SELECTION OF DROSOPHILA MELANOGASTER

# SELECTION OF ORGANOPHOSPHATE RESISTANT *DROSOPHILA MELANOGASTER* OVER TWELVE GENERATIONS

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ABSTRACT

Twenty-five different iso-chromosomal III lines of Drosophila melanogaster, with known haplotypes, were mixed and selected on malaoxon over twelve generations in order to find the importance of variants already present in the Drosophila genome relative to new mutations in the evolution of insecticide resistance.

Measurements of mixed function oxidase and acetylcholinesterase activities, as well as insecticide resistance, were made over the twelve generations of selection and on the the iso-chromasomal III lines extracted after the selection was completed. These measurements were compared to those made of the twenty-five lines before selection. This comparison indicated that 52A, one of the origional twenty-five lines, may have been selected during this experiment.

Comparisons of a possible cytochrome P-450 produced by a previously selected line of Drosophila called D23, and the DNA which is probably responsible for the production of this P-450, with the microsomal proteins and total genomic DNA of the selected lines were made using the techniques of Western and Southern blotting. The results of these procedures suggested that the mechanism of resistance used by the selected lines was not the same mechanism of resistance used by the D23 line.

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# INTRODUCTION:

## SECTION 1: EVOLUTION OF INSECTICIDE RESISTANCE

Some 400 species of insects and mites are known to have become resistant to one or more insecticides in the last forty years, and the rate of evolution to insecticide resistance is increasing (Georghiou, 1983). Almost 20% of the world's annual crop production is lost to insects, and this number would be much higher if insecticides were not used (Ware, 1983). New insecticides are becoming increasingly harder to discover, develop, register and manufacture (Metcalf, 1980). Less than two new insecticides are being developed per year, and resistance is evolving at a much greater rate than this (Ware, 1983). It is hoped that studying the mechanisms for the evolution of resistance might provide information to help develop methods to retard or avoid this process.

## SECTION 2: FIELD AND LABORATORY SELECTION

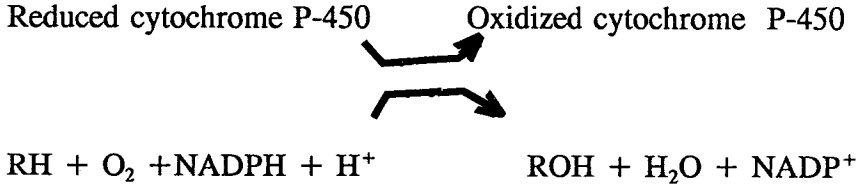
In many previous studies of the evolution of pesticide resistance carried out in the laboratory, selection for resistant insects resulted in the development of polygenically inherited resistance (Oppenoorth, 1984). In contrast, when resistant

insects were isolated from the field, monogenic resistance was selected (Roush and McKenzie, 1987). The different results may be explained as follows. In laboratory studies, the selecting doses of insecticide are chosen so as to allow some survival in each generation. This procedure favours the development of polygenic inheritance as many genes, each with small effects on resistance, accumulate with each generation. Rare alleles with high levels of resistance are not likely to be selected in the laboratory because sample sizes are not generally large enough to reliably detect resistance present at frequencies of less than ten percent (Roush and Miller, 1986). In field studies, the amount of insecticide used is meant to kill all individuals, and it is more likely that the insects that survive these conditions have rare resistance alleles. The mechanisms by which these alleles could lead to resistance may involve mutations in the target sites for the insecticide or in the insecticide metabolism within the insects (Roush and McKenzie, 1987). Although the populations selected for in laboratory studies may lack rare alleles with high levels of resistance, Houpt et al. (1988) suggested that the polygenic resistance genes found in laboratory selection can still be useful in determining the genetic basis of insecticide resistance, as these genes did originate in natural populations. One polygenic-controlled system in laboratory selected flies, which will be studied in this thesis, is that of cytochrome P-450.

## SECTION 3: CYTOCHROME P-450

Cytochrome P-450 is a component of the mixed function oxidase [MFO] system

involved in the detoxification of insecticides (Nerbert and Gonzalez, 1987). It is a carbon monoxide binding pigment present in the microsomes of insects (Hodgson, 1983). When this pigment is reduced and combined with carbon monoxide, its major absorption peak is approximately 450nm (Omura and Sato, 1964). The mono-oxygenase reaction of cytochrome P-450 occurs as follows (from Agosin, 1976):




where RH represents the substrate.

Cytochrome P-450 has a number of different functions in Drosophila melanogaster, such as BP hydroxylase, 7-EC deethylase, DMN demethylase and PNA demethylase activities (Hallstrom et al., 1983). Many different forms of cytochrome P-450 are known to exist. Three different forms of Drosophila cytochrome P-450, with molecular weights of 50,800, 51,750 and 54,800, were purified and characterized by Naquira et al. (1980). Hallstrom and Blanck (1985) used genetic mapping with marker strains to find a number of genes which control the activities of these cytochrome P-450 forms, and suggested that four or five genes regulate the cytochrome P-450 system in Drosophila.

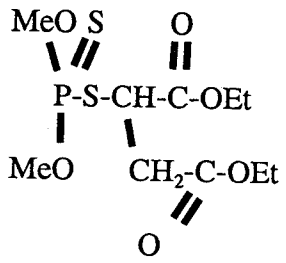
## SECTION 4: MIXED FUNCTION OXIDASE SYSTEM INVOLVED IN INSECTICIDE RESISTANCE IN Drosophila melanogaster

Mono-oxygenase systems, like the one dependant on cytochrome P-450, may be responsible for insecticide resistance of insects. Increases in oxidase activity and the amount of cytochrome P-450 may not be correlated, as no correlation between the two was found in insecticide resistant Musca domestica (Plapp and Casida, 1969). However, Morton and Holwerda (1985) found that a laboratory selected population of Drosophila melanogaster contained high levels of MFO activity, greater cytochrome P-450 content in microsomes, and increased capacity to oxidatively degrade malathion and malaoxon. Different forms of insect cytochrome P-450 exist, and are probably coded for by different genes (Hodgson, 1983). Levels of MFO activity found in insects may depend on variation in the cytochrome P-450 genes or genes which regulate these P-450 genes. Alterations in MFO activity may be caused by mutations in genes involved in related pathways, such as those controlling glutathion-6-phosphate dehydrogenase [G6PD] activity (Houpt et al., 1988). Morton and Holwerda (1985) found G6PD activity increased with insecticide resistance.

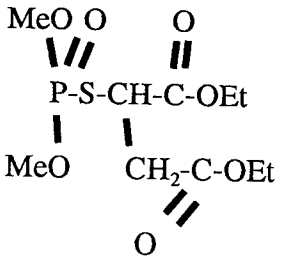
## SECTION 5: MIXED FUNCTION OXIDASE DETOXIFICATION OF MALATHION AND MALAOXON

A commonly used group of insecticides are organophosphorus compounds, which cause lethality in insects through the inhibition of acetylcholinesterase [AChE]. Malathion and malaoxon are two economically important organophosphorous insecticides used in this study. Their structures (Eto, 1979) are as follows:

Malathion




Malaoxon



Malaoxon is a much more potent inhibitor of cholinesterase than malathion (O'Brien, 1957). Both these insecticides can be degraded by mixed function oxidases. MFO acts by catalyzing an NADPH-dependant oxidation of malathion to malaoxon (Eto, 1979). Morton and Holwerda (1985) suggested that further NADPH-dependant oxidation of malaoxon can then occur, resulting in the formation of a major water soluble product, malaoxon B-monoacid, which is not toxic. G6PD is part of the oxidative pentose shunt pathway which increases NADPH levels in Drosophila melanogaster (Greer et al., 1979). High levels of G6PD lead to increased organophosphate resistance by increasing the rate of malaoxon detoxification (Morton and Holwerda, 1985).

## SECTION 6: MIXED FUNCTION OXIDASE GENES INVOLVED IN INSECTICIDE RESISTANCE

The number of genes involved in the malathion resistance of Drosophila melanogaster was studied by Halpern and Morton (1987) and Houpt et al. (1988). One major gene on chromosome II [2-64] and at least two genes on chromosome III [near 3-58] were all found to be involved in the control of increased MFO activity as well as malathion resistance. Microsomes isolated from strains carrying chromosome III resistance genes had more cytochrome P-450 and increased amounts of two heme staining protein bands [50kD and 54kD on SDS gels] than comparable susceptible

strains. Houpt et al. (1988) suggested that these latter two bands represent the polypeptide products of genes responsible for insecticide resistance. It is difficult to determine if this resistance is the result of altered levels of enzymes already present in the organisms studied, or a result of structural changes in these enzymes. This determination is made more difficult in the case of cytochrome P-450 as there are a variety of different enzymes present which cannot be distinguished by their substrate spectra (Taylor, 1986).

## SECTION 7: ACETYLCHOLINESTERASE ACTIVITY ASSOCIATED WITH INSECTICIDE RESISTANCE

In a laboratory experiment selecting for malathion resistance in Drosophila melanogaster, Morton and Singh (1982) found that increased resistance correlated with a decrease in AChE activity, probably caused by an altered AChE. AChE is a target molecule for organophosphorous insecticides (O'Brien, 1976). In the cholinergic synapses, acetylcholine, produced by the cholinergic nerve when the nerve fires its impulse to the next cell, must be subsequently broken down by AChE. Organophosphorous insecticides inhibit AChE activity by competing with acetylcholine for binding sites on AChE and, once bound, dissociate slowly (Augustinsson and

Nachmansohn, 1949). The organophosphorous insecticides [AB] react with AChE [EOH] according to the following mechanism:

$$EOH + AB \rightleftharpoons EOH\text{-}AB \rightarrow EOA \rightarrow EOH + AOH$$

where A represents the phosphorylating group, and B the leaving group, of an organophosphorous insecticide. The organophosphorous insecticide forms a complex with the AChE, and when a leaving group and a hydrogen are eliminated [BH], an AChE with a phosphorylated serine [EOA] remains (Corbett et al., 1984). Although the phosphorylated AChE is usually hydrolyzed back to its original form [EOH], this reaction is slow, preventing the AChE from efficiently breaking down acetylcholine which has been released into the synapse during the normal nervous activity of the insect (Reiner, 1971). This causes a build up of acetylcholine, and the fly suffers from hyperactivity followed by paralysis and death.

Insects can aquire resistance to organophosphorous insecticides through the evolution of altered AChE (Plapp, 1976). Resistance to insecticides through modified AChE was first noted by Smissaert (1964), who found that the red spider mite Tetranuchus urticae had decreased sensitivity to organophosphorous insecticides due to altered AChE. These resistant AChE were deficient in their ability to hydrolyze acetylcholine. Resistant strains of certain species of insects, such as Musca domestica, have been shown to carry different altered AChEs with differing sensitivities to

organophorous insecticides. Plapp (1976) suggested that resistance in insects may occur through the evolution of altered AChEs which have an altered active site to which acetylcholine, but not organophosphorous insecticides, can bind. Morton and Singh (1982) suggested that altered AChE may be a component in the malathion resistance found in Drosophila melanogaster.

## SECTION 8: MANY DIFFERENT GENES ARE INVOLVED IN INSECTICIDE RESISTANCE

Singh and Morton (1981) carried out a selection experiment for resistance to malathion in Drosophila melanogaster. A population made up of flies from forty iso-female lines collected from the Hamilton, Ontario area were used in this experiment. They found that the resistance in the selected lines was polygenic, as none of the initial iso-female lines used were as resistant as the selected lines. Morton and Singh (1982) found that while a decrease in AChE activity was correlated with the increase in the resistance of selected lines, the level of AChE activity stabilized while resistance continued to increase with selection. This result suggested that more genes than those resposible for AChE were involved in resistance (Morton and Singh, 1982). The correlation of resistance with increased MFO activity, caused by a number of genes, further comfirmed this (Morton and Holwerda, 1985). Morton and Singh (1982) concluded that genes at the structural Ace locus [3-52] are involved in the

aquisition of malathion resistance in Drosophila melanogaster.

## SECTION 9: PURPOSE OF THIS THESIS

Since the initial population used by Singh and Morton (1981) to select for malathion resistance was not characterized, no comparison of the properties of the resistant flies and the original unselected flies could be done. Such a comparison may have revealed the importance of variants already present in the Drosophila melanogaster population relative to new mutations in the evolution of increased insecticide resistance. A new selection experiment was performed in which the initial population was more thoroughly characterized and homozygous for chromosome III, as this chromosome was thought to carry genes involved in insecticide resistance. Twenty-five iso-chromosomal III lines of Drosophila, recieved from Dr. C. Aquadro, were characterized and then selected for resistance to malaoxon. After twelve generations of selection, the MFO and AChE activities and the insecticide resistance of the selected population, which was made homozygous for chromosome III, were compared with those of the initial population. These measurements were also made during the twelve generations of selection. These comparisons should help reveal the role of variants present in the initial population of Drosophila in the evolution of insecticide resistance.

# MATERIALS AND METHODS:

## SECTION 1: STRAINS OF FLIES USED

All twenty-five lines of iso-chromosomal III Drosphila melanogaster used in the selection experiment were obtained from Dr. C. Aquadro of Cornell University, New York, and were collected from the region of North Carolina. Wild type flies, Canton Special [CS], which are susceptible to organophosphates, and the third chromosome balancer strain, MKRS/TM3, were obtained from the Mid-American Stock Center, Bowling Green, Ohio. Balancer mutations were described by Lindsley and Grell (1969). Organophosphate resistant flies, D23, were obtained from a selection experiment done by Singh and Morton (1981), in which iso-female flies collected from the Hamilton, Ontario area were selected for malathion resistance over 110 generations. The D23 strain was then made homozygous for the third chromosome.

## SECTION 2: CHEMICALS USED

Malathion [96% pure] was a gift from the American Cyanamid Company. All

other chemicals used were purchased from Boehringer-Mannheim Canada or Sigma Chemical Company, unless otherwise stated.

## SECTION 3: MAINTENANCE OF FLY CULTURES

The Drosphila melanogaster strains used in the selection experiment were grown at 24°C with a cycle of a twelve hour day and twelve hour night. The flies were fed banana medium [12g of agar, 10g of malt powder, 20g of sugar, 40g of yeast, 1 banana, 25ml of tegosept and 1.2 litres of $H_2O$] in 80ml plastic vials or 350ml glass bottles. Before selection in population cages, flies were fed a mixture of sugar, yeast and water put on Kimwipe tissues in a plastic dish.

## SECTION 4: SIGNIFICANCE TESTS

The student t-test was used to find if the difference between the means of two independant samples was significant. The t value was calculated using the following formula (from Norman and Streiner, 1986):

t = difference between means/standard error of difference

The paired t-test was used to find if the difference between the means of two paired observations of matched individuals was significant. The paired t value was calculated using the following formula (from Norman and Streiner, 1986):

t = mean difference/standard error of differences

The t values obtained from the student t-tests and paired t-tests were examined on a table of the one-tailed t distribution, and were considered significant if the probability [p] was less than 0.02.

The t-ratio was used to find if there was significant correlation between two variables. The t-ratio was calculated using the following formula (Loftus and Loftus, 1982):

$$t = r \sqrt{\frac{N-2}{1-r^2}}$$

The correlation coefficient is represented by r, and N represents the number of samples. The t-ratio was examined on a table of the two-tailed t distribution, and was considered significant if the probability [p] was less than 0.05.

## SECTION 5: SELECTION EXPERIMENT

The twenty-five lines of iso-chromosomal III flies obtained from Dr. C. Aquadro were selected for insecticide resistance using the following procedure. Approximately twenty-five flies from each of the twenty-five lines were put in a plastic population cage [18"X12"X12"], allowed to mate, and their eggs were removed in eight glass bottles containing banana medium. The offspring of this original mating were considered generation zero [G=0]. These flies were allowed to mate in a population cage, and the bottles containing their offspring were grown in banana medium in four sets of glass bottles. One of these sets was made up of six bottles which had 1ml of 500uM malaoxon added to them when the flies reached the larval third instar stage of growth. Flies that emerged from these bottles were considered G=1 selected flies. Another two sets of bottles were made up of six bottles of flies selected with 125uM and 250uM malaoxon, and used as backups in case selection with 500uM malaoxon resulted in no survivors. The last set of bottles was made up of twelve bottles to which no malaoxon was added, and the flies that emerged from these bottles were considered G=1 control flies. This selection procedure was repeated each generation until the flies were G=12, with only the malaoxon concentration used being altered. In G=5 the malaoxon concentration used was raised to 1,000uM, and it was increased to 2,000uM in G=9. Flies in G=12 were selected on 4,000uM malaoxon. Egg survival, adult survival, AChE activity and MFO activity of flies in G=0, G=3, G=6, G=9, and G=12 were measured. To obtain enough flies to make accurate

measurements and to remove the immediate effects of the insecticide, the flies were grown for another generation without any selection before the measurements were made.

