MIXED FUNCTION OXIDASE ACTIVITY AND MALATHION RESISTANCE IN A SELECTED STRAIN OF *DROSOPHILA MELANOGASTER*

By

DANIEL R. HOUPT

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AUTHOR: Daniel R. Houpt, B.Sc. (University of Toronto).

SUPERVISOR: Professor R.A. Morton.

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Abstract

The genetic factors controlling Mixed Function Oxidase (MFO) Activity and malathion resistance was studied in the larva and the adult of a malathion resistant strain of Drosophila melanogaster. In addition, the developmental expression and tissue localization of high MFO activity was characterized in the larva and the adult.

Microsomal extracts from a strain with a resistant second chromosome were found to have increased amounts of protein with a relative molecular mass of 52 kD, while a strain with a resistant third chromosome was found to have increased amounts of proteins with relative molecular masses of 51 and 55 kD in the microsomal extract.

MFO activity associated with a strain with a resistant second chromosome was found to be most concentrated in the intestine and abdominal wall of the imago, while found primarily in the malpigian tubules and the fat body of the larva.

The mapping of genes on the second chromosome asociated with larval resistance to malathion suggested two loci, a major resistance gene at 2-64 cM and a second minor resistance gene. The mapping of genes on the second chromosome associated with adult resistance again suggested two loci, one at 2-64 cM and a second just to the left of the marker black (48.5 cM).

The mapping of genes on the second chromosome associated with high PNA demethylase activity (an MFO activity) in the adult

suggested a locus at 2-64 cM and a second between 54.5 and 60.0 cM. The locus at 2-64 cM and a third locus between 48.5 and 51 cM was found to be associated with high 7-EC hydroxylase activity (a second MFO activity) in the adult.

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DEDICATION

This work is lovingly dedicated to my grandparents, Samuel and Sylvia Ogus and the late Abraham and Esther Houpt.

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Introduction.

Section 1: Insecticide Resistance.

The first reports of insecticide resistance were written in 1897, when F.W. Card noted that the coddling moth, Laspeyresia pomonella (L.) failed to respond to the customary insecticidal spraying that had previously been successful in controlling this pest (Card, 1897, quoted by Forgash, 1984). Also in this year, J.B. Smith wrote that, "...washes that easily destroy the San Jose scale in California are ridiculously ineffective in the Atlantic states..." (Smith, 1897, quoted by Forgash, 1984). Melander (1914) first recognized resistance as it is now generally defined, when he noted an unusually high survival of the San Jose scale after attempts to control it with lime-sulfur treatments in Washington. Since then, insecticide resistance has been noted in 14 orders and 83 families and in 1981 totalled 428 different insects and acarines, of which 261 were of agricultural importance and the rest of medical/veterinary concern (Georghiou, 1981). Problems associated with insecticide resistance include increased vector-borne disease, such as malaria, crop losses, increased production costs, and increased pesticide hazards to the environment. The term "insecticide resistance" has been defined by the World Health Organization Expert Committee on Insecticides (1957) as,

"...the development of an ability in a strain of insects to tolerate doses of toxicants which would prove lethal to the majority of individuals in a normal population of the same species. The term 'behavioristic resistance' describes the ability to avoid a dose which would prove lethal".

Resistance may be due to an alteration of any of the processes that determine the penetration, distribution or target site interaction of an insecticide. Most important are: (i) an altered site of action (ii) reduced penetration (iii) increased detoxification.

1.1: Altered Site of Action.

The best understood examples of this type of resistance are resistance to organophosphates and carbamates found in insects with an altered acetylcholinesterase (AChE), the target enzyme of these compounds. Smissaert (1964) observed that in homogenates of OP-resistant spider mites Tetranychus urticae, the R-strain had an AChE with a much lower activity towards acetylcholine (ACh) and acetylthiocholine than did an S-strain. This altered AChE from the R-strain was inhibited very slowly by the organophosphate paraoxon, whereas the AChE from the S-strain was inhibited much more quickly. The rate of inhibition of the AChEs from resistant strains of insects may be reduced up to several hundred-fold, and in combination with detoxification enzymes, a

considerable degree of resistance can be produced. Resistance of this kind has been demonstrated for the Cattle tick Boophilus albimus (Hemingway and Georghiou, 1983), and the housefly Musca domestica (Tripathi and O'Brien, 1973; Devonshire and Sawicki, 1974) among other insects. Morton and Singh (1982) in addition reported that a strain of Drosophila melanogaster that had been selected for resistance to malathion had an altered acetylcholinesterase and that there was a significant negative correlation between malathion resistance and AChE activity among iso-female strains, with more resistant strains tending to have a lower AChE activity.

1.2: Reduced Penetration.

The mechanism of resistance due to reduced penetration of the insecticide into the insect (reviewed by Georghiou, 1972) is of significance because even a small reduction in the rate of entry of the insecticide may permit detoxification reactions to cope with the insecticide as it enters the system. A lower rate of penetration by various insecticides, usually about 50% of normal, has been demonstrated in various resistant strains of housefly, Musca domestica (El Bashir, 1967), mosquitos, Aedes aegypti (Matsumura and Brown, 1963), and Culex fatigans (Shrivastava et al, 1970) among other species. The best studied mechanism of reduced penetration has been determined using the housefly. This mechanism appears to be controlled by a number of

recessive genes, possibly allelic, such as pen and tin. These genes may control some aspect of the insect cuticle which reduces the penetration of the insecticide into the insect. Reduced penetration to a number of insecticides (such as DDT, dieldrin, parathion, malathion and others) is conferred by these genes. These genes work as intensifiers of resistance by allowing detoxification mechanisms a longer time in which to operate (Sawicki, 1970, 1973).

1.3: Increased Detoxification.

The most common mechanisms of resistance are those of an increased capacity for detoxification in the insect (reviewed by Oppenoorth and Welling, 1976 and Oppenoorth, 1984). A number of enzymes and enzyme systems are available that enable insects to cope with the many noxious chemicals in the environment, such as secondary plant substances. In some resistant strains, increased detoxification capacity is available due to the increased efficiency or amount of one or more detoxification enzymes. Enzymes such as esterases, oxidases and phosphatases among others may contribute to insecticide resistance.

Section 2: Malathion.

Malathion belongs to the general class of insecticides called organophosphates. These compounds are thought to cause

toxicity by inhibiting acetylcholinesterase (AChE), the enzyme which normally catalyzes the hydrolysis of the neurotransmitter acetylcholine (ACh). If AChE is inhibited, there is no degradation of ACh, and the post-synaptic membrane remains stimulated, such that normal synaptic transmission is interfered with. Because it is more extensively degraded in mammals and birds than in insects, malathion tends to be more toxic to insects. Oxidation of malathion can increase its toxicity by producing its oxon derivative, malaoxon, which is thought to be the actual toxicant (reviewed by Eto, 1979). This oxidation is performed by the Mixed Function Oxidase (MFO) system, which also is able to degrade malaoxon to less toxic derivatives. Both malathion and malaoxon can be degraded by numerous other enzymes present in the insect, such as carboxylesterases, phosphatases and glutathione-S-transferases. Resistance due to an increased malathion-carboxylesterase activity has been found in Culex tarsalis (Matsumura and Brown, 1961), Tetranychus urticae (Matsumura and Voss, 1964) and Musca domestica (Matsumura and Hogendijk, 1964) among others. In Cimex lectularis (Feroz, 1971) an increased phosphatase activity accounted for the majority of malathion degradation.

Section 3: Insecticide Resistance in Drosophila melanogaster.

Much of the work on insecticide resistance in Drosophila has

been done on resistance to Organophosphates (OP). A number of Japanese workers in the late 1950s suggested that larval resistance to OP was controlled by a major dominant gene (or perhaps a tightly linked cluster of genes) on the second chromosome at 2-64.5 cM (Kikkawa 1961). These workers found that Drosophila that were selected for DDT resistance were cross-resistant to a number of different insecticides such as parathion, malathion and BHC. The gene associated with this multiple resistance was named the RI gene (for Resistance to Insecticides). As well, a region on the third chromosome (near 3-50) which affects resistance to parathion (Kikkawa, 1961) and phenylthiourea (Ogita, 1958) was reported. Dapkus and Merrill (1977), by means of a chromosomal analysis concluded that a relatively small number of genes showing incomplete dominance were responsible for adult resistance to DDT in a strain of Drosophila selected for DDT resistance and that these were located on the second and third chromosomes. Hallstrom et al (1985) showed that resistance to DDT and other insecticides was associated with an increased MFO activity and they suggested that four or five genes might be responsible for regulation of the MFO system in Drosophila. Morton and Holwerda (1985) showed in a strain of Drosophila melanogaster selected for resistance to malathion, that metabolic resistance was associated with an increased MFO activity.

Section 4: The Mixed Function Oxidase System.

The MFO system performs a large number of biotransformations on most major groups of insecticides (Reviewed by Nakatsugawa and Morelli, 1976). The MFO System is characterized by its location in the endoplasmic reticulum and by a requirement for molecular oxygen and NADPH. Biochemical studies of this enzyme system are usually performed on the "microsomal fraction", defined as those vesicles precipitated by high-speed centrifugation (eg. 100,000 g for 60 minutes) of a post-mitochondrial suspension. The key component of this system is an enzyme called cytochrome P-450, named for the characteristic position of a peak in the optical difference spectrum of the carbon monoxide complex with the reduced cytochrome. Cytochrome P-450 acts as a terminal oxidase, introducing one atom of molecular oxygen into the substrate and reducing the other atom to water (H_2O). Electrons are transferred from NADPH to the cytochrome by NADPH cytochrome P-450 reductase. The oxidation of the substrate renders it sufficiently water soluble for immediate excretion or susceptible to further reaction with a conjugating system.

The MFO system is able to metabolize a large spectrum of endogenous substrates such as steroid hormones and fatty acids, as well as a wide variety of exogenous substrates such as drugs, pesticides and carcinogenic chemicals. The system's ability to activate many pre-carcinogens to more reactive compounds has

been the object of much study in mammals. MFO dependent metabolism has been observed in most tissues of mammals, although its greatest activity has been demonstrated in the liver. The ability of the MFO system to metabolize many different substrates is due, in part, to the multiplicity of cytochrome P-450 isozymes. These isozymes are inducible by compounds which themselves can act as substrates, although constituitive forms of the enzyme have also been demonstrated. Inducers of the enzyme fall into different classes of chemicals, such as pesticides, drugs, solvents, and polyaromatic hydrocarbons (PAH). Historically, the drug phenobarbitol (PB) and the PAH 3-methylcholanthrene have been used as inducers of different cytochrome P-450 species exhibiting different substrate specificities. Cytochrome P-450 is currently classified in terms of a genetic "superfamily" comprising at least 10 gene families. These families are distinguished based on differences in substrate specificity, the organism in which they were characterized, and their amino acid sequence (often deduced from the cDNA nucleotide sequence) (reviewed by Nebert and Gonzalez, 1987).

Section 5: The Insect Mixed Function Oxidase System.

The MFO System has also been demonstrated in insects (reviewed by Hodgson, 1983). In insects oxidase activity is usually found in cells along the gastrointestinal tract, in the

malpigian tubules and in the fat bodies, presumably tissues that represent the initial defence for compounds entering the body in the diet or by penetration of the cuticle (Wilkinson, 1983). A large number of functions have been ascribed to the MFO sytem in insects. These include the metabolism of juvenile hormone and ecdysone and their analogues, the production of pheremones, and as an adaptation to secondary plant substances (Hodgson, 1983). Much of the interest in the MFO system of insects is due to its role in the metabolism of insecticides, and the mode of action of synergists. It has been shown that the insect MFO system, like the mammalian liver counterpart, is capable of functioning as a non-specific xenobiotic metabolizing system, and while many of the MFO catalyzed reactions are part of an overall detoxification process, some are activation reactions, making the product more toxic than the parent compound. For instance, the organophosphorous insecticide thionates can be activated by the insect MFO system to the more toxic oxons. Oxons can, in turn, be degraded to ionic derivatives which are water soluble and much less toxic to the insect.

Increased MFO activity in insects has been shown to be associated with resistance to a variety of insecticides. Since the MFO system is of broad specificity, its high activity in many insecticide resistant strains is a likely cause of cross-resistance to other insecticides in addition to those for which resistance was originally selected. In addition to activating

organophosphates, the MFO system has the capacity to oxidatively degrade many organophosphate, carbamate, pyrethroid and chlorinated insecticides. For instance, the diazinon-selected Fc housefly strain showed increased ability to oxidize DDT (Oppenoorth and Houx, 1968), hydroxylate naphthalene and epoxidize aldrin (Schonbrod et al, 1968) and a dimethoate-selected strain of the same species showed increased capacity for MFO oxidation of juvenoids, aldrin, dihydroisodrin and p-nitroanisole (Hammock et al 1977). Insecticide resistance due to increased MFO activity can be blocked by the use of MFO inhibitors, such as piperonyl butoxide and sesamex. These compounds can thus act as synergists for insecticides normally degraded by the MFO system.

Much of the study of the insect MFO system has been performed on the housefly Musca domestica (Reviewed by Hodgson, 1983). Increased cytochrome P-450 levels have been reported for a number of different housefly strains possessing high oxidase levels. Although cytochrome P-450 may be present in increased quantities in a resistant strain, there is not necessarily a correlation between it and MFO activity. For example, the housefly strains Fc and R-Baygon have high NADPH-dependent oxidase activity, yet they do not have increased cytochrome P-450 levels over those of susceptible strains (Plapp and Casida, 1969). Hodgson (1983) reports that there has not been an insecticide resistant strain yet measured with more than a two-

fold increase in cytochrome P-450, whereas detoxification rates are often many times the susceptible values, as measured by enzymatic assays. This suggests that cytochrome P-450 may not be the limiting factor in MFO activity in resistant strains, or that some isozymes of cytochrome P-450 are elevated, while most remain constant.

Section 6: The Mixed Function Oxidase System in Drosophila melanogaster.

The mixed function oxidase system in Drosophila melanogaster has been shown to be inducible by a number of substances, to consist of several different isozymes, and to have the capacity to metabolize a number of substrates. Developmental differences in its inducibility and expression have been shown and a marked genetic variation of expression has been observed between different strains. Increases in specific protein bands in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) of strains with high expression of the MFO system have been shown and putative genetic locations responsible for its expression have been proposed. Increased expression of cytochrome P-450 has been associated with resistance to organophosphorous insecticides and various pleitropic effects of high expression have been observed.

Section 6.1: Induction

The cytochrome P-450 system in Drosophila is inducible just as that of mammals. Magnusson et al. (1978) found that treatment of Drosophila melanogaster males with vinyl chloride (VC) gave an increased frequency of complete and mosaic recessive lethals and that pretreatment with phenobarbitol (PB) increased the mutagenic effects of VC. This suggested that Drosophila possessed an MFO

system capable of activating vinyl chloride and that this system was inducible by PB, a classical inducer of the mammalian MFO system. Hallstrom et al (1981) found that larvae pretreated with P-450 inducers such as PB, B-naphthoflavone (BNF), or polychlorinated biphenyls (PCBs) had an increase in a number of cytochrome P-450 mediated enzyme activities as well as an increase in the amount of cytochrome P-450. A variability in the induction response was observed between different strains of the fly. Differences in the induction response have been observed between larval and adult stages. Hallstrom et al (1983) found that pretreatment with inducers increased larval P-450 content and various P-450 mediated activities up to 7-fold, while the effect of pretreatments in adult flies was weaker, never more than 3-fold, and often these reactions were unaffected by pretreatment of adults.

Section 6.2: Different Forms of Drosophila Cytochrome P-450.

Different molecular forms of the cytochrome P-450 enzyme have been observed in Drosophila. Naquira et al (1980) purified and characterized three different forms of the enzyme from untreated flies of a wild-type strain and found that they differed with respect to relative molecular mass (Mr) <values of 51,750, 50,800 and 54,800 were obtained>, spectral and catalytic properties.

Section 6.3: Substrates.

The cytochrome P-450 system in Drosophila melanogaster acts on a number of different substrates in vitro and thus mediates a number of different enzymatic activities. Hallstrom et al (1981) demonstrated the ability of subcellular fractions of Drosophila melanogaster to metabolically activate vinyl chloride, 2-aminoanthracene and benzo(a)pyrene to mutagens of Salmonella typhinurium in vitro. A large range of cytochrome P-450 enzyme activities have been demonstrated in Drosophila, such as BP hydroxylase, PNA demethylase and 7-EC deethylase activities (Hallstrom et al, 1983). This group demonstrated a DMN demethylase with levels of activity comparable to that found in uninduced rat liver in larval and adult forms of Drosophila. The expression of these enzyme activities has been found to be both genotype and age dependent (Waters et al, 1984).

Section 6.4: Strain Differences.

Differences in both inducibility and expression have been observed between different strains of Drosophila melanogaster. Insecticide resistant strains such as Hikone R have been shown to have relatively high constituitive levels of cytochrome P-450 and its mediated activities and to be poorly inducible, while an insecticide susceptible strain such as Berlin K was inducible, but had very low constituitive levels of P-450 activity (Zijlstra

et al, 1984).

Section 6.5: Microsomal Protein Differences.

A number of protein differences in SDS PAGE have been demonstrated among strains with high cytochrome P-450 levels. As mentioned, Naquira et al purified Drosophila microsomes and separated three different isozymes of cytochrome P-450 with molecular weights of 51,750, 50,800 and 54,800 kD. (Naquira et al, 1980). Hallstrom et al (1983) found that after pretreatment with inducers, microsomes from both adults and larvae exhibited increases in specific protein bands with apparent molecular weights of 51,000 to 58,000 kD. They also found that specific protein bands of 54,000 and 56,000 kD. were associated with insecticide resistant strains (Hallstrom and Blanck, 1984).

Section 6.6: Chromosomal Localization.

The genes responsible for controlling a number of cytochrome P-450 mediated activities in Drosophila melanogaster have been localized to the second and third chromosomes. Hallstrom et al (1982) found that a major gene responsible for the high metabolic activation capacity of the Hikone R strain could be localized to 67 cM on the second chromosome. They found that a dominantly inherited high PNA demethylation and BP-3-hydroxylation in insecticide resistant strains was regulated by a gene located

near 65 cM on the second chromosome. Hydroxylation of BP and deethylation of 7-EC were shown to be determined by two third chromosomal genes at 51 and 58 cM respectively. They found the capacity for BP hydroxylation is determined by two genes on the second chromosome, one near black (48.5 cM) and one at about 63 cM (Hallstrom, 1985). Altogether, they postulated that the regulation of the cytochrome P-450 system in Drosophila melanogaster is controlled by four or five genes which determine the capacity for several reactions.

Section 6.7: Association With Insecticide Resistance.

High levels of cytochrome P-450 mediated enzyme activities in Drosophila melanogaster have been associated with resistance to insecticides. Hallstrom et al (1984) found that two insecticide resistant strains (Hikone R and a selected strain of Oregon R(R)) had 3 to 17 fold higher PNA demethylase and BP-3-hydroxylase than did other strains susceptible to insecticides. Zijlstra et al (1984) found that PNA demethylation and AP demethylation were substantially higher in all insecticide resistant strains studied, but that there was no correlation between insecticide resistance and BP hydroxylating capacity or the cytochrome P-450 content of the microsomes from different strains. Morton and Holwerda (1985) found that increased MFO activity was highly correlated with resistance to malathion and malaoxon (two organophosphorous insecticides) in a strain of

Drosophila selected for resistance to malathion.

Section 6.8: Pleiotropic Effects of High MFO Activity.

A number of reproductive and developmental defects were associated with increased MFO activity in Drosophila melanogaster by Halpern and Morton (1987). They found that increased MFO activity, associated with chromosomes II and III, were associated with high sterility, decreased fecundity and lower egg to adult survival. Strains with increased MFO activity associated with chromosome III also had slower rates of larval development. Strains homozygous for resistant chromosomes II and III had 20 to 30 fold increased prepupal peaks of MFO activity, indicating that genes on both chromosomes affected activity during larval as well as adult stages. They postulated that genes controlling high MFO activity on both chromosomes have a pleiotropic effect on adult reproduction and larval development, possibly through disturbing juvenile hormone and/or ecdysone metabolism.

Section 7: The Purpose of this Work.

In this study, an attempt was made to determine the genetic factors controlling MFO activity and malathion resistance in the larva and the adult of a malathion-resistant strain of Drosophila melanogaster. In addition, the developmental expression and tissue localization of high MFO activity in the larva and the

adult was characterized.

This thesis is divided into three parts. Part I examines the differences in cytochrome P-450 and related MFO activities in resistant and susceptible strains and compares the development and tissue expression of MFO activity in the larva and the adult. Part II describes experiments to map the location of genes on the second chromosome responsible for resistance to malathion in the larva and the adult. Part III concerns the mapping of genes on the second chromosome responsible for increased MFO activities.

Materials and Methods.

Section 1: Cultures of Drosophila melanogaster.

The strains studied were derived from a selection experiment described by Singh and Morton (1981). A genetically heterogeneous population of Drosophila melanogaster (locally caught) was exposed to malathion in the growth medium for 110 generations. This resulting population (MH-110) was then removed from selection and grown thereafter on banana medium. A number of isochromosomal strains homozygous for the first, second and third "resistant" chromosomes were derived from the MH-110 population after it had been removed from selection for about five generations. The extraction procedure made these resistant chromosomes homozygous within a susceptible background. These strains are described by Halpern and Morton (1987).

Canton Special (C.S.), a wild-type, susceptible strain was a gift of Margaret Kidwell; bwD/CyO was synthesized from stocks obtained from the Bowling Green Stock Center. The "all" (homozygous recessive mutations al dp b pr c px on the second chromosome); rucuca (homozygous recessive mutations ru h th cu sr e on the third chromosome); bvg (homozygous recessive mutations b vg on the second chromosome); and ve h th gl (homozygous recessive mutations on the third chromosome) were obtained from the Bowling Green Stock Center; the ri ss strain (homozygous

recessive mutations on the third chromosome) was obtained from the stock center at Umea, Sweden. The multiply marked strains were malathion susceptible and had low MFO activity in comparison to the malathion-selected strains. For a description of mutations, see Lindsley and Grell (1968).

Section 2: Chemicals.

Malathion (96% pure) was a gift of American Cyanimid Company, N.J.; p-nitroanisole (PNA) was purchased from Aldrich Chemical Company, Milwaukee, WI. and was recrystallized twice from ethanol; Glucose-6-phosphate dehydrogenase (grade I, from yeast) was purchased from Boehringer Mannheim, Canada. Other biochemicals were purcased from Sigma Chemical Company and were reagent grade.

Section 3: Maintenance of Fly Stocks.

Cultures were maintained in 350 ml glass bottles or 80 ml plastic vials on banana medium (13.5 g agar(Difco), 10 g malt powder, 10 ml corn syrup, 40 g dried yeast powder, 2.4 g p-hydroxy-benzoic acid methyl ester(Tegosept) and one banana in 1.2 litres of double distilled water) at 24 degrees celsius with a 12 hr day, 12 hr night light cycle.

Section 4: p-Nitroanisole Colourimetric Assay.

Crude enzyme preparations were made by the rotary homogenization of adult flies or larvae (approximately 5 flies

per 50 ul extraction buffer). The extraction buffer used was ice-cold TKEG (50 mM Tris-HCl, 50 mM KCl, 1 mM sodium EDTA, 10% Glycerol, pH 7.4) and the homogenization was performed using a glass tissue homogenizer with a teflon pestle run by a drillpress. Approximately 30-40 gentle up and down strokes were required with the rheostat set at 40 volts (200 rpm). The homogenates were transfered to 1.5 ml Eppendorf centrifuge tubes and centrifuged for 15 minutes at 4 degrees celsius in an Eppendorf table-top centrifuge. The supernatant was used for the PNA assay and the Bradford protein determination. One hundred microlitres of the supernatant was added to a 1.5 ml centrifuge tube with a reaction mixture containing 100 ul of assay substrate solution(TKEG+ 1 mM p-nitranisole, 1 mM NaNADP, 5 mM sodium glucose-6-phosphate) and 1 ul Glucose-6-Phosphate-Dehydrogenase. The tubes were placed into a shaking water bath and incubated at 30 degrees celsius for 30 minutes. At the end of this time, the reactions were stopped by the addition of 50 ul of 1 M HCl and were placed on ice. The product(p-nitrophenol) was then extracted by the addition of 1 ml of chloroform, vortexed, centrifuged in an IEC clinical table top centrifuge at room temperature for 5 minutes at 3000 rpm. Then 750 ul of the chloroform layer was removed and added to 1.5 ml of 0.5 M NaOH in a 15 ml glass centrifuge tube. This was vortexed for 20 seconds and centrifuged in an IEC clinical table-top centrifuge at room temperature for 5 minutes at 3000 rpm. The amount of product (p-nitrophenol) was determined from the absorbance of the NaOH layer at 400 nm using

a Perkin-Elmer Lambda spectrophotometer. The activity was calculated in pKatal/ml(a Katal is one mole of product per second based on the extinction coefficient of pNP=0.0184). The specific activity of the preparation was calculated by dividing the activity by the concentration of protein (determined from the Bradford Assay).

Section 5: Bradford Protein Assay.

To determine the concentration of protein in the crude enzyme homogenates, 50 ul of the supernatant of each extract was placed into 3 ml of 0.06% Coomassie brilliant blue G-250 in 1.9% perchloric acid. The samples were read at 610 nm in a Horizon digital colorimeter using 50 ul of TKEG in the coomasie solution as a blank. A standard curve was constructed using different concentrations of bovine serum albumin as standards.

Section 6: Ethoxycoumarin Fluorimetric Assay.

Single flies were homogenized in 40 ul extraction buffer (50 mM Tris, 50 mM KCl, 1 mM Sodium EDTA, 10% Glycerol, 0.5 mg/ml ovalbumin, pH 8.6) in individual 0.5 ml plastic centrifuge tubes using a smooth metal drill bit of appropriate size to fill the tube. The bit was motor driven at approximately 200 rpm for 20-30 strokes in order to sufficiently grind the flies but not to overgrind or increase the temperature of the grinding tube. The

homogenate was centrifuged in an Eppendorf table-top centrifuge for 15 minutes (4 degrees celsius) and the supernatant was assayed for its activity in deethylating 7-ethoxycoumarin to produce 7-hydroxycoumarin. Thirty microlitres of the supernatant was added to a test tube containing 90 ul of assay solution (extraction buffer containing 0.5 mM NaNADP and 2.5 mM NaG6P + 3 uM 7-ethoxycoumarin + 1 ul G6PD, pH 8.6). The tubes were shaken in a water bath (30 degrees celsius) for one hour and stopped by the addition of 20 ul of 2 N HCl. The product was extracted with 90ul of chloroform, then re-extracted into 3 ml of 0.5 M Tris. Fluorescence was measured with a spectrophotometer constructed by R. Gilies of the McMaster Biology Department. Excitation was at 380 nm (0.5 nm band width), and fluorescence was measured through a 400 nm cut-off filter. The amount of product was determined from the fluorescence by comparison to that produced when a known amount (250 pmoles) of 7-hydroxycoumarin was included in a reaction mixture stopped at t=0. Specific activities were calculated in pmoles of product produced per second per milligram of protein.

Section 7: <u>Dissection of Adult Tissues.</u>

Flies between 4 and 10 days old were anaesthetized with ether and individually placed into small glass dissecting wells. A drop (30 ul) of ice cold extraction buffer was added, and the flies were sectioned into head, thorax and abdomen using a small

scalpel. Each of these sections was grouped until there were enough to assay (23 of each). Twelve whole flies were also assayed using the 7-EC fluorimetric assay.

After localizing the high MFO activity to the abdomen of the adult, a more specific dissection was undertaken. The flies were treated as above. Individual whole flies were dissected in a drop of ice-cold extraction buffer using fine dissection forceps, under a binocular dissecting microscope. The intestines, malpigian tubules, abdominal walls and remaining corpse were pooled and assayed separately. The liquid in which the flies were dissected was also assayed to determine if any activity could be detected. A comparable number of flies were also mock dissected, opened in dissection liquid, but not separated into tissues to determine the loss of activity due to dissection.

Section 8: <u>Dissection of Larval Tissues</u>.

