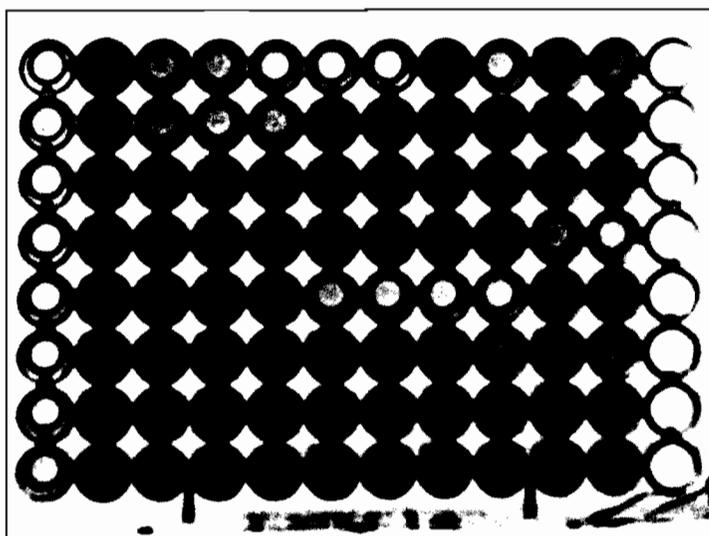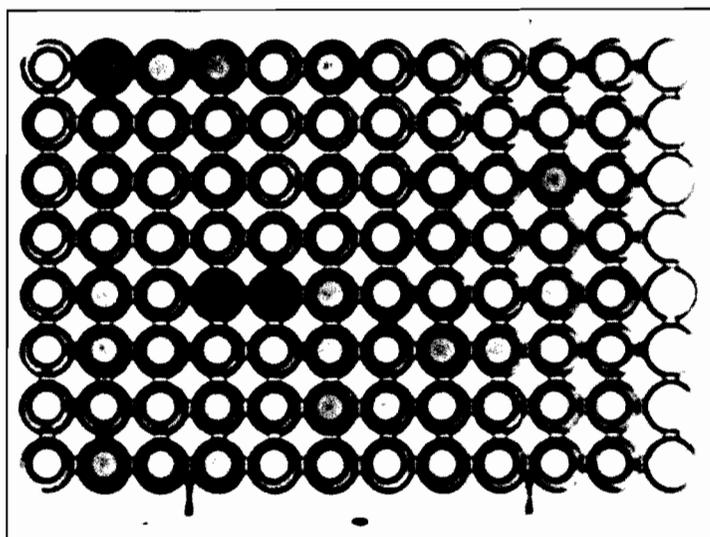# *Techniques to detect insecticide resistance mechanisms (field and laboratory manual)*

World Health Organization
Department of Disease Prevention & Control
WHO Communicable Diseases (CDS)

# TECHNIQUES TO DETECT

# INSECTICIDE RESISTANCE MECHANISMS

## (Field and laboratory manual)

*Prepared by:*
*Janet Hemingway*
*School of Pure & Applied Biology*
*University of Wales Cardiff*
*PO Box 915*
*CARDIFF CF1 3TL, U.K.*

*(with inputs from Dr William Brogdon*
*Entomology Branch F22*
*Division of Parasitic Diseases*
*Centre for Disease Control*
*Atlanta, Georgia 30333, U.S.A.)*

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# 1. INTRODUCTION

Insecticide resistance is an increasing problem faced by those who need insecticides to efficiently control medical, veterinary and agricultural insect pests. In many insects, the problem extends to all four major groups of insecticides. Resistance monitoring programmes should no longer rely on testing the response to one insecticide, with the intention of switching to another chemical when resistance levels rise above the threshold which affects disease control. Effective resistance management depends on early detection of the problem and rapid assimilation of information on the resistant insect population so that rational pesticide choices can be made.

The correct use of biochemical or immunological methods for resistance detection at a mechanistic level can provide a powerful tool for analyzing field and laboratory populations with the aim of improving resistance detection and management. This manual will be updated with new technologies and methodologies, as they become available. It attempts to outline the basic techniques and discusses their strengths and weaknesses. Clearly the biochemical assays provide more information about the insect population being analysed, but they also require more skill in interpretation, and those using this manual are urged to read the sections on interpretation of results carefully.

## 2. GENERAL OVERVIEW OF IMPORTANT RESISTANCE MECHANISMS IN ALL INSECTS

Where a mechanistic approach to resistance detection is being undertaken the investigator needs to have a basic understanding of the possible resistance mechanisms likely to be encountered. This manual currently deals only with the four major groups of insecticides, the organochlorines (with the exception of the cyclodienes), organophosphates, carbamates and pyrethroids. New compounds are clearly coming onto the market, such as insect growth regulators, but a mechanistic detection of resistance to these compounds has by necessity to be reactive rather than pro-active (i.e. we can only develop methods for resistance detection when we know the range of mechanisms selected in different insect populations). Hence monitoring for resistance to compounds outside the four well-characterised pesticide groups will still rely heavily on the standard susceptibility tests. There are four possible types of resistance mechanisms to the main insecticide groups in all insects analysed to date. These are:

♦ increased metabolism to non-toxic products
♦ decreased target site sensitivity
♦ decreased rates of insecticide penetration
♦ increased rates of insecticide excretion.

Of these four categories the first two are by far the most important. Penetration rate changes in isolation generally produce insignificant (<5-fold) levels of resistance, and only become important when found in combination with other resistance mechanisms. Increased rates of insecticide excretion are very uncommon and produce only low levels of resistance and are included in this list for completeness.

The first two categories of resistance mechanisms, which are the most common and produce the highest levels of resistance, can be sub-divided further. The enzyme groups involved in *insecticide metabolism* are:

1.  esterases
2.  monooxygenases
3.  glutathione-*S*-transferases

The target sites involved are the sodium ($Na^+$) channels for the pyrethroids and DDT, and acetylcholinesterase for the organophosphates and carbamates. (The target site for the cyclodienes, such as gamma HCH, is the GABA receptor, but detection of resistance due to changes in this target site is not dealt with in this manual).

The level of resistance conferred by the different mechanisms varies depending on the insecticide and the nature of the alteration in the enzyme system involved. Exact details of this are given in the text as each mechanism is considered.

There are two major ways that the metabolic enzymes can produce resistance:

❑ *overproduction* of the enzyme, leading to increased metabolism or *sequestration*;

❑ an *alteration in the catalytic centre activity* of the enzyme, increasing the rate at which an enzyme unit metabolizes the insecticide. These two routes are not mutually exclusive and an enzyme may be both *physically changed* and *over-produced.*

When an enzyme is overproduced but the pesticide is only slowly metabolised by that enzyme, the cause of resistance may be considered to be sequestration rather than metabolism, with the increased enzyme levels acting as a means of holding the pesticide and preventing it from reaching the target site within the insect. The level of resistance conferred is then roughly proportional to the increase in the quantity of enzyme produced.

Biochemical assays/techniques may be used to establish the mechanism involved in resistance. When a population is well characterised some of the biochemical assays can be used to measure changes in resistance gene frequencies in field populations under different selection pressures. It should be stressed that at present simple field biochemical assays do not exist for all resistance mechanisms. Biochemical assays are not complete substitutes for the standard susceptibility tests which are used to measure resistance.

## 3.    THE BIOCHEMICAL ASSAYS

Because the resistance mechanisms detected by these methods are common to all insects they are applicable across the range of insect pests.  However, it is important to note that the baselines may differ between insects and where possible a known susceptible strain of the same species as the field population being tested should be analysed at the same time.

To date biochemical assays have successfully been used on mosquitoes (*Culex, Anopheles* and *Aedes*), sand flies, cockroaches, houseflies and black flies as well as some agricultural pests.

Two main variants of two of the assays are in use.  To save repeating the arguments for and against each twice, these are outlined here, although both methods are later given in detail. One variant of the assays uses filter paper or another solid support media; the second variant is run in microtitre plates.  The 'filter' paper or nitrocellulose membrane assays generally use one mosquito per assay and are quantified visually or using a densitometer, but provide a permanent record which can be rechecked in the future.  The microtitre plate tests allow the same insect to be used for all assays and are quantified visually or with a spectrophotometer.  A permanent record can be made on paper by simply using a transfer plate, but this is not an automatic result of the test.

### 3.1    The microtitre plate tests

Once experienced with these techniques, it is practical to run assays for altered acetylcholinesterase, elevated esterase, glutathione-*S*-transferase and protein from the same insect.  It is not recommended that this is attempted initially, as it is easy for the novice to mix solutions up, and the time factor after homogenizing the insects is important, particularly if ice is not available to maintain the homogenates at a low temperature (i.e. the more rapidly the tests are run after insect homogenization, the more accurate the results will be).  It is recommended that anyone attempting these assays should familiarise themselves with each assay individually, and once they are confident and competent with the techniques, start to combine them for analysis of the same insect.

### 3.1.1 Equipment/supplies

Equipment required for these assays are:

*Microtitre plates* (preferably flat-bottomed if results are to be read spectrophotometrically): if affordable, these should be disposed of, after one use.  However, they can be re-used if thoroughly washed.  It is also recommended that the same set of plates be kept for the same assays (for example, plates used for the esterase and glutathione-*S*-transferase assays do not get contaminated with the propoxur used in the acetylcholinesterase assay).

