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Malathion Toxicity and Carboxylesterase Activity in *Drosophila melanogaster*

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Adults from nine strains of *Drosophila melanogaster* were exposed to a residual film of malathion. The LC_{50} values indicated that there was a fourfold difference in susceptibility to malathion among the strains. 3-Nonylthio-1,1,1-trifluoropropan-2-one (NTFP), a carboxylesterase inhibitor, synergized the toxicity of the insecticide when four fly strains were exposed to a mixture of the inhibitor and malathion. Whole-body homogenates of adults were analyzed by wide-range isoelectric focusing (IEF). Carboxylesterase activities on the gels were stained using common substrates such as α- and β-naphthyl acetate and *p*-nitrophenyl acetate. Less conventional substrates, ethyl and isobutyl carbonates of α-naphthol and *p*-nitrophenol, were synthesized and used for staining gels and kinetics in solution. Two major bands of activity (p*I* 4.4 and 4.0) were detected with naphthyl substrates. A major peak of malathion hydrolytic activity (p*I* 6.3) was detected by running spectrophotometric assays for carboxylesterase activity on malathion using IEF gel slices and a rapid, semiautomated assay method. The peak region of activity on malathion did not coincide with bands obtained when naphthyl substrates were used to stain the gels. NTFP inhibited malathion hydrolysis in a solution from the IEF peak of activity and also inhibited esterase activity on α-naphthyl acetate on the gels. Recovered carboxylesterase activities on malathion and *O*-ethyl carbonate of *p*-nitrophenol were higher in the cytosolic than in the microsomal or mitochondrial cell fractions.

INTRODUCTION

Malathion is a widely used organophosphate which has low mammalian toxicity and is effective against a broad range of insects. Various taxa have developed carboxylesterase-based resistance to malathion, including species of mosquitoes (1–3), a blowfly (4), heteropteran pests (5, 6), and the house fly (7, 8). Carboxylesterases (EC 3.1.1.1) are a highly variable and ubiquitous class of enzymes in mammals (9–11) and insects (12). They can be categorized by activity on synthetic substrates and by response to different classes of inhibitors, but there is no unambiguous classification scheme (9, 11). Esterases are difficult to classify and study, in part, because they have overlapping substrate specificities. Consequently, an increasingly attractive method of studying esterases is to distinguish the action of different isozymes by combining genetic and biochemical methods (13).

The purpose of this study was to investigate the biochemistry and initiate work on the genetics of the esterases that hydrolyze malathion. We have employed *Drosophila melanogaster,* which is a useful insect for biochemical genetics as well as an agronomic pest in some areas of the world. This work could lead to a practical method of counteracting malathion resistance in pest species or transferring resistance to beneficial insects.

The project included two avenues of research; the first was to assess the toxicity of malathion *in vivo,* with and without a novel carboxylesterase inhibitor, and the second included an *in vitro* characterization of carboxylesterases. Initially, a series of strains of *D. melanogaster* were assayed

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for malathion susceptibility. Lines that differed in their tolerance to malathion would be valuable for genetic analyses and biochemical comparisons. 3-Nonylthio-1,1,1-trifluoropropan-2-one (NTFP),[3] previously shown to be a carboxylesterase inhibitor in mammals and a malathion synergist in mice (14), was applied to a subset of the strains in combination with malathion. An inhibitor of malathion hydrolysis in an insect could be used as a synergist to counteract malathion resistance, or lead to the development of an affinity column to purify esterases.

Different esterases can metabolize malathion; thus the second part of our study concentrated on using substrates with a variety of structures to distinguish *D. melanogaster* carboxylesterases on isoelectric focusing gels. We used conventional substrates (naphthyl and *p*-nitrophenyl esters), a substrate of toxicological interest (malathion), and carbonates of naphthol and *p*-nitrophenol. We also studied the *in vitro* inhibition of carboxylesterase activities on α-naphthyl acetate and malathion. Finally, the distribution of carboxylesterase activities in subcellular fractions from homogenates of *D. melanogaster* was determined.

MATERIALS AND METHODS

Chemicals. Malathion (99.2%), *O,O*-dimethyl-*S*-1,2-bis(ethoxycarbonyl)ethyl phosphorodithioate, was a gift from Dr. M. Mallipudi, American Cyanamide Corp. (Princeton, NJ). *p*-Iodonitrophenyl tetrazolium violet (INT), alcohol dehydrogenase, NAD, NAD diaphorase, bovine serum albumin (fraction V), α- and β-naphthyl acetate, α- β-naphthyl butyrate, and fast blue were obtained from Sigma Chemical Co. (St. Louis, MO). *p*-Nitrophenyl acetate (*p*-NpAc), *p*-nitrophenol, α-naphthol, ethyl chloroformate, isobutyl chloroformate, and tributylamine were purchased from Aldrich Chemical Co. (Milwaukee, WI). Coomassie brilliant blue R was obtained from Eastman (Rochester, NY). Bio-Rad protein dye reagent was purchased from Bio-Rad Laboratories (Richmond, CA). NTFP was available from a previous synthesis (14). *O*-Ethyl and *O*-isobutyl carbonates of both α-naphthol and *p*-nitrophenol were synthesized and used as substrates. The chemical structures of the inhibitor and substrates used in this study are shown in Fig. 1.

Synthesis of O-ethyl and O-isobutyl carbonates of p-nitrophenol. To each of two flasks, 5.56 g (40 mmol) of *p*-nitrophenol was dissolved in 100 ml of dry diethyl ether. Then 5 ml (52.5 mmol) ethyl chloroformate was added to one flask and 6 ml (45 mmol) isobutyl chloroformate was added to the other (for the synthesis of *O*-ethyl and *O*-isobutyl carbonate, respectively), dropwise, over 5 min with ice cooling. Twelve milliliters (92.5 mmol) of tributylamine was added to each flask. The reaction was allowed to proceed at room temperature for 60 min and was monitored by thin-layer chromatography (TLC) developed in toluene:ethanol, 19:1. When judged complete by TLC, the reaction mixture was washed 3× with 5% HCl, 1× with water, and 1× with brine, and then 20 ml of hexane was added. The mixture was then filtered through a layer of anhydrous sodium sulfate which was washed 2× with hexane. The combined solvents were partially evaporated and 100 ml of warm hexane was added. The *O*-ethyl carbonate of *p*-nitrophenol almost immediately formed white needles from a pale yellow solution at room temperature. The first crop was recrystallized at room temperature overnight and washed with cold hexane to give white crystal needles (approximately 3 cm long) having a melting point of 67–68°C. The *O*-isobutyl carbonate of *p*-nitrophenol failed to crystallize at room temperature, 5°C, or −10°C. It did form a white wax in dry ice

[3] Abbreviations used: NTFP, 3-nonylthio-1,1,1-trifluoropropan-2-one; IEF, isoelectric focusing; INT, iodonitrophenyl tetrazolium violet; *p*-NpAc, *p*-nitrophenyl acetate; TCA, trichloroacetic acid; αNAE, α-naphthyl acetate esterase.