## SECTION 6: EXTRACTION OF THIRD CHROMOSOME

The third chromosomes of Drosophila in G=12 were extracted after the selection experiment was completed as this chromosome carries genes known to be involved in insecticide resistance. This procedure is summarized in Figure 1. Virgin G=12 females from both control and selected lines were mated with males from strains which carry third chromosome balancing mutations, MKRS and TM3. Virgin female offspring of this cross which carry TM3 were then crossed with MKRS/TM3 males. TM3 male and virgin female offspring from this cross were mated. The virgin progeny of this cross, which had wild type appearance, were mated to each other, resulting in an iso-chromosomal III line.

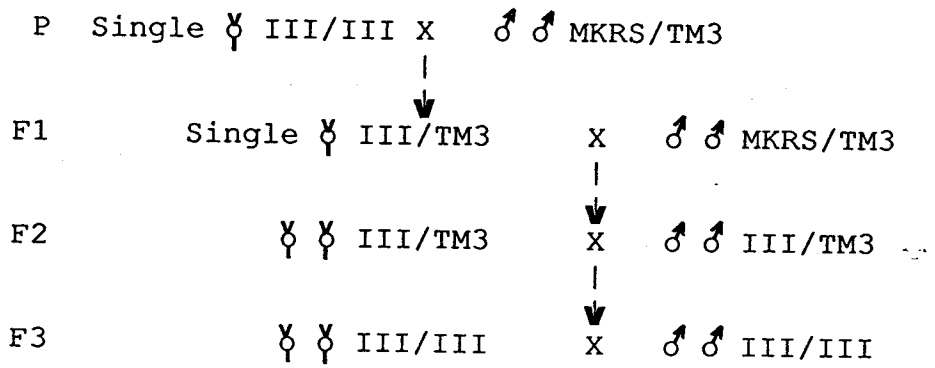
## SECTION 7: MEASUREMENT OF EGG SURVIVAL ON MALATHION

The procedure used for measuring insecticide resistance in Drosophila by finding the percentage egg to pupa survival on malathion was described by Houpt et

FIGURE 1

METHOD USED TO EXTRACT THE THIRD CHROMOSOME

MKRS/TM3 is a third chromosome balancer strain. The parental vigin females were taken from either control or selected populations.



al. (1988). Vials of banana medium, made one day earlier and containing the desired concentrations of malathion, were added to population cages containing flies and removed two to six hours later. The eggs were then counted, and after most of the flies had emerged, the number of pupa cases on the walls of the vials were counted as survivors. The number of survivors divided by the number of eggs gave the percentage of flies which survive on various malathion concentrations. This percentage may not actually represent the number of eggs that survive on the insectide, as Singh and Morton (1981) suggested that the eggs were resistant to malathion due to the impermeability of the egg membrane. What was actually measured with this assay was the number of larvae which fed on the poisoned medium and were able to survive to reach the pupa stage.

## SECTION 8: MEASUREMENT OF ADULT SURVIVAL ON MALATHION

The procedure used for measuring insecticide resistance in adult Drosophila was described by Houpt et al. (1988). Flies which were three to ten days old, grown on banana medium, were added to agar-sucrose medium containing different malathion concentrations. Agar-sucrose medium was a mixture of 1.5% agar and 1% sucrose. Both solutions were stirred, and solution A was heated, until dissolved. The solutions were then autoclaved for fifteen minutes and allowed to cool to 55°C, when they were

mixed together. When the temperature reached 50°C, the desired amount of malathion in 2-propanol was added to a vial containing 10ml of the agar sucrose medium. Groups of 10-100 flies, which were three to ten days old, were added to these vials. After twenty-four hours the number of flies that were still alive was recorded, and the percentage of flies which survived on various malathion concentrations computed.

## SECTION 9: MEASUREMENT OF PROTEIN CONCENTRATIONS

The Bradford and the Lowry assays were used to measure the concentrations of protein in samples.

In the Bradford assay, described by Bradford (1976), various amounts of fly extract supernatant were added to 3ml of 0.06% Coomassie brilliant blue stain in 1.9% perchloric acid and then vortexed. Absorbance measurements were made on a Horizon digital colorimeter at wavelength 610nm, as the binding of protein to Coomassie brilliant blue dye in this acidic solution caused the wavelength of the dye to shift from 465nm to 610nm. TKEG was used as a blank for these measurements. The protein concentrations of the samples were found on a standard calibration curve made using different concentrations of the bovine serum albumin.

The Lowry assay for protein concentration was used when isolating CHAPS solubilized microsomes, as CHAPS detergent interferes with the Bradford assay. In the

Lowry assay, described by Lowry et al. (1951), various amounts of protein were added to 0.5ml of $H_2O$ followed by the addition of 2.5ml of a solution containing a mixture of 49ml of 2% anhydrous $Na_2CO_3$ with 1ml of 0.5% $CuSO_4 \cdot 5H_2O$ and 1.2% $Na_3$ citrate. This was vortexed for ten seconds, and then allowed to sit at room temperature for 10 minutes. A volume of 0.25ml of 50% Folin phenol reagent was added, followed by vortexing to allow tyrosine and tryptophan residues in the protein to reduce the Folin phenol reagent. The mixture was then allowed to sit for sixty minutes. The reactions of protein mentioned above resulted in the solution becoming an intense blue colour. A Horizon digital colorimeter was used to measure the absorbance of the protein at wavelength 660nm. The measurements obtained were compared to values from a standard calibration curve made with known concentrations of bovine serum albumin.

## SECTION 10: MEASUREMENT OF MIXED FUNCTION OXIDASE ACTIVITY

MFO activities of Drosophila were measured by finding the rate of de-alkylation of 7-ethoxycoumarin to produce 7-hydroxycoumarin as described by Houpt et al. (1988). A group of fifteen male flies from each series to be measured were mixed with 350ul of extraction buffer [50mM Tris, 50uM KCl, 1uM sodium EDTA, 10% glycerol and 0.5 mg/ml of ovalbumin]. This mixture was then homogenized at room temperature and centrifuged in an Eppendorf centrifuge for 10 minutes at 4°C. To start

the de-alkylation reaction, 40uM of the supernatant was added to test tubes containing 90ul of freshly made assay mixture kept at 30°C in a water bath. The assay mixture consists of 25ul dissolved 7-ethoxycoumarin, 2.5ml assay solution [0.5uM sodium NADP, 2.5uM sodium glucose-6-phosphate and 10ml of extraction buffer] and 25ul G-6-PD enzyme. The reaction that occured was the de-ethylation of 7-ethoxycoumarin by the supernatant to produce 7-hydroxycoumarin. One tube in each series was made into a standard by adding 10ul of 7-hydroxycoumarin. The reaction was stopped at zero minutes for the blank and the standard, and at ten minute intervals up to thirty minutes for the other samples. To stop the reaction at certain times, 20ul of 2N HCl was added to the tube containing the sample, followed by 90ul of chloroform to extract the 7-hydroxycoumarin product. The tube was then vortexed, and then 3ml of 0.5M Tris was added to convert the 7-hydroxycoumarin to hydroxylate ion, which has greater florescence. The tubes were vortexed and then spun at 1,900rpm on the clinical centrifuge for two minutes. The measurement of the amount of 7-hydroxycoumarin product present was made on a fluorimeter [built by Rob Gilles, McMaster department of biology]. Excitation occured at a wavelength of 380nm [0.5nm band width]. The fluorescence of the product through a 400nm cut off blue filter was compared to that of a known amount of 7-hydroxycoumarin [247pmoles] in reaction mixture, which was used as the standard. The specific activities were then calculated as pmole product per second divided by ug protein, which was found using the Bradford assay.

## SECTION 11: MEASUREMENT OF ACETYCHOLINESTERASE ACTIVITY

Acetylcholinesterase activities in Drosophila were assayed using the technique described by Ellman et al. (1961), and modified by Morton et al. (1982). A group of ten fly heads of either sex were mixed with 100ul of extraction buffer [0.02M Tris-HCl with 0.5% Triton X-100, pH 7.4] and homogenized at room temperature. After an hour at room temperature, the samples were spun in a Eppendorf centrifuge at 2,000g for ten minutes. A volume of 10ul of supernatant was mixed with 1.0ml of 0.1M $KPO_4$ buffer [pH 8], 0.1ml of DTNB solution [39.6mg DTNB {5,5'-dithiobis-2-nitrobenzoic acid}, 15mg of $NaHCO_3$ and 10ml of 0.1 $KPO_4$ buffer, pH 7] and 20ul of ATC [21.7mg of acetylcholine iodide in 10ml of 0.1 $KPO_4$ buffer, pH 8] in a glass cuvet. The sample was then placed in the spectrophotometer, which was set to measure absorbance at 412nm and 25°C. A measurement of the rate of increase in absorbance over a five minute period was taken for all samples as well as for 10ul extraction buffer, which served as a background. The AChE activity was calculated using the following formula:

$$\text{AChE activity} = 1.225[V_t]A/[V_e]t$$

where A is the change in absorbance at wavelength 412nm, $V_t$ is the total volume used [ie. 1.13ml], $V_e$ is the volume of enzyme used [ie. 0.01ml] and t is the time in minutes. The specific AChE activity [units: nmoles product $s^{-1}$ $mg^{-1}$] was calculated

by dividing the AChE activity by the amount of protein in mg added to the cuvet.

## SECTION 12: MICROSOME PREPARATION BY SUCROSE GRADIENT CENTRIFUGATION

The procedure used to isolate the microsomes of Drosophila from a small number of flies has been described by Feyereisen et al. (1985). A sucrose gradient was made in a 5ml Beckman Quick-Seal tube, which contained 0.8ml 50% sucrose in 1XTKEN [diluted from a 2X stock solution of TKEN, which consisted of of 50uM Tris, 50uM KCL, 1uM EDTA and 10uM nicotinamide, pH 7.4], 1.6ml 35% sucrose in TKEN, and 2.5ml 25% sucrose in TKEN. On top of this gradient, 0.35ml of homogenate [supernatant of twenty flies homogenized in 0.5ml 10% sucrose in TKEN and then centrifuged at 2,000g on an Eppendorf centrifuge for ten minutes] was added. The tubes were then heat sealed and centrifuged on a VTi65.2 centrifuge at 50,000rpm for twenty minutes at 4°C. The lower 2ml were discarded, and the next 0.5ml which contained the microsome fraction was stored at -70°C (Patil et al. (1990). The concentration of protein in this fraction was found using a Lowry assay.

## SECTION 13: CHAPS-SOLUBILIZED MICROSOME PREPARATION

The procedure used to isolate microsomes of Drosophila from a large number of flies has been described by Houpt et al. (1988). This procedure solubilized the microsomes in CHAPS detergent, which separates the microsomal cytochrome P-450 in its monomeric form without denaturing it. Flies were homogenized in 6ml TKEG [0.05M Tris at pH 7.4, 0.05M KCl, 0.001M EDTA, 10% glycerol] per gram of flies and then centrifuged twice at 5,000rpm for five minutes at 4°C on the SW34 rotor in the Sorvall RC-2 centrifuge. The supernatant was then centrifuged twice at 12,000rpm for twenty minutes to remove the mitochondria. The supernatant of this spin was centrifuged on the SW50.1 rotor at 34,000rpm for thirty minutes to precipitate the microsomes. The supernatant was discarded and the microsomal pellet was resuspended in approximately 1ml of microsome phosphate buffer [100mM sodium phosphate, 20% glycerol, 1mM EDTA, and 0.1mM DTT at pH 7.25] and solubilized with 0.025ml of 20% CHAPS for every 2.5g of flies. After spinning this mixture on the SW50.1 rotor at 34,000rpm for forty-five minutes, the solubilizied microsomes were recovered in the supernatant. These microsome preparations were stored at -70°C for up to three months before analysis on SDS polyacrylamide gels. The concentration of protein in this microsome preparation was found using the Lowry assay, as CHAPS interferes with the Bradford assay.

## SECTION 14: PREPARATION OF CRUDE FLY EXTRACT FOR WESTERN BLOTS

In order to prepare crude fly extracts for Western blots, fifteen male flies, between two and six days of age, were homogenized in 75ul TKEG [pH 8.6] with 0.5% CHAPS in order to solubilize proteins. The extracts were then centrifuged for ten minutes at 2,000g in an Eppendorf centrifuge at 4°C. An equal mixture of 10ul supernatant and 10ul 2X sample buffer [20ml glycerol, 40ml $H_2O$, 1.52g TRIS, 20ml 20% SDS, 10ml B-mercaptoethanol and 10mg bromophenol blue]. After three minutes of boiling, these samples were loaded onto a SDS polyacrylamide gel for electrophoresis.

## SECTION 15: CYTOCHROME P-450 DIFFERENCE SPECTRA

The concentration of cytochrome P-450 in the microsomes of Drosophila was found by diluting CHAPS-solubilized microsome preparations in an equal volume of spectra buffer [300mM $KPO_4$ and 50% glycerol, pH 7.8]. The absorbance of samples were measured from wavelengths 400nm to 500nm on a Beckman DU 7 spectrophotometer. The spectrophotometer was blanked using the sample reduced with sodium dithionate. Carbon monoxide was bubbled through the sample for one minute, and the differential absorbance was recorded. The cytochrome P-450 content was

found using the Beer-Lambert law, which states that c=A/El. The cytochrome P-450 content, represented by c, is equal to the difference in absorbance between 450nm and 490nm [represented by A] divided by the path length [1cm, represented by l] and the extinction coefficient [91$mM^{-1}$ $cm^{-1}$, represented by E] (Omura and Sato, 1964).

## SECTION 16: ONE DIMENSIONAL SDS-PAGE

The procedure for analyzing proteins of Drosophila by one dimensional SDS polyacrylamide gel electrophoresis was described by Laemmli (1970) and modified by Coulthart (1986). SDS polyacrylamide gels [15X12cm, 0.15cm thick] were used, and contained 10% polyacrylamide resolution gel and a 4.75% polyacrylamide stacking gel. The protein standard used contained bovine erythrocyte carbonic anhydrase [30kD], pig heart fumarase [48kD], beef liver catalase [58kD], and bovine serum albumin [68kD]. After electrophoresis of the proteins on the gel for twelve to eighteen hours at 75 volts, the proteins were visualized by silver staining the gel with 0.1% $AgNO_3$ using the technique described by Morrissey (1981).

## SECTION 17: WESTERN BLOTTING

The technique of Western blotting was used to identify specific Drosophila

proteins which reacted with the antiserum directed towards Drosophila P-450. This procedure was described by a protocol in the Bio-Rad Alkaline Phosphatase Conjugate Immuno Blot Assay Kit. Following electrophoresis of proteins on an SDS polyacrylamide gel the gel was equilibrated in 500ml of Tris/glycine buffer. In order to transfer proteins to nitrocellulose the gel was placed on a piece of nitrocellulose paper, and together these were placed between two pieces of filter paper and two sponges in Bio-Rad transblot apparatus saturated in transfer buffer [25mM Tris, 60mM glycine, 20% methanol]. The apparatus was closed and placed in a transblot tank containing 3L transfer buffer. A current of 0.25 amps was passed through the gel side to the nitrocellulose side of the apparatus for four hours. The buffer in the tank was stirred and kept at 4°C. The nitrocellulose was then removed from the apparatus and blocked in 500ml of blotto [5% skim milk powder in $H_2O$] for half an hour and then washed in $H_2O$.

Primary antibody used in the Western blot procedure was a gift of J. C. Pursey, and had been prepared as follows (Pursey, 1989). A purified microsomal sample from resistant D23 flies was injected into a rabbit, and the rabbit was boosted by repeating the injection four weeks later. Blood was collected from the rabbit. The IgG fraction of the blood was column purified and D23 specific antibody was purified by absorption to and elution from CS microsomal proteins covalently attached to a sepharose column. The D23 specific antibody was collected and called Ab2-IgG.

The nitrocellulose paper containing absorbed proteins was immersed in a 1/1000 dilution of Ab2-IgG antibody in 0.05% Tween 20 in PBS and shaken for two hours at room temperature. The paper was then washed three separate times on the shaker for five minutes with 5ml aliquots of 0.05% Tween 20 in PBS. For the next two hours the paper was incubated at room temperature with a 1/5000 dilution of secondary antibody [goat anti-rabbit antibody conjugated to alkaline phosphatase from Inter Medico] in 0.05% Tween 20 in PBS. The washing described above was then repeated. A volume of 5ml of NBT/BCIP substrate [0.02ml 2M $MgCl_2$, 1ml NBT made up of 1mg/ml NBT in 0.1M Tris-HCl at pH 9.2, 0.1ml BCIP made up of 5mg/ml BCIP in DMF, and 9ml of 0.1M Tris-HCl at pH 9.2] was added to the paper and incubated at 37°C until bands were visible on the nitrocellulose paper. The nitrocellulose paper was then left at room temperature to dry.