*Homogenizing plates* - any plate suitable for grinding an individual mosquito in 200µl of solution can be used.  As 95 insects can be run per microtitre plate, a system for grinding this number of insects rapidly is beneficial (for details of one system used by some laboratories see ffrench-Constant & Devonshire, 1988).  Alternatively, insects can be ground directly in the microcentrifuge tubes, ready for centrifugation (where the glutathione-*S*-transferase assay is being used).

*Homogenizers* - glass rod or other device.

*Microcentrifuge tubes*

*Microfuge* (variable of fixed speed)

*Automatic pipettes* - (P20, P200 & P1000 variable volumes). Ideally 8 channel pipettes, but these are expensive. Single channel pipettes can be used, but slow down the procedure. Transfer plates are available which allow simultaneous treatment of all 96 wells and speeds up the system when large numbers of samples are being processed.

*Pipette tips (yellow and blue)* - These can be re-used if carefully washed. Those used for pipetting solutions containing insecticide should be kept separately.

*Microtitre plate reader* - This is essential only for the glutathione-*S*-transferase and protein assays, as the other assays can be scored visually. However, it is required for numerical quantification and publication of results. Ideally, a plate reader with a kinetic capability should be used so that reaction rates can be calculated, but end points can be measured on a basic plate reader if necessary. The minimum filter wavelengths needed are 340nm, 405nm, 570nm.

### 3.1.2 Solutions and reagents

All reagents listed are basic grade chemicals and are widely available from a range of suppliers. Unless otherwise stated, phosphate buffer refers to a sodium phosphate buffer system. ($NaH_2PO_4$ + $Na_2HPO_4$ solutions mixed in the correct molarities and ratios to give the stated pH and molar concentrations. Alternatively, the acid monobasic phosphate solution can be mixed with NaOH to give the correct pH. The molecular weight of the monobasic phosphate is 120 and the dibasic phosphate is 142.)

**i.    Acetylcholinesterase assay**

All solutions for this assay, with the exception of the 0.1M propoxur, should be made up freshly and used within 4 hours if left at room temperature or 4°C. The propoxur stock solution can be kept at 4°C in a tightly stoppered bottle for several months.

a)    10ml 0.01M dithiobis 2-nitrobenzoic acid (0.0396g DTNB + 10ml 0.1M phosphate buffer pH 7.0).
b)    20ml 0.01M acetylthiocholine iodide (0.0578g ASCHI + 20ml distilled water).
c)    10ml 0.1M propoxur (0.292g propoxur in 10ml acetone).
d)    Split the ASCHI solution into two 10ml aliquots, to one aliquot add 20μl of 0.1M propoxur.
e)    1% Triton X-100 in 0.1M phosphate buffer pH 7.8.

**ii.    Esterase assay**

*A.    Naphthyl acetate assay*

a)    30mM 1-naphthyl acetate (NA) (0.2793g 1-NA in 50ml acetone).
b)    30mM 2-naphthyl acetate made as above with 2-NA.

*Solutions a) and b) can be made up and stored separately in a tightly stoppered bottle at 4 °C for several months.*

c)    Working naphthyl acetate solutions 1 ml of 30 mM stock in 99 ml of phosphate buffer 0.02M pH 7.2.

d)    Stain (150 mg Fast blue B salt dissolved in 15 ml distilled water, then add 35 ml of 5% sodium lauryl sulphate (SDS)).

N.B.
It is important to first dissolve the stain in the water, as it will take much longer to dissolve directly in a solution containing SDS.

*Solutions c) and d) should be made up freshly and used within 1-2 hrs of preparation.*

**B.    PNPA rate reaction assay**

a)    100 mM para-nitrophenyl acetate stock solution in acetonitrile (N.B. molecular weight PNPA is 181.15).

b)    Working solution: dilute stock solution PNPA 1:100 with 50 mM sodium phosphate buffer pH 7.4.

Solution a) can be stored in a tightly stoppered bottle at 4°C for 1-2 months. Great care should be taken when handling the acetonitrile solution, as this is highly toxic. It is recommended that mouth pipetting of all chemical solutions is avoided, but this solution particularly should never be mouth pipetted.

### iii.    Glutathione-*S*-transferase assay

a)    10 mM GSH i.e. reduced glutathione 0.0081 g GSH in 2.5 ml 0.1 M phosphate buffer, pH 6.5).

b)    63 mM chlorodinitrobenzene (0.1278 g of CDNB in 10ml methanol).

c)    Working solution: add 125 µl of CDNB solution to 2.5 ml GSH solution.

*All solutions should be prepared freshly and used within 1-2 hrs.*

### iv.    Monooxygenase titration assay

a)    3% hydrogen peroxide.

b)    0.625M potassium phosphate buffer pH 7.2.

c)    0.01 gm $3,3^1,5,5^1$-tetramethyl benzidine in 5ml methanol.

d)    0.25M sodium acetate buffer pH 5.0.

### v.    Protein assay

Commercial kits are available from various manufacturers to measure protein concentrations. Alternatively the methods of Lowry *et al.* (1951) or Bradford (1976) can be followed.

### 3.1.3 Methods for biochemical assays with mosquitoes

The standard method here is given for adult, or 4th instar larvae of any mosquito species. For other insects or earlier instar mosquito larvae the initial homogenization volume may need to be increased or decreased depending on the size of the insect. Any stage of the insect can be used, but ideally this should be the life stage of the insect that is to be controlled with the pesticide, as resistance mechanisms do not necessarily operate throughout all life stages. For mosquitoes, the 4th instar larvae or young adults are preferable, as enzyme activity in younger larvae is proportional to their smaller size, and becomes increasingly more difficult to measure accurately in smaller larvae. If houseflies are being used, the head should be removed and used for the acetylcholinesterase assay, as the large amounts of pigment released from the eyes on homogenisation interfere with the other assays.

A.      Homogenize individual mosquitoes in 200 µl of distilled water on ice. Take 2 x 25 µl for the acetylcholinesterase assay and then spin the remainder of the homogenate at 14 K for 30 secs in a microfuge (or maximum speed on a fixed speed microfuge).

The microfuge step is essential only for the glutathione-*S*-transferase assay, as particulate matter in the crude homogenate has a significant impact on the absorption of light at 340 nm. The samples for the acetylcholinesterase assay are removed before spinning as being membrane bound, much of this enzyme may be pelleted and lost during centrifugation. The time and centrifuge speed are not critical, but in a non-refrigerated microfuge times of > 2 mins should be avoided or a large proportion of enzyme activity may be lost.

It is important when homogenizing the insects to ensure that all parts of the body are fully crushed. This is particularly important for the head, which contains a large proportion of the acetylcholinesterase activity. If the homogenate is not kept on ice, then enzyme activity loss will occur. As a general rule the higher the temperature the more rapid the enzyme activity loss. If ice is not available, then place the homogenate in the freezer compartment of a refrigerator when not in direct use. When frozen at -20°C directly after homogenisation the enzyme activity will remain relatively constant for several hours.

Live or frozen insects can be used for these assays. Whole insects stored at -20°C are viable for several months, while those stored at -70°C or in liquid nitrogen remain viable for years.

*Insects which have been dead for more than a few minutes at room temperature or those exposed directly to pesticides should not be used in these assays as the enzyme levels will have been seriously reduced and results will be meaningless.*

#### i.      Acetylcholinesterase assay

This assay can be used with adult or larval stages of mosquitoes; however, the intensity of colour formation is generally greater with adults, and where results are to be interpreted visually use of adults rather than larvae is recommended.

1.      Take 2 x 25 µl replicates of crude insect homogenate and place in separate wells of a microtitre plate. Make sure that large lumps of insect tissue are not transferred to the microtitre plate wells, as these will adversely affect results. (If results are to be interpreted visually rather than spectrophotometrically, then the two replicates from the same insect should be in adjacent wells for ease of comparison. If results are read

spectrophotometrically the location of each element of the pairs on the microtitre plates should be noted, so that values can be matched after reading.)

2. Add 15 m$\lambda$ of Triton phosphate buffer to each replicate. (This is used to solubilize the acetylcholinesterase. Mix the solution gently to avoid getting too many bubbles in the wells.)

3. Add 10 $\mu$l DTNB solution to each replicate.

4. Add 25 $\mu$l of ASCHI to *one* replicate and 25 $\mu$l of ASCHI + propoxur to the *other* replicate.

5. Always set up one or more blank wells per plate. These should contain 25 $\mu$l distilled water, 10 $\mu$l DTNB solution, 25 $\mu$l ASCHI solution and 145 $\mu$l Triton buffer but *no* mosquito homogenate. For most plate readers the A1 well on the microtitre plate is used as the blank well.

6. Read continuously at 405 nm for 5 minutes or leave for 1 hour and read as a fixed-point assay at 405 nm. Before reading, ensure that large numbers of small bubbles are not present in any of the wells, as these will affect the accuracy of the readings you obtain. If a fixed point is used the same time needs to be used for the inhibited and uninhibited wells for the same insect. If a continuous reading is used this must be completed no more than 20 minutes after the addition of the ASCHI solutions. Plate 1 is an example of a Microtitre analysis of an acetycholinesterase assay.

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*Notes:*
In this assay each insect acts as its own control. A bright yellow colour should appear in all the control wells, i.e. those with insect homogenate + ASCHI but not propoxur. If a yellow colour does not form, then:

a) the insects have had some pre-exposure to pesticide;
b) the homogenate has been allowed to stand too long before use;
c) the insects were dead for too long before freezing or homogenization;
d) the chemical reagents have expired.

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Generally, a) to c) are more likely, as degradation of the reagents (DTNB or ASCHI) in this assay will tend to produce a strong yellow colour in the plate blank which should normally be almost colourless. Results must be discarded for all insects where a yellow colour does not form in the control wells or for plates where the blank is strongly coloured.