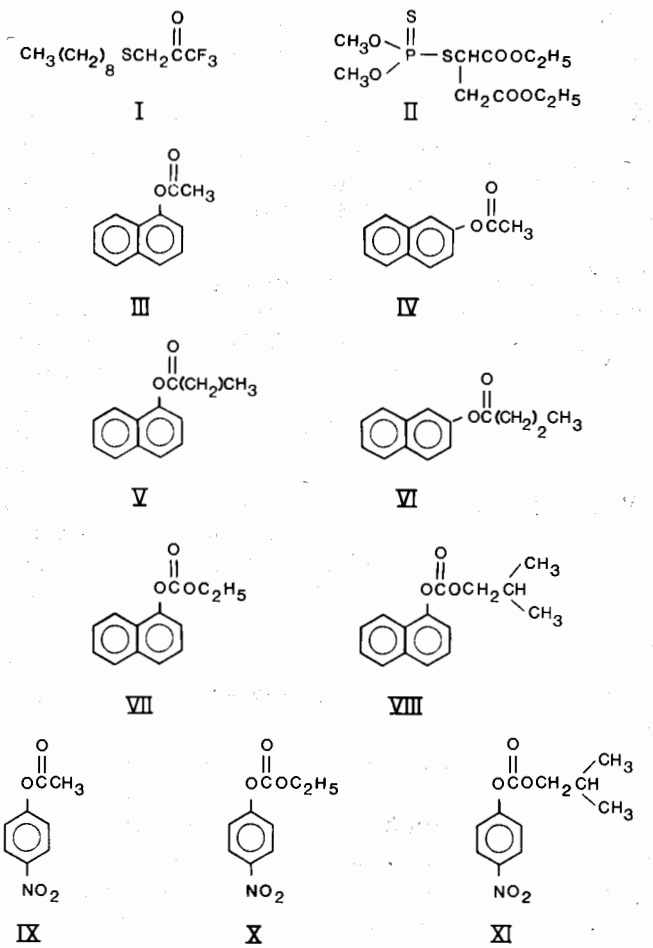

FIG. 1. *Chemical structures of the inhibitor and substrates used. I, 3-nonylthio-1,1,1-trifluoropropan-2-one (NTFP); II, malathion; III, α-naphthyl acetate; IV, β-naphthyl acetate; V, α-naphthyl butyrate; VI, β-naphthyl butyrate; VII, O-ethyl carbonate of α-naphthol; VIII, O-isobutyl carbonate of α-naphthol; IV, p-nitrophenyl acetate; X, O-ethyl carbonate of p-nitrophenol; and XI, O-isobutyl carbonate of p-nitrophenol.*

and subsequently the ether hexane was drained. The white waxy crystals were washed with cold hexane, melted at room temperature, and put on high-speed vacuum for 3 hr to give a tan nonviscous oil. The isolated yields were 91 and 92% for *O*-ethyl and *O*-isobutyl carbonates of *p*-nitrophenol, respectively. Each compound gave one spot on TLC and the structural assignment was verified by infrared (ir), nuclear magnetic resonance (NMR), and mass spectrum.

As an example, the structural assignment for *O*-ethyl carbonate of *p*-nitrophenol was as follows: ir (neat, NaCl) 1925 (m, *p*-phenyl), 1750 (vs, C=O), 1625 and 1600 (m, C=C aromatic), 1520 and 1330 (s–m, $-NO_2$), 1440 (q, $-CH_2$), 1375 (w, $-CH_3$), 1240 (vs, C–O), 900 and 860 (s, 1,4-phenyl) cm^{-1} using Perkin–Elmer 337 infrared spectrophotometer; ^1H NMR ($CDCL_3$) δ 8.15 (d, 2, ArH), 7.19 (d, 2, ArH), 4.31 (q, 2, CH_2), 1.39 (t, 3, CH_3) using a 90-MHz Varian EM-390; mass spectrum, *m/z* (rel intensity) 211 M^+ (3), 167 (9), 166 (7), 140 (13), 139 (100), 123 (8),

122 (15), 110 (5), 109 (66), 93 (29), 92 (7), 76 (14), 63 (29), 62 (7) using ZAB-HS-2F (VG Analytical, Wythenshawe, UK) with the following conditions: ionization (EI, 70 eV), resolution ($M/\Delta M$) = 10,000 (10% valley definition), accelerating voltage (8 kV), and sample introduction (solids probe).

Synthesis of O-ethyl and O-isobutyl carbonates of α-naphthol. Ethyl chloroformate (5 ml, 52.5 mmol) was added dropwise, with ice cooling, to a dry flask containing 5.76 g (40 mmol) α-naphthol dissolved in 100 ml benzene and 12 ml (92.5 mmol) tributylamine. The reaction, which was accompanied by a gradual change in color from a black to light tan solution, was allowed to proceed at room temperature for 60 min and was monitored by thin-layer chromatography (TLC) developed in toluene:ethanol (19:1). When judged complete by TLC, the reaction mixture was washed 3× with 5% HCl, 1× with water, and 1× with brine. The benzene was then filtered through celite and charcoal over a layer of anhydrous sodium sulfate which was washed 2× with hexane. The combined solvents were evaporated under high vacuum for 3 hr to give a red-brown nonviscous oil in 90.5% isolated yield, which gave one spot with several TLC systems.

O-Isobutyl carbonate of α-naphthol was synthesized in the same manner mentioned above except that isobutyl chloroformate was used instead of ethyl chloroformate. The product was a red-brown nonviscous oil in 56% isolated yield, which also gave one spot on TLC. The structural assignment for the two compounds was supported by ir, NMR, and mass spectrum, in above format.

Insect cultures. Nine stocks of *D. melanogaster* were used in this study. Four strains from Egypt were designated as +, QN (isogenic for the second chromosome), AL (Alexandria wild type), and AS (Assuit wild type). Standard laboratory stocks used were Hikone-R (HR) from the Mid-America Stock Center (Bowling Green, OH), as well as Canton-S (CS) and Oregon R-C (OR-C) from Cal Tech (Pasadena, CA). Two strains recently derived from the field were employed in this study. One was collected on campus at the University of California at Davis (PC2) and the other near Orange Cove, California (L).

All stocks were maintained in mass culture on fly medium for at least 10 generations before the start of the study. The medium consisted of cornmeal (6.2%), semolina (3.1%), sucrose (3.6%), dextrose (7.1%), agar (1.1%), and dead yeast (1.5%), with propionic acid (0.5%) added to suppress mold and bacteria. Flies were reared at room temperature (21–24°C) in half-pint bottles with 40 ml of medium and live yeast added to the food surface. Each bottle was initiated with 20 females and 20 males which were removed after 4 days.

Toxicity of malathion to D. melanogaster strains. All nine strains were employed to determine malathion toxicity. Flies were collected by discarding the first progeny to emerge in stock bottles and allowing the following emergents to accumulate for 2 to 3 days. These adults were anesthetized with ether and 50 individuals of each sex were transferred to an 8-dram shell vial containing 10 ml of laboratory medium with live yeast on the surface. After 1 to 2 days the flies were transferred to assay vials with an aspirator to avoid anesthetizing them immediately before exposure to malathion.

