The functional basis of pyrethroid resistance in the malaria vector, *Anopheles stephensi*

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Contents

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ABSTRACT

The functional basis of pyrethroid resistance in the malaria vector, *Anopheles stephensi*

Pyrethroid insecticides are increasingly being used for the control of malaria vectors. It is therefore important to understand the nature of resistance to this group of compounds. This thesis describes a series of studies designed to elucidate the functional basis of pyrethroid resistance in strains of the malaria vector *Anopheles stephensi* originating from the UAE.

Apart from the resistant strain from UAE, all other strains investigated, including ones originating from Iran, Iraq and India, were highly susceptible to all pyrethroids at both the larval and adult stage.

In vivo bioassays of adults of the pyrethroid-resistant DUB-APR strain employing enzyme inhibitors showed that cytochrome P-450s (=mixed function oxidases) and esterases contribute to its 8-fold permethrin resistance. However a portion of the observed resistance was due to other factors, postulated to include a form of target-site insensitivity.

Gel electrophoresis using α- and ß-naphthyl acetate as substrates showed no obvious differences in esterase activity associated with permethrin resistance.

Studies with ^{14}C-permethrin indicated that permethrin penetrated equally well in the resistant and susceptible strains.

To test the hypothesis that target-site insensitivity contributes to permethrin resistance in DUB-APR, neurophysiological studies were carried out on isolated thoracic nerves. A single dose application of 10^{-13} M permethrin rapidly produced a 3-fold increase in firing rate in the susceptible strain, whereas the resistant strain did not respond to this concentration. In a cumulative dose assay, the resistant strain required a dose 100,000-fold higher to produce the same rise in the frequency of action potentials.

Behaviour studies indicated that the resistant DUB-APR strain was 3-fold resistant to knock-down compared with the susceptible strain. The resistant strain was however 3-fold less irritable to permethrin. The resistant strain was also less responsive than the susceptible strain to the movement of an aspirator. If reduced irritability and reduced responsiveness to catch are consequences of the changes in the nervous system recorded in the neurophysiological studies, then such a form of resistance may be disadvantageous to mosquitoes in natural populations.

Re-selection of larvae of DUB-LPR with permethrin for 3 generations increased resistance to permethrin to a level of 204-fold and 100-fold, relative to BAN and IND-S strains respectively. From studies with TPP (an esterase inhibitor) and with gel electrophoresis of larval homogenates, it was concluded that hydrolysis plays only a minor role in the permethrin resistance of larvae. The highly synergistic effect of piperonyl butoxide (PB: a mixed-function oxidase inhibitor) demonstrates that oxidases play a major role in permethrin resistance in larvae.

The larvae of DUB-APR, a strain in which the adults had been selected with permethrin, were found to be resistant to pyrethroids. Similarly, adults of DUB-LPR, a strain that was selected at the larval stage, were also found to be resistant to pyrethroids. This, and the effects of PB,

suggest that some of the genes conferring resistance are active in both larval and adult stages, including those which govern the expression of mixed-function oxidases.

Strains selected with permethrin, were examined for cross-resistance to other classes of pyrethroids, DDT and malathion in the larval and adult stages. Permethrin-selected strains showed positive cross-resistance to deltamethrin, lambdacyhalothrin and DDT at both stages, but negative cross-resistance to malathion. This negative cross resistance is attributed to the enhanced activation of malathion to the oxon form by mixed function oxidases in the resistant strains.

Studies of the structure-activity relationship of pyrethroids in larvae revealed that deltamethrin, an α-cyano compound, had the most potency against susceptible larvae. Permethrin, the agent of selection, had the least toxicity to the DUB-LPR strain. PB synergised all pyrethroids with DUB-LPR and DUB-APR larvae, indicating that mixed-function oxidases are a common factor in resistance to all pyrethroids, whether α-cyano-derivatives or not.

Bioassays with permethrin, deltamethrin and lambdacyhalothrin after preconditioning resistant and susceptible adults at 5°C, showed that all these pyrethroids had a positive temperature coefficient. This is believed to result from the reduced penetration of the pyrethroids at lower temperatures.

The adults of both DUB-APR and DUB-LPR were strongly resistant to DDT but this resistance was unaffected by DMC (a dehydrochlorinase inhibitor) or PB suggesting that knock-down resistance (*kdr)* plays a major role in the DDT resistance.

Pyrethroid-resistance was not found to confer cross-resistance to the repellent deet. The susceptibility to *Bacillus thuringiensis* serotype H-14 of larvae of permethrin-resistant and susceptible strains was also not significantly different.

Prior exposure of adults and larvae of *An.stephensi* to a sub-lethal dose of permethrin increased their tolerance of permethrin in subsequent bioassays. A similar increase in tolerance (up to 3-fold) was seen when larvae were pre-exposed to peppermint and its active constituent, menthol. Menthol and its derivatives are known enzyme inducers. Sodium phenobarbital, another enzyme inducer, produced a negligible effect. Enzyme induction, by enhancing the breakdown of pyrethroids in the mosquito, may have a practical effect on the response of mosquitoes to pyrethroids, particularly in aquatic environments. Although induction may only be temporary, it is conceivable that enhanced inducibility is a heritable trait and hence of selective advantage in the field. Further studies are required to clarify this phenomenon.

Studies of the *in vitro* metabolism of ^{14}C-permethrin in larvae of permethrin-resistant a susceptible strains using HPLC, showed that the type of metabolite produced was similar resistant and susceptible strains, but the total activity was lower in the susceptible strain. The major metabolite produced by the two strains had the same retention time as that found with rat liver microsomes. Further work using mass spectrometry would be required to confirm the identity of this metabolite.

In view of these results, it can be concluded that resistance is not a simple matter, but it is multidimensional and hence flexible, and may vary in time and space. The effective management of resistance depends not only on understanding the genetical, biochemical and behavioural factors determining resistance in the mosquito but also on understanding how these interact with other factors in the environment including ecology and human interventions.

List of abbreviations

a	intercept of regression line
AChE	acetylcholinesterase
b	slope of regression line
BHC	benzene hexachloride
BSA	bovine serum albumine
C	carbamate insecticides
°C	degree centigrade
Ca^{++}	calcium ion
C.I.	confidence intervals
cm	centimetre
CNS	central nervous system
CO	carbon monoxide
DDT	dichlorodiphenyltrichloroethane
DEF	tributyl phosphorotrithionate
DNA	deoxyribonucleic acid
DMC	chlrofenethol
DPM	disintegration per minute
g	gram
GABA	gamma-aminobutyric acid
H^+	hydrogen ion

Hz	Hertz
HPLC	High Performance Liquid Chromatography
h	hour/s
IR	induction ratio
K^+	potassium ion
kdr	knock down
Kg	kilogram
LC50	concentration of insecticide required to kill 50% of larval population
LC90	concentration of insecticide required to kill 90% of larval population
LT50	time required to kill 50% of adult population at a given concentration of insecticide
LT90	time required to kill 90% of adult population at a given concentration of insecticide
mA	miliamper
M	molar
MFO	mixed function oxidase
min	minute/s
mg	miligram
Mg^{2+}	magnesium ion
ml	mililitre
mV	milivolt
Na^+	sodium ion
NADPH	nicotinamide adenine dinucleotide phosphate

nl	nanoliter
nm	nanometer
No.	number
OC	organochlorine insecticides
OP	organophosphate insecticides
PB	piperonyl butoxide
PCR	polymerase chain reaction
PNS	peripheral nervous system
PY	pyrethroid insecticides
RF	resistance factor
RH	relative humidity
RNA	ribonucleic acid
rpm	revolution per minute
(RR)	resistant strain
SD	standard deviation
Se	second
SE	standard error
SR	synergist ratio
(SS)	susceptible strain
T.C	temperature coefficient
TLC	thin layer chromatography
TPP	triphenyl phosphate

UV	ultra violet
v	voltage
V/V	volume to volume
WHO	World Health Organization
W/V	weight to volume

Chapter 1

INTRODUCTION

A number of vector-borne diseases are of great medical importance including malaria, filariasis, leishmaniasis, trypanosomiasis, yellow fever and dengue fever. Of these, malaria is the most important in terms of mortality and morbidity. It is major threat to health, blocking the path of economic development for individuals, communities and nations. It affects almost half the world's population, causing about 400 million clinical cases and is responsible for 1-2 million deaths annually (WHO, 1995), most of which occur in African children (Doherstyn, 1991). Malaria vectors are found in all continents, but they are more prevalent in the tropics where climatic conditions and weather make it optimal for their reproduction. Mosquitoes are the most widespread of all medically important insects.

Out of the 430 species of *Anopheles* mosquitoes throughout the world, about 70 species are known to be vectors of malaria (Service, 1996).

The control of mosquitoes is directed either towards the immature stages or the adults in an attempt to reduce the man-vector contact and transmission of parasites to man (Curtis et al., 1991). The methods available for control of vectors include: biological control, environmental management and chemical control. Chemical control through the use of insecticides has contributed enormously to vector control and hence to the control of mosquito-borne infections, thus the use of DDT house-spraying in the 1950s and 1960s greatly reduced malaria prevalence in most parts of Asia (Curtis, 1989).

The development of resistance by mosquitoes to the compounds used against them as

larvicides and adulticides was first observed in 1947, when the salt-marsh mosquitoes *Aedes taeniorhynchus* and *Ae. sollicitans* began to show resistance to DDT in Florida (WHO, 1963; Brown, 1986). Since that time populations which have shown resistance to the organochlorines (principally DDT) are known in an other 109 mosquito species; 58 species have developed resistance to organophosphate insecticides, 4 of which are not organochlorine resistant, 17 species have shown resistance to the carbamates propoxur and bendiocarb and 10 species have shown either resistance or cross-resistance to certain pyrethroids (WHO, 1992a). Multiple resistance has developed in a number of species, almost certainly, in part, as a result of the wide spread use of large quantities of pesticides in agricultural areas such as ricelands, where mosquitoes have frequently been found breeding in these habitats. The use of agricultural pesticides presents a serious problem in increasing the resistance in mosquito species. For example, some forms of insecticide resistance have been recorded in 48 species of *Anopheles* in 13 of which agricultural insecticides are presumed to have contributed to resistance in at least some populations (cited by Lines, 1988).

Anopheles stephensi is known to be an important malaria vector in the Persian Gulf, the Middle-East and Indian subcontinent. As a result of the continuous application of insecticides in these regions, *An.stephensi* populations are known to be resistant to DDT (Davidson, 1958; Davidson & Jackson, 1961; Mofidi et al., 1958), dieldrin (Davidson & Mason, 1963; Zaim, 1987) and malathion (Manouchehri et al., 1974, 1975, 1980; Rathor & Toqir, 1980; Hemingway, 1983; Scott & Georghiou, 1986; Herath & Davidson, 1981; Hemingway, 1982; Hemingway et al., 1984). There are some reports of pyrethroid resistance in *An.stephensi* based on laboratory selection

(Omer et al., 1980; Malcolm, 1988a; Chakravorthy & Kalyanasundaram, 1992; Sahgal et al., 1994), but only one report of pyrethroid resistance in field populations (Ladonni, 1988). In recent years the synthetic pyrethroids, with their high insecticidal activity, low mammalian toxicity, and biodegradability in the environment resulting in less detrimental effects on non-target organisms, have been used increasingly in vector control programmes, specially for impregnation of bednets.

Numerous projects are now in progress using pyrethroid-impregnated bednets in many parts of the tropical world including Africa, Asia, Western Pacific and the Americas (WHO, 1992a; Curtis, 1991; Curtis et al., 1991; Curtis, 1994a; Curtis et al., 1996; Roberts & Andre, 1994; Greenwood & Pickering, 1993). Based on trials in China (Wu et al., 1993), Thai-Burmese border (Luxemburger et al., 1994), Surinam (Rozendaal et al., 1989), Bangladesh (Hossain & Curtis, 1989), The Gambia (Greenwood & Pickering, 1993), Iran (M. Zaim, personal communication, 1995) and elsewhere, it seems that pyrethroid-impregnated bednets are effective in vector control.

Molecular and genetical investigations for control of malaria disease are being tested. These include the manipulation of the mosquito genome (Crampton et al., 1994; Crampton, 1994); release of sterile males (Davidson, 1974) and anti-malaria vaccine (Alonso et al., 1994). As we await for the effectiveness of these methods in the future, insecticides will continue as our main resource in vector control, particularly in developing countries.

Increase of problems of resistance in disease vectors have steadily diminished the choice of effective and economical alternative insecticides and we have at present no single, simple solution to the problem. It is important to recognize that only a limited

number of insecticides are available for use in public health programmes and they should be treated as a valuable resource. We need to use our present insecticide "resources" in the most effective way possible. This rational use of insecticides largely depends on a broad knowledge of the possible or probable resistance mechanisms. This knowledge enables us to take all necessary precautions to prevent the occurrence of resistance and to prepare in advance a plan for coping with it at the early stages of its development in the field.

This project attempts to determine the scope of the functional basis of resistance and the mechanisms underlying the resistance in *An.stephensi* populations to a number of insecticides, currently used in public health programmes, with a particular emphasis on pyrethroid compounds.

Aims & objectives

Specific objectives:

1. To identify the mechanisms of pyrethroid resistance in larvae and adults of selected strains of *An.stephensi*

2. To establish the extent of cross-resistance to different classes of pyrethroids, other insecticides and a biological agent

3. To examine the extent to which the enzymes involved in pyrethroid resistance may be inducible

4. To study the relationship between structure and activity of pyrethroids

5. To determine the temperature coefficient for resistance to different classes of pyrethroids

6. To develop techniques for separating metabolites of permethrin and elucidate the metabolism of radiolabelled permethrin in resistant and susceptible strains

Chapter 2

LITERATURE REVIEW

2.1 Historical development of insecticides

As a result of insect pests, man has always had to cope with disease, discomfort and great economic loss. Since the very beginning of civilization man has constantly endeavoured to improve his well-being, and this has been exemplified in his use of chemical agents in the control of insects responsible for both the transmission of disease and for the destruction of crops. Some of the methods for insect control date back many centuries. Ancient people relied almost entirely on the use of natural products and preparations derived from these (WHO, 1984). Before the 2nd world war, the chemicals used for destroying insect pests were largely inorganic chemicals such as compounds of lead and arsenic which are well known poisons. Some organic chemicals of plant origin, such as nicotine, pyrethrum and rotenone were also used for pest control.

The 1940's represent the beginning of the modern era of organic pesticides and may be called the "pesticide revolution" when DDT was first used as an insecticide. DDT was first synthesized by Zeidler in 1874, but its insecticidal properties were discovered by Paul Muller in 1939. He received a Nobel prize for medicine in 1948 for his discovery (Kumar, 1984). DDT was commercially manufactured in 1943 and soon became the most extensively used insecticide. Following the discovery and application of DDT, other new groups of synthetic insecticides have been manufactured and used against medically important insects. With the appearance of DDT, it was thought that the "magic bullet"

in malaria control had been found. Thereafter the WHO Assembly in 1955 voted for the eradication of malaria, excluding sub-saharan Africa, following on the success that insecticides had achieved in reducing malaria cases in many parts of the world. The insecticide euphoria was soon to end and in 1976 WHO officially began to speak in terms of malaria control instead of eradication. The major cause of this change of policy was the appearance of resistance in the vectors being sprayed.

2.2 Classification of insecticides

According to their chemical nature and origins, insecticides have been classified into the following groups:

2.2.1 Organochlorine (chlorinated hydrocarbons) (OC)

The organochlorine insecticides (OC) have been the most widely used in public health practice. This group of insecticides can be divided into three major sub-divisions, which are often considered as contact insecticides:

a) DDT and DDT analogues:

DDT is usually sprayed indoors on surfaces of walls and other potential resting places of mosquitoes. It may remain active on a surface for as long as a year. DDT had also been used as a larvicide to supplement the toxic action of oils, though its application as a larvicide is not acceptable these days (WHO, 1984). Four characteristics of this chemical that stimulated its use by workers were; the relative ease with which it could be manufactured, the long persistence of its residue on sprayed surfaces, its high toxicity

to insects together with low toxicity to man and the property of killing insects by simple contact. Though DDT is still very widely used in public health practice, its use is steadily decreasing because of its persistence in the environment and the development of resistance of some mosquitoes to it. Recently there has been a considerable debate about its potential harmfulness and the possible discontinuation of its application as the insecticide of choice for malaria control (Curtis, 1994b) and a reply from WHO (1994) asking for more evidence on harmful effects before any decision is taken to recommend that DDT no longer merits being promoted as the insecticide of choice. However, the increased trend of resistance to this group of chemicals will soon lead to its natural discontinuation. Following the success of DDT as a potent insecticide many insecticidal analogues of DDT were synthesized, the most important ones being methoxychlor, dicofol (kelthane), DDD (2, 4-dichlorophenoxy acetic acid), chlorobenzilate and chlorfenethol (DMC). DMC has been used as a dehydrochlorinase inhibitor. Fig. 2.1 shows the structure of DDT and some of its analogues.

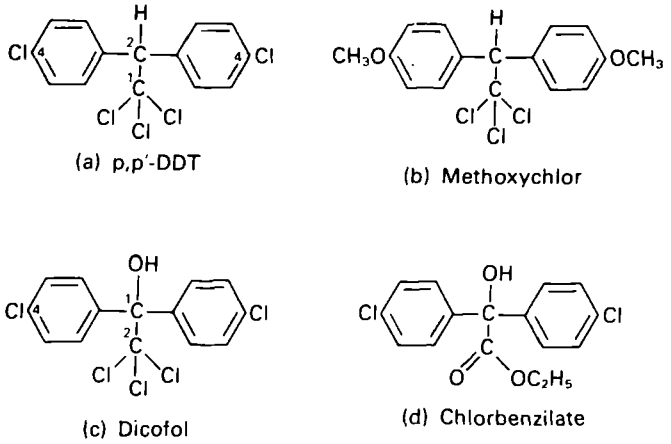


Fig. 2.1 Structure of DDT and its analogues

b) Hexachlorohexane (HCH):

This was first synthesized by Micheal Faraday in 1825 but its insecticidal properties were not recognized until 1942. Hexachlorohexane (HCH) (Fig. 2.2) has been used extensively against insects of medical importance since 1942. It has a strong vapour action, kills fast, and its persistence is short. This compound has also been used to replace DDT in areas where DDT resistance has occured, especially in India.

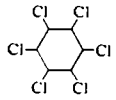


Fig. 2.2 Chemical structure of HCH

c) Cyclodiene compounds:

Several cyclodiene compounds were introduced after 1945. These insecticides include aldrin, chlordane, dieldrin, heptachlor, endrin and endosulphan (Fig. 2.3). After DDT, dieldrin has been the most extensively used in malaria control programmes. Dieldrin is more toxic than DDT and HCH to insects, human and animals, while it is less excito-repellent than DDT and also confers cross-resistance to other cyclodienes. Appearance of dieldrin resistance in DDT-resistant strains together with concern over the acute toxicity problems of this compound has put a constraint on its usage.

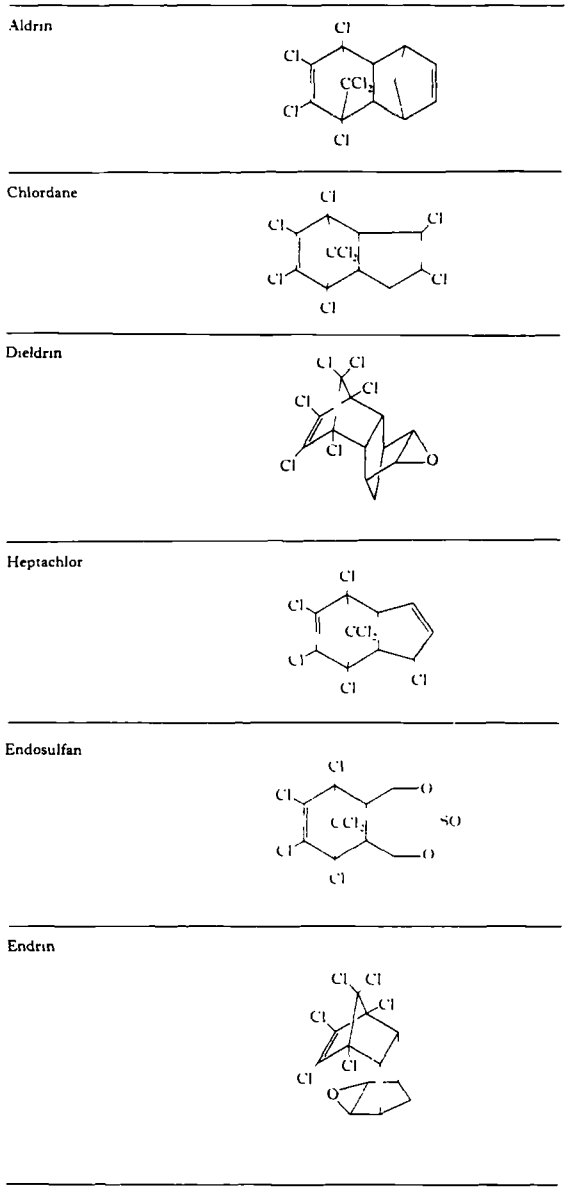


Fig. 2.3 Chemical structure of cyclodiene compounds (from Corbett et al., 1984)

2.2.2 Organophosphate insecticides (OP)

These compounds represent another extremely important class of organic insecticides. Compounds of this group were originally widely used for agricultural purposes but due to vector resistance to OCs, they are also now commonly used in public health practice. Their development was initiated during the 2nd world war by research on nerve gases by Gerald Schrader in Germany. Early examples included the potent systemic insecticide, schradan, in 1941 and tetraethyl pyrophosphate (TEPP) which was the first marketed OP insecticide. In 1944 parathion, a contact insecticide was also synthesised. Generally they are highly toxic to mammals as well as the target insects. As a consequence, research in this field was directed towards the discovery of more selective insecticides with lower toxicity for mammals. Malathion, discovered in 1950, was the first example of a broad-spectrum organophosphate insecticide with the added advantage of low mammalian toxicity and since then many other compounds have been developed. Most OP insecticides are esters or amides of organically bound phosphoric or pyrophosphoric acid. These compounds can be divided into five classes according to their phosphorous moiety (Eto, 1974). Among them two classes, phosphorothioates and phosphorothiolothioate esters contain important insecticides which have been widely used in mosquito control programmes. Fenthion (Baytex), temephos (Abate), chloropyrifos (Dursban), fenitrothion (Sumithion) and pirimiphos-methyl (Actellic) are the most important compound of phosphorothionate. Malathion (Fig. 2.4) is one of the most important insecticides in the class of phosphorothiolothioate and has been widely used in malaria control programmes. OPs compounds are currently used as stomach and contact poisons, fumigants, and

systemic insecticides, for nearly every type of insect. OPs are more expensive and much less stable than OCs.

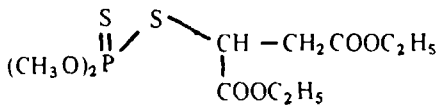


Fig. 2.4 Chemical structure of malathion

2.2.3 Carbamate insecticides (C)

Carbamates are acid esters that resemble in some ways OP insecticides. They were first discovered by the Geigy company in Switzerland in 1947, although the most generally effective member of the group, carbaryl, was not commercially introduced until almost a decade later. Since late 1995 the use of carbaryl has been restricted in UK. Another example of this group is propoxur (Baygon) (Fig. 2.5), which has been used widely in malaria control programmes, particularly in areas where resistance to DDT and malathion has emerged. The mode of action of carbamate insecticides is similar to OP insecticides and may affect acetylcholinesterase (AChE) receptors.

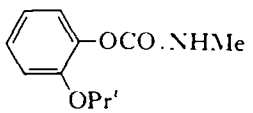


Fig. 2.5 Chemical structure of propoxur

2.2.4 Pyrethroid insecticides (PY)

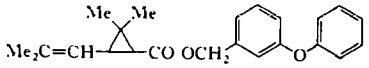
The pyrethroids constitute a new generation of highly potent synthetic insecticide inspired by from a group of insecticidal esters, the pyrethrins, extracted from the flower-heads of certain *Chrysanthemum* species mainly *Chrysanthemum cinerariaefolium*, grown commercially in part of Asia and Africa. The elucidation of structures of the pyrethrins was a process which continued over a period of 60 years. The six pyrethrins are esters of three cyclopentenolone alcohols, pyrethrolone, cinerolone and jasmolone with either chrysanthemic acid or pyrethric acid. Pyrethrum is used mainly in the form of a 10-25% extract of crushed dry flower in kerosene or other organic solvents. They are rapidly oxidized and inactivated in sunlight with a loss of insecticidal activity. The active insecticidal constituents of pyrethrum extract are known collectively as the pyrethrins. Pyrethroids are structural analogues related to the six biologically active compounds known as natural pyrethrins. They are all esters, of which both the alcohol and carboxylic acid moieties may have isomeric forms so that each pyrethroid may have several isomers. Commercially available insecticides now include the well-known natural pyrethrins and the synthetic pyrethroids. The first synthetic pyrethroid was discovered by Campbell and Schecter (Schecter et al., 1949). Synthetic analogues of the natural pyrethrins reached commercial success during the 1950s. The first commercial product, allethrin, is the ester of racemic allethrolone with racemic *cis/trans*-chrysanthemic acid. Allethrin was used in the public health market, especially for indoor space spray and vapour against mosquitoes. Other 'first-generation' synthetic pyrethroids such as phenothrin and tetramethrin, like the natural pyrethrins, are relatively unstable to light.

During the 1960s-1970s great progress was made with synthetic light-stable pyrethroids which led to fenvalerate. Work on esters of the dichlorovinyl analogues of chrysanthemic acid led to permethrin and cypermethrin. Subsequently "third" generation photostable analogues such as deltamethrin amongst others have been developed (see Fig. 2.6 for more details).

With the emergence of resistance to some of the organochlorine, organophosphates and carbamate insecticides, and recognition of their persistence in soil, the need for effective but safe and degradable insecticides was realised and attention was again focused on the pyrethroid insecticides. Currently pyrethroid insecticides represent important weapons against insect pests of both economic and medical importance. These products show remarkably high toxicity and rapid action against a wide range of insects, but relatively low mammalian toxicity. Pyrethroids more than any other classes of insecticides provide highly selective insect toxicity compared with mammalian toxicity. Selective ratios for rat oral LD50/ insect topical LD50 (mg/kg) for carbamate, organophosphate, organochlorine and pyrethroid insecticides are 16, 33, 91 and 4500, respectively (Elliot, 1989). Pyrethroid insecticides are also easily biodegraded to a harmless product, and hence do not accumulate in biological systems. They also have low volatility and low polarity, properties which restrict their movement in the air or soil from the site of application.

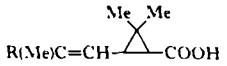
Fig. 2.6 Chemical structure of some of the most widely used pyrethroids (from Corbett et al., 1984)

Phenothrin



Pyrethrins or Pyrethrum

Esters from



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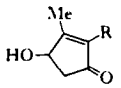
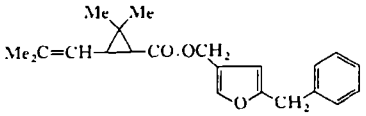


where R – Me and —COOMe
and R' – $-CH_2$ CH CH.CH CH_2
$-CH_2$ CH CH Me
and
$-CH_2$ CH—CH Et

Resmethrin



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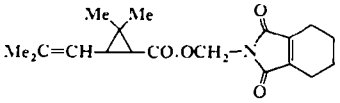
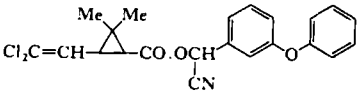
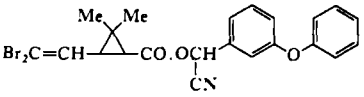


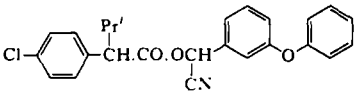
Cypermethrin



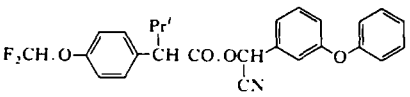
Deltamethrin



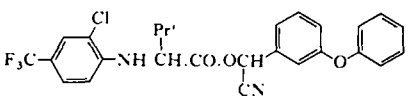
Fenvalerate



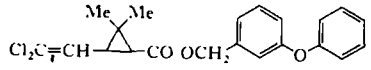
Flucythrinate



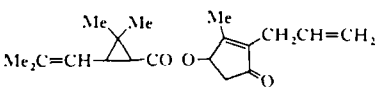
Fluvalinate



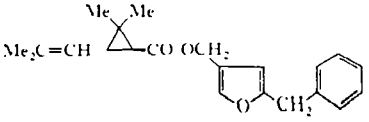
Permethrin



Allethrin



Bioresmethrin



2.2.4.1 The potential use of pyrethroids in pest and vector control

Synthetic pyrethroids with high insecticidal activity, low mammalian toxicity, safety in use, biodegradability in the environment resulting in less detrimental effects on non-target organisms and increased stability on inert surfaces, have significantly expanded their use in pest control programmes. An increasing use of the pyrethroids is in the control of various disease vectors including mosquitoes, tsetse flies (Barlow et al., 1977), lice (Nassif & Kamel, 1977), fleas (Lemke et al., 1989), house flies (Farnham, 1977), cockroach (Cochran, 1989, 1991, 1993, 1994), and biting flies (Blackman & Hodson, 1977). The pyrethroids are used in the form of domestic aerosols/sprays, coils mats, large-scale space sprays, aerial applications, surface treatments, larvicides, bednet treatments, curtain treatments and clothing treatments. Pyrethroids are also used for the control of veterinary pests such as ticks, face fly, stable fly and horn fly. The pyrethroids have long been used against a range of pests of stored products, wood pests, glass-house pests and agricultural pests (Elliot et al., 1978).

2.2.5 Biopesticides: The development of insecticides over the past 60 years provides a relatively simple tool for control of vectors of disease, especially in the vast rural areas of the tropics. However, the emergence and spread of insecticide resistance in many species of vectors and occurrence of multiple resistance to organochlorine, organophosphate, carbamate and pyrethroid insecticides in several insects, increasing attention has been directed towards natural pathogens. Among various pathogens, the bacterium *Bacillus thuringiensis* H-14 and *Bacillus sphaericus* produce proteins that are

toxic to mosquito and black fly larvae. The target site of the toxins is larval midgut cells, which in the presence of the toxin, undergo degradation and lysis, larvae undergo tremors, become sluggish, and eventually die (Rodcharoen & Mulla, 1994). Since the discovery of *B.thuringiensis* H-14 (*B.t.i*) in 1977, it has been found to have a potent effect against mosquito and black fly larvae in the laboratory and field conditions (Goldberg & Margalit, 1977; Margalit & Deam, 1985; Ramoska et al., 1982; Clark & Rowley, 1984; Fanara et al., 1984; Eldridge et al., 1985; Jones & LIoyd, 1985; Gharib & Hilsenhoff, 1988; Becker & Ludwig, 1993). So far there are no reports of resistance to *B.t.i* in field condition in mosquitoes. High selection pressure with *B.t.i* (IPS-82) applied in the laboratory in *Ae.aegypti* larvae has led to development of only low level (2-fold) resistance (Goldman et al., 1986). *B.sphaericus* like *B.t.i* is undergoing extensive laboratory and field trials for control of mosquito larvae (Karch et al., 1992; Hougard et al., 1993). A number of recent studies show that resistance development is possible in the laboratory. High and continuous exposure to *B.sphaericus* led to extremely high level of resistance in *Cx.quinquefasciatus* (Rodcharoen & Mulla, 1994; Nielsen-LeRoux & Charles, 1992; Georghiou et al., 1992). For the first time low-level resistance to *B.sphaericus* in a field-treated population of *Cx.quinquefasciatus* has been reported in Brazil (Silva-filha et al., 1995) and subsequently high level resistance (over 6000-fold at the LC50 level) in a field population of *Cx.quinquefasciatus* in India (Rao et al., 1995). Nielsen-LeRoux et al. (1995) demonstrated that resistance to *B.sphaericus* toxin is due to a change in the receptor on the midgut brush-border membrane.

2.3 Status of insecticide resistance in *An.stephensi*

An.stephensi is a major malaria vector in the Persian Gulf, the Middle-East and Indian subcontinent and the only urban vector of any importance. In 1955, DDT resistance was suspected in Saudi Arabia, after several years of DDT residual house spraying (Davidson, 1958). DDT resistance was reported in Iraq in 1957 (Davidson & Jackson, 1961). In *An.stephensi* from Iran, DDT resistance was detected in 1957 within 5-6 years of commencing residual application (Mofidi et al., 1958). Mahan & Singh (1965) selected an Indian strain of *An.stephensi* with DDT. Larvae and adults responded to the selection and exhibited cross-resistance to gamma-HCH and dieldrin. A strain of *An.stephensi* which originated from Iraq was also selected with DDT. Selection conferred highly specific resistance to DDT due to a dehydrochlorinase mechanism (Rongsriyam & Busvine, 1975). Synergist studies with PB, an MFO inhibitor, DMC and WARF, dehydrochlorinase inhibitors did not make the DDT-resistant strain of *An.stephensi* more susceptible, suggesting that nerve insensitivity was responsible for the resistance (Brown & Pal, 1971; Omer et al., 1980). Dieldrin resistance was first reported from Iran 1-2 years after starting application in 1959 (Zaim, 1987) and it was reported later from other countries (Davidson & Mason, 1963). In 1974 Manouchehri et al. reported that a field population of *An.stephensi* in Iran was resistant to DDT and dieldrin but susceptible to malathion. Subsequently they reported the development of malathion resistance in the population from Bandar Abbas, Iran (Manouchehri et al., 1976). Malathion resistance was detected in the Basrah area of Iraq in 1975 after six years of house spraying with malathion. Malathion resistance also has been reported from Iraq by Manouchehri et al.

(1980). Malathion and phenthoate resistance have been detected from Lahore, Pakistan (Rathor & Toqir, 1980; Hemingway, 1983), synergist studies suggesting the involvement of carboxylesterases in malathion resistance and with resistance inherited monofactorially (Hemingway, 1983). Scott & Georghiou (1986a) found that malathion and phenthoate resistance in larvae of *An.stephensi* was due to carboxylesterase activity. Malathion resistance due to an increased degradation at the carboxylester linkage has been implicated in *An.stephensi* (Herath & Davidson, 1981; Hemingway, 1982). Hemingway et al. (1984) showed that the larvae of a malathion resistant strain of *An.stephensi* were more susceptible to pirimiphos methyl than the susceptible strain (Resistance ratio=-2.7). The authors concluded that the malathion resistance gene only produces cross-resistance to compounds which contain a carboxylester bond such as phenthoate. Chitra & Pillai (1985) selected larvae of *An.stephensi* from India with various insecticides for 20 generations. Development of resistance was as follows: 234-fold to malathion, 36.5-fold to fenitrothion, 37.5-fold to fenthion, 65-fold to temephos and 7-fold to propoxur.

Thavaselvam et al. (1993) found that both the field and F1 adults of *An.stephensi* from India were resistant to DDT, dieldrin and malathion but were susceptible to fenitrothion. Similarly, larvae showed high resistance to DDT, fenitrothion and malathion but were susceptible to temephos.

For the first time Omer et al. (1980) found that the larvae of *An.stephensi* from Pakistan, initially showing low-level resistance to DDT but susceptible to pyrethroids, developed 18-fold cross-resistance to permethrin after six generations of selection with DDT, and 144-fold DDT resistance. Up to 23-fold cross-resistance to permethrin was obtained in

a sub-colony selected with DDT in conjunction with the synergist DMC. Synergist studies provided no evidence for enhanced metabolism due to dehydrochlorinase, or oxidase, of either insecticide in the resistant strains. Neurophysiological studies conducted by Omer et al. (1980), however, showed that the resistant strains required approximately 20 times more permethrin than the susceptible strain to induce an increase in the frequency of miniature endplate potentials. A difference in response to permethrin can be selected in larvae of *An.stephensi* of a similar magnitude to that found by Omer et al. (1980), which is also not reduced by oxidase or dehydrochlorinase-inhibiting synergists. However, this was shown to be subject to multigenic inheritance and not associated with the major source of DDT resistance which was controlled by a single gene located on chromosome III (Malcolm, 1988b). A study on pyrethroid resistance in *An.stephensi* demonstrated high level of resistance to peremthrin (at least 300-fold) in larvae and resistance can be blocked almost to the susceptible level by the synergist piperonyl butoxide indicating an oxidase-based resistance mechanism (Ladonni & Townson, in press, 1996). In another study larvae of *An.stephensi* were selected with deltamethrin and DDT for 40 generations (Kumar et al., 1991). They found that deltamethrin and DDT resistance was mainly due to MFO and dehydrochlorinase activity, respectively. In the work of Chakravorthy & Kalyanasundaram (1992) adults of *An.stephensi* were selected with permethrin in the laboratory in India. The selection resulted in the development of resistance in the F5 generation to the extent of 13-fold to permethrin, and cross-resistance of 7-fold to cypermethrin, 9-fold to alphamethrin and 10-fold to deltamethrin. The development of cross-resistance to 4% DDT was also

detected. Involvement of esterase-mediated hydrolysis as a mechanism of pyrethroid resistance in *An.stephensi* was investigated by Sahgal et al. (1994). Microplate assays of ß-esterases in individual larval and adult females and males revealed that there was a marginal role for esterases in the larvae of *An.stephensi*.

Table 2.1 shows the status of insecticide resistance in *An.stephensi* reported by WHO (1992a).

Table 2.1 Present status of pesticide resistance in *An.stephensi* in countries (WHO, 1992a)

DDT	OPs	Other compounds
Afghanistan, India, Iran, Oman, Pakistan, Saudi Arabia, United Arab Emirates, Yemen	India: malathion, fenitrothion, temephos, fenthion, iodofenphos Iraq: malathion, fenitrothion, pirimiphos methyl Iran: malathion, fenitrothion, iodofenphos, pirimiphos methyl, chlorphoxim, phoxim Pakistan: malathion, fenitrothion	Dubai: pyrethroids* India: pyrethroids, carbamates Pakistan: carbamates

* Ladonni & Townson (quoted in Malcolm, 1988)

2.4 Factors influencing development of resistance

Development of resistance in the field is multidimensional. It depends on the interaction of several factors. According to WHO (1980) and Wood & Bishop (1981) the multiple factors that influence the development of resistance to insecticides can be classified into the following categories:

A. Genetic

1. Mutation rate and frequency of R gene(s).
2. Penetrance, expressivity and dominance of R genes.
3. Relative fitness of genotypes.

B. Reproductive

1. Generations per year.
2. Rate of increase, and fluctuations in population size.
3. Monogamy/polygamy, parthenogenesis and other variations.

C. Behavioural/Ecological

1. Migration in and out of exposed population.
2. Avoidance of the insecticide.
3. Effects of age and natural inducers on degradative enzymes.
4. Endophagy/exophagy.

D. Operational

1. Relation of chemical to insecticides used earlier.
2. Proportion of population exposed to selective doses.
3. Dosage of insecticide taken up by exposed insects (heterozygotes killed?).

4. Persistence of insecticide.

5. Existence of unsprayed "refugia".

6. The exposure route.

7. Life stage exposed (before or after mating/oviposition).

8. Integration of insecticides with biological or genetic methods of control.

9. Use of insecticide mixtures; pattern application.

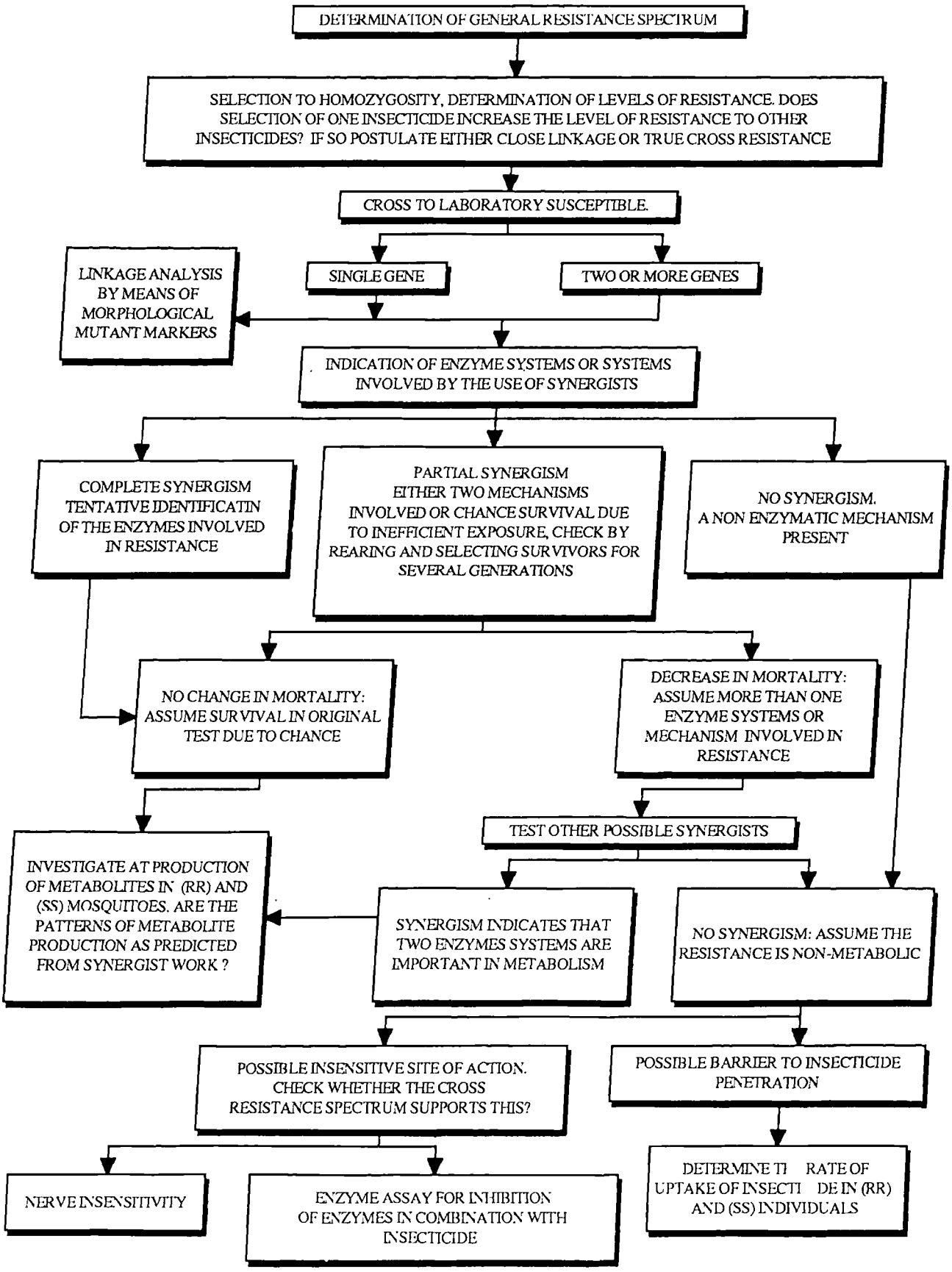
10. Release of susceptible males.

Among known and potential factors affecting the evolution of resistance, the operational factors are the only ones open to manipulation by man. Therefore, investigation on the development of resistance should ideally take account of all these factors.

2.5 The resistance spectra of mosquitoes

Determination of the general resistance spectra is the first stage in the investigation of any insecticide resistant population. Fig. 2.7 shows how to measure the spectrum of resistance in mosquito populations (Hemingway, 1981).

Fig. 2.7 Stages in the investigation of resistance mechanisms in mosquitoes (from Hemingway, 1981)



2.6 Methods of detecting resistance

Different approaches to detect the emergence of resistance are now possible. This is a basis for resistance management techniques to counteract resistance. The idea is to obtain base line susceptibility data, detect resistances in their early stages and monitor resistance levels over time (WHO, 1992a). These include:

a) WHO bioassay: These methods include WHO standard susceptibility tests in the laboratory. From these experiments the appropriate dosage required to kill 50% or 90% of populations can be calculated and be able to detect any changes in percentage mortality over a period of time as well as occurrence of resistance in the field (Brown, 1986; WHO, 1992a; Roberts & Andre, 1994). From such susceptibility tests one may be able to tell the inheritance patterns of resistance by crossing and testing progeny, and give a picture of the mechanisms conferring resistance.

b) Biochemical & immunological bioassays: It is now possible to detect enzymes associated with mechanism of resistance such as elevated esterases, P-450s and glutathione S-transferases. These include gel electrophoresis or immunologic tests. The advantage of biochemical tests include the ability to carry out multiple assay on a single insect to look for multiple resistance, and speed (Brown & Brgadon, 1987). A disadvantage of these methods are the expense and existence of difficulties in the field conditions.

c) DNA and RNA probes:

Molecular techniques are now being employed to detect resistance. Research is in progress to develop probes to detect resistance genes (Brown & Bragdon, 1987).

Polymerase Chain Reaction (PCR) based tests are available to detect cyclodiene resistance by picking up changes in GABA channels (Ffrench-Constant et al., 1995). Some examples of insecticide resistance detection using PCR methods are described by Ffrench-Constant et al. (1994, 1995).

The advantage of these over the above methods is that one may really be able to detect resistance even before it strikes. This would be possible by cloning genes which encode for resistance proteins and using radiolabelled tagged to an enzyme, it may be possible to detect the mutant allele that would confer resistance.

2.7 Mechanisms of resistance

Several years of intensive use of organic insecticides to control arthropod pests and disease vectors has led to the selection of pesticide resistance in some species. Today, resistance management in the context of integrated pest management has evolved as a favoured approach to prevent, delay or reduce the impact of insecticide resistance. Factors that induce resistance are numerous and the mechanisms adopted by organisms depend on the prevailing pressure and on the mode of action of the insecticide in use. Intoxication of an arthropod by a pesticide encompasses different levels of pharmacokinetic interaction: penetration of barrier tissue, distribution, storage, metabolism in internal tissue, and molecular interaction with the ultimate target site (Soderlund et al., 1989).

Many chemicals are being used against arthropods and there are hundreds of examples of resistance, a number of resistance mechanisms have been identified.

2.7.1 Reduced penetration (decreased absorption)

The composition of the insect's exoskeleton may become modified in ways that inhibit insecticide penetration. Decreased penetration of insecticides would allow ample time for detoxifying enzymes to metabolize the chemical which therefore would be less effective (Plapp, 1976). This kind of resistance was reported by Plapp & Hoyer (1968a) who found DDT and dieldrin resistance in *Musca domestica* was due to decreased penetration. Farnham (1971, 1973) showed that this mechanism is controlled by a gene on chromosome III that was given the name *pen* for penetration. Decreased cuticular penetration, possibly caused by a similar gene to *pen* was also found in a permethrin-selected strain of the house fly (DeVries & Georghiou, 1981a).

2.7.2 Metabolic resistance (degradation of insecticide)

In metabolic resistance, the metabolic pathways of the insect become modified in ways that detoxify the insecticide, or disallow metabolism of the applied compound into its toxic form. Metabolic resistance to insecticides is mediated by qualitative and quantitative changes in proteins that can often be difficult to define precisely at the biochemical level. Three broad enzyme classes are involved in insecticide detoxication, the mixed function oxidases (MFO), esterases and glutathione S-transferases. Their involvement in resistance is commonly identified by increases in the characteristic metabolites they produce. All three classes exist in multiple forms within each species and it is often not known whether increased activity arises from qualitative or quantitative changes in these enzyme complexes. Increased synthesis of these enzymes seem to result from gene

amplification (Devonshire et al., 1992).

2.7.2.1 Glutathione S-transferases

This group of enzymes catalyses the conjugation of glutathione with compounds having a reactive electrophilic centre, leading to the formulation of a water-soluble, less reactive product. Although there are many examples of increased metabolism of insecticide or model substrates by glutathione S-transferases of resistant insects, few are characterized at the molecular level. Metabolism mediated by these enzymes has been implicated in DDT and organophosphate resistance. Increased levels of DDT-dehydrochlorinase have been reported in different species resistant to DDT (Kimura & Brown, 1964; Rathor & Wood, 1981; Brealey et al., 1984; Hemingway et al., 1985; Herath et al., 1988; Amin & Hemingway, 1989). The natural function of DDTase is not known, although it is present in variuos insect organs including probably the peripheral nervous system where DDT appears to exert its major effect.

Studies on *Musca domestica* (Lewis & Sawicki, 1971; Yang et al., 1971; Motoyama & Dauterman, 1972) provided evidence for enhanced glutathione S-transferase activity in resistance to organophosphate insecticides. The glutathione S-transferase activity is controlled by a gene *g* on chromosome II of the house fly (Lewis & Sawicki, 1971). Two distinct glutathione S-transferases have been identified in *Musca domestica* (Fournier et al., 1992) and *Ae.aegypti* (Grant & Matsura, 1989). These classes of glutathione S-transferases are distinguished by substrate specificity and primary sequence characteristics of the enzymes. Recently a cDNA clone has been isolated from an adult, female-specific

An.gambiae library and has been identified as a member of the glutathione S-transferase family (Reiss & James, 1993). Prapanthadara et al. (1995) reported that DDT-resistance in *An.gambiae* from Zanzibar, Tanzania was based on increased DDT-dehydrochlorinase activity of glutathione S-transferases. Characterization of partially purified glutathione S-transferases from DDT susceptible and resistant strains demonstrated both quantitative and qualitative differences in the enzymes.

2.7.2.2 Mixed Function Oxidases (MFO)

MFO enzymes are of great significance both in mammals and arthropods in giving protection to a variety of insecticides, particularly to some chlorinated hydrocarbons, to many OPs and carbamates and to some pyrethroids. The mono-oxygenase, or mixed function oxidase (MFO), complex involves a reductase and one or more cytochrome P-450s and requires NADPH as cofactor (Devonshire et al., 1992). An increase in MFO activity is one of the most versatile mechanisms of resistance in insects. There are soluble forms of P-450 in bacteria and membrane bound forms in microsomes and mitochondria of eukaryotes. Insect P-450 enzymes also activate certain types of insecticides, for instance the conversion of phosphorothioates (P=S) to phosphate (P=O). This can result in an increased potency for inhibition of acetylcholinesterase by 3 or 4 orders of magnitude. P-450s are also involved in the biosynthesis of ecdysone, juvenile hormone and pheromone components (Feyereisen, 1995).

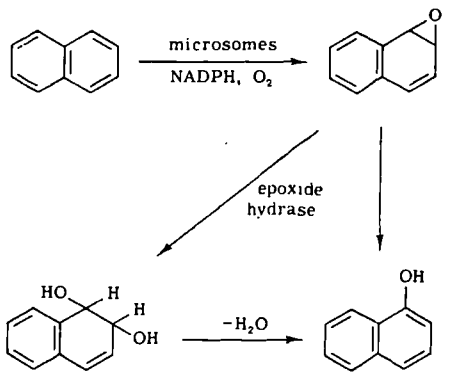
The work of Fenwick (1958), Agosin et al. (1961) and Arias & Terrier (1962) on the enzymology of insect microsomal oxidases, followed by the demonstration of insect

microsomal P-450 by Ray (1965), constitute the early, historical landmarks of insect P-450 research. The next part of this research, exhaustively reviewed by Agosin (1985) and Hodgson (1985), focused on the role of insect P-450 in the detoxification of insecticides with an emphasis on insecticide resistance and insecticide synergists. New aspects of P-450 research in insects have emerged during the last ten years. These include the discovery that P-450 enzymes are involved in insect hormone and pheromone metabolism. The number of different P-450 proteins present in any one insect species is not known. Ronis et al. (1988) were able to separate at least 7 forms of P-450 from adult house flies, and there are at least 32 distinct P-450 genes in the rat. It would not be surprising, therefore, to find several dozen P-450 genes in any one insect species. These enzymes have an extremely broad spectrum of substrates and catalyze a wide variety of biotransformations. These enzymes are subjected with enzyme induction (this will be discussed in more detail in chapter 6). Several factors including species, strain, sex, development stage, age and nutrition affecting mono-oxygenase activity. Cytochrome P-450 in insects is known principally from the Diptera and Lepidoptera. The mixed-function oxidase system has been shown to occur in the fat body, Malpighian tubules, and midgut. By far the most intensively studied mixed-function oxidase system is that of the house fly. Oxidative activity towards xenobiotics is always associated with the microsomal fraction of whole insect or organ homogenates. The microsomal fraction is the particulate fraction obtained by ultracentrifugation (100,000-200,000 g) of a postmitochondrial supernatant for an hour or more.

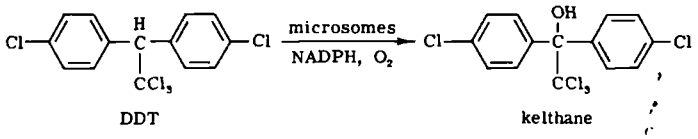
The processes by which xenobiotics are eliminated are divided by toxicologists into two

general groups, frequently referred to as phase I and phase II reactions. Oxidations, reductions and hydrolyses are typical of phase I reactions which introduce a hydrophilic functional group into the molecule (Gibson & Skett, 1986). Although the products of phase I reactions are more water soluble and may be excreted to some extent, they usually undergo one of the conjugation reactions typical of phase II. In the reactions the functional group introduced in phase I combines with a highly water-soluble endogenous metabolite such as glucose, glutathione, and various amino acids, to give rise to conjugation products which are readily excreted. Williams & Millburn (1975) have classified oxidative phase I reactions as follows:

Aromatic hydroxylation. The principal mechanism appears to be via epoxidation followed by subsequent hydration of the epoxide ring to yield a dihydrodiol or by rearrangement to yield a monohydroxy compound. This reaction sequence appears to be one of the routes for the metabolism of carbaryl (for more details see Eto, 1974).

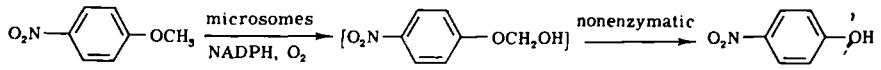


Aliphatic hydroxylation. This has been demonstrated *in vitro* using housefly microsomes in the presence of NADPH and O_2 and characteristically occurs at an aliphatic side chain. The oxidation of DDT to kelthane is probably the best known example.

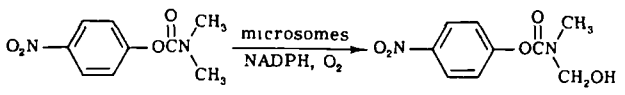


DDT kelthane

O-dealkylation. O-dealkylation of p-nitroanisole is commonly used as the basis of a rapid assay method for mixed-function oxidase activity since the product p-nitrophenol can be measured colorimetrically. The methylol derivative is believed to be an intermediate in the reaction.



N-dealkylation. N-dealkylation has been demonstrated *in vitro* using insect microsomal preparations, particularly in the case of N-methyl and N,N-di-methylcarbamate insecticides.

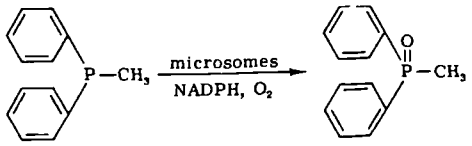


S-dealkylation has not been demonstrated *in vitro* with insect microsomes but *in vivo* investigations of aldicarb metabolism by house flies suggest that this reaction occurs.

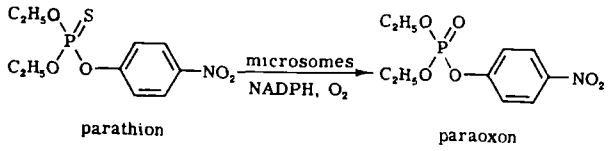
N-oxidation. The various nitrogen oxidations known from mammals, N-oxide formation, hydroxylamine formation and oxime formation, are catalyzed in mammalian liver either by flavoprotein enzymes or by cytochrome P-450. These reactions have not been investigated in insects.

S-oxidation. This reaction is not widely studied in insects but it does occur when mesorul (a kind of carbamate) is incubated with house fly microsomes and NADPH in the presence of oxygen, resulting in the formation of a sulfoxide.

P-oxidation. This reaction has only been described from studies using mammalian liver microsomes and it results in the formation of a phosphate from a trisubstituted phosphine. It has not yet been looked for in insects.



Replacement of S by O. This reaction is extremely important in insect toxicology since it results in the formation of highly potent phosphate cholinesterase inhibitors from the corresponding phosphorothioates. Although numerous examples are known, the one most studied is the formation of paraoxon from parathion.



parathion paraoxon

2.7.2.2.1 Mixed function oxidase and insecticide resistance

The study of the molecular genetics of P-450 in insecticide resistance is an interesting subject for scientists. Classical biochemical studies have indicated that resistant strains are often characterized by elevated P-450 levels and elevated P-450 enzyme activities. Moreover, the spectral characteristics of P-450 in resistant strains are often different from those in susceptible strains (Agosin, 1985; Hodgson, 1985). An increase in the level of some P-450 protein(s) best explains those biochemical data. Indirect data using antibodies to P-450 of resistant house flies suggest that P-450 increases from about 7% in a susceptible strain to about 70% of the total microsomal P-450 in the pyrethroid-resistant strain (Wheelock & Scott, 1989a). A single cytochrome P-450, termed P-450_{lpr}, is responsible for mono-oxygenase-mediated pyrethroid resistance in the LPR strain of house fly (Wheelock & Scott, 1992). P-450_{lpr} has been purified from resistant (LPR) and

susceptible strains of house fly (Wheelock & Scott, 1989a,b; Scott & Lee, 1993a,b). The specific content of P-450_{lpr} was estimated by quantitative immuno-electrophoresis to be 40-fold higher in the pyrethroid-resistant LPR strain than in an insecticide-susceptible strain. Immuno-inhibition studies demonstrated that P-450_{lpr} was responsible for the increased deltamethrin metabolism in LPR microsomes compared to those from a susceptible strain (Wheelock & Scott, 1992). The P-450 gene which is involved in pyrethroid resistance in house fly was sequenced and mapped to chromosome V (Feyereisen et al., 1995). The data from the work of Kumar et al. (1991) clearly suggest that deltamethrin resistance in the larvae of *Cx.quinquefasciatus, Ae.aegypti* and *An.stephensi* is mainly due to the detoxification of deltamethrin by microsomal mono-oxygenase. Table 2.2 shows the involvement of mixed function oxidase in pyrethroid resistance in different arthropods.

Table 2.2 Involvement of the mixed function oxidase system in pyrethroid resistance reported in different arthropods

Species	Insecticide	References
Musca domestica	permethrin	Scott & Georghiou (1986b)
M.domestica	deltamethrin	Wheelock & Scott (1992)
Blattella germanica	pyrethroids	Hemingway et al. (1993)
Aedes aegypti	pyrethroids	Rongsriyam & Busvine (1975)
Ae.aegypti	deltamethrin	Kumar et al. (1991)
Culex quinquefasciatus	deltamethrin	Kumar et al. (1991)
Cx.quinquefasciatus	permethrin	Priester & Georghiou (1980a)
Anopheles quadrimaculatus	pyrethroids	Rongsriyam & Busvine (1975)
Haematobia irritans	pyrethroids	Bull et al. (1988)

Table 2.2 (continued)

Species	Insecticide	References
Haematobia irritans	pyrethroids	Sheppard & Joyce (1992)
Heliothis virescens	cypermethrin	Little et al. (1989)
Helicoverpa armigera	pyrethroids	Daly & Fisk (1992)
Plutella xylostella	pyrethroids	Liu et al. (1981)
Leptinotarsa decemlineata	permethrin	Lee & Clark (1993)
Tribolium castaneum	pyrethroids	LIoyd & Ruczkowski (1980)
Frankliniella occidentalis	permethrin	Immanraju et al. (1992)
Cx. quinquefasciatus	pyrethroids	Rongsriyam & Busvine (1975)

2.7.2.3 Esterases and hydrolysis

Esterases are the most significant enzymes for insecticide detoxification in insects. Organophosphates, carbamates and pyrethroids contain carboxylester and phosphotriester bonds that are subject to attack by esterase enzymes. These esterases can often be separated into isozymes with different substrate specificities. Insect esterases are very diverse and can include monomers, dimers and multimers, which means that their relative molecular mass can cover a wide range. Polymorphism is a notable characteristics of insect esterases. Multiple forms of esterases are present in the soluble, cytosolic fraction of insect (Brattsten, 1992; Dauterman, 1985). Of the multiple forms of esterase isozymes that exist in insects, few participate in insecticide metabolism (Maa & Terrier, 1983). Each isozyme probably has a certain range of substrates. Unlike the mono-oxygenase reaction, esterases do not utilise high energy co-factors (Dauterman, 1985). Different types of esterases (A1, B1, A2, B2) have been recognized in OP resistant populations of the *Cx.pipiens* complex throughout the world (cited by Poirie et al., 1992) and overproduction of nonspecific esterases is a common mechanism of resistance. For esterase B1, resistance to OP insecticides has been shown to be due to sequestration of insecticide and overproduction of all esterase B is due to gene amplification (Callaghan et al., 1994). Jayawardena et al. (1994) showed that a strains *Cx.quinquefasciatus* with elevated A2 and B2 is resistant to a broad range of organophosphate insecticides. Enzyme assays suggested that sequestration rather than metabolism is the primary mode of operation of these esterases on malathion. The basis of malathion resistance in the adults of *An.arabiensis* from Sudan was a carboxylesterase (Hemingway, 1983). Malathion resistance due to an increase in degradation at the carboxylester linkage is a com on

detoxification pathway that has been implicated in *An.culicifacies* (Herath & Davidson, 1981), *An.stephensi* (Hemingway, 1982), *Blattella germanica* (Heuval & Cochran, 1965), *Cx.tarsalis* (Matsumura & Brown, 1963), *Tetranychus urtica* (Matsumura & Voss, 1964) and *Tribolium castaneum* (Dyte & Rowlands, 1968). Esterase cleavage has been implicated in OP and pyrethroid resistance in *M.domestica* (Funaki et al., 1994). Resistance to pyrethroids in *Blattella germanica* was found to be partly due to elevated esterases (Atkinson et al., 1991). Esterase dependent cross-resistance between OP and pyrethroids has been detected in several species. In OP resistant *M.domestica* and *Culex* mosquitoes, the esterases responsible for cross-resistance are thought to be involved in pyrethroid hydrolysis (Soderlund & Bloomquist, 1990). The best documented example of esterase based metabolic resistance to OP, carbamate and pyrethroid insecticides is found in the aphid *Myzus persica* (Devonshire & Moores, 1982) in which the overproduction of esterases FE4 and E4 responsible for insecticide hydrolysis and sequestration have been shown to be caused by amplification of a structural gene. The massive overproduction of any esterase protein by resistant *M.persica* may result in the detoxification of insecticidal esters first by sequestration and then by hydrolysis when the inhibited esterase reactivates (Devonshire & Field, 1991, 1995).

2.8 Metabolism of DDT

There are five principle routes of DDT metabolism in various organisms (Fig. 2.8): oxidation to DDA (route 1) or to kelthane (route 2) or dichlorobenzophenone (route 3), dehydrochlorination to DDE (route 4), or reductive dechlorination to DDD (route 5). In insects, the best known metabolite of DDT is DDE. In DDT-resistant h se

flies the enzyme responsible for this reaction is DDT dehydrochlorinase. Increased levels of DDT-dehydrochlorination have been reported in mosquitoes resistant to DDT (Kimura & Brown, 1964; McDonald & Wood, 1979a, b; Hemingway et al., 1985; Herath et al., 1988; Prapanthadara et al., 1995). These detoxification enzymes differ in quantitative and qualitative features in different species.

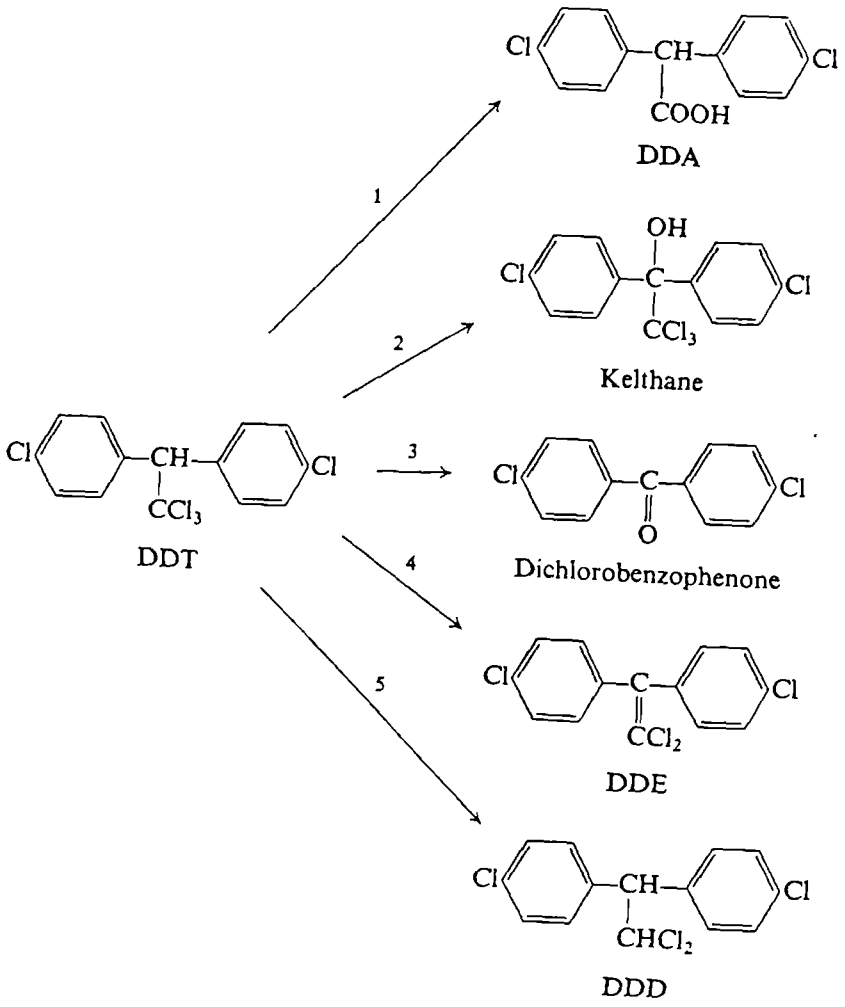
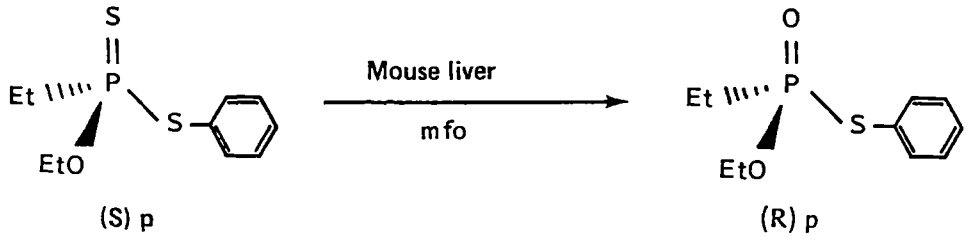


Fig. 2.8 Routes of DDT metabolism (from O'Brien, 1967)

2.9 Metabolism of malathion

Malathion is a dithiophosphate and the 'leaving group' is a succinic acid ester. It is metabolised either by activation or detoxification.

Activation: Malathion like other OPs insecticides contains a carboxylester linkage in the molecule (Eto, 1974). Conversion of P=S to P=O (Fig. 2.9) by an insect is an important step in activation. In this process the sulphur atom is replaced by oxygen, to yield a much more powerful cholinesterase inhibitor, malaoxon. Most insects possess a very active oxidative system. This can activate malathion by attacking the P=S linkage, so that the insect's own defence system actually helps to kill it. All insects and vertebrates possess both esterase and MFO systems; it is the balance of the action of these two systems which varies from one organism to another. Metcalf (1967) showed that the disulphuration reaction occurs in *Periplaneta americana* by MFO which requires NADPH and molecular oxygen and Mg^{2+} ions.



(S) p (R) p

Fig. 2.9 Conversion of P=S to P=O ester by the MFO system

Detoxification: OP insecticides are degraded by various hydrolytic pathways that lead to detoxification. Carboxylesterases are important in the inactivation of OPs such as malathion. This detoxification linkage reaction involves the hydrolysis of a carboxylester linkage resulting in a non-toxic product. In many insect species carboxylesterase activity is low or missing from susceptible populations (Hemingway et al., 1990). However, it is present in certain resistant insects and it is reasonable to presume that resistance to OPs is at least partly due to carboxylesterase activity. The process of activation and inactivation of malathion are shown in Fig. 2.10.

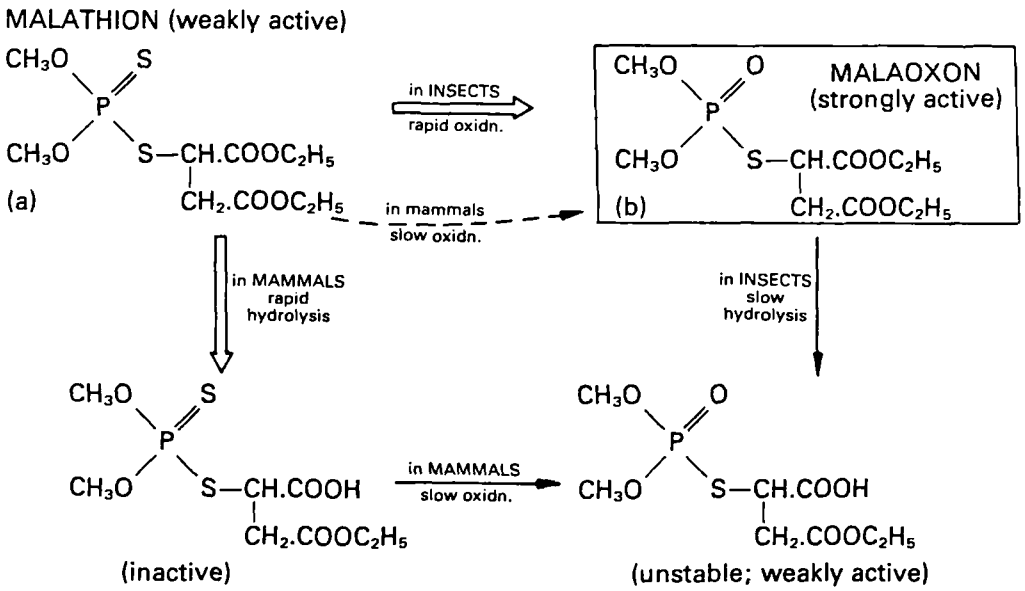


Fig. 2.10 Metabolic fate of malathion (from Hassall, 1982)

2.10 Metabolism of permethrin

2.10.1 Metabolism of permethrin in insects

Pyrethroid insecticides are metabolised by esterases and mixed function oxidases (MFO) causing hydrolytic and oxidative degradation respectively (see Fig. 2.11). In insects, the MFO system mediates methyl and aromatic hydroxylation and ester cleavage. This system is a characteristic component of the endoplasmic reticulum of cells from different insect tissues. They can be located in microsomes, the fraction of the cellular homogenate that is sedimented by high-speed centrifugation of the post-mitochondrial supernatant. In general under *in vitro* conditions, the MFO system is active only when the reaction medium is supplemented with the co-factor NADPH in the presence of molecular oxygen. The metabolic fate of permethrin has been studied in a number of insect species. The *in vivo* studies of Shono et al. (1978) on cockroach adults, house fly adults and cabbage looper larvae revealed that they metabolise the *cis*- and *trans*-permethrin isomers by a variety of pathways leading to 42 tentatively identified metabolites (see Fig. 2.12). These pathways are initiated by hydrolysis or hydroxylation at the geminal dimethyl group of the phenoxybenzyl moiety. The hydroxy esters or their ester cleavage products are further metabolized leading ultimately to a variety of conjugates. In all three insect species *cis*-permethrin is metabolised less readily than *trans*-permethrin. Ester cleavage and hydroxylation at the 4'-position of the alcohol moiety are the major metabolic process in all three insects, as is oxidation of 3-phenoxybenzyl alcohol to 3-phenoxybenzoic acid.

The major metabolic pathways in insects are generally similar to those in mammals.

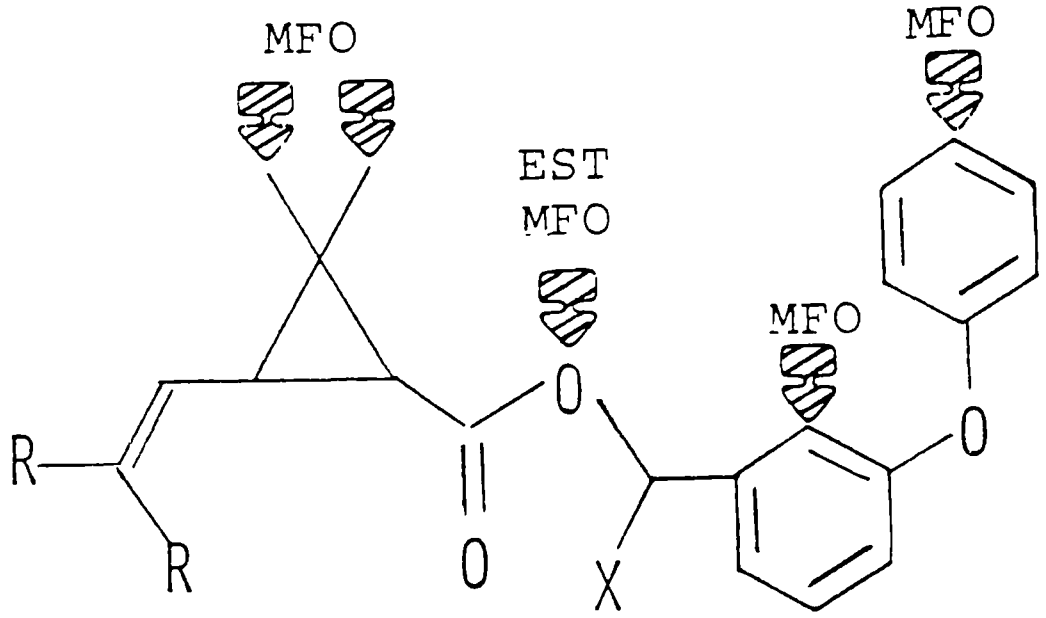
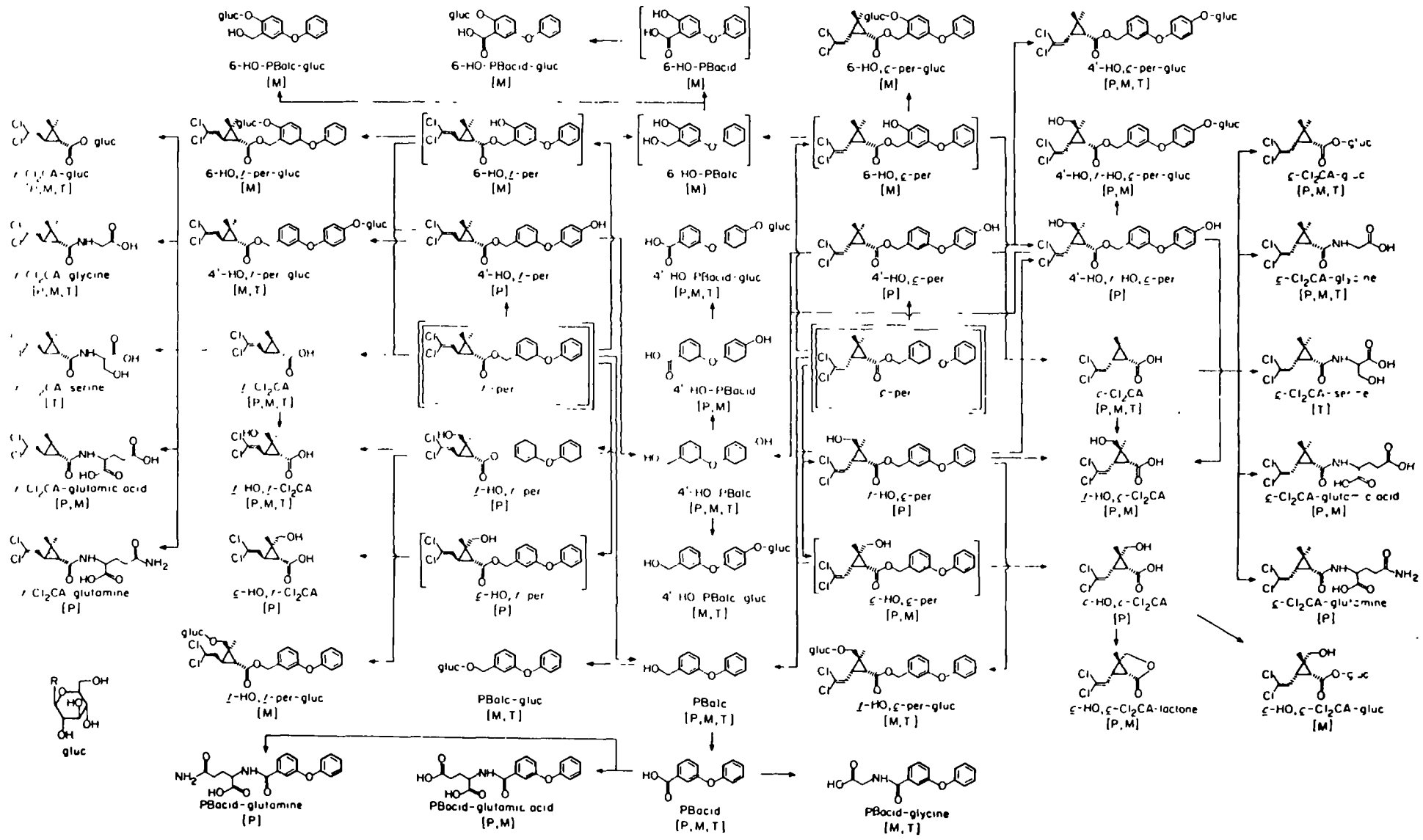


Fig. 2.11 Main sites of cleavage of pyrethroids by esterases (EST) and oxidation by mixed function oxidases (MFO) enzymes (from Zebra, 1988)

Fig. 2.12 ***In vivo*** **metabolism of permethrin by insects (from Shono et al., 1978). 42 metabolites have been tentatively identified**



2.10.2 Metabolism of permethrin in mammals

Most of the *in vivo* and *in vitro* studies of permethrin metabolism have been carried out using mice and rats as a laboratory model for toxicity assessments. The fate of permethrin has also been studied in livestock animals such as cows and chickens. A preliminary study in rats was carried out by Elliot et al. (1976) and this was followed by very detailed investigations in rats (Gaughan et al., 1977) and in isolated rat and mouse liver microsomes (Shono et al., 1979). The metabolic pathways detected in these studies are summarized in Fig. 2.13. The *in vitro* investigations established that permethrin is extensively metabolised by the rat such that very little permethrin is excreted unchanged. The *trans*-isomers were eliminated more readily than the *cis*-isomers. These differences between the *cis*- and *trans*-isomers were attributed to a much greater rate of metabolism for the *trans*-isomers. Such a differential rate of metabolism was confirmed by *in vitro* studies (Soderlund & Casida, 1977) where mouse liver microsomes were shown to metabolise *trans*-permethrin four times faster than *cis*-permethrin. The major *in vivo* route of metabolism for both *cis*- and *trans*-isomers is via ester cleavage. However, the cleavage of the *cis*-isomer is catalysed by an oxidase, not an esterase.

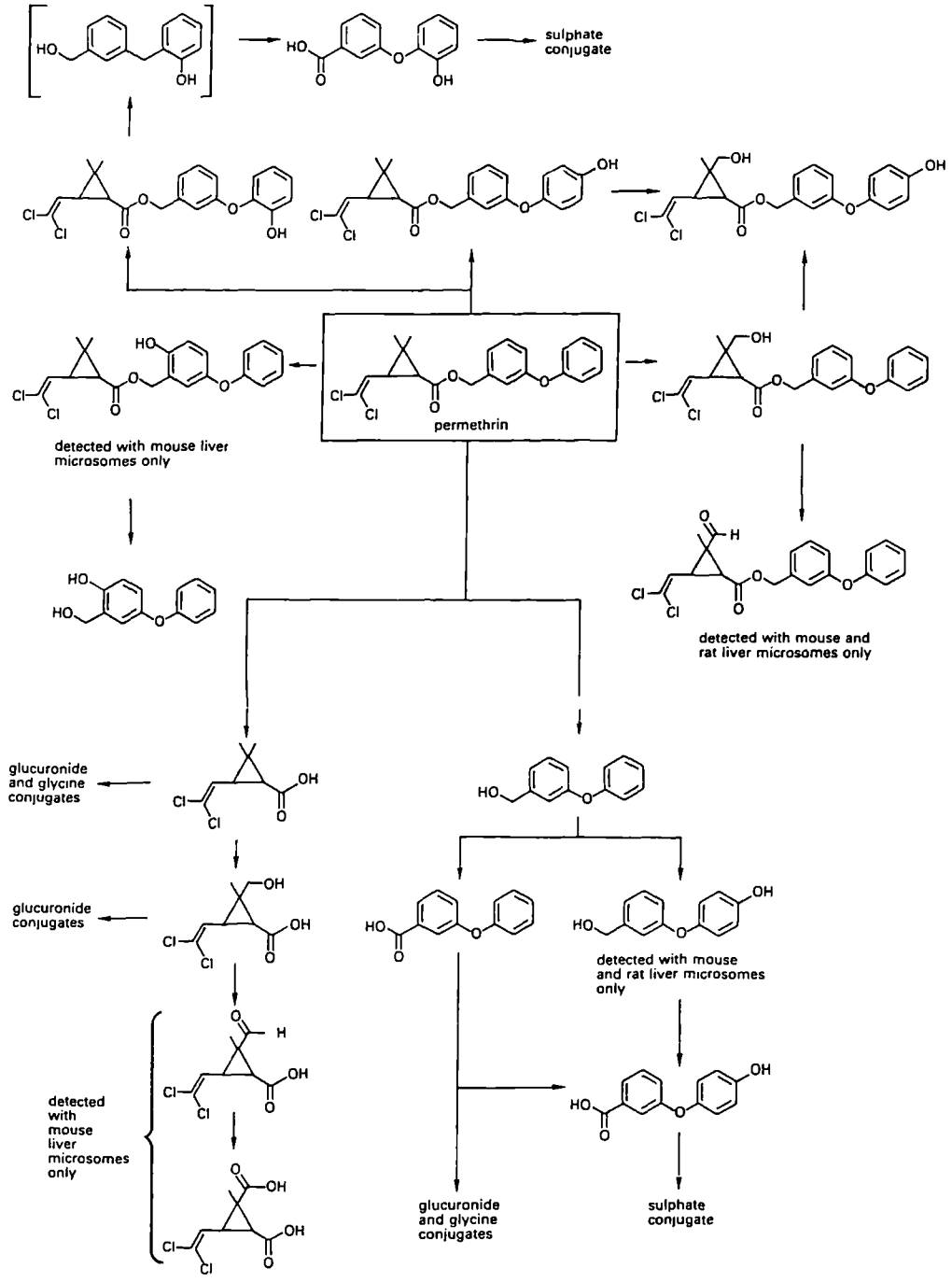


Fig. 2.13 Metabolism of permethrin in rats, and rat and mouse liver microsomes (from Leahey, 1985)

2.11 The importance of synergists in insecticide resistance studies

Synergists are compounds that are relatively non-toxic by themselves, but which serve to enhance the toxicity of an insecticide with which they are combined. Insecticide synergists act mainly by blocking the enzymes effecting insecticide detoxification. Enzyme inhibition can be broadly regarded as a decrease in the activity of an enzyme *in vitro* or *in vivo*, caused by a chemical compound. Inhibition may be divided into several types :

1. Synergists may alter the biological activity of the formulation by increasing the stabilization of aerosol droplet size (Metcalf, 1967)
2. Altering the rate of penetration through the cuticle. Sun & Johnson (1972) suggested that one of the mechanisms of sesamex synergism is the increased rate of penetration of carbamate insecticides.
3. Modifying the amount of insecticide picked up by the insect.
4. Interaction of the compound with the active site of the enzyme to give a complex.
5. Competitive inhibition by two different compounds at the same enzyme active site.
6. Destruction of the enzyme.
7. Reduced synthesis of the enzyme.

The main synergists used for insecticide resistance studies

Many compounds have been developed as synergists. They are involved in different reactions. The main compounds used as a synergists are:

a) Piperonyl butoxide (PB)

The best established mixed-function oxidase (MFO) inhibitors are methylenedioxyphenyl compounds (MDP), which were originally developed as synergists of pyrethroids. The best known of these compounds is piperonyl butoxid

It is active *in vivo* and *in vitro*. It has a fairly broad spectrum of P-450 inhibition and is not specific for particular isozymes. PB (Fig. 2.14) is widely used in aerosol sprays containing pyrethroids. It is unstable in UV light and, because of this, its usefulness in outdoor sprays is very restricted (WHO, 1992a).

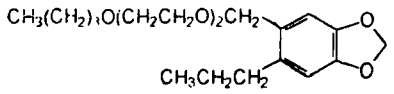


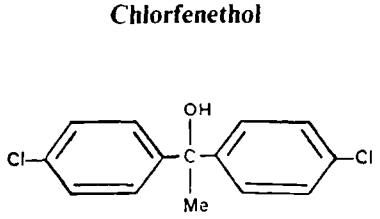
Fig. 2.14 Structure of piperonyl butoxide

b) Sesamine

Sesamine is one of the active principles of sesamine oil, which has long been known to synergize the action of pyrethrins (Cremlyn, 1978).

c) Chlorfenethol (DMC) & WARF

In certain DDT resistant strains in which enzymatic conversion of DDT to DDE by dehydrochlorinase is the major detoxication mechanisms, the compounds 1,1-bis(4-chlorophenyl) ethanol (DMC) and N-di-n-butyl-P-chlorobenzenesulphanamide (WARF) have been used as effective DDT synergists, by virtue of their capacity to inhibit *in vivo* activity of dehydrochlorinase (see Fig. 2.15).



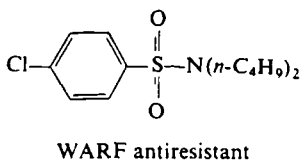


Fig. 2.15 Structures of DMC & WARF

d) Triphenyl phosphate (TPP); O,O,O, triphenylphosphate

e) DEF; S,S,S, tributyl phosphorotrithionate

TPP and DEF are potent inhibitors of esterases in insects and are widely used as synergists to indicate the involvement of esterases in the detoxification of insecticides (Plapp et al., 1963; Bell & Busvine, 1967; Priester & Georghiou 1978; Payne & Brown, 1984; Kotez & Sales, 1994).

2.12 Induction of detoxification enzymes

The three most important systems of detoxification in insects are the cytochrome P-450s, the glutathione S-transferases and the esterases. These enzymes have been detected in many insect species (Nakatsugawa & Morelli, 1976; Wilkinson & Brattsten, 1972; Bull et al., 1988; Callaghan et al., 1994; Jayawardena et al., 1994) and their involvement in resistance to insecticides has been demonstrated repeatedly (Plapp, 1976; Oppenoorth & Welling, 1976; Holden, 1979; Yu, 1990; Prapanthadara et al., 1995)). Their role in the biotransformation of xenobiotics allows insects to compete quite successfully in a hostile environment.

