Effects of the Synergists Piperonyl Butoxide and *S,S,S*-Tributyl Phosphorotrithioate on Propoxur Pharmacokinetics in *Blattella germanica* (Blattodea: Blattellidae)

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ABSTRACT Effects of the synergists piperonyl butoxide (PBO) and *S,S,S*-tributyl phosphorotrithioate (DEF) on propoxur pharmacokinetics were examined in the German cockroach, *Blattella germanica* (L.). Treatment of adult male German cockroaches with the cytochrome P450 monooxygenase inhibitor, PBO, or the esterase inhibitor, DEF, increased propoxur toxicity by 2- and 6.8-fold, respectively, implicating hydrolysis as a major detoxification route of propoxur in the German cockroach. However, significant hydrolytic metabolism could not be demonstrated conclusively in vitro resulting in a conflict between in situ bioassay data and in vitro metabolic studies. In vitro propoxur metabolism with NADPH-fortified microsomes produced at least nine metabolites. Formation of metabolites was NADPH-dependent; no quantifiable metabolism was detected with cytosolic fractions. However, microsomal fractions lacking an NADPH source did produce a low, but detectable, quantity of metabolites (1.6 pmol). PBO inhibited NADPH-dependent propoxur metabolism in a dose-dependent fashion, implicating cytochrome P450 monooxygenases as the enzyme system responsible for the metabolism. Interestingly, DEF also inhibited the NADPH-dependent metabolism of propoxur, albeit to a lower extent. Treatment with PBO or DEF also caused a significant reduction in the cuticular penetration rate of propoxur. The data demonstrate that unanticipated effects are possible with synergists and that caution must be exercised when interpreting synergist results.

KEY WORDS *Blattella germanica,* German cockroach, propoxur metabolism, pharmacokinetics, cuticular penetration, synergists

INSECTICIDE SYNERGISTS ARE powerful research tools for diagnosing resistance mechanisms, determining the conformation of target sites, and elucidating metabolic routes (Bernard and Philogène 1993). The synergists piperonyl butoxide (PBO) and *S,S,S*-tributyl phosphorotrithioate (DEF) have been used routinely in insect toxicological bioassays to provide a preliminary assessment of the contribution of detoxification enzyme systems such as mixed function oxidases and general esterases in insecticide resistant insects, respectively (Metcalf 1967, Bernard and Philogène 1993). However, occasionally synergists do not act as intended or expected and conclusions drawn from synergist bioassays are suspect until additional experiments are performed to confirm the results (Scott 1990).

The most common unanticipated effects from synergists are inhibition of an unintended enzyme (Scott 1990, Valles et al. 1997) and alteration of cuticular penetration rate of the insecticide (Sun and Johnson 1972, Bull and Pryor 1990, Kennaugh et al. 1993, Gunning et al. 1995). Although inhibition of nontarget enzyme systems has been reported (Valles et al. 1997), this effect typically occurs by non-specificity at comparatively high synergist concentrations (Scott 1990). Conversely, synergist-induced alterations in cuticular penetration rate have been reported frequently. The first demonstration of synergist-induced alterations in insecticide penetration was reported by Sun and Johnson (1972). They warned that some synergists cause synergism solely by an acceleration of insecticide across the cuticle. This effect was termed quasi-synergism. Similarly, Gunning et al. (1995) reported that the rate of penetration of esfenvalerate was increased in the presence of PBO in *Helicoverpa armigera* (Hübner). In contrast, Bull and Pryor (1990) reported that the cuticular penetration rate of fenvalerate decreased in the presence of PBO or DEF in house flies (*Musca domestica* L.). A similar conclusion was drawn by Kennaugh et al. (1993) who demonstrated that although permethrin resistance in a strain of *Helicoverpa armigera* (Hübner) could be eliminated with PBO, no evidence for increased permethrin detoxification in the resistant strain could be found. They suggested that a cytochrome P450 was involved in the process of permethrin penetration through the insect cuticle.

Synergists, such as PBO and DEF, have been used widely against the German cockroach to assess the

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contribution of metabolic insecticide resistance mechanisms. However, the effects of these synergists on insecticide pharmacokinetics (especially cuticular penetration) have not been fully explored in this species. Our objective was to examine the ability of the synergists, PBO and DEF to provide an accurate assessment of the metabolic route of propoxur in the German cockroach.