## SECTION 18: ANTIGENIC RESPONSE

Quantation of the stain in bands obtained from Western hybridization were found using a LKB 2222-020 laser densitometer. The reflectance peak of each band was detected by the densitometer, and the area under these bands was determined. This area provided a measurement of the relative antigenic response.

## SECTION 19: DNA EXTRACTION FOR SOUTHERN BLOTS

The procedure used to extract total Drosophila genomic DNA for examination with the Southern blotting technique was described by Jowett (1986) and modified by Morton (private communication). All solutions and equipment used in preparing DNA extractions and Southern blots were sterilized by autoclaving. Approximately 100 flies were knocked out on ice, and then homogenized in 2ml of 0.1M fly DNA buffer (0.1M NaCl, 0.2M Tris HCl, 0.05M EDTA and 0.5% SDS at pH 9.1) using glass homogenizers. After thirty minutes of incubation in a water bath at 65°C, 0.3ml of 8M K-Acetate was added and the homogenate incubated on ice for thirty minutes. This solution was then centrifuged on the Sorvall RC-2 centrifuge for ten minutes at 10,000g. The supernatant was mixed with an equal volume of EtOH and incubated for five minutes at room temperature before being centrifuged for another five minutes at 10,000g. The pellet was washed in 80% EtOH and left to dry. When completely dry, the pellet was resuspended in 200-400ul of TE buffer.

The pellet resuspended in TE buffer was treated with RNase (10ug/ml) to digest the RNA to oligonucleotides. Samples were incubated at room temperature for thirty minutes before being mixed with an equal volume of phenol. After spinning for five minutes in an Eppindorf centrifuge at 4°C, the supernatant was removed and shaken with an equal volume of a 24:1 dilution of chloroform was added to remove the remaining phenol. This solution was then spun at 4°C in an Eppindorf centrifuge for

two minutes, and the superatant was removed and mixed with one half volume of 7.5M ammonia acetate and three times the initial volume of absolute EtOH to precipitate the DNA. This mixture was kept at -70°C for at least one hour before being spun at 4°C in an Eppindorf centrifuge for ten minutes. The pellet was then washed in 70% EtOH, air dried and resuspended in TE buffer.

## SECTION 20: AGAROSE GEL ELECTROPHORESIS OF Drosophila DNA

Drosophila genomic DNA was analyzed on agarose gels prepared as described by Maniatis et al. (1982). A 0.5% solution of SeaKem HEEO agarose in 1XTBE buffer was boiled in a microwave oven. When it had cooled for a few minutes, the agarose was poured into a gel box. After the agarose solidified, the barriers around the gel were removed. 1XTBE was added to cover the gel to a depth of approximately 1mm and the comb was removed. Ten to fifteen ug of DNA was mixed with 5ul loading buffer [5-10% glycerol, 7% sucrose and 0.025% bromophenol blue] and heated at 65°C for ten minutes prior to loading into slots on the gel with a micropipettor. The DNA was separated on the gel by electrophoresis at 40-50 volts until the marker bands were approximately three quarters of the way down the gel. The gel was stained with EtBr to allow visualization of DNA bands on the gel under UV light. The gel was stained by immersing in a solution containing 0.5ug/ml of EtBr. After fifteen minutes of staining, the gel was briefly washed in $H_2O$ and photographed under a UV lamp.

## SECTION 21: TRANSFER OF DNA FROM AGAROSE GELS TO NITROCELLULOSE PAPER

The procedures for the transfer of DNA from agarose gels to nitrocellulose paper were described by Maniatis et al. (1982). After electrophoresis was completed, the gel was transferred to a glass baking dish and unused areas of the gel were trimmed away with a razor blade. The DNA on the gel was then denatured by shaking in a solution of 1.5M NaCl and 0.5M NaOH for one hour at room temperature. The gel was neutralized by shaking in a solution of 1M Tris-Cl [pH 8] and 1.5M NaCl for one hour at room temperature. A peice of Whatman 3MM paper, larger than the gel, was placed over a glass plate inside a glass baking dish. A solution of 10XSSC was added to the dish until it reached the top of the glass plate, and air bubbles in the 3MM paper were smoothed out with a pasteur pipette. The inverted gel was then placed on top of the filter paper, and the air bubbles smoothed out. A piece of nitrocellulose which was 1-2mm larger than the gel was placed on top of the gel after being saturated in 2XSSC. Then two pieces of Whatman 3MM paper the same size of the gel were placed on top of the nitrocellulose filter. A stack of paper towels, which had been cut to be just smaller than the gel, was placed over the Whatman 3MM paper. A glass plate with a 500g weight was placed on top of the paper towels to weigh them down. The gel was surrounded by Saran wrap to prevent short circuiting of fluid into the paper towels. Transfer proceeded for twelve to twenty-four hours, followed by the marking

of the lanes on the nitrocellulose paper with pencil. The gel was then discarded. After soaking for five minutes in 6XSSC at room temperature, the nitrocellulose paper was allowed to dry and then baked for two hours at 80°C.

## SECTION 22: PROBE USED FOR SOUTHERN HYBRIDIZATION

Probes used to study specific Drosophila DNA fragments using the technique of Southern hybridization were obtained from Jane Pursey. A lambda gt11 cDNA expression library made by Pursey (1989) contained cDNA fragments of a Drosophila lambda gt10 library in lambda gt11 arms. Three gt11 clones which reacted strongly with Ab2-IgG antibody were isolated. One of these clones produced a 16kd peptide fragment that was part of a 130kd fusion protein. A 1.5kb Kpn1/EcoR1 restriction fragment, which was made up of 1kb gt11 DNA and 0.5kb Drosophila cDNA, was taken from this clone and subcloned in pUC119, and was used in total Drosophila genomic DNA analysis. When total Drosophila genomic DNA was examined using the Southern hybridization technique with this insert as a probe, Pursey (1989) found polymorphism between the resistant D23 strain and the sensitive CS strain. When total D23 and CS genomic DNA was digested with EcoR1, each strain had a different band marked with the probe. D23 had a restriction fragment which was smaller than the CS restriction fragment labelled by the probe.

## SECTION 23: OLIGOLABELLING PROBES

Radiolabelled probe DNA was prepared by using the protocol of the Pharmacia oligolabelling kit. The Kpn1/EcoR1 restriction fragment described above was heat denatured for fifteen minutes at 90°C. The denatured DNA was then transferred to a 37°C water bath for five minutes. The DNA was mixed with 29ul $H_2O$, 2ul of dNTPs [25ug/ml], 10ul hexanucleotide oligomers, 2ul BSA, 5ul $^{32}P$ dCTP [3000Ci/mmole] and 2ul of Klenow polymerase. This mixture was incubated for two hours at room temperature before the reaction was terminated with 20ul of stop buffer in 180ul of $H_2O$.

The unincorporated $^{32}P$ dCTP was removed using the techniques described by Maniatis et al. (1982). A column of Sephadex G-50 equilibrated with TE (pH 8.0), and containing 0.1M NaCl (STE), was made up in a 1ml disposable syringe plugged with sterile glass wool. The syringe was inserted into a glass centrifuge tube and centrifuged at 1600g for 4 minutes. Sephadex G-50 was added to a volume of 0.9ml. A volume of 0.1ml STE was added and centrifugation carried out for four minutes at 1600g. This last step was then repeated. Using STE to make up the volume, 0.1ml of the sample DNA was then added to the column. An Eppendorf tube was used to collect 100ul of solution containing DNA labelled with $^{3}2P$ dCTP by centrifuging the Sephadex column for four minutes at 1600g.

## SECTION 24: HYBRIDIZATION OF SOUTHERN FILTERS

The procedures used to identify specific Drosophila DNA fragments were described by Maniatis et al. (1982). After baking, the nitrocellulose paper was saturated with 6XSSC, and moved with forceps to the bottom corner of a heat-sealable plastic bag. For each square centimeter of nitrocellulose filter, 0.2ml of prehybridization fluid [6XSSC, 0.5% SDS, 5XDenhardts solution and 100ug/ml denatured salmon sperm DNA in boiling $H_2O$ was added to the plastic bag. Air bubbles were pushed from the bag, and the bag was then sealed and incubated in a 68°C water bath for two to four hours. The prehybridization fluid was then removed from a cut corner of the bag, and hybridization fluid [6XSSC, 0.01M EDTA, 32P-labelled denatured DNA probe, 5XDenhardts solution, 0.5% SDS and 100ug/ml of denatured salmon sperm DNA at 68°C] was added. Air bubbles were pushed from the bag, which was then sealed and incubated in a 68°C water bath for approximately twenty-four hours.

The bag was removed from the water bath after the required hybridization period, and the nitrocellulose paper was removed and placed in 2XSSC and 0.5%SDS at room temperature for five minutes. The nitrocellulose paper was then submerged in a solution of 2XSSC and 0.5% SDS. After fifteen minutes the nitrocellulose paper was transferred to a solution of 0.1XSSC and 0.5% SDS where it was submerged for two hours at 68°C. The buffer was replaced, and incubation continued for thirty

minutes. The nitrocellulose paper was then dried on Whatman 3MM filter paper for autoradiography.

## SECTION 25: AUTORADIOGRAPHY

Specific fragments of Drosophila DNA identified by hybridization to an oligolabelled probe were visualized using the technique of autoradiography described by Maniatis et al. (1982). The nitrocellulose paper was placed under a plastic sheet in a metal film cassette. XAR Kodak diagnostic film [20.3X25.4cm] was then placed on top of the plastic sheet in a dark room. The cassette was closed and stored in the -70°C freezer for up to a week and then the film was developed.

## SECTION 26: PHOTOGRAPHY

Photographs of gels and blots were taken with 100 ASA black and white film. The composite was studied, and the most acceptable picture was blown up into a 5"X7" glossy print.

RESULTS:

SECTION 1: DESCRIPTION OF THE POPULATION OF Drosophila melanogaster USED IN THE SELECTION EXPERIMENT

E. C. Morton characterized a population of twenty-five iso-chromosomal III lines of Drosophila melanogaster, obtained from Dr. C. Aquadro of Cornell University, which were to be selected for resistance to malaoxon. The first step in characterization was the testing of the population for resistance to the insecticide malathion by making crude estimates of the LC-50. The LC-50 is the concentration of malathion in agar-sucrose medium which results in 50% survival of fifteen to thirty adult male and female flies after twenty-four hours. The lines were grouped into three different catagories of LC-50, class 1 [<5uM malathion], class 2 [5-9uM malathion] and class 3 [>9uM malathion]. There were seven lines in class 1, seven lines in class 2 and eleven lines in class 3. The specific activities of mixed function oxidase [MFO], acetylcholinesterase [AChE] and glutathion-S-transferase [GST] were measured for these flies [Table 1, data collected by E. C. Morton]. Class 3 had a mean MFO specific activity of 0.820 which was significantly higher than class 1 [student t-test, $t=14.12$, $p<0.001$, $df=18$] and class 2 [student t-test, $t=7.29$, $p<0.001$, $df=18$] which had mean specific activities of 0.620 and 0.610 respectively. There was no significant difference between the specific activities of class 1 and class 2 [student t-test, $t=0.42$, $p>0.25$, $df=12$]. This data indicated that MFO activity increased with increased

TABLE 1

ENZYME ACTIVITIES OF ISO-CHROMOSOMAL III STRAINS

| Line | Malathion Resist. LC50 Class | AChE Activity | MFO Activity | GST Activity |
|---|---|---|---|---|
| 18A | 1 | 7.2 | 0.620 | 5.3 |
| 54A | 1 | 9.4 | 0.520 | 4.8 |
| 109A | 1 | 12.4 | 0.680 | 4.5 |
| 130A | 1 | 10.9 | 0.760 | 4.7 |
| 130B | 1 | 9.6 | 0.950 | 4.9 |
| 135B | 1 | 11.4 | 0.210 | 4.4 |
| 228B | 1 | 9.3 | 0.580 | 3.7 |
| Mean Act. (Std. Error) | | 10.0 (0.6) | 0.62 (.09) | 4.7 (0.3) |
| 106A | 2 | 10.6 | 1.140 | 4.3 |
| 149A | 2 | 16.1 | 0.370 | 4.0 |
| 248A | 2 | 13.6 | 0.340 | 3.7 |
| 11B | 2 | 5.4 | 0.760 | 4.1 |
| 90B | 2 | 10.8 | 0.720 | 4.8 |
| 97B | 2 | 10.6 | 0.580 | 4.7 |
| 246B | 2 | 7.5 | 0.340 | 4.9 |
| Mean Act. (Std. Error) | | 10.6 (1.3) | 0.61 (.11) | 4.4 (0.2) |
| 52A | 3 | 12.6 | 1.580 | 6.6 |
| 56A | 3 | 8.0 | 0.840 | 4.1 |
| 57A | 3 | 8.5 | 0.700 | 4.9 |
| 95A | 3 | 10.7 | 0.415 | 6.1 |
| 215A | 3 | 6.9 | 0.830 | 4.4 |
| 232A | 3 | 9.7 | 0.680 | 3.8 |
| 5B | 3 | 4.7 | 0.890 | 4.5 |
| 73B | 3 | 10.3 | 0.620 | 5.3 |
| 85B | 3 | 13.2 | 0.480 | 4.6 |
| 204B | 3 | 7.8 | 1.380 | 4.9 |
| 234B | 3 | 12.6 | 0.580 | 5.1 |
| Mean Act. (Std. Error) | | 9.5 (0.8) | 0.82 (.11) | 4.9 (0.2) |
| CS | 1 | 12.5 | 0.400 | NOT DONE |
| IIID | 3 | 5.3 | 1.200 | 5.4 |

resistance [Figure 2]. In contrast, the GST and AChE activities were not significantly correlated with resistance. A negative correlation was found between MFO and AChE activities [t-test, t-ratio=-1.22, correlation coefficient=0.025, $p<0.15$, df=23] [Figure 3], suggesting that AChE activity may decrease with increasing resistance. GST activity may increase with increasing resistance, as a weak positive correlation was found between GST and MFO activities [t-test, t-ratio=1.74, correlation coefficient=0.034, $p<0.1$, df=23] [Figure 4].

## SECTION 2: SELECTION EXPERIMENT

### SECTION 2.1: DESCRIPTION OF SELECTION EXPERIMENT

Twenty-five flies from each of the twenty-five iso-chromosomal III lines were put into a population cage and allowed to mate. The offspring from this mating, which were considered generation zero [G=0], were allowed to mate in a population cage and a sample of these flies were tested for malathion resistance, MFO activity and AChE activity. The MFO activities of ninety male flies were measured in groups of fifteen flies each. The AChE activities in the heads of eighty flies were also measured in groups of ten heads each. Samples of ten, eleven, seventeen and thirty-four flies were measured to obtain the adult survival values on 15uM malathion, while samples of 160 and 235 flies were measured for the egg survival values on 4uM malathion. All of

FIGURE 2

MEAN MIXED FUNCTION OXIDASE ACTIVITIES OF THE THREE RESISTANCE CLASSES

The data used in this diagram was taken from Table 1. The twenty-five iso-chromosomal III lines used in the selection experiment were divided into three resistance classes, class 1 [LC-50 $<$ 5uM malathion], class 2 [LC-50 $=$ 5-9uM malathion] and class 3 [LC-50 $>$ 9uM malathion]. Class 3 had a significantly higher mean MFO activity than class 1 [student t-test, $t=14.12$, $p<0.001$, $df=18$] and class 2 [student t-test, $t=7.29$, $p<0.001$, $df=18$]. There was no significant difference between the mean MFO activities of class 1 and class 2 [student t-test, $t=0.42$, $p>0.25$, $df=12$].

