GENETICS AND BIOCHEMISTRY OF INSECTICIDE RESISTANCE IN ANOPHELINES.

by

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ABSTRACT

Eight populations of Anopheline mosquitoes showing resistance to one or more insecticide were investigated. *Anopheles albimanus* , with a Y-linked translocation of a resistance gene, showed broad spectrum organochlorine, organophosphate and carbamate resistance in the males, but only dieldrin resistance in the females. Synergist studies suggest that the translocated gene codes for an altered acetylcholinesterase as the basis of resistance. In *An. arabiensis* from Sudan synergist studies indicate a kdr-like gene producing DDT and permethrin resistance and an additional DDT-dehydrochlorinase mechanism. Malathion and phenthoate resistance in this species are inherited as a single gene, which appears to produce a qualitative change in a carboxylesterase enzyme. *An. atroparvus* from England was DDT resistant due to a DDT-dehydrochlorinase mechanism. *An. atroparvus* from Spain with broad spectrum organophosphate and carbamate resistance, all dependent on a single gene or several closely linked genes, possessed an altered acetylcholinesterase which always segregated with resistance. There was also an oxidase mediated propoxur detoxication mechanism in this population. Three *An. stephensi* populations from Iraq, Iran and Pakistan were resistant to malathion and phenthoate. A carboxylesterase mediated resistance mechanism is indicated in all three populations from synergist studies. In the Pakistani *An. stephensi* enzyme assays indicate a qualitative change in a carboxylesterase enzyme rather than a quantitative change. Metabolite production studies supported the supposition of a carboxylesterase mediated resistance mechanism. Fenitrothion resistance was found in *An. stephensi* from Iraq, it was not connected with the malathion resistance mechanism. *An. gambiae* from Nigeria showed DDT and permethrin resistance. DDT-dehydrochlorinase and a kdr-like mechanism are indicated as the cause of resistance on the basis of synergist studies.

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INTRODUCTION.

DDT was introduced commercially in 1946 and was greeted with great optimism. It was expected to completely eradicate insect pests. In 1955 the World Health Organisation (WHO) assembly proposed the global eradication of malaria using residual spraying at 1gm per m^2 of all human dwellings where malaria transmission occurred. However the insecticide euphoria was soon to end and in 1976 the 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.

The WHO expert committee on insecticides in 1975 reported that approximatly 256 million people in the American, Eastern mediterranean, European and South-east Asian regions were living in areas where vector resistance was causing problems in combating malaria. This estimate omitted the African region where DDT-resistance in *An. gambiae* has been noted.

Thus increasing resistance problems 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.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 and probable development of resistance mechanisms. This project attempts to determine the scope of resistance and the mechanisms underlying the resistances in a number of Anopheline populations with this view in mind.

LITERATURE REVIEW.

INTRODUCTION.

As resistance to organochlorine compounds has become more widespread, organophosphate, carbamate and pyrethroid insecticides have become more widely used against arthropod pests. However it is now clear that insects can also develop resistance to these compounds. The latest World Health Organisation document on resistance (WHO 1980) lists 51 species of Anophelines resistant to one or more insecticides.

HISTORY OF INSECTICIDE USE AGAINST THE COLONIES STUDIED.

Anopheles albimanus. The widespread use of a variety of insecticides against agricultural pests appears to have produced multiple resistance in *An.albimanus* (Georghiou 1974). Abnormally low mortalities on the discriminating dosages of malathion and propoxur were observed as early as 1969 in the central part of El Salvador. Laboratory selections in 1971 confirmed the presence of organophosphate (OP) and carbamate resistance genes in this population (Ariaratnam & Georghiou 1971). By 1974 OP and carbamate resistance had spread throughout most of the pacific coastal area of El Salvador and to Guatemala, Honduras and Nicaragua.

Anopheles arabiensis. Malathion was first used against *An. arabiensis* in a cotton growing area of the Gezira irrigated area of Sudan in 1975,and after four rounds of house spraying, survivors from adult exposures to 5% malathion for one hour were recorded in Barakat Falata 5km south of the Wad Medini. By the end of 1978 resistance could be detected in a 200km^2

area around this village.

Anopheles atroparvus. Organophosphate and carbamate resistance appeared in 1978 in a population of *An.atroparvus* from Cadiz, Spain. Propoxur has had only limited use in agriculture in this area, being sprayed as part of an insecticide mixture against maize crops. There has however been extensive use of propoxur in houses and stables over the last 14-15 years. Sevin, generally applied from the ground, has had widespread use against agricultural pests in the area (De Zulueta, personal communication).

Anopheles gambiae. Although we have no records of insecticide pressurization of *An.gambiae* in Iworo, Nigeria it is probable that DDT spraying has occurred in this area, as part of the state malaria control programme.

Anopheles stephensi. Manoucheri *et al* (1975) recorded the first evidence of malathion resistance in *An.stephensi* at Mamasani, Kazerdin in Iran in an area where no malathion had ever been used for agricultural purposes. Houses had been sprayed 2-3 times a year since 1964. Malathion resistance was also detected in the Basrah area of Iraq in 1975 after six years of house spraying with malathion (Iyengar 1977).

Rathor & Toqir (1980) detected malathion resistance in *An.stephensi* from Lahore, Pakistan in September 1978. There appears to have been three rounds of house spraying with malathion in the area since 1976. The quantity of insecticides used for agriculture in the area is unknown, but is likely to be minimal.

There is no routine adulticide spraying in Pondicherry, India for the control of *An.stephensi*. There are however a wide range of pesticides used regularly in the paddy fields in and around the town (Das *et al* 1980).

GENETICS OF INSECTICIDE RESISTANCE.

Genetic investigations of resistance have demonstrated over 40 examples of monofactorial inheritance of resistance in different species, as well as many examples of polyfactorial inheritance. The use of recessive marker genes has enabled workers to assign various resistance genes to particular linkage groups. This is particularly advanced in *Musca domestica* for which numerous markers are now available. While other pest insects are not quite as well understood as the housefly, marker genes have been used to determine the linkage groups in *Aedes aegypti*, (Kimura & Brown 1964), *Culex pipiens* (Tadano & Brown 1967) and *Blatella germanica*, (Ross & Cochran 1966).

The evidence for dividing resistance into different types is now well documented. The following types can be distinguished:-

1) Single resistance - conferred by a single mechanism of resistance which usually protects against the selecting compound and chemically related compounds, to give a specific resistance spectrum. The nature of the spectrum depends on the biochemical resistance mechanism and should be independent of the agent used for the selection.

2) Multiple resistance - which occurs when two or more distinct mechanisms, each protecting against different

insecticides are present.

3) Multiplicate resistance - which occurs when two or more mechanisms co-exist in the same organism and protect against the same insecticide.

One useful consequence of genetic analysis is that it has been possible to isolate, and study separately, multiplicate resistance genes, and to show that such systems are multiplying rather than additive.

The genetic parameters influencing the selection of insecticide resistance have been classified by Georghiou & Taylor (1977) as follows:-

1) Frequency of resistance(R) alleles.

2) Number of R alleles.

3) Dominance of R alleles.

4) Penetrance, expressivity and interactions of R alleles.

5) Past selection by other insecticides.

6) Extent of integration of R genome with fitness factors.

When an R allele is fully dominant, the dose-response curve for the RS phenotype is identical to that of the RR. When the S allele is fully dominant, the RS phenotype resembles the SS. Dominance is incomplete when the curve for the heterozygote is intermediate between the two homozygotes. All these possibilities have been observed in resistant populations, though partial dominance is the most common, (Georghiou 1969).

Field populations with no history of exposure to

insecticides are usually susceptible to all insecticides, although one notable exception to this may be dieldrin resistance, which has been found at high frequencies in unselected populations. The best example of this, is that detailed by Service and Davidson (1964) where 45% of *An. gambiae* tested from an area near Kaduna, Nigeria were homozygous for the dieldrin resistance gene. This may suggest that the dieldrin-R gene has a function other than that of dieldrin detoxication in the wild population. However generally the presence of R genes at low frequencies suggests that they exist because of recurrent mutation and are maintained at their low frequency by a balance between mutation and selection pressure.

Dominance has an important influence on the eventual fate of the R gene. When an allele is rare it occurs almost exclusively in the heterozygous state. Therefore in the early generations following mutation, when a population is under insecticidal selection pressure, susceptibility of the heterozygote is the main determinant of the R gene frequency. The R gene frequency increases most rapidly when the resistance gene is completely recessive, more slowly when intermediate and slowest when completely dominant, but computer simulations predict that in no case will dominance be a decisive factor in retarding the evolution of the resistance gene. Population size is however dependent on the dominance of the R allele. The rate of population increase under selection pressure is slowest when the R allele is recessive. The effect of

dominance is less pronounced when the initial R gene frequncy is high (Macdonald 1959, Georghiou 1969,Plapp 1970, Brown 1971, Georghiou & Taylor 1977, Taylor & Georghiou 1979).

DDT RESISTANCE.

The genetics of DDT resistance in Anopheline mosquitoes has been studied in the following eight species; *An.sundaicus* (Davidson 1958),*An.quadrimaculatus* (Davidson 1963),*An.stephensi* (Davidson & Jackson 1961,Davidson 1966),*An.albimanus* (Davidson 1963),*An.pharoensis* (Davidson 1964,Kamel *et al* 1970, Hamed *et al* 1973), *An.pseudopunctipennis* (Kitzmiller 1976) and *An.gambiae* species A and B (Haridi 1970,1971,1972). Resistance in most of the species investigated was due to a single incompletely dominant gene. However even within a species resistance may be dominant in one population and recessive in another.Ancillary genes influencing DDT resistance may be present, and other resistance genes may influence the expression of the DDT-R gene.

Davidson & Sawyer (1975) showed that the gene for DDT resistance in *An.albimanus* from El Salvador was linked to dieldrin resistance and the mutant marker *stripe* and that propoxur resistance assorted independently. In *An.gambiae* (species A) with two autosomal markers belonging to different linkage groups, *diamond* on linkage group II and *collarless* on linkage group III, the DDT resistance gene assorted independently of *collarless* and was linked to *diamond*. DDT and dieldrin resistance were also linked in this population (Haridi 1974).

ORGANOPHOSPHATE RESISTANCE.

MALATHION.

Crosses between a malathion resistant population of *An. culicifacies* from India and a susceptible one from Sri Lanka produced an F_1 with intermediate resistance. The first, second and third backcrosses to the susceptible strain showed mortalities of less than 50% and there was significant departure from the 1:1 ratio expected on the single gene hypothesis. If two incompletely linked resistance genes are assumed, each giving full protection against the malathion treatment on its own, a good fit to the observed mortalities at successive backcrosses is obtained (Herath & Davidson 1981).

Crosses of a malathion resistant and a susceptible strain of *Culex tarsalis* from California produced an F_1 which was incompletely dominant for the resistance character. Further studies showed that resistance was due to a single gene which assorted independently of the marker *white eye* (Calman & Georghiou 1970). Malathion resistance in *Aedes aegypti* was however shown to be polygenic (Apperson & Georghiou 1975).

FENTHION.

Resistance to fenthion and other related OPs was shown to be polygenic in *C.tarsalis* (Apperson & Georghiou 1975). However resistance to fenthion in *C.quinquefasciatus* was considered to be due to a single major gene and modifiers. The major gene was located on linkage group II (Dorval & Brown 1970).

CARBAMATE RESISTANCE.

A propoxur selected population of *An.albimanus* from

El Salvador which showed cross resistance to fenitrothion and malathion was reciprocally crossed with a susceptible population ; the hybrids showed intermediate resistance. Results of repeated backcrosses indicated a single gene mechanism (Davidson & Sawyer 1975). Georghiou *et al* (1966) in a separate population of *An.albimanus* from El Salvador found that a single major gene was responsible for both the carbamate and OP resistances, with only minor effects from ancillary genes. This major gene was almost fully dominant, the heterozygotes being nearly as resistant as the RR homozygotes.

Resistance to carbamate insecticides in the housefly *Musca domestica* has sometimes been attributed to a single gene and sometimes to polygenic systems (Georghiou & Garber 1965). Evidence for the major influence of a gene on chromosome II has been reported, (Kasai & Ogita 1965, Plapp & Hoyer 1967, Tsukamoto *et al* 1968, Plapp 1970) which confers resistance to a wide range of carbamates.

PYRETHROID RESISTANCE.

Plapp & Hoyer(1968) isolated kdr-O (knockdown resistance Orlando), a major gene conferring DDT resistance from a housefly population. They showed that this gene, located on chromosome III, was also responsible for a low level of resistance to pyrethroids. They also demonstrated a kdr-O-like gene in *C.tarsalis* which again conferred low level pyrethroid resistance.

Cochran (1973) demonstrated a single incompletely dominant gene responsible for pyrethroid resistance in the German

cockroach linked to the mutant marker *pallid eye* on chromosome VI. His data also suggests the presence of a modifying factor.

Priester & Georghiou (1979) studied the inheritance of pyrethroid resistance in two strains of *C.quinquefasciatus*. On the basis of dose-response lines for parental strains, F_1 and backcross progeny they concluded that permethrin resistance in the two strains studied was inherited polyfactorially.

INSECTICIDE BIOCHEMISTRY.

a) THE MODE OF ACTION OF INSECTICIDES.

Insecticides appear to act on insects, mammals and other groups in one of two ways. OPs and carbamates act as inhibitors of the enzyme acetylcholinesterase, which hydrolyses acetylcholine, terminating its synaptic action, whereas DDT and the pyrethroids act on the nerve sheath causing recurrent discharges of impulses.

The insecticide must first pass through the chemically complex natural barrier of the integument and finally arrive in sufficient concentration, either in an unaltered form or as an active derivative, at a critical site of action. Surprisingly little is known about the route taken by an insecticide through the integument to the site of action. It has been assumed that it passes into the haemolymph in which it circulates to the central nervous system (CNS) and other body tissues. Le Roux & Morrison (1954) concluded from topical application of C^{14}DDT to houseflies that the haemolymph appears to transport, but not to accumulate the DDT as radioactivity from the haemolymph accounted for only 1% of

the total recovery. Lewis (1965) showed that DDT very rapidly saturated the epicuticular wax layers of adult blowflies but did not diffuse freely into the haemolymph.

Matsumura (1963) showed that topically applied malathion was absorbed into the cuticle of the adult *Periplaneta americana*. Burt & Lord (1968) also showed that the haemolymph would carry moderately large quantities of the water soluble compound diazoxon.

THE ACTION OF ORGANOPHOSPHATES AND CARBAMATES.

In mammals the 'blood-brain' barrier (Lajatha 1962) consists of a complex system of structural and metabolic features (ie.enzyme and transport systems) which prevent the movement of a variety of electrolytes, colloids etc. into the CNS. It is generally conceded that such a barrier does not exist in the invertebrates with the exception of the insects. In insects there are both structural and metabolic elements protecting the CNS which should be viewed as a barrier system. Movement of OPs and carbamates across this barrier is important as in insects cholinergic nerve junctions are confined to the CNS. This probably accounts for the relatively greater toxicity of ionic anticholinesterase agents to vertebrates than to insects.

CHOLINESTERASES. The enzymes hydrolysing choline esters belong to the group of serine enzymes that are inhibited by the alkaloid, eserine. The work of Mendel and his collegues in 1943 originally suggested that these enzymes were of two main types which they called 'true cholinesterases' and 'pseudocholinesterases', these correspond to the two entries in the

present enzyme list; acetylcholinesterase (EC.3.1.1.7) and cholinesterase (EC.3.1.1.8). As well as kinetic and other differences the two groups have different specificities, although both rapidly hydrolyse acetylcholine. Some authors claim that pseudocholinesterases have not been found in insects (O'Brien 1967) although there seems to be some confusion on this point. The acetylcholinesterase (AChE) enzyme is however the one with which we are concerned.

The existence of AChE in oligomeric forms is well known. Eldefrawi *et al* (1970) have demonstrated seven different oligomers of housefly AChE, four in the head and three in the thorax, using polyacrylamide electrophoresis. Nolan *et al* (1972) demonstrated that the cattle tick *Boophilus microplus* has a heterogeneous soluble AChE system of at least 5 forms. However the fact that one appeared only after chromatography and ultrafiltration techniques had been used suggests that it was an aggregate of at least two other forms. Edwards and Gomez (1966) found three forms of AChE in the cricket *Acheta domesticus*; Knowles & Arurkar (1969) also showed three forms in the head of the face fly, *Musca autumnalis*

It is possible that all these reported forms are not isozymes, but in reality the same enzyme forms bound to various protein fractions (Grafuis *et al* 1971, Brodbeck *et al* 1973), or aggregations of sub-units (McIntosh & Plummer 1973). The interconvertibility of the different forms and the ratios of their molecular weights supports this latter hypothesis. Levinson & Ellory (1974) showed by irradiation inactivation

that the AChE from electric eel and erythrocytes behaves as a monomer when membrane bound and that the higher molecular weight forms in solublized preparations were aggregations of this monomer. In Triton X-100 they found the enzyme to behave predominantly as a dimer. Devonshire (1975) demonstrated the interconvertibility of the enzyme forms in the housefly head , although he made no attempt to determine the molecular weights of the different forms.

THE MECHANISMS OF CARBAMATE AND ORGANOPHOSPHATE INHIBITION OF AChE. OP compounds react with acetylcholinesterase to form a relatively stable phosphorylated enzyme. This reaction is temperature dependent. The reaction can be represented as follows

$$EH + AB \underset{K_{-1}}{\overset{K_{+1}}{\rightleftharpoons}} EHAB \xrightarrow{K_{+2}} EA + BH$$

Where EH = free enzyme

AB = ester of an organophosphorus acid .

EA = phosphorylated enzyme.

BH = first leaving product.

In the early studies the inhibition of AChE was considered to be irreversible, but it has been demonstrated that some of the phosphorylated enzyme derivatives are unstable and the equation should be extended to:-

$$EH + AB \underset{K_{-1}}{\overset{K_{+1}}{\rightleftharpoons}} EHAB \xrightarrow{K_{+2}} EA + BH \xrightarrow{K_{+3}} EH + AOH$$

As can be seen from this equation there are two discrete steps in the inhibition of AChE, the first being the binding of the inhibitor to the surface of the enzyme, which is governed by the dissociation constant (Kd). When the constant is very small the affinity is very high. The second K_2 step involves the actual phosphorylation or carbamylation of the enzyme with covalent bond formation between the enzyme and the inhibitor, leading to the inhibited AChE form. The overall rate constant (K_1) relates to these as follows:-

$$K_1 = {}^{K_2}/_{Kd}$$

It is now possible to measure Kd and K_2 separately. They should be measured separately as changes in the insecticide molecule which improve the Kd step may have an adverse effect on the K_2 step. Therefore it is important that the insecticide designers know what factors will improve Kd and K_2.

THE BINDING SITE OF AChE. It was originally thought that there were only two important binding sites on the AChE enzyme: the esteratic site, which is acetylated by acetylcholine, carbamylated by carbamates and phosphorylated by OPs, and the anionic site which is involved in the binding of the quaternary nitrogen of acetylcholine onto the enzymes surface. However later experiments showed that the binding site of acetylcholine in a mutant AChE was unchanged or only slightly changed, whereas the binding sites of the OPs and carbamates were greatly altered. O'Brien & Tripathi (1977) have produced evidence that there are three additional binding sites. These are a hydrophobic site, an indophenyl site and a charge-

transfer complex site. These could be arranged around the catalytic esteratic site, and there is indirect evidence for the involvement of the charge-transfer complex binding site in the binding of aromatic carbamates and OPs.

THE ACTION OF DDT AND PYRETHROIDS.

DDT.

When recordings are made from the various nerves of the DDT intoxicated insect, multiple and recurrent discharges of impulses can be observed. An increase in negative after potential caused by DDT has been observed in cockroach nerve. However no additions of negative after potentials are seen during repetitive excitation. This excludes the possibility that augmentation of the negative after potentials by DDT is caused by an increase accumulation of K^+ ions outside the nerve membrane (Wilkinson 1976).

PYRETHROIDS.

Insects intoxicated with pyrethroids develop hyperexcitation and tremors which are followed by paralysis. These symptoms imply that pyrethroids act primarily on the neuromuscular system. Pyrethroid nerve excitation is not directly dependent on metabolic activity and therefore pyrethroids are assumed to act directly on the nerve membrane where excitation takes place (Narahasi 1971). Under *in vivo* conditions repetitive discharges are related to the rate of change of threshold ie.the accomodation process. Bergman showed that the levels of sodium inactivation and potassium activation determined the degree of repetitive firing in

myelinated nerves. Pyrethroids may affect the same conduction parameters. For example, allethrin caused an increase in the negative after potential of the nerve which eventually suppressed the action potential. Partial recovery of the nerve after potential occurred slowly *in vitro* after washing with allethrin free medium, but the recovery was never complete. The mechanisms of pyrethroid action have been studied by voltage clamp techniques. These showed that allethrin inhibits the Na^+ permeability increase, thus suppressing the after potential, and inhibits both the Na^+ inactivation and the K^+ activation, thereby augmenting the prolonged negative after potential (Narahasi 1971, Wilkinson 1976).

Adams & Miller (1979) showed a similar effect of tetramethrin on the housefly. Their experiments strongly suggest that muscle discharges are a direct result of the action of tetramethrin on the motor nerve endings. They also demonstrated repetitive back-firing of the nerve impulses, impulses travelling towards the CNS as well as postsynaptically to the flight muscles. Such results have also been reported in crayfish motor units (Welsh & Gordon 1947, Barstad 1962, Laskowski & Dettborn 1975).

It has generally been observed in mammals, cockroach giant nerve axons and other preparations that the nerve endings are more sensitive to pyrethroids than the general nerve sheath. This may be because the nerve endings are closer to the threshold for repetitive firing than the other regions of the nerve.

b) THE BIOCHEMISTRY OF INSECTICIDE RESISTANCE.

1). RESISTANCE DUE TO TARGET SITE INSENSITIVITY.

The first indications that resistance might be associated with changes in the AChE molecule was the observation of Smissaert (1964) that in a resistant strain of the spider mite *Tetranychus urticae*, the activity of AChE towards acetylcholine and acetylthiocholine in an R strain was only a third of that in the S strain. Studies on the rate of inhibition of the enzyme by paraoxon and diazoxon showed that the bimolecular rate constants for paraoxon were 10^5 and 10^2 $1/mole^{-1}/min^{-1}$ and for diazoxon $3x10^6$ and $2x10^4$ in S and R strains respectively, indicating that the correlation between resistance and the altered esterase was causal. Further altered AChEs in other mite populations have also been reported (Ballantyne & Harrison 1967, Takahashi *et al* 1973, Van Asperen 1960). An altered AChE is also a major resistance factor in the cattle tick, *Boophilus microplus*. As in the spider mite strain, the AChEs of several R strains of the adult tick exhibit reduced substrate activity and a reduction in their rate of inhibition by OP insecticides (Lee & Batham 1966). Separation of the more insecticide sensitive forms of the AChE enzyme showed that the R strain derived its resistance from a modification of one of the AChE forms, which even in the S strain was a relatively insensitive form. Nolan *et al* (1972) suggested that the modification of these insensitive enzymes leads to the lower AChE activity of the R strain. The modification does not appear to have altered the electrophoretic patterns or column profile of the AChE forms, hence it must represent only a minor change in

chemical or physical structure of the molecule.

A resistant strain of the green rice leafhopper, *Nephotettix cincticeps* was shown to have an altered AChE which is less susceptible to inhibition by carbamates (Hama & Iwata 1971, Iwata & Hama 1972). The ratio of I_{50} (50% inhibition)of the AChE of the R to that of the S strain is 115 for propoxur and 43 for carbaryl, obtained by preincubating homogenates with the carbamates and subsequently incubating with acetylcholine. Eldefrawi *et al* (1970) showed that the isozymes they had isolated from the housefly differed significantly in their sensitivity to inhibition by OPs *in vitro*. However the range of sensitivities was not large, the greatest variation being 2.3 fold in the case of malaoxon. Studies of a population containing a mutant AChE showed that all the head isozymes were insensitive to OP and carbamate inhibition. As it is very unlikely that a whole series of mutations have occurred it is probable that all the head 'isozymes' are under the control of a single gene.

A strain of housefly has also been reported that is resistant to Rabon (O,O-Dimethyl-(2-chlor-1-(2,4,5-trichlorphenyl)vinyl phosphate)) due to the presence of an altered AChE (Tripathi & O'Brien 1973). The enzyme showed a considerably decreased rate of inhibition which was found to be mainly due to a greatly reduced affinity (increased dissociation constant) of the enzyme-insecticide complex. A second strain resistant to dimethoate has been found by Devonshire & Sawicki (1974).

A strain of *An.albimanus* has been found with a mutant AChE which is very resistant to parathion and propoxur but not to fenthion, (Ayad & Georghiou 1975). Until recently this was the only reported case of an altered AChE in mosquitoes.

The level of resistance conferred by altered AChEs differs with different species. In ticks the difference in enzyme sensitivity to inhibition is 1,000 fold. Altered housefly AChE confers only slight resistance (less than 20 fold when isolated genetically); however it can interact with other resistance mechanisms resulting in a high level of resistance (Devonshire & Sawicki 1974, Devonshire 1975).

II)RESISTANCE DUE TO METABOLISM OF INSECTICIDES.

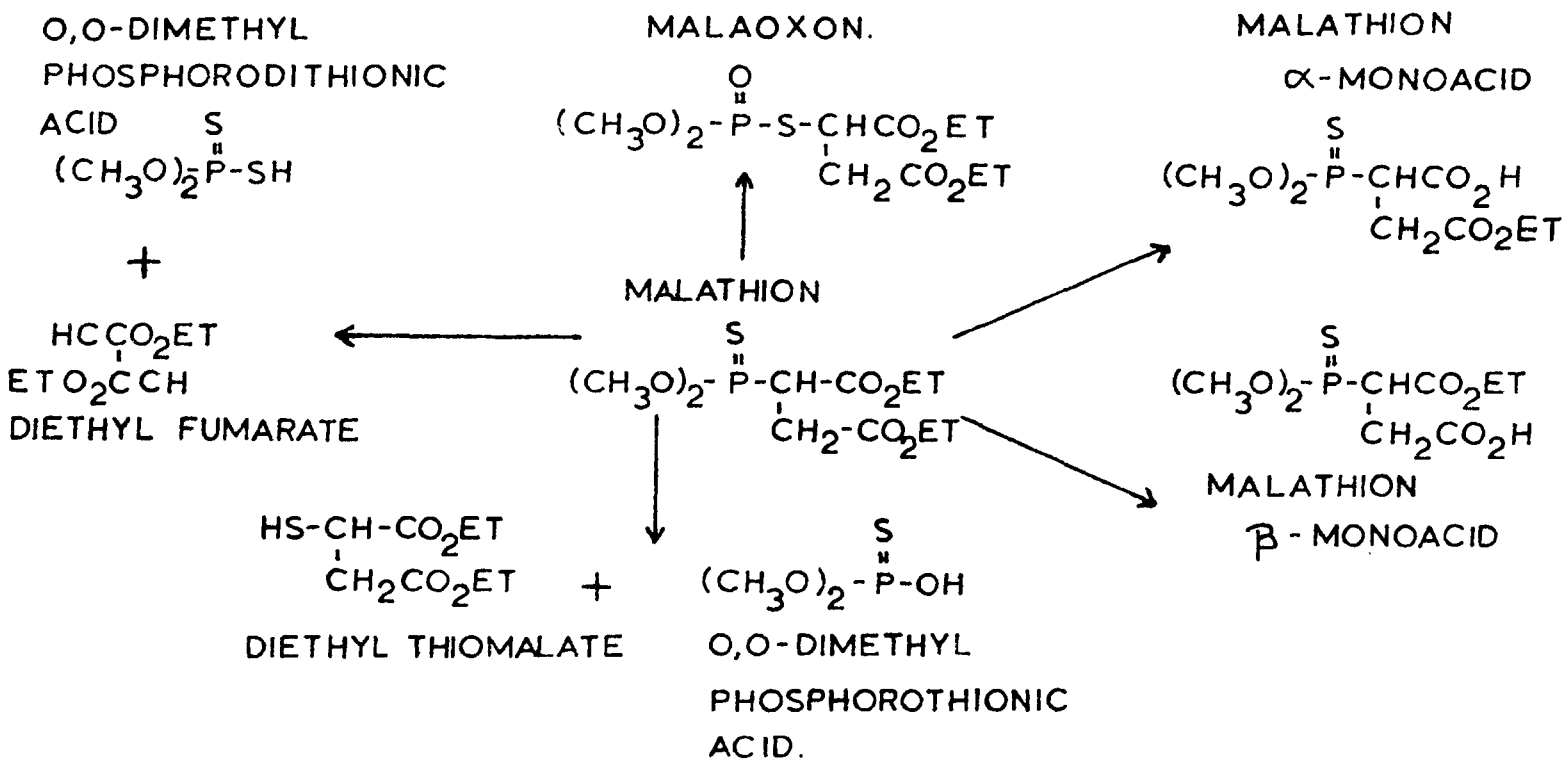
Biochemical research on various insect species has indicated that resistance can develop due to several metabolic pathways. Resistance may involve a quantitative or a qualitative change in one or more enzyme systems leading to an increased capacity for insecticide detoxication. These changes in the absence of selection pressure from insecticides may prove disadvantageous and some reversion of resistance may be expected when spraying is withdrawn.

ORGANOPHOSPHATE METABOLISM.

MALATHION METABOLISM.

Activation. Many of the useful organophosphates are phosphorothionates containing the P=S group. Because of the relatively lower electronegativity differences between P and S the phosphorus atom in such complexes is of low electrophilicity as compared to analogous P=O compounds, in which the

FIGURE 1. POTENTIAL CHEMICAL PATHWAYS FOR MALATHION DEGRADATION.



electronegativity difference is much greater. As a consequence P=S esters are 1,000x less reactive with cholinesterase and from 10-100x more stable to hydrolysis than the corresponding P=O compounds. Enzymatic conversion of P=S to P=O compounds is therefore an important step in activation. This was first demonstrated in mammals by Diggle and Gage (1951) and in *Periplaneta americana* by Metcalf and March (1953). More recent studies have shown that this 'desulphuration' reaction occurs via multi-function oxidases in mammalian liver and *P.americana* fat body and requires $NADPH_2$ and molecular oxygen and possibly magnesium ions (Metcalf 1967).

Detoxication. Most organophosphates are degraded to some extent by various hydrolases (Heath 1961, O'Brien 1960,1967). These enzymes may hydrolytically attack the phosphorus-ester bond or the anhydride bond, as well as an ester or amide bond in the leaving group. The disruption of any of these bonds to form a diester or a monocarboxylic acid is probably one of the most important mechanisms of inactivation or detoxication.

Carboxylesterases are important in the inactivation of OPs such as malathion, phenthoate, malaoxon and acethion.This detoxication reaction involves the hydrolysis of a carboxylester linkage resulting in a non-toxic ionic product, the mono-acid of malathion, (O'Brien 1960, Cook & Yip1958). In many insect species carboxylesterase activity is low or missing from susceptible populations. However it is present in certain resistant insects and it is reasonable to presume that

resistance to malathion is at least partly due to carboxylesterase activity in these populations, (Dauterman & Matsumura 1962, Matsumura & Brown 1963,Matsumura & Hogendijk 1964). Malaoxon is both a substrate and an inhibitor of carboxylesterase, (Main & Dauterman 1967).

Dyte & Rowlands (1968) suggested that malathion resistance in a population of *Tribolium castaneum* was due to enhanced detoxication by carboxylesterase on the basis of synergist work. Darrow & Plapp (1960) showed that at sub-lethal dosages larvae of malathion resistant and susceptible strains of *C.tarsalis* degraded malathion at a similar rate and manner, mainly through the formation of carboxylic acid derivatives. These studies were fairly crude as they were based on the water in which the larvae were treated rather than the larvae themselves as the P^{32}malathion they used was of low specific activity. Matsumura & Brown (1961) using enzymatic and radiometric techniques again employing P^{32} malathion, showed that resistant larvae degraded malathion and malaoxon twice as fast as susceptible larvae. By analysing whole larvae they found that the malathion resistant strain exhibited 3x more carboxylesterase activity than the susceptible strain. This difference was considered to be the main factor in the increased hydrolysis of malathion and malaoxnn.

With the use of labelled high specific activity malathion it was possible to quantitatively assess the production of individual metabolites from malathion. Using this method

Bigley & Plapp (1962) found that carboxylesterase activity was of primary importance in the degradation of malathion on the basis of metabolites found in the FRESNO strain of *C.tarsalis* larvae. They showed that the total water soluble radioactivity (representing malathion metabolites with the exception of malaoxon), present within the larvae, as well as excreted was higher in resistant larvae. Their *in vitro* results were comparable with the *in vivo* results. R larval homogenates degraded 2.3x more malathion than S larvae, and both S and R homogenates degraded malathion mainly by the formation of carboxylic acid derivatives. They concluded that the main difference between the S and the R strains was the greater production of metabolites by the R strain, formed by hydrolysis of the carbethoxy ester linkage.

Matsumura & Brown (1961) showed that in *C.tarsalis* interstrain differences in carboxylesterase activity were quantitative and not qualitative by plotting water soluble metabolite production against time to obtain a Michaelis constant (Km value = concentration of substrate giving half the maximal velocity). The Michaelis constant (0.11×10^{-4}M) was equal in both strains. They also indicated that the carboxylesterase increase and malathion resistance were causal, and not accidental, by crossing the resistant and susceptible strains and backcrossing the resultant F_1 to the susceptible for two generations. Each generation was then selected by exposure to 1ppm malathion for 24 hours, the susceptible homozygotes were eliminated and each backcross

larva was then assayed for carboxylesterase activity.

Matsumura & Hogendijk (1964) found a qualitative difference in the carboxylesterases of a housefly strain compared to the wild type susceptible strain. They demonstrated an additional carboxylesterase which was able to degrade malathion in this strain. Oppenoorth & Van Asperen (1960) considered the degradative enzymes in the resistant houseflies to be modified'aliesterases' (B-esterases according to the classification of Alderidge (1953)) because of low esterase activity levels in the OP resistant strain of housefly. Reduced 'aliesterase' activity has also been demonstrated in the CM strain of *Chrysomya putoria* (Townsend & Busvine 1969) and the Leverkusen-R strain of *Tetranychus urticae* (Smissaert 1964). There was no evidence of a change in B-esterase levels in R strains of *Nephotettix cincticeps*, *Laodelphax striatellus* & *C. fatigans*, (Kojima *et al* 1963, Ozaki 1969, Stone & Brown 1969). Needham & Sawicki (1971) found that resistance to OPs in *Myzus persicae* was associated with an increase in total carboxylesterase levels. Electrophoresis of several resistant populations showed that this increase was due to a single carboxylesterase enzyme (esterase 2). Beranek & Oppenoorth (1977) also found increased levels of esterase 2 in strains of *M. persicae*. They also looked for MFO and glutathion-S-transferase activity but were unable to find differences between resistant and susceptible strains. This may have been due to inhibition, (see discussion in Devonshire 1973).

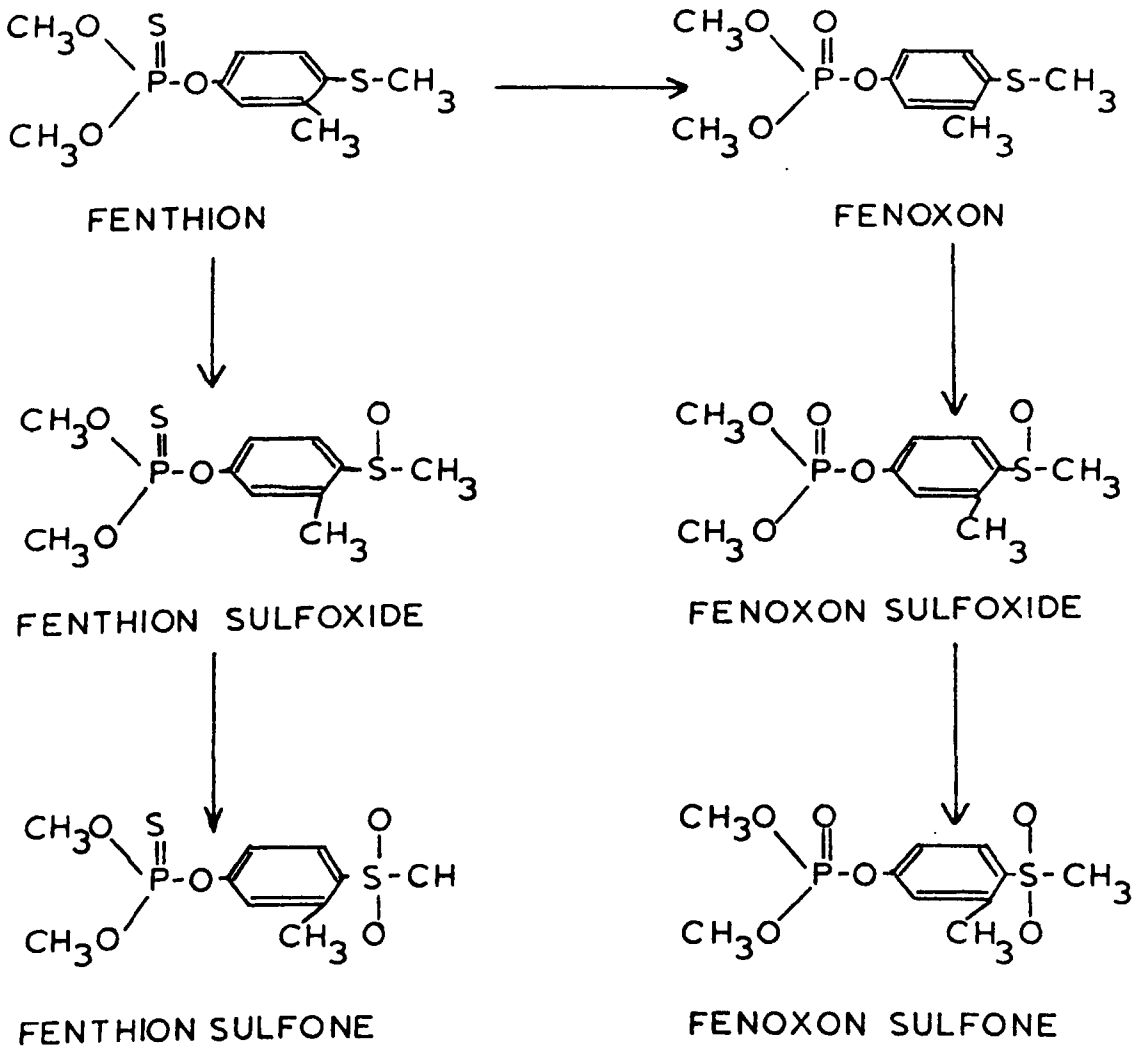
Matsumura & Voss (1964) showed that a strain of the two spotted spider mite had a superior ability to break down malathion and malaoxon *in vivo* as well as *in vitro*. In this case an increased phosphatase activity was observed in the resistant strain. However hydrolysis of malathion by carboxylesterase was still more important than the phosphatase mediated pathway. This was also true in the mosquito *C.tarsalis* The water soluble metabolites from the R larval homogenates contained a ratio of 11:1 carboxylesterase to phosphatase products, as compared to a 2:1 ratio in the S larvae (Bigley & Plapp 1962).

In the flour beetle, *Tribolium castaneum* a type of resistance to malathion has been found that is characterised by greater resistance to malaoxon than malathion (Dyte *et al* 1970). The R strain metabolizes malathion at approximately the same rate as the S strain, but in the R strain malaoxon was converted to desmethyl malaoxon. This resistance may therefore be due to increased oxidation.

FENTHION METABOLISM.

Brady & Arthur (1963) found that the German cockroach *Blattella germanica* metabolized 20% of applied fenthion to fenthion sulfone, and 40% to water soluble products within 4 hours. In the boll weevil 80% of the applied fenthion was metabolized to water soluble products within 4 hours. The main chloroform soluble metabolites are fenthion sulfoxide and fenoxon. In the NAIDM strain of *Musca domestica*, Metcalf *et al* (1963) found 75% hydrolysis of fenthion. Fenoxon was

FIGURE 2. OXIDATIVE METABOLITES OF FENTHION (after STONE 1969).



not detectable in the chloroform phase.

In the southern house mosquito, *C. fatigans*, Stone (1969) showed that fenoxon was detectable as the first metabolite in larvae exposed to a high dose (1.25ppm) of fenthion, but was not detectable in larvae exposed to a lower dosage (0.25ppm). At this dosage 35% of the fenthion was degraded after 1½ hours.

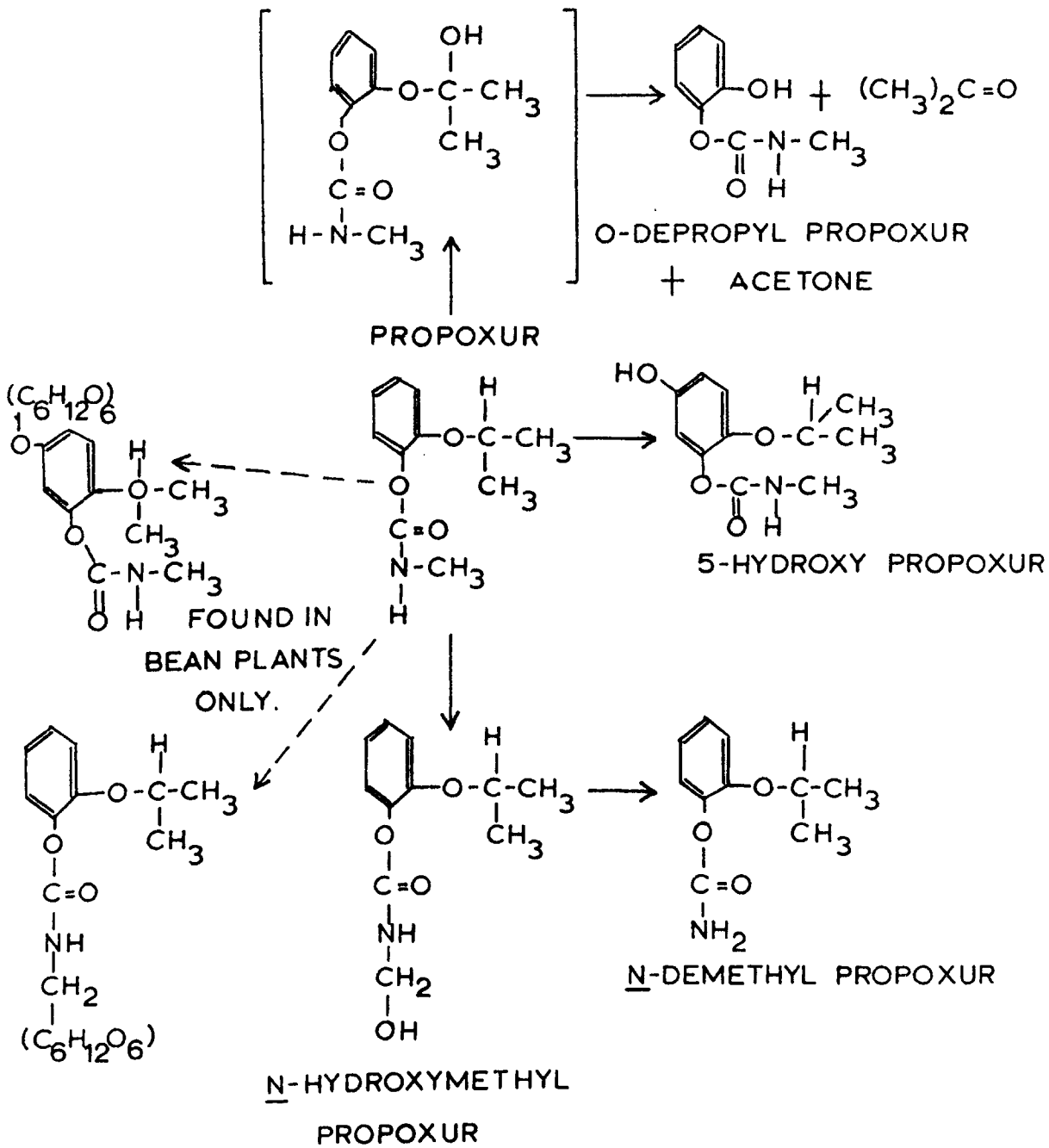
CARBAMATE METABOLISM.

Carbamates are absorbed and metabolized rapidly and completely in vertebrates. Commonly 70-90% of the dose is excreted within 24 hours in the form of various detoxication products. Mechanisms of metabolism of carbamates have been reviewed by Knaak (1971), Lykken & Casida (1969), Dorough (1970) & Kuhr (1970). Carbamate metabolism is commonly found to be very complicated. Oonithan & Casida (1968) reported at least 13 ether extractable metabolites of carbaryl formed by rat liver microsomes. Interpretation and comparison of pathways is difficult as many metabolites, particularly the conjugates are unidentified and others only tentatively identified. However the three basic processes, hydrolysis, oxidation and conjugation seem to form the basis of metabolism of carbamate insecticides in all the organisms studied.

PROPOXUR METABOLISM.

The majority of the propoxur breakdown reactions are catalysed by the microsomal-$NADPH_2$ dependent system and involve initial hydroxylation of the molecule at one of the three alternative sites. Shrivistava *et al* (1969) compared the metabolism of isopropyl-C^{14} labelled propoxur in eleven insect species *in vivo* using respirometry. The release of C^{14}-labelled

FIGURE 3. POSSIBLE METABOLIC PATHWAYS FOR PROPOXUR DETOXICATION.



volatile substances was taken , with caution, to relate to hydrolysis and ring O-dealkylation. They indicated that ester hydrolysis was of minor importance in propoxur metabolism in living houseflies or in fly enzyme preparations, as very little $C^{14}O_2$ was released from propoxur carbonyl-C^{14}, and because each major metabolite is recovered from propoxur-isopropyl-C^{14} and propoxur-N-methyl-C^{14} as well as from the carbonyl C^{14} with the exception of N-demethyl propoxur, from which the N-methyl-C^{14} label is removed and O-depropyl propoxur in which the isopropyl-C^{14} label is removed. The ratio of $C^{14}O_2$ liberation to acetone-C^{14} liberation from propoxur-carbonyl-C^{14} and propoxur-isopropyl-C^{14} preparations varies with insect species. A high ratio indicates that hydrolysis is predominant over O-depropylation. Hydrolysis was proportionately greater in the American cockroach, German cockroach, yellow mealworm and spruce budworm.

Metcalf *et al* (1967) found that both S and R houseflies metabolized the isopropoxy side chain of propoxur at a very rapid rate, producing from 28-44% $C^{14}O_2$ from the 1,3-C^{14} labelled isopropoxy group as compared to 2.2% in the dead control flies. This suggests that the major metabolic degradation of propoxur is O-dealkylation. There was however no significant difference in the total production of $C^{14}O_2$ from the various carbamates by the S-NAIDM, R-MIP and R-ronnel populations of houseflies. This suggests that ring hydroxylation is a more important avenue of enhanced detoxication in the carbamate resistant flies (Metcalf *et al* 1967).

Shrivistava *et al* (1969) demonstrated that the organosoluble metabolites of propoxur formed in living flies and in the housefly abdomen-$NADPH_2$ enzyme system are the same. In whole flies the major unconjugated propoxur metabolites in order of their decreasing amounts are; 5-hydroxy propoxur, N-hydroxymethyl propoxur or O-depropyl propoxur and N-demethyl propoxur. In the *in vitro* enzyme systems the order of decreasing amounts of identified metabolites is; 5-hydroxy propoxur, O-depropyl propoxur, N-demethyl propoxur and N-hydroxymethyl propoxur for the four susceptible strains and 5-hydroxy propoxur, O-depropyl propoxur, N-hydroxymethyl propoxur and N-demethyl propoxur for the two resistant strains.

The amount of water soluble metabolites of propoxur formed *in vitro* is very low as compared with *in vivo*.This is probably because co-factors are missing or present in insufficient quantities in the *in vitro* reaction. The authors reported that under *in vitro* conditions the amount of water-soluble radioactivity increased from 2-11% without bovine serum albumin (BSA) to 13-32% when the enzyme homogenate is prepared and incubated with BSA. The BSA in this case not only enhanced the enzyme activity but also reduced the variation in the degree of propoxur metabolism caused by several apparently identical enzyme preparations. However other workers have found that BSA can cause erratic and misleading results from *in vitro* carbamate metabolism studies (Kuhr 1975). Careful control experiments are therefore necessary where BSA is used.

PYRETHROID METABOLISM.

PERMETHRIN METABOLISM.

Very strong resistance to natural and synthetic pyrethroids developed twice in the late 1950s and again in the early 1970s in the housefly. It has been suggested that the trans-methyl group on the isobutyl side chain of chrysanthemic acid is a site for metabolic attack by multi-function oxidases (MFO) in the housefly. However this is unlikely in the case of permethrin which has a dichlorovinyl side chain. Resistance to pyrethroids tends to be complex involving a variety of interacting mechanisms in all cases studied to date (Sawicki 1975).

Cockroach adults, housefly adults and cabbage looper larvae metabolise the cis- and trans-permethrin isomers by a variety of pathways leading to 42 tentatively identified metabolites (see Fig 4). These pathways are initiated by hydrolysis or hydroxylation at the geminal dimethyl group or phenoxybenzyl moiety. The hydroxy esters or their ester cleavage products are further metabolized leading ultimately to a variety of conjugates. The products are identical in the three species with the following exceptions: the 6-HO derivatives are detected in houseflies only; the C-HO derivatives are found in cockroaches and houseflies but not in cabbage loopers. Hydroxylation at the 4'-position of the phenoxybenzyl group is the preferred site in all species studied. Hydroxylation at the 6-position in the alcohol moiety is restricted to houseflies and at the 2'-position to rats (Shono *et al* 1978).

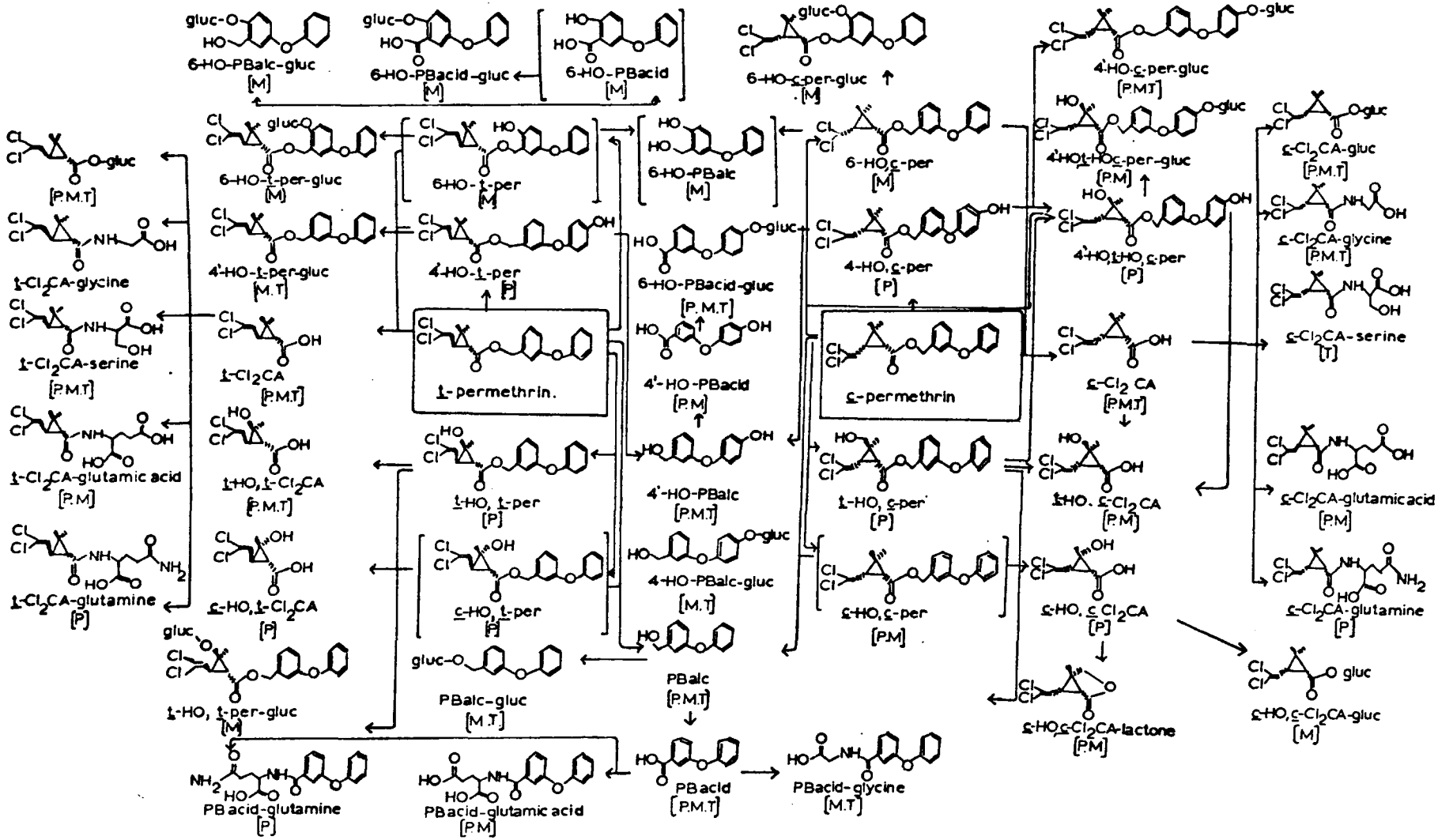


FIGURE 4. Metabolic pathways for the permethrin isomers (c- and t-per) detected with adult American cockroaches (P), adult houseflies (M) and cabbage looper larvae (T).

III) ENZYMES.

The Enzyme Commission in its report in 1961 devised a system for assigning code numbers to individual enzymes based on their classification, and these code numbers are widely used with the prefix EC. On their classification there are six main groups of enzymes of which members of three, the oxidoreductases (EC.1), transferases (EC.2) and hydrolases (EC.3) may be involved in insecticide metabolism.

ENZYMATIC INVOLVEMENT IN THE BREAKDOWN OF ORGANOPHOSPHATES.

