

Mechanisms of Fenvalerate Resistance in the German Cockroach, *Blattella germanica* (L.)

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Received November 7, 1997; accepted April 14, 1998

The Munsyana strain of German cockroach, originally collected in Muncie, Indiana, was found to have high-level resistance to fenvalerate, displaying 825-fold levels of resistance by topical application compared to a susceptible laboratory strain (Johnson Wax). Pretreatment with the cytochrome P450 monooxygenase inhibitors piperonyl butoxide (PBO) or MGK-264, or the esterase inhibitor *S,S,S*-tributyl phosphorotrithioate (DEF), reduced fenvalerate LD₅₀ resistance ratios by 12-fold, 55-fold, and 10-fold, respectively. Detoxication enzyme assays revealed elevated activity of microsomal oxidases (N-demethylation), esterases (PNPA hydrolysis), and glutathione-*S*-transferases (CDNB conjugation). *In vivo* penetration studies employing [¹⁴C]fenvalerate indicated reduced accumulation in the resistant strain relative to the susceptible strain. *In vitro* metabolism of [¹⁴C]fenvalerate by 105,000-g supernatant fractions with and without DEF inhibition suggested that hydrolysis was occurring. In the microsomal fraction, PBO also reduced the quantity of metabolites *in vitro*. Overall, results suggest that monooxygenase, hydrolase, glutathione-*S*-transferase and decreased cuticular penetration are involved in fenvalerate resistance in the Munsyana strain. However, resistance to fenvalerate was not completely eliminated by either PBO, MGK-246, or DEF, suggesting that additional mechanisms, possibly including sodium channel insensitivity, are involved in this resistance. ©1998 Academic Press

INTRODUCTION

Insecticide resistance in the German cockroach (*Blattella germanica*) has been well documented for many insecticide classes, including the organophosphates (1–4), the carbamates (2, 3, 5, 6), and the pyrethroids (3, 7–10). Additionally, it has been shown that resistance to multiple classes of insecticides is commonly due to multiple mechanisms (10–14). In the German cockroach, three classes of insecticide resistance have been identified; these are behavioral, physiological, and metabolic resistance. Previously, the two most prevalent metabolic resistance mechanisms to occur in the German cockroach have been identified in the Munsyana strain. These mechanisms include both cytochromes P450 and esterases (10, 14, 15). While there has been success

in identifying differences in these enzymatic detoxification enzyme mechanisms, it remains unclear which of these mechanisms are acting to cause pyrethroid resistance. The information provided by radiolabeled insecticide assays would allow us to answer this important question. Additionally, synergism and biochemical studies could facilitate the identification of compounds that would eventually restore the activity of pyrethroids. From a better understanding of these resistance mechanisms, we can begin development of formal resistance management strategies for the German cockroach.

MATERIALS AND METHODS

Chemicals. All chemicals were obtained from Sigma Chemical (St. Louis, MO) or US Biochemical (Cleveland, OH) unless otherwise specified. All inorganic reagents were analytical grade. 4-chloro-*N*-methylaniline, 2,4-dichlorodinitrobenzene (CDNB), and piperonyl butoxide (PBO) were obtained from Aldrich (Milwaukee, WI). DEF was obtained from Bayer Corporation,

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Agriculture Chemicals Division (Kansas City, MO). MGK-264 was obtained from MGK Inc. (Minneapolis, MN). [^{14}C]fenvalerate ($21.6 \mu\text{Ci mmol}^{-1}$) was a gift from Du Pont Chemical (Wilmington, DE).

Insects. Two strains of German cockroach were used in this study. The Johnson Wax (JWax) strain was isolated from a field population before the widespread use of synthetic organic insecticides and is susceptible (16). The JWax strain was used as a baseline in insecticide and biochemical assays. The Munsyana (MA) strain, collected from the Muncie Housing Authority (Muncie, IN), was exposed to Demon WP insecticide (cypermethrin) for 8 years prior to its collection in 1994. In previous work by Scharf *et al.* (10), toxicity tests revealed a high level of resistance to cypermethrin (LD_{50} resistance ratio = 82.2, LD_{95} resistance ratio = 136.5). Two generations prior to initiation of this study, the Munsyana strain ($n = 1000$) was selected by removing 90% of 5th–6th instar individuals with an exposure to cypermethrin residues of $1.8 \mu\text{g}/\text{cm}^2$.

One to 2-week-old adult male German cockroaches were used in all tests. In culture, test insects were maintained on 12:12 (L:D) photoperiod at a temperature of 26–28°C. All insecticide applications were made during the light phase of the photoperiod. Colonies of test insects were provided with unlimited food (Teklad-Rodent Diet No. 8604, Harlan Teklad, Madison, WI) and water. At 5–7 days prior to insecticide bioassays, test insects were isolated from culture and held under identical conditions with ample food and water. Average weights of each strain were calculated at 1 week postisolation.