Recent studies of detoxification in insects have revealed that further versatility in the adaptation of insects to their environment is provided by the phenomenon of induction (Hunter et al., 1994; Feng et al., 1995). This is the process in which a chemical stimulus enhances the activity of the detoxification system by the production of enzymes which are involved in degradation of xenobiotics. Induction provides energy economies by activating the detoxification system only when it is needed. The induction of xenobiotic metabolizing enzymes has been studied intensively in mammals during the last two decades, most of these studies being concentrated on the hepatic microsomal cytochrom

P-450 system. Nebert et al. (1981) summarized the steps involved in the induction process in mice (Fig. 2.16). In this phenomenon the enzyme inducer enters the cell and binds to a specific cytosilic protein, the receptor, and then the inducer-receptor complex is transferred to the nucleus and activates the appropriate structural gene. Transcription and translation result in the production of the appropriate cytochrome P-450 and associated factors

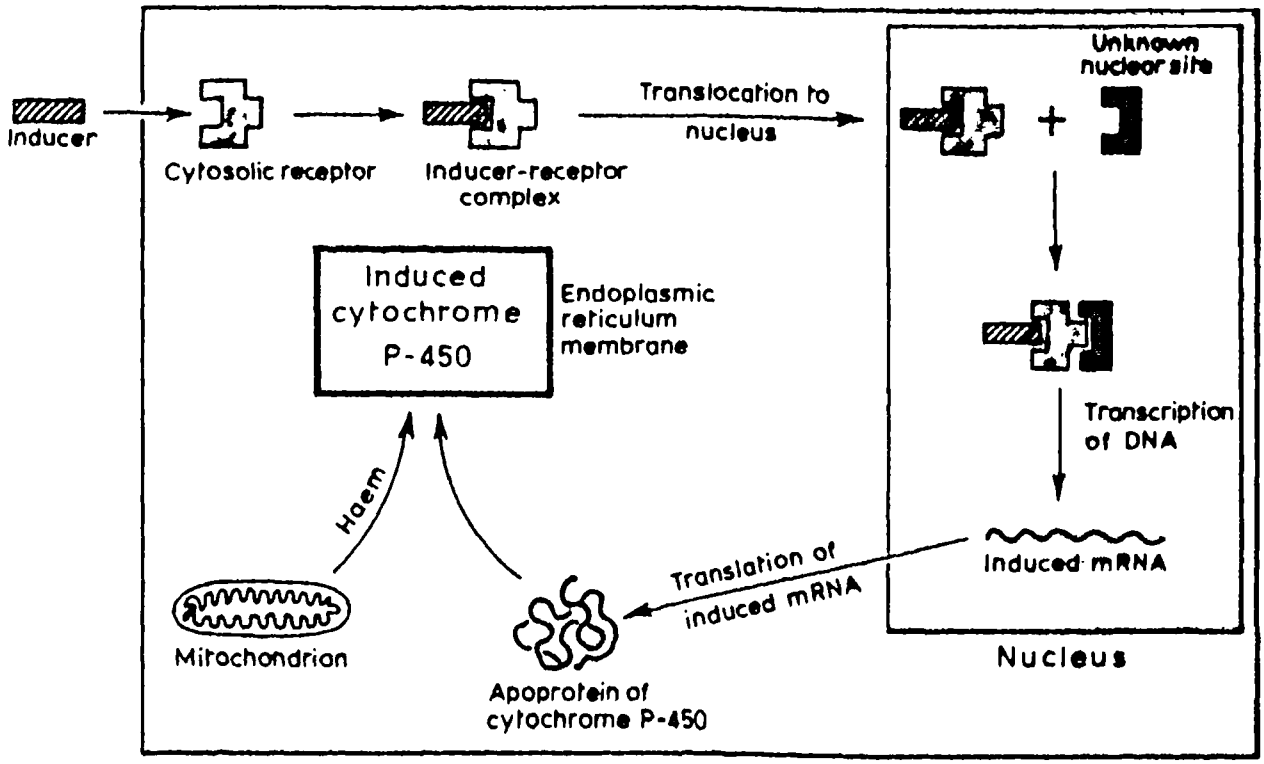


Fig. 2.16 Receptor-mediated induction of cytochrome P-450 by polycyclic aromatic hydrocarbons (from Gibson & Skett, 1986).

2.13 General outline of the insect nervous system

A basic knowledge of the structure of the tissue is an essential prerequisite to any biochemical investigation, and this is probably more true of nervous tissue than of any other in terms of the mode of action of insecticides. The nervous system of insects may be considered to consist of three main units: a dorsal anterior brain, a ventral nerve cord and a peripheral nervous system. The peripheral system may be further subdivided into three basic elements, the neuromuscular system, the sensory system and the stomatogastric system. The characteristic features of the nervous system of the house fly are shown in Fig. 2.17. The basic element in the nervous system is the nerve cell, or neurone. This consists of a cell body containing the nucleus and long cytoplasmic projections which extend to make contact with other neurones. The cell body is known as the soma or periokaryon, while the projections are known as axons. Frequently the axon has branches and ends in a terminal arborization. Nerve impulses are conducted from one cell to the next along the axon. Parts of each neurone are specialised for the reception of the stimuli which initiate conduction in the axon. This part, which is known as the dendrite, may arise directly from the periokaryon or represent the distal endings of an axon, in which case there is no anatomical differentiation between axon and dendrite. The site at which neurones are closely apposed so that the activity of one in influenced by the other is called the synapse. Fig. 2.18 illustrates the principle structural features of the nerve cell.

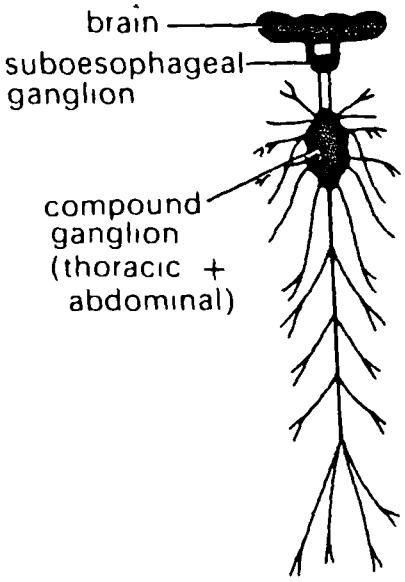


Fig. 2.17 Diagram of a house fly nervous system showing the ganglia of the central nervous system (CNS) and the fibres of the peripheral nervous system (PNS) (from Chapman, 1982)

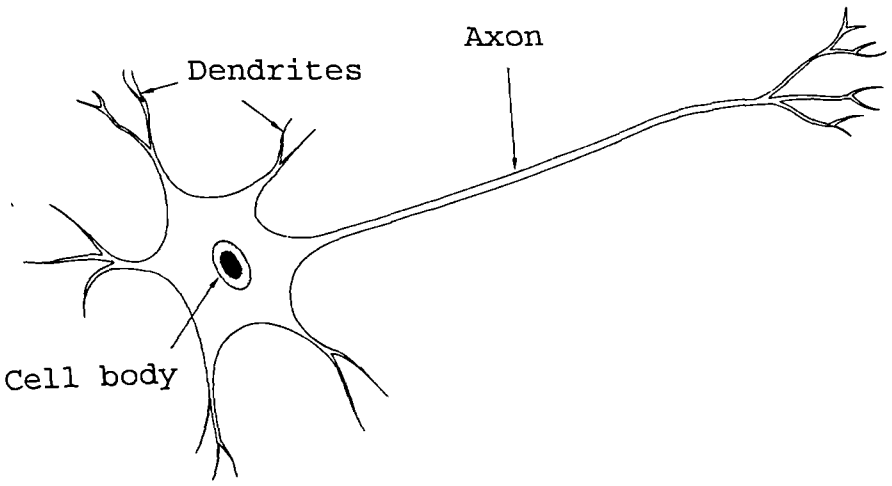


Fig. 2.18 Diagram to show the principle features of a nerve cell (from Dowson, 1977).

2.13.1 Mechanism of nerve excitation

In order to explore the mechanism of action of insecticides on the nerve membrane, one has to know how the nerve excitation occurs. According to Narahashi (1971a) the nerve cell and fibre generally contain a high concentration of potassium (K^+) and relatively low concentration of sodium (Na^+). Extracellular fluid is rich in Na^+ and Cl^-. In intracellular fluid, on the other hand, the main cation is K^+. In the external fluid, the K^+/Na^+ ratio is usually reversed. Thus, concentration gradients for sodium and potassium are established across the nerve membrane. At rest the nerve membrane is permeable to K^+, but only sparingly so to Na^+ and Cl^-. Therefore, the potential difference across the nerve membrane approaches the equilibrium potential for potassium (E_k). The values of the E_k and the resting potential are usually in the range of -50 mV to -100 mV, the inside being negative with respect to the outside. When the resting potential decreases owing to the application of an outward current across the nerve membrane, the permeability of the latter to sodium increases rapidly, so that the membrane now becomes almost exclusively permeable to sodium. Thus, the membrane potential is allowed to approach the equilibrium potential for sodium (E_{Na}), forming the rising phase of the action potential. Since E_{Na} is near +50 mV, the membrane potential is temporarily reversed in polarity during the action potential. Sodium ions enter the axon during this phase, according to the electrochemical gradient. Then the increased sodium permeability begins to decrease and the potassium permeability begins to increase beyond its resting value, so that the membrane again becomes almost exclusively permeable to potassium, bringing the membrane potential back to the original resting level. This is the falling phase of the

action potential. Potassium ions leave the axon during this phase (repolarization), according to electrochemical gradient (Fig 2.19). Changes in voltage gated channels during action potential are presented in Fig. 2.20.

Plasma membranes contain a variety of ion channels (pores) that may be closed or open (Fig. 2.20). Part of the protein that forms such channels may act as a gate, opening and closing on demand. Depending on the types of channels that are present, a portion of a neuron may be able to produce either a graded potential or an action potential (impulse). In addition to K^+ and Na^+ voltage-gated ion channels, there are chemically-gated ion channels that open or close in response to specific chemical stimuli. A wide variety of chemicals, such as neurotransmitters, hormones, and ions such as H^+ and Ca^{2+} , regulate chemically gated ion channels. Some Na^+/K^+ pumps expel three Na^+ for each two K^+ imported. Such pumps are said to be electrogenic (Tortora & Grahowski, 1993), which means they contribute to the negativity of the resting membrane potential. The total effect of such pumps, however, is very small, no more than -3 mV of the total resting membrane potential.

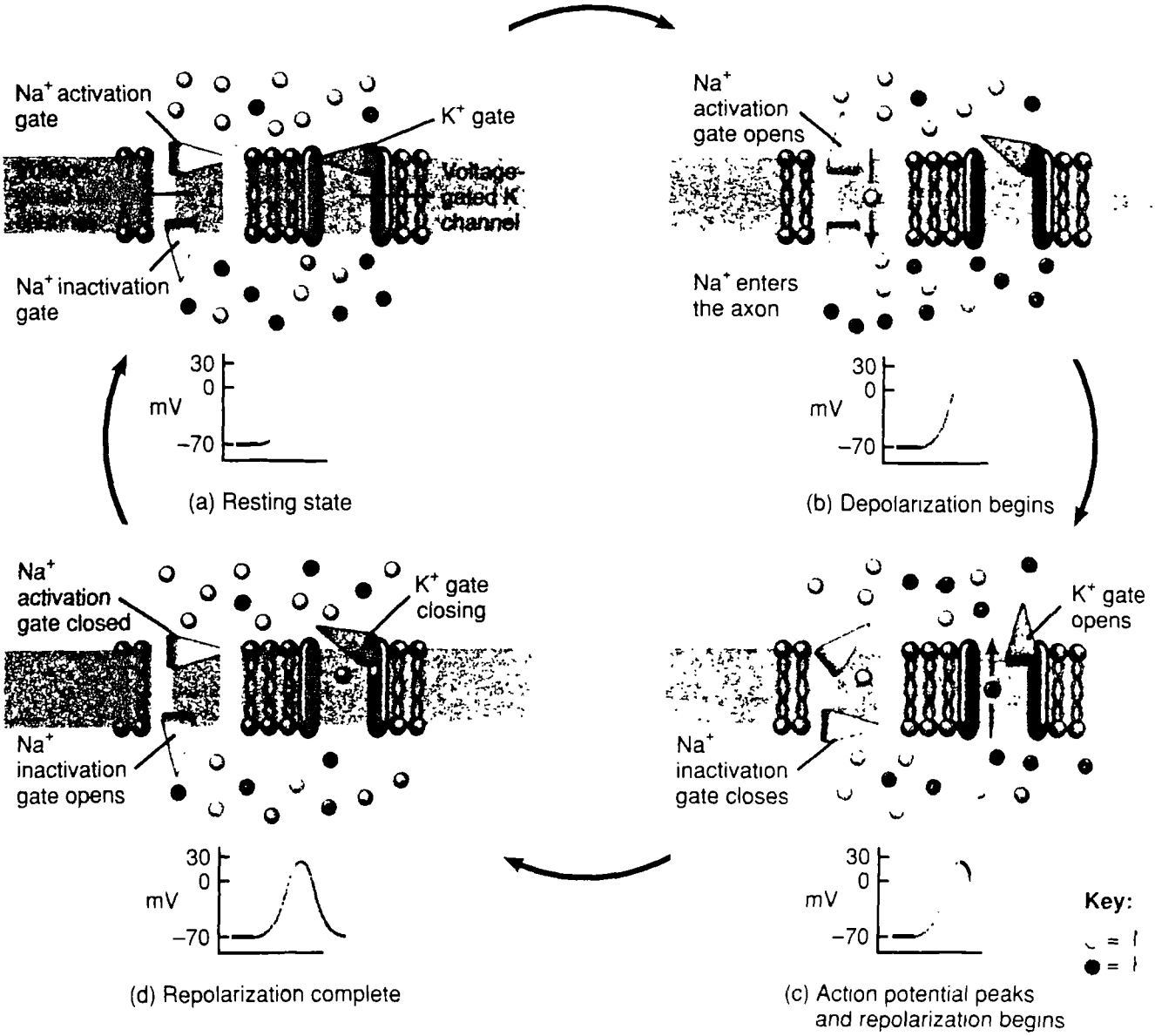


Fig. 2.19 Changes in voltage-gated channels during action potential depolarization and repolarization (from Tortora & Grahowski, 1993)

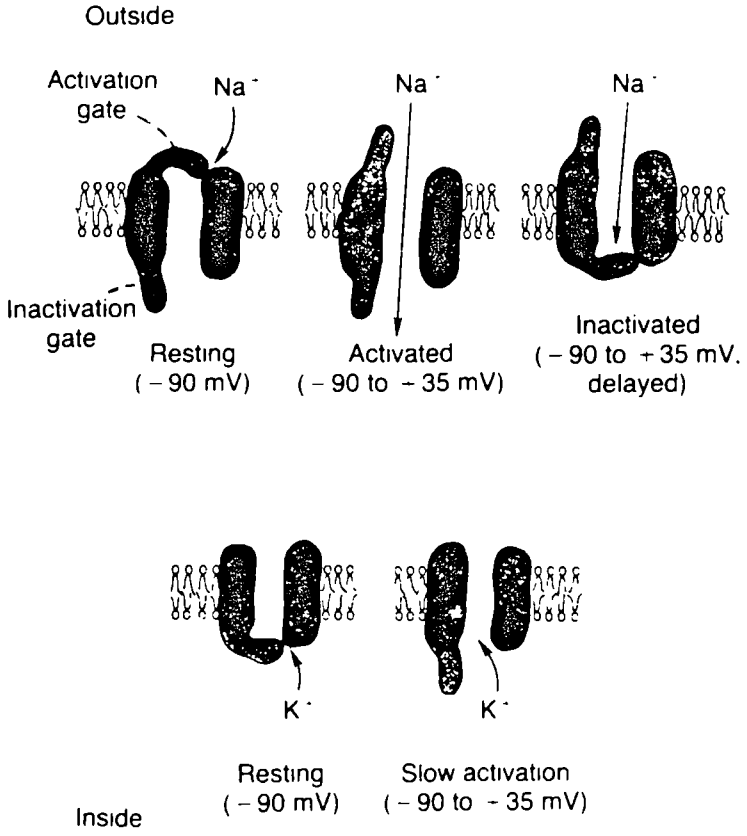


Fig. 2.20 Characteristics of the voltage-gated Na^+ and K^+ channels (from Guyton, 1992)

2.13.2 Conduction of action potential

Nerve impulses communicate information from one part of the body to another. To do this, they must travel from where they arise, at a trigger zone, to axon terminals. The special mode of impulse travel is called conduction. As Na^+ flows in, depolarization increases, and the depolarization opens voltage-gated Na^+ channels in adjacent patches of membrane. Thus the nerve impulse self-propagates along the membrane. The situation is like toppling a long row of dominos by pushing on the first one in the line . Also, since the membrane is refractory behind the leading edge of an action potential, an impulse normally moves only in one direction from where it arises at the trigger zone (see Fig. 2.21).

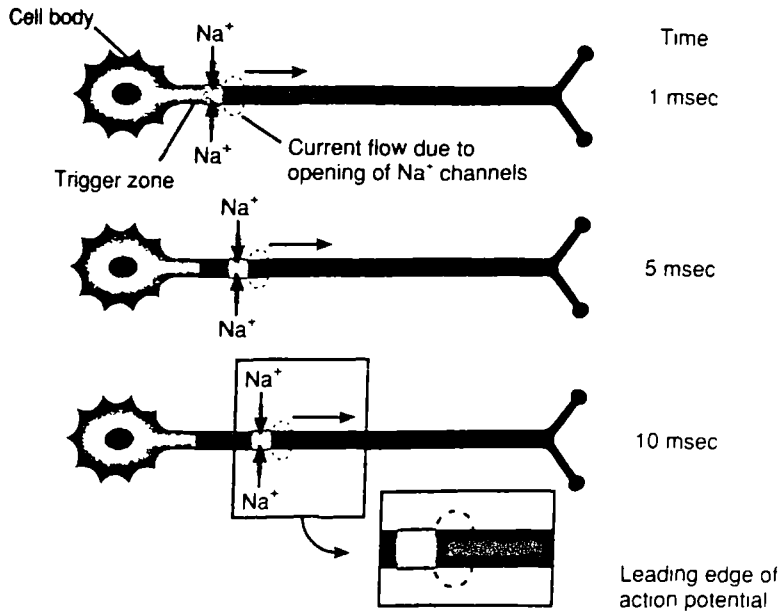


Fig. 2.21 Conduction of a nerve impulse after it arises at the trigger zone (from Tortora & Grahowski, 1993)

2.13.3 Crossing of nerve impulse from cell to cell

The function of the axon is to conduct the action potential to another excitable cell. The latter may be either another neuron if the axon is sensory (afferent), or the cells of an effector organ such as a muscle if the axon is motor (efferent). At the point of contact between the axon terminal and the receiving cell there is no structural continuity, and the gap between the two cells is usually too great for the impulse to jump across by purely electrical means. In the majority of cases, a chemically mediated communication process occurs between the two cells, and this process is known as synaptic transmission. Synapses are essential for homeostasis because they allow information to be integrated and filtered. Certain signals are transmitted while others are blocked. At a synapse, the neuron sending the signal is called the presynaptic neuron, and the neuron receiving the message is called postsynaptic neuron. The presynaptic neuron releases a neurotransmitter that diffuses across the synaptic cleft and acts on receptors in the plasma membrane of the postsynaptic neuron to produce a postsynaptic potential (see Fig .2.22). An indispensable part of any neurotransmitter system is a specific receptor for the transmitter located in the post synaptic membrane.

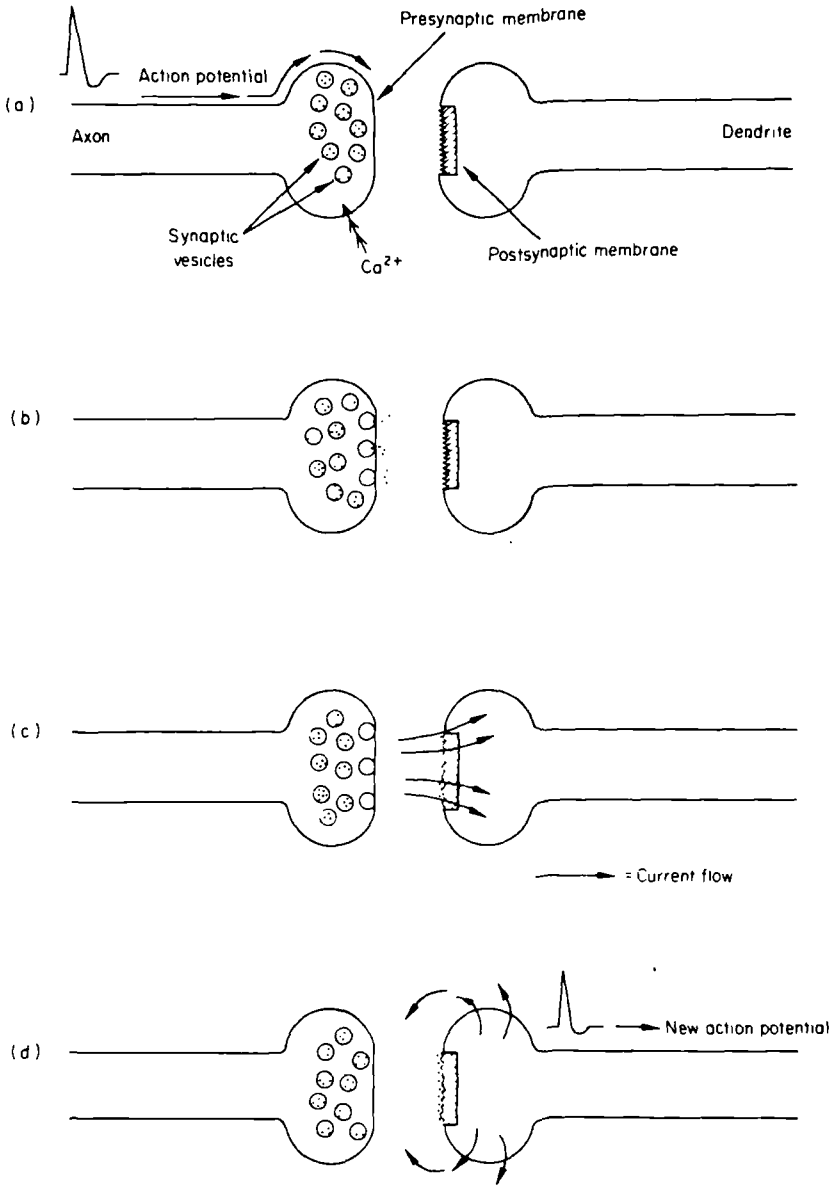


Fig. 2.22 Schematic diagram of transmission at an excitatory synapse (from Dowson, 1977)

2.13.4 Synaptic transmitters

In the synaptic cleft there are several types of neurotransmitter. Excitatory and inhibitory neurotransmitters are present in both the CNS and PNS. Acetylcholine (ACh) acts as an excitatory transmitter in insects as well as in vertebrates. One of the most studied inhibitory transmitters is gamma-amino butyric acid (GABA). The interaction of this transmitter with the postsynaptic receptor of an inhibitory synapse leads to an increase in the anionic (usually Cl^-) permeability of the membrane. Glutamate is an amino acid that plays a central role as an excitatory transmitter in the myoneural junction (Candy & Kilby, 1975). The neurotransmitter is removed in three basis ways: by diffusion, enzymatic degradation and re-uptake into cells.

2.14 The mode of action of insecticides

Studies on the mode of action of insecticides are very important from several points of view. Firstly, such knowledge is needed to understand the health hazards of these chemicals to man and other non-target organisms. Secondly, it helps chemists to design additional chemicals with similar modes of action. Thirdly, it could give scientists important clues as to the cause of resistance development in pests, particularly those involving target insensitivity, and thereby help in designing countermeasures to avoid resistance or reverse the development of resistance. Finally the knowledge gained by such studies yields valuable basic information on the nature of the target systems (e.g. the weakness of sensitive insects) in terms of physiological, biochemical and biophysical knowledge of a vital biological system (Matsumura, 1986).

2.14.1 Mode of action of organochlorine insecticides

The signs of poisoning in both insect and mammals, has been interpreted to indicate that the primary target of DDT is the nervous system. The well-known physiological signs of DDT poisoning of the insect nerve were first demonstrated in 1945 (a,b) by Yeager & Munson. The effects of DDT on the action potential have been analyzed using the voltage clamp method. It has been shown that DDT prolongs the inward sodium current and also suppresses the increase in potassium permeability. A combination of these effects leads to the prolonged falling phase, increases the negative after-potential, and leads, in turn, to repetitive activity (Hassall, 1982). Repetitive discharges could be induced in treated axons by a single stimulus and such repetitive activity has been recorded from various parts of nervous system of treated insects e.g. sensory neurons, labella hair receptors and motor neurons of the house fly, neurosecretory, peripheral and central nervous system of intact cockroaches (cited by Corbett et al., 1984). The treated insects rapidly become hypersensitive to external stimuli and develop tremors of the body and appendages. After a period of violent motion they fall on their backs and the continuous leg movement eventually becomes more spasmodic, being succeed finally by paralysis. In this respect DDT resembles the pyrethroids, but the kinetics of closing of the insecticide-modified channels are different. DDT can also affect the activity of ATPase (Matsumura, 1975).

2.14.2 Mode of action of organophosphate insecticides on AChE activity

Most of the insecticides in current use act by interfering with the passage of impulse in the insect nervous system. Insects depend, like mammals, on an integrated nervous system which enables external stimuli to be translated into effective action (Fig. 2.23). A number of different chemicals have been implicated in transmission synapse including acetylcholine. Acetylcholine is the transmitter at synapses in the central nervous system in insects. In order for the nervous system to operate properly it is necessary that, once the appropriate message has been passed, excess acetylcholine should be removed from the synapse, both to prevent repetitive firing and to allow a succeeding message to be transmitted. This removal is effected by the enzyme acetylcholinesterase, which catalyses hydrolysis of the ester bond. Organophosphate and carbamate insecticides inhibit the esterase. The result of this inhibition is that acetylcholine accumulates in the synapses so that nerve function is impaired. This leads ultimately to the death of the insect. Enzyme kinetics analysis has identified insensitive forms of acetylcholinesterase in insecticide resistant strains, often apparently involving just one mutant form of the enzyme (Hemingway et al., 1986), or as in the housefly, a family of alleles each conferring a distinct pattern of sensitivity (Devonshire, 1987). According to a review by Devonshire et al. (1992) *An.stephensi* is a mosquito species not yet reported to develop insensitive acetylcholinesterase.

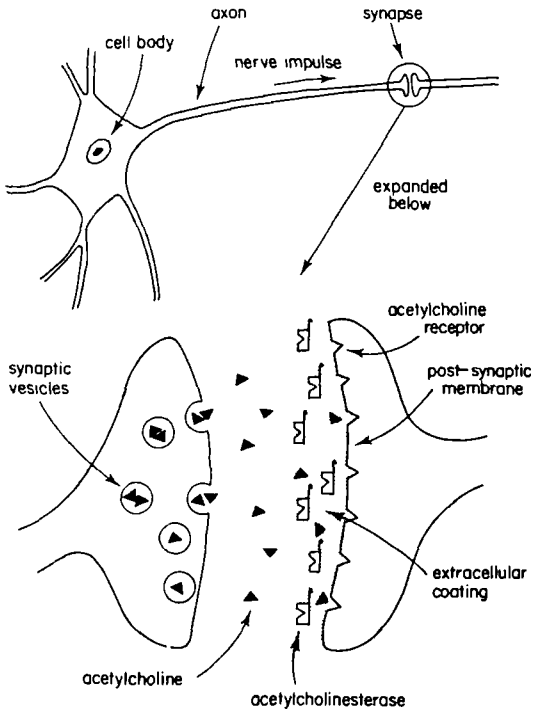


Fig. 2.23 Diagram of a nerve cell and a cholinergic synapse (from Corbett et al., 1984)

2.14.3 Mode of action of pyrethroids on the nervous system

Neurotoxicity of pyrethroids has been attributed to their activity on the nervous system. Ion channels are the primary target sites for several classes of natural and synthetic pyrethroids (Bloomquist, 1996). The pyrethroids appear to be acting at virtually every part of the insect nervous system: on sensory neurons (Roeder & Weiant, 1946) on interneurons (Narahashi, 1971b) on motor neurons (Yeager & Munson, 1945 a,b) and

on neurosecretion (Singh & Orchard, 1983). Through *in vivo* and *in vitro* assays, several enzymes and cellular processes have been proposed as targets of pyrethroid modification: synaptic neurotransmitter release, voltage-dependent sodium channels, potassium channels, calcium channels, calmodulin, peripheral benzodiazepine receptors, ATPase, nicotinic acetylcholine receptors, Na^+/Ca^{2+} exchangers, receptors for gamma-aminobutyric acid (cited by Rossignol, 1991; review of Bloomquist, 1996), ACh-receptor complex (Kiss & Osipenko, 1991) and release of neurohormones (Singh & Orchard, 1983).

There are a number different ideas concerning the action of pyrethroids that need clarification: Knock-down and kill; peripheral and central action; type I versus type II. The symptoms of poisoning with type I compounds like permethrin on *Periplaneta americana* are restlessness, incoordination, hyperactivity, prostration, and paralysis. Type II compounds such as cypermethrin, deltamethrin and fenvalerate (α-cyano groups) are said to induce repetitive firing but they cause a pronounced convulsion phase (Gammon et al., 1981). Recent neurophysiological studies on *Heliothis virescens* (McCaffery et al., 1995) using cypermethrin led to consistent, spontaneous repetitive nerve firing followed by nerve block suggesting that pyrethroid classification into type I and type II compounds may not always be appropriate for all experimental situations. At increased concentrations, type I pyrethroids produce effects on axons similar to those of type II compounds (see Bloomquist, 1996). It should be emphasized that pyrethroid action depends on the compound, the species, and the particular nerve preparation used. Peripheral actions are action on the peripheral nervous system which in insects consists

of sensory neurons and their axons, motor neurons and their terminals, and all neurosecretory axons and neurohaemal organs that lie outside of the ventral nerve cord and paired ganglia. The central nervous system (CNS) is considered to be the ganglia, connectives, and commissures from the brain to the terminal abdominal ganglia.

2.14.3.1 Effect on voltage-dependent sodium channel

The voltage-sensitive sodium channel is the major target site for DDT and pyrethroids. Recently, neurotoxic proteins from arthropod venoms, some of which specially attack the sodium channel, have been engineered into viruses to act as biopesticides (review of Bloomquist, 1996). The sodium channel is common to all insect nervous cells. Control of sodium permeability is so vital to nerve function that any long-term disruption here would have drastic consequences for the insect.

The basic cause for the neurotoxic action of pyrethroids is the alteration of the properties of the sodium channel. DDT and all pyrethroids affect the sodium channel in such a way that the channels close slowly after each action potential. Roeder & Weiant (1946) first observed that DDT caused repetitive firing of sensory cells of the cockroach. Since then, repetitive firing has been observed in nearly every type of nerve structure treated with DDT, DDT analogues, pyrethrins and type I pyrethroids. Narahashi (1971a) has made significant contributions to the elucidation of the mode of action of pyrethroids. Electrophysiological techniques were employed to find mechanisms of action of the nervous system of the cockroach, crayfish and squid. The nerve excitation occurs as a result of changes in nerve membrane permeabilities to sodium and potassium ions, and

any effect of pyrethroids can be interpreted in terms of such permeabilities. The pyrethroids first delay the closing of sodium channels (see Fig. 2.20). Essentially, modification of the open sodium channels during transmission of a nerve impulse results in an overall disruption in nervous transmission (see Fig. 2.24). Sodium current prolongation, acute lethality, and signs of intoxication are closely correlated in the action of pyrethroids (Bloomquist, 1996). Repetitive discharge has been recorded from the nervous system of many insects treated with pyrethroids, e.g. from cockroach (Gammon, 1978), *Musca domestica* (Adams & Miller, 1979), *Rhodnius prolixus* (Orchard & Osborne, 1979), *Carausius morosus* (Orchard, 1980) and *Schistocerca gregaria* (Clements & May, 1977). The nature of the response of nervous tissue to pyrethroids depends on properties of the neuron the structure of the insecticide, experimental conditions (methods of application) and temperature.

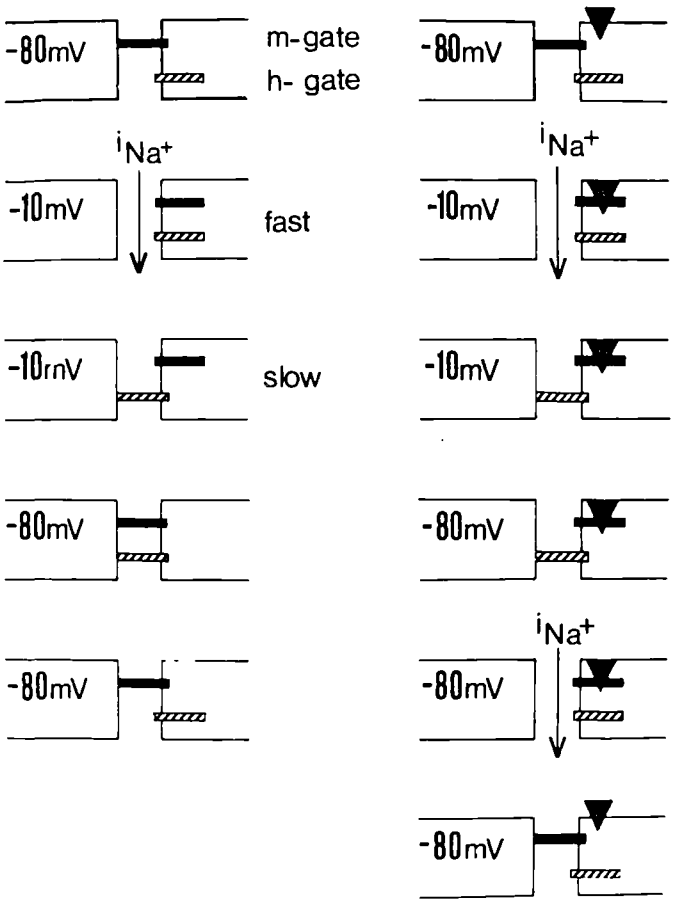


Fig. 2.24 Schematic representation of sodium channel gating in a normal node (left hand column), and in a node treated with allethrin or DDT (right hand column). Action of the insecticide indicated by arrowheads (from Van den Bercken, & Vijverberg, 1979)

2.14.3.2 Effect on potassium channel

In addition to the sodium channel, there may be other membrane components directly affected by pyrethroids. Narahashi (1976) showed that internal perfusion of allethrin in squid axons suppressed the potassium current. The toxicological significance of this effect on potassium channels has not been studied, as it is generally too small to be of importance in itself. However, when combined with the tendency of pyrethroids to keep the sodium channel open, suppression of the potassium current could enhance the ability of some pyrethroids to induce negative after-potential, repetitive firing, and nerve depolarisation (Miller & Salgado, 1985).

2.14.3.3 Effect on ATPase

Work of Clark (1981) and Clark & Matsumura (1982) on the effects of pyrethroids on the nerve system of the squid and *Periplaneta americana* revealed that pyrethrins and allethrin primarily inhibit Ca^{2+}-ATPase activity whereas cypermethrin and deltamethrin mainly inhibit $Ca^{2+}+Mg^{2+}$-ATPase. Permethrin was found to have an intermediate property in terms of its inhibitory potency to both Ca^{2+}-ATPase and $Ca^{2+}+Mg^{2+}$-ATPase activities. The toxicological significance of ATPase inhibition by pyrethroids is wholly unknown. The Ca^{2+}, Mg^{2+}-ATPase are thought to be involved in sequestering calcium, so their inhibition should increase intracellular calcium. It was suggested by Clark & Matsumura (1982) that this could account for the increase of spontaneous transmitter release by pyrethroids, but Salgado et al. (1983a) showed that the increase of spontaneous transmitter release by pyrethroids was due to depolarisation of the nerve

terminals by pyrethroid-induced sodium influx.

2.14.3.4 Effect on GABA-gated chloride channel

The gamma-aminobutyric acid (GABA)-gated channel is the proposed target in insects and mammals for several types of commercial and experimental insecticides. Gammon & Casida (1983) reported that insecticidal isomers of the type II pyrethroids, deltamethrin, cypermethrin, and fenvalerate blocked the GABA-activated conductance in crayfish muscles, while permethin, resmethrin and S-bioallethrin had no effect. GABA receptors are much less sensitive to pyrethroids than the sodium channels suggesting that their contribution to pyrethroid poisoning is normally of secondary importance (Charlmers et al., 1987). A more recent study on *Ae.aegypti* and *Drosophila melanogaster* demonstrated that cyclodiene insecticide resistance is associated with change in the GABA receptor/chloride ion channel (Ffrench-Constant et al., 1994). A recent review of Bloomquist (1996) stated that endosulfan like pyrethroids block the GABA-gated chloride channel.

2.15 Reports of nerve insensitivity in pyrethroid resistance

A review by Catteral (1988) showed that in *Drosophila melanogaster* and the rat there is more than one type of sodium channel gene. Mutants of the *para* gene in *D.melanogaster* show different sensitivities to both the lethal and knockdown effects of pyrethroids. Electrophysiological studies have shown that pyrethroids can trigger membrane depolarization by modifying only a small number of the total population of

voltage dependent sodium channels. It is likely that even a small reduction in the number of modified open channels confers resistance. In support of this conclusion, in *D.melanogaster* the *nap*^{ts} mutant shows marked knockdown resistance to pyrethroids and is associated with a 40% reduction in sodium channel density (Jackson et al., 1984). A reduction in number of channels would reduce rate of contact which in turn would lead to a delay in the onset of toxicity. In the field, selective advantage would be conferred on an individual that could delay onset of toxicity until the pyrethroid was metabolized by constitutive esterases or monooxygenases or perhaps such a delay would allow induction of metabolizing enzymes. Grubs et al. (1988) have established that the channel density in the sensitive and various *kdr* resistant strains of house flies, are the same but *kdr* may be conferred by alterations in the sequence and structure of a sodium channel protein that reduces its affinity for pyrethroids. Chialiang & Devonshire (1982) found that changes in membrane phospholipids of *kdr*-resistant house flies may plays a role in pyrethroid resistance.

The nature of target site or knock-down resistance to pyrethroids was studied by investigating specific binding of ^{14}C-permethrin to the membrane receptors from the heads of susceptible and resistant strains of the house fly (Chang & Plapp, 1983). *In vivo* studies showed that binding was much reduced in *kdr* flies in comparison with a susceptible strain. The nature of the target site insensitivity may relate to their having a reduced number of receptors for the insecticides. Work of Saldago et al. (1983a) demonstrated that the *kdr* gene in *kdr*-resistant house flies produced a modified sodium channel which was less sensitive to the action of pyrethroids. Dong & Scott (1991)

showed that there was no significant difference in the affinity or the number of binding sites for pyrethroids between susceptible and *kdr*-type resistant *Blattella germanica*. Their results suggest that the sodium channels in strains with the *kdr*-type of resistance are qualitatively different from those of susceptible strains. Decreased affinity of the voltage-gated sodium channel to deltamethrin has been reported in the housefly (Guo-Lei et al., 1992). The neurotoxicity of pyrethroids to knock-down resistant houseflies was less than to the wild type (Rossignol, 1988). The author demonstrated that a reduction in the number of sodium channels is a mechanism of resistance. Studies of the specific binding of [^{3}H] saxitoxin (a specific blocker of the voltage-sensitive sodium channel which has been used to measure the number of binding sites of sodium channels in nerve membrane) by brain membrane receptors of house flies showed a significant difference in the sodium channel between resistant and susceptible strains (Bull, 1992). The effect of type I and type II pyrethroids on susceptible *kdr* and super-*kdr* strains of house fly larvae revealed that *kdr* resistance comprises at least two site-insensitive areas within the nervous system. One involves insensitivity of the Na^+ channel which had similar efficacy in both *kdr* and super-*kdr* strains. The other is associated with the presynaptic terminals and is particularly effective in super-*kdr* resistance against type II pyrethroids. The latter could be associated with Ca^{2+} activated phosphorylation of proteins involved with neurotransmitter release (Pepper & Osborne, 1993). The different mechanisms of pyrethroid resistance in the nervous system of various arthropods are summarized in Table 2.3.

Table 2.3 Mechanism/s of pyrethroid resistance in nervous system reported in different arthropods

Species	Kind of resistance	References
Drosophila. melanogaster	Different type of sodium channel	Catteral, 1988
D.melanogaster	Reduction in sodium channel density	Jackson et al., 1984
M.domestica	Reduction in sodium channel density	Rossignol, 1988
M.domestica	Reduced affinity of sodium channel	Grubs et al., 1988
Blattella germanica	Qualitatively different sodium channel	Dong & Scott, 1991
M.domestica	Structural alteration of sodium channel	Soderlund et al., 1989
M.domestica	Modified sodium channel	Salgado et al., 1983
M.domestica	Reduced affinity of sodium channel	Guo-Lei et al., 1992
M.domestica	Decrease in sodium channel density	Bull, 1992
M.domestica	Changes in nerve membrane phospholipids	Chialiang & Devonshire, 1982
M.domestica	Change in sodium channel and Ca^{2+}-activated phosphorylation	Peppre & Osborne, 1993
M.domestica	Reduced binding	Chang & Plapp, 1983

2.16 Knock down and kill

The term "knock down" as applied in entomology denotes paralysis in insects, whether reversible or not. Non-recovery from knock-down is given a separate name, kill. The word "knock-down" was originally introduced to describe the rather quick induction of paralytic symptoms produced by pyrethrins, but its usage was eventually extended to all cases of insects paralysis by chemicals. It is most often used in connection with the time required for the onset of paralytic symptoms in a certain fraction of the test population (eg., KT_{50}, KT_{90}, etc.). Recovery from knock-down has been observed in a number of insect species. It is not been clarified whether recovery from knock-down is merely a matter of metabolism of the insecticide to a non-lethal level, or whether those poisoning processes leading to knock-down are the same as those that eventually lead to kill (Miller & Salgado, 1985). Speed and duration of knock-down, and the possibility and extent of recovery from it, are important considerations in pest control.

2.17 Behaviour changes

The irritant property of some insecticides can cause a proportion of insects to leave sprayed surfaces before acquiring a lethal dose, so that repeated contact is required before mortality occurs. The first observations of irritability by DDT were described as: "restlessness" (Buxton, 1945), it caused the insect to fly (Gahan & Lindquist, 1945). The increased activity of mosquitoes, caused by the insecticides is termed "irritability" (WHO, 1960). The disturbance of resting mosquitoes is the most obvious result of irritation; the term repellency (more often excito-repellency) is sometimes related to this phenomenon. Repellency is the stimulation by a chemical of oriented movements away from the source, or the prevention of the insect from approaching the insecticide. Both definitions imply that repellency is not only brought about by the contact of mosquitoes with an insecticide, but also by the fumigant action of the insecticide. It seems that direct contact with the irritant insecticides is generally required to cause the mosquitoes to leave the house, but Mattingly (1962) showed that irritability of the insecticide also depends on the solvent used while kerosene as a solvent causes a long distance repellency by the insecticide DDT. This irritability would produce heightened activity in the landing mosquito and will only remain on the treated surface for a short period of time.

Africa has been a continual source for reports of behavioural avoidance of DDT residues. In some cases, these behavioural response of vectors were interpreted to have a negative impact on control efforts (Brown et al., 1976; Muirhead-Thomson, 1947; Wilkinson, 1951). Macdonald (1972) reviewed the behavioural response of *An.quadrimaculatus, An.labranchiae, Ae.aegypti, An.punctulatus, An.farauti, An.sundaicus,*

An.pseudopunctipennis, An.atroparvous, An.sacharovi and the *An.gambiae* complex to DDT. He concluded that detection and definition of response were dependent on adequate methods of measurement. Shalaby (1966) showed that behavioral avoidance was an important type of action of DDT residues in India.

Although classifying resistance into biochemical, physiological and behavioural mechanisms represents a convenient way of viewing resistance, in reality these three groups represent an interrelated spectrum of biological responses. For example, a physiological response such as altered nerve insensitivity is the sum total of a series of biochemical events potentially involving changes in nerve structure. Likewise, a behavioural response is the sum total of a series of physiological and biochemical changes: behaviour may be sedcribed as observable physiology (Sparks et al., 1989). Many resistance studies have demonstrated a negative association between behaviour (measured as irritability) and physiological resistance (for example see Sparks et al., 1989). Georghiou (1972) has pointed out that insects must be physiologically susceptible to a pesticide to evolve stimulus dependent behavioural resistance and that, as physiological resistance evolves, irritability (behavioural resistance) frequently declines. The presence of physiological resistance and of refractory types of behaviour against residual insecticides is related to the properties of the insecticide used and those of the mosquitoes.

Repellency of permethrin-impregnated mosquito nets is either due to a volatile solvent (Lindsay et al., 1989) component of the emulsion or to contact repellency (H, Townson, personal communication, 1995). In a study conducted by Sorokin & Zharov (1992), a

high irritability response was observed with bendiocarb-impregnated paper in a population of *Cx.pipiens* collected from central Moscow, Russia. The authors concluded that the irritability was probably caused by the citric acid present on the paper.

The behavioural response of *An.gambiae* and *Cx.quinquefasciatus* to different insecticide-treated netting was assessed by Miller & Gibson (1994). The mixture of pirimiphos-methyl and permethrin was less irritating than permethrin alone. Lambdacyhalothrin was less irritating than permethrin and pirimiphos-methyl at 400 mg/m^2 was less irritating than all other treatments tested. The repellency effect of permethrin-impregnated nets was also reported on *An.albimanus* and *An.vestitipennis* from Guatemala (Richards et al., 1994). Biochemical/physiological resistance mechanisms to pesticides have been widely studied and are well understood and recognized. Conversely, behavioural mechanisms are usually undetectable with most bioassays used to monitor resistance. For example, topical bioassays are methodologically incapable to detecting differences in behaviour. It is also possible that a behavioural mechanism may not function outside the field (Sparks et al., 1989).

Behavioural resistance has been reported in both the presence and absence of physiological and biochemical resistance (Sparks et al., 1989). For example horn fly, *H.irritans*, developed both behavioural and physiological resistance to various pyrethroids soon after large scale control programmes with permethrin and fenvalerate were initiated (Lockwood et al., 1985). Behavioural and physiological resistance of horn flies to pyrethroids has severely hampered control efforts using pyrethroid-impregnated cattle ear tags in the USA (Spark et al., 1989).

Insecticide repellency could prevent vectors from entering human habitations treated with the insecticide. The behavioral responses may greatly reduce human-vector contact, which may be accompanied by sustained and reduced malaria transmission (Davidson, 1958; Ribbands, 1947; Smith & Webley, 1968). Specimens of *An.stephensi* were rapidly agitated by contact with the pyrethroid compound phthalthrin. This observation suggested that in the field, the numbers of mosquitoes leaving houses treated with synthetic pyrethroids and their subsequent survival rates would be high (Hadaway et al., 1970). In the long run this is likely to cause reduction in endophilic mosquitoes and an increase in the exophilic population. Cases of diseases such as malaria may fall, but the vector numbers may remain the same or even increase, a condition termed man-made anophelism-without malaria (Mattingly, 1962).

Studies on behavioral resistance are particularly critical for the use of pyrethroids in impregnated bednet applications. Indeed, the repellency response of malaria vectors to pyrethroid impregnated bednets may reduce malaria infection while simultaneously minimizing selective pressure for physiological resistance.

Pyrethoids which have low vapor pressure [($3.4x10^{-7}$mm/Hg for permethrin and $1.5x10^{-8}$ mm/Hg for deltamethrin) may repel insects due to air-borne repellency (dust) or contact, which raises the possibility that behavioural response might be important attributes of pyrethroids use. Bed nets and window screens have been used for decades to protect against mosquitoes. The efficacy of pyrethroid-impregnated bednets has been field-tested in Africa, Asia, the Western Pacific, and the Americas (Roberts & Andre, 1994). Widespread use of nets incrementally increases the selection pressure for resistance

among females mosquitoes attracted to occupied bed nets. Careful monitoring of both physiological and behavioural responses to pyrethroids will be essential in evaluating the merits of initiating or continuing large-scale, impregnated net programmes (Curtis, 1991; Roberts & Andre, 1994).

2.18 Excretion

Increased excretion is one of the mechanisms of resistance developed by insects. Abedi & Brown (1961) showed that larvae of a resistant strain of *Aedes aegypti* respond to DDT by excreting the insecticide into the perithrophic membrane, the production of which was sometimes increased so much that it protruded from the anus. This behaviour was more evident in some resistant strains than others and occurred to a much lesser extent in larvae of susceptible strains. It appeared to constitute a resistance mechanism for removing DDT from the alimentary canal and denying it to the body.

2.19 Mechanisms of pyrethroid resistance in arthropods

2.19.1 Mechanisms of pyrethroid resistance in *Musca domestica*

Pyrethroid-resistance in the house fly, *Musca domestica,* is widespread and the resistance in this species has been extensively studied by different authors (see Table 2.4) and the range of possible pyrethroid resistance mechanisms is well defined. These mechanisms are principally oxidation and hydrolysis by the MFO and esterases, as well as *kdr*-type insensitivity and reduced penetration. The evidence from their results supports the conclusion that several resistance factors including MFO, esterases and decreased sensitivity of the nervous system are the principal mechanisms of resistance in this species.

Table 2.4 Cases of pyrethroid resistance reported in the house fly (*Musca domestica*)

Origin	Insecticide	Mechanism/s of resistance	References
-	pyrethroids	nerve-insensitivity	Elliot et al., 1978
UK	permethrin	oxidative	Nicholson & Sawicki, 1982
-	permethrin	oxidative & hydrolysis	Shono et al., 1978
-	pyrethroids	reduced penetration, *kdr*, metabolic	Farnham (1973)
UK	natural pyrethrins, pyrethroids	delayed penetration, oxidation, *kdr*	Farnham (1971)
UK	pyrethoids	-	Farnham (1977)
UK	pyrethroids	*kdr*, oxidative	Sawicki et al., 1986
USA	permethrin	MFO, delayed penetration, *kdr*	Scott & Georghiou, 1986b
UK	pyrethroids	-	Farnham & Sawicki , 1976)

Table 2.4 (continued)

Origin	Insecticide	Mechanism/s of resistance	References
USA	deltamethrin	MFO, *kdr*, reduced penetration	Wheelock & Scott, 1992
Denmark	pyrethroids	-	Keiding (1976)
USA	permethrin	metabolic & non-metabolic, reduce penetration	DeVries & Georghiou, 1981a,b
Canada	permethrin	-	MacDonald et al., 1983
UK	pyrethroids	nerve-insensitivity	Pepper & Osborne, 1993

2.19.2 Mechanisms of pyrethroid resistance in *Aedes aegypti*

Penetration, metabolism and excretion of [^{3}H] *trans*-permethrin were studied in permethrin-resistant and susceptible strains of *Aedes aegypti* by Brealey et al. (1984). They found that there was no significant differences between resistant and susceptible strains in penetration and metabolism. It is concluded that permethrin-resistance is of the *kdr*-type. Different strains of *Ae.aegypti* from South America, resistant to DDT at the adult stage were examined in response to pyrethroids, and one strain showed 30-fold cross-resistance to permethrin. The results suggested that the pyrethroid resistance is due to the MFO system and the authors postulated that this mechanism interacts with another unknown mechanism (Prasittisuk & Busvine, 1977). In a study conducted by Mourya et al. (1993), adults of DDT and deltamethrin-resistant strains of *Ae.aegypti* were tested for glutathione S-transferases and glucose-6-phosphate dehydrogenase activities. The results did not show any significant differences in characteristics of these enzymes, suggesting a *kdr*-type nerve insensitivity in resistance. Comparison of the susceptibility of several Asian strains of adults of *Ae.aegypti* were made by Chadwick et al. (1977). Mosquitoes from Bangkok showed resistance to DDT and pyrethroids. Resistance in this strain was postulated to be due to oxidases, esterases and perhaps other resistance mechanisms. Kumar et al. (1991) found that deltamethrin resistance in larvae of *Ae.aegypti* was mainly due to MFO. The same mechanism of resistance was reported in DDT/pyrethroid resistant larvae of *Ae.aegypti* (Rongsriyam & Busvine, 1975). A summary of cases of pyrethroid resistance in *Ae.aegypti* is shown in Table 2.5

Table 2.5 Cases of pyrethroid resistance reported in *Aedes aegypti*

Origin	Insecticide	mechanism/s of resistance	References
-	*trans*-permethrin	*kdr*	Brealy et al., 1984
Trinidad,Puerto Rico	DDT/deltamethrin	*Kdr*	Mourya et al., 1993
Bangkok	permethrin	-	Malcolm & Wood, 1982
-	pyrethroids	MFO	Rongsriyam & Busvine, 1975
Tahiti	permethrin	-	Faicloux et al., 1994
India	deltamethrin	MFO	Kumar et al., 1991
Bangkok	pyrethroids	multiple factors	Chadwick et al., 1977
South America	pyrethroid	MFO	Prasittisuk & Busvine, 1977

2.19.3 Mechanisms of pyrethroid resistance in *Culex quinquefasciatus*

Priester & Georghiou (1978) found >4000-fold resistance to d-*trans* permethrin and 1021-fold to d-*cis* permethrin in larvae of *Culex quinquefasciatus*. The magnitude of resistance was partly due to the MFO and esterase inhibitors and penetration was not involved. They suggested that reduced sensitivity of the target site may be the primary source of resistance. Their succeeding investigations led to conclusion that resistance extended to all pyrethroids tested and it was due to *kdr*. The involvement of a *kdr*-like factor has been implicated in this strain from the strong correlation of DDT and pyrethroid resistance (Priester & Georghiou, 1980a,b). In contrast, Amin & Hemingway (1989) found that resistance to permethrin was mainly oxidative and there was no cross-resistance to lambdacyhalothrin. A summary of cases and mechanisms of pyrethroid resistance in *Cx.quinquefasciatus* is shown in Table 2.6.

Table 2.6 Cases of pyrethroid resistance reported in *Culex quinquefasciatus*

Origin	Insecticide	mechanism/s of resistance	References
USA	permethrin	*kdr*, oxidative , hydrolysis	Priester & Georghiou, (1978, 1979, 1980b)
USA	pyrethoids	*kdr*, oxidative, hydrolysis	Priester & Georghiou, 1980a
India	deltamethrin	MFO	Kumar et al., 1991
Saudi Arabia	permethrin	oxidative	Amin & Hemingway, 1989
China	deltamethrin	-	Kang et al., 1995
Cuba	pyrethroids	-	Bisset et al., 1991

2.19.4 Mechanisms of pyrethroid resistance in anopheline mosquitoes

Reports of resistance to pyrethroids in anophelines reviewed by Malcolm (1988a) and WHO, (1992a) include *An.albimanus* in South America, *An.arabiensis* in Sudan, *An.culicifacies* in Sri Lanka, *An.gambiae* in Nigeria and Burkina Faso, *An.multicolor* in Egypt, *An.nigerrimus* in Sri Lanka, *An.pseudopunctipennis* in Guatemala, *An.quadrimaculatus* in USA and *An.sacharovi* in Turkey and Syria. Of these only *An.gambiae* from Burkina Faso showed comparatively high tolerance (5.6-fold) to bioallethrin and the tolerance was reduced by piperonyl butoxide. There are some reports of pyrethroid resistance in the field in *An.albimanus* which is resistant to deltamethrin in Guatemala and Mexico and *An.sacharovi* resistant to permethrin in Syria. Pyrethroid resistance in *An.sacharovi* has also been reported following two rounds of winter fogging with a mixture of permethrin, bioallethrin and piperonyl butoxide in Turkey (Clark, 1985 quoted in WHO, 1992a). *An.albimanus* which was resistant to organophosphates, carbamates and DDT were found to possess a low level of cross-resistance to some of the pyrethroids (Priester et al., 1981). DDT/pyrethroid-resistance has been shown in *An.gambiae* (Prasittisuk & Curtis, 1982). Recently Vulule et al. (1994) have shown that, after only a year of village scale use of permethrin impregnated bednets, resistance has started to occur in *An.gambiae sensu lato*. Rongsriyam & Busvine (1975) reported that DDT/pyrethroid resistance in *An.gambiae* is mainly due to MFO. In contrast, in a laboratory study by Hemingway (1981) it has been shown that nerve insensitivity is the mechanism of DDT/permethrin resistance in *An.gambiae* from Nigeria. The same mechanism (*kdr*) has been postulated for *An.sacharovi* in Turkey by Herath (1979). Brogdon & Barber (1990) reported that an elevated esterase mechanism in Guatemalan *An.albimanus*

confers cross-resistance between organophosphate insecticides, fenitrothion, and the synthetic pyrethroid, deltamethrin.

The cases of pyrethroid resistance in anopheline mosquitoes have been shown in Table 2.7. The mechanisms of pyrethroid resistance in *An.stephensi* has previously been discussed in more detail in section 2.3.

Table 2.7 Cases of pyrethroid resistance reported in species of *Anopheles* mosquito

Species	Origin	Insecticide	Mechanism/s of resistance	References
An.gambiae	Nigeria	DDT/permethrin	nerve-insensitivity	Hemingway (1981)
An.sacharovi	Turkey	DDT/pyrethroids	nerve-insensitivity	Herath (1979)
An.albimanus	USA	pyrethroids	-	Priester et al. (1981)
An.albimanus	Guatemala	deltamethrin	esterases	Brogdon & Barber (1990)
An.gambiae	-	pyrethroids	MFO	Rongsriyam & Busvine (1975)
An.quadrimaculatus	-	pyrethroids	-	Prasittisuk & Busvine (1977)
An.arabiensis	Sudan	permethrin	-	Hemingway (1981)
An.albimanus	Guatemala, Mexico	pyrethroids	-	Shidrowi (1985)
An.gambiae	Kenya	permethrin	-	Vulule et al. (1994, 1996)
An.culicifacies	Sri Lanka	pyrethroids	-	WHO (1992a)

Table 2.7 (continued)

Species	Origin	Insecticide	Mechanism/s of resistance	References
An.nigerrimus	Sri Lanka	pyrethroids	-	WHO (1992a)
An.pseudopunctipennis	Guatemala	pyrethroids	-	WHO (1992a)
An.quadrimaculatus	USA	pyrethroids	-	WHO (1992a)
An.sacharovi	Turkey	pyrethroids	-	WHO (1992a)

2.19.5 Mechanisms of pyrethroid resistance in *Haematobia irritans*

Pyrethroid-impregnated cattle ear tags were introduced for the control of the horn fly, *Haematobia irritans,* in 1980 in USA. Resistance to pyrethroids appeared due to intense and constant exposure to pyrethroids via pyrethroid ear tags. The horn flies are resistant not only the pyrethroids used in the field (primarily permethrin and fenvalerate), but also to virtually every other pyrethroid. The reports of pyrethroid resistance in this species are summarized in Table. 2.8. In contrast, these horn flies possess little or negative cross-resistance to organophosphate or carbamate insecticides. Sheppard & Joyce (1992) reported that MFO play an important role in pyrethroid-resistance at the larval stage. Based on studies of Bull et al. (1988); Byford et al. (1985), Byford et al. (1987a,b) and Sparks et al. (1989) the mechanisms of pyrethroid resistance in adults of *H.irritans* have been associated with various combinations of altered penetration, *kdr*, enhanced metabolism via MFO and esterases, and altered behaviour. It is apparent that pyrethroid resistance in this insect is a multifactorial phenomenon involving biochemical, physiological, and behavioural factors.

Table 2.8 Cases of pyrethroid resistance reported in *Haematobia irritans*

Origin	Insecticide	Mechanism/s of resistance	References
USA	pyrethroids	*kdr*, MFO, esterases, behavioural resistance	Bull et al., 1988; Byford et al., 1985; Byford et al., 1987a,b; Sparks et al., 1989
USA	pyrethroids	MFO	Sheppard & Joyce, 1992
USA	permethrin	-	Kunz (1991)
USA	permethrin	target site insensitivity	Crosby et al., 1991
USA	pyrethroids	-	Cilek et al., (1991, 1995); Cilek & Knapp, 1993a,b

2.19.6 Mechanisms of pyrethroid resistance in *Blattella germanica*

Research on mechanisms of pyrethroid resistance in the German cockroach, *Blattella germanica,* has been limited to a few resistant strains, and results vary between strains. Scott & Matsumura (1983) discovered that nerve insensitivity was the only apparent mechanism of cross-resistance to pyrethroids in a DDT-resistant strain of German cockroach. PB and DEF had no effect on resistance and there was no significant difference in the *in vitro* metabolism of permethrin between susceptible and resistant strains. Scott et al. (1990) and Dong & Scott (1991) also concluded that in a pyrethroid-resistant strain *kdr* was the primary mechanism, with no significant metabolic contributions. Evidence for a metabolic basis for pyrethroid resistance involving both esterases and oxidases has also been reported by Atkinson et al. (1991) in one strain of *Blattella germanica.* On the basis of partial synergism they suggested that a nerve insensitivity type mechanism may also be involved. In a similar study by Hemingway et al. (1993) they tested different pyrethroids against thirty strains of *B.germanica* from three different continents. The elevated esterases and oxidase-based mechanisms were the most prevalent resistance mechanisms and *kdr* played a minor role in the resistance profile. The results of other authors on the mechanisms of pyrethroid resistance in *B.germanica* have been summarized in Table 2.9.

Table 2.9 Cases of pyrethroid resistance reported in German cockroach (*Blattella germanica*)

Origin	Insecticide	mechanism/s of resistance	References
USA	pyrethroids	metabolic & non-metabolic	Cochran (1994)
USA, Dubai, Denmark	pyrethroids	MFO, esterases, *kdr*	Hemingway et al., 1993
USA	pyrethroids	-	Cochran (1987, 1989, 1991, 1993)
USA	pyrethroids	-	Scharf et al., 1995
USA	cypermethrin	Physiological & behavioural resistance	Hostetler & Brenner, 1994
USA	pyrethroids	*kdr*	Scott et al., 1990
USA	pyrethroids	*kdr*	Dong & Scott, 1991
USA	pyrethroids	MFO, esterases, target site insensitivity	Atkinson et al., 1991
USA	pyrethroids	MFO, esterases, *kdr*, reduced penetration	Anspaugh et al., 1994

2.19.7 Mechanisms of pyrethroid resistance in veterinary and agricultural pests

Over 90% of all insecticides produced have been used for agricultural purposes, particularly for rice and cotton (Roberts & Andre, 1994). Over the last decade, control of veterinary and agricultural pests has been achieved primarily by the use of synthetic pyrethroids. A gradual increase in resistance to this class of insecticides in populations of these pests have been reported since their introduction. Mechanisms of resistance to pyrethroids may involve reduced penetration, target site insensitivity, oxidative and hydrolytic pathways (see Tables 2.10 and 2.11).

Table 2.10 Cases of pyrethroid resistance reported in *Heliothis virescens*

Origin	Insecticide	Mechanism/s of resistance	References
USA	pyrethroids	-	Luttrell et al., 1987
USA	pyrethroids	metabolic	Elzen et al., 1992
USA	pyrethroids	-	Plapp (1981)
USA	permethrin	-	Crowder et al., 1979
USA	fenvalerate	*kdr*, metabolic, excretion	Abd-Elghafar et al., 1994
Mexico	pyrethroids	-	Martinez-Carrillo et al., 1991
USA	cypermethrin	excretion, reduced penetration, MFO, esterases	Little et al., 1989

Table 2.11 Cases of pyrethroid resistance reported in veterinary and agricultural pests

Species	Origin	Insecticide	Mechanism/s of resistance	References
Helicoverpa zea	USA	pyrethroids	metabolic and non metabolic	Abd-Elghafar et al., 1993
Bovicola ovis	Australia	pyrethroids	-	Levot et al., 1995
Nilaparvata lugens	Taiwan	permethrin	-	Chung et al., 1982
Helicoverpa armigera	Australia	pyrethroids	MFO	Daly & Fisk., 1992
Helicoverpa armigera	India	cypermethrin	-	Armes et al., 1992
Plutella xylostella	Taiwan	pyrethroids	MFO, *kdr*	Liu et al., 1981
Leptinotarsa decemlineata	USA	permethrin	carboxylesterases, MFO, *kdr*	Lee & Clark, 1993
Tribolium castaneum	UK	pyrethroids	MFO	LIoyd & Ruczkowski, 1980
Spodoptera eridania	USA	permethrin	esterases	Abdel-Aal & Soderlund, 1980

Table 2.11 (continued)

Species	Origin	Insecticide	Mechanism/s of resistance	References
Frankliniella occidentalis	USA	permethrin	MFO	Immanraju et al., 1992
Spodoptera exigua	Guatemala	deltamethrin	delayed penetration, esterases	Delmore et al., 1988
Boophilus microplus	Australia	pyrethroids	*kdr*, reduced penetration, metabolic	Schnitzorling et al., 1983
Boophilus microplus	UK	permethrin	*Kdr*, esterases	Nicholson et al., 1979
Boophilus microplus	Australia	permethrin	carboxylesterases	Riddles et al., 1983
Trichoplusia ni	USA	pyrethroids	esterases	Ishaaya & Casida, 1981
Trichoplusia ni	-	permethrin	hydrolysis	Shono et al., 1978
Spodoptera littoralis	-	permethrin	nerve-insensitivity	Gammon. (1980)
Spodoptera littoralis	Egypt	pyrethroids	-	Riskallah et al., 1983

2.20 Ecology of *An.stephensi*

Knowledge of the ecology of the target species is critical to understanding the impact of mosquito control strategies relying principally on residual indoor spraying (Reisen & Milby, 1986) as well as using impregnated bed nets.

An.stephensi is an important vector of human malaria throughout much of South Asia including the Indo-Pakistan subcontinent extending westward through Iran and Iraq to the Middle-East (Manouchehri et al., 1976).

2.20.1 Larval habitat

An.stephensi breeds in a wide range of both urban and rural habitats throughout its distribution. Larvae can be collected from ponds, pools, stream margins, catch basins and seepage canals. According to Manouchehri et al. (1976) this species breeds readily in rice fields, especially in nurseries and newly-planted rice fields. It is found in water with high salinity, sometimes reaching or even exceeding that of sea water. Sharma et al. (1993) concluded that almost all types of water were suitable for *An.stephensi* breeding.

2.20.2 Seasonal activity and resting behaviour

Ecological studies carried out by Reisen et al. (1981), Reisen & Aslamkhan (1978, 1979), Reisen & Boreham (1979) and Reisen & Milby (1986) in Pakistan indicated that *An.stephensi* rests principally inside houses or cattle sheds, so it may be described as an endophilic mosquito. The overwintering strategy of this species is continued reproductive activity with delayed ovarian maturation. *An.stephensi* remained reproductively active throughout the year. Temporal changes in parity and

survivorship of this species were inversely related to temperature. It was more prevalent during the cooler temperatures of autumn and early winter (Reisen et al., 1981). Older mosquitoes were proportionately more prevalent during spring, late-fall and early winter. It remains gonotrophically active throughout the year with blood-fed, gravid, parous and virgin females during all months of year. Newly emerged adults may rest for a short period near the breeding site before flying to preferred indoor resting sites. Post-teneral adults were rarely collected resting outdoors in agricultural fields or herbaceous vegetation along drains. Most mating presumably occurs near the breeding places, en route or at indoor resting sites, or during the crepuscular egress for feeding (Reisen & Aslamkhan, 1979).

An.stephensi exhibited a marked seasonal shift in feeding times, with most biting occurring at dusk during the cold period and late at night during the warm period (Reisen & Aslamkhan, 1978). There is no evidence of hibernation or aestivation for this species in Iran (Manouchehri et al., 1976).

Temperature related changes in vectorial capacity, associated with seasonal modification of human behaviour, seemed to be responsible for the marked seasonality of malaria cases.

2.20.3 Anthropophilic and zoophilic index

Precipitin tests on specimens of *An.stephensi* from different parts of southern Iran showed that 15.7% were positive for human blood (Manouchehri et al., 1976). According to Reisen & Boreham (1979) this species is largely zoophilic and these authors could detect no human positive feeds in Pakistan.

2.21 Insecticide resistance and resistance management

Insecticide resistance, by definition, is an inherited characteristic that allows an insect to survive a dose of a pesticide that would normally prove fatal. According to WHO (1957) resistance has been defined as " the developed ability in a strain of insects to tolerate doses of toxicant which would prove lethal to the majority of individuals in a normal population of the same species". To ensure a long useful life for insecticides, it is essential to guard against the development of resistance. Resistance management consists of all measures designed to delay or prevent resistance levels rising to those at which the pesticide must be abandoned, while maintaining effective disease control. Management of resistance can help avoid resistance development in vector populations, slow the rate of resistance development, and cause resistant vector to "revert" to a more susceptible level. Tactics for management of resistance in vector populations according to (Curtis et al., 1993), WHO (1992a), Georghiou (1980 a,b) Croft (1990) and Taylor (1986) can include the following countermeasures:

1- Varying the dose or frequency of pesticide application

2- Using local rather than area wide application, i.e. limitation of insecticide use to areas with high levels of disease transmission.

3- Applying treatments locally only when endemic vector-borne diseases are present.

4- Using less persistent pesticides.

5- Treating only certain life stages of the vectors, for example use of methods that kill adult females, instead of both sexes or all stage of the life-cycle.

6- Using pesticide mixtures..

7- Using alternation, pre-planned rotation or sequences.

8- Using improved pesticides formulations.

9- Using synergists.

10- Exploiting unstable resistance (reduced fitness).

11- Avoidance of slow-release formulations.

12- Identify new pesticides with alternate site of activity.

13- Use of non-chemical control methods alone or an additional measure, in seasons or in areas where they are applicable and cost-effective.

Chapter 3

MATERIALS & METHODS

3.1 Materials

3.1.1 Insecticides

The main insecticides we used are:

DDT; 1,1,1-trichloro-2,2-di-(4-chlorophenyl)ethane, technical grade 98%

malathion; S-1, 2-bis (ethoxycarbonyl) ethyl, O, O-dimethyl phosphorodithioate, technical grade 95.5%

permethrin; 3-phenoxybenzyl-(1R)-*cis, trans*-3(2, 2-dichlorovinyl)-2, 2, dimethyl cyclopropanecarboxylate, technical grade 96.2%, density = 1.2 and *cis/trans* ratio 40/60

lambdacyhalothrin; (S)-α-cyano-3-phenoxybenzyl (1R)-*cis*-3-(2-chloro-3,3,3-triflouropropenyl)-2,2-dimethylcyclopropanecarboxylate, technical grade 98.3%

deltamethrin; (S)-α-cyano-3-phenoxybenzyl(1R)-*cis*-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate

cypermethrin; (RS)-α-cyano-3-phenoxybenzyl (1RS)-*cis, trans*-3-(2,2-dichlorovinyl)-2,2-dimethyl-cyclopropanecarboxylate

cyfluthrin; (RS)-α-cyano-4-flouro-3-phenoxybenzyll (1RS)-*cis, trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate

^{14}C-permethrin; provided by Zeneca

3.1.2 Other chemicals and materials

piperonyl butoxide (PB); 5-[2-(2-butoxyethoxy)ethoxymethyl)-6-propyl-1, 3-benzodioxole, technical grade 90%

silicon fluids; (DC 566), Dow corning 556, Ltd

O,O,O, triphenyl phosphate (TPP); technical grade 98%, $(C_6HsO)_3$ P(O)

acetone

absolute ethanol

olive oil; Sigma diagnostic, catalogue No. 0-1500

filter paper Whatman No. 1

menthol; (5-methyl-2-[1-methylethyl] cyclohexanel), Sigma Ltd

sodium phenobarbital; (5-ethyl-5-phenyl-2,4 ,6-trioxohexa-hydropyrimidine) Sigma Ltd

dried leaves of peppermint (provided by the Ministry of Agriculture, Iran)

chlorfenethol (DMC); 1,1-bis(4-chlorophenyl) ethanol

3.2 Methods

3.2.1 Mosquito strains used

The main *An.stephensi* strains used throughout this work are as follows:

1. DUB-S; a wild strain based on larvae, collected from Dubai (U.A.E), and colonised at Liverpool School of Tropical Medicine in May 1986.

2. DUB-LPR; a strain with larval permethrin resistance, selected originally at Liverpool School of Tropical Medicine (LSTM) from a sample collected in Dubai (U.A.E.) and colonised in 1986. Originally partly resistant but since 1992 this strain has been intermittently re-selected for permethrin resistance.

3. DUB-APR; a sub-strain from DUB-S, selected with permethrin at the adult stage in LSTM.

4. IND-S; a strain susceptible to permethrin, and a standard laboratory colony originating from New Delhi in 1947.

5. IRAQ; obtained from London School of Tropical Medicine & Hygiene and maintained at the LSTM. It is susceptible to pyrethroids.

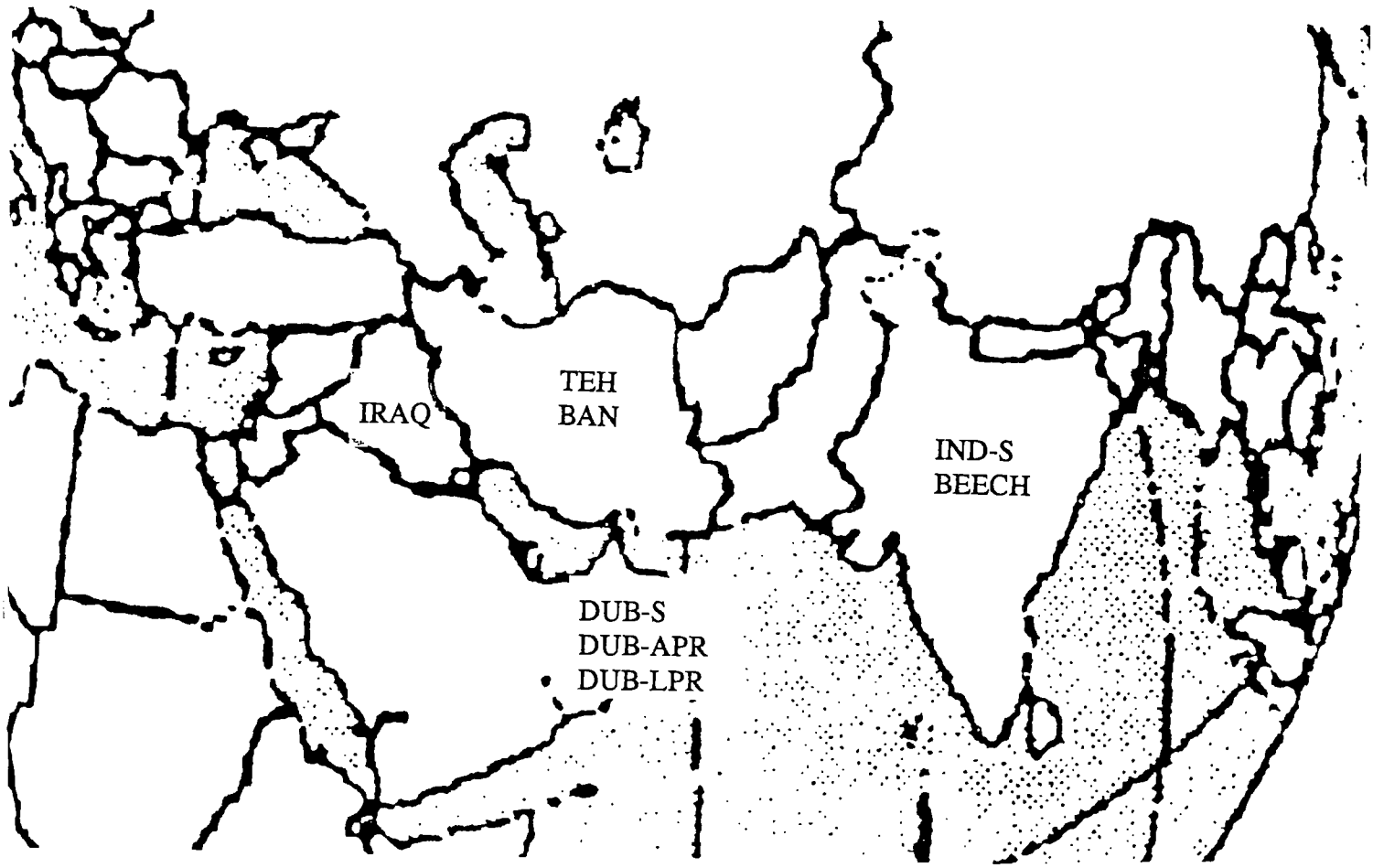
6. TEH; a laboratory strain that is resistant to both DDT and dieldrin at the adult stage and susceptible to all insecticides at the larval stage. This strain has been reared in an insectay for 300 generations. The eggs of this strain collected from Tehran University of Medical Sciences and colonised at the LSTM since November 1992.

7. BAN; a wild strain from Bandar Abbass in Iran. It is resistant to DDT, dieldrin and malathion (Manouchehri et al., 1975) at the adult stage and susceptible to all insecticides at the larval stage. The eggs of this strain were obtained from Tehran University of Medical Sciences and maintained at the LSTM since November 1992.

8. BEECH; a laboratory stock of Indian origin, from Beecham's laboratory.

The origin of these strains of *An.stephensi* are shown in Fig.3.1.

Fig.3.1 Geographical strains of *Anopheles stephensi* were used



3.2.2 Mosquito rearing

Mosquito rearing and maintenance was carried out in an insectary at 27±1°C and 75±5% RH, with a 12 h photoperiod regime. Rearing was carried out in tap water at a temperature of 23-25°C and larvae were fed with Tetramin® fish flakes. The optimum larval density was 200-300 per tray. The amount of food given depended on the number and stage of larvae in the breeding trays. Each day a small portion of Tetramin was spread on the surface of the water in the trays. Application of Tetramin in a powder form was found to be more suitable than the flake form. Adults were maintained in 30x30x30 cm cages. The females were allowed to feed two or three times a week on guinea pigs anaesthetized with 0.75 mg/kg body weight of Hypnorm and then 1.25 mg/kg body weight of diazepam. A pad of cotton wool soaked in water with 2 cubes of sucrose were placed on the top of each cage to serve as a source of carbohydrate.

3.2.3 Impregnation of papers with insecticides

In order to determine the susceptibility level of adults, insecticides impregnated papers were prepared in our laboratory according to the method of WHO (1970) as modified by Chadwick et al. (1977) and Ladonni (1988) (Fig. 3.2). Whatman No. 1 filter paper was used. The papers were cut into rectangles of 12x15 cm. An acetone solution of insecticide was diluted with an equal volume of oil (silicon oil for pyrethroids and olive oil for organochlorine and organophosphate compounds) and then 1.5 ml of this mixture was applied evenly by a pipette on to the paper (Fig. 3.2). The control papers were prepared by applying 1.5 ml of an equal volumes of a mixture of acetone and oil onto filter paper. These papers were stored for 4-5 h in

the dark to allow the oil to spread. Afterwards they were stored in sealed boxes and were put in the refrigerator. The following concentration of insecticides were prepared:

- 10% DDT (w/v)
- 5% malathion (v/v)
- 0.25% = 10 $\mu g/cm^2$ permethrin
- 0.025% = 1 $\mu g/cm^2$ deltamethrin
- 1.22 $\mu g/cm^2$ lambdacyhalothrin

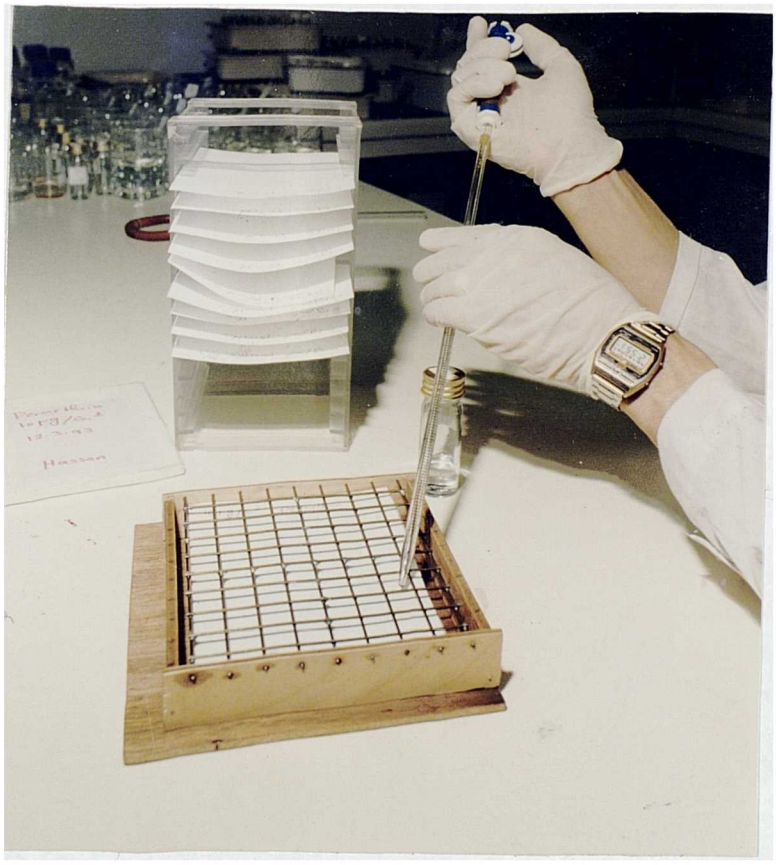


Fig. 3.2 Impregnation of papers with insecticides

3.2.4 Synergists impregnated papers

Piperonyl butoxide, an inhibitor of MFO (Mixed Function Oxidase), was prepared at a concentration of 20% (v/v). TPP, an inhibitor of carboxylesterases, also was prepared at 20% (w/v). DMC was prepared at 1% (v/v).

3.2.5 Insecticide testing method

3.2.5.1 Adults:

Tests on adults were carried out according to the methods of WHO (1970) as shown in Fig. 3.3 . At each exposure time 50-400 mosquitoes representing 2-16 individual replicates of 25 adults were tested. To reduce variability in the replicates, 2-3 day old sugar fed adults were used. Due to the knock-down effect of pyrethroids on the adults, the exposure tubes were held in a horizontal position during tests. The mortality rate was scored after a 24 h recovery period. Insecticide exposure took place in a room with a temperature of 25±1°C and holding tubes were held in a insectary under controlled conditions of 27±1°C and 75±5% relative humidity.

3.2.5.2 Larvae:

Larvae were tested with insecticides at the early 4th instar in a room with a temperature of 25±1°C according to the WHO method (1970) (Fig. 3.4). Mortality counts were made after 24 h exposure to the insecticide. In the analysis both dead and moribund larvae were considered as dead, and the live larvae were scored separately. Only larvae of uniform size were considered during all of the tests. Because of the appearance of delayed mortality in a number of larvae following the exposure to pyrethroids, larvae were transferred to fresh water after 24 h and then hold for a 24

h recovery period. At each concentration 50-200 mosquitoes representing individual replicates of 25 larvae were used.

Fig. 3.3 Insecticide testing method for adults according to WHO (1970) with some modification

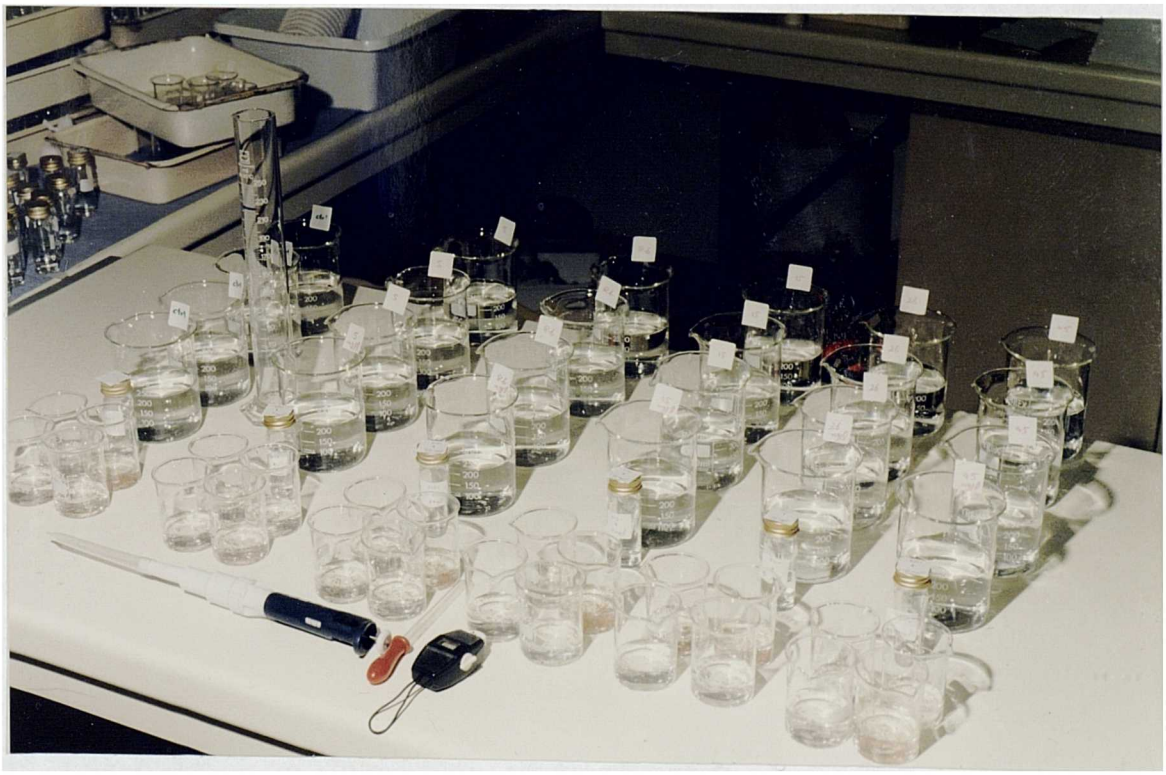


Fig. 3.4 Insecticide testing method for larvae according to WHO (1970)

3.2.6 Synergist testing method

3.2.6.1 Adults:

Adults were pretreated with the synergists piperonyl butoxide (PB), chlorfenethol (DMC), and triphenyl phosphates (TPP) at a sub-lethal dose for 30 minutes before exposure with insecticides. The synergist ratio (SR) was calculated by dividing the LT50 of insecticide alone by the LT50 of the insecticide in the presence of synergist. The resistant factor (RF) is the ratio of the LT50 of each strain compared with that of a susceptible strain.

3.2.6.2 Larvae:

The larvae were pretreated with synergist, PB and TPP at a sub-lethal dose of 5 mg/l and 10 mg/l, respectively for 4 h before exposure to insecticides. The effect of the synergists were assessed by means of synergist ratio (SR) which is the ratio of the LC50 of insecticide alone to the LC50 of the insecticide in the presence of the synergist. A value ≥ 1 indicates synergism, while a value < 1 indicates antagonism.

3.2.7 Pretreatment of mosquito with inducers to test for evidence of insecticide tolerance

3.2.7.1 Adults:

It was found that 10^{-4} molar of sodium phenobarbital was not toxic for adults, hence adult female mosquitoes were given access feed from a cotton wool soaked with water solution of sodium phenobarbital, plus 10% sucrose, plus a blue dye (blue food colouring), to enable a visual check that the compound was ingested (Fig. 3.5), 24 h before exposure to insecticide in a bioassay.