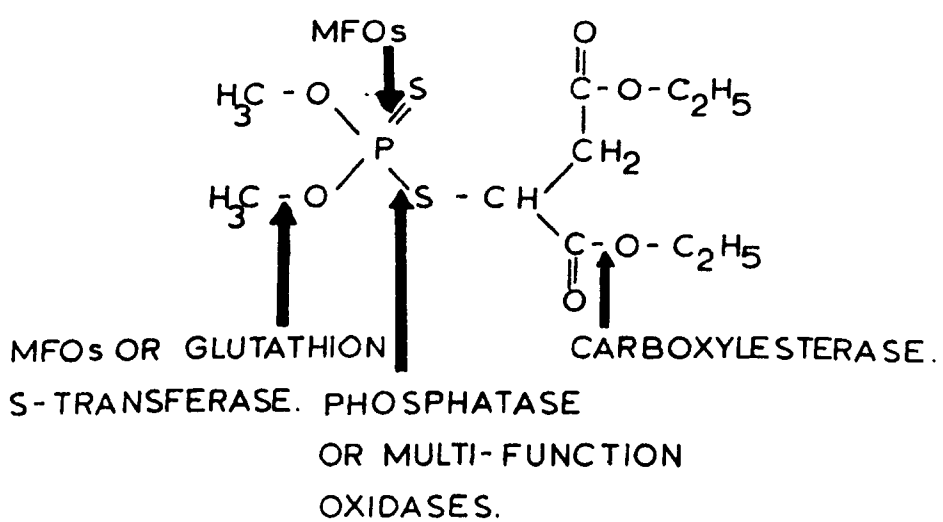
The enormous structural diversity of the organophosphate insecticides invites *in vivo* metabolism and detoxication by a variety of mechanisms and makes generalisation difficult. As all OP insecticides are esters of phosphoric or phosphonic acids, hydrolysis of the ester linkage is a ready avenue for metabolic breakdown. Degradative enzymes can act on malathion either by hydrolysing the phosphate ester bond or by hydrolysing the carboxylester bond. Care must be taken in inferring the nature of the detoxication mechanism since the activity of the hydrolases, glutathion dependent transferases and MFOs on a certain insecticide can often generate the same metabolic product, (Oppenoorth 1975).

HYDROLASES.

Hydrolases responsible for OP hydrolysis are present in many biological systems and are widely distributed in different organs and tissues, as well as being found in both soluble and sub-cellular fractions (Dauterman 1971). Hydrolytic mechanisms furnish the major pathways for the detoxication of the orthophosphate esters, and the comparatively strong acids

produced by any of the hydrolytic steps are poor cholinesterase inhibitors because of the strong negative charge introduced which greatly reduces the electrophilic character of the P atom and consequently its phosphorylating ability. Two major hydrolases are involved in OP resistance: phosphatases and carboxylesterases. The latter can only be important in OP compounds with a carboxylic ester such as malathion, malaoxon and phenthoate.

FIGURE 5 . POSSIBLE SITES OF ENZYMATIC ATTACK ON MALATHION.



CARBOXYLESTERASES (EC.3.I.I.I).

A number of enzymes in this group, particularly from mammalian liver, have been extensively purified and studied. They have been reviewed by Krisch (1971). Comparatively few detailed studies have been made of their specificity however. Changes in the acyl and alkyl parts of the substrate molecule affect enzyme specificity. Affinity tends to rise with an increase in chain length up to six carbon atoms.

The carboxylesterase enzymes hydrolyse the $-COOC_2H_5$ group in malathion. Matsumura & Brown (1961) established that a malathion resistant strain of *C.tarsalis* had greater carboxylesterase activity than the standard susceptible strain. The purified carboxylesterase had a molecular weight of 16,000 and its properties were identical in S and R larvae. The enzyme was concentrated about 13x in the R larvae and it was concluded that malathion resistance in *C.tarsalis* was associated with an allele of a gene which controlled synthesis of the enzyme (Matsumura and Brown 1963,1964).

In houseflies evidence of increased carboxylesterase activity was provided by experiments on cross resistance and synergism, (Matsumura & Hogendijk 1964). The situation is similar in mammals which are fairly tolerant to malathion due to the high malathion carboxylesterase activity of their tissues (Kreuger & O'Brien 1959, Seume & O'Brien 1960).

Welling & Blaakmeer (1971) found in both S and R strains of housefly that the carboxylesterase that attacked malathion was present in the soluble cell fraction. Also in

the R strain a particulate carboxylesterase was present with a 5x higher maximal activity than the soluble enzyme. *In vivo* the difference in activity may be greater than 5x, as the Km of the extra R enzyme is 10x lower ($3x10^{-6}$M) than that of the enzyme present in both strains.

The majority of carboxylesterase activity is found in the abdomen, particularly the gut in *Culex*. This tissue has also been implicated as the primary source of carboxylesterase in other insects (O'Brien 1967, Kuhr 1975).

PHOSPHATASES.

Both specific and non-specific phosphatases exist. Acid and alkaline phosphatases (EC.3.1.3.1. & 2) are like the carboxylesterases, a group of widely distributed enzymes. They act on a wide range of mono-esters of orthophosphoric acid.

The electrophilic phosphorus atom is thought to determine the susceptibility of OP compounds to phosphatase cleavage. Phosphatase activity could not be detected in S strains of housefly, but a low level of activity was found in homogenates of parathion and diazinon resistant strains, (Welling *et al* 1971, Lewis & Sawicki 1971). The enzyme studied by Welling *et al*(1971) had a Km for paraoxon of $4x10^{-9}$M. This is extremely low, and makes the phosphatase particularly suited to remove the last traces of inhibitor,and helps explain its relatively large contribution to resistance despite its low maximal detoxification capacity, the degradation rates being too low to explain the observed resistance levels.

In *C.tarsalis*, sodium fluoride which strongly inhibits phosphatase activity *in vivo* and *in vitro*, produces only slight synergism of malathion, indicating that in mosquitoes as well as houseflies phosphatases are not as important in malathion metabolism as carboxylesterases.

TRANSFERASES.

Hydrolysis of OP insecticides may also take place via glutathion transferase which is present in insects as a soluble enzyme system. In addition to dealkylation, glutathion transferase catalyses removal of the leaving group of OP insecticides.

As can be seen from figure 5 dialkyl phosphorothoic acid can be produced from malathion either oxidatively or by glutathion-S-alkyl transferase. Careful investigation is therefore required to determine which enzyme system is involved in metabolism, as the role of glutathion transferase in resistance is difficult to judge in the absence of inhibitors. The nature of the alkyl group in the OP molecule is important. Wilkinson (1979) has shown that glutathion-S-alkyl transferase is important in the metabolism of dimethyl phosphates and phosphorothiates such as fenitrothion. However diethyl compounds such as parathion and chlorfenvinphos with larger alkyl groups are more refractory to cleavage by glutathion-S-alkyl transferase (Hollingworth 1970) and dealkylation of these materials appears to result from $NADPH_2$-dependent microsomal oxidations.

OXIDOREDUCTASES.

Increased levels of oxidation in several resistant housefly strains have been reported eg. 6-16-fold for paraoxon and diazinon (El Bashir & Oppenoorth 1969), 4- and 6-fold for diazinon and diazoxon (Yang *et al* 1971) and approximately 9-fold for azinphosmethyl (Motoyama & Dauterman 1972). A thorough review of oxidase involvement in OP resistance was written by Wilkinson & Brattsten(1972).

Where subcellular fractionation has been performed oxidative activity has been associated with the microsomal fraction. The enzymes involved are mixed or multi-function oxidases which require $NADPH_2$ and oxygen, and are inhibited by methylenedioxyphenyl compounds and carbon monoxide.

A complex situation exists with multi-function oxidase (MFO) metabolism of phosphorothioates as MFOs are involved in both their activation and detoxication. A reduced level of activation is one theoretical mechanism for phosphorothioate resistance. In practice however in strains with an increased oxidative detoxication mechanism, activation is usually considerably increased. The situation is made more complex as small amounts of thiono-compounds appear to strongly inhibit the oxidative degradation of the oxon analogues, (Oppenoorth 1971). Resistance to thiono-compounds however differs only slightly from that of the oxon analogues in the majority of cases despite this inhibitory effect.

MFOs can also be involved in O-dealkylation yielding the same product as glutathion-S-alkyl transferase (see section on transferases for discussion).

ENZYMATIC INVOLVEMENT IN CARBAMATE DETOXICATION.

OXIDOREDUCTASES.

Synergist studies indicate the involvement of MFOs in carbamate detoxication (Casida 1963, Dorough *et al* 1963, Georghiou & Metcalf 1961, Metcalf & Fukuto 1965, Metcalf *et al* 1966). Mechanisms of metabolism of carbamates have been reviewed by Knaak (1971), Lykken & Casida (1969), Dorough (1970) and Kuhr (1970). The main difference in rates of carbamate metabolism between species are the relative contributions of hydrolysis, oxidation and conjugation, the sites of oxidative attack by MFO enzymes and the compounds used for conjugation.

Hydrolysis appears to be far more important in mammals than in insects. Oxidative attack can be at the ring, O-alkyl, N-methyl or ester groupings, with possible conjugation of products which have free hydroxyl groupings. The major conjugates in insects are often glucosides, whereas in mammals the glucuronides and sulfates are more common conjugates.

Casida (1969) investigated the effects of selection with carbamates on MFOs. He showed that the rates of many types of oxidations including epoxidations, hydroxylation and O- and N-demethylation were increased. Selection may therefore theoretically cause cross resistance to a large number of unrelated compounds.

N-DEALKYLATION. N-dealkylation was first characterised by Brodie *et al* (1958) as resulting from the attack of MFOs. The importance of N-dealkylation has been demonstrated

by Hodgson & Casida (1960) and by Dorough & Casida (1964). Dorough *et al* (1963) demonstrated N-dealkylation in the detoxication of carbaryl by the American cockroach, *Periplaneta americana* where 5-7% of 1-naphthyl-N-hydroxymethyl carbamate is formed, along with at least seven other metabolites, following injection of C^{14}-labelled carbaryl. Kuhr (1971) showed that hydroxymethyl carbamate was the major metabolite produced from carbaryl by cabbage looper larvae. This compound accounted for 70-80% of the total metabolites extracted from gut and fat body homogenates. *In vivo* studies appeared to show the existence of a steady state between oxidation of the carbaryl and conjugation of the ether soluble hydroxylated metabolites.

Studies by Metcalf *et al* (1967) suggest that the housefly is less able than the rat to metabolize N-methyl carbamates by N-dealkylation. Osman *et al* (1970) have shown that following topical application of propoxur to houseflies, 1-1.5% of the absorbed radioactivity is recovered after 24 hours as $C^{14}O_2$, thus indicating a low rate of N-dealkylation.

O-DEALKYLATION. Brodie *et al* (1958) showed involvement of MFOs in O-dealkylation of carbamates. Dealkylation of the O-alkyl groups of the ester or ether structures of insecticides is common, but does not take place by simple replacement of an alkyl group with a hydroxy group (Renson *et al* 1965). An unstable α-hydroxy intermediate is produced which spontaneously releases an aldehyde or a ketone. Thus the isopropyl group of propoxur is removed as acetone by the MFO system of rats and houseflies (Oonnithan & Casida 1968, Shrivistava *et al* 1969).

Metcalf *et al* (1967) on the basis of $C^{14}O_2$ liberation from propoxur suggested that most O-dealkylation takes place in the intact carbamate rather than from hydrolysed precursors. In *C. fatigans* larvae the MFO system plays an important role in propoxur metabolism (Shrivistava *et al* 1970) and metabolites formed *in vivo* and *in vitro* are similar to those found in the housefly.

RING HYDROXYLATION. The major metabolic attack on propoxur is by ring hydroxylation in the 5-position, with little hydroxylation occuring in the 4-position (Dorough & Casida 1964). This is in agreement with the results of Metcalf *et al* (1967) who, although they were unable to measure ring hydroxylation directly, showed that the housefly contains large amounts of phenolase enzyme, which *in vitro* hydroxylates a variety of aryl N-methylcarbamates.

ESTER HYDROLYSIS. Shrivistava *et al* (1969) showed that ester hydrolysis is of minor importance in propoxur metabolism in living houseflies as very little $C^{14}O_2$ is produced from propoxur-carbonyl-C^{14}.

A single microsomal preparation can therefore catalyse carbamate metabolism by a variety of pathways. It is not definitely known whether one or more basic enzymes are involved in the various reactions catalysed by fly microsome preparations. However there is some indication that the OP metabolizing MFO enzymes of flies differ from the carbamate metabolizing MFO enzymes.

ENZYMATIC INVOLVEMENT IN PYRETHROID RESISTANCE.

HYDROLASES.

It is known that (±)-trans-resmethrin is metabolized in rats by cleavage of the ester group and oxidation of the alcohol moiety. The presence of hydrolytic enzymes with varied substrate specificity has made studies of esterases difficult. Alderidge (1953) discovered two major groups of serum esterases, using an OP inhibitor, ie. the A or aryl esters which are unaffected by 10^{-3}M paraoxon and the B or 'aliesterases' which are inhibited by 10^{-7}M paraoxon. A third group of esterases (the C esterases) were described by Bergman & Rimon (1960) which are neither inhibited by or degrade OPs (Matsumura & Sakai 1968).

Preparation of milkweed bugs, cockroaches, houseflies, loopers, mealworms and mouse liver contain paraoxon sensitive esterases that hydrolyse the (+)-resmethrin and tetramethrin isomers. These preparations were in the form of acetone powders as fresh material appears to contain a factor(s) inhibiting (+)-trans-resmethrin hydrolysing esterases. The activity of the different enzyme sources decreases in the following order; mouse liver, milkweed bug, cockroach and housefly, looper and mealworm (Jao & Casida 1974).

OXIDOREDUCTASES.

The contribution of oxidative metabolism over and above the esterase attack can be evaluated by determining the effect of added $NADPH_2$ on the rate of pyrethroid metabolism by fresh microsomes. The low mammalian toxicity

of bioresmethrin appears from work on mice to result from the combined attack of esterase and oxidases that leads to rapid detoxication even when the activity of one or the other of the two enzyme systems is inhibited.

THE ROLE OF SYNERGISTS IN INSECTICIDE RESISTANCE STUDIES.

Synergists are compounds that are non-toxic by themselves, but which serve to enhance the toxicity of an insecticide with which they are combined. In addition to substantial economic advantages that can result from the synergism of expensive materials, the insecticidal activity of a given chemical can often be greatly modified when applied in combination with a synergist. In some cases synergists can extend the effectiveness of a certain insecticide by making possible the control of insect species or strains previously outside the activity spectrum of the insecticide alone, or conversely they may allow a greater degree of selectivity. A theoretically attractive way of utilizing the synergistic activity associated with certain groups is to incorporate them directly into the structure of the insecticide molecule. The success of this concept relies on the fact that the synergist grouping has no deleterious effects on the insecticide molecule into which it is incorporated. It may be that this is one reason that 'self synergism' has met with little success to date.

Strains of the housefly that have developed high levels of resistance to carbamates as a result of either selection pressure with the carbamates themselves or through cross-resist-

ance, usually remain relatively susceptible to synergized carbamate combinations. The development of resistance to carbamates is often greatly reduced or almost completely prevented if the insect population is selected with a carbamate/synergist combination (Georghiou 1962, Georghiou *et al* 1961, Moorefield 1960).

Insecticide synergists act by blocking the enzymes affecting insecticide detoxication. The chemical structure of a synergist for a given insecticide therefore depends on the rate-limiting enzyme(s) in the detoxication pathway. As well as this biochemical effect it has also been suggested that synergists may alter biological activity of formulations by increasing stability of insecticides, altering the rate of penetration through the cuticle or modifying the amount of insecticide picked up by the insect (Brown *et al* 1967, Metcalf 1967).

The main exploitation of synergists has been the synergism of pyrethrins with methylenedioxyphenyl compounds (MDP), mostly piperonyl butoxide. Widespread use of synergists in this case was fostered by the high cost of the insecticide. The synergist does not potentiate mammaliam toxicity and so has been used for fly control in domestic and industrial premises where foodstuffs are present and rapid knock-down of insects is required. Early work on pyrethroid synergism has been reviewed by Metcalf (1955) and Hewlett (1960). The effect of piperonyl butoxide on the toxicity of pyrethrins appears to vary between species and is most pronounced in houseflies (Hadaway *et al* 1963). In many species only small increases

in toxicity are noted after treatment with piperonyl butoxide and pyrethrins. Burnett (1961) showed a low factor of synergism (1.6x) with adult female tsetse flies and Hewlett *et al* (1961) recorded a factor of synergism of 3.2 with the lesser meal worm beetle. With adult females of the mosquitoes *An.stephensi* and *Aedes aegypti* the synergistic effect of piperonyl butoxide and pyrethrins was also low (2 and 2.7 respectively).

As we have already seen carbamates and organophosphates also undergo oxidative detoxication. Many synergists are therefore concerned with the microsomal oxidase enzymes. Synergists in this group also tend to contain a methylenedioxyphenyl (1,3-benzodioxole) ring. Compounds containing this group were originally developed as pyrethrin synergists. The exact mechanism by which the synergists work is still a matter of speculation (Casida 1970, Wilkinson 1968,1971,1979). There are three principle theories:-

1) Alternative substrates competitively inhibiting the metabolism of insecticides. For this the synergist must, for optimal activity, have a higher affinity (lower Km) than the insecticide for the active site of the MFO enzyme, with the result that binding of the synergist precludes the binding and subsequent metabolism of the insecticide. Relative turnover of the synergist must also be lower than that of the insecticide, or it will not be held at the site of metabolism long enough to work effectively. This question is considered in more detail by Rubin *et al* (1964), Wilkinson (1968), Wilkinson & Hicks (1969) and Casida (1970).

2). Hydrogen ion transfer (Hennessy 1965,1970). This would involve ligand displacement or addition at the haemochrome of cytochrome P^{450}, the terminal oxidase in the microsomal electron transport chain.

3). Formation of homolytic free radicals (Staudinger *et al* 1965).

Investigation of the structure/activity relationships of the MDP compounds show that maximum synergistic activity is associated with the intact MDP(1,3-aryldioxole) ring, and slight structural modifications of this ring result in either a decrease or a complete loss of potency. Carbaryl and propoxur toxicity is increased in housefly, cockroach, lepidopterous larvae and aphids among other species by piperonyl butoxide and aryl 2-propynyl ethers (Barnes & Fellig 1969, Brattsten & Metcalf 1970, Fukuto *et al* 1962, Shrivistava *et al* 1969). The optimum synergist for one methyl carbamate is sometimes different from that for another, as there is a considerable degree of selectivity with the synergists (Fahmy & Gordon 1965, Metcalf & Fukuto 1965, Wilkinson *et al* 1966).

Synergism of carbamates has not been used on any scale in commercial formulations, although extensive laboratory studies have been made. In such studies strongly insecticidal carbamates generally show a smaller response to the synergist than less effective or more easily detoxified carbamates. However the increase in insecticidal activity is often accompanied by an increase in mammalian toxicity.

Interactions of the phosphorothioate insecticides with inhibitors of microsomal oxidations presents a problem as these materials are subject to both activative and degradative metabolism by microsomal enzymes (Dahm & Nakatsugawa 1968). The antagonistic action of sesamex and other MDP compounds on the toxicity of several phosphorothioates can be explained by inhibition of the P=S to P=O conversion. Marked synergism may however be found, eg sesamex causes a 36-fold synergism of demeton and disulfton against houseflies and a lower degree of synergism with diazinon, (Sun *et al* 1967). The net result of synergistic interaction therefore depends on a metabolic balance between the critical pathways responsible for activation and degradation, and on the degree of inhibition by the synergist of these pathways.

In compounds with several groups which are potential sites for microsomal enzyme attack, it is not known which site will be preferentially attacked, nor is it known what type of microsomal reactions are most susceptible to synergist inhibition. The approach of treating with piperonyl butoxide or sesamex involves some danger of misrepresentation, as there is some risk in assuming that such substances never inhibit other detoxification mechanisms and are always effective against microsomal oxidations. Plapp (1970) noticed cross synergism, ie. a synergistic effect on different insecticides that are primarily degraded by different mechanisms (Oppenoorth 1971).

Organothiocyanates such as isobornyl thiocyanoacetate

and dodecyl thiocyanate are all synergistically active in combination with several carbamates. SKF525A and aryloxyalkylamine will synergise carbaryl and m-isopropyl phenyl in houseflies. A variety of tri-substituted aliphatic and aromatic phosphate esters are excellent synergists against housefly and mosquito larvae that have high malathion resistance (Plapp *et al* 1963, Plapp & Tong 1966). Dyte & Rowlands (1968) showed triphenyl phosphate to be a strong synergist in a strain of malathion resistant *Tribolium castaneum*. Plapp *et al* (1963) studied the synergism of malathion in the R_{Grothe} strain of housefly and in malathion resistant *C.tarsalis* larvae. Effective synergists were; tolyl phosphate, triphenyl phosphate, tributylphosphotrithioate, tributylphosphotrithioite and trimethylphosphotetrathioate. All of these caused an increase in the amount of malaoxon in the resistant mosquito larvae. The different spectra of synergistic activity in the flies and mosquitoes were attributed to the two types of malathion resistance ie. a mutant 'aliesterase' or B-esterase in the houseflies and an enhanced carboxylesterase in the mosquito larvae.

N-propyl paraoxon and EPNO are inhibitors of carboxylesterase. Oppenoorth & Van Asperen (1961) demonstrated a strong synergistic action of N-propyl paraoxon with malathion in the housefly G and H strains. Matsumura & Hogendijk (1964) also showed that EPN (O-ethyl-O-nitrophenyl phenylphosphonothioate) and EPNO are highly synergistic with the same strains but not against the susceptible strain. DEF (Tributyl-phosphorotrithioate) and EPN have also been reported as synergists for

dicrotophos, dimethoate and phorate against boll weevils (Bull *et al* 1965) suggesting that these synergists are able to inhibit enzymes other than carboxylesterases. Plapp *et al* (1963) reported that triphenyl phosphate decreases the toxicity of parathion to two strains of housefly. They attributed this to interference with parathion absorbtion , as topical application of synergist and insecticide on separate regions of the fly did not produce a decrease in toxicity.

MATERIALS AND METHODS.

ANOPHELINE SPECIES AND POPULATIONS STUDIED.

Anopheles albimanus.

MACHO. A population containing a Y-linked translocation of the propoxur resistance gene, kindly provided by Dr Seawright (1979). The original population from which this was selected came from El Salvador in 1974.

Anopheles arabiensis.

GI. A population from Gezira Sudan, colonised in 1978. Sub-colonies G MAL, G DDT and G PERM were derived from it.

JARAB. A population from McCarthy island, Gambiae 1974.

Anopheles atroparvus.

ATHOR. An insecticide susceptible colony from Kent england.

ATIG. A DDT resistant strain from the Isle of Grain, England, colonised in 1979.

AT SPA. A multiple resistant strain from an area around Cadiz, Spain, colonised in 1978. The sub-colonies ASFEN, ASP(2H),ASMAL, ASFW and ASPERM were derived from it.

Anopheles gambiae.

IAN. A population from Iworo, Nigeria, colonised in 1975. The sub-colonies IAN P10 and IAN P20 were derived from it.

Anopheles stephensi.

ST. An insecticide susecptible colony originating from near Delhi, India in the late 1940's.

ST POND. A strain from Pondicherry, India, colonised in 1979.

ST IRAN. A population from Roknabad, Bandar Abbas, southern Iran, colonised in 1976.

ST IRAQ. A population from Basrah, Iraq, colonised in 1978.

ST LA. A population from Lahore, Pakistan, colonised in 1979. The sub-colonies ST MAL and ST LAMA were selected from this population.

CHEMICALS USED.

INSECTICIDES.

Organochlorines
- DDT
- Dieldrin

Organophosphates
- Chlorphoxim
- Chlorpyriphos
- Dichlorvos
- Dimethoate
- Fenitrothion
- Fenthion
- Malathion (donated by Cheminova),
- Malaoxon
- Phenthoate
- Pirimiphos methyl (donated by ICI).

Carbamates
- Bendiocarb (donated by Fisons Ltd).
- Carbaryl
- Propoxur

Pyrethroids
- Deltamethrin
- Permethrin (pure cis- and trans-permethrin donated by ICI).

Insecticide impregnated papers of DDT, dieldrin, malathion, fenitrothion, fenthion, propoxur, deltamethrin and permethrin were provided by the World Health Organisation.

FUNGICIDES.

Kitazin P® (O-O-bis-isopropyl-S-phenyl-methyl-phosphorothioate). (Donated by Dr Miyata, Kasetsart University, Bangkok Thailand.

MALATHION METABOLITES.

Dimethyl phosphate

Malathion dicarboxylic acid (donated by Miss W.A.Matthews, Slough laboratories, Ministry of Agriculture, Fisheries and Food).

Malathion monocarboxylic acid.

SYNERGISTS.

Piperonyl butoxide (PB)

Sesamex

O,O-dimethyl O-phenyl phosphorothioate (SV_1)

Triphenyl phosphate (TPP)

S,S,S-tributyl phosphorotrithioate (DEF)

1.1-bis-(4-chlorophenyl)-2,2,2 trifluoroethyl (F-DMC).

GENERAL REARING OF MOSQUITOES.

Mosquito colonies were maintained in insectaries at 25-27°C and relative humidity of 70-80%. A 12 hour photoperiod was maintained by an automatic time switch. Larvae were reared in 12" diameter plastic bowls in tap water at the insectary temperature and fed on ground Farex,

a proprietary baby food, the quantity given depended on the number and age of the larvae.

Adults were maintained in 20 or 30cm cube cages, where 20% glucose solution was continuously available. Females were blood fed twice weekly on anaesthetised guinea pigs. Five inch diameter enamel bowls lined with filter paper and partially filled with tap water were provided as oviposition sites ands eggs were collected twice weekly.

PREPARATION OF IMPREGNATED PAPERS.

1). Cut Whatmans no 1 filter paper rectangles of 12 x 15cm.

2). Make up required concentration of insecticide solution or synergist in the relevant solvent (see below).

3). Place 0.7ml of the insecticide solution in a clean glass vial and add 1.2ml of acetone.

4). Mix the solution well and then pipette evenly over the rough side of the filter paper.

5). Allow 1-2 hours to dry before storing in sealed plastic boxes under refrigeration.

Solvents.

Unless otherwise stated the following non-volatile/ insecticide or synergist mixtures were used:-

4% DDT/Risella oil

4% Chlorphoxim/Olive oil

0.1% Dichlorvos/Olive oil

1.0% Dimethoate/Dioctyl-phthalate

1.0% Fenitrothion/Olive oil

2.5% Fenthion/Olive oil

5.0% Malathion/Olive oil

10% Phenthoate/Dioctyl-phthalate

0.031% Pirimiphos methyl/- (acetone only)

1.0% Bendiocarb/Dioctyl-phthalate

5.0% Carbaryl/Dioctyl-phthalate

0.1% Propoxur/Olive oil

0.25% Permethrin/Silicon oil

0.0025% Deltamethrin/Silicon oil

20% TPP/Silicon oil

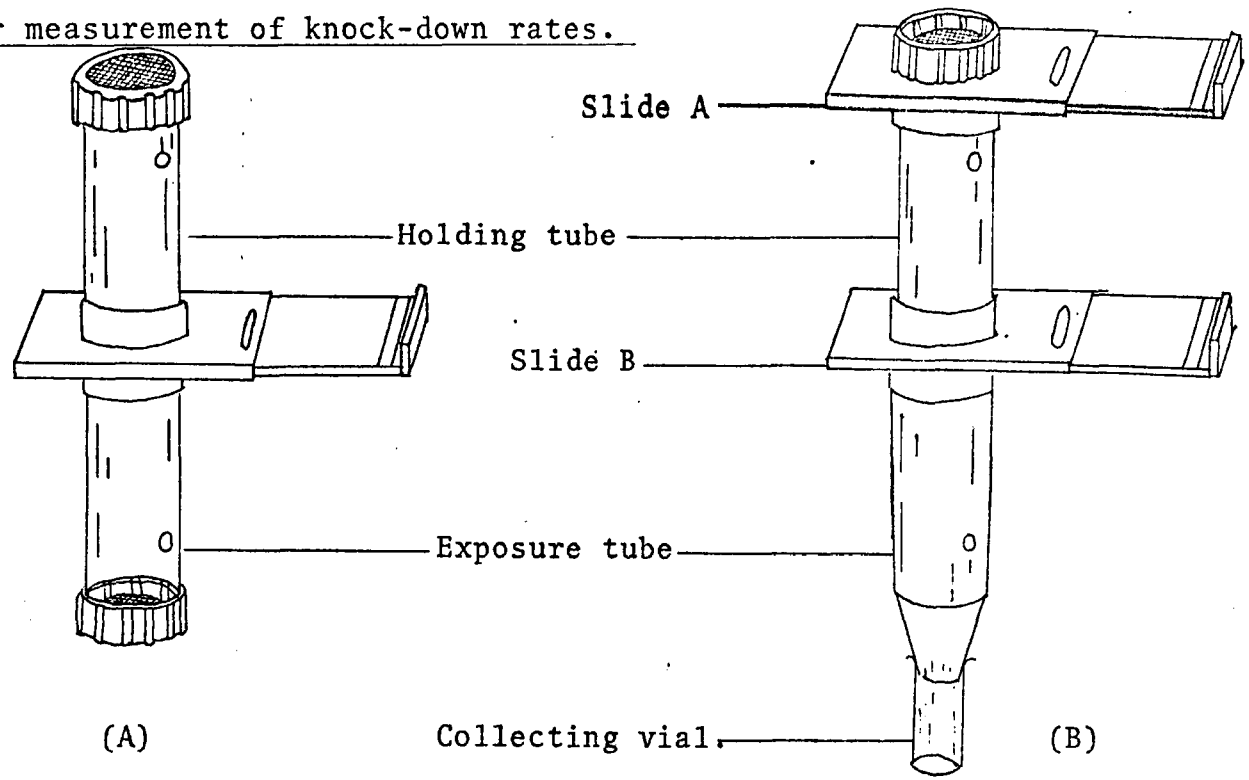
20% PB/silicon oil

1% F-DMC/Olive oil

GENERAL ADULT AND LARVAL INSECTICIDE TESTING.

The standard WHO adult and larval susceptibility tests were used in this study unless otherwise stated. In the larval test, the larvae are kept in 250ml distilled water containing a known concentration of insecticide for 24hours, at the end of which time mortality is scored. In the adult test the mosquitoes are confined for a certain time to a surface treated with a known concentration of insecticide, followed by confinement on an untreated surface for 24hours before mortality is scored. All tests employed one day old unfed males and females. A constant temperature of $25\pm2^{o}C$ and 70-80% relative humidity was maintained for all tests.

Figure 6. Standard WHO test kit (A) and modification of the WHO test kit (B) for measurement of knock-down rates.



MODIFICATION OF THE WHO TEST KIT FOR KNOCK-DOWN (KD) DETERMINATION.

The standard WHO test kit was modified to allow removal of knocked-down insects at regular time intervals (see Figure 6). The filter paper funnel at the base of the exposure (E) tube was impregnated with insecticide on its inner surface. This avoided the possibility of mosquitoes evading exposure to the insecticide by resting on an untreated surface during the test. Mosquitoes were placed in the holding (H) tube via slide A, this slide was then closed and a gauze placed in the inverted top. Mosquitoes were then blown into the B tube by opening slides A and B. The hole at the base of the funnel was covered with gauze during this process and for the first five minutes of the test. When the gauze at the base of the funnel was removed a collecting vial was placed directly underneath the funnel. Vials were changed every five minutes until all mosquitoes had been collected. Vials were covered with a cotton wool pad soaked with dilute glucose solution. Mortality in the collecting vials was then scored after 24hours. Survivors from each of the vials were kept separately and their progeny tested in the same way.

POLYACRYLAMIDE GEL TECHNIQUE.

Making the gel.

To make a 7.5% acrylamide gel;

Solutions	A	Methyl bis acrylamide	2.25g
		Acrylamide	75g
		Distilled water	500ml

B	Sucrose	25g
	TEMED	0.75ml
	buffer	250ml

(Make up buffer TEB pH 9.2.For 1 litre use:-

Tris	11.155g
di Na EDTA	0.96g
borate	0.73

Adjust to pH 8.6 using saturated barate).

C	Ammonium persulphate	0.4g
	Distilled water	250ml

METHOD.

1). Wash the glass plates with 2% 'Photoflo ' solution to give an even flow over the surface of the plates. Place the glass plates in a container with glass spacers of the required thickness between them.

2). Make up buffer to 1,000ml with distilled water.

3). Make up solutions A,B and C. Filter each of these separately.

4). Add solutions B and C to A. Mix thoroughly (without introducing too many bubbles).

5). Pour mixed solution into the container until the solution is 2cm above the glass plates.

6). Allow the gels to set and then separate them under water.

7). Store the gels under refrigeration in TEB buffer.

Preparing and running the gel.

1). Cut a horizontal line through the gel leaving 1cm at either side of the gel.

2). Homogenise individual mosquitoes on a ground glass slide using 20µl of ice-cold distilled water.

3). Take up the homogenate on a small piece of filter paper (Whatmans No 1).The filter paper should not show above the level of the gel when inserted).

4). Place insert into the slit in the gel. The last insert should be a visible marker of haemoglobin, bromophenol blue and albumin.

5). The gel should then be run in the usual way at 10mA for approximately 3 hours.

Staining.

1). Make up phosphate buffer from :-

a).0.9073g KH_2PO_4 in 100ml distilled water.

b).0.94g Na_2HPO_4 in 100ml distilled water.

Slowly add solution b) to a) until pH 6 is reached.

2). Make up the substrate by adding 0.1g acetylthiocholine iodide to 100ml phosphate buffer.

3). Then add the stain to the substrate in the following order:-

1) 0.354g sodium citrate

2) 0.114g Copper sulphate (anhydrous)

3) 0.039g Pottasium ferricyanide

4). Submerge the gel (after removing it from the electrodes) in the substrate/stain solution. Allow to stain overnight in an incubator at 20°C.

THE FILTER PAPER SPOT TECHNIQUE FOR TOTAL ESTERASE ACTIVITY.

Stock solutions.

a). Phosphate buffer (pH 6.5) made up from 4.8g Na_2HPO_4

+ 9.2g KH_2PO_4 in 1 litre distilled water.

b). Substrate solution- 1% α-naphthyl acetate in acetone.

c). Fixing solution - 10% acetic acid in water.

Working solutions.

a). 100ml phosphate buffer + 10ml substrate solution.

b). 300mg fast Garnett GBC salt in 100ml distilled water.

c). Fixing solution.

METHODS.

Mosquitoes were immobilised by placing at -20°C for 2-3minutes. Single mosquitoes were then crushed on a ground glass slide in 20µl of distilled water with a round based glass homogeniser. The base of the homogeniser was then firmly blotted onto Whatmans No 1 filter paper. When 10-20 mosquito homogenates had been deposited onto the filter paper, it was immersed in working solution a) for 90seconds. The paper was then blotted between two tissues and transferred to working solution b) for 60seconds. Finally it was dipped into solution c) and allowed to dry before recording results.

CARBOXYLESTERASE ASSAY.

Stock solutions.

a). 10^{-6}M eserine. 1ml 10^{-4}M eserine in 99ml distilled water.

b). 30mM α-naphthyl acetate. Dissolve 0.558g α-naphthyl acetate in 100ml acetone.

c). 5% sodium lauryl sulphate. 50g/l sodium lauryl sulphate in distilled water. (Prepare this the day before as it is slow to dissolve).

d). 0.02M pH 7 phosphate buffer. Dissolve 1.79g Na_2HPO_4 + 0.68g KH_2PO_4 in 500ml distilled water.

Working solutions.

A. Homogenising buffer. 20ml 10^{-6} eserine in 80ml phosphate buffer.

B. Substrate. 1ml 30mM α-naphthyl acetate in 99ml phosphate buffer (do not allow to stand more than ½hour before use).

C. Stain. Dissolve 150mg Fast blue B salts in 15ml distilled water, add 35ml 5% sodium lauryl sulphate and mix well.(This should be stored in the dark).

METHOD.

Immobilise the mosquitoes by placing at -20°C for 2-3 minutes. Weigh each mosquito individually and place in numbered tubes for homogenisation. Homogenise the mosquitoes in 1ml ice-cold homogenising buffer. Place 2.5ml substrate into a series of numbered test tubes, corresponding to the mosquito homogenisation tubes. Place the test tubes into a water bath at 25°C. Add 0.5ml of phosphate buffer to the control tubes at time 0, then at 15 second intervals add 0.5ml of mosquito homogenate to the similarly numbered substrate test tubes.

Thirty minutes after the start of the reaction add 0.5ml of stain to the control tube and then at 15 second intervals to each of the reaction test tubes. Fifteen minutes after adding the stain read the absorbance on a spectrophotometer at 605nm.

STARCH GEL ELECTROPHORESIS FOR CARBOXYLESTERASE STUDY.

Making the gel.

Solutions.

Electrode buffer : pH 8.6

20g tris (hydroxymethyl)aminomethane

2g di-Na ethylene diamintetra-acetic acid

3.8g boric acid

made up to 1,000ml with distilled water.

Gel buffer : pH 8.2

3 in 10 dilution of electrode buffer.

90ml electrode buffer

195ml distilled water

15ml boric acid solution (approximately).

METHOD.

1). Dissolve 33g of hydrolysed starch in 300ml of gel buffer.

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

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

4). Pour into plates (300ml should make 2 gels).

5). Allow the gels to cool and cover with cling film to prevent drying. Use within 24hours.

Preparing and running the gel.

1). Cut a horizontal line through the gel leaving 1cm at either side of the gel.

2). Homogenise insects in 5µl of distilled water. If the females are blood fed remove the abdomens before homogenising.

3). Take up the homogenate on small pieces of Whatmans 3M chromatography paper and insert the paper into the cut in the gel.

4). Use haemoglobin and bromophenol blue as visible markers on the last insert.

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

6). Run at 150volts for ½hour then increase the voltage to 300volts for 4-5hours., (gels are usually run under refrigeration at 8-9°C to avoid overheating).

Staining.

4ml 1% α-naphthyl acetate in acetone.

25mg Fast blue RR

50ml 0.1M tris maleate buffer pH 6.4.

Pour over the gel and allow to stain at room temperature until the bands are fully developed.

MALATHION METABOLITE SEPARATION TECHNIQUES.

Chemicals.

Malathion (greater than 99% pure) was donated by Cheminova.

Malaoxon

Malathion monocarboxylic acid was donated by W.A.Matthews, Ministry of Agriculture, Fisheries and Food.

Malathion dicarboxylic acid was prepared by the methods of March *et al* (1956).

2,6-dibromoquinone-4-chloromide was purchased from Koch light laboratories Ltd, Bucks.

C^{14}-labelled malathion was obtained from the Radiochemical centre, Amersham .(Activity 4.6mC/mM.). It was

labelled in both carbon atoms of the succinyl part of the molecule. The labelled malathion was diluted 1:2 with non-labelled malathion in absolute ethanol to give a working solution of 0.1% which had an activity of 841005.55 cpm/ml.

Pre-coated plastic sheets coated with 0.2mm silica gel N-HR/UV_{254} were purchased from Camlab, Cambridge.

The purity of all chemicals was assessed by thin layer chromatography. The plates were sprayed with 0.5% 2,6-Dibromoquinine-4-chloromide (DCQ) in cyclohexane and heated to 100°C to visualise the spots.

METHOD.

1). Expose batches of 100 4th instar larvae to either 2ppm malathion for 3hours or 0.125ppm malathion for 24hours.

2). Remove larvae from the test medium by pouring the test solution through a gauze filter. Wash the larvae and filter several times with distilled water.

3). Homogenise larvae in a small amount of sodium sulphate in 2x5ml hexane.

4). Filter the homogenate and wash the residue with a further 5ml of hexane.

5). Combine the filtrate and evaporate to dryness in a rotary evaporator.

6). Repeat this procedure using diethyl ether and then acetonitrile.

7). Extract metabolites from the larval test medium using hexane and diethyl ether as solvents.

8). Redissolve each sample in 100µl of the relevant solvent.

Analysis of the metabolites.

1). Pre-run TLC plates with chloroform for 1½hours prior to adding metabolites.

2). Spot out metabolites at equal distances along the origin line using a 5µl capillary tube.

3). Run the TLC plate using a solvent system of hexane: diethyl ether (1:3) (Matthews 1980).

4). Run standard metabolites on each plate to check the position of the metabolites (R_f values should be reproducible)

5). Stain TLC plate by spraying with 0.5% DCQ in cyclohexane and heating to 100°C.

Quantification of metabolites.

1). Expose batches of 100 4th instar larvae to 2ppm C^{14}-malathion for 20,40,60,80,120 or 180 minutes.

2). Separate metabolites by TLC as above but do not stain

3). Scrape into vials the regions of the TLC plate corresponding to the R_f value for each metabolite.

4). Re-extract the metabolites with 10ml of the relevant solvent.

5). Centrifuge, retain the liquid phase and evaporate to dryness in a rotary evaporator.

6). Re-dissolve metabolites in 0.1ml of either hexane, diethyl ether or acetonitrile.

7). Place solutions in clearly labelled counting vials and air dry.

8). Add 100µl absolute alcohol, followed by 40µl distilled water and 4ml scintillation fluid.(Packards scintillation

cocktail, Scintillator 299^{TM} was used).

9). Count in a scintillation counter for 10 minutes or up to 10,000 counts.

RESULTS.

SECTION I. ADULT RESISTANCE SPECTRA.

The adult resistance spectra of the original populations of each species studied were recorded. The standard World Health Organization (WHO) test kits were used and tests were carried out using the WHO recommended discriminating dosages. Comparisons were made between laboratory strains with no history of insecticide exposure, and the "resistant" strains which had been under insecticidal selection pressure in the field.

The resistance spectra of the colonies studied differed markedly. *An.albimanus* MACHO males showed broad spectrum resistance, including organochlorine, organophosphate and carbamate insecticides (see Table 1). Notable exceptions were the organophosphates fenthion, dichlorvos and phenthoate to which there was complete susceptiblity. MACHO females without the Y-linked propoxur gene (see materials section for details) only showed resistance to dieldrin and dimethoate. The low mortality with 1% dimethoate may have been due to too low a discriminating dosage or deterioration of the insecticide solution used to make up the papers, as other laboratory stocks with no history of insecticide exposure showed some survival on these papers.

The broad spectrum resistance in the males but not in the

Table 1 . Adult resistance spectra of Anopheles albimanus colony.

Insecticide	Conc	Exposure time	Colony tested					
			MACHO females			MACHO males		
Organochlorines			D	T	%	D	T	%
Dieldrin	0.4% 4.0%	1hr 2hr	25	45	55.6	25	45	55.6
DDT	4.0%	1hr	48	48	100	56	56	100
Organophosphates Chlorphoxim	4.0%	1hr	168	168	100	126	246	51.2
Dichlorvos	0.1%	1hr	54	54	100	67	67	100
Dimethoate	1.0%	1hr	7	47	14.9	1	49	2
Fenitrothion	1.0%	2hr	149	150	99.3	14	148	9.6
Fenthion	2.5%	1hr	660	660	100	542	542	100
Malathion	5.0%	1hr	133	133	100	29	127	22.8
Phenthoate	10.0%	1hr	70	70	1000	72	72	100
Pirimiphos methyl	1.0%	1hr						
Carbamates Bendiocarb	1.0%	1hr	360	360	100	1	410	0.24
Carbaryl	5.0%	1hr	102	102	100	3	58	5.2
Propoxur	0.1%	1hr	540	540	100	20	920	2.2
Pyrethroids Decamethrin	0.025% 0.0025	1hr 1hr	42	42	100	38	38	100
Permethrin	0.25%	1hr						

females of the MACHO population, indicate that all the OP and carbamate resistances are due to a single gene (or two closely linked genes). This suggests that the resistance is due to an altered acetylcholinesterase mechanism rather than a metabolic mechanism, which would be more specific.

The one MACHO female survivor of the fenitrothion treatment was hand mated to a MACHO male and one batch of eggs was obtained from her. Sixty four eggs were laid, out of which 49 larvae emerged and completed their development through to the adult stage. There were 19 females and 30 males. All males survived exposure to 5% malathion for one hour as expected, as they recieve their resistance gene(s) from the male parent. Seven females survived one hours exposure to 5% malathion. These must have recieved their resistance gene(s) from the female parent. This implies that the female which survived the original treatment was the result of a rare crossing-over event, involving the translocated Y-resistance gene. These would only be expected at a very low frequency as the translocated gene is covered by an inversion which should supress crossing-over.

The GI *An.arabiensis* colony from Sudan, showed organochlorine resistance. Organophosphate resistance in this species extended only to malathion and phenthoate and it is notable that these two OPs both contain a carboxylester bond. Survivors were also observed after exposure to the pyrethroid permethrin. The SB *An.arabiensis* colony showed only dieldrin resistance (see Table 2).

Table 2. Adult resistance spectra of *An. arabiensis* colonies.

Insecticide	Conc	Exposure time	Colony tested					
			SB			G1		
			D	T	%	D	T	%
Organochlorines								
Dieldrin	0.4%	1hr	150	249	60.2	9	360	2.5
	4.0%	2hr	140	150	93.3	31	192	16.1
DDT	4.0%	1hr	112	112	100	478	570	83.9
Organophosphate								
Chlorphoxim	4.0%	1hr				50	50	100
Dichlorvos	0.1%	1hr	106	106	100	242	242	100
Dimethoate	1.0%	1hr	113	113	100	321	321	100
Fenitrothion	1.0%	2hr	72	72	100	384	385	99.5
Fenthion	2.5%	1hr	210	210	100	644	644	100
Malathion	5.0%	1hr	162	162	100	130	463	28.1
Phenthoate	10.0%	1hr	52	52	100	40	220	18.2
Pirimiphos methyl	1.0%	1hr	55	55	100	105	105	100
Carbamates								
Bendiocarb	1.0%	1hr	41	41	100	79	79	100
Carbaryl	5.0%	1hr	67	67	100	172	172	100
Propoxur	0.1%	1hr	265	265	100	418	418	100
Pyrethroids								
Decamethrin (Deltamethrin)	0.025%	1hr	79	79	100	50	50	100
	.0025%	1hr	62	62	100	34	34	100
Permethrin	0.25%	1hr	46	46	100	30	99	30.3

Three colonies of *An.atroparvus* were studied (see Table 3). The ATHOR colony from England showed complete susceptibility to all the insecticides tested. ATIG a recently colonised English population was resistant only to DDT, though this was present at a fairly high level. In contrast to these populations the AT SPA colony from Spain was resistant to a range of organochlorines, OPs and carbamates, as well as showing some survival on 0.25% permethrin. The colony was however completely susceptible to phenthoate and dichlorvos. The resistance spectrum for this colony was very similar to that shown by the MACHO males, with the exception that in the AT SPA colony there is definite resistance to fenthion.

The IAN *An.gambiae* colony from Nigeria showed resistance to dieldrin, DDT and permethrin (see Table 4), it is however completely susceptible to the range of OPs and carbamates tested with the exception of dimethoate, and there are indications that survivals from these tests were due to failure of the papers rather than resistance in the mosquitoes.

Five colonies of *An.stephensi* were investigated (see Table 5). ST showed only low level organochlorine resistance but was completely susceptible to the range of OPs, carbamates and synthetic pyrethroids tested. ST POND from India showed some resistance to DDT but was susceptible to all other insecticides tested. ST IRAQ from Iraq was resistant to dieldrin DDT, fenitrothion, malathion and phenthoate. ST IRAN from Iran showed a similar resistance spectrum to that of ST IRAQ but there were fewer fenitrothion resistant individuals in

Table 3. Adult resistance spectra of *An. atroparvus* colonies.

Insecticide	Conc	Exposure	ATHOR			ATIG			AT SPA		
Organochlorines		time	D	T	%	D	T	%	D	T	%
Dieldrin	0.4%	1hr	139	139	100	62	62	100	20	32	62.5
	4.0%	2hrs	230	230	100				40	45	88.9
DDT	4.0%	1hr	230	230	100	12	120	10			
Organophosphate											
Chlorphoxim	4.0%	1hr	121	121	100	32	32	100	53	110	48.2
Dichlorvos	0.1%	1hr	49	49	100	28	28	100	32	32	100
Dimethoate	1.0%	1hr				63	63	100			
Fenitrothion	1.0%	2hrs	71	71	100	58	58	100	27	180	15
Fenthion	2.5%	1hr	62	62	100	48	48	100	84	102	82.4
Malathion	5.0%	1hr	61	61	100	146	146	100	57	122	46.7
Phenthoate	10.0%	1hr	32	32	100	46	46	100	47	47	100
Pirimiphos methyl	1.0%	1hr							240	280	85.7
Carbamates											
Bendiocarb	1.0%	1hr							45	99	45.5
Carbaryl	5.0%	1hr	62	62	100	46	46	100			
Propoxur	0.1%	1hr	128	128	100	62	62	100	57	114	50.4
Pyrethroids											
Decamethrin	0.025%	1hr	52	52	100	24	24	100			
Permethrin	0.25%	1hr	52	52	100	48	48	100	68	75	90.7

Table 4. Adult resistance spectrum of *An. gambiae* (IAN) colony.

Insecticide	Conc	Exposure time	Dead	Total	% mortality
Organochlorine					
Dieldrin	0.4%	1hr	1	47	2.1
	4.0%	2hrs	11	58	18.9
DDT	4.0%	1hr	27	183	14.8
Organophosphate					
Chlorphoxim	4.0%	1hr	129	129	100
Dichlorvos	0.1%	1hr	36	36	100
Dimethoate	1.0%	1hr	146	222	50
Fenitrothion	1.0%	1hr	55	55	100
Fenthion	2.5%	1hr	91	91	100
Malathion	5.0%	1hr	144	144	100
Phenthoate	10.0%	1hr	62	62	100
Carbamates					
Bendiocarb	1.0%	1hr	53	53	100
Propoxur	0.1%	1hr	121	121	100
Carbaryl	5.0%	1hr	185	185	100
Pyrethroids					
Decamethrin (Deltamethrin)	.0025%	1hr	211	211	100
Permethrin	0.25%	1hr	399	466	85.6

TABLE 5. ADULT RESISTANCE SPECTRA OF ANOPHELES STEPHENSI COLONIES.

INSECTICIDE	CONC	EXPOSURE TIME	COLONY TESTED														
			ST			ST POND			ST IRAQ			ST IRAN			ST LA		
			DEAD	TOTAL	%	DEAD	TOTAL	%	DEAD	TOTAL	%	DEAD	TOTAL	%	DEAD	TOTAL	%
ORGANOCHLORINES																	
DIELDRIN	0·4%	1hr	264	289	91·3	390	390	100	0	50	0						
	4%	2hrs				27	27	100	70	290	24·1	115	231	49·8	147	147	100
DDT	4%	1hr				35	41	85·4	6	33	18·2	1	45	2·2	22	61	36·1
ORGANOPHOSPHATES																	
CHLORPHOXIM	4%	1hr	76	76	100	61	61	100	42	42	100	160	160	100	142	142	100
DICHLORVOS	0·1%	1hr	110	110	100	77	77	100	46	46	100	18	18	100	162	162	100
DIMETHOATE	1%	1hr	42	42	100												
FENITROTHION	1%	1hr	112	112	100	142	142	100	127	150	84·6	1,766	1,795	98·4	540	540	100
FENTHION	2·5%	1hr	150	150	100	189	189	100	240	240	100	948	962	98·6	146	146	100
MALATHION	5%	1hr	145	145	100	169	169	100	981	2,709	36·2	558	1,126	49·6	0	92	0
PHENTHOATE	10%	1hr	160	160	100	170	170	100	21	72	29·2	162	278	58·3	14	292	4·8
PIRIMIPHOS METHYL	1%	1hr	380	380	100	82	82	100	198	198	100	195	195	100	164	164	100
CARBAMATES																	
BENDIOCARB	1%	1hr	75	75	100	59	59	100	62	62	100	48	48	100	123	123	100
CARBARYL	5%	1hr	72	72	100	75	75	100									
PROPOXUR	0·1%	1hr	135	135	100	156	156	100	153	153	100	168	168	100	139	139	100
PYRETHROIDS																	
DELTAMETHRIN	0·025	1hr															
	·0025	1hr	61	61	100	78	78	100	89	89	100	41	42	97·6	164	164	100
PERMETHRIN	0·25%	1hr	43	43	100	53	53	100	99	99	100	49	49	100			

the Iranian population. The fact that there is malathion and phenthoate resistance in these two populations may indicate the involvement of a carboxylesterase resistance mechanism This could not however be the cause of the fenitrothion resistance. ST IRAN also showed a few survivors after exposure to 2.5% fenthion for one hour.

ST LA from Pakistan was resistant to DDT, showed no mortality after one hour's exposure to 5% malathion and a very low level of mortality after exposure to 10% phenthoate for one hour. This colony was established from a single hand mated female and the first generation of her progeny were used for malathion testing, it was therefore necessary to carry out further selection and genetic analysis before it was certain whether individuals surviving this malathion treatment were heterozygous or homozygous for the malathion resistance gene(s). There was no fenitrothion resistance apparent in this population with 100% mortality after both one or two hours exposure to 1% fenitrothion.

SECTION I(b). LARVAL RESISTANCE.

Although the majority of this study is concerned with adult insecticide resistance, in some cases the larval response to particular insecticides was tested, in order to determine whether certain adult resistance genes were functional in the larval stages. This has important practical considerations in attempting to determine what selection pressures originally produced the observed resistance. ie.

it is unlikely that agricultural insecticide spraying will have produced a resistance which is only present in the adult stages, whereas a gene which is present in both the adult and larval stages may have been selected for by agricultural spraying and/or house spraying. There is however some contention on this point, as dense crops producing a high humidity beneath their foliage may provide resting places for adult mosquitoes, thus selection in the adult stages by agricultural spraying would be possible.

Larval testing as carried out here was not intended to give a comprehensive coverage of the possible larval resistance spectra of any particular population.

a) Larval resistance in An.stephensi from Pakistan.

Fourth instar larvae of the parental ST LA population were tested over a range of malathion concentrations using the standard WHO larval test method. Fourth instar larvae of the adult malathion susceptible ST population were exposed to the same range of dosages to act as a comparison.

Survivors of the 0.5ppm treatment of ST LA were allowed to complete their development and an F_1 generation was obtained from them. The F_1 generation larvae were exposed to malathion as fourth instars. Selection was then continued in this manner for five generations. The results are given in Table 6 , which shows a decrease in mortality after five generations. The question arises as to whether the larval malathion resistance is due to the same factor or factors as the adult malathion resistance in this population. After

five generations of larval selection, the progeny of ST LAMA (5) were allowed to complete their development and were tested against 5% malathion for 6 hours as one day old adults, they showed 15.2% mortality in these tests (sample size = 256).

Table 6. The mortalities of 4th instar ST and ST LA after exposure to various concentrations of malathion, and the response of ST LA after 5 generations of larval selection with malathion.

COLONY.	CONCENTRATION OF MALATHION (PPM)							
	0.125		0.25		0.5		1.0	
	DEAD/TOTAL	%	DEAD/TOTAL	%	DEAD/TOTAL	%	DEAD/TOTAL	%
ST	144 / 201	71.6	86/98	87.7	375/388	96.7	120/120	100
ST LA	303/ 513	59.1	548/703	77.9	243/263	92.4	113 / 114	99.1
ST LAMA	9/ 83	10.8	9/ 23	39.1				
ST LAMA (2)					164/192	85.4		
ST LAMA (3)					161/ 243	66.3		
ST LAMA (4)					80/231	34.6		
ST LAMA (5)					14/284	4.9		

A sample of ST LA adults tested against 5% malathion for 6hours at the start of the larval selection programme showed 58.4% mortality, while a sample of ST LA tested at the same time as

ST LAMA (6) showed 59.3% mortality. It therefore appears that larval selection with malathion also selects for the adult resistance gene(s). To check whether the converse was true a sample of ST MAL, the adult malathion selected population, was tested against a range of malathion concentrations as 4th instar larvae. At the time of testing the adults from this population showed 14.1% mortality to 5% malathion for 6hours. Results from the larval tests are given in Table 7.