Toxicity tests. Topical applications were made with an Arnold Automatic Microapplicator (Burkhard Mfg. Co., Ricksmanworth, England) equipped with a 1-ml glass syringe, by placing $1 \mu\text{l}$ of fenvalerate solution onto the intercoxal spaces of the ventral mesothorax of CO_2 -anesthetized cockroaches. For each strain, three replicates (10 insects per replicate) of four or five doses were treated with fenvalerate alone or with fenvalerate and synergists. Technical

grade fenvalerate, and the synergists PBO, DEF, and MGK-264 were dissolved in spectrophotometric grade acetone. Synergist studies were conducted by applying $1 \mu\text{l}$ of PBO ($100 \mu\text{g}/\mu\text{l}$), DEF ($30 \mu\text{g}/\mu\text{l}$), or MGK-264 ($100 \mu\text{g}/\mu\text{l}$) in an acetone solution onto the ventral abdomen 1 h before thoracic application of fenvalerate solutions. After insecticide application, test insects were placed in groups of 10 in 100-mm plastic Petri-dishes with a water vial and cardboard harborage. Mortality was recorded 72 h following treatment.

Data analysis. For the topical applications, all data were analyzed using SAS Probit (17). Each analysis yielded the following: sample size (n), χ^2 (with P values), slope (\pm SE), and 50 and 95% lethal dose values (with 95% fiducial limits). Resistance ratios were calculated using the method described by Robertson and Preisler (18). The components from SAS probit analyses used in each individual comparison included slope, intercept, and the three values of the estimated covariance matrix. Using this methodology, any ratio possessing a 95% CL which includes one (1.0) indicates equality; the point where no significant difference exists between each component of a ratio (18).

Enzyme preparation and protein quantification. The methods used are identical to those of Scharf *et al.* (10). Microsomal and soluble enzymes were prepared from homogenates of headless frozen adult male cockroaches. Fifteen insects were homogenized in 10 ml sodium phosphate buffer (0.1 M, pH 7.5) using a Kontes Duall 24 homogenizer (Vineland, NJ). Homogenates were centrifuged at 10,000g and 4°C for 10 min using a Sorvall RC-5B centrifuge. Supernatants were filtered through glass wool and spun at 105,000g and 4°C for 60 min in a Beckman LC-50B ultracentrifuge. Following ultracentrifugation, microsomal pellets were resuspended in 1.3 ml of homogenization buffer (pH 7.5) containing 30%, v/v, glycerol and frozen at -70°C . Microsomal preparations were always determined to be free of cytochrome P420 by carbon monoxide difference spectra.

The soluble enzymes (contained in the supernatant) were frozen at -70°C until assayed.

Protein was quantified according to the method of Smith *et al.* (19). In short, bicinchoninic acid (BCA) protein assay reagents A and B (Pierce, Rockford, IL) were mixed at a ratio of 50:1, then 900 μl of this mixture was added to 5 μl of protein sample (diluted 1:10) and 95 μl water. Water was used to dilute protein samples as glycerol contained in the resuspension buffer was found to interfere with the BCA assay. The reaction was allowed to proceed for 30 min at 37°C in a water bath before absorbance was measured at 562 nm. Absorbance was converted to protein concentration by comparison with a standard curve of bovine serum albumin.

Esterase activity assay. For each strain, 105,000-g supernatants from preparations were used to determine hydrolytic activity toward the model substrate *p*-nitrophenyl acetate (PNPA) (14). PNPA stock (4 μl of 200 mM in acetonitrile) was diluted in 800 μl 0.5 M sodium phosphate buffer (pH 7.4) in a spectrophotometer cuvette. Supernatant (40 μl) was pipetted into the cuvette and the rate of the reaction was measured for 2 min at 405 nm. For DEF inhibition assays, 25 μM DEF in ethanol was added to 40 μl sample and incubated on ice 10 min before testing. An extinction coefficient of $6.53\text{ mM}^{-1}\text{ cm}^{-1}$ was used to convert the absorbance to micromoles nitrophenol produced over 2 min. Blanks contained buffer alone (0.5 M sodium phosphate, pH 7.4) in place of supernatant. Protein concentration was estimated and used to convert activity to specific units (μmol 4-nitrophenol produced/min/mg protein). Activities were statistically compared using the Fisher least-significant difference (LSD) test ($\alpha = 0.05$, Ref. 17).

