Insect Pyrethroid-Hydrolyzing Esterases

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Esterases in acetone powder preparations of milkweed bugs, cockroaches, houseflies, cabbage loopers, mealworms, and mouse liver hydrolyze the (+)-trans- and (+)-cis- isomers of resmethrin and tetramethrin but they do not hydrolyze S-bioallethrin. Homogenate fractions are less suitable than acetone powders for assaying the insect esterases due to interfering reactions or inhibitors. The milkweed bug, looper and mouse liver esterases cleave the trans-isomers more rapidly than the cis-isomers of resmethrin and tetramethrin but this isomer specificity is less prominent or not present with the other esterase sources. Pyrethroid-hydrolyzing esterases are much less active in insect than in mouse liver preparations. 1-Naphthyl N-propylcarbamate is a more potent inhibitor than S,S,S-tributyl phosphorotrithioate for the insect esterases whereas the latter compound is more effective in inhibiting the mouse esterases. Both of these chemicals are noncompetitive inhibitors in each case suggesting that they carbamoylate and phosphorylate the detoxifying enzymes. Esterase inhibitors acting in the nmolar range may be useful synergists in species where pyrethroid detoxification by esterases limits the insecticidal action.

INTRODUCTION

Pyrethroids are synergized in their toxicity to insects by compounds known or suspected to act as both esterase and oxidase inhibitors (1-3). When applied to houseflies (Musca domestica L.), PB2 inhibits the allethrin-oxidizing enzymes (2) and O-(n-propyl) O-(2-propynyl) phenylphosphonate blocks the ester cleavage of tetramethrin (3). Mouse liver microsomal esterases acting on pyrethroid substrates are sensitive to in vitro inhibition by paraoxon2 and in vivo inhibition by DEF2 (4, 5). These mouse esterases preferentially

cleave (+)-trans-relative to (+)-cis-chrysanthemates of primary alcohols whereas chrysanthemates of secondary alcohols are not suitable substrates (4, 5).

An understanding of insect pyrethroid-hydrolyzing esterases and inhibitors of these enzymes may lay the background for a new type of pyrethroid synergist (6). An appropriate synergist must penetrate the insect and inhibit the esterases before the pyrethroid undergoes extensive ester cleavage. For target site selectivity, it must also be more potent in inhibiting insect esterases than mammalian esterases. It appears that some of these requirements are met by NPC2, an effective synergist for (+)-trans-resmethrin and -tetramethrin in large milkweed bugs (Oncopeltus fasciatus Dallas) and some other species (6).

The present investigation considers the assay, substrate specificity, and inhibition

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of insect esterases hydrolyzing the (+)-
trans- and (+)-cis-isomers of resmethrin
and tetramethrin (Fig. 1). Attention is
focused on but not restricted to the esterases
of the large milkweed bug and NPC as an
inhibitor.

MATERIALS AND METHODS

Chemicals. The [14C]acid- and [14C]-
phenyl-labeled pyrethroids investigated are
shown in Fig. 1. Their specific activities
range from 1.3 to 2.3 mCi/m mole (7, 8).
The source of the synergists [NPC, DEF, PB
and HPI] is previously described (6).
Paraaxon was provided by Dr. Eugene
Bellet of this laboratory.

Insects and mammals. The studies utilized
adult female large milkweed bugs, adult
male German cockroaches (Blattella germ-
icana L.), adult female houseflies, fifth-
instar larvae of the cabbage looper
(Tri-
choplusia ni (Hubner)) and the yellow
mealworm (Tenebrio molitor L.), adult male
and female lygus bugs (Lygus hesperus
Knight), and adult male Swiss-Webster
mice (6).

Enzyme preparations. Ten percent (w/v)
homogenates of whole insects, mouse liver,
and mouse brain in Tris-HCl buffer (0.1
M, pH 7.5) were centrifuged at 20,000 g for
15 min to obtain the supernatant fractions
referred to as "fresh preparations" of
enzyme. The acetone powder preparations
were made by dropwise addition of the
supernatant fractions of 25% (w/v) homo-

ogenates, prepared as above, into 10 vol of
dry acetone at -10°C, then following the
procedure of Morton (9). Acetone powder
preparations from frozen storage were re-
constituted in cold Tris-HCl buffer by
homogenization, holding at 5°C for 1 hr,
then shaking to resuspend any sediment.

The amounts of protein in the various
preparations relative to the original fresh
weight of organism or tissue were as follows:
fresh preparations—57–76 mg/g for the
insects and 129 mg/g for the liver; acetone
powder preparations—39–60 mg/g for the
insects and 64 mg/g for the liver (10).
Protein determinations were made by the
method of Lowry (11).