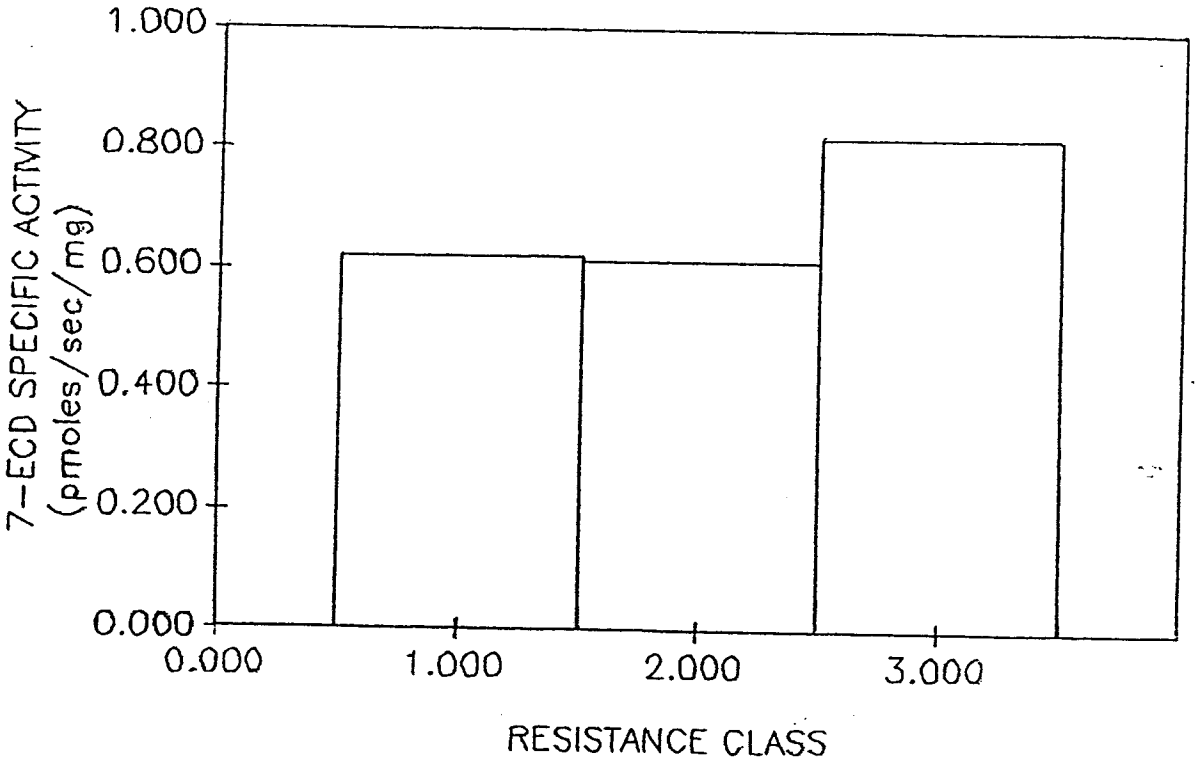

1.000
0.800
0.600
0.400
0.200
0.000
7-ECD SPECIFIC ACTIVITY
(pmoles/sec/mg)
0.000
1.000
2.000
3.000
RESISTANCE CLASS

FIGURE 3

CORRELATION OF MIXED FUNCTION OXIDASE AND ACETYLCHOLINESTERASE ACTIVITIES

The data used in this diagram was taken from Table 1. The MFO and AChE activities of the twenty-five iso-chromosomal III lines used in the selection experiment showed a negative correlation [t-test, t-ratio=-1.22, correlation coefficient=0.025, $p<0.15$, df=23].

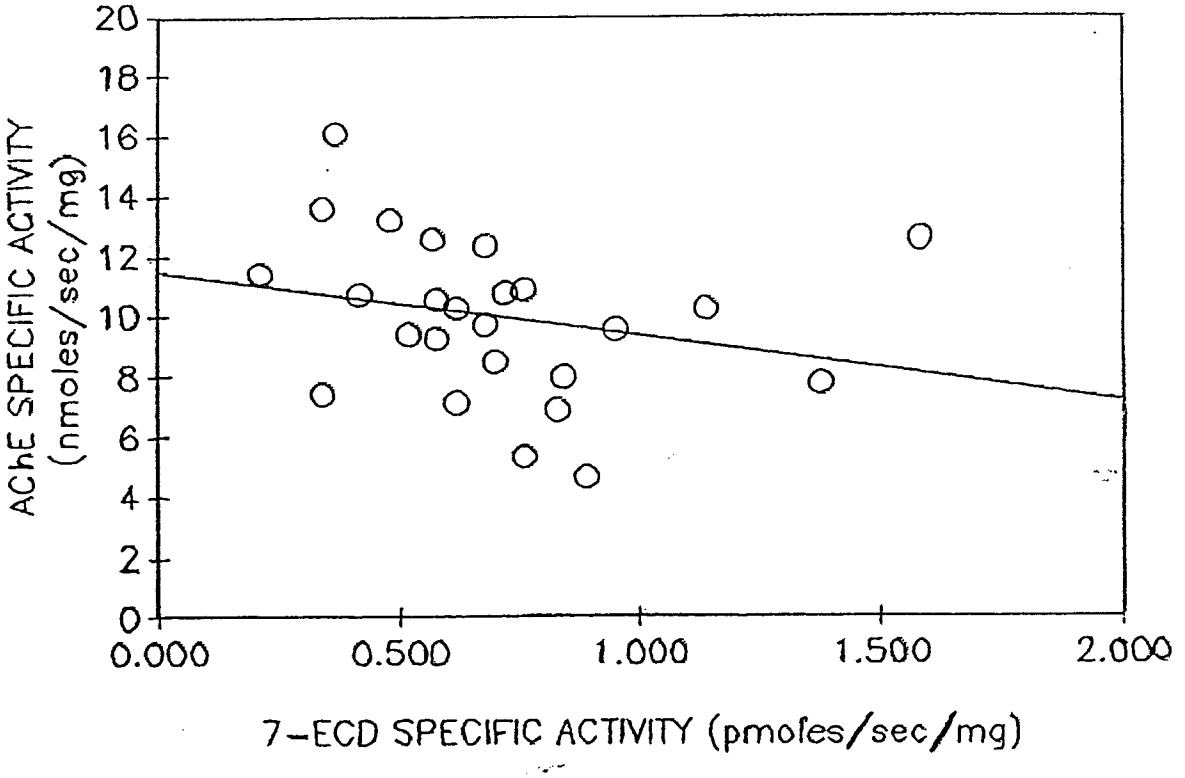
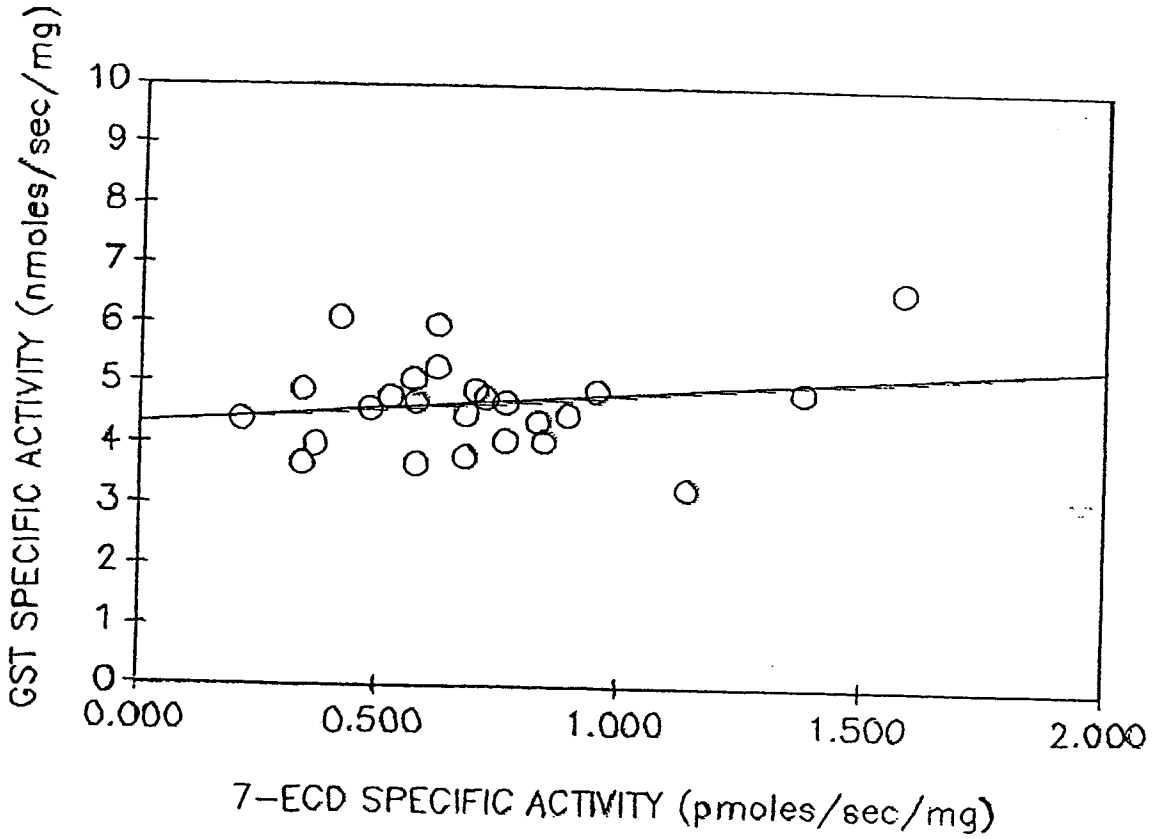

20
18
16
14
12
10
8
6
4
2
0
0.000
0.500
1.000
1.500
2.000
AChE SPECIFIC ACTIVITY (nmoles/sec/mg)
7-ECD SPECIFIC ACTIVITY (pmoles/sec/mg)

FIGURE 4

CORRELATION OF MIXED FUNCTION OXIDASE AND GLUTATHION-S-TRANSFERASE ACTIVITIES

The data used in this diagram was taken from Table 1. The MFO and GST activities of the twenty-five iso-chromosomal III lines used in the selection experiment showed a positive correlation [t-test, t-ratio=1.74, correlation coefficient=0.034, $p<0.1$, df=23].


GST SPECIFIC ACTIVITY (nmoles/sec/mg)
10
9
8
7
6
5
4
3
2
1
0
0.000
0.500
1.000
1.500
2.000
7-ECD SPECIFIC ACTIVITY (pmoles/sec/mg)

these measurements were recorded as G=0. The eggs of G=0 flies were removed in eight ounce bottles. Selection was then carried out in approximately half the bottles by adding 1.5ml of 500uM malaoxon. This concentration of malaoxon, which allowed approximately 10% larvae survival relative to the control line, was used in order to allow survival of the greatest possible number of even partly resistant recombinants at this stage. The selection procedures were repeated every generation, with the malaoxon concentration being doubled approximately every three generations. A sample of the offspring of selected flies and control flies from every third generation [G=3, G=6, G=9 and G=12] were measured for AChE activity and MFO activity as well as adult and egg survival.

## SECTION 2.2: MIXED FUNCTION OXIDASE ACTIVITIES

Selection produced increases in MFO activity between generations zero to three. This assay was done for every third generation of flies, as well as an additional measurement done on flies from generation eleven [Figure 5, Table 2]. As seen in this figure, the mean specific activities from generation three to generation twelve in the selected population was 1.290 with a standard error of 0.159. The MFO activity values of observed in the control flies showed a mean specific activity value of 0.592 with a standard error of 0.104 from generation three to generation twelve. The mean specific activities of the selected and control populations were significantly different after the

TABLE 2

MIXED FUNCTION OXIDASE ACTIVITIES OF CONTROL AND SELECTED POPULATIONS OVER TWELVE GENERATIONS OF SELECTION

The values represent the mixed function oxidase activities [pmoles/sec/mg] of fifteen male flies from control and selected populations. The number in brackets represents the standard error of this activity. The mean mixed function oxidase activity of both populations for each generation sampled was recorded, and the standard error of this activity was recorded in brackets.

TABLE 2

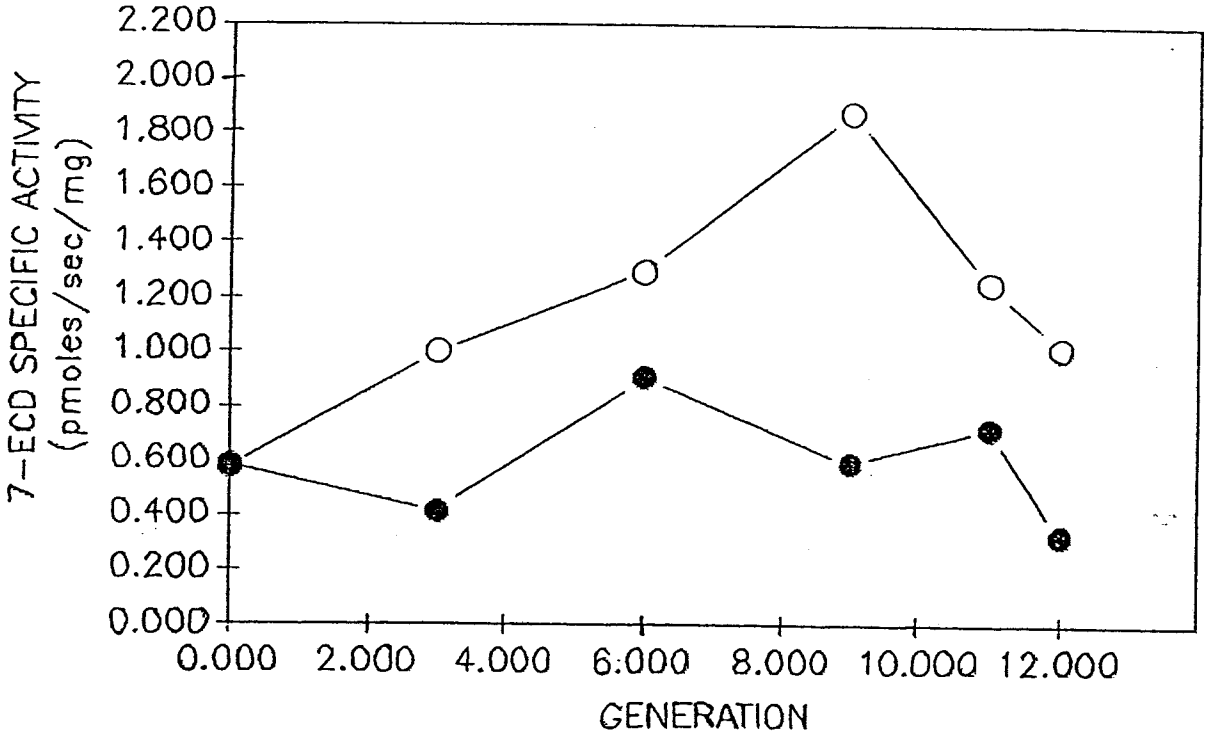
MIXED FUNCTION OXIDASE ACTIVITIES OF CONTROL AND SELECTED POPULATIONS OVER TWELVE GENERATIONS OF SELECTION

| GENERAT'N | CONTROL SPECIFIC ACTIVITY VALUE (STANDARD ERROR) | SELECTED SPECIFIC ACTIVITY VALUE (STANDARD ERROR) |
|---|---|---|
| 0 | .508(.046) .522(----) | |
| | .550(----) .568(.019) | |
| | .639(----) .720(.042) | |
| | MEAN: .585 (.033) | |
| 3 | .257(.012) .366(.032) | .765(.141) .933(.225) |
| | .391(.065) .412(.145) | 1.089(.202) 1.225(----) |
| | .455(.079) .623(.124) | |
| | MEAN: .417 (.049) | MEAN: 1.003 (.099) |
| 6 | .809(.040) .998(.072) | 1.183(.124) 1.407(.146) |
| | MEAN: .904 (.095) | MEAN: 1.295 (.112) |
| 9 | .423(----) .451(.085) | 1.727(.166) 1.808(----) |
| | .675(.010) .809(.134) | 1.826(.206) 2.144(.340) |
| | MEAN: .590 (.092) | MEAN: 1.876 (.092) |
| 11 | .652(----) .791(----) | 1.059(.155) 1.442(----) |
| | MEAN: .722 (.070) | MEAN: 1.251 (.192) |
| 12 | .184(.010) .359(.075) | .856(.010) .935(.120) |
| | .439(.079) | .936(.059) 1.324(.223) |
| | MEAN: .327 (.075) | MEAN: 1.013 (.105) |

FIGURE 5

COMPARISON OF MIXED FUNCTION OXIDASE ACTIVITIES IN CONTROL AND SELECTED POPULATIONS OVER TWELVE GENERATIONS OF SELECTION

The data used in this diagram was taken from Table 2. The filled circles represent the control population, while the empty circles represent the selected population. After the third generation of selection, the MFO activity of the selected population was significantly higher than the MFO activity of the control population [paired t-test, $t(\text{paired})=4.49$, $p<0.01$, $df=4$].


7-ECD SPECIFIC ACTIVITY
(pmoles/sec/mg)
2.200
2.000
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0.000
2.000
4.000
6.000
8.000
10.000
12.000
GENERATION

first three generations [paired t-test, t[paired]=4.49, $p<0.01$, df=4]. These results suggest that the significantly higher MFO activity of the selected population was maintained during the twelve generations of selection.

## SECTION 2.3: ACETYLCHOLINESTERASE ACTIVITIES

Selection for malaoxon resistance appeared to have little effect on the AChE activity [Figure 6, Table 3]. The mean specific activity of the selected flies from generation three to generation twelve was 10.31 with a standard error of 0.49, while the mean specific activity of the control flies was 11.42 with a standard error of 0.51. The mean AChE activities of the control and selected populations were not significantly different over the twelve generations of selection [paired t-test, t[paired]=1.113, $p>0.025$, df=3].