Materials and Methods

Insects. German cockroaches used throughout this study were originally collected from an infested institutional kitchen and cafeteria in Marietta, GA, in 1992 (Valles and Yu 1996a). Cockroaches were fed rodent diet # 5001 (PMI Feeds, St. Louis, MO). The colony was reared and maintained as described by Koehler and Patterson (1986) at 26°C, 50% RH, and a photoperiod of 12:12 (L:D) h.

Chemicals. Technical grade propoxur and DEF were purchased from ChemService (West Chester, PA). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, nicotinamide adenine dinucleotide phosphate (NADP), ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), and phenylmethylsulfonylfluoride (PMSF) were purchased from Sigma (St. Louis, MO). PBO and 1-phenyl-2-thiourea (PTU) were obtained from Aldrich (Milwaukee, WI).

[^{14}C]Propoxur with radiocarbon in the ring position (specific activity 23.5 mCi mmol^{-1}) was purified using two-dimensional thin-layer chromatography (TLC) plates (silica gel 60; Merck, Darmstadt, Germany) using hexane:ethyl acetate:ethanol (35:6:5 by volume) for the first dimension chromatogram. After drying, the plates were developed in the second dimension using benzene: ethyl acetate (6:1 by volume). The plates were exposed to Biomax MR-2 film (Eastman Kodak, Rochester, NY) for 3 d at −80°C. Propoxur was scraped and subsequently eluted from the silica gel with acetone. Purity was verified by TLC using propoxur standard (20 μg). [^{14}C] Propoxur and the propoxur metabolites, 2-isopropoxyphenyl N-hydroxymethyl carbamate, 2-isopropoxyphenyl carbamate, 2-hydroxyphenyl *N*-methyl carbamate and 2-isopropoxyphenol were supplied by Bayer (Kansas City, MO).

Insecticide Bioassays. Adult male German cockroaches (7–14 d old) were anesthetized with CO_2 and treated topically with propoxur and synergists dissolved in acetone. The propoxur solution (1 μl) was applied to the first abdominal sternite. At least five different concentrations that caused >0% and <100% mortality were used. At least three replications containing 10 cockroaches per dose (concentration) were conducted. PBO (100 μg per cockroach) or DEF (30 μg per cockroach) was applied to the first abdominal sternite 1 h before propoxur application. Mortality was recorded 24 h after treatment and cockroaches were considered dead if unable to right themselves in 15 s after being turned onto their dorsum.

Propoxur Penetration. Studies were conducted to determine how synergists might affect the rate of propoxur penetration. Insecticide penetration was determined by a method modified from Argentine et al. (1994). Cockroaches were treated topically with radioactive propoxur for varying periods of time. To assess penetration and in vivo metabolism, propoxur and metabolites were quantified from the external surface of the cockroach, the excreta (excreted) and from within the cockroach (internal). A sublethal dose of [^{14}C]propoxur (10,000 dpm, 0.04 μg) was applied to the first abdominal sternite of adult males (7–14 d old) in one μl of acetone. The cockroaches were dried briefly with gentle fanning, then placed individually into 20 ml glass scintillation vials for varying periods of time (15, 30, 60, and 120 min). After the specified time, two treated cockroaches were placed together into 5 ml of acetone and swirled gently for 15 s. This extraction process was repeated and the acetone extractions combined. For the 0 time point, treated cockroaches were extracted immediately after insecticide application by placing them directly into a scintillation vial containing 5 ml of acetone. Recovery from 0 time point cockroaches was considered 100% for subsequent extractions.

Internal propoxur was determined by homogenizing the solvent-rinsed cockroaches with a motor-driven Teflon pestle and glass mortar in 4 ml of acetone. The homogenate was then centrifuged at 500 × *g* for 3 min. The supernatant was removed by serological pipette and placed into a 20-ml scintillation vial. The extraction procedure was repeated two additional times and the extracts combined.

In Vivo Propoxur Metabolism. Production of metabolites from cockroach excreta was determined by adding 8 ml of acetone to the holding vials and spotting an aliquot of the extract on TLC plates. Three replicates of each time interval were conducted.

Propoxur and metabolites were identified and quantified from a 2.5 ml aliquot of the extraction solution (internal, external, and excreta). The aliquot was transferred to a 15 ml conical centrifuge tube and taken to dryness under a gentle stream of nitrogen. The remaining extract was counted by liquid scintillation spectrometry. The aliquot was resuspended in 100 μl of acetone, vortexed for a few seconds, and spotted onto a fluorescent 5 by 20-cm silica gel TLC plate (250 μm, Merck, Darmstadt, Germany). The tubes were rinsed three additional times with 100 μl of acetone, which was also spotted onto the TLC plate. The plates were developed twice in hexane:ethyl acetate:ethanol (35:6:5 by volume) as described by Shrivastava et al. (1969). After drying for 4 h, the plates were exposed to Biomax MR-2 film (Eastman Kodak, Rochester, NY) for 10 d at −80°C.