Native polyacrylamide gel electrophoresis (PAGE) and DEF inhibition. This method was modified from Scharf *et al.* (14). For each strain, 105,000-g supernatants from preparations were loaded onto 10% polyacrylamide gels with 4% stacking gels in a Bio-Rad Protein Mini Gel System (Richmond, CA). Equal amounts of protein, 50 μg mixed 1:1 with native PAGE sample

buffer, were loaded for all samples and electrophoresed in electrode buffer (242 mM Tris, 173 mM glycine) at 150 V. After electrophoresis, the gels were incubated in 100 ml of 200 mM sodium phosphate buffer (pH 6.8) containing 30 mM each α -naphthyl and β -naphthyl acetate (NA) for 5 min. Subsequently, 25 mg Fast Blue B salt (in 1.0 ml water) was added to visualize esterase bands. After the bands became clearly visible, the gels were removed and fixed in 10% acetic acid.

For DEF inhibition, 1 ml of 100 μM DEF (final concentration 1 μM) was added to 100 ml of 200 mM sodium phosphate buffer containing the gel. Five minutes later, α - and β -NA were added and the gels were stained as described above.

Glutathione S-transferase assay. Spectrophotometer cuvettes were filled with 805 μl glutathione solution (10.5 mM in 0.1 M sodium phosphate buffer pH 6.5, 40 μl CDNB (63 mM in methanol), and 5 μl of supernatant from the ultracentrifuged homogenate (14). Blanks contained buffer alone (0.1 M sodium phosphate, pH 6.5) in place of supernatant. The reaction rate was measured at 344 nm for 2 min in a spectrophotometer. Activity was calculated as conjugated glutathione/min/mg protein for the dinitrophenol extinction coefficient of $9.5\text{ mM}^{-1}\text{ cm}^{-1}$. Activities were statistically compared using the Fisher least-significant difference (LSD) test ($\alpha = 0.05$, Ref. 17).

4-CNMA cytochrome P450 assay. Demethylation of the model substrate 4-chloro-*N*-methylaniline was quantified following the method of Kupfer and Bruggerman (20). Five microsomal preparations were made and used as experimental replicates. For each replicate, 50 μl of protein was mixed with 400 μl of reaction mix (10 μM 4-chloro-*N*-methylaniline, 2.5 mM glucose-6-phosphate (G6P), 0.4 unit G6P-dehydrogenase (G6P-dh), 0.5 mM NADP^+ , and 7.5 mM MgCl_2) in a 1.5-ml microcentrifuge tube. The reaction proceeded at 37°C for 10 min in a water bath and was stopped with the addition of 750 μl of PDAB solution (*p*-dimethylaminobenzaldehyde in 1.0 N H_2SO_4). The contents of the tube were

centrifuged at 11,000g at 4°C for 15 min. The product, 4-chloroaniline, was quantified by comparing absorbance at 445 nm to a simultaneously determined standard curve (0–50 μmol). Demethylation activities were statistically compared using the Fisher least-significant difference (LSD) test ($\alpha = 0.05$, SAS Institute 1990; 17).

In vivo penetration. This method was modified from Siegfried *et al.* (21) and Bull *et al.* (22). A dose of [^{14}C]fenvalerate that is sublethal to adult males of resistant and susceptible strains was applied to the ventral thorax in 1 μl acetone (302 nmol; 1013 cpm). The cockroaches were dried briefly under a gentle stream of air and then held in 4.5-ml scintillation vials for 0, 0.5, 4, 8, or 24 h. Individual cockroaches were then transferred to vials containing 3.0 ml of acetone and gently swirled with a vortex mixer for 10 s. Insects were removed and the acetone was allowed to evaporate from the vials. Scintillation cocktail (3 ml) was added for liquid scintillation counting (LSC) of the external radioactivity. To the original vials that held cockroaches was added 3 ml of cocktail which facilitated the counting of radioactivity contained in frass.

To quantify internal accumulation radioactivity, groups of cockroaches above were homogenized by hand briefly in methanol (10 ml) in a glass tissue grinder. Homogenates were transferred to centrifuge tubes, chilled at -5°C , and centrifuged 10 min at 1000g. Solvent extracts were decanted and the precipitated solids were homogenized again with 10 ml of acetone. After centrifuging again (10 min at 1000g), the two solvent fractions were combined and evaporated to 3 ml. The fractions were transferred to scintillation vials and evaporated to dryness. Three milliliters of scintillation cocktail was added, and the internal radioactivity determined. The body tissues contained in the pellets then received 3 ml of scintillation cocktail and were counted for radioactivity. Three replicates for each time interval were conducted and the experiments were repeated three times. For PBO and DEF inhibition studies, 1 μl of PBO (100 $\mu\text{g}/\mu\text{l}$) or DEF (30 $\mu\text{g}/\mu\text{l}$) was applied to the abdomen 1

h prior to thoracic application of [^{14}C]fenvalerate.