## SECTION 2.4: EGG SURVIVAL VALUES

The greatest increase in egg to pupa survival on 15uM malathion was seen to occur sometime between the first and third generations of selection [Figure 7, Table 4]. The mean selected survival value was 50.9% with a standard error of 3.9% from generation three to generation twelve, while the mean survival value of the control flies

TABLE 3

ACETYLCHOLINESTERASE ACTIVITIES OF CONTROL AND SELECTED POPULATIONS OVER TWELVE GENERATIONS OF SELECTION

The values represent the mean acetylcholinesterase activities [nmoles/sec/mg] of two readings of heads from the same ten flies from control or selected populations. The mean acetylcholinesterase activities of the control and selected populations for each generation examined are summarized. The standard errors of these activities are recorded in brackets.

TABLE 3

ACETYLCHOLINESTERASE ACTIVITIES OF CONTROL AND SELECTED POPULATIONS OVER TWELVE GENERATIONS OF SELECTION

| GENERAT'N | CONTROL SPECIFIC ACTIVITY VALUE (STANDARD ERROR) | | SELECTED SPECIFIC ACTIVITY VALUE (STANDARD ERROR) | |
|---|---|---|---|---|
| 0 | 10.99 | 11.17 | | |
| | 11.49 | 12.16 | | |
| | 12.42 | 12.66 | | |
| | 13.01 | 13.41 | | |
| | MEAN: 12.16 (.36) | | | |
| 3 | 10.27 | 10.40 | 8.43 | 8.52 |
| | 11.33 | 14.04 | 9.01 | 9.46 |
| | | | 11.54 | 12.57 |
| | MEAN: 11.51 (.87) | | MEAN: 9.91 (.71) | |
| 6 | 8.70 | 10.28 | 8.24 | 9.54 |
| | 11.95 | | 10.52 | 11.66 |
| | MEAN: 10.31 (.94) | | MEAN: 9.99 (.73) | |
| 9 | 7.88 | 8.19 | 7.28 | 7.57 |
| | 11.43 | 12.31 | 9.13 | 9.48 |
| | 12.74 | 14.02 | 10.85 | 11.66 |
| | MEAN: 11.10 (1.03) | | MEAN: 9.59 (.91) | |
| 12 | 10.10 | 11.52 | 8.89 | 10.20 |
| | 12.07 | 12.56 | 10.56 | 10.58 |
| | 13.99 | 15.55 | 14.75 | 15.45 |
| | MEAN: 12.76 (.70) | | MEAN: 11.74 (1.11) | |

FIGURE 6

COMPARISON OF ACETYLCHOLINESTERASE ACTIVITIES OF CONTROL AND SELECTED POPULATIONS OVER TWELVE GENERATIONS OF SELECTION

The data used for this diagram was taken from Table 3. The filled circles represent the control populations, while the empty circles represent the selected populations. After the third generation, the mean AChE activities of the control and selected populations were not significantly different [paired t-test, $t(\text{paired})=3.8$, $p>0.02$, $df=3$].

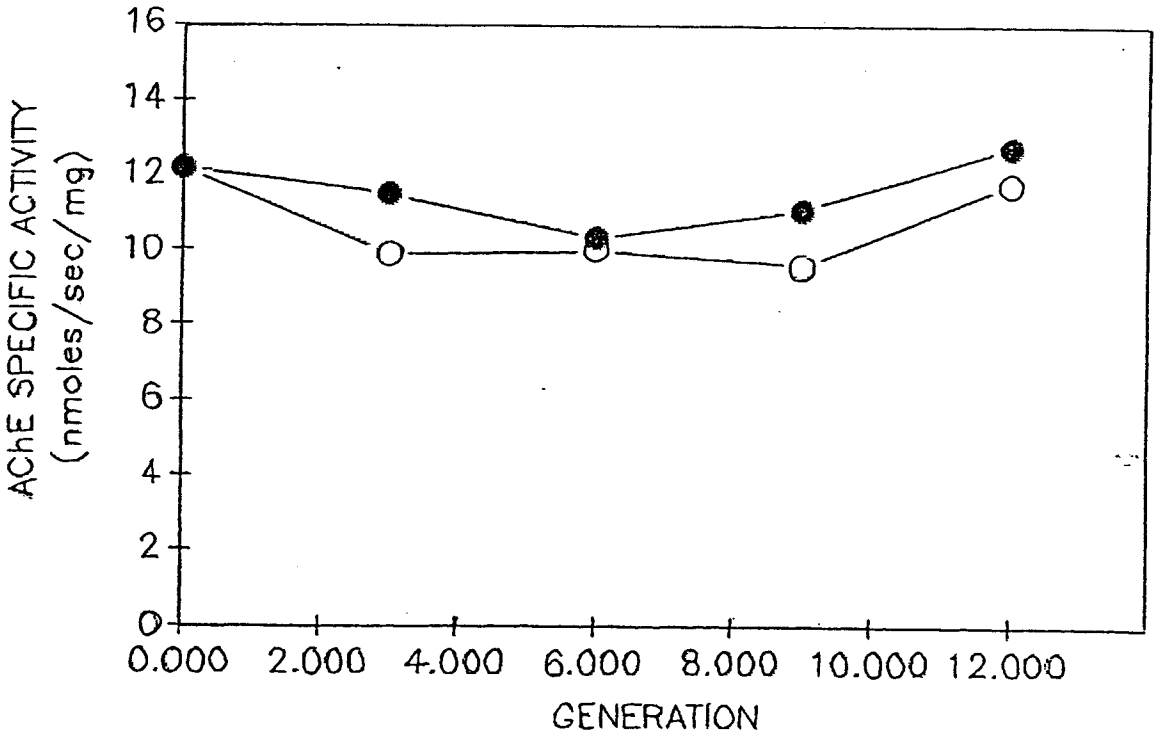

AChE SPECIFIC ACTIVITY
(nmoles/sec/mg)
16
14
12
10
8
6
4
2
0
0.000
2.000
4.000
6.000
8.000
10.000
12.000
GENERATION

TABLE 4

EGG TO PUPA SURVIVAL ON MALATHION FOR CONTROL AND SELECTED POPULATIONS OVER TWELVE GENERATIONS OF SELECTION

The values represent the number of number of flies which reach the pupa stage divided by the number of eggs laid on banana medium, containing 4uM malthion, by flies from the control or selected populations. The percentage of pupa which survive is recorded in brackets. The mean percentage of flies which survive to reach the pupa stage is recorded for each generation examined, and the standard error of these mean percentages is in brackets.

TABLE 4

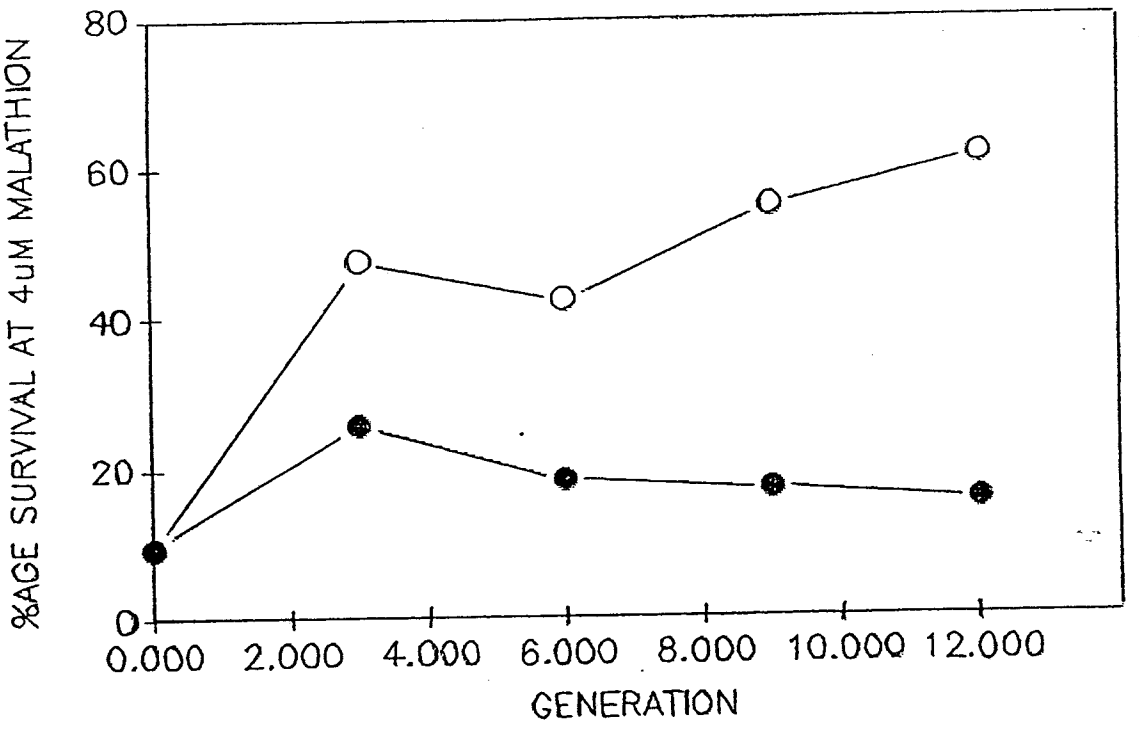
EGG TO PUPA SURVIVAL ON MALATHION FOR CONTROL AND SELECTED POPULATIONS OVER TWELVE GENERATIONS OF SELECTION

| GENER'N | CONTROL SURVIVORS/TOTAL AT 4uM MALATHION (PERCENTAGE SURVIVORS) | SELECTED SURVIVORS/TOTAL AT 4uM MALATHION (PERCENTAGE SURVIVORS) |
|---|---|---|
| 0 | 16/235 (6.8%) | |
| | 19/160 (11.9%) | |
| MEAN % (S.E.) | 9.4% (2.6%) | |
| 3 | 40/276 (14.5%) | 124/266 (46.6%) |
| | 37/100 (37.0%) | 140/290 (48.3%) |
| MEAN % (S.E.) | 25.8% (11.3%) | 47.5% (0.9%) |
| 6 | 14/107 (13.1%) | 16/38 (42.1%) |
| | 18/76 (23.7%) | 55/130 (42.3%) |
| MEAN % (S.E.) | 18.4% (5.3%) | 42.2% (0.1%) |
| 9 | 7/54 (12.9%) | 43/89 (48.3%) |
| | 17/79 (21.5%) | 36/59 (61.0%) |
| MEAN % (S.E.) | 17.2% (4.3%) | 54.7% (6.4%) |
| 12 | 7/60 (11.7%) | 26/47 (55.3%) |
| | 14/72 (19.4%) | 21/31 (67.7%) |
| MEAN % (S.E.) | 15.6% (3.9%) | 61.5% (6.2%) |

FIGURE 7

COMPARISON OF EGG TO PUPA SURVIVAL ON MALATHION FOR CONTROL AND SELECTED POPULATIONS OVER TWELVE GENERATIONS OF SELECTION

The data used in this diagram was taken from Table 4. The filled circles represent the control populations, while the empty circles represent the selected populations. The mean egg to pupa survival of the control and selected populations were significantly different after the first three generations of selection [paired t-test, $t(\text{paired}) = 5.61$, $p < 0.01$, $df = 3$].


%AGE SURVIVAL AT 4uM MALATHION
80
60
40
20
0
0.000
2.000
4.000
6.000
8.000
10.000
12.000
GENERATION

was 18.0% with a standard error of 1.0%. The mean survival values of the control and selected populations were significantly different after the first three generations [paired t-test, t[paired]=5.61, $p<0.01$, df=3]. These results suggest the significantly higher egg to pupa survival found in the selected population was maintained over the twelve generations of selection.

## SECTION 2.5: ADULT SURVIVAL VALUES

An increase in adult resistance to 4uM malathion to a survival value 3.5 times greater than that found in control populations was seen to occur during the first three generations of selection [Figure 8, Table 5]. This was the only significant change in resistance seen in the selected population. From generation three to generation twelve, the mean selected survival value was 86.0% with a standard error of 3.3%, while the mean control survival value was 22.3% with a standard error of 3.3%. The mean adult survival values of the control and selected populations were significantly different after the first three generations [paired t-test, t[paired]=26.92, df=3, $p<0.001$, df=3]. These results suggest that the significantly higher mean adult survival values of the selected population were maintained over twelve generations of selection.

TABLE 5

ADULT SURVIVAL ON MALATHION FOR CONTROL AND SELECTED POPULATIONS OVER TWELVE GENERATIONS OF SELECTION

The measurements shown to represent the number of adult survivors divided by the total number of flies from the control or selected populations exposed to 15uM malathion for twenty-four hours. The number in brackets represents the percentage of adult survivors. The mean percentage adult survivors of populations from each generation was recorded, with the standard error of this percentage in brackets.

TABLE 5

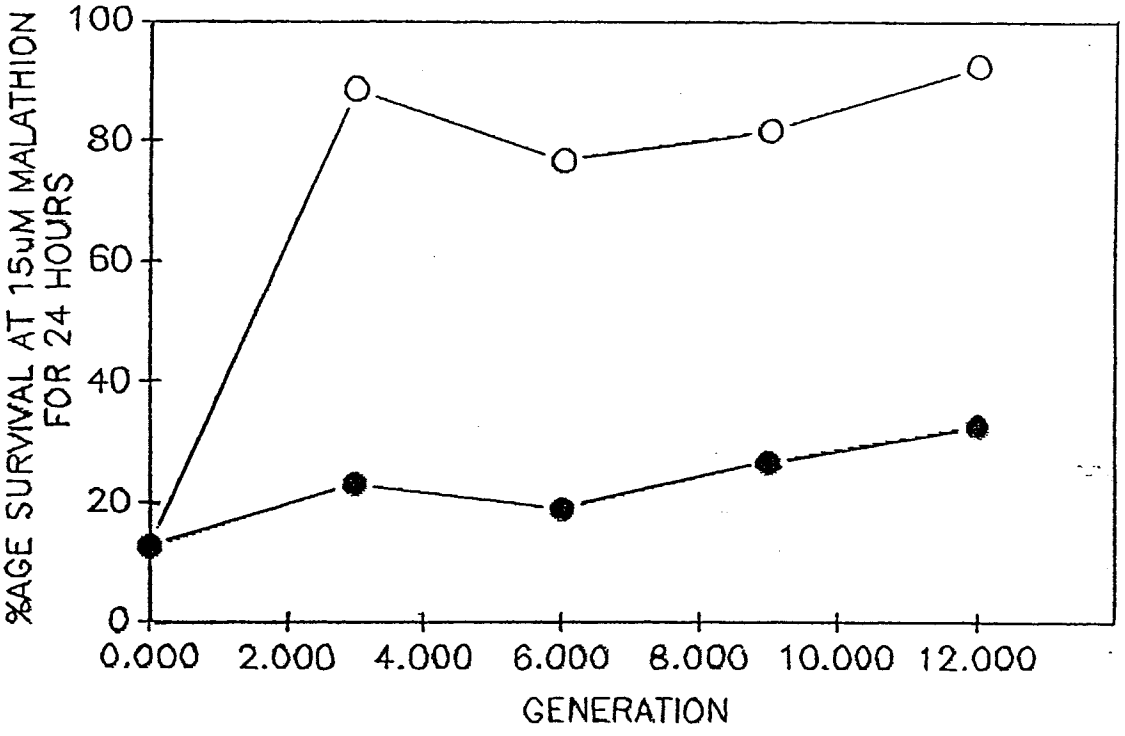
ADULT SURVIVAL ON MALATHION FOR CONTROL AND SELECTED POPULATIONS OVER TWELVE GENERATIONS OF SELECTION

| GENER'N | CONTROL SURVIVORS/TOTAL AT 15uM MALATHION (PERCENTAGE SURVIVORS) | SELECTED SURVIVORS/TOTAL AT 15uM MALATHION (PERCENTAGE SURVIVORS) |
|---|---|---|
| 0 | 0/17 (0.0%) | |
| | 4/34 (11.8%) | |
| | 2/11 (18.2%) | |
| | 2/10 (20.0%) | |
| MEAN % (S.E.) | 12.5% (4.5%) | |
| 3 | 3/19 (15.8%) | 33/39 (84.6%) |
| | 3/13 (23.1%) | 16/18 (88.9%) |
| | 10/33 (30.3%) | 12/13 (92.3%) |
| MEAN % (S.E.) | 23.1% (4.2%) | 88.6% (2.2%) |
| 6 | 1/8 (12.5%) | 7/10 (70.0%) |
| | 3/12 (25.0%) | 10/12 (83.3%) |
| MEAN % (S.E.) | 18.8% (6.3%) | 76.7% (6.7%) |
| 9 | 2/10 (20.0%) | 8/10 (80.0%) |
| | 2/6 (33.3%) | 15/18 (83.3%) |
| MEAN % (S.E.) | 26.7% (6.7%) | 81.7% (1.7%) |
| 12 | 15/55 (27.3%) | 21/23 (91.3%) |
| | 10/26 (38.5%) | 44/47 (93.6%) |
| MEAN % (S.E.) | 32.9% (5.6%) | 92.5% (1.2%) |

FIGURE 8

COMPARISON OF ADULT SURVIVAL ON MALATHION FOR CONTROL AND SELECTED POPULATIONS OVER TWELVE GENERATIONS OF SELECTION

The data used in this diagram was taken from Table 5. The filled circles represent the control population, while the empty circles represent the selected population. After the first three generations there was a significant difference between the mean adult survival values of the control and selected populations [paired t-test, $t(\text{paired}) = 26.92$, $p < 0.001$, $df = 3$].


