In Vitro Metabolism of Propetamphos by Housefly, Cockroach, and Mouse Liver Preparations

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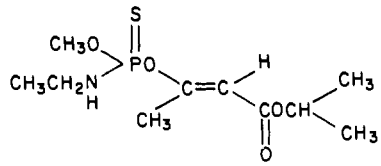
In vitro metabolism of propetamphos [(*E*)-*O*-2-(isopropoxycarbonyl)-1-methylvinyl *O*-methyl ethylphosphoramidothioate] in insecticide-resistant and -susceptible housefly preparations, and cockroach and mouse liver preparations, was investigated. Mouse liver preparations degraded more of the insecticide than the insect preparations, and the major metabolite was desisopropylpropetamphos. This was formed in all mouse liver subcellular fractions, particularly in the microsomal fraction with no cofactors added. The major metabolic route for propetamphos in the housefly is via glutathione conjugation and is directly proportional to the level of propetamphos resistance. Spectral analysis indicated that two conjugates were formed: *S*-3-(isopropylcrotonyl)glutathione and (*S*)-3-[[[[(ethylamino)methoxy]phosphino]-thioyl]oxy]butanoic acid, 1-methylethyl ester, glutathione. Phosphorotriesterase and mixed-function oxidase-catalyzed hydrolysis of propetamphos occurs in all the homogenates tested. Hydrolysis products detected were *O*-methyl ethylphosphoramidothioic acid (mouse, housefly, cockroach), isopropyl acetoacetate (mouse), and acetoacetic acid (mouse, cockroach). Oxidative desulfuration also occurred in mouse and housefly homogenates.

INTRODUCTION

Propetamphos [(*E*)-*O*-2-(isopropoxycarbonyl)-1-methylvinyl *O*-methyl ethylphosphoramidothioate] is a relatively new insecticide–acaricide developed by Sandoz, Inc. (Leber, 1972). Its metabolic fate in the rat and the



housefly, in vivo, has been studied (Sandoz, Inc., personal communication; Wells et al., 1985). In the rat, the breakdown of [^{14}C]propetamphos (labeled in the vinyl and carbonyl carbons) to $^{14}CO_2$ was the primary pathway at low doses. The other metabolites of consequence were glucuronides, presumably of the desisopropyl, desmethyl, and/or desisopropyl desmethyl analogues of propetamphos. Degradation in the housefly follows a similar pattern. CO_2 is the primary metabolite at low doses, while conjugation becomes important at higher doses. Conjugation in the housefly is probably via the glutathione *S*-transferases.

The following study was conducted to compare the in vitro metabolism of propetamphos using susceptible and multiresistant strtains of houseflies, as well as mouse liver and cockroach preparations.

MATERIALS AND METHODS

Chemicals. [^{14}C]Propetamphos [(*E*)-*O*-2-(isopropoxy-(^{14}C)carbonyl)-1-methyl(1-^{14}C)vinyl *O*-methyl ethylphosphoramidothioate] (sp act. = 39.6 μCi/mg), unlabeled propetamphos (*E* and *Z* isomers), and propetamphos oxon (the phosphoramidate analogue of propetamphos) were supplied by Sandoz, Inc., East Hanover, NJ. The desmethyl and *N*-desethyl analogues were synthesized as previously described (Wells et al., 1985). *O*-Methyl ethylphosphoramidothioic acid was obtained by adding 5 N NaOH to propetamphos and heating gently for 30 min. After dichloromethane extraction of propetamphos, the phosphoramidothioic acid was extracted from the aqueous layer on a Waters Sep-Pak. The structure was confirmed by NMR using D_2O as the solvent.

($^{14}CH_3O$)propetamphos [(*E*)-*O*-2-(isopropoxycarbonyl)-1-methylvinyl *O*-(^{14}C)methyl ethylphosphoramidothioate] was synthesized by first reacting thiophosphoryl chloride with isopropyl acetoacetate (1:1 molar ratio) while adding tributylamine dropwise (1 mol/mol of reactants) at 0 °C with stirring. After 2 h, the reaction mixture was applied to a silica column and eluted with toluene. The dichlorophosphorothioate intermediate (eluting in the first 300 mL of toluene) was next reacted with $^{14}CH_3OH$ (2 mmol of each reactant, 694.6 μCi) at 0 °C in 2 mL of acetone. After 2 h, 4 mmol of ethylamine (70% in H_2O) was added dropwise and allowed to react for 2 h at 0 °C. ($^{14}CH_3O$)Propetamphos was purified from the reaction mixture on TLC using the mobile phases hexane–ethanol (95:5 v/v) and hexane–ethyl acetate–methanol (94:4:2, v/v/v) (developed twice). The final product was 100% radiochemically pure and resulted in a single peak at 254 nm when analyzed on HPLC (Beckman Ultrasphere-ODS, 70% methanol–30% water). A specific activity of 0.86 μCi/mg was obtained. NMR spectroscopy was used to confirm the structure.

The methyl ester analogue of *cis*- and *trans*-propetamphos [(*E*)- and (*Z*)-*O*-2-(methoxycarbonyl)-1-methylvinyl *O*-methyl ethylphosphoramidothioate] were synthesized as described for ($^{14}CH_3O$)propetamphos above, with the following exceptions: (1) unlabeled methanol was used; (2) methyl acetoacetate was used instead of isopropyl acetoacetate for the cis isomer analogue; (3) the Na^+ salt of methyl acetoacetate was used instead of isopropyl acetoacetate for the trans isomer analogue.

All other chemicals and reagents were analytical grade, and all solvents were pesticide or HPLC grade.

NMR Spectroscopy. All NMR spectra were measured on a Varian EM 390 spectrometer (90 MHz) and/or a Bruker WM 250 spectrometer (250.13 MHz).