In vitro metabolism. Each individual reaction contained reaction mix (1.6 ml 0.1 M sodium phosphate buffer plus 30%, v/v, glycerol (pH 7.5), 30 μl of 2.5 mM G6P, 30 μl of 0.4 unit G6P-dh, 30 μl of 0.5 mM NADP $^+$, 30 μl of 7.5 mM MgCl_2 , and 2026 cpm of [^{14}C]fenvalerate in acetone). Protein (2.0 mg) was added in 200–400 μl and incubated for 120 min at 32°C in a shaking water bath. For *in vitro* synergism studies, 10 μg PBO or 10 μg DEF in 1 μl ethanol was added to protein aliquots and placed on ice for 5 min before [^{14}C]fenvalerate was added.

Solvent extraction methods were modified from Bull and Pryor (22). The metabolism reaction was stopped with 1 N HCl (75 μl). Contents were transferred to a 50-ml polypropylene tube, followed by three rinses of the original container with 1.0 ml acetone. The contents of the tube were homogenized briefly with a Ten Broek homogenizer and transferred to a 50-ml polypropylene tube. The homogenizer was rinsed nine times with 1.0 ml acetone, which was also added to the homogenate to give a total volume of 12 ml. The polypropylene tube was placed at -20°C overnight and then centrifuged at 2000g for 10 min to pellet solids. The supernatant was decanted and 10 μl scintillation counted to confirm the presence of radioactivity. The pellet was resuspended by adding 10 ml of water and vortexing; 10 μl was scintillation-counted and was always determined to contain less than 1% of the total radioactivity added to a given metabolism replicate. Acetone was evaporated from the supernatant, and to the remaining aqueous portion 15 ml dichloromethane was added. Using a wrist-action shaker, the mixture was agitated for 15 min, followed by centrifugation for 5 min at 2000g; both phases were scintillation-counted (the aqueous layer was always determined to essentially be free of radioactivity). The dichloromethane fraction was dried under N_2 at 40°C .

Prior to thin-layer chromatography (TLC), interfering lipid was removed from samples by

passing through a minicolumn (prepared by adding 75 mg silica gel on top of a thin layer of glass wool within a pasteur pipette). To dried samples, 1.0 ml of dichloromethane was added, and after waiting 2 min, the entire volume was loaded onto a minicolumn. The minicolumn was then washed with six, 1.0-ml-vol hexane:acetone [1:1] (Washes 1–4), acetone (Wash 5), and methanol (Wash 6). All radioactivity loaded onto minicolumns was recovered in the wash solutions, as determined by scintillation counting. All washes were collected in the same borosilicate tube and evaporated to dryness under N₂ at 40°C. The samples were redissolved in 20 µl dichloromethane and the entire volume spotted onto TLC plates. Plates were developed in one direction with hexane: acetone:acetic acid [50:50:1] for 45 min, then exposed to X-ray film for 1 month. Metabolites were quantified following the method of Wheelock and Scott (23). Autoradiograms of developed TLC plates were scanned on a Hewlett-Packard Scanjet 4C scanner, and images were printed on a 600 dpi laser printer. For each lane, printed images of metabolite and fenvalerate spots were excised and weighed on a balance sensitive to 10⁻⁵ g. Weights were used to calculate the percentage distribution of radioactivity among fenvalerate and its metabolites for each *in vitro* replicate.

RESULTS AND DISCUSSION

Toxicity test. Table 1 shows the results of fenvalerate topical applications. LD₅₀ for the Munsyana strain is 825-fold higher than in the susceptible strain. In the synergist study, PBO, MGK-264, and DEF substantially reduced resistance levels in the Munsyana strain (12-, 55-, and 10-fold, respectively). Partial suppression of resistance levels by PBO, MGK-264, and DEF suggest the involvement of cytochrome P450 monooxygenases and hydrolytic enzymes. Because resistance to fenvalerate was not completely eliminated by DEF, PBO, or MGK-264, it is likely that additional mechanisms are involved in fenvalerate resistance.

Esterase activity. PNPA hydrolysis activity in the Munsyana strain was significantly higher than the Jwax strain (Fig. 1). After DEF inhibition, there was no significant difference between Munsyana and JWax activity. This result along with the synergistic effect of DEF suggest that esterases may be involved in resistance.