3.2.7.2 Larvae:

10^{-8} molar sodium phenobarbital, 10^{-10} molar menthol, and 250 mg/l peppermint were applied into larval rearing trays, so that 1st, 2nd and 3rd instar of larvae were treated with inducers prior test with insecticides. Standard bioassay were used to determine probit regression lines.

Fig. 3.5 Female mosquitoes were given access to sodium phenobarbital plus a blue dye before tests with insecticide.

3.2.8 Pretreatment of mosquito with sub-lethal dose of insecticides

3.2.8.1 Adults:

Females were treated at the sub-lethal dose of permethrin and after 24 h they were exposed to permethrin at the LT50.

3.2.8.2 Larvae:

The late 3rd and 4th instar larvae were treated with a sub-lethal dose of permethrin for 24 h and followed by insecticide tests (sub-lethal dose was estimated from the probit regression line of each strain, which gives approximately 5-10% mortality).

3.2.9 Statistical methods

Dosage mortality regression lines were determined by the probit analysis method of Finney (1971), using the Probit 79 programme on an IBM computer. Goodness of fit of the points to a straight line were tested by Chi-square (X^2) analysis. Other statistical analysis were determined by Minitab and Glim programme on an IBM computer.

3.2.10 Impregnated nets

Impregnation of netting materials was carried out as described by WHO (1985). Pieces of netting were cut and the area of the net to be treated calculated. The amount of water necessary to saturate but not run off the net was determined by weighing dry pieces and then dipping in water. The excess water was squeezed out gently before weighing again. The difference in the two weights gave the amount of water absorbed by the net. The amount of permethrin needed to treat a net was measured. The pieces

of netting were dipped into prepared emulsion of permethrin in a non-absorbent container, the treatment being carried out with care to cover all surfaces, before the net was partially unfolded on a non-absorbent surface to air dry. Netting pieces with dosages of 500 mg/m^2 of permethrin were prepared.

3.2.11 Knock-down behaviour testing method

The knock-down behaviour of females of DUB-APR and IND-S strains exposed to 500mg/m^2 permethrin impregnated net was determined by using WHO plastic bioassay cones. Each cone was attached to a piece of netting using an elastic band, the hole in the cone was stopped with cotton wool (Fig. 3.6). Five female mosquitoes were released in to each cone. Mosquitoes were left in the cone and the time taken for each mosquito to be knocked-down were recorded. Mean and standard deviation of the first knock-down, the last knock-down and the median knock-down were recorded. For each strain 100 mosquitoes were tested represented in 20 replicates. Knock-down results for each individual female of both resistant and susceptible strains were graphically plotted.

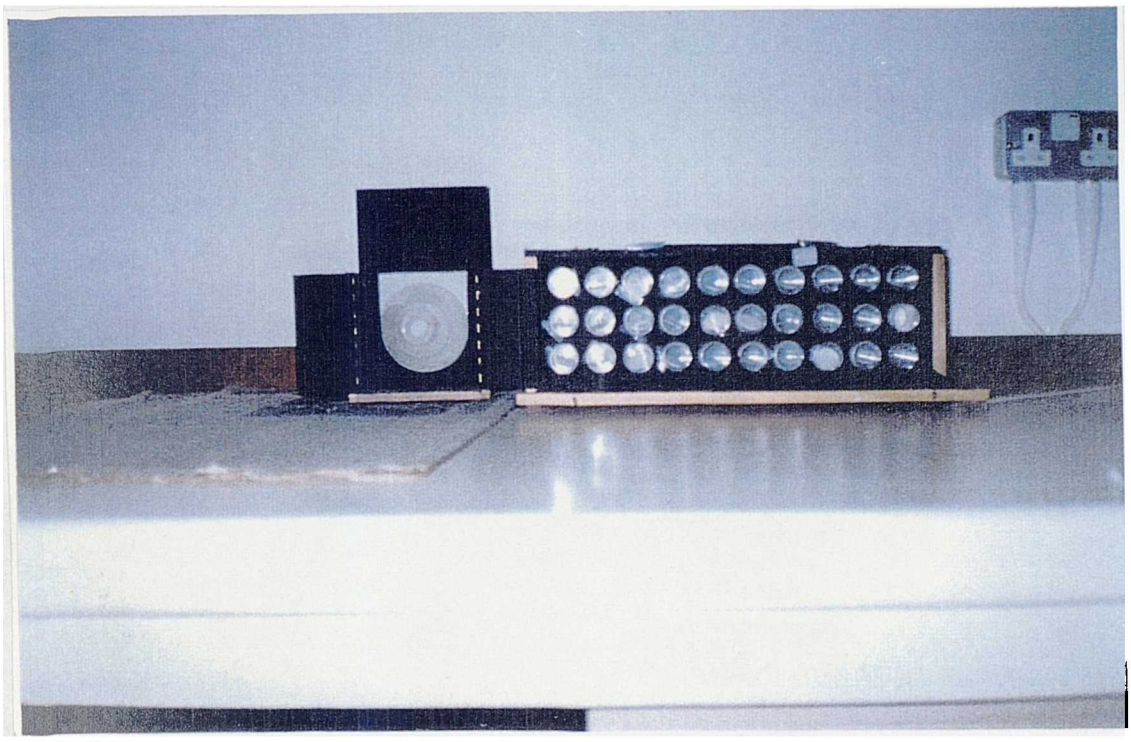
3.2.12 Irritability testing method

The level of irritability of mosquitoes was measured according to the method described by WHO (1963). 20 unfed 2-3 day old females of each strain (resistant and susceptible) were individually exposed to 10 μg/cm^2 permethrin in an exposure chamber and the number of take-offs were counted during a 15 minute exposure time. The differences between number of take-offs for (RR) and (SS) strains were calculated. The mean and standard deviation of number of take-offs for individuals

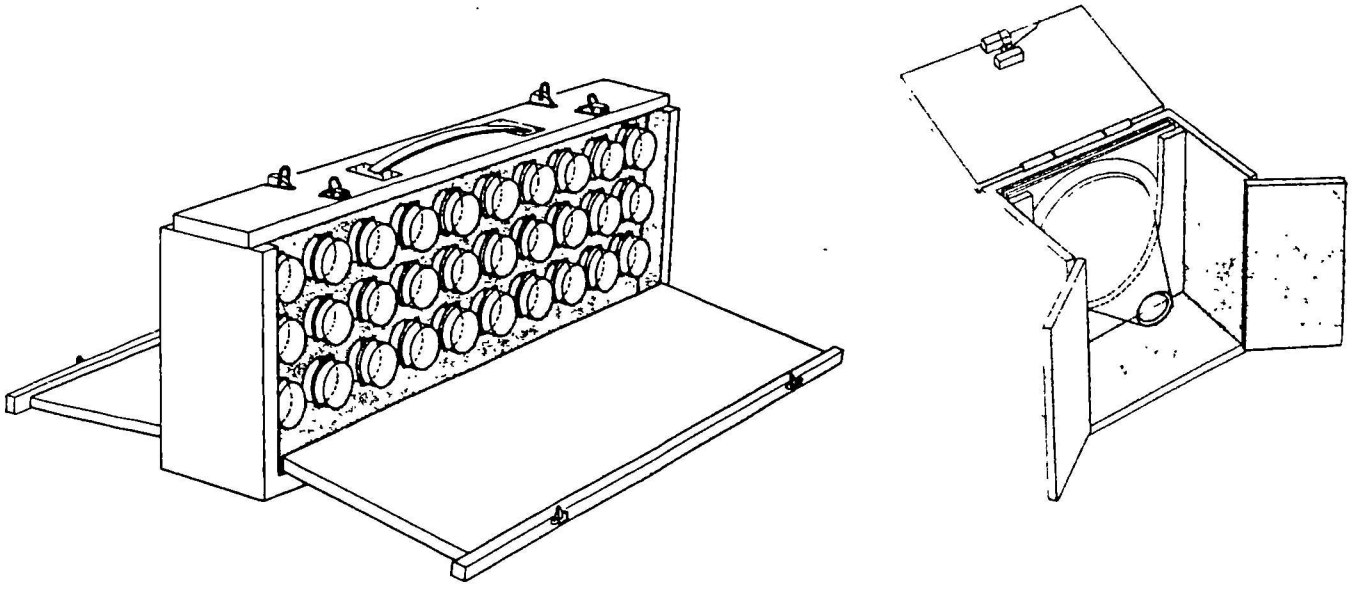
of each strain were calculated. The difference between susceptible and resistant strains in terms of irritability to permethrin was determined by analysis of variance. The experimental set-up for irritability testing are shown in Fig. 3.7.

Fig. 3.6 Knock-down behaviour testing method

Fig. 3.7 Experimental set-up for irritability testing, WHO (1963)



Tube-carrying box with pre-exposure tubes



3.2.13 Vapour effect testing method

The vapour effect of pyrethroids was assessed in our laboratory as shown in Fig 3.8. The impregnated papers were prepared as those used with the standard WHO irritability test cones, each cup being attached to a piece of netting using an elastic band. The distance between net and impregnated paper was 1 cm, so that the net prevented the mosquitoes coming into contact with impregnated paper (see Fig. 3.8). 25 mosquitoes were transferred into exposure cups and the vapour effect of pyrethroids assessed at the LT50 and LT90. Mortality was scored after a 24 h holding period.

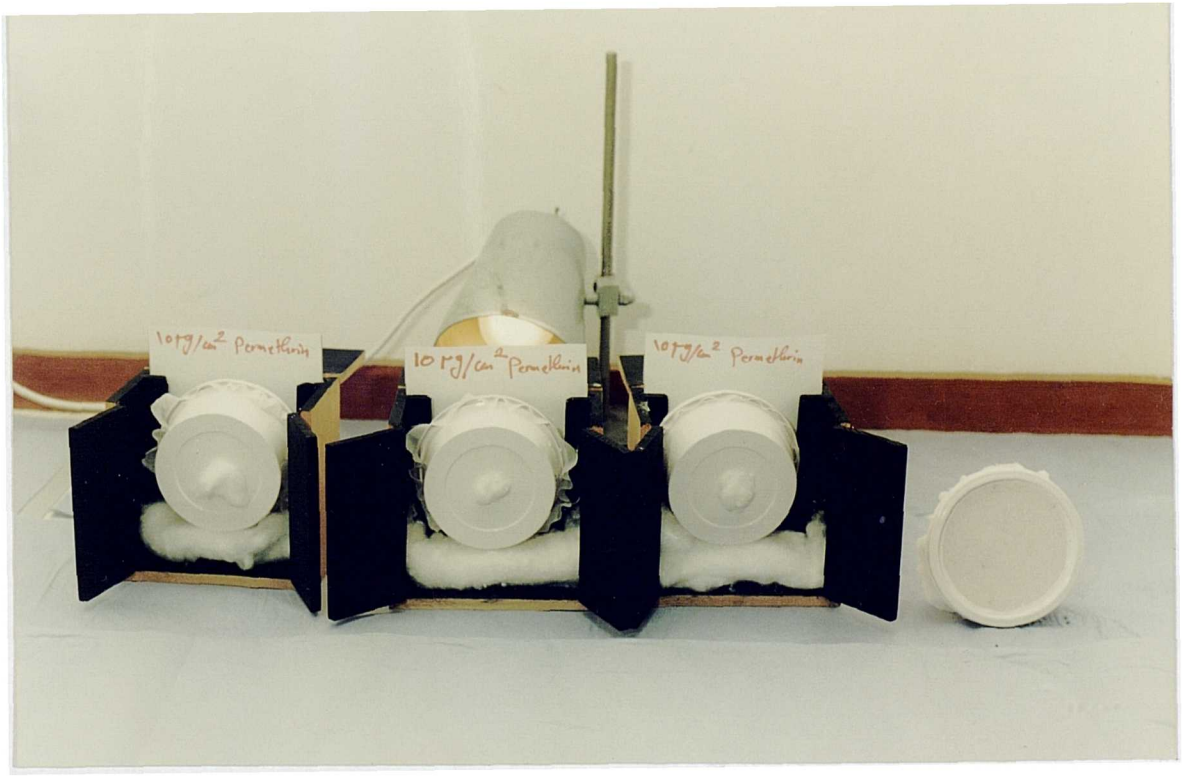


Fig. 3.8 Modified insecticide irritability chamber used for testing vapour effect

3.2.14 Measurement of the responsiveness of adult mosquitoes to movement

The ability of the DUB-APR and IND-S strains to respond to and avoid a moving "predator" was tested by assigning at random two unmarked population cages, containing known numbers of resistant and susceptible mosquitoes, to volunteers who cleared them as quickly as possible using mouth aspirators, the experiment was repeated 15 times for each strain, experiments were carried out with equal numbers of 10 mosquitoes in each cage and times of aspirator catch were recorded.

3.2.15 Bioassay of feeding success throughout repellent impregnated net

To assess the potency of deet impregnated netting materials in inhibiting the blood feeding of DUB-APR and IND-S strains, the following experiment was carried out. Nets were impregnated by dipping in a solution of a measured volume of repellent deet, dissolved in ethanol. The nets were dried and used for experiments. The concentration of deet used was 53 nl/cm^2 (as described by Curtis et al. 1987a). Batches of 2-3 day-old unfed mosquitoes were introduced into cylindrical cages which were lined on top with deet impregnated netting. Anaesthetized guinea pigs were gently placed on top of the nets, mosquitoes were left for 30 minutes. During the experiments the probing behaviour and total number engorged were recorded. At the end of the exposure period the mosquitoes were collected and tested for whether they had blood fed. Unimpregnated nets were used as a control.

3.2.16 Preconditioning of mosquitoes at cooler temperature to assess negative temperature coefficient of pyrethroids

To assess the influence of pretreatment temperature on the toxicity of pyrethroids, a trial was conducted using adult susceptibility tubes provided by WHO. In the holding tube 25 mosquitoes were released and transferred to a temperature of 5±1°C for 3 h, after which they were gently blown into exposure tubes and exposed to pyrethroids (permethrin, deltamethrin, lambdacyhalothrin) at the LT50 and LT90, while held at 5±1°C and the mortality scored after a 24 h holding period at 27±1°C. Both resistant (DUB-APR) and susceptible (TEH) strains of *An.stephensi* were used with 4-12 replicates.

3.2.17 Determination of cuticular penetration of ^{14}C-permethrin by adults of DUB-APR and IND-S strains

Some preliminary trials were conducted according to the method of Abd-Elghafar et al. (1994) with some modifications, using 2-3 day-old females of DUB-APR and IND-S strains to assess radiometrically the amount of insecticide (a) externally on adults (b) internally and (c) in the test containers after removing the mosquitoes. Mosquitoes were immobilized for 30 seconds in the cold and then were treated topically on the abdominal segments with 1 μl of methanol solution of ^{14}C-permethrin at 0.25 μg/mosquito, i.e 0.16 ng/mg of body weight (Fig. 3.9).

(a) External pick-up: After 0.5, 1, 2, 3, 4, 5, 6 h of exposure to the radiolabelled solution (Fig. 3.10), mosquitoes were removed to scintillation counting vials and rinsed with 5 washes of 1 ml methanol. The methanol rinse was then evaporated

under nitrogen free oxygen and the amount of radiolaballed permethrin counted with a scintillator.

(b) Internal pick-up: The rinsed mosquitoes were transferred to new scintillation counting vials. 700 μl of tissue solubiliser (N-ethyl-N-dodecyl-N,N-dimethyl ammonium hydroxide) were added into each vial and left to solubilise the tissues. After solubilisination, the amount of internal pick-up was counted by scintillator.

(c) Residue in containers: All test containers were rinsed carefully with 5 ml of methanol and the extract kept in scintillation counting vials. Methanol was then evaporated under nitrogen free oxygen and the residues in the counting vials dissolved in scintillating fluid and used for counting. The radioactive samples (^{14}C) were counted at about 94% efficiency by scintillation counter.

Fig. 3.9 Topical application of ^{14}C-permethrin on abdominal segments of adults

Fig. 3.10 Container were used to determine cuticular penetration of ^{14}C-permethrin

3.2.18 Gel electrophoresis for carboxylesterase study

Esterase activity was studied using horizontal starch gel electrophoresis, by the following the method of Townson (1969).

3.2.18.1 Making the gel

3.2.18.1.1 Materials:

- 27 g sieved potato starch (Sigma Electrostarch)
- 250 ml (10 ml of Tris EDTA Maleate buffer+240 ml DDH_2O)

buffer of Tris EDTA Maleate (TEM) included (23.8 g Maleic acid, 7.8 g EDTA, 4.0 g $MgCl_2$ and 30 g Tris)

Titrate to pH 7.4 with Tris and make up to 2 litres with DDH_2O for tank buffer.

3.2.18.1.2 Method:

a) Dissolve 27 g potato starch in 250 ml of mixture of 10 ml TEM and 240 ml DDH_2O.

b) Stir the mixture while heating to keep the starch in suspension.

c) When the gel begins to boil remove from the heat source and remove all air bubbles from the mixture by connecting to a vacuum pump.

d) Pour into the plates (the mixture should make 2 gels).

e) Allow the gels to cool and cover with cling film to prevent drying, use within 24 h.

3.2.18.2 Sample preparation:

a) Homogenise individual mosquitoes in 10 μl of tank buffer.

b) Soak the homogenate into small pieces (4mm x 4mm) of cellulose acetate paper.

c) Make slots in the gel using a stainless steel comb.

d) Insert the samples individually into the slots in the gel.

e) Use a small volume of bromophenol blue in one of the end slots as a visible marker during the run.

f) Cover each end of the gel with wicks dipped into electrode buffer and then cover the whole gel with cling film.

3.2.18.3 Running the gel:

a) With 2 gels per cooling plate, run at constant voltage of 200 v and a current of 70 mA.

b) Run until bromophenol blue reaches the end of the gel (marker migrates about 8 cm).

3.2.18.4 Staining:

3.2.18.4.1 Materials for staining of one gel:

- 50 mg Fast blue RR
- 60 ml of 0.15 molar phosphate buffer pH 6.5 (0.15 molar phosphate buffer contains 17.4 g KH_2PO_4, 12.8 g Na_2HPO_4 in 2 litter DDH_2O).
- 1 ml of 2% α-naphthyl acetate in acetone.

3.2.18.4.2 Method for staining

a) Mix fast blue RR with phosphate buffer.

b) Add substrate solution just before adding to the gel.

c) Pipette substrate solution beneath surface of buffer.

e) Incubate in a shaking water bath at 37°C until the bands are fully developed.

f) Wash gels with distilled water carefully.

j) Store the gels in 10% glacial acetic acid.

h) Take a photograph.

In this study frozen or fresh mosquitoes (adult and larvae) and supernatants of centrifuged mosquitoes were used. Two substrates, α and ß-naphthyl acetate, were used as substrate for A and B carboxylestrases, respectively.

3.2.19 Preparation of larval homogenate for permethrin metabolism studies The preparation of larval homogenate was carried out according the following steps as illustrated in Fig. 3.10.

1- Collect 4th instar larvae, wash with DDH_2O, add PBS+1% Glycerol, and store at -80°C until use.

2- To prepare larval homogenate, take out larvae from -80°C, thaw on ice.

3- Centrifuge at 3000 rpm for 10 minutes to pellet the larvae.

4- Remove PBS+1% Glycerol.

5- Add 1/15 M phosphate buffer pH 7.4, (0.89 g KH_2PO_4, 4.78 g Na_2HPO_4 made up to 500 ml with DDH_2O and adjusted to pH 7.4)

6- Rupture larvae with motor-driven cold teflon-pestle.

7- Add ice-cold phosphate buffer, transfer them into 25 ml MSE centrifuge tubes and accurately balance.

8- Centrifuge at 10800 g for 23 minutes at 4°C with ultracentrifuge.

9- Discard pellet and collect the supernatant (this supernatant was termed S9).

10- Add 1/15 M phosphate buffer to supernatant.

11- Centrifuge at 105000 g for 1.05 h at 4°C.

12- Remove supernatant and collect the pellet.

13- Add 1/15 M phosphate buffer to suspend pellet.

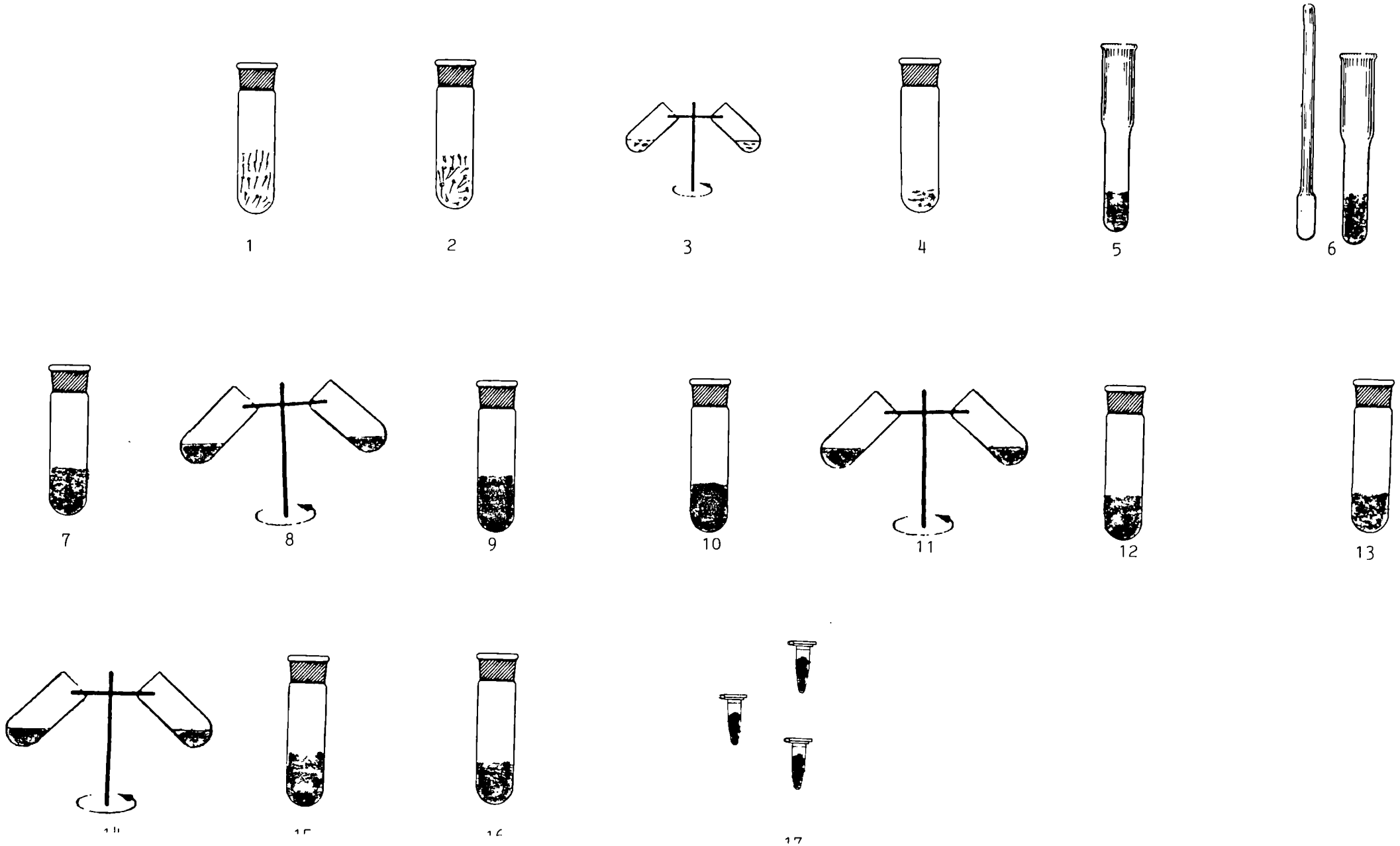
14- Centrifuge at 105000 g for 1.05 h at 4°C.

15- Remove supernatant and take pellet.

16- Add 1/15 M phosphate buffer to pellet and resuspend.

17- Pour the final liquid into the microtubes and store at -80°C.

Fig. 3.10 Scheme illustrating larval homogenate preparation



3.2.20 Preparation of rat liver microsomes

Rat liver microsomes which were used for the metabolism study were provided by the Pharmacology Dept, Liverpool University, according to the following process:

1- Adult rats weighing between 200-250 g were killed by cervical dislocation.

2- The liver was rapidly excised, washed in ice-cold 0.9 saline and stored on ice.

3- Liver weighed.

4- Add 1/15 M phosphate buffer with KCl into the liver container to prepare 25% homogenate.

5- Cut up liver using scissors.

6- Transfer liver into glass homogenising tube and homogenize using a motor-driven teflon pestle.

7- Transfer this liver homogenate into 25 ml MSE centrifuge tube and balance accurately.

8- Fit aluminium screw cups and place tubes in a pre-cooled (4°C) centrifuge head (MSE 8x25 ml).

9- Centrifuge at 13000 g for 20 minutes at 4°C to pellet intact cells, cell debris, nuclei and mitochondria.

10- Take supernatant and divide into MSE centrifuge tubes and balance the tubes, before placing in pre-cooled centrifuge head.

11- Centrifuge at 105000 g for 1.05 h.

12- Discard supernatant and resuspend pellet in phosphate buffer.

13- Centrifuge at 105000 g for 1.05 h.

14- Discard supernatant, take pellet, and place in microcentrifuge tubes.

15- Cover each pellet in a few drops of 1/15 M phosphate buffer and store at -80°C.

3.2.21 Determination of protein content

Enzyme activities are usually expressed against the protein content of the tissue or enzyme source and it was therefore necessary to measure the protein content of the liver and larval homogenates that were used. The most widely used assay for determination of protein content is a colorimetric method developed by Bradford (1976) using bovine serum albumin (BSA) as standard protein. This assay is based on the fact that the absorbence maximum for an acid solution of Coomassie brilliant blue G-250 shifts for 465 nm to 595 nm when protein binding occurs. A series of protein standards using BSA ranging in concentration from 0 μg/ml to 120 μg/ml was prepared. 1 ml of Coomassie agent [Coomassie agent is mixture of; 0.01%(w/v) Coomassie brilliant blue G-250, 4.7%(v/v) ethanol, 8.5%(v/v) orthophosphoric acid, diluted 1 in 5 with DDH_2O] was added into 200μl of each concentration of protein prepared, after allowing to stand for 15 minutes, absorbence at 595 nm was read with a CE6000 cecil spectrophotometer. A calibration curve of absorbence versus protein concentration was plotted and from this standard curve, the protein content of the samples were determined. A typical calibration curve is shown in Fig. 3.11.

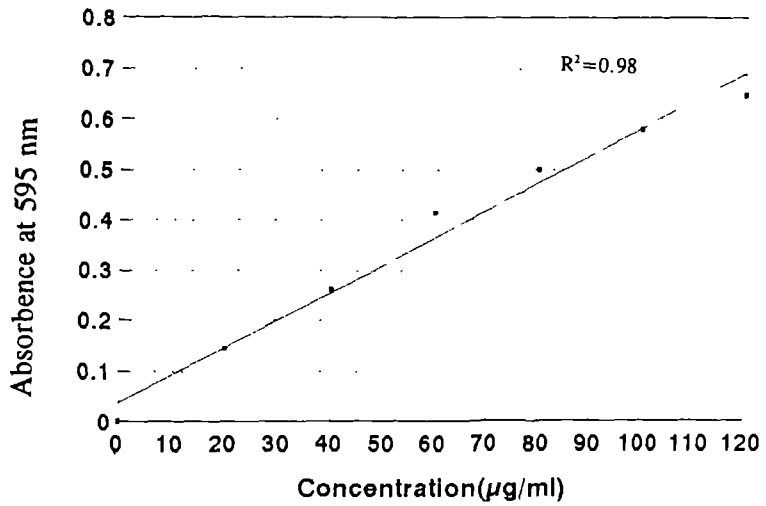


Fig. 3.11 A typical calibration curve for protein content by method of Bradford

3.2.22 Determination of cytochrome P-450 content

Spectrophotometric determination of P-450 content was carried out according to the method of Omura & Sato (1964). A characteristic absorption spectrum results when the haem iron of P-450 is reduced and complexed with carbon monoxide (CO). The reduced CO-complexed difference spectrum of P-450 absorbs maximally at around 450 nm and the extinction coefficient for the wavelength couple 450-490 nm has been accurately determined to be 91 nm/cm, thus allowing quantitative determination of P-450.

3.2.22.1 Method:

1 ml of rat liver microsomes or larval supernatant was placed in matched samples and reference 1 cm optical path length plastic cuvettes. Using a CE6000 double cecil beam spectrophotometer set at 1 nm band width, 5 nm/sec scan speed and a wavelength range of 400-500 nm, a baseline absorbence scan was recorded. A few grains of solid sodium dithionite were added to both sample and reference cuvettes with gentle stirring. To the sample cuvette only, CO was bubbled through gently for about 1 min. The spectrum was then rescanned from 400-500 nm and the difference spectra of P-450 recorded. A typical spectrum for P-450 is shown in Fig. 3.12.

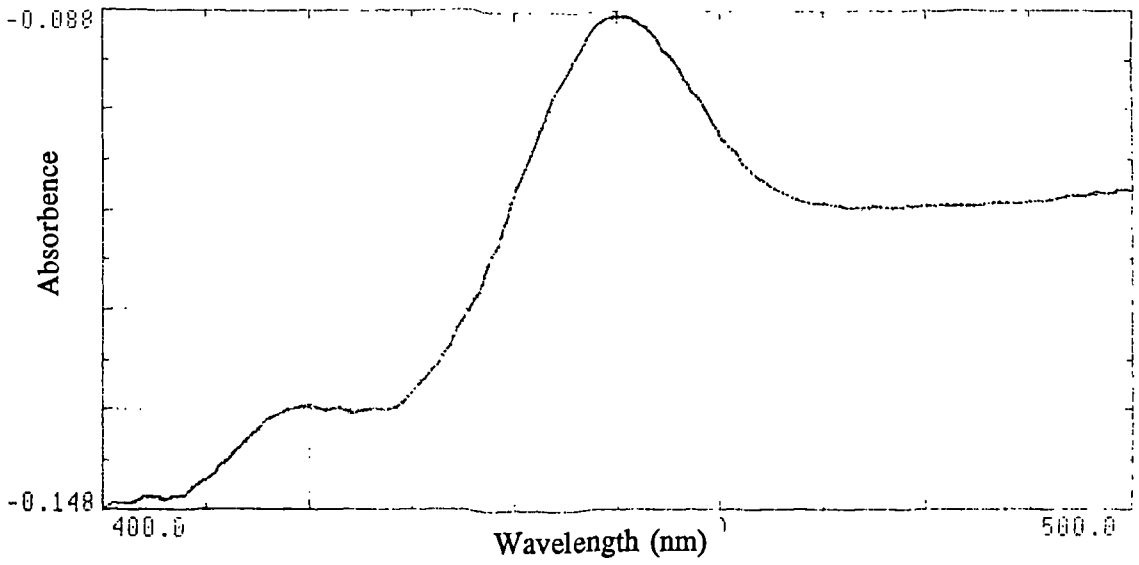


Fig. 3.12 Typical spectrum for P-450 content using rat liver microsome

3.2.23 Optimization of protein concentration and incubation time

In order to determine favourable conditions for permethrin turn over to quantifiable metabolites, initial incubations were carried out with rat liver microsome and larval homogenates. Incubations were repeated using protein concentration ranging from 0-3 mg, and incubation time of 0-60 minutes. From these optimisation studies it was determined that using a protein concentration of 0.1 mg and incubation time 10 minutes gave quantifable turn over of permethrin. The results of optimization for protein and incubation times are represented in Figs. 3.13 and 3.14, respectively.

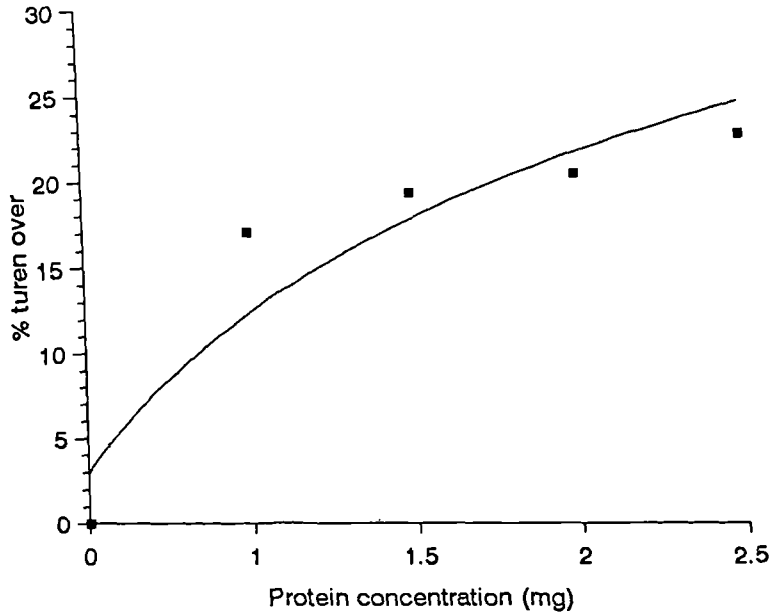


Fig. 3.13 Optimisation of protein concentration

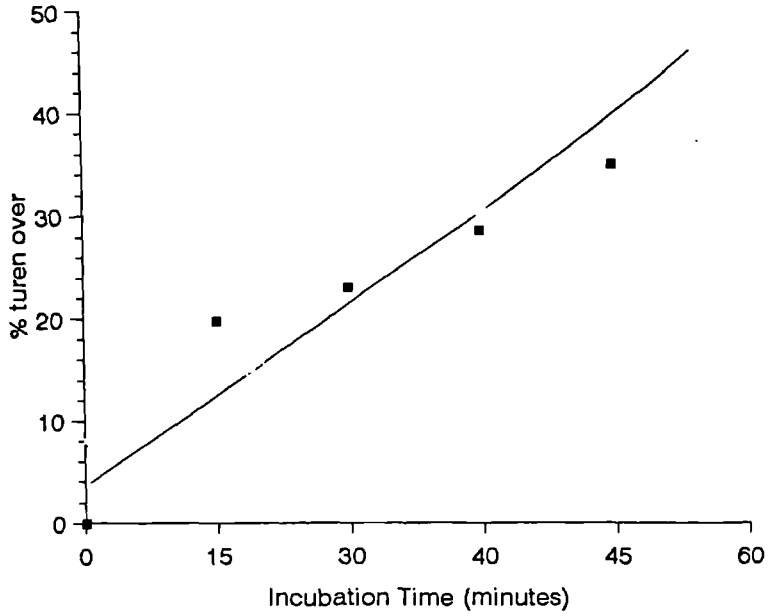


Fig. 3.14 Optimisation of incubation time

3.2.24 High Performance Liquid Chromatography (HPLC) technique

There are several methods capable of detecting metabolites from the parent insecticides including thin-layer chromatography (TLC), gas-liquid chromatography (GLC) and High Performance Liquid Chromatography (HPLC). HPLC was used in the studies described on permethrin metabolism. The HPLC apparatus (Fig. 3.15) consisted of:

a) A Rheodyne type 7125 injector fitted with a 50 μl sample loop.

b) Spherisorb carbon eighteen (C18) reverse phase column (100x4.6 mm) (Alletc, UK).

c) Spectra Physics SP8800 ternary HPLC pump.

d) Spectra Physics SP8450 UV/VIS detector (230 nm).

e) A-200 Flo-One\beta Radioactive flow detector (Radiomatic Packard) which was fitted with a 0.5 ml liquid scintillation flow cell.

f) Flo-Scint A, mixed with the HPLC mobile phase at a ratio of 3:1.

g) Radiomatic detector computer with Flo-One/Data software II and colour EGA graphic monitor.

h) Graphic printer.

i) The mobile phase which was degassed with helium comprised 60% methanol (HPLC), 40% distilled water with 1% triethylamine buffered to pH 7 with orthophosphoric acid. The flow rate was 2 ml/min.

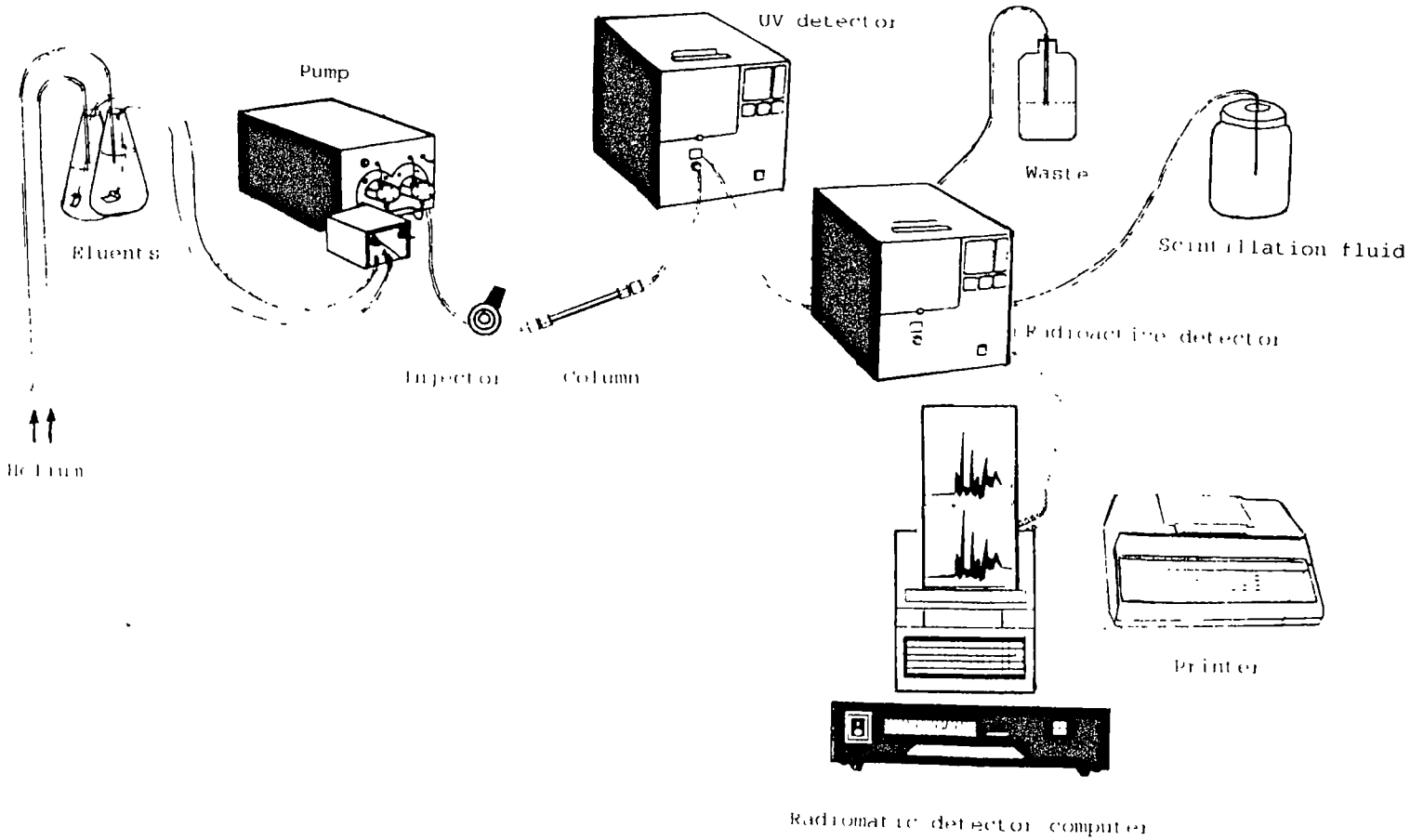
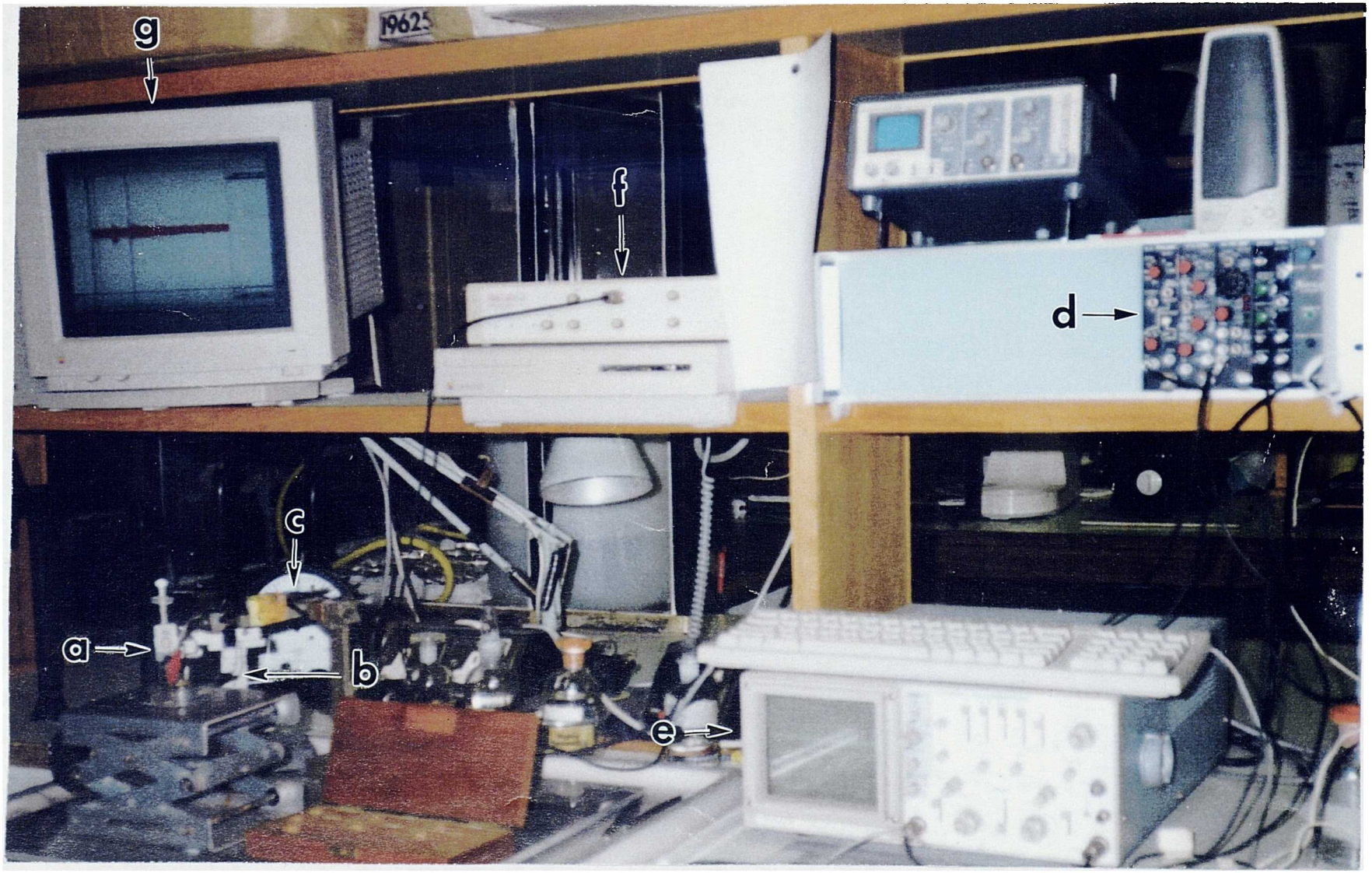


Fig. 3.15 High Performance Liquid Chromatography (HPLC) apparatus

3.2.25 Neurophysiology assay:

For the neurophysiology assay, 2-6 day old females of *An. stephensi* were chilled for 30 seconds and pinned out on a layer of saline-washed Sylgard resin (Dow Corning) in a 50mm plastic petri dish. Legs, wings and head were removed. The thorax was dissected and the nervous system was picked up with a 27 gauge stainless steel, hypodermic needle, insulated on its external surface. This needle served as a recording electrode. A stainless steel entomological pin (0.1 mm diameter x 15 mm length) grounded the preparation and served as a reference electrode. The preparation was located on a low-vibration table at a constant temperature (25 ± 1°C). The recording electrode was connected to the remote head of a Neurolog high-gain, amplifier and conditioning system (Neurolog; Digitimer, UK). Spontaneous nerve activity was monitored on an oscilloscope and activity recorded on Maclab/2e data recorder and analyzed on a Macintosh LCII computer (McCaffery et al., 1995) (see Fig. 3.16)

Fig. 3.16 Neurophysiological apparatus; a) Recording suction electrode, b) Reference electrode, c) Head amplifier, d) Neurolog amplifier, e) Oscilloscope, f) MacLab/2e data recording, g) Macintosh LCII computer



3.2.25.1 Materials & methods for neurophysiology assay

3.2.25.1.1 Insecticide:

Technical grade permethrin, [3-phenoxybenzyl (1RS)-*cis-trans*-3-(2,2,dichlorvinyl)-2,2,-dimethyl-cyclopropanecarboxylate], of 97% purity, and with a *cis/trans* ratio; 40/60 was used throughout the study.

3.2.25.1.2 Insecticide concentrations:

A stock solution of 10^{-5} M permethrin was prepared from technical grade permethrin dissolved in acetone (Analar grade) and diluted directly in saline to give a final logarithmic range of concentrations of 10^{-13} to 10^{-7} M. The concentration of acetone present in each solution never exceeded 1%.

3.2.25.1.3 Insect saline:

Insect saline (Osborne & Hart, 1979) was prepared each day.

3.2.25.1.4 Mosquitoes:

Newly emerged adults of permethrin resistant (DUB-APR) and susceptible (IND-S) strains of *An.stephensi* were transferred to an insectary of the Animal and Microbial Science Department, Reading University, supplied with 10% sucrose on a cotton pad. Unfed, 2-6 day old females were used for the neurophysiology assays.

3.2.25.1.5 Application of test compounds:

In a single dose assay, the preparations of susceptible and resistant strains were bathed in control saline for 1 h followed by saline solutions containing a

suprathreshold concentration of permethrin (10^{-13} M). In a cumulative dose assay, the number of action potentials in a 5 min control period was first recorded and then saline was removed with a disposable glass pipette. Preparations whose firing rate remained relatively stable over the control period were then treated with permethrin. An identical volume of fresh saline containing the test compound was applied, first the lowest concentration (10^{-13} M) and subsequently with replacement by successively higher concentrations up to 10^{-7} M. The average number of action potentials for each concentration were recorded and analyzed. For each strain, the frequency and SE of response at each concentration in the assay was displayed as a profile for the strain.

3.2.25.1.6 Coating of suction electrodes:

The external surface of the suction electrodes must be electrically insulated and this was done by using perspex particles dissolved in chloroform. Following coating, the tips of needles were cut to create a round tip.

3.2.25.1.7 Preparing of Sylgard dishes:

Sylgard dishes provide an inert resin for neurophysiology assays. Sylgard resin is a mixture of 1 Kg of Sylgard 184 silicon elastomer (Dow corning) in 100 g of mixing agent. The two compounds were mixed, then poured into a 50mm diameter plastic petri dish to a depth of 3-5 mm. Preparations were left to air dry for 48 h. Once dried, the dishes were placed in a bath of distilled water for a further 48 h, then washed once more to remove any remaining surface oil. The dried Sylgard dishes were then used for neurophysiology assays.

Chapter 4

MECHANISMS OF PERMETHRIN RESISTANCE IN SELECTED STRAINS OF *ANOPHELES STEPHENSI*

4.1 Introduction

Pesticide resistance has been documented in over 100 species of mosquitoes (Roberts & Andre, 1994). According to WHO (1992a), Miller (1988) and Malcolm (1988a) pyrethroid resistance has been reported in numerous species including *Aedes, Culex,* ticks, horn flies, bed bugs, the German cockroach and human lice. Unfortunately, this list includes some very important mosquito vectors of human disease, e.g. *Ae.aegypti, An.arabiensis, An.stephensi* and *Cx.tritaeniorhynchus.*

Increasing pyrethroid resistance is of particular concern since many countries are, or will be, using synthetic pyrethroids, such as deltamethrin in their vector control programmes, particularly the large-scale distribution of pyrethroid-impregnated bednets for malaria control (Roberts & Andre, 1994).

Today resistance management in the context of integrated pest management has evolved as the favoured approach to prevent, delay or reduce the impact of insecticide resistance (Soderlund et al., 1989).

In this study, strains of *An.stephensi* from Dubai, selected with permethrin in the laboratory were used. In one line selection was applied to adult (DUB-APR) and in another to larvae (DUB-LPR). An attempt was made to study the mechanisms involved in permethrin resistance at the adult and larval stages.

4.2 Base-line susceptibility of adult females of different geographical strains of *An.stephensi* to permethrin

Permethrin was studied in the laboratory for its comparative efficacy against females of different geographical strains of *An.stephensi*. Initially the susceptibility of TEH, BAN, IRAQ and BEECH strains was measured using 10 $\mu g/cm^2$ impregnated paper. Due to the high mortality rate, the concentration was reduced to 4.16 $\mu g/cm^2$ and all subsequent tests carried out with this concentration. DUB-APR, DUB-LPR, and IND-S strains were tested with 10 $\mu g/cm^2$ impregnated papers.

To eliminate the importance of early knock-down of adults during exposure, testing tubes were held in a horizontal position (Ladonni, 1988; WHO, 1992a). In each test 2-3 day-old females were tested with fresh laboratory-made impregnated papers. Regression line parameters including intercept (a), slope$\pm$ standard error (b$\pm$SE), heterogeneity about the regression line with degrees of freedom [X^2(df)], LT50$\pm$95% confidence interval (LT50$\pm$95%C.I.), LT90$\pm$95% confidence interval (LT90$\pm$95% C.I.) were determined from the dosage mortality regression lines as described by Finney (1971).

LT50 and LT90 represent respectively the times of exposure required to kill 50% and 90% of the population at a given concentration of pesticide. The LT50 is the main parameter used in this study to assess the relative toxicity of pesticides.

The results of the studies on the efficacy of permethrin against females of *An.stephensi* have been summarized in Table 4.1. Five strains TEH, BAN, IRAQ, IND-S and BEECH are susceptible to permethrin with LT50s around 22.4-33.6 minutes. The LT50 value from Table 4.1 showed that DUB-APR (strain selected with permethrin at the adult stage) is 8-fold more resistant to permethrin than the IND-S

strain which remains susceptible. Similarly DUB-LPR, that was selected at the larval stage showed resistance to permethrin compared to other strains. Comparative toxicity of permethrin at the LT50s are shown in Fig. 4.1 assuming that concentration x time=constant.

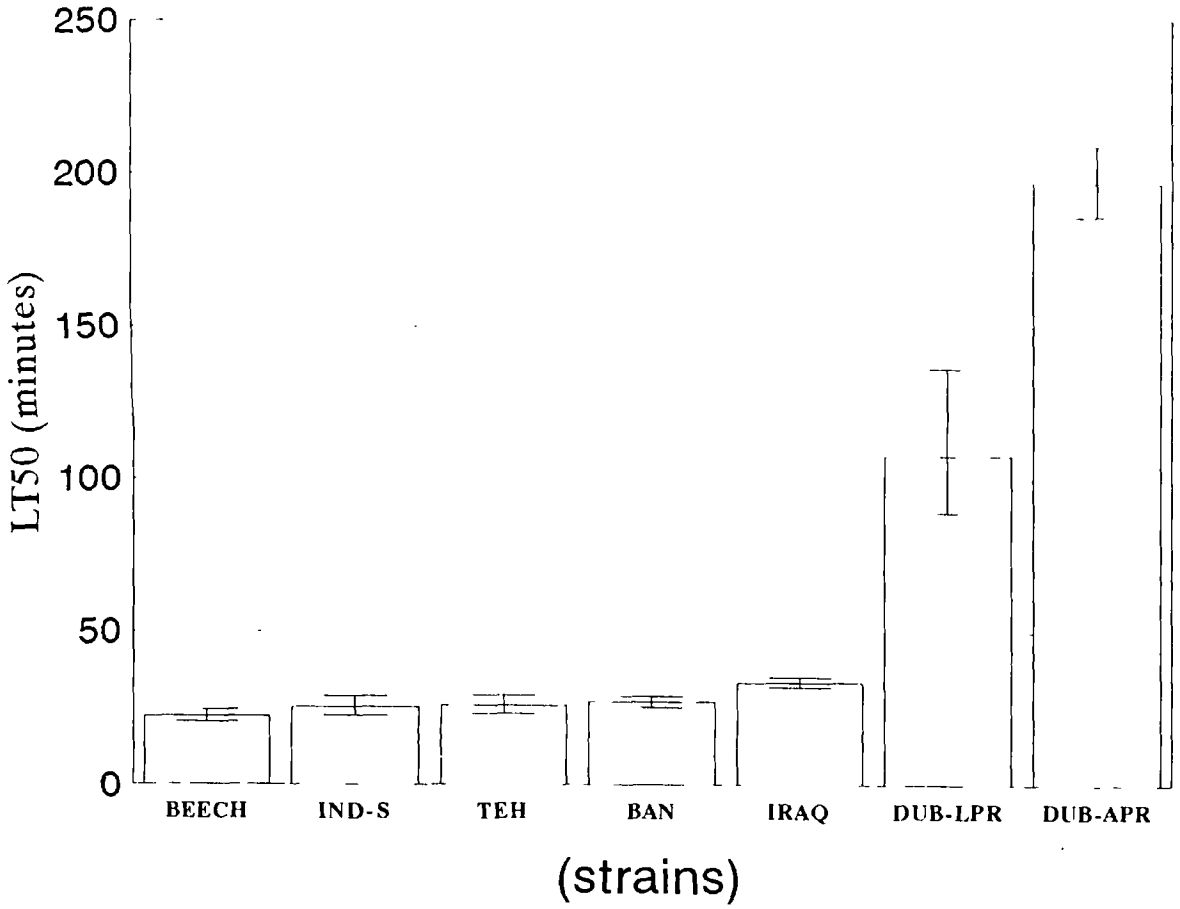
Table 4.1 Probit regression line parameters for adult females of *An.stephensi* tested with permethrin at 4.16 $\mu g/cm^2$

Strains	a	b±SE	LT50±95%C.I.	LT90±95%C.I.	X^2(df)	P
TEH	-5.99	4.24±0.57	23.4 25.9 29.4	42.3 51.9 72.4	4.12(3)	>0.05
BEECH	-7.27	5.38±0.64	20.6 22.4 24.6	33.7 38.7 47.9	11.16(3)	0.025>p>0.01
BAN	-8.60	5.98±0.63	25.5 27.1 29.1	39.8 44.4 52.2	19.78(4)	0.001>p>0.005
IRAQ	-10.13	6.64±0.61	31.9 33.6 35.3	49.0 52.5 57.5	3.10(3)	>0.05

Table 4.1 Probit regression line parameters for adult females of *An.stephensi* tested with permethrin at 10 $\mu g/cm^2$

Strains	a	b±SE	LT50±95%C.I.	LT90±95%C.I.	X^2(df)	P
DUB-APR	-10.70	5.57±0.41	77.4 82.0 87.0	126.5 139.2 157.4	4.95(5)	>0.05
DUB-LPR	-3.96	2.4±0.31	37.1 44.9 56.8	108.3 153.9 256.8	9.93(3)	0.025>p>0.001
IND-S	-3.84	3.74±0.42	9.4 10.6 12.1	19.4 23.4 30.4	5.21(3)	>0.05

Fig. 4.1 Relative toxicity of permethrin to females of different strains of *An.stephensi*. Vertical bars= 95% C.I.



Assuming the time x concentration=constant, the LT50 values for DUB-LPR, DUB-APR and IND-S strains were multiplied by 2.4 ($10\mu g/cm^2$ divided by $4.16\mu g/cm^2$)

4.3 *In vivo* synergist study for adults

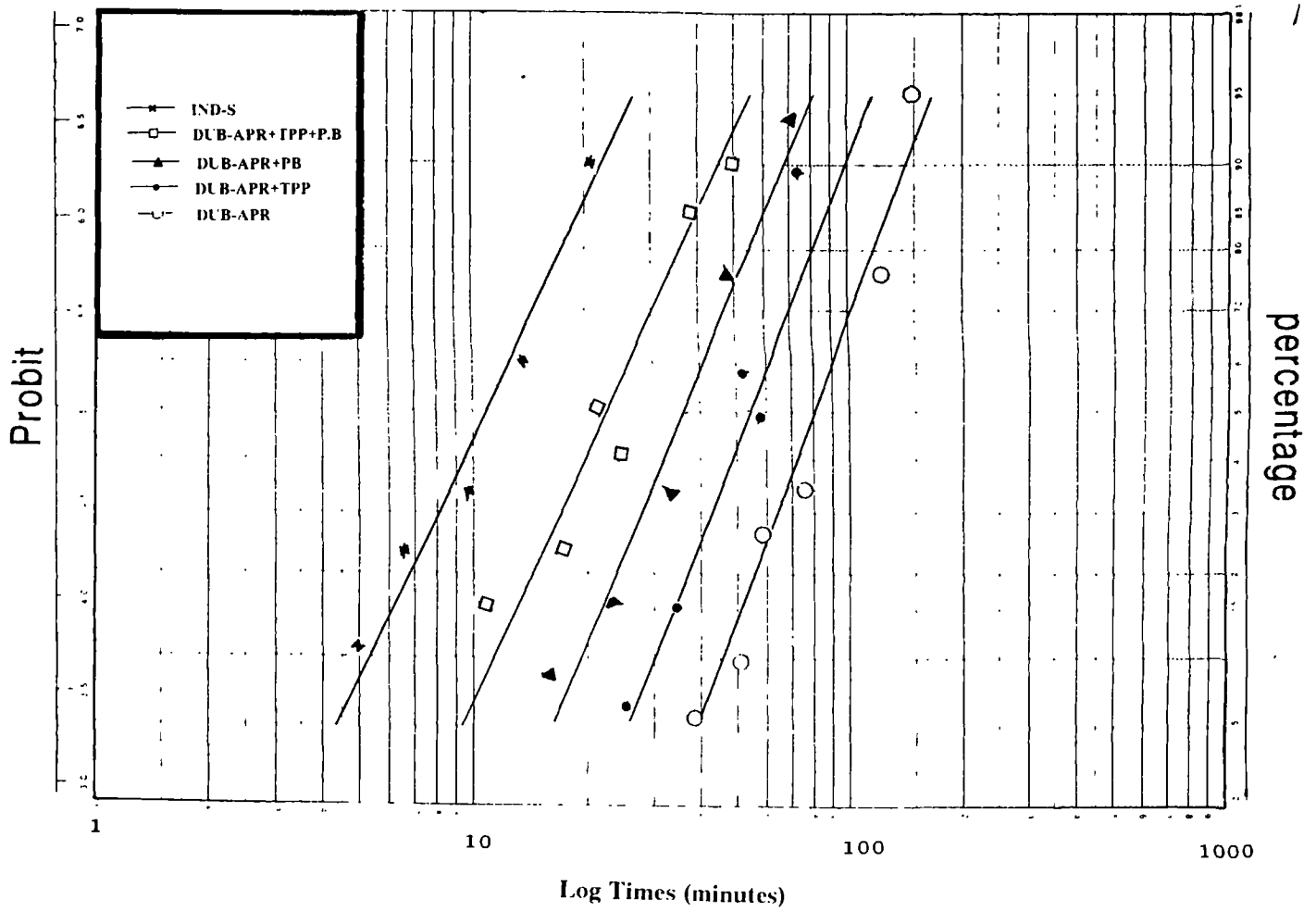
Esterases and mixed function oxidises (MFO) are the most important enzymes involved in pyrethroid metabolism (Soderlund & Bloomquist, 1990). Inhibitors of these enzymes can thereby synergize the activity of pyrethroid insecticides. Esterase activity is inhibited by triphenyl phosphate (TPP), and MFO by piperonyl butoxide (PB). Thus by carrying out *in vivo* insecticide assays in the presence and absence of synergists, it is possible to define the detoxification pathway underlying resistance. By studying the change in the probit regression lines, synergist ratios may be calculated, a synergist ratio being significantly different from 1 when the LT50's of the two lines differ significantly. The detailed methods are described in section 3.2.6. Essentially mosquitoes were pretreated with synergists at concentration determined to be non-toxic, then the mosquitoes exposed to permethrin in a standard *in vivo* bioassay. The regression lines of mosquitoes with and without such synergist pretreatment were then compared using probit analysis.

The results from synergist tests are shown in Table 4.2. Following pretreatment of DUB-APR with TPP and PB the LT50 declined 1.7 and 2.2-fold, respectively. Combination of TPP and PB of DUB-APR produced a synergist ratio of 3.4. The resistance factor (RF) of the DUB-APR strain was reduced from 8 fold to 2.2-fold in the presence of both synergists. The regression lines in Fig. 4.2 reveal that both synergists changed the LT50 of resistant strain moderately and indicate the existence of synergism. The most reasonable conclusion to be drawn from Fig. 4.2 is that in addition to enzyme involvement of esterases and MFO other mechanisms contribute to resistance, in the permethrin resistant strain. This is likely to be caused by reduced sensitivity of the active site, like a *kdr*-type mechanism.

Table 4.2 Probit regression line parameters of females of permethrin-resistant and susceptible strains of *An.stephensi* pretreated with synergists TPP and PB followed by exposure to 10 $\mu g/cm^2$ permethrin

strain	with/out	a	b±SE	LT50±95 %C.I	LT90±95 %C.I.	X^2 (df)	P	RF	SR
DUB-APR	-	-10.70	5.57± 0.41	77.4 82.0 87.0	126.5 139.2 157.4	4.95 (5)	>0.05	8	-
	TPP	-9.07	5.39± 0.61	44.4 48.4 53.1	73.1 83.7 102.0	11.5 (3)	0.01>P>0.005	4.6	1.7
	PB	-7.86	5.00± 0.42	35.1 37.5 40.4	61.3 68.0 77.8	7.83 (3)	0.05>P>0.025	3.5	2.2
	TPP+PB	-5.52	4.01± 0.41	21.3 23.8 26.5	42.5 49.7 61.9	9.22 (3)	0.025>P>0.01	2.2	3.4
IND-S	-	-3.84	3.74± 0.42	9.4 10.6 12.1	19.4 23.4 30.4	5.21 (3)	>0.05	-	-

Fig. 4.2 Probit regression lines of permethrin-resistant and susceptible strains of *An.stephensi* pretreated with synergists TPP and PB and then tested with permethrin



4.4 Knock-down behaviour study

We have established that in a permethrin resistant strain of *An.stephensi* PB and TPP reduced the level of resistance to permethrin. Since resistance was not completely eliminated by these synergists, it was speculated that other mechanisms were involved in resistance in addition to metabolism. It has been known for some time that the nerve membrane is the primary target of pyrethroid insecticides (Narahashi, 1976). A number of later studies have suggested that the voltage-gated nerve membrane sodium channels may be involved in the *kdr* mechanism (Dong & Scott, 1991; Bloomquist, 1996).

In this study knock down of females of permethrin-resistant (DUB-APR) and susceptible (IND-S) strains of *An.stephensi* was quantitated. The experimental methods are described in section 3.2.11. In summary, into each bioassay cone attached to netting impregnated with 500 mg/m^2 permethrin, five females were released and the knock-down time of each individual mosquito was recorded. The experiment was carried out with 100 mosquitoes of each strain, representing 20 replicate tests of 5 mosquitoes. The knock-down time of each mosquito was recorded. The results are presented in Table 4.3 and Fig. 4.3. Adult females of IND-S strain showed first knock-down after 5.1 minutes exposure and the last one knocked-down after 7.3 minutes, but first knock-down for DUB-APR took 14.5 minutes and last knock-down happened after 30.5 minutes. Average knock-down behaviour in IND-S strain was 6.1 minutes with standard error of 0.20, compared with 21 minutes with standard error of 1.06 for DUB-APR strain. Results from difference in time to knock-down of the 5th mosquito is 4.2-fold and on average there was a 3.4-fold difference in knock-down suggested that *kdr* is an important mechanism of resistance.

Table 4.3 The mean time to knock-down in minutes of females of DUB-APR and IND-S strains of *An.stephensi* exposed to mosquito netting impregnated with 500 mg/m^2 permethrin

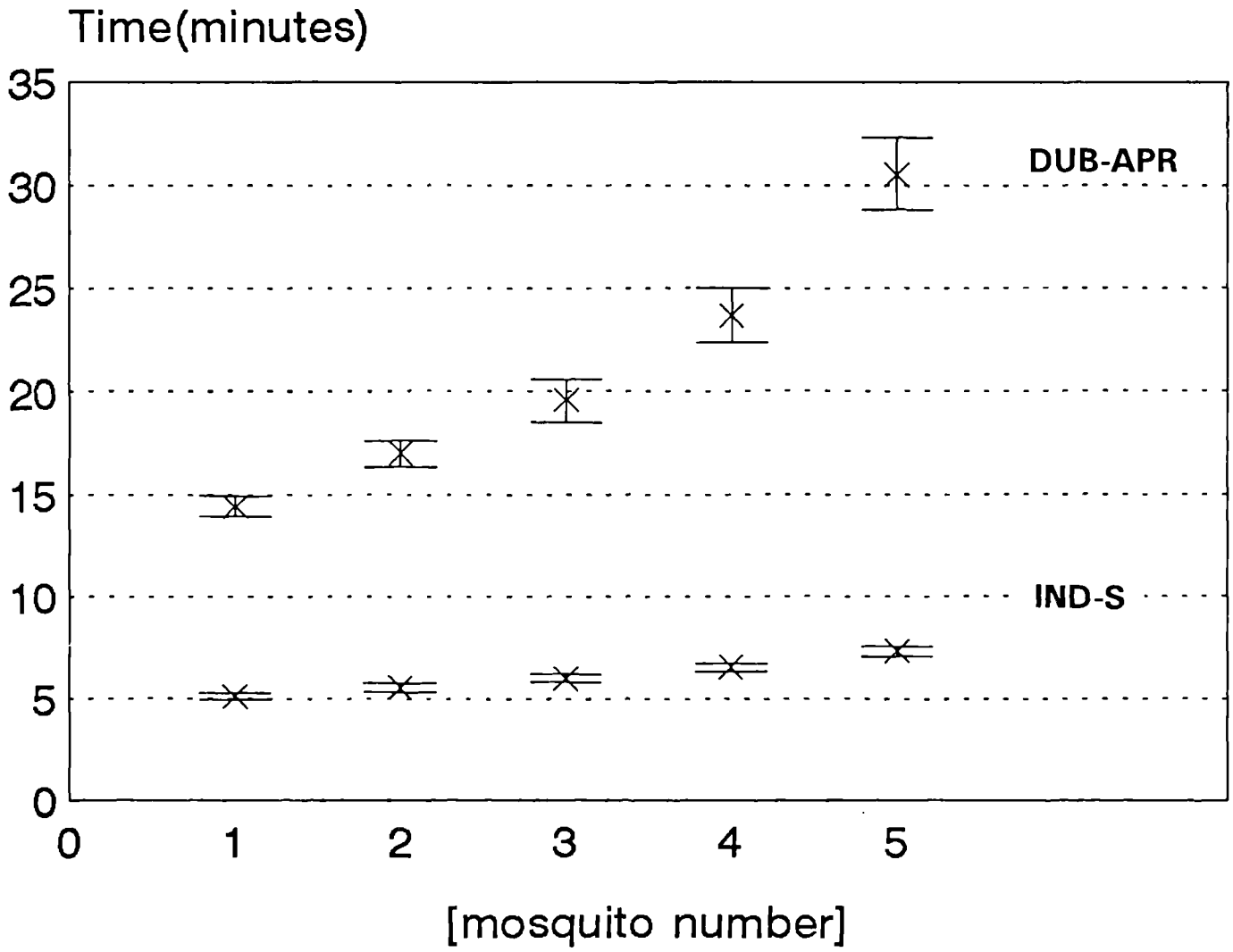
	Time in minutes to knock-down of mosquitoes 1-5					
Strain	1	2	3	4	5	Mean±SE
IND-S	5.1±0.18*	5.6±0.22	6.0±0.18	6.6±0.18	7.3±0.24	6.1±0.20
DUB-APR	14.5±0.50	17.0±0.64	19.6±1.07	23.8±1.33	30.5±1.77	21.0±1.06

Each test comprised 5 mosquitoes in 20 replicates

* ±SE

Fig. 4.3 Knock-down behaviour of females of resistant and susceptible strains of *An.stephensi* to permethrin impregnated net.

Vertival bars= SE



4.5 Irritability study

Experiments were performed on the IND-S and DUB-APR strains to determine the irritability level of adult females exposed to permethrin impregnated paper. The method used was according to WHO (1963). In summary 20 unfed 2-3 day old females of each strains were individually exposed to 10 $\mu g/cm^2$ permethrin in an exposure chamber and the number of take-offs were counted during 15 minutes exposure time. The results of irritability tests are summarized in Table 4.4 and illustrated in Fig. 4.4. During the exposure time of 15 minutes, the mean number of take-offs per female were 12.7 and 35.3 for DUB-APR and IND-S strains respectively. An analysis of variance of the number of take-offs for the two strains showed that DUB-APR is significantly less irritable to permethrin that the IND-S strain, $F=16.37_{(1,28)}$ $P<0.0001$ (Table 4.5). The resistant strain was 2.8 times less irritable to permethrin than the susceptible strain as measured by the number of take-offs.

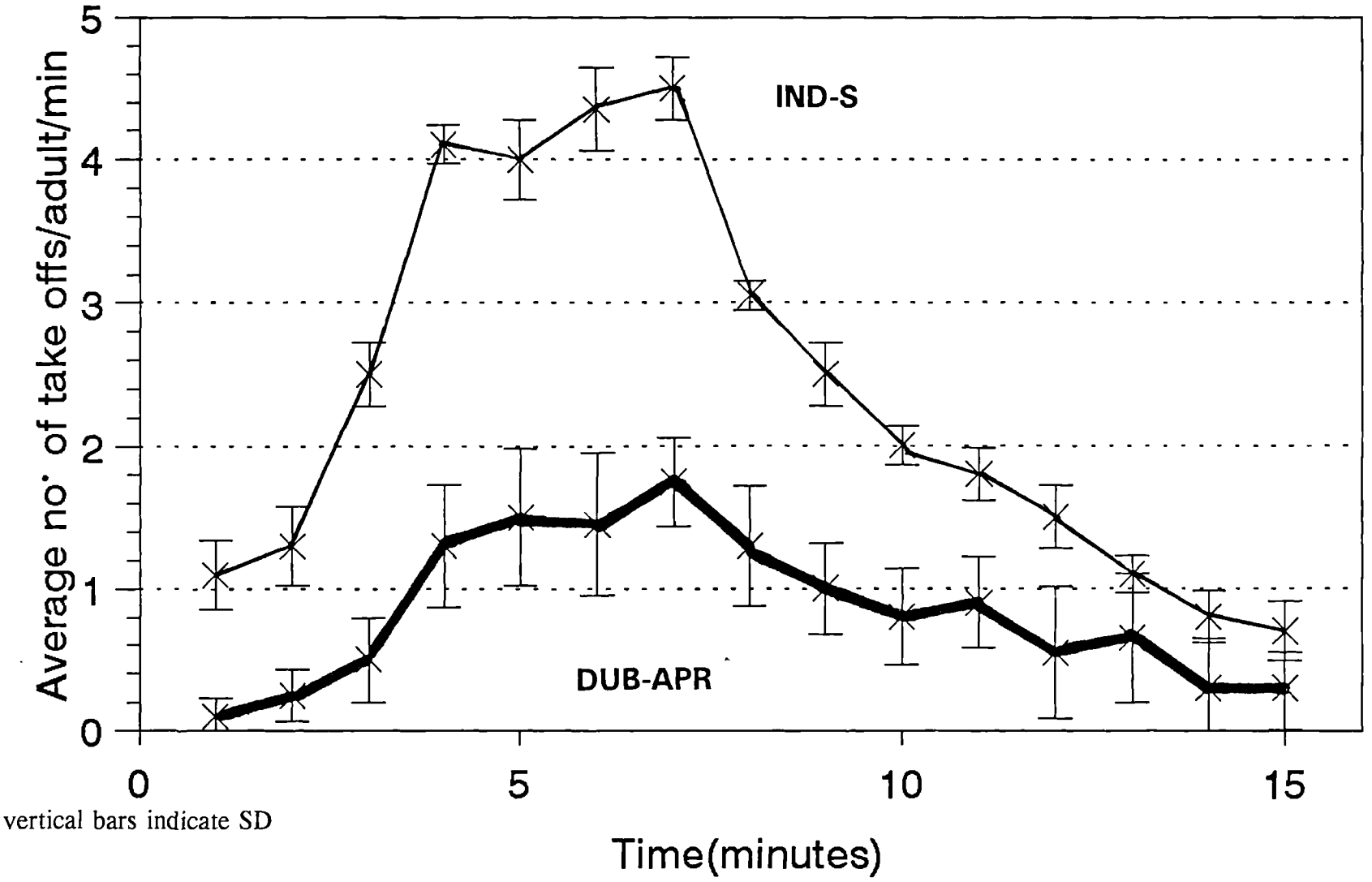
Table 4.4 Irritability level of females of resistant and susceptible strains of *An.stephensi* to permethrin

strains	No. of take-offs/20 female/15 min	No. of take-offs/female/15 min	No. of take-offs/female/min±SD
DUB-APR	254	12.7	0.846±0.52
IND-S	706	35.3	2.353±1.35

Table 4.5 Analysis of variance to compare irritability level of resistant and susceptible strains of *An.stephensi* to permethrin

Source	df	SS	MS	F	P
Factor	1	17.1	17.1	16.37	P<0.0001
Error	28	29.25	1.04		
Total	29	46.35			

Fig. 4.4 Irritability level of females of resistant and susceptible strains of *An.stephensi* to permethrin impregnated paper



vertical bars indicate SD

4.6 Responsiveness to external movement

Rowland (1991) reported some behavioural changes in cyclodiene resistant strains of *An.stephensi*, for instance egg production per oviposition, life time fecundity and flight activities of resistant females was less than those of susceptible strain.

Pyrethroid insecticides share many characteristics with DDT and DDT analogues, including knock-down and killing activity resulting from action against sodium channels of the peripheral and central nervous system (Zebra, 1988). In resistant strains various physiological mechanisms change the behavioural characteristics of the nervous system receptors and increase their permeability to chloride ions, causing hyperinhibition of the nervous system (Rowland, 1991).

In order to carry out further investigations on the behavioural changes in the permethrin-resistant strain, the responsiveness of IND-S and DUB-APR genotypes to external movement were determined. The experiments are described in detail in 3.2.14. In summary, volunteers were allowed to catch the populations of known number of resistant and susceptible mosquitoes from two unmarked cages. The experiments were repeated 15 times for each strain and the times of aspirator catches recorded. The results are shown in Table 4.6. Average time required to catch IND-S strain was 183.4 seconds, compared with DUB-APR which needed 118.2 seconds for collection. Two-way analysis of variance in Table 4.7 indicated that IND-S strain is more responsive than DUB-APR strain and was able to detect movement of the aspirator and took longer to catch.

Table 4.6 Responsiveness of resistant and susceptible genotype of *An.stephensi* to external movement. Experiment was carried out with equal numbers of 10 females in each cage and time of aspirator catch was recorded.

Replicates	IND-S	DUB-APR
1,2,3	194	101
4,5,6	193	105
7,8,9	169	129
10,11,12	178	126
13,14,15	183	130
Mean	183.4	118.2
SD	10.5	14.0

Table 4.7 Analysis of variance of responsiveness of resistant and susceptible strains of *An.stephensi* to external movement

Source	DF	SS	MS	F	P
Responsiveness	1	10628	10628	151	$P<0.0001$
Block	4	103	26	0.09	n.s.
Error	4	1125	281		
Total	9	11856			

4.7 Pharmacokinetics of penetration

Passive penetration of lipophilic pyrethroids through the insect cuticle is the primary event in the sequence of penetration. The insecticide can enter the insect haemolymph to be carried to all parts of body in solution, or bound to protein, or dissolved in lipid particles. Intoxication of an arthropod by a pesticide encompasses different levels of pharmacokinetic interaction: penetration of barrier tissue; distribution, storage, metabolism in internal tissues and molecular interaction with the ultimate target site (Soderlund et al., 1989). Delayed penetration of insecticide is a well known resistance mechanism for numerous insecticides. There is a general agreement that reduced penetration can be an important factor in resistance. The penetration barrier can be either at the cuticle level, resulting in a delayed penetration into the body, or at the level of target site. So a resistance mechanism restricting penetration through the cuticle is a possible explanation for some of the difference between resistant and susceptible strains.

The penetration of ^{14}C-permethrin throughout the integument of females of permethrin resistant (RR) and susceptible (SS) strains of *An.stephensi* was studied. The method is described in section 3.2.17. 2-3 day-old females of DUB-APR and IND-S strains were topically treated with ^{14}C-permethrin and after various times of exposure the amount of insecticide externally on adults, internally by a penetration and in the test containers were radiometrically assessed. The distribution of ^{14}C after topical treatment of resistant and susceptible females with ^{14}C-permethrin are summarized in Tables 4.8 and 4.9. Fig. 4.5 shows distribution of radiolabelled permethrin at total exposure times after topical application. Results from Tables 4.8 and 4.9 indicated that overall recoveries of radiolabelled permethrin in insect rinse for (RR) and (SS)

strains was 51.22±11.41 and 54.39±11.36 percent respectively. Levels of radiolabelled permethrin in the container rinse for resistant and susceptible strains was 40.28±15.75 and 35.02±12.94 percent respectively. Internal levels of permethrin were the same in both strains, i.e., 8.49±4.92 for resistant and 10.58±7.45 percent for susceptible.

The results of studies of the fate of ^{14}C-permethrin during internal penetration, suggest that there are no significance differences between susceptible and reistant strains with respect to the dynamics of permethrin penetration (Fig. 4.5).

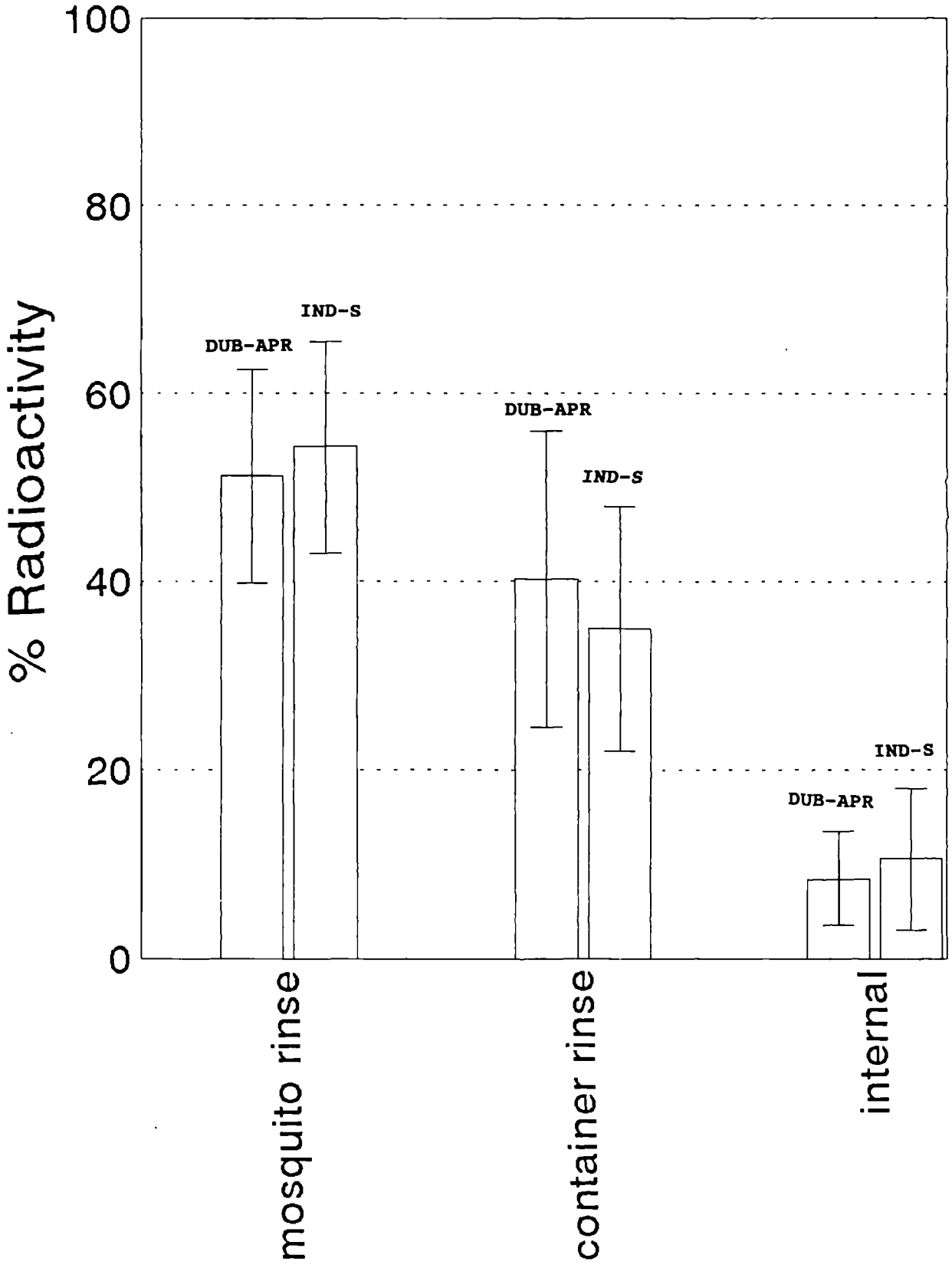
Table 4.8 Distribution and nature of average amount of ^{14}C-permethrin (DPM) for each individual mosquito in total time (21.5 hours) of exposure after topical treatment of permethrin-resistant (DUB-APR) and susceptible (IND-S) strains of *An.stephensi*

	mosquito external rinse	container rinse	internal penetration	Total
strain	Mean±SE	Mean±SE	Mean±SE	
DUB-APR	1487.0±331.30	1169.3±457.20	246.5±142.70	2902.80
IND-S	1616.2±337.60	1040.6±384.40	314.4±221.40	2971.20

Table 4.9 Distribution and nature of average amount of ^{14}C-permethrin (% radioactivity) for each individual mosquito in total time (21.5 hours) of exposure after topical treatment of permethrin-resistant and susceptible strains of *An.stephensi*

	mosquito external rinse	container rinse	internal penetration	Total
strain	Mean±SE	Mean±SE	Mean±SE	
DUB-APR	51.22±11.41	40.28±15.75	8.49±4.92	100%
IND-S	54.39±11.36	35.02±12.94	10.58±7.45	100%

Fig. 4.5 Distribution of ^{14}C-permethrin expressed as percent of radioactivity at total times (21.5 hours) after topical treatment of permethrin-resistant (DUB-APR) and susceptible (IND-S) strains of *An.stephensi*. Vertical bars= SE



4.8 Carboxylesterases enzyme assay

Esterases have been implicated in the metabolism and detoxification of some synthetic pyrethroids in vertebrates (Abernathy et al., 1973; Suzuki & Miyamoto, 1978) and in a variety of insect species (Jao & Casida, 1974 ; Shono et al., 1978 ; Ishaaya & Casida, 1981 ; Zebra, 1988).

From our previous *in vivo* synergist studies it was found that the synergist TPP increased the susceptibility level of permethrin resistant adults with synergist ratio of 1.7 (see Table 4.2 and Fig. 4.2). According to Devonshire et al. (1992) *An.stephensi* is a mosquito species not yet reported to develop insensitive acetylcolinesterase (AChE), so attention is focused on the toxicological significance of esterases for permethrin resistance. A series of experiments was carried out using starch gel electrophoresis and α and ß-naphthyl acetates as substrates for ''A'' and ''B'' carboxylesterases respectively. Assays were done with body homogenates of resistant and susceptible strains of *An. stephensi.* (for details see section 3.2.18). The results are presented in Figs. 4.6 and 4.7.

The results from Fig. 4.6 using α-naphthyl acetate revealed 5 zones of esterase activity, zone 1 is the area nearest the origin and is heavily stained. Relative activities of esterase from homogenate of IND-S and DUB-APR strains using ß-naphtyl acetate are shown in Fig. 4.7 revealed 2 zones. Although there were subtle differences in the activity of carboxylestrases towards the substrate used, there is no obvious difference in the esterase activity in the susceptible and resistant strains, when either α or ß-naphthyl acetate was used as a substrate. If permethrin resistance in DUB-APR female is partially due to detoxification of permethrin by a carboxylestrases enzyme there must be either a qualitative change of enzyme which attacks permethrin, or, it must

have very low activity with both α and ß-naphthyl acetate. These enzymes may be responsible for permethrin resistance through increased detoxification, or sequestration or both, or may be other classes of esterase isozymes are involved in permethrin detoxification that are not detected using these substrate-dye combinations.