To evaluate toxicity the 3- to 5-day-old flies were exposed to a residue of malathion in 8-dram shell vials. Malathion was added to vials in 0.5 ml of acetone and the vials were rotated so that the inside was coated with solution. The acetone was evaporated under nitrogen before 100 flies were added with an aspirator to each vial. The vials were plugged with cotton and two ml of H_2O was added to the plug to keep it wet and prevent desiccation of the flies. At least 5 concentrations of malathion were used and the amount of insecticide applied was expressed in micrograms of malathion

per vial. For each strain there were four experiments at each concentration and three to four replicate vials per experiment. The control vials were coated with acetone only. Mortality was recorded 24 hr after the flies were aspirated into the treated and control vials. LC_{50} values, their 95% confidence intervals, and slopes were determined using Finney's probit analysis on an Apple IIE computer (15).

The effect of NTFP on malathion toxicity was determined. This carboxylesterase inhibitor was used on four of the nine strains tested for malathion toxicity (HR, OR-C, CS, AL). The toxicity of the inhibitor alone to the flies was assessed by adding different amounts of NTFP in 0.5 ml of acetone to vials. The number and handling of the flies and replicated assays were conducted as previously described for malathion toxicity. An amount of NTFP which caused no mortality (2.7 μg/vial) was combined in the assay vials with various concentrations of malathion in 0.5 ml of acetone (total volume). The experiment was conducted and data were analyzed as specified for malathion alone.

Preparation of homogenate and subcellular fractions. Whole-body homogenates were obtained from 1- to 5-day-old adults, which were immobilized with ether, counted, and then weighed. Usually several hundred etherized flies were transferred to an all-glass Ten Broeck homogenizer on ice. One hundred microliters of 0.1 *M* sodium phosphate buffer (pH 7.4) was added per 100 mg of flies, which were homogenized by passing the pestle through the cylinder 30 times. The homogenate was poured over a thin layer of glass wool (to trap debris) into a centrifuge tube which was spun for 5 min at 1000*g* (16). The resulting supernatant was used for enzyme assays, applied to IEF gels, or used to isolate subcellular fractions.

To obtain subcellular fractions, homogenate from HR females was employed. The 1000*g* supernatant was spun at 12,000*g* for 15 min, and the pellet was resuspended in buffer and then spun again at 12,000*g*. The pellet (mitochondrial fraction) was resuspended in the same volume as the initial 1000*g* supernatant. The first and wash supernatant from the 12,000*g* spin were combined and centrifuged at 100,000*g* for 1 h. The supernatant was removed, and the pellet was resuspended in the starting volume of buffer and then again centrifuged at 100,000*g* for 1 h. The pellet (microsomal fraction) was resuspended in the starting volume of buffer. The first and second 100,000*g* supernatants were combined to give the cytosolic fraction. The procedures used to obtain cell fractions were derived from standard methods (17, 18). Protein concentration was determined for all cell fractions. Carboxylesterase activities were determined for the fractions using malathion and *O*-ethyl carbonate of *p*-nitrophenol as substrates.

Protein assay. Protein was measured by a dye-binding method according to Bradford (19) as modified by Bio-Rad, using bovine serum albumin (fraction V, Sigma) as standard protein. To a cuvette, 100 μl of protein was added followed by 400 μl of 5× diluted Bio-Rad reagent (20% in distilled water), and the mixture was incubated for 10 min at room temperature. Absorbance at 620 nm was then measured using a Gilford EIA manual ELISA reader interfaced with an Atari 400 microcomputer (programmed by R. Wixtrom, this laboratory).

Enzyme assays. Carboxylesterase activities on malathion and *O*-ethyl carbonate of *p*-nitrophenol were determined spectrophotometrically using a 96-well plate reader (Titertek Multiscan, Flow Laboratories) interfaced to a microcomputer. Standard curves run with both the Titertek and a Varian-Cary 219 spectrophotometer indicated that the two instruments showed similar sensitivity while the Titertek showed greater linearity at high absorbance values and better reproducibility. Initial rates determined with both instruments were not significantly different, so experimental de-

tails are given below for the Titertek only. For application to other assay systems see Ashour (20).

Carboxylesterase activity on malathion was determined according to Talcott (21). In this method the hydrolysis of the substrate was coupled to the reduction of a tetrazolium dye (INT) by alcohol dehydrogenase and NADH diaphorase. The reduction of INT was calculated from the linear portion of the change in absorption at 492 nm using an extinction coefficient of 13.8 mM^{-1} cm^{-1}. The housing chamber for the 96-well plate reader was 31 ± 0.5°C. The absorbance of all of the 96 wells in a plate could be determined in approximately 1 min and the rate of malathion hydrolysis was monitored repeatedly for 20 min by taking readings at 90-sec intervals. A total volume of 300 μl was used in each well which contained 150 μl of enzyme solution and 150 μl of reagent mixture in 0.1 *M* Tris–HCl buffer (pH 7.5). The reaction was started by injecting 3 μl of malathion in acetone (3×10^{-4} *M* final concentration) into a well using a Hamilton repeating dispenser and stirring the well contents with the syringe needle. As controls, reagent and enzyme blanks were run with the reaction mixture minus enzyme solution and substrate, respectively. Data were collected using the PC-EIA program (Dorian Software), and then translated by an in-house program called TRANSFER into a format suitable for Lotus 1-2-3 (Lotus Development Incorporated). Lotus was used for data management and regression analysis.

The hydrolysis of the *O*-ethyl carbonate of *p*-nitrophenol was determined according to Ljungquist and Augustinsson (22). The liberation of *p*-nitrophenol was monitored repeatedly for 20 min at 405 nm. The sample well contained enzyme solution in 300 μl of 0.1 *M* Tris–HCl buffer (pH 7.5), and 3 μl of an acetone solution of the substrate was injected into the well yielding 1.5×10^{-4} *M* final concentration. Substrate and enzyme blanks were run, and the data were managed as described previously.

Isoelectric focusing (IEF). Supernatants from whole-body homogenates of flies were analyzed by wide-range IEF (pH 3.5–9.5) on an LKB multiphor apparatus using LKB Ampholine PAG plate ready-made polyacrylamide gels containing Ampholine carrier ampholytes (23). The gels were prefocused for 30 min at 30 W. Samples were applied as 10 μl of homogenate (50 μg protein) to Whatman No. 3 filter wicks (1.0 × 0.5 cm) and then focused at 30 W for 1 hr at 5°C. The filter wicks were removed after approximately half the focusing time. Ten micrograms of bovine serum albumin (fraction V, Sigma) was used as a standard. All samples were run at least three times and the gels were stained for either general protein or esterase activities. Malathion hydrolysis was analyzed by slicing gel lanes into 5-mm sections, which were incubated overnight in 0.6 ml of 0.1 *M* Tris–HCl buffer (pH 7.5) followed by enzyme assay as described previously. Preliminary experiments indicated that there was no loss of enzyme activity during overnight incubation. The pH gradient was determined by soaking gel slices (5 mm) for 24 hr in 0.5 ml of glass distilled water at 4°C, and measuring the pH of the solution using a Corning (model 125) pH meter. The meter was standardized with a buffer of known pH (Fisher Certified Buffer) before each reading.