Identification of metabolites and propoxur were made by comparing the positions of unknown autoradiographic bands with known metabolites under UV light. Propoxur was scraped from the TLC plate and quantified by liquid scintillation spectrometry. The metabolites were scraped from the TLC plate, combined and quantified by liquid scintillation spectrometry. The total radioactivity was determined by summing the radioactivity present on the TLC plate, in the

aqueous fraction, and in the remaining extract. The liquid scintillation counter was a Packard model 4530 (Downers Grove, IL) with 95% efficiency for ^{14}C. Metabolism was expressed as a percentage of the total recovery of radioisotope. A quench curve was established with an extended series of quenched samples and used for all radioactivity quantifications. All values were converted to disintegrations per minute (dpm).

In Vitro Propoxur Metabolism. In vitro metabolism of propoxur was studied using a method described by Valles et al. (2000) using microsomal enzymes derived from the thorax and abdomen of adult males (7–14 d old) of the Marietta strain of German cockroach. For microsomes preparation, adult males were decapitated, then cut with scissors to remove the last three abdominal segments. The entire alimentary canal was gently removed from the posterior opening and placed into iced-cold 1.15% KCl. The contents of the foregut and midgut were removed by teasing open the tissues longitudinally with fine forceps to minimize detoxification enzyme inhibition by liberated digestive proteins (Valles and Yu 1996b). The tissues were recombined (less the head) and homogenized for 30 s in a protected buffer (0.1 M sodium phosphate, pH 7.5, containing 10% glycerol, 0.1 mM DTT, 1 mM EDTA, one mM PMSF, and 1 mM PTU) using a motor-driven Teflon pestle and glass mortar.

The homogenate was filtered through two layers of cheesecloth, then centrifuged at 10,000 $\times$ g_{Max}. The supernatant was filtered through glass wool and further centrifuged at 105,000 $\times$ g_{Max} for 1 h. The resulting pellet (microsomes) was rinsed and suspended in 0.1 M sodium phosphate buffer, pH 7.5. The above procedures were performed at 0–4°C.

When oxidative detoxification was assessed, the 2-ml incubation mixture contained 1 mg of microsomal protein suspended in 0.1 M sodium phosphate buffer, pH 7.5, and 0.04 ml methyl cellosolve containing 0.128 μg [^{14}C]propoxur (32,000 dpm). The reaction mixture was fortified with an NADPH-generating system composed of 1.8 μmol NADP, 1.8 μmol glucose-6-phosphate, and 1 U of glucose-6-phosphate dehydrogenase. When DEF was used, 20 μl of an acetone solution was first added to a reaction vessel and allowed to dry. Reactions were repeated in the presence and absence of an NADPH-generating source to verify oxidation by microsomal monooxygenases.

Hydrolytic detoxification was assessed with the 105,000 $\times$ g_{Max} supernatant (soluble fraction) and pellet (microsomes) as sources of hydrolytic enzymes. Enzyme preparation was accomplished as described above with the exception of using 0.1 M sodium phosphate buffer, pH 7.5. To evaluate propoxur metabolism catalyzed by microsomal and soluble esterases, the 2-ml incubation mixture contained microsomal or soluble fraction protein (1 mg) suspended in 0.1 M sodium phosphate buffer, pH 7.5, and 0.04 ml methyl cellosolve containing 0.128 μg [^{14}C]propoxur (32,000 dpm).

Duplicate incubations were carried out at 30°C in a water bath with shaking for 2 h. The reaction was terminated by the addition of 0.2 ml of four M HCl followed by plunging the reaction vessel in ice. Reactions were initiated by the addition of the enzyme source. In all instances, boiled equivalent tissue was used as a blank to correct for nonenzymatic propoxur degradation which averaged 6.3 $\pm$ 1.6%.