%AGE SURVIVAL AT 15uM MALATHION FOR 24 HOURS
100
80
60
40
20
0
0.000
2.000
4.000
6.000
8.000
10.000
12.000
GENERATION

## SECTION 2.6: MICROSOMAL PROTEIN CONTENT CHANGES WITH SELECTION

Sucrose gradient microsomes prepared from thirty flies from the control and selected populations from generations zero, three, six, nine and twelve, were analyzed on 1-D SDS polyacrylimide gels in order to examine the spectrum of polypeptide bands in the microsomes of these populations. There appeared to be no significant changes in microsomal protein content observed from generation to generation in either population, and the control and selected populations appeared the same [data not shown]. In view of these results, a more thorough comparison was done using a large scale microsomal preparation of flies from generation twelve.

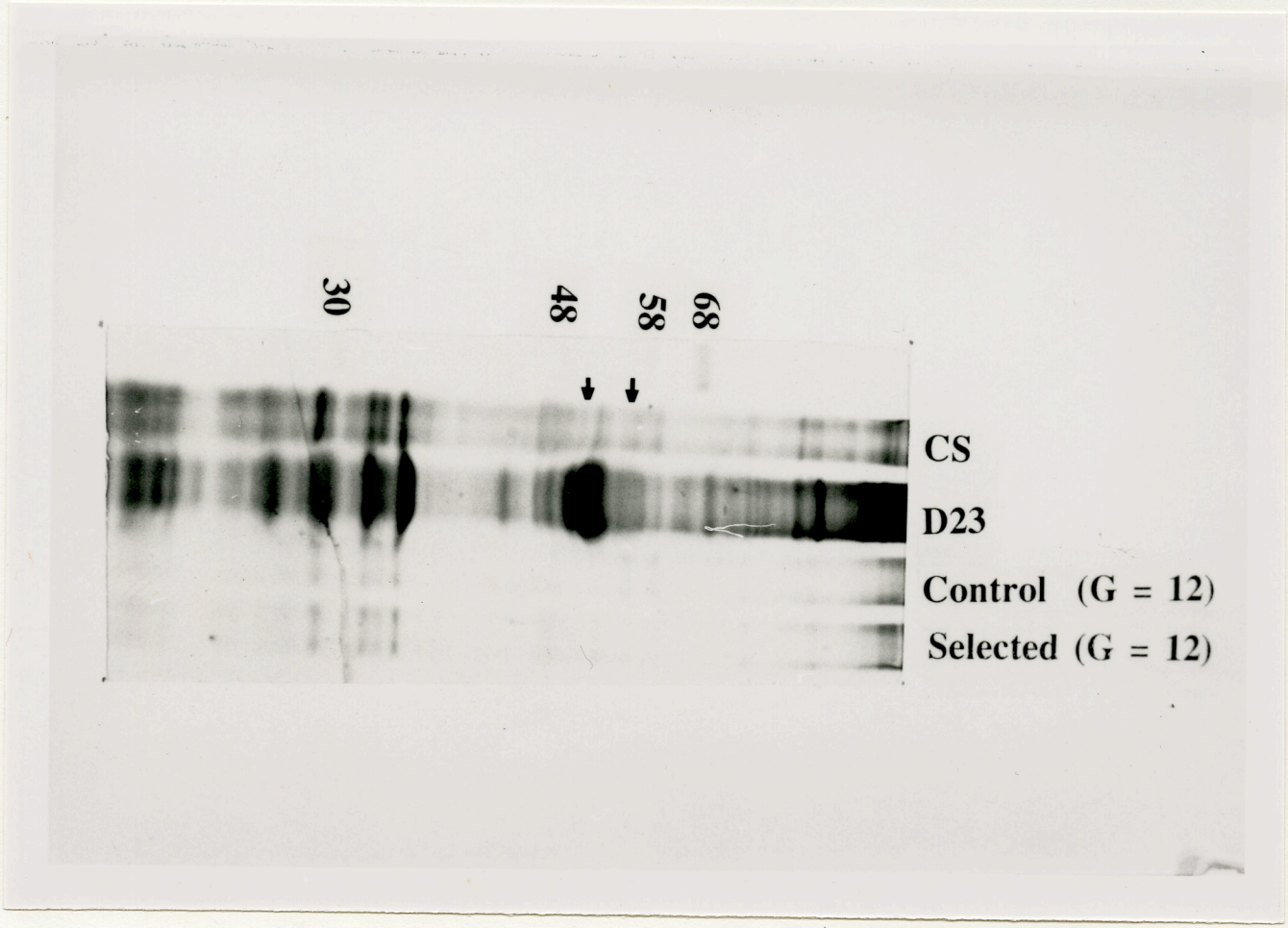
CHAPS-solubilized microsome preparations from control and selected populations were analyzed on SDS gels and the proteins visualized by silver staining. Microsomal preparations were made from flies in generation twelve, and were compared to microsome preparations of both CS and D23 flies. No significant difference in microsomal protein content was found between control and selected populations. The control and selected populations were similar to the wild type CS population, as none of these populations showed the enhanced 54kD protein band found in the insecticide resistant D23 population [Figure 9].

The immunological reactivity of microsomal proteins with the antibody Ab2-IgG,

FIGURE 9

ONE DIMENSIONAL SDS POLYACRYLAMIDE GEL ELECTROPHORESIS OF MICROSOMAL PROTEINS FROM CONTROL AND SELECTED POPULATIONS AFTER TWELVE GENERATIONS OF SELECTION

CHAPS-solubilized microsome preparations were electrophoresed on a 10% SDS polyacrylamide gel and silver stained. The molecular weight markers used were bovine erythrocyte carbonic anhydrase [30kD], pig heart fumerase [48kD], beef liver catalase [58kD] and bovine serum albumen [68kD]. Quantities of 15ug of protein were loaded into each lane. The arrows indicate the 50kD and the 54kD protein bands which were enhanced in the resistant D23 line. The left lane represents the polypeptides of the sensitive CS line next to the lane representing the polypeptides fo the resistant D23 line. The band on the far right represents the polypeptides of flies from the selected population in generation twelve. The remaining band represents the polypeptides of flies from the control population in generation twelve.


30
48
58
68
CS
D23
Control (G = 12)
Selected (G = 12)

which was directed against the 54kD protein band enhanced in the resistant IIID line (Pursey, 1989), was examined [Figure 10]. Densitometer readings of these protein bands were taken, and the insecticide resistant D23 line was the most intensely stained band with a reading for the 54kD protein band of $13.55X10^{-3}$ AUXmm/ug. The control and selected lines had much less intense readings of $1.91X10^{-3}$ AUXmm/ug and $3.88X10^{-3}$ AUXmm/ug, respectively, which were similar to the reading of $0.87X10^{-3}$ AUXmm/ug found for the insecticide sensitive CS line.

## SECTION 3: STUDY OF ISO-CHROMOSOMAL III LINES OF CONTROL AND SELECTED POPULATIONS

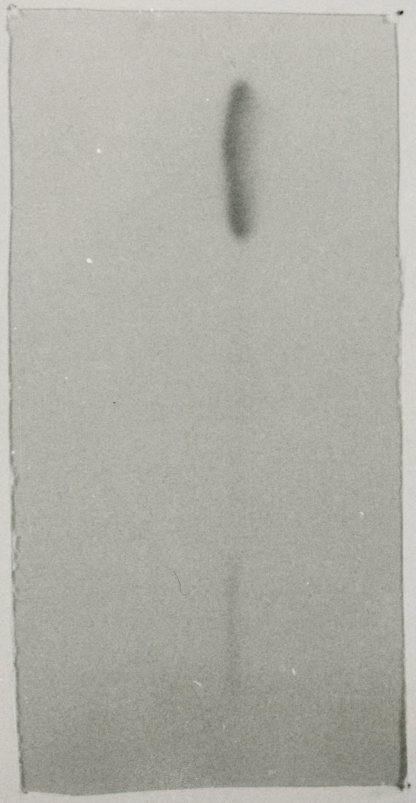
### SECTION 3.1: CONTROL AND SELECTED ISO-CHROMOSOMAL III LINES

After twelve generations of selection had passed, the third chromosome of twenty female flies from both control and selected lines were extracted to produce iso-chromosomal III lines [Figure 1]. From these lines, five selected and nine control lines were eventually made homozygous [Table 6], suggesting that the selected population carried a greater number of genes detrimental to fertility than the control population. Nine selected and three control lines could survive in the heterozygous state with the TM3 balancer chromosome, and the other lines were lost. One selected line, S5, and two control lines, C11 and C16, had low homozygous viability, and could not

FIGURE 10

WESTERN HYBRIDIZATION OF D23 SPECIFIC ANTIBODY TO MICROSOMAL PROTEINS FROM CONTROL AND SELECTED POPULATION TWELVE GENERATIONS OF SELECTION

Ab2-IgG antibody, which hybridized to the 54kD protein band enhanced in the resistant D23 line, was hybridized to CHAPS-solubilized microsomal proteins after these proteins had been separated by 1-D SDS polyacrylamide gel electrophoresis. Quantities of 15ug of protein were loaded into each lane. The 54kD protein band is visible. The left lane represents 54kD protein band of the resistant D23 line next to a lane which represents the 54kD protein band of the sensitive CS line. The lane on the far right represents the 54kD protein band of flies from the selected population, and the remaining lane represents the 54kD protein band of flies from the control population.



D23

CS

Control (G = 12)

Selected (G = 12)

TABLE 6

ISO-CHROMOSOMAL III STRAINS

The heterozygous lines carried one third chromosome from selected or control lines heterozygous with the TM3 third chromosome balancer [Figure 1]. The homozygous viability was obtained by dividing the observed frequency of homozygous offspring by the expected frequency of homozygous offspring [1/3].

TABLE 6

ISO-CHROMOSOMAL III STRAINS

FROM SELECTED POPULATION

| Homozygous Lines | Heterozygous Lines | Homozygotes Total Flies | Homozygous Viability |
|---|---|---|---|
| S1 | S5 | 17/73 | .699 |
| S7 | S8 | 0/24 | .000 |
| S10 | S11 | 0/79 | .000 |
| S24 | S12 | 0/39 | .000 |
| S25 | S14 | 0/34 | .000 |
| | S15 | 0/86 | .000 |
| | S16 | 0/90 | .000 |
| | S18 | 0/31 | .000 |
| | S21 | 0/54 | .000 |
| | S27 | 0/97 | .000 |

FROM CONTROL POPULATION

| Homozygous Lines | Heterozygous Lines | Homozygotes Total Flies | Homozygous Viability |
|---|---|---|---|
| C2 | C11 | 9/45 | .600 |
| C5 | C16 | 4/72 | .167 |
| C7 | C22 | 0/99 | .000 |
| C8 | C25 | 0/32 | .000 |
| C9 | C26 | 0/47 | .000 |
| C12 | | | |
| C17 | | | |
| C21 | | | |
| C24 | | | |

be maintained in the homozygous state. No homozygous flies were recovered from the other heterozygous lines.

## SECTION 3.2: MIXED FUNCTION OXIDASE ACTIVITIES [ISO-CHROMOSOMAL III LINES]

The MFO specific activities for the iso-chromosome III control and selected lines were measured to allow comparison with the control and selected populations from which the iso-chromosome III lines were extracted [Figure 11, Table 7]. At least three measurements were made for each line and it was found that the control iso-chromosomal III lines mean MFO activities were significantly lower than the mean MFO activities of the selected iso-chromosomal III lines [student t-test, $t=3.21$, $p<0.005$, $df=12$]. The mean specific activities of the $G=12$ control and selected populations showed an even greater significant difference [student t-test, $t=5.29$, $p<0.001$, $df=10$] than the iso-chromosomal III lines. These results suggested that the iso-chromosomal III lines were not a representative sample of the $G=12$ population from which the iso-chromosomal III lines were extracted.

TABLE 7

MIXED FUNCTION OXIDASE ACTIVITIES FOR CONTROL AND SELECTED ISO-CHROMOSOME III POPULATIONS

Each measurment represents the MFO-specific activity [pmoles/sec/mg] of the fifteen male Drosophila. The numbers in brackets represent the standard error of the specific activity measurments.

TABLE 7

MIXED FUNCTION OXIDASE ACTIVITIES FOR CONTROL AND SELECTED ISO-CHROMOSOME III POPULATIONS

FROM CONTROL POPULATION

| LINE: | C2 | C5 | C7 |
|---|---|---|---|
| | .418(.045) | .278(.128) | .413(.011) |
| MFO-SPECIFIC | .562(.002) | .435(.072) | .507(.088) |
| ACTIVITY | .593(.070) | .616(.035) | .650(.037) |
| (STD. ERROR) | .743(.107) | | |
| MEAN S. A. | .579(.067) | .443(.098) | .523(.069) |

| LINE: | C8 | C9 | C12 |
|---|---|---|---|
| | .348(.062) | .271(.156) | .617(.218) |
| MFO-SPECIFIC | .634(.106) | .407(.051) | .662(.055) |
| ACTIVITY | .650(.037) | .484(.116) | .759(.161) |
| (STD. ERROR) | | | |
| MEAN S. A. | .544(.098) | .387(.062) | .679(.042) |

| LINE: | C17 | C21 | C24 |
|---|---|---|---|
| | .354(----) | .499(----) | .466(.206) |
| MFO-SPECIFIC | .543(.061) | .809(.182) | .484(.094) |
| ACTIVITY | .620(.000) | .875(.165) | .613(.070) |
| (STD. ERROR) | | | |
| MEAN S. A. | .506(.079) | .728(.116) | .521(.046) |

FROM SELECTED POPULATION

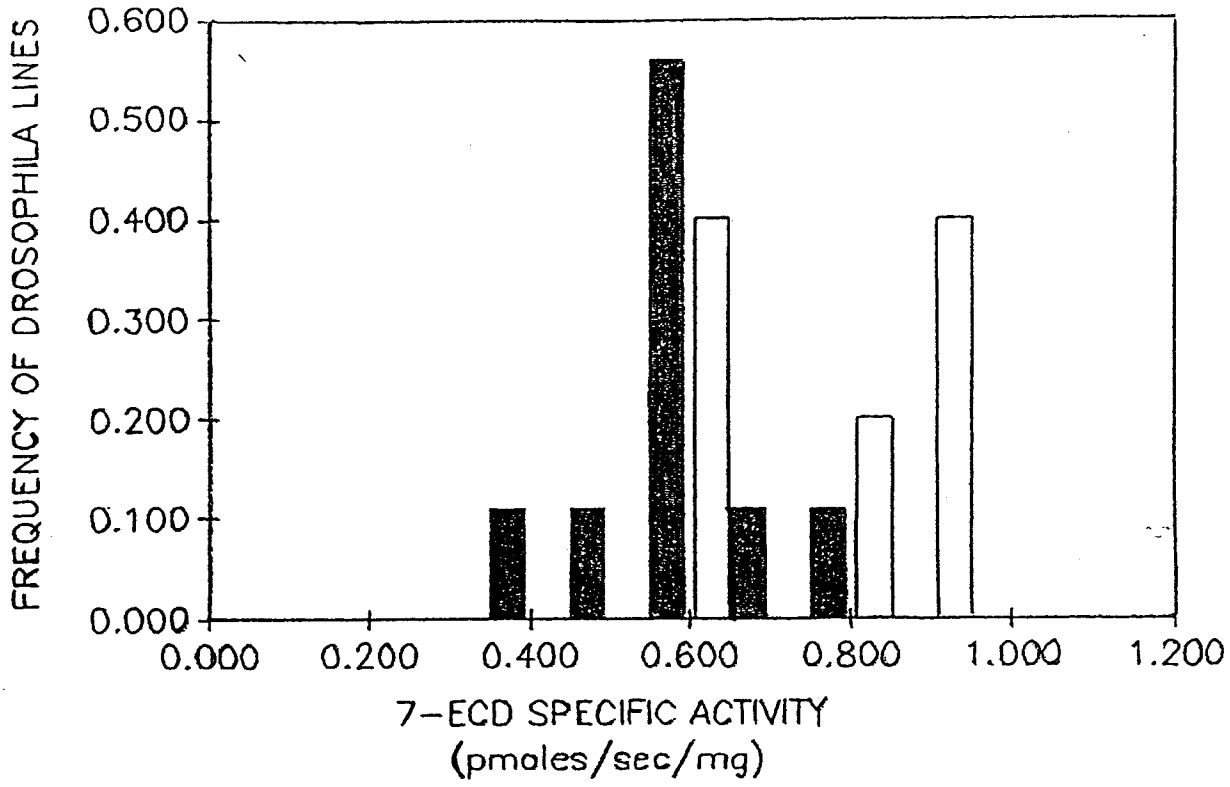
| LINE: | S1 | S7 | S10 |
|---|---|---|---|
| | .549(.286) | .464(.077) | .580(----) |
| MFO-SPECIFIC | .958(.157) | .698(----) | .639(.040) |
| ACTIVITY | 1.012(.007) | .744(----) | .647(.159) |
| (STD. ERROR) | 1.025(.195) | | |
| | 1.214(.170) | | |
| MEAN S. A. | .952(.110) | .635(.087) | .622(.021) |

| LINE: | S24 | S25 |
|---|---|---|
| | .673(.102) | .505(----) |
| MFO-SPECIFIC | .982(.187) | .857(.088) |
| ACTIVITY | 1.011(.115) | 1.122(.220) |
| (STD. ERROR) | | 1.161(.037) |
| MEAN S. A. | .889(.108) | .911(.151) |

FIGURE 11

COMPARISON OF MIXED FUNCTION OXIDASE ACTIVITIES OF CONTROL AND SELECTED ISO-CHROMOSOME III POPULATIONS

The data used in this diagram was taken from Table 7. The filled bars represent the control lines, while the empty bars represent the selected lines. The mean MFO activities of the control and selected iso-chromosomal III lines were significantly different [student t-test, $t=3.21$, $p<0.005$, $df=12$].