GC–MS. Samples were analyzed on a Hewlett-Packard 5984B MS. The hydrogenolysis reaction was injected onto a 30-m DB5 column (35 °C for 5 min–200 °C at 10 °C/min), scanned 40–300 amu). Other samples were injected on to a 15-m fused silica capillary column (50 °C for 1

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Table I. R_f Values of Propetamphos and Possible Metabolites

compd	solvent systems[a]					
	A	B	C	D	E	F
(*E*)-*O*-2-(isopropoxycarbonyl)-1-methylvinyl *O*-methyl ethylphosphoramidothioate (propetamphos)	0.33	0.88	0.90	0.92	0.92	0.89
(*E*)-*O*-2-(isopropoxycarbonyl)-1-methylvinyl *O*-methyl ethylphosphoramidate (propetamphos oxon)	0.18	0.40	0.86	0.90	0.90	0.67
(*E*)-*O*-2-(isopropoxycarbonyl)-1-methylvinyl ethylphosphoramidothioate (desmethylpropetamphos)	0.00	0.00	0.44	0.85	0.88	0.10
(*E*)-*O*-2-(isopropoxycarbonyl)-1-methylvinyl *O*-methyl phosphoramidothioate (*N*-desethylpropetamphos)	0.11	0.84	0.90	0.92	0.90	0.87
(*E*)-*O*-2-carboxy-1-methylvinyl *O*-methyl ethylphosphoramidothioate (desisopropylpropetamphos)	0.0–0.15	0.00	0.85	0.89	0.88	0.62
O-methyl ethylphosphoramidothioic acid	0.00	0.00	0.0–0.07	0.21–0.32	0.24	0.00–0.04
isopropyl acetoacetate	0.35	0.85	0.90	0.90	0.91	0.90
acetoacetic acid	0.00	0.00	0.00	0.14	0.16	0.40
unknown conjugate	0.00	0.00	0.00	0.55	0.55	0.00

[a] A, hexane–water (95:5, v/v); B, benzene–ethyl acetate–hexane (1:4:2, v/v/v); C, acetonitrile–water (95:5, v/v); D, acetonitrile–water (75:25, v/v); E, butanol–acetic acid–water (11:4:5, v/v/v); F, benzene–acetonitrile–acetic acid (49:50:1, v/v/v).

min–250 °C at 10 C/min, scanned 40–400 amu). In all cases the injector temperature was 250 °C, the source temperature was 200 °C, and the flow rate was 1 mL/min.

Animals. Three- to six-day adult female houseflies (*Musca domestica*) were used. The strains of houseflies, described previously (Hayaoka et al., 1982), are as follows: (1) CSMA, a strain susceptible to most insecticides; (2) Rutgers, a multiresistant strain characterized by high mixed-function oxidase and glutathione *S*-transferase activity; (3) Cornell-R, an organophosphorus-resistant strain possessing very high levels of glutathione *S*-transferase activity and altered cholinesterase; (4) Hirokawa, an organophosphorus-resistant strain reported to have altered cholinesterase and increased glutathione *S*-transferase and carboxylesterase levels. The flies were reared on a diet of milk and sugar at 27 °C and 80% RH.

Two species of adult female cockroaches were used; the German (*Blatella germanica*) and American (*Periplaneta americana*) cockroach. They were reared on a diet of dog chow at 27 °C and 80% RH.

The mice used for these studies were 30-g (±2 g) male Dublin ICR mice (Dominion Labs, Dublin, VA). Male Dublin Sprague–Dawley rats, 175 g (±10 g), were also obtained from Dominion Labs.

Toxicity Testing. Various concentrations of propetamphos, in 0.5 μL of acetone, were applied topically to the dorsal thorax of individual houseflies and cockroaches from each strain or species using a 25-μL Hamilton syringe in a repeating dispenser (±1% error). Mice were treated by gastric intubation of 0.2 mL of corn oil containing the various concentrations of propetamphos. All mice were starved for 18 h prior to intubation. Food was returned 6 h after dosing. Some mice were pretreated with tri-*o*-cresyl phosphate (TOCP) (0.5 g/kg) in corn oil (0.2 mL total) 4 h before administering propetamphos. Mortality of houseflies, cockroaches, and mice was determined 24 h after propetamphos treatment, and the LD_{50} was then calculated (Litchfield and Wilcoxon, 1949).

In Vitro Metabolism. Abdomens of houseflies and cockroaches were homogenized in 0.1 M potassium phosphate buffer, pH 7.4, containing 1% BSA. Mouse liver was homogenized in the same buffer without the addition of BSA. All preparations were approximately 10% (w/v). After filtration through cheesecloth, the subcellular fractions, 10800*g* supernatant, 105000*g* supernatant, and microsomes, were obtained by differential centrifugation as previously described (Motoyama and Dauterman, 1972). The microsomes were then resuspended in the same buffer at a volume equal to that of the 105000*g* supernatant. All fractions were dialyzed overnight against 100 volumes of the same buffer, changed twice. Dialysis did not ressult in any qualitative differences in metabolite formation after cofactors were added. At all times during preparation, the homogenates were kept on ice.

Dialyzed subcellular fractions (0.5 mL) were incubated with propetamphos in the presence of either no cofactor, 4 mM GSH, or 1.1 mM NADPH (added as 0.0, 8.0, or 2.2 mM, resp., in 0.5 mL of the same buffer). For the insect preparations, 2.5 μM (*crotonyl*-^{14}C) propetamphos was allowed to incubate at 25 °C for 2 h. For the mouse preparations, 25 μM (*crotonyl*-^{14}C)propetamphos was allowed to incubate at 37 °C for 1 h. The mouse incubations were repeated using ($^{14}CH_3O$)propetamphos. When ($^{14}CH_3O$)propetamphos was used as a substrate for insect reactions, 5 μM ($^{14}CH_3O$)propetamphos was added to 4.5 mL of the enzyme preparations +0.5 mL of buffer containing cofactors (no cofactor, 4 mM GSH, or 1.1 mM NADPH final concentrations). All reactions, except the insect-($^{14}CH_3O$)propetamphos incubations, were terminated by the addition of 50 μL of ethanol to 50-μL aliquots of the incubation mixture and applying these to Brinkman Polygram Sil-NHR TLC plates. That portion of each incubation mixture that was not used for TLC analysis was immediately passed through a reversed-phase Sep-Pak (Waters Associates, Milford, MA). The radioactive metabolites were washed from the cartridge with methanol, which was then concentrated under nitrogen and subjected to HPLC analysis. Because of the low specific activity of ($^{14}CH_3O$)propetamphos, insect incubations using this substrate could not be directly analyzed by TLC. Metabolites from these incubation mixtures had to be concentrated on Sep-Paks, as described above, before TLC and HPLC analysis. More than one cartridge was sometimes necessary to remove all the radioactivity from the aqueous phase. Boiled enzyme preparations were used in all cases to correct for nonenzymatic breakdown of propetamphos.