Propoxur and its metabolites were extracted by adding 4 ml of diethyl ether to the incubate and shaking for 10 min on a rotary mixer. The shaken incubate was centrifuged for 3 min at 500 $\times$ g and the ether phase was removed by pipette. The extraction process was repeated three additional times. The combined ether extract was taken to dryness under a gentle stream of nitrogen and resuspended in 100 μl of acetone. The entire extract was spotted onto a 5 by 20-cm TLC plate along with known propoxur metabolites. The plates were developed, and propoxur and its metabolites were quantified as described above for the in vivo assay. Propoxur metabolism was quantified by subtracting the quantity of metabolites produced in the boiled incubate (control) from the treatment. Propoxur metabolism was expressed as pmol/2 h/mg protein. Total recovery of radioactivity was determined by summing all radioactivity from the TLC plate and from the aqueous fraction.

To verify cytochrome P450 monooxygenases as the enzyme system responsible for propoxur metabolism, an experiment was performed using increasing concentrations of PBO and NADPH-fortified microsomes as enzyme source, as described above. Final concentrations of PBO in the incubation tubes were 10^{-4}, 10^{-5}, and 10^{-6} M.

Protein determinations were made by the method of Bradford (1976) using bovine serum albumin as the standard. All enzyme assays were conducted three times.

Statistical Analysis. Insecticide bioassay data were analyzed by probit analysis (SAS Institute 1988). Treatment means in the penetration study (with synergist) were compared with control values (without synergist) by Student's *t*-test. Inhibition of propoxur metabolism by synergists was analyzed by analysis of variance (ANOVA) followed by Scheffé multiple comparison procedure when appropriate.

Results

Synergism. Treatment of adult male German cockroaches of the Marietta strain with the cytochrome P450 monooxygenase inhibitor, PBO, or the esterase inhibitor, DEF, increased propoxur toxicity by 2- and 6.8-fold, respectively (Table 1). Increased toxicity by the synergists suggested that cytochrome P450 monooxygenases and hydrolytic enzymes were contributing to the detoxification of propoxur. Furthermore, the synergist bioassay data implicated that hydrolysis may be a major detoxification route because greater synergism occurred with DEF.

Table 1. LD_{50} of topically applied propoxur and propoxur + synergists to German cockroach adult males

Insecticide and Synergist	n	Slope ± SE	LD_{50} (95% CL)[a]	χ^2	SR[b]
Propoxur	150	2.43 ± 0.33	1.69 (1.32–2.13)	1.17	—
Propoxur + PBO	180	3.97 ± 0.58	0.83 (0.74–0.96)	4.15	2.03
Propoxur + DEF	120	1.44 ± 0.44	0.25 (0.20–0.32)	4.18	6.76

[a] μg propoxur per insect.
[b] Synergist ratio, LD_{50} propoxur/LD_{50} propoxur + synergist.

Cuticular Penetration of Propoxur Impacted by Synergists. Figs. 1 and 2 summarize propoxur partitioning in the presence and absence of PBO and DEF. Propoxur penetrated the cuticle rapidly in the absence of synergists (Fig. 1A). However, when cockroaches were treated with PBO or DEF, the rate of propoxur penetration slowed significantly. Decreased quantities of propoxur were found inside the cockroaches and in cockroach excreta at all time points when compared with non-synergist-treated cockroaches (Fig. 1 B and C). Despite reduced insecticide penetration caused by PBO and DEF, the synergists nevertheless caused a significant increase in propoxur toxicity in bioassay experiments indicating that they were effectively penetrating the cuticle and inhibiting detoxification. In fact, verification of in vivo enzyme inhibition by the synergists was evidenced by a significantly lower quantity of metabolites inside the cockroaches compared with those not treated with a synergist (Fig. 2B).

In Vivo Metabolism. There were no significant differences in metabolite production from external rinses among all treatments and at all time points (Fig. 2A). However, metabolite production was significantly reduced within the cockroaches (Fig. 2B) when treated with PBO or DEF. Interestingly, both PBO and DEF caused an equivalent reduction in metabolite production (Fig. 2B).

Radiolabel recovery for the in vivo experiments is summarized in Table 2. Recovery declined over time in all treatments and was the same for propoxur and synergist treated cockroaches 15 and 30 min after treatment. However, recovery from DEF-and PBO-treated cockroaches was higher than the control at 60 and 120 min.

