PESTICIDE BIOCHEMISTRY AND PHYSIOLOGY 7, 261–272 (1977)

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Mechanisms of Resistance to the Juvenoid Methoprene in the House Fly *Musca domestica* L.

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Received April 27, 1976; accepted August 3, 1976

The mechanisms of resistance and cross resistance to the juvenoids methoprene and R-20458 in the house fly, *Musca domestica*, were examined. Radiolabeled methoprene was found to be metabolized faster in resistant and cross-resistant house fly larvae than in susceptible larvae, and methoprene and R-20458 penetrated more slowly into larvae of the resistant strain. *In vivo* and *in vitro* metabolism of methoprene was largely by oxidative pathways followed by conjugation in all strains examined, and little or no ester change of methoprene was noted *in vitro*. *In vitro* oxidative metabolism of methoprene, R-20458, juvenile hormone I, and several model substrates was higher in resistant and cross-resistant larvae than in susceptible larvae. Juvenoid functionalities susceptible to metabolic attack by resistant strains are indicated.

INTRODUCTION

The juvenile hormone mimics, or juvenoids, are new compounds which hold promise for the control of dipteran pests and other insect groups. It was hoped that the juvenoids would circumvent resistance to classical pesticides, but cross resistance between classical pesticides and some juvenoids was soon noted (1, 2), and there are now many reports in the literature on cross resistance to juvenoids. At least two laboratories have reported the development of moderate levels of resistance in mosquitoes with juvenoids (3, 4), and, subsequently, extremely high resistance to juvenoids was induced in the house fly, *Musca domestica*, by selection (5). The resistance mechanisms of the house fly, *Musca domestica*, L., to juvenoids were examined by comparing the *in vivo* and *in vitro* metabolism and penetration of the dienoate ester juvenoid, methoprene (Fig. 1) (Zöecon Corp.) (6), and comparing it with the penetration and *in vitro* metabolism of the geranyl phenyl ether juvenoid, R-20458 (Fig. 1) (Stauffer Chemical Co.) (7), in susceptible (S-NAIDM), cross-resistant (R-dimethoate), and resistant (R-methoprene) strains of house flies. The *in vitro* metabolism of juvenile hormone I (JH I)[1] (Fig. 1) was also examined in the three strains. Insect metabolism of juvenoids has been recently reviewed (8), and several previous studies of juvenoid metabolism in *M. domestica* have been made (9–13).

MATERIALS AND METHODS

Chemicals. Methoprene (Altosid, ZR-515, Zöecon Corp.) was labeled with ^{14}C (5 position, 97.9% 2*E*, 5.0 mCi/mmol) or

[1] Abbreviations used: JH, juvenile hormone(s); tlc, thin-layer chromatography; lsc, liquid scintillation counting; hrlc, high resolution liquid chromatography; HEt, hexane–ethyl acetate; PTU, phenylthiourea; and uv, ultraviolet light. Abbreviations utilized for reference standards appear in Fig. 1, and additional tlc solvent systems utilized are listed in the legend to Fig. 2.

ISSN 0048-3575

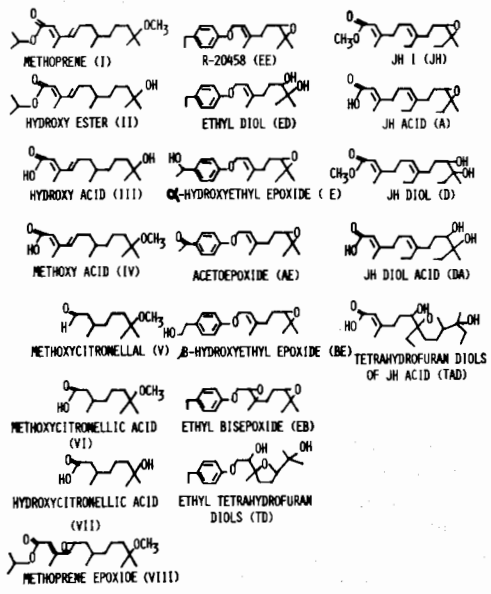

FIG. 1. *Structures of substrates and nonradioactive standards of methoprene, R-20458, and juvenile hormone. Roman numerals or capital letters in parentheses and trivial names are used elsewhere in the text to indicate the appropriate structure. The s-cis diene conformation shown here for methoprene is less important than the s-trans conformation.*

with ^{3}H (10 position, >97% 2*E*, 1960 mCi/mmol) (14). R-20458 (Stauffer Chemical Co.) was ring-labeled either with ^{3}H (>96% 2*E*, 600 mCi/mmol) (15) or with ^{14}C (>96% 2*E*, 17 mCi/mmol) (16), and JH I was ^{3}H-labeled (10 position, >98% 2*E*, 6*E*, 10*Z*, 10 Ci/mmol) (New England Nuclear Corp.). Authentic standards of methoprene metabolites were provided by Zöecon Corp., while standards for R-20458 and JH I were synthesized as described earlier (17, 18).