FREQUENCY OF DROSOPHILA LINES
0.600
0.500
0.400
0.300
0.200
0.100
0.000
0.000
0.200
0.400
0.600
0.800
1.000
1.200
7-ECD SPECIFIC ACTIVITY
(pmoles/sec/mg)

## SECTION 3.3: ADULT SURVIVAL VALUES [ISO-CHROMOSOMAL III LINES]

The adult malathion resistance of iso-chromosomal III lines was measured to allow comparison with the control and selected populations from which these lines were extracted [Figure 12, Table 8]. The mean adult survival values of the iso-chromosomal III control and selected lines were significantly different [student t-test, $t=2.19$, $p<0.025$, $df=12$]. The difference between the mean adult survival values of the control and selected lines after generation three showed an even greater significant difference [paired t-test, $t[paired]=26.92$, $p<0.001$, $df=3$] than the iso-chromosomal III lines. These results suggest that the alleles of the iso-chromosomal III lines were not a representative sample of the alleles of the control and selected populations from which the iso-chromosomal III lines were extracted.

## SECTION 3.4: ADULT RESISTANCE CORRELATED WITH MFO ACTIVITY

Increased resistance to malathion was found to be correlated with increased MFO activity in the iso-chromosome III lines [t-test, $t\text{-ratio}=1.96$, correlation $coefficient=0.049$, $p<0.05$, $df=12$]. This result suggested that increased MFO activity may have caused increased insecticide resistance in the iso-chromosome III lines. The iso-chromosomal lines could be divided into two distinct groups with regard to resistance [Figure 12 and Figure 13]. The less resistant of the two groups contained

TABLE 8

ADULT SURVIVAL ON MALATHION FOR CONTROL AND SELECTED ISO-CHROMOSOME III POPULATIONS

Each value represents the number of adult flies that survive exposure to 15uM malathion for 24 hours divided by the total number of flies exposed to malathion. The numbers in brackets represent the percentage of flies the survive on the malathion. The mean percentage survival for each line is also recorded, followed by the standard error of this percentage in brackets.

TABLE 8

ADULT SURVIVAL ON MALATHION FOR CONTROL AND SELECTED ISO-CHROMOSOME III POPULATIONS

FROM CONTROL POPULATION

| LINE | C2 | C5 | C7 |
|---|---|---|---|
| SURVIVORS/TOTAL | 2/34 (5.9) | 4/46 (8.7) | 30/54 (55.6) |
| (%AGE SURVIVORS) | 10/51 (19.6) | 23/66 (34.5) | 33/58 (56.9) |
| | 10/36 (27.8) | 13/31 (41.9) | 20/28 (71.4) |
| MEAN %AGE (S.E.) | 17.8% (6.3) | 28.4% (10.1) | 61.3% (5.1) |

| LINE | C8 | C9 | C12 |
|---|---|---|---|
| SURVIVORS/TOTAL | 19/27 (70.4) | 5/20 (25.0) | 1/31 (3.2) |
| (%AGE SURVIVORS) | 21/28 (75.0) | 8/24 (33.3) | 4/21 (14.3) |
| | 15/20 (75.0) | | 16/68 (23.5) |
| MEAN %AGE (S.E.) | 73.5% (1.5) | 29.2% (4.2) | 13.7% (5.9) |

| LINE | C17 | C21 | C24 |
|---|---|---|---|
| SURVIVORS/TOTAL | 0/16 (0.0) | 4/28 (14.3) | 1/23 (4.4) |
| (%AGE SURVIVORS) | 3/17 (17.7) | 6/37 (16.2) | 14/36 (38.9) |
| | 8/40 (20.0) | 10/28 (35.7) | 17/36 (47.2) |
| MEAN %AGE (S.E.) | 12.6% (6.3) | 22.1% (6.8) | 30.2% (13.1) |

FROM SELECTED POPULATION

| LINE | S1 | S7 | S10 |
|---|---|---|---|
| SURVIVORS/TOTAL | 36/48 (75.0) | 25/40 (62.5) | 2/18 (11.1) |
| (%AGE SURVIVORS) | 12/14 (85.7) | 30/45 (66.7) | 15/68 (22.1) |
| | 24/28 (85.7) | 49/57 (86.0) | 6/24 (25.0) |
| MEAN %AGE (S.E.) | 82.1% (3.6) | 71.7% (7.2) | 19.4% (4.2) |

| LINE | S24 | S25 |
|---|---|---|
| SURVIVORS/TOTAL | 20/35 (57.1) | 76/120 (63.3) |
| (%AGE SURVIVORS) | 17/28 (60.7) | 30/44 (68.2) |
| | 22/32 (68.8) | |
| MEAN %AGE (S.E.) | 62.2% (3.5) | 65.8% (2.5) |

FIGURE 12

COMPARISON OF ADULT SURVIVAL ON MALATHION FOR CONTROL AND SELECTED ISO-CHROMOSOME III POPULATIONS

The data used in this diagram was taken from Table 8.

The filled bars represent strains from the control iso-chromosomal III lines, while the open bars represent strains from the selected iso-chromosomal III lines.

The mean adult survival values of the iso-chromosomal III control and selected lines were significantly different [student t-test, $t=2.19$, $p<0.025$, $df=12$].

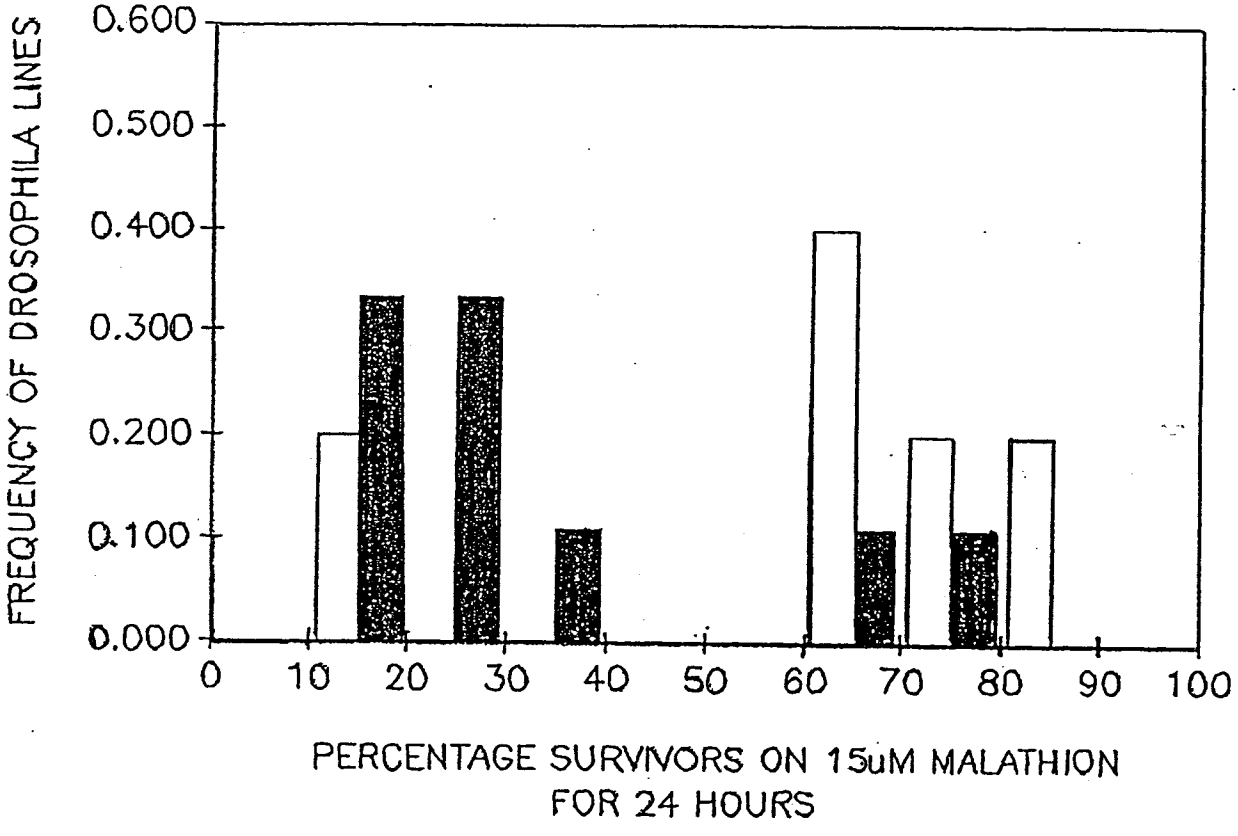
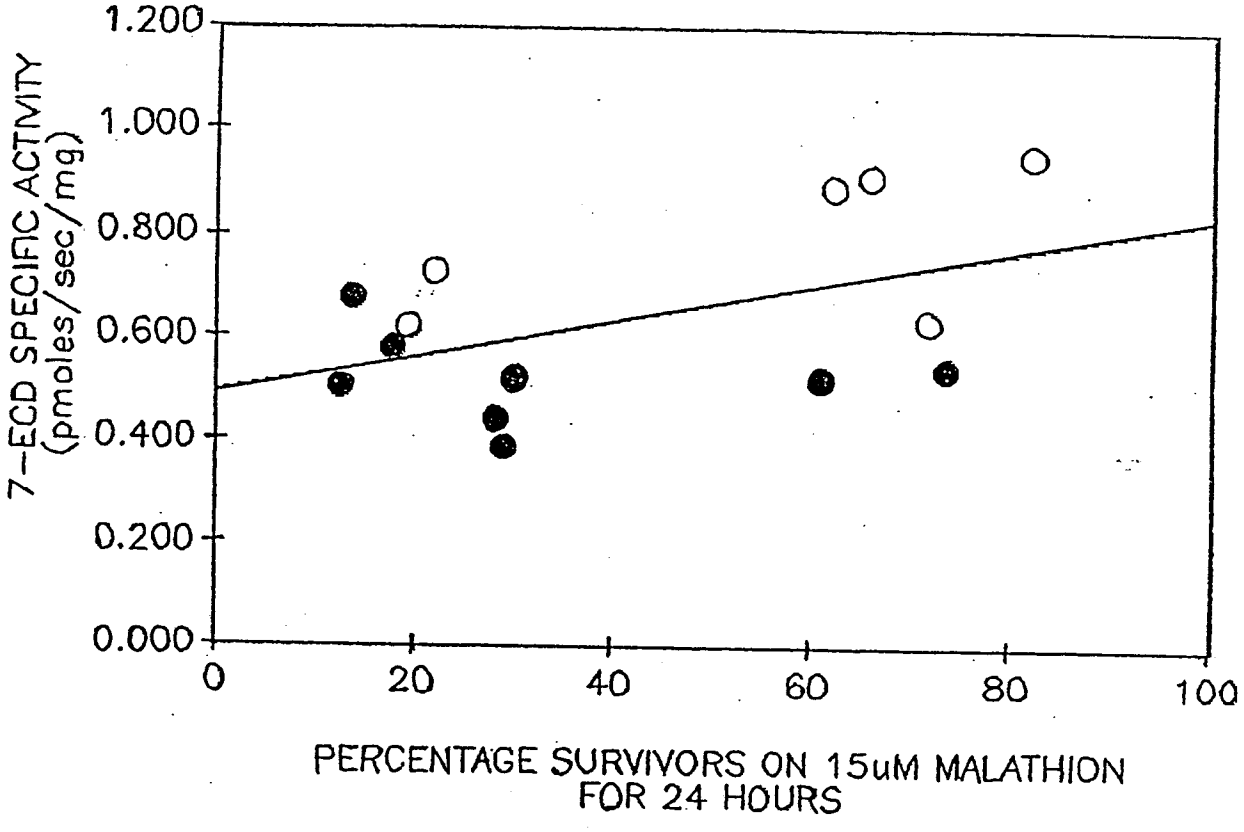

FREQUENCY OF DROSOPHILA LINES
0.600
0.500
0.400
0.300
0.200
0.100
0.000
0
10
20
30
40
50
60
70
80
90
100
PERCENTAGE SURVIVORS ON 15uM MALATHION
FOR 24 HOURS

FIGURE 13

CORRELATION OF MIXED FUNCTION OXIDASE ACTIVITY AND MALATHION RESISTANCE FOR CONTROL AND SELECTED ISO-CHROMOSOME III POPULATIONS

The data used in this diagram was taken from Tables 7 and 8. The filled circles represent the control iso-chromosomal III lines, while the empty circles represent the selected iso-chromosomal III lines. There was a correlation between the mean MFO activities and the adult survival values [t-test, t-ratio=1.96, correlation coefficient=0.049, $p<0.05$ df=12].


7-ECD SPECIFIC ACTIVITY
(pmoles/sec/mg)
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
20
40
60
80
100
PERCENTAGE SURVIVORS ON 15uM MALATHION
FOR 24 HOURS

the C2, C5, C9, C12, C17, C21, C24 and S10 lines, while the more resistant group contained the C7, C8 S1, S7, S24 and S25 lines. The mean adult survival values of the two groups on 15uM malathion were significantly different [student t-test, $t=11.74$, $p<0.001$, $df=10.1$]. The two groups had no significant difference in MFO activity [student t-test, $t=2.04$, $p=0.08$, $df=7.6$]. These results suggested that the MFO activity was not responsible for the difference in insecticide resistance between the two groups.

## SECTION 3.5: MICROSOMAL PROTEINS [ISO-CHROMOSOMAL III LINES]

CHAPS-solubilized microsome preparations of one representative sample from both the less resistant and more resistant iso-chromosome III groups, the C12 and S25 lines respectively, were prepared and analyzed on a 1-D SDS polyacrylamide gel to see if there was a difference between the microsomal proteins of the two groups. No difference could be seen between the control and selected iso-chromosome III lines when the proteins were visualized with silver staining [Figure 14].

The microsomal preparations of the C12 and the S25 were analyzed using the Western blot technique with the Ab2-IgG antibody directed against the 54kD protein enhanced in the resistant D23 line [Figure 15]. The intesity of staining of the bands on the blots was then examined using a densitometer. The 54kD protein band of the S25

FIGURE 14

ONE DIMENSIONAL SDS POLYACRYLAMIDE GEL ELECTROPHORESIS OF MICROSOMAL PROTEINS FROM ISO-CHROMOSOME III CONTROL AND SELECTED LINES

CHAPS-solubilized microsome preparations were electrophorised on a 10% SDS polyacrylamide gel and silver stained. The molecular weight markers used were bovine erythrocyte carbonic anhydrase [30kD], pig heart fumerase [48kD], beef liver catalase [58kD] and bovine serum albumin [68kD]. Quantities of 15ug of protein were loaded intothe control and selected lanes, and approximately 30ug of protein were loaded into the CS and D23 lanes on the left. The arrows indicate the 50kD and 54kD protein bands, which were enhanced in the resistant D23 line. The left two lanes represent the polypeptides present in the sensitive CS line next to a lane which represents the polypeptides present in the resistant D23 line. The lane on the far right represents the polypeptides present in the resistant S25 iso-chromosomal III line, and the remaining lane represents the polypeptides present in the sensitive C12 iso-chromosomal III line.