Propoxur is used in this assay as described, as in all cases of altered acetylcholinesterase documented in mosquitoes resistance to this pesticide occurs even though it may not have been involved in the field selection of the resistance. The concentration of propoxur has been set so that at least 70% of the total acetylcholinesterase activity is inhibited in the susceptible insect and the concentration is valid for analytical grade propoxur. If technical grade material is used the amount of propoxur used may need to be increased slightly. The concentration given produces a clear distinction between resistant and susceptible insects both visually and spectrophotometrically. If an end point assay is used, then susceptible (SS) individuals can be easily differentiated from heterozygote (RS) and homozygote (RR) resistants, but differentiating some RS and RR is difficult. If a rate assay is used, then all three genotypes can be clearly distinguished. For insects other than mosquitoes, the concentration of propoxur may

need to be adjusted slightly, by experimentation with the susceptible strain of the test insect, to achieve at least 70% inhibition of acetylcholinesterase activity. If propoxur is not available another carbamate can be substituted or the *oxon* analogue of an organophosphorus insecticide (e.g. malaoxon or paraoxon), although again the concentration of the insecticide will need to be determined. *You must not use the organophosphorus insecticides themselves* as they do not interact directly with acetylcholinesterase and should not give inhibition even at high concentrations.

If initial homogenization of the insects was poor and large pieces of tissue are subsequently transferred to the microtitre plates in the homogenate, the acetylcholinesterase contained in the tissue will cause increased colour formation in the well. Transfer of pieces of tissue should be avoided, as this will not be equal in insecticide treated and untreated wells and will lead to poor reproducibility of results.

**3**

**Plate 1.**

Microtitre plate analysis of acetylochlinesterase assay. The individual mosquitoes represent the three genotypes: SS = homozygous susceptible; RS = hetrozygote resistant; and RR = homozygous resistant. The reaction rate in the graphs above the microtitre plate represent all three genotypes, each represents one microtitre plate well in the same as the 96 format.



Figure

Graphical output from kinetic microtitre plate reader for SS, RS and RR *An. albimanus* (A) with associated microtitre assay plate (B). 96 graphs of optical density (maximum limit = 0.1) against time (5 minutes overall), for all 96 wells of the microtitre plate. Horizontal pairs represent aliquots from the same individual, U= uninhibited, I= inhibited by addition of 100 mM propoxur.

## ii.    Esterase assays

## A.    Naphthyl acetate end point assays

1.    Take 2 x 20 µl replicates of homogenate and place in separate wells in a microtitre plate.

2.    Add 200 µl of 1-NA working solution to one replicate, and 200 µl of 2-NA to the second replicate. Leave at room temperature for 15 mins.

3.    Add 50 µl of fast blue stain solution.

4.    One or more plate blank should be included per plate. This should contain 20 µl distilled water, 200 µl of 1-NA or 2-NA solution and 50 µl of stain.

5.    Read at 570 nm as an end point.

> *Notes:*
>     The 15-minute time point is an arbitrary one. However, for results to be comparable it is necessary to adhere to a single time interval (e.g. not to leave for 10 mins one day, for 15 mins the next). Extending the time to more than 15 minutes may produce such an intense colour in insects which have a highly amplified esterase, that spectrophotometric readings become inaccurate. Occasionally, where esterases are greatly overproduced, even a time of 10 mins has given a production of naphthol too dark to read on a plate reader. This problem can be overcome by shortening the incubation time or alternatively by diluting the homogenate or taking a smaller volume (e.g. 5 µl) of homogenate.

The colour formation with 1-NA after addition of the stain tends to initially go pink and then change over 2-5 minutes to a purple/blue colour. The microtitre plates should be left for 5 minutes after addition to the stain, before reading the results, to allow this colour transformation to occur. Plate 2 is an example of microtitre tables showing the esterase assays with a blue (alpha) and red (beta) naphthyl acetate.

This assay measures esterase activity directly, and linking this to resistance requires confirmation via synergist and/or electrophoretic work. (See section on interpretation for further details.) The assay as detailed above uses 2 substrates. In mosquitoes elevated esterases involved in resistance are generally active with both substrates. (Hence a positive result with one substrate should be confirmed by the second substrates; alternatively, 2 replicates with a single substrate can be used.) In other insects, such as *Blattella germanica*, there can be a marked preference for one of the two substrates used.

On addition of the stain, the plate blank well(s) should remain light yellow in colour, or have only a slight pink colour. If significant colour formation occurs in the blank, then either:

1.    The working solutions of 1-NA or 2-NA have been made up too long, *or*

2.    The stock solutions of 1-NA or 2-NA need replacing.

For accurate comparison of results between laboratories, the absorbance values for individual insects should ideally be converted to nmoles of product produced. For 1-NA the

product is 1-naphthol (or 2-NA, 2-naphthol). Both chemicals are commercially available, and standard curves of absorbance of known concentrations stained as above with fast blue should be produced for each microtitre plate reader, to quantify results accurately.

**Plate 2**

Two microtitre plates showing the esterase assays with α blue and β red – naphtyl acetate. A1-H1 are controls without mosquito homogenate The upper plate is a fully susceptible strain of mosquito. The lower plate is mixed resistant population with resistant mosquitoes showing the dark blue and red coloration.

**3**
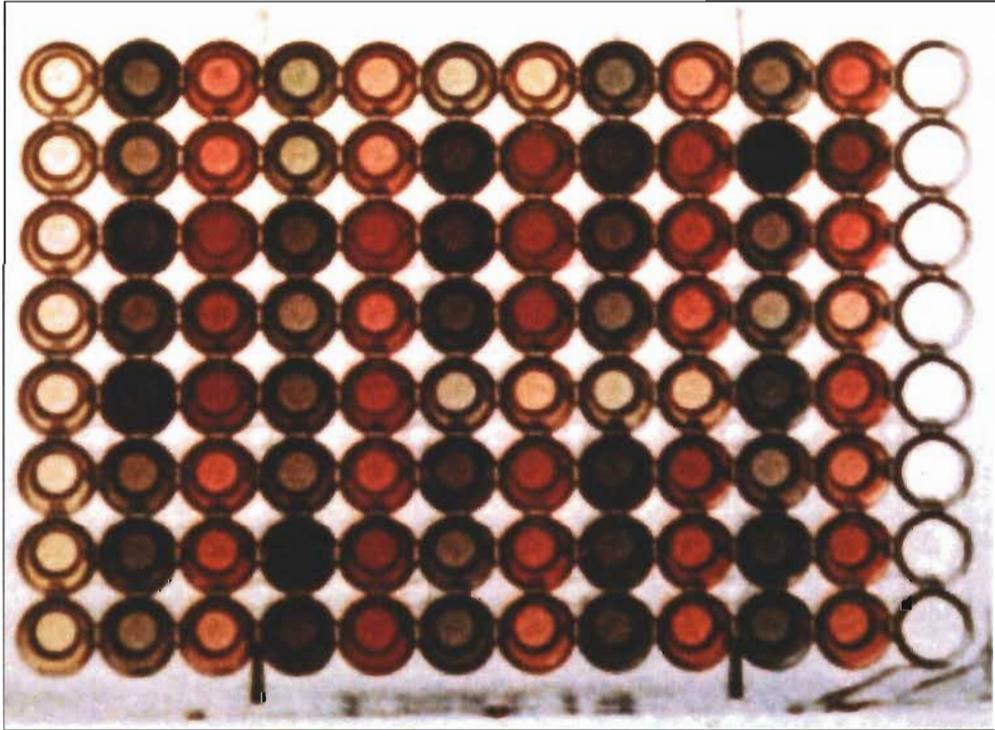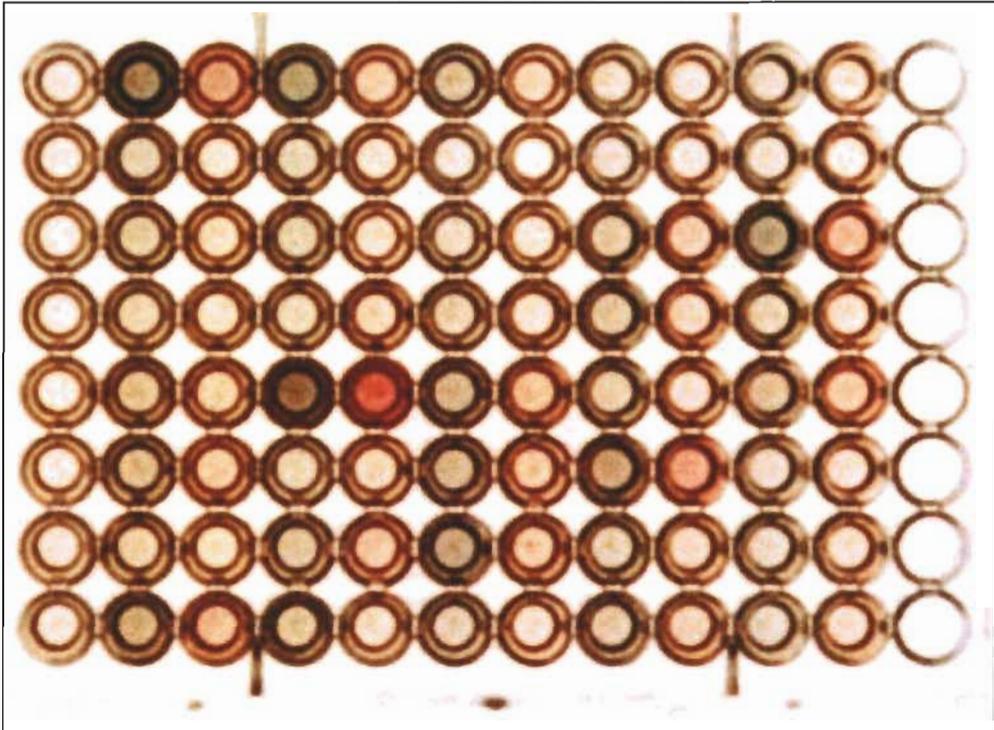
**B.    PNPA rate reaction assay**

1.    Take 2 x 10 µl replicates of homogenate and put into separate wells of a microtitre plate.

2.    Add 200 µl of PNPA working solution.

3.    One or more plate blanks should be included per microtitre plate.  These should include 10 µl of distilled water and 200 µl working PNPA solution.

