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P450 Monooxygenases Are an Important Mechanism of Permethrin Resistance in *Culex quinquefasciatus* Say Larvae

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Culex quinquefasciatus **Say from Saudi Arabia (JPal-per) showed high levels of resistance (2,500-fold) to permethrin. A major contribution of cytochrome P450 monooxygenases as a mechanism of resistance was suggested by the large synergistic effects of oxidase inhibitors such as 2-propynyl 2,3,6-trichlorophenyl ether (PTPE) and piperonyl butoxide (PBO) on the toxicity of permethrin in the resistant strain. Contents of cytochrome P450 and b_5 were about 2.5 times higher in the JPal-per strain than those in the susceptible strain. P450 monooxygenase in the microsomes of gut and other parts of the body metabolized permethrin to 4´-HO-permethrin and other metabolites. Degradation of permethrin by microsomal P450 enzymes was much greater in the resistant JPal-per strain and was inhibited by PBO and PTPE. In addition to a kdr type mechanism which seems to work in the larvae, cytochrome P450 monooxygenase is clearly shown to play an important role as a mechanism of permethrin resistance in JPal-per strain larvae. Arch. Insect Biochem. Physiol. 37:47–56, 1998.** © 1998 Wiley-Liss, Inc.

Key words: resistance; pyrethroid; permethrin; mosquito; ***Culex quinquefasciatus*****; metabolism**

INTRODUCTION

Culex quinquefaciatus Say is a predominant house resting mosquito in many tropical countries. It is important as a vector of filariasis in some countries as well as a nuisance mosquito. Mosquitoes breed in polluted waters such as blocked drains, damaged septic tanks, or soak-

Abbreviations used: DEF = *S*-*S*-*S*-tributylphosphorotrithioate; DMC = 1,1-bis (*p*-chlorophenyl) ethanol; DTT = threo-1,4-dimercapto-2,3-butanediol; *p*-APMSF = *p*-amidinophenyl-methanesulfonyl fluoride hydrochloride; PB acid = 3-phenoxy-benzyl acid; PB alc. = 3-phenoxy-benzyl alcohol; PBO = piperonyl butoxide; PTPE = 2-propynyl 2,3,6-trichlorophenyl ether; PTU = 1-phenyl-2-thiourea.

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age pools close to human habitations. The use of insecticides in the control of larvae has resulted in the development of resistance to organophosphates and carbamates (Bisset et al., 1990; Peiris and Hemingway, 1990; Khayrandish and Wood, 1993a).

Photostable pyrethroid insecticides have been widely used to control agricultural pests and vectors of disease since about the middle of the 1970s. This extensive use of pyrethroids brought the development of resistance in many insects of agriculturally and medically important insects (Liu et al., 1981; Scott and Georghiou, 1985; Pree et al., 1990; Sheppard and Joyce, 1992), including mosquitoes (Umeda et al., 1990; Bisset et al., 1991). Two major mechanisms of resistance to pyrethroids are well known. The first is low nerve sensitivity to chemicals, the so-called kdr type mechanism (Shono, 1985; Osborne and Pepper, 1992), and the second is high activity of P450 monooxygenases. Increased activity of P450 monooxygenase leads to the detoxification of insecticides and thus works as a mechanism of resistance for many classes of insecticides including pyrethroids in a large number of insects (Wilkinson, 1983; Brattsten et al., 1986; Scott and Wheelock, 1992).

There is one study on P450 monooxygenase as a mechanism of resistance in mosquito larvae (Shrivastava et al., 1970). In this study carbamate-resistant *Culex pipiens fatigans* metabolized more propoxur to hydroxy and *N*-demethyl metabolites than the susceptible strain. P450 monooxygenases have been implicated as a mechanism of resistance of pyrethroids in mosquitoes on the basis of results with synergists (Priester and Georghiou, 1978; Kumar et al.,1991). However, there are no reports of pyrethroid metabolism studies in mosquitoes even though pyrethroid insecticides have been shown to be metabolized by P450 monooxygenase in other insects (Shono et al., 1979). The present work was undertaken to clarify the role of P450 monooxygenase in permethrin resistance by examining synergist activity and in vitro metabolism of the chemical in larvae of resistant *Culex quinquefasciatus* collected from Saudi Arabia (Amin and Hemingway, 1989).