Third instar larvae (4 days old, still feeding in the medium) were removed from the medium, rinsed with extraction buffer and dissected individually in a drop(30ul) of ice cold extraction buffer under a binocular dissecting microscope, using fine dissection forceps. A mock dissection where the larva was opened in dissection liquid but not separated into different tissues was also performed to determine the loss of activity due to dissection. The larval intestine, malpigian tubules, fat body and remaining corpse were pooled and assayed separately as was

the dissection liquid.

Section 9: Adult Survival Test.

The adult survival test was used to determine what percentage of adult flies of a particular genotype would survive exposure to different concentrations of malathion. Young adults (2-5 days post eclosion) were maintained on fresh banana medium seeded with live yeast. Groups of flies of the genotype(s) to be tested were quickly anaesthetized with carbon dioxide and were placed in groups of 15-20 into 15ml plastic centrifuge tubes containing 3ml of 1.5% agar- 1% sucrose made 24 hr previously containing varying concentrations of malathion. The tubes were stoppered with sponge and an initial observation was made to determine that all flies had survived the anaesthesia and were moving. If any flies had not survived, this was noted. The tubes were left stoppered for 24 hrs at 24 degrees celsius (12 hr day, 12 hr night) and after this time period, the number of survivors was counted. The percentage of the number of survivors of each genotype at each concentration was then calculated.

Section 10: Egg to Pupa Survival Test.

The egg to pupa survival test was used to determine the viability of progeny from different crosses exposed to various concentrations of malathion. It differed from the adult survival

test in that in this test, the actively feeding larvae were exposed to malathion in their food. Groups of male and virgin female flies were crossed and placed on vials of normal banana medium for one to two days. They were then transferred to vials of banana medium containing different concentrations of malathion (0,1,2,3 or 4 uM) which had been prepared the previous day. The females were allowed to lay eggs for a period of approximately 8 hours, whereupon the vials were cleared and the number of eggs per vial counted. The vials were then incubated at 24 C and the eggs were allowed to develop until pupation. Seven days after egg deposition, the number of pupae in each vial was counted and the percentage survival of each genotype at each concentration of malathion was calculated. The vials that had no malathion added to them were used as controls, because in a number of the resistant lines, egg survival was significantly below 95-100%.

Section 11: <u>Microsome Preparations</u>.

All procedures were performed at 2-4 degrees celsius. One to two grams of etherized adult flies were gently homogenized in 15 ml of TKE buffer (0.05M Tris, 0.05M KCL, 0.001M EDTA, pH 7.4) for approximately one minute using a 40 ml glass homogenizing vessel with a teflon pestle. The pestle was motor driven at approximately 100 rpm. The homogenate was centrifuged at 3000g (5000 rpm) for 5 minutes, then the supernatant was removed and centrifuged twice at 13,000 rpm (20,0000g) for 20 minutes to

remove mitochondria (Servall SS-34 rotor). The resulting "post-mitochondrial" supernatant was ultracentrifuged at 135,000g (27,000 rpm, SW 27.1 rotor) for 90 minutes to precipitate out the microsomes. The supernatant was discarded and the microsomal pellet was gently resupended in 0.5 to 1.0 ml (depending on amount of flies used) of "microsome-phosphate buffer" (60 mM sodium phosphate buffer pH 7.4, 2 mM MgCl, 2% glycerol) to yield the microsome preparation. This was either used immediately for P-450 content calculations, ethoxycoumarin assay or 1-D gels, or else was frozen and stored at -70 C for up to 3 months. The protein yield from such an unwashed microsome preparation was approximately 5 mg per gram of flies.

Section 12: <u>Assay of Cytochrome P-450 Content</u>.

Cytochrome P-450 content was measured as described previously by Brattstein et al (1980). Microsome preparations resuspended in microsome phosphate buffer (60 mM sodium phosphate pH 7.8, 2 mM MgCl, 2% Glycerol) were diluted 1:1 with 0.3 M potassium phosphate buffer pH 7.8 containing 50% glycerol. Approximately 2 mg of sodium dithionate was added to reduce the cytochrome P-450. The reduced solution was placed in a 3 ml quartz cuvette(1 cm pathlength) and a baseline spectrum was recorded from 500 to 400 nm using a Beckman DU 7 spectrophotometer. Carbon monoxide was then gently bubbled through the sample and a second, CO bound reduced spectrum was

recorded. Cytochrome P-450 content was calculated based on the difference in absorbance between 450 and 490 nm using an extinction coefficient of 91 mM^{-1} cm^{-1} as described by Omura and Sato(1964).

Section 13: SDS Polyacrylamide Gel Electrophoresis.

SDS polyacrlamide gel electrophoresis was performed according to the method originally described by Laemmli (1970), and later modified by Coulthart (1985). The stacking gel contained 4.75% and the separating gel 10% polyacrylamide. Approximately 0.1 ug of microsomal protein was applied to each well. The gels were stained with silver according to the method of Morrisey (1981) as modified by Coulthart (1985).

Section 14: Calculations.

The following are calculations of map postions for two genes for high PNA demethylation in the region from black (48.5 cM) to vestigial (67.0 cM) that are consistent with the recombinant data (see Results Section 3.3). A2 (the second activity gene) is assumed to be at 64 cM from previous data, calculations shown here are for the position of A1.

Class	Activity	S.E.
b+ vg+	6.00	1.13
b vg+	4.35	0.65
b+ vg	3.40	0.55
b vg	2.50	0.30

Intermediate Activity between b+ vg+ and b vg activities= 4.25

Map Distance between black and vestigial= 18.5 cM.

Assume if recombinant receives A1 or A2, activity=4.25

If recombinant receives neither A1 nor A2, activity= 2.50

If recombinant receives both A1 and A2, activity= 6.00

Model 1: A1 at 54.5 cM, A2 at 64 cM.

b	A1	A2	vg
48.5	54.5	64	67
6	9.5	3	

b vg+ recombinant class		b+ vg recombinant class	
6/18.5 (6.00)=	1.94	6/18.5 (2.5)=	0.81
9.5/18.5 (4.25)=	2.18	9.5/18.5 (4.25)=	2.18
3/18.5 (2.5)=	0.40	3/18.5 (6.00)=	0.97
	4.52		3.96

4.52 is consistent with the actual b vg+ activity; 3.96 is just consistent with the b+ vg activity. 54.5 cM is thus the mininum value for the position of A1 that is consistent with both recombinants.

Model 2: A1 at 60.0 cM, A2 at 64 cM.

b		A1		A2		vg
48.5		60		64		67
	11.5		4		3	

b vg+ recombinant class		b+ vg recombinant class	
11.5/18.5 (6.00)-	3.73	11.5/18.5 (2.5)-	1.55
4/18.5 (4.25)-	0.92	4/18.5 (4.25)-	0.92
3/18.5 (2.5)-	0.40	3/18.5 (6.00)-	0.97
	5.05		3.44

5.05 is just consistent with the actual b vg+ activity; 3.44 is consistent with the b+ vg activity. 60.0 cM is thus the maximum value for the position of A1 that is consistent with both recombinants.

The following are calculations of map positions for two genes for high 7-EC hydroxylase activity in the region from black (48.5 cM) to vestigial (67.0 cM) that are consitent with the single-fly recombinant data (see results Section 3.4). A2 (the second high activity gene is assumed to be at 64 cM from previous data; calculations shown here are for the position of A1.

Class	Activity	S.E.
b+ vg+	0.0321	0.0011
b vg+	0.0231	0.0010
b+ vg	0.0173	0.0010
b vg	0.0170	0.0007

Intermediate activity between b+ vg+ and b vg activities= 0.0245

Map distance between black and vestigial= 18.5 cM.

Assume if recombinant receives both A1 and A2, activity= 0.0321

If recombinant receives A1 or A2, activity= 0.0245

If recombinant receives neither A1 nor A2, activity= 0.0170

From prior calculations, it was found that there is no position for A1 that is in agreement with both b vg+ and b+ vg data. What is shown are separate calculations for each recombinant class for a map position for A1 that is consistent with its data.

<u>b vg+ recombinant class</u>:

Model 1: A1 at 48.5 cM; A2 at 64 cM.

b		A1		A2		vg
48.5				64		67
	0		15.5		3	

0/18.5 (0.0321)= 0

15.5/18.5 (0.0245)= 0.0205

3/18.5 (0.0170)= 0.0027

0.0232

This value is consistent with the b vg+ data.

Model 2: A1 at 51 cM; A2 at 64 cM.

b		A1		A2		vg
48.5		51		64		67
	2.5		13		3	

2.5/18.5 (0.0321)= 0.0043

13/18.5 (0.0245)= 0.0172

3/18.5 (0.0170)= 0.0027

0.0242

This value is just consistent with the b vg+ data, hence 51 cM is the maximum value for the position of A1 that is consistent with the b vg+ data.

<u>b+ vg recombinant class</u>:

Model 1: A1 at 64 cM, A2 at 66 cM.

b	A1	A2	vg
48.5	64	66	67
15.5	2	1	

15.5/18.5 (0.0170)= 0.0142

2/18.5 (0.0245)= 0.0026

1/18.5 (0.0321)= 0.0017

0.0185

This value is just consistent with the b+ vg data, hence a locus at 66 cM is the mininum locus for A2 that is consistent with this data.

Model 2: A1 at 64 cM, A2 at 67 cM.

b	A1	A2,vg
48.5	64	67
15.5	3	0

15.5/18.5 (0.0170) = 0.0142

3/18.5 (0.0245) = 0.0245

0/18.5 (0.0321) = 0

0.0181

This value is consistent with the actual b+ vg data and is thus the maximum possible locus for A2 within the interval that is consistent with this data.

RESULTS

Part I: Expression of Cytochrome P-450 and Related MFO Activities.

Section 1.1 Malathion Resistance and MFO Activity of Selected Strains.

Halpern and Morton (1987) derived strains which were homozygous for either second or third chromosomes from a population which had been selected in the laboratory for resistance to malathion. Increased MFO activity and malathion resistance (relative to a control population or the susceptible strain Canton S) were found to be associated with genes on both chromosomes 2 and 3. Their data is summarized in Table 1. Microsomal proteins from several of their strains, homozygous for chromosomes 2 or 3, were examined by SDS PAGE. Increased amounts of protein having a relative molecular mass of 52 Kd were found in microsomes from chromosome 2 strains, and increased amounts of microsomal proteins of relative molecular mass of 51 and 55 Kd were found in chromosome 3 strains (see Figure 1). These data are also summarized in Table 1. These proteins are of the appropriate molecular weight to be cytochrome P-450 species and it is possible that increased cytochrome P-450 content, controlled by genes on the second and third chromosomes contributed to the resistance of these strains to malathion.

Figure 1.

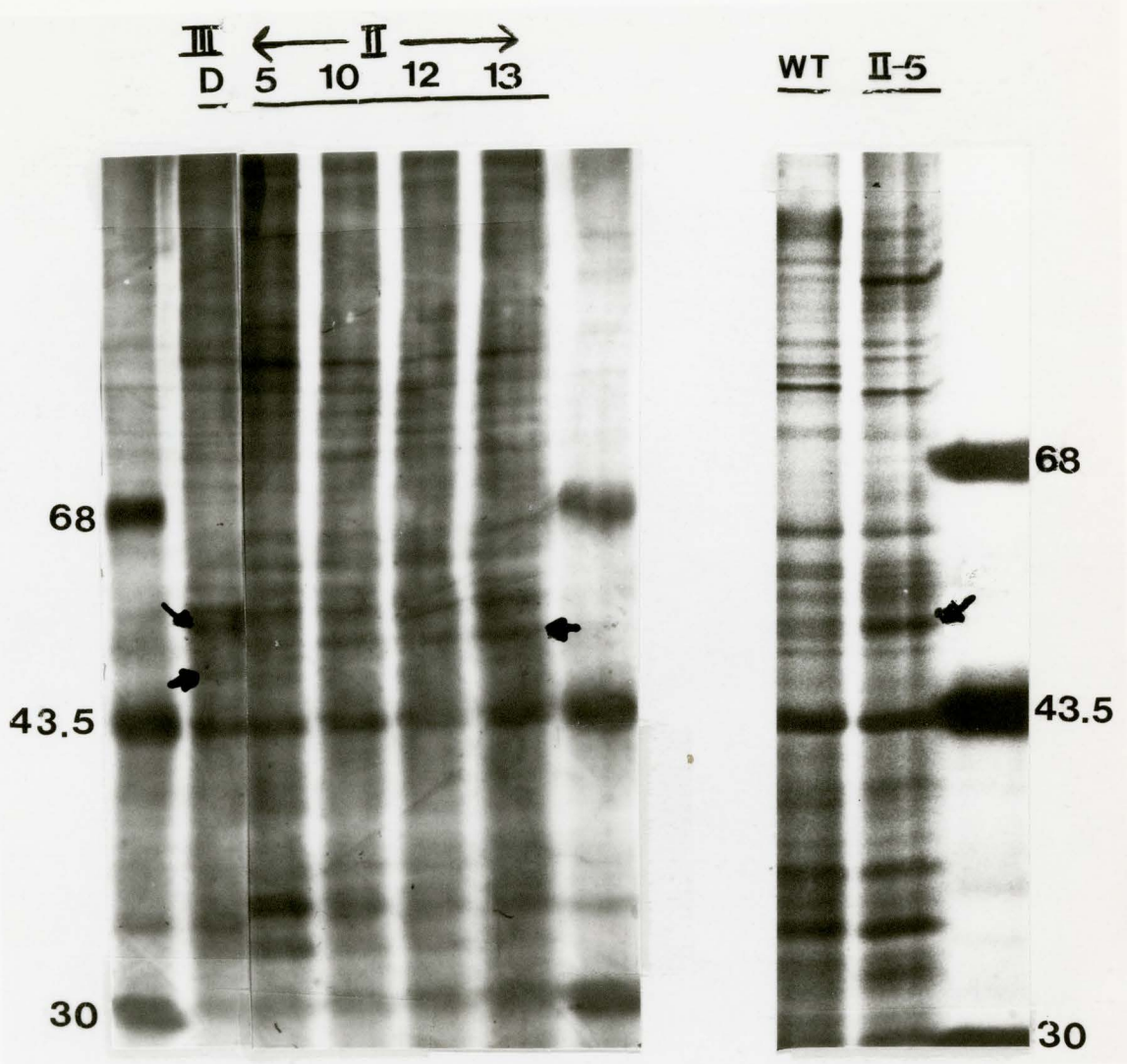
One Dimensional Polyacrylamide Gel Electrophoresis of Resistant and Susceptible Strains.

Microsomal preparations were electrophoresed on a 10% SDS Polyacrylamide gel; Molecular weight markers are: Bovine serum albumin (68 kD), Ovalbumin (43.5 kD) and Bovine carbonic anhydrase (30 kD). Wild Type (W.T. -Canton S) and II-5 lanes were provided by J. Pursey (unpublished results). The gels were silver stained according to the method of Morrisey (1981) as modified by Coulthart (1985). The arrows indicate bands of increased amounts of protein associated with resistant second and third chromosome strains.

Figure 2.

The Cytochrome P-450 Content of Resistant and Susceptible Strains.

Cytochrome P-450 content was determined on unwashed microsomal preparations and calculated from the difference in absorbance between 450 and 490 nm using an extinction coefficient of 91 /mM cm.


III
D
II
5
10
12
13
WT
II-5
68
43.5
30
68
43.5
30

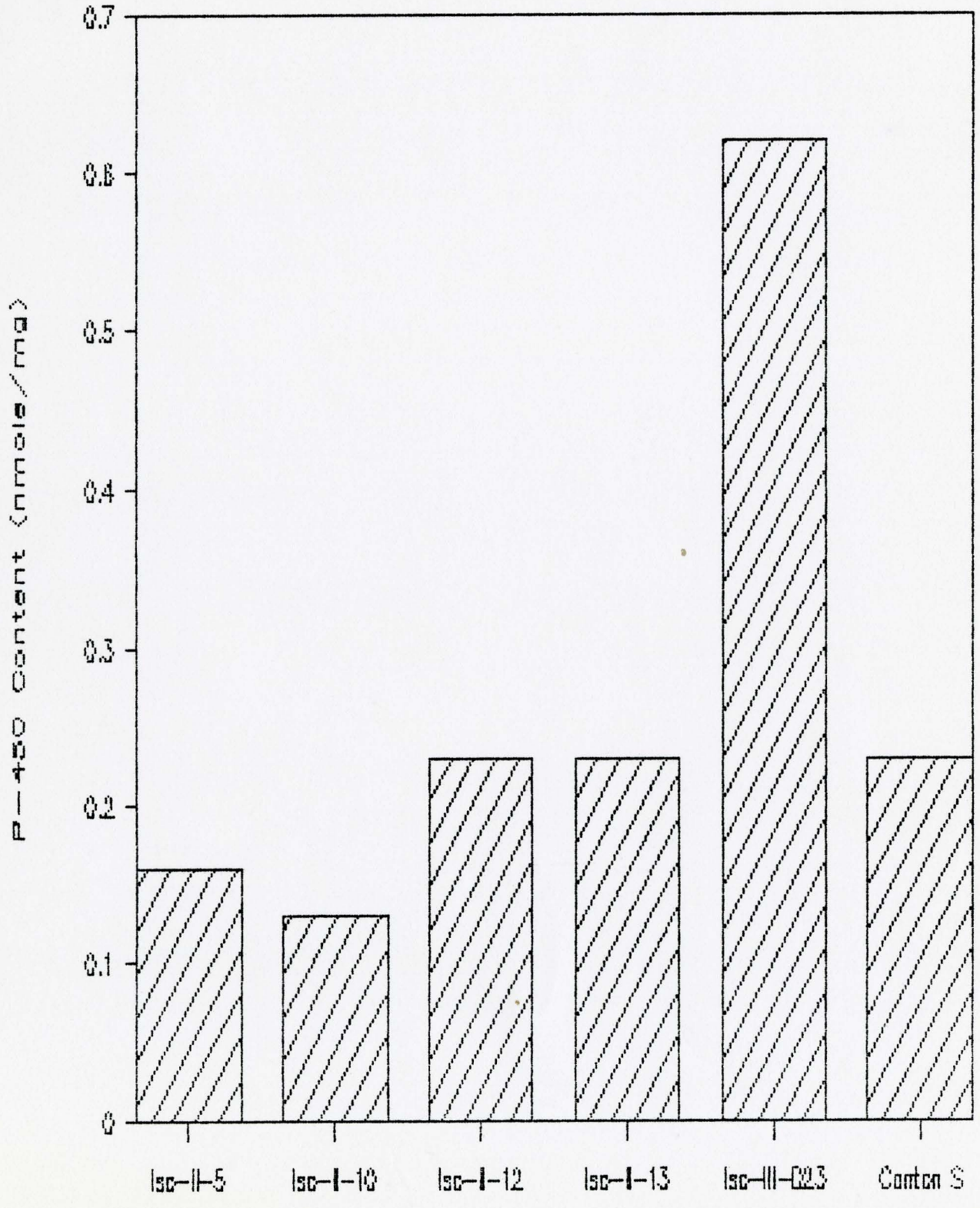

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
P-450 Content (nmole/mg)
Isc-II-5
Isc-I-10
Isc-I-12
Isc-I-13
Isc-III-B2.3
Canton S
Strain

Table 1: Malathion Resistance, MFO Activity of Adults and Associated Increased Protein Bands of D. melanogaster Strains.

Strain	N[b]	LC-50[a]	MFO Activity[a,c]	Microsomal Protein[d]
Control	-	13(8-13)	6.2(8-13)	N.D.
Canton S	-	9	1.9(0.8-2.4)	-
Iso-chrom. II	9	32(23-49)	8.9(7-12)	52 Kd.
Iso-chrom. III	8	80(48-150)	11.3(7-15)	51,55 Kd.

a
LC50 and MFO Data from Halpern and Morton (1987).

b
N is the number of independently isolated iso-chromosomal strains tested. For the Control and Canton S strains, the range of replicated measurements is shown in parentheses; for the iso-chromosomal strains the number in parentheses is the range of intra-strain means.

c
MFO Specific Activity as measured by the rate of O-demethylation of p-nitroanisole in pmoles/sec mg measured using crude fly extracts.

d
Relative Molecular Mass (Mr) of increased microsomal proteins as determined from Figure 1. SDS PAGE spectrum of microsomal proteins from the Canton S strain was provided by J. Pursey (unpublished results).

Section 1.2 Microsomal Cytochrome P-450 and MFO Activity.

The total cytochrome P-450 content of microsomes from several different strains homozygous for resistant second chromosomes from the original selected population were compared to one strain (IIID) homozygous for a resistant third chromosome and to the susceptible Canton S strain (homozygous susceptible). These data are summarized in Figure 2. Although crude extracts of Iso-chromosomal-II strains did not have an elevated cytochrome P-450 content relative to the susceptible Canton S, increased MFO activity was measured in their microsomal preparations using the 7-EC MFO assay (data not shown). On the other hand the resistant Iso-III-D (IIID) strain had a markedly elevated cytochrome P-450 content and MFO activity (data not shown). Therefore, genes on chromosomes 2 and 3 control different microsomal proteins and probably different P-450 species.

Section 1.3 Comparison of PNA and 7-EC MFO Assays.

This research has concentrated on the chromosome 2 gene(s) controlling resistance and their relation to MFO activity. During the course of this work, two different substrates for MFO activity were used and it was important to determine if the increased MFO activity associated with either chromosome 2 or chromosome 3 behaved differently with respect to these two substrates. Therefore, relative activities from males and females

of a resistant chromosome 2 strain and a resistant chromosome 3 strain were compared for both substrates. The results are shown in Table 2. The specific activities measured by PNA demethylation in this experiment were abnormally high (eg. compare with table 1). This could be due to the 7-EC extraction buffer used in the grinding of the flies which has a higher pH than the regular PNA extraction buffer. The only significant difference between those of the same gender (t-test, 95% confidence level) is between females of strain II-10 and III-D for the PNA substrate. In addition, however, there is a general trend for strain III-D to have a greater MFO activity for either substrate. There is no indication that the MFO system associated with either chromosome 2 or 3 has a preference for one of these substrates.

Table 2: Comparison of PNA and 7-EC MFO Assays.[a]

Strain	Assay: PNA[b] Average	PNA S.E.	PNA N[d]	Assay: 7-EC[c] Average	7-EC S.E.	7-EC N
Iso-II-10						
Males	22.4	(2.2)	12	1.15	(0.20)	3
Females	19.7	(0.5)	10	1.10	(0.10)	4
Iso-III-D23						
Males	27.2	(2.2)	12	1.15	(0.16)	4
Females	27.9	(1.0)	10	1.44	(0.25)	4

[a] Adult flies were extracted in 7-EC extraction buffer and assays for PNA and 7-EC MFO Activity were performed as described (see materials and methods).

[b] PNA Specific Activity is the rate of O-demethylation of p-nitroanisole in pmoles/sec mg as measured using crude fly extracts.

[c] 7-EC Specific Activity is the rate of deethylation of 7-ethoxycoumarin in pmoles/sec mg as measured using the same crude fly extracts.

[d] Number of replicate measurements.

Section 1.4 Developmental Profile of MFO Activity.

The developmental profile of MFO activity vs age, associated with a resistant chromosome 2 strain was characterized. Two to ten day old Iso-II-10 flies, raised on banana medium seeded with live yeast were placed on fresh cultures seeded with live yeast for periods of six to eight hours. Eggs or larvae from these cultures were sampled at different time intervals and groups of each were assayed using the 7-EC MFO assay to determine their MFO specific activities. The results are shown in Figure 3a. The specific activity of the 6 hour old egg was very low. The specific activity of the developing larva rose continuously to a peak at 4 days old (3rd instar larvae in medium) and then dropped precipitously as the larvae began to pupariate. The activity of the pupae eventually became too low to measure at 7 days (2 day old pupae). If, because of crowded conditions, the larvae had not yet pupated by 5 days (exhibiting a developmental delay), their activity continued to be very high (1.55 pmoles/sec mg), while if they pupated, their activity dropped to 0.30 pmoles/sec mg. This indicates that developmental stage (larva vs pupa), rather than strictly the age of the organism has a more profound effect on the MFO specific activity. Figure 3b indicates the cumulative number of larvae that had pupated by the time indicated. Most larvae pupariate between the fifth and sixth days, with all of them pupating by the ninth day (under the conditions sampled).

To characterize the changes of MFO activity following

Figure 3a.

The Developmental Profile of MFO Activity in Larvae.

The age is the average time between egg deposition and measurement. The larvae pupated at approximately the fifth day. MFO activity was measured using the 7-EC Fluorimetric Assay. Larvae used were of the Iso-II-10 strain. Specific Activity is measured in pmoles sec^{-1} mg^{-1}.