4.    Read at 405 nm continuously for 2 mins.

> *Notes:*
> This assay is simpler and quicker than the naphthyl acetate assays and equally accurate. However, it *cannot* be used as an end point assay.  If the plate reader does not do kinetic measurements, it is preferable to use the naphthyl acetate assay.  This assay also measures only elevation of esterase activity and the association of this with resistance still needs confirming, as with the naphthyl acetate assays, by electrophoresis and/or synergist work.

Visually results can be differentiated on the intensity of the yellow colour produced, but if results are only to be scored visually, the naphthyl acetate assays are easier to interpret.  It is also important when using this assay to ensure that the PNPA working solution is made up immediately before use, as significant yellow colour will result from auto-hydrolysis if the solution is allowed to stand.

**iii.    Glutathione-*S*-transferase assay**

1.    Take 2 x 10 µl replicates of homogenate and place in separate wells of a microtitre plate.

2.    Add 200 µl of the GSH/CDNB working solution.

3.    One or more plate blanks should be used per microtitre plate.  These should contain 10 µl distilled water + 200 µl of the GSH/CDNB working solution.

4.    Read at 340 nm continuously for 5 mins or leave at room temperature for 20 mins and then read at 340 nm as an end point.

> *Notes:*
> This assay must be read spectrophotometrically as the change in absorbance is in the UV range (i.e. a colour change in the wells will not be seen).  Ideally, the assay should be read as a rate, as this gives a much better differentiation between resistant and susceptible insects with this mechanism.  If the assay is used at a fixed point, an exact time point must be used every time as the reaction continues over time, until becoming rate limited by the substrate concentration.  Generally this reaction is linear for approximately 20 minutes after the addition of the GSH/CDNB solution.  Hence the rates should be measured within this period.

Dichloronitrobenzene can be used rather than chlorodinitrobenzene.  *However,* the amount of insect homogenate will need to be increased, as the baseline activity with DCNB is much lower than that with CDNB for mosquitoes.

## iv. Monooxygenase titration assay

1.  Take 2 x 2 µl replicates of homogenate and place in separate wells of a microtitre plate.

2.  Add 80 µl of 0.625 M potassium phosphate buffer (pH 7.2) to each replicate.

3.  Mix the 5 ml methanol solution of tetramethyl benzidine with 15 ml of 0.25 M sodium acetate buffer (pH 5.0).

4.  Add 200 µl of the above mixture to each replicate.

5.  Add 25 µl of 3% hydrogen peroxide to each replicate.

6.  Leave the mixture for 2 hours at room temperature before reading absorbance at 650 nm.

7.  Controls should be run with 20 µl of buffer in place of the insect homogenate.

> *Notes:*
> This assay is based on that of Brogden *et al* (1997). The assay measures the haem content of the insect, the majority of which is associated with cytochrome $P^{450}$ in the non-bloodfed insects.

> *N.B.*
> It is obviously important that bloodfed insects are not used for this assay. The assay is, therefore, a rough means of titrating $P^{450}$ content. It is not a measure of $P^{450}$ activity in the insect.

## v. Protein assay

1.  Take 2 x 10 µl replicates of homogenate and place in separate wells of a microtitre plate.

2.  Add 300 µl of Bio Rad or Pierce BCA protein reagent.

3.  Read Bio Rad at 570 nm after 5 mins at room temperature. Read BCA at 570 nm after 30 mins at room temperature.

> *Notes:*
> Various commercial kits for measurement of protein concentrations are available, or the published methods such as Lowry *et al*. (1951) can be used. The protein concentrations are used in calculation of the esterase and glutathione-*S*-transferase data. It allows different sizes of insect to be used, e.g. 2nd or 4th instar larvae, so that results can be directly compared in the same units (such as nmoles product formed/minute/mg protein). If the assays are being interpreted visually rather than spectrophotometrically there is no need to run the protein assays.

> *N.B.*
> As all protein assays do not measure protein concentration in the same way, there should be no switch between different methods, especially where results need to be directly compared. If the Bio-rad kit is used, absorbance should be read within 30 minutes of adding the working solution, as a precipitate forms in the sample when left to stand for longer periods.

A standard curve using different concentrations of bovine serum albumin should be run under the conditions used above, so that absorbance values can be converted into μg of protein.

### 3.1.4 Analysis and interpretation of results

#### i.  Acetylcholinesterase assay

#### A.  Visual interpretation of results

1.  Is your plate blank almost colourless?  If so, go on to interpret the results: if not, discard the plate and make up fresh reagents, as results will not be accurate.

2.  Is there a strong yellow colour in all wells with insect homogenate but *no* propoxur?  If not, discard results for pairs with low or no yellow colour, as results will be inaccurate.

3.  Compare each pair of wells with and without propoxur for the same insect.  Is the intensity of the yellow colour almost the same in both or even slightly stronger in the well with propoxur?  If so, this represents a resistant insect containing the altered acetylcholinesterase mechanism and probably indicates a resistant homozygote.

4.  Is the yellow colour in the well without propoxur strong and that with propoxur the same or only slightly higher than the plate blank?  If so, this represents an insect which does not have the altered acetylcholinesterase based mechanism, i.e. a homozygous susceptible individual with respect to this mechanism.

5.  Is the yellow colour in the well without propoxur strong and that with propoxur much higher than the plate blank but lower than that in the replicate well?  If so, this is an individual heterozygous for the altered acetylcholinesterase based mechanism.  Visually it may be difficult to distinguish some of these individuals from those in 3 above, but they should be clearly different for those in 4 above.

#### B.  Spectrophotometrical interpretation of results

Whether a rate or fixed time point value has been obtained, there should be 2 values for each insect (one with, one without propoxur).  Discard any pairs where the value for the well *without* propoxur is the same, or only marginally higher than the plate blank.

Divide the value for the well with propoxur by that without propoxur for the *same* insect, i.e. *rate or end point with propoxur* x 100 = % remaining activity in propoxur inhibited replicate rate or end point without propoxur.

Resistant insects should have a % value greater than 30%.  The accuracy of the results should be evident, if the data for the population is expressed graphically, as shown in Figure 1. A known susceptible population should be used to check that 30% is an accurate cut-off point. Values for homozygous resistant insects may be substantially higher than 100%.  This is partially due to the absorbance of propoxur in the microtitre plate well and is normal in resistant strains.

**FIGURE 1**

3
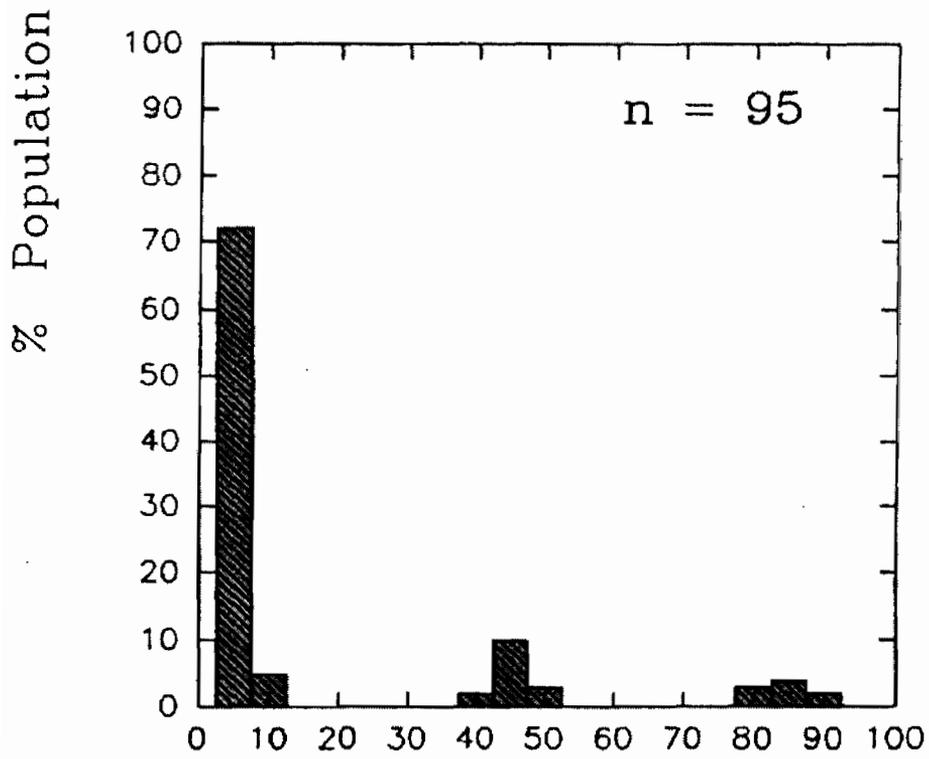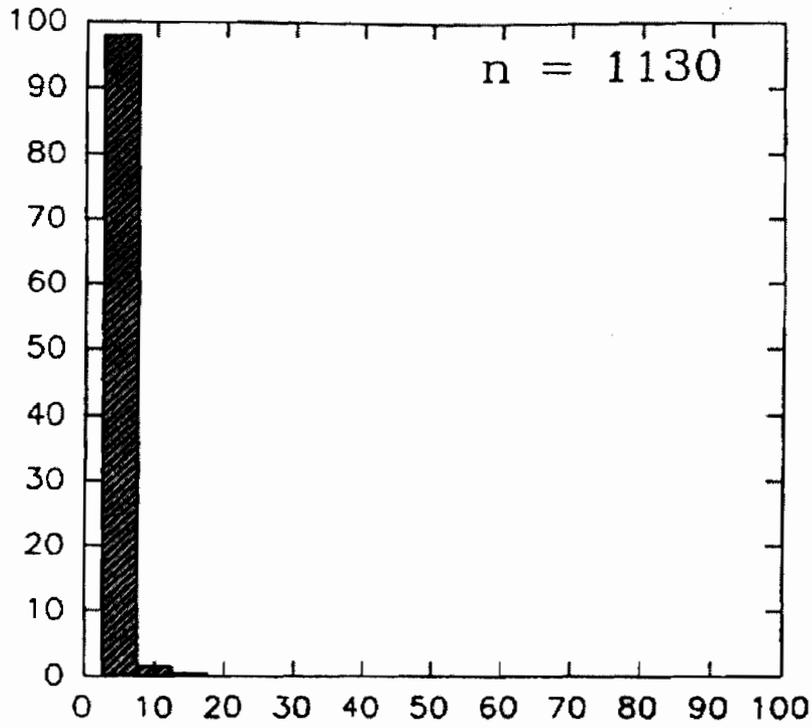