MATERIALS AND METHODS

Mosquito Strains

The pyrethroid-resistant strain of *Culex quinquefasciatus* Say was obtained from Dr. J. Hemingway (College of Cardiff, University of Wales). This strain was collected in Saudi Arabia and selected by permethrin for 20 consecutive generations at a mortality level of 60–75% (Amin and Hemingway, 1989). The susceptible strain was collected in Chichijima, Ogasawara Islands, Japan, in 1968 and cultured without exposure to insecticides. Larvae were fed a ground diet of rat pellets (Oriental Yeast Co., Ltd., Tokyo, Japan). Adults were maintained on 10% sucrose, and females were given blood meals from a mouse. Both strains were reared at 27 ± 1°C with a photoperiod of 16:8 (L:D)h.

Chemicals

The following insecticides were used: permethrin (91.2%, *cis:trans* = 55:45), *p,p´*-DDT* (>90%), fenitrothion (99.9%), profenofos (95.0%), parathion (>90%), carbaryl (95.0%), and pyriproxyfen (96.6%). [^{14}C] (1RS)-*trans*-permethrin (sp. act. 57 mCi/m mol, purity >99%) labeled at the α-carbon of phenoxy benzyl group (Shono et al., 1979), and the following authentic standards were generously provided by Sumitomo Chemical Co., Ltd. (Osaka, Japan): 4´-hydroxy-3-phenoxy-benzyl 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-carboxylate (4´-HO-permethrin), 3-phenoxy-benzyl alcohol (PB alc.), and 3-phenoxy-benzyl acid (PB acid). Piperonyl butoxide (PBO) was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). *S,S,S*-tributyl phosphorotrithioate (DEF) and 2-propynyl 2,3,6-trichlorophenyl ether (PTPE) were kindly provided by Nihon Bayer Agrochem K.K. (Yuki, Japan). DMC, 1,1-bis (*p*-chlorophenyl) ethanol, was a gift from Nippon Soda Co. (Kanagawa, Japan). All other chemicals used were of the highest grade commercially available.

Larval Bioassay

Larval bioassays were carried out using standard bioassay techniques for mosquito larvae (WHO, 1981). Twenty to thirty early fourth instar larvae were exposed to different concentrations of insecticides in 50 ml of distilled water. The mortality was counted after a 24 h exposure period (except for pyriproxyfen) at 27 ± 1°C. Alcohol solution of insecticides were added to the water, but DDT and pyriproxyfen were dissolved in acetone and distilled water, respectively. The alcohol and acetone concentration never exceeded 1% of the total volume. Controls were run with alcohol or acetone alone. At least three replicates were run for each insecticide concentration. LC_{50} values for each strain and for each insecticide were calculated using log-probit mortality regression analysis (Finney, 1971). The re-

sistance ratio for each insecticide was calculated by dividing the LC_{50} values of the resistant strain by that of the susceptible strain. For determining the mortality to pyriproxyfen, we exposed the larvae until all of them emerged or died, and the mortalities were expressed as the percentage of emergence inhibition.

For estimating the synergistic effects, the larvae were tested with permethrin, fenitrothion, and DDT in combination with PBO, PTPE, DEF, or DMC. The mortality of *C. quinquefasciatus* larvae to each synergist was studied to determine its toxicity to the insect. We selected the highest concentration of synergists that caused no mortality to mosquito larvae to maximize the potential for synergism (Scott, 1990). Although the maximum sublethal concentration of PBO to the resistant strain (JPal-per) was 5.0 μg/ml, we examined also 0.5 μg/ml, which was the maximum sublethal concentration of the susceptible strain, and 1.0 μg/ml.

Assay of Carboxylesterase Activity

The activity of carboxylesterase was assayed with the Gomori method (1953) as modified by van Asperen (1962) for houseflies. Two noninsecticidal substrates, α-and β-naphthyl acetate were used. Larvae were homogenized in Tris-HCl buffer (0.01 M, pH 8.0) and centrifuged at 10,000*g* for 15 min, and the supernatant fraction was used as an enzyme source. The reaction mixture (3 ml) consisted of 0.02 ml of naphthyl acetate (0.03 M), 1.0 ml of supernatant, and 1.8 ml buffer and was incubated at 30°C. The reaction was stopped at different time intervals, and color was developed by the addition of 1.0 ml water solution of 1.0% fast violet BB salt and 5.0% sodium lauryl sulfate. The absorbances were read at 600 nm for α-naphthyl acetate and 550 nm for β-naphthyl acetate with a Shimadzu UV-160A (Kyoto, Japan) spectrophotometer. The esterase activity was determined by α- and β-naphthol production based on a standard curve.