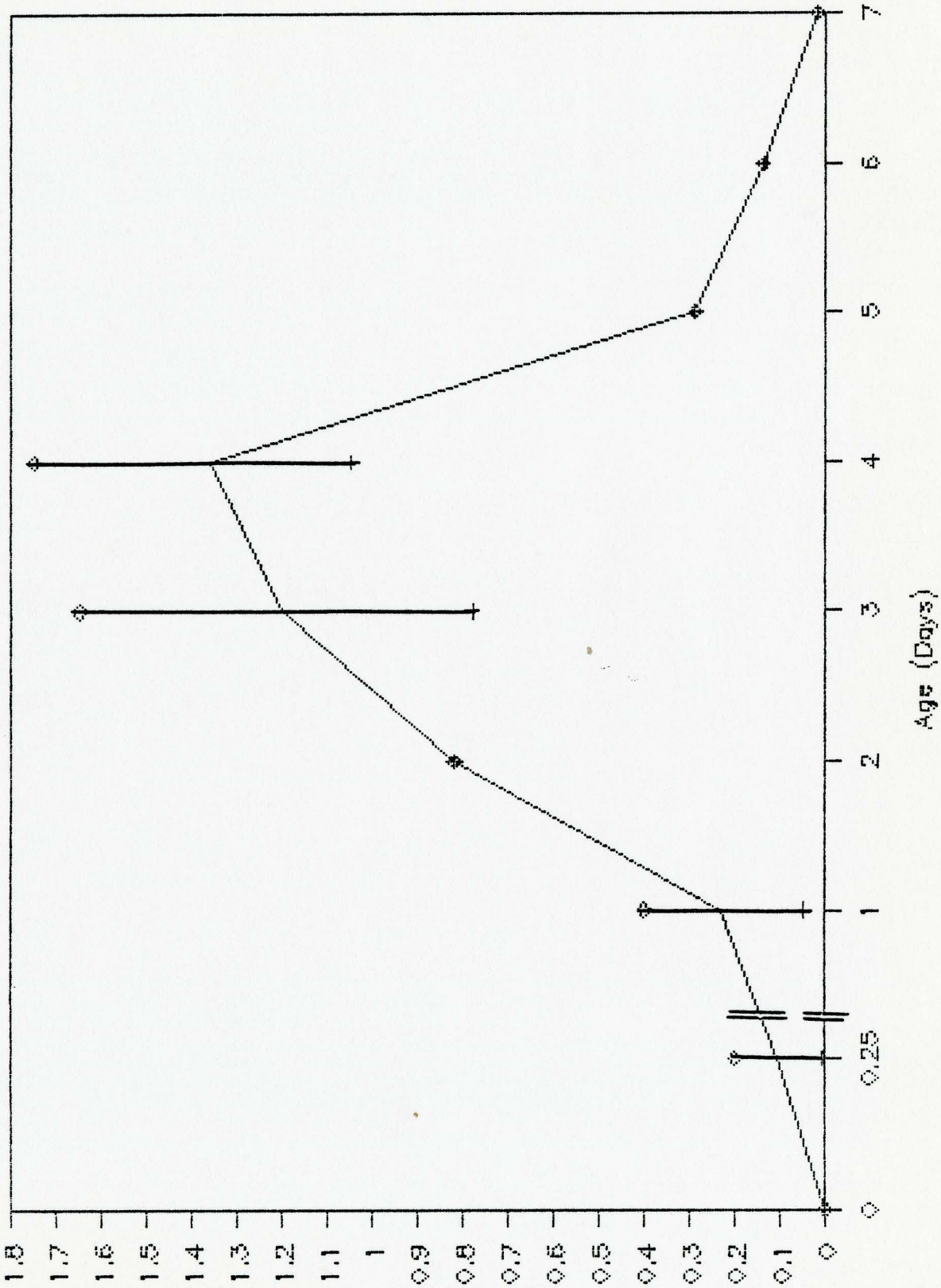
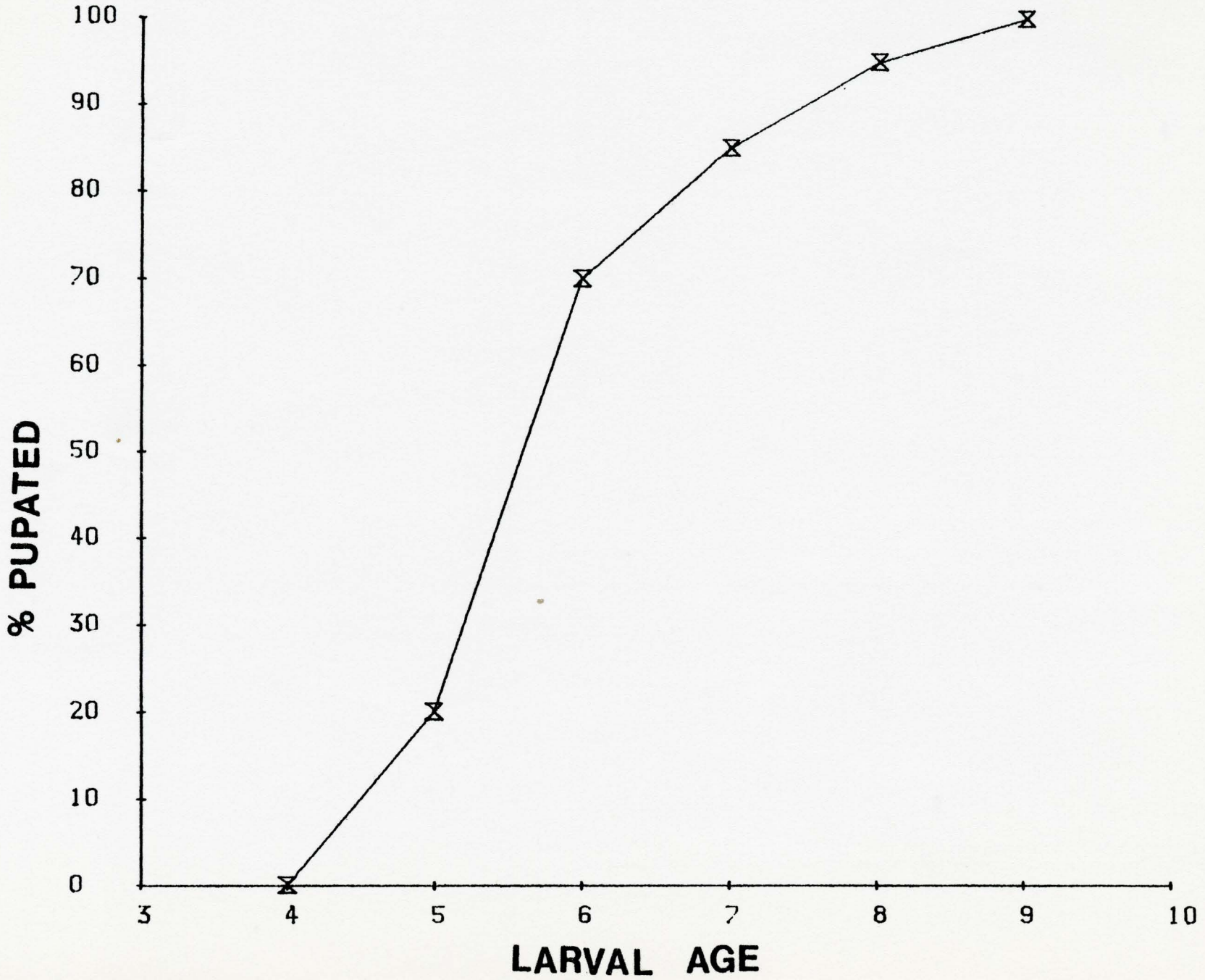

1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.25
1
2
3
4
5
6
7
Age (Days)

Figure 3b.

Pupation of Larvae of Iso-II-10 Strain.

The plot shows the cumulative percentage of larvae pupating over time for the Iso-II-10 strain. Larvae were maintained at 24 C with a 12 hr day/ 12 hr night cycle.

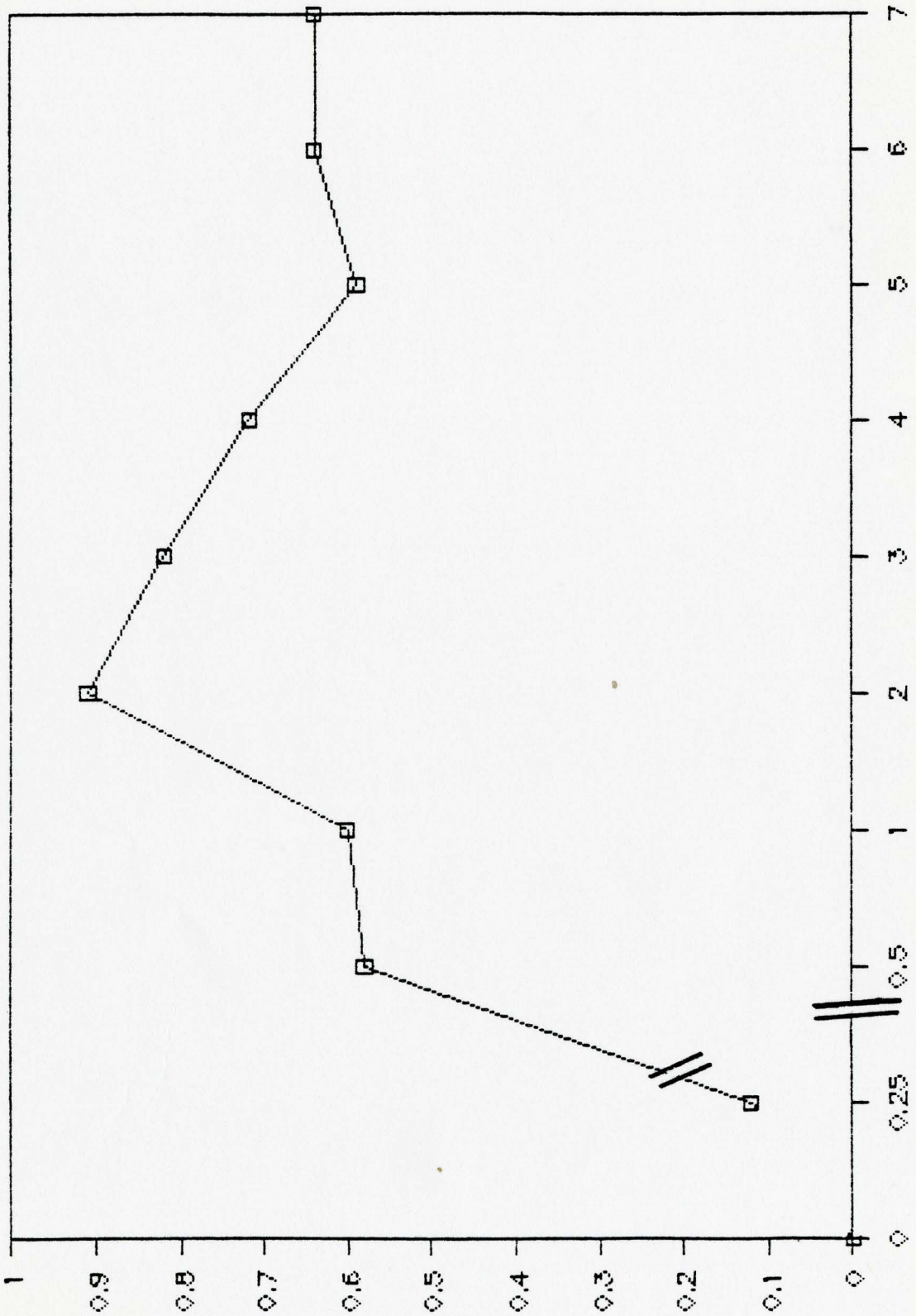

100
90
80
70
60
50
40
30
20
10
0
% PUPATED
3
4
5
6
7
8
9
10
LARVAL AGE

eclosion in the imago, groups of increasingly older Iso-II-10 flies were assayed. Newly eclosed adults were taken from their original media and placed on fresh banana medium seeded with live brewers yeast. Groups of females were assayed at different intervals post eclosion using the 7-EC MFO assay. The results are shown in Figure 4. The lowest activities were found in very young flies (<12 hours old). The MFO specific activity rose to a peak at 2 days and then slowly dropped, levelling off at about 0.65 pmoles/sec mg at 5 days post eclosion.

Figure 4.

The Developmental Profile of MFO Activity in the Adult.

Iso-II-10 adults were assayed for MFO activity using the 7-EC Fluorimetric Assay on crude extracts. MFO specific activity measured in pmoles hydroxycoumarin produced per second per mg protein.


MFO Specific activity
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.25
0.5
1
2
3
4
5
6
7
Days Post Eclosion

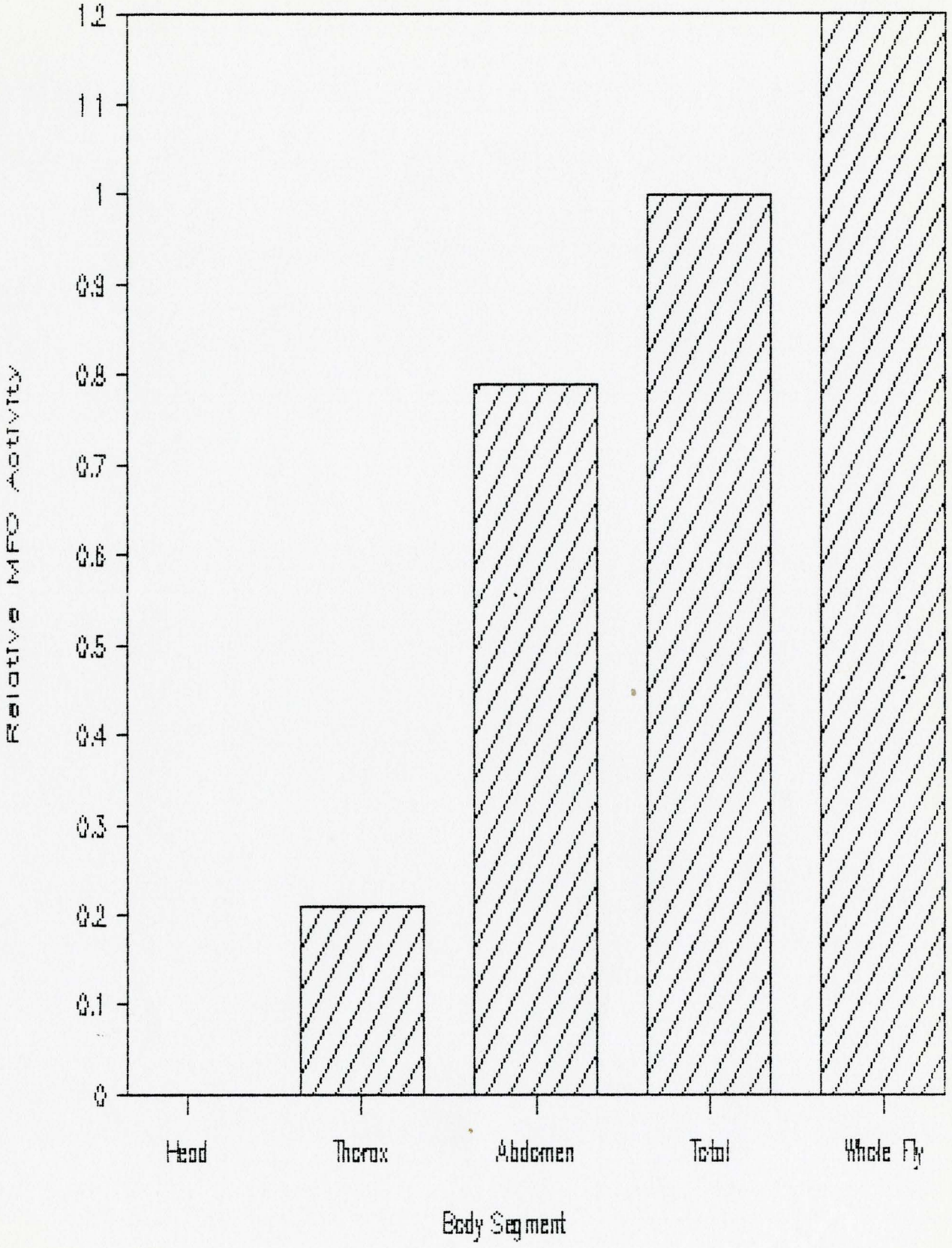
Section 1.5 Distribution of MFO Activity in Larval and Adult Tissues.

In order to determine whether there were tissue differences in expression of MFO Activity in a strain of *Drosophila* that was homozygous for a resistant second chromosome, an attempt was made to determine the tissue location of the MFO activity of the larvae and the adult of Iso-II-10. Initially it was found useful to determine which section of the adult (head, thorax or abdomen) showed the greatest contribution to the total activity of the adult. Twenty-three flies between 4 and 10 days old were ether anaesthetized and individually sectioned into head, thorax and abdomen. Each of these sections was grouped until there were enough to assay (23 of each section). Twelve whole flies were also assayed for MFO activity using the 7-EC fluorimetric assay. The results are shown in Figure 5. It can be seen that the majority of the activity (80%) was localized to the abdomen of the fly, the remainder (20%) was found in the thorax. No activity could be measured in the heads of the flies. Since fewer whole flies than dissected flies were assayed, the total activity (and total protein content) of the whole flies was lower than that of the dissected parts. The activities were adjusted by correcting the total extract protein measurements of the whole fly to that of the dissected parts. In this way, the 'whole fly' and the 'dissected parts' activities could then be compared. This

Figure 5.

MFO Activity of Segments of Adult Fly.

Twenty-three Iso-II-10 adult females were dissected in ice-cold 7-EC extraction buffer; 12 whole flies were used to measure 'whole-fly' activities. MFO activity was measured using the 7-EC Fluorimetric Assay. The activities were normalized to 1.0 for the total of the three body segments. The "whole flies" were corrected for the same total protein.


Relative MFO Activity
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Head
Thorax
Abdomen
Total
Whole Fly
Body Segment

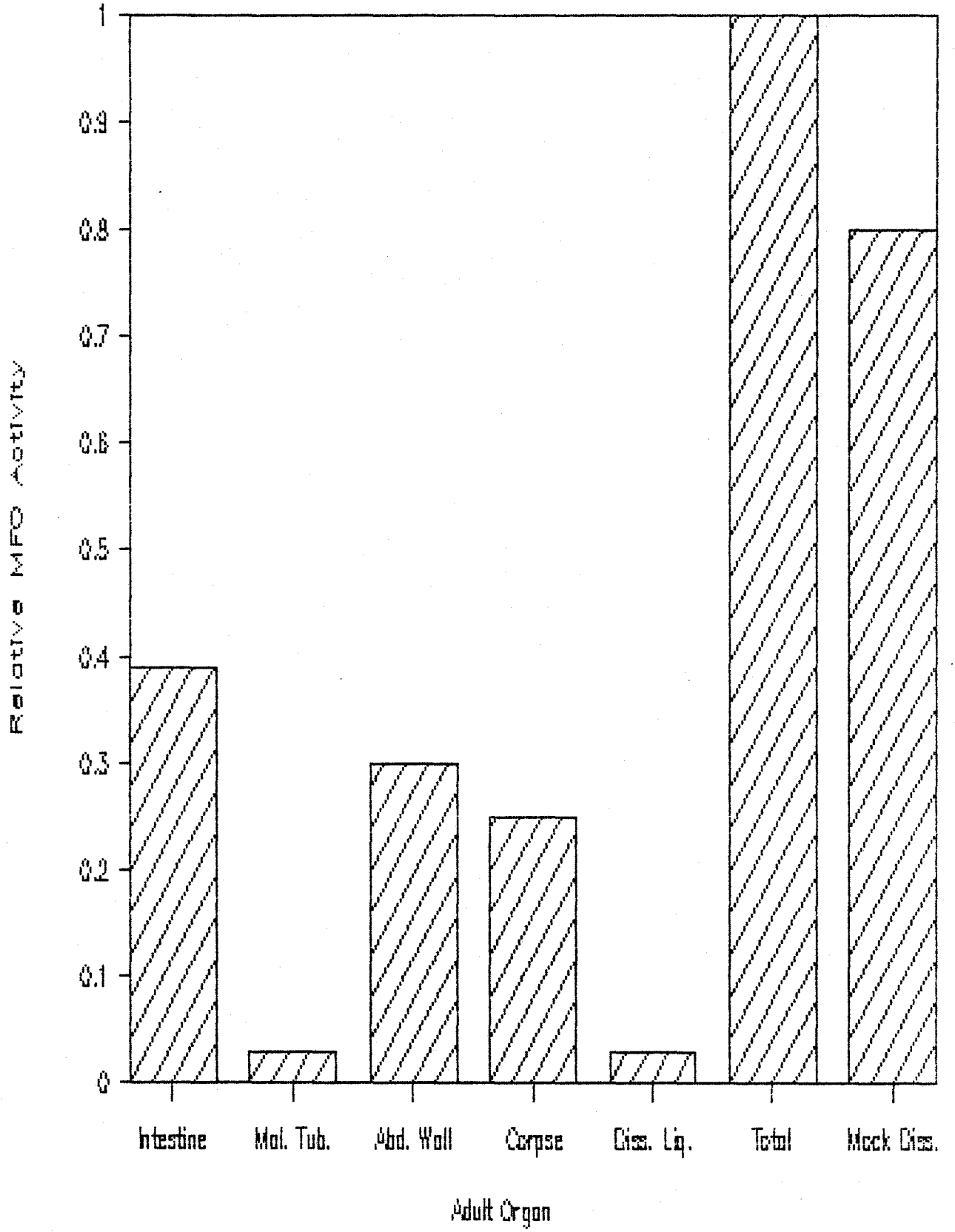
comparison showed that only 85% of the activity could be recovered after the dissections. In terms of specific activity, it was found that just as for the total activity, the abdomen had the highest specific activity, although the difference between it and the thorax was not as great as the total activity difference. This suggests that one reason the abdomen contributed so much to the total activity was due to its large contribution to the total protein.

After establishing that the abdomen contributed the most to the total MFO activity of the adult, a more stringent determination of the tissue distribution of activity was undertaken. Figure 6 shows the results. Twenty individual whole female flies were dissected in ice cold extraction buffer. In the determination of the tissue distribution of MFO activity, it was assumed that activity is proportional to the number of flies per sample. The intestines, malpigian tubules, abdominal walls and remaining corpse were assayed separately. The liquid in which the flies were dissected was also assayed to see if activity could be measured. A comparable number of flies were also mock dissected to determine how much activity was lost due to dissection into tissues. This "mock dissection" involved opening individual flies, not separating the organs, but rather assaying groups of complete mock dissected flies. More activity was recovered from the total of all the dissected parts than from the mock dissection. This could be due to proteolytic degradation taking place in the mock dissection to a greater extent than occured to

Figure 6.

MFO Activity of Organs of the Adult.

Twenty Iso-II-10 adult females were used for dissections and twenty females were "mock-dissected" and then assayed to determine the loss of activity due to dissection. The flies were individually dissected in ice-cold 7-EC extraction buffer. MFO Activity was measured using the 7-EC Fluorimetric Assay. The activities were normalized to 1.0 for the total of the body parts. The mock dissection activity was corrected for the same total protein. (Mal. Tub.= Malpigian Tubules; Abd. Wall= Abdominal Wall; Diss. Liq.= Liquid in which flies were dissected; Mock Diss.= Mock Dissection).


Relative MFO Activity
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Intestine
Mal. Tub.
Abd. Wall
Carpse
Class. Liq.
Total
Mock Class.
Adult Organ

the dissected tissues. Because the mock dissection disrupted more tissues than did the more methodical dissection of individual organs which left the organs intact, it is possible that the mock dissected flies were exposed to proteolytic digestive enzymes to a greater extent. The specific activity of the mock dissection was approximately 1/2 that of normally assayed whole flies, indicating that considerable activity was lost due to the process of opening the flies in dissection fluid (Figure 7). As can be seen, the intestine and abdominal walls of the adult seem to contribute the most to the high activity of this strain. The probable origin of the high activity in the abdominal wall is the fat body, but due to the difficulty in isolating this "organ", this hypothesis is difficult to prove. Figure 8 shows the data of Figure 6 re-expressed on a percentage basis. The intestine contributes close to 40% of the total activity of the fly(imago). Figure 7 shows that the ability to de-ethylate 7-EC was found to be most concentrated in the intestine which had almost triple the specific activity of any other tissue. The next highest contribution comes from the abdominal wall (probably the fat body) and it too has a fairly high specific activity. It can be seen that almost a quarter of the total activity was still found to be associated with the "corpse" after dissection. This could possibly have been due to fat body that was not removed along with the abdominal wall.

A similar approach was used to discern the general area of MFO activity in the larva. To localize the activity to different

Figure 7.

Distribution of MFO Specific Activity in the Adult.

Twenty Iso-II-10 adult females were dissected in ice-cold 7-EC extraction buffer; MFO activity was measured using the 7-EC Fluorimetric Assay; Protein was measured using the Bradford Protein Assay.

Mal. Tub.= Malpigian Tubules; Abd. Wall= Abdominal Wall; Diss. Liq. = Dissection Liquid.

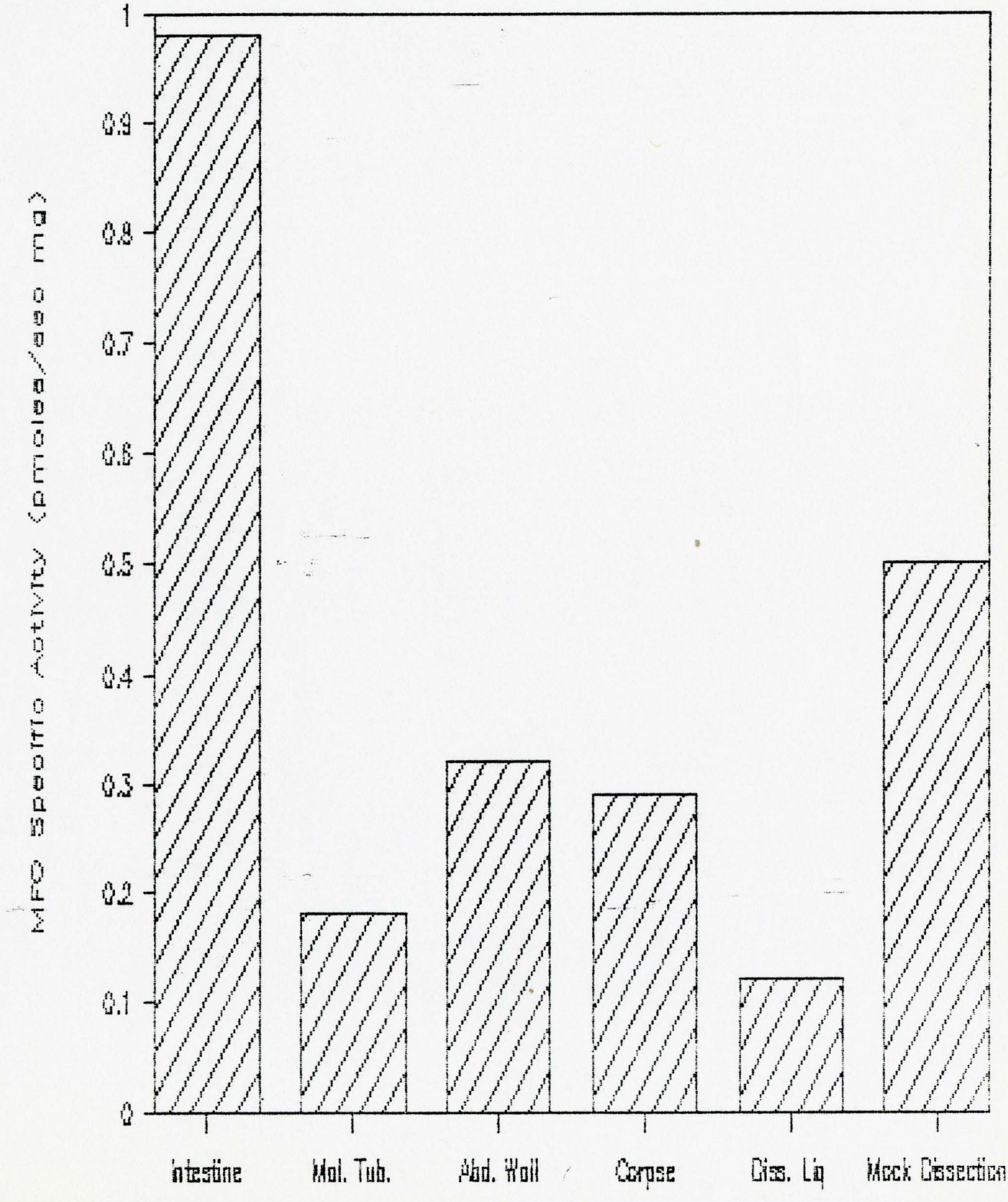
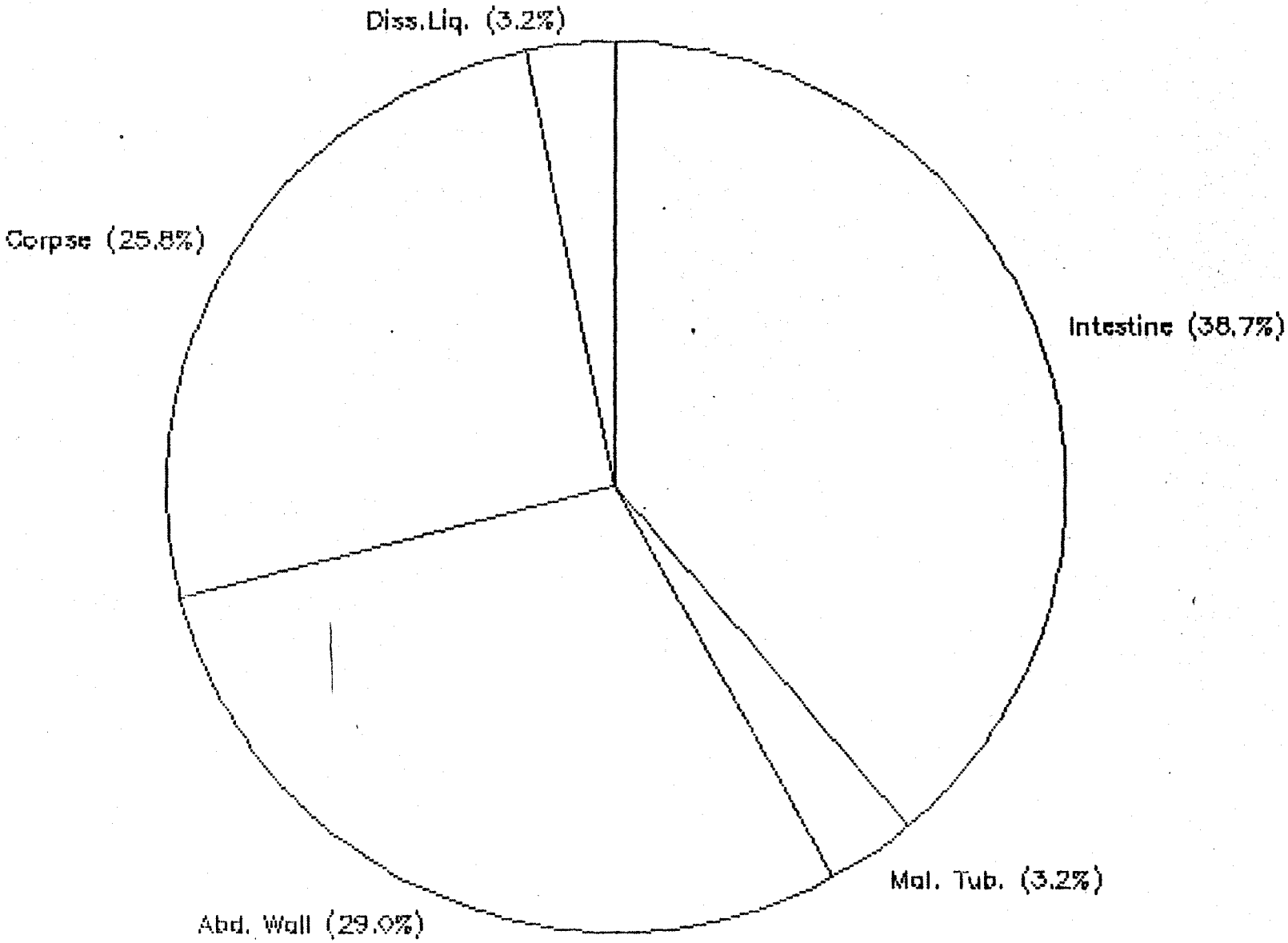

MFO Specific Activity (pmoles/sec mg)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Intestine
Mal. Tub.
Abd. Wall
Corpse
Diss. Liq
Mock Dissection

Figure 8.