% Activity in Propoxur Inhibited Fraction.

## ii. Esterases

### A. Visual interpretation of results

Individuals with non-elevated levels of esterase activity should have a pale blue or pink colour with 1-NA or 2-NA respectively. If a known insecticide susceptible population is available, this should be used as the baseline for comparison. Individuals with elevated esterase activity show an intense blue/black or pink/red colour with 1-NA and 2-NA respectively.

> *N.B.*
> Even in the susceptible strain there will be some variation in esterase activity levels, hence it is important to be familiarized with the baseline levels of activity in the susceptible strain of the insect species you are working with. (See Figure 2 for presentation of results)

### B. Spectrophotometrical interpretation of results

The products of the reactions with 1-NA and 2-NA are 1-naphthol and 2-naphthol respectively. These chemicals are commercially available from a number of sources. Standard curves should be set up for the two chemicals (i.e. a range of concentrations of the chemical in 220 μl 0.02 M phosphate buffer pH 7.2 mixed with 50 μl of stain solution). Absorbance values obtained from the mosquito samples can then be converted to nmoles 1-naphthol or 2-naphthol using the relevant standard curves.

Once results have been converted, the values in units of nmoles 1- or 2-naphthol/min/mg protein can be calculated as follows:

❑ Read off protein value for the insect from the bovine serum albumin standard curve.

> *N.B.*
> If the method given has been followed, this is for 10 μl of homogenate. If there are two protein values (i.e. you have done two replicates for the protein estimation), the mean value can be taken.

❑ Take the nmoles 1- or 2-naphthol value and divide it by the number of minutes the homogenate and substrate were incubated for *before* the stain was added. (If the method given is followed, this would be nmoles 1-naphthol/15 as a 15 minute incubation is used.)

❑ Take the protein value calculated from the standard curve in a) and times by 2 (to give the amount of protein expected in 20 μl homogenate). This protein value should be in μg.

❑ Take the value obtained in b), divide by the value in c) and times this by 1000. The units you now have are nmoles 1- or 2-naphthol/min/mg protein.

❑ These values for the population of insects analysed can then be graphed as in Figure 2.

If there is evidence of an elevated esterase it is important to go on and demonstrate the association of this with insecticide resistance by electrophoresis and/or synergist work.

**FIGURE 2**

3

Activity \ min \ mg protein
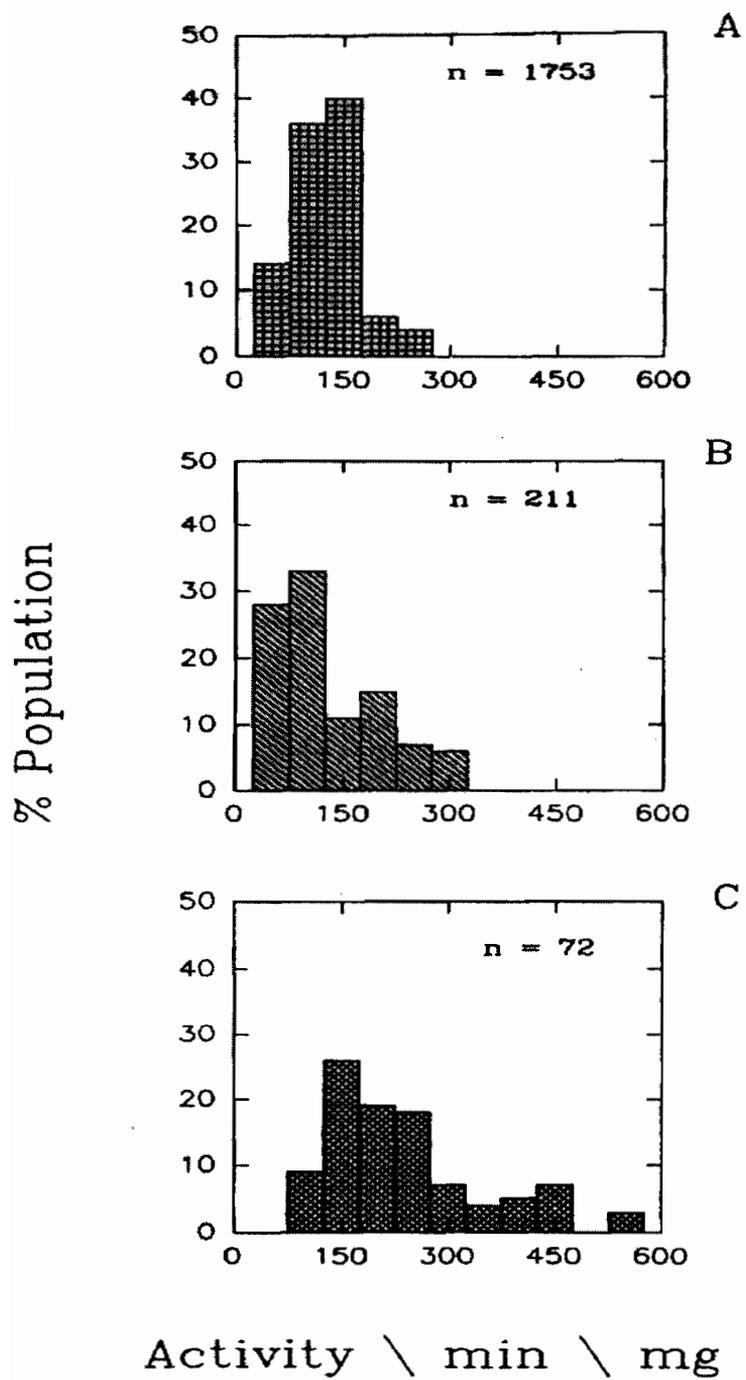
### iii. Glutathione-*S*-transferases

Visual interpretation of the results of this assay is not possible as absorbance is in the UV range.

### iv. Spectrophotometrical interpretation of results

To use the assay with CDNB as given, the extinction coefficient of the product of the reaction (3-(2-chloro-4-nitrophenyl)-glutathione) has been determined at 340 nm. The extinction coefficient = 4.39 mM$^{-1}$. This value does not include a unit of path length, as path length is a function of well volume. In the microtitre plates we use, with a constant assay volume of 210 μl, the path length (i.e. depth of solution in the microtitre plate well) = 0.6 cm.

To calculate the activity in mMole/min/mg protein the absorption value can be converted, assuming that absorbance is following Beer's law:

i.e.     $A = \varepsilon c l$
where    A = absorbance
         ε = extinction coefficient
         c = concentration
         l = path length

*N.B.*
The value of mMoles or μMoles/min can be converted to protein units by dividing by the protein value calculated in a) for the esterases and x 1000, as 10 μl homogenate volumes were used for both protein and glutathione-*S*-transferase assays.

The results for the population of insects can then be presented graphically as shown in Figure 3.

