MALATHION RESISTANCE IN *DROSOPHILA MELANOGASTER*

THE *IN VITRO* DEGRADATION OF [^{14}C]-MALATHION AND [^{14}C]-MALAOXON IN RESISTANT AND SUSCEPTIBLE STRAINS OF *DROSOPHILA MELANOGASTER*

By

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TITLE: The *In Vitro* Degradation of [^{14}C]-Malathion and [^{14}C]-Malaoxon in Resistant and Susceptible Strains of *Drosophila melanogaster*.

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ABSTRACT

Malathion-resistance in *Drosophila melanogaster* was studied in susceptible laboratory strains (CS and OR), a heterogeneous intermediate-resistant strain (C1-39), and a more resistant, artificially selected (with malathion) strain (MH19) by comparing the *in vitro* metabolism of [^{14}C]-malathion and [^{14}C]-malaoxon in crude enzyme preparations made from adult flies.

Extracts from all strains were found to contain two enzymatic activities that metabolized malathion and/or malaoxon. One activity degraded malathion to its α- and β-monocarboxylic acids and was designated as malathion-carboxylesterase activity (ME-activity). ME-activity was progressively lost in CS-extracts during reaction with [^{14}C]-malathion due to inhibition of the enzyme(s) by a tightly bound [^{14}C]-labeled molecule (not identified) that could not be removed by chromatography on Sephadex G-25. ME-activity, based on initial (0-1 min) rates with or without metyrapone present was similar in all strains and furthermore, the carboxylesterase inhibitors TPP and DEF did not synergize malathion toxicity in either resistant or susceptible strains. It was concluded that carboxylesterase-mediated degradation of malathion was not a factor in the resistance of the C1-39 and MH19 strains.

A second enzyme system, microsomal mixed-function oxidases (MFO), converted malathion to malaoxon (activation) and degraded malaoxon to at least two products that were tentatively identified (malaoxon α- and

β-monoacids and demethyl-malaoxon). The rate of conversion of malathion to malaoxon was highest in crude extracts of the most resistant MH19 flies, intermediate in C1-39 and could not be detected in the susceptible CS flies while the rate of malaoxon degradation was similar between MH19 and C1-39, but higher than that in the susceptible OR flies. Furthermore, malaoxon (but not malathion) toxicity was most strongly synergized by the MFO-inhibitor MTP in the more resistant strains (MH19 and C1-39). These results were used along with a previous result that MH19 strain possesses a less sensitive form of the target enzyme, acetylcholinesterase (R.A. Morton, personal communication), to propose a biochemical mechanism that accounts for the increased malathion-resistance of the C1-39 and MH19 strains.

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Most importantly, however, I would like to extend a loving thank you to my wife, Helene, who expressed limitless patience and understanding when the nights were late and the outlook was dim.

DEDICATION

For Tony...

....still takin' Pal.....this one's for you Bones...

Every increase of needs tends to increase one's dependence on outside forces over which one cannot have control, and therefore increases existential fear.

- Schumacher

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I. INTRODUCTION

A. Insecticide Resistance

Insecticide resistance has been a focus of much research since the end of the Second World War when the widespread use of chemicals to control insects began. The term "resistance" is somewhat ambiguous since it may be confused with a phenomenon such as "refractoriness", which is not a developed attribute, but a pre-existing condition (Brown, 1958). To clarify any possible confusion, the World Health Organization Expert Committee on Insecticides (1957) defined insecticide resistance as follows:

> "Resistance to insecticides is 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".

Insecticide resistance presently poses a serious agricultural and public health problem in many areas of the world. The United Nations Environment Programme (UNEP) (cited in Agarwal, 1979) has reported that insecticide resistance is increasing in terms of the numbers of species and geographic range reported for resistant insects. The UNEP report states that, according to the Food and Agriculture Organization (FAO) figures, the number of agricultural insect pests had risen from 182 in 1965 to 364 species in 1977. Furthermore, comparison of two recent World Health Organization (WHO) reports on pesticide resistance (World Health Organization, 1976 and 1980) reveals that insects posing a public health threat are also becoming more resistant. In 1976, 43

species of Anopheline mosquitoes, 40 species of Culicine mosquitoes and 38 species of other insects had developed resistance (WHO, 1976). The WHO figures reported in 1980 were 51, 52 and 41 for the respective pests (WHO, 1980). These numbers may not seem great compared to the above FAO figures, but the geographic range of these resistant insects has also increased (WHO, 1980). Most worrying are facts such as the following: Anopheline mosquito resistance-increases have been accompanied by a 30- to 40-fold increase in the occurance of malaria compared to figures in 1969-1970 (UNEP, cited in Agarwal, 1979).

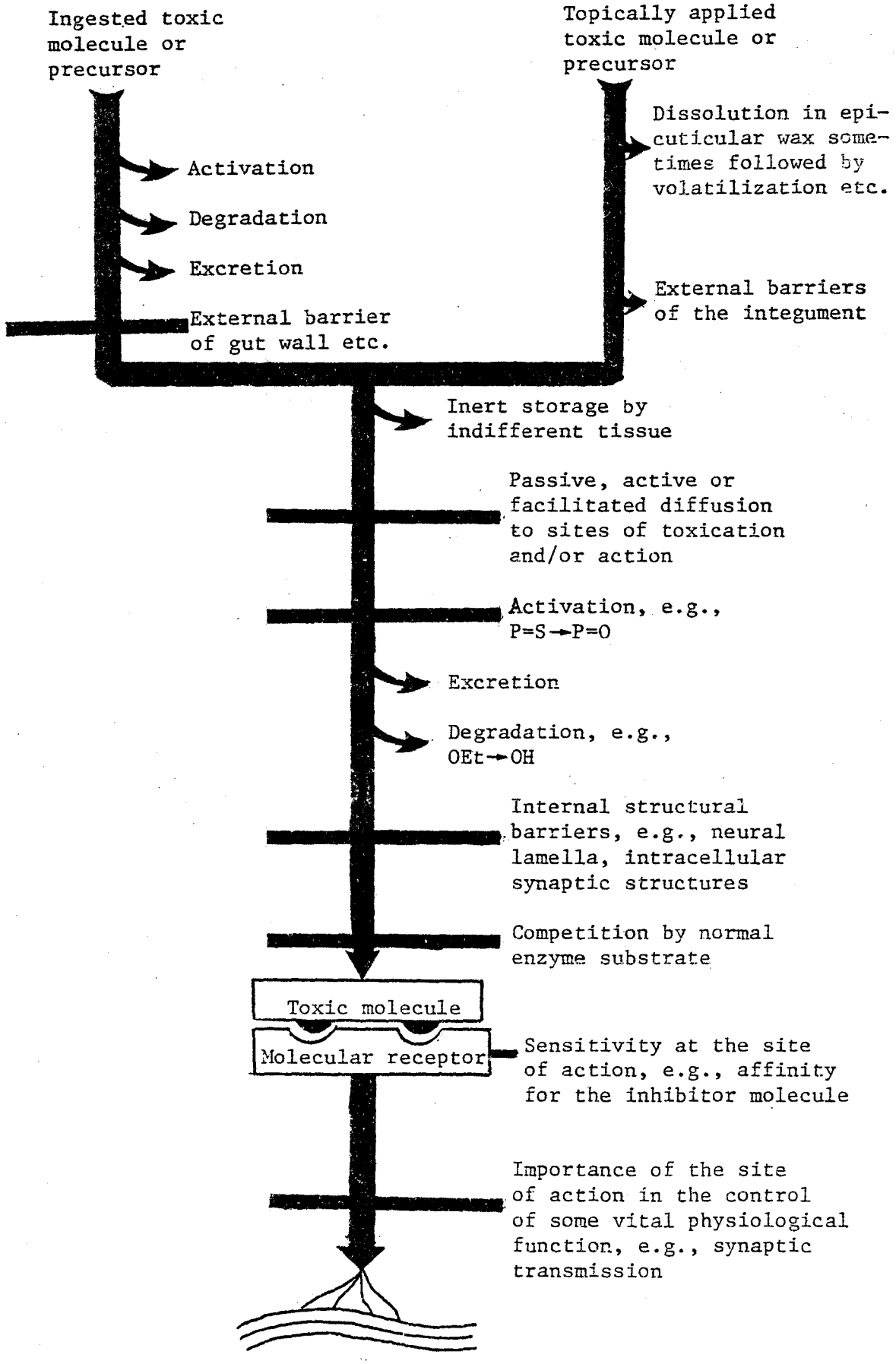
In order to gain an understanding of the mechanisms by which populations of insects become less susceptible to the action of an insecticide it is important to characterize the events that occur when a given insect comes into contact with a given insecticide. Figure 1 is a diagrammatic representation of biochemical and physiological rate-limiting steps and effective barriers in the toxicity of an either ingested or topically applied insecticide (modified from Winteringham, 1969). The thick vertical arrow in Figure 1 represents the successful formation and persistance of toxic molecules reaching the "target" molecular receptors of the "site of action". The target of organophosphate insecticides is thought to be acetylcholinesterase (AChE) (reviewed by O'Brien, 1976) whereas the target for pyrethroid insecticides is thought to be Na^+/K^+ channels in axons (reviewed by Narahashi, 1971 and 1976). Both of these insecticides interfere with the normal neurophysiology of the insect; organophosphates inhibit AChE and thereby block synaptic transmission, whereas pyrethroids block Na^+/K^+ channels and

FIGURE 1

Processes Mediating The Toxicity of Topically Applied or Ingested Insecticides*

The vertical arrow represents the successful formation and survival of toxic molecules as far as the molecular receptors of the "site of action". Horizontal arrows represent competing processes of inert storage, degradation, excretion, etc. Horizontal bars represent rate-limiting steps or barriers in the overall process of intoxication.

*Modified from Winteringham (1969).


Ingested toxic molecule or precursor
Topically applied toxic molecule or precursor
Dissolution in epicuticular wax sometimes followed by volatilization etc.
Activation
Degradation
Excretion
External barriers of the integument
External barrier of gut wall etc.
Inert storage by indifferent tissue
Passive, active or facilitated diffusion to sites of toxication and/or action
Activation, e.g., P=S→P=O
Excretion
Degradation, e.g., OEt→OH
Internal structural barriers, e.g., neural lamella, intracellular synaptic structures
Competition by normal enzyme substrate
Toxic molecule
Molecular receptor
Sensitivity at the site of action, e.g., affinity for the inhibitor molecule
Importance of the site of action in the control of some vital physiological function, e.g., synaptic transmission

thus interfere with the conduction of action potentials along axons. Devonshire (1973), in reference to a diagram similar to Figure 1, pointed out that only a small change in any process that can alter the amount of toxic molecules (insecticide) reaching the target can cause a large change in the required lethal dose (resistance). Studies on insecticide resistance are generally directed at the discovery of what particular processes (Figure 1) have been altered and the contribution these changes make to resistance.

B. Non-metabolic Mechanisms of Resistance

The present study deals only with the role of differences in insecticide metabolism in resistance, but there are processes other than metabolism (Figure 1) that can be altered in such a way to cause resistance. The metabolism of insecticides and the role of alterations in such processes in resistance is discussed later, with particular attention to malathion. Three examples of non-metabolic mechanisms of resistance are discussed here.

B(i). Decreased Adsorption of Insecticide Through the Integument

Resistance can be caused by a change in the integument that retards the entry of topically applied insecticide. Plapp and Hoyer (1968), using houseflies, *Musca domestica*, showed that a gene, "tin", acts as an intensifier of resistance by causing a decreased rate of absorption of insecticide through the insect cuticle. By itself, the "tin" gene had a small effect on resistance, but in combination with other resistance mechanisms it significantly increased resistance above levels seen in resistant flies not possessing "tin". As shown by this

example, limiting the rate of absorption affords the insect more opportunity to degrade the insecticide (cf. Figure 1).

B(ii). Increased Excretion of Insecticide

An insecticide can not very well exert a toxic effect if it is excreted before reaching its target (cf. Figure 1). Matthews (1980) found an increased excretion rate of intact insecticide (malathion) in a resistant strain of the lesser grain borer, *Rhyzopertha dominica*, but could not detect any differences with respect to *in vivo* degradation. Increased excretion as a mechanism of resistance could possibly[1] be quite complex, since differences in excretion rates were found when the insecticide was topically applied and therefore it must have passed through several different tissues before reaching the gut from where it was finally discharged.

B(iii). Decreased Sensitivity of Target

If the target molecule is less sensitive to the inhibitory effect of the toxic molecule (insecticide), more insecticide will be necessary to kill the insect (cf. Figure 1). Acetylcholinesterase (AChE), the presumed target of organophosphate insecticides, that is less sensitive to inhibition has been shown to contribute to resistance in spider mites, *Tetranychus urticae*, (Voss and Matsumura, 1964 and Smissaert, 1964) and more recently in the mosquito, *Anopheles albimanus*, (Hemingway and Georghiou, 1983).

In summary, if genetic variability exists in any of the processes that modulate the ultimate toxic effect of an insecticide (this includes alterations of the target), it may well become an important factor in the evolution of resistance by a population of insects pressured by an

insecticide. Variants that reduce the amount of insecticide reaching the target or possess a less sensitive target will be favourably selected. If the selected attributes are genetically controlled, succeeding generations will manifest resistance. Many of the resistance mechanisms that are related to single enzymatic alterations have been genetically mapped (AChE, tin and many of the insecticide degrading enzymes discussed later) and are generally inherited as co-dominant characters that are inseparable from resistance (reviewed by Plapp, 1976).

C. Organophosphate Insecticides

Gerhard Schrader is probably the most important figure in the development of insecticidal organophosphorus compounds (reviewd by Eto, 1979a). In 1937, Schrader and his co-workers discovered that certain neutral organic phosphorus esters had contact insecticidal activity. An important advance in this field was made in 1944, when Schrader discovered compound No. 605, named parathion (Figure 2). From that time to the present, innumerable other organophosphate insecticides have been developed that possess the general structure as illustrated in Fig. 2. As mentioned earlier, these compounds are thought to cause toxicity by inhibiting AChE. The thiono-analogues (P=S compounds) are poor inhibitors of AChE, whereas the oxo-analogues (P=O compounds) are potent AChE inhibitors (reviewed by Eto, 1979b). The toxicity exhibited by the thiono-compounds, which are the form generally used as insecticides, is due to oxidative desulfuration of the thiophosphoryl group (P=S) *in vivo* forming the phosphoryl group (P=O). This reaction is often referred to as "activation".

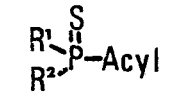
FIGURE 2

General Structure and Examples of Organophosphate Insecticides*

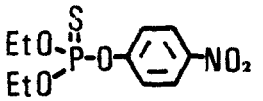
R^1 and R^2: alkyl, alkoxy or amino groups

Acyl: any acid residue

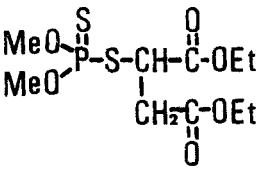
* General Structure taken from Eto (1979b).



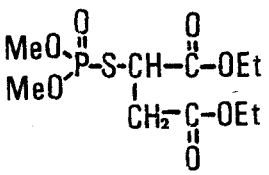
general structure



parathion



malathion



malaoxon

C(i). Malathion

The organophosphate insecticide, malathion (Figure 2), was discovered by the American Cyanimid Co. in 1950. Compared to compounds of similar structure (i.e. 0,0-diethyl-analogues of malathion, see Figure 2), malathion exhibits the highest selective toxicity towards insects (Johnson *et al.*, 1952). It was found to be more extensively degraded in mammals and birds compared to insects, thus accounting for its selectivity (March *et al.*, 1956, Krueger *et al.*, 1959). Cognate with other organophosphate insecticides, malathion is activated, *in vivo* to its oxon-analogue, malaoxon (Figure 2). O'Brien (1957) first discovered this reaction in the cockroach, *Periplaneta americana*, and showed that malaoxon has about 4000-fold higher anticholinesterase-activity than malathion (based on inhibition of housefly AChE). Since malathion and malaoxon are both potentially toxic to the insect, it is important to characterize the metabolism of both compounds in studies of malathion resistance.

D. The Metabolism of Malathion in Resistant and Susceptible Insects

This section is intended to serve as an introduction and summary of the known mechanisms of malathion metabolism in resistant and susceptible insects. Malathion is activated by mixed function oxidases and both malathion and malaoxon can be degraded by numerous enzymes such as carboxylesterases, phosphatases, mixed function oxidases and glutathion-S-alkyltransferases.

D(i). Activation

Conversion of malathion to malaoxon is mediated by microsomal

mixed function oxidases (reviewed by Eto, 1979b). Since malaoxon is more toxic than malathion, a strain of insects that accumulates less malaoxon on exposure to malathion will be less susceptible to its toxic effects. This has been shown in resistant strains of mosquitoes, *Culex tarsalis* (Matsumura and Brown, 1961), houseflies, *Musca domestica* (Matsumura and Hogendijk, 1964) and bedbugs, *Cimex lectularius* (Feroz, 1971). Since malaoxon was degraded more extensively by the resistant insects in these studies, less accumulated malaoxon more likely reflected increased rates of malaoxon degradation and not decreased rates of malaoxon production.

D(ii). Degradation

Reactions in which malathion or malaoxon is changed to an ionic derivative (at physiological pH) are detoxication reactions. Ionic derivatives of organophosphate insecticides are extremely poor inhibitors of AChE (reviewed by O'Brien, 1976) and thus, increased capacity of an insect to convert malathion and malaoxon to ionic derivative will impart resistance.

D(iia). Carboxylesterases

Malathion is unique compared to the majority of organophosphates. It possesses two carboxylester bonds and is therefore labile to attack by carboxylesterases forming the di- and monocarboxylic acids of malathion (Figure 3) (reviewed by Oppenoorth and Welling, 1976a). Since esterases are among the most polymorphic of enzymes in insect populations (Powell, 1975), it might be expected that evolution of malathion resistance would involve the selection of genotypes having increased activity towards the carboxylester bonds of malathion. Increased malathion-

FIGURE 3

Chemical Structures of Known Degradation Products of Malathion and Malaoxon

Superscript figures in brackets represent the enzyme(s) involved in the formation of the respective product; 1 = carboxylesterases; 2 = phosphatases; 3 = mixed function oxidases; 4 = glutathion-S-alkyltransferases. (0) represents the corresponding product formed from malathion. References and more details are given in part D(ii) of INTRODUCTION.

(i) malathion α-monoacid[1]

(ii) malathion β-monoacid[1]

(iii) malathion α, β-diacid[1]

(iv) 0,0-dimethyl phosphorothionic acid[2] (from malathion)
0,0-dimethyl phosphoric acid[2] (from malaoxon)

(v) diethylthiosuccinic acid [2]

(vi) 0,0-dimethyl phosphorothiolothionic acid[2] (from malathion)
0,0-dimethyl phosphorothiolic acid [2] (from malaoxon)

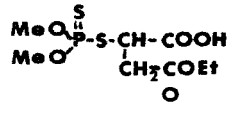
(vii) diethyl malic acid[2]

(viii) malaoxon α-monoacid[1]

(ix) malaoxon β-monoacid[1,2]

(x) demethyl-malathion[2,4]

(xi) demethyl-malaoxon[2,3,4]



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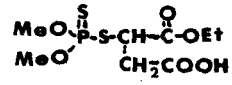

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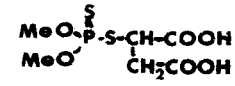

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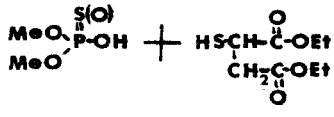

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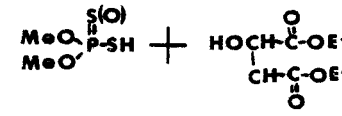

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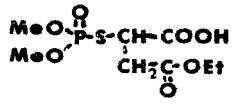

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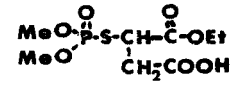

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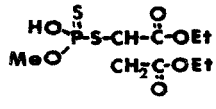

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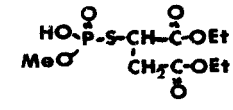


xi

carboxylesterase activity has been found in resistant strains of the mosquito species, *Culex tarsalis* (Matsumura and Brown, 1961; Bigley and Plapp, 1962) and *Anopheles stephensi* (Hemingway, 1982), spider mites, *Tetranychus urticae* (Matsumura and Voss, 1964), houseflies, *Musca domestica* (Matsumura and Hogendijk, 1964; Niwa *et al.*, 1964), blowflies, *Chrysomya putoria* (Townsend and Busvine, 1969), bedbugs, *Cimex lectularius* (Feroz, 1971) and smaller brown leaf hoppers, *Laodelphax striatellus* (Miyata *et al.*, 1976). In insects where increased carboxylesterase activity is the sole mechanism of malathion resistance there is no appreciable cross-resistance to other organophosphates not possessing carboxylester bonds (Matsumura and Brown, 1961; Bigley and Plapp, 1962). Furthermore, in cases where carboxylesterase activity is an important component of resistance, carboxylesterase inhibitors such as triphenyl phosphate (TPP) and S,S,S-tributyl trithiophosphate (DEF, for "defoliant") greatly decrease resistance to malathion. The degradation of malaoxon by carboxylesterases seems to play a minor role in resistance since malaoxon is both a substrate and a potent inhibitor of these enzymes (Main and Dauterman, 1967).