Esterase and protein staining. Gels were stained for general protein using a standard method which employs Coomassie blue dye. The gels were first fixed in a solution containing 57 g of trichloroacetic acid (TCA) and 17 g sulfosalicylic acid in 500 ml distilled water. After 1 hr the gels were removed from the fixative and transferred to a staining solution (0.46 g Coomassie brilliant blue R in 500 ml ethanol, 160 ml acetic acid, to 2 liters with distilled water). Gels were stained with α- and β-naphthyl acetate and butyrate as well as *O*-ethyl and *O*-isobutyl carbonate of α-naphthol as substrates. The gels were preincubated in 0.04 *M* Tris–HCl buffer, pH 6.6, for 10 min at

22°C and then transferred to the staining solution (50 ml of 0.04 *M* Tris–HCl buffer, pH 6.6, 50 mg of fast blue dye, and 2 ml of 1% of the substrate in acetone) for 1 hr at 22°C, followed by storage in 10% acetic acid. Gels stained with *p*-nitrophenyl acetate as well as *O*-ethyl and *O*-isobutyl carbonate of *p*-nitrophenol were stained in the same manner except that fast blue was not included.

In vitro inhibition of α-naphthyl acetate and malathion carboxylesterase activities. Whole-body homogenates of females from the HR strain of *D. melanogaster* were analyzed by IEF. Gels were preincubated in 0.04 *M* Tris–HCl buffer, pH 6.6, for 10 min at 5°C. The gels were then incubated in a final concentration of 1×10^{-5} or 1×10^{-7} *M* NTFP in 50 ml of the above buffer for 30 min at 5°C. They were next stained with α-naphthyl acetate by adding 50 mg of fast blue and 2 ml of 1% α-naphthyl acetate dissolved in acetone to the incubation solution and were placed on a shaker for 60 min at 22°C (24). Controls were gels stained without the inhibitor.

The enzyme solution from the gel slice that gave the highest activity for malathion hydrolysis (Fig. 3, this study) was used to study the inhibitor potency of NTFP on malathion-metabolizing carboxylesterase(s). In each well, 150 μl of the enzyme solution and 3 μl of acetone solution of the inhibitor (1×10^{-7} *M* final concentration) were incubated for 10 min at 31°C (in the Titertek plate chamber), and then 150 μl of the reagent mixture was added. The reaction was started by injecting 3 μl of 3×10^{-2} *M* malathion in acetone into the well. Acetone control and enzyme blanks were done and malathion hydrolysis was performed using the Titertek as mentioned before.

TABLE 2
Toxicity of 3-Nonylthio-1,1,1-trifluoropropan-2-one to Drosophila melanogaster Strains

	Percentage mortality			
Dose[a]	HR	CS	OR-C	AL
2.7	0	0	0	0
4.0	0	3	0	2
5.4	0	4	0	4
8.1	0	14	10	7
10.8	0	17	18	10

[a] μg NTFP per vial. The inhibitor was added to vials in 0.5 ml acetone. The acetone was evaporated under nitrogen and then 100 flies were transferred to the vials. For each strain, there were at least four replicates per dose.

TABLE 1
Comparative Toxicity of Malthion and the Synergistic Effect of 3-Nonylthio-1,1,1-trifluoropropan-2-one (NTFP) among Strains of Drosophila melanogaster

	Malathion			Malathion + NTFP			
Strain	LC_{50}[b]	Slope	95% C.I. to LC_{50}	LC_{50}[b]	Slope	95% CI to LC_{50}	*SR*[a]
HR	3.00	7.8	2.87–3.12	2.00	9.3	1.93–2.06	1.50
OR-C	1.48	3.6	1.38–1.63	0.99	6.9	0.95–1.03	1.49
CS	1.05	4.8	0.99–1.13	0.66	6.1	0.63–0.69	1.59
AL	0.75	3.7	0.69–0.81	0.51	4.9	0.46–0.54	1.47
AS	1.70	4.5	1.53–1.78	—[c]	—	—	—
QN	1.50	2.5	1.38–1.68	—	—	—	—
+	0.77	3.3	0.70–0.83	—	—	—	—
PC2	2.30	5.4	2.16–2.43	—	—	—	—
L	1.60	2.8	1.39–1.79	—	—	—	—

[a] Synergistic ratio (*SR*) = LC_{50} of malathion alone/LC_{50} of malathion + NTFP.
[b] μg malathion/vial.
[c] The effect of NTFP on malathion toxicity was not evaluated in five of the strains.

TABLE 3
Carboxylesterase Activities in Subcellular Fractions of Drosophila melanogaster[a]

Fraction	Specific activity[b] (nmol/min/mg protein)		Total activity[b] (nmol/min)	
	Malathion	*O*-Ethylcarbonate of *p*-nitrophenol	Malathion	*O*-Ethylcarbonate of *p*-nitrophenol
1,000*g* supernatant	1.15 ± 0.25	4.68 ± 0.28	77.3 ± 16.5	313.5 ± 19.1
12,000*g* pellet	0.07 ± 0.01	0.63 ± 0.04	1.2 ± 0.1	11.9 ± 0.8
100,000*g* supernatant	0.81 ± 0.06	4.32 ± 0.15	19.0 ± 1.3	101.1 ± 3.5
100,000*g* pellet	0.32 ± 0.09	1.77 ± 0.05	1.9 ± 0.5	10.5 ± 0.3

[a] Cell fractions were obtained from females of the HR strain.

[b] The values are means ± SD for three determinations. Substrate concentrations were 3×10^{-4} *M* for malathion and 1×10^{-4} *M* for *O*-ethyl carbonate of *p*-nitrophenol. Protein concentrations were 0.5, 2.0, 0.29, and 0.64 mg/ml for the 1000*g* supernatant, 12,000*g* pellet, 100,000*g* supernatant, and 100,000*g* pellet, respectively.

RESULTS

Toxicity of malathion to D. melanogaster strains. Toxicity of malathion to field and laboratory strains of *D. melanogaster* and the synergistic effect of NTFP, a carboxylesterase inhibitor, are shown in Table 1. On the basis of LC_{50} values and their 95% confidence intervals, it is clear that there are differences in the susceptibility of the strains tested against malathion. The susceptibility of the strains is arranged descendingly as follows: AL, +, CS, OR-C, QN, L, AS, PC2, and HR.

Four strains that represented low to high susceptibility to malathion (HR, OR-C, CS, and AL) were chosen to study the *in vivo* effect of NTFP on malathion toxicity. The toxicity of different concentrations of NTFP on the four strains is shown in Table 2. The lack of mortality at 2.7 μg NTFP/vial enabled us to study the effect of this compound on malathion toxicity. A dose–response study was carried out to determine LC_{50} for malathion in the presence of the inhibitor (Table 1). This treatment resulted in lower LC_{50} values compared with the corresponding values for malathion alone. The toxicity of malathion to the four strains used was enhanced about 1.5 times as a result of NTFP treatment.