Chromatography. All thin-layer chromatography (tlc) utilized silica gel F254 chromatoplates (0.25-mm gel thickness, EM Laboratories) developed in one or two dimensions with solvent systems discussed later or shown in Fig. 2. Conjugated or aromatic standards were visualized by their quenching of gel fluorescence under ultraviolet (uv) light, while other standards were visualized by vanillin–sulfuric acid or anisaldehyde–sulfuric acid sprays (19). Thin-layer chromatographic plates were scanned (Berthold LB 2723 tlc scanner) or exposed to X-ray film after coating the tlc plate with a scintillation fluor for ^{3}H (20) for the detection of radioactive metabolites which were quantified by liquid scintillation counting (lsc). For qualitative studies enough radioactivity was applied to the tlc plate to allow the detection of a distinct spot accounting for less than 0.5% of the applied material.

High resolution liquid chromatography (hrlc) employed a Waters Associates (M-6000) or Spectra Physics (M-3500B) pump equipped with a loop injector (50 μl, Valco), and a uv monitor (Spectra Physics, Model 230). Radioactive metabolites or their methyl esters were mixed with cold standards and separated on a prepacked ODS permaphase column (2 × 250 mm, 5 μm DuPont) by eluting with 25% aqueous methanol (12).

Insects. The three strains of *M. domestica* used in this study were reared as previously described (21). The strains included a susceptible (S-NAIDM) strain, an organophosphate-resistant strain (R-dimethoate), and a juvenoid-resistant strain (R-methoprene). The ED_{50}s of methoprene (0.0033, 0.13, ~17 μg/pupa) and R-20458 (5, >100, >100 μg/pupa) to the S-NAIDM, R-dimethoate, and R-methoprene strains, respectively, are further discussed in other papers (5, 22). The stages utilized include white larvae (postfeeding last-instar larvae which had over half but not all of their gut cleared), white pupae (pupae which had not yet tanned), and adults (females 3–5 days old). The weights of white larvae of the three strains were 25.5 ± 1.1, 25.5 ± 1.0, and 25.3 ± 0.3 mg for S-NAIDM, R-dimethoate, and R-methoprene, respectively.

In vivo metabolism by housefly larvae. White larvae (100) of each of the three strains were treated topically on the posterior end with [5-^{14}C]methoprene (360 ng in 1 μl of acetone) as described by

Quistad *et al.* (12) and held in a metabolism chamber (constant light, 22°C). Control larvae were killed by immersion in boiling water for 2 min prior to treatment. A vacuum was applied to draw air through the chamber, first into two cold fingers in series for the collection of volatile organic materials and then through two CO_2 traps charged with ethylene glycol monomethyl ether and ethanolamine (1:1). Samples of the CO_2 traps from each strain were analyzed by lsc at 1, 2, 4, 8, 12, and 24 hr. After 24 hr the cold traps were washed with acetone and an aliquot was analyzed by lsc. The larvae were washed and homogenized in methanol, and their metabolites were characterized as described by Quistad *et al.* (12). The cage was rinsed as described below.

For qualitative examination of metabolites, white larvae (10 per treatment) were treated as above with [10-^{3}H]methoprene and held in vials for varying periods of time (the data presented here are for 3 hr). The larvae were washed (acetone) and homogenized in distilled acetonitrile (4 ml) containing granular sodium chloride for more efficient homogenization of the cuticle. The acetonitrile was extracted with hexane (4 ml, twice), and the hexane phase was dried (Na_2SO_4) and analyzed by tlc and lsc (Fig. 2). When partitioned between equal volumes of acetonitrile-saturated hexane and hexane-saturated acetonitrile, 64% of the radiolabeled methoprene is found in the hexane phase. Water (4 ml) was added to the acetonitrile phase which was extracted with peroxide-free ether (8 ml, three times). The ether acetonitrile layer contains all of the cold hydroxy acid (III) added as a standard. The organic phase was dried (Na_2SO_4) and the extracted metabolites analyzed by tlc and lsc (Fig. 2). The aqueous residue was treated in one of four ways as shown in Table 1. The enzymatic method utilized sulfatase, β-glucuronidase, phosphatase, and β-glucosidase as previously described (12). The highest proportion of organosoluble radioactivity was found after the addition of HCl to pH 2.0 and incubation at 40°C (18 hr) followed by ether extraction. This procedure resulted in the recovery of 87–94% of the radioactivity in the organic phase. The ether extracts were analyzed by tlc (Fig. 2). At each stage in the above procedure the distribution of radioactivity between phases was monitored by lsc. The acetone from the surface wash of the larvae was analyzed by lsc to determine penetration, while the acetone wash of the cage was analyzed to determine loss of label by abrasion and excretion. For determination of 2*Z*:2*E* ratios, radioactive fractions cochromatographing with known methoprene (I) or the hydroxy ester (II) on tlc were analyzed by reversed phase hrlc, while products apparently cochromatographing with the methoxy (IV) or hydroxy (III) acids were treated with diazomethane (23) prior to hrlc analysis. Fractions corresponding to the retention volumes of conjugated standards were collected, an equal volume of saturated aqueous brine was added, and the fraction was extracted with toluene and analyzed by lsc. This procedure removes essentially all of the radioactivity from the aqueous fraction.