D(iib). Phosphatases

Phosphoric triester hydrolases (phosphatases) can cleave the phosphorus ester bonds (C-O-P and P-S-C) of malathion and malaoxon to form several less toxic acidic products (Figure 3). Increased malathion and/or malaoxon phosphatase activity has been found in malathion-resistant strains of mosquitoes, *Culex tarsalis* (Matsumura and Brown, 1961; Bigley and Plapp, 1962), houseflies, *Musca domestica* (Matsumura and Hogendijk, 1964; Niwa *et al.*, 1977), begbugs, *Cimex lecturius*

(Feroz, 1971) and spider mites, *Tetranychus urticae* (Matsumura and Voss, 1964). In all of these studies increased carboxylesterase activity was found together with increased phosphatase activity. With the exception of *Cimex lectularius* (Feroz, 1971), carboxylesterase products accounted for a major part of malathion degradation. Inasmuch as phosphorus ester bonds are a common characteristic of organophosphate insecticides, increased phosphatase activity may confer some cross-resistance to other organophosphates. In *Cimex lectularius* (Feroz, 1971), where increase phosphatase activity accounts for the majority of malathion degradation, the resistant strain shows moderate cross-resistance to other organophosphates.

D(iic). Microsomal Mixed Function Oxidases

Microsomal mixed function oxidases (MFO) perform a large variety of transformations on most major groups of insecticides including organophosphates (reviewed by Terriere, 1968; and Nakatsugawa and Morelli, 1976). MFO-mediated reactions are characterized by their location in the endoplasmic reticulum and the absolute requirement for NADPH and molecular oxygen. A key component of the MFO-system is the terminal oxidase, cytochrome P-450, which is responsible for the binding and oxidation of the substrate. The name "P-450" derives from the characteristic absorption of light (Soret peak) at about 450 nm when dithionite-reduced microsomes are treated with carbon monoxide (Omura and Sato, 1964). Insect cytochrome P-450 has been most thoroughly characterized in the housefly, *Musca domestica* (reviewed by Hodgson *et al.*, 1974; and Agosin, 1976). Different forms of cytochrome P-450, defined mainly by spectral ligand-binding properties,

have been found in resistant and susceptible strains of houseflies. Moreover, certain quantitative and qualitative changes in cytochrome P-450 have been shown to be genetically inseparable from insecticide resistance. Tate *et al.* (1974) have shown, in *Musca domestica*, that increased cytochrome P-450 levels and resistance were associated with a gene(s) on chromosome II. Futhermore, they showed that certain qualitative changes in cytochrome P-450 (as measured by ligand-binding spectra) and resistance mapped to genes on chromosomes II and V.

In addition to activation of organophosphates (discussed above), the MFO system is also responsible for the oxidative degradation of a large number of organophosphate, carbamate, pyrethroid and chlorinated insecticides and therefore, insects that are resistant by virtue of increased MFO-activity are generally cross-resistant to several classes of insecticides (reviewed by Oppenoorth and Welling, 1976). O-dealkylation (cleavage of P-O-C bonds) of organophosphates by the MFO-system is a common mode of degradation. To date, this reaction has not been demonstrated for malathion in any insect species. However, oxidative O-demethylation of malaoxon may be operative in a malathion resistant strain of the flour beetle, *Tribolium castaneum* (Dyte *et al.*, 1970a, cited in Oppenoorth and Welling, 1976). In addition, the resistant flour beetles were cross-resistant to a wide range of other organophosphates. Welling *et al.* (1974) showed that malaoxon was oxidatively degraded by cleavage of a carboxylester bond forming its β-monocarboxylic acid (Figure 3) in a malathion resistant strain of houseflies, *Musca domestica*. In both of these latter studies, the resistant strains were more tolerant to malaoxon than

malathion. This is in direct contrast to situations where malathion resistance is primarily due to increased carboxylesterase (see part D(iia) carboxylesterases). In cases where malathion-resistance is mediated by carboxylesterases, resistant insects were less tolerant to malaoxon than malathion (Matsumura and Brown, 1961; Matsumura and Voss, 1964; Matsumura and Hogendijk, 1964). Relative resistance to malathion *versus* malaoxon is discussed further in the discussion section of the thesis.

The action of mixed function oxidases is blocked by certain methylene dioxyphenyl compounds such as piperonyl butoxide and sesamex, which specifically inhibit cytochrome P-450 (reviewed by Eto, 1979c and Testa and Jenner, 1981). Where increased MFO-activity plays a major part in resistance, inhibitors of cytochrome P-450 can abate this acquired tolerance by blocking the degradation of the insecticide (reviewed by Wilkinson, 1971; and Oppenoorth, 1971).

D(iid). Glutathione-S-alkyltransferases

Glutathione-S-alkyltransferases are known to catalyse dealkylation (cleavage of P-O-C bond) of several organophosphates (reviewed by Yang, 1976). They are soluble enzymes which require reduced glutathione (GSH) for activity. The leaving group (alkyl) of the organophosphate becomes covalently bound to GSH. The only conclusive report of malathion and malaoxon degradation by GSH-transferases is that of Houx *et al.* (1979). They showed that malathion and malaoxon are converted to demethyl-malathion and demethyl-malaoxon (Figure 3), respectively, by housefly, *Musca domestica*, homogenates fortified with GSH. However, the role of these reactions with respect to resistance was not investigated.

D(iie). Summary

The metabolism of malathion (or any organophosphate) can be divided into two major catagories: activation and degradation. It is the balance of these processes including the sensitivity of AChE to inhibition that will determine the survival of an insect when challenged with an insecticide. Furthermore, the relationship of these two processes with respect to each other and the target (AChE) is of extreme importance. Since some metabolites can be produced by more than one route (e.g. demethyl-malaoxon and malaoxon β-monoacid), subcellular location, cofactor requirements and specific inhibitors must be characterized to unquestionably identify the enzyme(s) involved in converting substrate to product. *In vitro* studies are necessary to determine the enzymology of a given metabolic transformation whereas *in vivo* studies are necessary to determine the contribution of the reaction to the survival of the insecticide exposed insect.

E. The Purpose of This Work

In this study, an attempt was made to determine the role of *in vitro* malathion and malaoxon degradation in a resistant strain of *Drosophila melanogaster* that was artificially selected from a genetically heterogeneous population. Although *D. melanogaster* is not a serious insect pest, it is genetically well characterized. This provides the possibility to identify and map resistance genes which are responsible for the appropriate biochemical results. The method of approach was as follows: radioactively labeled (^{14}C) malathion and malaoxon were used as tracers in experiments; crude enzyme preparations and microsomes from

resistant and susceptible flies provided the sources of material used to characterize the degradation data reported here.

This thesis is divided into two sections. Section I describes the comparative degradation of [^{14}C]-malathion by carboxylesterase(s) in resistant and susceptible flies and provides evidence that this activity is not important to resistance. These experiments examine relative activity, inhibition and the synergism of malathion toxicity by carboxylesterase inhibitors.

The results in Section II provide evidence that differences in mixed function oxidases are responsible for a major part of the malathion resistance in *D. melanogaster*. The experiments reported in this section examine synergism of malathion and malaoxon toxicity by MFO-inhibitors, relative MFO-activity (p-nitroanisole O-demethylase activity), cytochrome P-450 content, [^{14}C]-malaoxon degradation and their role in resistance.

II. MATERIALS AND METHODS

A. Culture of *Drosophila melanogaster*

Drosophila melanogaster strains were maintained in 8 oz. glass bottles on a banana-agar medium containing the following components: water, 1.8 ℓ; agar (Difco), 20 g; malt powder, 1.5 tablespoons; corn syrup, 2 tablespoons; bananas (peeled), 160 g; dried yeast powder, 60 g; 95% ethanol, 40 ml; 10% p-hydroxybenzoic acid methyl ester (methylparaben) in 95% ethanol, 36 ml.

The strains under study were derived from a selection experiment described by Singh and Morton (1981). A genetically heterogeneous population of *D. melanogaster* (caught locally) was exposed to malathion in the growth media for 19 generations to produce the resistant strain called MH19, which afterwards was maintained without further selection on banana medium not containing malathion. A control population, C1-39, was maintained in parallel without exposure to insecticide. Two laboratory stock strains, Canton Special (CS) and Oregon Regular (OR), were obtained from the Drosophila Stock Centres at the California Institute of Technology and Bowling Green State University, respectively.

B. Chemicals

[^{14}C]-malathion [0, 0-dimethyl-S-1, 2-bis(carboethoxy) ethyl phosphorodithioate] (labeled at both succinyl carbons) was purchased from Amersham. Malathion and malaoxon [0, 0-dimethyl-S-1, 2-bis (carboethoxy)ethyl phosphorothioate] analytical standards were a gift

from American Cyanimid Co., N.J. Metyrapone [2-methyl-1, 2-di-3-pyridyl-1-propanone], p-nitrophenol (spectrophotometer grade), bovine serum albumin (fraction V) and D-glucose-6-phosphate (monosodium salt) were purchased from Sigma Chemical Co., St. Louis, Mo. M-chloroperbenzoic acid (purified grade) was purchased from Fischer Scientific Co., Toronto, Ont. DEF [S, S, S-tributyl phosphorotrithioate], parathion [0, 0-diethyl-0-4-nitrophenyl phosphorothioate], diazinon [0, 0-diethyl-0-(2-isopropyl-4-methyl-6-pyrimidinyl) phosphorothioate], permethrin [(3-phenoxybenzyl)-3-(2, 2-dichlorovinyl)-2, 2-dimethylcyclopropane-carboxylate; 41%/59%, cis/trans], and carbaryl [1-naphthyl methylcarbamate] were gifts from Dr. David Pree, Agriculture Canada, Vineland, Ontario. Triphenylphosphate (TPP), 2, 6-dibromo-N-chloro-p-benzoquinoneimine (DQC) and Coomassie brilliant blue G-250 were purchased from Eastman Chemicals, Rochester, N.Y. p-Nitroanisole (pNA) was purchased from Aldrich Chemical Co., Milwaukee, Wisconsin and was recrystalized twice from ethanol. Piperonyl butoxide (PBO) [3, 4-methylenedioxy-6-propylbenzyl n-butyl diethyleneglycol ether], 80%, was purchased from ICN Pharmaceuticals, Plainview, N.Y. Glucose-6-phosphate dehydrogenase (grade I, from yeast) was purchased from Boehringer-Mannheim, Canada. All other chemicals were reagent grade.

C. Synthesis of [^{14}C]-malaoxon

[^{14}C]-malaoxon (labeled at both succinyl carbons) was synthesized according to Welling *et al.* (1974) with minor modifications. Approximately 100 µg (80 nℓ) of [^{14}C]-malathion was dissolved in 8 mℓ of reagent grade methylene chloride, and to this solution, 2 mℓ of a

solution of m-chloroperbenzoic acid (0.11 mmoles) in methylene chloride was added. The mixture was refluxed in a 20 mℓ round bottom flask fitted to a water cooled condenser for 2 h. After cooling, the solution was washed with 5 mℓ volumes of, first, 2% $NaHSO_3$ (1X), second, 2% $NaHCO_3$ (1X) and last 3% Na_2SO_4 (4X). The methylene chloride layer was reduced to 0.5 mℓ by use of an aspirator and applied to the preadsorbant area of a preparative thin layer chromatographic plate (Whatman Linear-K, silica gel, 20 X 20 cm, 1000 μm layer). The plate was developed with benzene-ethylacetate (1:2) in a paper lined and equilibrated glass tank at room temperature. The location of [^{14}C]-malaoxon was identified by running a standard on the same plate. The standard was visualized using DQC reagent (see "Thin Layer Chromatography"). The appropriate area (approximately R_f 0.6) was scraped from the plate by use of an aspirator attached to a glass tube (1 cm X 8 cm) plugged with a glass wool filter. The silica gel scraping was extracted with 2 mℓ of the developing solvent (8X, total 16 mℓ) in a conical centrifuge tube. The extracts were combined and reduced to dryness on a rotary evaporator. The residue was dissolved in 5 mℓ of chloroform and washed with 10 mℓ of 50 mM Tris-HCℓ buffer, pH 7.1. The chloroform layer was reduced to dryness and the residue dissolved in 1 mℓ of ethanol. The purity was checked by thin layer chromatography against a malaoxon standard (see "Thin Layer Chromatography"); radioactivity was found only in the area of the plate corresponding to the malaoxon standard. The yield of [^{14}C]-malaoxon varied in different preparations from 10% to 40% based on the initial amount of [^{14}C]-malathion used.

D. Assay of Insecticide Resistance

Two methods were used to assay the resistance of adult flies to malathion. In one, a feeding assay (Singh and Morton, 1981), about 15 to 30 flies (3 to 10 days post-eclosion, males and females mixed) were placed in a 50 mℓ plastic vial containing approximately 5 mℓ of 1% sucrose, 1.5% agar and the insecticide preparation to be tested. Survival curves were determined by plotting probit survival against log time and fitting the data to a straight line using linear regression. In this manner a LT50 (for 50% lethal time) was obtained. The other method, a vacuum injection assay, which provides an even coating of insecticide over the external body surface, was carried out with adult male flies as described previously (Pluthero and Threlkeld, 1980). Briefly, 1 µl of a 2% solution of malathion in 2-propanol was injected at low pressure (50 cm Hg) into a 10 mℓ serum bottle containing 20 male flies. After 2 min., the seal was broken and survivors were counted at timed intervals. The LT50 was determined in the same manner as the feeding assay (see Pluthero and Threlkeld, 1980 for more details).

E. *In vitro* Degradation of [^{14}C]-Malathion

Adult flies (40 mg fresh weigh/mℓ buffer) were homogenized in 50 mM Tris-HCℓ buffer, pH 7.1 using a glass tissue homogenizer. The homogenate was centrifuged for 20 min. at 10000 rpm (12000X g, Servall SS-34 rotor) and the supernatant used as crude enzyme preparation. All operations were carried out at 0-2°C.

The desired amount of [^{14}C]-malathion (2-4 nmoles at 0.6 nmol/ml in ethanol) was placed in a test tube. The ethanol was removed with N_2 and the residue dissolved with

1.0 mℓ of 50 mM Tris-HCℓ buffer, pH 7.1. The reaction was initiated by adding 1.0 mℓ of the crude enzyme preparation and was maintained at 30(±1)°C with continuous shaking. After timed intervals, 0.2 mℓ aliquots of the reaction mixture were removed and mixed with 1 mℓ of $CHC\ell_3$ and 0.8 mℓ of Tris-HCℓ buffer, pH 7.1 in a 15 mℓ conical centrifuge tube. The phases were separated by centrifuging at 4500 rpm in a table-top centrifuge and the lower ($CHC\ell_3$) layer removed. The extraction was repeated with another 1 mℓ of $CHC\ell_3$ and the two $CHC\ell_3$ extracts were combined ("neutral-$CHC\ell_3$ fraction"). The aqueous phase was acidified to approximately pH 2 with 0.1 mℓ of 0.2N HCℓ and extracted twice with 1 mℓ of $CHC\ell_3$. These $CHC\ell_3$ extracts were combined (2 mℓ total) to give the "acidic-$CHC\ell_3$ fraction". The remaining aqueous phase has been called the "residual fraction".

F. *In vitro* Degradation of [^{14}C]-Malaoxon

Whole adult flies (120 mg fresh weight/mℓ buffer) were homogenized in 50 mM Tris-HCℓ buffer, pH 7.9 containing 5 mg/mℓ BSA and 7.5 mM $MgC\ell_2$ in a glass tissue homogenizer. The homogenate was centrifuged at 10000 rpm for 15 min (12000X G, Servall SS-34 rotor) and the supernatant used as a crude enzyme preparation. All operations were carried out at 0-2°C.

A substrate solution was prepared by placing the desired amount (3-6 nmol) of [^{14}C]-malaoxon (0.5 nmol/μℓ in ethanol) in a test tube and removing the solvent with N_2. The residue was dissolved in 1.0 mℓ of Tris-HCℓ buffer, pH 7.9 containing 5 mg/mℓ BSA, 7.5 mM $MgC\ell_2$, 1.0 mM NADP and 5 mM glucose-6-phosphate. The reaction was initiated by mixing

equal volumes of the crude enzyme preparation (see above) and the substrate solution followed by the immediate addition of glucose-6-phosphate dehydrogenase (0.35 units/mℓ of reaction mixture). The reaction was maintained at 30(±1)°C with continuous shaking. At various times during the reaction aliquots (0.1 mℓ) were removed and added to 1.0 mℓ of $CHC\ell_3$. After vigorous mixing, 0.9 mℓ of buffer (50 mM Tris-HCℓ, pH 7.1) was added to the extraction mixture to bring the total aqueous volume to 1.0 mℓ . The phases were separated by centrifugation at 4500 rpm. The $CHC\ell_3$ phase contained unreacted malaoxon and the aqueous phase contained the acidic products.

In some experiments aliquots (0.2 mℓ) were removed at 180 min. after the start of the reaction in order to analyze the reaction products. Samples were extracted twice with 1.5 mℓ of $CHC\ell_3$ and the neutral $CHC\ell_3$ extracts were pooled. The aqueous phase was acidified to pH 2 by the addition of 0.2N HCℓ and extracted with $CHC\ell_3$ as above. The acidic $CHC\ell_3$ fraction contained weakly acidic products ($pK_a > 2$). The remaining aqueous phase contained very acidic products ($pK_a < 2$).

G. p-Nitroanisole O-demethylase Assay

Mixed function oxidase activity was characterized by the assay of p-nitranisole O-demethylase activity (pNA-demethylase activity) (Hansen *et al.*, 1971). Reactions were initiated by mixing crude enzyme preparations (1.0 mℓ, see below) or washed microsomes (50 µℓ, see below) with 1.0 mℓ of substrate solution (1.0 mM p-nitroanisole, 1.0 mM NADP and 5 mM glucose-6-phosphate in TKE buffer containing 10% glycerol). Glucose-6-phosphate dehydrogenase (0.7 units) was added immediately

after mixing the enzyme source with the substrate solution and the reaction was maintained at 30 (±1)°C for 60 min with continuous shaking. The reaction was terminated by the addition of 0.5 mℓ of 1N HCℓ followed by extraction with 2.5 mℓ of $CHC\ell_3$. The phases were separated by centrifugation at 4500 rpm in a table-top centrifuge. A portion (1.5 mℓ) of the $CHC\ell_3$ layer was removed and extracted with 1.5 mℓ of 0.5N NaOH. The amount of product (p-nitrophenol) was determined from the absorbance of the NaOH layer at 400 nm. A standard curve was obtained by dissolving known concentrations of p-nitrophenol in 0.5N NaOH.

Crude enzyme preparations for the measurement of pNA-demethylase activity were made by manual homogenization of adult flies (100 mg fresh weight/mℓ buffer) in TKE buffer (50 mM Tris-HCℓ buffer, 50 mM KCℓ, 1 mM disodium EDTA, 10% glycerol, pH 7.4) using a glass tissue homogenizer. The homogenate was centrifuged for 30 min at 10000 rpm (12000X g, Servall SS-34 rotor) and the supernatant used in the pNA-demethylase assay. All operations were carried out at 0-2°C.

Microsomal pNA-demethylase activity was determined by using 50 µℓ of washed microsomes (resuspended in 60 mM sodium phosphate buffer, pH 7.4 containing 2 mM $MgC\ell_2$ and 2% glycerol) in place of the crude enzyme preparation (see "Microsome Preparation").