Subcellular distribution of carboxylesterase activity in D. melanogaster. Subcellular distributions of carboxylesterase activities on malathion and *O*-ethyl carbonate of *p*-nitrophenol in female flies of *D. melanogaster* (HR strain) are shown in Table 3. Enzyme activities were found in the 1000*g* and 100,000*g* supernatants and the 12,000*g* and 100,000*g* pellets. The highest specific activity was recovered in the 1000*g* supernatant which represents the whole-body homogenate except the nuclei and debris. The cytosolic fraction (100,000*g* supernatant) hydrolyzed the two substrates much faster than the microsomal fraction (100,000*g* pellet). The mitochondrial fraction (12,000*g* pellet) had the lowest activity. In all fractions, *O*-ethyl carbonate of *p*-nitrophenol was hydrolyzed more rapidly than malathion. Of the total malathion-metabolizing carboxylesterase activity recovered in the fractions, 77.7, 19.1, 2.0, or 1.3% of the activity was present in the 1000*g* or 100,000*g* supernatant or the 100,000*g* or 12,000*g* pellet, respectively. A similar distribution of the enzyme activity on the *p*-nitrophenyl carbonate ester was

found and the respective fractions had 71.7, 23.1, 2.4, or 2.7% of the total activity.

As expected from their higher chemical stability, the carbonates yielded a lower background than the corresponding acetate. Although the sensitivity of an esterase assay will vary, largely depending upon the k_{cat} for the substrate, the carbonates offer the possibility of higher assay sensitivity by reducing nonenzymatic hydrolysis.

Naphthyl and p-nitrophenyl ester staining. The esterase bands from adults (HR) after staining IEF gels using different naphthyl esters are shown in Fig. 2. Staining gels with the naphthyl esters (Compounds III–VIII, Fig. 1) resulted in two major bands of esterase activity which correspond to esterase-6 and esterase-C in *D. melanogaster*. Esterase-6 focuses at a higher p*I* and was identified by the observation that it was relatively more abundant in males than in females (Fig. 2, lanes a and b, respectively). These activities were substrate dependent and high activities for the two bands were detected on gels stained with α-naphthyl acetate, α-naphthyl butyrate, and *O*-ethyl carbonate of α-naphthol (Fig. 2, lanes b, d and F). Using β-naphthyl butyrate, esterase-6 and esterase-C were detected as very weak bands; β-naphthyl acetate stained better than β-naphthyl butyrate (Fig. 2, lanes c and e). Faint bands were detected on gels stained with *O*-isobutyl carbonate of α-naphthol (Fig. 2, lane g).

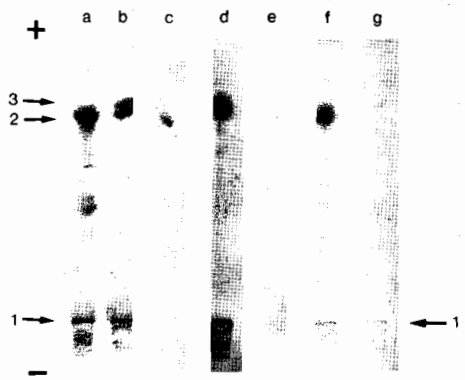

FIG. 2. *Gel lanes from wide-range IEF run with whole-body homogenate from the Hikone-R (HR) strain and stained with different substrates for carboxylesterase activity. Lanes a and b were loaded with the same amount of protein from homogenized males and females (respectively) then stained using α-naphthyl acetate. Female homogenate was run and stained with the following substrates: β-naphthyl acetate (lane c), α-naphthyl butyrate (lane d), β-naphthyl butyrate (lane e), O-ethyl carbonate of α-naphthol (lane f), and O-isobutyl carbonate of α-naphthol (lane g). Arrow 1 shows where the sample wicks were placed on the gel, arrow 2 points to esterase-6, and arrow 3 points to esterase-C.*

Under the conditions used in the present study, superficial but transient bands of esterase activity were noticed near the esterase-6, esterase-C, and cathodal regions on gels stained with acetate and carbonates of *p*-nitrophenol. However, it was difficult to determine the exact location of esterase activity as the yellow color rapidly diffused out of the gel.

Malathion carboxylesterase activity. The isoelectric points for malathion hydrolytic activity was determined by sectioning the IEF gels and measuring the activity spectrophotometrically using a 96-well plate reader (Titertek). The use of this procedure enabled us to monitor the rate of malathion hydrolysis in large numbers of gel slices in a short time. Reaction rates with malathion obtained by Titertek were confirmed by similar rates obtained with a Varian-Cary 219 spectrophotometer. Several regions of malathion hydrolytic activity with one major peak were obtained (Fig. 3) from gel slices. Interestingly, this major peak, which has p*I* value of approximately 6.3, did not stain with any of the naphthyl substrates used. There were two minor peaks (p *I* 4.4 and 4.0) showing almost the same activity and corresponding to esterase-6 and esterase-C as detected on the gels stained with the naphthyl substrates. The region of the major peak of malathion hydrolytic activity matched one of three abundant proteins in the body of HR, CS, and AL strains of *D. melanogaster* as judged by Coomassie blue stains (Fig. 4). Female ho-

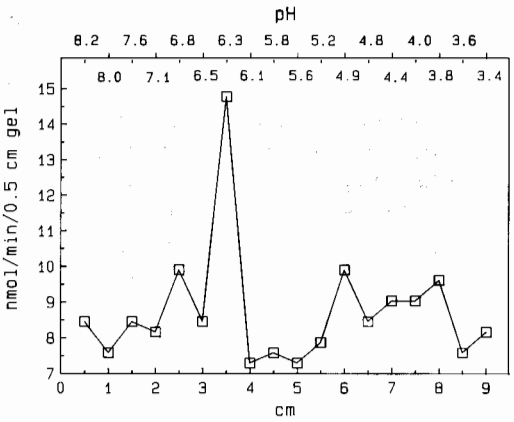

FIG. 3. *Profile of total malathion carboxylesterase activity (nmol/min/0.5 cm) from slices of wide-range IEF gels. The abscissa presents the distance (cm) along the gel from anode to cathode and the pH is shown for each gel slice.*

mogenates from the HR, CS, and AL strains, which differ in their susceptibility to malathion toxicity (Table 1), were run on IEF gels. The regions that correspond to the malathion major peak, and naphthyl bands, were sectioned and assayed for malathion hydrolytic activity. There was a suggestion of differences in enzyme activity among the three strains (Fig. 5).