For time courses, white larvae were treated with 17 ng of methoprene and analyzed as above at various time intervals

TABLE 1

Percentage Recovery of Water-Soluble Radioactive Methoprene Metabolites from Aqueous Medium by Ether Extraction following Various Treatments

Strain	Procedure				
	Immediate extraction	18 hr, 30°C	18 hr, enzyme[a]	18 hr, pH 2, 30°C	18 hr, pH 2, 40°C
S-NAIDM	5	69	87	82	94
R-dimethoate	3	55	79	85	87
R-methoprene	3	43	73	76	92

[a] The enzyme treatment utilized sulfatase, β-glucuronidase, phosphatase, and β-glucosidase as described by Quistad *et al.* (12).

(0–12 hr) with the following exceptions. After the initial homogenization in acetonitrile, water was added to give 50% aqueous acetonitrile and this aqueous acetonitrile was extracted with hexane. This procedure quantitatively extracts radioactive methoprene from the aqueous phase. The hexane fraction was analyzed by 1-dimensional tlc (HEt 3:2) and lsc.

Penetration studies. Penetration studies were run on white larvae by treating the larvae topically with 360 ng of [5-^{14}C]-methoprene or [ring-^{14}C]R-20458 in 0.5 μl of distilled acetone. The larvae were dried briefly under a stream of air and then held in scintillation vials for varying periods of time (10, 20, and 30 min and 1, 2, 4, 8, 12, and 24 hr). The larvae were then transferred to a new scintillation vial containing acetone (1 ml) and swirled for 1 min. Following a second identical wash, the larvae from each time period were combusted in a Packard 306 Tri-Carb sample oxidizer and analyzed by lsc. Scintillation solution was added to the original holding vial to monitor excreted and abraded radioactivity and to wash vials after the acetone was carefully evaporated to determine the quantity of radioactivity removed by surface washes of the larvae by lsc. Initial rates of penetration (expressed as the time required for half of the material to penetrate, $t_{\frac{1}{2}}$) were extrapolated from the 0- to 30-min values (Table 3). Similar studies were run for white pupae of each strain.

In vitro metabolism. Unless otherwise specified, *M. domestica* larvae (five/ml) of each strain were homogenized in phosphate buffer (pH 7.4, 0.1 *M*) containing bovine serum albumin (1%, w/v) and phenylthiourea (PTU, 0.01%, w/v). Adult female abdomens were homogenized in the same buffer with no PTU. The homogenates were fractionated by differential centrifugation as follows: "nuclei and cellular debris" (2000*g*, 10 min), "mitochondria" (12,000*g*, 15 min), "microsomes" (100,000*g*, 60 min), and soluble (100,000*g* supernatant). In some cases the precipitated fractions were rehomogenized in buffer without PTU and resedimented to remove the chance of esterase inhibition by PTU, while the soluble fraction was chromatographed on a Sephadex G-25, G-75, or G-150 column. Each fraction was assayed for esterase activity as has been done for several other insect species (24, 25).

All incubations were carried out in Carbowaxed glass vials in a shaking incubator (30°C) for varying periods of time (0–120 min). The substrates were routinely added in ethanol (to 1% final volume), but incubations were also run when the substrate was added to the incubation vial in hexane; the solvent was removed under N_2, and the enzyme was then added. Reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (to 5×10^{-4} *M*) was added in distilled water (10 μl) where appropriate.

Previous studies utilizing surface tension measurements (26) have shown that methoprene and R-20458 are no longer in true solution above 1.3×10^{-5} *M*, while JH I begins to form micelles at about 1.2×10^{-5} *M*, so no *in vitro* studies were run at substrate concentrations higher than 1×10^{-5} *M*. Comparisons of metabolism of methoprene and R-20458 were run at concentrations of 1×10^{-8} to 1×10^{-5} *M*, while all JH I incubations were run at 1×10^{-8} *M*.