In Vitro Metabolism. In vitro propoxur metabolism with NADPH-fortified microsomes produced at least nine metabolites (Fig. 3). The three most abundant putative metabolites identified by cochromatography with known standards were 2-isopropoxyphenyl *N*-hydroxymethyl carbamate (R_f = 0.31), 2-hydroxyphenyl *N*-methyl carbamate (R_f = 0.29), and 2-isopropoxyphenyl carbamate (R_f = 0.34, Fig. 3). Additional unidentified metabolites were produced in smaller quantities. Formation of metabolites was primarily NADPH-dependant. However, a low level of propoxur metabolism was catalyzed by microsomal esterases (Table 3). PBO inhibited NADPH-dependent propoxur metabolism in a dose dependant fashion (Fig. 4), implicating cytochrome P450 monooxygenases as the enzyme system responsible for the metabolism. DEF also inhibited the NADPH-depen-

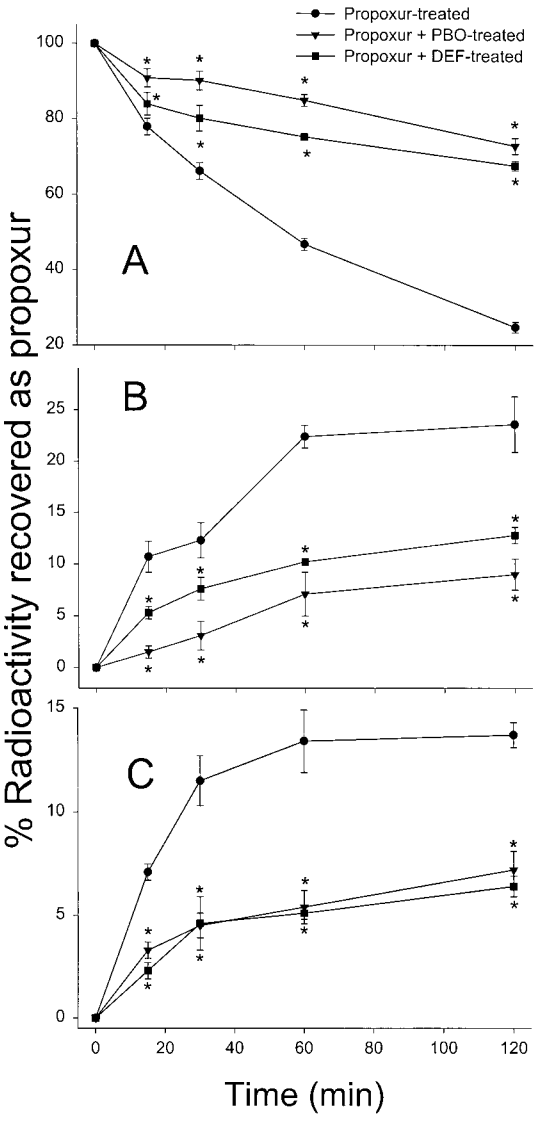

Fig. 1. Recovery of [^{14}C]propoxur from adult male German cockroaches (Marietta strain) at various times after [^{14}C]propoxur application. Cockroaches were treated with acetone (control), PBO, or DEF, and subsequently treated topically with [^{14}C]propoxur (10,000 dpm; 0.04 μg) 1 h later. Unmetabolized propoxur was separated from metabolites by TLC as described. (A) External rinse; (B) internal extract; (C) fecal extract. *, value significantly different ($P < 0.05$) from the control (propoxur-treated).