Preparation of Microsomes

The fourth instar larvae of *C. quinquefasciatus* were dissected and separated into the gut and the other body parts. Contents of the gut were removed, because midgut contents inhibit P450 monooxygenase activity (Kasai, unreported data). Several hundred midguts and body parts were homogenized in 2–3 ml of 0.1 M sodium phosphate buffer (pH 7.5) containing 1 mM EDTA, 1 mM (*p*-amidinophenyl)-methanesulfonyl fluoride hydrochloride (*p*-APMSF), 0.1 mM dithiothreitol (DTT), and 1 mM 1-phenyl-2-thiourea (PTU) (dissolved in ethanol) (Ronis et al., 1988). The homogenate was centrifuged at 10,000*g* for 15 min, and supernatant was centrifuged again at 110,000*g* for 1 h. The microsomal pellet obtained was resuspended in 6 ml of sodium phosphate buffer (pH 7.5, 0.1 M) containing 1 mM EDTA, 0.1 mM DTT, and 1 mM *p*-APMSF. All the procedures were carried out at 4°C, and extracts were used on the same day of preparation.

Protein Determination

Protein was determined according to the method of Bradford (1976). Bovine serum albumin was used as a standard.

Cytochrome P450 Levels

Cytochrome P450 and b_5 contents in larval microsomes of whole body were quantitatively analyzed in triplicate using the dithionite reduced carbon monoxide difference spectrum, according to the method of Omura and Sato (1964). After treating the test sample with a small amount of sodium dithionite, the cytochrome b_5 spectrum was obtained at 400–500 nm using a Shimadzu UV-160A spectrophotometer. Thereafter the reference sample was also treated with sodium dithionite to obtain the baseline absorbance before the spectrum for cytochrome P450 was obtained. The test sample was then gently bubbled with CO for 30 s and rescanned at 400–500 nm to get the spectrum for cytochrome P450. The extinction coefficient for the P450 dithionite-reduced CO spectrum was taken as 91 mM cm^{-1}. Cytochrome b_5 contents were measured from the oxidized vs. dithionite-reduced difference spectrum using an extinction coefficient of 185 mM cm^{-1} between 423 and 409 nm (Ronis et al., 1988).

In Vitro Metabolism

In vitro metabolism was studied by the modified method of Shono et al. (1979). Each incubation mixture in a 10 ml glass tube (PYREX; Iwaki Glass, Chiba, Japan) contained 0.8 ml of 0.1 M sodium phosphate resuspension buffer, 1 ml of enzyme (microsome or supernatant) equivalent to 50 guts or the other body parts, 0.2 ml of 10 mM β-NADPH, and approximately 110,000 dpm (0.34 μg) of [^{14}C] permethrin in 10 μl of ethanol. An incubation mixture without β-NADPH served as the control. For inhibition studies, 10 μl of PBO, PTPE, or DEF (0.1 mM final concentration) or paraoxon (0.01 mM final concentration) were added to the mixture.

The mixtures were incubated for 2 h at 25°C with shaking, and then incubation was terminated by adding 0.2 ml of 1 N HCl and 1.0 g of $(NH_4)SO_4$. The incubation mixtures were extracted three times with 4 ml aliquots of diethyl ether. The ether extracts were dried with Na_2SO_4 (0.5 g) overnight. The extracts were dried under N_2 and adjusted to 10,000 dpm/10 µl by methanol. The extraction efficiency was approximately 90% under the conditions stated. Ten microliters of each sample were spotted on TLC plates (Merck (Darmstadt, Germany) plates of silica gel 60 F254 [20 x 20 cm; layer thickness, 0.25 mm]) and developed in TEM solvent (toluene/ethyl acetate/methanol, 15:5:1) with unlabelled authentic compounds. Unlabelled authentic compounds were viewed under ultraviolet light (254 nm). Radioactive spots were identified by autoradiography for 12 h using a BAS-III Fuji Imaging Plate (Fuji Photo Film Co., Ltd., Tokyo, Japan), and areas of radioactivity were located and quantified using a Bas 2000 Bio Image analyzer. The relative amounts of radioactivity were determined using a standard curve. Metabolites were identified by cochromatography with the authentic compounds.