Enzyme reactions were halted by the addition of salt and the metabolites were extracted with ethyl acetate (three times, 1 vol), the ethyl acetate was dried (Na_2SO_4), concentrated (N_2), and cospotted on tlc with the appropriate standards. The metabolites were separated by 1 or 2 dimensional tlc followed by lsc quantitation. A single ethyl acetate extraction removes >96% of the radioactivity from the aqueous fraction. For further comparison of the metabolic activity of the three strains, *O*-demethylation of *p*-nitroanisole, epoxidation of aldrin, and hydroxylation of

TABLE 2

Distribution of in Vivo Larval Metabolites 3 hr following Treatment with Radioactive Methoprene (17 ng)

Metabolite[a]	Organosoluble radioactivity recovered (%) cochromatographing with the appropriate standard					
	S-NAIDM strain		R-dimethoate strain		R-methoprene strain	
	Organo-soluble[b] 44%[c]	Aqueous[b] 22% (1)[d]	Organo-soluble 29%	Aqueous 27% (4)	Organo-soluble 26%	Aqueous 36% (3)
Methoprene I	30	0.6	17	0.3	15	0.3
Methoprene epoxide VIII	6	0.1	2	0.1	3	0.1
Hydroxy ester II	32	13	13	8	11	10
Methoxy acid IV	0.9	0.4	1	0.6	1	1
Hydroxy acid III	0.9	0.9[e]	2	0.6[e]	1	0.5[e]
Hydroxycitronellic acid VII	3	8	5	10	13	10
Unknown A	2	9	2	3	2	4
Unknown B	14	13	23	19	24	35
Origin	3	8	2	20	3	13
Other	8	47	33	38	27	26

[a] Metabolites are tentatively identified based solely on cochromatography.

[b] Organosoluble fraction = hexane and ether extracts, while aqueous fraction refers to those metabolites extracted with ether following acid treatment.

[c] Percentage of applied radioactivity.

[d] The number in parentheses indicates the percentage of nonextractable metabolites following acid treatment.

[e] An unknown radioactive metabolite partially overlaps with the hydroxy acid (III).

dihydroisodrin were examined by standard procedures described elsewhere (27–30).

RESULTS AND DISCUSSION

In Vivo Metabolism by House Fly Larvae

When white larvae of the three strains of *M. domestica* were treated as previously described (12) with 360 ng of ^{14}C-labeled methoprene, no detectable differences were found among the strains with regard to volatile ^{14}C released. Neither the material in the cold traps nor in the carbon dioxide traps accounted for as much as 1% of the total dose after 24 hr in any strain. These findings are in agreement with the results of Quistad *et al.* (12) who found less than 1% of the applied dose in the volatile fraction after 24 hr. The ^{14}C-radiolabeled metabolites were analyzed as below except that methanol was used as the homogenization medium. The metabolites found were identical to the ones described below for lower dose levels, except that no distinct radioactive metabolite was found to cochromatograph with the hydroxy acid (III).

As shown in Fig. 2 and Table 2, the initial hexane extraction of acetonitrile homogenates of the three strains yields methoprene (I) and other relatively nonpolar metabolites. This procedure of hexane extraction permits the use of tlc solvent systems which remove interfering lipids from the methoprene region of the tlc plate and allow rather nonpolar solvent systems to be used to resolve compounds with polarity similar to methoprene such as methoprene epoxide (VIII). The ether extracts of the aqueous acetonitrile phase indicate that the hydroxyester (II) is a predominant metabolite, while under no circumstances were