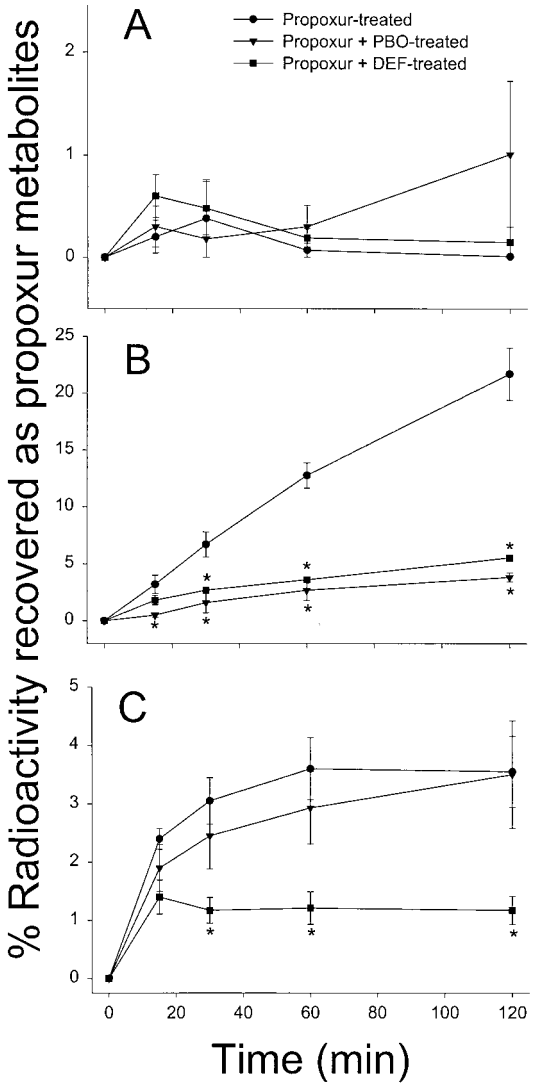

Fig. 2. Recovery of [^{14}C]propoxur metabolites from adult male German cockroaches (Marietta strain) at various time points after [^{14}C]propoxur application. Cockroaches were treated with acetone (control), PBO, or DEF, and subsequently treated topically with [^{14}C]propoxur (10,000 dpm; 0. 04 μg) 1 h later. [^{14}C]Metabolites were separated from [^{14}C]propoxur by TLC as described. (A) External rinse; (B) internal extract; (C) fecal extract. *, value significantly different ($P < 0.05$) from the control (propoxur-treated).

dent metabolism of propoxur (Fig. 4). However, high concentrations of DEF were required to achieve inhibition. For example, at 10^{-3} M, PBO caused a 90% reduction in propoxur metabolism while DEF (10^{-3} M) only inhibited the rate by 40%. Furthermore, even at 10^{-2} M, DEF only inhibited propoxur metabolism to 40% of the control value.

No detectable propoxur metabolites were observed from soluble enzyme preparations (cytosol). With this enzyme preparation, the autoradiogram and respective propoxur and metabolite counts were not significantly different from boiled blanks.

Table 2. Total recovery (%) of radioactivity from adult males of the Marietta German cockroach strain after topical treatment with [^{14}C]propoxur

Treatment	Time (min)			
	15	30	60	120
Propoxur	95.6 ± 1.5	89.9 ± 7.2	82.6 ± 0.9	61.6 ± 2.7
Propoxur + PBO	95.7 ± 1.2	97.8 ± 2.8	97.4 ± 1.9	88.9 ± 2.2
Propoxur + DEF	95.8 ± 3.4	89.9 ± 3.2	92.4 ± 3.2	84.3 ± 1.8

Values are mean percentage (±SE) from three experiments, each with duplicate determinations. Recovery at time 0 was considered 100%.

Discussion

The synergists PBO and DEF had a profound effect on the ability of propoxur to penetrate through the cuticle of the German cockroach. The amount of propoxur reaching the inside of the synergist-treated cockroaches was less than half that of cockroaches not treated with a synergist (Figs. 1 and 2). This observation is not without precedent. Scott and Georghiou (1985) and Bull and Pryor (1990) also provided evidence that pretreatment with synergists slowed permethrin and fenvalerate cuticular penetration in house flies, respectively. Furthermore, Sun and Johnson (1972) were the first to describe quasi-synergism, an increase in insecticide toxicity due to accelerated

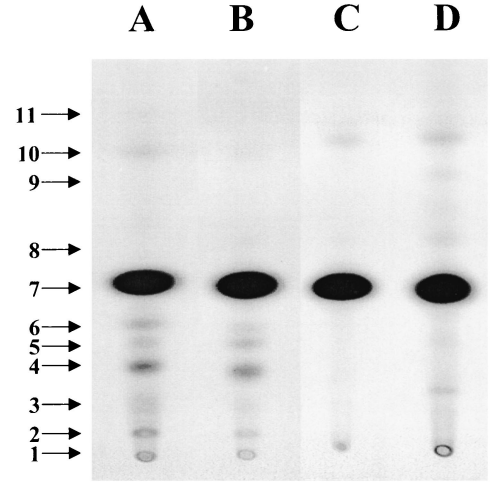

Fig. 3. Autoradiograms of TLC separations of [^{14}C]propoxur metabolites from in vitro metabolism experiments using microsomes fortified with an NADPH source (A), microsomes fortified with an NADPH source and DEF (10^{-4} M) (B), soluble fraction (C), and microsomes without an NADPH source (D). Metabolite identification and R_f values are as follows: (1) origin; (2) unknown A, 0.06; (3) unknown B, 0.11; (4) 2-hydroxyphenyl *N*-methyl carbamate, 0.22; (5) 2-isopropoxyphenyl *N*-hydroxymethyl carbamate, 0.28; (6) 2-isopropoxyphenyl carbamate, 0.32; (7) propoxur, 0.43; (8) unknown C, 0.53; (9) unknown D, 0.67; (10) 2-isopropoxyphenol, 0.73; (11) unknown E, 0.83.