### 3.2     The dot-blot tests

### 3.2.1     Supplies / equipment
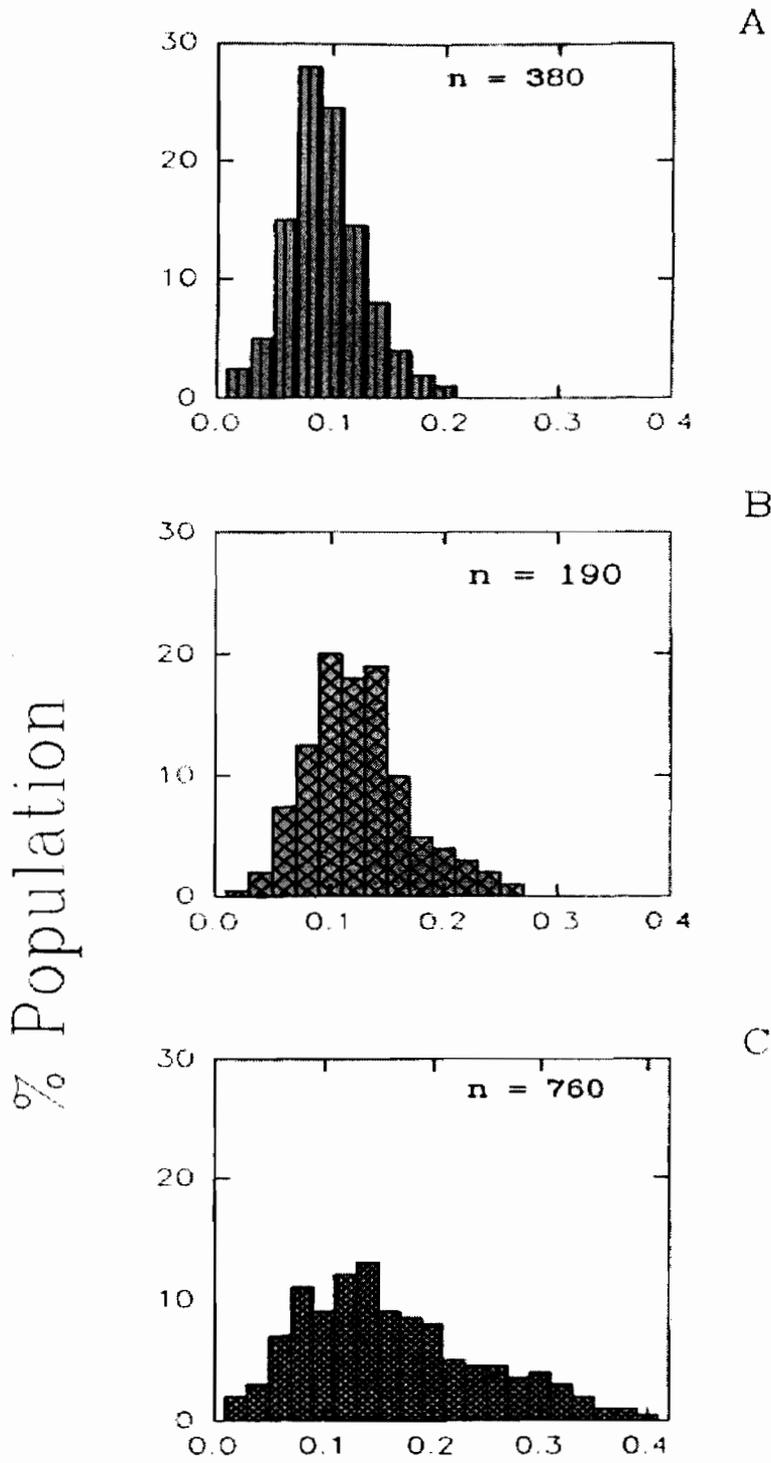
- Nitrocellulose membranes
- Whatmans filter paper No. 2 (or No. 42)
- One 10 ml test tube
- Porcelain plate with 12 cavities
- Automatic pipettes (P20 and P200 variable volumes)
- Forceps
- Glass Petri dishes or similar containers
- Tissue paper
- Densitometer (e.g. RCX model, Tobias associates, Ivyland, Pa.)
- pH meter

**FIGURE 3**

**3**



nmoles 1-naphthol\min\mg protein

**Solutions and reagents needed**

All reagents listed are basic grade chemicals and are widely available from a range of suppliers.

**For esterase assay**

a)    0.1 M monobasic sodium phosphate $NaH_2PO_4$.
b)    0.1 M dibasic sodium phosphate $Na_2HPO_4$.
c)    Phosphate buffer (0.1M pH 6.5) made from mixing solutions a) and b) in the proportions to give the correct pH.
d)    Homogenization buffer: buffer c) above containing 0.5% Triton X-100.
e)    Substrate: 0.2 gm 1-naphthyl acetate in 10 ml ethanol.
f)    Solution A: 5 ml substrate e) in 95 ml phosphate buffer c).
g)    Solution B: 150 mg Fast Garnet GBC in 100 ml distilled water.
h)    Solution C: 500 ml distilled water.

Solutions a-e can be made up and stored in tightly stoppered bottles at 4°C.  Solution A should be prepared just before use, and solution B should be prepared daily as required.

**For altered acetylcholinesterase**

a)    Phosphate buffer 0.1 M pH 6.5 (as above).
b)    100 mM propoxur in ethanol.
c)    10 mM paraoxon in ethanol.
d)    Developing solution (17 ml distilled water, 25 ml phosphate buffer a).  1 ml 100 mM sodium citrate, 2 ml 30 mM cupric sulphate, 4 ml 5 mM potassium ferricyanide (prepared freshly) and 1 ml 100 mM acetylthiocholine iodide (prepared freshly).

### 3.2.2 Methods for dot-blot assays with mosquitoes

**i.    Elevated esterases**

1.    Homogenize individual insects in 100 µl of homogenization buffer in the wells of porcelain plate using the base of the test tube.  (The tube should be wiped after processing each insect to avoid contamination between samples.)

2.    Place a small (1 cm x 1 cm) piece of a single layer of tissue paper on top of the homogenate in each well.

3.    Take 2 µl of homogenate through the layer of tissue paper.

4.    Deposit the 2 µl of homogenate onto a strip of Whatmans filter paper by bringing the tip of the automatic pipette lightly in contact with the filter.

5.    After processing a maximum of 10-20 homogenates (which should take approximately 2 minutes), immerse the filter paper in Solution A for 60 seconds.

6.    Remove the paper and blot between 2 layers of tissue paper.

7.    Transfer the filter to solution B for 60 seconds.

8. Dip the filter paper briefly in solution C.

9. Allow to dry on tissue paper.

10. Repeat the above method with a series of concentrations of 1-naphthol replacing the mosquito homogenate, to produce a 'standard curve' on the filter paper.

**ii. Altered acetylcholinesterase**

*This assay cannot be used with mosquito larvae.*

1. Homogenize whole mosquito adults in 60 μl of phosphate buffer (for houseflies use individual heads only and homogenize in 100 μl buffer), as for esterase assay.

2. Place a 1 cm x 1cm piece of tissue on top of the homogenate in each well.

3. Aspirate 5 μl (the original paper, Dary *et al*, 1991, says 5 ml, presumably a misprint) of homogenate through the tissue and transfer to a nitrocellulose membrane placed on a glass plate. At least 35 ml replicates from each insect should be aspirated onto these different membranes.

4. Air dry the blots and use immediately or store in the dark at room temperature for up to 3 weeks.

5. Place the blotted membranes into a plastic bag containing 50 ml distilled water.

6. Place the three membranes spotted with the same insect replicates into 3 different plastic bags. To bag A add 50 ml distilled water + 25 μl ethanol; to bag B add 50 ml distilled water + 25 μl 100 mM propoxur; to bag C add 50 ml distilled water + 25 μl 10 mM paraoxon.

7. Leave for 15 minutes at room temperature. Remove membranes and rinse with distilled water.

8. Immerse the membranes in developing solution and leave for 3-4 hrs at room temperature with occasional shaking. (N.B. The developing solution for the propoxur treated membranes should contain 25 μl 10 mM propoxur to control for reactivation of the inhibited acetylcholinesterase during this incubation period.)

9. Red/brown spots should appear on all control membranes from bag A and from insects containing an altered acetylcholinesterase from bags B and C.

10. Air dry the membranes after rinsing in distilled water and store in paper envelopes. (Nitrocellulose membranes will disintegrate if stored in plastic.)

### 3.2.3 Analysis and interpretation of results

**i. Esterase assay**

**A. Visual interpretation**

Insects which have elevated esterase activity should show a distinct purple colour where they have been spotted onto the filter paper. Insects without the elevated esterase should show little or no colour formation. A known susceptible insect strain of the same species should be used for comparison of field material.

**B. Densitometric interpretation**

Dry filter papers should be used. Blank the densitometer on an area of the filter paper where there is no insect homogenate. For each filter paper measure the optical density of each spot that was developed from each homogenate.

As with the microtitre plate version of this assay, this measures <u>only</u> elevated esterase activity and the association of this with resistance needs to be shown by electrophoresis and/or synergist studies.

**ii. Altered acetylcholinesterase assay**

**A. Visual interpretation**

1. Are all the homogenate spots on the control membrane (treated in bag A) red/brown? If not, the results for that insect should be discarded, as either:
   - ❑ the reagents are inactive
   - ❑ the insects have been pre-exposed to pesticide, or
   - ❑ the insects have been incorrectly handled prior to homogenization.

2. Is there a red/brown spot on the membrane with the replicate of homogenate from bag A but not those from bags B or C? If so, this represents an insect which does not have the altered acetylcholinesterase mechanism.

3. Is there a red/brown spot on all three membranes or on either that from B or C plus a spot on the Bag A membrane? This represents an insect with the altered acetylcholinesterase mechanism.

   *N.B.*
   In some insects this mechanism gives cross-resistance to organophosphates and carbamates, e.g. in *Culex pipiens* and *Anopheles albimanus*, in others resistance may only occur to organophosphates but not to carbamates, e.g. *Musca domestica*. The use of a carbamate and the oxon analogue of parathion in this assay allows detection of either type of altered acetylcholinesterase.

# 4. IMMUNOLOGICAL DETECTION METHODS

Currently this method is available only for specific elevated esterases in collaboration with laboratories that have access to the antiserum. There are no monoclonal antibodies, as yet, available for this purpose.