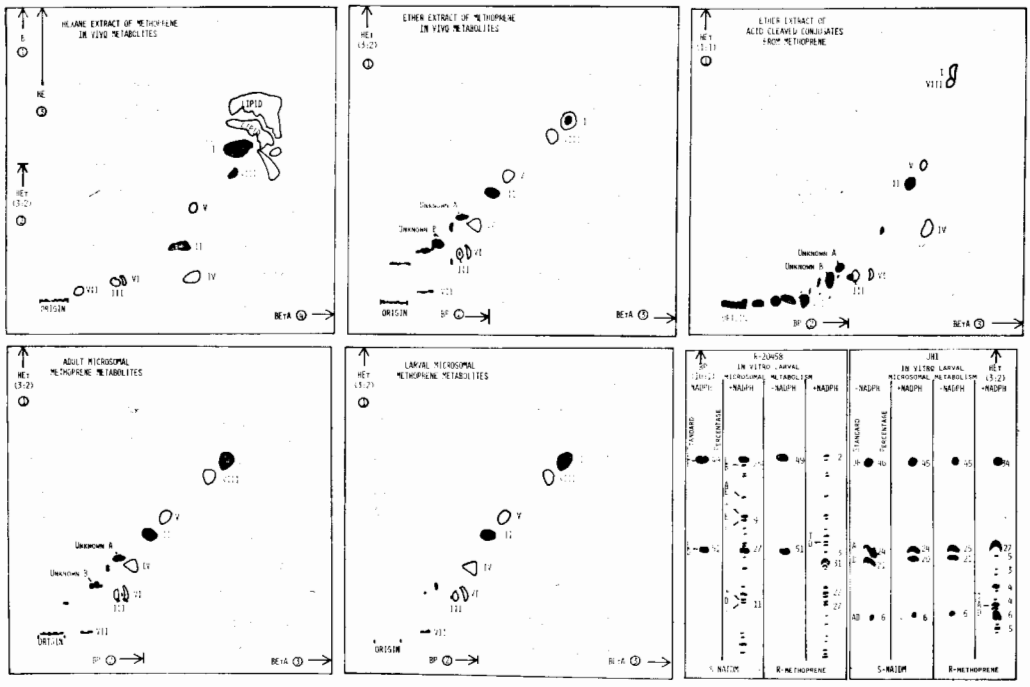

FIG. 2. *The tlc distribution of standards and metabolites with various solvent systems. Darkened spots show the position of radioactive metabolites, open circles indicate the position of standards not cochromatographing with metabolites, while Roman numerals or capital letters indicate the identity of standards (see Fig. 1). The order and direction of solvent development are shown by a circled number and arrow, respectively. The solvents utilized are: B = benzene, HE = hexane–ether (5:1), HEt = hexane–ethyl acetate in varying ratios, BP = benzene-n-propanol in varying ratios, and BEtA = benzene–ethyl acetate–acetic acid (50:25:2).*

radioactive spots found to cochromatograph with the methoxy acid (IV), and only trace amounts of the hydroxy acid (III) metabolite were found. This is in contrast to the findings by Quistad *et al.* (12) who reported that the hydroxy acid is the major nonpolar metabolite. The discrepancy may be due to differences in the strains used, differences in the times that larvae were held prior to analysis, or failure to resolve the hydroxy acid from unknown metabolites shown in Fig. 2. The radioactive metabolites cochromatographing with the hydroxy acid in a one-dimensional tlc solvent system (HEt 3:2) failed to cochromatograph on hrlc after methylation. Our standard of the hydroxy acid (III) was found to contain at least 22 very minor impurities which could be detected by a vanillin–sulfuric acid spray. Some of these minor spots cochromatograph with unknown metabolites.

Three ether extracts of the aqueous phase apparently removed most of the organosoluble radioactivity, because a subsequent extraction in each case recovered less than 5% additional radioactivity from the aqueous phase. Some of the conjugates are rather unstable in water as incubation for 18 hr results in 43–69% of the radioactivity becoming organosoluble (Table 1). The differences noted among the four methods include consistently more radioactivity at the origin in the case of the fractions allowed to incubate overnight with no additional treatment and a higher proportion of the hydroxyester (II) detected following acid treatment. The cleaved conjugates include material co-

chromatographing with the hydroxyester (II), but no radioactive spot was found to cochromatograph with the methoxy (IV) or hydroxy (III) acid. The major metabolites from conjugate cleavage, other than the hydroxy ester, are unknown, but one of them (Fig. 2) chromatographs in the region of hydroxycitronellic acid (VII).

The time course shown in Fig. 3 illustrates the least total methoprene in the larvae of the R-methoprene followed by the R-dimethoate and the most methoprene in the S-NAIDM strains for up to 6 hr. Five separate studies were run for varying times after applying 17–360 ng of methoprene. In all cases the level of radioactivity cochromatographing with methoprene was highest in the S-NAIDM followed by the R-dimethoate and finally the R-methoprene strains.

The isomer ratios at the 2Z double bond are very similar in the three strains after 6 hr. Methoprene is isomerized to the extent of 34, 35, and 34%, and the hydroxy ester is isomerized to the extent of 42, 41, and 41% in the S-NAIDM, R-dimethoate, and R-methoprene strains respectively, while controls show under 5% isomerization.