H. Microsome Preparation

Microsomes were prepared following the method described by Waters *et al.* (1982). All operations were carried out at 0-2°C. Whole adult flies (2.0 g) were very gently homogenized in 15 mℓ of TKE buffer

using a 40 mℓ glass homogenizing vessel with a teflon pestle having a serrated tip and a diameter 0.03 inches less than the vessel. The pestle was motor driven at approximately 100 rpm for no more than 25 strokes in order to minimize disruption of mitochondria. Cytochrome oxidase released by mitochondrial disruption can interfere with measurement of cytochrome P-450 difference spectra (Agosin, 1976). The resulting homogenate was centrifuged at 3000 rpm for 5 min to remove large debris and the supernatant was centrifuged twice at 20000X g for 20 min (13000 rpm, Servall SS-34 rotor). The resulting (post-mitochondrial) supernatant was removed (taking care not to disturb the loose mitochondrial fragments) and microsomes were precipitated by centrifugation at 140000X g for 90 min (39000 rpm, Spinco 50 Ti rotor). Washed microsomes were prepared by resuspending the pellet in TKE buffer containing 10% glycerol (using a 1 mℓ glass-teflon tissue homogenizer) and re-centrifuging at 140000X g for 90 min. The final microsomal pellet was resuspended in 1.0 mℓ of 60 mM sodium phosphate buffer, pH 7.4 containing 2 mM $MgC\ell_2$ and 2% glycerol.

I. Assay of Cytochrome P-450 Content

Cytochrome P-450 content was measured as described previously by Brattsten *et al.* (1980). Washed microsomes resuspended in 60 mM sodium phosphate buffer, pH 7.4 containing 2 mM $MgC\ell_2$ and 2% glycerol were diluted by the addition of 5.0 mℓ of 0.3 M potassium phosphate buffer, pH 7.8 containing 50% glycerol. Cytochrome P-450 was reduced by the addition of approximately 2 mg of sodium dithionite to the diluted suspension. The reduced solution was divided equally between two 3 mℓ

glass cuvettes (1 cm pathlength) and a baseline spectrum recorded from 500 nm to 400 nm (Perkin-Elmer model Lambda 3 spectrophotometer with a model 561 recorder). Carbon monoxide was gently bubbled through the sample cuvette for 60 s and the spectrum again recorded. Cytochrome P-450 content was calculated from the difference in absorbance between 450 nm and 490 nm using an extinction coefficient of 91 $mM^{-1}cm^{-1}$ as described by Omura and Sato (1964).

J. Thin-Layer Chromatography

Thin-layer chromatography was performed by applying the concentrated $CHC\ell_3$ extracts to glass silica gel plates (Whatman Linear-K, 250 μm), which were then developed at room temperature in tanks (20 X 20 X 5 cm) lined with solvent-saturated paper. Two solvent systems were used: (A) 8:2:1 benzene, diethyl ether, acetic acid (Suzuki and Miyamoto, 1978) and (B) 200:1 acetone, acetic acid (Welling *et al.*, 1970). The plates for system B were activated by thoroughly washing with acetone, drying and heating at 90°C for 15 min. Compounds were visualized by exposure to iodine vapor or by using DQC reagent (0.5% in cyclohexane) (Menn *et al.*, 1957). The α- and β-monoacid derivatives of malathion used as standards were prepared by base hydrolysis of malathion and identification was made by comparison with previously published R_f values (Suzuki and Miyamoto, 1978; Welling *et al.*, 1970).

K. Molecular Exclusion Chromatography

A Sephadex G-25 column (180 X 10 mm) was used to remove small molecules from the reaction mixtures while a calibrated Sephadex G-150

column (90 X 2 cm) was used to resolve proteins in the molecular weight range 200000 to 20000. Both columns were eluted at 4°C with 50 mM Tris-HCℓ buffer, pH 7.1. Fractions collected from these columns were assayed for carboxylesterase activity using α-naphthyl acetate as a substrate (NAE-activity) according to the procedure of Sheehan *et al.* (1979). The ability of fractions to degrade [^{14}C]-malathion (ME-activity) was assayed as follows: 0.05 mℓ to 1.0 mℓ of the fraction was incubated in 0.5 mℓ of 50 mM Tris-HCℓ buffer, pH 7.1 with 1.0 µg of [^{14}C]-malathion for 2-45 min at 30(±1)°C. This reaction mixture was rapidly extracted at pH 7 with $CHC\ell_3$. The amount of [^{14}C] remaining in the aqueous phase (malathion monoacid products) was converted to a rate and used as a relative measure of ME-activity.

L. Scintillation Techniques

[^{14}C]-labeled compounds were counted by liquid scintillation using either toluene based cocktail (Omnifluor, New England Nuclear: 3.92 g PPO, 0.08 g bis-MSB in 1 ℓ scintillation grade toluene) for non-aqueous samples or with 2 parts of the above cocktail with one part Triton X-100 for aqueous samples. $CHC\ell_3$-containing samples were evaporated to dryness under reduced pressure in a scintillation vial before addition of cocktail. The efficiency of counting was determined by the channels ratio techniques, and results are reported in dpm, %-total [^{14}C] or converted to the equivalent amount of [^{14}C]-malathion using the original specific radioactivity.

M. Protein Determinations

Protein determinations were performed by a modification of the

method of Sedmak and Grossberg (1977). Protein solutions (50 µl) were added to 1.0 mℓ of a Coomassie reagent (0.06% Coomassie brilliant blue G-250 in 1.9% perchloric acid), mixed on a vortex mixer for 5 s and the absorbance read at 620 nm. The coloured product formed rapidly (within a few seconds) and was stable for several hours (see Sedmak and Grossberg, 1977 for details). Bovine Serum Albumin was used to construct a standard curve.

III. RESULTS

SECTION I. The Degradation of [^{14}C]-Malathion by Carboxylesterases and Their Role in Malathion Resistance.

A. The Degradation of [^{14}C]- Malathion by Canton S Crude Enzyme Preparations.

As an initial approach to the study of *in vitro* malathion degradation in *Drosophila melanogaster*, [^{14}C]-malathion was added to crude extracts made from the susceptible laboratory flies. The results of these experiments served as a basis for comparison with the more resistant strains (MH19 and C1-39). Figure 4 shows the effect of a crude enzyme preparation from the malathion-susceptible, laboratory strain Canton S (CS) on [^{14}C]-malathion at 30°C and pH 7.1. During the first hour approximately 50% of the total radioactivity disappeared from the neutral $CHC\ell_3$ fraction. Almost all of this radioactivity could be recovered in the acidic $CHC\ell_3$ fraction. Over a period of 24 hr, 90% of the original radioactivity in the neutral fraction disappeared and was recovered in the acidic fraction. The nature of the products in each of the $CHC\ell_3$ fractions was examined by thin layer chromatography (TLC). These results are shown in Figure 5 and summarized in Table 1. Thirty minutes after starting the reaction by adding [^{14}C]-malathion, the neutral $CHC\ell_3$ fraction contained only unreacted [^{14}C]-malathion. No other products (such as malaoxon) were detected in this fraction. On the other hand, the acidic $CHC\ell_3$ fraction contained the α- and β-monocarboxylic acids of malathion (Figure 5). The identification of

FIGURE 4

Recovery of [^{14}C]- from a Reaction Mixture Containing [^{14}C]-Malathion and Canton S Crude Extract

A: Buffer control without crude extract; B: Control with extract heated at 100°C for 10 min; C: Neutral $CHC\ell_3$ fraction; D: Acidic $CHC\ell_3$ fraction; E: Residual fraction.

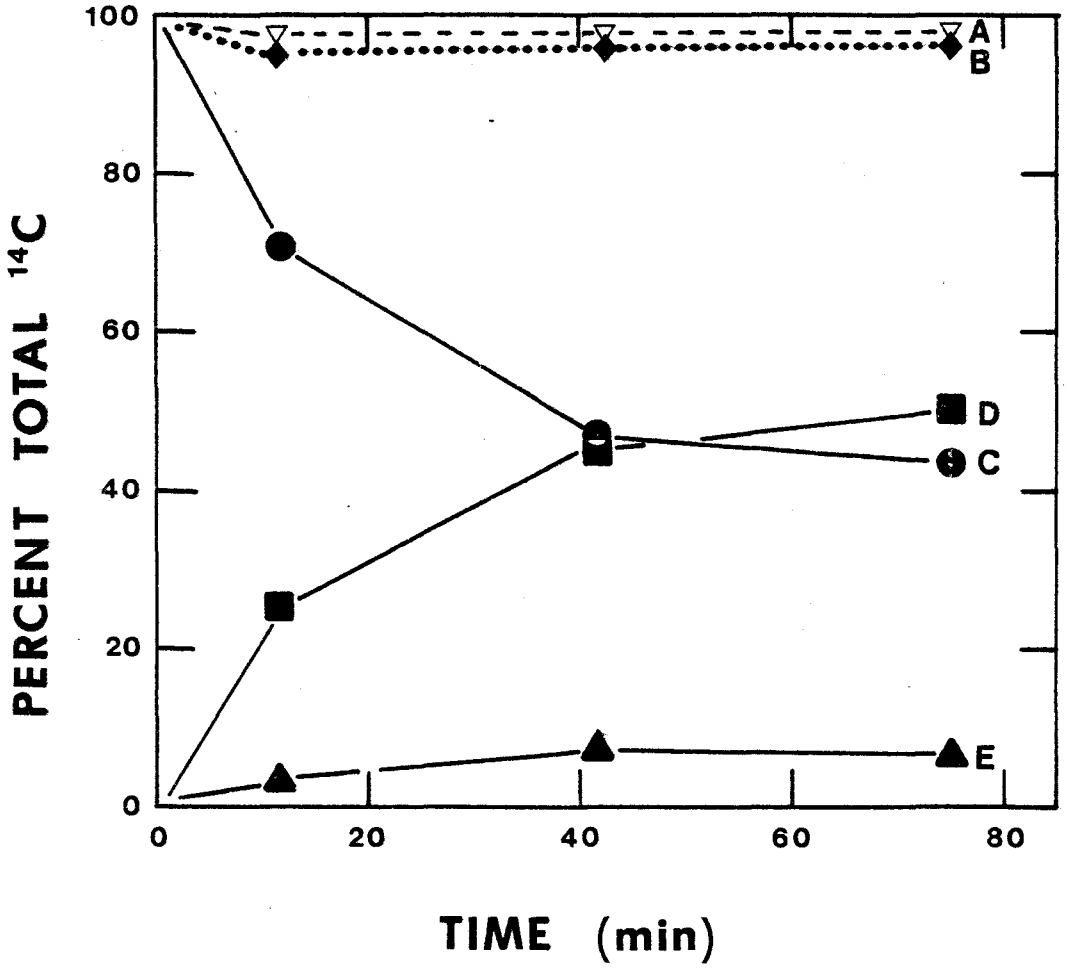

100
80
60
40
20
0
0
20
40
60
80
PERCENT TOTAL ^{14}C
TIME (min)
A
B
C
D
E

FIGURE 5

Radioactivity-Profiles from Thin Layer Chromatography of Chloroform Fractions from Canton S Reaction Mixtures with [^{14}C]-Malathion

Top panels: $CHC\ell_3$ fraction obtained by acidifying a reaction mixture to pH 2 and immediately extracting with $CHC\ell_3$; Middle panels: Neutral $CHC\ell_3$ fraction; Bottom panels: Acidic $CHC\ell_3$ fraction; A: Solvent system was benzene/diethyl ether/acetic acid (8:2:1); B: Solvent system was acetone/acetic acid (200:1); MA: Malathion; MX: malaoxon; α: malathion α-monoacid; β: malathion β-monoacid.

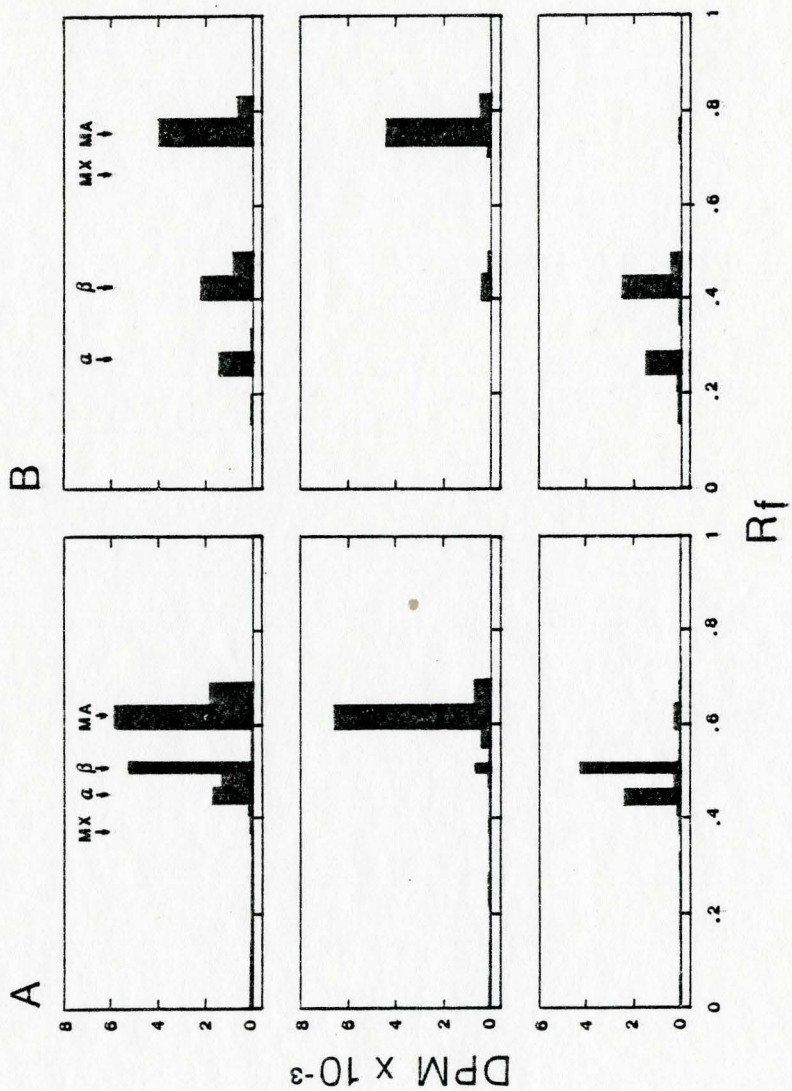

A
B
MX
α
β
MA
DPM x 10-3
Rf

TABLE 1

Compounds Recovered After 30 min *In Vitro* Degradation of [^{14}C]-Malathion

Compound	Solvent System	R_f	Relative Percent		
			Canton S	C1-39	MH19
malathion	A	0.62	48	50	63
	B	0.75	50	54	67
malaoxon	A	0.39	∿0	2.5	8
	B	0.65	∿0	2.0	7
β-monoacid	A	0.49	37	37	17
	B	0.44	33	33	15
α-monoacid	A	0.45	15	11	12
	B	0.26	17	11	10

these products was based on their solubility properties (both were extracted into $CHC\ell_3$ only from an acidified aqueous phase) and their R_f values in two different solvent systems. On the basis of this identification, it was concluded that the CS crude enzyme preparation catalysed the hydrolysis of [^{14}C]-malathion at the carboxylester bonds and hence was tentatively designated as containing malathion-carboxylesterase activity (ME-activity).

The kinetics of malathion degradation (Figure 4) were unusual in that there was an apparent loss of ME-activity as the reaction progressed. It was desirable to further characterize this loss of activity in order to make more meaningful comparisons of ME-acitivity between resistant and susceptible strains. The lability of ME-activity at 30°C was tested by comparing a CS crude enzyme preparation that was pre-incubated (30 min) prior to the addition of [^{14}C]-malathion to a control preparation that was not pre-incubated. Pre-incubation did not decrease ME-activity (Table 2). Another possibility examined as a cause for the apparent loss of ME-activity was substrate depletion. This was tested by adding more [^{14}C]-malathion to a reaction mixture 18 min after initiation of the reaction. The results in Table 3 indicate that the initial rate of reaction could not be restored even though the additional [^{14}C]-malathion brought the total amount of [^{14}C]-malathion slightly above its initial concentration. In summary, these results indicate that the apparent loss of ME-activity was not due to lability of the enzyme or substrate depletion.

The possibility that the loss of ME-activity was caused by the depletion of a low molecular weight cofactor or the accumulation of an

TABLE 2

The Effect of Pre-incubation on the Rate of [^{14}C]-Malathion Degradation by Extracts from Canton S Flies

Time Interval (min)	Rate (nmole $ml^{-1}min^{-1}$) No pre-incubation	Pre-incubation
0 - 5	.168	.158
5 - 10	.092	.090
10 - 15	.062	.072
15 - 20	.036	.046

TABLE 3

The Effect of Restoring the Initial [^{14}C]-Malathion Concentration After 18 Minutes of Reaction with Canton S Extracts

Time Interval (min)	Rate (nmol $ml^{-1}min^{-1}$)	Relative Percent
0 - 5	.104	100
18 - 23	.068	65

inhibitor was investigated by molecular exclusion chromatography using a Sephadex G-25 column in order to remove small molecules from the enzyme preparations. The malathion carboxylesterase reaction did not require a soluble cofactor as shown by the similarity of reaction rates between a control enzyme preparation (not applied to the column) and a preparation that had been passed through the column (Table 4). When potential inhibitors were removed from a 30 min reaction mixture ([^{14}C]-malathion plus CS extract) by G-25 chromatography only 64% to 66% (two experiments) of the original (control) ME-activity could be recovered (Table 4). The elution profile (Figure 6) indicated that a small amount of radioactivity was found in the void volume (which also contained the protein), equivalent to approximately 1.3×10^{-2} nmol of malathion per mg total protein. These results suggest that the loss of ME-activity is due to inhibition by [^{14}C]-labeled molecules that bind to the enzyme (either malathion, an impurity in the starting material, or a product enzymatically formed during the reaction). The nature of the inhibitor or its mode of binding was not investigated further.

ME-activity in the CS crude enzyme preparation was further characterized by chromatography on a Sephadex G-150 column (Figure 7). Activity was found only in the void volume and was not associated with either of the major soluble carboxylesterase activities of *D. melanogaster*, Esterase-6 and Esterase-C. The latter were found in fractions 75-80, corresponding to a molecular weight of approximately 65000. Since ME-activity eluted in the void volume, it was uncertain if it represented a single enzyme species.

TABLE 4

The Recovery of Malathion Carboxylesterase Activity in Canton S Extracts After Chromatography on a Sephadex G-25 Column

Conditions	Experiment	Rate (nmol $ml^{-1}min^{-1}$)	Relative Percent[a]
Control: not exposed to [^{14}C-malathion] or passed through column	1	.197	100
	2	.150	100
Passed through column but not exposed to [^{14}C]-malathion	1	.200	∿100
	2	ND[b]	ND
Exposed to [^{14}C]-malathion and passed through column	1	.127	64
	2	.099	66

[a]For the respective experiments

[b]not done

FIGURE 6

Sephadex G-25 Elution Profile of a 30 min Canton S Reaction Mixture

Relative dpm [^{14}C] and ME-activity are shown. V_o indicates the column void volume.