Percent Distribution of MFO Activity in the Adult.

Twenty Iso-II-10 adult females were dissected in ice-cold 7-EC extraction buffer; MFO activity was measured using the 7-EC Fluorimetric Assay.


Diss.Liq. (3.2%)
Corpse (25.8%)
Intestine (38.7%)
Mal. Tub. (3.2%)
Abd. Wall (29.0%)

tissues, twenty third instar larvae (4 days old, still feeding) were dissected individually in ice cold extraction buffer and a mock dissection of ten larvae was also performed to determine how much activity was lost due to dissection into tissues. The larval intestine, malpigian tubules, fat body and remaining corpse were assayed separately as was the dissection liquid. It was found, in contrast to the adult, that the larval malpigian tubules made a significant contribution to the total activity (20%) while the intestinal contribution was much lower (10%)- see Figures 9 & 11. This is in contrast with the adult, where the intestine contributed nearly 40% of the total activity. Figure 10 shows that the specific activity of the malpigian tubules was very high, 1.8 pmoles/sec mg while the intestinal specific activity was much lower 0.12 pmoles/sec mg. Again, the fat body appeared to make a substantial (30%) contribution to the total activity. The corpse again seemed to have considerable activity perhaps due to residual fat body. Table 3 compares the two stages with respect to specific activity. As was found in the adult, the MFO specific activity of the larval mock dissection was less than half that found when larvae are assayed normally without previous dissection. This indicates that a substantial amount of activity was lost due to dissection, possibly due to proteolytic degradation of the MFO enzymes.

Figure 9.

MFO Activity in Organs of the Larva.

Twenty Iso-II-10 third-instar larvae were dissected in ice-cold 7-EC extraction buffer. MFO Activity was measured using the 7-EC Fluorimetric Assay. Ten larvae were also "mock-dissected" and then assayed to determine the loss of activity due to dissection. Units are in pmoles of hydroxycoumarin formed per second per 20 dissected organs, 10 whole larvae. The activities were normalized to 1.0 for the total of the body parts. The mock dissection activity was corrected for the same total protein. Diss. Liq. =Dissection Liquid; Mock Diss. = Mock Dissection.

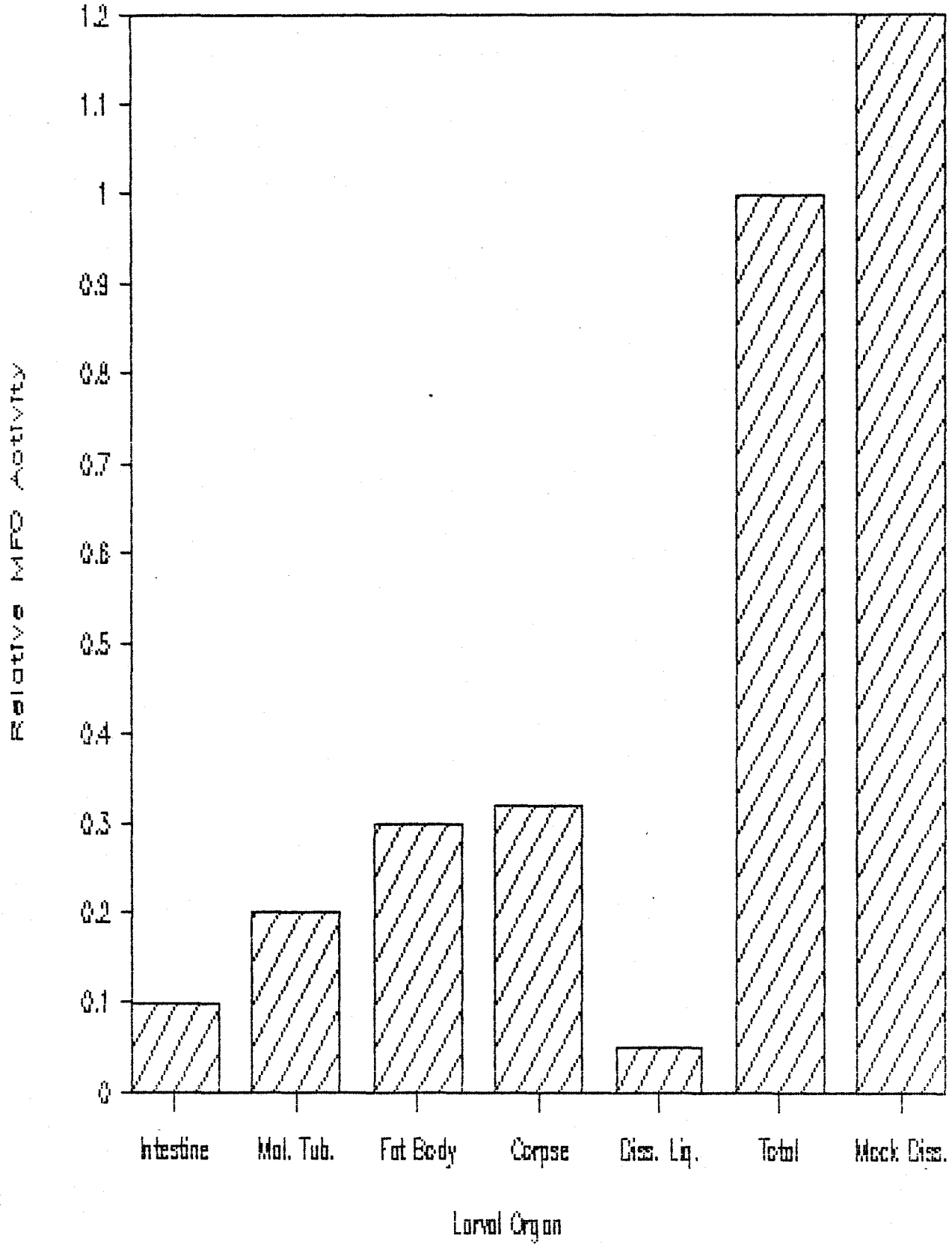

Relative MFO Activity
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Intestine
Mal. Tub.
Fat Body
Carpse
Diss. Liq.
Total
Mock Diss.
Larval Organ

Figure 10.

Distribution of MFO Specific Activity in the Larva.

Twenty Iso-II-10 third-instar larvae were dissected in ice-cold 7-EC extraction buffer; twenty larvae were also "mock-dissected" and then assayed to determine the loss of activity due to dissection. MFO activity was measured using the 7-EC Fluorimetric Assay; Units are in pmoles of hydroxycoumarin formed per second per mg of protein; Protein was measured using the Bradford Protein Assay.

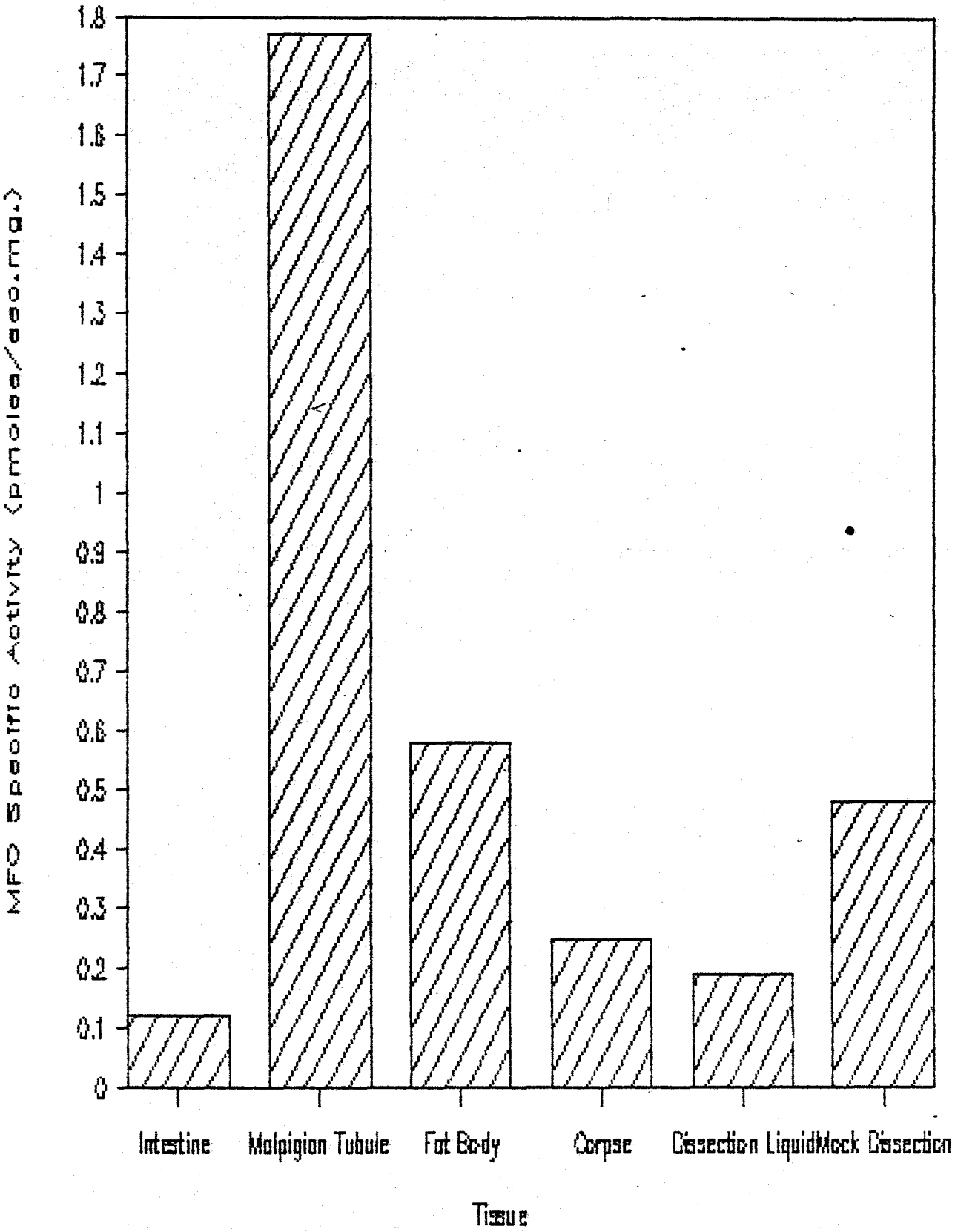

MFO Specific Activity (pmoles/min.mg.)
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Intestine
Malpigian Tubule
Fat Body
Carcass
Dissection Liquid
Mock Dissection
Tissue

Figure 11.

Percent Distibution of Larval MFO Activity.

Twenty Iso-II-10 third-instar larvae were dissected in ice-cold 7-EC extraction buffer and then assayed using the 7-EC Fluorimetric Assay.

Mal. Tub.= Malpigian Tubules; Diss. Liq. = Dissection Liquid.

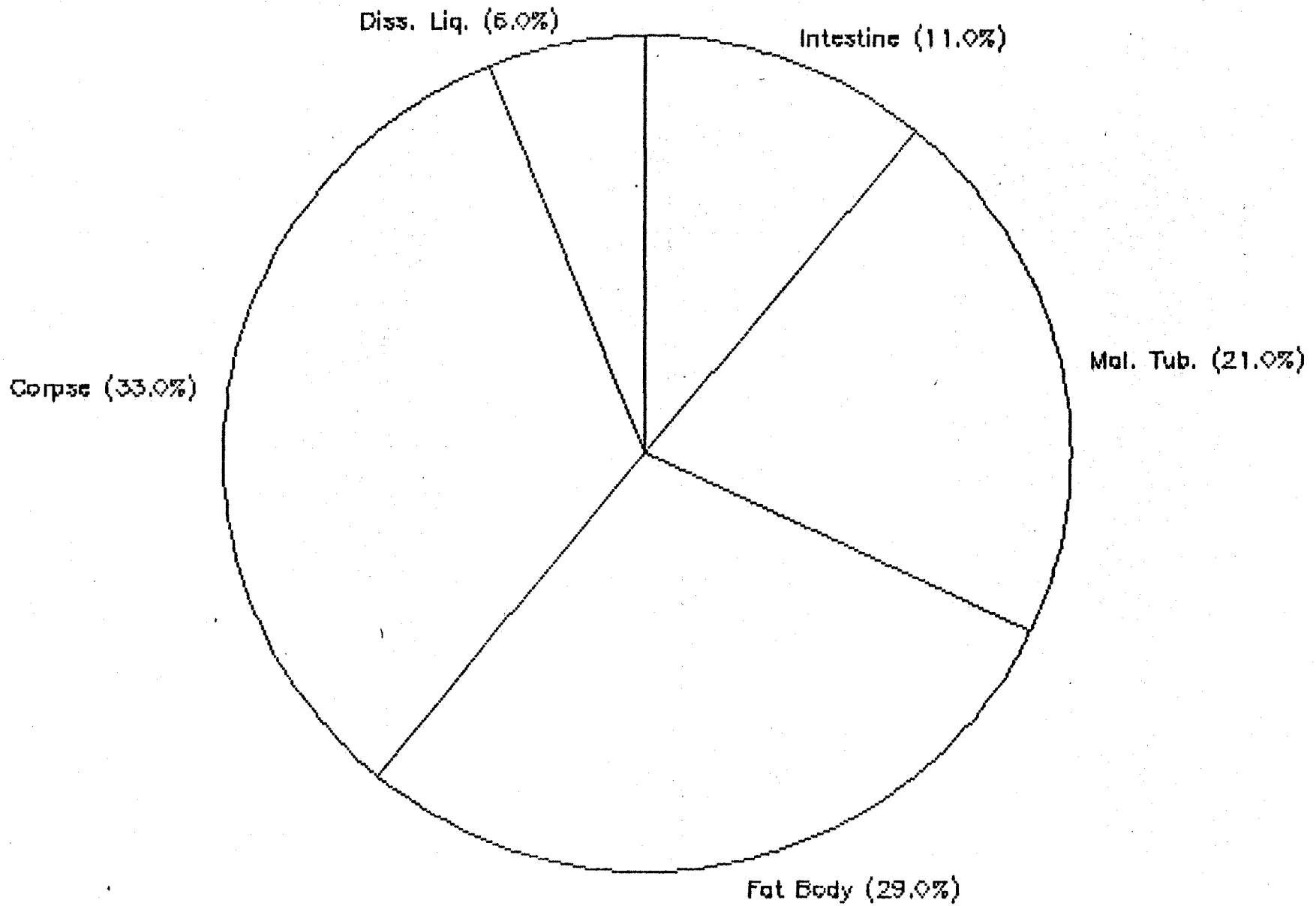

Diss. Liq. (6.0%)
Intestine (11.0%)
Mal. Tub. (21.0%)
Fat Body (29.0%)
Corpse (33.0%)

Table 3: A Comparison of MFO Specific Activity in Tissues of Larva and Adult.

	Tissue					
Stage	M.T.	Int.	Fat Body	Resid.	Mock	Whole
Adult	0.18	0.98	0.32	0.30	0.50	1.00
Larva	1.78	0.12	0.58	0.45	0.55	1.40

Specific Activity was measured using the 7-EC Fluorimetric Assay. The units are in pmoles/sec mg.

M.T.=Malpigian Tubules.
Int.=Intestine.
Resid.= Residual liquid after dissection.
Mock=Mock Dissection.
Whole=Activity of non-dissected whole adult or larva.

Part II: Genetics of Resistance to Malathion.

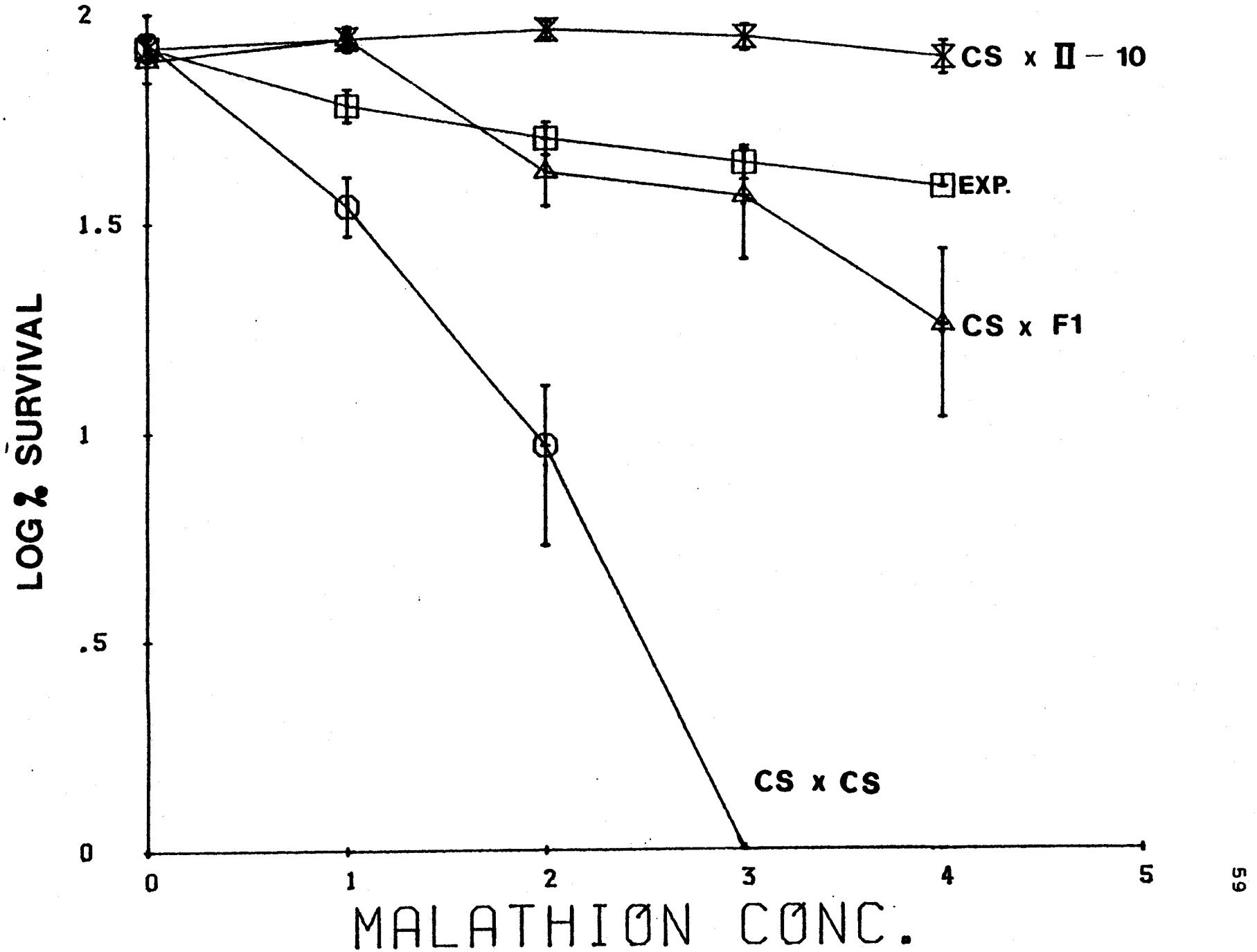
Section 2.1: Differential Survival on Exposure to Malathion of Larvae of Different Genotype.

A number of approaches were taken in order to map the location of gene(s) responsible for malathion resistance on the second chromosome. In order to map the gene(s) for larval resistance, a concentration of malathion was sought which would discriminate between larvae homozygous susceptible and those heterozygous for the resistance gene(s). Figure 12 shows the results of such an experiment. Groups of Canton S (non-resistant) flies were crossed to one of three types of mate: Canton S(non-resistant), Iso-II-10(homozygous resistant) or the heterozygous F1 (II-10/b vg) of a cross between Iso-II-10 and a suceptible strain (b vg) carrying the recessive mutations black (b, 2-48.5) and vestigial (vg, 2-67.0). The crossed flies were then placed on vials of banana medium containing different concentrations of malathion (0,1,2,3 or 4um) and the ability of the larvae to survive on these concentrations of malathion was determined (see Egg to Pupa Survival Test, Materials and Methods). Figure 12 shows the results. It can be seen that the Canton S/ Canton S cross progeny were most affected by the concentration of malathion in the medium. The genotype of these progeny would be homozygous susceptible for any genes on the second chromosome affecting resistance. The cross between

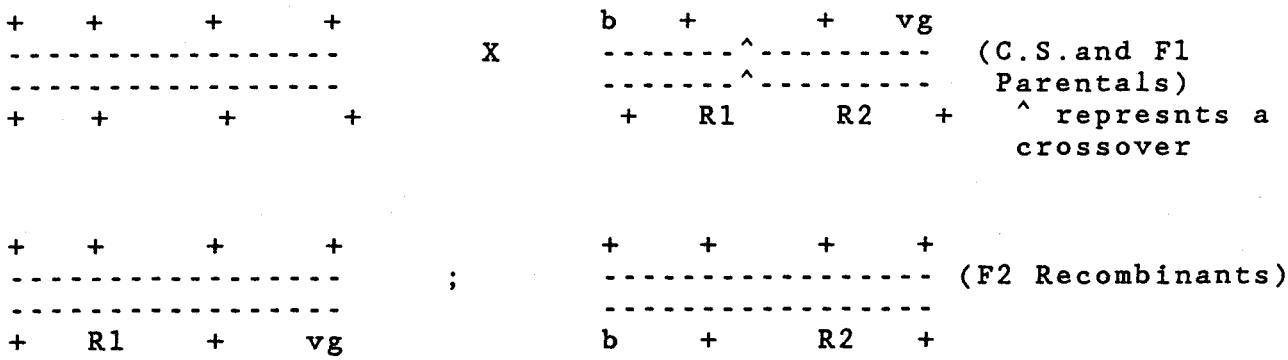
Figure 12.

Larval Survival on Malathion Medium.

Survival is plotted on a logarithmic scale. X represent the survival of progeny of a Canton S x Iso-II-10 cross; Δ represents the survival of the progeny (F2) from a bvg/II-10 x CS/CS cross; O represents the survival of the progeny of a Canton S x Canton S cross and □ (EXP.) represent the expected survival of the F2 progeny based a single larval resistance gene on the second chromosome. Survival could not be detected for progeny of the C.S. x C.S. cross at 3uM or 4uM malathion concentrations based on n=300 eggs counted.


2
1.5
1
.5
0
LOG 2 SURVIVAL
CS x II – 10
EXP.
CS x F1
CS x CS
0
1
2
3
4
5
MALATHION CONC.

Canton S and the Iso-II-10 resistant line gave very different results. The heterozygous progeny were not greatly affected by these concentrations of malathion, showing greater than 80% survival until 4 uM. malathion, where the survival dropped to 76%. At 3 uM., the survival of the progeny was 86% and this dose was decided appropriate in order to discriminate between larvae heterozygous for any resistance gene(s) and larvae homozygous susceptible. The backcross between the Canton S and the F1 heterozygotes produced progeny intermediate in resistance between heterozygote and homozygote non-resistant, as expected, although the survival curve of these progeny was not consistent with one calculated on the basis of there being one larval resistance gene on the second chromosome. If there were only one resistance gene, one would expect of the progeny of the C.S. x F1 backcross, half would be heterozygous resistant and half to be homozygous susceptible (S/S X S/R = 1/2 S/S : 1/2 S/R). If there were more than one resistance gene on the second chromosome, the results would be more difficult to predict, but one would expect the progeny to collectively show lower resistance to extreme doses than on the basis of a one-gene model. This is because if there is a recombination event, the resulting progeny might only receive one of the resistance genes necessary for the heterozygote level of resistance. For instance:

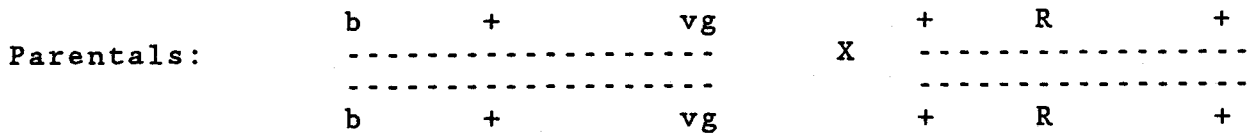


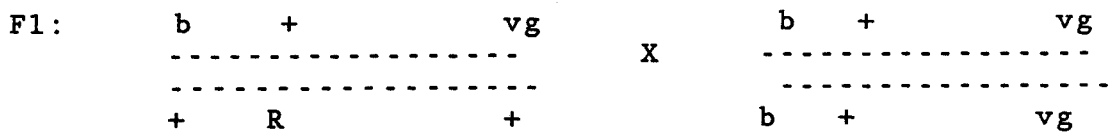
Since these two recombinant classes only receive one of the putative larval resistance genes, larvae in these classes are expected to have less than the full heterozygote level of resistance. If it were the case that there were in fact more than one larval resistance gene in this interval, then the survival curve of the progeny between a heterozygote resistant fly and a homozygous susceptible one might not be the average of the two parental curves as expected if there were one resistance gene. The results shown in Figure 12 appear to be more consistent with there being more than one larval resistance gene on the second chromosome.

Section 2.2 Larval Survival at a Discriminating Dose by Genotype

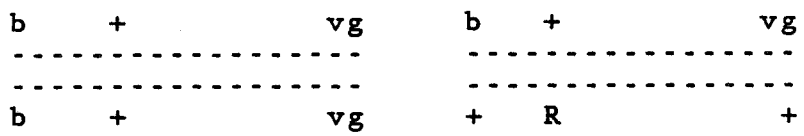
Once a dose of 3 uM malathion had been established to distinguish between progeny heterozygous for the resistance gene(s) and those homozygous susceptible, this dosage was used to map the gene for larval survival on the second chromosome. Iso-II-10 flies (homozygous resistant) were crossed to bvg flies and

the F1 generation were backcrossed to the bvg strain. The resulting progeny were sorted into the four pssible phenotypic classes: b+vg+ (wild type parental); bvg+; b+vg recombinants; and bvg (mutant type parental). Males from these classes were single mated to either Canton S virgin females (homozygous non-resistant) or to a non-resistant strain carrying bwD and the balancer CyO. After allowing a few days for mating, the Egg to Pupa Survival Test at 3 uM malathion (see materials and methods) was conducted on the crosses. The number of males of each genotype producing viable progeny was compared to the total number of males tested of each genotype. The results are shown in Table 4. The theory behind this type of recombinant mapping is as follows:





F2 Generation:



(parentals)

('b' recombinants)
(black phenotype)

('vg' recombinants)
(vestigial phenotype)

If there is one larval resistance gene, the closer the gene is to the marker 'black', the fewer recombinants with the black phenotype will receive the gene, and therefore have the heterozygous level of resistance, while the more recombinants with the vestigial phenotype will receive the gene and be resistant. Conversely. if the resistance gene is close to vestigial , the fewer recombinants with the vestigial phenotype and the more recombinants with the black phenotype will receive

the resistance gene. A map position for the larval resistance gene can be calculated from the fraction of the bvg+ or b+vg recombinants surviving the discriminating dose of malathion.

All the tubes resulting from a mating with b+vg+ males contained viable progeny and none of the tubes resulting from a mating using bvg males contained viable progeny. This indicates that the resistance gene(s) are likely in the interval between b and vg, for if they were not, one would expect some progeny from the b+vg+ males to be non-resistant and some progeny from the bvg males to be resistant. The resistance gene(s) could also be just outside of the interval, close enough so that there was little or no recombination between them and either of the markers. An average map position of 63.8cM (95% confidence interval of 61-65cM) for the larval resistance gene on the second chromosome was calculated. As shown in Table 4, there was good agreement between the two recombinant types. Since there are no phenotypic markers available for larvae, a map position based on recombination between known markers could not be determined as it was for the adult.

Table 4: Egg Survival at 3 uM Malathion.