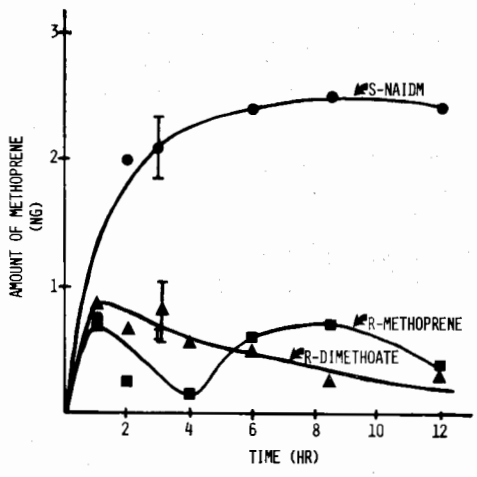

FIG. 3. *The amount of material inside white larvae cochromatographing with methoprene as a function of time following topical application of 17 ng of methoprene (●, S-NAIDM strain; ▲, R-dimethoate strain; ■, R-methoprene). The 3-hr values are averages of four separate runs made on a day different from that of the rest of the time course. Vertical bars indicate standard deviation. The data in Table 2 were obtained from a different experiment run on a different day and also indicate that the S-NAIDM strain contains the most and R-methoprene strain the least internal methoprene 3-hr post-treatment.*

TABLE 3

Penetration of Two Juvenoids into M. Domestica White Larvae[a]

Strain	Compound	$t_{\frac{1}{2}}$ (min)[b]	Percentage of total penetrated (120 min)
S-NAIDM	Methoprene	81	40
	R-20458	27	70
R-dimethoate	Methoprene	70	34
	R-20458	28	69
R-methoprene	Methoprene	93	24
	R-20458	48	46

[a] Flies treated with 360 ng/larva.

[b] The time required for 50% penetration extrapolated from 0- to 30-min data.

Penetration

The data in Table 3 indicate that, when monitored by acetone washes, in all cases R-20458 penetrates faster than methoprene. When plotted on semilog paper, the penetration rates were relatively linear through 30 min, but thereafter the rates decreased. The R-methoprene strain demonstrates a longer $t_{\frac{1}{2}}$ for R-20458 or methoprene penetration than the R-dimethoate or S-NAIDM strain, and it also has less total penetration of the two compounds at most time intervals assayed up to 24 hr. When dosed at 17 ng, two acetone washes after 3 hr removed 7.3 ± 0.3, 8.3 ± 0.8, and 10.0 ± 1.0 ng of methoprene equivalents from the surface of four replicates of S-NAIDM, R-dimethoate, and R-methoprene larvae. During the same time period, the larvae had abraded or eliminated 1.5 ± 0.2, 2.0 ± 0.3, and 2.2 ± 0.2 ng, respectively. In five other penetration experiments with doses of 17–360 ng, the R-methoprene strain demonstrated less penetration at most time intervals. As found earlier (22), there is little difference

TABLE 4

In Vitro Metabolism of Methoprene by House Fly Enzymes[a]

Metabolite	Organosoluble radioactivity recovered (%) cochromatographing with the appropriate standard															
	S-NAIDM strain					R-dimethoate strain					R-methoprene strain					Control
Stage	W	W[b]	W	W[c]	A	W	W[b]	W	W[c]	A	W	W[b]	W	W[c]	A	
NADPH	+	−	−	−	+	+	−	−	−	+	+	−	−	−	+	
Fraction	M	M	M + S	S	M	M	M	M + S	S	M	M	M	M + S	S	M	
Methoprene I	93 (±3)	97	95	98	94 (±2)	76 (±4)	98	72	97	71 (±5)	60 (±6)	97	75	98	81 (±4)	97
Hydroxy ester II	3 (±1)	1	2	0.4	1 (± <1)	16 (±3)	1	16	0.6	15 (±3)	22 (±4)	1	16	0.4	11 (±2)	0.5
Methoxy acid IV	0.8	0.5	0.6	0.5	0.2	1	0.5	0.8	0.3	0.4	0.5	0.6	0.7	0.2	0.9	1
Hydroxy acid III	0.7	0.7	0.2	0.2	0.2	1[d]	0.3	0.2	0.5	0.2	2[d]	0.5	0.2	0.2	0.2	0.2
Hydroxycitronellic acid VII	0.1	0.1	0.2	—	0.1	0.5	—	1	0.1	3	3	0.2	1	0.1	2	0.1
Unknown A	0.8	0.2	0.5	—	0.3	2	—	2	0.1	3	1	0.2	1	0.1	0.2	—
Unknown B	0.6	0.3	0.6	—	0.5	1	0.1	3	0.3	5	6	0.3	5	0.4	0.3	—
Origin	0.1	0.1	0.2	—	1	0.3	0.1	0.5	0.2	0.6	1	0.1	0.2	0.2	0.8	1
Other	0.9	0.1	0.7	0.9	3	2	0.1	4	1	2	4	0.1	1	0.4	3	0.2

[a] Abbreviations: W = white larvae, A = adult female, + or − indicates the presence or absence of NADPH (5×10^{-4} *M*), M = microsomal fraction, and S = 100,000*g* soluble fraction. All incubations were at 30°C for 60 min with methoprene (5×10^{-6} *M*). Extraction efficiency was >96% in all cases. All incubations were run at least three times on two separate microsome preparations, and standard deviations (SD) are given for three replicates of microsome + NADPH values.