Fig. 4.6 Gel electrophoresis of esterases from permethrin susceptible (IND-S) and resistant (DUB-APR) strains of *An.stephensi* females. Esterase activity was visualized with α-naphthyl acetate as a substrate

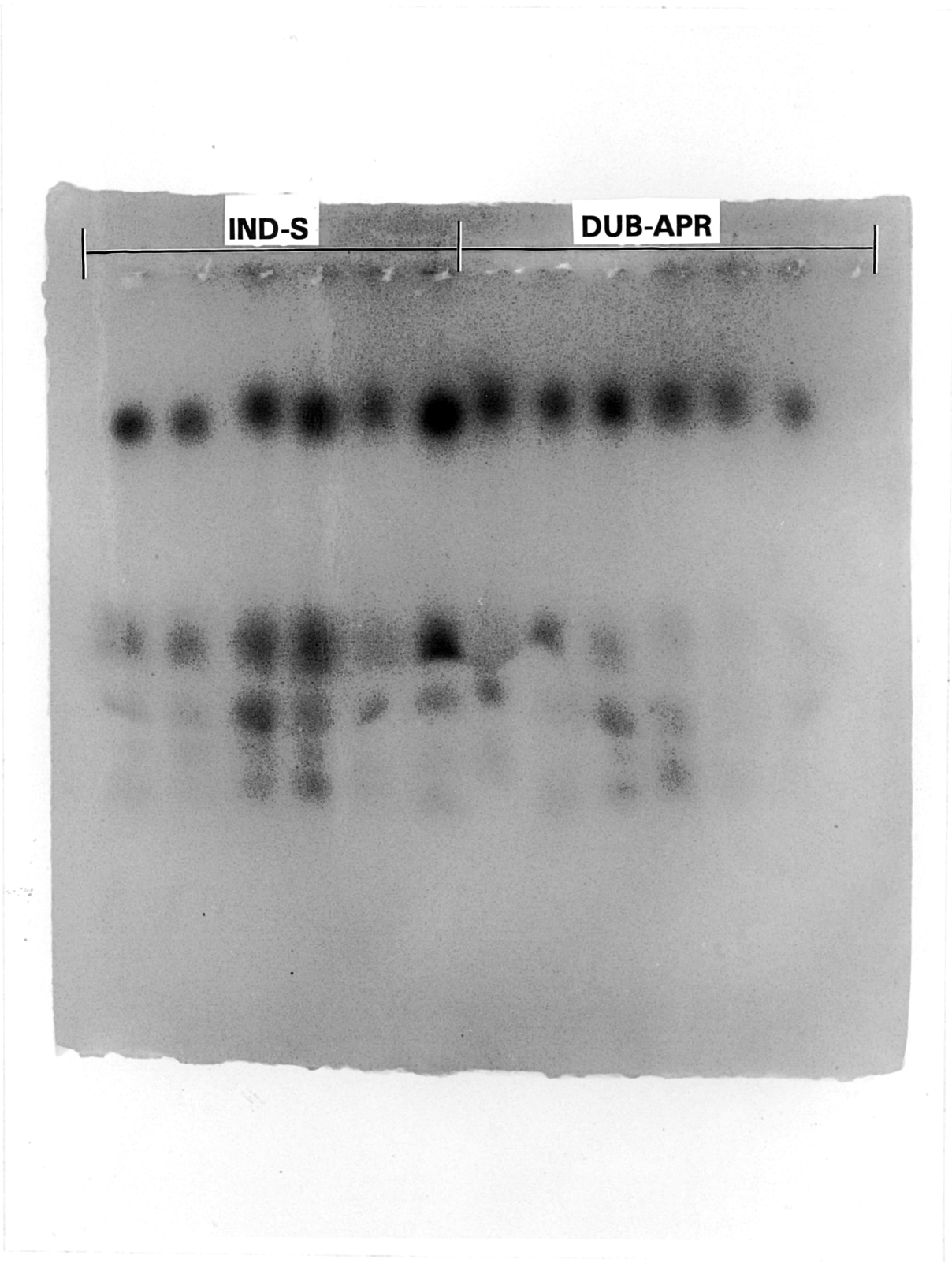
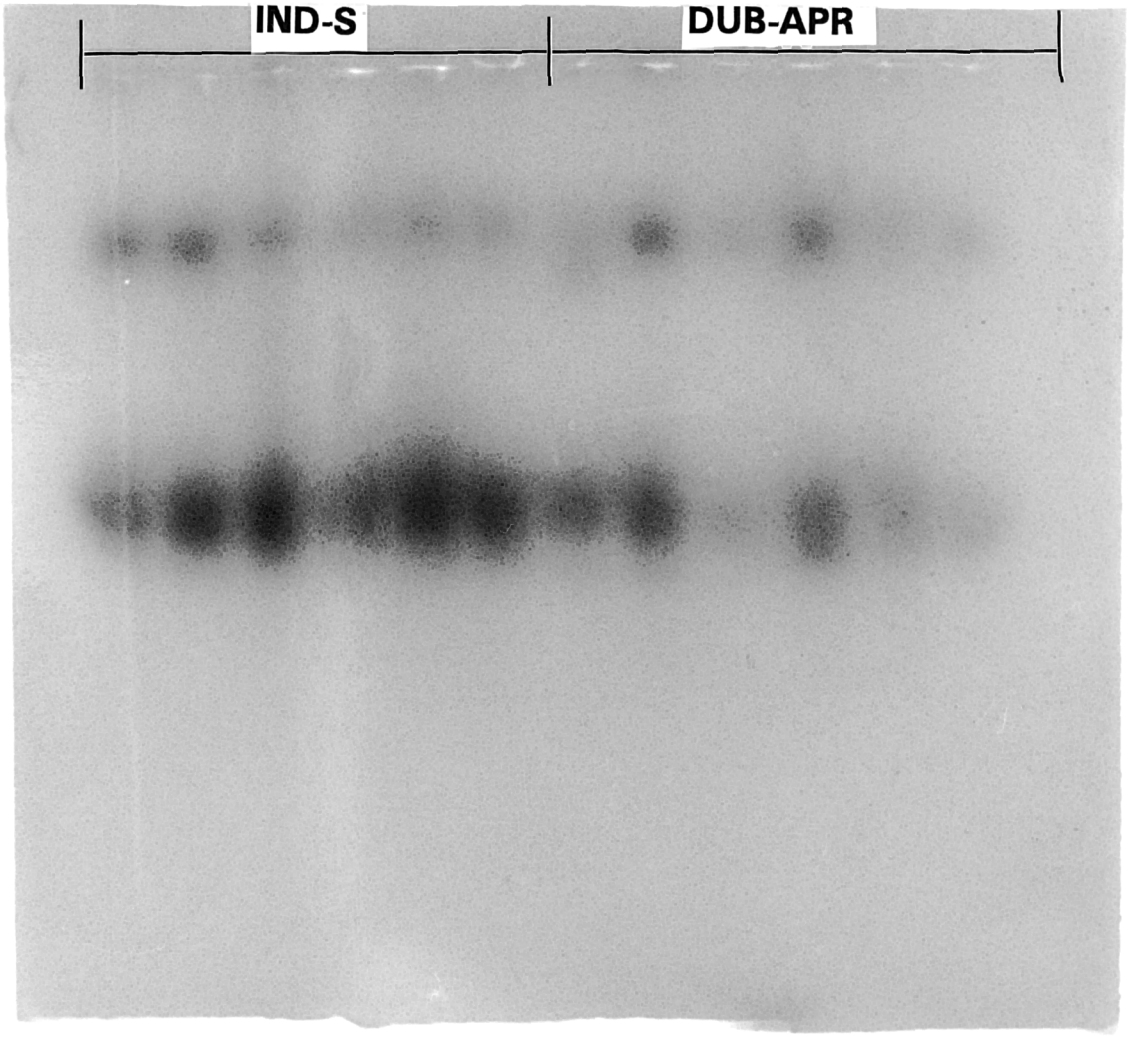


Fig. 4.7 Results of gel electrophoresis of esterases from permethrin susceptible (IND-S) and resistant (DUB-APR) strains of *An.stephensi* females. Esterase activity was visualized with ß-naphthyl acetate as a substrate



4.9 Electrophysiological investigation of target-site insensitivity mechanisms in permethrin-resistant and susceptible strains of *An.stephensi* adults

It has been known for many years that the nervous system of insects is highly susceptible to poisoning by pyrethroids. Although the precise sites of lethal actions of these compounds are poorly defined, effects upon both central and peripheral nervous activity are well documented. Pyrethroids show neurotoxicological activity on sensory neurones, motor neurones, and interneurons. At the molecular level, they have been found to affect several types of ion channel including GABA-chloride channels, voltage-sensitive Na^+ and K^+ channels, Ca^{2+} channels, and membrane pumps such as the Ca^{2+} and Ca^{2+}-Mg^{2+}-ATPase (Seabrook et al., 1988; Bloomquist, 1996).

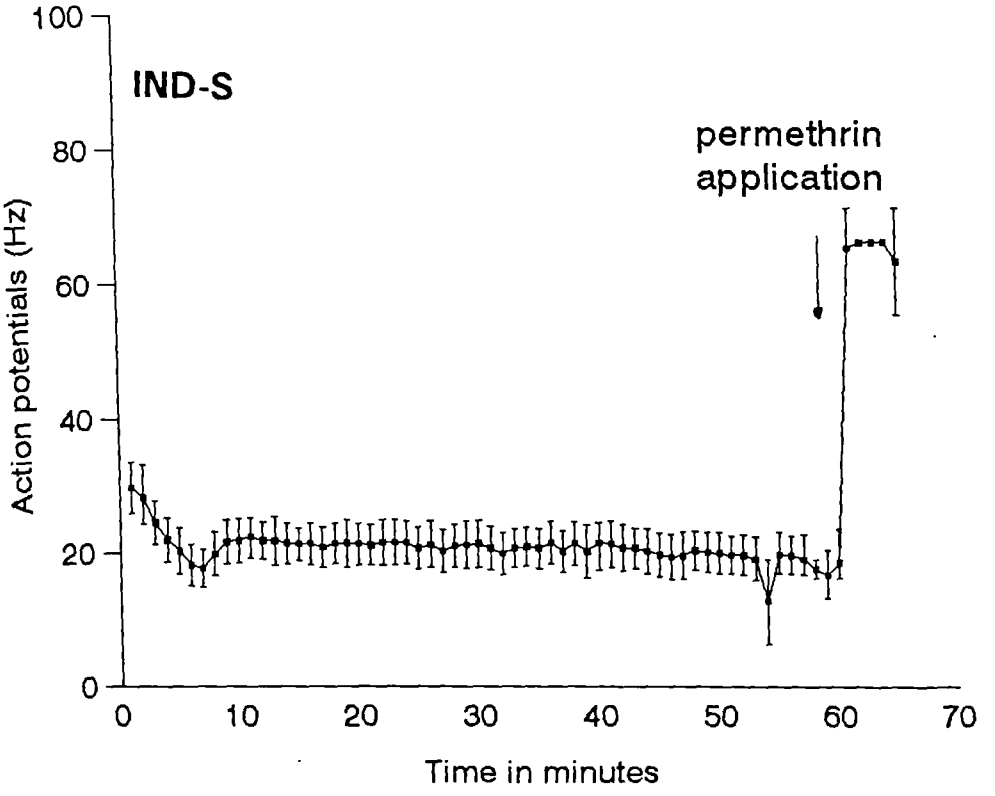
Several forms of resistance to pyrethroids have been found in insects. One such, knock- down resistance is associated with nerve insensitivity. Knock-down resistance (*kdr*) was first recognised by Busvine in 1951. Although various kinds of indirect evidence have been taken to indicate the involvement of nerve insensitivity in pyrethroid resistance including the existence of cross-resistance between DDT and pyrethroids, ineffectiveness of synergists, lack of evidence of delayed penetration or metabolic factors, presence of high levels of unmetabolized insecticides at the target site, and absence of behavioural symptoms of toxicology in the initial post-treatment period (Bloomquist & Miller, 1985), a more informative approach is to study the effect of insecticide directly on nerve preparations, thereby bypassing behavioural, penetration and metabolic resistance mechanisms.

In our previous experiments we have established that permethrin resistance in adults of *An.stephensi* is, in part due to esterases and cytochrome P-450s (see Table 4.2 and

Fig. 4.2). It has also been shown that ^{14}C-permethrin penetrates equally well in resistant and susceptible strains (Fig. 4.5). The fact that some resistance remains following synergism by inhibitors of these enzymes suggests that another component of resistance exists and we have postulated that this is a site insensitivity-type mechanism. To investigate this possibility, a neurophysiology study was undertaken to study the response of the nervous system to permethrin in resistant and susceptible mosquitoes. The methods are describe in section 3.2.25.

The results of a single dose application of permethrin indicated that the nervous system of the susceptible strain is very sensitive to permethrin, being affected by concentrations as low as 10^{-13} M. In contrast, the nervous system of the resistant strain is insensitive to this insecticide (Fig. 4.8). In a cumulative dose assay, the susceptible strain responded to the lowest concentration of permethrin (10^{-13} M), but the resistant strain required a greatly increased dose (10^{-8} - 10^{-7} M) to produce the same rise in frequency of action potentials (Fig. 4.9).

Fig. 4.8 Neural response of IND-S and DUB-APR strains of *An.stephensi* to a single dose (10^{-13} M) of permethrin. Vertical bars=SE



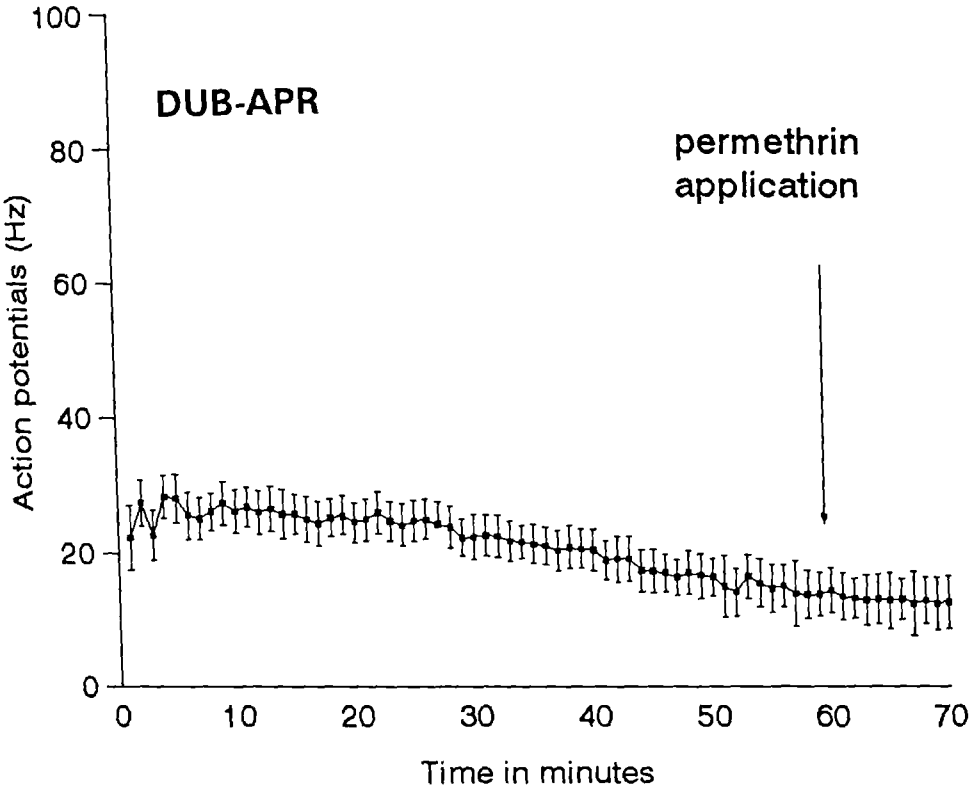
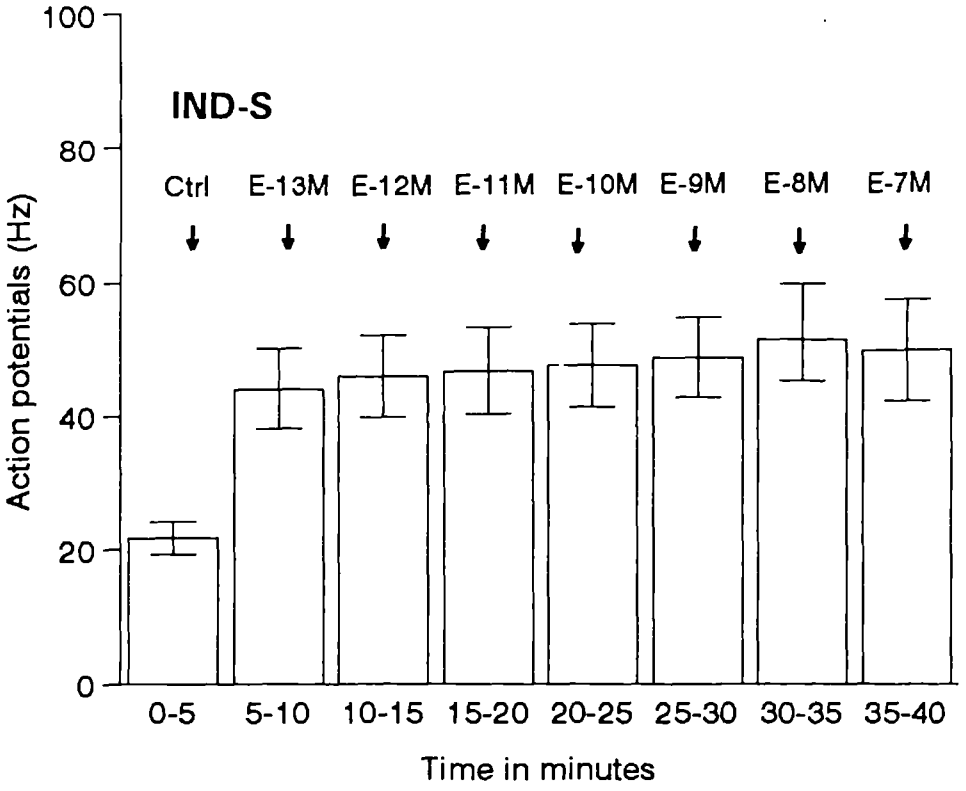
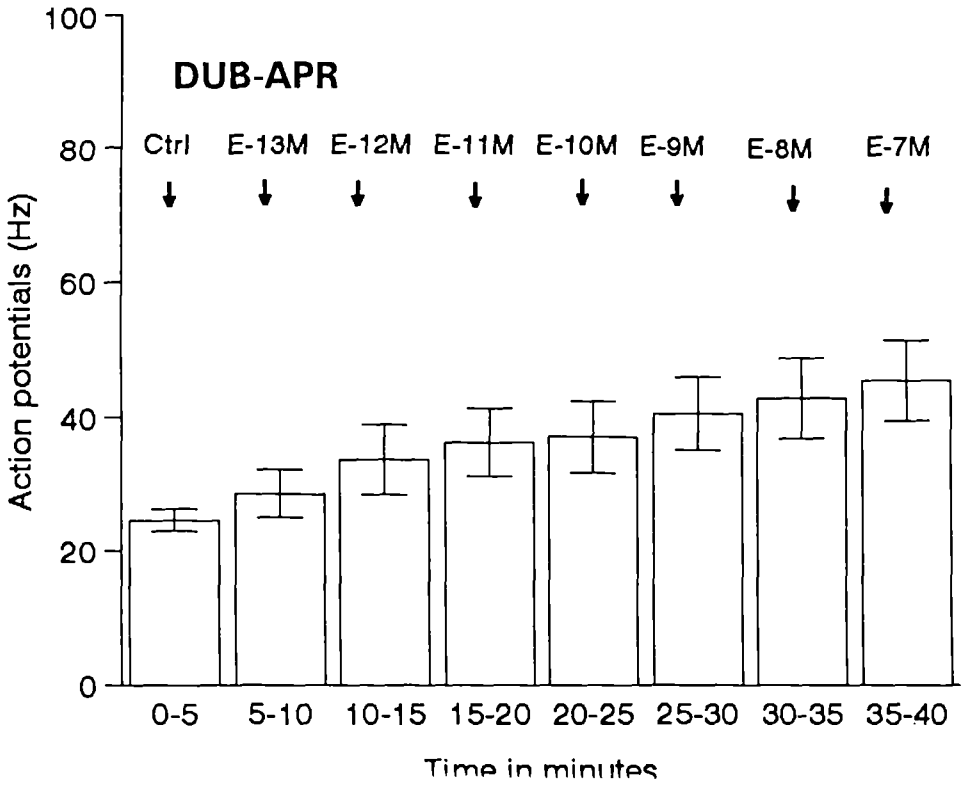


Fig. 4.9 Neural response of IND-S and DUB-APR strains of *An.stephensi* to a cumulative dose of permethrin. Dose of permethrin ranged from 10^{-13} to 10^{-7} M. Vertical bars=SE





4.10 Larval selection

The procedure of larval reselection were established by exposing about 200-250 4th instar larvae of DUB-LPR in 250 ml of tap water to which had been added 1 ml of permethrin to give the appropriate concentration (for initiating the re-selection 5 mg/l of permethrin was applied). After 24 h exposure time, live larvae were washed with tap water and maintained to produce the next progeny. After three generations of selection about 204-fold resistance relative to BAN strain and 100-fold relative to IND-S, a laboratory strain, were obtained (Tables 4.10 and 4.11). Enzyme studies to measure the mechanisms involved to permethrin resistance were carried out with F_3 generation of the selected strain, DUB-LPR.

Table 4.10 Summary of permethrin re-selection on larvae of DUB-LPR strain

Generations	Permethrin conc. (mg/l)	No. individuals exposed	No. survivors	Selection pressure: % mortality
F1	5	3900	807	79.3
F2	8	6400	2733	57.3
F3	13	5000	2230	55.4

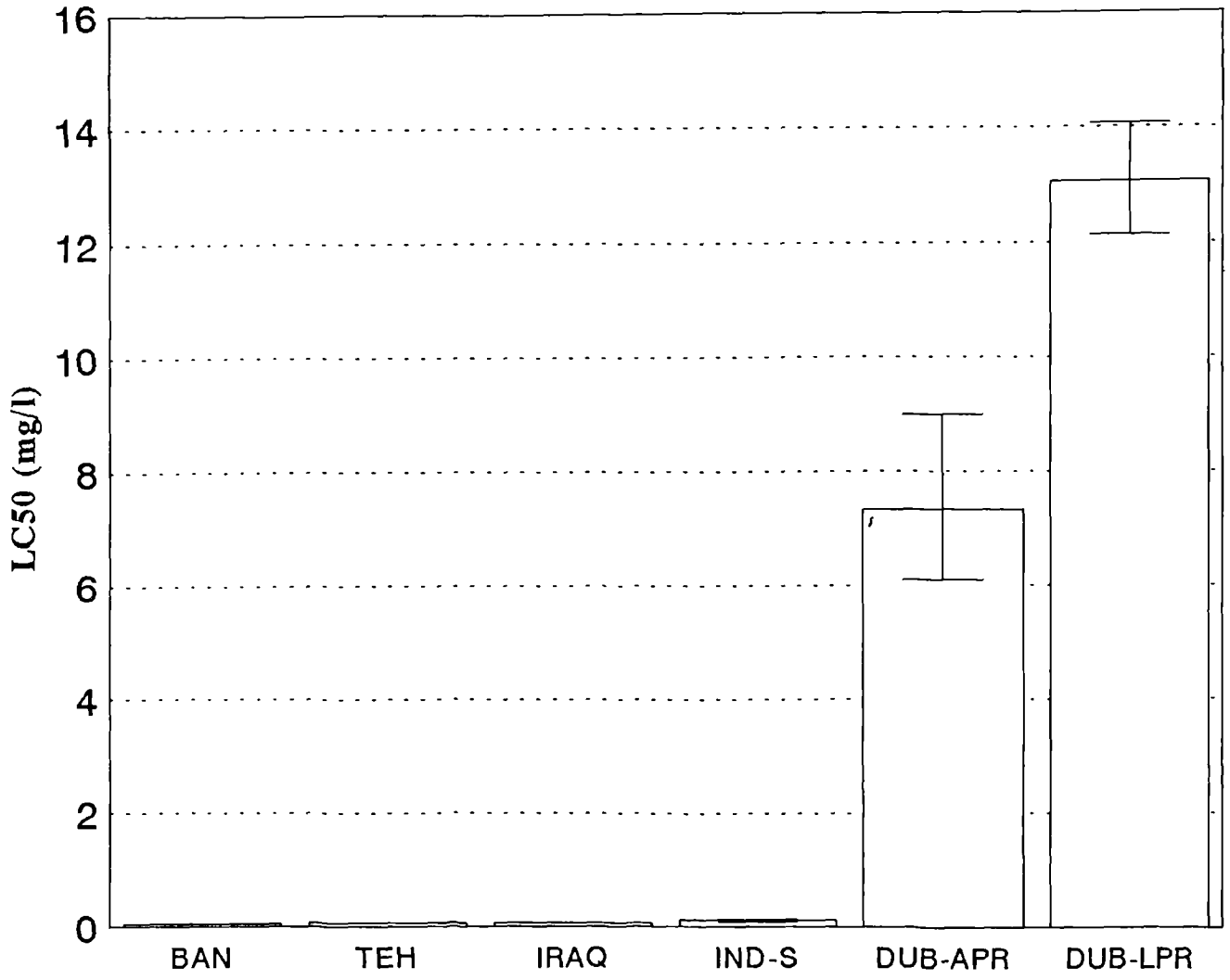
4.11 Larval susceptibility test to permethrin

Studies were carried out to determine the susceptibility status of mosquito larvae to permethrin. Tests were carried out according to the following standard procedures (see section 3.2.5). Data from tests were subjected to probit analysis, and the resistance ratio calculated by comparing the LC50's of the resistant strains to that of a susceptible strain. The results which are tabulated in Table 4.11 revealed that at the LC50 level DUB-LPR strain is 204 and 100 times more resistant to permethrin in comparison to BAN and IND-S strains respectively. The comparative efficacy of permethrin against larvae of different geographical strains is shown in Fig. 4.10. BAN, TEH, IRAQ and IND-S strains all showed a high level of susceptibility to permethrin. Larvae of the DUB-APR showed resistance to permethrin with resistance ratios of 115 and 56 fold compared to BAN and IND-S strains respectively, roughly half the level of resistance seen in the DUB-LPR strain.

Table 4.11 Probit regression line parameters of different strains of *An.stephensi* larvae to permethrin

Strains	a	b±SE	LC50±95%C.I	LC90±95%C.I	X^2(df)	P
TEH	12.83	11.19±0.10	0.069 0.070 0.073	0.089 0.093 0.099	13.89(6)	0.05>p>0.025
BAN	16.43	13.79±1.09	0.063 0.064 0.066	0.077 0.080 0.083	9.79(5)	>0.05
IRAQ	20.05	18.26±1.78	0.078 0.080 0.082	0.090 0.094 0.098	20.02(5)	<0.005
DUB-APR	-1.22	1.41±0.11	6.110 7.330 8.990	30.440 32.140 35.400	3.75(8)	>0.05
IND-S	2.16	2.44±0.02	0.110 0.130 0.150	0.340 0.440 0.610	5.35(3)	>0.05
DUB-LPR	-3.17	2.84±0.14	12.14 13.06 14.07	32.76 36.90 42.38	1.51(3)	>0.05

Fig. 4.10 Relative toxicity of permethrin to larvae of different strains of *An.stephensi*. Vertical bars=95%C.I.



4.12 Synergist studies with larvae

From permethrin insecticide tests it has been found that DUB-LPR is 100-fold resistant compared with IND-S strain. Oxidation and hydrolysis mediated by mono-oxygenases and esterases are both pathways of pyrethroid detoxification. An indirect method of quantitating the involvement of mono-oxygenases and esterases is by the use of the mono-oxygenase inhibitor, piperonyl butoxide (PB) and the esterase inhibitor, triphenyl phosphate (TPP).

The synergistic ratio in each case may be calculated by dividing the LC50 value of the insecticide alone by the LC50 value of the insecticide when used along with the synergist. A value greater than one indicates the involvement of a detoxification mechanism.

The efficacy of PB and TPP, in the profile of permethrin resistance were tested on larvae of the IND-S and DUB-LPR strains. The results are presented in Table 4.12. TPP had a very weak synergistic effect on resistant and susceptible strains with synergist ratios of 1.30 and 1.24. This indicates that esterase-related hydrolysis plays only a minor role in permethrin resistance in this strain. Strong synergist ratios were obtained when the resistant strain was tested with permethrin in the presence of PB. PB synergized permethrin in both resistant and susceptible strains to varying degrees. Pretreatment of resistant strain with PB increased the effectiveness of permethrin by 131 fold, whereas for susceptible strain it was 32.5 fold.

The results from Figs. 4.11 and 4.12 clearly indicated not only that PB increased the susceptibility of DUB-LPR strain towards that of the IND-S strain, but it completely cover the gap between the regression lines of the resistant and susceptible strains. Enhanced detoxication can theoretically be due to the production of a more active

enzyme or to an increased amount of an enzyme. In the case of the mono-oxygenase system, both situations can occur (Oppenoorth & Welling, 1976). The presence of PB synergism of permethrin in the IND-S strain (32.5-fold) and a high synergism in the DUB-LPR strain (131-fold) suggest that either a different or an altered form of the mono-oxygenase(s) or higher titers of mono-oxygenase(s) may be associated with the increased detoxification of permethrin. As in our synergist studies Priester & Georghiou (1978) have found synergist ratios of 5 and 4.5-fold in a susceptible strain of *Cx. quinquefasciatus* to *trans* and *cis*-permethrin when pretreated with PB and DEF. The synergistic effect of PB to susceptible strains of *Helicoverpa armigera* in the presence of *cis* and *trans*-cypermethrin and fenvalerate has also been reported by Ahmed & McCaffery (1991). A low synergist ratio with PB to deltamethrin has been reported in a susceptible strain of *Lucilia cuprina* (Kotez & Sales, 1994).

Table 4.12 The effect of synergists TPP and PB on larvae of *An. stephensi* strains tested with permethrin

Strains	with/out agent	LC_{50}±95%C.I.	LC_{90}±95% C.I.	a	b±SE	X^2 (df)	P	SR*
DUB-LPR	TPP	8.780 9.900 11.05	24.600 28.320 35.390	-2.75	2.76±0.23	4.57 (3)	>0.05	1.30
	PB	0.090 0.100 0.130	0.310 0.400 0.600	2.15	2.26±0.26	3.20 (3)	>0.05	131.0
	-	12.140 13.060 14.070	32.760 36.900 42.380	-3.17	2.84±0.14	1.51 (3)	>0.05	-
IND-S	TPP	0.086 0.105 0.129	0.620 0.880 1.390	1.36	1.38±0.1	16.04 (6)	>0.05	1.24
	PB	0.0030 0.0040 0.0045	0.009 0.015 0.011	6.3	2.56±0.24	12.43 (3)	<0.005	32.50
	-	0.110 0.130 0.150	0.340 0.440 0.610	2.16	2.44±0.02	5.35 (3)	>0.05	-

SR= LC50 insecticide/LC50 insecticide+ PB

Fig. 4.11 The effect of synergists TPP and PB on the profile of permethrin resistance in larvae of *An.stephensi*

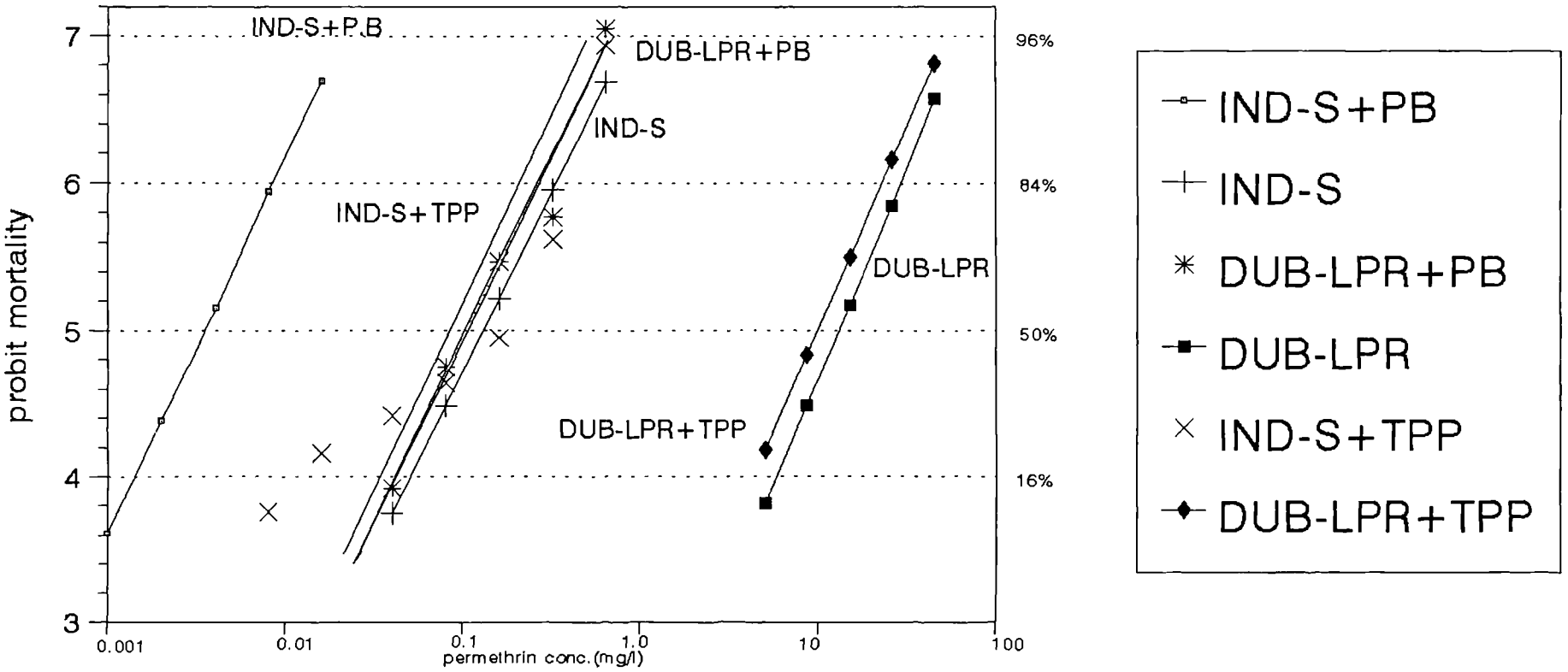
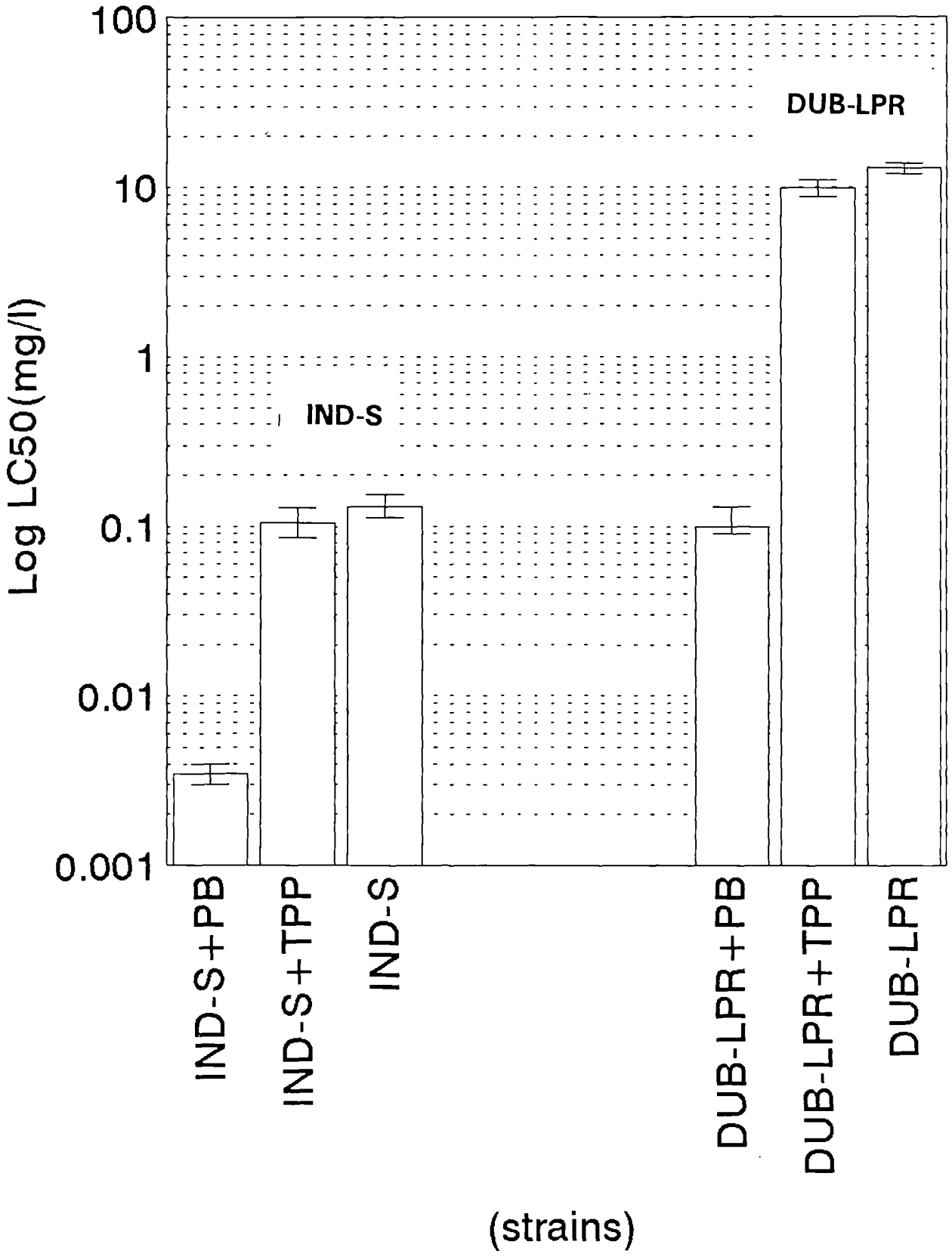


Fig. 4.12 The effect of synergists TPP and PB on the profile of permethrin resistance in larvae of *An.stephensi.* Vertical bars= 95% C.I.



4.13 Carboxylesterases enzyme assay for larvae

In our previous *in vivo* synergist studies we have established that esterases had a very weak effect on larvae of the permethrin-resistant strain with calculated synergist ratio of 1.3, so a series of experiments was carried out to measure esterase activity.

Esterase activity in larvae was assayed in a manner similar to that previously described for adults, using α and ß naphthyl-acetate as substrates of "A" and "B" esterases respectively. The results are shown in Figs. 4.13 and 4.14. As with adults, larval homogenates revealed 5 zones of esterase activity when α-naphthyl acetate was used. Relative activities of esterases from homogenate of resistant and susceptible strains by using ß-naphthyl acetate that are shown in Fig 4.14 revealed 2 zones. The pattern of ''A'' and ''B'' esterases is significantly different, but there is no obvious differences in the esterase activities among the strains, when either α or ß-naphthyl acetate was used as a substrate.

Fig. 4.13 Gel electrophoresis of esterases from larvae of permethrin-resistant (DUB-LPR) and susceptible (IND-S) strains of *An.stephensi*. Esterase activity was visualized with α-naphthyl acetate as a substrate

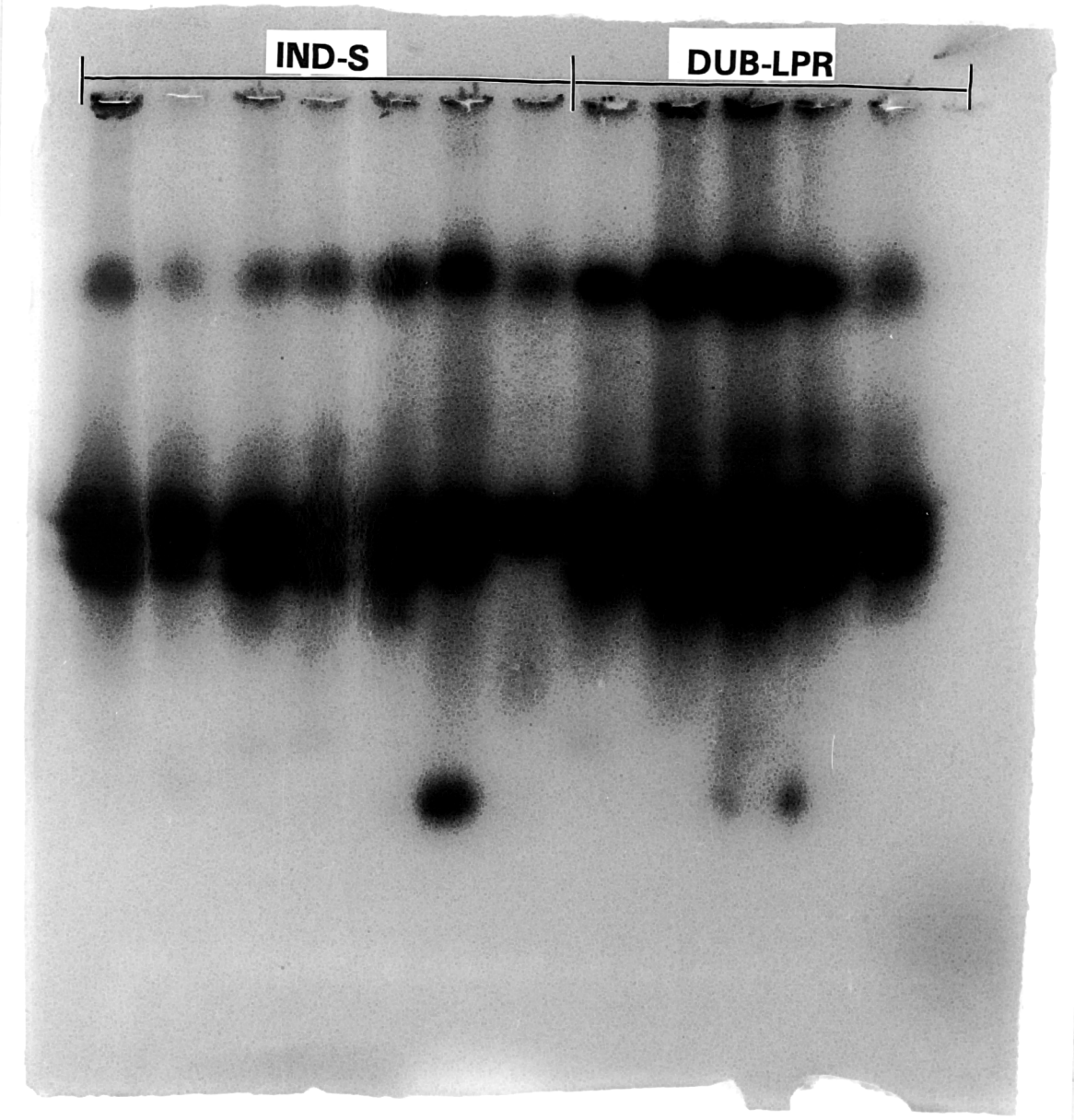
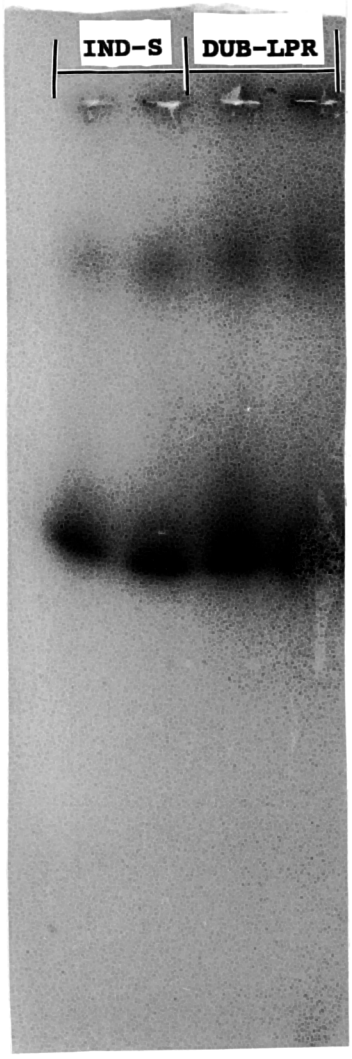


Fig. 4.14 Results of gel electrophoresis of esterases from larvae of permethrin-resistant (DUB-LPR) and susceptible (IND-S) strains of *An.stephensi*. Esterase activity was visualized with ß-naphthyl acetate as a substrate



4.14 Discussion

In this study permethrin has been studied in the laboratory for its comparative efficacy against different geographical strains of *An.stephensi*. Results from Tables 4.1 , 4.2 and Fig. 4.1 showed that geographical strains can be divided in two distinct groups, the susceptible and the resistant. Among those the BAN, TEH, IRAQ, BEECH, IND-S, can be considered as susceptible and DUB-APR, DUB-LPR as resistant strains. The LT50 value of DUB-APR strain which had been selected by Ladonni (1988) was 300 minutes with a resistance ratio of 10-fold compared with susceptible strain, IND-S. The DUB-APR strain was provided to the London School of Tropical Medicine & Hygiene in 1988. Subsequently similar *in vivo* study using 10μg/cm^2 impregnated paper were conducted by Magesa et al. (1994). They found 12.3% mortality after 4 h exposure in 1988, but in 1994 the same exposure time caused 41.8% mortality, i.e. 3.4-fold decline in resistance. After 3 years release of selection we have found that LT50 value for DUB-APR strain has declined to 82 minutes and the resistance ratio was reduced to 8-fold. Prolonged periods of no permethrin selection resulted in 3.7 and 1.25-fold declines in the LT50 and resistance ratio respectively. Resistance to permethrin in DUB-APR is controlled by a recessive genes (Ladonni, 1988; Curtis et al., 1990). This stepwise pattern for the reduction of pyrethroid resistance which is controlled by recessive genes was reported in a German cockroach population (Cochran, 1993). MacDonald et al. (1983) observed that in permethrin-resistant *M.domestica* held without selection for 18 months the level of resistance declined from 73-fold to 40-42-fold. The same decline in the horn fly, *H.irritans,* was reported by Kunz (1991). Results of his studies indicated that a low level of resistance will remain for extended periods, and re-exposure to pyrethroids

will result in rapid increase in resistance. Consideration must be given to how our results would affect *An.stephensi* control in resistance management schemes. In this respect Curtis et al. (1978) pointed out that populations of insects containing a high frequency of alleles for cyclodiene resistance often revert towards susceptibility when the insecticide is withdrawn from use. If reversion goes far enough it may be possible to effectively re-introduce the insecticide until re-selection of resistance cause control problem once again (Curtis, 1987b).

To monitor the involvement of enzyme activity, synergist studies were conducted using inhibitors of esterases and mixed function oxidase TPP and PB. Results from Table 4.2 and Fig. 4.2 indicated that cytochrome P-450s and esterases are responsible for two-thirds of the 8-fold enhanced tolerance of the DUB-APR resistant strain as compared with a sensitive strain, IND-S. The possibility of *kdr*-type resistance was postulated in addition to enzyme involvement. The knock-down behaviour of resistant and susceptible strains which is summarized in Table 4.3 and illustrated in Fig. 4.3, revealed that the resistant strain is 3.4-fold resistant to knock-down compared to the susceptible strain. As in our study, knock-down behaviour of pyrethroid-resistant and susceptible strains of larvae of *H.irritans* was determined when larvae were exposed to filter paper treated with permethrin (Crosby et al., 1991). Their study showed that resistant horn fly larvae were 42-times more resistant to knock-down than the susceptible larvae. Neurophysiological studies demonstrated that the basis for *kdr* is a reduced sensitivity of the nervous system to the neurotoxic action of pyrethroids. Magesa et al. (1994) exposed the DUB-APR and BEECH strains of *An.stephensi* for 2 minutes to a range of doses of permethrin on nylon netting and knock-down was measured 1 h after treatment. A resistance ratio of 370.1 was observed and exposure

for 2 minutes to 200mg/m^2 almost discriminated between the two strains.

Irritability studies of resistant and susceptible strains, the results of which are shown in Table 4.4 and Fig. 4.4, indicated that the resistant strain is 2.8 times less irritable to permethrin in comparison with susceptible ones. In contrast to our irritability results, Lockwood et al. (1985) have found that pyrethroid-resistant populations of the horn fly were significantly more irritated by permethrin at lower doses than the susceptible populations. However, the form of behavioural resistance to deltamethrin was different from that found with permethrin. The threshold for response in the resistant strain was increased with deltamethrin. They concluded that the behavioural response of resistant horn fly to permethrin would be selectively advantageous. In populations of horn fly two classes of behavioural resistance are recognized: stimulus-dependent, which requires sensory stimulation of the insect for avoidance to occur. Stimulus-dependent behavioural resistance includes irritability, in which an insect is stimulated to leave a toxic environment upon contact, or before contact with a treated surface (Lockwood et al., 1984). Stimulus-independent behavioural resistance generally involves some forms of exophily, in which an insect avoids exposure to the toxicant by prolonged occupation of a nontoxic habitat (Lockwood et al., 1984). In populations with stimulus-independent behaviour the resistant strain uses some cue (other than the toxicant) to identify consistently untreated habitats (Byford et al., 1987b).

In order to carry out further investigation on behavioural changes in terms of responsiveness to being caught with an aspirator the method of Rowland (1991) was followed. Results from Table 4.6 showed that the resistant strain is less responsive (1.5-fold) than the IND-S strain to the movement of an aspirator and this is in

agreement with the study of Rowland (1991) who found that cyclodiene-resistant *An.stephensi* are 1.6-fold less responsive to movement. Many resistance studies have demonstrated a negative association between behavioural (measured as irritability) and physiological resistance (Sparks et al., 1989). Trapido (1954) and Georghiou (1972) have postulated that insects must be physiologically susceptible to a pesticide to evolve stimulus-dependent behavioural resistance and that, as physiological resistance evolves, behavioral resistance declines. In our study the physiologically resistant strain of *An.stephensi* (DUB-APR) was shown to be at a behavioural disadvantage and fitness costs may be associated with physiological resistance.

Our *in vivo* experiments showed that permethrin resistance in adult *An.stephensi* is due to at least three separate mechanisms. Cytochrome P-450s and esterases are responsible for two-thirds of the 8-fold enhanced tolerance of the DUB-APR resistant strain as compared with a sensitive strain, IND-S. In addition, from all component tests on adult females of DUB-APR strain including knock-down behaviour test, irritability test and responsiveness to movement, we have postulated that target site insensitivity is also involved. To test this hypothesis we conducted a neurophysiological study with the thoracic nerves of adult female mosquitoes under perfusion with saline. Spontaneous neural activity was recorded with saline alone and in the presence of permethrin. There was no change in the frequency of spontaneous firing during a 1h application of saline in either strain. A single dose application of 10^{-13} M permethrin rapidly produced a 3-fold increase in firing rate in the susceptible strain. In contrast, the resistant strain did not respond to this concentration (Fig. 4.8). In a cumulative dose assay, the susceptible strain responded to the lowest concentration of permethrin (10^{-13} M), but the resistant strain required a much higher

dose (10^{-8} - 10^{-7} M) to produce the same rise in frequency of action potentials (Fig. 4.9). This strongly suggests that nerve insensitivity contributes to permethrin resistance in this mosquito.

Nicholson & Miller (1985) found that insensitive or tolerant nerves of *Heliothis virescens* either required longer periods of exposure to induce poisoning symptoms than susceptible preparations, or require a higher concentration of insecticide to induce the same poisoning symptoms as susceptible preparations.

Our neurophysiology results are in agreement with studies upon susceptible and *kdr* strains of house fly, *Musca domestica*, (Nicholson et al., 1979b; Osborne & Hart, 1979). These authors found that the lowest concentration of permethrin required to elicit a response in a susceptible strain could be below 10^{-10} M, and as high as 10^{-7} M for *kdr* larvae. Thus the resistance to permethrin conferred on the *kdr* strain was over 1000-fold. Salgado et al. (1983b) showed that larvae of a permethrin-resistant strain of *An.stephensi* required a 20-fold higher concentration of insecticide to bring about an increase in the spontaneous action potential rate in comparison with a susceptible strain. For larvae of resistant and susceptible strains of *Culex quinquefasciatus* the equivalent figure was 2000-fold. Neurophysiological studies on the *kdr*-type resistant larvae of *An. stephensi* (Omer et al., 1980) revealed that the resistant strain needed 20 times more permethrin than the susceptible strain to induce an increase in the frequency of miniature end-plate potentials. In a neurophysiology assay the EC_{50} value for the susceptible strain of house fly adult was found to be 10^{-12} M, the value for *kdr* strain being 10^{-7} M to permethrin (Gibson et al., 1988).

In our study we found a very wide difference in nerve sensitivity between the resistant and susceptible strains. This difference in sensitivity is much greater than the

8-fold resistance shown by *in vivo* bioassays and is comparable to data obtained with *Musca domestica* (Smallcombe, 1981). This difference could reside in factors present in the intact insect and enhanced by *in vitro* direct application.

Further studies are needed to find exactly where in the nervous system permethrin acts to cause the neurophysiological effects observed. The neurophysiology extracellular recording technique developed here is not specific for a single nerve cell. Action potentials of several nerves are recorded and nerve insensitivity detected by the assay probably is a function of ion channels or synaptic neurotransmitter release or both.

Results on rate of penetration of ^{14}C-permethrin (Tables 4.8, 4.9) and (Fig. 4.5) indicated that radiolabelled permethrin penetrated equally well in resistant and susceptible strains.

In an study which was conducted by Chakravorthy & Kalyanasundaram (1992), the laboratory strain of *An.stephensi* adults was selected with permethrin. The selection resulted in the development of resistance in F5 generation by 13-fold to permethrin, and cross-resistance of 7-fold to cypermethrin, 9-fold to alphamethrin and 10-fold to deltamethrin. The development of cross-resistance to 4% DDT was also detected. To investigate the mechanisms of permethrin-resistance an *in vivo* bioassay was conducted using piperonyl butoxide. The synergistic effect of piperonyl butoxide with permethrin (synergist ratio=2-fold) did not overcome the development of resistance suggesting the involvement of a *kdr*-type mechanism. In our study only one-third of resistance to permethrin in DUB-APR was due to target site insensitivity.

Genetics of resistance of pyrethroid-selected house flies has been extensively studied by Farnham (1973, 1977). Four resistance factors were isolated genetically from the

NPR strain of house flies. The four factors were; *pen*, which reduces the rate of penetration of insecticide through the cuticle. *kdr-NPR*, a general pyrethroid resistance mechanism unaffected by the synergist sesamex, *py-ses*, a mechanism of resistance to natural pyrethrins that can be suppressed by sesamex; and *py-ex*, a factor that gives strong resistance to synergized natural pyrethrins and to the new synthetic esters.

In a relatively similar study to our works a strain of house fly (147-R) was selected with *trans*-permethrin to level of 97-fold by DeVries & Geroghiou (1981a) and the mechanisms of permethrin resistance were studied in this strain. The *in vivo* tests with esterase inhibitor produced little synergism (1.6-fold). 12-fold resistance synergism was obtained with piperonyl butoxide. The application of both synergists yielded 13-fold synergist ratio. *In vitro* studies also revealed 2-fold increase in the amount of cytochrome *b5* and P450 in resistant strain. Electrophysiological assay demonstrated that the CNS of the resistant strain was not affected by dosage of pyrethroid that caused uncoupling in the susceptible strain. The accumulation study showed that *trans*-[^{14}C]permethrin penetrated more slowly into the resistant strain. The evidence from their results supports the conclusion that several resistant factors including MFO, esterases and mainly decreased sensitivity of the nervous system are the principal mechanisms of permethrin resistance in house fly which are in agreement to our results in *An.stephensi* adult.

In another study the mechanisms responsible for >6000-fold permethrin resistance in a pyrethroid-resistant strain of house fly, Learn-PYR, were investigated by Scott & Georghiou (1986b). Through electrophysiological studies, *in vitro* metabolism, *in vivo* penetration and synergists studies, it was demonstrated that the resistance mechanisms consisted of enhanced metabolic detoxification via MFO system, target

site insensitivity and decreased cuticular penetration. Although the involvement of esterases enzymes has not been mentioned by the authors, 2.3-fold synergist ratio has been detected in the presence of esterase inhibitor, DEF. In contrast to the findings of Farnham (1973, 1977), Scott & Gerorghiou (1986b) and DeVries & Georghiou (1981a,b) lack of *kdr* or *super-kdr* and instead the presence of an oxidative pathway in the field-collected permethrin-resistant strains of house flies has been implicated by Nicholson & Sawicki (1982). They concluded that prolonged treatment of buildings with pyrethrins senergized with PB play a major role in selecting for a permethrin-resistant strain through the oxidative mechanism.

In a study by Bull et al. (1988) pyrethroid resistance developed rapidly in a laboratory strain of the horn fly (*H.irritans*), which was selected with permethrin. After 30 generations of selection, LC50's for the *cis* and *trans*-permethrin isomers increased 48.9 and 70.5 times, respectively. The resistant flies were cross-resistant to DDT. Studies of factors influencing development of resistance to *trans*-permethrin indicated no important differences between susceptible and resistant strains in rates of absorption, internal accumulation, or excretion following topical application with ^{14}C-permethrin. Tests of the topical toxicity of *trans*-permethrin to resistant flies demonstrated that co-administration of PB caused a 10-fold reduction in the LC50 and DEF (an esterase inhibitor) caused 4-fold reduction. However, the synergists did not overcome the development of resistance. From their results it was apparent that target site insensitivity was the major resistance factor. Based on studies of Bull et al. (1988); Byford et al. (1985); Byford et al. (1987a,b); Sparks et al. (1989) the mechanisms of pyrethroid resistance in adults of *H.irritans* have been associated with various combinations of altered penetration, *kdr*, enhanced metabolism via MFO and

esterases, and altered behaviour. It is apparent that pyrethroid resistance in this insect is a multifactorial phenomenon involving biochemical, physiological, and behavioural factors.

A pyrethroid-resistant (>100-fold) German cockroach (*B.germanica*) strain was compared to a susceptible strain in respect of possible mechanisms of permethrin resistance by Anspaugh et al. (1994). Based on topical application, the resistant strain was 20-times more resistant to knock-down. The cuticular penetration effect was perceived as minimal, only a 1.4-fold ratio in penetration was observed. Data from *in vivo* bioassays with synergists suggest that increased metabolism may be a component of resistance in this strain. An *in vivo* experiment was done to further examine the role of metabolism, P-450 concentration and mono-oxygenase activity. Results from *in vitro* metabolism showed that 2.1-fold increase in P-450 concentration and 6.9-fold in mono-oxygenase activity in resistant strains as compared to the susceptible one. By employing polyacrylamide gel electrophoresis and α-naphthyl acetate, three different esterase isozymes (E1, E3, E4) have been found in both strains, but a novel esterase , E2, was discovered in the resistant cockroach which was absent in the susceptible strain. From the colorimetric diagnostic assay they concluded that the elevation in esterase activity in resistant cockroaches could be the result of an increase in constitutive esterases and/or because of the appearance of a novel esterase. In our starch gel electrophoresis assay using α-naphthyl acetate as a substrate we have found 5 zones of esterase activity which are present in both resistant and susceptible strains. In a similar study Hemingway et al. (1993) tested different pyrethroids against 30 strains of *B.germanica* from 3 different continents. A full ranges of resistance mechanisms including elevated esterases, an oxidase-based

mechanism and *kdr* were involved in resistance.

Several Asian strains of adults of *Aedes aegypti* were tested with different insecticides by Chadwick et al. (1977). Mosquitoes from Bangkok showed resistance to DDT and pyrethroids. Resistance in this strain was postulated to be due to oxidases, esterases and perhaps other resistance mechanisms. Whether *kdr* and reduced penetration was present in this strain was not clear. Similarly *kdr* resistance has been suggested in DDT-deltamethrin resistant adults of *Ae.aegypti* (Mourya et al., 1993).

Brealy et al. (1984) did not find any significant differences in penetration, metabolism and excretion of [^{3}H] *trans*-permethrin in permethrin-resistant and susceptible strains of *Ae.eagypti*. Although there was no *in vivo* study of mixed function oxidase and esterase inhibitors, but they concluded that permethrin resistance was due to *kdr*-type mechanism.

The result from our esterase gel electrophoresis using α- and ß-naphthyl acetate as a substrate of A and B groups of esterases are presented in Figs. 4.6 and 4.7. Although there were subtle difference in the activity of esterases toward the substrate used, there is no obvious significant differences in esterase activity between permethrin-resistant and susceptible strains of *An.stephensi*. Townson (1969) recorded the differentiation of different strains of *Ae.aegypti* adults by means of electrophoresis of protein using α-naphthyl acetate. Six different esterase bands have been detected, although all six were rarely present in one mosquito. An electrophoresis survey of strains of *Cx.tritaeniorhynchus* adults has revealed 5 areas of esterase activity toward α-naphthyl acetate which were designated areas 1, 2, 3, 4 and 5 in ascending order toward the anode (Iqbal et al., 1973). In a study by Garnett & French (1971) wild populations of *Cx.quinquefasciatus* were chosen for a study of esterase enzyme system

by means of acrylamide gel electrophoresis using α-naphthyl acetate. 13 different forms of esterases were observed in adult populations. In a study by Sahgal et al. (1994) adult females of *An.stephensi* were collected from the field in India and maintained in an insectary. Permethrin selection was applied to larvae. Selection for 25 generations caused 37-fold resistance to permethrin at the LC50 level. The possibility of ester hydrolysis as a mechanism of permethrin resistance has been investigated by estimating the levels of non-specific ß-esterases in 4th instar larvae and 3-day old males and females using microplate assays and ß-naphthyl acetate as a substrate. The elevated esterase activity in the larvae of the pyrethroid-resistant strain showed a marginal increase as the frequency increased from 4.6% in the susceptible strain to 6.7% in the permethrin-resistant strain. In the case of adult males the esterase level did not show any distinction between the resistant and susceptible strains. However, female adults exhibited more esterase activity as compared to males. In another experiment which was conducted by the same authors there were no distinct differences between pyrethroid-resistant and susceptible larvae and adults of *Ae.aegypti* in terms of ß-esterase activity. In the present study using starch gel electrophoresis and ß-naphthyl acetate there were no significant differences between permethrin-resistant and susceptible larvae and adults in the pattern of bands.

Polyacrylamide gel electrophoresis of specimens of permethrin selected and susceptible strains of *Cx.quinquefasciatus* was undertaken by Hemingway et al. (1990) to determine the pattern of bands which were stained with a mixture of α- and ß-naphthyl acetate. Two bands of esterase activity were found in each strain. The same pattern of bands have also been found in organophosphate-selected and susceptible strains. Similarly the same pattern of esterase bands has been observed in our gel

electrophoresis assay in both larvae and adults of permethrin-resistant and susceptible strains of *An.stephensi* when ß-naphthyl acetate was used.

Zymograms of soluble esterases from permethrin-resistant and susceptible strains of the mite *Amblyseius fallacis* showed that at least 15 esterases (E1-E15) sensitive to α-naphthyl acetate could be seen in a resistant strain, but only E6 and E10 esterases were found from a susceptible strain. Five esterases (E6-E10) in the resistant strain showed remarkably high permethrin hydrolyzing activity compared to the susceptible strain (Chang & Whalon, 1986).

There are several reports on esterase conferring organophosphate and carbamate resistance in insects. For example in *Cx.tarsalis* (Whyard et al., 1994); *Cx.quinquefasciatus* (Jayawardena et al., 1994), *Cx.pipiens* complex (Callaghan et al., 1994) and *B.germanica* (Prabhakaran & Kamble, 1994). Similarly the role of esterases in pyrethroid resistance has been established in several arthropods (Abdel-Aal & Soderlund, 1980; Jao & Casida, 1974; Riskallah et al., 1983; Riddles et al., 1983; Holden, 1979; Yu, 1990; Dowd & Sparks, 1987; Ishaaya & Casida, 1980; Ishaaya et al., 1987; Delmore et al., 1988). Esterase dependent cross-resistance between OPs and pyrethroids has been detected in several species. In OP resistant *M.domestica* and *Culex* mosquitoes, the esterases responsible for cross-resistance are thought to be involved in pyrethroid hydrolysis (Soderlund & Bloomquist, 1990).

In a bioassay with multiresistant larvae of Guatemalan *An.albimanus* which was carried out by Brogdon & Barber (1990) the LC50 values for fenitrothion and deltamethrin were 24- and 57-fold greater than in the susceptible strain, respectively. Resistance to both insecticides was nearly abolished by the esterase inhibitor, DEF. Hence it was concluded that an elevated non-specific esterase mechanism conferred

cross-resistance between an organophosphate insecticide, fenitrothion, and a pyrethroid, deltamethrin. They concluded that there was a threat to malaria vector control due to the existence of a common resistance mechanism to both insecticides. Many esterases exist as multiple forms (isozymes), some of which degrade insecticides, but most of the isozymes have not been assigned any particular physiological or biochemical function. Each isozyme probably has a certain range of substrates, which explains why some isozymes are responsible for certain types of insecticide resistance (Xu & Brindley, 1994).

In this study, larval reselection of DUB-LPR with permethrin for 3 generations increased resistance to permethrin to a level of 204- and 100-fold relative to BAN and IND-S strains, respectively. Comparison of the efficacy of permethrin against larvae of different geographical strains showed a high a level of susceptibility to permethrin in BAN, TEH, IRAQ, and IND-S strains. In contrast, larvae of DUB-LPR and DUB-APR are highly resistant to this insecticide (see Table 4.11 and Fig. 4.10).

The efficacy of PB and TPP on the level of permethrin resistance were tested on the IND-S and DUB-LPR strains. The results are presented in Table 4.12 and Fig. 4.11. TPP had very weak synergistic effect on resistant and susceptible strains with synergist ratios of 1.30 and 1.24, respectively. This indicates that hydrolysis may plays a minor role in the permethrin resistance. Strong synergism was observed when the resistant strain was tested with permethrin in the presence of PB. PB synergized permethrin in resistant and susceptible strains to different degrees. Pretreatment of DUB-LPR strain with PB increased the effectiveness of permethrin by 131-fold, whereas for IND-S strain the increase was 32.5-fold.

The results in Fig. 4.12 clearly indicated that PB increased the susceptibility of DUB-

LPR strain rendering it virtually as susceptible as the susceptible IND-S strain. Results from Fig. 4.12 showed that permethrin resistance can be reduced almost to the susceptible level with PB.

Larvae of *Ae.aegypti* and *An.gambiae* showed an increase in tolerance to pyrethroids (Rongsriyam & Busvine, 1975). The effect of PB was assessed in both tolerant and susceptible larvae. Synergist ratios 1.04 and 14.5 were obtained with susceptible strains of *Ae.aegypti* and *An.gambiae* respectively, but for pyrethroid tolerant strains the synergist ratios were 4.14 and 21.30-fold respectively. The presence of synergistic action by PB suggested that the tolerance depended on the MFO system. The results obtained with PB in *Ae.aegypti* and *An.gambiae* larvae (Rongsriyam & Busvine, 1975) indicated that synergism was more effective against tolerant larvae than in the susceptible strains. The same effect of PB on susceptible strains has been reported in *Cx.quinquefasciatus* (Priester & Georghiou, 1978, 1980a), *M.domestica* (DeVries & Georghiou, 1981b), *Heliothis armigera* (Ahmad & McCaffery, 1991), and *Lucilia cuprina* (Kotze & Sales, 1994) when tested with pyrethroids. In our study PB synergism was slight with permethrin in the susceptible strain, IND-S. The susceptible strain has its own detoxification machinery but it is in less active than in the resistant strain. The presence of synergism in the susceptible strain could be explained in this way.

Kumar et al. (1991) selected larvae of *Ae.aegypti* with deltamethrin. Selection yielded a 703-fold resistance ratio after 40 generations. Treatment with PB showed evidence of synergism in the resistant larvae (SR= 9.01) and resistance was mainly due to the MFO system. The possibility of ester hydrolysis as a mechanism of deltamethrin resistance has been investigated by estimating the levels of non-specific ß-esterases.

The results indicated no significant differences between the two strains in esterase activity (Sahgal et al., 1994)

Priester & Georghiou (1978) selected larvae of *Cx.quinquefasciatus* with d-*trans* permethrin for 18 generations in the laboratory. A high level of resistance (>4000-fold) has been induced in this species. The d-*trans*-permethrin selected strain also manifested a high level of cross-resistance (1021-fold) to d-*cis* permethrin. *In vivo* studies using esterase inhibitor (DEF) and MFO inhibitor (PB) were undertaken to find the mechanisms of d-*trans* permethrin resistance in this strain. Both resistant and susceptible strains were pretreated with DEF and PB followed by a permethrin test. DEF synergized permethrin in susceptible and resistant strains, with synergist ratios of 3.7 and 2-fold respectively. The effect of PB was more pronounced in the resistant strain, being 16-fold compared with 5.5-fold in the susceptible strain. Additional data, based on cross-resistance characteristics to DDT and other pyrethroids and lack of penetration differences (Priester & Georghiou, 1980 a,b) suggested that oxidative and hydrolysis mechanisms may play only a minor role in the resistance and they conclude that reduced sensitivity of the target site is the primary mechanism of resistance. In a similar study to that of Priester & Georghiou (1978), Amin & Hemingway (1989) selected a strain of *Cx.quinquefasciatus* from Saudi Arabia with permethrin. After 20 generations a high level (>1000-fold) of resistance to permethrin was detected. The permethrin selected strain also showed cross-resistance to deltamethrin and DDT but not to lambdacyhalothrin. They suggested that resistance in *Cx.quinquefasciatus* was mainly oxidative which contrasts with the finding of Priester & Georghiou (1978).

Omer et al. (1980) found that larvae of *An.stephensi* from Pakistan initially showed

a low level resistance to DDT but was susceptible to pyrethroids. Six generations of selection with DDT produced 144-fold resistance to it. The DDT-selected strain developed cross-resistance to *trans*-permethrin (12-fold) and to *cis*-permethrin (18-fold). The highest cross-resistance to *trans*-permethrin (19-fold) and to *cis*-permethrin (23-fold) was detected in a sub-strain that had been selected with DDT in conjunction with the synergist DMC. Synergist studies provided no evidence for enhanced metabolism due to dehydrochlorinase or oxidase, of either insecticide in the resistant strains. Neurophysiological studies carried out by Omer et al. (1980), however, showed that the resistant strains required approximately 20 times more permethrin than the susceptible strain to induce an increase in the frequency of miniature endplate potentials. Although there were no additional experiments on the role of esterases and reduced penetration in the resistant strain, they concluded that resistance in larvae of *An.stephensi* was mainly of the *kdr* type. Work of Sahgal et al. (1994) proved that ß-esterase plays a role in permethrin resistance larvae of *An.stephensi* from India. Ladonni (1988) selected a strain of *An.stephensi* from Dubai which was resistant to DDT. At the F7 generations the LC50 of the selected strain had increased 138-fold relative to its parent and 1030-fold compared to the IND-S strains. Although PB could not eliminate the resistance completely (SR=426-fold), an oxidase-based mechanism was postulated as an important factor in resistance. He showed that permethrin resistance in larvae was inherited as a monofactorial character. A difference in response to permethrin can be selected in larvae of *An.stephensi* of a similar magnitude to that found by Omer et al. (1980), which is also not reduced by oxidase or dehydrochlorinase-inhibiting synergists. However, this was shown to be subject to multigenic inheritance and not associated with the major source of DDT resistance

which was controlled by a single gene located on chromosome III (Malcolm, 1988b). Kumar et al. (1991) selected a field-collected strain of *An.stephensi* larvae with deltamethrin. Selection yielded a 60-fold resistance ratio after 40 generations. When PB was assessed *in vivo,* there was evidence of synergism in the resistant strain (SR=7.86). Since the enzyme, glucose-6-phosphate dehydrogenase (G6PD) is the NADPH generating system for MFO, the activity of this enzyme was measured in deltamethrin resistant, as well as in susceptible,individuals. The resistant strain demonstrated high enzyme activity as compared to the susceptible strain. The enzyme activity exhibited a positive correlation with LC50 levels to deltamethrin. Subsequently Sahgal et al. (1994) carried out microplate assays to investigate the involvement of esterase-mediated hydrolysis in this resistant strain. This was accomplished by estimating levels of non-specific ß-esterase in larvae. The elevated esterase activity in the resistant larvae showed a marginal increase as the frequency increased from 4.6% in the susceptible strain to 7.4% in the resistant strain. The results of Kumar et al. (1991) and Sahgal et al. (1994) indicated that resistance to deltamethrin in larvae of *An.stephensi* was due to the MFO system. However, a marginal role of esterase-mediated hydrolysis was evident.

In the present study PB had a strong synergistic effect on permethrin in the DUB-LPR strain. PB completely covered the gap between the two regression lines of DUB-LPR and IND-S strains which is an indication of a MFO system of resistance. But if comparison was made with another strain such as BAN (the relation to which the resistance factor was 204-fold), PB could not eliminate the resistance completely in the resistant strain, suggesting that apart from MFO and esterases probably another mechanism/s such as target site insensitivity was involved. As already stated this was

the conclusion in *Cx.quinquefasciatus* larvae (Priester & Gerorghiou, 1980a) and *An.stephensi* larvae (Omer et al., 1980) and it may be true in DUB-LPR larvae also. Further penetration and neurophysiological experiments are needed to find the role of other mechanisms such as reduced penetration and target site insensitivity in larvae of this permethrin-resistant strain of *An.stephensi*.

Chapter 5

CROSS-RESISTANCE SPECTRUM IN PERMETHRIN RESISTANT *ANOPHELES STEPHENSI*

5.1 Introduction

Pyrethroids are increasingly used in malaria control, particularly in the form of impregnated mosquito bednets but also, in indoor residual applications. However, the emergence of resistance could jeopardize the gains achieved by using pyrethroids. It is therefore important to investigate the possible containment measures that may be adopted where resistance develops, including the use of alternative chemicals, or of synergists that may potentiate the effects of pyrethroids. A rational process for selecting such compounds requires an understanding of the biochemical basis of resistance. Renewed interest in these naturally occurring insecticides has been accompanied by the development of many synthetic pyrethroids showing a wide range of insecticidal properties. Genetic and biochemical studies have contributed greatly to our understanding of pyrethroid resistance in insects. Insects which have become resistant to an insecticide may be controlled by finding alternative agents from other groups of insecticides, or interfering with resistance mechanisms by using an additive, or altering the chemical structure of the insecticides to restore its toxicity. To choose replacement chemicals rationally, information on the cross-tolerance/resistance of the resistant population to different insecticides must be obtained while characterisation of individual resistance mechanisms is desirable.

This study deals with the patterns of cross-resistance seen in permethrin-resistant strains of *An.stephensi* to the compounds commonly used in malaria control programmes.

5.2 Cross-resistance to pyrethroids

a) deltamethrin

The susceptibility of females of different strains to deltamethrin is summarized in Table 5.1 and illustrated in Fig. 5.1. The development of permethrin resistance has been accompanied by cross-resistance to deltamethrin. DUB-APR showed 14 and 6-fold resistance to deltamethrin compared with TEH and IND-S strains, respectively. Similarly at the LT50, DUB-LPR females exhibited 13 and 5.7-fold cross-resistance to deltamethrin compared with the TEH and IND-S strains. Results obtained for deltamethrin against males of various strains are shown in Table 5.2 and Fig. 5.2. The marked differences in response of these strains to deltamethrin clearly showed that males of DUB-APR were resistant to this compound and the level of resistance was 5.3-fold compared to TEH strain. Toxicological investigation in the laboratory demonstrated that larvae of DUB-LPR and DUB-APR exhibited a marked cross-resistance to deltamethrin (see Table 5.3 and Fig. 5.1). In decreasing order of toxicity, deltamethrin was the least effective against DUB-LPR and DUB-APR strains and was the most toxic to TEH, IRAQ, BAN, and IND-S strains. LC50s of the resistant population (DUB-LPR) for deltamethrin was 256-fold greater than IND-S strain.

Table 5.1 Probit regression line parameters of females of different strains of *An.stephensi* to 0.025% deltamethrin

Strains	a	b±SE	LT_{50}±95%C.I.	LT_{90}±95%C.I.	X^2(df)	P
TEH	-0.89	1.52±0.30	2.8 3.9 4.9	15.9 27.0 35.3	0.95(3)	>0.05
BAN	-4.03	2.86±0.36	4.9 5.6 6.5	12.1 14.7 23.7	2.12(3)	>0.05
IRAQ	-2.59	2.58±0.34	8.6 10.1 11.9	24.0 31.7 49.2	1.84(3)	>0.05
DUB-APR	-8.33	4.83±0.40	50.0 53.2 56.4	89.5 98.1 110.6	2.33(4)	>0.05
IND-S	-1.65	1.67±0.20	7.3 8.7 10.2	35.1 46.3 67.9	3.92(3)	>0.05
DUB-LPR	-8.21	4.84±0.37	46.6 49.7 52.9	83.6 91.5 102.4	4.38(3)	>0.05

Table 5.2 Probit regression line parameters of males of different strains of *An.stephensi* tested with 0.025% deltamethrin

Strains	a	b±SE	LT_{50}±95%C.I.	LT_{90}±95%C.I.	X^2(df)	P
TEH	-2.20	3.06±0.30	4.6 5.2 5.9	11.4 13.7 17.6	11.48(4)	0.025>P>0.01
BAN	-1.30	2.37±0.26	2.9 3.6 4.2	9.9 12.3 16.5	0.85(3)	>0.05
IRAQ	-3.12	3.43±0.33	7.1 8.1 9.2	16.5 19.3 23.6	0.99(4)	>0.05
DUB-APR	-9.19	6.40±0.62	25.8 27.5 29.4	39.7 43.7 49.9	8.24(4)	>0.05

Table 5.3 Probit regression line parameters of fourth instar larvae of different strains of *An.stephensi* to deltamethrin

Strains	a	b±SE	LC_{50}±95%C.I.	LC_{90}±95%C.I.	X^2(df)	P
TEH	4.84	2.06±0.16	0.0040 0.0045 0.0050	0.015 0.019 0.025	6.12(7)	>0.05
BAN	5.77	2.60±0.24	0.005 0.006 0.007	0.015 0.019 0.025	5.03(4)	>0.05
IRAQ	7.52	3.22±0.40	0.0040 0.0050 0.0055	0.010 0.012 0.016	2.80(3)	>0.05
IND-S	1.84	1.16±0.16	0.019* 0.026 0.040	0.16 0.33 1.17	19.07(4)	0.001>p>0.0005
DUB-APR	-1.62	2.17±0.25	4.60* 5.58 6.88	15.68 21.80 35.57	12.95(3)	0.005>p>0.001
DUB-LPR	-2.47	3.0±0.43	6.00 6.65 7.31	15.49 17.80 21.24	8.58(3)	0.05>p>0.025

* 95% C.I. not valid because of significant heterogeneity

b) lambdacyhalothrin

Impregnated papers with a concentration of 1.22 $\mu g/cm^2$ of lambdacyhalothrin were prepared and all of the tests for adults were carried out with this concentration. Exposure tubes were held in a horizontal position. The results are summarized in Table 5.4 and illustrated in Fig. 5.1. The permethrin selected strains (DUB-APR and DUB-LPR) showed high resistance to this compound. DUB-APR was 11 and 7-times less susceptible than the TEH and IND-S strains, respectively. DUB-LPR females exhibited 7.4 and 5-fold resistance to lambdacyhalothrin compared with TEH and IND-S strains, respectively. Similarly the degree of cross-resistance of males of selected strains was determined. The results are shown in Table 5.5 and Fig. 5.2. The BAN strain showed an initial LT50 of 5 minutes, DUB-APR male showed a LT50 of 92.4 minutes, i.e., cross-resistance of 18.5-fold. At the LT50 level DUB-LPR male was 16.6-fold more resistant to lambdacyhalothrin compared with BAN strain.

The larvicidal potential of lambdacyhalothrin was evaluated against different strains in the laboratory. The results and probit analysis data are given in Table 5.6. Efficacy of lambdacyhalothrin as a larvicide was found to be highest against TEH, BAN, IND-S and IRAQ strains. The LC50 values against DUB-LPR strain were 231 and 104 times higher than that of BAN and IND-S strains, respectively. The LC50 value estimated in the present study for DUB-APR larvae was 3.6 mg/l which is 200 times more than the LC50 of BAN and 90 times more than the IND-S strains (see Fig. 5.1).

Table 5.4 Probit regression line parameters of females of different strains of *An.stephensi* to 1.22 μg/cm² lambdacyhalothrin

Strains	a	b±SE	LT_{50}±95%C.I.	LT_{90}±95%C.I.	X^2(df)	P
TEH	-3.30	2.99±0.35	10.6 12.7 14.7	28.4 34.1 44.0	12.5(3)	0.01>p>0.005
BAN	-3.17	2.77±0.39	12.0 13.9 16.8	29.7 40.4 67.7	4.4(3)	>0.05
IRAQ	-3.78	2.92±0.45	17.2 19.8 23.9	39.7 54.3 95.5	1.5(3)	>0.05
DUB-APR	-8.69	4.04±0.52	127.8 141.0 158.0	242.0 293.0 396.0	0.2(3)	>0.05
IND-S	-4.16	3.20±0.30	17.6 19.7 21.9	42.4 49.4 61.4	6.7(3)	>0.05
DUB-LPR	-9.19	4.66±0.50	85.7 94.1 103.0	155.0 177.0 215.0	1.0(3)	>0.05

Table 5.5 Probit regression line parameters of males of different strains of *An.stephensi* to 1.22 μg/cm² lambdacyhalothrin

Strains	a	b±SE	LT_{50}±95%C.I.	LT_{90}±95%C.I.	X^2(df)	P
TEH	-2.92	2.85±0.35	9.2 10.6 12.3	23.3 29.9 44.0	0.25(3)	>0.05
BAN	-1.30	1.87±0.30	3.6 5.0 6.3	17.7 24.5 43.2	3.52(3)	>0.05
IRAQ	-3.10	2.26±0.26	19.8* 19.9 29.1	60.7 86.4 148.0	18.97(6)	0.005>p>0.001
DUB-APR	-12.72	6.47±0.70	85.4 92.4 99.4	132.4 145.8 166.7	6.18(2)	>0.05
DUB-LPR	-10.70	5.57±0.51	77.7 83.2 89.3	126.6 141.2 163.9	4.53(3)	>0.05

* 95% C.I. not valid because of significant heterogeneity

Table 5.6 Probit regression line parameters of larvae of different strains of *An.stephensi* to lambdacyhalothrin

Strains	a	b±SE	LC_{50}±95%C.I.	LC_{90}±95%C.I.	X^2(df)	P
TEH	2.76	1.76±0.12	0.023 0.027 0.032	0.115 0.140 0.190	6.12(7)	>0.05
BAN	4.23	2.42±0.23	0.015 0.018 0.020	0.048 0.060 0.083	0.76(3)	>0.05
IRAQ	1.07	1.68±0.13	0.020 0.023 0.027	0.105 0.135 0.186	11.70(8)	>0.05
IND-S	2.86	2.05±0.20	0.033 0.040 0.050	0.13 0.17 0.26	3.80(4)	>0.05
DUB-APR	-1.66	2.98±0.30	3.16 3.60 4.12	7.78 9.66 13.16	14.78(4)	0.01>p>0.005
DUB-LPR	-1.03	1.67±0.18	3.33 4.16 5.25	16.67 24.37 42.27	2.82(4)	>0.05

Fig. 5.1 Relative toxicity of pyrethroids to different geographical strains of *An.stephensi*. Vertical bars= 95% C.I.

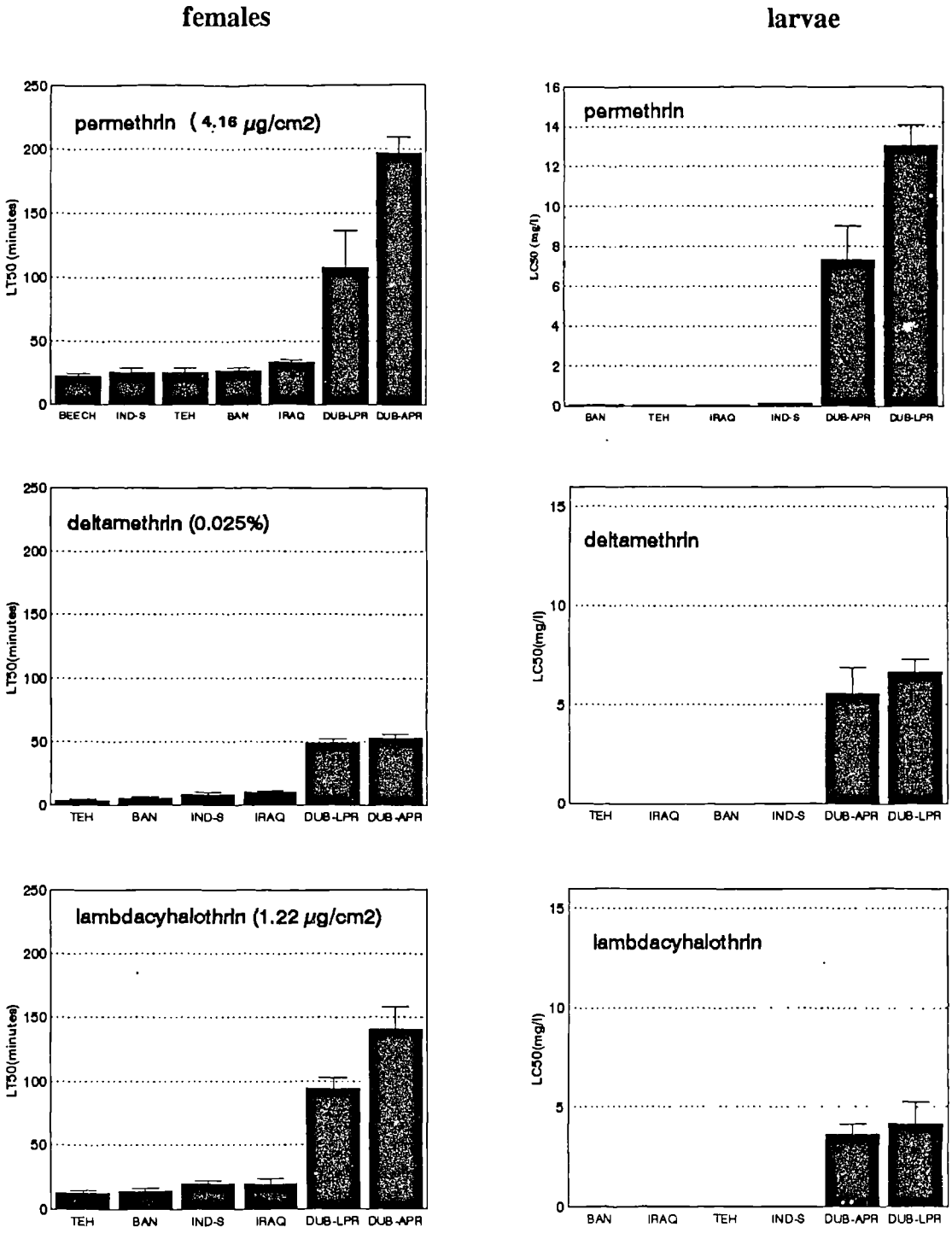
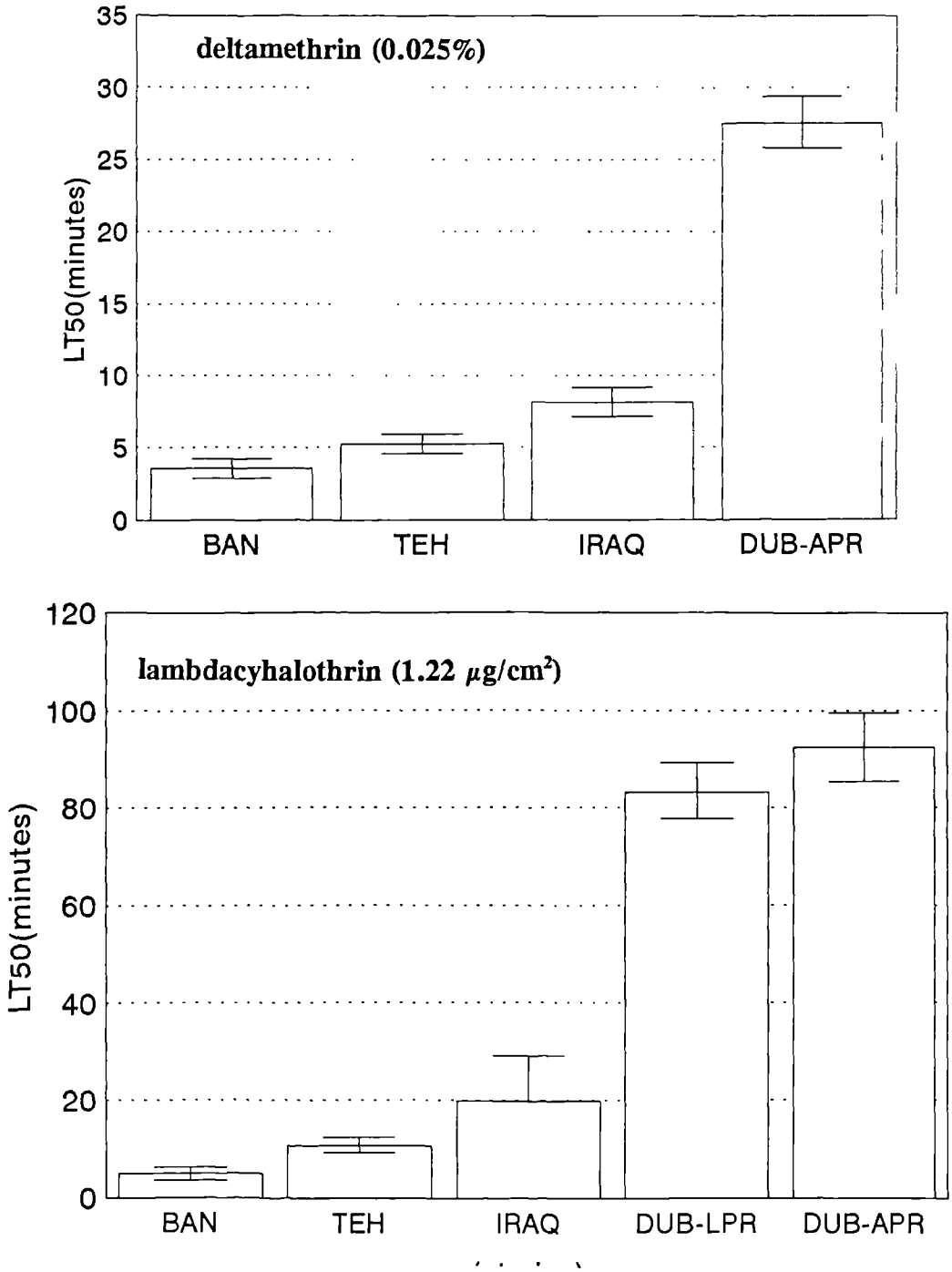


Fig. 5.2 Relative toxicity of pyrethroids to males of different geographical strains of *An.stephensi*. Vertical bars= 95% C.I.