RESULTS

Bioassay

The toxicities of permethrin and the effects of PBO, PTPE, and DEF against fourth instar larvae are presented in Table 1. The JPal-per strain showed high resistance to permethrin (2,500-fold) compared to the susceptible strain. The involvement of oxidases and esterases in permethrin resistance were investigated by using their inhibitors as synergists. The synergistic effects of three different concentrations of PBO (0.5, 1.0, 5.0 µg/ml) on permethrin were tested with the JPal-per strain, but for the susceptible strain only the lowest concentration of PBO (0.5 µg/ml) was used because of high control mortality at the other two concentrations. The PBO showed a significant effect on permethrin toxicity in both strains (Table 1). The resistance ratios for permethrin were reduced from 2,500-fold to 550-, 180-, and 43-fold with 0.5, 1.0, and 5.0 µg/ml of PBO, respectively. The mortality regression line of the JPal-per strain shifted to the left as the concentration of PBO increased (Fig. 1). Another P450 monooxygenase inhibitor, PTPE (Casida, 1970; Brown et al., 1996), also decreased the resistance level from 2,500-fold to fifteen fold when larvae were treated at the maximum sublethal concentration (i.e., 4 µg/ml for the JPal-per and 1 µg/ml for the susceptible strain). Tests with permethrin plus the esterase inhibitor, DEF, showed that the resistance level remained high (1,300-fold), and this indicates that the contribution of esterases to the resistance of permethrin is very low or minimal.

The toxicities of six insecticides belonging to organochlorine, organophosphate, carbamate, and insect growth regulator (IGR) groups are shown in Table 2. The JPal-per strain showed 300-fold resistance to DDT, but oxidase inhibitors (PBO, PTPE) or dehydrochlorinase inhibitor (DMC) did not enhance the toxicity of DDT significantly, suggesting that there is no detoxification system involved in DDT resistance of JPal-per strain. Modest cross-resistance was observed to organophosphate and carbamate insecticides in the JPal-per larvae, and no cross-resistance was found to an IGR insecticide, pyriproxyfen.

TABLE 1. Permethrin Toxicities to the Resistant JPal-Per and the Susceptible *Culex quinquefasciatus* Larvae and Synergistic Effects of PBO, PTPE, and DEF

	Susceptible (S)				JPal-per				
Insecticide	n[a]	Slope ± SE	LC_{50} (µg/ml)	95% CL	n	Slope ± SE	LC_{50} (µg/ml)	95% CL	RR[b]
Permethrin	287	5.3 ± 0.02	0.0040	0.0030–0.0040	290	2.6 ± 0.33	10.0	8.4–12	2,500
+ PBO (0.5 µg/ml)	404	3.2 ± 0.72	0.00044	0.00024–0.00065	295	2.6 ± 0.19	0.24	0.20–0.30	550
+ PBO (1.0 µg/ml)	—	—	—	—	333	2.7 ± 0.13	0.079	0.065 –0.096	180[c]
+ PBO (5.0 µg/ml)	—	—	—	—	508	3.2 ± 0.11	0.019	0.017–0.023	43[c]
+ PTPE[d]	258	4.6 ± 1.10	0.0046	0.0031–0.0051	420	3.2 ± 0.17	0.071	0.064–0.080	15
+ DEF (1.0 µg/ml)	767	5.0 ± 0.43	0.0031	0.0030–0.0033	540	1.0 ± 0.10	3.9	3.0–5.1	1,300

[a]Total number of larvae used.
[b]Resistance ratio = LC_{50} (JPal-per) / LC_{50} (S).
[c]LC_{50} (JPal-per)/ LC_{50} (S + PBO 0.5 µg/ml).
[d]Treated 4.0 µg/ml to JPal-per, 1.0 µg/ml to S strain.