Esterase assays. The [14C]acid-labeled
pyrethroid (2000 pmole) was injected in
ethanol (20 μl) into the enzyme incubation
mixture (2.0 ml; "fresh preparation" or
reconstituted acetone powder) in a 30-ml
centrifuge tube, with swirling then
thorough mixing. The tube was sealed
with parafilm and held in a shaking incu-
barator at 37°C for 2 hr, in the case of
[14C]resmethrin or -allethrin, or 20 min
with [14C]tetramethrin.

The extent of [14C]pyrethroid hydrolysis
was determined by addition of 2 N NaOH
to bring the pH to 8.5 in the case of [14C]-
resmethrin or -allethrin or without pH
adjustment in the case of [14C]tetra-
methrin, extraction with hexane (2 × 6 ml)
then chloroform (2.5 ml) utilizing centrifu-
gation to separate the phases in each case,
and finally determination of the radio-
carbon content of the aqueous phase using
duplicate samples of 0.2 ml for liquid
scintillation counting. In this assay, the
aqueous phase contains 95–99% of the
[14C]ehrsanthenic acid liberated on hy-
drolysis and no [14C]resmethrin, -tetra-
methrin or -allethrin remaining unhys-
droyzed (10). The amount of pyrethroid
cleavage on incubation was calculated from
the percentage of the radioactivity present
in the aqueous phase as [14C]ehrsanthenic
acid relative to the total radioactivity in
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Fig. 2. Activity of "fresh preparations" and acetone powders of whole insects and mouse liver in hydrolyzing (+)-trans-resmethrin.

the incubation mixture. The spontaneous hydrolysis rate of tetramethrin is higher than that of resmethrin, so the incubation time was shorter for [14C]tetramethrin and the pH was not adjusted prior to extraction. Correction was made for nonenzymatic substrate hydrolysis which was always less than 1% with [14C]resmethrin within 2 hr and averaged 4.8% with [14C]-(+)-cis-tetramethrin and 12.2% with [14C]-(+)-trans-tetramethrin at 20 min at the pyrethroid concentration of 10^{-4} M (10).

All studies were repeated two or more times with separate enzyme preparations and involved duplicate determinations of protein level and extent of pyrethroid hydrolysis in each case. The deviation of individual results from the mean for each preparation was 2–5% for the protein levels and 10–15% for the hydrolysis rates. The results given are the average values for pmole pyrethroid hydrolyzed/mg protein/min.

In the studies with synergists and paraoxon, the inhibitor was deposited on the bottom of the tube by evaporation of 50 μl of an acetone solution immediately prior to addition of the enzyme source. The enzyme and inhibitor were incubated at 37°C for 15 min, then the [14C]pyrethroid was added and the analysis continued as above. The type of inhibition was determined in each case by the procedure of Dixon (12) with four different inhibitor concentrations at each of two resmethrin levels (1.0 and 5.0 × 10^{-6} M). The molar inhibitor concentration for 50% inhibition (I_{50}) was established under the same conditions with 1 × 10^{-6} M substrate.

RESULTS

Esterases hydrolyzing (+)-trans-resmethrin. Milkweed bug homogenate contains a heat- and paraoxon-sensitive component(s) that hydrolyzes (+)-trans-resmethrin and that resides to the extent of greater than 95% in the microsome-plus-soluble fraction. However, the microsome-plus-soluble fraction or "fresh preparation" is not suitable for routine assays since the extent of (+)-trans-resmethrin hydrolysis is not proportional to protein level, at least above 0.1 mg per 2 ml of incubation mixture (Fig. 2). Addition of the milkweed bug fresh preparation to the comparable mouse liver preparation results in extensive inhibition of (+)-trans-resmethrin hydrolysis by mouse liver esterases; this inhibition is partially overcome by the addition of bovine serum albumin (1%, w/v) (10). Thus, the milkweed bug microsome-plus-soluble fraction contains a factor(s) in-
TABLE 1
Hydrolysis Rates for Five (+)-Chrysanthemates Incubated with Insect and Mouse Liver Esterases

<table>
<thead>
<tr>
<th>Enzyme source, acetone powder</th>
<th>pMole/mg protein/min</th>
<th>Ratio, trans/cis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resmethrin (trans)</td>
<td>Tetramethrin (trans)</td>
</tr>
<tr>
<td></td>
<td>Resmethrin (cis)</td>
<td>Tetramethrin (cis)</td>
</tr>
<tr>
<td>Insect</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milkweed bug</td>
<td>12.0</td>
<td>26.8</td>
</tr>
<tr>
<td>Cockroach</td>
<td>7.5</td>
<td>6.3</td>
</tr>
<tr>
<td>Housefly</td>
<td>3.6</td>
<td>10.1</td>
</tr>
<tr>
<td>Looper</td>
<td>4.5</td>
<td>8.0</td>
</tr>
<tr>
<td>Mealworm</td>
<td>1.6</td>
<td>3.4</td>
</tr>
<tr>
<td>Mouse liver</td>
<td>200</td>
<td>57</td>
</tr>
</tbody>
</table>

* Determined at an enzyme concentration of 1.0 mg insect protein or 0.10 mg liver protein per 2 ml incubation mixture. The hydrolysis rate of each substrate is decreased to less than 0.2 pmole/mg protein/min with each enzyme source preincubated with 1 × 10⁻⁶ M paraoxon. S-Bioallethrin does not undergo significant hydrolysis, even in the absence of paraoxon, averaging 0.3 pmole/mg protein/min for the six enzyme sources.