Table 7. The larval resistance level of a malathion resistant adult selected population (ST MAL) of *An.stephensi*, as compared to an adult malathion susceptible strain (ST) and the parental population (ST LA) of ST MAL.

COLONY.	CONCENTRATION OF MALATHION (PPM).							
	0·125		0·25		0·5		1·0	
	DEAD/TOTAL	%	DEAD/TOTAL	%	DEAD/TOTAL	%	DEAD/TOTAL	%
ST	186/273	68·1	82/91	90·1	40/40	100	60/60	100
ST LA	141/260	54·2	462/498	92·8	183/198	92·4	20/20	100
ST MAL	16/290	5·5	21/266	7·9	6/150	4·0	12/40	30

It appears from these results that selection with malathion in the adult stage also increases the larval

malathion resistance level. It can therefore be concluded that the factor(s) involved in malathion resistance in this *An.stephensi* population are already operative at the 4th instar stage and continue to operate into adult life.

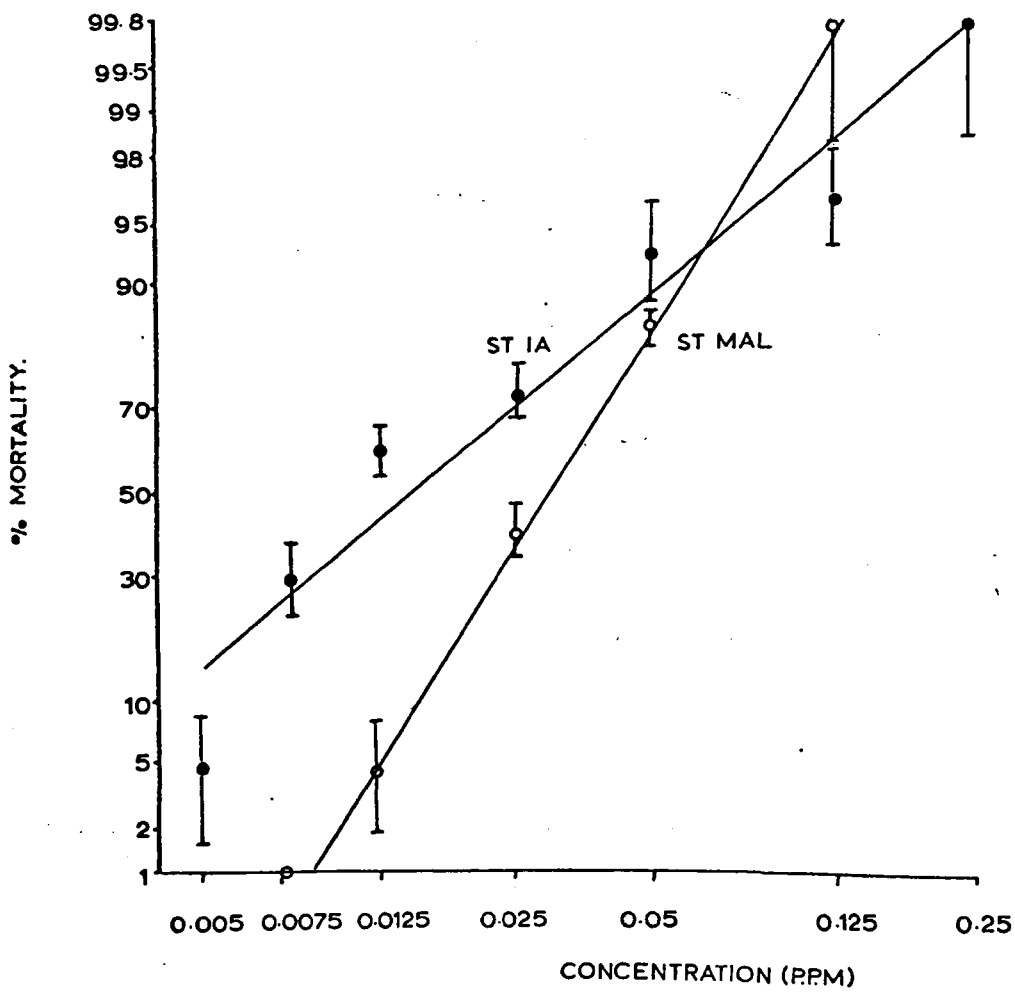
Fourth instar larvae of the malathion adult selected line of *An.stephensi* (ST MAL) were tested against a range of concentrations of abate (temephos) and the results were compared against results from a pooled population of malathion susceptible *An.stephensi* from North India and Afghanistan (ST IA) (see Table 8).

Table 8. The mortalities of 4th instar ST IA and ST MAL after exposure to various concentrations of abate.

CONCENTRATION OF ABATE (PPM)	COLONY TESTED.			
	ST IA *		ST MAL	
	DEAD/TOTAL	%	DEAD/TOTAL	%
0·005	6/142	4.2	0/100	0
0·0075	43/143	30	0/150	0
0·0125	134/220	60.9	7/149	4.7
0·025	162/221	73.3	75/183	41
0·05	136/147	92.5	193/230	83.9
0·125	261/271	96.3	162/162	100
0·25	248/248	100	72/72	100

* Data obtained in collaboration with C.F. Curtis.

FIGURE 7. LOG-DOSAGE PROBIT-MORTALITY LINES FOR MALATHION RESISTANT (ST MAL) AND SUSCEPTIBLE (ST IA) LINES OF AN. STEPHENSI AFTER EXPOSURE TO ABATE (TEMEPHOS).



(Lines fitted by maximum-likelihood analysis. ST MAL P = ns. ST IA P = 0.01).

The WHO recommended discriminating dosage for abate was 0.625ppm, it can be seen from these results that this dosage appears to be too high for *An.stephensi*. This was supported by a collaborative experiment organised by the WHO VBC division where the majority of anophelines exhibited 100% mortality at concentrations equal to or below 0.125ppm. The WHO recommended dosage was therefore changed to 0.25ppm (WHO TRS 655 1980).

From figure 7 it can be seen that the LDP lines of malathion resistant and susceptible *An.stephensi* cross. ie. at lower dosages of abate the resistant strain is more 'tolerant' while at higher dosages (above 0.125ppm) the susceptible strain is more'tolerant'. It would seem reasonable to assume that the malathion resistance gene in ST MAL is not producing a large degree, if any, cross resistance to abate, although the LD_{50} values of the malathion resistant and susceptible populations differ significantly.

b). Larval resistance in An.atroparvus from Spain.

Fourth instar larvae were tested against a range of concentrations of the organophosphates Dursban and malathion and the carbamate propoxur. Comparisons were made between AT SPA, an *An.atroparvus* population with broad spectrum organophosphate and carbamate resistance and ATHOR, an adult susceptible population (see Table 9). It is clear from these results that there is propoxur resistance in the AT SPA larvae. To determine whether the propoxur resistance in the larvae is controlled by the same factor or factors that control propoxur resistance in the adult, larval

Table 9. Larval mortalities of An.atroparvus after 24hours exposure to various insecticides at a range of dosages.

INSECTICIDE	CONCENTRATION (PPM)	AT SPA		ATHOR	
		MORTALITY	%	MORTALITY	%
DURSBAN	0·02	70/90	77.7	100/100	100
	0·04	90/90	100	100/100	100
	0·05	90/90	100	99/99	100
	0·1	90/90	100	100/100	100
PROPOXUR	0.5	0/80	0	60/90	66.7
	1·0	0/80	0	90/90	100
	2·0	10/80	12.5	90/90	100
MALATHION	0.5	10/60	16.7	8/60	13.3
	1·0	48/60	80	56/60	93.3
	2·0	60/60	100	60/60	100

selection at 2ppm was carried out for two generations with AT SPA. The first generation showed 10.5% mortality and the second generation showed 1.1% mortality (sample size 57 and 90 respectively). Half of the F_3 generation were then tested on 0.1% propoxur for 2hours and half on 0.1% propoxur for 6hours, and the results were compared with those from similar tests with the parental strain,AT SPA. The results in table 10 show that selection at the larval stage has an effect on the level of adult resistance when measured by a two hour exposure period but has little or no effect when measured by a six hour exposure period.A population of *An.atroparvus* selected for 20 generations in the adult stage

with 0.1% propoxur (see Table 15 for details of selection) showed no mortality of 4th instar larvae when exposed to 2ppm propoxur for 24hours. The results of this study are rather difficult to interpret in isolation. Selection for

Table 10. Adult propoxur resistance levels in a population of *An.atroparvus* selected for larval resistance to propoxur for three generations.

COLONY	0.1% PROPOXUR			
	2 HOURS		6 HOURS	
	DEAD/TOTAL	%	DEAD/TOTAL	%
AT SPA	26/38	68.4	61/69	88.4
AT SPA (F_3)	11/67	16.4	59/66	89.4

larval resistance to propoxur appears to affect adult resistance at a low level. From this we may tentatively conclude that more than one factor is involved in the propoxur resistance and that one or more factors are operative at the larval stages and at the adult stage, while a further factor or factors are involved in the adult resistance and are probably not operative at the larval stage.

It can be seen from Table 9 that there is no significant difference between the mortalities of adult malathion resistant and susceptible stocks of *An.atroparvus* after

exposure to a range of malathion dosages at the 4th larval instar. It can therefore be concluded that the adult malathion resistance gene(s) are not operative in the larval stage in this population. This is in contrast to the situation found with malathion resistance in *An.stephensi* from Pakistan (see pp 95–97).

c). *Larval resistance in An.stephensi from Iraq, Iran and India.*

First and fourth instar larvae of ST IRAQ, ST IRAN and ST POND were exposed to various concentrations of malathion (see Table 11). From the adult resistance spectra (see Table 5) it is clear that ST IRAQ and ST IRAN are resistant to malathion and ST POND is susceptible. If the adult malathion

Table 11. The 1st and 4th instar larval response to malathion in adult malathion resistant (ST IRAN, ST IRAQ) and susceptible (ST POND) populations of *An.stephensi.*

INSTAR	CONCENTRATION (PPM)	ST IRAQ		ST POND		ST IRAN	
		Dead/Total	%	Dead/Total	%	Dead/Total	%
1st	0.0125	0/200	0	0/200	0	0/40	0
1st	0.025	130/200	65	0/200	0	19/40	47.5
1st	0.05	200/200	100	200/200	100	40/40	100
1st	0.1	200/200	100	200/200	100	40/40	100
1st	0.2	200/200	100	200/200	100	40/40	100
4th	0.5	38/100	38	41/120	34.2	43/120	35.8
4th	1.0	68/100	68	130/130	100	50/90	55.6
4th	2.0	60/60	100	170/170	100	150/150	100

resistance gene(s) are operative in the larval stages one would expect ST IRAQ and ST IRAN to be more tolerant to malathion than ST POND. This is the case with 4th instar larvae, where there are some survivors from ST IRAQ and ST IRAN when treated with 1ppm malathion for 24hours but no survivors from the ST POND population. However the reverse appears to be true of the first instar larvae, as ST POND showed no mortality when exposed to 0.025ppm malathion for 24hours, whereas ST IRAQ and ST IRAN showed approximately 50% mortality at this dosage. From this study we do not know whether the observed 4th instar malathion resistance mechanism is the same as that observed in the adults, though this is likely. However from the results of the 1st instar larval tests it appears that the malathion resistance mechanism(s) are not 'switched on' at this stage, as ST POND showed a higher 'tolerance' to malathion in the 1st instar. The malathion resistance mechanism(s) must therefore become operative between the end of the first instar and the begining of the 4th instar.

d). Larval resistance in An.arabiensis from Sudan.

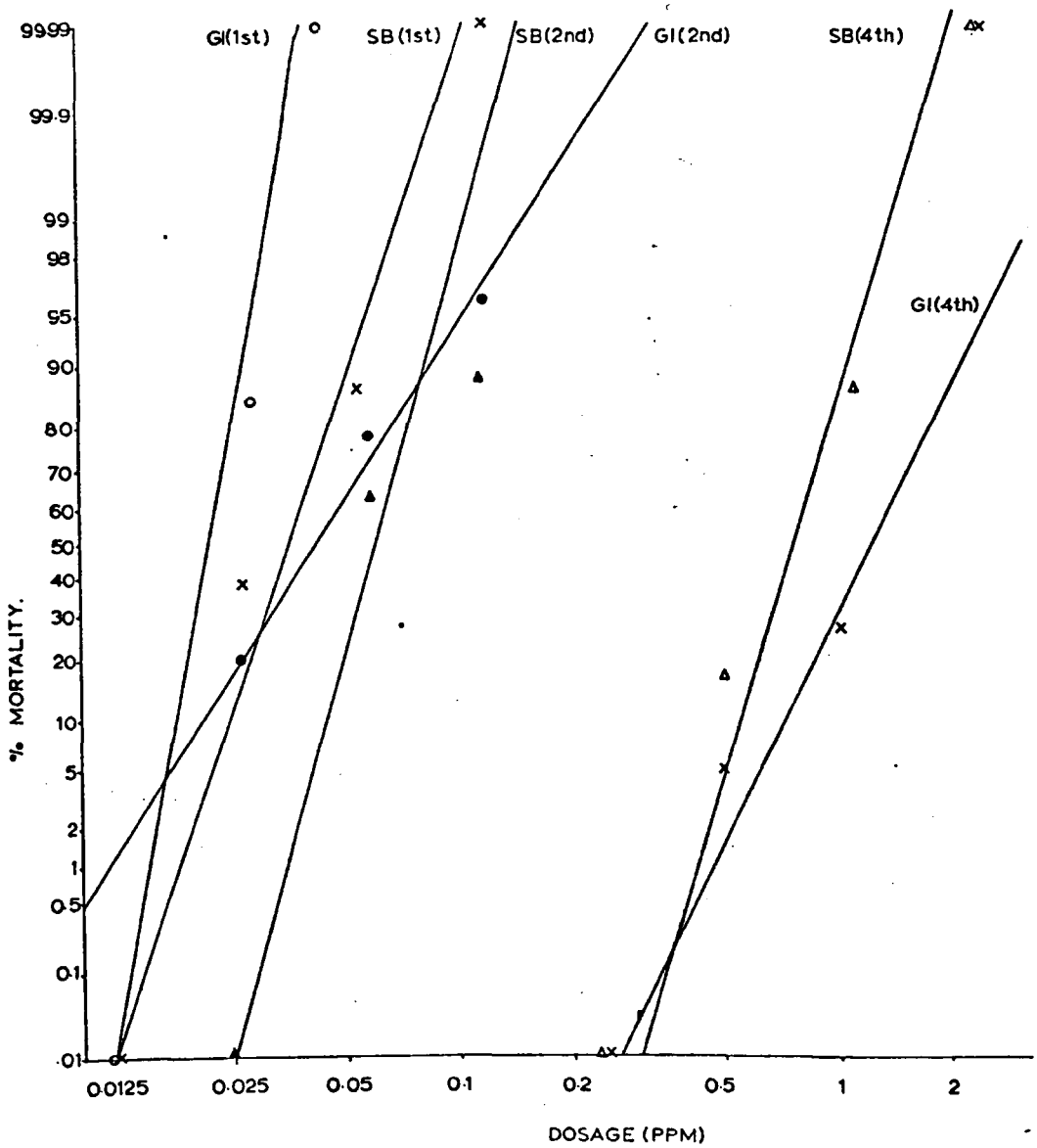
Larval resistance to malathion in 1st, 2nd and 4th instar larvae was investigated. The adult malathion susceptible colony SB was used as a comparison. First instar larvae of the Sudanese G1 and SB showed no mortality at 0.0125ppm malathion. At 0.05ppm 1st instar larvae of G1 showed 100% mortality, while there was still some survival of SB larvae. Second instar larvae of G1 and SB showed similar responses

Table 12. Larval mortalities of 1st, 2nd and 4th instar An.arabiensis after 24hours exposure to malathion over a range of dosages.

INSTAR	CONCENTRATION	G1		SB	
		Dead / Total	%	Dead/Total	%
1st	0.0125	0/60	0	0/60	0
1st	0.025	58/70	82.9	28/80	35
1st	0.05	60/60	100	65/80	81.3
1st	0.1	80/80	100	80/80	100
1st	0.2	100/100	100	80/80	100
2nd	0.025	15/80	18.8	0/80	0
2nd	0.05	61/80	76.3	49/80	61.3
2nd	0.1	75/80	93.8	70/80	87.5
2nd	0.5	80/80	100	80/80	100
4th	0.2	0/60	0	0/60	0
4th	0.5	4/100	4	14/100	14
4th	1.0	27/100	27	82/100	82
4th	2.0	100/100	100	100/100	100

over a range of dosages between 0.025ppm and 0.5ppm, while the fourth instar larvae of G1 appeared slightly more tolerant to malathion than SB. However the differences between the two populations were not great enough to justify giving resistance status to the G1 larvae, (see Table 12 and Figure 8). It therefore appears from this study that the malathion resistance which is apparent in the adult stages of *An.arabiensis* from Sudan is not operative in the larval stages. There are slight differences in 'tolerance' between the two populations tested at the first and fourth instars but these are within the normal range of variation expected

FIGURE 8. LOG-DOSAGE PROBIT MORTALITY LINES FOR 1st, 2nd AND 4th INSTAR AN. ARABIENSIS EXPOSED TO MALATHION (GI=adult malathion resistant population, SB=adult malathion susceptible population).



from this test.

The response of 4th instar larvae of the SB and G1 colonies to abate (temephos) was also tested.

Table 13 Larval mortalities of fourth instar *An. arabiensis* after 24hours exposure to abate over a range of dosages.

CONCENTRATION	G1		SB	
	DEAD/TOTAL	%	DEAD/TOTAL	%
0.0025	0/60	0	2/60	3.3
0.005	12/60	20	15/60	25
0.006	42/90	46.7	30/80	37.5
0.0075	82/89	92.1	81/85	95.3
0.01	56/58	96.6	42/45	93.3
0.02	80/80	100	79/79	100

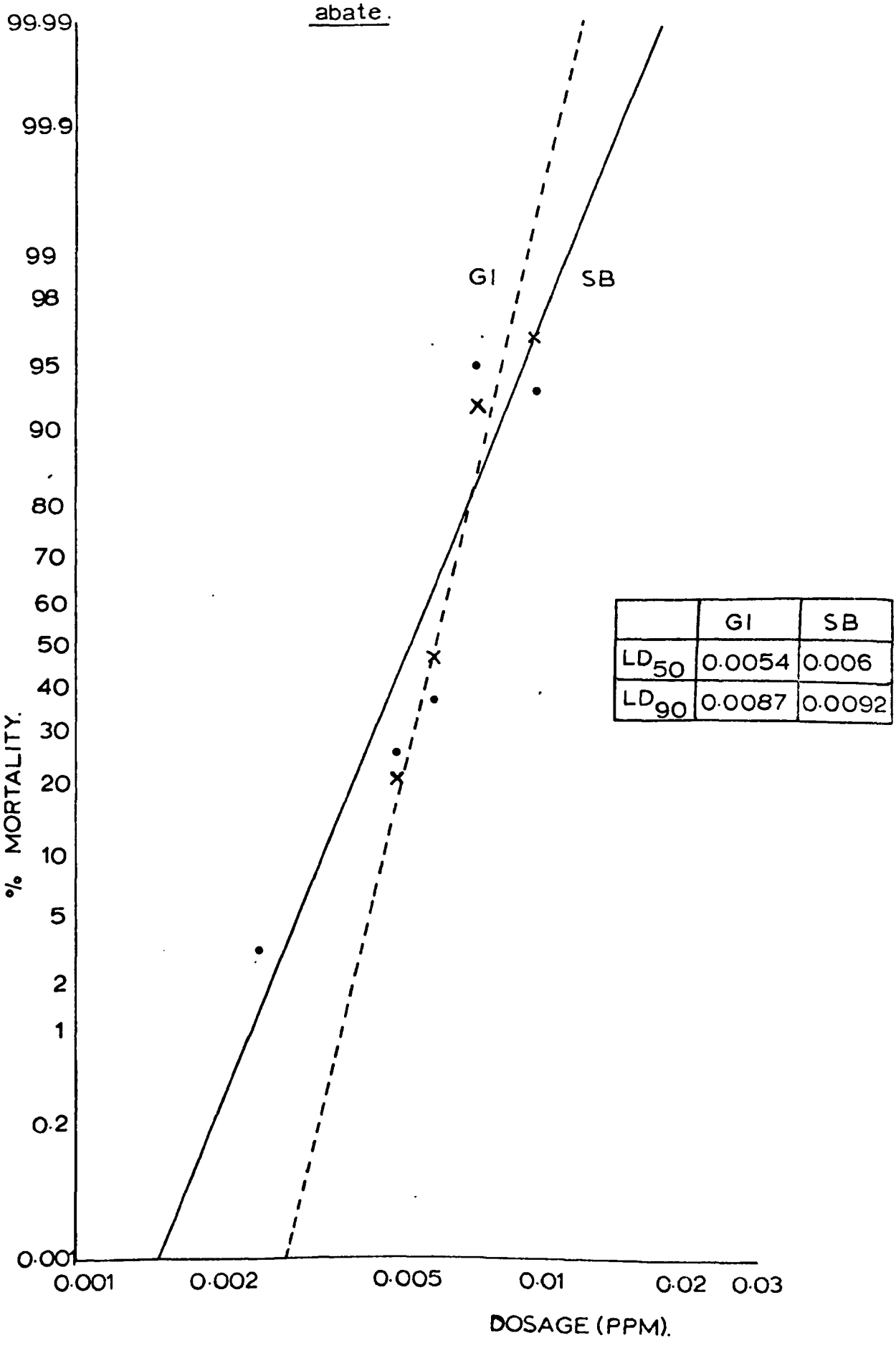
The results show that both G1 and SB colonies are susceptible to abate. One hundred percent mortality was obtained for both colonies at a concentration of abate well below the level of the WHO recommended discriminating dosage of 0.25ppm (see Figure 9 and Table 13).

SECTION II. SELECTION OF HOMOZYGOUS RESISTANT STOCKS.

After determination of the adult resistance spectra it was necessary to select stocks for homozygosity for the various insecticide resistances. This had two major functions

a) To confirm resistance where initial mortalities were high and survival may have been due to slight changes in the efficacy of the papers.

Figure 9. Log-dosage-probit lines for 4th instar An. arabiensis against abate.



	GI	SB
LD_{50}	0.0054	0.006
LD_{90}	0.0087	0.0092

b) To prepare the stocks for further genetical and biochemical studies of the resistance mechanisms.

As it was not possible at this stage to determine what genotypes were represented after each selected generation, selection pressure was increased when a general decrease in the % mortality was observed over several generations. Selection pressure was usually increased by increasing the exposure time, generally at hourly intervals, unless this produced greater than 98% mortality, in which case half hourly intervals were used. All emergents were selected in every generation in an attempt to maintain a constant selection pressure.

General key for Tables 14-18

GEN = generation of selection.
E = exposure time in hours

D = number of mosquitoes dead after insecticide exposure and a 24hour recovery period.

T = total number of mosquitoes tested.

% = percentage mortality.

C = concentration (% solution spread at 3.6 $\mu g/cm^2$) of insecticide (where insecticide concentration rather than time was varied during selection).

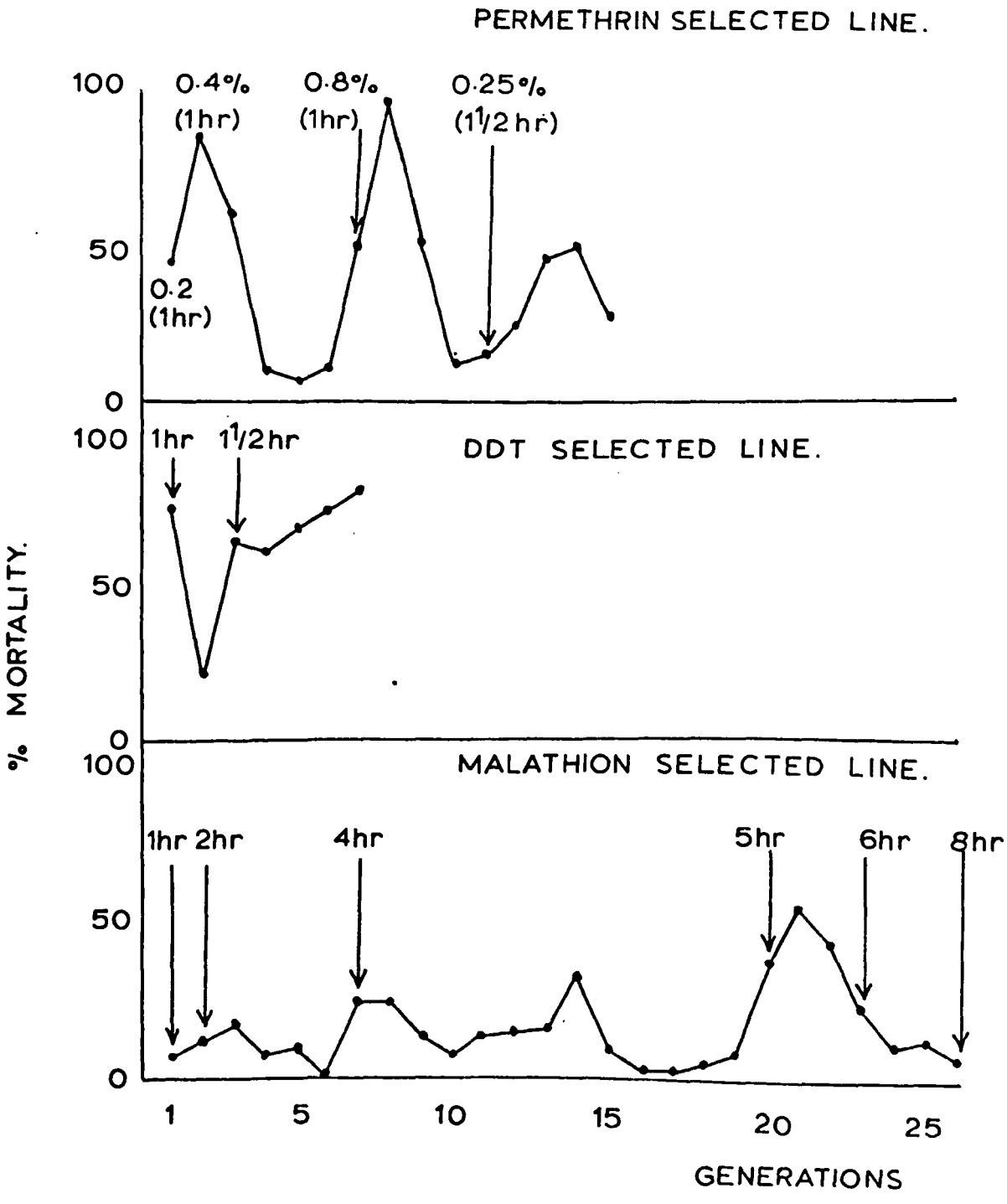
Arrows and times on Figures 10-14 indicate the start of selection with a particular length of exposure time.

Initial mortalities of *An.arabiensis* (G1) with 5% malathion for 1hour were low and after one generation the

Table 14. Selection of *An. arabiensis* (G1) with malathion DDT and permethrin.

Generations	Permethrin				DDT 4%			Malathion 5%		
	E	C	D/T	%	E	D/T	%	E	D/T	%
1	1	0.2	29/65	44.6	1	159/214	74.3	1	63/815	7.7
2	1	0.4	351/406	86.5	1	10/43	23.3	2	89/698	12.8
3	1	0.4	34/55	61.8	1½	80/123	65	2	36/187	19.3
4	1	0.4	20/184	10.9	1½	150/235	63.8	2	33/335	9.9
5	1	0.4	20/237	8.4	1½	29/42	69	2	16/146	10.9
6	1	0.4	27/240	11.3	1½	159/210	75.7	2	0/152	0
7	1	0.8	32/62	51.6	1½	25/30	83.3	4	16/58	27.6
8	1	0.8	36/37	97.3				4	35/140	25
9	1	0.8	49/91	53.8				4	47/316	14.9
10	1	0.8	7/56	12.5				4	57/679	8.4
11	1	0.25	6/36	16.7				4	26/191	13.6
12	1½	0.25	75/292	25.7				4	6/41	14.6
13	1½	0.25	15/31	48.4				4	20/137	14.6
14	1½	0.25	150/294	51				4	51/154	33.1
15	1½	0.25	10/35	28.6				4	22/240	9.2
16								4	2/56	3.6
17								4	14/361	3.9
18								4	2/40	5
19								4	26/268	9.2
20								5	240/606	39.6
21								5	209/386	54.2
22								5	440/1023	43
23								6	212/903	23.5
24								6	11/103	10.7
25								6	19/143	13.3
26								8	13/135	9.6

FIGURE 10. SELECTION OF AN. ARABIENSIS (GI) WITH MALATHION, DDT AND PERMETHRIN.



selection pressure was increased to 5% malathion for two hours resulting in a slight increase in mortality (see Table 14 and Figure 10). Changes in the exposure time to four and five hours at generations 7 and 20 respectively also showed slight increases in mortality, however increasing the exposure time to six and eight hours at generations 23 and 26 respectively produced no resultant increase in mortality. At this stage the selected line was assumed to be homozygous with regard to malathion resistance.

DDT selection of G1 with 4% DDT for one hour produced a drop in mortality after one generation, but increasing the exposure time to 1½hours did not produce a decrease in mortality over five generations (see Figure 10). An increase in the exposure time to 2hours resulted in 100% mortality.

Selection of *An.atroparvus* (AT SPA) with 1% fenthion for 1hour produced a drop in mortality from 82.4% to 0% in five generations (see Table 15 and Figure 11), increasing the exposure time to 2hours produced 100% mortality. The selected line was therefore considered to be homozygous for low level fenthion resistance.

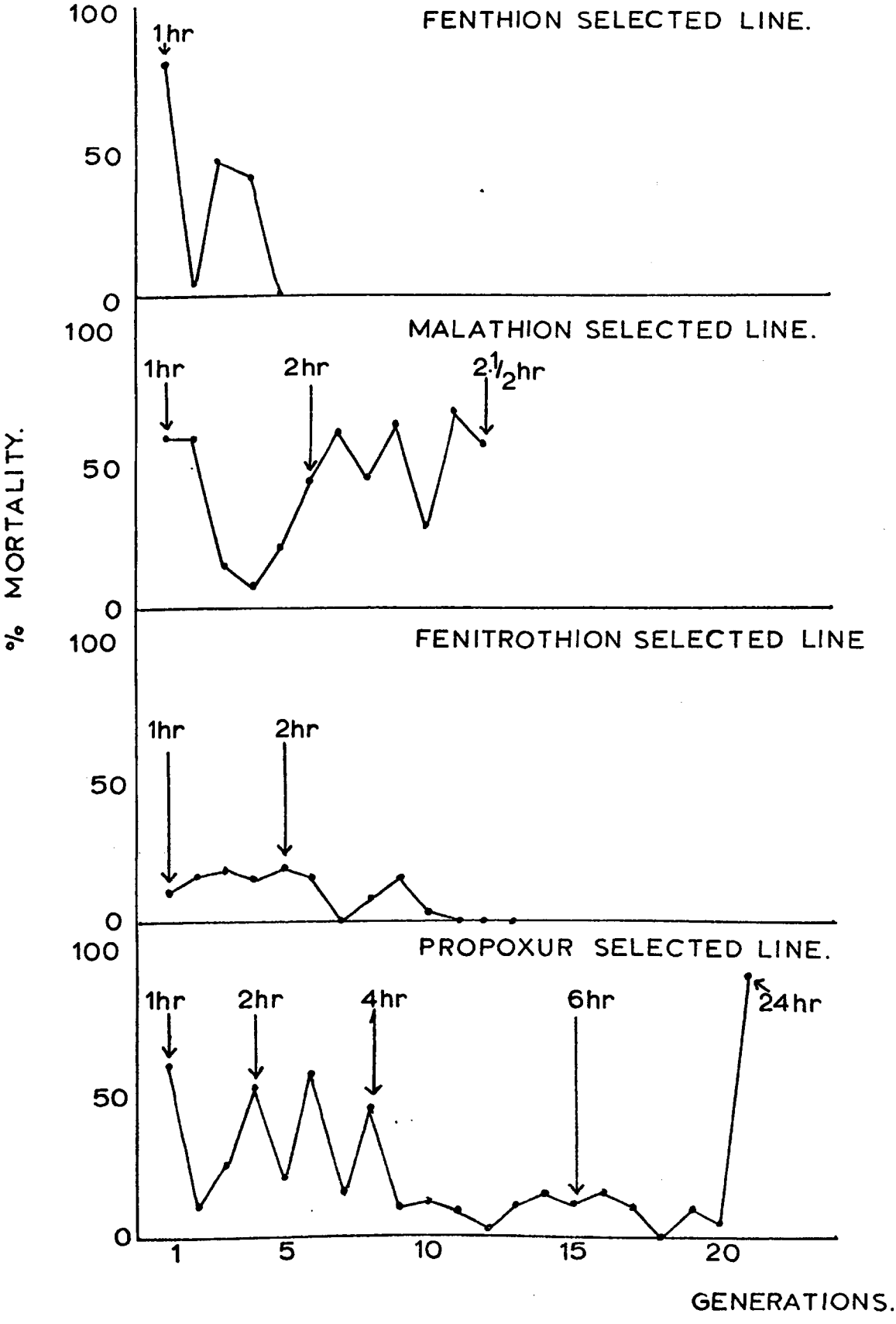
Increasing the insecticide exposure time in both the malathion and fenitrothion selected lines to 3½hours resulted in 100% mortality. After 12 generations of selection (see Figure 11) the fenitrothion line was considered to be homozygous for low level resistance. This was later confirmed by genetic studies (see Figure 23).

Propoxur resistance in the AT SPA population appeared

Table 15. Selection of adult *An. atroparvus* (AT SPA) with propoxur, malathion, fenthion and fenitrothion.

Gen	2.5% Fenthion			1% Fenitrothion			5% Malathion			0.1% Propoxur		
	E	D/T	%	E	D/T	%	E	D/T	%	E	D/T	%
1	1	84/102	82.4	1	20/156	12.8	1	56/92	60.9	1	33/54	61.0
2	1	1/23	4.3	1	3/17	17.6	1	37/61	60.6	1	14/108	12.9
3	1	26/53	49.1	1	35/179	19.6	1	35/212	16.5	1	53/206	25.7
4	1	24/55	43.6	1	26/168	15.5	1	16/195	8.2	2	90/173	52.0
5	1	0/6	0	2	64/320	20.0	1	5/21	23.8	2	22/102	21.6
6	1	0/41	0	2	15/96	15.6	2	6/13	46.2	2	61/103	59.2
7				2	0/30	0	2	81/128	63.3	2	21/127	16.5
8				2	3/37	8.1	2	44/94	46.8	4	130/289	45.0
9				2	12/74	16.2	2	40/61	65.6	4	28/251	11.2
10				2	4/101	4.0	2	50/170	29.4	4	24/188	12.8
11				2	0/40	0	2	150/210	71.4	4	25/279	9
12				2	0/30	0	2½	116/196	59.2	4	1/42	2.4
13										4	9/76	11.8
14										4	11/73	15.1
15										6	12/100	12.0
16										6	9/58	15.5
17										6	11/97	11.3
18										6	0/20	0
19										6	10/100	10
20										6	3/54	5.6
21										24	46/50	92.0

FIGURE 11. SELECTION OF AN. ATROPARVUS (AT SPA) WITH PROPOXUR, FENITROTHION, FENTHION AND MALATHION.



to be of a much higher level than the OP resistances, as the colony showed only low mortality when exposed to 0.1% propoxur for 6hours, and there were some survivors of the 20th selected generation after exposure to 0.1% propoxur for 24hours (see Table 15).

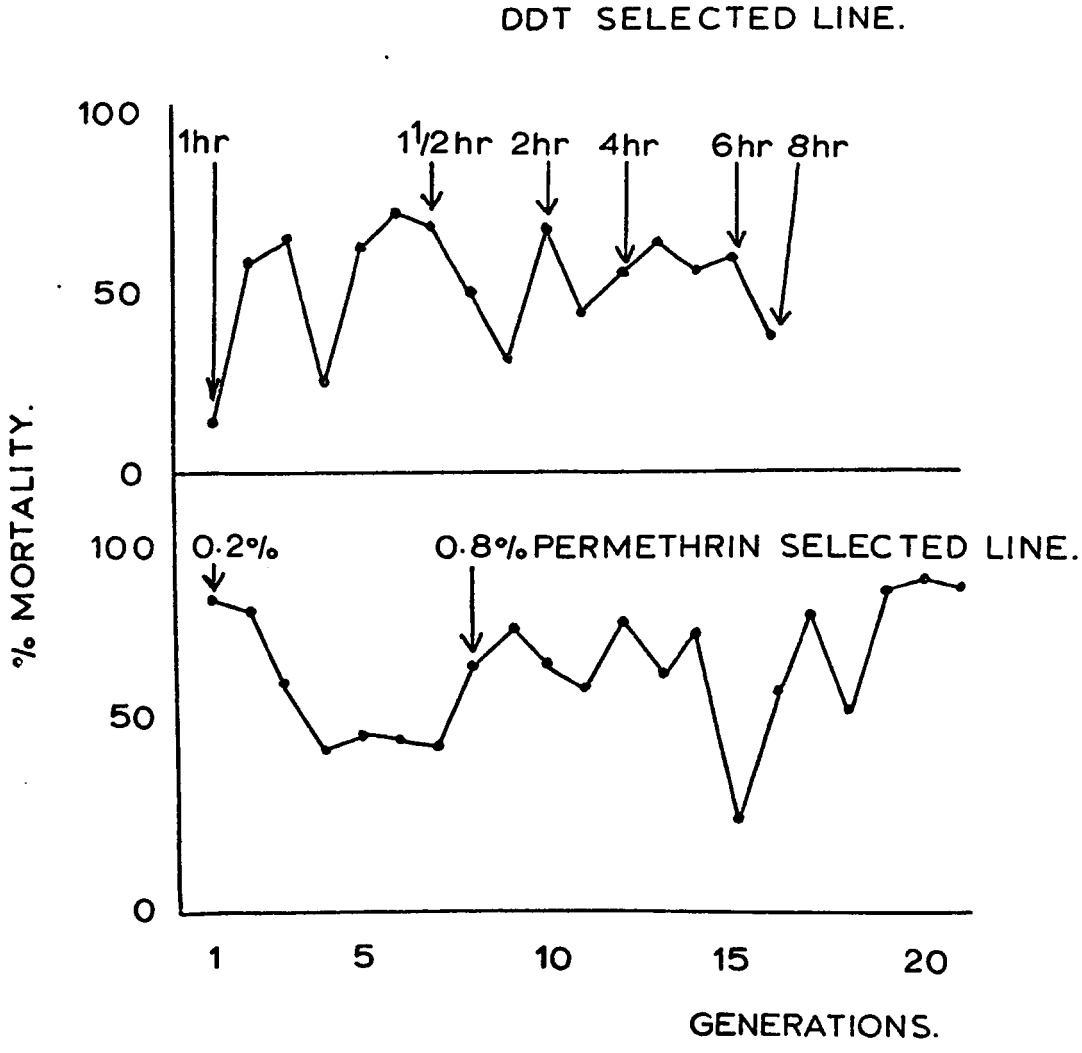
It can be seen from Figure 12 that difficulties were encountered in selecting out a pure permethrin resistant stock from the IAN strain of *An.gambiae* using the standard WHO test method. Because of the very rapid action of permethrin, the general selection method of increasing the exposure time could not be used with this insecticide, as mosquitoes were normally knocked-down during the initial hours exposure time. Therefore an increase in dosage was used to try and increase the selection pressure. This had very little effect on the IAN strain over 12 generations, the mortality showing no definite downward trend. With *An.arabiensis* (G1) this selection method was a little more successful as this population showed a decrease in % mortality over several generations after each increase in the dosage of insecticide used. However due to the problems of selection, particularly with the IAN colony and the AT SPA colony, a different method of selection using the criteria of knock-down and mortality was adopted (details of results from this type of selection are given in Section VII pp 201 – 208).

The IAN colony did however show a 'normal' response to DDT selection. Selection pressure was gradually increased from 1hour to 8hours over 16 generations. This selection

Table 16. Selection of *An.gambiae* (IAN) with permethrin and DDT

Gen	4% DDT			Permethrin			
	E	D/T	%	E	C	D/T	%
1	1	27/183	14.8	1	0.2	399/466	85.6
2	1	126/214	58.8	1	0.2	989/1193	82.9
3	1	126/196	64.3	1	0.2	1107/1814	61.0
4	1	27/111	24.3	1	0.2	212/473	44.8
5	1	15/24	62.5	1	0.2	145/295	49.3
6	1	161/229	70.3	1	0.2	126/265	47.5
7	1½	275/408	67.4	1	0.2	320/690	46.4
8	1½	145/293	49.5	1	0.8	961/1424	67.5
9	1½	118/383	30.8	1	0.8	546/697	78.3
10	2½	254/377	67.4	1	0.8	480/720	66.6
11	2½	148/350	42.3	1	0.8	209/339	61.7
12	4	730/1365	53.5	1	0.8	546/696	78.3
13	4	463/737	62.8	1	0.8	756/1170	64.6
14	4	139/253	54.9	1	0.8	742/968	76.6
15	6	44/75	58.7	1	0.8	27/108	25.0
16	8	13/35	37.1	1	0.8	111/197	56.4
17				1	0.8	171/212	80.7
18				1	0.8	29/53	54.7
19				1	0.8	393/445	88.3
20				1	0.8	400/444	90.1

FIGURE 12. SELECTION OF AN. GAMBIAE (IAN PIO) WITH PERMETHRIN AND DDT.



however did not appear to have produced a homozygous resistant stock. As there was some mortality on one hours exposure to 4% DDT after the colony had been left unselected for four generations. Prassitisuk (1979) selecting a similar colony of *An.gambiae* with 4% DDT showed 97.5% mortality in IAN P7 (7th selected generation) and was unable to select at a higher level.

Table 17. Selection of *An.stephensi* (ST IRAQ and ST IRAN) with malathion and fenitrothion.

ST IRAQ	Malathion			Fenitrothion		
Gen	E	D/T	%	E	D/T	%
1	1	194/740	26.2	1	359/486	73.9
2	1	195/613	31.8	1	290/455	63.7
3	1½	411/776	53.0	1	138/303	45.5
4	1½	114/337	33.8	1	182/460	39.6

ST IRAN	Malathion			Fenitrothion		
Gen	E	D/T	%	E	D/T	%
1	1	438/1216	36.0	1	1766/1795	98.4
2	1	203/809	25.1	1	915/925	98.9
3	1	424/1830	23.2	1	754/770	97.9
4	1	126/448	28.1	1	938/952	98.5
5	1	55/445	12.4			
6	1½	154/634	24.3			
7	1½	76/219	34.7			
8	1½	304/1055	28.8			
9	1½	22/149	14.8			

FIGURE 13. SELECTION OF AN. STEPHENSI (ST IRAN AND ST IRAQ) WITH MALATHION AND FENITROTHION.

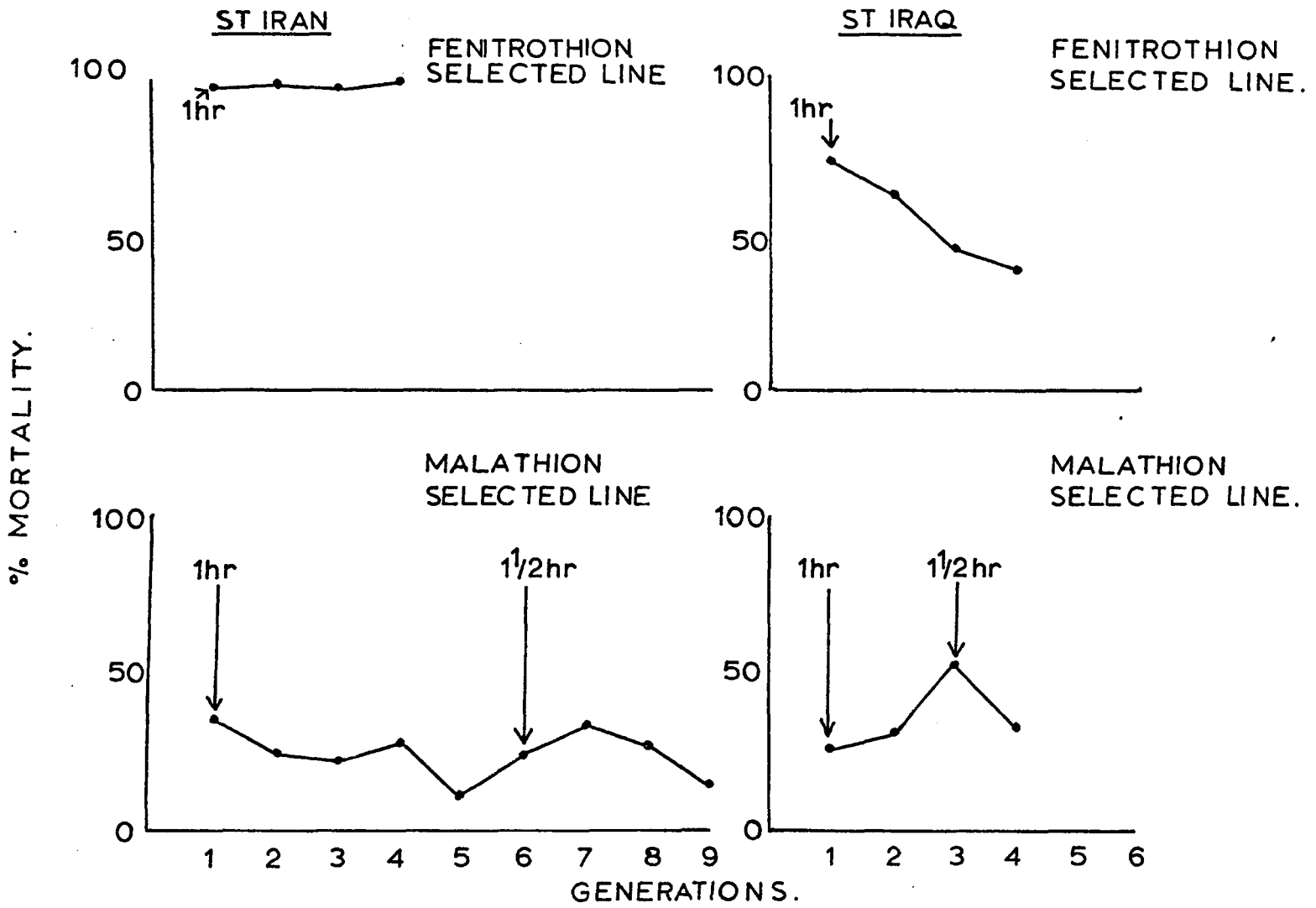
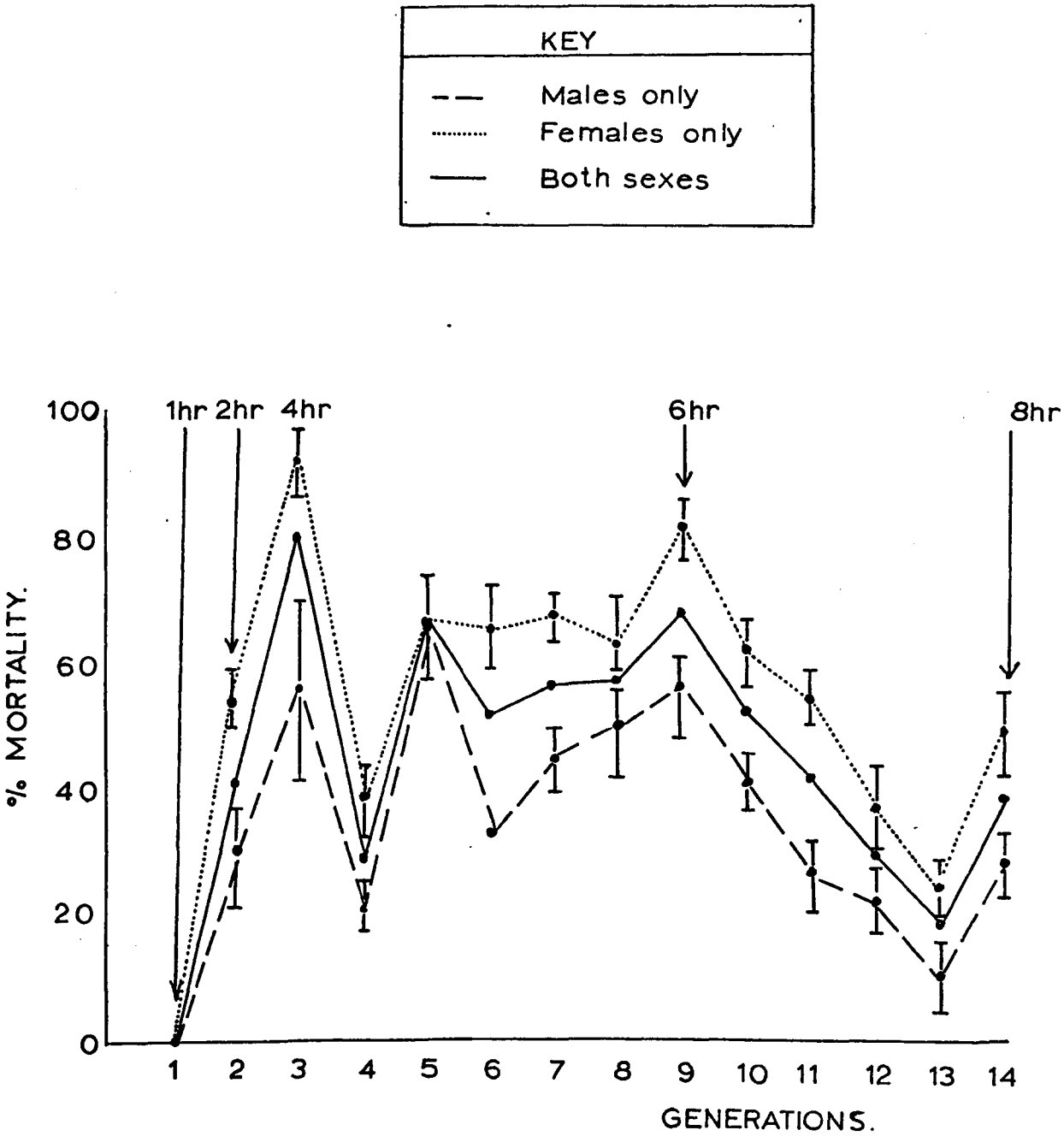


Table 18: Selection of *An.stephensi* (ST LA) with malathion.

GEN	MALE			FEMALE			COMBINED MORTALITY	
	E	D/T	%	E	D/T	%	D/T	%
1	1	0/47	0	1	0/45	0	0/92	0
2	2	29/96	30.2	2	44/84	52.4	73/180	40.7
3	4	145/263	55.1	4	406/441	92.1	551/704	78.3
4	4	70/320	21.9	4	130/370	35.1	200/690	29.0
5	4	216/323	66.9	4	231/347	66.6	447/670	66.7
6	4	35/110	31.8	4	110/171	64.3	145/281	51.6
7	4	93/210	44.3	4	172/258	66.7	265/468	56.6
8	4	234/469	49.9	4	365/578	63.2	599/1047	57.2
9	6	58/103	56.3	6	63/76	82.9	121/179	67.6
10	6	316/749	42.2	6	455/756	60.2	771/1505	51.2
11	6	107/409	26.2	6	266/492	54.1	373/901	41.4
12	6	57/253	22.5	6	94/261	36.0	151/514	29.4
13	6	12/117	10.2	6	37/152	24.3	49/269	18.2
14	8	83/289	28.7	8	157/324	48.5	240/613	39.2

FIGURE 14. SELECTION OF AN. STEPHENSI (ST LA) WITH MALATHION. (Male and female % mortality lines are given separatly with 95% confidence limits).



Fenitrothion selection of ST IRAN was stopped after 4 generations as the population had shown no decrease in mortality after this selection, and the very high mortality rate may indicate that survivors were due to slight differences in 'tolerance' rather than the presence of a resistance mechanism in the population. In contrast the fenitrothion selected line of ST IRAQ showed a decrease in mortality over 4 selected generations. Low mortalities were obtained for both ST IRAN and ST IRAQ after selection for several generations with 1½hours 5% malathion (see Figure 13 and Table 17) but increasing the exposure time to 2hours produced 100% mortality in both colonies.

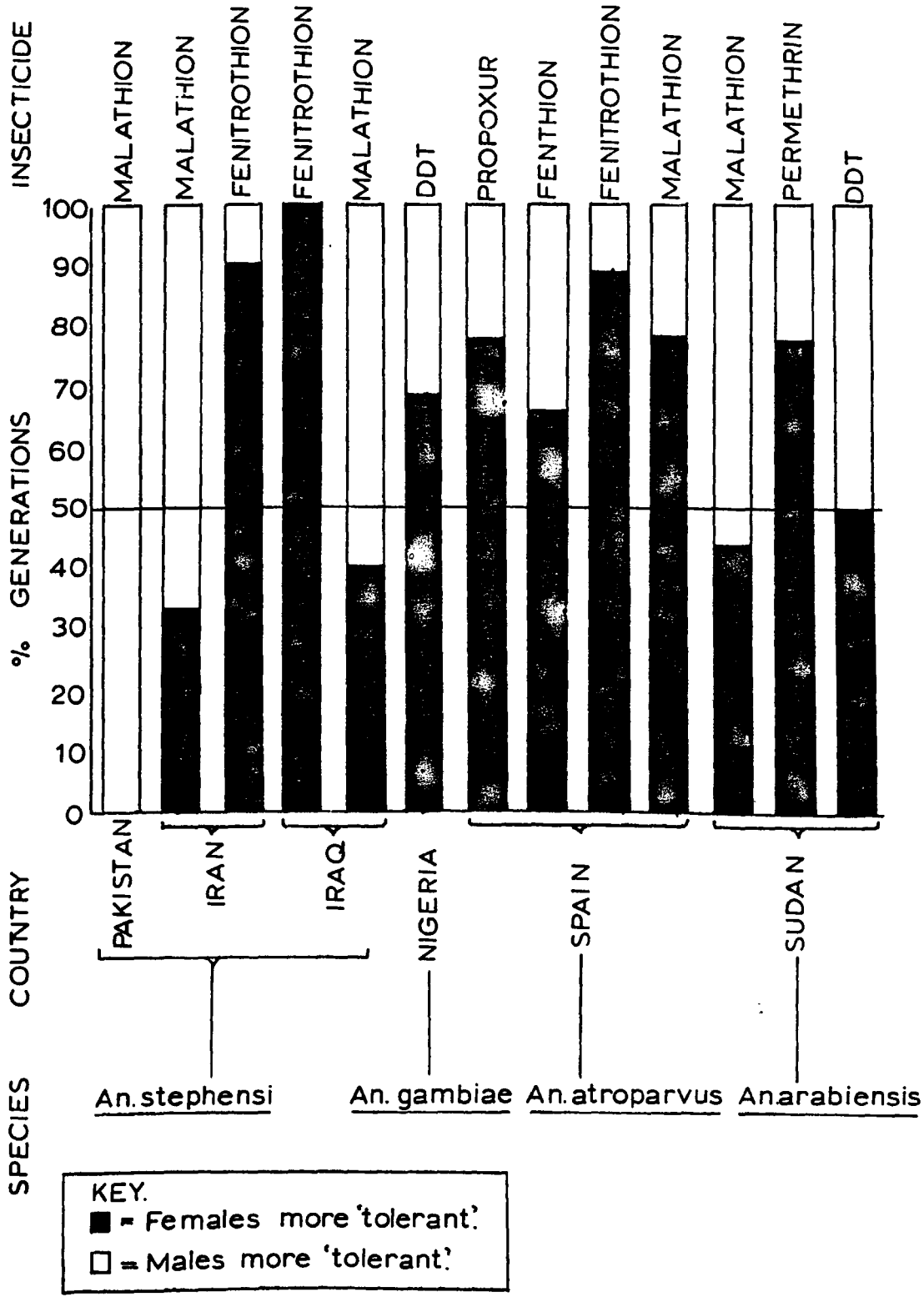
Malathion resistance in *An.stephensi* from Pakistan (ST LA) is of a higher level than ST IRAN or ST IRAQ as after 14 generations of selection of the ST LA population only 39.2% mortality was observed after exposure to 8hours 5% malathion. A few survivors (males only) were observed after a 24 hour exposure period to 5% malathion (95% mortality sample size 279).