5.3 Resistance to DDT

Initially the susceptibility of adults of different strains was measured using WHO impregnated papers (4%) and testing tubes were held in a vertical position. In such tests the mortality rate was very low, therefore papers with a concentration of 10% DDT were prepared in our laboratory, and all subsequent tests were done with 10% DDT. The results of probit regression lines are presented in Table 5.7. Results of adult tests with DDT showed that DUB-APR has the highest level of resistance and IND-S strain the lowest level of resistance. BAN, TEH and IRAQ strains had a moderate level of resistance. At the LT50 DUB-APR was 13-fold resistant to DDT compared to IND-S strain (see Fig. 5.3). Results from larval tests (Table 5.8) showed that the IND-S strain had the lowest LC50 and DUB-LPR had the highest LC50. Larvae of DUB-LPR showed 8-fold cross-resistance to DDT in comparison with IND-S strain (Fig. 5.3).

Synergist study: At the adult stage DDT was tested in the presence and absence of piperonyl butoxide (PB) (MFO inhibitor) and DMC as a dehydrochlorinase inhibitor. The results are presented in Tables 5.9 and 5.10. The results from PB tests suggested that MFO does not contribute to DDT resistance. DMC had no effect. From results with synergist tests it is suggested that DDT resistance was due to non-metabolic factors, and it was postulated that reduced sensitivity of the active site was the major factor responsible for the observed resistance. Resistance to DDT in DUB-APR strain is unlikely to be a result of a *kdr* gene. Ladonni (1988) pointed out that tolerance to DDT in larvae of *An.stephensi* originating from Dubai was probabely due to different genes than those which caused permethrin resistance, i.e. there was multi-resistance, not cross-ressitance in this strain.

Table 5.7 Probit regression line parameters of females of different strains of *An.stephensi* tested with 10% DDT.

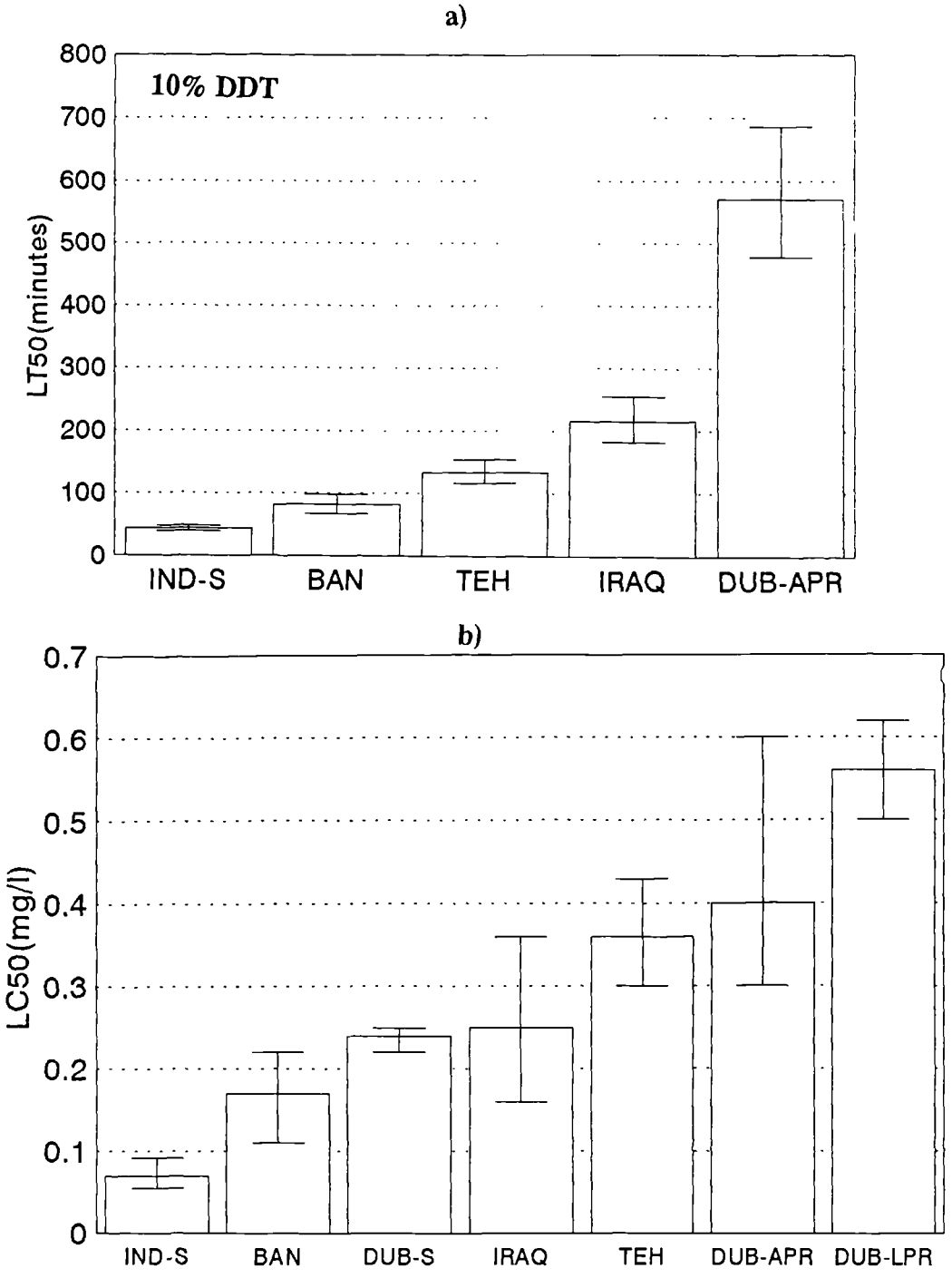
Strains	a	b±SE	LT_{50}±95%C.I.	LT_{90}±95%C.I.	X^2(df)	P
TEH	-5.97	2.81±0.33	116.8 133.3 153.7	299.2 381.5 550.4	8.40(4)	>0.05
BAN	-4.03	2.10±0.25	66.8 82.5 98.4	259.9 335.8 488.5	0.20(4)	>0.05
IRAQ	-5.28	2.26±0.25	183.0 216.2 255.6	577.3 797.0 1284.9	4.60(3)	>0.05
DUB-APR	-5.55	2.01±0.2	477.5 570.7 686.0	1840.0 2472.4 3727.9	1.06(4)	>0.05
IND-S	-5.68	3.57±0.38	39.7 43.8 48.2	85.4 100.2 126.0	6.88(4)	>0.05

Table 5.8 Probit regression line parameters of larvae of different strains of *An.stephensi* to DDT

Strains	a	b±SE	LC_{50}±95%C.I.	LC_{90}±95%C.I.	X^2(df)	P
TEH	1.00	5.25±0.25	0.30 0.36 0.43	0.99 1.33 2.04	1.29(3)	>0.05
BAN	1.56	2.02±0.31	0.11 0.17 0.22	0.56 0.73 1.10	1.53(3)	>0.05
IRAQ	0.62	1.02±0.20	0.16 0.25 0.36	2.0 4.42 4.80	0.18(3)	>0.05
IND-S	1.66	1.45±0.15	0.055 0.070 0.092	0.39 0.55 0.90	14.48(5)	0.025>p>0.01
DUB-APR	0.91	2.27±0.32	0.30 0.40 0.60	0.90 1.45 4.00	9.73(4)	0.05>p>0.025
DUB-LPR	0.47	1.87±0.11	0.50 0.56 0.62	2.45 2.70 3.37	1.70(3)	p>0.05
DUB-S	4.65	7.40±0.67	0.22 0.24 0.25	0.33 0.35 0.38	12.95(4)	0.025>p>0.01

Fig. 5.3 Relative toxicity of DDT to different geographical strains of *An.stephensi*

a) females; b) larvae



Vertical bars= 95%C.I.

Table 5.9 The effect of synergist piperonyl butoxide (PB) on the mortality of females of *An.stephensi* tested with 10% DDT

Strains	DDT/PB	Mortality
DUB-LPR	PB (30 min)	0/50=0%
	DDT (60 min)	0/100=0%
	PB + DDT	2/100=2%
DUB-APR	PB (30 min)	0/50=0%
	DDT (60 min)	1/100=1%
	PB + DDT	1/100=1%

Table 5.10 The effect of DMC (dehydrochlorinase inhibitor) on mortality of adults of *An.stephensi* followed by exposure to 10% DDT

Strains	Sex	DDT/DMC	Exposure time (minutes)	Mortality
DUB-APR	♀	DMC	30	0/100=0%
		DDT	60	0/100=0%
		DMC+DDT	30+60	0/100=0%
	♂	DMC	30	0/100=0%
		DDT	60	0/100=0%
		DMC+DDT	30+60	0/100=0%
DUB-LPR	♀	DMC	30	0/100=0%
		DDT	60	0/100=0%
		DMC+DDT	30+60	1/100=1%
	♂	DMC	30	0/100=0%
		DDT	60	0/100=0%
		DMC+DDT	30+60	0/100=0%

5.4 Base-line susceptibility of different strains of *An.stephensi* to malathion

Malathion resistance in *An.stephensi* was first reported in Iran from the coast (Manouchehri, 1976) and interior (Eshghy, 1978), then in Pakistan (Rathor & Toqir, 1980) and in Iraq in Basreh province (Manouchehri et al., 1980). Synergist tests with TPP suggested involvement of a carboxylesterase enzyme in malathion resistance. Piperonyl butoxide (PB) had antagonist effects at all dosages tested indicating that MFO were involved in the activation of malathion to toxic malaoxon. In *An.stephensi* from Pakistan, synergist studies suggested involvement of a carboxylesterases enzyme in malathion resistance. PB, an MFO inhibitor, had a slight antagonistic effect on malathion resistance (Hemingway, 1981).

Our investigations were done to determine if resistance to pyrethroids and susceptibility to malathion in this species was correlated. Therefore, we exposed pyrethroid resistant strains to malathion. Results from tests with malathion showed that females of *An.stephensi* strains resistant to pyrethroid (permethrin, deltamethrin, and lambdacyhalothrin) (DUB-APR and DUB-LPR) were more susceptible than the TEH, BAN, IRAQ, and DUB-S strains (see Table 5.11 and Fig. 5.4). At the LT50 DUB-APR was 4.7 and 2.5 times more susceptible than the IRAQ and DUB-S strains, respectively. Malathion was 4.2 and 2.2 times more toxic to DUB-LPR than to the IRAQ and DUB-S strains respectively, indicating negative cross-resistance to malathion. The larval tests with malathion presented in Table 5.12 and Fig. 5.4 showed that DUB-APR and DUB-LPR larvae were more susceptible to malathion than the other strains tested. Resistance ratios less than 1 were obtained with larvae of both pyrethroid-resistant populations compared to other strains when tested with malathion, indicating negative cross-resistance. Our results indicated that the response of

pyrethroid resistant *An.stephensi* was correlated negatively with malathion toxicity (i.e., as pyrethroid susceptibility decreased, malathion toxicity increased). Many useful insecticides such as phosphorothionates and phosphorothiolothionates e.g. pirimiphos methyl and diazinon contain P=S groups activated by the oxidative reaction of MFO systems, the *in vivo* activity being the result of metabolic activation of P=S to P=O (oxo-analogues). Hence biological modification of organophosphate compounds often causes a change to a more toxic product. In chapter 4 we have established that MFO play a role in the permethrin-resistant populations. Increased mixed function oxidase activity may be the common metabolic mechanism responsible for malathion negative cross-resistance in pyrethroid-resistant *An.stephensi* by enhanced metabolism of malathion to the oxon form, a toxic metabolite. In the context of insecticide resistance, the phenomenon has been referred to as negative cross-resistance, i.e resistance to one insecticide class confers increased toxicity of an unrelated class compared with a strain not exposed to insecticides.

An attempt was made to test the effect of piperonyl butoxide (PB) as an MFO inhibitor on the profile of malathion action. Females of pyrethroid resistant strains (DUB-APR and DUB-LPR) and DUB-S, as a parent of selected strains, were pretreated with PB and then tested with 5% malathion. Results are presented in Table 5.13 and Fig. 5.5. PB had no significant synergistic effect on the parental strain, whereas the synergist PB decreased the effectiveness of malathion to resistant strains (i.e. PB antagonized rather than synergized the organophosphate insecticide, malathion). PB increased the LT50s of DUB-APR and DUB-LPR 4- and 3.5-fold, respectively, indicating the possibility blockage of MFO by PB and resulting decrease in the toxicity of malathion (see Table 5.13).

Table 5.11 Probit regression line parameters of females of different strains of *An.stephensi* to 5% malathion

Strains	a	b±SE	LT_{50}±95%C.I.	LT_{90}±95%C.I.	X^2(df)	P
TEH	-4.35	3.17±0.35	20.7* 23.5 27.0	47.7 59.6 82.4	34.60(4)	<0.0005
BAN	-4.37	4.05±0.32	10.9 12.0 13.1	22.0 24.8 28.9	6.10(4)	>0.05
IRAQ	-6.37	4.49±0.42	23.9* 26.3 28.9	44.5 50.8 60.7	15.80(5)	0.01>p>0.005
DUB-APR	-1.90	2.52±0.16	5.0 5.6 6.2	15.5 17.9 21.3	9.90(5)	>0.05
DUB-S	-3.70	3.47±0.20	13.0 14.0 15.1	29.0 32.7 37.9	26.50(5)	<0.0005
DUB-LPR	-2.16	2.70±0.17	5.7 6.3 6.9	16.1 18.5 22.2	7.40(5)	>0.05

* 95% C.I. not valid because of significant heterogeneity

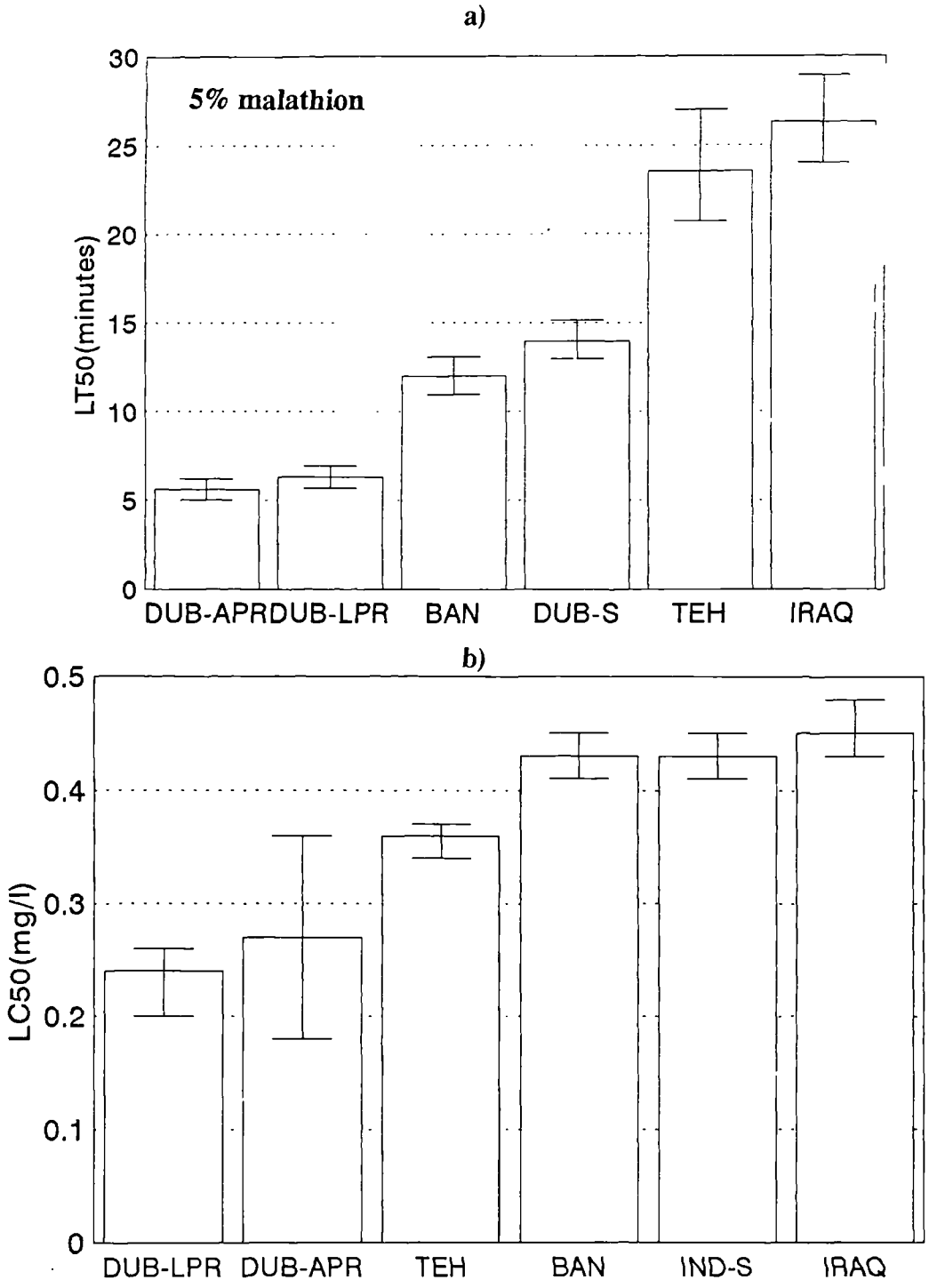
Table 5.12 Probit regression line parameters of larvae of different strains of *An.stephensi* to malathion

Strains	a	b±SE	LC_{50}±95%C.I.	LC_{90}±95%C.I.	X^2(df)	P
TEH	4.18	9.38±0.80	0.34 0.36 0.37	0.47 0.49 0.52	7.91(6)	>0.05
BAN	2.87	7.80±0.71	0.41* 0.43 0.45	0.59 0.63 0.69	19.60(6)	0.005>p>0.001
IRAQ	2.65	7.72±0.71	0.43 0.45 0.48	0.62 0.66 0.72	11.10(6)	>0.05
IND-S	3.09	8.38±1.02	0.41 0.43 0.45	0.56 0.61 0.70	2.30(3)	>0.05
DUB-APR	6.94	12.12±3.4	0.18* 0.27 0.36	0.29 0.34 2.15	18.82(3)	>0.0005
DUB-LPR	4.65	7.40±1.21	0.20 0.24 0.26	0.30 0.35 0.47	12.95(4)	0.025>P>0.01

* 95% C.I. not valid because of significant heterogeneity

Fig. 5.4 Relative toxicity of malathion to different geographical strains of *An.stephensi*

a) females; b) larvae

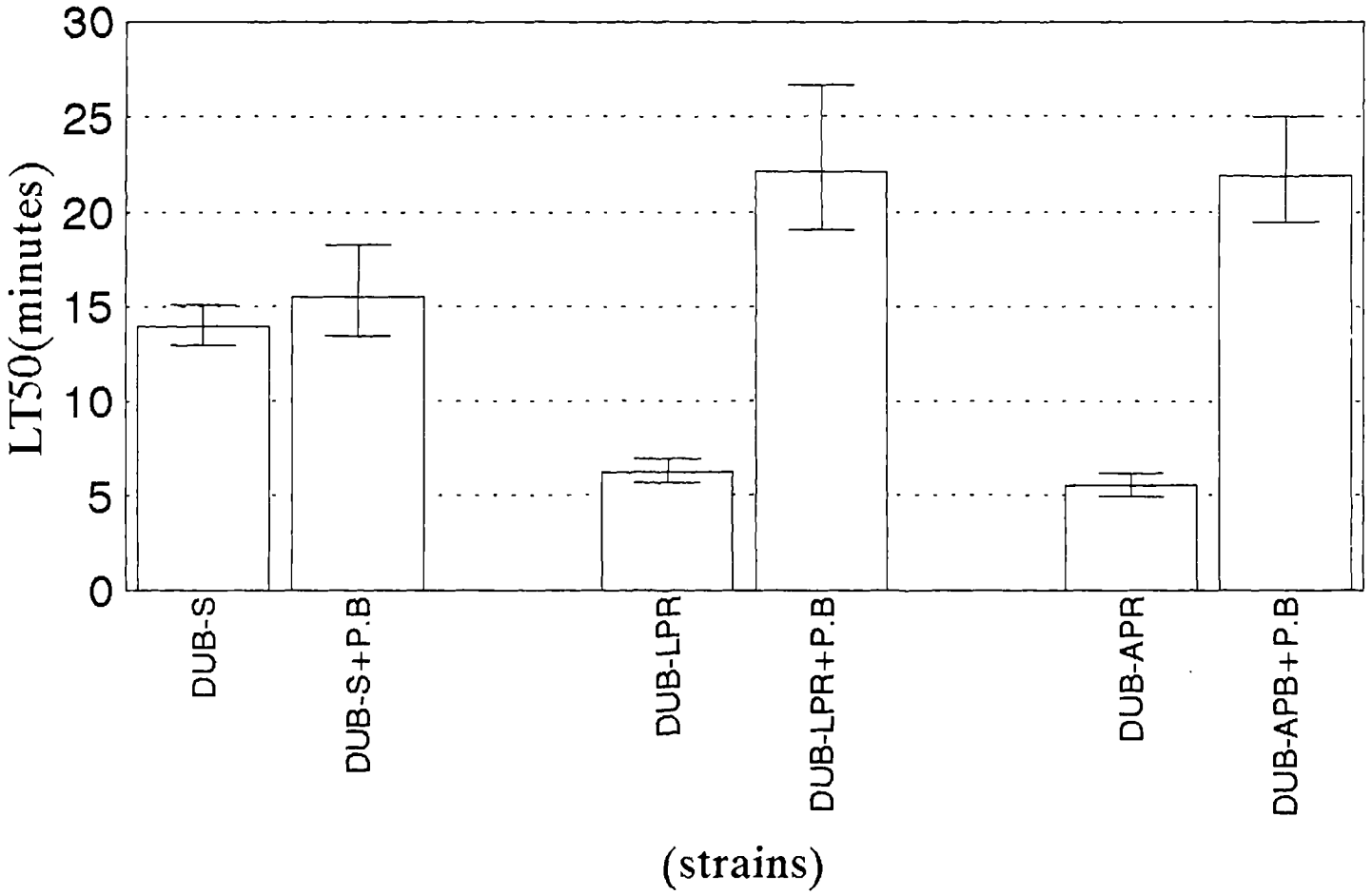


Vertical bars= 95%C.I.

Table 5.13 Probit regression line parameters of females of *An.stephensi* to 5% malathion in the presence and absence of PB

Strains	with/out PB	a	b±SE	LT50±95%C.I	LT90±95%C.I	X^2(df)	P	SR	RF
DUB-S	-	-3.70	3.47±0.20	13.0 14.0 15.1	29.0 32.7 37.9	26.50 (5)	<0.0005	0.9	-
	+	-2.10	1.77±0.14	13.4 15.5 18.3	60.6 82.1 123.0	27.48 (5)	<0.0005		
DUB-LPR	-	-2.16	2.70±0.17	5.7 6.3 6.9	16.1 18.5 22.2	7.40 (5)	>0.05	0.29	0.45
	+	-2.76	2.05±0.18	19.0 22.1 26.7	68.1 93.4 144.0	60.10 (5)	<0.0005		
DUB-APR	-	-1.90	2.52±0.16	5.0 5.6 6.2	15.5 17.9 21.3	9.90 (5)	>0.05	0.26	0.40
	+	-4.12	3.07±0.32	19.5 21.9 25.0	45.7 57.1 79.0	6.80 (3)	>0.05		

Fig. 5.5 The effect of piperonyl butoxide on females of permehrin-resistant and susceptible strains of *An.stephensi* tested with 5% malathion. Vertical bars= 95% C.I.



5.5 Susceptibility status of *An.stephensi* to biological control agent , *Bacillus thuringiensis* serotype H-14

The bacterium *Bacillus thuringiensis* produces a large and variable family of pesticidal proteins. Many of these are toxic to agricultural pests, especially Lepidoptera, but *B.thuringiensis* serotype H-14 produces proteins that are toxic to mosquito and black fly larvae. *B.t.*H-14 owes its activity to four different proteins with different molecular masses, and has been in commercial use for several years. Since the discovery of *B.t.*H-14 , it has been reported to be a potent agent. Margalit & Deam (1985) reviewed the efficacy of *B.t.*H-14 against black fly and mosquito larvae including 21 species of *Aedes*. To evaluate the efficacy of *B.t.*H-14 against permethrin-resistant and susceptible strains of *An.stephensi,* the present study was carried out using a liquid formulation of *B.t.*H-14 (Skeetal, Flowable concentrate, Novo Nordisk A/S Denmark). A bioassay procedure similar to those employed for insecticide tests was used. The results are summarized in Table 5.14. LC50 values of *B.t.*H-14 for IND-S, BEECH, and DUB-LPR larvae were 0.104, 0.108, and 0.107 mg/l, respectively. Results clearly showed that the susceptibility of permethrin-resistant and susceptible larvae was not significantly different. It is clear from this study and other published data that insecticide-resistant strains of mosquitoes and black flies are not cross-resistant to *B.t.*H-14. Its performance is therefore unaffected by the various resistance mechanisms that have been developed in these insects by the use of conventional insecticides.

Table 5.14 Probit regression line parameters of *An.stephensi* larvae tested with *B.t.*H-14

Strains	a	b±S.E	LC50±95%C.I.	LC90±95%C.I.	X^2(df)	P
IND-S	3.4	3.45±0.19	0.083 0.104 0.129	0.186* 0.244 0.377	8.86(3)	0.005>P>0.025
BEECH	3.6	3.70±0.17	0.087 0.108 0.131	0.190 0.240 0.347	14.26(3)	0.005>P>0.0001
DUB-LPR	3.7	3.80±0.20	0.073 0.107 0.153	0.159 0.230 0.546	26.10(3)	P<0.0005

* 95% C.I. not valid because of significant heterogeneity

5.6 The effect of the repellent, deet, on the feeding activity of females of permethrin-resistant and susceptible strains of *An.stephensi*

There are many reports on the efficacy of chemical repellents against mosquitoes (Rutledge et al., 1978). Among chemical repellents, deet (N, N-diethyl-m-toluamide) is widely used for protection against mosquito bites. The methods of using deet against mosquito bites are described by Curtis (1991). The assay which is described in section 3.2.15, has been developed to examine whether development of permethrin resistance due to target site insensitivity can affect the feeding behaviour of a resistant strain to this repellent in comparison with a susceptible one. Batches of 2-3 day-old unfed females were introduced into cylindrical cages which were lined on top with deet impregnated netting at 53 nl/cm^2. Anaesthetized guinea pigs were gently placed on top of the netting for blood feeding and, mosquitoes were left for 30 minutes. During the experiments the probing behaviour and total number of engorged mosquitoes were recorded. At the end of the exposure period the mosquitoes were collected and examined for the presence of blood. Unimpregnated nets were used as a control. Experiments were carried out in 4 replicates on different days. The results are summarized in Table 5.15. In the control group with ethanol impregnated net, following introduction of the mosquitoes all of them fed on the guinea pig, but none of the mosquitoes which was introduced to cages with deet impregnated net had fed. Results showed that the concentration of 53 nl/cm^2 protected against feeding for 30 minutes and there was no significant difference between resistant and susceptible strains in terms of feeding behaviour. Further experiments are needed to establish the correlation between feeding behaviour and different concentrations of deet for resistant and susceptible strains.

Table 5.15 Feeding activity of females of permethrin-resistant and susceptible strains of *An.stephensi* on guinea pig using deet impregnated nets

Strains	Control	Deet impregnated net
(IND-S)	55/55	0/45
(DUB-APR)	45/45	0/45

Time of feeding on guinea pig= 30 minutes

Deet concentration= 53 nl/cm^2 according to Curtis et al. (1987a)

5.7 Discussion

In this study, strains of *An.stephensi* from Dubai which had been selected with permethrin, were examined in the larval and adult stage for cross-resistance to other classes of pyrethroids, DDT and malathion. Permethrin resistant adults were cross-resistant to deltamethrin (RF=14) (Fig. 5.1), lambdacyhalothrin (RF=11) (Fig. 5.1), and DDT (RF=13) (Fig. 5.3). In contrast malathion was more toxic to the resistant strain (RF<1) (Fig. 5.4). Permethrin resistant larvae (RF>100) showed high cross-resistance to deltamethrin (RF=256) (Fig. 5.1), lambdacyhalothrin (RF=231) (Fig. 5.1) and DDT (RF=8) (Fig. 5.3), whereas they were more susceptible to malathion (RF<1 ; i.e. negative cross-resistance). Piperonyl butoxide antagonized malathion (Fig. 5.5) and its antagonism by piperonyl butoxide indicates that, as well as conferring resistance to pyrethroids, mixed function oxidases also metabolise malathion to its more active oxon form. Synergist tests with DMC (dehydrochlorinase inhibitor) and PB showed that metabolic pathways were not involved in DDT resistance in adults and *kdr* plays a major role (Tables 5.9, 5.10).

A relationship between DDT and pyrethroid resistance has previously been reported in bed bug, *Cimex* spp. (Busvine, 1958), ticks (*Boophilus decoloratus*) (Whitehead, 1959), house flies (*Musca domestica*) (Fine, 1961 ; Plapp & Hoyer, 1968b) and body lice (*Pediculus humanus humanus*) (Cole & Clark, 1961). The evidence suggested that resistance to DDT and to the pyrethrins is controlled by the same gene.

The *Blattella germanica* resistant strain was cross-resistant to three pyrethroid insecticides tested by injection when compared to the isogenic susceptible strain: bioallethrin (48-fold), deltamethrin (17-fold) and fenvalerate (59-fold) (Dong & Scot, 1991). In another study *Blattella germanica* selected with permethrin and fenvalerate

showed cross-resistance to other classes of pyrethroids (allethrin, phenothrin, cyfluthrin, and cypermethrin) and several independent mechanisms exist for pyrethroid resistance in this species (Cochran, 1991). In contrast, deltamethrin did not confer cross-resistance in a DDT-resistant population of *Blattella germanica* (Matsumura, 1985). DDT and dieldrin resistance in *An.culicifacies* did not confer cross-resistance to deltamethrin (Das et al., 1986). These authors did not mention the role of *kdr* gene in the multiresistant field population of *An.culicifacies*.

The organochlorine and malathion resistant *An.arabiensis* in the Gezira, Sudan was cross-resistant to pyrethroids (Davidson & Curtis, 1979). Selection for DDT resistance in *An.gambiae* produced appreciable cross-resistance to permethrin, but selection with permethrin does not confer cross-resistance to DDT (Prasittisuk & Curtis, 1982). Permethrin resistant strains of *Cx.quinquefasciatus* were also variously cross-resistant to other pyrethroids (Priester et al., 1981). Priester & Georghiou (1978) have reported high levels of resistance to (IR)-*trans*-permethrin in larvae of *Cx.quinquefasciatus*. Cross-resistance extends to all pyrethroids tested. Their succeeding investigations led to the conclusion that resistance extended to all pyrethroids was due to *kdr*. Larval cross-resistance to pyrethroids has been shown by DDT resistant populations of *Ae.aegypti* in Guyana (Prasittisuk & Busvine, 1977) and in Bangkok (Chadwick et al., 1977). A resistant population of the horn fly, *H.irritans* demonstrated that resistance to one pyrethroid conferred cross-resistance to all the pyrethroids tested and conferred cross-resistance to methoxychlor (Cilek et al., 1991). Larvae of permethrin resistant horn flies demonstrated high levels of resistance to pyrethroids (permethrin, fenvalerate, cyhalothrin) and DDT, but a lack of cross-resistance to diazinon. In *Musca domestica* and *Cx.tarsalis* resistance to DDT and

cross-resistance to pyrethrins was due to the *kdr* recessive gene (Plapp & Hoyer, 1968b). Organophosphate (azamethiphos) resistant *Musca domestica* showed cross-resistance to most organophosphates, carbamates and a low level of resistance to DDT, fenvalerate and permethrin (Saito et al., 1991). The *kdr* gene that located on chromosome III is known to confer cross-resistance to DDT, DDT analogues and pyrethroids in *Musca domestica* (Farnham, 1977).

Among the Indian anophelines tested as larvae, those of *An.stephensi* which were resistant to DDT showed the highest LC50 value against deltamethrin (Rajvanshi et al., 1982). A DDT resistant strain of *An.stephensi* from India quickly developed *kdr* resistance against fenvalerate (Verma & Rahman, 1984). Studies on larvae of *An.stephensi* from Pakistan demonstrated a major DDT resistance factor that does not confer cross-resistance to pyrethroids (Malcolm, 1988b). Involvement of the *kdr* gene in the manifestation of resistance to DDT and pyrethroid compounds is well recognized in insects including mosquitoes (Halliday & Georghiou, 1985a, 1985b). The results of our studies on the mechanisms of permethrin and DDT resistance suggest that *kdr* may be a component for cross-resistance to DDT and pyrethroids in adults of *An.stephensi*.

Tests with malathion revealed that pyrethroid-resistant strains were more sensitive than the susceptible ones (Fig. 5.4) indicating negative cross-resistance. We have previously established that MFO plays a role in the permethrin resistant population. Induced MFO activity may be the common metabolic mechanism responsible for malathion negative cross-resistance in pyrethroid resistant population by enhanced metabolism of malathion to the oxon form, a toxic metabolite. The first report of enhanced toxicity of chemicals to insecticide resistant arthropods was the increase

in oral toxicity of potassium bromide in the organophosphate-resistant strains of house fly, compared with the response of an organophosphate-susceptible strain (Ascher & Kocher, 1954). Studies by Chapman & Penman (1979) demonstrated that an organophosphate (azinphosmethyl)-resistant strain of *Tetranychus urticae* was more susceptible to pyrethroid fenvalerate than an azinphosmethyl-susceptible strain. Kurtak et al. (1987) found three organophosphate resistant sibling species of *Simulium damnosum* in West Africa, had up to 11-fold increased susceptibility to permethrin. These studies suggested that organophosphate resistance in certain arthropods could confer negative cross resistance to pyrethroids. Priester et al. (1981) reported that a temephos-resistant strain of *Culex quinquefasciatus* and methyl parathion-resistant of *Culex tarsalis* were more susceptible to several pyrethroid insecticides than the organophosphate-susceptible strain. Cilek (1989), Sparks et al. (1990), and Cilek & Knapp (1993a) demonstrated increased pyrethroid metabolism by MFO in several populations of horn flies resistant to permethrin. Increased MFO activity may be the common metabolic mechanism responsible for diazinon negative cross-resistance to pyrethroid-resistant horn flies by enhanced metabolism of diazinon to diazoxon, a toxic metabolite. Some geographical populations of horn flies, *Haematobia irritans,* which were resistant to permethrin were more susceptible to diazinon and pirimiphos methyl (Cilek et al., 1991). In another study horn flies which were selected for a high level of pyrethroid resistance with lambdacyhalothrin remained highly susceptible to diazinon with a resistance ratio <1 (Sheppard & Joyce, 1992). Cilek & Knapp (1993b) demonstrated that diazinon toxicity was negatively correlated with horn fly, *H.irritans* resistance to permethrin. Results from their field trails showed greater reduction in the frequency of pyrethroid-resistant flies when flies were exposed to

cattle tagged with diazinon-impregnated ear tags compared with other resistance management practices. Sheppard & Marchiondo (1987) reported enhanced toxicity of the organophosphate diazinon to a fenvalerate resistant laboratory horn fly population. Sawicki (1985) suggested that negative cross-resistance may be the result of a polymorphic enzyme(s) that would degrade compounds of one chemical group much faster while conferring sensitivity to compounds of others.

From these results it can be seen that permethrin-resistant strains of *An.stephensi* exhibited positive cross-resistance to other pyrethroids and DDT, but negative cross-resistance to malathion. Further experiments are needed to determine if the negative cross-resistance is operationally useful.

Curtis et al. (1993) proposed that a mixture of pyrethroid and an insecticide with no positive cross-resistance could delay the build up of resistance to insecticides in impregnated bednets. It should be emphasized that this form of mixture will only work prior to the development of full pyrethroid resistance in the field populations.

Results with *Bacillus thuringiensis* clearly showed that the susceptibility of permethrin-resistant and susceptible larvae was not significantly different. It is likely that periodic use of *B.t.H-14* in mosquito control in rotation with other conventional insecticides, may prevent or delay the onset of resistance to them, so long as there is reduced fitness associated with pyrethroid resistance.

Chapter 6

INDUCTION OF DETOXIFICATION ENZYMES

6.1 Introduction

In chapter 4 we have established that the DUB-APR strain of *An.stephensi* is resistant to pyrethroid insecticides and this resistance is polyfactorial. Synergist tests suggest that MFO and carboxylestrase enzymes are involved in the resistance but the target site insensitivity (*kdr*) plays a major role. The role of MFO as a mechanism of resistance to permethrin in the larvae of *An.stephensi* was developed by selection with permethrin. Synergist tests with piperonyl butoxide clearly suggest that permethrin resistance in the larvae of DUB-LPR strain is mainly due to detoxification of permethrin by MFO.

Development of resistance in the field is multidimensional. It depends on the interaction of several important factors such as potentiating (genetic, reproductive, behavioural, ecological) and operational factors. Therefore investigation on the development of resistance should ideally take account of all these factors.

A wide variety of chemicals have been shown to elicit induction of detoxification enzymes. Those which are of interest in entomology include the allelochemicals of plants, insecticides, insect hormones and their analogues, as well as the experimentally useful barbiturate phenobarbital. One reason for our research into induction is the possibility, which has been mentioned by some authors, that induction may have had an influence on the development of resistance and that it may still complicate the control of insects by chemical pesticides. These inducers can "turn on" the detoxifying enzymes, they could enhance the existing detoxification machinery,

speed the development of resistance and cause cross-tolerance to other pesticides under field conditions. Pretreatment of mosquitoes with inducers have been described in chapter 3. In summary, for studies of induction in larvae, sodium phenobarbital was applied to rearing trays at a concentration of 10^{-8} molar from the 1st instar to the 3rd, followed by bioassay of 4th instar larvae with permethrin. Similar pretreatments were carried out with 10^{-10} M menthol and 250 mg/l peppermint leaves. To test whether permethrin itself acts as inducer, late 3rd and 4th instar larvae were treated with a sub-lethal dose of permethrin for 24 h bioassay with a range of permethrin concentrations.

For studies of induction in adult female mosquitoes were given access to 10^{-4} M sodium phenobarbital plus 10% sucrose plus a blue dye (food colouring) (to enable a visual check that the compound had been ingested) 24 h before exposure to insecticide in a bioassay (see Fig. 3.5).

To study the inducing effect of permethrin, adult female mosquitoes were treated with a sub-lethal dose of permethrin and after 24 h, exposed to permethrin at the LT50. Standard bioassays were used to determine insecticide resistance in larvae and adults.

6.2 Results & discussion

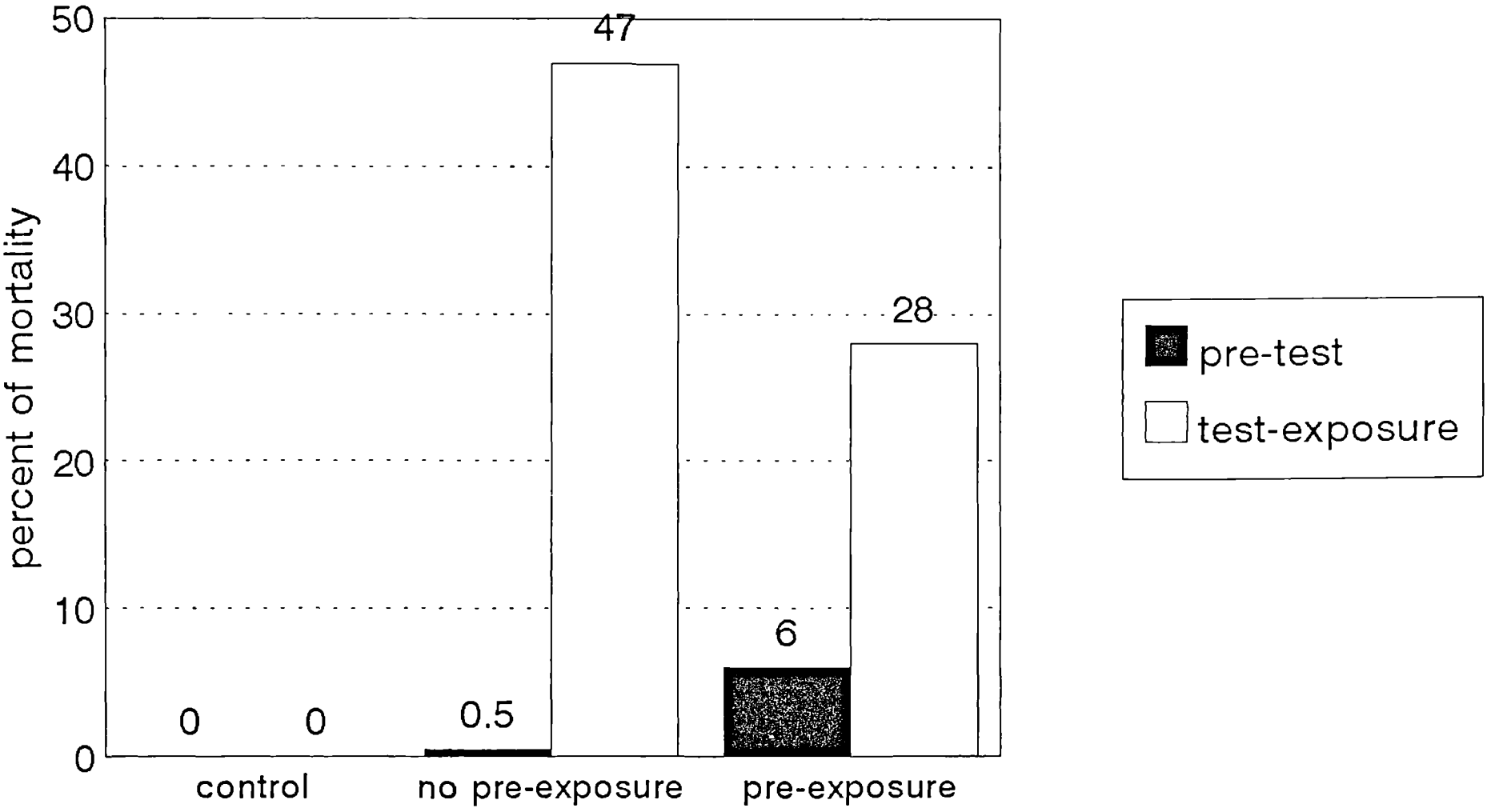
6.2.1 Effect of sub-lethal dose of permethrin on the tolerance level of mosquito adults to subsequent exposure

When insecticides are applied to the control of mosquitoes in their environment, it is inevitable that the target population will be in contact with varying amounts, over the whole range from the negligible (usually weathered or decomposed) to freshly deposited highly toxic levels. In the field, the importance of sub-lethal effects depends on the frequency of application and the persistence of the insecticide. Sub-lethal doses of insecticides have been shown to cause latent toxicity, enzyme induction, stimulatory or inhibitory effects on reproduction, altered behaviour and insect physiology (Kumar & Chapman, 1984). These sub-lethal effects must occur in a proportion of insect populations during exposure to chemical control agents, hence such phenomena must be considered when the overall efficacies of control measures are assessed.

To investigate the effect of sub-lethal doses of permethrin on tolerance level of *An. stephensi* to the same insecticide, the IRAQ strain (permethrin susceptible) was used. One group of females was pretreated with permethrin at a sub-lethal dose (< 10% mortality), and subsequently were held for 24 h. The mortality rate was scored after 24 h. The expected mortality having been obtained (5-10%), the remaining adults were exposed at the LT50 of permethrin and then held for another 24 h. A second control group was not pretreated with permethrin, hence they were exposed at the LT50 of permethrin after a 24 h holding period. The mortality rates were then scored after a further 24 h holding period. The results of pretreatment with a sub-lethal dose

of permethrin are illustrated in Fig. 6.1. There is a significant difference between the two groups. Adults of the permethrin sensitive strain (IRAQ) that were pre-exposed to a sub-lethal dose of permethrin showed a 28% mortality (n=377) on subsequent exposure to a dose of permethrin close to the LT50, compared to a mortality of 47% in the control group (n=398); a 65% enhancement of permethrin tolerance has resulted from the pre-treatment ($X^2_{(1)} = 27$, $P < 0.0001$).

Fig. 6.1 Induction of enhanced tolerance in adults of IRAQ *An.stephensi* by pre-exposure to sub-lethal dose of permethrin



6.2.2 Effect of sub-lethal dose of permethrin on the tolerance level of mosquito larvae to subsequent exposure

In previous experiments we established that there is enhanced P-450 activity *in vivo* in the permethrin resistant larvae of the anopheline strain DUB-LPR compared to susceptible strains. A synergist test with the P-450 inhibitor, piperonyl butoxide (PB), suggested that P-450s have a significant role in permethrin resistance. Although IND-S strain is considered to be a susceptible strain, a synergist test indicated the presence of MFO enzymes in this strain which enhance its survival on exposure to permethrin albeit to a lesser extent than with the DUB-LPR strain. The synergist ratio for DUB-LPR and IND-S strains were 131 and 32.5-fold, respectively (see chapter 4).

A series of experiments were carried out to look for evidence of induction in larvae exposed to a sub-lethal concentrations of permethrin. The DUB-LPR and IND-S strains were pretreated with sub-lethal doses of permethrin before the 4th instar and at the early fourth instar stage were subjected to permethrin. Pre-exposure of DUB-LPR larvae to a sub-lethal dose of permethrin resulted in a 50% increase in the LC_{50} ($P<0.05$) and a two-fold increase in the LC90 ($P<0.05$) in a bioassay with permethrin. For the sensitive IND-S strain there appeared to be an inductive effect but it was not significant (Table 6.1 and Fig. 6.2).

Sub-lethal doses of permethrin have been found to induce enhanced tolerance in both adults (IRAQ strain) and larvae of IND-S and DUB-LPR strains. This appears to be the first report of induction in *An.stephensi*. The results showed an increased in tolerance to permethrin in pretreated mosquitoes. Since the induced mosquito becomes more tolerant to subsequent exposure to permethrin, there are two possibilities to explain these observations. One is that pre-exposure to permethrin might result in

selecting the more resistant individual within the strains. However, since permethrin was used at a concentration that killed less than 10% of exposed mosquitoes, selection is most unlikely to explain the results. The other possibility is that sub-lethal doses of permethrin turn on the detoxification machinery in the mosquito and give them some protection against subsequent exposure. Vulule et al. (1994) reported a rise in level of pyrethroid tolerance in *An.gambiae* population during a village scale trial of impregnated nets in Kenya. However, recently they reported that long-term use of permethrin-impregnated nets did not further increase permethrin tolerance in this population (Vulule et al., 1996). The low level permethrin tolerance (2.5-fold) in adults of this species collected from Kenyan villages could be due to earlier contact with impregnated nets in the village causing enzyme induction from sub-lethal dose of permethrin which gave them some protection to subsequent exposure. Apart from this possible non-genetic form of permethrin tolerance, Vulule et al, (1994) subsequently produced a strain with genetic tolerance to permethrin by laboratory selection.

Table 6.1 The influence of pre-exposure of larvae of *An.stephensi* to a sub-lethal dose of permethrin followed by bioassay with permethrin

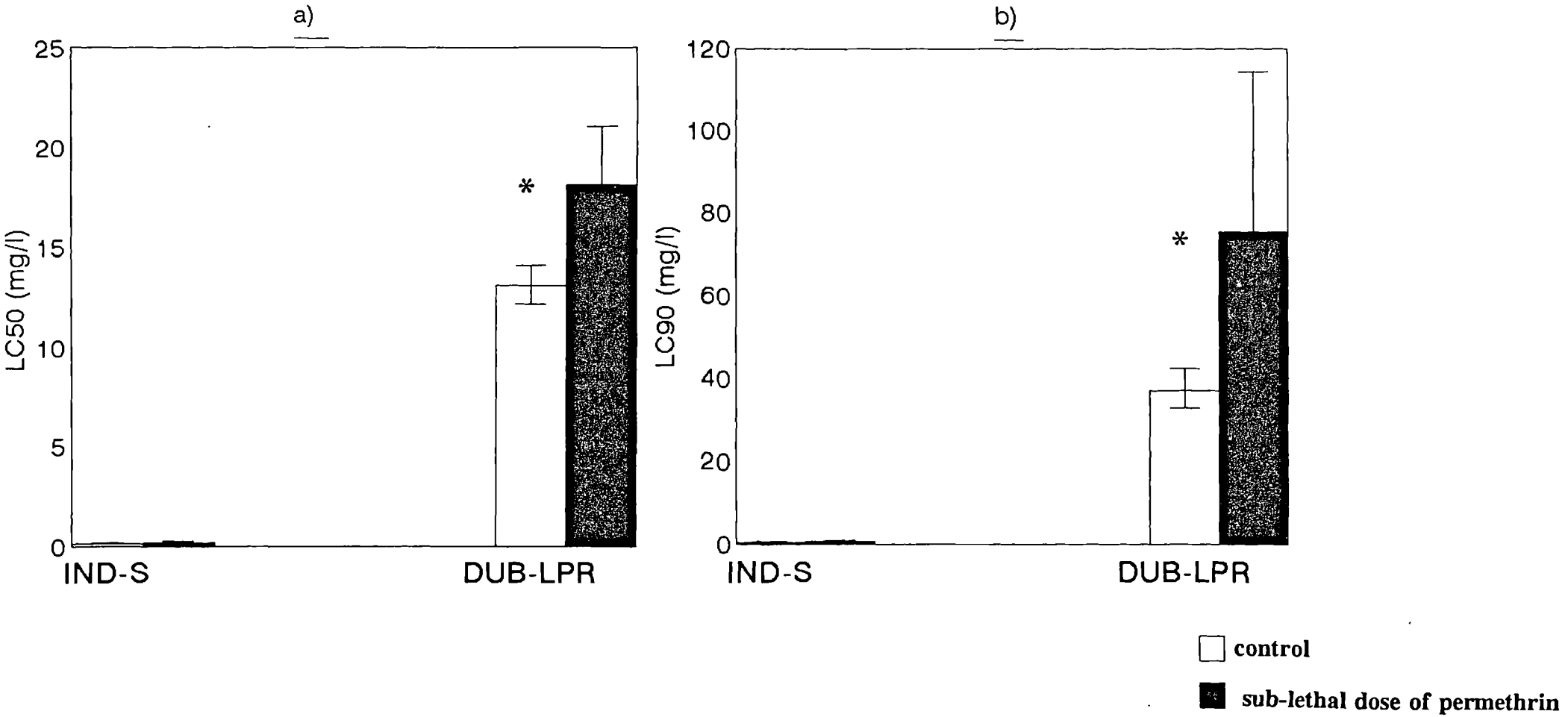
(IR=induction ratio)

Strains	Pre-exposure with permethrin	LC_{50} (mg/l)	LC_{90} (mg/l)
DUB-LPR	+	18.1	75.5
	-	13.06	36.9
IR		1.39	2.04
P		<0.05	<0.05
IND-S	+	0.16	0.59
	-	0.13	0.44
IR		1.23	1.35
P		n.s.	n.s.

Fig. 6.2 The influence of sub-lethal dose of permethrin on tolerance of *An.stephensi* larvae to permethrin. a) at the LC50; b) at the LC90

Asterisks indicate significant difference between control and treatment ($^{*}P<0.05$)

Vertical bars= 95%C.I.



Enzyme induction was reported in *Triatoma infestans* (Agosin & Dinamarca, 1963) where treatment with DDT increased the level of NAD-kinase. Further confirmation of microsomal enzyme induction was provided by Morello (1964) who showed that treatment of *T.infestans* with 3-methylcholanthrene led to an increase in the *in vivo* production of polar metabolites from DDT and at the same time caused a decrease in mortality from DDT poisoning.

By the late 1970's at least 12 species of insects had been shown to respond to inducers by producing increased levels of such enzymes as cytochrome P-450s, DDT dehydrochlorinase, phosphotransferase, carboxyesterase, epoxide hydratase, and sulphotransferase. Cytochrome P-450s are induced by many lipophilic xenobiotics in the *M.domestica* (Plapp & Casida, 1970), *Phormia regina* (Vincent et al., 1985), *D.melanogaster* (Magnusson et al., 1979), *Spodoptera eridania* and *S.fragiperda* (Brattsten et al., 1984; Yu & Ing, 1984), *Peridarma saucia* (Yu et al., 1979), *Californica autographica* (Farnsworth et al., 1981), *Heliothis puntiger* (Collins, 1985) and others. Glutathione transferase activity is inducible in *M.domestica* (Ottea & Plapp, 1984), *S.fragiperda* (Brattsten et al., 1984; Yu, 1982) and in other species. Induced esterase activity has been demonstrated in *S.fragipedra* (Yu & Hsu, 1985). Early evidence that the insect's microsome systems was inducible was obtained with various insecticides and phenobarbital. Hayaoka & Dauterman (1982) reported that DDT caused a 150% increase in the activity of glutathione S-transferase in *M.domestica.*

6.2.3 Pretreatment of mosquito adults with sodium phenobarbital

Many substances have been shown to be inducers, such as phenobarbitone which

induces particular P-450 isozymes. There is a similarity in the induction of detoxificating enzymes by phenobarbital in insects and mammals. Phenobarbital induces glutathione S-transferases (Ottea & Plapp, 1981) and MFO (Terrier et al., 1971) in *M.domestica*. These two reactions are responsible for detoxication and activation and are increased in the phenobarbital pretreated housefly.

The results of bioassays of resistant and susceptible *An.stephensi* females following pretreatment with 10^{-4} molar sodium phenobarbital and subsequent exposure to permethrin, deltamethrin and malathion are presented in Tables 6.2, 6.3 and 6.4, respectively. The results from Tables 6.2, 6.3 and Figs. 6.3 and 6.4 indicate that sodium phenobarbital increases the pyrethroid tolerance level of the IND-S strain to a greater extent than the DUB-APR strain. We reasoned that the reduced effect of inducers on the resistant strain is the consequence of the initially higher enzyme activity in this strain. The phenomenon of a pyrethroid resistant strain being less sensitive to phenobarbital induction compared to susceptible flies has been noted by Scott & Lee (1993b). They found that pretreatment of pyrethroid-resistant house flies with phenobarbital caused no, or only a small increase in cytochrome P-450, total cytochrome P-450s and P-450 monooxygenase activities. In contrast, phenobarbital increased the cytochrome P-450 content in tissues of a susceptible strains to levels similar to those found in tissues of a resistant strain. The low responsiveness to phenobarbital in tissues from resistant strains suggests that production of cytochrome P-450 may be constitutively expressed in them at near maximal level.

There is also some evidence of protection against malathion in females of IND-S and DUB-APR strains that have been pretreated with sodium phenobarbital (Table 6.4, Fig. 6.5).

Table 6.2 The influence of pretreatment of females of *An.stephensi* to 10^{-4} M sodium phenobarbital followed by exposure to $10\mu g/cm^2$ permethrin (IR=induction ratio)

Strains	Pretreatment with sodium phenobarbital	LT_{50} (minutes)	LT_{90} (minutes)
DUB-APR	+	62.3	110.2
	-	82.0	139.2
IR		0.76	0.79
P		n.s.	n.s.
IND-S	+	16.0	53.5
	-	10.6	23.4
IR		1.5	2.29
P		<0.05	<0.01

Fig. 6.3 The influence of sodium phenobarbital (10^{-4} M) on tolerance of *An.stephensi* females to permethrin

a) at the LT50; b) at the LT90

Asterisks indicate significant difference between control and treatment ($^{*}P<0.05$, $^{**}P<0.01$)

Vertical bars= 95%C.I.

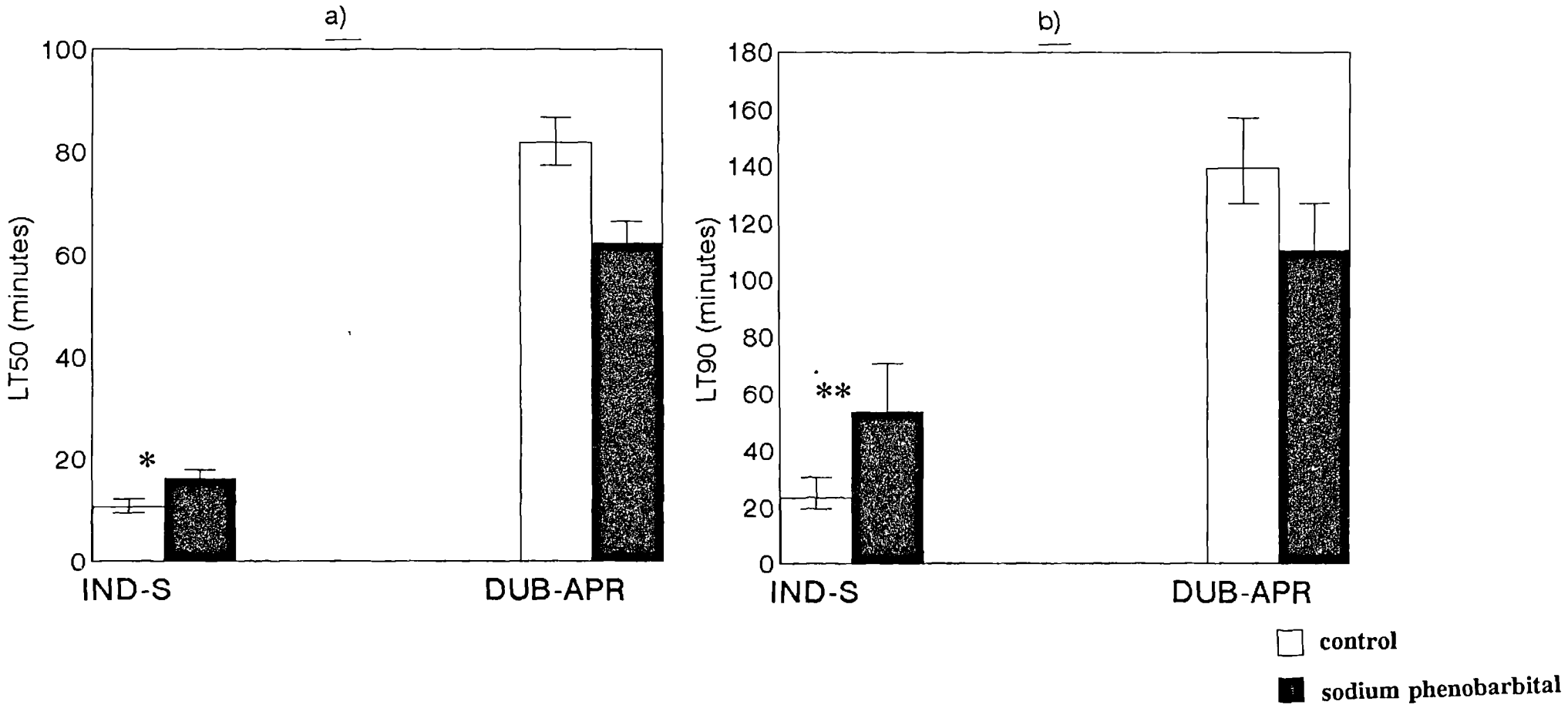


Table 6.3 The influence of pretreatment of females of *An.stephensi* to 10^{-4} M sodium phenobarbital followed by exposure to 0.025% deltamethrin (IR=induction ratio)

Strains	Pretreatment with sodium phenobarbital	LT_{50} (minutes)	LT_{90} (minutes)
DUB-APR	+	41.2	84.2
	-	53.2	98.1
IR		0.77	0.86
P		n.s.	n.s.
IND-S	+	10.2	64.1
	-	8.7	46.3
IR		1.17	1.38
P		n.s.	n.s.

Fig. 6.4 The influence of sodium phenobarbital (10^{-4} M) on tolerance of *An.stephensi* females to deltamethrin

a) at the LT50; b) at the LT90

Vertical bars= 95%C.I.

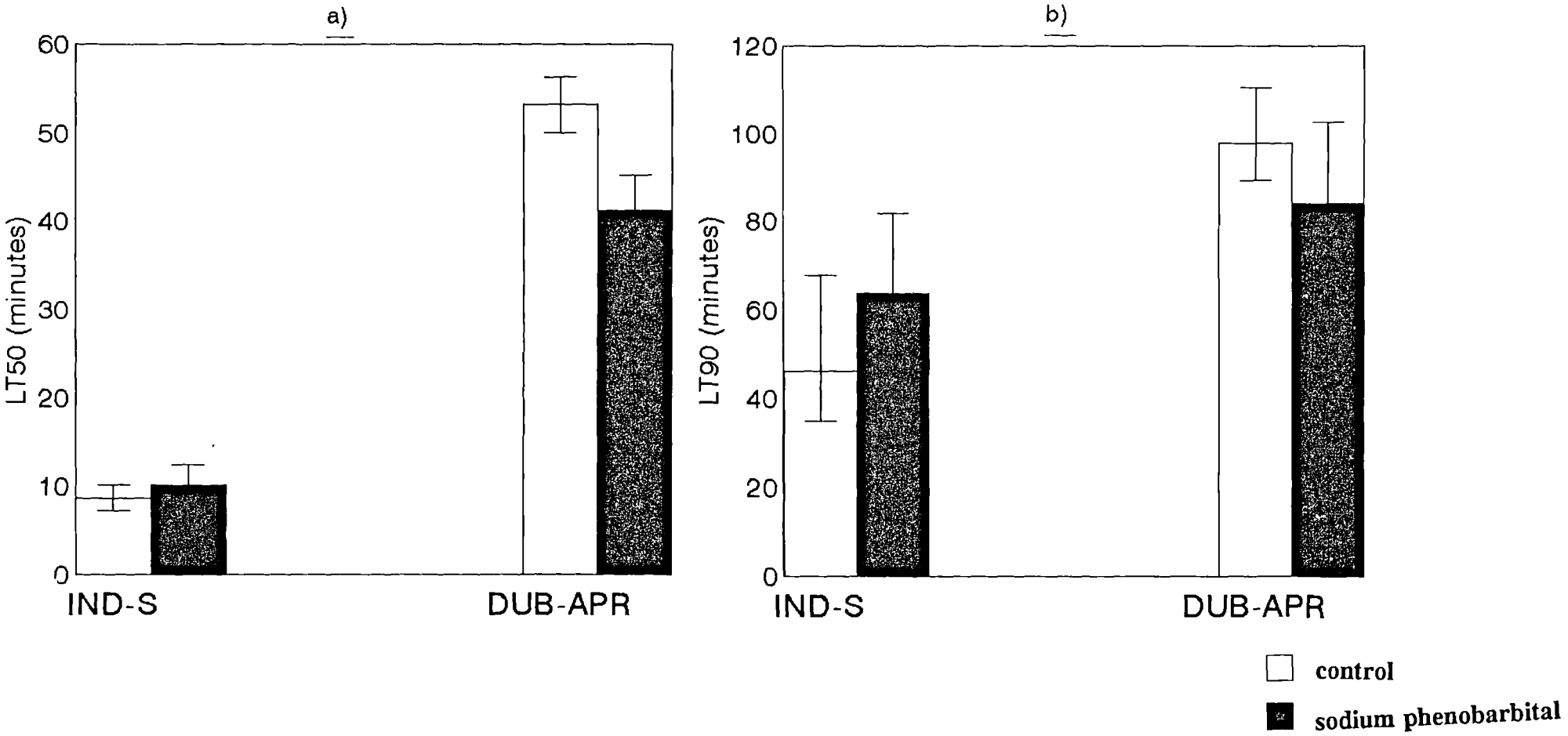


Table 6.4 The influence of pretreatment of females of *An.stephensi* to 10^{-4} M sodium phenobarbital followed by exposure to 5% malathion

(IR=induction ratio)

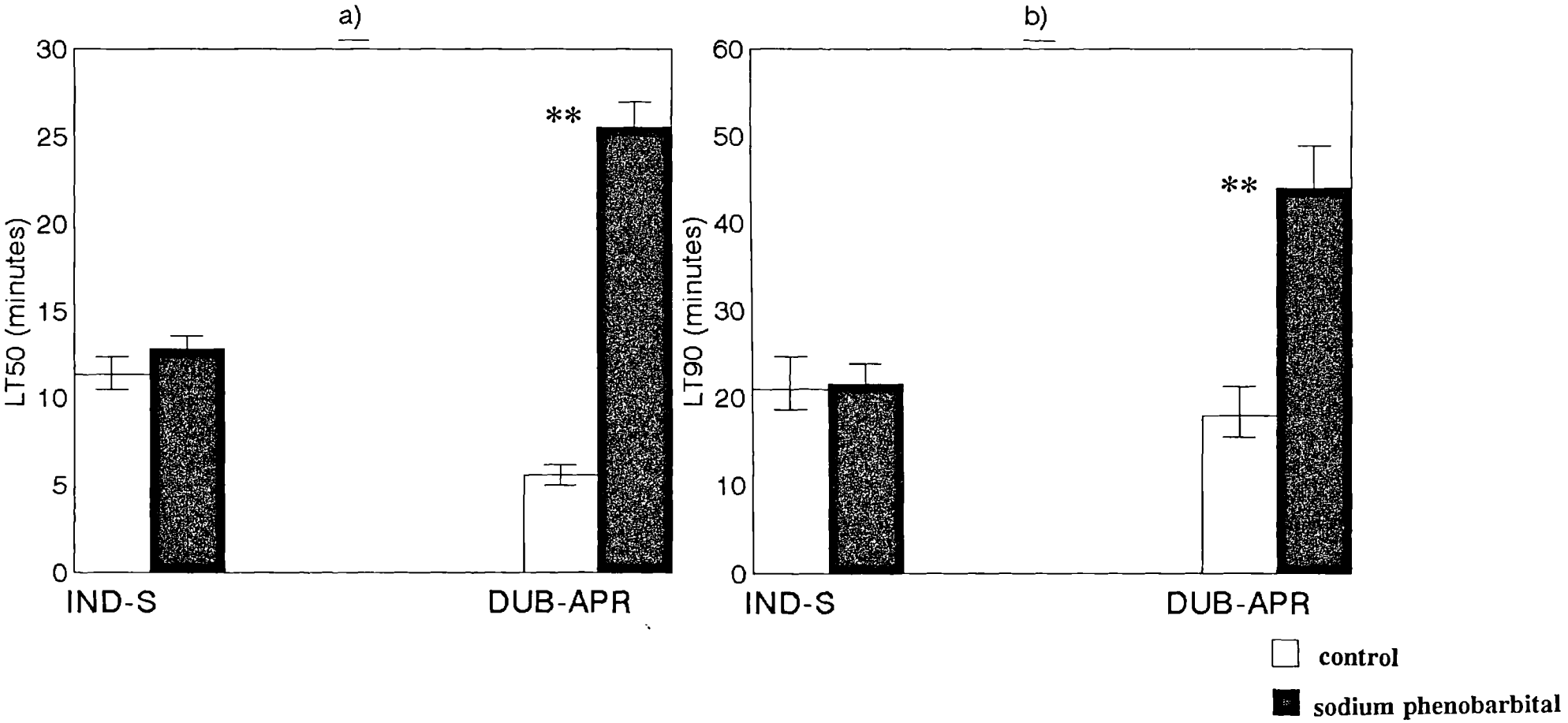
Strains	Pretreatment with sodium phenobarbital	LT_{50} (minutes)	LT_{90} (minutes)
DUB-APR	+	25.5	44.0
	-	5.6	17.9
IR		4.6	2.5
P		<0.01	<0.01
IND-S	+	12.8	21.5
	-	11.4	21.0
IR		1.12	1.0
P		n.s.	n.s.

Fig. 6.5 The influence of sodium phenobarbital (10^{-4} M) on tolerance of *An.stephensi* females to malathion

a) at the LT50; b) at the LT90

Asterisks indicate significant difference between control and treatment ($^{*}P<0.05$, $^{**}P<0.01$)

Vertical bars= 95%C.I.



6.2.4 Pretreatment of mosquito larvae with sodium phenobarbital

Most of the work on induction has concentrated on *M.domestica* and *Phormia regina*, with which some progress has been made in resolving and characterising the multiple forms of P-450 (Yu & Terriere, 1973 ; Terriere & Yu 1979; Molderke & Terriere, 1981; Feyereisen & Vincent, 1984; Feyereisen et al., 1995).

The results of Clark et al. (1986) showed that in the house fly there are multiple glutathione S-transferases having varied substance specificities. The relative activities of these multiple isozymes are different with different insecticides.

The MFO system is known to be inducible in the house fly by phenobarbital (cited by Ottea & Plapp, 1981). Evidence of glutathione S-transferase inducibility by phenobarbital in rats has also been reported (Kaplowitz et al., 1975 ; Hales & Neims, 1977). Terriere et al. (1971) reported that the net increase in MFO activity on phenobarbital induction was greater in a house fly strain initially high in oxidase activity than in a strain low in this respect. The induction of MFO by phenobarbital in the house fly was affected by age, sex, strain and dose of inducer (Walker & Terriere, 1970).

A series of experiments was carried out to look for evidence of induction of insecticide tolerance in larvae of *An.stephensi* by enzyme inducers, such as sodium phenobarbital. There was some evidence of enhancement of permethrin tolerance in the larvae of DUB-LPR and IND-S strains when they were pretreated with 10^{-8} M sodium phenobarbital (Table 6.5, Fig. 6.6). It was shown that sodium phenobarbital enhanced the tolerance of both strains to malathion with induction ratios of 1.29 and 1.23 for DUB-LPR and IND-S strains respectively (Table 6.6, Fig. 6.7).

Table 6.5 The influence of pre-exposure of larvae of *An.stephensi* to 10^{-8} M sodium phenobarbital on subsequent tolerance to permethrin

(IR=induction ratio)

Strains	Pre-exposure with sodium phenobarbital	LC_{50} (mg/l)	LC_{90} (mg/l)
DUB-LPR	+	16.3	64.8
	-	13.06	36.9
IR		1.25	1.80
P		<0.05	<0.05
IND-S	+	0.11	0.57
	-	0.13	0.44
IR		0.88	1.30
P		n.s.	<0.05

Fig. 6.6 The influence of sodium phenobarbital (10^{-8} M) on tolerance of *An.stephensi* larvae to permethrin

a) at the LC50; b) at the LC90

Asterisks indicate significant difference between control and treatment (*P<0.05)

Vertical bars= 95%C.I.

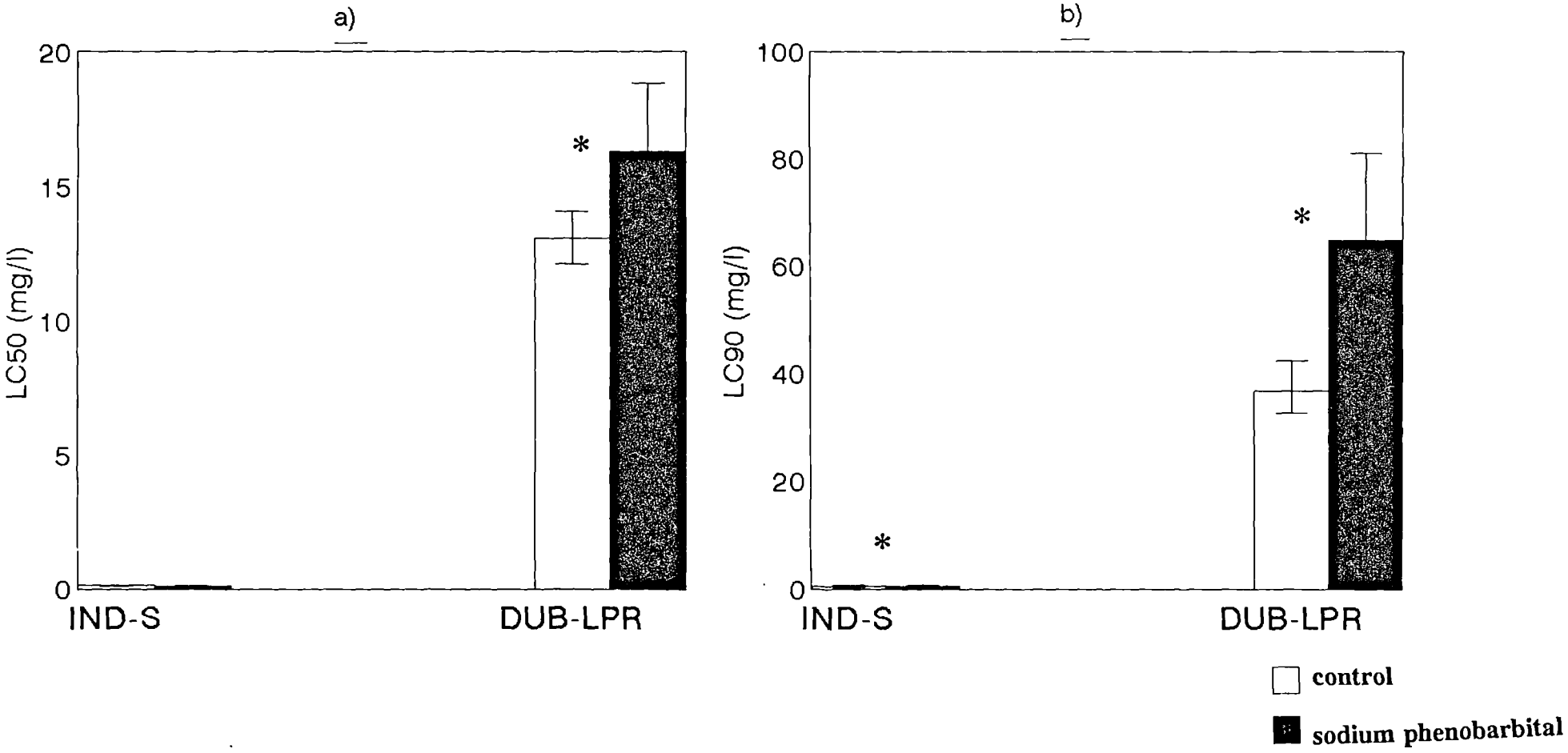


Table. 6.6 The influence of pre-exposure of larvae of *An.stephensi* to 10^{-8} M sodium phenobarbital on subsequent tolerance to malathion

(IR=induction ratio)

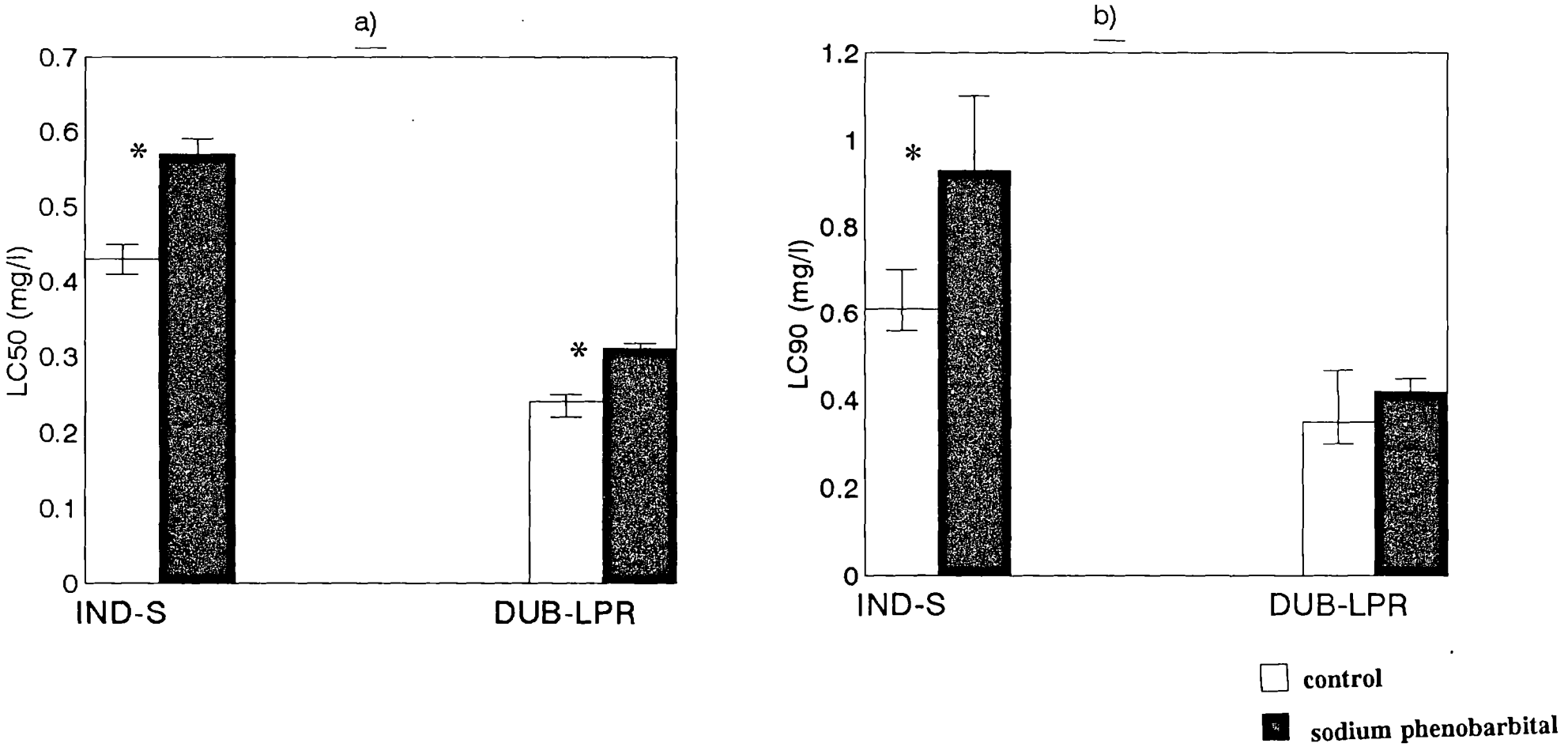
Strains	Pre-exposure with sodium phenobarbital	LC_{50} (mg/l)	LC_{90} (mg/l)
DUB-LPR	+	0.31	0.42
	-	0.24	0.35
IR		1.29	1.20
P		P<0.05	n.s.
IND-S	+	0.57	0.93
	-	0.43	0.61
IR		1.23	1.52
P		p<0.05	P<0.05

Fig. 6.7 The influence of sodium phenobarbital (10^{-8} M) on tolerance of *An.stephensi* larvae to malathion

a) at the LC50; b) at the LC90

Asterisks indicate significant difference between control and treatment ($^{*}P<0.05$)

Vertical bars= 95%C.I.



Pretreatment of house flies with phenobarbital provides some protection against methyl parathion, methyl paraoxon, azinphosmethyl, and methidathion toxicity. The LD50 value of these insecticides increased 1.86, 2.8, 1.33 and 1.95-fold, respectively in pretreated house flies (Hayaoka & Duaterman, 1982). Pretreatment of rats with barbiturates protects animals from the lethal effects of warfarin, meprobamate and strychnine by stimulating the metabolism of these compounds to nontoxic products but this same treatment increased the toxicity of schradan (cited by Conny, 1986).

Phenobarbital and 3-methylcholanthrene treatment of rats lead to 2 to 3-fold increase in the microsomal content of cytochrome P-450, NADPH cytochrome P-450 reductase, epoxide hydrase, glutathione S-transferases and aldehyde dehydrogenase proteins (cited by Adesnik et al., 1981).

Our finding indicates that the detoxification reaction was accelerated by sodium phenobarbital and exceeded the activation reaction. Data obtained by Vincent et al. (1985) suggested that phenobarbital induced several P-450's in the blow fly, *Phormia regina.*

A key feature of the biochemistry of P-450 is that there are multiple forms of these enzymes; different forms (isozymes) are induced by different inducers or induced under different physiological conditions. For example one inducer may induce several forms of P-450s and affect different P-450s differently, structurally unrelated compounds can induce the same P-450 isozymes and there are sex differences in P-450 isozymes distributions.

6.2.5 Pretreatment of mosquito with menthol-related compounds

A wide variety of allelochemicals of plants such as menthol and menthone, are potent and effective at dietary doses in eliciting induction. The first evidence that plant allelochemicals could induce enzyme systems was presented by Brattsten et al. (1984); the treated larvae of *Spodoptera eridania* were shown to be less susceptible to nicotine. Tolerance to malathion was greater in larvae of *Peridrona saucia* that were fed peppermint leaves. Similar results were obtained with last instar larvae of *Trichoplusia ni* fed peppermint. Peppermint was active in stimulating aldrin epoxidase and P-450. Bioassays of larvae indicated that tolerance to carbaryl and methonyl was greater than with control larvae. Ronis et al. (1988) reported that MFO activity is dramatically higher in polyphagous Lepidoptera, which can encounter a broader spectrum of plant derived toxicants, than in mono- or oligo-phagous species.

Relating all of this to our work, a study in our laboratory was directed to determine whether monoterpens such as menthol which occurs in peppermint leaves enhances the tolerance of *An.stephensi* larvae to permethrin. Larvae of DUB-LPR and IND-S strains pretreated with 10^{-10} M menthol exhibited a small but significant enhanced tolerance to permethrin (Table 6.7). Pre-exposure of larvae of a sensitive strain (IND-S) to 10^{-10} molar menthol produced a 1.7-fold increase ($P<0.05$) in the LC50 and a 2.5-fold increase ($P<0.05$) in the LC90. For the resistant strain (DUB-LPR) the inductive effect was less pronounced (Figs. 6.8 and 6.9).

According to results summarized in Table 6.8 more enhanced tolerance was observed in larvae pretreated with 250 mg/l of peppermint leaves. When peppermint leaves were used as the inducer, the LC50 of the sensitive strain (IND-S) increased 2-fold ($P<0.01$) and LC90 2.6-fold ($P<0.01$). For the resistant strain (DUB-LPR) the LC50 increased 2.9-fold ($P<0.01$) and the LC90 3.3-fold ($P<0.01$) (Figs. 6.10 and

6.11).

In an experiment by Farnsworth et al. (1981), larvae of alfalfa looper (*California autographica*) and cabbage looper (*Trichoplusia ni*) reared on peppermint indicated that stimulation of microsomal oxidase activity by the peppermint constituents provided increased tolerance for carbaryl and methomyl but not the organophosphate, acephate. The difference in insecticide susceptibility in comparison with control larvae was attributed to increased microsomal oxidase activity as indicated by a nearly 10-fold increase in aldrin epioxidation by the midgut microsome of mint-fed larvae. Some allelochemicals of plants inhibit, rather than induce, detoxication enzymes. The most notable examples of this are the 1,3-benzodioxy compounds such as safrole, isosafrole and myristicine. These compounds are potent inhibitors of the MFO and could prevent metabolism by this system.

Table 6.7 The influence of pre-exposure of larvae of *An.stephensi* to 10^{-10} M menthol on subsequent tolerance to permethrin

(IR=induction ratio)

Strains	Pre-exposure with menthol	LC_{50} (mg/l)	LC_{90} (mg/l)
DUB-LPR	+	14.40	67.73
	-	13.06	36.90
IR		1.1	1.84
P		n.s.	$p<0.05$
IND-S	+	0.22	1.08
	–	0.13	0.44
IR		1.70	2.5
P		$p<0.05$	$P<0.05$

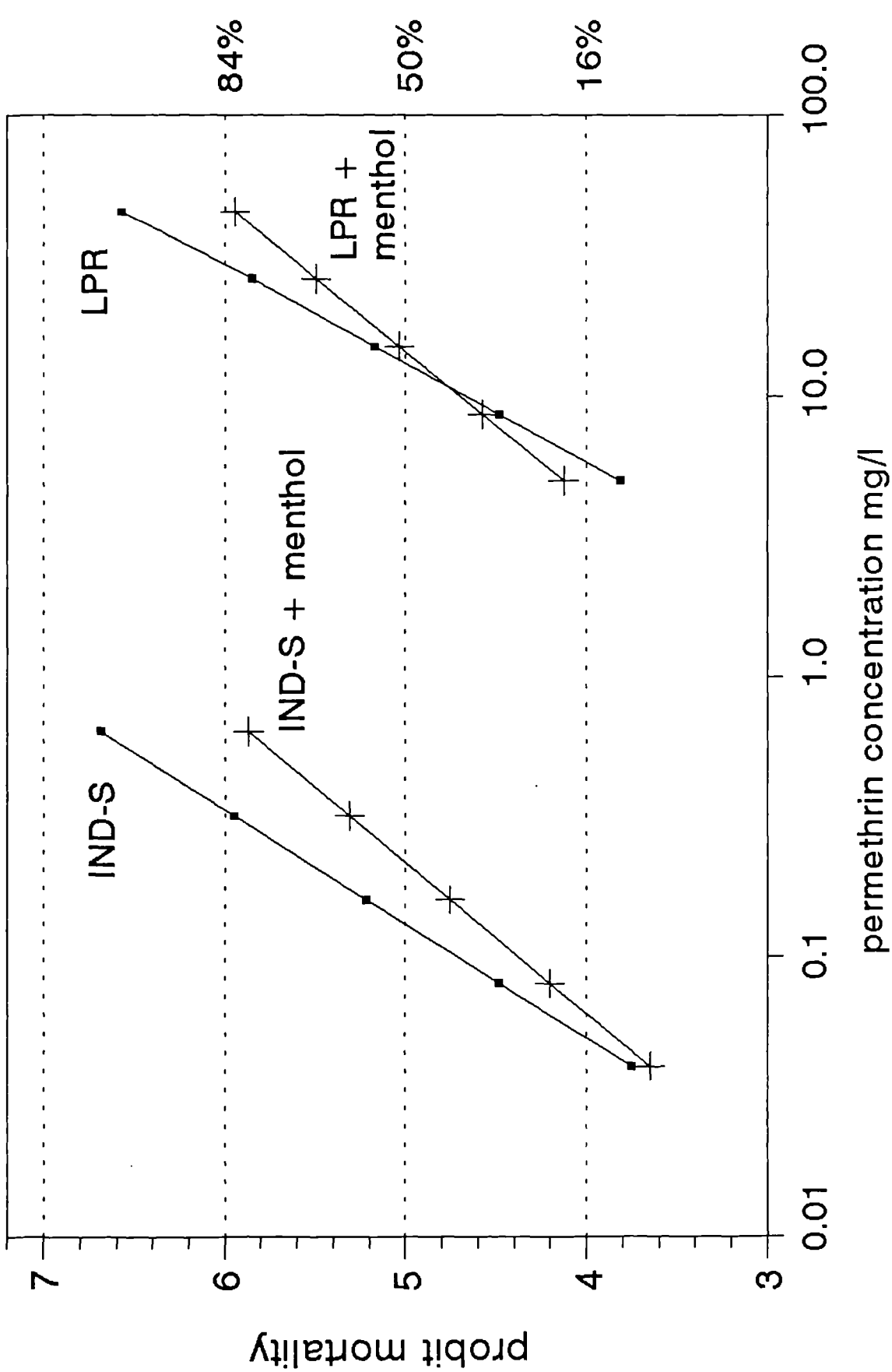


Fig. 6.8 Effect of pre-exposure to menthol (10^{-10} M) on the mortality of larvae subsequently exposed to permethrin

Fig. 6.9 The influence of menthol (10^{10} M) on tolerance of *An.stephensi* larvae to permethrin

a) at the LC50; b) at the LC90

Asterisks indicate significant difference between control and treatment ($^{*}P<0.05$)

Vertical bars= 95%C.I.