Cross	# Resistant	Total	% Resistant
C.S.x b+vg+ F2	10	10	100
C.S.x bvg F2	0	14	0
C.S.x bvg+ F2	49	56	87.5
C.S.x b+vg F2	11	51	21.6

Calculation of Map Position (M.P.).

bvg+ M.P.= .875(18.5) +48.5=64.6cM (95% confidence range from 62.5 to 66.0 cM).

b+vg M.P.= 67-.215(18.5)=63.0cM. (95% confidence range from 60.4 to 64.9 cM).

Average= 63.8cM with approximate 95% confidence range of 61 to 65 cM.

The 95% confidence interval was calculated using the binomial distribution of the fraction of the resistant to the total according to the method described by Colquhoum (1971).

Section 2.3 Adult Survival on Exposure to Malathion by Genotype.

In order to map adult resistance using a similar principle to that used for larval resistance, a concentration of malathion that would differentiate between adults heterozygous for the resistance gene and those homozygous susceptible was found. Adult flies homozygous resistant and homozygous susceptible were tested using the Adult Survival Test (see materials and methods).

The flies tested to determine the discriminant concentration of malathion were the bvg strain (homozygous susceptible), the F1 progeny of a bvg x Iso-II-10 (resistant) mating and the F2 progeny from such females backcrossed to the bvg strain. The results are shown in Figure 13. A dosage of 18 uM (log18= 1.255) malathion was chosen to discriminate as over 98% of those heterozygous for the resistance gene(s) survived this dose and less than 3% of those homozygous susceptible survived it. The data points for the F2 survival were fit to various hypothetical models in order to determine whether the points were consistent with one or two resistance genes on the second chromosome. These curves are shown in Figure 14 and the analysis is described in the next section.

Figure 13.

Adult Survival on Malathion Medium.

Survival is plotted on a probit scale; malathion concentration is plotted on a logarithmic scale. X- b vg strain; O- F1 of an Iso-II-10 x b vg cross; Δ- F2 recombinants of a backcross of the F1 x b vg strains. The lines were fit by a least square linear regression analysis.

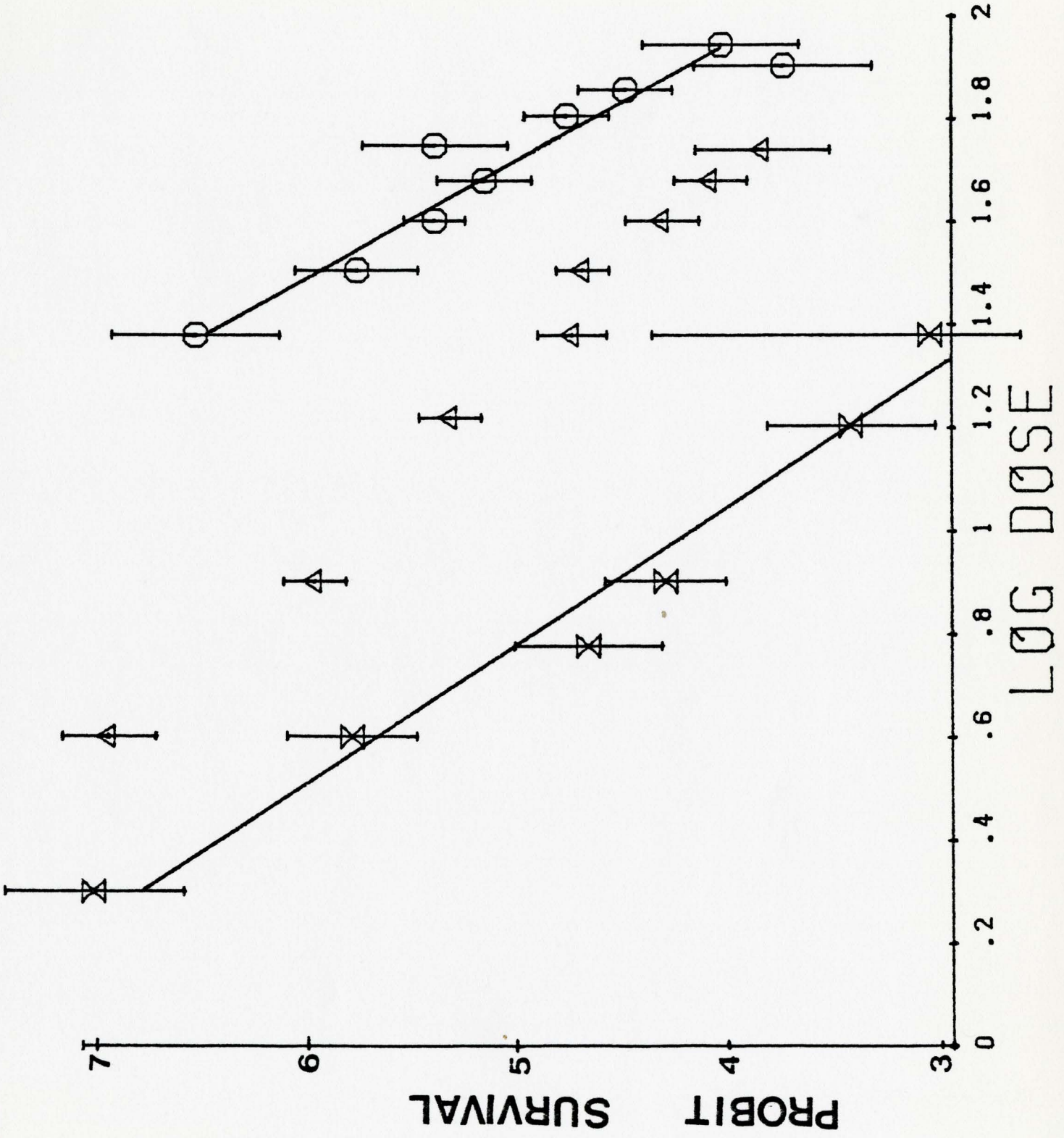
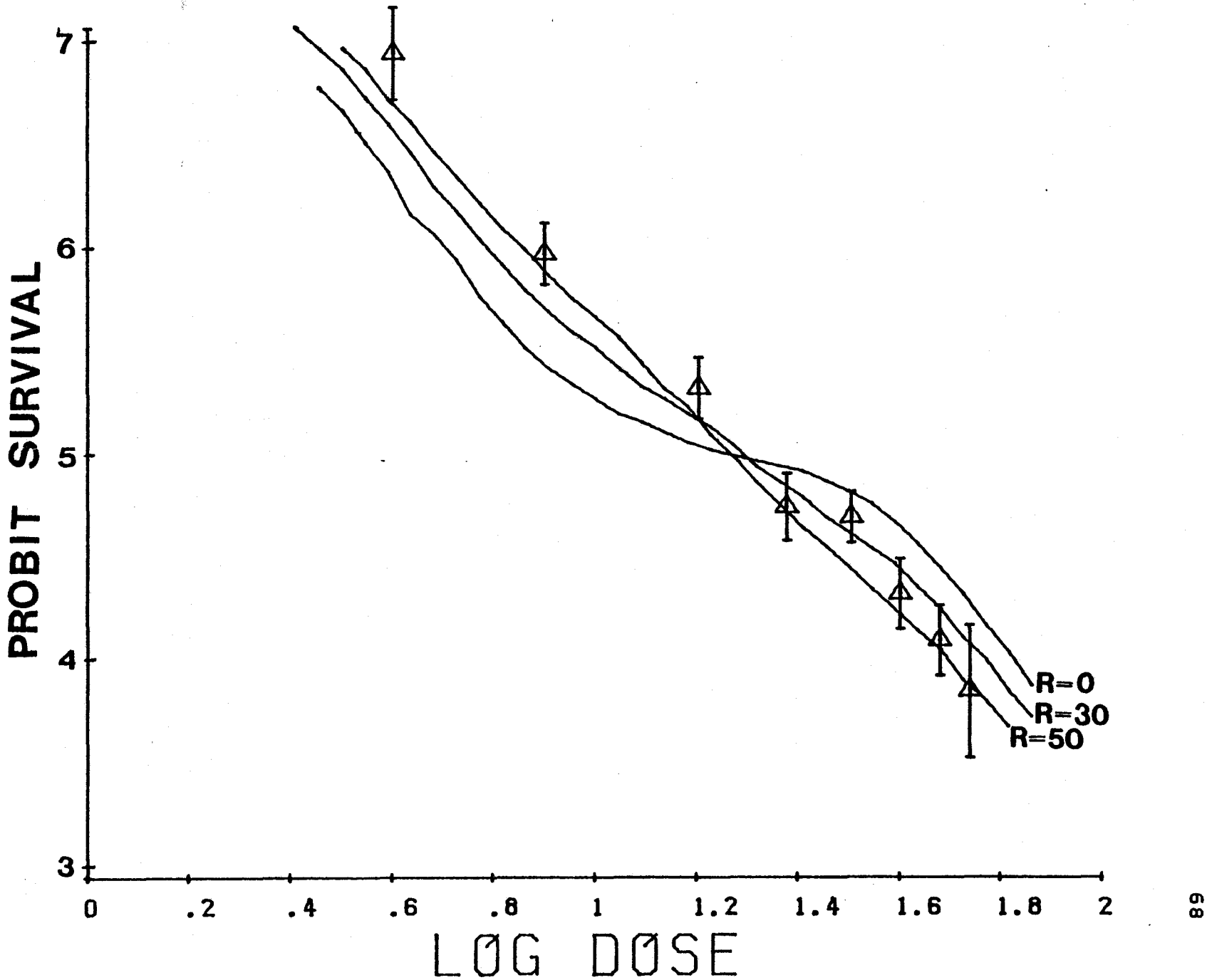

PROBIT SURVIVAL
7
6
5
4
3
0
.2
.4
.6
.8
1
1.2
1.4
1.6
1.8
2
LOG DOSE

Figure 14.

Test of One Gene Model for Adult Resistance.

Survival is plotted on a probit scale; malathion concentration is plotted on a logarithmic scale. Data points (Δ) are the same as in figure 13; curves illustrated are theoretical combinations of the b vg and F1 survival curves shown in figure 13 based on one or two resistance genes. R=0 is a curve based on one resistance gene or two closely linked genes with no recombination; R=30% is a curve based on two genes with 30% recombination; R=50% is a curve based on genes with 50% recombination (unlinked). All curves shown assume a multiplicative combination of the two parental curves.


PROBIT SURVIVAL
7
6
5
4
3
0
.2
.4
.6
.8
1
1.2
1.4
1.6
1.8
2
LØG DØSE
R=0
R=30
R=50

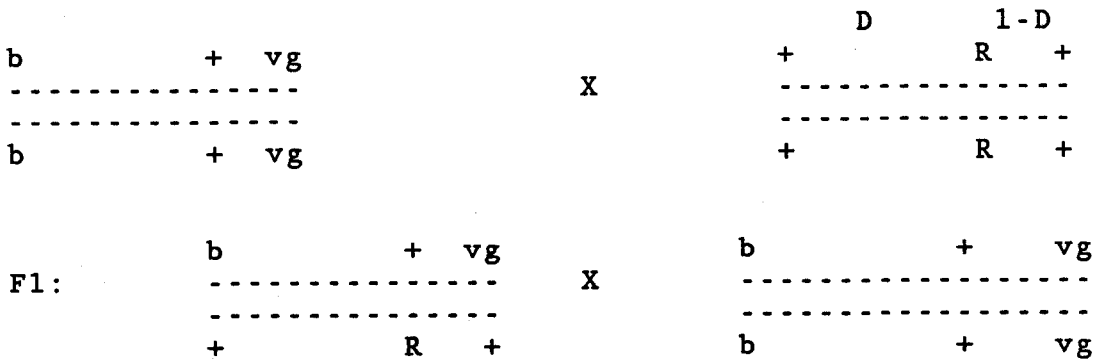
Section 2.4 Analysis of One and Two Gene Models for Adult Resistance.

Using the experimental survival curves fitted to the bvg (homozygous susceptible) and the F1 progeny survival of a bvg x Iso-II-10 (homozygous resistant), an attempt was made to fit various genetic models to the data for the F2 of a bvg x F1 cross. Three models are shown in Fig. 14: (1) A single resistance gene (or the equivalent, two closely linked genes without recombination); (2) Two unlinked (R(recombination)= 50%) genes, each exerting equal intermediate effects; (3) Two genes on the same chromosome, exerting equal intermediate effects, but with a recombination frequency of 30%. Based on a chi square goodness of fit analysis of these models, both curves (2) and (3) fit within 95% confidence limits.

Section 2.5 Recombinant Mapping Using a Discriminating Dose of Malathion.

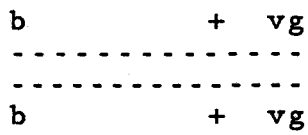
After choosing 18 uM malathion as an appropriate dose for discriminating between adults heterozygous for the resistance gene(s) and those homozygous non-resistant, an attempt was made to map an adult resistance gene in the interval from black (48.5 cM) to vestigial (67.0 cM). Crossing the Iso-II-10 (homozygous resistant) to the bvg strain (homozygous non-resistant), the F1 virgin females were backcrossed to the bvg stain and the F2

recombinants sorted according to phenotype (b+vg+, wild type parental; bvg+; b+vg recombinants and bvg, mutant type parental). Groups of 10-20 flies of each class were placed in tubes on top of solidified agar-sucrose containing 18 uM malathion (see Adult Survival Test- Materials and Methods) The numbers of survivors were compared to the total number in each class. The results are shown in Table 5. Flies in the phenotypic class b+vg+ showed 100% survival as expected. If the gene(s) are in the interval from b to vg, all these flies should be heterozygous and should show 100% survival at that concentration (ignoring double recombinants). The bvg class had 18% survival which is considerably higher than one would expect. Assuming the resistance gene(s) are in this interval and barring double recombination, these flies should lack the resistance genes and hence show less than 3% survival at that concentration (see Fig. 13). The bvg+ class showed 50% survival and the b+vg class only 16% survival which is not consistent with a single gene in that interval (chi square= 116.7, $p<.01$, 2 df). If there were a single gene responsible for adult resistance, one should expect the difference in survival between the b+vg+ and the bvg+ classes to be the same as the difference in survival between the bvg and the b+vg types. A diagram illustrating this theory is shown below:

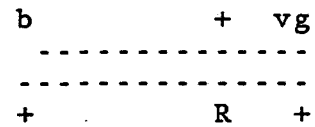


F2 Parentals.

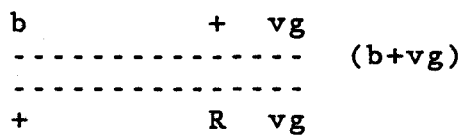
Homozygous Susceptible

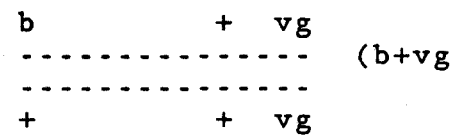


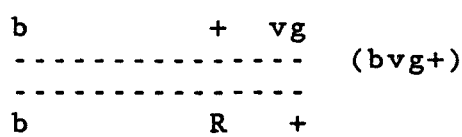
Heterozygous Resistant

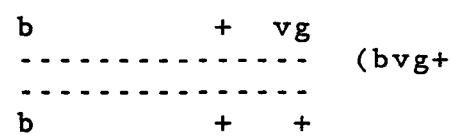


F2 Recombinants.









The proportion of bvg+ resistant recombinants to the total number of bvg+ recombinants should be equal to the proportional distance (D) of the resistance gene (R) from the marker 'b'. Similarly, the number of b+vg resistant recombinants to the total number of b+vg recombinants should be proportional to the

distance (1-D) of the R-gene from 'vg'. This was not so (chi square=116.7 $p<.01$,2df). A possible explanation is that there is more than one gene responsible for adult survival to the discriminating dose of malathion. As the larval mapping experiments were consistent with one gene located at 2-64.5, the adult mapping results indicate that there are differences in genes controlling resistance in the larva and the adult. The adult data suggests that there is more than one gene in this interval responsible for adult resistance to malathion (see discussion section).

Table 5: Recombinant Mapping of Adults Using F2 Backcross for a Discriminating Dosage of Malathion (18 uM).

Class	# Survivors	Total	% Survival
b+vg F2	8	50	16
bvg+ F2	26	52	50
b+vg+ F2	15	15	100
bvg F2	2	11	18

Calculation of Map Position (M.P.) (One Gene).

bvg+ M.P.= .50(18.5) +48.5= 57.3cM. (With a 95% confidence range of 55.1 to 60.3 cM.)

b+vg M.P.= 67- .16(18.5)= 64cM. (With a 95% confidence range of 61.6 to 65.66 cM.)

Average= 60.6cM.

The ranges were calculated using the binomial distribution of the fraction of the survivors to the total.

Part III: Map Location of Elevated MFO Activity Due to Genes on the Second Chromosome.

Section 3.1 Chromosome Two Recombinant Mapping.

In order to assign the location of the resistance gene(s) responsible for high MFO activity to a particular interval on the second chromosome, the following approach was taken. Resistant Iso-II-10 and Iso-II-14 flies were crossed to the "all" strain, a non-resistant, low MFO activity laboratory stock carrying multiple homozygous recessive mutations on the second chromosome. The MFO activity of the heterozygous F1 was measured using the p-nitroanisole MFO assay. Heterozygous F1 virgin females were backcrossed to "all" males. Each combination of F2 phenotypes that involved either none or one recombination event was sorted and assayed for MFO activity by the PNA Assay. These F2 flies showing evidence of 2 or more recombinations were discarded. By analyzing the recombinant group activities, putative map locations for the activity gene(s) can be assigned. Figures 17 to 19 show three different interpretations of the data. Each graph has been plotted as follows: Points plotted with an X (lettered points) represents a different recombinant group and is plotted at the locus to the right of which a putative recombination event took place. These groups had successively larger sections of the non-resistant "all" second chromosome substituted into the resistant second chromosome by recombination from the left end of the chromosome. The points plotted with a 0 (numbered points)

also represent different recombinant groups and are also plotted at the locus to the right of which a putative recombination event has taken place, though these groups had successively larger portions of the resistant second chromosome substituted into the non-resistant second chromosome by recombination from the left end of the chromosome. The homologous second chromosome in both cases (X and 0) is the non-resistant low activity "all" second chromosome.

Section 3.2: Recombinant Mapping Model.

The data for the preliminary recombinant mapping of MFO activity gene(s) on the second chromosome were interpreted in the following way: Examining the recombinants identified by letters from the left end of the chromosome, an activity level similar to the F1 heterozygous level of activity is expected until the interval within which there is an activity gene. If there is only one activity gene on the chromosome, it is expected that the level of activity will drop from the F1 heterozygote level at the left-most marker of the interval, to the homozygous low activity level (the "all" level) at the right most marker of the interval. The points representing the left-most markers are then placed on the theoretical activity lines to indicate a probable location of the activity gene. For the lettered points, the closer the activity gene is to the left-most marker of the interval, the closer to the F1 heterozygous level of activity

will be the point representing it. The theory behind the points represented by numbers is similar, although for these points, the analysis takes place from the left end of the chromosome starting with the "all" level of activity and increasing to the F1 heterozygous level of activity. If there are two activity genes between different pairs of markers on the second chromosome, it is assumed that the total activity could be split between the two genes and instead of the activity decreasing to the "all" level of activity at the right end of the interval in which the first activity gene is encountered (in a single step), the activity only decreases to a level midway between the F1 heterozygous level and the "all" level. The further decrease then is seen in the interval in which the second activity gene is encountered.

Sections (A) to (C) discuss three possible models by which the data of the preliminary mapping of the second chromosome activity gene(s) may be interpreted.

(A) Two Loci, Each With 50% Activity, One to the Left of b(48.5 cM) and the Other Near vg(67.0 cM) (Fig. 15).

The solid lines of Figure 15 follow this interpretation. Starting from point **A**, no effect of substituting portions of the non-resistant "all" chromosome is seen until the interval between dp(13.0 cM) and b(48.5 cM). At this point, the activity appears to decrease to a level midway between the heterozygote level (6.0 pmoles/sec mg) and the "all" level (1.7 pmoles/sec mg). This

model is drawn according to the assumption there is a resistance gene somewhere in that interval contributing one half of the increased activity. There is no change seen between b (48.5 cM) and pr (54.5 cM). A second postulated resistance gene then exerts its effect on recombinants between pr (54.5 cM) and c (75.5 cM). It is in that interval that the activity will drop from an intermediate level between the heterozygote and "all" to that of the homozygous "all" strain. Substituting more of the non-resistant second chromosome to the right of c (75.5 cM) does not decrease the activity any further. The reciprocal classes (substituting non-resistant second chromosome from the right end of the chromosome) seem to be consistent with this interpretation. One inconsistency with this model are the activities of the recombinant classes represented by points C (al dp + + + +) and 3 (+ + b pr c px). Point C would suggest by its high activity that if there is indeed a gene in the interval between dp and b giving 50% of the high activity, then this gene should be closely linked to dp. This is because the majority of class C flies appear to have this higher activity (above the 50% intermediate activity caused by the second gene between pr and c). If this were the case, then one should expect point 3 to have much higher activity, because if the gene is closely linked to dp, most flies of the recombinant class (+ + b pr c px) should have this gene. This class should therefore have the full 50% increase in activity caused by that gene. Point 4 should then have the full 50% increase in activity, because all flies falling

into this class should have the first resistance gene. It was on this basis that an interpretation suggesting a resistance gene to the left of b (48.5 cM) was discarded.

Figure 15.

MFO activity of F2 testcrossed flies. The model is:

A: Two Loci, Each With 50% Activity, One to the Left of black (48.5cM.) and One Near Vestigial (67.0 cM.)

Iso-II-10 was crossed with "all" and the F1 virgin females were testcrossed to the "all" strain. The F2 were then sorted into recombinant classes which were assayed for MFO activity using the PNA colourimetric assay. MFO specific activity is given in pmoles of p-nitrophenol formed per second per mg of crude extract protein. The data is plotted as follows: Points which represent the activity of the particular class of recombinant are plotted at the locus to the right of which a putative recombination event has taken place. The points plotted with an X (lettered points) had successively larger sections of the low activity second chromosome substituted into the high activity second chromosome by recombination from the left end of the chromosome. The points plotted with an O (numbered points) had successively larger sections of the high activity second chromosome substituted into the low activity second chromosome by recombination from the left end of the chromosome. Points off the scale (A and 1) represent flies with no recombination, ie. parental types. Points 4 and D, and 5 and E were shifted such that they would fall on the theoretical activity lines, indicating possible locations of genes responsible for high activity. The position of points 4 and

D along the x-axis correspond to the location(s) of one putative activity gene; points 5 and E correspond to the other.

The recessive markers from the "all" strain are as follows: al-0.0 cM.; dp-13.0 cM.; b-48.5 cM.; pr-54.5 cM.; c-75.5 cM.; px-100.5cM.

The phenotypes of the points are the following:

A: + + + + + + + ; B: al + + + + + + ; C: al dp + + + +

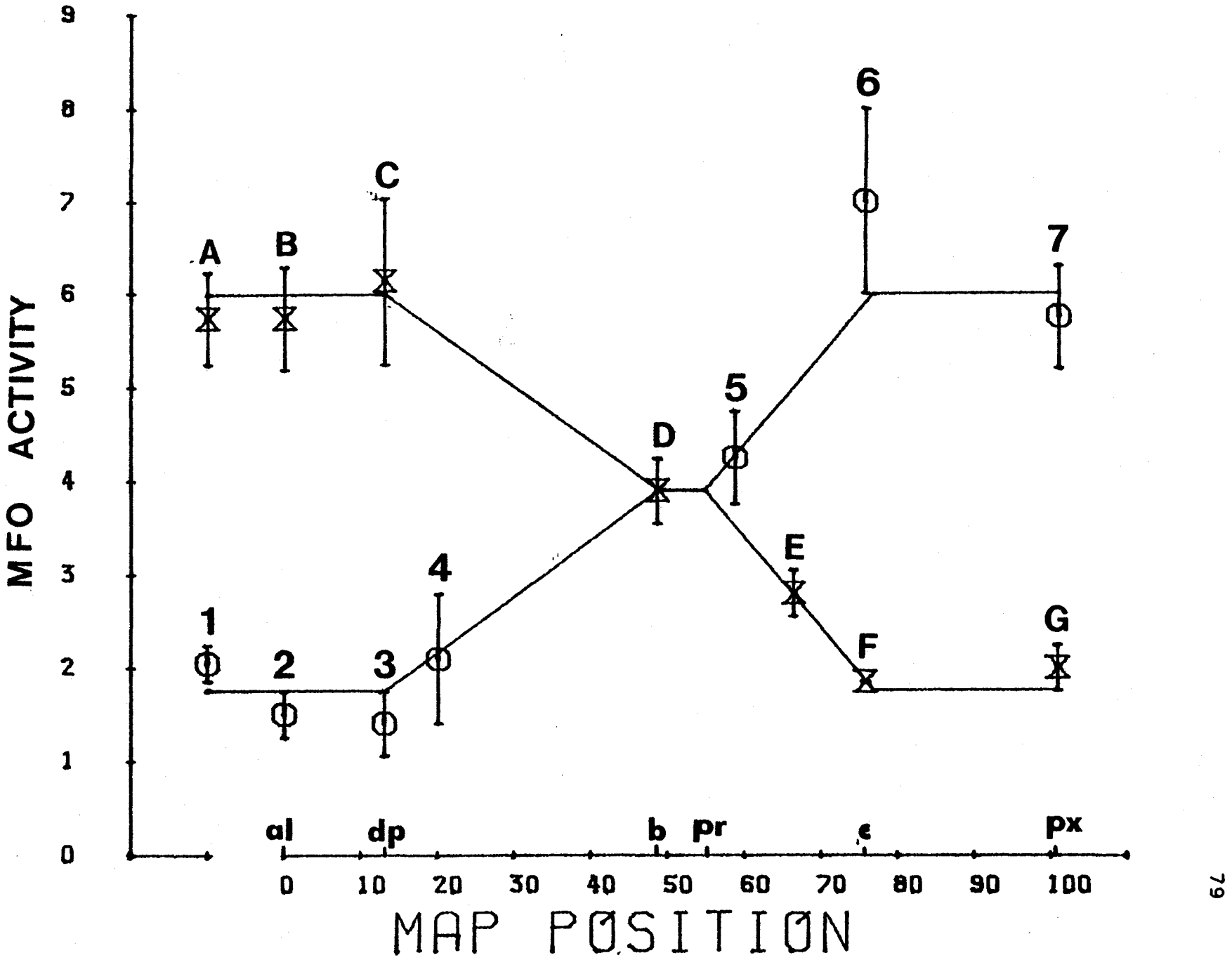
D; al dp b + + + ; E: al dp b pr + + ; F: al dp b pr c +

G: al dp b pr c px.

1: al dp b pr c px ; 2: + dp b pr c px ; 3: + + b pr c px

4: + + + pr c px ; 5: + + + + c px ; 6: + + + + + px

7: + + + + + +.


MFO ACTIVITY
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al
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b
pr
e
px
0
10
20
30
40
50
60
70
80
90
100
MAP POSITION

(B): Two Loci Each With 50% Activity, One to the Right of b(48.5 cM) and One Between pr(54.5 cM) and c(74.5 cM) (Fig.16).