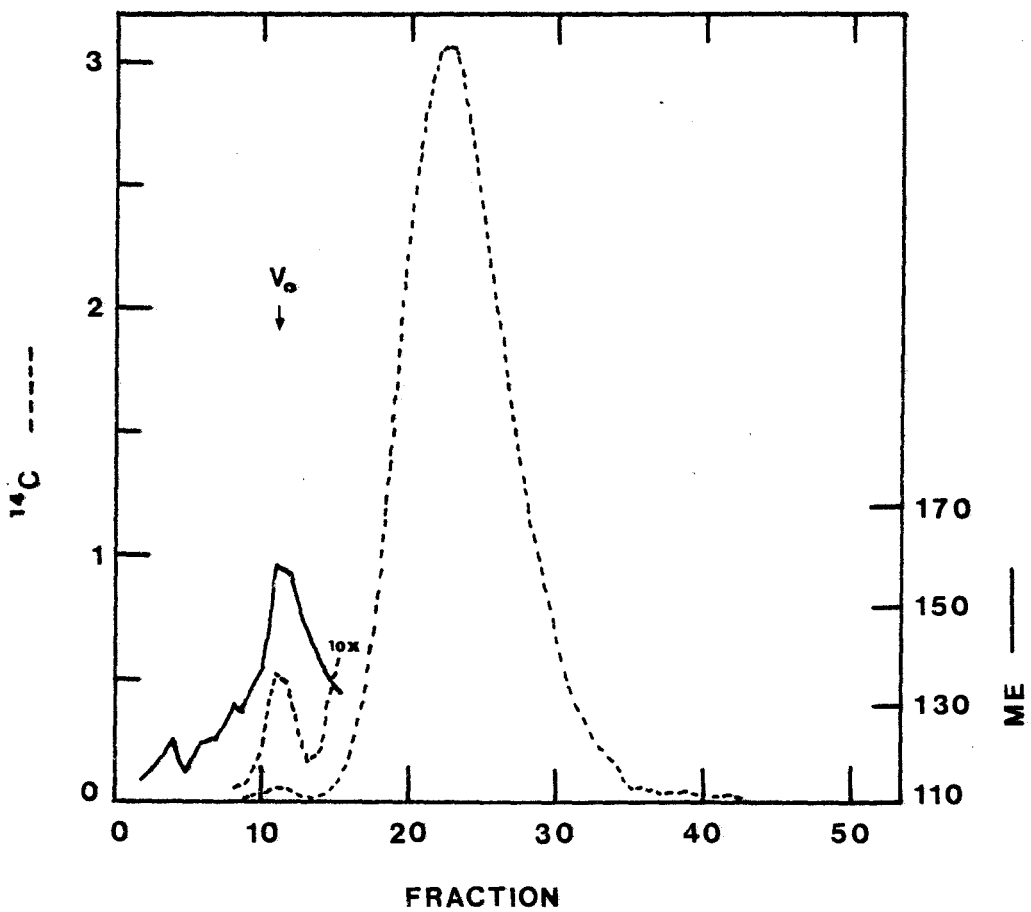
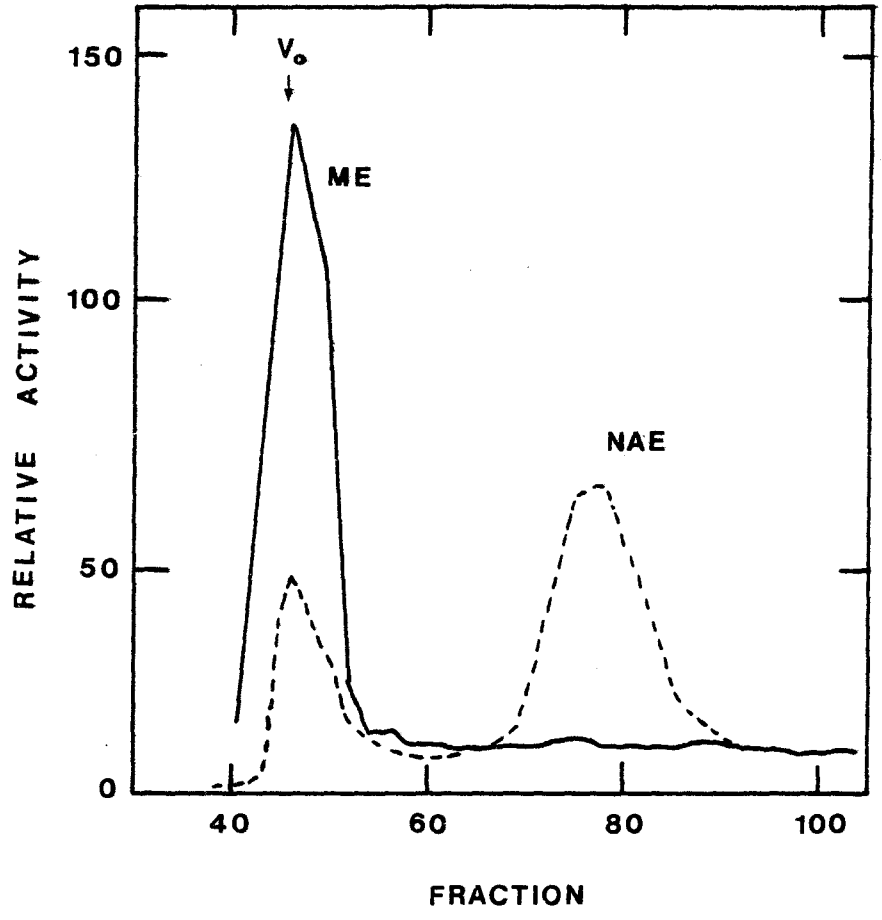

3
2
1
0
V_o
^{14}C ----
10x
170
150
130
110
ME ——
0
10
20
30
40
50
FRACTION

FIGURE 7

Sephadex G-150 Elution Profile of a Canton S Crude Extract

ME: malathion-carboxylesterase activity; NAE, α-naphthylacetate esterase activity. V_o indicates the column void volume.


150
100
50
0
V_o
ME
NAE
RELATIVE ACTIVITY
40
60
80
100
FRACTION

B. Comparison of [^{14}C]-malathion Degradation by Extracts from Resistant and Susceptible Strains

The resistance of *D. melanogaster* strains was tested by both the feeding and vacuum injection assays. The results are summarized in Table 5 and typical survival curves for a vacuum injection assay are shown in Figure 8. The MH19 strain was most resistant to malathion, while the control population (C1-39) was intermediate between the laboratory populations (OR and CS) and MH19 by both feeding and vacuum assays.

The degradation of [^{14}C]-malathion by crude enzyme preparations from resistant, MH19 flies is shown in Figure 9. The results were similar to those obtained from CS preparations (see Figure 4) except that the inhibition of reaction rate was more pronounced and, therefore, less malathion was degraded during a given time interval. The products of the reaction were examined by TLC. These results are shown in Figure 10 and summarized in Table 1. The major products of the reaction were the α- and β-monoacids of malathion, but in contrast to CS, the MH19 preparations produced significant amounts of a product tentatively identified as malaoxon on the basis of co-migration with a standard in two different solvent systems. Since malaoxon is a known inhibitor of carboxylesterases (Main and Dauterman, 1967) it seemed likely that it was responsible for the more pronounced inhibition of MH19 relative to CS. The inhibition of ME-activity by malaoxon was verified in all strains by pre-incubation of crude enzyme preparations with malaoxon at a concentration comparable to that accumulated after 30 min of MH19 reaction mixtures. Treatment with malaoxon in this manner caused 40%

TABLE 5

Resistance of *Drosophila melanogaster* Strains to Malathion

Strain	LT50[a] Feeding Assay (hours)	LT50[a] Vacuum Injection Assay (min)
CS	ND[b]	9.1(.3)
OR	2.4(.1)	ND
Cl-39	6.0(.1)	20.7(.7)
MH19	10.8(.7)	220(45)

[a]The standard error determined from three replicates is in brackets

[b]not done

FIGURE 8

Survival of *Drosophila Melanogaster* Strains Following Administration of Malathion by Vacuum Injection

Data are plotted as probit survival *versus* log time after releasing the partial vacuum.

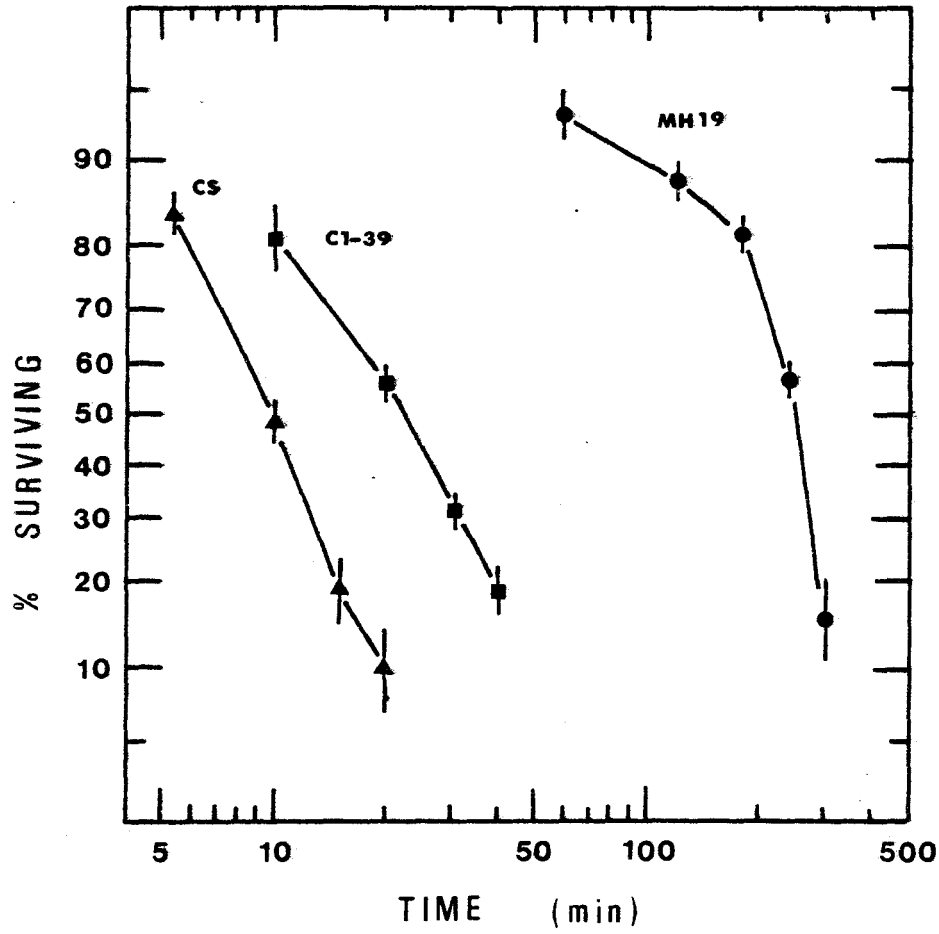

MH19
CS
C1-39
90
80
70
60
50
40
30
20
10
% SURVIVING
5
10
50
100
500
TIME (min)

FIGURE 9

Recovery of [^{14}C] from a Reaction Mixture Containing [^{14}C]-Malathion and MH19 Crude Extract

A: Control with extract heated at 100°C for 10 min; B: Neutral $CHC\ell_3$ fraction; C: Acidic $CHC\ell_3$ fraction; D: Residual fraction.

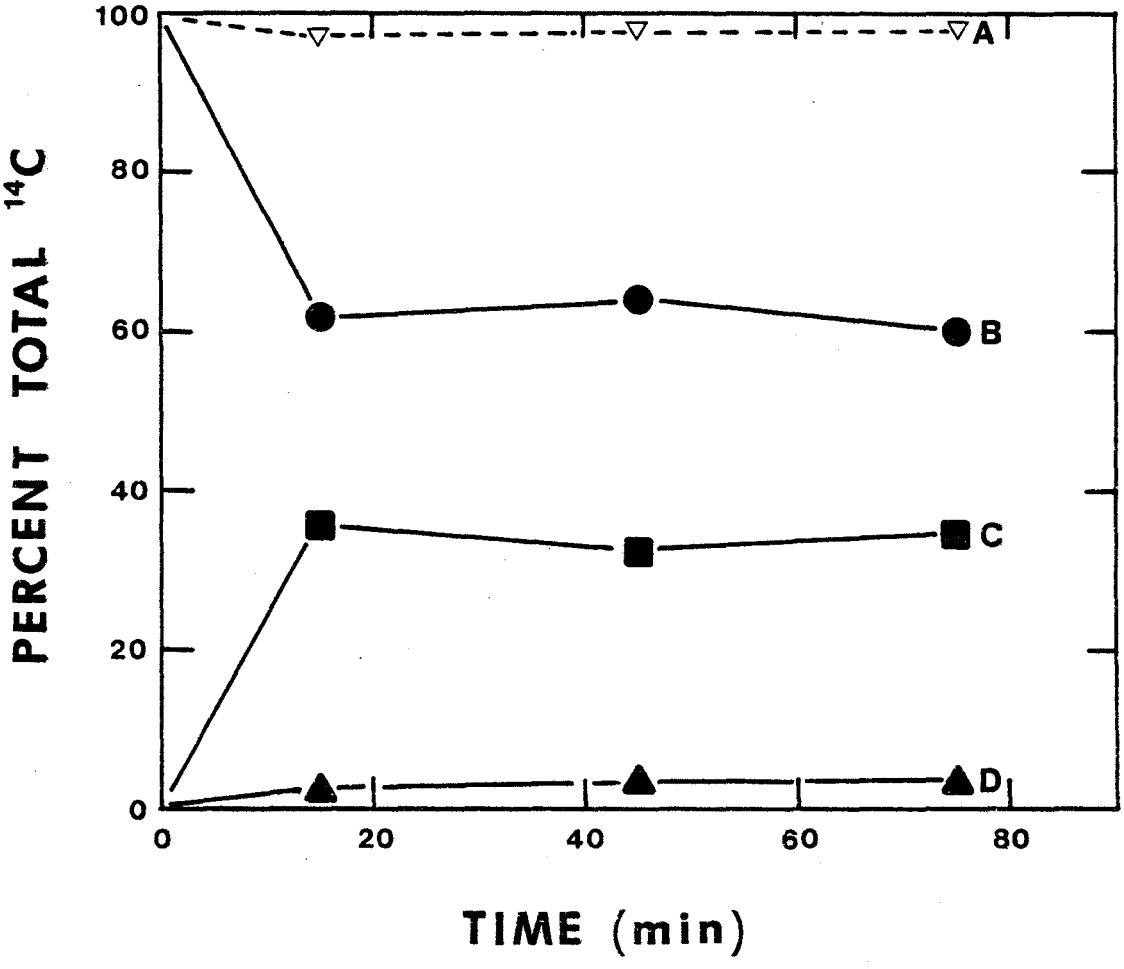
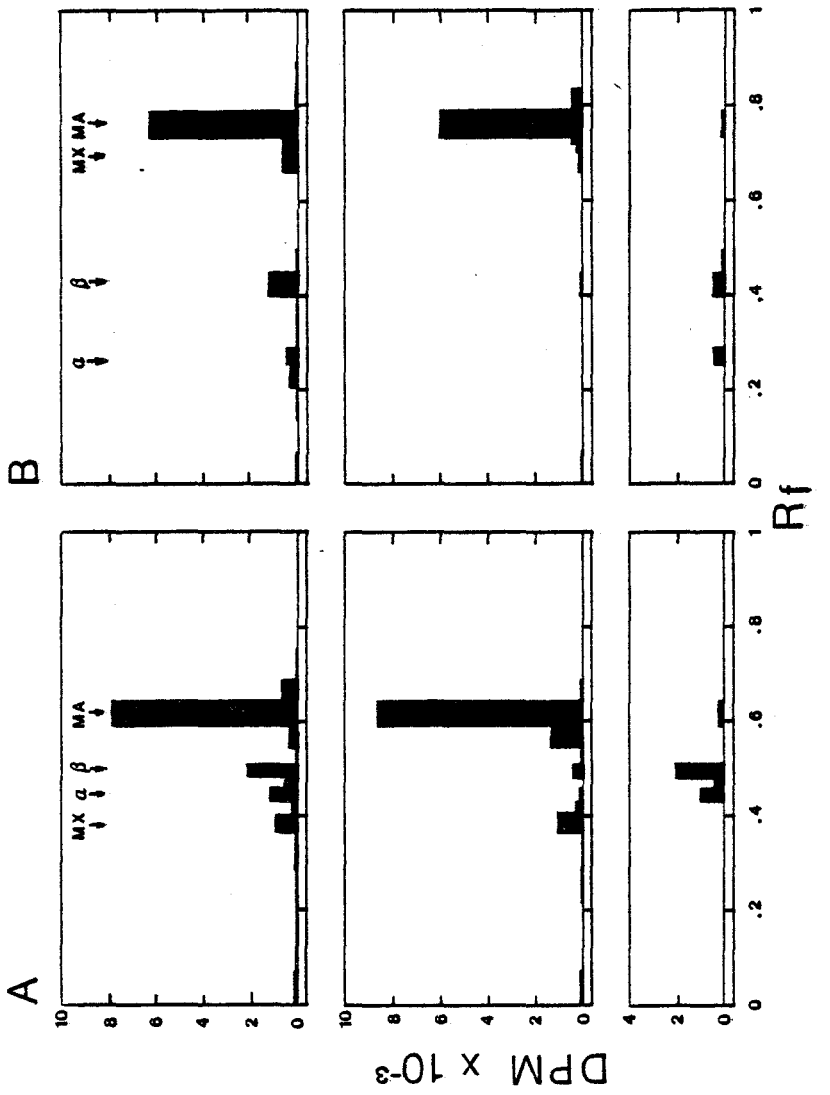

PERCENT TOTAL ^{14}C
100
80
60
40
20
0
0
20
40
60
80
TIME (min)
A
B
C
D

FIGURE 10

Radioactivity-Profiles from Thin-Layer Chromatography of Chloroform Fractions from MH19 Reaction Mixtures with [^{14}C]-Malathion

Top panels: $CHC\ell_3$ fraction obtained by acidifying a reaction mixture to pH 2 and immediately extracting with $CHC\ell_3$; Middle panels: Neutral $CHC\ell_3$ fraction; Bottom panels: Acidic $CHC\ell_3$ fraction; A: Solvent system was benzene/diethyl ether/acetic acid, (8:2:1); B: Solvent system was acetone/acetic acid, (200:1); MA: malathion; MX: malaoxon; α: malathion α-monoacid; β: malathion β-monoacid.


A
B
MX
α
β
MA
DPM x 10-3
Rf

to 47% (over all strains) inhibition of ME-activity (Table 6).

The degradation of [^{14}C]-malathion by enzyme preparations from the control strain (Cl-39) followed a curve intermediate to those of the CS and MH19 strains (Figure 11). Again the major products of the reaction were the α- and β-monoacids of malathion. A small amount of malaoxon was produced, although consistantly less than that from MH19 preparations (Figure 12 and Table 1).

In order to accurately assess the relative ability of extracts from the three strains to degrade [^{14}C]-malathion *in vitro*, it was necessary to compare the initial rates of reaction. Measurements over a long time interval were influenced by the inhibition of enzyme activity as described above, and this inhibition was found to be different for each of the three strains. Therefore the degradation rate was calculated from the [^{14}C] remaining in the aqueous fraction after 1 min. The results are given in Figure 13 and summarized in Table 7. No consistent differences in ME-activity were found between the three strains.

The observations that extracts from the more resistant strains (MH19 and Cl-39) produced noticeable amounts of malaoxon and that malaoxon inhibits ME-activity raised the possibility that malaoxon production could interfere with the detection of differences in ME-activity between strains. Therefore, the rate of malathion degradation was determined in the presence of the microsomal oxidase inhibitor metyrapone (MTP) (Testa and Jenner, 1981). The results are shown in Table 8. Control experiments (data not shown) indicated that the concentration of

TABLE 6

Malathion-Carboxylesterase Determined in the Presence of 0.16 mM Malaoxon[a]

Strain	Control Rate	Rate with Malaoxon
CS	.024	.010
C1-39	.020	.008
MH19	.024	.011

[a]Extracts were pre-incubated for 10 min at 30°C with or without malaoxon. Units are nmol $ml^{-1}min^{-1}$.