Solvent systems used for metabolite separation on TLC were (A) hexane–ethanol (95:5, v/v), (B) benzene–ethyl acetate–hexane (1:4:2, v/v/v), (C) and (D) acetonitrile–water (95:5 and 95:25, v/v), (E) butanol–acetic acid–water (11:4:5, v/v/v), and (F) benzene–acetonitrile–acetic acid (49:50:1, v/v/v) (see Table I). After development of incubation samples and authentic standards of possible metabolites, the TLC plates were cut into 0.5-cm strips and put into scintillation vials and the radioactivity was quantitated on a Packard 3330 scintillation spectrometer. Nonradioactive standards were visualized by UV light, by exposure to iodine vapors, and/or by spraying with DBQ (0.5%, 2,6-dibromo-*N*-chloro-*p*-benzoquinonimine in cy-

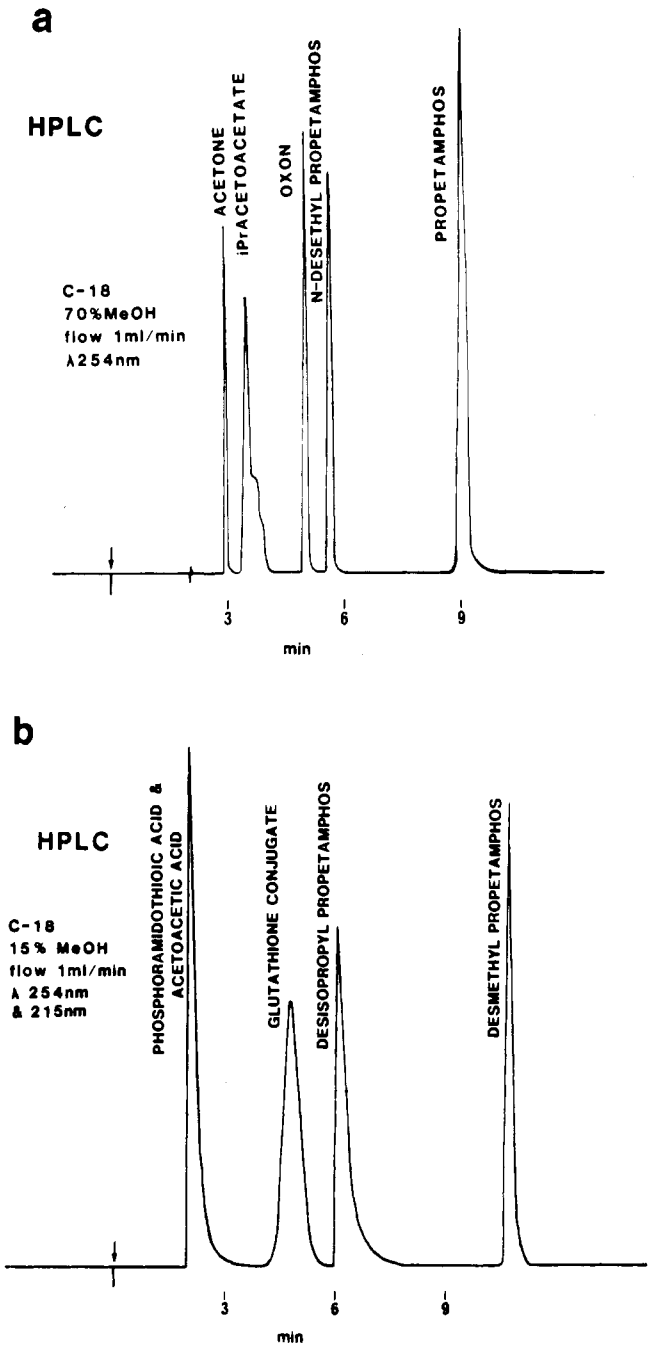

Figure 1. HPLC chromatograms of propetamphos and possible metabolites: (a) mobile phase of 70% methanol–30% water; (b) mobile phase of 15% methanol–85% water.

clohexane) with heating (110 °C) for 10 min (Menn et al., 1957), or by spraying with $PdCl_2$ (250 mg/100 mL of 0.2 N HCl).

HPLC analysis was carried out on a Beckman Ultrasphere-ODS column (dp 5 μm, 4.6 mm × 25 cm) and mobile phases of 70% methanol–30% water and 15% methanol–85% water, at 1 mL/min (Figure 1). Fractions were collected every 0.25 min and quantitated by liquid scintillation counting, as described above. Nonradioactive standards were visualized at 254 and 215 nm.

Glutathione Conjugate. Cornell-R strain glutathione *S*-transferase was partially purified by using the method of Motoyama and Dauterman (1978) in which the hydroxylapatite purification steps were excluded. Flies (10 g) were homogenized in 50 mL of 10 mM potassium phosphate buffer, pH 7.4, containing 1% BSA. After centrifugation at 10800*g* (10 min) and 105000*g* (1 h), and 105000*g* supernatant was passed over DEAE–cellulose. The void volume was subjected to ammonium sulfate precipitation, and the precipitate was resuspended in a minimum volume of 10 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol, 2 mM GSH, and 0.1 mM EDTA. The sample was applied to a Sephadex G-200 column and eluted with the same buffer. The active fractions collected from the column were concentrated by ultrafiltration (Diaflo UM-10, Amicon Corp., Lexington, MA). The final volume was 5.2 mL (8.3 mg of protein/mL) with a specific activity of about 1.8 μmol of dichloronitrobenzene conjugated/min per mg protein. Activity was measured as described in Motoyama and Dauterman (1978) and protein was determined by the fluorescamine method of Bohlen et al. (1973).

One-milliliter (8.3 mg of protein) aliquots of the purified enzyme were incubated with 1 mg of propetamphos (containing 1% [^{4}C]propetamphos) and 12 mM GSH at 25 °C for 2 h. Pooling four incubations yielded about 1.2 mg (propetamphos equivalents) of conjugate. This was determined by quantitation of the radioactivity contained in the water phase after three extractions with equal volumes of methylene chloride to remove unreacted propetamphos. Protein was removed from the water phase by ultrafiltration (Centrifree, Amicon Corp., Lexington, MA). The conjugate was then applied to a QAE-A25 Sephadex column (anion exchange) and eluted with a 0–250 mM KBr gradient. The radioactivity was recovered in the 65–95 mM KBr fractions, with a recovery of about 95% of that measured in the water phase after methylene chloride partitioning.