Esterase native PAGE. Native PAGE was used for nonspecific esterase visualization (13, 24–27). The native PAGE results indicate that the difference in esterase activity between the JWax and Munsyana strains is caused by four electromorphs (Fig. 2). Specifically, bands E3

TABLE 1

Lethal Dose Determinations from Topical Applications of Fenvalerate and Fenvalerate plus Synergists, to Insecticide-Susceptible and -Resistant Adult Male German Cockroaches

Strain ^a	Synergist	n ^b	Slope(±SE)	χ ^{2c}	LD ₅₀ (95% CI) ^d	LD ₉₅ (95% CI) ^d	RR ₅₀ ^e
JW	—	150	7.06(1.27)	1.05	0.15(0.14–0.17)	0.26(0.22–0.34)	—
MA	—	150	4.10(1.10)	7.28	125.45(36.8–193.8)	316.06(200.9–>500)	825.00
JW	PBO	150	3.69(0.70)	1.51	0.10(0.08–0.14)	0.30(0.30–0.50)	—
MA	PBO	150	3.24(0.91)	9.80	7.03(0.43–12.31)	22.66(12.76–>500)	70.30
JW	MGK-264	150	5.11(1.10)	0.22	0.73(0.60–0.70)	1.42(1.10–2.10)	—
MA	MGK-264	150	2.15(0.41)	5.17	10.72(8.28–13.60)	62.31(37.72–178.60)	14.99
JW	DEF	150	4.26(0.81)	3.20	0.71(0.61–0.84)	1.65(1.32–2.56)	—
MA	DEF	150	2.78(0.37)	3.07	57.20(47.55–70.26)	221.28(162.73–356.41)	80.56

^a JW, Johnson Wax (susceptible); MA, Munsyana (resistant).

^b Number of insects in the probit analysis.

^c Pearson's chi-square-goodness-of-fit test. All *P* values are >0.100 indicating goodness-of-fit.

^d LD₅₀ and LD₉₅ in microgram insecticide per gram of insect, with 95% confidence intervals.

^e LD₅₀ resistance ratio. Ratios are significant, as described by Robertson and Preisler (18).

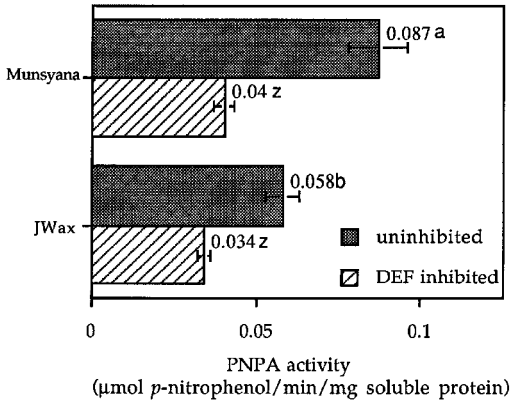


FIG. 1. Comparison of average ($n = 3$) p-nitrophenyl acetate (PNPA) activity from supernatants of susceptible (JWax) and resistant (Munsyana) strains of German cockroach. Means followed by the same letter are not significantly different by the Fisher least-significant difference (LSD) test ($\alpha = 0.05$, SAS Institute 1990).

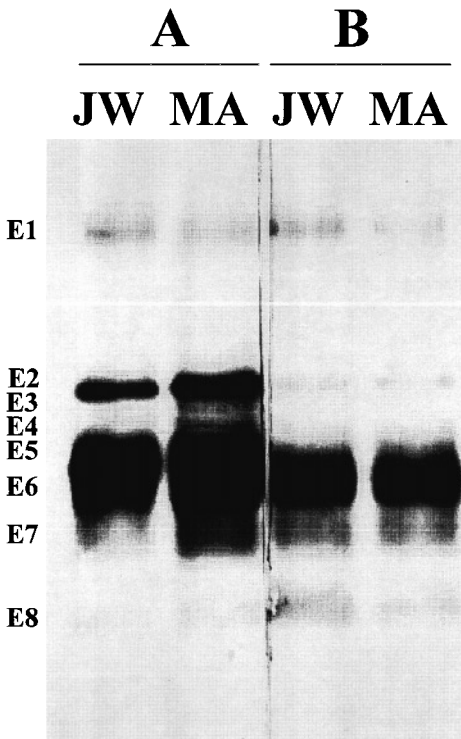


FIG. 2. Native polyacrylamide gel electrophoresis results of (A) uninhibited and (B) DEF-inhibited esterases from *B. germanica*. JW, Johnson Wax strain (susceptible); MA, Munsyana strain (resistant).

and E4 were visible in the Munsyana strain but not in the JWax strain. Bands E2 and E6 were more intense in the Munsyana strain than in the JWax strain. After DEF inhibition, these bands that previously showed elevated activity were not visible.