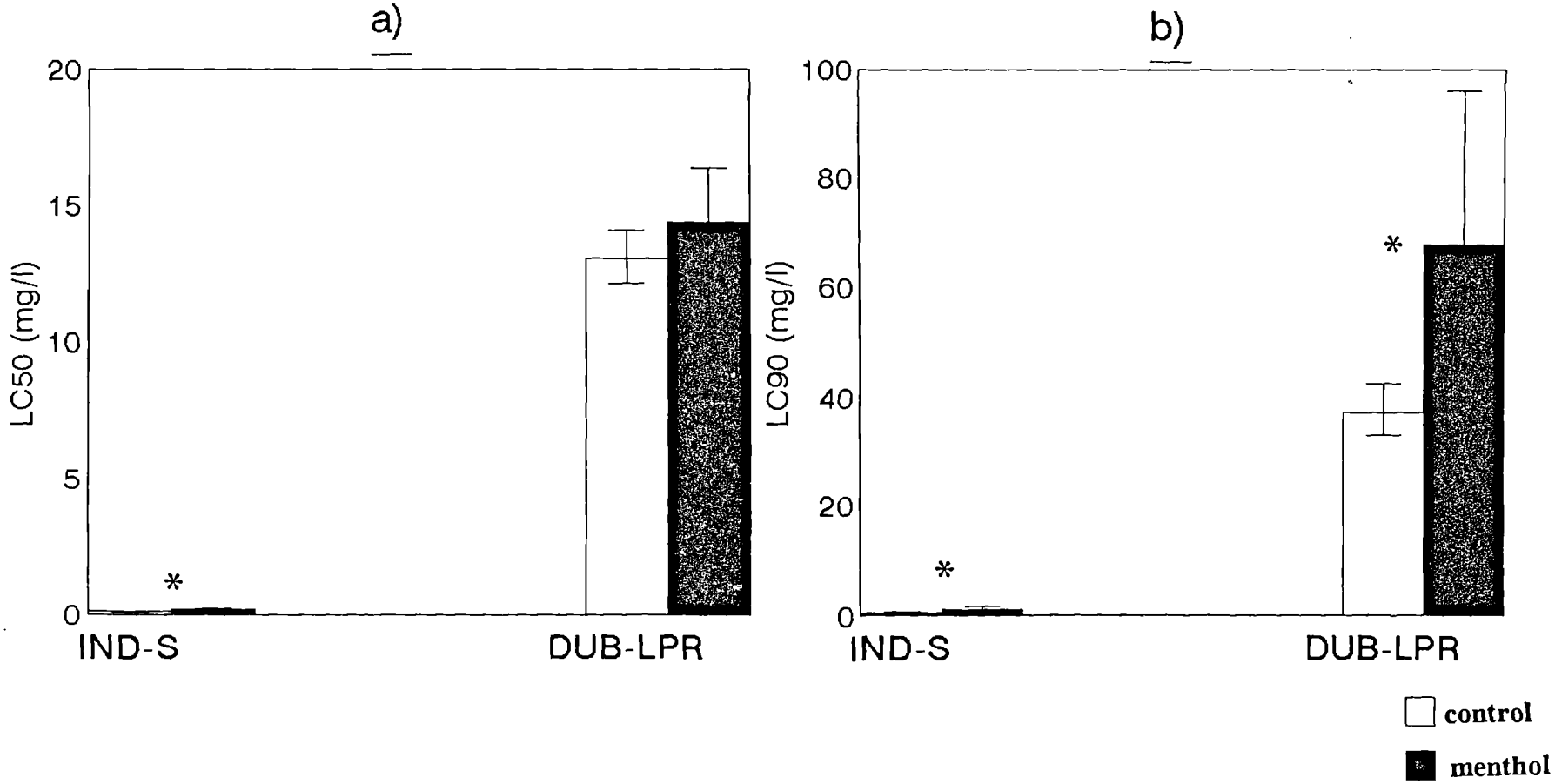


Table 6.8 The influence of pre-exposure of larvae of *An.stephensi* to peppermint (250 mg/l) on subsequent tolerance to permethrin

(IR=induction ratio)

Strains	Pre-exposure with peppermint	LC_{50} (mg/l)	LC_{90} (mg/l)
DUB-LPR	+	37.75	122.10
	-	13.06	36.90
IR		2.9	3.3
P		P<0.01	p<0.01
IND-S	+	0.27	1.16
	–	0.13	0.44
IR		2.0	2.6
P		p<0.01	P<0.01

Fig. 6.10 Effect of pre-exposure to peppermint (250 mg/l) on the mortality of larvae subsequently exposed to permethrin

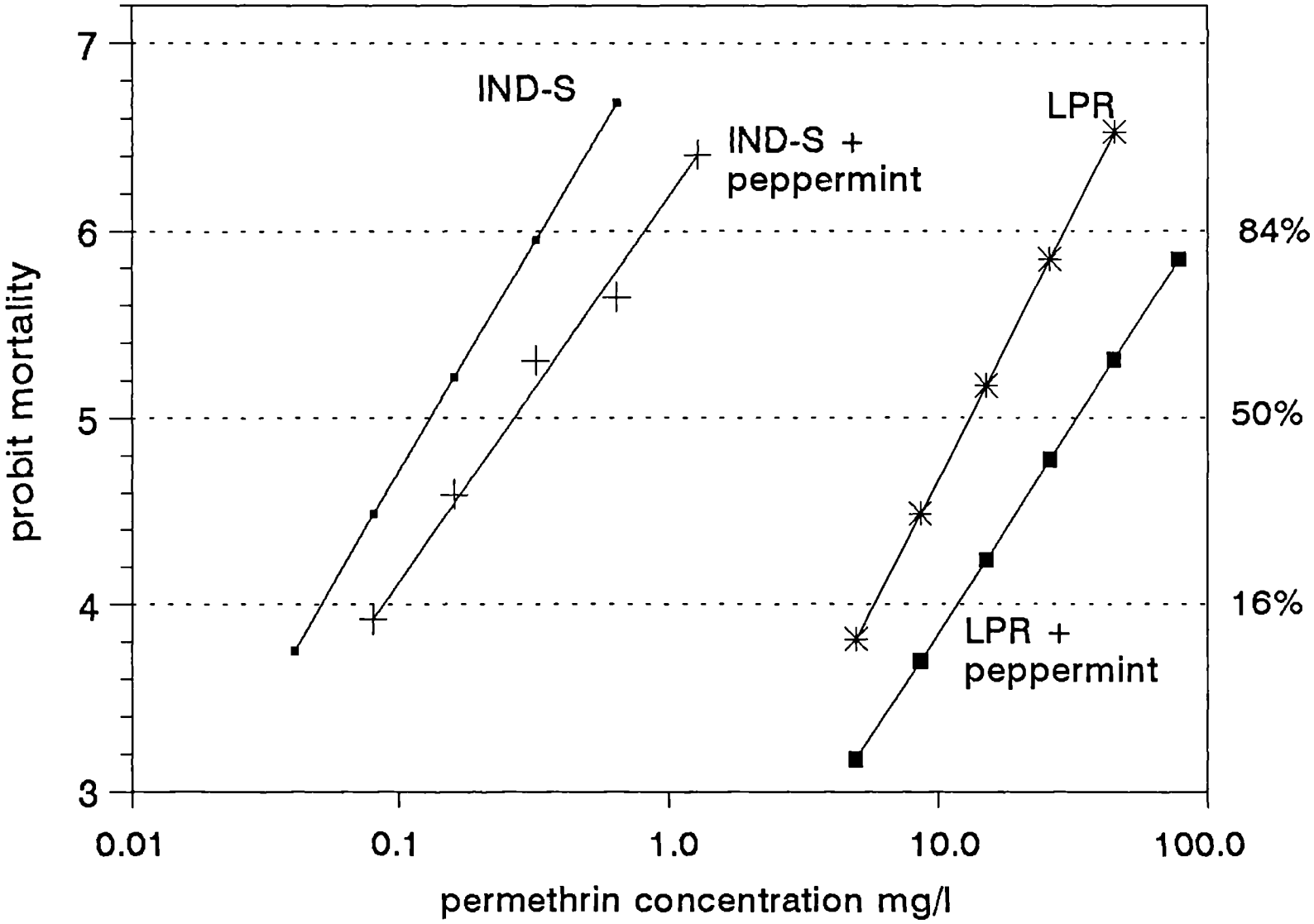
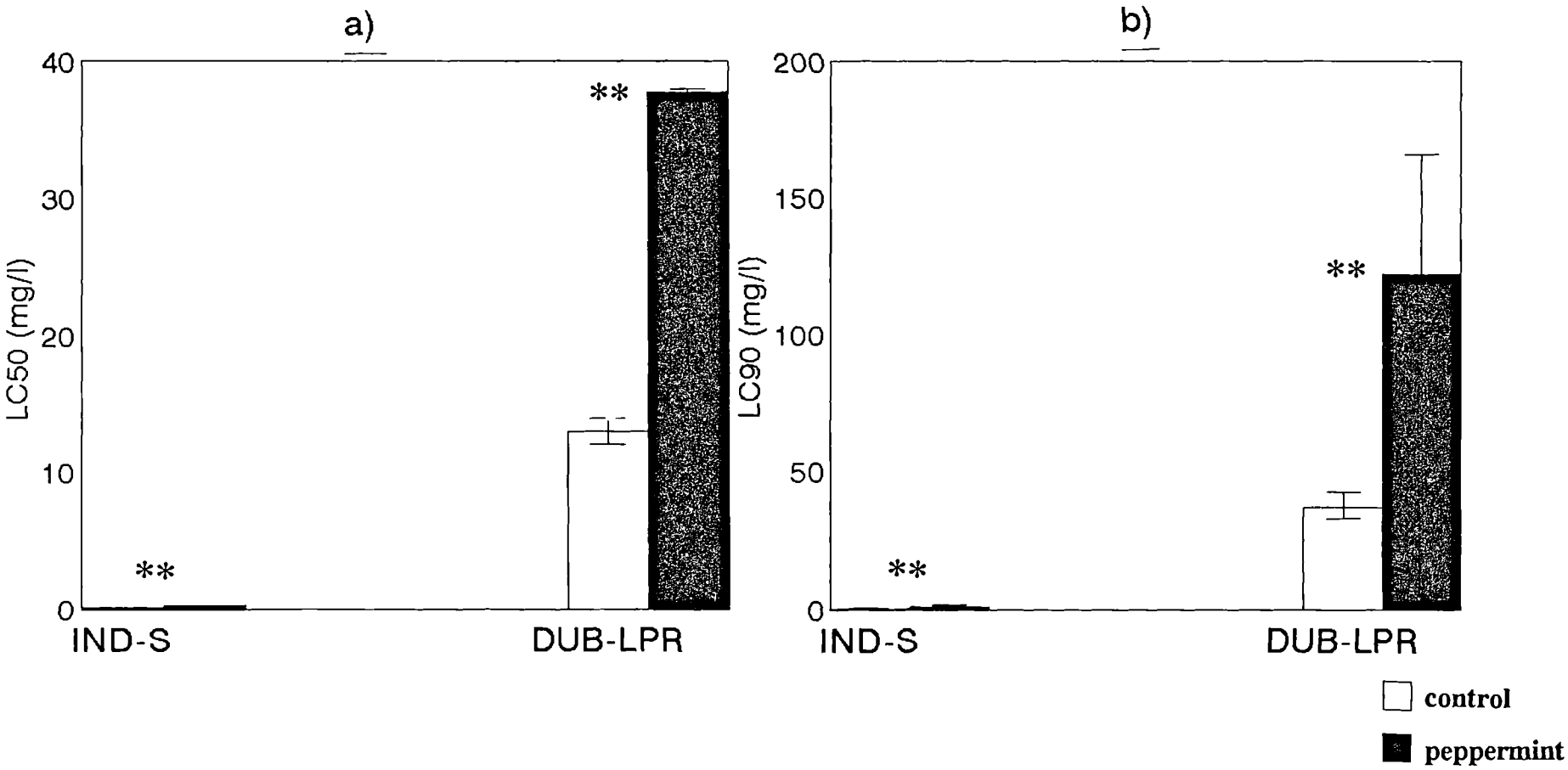


Fig. 6.11 The influence of peppermint (250 mg/l) on tolerance of *An.stephensi* larvae to permethrin

a) at the LC50; b) at the LC90

Asterisks indicate significant difference between control and treatment ($^{*}P<0.05$, $^{**}P<0.01$)

Vertical bars= 95%C.I.



6.3 Conclusions

Larval resistance to permethrin in the DUB-LPR strain is known to be due to a single autosomal gene (Ladonni & Townson, 1996). The inhibition of this resistance by piperonyl butoxide shows that this resistance is mediated through cytochrome P-450 enzymes (Ladonni & Townson, 1996). In DUB-APR resistance is more complicated, being polyfactorial and involving P-450s, esterases and target site insensitivity (see chapter 4).

The treatments described in this chapter were able to induce a significant level of enhanced tolerance, as high as 3-fold in the case of larvae of DUB-LPR exposed to breeding water containing peppermint leaves. Siegfried & Young (1993) found that different detoxification enzymes including general esterases, permethrin hydrolysis, total cytochrome P-450, aldrin epoxidase and glutathione S-transferases were present in aquatic insects such as black fly, damsel fly and dragon fly. They concluded that these enzyme activities in the aquatic insects were influenced by the presence of environmental contaminants in the aquatic habitats. Such influences (e.g. induction) might explain the high activities of detoxification enzymes in aquatic insects.

Some insecticides, instead of being inactivated by detoxification, are made more toxic, at least temporarily, it would seem that enhancement of such metabolism could be advantageous (Terriere, 1984). Some inducers are more potent than others. Correspondingly, we would expect differences in the amount of a pesticide required to control a given insect in different habitats. Enzyme induction depends on species and life stage. For example Feng et al. (1995) showed that malathion toxicity to the larvae of *Spodoptera litura* which were fed on the tree *Melia toosendan* was reduced during the first 2 days and increased on the subsequent 2 days. This phenomenon was associated with increased midgut esterase activity during the first 2 days and

decreased esterase activity thereafter.

Since a number of enzyme systems involved in insecticide resistance are known to be inducible (Terriere, 1984), it cannot be concluded with certainty that the increased tolerance is mediated solely through enhanced P-450 activity. Further immunological and synergistic assays suggest enzyme differences between induced and noninduced mosquitoes. However, according to studies undertaken by Sivananthan (1993) enzyme assays which reveal recognition of proteins related to P-450 classes IA1 and IIC in *An. stephensi* strains which have been used in our studies.

Overall, based on our studies, we suggest that mosquitoes possess a battery of detoxification enzymes of differing substance specificities and that these may be expressed differentially in different strains of *An.stephensi*. The ability to detoxify a given substance by reactions mediated by the detoxification enzymes may thus represent the expression of a number of different genes.

Knowledge gained in the area of induction has the potential for exploitation in vector control programmes. The inductive effect of some inducers which are present in habitats of mosquitoes may turn on the detoxification mechanisms in insects and result in a higher pesticide degradation rate in induced insects than the non-induced populations.

Chapter 7

THE STRUCTURE-ACTIVITY RELATIONSHIPS OF PYRETHROIDS

7.1 Introduction

The natural pyrethrins and some synthetic pyrethroids have been used for many years in insect pest control because of their lower mammalian toxicity than those of other classes of insecticides (Elliot, 1989) (Table 7.1), outstanding insecticide efficiency and degradation to innocuous residues. Their cost and instability, however, have excluded them from many other applications. Nowadays there is considerable scope for developing new pyrethroid insecticides with little hazard for man and mammals (low mammalian toxicity is particularly important) by modifying the structures of the natural pyrethrins. New compounds already synthesized are more effective against some insect species than the natural compounds and, are even less hazardous to mammals (Elliot et al., 1978). Absolute values for likely field performance cannot be predicted for individual compounds from laboratory experiments but comparative tests should enable it to be decided which compounds are likely to suit a particular application and particular vectors.

The structure-activity approach contributes to a better understanding of the specific substituents on pyrethroid compounds that will be optimal for a particular desired property. That knowledge may be useful for the development of novel compounds that could play a role in the control of insects. The activities of pyrethroids to insects, mammals and other groups, depend on the optical (1R/1S) and geometrical (*cis/trans*) configurations of their acidic and alcoholic compounds. Structure-activity relationship studies are useful as they give an indication of the relative importance of the different

parts of molecules in terms of both their activity and their metabolism (Davies, 1985). The aim of structure-activity relationship studies is to determine whether changes in the structure of the pyrethroids markedly affect their potency as insecticides towards the different strains of *An.stephensi* and to examine the response of permethrin-resistant and susceptible strains to different classes of pyrethroids. It should be noted that all pyrethroids are carboxylic acid esters and generally have no more than three chiral centres, located on carbon 1 and 3 of the cyclopropane ring and at the α-carbon of the alcohol moiety (Fig. 7.1). i.e., asymmetric carbon atoms about which different isomeric arrangement can occur.

An attempt was made to carry out a study using a range of pyrethroids to assess structure-activity relationships. In our study the following pyrethroids were chosen: cypermethrin, permethrin, deltamethrin, cyfluthrin and lambdacyhalothrin. The structure and substitutions of these compounds are shown in Fig. 7.2. All compounds are based on a common generic structure but have various substitutions. Except permethrin all have α-cyano substitution. Cypermethrin has a similar structure to deltamethrin but with a dichloro substitution rather than a dibromo substitution. Cyfluthrin has a flouro substitution on one of the aromatic rings and lambdacyhalothrin has a triflouromethyl substitution at the end of the acid moiety. Bioassays were conducted with these compounds and the LC50s values determined by probit analysis as described by Finney (1971). Standard bioassay procedures were used as described in section 3.2.5. Permethrin susceptible and resistant strains of *An.stephensi* including TEH, BAN, IND-S, IRAQ, DUB-LPR, and DUB-APR were used.

Table 7.1 Rat oral toxicity of pyrethroids compared with other insecticides

Pyrethroids	Rat acute oral LD_{50} (mg/kg)	References
permethrin	430-4000	The Pesticide Manual, 1987
cypermethrin	251-4123	"
cyfluthrin	500-1200	"
deltamethrin	135-5000	"
lambdacyhalothrin	231-483	Parasitology Today, 1988
parathion	5	Elliot, 1989
carbaryl	540	Elliot, 1989
DDT	113-118	The Pesticide Manual, 1987

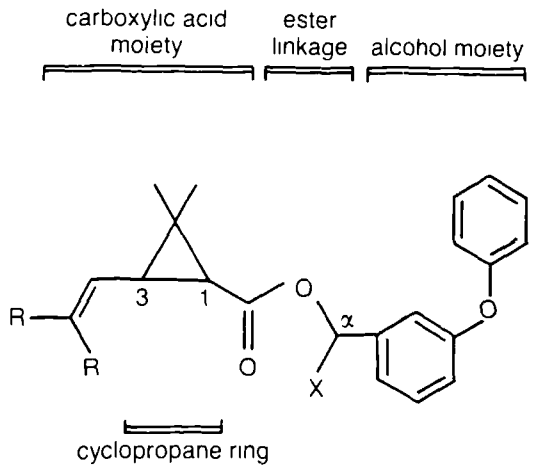
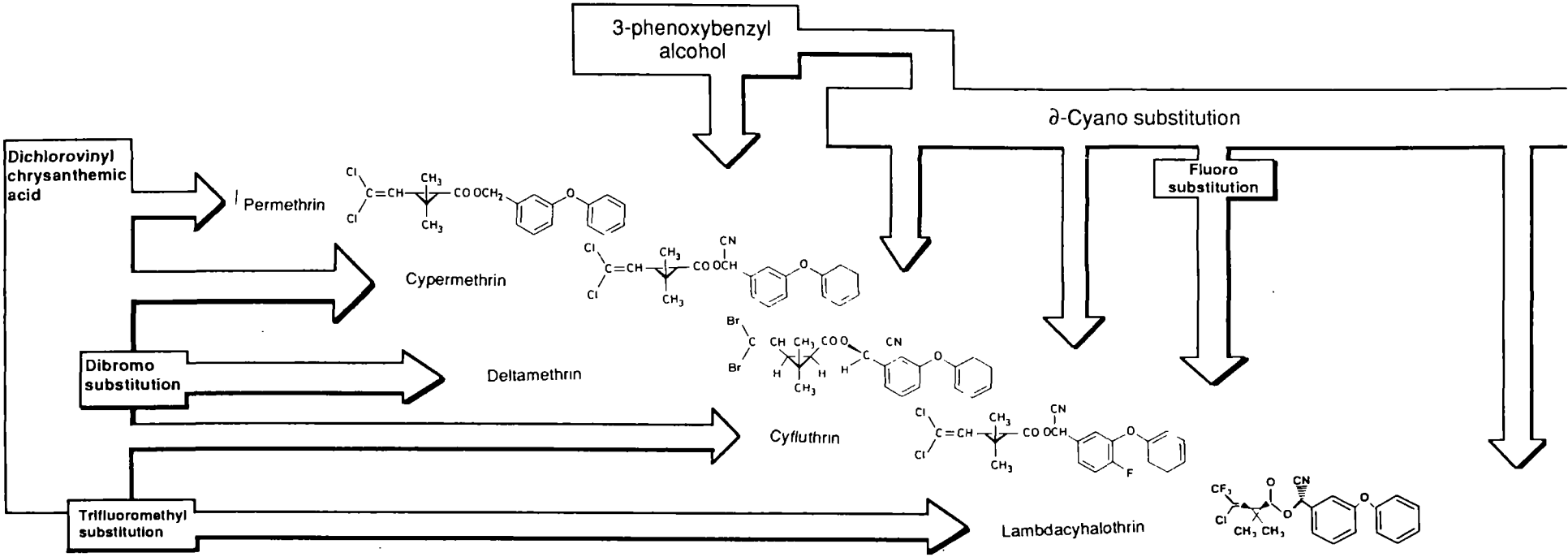


Fig. 7.1 General structure of a synthetic pyrethroid

Fig. 7.2 Structure of the pyrethroids which were used (from Miller, 1988)



7.2 Results

7.2.1 Structure-activity relationship of pyrethroids to larvae of susceptible strains

a) IND-S

Deltamethrin was by far the most toxic pyrethroid with an LC50 value of 0.026 mg/l. Cypermethrin and cyfluthrin with LC50s of 0.57 and 0.32 mg/l were the least toxic, with over 22 and 12 fold difference in toxicity compare to deltamethrin (Tables 7.2 and 7.3). Permethrin with an LC50 value of 0.13 mg/l had a moderate effect. Lambdacyhalothrin with LC50 value of 0.04 was 1.5 times less toxic than deltamethrin.

b) IRAQ

The potency of 5 pyrethroids, as toxicants for larvae of IRAQ strain increased in the order deltamethrin > lambdacyhalothrin > permethrin > cypermethrin > cyfluthrin.

c) BAN strain

The pyrethroid sensitivity assay with BAN strain revealed that among pyrethroids again deltamethrin is the most toxic compound with an LC50 value of 0.006mg/l (Table 7.2). Deltamethrin showed a 78, 72, 11 and 3-fold higher toxicity compared with cypermethrin, cyfluthrin, permethrin and lambdacyhalothrin respectively (Table 7.3).

d) TEH

Bioassay for TEH strain is shown in Table 7.2. Deltamethrin was most toxic with LC50 value of 0.0045 mg/l and cypermethrin the least toxic with LC50 value of 0.16 mg/l, i.e. 36 times different in terms of toxicity (Table 7.3). The remaining

pyrethroids showed moderate toxicity in the order lambdacyhalothrin > permethrin > cyfluthrin. Relative toxicity of pyrethroids against susceptible strains are shown in Fig. 7.3.

7.2.2 Structure-activity relationship of pyrethroids in larvae of permethrin-resistant strains

a) DUB-APR

In DUB-APR larvae lambdacyhalothrin was the most toxic pyrethroid with an LC50 value of 3.6 mg/l (Table 7.2). Cyfluthrin with an LC50 of 13.05 mg/l was the least toxic with a 3.6 fold difference in toxicity compared to lambdacyhalothrin. The other pyrethroids had different LC50 values with order of toxicity deltamethrin > permethrin > cypermethrin. At the LC50 level deltamethrin was 0.6 fold less toxic than lambdacyhalothrin and 2, 2.3 and 1.3 fold more toxic than cypermethrin, cyfluthrin and permethrin respectively (Table 7.3). The comparative toxicity of pyrethroids is shown in Fig. 7.4.

b) DUB-LPR

Larvae of DUB-LPR strain have become highly resistant to their selecting chemical as indicated by the high insecticide concentration needed for mortality (LC50=13.06 mg/l) (Table 7.2). Cyfluthrin was the most toxic with an LC50 value of 1.31 mg/l. Results from bioassay data in Table 7.2 and Fig. 7.4 indicate that resistance is affected by the presence of the α-cyano group. Cyfluthrin, cypermethrin, lambdacyhalothrin and deltamethrin had more potency against larvae of DUB-LPR in comparison with permethrin with potencies of 10, 7.5, 3 and 2-fold more than that of permethrin.

Table 7.2 LC_{50}±95%C.I with various pyrethroids for larvae of different strains of *An.stephensi*

Pyrethroids Strains	deltamethrin	lambdacyhalothrin	permethrin	cyfluthrin	cypermethrin
IND-S	0.019 0.026 0.040	0.033 0.040 0.050	0.110 0.130 0.150	0.25 0.32 0.39	0.47 0.57 0.68
IRAQ	0.0040 0.0050 0.0055	0.020 0.023 0.027	0.078 0.080 0.082	0.47 0.55 0.65	0.41 0.49 0.58
BAN	0.005 0.006 0.007	0.015 0.018 0.020	0.063 0.064 0.066	0.36 0.43 0.51	0.39 0.47 0.56
TEH	0.0040 0.0045 0.0050	0.023 0.027 0.032	0.069 0.070 0.073	0.09 0.12 0.15	0.13 0.16 0.20
DUB-APR	4.60 5.58 6.88	3.16 3.60 4.12	6.11 7.33 8.99	11.58 13.05 14.85	9.51 10.53 11.70
DUB-LPR	6.00 6.65 7.31	3.33 4.16 5.25	12.14 13.06 14.07	1.14 1.31 1.50	1.52 1.74 2.00

Table 7.3 Relative activity of pyrethroids in comparison with deltamethrin for larvae of different strains of *An.stephensi*

Pyrethroids Strains	lambdacyhalothrin	permethrin	cyfluthrin	cypermethrin
IND-S	1.5	5	12	22*
IRAQ	4.6	16	110	98
BAN	3	11	72	78
TEH	6	16	27	36
DUB-APR	0.6	1.3	2.3	2
DUB-LPR	0.6	2	0.2	0.3

* At the LC_{50} level, the value for cypermethrin is 22 times higher than for deltamethrin with the IND-S strain

Fig. 7.3 Relat ve toxicity of pyrethroids to larvae of susceptible strains of *An.stephensi*. Vertical bars=95%C.I.

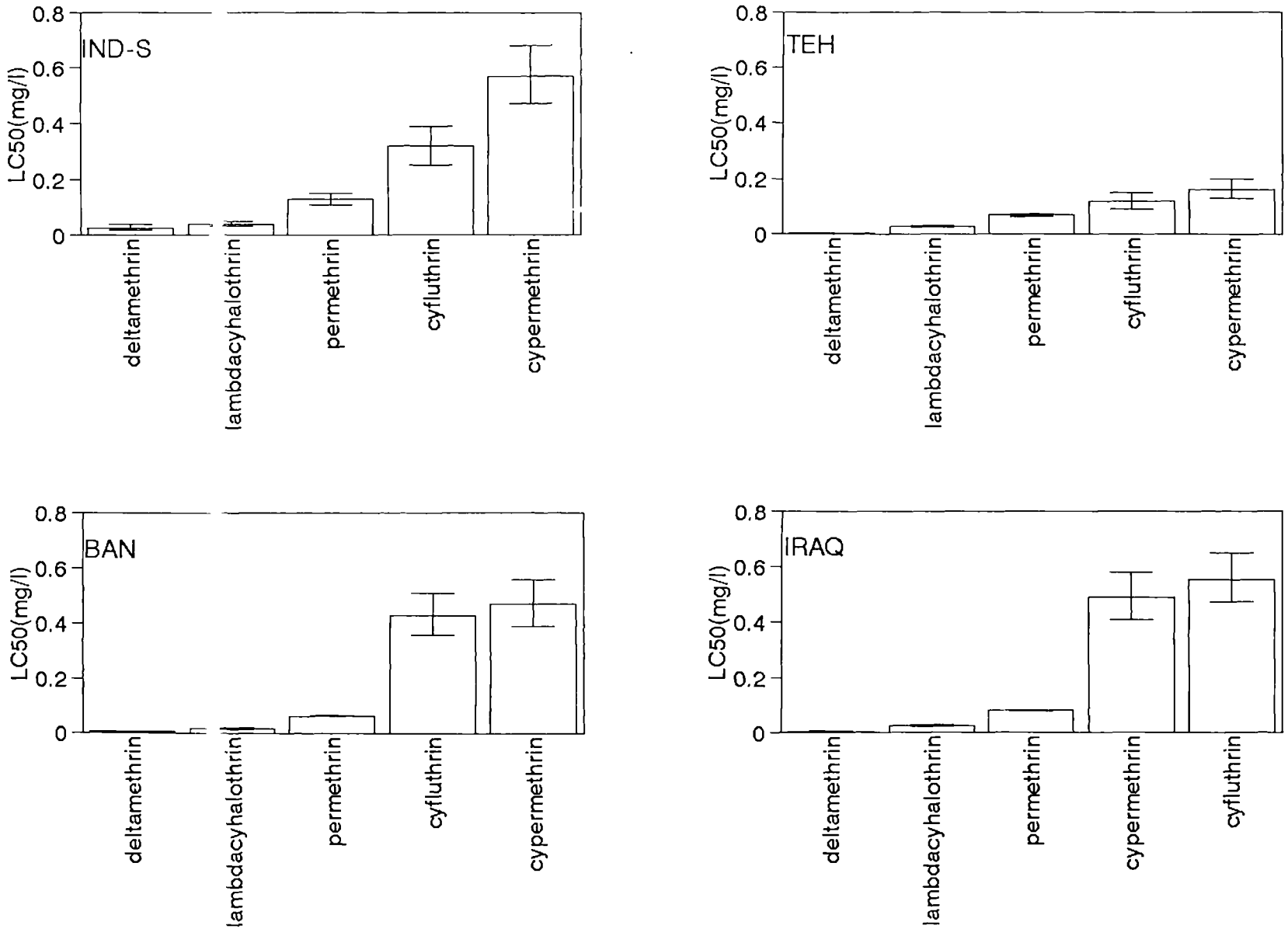
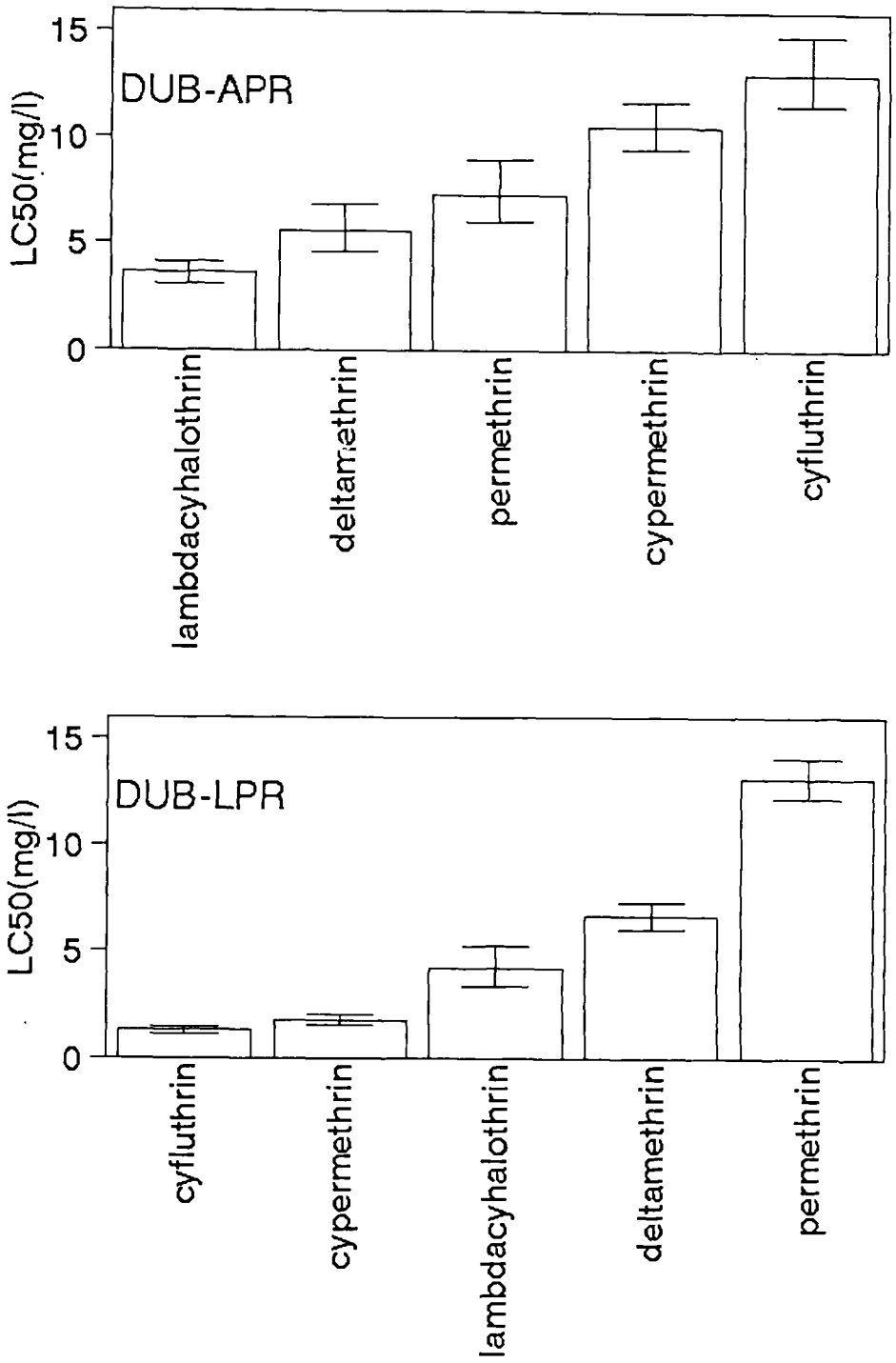


Fig. 7.4 Relative toxicity of pyrethroids to larvae of permethrin-resistant strains of *An.stephensi*. Vertical bars=95%C.I.



7.3 Synergist study

Because DUB-LPR and DUB-APR larvae have developed resistance to permethrin and this resistance was mainly due to P-450 dependent basis, it is important to consider the biochemical mechanism of resistance to other classes of pyrethroids to indicate whether similar mechanism/s are involved in each resistant strain. Therefore the effects of the mono-oxygenase synergist, piperonyl butoxide (PB) were examined. The results of synergist studies are summarized in Tables 7.4 and 7.5.

In larvae of DUB-LPR and DUB-APR strains, permethrin was strongly synergized by PB with synergist ratios of 131 and 73-fold, respectively. PB significantly reduced the resistance factor (RF), causing it to drop from 100-fold and 56-fold for DUB-LPR and DUB-APR respectively to the level of the LC50 of the IND-S strain. In DUB-LPR strain PB produced a high synergism (85-fold) of deltamethrin and 87-fold of lambdacyhalothrin. The application of synergist with cypermethrin and cyfluthrin gave SRs of 19 and 21-fold at the LC50 level (Table 7.4).

The synergist studies revealed that a slight change (4-fold) occurred in the DUB-APR strain response to lambdacyhalothrin when PB was added but PB synergism was relatively high with other pyrethroids: 73-fold for permethrin, 51-fold for deltamethrin, 11-fold for cypermethrin and 13-fold for cyfluthrin at the LC50 level (Table 7.5). This work clearly showed that, with most of pyrethroids tested, the synergist made a difference in the response of resistant larvae, i.e. after synergism by PB, the resistance factor for all of the pyrethroids declined. The synergism of permethrin and deltamethrin in either resistant strain was similar, but synergism for cypermethrin and cyfluthrin is less than seen with deltamethrin and permethrin. This seems unrelated to α-cyano substitution. Davies (1985) pointed out that a given

modification of structure may, or may not, produce a change in intrinsic activity, but even if such a change is produced it may be obscured by changes in routes and rates of metabolism, and by changes in penetration and distribution consequent upon changes in physical properties. All of these variables will affect the ease of access of a compound to its site of action and thus its effective toxicity. However, the total effect need not to be the same for different insect strains, nor need a given modification applied to a series of related compounds produce the same modification of biological activity in each case.

Table 7.4 Effect of the synergist piperonyl butoxide (PB) on the status of pyrethroid resistance in larvae of DUB-LPR strain of *An.stephensi*

Pyrethroids	with/out PB	LC_{50}±95% C.I	SR*
permethrin	+	0.09 0.10 0.13	131
	-	12.14 13.06 14.07	
deltamethrin	+	0.066 0.078 0.093	85
	-	6.00 6.65 7.31	
lambdacyhalothrin	+	0.041 0.048 0.560	87
	-	3.33 4.16 5.25	
cypermethrin	+	0.074 0.092 0.120	19
	-	1.52 1.74 2.00	
cyfluthrin	+	0.052 0.062 0.075	21
	-	1.14 1.31 1.50	

* SR= LC50 of insecticide alone/ LC50 of insecticide+synergist

Table 7.5 Effect of the synergist piperonyl butoxide (PB) on the status of pyrethroid resistance in larvae of DUB-APR strain of *An.stephensi*

Pyrethroids	with/out PB	LC_{50}±95% C.I	SR*
permethrin	+	0.076 0.100 0.140	73
	-	6.11 7.33 8.99	
deltamethrin	+	0.09 0.11 0.13	51
	-	4.60 5.58 6.88	
lambdacyhalothrin	+	0.85 1.00 1.22	4
	-	3.16 3.60 4.12	
cypermethrin	+	0.74 0.92 1.14	11
	-	9.51 10.53 11.70	
cyfluthrin	+	0.80 0.99 1.23	13
	-	11.58 13.05 14.84	

* SR= LC50 of insecticide alone/ LC50 of insecticide+synergist

Fig. 7.5 Log insecticide concentration/probit response lines for IND-S (♦) and DUB-APR (◘) larvae treated with pyrethroids in combination with the synergist piperonyl butoxide (PB) (☼).

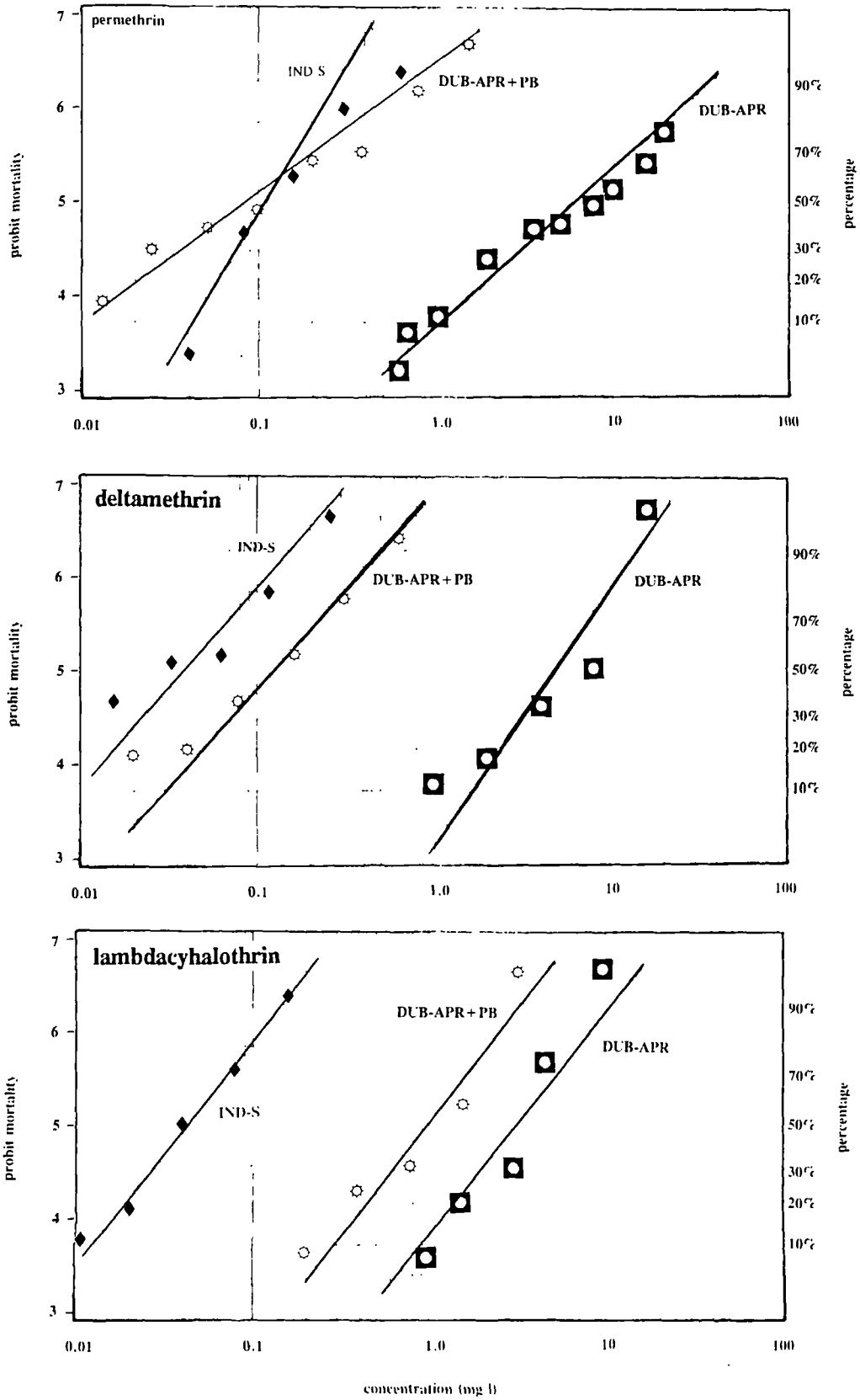


Fig. 7.5 (continued)

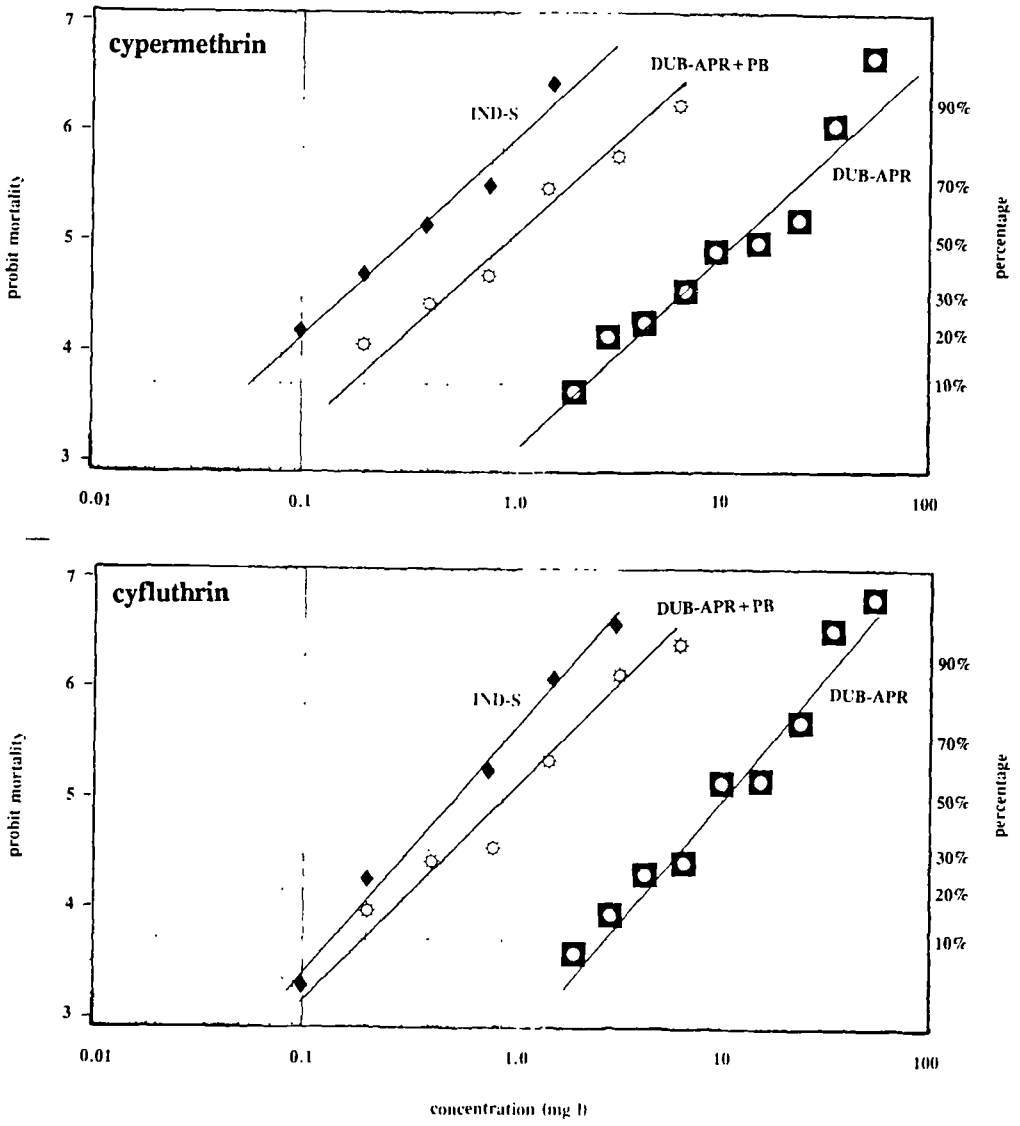


Fig. 7.6 Log insecticide concentration/probit response lines for IND-S (♦) and DUB-LPR (◘) larvae treated with pyrethroids in combination with the synergist piperonyl butoxide (PB) (☼).

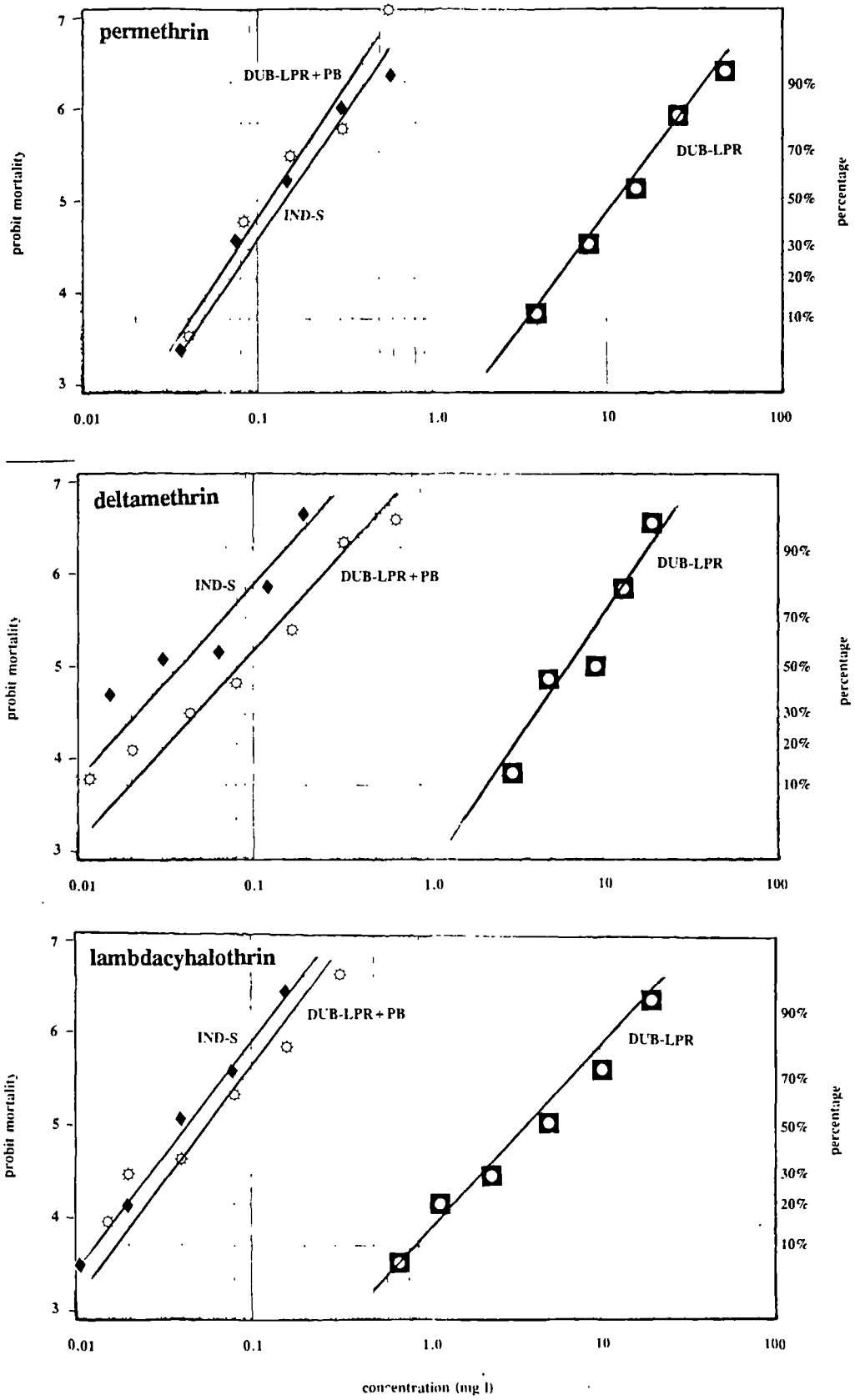
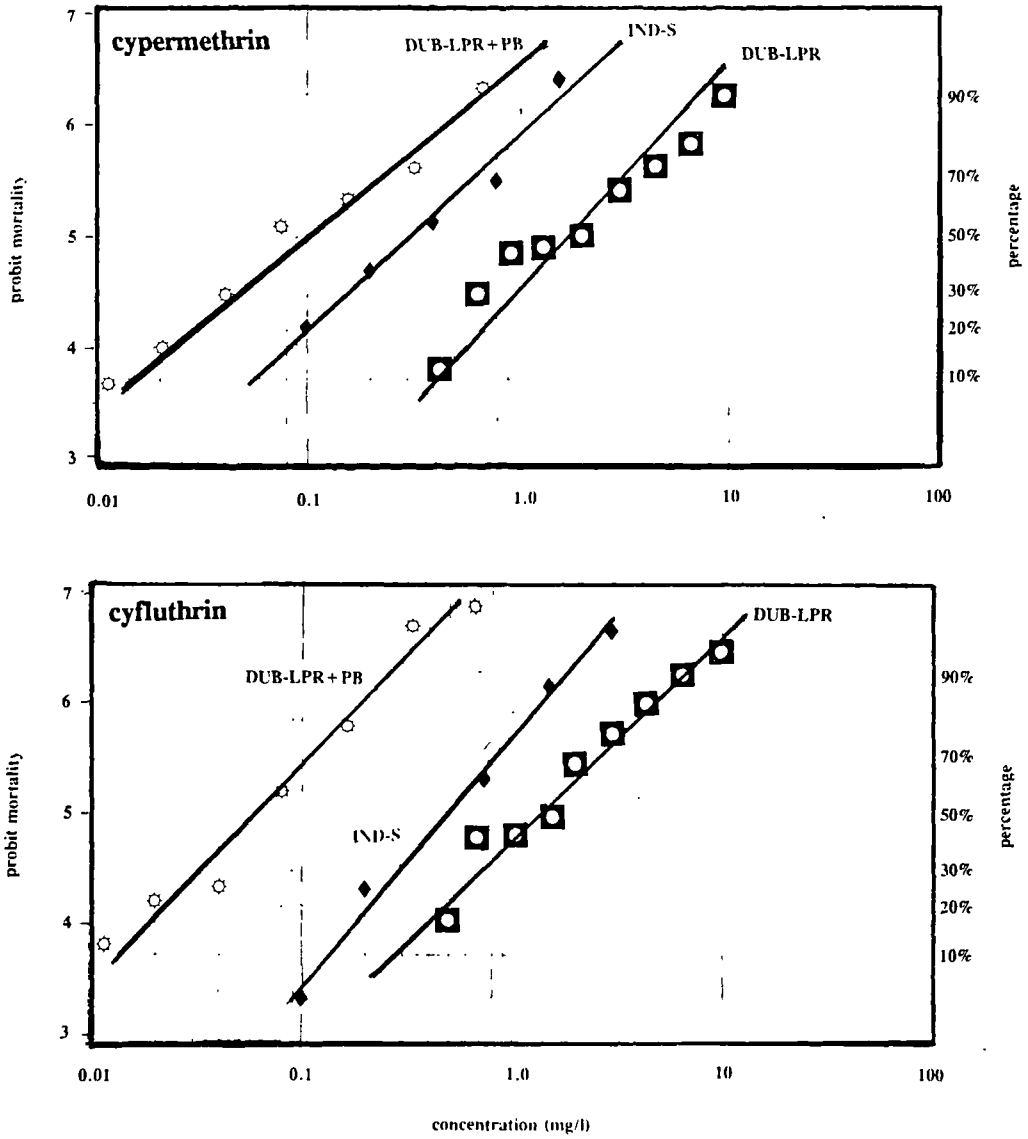


Fig. 7.6 (continued)



7.4 Discussions

The results of sensitivity assays for susceptible and resistant strains are illustrated in Figs. 7.3 and 7.4, respectively. The order of toxicity among the pyrethroids depends on the target strain but in general, deltamethrin, an α-cyano pyrethroid with dibromo substitution, was the most potent compound against susceptible larvae. Cypermethrin and cyfluthrin were the least toxic, while lambdacyhalothrin and permethrin had moderate toxicity. The range of pyrethroid toxicity at the LC50 between the two extremes (deltamethrin and cypermethrin) was greatest for the susceptible strains. For example deltamethrin was 98, 78, 36 and 22 times more potent than cypermethrin for larvae of IRAQ, BAN, TEH and IND-S strains and the pattern of sensitivity to pyrethroids was applied only to susceptible strains. In contrast, with the DUB-APR strain the difference between highest and lowest potency (lambdacyhalothrin and cyfluthrin) did not exceed 3.6-fold. The ranking order in toxicity of pyrethroids to DUB-LPR strain was different from that of DUB-APR larvae. Permethrin as a selection agent had the least potency and cyfluthrin the most. Similarly other α-cyano pyrethroids (deltamethrin, lambdacyhalothrin, cypermethrin) were more toxic than permethrin. The range of toxicity at the LC50 between the two extremes (cyfluthrin and permethrin) was around 10-fold for DUB-LPR strain (see Fig. 7.4). In the DUB-LPR strain resistance was affected by α-cyano substitution. In the resistant strains (DUB-APR and DUB-LPR) a compression in the range of LC50 values was evident for all the pyrethroids (see Table 7.3).

Structure-activity relationships for 10 pyrethroids against susceptible, *kdr* and super-*kdr* strains of house flies were investigated by Farnham et al. (1987). They found that deltamethrin was more potent than cypermethrin and permethrin to susceptible strains.

The range of pyrethroid toxicity at the LD50 between the two extremes (deltamethrin and permethrin) was greatest for the susceptible strains, intermediate for *kdr* strains, and least for strains with super-*kdr*. Among the *kdr* strains there was no uniform and consistent pattern in structure-activity relationship. The same inconsistent pattern of structure-activity relationship of pyrethroids can be seen in DUB-APR and DUB-LPR strains, where cyfluthrin was the most potent for DUB-LPR and least for DUB-APR. Davies (1985) pointed out that pyrethroids act at many different sites and different compounds can produce different symptoms of toxicity.

From the resistance factors (RF), it can be concluded that the relative activities of different pyrethroids are not the same. The range of RF for DUB-LPR varied. A high RF was obtained with deltamethrin, where it was 256-fold, while the lowest was for cypermethrin with an RF of 3-fold. A similar trend can be seen with the DUB-APR strain (see Table 7.6). In general the RF can be categorized within 3 groups; deltamethrin with high RF, permethrin and lambdacyhalothin with moderate RF, cypermethrin and cyfluthrin with low RF for both resistant strains (Fig. 7.8). Rate of penetration, transport to the site of action, susceptibility to detoxication and fit of the molecular structure at the site of action are possible reasons for such differences in observed resistance factor. It is important to determine whether such differences arise through selective penetration, transport and/or metabolism, or through preferential action in the nervous system.

The trend for high toxicity of α-cyano compounds has been well documented in house flies (Elliot et al., 1974; DeVries & Georghiou, 1980), and in a predatory mite *Amblyseius fallacis* (Croft et al., 1982). Soderlund & Casida (1977) pointed out that the addition of a cyanide group to the α-carbon of the pyrethroids derived from 3-

phenoxybenzyl alcohol reduces the susceptibility of the molecule to both hydrolytic and oxidative metabolism. Structure activity relationships of pyrethroids against larvae and adults of *Cx.quinquefasciatus* dependent on the nature and position of the substitutent in the phenyl ring of the acid moiety and also the type of alcohol moiety. The larvicidal activity was found to increase in the following order: bromo< chloro< fluoro, whereas the adulticide activity was found be vary in an the opposite way to that of larvae (Nisha & Kalyanasundaram, 1992). Studies of Clements & May (1977) and Nishimura & Narahashi (1978) using a variety of pyrethroids have failed to show a clear correlation between the structure activity relationship found in neurophysiological preparations in *Periplaneta americana* and crayfish.

In our study permethrin had the least activity against the DUB-LPR strain of all the pyrethroids.

The synergism shown by PB (Figs 7.5 and 7.6) suggests that each resistant strain possesses mono-oxygenase-mediated resistance mechanisms, at least in part, for the pyrethroid resistance. It appears likely, therefore, that permethrin-selection may have led to increases in cytochrome P-450s specific for detoxication of these compounds and leading to the variable cross-resistance observed, i.e., there is at least a common resistance mechanism via P-450s for all the pyrethroids. Moreover, results from Table 7.6 and Fig. 7.8 indicate that relative activities of different pyrethroids are not the same, suggesting that another resistance mechanism(s) may play a role. Although the ability to metabolize pyrethroids varies greatly with compound and insect species, the extent to which this affects insecticidal potency is not yet precisely defined. The situation in resistant strains is further complicated by interaction between the various factors of resistance. Therefore further work is needed to determine the possibility of

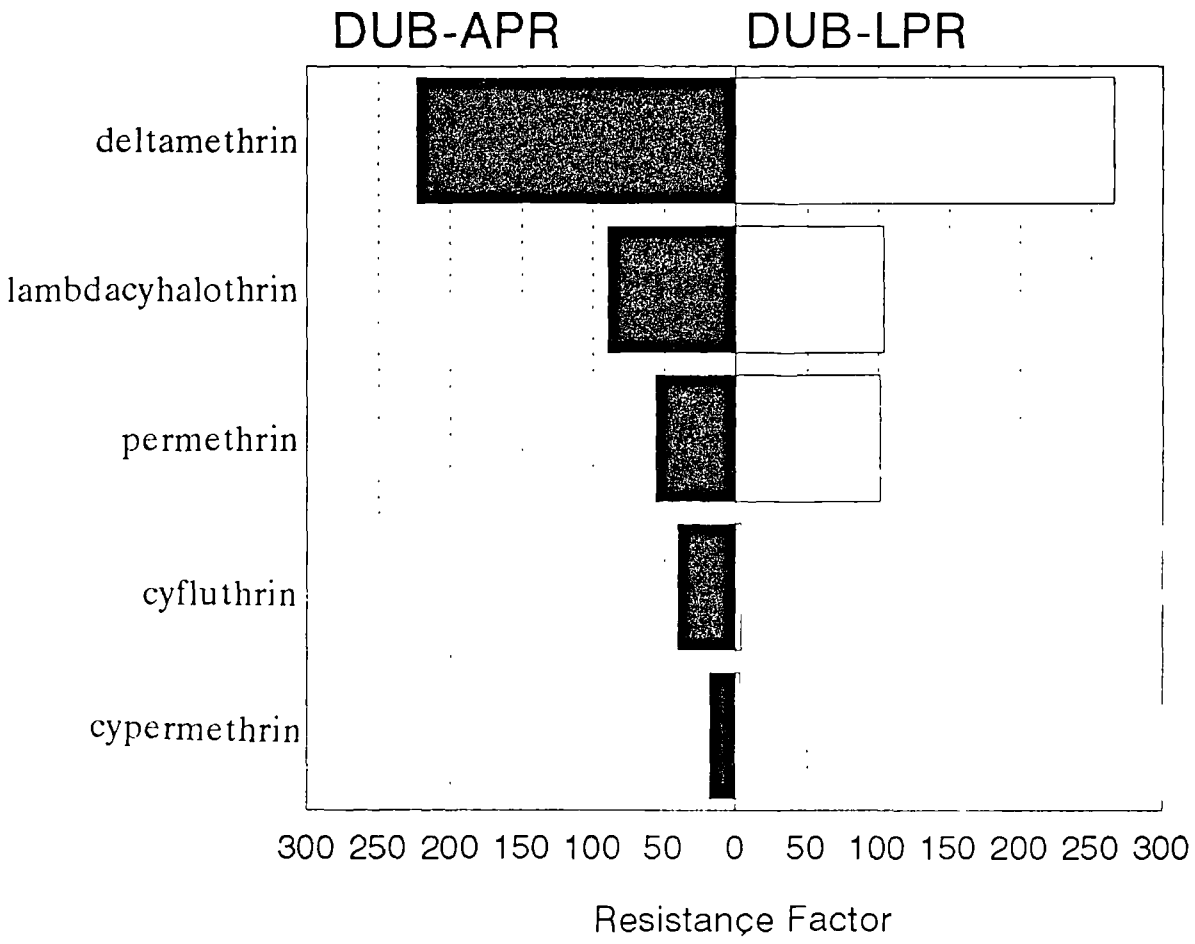
quantitative differences in the mono-oxygenase systems in each selected strains. An understanding of such differences between strains will be an important to the development of insecticide-use strategies to enable us to prevent resistance problems with synthetic pyrethroids.

Table 7.6 Resistance Factor (RF) of pyrethroids for larvae of *An.stephensi*

Strains	Pyrethroids	RF*
DUB-LPR	deltamethrin	**256**
	lambdacyhalothrin	**104**
	permethrin	**100**
	cyfluthrin	**4**
	cypermethrin	**3**
DUB-APR	deltamethrin	**215**
	lambdacyhalothrin	**90**
	permethrin	**56**
	cyfluthrin	**41**
	cypermethrin	**18**

***RF= LC50 of permethrin-resistant strain/LC50 of susceptible strain (IND-S)**

Fig. 7.8 Resistance Factor (RF) of pyrethroids for larvae of permethrin-resistant strains (DUB-APR and DUB-LPR) in comparison with IND-S strain



***RF= LC50 of permethrin-resistant strain/LC50 of susceptible strain (IND-S)**

Chapter 8

TEMPERATURE COEFFICIENT OF PYRETHROIDS

8.1 Introduction

Numerous factors influence insecticide toxicity. Temperature is one of the most important factors which influences the toxicity of insecticide to the target site of the insect. Consideration of the toxicity of pyrethroids at various temperatures should be a vital part of any pest control practice. Since resistance development is accelerated by prolonged exposure of populations to suboptimal dosage of insecticide (Georghiou, 1980a,b), negative temperature mortality relationships may affect the use of pyrethroids in mosquito control programmes. If the pyrethroid functions best at lower temperature, this feature could provide explanation for the increased rate of pyrethroid resistance. If the effectiveness of such an insecticide would be diminished at a higher temperature (becoming a suboptimal dose), mosquitoes with resistant traits would be possibly selected and then resistance rapidly develop. Any alteration of resistance level produced by temperature changes could provide an insight into the importance of this factor in the evolution of resistance in the field.

8.2 Results & discussion

The influence of temperature on the toxicity of the pyrethroids (permethrin, deltamethrin, and lambdacyhalothrin) to adults of both permethrin-resistant (RR) and susceptible (SS) strains of *An.stephensi* was determined based on exposing adults at the LT50 and LT90 at two different temperatures. Each test was repeated at two temperatures (25 ± 1°C and 5 ± 1°C) (for more details see 3.2.16). The exposure of

adults to 5±1°C in our experiments was due to availability of a room with this temperature and such temperature naturally occurs in the spring, autumn, and daily temperature of winter, when *An.stephensi* is still active.

The comparative toxicity of permethrin against females of DUB-APR and TEH strains are represented in Table 8.1 and Fig. 8.1. The results of X^2 test indicated that at the LT50, toxicity of permethrin increased 7.8 and 5.1 fold for DUB-APR and TEH strains respectively, when mosquitoes were held at 25±1°C rather than 5±1°C. A positive temperature coefficient was observed at the LT90, the toxicity of permethrin increased 12.6 and 10 fold for DUB-APR and TEH strains from 5±1°C to 25±1°C. Results of tests with deltamethrin are presented in Table 8.2 and Fig. 8.2. At the LT50 the toxicity of deltamethrin decreased 12.8 and 4.3 fold for DUB-APR and TEH strains respectively at lower temperatures. X^2 test indicated that mortality for deltamethrin at the LT90 was greater ($p<0.0001$) at 25±1°C than at 5±1°C with temperature coefficient of 11.5 and 3.7 for DUB-APR and TEH strains respectively. Temperature coefficients of lambdacyhalothrin against DUB-APR and TEH strains are tabulated in Table 8.3, and the comparative toxicities are shown in Fig. 8.3. At the LT50, lambdacyhalothrin was most effective against both strains at higher temperatures. Similar trends of mortality at two different temperatures were observed at the LT90. Increased fumigant action at the higher temperature may be one of the several factors involved in this increased toxicity of pyrethroids. To assess the fumigant effect of pyrethroids experiments were carried out as described in section 3.2.13. Results are summarized in Table 8.4. However, according to these results, we considered the possibility of any fumigant action and air-borne repellency (dust) of pyrethroids at 25±1°C to be unlikely.

Table 8.1 Effect of temperature on the mortality of females of permethrin-resistant (DUB-APR) and susceptible (TEH) strains of *An.stephensi* followed by exposure to permethrin at the LT_{50} and LT_{90}

	Mortality at the LT_{50}±SD					Mortality at the LT_{90}±SD				
Strain	25±1°C	5±1°C	T.C	X^2(df)	P	25±1°C	5±1°C	T.C	X^2(df)	P
DUB-APR	149(300) =50.00±4.65	16(250) =6.40±2.06	+7.8	121(1)	**	177(200) =88.50±3.33	14(200) =7.00±3.54	+12.6	266(1)	**
TEH	51(100) =51.00±2.00	15(150) =10.00±2.30	+5.1	52(1)	**	137(150) =91.00±1.63	14(150) =9.00±2.06	+10	202(1)	**

T.C= Temperature coefficient= mortality rate at high temperature/mortality rate at low temperature

Number in parenthesis represent number of mosquito tested

Table 8.2 Effect of temperature on the mortality of females of permethrin-resistant (DUB-APR) and susceptible (TEH) strains of *An.stephensi* followed by exposure to deltamethrin at the LT_{50} and LT_{90}

	Mortality at the LT_{50}±SD					Mortality at the LT_{90}±SD				
strain	25±1°C	5±1°C	T.C	X^2 (df)	P	25±1°C	5±1°C	T.C	X^2 (df)	P
DUB-APR	77(150) =51.00±1.63	6(150) =4.00±2.52	+12.8	84(1)	**	184(200) =92.00±3.02	16(200) =8.00±5.23	+11.5	282(1)	**
TEH	102(200) =51.00±4.65	24(200) =12.00±3.02	+4.3	70(1)	**	138(150) =92.00±2.52	37(150) =25.00±9.9	+3.7	140(1)	**

T.C= Temperature coefficient= mortality rate at high temperature/mortality rate at low temperature

Number in parenthesis represent number of mosquito tested

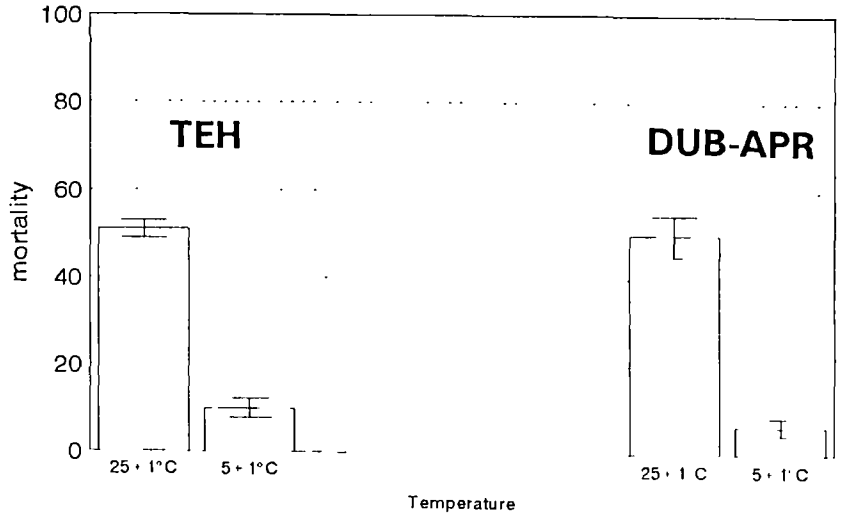
Table 8.3 Effect of temperature on the mortality of females of permethrin-resistant (DUB-APR) and susceptible (TEH) strains of *An.stephensi* followed by exposure to lambdacyhalothrin at the LT_{50} and LT_{90}

	Mortality at the LT_{50}±SD					Mortality at the LT_{90}±SD				
strain	25±1°C	5±1°C	T.C	X^2 (df)	P	25±1°C	5±1°C	T.C	X^2 (df)	P
DUB-APR	94(200) =47.00±2.82	18(200) =9.00±2.82	+5.2	72(1)	**	181(200) =91.00±2.13	26(200) =13.00±7.01	+7	241(1)	**
TEH	66(125) =53.00±9.54	15(125) =12.00±4.00	+4.4	48(1)	**	111(125) =89.00±6.00	16(125) =13.00±4.38	+6.8	144(1)	**

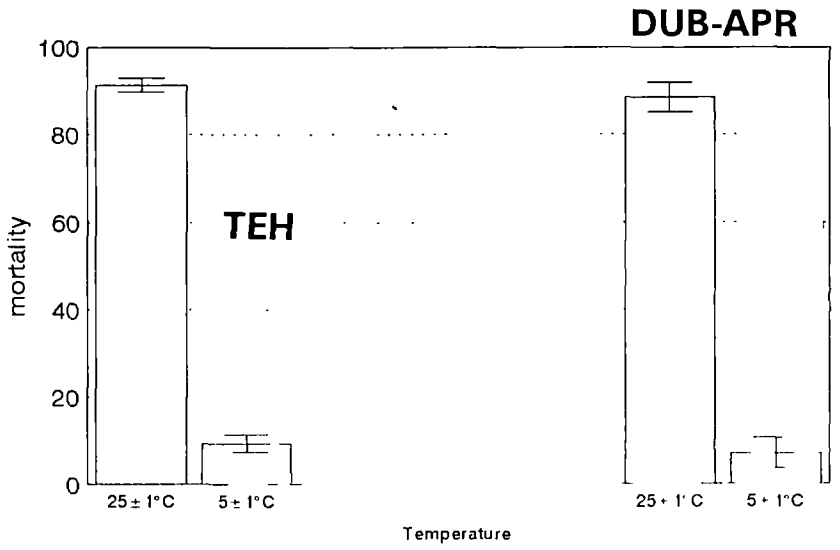
T.C=Temperature coefficient=mortality rate at high temperature/mortality rate at low temperature

Number in parenthesis represent number of mosquito tested

Fig. 8.1 Mean percent mortality of females of DUB-APR and TEH strains of *An.stephensi* tested with permethrin at different temperatures. a) mortality at the LT_{50}; b) mortality at the LT_{90}



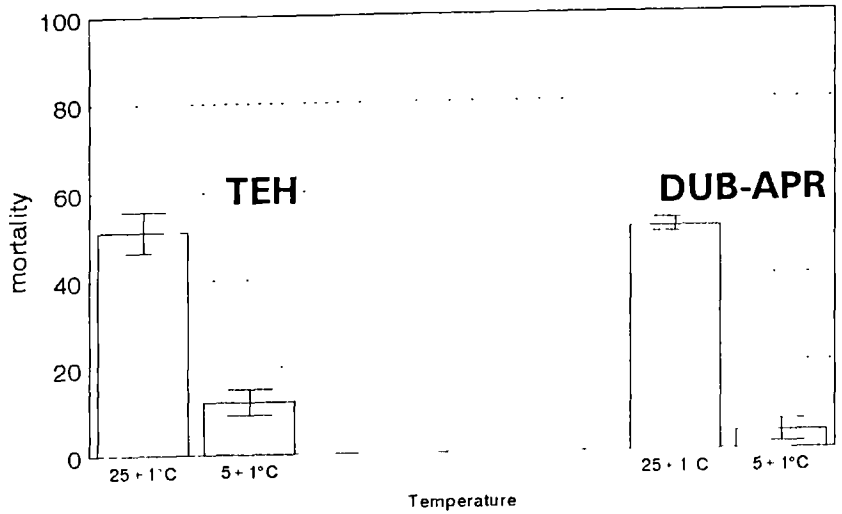
a)



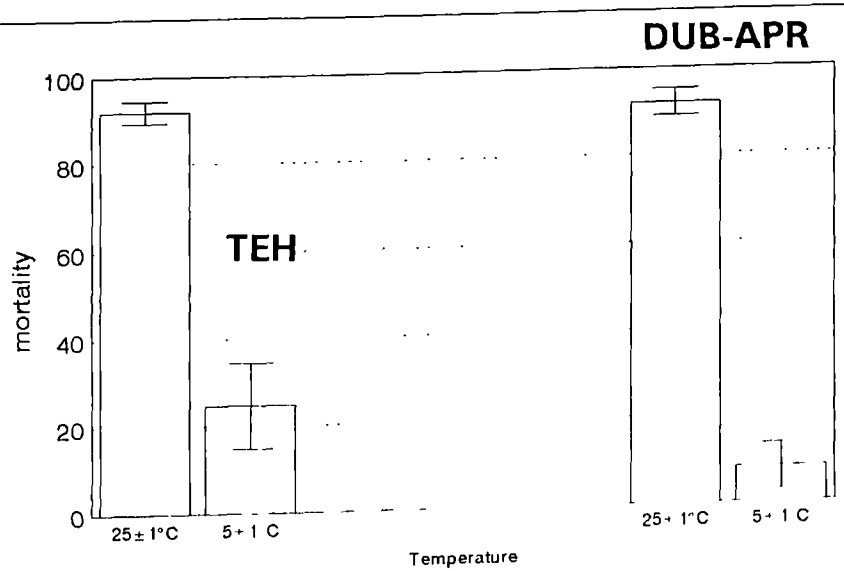
b)

Vertical bars=SD

Fig. 8.2 Mean percent mortality of females of DUB-APR and TEH strains of *An.stephensi* tested with deltamehtrin at different temperatures. a) mortality at the LT_{50}; b) mortality at the LT_{90}



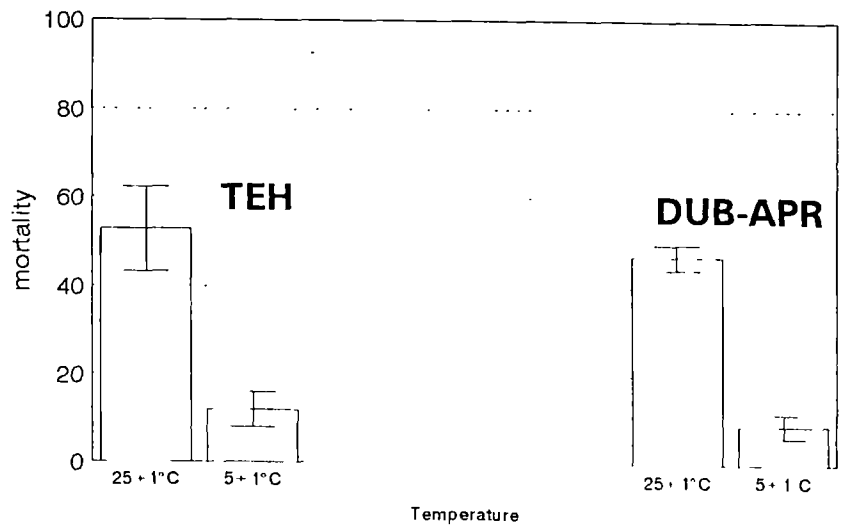
a)



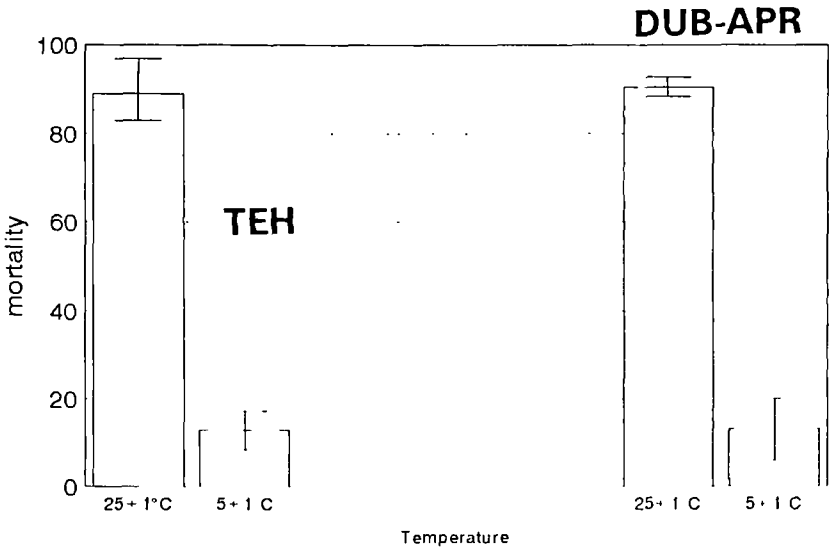
b)

Vertical bars=SD

Fig. 8.3 Mean percent mortality of females of DUB-APR and TEH strains of *An.stephensi* tested with lambdacyhalothrin at different temperatures. a) mortality at the LT_{50}; b) mortality at the LT_{90}



a)



b)

Vertical bars=SD

Table 8.4 Fumigant effect of pyrethroids to females of *An.stephensi*

Strains	Pesticide concentration	Exposure time at the LT_{50} (min)	Mortality rate	Exposure time at the LT_{90} (min)	Mortality rate
DUB-APR	permethrin, 10 $\mu g/cm^2$	82.0	0/(75)	139.2	1/(75)
TEH	permethrin, 4.16 $\mu g/cm^2$	25.9	0/(75)	51.9	1/(75)
DUB-APR	deltamethrin, 0.025%	53.2	0/(75)	98.1	0/(75)
TEH	deltamethrin, 0.025%	3.9	0/(75)	27.0	0/(75)
DUB-APR	lambdacyhalothrin, 1.22 $\mu g/cm^2$	141.0	1/(75)	293.0	0/(75)
TEH	lambdacyhalothrin, 1.22 $\mu g/cm^2$	12.7	0/(75)	34.1	0/(75)

Numbers in parenthesis represent number of mosquito tested

An.stephensi is an endophilic mosquito. Endophilic mosquitoes which include many principal malaria vectors, are expected to be most affected by residual insecticide applications. Pyrethroid insecticides are being used for malaria control programmes. Pyrethroids also are being applied on a large scale for agricultural and veterinary pest control. Low temperatures could have important practical implications in controlling vectors which can appear during cold weather conditions encountere during the spring and fall.

Pyrethroid insecticides share many characteristics with DDT including in many cases a negative temperature coefficient, i.e., they tend to be toxic at higher temperature or more active at lower temperature (Zebra, 1988). In contrast, the organophosphate and carbamate insecticides are usually more toxic at higher temperature.

Since mosquitoes are controlled with pyrethroids principally by residues rather than aerial droplets, so residual effectiveness and toxicity of pyrethroids in the field may be affected by temperature.

In 1949 Hoffman & Lindquist and subsequently in 1950 Guthrie stated that the most remarkable feature of insect poisoning by DDT is the so-called ''negative temperature coefficient of intoxication'', whereas pertinent kinetic parameters such as penetration and detoxication have a positive temperature coefficient.

The effect of temperature on the toxicity of five organic insecticides applied topically to the German cockroach (*Blattella germanica*) has been determined by Guthrie (1950). DDT, pyrethrum, and lindane gave better results at a lower temperature and dieldrin and aldrin gave better results at a higher temperature. DDT was about 20 times more effective at the lower temperature. Aldrin and dieldrin were about 3 times more effective at a higher temperature.

Temperature and the mode of action of pyrethroids have been inseparably connected since Vinson & Kearns (1952) concluded that DDT was more toxic at a colder temperature by virtue of an intrinsic susceptibility of some physiological system other than penetration or metabolism; the same conclusion was reached by Blum & Kearns (1956) concerning the action of pyrethrum on the American cockroach. Topically applied pyrethrum showed a negative temperature coefficient of action between 15°C and 35°C. However at 35°C the rate of penetration was more than twice that which occurred at 15°C.

Allethrin was applied topically to the dorsal surface of the abdomen of *Periplaneta americana* by Gommon (1978). The results obtained from experiments showed that the LD95 was 1.78 and 17 μg/cockroach at 15°C and 32°C respectively, indicating a negative temperature coefficient of allethrin.

Electrophysiological study of Scott & Matsumura (1982) on *B.germanica* revealed that there were two distinctly different types of pyrethroid actions. Pyrethrins and structurally related pyrethroids have predominantly type I action, cypermethrin and deltamethrin exhibit predominantly type II action. Type I action is represented by symptoms which exhibit a distinct negative temperature correlation for both knock-down and mortality factors, while type II action showed no temperature correlation for knock-down and positive temperature correlation for mortality. Hence cypermethrin and deltamethrin gave a positive correlation of toxicity with temperature and allethrin a negative correlation. At the LD50 level cypermethrin was > 6.6 times less toxic at 11°C than at 24°C, and deltamethrin showed > 1.8 times less toxicity at a lower temperature. Recently, however some differences among type I and type II pyrethroids in their modes of action have been reported in *Heliothis virescens*

(McCaffery et al., 1995).

The toxicity of two pyrethroid insecticides, S-bioallethrin and cypermethrin was investigated over time at 12, 25, and 31°C in susceptible and *kdr* resistant strains of *B.germanica* by Scott (1987). Both strains showed a negative temperature coefficient for S-bioallethrin, the susceptible strain had a negative temperature coefficient for knock-down, but a positive temperature coefficient for mortality towards cypermethrin. The resistant strain had a negative temperature coefficient toward cypermethrin at all times. Resistance to cypermethrin was significantly less at 12°C that at 25 or 31°C.

Applying 10 pyrethroids with and without α-cyano moiety against males of *B.germanica* revealed that at the LC50, toxicity was negatively related to temperature for all pyrethroids used (Wadeligh et al., 1991).

Narahashi (1971a) concluded that the nerve blocking action of allethrin in insects increased at low temperature. Yoke & Sudderoddin (1975) noted that bioresmethrin had a negative temperature coefficient of toxicity against *Musca domestica.*

It has been reported that the *kdr* house flies showed a negative temperature coefficient, whereas the *super-kdr* house flies showed a positive effect to DDT (Sawicki, 1978).

Bioresmethrin was used by DeVries & Georghiou (1979) against resistant and susceptible strains of house fly, the experimental temperatures were 15 and 25°C. Bioresmethrin manifested greater toxicity at 15°C. This effect was more pronounced in the resistant strain.

The pyrethroid analoges examined by Miller et al. in 1979 had a negative temperature coefficient of action on the exposed thoracic ganglia from house flies. DDT showed

a positive temperature coefficient of action on the exposed thoracic ganglion at temperatures ranging between 15 and 30°C.

Knock-down, toxicity, and resistance to bioallethrin, permethrin, flucythrinate, and cypermethrin in *Musca domestica* were examined at 18, 25, and 32°C in a resistant strain with *kdr* resistance mechanism and susceptible strains by Scott & Georghiou (1984). They found resistance to flucythrinate and cypermethrin was positively correlated with temperatures during the first 2 h, but negatively correlated thereafter. The action of DDT and some pyrethroids on the susceptible and *kdr*-type house flies at different temperatures ranging from 15 to 35°C were examined by Ahn et al. (1987). The two classes of insecticides showed a negative temperature coefficient for both strains and the penetration of (IRS)-*trans*-^{14}C-permethrin was positively correlated to temperature.

The effect of temperature on toxicity of some insecticides on agricultural pests was studied by Harris & Kinoshita (1976). They found that pyrethrins, resmethrin, carbaryl, and DDT had a negative temperature coefficient, while endosulfan showed a positive temperature coefficient, being 3.2 times more toxic at 32°C as compared to 15°C.