The sample was prepared for HPLC by using a Waters Sep-Pak to concentrate the sample and remove KBr. Greater than 96% of the radioactivity was retained on the cartridge until being eluted with methanol. The methanol was concentrated under nitrogen and injected on to a Beckman Ultrasphere-ODS column (p 5 μm, 4.6 mm × 25 cm) in 100-μg portions. The mobile phase employed was 15% methanol–85% water. The conjugate was collected between 4 and 8 min after injection. A total recovery of only about 47% (0.5 mg of propetamphos equivalents) could not be explained. The water–methanol mixture was evaporated under nitrogen at 40 °C and the residue dissolved in methanol-d_4 for high-field FT NMR. Impurities in the sample required another HPLC purification step, this time with a mobile phase of 95% water–5% methanol. The radioactivity was recovered about 40–48 min after injection. Recovery was about 53% (<0.3 mg equiv).

FT NMR was carried out on this sample in methanol-d_4. The methanol-d_4 was then slowly evaporated under nitrogen at room temperature while diluting with methanol (nondeuterated). About 100 μg equiv was dissolved in absolute ethanol with an excess of Raney nickel. This was refluxed in a sealed vial for 24 h at 85 °C. The reaction was then placed in the freezer for 24 h. After filtering the nickel from suspension with a plug of glass wool, the ethanol solution was subjected to GC–MS.

Major Mouse Metabolite. A 12-g sample of mouse liver was homogenized in 0.1 M potassium phosphate buffer, pH 7.4. The 20% (w/v) homogenate was centrifuged at 10800*g* for 10 min. The resulting supernatant was incubated with 8 mg of propetamphos (0.67 mg/5 mL incubation, no cofactor) for 2 h at 37 °C. The reaction mixture was then partitioned with 2 volumes of methylene chloride, three times, at pH 7.4. The pH was then adjusted to 2, using 1 N HCl, and extracted again with methylene chloride. The organic extract (pH 2) was dried over anhydrous sodium sulfate and concentrated on a rotary

Table II. Propetamphos Toxicity to the Housefly and Cockroach[a]

housefly strain	LD_{50}, μg/fly	95CL[b] (lower–upper)	LD_{50}, μg/g
CSMA	0.078	0.073–0.083	4.3
Rutgers	0.188	0.168–0.210	10.4
Cornell-R	0.274	0.257–0.292	15.2
Hirokawa	0.323	0.294–0.354	17.9
cockroach species	LD_{50}, μg/roach	95CL[b] (lower–upper)	LD_{50}, μg/g
American	0.455	0.422–0.486	4.6
German	5.65	5.05–6.13	4.5

[a] Based on 100 insects/dose, in triplicate. [b] 95% confidence limits.

Table III. Toxicity of Cis and Trans Isomers of Propetamphos to the Mouse and the Effect of TOCP Pretreatment[a]

	LD_{50}, mg/kg	95CL[b]	0.5 g/kg of TOCP pretreatment: LD_{50}, mg/kg	95 CL[b]
cis isomer	49	40–59	50	41–62
trans isomer	18	15–22	20	17–24

[a] 10 mice/dose, done in triplicate. [b] 95% confidence limits.

evaporator at 40 °C. The residue (2 mg equiv) was dissolved in a minimum amount of methanol and injected onto the previously described HPLC column in 200 μg equiv portions (~2 mg total). Using a mobile phase of 100% H_2O, the metabolite was collected after 28–36 min. The metabolite was extracted from the water by acidifying with HCl and partitioning with methylene chloride. After concentrating to dryness, the metabolite (~1.9 mg equiv) was dissolved in chloroform-*d*, for FT NMR spectroscopy. Methylation of the metabolite was achieved by dissolving it in ether and allowing it to react with an ethereal solution of diazomethane (MNNG–diazomethane apparatus, Aldrich Chemical Co.) overnight. This methylating procedure was carried out three times in order to acquire about 0.5 mg equiv of the methylated product. The ether was then carefully evaporated with a gentle stream of nitrogen. Buffer, pH 7.4, was added to the residue and partitioned with 2 volumes of methylene chloride, three times. The methylene chloride was evaporated and the methylated metabolite dissolved in chloroform-*d* for FT NMR spectroscopy. The nonmethylated and methylated metabolites were also analyzed by GC–MS.

RESULTS AND DISCUSSION

Toxicity. The toxicity of propetamphos to the housefly and cockroach is presented in Table II. The CSMA strain is the most susceptible of the housefly strains. Both species of cockroach are just as susceptible as the CSMA strain when body weight is considered. There is only about a 3.5–4-fold resistance in the most resistant strains.

The toxicity of propetamphos, when administered orally to the mouse, is recorded in Table III. The difference in toxicity between the cis and trans isomers should be noted. Although not presented in Table II, there is very little difference in toxicity between the isomers toward the insects tested in this study. These toxicity–isomer relationships are consistent with the first report on propetamphos (Leber, 1972), except that the reported difference between the toxicity of the isomers to the rat is greater than that observed here for the mouse. The effect of TOCP on propetamphos toxicity will be discussed later.

Metabolism. When the 10800*g* supernatant preparations from mouse liver, housefly abdomens, and cockroach abdomens are incubated with 2.5 μM (*crotonyl*-^{14}C)propetamphos, considerably less water-soluble products are formed by insect preparations (Figure 2). In fact, more than 10 times as much substrate can be metabolized by the mouse preparations.

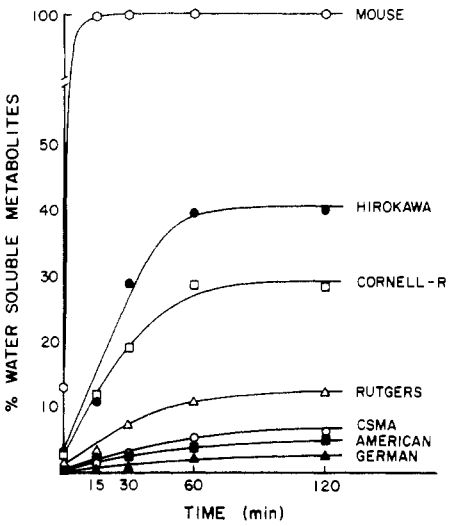

Figure 2. Degradation of 2.5 μm propetamphos to water-soluble metabolites by 10800*g* preparations (4 mM GSH and 1.1 mM $NADPH_2$ added) from mouse liver, housefly abdomens, and cockroach abdomens.