In vitro inhibition of α-naphthyl acetate and malathion carboxylesterase activities. The inhibition effect of NTFP on α-naphthyl acetate and malathion carboxylesterases was investigated, and Fig. 6 shows the inhibition potency of NTFP on IEF gels run with whole-body homogenate of HR strain females. Gel lanes were preincubated with 1×10^{-7} and 1×10^{-5} *M* NTFP and stained for α-naphthyl acetate esterase (α-NAE) activity (Fig. 6). Compared to control lanes, NTFP at the two concentrations used reduced the intensity of α-NAE bands. Esterase-6 was more sensitive to NTFP than esterase-C. Some minor bands appeared in the α-NAE region of activity as a result of the reduction of the enzyme activity. The inhibitory effect of NTFP on malathion-metabolizing carboxylesterase was tested by incubating an enzyme solution from the major peak with 1×10^{-7} *M* NTFP and measuring the enzyme activity as described previously. This treatment resulted in 67.2% inhibition of

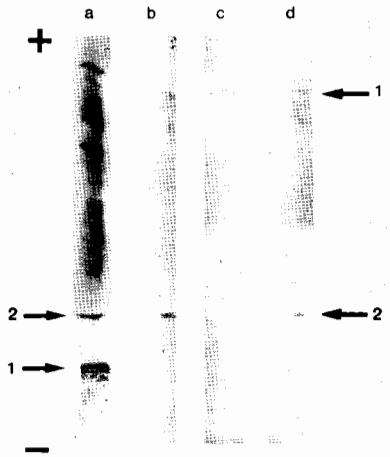

FIG. 4. *Whole-body homogenates of females from three strains of* Drosophila melanogaster *run on wide-range IEF gel lanes stained for general protein using Coomassie blue. Lane a was loaded with 55 μg of protein and stained overnight with Coomassie blue. Lanes b, c, and d were loaded with 70 μg of protein from the HR, CS, and AL strains (respectively). These three lanes were stained for less than 1 hr using Coomassie blue. Arrow 1 shows where the sample wicks were placed on the gel and arrow 2 points to a prominent protein band that coincides with the position of the peak of malathion hydrolysis on the gels.*

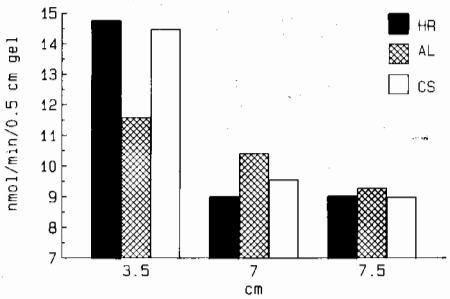

FIG. 5. *Total malathion carboxylesterase activity (nmol/min/0.5 cm) in the HR, CS, and AL strains from three slices of a wide-range IEF gel corresponding to the peak of malathion hydrolysis (3.5 cm, pI 6.3), esterase-6 (7 cm, pI 4.4), and esterase-C (7.5 cm, pI 4.0). The abscissa presents the distance (cm) along the gel.*

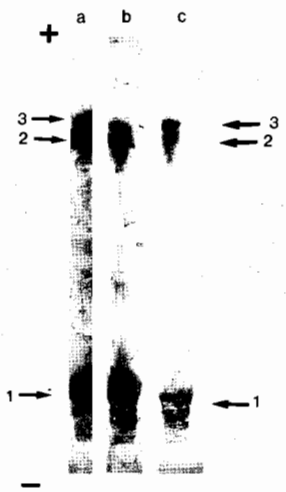

FIG. 6. *Wide-range IEF gel lanes demonstrating inhibition of carboxylesterase activity on α-naphthyl acetate by NTFP. All lanes were loaded with the same amount of protein in whole-body homogenate from HR females. Lane a was stained using α-naphthyl acetate alone, lanes b and c were stained using α-naphthyl acetate with* 1×10^{-7} *and* 1×10^{-5} *M NTFP, respectively. Arrow 1 shows where the sample wicks were placed in the gel, arrow 2 points to esterase-6, and arrow 3 points to esterase-C.*

the activity of malathion-hydrolyzing enzyme(s).

DISCUSSION

Carboxylesterases are highly efficient catalysts for the hydrolysis of a wide range of esters. Apart from the metabolism of xenobiotics, there is lack of information available regarding the role of these enzymes as well as the toxicological significance of the numerous isozymes present. *D. melanogaster* has been well studied genetically and possesses a useful transformation system. Thus, this insect is especially useful as a model for evaluating the biological and toxicological role of esterases.

The present study indicates that there is variation for susceptibility to malathion toxicity among lines of *D. melanogaster*. Since all the lines used have been reared and handled in the same manner, the among-line differences probably have a genetic basis. The Hikone-R (HR) strain is the least susceptible strain to malathion. This strain has not been selected for insecticide resistance for many generations; however, its resistance to DDT, parathion, nicotine sulfate, and other insecticides has been reported (25). The strains recently derived from the field (PC2, L) appear, on average, to be less susceptible than the rest of the strains, which have been kept much longer on laboratory medium.

Simultaneous treatment with a nontoxic dose of NTFP increases malathion toxicity to all the strains tested (HR, CS, OR-C, and AL). The high inhibitory potency *in vitro* of NTFP on α-NAE bands (IEF gels) as well as on malathion hydrolytic activity in enzyme solution from the major peak of activity supports the argument that NTFP synergizes malathion by *in vivo* inhibition of carboxylesterase(s). In contrast, Holwerda and Morton (26) found that the carboxylesterase inhibitors *S,S,S*-tributylphosphorotrithioate and triphenylphosphate decreased the toxicity of malathion to susceptible and resistant strains of *D. melanogaster*. Ashour and Hammock (14) have reported that NTFP inhibited mouse liver microsomal carboxylesterase activity on malathion *in vitro* and synergized its toxicity to mice. They added that, besides the *in vivo* inhibition of liver carboxylesterase, NTFP may potentiate malathion toxicity as they found that high doses of NTFP were toxic to mice. Various investigators have employed selective inhibitors to synergize malathion toxicity (27–29). Brodbeck *et al.* (30) and Gelb *et al.* (31) have demonstrated that other trifluoroketones inhibited acetylcholinesterase activity in mammals. In the present study the differences in the slope values between malathion alone and malathion with inhibitor may be due to an unknown mechanism for the malathion and NTFP mixture or to heterogeneity among or within the strains.