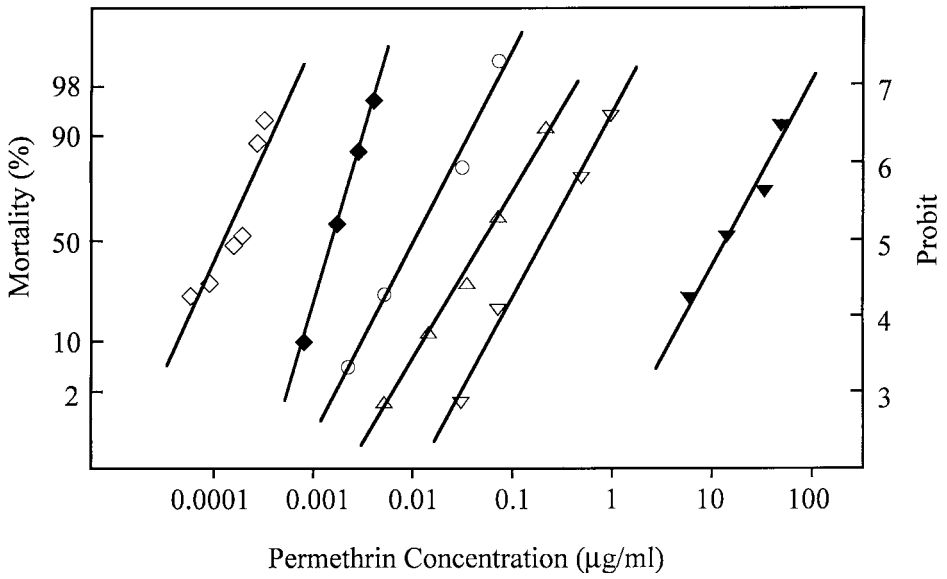

Fig. 1. Dosage-mortality regression lines for permethrin alone and with PBO against susceptible (S) and resistant JPal-per larvae of *C. quinquefasciatus*. ◆, S permethrin only; ◇, S + PBO 0.5 μg/ml; ▼, JPal-per permethrin only; ▽, JPal-per + PBO 0.5 μg/ml; △, JPal-per + PBO 1.0 μg/ml; ○, JPal-per + PBO 5.0 μg/ml.

Esterase Activity

The activity of esterases was measured as μmol/min/larva and μmol/min/mg protein and the results are given in Table 3. The JPal-per strain was shown to have elevated esterase activity against both α- and β-naphthyl acetate compared with the susceptible strain. The JPal-per strain showed 5.3- and 5.2-fold activity per larva and per milligram of protein, respectively, for α-naphthyl acetate, while for β-naphthyl acetate the activity was 5.7- and 5.6-fold, respectively. High activity of carboxylesterase in the

TABLE 2. Toxicities of Various Insecticides With and Without Synergists to the Resistant JPal-Per and the Susceptible *Culex quinquefasciatus* Larvae

	Susceptible (S)				JPal-per (R)				
Insecticide	n[a]	Slope ± SE	LC_{50} (μg/ml)	95% CL	n	Slope ± SE	LC_{50} (μg/ml)	95% CL	RR[b]
Organochloride									
DDT	504	4.2 ± 0.40	0.42	0.39–0.45	343	3.8 ± 0.81	124	94.0–159	300
DDT + PBO[c]	585	5.1 ± 0.60	0.44	0.39–0.50	390	2.9 ± 0.52	70.5	55.4–103	160
DDT + PTPE[d]	514	4.2 ± 0.29	0.17	0.16–0.19	869	2.8 ± 0.28	141	122–158	830
DDT + DMC (1.0 μg/ml)	438	7.4 ± 0.67	0.14	0.13–0.15	573	3.9 ± 0.42	86.9	79.7–94.7	620
Organophosphate									
Fenitrothion	389	11.5 ± 1.4	0.0048	0.0043–0.0054	575	7.3 ± 1.4	0.024	0.021–0.031	5.0
Fenitrothion + DEF	416	3.7 ± 0.09	0.0007	0.00063–0.00076	757	5.9 ± 1.0	0.0056	0.0052–0.0065	8.0
Profenofos	474	8.8 ± 2.0	0.0054	0.0048–0.0061	318	7.8 ± 0.22	0.026	0.025–0.028	4.8
Parathion	425	12.5 ± 0.66	0.0028	0.0026–0.0029	264	7.1 ± 2.2	0.013	0.011–0.019	4.6
Carbamate									
Carbaryl	632	3.5 ± 0.53	0.20	0.15–0.26	394	6.5 ± 0.97	1.27	1.09–1.53	6.4
IGR									
Pryiproxyfen	890	2.0 ± 0.28	0.083[e]	0.063–0.12	895	2.7 ± 0.30	0.15[e]	0.12–0.19	1.8

[a]Total number of larvae used.
[b]Resistance ratio = LC_{50} (R)/LC_{50} (S)
[c]Treated 5.0 μg/ml to R, 0.5 μg/ml to S strain.
[d]Treated 4.0 μg/ml to R, 1.0 μg/ml to S strain.
[e]Expressed as ng/ml.