Glutathione-S-transferase activity. Nearly all the studies of the role of glutathione-S-transferase in insecticide resistance involve various OP-resistant strains of the house fly (28). Hemingway *et al.* (12) reported high frequencies of individuals with elevated GST activity in four German cockroach strains. Results of the present study showed 0.91 mmol/min/mg protein in Munsyana and 0.69 mmol/min/mg protein in the JWax strain, a 1.3-fold elevation in GST activity in the Munsyana strain. Statistical analyses indicated significant differences between the MA and JWax strains ($\alpha = 0.05$).

Cytochrome P450 assay. Our results indicated 2.3-fold higher *N*-demethylase activity in the resistant Munsyana strain than in the susceptible JWax strain (Fig. 3). The lack of *N*-demethylase activity without an NADPH-generating system showed that a cytochrome P450 monooxygenase metabolizes this model substrate. Cytochrome P450 content has also been well-

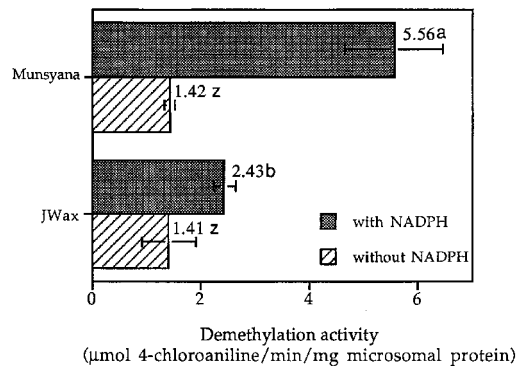
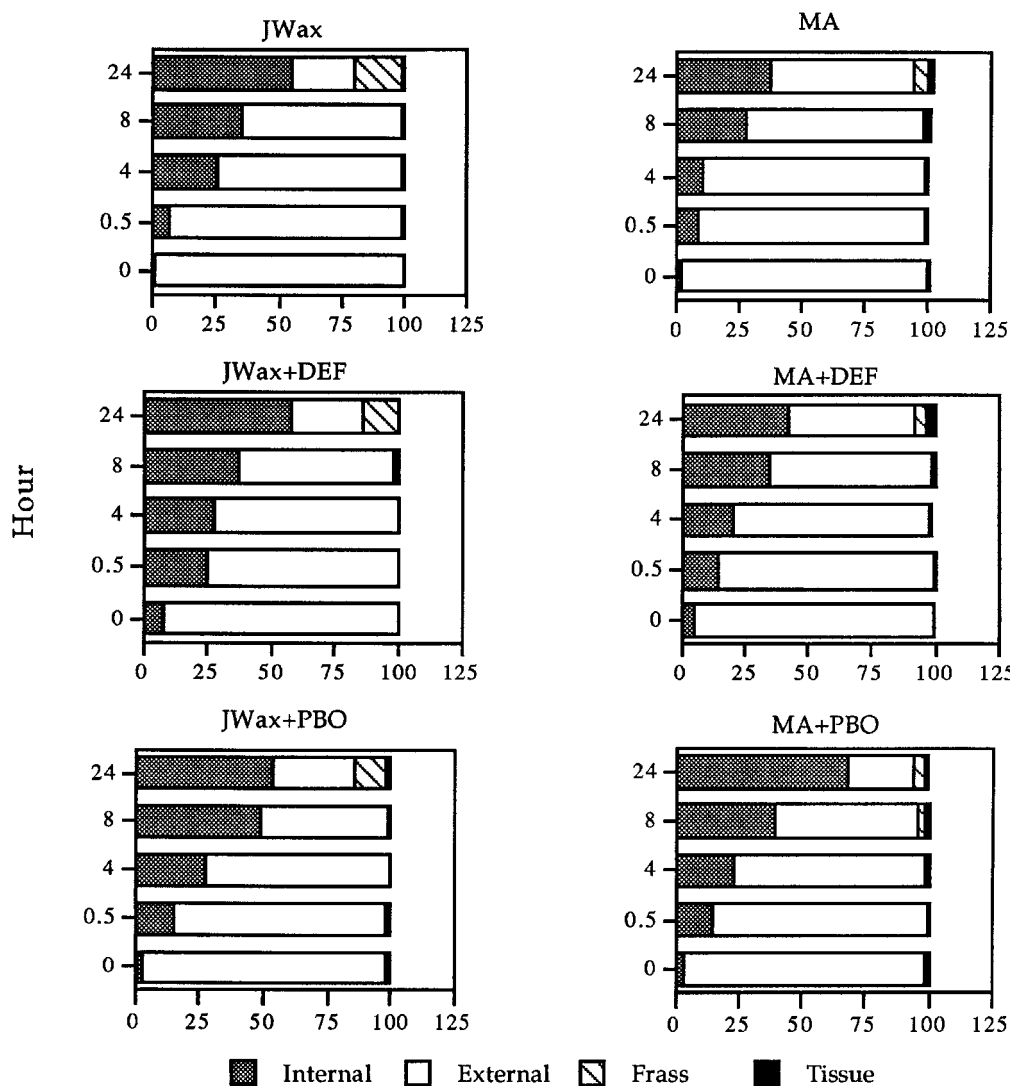