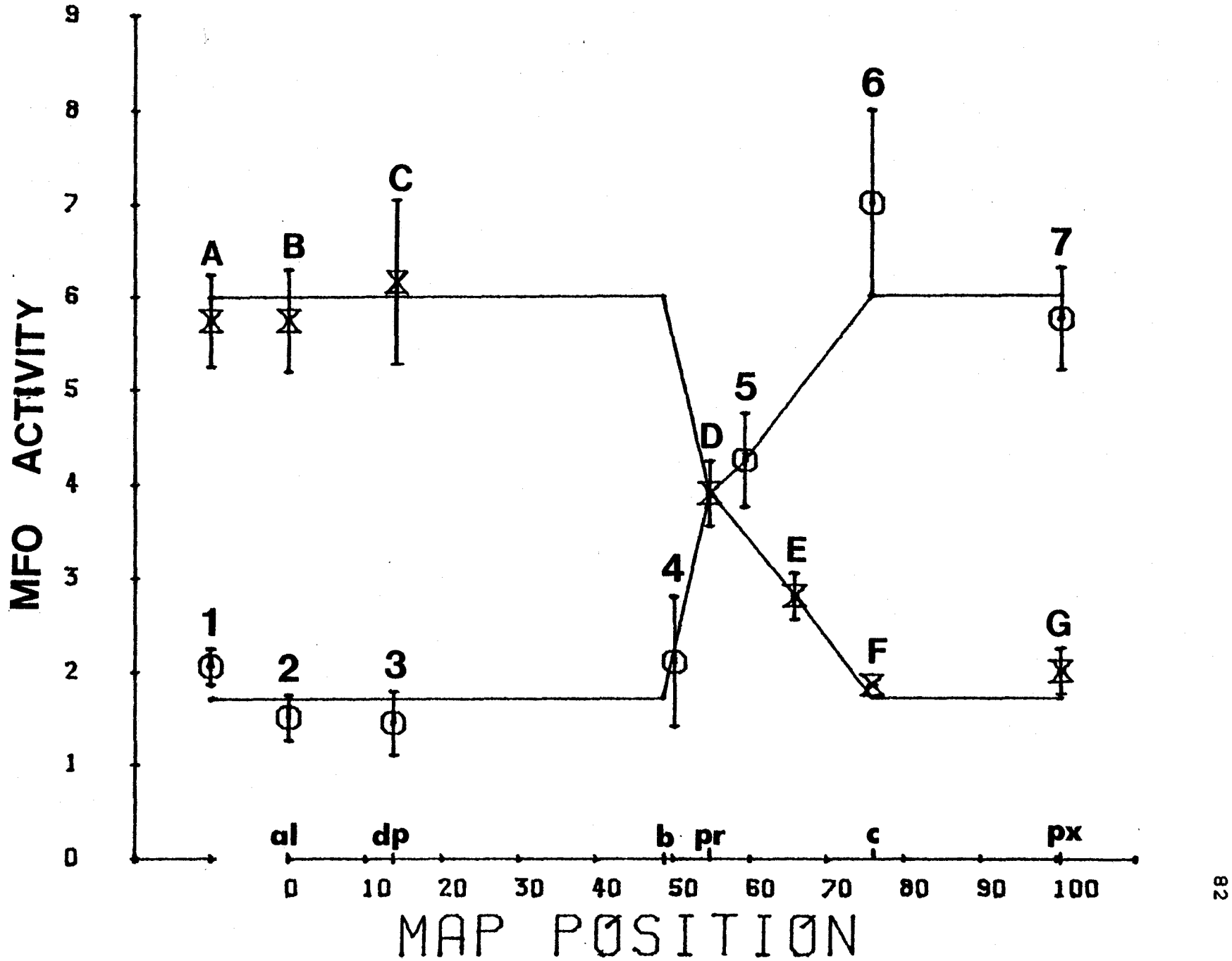
According to this model, there one activity controlling gene between b and pr giving 50% of the increased activity and another activity controlling gene between pr and c giving the other 50% of the increase in activity. Starting from point A, substituting increasing amounts of the non-resistant chromosome from the left end of the chromosome makes no difference to the activity until the interval between b (48.5 cM) and pr (54.5 cM). Between b and pr, the activity decreases to a level midway between the F1 heterozygote activity and the activity of the "all" strain. From pr to c (75.5 cM) there is a further decrease to the "all" activity level. Past c, there was no effect on the activity by increasing the substitution of more of the non-resistant chromosome for the resistant chromosome. The reciprocal recombinants (0-numbered points) are consistent with this interpretation. An inconsistency with this interpretation is that of the recombinant classes represented by points D and 4. Because each represents a crossover in the interval from b to pr, one should expect the activity differences from baseline to be consistent for reciprocal recombinants. In other words, the activity difference between point D and its baseline activity (the F1 heterozygote activity) should be the same as the difference between point 4 and its baseline activity (that of the

non-resistant "all" chromosome). Since the recombinant class represented by point 4 had a marginally greater activity than the "all" baseline (suggesting a locus close to b), the recombinant class represented by point D should have an activity just slightly less than the F1 heterozygote activity. Instead, it had an activity very close to the 50% point between the F1 and the "all" activities, suggesting a locus closely linked to pr. A possible explanation for this discrepancy was the difficulty in scoring pr (purple-eyed) flies due to their resemblance to the wild-type. It is possible that a number of pr flies were scored wild-type and vice versa. This was an unfortunate problem and one which might suggest reasons for the inconsistent data.

Figure 16.

B: Two Loci, Each With 50% Activity, One to the Right of black (48.5 cM.) and One Between purple (54.5 cM.) and curved (75.5 cM.)

F2 flies were analyzed and plotted as in Fig. 15. Again, points 4 and D have been plotted such that they fall on the activity lines and their position along the x-axis indicates a possible location of one of the activity genes and points 5 and E correspond to the location of the other (according to the model of section 3.1).


MFO ACTIVITY
MAP POSITION
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(C): <u>One Locus Between pr(54.5 cM) and c(75.5 cM) (Fig. 17)</u>.

According to this model, there is only one high activity gene in the interval between pr (54.5 cM) and c (75.5 cM) giving 100% of the increase in activity. Substituting increasing amounts of the resistant second chromosome for the non-resistant second chromosome beginning from the left end of the chromosome did not affect the activity until the interval between pr and c, whereupon there was a change from the baseline "all" activity to the F1 heterozygote level of activity. Any further substitution past the c locus failed to raise the activity. This observation should indicate a high activity locus between pr (54.5 cM) and c (75.5 cM). The reciprocal recombinants are consistent with this interpretation except for point D which exhibits lower activity than one would expect. According to this interpretation, point D flies (al dp b + + +) should contain the gene 100% 0f the time (barring double recombination) and should therefore have the F1 heterozygote level of activity. Instead of showing such a high activity, it shows a level midway between the F1 level and the "all" level. A possible explanation for this discrepancy is again the miscoring of pr flies. If a number of flies in the D class were pr instead of being wild type, this might explain why the activity of this class was so low.

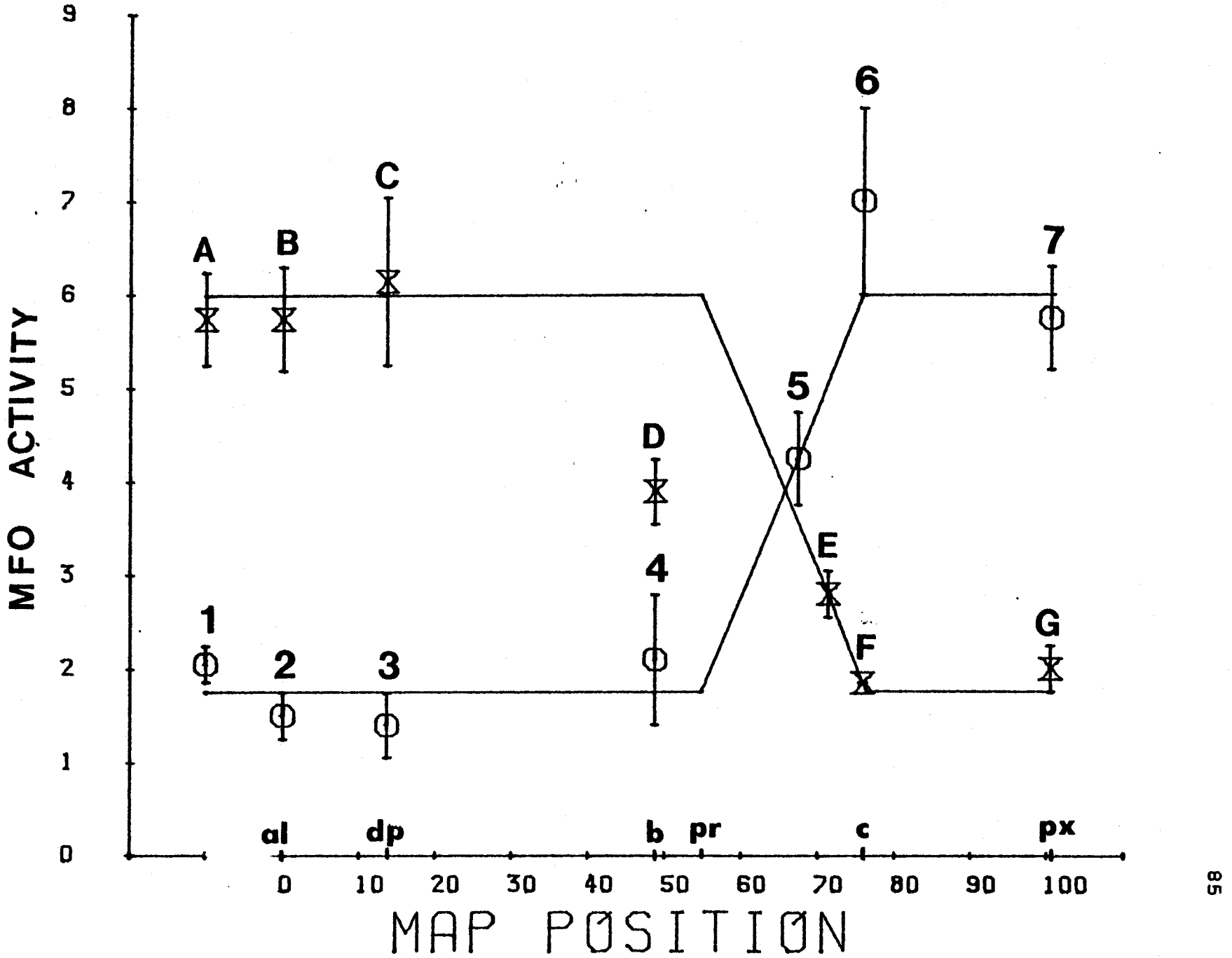
Since all three models of the data suggest that the high MFO activity gene(s) fall in an interval approximately between black (48.5 cM) and vestigial (67.0 cM), a more precise mapping of the

gene(s) was attempted, concentating on the area of the second chromosome falling in the interval between these two markers. Initially mapping was done using the results from the PNA demethlase assay, later the more sensitive 7-EC hydroxylase assay was used. This assay was able to measure the MFO activity of a single fly.

Figure 17.

C: One Locus Between purple (54.5 cM.) and curved (75.5 cM.).

F2 flies were analyzed and plotted as in Fig. 15. Points 5 and E have been shifted to fall on the activity lines and they correspond to the location(s) of the high activity gene.


MFO ACTIVITY
MAP POSITION
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Section 3.3: Mapping of Activity Gene(s) in the Interval Between black (48.5 cM) and vestigial (67.0 cM).

A more precise localization of the gene(s) for high MFO activity in the interval between black (48.5 cM) and vestigial (67.0 cM) was performed. One method used was to create F2 recombinant classes in a certain interval along the second chromosome that contained different proportions of the heterozygous resistant, and susceptible second chromosome. Measuring the MFO activity of these classes could then show the relative distance of the activity gene(s) from markers on the chromosome. The first such attempt was done using the PNA assay on groups of flies with the recombinant event between b (48.5cM) and vg (67.0cM). Resistant Iso-II-10 flies were crossed to non-resistant bvg flies, and the F1 virgin females from this cross were then backcrossed to bvg males. The resulting F2 generation were sorted into phenotypic recombinant classes (b+vg+, wild type parental; bvg+, b+vg recombinants and bvg, mutant type parental). Each group was assayed using the PNA MFO Assay and the results shown in Table 6. The b+vg+ class had the highest activity followed by the bvg+ class, the b+vg class and finally the bvg class. Using these results, a locus of approximately 58 cM for the b vg+ recombinants and 62 cM for the b+ vg recombinants was calculated, however the agreement obtained from reciprocal classes was poor (see discussion).

If one assumes that there are two genes responsible for PNA

MFO activity in the interval between black and vestigial, a different method of calculation is used. This method assumes that if recombinants in this interval receive one of the two activity genes, their activity will be the intermediate of the F2 parental type activities (b+ vg+=6.00; b vg=2.50; intermediate=4.25; see table 6). The activity of the recombinant classes (b vg+ and b+ vg) will depend on the fraction of flies receiving no activity genes (activity= 2.50); one activity gene (activity= 4.25) or both activity genes (activity= 6.00). This fraction will depend on the distance that the putative activity genes are from each of the markers (black and vestigial). Since the preliminary recombinant mapping had identified one putative activity gene to the right of black (48.5 cM) and one between purple (54.5 cM) and curved (75.5 cM), analysis concentrated on assigning loci in these intervals. The calculations for two genes in this interval are shown in section 14 of the materials and methods.

Table 6: MFO Activity of bvg Recombinants.

F2 Recombinant Class	S.A. (pmoles/sec.mg)	(S.E.)
F1 Heterozygote	5.75	0.5
"b+vg+" F2	6.00	1.13
"bvg+" F2	4.35	0.65
"b+vg" F2	3.40	0.55
"bvg" F2	2.50	0.30
bvg parental strain	2.00	0.30

Calculation of Map Position (M.P.) (One Gene).

bvg+ M.P.= 48.5 + .53(18.5)= 58.2cM (with a 95% confidence range of 54.9 cM to 61.7 cM).

b+vg M.P.= 67- .25(18.5) =62.2cM (with a 95% confidence range of 60.1 cM to 64.3 cM).

Average= 60.2cM.

Section 3.4: Single Fly Mapping of bvg F2 Recombinants Using 7-EC Assay.

A second attempt was made to map gene(s) responsible for adult MFO activity on the second chromosome in the interval between black (48.5cM) and vestigial (67.0cM). The method of analysis was similar to other recombinant mapping experiments, determining the frequency of recombination between a known phenotypic marker and a putative activity gene to assign a map position. Utilizing the sensitivity of the 7-EC MFO assay, single flies were assayed for their MFO activity. In order to determine the variation of activity from resistant and susceptible strains, individual flies from the Iso-II-10 line (homozygous resistant) and the bvg strain (homozygous susceptible) were first measured. The results are shown in Figure 18, top and bottom. In these assays only the activity of females was measured because male flies had a much lower activity that was often difficult to measure against background (the high background of male flies may

Figure 18.

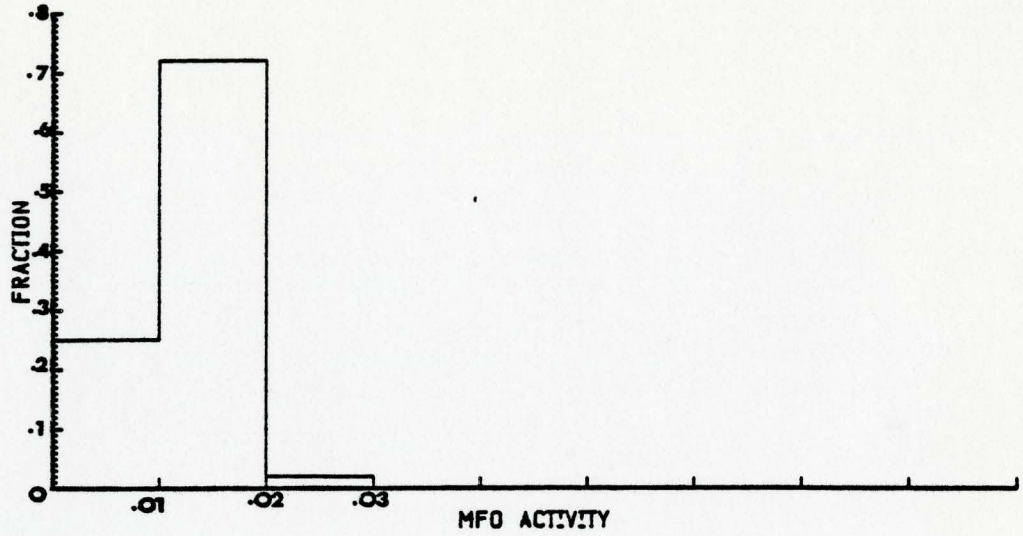
Single Fly MFO Activity Distribution of Different Strains.

MFO Activity was measured using the 7-EC Fluorimetric Assay on single female adults. Units are in pmoles of hydroxycoumarin formed per second. (a) b vg strain x=0.0118 (0.000606) N=48

(b) II-10/b vg F1 heterozygote x=0.0232 (0.00078) N=83

(c) II-10 strain x=0.0630 (0.004) N=40.

Standard Errors in brackets.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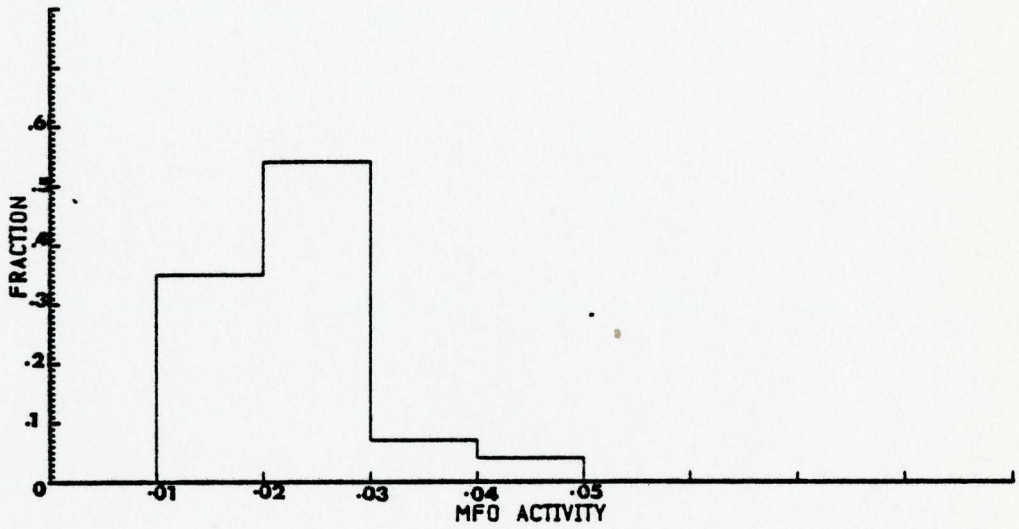
MFO ACTIVITY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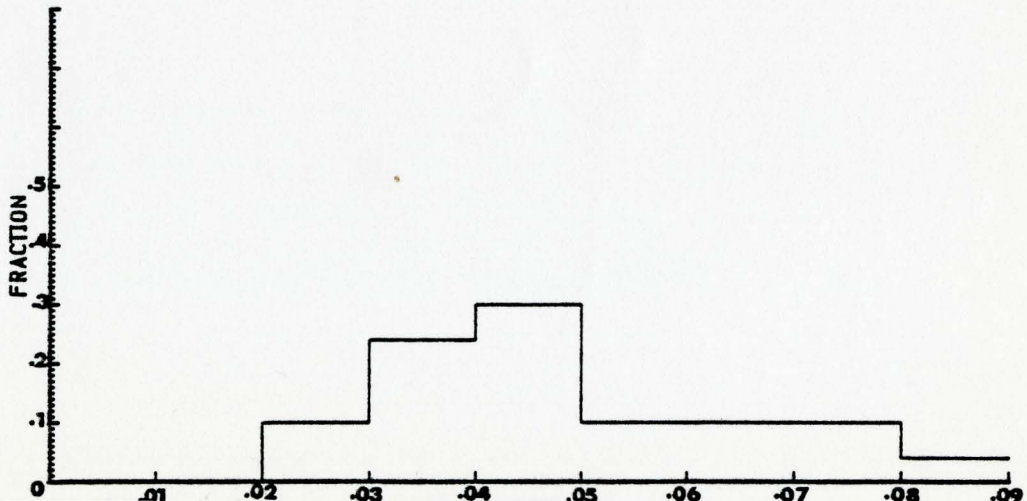
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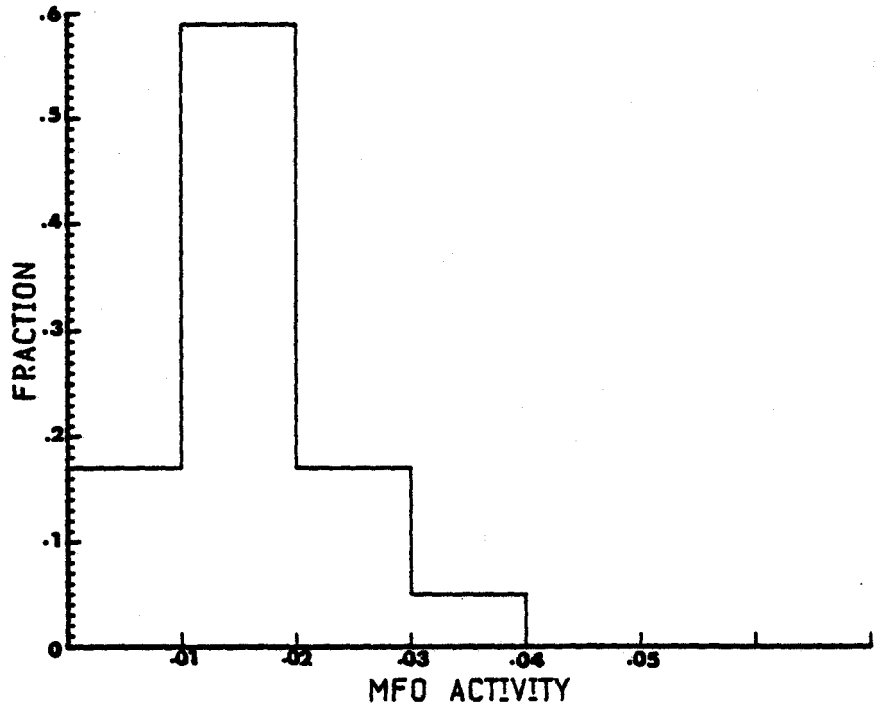
be due to the natural fluorescence of the testes). The Iso-II-10 and b vg strains were crossed and the F1 females were assayed, the results shown in Figure 18 middle. One would expect the F1 activity to be intermediate between the two parental types, its activity being the sum of one resistant and one non-resistant second chromosomes. As can be seen, the activity of the heterozygote was much less (.0232 pmoles/sec.mg.) than the anticipated average (.0374).

The F1 virgin heterozygous females were backcrossed to bvg parental males and the F2 recombinants sorted into the four phenotypic classes: b+vg+, bvg+, b+vg and bvg. One would expect the b+vg+ class to have the F1 heterozygote activity, because, assuming the resistance gene(s) are in the interval between black(48.5 cM.) and vestigial(67.0 cM.) and barring double recombination, all b+vg+ should have one resistant second chromosome (from the Iso-II-10) and one non-resistant second chromosome (from the bvg strain). The bvg+ (Fig. 19c) and b+vg (Fig. 19b) recombinants should show intermediate activity between the two classes (b+vg+ and bvg) depending on the distance that the activity gene(s) are from the markers. The results shown in Figures 19 a,b,c,d are plotted as histograms of the fractions of flies falling into different activity classes for each recombinant type. The averages, standard errors and sample sizes are also indicated. There are a number of methods of analysis of these results. One (as was done for the pooled pNA activities in the previous section) is to take the difference in average

Figure 19a,b.

Single-Fly MFO Activity Distribution of F2 Recombinants.

MFO Activity was measured using the 7-EC Fluorimetric Assay on single female adults. Units are in pmoles of hydroxycoumarin formed per second. F2 recombinants were from a b vg/II-10 x bvg/bvg backcross. (a) (top) "b vg" F2 recombinant x=0.0170 (0.00072) N=97 (b) (bottom) "b+vg" F2 recombinant x=0.0173 (0.0010) N=87.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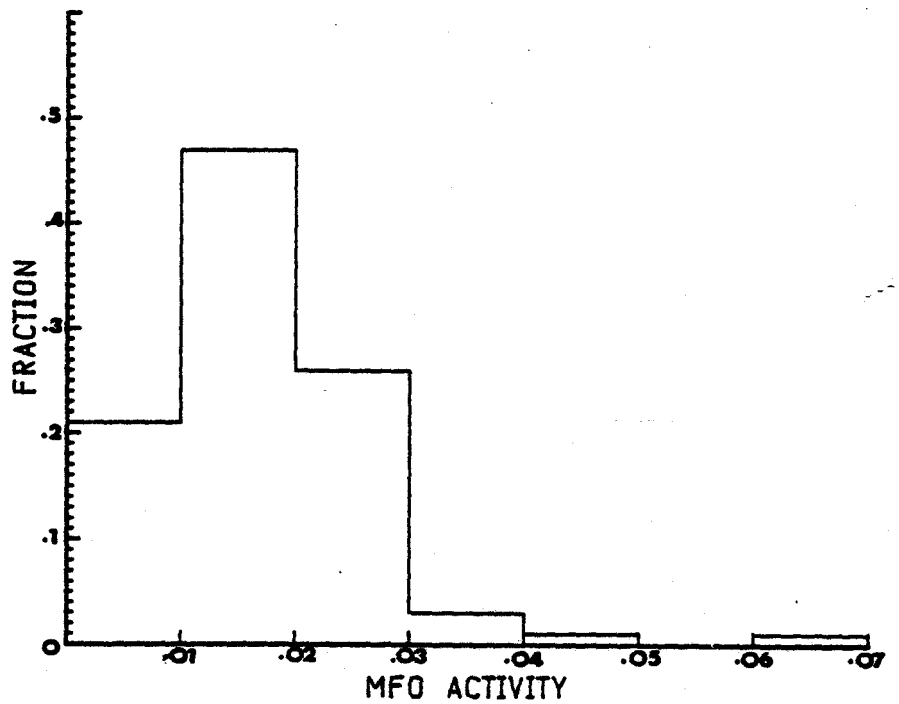
MFO ACTIVITY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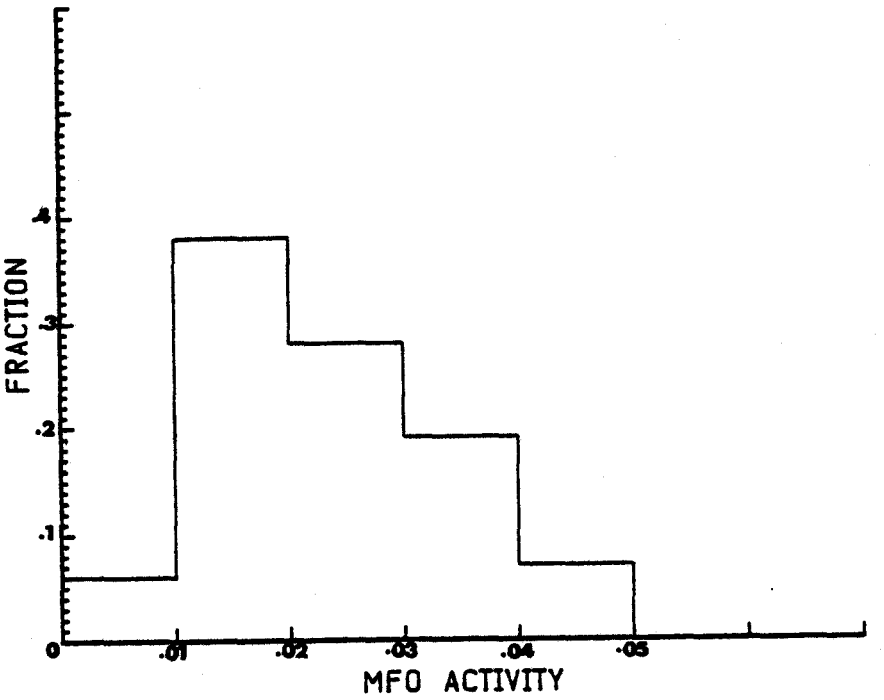
MFO ACTIVITY

Figure 19c,d.

Single-Fly MFO Activity Distribution of F2 Recombinants.