Staining for *D. melanogaster* carboxylesterase activities on IEF gels with different naphthyl substrates, including car-

bonates, results in two major bands of activity with approximate p*I* values of 4.4 and 4.0, which correspond to esterase-6 and esterase-C in *D. melanogaster*. The intensity of the two bands is substrate dependent. *p*-Nitrophenyl carbonates and acetate yielded a transient stain in the same gel region. Carboxylesterase activity (in IEF gel slices) on malathion indicates several regions of activity with one major peak which has a p*I* of 6.3 and does not respond to staining with any of the substrates used to detect "general esterases." However, it matches one of the most abundant proteins in the homogenates of all three strains studied. The p*I* for the peak of malathion activity is close to that of malathion esterase in mammals (32). Two minor peaks are found to correspond to esterase-6 and esterase-C. Our results suggest that malathion is hydrolyzed mainly by an enzyme which differs from that hydrolyzing naphthyl substrates. Consequently, caution should be used in generalizing from the results obtained using "general esterase substrates" to characterize malathion carboxylesterase activities in different species or in resistant and susceptible strains. Holwerda and Morton (26) separated malathion carboxylesterase activity from α-naphthylacetate esterase activity in *D. melanogaster*. Hemingway (3) has demonstrated that there was no difference between semipurified α- or β-naphthyl acetate esterases of the resistant and susceptible strains of *Anopheles arabiensis* in either the adults or larvae. However, with a malathion-specific assay a second peak of activity was observed in the resistant-strain adults which was not present in either the larvae of this strain or the larvae and adults of the susceptible strain. Dowd and Sparks (16) have pointed out that the hydrolysis of *trans*-permethrin in the midgut of *Pseudoplusia includens* (Walker) was distinct from the majority of "general esterase" activity when enzyme activity was separated by electrophoretic techniques. In another investigation, inhibition data using rodent and primate microsomes gave an indication that acetates were hydrolyzed by a somewhat different family of carboxylesterase than was malathion (14). Our work in the subcellular distribution of carboxylesterases from *D. melanogaster* indicates that the cytosol has higher activities than the microsomes on malathion and *O*-ethyl carbonate of *p*-nitrophenol. However, only 29 and 39% of the total activity on malathion and *O*-ethyl carbonate of *p*-nitrophenol, respectively, were recovered in the fractions derived from the 1000*g* fraction. In all fractions the enzyme(s) hydrolyzes the ethyl carbonate ester much faster than malathion. Imamura and Hasegawa (17) have demonstrated that the highest total activity of malathion carboxylesterase is encountered in the cytosolic fraction of rat lung while in liver the microsomal fraction gave a much higher activity of the enzymes.

Model substrates such as α-naphthyl acetate or *p*-nitrophenyl acetate are commonly used to indicate esterase activity on substrates of toxicological or pharmacological significance with the idea of employing a wide variety of different substrates as one goes from α- to β-naphthol, or from acetate to butyrate. Comparing the structures of malathion, Fig. 1, compound **II** (an ester with a small alcohol and a large acid), with α-naphthyl acetate, Fig. 1, compound **III** (an ester with a small acid and a large alcohol), one hardly would expect the same enzyme(s) to metabolize both compounds. This is confirmed by our finding that the major enzyme hydrolyzing malathion in *D. melanogaster* is not active on a variety of esterases of α- and β-naphthols. Hopkinson *et al.* (33) detected a new human esterase, which cannot be detected by conventional naphthyl esterase azo dye-coupling techniques, when 4-methylumbelliferone was used for staining after electrophoresis. The carboxylic acid esters of α-naphthol and *p*-nitrophenol are in wide use, but all are esters of a large alcohol. However, they are in wide use because

common endpoints can be used with the different esters. Most other model substrates require the use of alternative techniques for detection. The carbonates are useful in that the carbonic acid derivative formed following acid hydrolysis is unstable. It spontaneously decomposes releasing carbon dioxide (which is readily detected) as well as the chromophores (α-naphthol, *p*-nitrophenol, methylumbelliferone) commonly detected in esterase work. Carbonates of the above three phenols can be used to stain gels, provide a simple continuous colorimetric assay, or provide a highly sensitive fluorescence assay based on the same technology in common use with the corresponding acetates. It was disappointing that the ethyl carbonates of α-naphthol failed to detect the malathion esterase with a p*I* of 6.3; however, these substrates have been shown to detect esterases not seen with general stains in other species. They have great promise as simple model substrates for esterase detection.

The presence of esterase variation in *D. melanogaster* has been documented (34, 35). Esterase-6 allozymes have a p*I* of 4.3 (36) which agrees with our value for the p*I* of this enzyme. Danford and Beardmore (37) tested esterase-6 genotypes with chlorfenvinphos, dichlorvos, malathion, and eserine. Only with dichlorvos was there a difference among genotypes. We found that the esterase-6 region from IEF gels hydrolyzed malathion at a relatively low rate. Ogita (38) determined that insecticide resistance in the HR strain was not correlated with ali-esterase level. Similarly, we did not find the HR strain to have relatively high levels of malathion carboxylesterase activity in IEF gel slices that corresponded to the location of esterase-6 or esterase-C. In addition, at p*I* 6.3 the malathion carboxylesterase activity was not substantially higher in the HR strain than in the AL strain.

It may be possible to purify the major enzyme(s) responsible for malathion degradation using the trifluoroketone in an affinity column following the procedure developed for the purification of juvenile hormone esterase (39). Once an enzyme is purified, peptide fragments can be used to generate sequences for oligonucleotide probes to clone a malathion carboxylesterase gene. The goal using *D. melanogaster* in this research is to isolate and transfer insecticide-metabolizing genes to beneficial insects. A highly resistant gene could be transferred or multiple copies of a relatively inefficient gene could be integrated into the target genome. This latter approach has not been previously considered, but it would appear feasible since organophosphate resistance has arisen in the field by esterase gene duplication (40, 41). The purpose of this work would be to increase the survival of pest predators and parasites in sprayed areas to augment integrated pest management programs.

The use of the 96-well plate format, perhaps with a robotic work station, is promising for the very rapid analysis of multiple enzyme activities using a battery of substrates. Because of its simplicity, the format may be useful for monitoring enzyme activities in field population preparations. The small volume of the enzyme solution required and the speed of the assays make it possible to sample a relatively large number of individuals. Consequently, a population can be surveyed for high levels of activity of different classes of detoxifying enzymes. This approach could be useful in both laboratory and field studies for detecting uncommon resistance genes and deciding what alternate pesticides to use based on the "metabolic profile" of the population. Thus, the Titertek procedure could aid in managing insecticide resistance in the field.