Table 3. In vitro propoxur metabolism by various enzyme sources from the Marietta strain of German cockroach

Enzyme source	Protein quantity, mg	NADPH	DEF (10^{-4}M)	pmol recovered as[a]		Total recovery, %
				Propoxur	Metabolites	
Microsomes	1	+	−	365.3 ± 10.4	63.7 ± 4.8	85.2 ± 1.6
Microsomes	1	+	+	393.7 ± 6.8	27.5 ± 3.5	84.0 ± 1.1
Microsomes	1	−	−	407.1 ± 7.6	1.6 ± 0.9	80.9 ± 1.4
Soluble fraction	1	−	−	364.1 ± 7.1	ND[b]	74.2 ± 1.1

[a] Less than 1% of the initial dose of propoxur was recovered from the aqueous fraction after extraction with diethyl ether.
[b] None detected.

penetration across the cuticular barrier as opposed to inhibition of detoxification enzymes. Although the effect of PBO and DEF on propoxur penetration in the Marietta German cockroach strain was different than that observed by Sun and Johnson (1972), it nonetheless illustrates penetration rate changes attributed to coapplication of chemicals.

Topical bioassay data revealed that treatment with either synergist increased the toxicity of propoxur in the Marietta strain of German cockroach (Table 1). The level of propoxur tolerance was reduced 1.8-fold when cockroaches were treated with PBO (a cytochrome monooxygenase inhibitor) and 6.8-fold when treated with DEF (an esterase inhibitor). These data suggested that both oxidative and hydrolytic enzymes participated in the detoxification of propoxur. Based on the bioassay results, hydrolysis appeared to be the primary detoxification route of propoxur in the German cockroach because synergism was over 3-fold greater with DEF compared with PBO. However, we were unable to detect any propoxur metabolites when the cytosolic fraction was used as the enzyme source. Also, propoxur metabolism was very low by microsomes without an NADPH source (Table 3). Conversely, propoxur was readily metabolized by microsomal enzymes fortified with NADPH, indicating detoxification catalyzed by cytochrome P450 monooxygenases. Indeed, this notion was confirmed by inhibition of propoxur metabolism (with NADPH-fortified microsomes) by PBO, both in vivo and in vitro (Fig. 4).

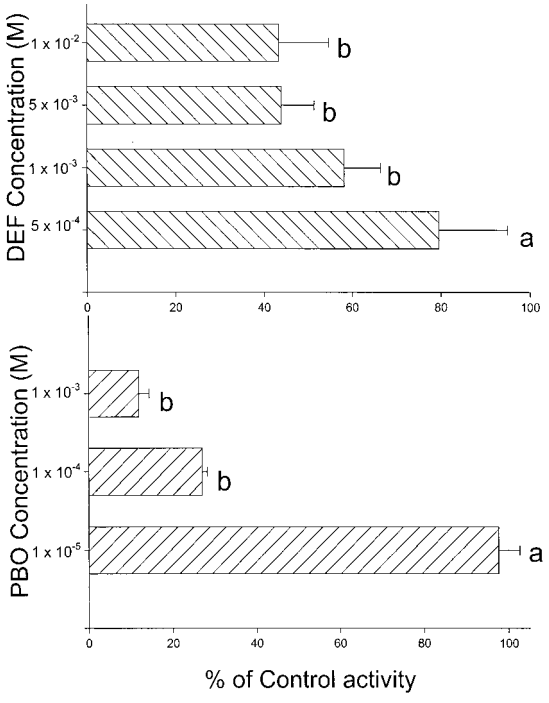

Fig. 4. Effects of increasing DEF or PBO concentrations on propoxur metabolism by NADPH-fortified microsomes prepared from adult males of the Marietta strain of German cockroach. Bars with different letters are significantly different by Scheffé multiple comparison procedure ($P < 0.05$). Control activity was 56.0 ± 5.2 pmol/2 h/mg protein for DEF treatment, and 51.4 ± 2.5 pmol/2 h/mg protein for PBO treatment.