MFO Activity was measured using the 7-EC Fluorimetric Assay on single female adults. Units are in pmoles of hydroxycoumarin formed per second. F2 recombinants were from a b vg/II-10 x bvg/bvg backcross. (c) (top) "b vg+" F2 recombinant x=0.0231 (0.0010) N=96. (d) (bottom) "b+vg+" F2 recombinant x=0.0321 (0.0011) N=113.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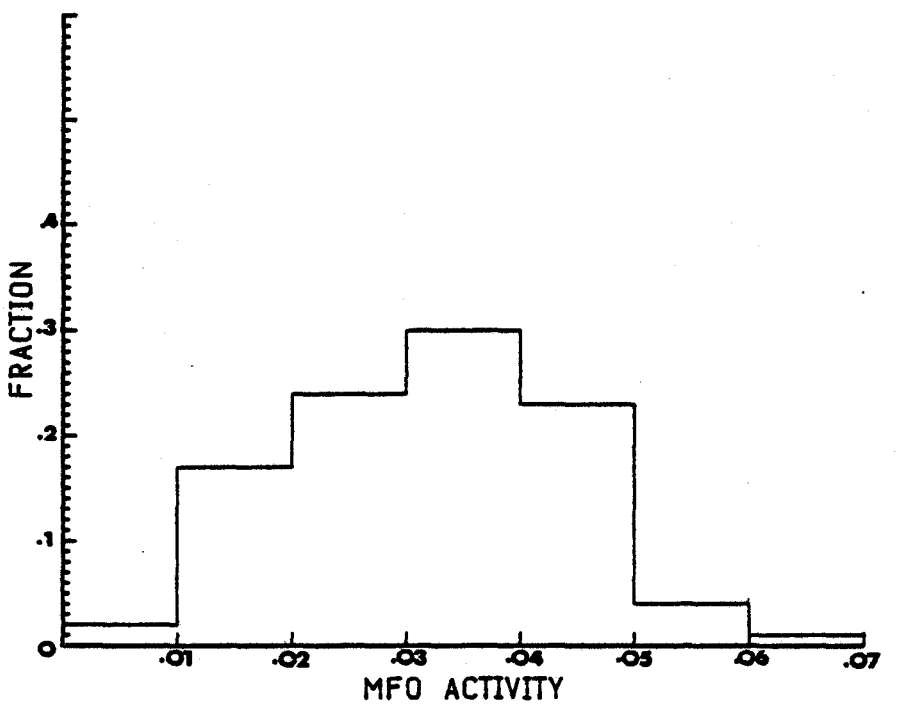
MFO ACTIVITY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MFO ACTIVITY

activity levels between each recombinant class and the mutant parental type (the b vg class), and to divide this by the difference in average activity levels between the wild type parental (the b+ vg+ class) and the mutant type parental (b vg), and to calculate a possible map position based on this result. Using this method, the bvg+ average map position is 55.9 + 1.3 cM (95% confidence interval) and the b+vg average map position is 66.6 + 1.2 cM (95% confidence interval). The map positions do not agree with each other within 95% confidence limits. Another method of analyzing the data is to calculate the number of "high activity" and "low activity" flies in each recombinant activity class and to calculate an overall expected value for the fraction of "high activity" flies for each recombinant class ("F"). One may then assign a map position based on these calculations. These calculations are shown in tables 7 and 8 and yield a 95% confidence interval from 56 to 61.5 cM for the b vg+ recombinant data and the b+ vg recombinant data is consistent with a 95% confidence interval of from 63.7 to 67.0 cM.

If one assumes that there are two genes responsible for 7-EC MFO activity in this interval, a method of calculation similar to that used in Section 3.3 to calculate putative map locations for genes for high PNA MFO activity can be used. Based on these calculations, the b vg+ recombinants are consistent with map positions of 51 cM and 64 cM, while the b+ vg recombinants are consistent only with two very closely linked genes (or one gene) very close to vestigial (67.0 cM) (the calculations are shown in

section 14 of the materials and methods) Further interpretation of this data will be given in the discussion section

Table 7: Calculation of Expected Fraction (F) of 'High Activity' Flies in b vg+ Recombinant Class.

Activity Interval	Contributions to Expected Totals			Observed Totals
	R		S	
0-0.01	0.0177(F)*96	+	0.175(1-F)*96	6
0.01-0.02	0.168(F)*96	+	0.598(1-F)*96	37
0.02-0.03	0.238(F)*96	+	0.175(1-F)*96	27
0.03-0.04	0.301(F)*96	+	0.0515(1-F)*96	19
0.04-0.05	0.23(F)*96	+	0	7

"F" Value	Chi Square	
0.3	11.04	(p<.05, 4 df)
0.4	7.27	
0.5	5.43	
0.6	6.01	
0.65	7.25	
0.7	9.16	
0.75	11.97	(p<.05, 4 df)

"F" Values with non-significant deviations from observed- 0.4 to 0.7.

Map Position with F(0.4)= 55.9 cM
Map Position with F(0.7)= 61.5 cM.

Proportions in the "R" column are the frequencies of the b+ vg+ (high activity) distribution in each activity interval.

Proportions in the "S" column are the frequencies of the b vg (low activity) distribution in each activity interval.

"F" represents the fraction of "high activity" flies in the b+ vg recombinant class; 96 is the number of flies sampled.

Table 8: Calculation of Expected Fraction of 'High Activity' Flies in b+ vg Recombinant Class.

Activity Interval	Contributions to Expected Totals			Observed Totals
	R		S	
0-0.01	0.0177(F)*87	+	0.175(1-F)*87	18
0.01-0.02	0.168(F)*87	+	0.598(1-F)*87	41
0.02-0.03	0.238(F)*87	+	0.175(1-F)*87	23
0.03-0.04	0.301(F)*87	+	0.0515(1-F)*87	3
0.04-0.05	0.23(F)*87	+	0	1
0.05-0.06	0.088(F)*87	+	0	1

"F" Value	Chi Square	
0	7.25	
0.1	8.3	
0.15	9.72	
0.175	10.54	
0.2	12.7	($p<.05$, 5 df)
0.3	15.24	($p<.05$, 5 df)

"F" Values with non-significant deviations from observed= 0 to 0.175.

Map Position with F(0)= 67.0 cM
Map Position with F(0.175)= 63.7 cM.

Proportions in the "R" column are the frequencies of the b+ vg+ (high activity) distribution in each activity interval.

Proportions in the "S" column are the frequencies of the b vg (low activity) distribution in each activity interval.

"F" represents the fraction of "high activity" flies in the b+ vg recombinant class; 87 is the number of flies sampled.

DISCUSSION

Part I: Expression of Cytochrome P-450 and Related MFO Activities.

Section 1.1: Expression Associated With Chromosomes Two and Three.

Insecticide resistance in D. melanogaster has been shown to be associated with increases in total cytochrome P-450 content or increases in particular species of the enzyme (Hallstrom, 1983). This study of a malathion-selected, resistant strain found that there are at least two increased protein bands associated with an insecticide-resistant third chromosome and one increased protein band associated with a resistant second chromosome that are not increased in a susceptible strain (Figure 1). These bands, from microsomal preparations are of the appropriate molecular weights (51 and 55 kD from chromosome III; 53 kD from chromosome II) to be cytochrome P-450 species. Hallstrom et al (1983) found increases in protein bands of apparent molecular weights of 51 to 58 kD to be associated with the induction of cytochrome P-450, and specifically bands of 54 kD and 56 kD to be associated with the second chromosome. It is possible that the band of 53 kD shown in Fig. 1 and the band of 54 kD demonstrated by Hallstrom could be due to the same P-450 species. Although the increased protein in microsomal extracts from resistant strains (Figure 1) could be cytochrome P-450 species, they could also be an unidentified protein also associated with resistance to malathion. Although the total amount of cytochrome P-450 was

elevated in the strain with the resistant third chromosome, it was not increased in strains with resistant second chromosomes compared with a wild-type strain (see Figure 2). This could indicate that the resistance and increased MFO activity associated with the third chromosome could be due to an increase in total cytochrome P-450. The smaller increase in resistance and MFO activity associated with the second chromosome could be associated with specific isozymes of cytochrome P-450 that make up a lesser fraction of the total. Perhaps the increase of these specific isozymes of cytochrome P-450 do not increase the total cytochrome P-450. Alternatively, the increased resistance and increased MFO activity associated with the second chromosome could be due to a qualitative rather than a quantitative change in one or more P-450 isozymes. Although there was no increase in total cytochrome P-450 associated with the resistant second chromosome, there was a 4.5 fold increase in PNA demethylase activity (see Table 1). There could be an altered cytochrome P-450 associated with the second chromosome that increases MFO activities without appearing in greater quantities. The increased protein band observed could therefore represent proteins unrelated to the increase in MFO activity. The same could be true of the cytochrome P-450(s) associated with the third chromosome, for although there is a two-fold increase in total cytochrome P-450 over the wild-type Canton S strain, there is an over 5-fold increase in PNA demethylation (see Table 1). Zijlstra et al(1984) has shown no correlation between MFO activity and total

cytochrome P-450 content. These results could indicate that something other than an increase in total P-450 is responsible for the increased MFO activity associated with chromosomes II and III. For example, differences in other components of the mixed function oxidase enzyme system such as an altered cytochrome P-450 reductase, or increased amounts of cofactors of cytochrome P-450 (such as NADPH) may increase MFO activity without affecting the total P-450 content.

Although the strains with a resistant third chromosome had a higher MFO activity than did those with a resistant second chromosome, extracts from either strain were capable of both MFO activities (7-EC hydroxylase and PNA-demethylase) (see Table 2). Hallstrom et al (1985) had found that increased PNA demethylation was associated with the second chromosome in insecticide resistant strains of Drosophila melanogaster, while 7-EC hydroxylase activity was associated a resistant third chromosome. The results demonstrated in Table 2 indicate that both MFO activities are associated with both resistant chromosomes II and III. This indicates that there are genes on both chromosomes regulating these two activities. These genes could control an enzyme with overlapping substrate specificity, or they could regulate the production of different isozymes altogether. An hypothesis for the difference in chromosomal determination of genes regulating these different MFO activities is that the strains used in the present study were derived from a population selected for malathion resistance, whereas the strains used by

Hallstrom were selected for resistance to DDT. It is possible that as a result, these strains exhibit a different genetic regulation of MFO activities.

Section 1.2: Developmental Expression of MFO Activity and Tissue Localization.

The expression of MFO activity associated with gene(s) on chromosome II of *Drosophila melanogaster* is not constant, but rather there are temporal differences in expression in the larva and the adult, and differences in the tissue localization of MFO activity. In the larva, MFO activity gradually increases until the third instar stage at four days (Figure 3a). After that it decreases, until pupation proceeds after which the activity is very low until eclosion. Because larvae were assayed at daily intervals, important changes in MFO activity such as those occuring at the first and second molts may not have been measured. (Also, synchronization of the larval stages was not adequate so that these changes may have been averaged out). As larvae prepare to molt into a new instar, they usually enter a period of quiessence and reduce feeding. In many insects, such as the southern armyworm *P.aridania* (Krieger and Wilkinson, 1969); the house cricket *A. domesticus* (Benke and Wilkinson, 1971) and the Madagascar cockroach *G. portentosa* (Benke et al, 1972), this period is marked by a sudden drop in many MFO activities, and these activities remain at a low level for a period that

extends through the molt and into the early part of the next stage before increasing again (Wilkinson and Brattstein, 1972). Since the larvae were likely assayed at times when they were not molting, any sudden decreases in MFO activity may have been missed. This may explain why only a gradual increase in MFO activity, without any sudden decreases until pupation was observed. The decline in MFO activity observed as the larvae begin to pupate could be caused by hormonal factors which are necessary for pupation. If the pupation is delayed, due to crowding of the medium, and the larvae continue to feed, the MFO activity remains at high levels (data not shown).

In the adult, the situation is different. The expression of 7-EC MFO activity appears to increase post-eclosion for about two days, after which it levels off. Waters et al (1984) observed similar results in an insecticide resistant strain of Drosophila melanogaster that had high expression of 7-EC hydroxylase activity. They found that the peak of expression occurred about 12 days post-eclosion, while the present study found a peak after only two days, and a decline thereafter. Regardless of this difference, both studies indicate temporal control of the enzyme in the adult. These changes could be in response to changes in endogenous substrates (such as hormones) in the adult.

Differences in tissue localization of MFO activity were found in both the larva and the adult of strains homozygous for a

resistant second chromosome. Although the overall specific activities of the larva and the adult were comparable (1.3 pmoles/mg sec for the larva; 0.9 pmoles/mg sec for the adult), there were differences in regard to which tissues showed expression of the high activity. In the adult, although the total MFO activity is divided fairly evenly among the intestine, abdominal wall, and the rest of the fly (corpse), the intestine had a much higher specific activity, indicating that as a tissue, the intestine is relatively rich in this enzyme system. The larval total MFO activity is distributed among the malpigian tubules, fat body and corpse, although as a tissue, the malpigian tubules are a relatively rich source of this enzyme system. These results indicate that, not only is the MFO system expressed differently in the tissues of both the larva and the adult, but the specific tissues in which the system is most active are also different. Since the endogenous function of the MFO system in Drosophila is still unclear, these results are somewhat difficult to interpret. The larval stage of the insect is one in which tremendous feeding and growth occurs. The MFO activity found in various tissues of the larvae may be involved in detoxification of xenobiotics and other compounds in the food that the larva ingests. The larvae that were measured in this study were derived from a strain of Drosophila that had been selected for resistance to malathion. Part of this resistance might be the increased capacity of the larvae to detoxify malathion using increased MFO system enzymes. The larval fat body was responsible for a third

of the total MFO activity of the larva, and since the insect fat body is considered analogous to the mammalian liver, the fat body may be where malathion detoxification occurs in the larva. The malpigian tubules, which function as an excretory organ also were rich in MFO activity and this could be another site of malathion detoxification (Hodgson, 1983). In the adult, the function of the MFO system may be involved in hormone metabolism as well as detoxification. The high MFO activity measured in the adult intestine may suggest that this is a site for malathion detoxification. The high MFO activity measured in the abdominal wall of the adult may actually be the activity of the adult fat body which was difficult to remove from the abdominal wall. The high activity in the adult may suggest that the fat body in the adult has some endogenous function involving hormone metabolism. However, this insecticide-resistant strain may not reflect the tissue location of MFO activity in a wild-type, non-selected strain of Drosophila. Rather than suggesting that malathion selection changed the distribution of cytochrome P-450 expression, it is more likely that the P-450 species selected by malathion exposure may be those that exhibit the observed distribution.

Part II: Genetics of Resistance to Malathion:

Section 2.1: Larval Resistance.

The mapping of genes on the second chromosome responsible

for larval resistance to malathion suggested that resistance associated with the second chromosome is caused by more than a single gene. Figure 12 indicates that the results of a larval survival test using the F2 of a cross between a susceptible strain (Canton S) and a heterozygous resistant strain (b vg/II-10) are not consistent with an expected curve for larval survival based on a single resistance gene on the second chromosome. At a low dosage of malathion (1 uM), the survival of the F2 larvae were greater than what would be expected from a one gene model, while at a higher dose of malathion (4 uM), the survival of the F2 is lower than would be expected from a one-gene model. If there were only one larval resistance gene on the second chromosome, the F2 generation should consist of 50% of larvae heterozygous for the resistance gene and 50% of larvae homozygous susceptible. This model takes into account recombinations of the second chromosome, because any recombinations that end up recombining out the resistance gene will be balanced out by reciprocal recombinations recombining in the resistance gene. Hence, the expected curve for a one resistance gene model is the arithmetic average of the F1 heterozygous resistant (C.S./II-10) and the homozygous susceptible (C.S./C.S.) curves, respectively. If there is more than one resistance gene, then the expected curve is more difficult to predict. For instance, assuming two resistance genes, if there is no recombination on the second chromosome, the F2 will again consist of 50% heterozygous resistant (one resistant second chromosome and one susceptible)

and 50% homozygous susceptible. If there is recombination along the second chromosome, then there will be a certain fraction of the F2 that receive both resistance genes (and behave similarly to the heterozygous resistant larvae); some that receive one of the two resistance genes (and have some intermediate resistance greater than the susceptible) and some that receive no resistance genes and behave like the homozygous susceptible strain. The probability of the F2 receiving at least one of the two resistance genes is greater in a two-gene model than in a one-gene model simply because there are more genes to "pick-up" or to lose by recombination. Because of this, one would expect the F2, in a two-gene model, to have greater resistance at low doses of malathion than in a one-gene model, because there is a greater probability of the F2 having at least one of the two resistance genes (and fewer F2 larvae lacking any resistance genes). At higher doses, the situation would be reversed. In a one-gene model, the F2 larvae either receive the resistance gene or they do not. In a two gene model, some receive both genes, some receive none, but some receive only one of the two resistance genes, and these larvae may not show full F1 heterozygote resistance at high doses of malathion. Hence, at high doses which are able to discriminate between larvae with one of the two resistance genes and larvae with both genes, F2 larvae in a two-gene model would show lower resistance than would F2 larvae in a one gene model. The results shown in Figure 12 are consistent with there being more than one larval resistance gene on the

second chromosome.

Although the results discussed above are consistent with more than one larval resistance gene on the second chromosome, the results of mapping the larval resistance gene(s) in the interval between black (48.5 cM) and vestigial (67.0 cM) on the second chromosome can be interpreted in a number of ways (refer to table 4). If one is to assume a one-resistance-gene model in that interval, the two reciprocal crosses are consistent with this and localize the gene to 64 cM. However, even with a two-gene model, this location may still be appropriate for a major resistance gene. Since a dosage of 3 uM malathion was used to differentiate between heterozygous resistant and homozygous susceptible larvae, there may have been no discrimination between those larvae with one major resistance gene and those with both a major and a minor resistance genes. As Fig. 12 indicates, a dosage of 3 uM malathion does not discriminate between F2 larvae in a one-gene model and those in a putative two-gene model. In other words, 3 uM will <u>not</u> allow larvae with <u>only</u> the <u>minor</u> gene to survive, while a dose of 1 uM will not discriminate between those larvae with the major gene and those with the minor gene, as both will survive. If this were the case, then even in a two-gene model, a map position for <u>one</u> of the two larval resistance genes (the major gene) of 64 cM may be correct, while the location of the other gene (the minor gene) would not be determined. This second resistance gene could lie anywhere along

the second chromosome, although it is more likely to lie some distance from the 'major' gene, because its independent effect was detected by recombination as shown in Figure 12. If it were very close to the major gene, with very little recombination between the two, it is unlikely that its own effect would have been discerned.

The data supports the localization of a major gene for larval resistance to malathion at 2-64 cM and at least one other minor gene. This interpretation agrees well with a number of previous findings for genes responsible for larval insecticide resistance in Drosophila melanogaster. Tsukamoto and Ogaki (1953,1954) found that larval resistance to DDT and BHC were caused by a gene on the second chromosome near vestigial (67.0 cM). Ogita (1958) found that a recessive gene for PTU resistance and a dominant gene for phenylurea (PU) resistance was located in the region of 64-66 cM. Kikkawa (1961) showed that a dominant gene at 64.5 cM played the most significant role in parathion resistance in larvae of Drosophila melanogaster.

As well as being implicated in larval resistance to a wide variety of insecticides, a locus at approximately 64 cM has been found to be associated with a number of increased MFO activities in adult Drosophila melanogaster. Hallstrom (1985) showed that the genes regulating a dominantly inherited high PNA demethylation and biphenyl 3-hydroxylation in insecticide resistant strains were located around 2-65 cM. Hallstrom et al

(1982) found that a gene responsible for high metabolic activation of vinyl chloride could also be localized to near 67.0 cM on the second chromosome. These findings might suggest that a possible mode of cross resistance to several classes of insecticides in Drosophila is through increased MFO activities which are able to detoxify these compounds. The larval resistance to malathion associated with a semi-dominantly inherited gene at 64 cM on the second chromosome found in the present study could be due to one or more increased MFO activities.

Section 2.2: Adult Resistance.

The mapping of the gene(s) responsible for adult resistance to malathion also indicated that there were likely more than one resistance gene on the second chromosome affecting adult survival. Again, as for larval survival, a single-gene model is not able to account for adult survival to a dosage of malathion able to discriminate between adults heterozygous resistant and those homozygous susceptible for resistance gene(s). Two different two-resistance gene models, with resistance genes exhibiting 30% and 50% recombination, respectively, were consistent with the data. This would imply that, though there may be one resistance gene in the interval between black (48.5 cM) and vestigial (67.0 cM), other resistance gene(s) likely lie outside of this interval. This is because the interval (18.5 cM) is smaller than the minimum recombination frequency between

resistance genes that is consistent with the data (30% recombination). The data shown in Table 5 also suggests that there is more than one resistance gene on the second chromosome. If there were only one resistance gene in the interval between black and vestigial, one would expect the map positions suggested by the reciprocal F2 recombinants (b vg+ and b+vg) to be similar, which they were not. As discussed in the results section, if there is a single resistance gene in this interval, then the difference in the fractional survival between the b+ vg F2 recombinant class and the b vg class should be equal to the difference in % survival between the b vg+ and the b+ vg+ classes. This was not the case (chi square=116.7, $p<.01$, 2df). Also, the b vg class had 18% survival on a dose of malathion that should have killed them all if there were only a single resistance gene. If there were a single resistance gene in this interval, this class should not have the resistance gene and (barring double recombination) should therefore be susceptible to this dose (see Figure 13). The survival of 18% might indicate that there are other resistance genes outside of this interval and that those flies of the b vg class that were resistant to 18 uM malathion had these resistance genes. The low survival of the b+ vg class might suggest that at least one of the resistance genes is close to vestigial, because if so, few b+ vg class flies would have the gene and show resistance. If this were the case, one would expect the b vg+ flies to predominantly have this gene and show high survival. It is possible that a second resistance

gene, perhaps located to the left of the black marker is needed for full expression of adult resistance in this strain. In summary, one could postulate two resistance genes, one near vestigial at around 64 cM and another probably to the left of black as shown by the diagram below. This gene is placed to the left of black, rather than to the right of vestigial because this best explains the low survival (50%) of the b vg+ recombinant class. If it were to the right of vestigial, most b vg+ recombinants would likely receive <u>both</u> resistance genes and should then show much higher survival than was found. Without a more accurate discrimination, it is difficult to more precisely localize this second adult resistance gene. Both resistance genes would be necessary for full expression of adult resistance to 18 uM malathion, although the presence of one of these resistance genes may be sufficient for partial resistance.

R1	b	R2	vg
?	48.5	64	67

If the suggested loci for adult resistance genes is correct, this might suggest that larval and adult resistance is affected by the same set of genes on the second chromosome. What was a minor resistance gene in the larva might be comparable in effect in the adult or perhaps exert a greater effect. The adults showed a greater resistance to malathion than did the larvae, however this finding could be due to the different methods of exposure of the two stages to malathion.

Part III: Map Location of Elevated MFO Activity Due to Genes on the Second Chromosome.

Section 3.1: Preliminary Recombinant Mapping.

MFO activity is controlled by genes on the second chromosome exerting a semi-dominant effect. This was shown by data indicating that the F1 generation of a cross between a high activity strain (Iso-II-10) and a low activity strain ("all") had an MFO activity that was the arithmetic average of the parental strains (data not shown). There was no dominance nor recessiveness of high MFO activity over the low MFO activity phenotype. This was in contrast to MFO genes on the third chromosome which were expressed with incomplete dominance with respect to their low activity alleles (Appendix A). The mapping data indicates that there were one or more genes responsible for high MFO activity situated near the center of the second chromosome, in the region between 40 cM and 70 cM. The most likely model to account for the recombinant data is shown in Figure 16. This model suggests that there are two semi-dominant genes responsible for high MFO activity and each of them exert 50% of the total high MFO activity effect. The first is located just to the right of black(48.5 cM) at around 51 cM and the second is between purple(54.5 cM) and curved(75.5 cM) at around 64 cM. The following is a diagram illustrating possible map locations for genes for high PNA demethylase activity:

b	A1	A2	vg
48.5	51	64	67

Section 3.2: Pooled Fly Mapping in Interval Between black(48.5 cM) and vestigial(67.0 cM).

The preliminary classification of recombinants along the second chromosome for PNA demethylase activity suggested that two genes are responsible for this MFO activity. The results from the mapping in the interval between black (48.5 cM) and vestigial (67.0 cM) of genes responsible for high PNA demethylation can be interpreted in a number of ways. One method is to assume that there is only one gene in this interval responsible for high PNA demethylation, and to calculate a putative map location of this gene based on the fraction of the difference in activity between F2 parental types (b+vg+ and b vg) that each recombinant class (b vg+ and b+ vg) exhibits. The fraction of this difference that a recombinant class exhibits will be inversely proportional to the map distance that the high activity gene is from the marker that is wild-type (vestigial for b vg+ and black for b+ vg recombinants respectively). Based on this method, a map position of 58 cM (with 95% confidence limits of 56 to 60 cM) was calculated for the b vg+ recombinants and a map position of 62 cM (with 95% confidence limits of 60 to 64 cM) was calculated for the b+ vg recombinants (see Table 6). The average map position for the reciprocal recombinants is approximately 60 cM for this single high activity gene. A second method of calculation would

be to assume that there are two activity genes in this interval as suggested by the preliminary mapping using the "all" strain as the low activity marker strain. Using this model, the data shown in Table 6 is consistent with there being two genes responsible for high demethylation on the second chromosome, one to the right of black(48.5 cM) between 54.5 cM and 60 cM and one to the left of vestigial at 64 cM. The position of the second activity gene at 64 cM is consistent with the earlier preliminary mapping while the position of the first activity gene in the region between 54.5 and 60 cM places it to the right of the earlier suggested locus at around 51 cM.

b	A1	A2	vg
48.5	54.5-60	64	67

Section 3.3: Single Fly Mapping in Interval Between black (48.5 cM) and vestigial (67.0 cM).

When the single-fly mapping of genes for high MFO activity in the interval between black (48.5 cM) and vestigial (67.0 cM) was first initiated, it was hoped that the activity distributions of the b vg F2 phenotypic class and the b+ vg+ F2 phenotypic class would not overlap. If this were the case, single fly recombinants of the b+ vg class or the b vg+ class could be scored either as a high activity fly (containing the high activity R-gene) or as a low activity fly (lacking the high

activity R-gene). In this way, the activity gene(s) in this interval could be mapped based on the fraction of b vg+ recombinants (or the reciprocal b+ vg recombinants) that fell into each of the low activity or high activity distributions. For instance, if 3/4 of the b vg+ class were of high activity (purportedly having the R-gene), this would indicate that the R-gene was 3/4 of the interval (18.5 mu) from black (48.5 cM) or at approximately 62.3 cM. Similarly, if 1/4 of the b+ vg recombinants were of high activity, this would indicate that the R-gene would be 1/4 of the interval from vestigial (67.0 cM), also at 62.3 cM. The actual results, though, showed that the activity distributions of the low activity class b vg (flies that should lack any high activity genes in this interval) overlapped to a considerable extent with that of the high activity class b+ vg+ (flies that should contain any high activity genes in this interval). For this reason, other methods of interpreting the data which took into account the this overlap were devised.

One method of analyzing the data, shown in Tables 7 and 8 is to assume that there is one high activity gene in this interval and to calculate the number of high activity and low activity flies in each recombinant class based on the probability that any recombinant falls into the high or low activity distributions. The fraction of high activity flies in each recombinant class is called the "F" value and this can be used to calculate a map position for each recombinant class. Although the activity distributions of the low activity b vg class and the high

activity b+ vg+ class overlap, there is enough of a difference between the two distributions to allow such an analysis. Based on this analysis, the b vg+ recombinant data is consistent with a map position of from 56 to 61.5 cM and the b+ vg recombinant data is consistent with a map position of from 63.7 to 67.0 cM. Although these calculations suggest a wide area in which the putative activity gene may be localized, the reciprocal recombinants do not overlap, indicating that there is a discrepancy between the reciprocal recombinant data and that such a calculation of one map position is not satisfactory. This suggests that there is more than one locus resposible for high 7-EC hydroxylase activity in this interval. If this is indeed the case, one may suggest possible map locations for two genes responsible for high activity in this interval based on the average activities of the reciprocal recombinants. Based on such analysis, the data for the b vg+ recombinants would suggest two loci, one in the interval between 48.5 cM and 51 cM and a second at approximately 64 cM. The data from the b+ vg recombinant class would be consistent only with two very closely linked genes (or one gene) very close to vestigial at 67 cM (calculations are shown in section 14 of materials and methods). Although the two reciprocal recombinants again suggest different map positions, the earlier findings suggesting loci for high PNA demethylation at 51 and 64 cM are consistent with those suggested by the b vg+ recombinant class data for high 7-EC hydroxylase activity. The activity of the single flies tested were extremely variable (the

range of readings for the activity of the Iso-II-10 strain was from .02 to .09). Because of the variability of the 7-EC MFO activity of individual flies, some recombinant flies that lack the putative high activity gene(s) might still manifest high activity (flies from the non-resistant, low activity b vg strain had readings as high as .03), while other recombinants which should have the high activity gene(s) might manifest low activity (as did some of the Iso-II-10 flies tested which should be homozygous for any high activity genes on the second chromosome). This might explain why the b+ vg recombinant class data was inconsistent with its reciprocal recombinant, the b vg+ recombinant class.