THE RELATIVE INSECTICIDE SUSCEPTIBILITY OF MALES AND FEMALES.

The mortality of males and females, after insecticide exposure and a 24hour holding period, were routinely scored separately, so that male and female mortality could be calculated for each generation tested. The number of generations in which one sex showed a higher mortality than the other is represented in Figure 15.

The generally expected pattern is that males are slightly

Figure 15. The percentage of generations in which each sex shows a higher mortality than the other sex for all the species studied.



less tolerant than females to most insecticides, as the females are slightly larger than the males on average. It can be seen from Figure 15 that this is usually the case in this study. The males are slightly less tolerant to all insecticides than the females with the exception of malathion resistant *An.stephensi* (ST IRAN, ST IRAQ, ST LA) and *An.arabiensis* (G1). The most extreme example of males being more tolerant than the females to malathion is that of *An.stephensi* from Lahore, Pakistan where all 14 selected generations show this dichotomy (see Figure 15). This difference in response of the sexes was true at all five selection times used.

Investigation of the malathion susceptible *An.stephensi* population ST and the malathion susceptible *An.arabiensis* SENN showed that there was no difference in the response of the sexes to malathion in ST but a similar response to that of the resistant population was seen in the SENN population. With such a small sample of susceptible populations it is impossible to say whether the difference in the male and female response to malathion is connected with the resistance gene(s) or whether it is an inate characteristic of the species (see also Section VI).

Malathion resistance in *An.atroparvus*, AT SPA shows the generally expected pattern of the males being less tolerant than the females. This is the only malathion selected resistance in this study to follow this pattern.

SECTION III. CROSS-RESISTANCE SPECTRA.

From Section I it can be seen that resistance to more than one chemical or group of chemicals can occur in any one population. It is important to find out whether two or more mechanisms are present in the population ie. multiple resistance or if there is a single resistance mechanism responsible for the entire resistance spectrum ie. cross-resistance. By selecting separate lines to homozygosity or near homozygosity with each insecticide and then comparing the resistance levels to other insecticides in the selected lines with the original levels in the parental population, multiple and cross-resistance can be distinguished.

In the case of the MACHO population,where the resistance gene has been translocated to the Y chromosome, the resistance spectrum of the males and females are compared to demonstrate the cross-resistance spectrum conferred by the translocated propoxur-selected resistance gene. It can be seen from Figure 16 that the translocated gene(s) confers resistance to a broad range of OPs and carbamates, but not to the organochlorines, as there is no difference between the male and female response to 0.4% dieldrin. The low mortality for both males and females exposed to 1% dimethoate is unusual as there appears to be no cross-resistance in the female to other OPs. It may be that the papers were not impregnated with the correct amount of active ingredient for these tests

FIGURE 16. Cross resistance patterns in the MACHO males compared with the MACHO females.

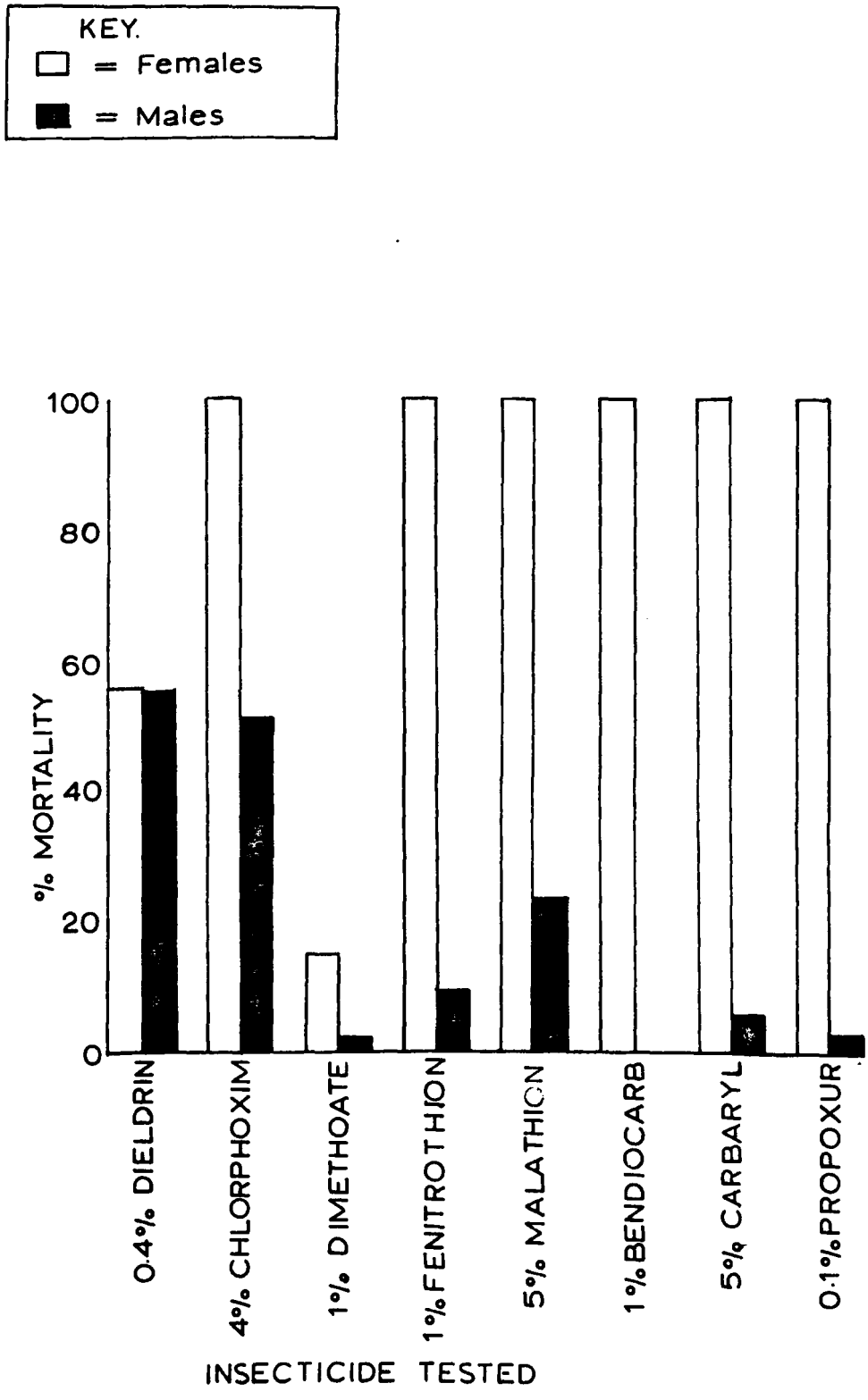
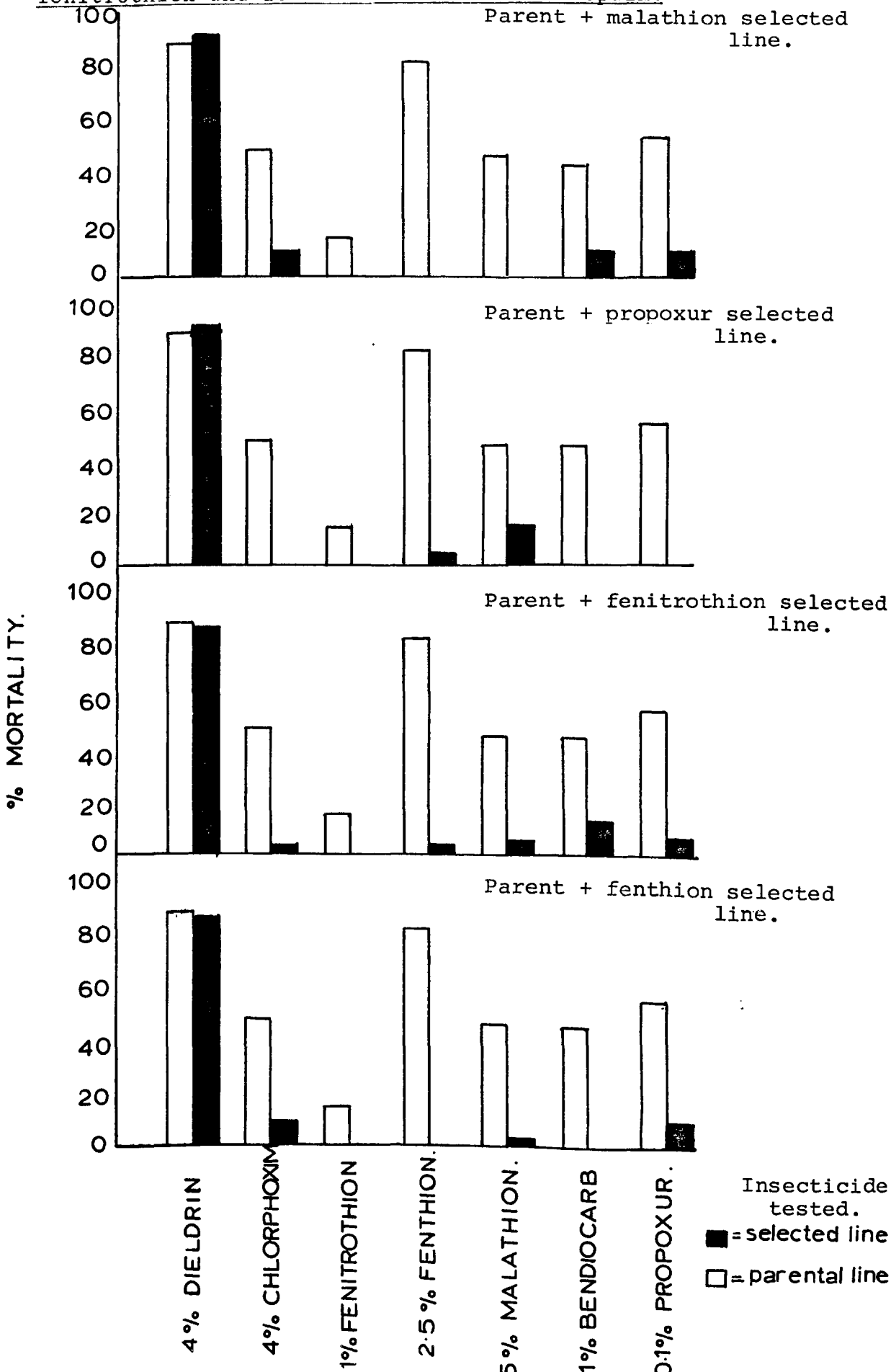


Table 19. The effect of selection with malathion, propoxur, fenitrothion and fenthion in the broad-spectrum resistant strain of An. atroparvus (AT SPA) from Spain.

INSECTICIDE TESTED	PARENT	SELECTING COMPOUND			
		MALATHION	PROPOXUR	FENITROTHION	FENTHION
4 % DIELDRIN	88.9 (45)	92.9 (56)	91.3 (69)	86.1 (36)	87.2 (39)
4% CHLORPHOXIM	48.2 (110)	10 (20)	0 (27)	1.8 (55)	9.5 (21)
1% FENITROTHION	15 (180)	2.9 (35)	0 (35)		0 (27)
2.5% FENTHION	82.4 (102)	4.5 (44)	4 (25)	2.9 (69)	
5% MALATHION	46.7 (122)		15.4 (43)	4.5 (22)	3.1 (32)
1% BENDIOCARB	45.5 (99)	8.1 (37)	0 (62)	12.2 (41)	0 (39)
0.1% PROPOXUR	50.4 (114)	9.3 (43)		6.3 (16)	8.9 (45)

(Figures in parenthesis are numbers tested).

Figure 17. The effect of selection with malathion, propoxur fenitrothion and fenthion in AT SPA from Spain.



as some difficulty was encountered in trying to get this insecticide into solution. It should be noted that the IAN colony also gave only 50% mortality with this insecticide and there is no reason to suppose that this population has been exposed to dimethoate prior to laboratory colonisation.

The propoxur selected resistance gene does not confer cross-resistance to the synthetic pyrethroids, as 100% mortality was observed in both males and females exposed to permethrin and decamethrin (deltamethrin).

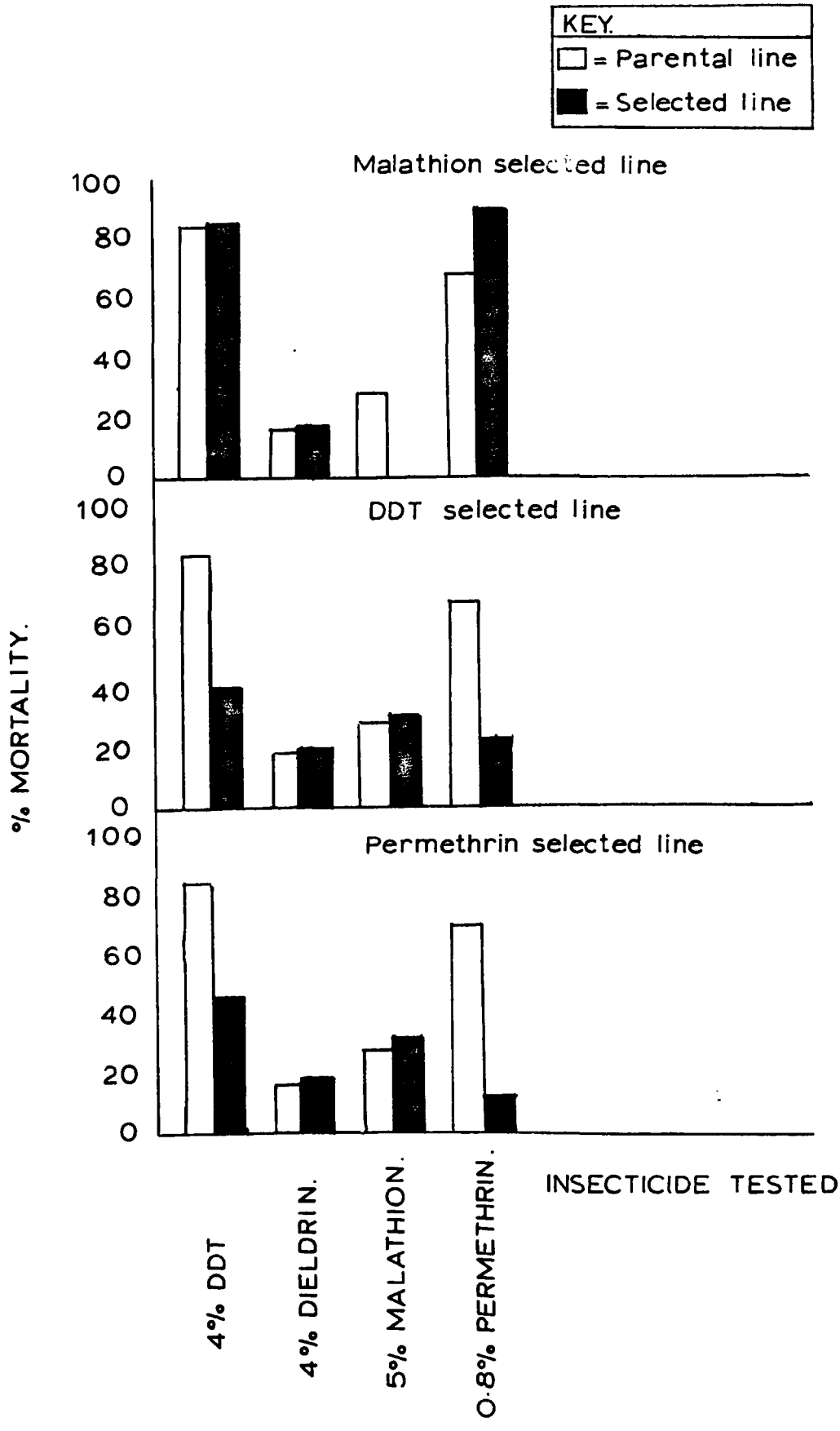
It can be seen from Figure 17 and Table 19 that laboratory selection with malathion, fenitrothion, fenthion or propoxur increases the proportion of resistant individuals to the range of OPs and carbamates tested in comparison to the proportion of resistant individuals in the parental population. It has no effect on the proportion of dieldrin resistant individuals however. This therefore indicates that there are at least two resistance mechanisms in this population, one responsible for resistance to dieldrin and a second non-specific mechanism which produces resistance to a number of OPs and carbamates. This is an identical pattern to that seen in the MACHO population, with the exception of resistance to fenthion which though found in the AT SPA population is absent in the MACHO population.

Table 20 shows that there is no cross-resistance from malathion to permethrin or DDT in *An.arabiensis* from Sudan, there is however good evidence in the permethrin selected line of cross-resistance to DDT and similarly with the DDT selected

Table 20. The effect of selection with malathion DDT and permethrin of the resistant strain of An. arabiensis from the Sudan (GI).

INSECTICIDE AND CONCENTRATION	PARENT	SELECTING INSECTICIDE		
		MALATHION	DDT	PERMETHRIN
4% DDT	83.9 (570)	85.2 (263)		45.8 (144)
4 % DIELDRIN	16.1 (192)	16.4 (116)	18.3 (142)	18.5 (211)
5% MALATHION	28.1 (463)		28.4 (321)	29.2 (72)
0.8% PERMETHRIN	68.7 (99)	89 (73)	22.5 (89)	

Figure 18. The effect of selection with malathion, DDT and permethrin on the levels of resistance to other chemically related and unrelated insecticides in An. arabiensis (G1).



line of cross-resistance to permethrin. As there is no evidence of permethrin spraying against *An.arabiensis* in the Gezira, it is likely that the permethrin resistance has arisen as a result of DDT spraying.

In the case of *An.stephensi* from Iraq and Iran two lines were selected in the laboratory using malathion and fenitrothion as the selecting agents. Fenitrothion resistance was not observed in *An.stephensi* from Pakistan; selected lines from this population were established using DDT, malathion and phenthoate as the selecting agents. It can be seen from Tables 21 and 22 that DDT, dieldrin, malathion and fenitrothion resistance are unrelated in the three populations, as the levels of resistance to the three unselected insecticides in each selected line are similar to the resistance levels in the original parental populations. It can therefore be concluded that malathion and fenitrothion resistance in the ST IRAQ and ST IRAN colonies are not controlled by the same gene or genes.

There appears to be a relationship between malathion resistance and phenthoate resistance in the ST LA colony (see Table 22) as the malathion selected line shows a decrease in mortality when compared with the parental population exposed to phenthoate. The phenthoate selected line showed no mortality on 5% malathion for one hours exposure, as did the parental population, while the DDT selected line showed a low mortality after exposure to 5% malathion. However from this information it is not possible to say whether phenthoate

Table 21. The effect of selection with malathion and fenitrothion on the resistant strains of An. stephensi from Iraq and Iran. (figures in parenthesis are numbers tested).

INSECTICIDE AND CONCENTRATION.	PARENT	SELECTING COMPOUND	
		MALATHION	FENITROTHION
4% DDT	18.2 (33)	20.4 (78)	19.8 (103)
4% DIELDRIN	24.1 (290)	31.2 (168)	25.7 (129)
5% MALATHION	36.2(2,709)		40.1 (611)
1% FENITROTHION	84.6 (150)	87.4 (245)	

ST IRAQ.

INSECTICIDE AND CONCENTRATION	PARENT	SELECTING COMPOUND	
		MALATHION	FENITROTHION
4% DDT	2.2 (22)	4.2 (48)	3.2 (31)
4% DIELDRIN	49.8(231)	52.9 (102)	61.7 (47)
5 % MALATHION	49.6(1,126)		51.3 (39)
1% FENITROTHION	98.4(1,795)	93.1 (247)	

ST IRAN.

Figure 19. The effect of selection with one chemical on the levels of resistance to other chemically related and unrelated compounds, compared with the parental resistance levels in An. stephensi (ST IRAQ and ST IRAN).

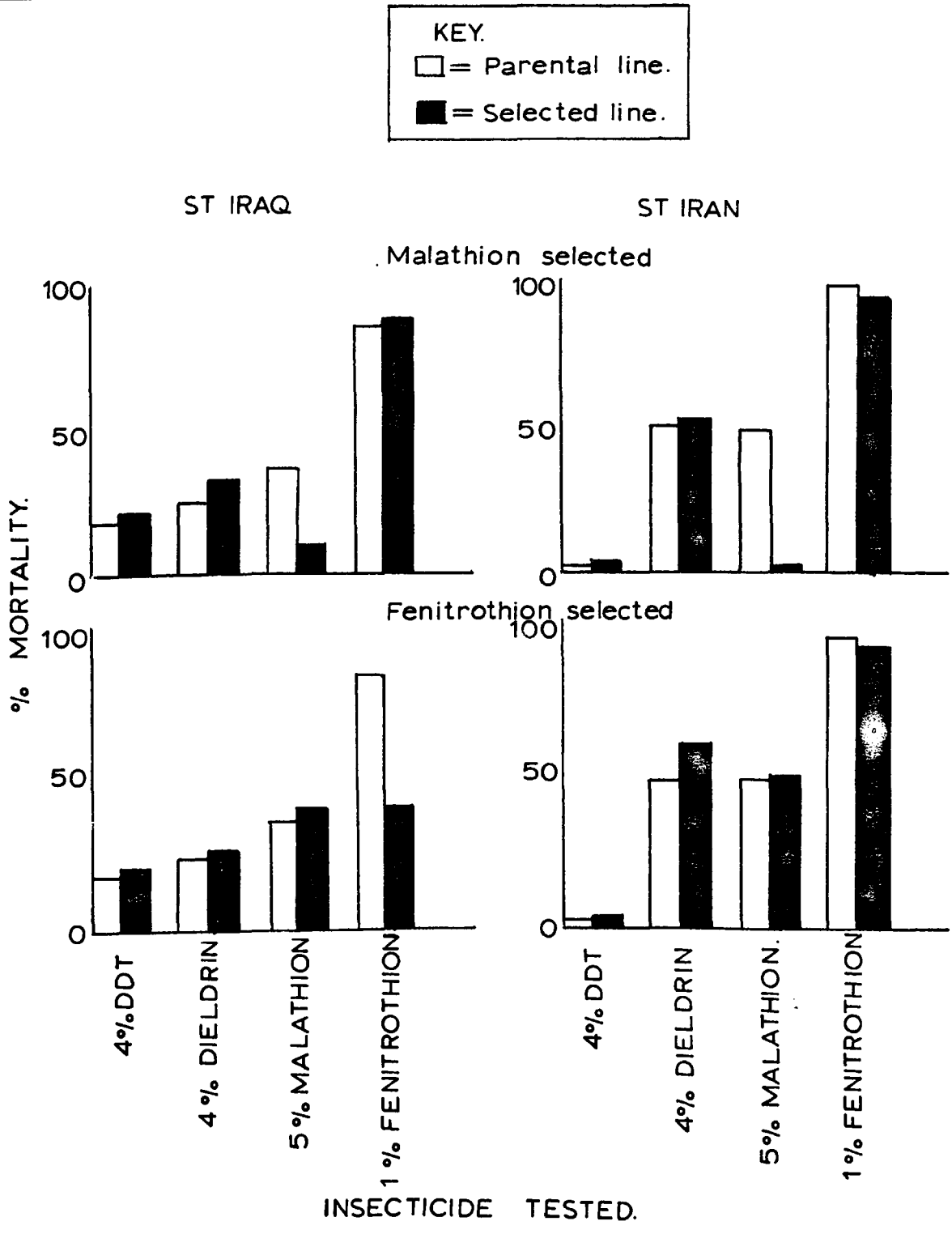


Table 22. The effect of selection with DDT, malathion and phenthoate on the resistant strain of An. stephensi (ST LA) from Pakistan. (Figures in parenthesis are numbers tested).

INSECTICIDE AND CONCENTRATION	PARENT	SELECTING COMPOUND		
		MALATHION	DDT	PHENTHOATE
4% DDT	36.1 (61)	45.2 (42)		37.5 (56)
4% DIELDRIN	100 (147)	100 (32)	100 (21)	100 (36)
5% MALATHION	0 (92)		7.2(69)	0 (261)
1 % FENITROTHION	100 (540)	100 (27)	100 (46)	100 (52)
10% PHENTHOATE	4.8(292)	0 (211)	12.5(96)	

selection is also selecting for malathion resistance. The malathion selection experiments (see Figure 14) indicated that malathion resistant individuals of the ST LA colony would survive 6hours exposure to 5% malathion. The DDT and phenthoate selected lines were therefore tested on 5% malathion for 6hours. The results were as follows;-

Insecticide		selected line	
	Parent	DDT	Phenthoate
1hr 5% malathion	0 (92)	7.2 (69)	0 (261)
6hr 5% malathion	53.1 (98)	65.3 (101)	12.5(144)

% Mortality (number tested).

The results for a 6hour exposure to 5% malathion strongly indicate that phenthoate selection has increased the level of malathion resistance when compared with the parental line, so that either phenthoate and malathion resistance are controlled by the same gene(s) or they are controlled by genes which are very closely linked together.

SECTION IV. GENETICS OF INSECTICIDE RESISTANCE.

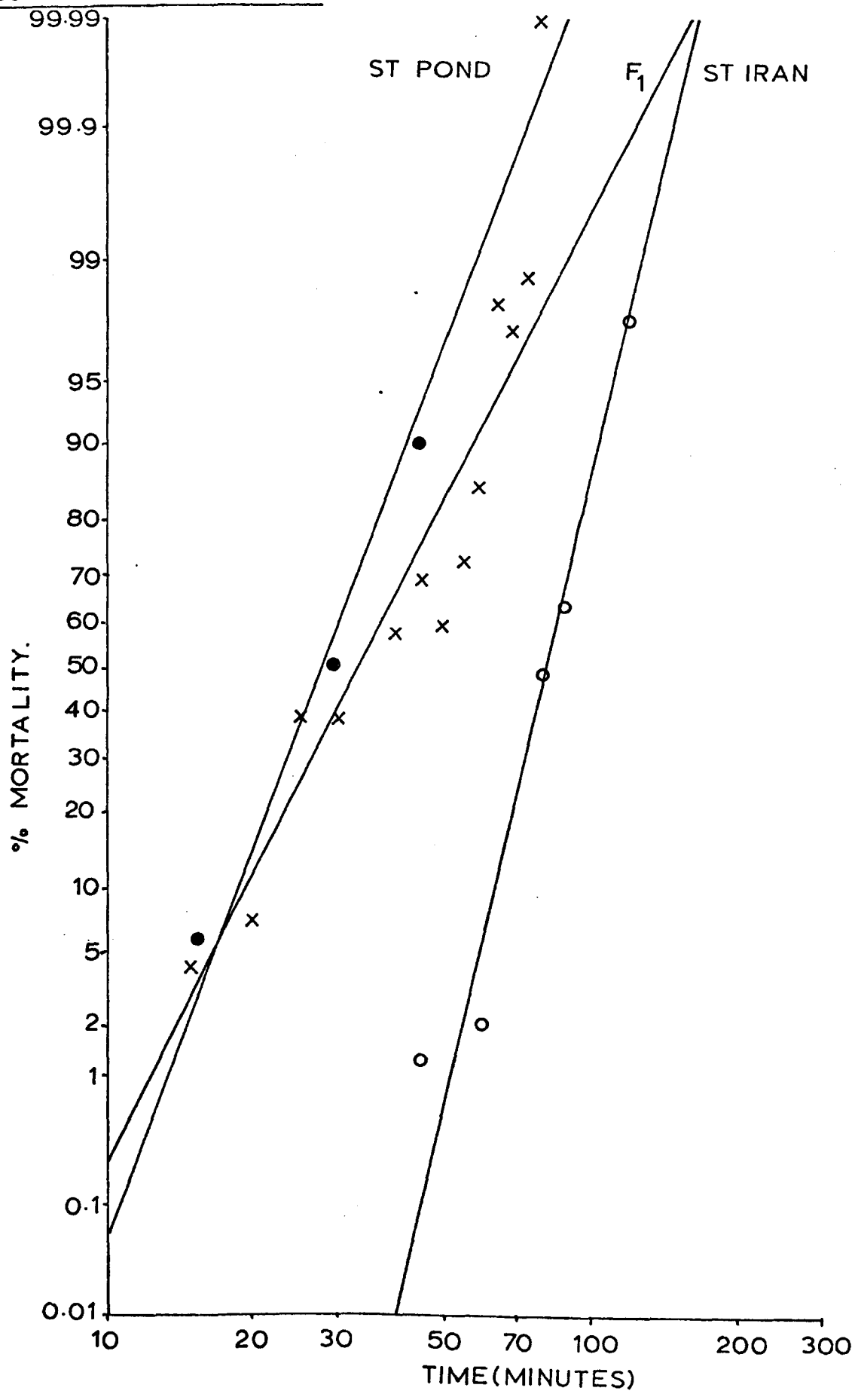
a) Malathion resistance in An.stephensi from Iran.

A reciprocal mass cross was carried out between ST POND a malathion susceptible strain from Pondicherry, India, and ST IRAN, a malathion resistant population from Bandar Abbas, Iran, (see Figure 13 for details of laboratory selection). At the time of crossing the ST IRAN colony was showing approximately 30% mortality of adults after 1½ hours exposure to

TABLE 23. Percentage mortalities after exposure of ST POND, ST IRAN and the reciprocal F_1 generations and first backross progeny to 5% malathion for various times.

TIME (MINS)	ST POND		F_1		BACKCROSS		ST IRAN	
	DEAD/TOTAL	%	DEAD/TOTAL	%	DEAD/TOTAL	%	DEAD/TOTAL	%
15	4/78	5.1	3/72	4.2	4/80	5	0/40	0
20			6/83	7.2	7/50	14		
25			36/94	38.3				
30	40/80	50	35/93	37.6	36/82	43.9	0/60	0
35			81/147	55.1				
40			35/61	57.4				
45	198/220	90	91/131	69.5	148/196	75.5	1/80	1.25
50			34/57	59.5				
55			24/33	72.7				
60	627/627	100	194/227	85.5	127/129	91.4	13/629	2
65			50/51	98				
70			70/72	97.2				
75			68/69	98.6				
80	149/149	100	78/78	100	60/60	100	140/296	47.3
90	19/19	100					510/796	64
120	40/40	100	40/40	100			279/286	97.6

Figure 20. Log-time probit-mortality lines for An. stephensi (ST POND, ST IRAN and reciprocal F_1 generations) after exposure to 5% malathion.



5% malathion. Log-time probit mortality lines were constructed for both ST POND and ST IRAN and for the F_1 progeny of the reciprocal crosses. It can be seen from Figure 20 that the F_1 shows intermediate resistance, which is slightly closer to the susceptible parental line than the resistant line. From Figure 20 the LT_{50} and LT_{90} values were as follows:-

Colony	LT_{50}	LT_{90}
ST POND	28.6	42.5
ST IRAN	83.7	105.5
F_1	33.9	62.8

(numbers are time in minutes).

There was no difference in the response to malathion testing of the two reciprocal F_1 generations indicating that the resistance is autosomally inherited.

Virgin F_1 females and F_1 males were reciprocally backcrossed to the susceptible parental strain and the resulting progeny tested on 5% malathion for one hour. The expected mortality on a single gene hypothesis would be 92.8%, the observed mortality was 91.4%. A Chi square test shows that this result is not significantly different from that expected assuming a single gene is responsible for malathion resistance in this population,

An F_2 generation was produced by allowing 100 F_1 individuals to interbreed. All the resulting F_2 progeny were tested on 5% malathion for one hour and gave 85.4%

mortality (sample size 610). On a single gene hypothesis you would expect 68% mortality (P = 0.001) and this result for the F_2 is therefore significantly different from that expected on the single gene theory.

Values for the slopes of the lines, chi squares for goodness of fit of the points to a straight line and the number of degrees of freedom are given in Table 24 . These

Table 24. Values for maximum likelihood fitting of LT-P lines of ST POND, ST IRAN and the reciprocal F_1 generations after exposure to 5% malathion.

Population	Slope line(± SD)	Chi square	Df	P
ST POND	6.29 (± 0.015)	1.32	I	ns
ST IRAN	12.78 (±0.003)	51.79	3	0.001
F_1	4.79 (± 0.010)	17.34	11	.05-0.01

results indicate that the points for ST IRAN and the reciprocal F_1 generations both deviate significantly from a straight line after exposure to 5% malathion. The ST IRAN response may indicate that this population was not homozygous for malathion resistance at the time of crossing. This may also account for the non-linear response of the F_1 generation and the high mortality in the F_2 generation. However the points for the F_1 do not follow the classical pattern expected from a heterogenious population. Further backcrosses and a repeat

of the original experiment after further insecticide selection of the resistant parental line should have been carried out on this population. However this was not possible as the colony was lost due to a thermostat failure in the insectary.

b) Malathion resistance in An. stephensi from Iraq.

A reciprocal mass cross was carried out between ST POND and ST IRAQ a malathion resistant strain of *An.stephensi* from Basrah, Iraq. At the time of crossing the ST IRAQ colony was showing approximately 17% mortality after 1½ hours exposure of one day old adults to 5% malathion (for details of selection see figure 13). The ST IRAQ colony showed 4.2% mortality at this stage after 1hours exposure to 5% malathion. Log-time probit-mortality lines were constructed for each of the parental lines and for the resulting F_1 generation. There was no difference in the response of the reciprocal F_1s to exposure to 5% malathion , indicating that the resistance is autosomally inherited.

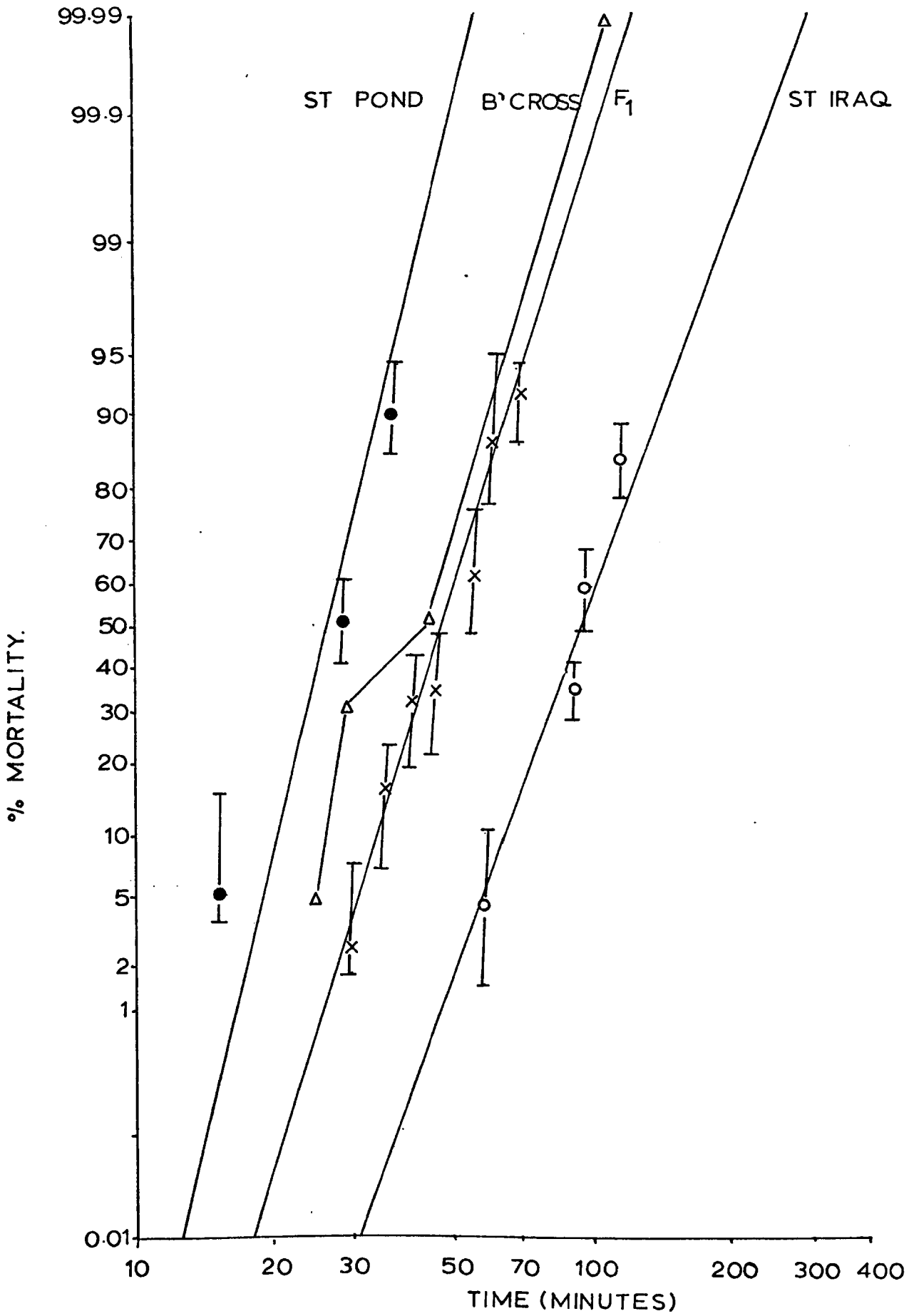
The F_1 progeny showed intermediate resistance, which was slightly closer to the susceptible parental line than the resistant line. The LT_{50} and LT_{90} values for the three populations from Figure 21 were as follows:-

COLONY	LT_{50}	LT_{90}
ST POND	28.6	42.5
ST IRAQ	96.7	132.6
F_1	47.9	68.2

Table 25. Percentage mortalities resulting from exposure of ST POND, ST IRAQ, reciprocal F_1s and (R x S) x S backcross progeny to 5% malathion for various times.

TIME (MINS)	ST POND		F_1		BACKCROSS		ST IRAQ	
	DEAD/TOTAL	%	DEAD/TOTAL	%	DEAD/TOTAL	%	DEAD/TOTAL	%
15	4/78	5.1	0/20	0	0/20	0	0/60	0
25			0/40.	0	2/40	5		
30	40/80	50	1/40	2.5	12/39	30.8	0/45	0
35			11/73	15.1				
40			16/52	30.8				
45	198/220	90	19/56	33.9	27/52	51.9	0/40	0
55			29/47	61.7			0/20	0
60	627/627	100	43/50	86	63/70	90	10/240	4.2
70			24/26	92.3				
95	19/19	100	20/20	100	20/20	100	114/337	33.8
100							77/130	59.2
120	40/40	100	10/10	100			164/196	83.7

Figure 21. Log time probit mortality lines for ST POND, ST IRAQ the reciprocal F_1 generation and first backcross progeny after exposure to 5% malathion.



Values for the slopes of the line, Chi squares for goodness of fit of the points to a straight line and the numbers of degrees of freedom are given in Table 26.

Table 26. Values for maximum likelihood fitting of LT-P lines of ST POND, ST IRAQ and the reciprocal F_1 generations after exposure to 5% malathion.

Population	Slope line (± SD)	Chi square	Df	P
ST POND	6.29 (±0.015)	1.32	1	ns
ST IRAQ	9.28 (±0.005)	23.63	2	<0.001
F_1	8.37 (±0.010)	5.22	5	ns

Reciprocal backcrosses were carried out in the following way :-

Virgin male and female progeny of the F_1 x susceptible reciprocal crosses were separated less than 24 hours after emergence and tested separately. The progeny were exposed to the insecticide for the minimum time that killed 100% of the parental susceptible population. Survivors of this treatment were used to produce a second backcross generation to the susceptible line and their progeny were treated as above.

Three generations of reciprocal backcrosses were carried out. The progeny were tested on 5% malathion for 1hour. Some of the progeny from the (R x S)♀ x S ♂ backcross were exposed for various times to 5% malathion to produce a log-dosage probit mortality line.

The results for 1hours exposure to 5% malathion of all four possible backcrosses are given in Table 27.

Table 27. Results of repeated reciprocal backcrosses to ST POND.

	(S x R) x S (R x S) x S		S x (S x R) S x (R x S)	
	Dead/Total	%	Dead/Total	%
1st backcross	63/70	90	80/98	81.6
2nd backcross	60/68	88.2	92/102	90.2
3rd backcross	79/84	94	48/53	90.6

The expected mortality in all backcrosses on the single gene hypothesis is 93%. Chi square tests, comparing observed and expected data showed that the values of P in all cases were not significant. i.e. The results of the backcrosses did not differ significantly from those expected if a single gene were responsible for malathion resistance in this population.

c) *Malathion resistance in An. stephensi from Pakistan.*

Log-time probit mortality regression lines were produced for the malathion susceptible ST and the malathion resistant ST MAL colonies (see Figure 22 and Table 28). The F_1 generation produced from a cross of ST and ST MAL gave a line lying between the two parentals (see Figure 22), indicating that the resistance is partially dominant. There was no significant difference between the response of the reciprocal F_1s indicating that the resistance is autosomally

Table 28.Percentage mortalities of ST and ST MAL after exposure to 5% malathion for various time intervals. (Figures in parenthesis are numbers tested).

Exposure time(minutes)	Colony tested	
	ST	ST MAL
15	51.6(124)	
20	62.4(85)	
25	88.4(259)	
30	90 (110)	
40	96.2(239)	
45	98.3(175)	
60	100 (145)	0 (52)
120		0 (40)
180		0 (240)
240		0.91(773)
270		3.2 (95)
300		1.6 (381)
400		13.1 (222)
480		23.2 (155)
600		73.1 (145)

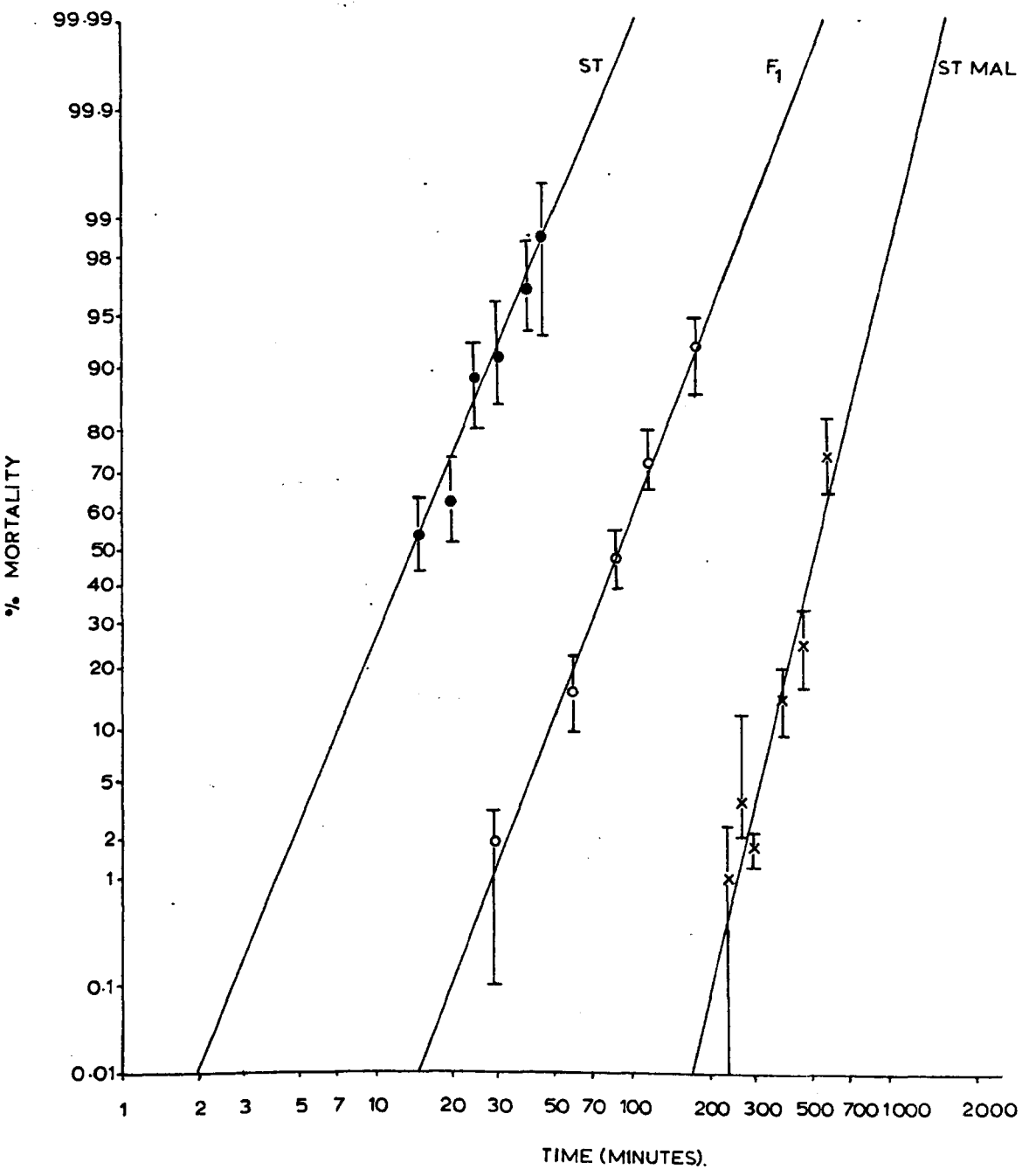
TABLE 29. Percentage mortalities of the F_1 generations from reciprocal crosses between ST and ST MAL strains of *An. stephensi* after exposure to 5% malathion for various times. (Figures in parenthesis are numbers tested).

TIME (MINS)	ST x ST MAL			ST MAL x ST		
	♂	♀	TOTAL	♂	♀	TOTAL
15	0(24)	0 (28)	0 (52)	0 (16)	0 (20)	0 (36)
30	0(40)	2.4 (42)	1.2 (82)	0 (24)	5 (20)	2.2 (44)
60	10.5(38)	16.7 (36)	13.5(74)	14.3(35)	18.9(37)	16.7 (72)
90	42.9(42)	50 (44)	46.5(86)	40 (40)	53.8(39)	46.8(79)
120	65.9 (41)	79.5(39)	76.3 (80)	61.6(39)	73.6(38)	67.5(77)
180	90 (40)	95 (40)	92.5(80)	82.9(35)	92.3(39)	87.8 (74)

Additive figures for the reciprocal F_1s are:-

TIME (MINS)	% MORTALITY (Number tested)
15	0 (88)
30	1.6 (126)
60	15.1 (146)
90	46.7 (165)
120	70.1 (157)
180	90.3 (154)

FIGURE 22. LOG–TIME PROBIT–MORTALITY LINES FOR ANOPHELES STEPHENSI, (ST, ST MAL AND RECIPROCAL F_1 GENERATIONS) AFTER EXPOSURE TO 5% MALATHION.



inherited. However the males in both F_1 generations were more 'tolerant' than the females (see Table 29), a phenomenon which was also noted in the ST MAL parental population (see Figure 14). The LT_{50} and LT_{90} values for the three populations from figure 22 were as follows:-

COLONY	LT_{50}	LT_{90}
ST	14.5	35
ST MAL	545	900
F_1	94.2	278

(figures are time in minutes).

Values for the slopes of the line, Chi squares for goodness of fit of the points to a straight line and the number of degrees of freedom are given in table 30.

Table 30. Values for maximum likelihood fitting of LT-P lines of ST, ST MAL and the reciprocal F_1s after exposure to 5% malathion.

Population	Slope line (± SD)	Chi square	Df	P
ST	4.4 (± 0.02)	8.07	4	ns
ST MAL	7.5 (± 0.009)	27.6	4	<0.001
F_1	4.7 (± 0.012	1.42	3	ns

These show that the points for ST and the reciprocal

Table 31. Percentage mortality in reciprocal backcrosses of the F_1 generation to the susceptible ST parent. All tests were with 5% malathion for one hour (figures in parentheses are numbers tested).

Backcross	% mortality
ST x (ST x ST MAL)	51.9 (189)
ST x (ST MAL x ST)	61.3 (199)
(ST x ST MAL) x ST	54.0 (187)
(ST MAL x ST) x ST	53.1 (130)

Table 32. Percentage mortalities of the four pooled backcross generations of the ST x ST MAL cross.

	% mortality (numbers tested)
1st backcross	55.3 (705)
2nd backcross	60.2 (642)
3rd backcross	54.8 (453)
4th backcross	56.6 (286)

Table 33. Chi-square values for goodness of fit of each of the four pooled backcross generations to the single gene hypothesis.

	χ^2	Df	P
1st backcross	1.2	1	ns
2nd backcross	1.73	1	ns
3rd backcross	1.195	1	ns
4th backcross	0.073	1	ns

F_1s conform to a straight line. The points for the ST MAL population however deviate significantly from a straight line, the observed deviation appears to be due to point scatter rather than a curvilinear response of log-time (see Figure 22).

Reciprocal backcrosses to the susceptible ST parents were performed and the resultant progeny were tested on 5% malathion for 1hour, as this was the minimum dosage that gave 100% kill of the susceptible parentals. The results for the reciprocal backcrosses are given in Table 32 . On the basis of a single gene hypothesis you would expect 57.5% mortality in each of the backcross generations. A Chi square test was used to test the goodness of fit of the results obtained for the four pooled backcross generations; the results are given in Table 33 . These results are in agreement with the single gene hypothesis. It can therefore be concluded that malathion resistance in *An. stephensi* from Pakistan is dependent on a single autosomal partially dominant gene.

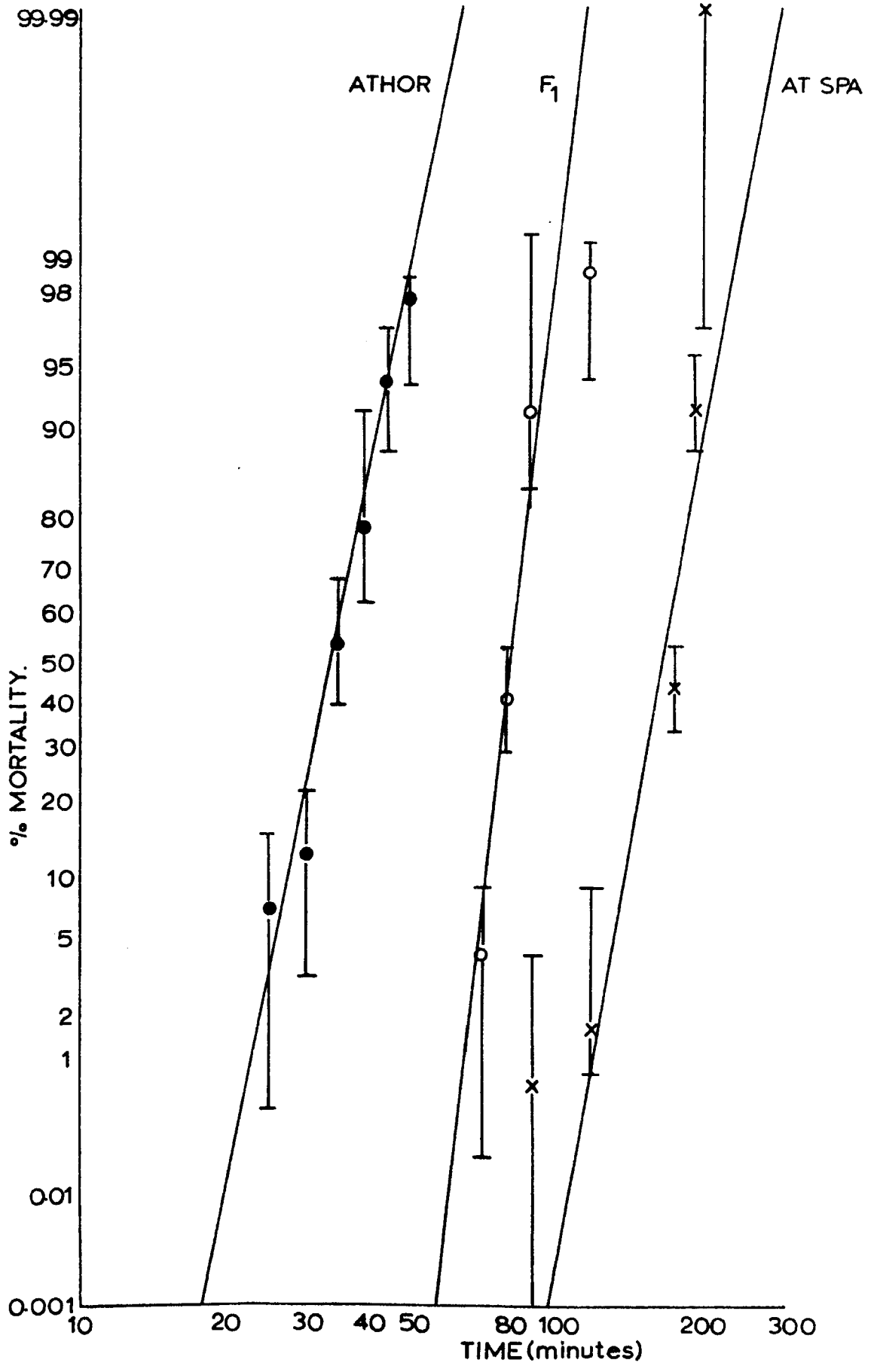
d) Fenitrothion resistance in An. atroparvus from Spain.

A reciprocal mass cross was carried out between ATHOR, a fenitrothion susceptible strain from England and AT SPA, a population with broad spectrum organophosphate and carbamate resistance from Spain, which on the basis of cross resistance patterns appear to be due to the same gene or genes (see pp126-129 for details). At the time of crossing, the AT SPA colony showed only low mortality after 2hours exposure to 1%

Table 34. Percentage mortalities resulting from exposure of ATHOR, AT SPA and the reciprocal F_1 generations to 1% fenitrothion for various time exposures.

TIME	ATHOR		F_1		AT SPA	
(MIN)	Dead/Total	%	Dead/Total	%	Dead/Total	%
15	0/62	0				
20	0/24	0				
25	4/52	7.7				
30	7/55	12.7				
35	32/60	53.3				
40	24/31	77.5			0/40	0
45	33/35	94.3				
50	49/50	98			0/40	0
55	40/40	100				
60	61/61	100	0/69	0	0/110	0
70			4/82	4.88		
80			29/71	40.8		
90			48/52	92.3	1/145	0.7
120			78/79	98.7	3/175	1.7
180			80/80	100	89/211	42.2
200			60/60	100	185/201	92
210					140/140	100

Figure 23. Log-time probit-mortality lines for An.atroparvus, (AT SPA, ATHOR and the reciprocal F_1 generation) after exposure to 1% fenitrothion.



fenitrothion and had shown no segregation of the susceptible phenotype for three generations. Log-time probit-mortality lines were constructed for both ATHOR and AT SPA and for the F_1 generation of the cross, by various timed exposures to 1% fenitrothion. The F_1 generation showed intermediate resistance. From Figure 23 the LT_{50} and LT_{90} values were as follows:-

COLONY	LT_{50}	LT_{90}
ATHOR	34.73	43.86
AT SPA	174.9	214.8
F_1	81.65	93.36

(Figures given as time in minutes).