In certain cases, the in vivo degradation pathway of an insecticide may differ from that observed in in vitro enzyme systems (Tsukamoto and Casida 1967). A similar contradiction between bioassay and in vitro metabolism data were reported by Siegfried et al. (1990). They found that resistance to chlorpyrifos in the Dursban-R strain of German cockroach was partially suppressed by DEF, but, were unable to detect in vitro metabolism of chlorpyrifos with cytosolic fractions. However, high levels of hydrolysis were detected against the activated product, chlorpyrifosoxon, which explained the synergism by DEF. Additionally, Kennaugh et al. (1993) reported a similar result with PBO in *Helicoverpa armigera* (Hübner). They found that a 20-fold resistance level toward permethrin could be eliminated by PBO, however no evidence for increased permethrin detoxification by cytochrome P450 monooxygenases in the resistant strain was found. They concluded that synergism of pyrethroid toxicity by PBO was not necessarily an indication of resistance due to increased detoxification by cytochromes P450.

Topical bioassays in the Marietta strain of German cockroach implied that propoxur hydrolysis was a major route of detoxification, but in vitro metabolic studies did not support these data. Siegfried and Scott (1991) also showed that DEF synergized propoxur toxicity in the Baygon R strain of German cockroach. However, they identified a single metabolite with an R_f value slightly lower than *o*-hydroxy propoxur (2-hydroxyphenyl *N*-methyl carbamate) from in vitro assays. Further, they contended that propoxur disappearance from the reaction mixture was sufficiently high to indicate metabolism. However, they did not

indicate whether or not a boiled control was used to discern nonenzymatic hydrolysis. Conversely, we could not detect any metabolites (beyond boiled controls) when the cytosolic fraction was used as the enzyme source. Also, although metabolism was detected in microsomal fractions without an NADPH source (i.e., microsomal esterases), the rate was low, comparatively. Finally, we did not observe a significantly higher loss of propoxur from the esterase fractions compared with boiled controls. Because the rate of metabolism was very low, we felt that using substrate disappearance for quantification was unreliable. 2-Isopropoxyphenol, an anticipated hydrolytic metabolite, is volatile, so it is possible that it was lost during the incubation, extraction, and separation processes. Alternatively, 2-isopropoxyphenol could have undergone complete decomposition as suggested by Shrivastava et al. (1969). However, the amount of propoxur and its metabolites recovered from active cytosolic and non-NADPH microsomal fractions were not different from the boiled controls. Hence, these data suggest that hydrolysis of propoxur did not readily occur in vitro. Indeed, hydrolysis has been shown to be an unimportant reaction in the metabolism of propoxur in the housefly, *Musca domestica* L. (Shrivastava et al. 1969), and cabbage looper, *Trichoplusia ni* (Hübner) (Kuhr 1970).

Finally, the data suggest that the esterase inhibitor, DEF, may inhibit microsomal monooxygenases as observed previously (Valles et al. 1997, Scott 1990). In vitro inhibition of microsomal monooxygenases did occur, albeit at fairly high DEF concentrations (Fig. 4). Furthermore, propoxur metabolism was apparently inhibited equally by PBO and DEF in vivo (Fig. 4). However, these effects were still unable to account for the greater synergism (3-fold) by DEF in the propoxur bioassay.

Several hypotheses could reconcile the DEF synergism of propoxur toxicity with no apparent metabolism by esterases (in vitro). First, as suggested by Satoh (1987), competition for nonvital proteins may accelerate the rate and level of insecticide reaching the target site. For example, if such a protein had greater affinity toward DEF compared with propoxur, then it may be possible to achieve a greater concentration of propoxur at the target site in the presence of DEF. Second, DEF may produce an additive or synergistic inhibition of acetylcholinesterase. However, we found that DEF did not inhibit acetylcholinesterase in the Marietta strain of German cockroach nor did DEF enhance propoxur inhibition of acetylcholinesterase in vitro (data not shown). Finally, DEF has been reported to be activated by cytochrome P450 monooxygenases (Hur et al. 1992). The sulfoxide and sulfone metabolites were found to be potent inhibitors of mouse acetylcholinesterase. Hence, it is possible that DEF was being activated in the German cockroach which resulted in enhanced toxicity (synergism) of propoxur. However, the acetylcholinesterase activity from DEF-treated cockroaches (24 h) was not significantly different from controls indicating that DEF activation was not occurring in vivo (data not shown).

We have shown that the addition of PBO or DEF profoundly reduced the cuticular penetration rate of propoxur in the German cockroach. Furthermore, synergist bioassay data conflicted with the metabolism of propoxur in vitro. The use of synergists are invaluable tools for the study of metabolic insecticide resistance mechanisms and detoxification pathways in insects. However, as evidenced by the data presented herein, caution must be exercised when using these chemicals because unanticipated or unexplained effects are possible.

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