FIGURE 11

Recovery of [^{14}C]- from a Reaction Mixture Containing [^{14}C]-Malathion and C1-39 Crude Extract

A: Boiled Control; B: Neutral $CHC\ell_3$ fraction; C: Acidic $CHC\ell_3$ fraction; D: Residual Fraction

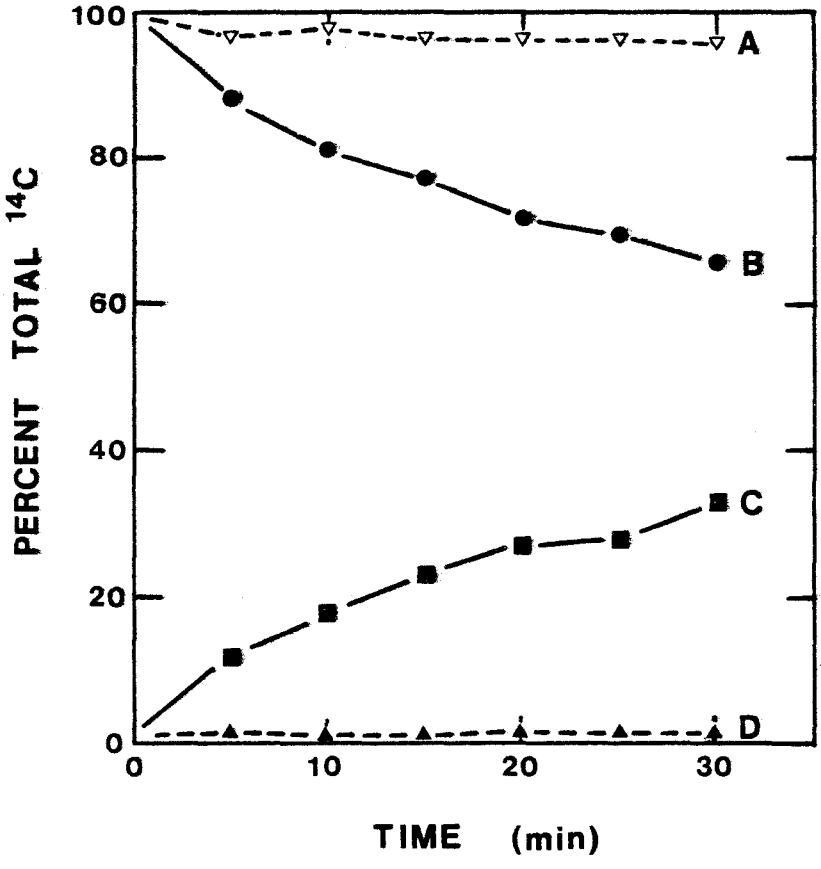

100
80
60
40
20
0
PERCENT TOTAL ^{14}C
A
B
C
D
0
10
20
30
TIME (min)

FIGURE 12

Radioactivity-Profile from Thin-Layer Chromatography of a Chloroform Fraction from Cl-39 Reaction Mixtures with [^{14}C]-Malathion

The $CHC\ell_3$ fraction was obtained by acidifying a reaction mixture to pH 2 and immediately extracting with $CHC\ell_3$. The solvent system was benzene/diethyl ether/acetic acid, (8:2:1). MA: malathion; MX: malaoxon; α: malathion α-monoacid; β: malathion β-monoacid.

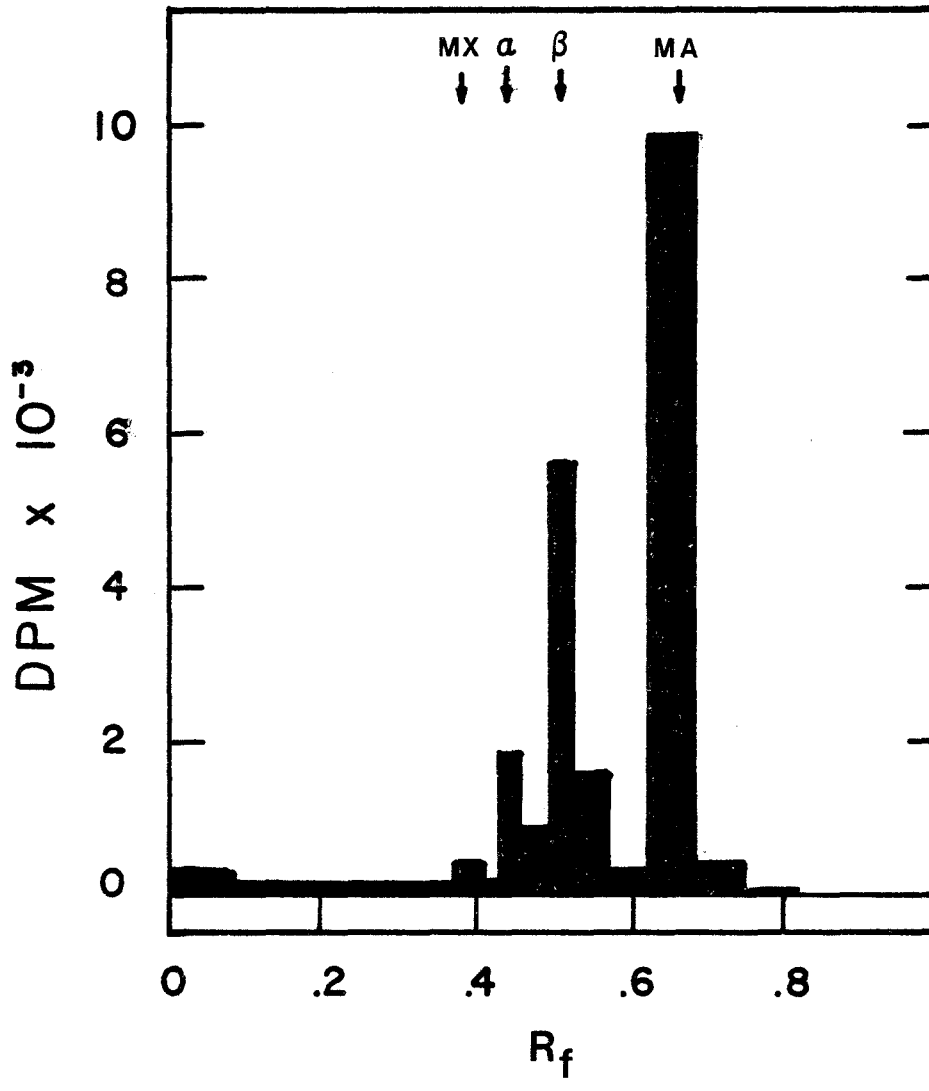

MX
α
β
MA
10
8
6
4
2
0
DPM x 10⁻³
0
.2
.4
.6
.8
1
Rf

FIGURE 13

Malathion-Carboxylesterase Activity as Measured by Initial Rates (0-1 min) in Resistant and Susceptible Strains of *Drosophila melanogaster*.

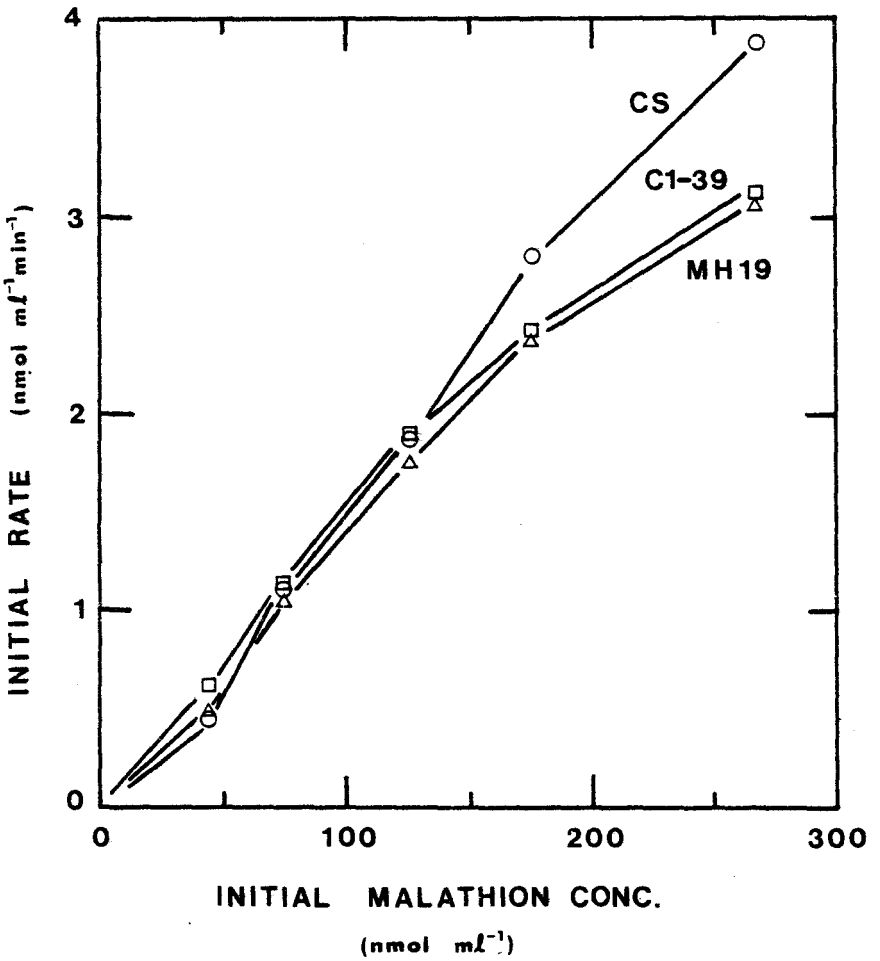

4
3
2
1
0
0
100
200
300
CS
C1-39
MH19
INITIAL RATE (nmol mℓ⁻¹min⁻¹)
INITIAL MALATHION CONC.
(nmol mℓ⁻¹)

TABLE 7

Malathion Carboxylesterase Activity as Measured by Initial Rates[a]

Initial [^{14}C]-malathion concentration (nmol ml^{-1})	Rate (nmol ml^{-1}min^{-1}) CS	C1-39	MH19
24.4	.470	.607	.483
75.2	1.11	1.16	1.08
125	1.84	1.85	1.75
176	2.80	2.37	2.35
268	3.88	3.12	3.04

[a]Data from Figure 13

TABLE 8

Malathion Carboxylesterase Activity Determined in the Presence of 0.5 mM Metyrapone[a]

Strain	Control Rate	Rate with Metyrapone
CS	0.14 (.01)	0.13 (.01)
Cl-39	0.16 (.01)	0.15 (< .01)
MH19	0.14 (.02)	0.12 (< .01)

[a]The standard error of 3 replicates is in brackets. Units are nmol $min^{-1}ml^{-1}$.

MTP used (1.0 mM) inhibited mixed function oxidase activity (as measured by p-nitroanisole O-demethylase activity) by greater than 85%. It had little effect, however, on ME-activity and even in its presence, there were no consistant differences between strains.

Although the measurements of ME-activity *in vitro* failed to show any differences between resistant and susceptible flies, it remained important to test the relevance of this enzyme activity to *in vivo* malathion resistance. This was done by measuring malathion survival (feeding assay) in the presence of the carboxylesterase inhibitors DEF and TPP (Plapp *et al.*, 1963). The presence of these inhibitors did not decrease the malathion LT50 as might be expected if carboxylesterase activity were important to resistance (Table 9). On the contrary, they antagonized (i.e., produced a protective effect) malathion toxicity.

All of the above results are consistent with the hypothesis that the malathion resistance of the C1-39 and MH19 strains was not due to an activity increase of an enzyme that specifically degrades malathion by means of carboxylester hydrolysis (ME-activity). It was evident that other pathways of detoxication existed in the more resistant MH19 and C1-39 strains.

TABLE 9

Resistance of *Drosophila melanogaster* Strains to Malathion in the Presence of the Carboxylesterase-Inhibitors DEF and TPP

Strain	LT50[a] Malathion[b]	Malathion plus DEF[c]	Malathion plus TPP[c]
OR	2.4(.1)	3.2(.1)	3.5(.1)
C1-39	6.0(.1)	8.7(.4)	5.0(.2)
MH19	10.8(.7)	11.1(.4)	11.5(.5)

[a]Feeding assay; The standard error of 3 replicates in in brackets; Units are hours.

[b]50 ppm

[c]250 ppm

SECTION II. Characterization of Mixed Function Oxidases and Their Role in Resistance

A. Cross-Resistance and the Synergism of Malathion and Malaoxon by Mixed Function Oxidase Inhibitors

The existance of other pathways can be investigated by testing the cross-resistance of MH19 and Cl-39 to other insecticides not likely to be degraded by carboxylesterase-dependant pathways. The results in Table 10 indicate that the MH19 strain was the most tolerant (compared to Cl-39 and OR) to three of the insecticides tested (parathion, diazinon and carbaryl), but was the least tolerant to permethrin. Futhermore, the malathion susceptible strain (CS) was most resistant to permethrin while least resistant to the other insecticides. All of these insecticides can be degraded by mixed function oxidase (MFO) mediated reactions in insects (reviewed by Eto, 1979d; Oppenoorth and Welling, 1976; and Nakatsugawa and Morelli, 1976), but the presumed target of permethrin (a pyrethroid) differs from that of organophosphates (parathion and diazinon) and carbamates (carbaryl). The mode of action of pyrethroids is thought to be through blockage at Na^+/K^+ channels in nerve axons (reviewed by Narahashi, 1971 and 1976) whereas organophosphates and carbamates act by inhibition of acetylcholinesterase (AChE) (reviewed by O'Brien, 1976). Hence, the specificity of MH19 cross-resistance to organophosphate and carbamate insecticides may reflect the existance of a less sensitive AChE (to inhibition) in this strain whereas the greater resistance of CS to permethrin could be indicative of decreased target

TABLE 10

Cross-Resistance of *Drosophila melanogaster* Strains

Strain	LT50[a] Malathion (50 ppm)	Parathion (20 ppm)	Diazinon (5 ppm)	Carbaryl (500 ppm)	Permethrin (100 ppm)
CS	ND	ND	ND	ND	12.5(.8)
OR	2.4(.1)	0.9(.1)	1.5(.08)	25(2)	ND
C1-39	6.0(.1)	2.5(.2)	2.8(.1)	45(5)	8.5(.6)
MH19	10.8(.7)	8.0(.2)	4.8(.1)	105(4)	7.2(.6)

[a]Feeding assay; The standard error of 3 replicates is in brackets; Units are hours.

sensitivity (possibly Na^+/K^+ channels) in this strain. Since mixed function oxidases are known to degrade a large number of lipophilic xenobiotics in insects (reviewed by Terriere, 1968; and Wilkinson and Brattsten, 1972) increased activity of the enzymes could account for a major part of the malathion resistance of the C1-39 and MH19 strains. The contribution of increased MFO activity to malathion resistance was further examined by comparing the survival (LT50, feeding assay) of the strains on malathion alone and malathion plus the MFO inhibitors PBO and MTP. However, addition of these inhibitors to the test media at concentrations 5 or 10 times that of the malathion failed to cause any significant change in the LT50 values (Table 11). This result is in contradiction to the idea that mixed function oxidases are an important factor in the resistance of these strains. Since the more resistant strains produced malaoxon *in vitro* (Table 1), their resistance to malaoxon was also tested, with and without MFO inhibitors present. All strains were more resistant to malaoxon than malathion (Table 12). The pattern of resistance to malaoxon was similar to that for malathion in that the MH19 strain was most resistant while the control strain (C1-39) was intermediate between MH19 and the laboratory strain (OR). The presence of MFO inhibitors (PBO or MTP) with malaoxon in the test media generally decreased the survival times of all strains by about 50%. The effects of the two inhibitors, MTP and PBO, on malaoxon toxicity were similar except in the case of the C1-39 strain. Inasmuch as MTP synergized the toxicity of malaoxon to C1-39 flies, PBO failed to produce a similar effect in this strain. The basis of this apparent anomaly was not investigated. The LT50 results for malathion and malaoxon are

TABLE 11

Resistance of *Drosophila melanogaster* Strains to Malathion in the Presence of Mixed Function Oxidase Inhibitors PBO and MTP

Strain	LT50[a]				
	Malathion[b] (50 ppm)	plus PBO (250 ppm)	plus PBO (500 ppm)	plus MTP (250 ppm)	plus MTP (500 ppm)
OR	3.7(.1)	4.0(.1)	3.5(.1)	3.4(.1)	4.1(.1)
C1-39	10.1(.5)	9.4(.2)	9.7(.4)	9.0(.5)	8.9(.5)
MH19	13.1(.2)	11.1(.3)	12.0(.3)	12.4(.2)	12.7(.3)

[a]Feeding assay; The standard error of 3 replicates is in brackets; Units are hours.

[b]Malathion (50 ppm) was present under all conditions.

TABLE 12

Resistance of *Drosophila melanogaster* Strains to Malaoxon in the Presence of the Mixed Function Oxidase Inhibitors PBO and DEF

Strain	Malaoxon[b] (50 ppm)	LT50[a] plus PBO (250 ppm)	LT50[a] plus MTP (250 ppm)
OR	8.0(.2)	4.7(.1)	5.6(.4)
C1-39	20.2(.5)	20.6(.9)	11.4(.3)
MH19	41.3(1.4)	21.6(.2)	20.7(1.2)

[a]Feeding assay; The standard error of 3 replicates is in brackets; Units are hours.

[b]Malaoxon (50 ppm) was present under all conditions.

summarized with respect to the synergistic effects of MTP by means of a bar graph in Figure 14. From this diagram it is clear that all strains were more resistant to malaoxon than malathion and that only malaoxon toxicity was strongly synergized by the MFO inhibitor MTP. These results suggest that mixed function oxidases play an important role in malathion resistance by degrading malaoxon.

B. Characterization of Mixed Function Oxidase Activity and Cytochrome P-450 Content

The MFO systems of all strains were characterized with respect to the level of activity and cytochrome P-450 content. The rate of O-demethylation of p-nitroanisole (pNA-demethylase activity) by crude fly extracts was used as an indicator of MFO-activity. The most resistant MH19 strain had the highest level while the less resistant strain, C1-39, was intermediate between MH19 and the susceptible OR strain (Table 13). Though these results indicate that the more resistant strains apparently possessed higher MFO-activity it was important to isolate microsomes from crude homogenates in order to confirm that the activity was indeed microsomal in nature. Washed microsome preparations were used to measure cytochrome P-450 content and pNA-demethylase activity. The post-microsomal supernatant (after the first centrifugation at 140000X g, before washing; see "Microsome Preparation" in "MATERIALS AND METHODS") did not contain significant pNA-demethylase activity (data not shown). A typical cytochrome P-450 carbon monoxide difference spectrum of microsomes prepared from C1-39 flies is shown in Figure 15. Spectra varied between strains with respect to peak height at 450 nm, but were

FIGURE 14

Diagrammatic Comparison of Malathion and Malaoxon Resistance Levels in *Drosophila melanogaster* Strains

The data used in this diagram are from Tables 11 and 12.

■ 50 ppm malaoxon or malathion.

□ 500 ppm malaoxon/malathion plus 250 ppm MTP.