The two reciprocal F_1 generations showed no significant difference in their response to testing against 1% fenitrothion indicating that this resistance is autosomally inherited. LT-P lines were fitted using maximum likelihood analysis; values were corrected for control mortalities using Abbotts formula before the figures were computed. The major effect of this was to remove the point for 90 minutes exposure of AT SPA (see Table 34). Values for the slopes of the lines, Chi squares for goodness of fit of the points to a straight line and the number of degrees of freedom are given in Table 35 .These show that the points for ATHOR conform to a straight line. The points for the F_1 generation and the AT SPA population however deviate significantly from a straight

line. In the case of the F_1 this deviation was caused by the one survivor of the 120 minute exposure to 1% fenitrothion (see Table 34).

Virgin F_1 females and F_1 males were reciprocally backcrossed to the fenitrothion susceptible ATHOR stock, the resulting progeny were tested on 1% fenitrothion for 1hour, which was the minimum time that gave 100% kill of the susceptible parents. On the basis of a single gene hypothesis the expected mortality in the backcross progeny from this treatment would be 50%. Results of tests of four generations of backcross progeny are given in Table 36. All four reciprocal backcrosses were carried out, and gave results which were not significantly different from one another. Results for each of the backcross generations are therefore given as pooled figures.

Table 35. Values for maximum likelihood fitting of LT-P lines for ATHOR, AT SPA and the reciprocal F_1s after exposure to fenitrothion.

Population	Slope line(± SD)	Chi square	Df	P
ATHOR	12.65(± 0.008)	5.2	4	ns
AT SPA	17.61(±0.005)	56.2	1	<0.001
F_1	22.01(± 0.005)	111.2	2	<0.001

Table 36. Results of reciprocal backcrosses of the F_1 generation of AT SPA x ATHOR to the fenitrothion susceptible ATHOR parent.

	% mortality(numbers tested).
First backcross	45.4 (238)
Second backcross	51.7 (205)
Third backcross	51.6 (91)
Fourth backcross	45.7 (70)

Chi square tests indicated that the results from each of the four backcross generations did not deviate significantly from those expected on the single gene hypothesis. It can therefore be concluded that fenitrothion resistance in *An. atroparvus* from Spain is inherited as a single semi-dominant autosomal gene.

Table 37. χ^2 values for each of the four pooled backcross generations.

	χ^2	Df	P
First backcross	2.03	1	ns
Second backcross	0.12	1	ns
Third backcross	0.098	1	ns
Fourth backcross	0.514	1	ns

e). Penitrothion resistance and linkage studies in An. atroparvus from Spain.

The visible larval mutant *stripe* is present in both the English and Spanish populations of *An. atroparvus* studied. The *stripe* mutant has been described in several species of anophelines (Kitzmiller and Mason 1967). *Stripe* is inherited as a simple autosomal dominant over *non-stripe*, with excellent penetrance and expressivity of both phenotypes. Both phenotypes are easily recognisable in larvae and pupae.

Linkage of fenitrothion resistance and the marker *stripe* was studied. Two strains were selected from the fenitrothion resistant AT SPA and the fenitrothion susceptible ATHOR colonies. A population of ATHOR was obtained which was homozygous for *non-stripe* (*st*) and fenitrothion susceptibility (*r*) and a population of AT SPA was obtained which was homozygous for fenitrothion resistance (*R*)and for *stripe*(*ST*).

The ATHOR *st/st,r/r* colony was obtained by mass selection of non-stripe larvae, the selected line was then checked for three generations to ensure that there was no segregation of the stripe phenotype. The AT SPA *ST/ST,R/R* colony was obtained by selecting a homozygous R/R population for stripe with five generations of sib-mating of individual families. At each generation families showing segregation of *non-stripe* were discarded. After selection the resultant population (ASFS) bred true for three generations without selection.

Reciprocal F_1s were produced from the two selected

populations. All F_1 progeny showed the stripe phenotype and, at the dosage of fenitrothion used (1% for 1hour), were phenotypically resistant. Reciprocal backcrosses to the ATHOR *st/st, +/+* were performed. The results are given in Table 38 .

Table 38. Segregation of the stripe and fenitrothion resistance genotype in backcrosses of (ATHOR x ASFS) x ATHOR.

Backcross ♀ x ♂	Larval type	ND	Susc *+/+*	Resis *R/+*	d^2	P
(ASFS x ATHOR) x ATHOR	*ST/st*	4	66	72	4.88	ns
	st/st	1	81	92		
(ATHOR x ASFS) x ATHOR	*ST/st*	8	25	31	1.11	ns
	st/st	4	28	24		
ATHOR x (ASFS x ATHOR)	*ST/st*	1	56	60	1.93	ns
	st/st	0	46	54		
ATHOR x (ATHOR x ASFS)	*ST/st*	0	37	10	21.2	<0.01
	st/st	4	42	41		

ND = larvae which did not complete their development through to the adult stage.

All four classes of data for the backcrosses cannot be pooled as the homogeneity factor (d^2 = 24.9 (8Df) P = 0.01) is highly significant; the first three classes of data could however be pooled. Data in the fourth

class is deficient in the number of stripe larvae and resistant adult phenotypes. The reason for the deficiency is unknown. It is not however due to differential mortality in the larvae during rearing, as the four larvae which did not complete their development were all of the non-stripe phenotype. Abnormal egg hatch may have been the cause of the phenotype deficiencies. This was not checked, though it is unusual that this should occur in only one of the four backcrosses.

On the basis of the results obtained in backcrosses 1-3 it can be tentatively concluded that the two characters *stripe* and fenitrothion resistance are on separate linkage groups, as analysis of the data shows that the two genes are independently assorting.

6). Propoxur resistance in An. atroparvus from Spain.

A reciprocal cross was carried out between the propoxur selected resistance line of AT SPA and the propoxur susceptible English population ATHOR. Cross resistance analysis shows that propoxur and fenitrothion resistance are connected in the AT SPA population (see Figure 17) as selection with either causes an increase in the number of individuals resistant to the other.

At the time of crossing, the AT SPA propoxur line showed 0-10% mortality after exposure to 0.1% propoxur for 6 hours. Log time probit mortality lines were constructed for ATHOR, AT SPA and the pooled reciprocal F_1 generations of the cross after various timed exposures to 0.1% propoxur.

Table 39.Percentage mortalities for ATHOR, AT SPA and the reciprocal F_1 generations after exposure to 0.1% propoxur for various times.

TIME	ATHOR		F_1		AT SPA	
	Dead/Total	%	Dead/Total	%	Dead/Total	%
10	1/60	1.6				
15	9/60	15				
20	16/54	29.6				
25	42/59	71.2				
30	46/49	93.9				
40	57/58	98.3				
45	60/60	100				
60	60/60	100	44/214	20.6	0/124	0
120			144/170	84.7		
160			162/165	98.2		
240			60/60	100	4/169	2.4
300					24/69	34.8
360					51/114	44.7
420					62/122	50.8
480					78/104	75
540					66/71	93

The F_1 generation showed intermediate resistance. The LT_{50} and LT_{90} values derived from Figure 24 are as follows:-

COLONY	LT_{50}	LT_{90}
ATHOR	21.2	31.1
AT SPA	386.6	564
F_1	80.9	128

(Time given in minutes).

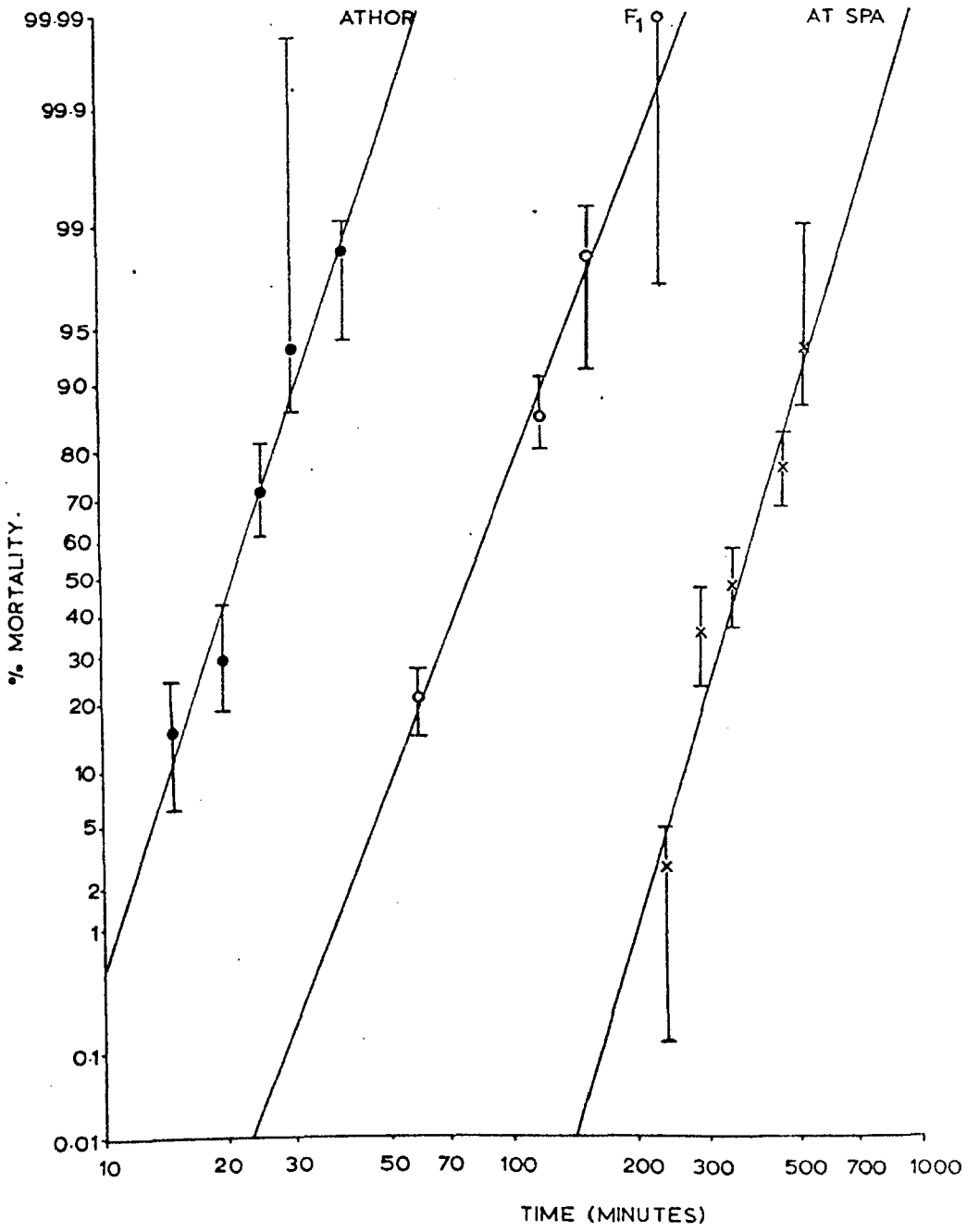
The two reciprocal F_1 generations showed no significant differences in their response to the propoxur treatment indicating that the resistance gene(s) are autosomally inherited.

Table 40 shows that while the points for the ATHOR colony and the F_1 generation correspond to a straight line the points for the AT SPA colony deviate significantly. The pattern of these points may suggest that even after 20 generations of propoxur selection the colony was not homozygous for all the factors involved in propoxur resistance (see Figure 11).

Table 40. Values for fitting of the LT-P lines for AT SPA, ATHOR and the reciprocal F_1 generations after propoxur exposure.

Population	Slope line (±SD)	Chi Square	Df	P
ATHOR	7.75 (± 0.012)	6.88	4	ns
AT SPA	7.81 (± 0.007)	21.77	4	<0.001
F_1	6.44 (± 0.011)	1.09	1	ns

FIGURE 24. LOG-TIME PROBIT-MORTALITY LINES FOR ATHOR, AT SPA AND THE RECIPROCAL F_1 GENERATIONS AFTER EXPOSURE TO 0·1% PROPOXUR.



Virgin F_1 females and males were reciprocally backcrossed to the propoxur susceptible ATHOR population, the resulting progeny were tested on 0.1% propoxur for 1hour, the minimum dosage that produced 100% mortality of the susceptible parents. Survivors of this treatment were again backcrossed to the susceptible stock. This selection was maintained for four backcross generations. The results are given in table 41 .

Table 41. Results of reciprocal backcrosses to the propoxur susceptible ATHOR parent.

	% MORTALITY (Number tested)
FIRST BACKCROSS	59.6 (166)
SECOND BACKCROSS	62.5 (174)
THIRD BACKCROSS	77.5 (80)
FOURTH BACKCROSS	87.5 (40)

All four reciprocal backcrosses of the F_1s to the susceptible parentals were carried out and as the results from each of the backcrosses were not significantly different from one another, results for each backcross generation are pooled.

On the single gene hypothesis 60% mortality would be expected in each of the backcross generations. χ^2 tests indicate (see Table 42) that the results of the first two backcrosses do not deviate significantly from those expected were a single gene responsible for the resistance

but the mortalities in the third and fourth backcross generations are higher than expected on the single gene hypothesis. It is also notable that the mortalities show an increasing trend over the four generations tested, which is indicative of polyfactorial inheritance. From these crosses it therefore appears that propoxur resistance is dependent on more than one gene. Results from the F_1 generation indicate that these genes are autosomally inherited.

Table 42. χ^2 values for each of the four pooled backcross generations.

	χ^2	Df	P
FIRST BACKCROSS	0.0072	1	ns
SECOND BACKCROSS	0.405	1	ns
THIRD BACKCROSS	8.2	1	0.01
FOURTH BACKCROSS	10.1	1	0.01

As we have already seen that fenitrothion segregates as a single gene in the AT SPA colony (see Table 37) and that there is evidence of cross-resistance between propoxur and fenitrothion, (see pp 126 - 129) it is, therefore, likely that either one of the propoxur resistance genes also confers resistance to fenitrothion, or that the propoxur and fenitrothion resistance genes are closely linked. This will be discussed further when considering synergism (see pp 173-177).

SECTION V. SYNERGIST STUDIES.

Indications of biochemical pathways involved in particular cases of resistance may be obtained by the use of synergists (see pp 63-69). Where two resistance mechanisms to one insecticide are suspected, selection of the survivors of the insecticide/synergist treatment should confirm this, a larger percentage survival occurring with each synergist/insecticide selected generation. Synergist studies should not however be taken as definitive (see pp 68-69).

All synergist impregnated papers were prepared in the laboratory. Preliminary experiments were carried out to determine the active life of these papers. The results for 20% triphenyl phosphate (TPP) impregnated papers used against malathion resistant *An. stephensi* were as follows:-

Age of TPP papers (days)	Age of malathion papers (days)	% mortality for TPP + malathion
3	3	100(76)
13	13	100(26)
17	2	96(49)
24	9	98(63)
28	13	57(119)
29	1	86(73)
30	2	41(36)
1	13	100(69)
2	1	100(75)
3	2	100(31)

From the results of this experiment it can be seen that the efficacy of the TPP papers appears to decline after 13-17 days under insectary conditions, with a fairly rapid decline in activity between 24-28 days. Subsequent experiments with TPP therefore always employed papers which were less than 20 days old. Papers could be stored under refrigeration at 8°C for up to 1½ months without any observable decline in efficacy.

Similar studies were carried out with all the other synergists used, to ensure that all papers were only used while showing optimal activity.

Table 43 gives the percentage mortality figures for TPP, malathion and TPP + malathion treatments respectively in *An. stephensi* from Iraq, Iran and Pakistan and *An. arabiensis* from Sudan. The survivors of the original TPP + malathion treatment were allowed to breed and their progeny were again tested with the TPP + malathion mixture. In the case of *An. arabiensis* from Sudan it was impossible to get the one surviving male of the first selected generation to mate. Reselection was tried on a further stock of the malathion selected line. Progeny from the survivors of this second generation were all killed by the TPP+ malathion treatment.

The survivors of the second and third selected generations of ST LA exposed to TPP + malathion were all males. This may reflect the generally higher resistance observed in the males of this population under selection pressure with

Table 43.

Percentage mortalities in successive generations of the malathion selected lines of *An. stephensi* (ST LA, ST IRAQ , ST IRAN) and *An. arabiensis* (G1) after exposure to TPP + malathion. (Figures in parentheses are numbers tested).

Generation	ST IRAN			ST IRAQ			ST LA			G1		
	TPP	MAL	TPP+MAL	TPP	MAL	TPP+MAL	TPP	MAL	TPP+MAL	TPP	MAL	TPP+MAL
	(20%)	(1hr)		(20%)	(4hr)		(20%)	(6hr)		(20%)	(4hr)	
1	0	25.1	89.4	0	26.2	94.1	0	24.1	95	0.8	12.8	99.3
	(25)		(198)	(25)		(358)	(46)		(400)	(25)		(143)
2	0	23.2	94.4	2	31.8	98.1	0	28.2	95.3	RESELECTION		
	(25)		(261)	(25)		(158)	(42)		(192)			
3	0	28.1	77.2	0	33.8	93.8	0	26.3	98.2	0.1	19.3	98.5
	(25)		(281)	(25)		(211)	(18)		(216)	(25)		(272)
4	0	24.1	97.8	0	29.4	95.2	-	30.1	92.5	0	9.9	100
	(25)		(910)	(30)		(167)			(40)	(6)		(22)
5	0	12.4	99.0	0	26.0	100	0	29.0	100			
	(25)		(198)	(5)		(26)	(41)		(10)			

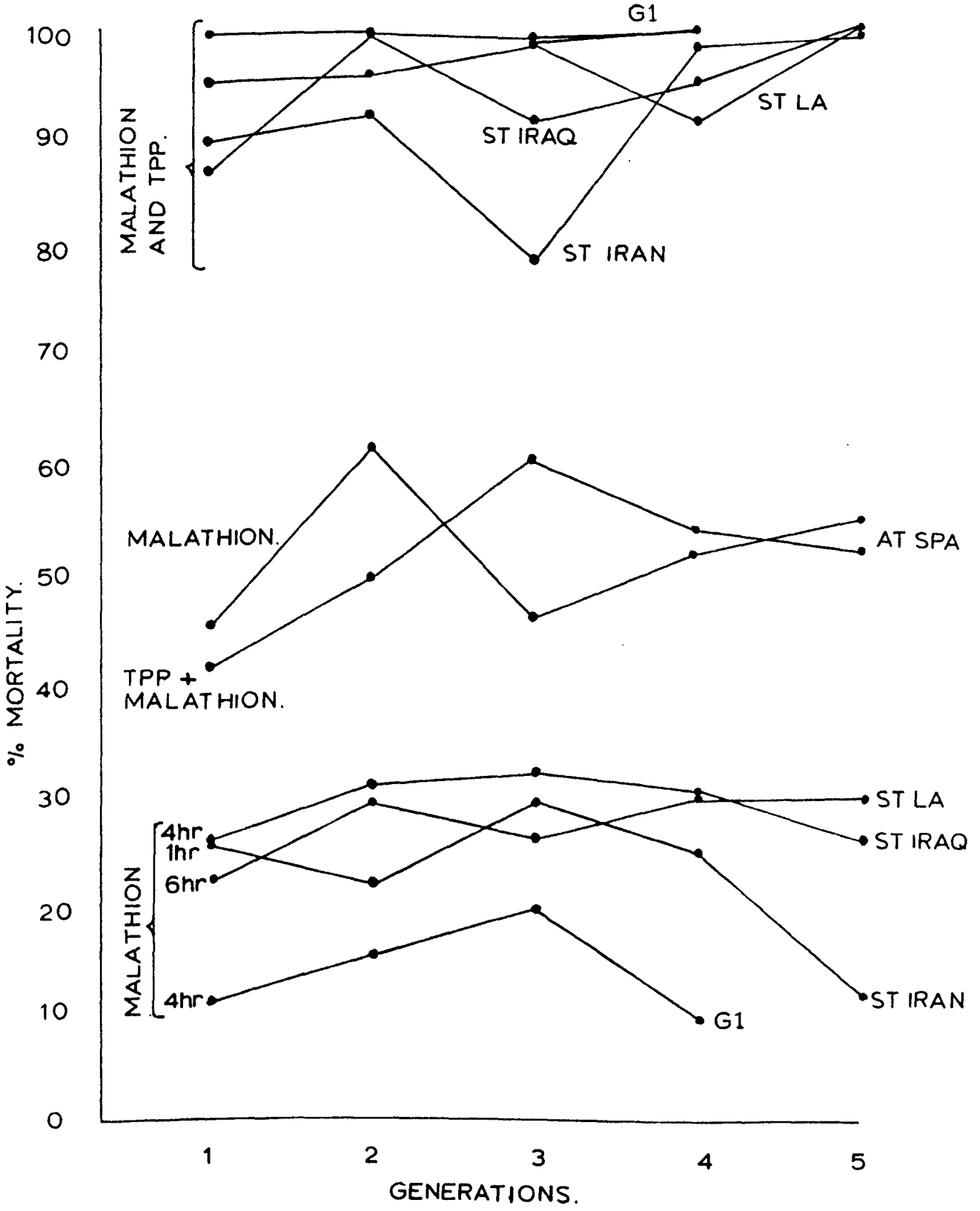
5% malathion (see Figure 14). Surviving males from the second and third generation were hand mated to virgin females of the malathion selected line after the females had been exposed to 5% malathion for 6 hours.

The results show that in *An. stephensi* from Iraq, Iran and Pakistan there was no increase in the percentage survival over 5 generations, of progeny that had come from the original TPP + malathion selection, when they were subjected to the same selection pressure as their parents. In *An. arabiensis* from Sudan the high level of mortality from TPP + malathion selection precluded following the progeny over more than 2 generations.

Therefore in *An. stephensi* from Iraq, Iran and Pakistan and *An. arabiensis* from Sudan there appears to be only one mechanism operative in malathion resistance. This mechanism is blocked by TPP treatment which restores almost complete susceptibility to the malathion resistant lines. As TPP is known to block the carboxylesterase enzyme system it may be tentatively concluded that malathion resistance in all four strains involves a quantitative or a qualitative difference between the carboxylesterase enzymes of resistant and susceptible stocks. This is compatible with the cross-resistance spectra observed in these species (see Figures 18 and 19).

In contrast in *An. atroparvus* from Spain, TPP + malathion treatment did not increase the AT SPA's susceptibility to malathion, as the percentage mortality for TPP + malathion treatment does not differ significantly from mortality with

Figure 25. The effect of TPP pretreatment on malathion resistance in *An. stephensi* (ST IRAQ, ST IRAN, ST LA), *An. arabiensis* (GI) and *An. atroparvus* (AT SPA).



malathion alone over three generations. This suggests that a malathion specific carboxylesterase mediated mechanism is not responsible for malathion resistance in this population of *An. atroparvus*. The differences between the response of the *An. stephensi* and *An. arabiensis* populations to TPP + malathion treatment and that of *An. atroparvus* are illustrated by Figure 25.

Table 44. Percentage mortalities in successive generations of AT SPA selected with TPP + malathion (figures in parenthesis are numbers tested).

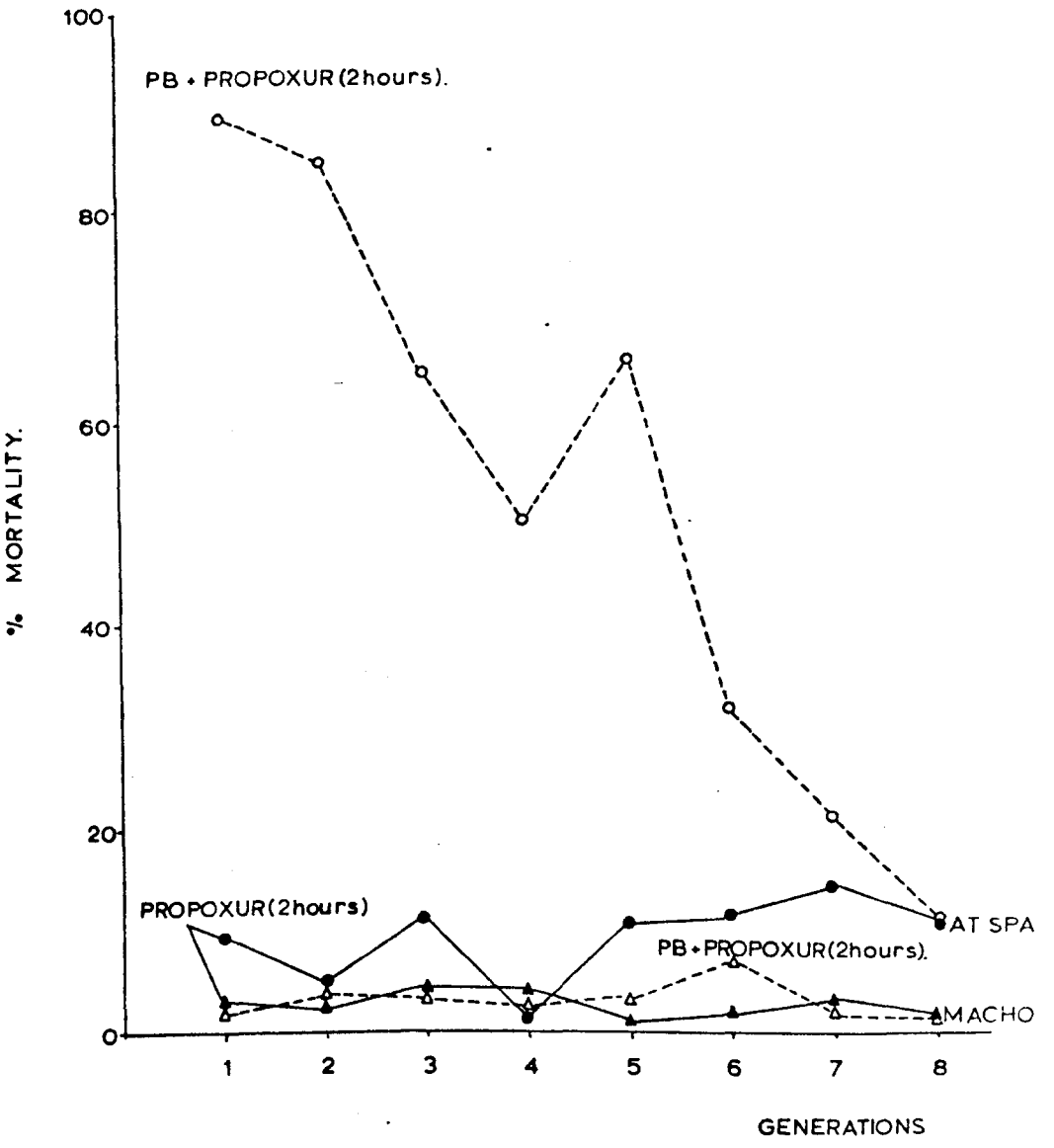
Generation	TPP(1hr)	Mal(2hr)	TPP + Mal(2hr)
1	0	46.2(13)	43.5(46)
2	0	63.3(128)	49.7(65)
3	0	46.8(94)	61.7(60)
4	0	52.5(59)	53.3(45)
5	0	55 (60)	51.9(54)

Table 45 shows the effect of piperonyl butoxide (PB) treatment on a propoxur selected line of *An. atroparvus* that had been under selection pressure for four generations with 0.1% propoxur for 1hour and two generations with 0.1% propoxur for 2hours. It can be seen that there is a significant decrease in the percentage mortality in progeny from the piperonyl butoxide/propoxur selected line over eight

Table 45.Percentage mortalities in successive generations of the propoxur selected lines of *An. albimanus* (MACHO males) and *An. atroparvus* (AT SPA) after PB/propoxur treatment.

GENERATIONS	AT SPA			MACHO		
	PB(20%)	Propoxur	PB+Prop	PB(20%)	Propoxur	PB+Prop
1	0	9.1	89.1(46)	0	2.1	2.8(72)
2	0.7	5.6	85.5(62)	0	4.2	2.3(89)
3	0	12.8	64.3(143)	0	3.6	4.3(94)
4	0	2.4	50 (40)	0	2.5	4.5(112)
5	0	11.6	66.7(63)	0	3.2	1.0(104)
6	0	12.4	33.3(60)	0	6.8	1.9(106)
7	0	14.5	23.4(64)	0	2.4	3.1(97)
8	0.5	13.7	13.9(72)	0	1.8	1.9(211)

FIGURE 26. THE EFFECT OF PIPERONYL BUTOXIDE (PB) PRETREATMENT ON PROPOXUR RESISTANT AN. ATROPARVUS (AT SPA) AND AN. ALBIMANUS (MACHO MALES).



generations. In the eighth generation mortality from the PB/propoxur treatment was similar to that of the control tested with propoxur alone. These results strongly suggest that at least two factors are involved in propoxur resistance in this population. One mechanism is blocked by piperonyl butoxide and appears to be at a fairly high frequency. It can be tentatively concluded that this mechanism is due to the involvement of multi-function oxidases in the resistance. The second mechanism appears to be unaffected by PB treatment and is at a low frequency at the start of the synergist/insecticide selection. The exact frequency of the genes controlling this mechanism are however impossible to assess without knowing the dominance relationship of the gene(s) . However it is probable that this mechanism is that which is represented by the broad spectrum OP/carbamate resistance in AT SPA. If this is the case the AT SPA x ATHOR cross with fenitrothion as the selecting agent has shown that this gene is effectively dominant for fenitrothion (at 1% for 1hour). Its effective dominance for 0.1% propoxur for 2hours is however doubtful.

In contrast to AT SPA (see Figure 26) PB/propoxur treatment of MACHO males shows that PB treatment has no effect on the propoxur resistance in this stock. This translocated gene also confers broad spectrum OP and carbamate resistance to the MACHO males.

After further selection of the propoxur resistant line of AT SPA with 0.1% propoxur for 6hours over five

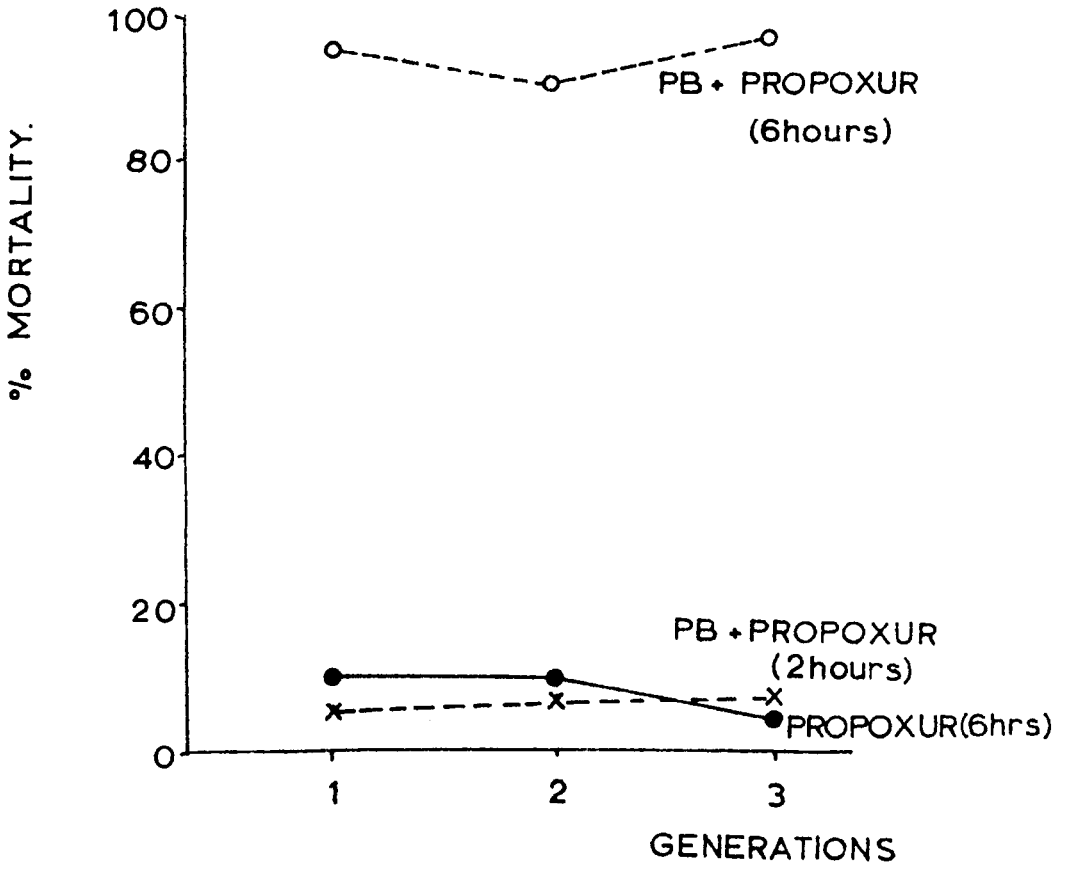
generations (see Figure 11 for details of selection), the response of the resistant selected line to PB/propoxur treatment was again tested. The results are given in Table 46.

Table 46. The effect of piperonyl butoxide pretreatment on a propoxur selected line of *An. atroparvus* (AT SPA) using different propoxur exposure times in conjunction with PB pretreatment.

Generation	Percentage mortalities(number tested)			
	PB	Propoxur	PB/propoxur	PB/propoxur
	(20%)	(0.1% 6hr)	(2hr)	(6hr)
1	0	10	6.8(44)	96.7(91)
2	0	9.2	8.2(49)	91.2(68)
3	0	4.6	6.1(99)	97.6(41)

This confirms that the line originally used for the PB/propoxur treatment (see Figure 26) was not homozygous for all factors involved in the resistance. This experiment demonstrates that PB pretreatment has no effect on propoxur resistance when selection with 0.1% propoxur for 2hours is used, but PB has a marked synergistic effect when followed by 0.1% propoxur for 6hours (see Figure 27). The fact that there is no significant decrease in the percentage mortality

FIGURE 27. The effect of piperonyl butoxide pretreatment followed by 2 or 6 hours exposure to 0.1% propoxur, compared to mortality with 0.1% propoxure alone for 6hours in adult An. atroparvus (AT SPA).



with PB/propoxur(6hours) over three generations suggests that the stock is now homozygous and that the mechanism or mechanisms which are independent of multi-function oxidases are insufficent by themselves to produce the very high propoxur resistance observed in the selected line. It can also be confirmed that the non-MFO mediated mechanism(s) were present at a low level in the stock originally used for the PB/propoxur treatment, as the original stock showed 89% mortality when first treated with PB + propoxur (2hrs) (see Table 45). Selection with four or six hours 0.1% propoxur for 12 generations (see Figure 11) has however dramatically increased the frequency of the non-MFO mediated resistance mechanism in the propoxur selected resistant line of AT SPA, as initial selection of this line with PB + propoxur (2hrs) showed only 6.8% mortality (see Table 46).

The effect of SV_1 and sesamex pretreatment on the propoxur resistance in the AT SPA colony was also checked. Results are given in Table 47 . These show that both sesamex and SV_1 show a similar synergistic pattern to piperonyl butoxide, as synergism occurs with all three compounds when followed by 6hours exposure to 0.1% propoxur, but not when followed by 2hours exposure to 0.1% propoxur.

The synergistic action of FDMC against the three DDT selected resistant lines of *An. atroparvus* (ATIG), *An.gambiae* (IAN) and *An. arabiensis* (GI) were investigated (see Table 48). One hundred percent mortality of ATIG was obtained after exposure to 20% FDMC for 1hour followed by exposure

Table 47. Percentage mortalities in successive generations of AT SPA after SV_1 or sesamex pretreatment followed by different propoxur exposure times (figures in parenthesis are numbers tested).

Generation	SV_1	Sesamex	Propoxur (6hrs)	SV_1+Propoxur (2hrs)	SV_1+Propoxur (6hrs)	sesamex+Propoxur (2hrs)	Sesamex+Propoxur (6hrs)
1	0	0	10	6.9(29)	58.3(36)	13.6(22)	84.2(38)
2	1	0	9.2	11.4(35)	85.7(35)	6.7(30)	72.4(29)
3	0	0	4.6	16.7(36)	57.1(28)	11.1(36)	95.8(24)

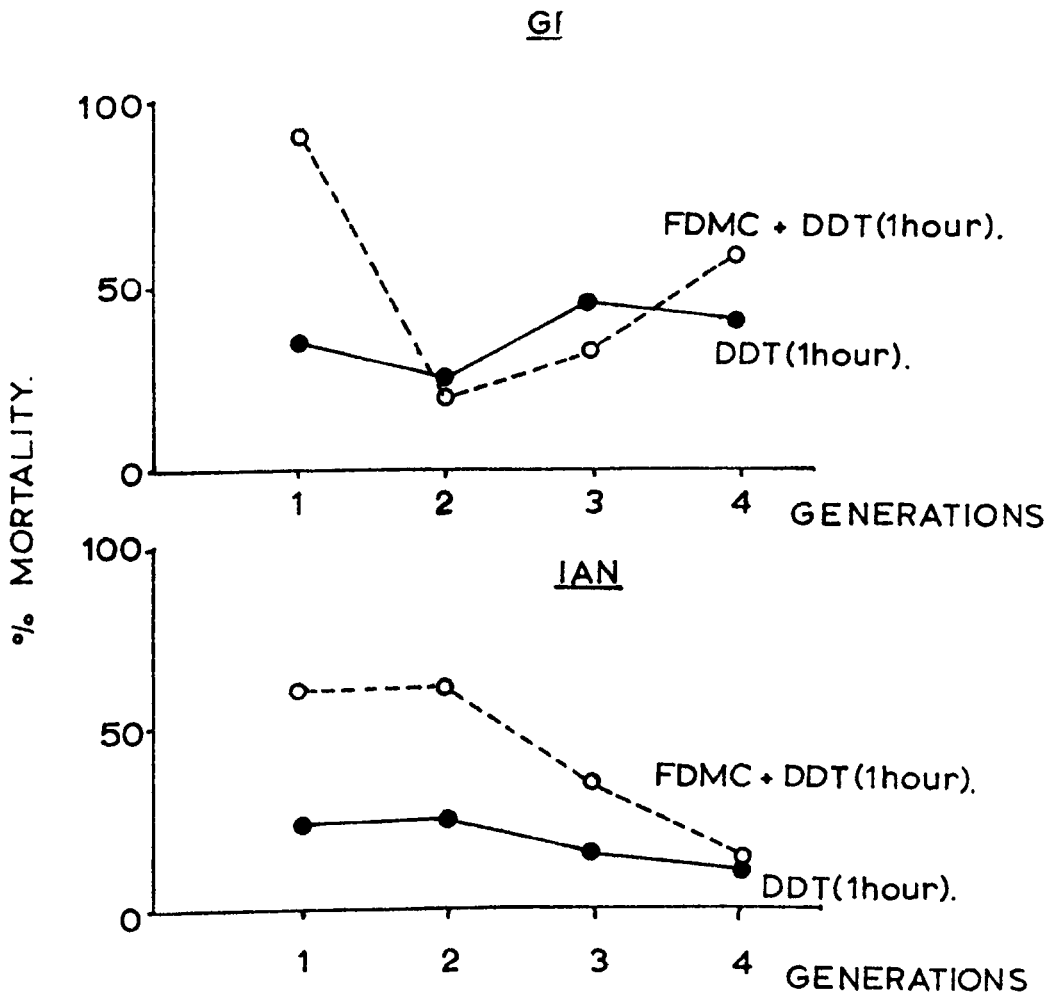
to 4% DDT for 1hour in three separate experiments. However in IAN and GI only partial synergism with FDMC was observed. The survivors of the FDMC/DDT treatment were allowed to breed and their progeny were again tested with FDMC + DDT. In IAN a decrease in the percentage mortality of the FDMC/DDT line was observed after selection, such that the mortality in the FDMC/DDT line was similar to that observed with DDT alone after 4 generations. The GI stock however showed an initial decline in the percentage mortality in the 2nd FDMC/DDT selected generation and then a gradual increase in mortality for the next two selected generations (see Figure 28). This trend was to some extent paralleled by an increasing mortality in the line selected with DDT alone.

From these results it appears that DDT resistance in the ATIG colony is dependent on a DDT-dehydrochlorinase mechanism which is blocked by the action of FDMC. The results for the IAN colony may indicate the presence of two DDT resistance mechanisms in this population. One of which is dependent on DDT-dehydrochlorinase and a second which is not synergisable by FDMC, as there is a decreasing trend in mortality over 4 generations in the FDMC/DDT selected line. This is supported by the fact that 100% mortality is obtained by treatment with FDMC + 4% DDT(8hrs), which indicates that both mechanisms are necessary for survival of this high level DDT selection. Evidence of cross-resistance between DDT and permethrin in this stock

Table 48. Percentage mortalities in successive generations after exposure to FDMC + DDT of ATIG, IAN and G1 colonies (figures in parenthesis are numbers tested).

Gener-	ATIG			IAN			G1		
ations	FDMC	DDT	FDMC+DDT	FDMC	DDT	FDMC+DDT	FDMC	DDT	FDMC+DDT
1	0	4.2	100	0	24.2	60.6	0	37.6	89.2
	(22)	(24)	(62)	(25)	(62)	(132)	(20)	(149)	(139)
2	RESELECTION								
	0	0	100	0	24.5	62.8	0	24.5	20
	(10)	(8)	(26)	(25)	(49)	(62)	(40)	(98)	(210)
3	RESELECTION								
	0	5.1	100	0	16	36	0	46.7	33.3
	(4)	(39)	(48)	(4)	(25)	(76)	(30)	(122)	(96)
4				0	12.5	15.5	0	40.9	58.6
				(20)	(64)	(58)	(20)	(154)	(152)

Figure 28. The effect of FDMC pretreatment on DDT resistance in An. arabiensis (GI) and An. gambiae (IAN).



also indicates that there is a non-DDT-dehydrochlorinase DDT resistance mechanism in this population.

The situation in the GI colony is however more complex. Again the synergist/insecticide combination appears to indicate two mechanisms for DDT resistance in this population. However the level of DDT resistance in this population is low, the DDT selected line showing 100% mortality after exposure to 4% DDT for 2hours (see Figure 10) and it seems unlikely that two major resistance mechanisms would produce such a low resistance. The presence of a non-FDMC-synergisable DDT resistance mechanism is suggested by the DDT-permethrin cross-resistance in this population.

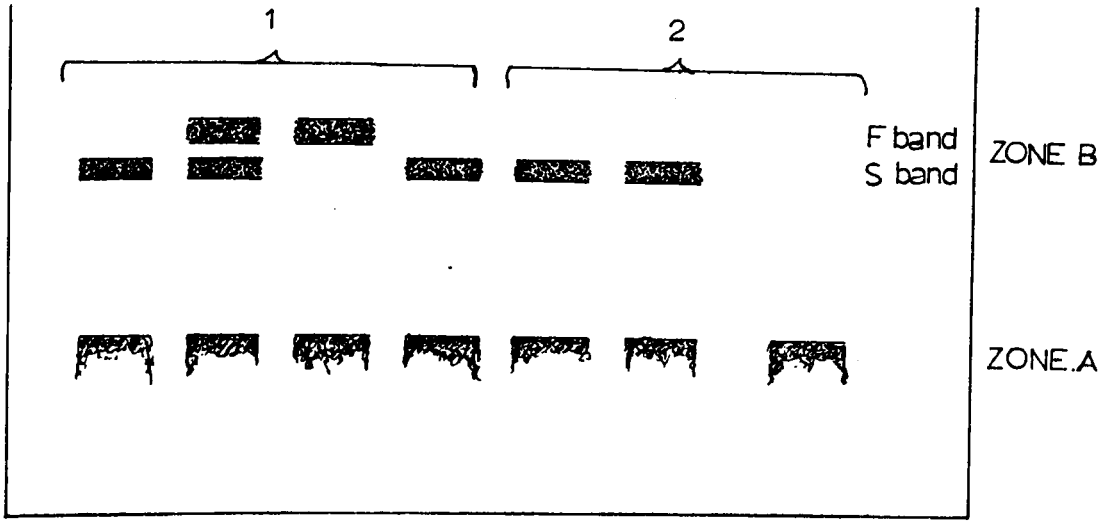
SECTION VI. FURTHER BIOCHEMICAL STUDIES OF RESISTANCE.

A). Acetylcholinesterase studies in An. atroparvus and An. albimanus.

Acetylcholinesterase isozyme phenotypes of individual insects of AT SPA and ATHOR (*An. atroparvus*) were visualised by continuous horizontal polyacrylamide gel electrophoresis. When individuals were homogenised in Triton^{R} X-100 two zones, A and B, were apparent after electrophoresis (see Figure 29). Zone A consisted of a slow-moving set of bands and zone B of a weakly-staining faster-migrating band. When individuals were homogenised in ice-cold distilled water an additional faster migrating band appeared in zone B (see Figure 29).

The Triton X-100 appears to inhibit one of the zone B bands (the F band). It may be that the non-polar Triton

Figure 29. Diagramatic representation of AT SPA acetylcholinesterase after homogenisation in; 1. distilled water and 2. Triton^{R} X-100.



coats the acetylcholinesterase molecules and stops them reacting with the substrate. Homogenisation with distilled water was therefore used routinely.

Both zone B isozymes appeared in the parental AT SPA population (see Plate 1a). The faster of the two isozymes (the F band) was the only band present in all the selected insecticide resistant stocks, whereas the slower isozyme (the S band) was the only form found in the insecticide susceptible English ATHOR population (see Table 51).

Acetylcholinesterase inhibition. A 10^{-4} M solution of eserine inhibited the bands in both zones A and B, indicating that true cholinesterases were being visualised.

Propoxur inhibition of the acetylcholinesterase was

Plate 1a. Acetylcholinesterase phenotypes in *An. atroparvus* (AT SPA).

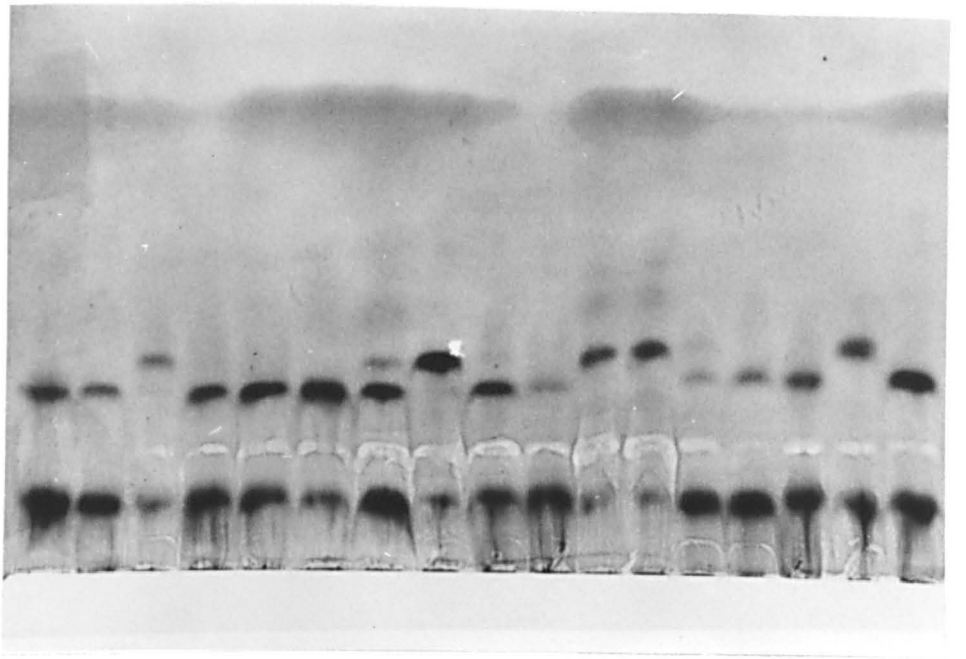


Plate 1b. Acetylcholinesterase phenotypes in *An. albimanus* (MACHO males and females).

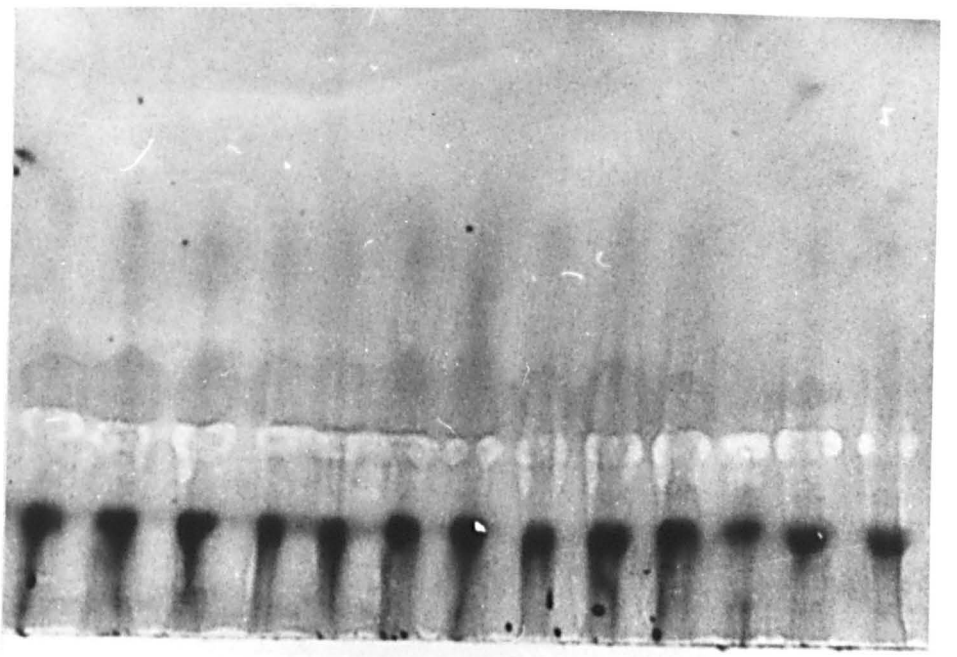


Table 51. The percentage of SS, SF and FF AChE isozyme phenotypes in various populations of *An. atroparvus*, (see Materials and Methods for details of populations).

Population	SS	FS	FF
ATHOR	100(79)	0	0
AT SPA	28.6(49)	24.5(49)	46.9(49)
ASP(2H)	0	0	100(53)
ASMAL	0	0	100(26)
ASFEN	0	0	100(19)
ASFW	0	0	100(24)

(Figures in parenthesis are numbers tested).

attempted in several ways. The S band appeared to be more susceptible to inhibition than the F band, but no attempt was made to quantify the staining intensities of the S and F bands after inhibition,as there was variation in the staining intensity when propoxur was not present.

Synergist and cross-resistance studies indicate that AChE insensitivity is the basis of the broad spectrum resistance in *An. atroparvus* (AT SPA). The F band appears to represent this altered acetylcholinesterase. If the bands in zone B represent a single locus at which two alleles, F and S, are segregating, then the percentage of heterozygotes (FS) in the parental population is lower than expected from the Hardy-Weinberg equilibrium (see Table 52). This deficiency of FS individuals may be due to inaccurate scoring of heterozygotes, as the staining

intensity of S and F bands varied. Alternatively the bands do not represent alleles.

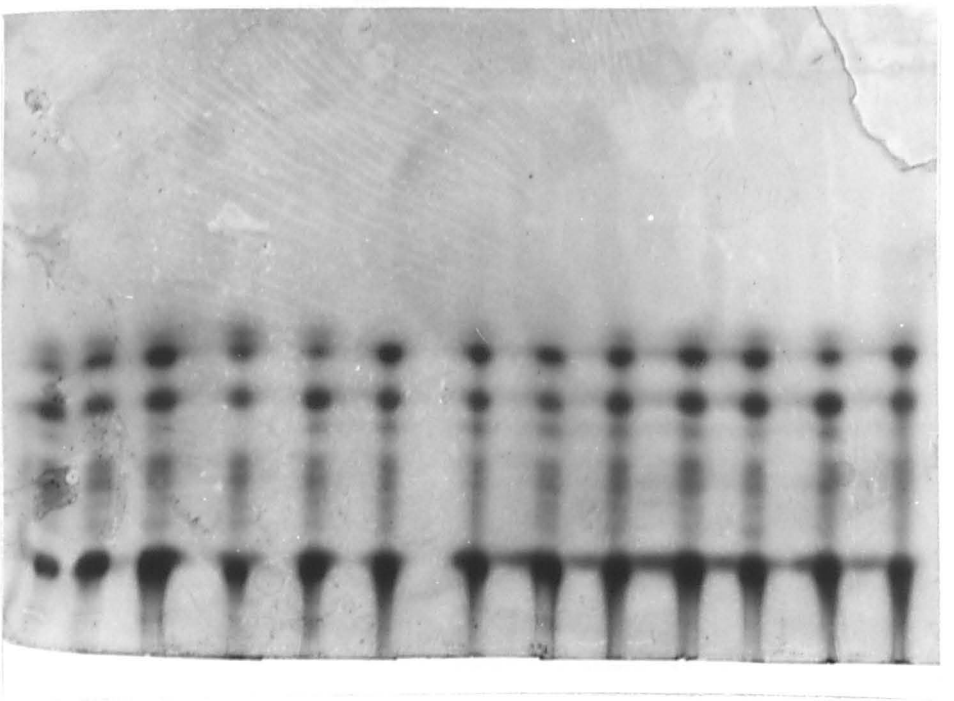
Table 52. Chi-square test for goodness of fit between zone B bands and Hardy-Weinberg equilibrium in AT SPA.

	SS	FS	FF	Total
Observed	14	12	23	49
O frequency	.29	.24	.47	
E frequency	$.168(p^2)$	$.49(2pq)$	$.34(q^2)$	
Expected	8	24	17	49
$\frac{(O-E)^2}{E}$	4.5	8.5	2.1	
$d^2 = 15.1$ $\chi^2 (1,0.001) = 10.83$				

The inheritance of the F and S bands was investigated by reciprocal crossing of ASFEN (homozygous for the F band) and ATHOR (homozygous for S). The resulting progeny from three single families were all FS, although there were individuals which showed marked differential staining of the F and S bands (see Plate 2). The F_1 individuals should have been inbred and the genotype frequencies of the F_2 generation scored, but this was not possible.

The broad spectrum resistance in the MACHO males (*An. albimanus*) also appears to be due to an altered acetylcholinesterase. MACHO males and females homogenised in either distilled water or Triton X-100 showed bands of acetylcholinesterase activity equivalent to zone A

Plate 2. Acetylcholinesterase phenotypes in the F_1 generation of AT SPA x ATHOR.



(see Plate 1b). These results are similar to those obtained by Georghiou & Pasteur (1978) for a different propoxur resistant colony of *An. albimanus*. The molecular difference between the *An. atroparvus* and *An. albimanus* acetylcholinesterase is unknown.

B). Carboxylesterase studies in An. stephensi.

i) Non-specific esterase activity.

The total esterase activity of the malathion resistant *An. stephensi*, ST MAL and the malathion susceptible ST was investigated using the filter paper spot method of Pasteur and Georghiou (1980). One day old individuals which had not been exposed to insecticide were used in all tests.