[b] Similar to adult microsomal metabolites without NADPH.

[c] Addition of NADPH caused no significant change in metabolism.

[d] The hydroxy acid partially overlaps an unknown metabolite.

in methoprene or R-20458 penetration into white pupae of the three strains.

In Vitro Metabolism

As shown in Fig. 2 and Table 4, the hydroxy ester (II) is the major *in vitro* metabolite of methroprene in both larval and adult *M. domestica* microsomes, while the nuclear and mitochrondrial fractions have much lower *O*-demethylase activity. Most other minor *in vitro* metabolites show R_fs similar to the *in vivo* metabolites. Qualitatively, the methoprene metabolites from adult and larval microsomes are similar, as is also the case for metabolites of R-20458. The addition of NADPH is required for the appearance of any metabolites of methoprene except when low substrate levels are used with unwashed microsomes or the 12,000*g* supernatant. In these cases some cofactor is probably present and the hydroxy ester (II) is the predominant metabolite. In microsomes from white larvae, NADPH-dependent metabolism of methoprene is fastest in the R-methoprene strain and slowest in the S-NAIDM strain.

Since it was previously reported that ester cleavage is a major route of methoprene metabolism (12), some effort was made to demonstrate this pathway *in vitro*. Under no circumstances were we able to demonstrate a distinct radioactive spot cochromatographing with the methoxy (IV) or hydroxy (III) acid following incubation of methoprene with the crude homogenate, any subcellular fraction, or recombined fractions from larvae, pupae, or adults of any of the three strains examined. This finding is in agreement with work by Yu and Terriere (13) who found very little hydrolytic metabolism of methoprene. Incubations were run at a variety of substrate concentrations (from 5×10^{-8} to 5×10^{-6} *M*). In some cases NADPH was added to examine for the occurrence of oxidative ester cleavage, but no radioactive spot was found to cochromatograph with the two acids (Table 2). Washing of subcellular fractions was not found to increase the hydrolytic metabolism of methoprene. Gel filtration was utilized in an attempt to remove possible inhibitory substances, but none of the fractions from Sephadex G-25, G-75, or G-150 columns showed significant hydrolytic activity on methoprene, although high esterase activity monitored on α-naphthyl acetate and *p*-nitrophenyl acetate was recovered from the column (25). If inhibitory substances are present, they must have a molecular weight similar to the esterases involved. When ethanol was not used to add radioactive methoprene to the incubation vial, no increase in hydrolytic activity was found.

When microsomes of white larvae were incubated with methoprene (5×10^{-6} *M*) but without NADPH, less than 0.5% of the radioactivity cochromatographed with the methoxy acid (IV) after 60 min. There was a linear increase in this very small amount of radioactivity cochromatographing with the methoxy acid after 0, 15, 30, and 60 min and the increase was identical (less than 5% difference in the three strains at each time interval) in the three strains while slightly exceeding hydrolysis in heat-treated controls. Even if this very minor level of metabolism is due to enzymatic ester hydrolysis, there was no difference detected in the strains examined.

Epoxide hydration of R-20458 seems very similar in adults and larvae of the three strains; however, as found earlier for propoxur (Bagon)-resistant strains (10), oxidative metabolism of R-20458 is much higher in the resistant strains. At least 22 metabolites can be detected from R-20458 incubation in microsomes from the R-dimethoate and R-methoprene strains. S-NAIDM microsomes yield the ethyl-diol (ED) as the predominant metabolite, while α- and β-hydroxylation on the ethyl side chain are predominant in the resistant strains (Fig. 2). Oxidation at the 2*Z* double bond to yield bis-epoxides (BE) and tetra-

TABLE 5

Mixed Function Oxidase Activity in Adult House Fly Abdomen Microsomes

Strain	Enzyme monitored		
	O-demethylase[a]	Epoxidase[b]	Hydroxylase[c]
S-NAIDM	0.43 ± 0.10	0.038 ± 0.008	0.015 ± 0.006
R-dimethoate	2.35 ± 0.42	0.127 ± 0.015	0.037 ± 0.008
R-methoprene	1.96 ± 0.35	0.145 ± 0.032	0.058 ± 0.005

[a] Activity expressed in nanomoles of *p*-nitrophenol formed per milligram of protein per minute ±SD using *p*-nitroanisole as model substrate.

[b] Activity expressed in nanomoles of dieldrin formed per milligram of protein per minute ±SD using aldrin as model substrate.

[c] Activity expressed in nanomoles of hydroxydihydroisodrin formed per milligram of protein per minute ±SD using 6,7-dihydroisodrin as model substrate.

hydrofuran diols (TD) are minor pathways, while the structures of many of the metabolites are not known. Possibly allylic hydroxylation plays a role as many of the unknown metabolites react with acetic anhydride in pyridine (10, 17).