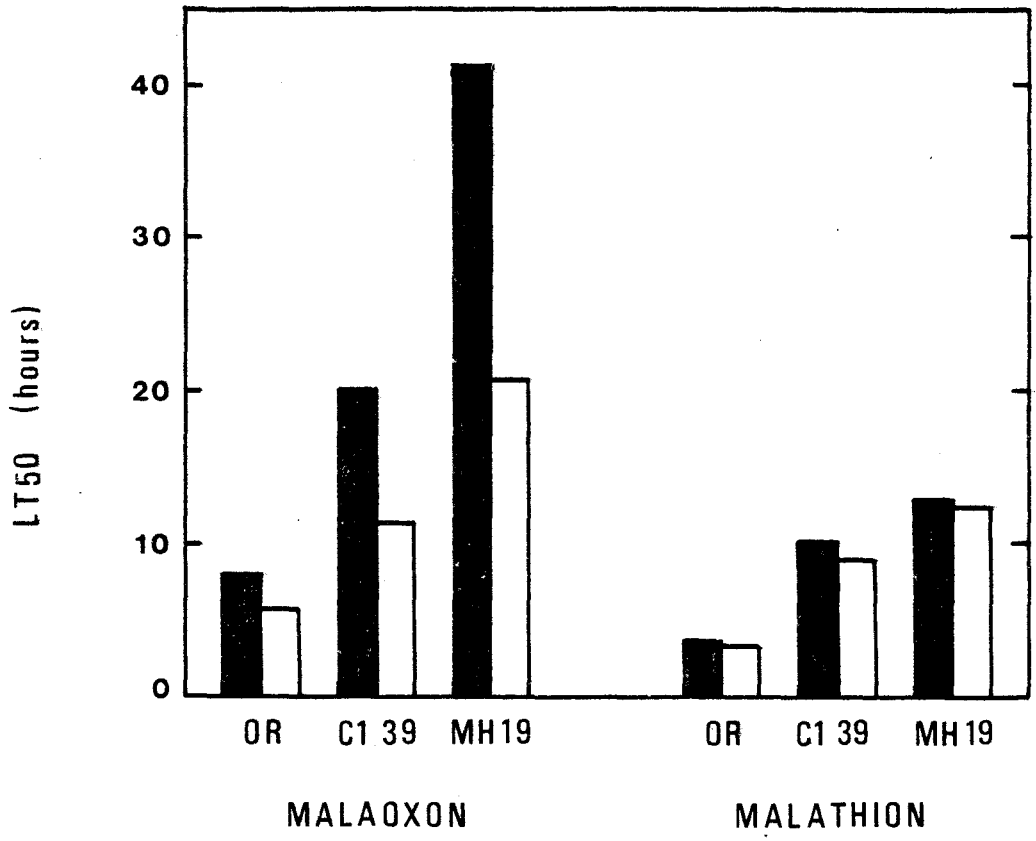

LT50 (hours)
40
30
20
10
0
OR
C1 39
MH19
OR
C1 39
MH19
MALAOXON
MALATHION

TABLE 13

p-Nitroanisole O-demethylase Activity in Crude Extracts from *Drosophila melanogaster* Strains

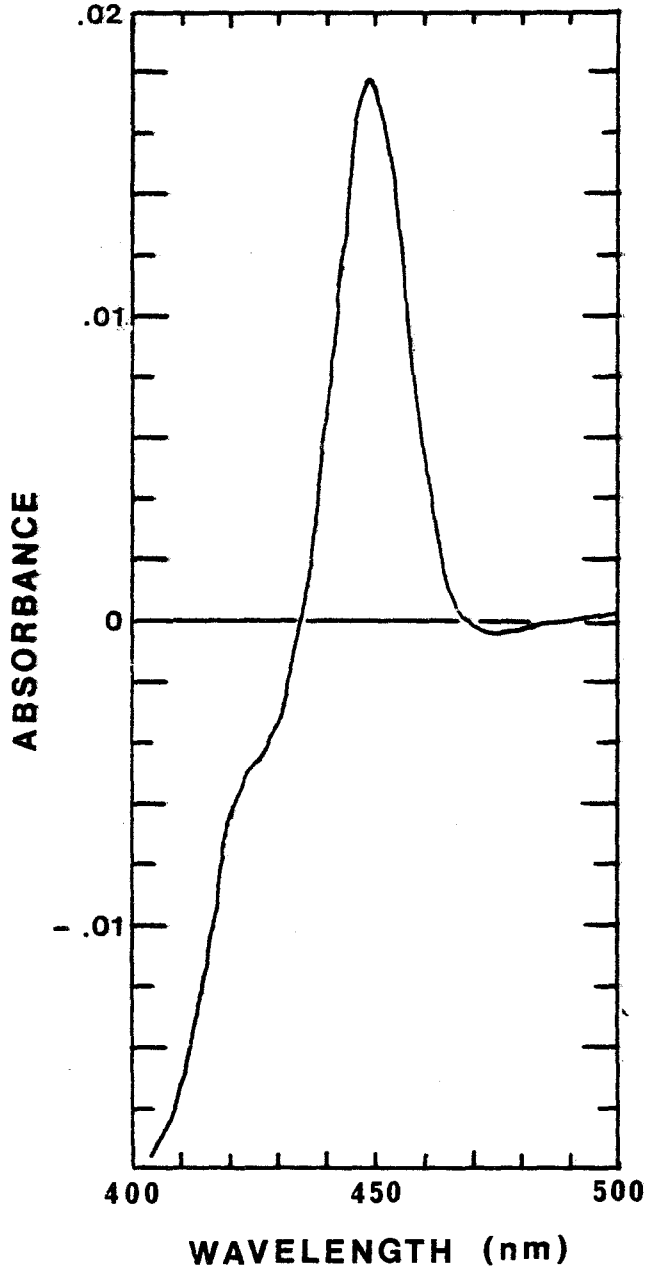
Strain	Rate[a]
CS	5.17
C1-39	16.1
MH19	41.7

[a]Units are: nmol p-nitrophenol $hr^{-1}mg^{-1}$ protein

FIGURE 15

Reduced-CO Difference Spectrum of Cytochrome P-450 from C1-39 Microsomes

Spectra for all strains of *D. melanogaster* were similar with respect to shape and absorption maxima at 450 nm.


.02
.01
0
- .01
ABSORBANCE
400
450
500
WAVELENGTH (nm)

similar in all other respects. The amount of cytochrome P-450 (nmol P-450/mg microsomal protein) was similar for all strains (Table 14), but the pNA-demethylase activity of washed microsomes from each strain followed the same pattern as found for crude extracts (see Table 14). Thus, although resistant and susceptible strains contain similar amounts of cytochrome P-450, the activity of this enzyme (as measured by pNA-demethylase activity) was higher in the more resistant strains.

C. Comparison of [^{14}C]-Malaoxon Degradation by Extracts from Resistant and Susceptible Strains

The finding that the MFO inhibitor MTP increased the sensitivity of flies to malaoxon, but not to malathion (Figure 14), suggests that malaoxon might be degraded by the microsomal MFO system. Malaoxon is known to be a more potent anticholinesterase than malathion (O'Brien, 1957), and therefore presumably more toxic to the insect. The more resistant strains (MH19 and C1-39) showed a more pronounced synergistic effect with MTP (Figure 14) and it is possible that their increased resistance was due to a more active malaoxon degradation system. These ideas were tested by examining the *in vitro* metabolism of [^{14}C]-malaoxon in crude enzyme preparations of the most resistant MH19 flies. The amount of [^{14}C]-malaoxon degraded was determined from the amount of [^{14}C] remaining in the aqueous fraction after extraction of the pH 7 reaction mixture with $CHC\ell_3$. [^{14}C]-malaoxon was converted to a water soluble derivative(s) when enzyme extracts were supplemented with an NADPH generating system (Figure 16, compare Lines A and B). Under these conditions approximately 65% of the initial [^{14}C]-malaoxon was degraded

TABLE 14

Cytochrome P-450 Content and p-Nitroanisole O-demethylase Activity of Washed Microsome Preparations from *Drosophila melanogaster* Strains

Strain	Cytochrome P-450 Content (nmol mg^{-1})[b]	Rate[a] (nmol pNP $hr^{-1}mg^{-1}$)[c]	Rate[a] (nmol pNP hr^{-1}nmol P-450^{-1})[d]
OR	.33	29.5	88.9
Cl-39	.34	49.5(2.4)	146 (7.0)
MH19	.34	63.5(8.4)	189 (25)

[a]The standard deviation determined from 3 replicates is in brackets; where there are no brackets one replicate was done.

[b]Expressed as: nmol of cytochrome P-450 per mg microsomal protein.

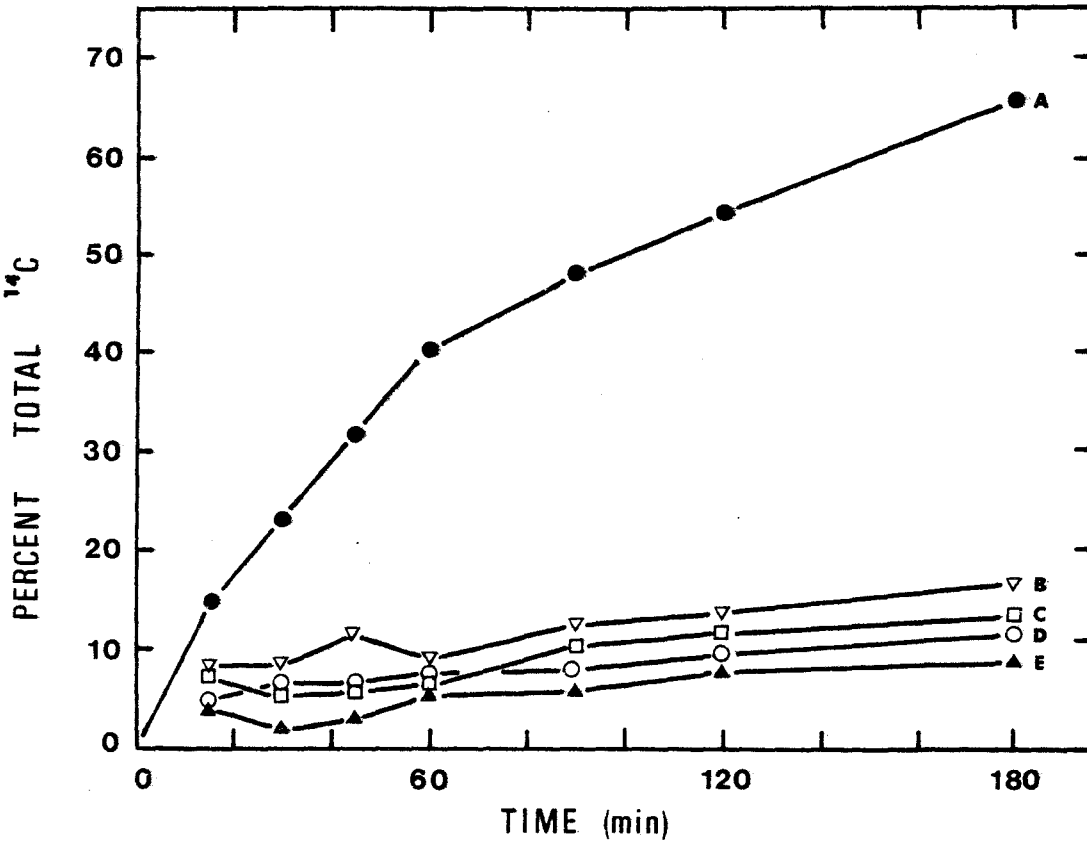
[c]Expressed as: nmol of p-nitrophenol formed per hr per mg microsomal protein.

[d]Expressed as: nmol of p-nitrophenol formed per hr per nmol of cytochrome P-450.

FIGURE 16

Recovery of Aqueous [^{14}C]- After $CHC\ell_3$ Extraction at pH 7 of Reaction Mixtures Containing [^{14}C]- Malaoxon and MH19 Crude Extracts

A: NADPH generating system included in reaction; B: No NADPH generating system included; C: Same as A but including malathion (3.5 nmol ml^{-1}); D: Same as A, but including MTP (0.5 mM); E: Boiled control.


PERCENT TOTAL ^{14}C
70
60
50
40
30
20
10
0
0
60
120
180
TIME (min)
A
B
C
D
E

in 180 min. A small amount of radioactivity (9% of original) was recovered in the boiled control (Figure 16, Line E). This could be due to the fact that malaoxon is chemically more polar in nature than malathion and hence a small amount of malaoxon tends to remain in the more polar aqueous fraction upon $CHC\ell_3$ partitioning. Malaoxon has a chloroform-water partition coefficient equal to 5.8 whereas the corresponding value for malathion is 64 (O'Brien, 1957). Using this value for malaoxon (5.8), 17% of the original [^{14}C]-malaoxon would be expected to remain in water after partitioning with an equal volume of $CHC\ell_3$; aliquots of the reaction mixture were extracted with equal volumes of $CHC\ell_3$ at pH 7 in all [^{14}C]-malaoxon degradation experiments (see "*In Vitro* Degradation of [^{14}C]-Malaoxon" in "MATERIALS AND METHODS"). The difference between the expected amount remaining in water (17%) and that found in the MH19 boiled control (9% of original [^{14}C]-malaoxon, Figure 16, Line E) may reflect differences in the solubility of malaoxon in enzyme extracts and pure water. In the absence of the MFO cofactor, NADPH, (Figure 16, Line B) or in the presence of an MFO inhibitor, MTP, (Figure 16, Line D) only 17% and 13%, respectively, of the original radioactivity was found in the aqueous fraction after 180 min. Since the major part of the malaoxon conversion to more acidic products required NADPH and was inhibited by MTP, it was concluded that [^{14}C]-malaoxon was degraded by mixed function oxidases. When malathion (3 nmol/ml) was added to the reaction mixture (Figure 16, Line C) only 12% of the initial [^{14}C] was recovered in the aqueous fraction. This indicates that malathion acted as an inhibitor of the oxidative degradation of malaoxon. The inhibition of MFO activity

by malathion may explain why [^{14}C]-malaoxon degradation was not detected in reaction mixtures when [^{14}C]-malathion was used as substrate. Thus, although crude enzyme preparation from MH19 and C1-39 flies produced significant amounts of malaoxon from [^{14}C]-malathion, no further degradation products of malaoxon were detected (Table 1).

The nature of the products from the degradation of [^{14}C]-malaoxon by MH19 crude extracts was examined by their solubility properties. The aqueous fraction (after extraction at pH 7 with $CHC\ell_3$) was acidified to pH 2 and extracted with $CHC\ell_3$ (see "*In Vitro* Degradation of [^{14}C]-Malaoxon" in "MATERIALS AND METHODS"). The amount of radioactivity in the resulting $CHC\ell_3$ fraction represents weakly acidic products ($pK_\alpha > 2$) and that remaining in the aqueous phase represents strongly acidic products ($pK_\alpha < 2$). The results in Table 15 show that the majority (64%) of the products of the MH19 reaction are weakly acidic (possibly malaoxon monocarboxylic acid(s)), while the remainder (36%) are strongly acidic in nature (possibly demethyl-malaoxon).

[^{14}C]-malaoxon degradation activity was compared between resistant and susceptible strains by measuring the amount of radioactivity remaining in the aqueous phase after extraction of pH 7 reaction mixtures with $CHC\ell_3$ (Figure 17). In spite of the observation that the microsomal pNA-demethylase activity was about 1.3X greater in the MH19 strain than the C1-39 strain (Table 14), there were no differences in MFO-mediated [^{14}C]-malaoxon degradation. The MH19 and C1-39 strains degraded 79% and 81%, respectively, of the original [^{14}C]-malaoxon in 180 min. On the other hand, enzyme preparations from the OR flies degraded only 35% of

TABLE 15

Compounds Recovered After 180 min *In Vitro* Degradation of [^{14}C]-Malaoxon

Strain	Relative Percent		
	Malaoxon	Weak-Acidic[a]	Strong-Acidic[b]
OR	65	7	28
Cl-39	20	54	26
MH19	20	51	29

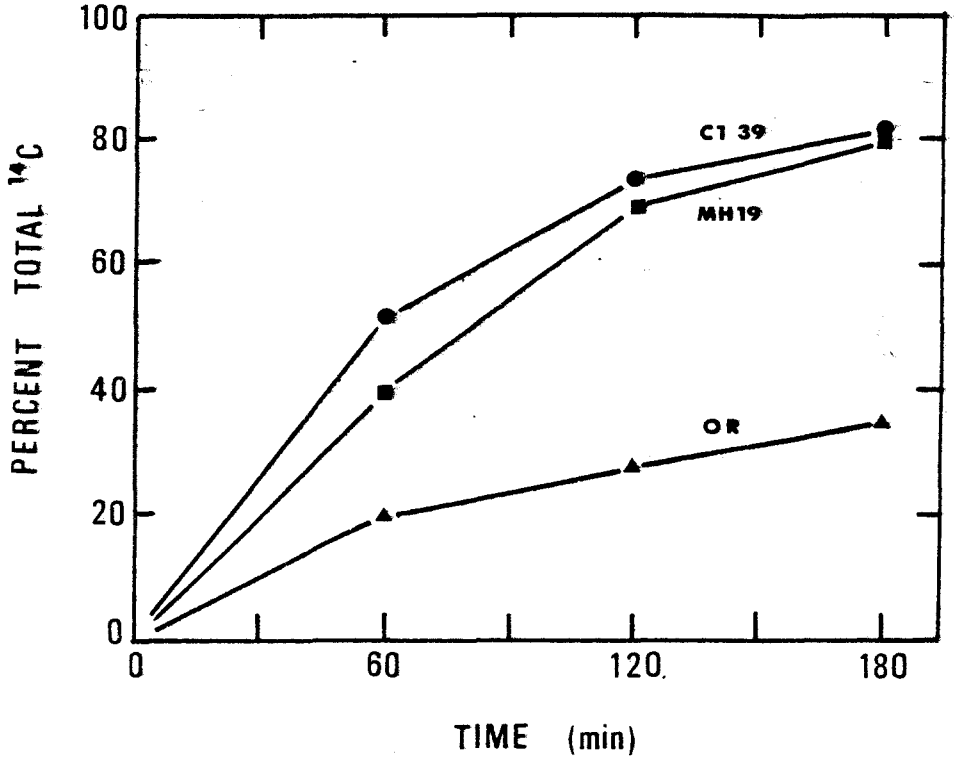
[a]Possibly malaoxon monoacid(s)

[b]Possibly demethyl-malaoxon

FIGURE 17

Recovery of Aqueous [^{14}C]- After $CHC\ell_3$ Extraction at pH7 of Reaction Mixtures Containing [^{14}C]-Malaoxon and Crude Extracts from *Drosophila melanogaster* Strains

All reaction mixtures were supplied with an NADPH generating system.


100
80
60
40
20
0
PERCENT TOTAL ^{14}C
0
60
120
180
TIME (min)
C1 39
MH19
OR

the [^{14}C]-malaoxon in the same time period. The nature of the [^{14}C]-malaoxon degradation products was examined by partitioning the aqueous fraction (after extraction of reaction mixtures at pH 7 with $CHC\ell_3$) against $CHC\ell_3$ at pH 2 and measuring radioactivity in the separate phases. The differences observed in [^{14}C]-malaoxon degradation between the more resistant strains (MH19 and C1-39) and the susceptible OR strain were due to differences in the production of weakly acidic products (Table 15). These could be malaoxon carboxylic acid(s). The C1-39 and MH19 flies converted 54% and 51%, respectively, of the [^{14}C]-malaoxon to weakly acidic products whereas the OR flies converted only 7% of the substrate to these products. However, the resistant and susceptible strains did not differ in their capacity to convert [^{14}C]-malaoxon to strongly acidic products (possibly demethyl-malaoxon). All strains transformed 26% to 29% of the original [^{14}C]-malaoxon to strongly acidic products (Table 5). In summary, these results indicate that the more resistant strains (MH19 and C1-39) possess an increased level of MFO-activity (compared to OR) that degrades malaoxon to a weakly acidic derivative, presumably malaoxon carboxylic acid(s). They do not however clarify the basis of the differences in malathion and malaoxon sensitivity of the MH19 and C1-39 strains.

IV. DISCUSSION

Section I. Carboxylesterase Mediated Hydrolysis of [^{14}C]-Malathion and Its Role in Resistance

A. Malathion Carboxylesterase Activity in Canton S Crude Extracts

The primary products of [^{14}C]-malathion degradation by crude enzyme preparations from the susceptible CS-flies were found to be the α- and β-monoacids of malathion (Table 1 and Figure 5). Identification of these products was based upon two criteria. First, both products were completely extracted into $CHC\ell_3$ only under acidic conditions (i.e. pH 2) (cf. Figure 5); the pK_a values for the α- and β-monoacids of malathion are 3.62 and 3.74, respectively (Welling *et al.*, 1970). Second, the relative mobilities (R_f values) of the products on thin-layer chromatograms were similar to those reported for the α- and β-monoacids of malathion in two different solvent systems (Welling *et al.*, 1970; Suzuki and Miyamoto, 1978). Since the boiled CS crude extracts did not degrade [^{14}C]-malathion (Figure 4) it was concluded that the α- and β-monoacids of malathion were enzymatically formed. Recovery of approximately 100% of the control ME-activity when CS-extracts were passed through Sephadex G-25 (Table 4) indicated that ME-activity required no cofactor, which is consistant with a carboxylester hydrolase (Dixon and Webb, 1979a and 1979b). Therefore, it was concluded that the CS-extracts contained malathion carboxylesterase activity (ME-activity) (Figure 18, I).

It was observed that ME-activity apparently decreased during the course of reaction with [^{14}C]- malathion (Figure 4). This decrease in

ME-activity was not due to instability of the enzyme(s) (Table 2) or the depletion of substrate ([^{14}C]-malathion) (Table 3). The fact that only 65% of the original ME-activity was regained when more [^{14}C]-malathion was added to a CS-reaction mixture (Table 3) suggested that the apparent loss of activity was due to either depletion of a cofactor or inhibition of the enzyme(s). A Sephadex G-25 chromatographic column was utilized in order to separate large molecular weight components from small molecular weight components (possible inhibitors and cofactors), as Sephadex G-25 discriminates only molecular species with a molecular weight below 5000. Thus, proteins (enzymes) will not be adsorbed and therefore elute in the void volume while smaller molecules (molecular weight < 5000) will be retarded. As discussed above, loss of ME-activity was not due to depletion of a cofactor (Table 4). However, when CS-extracts were exposed to [^{14}C]-malathion for 30 min prior to passage through Sephadex G-25 only 65% of the original control ME-activity was recovered (Table 4). Furthermore, the elution profile of such an extract indicated that a small amount of radioactivity remained associated with the protein fractions. Since the inhibitor could not be removed by Sephadex G-25 chromatography, it was concluded that ME-activity was inhibited by a tightly bound, [^{14}C]-labeled molecule, possibly at the active site of the enzyme. Though the chemical nature of the inhibitor was not further investigated, these results served to indicate that comparisons of ME-activity between resistant and susceptible strains would of necessity have to be made on initial reaction rates since inhibition was progressive over the reaction period.