In Figure 29a it can be seen that, from this rather crude measure of esterase levels, that there is no obvious difference between ST and ST MAL individuals.

Figure 29a. Total esterase activity in single *An. stephensi* mosquitoes of malathion resistant (ST MAL) and susceptible (ST) individuals.

ii). Carboxylesterase assay.

Carboxylesterase activity in ST MAL and ST was assayed using the technique of Van Asperen (1964).

. One day old individuals which had not been exposed to insecticides were used in the test. Absorbance values for single insects from the spectrophotometer, read at 605nm, were corrected for weight of the insect and amount of enzyme used by the formula;

$$\frac{\text{Spectrophotometer reading} \times \frac{\text{Total volume of mosquito}}{\text{Volume of mosquito used}}}{\text{Weight of mosquito.}}$$

One hundred individual insects from each colony were tested. Both populations showed a normal distribution of readings, therefore the mean value for each population was taken. The values were as follows:-

POPULATION	MEAN ABSORBANCE
ST	632.4 ± 10.9
ST MAL	621.7 ± 9.9

From this result it is clear that there is no quantitative difference in the amount of carboxylesterase enzyme present in the two populations. This is in agreement with the results from the filter paper spot test which also showed no gross differences between the total esterase activity of the malathion resistant and susceptible *An. stephensi* populations

iii). Starch gel electrophoresis of esterases.

Studies on the cross-resistance patterns of *An. stephensi* from Pakistan indicated that resistance only extended to organophosphates possessing a carboxylester bond, and synergist studies indicated that TPP almost completely restored suceptibility in the malathion resistant ST MAL strain. This evidence indicates either a quantitative or a qualitative change in the carboxylesterase enzymes of the resistant strain as compared with the susceptible. Enzyme assays have shown that there is no gross quantitative change in the total esterase activity of the resistant strain. Starch gel electrophoresis was therefore carried out to determine whether a qualitative change in a carboxylesterase enzyme had produced a charge difference between 'resistant' and 'susceptible' enzymes.

A distinct polymorphism in electrophoretic mobility in *Esterase-1* (Bianchi 1969) between the malathion susceptible ST and resistant ST MAL populations was observed (see Figure 30). Frequencies of fast and slow bands in the ST MAL parental population (ST LA) were scored (see Table 53)

Table 54. Chi-square test for goodness of fit between esterase-1 electromorph genotypes in *An. stephensi* (ST LA) and those predicted from the Hardy-Weinberg equilibrium.

	SS	FS	FF
Observed	12	4	0
Expected	12.25	3.5	0.25
χ^2	0.005	0.07	0.25

Figure 30. Esterase 1 bands in ST and ST MAL populations of *An. stephensi* after starch gel electrophoresis.

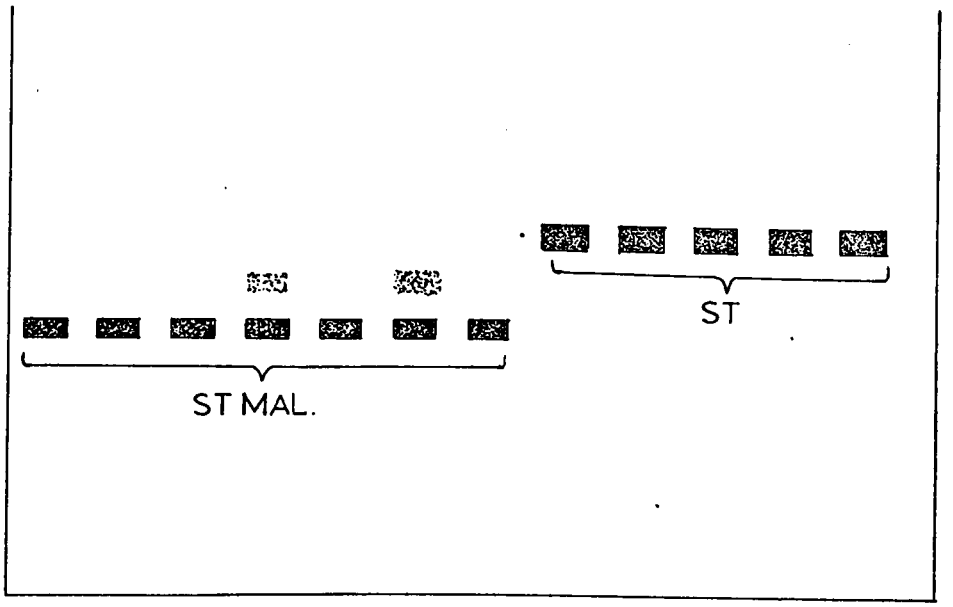


Table 53. Percentages of SS, SF, and FF individuals for esterase 1 in malathion resistant and susceptible colonies of *An. stephensi*. (Figures in parenthesis are numbers tested).

	SS	SF	FF
ST	-	-	100(36)
ST LA	75(16)	25(16)	-
ST MAL	100(26)	-	-

$d^2 = 0.325$ $\chi^2_{(2,0.05)} = 3.15)$ P is not significant.

The high frequency of SS individuals in the parental (ST LA) population is probably due to the high level of resistance in this population at the time the colony was sampled (0% mortality after exposure to 5% malathion for 1 hour, sample size 42).

Carboxylesterase studies in An. arabiensis.

i), Non-specific esterase activity.

Esterase activity in the malathion resistant *An. arabiensis* colony, G1 and the malathion susceptible, SENN were investigated using the filter paper spot technique. One day old adult mosquitoes which had not been exposed to insecticides were used in all tests.

From Figure 30a it can be seen that there was no difference in total esterase activity in these two populations and activity in males and females in the same population. This result is similar to that obtained with the malathion resistant and susceptible *An. stephensi* populations.

Figure 30a. Total esterase activity in single *An. arabiensis* mosquitoes of malathion resistant (G1) and susceptible (SENN) individuals.

ii). Carboxylesterase assay.

Carboxylesterase activity in the G1 and SENN colonies was assayed using the technique of Van Asperen (1964). . One day old adults which had not been exposed to insecticide were used for the assay. Absorbance was read at 605nm and values were corrected for weight of the insect and amount of the enzyme preparation used. A sample of 60 SENN and 58 G1 individuals were tested. The mean absorbance values were as follows:-

POPULATION	SEX	MEAN ABSORBANCE
SENN	♂	625 ± 8.2
	♀	632 ± 7.8
G1	♂	599 ± 10.4
	♀	750 ± 9.6

From this result it appears that there is no distinct quantitative difference between the carboxylesterase activity of the resistant and susceptible colonies. Although the activity of the G1 females is higher than that of the SENN females, the activity of the G1 males is lower than that of the SENN males. It has already been shown (see Figure 15.) that the G1 males tend to be more resistant than the females. The lack of a quantitative difference in carboxylesterase activity between resistant and susceptible colonies of *An. arabiensis* is in agreement with the results for the filter paper spot test.

C). *Malathion metabolism in An. stephensi from Pakistan.*

The R_f values of malathion and its metabolites on TLC plates were determined using standard metabolites. The R_f values were reproducible over several runs.

Table 55. Summary of R_f values of standard metabolites of malathion.

METABOLITE	SOLVENT Hexane/diethyl ether 1:3
Malathion	0.64
Malaoxon	0.33
Malathion monoacid	0.46
Malathion diacid	0.28
O'O-dimethyl phosphoro-dithionic acid (DMPDT).	0.42
O,O-dimethylphospororothionic acid.(DMPT)	0
O,O-dimethylphosphate (DMP).	0

When a concentrated sample of malathion was applied directly to the TLC plate only one spot appeared. However, if the malathion was first dissolved in hexane, in a 1:1 ratio, two spots appeared on the TLC plate. The slower and darker staining of the two spots had an R_f value corresponding to the value of concentrated malathion alone, while the faster, lighter staining spot had an R_f value of approximately 0.8. Application of hexane to the TLC plate did not produce a spot. The biochemical nature of the faster spot

Table 56. Presence(✓) or absence (x) of malathion and its metabolites in ST MAL and ST larvae and the test medium after various timed exposures to 2ppm malathion.

METABOLITE	MINUTES EXPOSURE TO 2 PPM MALATHION																							
	20				40				60				80				120				180			
	ST		ST MAL		ST		ST MAL		ST		ST MAL		ST		ST MAL		ST		ST MAL		ST		ST MAL	
	larvae	H_2O	larvae	H_2O	larvae	H_2O	larvae	H_2O	larvae	H_2O	larvae	H_2O	larvae	H_2O	larvae	H_2O	larvae	H_2O	larvae	H_2O	larvae	H_2O	larvae	H_2O
Malathion	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Malaoxon	x		x		x		x		x		x		✓		✓		✓		✓		✓		✓	
Malathion monoacid	x	x	x	x	x	x	x	✓	x	✓	✓	✓	x	✓	✓	✓	x	✓	✓	✓	x	✓	✓	✓
Malathion diacid	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
DMPDT	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	✓	x	✓	x	✓	x	✓
DMPT	x	x	x	x	x	x	x	x	x	x	x	x	x	✓	x	✓	x	✓	x	✓	✓	✓	✓	✓
DMP																								

was not investigated. It may have been a contaminant of the malathion.

Larval tests were carried out in triplicate using unlabelled malathion. 100 4th instar larvae were used in each test . The presence or absence of metabolites was noted for each test. Results are given in table 56. These results show that the malathion resistant ST MAL colony produced malathion monoacid more rapidly than the malathion susceptible ST colony.

Faint spots visible at the origin in both ST and ST MAL after 80 minutes exposure to 2ppm malathion were tentatively taken to be DMPT and/or DMP. Results for 24 hours exposure of larvae to 0.125ppm malathion are given in Table 57.

Table 57. Presence (✓) or absence (x) of malathion and its metabolites in larvae of ST and ST MAL after 24 hours exposure to 0.125ppm malathion.

Metabolite	ST		ST MAL	
	Larvae	water	larvae	water
Malathion	✓	✓	✓	✓
Malaoxon	✓		✓	
Malathion monoacid	✓	✓	✓	✓
Malathion diacid	X	X	X	X
DMPDT	✓	✓	✓	✓
DMPT	✓	✓	✓	✓
DMP				

These results show that there is no qualitative difference in the metabolites produced by susceptible and resistant larvae.

A quantitative measure of metabolite production was obtained by exposing two lots of 100 4th instar larvae of each of ST and ST MAL to 2ppm of C^{14}-malathion for up to 3 hours. The total recoveries of radioactivity were 79 and 82% respectively in resistant and susceptible strains. As recoveries from both stocks were similar comparisons of metabolite production were possible. These results are given in Table 58. The mono-carboxylic acid is the major breakdown product of malathion in both the susceptible and resistant stocks from both larval and test media extractions.

No attempt was made to separate malathion and malaoxon due to the large excess of malathion present in the hexane extract. However the joint figures for malathion and malaoxon in the larval homogenate extractions indicate that malathion resistance in ST MAL is not due to reduced penetration. A slightly higher percentage (3.35%) of the total radioactivity was recovered from the resistant larvae compared to the susceptible (2.2% recovery).

There was more unmetabolised malathion remaining in the ST test medium than in the ST MAL test medium at the conclusion of the larval exposure period. Seventy percent of the total radioactivity recovered from the ST test was unmetabolised malathion whereas 42% of the malathion remained unmetabolised in the ST MAL test.

Table 58. Amounts of malathion and various metabolites (expressed as percentages of the total recovery) in larvae and the test medium after exposing 4th instar larvae of ST and ST MAL to 2ppm malathion for 3 hours.

COMPOUND	SUSCEPTIBLE		RESISTANT	
	LARVAE	WATER	LARVAE	WATER
Malathion/oxon	1.05	69.6	1.1	40.99
Malathion monoacid	0.63	25.4	1.5	52.38
DMPDT	0.33	1.34	0.52	0.71
DMPT DMP	0.2	1.41	0.23	2.55

Table 59. Total amounts of malathion and its metabolites (expressed as percentages of the total recovery) in ST and ST MAL.

COMPOUND	SUSCEPTIBLE	RESISTANT
Malathion/malaoxon	70.68	42.1
Malathion monoacid	26.03	53.9
DMPDT	1.67	1.23
DMPT DMP	1.61	2.78
Total metabolites	29.31	57.91

These results indicate a higher rate of metabolism of malathion in *An. stephensi* compared with results obtained for *Culex tarsalis* by Darrow and Plapp (1960). They found 94 and 89% of the malathion unmetabolised in resistant and susceptible strains respectively, after four hours exposure of 4th instar larvae to 1ppm malathion.

An alternative extraction method was attempted to try and increase the total recovery rate of radioactivity. At the conclusion of the larval test the pH of the test medium was adjusted to pH7 with 0.1N sodium hydroxide and hydrochloric acid. Any unmetabolised insecticide was then partitioned into chloroform. The water was acidified to pH2.5 with hydrochloric acid and again partitioned with chloroform to remove the α- and β-monocarboxylic acids, DMPDT, DMPT and DMP. Larvae were homogenised in 3ml of distilled water and extraction carried out in the same way. With this method 89 and 91% recovery of radioactivity was achieved in resistant and susceptible strains respectively. Results are given in Table 60.

These results show a similar pattern to those in Table 59. Again, using this extraction procedure, there is no evidence of reduced penetration in the malathion resistant population, 4.4% of total radioactivity being recovered from R larvae and 2.6% of activity recovered from S larvae. Total malathion/malaoxon recovery from S and R larvae was similar. However total malathion/malaoxon recovery from test medium and larvae was greater in the ST test than in the ST MAL test. Proportions of unmetabolised malathion

remaining after the conclusion of the test were similar for both extraction procedures; the same applied to the proportions of metabolites produced by the ST and ST MAL colonies. These results strongly indicate that the primary metabolic pathway for both ST and ST MAL is production of malathion monoacid. Malathion breakdown to the monocarboxylic acid is generally considered to be catalysed by a carboxylesterase enzyme. However, it has been noted by Welling *et al*(1974) that monocarboxylic acid can be produced by MFO action. Evidence from cross-resistance and synergist studies on ST MAL indicate that MFO action is not involved however.

Table 60. Amount of radioactivity in various extracts (expressed as percentages of the total recovery) in larvae and test medium, after exposing 4th instar larvae of ST and ST MAL to 2ppm malathion for 3 hours.

EXTRACT	ST		ST MAL	
	Larvae	Water	Larvae	Water
Chloroform(pH7)	1.14	74.6	1.16	46.8
Chloroform(pH2.5)	1.2	21.4	2.87	47.9
Water	0.22	0.9	0.24	0.45
Larval residue	0.54		0.58	

Malathion metabolism in An. arabiensis from Sudan.

As malathion resistance in the *An. arabiensis* G1 colony from Sudan is only expressed at the adult stage a comparative study between resistant and susceptible

colonies using larvae could not be done. Studies on adults could not be made as the recovery of malathion and its metabolites using the adult test was inefficient. However one experiment using unlabelled malathion was carried out to determine whether there was any carboxyl-esterase activity present in the larvae of the adult malathion resistant G1 and susceptible SENN populations. The results are given in Table 61. The pattern of metabolite production is similar in both populations. Malathion monoacid was present after 24 hours exposure to 0.125ppm malathion, but not after 3hours exposure to 2ppm malathion in both G1 and SENN. This indicates that there is some carboxylesterase activity in both the adult malathion susceptible and resistant larvae. As synergist and cross-resistance studies indicate that a carboxyles-terase enzyme is the basis of the G1 adult resistance then either the particular carboxylesterase gene involved is not 'switched on' in the G1 larvae or it is producing enzyme in such minute amounts that it has little effect on the larval response to malathion.

Table 61. Presence (✓) or absence (X) of malathion and its metabolites after exposure of 4th instar larvae of G1 and SENN to either 2ppm malathion for 3 hours or 0.125 ppm malathion for 24 hours.

Metabolite	3hrs 2ppm		24hrs 0.125ppm	
	G1	SENN	G1	SENN
Malathion	✓	✓	✓	✓
Malaoxon	✓	✓	✓	✓
monoacid	X	X	✓	✓
DMPDT	X	X	✓	✓
DMPT } DMP }	✓	✓	✓	✓

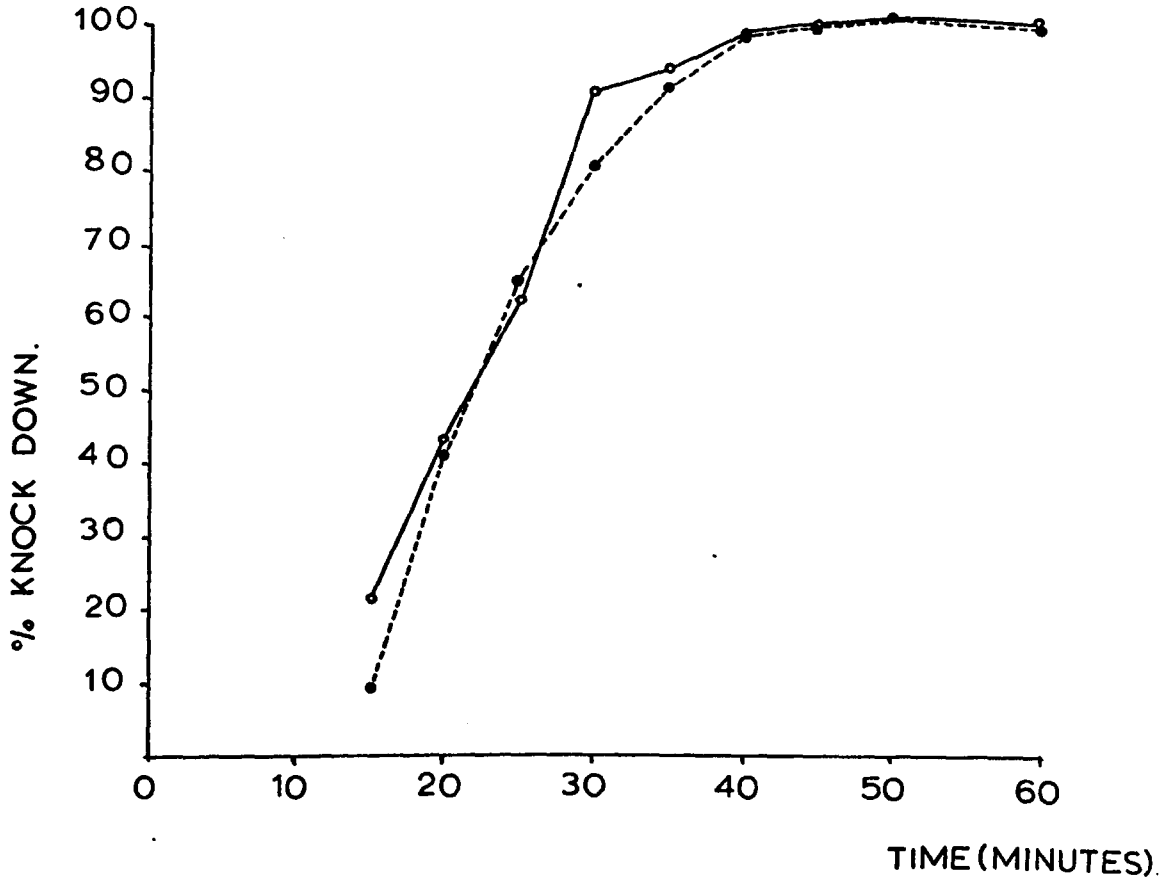
SECTION VII. A. THE USE OF PERMETHRIN IN ADULT TESTING.

Due to the rapid action of the residual pyrethroids leading to knock-down (KD) of adult mosquitoes during the test period in the standard WHO test, the amount of insecticide picked up by the insect is not necessarily related to the length of the 'exposure' time. Resistance is usually confirmed by allowing the survivors of the tests to breed and exposing their offspring to the insecticide over several generations, under constant selection pressure. The percentage mortality is then expected to decline until it reaches zero. However with permethrin selection of several anophelines this was not observed (see Figures 10 and 12). The rate of KD in conjunction with kill was therefore measured to see if this aided the selection of resistant stocks.

Experiments to determine the rate of KD were carried out on four species, *An. gambiae*, *An. arabiensis* *An. atroparvus* and *An. albimanus*. The five populations tested all gave similar results to those in Figure 31. There was no difference between the rate of KD in males and females.

Recoveries from early KD (KD 0-20 minutes after the start of the test) were reared separately from late KD (21-60 minutes after the start of the test) and their progeny were tested separately in the same way as the parents. During selection progeny of early KDs showing late KD were discarded after testing, while progeny of

FIGURE 31 The rate of knockdown of An. gambiae (IAN) on exposure to 0.8% permethrin.



KEY

----- = FEMALES

—— = MALES

Table 62 . The rate of knock-down of *An. gambiae* (IAN) on 0.8% permethrin.

Minutes	Male	Female
10	0 (163)	0 (145)
15	20.9(163)	8.9 (145)
20	42.9(163)	39.3 (145)
25	61.4(163)	64.1 (145)
30	90.2(163)	80 (145)
35	93.3(163)	90.3 (145)
40	98.2(163)	97.2 (145)
45	99.4(163)	98.6 (145)
50	100 (163)	100 (145)

late KDs showing early KD were discarded after testing. Figures 32 and 33 show that mortality in the two KD groups differed markedly.

The mortality of the early KD group of *An. gambiae* (IAN) and *An. arabiensis* (G1) show a pattern similar to that in ATIG, AT SPA and MACHO. The early KD group in IAN and G1 approached 100% mortality and went to extinction in seven generations. In *An. albimanus* (MACHO) and *An. atroparvus* (ATIG and AT SPA) the progeny of the original early KD survivors showed 92-100% mortality in all KD tests (see Figure 33). The late KD group from these three populations showed 100% mortality in all cases.

In IAN and G1 the late KD group approached 0% mortality remaining stable at 2.4-7.7% mortality for 6 generations (see Figure 32).

Table 63. Percentage mortality in early (0-20 minutes) and late (21-60 minutes) knock-down fractions in two colonies of anophelines selected for late knock-down.

Generations	colony tested			
	LAN		GI	
	0-20	21-60	0-20	21-60
1	80.7(212)	7.2(866)	95(20)	7.7(349)
2	56.4(197)	4.8(454)	80.9(42)	3.1(294)
3	55.2(297)	2.6(442)	95.5(22)	6.7(328)
4	87.6(169)	4.7(386)	81(126)	3.6(110)
5	88.6(140)	4.1(296)	66.6(3)	4.4(114)
6	89(182)	6.2(161)	57.1(28)	4.8(42)
7	85.3(204)	6.9(348)		

Figure 32 Selection of late KD from permethrin exposure in *An. gambiae* (IAN) and *An. arabiensis* (G1).

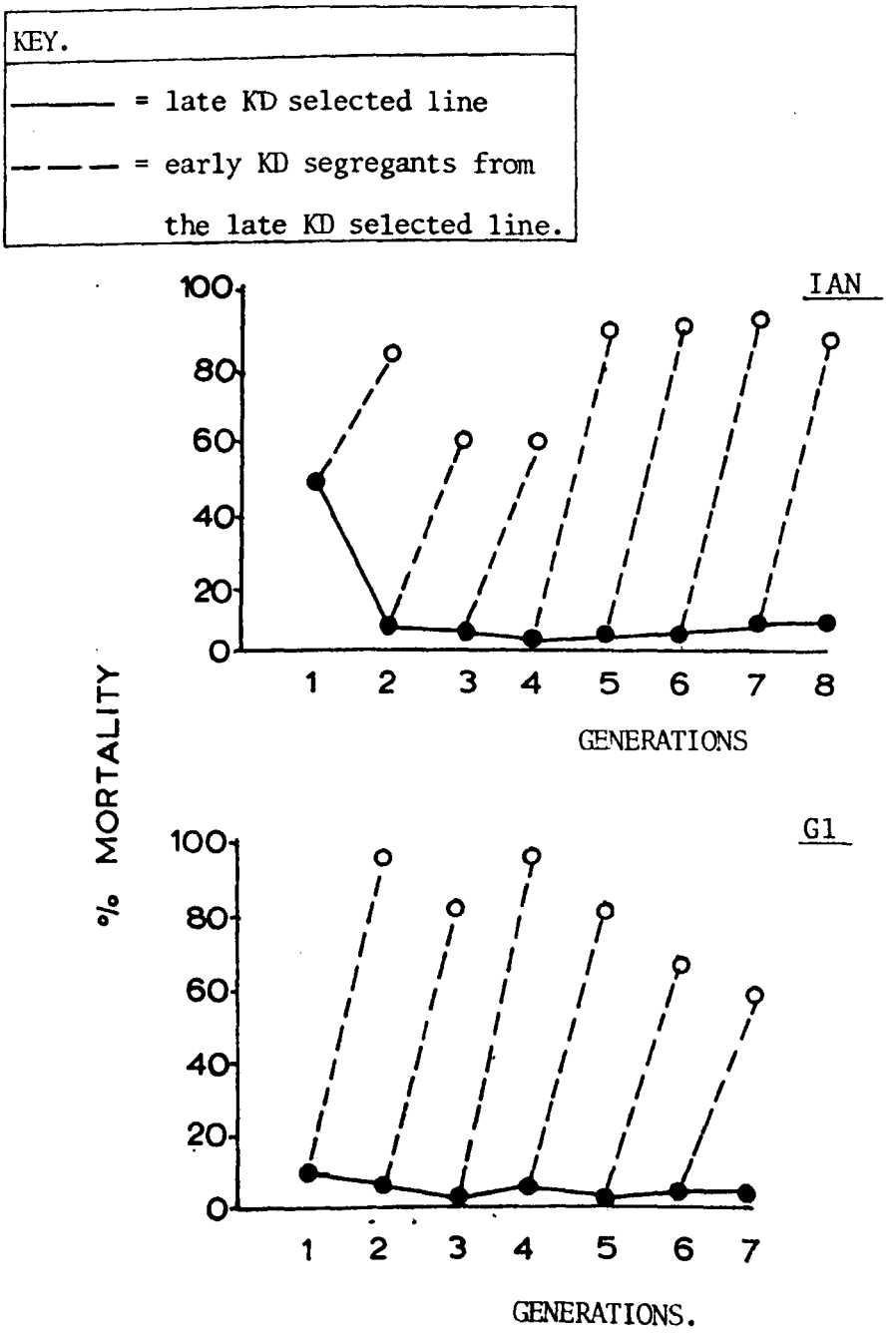
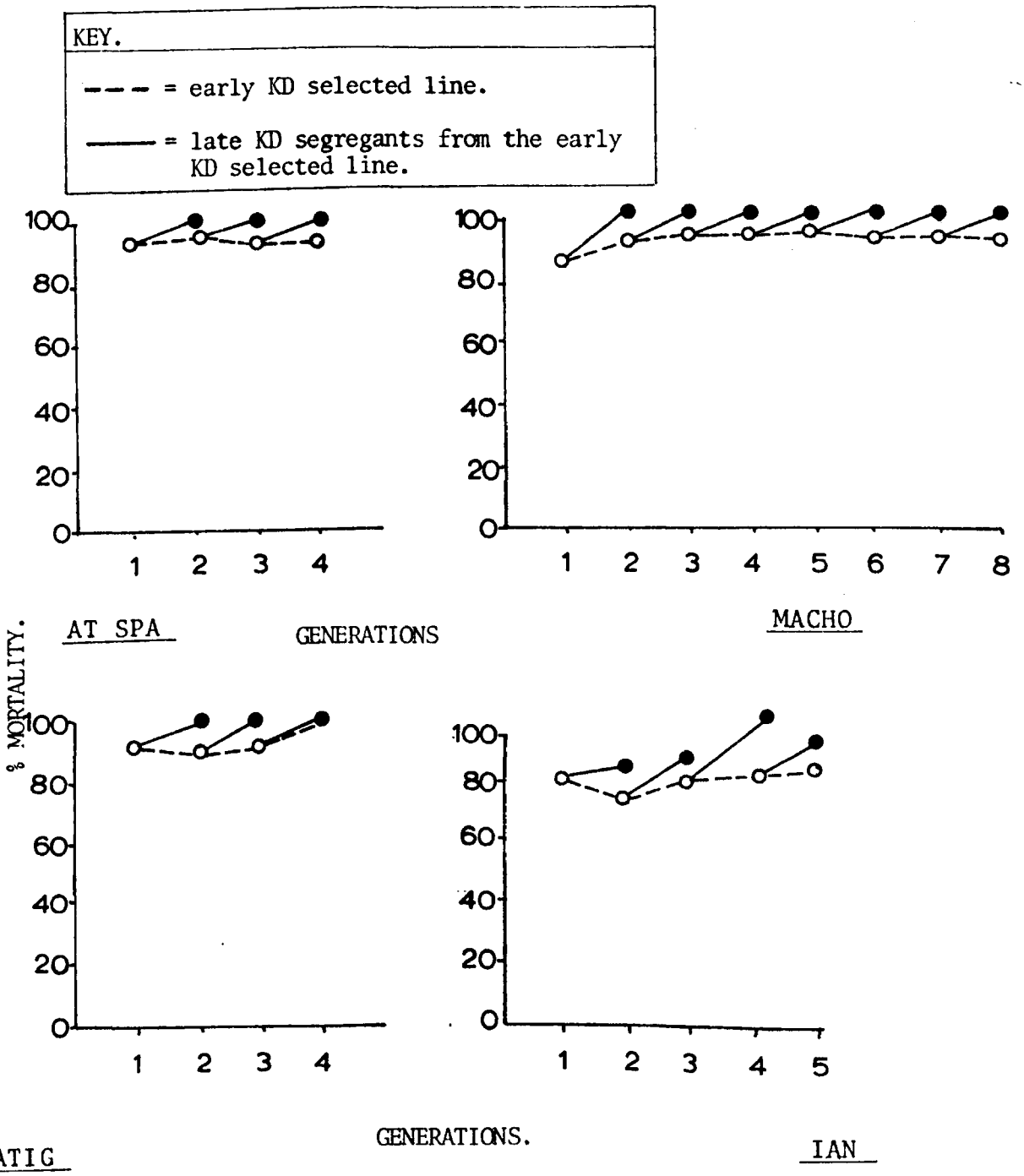


Table 64 Percentage mortality in early (0-20 minutes) and late (21-60 minutes) knock-down fractions in four colonies of anophelines selected for early knock-down.

Generations	Colony tested							
	IAN		MACHO		AT SPA		ATIG	
	0-20	21-60	0-20	21-60	0-20	21-60	0-20	21-60
1	79.5(151)		91.5(47)	100(82)	95.3(64)	100(80)	93.3(45)	100(102)
2	72.5(142)	81(131)	92.4(66)	100(74)	92.9(28)	100(42)	94.4(72)	100(62)
3	79.1(115)	84.8(66)	93.3(75)	100(91)	93.3(15)	100(21)	100(41)	100(38)
4	81.1(74)	96.3(27)	98.3(114)	100(104)				
5	82.4(17)	90.5(21)	92.3(26)	100(30)				
6			93.3(30)	100(16)				
7			93.3(16)	100(28)				

Figure 33. Selection of early KD from permethrin exposure of *An. albimanus* (MACHO), *An. gambiae* (IAN) and *An. atroparvus* (ATIG and AT SPA).



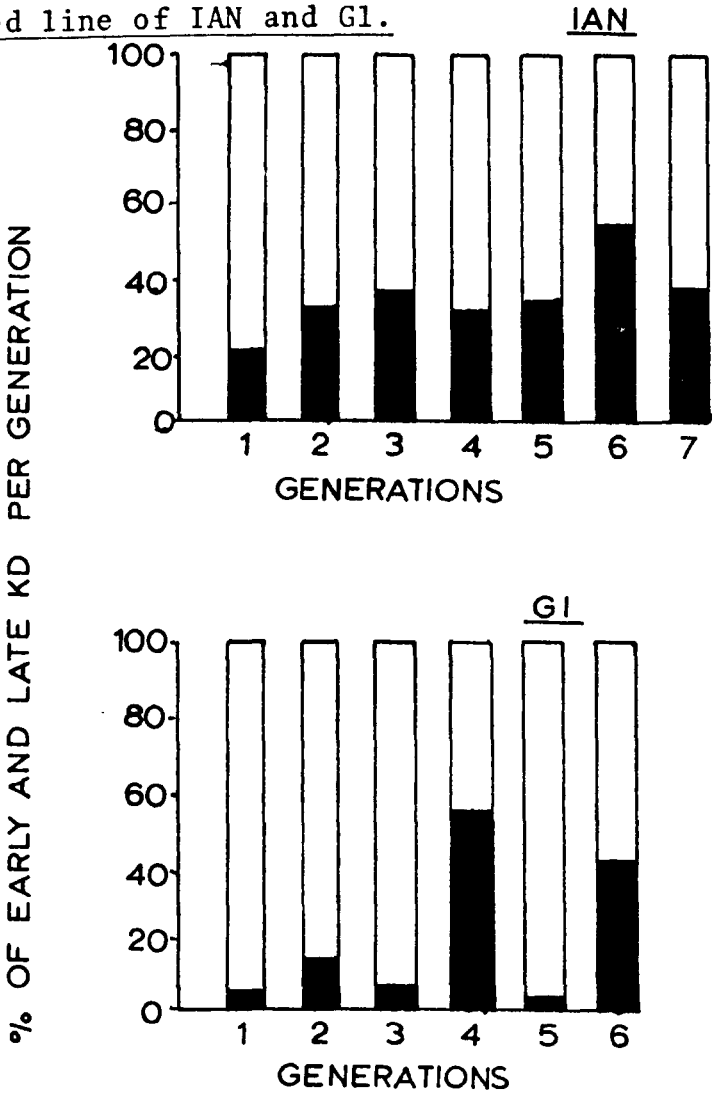
The survivors in *An. albimanus* and *An. atroparvus* appear to be due to rapid KD, which does not allow a lethal dose of the insecticide to penetrate into the insect. Some insects in this category would be expected in each batch of tests (as confirmed by the MACHO, ATIG and AT SPA selections). These insects should not be considered as resistant, as analysis of the progeny shows that survival appears to be due to chance.

However selection of a homozygous resistant colony does not appear to have occurred using the late KD selection. If only resistant individuals survived the late KD selection, the percentage of early KD individuals segregating in the progeny of the late KD line would be expected to decline with selection. It can be seen from Figure 34 that there is no obvious decline in the early KD class, as a percentage of the total number of insects tested, in either the IAN or the G1 colony. In IAN 30-40% early KDs segregated out from the late KD selected line in every generation for seven generations. In G1 the level of early KD segregation from the late KD selected line was lower than in IAN for 4 generations, but 2 generations showed an unusually high segregation of early KD individuals.

B. THE USE OF VOLATILE AND NON-VOLATILE SOLVENTS IN THE PREPARATION OF INSECTICIDE IMPREGNATED PAPERS.

Traditionally insecticide impregnated papers have been prepared using a non-volatile solvent as a carrier for the insecticide. This was advisable where solid or

Figure 34. Histograms to show the percentage of early and late KD fractions in every selected generation of the late KD selected line of IAN and G1.



powder forms of insecticide such as DDT were involved, as it facilitated even spreading of the insecticide on the filter paper. The practice has also been used for liquid insecticides such as pirimiphos-methyl and malathion, where even spreading of the active ingredient should not be a problem.

Three different samples of pirimiphos-methyl impregnated papers (1% in olive oil) from the WHO failed to give complete kill of laboratory susceptible strains after 1-4 hours exposure period. Papers prepared in our laboratories from 1% active ingredient solutions in dioctylphtallate (DOP) also failed to give complete kill after 1 hours exposure. Poor results were also obtained with similar papers tested in the field in Iraq. The effect of different solvents on the activity of pirimiphos-methyl were therefore examined in an attempt to produce a paper that would give reproducible results. The non-volatile solvents DOP and olive oil were compared with the two volatile solvents ethanol and acetone. The efficacy of the papers was tested using susceptible and malathion resistant *An. stephensi* colonies.

Pirimiphos-methyl papers prepared using either acetone or ethanol gave complete kills following a 1 hour exposure period to a 1% solution spread at 39$\mu g/cm^2$ and at serial dosages down to 4.9$\mu g/cm^2$. However only the acetone papers gave 100% kill at 2.4$\mu g/cm^2$ (see Table 65). Filter papers treated with pirimiphos-methyl from volatile solvent solutions therefore show more than an 8-fold

Table 65 Percentage mortalities of *An. stephensi* on filter papers treated with pirimiphos methyl at various concentrations in acetone or ethanol.

Deposit ug/cm^2	Exposure time(mins)	% mortality (number tested in parenthesis)			
		Acetone		Ethanol	
		ST	ST LA	ST	ST LA
38.9	60	100(62)	100(189)	100(20)	100(26)
	40	100(30)	100(34)		
	20	100(42)	100(85)		
	10	100(52)	100(52)		
	5		100(88)		
	2		100(56)		
19.5	60	100(28)	100(47)	100(40)	100(40)
	20	100(60)	100(24)		
9.7	60	100(68)	100(22)	100(32)	100(41)
	20	100(54)	100(46)	100(27)	100(36)
4.88	60	100(81)	100(68)	100(46)	100(52)
	45	100(89)	100(42)	100(28)	100(62)
	35	100(61)	100(47)	50(42)	91.8(61)
	20	53.3(150)	94.9(59)	8.7(23)	13.9(43)
2.44	60	100(74)	100(46)	59.1(44)	83.7(49)
	35	91.3(46)	100(81)		
	20	2.4(87)	65(40)		
Control	60	0(24)	0(24)	0(52)	0(46)

increase in activity over those prepared from DOP solutions.

The storage life of the acetone/pirimiphos methyl impregnated papers was then determined, as papers must be stable for a reasonable amount of time to be practically useful. Papers impregnated at 2.4µg/cm^2 were effective against *An. stephensi* after 8 weeks storage in aluminium foil at room temperature and after 18 weeks storage in aluminium foil at 10°C. After this toxicity declined slowly.

The effect of solvents on the toxicity of malathion and fenitrothion was also determined. Figure 35 shows that olive oil decreases the toxicity of malathion impregnated papers against susceptible (ST) and resistant (ST MAL) populations of *An. stephensi*. The LD_{50} values for the malathion/acetone and malathion/olive oil/acetone impregnated papers were as follows:-

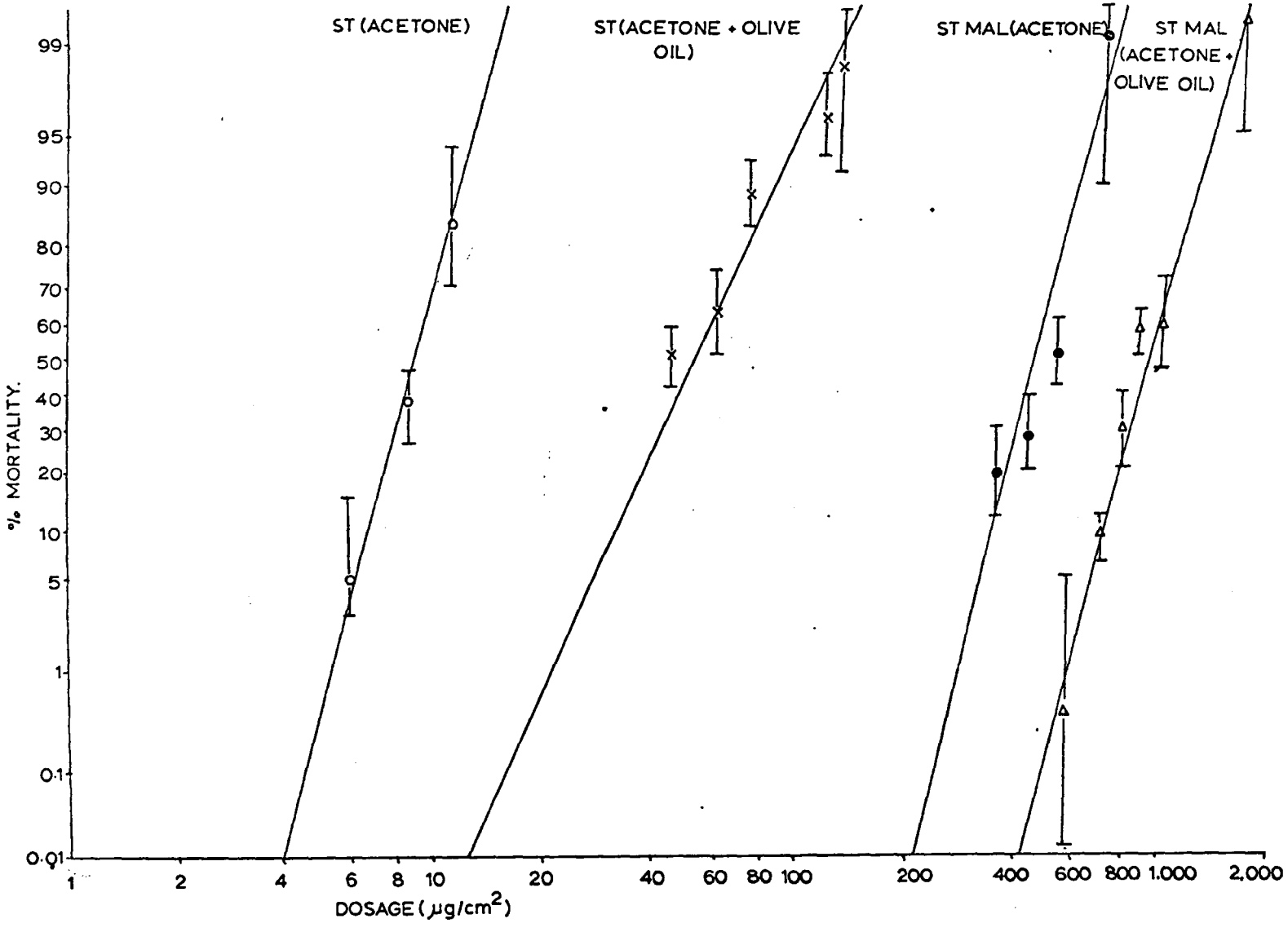
	Malathion/acetone		Malathion/olive oil	
	ST	ST MAL	ST	ST MAL
LD_{50}	9.5µg/cm^2	500µg/cm^2	45.4µg/cm^2	1,037µg/cm^2
LD_{95}	15 µg/cm^2	748µg/cm^2	116.6µg/cm^2	1,490µg/cm^2

If the resistance ratios are considered at the LD_{50} level for the resistant and susceptible strains, ST MAL would be considered 52.6x more resistant than ST when assayed against the malathion/acetone papers and only 22.8x more resistant than ST when assayed against the malathion/olive oil papers. It is therefore important to consider what kind of impregnated papers have been used in resistance studies when comparing the levels of resistance in different species or different populations

Table 66. Percentage mortalities of *An. stephensi* on filter papers treated with malathion at various concentrations in acetone or olive oil.

Deposit ug/cm^2	% mortality (no.tested in parenthesis).			
	Olive oil/acetone		Acetone only	
	ST	ST MAL	ST	ST MAL
1944.4		99.3(145)		
1166.4		56.6(53) .	100(20)	100(80)
972.2		56.2(381)		
875		29.5(95)		
777.8		9.9(773)	100(20)	99.2(130)
583.3		0.42(240)	100(20)	52.5(80)
486			100(20)	27.5(80)
388.9		0(40)	100(20)	20(80)
194.4	100(145)	0(52)	100(80)	0(80)
145.8	98.3(174)			
129.6	96.2(239)			
97.2	90(110)		100(69)	0(40)
81	88.4(259)			
64.8	63.4(85)			
48.6	51.6(124)		100(40)	0(40)
24.3			100(40)	
12.2			85(60)	0(40)
9.1			38(100)	0(80)
6.11			5(80)	0(40)
3.9			0(80)	0(40)

FIGURE 35. LOG DOSAGE PROBIT MORTALITY FOR RESISTANT (ST MAL) AND SUSCEPTIBLE (ST) AN. STEPHENSI POPULATIONS EXPOSED TO MALATHION PAPERS IMPREGNATED WITH AND WITHOUT OLIVE OIL AS A NON-VOLATILE SOLVENT.



of the same species.

SECTION VIII. THE USE OF MIXTURES. A. THE JOINT ACTION OF MALATHION AND FENTITROTHION.

Adult mosquitoes of ST and ST MAL were exposed to papers impregnated with serial dilutions of malathion, fenitrothion, and 1:1 and 3:1 mixtures of malathion and fenitrothion. Results are given in Table 67. Log-dosage probit-mortality plots were produced from these figures (see Figures 36 and 37). The lines were fitted using maximum likelihood analysis. LD_{50} and LD_{90} values and values for goodness of fit are given in Table 68. 95% confidence limits are given for each point; for mortality values less than 5% or greater than 95% limits were calculated from Stevens table (Stevens 1943).

Figures 36 and 37 show that the log-dosage probit (LDP) lines for the mixtures of malathion and fenitrothion for the malathion resistant ST MAL colony, lie between the LDP lines for malathion and fenitrothion alone. In the ST colony the LDP lines for the 3:1 malathion:fenitrothion mixture and that for malathion are very close together. The LD_{50} value for the 3:1 mixture is greater than that of malathion indicating some degree of antagonism.

The LD_{50} values for each of the LDP lines were plotted on the isobolograms in Figures 38 and 39 to determine whether potentiation or antagonism of insecticide action

Table 67 Percentage mortalities of ST and ST MAL after exposure to varying concentrations of :-

a) Malathion/acetone impregnated papers.

(figures in parenthesis are numbers tested).

Dosage ($\mu g/cm^2$)	ST	ST MAL
1166.4	100(20)	100(80)
777.6	100(20)	99.2(130)
583.2	100(20)	52.5(80)
486	100(20)	27.5(80)
388.8	100(20)	20 (80)
194.4	100(20)	0 (80)
12.15	85(60)	0 (40)
9.1	38(100)	0 (40)
6.08	5(80)	0 (20)
3.89	0(80)	0 (20)

b) Fenitrothion/acetone impregnated papers.

Dosage ($\mu g/cm^2$)	ST	ST MAL
1.95	100(80)	100(80)
1.14	100(40)	97.7(88)
0.98	100(80)	-
0.49	100(80)	59.3(59)
0.32	83.9(93)	10 (150)
0.24	48(80)	0 (80)
0.16	5.3(76)	0 (40)

Table 67 (continued).

c) Malathion:fenitrothion 3:1/acetone impregnated papers.

Dosage ($\mu g/cm^2$)	ST	ST MAL
24.3	100(80)	100(80)
20	100(40)	97.5(80)
18.2	93.44(122)	88 (100)
15	-	5 (80)
12.2	59.9(157)	0 (97)
9.1	10 (80)	0 (40)
6.1	0 (80)	0 (86)

d) Malathion:fenitrothion 1:1/acetone impregnated papers.

Dosage ($\mu g/cm^2$)	ST	ST MAL
6.1	100(80)	100(80)
3.9	100(80)	99.3(140)
1.95	95.4(130)	58.8(80)
0.973	62.5(160)	2.3(89)
0.486	1.4(70)	0 (80)
0.162	0 (40)	0 (40)

Figure 36. Log dosage probit mortality lines for An. stephensi (ST) after exposure to malathion, fenitrothion and mixtures of the two insecticides.

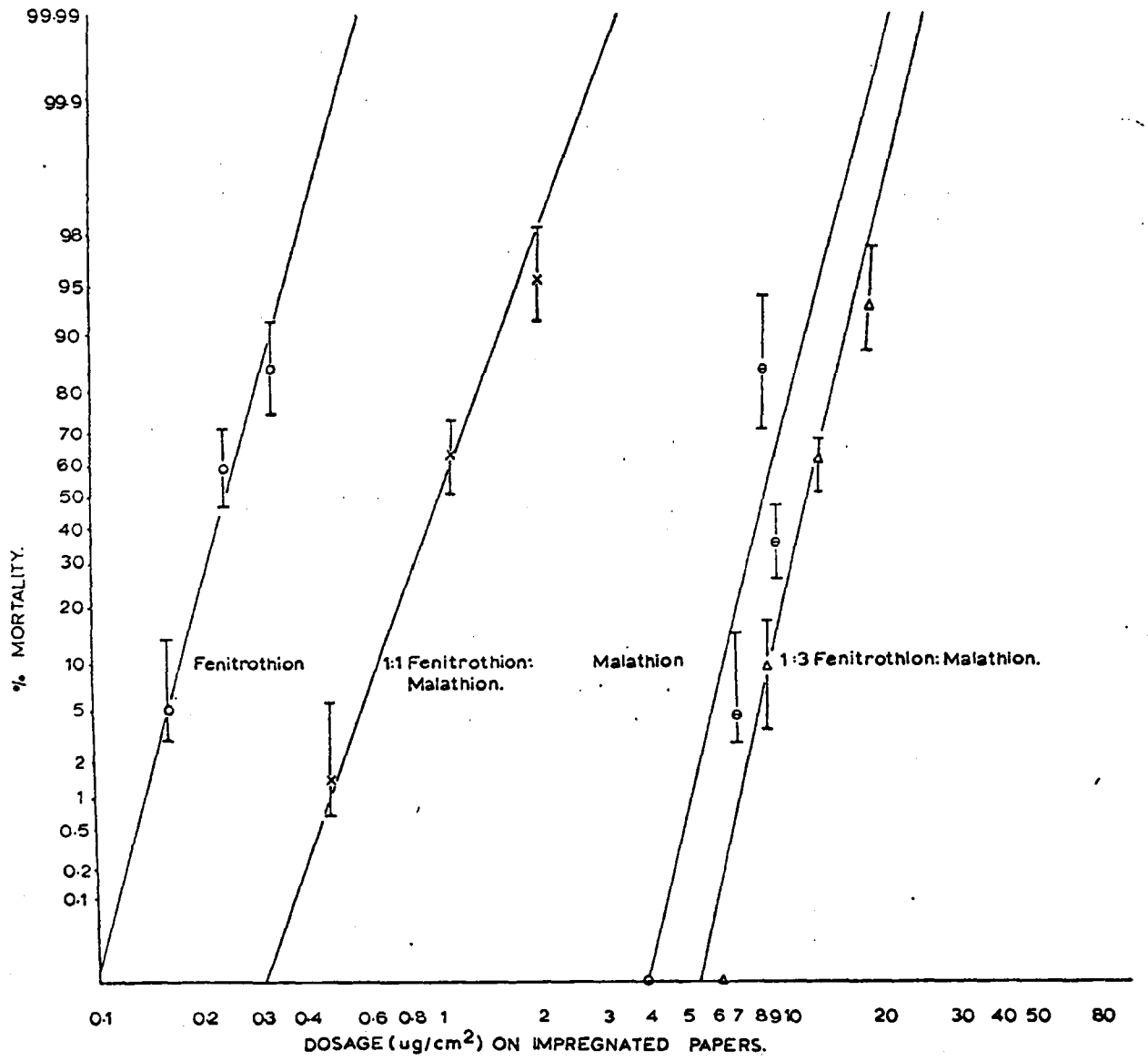


Figure 37. Log dosage probit mortality lines for An. stephensi (ST MAL) after exposure to malathion, fenitrothion and mixtures of the two insecticides.

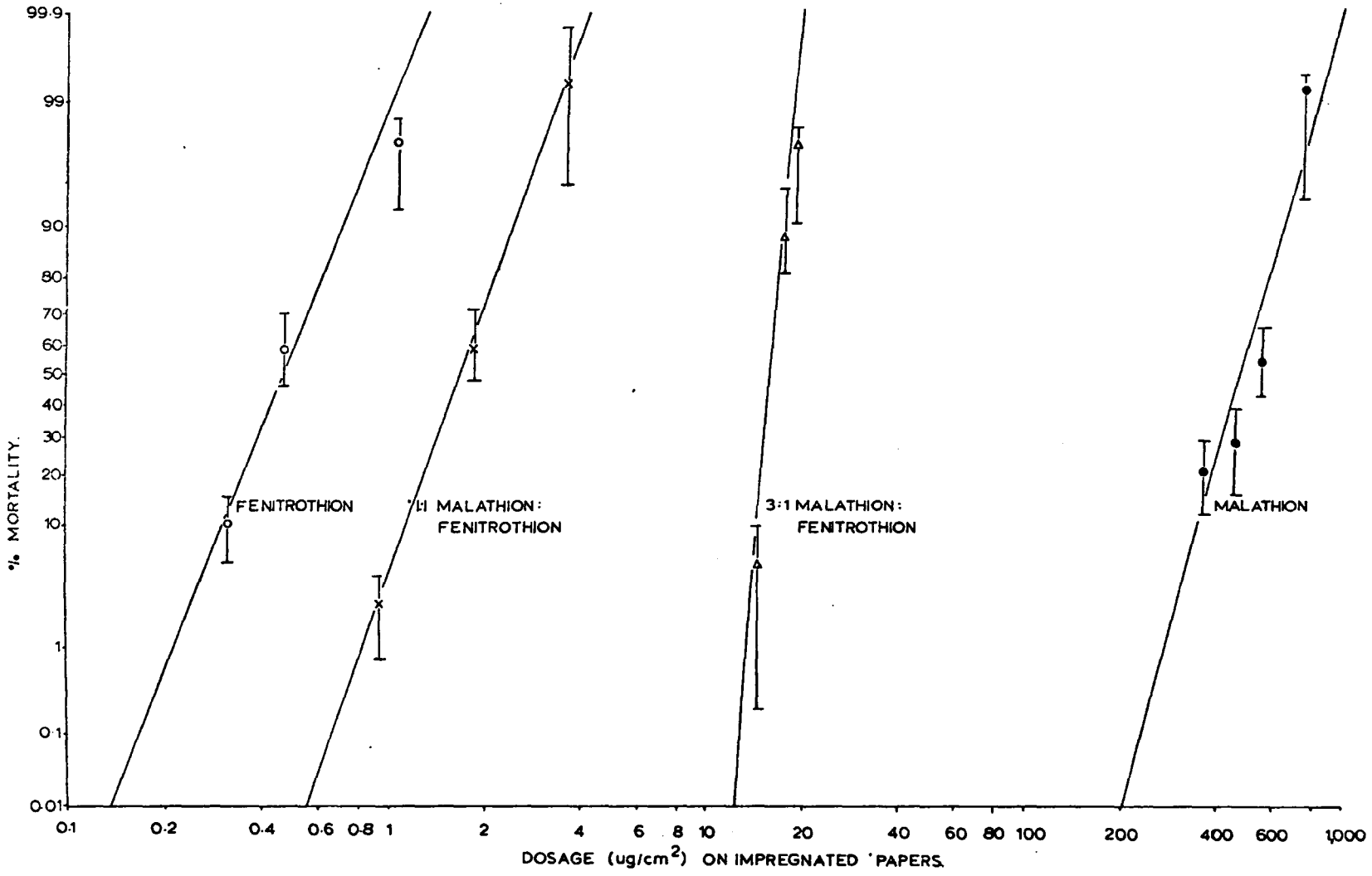


Table 68. LD_{50} and LD_{90} values for malathion, fenitrothion and mixtures of the two insecticides against malathion resistant (ST MAL) and susceptible (ST) *An. stephensi*. Values for goodness of fit of the points to straight lines are given.