TABLE 3. Carboxylesterase Activities in the Resistant JPal-Per and the Susceptible *Culex quinquefasciatus* Larvae*

	μmol/min/larva ± SE			μmol/min/mg ± SE		
Substrate	Susceptible	JPal-per	Ratio	Susceptible	JPal-per	Ratio
α-naphthyl acetate	0.052 ± 0.001	0.274 ± 0.002	5.3	0.439 ± 0.009	2.30 ± 0.01	5.2
β-naphthyl acetate	0.035 ± 0.003	0.200 ± 0.007	5.7	0.300 ± 0.027	1.68 ± 0.06	5.6

*All values are mean ± SE of three replicates.

JPal-per strain may contribute to modest resistance to organophosphates and carbamate insecticides.

Cytochrome P450 and b_5 Levels

Levels of cytochrome P450 and cytochrome b_5 in whole body of both susceptible and resistant larvae are shown in Table 4. The cytochrome P450 content of JPal-per was 2.46 times higher than that of the susceptible strain. JPal-per strain also showed 2.60-fold higher content of cytochrome b_5 than that of the susceptible strain.

In Vitro Metabolism of Permethrin

Metabolism of permethrin was studied using microsomes of guts and other body parts of larvae with and without NADPH. The data in Table 5 clearly show that JPal-per microsomes metabolized *trans*-permethrin at a much higher rate than the susceptible strain. In the presence of NADPH, 62.0 and 46.2% of permethrin were metabolized by microsomal fraction of gut and the other body parts, respectively, in the JPal-per strain, whereas 12.1 and 2.3% were metabolized by gut and other body parts, respectively, of the susceptible strain. In both strains, the activity of permethrin degradation was higher in the gut microsome than that of the other body parts. Metabolites, 4´-HO-permethrin, PB alc., and PB acid were identified by cochromatography with authentic standards. The major metabolite in the JPal-per was 4´-HO-permethrin, which was also found in small quantities in the susceptible strain. Large amounts of metabolite "unknown 1" were also found in the gut and the other body parts of JPal-per strain. Unknown 1 and 2 metabolites were not identified because other authentic standards were not available. Less than 10% of permethrin was metabolized in all of the microsomal incubations without addition of NADPH.

The gut supernatant of both strains produced large amounts of metabolites, whereas small amounts of metabolites were formed by supernatant from the body parts other than the gut (Table 6). Total metabolites produced by the gut supernatant were 19.0% and 24.6% from susceptible and JPal-per strains, respectively. There were consequently no meaningful differences between the two strains in permethrin degradation by gut supernatant fractions. Differences between the two strains in the amounts of PB alc. and PB acid produced were not great because PB alc. can be easily converted to PB acid during the analytical process.

Addition of cytochrome P450 inhibitors, PBO or PTPE, to the enzyme mixture inhibited the activities of gut microsomal enzymes in the JPal-per strain, and thus the metabolized permethrin decreased to less than 10% (Table 7). Furthermore, the amount of all metabolites decreased by addition of PBO and PTPE. Paraoxon slightly inhibited microsomal metabolism of permethrin. Permethrin degradation by soluble fractions was extremely inhibited by esterase inhibitors, DEF, and paraoxon.

TABLE 4. Contents of Cytochrome P450 and b_5 in Whole Body of Resistant JPal-Per and the Susceptible Larvae of *Culex quinquefasciatus**

	nmol/mg protein		
Components	Susceptible	JPal-per	Ratio
Cytochrome P450	0.0667 ± 0.0054	0.164 ± 0.0098	2.46
Cytochrome b_5	0.0392 ± 0.0024	0.102 ± 0.0082	2.60

*All values are mean ± SE of three replicates.