Several other authors have investigated the inhibition of

carboxylesterases by impurities present in technical malathion formulations (Umetsu *et al.*, 1977 and 1981; Toia *et al.*, 1980; Talcott *et al.*, 1979). In these studies it was found that impurities which accumulated upon storage (presumably due to chemical transformations and breakdown of malathion) significantly increased the mammalian toxicity of these formulations by inhibiting carboxylesterases that normally degrade malathion. The most potent carboxylesterase inhibitors found as impurities were iso-malathion (0,S-dimethyl S-1,2-bis(carboethyoxy)ethyl phosphorodithioate) (Umetsu *et al.*, 1977; Talcott *et al.*, 1979) and 0,0,S-trimethyl phosphorotrithioate (Umetsu *et al.*, 1977 and 1981; Toia *et al.*, 1980). The [^{14}C]-malathion formulation used in the present study was characterized as greater than 99.7% pure according to the supplier, but it had been stored for a few months at room temperature by the manufacturer and in the laboratory at about 3^{o}C for several months before it was used in the above experiments. Periodic tests of purity were made on the stock [^{14}C]-malathion by TLC and no other radioactivity was ever detected that did not have the same relative mobility as an analytical standard of malathion. In spite of the failure to detect radioactive impurities in the [^{14}C]-malathion used in this study, it remains possible that impurities such as the potent carboxylesterase inhibitor 0,0,S-trimethyl phosophorotrithioate were present since this compound would not have been radioactively labeled ([^{14}C]-malathion was labeled only at the two succinyl carbon atoms) and therefore would not have been detected.

Another possibility for the observed inhibition of ME-activity could have been the formation of small amounts of [^{14}C]-malaoxon in the

CS-reaction mixtures. Though malaoxon was not detected in CS reaction mixtures on TLCs (Table 1 and Figure 5), minute amounts may have been formed. Malaoxon is both a substrate and a potent inhibitor of malathion carboxylesterase (Main and Dauterman, 1967; Dauterman, 1976) and thus, if present, may have caused the inhibition (Figure 4). A more sensitive technique such as gas chromatography is necessary to detect trace quantities of potential inhibitors.

All of the potential inhibitors discussed above are neutral esters of phosphoric acid. Such compounds are characterized by their reactivity with esterases and it can be speculated that the inhibition of ME-activity was by phosphorylation of a serine or cysteine residue at the active "esteratic" site of the enzyme, analogous to the action of DFP (di-isopropyl fluorophosphate; Dixon and Webb, 1979b). The fact that radioactivity remained associated with protein after Sephadex G-25 chromatography (Figure 6) supports this speculation.

Chromatography on Sephadex G-150, which discriminates in the molecular weight range 10000 to 150000, indicated that the two major soluble esterase activities of *D. melanogaster* were not responsible for ME-activity (Figure 7). The fact that ME-activity was found exclusively in the void volume of the elution (Figure 7) suggests that the enzyme(s) responsible are either very large (molecular weight >150000) or associated with a particulate subcellular fraction such as microsomes or mitochondria. Matsumura and Brown (1963) partially purified a malathion-carboxylesterase from the mosquito, *Culex tarsalis*. They found that the enzyme was soluble and had a molecular weight of approximately 16000. A soluble malathion-carboxylesterase was also found in the

housefly, *Musca domestica*, (Matsumura and Hogendijk, 1964). In contrast to these reports is the localization of ME-activity in a microsomal fraction from the blowfly, *Chrymosomya putoria* (Townsend and Busvine, 1969). Based on the results of these two latter reports and the elution behaviour of ME-activity from Sephadex G-150 (Figure 7) it can be speculated that the enzyme has a molecular weight less than 150000 and is associated with a particulate subcellular fraction such as microsomes. Differential centrifugation of CS-extracts is necessary to establish the subcellular location of ME-activity and if associated with a particulate fraction, the enzyme must be solubilized to determine its molecular weight.

B. Malathion-Carboxylesterase Activity in MH19 and Cl-39 Extracts

Assays of malathion resistance showed that the most resistant strain was MH19 followed by Cl-39 and lastly the laboratory strains, OR and CS (Table 5 and Figure 9). Though the above trend was the same in both assays, the LT50 values for all strains were much larger in the feeding assay than the vacuum assay (cf. Table 5). In the vacuum assay, the insecticide is thought to enter the insect directly through the tracheal system aided by the sharp increase in air pressure when the partial vacuum is released (see "Assay of Insecticide Resistance" in "MATERIALS AND METHODS") (Sega and Lee, 1979, cited in Pluthero and Threlkeld, 1980). In the feeding assay, the insecticide is subject to processes such as absorption, excretion and possible degradation before it can reach target sites within the insect. O'Brien (1957) has shown that malathion is degraded by isolated fore-, mid- and hind-gut prepara-

tions from the American cockroach, *Periplaneta americana*. These considerations may explain the differences in LT50 values between the two techniques (feeding *versus* vacuum assays), but they do not explain interstrain differences encountered between the Cl-39 and MH19. In the feeding assay the MH19 flies were 1.8X more resistant than Cl-39 flies whereas in the vacuum assay MH19 were 10.6X more resistant than Cl-39 (Table 5).

Both MH19 and Cl-39 reaction mixtures degraded [^{14}C]-malathion primarily to the α- and β-malathion monoacids (Figures 10 and 12; summary in Table 1, see Figure 18, I). However, in contrast to CS extracts these strains produced significant amounts of malaoxon (Figure 18, II) whereas this product was not detected in CS-extracts incubated with [^{14}C]-malathion (Table 1). Furthermore, the most resistant strain (MH19) formed more malaoxon (7-8% of total [^{14}C]) than the intermediately resistant Cl-39 strain (2-2.5% of total [^{14}C]).

The increased production of malaoxon from malathion in the more resistant MH19 and Cl-39 strains of *D. melanogaster* is in contrast to other comparative studies of malathion metabolism in insects. Feroz (1971) found that a resistant strain of the bedbug, *Cimex lectularius*, produced less malaoxon when crude extracts were incubated with [^{14}C]-malathion. *In vivo* studies on a resistant strain ("G") of housefly, *Musca domestica* (Matsumura and Hogendijk, 1964) and a resistant strain (Fresno) of the mosquito, *Culex tarsalis* (Matsumura and Brown, 1961), showed that both accumulated less malaoxon than susceptible strains when malathion was topically applied. Furthermore, the resistant strains of

M. domestica and *C. tarsalis* were shown to possess an elevated level of a phosphatase that degraded malaoxon *in vitro*. The MH19 and C1-39 reaction mixtures did not form any detectable malaoxon degradation products even though both produced malaoxon when incubated with [^{14}C]-malathion.

Since AChE is the presumed target of organophosphate insecticide poisoning (O'Brien, 1976) and malaoxon is a more potent anticholinesterase than malathion (O'Brien, 1957), it is puzzling why the more resistant strains of *D. melanogaster* (MH19 and C1-39) would have an increased capacity to produce malaoxon. If AChE-inhibition, through conversion to malaoxon, accounts for the toxicity of malathion, it would seem that the malaoxon is produced at a site within the fly (especially the MH19 and C1-39 strains) that is far removed from crucial AChE-sites. Malaoxon may, for example, be produced only in the gut and excreted immediately as suggested by a study on malathion-resistance in the lesser grain borer, *Rhyzopertha dominica* (Matthews, 1980) in which it was observed that more malaoxon was excreted by a resistant strain. It is possible that such a mechanism of resistance may be operative in the more resistant (MH19 and C1-39) strains of *D. melanogaster* studied here.

MH19-extracts showed a more pronounced inhibition of ME-activity than those of CS (compare Figures 9 and 4). Pre-incubation of crude enzyme preparations from all strains with malaoxon resulted in an average (for all strains) of 57% inhibition of ME-activity (Table 6). The concentration of malaoxon used in these experiments was approximately 75% of that which accumulated after 30 min when MH19-extracts were

incubated with [^{14}C]-malathion (Table 1). Thus, the greater inhibition of MH19 ME-activity could be accounted for by the increase production of malaoxon in these reaction mixtures. It is difficult to estimate the effect of malaoxon inhibition of malathion-esterase *in vivo* on resistance. Since malathion carboxylesterase activity probably contributes to protecting the insect, one can only suppose that the sites within the insect of ME-activity and malaoxon production are different if this degradative route is important to resistance.

The *in vitro* degradation of [^{14}C]-malathion by C1-39 flies followed a curve that was intermediate between MH19 and CS (compare Figure 11 with Figures 9 and 4). The intermediate inhibition of C1-39 ME-activity is most likely due to the fact that C1-39 flies produced an intermediate amount of malaoxon *in vitro* compared to MH19 and CS reaction mixtures (Table 1).

C. Comparison of Malathion-Carboxylesterase Activity Between Resistant and Susceptible Strains of *Drosophila melanogaster*

Because of inter-strain differences in ME-activity initial (0-1 min) rates were used to compare activity between the strains. No significant differences were found between the resistant and susceptible strains at any malathion concentration tested (Figure 13 and Table 7). The fact that CS-extract had a slightly higher level of ME-activity at higher concentrations of [^{14}C]-malathion may have been due to a greater accumulation of malaoxon in the more resistant strains (MH19 and C1-39) (Figure 3 and Table 7). The MFO-inhibitor metyrapone was included in reaction mixtures along with [^{14}C]-malathion to block the formation of

of malaoxon. Even in the presence of metyrapone at a concentration that inhibited p-nitroanisole O-demethylase activity by greater than 85%, significant differences in ME-activity could not be detected between the resistant and susceptible strains (Table 8). Since ME-activity was the same in all strains it was concluded that it was not an important contributing factor to the increased resistance of the MH19 and C1-39 strains. However, it should be pointed out that despite the fact that metyrapone was used to inhibit the formation of malaoxon, only the inhibition of pNA-demethylase activity was examined. Though metyrapone generally possesses good inhibitory activity for most MFO-mediated reactions, it is less effective in a few isolated cases (Testa and Jenner, 1981). For example, Jonen *et al.* (1974) showed that while metyrapone was a potent inhibitor of pNA-demethylase activity in microsomes from phenobarbitol-induced rats it was a poor inhibitor of aniline-hydroxylation. Thus, it is still possible that differences in ME-activity were being masked by the production of malaoxon in the more resistant strains (MH19 and C1-39).

The failure to find consistant differences in malathion carboxylesterase activity *in vitro* may not reflect the *in vivo* contribution of this activity to malathion resistance. To this end, the resistance of the strains was tested in the presence of the carboxylesterase inhibitors (synergists) DEF and TPP (Plapp *et al.*, 1963). The inclusion of DEF and TPP with malathion in the test media did not synergize the toxicity of malathion in either the resistant or susceptible strains (Table 9). This finding is consistent with the failure to detect any inter-strain difference in ME-activity (Tables 7 and 8). What is puzzling, however,

is that DEF and TPP antagonized the toxicity of malathion in all strains (Table 9). As will be discussed below, all strains degrade malathion by way of an MFO-mediated pathway. In view of the previous finding that both DEF and TPP can induce the formation of cytochrome P-450 (Perry *et al.*, 1971) it was possible that MFO-mediated degradation of malathion was induced by the presence of DEF and TPP. Thus, antagonism of malathion toxicity seems reasonable. It might be expected that DEF and TPP should exert a small synergistic effect, roughly equal in all strains, since ME-activity (malathion degradation) was similar for all strains *in vitro*. The possible induction of MFO-mediated degradation by DEF and TPP (discussed above) may have masked this expected outcome.

D. The Contribution of Malathion-Carboxylesterase Activity to The Resistance of *Drosophila melanogaster* Strains

The results discussed so far provide strong evidence for the conclusion that the resistant strains (MH19 and C1-39) do not possess an increased capacity to degrade malathion by way of carboxylester hydrolysis (ME-activity). This discovery is somewhat surprising in view of the facts that, firstly, esterases are among the most polymorphic of enzymes in insect populations (Powell, 1975) and secondly, the MH19 strain was selected from a (presumably) genetically heterogeneous population established by combining 40 isofemale lines that were caught in the wild (Singh and Morton, 1981). One might have expected that carboxylesterase allozyme variants possessing increase activity towards malathion would have been present in the original heterogeneous

population of *D. melanogaster* and therefore been selected when pressured with malathion. Furthermore, the lack of increased ME-activity associated with malathion resistance is in contrast to many other reports of carboxylesterase-mediated malathion-resistance. Increased ME-activity, *in vitro*, has been reported in resistant strains of the bedbug, *Cimex lectularius* (Feroz, 1971), smaller brown leaf hopper, *Laodelphax striatellus* (Miyata *et al.*, 1976), housefly, *Musca domestica* (Niwa *et al.*, 1977); *in vivo* in the mosquito, *Anopheles stephensi* (Hemingway, 1982), and both *in vivo* and *in vitro* in the mosquito, *Culex tarsalis* (Matsumara and Brown, 1961; Bigley and Plapp, 1962), the housefly, *M. domestica* (Matsumura and Hogendijk, 1964) and the blowfly, *Chrysomya putoria* (Townsend and Busvine, 1969). In many of these instances carboxylesterase inhibitors synergized the toxicity of malathion. In particular, the following inhibitors were found to synergize the toxicity of malathion: TPP in *A. culicifacies* (Hearth and Davidson, 1981) and *A. stephensi* (Hemingway, 1982); TPP and DEF in the Grothe strain of *M. domestica* and the Fresno strain of *C. tarsalis* (Plapp *et al.*, 1963); EPN and EPNO in the G strain of *M. domestica* (Matsumura and Hogendijk, 1964); and TPP, DEF, TOTP and EPN in the CM strain of *C. putoria* (Townsend and Busvine, 1969).

In summary, the failure to find an increase in carboxylesterase-mediated [^{14}C]-malathion degradation *in vitro* and synergism of malathion toxicity by carboxylesterase inhibitors *in vivo* provided ample evidence to conclude that malathion-resistance in the MH19 and C1-39 strains of *D. melanogaster* is not associated with increased carboxylesterase activity.

Section II. Microsomal Mixed Function Oxidases and Their Role in Resistance

A. The Implications of Cross-Resistance to Other Insecticides

The fact that the MH19 and C1-39 strains, which had been selected for resistance to malathion, were cross-resistant to other insecticides (Table 10) further strengthened the conclusion that carboxylesterase activity had a minor role in the increased resistance of these strains. Since malathion has a unique structure compared to other organophosphate insecticides (two carboxylester bonds), carboxylesterase-mediated resistance should be relatively specific to malathion. In cases where increased malathion-carboxylesterase activity accounted for a major part of malathion-resistance, little or no cross-resistance to other organophosphates not possessing a carboxylester moiety has been found. Such, for example, was found in the Fresno strain of the mosquito, *Culex tarsalis* (Matsumura and Brown, 1961), the Sapporo and Moiwa strains of the housefly, *Musca domestica* (Niwa *et al.*, 1977), the bedbug, *Chrysomya putoria* (Townsend and Busvine, 1969) and the ST-MAL strain of the mosquito, *Anopheles stephensi* (Hemingway, 1982). It is interesting to note that the resistant strain of *C. putoria* was cross-resistant to acethion, which contains a carboxylamide group.

Increased levels of phosphatase activity could be eliminated as a factor in the cross-resistance of MH19 and C1-39 flies on the basis of two points. First, phosphatase products of [^{14}C]-malathion degradation were not detected in MH19 (Figure 9 and 10) or C1-39 (Figures 11 and 12) reaction mixtures. Unreacted [^{14}C]- malathion, malaoxon and the α- and

β-malathion monoacids accounted for 100% of the input radioactivity (Table 1). Second, increased phosphatase activity as a mechanism of resistance is specific to organophosphate insecticides whereas the observed cross-resistance of MH19 and C1-39 extended to the carbamate insecticide, carbaryl (Table 10). Feroz (1971) found that malathion resistance in a strain of *Cimex lectularius* was associated with increased phosphatase activity and that cross-resistance extended to other organophosphates, but not to carbamates or organochlorine insecticides.

Mixed function oxidases (MFO) are known to degrade most major types of insecticides including organophosphates, carbamates and pyrethroids (reviewed by Oppenoorth and Welling, 1976; and Nakatsugawa and Morelli, 1976). The role of the MFO-system in the resistance of MH19 and C1-39 strains seems questionable, since these strains were less resistant to permethrin (a pyrethroid) than the otherwise susceptible CS strain (Table 10). Plapp and Casida (1969) using the resistant "Fc" strain of houseflies, *Musca domestica*, showed that cross-resistance to diazinon, malathion, propoxur (a carbamate) and allethrin (a pyrethroid) was genetically inseparable from a gene that caused increased MFO-activity. The pyrethoid insecticide permethrin possesses a carboxyl-ester moiety as with all compounds in this class, but carboxylesterase-mediated degradation of permethrin (specifically trans-permethrin) seems to be minor in insects such as cabbage looper larvae, *Trichoplusia ni* (Gaughan *et al.*, 1980) and houseflies, *Musca domestica* (DeVries and Georghiou, 1981). In both these instances, trans-permethrin toxicity was only weakly enhanced by simultaneous treatment with the carboxyl-

esterase inhibitor DEF. In view of these results and the fact that MH19 and Cl-39 were not cross-resistant to permethrin, the possibility that increased MFO-activity was not responsible for increased resistance of MH19 and Cl-39 relative to CS must be considered.

Another factor to be considered, with regard to cross-resistance, is that the target of pyrethroid insecticides differs from that of organophosphates. Pyrethroids apparently exert their toxic effects by blocking Na^+/K^+ channels in axons (reviewed by Narahashi, 1971 and 1976) whereas organophosphates and carbamates act by inhibition of AChE (reviewed by O'Brien, 1976). The lack of resistance to permethrin by MH19 and Cl-39 flies may reflect the existance of a more sensitive pyrethroid target (Na^+/K^+ channels) compared to CS flies. DeVries and Georghiou (1981) found the amount of *in vitro* degradation of trans-permethrin to be similar in resistant ("147") and susceptible (NAIDM) strains of houseflies, *Musca domestica*, and concluded that the principle mechanism of resistance was decreased sensitivity of the nervous system. The differences in resistance to permethrin exhibited by the strains of *D. melanogaster* in the present study (especially CS) may be due to differences in target site sensitivity.

The cross-resistance data (Table 10) indicate that the Cl-39 and MH19 strains were resistant to organophosphate (malathion, parathion and diazinon) and carbamate (carbaryl) insecticides which supposedly act by AChE-inhibition (O'Brien, 1976). By itself, this result suggests that more resistant strains of *D. melanogaster* possess an insensitive (to inhibition) form of AChE. Morton and Singh (1981) reported the MH19 strain (used here) possessed an altered form of AChE relative to

CS and Cl-39. Further work (R.A. Morton, personal communication) on the AChE of these strains has revealed that the MH19 enzyme is much less sensitive to inhibition by malaoxon than both the CS and Cl-39 enzymes. The CS and Cl-39 AChE forms were similar in their sensitivity to malaoxon. Though an insensitive form of AChE may account for the cross-resistance of MH19, the similar AChE-sensitivities of Cl-39 and CS do not account for the greater resistance of Cl-39 relative to CS. Increased MFO-activity seemed the most likely candidate to account for the malathion resistance of Cl-39 whereas decreased sensitivity of AChE to inhibition may account for the resistance of MH19.

B. The Synergism of Malathion and Malaoxon Toxicity by Mixed Function Oxidase Inhibitors

The role of mixed function oxidases (MFO) in malathion resistance *in vivo*, was tested more directly by including the MFO-inhibitors PBO and MTP in test media. Malathion toxicity was not synergized by either PBO or MTP in any of the resistant or susceptible strains (Table 11). This result seemingly contradicts the hypothesis that increased levels of MFO-activity contribute to resistance, but it must be remembered that the MFO-system mediates the activation of malathion to the more potent anticholinesterase, malaoxon (O'Brien, 1957). Since activation of malathion and degradation of malaoxon are both accomplished by the MFO-system it is difficult to predict the synergistic effect of PBO and MTP when used in conjuction with malathion.

The MH19 strain was most resistant to malaoxon followed by Cl-39, whereas OR was least resistant. The fact that the toxicity of malaoxon

was strongly synergized by the MFO-inhibitors PBO and MTP in all three strains suggested that malaoxon degradation by MFOs was relevant to their resistance to malaoxon and, by inference, malathion. Malaoxon toxicity was synergized in both resistant and susceptible strains, but the effect was more pronounced for the more resistant strains (in terms of absolute decrease in LT50). This suggested that the more resistant strains likely had an increased capacity to oxidatively degrade malaoxon compared to the OR strain.