	Malathion		Fenitrothion		Malathion: Fenitrothion (3:1)		Malathion: Fenitrothion (1:1)	
LD_{50} ($\mu g/cm^2$)	ST	ST MAL	ST	ST MAL	ST	ST MAL	ST	ST MAL
	9.62	528.9	0.154	0.641	0.891	1.815	11.81	16.85
LD_{90} ($\mu g/cm^2$)	13.4	721.9	0.224	1.235	1.307	2.705	16.38	18.53
Slope (SD)	8.96 ±.011	9.43 ±.009	7.98 ±.021	4.5 ±.013	7.71 ±.016	7.4 ±.009	9.0 ±.012	31 ±.004
χ^2 (d,f)	1.28 (1)	24.4 (4)	5.18 (1)	23.6 (1)	0.53 (1)	0.00 (1)	4.9 (1)	2.19 (1)
p	ns	<0.001	ns	<0.001	ns	ns	.05-.01	ns

was occurring in the mixtures. In the case of ST MAL the differences between the toxicities of fenitrothion and malathion, and the non-linearity of the ST MAL malathion LDP line (see Table 68), meant that a more meaningful plot was obtained by assuming that malathion is non-toxic to the ST MAL colony (see Figure 39).

It can be shown mathematically that points which lie below the fenitrothion LD_{50} line in Figure 39 indicate that malathion is synergising fenitrothion, whereas points which lie above the fenitrothion LD_{50} line demonstrate that malathion is antagonising the action of fenitrothion. Figure 39 shows that malathion is having a strong antagonistic effect on the action of fenitrothion in the malathion resistant stock.

The isobologram for ST assumes that malathion and fenitrothion are toxic. The points for the LDP lines for exposure of ST to malathion and fenitrothion do not deviate significantly from a straight line, so the LD_{50} values from these lines were used to produce the isobologram in Figure 38 .This again shows that malathion is strongly antagonistic to fenitrothion. However because of the proportions of the active ingredients used and the higher inate toxicity of fenitrothion, as measured by this test, it is difficult to say what if any effect fenitrothion is having on the action of malathion.

B. THE JOINT ACTION OF MALATHION AND KITAZIN.

The fungicide Kitazin (O,O-bis-isopropyl-S-phenyl

Figure 38. Isobologram for ST with LD_{50} values for fenitrothion, malathion and 3:1 and 1:1 mixtures of malathion and fenitrothion.

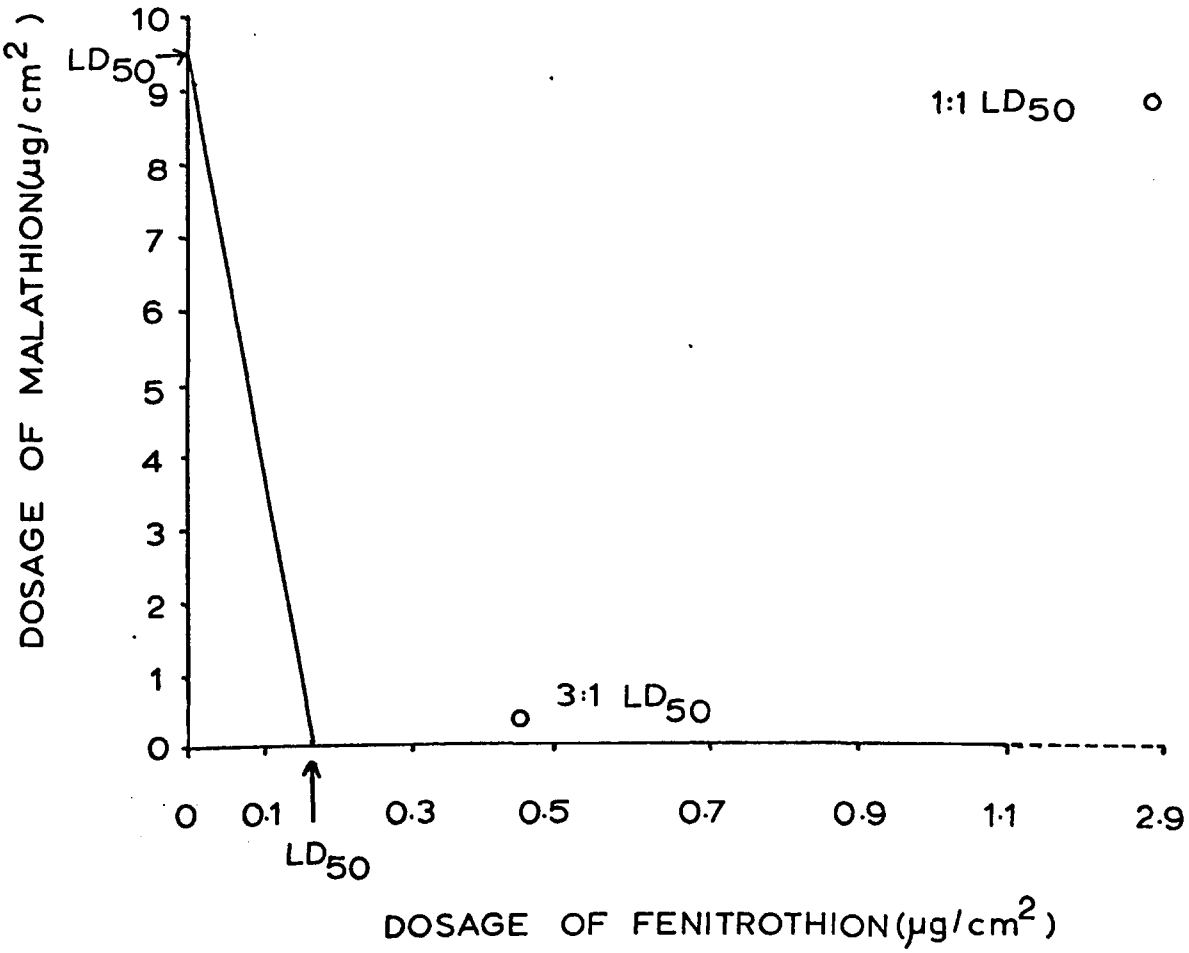
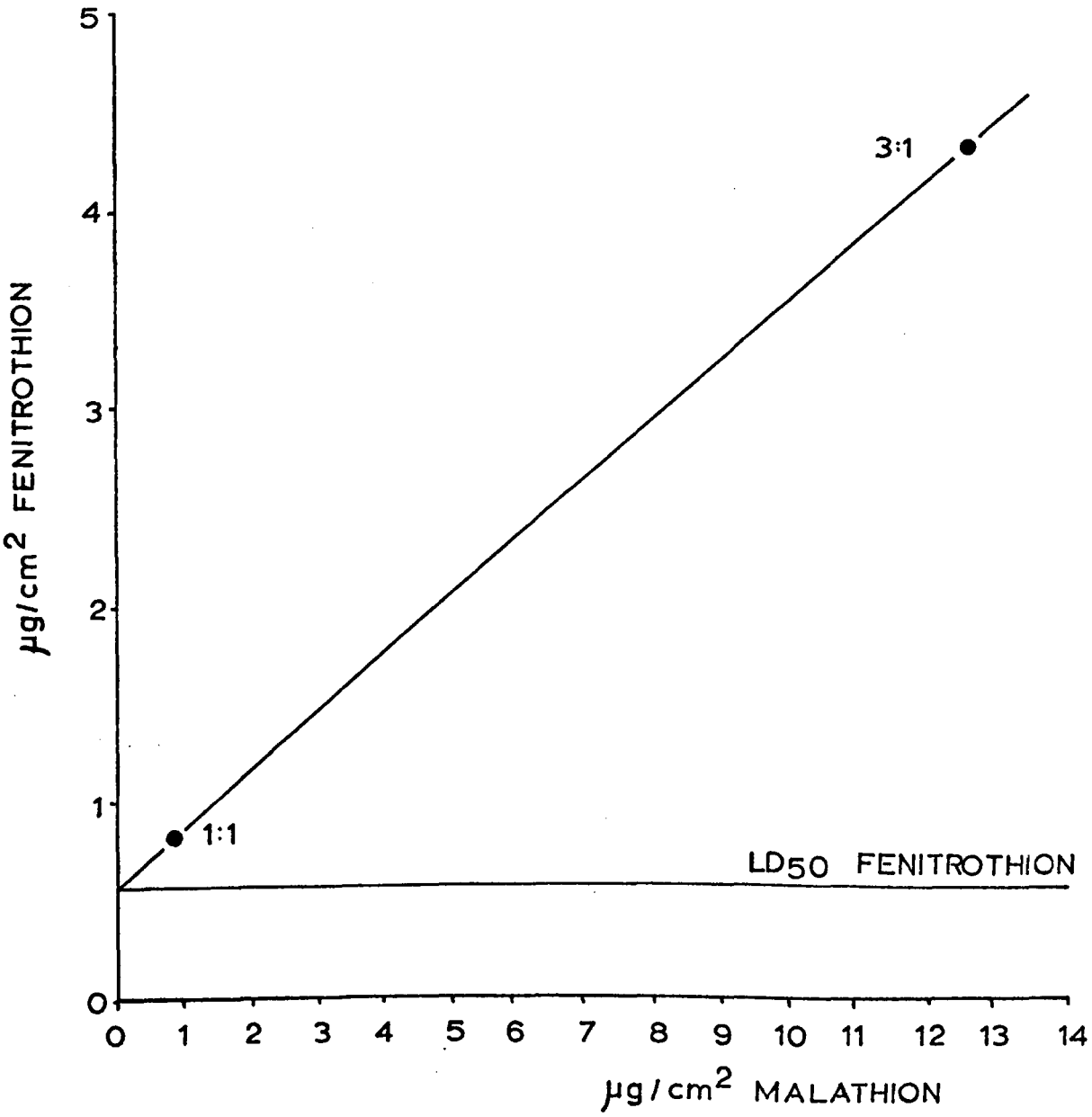


Figure 39. Isobologram for fenitrothion tested against ST MAL. Points for LD_{50} values of a 3:1 and a 1:1 mixture of malathion and fenitrothion against ST MAL are plotted.



-methyl-phosphorothioate) has been loosely described as a synergist by the Japanese authors Miyata and Saito (1980), due to its joint toxic action with malathion against malathion resistant green rice leafhoppers. They reported that this joint action was observed in the resistant strains but not in the susceptible strains and suggested that the action may be caused by inhibition of carboxylesterase by kitazin.

Serial dilutions of kitazin impregnated papers were prepared using acetone as a volatile solvent (no non-volatile solvent was used as pure kitazin is a liquid). The toxicity of kitazin to malathion resistant (ST MAL) and susceptible (ST) *An. stephensi* populations was then determined. The results are given in Table 69 . These results were used to produce LDP-mortality lines for ST and ST MAL. From Figure 40 it is clear that the malathion resistant and susceptible populations do not show the same 'susceptibility' to kitazin. The malathion resistant ST MAL is more 'tolerant' to kitazin impregnated papers than the malathion susceptible ST population. However the males of ST MAL appear more susceptible to kitazin than the females (see Table 69) whereas the males of this stock are more resistant to malathion than the females (see Figure 15).

Maximum-likelihood fitting of LDP mortality lines showed that the ST colony produced a linear response to both malathion and kitazin exposures. The ST MAL colony shows a non-linear response for malathion ($p = <0.001$) and

Table 69. Percentage mortalities of ST and ST MAL after exposure to various concentrations of malathion or kitazin (figures in parenthesis are numbers tested).

Dosage ($\mu g/cm^2$)	Malathion		Kitazin			
	ST	ST MAL	ST		ST MAL	
			♂	♀	♂	♀
1166.4	100(20)	100(80)				
777.6	100(20)	99.2(130)				
583.2	100(20)	52.5(80)				
486	100(20)	27.5(80)				
388.8	100(80)	20 (80)				
220.2	100(80)	0 (80)	100(71)	100(68)	100(113)	95(100)
194.4	100(80)	0 (80)	100(42)	100(56)	100(142)	73(110)
110	100(40)	0 (20)	100(42)	100(44)	88(159)	17(110)
97.2			100(36)	100(51)	54(69)	8(62)
64.8			72(51)	67(49)	0(42)	0(37)
55.1			25(53)	32(47)	0(61)	0(49)
48.6			5(41)	7(61)		
32.4			0(47)	0(51)		
18.2	100(60)	0(80)				
12.2	85(60)	0(40)				
9.1	38(100)	0(40)				
6.1	5(80)	0(20)				
3.9	0(80)	0(40)				

FIGURE 40. LOG-DOSAGE PROBIT-MORTALITY LINES FOR ST AND ST MAL AFTER EXPOSURE TO MALATHION OR KITAZIN.

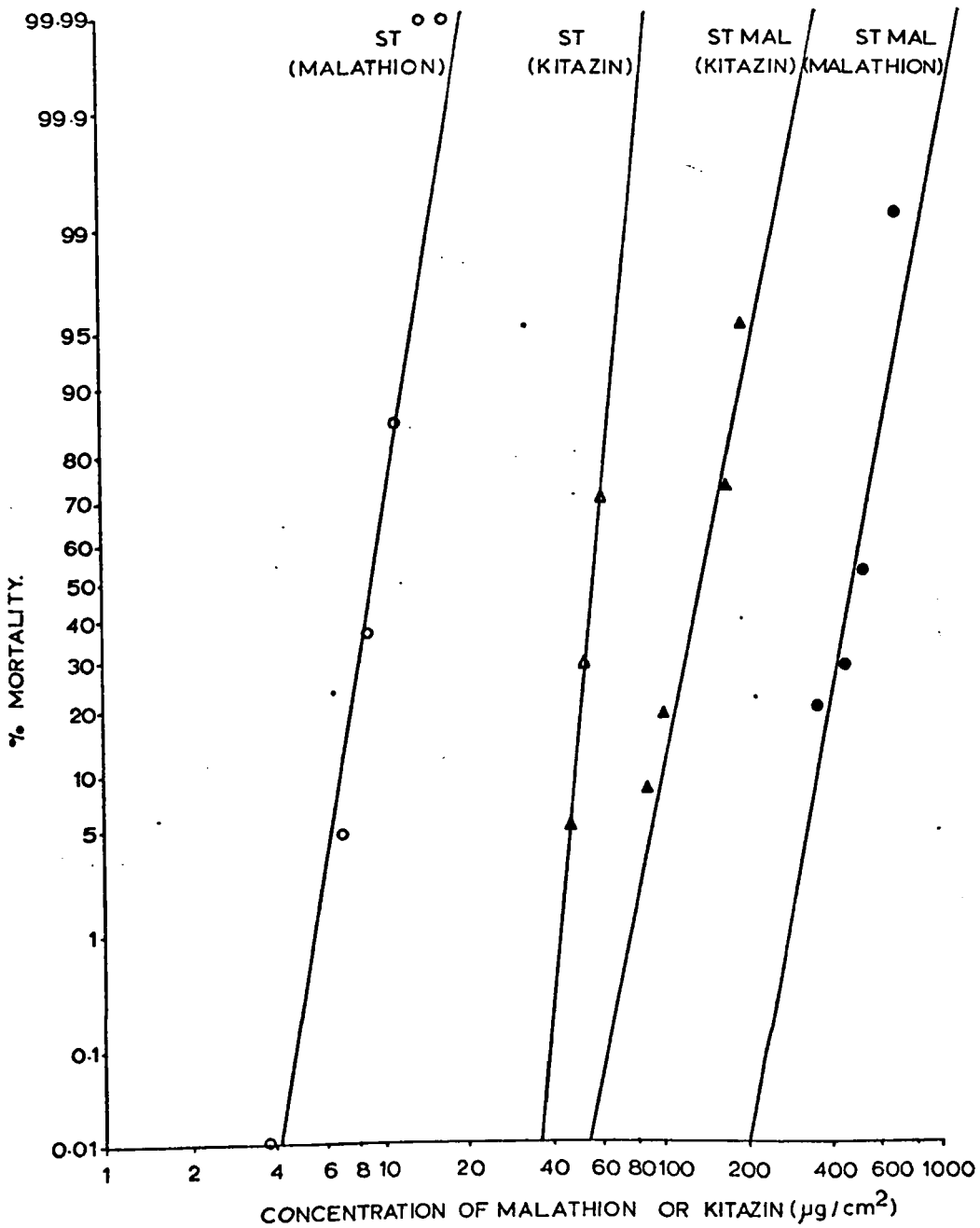


Table 70. LD_{50} and LD_{90} values for ST and ST MAL colonies of *An. stephensi* after exposure to malathion or kitazin. (Values for goodness of fit of the data to a straight line are given).

	Malathion		Kitazin	
	ST	ST MAL	ST	ST MAL
LD_{50} ($\mu g/cm^2$)	9.61	528.9	59.95	149.9
LD_{90} ($\mu g/cm^2$)	13.4	723.2	71.5	223.3
Slope (SD)	8.9(.01)	9.4 (.009)	16.8(.005)	7.4(.011)
χ^2(df)	1.28(1)	22.7(2)	.01(1)	7.1(2)
p	ns	<0.001	ns	o.5-0.1

kitazin (p = 0.5-0.1). The points on these plots suggest some degree of heterogeneity in the ST MAL population (see Figure 40).

The LD_{50} values of ST and ST MAL for both malathion and kitazin were used to produce the isobolograms in Figures 43 and 45 and three equidistant points were plotted on these isoboles. The proportions of the two insecticides were then read from the isobole for each of the three mixtures. The predicted proportions in the mixtures to give LD_{50} values are given in the inset tables in Figures 43 and 45 . Serial dilutions of these mixtures were then prepared. The proportions of each of the active ingredients in the mixtures are given in Table 71 .

In addition a fourth set of mixtures of 45$\mu g/cm^2$ kitazin and varying concentrations of malathion were tested against ST and 108$\mu g/cm^2$ kitazin and varying

Table 71 . The relative proportions of malathion and kitazin in the mixtures tested against ST and ST MAL.

Mixture	ST	ST MAL
	malathion:kitazin	Malathion:kitazin
I	1:2	11.43:1
II	1:6.25	4:1
III	1:18	1.39:1

concentrations of malathion against ST MAL.

Results of exposures to mixtures I to III for ST are given in Table 72 . These results were used to produce LDP lines for each of the three mixtures (see Figure 41). All lines were fitted by maximum-likelihood analysis. LD_{50} and LD_{90} values and values for goodness of fit to a straight line are given in Table 73 . From these figures it is evident that the points for the three mixtures all conform to straight lines. The proportions of malathion and kitazin at the LD_{50} values for each of the three mixtures were calculated and plotted on the isobole in Figure 43 .

Results for exposure of ST against mixture IV , are given in Table 74 . A log-dosage probit-mortality line for this mixture is plotted in Figure 44 .These points fitted a straight line. The LD_{50} value for mixture IV was plotted on the isobole in Figure 43 .

If the LD_{50} values for mixtures I-IV lie below the predicted isobol line then potentiation has occurred

Table 72. Percentage mortalities for mixtures of malathion/ kitazin against ST (figures in parenthesis are numbers tested).

Dosage $\mu g/cm^2$	I	II	III
47.5			93.2(44)
34.8		98.6(71)	
23.75			86.4(44)
23.2		89.5(67)	
22.5	100(82)		
17.4		90.9(44)	
15.8			81.9(72)
15	100(123)		
14.5		82.5(57)	
11.9			70.2(47)
11.6		65.7(70)	
5.6	96.2(59)		
4.1	85.6(95)		
2	70.8(72)		
1.35	49.5(190)		
0.675	11.43(35)		

FIGURE 41. LOG-DOSAGE PROBIT MORTALITY LINES FOR ST AFTER EXPOSURE TO MIXTURES I,II AND III OF MALATHION AND KITAZIN.

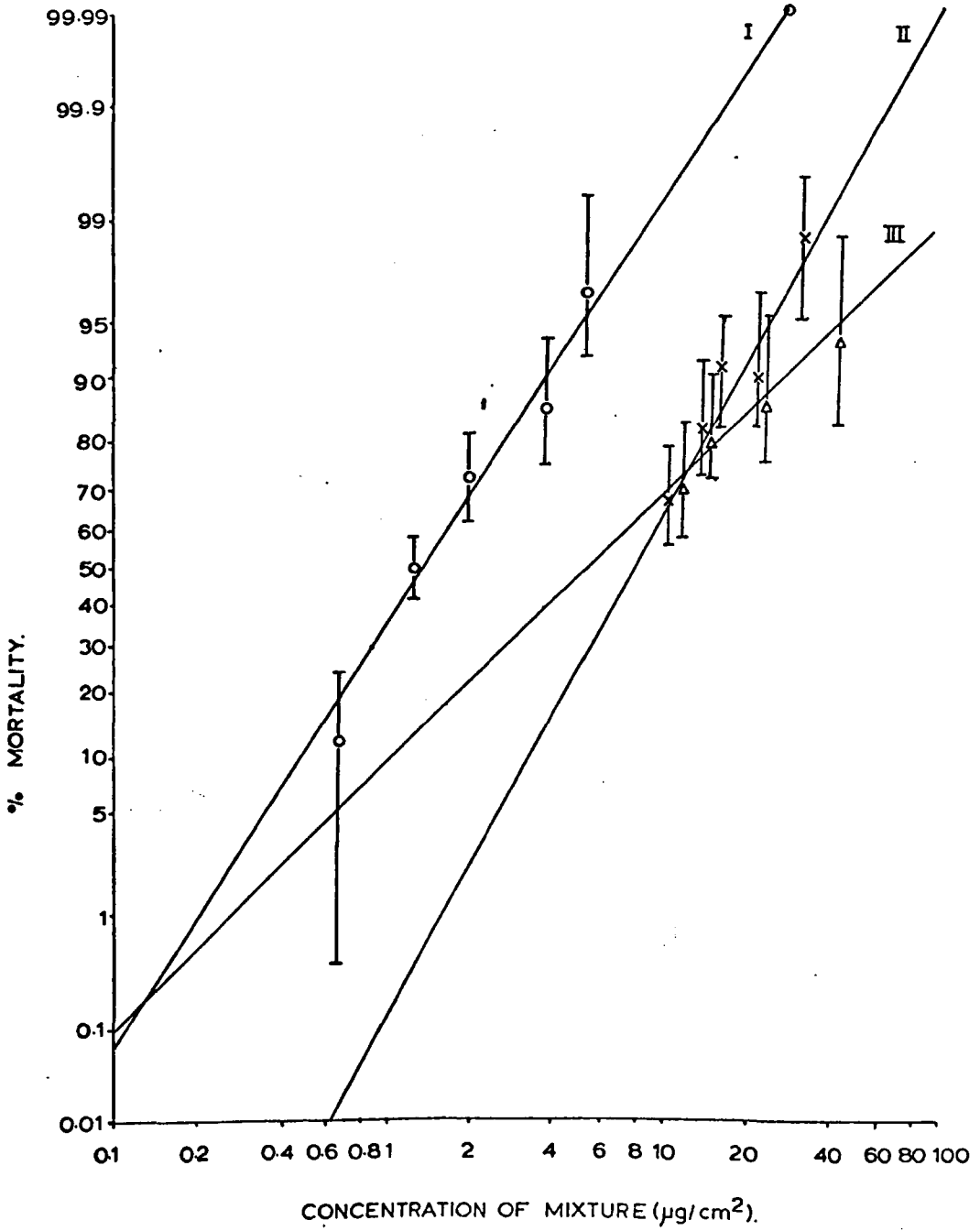


Table 73 . LD_{50} and LD_{90} values for ST after exposure to malathion and kitazin mixtures. (Values for goodness of fit of the points to a straight line are given).

	Mixture tested.		
	I	II	III
LD_{50} ($\mu g/cm^2$)	1.4	8.3	4.6
LD_{90} ($\mu g/cm^2$)	4.2	20.1	31.7
Slope (SD)	2.7(.026)	3.3(.063)	1.5(.22)
χ^2(df)	4.2(3)	3.1(3)	0.6(2)
p	ns	ns	ns

ie. the mixtures are more effective than either compound on their own. This is clearly the case with the kitazin/ malathion mixtures (see Figure 43).

The mixtures for bioassay against ST MAL were treated in the same way as those for ST. Results of exposures of ST MAL to mixtures I(a)-III(a) are given in Table 75 . These results were used to produce log-dosage probit-mortality lines for each of the mixtures (see Figure 45). All lines were fitted by maximum-likelihood analysis. LD_{50} and LD_{90} values and values for goodness of fit of the points to straight lines are given in Table 76 . From these figures it is evident that the response to all the mixtures is non-linear. This may reflect the non-linearity of the kitazin and malathion response in this population (see Table 70).

Results of exposure of ST MAL to mixture IV(a) are

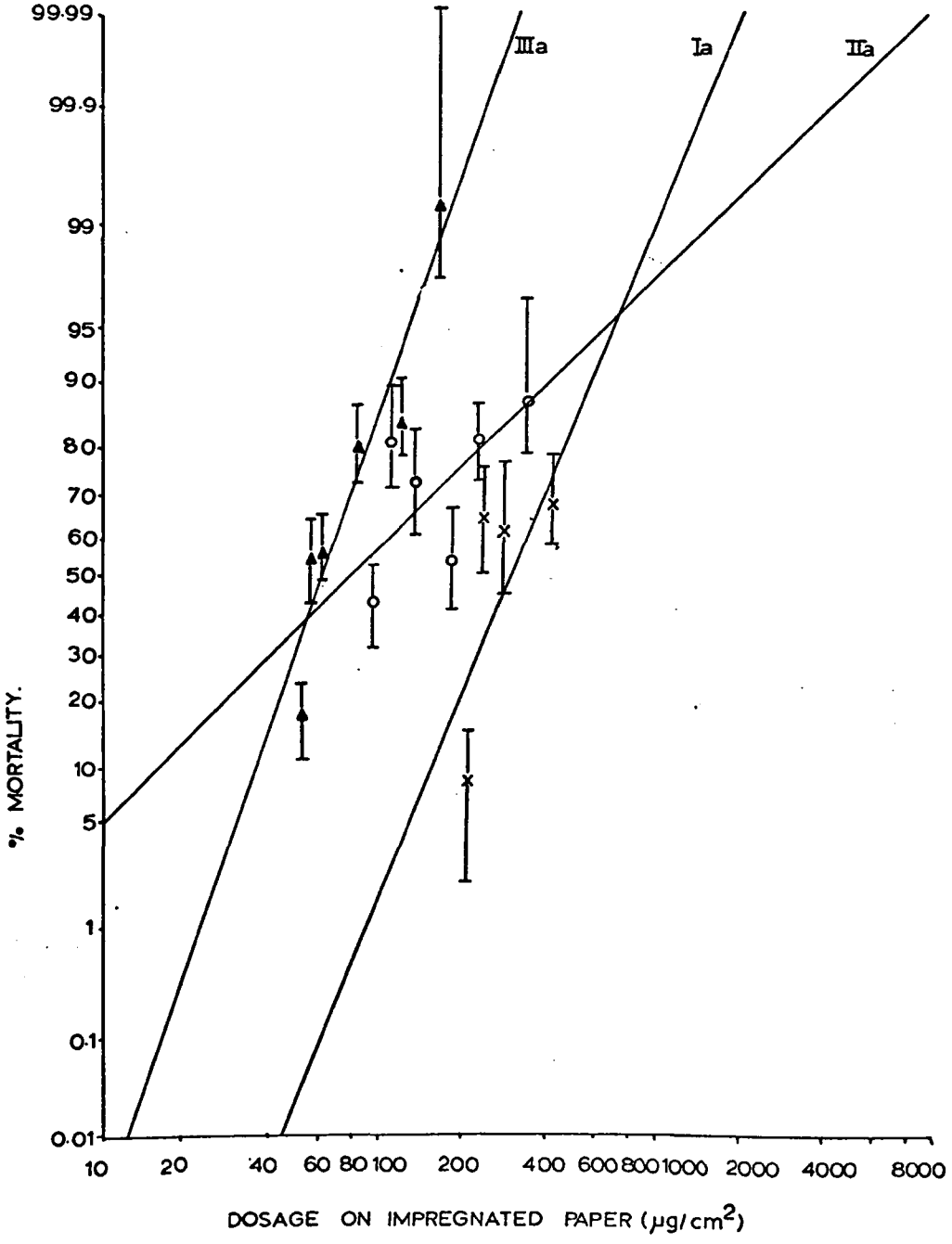
Table 74. Percentage mortalities of ST after exposure to papers impregnated with mixtures of 45µg/cm^2 kitazin + various concentrations of malathion (figures in parenthesis are numbers tested).

Concentration of malathion	Concentration of kitazin	% mortality
2.5µg/cm^2	45µg/cm^2	100(72)
2 µg/cm^2	45µg/cm^2	100(80)
1 µg/cm^2	45µg/cm^2	98.4(123)
0.5µg/cm^2	45µg/cm^2	66.2(142)
0.4µg/cm^2	45µg/cm^2	61.1(144)
0.3µg/cm^2	45µg/cm^2	49.6(131)
0.2µg/cm^2	45µg/cm^2	21.3(122)

Table 75. Percentage mortalities for ST MAL exposed to various concentrations of mixtures of malathion and kitazin.

Dosage ($\mu g/cm^2$)	I(a)	II(a)	III(a)
435	66.4(122)		
350		87.3(55)	
290	60.5(43)		
258			100(62)
253	64.4(59)		
233.3		81(100)	
217.5	8.9(112)		
175		53.8(65)	
172			99.2(125)
145.8		72.5(40)	
129			84.4(122)
116.7		81.7(60)	
87.5		44.3(79)	
86			80.8(130)
64.5			56.2(146)
60			53.3(90)
53.5			17.1(123)
40.2			0 (140)

FIGURE 42. LOG-DOSAGE PROBIT-MORTALITY LINES FOR ST MAL AFTER EXPOSURE TO MIXTURES Ia, IIa AND IIIa OF KITAZIN AND MALATHION.



	MIXTURES MAL/KIT		
	1	2	3
PREDICTED LD_{50}	7·5/15	4·8/30	2·5/45
ACTUAL LD_{50}	0·47/0·93	1·14/7·14	0·24/4·37
	I	II	III

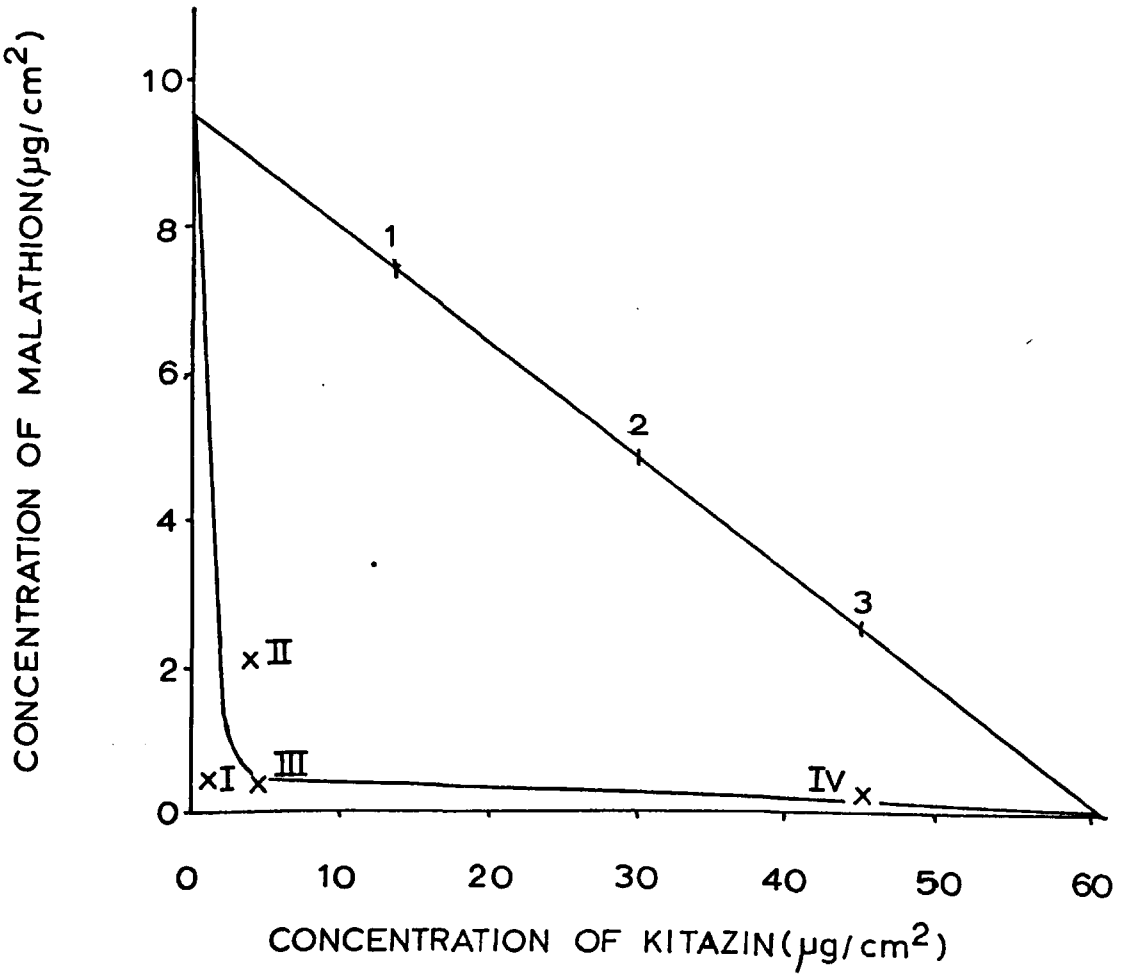


Figure 43. Isobologram for malathion and kitazin showing the predicted and actual LD_{50} values of the four mixtures tested against ST.

Table 76 . LD_{50} and LD_{90} values for ST MAL after exposure to mixtures of malathion and kitazin. (Values for goodness of fit of the points to straight lines are given).

	Mixtures tested		
	I(a)	II(a)	III(a)
$LD_{50}(\mu g/cm^2)$	316.9	81.2	65.22
$LD_{90}(\mu g/cm^2)$	638.2	451.1	119.3
Slope (SD)	4.2(.017)	1.7(.07)	4.8(.01)
χ^2(df)	45.9(2)	24.3(2)	39.8(2)
p	<0.001	<0.001	<0.001

given in Table 77. A log-dosage probit-mortality line for these results is plotted in Figure 44 . These points again deviate significantly from a straight line (p = <0.001). The fact that ST MAL shows a non-linear response when tested against all four mixtures of malathion and kitazin, as well as both compounds on their own is a strong indication that this colony is not yet homozygous for kitazin or malathion resistance. However this is not supported by genetic studies on malathion resistance in this popualtion.

Although the points for the mixtures significantly deviate from a straight line the computed LD_{50} values have been plotted on the isobologram in Figure 45 to give an indication of whether potentiation, additive joint action or antagonism of kitazin and malathion is occurring.

Table 77. Percentage mortalities of ST MAL after exposure to papers impregnated with 108µg/cm^2 kitazin and varying concentrations of malathion. (Figures in parenthesis are numbers tested).

Concentration of malathion	Concentration of kitazin	% mortality
100µg/cm^2	108µg/cm^2	100(64)
80µg/cm^2	108µg/cm^2	100(45)
75µg/cm^2	108µg/cm^2	89.9(99)
70µg/cm^2	108µg/cm^2	84.9(132)
65µg/cm^2	108µg/cm^2	54.8(115)
60µg/cm^2	108µg/cm^2	48.9(92)
50µg/cm^2	108µg/cm^2	49.1(106)
40µg/cm^2	108µg/cm^2	37.5(32)
30µg/cm^2	108µg/cm^2	0 (42)
20µg/cm^2	108µg/cm^2	0 (105)

Figure 44. Log-dosage probit-mortality lines for ST MAL and ST after exposure to mixture IV and IVa of malathion and kitazin.

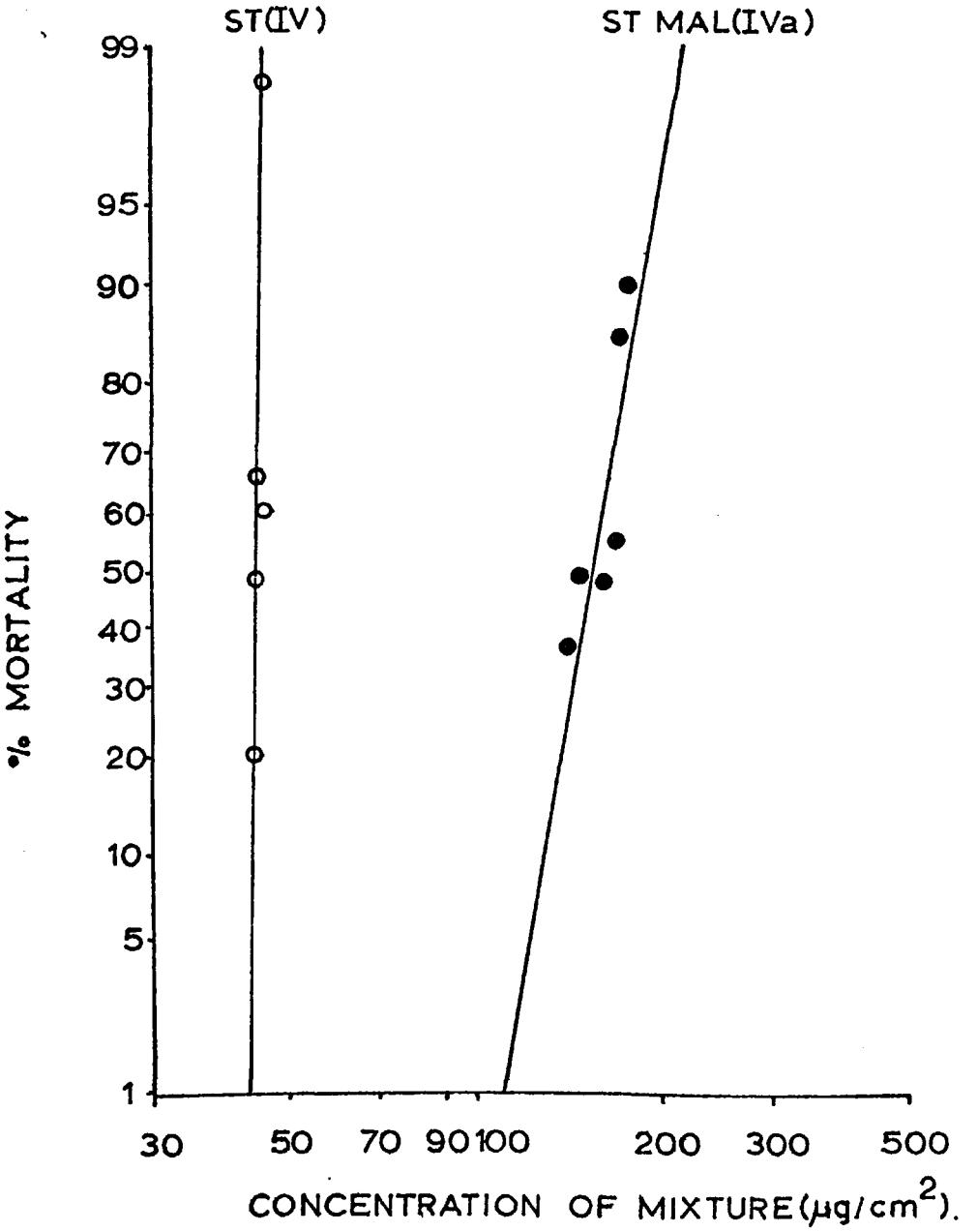
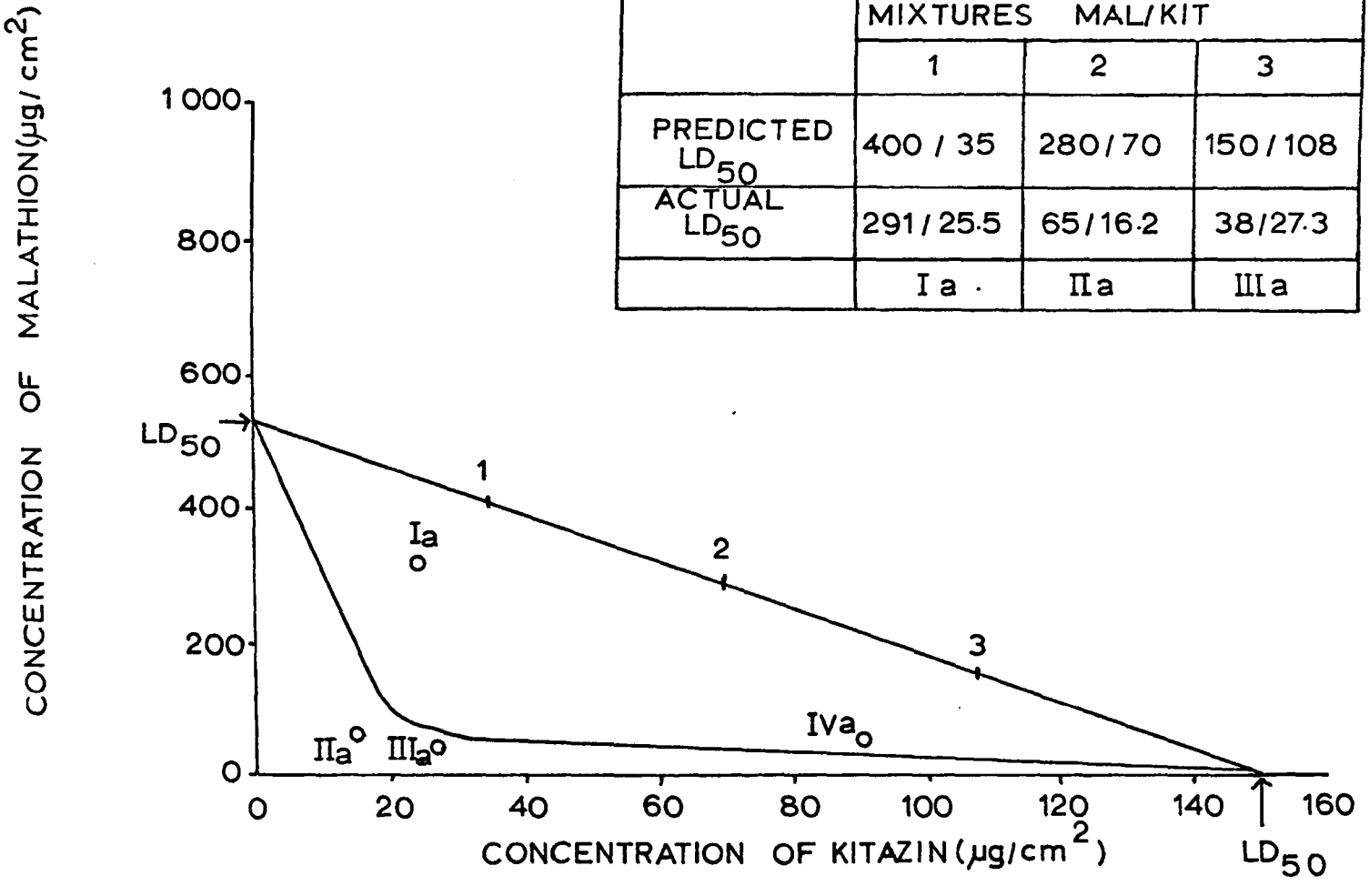


Figure 45. Isobologram for malathion and kitazin showing the predicted and actual LD_{50} values of the four mixtures tested against ST MAL.

	MIXTURES MAL/KIT		
	1	2	3
PREDICTED LD_{50}	400 / 35	280/70	150/108
ACTUAL LD_{50}	291/25.5	65/16.2	38/27.3
	I a	II a	III a



As in the case of ST, all the actual LD_{50} values lay below the predicted line indicating that the mixtures are more effective than either malathion or kitazin alone.

It can be concluded that malathion and kitazin act to increase the toxicity of each other in mixtures, in both the malathion resistant and susceptible *An. stephensi* populations tested. This is in contrast to the mixtures of malathion and fenitrothion tested against the same populations of *An. stephensi*, where malathion acted to decrease the toxicity of fenitrothion to both the malathion resistant and susceptible populations.

The observed resistance to kitazin in the ST MAL population as compared to the ST population, may indicate that the carboxylesterase resistance mechanism in the ST MAL population is able to detoxify some of the applied kitazin. If this is the case however, it is less efficient at detoxifying kitazin than malathion, as there is a much higher level of malathion resistance in this population if the LD_{50} values of ST and ST MAL for malathion and kitazin are compared.

"Synergistic" ratios from isobolograms in Figures 43 and 45 are 1:4.5 for ST MAL and 1:9.2 for ST indicating that there is greater potentiation in ST than in ST MAL. This is not the case in the green rice leaf hopper where the effect on the malathion resistant strain was greater than that on the susceptible strain. However too much weight cannot be put on these ratios due to the non-linear response of ST MAL to the mixtures.

DISCUSSION.

A. LABORATORY STUDIES OF INSECTICIDE RESISTANCE.

The study undertaken here attempts to answer the following questions:-

a) To what insecticides are the adult anophelines resistant?

b) Is resistance in the adults also expressed in the larvae?

c) What is the genetic basis of the resistance?

d) What is the biochemical mechanism underlying the resistance?

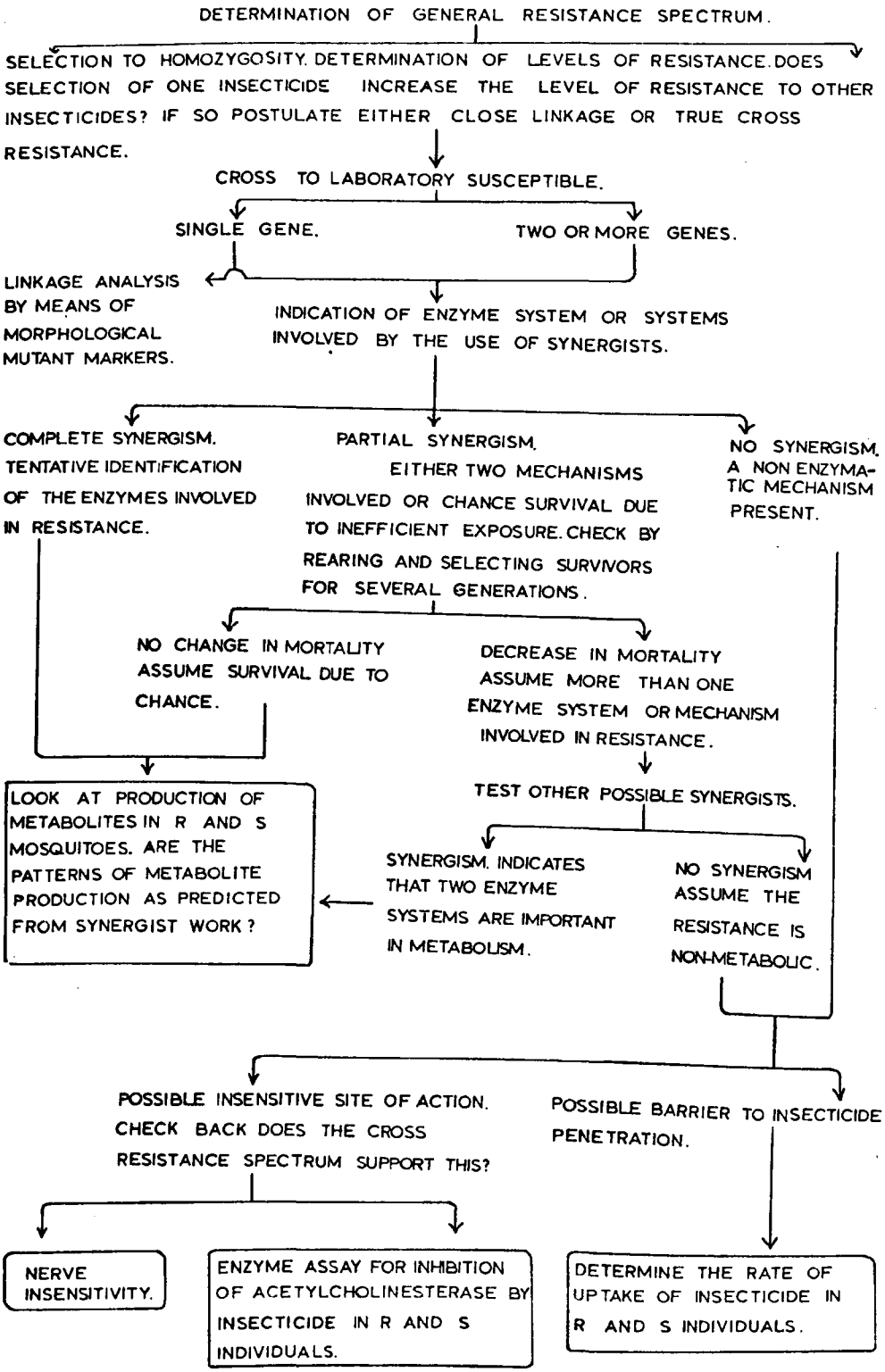
The stages of the investigation can be represented by a general flow chart (see Figure 46).

The resistance spectra of mosquitoes.

Determination of the general resistance spectra is the first stage in the investigation of any insecticide resistant population (see Figure 46). The spectrum of insecticides tested against adult mosquitoes was chosen to cover all four major groups of insecticides.

The presence of patterns of resistance within or between groups was noted. For example within the organophosphate group what combination, if any, of phenthoate, malathion and fenitrothion resistance occurred? The presence of particular patterns of resistance then gave the first indication of the mechanism underlying the resistance(s). The high level of malathion and phenthoate resistance in *An. arabiensis* from Sudan, and *An. stephensi* from Iraq, Iran and Pakistan coupled with no, or low level,

FIGURE 46. STAGES IN THE INVESTIGATION OF RESISTANCE MECHANISMS IN ANOPHELINE MOSQUITOES.



fenitrothion resistance indicated the possibility of a carboxylesterase resistance mechanism in these populations. Emphasis should not be placed on the existance of similar levels of resistance as measured by discriminating dosages at this stage, as the exact genotypes surviving the discriminating dosages are unknown and it is probable that the discriminating dosages of different insecticides may affect different genotypes. At present only one insecticide, dieldrin, can be used with a reasonable degree of confidence to differentiate susceptible, heterozygous resistant and homozygous resistant mosquitoes using two standard discriminating dosages.

The MACHO population was produced from the FERNS/RR population by translocating a propoxur-selected resistance gene from an autosome to the Y chromosome, then backcrossing to a susceptible population, while selecting for male resistance (Seawright *et al* 1978). The MACHO population therefore has males which are heterozygous for the propoxur resistance gene and females which are homozygous for the susceptible allele. If the resistance spectrum of the FERNS/RR colony (Herath & Davidson 1981) and the MACHO males are compared, the only difference between the two is the presence of phenthoate resistance in the FERNS/RR population but not in the MACHO strain.

The MACHO system is ideal for studying the resistance spectrum conferred by the translocated gene in its heterozygous form. Any resistance due to the translocated gene will only be apparent in the males, the females of

this stock can then be considered as controls. These females should show 100% mortality for any resistance caused by the translocated gene, unless crossing-over has occurred between the translocated gene and the Y-chromosome. The broad resistance spectrum in the MACHO males but not in the females indicates that the resistance factor involved is an altered acetylcholinesterase, rather than a metabolic enzyme which would detoxify the insecticides. Ariaratnam & Georghiou (1975) also worked with a population of *An. albimanus* from El Salvador which showed high organophosphate (OP) and carbamate resistance following laboratory selection with propoxur. The broad spectrum resistance in this population was shown to be due to an altered acetylcholinesterase (AChE) rather than metabolic breakdown of the insecticides. Ayad & Georghiou (1973) investigated the inhibition of the AChE by various insecticides in *An. albimanus*. They showed that the AChE was 25,300x less sensitive to propoxur and 371x less sensitive to paraoxon than the susceptible AChE strain. However the altered AChE was only 1.6x less sensitive to fenoxon.

The resistance spectrum of the population studied by Ariaratnam & Georghiou (1971) and Ayad & Georghiou (1973) and that seen in the MACHO population are very similar, supporting the altered AChE hypothesis in the MACHO population. However if the same gene is present in both colonies either the heterozygote's "susceptibility" level is very much closer to the homozygote susceptible

level, as MACHO males are 20x more resistant to propoxur than the females, or the altered AChE of this population is less insensitive to inhibition than that studied by Ayad & Georghiou (1973).

The presence of phenthoate resistance in FERNS/RR but not in the MACHO strain may indicate that there was originally a carboxylesterase resistance mechanism in this population, as well as the more general resistance conferred by the translocated gene.

The resistance spectra of various mosquito species assimilated from a number of authors are given in Table 78 . It is unfortunate that the majority of these studies did not include phenthoate, acethion, dimethoate or malaoxon testing in conjunction with malathion testing, where malathion resistance was found, as lack of this information makes comparisons of resistance spectra of a number of species difficult. Of the species studied two anophelines showed malathion and fenitrothion resistance but no phenthoate resistance, four anophelines showed malathion, fenitrothion and phenthoate resistance, while two showed malathion and phenthoate resistance but no fenitrothion resistance. Of the other resistance spectra given two anophelines, three aedine and eight culicine species had malathion and fenitrothion resistance while two Aedes species were resistant to malathion but susceptible to fenitrothion. In cases where malathion and fenitrothion resistance have been reported very few studies go on to show whether the two resistances are dependent

Table 78. The resistance spectra of a number of anopheline, aedine and culicine mosquitoes.

SPECIES	LOCATION	Organophosphates										Carbamates		Pyrethroids		REFERENCES
		Malathion	Malaoxon	Phenthoate	Fenitrothion	Pirimiphos methyl	Dimethoate	Fenthion	Chlorpyrifos	Parathion	Abate	Propoxur	Carbaryl	Permethrin	Deltamethrin	
An. albimanus	El Salvador	R	R	R	R	R	R	R	R	R	S	R	R		S	115
An. albimanus	El Salvador	R	R	S	R	R	R	R	R	R	S	R	R		S	11,15 present study (ps)
An. atroparvus	Spain	R		S	R	S		R	S			R	R	S		ps
An. freeborni	U.S.A (Californ)							S		S	S					276
An. culicifacies	India	R	R		R	R	S	S				S		S		117
An. stephensi	Iraq	R	R	R	R	S		S				S		S	S	ps
	Iran (I)	R	R	R	R	S		S				S	S			
	Iran (II)	R	R	R	R							R				55
	Pakistan (I)	R		R	S	S		S				S	S	S	S	ps
	Pakistan (II)													R(D)		190
An. sinensis	Japan	R			R			R								55
	China	R														
An. messeae	Romania	R						R								
An. arabiensis	Sudan	R		R	S	S	S	S			S	S	S	R(D)	S	ps
An. gambiae	Nigeria	S	S	S	S		R?	S				S	S	R(D)	S	ps
An. sacharovi	Lebanon	S			R											238
	Syria	S			R											238
	Turkey	S			R	S	R	R	R	R		R	R	R		215
	Greece											R				
An. maculipennis	Turkey (I)	S			R	S		S				R	R			215
	Turkey (II)	S			S	S		R				R				215
An. hyrcanus	Turkey	S			R	R		R		S		S	S			117
An. sergenti	Jordan										R					238
An. d'thali	Jordan										R					238
An. multicolor	Jordan										R					238

(D) = associated with DDT resistance.

Table 78 continued.