The temperature dependence of toxicity of some pyrethroids (phenothrin, permethrin, cypermethrin, tetramethrin, deltamethrin, fenvalerate and flucythrinate) showed interesting variations in tobacco budworm (*Heliothis virescens*). Some 3-phenoxybenzyl pyrethroids lacking α-cyano substitutions (permethrin, phenothrin) showed high negative temperature coefficients similar to DDT. However, α-cyano groups are weakly or moderately positively correlated with toxicity (tralomethrin, deltamethrin, and fenvalerate) or slightly negatively correlated (flucythrinate, and

cypermethrin) (Sparks et al., 1982, 1983).

Permethrin, fenvalerate and deltamethrin were bioassayed on adults of the boll weevil, *Anthonomus grandis grandis*, at 15.6, 26.7 and 37.8°C. All three pyrethroids displayed a negative temperature coefficient (Sparks et al., 1983).

Among three pyrethroids which were applied to the red flour beetle (*Tribolium castaneum*), cypermethrin and fenvalerate often resulted in significantly higher mortalities at 20°C than at 10 or 30°C, whereas permethrin generally resulted in mortalities which increased with temperature (Watters et al., 1983).

Cis-permethrin showed a strong negative correlation of toxicity with temperature in larvae of the pyrethroid-susceptible and pyrethroid-DDT resistant strains of the cattle tick, *Boophilus microplus* as reported by Schnitzerling (1985).

Data from the study of Grafius (1986) demonstrated that between 14°C and 30°C all pyrethroids (cypermethrin, fenvalerate, flucythrinate, permethrin) showed decrease in toxicity at higher temperatures on the potato beetle *Leptinotarsa decemlineata.*

In an experiment by Burgess & Hinks (1986) which is parallel to our study, flea beetles, *Phyllotetra cruciferae,* were collected from the field and were held at 7±1°C to minimize the mortality until use the next day. On the following day beetles were sprayed with cypermethrin. Immediately after spraying the beetles were held at 10, 21, and 32°C. Mortality was assessed after 24 h. It was found that cypermethrin was more toxic at 32°C than 21°C or 10°C, with positive temperature coefficient of 13 and 2.5 at 32°C and 21°C respectively. The authors did not mention the effect of a low holding temperature (7±1°C) on cypermethrin toxicity.

Subramanyam & Cutkomp (1987) examined the effect of temperature on the toxicity of five synthetic pyrethroids on *Tetranychus urticae*. They found that bioallethrin, d-

phenothrin, and fenvalerate, were more toxic to females at 30°C than 20°C, two other pyrethroids, flucythrinate and cyfluthrin, gave similar toxicity values at these two temperatures. The conclusion of this study was that there was greater sensitivity of mites at a warmer temperature to the pyrethroids.

The toxicity of *cis*- and *trans*-permethrin to larvae of cabbage loopers, *Trichoplusia ni*, was determined at 15.6, 26.7 and 37.8°C by two treatment techniques. Both isomers showed negative temperature coefficient between 15.6 and 37.8°C. However, between 26.7 and 37.8°C the compounds had smaller positive temperature coefficients at the LT50 and negative temperature coefficients at the LT90 (Toth & Sparks, 1988).

Schmidt & Robertson (1986) and Hinkle et al. (1989) demonstrated a positive temperature effect on toxicity of pyrethroids between 21 and 27°C and no significant temperature effect on toxicity between 27 and 32°C against horn flies. Schmidt & Robertson (1986) reported that the temperature effect depends on the test method in the horn fly; permethrin had a negative temperature coefficient when applied topically and a positive one when applied to a treated cloth surface.

Cutkomp & Subramanyam (1986) found that the toxicities of some pyrethroids (cypermethrin, permethrin, fenvalerate, phenothrin, flucythrinate and bioallethrin) against larvae of *Aedes aegypti* was 1.33-3.63 fold greater at 20°C than at 30°C, revealing negative temperature coefficients of pyrethroids.

Mittal et al. (1993) found that *Bacillus thuringiensis* (H-14) and *B.sphaericus* were more toxic at 31±2°C than at 21±2°C against larvae of *An.culicifacies* and *An.stephensi*. A positive relationship between temperature and larvicide activity of *Bacillus thuringiensis subsp.darmstadiensis* (H-10) against larvae of *Culex*

quiquefasciatus was found by Lacey & Oldacre in 1983. Mortality at 31°C was 2.4 times more than at 18°C.

A brief summary of the effect of temperature on the toxicity of a number of insecticides including pyrethroids as reported by several workers indicates two different general trends of the effect of temperature on toxicity, one is an increase in toxicity at the lower temperature (negative temperature coefficient) and the other an increase in toxicity at a higher temperature (positive temperature coefficient). Although a negative or positive temperature coefficient of action is known for several insecticides, pharmacological and biochemical consideration of this phenomenon have received very little consideration.

According to papers cited above various hypotheses have been proposed to explain temperature/toxicity relationships. These can be summarized into the following categories:

- Characteristics of insecticide involved (vapour effect, viscosity, solubility, kind of solvent, lipophilicity)
- Method of insecticide application
- Temperature range
- Species of insect
- Physiology and biological response of insect (activity at different temperature, knock-down, different site of actions, mechanism of resistance, penetration of insecticide through the cuticle, amount of fat body, saturated/unsaturated ratio of insect's lipids, affinity of receptors at the target site, e.g., peripheral or central nervous system).

The result of the present study indicated that the toxicity of pyrethroids increased at

higher temperature and the positive effect of temperature was greater in the resistant strain. Results suggested that low temperatures may cause control problems in the field, but in the light of these observations the pyrethroids should prove highly effective in tropical climates.

To fully understand the relationship between mortality and temperature it is suggested that one should calculate predictive models to describe a best-fit regression equation. Negative temperature coefficients of pyrethroids are usually reported from the neurophysiological assays. To clarify the precise interaction of temperature and mode of action, further studies on the mode of action of pyrethroids on the nervous system are needed. It has been hypothesised by Munson (1953) that temperature affects the amount of saturated/unsaturated lipids in the insect's body. Pharmacokinetics of penetration and amount of uptake are also related to temperature. Further experiments are needed to measure the amount of fat body in relation to penetration at different temperatures.

Chapter 9

IN VITRO METABOLISM OF ^{14}C-PERMETHRIN

9.1 Introduction

One of the major factors governing the toxicity of insecticides is the metabolism in target and nontarget organisms leading to the formation of either more active compounds or detoxified products. Because pyrethroids generally have remarkable efficacy against various species of insects while exhibiting low toxicity to mammals, elucidation of metabolic pathways of this unique group of compounds is a prerequisite to the understanding of selective toxicity.

The previous *in vivo* synergism experiments (chapter 4) provided some indirect evidence for a role for detoxification enzyme/s in permethrin-resistant larvae of *An.stephensi*. This chapter describes investigations into the *in vitro* metabolism of ^{14}C-permethrin by larval homogenates and by rat liver microsomes. The primary aim of the following experiments was to quantify metabolite formation by the permethrin-resistant (DUB-LPR) and susceptible (IND-S) strains of *An.stephensi* larvae as well as to find differences in the pattern and extent of metabolism between the two strains. If the difference in cytochrome P-450 content between resistant and susceptible strains determines the degree of metabolism of permethrin to inactive compounds, then quantification of the amount of metabolite formation by the resistant and susceptible strains should provide further evidence to support this hypothesis, as the resistant strain would be expected to produce more metabolite/s than the susceptible strain.

In vitro studies involve the preparation of an enzyme source from insect tissues and

its incubation with insecticide, together with co-factors, in order to determine the routes of metabolism and the specific enzymes involved in detoxification.

In this study enzyme preparations from whole larvae were made by homogenisation and differential centrifugation. Crude preparations of insect homogenate (S9) were made by centrifugation at 10,000 g. ^{14}C-permethrin metabolism in this fraction was compared with that of 100,000 g supernatant (2nd product from centrifuge) containing soluble enzymes (predominantly esterases) and that of microsomal pellets containing membrane-bound enzymes (predominantly mono-oxygenases along with lesser amounts of membrane bound esterases) (Mullin & Scott, 1992). The aim of this experiment was to compare the fate of ^{14}C-permethrin incubated with these 3 fractions (see Fig. 3.10).

9.2 Materials & methods

9.2.1 Materials: ^{14}C-permethrin labelled in the alcohol position was supplied by Zeneca Agrochemical Ltd, UK. The compound (90-J8) labelled at the terminal aromatic ring on the alcohol moiety had a specific activity of 1.97 GBq/mmol. The position of the label is shown in Fig 9.1. All solvents including methanol, ethyl acetate and triethylamine phosphate (TEA) were of HPLC or Analar grade as appropriate. NADPH was supplied as Analar grade reagents by Sigma (UK). Coomassie reagent and bovine serum albumin were obtained in a rapid protein kit.

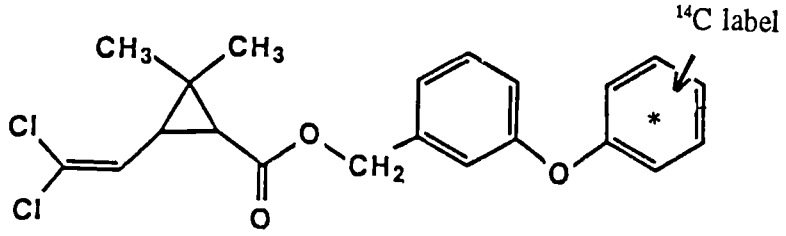


Fig. 9.1 ^{14}C-permethrin was used for metabolism studies (information on exact position not available)

9.2.2 Methods:

In vitro metabolism studies involve the preparation of an enzyme source. Mosquito larval homogenates and rat liver microsomes were prepared as described in sections 3.2.19 and 3.2.20, respectively. The protein content was determined as described by Bradford (1976), bioassay (see section 3.2.21) and spectrophotometric determination of P-450 content, carried out according to the method of Omura & Sato (1964) (see section 3.2.22). Optimization results for protein and incubation time revealed that 1 mg protein incubated for 45 minutes gives favourable turn-over of ^{14}C-permethrin to a quantifiable metabolism (see section 3.2.23). The incubation procedure using radiolabelled permethrin was carried out with rat liver microsomes as a control enzyme source and with larval homogenate of DUB-LPR and IND-S strains of *An.stephensi*. The steps of this procedure are as follows:

1- Add 100 μl (1 μl=10,000 DPM) of cold (10 ^{6}nM) and radiolabelled permethrin into 15 ml glass tubes.

2- Evaporate methanol under a stream of nitrogen free oxygen.

3- Add 1/15 M phosphate buffer.

4- Add protein source (1 mg/ml).

In the control incubations the enzyme resource was omitted.

5- Before adding 10 mM co-factor (NADPH), take 3x10 μl of each tube, add 2 ml scintillation fluid and count it with scintillator.

6- Add 10 mM NADPH, makings up a total volume up to 2 ml with 1/15 M phosphate buffer.

7- Incubate at 37°C for 45 minutes.

8- Terminate incubation by adding of 1 ml ice-cold methanol following immediate storage on ice.

9- Add 5 ml ethyl acetate into each tube, and vortex.

10- Centrifuge the tube for 5 minutes at 1000 g in a bench-top centrifuge. In this step the aqueous layer was decanted off to leave the pellet.

11- Take aqueous phase with a glass pipette and pour into another fresh glass tube.

12- Repeat the extraction procedure (step 10), take the aqueous phase and add into the glass tube used in step 11.

13- Take 3x10 μl of pellet and count it with scintillant and measure radioactivity in a scintillation counter (in this step all of the radioactive compound should be extracted by ethyl acetate).

14- Aspirate the supernatant organic phase with a glass pipette into a fresh glass tube.

15- Evaporate the ethyl acetate containing metabolites and parent permethrin under a stream of nitrogen free oxygen.

16- Add 100 μl methanol (HPLC grade) into each tube and agitate it by hand.

17- Inject a 20 μl aliquot on to the HPLC column and chromatograph.

In order to reduce chromatographic cross-contamination on the column sample injections were interspersed with blank injections of 50 μl methanol.

9.3 Results

9.3.1 Metabolism of permethrin by rat liver microsomes

Control sample injected into HPLC showed that standard permethrin was unaffected by incubation conditions and extraction procedures, and was 98% pure. A radiochromatogram of ^{14}C-permethrin is shown in Fig. 9.2. A sample chromatogram showing an enhanced turnover from an incubation of permethrin with rat liver microsomes is given in Fig. 9.3. From these studies using rat liver microsomes it was found that approximately 16.6% of radiolabelled permethrin was converted to a metabolite which had a retention time of 0.4 minutes.

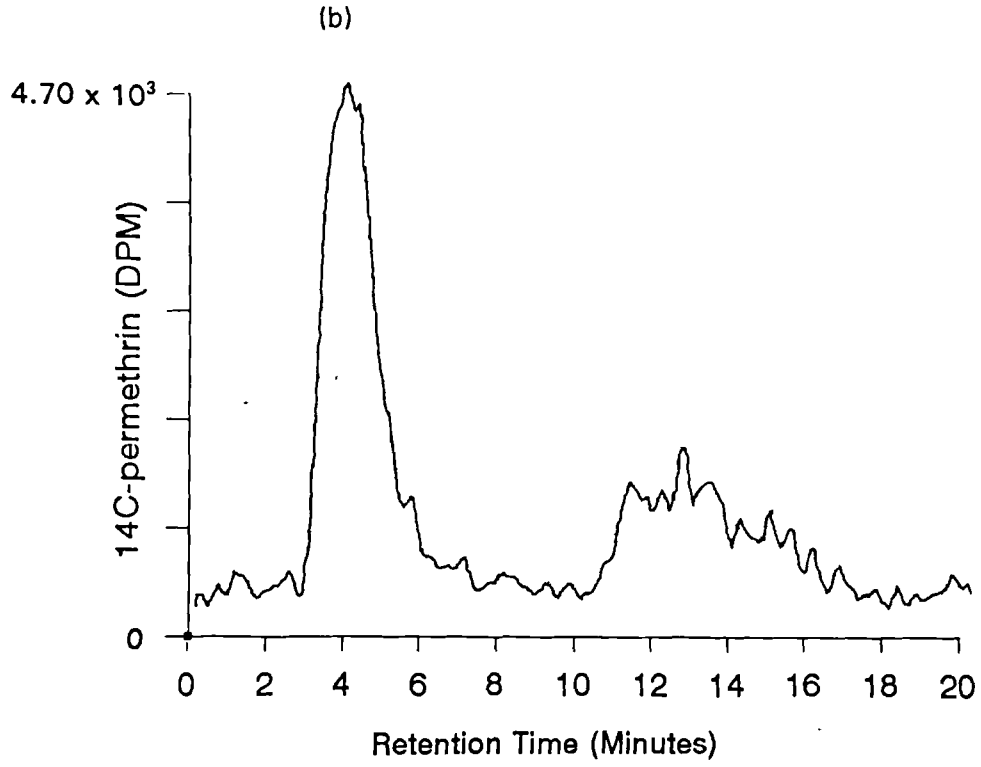


Fig. 9.2 HPLC radiogram of ^{14}C-permethrin following incubation and extraction

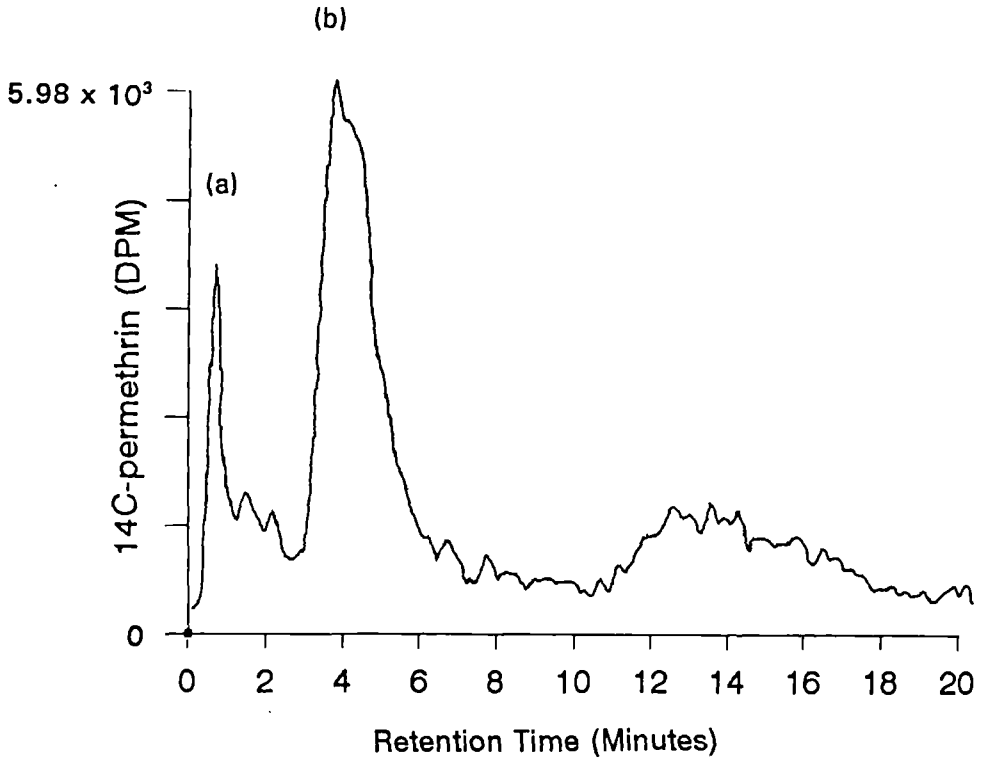


Fig. 9.3 HPLC radiogram of metabolite (a) and ^{14}C-permethrin (b) following incubation with rat liver microsomes

9.3.2 Metabolism of permethrin by mosquito larval homogenates

In vitro metabolism of ^{14}C-permethrin by homogenate preparations of larvae of resistant and susceptible strains of *An.stephensi* are summarized in Table 9.1.

The amount and pattern of metabolite formation was similar in the two strains and was quantitatively dependent on subcellular fraction. Larval S9 incubation resulted in 11% turn-over in the resistant strain and 7.6% in the susceptible strain (Fig. 9.4). At equivalent protein concentration, the greatest turn-over was seen with the 100,000 g supernatant in both strains (resistant 32.4% and susceptible 31.9% of the total radioactivity) (Fig. 9.5). The least active fraction in the both strains was the microsomal pellet which metabolised a total of 7.4% and 7.0% of the ^{14}C-permethrin in resistant and susceptible strains, respectively (Fig. 9.6).

Table 9.1 Quantitative *in vitro* metabolism of ^{14}C-permethrin by different fraction of larval homogenates from resistant and susceptible strains of *An.stephensi*

Strains	Larval homogenate fractions	% Turnover
IND-S	S9	7.6
	100,000 g	31.9
	Microsomes	7.0
DUB-LPR	S9	11.0
	100,000 g	32.4
	Microsomes	7.4

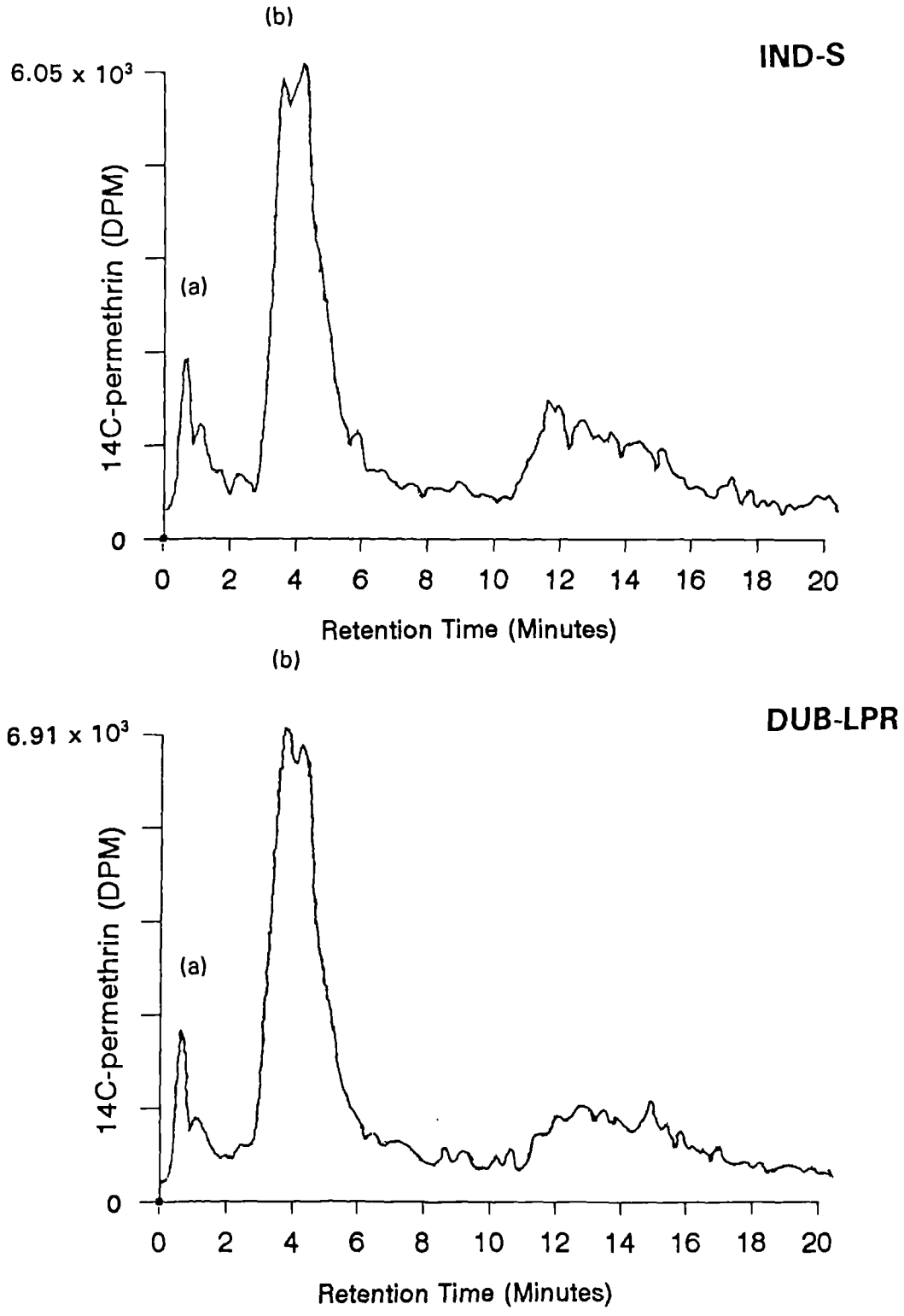


Fig. 9.4 HPLC radiogram of ^{14}C-permethrin (b) and metabolite (a) following incubation with S9 fractions of larval homogenates of resistant and susceptible strains

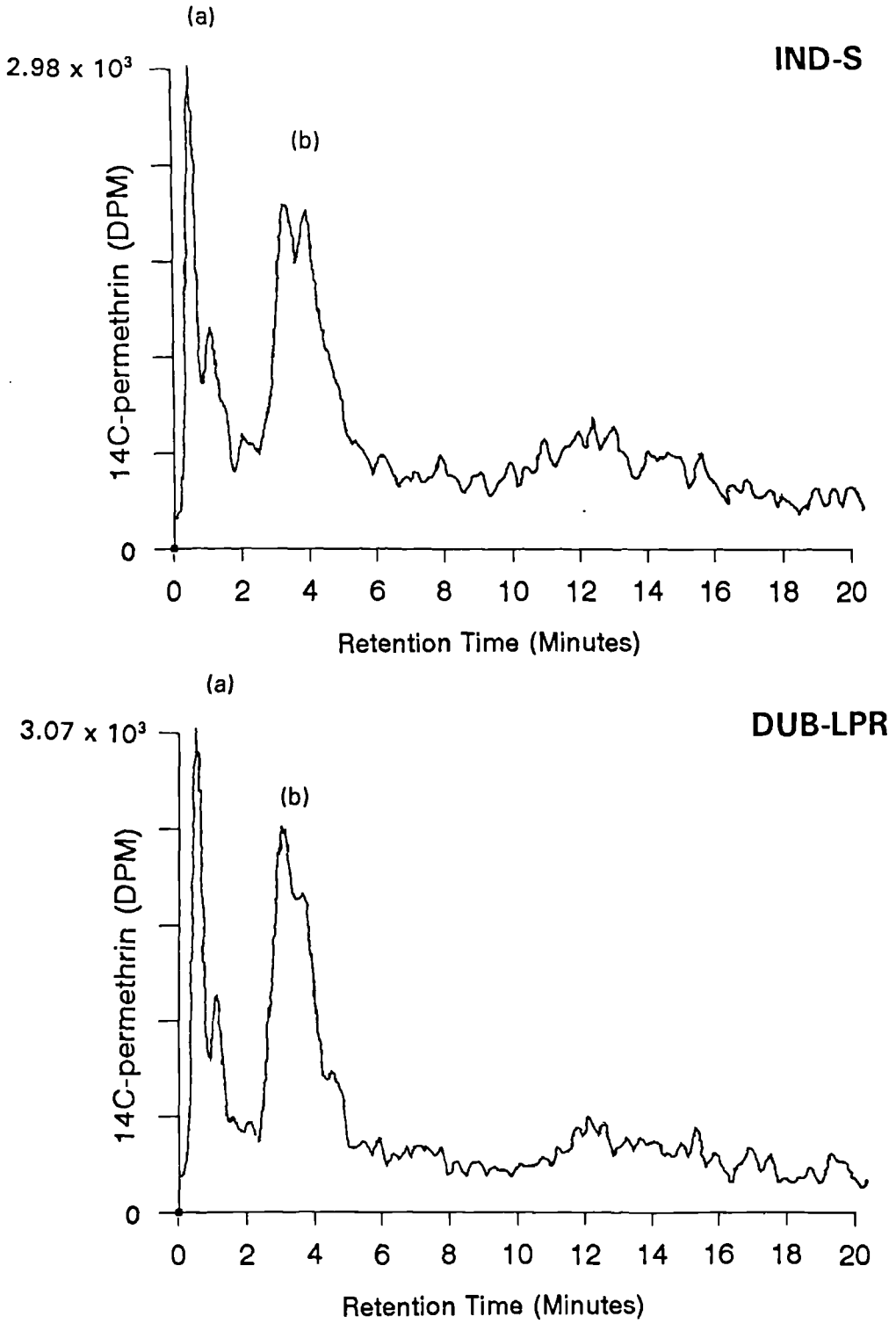


Fig. 9.5 HPLC radiogram of ^{14}C-permethrin (b) and metabolite (a) following incubation with 100,000 g fraction of larval homogenates of resistant and susceptible strains

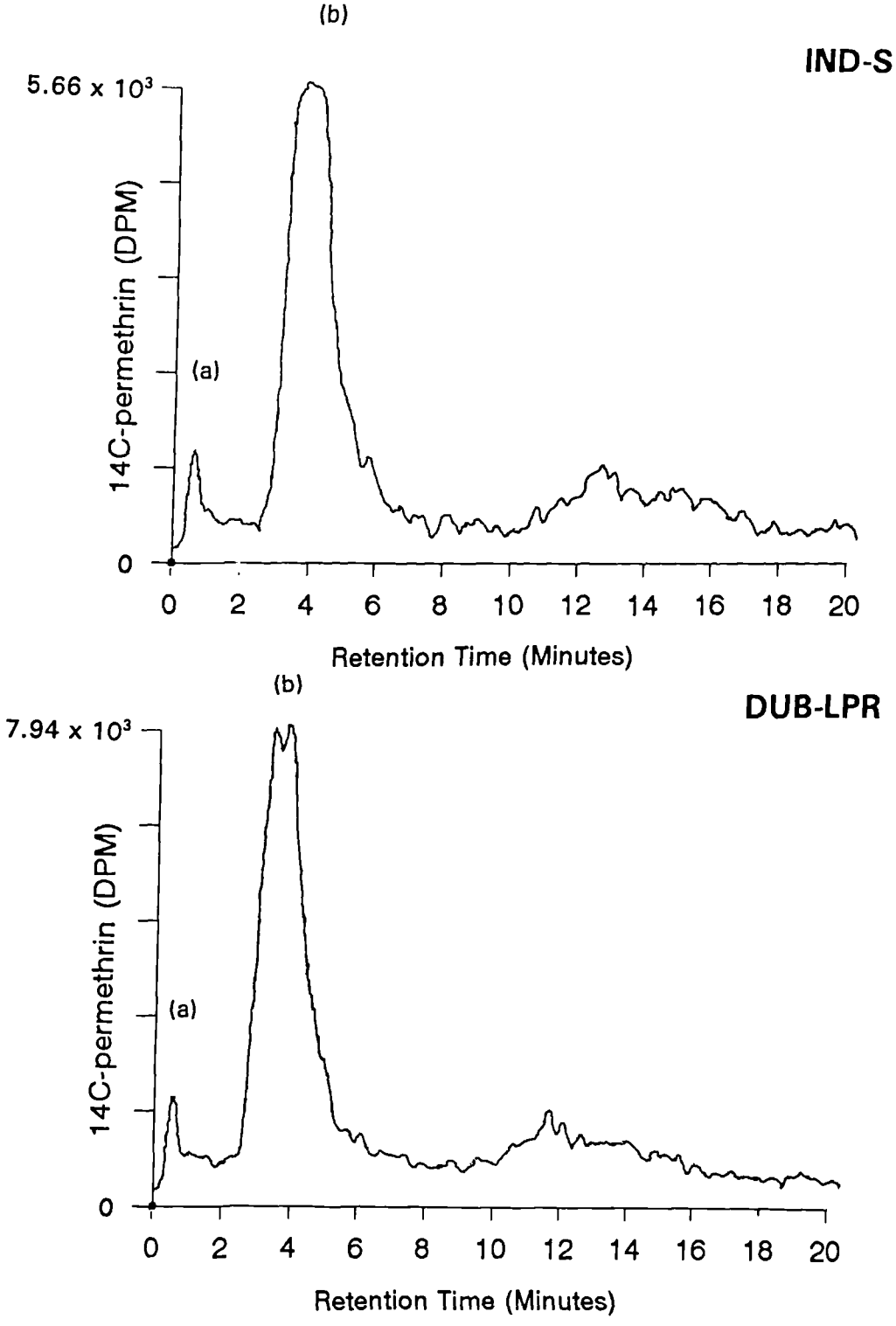


Fig. 9.6 HPLC radiogram of ^{14}C-permethrin (b) and metabolite (a) following incubation with microsomes fraction of larval homogenates of resistant and susceptible strains

9.4 Discussion

From our HPLC chromatographs it could be seen that permethrin was turned-over to a single peak of radioactivity which appeared before the parental compound, permethrin, and the only metabolite produced by the two strains had the same retention time (0.4 minutes) as the rat liver metabolite, suggesting that it was the same compound.

In recent years considerable information on the metabolism of insecticides has been obtained through *in vitro* studies employing suitably fortified homogenates or microsomal fractions from both insects and mammalian liver.

As in our study DeVries & Georghiou (1981b) conducted an *in vivo* synergism assay of *trans*-permethrin with resistant and susceptible strains of house fly. When *trans*-permethrin was applied alone, the resistance ratio was 97-fold. The addition of piperonyl butoxide produced 12-fold synergism in the resistant strain and 2.7-fold in the susceptible strain. When an esterase inhibitor was applied, the resistant strain was synergized 1.6-fold and the susceptible strain 1.9-fold. When the *in vivo* results were compared with *in vitro* observations, they found no significant differences between the resistant and susceptible strains in their overall metabolism of permethrin. They suggested that one possible explanation for the synergism caused by piperonyl butoxide in *in vivo* experiments was an increase in the rate of penetration rather than the inhibition of oxidative degradation. A similar suggestion was put forward by Farnham (1973) who found that piperonyl butoxide increased the toxicity of the pyrethroids more against pyrethroid-resistant than the susceptible strains of *M.domestica*. Permethrin metabolism was also studied by Nicholson & Sawicki (1982) in the weakly resistant (2.6-fold) R2 strain of *Musca domestica*, lacking *kdr*,

in comparison with the susceptible strain. The R2 strain degraded injected *trans*- and *cis*-permethrin slightly faster than the susceptible strain, and both strains metabolized more of the *trans* than the *cis* isomer. The total amount of metabolites produced was 6.2% and 5.4% in resistant and susceptible strains, respectively. Other polar metabolites (11% in R2 and 5% in susceptible strains) remained unidentified. *In vitro* studies on the metabolism of ^{14}C-permethrin using abdomen homogenates of *M.domestica* revealed that there were only minor differences between permethrin-resistant and susceptible strains in their capability for metabolic detoxification (Bull, 1992). The *in vivo* studies of Shono et al. (1978) on cockroach adults, house fly adults and cabagge looper larvae revealed that they metabolize permethrin by a variety of pathways leading to 42 tentatively identified metabolites and house flies excreted permethrin metabolites as > 80% conjugates. In our *in vitro* bioassay we have found only one single peak. It may be that this peak does not represent a single distinct chemical entity but could be several overlapped peaks due to short retention time. In another experiment on the metabolic fate of *trans* and *cis*-permethrin on the cattle tick, *Boophilus microplus*, it was found that there were no significant metabolic differences between pyrethroid-resistant and susceptible strains (Nicholson et al., 1979a).

Carboxylesterase E4, the enzyme which in the aphid, *Myzus persica*, is linked with OP and carbamate resistance, hydrolysed the (1S)-*trans* isomer of permethrin (Devonshire & Moores, 1982) indicating that esterases confer cross-resistance between OPs and pyrethroids.

The penetration, degradation and excretion of [^{3}H] *trans*-permethrin in susceptible and pyrethroid-resistant strains of *Heliothis virescens* was examined by Nicholson &

Miller (1985). A considerably lower internal concentration of the parent compound was present in resistant compared with susceptible larvae after 24 h, suggesting a greater degradative capacity in the resistant strain.

Absorption and metabolism of radiolabelled permethrin was studied in *Periplaneta americana* by Holden (1979). Results indicated that permethrin was metabolised by attack at the ester bond, by adult cockroaches *in vivo* and by esterase preparations *in vitro*. In *in vivo* studies the major metabolites formed from alcohol-labelled ^{14}C-permetrhin were 3-phenoxybenzyl alcohol and phenoxybenzoic acid. Using permethrin labelled in both positions there was no significant formation of any metabolite retaining the ester linkage from any of the permethrin isomers. Results indicate that the ester-bond in permethrin is the site which undergoes the initial metabolic attack. From *in vivo* studies the overall metabolism of *trans*- and *cis*-permethrin was 75% and 28%, respectively. *In vitro* studies using the *cis*-isomer showed that only 6% was metabolised. *In vitro* and *in vivo* studies with synergists (piperonyl butoxide as a oxidase inhibitor and triphenyl phosphate an esterase inhibitor) indicated that cleavage of the ester bond in permethrin can be both oxidative for the *cis*-isomer and hydrolytic for *trans*-permethrin (Holden, 1979).

TLC analysis of radioactive *cis*- and *trans*-permethrin in internal extracts from the pyrethroid-resistant and susceptible strains of the horn fly, *H.irritans*, indicated that the relative distribution of radioactive metabolites was similar at all times after treatment (Bull et al., 1988).

Permethrin esterase activity was measured using unlabelled *trans*-permethrin in larval preparations of four entomophagous insects by Yu (1990). 3-phenoxybenzyl alcohol was quantified by HPLC as a metabolite of permethrin. Activity of esterases was

inhibited by paraoxon and DEF, revealing hydrolytic degradation of permethrin. Esterases activity has been implicated in the permethrin-resistant whitefly, *Bemisia tabacci*. *Trans*-permethrin was hydrolysed faster than its *cis*-isomer. The results of Dowd & Sparks (1987) indicated that the hydrolysis of *cis*- and *trans*-permethrin can occur in all stages of *Pseudopulsia includens* and *Heliothis virescens*.

In vitro metabolism of permethrin in larvae of susceptible and resistant strains of the cattle tick *Boophilus microplus* was studied by Schnitzerling et al. (1983). An HPLC technique indicated that metabolic detoxification was mainly esteratic and this phenomenon was faster in susceptible larvae, the major radioactive metabolite being 3-phenoxybenzyl alcohol.

Esterases derived from the gut of the cabbage looper (*Trichoplusia ni*) hydrolysed the *cis*-and *trans*-isomers of permethrin and were inhibited by DEF and carbaryl both in *in vitro* and *in vivo* conditions (Ishaaya & Casida, 1980).

Pyrethroid-hydrolyzing activity in whole body homogenates of permethrin-resistant and susceptible strains of the predatory mite, *Amblyseius fallacis,* has been examined *in vitro*. The resistant strain had the highest esterases and hydrolysed both isomers of permethrin. DEF and piperonyl butoxide moderately inhibited permethrin hydrolysis (Chang & Whalon, 1986). The penetration, excretion and metabolism of topically applied ^{14}C-permethrin have been examined in larvae of the moth *Wiseana cervinata* (Chang & Jordan, 1982). Metabolism was by hydrolysis and to a lesser extent oxidation and the primary metabolites were quickly conjugated to water-soluble products. The level of unchanged permethrin were enhanced by pretreatment of larvae with the metabolic inhibitors carbaryl and piperonyl butoxide. Esterase activity hydrolyzing both *cis*- and *trans*-permethrin was detected in homogenates of the

armyworm *Spodoptera eridania* larvae by Abdel-Aal & Soderlund (1980). The results demonstrated that hydrolysing activity is broadly distributed in insect tissues and permethrin is hydrolysed by several enzyme with different properties.

The results of our study suggest that *in vitro* metabolism of ^{14}C-permethrin in *An.stephensi* larvae results in the formation of a single metabolite. This could be the results of either MFO or esterase activity, a conclusion put forward based on our previous *in vivo* studies (see chapter 4). However, further *in vitro* synergist studies are necessary to confirm this. Total metabolic activity in a larval preparation of the susceptible strain was lower than in a resistant strain (46.5% in susceptible and 50.8% in resistant strain).

In vitro preparations from both larval and adult stages of insects are very labile and extremely sensitive to inhibition by endogenous inhibitors, a notorious problem in the study of microsomal oxidation in insects (Wilkinson & Brattsten, 1972; Wilson & Hodgson, 1972). One powerful inhibitor, the pigment xanthommatin is present in the eye and also possibly in the integument of insects, and at very low concentrations interferes with the electron flow between cytochrome P-450 and cytochrome P-450 reductase by acting as an electron sink. Potent inhibitors of microsomal oxidation have also been reported from the gut contents of insects and appear to be proteolytic enzymes which solubilize the flavoprotein NADPH cytochrome c reductase from the microsomal membrane, thus interfering with microsomal electron transport (Wilkinson & Brattsten, 1972).

Evidence from chapter 4 is available that synergists reduce the *in vivo* rate of permethrin metabolism. Further studies are necessary on this relationship by *in vitro* studies. To investigate the relative contribution of the esterase and mono-oxygenase

routes of metabolism by blocking them both independently and together in *in vitro* conditions with pre-application of esterase inhibitors such as paraoxon, DEF, and TPP and the mono-oxygenase inhibitor piperonyl butoxide (PB) are needed.

The *in vitro* assay described here could be suitable for detecting metabolites but it has the inherent difficulties of preparation instability and endogenous inhibition difficulties that are likely to persist. In addition the financial cost of materials such as NADPH and radiolabelled ^{14}C-permethrin (with the concomitant safety problems of a high specific activity) are limitations of this assay.

Chapter 10

GENERAL DISCUSSION & CONCLUSIONS

10.1 The impact of pyrethroid-resistance and cross-resistance on vector control strategy

To ensure a long, useful life for insecticides, it is essential to guard against the development of resistance. By management of insecticide usage it may be possible to avoid resistance in vector populations, slow the rate of resistance development, or cause resistant vectors to "revert" to a more susceptible level. Broad knowledge of the possible and probable resistance mechanisms may enable us to choose the appropriate technology in vector control.

This study revealed that populations of *An.stephensi* from Iran, Iraq and India have broadly similar levels of susceptibility to permethrin at the larval and adult stages, in spite of the fact that these populations have different histories of field exposure to insecticides. Only in the population from Dubai have resistance genes been discovered and it is from this population that the highly resistant DUB-APR and DUB-LPR strains have been selected. The resistance in this population is thought to result from extensive use of a wide range of insecticide compounds (Ladonni, 1988). Laboratory exposure of adult females for 30 minutes to 10μg/cm^2 permethrin-impregnated papers readily discriminated the susceptible BAN, TEH, IRAQ, BEECH, and IND-S strains from the selected DUB-APR.

An improved understanding of the biochemical basis of resistance mechanisms is required

for the development of an effective control strategy. To monitor the involvement of enzyme activity, synergist studies using inhibitors of esterases (TPP) and mixed function oxidases (PB) were performed. The results revealed that cytochrome P-450s and esterases are responsible for two-thirds of the 8-fold enhanced tolerance of the DUB-APR resistant strain as compared with a sensitive strain, IND-S.

In addition to enzyme involvement the possibility of *kdr*-type resistance was also postulated. Studies of the response of resistant and susceptible strains to permethrin-impregnated netting (500mg/m^2) showed that the resistant strain is 3.4-fold resistant to knock-down compared to the susceptible strain. The resistant strain was found to be 2.8-fold less irritable to permethrin. These results parallel those of Lockwood et al. (1985) who found that pyrethroid-resistant populations of horn fly are significantly less irritated by deltamethrin at low doses than are susceptible populations. However, the form of behavioural resistance to permethrin was the opposite to that of our irritability study (i.e. pyrethroid-resistant horn flies were irritated more rapidly by permethrin than the susceptible flies). They concluded that a behavioural response of resistant horn fly to permethrin would be selectively advantageous to the fly. Under field conditions, for a susceptible population to survive, rapid irritation is necessary so as to prevent the acquisition of a lethal dose upon contact with treated surfaces. In contrast, in DUB-APR strain physiological resistance to permethrin resulted in a decline in irritability to this insecticide. Thus resistant mosquitoes will spend more time in the treated surface than the susceptible strains, resulting in the acquisition of more insecticide. Sparks et al. (1989) have demonstrated a negative association between behavioural (measured as

irritability) and physiological resistance. Trapido (1954) and Georghiou (1972) have postulated that insects must be physiologically susceptible to a pesticide to evolve stimulus-dependent behavioural resistance and that, as physiological resistance evolves, behavioural resistance declines.

To further investigate behavioural changes the method of Rowland (1991) was followed. Results showed that the resistant DUB-APR strain is less responsive (1.5-fold) than the IND-S strain to the movement of an aspirator and this is in agreement with the study of Rowland (1991) who found that cyclodiene-resistant *An.stephensi* is 1.6-fold less responsive to movement. Moreover he pointed out that resistant females were less responsive to oviposition stimuli, they produce fewer eggs per unit of blood, they fly less during the periods available for seeking hosts or oviposition sites, they seem incapable of responding to or taking advantage of 'moonlight' by prolonging activity, they respond more slowly to 'predators' and resistant males were less successful at competing for females than were those of a susceptible strain.

These studies of irritability and responsiveness to movement showed that the resistant strain of *An.stephensi* (DUB-APR) was less active than the IND-S strain. In the confines of a laboratory population cage this changed activity would be of little importance, but in nature resistant females would be at a great fitness disadvantage if they spent less time searching for hosts or good oviposition sites or if they were less responsive to predators. Behavioural disadvantage and fitness costs may be associated with such resistance. The link between these behavioural characteristics and resistance is presumably closely connected with the neurophysiological basis of the resistance mechanism that is, the

reduced irritability and reduced activity in the DUB-APR strain seems to be a consequence of the reduced sensitivity of the nervous system recorded in the neuorophysiological studies.

The mode of behavioural resistance that occurs in the field is extremely important with regard to management strategies. If behavioural resistance is due to a physiologically potentiated mechanism (stimulus-dependent), management of behavioural resistance is simply a matter of increasing physiological susceptibility. However, if behavioural resistance occurs by an independent selected mechanism (stimulus-independent), then increasing toxicity may do nothing to increase a compound's efficacy in the field. Stimulus-independent behavioural resistance as reported in horn fly populations by Byford et al. (1987c), is effectively resistance to an application system. i.e., the insect avoids a consistently toxic habitat without interacting with any particular toxicant. Therefore, in such a situation, if the choice of the resting places continues to evolve, the application of insecticide will become ineffective, regardless of the insecticide being used.

The relationship between permethrin and DDT resistance was studied in the DUB-APR strain. DMC (a dehydrochlorinase inhibitor) and PB (an MFO inhibitor) had no synergistic effect on DDT in this strain. From these results it is suggested that DDT resistance in DUB-APR was due to non-metabolic factors, and it was postulated that reduced sensitivity of the active site was the major factor responsible for observed resistance. Involvement of the *kdr* gene in the manifestation of resistance to DDT and pyrethroid compounds is well recognized in insects including mosquitoes (Halliday & Georghiou, 1985a,b). In contrast, DDT and dieldrin resistance in *An.culicifacies* did not

confer cross-resistance to deltamethrin (Das et al., 1986). Similarly, studies on larvae of *An.stephensi* from Pakistan demonstrated a major DDT resistance factor that does not confer cross-resistance to pyrethroids (Malcolm, 1988b) nor does DDT resistance in *An.gambiae* confer cross-resistance to pyrethroids (Prapanthadara et al., 1995).

The results of our studies on the mechanisms of permethrin and DDT resistance suggest that *kdr* may be a component of cross-resistance to DDT and pyrethroids in adults.

From all the tests on adult females of the DUB-APR strain, including synergism and cross-resistance studies, *kdr* behaviour tests, irritability experiments, and responsiveness to movement, we have postulated that target site insensitivity is also involved in permethrin resistance in DUB-APR. To test this hypothesis a direct neurophysiological study with the thoracic nerves of adult female mosquitoes under perfusion with saline was conducted. These experiments showed that the nervous system of the resistant strain was around 100,000 times less sensitive to permethrin than that of the susceptible strain. The very wide difference in nerve sensitivity between the resistant and susceptible strains seen in our neurophysiological study is much greater than the 8-fold resistance shown by *in vivo* bioassays but is comparable to data obtained with *Musca domestica* (Smallcombe, 1981). This difference could reside in factors present in the intact insect and enhanced in *in vitro* direct application. It is not known exactly where in the nervous system permethrin acts to cause the neurophysiological effects observed. The extracellular recording technique developed here is not specific for a single nerve cell, action potentials of several nerves could be recorded and nerve insensitivity detected by the assay may be a function of ion channels or synaptic neurotransmitter release or both. The

neurophysiological technique developed during the present study may prove useful in the identification of resistant individuals in field populations where nerve insensitivity contributes to resistance.

Delayed penetration of insecticide is a well known mechanism of resistance to numerous insecticides. It had been thought possible that a resistance mechanism, restricting penetration through the cuticle, may have been responsible for some of the differences seen between resistant and susceptible strains of *An.stephensi*. However, when the penetration of ^{14}C-permethrin through the integument of females of the DUB-APR and IND-S strains was studied, the results showed that there was no significant difference in the extent of permethrin penetration.

From our *in vivo* synergist studies it was found that esterases play a minor role in permethrin resistance in DUB-APR (SR=1.7). In an attempt to define more closely the nature of these esterases, a series of experiments were carried out using starch gel electrophoresis and α- and ß-naphthyl acetate as substrates. The results using α-naphthyl acetate revealed 5 zones of esterase activity while ß-naphthyl acetate revealed 2 zones. Although there were subtle differences in the activity of esterases towards the substrates used, there were no obvious differences in esterase activity between resistant and susceptible strains. We postulated that partial synergism of DUB-APR with TPP may be due to a quantitative change of enzyme; if so, either these changes are too subtle to be detected with the electrophoretic technique involved or other classes of esterases isozymes are involved in permethrin detoxification that are not detected using these substrates.

Esterase dependent cross-resistance between OPs and pyrethroids has been detected in several species including *M.domestica* and *Culex* (Soderlund & Bloomquist, 1990) and Guatemalan *An.albimanus* (Brogdon & Barber, 1990). Any common resistance mechanism to both insecticides due to non-specific esterases would be a threat to vector control. The low levels of esterase-based resistance seen in the present study may be less of a threat, particularly since the overall resistance to malathion is greater in the susceptible strains. In a study conducted by Chakravorthy & Kalyanasundaram (1992), the synergistic effect of piperonyl butoxide (PB) with permethrin (synergist ratio=2-fold) did not overcome the development of resistance in permethrin-resistant (13-fold) adults of *An.stephensi*, suggesting the involvement of a *kdr*-type mechanism. It seems that selection with permethrin has resulted in a common resistant mechanism in the strains from Dubai and India with different genetical backgrounds, although target-site insensitivity contributes only one-third of the resistance seen in DUB-APR.

In our studies, larval reselection of DUB-LPR with permethrin for 3 generations increased resistance to permethrin to a level of 204-fold relative to BAN and 100-fold relative to IND-S. PB had a strong synergistic effect on permethrin in *in vivo* tests with DUB-LPR. PB completely covered the gap between the two regression lines for larvae of DUB-LPR and IND-S strains which is indication of a MFO system of resistance. But if another strain such as BAN (with resistance factor of 204-fold) is considered as the susceptible strain and comparison made with this strain, it can be seen that PB does not eliminate the resistance completely in the DUB-LPR strain suggesting that apart from MFO and esterases, another mechanism/s such as target site insensitivity may be

involved as postulated in *Cx.quinquefasciatus* larvae (Priester & Georghiou, 1980a) and *An.stephensi* larvae (Omer et al., 1980). Further penetration and neurophysiological experiments are needed to understand fully the role of other mechanisms such as reduced penetration and *kdr* in larvae of the permethrin-resistant strain.

An.stephensi larvae are found in a wide range of habitats including: pools, ponds, stream margins, catch basins, seepage canals and rice fields where they become exposed to agricultural pesticides. Agricultural chemicals can indeed select for resistance in mosquitoes, for some form of insecticide resistance has been recorded in 48 species of *Anopheles*, in 13 of which agricultural insecticides are presumed to have contributed to selection for resistance in at least some populations (Lines, 1988). In our study, adults of a strain (DUB-LPR) that was selected at the larval stage were also resistant to pyrethroids. Ideally, in order to preserve the effectiveness of pyrethroids for anti-malaria spraying, the application of these insecticides against agricultural pests should be limited or prohibited from malarious areas. Whether this is politically and economically realistic is another matter.

Our results with *Bacillus thuringiensis* showed that there is no cross-resistance between pyrethroids and *B.t.*H-14 in DUB-LPR larvae. To overcome the pyrethroid-resistance, it is likely that periodic use of *B.t.*H-14 as a larvicide in mosquito control, in rotation with conventional insecticides, may prevent or delay the onset of resistance to them. There are good practical reasons for alternating *B.t.i* in the control of *Simulium damnosum* in the dry season with permethrin and/or carbusolfan in the wet season (Kurtak et al., 1987, quoted in Curtis et al., 1993). Another control measure for larvae

is polystyrene beads applied in breeding places of larvae. Sharma (1992) and Sharma (quoted by Curtis & Minjas, 1985) has investigated the use of layers of polystyrene beads in water tanks in India for control of important urban vectors such as *An.stephensi* and *Ae.aegypti*

Possible future patterns of cross-resistant within and among chemical classes of insecticides and their relationship to previous insecticide pressure should be considered as an integral part of any strategy for the delay, avoidance, or management of insecticide resistance (Cilek et al., 1991).

In this study permethrin resistant adults (DUB-APR) were cross-resistant to deltamethrin, lambdacyhalothrin and DDT. In contrast malathion was more toxic to the resistant strain. Permethrin resistant larvae (DUB-LPR) showed high cross-resistance to deltamethrin, lambdacyhalothrin and DDT, whereas they were more susceptible to malathion ($RF < 1$; i.e. negative cross-resistance). Multifactorial resistance involves a suite of co-existing mechanisms with contrasting cross-resistance patterns that can protect an insect from a range of insecticides from the same or different classes and may predispose them to resist new and as yet unused compounds (Denholm & Rowland, 1992).

In spite of the development of malathion resistance in populations of *An.stephensi* which is reported from Iran and Iraq, our results indicated that these different geographical strains have nearly the same susceptibility to malathion at the adult and larval stages. Permethrin resistant strains (DUB-APR and DUB-LPR) exhibited more sensitivity to malathion but the susceptibility level of adults reached that of other pyrethroid-susceptible strains when they were pretreated with PB. Induced MFO activity may be the common

metabolic mechanism responsible for malathion negative cross-resistance in the pyrethroid resistant population by enhancing metabolism of malathion to the oxon form, a toxic metabolite. Negative cross-resistance between organophosphates and pyrethroids has been reported in *Tetranychus urticae* (Chapman & Penman, 1979), *Simulium damnosum* (Kurtak et al,. 1987), *Culex quinquefasciatus* and *Culex tarsalis* (Priester et al,. 1981), *Haematobia irritans* (Cilek et al., 1991; Sheppard & Joyce, 1992; Cilek & Knapp, 1993a,b; Sheppard & Marchiondo, 1987) and *Spodoptera littoralis* (Riskallah et al,. 1983). Negative cross-resistance to malathion in pyrethroid-resistant insects may be useful in reducing the number of pyrethroid-resistant individuals in a population, but further experiments are needed to determine if the negative cross-resistance found in this study is likely to be operationally useful.

Mosquito control programmes have encountered a number of serious problems such as increased exophilic behaviour of the vector, poor quality insecticide application, inadequate operational methods, low rates of coverage and refusal of permission to spray by householders. In malaria control programmes it appears that personal protection using pyrethroid-impregnated nets is more acceptable and affordable to both national vector control staff and communities in Asia and the Pacific (Rozendaal & Curtis, 1989) and Africa (Curtis et al., 1996). Bednets exploit the attractiveness of mosquitoes to CO_2 emitted by a breathing person who acts as an attractant to the treated net, making it a very effective trap. Also most vectors bite at night when people are likely to be in bed and this is an advantage for treated bednets where vectors are endophagic. The use of bednets for vector control is quite selective. It only kills adult female mosquitoes,

reducing their vectorial capacity, but male mosquitoes will be free from the exposure to pyrethroid-impregnated nets and in the event of female resistance, they will act as a dilution resource. There are several reports on the effectiveness of pyrethroid-impregnated bed nets and curtains in the laboratory as well as field conditions in different regions such as Asia (Iran, M. Zaim, personal communications, 1995; India, Sharma & Yadav, 1995), Malaysia (Lin, 1991), Africa, Suriname and central America (Rozendaal & Curtis, 1989; Elissa & Curtis, 1995; Njunwa et al., 1991; Lyimo et al., 1991; Miller et al., 1995; Hossain et al, 1989; Stich et al., 1994; Pleass et al., 1993; Msuya & Curtis, 1991; Snow et al., 1987; Lines et al., 1987; ; Lindsay et al., 1989; Majori et al., 1987). There are some reports of pyrethroid resistance in *An.stephensi* based on laboratory selection (Omer et al., 1980; Malcolm, 1988b; Chakravorthy & Kalyanasundaram, 1992; Sahgal et al., 1994), but only one report of pyrethroid resistance in a field population of *An.stephensi* (Ladonni, 1988). Vulule et al. (1994) reported a rise in level of pyrethroid tolerance in *An.gambiae* population during a village-scale trial of impregnated nets in Kenya. However, recently they reported that long-term use of permethrin-impregnated nets did not further increase further permethrin tolerance in this population (Vulule et al., 1996).

We have found that different geographical strains of *An.stephensi* from Iran, Iraq and India are susceptible to pyrethroids, hence these insecticides could be candidates for impregnated bed-nets in those regions. Ladonni (1988) found that resistance to permethrin in DUB-APR is controlled by recessive genes. Subsequently Curtis et al., (1990) reported that this example of resistance had only limited impact on the

effectiveness of impregnated nets under realistic conditions. The extent to which different resistance mechanisms are expressed in vector populations depends on ecological and genetic factors and may vary in time and space. Oppenoorth & Welling (1976) stated that it is not possible to predict whether resistance will develop in any particular situation, since this will depend on the insecticide selection pressure applied as well as on unknown characteristics of the insect populations.

It is obvious that the effective management of resistance is dependent on understanding the relevant biology and ecology of *An.stephensi*. Movement and dispersal of a vector can have a significant impact on the evolution of resistance to insecticides, because immigration of susceptible individuals can counter the evolutionary effects of insecticidal selection. Regular monitoring of the frequency of resistance to pyrethroids in field conditions may allow selection of appropriate control strategies. Reversion to susceptibility in DUB-APR strain has been pointed out in chapter 4. In this respect Farnham (1977) proposed that as *kdr,* which confers cross-resistance to DDT and pyrethroids in the house fly, confers a recessive trait, natural dilution of the population on removal of selection pressure would help to increase greatly the number of heterozygotes which are susceptible to DDT or pyrethroids. Curtis et al. (1978) pointed out that populations of insects containing a high frequency of alleles for cyclodiene resistance often revert towards susceptibility when the cyclodiene is withdrawn from use and re-exposure to pyrethroids will result in rapid increase in resistance. Migration of insects from populations untreated with a given insecticide would tend to delay the evolution of resistance in a neighbouring treated population. It has therefore been

proposed that an insecticide should not be applied uniformly but in alternate sectors of a 'grid' (Curtis et al., 1978). It is sometimes erroneously stated or implied that reversions could result merely from the recessiveness of resistance genes. In fact, however reversion could result only from selection against the resistance gene because of associated reduced survival or fecundity (i.e. reduced fitness), or from immigration from more susceptible populations or a combination of the two (Curtis et al., 1978). A stepwise pattern of reduction of pyrethroid resistance which is controlled by recessive genes was reported in a German cockroach population (Cochran, 1993; Zhai & Robinson, 1996), *M.domestica* (MacDonald et al., 1983), and *H.irritans* (Kunz, 1991). Instability of pyrethroid-resistance after 13 generations of withdrawal of pyrethroid application has been reported in field populations of the German cockroach (Zhai & Robinson, 1996). Mofidi (quoted by Curtis et al., 1978) reported that DDT-resistant populations of *An.stephensi* from Iran showed slow reversion towards susceptibility over a period of up to 6 years (approximately 72-generations) when DDT spraying had been withdrawn. Reversion of permethrin-resistance has been reported in *Pectinophora gossypiella* from Arizona (Osman et al., 1991). If reversion goes far enough it may be possible to effectively re-introduce the insecticide until re-selection of resistance cause control problems once again, a type of resistance management known as insecticide rotation (Curtis, 1987b)

In insects like those of the DUB-APR strain of *An.stephensi* with multifactorial mechanisms of resistance, not all resistance mechanisms can be expected to be equally expressed throughout the life cycle. The patterns of expression of the detoxification

enzymes may differ between the larval and adult stage. For example in the present study larval resistance to permethrin in DUB-LPR could be fully blocked by piperonyl butoxide and a single major gene plays an important role in the resistance (Ladonni & Townson in press), but target site insensitivity is a major resistance factor in adults (DUB-APR) and inheritance of resistance appears to be polygenic. The relative stability of each resistance mechanism is of considerable importance to pyrethroid restriction and rotation. For example uncontrolled pyrethroid use against *Heliothis armigera* in Australia was initially selected for nerve insensitivity resistance (Gunning et al., 1991). The frequency of nerve insensitivity then declined, this resistance mechanism being suppressed by enhanced mono-oxygenase and esterase-based metabolism and decreased penetration (Kennaugh et al., 1993). The instability of nerve insensitivity following insecticide withdrawal may be an important factor in developing a effective strategy for the management of this resistance. Elsewhere I have shown that nerve insensitivity in *An.stephensi* is associated with reduced responsiveness to stimuli, making this form of resistance highly disadvantageous in the absence of selection pressure from the insecticide. However rotation strategies will not succeed against stable resistance mechanisms. The stability of penetration, metabolic and target site insensitivity in *An.stephensi* requires further study.

The length of time a field population has been exposed to insecticide selection pressure can influence the stability of resistance. Other factors that can influence stability include the size and geographic area occupied by the resistant population, the presence of unexposed segments of the population, and opportunities for movement within the

geographical area (Zhai & Robinson, 1996).

Several methods have been proposed as solutions to the resistance problem including the use of synergists like PB. Two considerations are critical in selecting an appropriate synergist-plus-insecticide combination: the resistance mechanism should be limited to the detoxification pathway involving the enzyme system affected by the synergist, and the mixture should be stable. In practice, piperonyl butoxide (a mixed function oxidase inhibitor), may fail to meets these requirements with respect to permethrin because of the possible importance of a *kdr*-gene involving target site insensitivity at the site of action in the house fly (Farnham, 1977), rather than oxidative metabolism. In contrast to the findings of Farnham (1977), Macdonald et al. (1983) recorded the involvement of MFO in a strain of permethrin-resistant house fly and stated that a 10:1 mixture of piperonyl butoxide and permethrin increased permethrin susceptibility 5-fold and reduced heterogeneity in the resistant strain. When considering the field use of such synergists it should be noted that PB is unstable to light (WHO, 1992a).

Theoretical studies (Curtis et al., 1993) suggested that mixtures of unrelated compounds (with no cross-resistance) on nets might delay the appearance of resistance. The principle of this idea is that insects resistant to compound A will be killed by B, those resistant to B will be killed by A, and double resistance will be exceedingly rare at the early stage when each resistance gene(s) is still at a low frequency (Curtis et al., 1991). Byford et al. (1987a) reported that mixtures of pyrethroid-PB-chloropyrifos reduced the level of pyrethroid resistance in *H.irritans* in the laboratory and field in the short term. In contrast, the failure of a mixture of insecticides in control of pyrethroid-resistant

(polyfactorial resistance) horn fly has been reported by Cilek & Knapp (1993). In their studies, ear tags impregnated with a mixture of cyhalothrin-PB-DEF or cypermethrin-PB-chlorpyrifos were evaluated. After 14 weeks of exposure, the frequency of pyrethroid-resistant individuals remaining in each of the treated horn fly populations was 100%. Because the mixtures involved the use of pyrethroids to which resistance had already been documented, their inability to greatly reduce the level of resistance present and their lack of efficacy in the field was not surprising. Sparks & Byford (1988) demonstrated that insecticide mixtures in the form of pyrethroid+synergist combinations can be effective, at least in the short term, for the control of pyrethroid-resistant horn flies.

Larvae of *Heliothis virescens* selected with permethrin in the laboratory developed resistance within 30 generations (Crowder et al., 1984 quoted in Sparks & Byford, 1988). When these permethrin-resistant larvae were bioassayed with a permethrin+ chlordimeform mixture, the level of resistance was reduced by no more than one half. However, when larvae were selected from the outset with a combination of permethrin+chlordimeform, resistance failed to develop. Thus the use of a mixture after resistance has developed to one of the compounds negates the primary advantage of a mixture.

Another possible candidate to mix with a pyrethroid would be an insect growth regulator (IGR) (Miller & Curtis, 1995) which has a sterilizing action on females insects but is harmless to mammals. The intention would be to ensure that any pyrethroid-resistant mutant which survived contact with the net was sterilized and unable to pass on its mutant genes. This integration would allow for the control of

immature pyrethroid resistant forms, thus reducing the production of a resistant population and allowing the influence of susceptible adult immigrants to reduce further the resistance in a given area. Bendiocarb-sprayed curtains may be a possible alternative impregnated material in the event of the failure of pyrethroids or mixtures of unrelated compounds (Curtis et al., 1996).

Before planning any actual usage of a mixture, it is obviously necessary to check that the rates of decay of the two compounds are similar and to test in the laboratory and/or field for interaction effects in their lethality to different resistance genotypes of the target species and also with regard to their safety for man and other non-target organisms (Curtis, 1985). Formulation technology must address the need for similar release and decay rates of the components in a mixture so that a pest population is not selected with a single compounds for an extended period of time. The problems of chemical compatibility and the limitations of formulation technology may be solved. Moreover, the insecticide/synergist ratio should be considered in a mixture. It should be noted that it is essential to the success of a scheme using mixtures or alternation of insecticides that the components exhibit little or no cross-resistance. Should this criterion not be satisfied, the potential for resistance rapidly developing to both compounds is considerable (Georghiou, 1980, cited by Macdonald et al., 1983).

Curtis & Rawlings (1980) have proposed "mosaic" spraying patterns, in which the natural dispersal from sectors of the mosaic where a given insecticide is not being sprayed will dilute the build up of resistance in sectors where this insecticide is being used. Another possibility is the localized eradication of mosquitoes in an area where a new focus of resistance has just been detected, in order to destroy the resistance gene before it can spread. By limiting use of pyrethroids in a pest control

management programme to populations with a history of pyrethroid-resistance, the effectiveness of these insecticides could be maintained.

In this study it was shown that permethrin resistance in adults of *An.stephensi* did not confer cross-tolerance to the repellent, deet thus could continue to be used as a repellent on the skin or on cotton fabric to prevent mosquitoes biting even in the presence of permethrin resistance.

10.2 Enzyme induction and control management

Mosquito larvae and eggs share their habitats with a diverse flora and fauna. The presence of such flora and fauna may have a positive or negative effect on larval and adult development as well as on the insecticide toxicity. For example, decomposition of some vegetation in the breeding places may release plant allelochemicals which are capable of inducing detoxification enzymes in the mosquito and this may be sufficient to increase the tolerance of the insect to various insecticides. Alternatively some of these compound may inhibit rather than induce the action of insect enzymes.

The taking of a blood meal by female mosquitoes is, in most species, a necessary pre-requisite for egg production. During the oocyte development, some hormonal regulation occurs inside the body and these process required enzyme activation. Thus Bladridge & Feyereisen (1986) showed that total cytochrome P-450 activity in blood-fed females of *Cx.pipiens* is different from that in non-blood-fed insects. It is conceivable those P-450s which are enhanced in blood-fed mosquitoes may also detoxify the insecticides. Apart from the attraction of female mosquitoes to blood sources, both sexes visit flowers for nectar and respond to floral extracts (Jepson & Healy quoted in Curtis, 1986). A wide variety of chemicals such as allelochemicals

of plants, insecticides, insect hormones and their analogues, together with the barbiturate phenobarbital have been shown to elicit the induction of detoxification enzymes. The question whether exposure of *An.stephensi* in nature, to compounds which induce detoxification enzymes, could lead to enhanced tolerance of insecticides led to the experiments on enzyme induction reported in chapter 6. Moreover, the concept of induction is of particular importance in the field situation.

Pretreatment of adults of *An.stephensi* with a sub-lethal dose of permethrin resulted in a 65% enhancement of tolerance to this insecticide. Similarly pre-exposure of DUB-LPR larvae to a sub-lethal dose of permethrin resulted in increased tolerance to permethrin. For the sensitive IND-S strain, the inductive effect was smaller. It seems that sub-lethal doses of permethrin turn on the detoxification machinery in the mosquito and give them some protection against subsequent exposure. These sub-lethal effects must occur in a proportion of insect populations during exposure to chemical control agents, hence such phenomena must be considered when the overall efficacies of control measures are being assessed. The results of bioassays of resistant and susceptible *An.stephensi* females following pretreatment with sodium phenobarbital and subsequent exposure to permethrin and deltamethrin indicated that sodium phenobarbital increases the pyrethroid tolerance level of the IND-S strain to a greater extent than the DUB-APR strain. We reasoned that the reduced effect of inducers on the resistant strain is a consequence of the initially higher enzyme activity of this strain. The phenomenon of a pyrethroid-resistant strain being less sensitive to phenobarbital induction compared to susceptible flies has also been noted by Scott & Lee (1993b). Our finding indicates that the detoxification reaction was accelerated by sodium phenobarbital and exceeded the activation reaction.

Larvae of DUB-LPR and IND-S strains pretreated with menthol-related compounds exhibited a significantly enhanced tolerance to permethrin. Treatment with menthol-related compound was able to induce a significant level of enhanced tolerance, as high as 3-fold in the case of larvae exposed to breeding water containing peppermint leaves in which menthol is probably the main active compound. Correspondingly, we would expect differences in the amount of a pesticide required for control to be effective in different habitats. The inductive effect of natural compounds which are present in the habitats of mosquitoes may turn on the detoxification mechanisms and required a higher pesticide treatment rate for induced than non-induced populations.

10.3 Structure-activity relationship of pyrethroids

The aim of our studies of structure-activity relationships was to determine whether changes in the structure of pyrethroids markedly affect their potency as insecticides towards the different strains of *An.stephensi* and to examine the response of permethrin-resistant and susceptible strains to different classes of pyrethroids.

The order of toxicity among the pyrethroids depends on the target strain but in general deltamethrin, an α-cyano pyrethroid with dibromo substitution was the most potent compound against permethrin-susceptible larvae. Cypermethrin and cyfluthrin were the least toxic, while lambdacyhalothrin and permethrin had moderate toxicity. The range of pyrethroid toxicity at the LC50 between the two extremes (deltamethrin and cypermethrin) was greatest for the susceptible strains. In contrast, with the DUB-APR strain, the difference in highest and lowest potency (lambdacyhalothrin and cyfluthrin) did not exceed 3.6-fold. In the DUB-LPR strain resistance appears to be affected by α-cyano substitution.

In larvae of DUB-LPR and DUB-APR strains, pyrethroids were synergized by PB with various synergist ratios suggesting that each resistant strain possesses mono-oxygenase-mediated resistance mechanisms responsible, at least in part, for the resistance. It appears likely, therefore, that permethrin-selection may have led to an increase in cytochrome P-450s specific for detoxification of these compounds, leading to the cross-resistance as observed. This suggests that there is at least a common resistance mechanism via P-450s for all the pyrethroids. Moreover, since the relative activities of different pyrethroids are not the same this suggests that another resistance mechanism(s) also plays a role. Further work is needed to clarify the possibility of quantitative differences in the mon-ooxygenase systems in each selected strain. An understanding of such differences will be an important part of developing strategies of insecticide usage that delay or prevent the development of resistance to synthetic pyrethroids.

10.4 The effect of temperature on the pyrethroid application

Georghiou (1980a,b) stated that since resistance development is accelerated by prolonged exposure of populations to suboptimal dosages of insecticide, negative temperature mortality relationships may affect the use of pyrethroids in mosquito control programmes. Any alteration of resistance by temperature changes could provide an insight into the importance of this factor in the evolution of resistance in the field. The efficacy of some pyrethroids may be greater at low temperatures (i.e., negative temperature coefficient), while for others it may be decreased at lower temperature (i.e., positive temperature coefficient).

Our exposure method in studies of the influence of temperature on the toxicity of the

pyrethroids was similar to the method of Hinkle et al. (1989), who used pyreythroids at the LC70 for temperature coefficient assays in horn fly populations. Our results indicated that pyrethroids were more effective against both strains at a temperature of 25±1°C than at the lower temperature of 5±1°C and mortality was more pronounced for the susceptible strain at the lower temperature. In experiments conducted by Burgess & Hinks (1986), a positive temperature coefficient for cypermethrin was found in the flea beetle, *Phyllotetra crucifera*. The LD50 at 10° and 32°C was 477 g/ha (95% F.L.=170-10730) and 38 g/ha (95% F.L.=29-47) respectively. This LD50 was 13-fold greater at low temperature, which is similar to our results.

Although no experiments on the pharmacokinetics of insecticide penetration at different temperatures were conducted with *An.stephensi*, we postulated that the increased mortality of both resistant and susceptible strains of *An.stephensi* at higher temperature is not related to the structure of compounds or the physiological resistance of the strains but rather a consequence of the kinetics of insecticide penetration; at higher temperature more insecticide gets into the insect. Thus, Blum & Kearns (1956) found that at 35°C the rate of penetration of pyrethrum in the American cockroach was more than twice that which occurred at 15°C. Similarly penetration of (IRS)-*trans*-^{14}C-permethrin was positively correlated to temperature in *M.domestica* (Ahn et al., 1987).

The result of this study indicated that pyrethroid toxicity increased at higher temperature suggesting that low temperatures may cause control problems in the field. However, in the light of these observations, pyrethroids should prove highly effective in tropical climates.

10.5 The importance of metabolic pathways

The aim of the metabolism studies involving HPLC was to compare and quantify the route and rate of ^{14}C-permethrin metabolism between permethrin-resistant and susceptible strains. Results showed that the production and the pattern of metabolite distribution was similar in DUB-LPR and IND-S strains, but quantitatively dependent on the type of cell fraction. The total metabolic activity in larval preparations of the susceptible strain was slightly lower than in resistant strains. Our study suggests that *in vitro* metabolism of ^{14}C-permethrin in *An.stephensi* results in the formation of one single metabolite (but see below) and this is probably via both oxidative and esterases pathways, a conclusion put forward by our previous *in vivo* studies. *In vitro* studies on the metabolism of ^{14}C-permethrin using abdomen homogenates of *M.domestica* revealed that there were only minor differences between permethrin-resistant and susceptible strains in their capability for metabolic detoxification (Bull, 1992). In other experiments on the metabolic fate of *trans* and *cis*-permethrin on the cattle tick, *Boophilus microplus*, it was found that there were no major metabolic differences between pyrethroid-resistant and susceptible strains (Nicholson et al., 1979a).

To investigate further the relative contribution of esterases and monooxygenases in determining the metabolites produced would require blocking them both independently by pre-application of esterase inhibitors such as paraoxon, DEF, and TPP and the monooxygenase inhibitor piperonyl butoxide (PB). Further work should focus on the multiplicity and specificity of detoxifying oxidases and esterases, purification of cytochrome P-450 from the resistant and susceptible strains of *An.stephensi* and comparison between them, and identification of metabolic product(s). Moreover, It will be important to determine whether the single peak of radiolabelled metabolite

which has a 0.4 minutes retention time is indeed a single metabolite or results from the overlapping of different metabolites not separated under these HPLC conditions. To answer this question, this peak should be identified by mass spectrometry.

If there were no real differences between the two strains in the amount of permethrin metabolism, what could account for the 131-fold synergism shown by the *in vivo* studies? Sun & Johnson (1972) have suggested that one of the mechanisms of sesamex synergism of carbamates is that sesamex increases the rate of penetration of insecticide. Possibly therefore penetration of PB may increase the rate of penetration of permethrin in the permethrin-resistant DUB-LPR. It is also likely that target site insensitivity, which has been postulated in permethrin-resistant larvae of *An.stephensi* (Omer et al., 1980) and *Cx.quinquefasciatus* (Priester & Georghiou, 1980a) is involved in the DUB-LPR strain to a limited extent. In this respect further penetration and neurophysiological experiment are needed to understand fully the basis of resistance in larvae of the DUB-LPR strain.

REFERENCES

Abd-Elghafar, S. F., Abo-Elghar, G. E. & Knowles, C. O. (1994). Fenvalerate penetration, metabolism and excretion in pyrethroid-susceptible and resistant *Heliothis virescens* (Lepidoptera: Noctuidae). J. Econ. Entomol. **87**: 872-878.

Abd-Elghafar, S. F., Knowels, C. O. & Wall, M. L. (1993). Pyrethroid resistance in two field strains of *Helicoverpa zea* (Lepidoptera: Noctuidae). J. Econ. Entomol. **86**: 1651-1655.

Abdel-Aal, Y. A. & Soderlund, D. M. (1980). Pyrethroid-hydrolysing esterases in southern armyworm larvae: tissue distribution, kinetic properties, and selective inhibition. Pestic. Biochem. Physiol. **14**: 282-289.

Abedi, Z. H. & Brown, A. W. A. (1961). Peritrophic membrane as vehicle for DDT and DDE excretion in *Aedes aegypti* larvae. Ann. Entomol. Soc. Am. **54**: 539-542.

Abernathy, C. O., Ueda, K., Engel, J. L., Gaughan, L. C. & Casida, J. E. (1973). Substrate-specificity and toxicological significance of pyrethroid-hydrolysing esterases of mouse liver microsomes. Pestic. Biochem. Physiol. **3**: 300-311.

Adams, M. E. & Miller, T. A. (1979). Site of action of pyrethroids: Repetitive "Backfiring" in flight motor units of house fly. Pestic. Biochem. Physiolo. **11**: 218-231.

Adesnik, M., Bar-nut, S., Maschio, F., Zunich, M., Lippman, A. & Bard, E. (1981). Mechanism of induction of cytochrome P-450 by phenobarbital. J. Biol. Chem. **256**: 10340-10345.

Agosin, M. (1985). Role of microsomal oxidation in insecticide degradation. pp. 647-712. In: Comprehensive Insect Physiology, Biochemistry and Pharmacology. Vol 12. Kerkuk, G. A., & Gilbert, L. I. [eds]. Pergamon Press, Oxford.

Agosin, M. & Dinamarca, M. L. (1963). The effect of DDT on the level of di- and triphosphopyridine nucleotides in *Triatoma infestans*. Exp. Parasitol. **13**: 199-203.

Agosin, M., Michaeli, D., Miskus, R., Nagasawa, S. & Hoskins, W. (1961). A new DDT-metabolizing enzyme in the German cockroach. J. Econ. Entomol. **54**: 340-342.

Ahmed, M. & McCaffery, A. R. (1991). Elucidation of detoxication mechanisms

involved in resistance to insecticides in the third instar larvae of a field-selected strains of *Heliothis armigera* with the use of synergists. Pestic. Biochem. Physiol. **41**: 41-52.

Ahn, J. Y., Shono, T. & Fukami, J. I. (1987). Effect of temperature on pyrethroid action to *kdr*-type house fly adults. Pestic. Biochem. Physiol. **28**: 301-307.

Alonso, P. L., Smithy, T., Armstrong Schellenberg , R. M., Masanya. H., Mwankusye, S., Urassa, H., Bason de Azevedo, I., Chongela, J., Kobera, S., Mendenz, C., Hurt, H., Thomas, M. C., Lyimo, E., Weiss, N. A., Hayes, R., Kitua, A. Y., Lopez, M. C., Kilama, W. L., Teuscher, T. & Tanner, M. (1994). Randomized trail of efficacy of SPf66 vaccine against *Plasmodium falciparum* malaria in children in southern Tanzania. Lancet. **344**: 1175-1181.

Amin, J. M. & Hemingway, J. (1989). Preliminary investigation of the mechanisms of DDT and pyrethroid resistance in *Culex quinquefasciatus* Say (Diptera: Culicidae) from Saudi Arabia. Bull. Entomol. Res. **79**: 361-366.

Anspaugh, D. D., Randly, L., Rose, P., Koehler, G., Hodgson, E. & Roe, R. M. (1994). Multiple mechanisms of pyrethroid resistance in German cockroach, *Blattella germanica* (L.). Pestic. Biochem. Physiol. **50**: 138-148.

Arias, R. O. & Terrier, L. C. (1962). The hydroxilation of naphthalate-C14 by housefly microsomes. J. Econ. Entomol. **55**: 925-929.

Armes, N. J., Jadhav, D. R., Bond, G. S. & King., A. B. S. (1992). Insecticide resistance in *Helicoverpa armigera* in South India. Pestic. Sci. **34**: 355-364.

Ascher, K. R. S. & Kocher, C. (1954). Enhanced susceptibility of highly resistant strain of house fly to ingestion to potassium bromide. Experientia. **10** : 465-466.

Atkinson, T. H., Wadleigh, R. A., Koehler, P. G. & Patterson, R. S. (1991). Pyrethroid resistance and synergism in a field strain of the German cockroach (Dictyoptera: Blattellidae). J. Econ. Entomol. **84**: 1247-1250.

Barlow, F., Hadaway, B., Flower, L. S., Grose, J. E. H. & Turner, C. R. (1977). Some laboratory investigations relevant to the possible use of new pyrethroids in control of mosquitoes and tsetse flies. Pestic. Sci. **8**: 291-300.

Becker, N. & Ludwig, M. (1993). Investigations on possible resistance in *Aedes vexans* field populations after a 10-year application of *Bacillus thuringiensis israelensis*. J. Am. Mosq. Control Assoc. **9**: 221-224.

Bell, J. D. & Busvine, J. R. (1967). Synergism of organophosphate in *Musca domestica*

and *Chrysomyia putori*. Entomol. Exp. Appl. **10**: 263-269.

Bisset, J. A., Rodriguez, M. M., Hemingway, J. Diaz, C., Small, C. J. & Ortiz, E. (1991). Malathion and pyrethroid resistance in *Culex quinquefasciatus* from Cuba: Efficacy of pirimiphos-methyl in the presence of at least three resistance mechanisms. Med. Vet. Entomol. **5**: 223-228.

Blackman, G. G. & Hodson, M. J. (1977). Further evaluation of permethrin for biting-fly control. Pestic. Sci. **8**: 270-273.

Bladridge, G. D. & Feyereisen, R. (1986). Blood meal and cytochrome P-450 monooxygenases in the northern house mosquito, *Culex pipiens*. Pestic. Biochem. Physiol. **25**: 407-413.

Bloomquist, J. R. (1996). Ion channels as targets for insecticides. Annu. Rev. Entomol. **41**: 163-190.

Bloomquist, J. R. & Miller, T. A. (1985). A simple bioassay for detecting and characterising insecticide resistance. Pestic. Sci. **16**: 611-614.

Blum, M. S. & Kearns, C. W. (1956). Temperature and the action of pyrethrum in the American cockroach. J. Econ. Entomol. **49**: 862-865.

Bradford, M. M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-binding. Ann. Biochem. **72**: 248-254.

Brattsten, L. B. (1992). Potential role of plant allelochemicals in development of insecticide resistance. pp. 313-348 In: Molecular mechanism of insecticide resistance, diversity among insects. American Chemical Society Symposium Series. Mullin, C. A., & Scott, J. G. [eds]. Washington DC.

Brattsten, L. B., Evans, C. K., Bonetti, S. & Zalkow, L. H. (1984). Induction by carrot allelochemicals of insecticide-metabolizing enzymes in the southern armyworm (*Spodoptera eridania*). Comp. Biochem. Physiol. **77**: 29-37.

Brealey, C. J., Crampton. P. L, Chadwick, P. R. & Rickett, F. E. (1984). Resistance mechanisms to DDT and *trans*-permethrin in *Aedes aegypti*. Pestic. Sci. **15**: 121-132.

Brogdon, W. G. & Barber, A. (1990). Fenitrothion-deltamethrin cross-resistance conferred by esterases in Guatemalan *Anopheles albimanus*. Pestic. Biochem. Physiol. **37**: 130-139.

Brown, A. W. A. (1986). Insecticide resistance in mosquitoes: A pragmatic review. J. Am. Mosq. Control Assoc. **2**: 123-140.

Brown, T. & Bragdon, W. G. (1987). Improved detection of insecticide resistance through conventional and molecular techniques. Annu. Rev. Entomol. **32**: 145-162.

Brown. A. W. A., Haworth, J. & Zahar, A. R. (1976). Malaria eradication and control from a global standpoint. J. Med. Entomol. **13**: 1-25.

Brown, A. W. A. & Pal. R. (1971). Insecticide resistance in arthropods. Bull. WHO. Ser. **38**. pp. 491.

Bull, D. L. (1992). Target site and enzyme changes associated with selection of subcolonies of a multiresistant house fly strain with methyl parathion or permethrin. Pestic. Biochem. Physiol. **42**: 211-226.

Bull, D. L., Harris, R. L. & Pryor, N. W. (1988). The contribution of metabolism to pyrethroid and DDT resistance in the horn fly (Diptera: Muscidae). J. Econ. Entomol. **81**: 449-458.

Burgess, L. & Hinks, C. F. (1986). Effect of post-treatment on the contact toxicity of cypermethrin spray to adults of the flea beetle, *Phyllotreta cruciferae* (Goeze). Canad. Ent. **118**: 79-80.

Busvine, J. R. (1951). Mechanism of resistance to insecticides in house flies. Nature, **168**: 193-195.

Busvine, J. R. (1958). Forms of insecticide resistance in house flies and body lice. Nature. **171**: 118-119.

Buxton, P. A. (1945). The use of the new insecticide DDT in relation to the problems of tropical medicine. Trans. R. Soc. Trop. Med. Hyg. **38**: 267.

Byford, R. L., Lockwood, J. A., Smith, S. M. & Franke, D. E. (1987b). Redistribution of behaviorally resistant horn flies (Diptera: Muscidae) on cattle treated with pyrethroid-impregnated ear tags. Environ. Entomol. **16**: 467-470.

Byford, R. L., Lockwood, J. A. & Sparks, T. C. (1987c). A novel resistance management strategy for horn flies (Diptera: Muscidae). J. Econ. Entomol. **80**: 291-296.

Byford, R. L., Lockwood, J. A., Smith, S. M., Sparks, T. C. & Luther, D. C. (1987a). Insecticide mixtures as an approach to the management of pyrethroid resistant

horn flies (Diptera: Muscidae). J. Econ. Entomol. **80**: 111-116.

Byford, R. L., Quisenberry, S. S., Sparks, T. C. & Lockwood, J. A. (1985). Spectrum of insecticide cross-resistance in pyrethroid-resistant population of *Haematobia irritans* (Diptera: Muscidae). J. Econ. Entomol. **78**: 768-773.

Callaghan, A., Boiroux, V., Raymond, M. & Pasteur, N. (1994). Prevention of changes in the electrophoretic mobility of overproduced esterases from organophosphate-resistant mosquitoes of *Culex pipiens* complex. Med. Vet. Entomol. **8**: 391-394.

Candy, D.J. & Kilby, B. A. (1975). Insect biochemistry and function. John Wiley & Sons. Inc. New York. pp. 314.

Catteral, W. A. (1988). Structure and function of voltage-sensitive ion channels. Science. **242**: 50-61.

Chadwick, P. R., Invest, J. F. & Brown, M. J. (1977). An example of cross-resistance to pyrethroids in DDT-resistant *Aedes aegypti*. Pestic. Sci. **8**: 618-624.

Chakravorthy, B. C. & Kalyanasundaram, M. (1992). Selection of permethrin resistance in the malaria vector *Anopheles stephensi*. Indian. J. Malariol. **29**: 161-165.

Chang, C. P. & Plapp, F. W. (1983). DDT and pyrethroids: receptor binding in relation to knock-down resistance (*kdr*) in the house fly. Pestic. Biochem. Physiol. **20** : 86-91.

Chang, C. K. & Jordan, T. W. (1982). Penetration and metabolism of topically applied permethrin and cypermethrin in pyrethroid-tolerant *Wiseana cervinata* larvae. Pestic. Biochem. Physiol. **17**: 196-204.

Chang, C. K. & Whalon, M. E. (1986). Hydrolysis of permethrin by pyrethroid esterases from resistant and susceptible strains of *Amblyseius fallacis* (Acari: Phytoseiidae). Pestic. Biochem. Physiol. **25**: 446-452.

Chapman, R. B. & Penman, D. E. (1979). Negative correlated cross-resistance to synthetic pyrethroids in organophosphorus-resistant *Tetranychus urtica*. Nature. **281**: 298-299.

Chapman, R. B. & Penman, D. E. (1980). The toxicity of mixtures of pyrethroids and organophosphorus insecticides to *Tetranychus urtica* Kock. Pestic. Sci. **11**: 600-604.

Chapman, R. F. (1982). The insects. Structure and function. 3rd edition. MacMillan India Ltd, Bangalore. pp. 919.

Charlmers, A. E., Miller, T. A. & Olsen, R. W. (1987). Deltamethrin: a neurophysiological study of the site of action. Pestic. Biochem. Physiol. **27**: 36-41.

Chialiang, C. & Devonshire, A. L. (1982). Changes in membrane phospholipids, identified by arrhenius plots of acetylcholinesterase and associated with pyrethroid resistance (*kdr*) in house flies (*Musca domestica*). Pestic. Sci. **13**: 156-160.