An antiserum has been prepared against the E4 carboxylesterase in the aphid *Myzus persicae*. An affinity purified 1 gG fraction from this antiserum has been used in a simple immunoplate assay to discriminate between the three common resistant variants of *Myzus persicae* found in UK field populations (Devonshire *et al.*, 1986). The sensitivity of this assay is such that it gives a clearer differentiation of resistant phenotypes than the esterase microplate assay (Devonshire *et al*, 1992).

Various antisera have been raised to the $A_2$, $B_1$ and $B_2$ elevated esterases in *Culex*. The literature suggests that sera raised to any of the 'B' esterases will detect other 'B', but not 'A' esterases and *vice versa* (Beyssat-Arnaouty *et al*, 1989, Hemingway *et al.*, 1986, Mouches *et al*, 1987). This has recently been shown to be incorrect, as sera raised to purified $A_2$ will in fact bind to $B_2$, although the sensitivity is 60-fold lower to the latter enzyme (Karunaratne *et al*, in press). The aphid 1 gG fraction used in the immunoplate assay is specific for the $E_4$ enzyme and the closely related $FE_4$; it shows no cross-reactivity with any of the elevated *Culex* esterases. The antiserum raised to the $B_1$ esterase also detects other immunologically related proteins in *Aedes aegypti* and *Musca domestica* (Mouches *et al*, 1987).

N.B.
The 'A' and 'B' classification of *Culex* esterases referred to here is with respect to their preference for 1- or 2-naphthyl acetate as described by Raymond *et al.* (1987), and independent of the earlier classification of Aldridge (1953). Under Aldridge's classification these *Culex* esterases are all B-type serine esterases.

## 5. DETECTION OF QUALITATIVELY ALTERED MALATHION CARBOXYLESTERASE

Where quantitative changes in esterases have occurred, these can be detected by the elevated esterase microplate or filter paper tests. However, in a significant number of *Anopheles* species malathion exposure in the field has resulted in selection of a quantitatively altered esterase, which is specifically able to metabolize malathion (and/or malaoxon) more rapidly than the susceptible esterase. This mechanism will *not* be detected by the microplate or filter paper assays, as it does not result in any increase in activity with either 1- or 2-naphthyl acetate. This mechanism is characterised by its limited cross-resistance spectra (usually only malathion and phenthoate, but not any of the other organophosphorus insecticides). It can be synergised by both triphenyl phosphate and DEF, and can be detected directly by malathion metabolism studies. In resistant insects there is an increase in production of one or both of the malathion monoacids and/or the malathion diacid compared to the susceptible strain.

If synergist studies are to be undertaken, it should be noted that this mechanism in *Anopheles* is most effective in young (1-3 day old) adult insects. It is often poorly expressed in larvae, and declines rapidly with increasing age of the adult insects. Hence, synergist and/or metabolism studies should be done on young adults.

# 6. DETECTION OF MONOOXYGENASE (CYTOCHROME P$^{450}$) - BASED INSECTICIDE RESISTANCE USING SYNERGIST PRE-EXPOSURE

The levels of oxidase activity in individual mosquitoes are relatively low, and no reliable microtitre plate or dot-blot assay has yet been developed to measure P$^{450}$ activity in single insects. The P$^{450}$s are also a complex family of enzymes, and it appears that different Cytochromes P$^{450}$s produce resistance to different insecticides. A simple way to obtain an indication of the presence of a monooxygenase-based mechanism is to use pre-exposure to the synergist piperonyl butoxide (PB) followed by exposure to an insecticide within the class you wish to detect resistance to (e.g. a pyrethroid, carbamate or organophosphorus insecticide). The dosage of synergist used should be the maximum that results in no mortality after exposure to the synergist alone. This dose varies between species and insect groups and should be determined for your species of interest by pre-exposing a sample of insects to a range of different concentrations. For larvae 1-4 hours pre-exposure can be used, for adults a 1 hour pre-exposure is usually sufficient. Insects should be held for 24 hours after exposure before mortality is assessed.

For the larvae, a range of PB concentrations can be made in acetone, and added to the test water to give the required final concentration for the standard WHO larval susceptibility test. For the adult tests synergist impregnated papers need to be made. These should be made by making a series of concentrations of piperonyl butoxide in olive oil. 0.7 ml of the oil solution should then be evenly spread on 12 x 15 cm rectangles of Whatmans no. 1 filter paper. This will give a paper with a percentage equal to that of the oil solution spread at the standard WHO rate for impregnated papers. Adult tests are then done exactly as for the standard WHO susceptibility test using a 1 hour exposure period. After the concentration of synergist to be used has been determined, insects are tested by pre-exposure to the synergist followed by direct transfer to the insecticide. (N.B. For larvae it is possible to use a simultaneous exposure to synergist + insecticide. In this case the dose of synergist used should be that which gives no mortality after 24 hours exposure).

## Interpretation of results

Care needs to be taken in interpretation of synergist results, as there is some baseline oxidase activity even in the susceptible strain. Hence, results for the resistant or field strain should be compared to those for a known susceptible strain of the same species subjected to the same treatment.

The interpretation of results for organophosphorus insecticides can be difficult, as the oxidases are involved in both activation and detoxication of these compounds. Hence, piperonyl butoxide can lower the toxicity of OPs to both susceptible and resistant strains without an oxidase-based OP resistance mechanism.

# 7.   DETECTION OF 'KDR'-BASED PYRETHROID/DDT RESISTANCE

A simple molecular detection system for 'kdr'-like resistance in mosquitoes is under development. Details of the primers to be used in this PCR assay will be generally available shortly. This resistance mechanism is based on physical changes in the sodium channel protein, and as such is not amenable to the same enzyme based assay systems as the other mechanisms. Currently 'kdr'-like mechanisms can be implied by default if access to complex and expensive neurophysiological equipment is not possible. To imply 'kdr' by default you need the following information:

1.    Is your population resistant to both DDT, and a range of pyrethroids?
[If it is not, then you do not have 'kdr', as this is the only mechanism which gives cross-resistance between DDT and the pyrethroids.]

2.    If you select your population with DDT, do you increase the level of pyrethroid resistance and vice versa? [If you do, then you probably have 'kdr'-like resistance. If you do not, then the pyrethroid and DDT resistance are due to different mechanisms and you do not have 'kdr'].

3.    Have you checked for glutathione-S-transferase based DDT resistance? (Preferably by looking for increased rates of DDT metabolism.) [If the rates of metabolism of DDT are the same in the susceptible and resistant strains, and your population is also pyrethroid resistant, you have 'kdr'].

You should remember that it is possible to have more than one resistance mechanism in your population. If you have 'kdr' plus a metabolic DDT resistance mechanism, you will have very high (>20-fold) levels of DDT-resistance.

A PCR-based molecular technique has now been devised for 'field' detection of 'kdr' in individual insects. This is currently being tested against a number of insects to see whether it is practical to put forward a generalized PCR-based detection method for this resistance mechanism.

## 8. ADDITIONAL METHODS REQUIRING MORE SOPHISITICATED LABORATORY FACILITIES
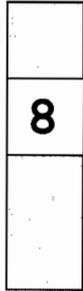
### A. Quantification of cytochrome $P^{450}$

At present, the sensitivity of the method for this is not sufficient to allow activity measurements in individual insects. A crude measure of the involvement of monooxygenases oxidases (of which Cytochrome $P^{450}$ is a key component) in resistance can be obtained using the synergist piperonyl butoxide (see page 29 on synergist work for exact details of methods).

1. Homogenize 0.2-2 gms of insects in 2-4 ml of potassium phosphate buffer (pH 7.6 50 mM). This is usually done on ice using a motor driven teflon homogenizer.

2. Spin at 10 000 g for 15 mins 4°C.

3. Remove the supernatant and spin at 100 000g for 40-90 mins at 4°C.

4. Take the pellet and resuspend in 4 ml Tris buffer. (0.25 M sucrose, 0.25 mM phenyl-methylsulfonyl fluoride, 1 mM dithiothreitol, 1 mM EDTA, 1% polyvinylpyrrolidone adjusted to pH 7.6 with 0.5 mM Tris.HC1.)

5. Take 2 ml of the resuspension and bubble briefly with carbon monoxide. Scan the CO and non-CO treated fractions from 300-500 nm.

6. The difference spectra between the two fractions should show a peak with an optima at 450 nm. The area under the peak gives a measure of the amount of $P^{450}$ present. If a significant peak occurs with a maximum absorption of 420 nm, it is likely that degradation of the $P^{450}$ has occurred during processing and calculation of the amount of $P^{450}$ will be inaccurate.

For further details of method see Omura and Sato (1964).

### B. Electrophoresis (PAGE) for identification of specific esterases

Starch or acrylamide can be used as the matrix for the electrophoresis. The latter gives much better resolution but needs more careful handling. The acrylamide method is given here, for details of the starch method see Georghiou & Pasteur (1978).

A vertical gel electrophoresis unit is required for this method. A 7.5% gel gives good resolution of all esterases involved in resistance in *Culex* with the conditions described below:

Gel buffer (Tris borate/EDTA buffer pH 8.6)
0.1M Tris
0.l0025 M EDTA (disodium salt)
0.04M boric acid
(adjust pH with saturated boric acid if required)

Solution A (30% acrylamide)
0.03M methyl bis acrylamide
2.10M Acrylamide
Filter through Whatmans no. 1 filter paper and store in a dark bottle at 4°C.

Solution B.  10% sucrose in gel buffer

Solution C.  0.007 M ammonium persulphate (make fresh).