Species	Location	Organophosphates										Carbamates		Pyrethroids		References
		Malathion	Malaoxon	Phenthoate	Fenitrothion	Pirimiphos methyl	Dimethoate	Fenthion	Chlorpyrifos	Parathion	Abate	Propoxur	Carbaryl	Permethrin	Deltamethrin	
Aedes aegypti	Thailand	R			S							R		R(D)		42
	Jakarta	R			R							R		R(D)		42
	Singapore	R			S							R		S		42
	Trinidad	R						R	R		R			R		220
	Caribbean	R			R			S	S			S		S		55
	Guyana	R												R(D)		211
Ae. albopictus	Madagascar				R			R								119
	Vietnam	R						S								244, 119
Ae. dorsalis	Utah	R						R		R						111
Ae. nigromaculis	USA(California)	R			R			R	R	R	R	S				55, 229
Ae. sollicitans	USA(Virginia)	R									R					55, 253
Ae. taeniorhynchus	USA(Florida)	R						S	S			S		S		22, 181
Ae. togai									R	R						22
Ae. vexans									R							22
Culex nigripalpus	USA(Florida)	R														22
C. fuscocephalus	Taiwan	R			R			R	R		R					55
C. pallens	Far East	R			R			R	R	R	R					55
C. pipiens	France	R						R	R	R	R	S		S	S	55
	Egypt	R						R	R	R	R	S		S	S	55
	USA(California)	S						R	S	S	S					89
	USA(Tennessee)	R						R						S		178
	Puerto Rico	R														180
C. pens	USA	R						R				R				180
C. quinquefasciatus	USA(Louisiana)	R							R							246
	Japan	R							R		R					180
	USA(California)								R	R	R	R				98
	USA(California)	R			R			R	R	R	R	S		S		90
	USA(California)	R			R			R				R				85
	Tanzania	R				R		R	R							45
	Burma	R						R								254
	Brazil	S			R			R	R		R	R				55
	USA(Texas)	R			R			R	R	R	R					175
C. tritaeniorhyncus	Japan	R						R	R							
	Korea	R			R			R								
C. tarsalis	USA(California)	R			R			R	R	R	R	S	S			7, 8
	USA(Coachella)	R			R			R	R		R	S	S			88

(D) = associated with DDT resistance.

on the same resistance mechanism.

It is desirable to select the population to, or near to, homozygosity with respect to the resistance gene or genes, as this simplifies the interpretation of future results. Selection is particularly useful in confirming resistance where initial numbers of survivors of a particular test are low. For example there was 98.4% mortality on 1% fenitrothion after one hours exposure of the ST IRAN colony of *An. stephensi* (see Figure 13). Selection of the survivors of this treatment for four generations with fenitrothion indicated that fenitrothion resistance was not present in this population. This is not consistent with the data of Herath (1979) who originally showed fenitrothion resistance in the same laboratory population. This is probably an example of a resistance gene or genes being lost from a laboratory population in the absence of positive selection pressure for resistance over several generations and illustrates the point that new material should be brought in from the field and that laboratory and field tests should be run concurrently,

Problems were encountered both in determining the presence of permethrin resistance and in selecting the resistance to homozygosity. Development of a test method utilising knock-down and mortality satisfactorily demonstrated the presence or absence of permethrin resistance in a population. However this method of selection was not very successful in selecting the permethrin resistance mechanism to homozygosity. Similar results have been obtained with

Aedes aegypti using knock-down as a criteria for selection (Malcolm personal communication).

Cross-resistance patterns.

Determination of the cross-resistance spectrum after selection for resistance is an invaluable tool which shows whether a single or a multiple resistance mechanism is present in a population and should form the second stage in the investigation of insecticide resistance (see Figure 46).

Cross-resistance studies indicated that a single resistance mechanism is responsible for the broad spectrum OP and carbamate resistance seen in *An. atroparvus* (AT SPA) but that the population should be described as containing multiple resistance as dieldrin and DDT resistance in this population are caused by two further unrelated single resistance mechanisms. A summary of the presence of single or multiple resistances in the populations studied is given in Table 79 . This table also shows the minimum number and scope of resistances that make up each multiple resistance mechanism. It is clear from this that the majority of populations studied have more than one resistance mechanism. What is not clear from the cross-resistance patterns is whether there is multiplicate resistance in a population, as it is impossible to tell what genotypes are affected by the insecticide dosage used in the cross-resistance studies.

It should be noted that of the six populations which showed malathion resistance four were associated

Table 79. Summary of multiple and single resistance mechanisms in the populations studied.

COLONY	Single (S) or multiple (M) resistance.	Minimum number of mechanisms	Spectrum of each resistance mechanism.
AT SPA	M	3	1.DDT 2.Dieldrin 3.OPs + carbamates
ATIG	S	1	1.DDT
MACHO	M	2	1.Dieldrin 2.OPs + carbamates
G1	M	3	1.DDT + permethrin. 2.Dieldrin 3.Malathion + phenthoate.
SB	S	I	1.DDT
IAN	M	2	1.DDT + permethrin. 2.Dieldrin
ST POND	S	1	1.DDT
ST IRAQ	M	4	1.DDT 2.Dieldrin 3.Malathion + phenthoate 4.Fenitrothion
ST IRAN and ST LA	M	3	1.DDT 2.Dieldrin 3.Malathion + phenthoate.

with phenthoate resistance, while the other two malathion resistances were associated with fairly broad spectrum OP and carbamate resistance which in neither case involved phenthoate. As both phenthoate and malathion contain a carboxylester bond, the connection between malathion- and phenthoate-resistance is a strong indication of the involvement of a carboxylesterase enzyme. However the lack of cross-resistance between chemicals containing a carboxylester bond should not be taken as definitive evidence that a carboxylesterase enzyme is not involved in the resistance. O'Brien (1967) has shown that acethion and malathion, which both contain a carboxylester bond and are fairly closely chemically related, are degraded by different carboxylesterase enzymes in many mammal and insect species. There are instances where both insecticides are degraded by the same enzyme however. Bigley & Plapp (1962) showed cross-resistance between malathion and acethion in the FRESNO strain of *Culex tarsalis*, but Darrow & Plapp (1960) working on a similar colony showed no difference in the susceptibility of malathion resistant and susceptible strains to acethion. Malathion/acethion cross-resistance has been reported in *Chrysomya putoria* (Townsend & Busvine 1969).

There was an association of DDT and permethrin resistance in two cases but not in the other seven DDT resistant colonies studied. The mode of action of DDT and pyrethroids are similar although they are not chemically related. Both DDT and pyrethroids show a negative temperature

co-efficient; that is toxicity is more intense at low temperatures. However the poisoning symptoms of the two differ. Adams & Miller (1980) showed that pyrethroids caused knock-down in houseflies which was characterised by loss of co-ordinated movements without pronounced excitation, i.e. paralysis of some sort, whereas flies poisoned with DDT went through a period of hyperexcitation which continued long after prostration. Both groups have been shown to act on the nerve sheath. It is therefore probable that a resistance mechanism associated with both compounds would be due to blockage of their action rather than metabolic breakdown of the insecticides.

Busvine (1951) first noted that an Italian DDT-resistant strain of housefly showed cross-resistance to pyrethrins. Since then cases of DDT/pyrethroid cross-resistance have been noted in *Culex tarsalis* (Plapp & Hoyer 1968), stableflies (Stenerson 1965) and cattle ticks (Wharton & Roulston 1970).

Genetics of resistance.

Before going on to detailed biochemical analysis it is helpful to determine whether resistance is monofactorially or polyfactorially inherited. This information indicates whether one biochemical mechanism forms the basis of the particular resistance studied, or whether several biochemical mechanisms are likely to be involved each having an additive or multiplicative effect on the level of resistance.

Unfortunately the genetic analyses of *An. stephensi* from Iraq and Iran are incomplete, as the colonies were

lost due to thermostat failure in the insectaries towards the close of these experiments. So only tentative conclusions about the inheritance of malathion resistance in these colonies can be drawn. In some cases it was necessary to repeat crosses, as only after the stocks had been crossed did it become clear that the selected resistant parental lines were not homozygous for the resistance gene(s) at the time of the original cross.

The genetic analysis of the populations studied can be summarised as follows:-

COLONY	INSECTICIDE	Mono- or Polyfactorial
AT SPA	Fenitrothion	M
AT SPA	Propoxur	P
ST IRAN	Malathion	M?
ST IRAQ	Malathion	M
ST LA	Malathion	M

Initially the results for the genetic analysis of propoxur resistance in AT SPA seem to contradict the cross-resistance patterns, which showed that propoxur and fenitrothion resistance in this population were due to the same factor(s). This may be explained by assuming that there is multiplicate resistance in this population with regard to propoxur, which would not be apparent in the cross-resistance studies.

Monofactorial inheritance of malathion resistance in the Iranian population of *An. stephensi* was tentatively

concluded by Herath (1979). However it should be noted that in all three of her backcrosses to the susceptible parental stock there was a significant departure from the expected ratios, due in all backcrosses to a lack of the susceptible phenotype.

The results of the crosses of ST IRAN and ST POND given here show that the results of the first backcross are very close to those expected on the single gene hypothesis, though further backcrosses were not performed. The results from the F_2 are significantly different from those expected on the single gene hypothesis. It is therefore impossible to conclude with any certainty from the genetic analysis alone whether the malathion resistance in this population is due to one or more than one gene.

Monofactorial inheritance of malathion resistance in *An. stephensi* from Iraq and Pakistan is indicated. The resistance gene in both colonies is partially dominant. The level of malathion resistance in the two colonies is very different. ST MAL, from Pakistan, was 37.5x as resistant as the ST susceptible, whereas ST IRAQ, from Iraq, was only 6.7x resistant. This is reflected in the resistance levels of the F_1 generation as the ST x ST MAL F_1 showed a higher level of malathion resistance than the ST IRAQ selected line. It would be interesting to know whether the resistance genes in these two populations are allelic as their cross-resistance patterns are similar, both appearing to have a carboxylesterase mediated resistance mechanism. Simple genetic crosses between ST IRAQ and ST

MAL could be used to test for allelism.

The genetics of malathion resistance in a population of *An. stephensi* from Pakistan was investigated by Rathor & Toqir (1980), who concluded that malathion resistance was due to a single semi-dominant gene. However there are anomalies between their results and those obtained here. Rathor & Toqir's study used papers prepared in their laboratory with acetone and absolute alcohol as the only solvents. The levels of resistance they reported were 25x higher than those for ST MAL tested with acetone/malathion impregnated papers. The susceptible colony they used also required twice the concentration of malathion to give 100% kill. The reason for the differences are unknown, as both their resistant colony and ST MAL came from the same parental stock and it is very unlikely that differences in genetic background would have such extensive effects on the levels of resistance conferred by a single resistance gene.

Lines (personal communication) crossed the Sudanese G1 to the malathion susceptible SENN population of *An. arabiensis*. His results indicated a single dominant autosomal gene was responsible for the malathion resistance in the G1 population.

The results of genetic analysis of insecticide resistance in a range of mosquito species are given in Table 80. This shows that malathion resistance tends to be inherited monofactorially with the exception of *Aedes aegypti* where three populations showing polyfactorial inheritance of malathion resistance have been described

Table 80. Monofactorial (M) or polyfactorial (P) inheritance of insecticide resistance in mosquitoes.

SPECIES	LOCATION	INSECTICIDE	MODE OF INHERITANCE	REFERENCE
Anopheles arabiensis	Sudan	Malathion	M(SD)	Lines(pers comm)
An. culicifacies	India	Malathion	P(?)	115
An. stephensi	Iran	Malathion	M(?)(SD)	Present study (ps)
An. stephensi	Iraq	Malathion	M (SD)	ps
An. stephensi	Pakistan	Malathion	M (SD)	ps , 218
Aedes aegypti	Trinidad	Malathion	P	158
Ae. aegypti	Malaya	Malathion	P	160
Ae. aegypti		Malathion	P	7
Ae. taeniorhynchus	USA (Florida)	Malathion	M	232
Culex tarsalis	USA(California)	Malathion	M(SD)	159
C. tarsalis	USA(California)	Malathion	M(SD)	37
An. atroparvus	Spain	Fenitrothion	M(SD)	ps
C. quinquefasciatus		Fenthion	M	67
C. tarsalis	USA(California)	Fenthion	P	9
C. pipiens	France	Chlorpyrifos	M(SD)	203
C. quinquefasciatus	Tanzania	Chlorpyrifos	M(SD)	45
C. quinquefasciatus	USA(California)	Abate	M	76
An. albimanus	El Salvador	Propoxur(OPs)	M(SD)	89
An. albimanus	El Salvador	Propoxur	M(SD)	57
An. atroparvus	Spain	Propoxur	P	ps
C. quinquefasciatus		Propoxur	P	84 85
C. tarsalis	(Orlando strain)	DDT/Pyrethroids	M(D)	208
C. quinquefasciatus	USA(California)	Permethrin	P	213

KEY
M = Monofactorial inheritance
SD = Semi-dominant
P = Polyfactorial inheritance
D = Dominant

In the majority of cases of monofactorial inheritance studied the F_1 generation showed a level of resistance intermediate between the susceptible and resistant parental lines. This was the case in all the populations in this study.

Georghiou (1965) showed polyfactorial inheritance of propoxur resistance in *Culex quinquefasciatus* which was also shown in the AT SPA colony of *An. atroparvus*. The resistance spectra of these two populations (see Table 78) are however very different. Monofactorial inheritance of propoxur resistance was recorded in *An. albimanus* (Georghiou *et al* 1974, Davidson & Sawyer 1975).

Linkage analysis of a resistance gene and a visible marker was only attempted in *An. atroparvus* as this was the only stock where a marker was readily available. Analysis showed that fenitrothion resistance and the marker *stripe* segregated independently. As both genes are not sex-linked it is probable that the fenitrothion resistance and *stripe* genes are on the remaining two autosomes, although linkage of the two genes at a distance of greater than 50 map units apart cannot be entirely discounted. Further linkage analysis in *An. atroparvus* could utilise both fenitrothion resistance and *stripe* as both genes are dominant and show excellent penetrance with the dosage of fenitrothion used in this study.

Linkage analysis in *Anopheles* species is, at present, limited due to a lack of suitable markers. However further studies of linkage should eventually provide a workable

genetic map which could be used to check allelism for resistance genes arising in different populations of the same species, and would be a useful step in furthering our understanding of resistance.

Genetic analysis of permethrin resistance in *An. gambiae* (IAN) and *An. arabiensis* (G1) was not attempted due to the difficulties in selecting homozygous resistant stocks. However synergist studies would favour polyfactorial inheritance in *An. gambiae*.

Synergism.

After determining the inheritance patterns of various resistances within the populations, synergist studies were undertaken to indicate the biochemical mechanisms underlying resistance. The maximum dose of synergist which gave no kill of the test insects was determined before experiments with the synergist/insecticide mixtures were carried out. In most cases it was rare that the synergist/insecticide tests gave 100% kill. Selection of survivors of the synergist/insecticide treatment was employed for several generations to demonstrate the presence or absence of further resistance mechanisms, when there was an obvious effect of the synergist in the original test. The effects of various synergists on the resistances in the populations studied are given in Table 81. From this it is clear that synergist studies support the indications that carboxylesterase is involved in the malathion/phenthoate resistance shown in the cross-resistance studies in ST IRAQ, ST LA and G1, as there is strong evidence of synergism with triphenyl phosphate (TPP) but no

Table 81. The effect of pretreatment with synergists on various resistances in the populations studied.

Presence (✓) or absence (x) of synergism.

Colony	Insecticide	Synergist				
		TPP	F-DMC	PB	SV_1	SESAMEX
ST IRAQ	Malathion	✓		X		
ST LA	Malathion	✓		X	✓	
G1	Malathion	✓		X	✓	
	DDT		✓			
AT SPA	Malathion	X		X	X	X
	Fenitrothion	X		X	X	X
	Propoxur(1hr)	X		X	X	X
	Propoxur(6hr)	X		✓	✓(?)	✓
MACHO	Propoxur			X		
ATIG	DDT		✓			
IAN	DDT		✓			
ST IRAN	Malathion	✓		X		

evidence of synergism with PB. The fact that no further mechanism could be selected out, over several generations of selection, from the TPP/malathion survivors also supports the evidence from genetic studies that there is a single major gene involved in the malathion/phenthoate resistance in all these four populations.

A summary of malathion-selected resistance mechanisms in arthropod species is given in Table 82 . This shows that malathion strongly selects for a carboxylesterase resistance mechanism, as in all cases but that of *Rhyzopertha dominica* from Kuwait (Matthews 1980), carboxylesterase enzymes are implicated in the detoxication of malathion. However a note of caution should be introduced when considering this data, as Welling *et al* (1974) showed that a strain of resistant housefly could oxidatively degrade malaoxon to form the mono-acid, formerly considered to be strictly a carboxylesterase produced degradative product. This particular enzyme was specific to malaoxon; malathion was not an alternative substrate. But this study does raise doubts on earlier work which based their conclusions on metabolite production, without information on the effect of synergists on the resistance mechanism. However:

a) the malaoxon induced MFO-mediated resistance mechanism in *Tribolium castaneum* produced only a slight increase in tolerance to malathion (Dyte *et al* 1970) and

b) studies after 1974 have not produced evidence of malathion-induced resistance producing MFO-mediated mechanisms.

It therefore seems likely that although MFO degradation of malathion is theoretically possible (see pp 54) it is

KEY TO TABLES 82-85.

S	= synergist studies.
B IV	= biochemical studies in vivo.
B IV	= biochemical studies in vitro.
electroph	= electrophoretic inves-tigations.
chromat	= chromatography.

Table 82. Summary of esterase mediated resistance mechanisms in a number of arthropod species.

SPECIES	LOCATION	INSECTICIDE	MECHANISM	Mode of detection.	REFERENCE
Anopheles albimanus	El Salvador	Malathion	Carboxylesterase	S	115
An.culicifacies		Malathion	Carboxylesterase	S	115 ,116
An.stephensi	Iraq	Malathion	Carboxylesterase	S	present study
" "	Iran	Malathion	Carboxylesterase	S	" "
" "	Pakistan	Malathion	Carboxylesterase	S, B IV	" "
Blatella germanica		Malathion	Carboxylesterase		120
B.germanica		Tetramethrin	Hydrolytic esterases.	B IV	129
Cimex lectularius		Malathion	Carboxylesterase	B IV	79
Culex pipiens	France	Chlorpyrifos	B-esterases	electroph	201,202,203
C. pallens	Japan	OPs	Esterases		
C.quinquefasciatus	USA(California)	Chlorpyrifos	Hydrolytic esterases	S	90
" "	USA(California	OPs	Esterases	S	97
C.tarsalis		Parathion/ Malathion	Carboxylesterase	S, B IV	21,205,209
" "		Malathion	Carboxylesterase	B IV	46
" "	USA(California)	Malathion	Carboxylesterase	B IV	158, 159
" "	USA(California)	Parathion / Fenthion.	Esterases	S	7
Chrysomya putoria.	Congo	Malathion	Carboxylesterase	S, B IV	259
Dermestes maculatus		Malathion	Carboxylesterase		68
Hellothis virescens		OPs	Carboxylesterase		32
Laodelphax striatellus		Malathion	Carboxylesterase		198
Musca domestica	USA (Georgia)	Malathion	Carboxylesterase	S B IV	266
M.domestica		Malathion	Carboxylesterase	B IV	162
M.domestica		Tetramethrin	Hydrolytic esterases	B IV	129
Myzus persicae	England	Methyl-parathion	Carboxylesterase		18
Nephotettix cincticeps		Malathion	Carboxylesterase	B IV	138, 199
" "		Malathion	Carboxylesterase		198
Oncopeltus fasciatus		Tetramethrin	Hydrolytic esterases	B IV	129
Periplaneta americana		Malathion	Carboxylesterase	B IV	163
Plodia interpunctella	India	Malathion	Carboxylesterase	S	14
Rhyzopertha dominica	Kuwait	Malathion	excretion	B IV	165
Tenebrio molitor		Tetramethrin	Hydrolytic esterases	B IV	129
Tetranychus urticae		Malathion/oxon	Carboxylesterase	B IV	164
Tribolium castaneum		Malathion	Carboxylesterase	S B IV	70

very rare, if at all, that malathion induced resistance mechanisms involve the oxidase systems, the alternative carboxylesterase mediated degradation being a more readily available avenue for attack in a wide range of arthropod species.

In contrast to the cases in Table 82 malathion resistance in *An. atroparvus* (AT SPA) and *An. albimanus* (MACHO males) showed no synergism with TPP, SV_1, PB or sesamex indicating that the carboxylesterase and oxidase mediated degradation of malathion are not the basis of this resistance. It should be stressed that in contrast to the other four malathion resistant populations studied, there is no evidence in AT SPA or MACHO that malathion induced these resistances, rather than the malathion resistance being a product of cross-resistance from another selecting chemical (see pp 22 - 23).

A general lack of synergism with all compounds tested was also the case in the fenitrothion selected line of AT SPA and in the propoxur selected line when a combination of synergist + 2hours exposure to propoxur was used. However if the synergist treatment was followed by 6 hours on 0.1% propoxur synergism was observed with PB, sesamex and to a limited extent with SV_1. This indicates that there are two 'levels' of resistance in the propoxur selected population and can be correlated with the multiplicate resistance which was indicated by the genetic studies on the propoxur-selected resistant line of this population.

The propoxur resistance mechanism which is unaffected by any of the synergists is presumably that which is controlled by the same gene as the broad spectrum OP resistance in this population, (on the evidence of cross-resistance studies), while the PB, SV_1, sesamex synergisable mechanism is 'super-imposed' on this, to produce a higher level of resistance to propoxur. The level of propoxur resistance due to the general mechanism would then be approximately the same level as that of the other OPs and carbamates. This fairly low resistance (6-8x) is characteristic of resistance based on an altered acetylcholinesterase in some species, where inhibition of the altered acetylcholinesterase (AChE) by the insecticide still occurs when the insecticide is present in high concentrations. Devonshire & Sawicki (1974) reported an altered AChE in the housefly which conferred only slight resistance (less than 20-fold) when isolated genetically. They also showed that this mechanism could interact with other resistance mechanisms resulting in very high levels of resistance. Altered AChEs producing only 1.5-fold resistance to diazinon in *Lucilia cuprina* (Schunter & Roulston 1968) and 1.2-fold resistance to phosphamidon in *Ceratitis capitata* (Zahavi & Tahon 1970), have been isolated. In both cases the altered AChE had pronounced effects in conjunction with other resistance mechanisms.

A low resistance level due to an altered AChE is not always the case in arthropods. Resistances ranging from 18-115-fold have been recorded for carbaryl, propoxur and

malathion in the green rice leafhopper *Nephotettix cincticeps* (Hama & Iwata 1978) and resistances of the order of 1,000-fold have been reported in ticks and mites.

The very broad spectrum cross-resistance of AT SPA supports the hypothesis that an altered AChE may be involved in this resistance, as does the lack of synergism with inhibitors of enzyme systems commonly involved in particular insecticide detoxication pathways. As PB and sesamex are both oxidase inhibitors it seems likely that the synergisable resistance mechanism is based on oxidative metabolism of propoxur (see literature review for possible degradation pathways). There was also some synergism with SV_1. This is primarily considered as a carboxylesterase inhibitor, and as such should have no effect on propoxur, which has no carboxylester bond. It is therefore presumed that SV_1 is inhibiting enzymes other than carboxylesterases. It is not clear whether SV_1 is inhibiting the oxidase mediated mechanism, the general mechanism or a third as yet uncharacterised mechanism. There are many cases of propoxur-induced resistance producing an MFO-mediated mechanism (see Table 83). This indicates that carbamate resistance in arthropods is generally due to oxidative degradation and that mechanisms involving esterases, though theoretically possible, are of little practical importance. Details of the role of oxidation in propoxur resistance in houseflies and *Culex quinquefasciatus* are well documented (see Literature review for details).

Three DDT resistant populations ATIG, IAN and G1

Table 83. The involvement of oxidases in insecticide resistance in a number of arthropod species.

SPECIES	LOCATION	INSECTICIDE	MECHANISM	Mode of detection.	REFERENCE
Anopheles atroparvus	Spain	Propoxur	MFO	S	present study
An.sacharovi	Turkey	Fenitrothion	MFO	S	115
Culex quinquefasciatus	USA(California)	Propoxur	MFO	S	95
C.quinquefasciatus	USA(California)	Propoxur	MFO	BIV	242
Cquinquefasciatus	USA(California)	Abate	MFO	S	76
Cquinquefasciatus		Carbamates	MFO		241
Musca domestica		Aldicarb	MFO	BIV	174
M.domestica	Denmark	Diazinon	MFO	S	193
M.domestica		Propoxur	MFO	BIV	162
M.domestica		Propoxur	MFO	B	41
M.domestica	(R_{BAYGON})	Propoxur	MFO	BIV	242
M.domestica	(Fc strain)	Propoxur	MFO	GB	258
M.domestica	(Fc strain)	Diazinon	MFO	S	193
M.domestica		Tetrachlorvinfos	MFO	S	224
M.domestica		Dimethoate	MFO	S	224
M.domestica	(SKA strain)	DDT	MFO	S	227
M.domestica		Pyrethroids	MFO	BIV	278
M.domestica	(Fc strain)	DDT	MFO	S	192
M.domestica		Carbaryl	MFO	S	74
M.domestica		Malaoxon	MFO	BIV	266
M.domestica		Paraoxon Diazoxon	MFO	BIV	73
M.domestica		Diazinon	MFO		277
M.domestica		Allethrin	MFO		206
Plodia interpunctella		Dichlorvos Fenitrothion	MFO		14
Tribolium castaneum		Malaoxon	MFO	BIV	71
Trichoplusia ni	USA	Carbaryl	MFO	BIV	142

were tested with the synergist F-DMC. F-DMC is primarily an inhibitor of DDT-dehydrochlorinase. The ATIG colony gave 100% mortality on three separate occasions with F-DMC + DDT, indicating that DDT-dehydrochlorinase is the sole resistance mechanism in this population. In contrast results with F-DMC + DDT tested against IAN and G1 showed a relatively high level of synergism in the original tests. In the IAN colony selection of the survivors of the F-DMC/DDT treatment over four generations produced a significant decrease in mortality. The mortality for F-DMC + DDT in the fourth selected generation was similar to the mortality observed for DDT alone. This can be tentatively taken to indicate that there is multiplicate resistance with regard to DDT in this population.

One generation of F-DMC + DDT selection of the G1 population produced a rapid drop in mortality to below the level of mortality observed with DDT alone in this population. Mortality with DDT/F-DMC then gradually increased for two further selected generations. Again this appears to indicate multiplicate resistance with regard to DDT in this population. However the presence of multiplicate resistance is not supported by the level of DDT resistance in this population which is relatively low (x3).

The evidence of cross-resistance studies indicate that in the IAN and G1 colonies there is a connection between DDT and permethrin resistance. As F-DMC should have no effect on permethrin it is probable that a non-F-DMC synergisable mechanism is responsible for the

DDT/permethrin resistance in both populations. There is a great deal of evidence in the literature for the association between DDT and pyrethroid resistance. The DDT/pyrethroid cross-resistance often appears correlated to the degree of DDT-resistance. Plapp & Hoyer (1968) found that a DDT resistant strain (x66) of *Culex tarsalis* possessed cross-resistance of 6.5x to piperonyl butoxide synergised pyrethrins. They attributed this cross-resistance to a monofactorial trait represented by a knock-down resistant (kdr) -like mechanism. Chadwick *et al*(1977) and Prasittisuk & Busvine (1977) reported DDT-resistance in field strains of *Aedes aegypti* (RR = 19-73x) which had as much as a 30x cross-resistance to pyrethroids. Conversely laboratory selection with pyrethroids has been shown to enhance the levels of DDT resistance which pre-existed in the strains under investigation.

Increasing evidence is accumulating that the DDT-kdr mechanisms are involved in pyrethroid resistance in houseflies and mosquitoes. Recently a lower sensitivity of the nervous system to poisoning by pyrethroids was was demonstrated in *An. stephensi* by intracellular recordings from larval muscles during exposure to permethrin, confirming that resistance was due to a kdr-like factor that imparted reduced sensitivity at the site of action (Omer *et al*1980). It is possible that the non-synergisable DDT-resistance mechanisms involved in the IAN and G1 colonies are kdr-like mechanisms.

In contrast to the results obtained here Prasittisuk (1979) with IAN 7 (a strain derived from IAN) could

show little, if any, correlated increase in permethrin tolerance due to DDT selection. With permethrin selection he obtained a 4.8-fold increase in resistance to permethrin, which was greater than that arising from DDT selection. Cross-resistance studies showed no increase in DDT resistance in the permethrin selected line. The discrepancy between the two studies may have been caused by the different selection methods employed, or to preferential selection of the F-DMC synergisable DDT resistance mechanism for some reason in Prasittisuk's study. The latter cannot be checked as synergist studies were not carried out on his selected stock.

A summary of biochemical, synergist and cross-resistance studies indicating nerve insensitivity as the basis for DDT/ pyrethroid resistance is given in Table 84 . From this it is clear that DDT/pyrethroid resistance occurs in a wide range of arthropod species.

Further biochemical studies.

After showing that there was a generalised non-synergisable mechanism for OP and carbamate resistance in AT SPA, the next logical step in the investigation of the resistance mechanism in this population,would be to carry out a comparative study of the kinetics of enzyme inhibition by the insecticide in resistant and susceptible stocks. However this requires fairly large numbers of mosquitoes of both AT SPA and ATHOR. Producing these stocks synchronously in large numbers was not feasible. Therefore the AChEs of individual insects were

Table 84. DDT and pyrethroid resistance mechanisms in various arthropod species.

SPECIES	LOCATION	INSECTICIDE	MECHANISM	MODE OF DETECTION	REFERENCE
Anopheles gambiae	Nigeria	DDT	DDT-dehydrochlor	S	Present study (ps)
An. gambiae	Nigeria	DDT/Permethrin	Nerve insensitivity	S	ps
An. sacharovi	Turkey	DDT	DDT-dehydrochlor	S	115
An. sacharovi	Turkey	DDT/Pyrethroids	Nerve insensitivity	S	115
An. stephensi		Pyrethroids	Nerve insensitivity	B IV	190
Aedes aegypti	Guyana	DDT/Pyrethroids	Nerve insensitivity	S	211
Ae. aegypti		DDT	DDT-dehydrochlor		134
Blatella germanica		DDT	MFO	S	3
Culex quinquefasciatus	USA(California)	Permethrin	Nerve insensitivity	S	212
C.tarsalis		DDT/Pyrethroids	Nerve insensitivity		208
Musca domestica	(SKA strain)	DDT	Reduced penetration		228
M.domestica	USA(Illinois)	DDT	DDT dehydrochlor	S	193
M. domestica	(Fc strain)	DDT	MFO	S	101
M. domestica		Permethrin	Hydrolysis	BTV	239
Periplaneta americana		Permethrin	Hydrolysis	BTV	239
Sitophilus granarius		DDT	MFO	S	68
Spodoptera littoralis		Permethrin	Nerve insensitivity	BTV	82
Tribolium castaneum		DDT	MFO	S	68
Trichoplusia ni		Permethrin	Hydrolysis	BTV	239

visualised electrophoretically to determine whether there were any charge differences between the AChE of the resistant and susceptible populations. The MACHO colony was also investigated electrophoretically to find out whether there were any charge differences between the AChE of males and females in this population.There was no difference between the AChEs of MACHO males and females as seen by polyacrylamide electrophoresis. For some reason the 'monomer' band of AChE did not appear in any individual of the MACHO strain. Inability to distinguish the AChE forms of resistant and susceptible individuals of the MACHO population electrophoretically does not neccesarily indicate that an altered AChE is not the basis of resistance. Devonshire (1975) could show no differences between the AChE from heads of resistant and susceptible houseflies using polyacrylamide disc gel electrophoresis. However he showed that the bimolecular rate constants (ki) from R and S flies differed by approximately 4-20-fold for a range of OP compounds.

The 'monomeric' form of the AChE enzymes of AT SPA and ATHOR appears to form readily. Two isozymes of the 'monomeric' form were found in the parental AT SPA population. The ATHOR colony showed only the slow migrating isozyme while all the AT SPA selected lines had only the fast migrating isozyme. This strong correlation between the fast isozyme and resistance again supports the hypothesis that the broad spectrum OP and carbamate

resistance in this population is due to an altered AChE, which is insensitive to insecticide inhibition compared with the wild-type AChE. Insecticide inhibition of the AChE bands on the gel was attempted by various methods to determine whether the AChE of the insecticide resistant population was less susceptible to inhibition than the insecticide susceptible strain as judged by a weakened staining response of the enzyme on the gels. Although there appeared to be a difference in the inhibition of S and R individuals it is unwise to draw any firm conclusions from these experiments as; a) no quantitative measure of activity could be taken, b) the activity of the bands when no insecticide was present varied. The frequency of each of the isozymes in the AT SPA parental population was significantly different from that predicted by the Hardy-Weinberg equilibrium if a single gene with two alleles was present on the gels. The discrepancy was probably caused by scoring of heterozygous individuals as SS or FF homozygotes due to the unequal staining of the S and F bands in some cases.

That the broad spectrum resistance in the AT SPA population is due to an altered AChE agrees with the synergist and genetic evidence, which suggests monofactorial inheritance of a non-metabolic resistance mechanism. Altered AChE resistance mechanisms have been reported in a number of arthropod species. A summary is given in Table 85 . This shows that a variety of insecticides have selected for an altered AChE. However only two anophelines

Table 85. Insecticides selecting for altered acetylcholinesterase mechansims in a number of arthropod species.

SPECIES	LOCATION	INSECTICIDE	MECHANISM	Mode of detection.	REFERENCE
Anopheles albimanus	El Salvador	Propoxur	AChE	BIV	15
An. atroparvus	Spain	Propoxur	AChE	S, electro	present study
Boophilus microplus	Australia	OPs & carbamates	AChE	BIV	146
B. microplus	Australia	OPs & carbamates	AChE	BIV	222
Ceratitis capitata		Phosphamidon	AChE		279
Lucilia cuprina	Australia	Diazinon	AChE		230
Musca domestica	Denmark	Dimethoate	AChE	S,B	59, 60
M.domestica		Malaoxon	AChE	BIV	75
M.domestica	USA	Rabon	AChE		261
M.domestica	USA	Tetrachlorvinphos	AChE	B	261
Nephotettix cincticeps	Japan	Methyl carbamates	AChE	Chromat	278
Nephotettix cincticeps	Japan	OPs & carbamates	AChE	BIV	104 127
Spodoptera littoralis	Egypt	Methyl parathion	AChE		63
Tetranychus pacificus	USA(California)	Parathion	AChE		
T. urticae	West Germany	Paraoxon Diazinon	AChE	BIV	243
T.urticae	New Zealand	Parathion	AChE		16
T.urticae	Israel		AChE	ID_{50}	281

show an altered AChE and both appear to have arisen from propoxur selection. The strength of propoxur selection in *An. atroparvus* from Spain is unknown, and there are suggestions that it may be insufficient to account for two propoxur resistance mechanisms.

If the resistance spectra of arthropods are compared there are indications that several different alterations to the binding site of OPs and carbamates to the AChE molecule have taken place, leading to several different cross-resistance patterns. This is apparent in *An. albimanus* and *An. atroparvus* where the former is susceptible to fenthion while the latter species is resistant.

A decrease in the rate of AChE inhibition by itself will only serve to postpone death in the absence of other processes to reduce the amount of inhibitor present. It may be that a second resistance mechanism is present in the population, as with propoxur resistance in AT SPA, or that the low level detoxication capacity present in most 'susceptible' insects is sufficient to remove the toxicant in the extended survival period caused by the decreased rate of AChE inhibition in the resistant strains, which appears to be the case in the MACHO strain.

The carboxylesterases of ST LA and G1 were investigated using the filter paper spot technique of Pasteur and Georghiou (1980) and the spectrophotometric esterase assay of Van Asperen (1964).
Synergist, biochemical and cross-resistance studies

indicated the involvement of the carboxylesterase enzyme in the malathion/phenthoate specific resistance in these populations. The crude filter paper spot technique showed no differences in total esterase content of resistant and susceptible individuals. The total esterase assay also showed no difference between the absorption in resistant and susceptible individuals again indicating that there is no quantitative difference in the amount of ca·boxylesterase enzyme present in the resistant and susceptible stocks. There was no difference between male and female resistant individuals despite the higher level of resistance in the males. Therefore if the carboxylesterase enzyme is responsible for malathion resistance in the ST MAL and G1 colonies then the enzyme must have been altered qualitatively in the resistant population, such that its affinity for malathion is greater in comparison to that of the susceptible population. This resembles the situation in the FRESNO strain of *Culex tarsalis* (Matsumura & Brown 1963), the malathion 'tolerant' Penang strain of *Aedes aegypti* (Matsumura & Brown 1961), and the malathion resistant *Cimex lectularius* (Feroz 1971). However it differs from the situation in other arthropod strains where esterase levels are changed with OP resistance. Reduced esterase activity has been demonstrated in several strains of *Musca domestica* (Van Asperen & Oppenoorth 1959), the CM strain of *Chrysomya putoria* (Townsend & Busvine 1969) and the Leverkusen-R strain of *Tetranychus urticae* (Smissaert 1965). Increase levels of

esterase in OP resistant strains have been shown in *Nephotettix cincticeps*, *Laodelphax striatellus*, *Myzus persicae*, *Culex pipiens* and *C. quinquefasciatus* (Kojima *et al* 1963, Ozaki 1969, Stone & Brown 1969, Pasteur & Singre 1975, Georghiou *et al* 1980, Needham and Sawicki 1971, Devonshire & Sawicki 1979).

The esterases of resistant and susceptible *An. stephensi* populations were visualised by starch gel electrophoresis using α- and β-naphthyl acetate as substrates to determine whether the predicted qualitative change in the resistant strain carboxylesterase enzyme had resulted in a charge difference between the resistant and susceptible carboxylesterase enzymes. The presence of a particular isozyme of Esterase-1 (Bianchi 1968) was strongly correlated with resistance. No other isozyme of other esterase loci segregated with resistance. No attempt was made to establish whether the association of the slow isozyme of Esterase-1 represented the esterase responsible for malathion resistance. Further studies involving elution of the enzyme and determination of its activity with malathion as a substrate may be feasible. Wild populations should be sampled, as the resistant and susceptible laboratory populations are obviously highly inbred and correlations of enzyme loci with resistance may prove spurious.

Cross-resistance, synergist, genetic and electrophoretic studies all indicate that a carboxylesterase enzyme catalysing the breakdown of malathion is

the major resistance mechanism in the ST MAL population. Metabolic studies were attempted to determine the major malathion metabolites formed by resistant and susceptible individuals. Although the larval resistance in ST MAL was lower than that of the adult, metabolic studies were carried out at the larval stage, as recovery of excreted metabolites and applied malathion was more efficient from water than from impregnated filter paper.

Results of the metabolic studies strongly suggest that the monocarboxylic acids were the primary metabolic products of malathion in both ST and ST MAL larvae. No attempt was made to separate the α- and β-monocarboxylic acids. The dicarboxylic acid was not produced by either the resistant or susceptible larvae, or if present was in quantities below the resolution of the TLC system used. The small amount of radioactivity in the water fraction after chloroform extraction may however indicate that there are traces of the diacid produced by both resistant and susceptible larvae, as with the extraction procedure used the diacid would not be expected to partition into the chloroform phase, being a strong acid. The lack of diacid production has also been noted in *Chrysomya putoria* (Townsend & Busvine 1969) and *Tetranychus urticae* (Matsumura & Voss 1964). The presence of the diacid was noted in *Culex tarsalis* (Bigley & Plapp 1962) and *Cimex lectularis* (Feroz 1971).

B. THE APPLICATION OF LABORATORY STUDIES OF INSECTICIDE RESISTANCE TO RESISTANCE PROBLEMS IN THE FIELD.

This entire study was laboratory based, using

populations which had been colonised for varying lengths of time. The main advantage of this is the ability to genetically standardise the test material and the test conditions. A lack of genetic variability, though advantagious in investigating a particular resistance mechanism, may mean that results obtained in the laboratory do not reflect the situation in the field. For this reason laboratory studies may be more beneficial when carried out in conjunction with field tests. However, various aspects of a laboratory study may be useful in predicting the effect of an increase in frequency of a resistance gene in the field. Such studies can also be used to suggest the most effective alternative insecticide control strategies.

Levels of resistance in the populations studied.

The level of resistance conferred by a resistance gene is important in determining the effect of resistance on control measures. Ideally the levels of resistance in a population should be a measure of the increase in tolerance by comparison of the susceptibility level of the population before exposure to an insecticide with the same population after selection for resistance to an insecticide. However in practice base-line data for a resistant population are rarely available and comparisons have to be made with susceptible populations of the same species from other geographical areas. This was the case in the present study.

A summary of the levels of resistance after laboratory selection in each of the populations studied is given in Table 86 . This shows that the resistances were generally of a fairly low order (3-6x). Of the five malathion resistant populations, three had a low level resistance (AT SPA, ST IRAQ and ST IRAN) while two populations (G1

Table 86. The relative resistance levels in the populations studied compared to laboratory susceptible populations of the same species.

Population	Insecticide resistance	Mechanism	Level of resistance
An. atroparvus			
AT SPA	Malathion	Altered AChE	5.2x
	Fenitrothion	Altered AChE	4.8x
	Fenthion	Altered AChE	3x
	Propoxur	Altered AChE	6x } 72x
		Multi-function oxidase	? }
An. arabiensis			
G1	Malathion	Carboxylesterase	24.6x
	DDT	DDT-dehydrochlorinase + kdr	4.6x
An. gambiae			
IAN	DDT	DDT-dehydrochlorinase + kdr	12x
An. stephensi			
ST IRAQ	Malathion	Carboxylesterase	4.5x
ST IRAN	Malathion	Carboxylesterase	4x
ST LA	Malathion	Carboxylesterase	24x
An. albimanus			
MACHO males	Propoxur	Altered AChE	20x

and ST MAL) showed a relatively high level of resistance to malathion (24x). Both populations with a high level of malathion resistance appear to have a qualitative change in a carboxylesterase enzyme. However ST IRAQ and ST IRAN also appear from synergist and cross-resistance studies to have a carboxylesterase based resistance mechanism. It is not known whether a qualitative or a quantitative change in the carboxylesterase enzymes has occurred in these strains.

In general malathion resistance levels for the anophelines studied are lower than the resistance levels found in other species where resistance is due to a carboxylesterase mediated mechanism. Matsumura & Voss (1964), using a dip test, showed that the Blauvelt strain of *Tetranychus urticae* was 60x as malathion resistant as the Niagara strain. Townsend & Busvine (1969), using topical application, showed that the CM strain of *Chrysomya putoria* from the Congo was 168x more resistant to malathion than the CS strain. Strain G of *Musca domestica* was 80x as resistant to malathion as the susceptible SRS strain (Welling *et al* 1974) and 4th instar larval tests of *Culex tarsalis* showed the resistant strain was 60x more resistant than the susceptible strain (Darrow & Plapp 1960). Too much emphasis should not be placed on such comparisons of resistance levels, as levels of resistance vary with the test method. For example the malathion resistance levels in *An. stephensi* (ST and ST MAL) differed depending on whether the insecticide impregnated papers were spread with or without a non-volatile solvent.

The cross-resistance between DDT and permethrin in the IAN and G1 colonies produced low level (2-4x) permethrin resistance in both cases. In houseflies DDT-kdr resistance also confers moderate (10x) to weak (1.5x) resistance to many pyrethroids (Sawicki 1975a).

Although the levels of resistance in *An. stephensi* (ST MAL) and *An. arabiensis* (G1) are similar, the malathion resistance patterns throughout the life-cycle differ between the two populations. The ST MAL colony shows larval resistance to malathion at the fourth larval instar. Separate selection of resistant lines by pressurisation of larvae or adults demonstrates that the same malathion resistance gene is operative at both stages. The G1 colony shows no evidence of malathion resistance in any of the larval instars. This was confirmed by Curtis and Lines (personal communication). It has often been noted that some species display strong resistance in the adult state, whereas their larvae are almost susceptible. *Culex quinquefasciatus* in Africa and *C. tritaeniorhynchus* in Korea were OP resistant in the adult stage whereas the larvae were susceptible (Hamon & Mouchet 1967, Self *et al* 1974). In Florida adulticide treatment with malathion produced 500-fold resistance in the adult *Aedes taeniorhynchus*, but not more than 10-fold resistance in the larvae (Boike *et al* 1978).

Table 87. Presence (✓) or absence (x) of adult and larval resistance to malathion in the populations studied.

COLONY	LARVAL INSTAR			ADULT
	1st	3rd	4th	
ST LA			✓	✓
ST IRAQ	X	✓	✓	✓
ST IRAN	X	✓	✓	✓
AT SPA	X	X	X	✓
G1	X	X	X	✓

Although ST MAL has a much higher level of adult malathion resistance than ST IRAQ or ST IRAN, the larval resistance levels of the three populations studied are very similar, all three colonies show 100% mortality after exposure to 2ppm malathion for 24 hours as fourth instar larvae. The reason for the lack of malathion resistance in the G1 larvae is unclear. Preliminary biochemical studies on the production of metabolites from G1 and SENN fourth instar larvae using thin layer chromatography, indicated that malathion monoacid is produced by both sets of larvae. Therefore there must be some carboxylesterase activity in the larvae. One has to conclude that either the gene for the particular carboxylesterase which produces the high level of resistance in the G1 adult is not 'switched-on' in the larval stages, or that this carboxylesterase is produced in such minute quantities that it has no effect on the level of larval susceptibility. However in either case a second carboxylesterase enzyme must be producing some of the malathion monoacid in the larvae as the sensitivity of the TLC system used is such that relatively large quantities of the monoacid are required for visualisation.

The larval resistance spectrum of *An. atroparvus* (AT SPA) supports the hypothesis that at least two genes are involved in the propoxur resistance in this population, as fourth instar larvae from this colony show propoxur resistance but not malathion resistance. However there is strong evidence of malathion/propoxur cross-resistance in the adult. Larval selection with propoxur for three generations increased the percentage survival of adults in this line exposed to 0.1% propoxur for 2 hours, but had no effect on the percentage of adults surviving 6 hours exposure to 0.1% propoxur.

This suggests that the propoxur specific oxidase resistance mechanism is operative at the larval and adult stages, whereas the altered acetylcholinesterase mechanism is only operative in the adults. This is difficult to envisage as presumably cholinergic nerve junctions are present in the larvae. If not OPs and carbamates would be ineffective at this stage. This may be evidence that more than one AChE form occurs in *An. atroparvus*.

The impact of resistance in control of the populations studied.

Laboratory based studies such as these are of limited value by themselves in providing information on the impact of a resistance gene or genes on vector control. They should be used in conjunction with field studies on the operational and epidemiological implications of resistance to give a broader view of the problems facing control programmes.

The percentage of resistant individuals in a population and the level of resistance conferred by the resistance gene in its heterozygous form are of primary importance in the rate of evolution of resistance, (see also pp 26-27). Laboratory studies indicate the level of resistance conferred by an R gene, but it is not possible to judge from this whether the dose of insecticide experienced by the mosquito in the field is above or below the lethal dose for the heterozygote. i.e. whether resistance in the field is effectively dominant or recessive. There is also the added complication that the effective dominance of a resistance gene for a residual insecticide may change as the chemical decays. However the occurrence and increase in frequency of a resistance gene in a population must indicate that individuals carrying the resistance gene in the homozygous, and possibly the heterozygous form, are at an advantage over the susceptible homozygotes in the presence of insecticide

selection. Whether this selective advantage affects the level of malaria transmission must be determined.

Failure to control malaria transmission due to organochlorine resistance originally caused the replacement of DDT and HCH by malathion in Iraq, Iran, Pakistan and Sudan. The recent development of malathion resistance in mosquito vectors in these four countries may be considered as a further threat to the effectiveness of future control programmes. However malathion resistance in *An. stephensi* in the Basrah province of Iraq does not appear to have impeded malaria control operations to date, perhaps not surprisingly in view of the low level of malathion resistance (4-5x) conferred by the resistance gene even when homozygous.

In Pakistan malathion has replaced DDT for all malaria control operations. The impact of malathion resistance in *An. stephensi* in Pakistan has yet to be fully assessed, but it may be that this resistance will have more of an adverse effect on control than in Iraq, due to the higher level of resistance conferred by the resistance gene in Pakistan.

In Sudan, where malaria control operations are only carried out in the Gezira province, organochlorines were replaced by malathion in 1975 and weekly abate larviciding was introduced as a supplementary measure from 1976 onwards. Recent evaluation (Shidrawi 1979) of the impact of malathion resistance in Gezira indicated that, wherever good coverage of malathion was attained, it resulted in satisfactory control of *An. arabiensis*.

An. albimanus developed resistance to DDT in the early 1960s in Guatemala, El Salvador, Honduras and Nicaragua. DDT was replaced by propoxur in areas of high DDT resistance and the incidence of

malaria was reduced to a low level by 1973. However the production of a broad spectrum of resistance in *An. albimanus* has lead to serious problems in control, resulting in a resurgence of malaria, which reached a high level by 1975 and remains high to date.

The use of insecticide mixtures.

One suggestion put forward as a control measure is the use of mixtures of insecticides. These could either be used on a previously unsprayed population to attempt to delay the emergence of resistance, or where resistance to one chemical has appeared, to enhance insecticidal activity. As ST MAL contained a high level of malathion resistance this population was used to investigate the effect of mixtures of malathion and fenitrothion or kitazin on the malathion resistance. Results showed that malathion antagonised the action of fenitrothion in both the ST and ST MAL colonies. There was however potentiation of the action of malathion and kitazin when these chemicals were used against both ST and ST MAL in mixtures, though the effect was greater in ST.

One of the fundamental problems of studies of insecticide mixtures is that of relating the observed biological effect to the primary cause of the response. Since the concentration of any insecticide reaching a particular target site is governed by a complex series of inter-related equilibria associated with absorption, distribution, activation and detoxication reactions, and excretion, there are many potential sites at which the insecticides may interact. The ability of a second insecticide to cause a shift in the equilibria of one or more of these events is likely to be reflected by some change in the biological activity of the first insecticide when the two are combined. In addition there is also the possibility of an interaction occurring at the target site itself.

No attempt was made in this study to determine the interactions of the insecticides used in mixtures. There has been speculation that malathion and kitazin both act as substrates for the same carboxylesterase enzyme (Miyato & Saito 1980). This may explain the greater toxicity of kitazin to ST rather than the ST MAL colony, as malathion resistance in ST MAL appears to be due to a qualitative change in a carboxylesterase enzyme.

Results of the testing of malathion/kitazin mixtures against *An. stephensi* differ from those obtained with the green rice leafhopper, *Nephotettix cincticeps* and the aphid *Myzus persicae*. In the latter two species the 'synergistic effect' of kitazin was far greater in the resistant than the susceptible colonies. Kitazin synergised demeton-S-methyl about 70-fold against R_2 aphids but only 6-fold against S aphids (Sawicki personal communication). In *An. stephensi* the S strain was synergised 9.2-fold while the R strain was only synergised 4.5-fold. Both *N. cincticeps* and *M. persicae*'s OP resistance appears to be due to a quantitative change in the amount of carboxylesterase enzyme, rather than a qualitative change as in *An. stephensi* (ST MAL).

These experiments demonstrate the importance of looking for mixtures of chemicals which are likely to interact favourably together to produce a greater joint toxicity than either compound alone. This would be particularly valuable where both compounds involved are fairly cheap to produce, resulting in an economically viable proposition, and where the mixtures of insecticides had no or very low mammalian toxicity. Results obtained in this study indicate that mixtures of malathion and fenitrothion are not a viable proposition, as the two compounds antagonise each others action. Mixtures of

malathion and kitazin may however be more viable, as potentiation of each compound's action occurs in a mixture of the two. The economics of production of this type of mixture are not, however, within the scope of this study.

It is possible that as fewer new insecticides are produced and problems of resistance increase, more emphasis will be put on determination of the joint action of mixtures of insecticides.

Sequential use of insecticides.

Determination of an optimum strategy for sequential usage of insecticides is complicated by the fact that certain insecticides can select for different mechanisms of resistance. In anophelines, as well as many other pest insects DDT was the first insecticide used for control, followed by dieldrin and BHC. It has been shown that DDT selected DDT-dehydrochlorinase and kdr-type mechanisms in various populations. Both these mechanisms are represented in this study, which also demonstrates the characteristic DDT/pyrethroid cross-resistance conferred by the kdr-like mechanism. The use of dieldrin appears to have produced little or no cross-resistance outside the organochlorines.

A variety of OPs and carbamates have been used in place of the organochlorines. In houseflies parathion was used, which in turn was replaced by diazinon, dimethoate, fenthion and pyrethroids. In anophelines the organochlorines were generally replaced by malathion, fenitrothion or propoxur as organochlorine resistance became a problem.

It has been suggested that fenitrothion should be used before malathion, as it is theoretically possible to get cross-resistance to fenitrothion by a malathion-induced MFO resistance mechanism.

However this makes the assumption that the particular oxidase conferring one resistance is the same oxidase conferring resistance to the second insecticide, and if this were the case fenitrothion would probably select the same mechanism and produce malathion cross-resistance. Evidence to date from laboratory studies also strongly suggests that malathion, in many species, primarily selects a carboxylesterase mediated mechanism. This is supported by the present study: four populations of anophelines all having malathion resistance which does not produce fenitrothion cross-resistance. This is therefore a strong case for using malathion before fenitrothion in public health.

Two species of *Anopheles*, *An. albimanus* and *An. atroparvus* exposed to propoxur showed an altered site of insecticide action. These are the only two species of anophelines which show an altered AChE to date. Such a resistance mechanism has a very broad cross-resistance spectrum encompassing many OPs and carbamates. As the levels of resistance conferred by the altered AChE are fairly low in the two species studied, the mechanism itself may appear of minor importance. However interactions between an altered AChE and a metabolic resistance mechanism may produce very high levels of resistance. This is demonstrated by propoxur resistance in *An. atroparvus* (AT SPA) where the AChE mechanism alone confers 6x resistance whereas the AChE + MFO mechanism produces a level of resistance 72x greater than the susceptible English ATHOR population. Chemicals which are likely to select an altered AChE mechanism should therefore be used late in the sequence of insecticides. Further information on the effect of propoxur selection on anophelines would be of value as, to date, laboratory studies have only been carried out on *An. albimanus* and *An. atroparvus*. In *Culex* species, cases of propoxur selection resulting in MFO-mediated

mechanisms have been recorded (Georghiou *et al* 1966), and there are many instances of MFO-mediated mechanisms produced by propoxur selection in *Musca domestica* (Matsumura *et al* 1967, Casida *et al* 1968, Shrivistava *et al* 1969, Terriere & Schonbrod 1976).

Pyrethroids have not been used to any large extent against anophelines. Cross-resistance between DDT and the pyrethroids, coupled with the irritational properties of the pyrethroids and their high production costs, may however limit there usefulness.

Decisions on sequential use of insecticides may be complicated by agricultural spraying in areas where malaria control teams are operating. The effect of agricultural spraying in the development of resistance in anophelines in the larvae and/or the adult stages has yet to be satisfactorily determined. Co-operation between agricultural and public health authorities as in Sri Lanka, where malathion can only be used in public health and not in agriculture, may have long term benefits if agricultural spraying is unequivocally shown to be important in anopheline resistance.

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