Table IV. Propetamphos Metabolites from Subcellular Fractions of Housefly and Cockroach Homogenates Using (*Crotonyl*-^{14}C)propetamphos[a]

housefly strain	105000*g* supernat + GSH: % glutathione conj	microsomes + NADPH: % propetamphos oxon
Hirokawa	39.8 ± 0.34	9.3 ± 0.20
Cornell-R	28.9 ± 0.52	11.8 ± 2.00
Rutgers	12.5 ± 1.12	14.4 ± 1.03
CSMA	6.4 ± 0.15	8.3 ± 1.67

	% acetoacetic acid			
cockroach species	105000*g* supernatant		microsomes	
	no cofactor	GSH	no cofactor	NADPH
American	3.6 ± 0.25	3.9 ± 0.15	4.0 ± 0.18	6.7 ± 0.15
German	2.7 ± 0.10	2.5 ± 0.03	3.1 ± 0.39	4.9 ± 0.18

[a] All values expressed as a percent of the total substrate and are the average of three experiments (mean ± SE).

When housefly preparations are incubated with (*crotonyl*-^{14}C)propetamphos, two major metabolites can be detected (Table IV). One of these is not very water soluble and cochromatographs, on HPLC and TLC, with propetamphos oxon. It is formed in the 10800*g* and microsomal fractions only when NADPH is needed. There is not a big difference in the rate of formation observed between strains.

The second major housefly metabolite detected is very water soluble and only occurs when reduced glutathione is added to the 105000*g* or 10800*g* supernatants. Its rate of formation is greater in those strains less susceptible to propetamphos. Its rate of formation is also greater in those strains with higher reported glutathione *S*-transferase activity (Hayaoka and Dauterman, 1982). When larger quantities of this metabolite were produced, using purified Cornell-R glutathione *S*-transferase, the NMR spectrum in Figure 3 was obtained. In order to get a radiolabeled glutathione conjugate from (*crotonyl*-^{14}C)propetamphos, the most logical reaction is at the vinyl bond. A substitution reaction would result in *S*-(isopropyl[^{14}C]crotonyl)glutathione, while an addition reaction would result

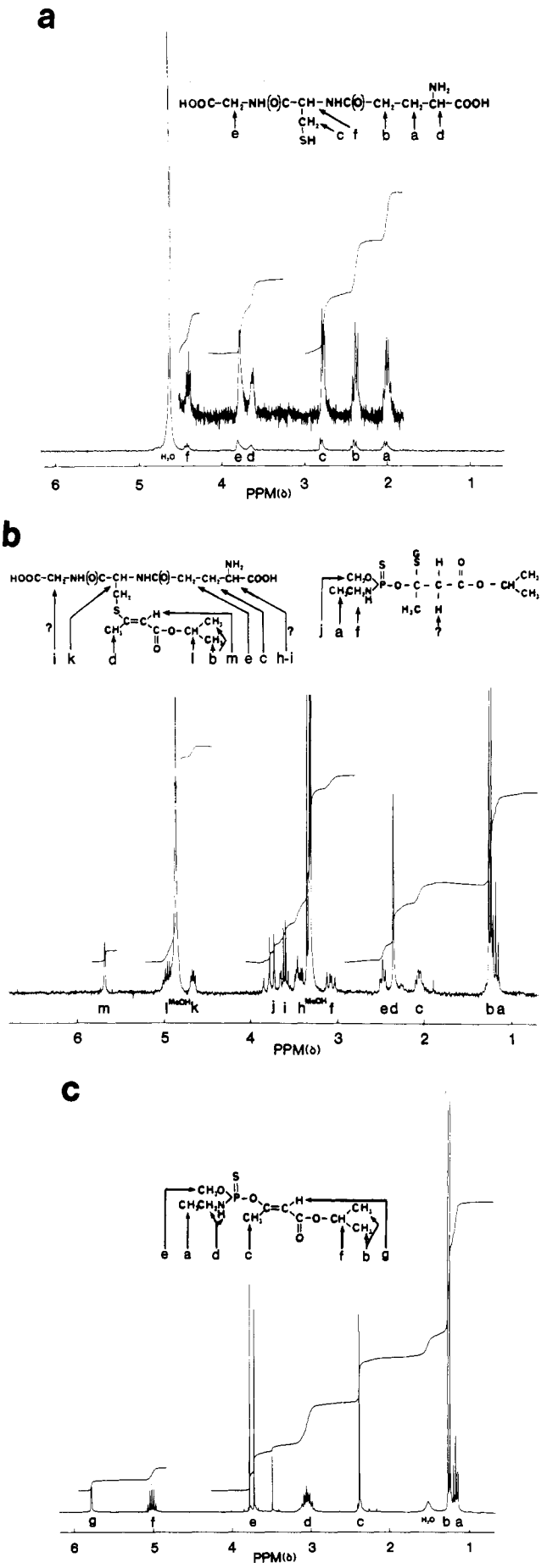

Figure 3. FT NMR (^{1}H) spectra: (a) glutathione in D_2O; (b) housefly glutathione conjugate in CD_3OD; (c) propetamphos in $CDCl_3$.

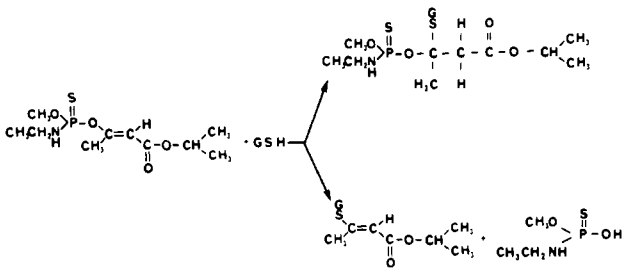

Figure 4. Proposed conjugation of propetamphos by housefly glutathione *S*-transferase plus glutathione (GSH).