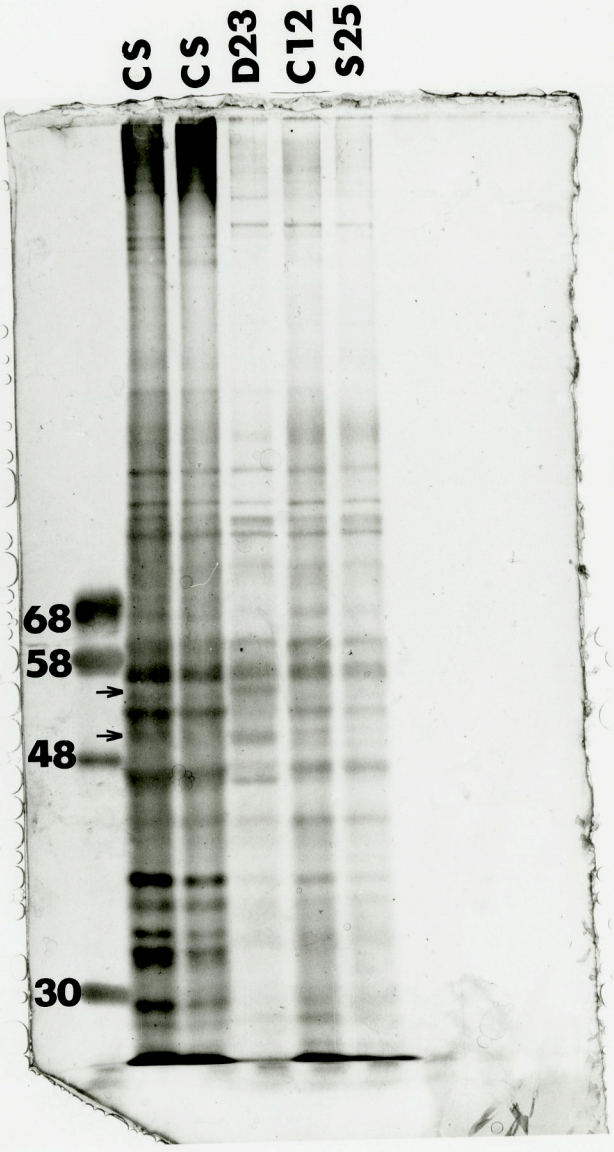
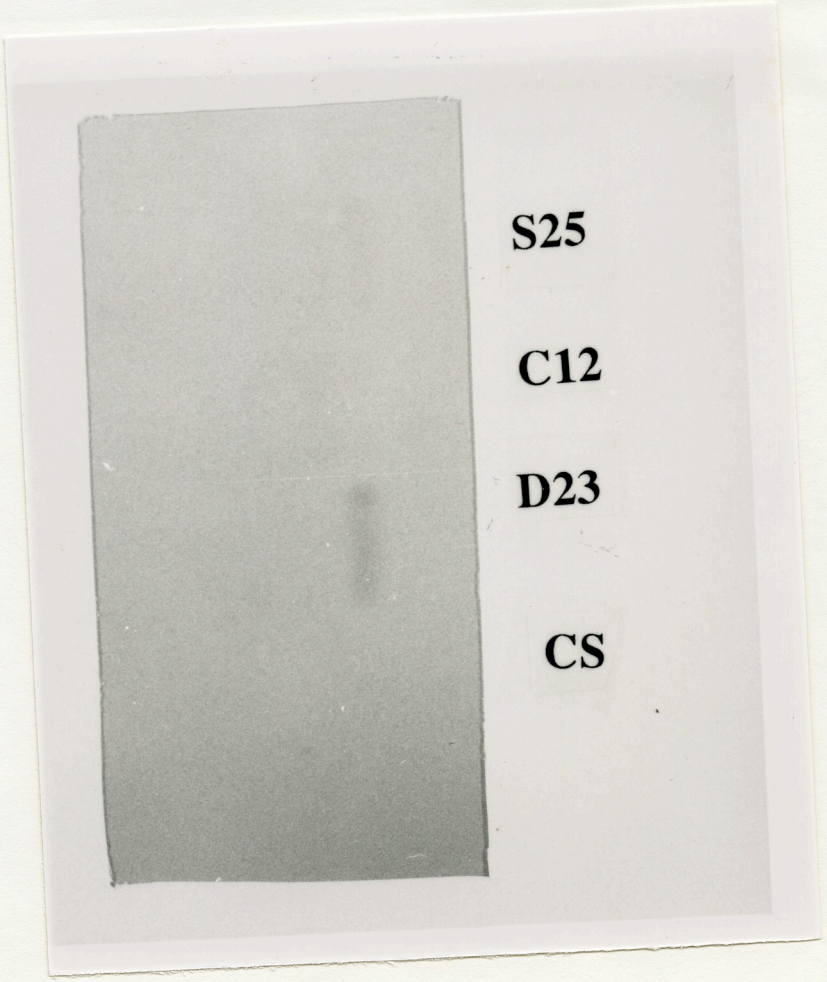

CS
CS
D23
C12
S25
68
58
48
30

FIGURE 15

WESTERN HYBRIDIZATION OF D23 SPECIFIC ANTIBODY TO MICROSOMAL PROTEINS FROM ISO-CHROMOSOME III CONTROL AND SELECTED LINES

Ab2-IgG antibody, which hybridized to the 54kD protein band enhanced in the resistant IIID line, was hybridized to CHAPS-solubilized microsomal proteins after these proteins had been separated by 1-D SDS polyacrylamide gel electrophoresis. Quantities of 15ug of protein were loaded into each lane. The 54kD protein band is visible. The lane on the far right represents the 54kD protein band of the sensitive CS line, next to the lane which represents the 54kD protein band of the resistant D23 line. The lane on the far left represents the 54kD protein band of the resistant S25 iso-chromosomal III line, and the remaining lane represents the 54kD protein band of the sensitive C12 iso-chromosomal III line.


S25
C12
D23
CS

line had a densitometer reading of $10.40X10^{-3}$ AUXmm/ug and the 54kD band of the C12 line had a higher densitometer reading of $5.87X10^{-3}$ AUXmm/ug. These values are lower than the $16.00X10^{-3}$ AUXmm/ug reading found for the insecticide resistant line, D23, as is the $1.40X10^{-3}$ AUXmm/ug reading found for the insecticide sensitive CS line.

## SECTION 3.6: RESTRICTION FRAGMENT LENGTH POLYMORPHISM

A probe specific for a Drosophila DNA fragment, which coded for a polypeptide fragment that was antigenic towards the Ab2-IgG antibody, revealed EcoR1 restriction fragment polymorphism in the CS and D23 lines, with the probe binding to a slow band in CS and a faster band in D23 (Pursey, 1989). Similar polymorphism in the control and selected iso-chromosomal III lines were looked for using the technique of Southern blotting. The EcoR1 restriction fragments of all the control and selected iso-chromosomal III lines were labelled with the probe and compared with those of the CS and D23 line, and only the fast D23 fragment was seen in the control and selected lines (data not shown).

# DISCUSSION:

## SECTION 1: CHARACTERISTICS OF THE ORIGINAL TWENTY-FIVE LINES

The twenty-five iso-chromosomal III lines forming the control and selected populations of Drosophila melanogaster that were selected over twelve generations for insecticide resistance had a wide range of insecticide resistance before selection, as well as a wide range of MFO, AChE and GST activities. This allowed selection to act on many different alleles, including some combinations formed when twenty-five flies from each line line were put in a population cage and allowed to mate. The MFO activities of the twenty-five isochromosomal III lines ranged from 0.340 for the 248A line to 1.580 for the 52A line. Resistance of these lines appeared to correlate to MFO activity [Figure 2], suggesting that increased MFO activity may have been responsible for resistance in some of these lines. A correlation between MFO activity and resistance in Drosophila had previously been found by Morton and Holwerda (1985). The AChE activities of the twenty-five iso-chromosomal III lines ranged from 5.4 for the 11B line to 13.2 for the 248A line. The negative correlation between AChE activity and MFO activity [Figure 3] indirectly suggested that increased insecticide resistance in some lines may have been due to reduced AChE activity. A correlation between AChE activity and resistance had previously been found by Morton and Singh (1982). The GST

activities of the twenty-five iso-chromosomal III lines ranged from 3.7 for the 248A and 228B lines to 6.6 for the 52A line. Greater levels of resistance in some lines may have been due to increased GST activity, as was indirectly suggested by the positive correlation found between GST activity and MFO activity [Figure 4]. A correlation between GST activity and resistance was previously found by Morton and Holwerda (1985). Only AChE and MFO activity were examined in the selection experiment, as the correlation between increased GST activity and increased resistance had not been verified by the time the experiment was begun.

## SECTION 2: AQUISITON OF RESISTANCE THROUGH SELECTION

There were three ways by which insecticide resistance could have been aquired during the selection experiment. A new mutation may have been selected. Selection for a new allelic combination of a number of the original alleles could have occured. The unchanged resistant alleles of one of the original lines also have been selected.

The results in this thesis suggest that the resistant flies were selected within the first three generations, and that no further increase in resistance occured in later generations. In the selected population the MFO specific activity, egg survival and adult survival values all increased to values much greater than those observed in the control line during the first three generations of selection [Figures 5,6,7 and 8]. After

this initial selection the values seemed to level off, and any fluctuations in the egg and adult survival of the selected population were found to correlate with fluctuations observed in the control population. This leveling off occured even though the concentration of malathion used for selection was doubled every three generations, suggesting that the a new mutation had not been selected. If a new resistant mutation had arisen in the first three generations, further increases in malathion concentration may have resulted in the selection of new mutations. These new mutations would have resulted in increased resistance, which was not seen in the results.

The results suggested that selection for a new allelic combination of a number of the original haplotypes or selection for an unchanged resistant haplotype from one of the twenty-five iso-chromosomal III lines forming the control and selected populations may have occured. The LC-50 for adults of the selected population was between 30-50uM malathion. While eleven iso-chromosomal III lines had LC-50 values greater than 9uM, only 52A had an LC-50 as high as the selected line, with a value of 20-50uM malathion. The mean AChE and MFO values of the selected population were 10.3 and 1.29, respectively. These mean specific activities were similar to the AChE value of 12.6 and MFO value of 1.58 found for the 52A line [Table 1]. Only one other line, 106A, had a combination of AChE and MFO values similar to those of the selected population, with values of 10.3 and 1.14 respectively [Table 1]. However, this 106A line and the selected population were dissimilar, as 106A had an LC-50 of 5-9uM. The results suggested that selection of alleles or new combinations of alleles

with properties similar to those found in the 52A line occured.

The iso-chromosomal III lines extracted from the selected population were unlikely to carry the complete third chromosome of one of the twenty-five lines used to the control and selected populations. Only twenty-five percent of the flies from the iso-chromosomal III selected population and forty-five percent of flies from the iso-chromosomal III control population were homozygous viable. A large sample of flies in the control and selected populations were only viable in the heterozygous state, while all of the iso-chromosomal III lines which formed these populations were homozygous viable, suggesting that new combinations of alleles existed in the selected population which were not homozygous viable. Many combinations of alleles which were on other chromosomes or which were not homozygous viable, some which may be involved in resistance, may have been lost when the third chromosome was extracted from the selected population, suggesting the iso-chromosomal III lines extracted from the selected population did not carry a representative sample of the alleles involved in resistance. Resistance in the iso-chromosomal III lines extracted from the selected population may have been caused by a non-representative sample of alleles on the third chromosome, or alleles on another chromosome. This conclusion was further enforced by the fact that the mean MFO activities and insecticide resistance of the iso-chromosomal III selected and control populations and the G=12 selected and control populations were different.

## SECTION 3: MECHANISM OF INSECTICIDE RESISTANCE

The insecticide resistance of the selected population may have been due to the detoxification of insecticide by the fly, possibly through increased MFO activity. The level of insecticide resistance in the selected population was much higher than that found in the sensitive CS strain and similar to that found in the resistant D23 strain. An altered AChE may not have played a role in the resistance of the selected population, as the selected population had AChE activities similar to the CS strain and much higher than the D23 strain [Table 1]. This result suggested the mechanism of resistance was different than that of the resistant D23 strain, as Morton and Holwerda (1985) found that resitance in D23 was caused by high MFO activity coupled with altered AChE.

Silver staining of the microsomal proteins of D23 revealed enhanced 50kD and 54kD protein bands which appeared to represent cytochrome P-450s (Houpt et al., 1988). No evidence of enhancement of the 50kD and 54kD bands in the selected population was seen in silver stains and Western blots of the microsomal proteins of flies from the selected population [Figure 9 and Figure 10]. When the Western blotting technique was done, using the antibody Ab2-IgG which was directed against the enhanced 54kD band of the D23 strain, Ab2-IgG did not stain the microsomal proteins of the control and selected population strongly. When the spectrum of polypeptide bands in microsomes from one representative of both the resistant and sensitive iso-chromosomal III groups extracted after selection were examined with silver stains

and western blotting, the staining of the 54kD band was much less intense in the iso-chromosomal III lines than in the D23 strain [Figure 14 and Figure 15]. There are four possible explainations for the different cytochrome P-450 concentrations between the selected population and the D23 strain. One possible explanation could have been that the selected population may have produced a cytochrome P-450 type, different than that produced by D23, which caused insecticide resistance but could not be detected by antibody directed against the cytochrome P-450 of D23. An altered cytochrome P-450 may be present at lower concentrations in the selected population which is just as effective at detoxifying insecticides as high concentrations of cytochrome P-450 from the D23 strain. Increased levels of NADPH could have been responsible for the insecticide resistance of the selected population, with no increase in cytochrome P-450 levels being necessary. The 50kD and 54kD polypeptide bands of the IIID strain may not represent cytochrome P-450, and changes the concentration of these proteins may not affect resistance.

The MFO activities of the more resistance iso-chromosomal III group overlapped with the MFO activities of the less resistant iso-chromosomal III group [Figure 13], suggesting that the resistant alleles of the iso-chromosomal III lines were not related to MFO activity. Morton and Holwerda (1985) found insecticide resistance and MFO activities correlated in the resistant D23 strain, suggesting that the selected population and D23 had a different mechanism of resistance. The restriction site analysis of the genomic DNA of D23 and the control and selected iso-chromosomal III lines revealed

that the iso-chromosomal III lines and the D23 strain all carried a fast restriction fragment which was labelled by the probe. This result suggested that the genes on the fast restriction fragment may not be responsible for enhancement of the 54kD protein.

Differences between the characteristics of the resistant D23 strain and the selected population have been caused by the different methods of selection used. The D23 strain was derived after 110 generations of selection (Singh and Morton, 1981), while the selected population was only selected for twelve generations, suggesting the additional selection by Singh and Morton (1981) may have resulted in the selection of resistant characteristics not found in the selected population. However, resistance in the population selected by Singh and Morton (1981) was noticed after the first nineteen generations of selection, and selection after this period may have had no additional effects. The D23 strain was continually exposed to insecticide during selection, while the selected population studied in this thesis was only exposed to insecticide during the larval stage, suggesting that alleles only important in the larval resistance may have been selected in the selected population. However, both the selected population and the D23 strain were resistant to insecticides in both the larval and adult stages [Figure 7 and Figure 8], suggesting the selected population carried alleles providing insecticide resistance in all the stages of the Drosophila's life cycle. Singh and Morton (1981) selected for alleles with resistance to malathion, while the experiment described in this thesis selected for alleles with resistance to malaoxon. The alleles selected to provide

malathion and malaoxon resistance may not have been the same.

## SECTION 4: FURTHER RESEARCH

The mechanism of resistance in the iso-chromosome III lines extracted after selection was not the MFO activity related resistance mechanism found in D23 (Morton and Holwerda, 1985). The first step in finding the mechanism of resistance used by the selected iso-chromosomal III lines would be to determine what proteins were specifically involved in this resistance. The resistant proteins could be used to determine which chromosome was responsible for resistance, and which genes were responsible for production of these proteins. The proteins could be characterized to determine if they were cytochrome P-450's, or some other proteins involved in the detoxification of insecticides. Once the proteins involved in resistance had been determined, different mechanisms of insecticide resistance could be hypothesized and investigated.

Further evidence proving resistance was aquired through the selection of a new combination of alleles present in the population before selection could be found be comparing the haplotypes of the twenty-five iso-chromosomal III lines used to form the control and selected populations with those of the iso-chromosomal III control and selected lines. Dr. C. Aquadro has found the haplotype of each of the twenty-five lines used to form the control and selected populations by recording the presence or absence

approximately twenty restriction sites along the third chromosome (unpublished results). Restriction analysis of the iso-chromosomal III control and selected lines could be done, and the data compared with the haplotypes of the twenty-five iso-chromosomal III lines used to form the control and selected populations. The average MFO activity or percentage adult survival could be found for the twenty-five iso-chromosomal III lines which have or lack each restriction site. This would identify if a certain restriction site polymorphism was associated with increased resistance and MFO activity. The haplotypes of the selected populations could then be studied to see if they carry a restriction site polymorphism present in the twenty-five iso-chromosomal III lines. Ordering of the restriction sites on a genetic map could then be carried out, and the location of the region resposible for the selected population's increased resistance could be found on this map. If the selected population doesn't have same the haplotype as one of the lines used to form this population, the results would suggest that resistance was due to the aquisition of a new combination of alleles present in the iso-chromosomal III lines used to form the control and selected populations.

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