FIG. 3. Comparison of average ($n = 5$) 4-chloro-*N*-methylaniline demethylation activity between susceptible (JWax) and resistant (Munsyana) German cockroach strains. Abbreviation: -NADP, no NADPH-generating system; +NADP, added NADPH-generating system. Means followed by the same letter are not significantly different by the Fisher least-significant difference (LSD) test ($\alpha = 0.05$, SAS Institute 1990).



Percentage distribution of radioactivity

FIG. 4. Distribution of radioactivity (by percentage) after topical treatment of susceptible (JWax) and resistant (Munस्याna) German cockroach with [^{14}C]fenvalerate. Abbreviations: +PBO or +DEF, applied PBO or DEF on the abdomen of insects 1 h prior to fenvalerate.

established to be elevated in the Munस्याna strain by previous investigations (10, 14, 15), and cytochromes P450 appear to have a significant contribution to fenvalerate resistance, as is shown by synergism data. Several reports have found both cytochrome P450 and hydrolytic enzymes

to be elevated in resistant German cockroaches (12, 15, 24, 26, 29, 30). The results of this investigation are consistent with those of previous studies.

In vivo accumulation. Accumulation of

radiolabelled fenvalerate and appearance of absorbed radioactivity in frass were slower in the resistant strain (Fig. 4). Delayed penetration can be a contributing factor in resistance depending on the extent of the interaction of penetration with detoxication mechanisms (31). Slower entry of toxicant into the insect's system might allow different defensive mechanisms (e.g., metabolic degradation or partitioning of unmetabolized toxicant into storage areas) more time to act (32). This study identified statistically significant differences between the resistant (37.6 ± 6.3 , 27.5 ± 4.3 , $10.8 \pm 1.9\%$) and susceptible (54.9 ± 1.7 , 35.2 ± 3.4 , $25.7 \pm 8.7\%$) strains in the internal accumulation of fenvalerate at 24, 8, and 4 h incubation ($\alpha = 0.05$, $n = 9$), but no significant differences in internal accumulation at 0.5 h. The internal accumulation in late time points could be contributed by a combination of penetraton, metabolism, sequestration, and excretion. However, a significant increase in accumulation was observed in the resistant strain (67.8 ± 6.3 , 38.9 ± 1.8 , $23.2 \pm 3.29\%$) at 24, 8, and 4 h, compared to the susceptible strain, following pretreatment with the synergist PBO ($\alpha = 0.05$, $n = 6$) (Fig. 4).

By all rights, increased metabolism should lead to increased excretion. However, there may be some effect of fenvalerate on excretion rate due to sublethal poisoning, as larger amounts of frass were produced in the susceptible strain. For the resistant strain, there was also more internal radioactivity identified after treatment with synergists; and in nonsynergized treatments, more radioactivity was present on the outside of insects, and not in the frass. If there were no penetration differences between fenvalerate and synergist pretreated insects, then radioactivity could be expected to be excreted via the frass at similar rates between resistant and susceptible insects. However, because more radioactivity appeared on the exterior of resistant insects, it is possible that there is some mechanism(s) to excrete either fenvalerate or its metabolites that was not associated with the hind-gut and/or frass.

In vitro metabolism. There were five metabolites visible following incubation of fenvalerate

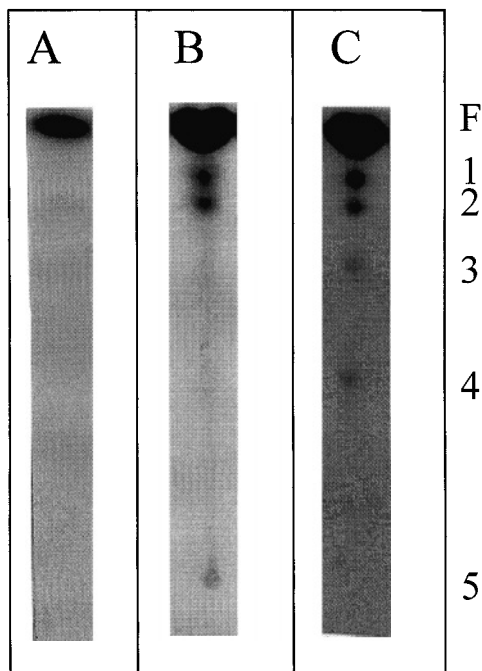


FIG. 5. Representative autoradiograms of [^{14}C]fenvalerate (A) and metabolites following incubation with either resuspended microsomes (B) or 105,000 g supernatants (C). Separations were performed using thin-layer chromatography; by developing in one direction for 45 min with hexane:acetone:acetic acid (50:50:1).