C. Microsomal Mixed Function Oxidase Activity and Cytochrome P-450 Content

Direct measurement of MFO-activities (pNA-demethylase activity) showed that it was highest in crude extracts and microsome preparations of the MH19 flies followed by C1-39 and lowest in the susceptible OR strain (Tables 13 and 14). Furthermore, cytochrome P-450 contents, as determined by the reduced-CO spectrum (Table 14 and Figure 15) were indistinguishable between resistant and susceptible strains. Therefore, microsomes from the more resistant strains have higher activity although the cytochrome P-450 content is the same for all strains. Since the MFO-system consists of a series of coupled enzymatic steps, higher MFO-activity could be a reflection of an activity change in one or more of the component enzymes (for review of MFO-system in insects see Wilkinson and Brattsten, 1972). No further attempt was made in this study to determine the enzymes responsible for higher MFO-activity in the MH19 and C1-39 strains. Kulkarni *et al.* (1976) reported that in the resistant Fc strain of houseflies, *Musca domestica* increased microsomal pNA-

demethylase activity was correlated with increased levels of NADPH-cytochrome C(P-450) reductase, cytochrome C oxidase and cytochrome P-450. Higher MFO-activity, as measured by several different assays, has also been found in the following multi-resistant strains of *M. domestica*: Rutgers (Diazinon-R) strain, aldrin epoxidase, heptachlor epoxidase (Moldenke and Terriere, 1981) and Baygon hydroxylation (Perry *et al.*, 1971); Rutgers and Fc strains, amino-pyrene N-demethylase, aniline-hydroxylase, aldrin-epoxidase, and pNA-demethylase (Chang and Hodgson, 1975). The fact that MFO-activity was consistently higher in these resistant *M. domestica* strains using several quite different substrates and reactions, suggests that the increased pNA-demethylase activity of the MH19 and C1-39 strains is an indication of increased MFO-mediated detoxication of insecticide.

The fact that cytochrome P-450 content was similar for both resistant and susceptible strains of *D. melanogaster* is in contrast to numerous reports of increased levels of this enzyme in resistant insects. Table 16 summarizes some of these reports, the majority of which have used houseflies, *M. domestica*. Cytochrome P-450 content varies considerably in different studies of the same strain. Kulkarni and Hodgson (1975) reported that the yield of cytochrome P-450 depended upon the method used to prepare microsomes. This suggests that differences between studies of the same strain may reflect differences in preparation techniques. However, inter-strain comparisons of cytochrome P-450 content within a given study are reasonable, since microsome preparation techniques would be similar for all strains.

TABLE 16

Summary of Cytochrome P-450 Levels in *Musca domestica* and *Drosophila Melanogaster* as Reported by the Present and Previous Studies

Species	Strain	Resistance[a]	Cytochrome P-450 Content (nmol/mg)[b]	Reference
M. domestica	NAIDM	S	.23	Perry *et al.* (1971)
	Rutgers	R	.57	
	DDT-R	R	.33	
	Malathion-R	R	.28	
	Fc	R	.27	Morello *et al.* (1971)
	NAIDM	S	.16	Moldenke & Terriere
	CSMA	S	.14	(1981)
	Rutgers	R	.28	
D. melanogaster	Hikone-R	S	.23	Waters *et al.* (1982)
	Hikone-R	S	.18	Hallström &
	Karsnäs-60	S	.05	Gräfström (1981)
	Berlin-K	S	.05	
	OR	S	.33	Present Study
	C1-39	R	.34	
	MH19	R	.34	

[a] S: susceptible; R: resistant

[b] nmol cytochrome P-450 per mg microsomal protein

Spectral shifts of the major Soret band (near 450 nm) have been reported for reduced CO-found cytochrome P-450 between resistant and susceptible strains of *M. domestica*. These and other differences in ligand-binding spectra have been interpreted as evidence of an altered form of cytochrome P-450 associated with increased activity and hence, resistance (reviewed by Hodgson *et al.*, 1974). That no differences in the absorption maxima of cytochrome P-450 were found between resistant and susceptible strains of *D. melanogaster* does not necessarily mean that the enzyme is the same in all strains. Additional (high resolution) filters were not available and the differences cited above for *M. domestica* were in the range of 448 nm to 451 nm. It is possible that differences in cytochrome P-450 (as detected by spectral differences) may exist between resistant and suceptible strains.

D. Mixed Function Oxidase Mediated Degradation of [^{14}C]-Malaoxon

Using crude extracts of MH19 flies it was found that [^{14}C]-malaoxon was degraded to at least two products (weakly acidic and strongly acidic) by an enzyme(s) that required NADPH and was inhibited by MTP (Figure 16). On the basis of these results it was concluded that [^{14}C]-malaoxon was degraded by the MFO-system.

The products of [^{14}C]-malaoxon degradation were characterized only with respect to their solubility properties in aqueous-$CHC\ell_3$ mixtures at pH 2 (Table 15). The radioactive product(s) that partitioned into $CHC\ell_3$ at pH 2 was possibly the α-and/or β-monoacid(s) of malaoxon (Figure 18, III). It was assumed that the monoacids of malaoxon have pK_a values around 3.5 similar to the monoacids of malathion and hence

would have partitioned into $CHC\ell_3$ at pH 2. However, it cannot be assumed that the malaoxon monoacids were completely extracted into $CHC\ell_3$ at pH 2, since the partition coefficients ($CHC\ell_3$:H_2O) of these compounds may be lower than those of the malathion monoacids. Malathion monoacids were almost completely removed from reaction mixtures by extraction with $CHC\ell_3$ at pH 2 (cf. Figures 4, 9, and 11) and therefore were far more soluble in $CHC\ell_3$ than water at this pH. Malaoxon monoacids may have lower solubility in $CHC\ell_3$, since malaoxon is less lipophilic than malathion (O'Brien, 1957). Small amounts of radioactive malaoxon may have remained in the aqueous phase and thus would have been mistakenly identified as strong-acidic products. Despite this possibility, a substantial amount of radioactivity was found in $CHC\ell_3$ extracts at pH 2 (Table 15) and was probably the α- and/or β-monoacids of malaoxon (Figure 18, III). The more strongly acidic product of [^{14}C]-malaoxon degradation is possibly demethyl-malaoxon (Figure 18, IV). Since demethyl-malaoxon is a phosphoric acid derivative and hence a strong acid, it would remain in the aqueous phase after extraction with $CHC\ell_3$ at pH 2. Although it is possible that some of the radioactivity found in this aqueous phase was due to unextracted (radioactive) malaoxon monoacid(s), it was concluded that the majority of radioactivity in this fraction (Table 15) was due to a more strongly acidic product.

Malathion was found to be a potent inhibitor of MFO-mediated [^{14}C]- malaoxon degradation (Figure 16). This inhibition of malaoxon degradation may have accounted for the fact that no degradation of malaoxon was detected in MH19 and C1-39 reaction mixtures where [^{14}C]-malathion was used as substrate (Table 1). Further implications of this

inhibition with respect to malathion resistance are discussed later (Section II. E).

When [^{14}C]- malaoxon degradation was examined in crude extracts from all strains (Figure 17), the more resistant MH19 and C1-39 flies had similar, but higher levels of activity than the susceptible OR flies. Examination of the products (Table 15) revealed that all strains produced similar amounts of the very acidic product (probably demethyl-malaoxon) whereas the more resistant strains (MH19 and C1-39) produced 7.5X greater amounts of the weak-acid product (probably malaoxon mono-acid(s)) than the susceptible OR strain.

Overall, the studies on malathion resistance in *D. melanogaster* are quite similar to two different studies on malathion resistance in other insects. Welling *et al.* (1974) reported that the resistant (E1) strain of the housefly, *Musca domestica*, was more resistant to malaoxon than malathion, that malathion toxicity was not synergized by either TPP (a carboxylesterase inhibitor) or sesamex (an MFO-inhibitor), that microsomes prepared from E1 oxidatively degraded malaoxon at 16X the rate of the susceptible SRS strain and that oxidative malaoxon degradation was strongly inhibited by malathion. The product of oxidative degradation of malaoxon in strain E1 was malaoxon β-monoacid both *in vitro* and *in vivo*. Though Welling *et al.* (1974) did not report on the cross-resistance of the E1 strain, it was derived as a sub-strain of strain C that was originally described by Oppenoorth (1959). Oppenoorth (1959) examined the cross-resistance of strain C and found that it was tolerant to a wide range of organophosphate insecticides. Of the insecticides tested, strain C was more tolerant to the oxon-analogues

(P=0) of the respective thiono-organophosphates (P=S).

In another study of malathion resistance in the flour beetle, *Tribolium castaneum*, Dyte *et al.* (1970a, cited in Oppenoorth and Welling, 1976; 1970b and 1971, cited in Welling *et al.*, 1974) found that the resistant strain was more resistant to malaoxon than malathion, that neither TPP or PBO synergized malathion toxicity, that the resistant strain showed a broad cross-resistance particularly to oxon-analogues of dimethoxy- and diethoxyphosphorothionates (i.e. organophosphates such as malathion, parathion and diazinon) and that both the resistant and susceptible strains metabolized malathion at about the same rate. However, in contrast to the present study and that of Welling *et al.* (1974), PBO failed to synergize the toxicity of malaoxon. Oppenoorth and Welling (1976) suggested this lack of synergism may have been due to the fact the PBO was rapidly oxidized by the resistant strain of *T. castaneum*. In further contrast to the resistant *M. domestica* and *D. melanogaster* strains, the resistant strain of *T. castaneum* produced only demethyl-malaoxon (i.e. a strong-acidic product), *in vivo*. The malaoxon degradation reaction in *T. castaneum* was not characterized *in vitro* so it cannot be concluded with certainty that it was MFO-mediated.

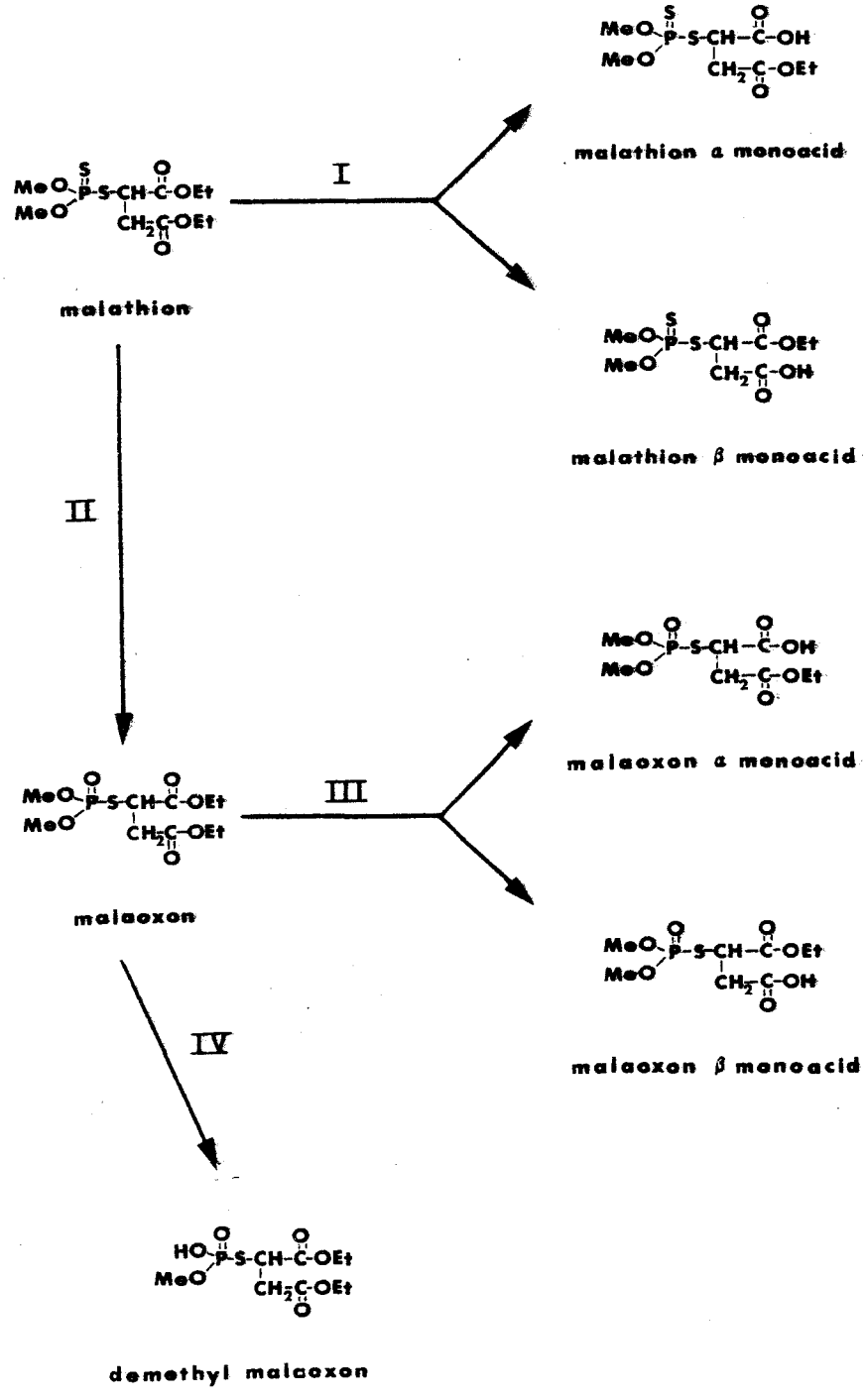
E. The Mechanisms of Malathion Resistance in *Drosophila melanogaster*

As a summary, the pathways of malathion degradation are shown in Figure 18. In view of the fact that the resistant strains (MH19 and C1-39) had increased levels of activity that converted malathion to malaoxon and degraded malaoxon *in vitro*, it was concluded that this

FIGURE 18

Summary of Pathways of Malathion Degradation in *Drosophila melanogaster*

Step I is mediated by a carboxylesterase; Steps II, III and IV are mediated by mixed function oxidases.


malathion
I
malathion α monoacid
malathion β monoacid
II
malaoxon
III
malaoxon α monoacid
malaoxon β monoacid
IV
demethyl malaoxon

pathway (Figure 18, II→III) was a major factor in malathion-resistance. This proposed resistance mechanism does not fully account for all of the differences between the MH19 and C1-39 strains. The MH19 strain showed an increased ability, over C1-39, to produce malaoxon (Table 1) while both strains degraded malaoxon at similar rates (Figure 17) to similar products (Table 15). It is conceivable that the difference in malathion-resistance between these two strains was due partly to the increased ability of MH19 flies to convert malathion to malaoxon over that in the C1-39 flies. In reference to Figure 18, pathway II (malathion >--> malaoxon) is faster in MH19 while pathway III (malaoxon >--> malaoxon monoacids) is the same rate in both MH19 and C1-39. This resistance presupposes that malaoxon formation occurs at a site(s) removed from crutial AChE target-sites, since malaoxon is a potent inhibitor of AChE-activity (O'Brien, 1957). Inhibition of AChE is thought to be the basis of organophosphate (malathion) toxicity (reviewed by O'Brien, 1976). O'Brien (1957) has shown that malaoxon formation and degradation both occur in isolated fatbody tissue from the cockroach, *Periplaneta americana*. In view of this, the hypothesis of malathion degradation via malaoxon formation is feasible if both processes (malaoxon formation and degradation) occur in a tissue such as fatbody.

Although this mechanism can account for the differences in malathion resistance, it cannot, however, account for the difference in malaoxon resistance between MH19 and C1-39 (Figure 14). Since the *in vitro* [^{14}C]-malaoxon degradation activity is similar in both strains, there must exist another factor accounting for the greater tolerance

of MH19 to malaoxon over C1-39. As discussed earlier (Section II.A) MH19 flies possess an altered form of AChE (Morton and Singh, 1981) that is less sensitive to inhibition by malaoxon (R.A. Morton, personal communication) and this fact may well account of the greater resistance of MH19 to malaoxon. This sensitive AChE probably contributes to the greater malathion resistance of MH19, but to what degree this occurs is not known.

Further comment is necessary on the inhibition of MFO-mediated degradation of malaoxon by malathion (Figure 16) with respect to the physiological implications that this inhibition has to the survival of resistant insects. The results of the present study are not the first to report increased oxon-analogue production from the corresponding thiono-organophosphate insecticide in resistant insects. ElBashir and Oppenoorth (1969) reported the microsomes from the resistant Fc and Nic strains of *Musca domestica* converted more parathion and diazinon (in separate experiments) to their respective oxon-analogues (paraoxon and diazoxon) than the susceptible SRS and ACR strains. The resistant strains (Fc and Nic) also had increased capacity to oxidatively degrade paraoxon and diazoxon. Oppenoorth *et al.* (1971) demonstrated that the oxidative degradation of paraoxon in Fc microsomes was inhibited by both parathion and diazinon. Thus, it seems possible that inhibition of MFO-mediated oxon-analogue degradation by thiono-organophosphate insecticides may be common to insects that are resistant by virtue of increased MFO-activity.

In cases where increased MFO-activity causes resistance, a balance must exist between activation (oxon-formation) and oxon-

analogue degradation. In an attempt to find where an *in vivo* difference in the formation of oxon-analogues could be detected, ElBashir and Oppenoorth (1969) injected the parathion and diazinon (separately) into resistant (Fc and Nic) and susceptible (ACR) strains of *M. domestica*. Their idea was to flood the MFO-system with thion-compounds and see if the increased capacity of the resistant flies to activate parathion and diazinon could be a disadvantage. The result was quite interesting: the resistant flies were knocked-down (presumably killed) faster than the otherwise susceptible flies. This effect was not seen with diazinon and may have been due to a 20-fold greater microsomal diazoxon degradation (relative to that for paraoxon). Since ElBashir and Oppenoorth (1969) were not aware of the inhibitory effect of thiono-compounds on MFO-mediated oxon-degradation, they could only conclude that it represented the importance of "opportunity factors" of activation and degradation in insects. In light of later work by Oppenoorth *et al.* (1971), who demonstrated the inhibitory activity of thiono-compounds, the interpretation seems clear. The injected thiono-compounds acted as a substrate for activation, but their excess inhibited the subsequent degradation of the oxon-derivative. The resistant flies produced more oxon (P=0) *in vivo* and since oxo-analogues are more potent anticholinesterases these flies were killed faster.

Although insecticides are not injected in field control programs, Oppenoorth *et al.* (1971) suggested that thiono-compounds act as their own synergists because they can inhibit their own degrdation. The results of the present study show that the resistant *D. melanogaster*

strains (MH19 and C1-39) were far more resistant to malaoxon than to malathion (Figure 14). It seems probable that malathion synergized its own toxicity by inhibiting the degradation of malaoxon. A similar explanation was put forward by Welling *et al.* (1974) to explain the greater malaoxon resistance of *M. domestica* strain E1. A contributing factor to the greater malathion tolerance of MH19 is that it converts more malathion to malaoxon and thus, decreases the chances that malaoxon degradation will be inhibited by malathion.

F. Proposed Future Work

As discussed above, the more resistant MH19 strain differed from C1-39 in two respects. Firstly, MH19 extracts converted more malathion to malaoxon and secondly, MH19 possessed a less sensitive (to malaoxon inhibition) form of AChE (R.A. Morton, personal communication). Because these factors probably simultaneously contribute to the survival of MH19 flies, it is impossible to estimate the individual importance of these mechanisms to the increased malathion resistance of MH19 over C1-39 without separating them genetically. As a first approach, it may be useful to construct iso-chromosomal sub-strains from MH19 and test these sub-strains with respect to malathion and malaoxon resistance, malaoxon formation and degradation and AChE sensitivity. To this end, a sub-strain possessing the C1-39 AChE genotype and the MH19 genotype with respect to increased capacity to convert malathion to malaoxon would be ideally suited to establish the contribution of increased malaoxon formation in malathion resistance.

In vitro studies on metabolism are useful in elucidating the enzymology and relative rates of a particular reaction, but *in vivo* studies of malathion metabolism are necessary to establish the capacity of such reactions in the live insect. Although topical application of malathion to insects has been routinely used in several previous studies, the results from feeding radioactive malathion would be more readily interpretable since the MH19 strain was selected (Singh and Morton, 1981), and resistance was generally measured in this manner.

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