DISCUSSION

It is well established that cytochrome P450 monooxygenase plays a very important role as a mechanism of resistance to various insecticides in many resistant species of pest insects (Wilkinson, 1983; Brattsten et al., 1986). The higher activity of the enzyme is usually demonstrated by in vitro studies using microsomal fractions of insect homogenates. Contribution of P450 enzymes to resistance in mosquito larvae has, however, mostly been shown indirectly by the synergistic effects of the P450 monooxygenase inhibitor, PBO, in toxicity tests (Priester and Georghiou, 1978; Kumar et al., 1991; Khayrandish and Wood, 1993b). When in vitro metabolism studies have been un-

TABLE 5. Metabolism of ^{14}C-Permethrin by Microsomal Fractions of the Resistant JPal-Per and the Susceptible *Culex quinquefasciatus* Larvae*

	Susceptible				JPal-per			
	Gut		Other body parts		Gut		Other body parts	
Metabolite	NADPH –	+	–	+	–	+	–	+
Permethrin	98.1 (0.35)	87.9 (0.58)	99.1 (0.03)	97.7 (0.12)	94.0 (0.94)	38.0 (1.66)	98.7 (0.13)	53.8 (0.55)
Unknown 1	—	3.5 (0.20)	—	—	—	12.2 (0.30)	—	19.8 (0.15)
4´-HO-permethrin	—	4.9 (0.06)	—	1.8 (0.09)	—	15.9 (0.45)	—	13.0 (0.25)
PB alcohol	—	—	—	—	—	3.3 (0.03)	—	2.3 (0.00)
Unknown 2	—	—	—	—	—	2.8 (0.06)	—	1.7 (0.12)
PB acid	1.2 (0.15)	1.7 (0.33)	0.9 (0.03)	0.5 (0.06)	2.9 (0.46)	8.9 (0.20)	—	2.5 (0.15)
Origin	0.7 (0.20)	2.0 (0.43)	—	—	3.1 (0.52)	18.9 (0.80)	1.3 (0.13)	6.9 (1.02)
% of total metabolites	1.9	12.1	0.9	2.3	6.0	62.0	1.3	46.2

*Results are expressed as a percentage of the recovered dose. All values are mean of three replicates (± SE in parentheses).

dertaken, whole body homogenates have been used as the enzyme source (Shrivastava et al., 1970, 1971) instead of the microsomal fraction which is commonly utilized in most animals. Whole body homogenates of mosquito larvae are thought to bring insufficient oxidase activity because of the existence of oxidase inhibitors in the midgut contents and no separation of the microsomal fraction. Removal of the midgut contents and separation of microsomal fractions in our experiments clearly demonstrated in vitro metabolism of permethrin by P450 monooxygenase in mosquito larvae. Increased degradation of permethrin by micorsomal fractions of gut and other body parts of JPal-per larvae in the presence of NADPH distinctly indicated P450 monooxygenase works as a mechanism of resistance in the JPal-per strain of this insect.

Cytochrome P450 and b_5 contents in the resistant JPal-per strain were about 2.5 times higher than that of the susceptible strain (Table 4). Permethrin in the presence of NADPH was metabolized 12.1% and 62.0% by the gut microsomes of the susceptible and the resistant strains, respectively, and 2.3% and 46.2% by the microsomes from the other body parts of the susceptible and resistant strains, respectively (Table 5). The P450 monooxygenase of the JPal-per strain metabolized five times more and about 20 times more permethrin in the gut and in the other body parts than the susceptible strain, even though the microsomes of JPal-per larvae contained only about 2.5 times as much cytochrome P450 and b_5. The pyrethroid-resistant LPR strain of the housefly contains $P450_{lpr}$, which effectively metabolizes pyrethroid insecticides and works as one of the major mechanisms for pyrethroid resistance in this strain (Wheelock and Scott, 1992). The amount of $P450_{lpr}$ reached 68% of total P450 in the LPR housefly, but the amount of $P450_{lpr}$ in the susceptible housefly was only 6.5% of the total P450 (Wheelock and Scott, 1990). It may be concluded that an isozyme of cytochrome P450 which is responsible for metabolism of an insecticide is overexpressed in the insects which show

TABLE 6. Metabolism of ^{14}C-Permethrin by Supernatant Fractions of the Resistant JPal-Per and the Susceptible *Culex quinquefasciatus* Larvae*

	Susceptible		JPal-per	
	Gut	Other	Gut	Other
Permethrin	81.0 (0.90)	98.9 (0.06)	75.4 (0.15)	96.3 (0.12)
Unknown 1	—	—	—	—
4´-HO-permethrin	—	—	—	—
PB alcohol	2.9 (0.23)	0.3 (0.03)	20.0 (0.12)	1.3 (0.22)
Unknown 2	—	—	—	—
PB acid	16.1 (0.66)	0.8 (0.00)	4.1 (0.07)	1.4 (0.22)
Origin	—	—	0.5 (0.12)	1.0 (0.18)
% of total metabolites	19.0	1.1	24.6	3.7

*Results are expressed as a percentage of recovered radioactivity. All values are mean of three replicates (± SE in parentheses).