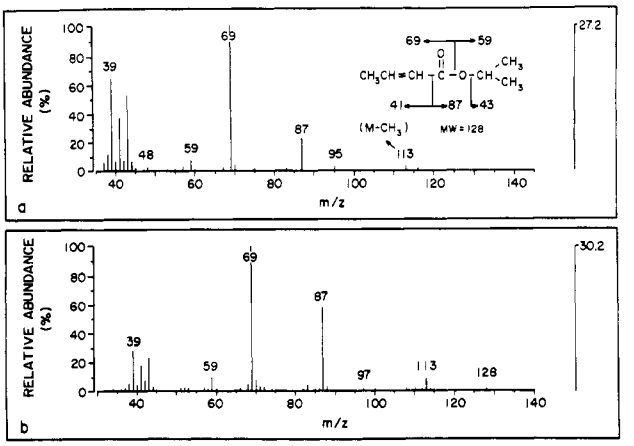

Figure 5. Mass spectra (EI): (a) isopropyl crotonate standard; (b) Ni hydrogenolysis of housefly glutathione conjugate.

in a reduction of the vinyl bond and conjugation of the entire parent molecule (Figure 4). The NMR spectrum would indicate both are present since a substitution reaction would result in the loss of $POCH_3$ peak (peak e in the propetamphos spectrum, Figure 3c) while an addition reaction would result in the loss of the vinyl proton peak (peak g in the propetamphos spectrum, Figure 3c). Both of these peaks are present in the conjugate spectrum, but their integration relative to each other has changed considerably. The ratio of approximately 1:3 ($C{=}CH{:}OCH_3$) in the propetamphos spectrum changes to a ratio of approximate 1:1 ($C{=}CH{:}OCH_3$) in the conjugate spectrum. Further attempts to separate the conjugate were unsuccessful.

Nickel is a known catalyst for sulfide bond cleavage and has been used to cleave glutathione conjugates (Boyland and Sims, 1960; Booth et al., 1960; Lamoureux et al., 1970; Watabe et al., 1981). When the housefly conjugate was subjected to these conditions of hydrogenolysis, isopropyl crotonate was identified in the reaction mixture by GC–MS (Figure 5b). GC–MS of the isopropyl crotonate standard, subjected to the same hydrogenolysis conditions, produced the same spectrum (Figure 5a).

When ($^{14}CH_3O$)propetamphos is allowed to react with the purified glutathione *S*-transferase in a manner identical with that of the (*crotonyl*-^{14}C)propetamphos, different ^{14}C-labeled products are formed. HPLC analysis results in the detection of two radioactive peaks when ($^{14}CH_3O$)propetamphos is used. One of these products cochromatographs with phosphoramidothioic acid, while the other cochromatographs with the single peak of the (*crotonyl*-^{14}C)propetamphos reaction. The ratio of the two products is about 85:15 (phosphoramidothioic acid: conjugate). These results are consistent with the previous data, suggesting two conjugates, one involving isopropyl crotonate and one involving the whole insecticide.

There appears to be a correlation between the glutathione conjugation and the toxicity of propetamphos in

Table V. Phosphoramidothioic Acid Recovered from Subcellular Fractions of Housefly and Cockroach Homogenates Using ($^{14}CH_3O$)Propetamphos[a]

housefly strain	105000g supernatant		microsomes	
	no cofactor	GSH	no cofactor	NADPH
Hirokawa	ND[b]	30.2 ± 0.34	3.8 ± 0.21	6.1 ± 0.21
Cornell-R	ND	21.1 ± 0.52	3.7 ± 0.07	6.4 ± 0.24
Rutgers	ND	9.7 ± 1.12	4.8 ± 0.15	10.3 ± 0.49
CSMA	ND	5.0 ± 0.15	4.1 ± 0.41	6.0 ± 0.35
cockroach species	105000g supernatant		microsomes	
	no cofactor	GSH	no cofactor	NADPH
American	4.5 ± 0.47	5.6 ± 0.35	7.1 ± 0.17	12.2 ± 0.66
German	3.8 ± 0.22	3.9 ± 0.09	4.9 ± 0.27	6.5 ± 0.48

[a] All values represent phosphoramidothioic acid expressed as a percent of the total substrate and averaged from three experiments (mean ± SE). [b] ND, not detected.

the housefly. Those housefly strains in this study that possess higher levels of glutathione *S*-transferase activity (Motoyama and Dauterman, 1977, 1978; Clark and Dauterman, 1982; Hayaoka and Dauterman, 1982) have a greater capacity to conjugate propetamphos and are less susceptible to propetamphos toxicity. There is a similar correlation in vivo (Wells et al., 1985), in which the production of a conjugate with similar properties was proportional to propetamphos resistance.

Another interesting aspect of this conjugation is its uniqueness in organophosphorus insecticide metabolism. Glutathione conjugation of organophosphate insecticides generally follows the patterns outlined by Motoyama and Dauterman (1980). Although it is common for glutathione to react with the "leaving group", causing a hydrolysis of the phosphate ester bond, the leaving group is always an aryl moiety. There are only a few reports of vinyl phosphate insecticides reacting with glutathione at the vinyl bond, and they involve dechlorination (Hutson et al., 1976; Akhtar, 1979) or epoxide reduction (Crawford et al., 1976). This may be the first example of a vinyl phosphate insecticide in which there is a direct substitution or addition reaction of glutathione with a nonhalogenated alkene leaving group.

Although no [^{14}C]isopropyl acetoacetate was detected when housefly homogenates were incubated with (*crotonyl*-^{14}C)propetamphos, microsomal hydrolysis was demonstrated with ($^{14}CH_3O$)propetamphos (Table V) via the formation of nonvolatile [^{14}C]phosphoramidothioic acid. The volatile nature of [^{14}C]isopropyl acetoacetate makes its detection difficult at the submicrogram levels using the procedures outlined in this study. Hydrolytic activity is low in these preparations; however, it was found to be an important pathway, in vivo, for the degradation of propetamphos by the housefly, especially at low dose levels (Wells et al., 1985).