Chitra, S. & Pillai, M. K. K. (1985). Role of esterases in organophosphorus and carbamate resistance in Indian strain of *Anopheles stephensi* Liston. Indian. J. Exp. Biol. **23**: 576-584.

Chung, T. C, Sun, C. N. & Hung, C. Y. (1982). Resistance of *Nilaparvata lugens* to six insecticides in Taiwan. J. Econ. Entomol. **75**: 199-200.

Cilek, J. E. (1989). Horn fly, *Haematobia irritans* (L.) resistance to insecticides in Kentuky, Ph.D Thesis. University of Kentuky, Lexington. USA.

Cilek, J. E., Dahlman, D. L. & Knapp, F. W. (1995). Possible mechanism of diazinon negative cross-resistance in pyrethroid-resistant horn flies (Diptera: Muscidae). J. Econ. Entomol. **88**: 520-524.

Cilek, J. E. & Knapp, F. W. (1993a). Influence of insecticide mixtures impregnated in cattle ear tags for management of pyrethroid-resistant horn flies (Diptera: Muscidae). J. Econ. Entomol. **86**: 444-449.

Cilek, J. E. & Knapp, F. W. (1993b). Enhanced diazinon susceptibility in pyrethroid-resistant horn flies (Diptera: Muscidae): Potential for insecticide resistance management. J. Econ. Entomol. **86**: 1303-1307.

Cilek, J. E., Steelman, C. D. & Knapp, F. W. (1991). Horn fly (Diptera: Muscidae) insecticide resistance in Kentucky and Arkansas. J. Econ. Entomol. **84**: 756-762.

Clark, J. L. & Rowley, W. A. (1984). Evaluation of granular *Bacillus thuringiensis* var. *israelensis* (serotype H-14) formulations against mosquito larvae in central Iowa. Mosq. News. **44**: 502-505.

Clark, J. M. (1981). Pyrethroid inhibition of neural ATPase. Ph.D Thesis. Michigan State University. USA. pp. 113.

Clark, J. M. & Matsumura, F. (1982). Two different types of inhibitory effects of pyrethroids on nerve Ca^{2+} and $Ca^{2+}+Mg^{2+}$-ATPase activity in the squid, *Loligo pealei*. Pestic. Biochem. Physiol. **18**: 180-190.

Clark, A. G., Shammaan, N. A., Sinelain, M. D. & Dauterman, W. C. (1986). Insecticide metabolism by multiple glutathione S-transferases in two strains of the house fly, *Musca domestica* (L). Pestic. Biochem. Physiol. **25**: 169-175.

Clements, A. N. & May, T. E. (1977). The actions of pyrethroids upon the peripheral nervous system of the cockroach *Periplaneta americana* L. Pestic. Sci. **8**: 661-668.

Cochran, D. G. (1987). Selection for pyrethroid resistance in the German cockroach, *Blattella germanica* (L.) (Dictyoptera: Blattellidae). J. Econ. Entomol. **80**: 1117-1121.

Cochran, D. G. (1989). Monitoring for insecticide resistance in field-selected strains of the German cockroach (Dictyoptera: Blattellidae). J. Econ. Entomol. **82**: 336-341.

Cochran, D. G. (1991). Extended selection for pyrethroid resistance in the German cockroach (Dictyoptera: Blatellidae). J. Econ. Entomol. **84**: 1412-1416.

Cochran, D. G. (1993). Decline of pyrethroid resistance in the absence of selection pressure in a population of German cockroach (Dictyoptera: Blattellidae). J. Econ. Entomol. **86**: 1639-1644.

Cochran, D. G. (1994). Effects of three synergists on pyrethroid resistance in the German cockroach (Dictyoptera: Blattellidae). J. Econ. Entomol. **87**: 879-884.

Cole, M. M. & Clark, P. H. (1961). Development of resistance to synergize pyrethrins in body lice and cross-resistance to DDT. J. Econ. Entomol. **54**: 649-651.

Collins, P. J. (1985). Induction of polysubstrate monoxygenase system of native budworm *Heliothis punctiger* (Wallengren) (Lepidoptera: Noctuidae). Insect. Biochem. **15**: 551-557.

Conney, A. H. (1986). Induction of microsomal cytochrome P-450 enzymes. Life Sci. **39**: 2493-2518.

Corbett, J. R., Wright, K. & Ballie, A. C. (1984). The biochemical mode of action of pesticides. Academic Press, London. pp. 382.

Crampton, J. M. (1994). Approaches to vector control; New and trust: 3. Prospects for genetic manipulation of insect vector. Trans. R. Soc. Trop. Med. Hyg. **88**: 141-143.

Crampton, J. M., Warren, G. J., Lycett, M. A., Hughes, I. P., Comely, P. & Eggleston, P. (1994). Genetic manipulation of insect vectors as a strategy for the control of vector-borne disease. Ann. Trop. Med. Parasitol. **88**: 3-12.

Cremlyn, R. (1978). Pesticides; preparation and mode of action. John Wiley & Son. Chichester, New York, Brisbane, Toronto, USA. pp. 142-149.

Croft, B. A. (1990). Developing a philosophy and programme of pesticide resistance management. In: Pesticide resistance in arthropods. Roush, R.T. & Tabashnik, B. E. [eds]. Chapman and Hall, New York and London.

Croft, B. A., Wagner, S. W. & Scott, J. G. (1982). Multiple and cross-resistance to insecticides in pyrethroid resistance strains of the predatory mite *Amblyseius fallacis*. Environ. Entomol. **11**: 161-164.

Crosby, B. L., Byford, R. L. & Spark, T. C. (1991). Bioassay for detecting active site insensitivity in horn fly (Diptera: Muscidae) larvae. J. Econ. Entomol. **84**: 367-370.

Crowder, L. A., Touefson, M. S. & Watson, T. F. (1979). Dosage-mortality studies of synthetic pyrethroids and methyl parathion on the tobacco budworm in central Arizona. J. Econ. Entomol. **72**: 1-3.

Crubs, R. E., Adams. P. M. & Soderlund. D. M. (1988). Binding of [^{3}H] saxitoxin to head membrane preparation from susceptible and knock-down resistant house flies. Pestic. Biochem. Physiol. **32**: 217-223.

Curtis, C. F. (1985). Theorical models of the use of insecticide mixtures for the management of resistance. Bull. Entomol. Res. **75**: 259-265.

Curtis, C. F. (1986). Fact and fiction in mosquito attraction and repulsion. Parasitol. Today. **11**: 316-318.

Curtis, C. F. (1987b). Genetic aspects of selection for resistance. pp. 150-161. In: Combating resistance to xenobiotics. Ford, M. G., Holloman, D. W., Khambay, B. P. S. & Sawicki, R. M. [eds]. Ellis Harwood, Chichester.

Curtis, C. F. (1989). Appropriate technology and why it is needed. pp. 1-5. In: Appropriate Technology in vector control. Curtis, C. F. [ed]. CRC press. Boca Raton

Curtis, C. F. (1991). Control of disease vectors in the community. CRC Press. Boca Raton. pp. 233.

Curtis, C. F. (1992). Spraying bednets with deltamethrin in Sichuan, China. Trop. Dis. Bull. **89**: R1-R6.

Curtis, C. F. (1994a). Approach to vector control: New and trusted; 4, Appropriate technology for vector control: Impregnated bednets, polystyrene beads and fly traps. Trans. R. Soc. Trop. Med. Hyg. **88**: 144-146.

Curtis, C. F. (1994b). Should DDT continue to be recommended for malaria vector control?. Med. Vet. Entomol. **8**: 107-112.

Curtis, C. F., Cook, L. M. & Wood, R. J. (1978). Selection for and against insecticide resistance and possible methods of inhibition the evolution of resistance in mosquitoes. Ecol. Entomol. **3**: 273-287.

Curtis, C. F., Hill, N. & Kasim, S. H. (1993). Are there effective resistance management strategies for vectors of human disease? Biol. J. Linn. Soc. **48**: 3-18

Curtis. C. F., Hill, N., Ulloa, M. & Magesa, S. (1990). The possible impact of resistance on the effectiveness of pyrethroid-impregnated bednets. Trans. R. Soc. Trop. Med. Hyg. **84**: 323-466.

Curtis, C. F., Lines. J. D., Carnevale, P., Robert, V., Boudin, C., Halna, J. M.,Pazart, L., Gazine, P., Richard, A., Mouchet, J., Charlwood, J. D., Graves, P. M., Hossain, M. I., Kurihara, T., Ichimori, K., Zuzi, L., Baloin, L., Majori, G., Sabatinelli, G., Coluzzi, M., Njunwa, K. J., Wilkes, T. J., Snow, R. W. & Lindsay, S. W. (1991). Impregnated bed nets and curtains against malaria mosquitoes. pp. 5-46. In: Control of disease vectors in the community. Curtis, C. F. [ed]. CRC press. Boca Raton

Curtis, C. F., Lines, J. D., Ijumba, J., Callaghan, N., Hill, N. & Karimzad, M.A. (1987a). The relative efficacy of repellents against mosquito vectors of disease. Med. Vet. Entomol. **1**: 109-119.

Curtis, C. F., Mayamba, J. & Wilkes, T. J. (1996). Comparison of different insecticides and fabrics for anti-mosquito bednets and curtains. Med. Vet. Entomol. **10**: 1-11.

Curtis, C. F. & Minjas, J. (1985). Expanded polystyrene for mosquito control. Parasitol. Today. **1**: 36.

Curtis, C. F. & Rawlings, P. (1980). A preliminary study of dispersal and survival of *Anopheles culicifacies* in relation to the possibility of inhibiting the spread of insecticide resistance. Ecol. Entomol. **5**: 11-17.

Cutkomp, L. K. & Subramanyam, B. (1986). Toxicity of pyrethroids to *Aedes aegypti*

larvae in relation to temperature. J. Am. Mosq. Control Assoc. **2**: 347-349.

Daly, J. C. & Fisk, J. H. (1992). Inheritance of metabolic resistance to synthetic pyrethroids in Australian *Helicoverpa armigera* (Lepidoptera: Noctuidae). Bull. Entomol. Res. **82**: 5-12.

Das, M., Srivastava, S. P., Khamer, J. S. & Deshpande, L. B. (1986). Susceptibility of DDT, dieldrin and malathion resistant *Anopheles culicifacies* to deltamethrin. J. Am. Mosq. Control Assoc. **2**: 553-555.

Dauterman, W. C. (1985). Insect metabolism: extramicrosomal. pp. 713-730. In: Comprehensive Insect Physiology, Biochemistry and Pharmacology. KerkuK. G. A. & Gilbert, L. I. [eds]. Pergamon Press, Oxford.

Davidson, G. (1958). Studies on insecticide resistance in anopheline mosquitoes. Bull. WHO. **18**: 579-621.

Davidson, G. (1974). Genetic control of insect pests. Academic Press, London.

Davidson, G. & Curtis, C. F. (1979). Insecticide resistance and the upsurge of malaria. Annual Report of London School of Tropical Medicine & Hygiene. 1978-79. pp. 78-82.

Davidson, G. & Jackson, C. G. (1961). DDT-resistance in *Anopheles stephensi*. Bull. WHO. **25**: 209-217.

Davidson, G. & Mason, G. F. (1963). Genetics of mosquitoes. Annu. Rev. Entomol. **8**: 177-196.

Davies, J. H. (1985). The pyrethroids: an historical introduction. pp. 1-41. In: The pyrethroid insecticides. Leahey, J. D. [ed]. Taylor & Francis Ltd. London and Philadelphia. pp. 440.

Delmore, R., Fournier, D., Chaufaux, J., Cuany, A., Bride, J. M., Auge, D. & Berge. J. B. (1988). Esterase metabolism and reduced penetration are causes of resistance to deltamethrin in *Spodoptera exigua* HUB (Noctuidae: Lepidoptera). Pestic. Biochem. Physiol. **32**: 240-246.

Denholm, I. & Rowland, M. W. (1992). Tactics for managing pesticide resistance in arthropods: Theory and practice. Annu. Rev. Entomol. **37**: 91-112.

Devonshire, A. L. (1987). In: Combating resistance to xenobiotics- biological and chemical approach. Ford, M. G, Hollomon, D. W, Khambay. B. P. S. & Sawicki, R. M. [eds]. Ellis Horwood, Chichester. pp. 227-238.

Devonshire, A. L. & Field, L. M. (1991). Gene amplification and insecticide resistance. Ann. Rev. Entomol. **36**: 1-23.

Devonshire, A. L., Field, L. M. & Williams, M. S. (1992). Molecular biology of insecticide resistance. pp. 173-183. In: Insect molecular science. Crampton, J. M. & Egglesten, P. [eds]. Academic Press, London.

Devonshire, A. L. & Field, L. M. (1995). Esterases in resistant peach-potato aphids, *Myzus persica* (Sulzer). In: Towards the genetic manipulation of insects. Keyston Symposium on Molecular Cellular Biology. Tammarron, Coloroda, USA. pp. 192.

Devonshire, A. L. & Moores, G. D. (1982). A carboxylesterase with broad substrate specificity causes organophosphorus, carbamates and pyrethroid resistance in peach-potato aphids (*Myzus persicae*). Pestic. Biochem. Physiol. **18**: 235-246.

DeVries, D. H. & Georghiou, G. P. (1979). Influence of temperature on the toxicity of insecticides to susceptible and resistant house flies. J. Econ. Entomol. **72**: 48-50.

DeVries, D. H. & Georghiou, G. P. (1980). A wide spectrum of resistance to pyrethroid insecticides in *Musca domestica*. Experientia. **36**: 226-227.

DeVries, D. H. & Georghiou, G. P. (1981a). Decreased nerve sensitivity and decreased cuticular penetration as mechanism of resistance to pyrethroids in a (IR)-*trans*-permethrin-selected strains of the house fly. Pest. Biochem. Physiol. **15**: 234-241.

DeVries, D. H. & Georghiou, G. P. (1981b). Absence of enhanced detoxication of permethrin in pyrethroid-resistant house flies. Pestic. Biochem. Physiol. **15**: 242-252.

Doherstyn, E. B. (1991). Malaria research and control. Acta Leiden, **60**: 9-110.

Dong, K. E. & Scott, J. G. (1991). Neuropharmacology and genetics of *kdr*-type resistance in the German cockroach, *Blattella germanica* (L.). Pestic. Biochem. Physiol. **41**: 159-169.

Dowd, P. F. & Sparks, T. C. (1987). Comparative hydrolysis of permethrin and fenvalerate in *Pseudoplusia includens* (Walker) and *Heliothis virescens* (F.). Pestic. Biochem. Physiol. **27**: 123-131.

Dowson, R. J. (1977). An introduction to the principle of neurophysiology. Pestic. Sci. **8**: 651-660.

Dyte, C. E. & Rowlands, D. G. (1968). The metabolism and synergism of malathion in resistant and susceptible strains of *Tribolium castaneum* (Herbst) (Coleoptera). J. Stored. Prod. Res. **4**: 157-173.

Eldridge, B. F., Washino, R. K. & Henneberger, D. (1985). Control of snow pool mosquitoes with *Bacillus thuringiensis* serotype H-14 in mountain environments in California and Oregon. J. Am. Mosq. Control Assoc. **1**: 69-75.

Elissa, N. & Curtis, C. F. (1995). Evaluation of different formulation of deltamethrin in comparison with permethrin for impregnation of netting. Pestic. Sci. **44**: 363-367.

Elliot, M., Farnham, A. W., Janes, N. F., Needham, P. H. & Pullman, D. A. (1974). Synthetic pyrethroids with new order of activity. Nature. **248**: 710-711.

Elliot, M., James, N. F., Pulman., D. A. Gaughan, L. C., Unai, T. & Casida, J. E. (1976). Radiosynthesis and metabolism in rats of the 1R isomers of the insecticide permethrin. J. Agr. Food Chem. **24**: 270-276.

Elliot, M., James, N. F. & Potter, C. (1978). The future of pyrethroids in insect control. Annu. Rev. Entomol. **23**: 443-469.

Elliot, M. (1989). The pyrethroids: Early discovery, recent advantages and the future. Pestic. Sci. **27**: 337-351.

Elzen, G. W., Leonard, B. R., Graves, J. B., Burris, E. & Micinski, S. (1992). Resistance to pyrethroid, carbamate, and organophosphate insecticides in field population of tobacco budworm (Lepidoptera: Noctuidae) in 1990. J. Econ. Entomol. **85**: 2064-2071.

Eshghy, N. (1978). Tolerance of *Anopheles stephensi* to malathion in the province of Fars, southern Iran. Mosq. News. **38**: 580-583.

Eto, M. (1974). Organophosphorus pesticides: Organic and biological chemistry. CRC Press, Cleveland, Ohio. USA. pp. 387.

Faicloux, A., Umg, A., Raymond, M. & Pasteur, N. (1994). Insecticide susceptibility in mosquitoes (Diptera: Culicidae) from French Polynesia. J. Med. Entomol. **31**: 639-644.

Fanara, D. M., Knepper, R. G. & Ross. D. H. (1984). Field tests of two granular *Bacillus thuringiensis* (H-14) formulations against snowpool *Aedes* spp. Mosq. News. **44**: 236-239.

Farnham, A. W. (1971). Changes in cross-resistance patterns of house flies selected with natural pyrethrins or resmethrin (5-benzyl-3-furylmethyl-*cis-trans*-chrysanthemate). Pestic. Sci. **2**: 138-143.

Farnham, A. W. (1973). Genetics of resistance of pyrethroid selected house flies, *Musca domestica* L. Pestic. Sci. **4**: 513-520.

Farnham, A. W. (1977). Genetics of resistance of house flies (*Musca domestica* L.) to pyrethroids. I. Knock-down resistance. Pestic. Sci. **8**: 631-636.

Farnham, A. W., Murray, W. A., Sawicki, R. M., Denholm, I. & White, J. C. (1987). Characterization of the structure-activity relationship of *kdr* and two variants of *super-kdr* to pyrethroids in the house fly (*Musca domestica* L.). Pestic. Sci. **19**: 209-220.

Farnham, A. W. & Sawicki, R. M. (1976). Development of resistance to pyrethroids in insects resistant to other insecticides. Pestic. Sci. **7**: 278-282.

Farnsworth, D. E., Berry, R. E., Yu, S. J. & Terriere, L. C. (1981). Aldrin epoxidase activity and cytochrome P-450 content of microsomes prepared from alfalfa and cabbage looper larvae fed various plant diets. Pestic. Biochem. Physiol. **15**: 158-165.

Feng, R., Chen, W. & Isman, M. B. (1995). Synergism of malathion and inhibition of midgut esterase activities by an extract from *Melia toosendan* (Meliacae). Pestic. Biochem. Physiol. **53**: 34-41.

Fenwick, M. L. (1958). The production of an esterase inhibitor from schradan in the fat body of the desert locust. Biochem. J. **70**: 373-381.

Feyereisen, R. (1995). P-450 mediated resistance in the house fly. pp. 192. In: Toward the genetic manipulation of insects. Keyston Symposium on Molecular Cellular Biology. Tammarron, Coloroda, USA.

Feyereisen, R., Andersen, J. F., Carino, F. A., Cohen, M. B. & Koener, J. F. (1995). Cytochrome P450 in house fly: structure, catalytic activity and regulation of expression of CYP6A1 in an insecticide-resistant strain. Pestic. Sci. **43**: 233-239.

Feyereisen, R. & Vincent, D. R. (1984). Characterisation of antibodies to house fly NADPH - cytochrome P-450 reductase. Insect Biochem. **14**: 163-168.

Ffrench-Constant, R. H., Aronstein, K., Anthony, N. & Coustau, C. (1995). Polymerase Chain Reaction-based monitoring techniques for detection of insecticide resistance-associated points mutations and their potential applications. Pestic. Sci.

43: 195-200.

Ffrench-Constant, R. H., Steichen, J. C. & Shotkoski, F. (1994). Polymerase Chain Reaction diagnostic for cyclodiene insecticides resistance in the mosquito *Aedes aegypti*. Med. Vet. Entomol. **8**: 99-100.

Fine, B. C. (1961). Pattern of pyrethrin-resistance in house flies. Nature. **191**: 884-885.

Finney, D. J. (1971). Probit analysis. 3rd edition. Cambridge University Press. Cambridge.

Fournier, D., Bride, J. M., Poirie, M., Berge, J. B. & Plapp. F. W. (1992). Insect glutathione S-transferases. J. Biol. Chem. **267**: 1840-1845.

Funaki, E., Dauterman, W. C. & Motoyama, N. (1994). *In vivo* and *In vitro* metabolism of fenvalerate in pyrethroid resistant houseflies. Pestic. Sci. **19**: 43-52.

Gahan, J. B. & Lindquist, A. W. (1945). DDT-residual sprays applied in building to control *Anopheles quadrimaculatus*. J. Econ. Entomol. **38**: 236.

Gammon, D. W. (1978). Neural effects of allethrin on the free walking cockroach *Periplaneta americana*: an investigation using defined doses at 15 and 32°C. Pestic. Sci. **9**: 79-91.

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. & Casida, J. E. (1983). Pyrethroids of the most potent class antagonize GABA action on the crayfish neuromuscular junction. Neurosci. Lett. **40**: 163-168.

Gammon, D. W., Brown, M. A. & Casida, J. E. (1981). Two class of pyrethroid action in the cockroach. Pestic. Biochem. Physiol. **15**: 181-191.

Garnett, P. & French, W. L. (1971). A genetic study of an esterase in *Culex pipiens quinquefasciatus*. Mosq. News. **31**: 379-386.

Gaughan, L. C., Unai, T. & Casida, J. E. (1977). Permethrin metabolism in rats. J. Agric. Food Chem. **26**: 525-528.

Georghiou, G. P. (1972). The evolution of resistance to pesticides. Annu. Rev. Ecol. **3**: 133-168.

Georghiou, G. P. (1980a). Insecticide resistance and prospects for its management. Residue Rev. **76**: 131-145.

Georghiou, G. P. (1980b). Implication of the development of resistance to pesticides: basic principles and consideration of countermeasures. pp. 116-129. In: Proceeding of seminar and workshop, Barbados. Pest and Pesticide management in the Caribbean.

Georghiou, G. P., Malik, J. I., Wirth, M. & Sainato. K. (1992). Characterization of resistance of *Culex quinquefasciatus* to the insecticidal toxins of *Bacillus sphaericus* (strain 2362). pp. 34-35. In: Annual Report Mosquito Control Research, University of California.

Gharib, A. H. & Hilsenhoff, W. (1988). Efficacy of two formulations of *Bacillus thuringiensis* var. *israelensis* (H-14) against *Aedes vexans* and safety to non-target macroinvertebrates. J. Am. Mosq. Control Assoc. **4**: 252-255.

Gibson, A. J. Osborne, M. P. & Ross, H. F. (1988). An electrophysiological investigation of the susceptible (Cooper) and resistant (*kdr*; *super kdr*) strains of adult house fly, *Musca domestica*. Pestic. Sci, **23**: 283-292.

Gibson, G. G. & Skett, P. (1986). Introduction to drug metabolism. Chapman & Hall, London. pp. 292.

Goldberg, L. J. & Margalit, J. (1977). A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii, Uranotaenia unguiniculata, Culex univittatus, Aedes aegypti* and *Culex pipiens*. Mosq. News. **37**: 355-358.

Goldman, I. F., Arnold, J. & Carlton, B. (1986). Selection for resistance to *Bacillus thuringiensis* subspecies. *israelensis* in field and laboratory populations of the mosquito *Aedes aegypti*. J. Inver. Pathol. **47**: 317-324.

Grafius, E. (1986). Effects of temperature on pyrethroid toxicity to Colorado potato beetle (Coleoptera: Chrysomelidae). J. Econ. Entomol. **79**: 588-590.

Grant, D. F. & Matsura, F. (1989). Glutathione S-transferase-1 and 2 in susceptible and insecticide resistant *Aedes aegypti*. Pestic. Biochem. Physiol. **33**: 132-143.

Greenwood, B. M. & Pickering, H. (1993). A malaria control trail using insecticide-treated bednets and targeted chemoprophylaxis in a rural area of The Gambia, West Africa, 1: a review of the epidemiology and control of malaria in The Gambia, West Africa. Trans. R. Soc. Trop. Med. Hyg. **87**: S2/3-S211.

Gunning, R. V., Easton, C. S., Balfe, M. E. & Ferris, J. G. (1991). Pyrethroid

resistance mechanisms in Australian *Heliothis armigera*. Pestic. Sci. **33**: 473-490.

Guo-Lei, F., Marion, J. R. & Clark, J. M. (1992). Suppression of pyrethroid-dependent neurotransmitter release from synaptosomes of knockdown-resistant houseflies under pulsed-depolarization conditions during continuous perfusion. Pestic. Biochem. Physiol. **42**: 64-77.

Guthrie, F. E. (1950). Effect of temperature on toxicity of certain organic insecticides. J. Econ. Entomol. **43**: 559-560.

Guyton, A. R. (1992). Human physiology and mechanism of disease. Harcourt Bruce Jovanovish Inc. PP. 690.

Hadaway, A. B., Barlow, F., Grose, J. E. H., Turner, C. R. & Flower, L. S. (1970). Evaluation of compounds for insecticidal activity on adult mosquitoes. 1. Response of adult mosquitoes to some new insecticides. Bull. WHO. **42**: 353-366.

Hales, B. F. & Neims, A. H. (1977). Induction of rat hepatic glutathione S-transferase by the phenobarbital and 3-methycholauthrene. Biochem. Pharmacol. **26**: 553-559.

Halliday, W. R. & Georghiou, G. P. (1985a). Inheritance of resistance to permethrin and DDT in the southern house mosquito (Diptera: Culicidae). J. Econ . Entomol. **78**: 762-767.

Halliday, W. R. & Georghiou, G. P. (1985b). Linkage relationships of the knockdown resistance gene (*Kdr*) in larvae of *Culex quinquefasciatus* (Diptera: Culicidae). J. Med. Entomol. **22**: 572-576.

Harris, C. R. & Kinoshita, G. B. (1976). Influence of posttreatment temperature on the toxicity of pyrethroid insecticides. J. Econ. Entomol. **70**: 215-218.

Hassall, K. A. (1982). The chemistry of pesticides. The Macmillan Press. London & Basingstoke. pp. 372.

Hayaoka, T. & Dauterman, W. C. (1981). Induction of glutathione S-transferase by phenobarbital and pesticides in various house fly strain and its effect on toxicity. Pestic. Biochem. Physiol. **17**: 113-119.

Hemingway, J. (1981). Genetics and biochemistry of insecticide resistance in Anophelines. Ph.D Thesis. University of London. pp. 310.

Hemingway, J. (1982). The biochemical nature of malathion resistance in *Anopheles*

stephensi from Pakistan. Pestic. Biochem. Physiol. **17**: 149-155.

Hemingway, J. (1983). The genetics of malathion resistance in *Anopheles stephensi* from Pakistan. Trans. Ro. Soc. Trop. Med. Hyg. **77**: 106-108.

Hemingway, J. Callaghan, A. & Amin, J. M. (1990). Mechanisms of organophosphate and carbamate resistance in *Culex quinquefasciatus* from Saudi Arabia. Med. Vet. Entomol. **4**: 275-282.

Hemingway, J., Dunbar, S. J., Monro, A. C. & Small, G. J. (1993). Pyrethroid resistance in German cockroach (Dictyoptera: Blattellidae): Resistance levels and underlying mechanisms. J. Econ. Entomol. **86**: 1631-1638.

Hemingway, J., Malcolm, C. A, Kisson, K. E., Boddington, R. G., Curtis, C. F. & Hill, N. (1985). The biochemistry of insecticide resistance in *Anopheles sacharovi*. Comparative studies with a range of insecticide susceptible and resistance *Anopheles* and *Culex* species. Pestic. Biochem. Physiol. **24**: 68-76.

Hemingway, J., Rowland, M. & Kisson, K. E. (1984). Efficacy of pirimiphos methyl as a larvicide or adulticide against insecticide resistance and susceptible mosquitoes (Diptera: Culicidae). J. Econ. Entomol. **77**: 868-871.

Hemingway, J ., Smith. C., Jayawardena, K. G. I. & Herath, P. R. J. (1986). Field and laboratory detection of the altered acetylcholinesterase resistance genes which confer organophosphate and carbamate resistance in mosquitoes (Diptera: Culicidae). Bull. Entomol. Res. **76**: 559-565.

Herath, P. R. J., Jayawardena, K. G. I., Hemingway, J. & Harris, J. (1988). DDT resistance in *Anopheles culicifacies* Giles and *An.subpictus* Grassi (Diptera: Culicidae) from Sri Lanka: a field study on the mechanisms and changes in gene frequency after cessation of DDT spraying. Bull. Entomol. Res. **78**: 717-723.

Herath, P. R. J. (1979). Studies on insecticide resistance in anopheline mosquitoes. Ph.D Thesis. University of London.

Herath, P. R. J. & Davidson, G. (1981). Studies on the nature of malathion resistance in population of *Anopheles stephensi* from southern Iran. Mosq. News. **41**: 531-534.

Heuval, M. J. & Cochran, D. G. (1965). Cross-resistance to organophosphorus compounds in malathion and diazinon-resistant strains of *Blattella germanica* . J. Econ. Entomol. **58**: 872-874.

Hinkle, N. C., Sheppard, D. C., Bondari, K. & Butler, J. F. (1989). Effect of

temperature on toxicity of three pyrethroids to hornflies. Med. Vet. Entomol. **3**: 435-439.

Hodgson, E. (1985). Microsomal mono-oxygenase. pp. 225-321 In: Comprehensive Insect Physiology , Biochemistry and Pharmacology. Vol 11. Kerkuk, G. A , & Gilbert, L. I. [eds]. Pergamon Press, Oxford.

Hoffman, R. A. & Lindquist, A. W. (1949). Temperature coefficient for chlorinated hydrocarbons. J. Econ. Entomol. **42**: 891.

Holden, J. S. (1979). Absorbtion and metabolism of permethrin and cypermethrin in the cockroach and the cotton-leafworm larvae. Pestic. Sci. **10**: 295-307.

Hossain, M. I., Curtis, C. F. & Heekin, J. P. (1989). Assays of permethrin-impregnated fabrics and bioassays with mosquitoes (Diptera: Culicidae). Bull. Entomol. Res. **79**: 299-308.

Hossain, M. I. & Curtis, C. F. (1989). Permethrin-impregnated bed nets: behaviourial and killing effects on mosquitoes. Med. Vet. Entomol. **3**: 367-376.

Hostetler, M. E. & Brenner, R. J. (1994). Behavioural and physiological resistance to insecticides in the German cockroach (Dictyoptera: Blattellidae): An experimental reevaluation. J. Econ. Entomol. **87**: 885-893.

Hougard, J. M., Mbentengam, R., Lochouran, L., Escaffer, H., Darriet, F., Barbazan, P. & Quillevere. D. (1993). Control of *Culex quinquefasciatus* by *Bacillus thuringiensis*: results of a pilot campaign in a large urban area in equatorial Africa. Bull. WHO. **71**: 367-375.

Hunter, M. D., Biddinger, D. J., Carlini, E. J., McPheron, B. A. & Hull. L. A. (1994). Effects of apple leaf allelochemistry on tufted apple bud moth (Lepidoptera: Tortricidae) resistance to azinphosmethyl. J. Econ. Entomol. **87**: 1423-1429.

Immanraju, J. A., Paine, T. D., Bethke, J. A, Rob, K. L. & Newman, J. P. (1992). Western flower thrips (Thysanoptera: Thripidae) resistance to insecticides in coastal California green houses. J. Econ. Entomol. **85**: 9-14.

Iqbal, M. P., Sakai, R. K. & Baker, R. H. (1973). The genetics of an esterase in *Culex tritaeniorhynchus*. Mosq. News. **33**: 72-75.

Ishaaya, I. & Casida, J. E. (1980). Properties and toxicological significance of esterase hydrolysing permethrin and cypermethrin in *Trichoplusia ni* larvae gut and integument. Pestic. Biochem. Physiol. **14**: 178-184.

Ishaaya, I. & Casida, J. E. (1981). Pyrethroid esterase(s) may contribute to natural pyrehtroid tolerance to larvae of the common green lacewing. Environ. Entomol. **10**: 681-684.

Ishaaya, I., Mendelson, Z., Ascher, K. R. S. & Casida, J. E. (1987). Cypermethrin synergism by pyrethroid esterase inhibitors in adults of the whitefly *Bemisia tabaci*. Pestic. Biochem. Physiol. **28**: 155-162.

Jackson, F. R., Wilson, S. D., Strichartz, G. R. & Hall, L. M. (1984). Two types of mutants affecting voltage-sensitive sodium channels in *Drosophila melanogaster*. Nature. **308**: 189-191.

Jao, L. T. & Casida, J. E. (1974). Insect pyrethroid- hydrolysing esterases. Pestic. Biochem. Physiol. **4**: 465-472.

Jayawardena, K. G. I., Karunaratue, S. H. P. P., Ketterman, A. J. & Hemingway, J. (1994). Determination of the role of elevated B2 esterase in insecticide resistance in *Culex quinquefasciatus* (Diptera: Culicidae) from studies on the purified enzyme. Bull. Entomol. Res. **84**: 39-44.

Jones, C. J. & LIoyd, J. E. (1985). Efficacy of *Bacillus thuringiensis* (H-14) for larval *Aedes* mosquito control in intermountain meadows in Wyoming. J. Am. Mosq. Control Assoc. **1**: 51-55.

Kang, W., Gao, B., Jiang, H., Wang, H., Yu, P., Xu, B. & Curtis, C. F. (1995). Tests for possible effects of selection by domestic pyrethroids for resistance in culicine and anopheline mosquitoes in Sichuan and Hubei, China. Ann. Trop. Med. Parasitol. **6**: 677-684.

Kaplowitz, N., Kuhlenman, P. J. & Clifton, G. (1975). Drug induction of hepatic glutathione S-transferases in male and female rats. Biochem. J. **146**: 351-355.

Karch, S., Asidi, N., Mazambi, M. & Salaum, J. J. (1992). Efficacy of *Bacillus sphaericus* against the malaria vector *Anopheles gambiae* and other mosquitoes in swamps and rice fields in Zair. J. Am. Mosq. Control Assoc. **8**: 376-380.

Keiding, J. (1976). Development of resistance to pyrethroids in field populations of Danish house flies. Pestic. Sci. **7**: 283-291.

Kennaugh, L., Pearce, D., Daly, J. C. & Hobbs, A. A. (1993). A piperonyl butoxide synergisable resistance to permethrin in *Helicoverpa armigera* which is not due to increased detoxification by cytochrome P-450. Pestic. Biochem. Physiol. **45**: 234-241.

Kimura, T. & Brown, A. W. A. (1964). DDT-dehydrochlorinase in *Aedes aegypti*. J. Econ. Entomol. **57**: 710-716.

Kiss, T. & Osipenko, O. (1991). Deltamethrin depress acetylcholine-activated currents in snail neurons. Pestic. Sci. **32**: 392-395.

Kotez, A. C. & Sales, N. (1994). Cross-resistance spectra and effects of synergists in insecticide-resistance strains of *Lucilia cuprina* (Diptera: Calliphoridae). Bull. Entomol. Res. **84**: 355-360.

Kumar, R. (1984). Insect pest control with special reference to African agriculture. Edward Arnold Ltd. London.

Kumar, K. & Chapman. R. B. (1984). Sub-lethal effects of insecticides on the diamond back moth *Plutella xylostella* (L.). Pestic. Sci. **15** :344-352.

Kumar, S., Thomas, A. & Pillai, M. K. K. (1991). Involvement of mono-oxygenases as a major mechanism of deltamethrin-resistance in larvae of three species of mosquitoes. Indian. J. Exper. Biol. **29**: 379-384.

Kunz, S. E. (1991). Dynamics of permethrin resistance in a colony of horn flies (Diptera: Muscidae). J. Med. Entomol. **28**: 63-66.

Kurtak, D., Meyer, R ., Ocran, M., Ouedraogo, M., Renaud, P., Sawadogo, R. O. & Tele, B. (1987). Management of insecticide resistance in control of *Simulium damnosum* complex in by the Onchocerciasis Control Programme, West Africa: Potential use of negative correlation between organophosphate resistance and pyrethroid susceptibility. Med. Vet. Entomol. **1**: 137-146.

Lacey, L. A. & Oldacre, S. L. (1983). The effect of temperature, larval age, and species of mosquito on the activity of an isolate of *Baccillus thuringiensis var darmstadiensis* toxic for mosquito larvae. Mosq. News. **43**: 176-180.

Ladonni, H. (1988). Genetics and biochemistry of insecticide resistance in *Anopheles stephensi*. Ph.D Thesis. University of Liverpool. pp. 221.

Leahey, J. P. (1985). The pyrethroid insecticides. Taylor & Francis Press. London & Philadelphia. pp. 440.

Lee, S. & Clark, J. M. (1993). Multiple forms of carboxylesterases from *Leptinotarsa decemelineata* haemolymph associated with permethrin resistance. Extended summaries-8th International Congress of Pesticide Chemistry. pp. 247-250.

Lemke, L. A., Koehler, P. G. & Patterson, R. S. (1989). Susceptibility of the cat flea

(Siphonaptera: Pulicidae) to pyrethroids. J. Econ. Entomol. **82**: 839-841.

Levot, G. W., Johnson. P. W., Hughes, P. B., Powis, K. J., Boray, J. C. & Dawson, K. L. (1995). Pyrethroid resistance in Australian field populations of the sheep louse, *Bovicola* (*Domalinia*) *ovis*. Med. Vet. Entomol. **9**: 59-65.

Lewis, J. B. & Sawicki, R. M. (1971). Characterization of resistance mechanisms to diazinon, parathion and diazoxon in the organophosphorus-resistant SKA strain of houseflies (*Musca domestica* L.). Pestic. Sci. **1**: 275-285.

Lin, L. B. (1991). Bednet treated with pyrethroids for malaria control. pp. 67-82. In : Malaria- waiting for the vaccine. Target, G. A. T. [ed]. John Wiley & Sons. Chichester. England.

Lindsay, S. W., Snow, R. W., Armstrong, J. R. M. & Greenwood, B. M. (1989). Permethrin-impregnated bednets reduce arthropods in Gambian houses. Med. Vet. Entomol. **3**: 377-383.

Lines, J. D., Myamba, J. & Curtis, C. F. (1987). Experimental hut trails of permethrin-impregnated mosquito nets and eave curtains against malaria vectors in Tanzania. Med. Vet. Entomol. **1**: 37-51.

Lines, J. D. (1988). Do agricultural insecticides select for insecticide resistance in mosquitoes? A look at the evidence. Parasitol. Today. **4**: S17-S20.

LIoyd, C. J. & Ruczkowski, G. E. (1980). The cross-resistance to pyrethrins and eight synthetic pyrethroids of an organophosphorus-resistant strain of the rust-red flour beetle *Tribolium castaneum* (Herbst). Pestic. Sci. **11**: 331-340.

Little, E. J., McCaffery, R. A., Walker. C. H. & Parker. T. (1989). Evidence for an enhanced metabolism of cypermethrin by a monooxygenase in a pyrethroid-resistant strain of the tobacco budworm (*Helothis virescens* F.). Pestic. Biochem. Physiol. **34**: 58-68.

Liu, M. Y., Tzeng, Y. J. & Sun, C. N. (1981). Diamond back moth resistance to several synthetic pyrethroids. J. Econ. Entomol. **74**: 393-396.

Lockwood, J. A., Story, R. N., Bayford, R. L., , Sparks, T. C. & Quisenberry, S. S. (1985). Behavioral resistance to the pyrethroids in the horn fly *Haematobia irritans* (Diptera: Muscidae). Environ. Entomol. **14**: 873-880.

Lockwood, J. A., Sparks, T. C. & Story, R. N. (1984). Evolution of insect resistance to insecticides: a reevaluation of the roles of physiology and behavior. Bull. Entomol. Soc. Am. **30**: 41-50.

Lutterell, R. C., Roush, R. T., Mink, A. A. J. S., Reid, M. R. & Soderlund, C. L. (1987). Pyrethroid resistance in field population of *Heliothis virescens* (Lepidoptera: Noctuidae) in Mississippi in 1986. J. Econ. Entomol. **80**: 985-989.

Luxemberger, C., Perea, W. A., Delmas, G., Prujua, C., Pecoul, B. & Moren, A. (1994). Permethrin-impregnated bednets for the prevention of malaria in schoolchildren on the Thai-Burmese border. Trans. R. Soc. Trop. Med. Hyg. **88**: 155-159.

Lyimo, E. O., Msuya, F. H. M., Rwegoshora, R. T., Nicholson, E. A., Mnzava, A. E. P., Lines, J. D. & Curtis, C. F. (1991). Trail of pyrethroid impregnated bednets in an area of Tanzania holoendemic for malaria. Part 3. Effects on the prevalence of malaria parasitemia and fever. Acta Trop. **49**: 157-163.

Maa, W. C. & Terrier, L. C. (1983). Age dependent variation in enzymic and electrophoretic properties of house fly (*Musca domestica*) carboxylesterases. Comp. Biochem. Physiol. **74**: 461-467.

Macdonald, W. W. (1972). The response of vectors and of nature to current eradication and control measures. pp. 41-53. In: Vector control and the recrudescence of vector-borne disease. Pan American Health Organization.

MacDonald, R. S., Surgeoner, C. A., Soloman, K. R. & Haris. C. R. (1983). Effect of four spray regimes on the development of permethrin and dichlorvos resistance in the laboratory by the house fly (Diptera: Muscidae). J. Econ. Entomol. **76**: 417-422.

Magnusson, J., Hallstrom, I. & Ramel, C. (1979). Studies on metabolic activities of vinyl chloride in *Drosophila melanogaster* after pretreatment with phenobarbital and polychlorinated biphenyls. Chem. Biol. Interact. **24**: 287-295.

Mahan, B. N. & Singh, N. N. (1965). Studies on selection and inheritance of insecticides resistance in *Anopheles stephensi*. Indian. J. Genet. **25**: 266-278.

Majori, G., Sabatinelli, G. & Coluzz, M. (1987). Efficacy of permethrin-impregnated curtains for malaria vector control. Med. Vet. Entomol. **1**: 185-192.

Malcolm, C. A. (1988a). Current status of pyrethroids resistance in Anophelines. Parasitol. Today. **4**: S13-S15.

Malcolm, C. A. (1988b). Reduced susceptibility to permethrin and its relationship to DDT resistance in larvae of *Anopheles stephensi*. Med. Vet. Entomol. **2**: 37-46.

Malcolm, C. A. & Wood, R. J. (1982). The establishment of a laboratory strain of *Aedes aegypti* homogenous for high resistance to permethrin. Pestic. Sci. **13**: 104-108.

Manouchehri, A. V., Djavadian, E., Eshghy, N. & Motabar, M. (1976). Ecology of *Anopheles stephensi* in southern Iran. Trop. Geogr. Med. **28**: 228-232.

Manouchehri, A. V., Shalli, A. K., Al-Saadi, S. H. & Al-Okailv, A. K. (1980). Status of resistance of *Anopheles* mosquitoes in Iraq. Mosq. News. **10**: 535-540.

Manouchehri, A. V., Djavadian, E. & Rouhani, F. (1976). Studies on the resistance of *Anopheles stephensi* to malathion in Bandar Abbas, Iran. Mosq. News. **36**: 320-322.

Manouchehri, A. V., Zaini, A. & Yazdanpanah, H. (1975). Selection for resistance to malathion in *Anopheles stephensi mysorensis*. Mosq. News. **35**: 278-280.

Manouchehri, A. V., Eshgy, N. & Rouhani, F. (1974). Malathion susceptibility test of *Anophelse stephensi mysorensis* in southern Iran. Mosq. News. **34**: 440-442.

Margalit, J. & Deam, D. (1985). The story of *Bacillus thuringiensis* var. *israelensis* (*B.t.i.*). J. Am. Mosq. Control Assoc. **1**: 1-7.

Martinez-Carrillo, J. L., Schouest, L. P. & Miller, T. A. (1991). Response of populations of the tobacco budworm (Lepidoptera: Noctuidae) from North West Mexico to pyrethroids. J. Econ. Entomol. **84**: 363-366.

Matsumura, F. & Voss, G. (1964). Mechanism of malathion and parathion resistance in the two-spotted spider mite, *Tetranychus urtica*. J. Econ. Entomol. **57**: 911-917.

Matsumura, F. & Brown, A. W. A. (1963). Studies on carboxylesterase in malathion-resistant *Culex tarsalis*. J. Econ. Entomol. **56**: 381-388.

Matsumura, F. (1975). Toxicology of insecticides. Plenum Press, New York. pp. 503.

Matsumura, F. (1986). Advances in understanding insecticide modes of action. pp. 151-159. In: pesticide science and biotechnology, Sixth IUPAC congress of pesticides chemistry.

Matsumura, F. (1985). Toxicity of insecticides. 2nd Edition. Plenum Press. New York. pp. 149.

Mattingly, P. F. (1962). Mosquito behaviour in relation to disease eradication programmes. Annu. Rev. Entomol. **7**: 416-436.

McCaffery, A. R., Holloway, J. W. & Gladwell, R. T. (1995). Nerve insensitivity resistance to cypermethrin in larvae of the tobacco budworm *Heliothis virescens* form USA cotton field population. Pestic. Sci, **44**: 237-247.

McDonald, A. E. & Wood, R. J. (1979a). Mechanism of DDT resistance in larvae of the mosquito *Aedes aegypti* L. Pestic. Sci. **10**: 375-382.

McDonald, A. E. & Wood, R. J. (1979b). Mechanism of DDT resistance in larvae of the mosquito *Aedes aegypti* L.; the effect of DDT selection. Pestic. Sci. **10**: 383-388.

Megesa, S. M., Aina, O. & Curtis, C. F. (1994). Detection of pyrethroid resistance in *Anopheles* mosquitoes. Bull. WHO. **72**: 737-740.

Metcalf, R. L. (1967). Mode of action of insecticide synergists. Annu. Rev. Entomol. **12**: 228-256.

Metcalf, R. L., Osman, M. F. & Fukuto, T. R. (1967). Metabolism of ^{14}C-labelled carbamate insecticides to $^{14}C\ O_2$ in the house fly. J. Econ. Entomol. **60**: 445-450.

Miller, T. A. (1988). Mechanism of resistance to pyrethroid insecticides. Parasitol. Today. **4**: S8-S12.

Miller, J. E. & Curtis, C. F. (1995). Testing net impregnating agents with mosquitoes released to fly freely in a room. Trans. R. Soc. Trop. Med. Hyg. **89**: 244-245

Miller, T. A., Kenedy, J. M. & Collins, C. (1979). CNS insensitivity to pyrethroids in the resistant *kdr* strain of house flies. Pestic. Biochem. Physiol. **12**: 224-229.

Miller, J. E. & Gibson, G. (1994). Behavioural response of host-seeking mosquitoes (Diptera: Culicidae) to insecticide-impregnated bed netting: a new approach to insecticide bioassays. J. Med. Entomol. **31**: 114-122.

Miller, J. E., Lindsay, S. W., Armstrong Schellenberg, J. R. M. Adiamah, J., Jawara, M. & Curtis, C. F. (1995). Village trail of bednets impregnated with wash-resistant permethrin compared with other pyrethroid formulations. Med. Vet. Entomol. **9**: 43-49.

Miller, T. A. & Salgado, V. L. (1985). The mode of action of pyrethroids on insects. In: The pyrethroid insecticides. Leahey. J. P. [ed]. Taylor & Francis Publ, London & Philadelphia. pp. 440.

Mittal, P. K., Adak, T. & Sharma, V. P. (1993). Effect of temperature on toxicity of two bioinsecticide *Sphaericus* (*Baccillus sphaericus*) and bactoculicidae (*Baccillus*

thuringiensis) against larvae of four vector mosquitoes. Indian. J. Malariol. **30**: 37-41.

Mofidi, C. H. & Samini, B. (1960). Resistance of *Anopheles stephensi* to dieldrin in Iran. Inst. Parasitol. Malariol., Tehran. Iran, Publ. 650.

Mofidi, C. H. & Samini, B., Eshghy, N. & Ghiassedin, M. (1958). Further studies of anopheline susceptibility to insecticide in Iran. Results of Busvine and Nash method. Inst. Parasitol. Malariol., Tehran. Iran, Publ. 585.

Molderke, A. F. & Terriere, L. C. (1981). Cytochrome P-450 in insects. 3. Increase in substrate binding by microsomes from phenobarbital-induced house flies. Pestic. Biochem. Physiol. **16**: 222-230.

Morello, A. (1964). Role of DDT-hydroxylation in resistance. Nature (London). **203**: 785-786.

Motoyama, M. & Dauterman, W. C. (1972). *In vitro* metabolism of azinphosmethyl in susceptible and resistant houseflies. Pest. Biochem. Physiol. **2**: 113-122.

Mourya, D. T., Hemingway, J. & Leake, C. J. (1993). Changes in enzyme titres with age in four geographical strains of *Aedes aegypti* and their association with insecticide resistance. Med. Vet. Entomol. **7**: 11-16.

Msuya, F. H. M. & Curtis, C. F. (1991). Trail of pyrethroid impregnated bednets in an area of Tanzania holoendemic for malaria. Part 4. Effects on incidence of malaria infection. Acta Trop. **49**: 165-171.

Muirhead-Thomson, R. C. (1947). The effect of house spraying with pyrethrum and with DDT on *Anopheles gambiae* and *A.melas* in West Africa. Bull. Entomol. Res. **38**: 449-464.

Mullin, C. A. & Scott, J. G. (1992). Biomolecular basis for insecticide resistance classification and comparisons. pp. 1-13. In: Molecular mechanisms of insecticide resistance. Mullin, C. A. & Scott, J. G. [eds]. American Chemical Society.

Munson, S. C. (1953). Some effects of storage at different temperatures on the lipids of the American roach and on the resistance of this insect to heat. J. Econ. Entomol. **46**: 657-666.

Nakatsugawa, T. & Morelli, M. A. (1976). Insecticide biochemistry and physiology. pp. 61-114. In: Microsomal oxidation and insecticide metabolism. Wilkinson, C. F. [ed]. Plenum Press, New York.

Narahashi, T. (1971a). Mode of action of pyrethroids. Bull. WHO. **44**: 337-345.

Narahashi, T. (1971b). Effects of insecticides on excitable tissue. Adv. Insect Physiol. **8**: 1-93.

Narahashi, T. (1976). Nerve membrane as a target of pyrethroids. Pestic. Sci. **7**: 267-272.

Nassif, M. & Kamel, N. (1977). A field trail with permethrin against body lice, *Pediculus humanus* in Egypt in 1976. Pestic. Sci. **8**: 301-304.

Nebert, D. W., Eisen, H. J., Negishi, M., Lang, M. A. & Hjelmeland, L. M. (1981). Genetic mechanisms controlling the induction of polysubstrate monooxygenase (P-450) activities. Ann. Rev. Pharmacol. Toxicol. **21**: 431-462.

Nicholson, A. R., Chalmers, A. E., Hart, R. J. & Wilson, R. G. (1979). Pyrethroid action and degradation in the cattle tick (*Boophilus microplus*). pp. 289-295. In :Insect Neurobiology and Pesticide Action (Neurotox 79). Proceedings of a Society of Chemical Industry Symposium. University of York, England, 1979.

Nicholson, R. A. & Miller, T. A. (1985). Multifactorial resistance to *trans*-permethrin in field-collected strains of the tobacco budworm *Helothis virescens* F. Pestic. Sci. **16**: 561-570.

Nicholson, R. A., Hart, R. J. & Osborne, M. P. (1979a). Mechanism involved in the development of resistance to pyrethroids with particular reference to knock-down resistance in house flies. Insect Neurobiology and Pesticide Action (Neurotox 79). Society of Chemical Industry. London. pp. 466-471.

Nicholson, R. A., Hart, R. J. & Osborne, M. P. (1979b). Insect Neurobiology and Pesticide Action (Neurotox 79). Society of Chemical Industry. London. pp. 466-471.

Nicholson, R. A. & Sawicki, R. M. (1982). Genetic and biochemical studies of resistance to permethrin in a pyrethroid-resistant strain of the house fly (*Musca domestica* L.). Pestic. Sci. **13**: 357-366.

Nielsen-LeRoux, C. & Charles, J. F. (1992). Binding of *Bacillus thuringiensis* binary toxin to a specific receptor on midgut brush-border membranes from mosquito larvae. Eur. J. Biochem. **210**: 585-590.

Nielsen-LeRoux, C., Charles, J. F., Thiery, I. & Georghiou, G. P. (1995). Resistance in a laboratory population of *Culex quinquefasciatus* (Diptera: Culicidae) to *Bacillus sphaericus* binary toxin is due to a change in the receptor on midgut

brush-border membranes. Eur. J. Biochem. **228**: 206-210.

Nisha, G. & Kalyanasundaram, M. (1992). Structure-activity relationship in pyrethroid esters derived from substituted 2-phenoxy-3-methybutanoic acid. Pestic. Sci. **35**: 21-25.

Nishimura, K. & Narahashi, T. (1978). Structure-activity relationships of pyrethroids based on direct action on the nerve. Pestic. Biochem. Physiol. **8**: 53-68.

Njunwa, K. J., Lines, J. D., Megesa, S. M., Mnzava, A. E. P., Wilkes, T. J., Alilio, M., Kivumbi, K. & Curtis, C. F. (1991). Trail of pyrethroid impregnated bednets in an area of Tanzania holoendemic for malaria. Part 1. Operational methods and acceptability. Acta Trop. **49**: 87-96.

O'Brien. R. D. (1967). Insecticides action and metabolism. Academic Press. New York & London. pp. 332.

Omer, S. M., Georghiou, G. P. & Irving, S. N. (1980). DDT/pyrethroid resistance inter-relationship in *Anopheles stephensi*. Mosq. News. **40**: 200-209.

Omura, T. & Sato, R. (1964). The carbon monoxide-binding pigment of liver microsome. I. Evidence for its hemoprotein nature. J. Biol. Chem. **239**: 2370-2378.

Oppenoorth, F. J. & Welling, W. (1976). Insecticide Biochemistry and Physiology. pp. 507-551. In: Biochemistry and physiology of resistance. Wilkinson, C. F. [ed]. Plenum Press, New York.

Orchard, I. (1980). The action of insecticides on neurosecretory neurones in the stick insect, *Carausius morosus*. Pestic. Biochem. Physiol. **13**: 220-226.

Orchard, I. & Osborne, M. P. (1979). The effects of pyrethroids on the electrical activity of neurosecretory cells from the brain of *Rhodnius prolixus*. Pestic. Biochem. Physiol. **10**: 197-202.

Osborne, M. P. & Hart, R. J. (1979). Neurophysiological studies of the effects of permethrin upon pyrethroid resistant (*kdr*) and susceptible strains of dipteran larvae. Pestic. Sci. **10**: 407-413.

Osman, A. A., Watson, T. F. & Sivasupramaniam, S. (1991). Reversion of permethrin resistance in field strains and selection for azinphosmethyl and permethrin resistance in pink bollworm (Lepidoptera: Gelechiidae). J. Econ. Entomol. **84**: 353-357.

Ottea, J. A. & Plapp, F. W. (1981). Induction of glutathione S-transferase by phenobarbital in the house fly. Pestic. Biochem. Physiol. **15**: 10-13.

Ottea, J. A. & Plapp, F. W. (1984). Glutathione S-transferase in the house fly: biochemical and genetic changes associated with induction and insecticide resistance. Pestic. Biochem. Physiol. **22**: 203-208.

Payne, G. T. & Brown, T. M. (1984). EPN and S,S,S-tributyl phosphorotrithioate as synergists of methyl parathion in resistant tobacco budworm in South Carolina. J. Agr. Entomol. **2**: 85-92.

Pepper, D. R. & Osborne, M. P. (1993). Electrophysiological identification of site insensitive mechanisms in knock-down resistant strains (*kdr*, super-*kdr*) of the house fly larvae (*Musca domestica*). Pestic. Sci. **39**: 279-286.

Plapp, F. W. (1976). Biochemical genetics of insecticide resistance. Annu. Rev. Entomol. **21**: 179-197.

Plapp, F. W. (1981). Toxicity of synthetic pyrethroids to laboratory and field populations of the tobacco budworm in central Texas. J. Econ. Entomol. **74**: 207-209.

Plapp, F. W., Bigley, W. S., Chapman, A. & Eddy, G. W. (1963). Synergism of malathion against resistant houseflies and mosquitoes. J. Econ. Entomol. **56**: 643-649.

Plapp, F. W. & Casida, J. E. (1970). Induction by DDT and dieldrin of insecticide metabolism by house fly enzymes. J. Econ. Entomol. **63**: 1091-1092.

Plapp, F. W. & Hoyer, R. F. (1968a). Insecticide resistance in houseflies: decreased rate of absorption as the mechanism of action of a gene that acts as an intensifier of resistance. J. Econ. Entomol. **61**: 1298.

Plapp, F. W. & Hoyer, R. F. (1968b). Possible pleiotropism of a gene conferring resistance to DDT, DDT analogues and pyrehrins in the house fly and *Culex tarsalis*. J. Econ. Entomol. **61**: 761-765.

Pleass, R. J., Armstrong, J. R. M., Curtis, C. F., Jawara, M. & Lindsay, S. W. (1993). Comparison of permethrin treatments for bednets in The Gambia. Bull. Entomol. Res. **83**: 133-140.

Poirie, M., Raymond, M. & Pasteur, N. (1992). Identification of two distinct amplification of the esterases B locus in *Culex pipiens* (L.) mosquitoes from Mediterranean countries. Bioch Gen. **30**: 13-26.

Prabhakaran, S. K. & Kamble, S. T. (1994). Subcellular distribution and characterization of esterase isozymes from insecticide-resistant and susceptible strains of German cockroach (Dictyoptera: Blattellidae). J. Econ. Entomol. **87**: 541-545.

Prapanthadara , L., Hemingway, J. & Ketterman, A. J. (1995). DDT-resistance in *Anopheles gambiae* (Diptera: Culicidae) from Zanzibar, Tanzania, based on increased DDT-dehydrochlorinase activity of glutathione S-transferases. Bull. Entomol. Res. **85**: 267-274.

Prasittisuk, C. & Busvine, J. R. (1977). DDT-resistant mosquito strains with cross-resistance to pyrethroids. Pestic. Sci. **8**: 527-533.

Prasittisuk, C. & Curtis. C. F. (1982). Further study of DDT resistance in *Anopheles gambiae* Giles (Diptera: Culicidae) and a cage test of elimination of resistance from a population by male release. Bull. Entomol. Res. **72**: 335-344.

Priester, T. M. & Georghiou, G. P. (1978). Induction of high resistance to permethrin in *Culex quinquefasciatus*. J. Econ. Entomol. **71**: 197-200.

Priester, T. M. & Georghiou, G. P. (1979). Inheritance of resistance to permethrin in *Culex quinquefasciatus*. J. Econ. Entomol. **72**: 124-127.

Priester, T. M. & Georghiou. G. P. (1980a). Penetration of permethrin and knockdown in larvae of pyrethroid-resistant and susceptible strains of the southern house mosquitoes. J. Econ. Entomol. **73**: 165-167.

Priester, T. M. & Georghiou. G. P. (1980b). Cross-resistance spectrum in pyrethroid resistance *Culex quinquefasciatus*. Pestic. Sci. **11**: 617-624.

Priester, T. M., Georghiou, G. P., Hawley, M. K. & Pasternak, M. E. (1981). Toxicity of pyrethroids to organophosphate, carbamate and DDT resistant mosquitoes. Mosq. News. **41**: 143-150.

Rajvanshi, A. C., Wattal, B. L. M. & Joshi, G. C. (1982). Laboratory evaluation of two synthetic pyrethroids against larvae of five vector mosquitoes. J. Commun. Dis. **14**: 52-56.

Ramoska, W. A., McCollim, W. A. & Quinckenden, K. L. (1982). Field tests of two commercial formulations of *Bacillus thuringiensis* serotype H-14 against *Aedes* mosquito larvae in Montana pastureland. Mosq. News. **42**: 251-254.

Rao, D. R., Mani, T. R., Rajendram. R., Joseph, A. S., Gajanana, A. & Reuben, R. (1995). Development of a high level resistance to *Bacillus sphaericus* in a field population of *Culex quinquefasciatus* from Kochi, India. J. Am. Mosq. Control

Assoc. **11**: 1-5.

Rathor, M. S. & Toqir, A. (1980). Malathion resistance in *An.stephensi* in Lahore, Pakistan. Mosq. News. **40**: 526-531.

Rathor, H. R & Wood, R. J. (1981). *In vivo* and *in vitro* studies on DDT uptake and metabolism in susceptible and resistant strains of the mosquito *Aedes aegypti* L. Pestic. Sci. **12**: 255-264.

Ray, J. W. (1965). Pest Infestation Research. HMSO. London. pp. 59

Reisen, W. K. & Aslamkhan, M. (1978). Biting rhythms of some Pakistan mosquitoes (Diptera: Culicidae). Bull. Entomol. Res. **68**: 313-330.

Reisen, W. K. & Aslamkhan, M. (1979). A release-recapture experiment with the malaria vector, *Anopheles stephensi* Liston, with observations on dispersal, survivorship, population size, genotrophic rhythm and mating behaviour. Ann. Trop. Med. Parasitol. **73**: 251-269.

Reisen, W. K. & Boreham, P. F. L. (1979). Host selection patterns of some Pakistan mosquitoes. Am. J. Trop. Med. Hyg. **28**: 408-421.

Reisen, W. K. & Milby, M. M. (1986). Population dynamics of some Pakistan mosquitoes; changes in adult relative abundance over time and space. Ann. Trop. Med. Parasitol. **80**: 53-68.

Reisen, W. K., Siddiqui, T. F., Aslamkhan, M. & Malik, G. M. (1981). Larval interspecific associations and physio-chemical relationships of the ground-water breeding mosquitoes of Lahore. Pakistanian Journal of Scientific Research. **3**: 1-23.

Reiss, R. A. & James, A. A. (1993). A glutathione S-transferase gene of the vector mosquito, *Anopheles gambiae*. Pestic. Biochem. Physiol. **2**: 25-32.

Ribbands, C. R. (1947). The use of residual films of DDT and gammexane in malaria control. Bull. Entomol. Res. **37**: 567-592.

Richards, F. O-J. R., Zea-Flores, R., Sexton, J. D., Beach, R. F., Mount, D. L., Cordon-rosales. C., Gatica. M., Klein, R. E., Flores, R. Z. & Rosales, C. C. (1994). Effects of permethrin impregnated bed nets on malaria vectors of northern Guatemala. Bull. Pan. Am. Health Organ. **28**: 112-121.

Riddles, P. W., Davey, P. A. & Nolan, J. (1983). Carboxylesterases from *Boophilus microplus* hydrolyse *trans*-permethrin. Pestic. Biochem. Physiol. **20**: 133-140.

Riskallah, M. R., Abd-Elghafar, S. F., Abo-Elghar, M. R. & Nassar, M. E. (1983). Development of resistance and cross-resistance in fenvalerate and deltamethrin selected strains of *Spodoptera littoralis* (Boisd). Pestic. Sci. **14**: 508-512.

Roberts, D. R. & Andre, R. G. (1994). Insecticide resistance issue in vector-borne disease control. Am. J. Trop. Med. Hyg. **50**: 21-34.

Rodcharoen, J. & Mulla, M. S. (1994). Resistance development in *Culex quinquefasciatus* (Diptera: Culicidae) to the microbial agent *Bacillus sphaericus*. J. Econ. Entomol. **87**: 1133-1140.

Roeder, K. D. & Weiant, E. A. (1946). The site of action of DDT in the cockroach. Science. **130**: 304-306.

Rongsriyam, Y. & Busvine, J. R. (1975). Cross-resistance in DDT-resistance strains of various mosquitoes (Diptera: Culicidae). Bull. Entomol. Res. **65**: 459-471.

Ronis, M. J. J., Hodgson, E. & Deuterman, W. C. (1988). Characterization of multiple forms of cytochrome P-450 from an insecticide resistant strain of house fly (*Musca domestica*). Pestic. Biochem. Physiol. **32**: 74-90.

Rossignol, D. P. (1991). Analysis of pyrethroid binding by use of photoreactive analogue: possible role of GTP-binding proteins in pyrethroid activity. Pestic. Biochem. Physiol. **41**: 103-120.

Rossignol, D. P. (1988). Reduction in number of nerve membrane sodium channels in pyrethroid resistant house flies. Pestic. Biochem. Physiol. **32**: 146-152.

Rowland, M. (1991). Behaviour and fitness of HCH/dieldrin resistant and susceptible females *Anopheles gambiae* and *Anopheles stephensi* mosquitoes in the absence of insecticides. Med. Vet. Entomol. **5**: 193-206.

Rozendaal, J. A., Voorham, J., Van Hoof, J. P. M. & Oostburg, B. F. J. (1989). Efficacy of mosquito nets treated with permethrin in Surinam. Med. Vet. Entomol. **3**: 353-365.

Rozendaal, J. A. & Curtis, C. F. (1989). Recent research on impregnated mosquito nets. J. Am. Mosq. Control Assoc. **4**: 500-507.

Rutledge, L. C., Sofield, R. K. & Moussa, M. A. (1978). A bibliography of diethyl tolumide. Bull. Entomol. Soc. Am. **24**: 431-439.

Sahgal, A., Kumar, S. & Pillai, M. K. K. (1994). Microplate assay of elevated esterases activity in individual pyrethroid-resistant mosquitoes. J. Biosci. **19**: 193-199.

Saito, K., Motoyama, N. & Dauterman, W. C. (1991). Studies on the resistance to various insecticides of a house fly strain (Diptera: Muscidae) selected with azamethiphos. J. Econ. Entomol. **84**: 1635-1637.

Salgado, V. L., Irving, S. N. & Miller, T. A. (1983b). The importance of nerve terminal depolarization in pyrethroid poisoning of insects. Pestic. Biochem. Physiol. **20**: 169-182.

Salgado, V. L., Irving, S. N. & Miller, T. A. (1983a). Depolarization of motor nerve terminals by pyrethroids in susceptible and *kdr*-resistant houseflies. Pestic. Biochem. Physiol. **20**: 100-114.

Sawicki, R. M. (1985). Resistance to pyrethroid insecticides in arthropods. pp. 143-192. In: Progress in pesticide biochemistry and toxicology. Hutson, D. H. & Roberts, E. [eds]. Willey, New York.

Sawicki, R. M., Farnham, A. W., Denholm, I. & Church, V. J. (1986). Potentiation of super-*kdr* resistance to deltamethrin and other pyrethroids by an intensifier (factor 161) on autosome 2 in the house fly (*Musca domestica* L.). Pestic. Sci. **17**: 483-488.

Sawicki, R. M. (1978). Unusual response of DDT-resistance house flies to carbinol analogues of DDT. Nature (London). **275**: 443-444.

Scharf, M. E., Bennett, G. W., Reid, B. L. & Qui, C. (1995). Comparison of three insecticide resistance detection methods for the German cockroach (Dictyoptera: Blattellidae). J. Econ. Entomol. **88**: 536-542.

Schecter, M. S., Green, N. & Laforge, F. B. (1949). Constituents of pyrethrum flowers. XXIII. Cinerolone and the synthesis of related cylopenlenolones. J. Amer. Chem. Soc. **71** : 3165-3173.

Schmidt, C. D. & Robertson, J. L. (1986). Effects of treatment technique on response of horn flies (Diptera: Muscidae) to permethrin at the different temperature, J. Econ. Entomol. **79**: 684-687.

Schnitzerling, H. J. (1985). A simple binding mechanism accounts for the temperature-dependent toxicity of *cis*-permethrin to larvae of the cattle tick, *Boophilus microplus*. Pestic. Biochem. Physiol. **24**: 362-367.

Schnitzerling, H. J., Nolan, J. & Hughes, S. (1983). Toxicology and metabolism of some synthetic pyrethroids in larvae of susceptible and resistant strains of the cattle tick *Boophilus microplus* (Can.). Pestic. Sci. **14**: 64-72.

Scott, J. G. (1987). Effect of temperature on the toxicity of s-bioalethrin and cypermethrin to susceptible and *kdr*-resistant strains of *Blattella germanica* (L.) (Dictyoptera: Blattellidae). Bull. Entomol. Res. **77**: 431-435.

Scott, J. G. & Georghiou, G. P. (1984). Influence of temperature on knock-down, toxicity and resistance to pyrethroids in the house fly, *Musca domestica*. Pestic. Biochem. Physiol. **21**: 53-62.

Scott, J. C. & Georghiou, G. P. (1986a). Malathion-specific resistance in *Anopheles stephensi* from Pakistan. J. Am. Mosq. Control Assoc. **2**: 29-32.

Scott, J. G. & Georghiou, G. P. (1986b). Mechanisms responsible for high level of permethrin resistance in house fly. Pestic. Sci. **17**: 195-206.

Scott, J. G., Cochran, D. G. & Siefrried, B. D. (1990). Insecticide toxicity, synergism and resistance in the German cockroach (Dictyoptera: Blattellidae). J. Econ. Entomol. **85**: 1698-1703.

Scott, J. G. & Lee, S. S. T. (1993a). Purification and characterization of a cytochrome P-450 from insecticide-susceptible and resistant strains of housefly *Musca domestica* L., before and after phenobarbital exposure. Arch. Insect Biochem. Physiol. **24**: 1-19.

Scott, J. G. & Matsumura, F. (1982). Evidence for two types of toxic actions of pyrethroids on susceptible and DDT-resistant German cockroach. Pestic. Biochem. Physiol. **19**: 141-150.

Scott, J. G., & Lee, S. S. T. (1993b). Tissue distribution of microsomal cytochrome p-450 monooxygenases and their inducibility by phenobarbital in the insecticide resistant LPR strain of house fly, *Musca domestica* L. Insect Biochem. Mol. Biol. **23**: 729-738.

Seabrook, G. R, Duce, I. R, & Irving, S. N. (1988). Effects of pyrethroid cypermethrin on L-glutamate-induced changes in the input conductance of the ventrolateral muscles of the larvae house fly, *Musca domestica*. Pestic. Biochem. Physiol. **32**: 232-239.

Service, M. W. (1996). Medical Entomology for Students. Chapman & Hall publisher, pp. 278.

Shalaby, A. M. (1966). Observations on some response of *Anopheles culicifacies* to DDT in experimental huts in Gujarat Atate, India. Ann. Entomol. Soc. Am. **59**: 938-944.

Sharma, V. P. (1992). Environmental management in malaria control in India. pp. 49-66. In: Malaria waiting for the vaccine. Targett, G. A. T. [ed]. John Wiley & Sons publisher. England.

Sharma, S. N., Subbarao, S. K., Chondhury, D .S. & Pandey, K. C. (1993). Role of *An.culicifacies* and *An.stephensi* in malaria transmission in urban Delhi. Indian. J. Malariol. **30**: 155-168.

Sharma, V. P. & Yadav, R. S. (1995). Impregnatting mosquito net with cyfluthrin, study in the minig settlements of Orissa, India, to control malaria. Public Health, Bayer. 10-17.

Sheppard, D. C., & Joyce, J. A. (1992). High level of pyrethroid resistance in horn flies (Diptera: Muscidae) selected with cyhalothrin. J. Econ. Entomol. **85**: 1587-1593.

Sheppard, D. C. & Marchinodo, A. A. (1987). Toxicity of diazinon to pyrethroid-resistant and susceptible horn flies, *Haematobia irritans* (L.): laboratory studies and field trails. J. Agr. Entomol. **4**: 262-270.

Shidrawi, G. R. (1985). WHO document. VBC/ECV/EC/ 85, 8.

Shono, T., Oshawa, K. & Casida, J. E. (1979). Metabolism of *trans*-and *cis*-permethrin, *trans*- and *cis*-cypermethrin and decamethrin by microsomal enzymes. J. Agr. Food Chem. **27**: 316-325.

Shono, T., Unai, T. & Casida, J. E. (1978). Metabolism of permethrin isomers in the American cockroach, house fly adults and cabbage looper larvae. Pestic. Biochem. Physiol. **9**: 96-106.

Siegfried, B. D. & Young, L. J. (1993). Activity of detoxification enzymes in aquatic and terrestrial insects. Environ. Entomol. **22**: 958-964.

Silva-filha, M. H., Regis, L., Nielsen-LeRoux, C. & Charles, J. F. (1995). Low-level resistance to *Bacillus sphaericus* in a field-treated population of *Culex quinquefasciatus* (Diptera: Culicidae). J. Econ. Entomol. **88**: 525-530.

Singh, G. J. P. & Orchard, I. (1983). Action of bioresmethrin on the corpus cardiacum of *Locusta migratoria*. Pestic. Sci. **14**: 229-234.

Sivananthan, T. (1993). Pyrethroid resistance in the mosquito, *Anopheles stephensi* and the role of cytochrome P-450 enzymes. Ph.D Thesis. University of Liverpool. pp. 184.

Smallcombe, A. (1981). Some investigations into knock-down resistance in house flies

(*Musca domestica*). Ph.D Thesis. University of Birmingham. pp-141.

Smith, A., Webley, D. J. (1968). A veranadah-type hut for studying the house-frequenting habits of mosquitoes and for assesing insecticides. III. The effect of DDT on behaviour and mortality. Bull. Entomol. Res. **59**: 33-46.

Soderlund, D. M., & Bloomquist, J. R. (1990). Molecular mechanism of insecticide resistance. pp. 237-260. In: Pesticide resistance in arthropods. Roush, R. T. & Tabashnic, B. E. [eds]. Champan & Hall. London.

Soderlund, D. M., Bloomquist, J. R., Wong, F., Payne, L. L. & Knipple, D. C. (1989). Molecular Neurobilogy: implication for insecticide action and resistance. Pestic. Sci. **26**: 359-374.

Soderlund, D. M., & Casida, J. E. (1977). Effects of pyrethroid structure on rates of hydrolysis and oxidation by mouse liver microsomes enzymes. Pestic. Biochem. Physiol. **7**: 391-410.

Sorokin, N. N. & Zharov, A. A. (1992). Resistance and irritability to insecticides exhibited by *Culex pipiens* in Moscow. Meditsinskaya-Parazitologiya-i-Parazitaryne-Bolenzi. **3**: 35-38.

Snow, R. W., Jawara, M. & Curtis, C. F. (1987). Observation on *Anopheles gambiae* Giles s.l. (Diptera: Culicidae) during a trial of permethrin-treated bed nets in The Gambia. Bull. Entomol. Res. **77**: 279-286.

Sparks, T. C. & Byford, R. L. (1988). Pyrethroid-synergist mixtures: Toxicity, resistance, and field efficacy toward pyrethroid-resistant horn flies (Diptera: Muscidae). J. Econ. Entomol. **81**: 1567-1574.

Sparks, T. C., Byford, R. L., Craig, M. E., Crosby, B. L. & Mekenzi, C. (1990). Permethrin metabolism in pyrethroid-resistant adults of horn fly (Diptera: Muscidae). J. Econ. Entomol. **83** : 662-665.

Sparks, T. C., Lockwood, J. A., Byford, R. L., Graves, J. B. & Leonard, B. R. (1989). The role of behaviour in insecticide resistance. Pestic. Sci. **26**: 383-399.

Sparks, T. C., Pavloff, A. M., Rose, R. L. & Clower, D. F. (1983). Temperature-toxicity relationships of pyrethroids on *Heliothis virescens* (F.) (Lepidoptera: Noctuidae) and *Authonomus grandis grandis* Boheman (Coleoptera: Curculionidae). J. Econ. Entomol. **76**: 243-246.

Sparks, T. C., Shour, M. H. & Wellemyer, E. G. (1982). Temperature-toxicity relationships of pyrethroids on three Lepidopterans. J. Econ. Entomol. **75**:

643-646.

Stich, A. H. R., Maxwell. C. A., Haji, A. A., Haji, D. M., Machano, A. Y. Mussa, J. K., Matteelli, A., Haji, H. & Curtis, C. F. (1994). Insecticide-impregnated bed nets reduce malaria transmission in rural Zanzibar. Trans. R. Soc. Trop. Med. Hyg. **88**: 150-154.

Subramanyam, B. & Cutkomp, L. K. (1987). Effect of posttreatment temperature on the toxicity of five synthetic pyrethroids to *Tetranychus urtica* Koch (Acari: Tetranychidae). Exp. Appl. Acarol. **3**: 109-113.

Sun, Y. P. & Johnson, E. R. (1972). Quasi-synergism and penetration of insecticides. J. Econ. Entomol. **65**: 349-353.

Suzuki, T. & Miyamoto, J. (1978). Purification and properties of pyrethroid carboxylesterase in rat liver microsomes. Pestic. Biochem. Physiol. **8**: 186-198.

Taylor, C. E. (1986). Genetics and evaluation of resistance to insecticides. Biol. J. Linn. Soc. **27**: 103-112.

Terriere, L. C. (1984). Induction of detoxication enzymes in insects. Annu. Rev. Entomol. **29**: 71-88.

Terriere, L. C. & Yu, S. J. (1979). Cytochrome P-450 in insects. 2. Multiple forms in the flesh fly *Sarcophaga bullata* and the blow fly *Phormia regina*. Pestic. Biochem. Physiol. **12**: 294-256.

Terriere, L. C., Yu, S. J. & Hoyer, R. F. (1971). Induction of microsomal oxidase in F_1 hybrids of a high and low oxidase house fly strain. Science. **121**: 581-583.

Thavaselvam, D., Kumar, A. & Sumodan, P. K. (1993). Insecticide susceptibility status of *Anopheles stephensi*, *Culex quinquefasciatus*, and *Aedes aegypti* in Panaji, Goa. Indian. J. Malariol. **30**: 75-79.

Tortora, G. J. & Grahowski, S. R. (1993). Principles of anatomy and physiology. Harper College publishers. New York. pp. 999.

Toth, S. J. & Sparks, T. G. (1988). Influence of treatment technique on the temperature-toxicity relationships of *cis*- and *trans*-permethrin in the cabbage looper (Lepidoptera: Noctuidae). J. Econ. Entomol. **81**: 115-118.

Townson , H. (1969). Esterases isoenzymes of individual *Aedes aegypti*. Ann. Trop. Med. Parasitol. **63**: 413-418.

Townson, H. & Ladonni, H. (1996). A major gene conferring insecticide resistance in the larvae of malaria vector *Anopheles stephensi*. Bull. Entomol. Res. (in press).

Trapido, H. (1954). Recent experiments on possible resistance to DDT by *Anopheles labranchiae* Falleroni in the Geremaes Valley Sardina. Am. J. Trop. Med. Hyg. **3**: 658-676.

Van den Bercken, J. & Vijverberg, H. P. M. (1979). Voltage clamp studies on the effects of allethrin and DDT on the sodium channels ion from melinated nerve membrane. pp. 79-85. In: Insect Neurobiology and Pesticide Action (Neurotex 79 Symposium).

Verma, K. V. S. & Rahman, S. J. (1984). Comparative efficacy of synthetic pyrethroids, natural pyrethrins and DDT against mosquito larvae. J. Commun. Dis. **16**: 144-147.

Vincent, D. R., Moldenke, A. F., Farnsworth, D. E. & Terriere, L. C. (1985). Cytochrome P-450 in insects. 7. age dependency and phenobarbital induction of cytochrome P-450, P-450 reductase, and monooxygenase activities in the black blow fly (*Phormia regina*, Meigen). Pestic. Biochem. Physiol. **23**: 182-189.

Vinson, E. B. & Kearns, C. W. (1952). Temperature and the action of DDT on the American cockroach. J. Econ. Entomol. **45**: 484-490.

Vulule, J. M., Beach, R. F., Atieli, F. K., Roberts. J. M., Mount, D. L. & Mwangi, R. W. (1994). Reduced susceptibility of *Anopheles gambiae* to permethrin associated with the use of permethrin-impregnated bednets and curtains in Kenya. Med. Vet. Entomol. **8**: 71-75.

Vulule, J. M., Beach, R. F., Atieli, F. K., Roberts. J. M., Mount, D. L. & Mwangi, R. W. (1996). Long-term use of permethrin-impregnated nets does not increase *Anopheles gambiae* permethrin tolerance. Med. Vet. Entomol. **10**: 71-79.

Wadleigh, R. W., Koehlier, P. G., Preisler, H. K., Patterson, R. S. & Robertson, J. L. (1991). Effect of temperature on the toxicities of ten pyrethroids to German cockroach (Dictyoptera: Blattellidae). J. Econ. Entomol. **84**: 1433-1436.

Walker, C. R. & Terriere, L. C. (1970). Induction of microsomal oxidase by dieldrin in *Musca domestica*. Entomol. Exp. Appl. **13**: 260-264.

Watters, F. C., White, N. D. G. & Cote, D. (1983). Effect of temperature on toxicity and persistence of three pyrethroid insecticides applied to fir plywood for the control of red flour beetle (Coleoptera: Tenebrionidae). J. Econ. Entomol. **76**:

11-16.

Wheelock, G. D. & Scott, J. G. (1989a). Simultaneous purification of cytochrome P-450 and cytochrome *b5* from the house fly, *Musca domestica* L. Insect Biochem. **19**: 481-489.

Wheelock, G. D. & Scott, J. G. (1989b). Purification and initial characterization of the major cytochrome P-450 from a pyrethroid resistant house fly. In: Molecular Insect Science. Hagedorn, H. H., Hildebrand, J. G., Kidwell, M. G. & Law, J. H. [eds]. Plenum, New York.

Wheelock, G. D. & Scott, J. G. (1992). The role of cytochrome $P450_{lpr}$ in deltamethrin metabolism by pyrethroid-resistant and susceptible strains of house flies. Pestic. Biochem. Physiol. **43**: 67-77.

Whitehead, G. B. (1959). Pyrethrum resistance conferred by resistance to DDT in the blue tick. Nature. **184**: 378-379.

WHO. (1957). Insecticides. 7th report of the expert committee on insecticides. WHO. Tech. Rep. Ser. **125**.

WHO. (1960). Insecticide resistance and vector control. 10th report of expert committee on insecticides. WHO. Tech. Rep. Ser. **191**.

WHO. (1963). Insecticide resistance and vector control. 13th report of the expert committee on insecticides. WHO. Tech. Rep. Ser. **265**.

WHO. (1970). Insecticide resistance and vector control. 17th report of the export committee on insecticides. WHO. Tech. Rep. Ser. **265**.

WHO. (1980). Resistance of vectors of disease to pesticides: 5th report of expert committee on vector biology and control. WHO. Tech. Rep. Ser. **655**.

WHO. (1984). Prevention, diagnosis and treatment of insecticide poisoning. WHO/VBC/84. 889.

WHO. (1985). Treating mosquito nets for better protection from bites and mosquito-borne disease. WHO/VBC/85.914.

WHO. (1992a). Vector resistance to pesticides. 15th report of the export committee on vector biology and control. WHO. Tech. Rep. Ser. **818**.

WHO. (1992b). The ministerial conference on malaria meeting in Amsterdam. World reclamation on control of malaria, WHO/CTD/MCM. **6**: 9-120.

WHO. (1994). Use of DDT in vector control. Med. Vet. Entomol. **8** :113.

WHO. (1995). Vector control for malaria and other mosquito-born disease. Tech. Rep. Ser. **857**.

Whyard, S., Downe, A. E. R. & Walker, V. K. (1994). Isolation of an esterase conferring insecticide resistance in the mosquito *Culex tarsalis*. Insect Biochem. Mol. Biol. **24**: 819-827.

Wilkinson, C. F. & Brattsten, L. B. (1972). Microsomal drug metabolising enzymes in insects. Drug metabolism review. **1**: 153-227.

Wilkinson, P. R. (1951). Distribution and fate of *Anopheles gambiae* and *An.funestus* in two different types of huts treated with DDT and BHC in Uganda. Bull. Entomol. Res. **42**; 45-54.

Williams, R. T. & Millburn, P. (1975). Detoxication mechanisms, the biochemistry of foreign compounds. pp. 211-226. In: Physiological and Pharmacological Biochemistry. Blaschko, H. K. F. [ed]. Series 1, Vol 12 . University Park Press, Baltimore, Maryland.

Wilson, T. G. & Hodgson, E. (1972). Mechanism of microsomal mixed-function oxidase inhibitor from the house fly *Musca domestica* L. Pestic. Biochem. Physiol. **2**: 64-71.

Wood, R. J. & Bishop, R. J. (1981). Insecticide resistance: populations and evolution. In: Genetic of consequences of man made change. Bishop, J. A. & Cook, L. M. [eds]. Academic Press. pp. 400.

Worthing, C. R. & Walker, S. B. (1987). The pesticide manual, 8th edition. The British Plant Protection Council publisher. pp. 1801.

Wu. N., Liexien, Q., Gouhou, L., Weimin, Z., Yuming, S., Yi, T. & Kuanren, Z. (1993). Field evaluation of bednets impregnated with deltamethrin for malaria control. Southeast Asian. J. Trop. Med. Public Health. **24**: 664-671.

Xu, G. & Brindley, W. A. (1994). Esterase isozymes in *Lygus hesperus*: Characterization and relationship with organophosphate resistance. Pestic. Sci. **42**: 273-280.

Yang, R. S. H., Hodgson, E. & Dauterman, W. C. (1971). Metabolism *in vitro* of diazinon in susceptible and resistant houseflies. J. Agr. Food Chem. **19**: 14.

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

Yeager, J. F. & Munson, S. C. (1945b). Site of action of DDT in Periplaneta. Science. **102**: 305.

Yoke, O. P. & Suddroddin, K. I. (1975). Toxicological studies of four insecticides against *Musca domestica* L. Southeast Asian J. Trop. Med. Public Health. **6**: 525-531.

Yu, S. J. (1982). Host plant induction of glutathione S-transferase in the fall armyworm, *Spodoptera frugiperda* (J.E. Smith), Pestic. Biochem. Physiol. **18**: 101-106.

Yu, S. J. (1990). Liquid chromatographic determination of permethrin esterase activity in six phytophagous and entomophagous insects. Pestic. Biochem. Physiol. **36**: 237-241.

Yu, S. J. & Ing, R. T. (1984). Microsomal biphenyl hydroxylase of fall armyworm larvae and its induction by allelochemicals and host plants. Comp. Biochem. Physiol. **78**: 145-152.

Yu, S. J. & Terriere, L. C. (1973). Phenobarbital induction of detoxifying enzymes in resistant and susceptible house flies. Pestic. Biochem. Physiol. **3**: 141-148.

Yu, S. J., Berry, R. E. & Terrier, L. C. (1979). Host plant stimulation of detoxifying enzymes in a phytophagous insect. Pestic. Biochem. Physiol. **12**: 280-284.

Yu, S. J. & Hsu, E. L. (1985). Induction of hydrolyses by allelochemicals and host plants in fall armyworm (Lepidoptera: Nuctoidae) larvae. Environ. Entomol. **14**: 512- 515.

Zaim, M. (1987). Malaria control in Iran: Present and future. J. Am. Mosq. Control Assoc. **3**: 392-396.

Zebra, E. (1988). Insecticide activity of pyrethroids on insects of medical importance. Parasitol. Today. **4**: S3-S7.

Zhai, J. & Robinson, W. H. (1996). Instability of cypermethrin resistance in a field population of the German cockroach (Orthoptera: Blattellidae). J. Econ. Entomol. **89**: 332-336.