activity
 $\text{S.A.} = 0.0294 \text{ mCi/mg.}$

since, $1 \text{ mCi} = 2.22 \times 10^9 \text{ dpm}$

then, $0.0294 \text{ mCi} = 6.53 \times 10^7 \text{ dpm}$

restating the Specific Activity, $\text{S.A.} = 6.53 \times 10^7 \text{ dpm/mg}$

In other words, for each milligram of DDT there is
 an associated $6.53 \times 10^7 \text{ dpm.}$

Knowing this ratio enables the calculation of the
 amount of DDT in a 1 ul sample containing
 $1.25 \times 10^5 \text{ dpm}$ (i.e., the
 average amount injected into each cockroach):

$$6.53 \times 10^7 \text{ dpm/mg} = 1.25 \times 10^5 \text{ dpm/x mg}$$

solving for x:

$$\begin{aligned} x &= \frac{1.25 \times 10^5}{6.53 \times 10^7} \\ &= 1.91 \times 10^{-3} \text{ mg} \end{aligned}$$

The molecular weight of p,p'-DDT = 354.49 Daltons.

(Note that the molecular weight
 of ^{14}C -DDT = 356.49. Since the number of molecules
 of unlabelled DDT was far greater than the number
 containing the ^{14}C atom, 354.49 will be used.)

Therefore, the number of moles of DDT in a 1 ul sample
 containing ca. $1.25 \times 10^5 \text{ dpm}$ was:

$$\begin{aligned} \# \text{moles} &= \frac{1.91 \times 10^{-3}}{354.49} \\ &= 5.39 \times 10^{-6} \end{aligned}$$

$$= 5.39 \times 10^{-9} \text{ moles p,p'-DDT}$$

Similarly, the weight (mg) of DDT associated with the membranal, mitochondrial, and microsomal fractions were determined from the total dpm associated with each fraction. This was followed by the calculation of the number of moles.

Membranal Fraction: Total Mean dpm = 845 dpm

$$6.53 \times 10^{+7} \text{ dpm/mg} = 845 \text{ dpm/x mg}$$

solving for x:

$$\begin{aligned} x &= \frac{8.45 \times 10^{+2}}{6.53 \times 10^{+7}} \\ &= 1.29 \times 10^{-5} \text{ mg} \end{aligned}$$

Therefore, the total number of moles in 30 CNSs = 3.64×10^{-11} p,p'-DDT. Thus, 1 VNC would contain 1.21×10^{-12} moles p,p'-DDT in its membranal fraction.

Mitochondrial Fraction: Total Mean dpm = 81 dpm

solving for x:

$$\begin{aligned} x &= \frac{8.10 \times 10^{+1}}{6.53 \times 10^{+7}} \\ &= 1.24 \times 10^{-6} \text{ mg} \end{aligned}$$

Therefore, the total number of moles in 30 CNSs = 3.50×10^{-12} moles p,p'-DDT.

Thus, 1 CNS would contain 1.17×10^{-13} moles

p,p'-DDT in its mitochondrial fraction.

Microsomal Fraction: Total Mean dpm = 53 dpm

solving for x:

$$\begin{aligned}x &= \frac{5.30 \times 10^{+1}}{6.53 \times 10^{+7}} \\&= 8.12 \times 10^{-7} \text{ mg}\end{aligned}$$

Therefore, the total number of moles in 30 CNSs =

2.29×10^{-12} moles p,p'-DDT.

Thus, 1 CNS would contain 7.63×10^{-14} moles p,p'-DDT
in its microsomal fraction.

Appendix H: Methods of Protein Determination

Protein determinations employing the Lowry and Coomassie Blue Methods revealed that, depending upon the method employed, membrane protein content was either high (Lowry Method) or low (Coomassie Blue Method) (Fig. 32). The sample of membrane protein was taken from the same test-tube. Samples were usually re-run to ensure that an error was not made during the assay procedure. In all cases the same results were obtained. Therefore, the difference in value for membrane protein between the Lowry and Coomassie Blue Methods must be explainable by different inherent sensitivities. Indeed, the Methods employ two entirely different chemical reactions to detect the presence of protein. In the Lowry Method of protein determination, two distinct steps lead to the final colour of the protein solution resulting from the reagents interaction with protein: 1/ reaction of the protein with copper in alkali; and 2/ reduction of the phosphomolybdic-phosphotungstic reagent by the copper treated protein (Lowry et al., 1951). Although the copper reaction involves the proteins as a whole, free tyrosine and tryptophan (aromatic amino acids) in the protein solution will react with the protein reagents and contribute to the final colour of the solution. Thus, the amount of colour development is not strictly proportional to the protein concentration. This is quite different from the Coomassie Blue chemical reaction which

depends upon intact protein structures (only) for colour development. Conversion of the Coomassie Blue dye from a brownish-orange to an intense blue colour depends upon the interaction of the dye-anion with the NH_3^+ groups on proteins (Bradford, 1976; Sedmak and Grossberg, 1977). It does not react with free amino acids in the protein solution. But, it should be noted that all proteins do not have the same proportion of NH_3^+ groups and not all NH_3^+ groups interact identically with the dye. Thus, the amount of colour development depends upon the protein used. However, colour development in the Lowry Method also depends upon the protein being assayed.

The membrane fraction was unique because it contained a large proportion of collagenous material (from the neural lamella). In mammals, collagen normally forms a triple helix of linear polypeptide strands of glycine, alanine, proline, and hydroxyproline (Lehninger, 1975). The component amino acids vary slightly among species. Its composition in cockroach nerve cord has not been elucidated. Therefore, it may contain other amino acids (such as tyrosine and tryptophan). If this were the case, then it is possible that some of these residues would be released during the extraction procedure. Thus, colour development in the Lowry determination of protein would reflect this by a greater final absorbance. Since the Coomassie Blue Method does not recognize free amino acids, colour intensity (and

thus absorbance) would not be similarly increased.

Tyrosine (or tryptophan) could have been cleaved from another protein that was unique to the membrane fraction. In fact, evidence indicates that a tyrosine residue is a component of the lipoprotein complex responsible for the Na-inactivation mechanism (Oxford et al., 1978). This lipoprotein complex is characteristic of excitable membranes, and not mitochondria (Oxford et al., 1978).

However, there is another possibility regarding the low value of protein determined for the membrane fraction by the Coomassie Blue Method. Sedmak and Grossberg (1977) claimed that the Coomassie Blue dye would not react with proteins of molecular weight less than 3000 Daltons. Collagen strands separate and undergo some hydrolysis when boiled in water. If the strands separated into fragments of molecular weight less than 3000 Daltons then the Coomassie Blue dye would not react. However, these proteins would be detected by the Lowry Method. This may have contributed to the lower value of protein content determined using the Coomassie Blue Method.

The value for protein contained in the mitochondrial and microsomal fractions were found not to be dependent upon the method of protein determination employed. This supported the hypothesis that there was a protein(s) contained in the membrane fraction that was unique to that fraction. That protein would then cause the discrepancy in the values of protein described above.

END

1 0 1 0 9 H 8 4

FIN