In addition to phosphoroamidothioic acid, propetamphos oxon was also detected in the microsomal plus NADPH fractions. The formation of the oxon was 10.7%, 10.1%, 13.6%, and 9.4% of the total substrate for Hirokawa, Cornell-R, Rutgers, and CSMA incubations, respectively. These are approximately the same values found when (*crotonyl*-^{14}C)propetamphos was used as the substrate. Also not listed in Table V is a metabolite that was found in the 105000*g* supernatant plus GSH fraction of Hirokawa and Cornell-R strain preparations. This metabolite cochromatographs with the glutathione conjugate and represents 5.6% and 4.2% of the total substrate for Hirokawa and Cornell-R incubations, respectively. This is consistent with the proposal that two glutathione conjugates are formed. Recovery of this conjugate was probably too low to detect for the Rutgers and CSMA incubations.

Low levels of hydrolysis can also be detected in cockroach preparations (Tables IV and V). Acetoacetic acid and phosphoramidothioic acid were detected in all fractions incubated with (*crotonyl*-^{14}C)propetamphos and ($^{14}CH_3O$)propetamphos, respectively. In addition, they were found to a greater extend in the microsomal plus NADPH fraction, indicating that the mixed-function oxidases, as well as phosphotriesterases, play a role in propetamphos hydrolysis. This type of oxidative hydrolysis, as first observed with parathion (Nakatsugawa and Dahm, 1967; Neal, 1967), is a reaction that occurs with phosphorothionates and is postulated to share a common intermediate with the oxidative desulfuration reaction, leading to the phosphate (oxygen analogue) (Ptashne et al., 1971). The detection of acetoacetic acid also suggests carboxylesterase activity, yet no desisopropylpropetamphos was found. No conjugative activity was detected in the soluble cockroach fractions. This minimal ability of cockroach homogenates to degrade propetamphos is consistent with the high toxicity of propetamphos toward this insect.

As previously stated, mouse liver homogenates are capable of degrading much more propetamphos than housefly homogenates. In fact, 10 times as much propetamphos was used for mouse liver incubations as was for insect incubations, and the mouse microsomes still degraded 100% of the propetamphos within 30 min (Table VI). One major metabolite was formed to a much greater degree than any other. The enzyme activity responsible for its formation was found to a greater extent in the

Table VI. Propetamphos Metabolites from Subcellular Fractions of Mouse Liver Homogenates[a]

	subcellular fraction			
	105000*g* supernatant	105000*g* supernatant + GSH	microsomes	microsomes + NADPH
	(*Crotonyl*-^{14}C)propetamphos			
propetamphos oxon	ND	ND	ND	3.8 ± 0.30
acetoacetic acid	ND	ND	ND	5.4 ± 0.10
isopropyl acetoacetate	1.1 ± 0.38	0.8 ± 0.41	2.7 ± 0.69	21.1[b] ± 4.01
desisopropylpropetamphos	15.4 ± 0.51	13.9 ± 0.49	92.9 ± 1.64	58.1 ± 1.10
glutathione conjugate	0.2 ± 0.03	4.2 ± 0.15	ND	ND
	($^{14}CH_3O$)Propetamphos			
propetamphos oxon	ND	ND	ND	4.7 ± 0.28
phosphoramidothioic acid	3.1 ± 0.34	6.2 ± 0.64	ND	31.4 ± 0.97
desisopropylpropetamphos	19.5 ± 0.98	19.2 ± 0.45	100	48.6 ± 2.52
glutathione conjugate	ND	ND	ND	ND

[a] All values are expressed as a percent of the total substrate and are the average of three experiments (mean ± SE). ND, not detected. [b] Recoveries for microsomes + NADPH fractions were poor, probably due to the volatile nature of isopropyl acetoacetate.

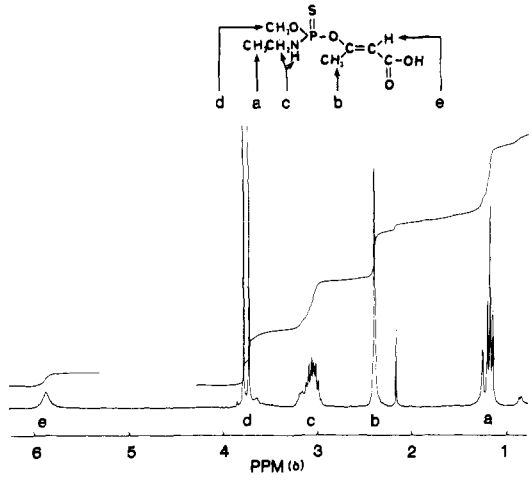

Figure 6. FT NMR (^{1}H) spectrum of major metabolite of propetamphos from mouse liver preparations (nonmethylated).

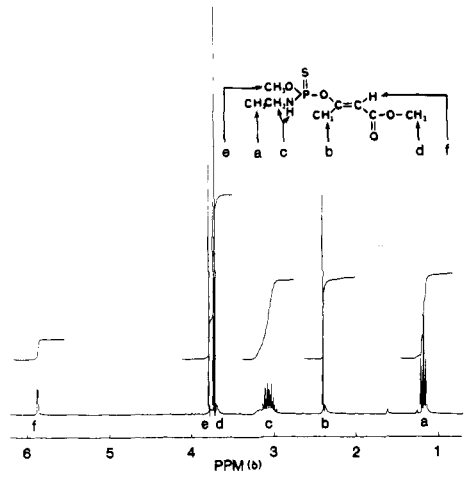

Figure 7. FT NMR (^{1}H) spectrum of major metabolite of propetamphos from mouse liver preparations (methylated).

microsomal fraction and required no cofactor. The metabolite was extractable with organic solvents when the pH was adjusted to below 3. In order to characterize this metabolite, a larger reaction was employed, using 12 g of mouse liver and 8 mg of propetamphos and yielding about 2 mg equiv of the metabolite. After dichloromethane extraction at pH 2 and HPLC purification, the NMR spectrum shown in Figure 6 was obtained. This spectrum clearly shows the loss of the isopropyl moiety, suggesting that desisopropylpropetamphos is the metabolite. The carboxylic acid proton, however, was absent from the spectrum, even as far as 14.5 ppm (δ). Methylation of the metabolite leads to a NMR spectrum identical with that of the synthesized standard of the methyl ester analogue of propetamphos (Figure 7). This supports the evidence that the metabolite is desisopropylpropetamphos. Analyses of the nonmethylated and methylated metabolites by GC–MS are shown in Figures 8 and 9.