TABLE 2
Kinetic Parameters for Insect and Mouse Liver Esterases Hydrolyzing (+)-trans-Resmethrin in the Presence or Absence of Inhibitors

<table>
<thead>
<tr>
<th>Enzyme source, acetone powder</th>
<th>Normal esterase</th>
<th>Inhibited esterase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kₘ (M × 10⁻⁷)</td>
<td>Vₘₐₓ (pmole/mg protein/min)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insect</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milkweed bug</td>
<td>25</td>
<td>66</td>
</tr>
<tr>
<td>Cockroach</td>
<td>83</td>
<td>58</td>
</tr>
<tr>
<td>Housefly</td>
<td>63</td>
<td>30</td>
</tr>
<tr>
<td>Looper</td>
<td>111</td>
<td>60</td>
</tr>
<tr>
<td>Mouse liver</td>
<td>125</td>
<td>2083</td>
</tr>
</tbody>
</table>

* Assay conditions other than those specified in the text are as follows: enzyme concentration, 1.0 mg protein/2 ml incubation mixture; incubation time after substrate addition, 1.0 hr with insect esterases and 0.5 hr with mouse liver esterases.
roaches, and houseflies but not from mouse liver (Fig. 2). Thus, the acetone powder preparations are more suitable for the studies with insects; so, they were used in all subsequent investigations.

Incubation of $[^{14}C]$resmethrin with milkweed bug and cockroach acetone powder preparations yields only two products as determined by appropriate thin-layer chromatographic techniques (7, 10); $[^{14}C]$chrysanthemic acid from the acid-labeled compound; $[^{14}C]$-5-benzy1-3-furylmethanol from the alcohol-labeled compound. These hydrolysis products are not liberated by similar preparations preincubated with paraoxon.

The extent of (+)-trans-resmethrin hydrolysis by acetone powder preparations from whole insects and mouse liver is proportional to both enzyme concentration and time (Figs. 2 and 3). A similar situation is expected to exist for the hydrolysis of the other $[^{14}C]$pyrethroids. Mouse brain microsome-plus-soluble fraction and its acetone powder hydrolyze (+)-trans-resmethrin with an activity of 3.8 pmoles/mg protein/min for the fresh preparation and 2.4 for the acetone powder preparation.

Substrate specificity of esterases. Each enzyme source hydrolyzes the (+)-trans- and (+)-cis-isomers of resmethrin and tetramethrin at a measurable rate but not any one of them hydrolyzes S-bioallethrin (Table 1). Mouse liver enzyme hydrolyzes the trans-isomer of resmethrin 14 times more rapidly than the cis-isomer, but isomer specificity of this magnitude is not evident with insect enzymes acting on resmethrin or any of the enzymes acting on tetramethrin. The only case where the cis-isomer is hydrolyzed appreciably faster than the corresponding trans-isomer is with housefly enzyme and resmethrin. (+)-trans-Tetramethrin is hydrolyzed more readily than (+)-trans-resmethrin by all enzyme sources except mouse liver and cockroaches while (+)-cis-tetramethrin is more readily cleaved than (+)-cis-resmethrin by all enzymes except that from the cockroach. The component(s) in the acetone powders active in (+)-trans-resmethrin hydrolysis is probably an esterase in each case, being sensitive to almost complete inhibition by paraoxon (Table 1). It is likely that this same esterase or a similar one is involved in each case in hydrolyzing (+)-cis-resmethrin and the tetramethrin isomers. The activity of the different enzyme sources generally decreases in the following order: mouse liver >> milkweed bug > cockroach or housefly > looper > mealworm.

The various enzyme sources differ in their $K_m$ and $V_{max}$ values obtained with (+)-trans-resmethrin as the substrate (Table 2, Fig. 4, ref. 10). These findings and those shown in Table 1 indicate the high activity of the mouse liver enzyme for hydrolytic detoxification of (+)-trans-resmethrin relative to the insect enzymes and particularly those from houseflies, loopers, and mealworms.

Inhibition of esterases. The insect esterases are very sensitive to inhibition by NPC relative to the mouse liver esterases (Table 2). DEF is also a potent inhibitor but lacks the specificity of NPC in differentiating the insect and mammalian esterase sources. The other candidate synergists, MPP and PB, are relatively poor inhibitors of (+)-trans-
resmethrin hydrolysis regardless of the enzyme source.