As earlier mentioned, loci in the interval between 48.5 and 51 cM and at 64 cM appear to be responsible for increased 7-EC hydroxylase activity, as well as increased PNA demethylase activity shown previously. This second locus again coincides with the locus previously suggested for high PNA demethylation (Hallstrom, 1985) and for cross resistance to several classes of insecticides (Kikkawa, 1961). The other locus to the right of black between 48.5 and 51 cM may represent a locus not previously associated with high MFO activity (specifically high PNA demethylation) and may be something unique to this strain of Drosophila that was derived from a population selected for resistance to malathion. An important difference between these findings and those of Hallstrom and Blanck (1985) with respect to the high PNA demethylation of insecticide resistant strains is

that those researchers found the gene(s) for high PNA demethylation to be dominantly expressed whereas this study has shown these genes to be semi-dominantly expressed. This discrepancy might suggest that the strain used in this study and those used in their study (Hallstrom and Blanck, 1985) have some unknown difference in the regulatory mechanisms of these gene(s).

The finding that there are genes on the second chromosome that are responsible for increased 7-EC hydroxylase activity is also in contrast with that of Hallstrom and Blanck (1985) that showed that this enzyme activity was associated only with the third chromosome in insecticide resistant strains of Drosophila melanogaster. If indeed there are loci on the second chromosome responsible for both increased PNA demethylase and 7-EC hydroxylase activities, and if at least one of these loci (at 64 cM) is near or identical with the RI gene (resistance to insecticides) and consistent with a locus found for larval resistance to malathion, this might suggest that insecticide resistance is achieved by a mutation giving rise to an increased capacity of cytochrome P-450 dependent metabolic detoxification of the insecticide. The position of those genes responsible for malathion resistance in the adult (one to the left of black, one at 64 cM) found in this study are similar to those that Hallstrom (1985) showed were associated with various MFO activities. In that study, high PNA demethylation and biphenyl 3-hydroxylation in insecticide resistant strains were shown to be regulated by genes located around 65 cM and the capacity for biphenyl 4-

hydroxylation was associated with a locus to the left of black. It is possible that the gene(s) at these loci control one or more MFO activities which enables both larval and adult resistance to malathion through MFO mediated detoxification of the chemical.

Although it is possible that genes for both increased PNA demethylase and 7-EC hydroxylase enzyme activities were fortuitously selected for when the original population was selected for resistance to malathion, it seems more likely that a mutation in a regulatory gene controlling these and perhaps other P-450 mediated enzyme activities was selected when selection for malathion resistance took place. This mutation could have increased the transcription of genes for these MFO activities, increasing the expression of these enzymes. Since total cytochrome P-450 was not increased in strains with a resistant second chromosome, the cytochrome P-450 species that perform the PNA and 7-EC MFO activities likely make up a relatively small fraction of total cytochrome P-450.

It has been suggested that adaptive evolution depends predominantly on regulatory gene mutations (Wilson, 1976) and variation in such genes affecting enzyme activities in Drosophila has been shown to occur in nature (McDonald and Ayala, 1978). Plapp (1984) has proposed that a regulatory gene mutation is the basis for insecticide resistance in houseflies and a change in a regulatory gene affecting the cytochrome P-450 enzyme system has been observed in mice (Nebert and Jensen, 1979).

Section 3.4: Proposed Future Work:

There are a number of ways in which this research might be continued. In order to determine the precise locations of resistance and/or high MFO activity genes on the second chromosome, segmental aneuploidy stocks could be used to construct strains with different segments of a susceptible second chromosome replacing segments of a resistant second chromosome. By constructing many such strains overlapping different segments of the resistant chromosome and comparing the malathion resistance (and MFO activity) of these strains with other strains homozygous and heterozygous for a resistant chromosome, more precise localizations for gene(s) controlling malathion resistance and MFO activity could be determined.

In order to elucidate the nature of the genetic differences between resistant and susceptible strains, it would also be useful to determine whether these strains are inducible for cytochrome P-450 mediated enzyme activities and for actual cytochrome P-450 content. Hallstrom et al(1982) found that a number of insecticide susceptible strains of *Drosophila melanogaster* were inducible for certain cytochrome P-450 mediated enzyme activities, while insecticide resistant strains were not inducible, behaving like constituitive mutants. It would be interesting to determine if the malathion-resistant strains used in the present study were inducible in relation to strains not selected for malathion resistance, and if not, whether this would indicate that these malathion resistant strains were also

behaving like constituitive mutants of a regulatory gene. This might then show that a regulatory gene mutation is the basis for malathion resistance due to detoxification of the substance and for increased cytochrome P-450 mediated enzyme activities.

Appendix A: Recombinant Mapping of Third Chromosome Activity Gene(s).

Although extensive mapping work was not done on third chromosome genes controlling increased MFO activity, an attempt was made to localize these gene(s) to a particular interval between known markers. A similar approach to that used for preliminary recombinant mapping of the activity gene(s) on the second chromosome (section 3.1) was used. Resistant, high activity MFO Iso-III-D flies were crossed to the "rucuca" strain, a non-resistant, low MFO activity laboratory stock carrying multiple homozygous recessive mutations on the third chromosome. The F1 generation was assayed for MFO activity as measured by the PNA Assay. F1 virgin females were then backcrossed to the "rucuca" strain and the F2 were examined. Each different combination of F2 phenotype that involved none or one recombination event was sorted and each group assayed for its respective MFO activity (PNA Assay). Figure 20 shows the results.

Figure 20.

Map Location of Third Chromosome Activity Gene.

Iso-III-D was crossed with "rucuca" and the F2 virgin females were backcrossed to the "rucuca" strain The F2 were then sorted into different recombinat classes and respectively assayed for MFO activity using the PNA colorimetric assay. MFO specific activity is measured in pmoles of p-nitrophenol formed per second per mg. of crude extract protein. The data is plotted as follows: All points are plotted at the locus to the right of which a putative recombination event took place. The **X** points (lettered) had successively larger sections of the low activity third chromosome substituted into the high activity third chromosome by recombination from the left end of the chromosome. The points plotted with an **O** (numbered) had successively larger sections of the high activity third chromosome substituted into the low activity background by recombination from the left end of the chromosome. Points off the scale (A and 1) represent classes with no recombination events ie. parental types. Points 5 and E represent a putative location of the third chromosome high activity gene.

The recessive markers from the "rucuca" strain are the follows: ru: 0.0 cM; h: 24.5 cM; th: 43.5 cM; cu: 50.0 cM; sr: 62.0 cM; e:70.5 cM.

The phenotypes of the points are as follows:

A: + + + + + + ; B: ru + + + + + ; C: ru h + + + +

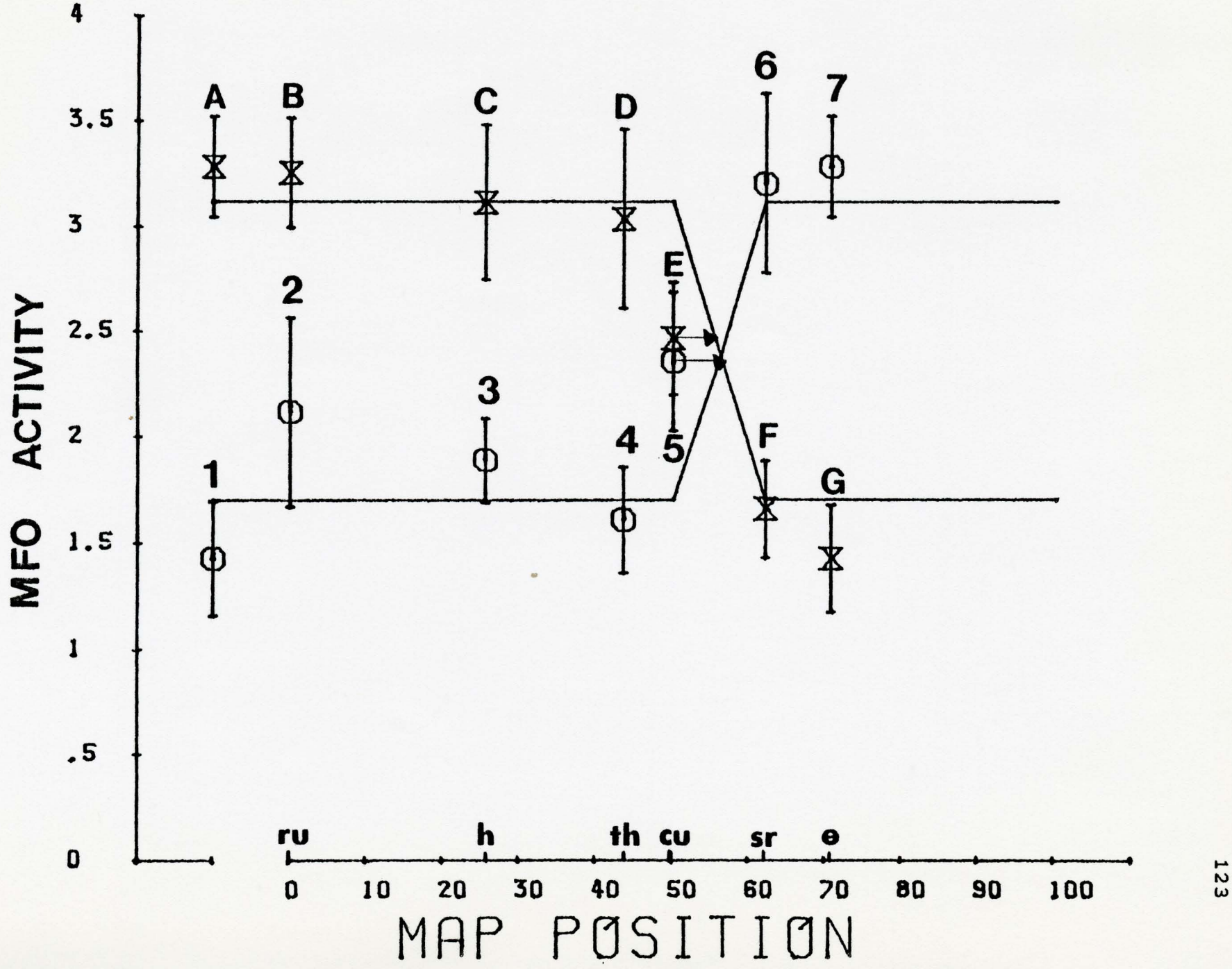
D: ru h th + + + ; E: ru h th cu + + ; F: ru h th cu sr +

G: ru h th cu sr e.

1: ru h th cu sr e; 2: + h th cu sr e ; 3: + + th cu sr e

4: + + + cu sr e ; 5: + + + + sr e ; 6: + + + + + e

7: + + + + + + +.


MFO ACTIVITY
4
3.5
3
2.5
2
1.5
1
.5
0
A
B
C
D
E
F
G
1
2
3
4
5
6
7
ru
h
th
cu
sr
e
0
10
20
30
40
50
60
70
80
90
100
MAP POSITION

Each point plotted with an X (lettered points) is plotted at the locus to the right of which a putative recombination event has taken place. These groups had successively larger sections of the non-resistant "rucuca" third chromosome substituted into the resistant third chromosome by recombination from the left end of the chromosome. The points plotted with an O (numbered points) are also plotted at the locus to the right of which a putative recombination event has taken place though these flies had successively larger portions of the resistant third chromosome substituted into the non-resistant third chromosome by recombination from the left end of the chromosome. The homologous third chromosome in both cases (X and O) is the non-resistant, low MFO activity third chromosome. It can be observed from the X points that substituting increasing sections of the left end of the non-resistant third chromosome in for the resistant third chromosome background makes no difference up until the cu (50.5 cM) locus. Up until this point, there is a constant high activity, indicating that the resistance gene is likely somewhere to the right of cu. As sections of the non-resistant third chromosome are substituted in between cu (50.5 cM) and sr (62.0 cM), the activity drops until the right of sr. At this point, any substitution of the non-resistant third chromosome for the resistant third chromosome did not drop the activity any further. The reciprocal points (O-numbered points) were consistent with this pattern. These results are consistent with an interpretation of one gene resposible for increased activity located on the

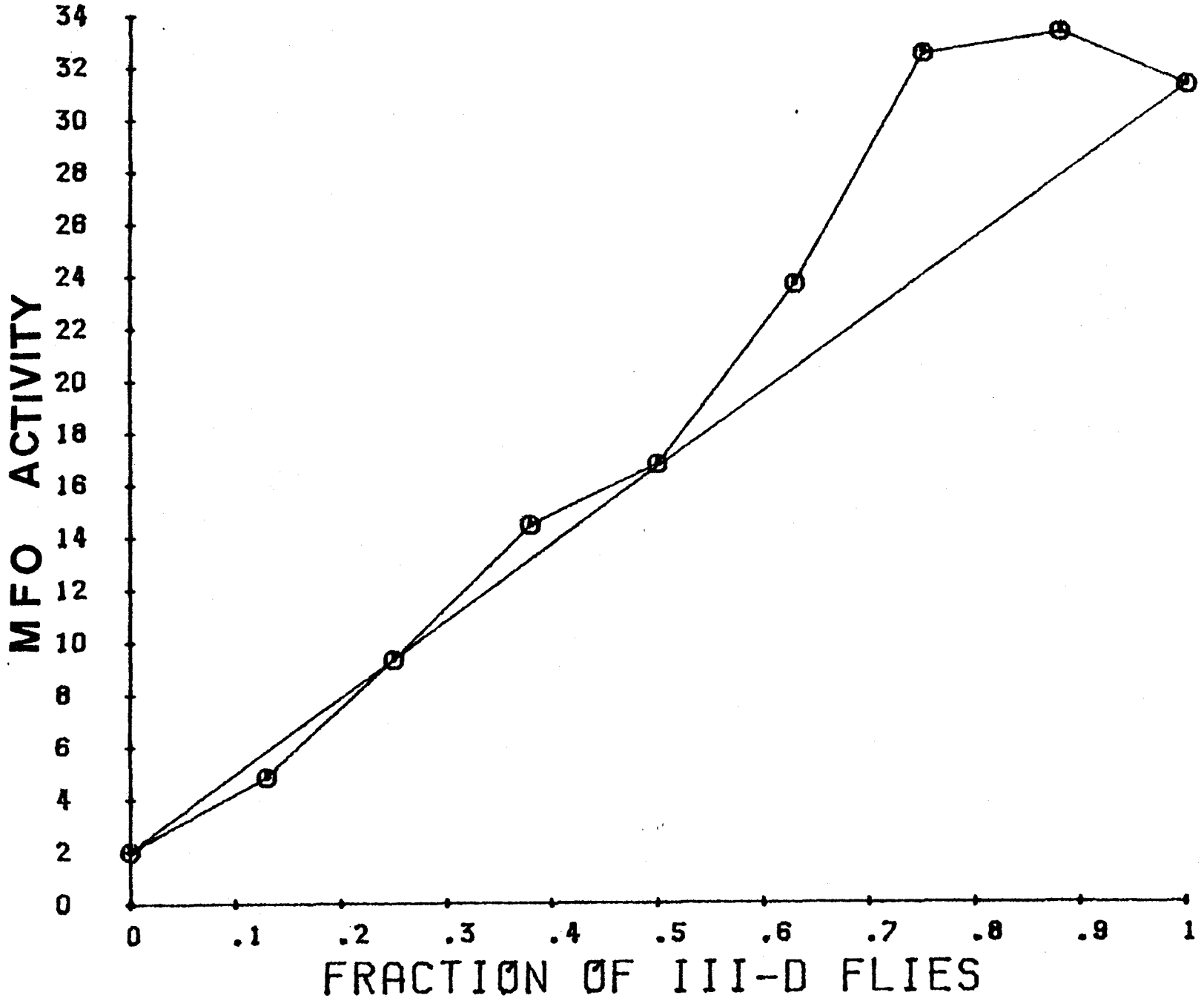
third chromosome between the recessive mutant markers cu(50.5 cM) and sr(62.0 cM). The approximate map position of this gene would be 54 cM. There is an inconsistency with this interpretation, though. According to the hypothesis of one major resistance gene exerting its effect on the third chromosome, those recombinant classes containing the portion of the third chromosome with the gene should have the same MFO activity as the F1 heterozygote. The activity of the F1 heterozygote should be made up of one resistant, high activity chromosome (from the Iso-III-D1 parental=8.4 pmoles/sec mg) and one non-resistant, low activity chromosome (from the "rucuca strain=0.7 pmoles/sec mg) totalling approximatel 9.0 pmoles/sec mg. In fact, the average MFO activities for those "resistant" F2 recombinants was only 3.1 pmoles/sec mg. This indicated that the resistant third chromosome was somehow not exerting its anticipated effect. There are a number of possibilities as to why this might be so.

(a) One is that there are gene products produced by the "rucuca" strain that inhibit MFO activity in the PNA Assay. This hypothesis was tested in a mixing experiment (Fig. 21). Different proportions of Iso-III-D1 and "rucuca" flies were assayed. The results are plotted against an expected curve, based on the expected contribution of third chromosomes from each type of fly. It was observed that there was no inhibition of activity by the rucuca extracts.

Figure 21.

Chromosome III "Mixing Experiment".

Different proportions of Iso-III-D1 and "rucuca" adults were assayed together using the PNA colorimetric assay. The O points are the actual results, the straight line represents what would be expected if the III-D1 contribution to total activity was proportional to its respective fraction of flies. MFO activity is measured in pmoles of p-nitrophenol formed per second per mg. of crude extract protein.


MFO ACTIVITY
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1
FRACTION OF III-D FLIES

(b) Another possibility is that the activity gene(s) on the third chromosome are not co-dominantly expressed. A test of dominance was carried out. Working under the assumption that that the gene products controlled by the two resistant third chromosomes were additive to produce the high MFO activity of the III-D strain, it was expected that the F1 activity should be made up of one high activity gene product (from the III-D strain) and one low activity gene product (from the rucuca strain) and hence should be the average of the two. This was not found. The average activity of the F1 generation was much lower than the anticipated average (data not shown). It is possible that high activity genes from the resistant third chromosome were not dominant to the low activity genes from the low activity strain. To test this, the III-D strain was crossed to a number of non-resistant, low activity control strains and the F1 generations were assayed for their respective MFO activities using the PNA assay. The results are shown in Figure 22. It is apparent that the activities of the various heterozygous F1 do not equal the expected F1 activity (based on the hypothesis that the activities of the resistant and non-resistant chromosomes are additive). One conclusion which may be reached is that the high activity gene on the third chromosome from the resistant strain is incompletely dominant with respect to the low activity gene from the non-resistant strain.

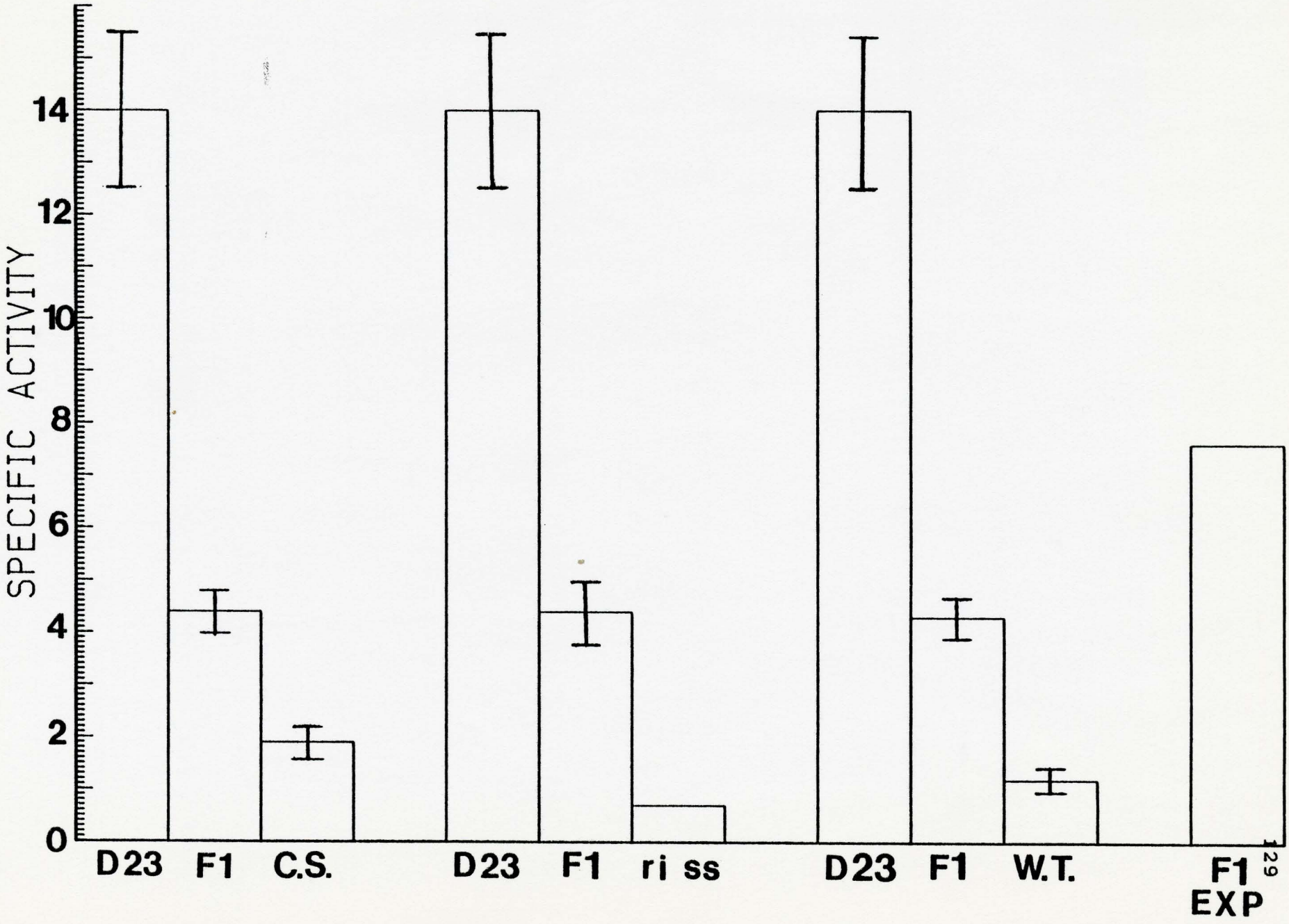
Although no further mapping of the gene(s) for high PNA demethylase activity on the third chromosome was performed, the

results shown in Figure 20 are consistent with a single locus of high activity between cu(50.5 cM) and sr (62.0 cM), at approximately 54 cM. Hallstrom (1985) had previously found that PNA demethylase activity was associated only with the second chromosome, although this researcher also found that other MFO enzyme activities (BP hydroxylation and 7-EC deethylation) were associated with loci on the third chromosome, at 51 cM and 58 cM respectively. It is possible that the locus associated with high PNA demethylase activity found in the present study is the same as that found by Hallstrom for 7-EC deethylase activity. Without further mapping of the third chromosome, this is just speculation.

Figure 22.

Test of Dominance of Activity Gene(s) on Third Chromosome.

Strain Iso-III-D was crossed to a number of low activity strains and these strains and the F1 were assayed using the PNA colourimetric assay. MFO activity is measured in pmoles of p-nitrophenol formed per second per mg of crude extract protein.


SPECIFIC ACTIVITY
0
2
4
6
8
10
12
14
D23
F1
C.S.
D23
F1
ri ss
D23
F1
W.T.
F1
EXP

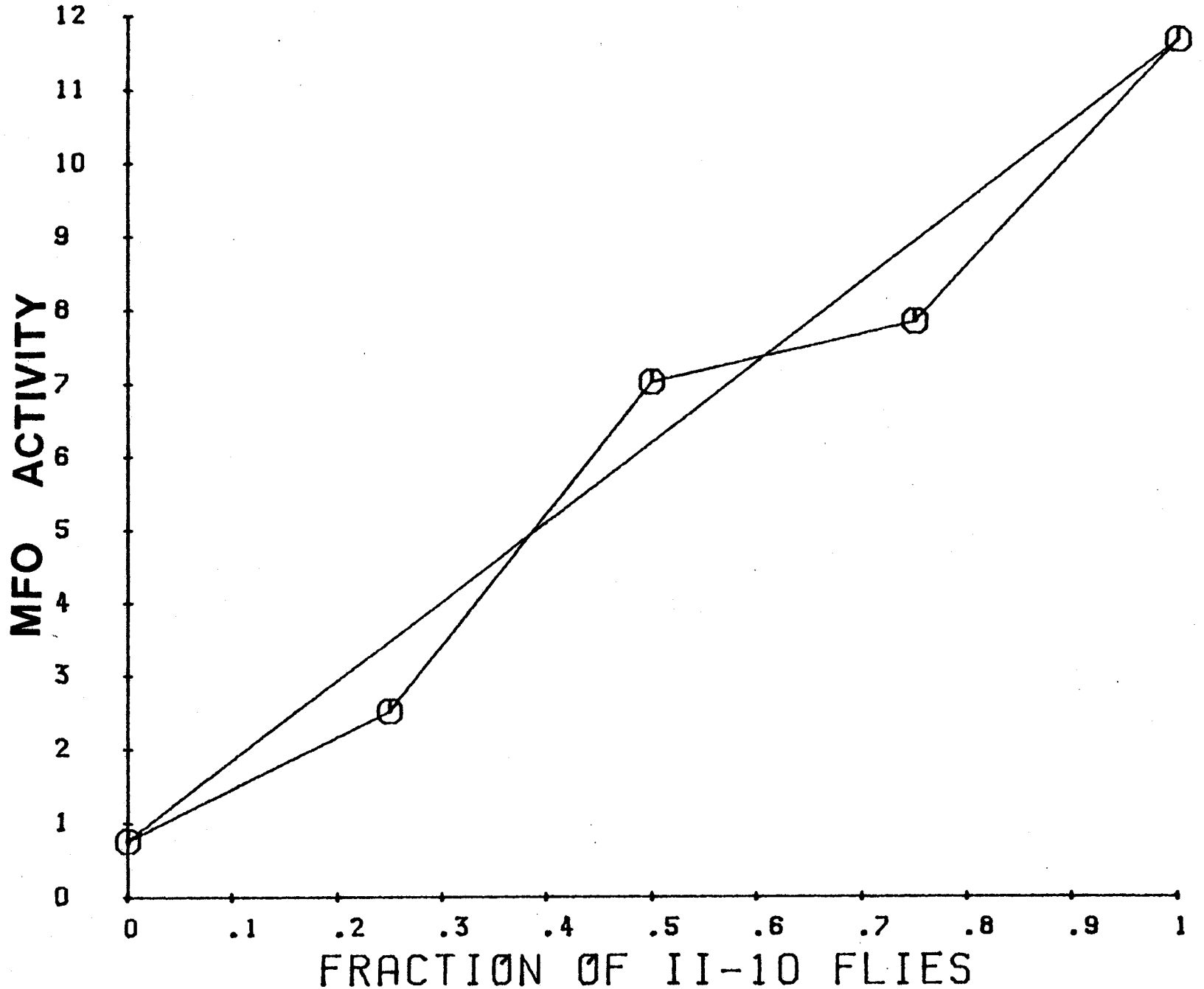
Appendix B: Second Chromosome 'Mixing' Experiment.

In order to determine whether the "all" strain produced any gene product that affected the MFO activity of F2 recombinant flies (resulting from a cross between "all" and the Iso-II-10 strain), the following experiment was performed. Different ratios of "all" flies were assayed with Iso-II-10 flies using the PNA MFO assay and the results were plotted with respect to an expected curve, based on the relative contribution of each genotype per ratio. No unexpected effect of the "all" flies was observed (see Figure 23).

Figure 23.

Chromosome II "Mixing Experiment".

Different proportions of Iso-II-10 and "all" adults were assayed together using the PNA colorimetric assay. The O points are the results, the straight line represents what would be expected if the Iso-II-10 contribution to total MFO activity was proportional to its respective fraction of flies. MFO activity is measured in pmoles of p-nitrophenol formed per second per mg. of crude extract protein.


MFO ACTIVITY
12
11
10
9
8
7
6
5
4
3
2
1
0
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1
FRACTION OF II-10 FLIES

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