As with the insect reactions, hydrolysis products were detected in the mouse incubations (Table VI). The oxygen analogue of propetamphos and a glutathione conjugate were also found in small quantities. It would appear, from the in vitro metabolic profile, that carboxylesterase activity, resulting in desisopropylpropetamphos, plays an important role in the detoxification of propetamphos in the mouse. This prompted the speculation that differences in toxicity between the cis and trans isomers in the rat (Leber, 1972) and the mouse (Table III) might be due to substrate specificity of carboxylesterase.

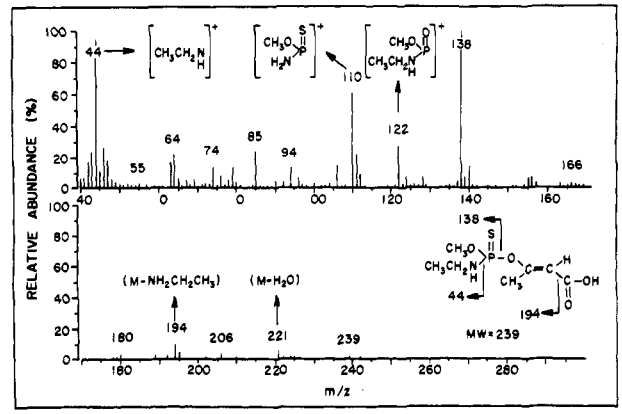

Figure 8. Mass spectrum (EI) of major metabolite of propetamphos from mouse liver prepartions (nonmethylated).

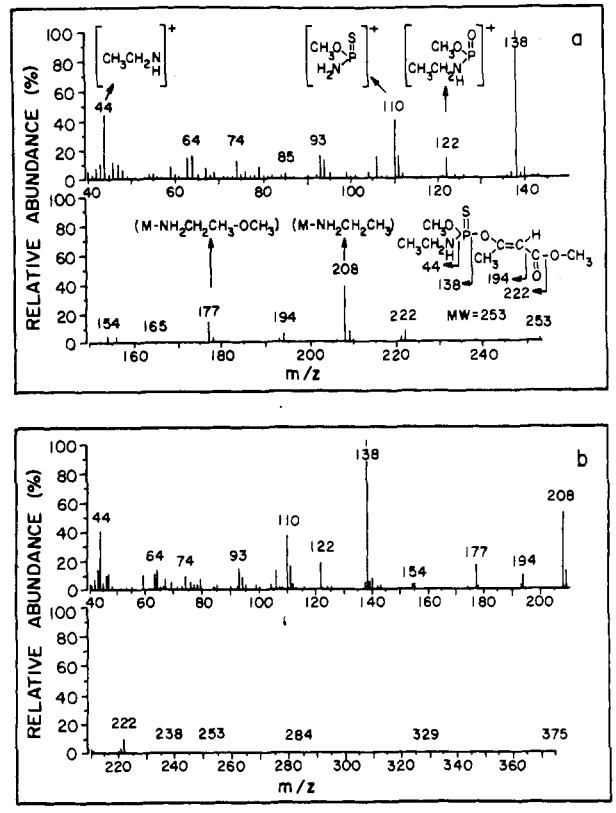

Figure 9. Mass spectra (EI): (a) (*E*)-*O*-2-(methylcarbonyl)-1-methylvinyl *O*-methyl ethylphosphoramidothioate standard; (b) major metabolite of propetamphos from mouse liver preparations (methylated).

Rat liver microsomal preparations were permitted to incubate with propetamphos as described for the mouse. As with the mouse, desisopropylpropetamphos was produced in abundance. Mouse liver microsomes were incubated for 1 h with *cis*-(*crotonyl*-^{14}C)propetamphos as described earlier, and also with unlabeled *trans*-propetamphos. Each incubation was then acidified with 1 N HCl to pH 2 and extracted three times with equal volumes of dichloromethane and the organic layer dried with anhydrous sodium sulfate and concentrated under nitrogen. HPLC analysis (15% methanol–85% water) resulted in the UV detection of desisopropylpropetamphos in those incubations in which *cis*-propetamphos was used. Measurements or radioactivity indicated that greater than 96% of the substrate added was recovered as the desisopropyl analogue. No product was detected by using the same conditions for the trans isomer. The organic extracts were then dried under nitrogen at room temperature. The

residue was dissolved in ether and alowed to react with an ethereal solution of diazomethane overnight. The reaction products were then analyzed on HPLC (70% methanol–30% water). The *cis*-propetamphos incubations had one UV peak at the solvent front (desisopropylpropetamphos) and a second peak at 8-min retention. The second peak cochromatographs with the synthesized standard of the methyl ester analogue of propetamphos. The trans isomer preparation had neither a comparable peak at the solvent front nor one cochromatographing with the methyl ester analogue of *trans*-propetamphos.

The previously described results strongly suggest that the carboxylesterase degradation of propetamphos is an important factor in the toxicity differences between the cis and trans isomers in mice and probably rats. If this is the case, tri-*o*-cresyl phosphate (TOCP), a carboxylesterase inhibitor, should potentiate *cis*-propetamphos toxicity in mice, as it has been shown to do with other carboxyl ester containing organophosphate insecticides (Seume and O'Brien, 1960; Dauterman and Main, 1966; Chiu et al., 1968). The results of TOCP potentiation experiments with propetamphos are summarized in Table II. TOCP pretreatment appears to have no effect on propetamphos toxicity in these studies.

In summary, all of the animals studied demonstrate some ability to hydrolyze the phosphoric anhydride bond of propetamphos in vitro. This is consistent with the results of propetamphos metabolism studies, in vivo, for the housefly (Wells et al., 1985) and the rat (Sandoz Inc., personal communication), as well as for all other vinyl phosphate insecticides in numerous animal species (Benyon et al., 1973). Glutathione conjugation, involving a reaction at the vinyl bond of propetamphos, may be an important detoxification reaction for less susceptible houseflies. Carboxylesterase activity leading to desisopropylpropetamphos is the predominate pathway for the degradation of *cis*-propetamphos in mouse liver preparations. The significance of this reaction in vivo, however, is unclear since TOCP, a carboxylesterase inhibitor, has no effect on toxicity.

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