The metabolism of JH I was similar with and without NADPH in microsomes from larvae or adult females of the S-NAIDM strain. The principal metabolites cochromatographed with JH acid (A) and JH diol (D) with some JH diol acid (DA) formed, as shown in Fig. 2. This is in contrast to the findings in *Manduca sexta* fatbody microsomes where the acid and diol acid are major JH I metabolites (18), and these findings provide further support for the earlier data of Slade and Zibitt (31) that epoxide hydration is an important primary step in JH metabolism in the Diptera. Hydrolytic metabolism of JH by the microsomes from the resistant strains is similar to that by S-NAIDM microsomes. When NADPH is added, JH metabolism is much more rapid in the microsomes of the resistant strains. The amount of JH acid is similar in each strain; however, very little JH diol remains and the proportion of radioactivity cochromatographing with the JH diol acid increases as do unknown metabolites, two of which cochromatograph with tetrahydrofuran diols of JH acid (TAD).

For comparison with other strains, the metabolism of model substrates by adult female abdomen microsomes was examined in the three strains. Whether calculated on the basis of activity per fly or as specific activity, *O*-demethylase activity was lowest in the S-NAIDM, and the activity was found to be a little higher in the R-dimethoate strain than the R-methoprene strain. Epoxidation of aldrin and hydroxylation of dihydroisodrin were also lowest in the S-NAIDM but were slightly higher in the R-methoprene than the R-dimethoate strains (Table 5).

CONCLUSIONS

The resistance and cross resistance of the R-dimethoate and R-methoprene strains to methoprene and R-20458 are apparently not due to a large increase in hydrolytic enzymes (esterases and epoxide hydratases) or isomerization of the 2*Z* bond but, rather, in part, to an increase in juvenoid degradation due to oxidative metabolism as predicted by earlier work (2, 4, 8–10, 13, 22, 32). *In vivo* data indicate that excretion of methoprene is slightly faster in the R-methoprene than the other strains while penetration into white larvae is slower. Reduced penetration, increased metabolism, and increased excretion lead to a smaller amount of methoprene in resistant larvae during the most sensitive period. It is likely that a complete explanation for the juvenoid resistance present in the R-methoprene strain has not been found. Changes in the JH active site, variations in juvenoid distribution, or a shorter window of sensitivity may provide other resistance mechanisms.

One would expect some cross resistance in the R-methoprene strain to many juvenoids due to decreased penetration, although the degree of resistance is difficult to predict. Any juvenoid which has alkoxide moieties, benzylic carbons, olefinic bonds, or other functionalities labile to oxidative attack may experience cross resistance in

the R-methoprene strain, R-dimethoate strain, or field strains which have been selected for high levels of oxidative enzymes. From a cursory examination of JH metabolism in resistant and nonresistant strains, it is clear that strains of flies can be selected which rapidly metabolize not only juvenoids or hormone mimics but also the natural hormones.

The cross resistance of the R-dimethoate strain to several juvenoids indicates that degradative metabolism is an important resistance factor, and the development of juvenoids refractory to oxidative degradation or the use of synergists which inhibit xenobiotic oxidation may partially circumvent resistance. The isopropyl ester of methoprene is apparently quite stable in these insects; however, the methoxy and possibly the olefinic moieties are labile to degradation, as predicted earlier (12) for another series of juvenoids. Since epoxide hydratase levels do not appear to be higher in the resistant strains, an epoxide which is relatively stable to intrinsic epoxide hydratases may be a practical replacement for the methoxy moiety. The *p*-ethyl moiety and the 2,3-olefin and adjacent carbons are probably sites for oxidative attack on R-20458 in the resistant *M. domestica* examined. Replacement of these groups with functionalities less labile to oxidative degradation will possibly yield compounds active on resistant insects. Even compounds refractory to degradative metabolism are not likely to circumvent totally the development of resistance by alternative mechanisms if pest insects are exposed to intense selection pressure in the field.

ACKNOWLEDGMENTS

The facilities to perform the early part of this work were provided by T. R. Fukuto, the manuscript and figures were prepared by P. Kaplan, and the house fly strains were developed and maintained under the direction of G. P. Georghiou by M. K. Hawley and S. Lee of the Division of Toxicology and Physiology, Department of Entomology, University of California, Riverside. G. B. Quistad of Zöecon Corporation, Palo Alto, California, provided radiolabeled methoprene and unlabeled standards in addition to suggesting many of the techniques used in this study. J. B. McBain of Stauffer Chemical Company supplied the carbon-labeled R-20458 for this work.

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