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REFERENCES

1. F. Matsumura and A. W. A. Brown, Biochemistry of malathion resistance in *Culex tarsalis*, *J. Econ. Entomol.* **54**, 1176 (1961).
2. F. Matsumura and A. W. A. Brown, Studies on carboxylesterase in malathion-resistant *Culex tarsalis* mosquitoes, *J. Econ. Entomol.* **56**, 381 (1963).
3. J. Hemingway, Malathion carboxylesterase enzymes in *Anopheles arabiensis* from Sudan, *Pestic. Biochem. Physiol.* **23**, 309 (1985).
4. M. G. Townsend and J. R. Busvine, The mechanism of malathion resistance in the blowfly. *Chrysoma putoria*, *Entomol. Exp. Appl.* **12**, 243 (1969).
5. K. Osaki and T. Kasai, Biochemical genetics of malathion resistance in the smaller brown plant hopper *Laodelphax striatellis*, *Entomol. Exp. Appl.* **13**, 162 (1970).
6. E. J. Wood, M. I. P. de Villar, and E. N. Zerba, Role of a microsomal carboxylesterase in reducing the action of malathion in eggs of *Triattoma infestans*, *Pestic. Biochem. Physiol.* **23**, 24 (1985).
7. L. R. Kao, N. Motoyama, and W. C. Dauterman, Studies on hydrolases in various house fly strains and their role in malathion resistance, *Pestic. Biochem. Physiol.* **22**, 86 (1984).
8. L. R. Kao, N. Motoyama, and W. C. Dauterman, The purification and characterization of esterases from insecticide-resistant and susceptible house flies. *Pestic. Biochem. Physiol.* **23**, 228 (1985).
9. W. Junge and K. Krisch, The carboxylesterases/amidases of mammalian liver and their possible significance, *in* "Critical Reviews in Toxicology" (L. Goldberg, Ed.), Vol. 3, p. 371, CRC Press, Cleveland, 1975.
10. E. Heymann, Carboxylesterases and amidases, *in* "The Enzymatic Basis of Detoxication" (W. B. Jakoby, Ed.), Vol. 2, p. 291, Academic Press, New York, 1980.
11. E. Heymann, Hydrolysis of carboxylic esters and amides, *in* "Metabolic Basis of Detoxication" (W. B. Jakoby, J. R. Bend, and J. Caldwell, Eds.), p. 229, Academic Press, New York, 1982.
12. K. I. Ogita and T. Kasai, Genetico-biochemical analysis of specific esterases in *Musca domestica*, *Japan. J. Genet.* **40**, 173 (1965).
13. J. Peters, Nonspecific esterases of *Mus musculus*, *Biochem. Genet.* **20**, 585 (1982).
14. M.-B. A. Ashour and B. D. Hammock, Substituted trifluoroketones as potent, selective inhibitors of mammalian carboxylesterases, *Biochem. Pharmacol.* **36**, 1869 (1987).
15. H. R. Lieberman, Estimating LD_{50} using the probit technique: A basic computer program, *Drug Chem. Toxicol.* **6**, 111 (1983).
16. P. F. Dowd and T. C. Sparks, Characterization of a *trans*-permethrin hydrolyzing enzyme from the midgut of *Pseudoplasia includens* (Walker), *Pestic. Biochem. Physiol.* **25**, 73 (1986).
17. T. Imamura and L. Hasegawa, Subcellular distribution of malathion, phenthoate, and diethyl succinate carboxylesterases in rat lung, *Pestic. Biochem. Physiol.* **22**, 321 (1984).
18. M. Ugaki, T. Shono, and J.-I. Fukami, Metabolism of fenitrothion by organophosphorus-resistant and -susceptible house flies, *Musca domestica* L., *Pestic. Biochem. Physiol.* **23**, 33 (1985).
19. M. M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.* **72**, 248 (1976).
20. M.-B. A. Ashour, S. J. Gee, and B. D. Hammock, Use of a 96-well microplate reader for measuring routine enzyme activities, *Anal. Biochem.*, in press.
21. R. E. Talcott, Hepatic and extrahepatic malathion carboxylesterases. Assay and localization in the rat, *Toxicol. Appl. Pharmacol.* **47**, 145 (1979).
22. A. Ljungquist and K.-B. Augustinsson, Purification and properties of two carboxylesterases from rat-liver microsomes, *Eur. J. Biochem.* **23**, 303 (1971).
23. A. Winter, K. Elk, and U.-B. Andersson, "Analytical Electrofocusing in Thin Layers of Polyacrylamide Gels," Application note 250, LKB-Produkter AB, Bromma, Sweden, 1977.
24. P. M. Coates, M. A. Mestriner, and D. A. Hopkinson, A preliminary genetic interpretation of the esterase isozymes of human tissues, *Ann. Hum. Genet.* **39**, 1 (1975).
25. Z. Ogita, The genetical relation between resistance to insecticides in general and that to phenylthiourea (PTU) and phenylurea (PU) in *Drosophila melanogaster*, *Bochu-Kagaku* **23**, 188 (1958).
26. B. C. Holwerda and R. H. Morton, The *in vitro* degradation of [^{14}C]malathion by enzymatic extracts from resistant and susceptible strains of *Drosophila melanogaster*, *Pestic. Biochem. Physiol.* **20**, 151 (1983).
27. S. D. Murphy, R. L. Anderson, and K. P. DeBois, Potentiation of toxicity of malathion by triorthotolyl phosphate, *Proc. Soc. Exp. Biol. Med.* **100**, 483 (1959).
28. J. E. Casida, R. L. Baron, M. Eto, and J. L. Engel, Potentiation and neurotoxicity induced by certain organophosphates, *Biochem. Pharmacol.* **12**, 73 (1963).
29. W. Welling and J. W. deVries, Synergism of or-

ganophosphorus insecticides by diethyl maleate and related compounds in house flies, *Pestic. Biochem. Physiol.* 23, 358 (1985).
30. V. Brodbeck, K. Schweikert, R. Gentinetta, and M. Rottenberg, Fluorinated aldehydes and ketones acting as quasi-substrate inhibitors of acetylcholesterase, *Biochim. Biophys. Acta* 567, 357 (1979).
31. M. H. Gelb, J. P. Svaren, and R. H. Abeles, Fluoroketone inhibitors of hydrolytic enzymes, *Biochemistry* 24, 1813 (1985).
32. L. R. Kao, N. Motoyama, and W. C. Dauterman, Multiple forms of esterases in mouse, rat, and rabbit liver and their role in hydrolysis of organophosphorus and pyrethroid insecticides, *Pestic. Biochem. Physiol.* 23, 66 (1985).
33. D. A. Hopkinson, M. A. Mestriner, J. Cortner, and H. Harris, Esterase-D: A new human polymorphism, *Ann. Hum. Genet.* 37, 119 (1973).
34. T. R. F. Wright, The genetics of an esterase in *Drosophila melanogaster*, *Genetics* 48, 787 (1963).
35. L. Beckman and F. M. Johnson, Esterase variations in *Drosophila melanogaster*, *Hereditas* 51, 212 (1964).
36. S. D. Mane, C. S. Tepper, and R. C. Richmond, Studies of esterase-6 in *Drosophila melanogaster*. XIII. Purification and characterization of the two major isozymes, *Biochem. Genet.* 21, 1019 (1983).
37. N. D. Danford and J. A. Beardmore, Effects of insecticides *in vitro* and *in vivo* on esterase-6 in *Drosophila melanogaster*, *Comp. Biochem. Physiol. C.* 61, 47 (1978).
38. Z. Ogita, Genetical relationship between ali-esterase activity and insecticide-resistance in *Drosophila melanogaster*, *Bochu-Kagaku* 26, 93 (1961).
39. Y. A. I. Abdel-Aal and B. D. Hammock, Transition state analogs as ligands for affinity purification of juvenile hormone esterase, *Science* 223, 1073 (1986).
40. A. L. Devonshire and G. D. Moores, A carboxylesterase with broad specificity causes organophosphate, carbamate, and pyrethroid resistance in peach–potato aphids (*Myzus persicae*), *Pestic. Biochem. Physiol.* 18, 235 (1982).
41. C. Mouchès, N. Pasteur, J. B. Bergé, O. Hyrien, M. Raymond, B. R. S. Vincent, M. Silvestri, and G. P. Georghiou, Amplification of an esterase gene is responsible for insecticide resistance in a California *Culex* mosquito, *Science* 233, 778 (1986).