The graphic method used in determining the type of inhibition is illustrated in Fig. 5 with NPC and the mouse liver and milkweed bug esterases and by Jao (10) for DEF with these two enzymes and both NPC and DEF with cockroach and looper enzymes. In each of these cases, kinetic treatment indicates that noncompetitive inhibition is involved; this is as expected since the inhibitor was preincubated with the enzyme for 15 min prior to substrate addition.

**DISCUSSION**

Synergist studies indicate that milkweed bugs, cockroaches, and some other insects are likely to contain esterases important in pyrethroid detoxification, acting on primary but not secondary alcohol chrysanthemates (6). The activity, nature, and inhibition of pyrethroid-hydrolyzing insect esterases is therefore of interest.

Acetone powder preparations of milkweed bugs, cockroaches, houseflies, loopers, mealworms, and mouse liver contain paraoxon-sensitive components that hydrolyze the (+)-resmethrin and -tetramethrin isomers but not S-bioallethrin. The esterase activity is very low in acetone powder preparations from mealworms and lygus bugs assay with (+)-trans-resmethrin so no further studies were made with these insects. Fresh insect enzyme preparations are less favorable than the corresponding

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**Fig. 4. Lineweaver-Burk plots showing $K_m$ and $V_{max}$ for (+)-trans-resmethrin hydrolysis by acetone powders of mouse liver and whole milkweed bugs at 0.1 and 1.0 mg protein/2 ml incubation mixture, respectively, with 1.0-hr incubation.**

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**Fig. 5. Dixon plots for 1-naphthyl N-propylcarbamate inhibition of (+)-trans-resmethrin hydrolysis by acetone powders of mouse liver and whole milkweed bugs at 1.0 mg protein/2 ml incubation mixture with 1.0-hr incubation for insect esterases and 0.5 hr for mouse esterases.**
acetone powders for esterase assays because of the presence of interfering reactions or inhibitors. The insect preparations are all less active than mouse liver when assayed on a comparable basis; in fact, the mouse liver preparation is 17 times more active than the best insect preparation in hydrolyzing (+)-trans-resmethrin.

The esterases are all assayed under the conditions found to approach an optimum with milkweed bugs. The pH optimum is probably higher than the normal assay pH since milkweed bug enzymes hydrolyze (+)-trans-resmethrin three times more rapidly at pH 9 than at 7.5. It was not practical in the present study to use the higher pH value because the tetramethrin isomers undergo very rapid spontaneous hydrolysis at pH 9.

Mouse liver enzymes show the greatest rate difference in hydrolyzing isomeric pyrethroids, cleaving (+) trans-resmethrin 14 times more rapidly than (+)-cis-resmethrin (see also refs. 4 and 5). The trans/cis isomer specificity is less pronounced with milkweed bug and looper enzymes and is almost absent with the other insects. A possible exception is the reverse isomer specificity with houseflies since these enzyme preparations hydrolyze the cis-isomer of (+)-resmethrin more rapidly than the trans-isomer.

The V_max value for (+)-trans-resmethrin hydrolysis is more than 30-fold greater for mouse liver than the insect esterases. The K_m values differ 5-fold with the various enzyme sources, from a low for milkweed bugs to a high for mouse liver. The low K_m relative to the V_max of the milkweed bug esterases indicates their high efficiency in (+)-trans-resmethrin hydrolysis.

Studies on the potency of various inhibitors with (+)-trans-resmethrin as the substrate also establish differences between the various enzyme sources. NPC and DEF are noncompetitive inhibitors with each esterase source. The relatively low activity of DEF acting on the milkweed bug esterases is worthy of note. NPC is the most potent inhibitor with each insect enzyme but DEF is more potent with mouse liver enzymes. This finding is in agreement with the synergistic activity of NPC and DEF observed with pyrethroid-treated insects and mice (4-6). MPP and PB are not potent esterase inhibitors under the conditions of assay with each enzyme source. Thus, if they act in vivo by esterase inhibition then it is likely that a metabolic activation step forming a more potent inhibitor is involved with the living organisms. No explanation is available for the moderate inhibitory activity of PB acting on the milkweed bug esterases in contrast to the other esterase sources. An esterase inhibitor of high selectivity for insect pyrethroid-hydrolyzing esterases is preferred for use as a synergist; NPC best fits this specification.

Esterase detoxification is only one of many factors important in the insecticidal activity of pyrethroids. Oxidase detoxification is even more significant in some species. Effective oxidase inhibitors are already available. The finding of effective inhibitors for insect pyrethroid-hydrolyzing esterases permits their use in better evaluating the relative importance of esterases and oxidases in pyrethroid detoxification in insects.

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