Mix solutions A:B:C in the ratios 2A:1B:1C and degas.  Add 0.75 µl temed and pour into the cassette, leaving a 1-2 cm gap for the stacking gel.  Layer water saturated butanol on top of the gel to ensure an even surface while the gel polymerizes.

*Stacking gel (3%)*

Solution A 0.01M Methyl bis acrylamide

0.88M Acrylamide

Solutions B and C as above.  Add temed after mixing in same ratios as above.  Pour off the butanol from the top of the running gel and rinse with distilled water.  Pour stacking gel onto top of the running gel, carefully place the gel combs into the top of the cassette to avoid trapping bubbles between the comb and the gel.  Layer with butanol and allow to polymerize.

*Electrode buffer* for both upper and lower electrophoresis tanks.

0.1 M Trizma base

0.002 M EDTA

0.07 M boric acid

Adjust to pH 8.0 with saturated boric acid.  Pre-run gels at 150 volts for 30 mins.  Mix 100 µl of insect homogenate with 20 µl of marker (0.02% xylene cyanol in 15% glycerol (v/v) electrode buffer) and load into individual wells with a syringe or glass pipette.  Run the gel for 5 mins at 200 V until the marker has clearly entered the gel.  Flush out the wells with electrode buffer and re-start the electrophoresis at 200V.  When the marker reaches the running gel reduce to 180V and continue running until the visible marker is 3/4 of the way down the gel.

*N.B.*

Mini systems can be used in which case 15 µl of homogenate with 5 µl of marker is used.

Staining of the gels

Staining buffer:    0.07 M $KH_2PO_4$

0.033 M $Na_2HPO_4$

Staining solution:

0.0005 M Fast blue B salt

1.2 mM 1- and 2-naphthyl acetate prepared by adding 30 mM acetone stock solution (see esterase microtitre plate solutions) to staining buffer.

Incubate the gels at room temperature in the staining solution until the desired intensity of bands is apparent.

Fix the gels in 10% acetic acid.

*Interpretation of results*

With the method described above very intensely stained red/purple or purple/blue bands should appear on the gel within minutes of esterases being elevated.  1-naphthyl acetate specific bands are more blue coloured than 2-naphthyl acetate specific bands, which are more pink/red coloured.

Individual bands can be identified by measuring their mobility from the origin of the gel relative to the xylene cyanol marker.  Ideally, insect strains with known esterase bands, e.g. $A_2/B_2$ or $B_1$ in *Culex* or $E_4$ in Aphids should be run on the same gel if these bands are to be accurately identified.

## C.  DDT metabolism

DDT is converted to DDE by the action of a number of glutathione-*S*-transferases (Clarke & Shamaan, 1984, Prapanthadara *et al.*, in press).  This assay allows accurate quantification of DDE production and can be used to confirm that elevated levels of glutathione-*S*-transferase seen in the microplate assay are involved in DDT resistance.

*Solutions*
   a)   0.1 M sodium phosphate buffer pH 6.5.
   b)   4 mM DDT in methanol.
   c)   0.1 M reduced glutathione (GSH) in phosphate buffer.
   d)   DDT and DDE standards (available from British Greyhound, UK).

*Method*

This assay is not sensitive enough to run on individual insects and requires pooled homogenates from at least 25 mosquitoes.  Where a population is to be analyzed, ideally a large number (several gms) of insects should be used on the crude homogenate to remove material which may otherwise interfere with the assay.

To clean up the sample homogenize the insects in 25 mM bis-Tris propane (BTP) buffer pH 6.5 containing 15 mM dithiothreitol.  (Homogenize in ratio 1 gm insects/5 ml buffer). Centrifuge at 10 000g for 20 mins at 4°C and filter through Whatmans no. 1 filter paper to remove lipid material.  Additionally, Q-Sepharose anion exchange chromatography can be used.  Adjust the conductivity of the supernatant to 3 mS cm$^{-1}$, pH 6.5 with dilute hydrochloric acid and water and apply to a Q-Sepharose column containing 10 mM dithiothreitol at a flow rate of 3 ml/min.  (Column length will depend on the amount of homogenate to be used; a 4.4 x 6.5 cm column can be loaded with supernatant from 30 gms of mosquito larvae.)  Wash with 10 column volumes of BTP buffer, then eluted glutathione-*S*-transferase (GST) activity with a linear gradient of BTP buffer containing 0 - 0.35 M NaCl.  In our experience with *Anopheles* a peak of GST activity washes through with the BTP, but this has no associated DDT dehydrochlorinase activity and can be discarded.  The GSTs associated with resistance are eluted in the gradient and can be detected in small (10 μl) aliquots of each fraction using the GST microplate assay as described earlier.

Dilute an appropriate amount of the enzyme source to give a total volume of 1.7 ml in 0.1 M sodium phosphate buffer pH 6.5.  (An equal amount of enzyme with regard to the CDNB activity should be used from both the susceptible and resistant strains.  The dilution factor will depend on the amount of insect material in the initial homogenate.)  Add 100 μl 4 mM DDT and 0.2 ml 0.1 M GSH.  Leave at 28°C for 2 hours.  A control without enzyme should also be run.

After incubation extract the reaction mixture with 4 x 2 ml chloroform.  Pool the extracts and air dry.  Store the air dried tube at -20°C until analysis.

*To analyse:* Take up extract in 100 μl isopropanol for HPLC quantification on an ODS Ultrasphere reverse phase column using methanol:acetonitrile: water (72.5:12.5:15) as the mobile phase with a flow rate of 0.8 ml/min.  Peaks of DDT and DDE are integrated at the absorption optima for both compounds of 236 nm.  The amount of DDE produced (μmole/mg protein) can be calculated based on a standard curve established by injecting known

concentrations of standard DDE into the HPLC column and quantifying the detected peak areas. Detection of as little as 25 nmoles of DDT or DDE after HPLC analysis is possible.

## D.    Malathion metabolism study

Malathion is converted into the mono-carboxylic acids and/or the dicarboxylic acid by the action of malathion carboxylesterases. In the mosquito this resistance mechanism is characterised by the lack of cross-resistance to other organophosphorus insecticides, with the notable exception of phenthoate. Generally, there is no increase in activity associated with either 1- or 2-naphthyl acetate or *p*-nitrophenyl acetate (unlike the elevated esterases found in *Culex* and aphids, which sequester rather than metabolize malathion).

*Solutions*
     0.1M Phosphate buffer pH 7.0
     4 mM[1] C[14] malathion in methanol or acetone
     Malathion metabolic standards
     Metabolic standards
     malaoxon (0,0-dimethyl-S-(1,2-di-(ethoxycarbonyl)-ethyl)phosphorothioate)
     malathion monocarboxylic acids
     malathion dicarboxylic acid
     DMPDT (0,0-dimethyl phosphorodithionic acid)
     DMPT (0,0-dimethyl phosphorothionic acid)
     DMP (0,0-dimethyl phosphate)
     Thin layer chromatography plates 0.2 mm silica gel

*Method*

This method cannot be run accurately on individual insects and requires mass homogenates of 25 or more adult mosquitoes.

Homogenize insects in phosphate buffer (at a ratio 1 gm/ wet weight insects/5 ml buffer). Centrifuge at 10 k for 5 mins 4°C and take the supernatant.

Add 50 µl 4 mM malathion to 1.5 ml enzyme source (supernatant) and leave for 3 hours at 28°C. A control without enzyme (+ phosphate buffer) should also be incubated for the same period.

After incubation extract the reaction mixtures with 2 x 5 ml washes of hexane, after adding a small amount of anhydrous sodium sulphate. Combine the hexane extracts and evaporate to dryness under a stream of nitrogen. Repeat the extraction of the reaction mixtures with diethyl ether and acetonitrile as above.

Pre-run TLC plates in chloroform for 1 hour, then air dry. Take up the hexane, ether and acetonitrile fractions in 0.1 ml of the same solvent and spot out onto individual tracks on the TLC plate. Standard metabolites should also be spotted.

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[1] Quantification of the metabolites is by scintillation counting. C[14]-labelled malathion with a specific activity of 4.6mCi/mM, labelled in both carbon atoms of the succinyl part of the molecule, diluted at least 1:2 with unlabelled malathion, can be used.

Run the TLC plates with a hexane: diethyether (1:3) solvent system until the solvent almost reaches the top of the plate. Remove the TLC plate and air dry. Spray the tracks of the TLC plate containing the standard with 0.5% 2,6-dibromoquinone-4-chlorimide in cyclohexane. Heat the plate to 100°C. All standards and malathion should be clearly visible as red/brown spots.

Scrape the areas of the TLC plate on the test tracks corresponding to the Rf values of the standard metabolites. Re-elute the metabolites from the silica with hexane or ether, place the eluate into scintillation vials, air dry, then add 100 μl ethanol 40 μl water and 4 ml scintillation fluid to each vial and $C^{14}$-count. (Volumes may need to be adjusted according to the vials, counter and make of scintillation fluid used.)

A protein reading of the original supernatant should be used along with the metabolism data to compare metabolic rates from the resistant and susceptible strains.

## E.    DNA dot-blots for esterase gene quantification

DNA-based detection systems are not yet generally available. However, the recent emphasis on molecular studies of all the major insecticide resistance genes suggests that these are likely to become more accessible over the next few years. A dot-blot detection system combined with an immunoassay to identify resistant aphids, which have reverted to susceptibility, has been described by Field *et al.* (1989).

Probes are now available for the B1 and B2 elevated esterases commonly found in the *C. pipiens* complex, where a similar system can be employed.

## 9. REFERENCES

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