TABLE 7. Effect of Synergists on ^{14}C-Permethrin Metabolism in the JPal-Per Strain of *Culex quinquefasciatus* Larvae*

	Gut (microsome) + NADPH				Gut (supernatant)		
Metabolite	Control	PBO	PTPE	Paraoxon	Control	DEF	Paraoxon
Permethrin	38.0 (1.66)	90.3 (0.74)	94.6 (0.87)	54.5 (0.42)	75.4 (0.15)	97.0 (0.12)	97.8 (0.12)
Unknown 1	12.2 (0.30)	5.5 (0.49)	0.7 (0.06)	13.3 (0.18)	—	—	—
4´-HO-permethrin	15.9 (0.45)	1.2 (0.12)	—	7.6 (0.09)	—	—	—
PB alcohol	3.3 (0.03)	—	—	2.0 (0.07)	20.0 (0.12)	0.4 (0.00)	0.3 (0.03)
Unknown 2	2.8 (0.06)	—	—	2.6 (0.00)	—	—	—
PB acid	8.9 (0.20)	2.1 (0.13)	2.0 (0.03)	5.7 (0.41)	4.1 (0.07)	2.6 (0.10)	1.9 (0.10)
Origin	18.9 (0.80)	0.9 (0.07)	2.7 (0.95)	14.3 (0.26)	0.5 (0.12)	—	—
% of total metabolites	62.0	9.7	5.4	45.5	24.6	3.0	2.2

*Results are expressed as a percentage of radioactivity. All values are man of three replicates (± SE in parentheses).

resistance to the insecticide. The isozyme which metabolizes permethrin probably increased its fraction in the total P450 and brought greater metabolism of permethrin in the JPal-per strain. Purification of the P450 isozyme that elevates permethrin metabolism in JPal-per mosquito larvae is being undertaken now.

It has been reported that esterase also works as a mechanism of pyrethroid resistance (Devonshire and Moors, 1982; Ishaaya and Casida, 1981; Riskallah, 1983). Permethrin degradation of microsomal fractions by esterase is shown as total metabolites with no addition of NADPH (Table 5). The amount of degradation by microsomes of the gut in susceptible and resistant JPal-per strains were 1.9% and 8.9%, respectively. There was a 4.7 times difference between the resistant and susceptible strains, but 8.9% metabolism in JPal-per gut microsomes seems to be inconsequential when total metabolism increased 62% with the addition of NADPH to the same microsomes. Total metabolites of the supernatant fractions of the gut were relatively high (Table 6), but there was no great difference between the resistant and susceptible strains. High carboxylesterase, therefore, measured using α- and β-naphthyl acetate as substrates in the JPal-per strain is thought not to be concerned with permethrin resistance. High carboxylesterase activity in the JPal-per strain originated from the progenitor JPal strain and remained during permethrin selection in the laboratory. The original JPal strain showed resistance to organophosphorus insecticides (Amin and Peiris, 1990), and organophosphate-resistant mosquito larvae usually possess high carboxylesterase activity.

Permethrin resistance in the JPal-per strain was not completely blocked by P450 monooxygenase inhibitors, PBO and PTPE, and an esterase inhibitor, DEF (Table 1). DDT resistance in the JPal-per strain was not affected by either P450 monooxygenase inhibitors or DMC, which inhibits DDT dehydrochlorinase, a major metabolic enzyme of DDT (Table 2). The results of the toxicity tests with metabolic inhibitors as synergists, shown above, suggested that JPal-per larvae have a mechanism other than metabolic enzymes. The kdr-type mechanism (low sensitivity to DDT and pyrethroids in voltage-dependent sodium channel) is a very common mechanism for resistance to the chemicals in many insects (Shono, 1985; Osborne and Pepper, 1992), and this mechanism has also been found in *Culex quinquefasciatus* (Salgado et al., 1983). JPal-per larvae probably possess the kdr-type mechanism for permethrin and DDT resistance in addition to high activity of P450 monooxygenase. It has been confirmed neurophysiologically that adult mosquitos of the JPal-per strain show low nerve sensitivity to permethrin (Umeda and Shono, unreported data).

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