with supernatant fractions (1, 2, 3, 4, and 5; Fig. 5). After inhibition with DEF, unmetabolized fenvalerate quantities increased in the resistant strain and metabolites 3 and 4 substantially reduced in intensity (Table 2). Only three metabolites were identified in *in vitro* microsomal metabolism studies (1, 2, and 5; Fig. 5). In microsomal reactions with no NADPH-generating system, PBO, or DEF inhibition, the amount of unmetabolized fenvalerate increased, while each of the three metabolite quantities decreased (Table 2). It was also observed that the effects of each synergist were more substantial in the resistant Munsyana strain, further supporting that enhanced metabolism is involved in resistance.

Combining all the results from this study, our analyses of the mechanisms of fenvalerate resistance indicate an involvement of cytochromes

TABLE 2

In Vitro Metabolism of [¹⁴C] Fenvalerate by Insecticide-Resistant and -Susceptible German Cockroach Preparations

Strain ^a	n	NADPH	Synergist	Distribution of radioactivity on developed TLC plates (% ± SE) ^b					
				Fenvalerate	1	2	3	4	5
Resuspended microsomes									
JW	3	Yes		61.84(3.96)	16.04(5.61)	13.33 (6.09)	0.0	0.0	8.79(3.17)
MA	3	Yes		52.97(3.92)	15.72(3.79)	17.30(1.43)	0.0	0.0	14.02(6.53)
MA	2	No		67.53(4.31)	14.40(3.88)	11.47(0.42)	0.0	0.0	6.59(0.01)
JW	1	Yes	PBO	64.9	28.86	3.61	0.0	0.0	2.60
MA	3	Yes	PBO	65.44(6.03)	13.01(4.32)	7.95(1.80)	0.0	0.0	13.59(6.00)
JW	1	Yes	DEF	73.68	11.38	10.71	0.0	0.0	4.23
MA	2	Yes	DEF	58.57(11.72)	21.43(8.78)	14.52(5.23)	0.0	0.0	5.47(2.52)
100,000 g supernatant									
JW	2	No		69.58(2.14)	11.00(3.49)	10.22(1.06)	3.74(0.23)	2.70(1.95)	2.77(0.23)
MA	2	No		62.50(3.80)	13.72(1.89)	9.16(2.56)	6.14(0.36)	5.29(0.49)	3.20(0.52)
MA	2	No	DEF	74.22(3.19)	9.59(1.91)	13.95(2.65)	0.0	0.0	1.37

^a JW, Johnson Wax strain (susceptible); MA, Munsyana strain (resistant).

^b Distribution of radioactivity following incubation with either resuspended microsomes or 100,000 g supernatants, following separation on TLC plates. Radioactivity partitioned into 1 of 6 TLC zones: fenvalerate (parent compound), metabolites 1–4, or 5 (polar metabolite at origin). See Fig. 5 for positions of TLC zones.

P450, esterases, decreased penetration and suggest the involvement of glutathione- *S*-transferases. It may be possible that both microsomal and cytosolic esterases (26) play a role in fenvalerate metabolism; however, if DEF is inhibiting microsomal cytochromes P450 (33) this would not be the case. Regardless, these mechanisms likely only play a part in the overall resistance. Dong (34) suggested that a single amino acid change from Leu-993 to Phe-993 in the para sodium channel gene is also responsible for the *kdr*-type resistance in German cockroach. Miyazaki *et al.* (35) also similarly reported cloning and sequencing of the para-type sodium channel gene from susceptible and *kdr*-resistant German cockroaches. The overall structure of the open reading frame region of this *B. germanica* gene is very similar to that of the para gene of *Drosophila melanogaster*. A recent study showed the *kdr*-type nerve insensitivity resistance mechanism is present in the Munsyana strain (36).

In summary, enhanced metabolism, reduced penetration, and sodium channel insensitivity have now been identified in the Munsyana strain. While the specific role of each of these factors in the 825-fold fenvalerate resistance remains unclear, it is likely that more than one of these mechanisms is responsible.

ACKNOWLEDGMENTS

We thank Du Pont for their generous gift of [¹⁴C]fenvalerate; Dean Brad for insect rearing and technical assistance; Dr. Jeff Stuart and Dr. Ron Coolbaugh for the manuscript review. This is Journal Paper Number 15578 of the Agriculture Research Program of Purdue University, West Lafayette, Indiana.

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