Oxidative Metabolism of Pyrethroids in Houseflies

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Allethrin is metabolized in living houseflies (*Musca domestica* L.) and in the housefly mixed-function oxidase system by attack at the *trans*-(major site) and *cis*-(minor site) methyl groups of the isobutenyl side chain in the acid forming, in succession, the corresponding hydroxymethyl, aldehyde, and acid compounds. No hydrolysis or attack on the alcoholic part of the ester is detected, but there are trace amounts of unidentified metabolites. Piperonyl butoxide inhibits hydroxylation of the methyl groups by the mixed-function oxidase system. Living houseflies conjugate and excrete the hydroxymethyl compounds, probably as glucosides. Pyrethrin I, phthalthrin, and dimethrin are similarly metabolized, *in vivo* and *in vitro*, by oxidation of the *trans*-methyl group.

Natural and synthetic pyrethroids are relatively harmless to mammals but are extremely toxic to houseflies, mosquitoes, and many other insect pests, especially in the presence of synergists. Despite these favorable properties, early attempts to elucidate their penetration characteristics and metabolic fate in houseflies (Bridges, 1957; Chamberlain, 1950; Chang and Kearns, 1964; Fine *et al.*, 1967; Hayashi *et al.*, 1968; Hopkins and Robbins, 1957; Winteringham *et al.*, 1955) and cockroaches (Zeid *et al.*, 1953) showed only that pyrethroids are rapidly metabolized and that synergists reduce the rate of metabolism; no adequate characterization of metabolites or definition of metabolic pathways were developed. These studies had limited success because the radiolabeled compounds used had either low specific activity, in the case of biosynthesized random-labeled pyrethrum constituents (Chang and Kearns, 1964), or were mixtures of isomers with the C^{14}- and H^3-labeled preparations of allethrin and phthalthrin. It is difficult to determine the metabolic fate of one compound in a mixture, and high purity is of particular importance with pyrethroids in which the different stereoisomers vary markedly in biological activity. Several C^{14}-labeled pyrethroids, stereochemically pure isomers of high specific activity, were recently synthesized (Nishizawa and Casida, 1965; Yamamoto and Casida, 1968) and this paper describes their use to define the metabolic pathway of allethrin and some other pyrethroids in houseflies. Earlier, Yamamoto and Casida (1966) showed that the *trans*-methyl group of the isobutenyl side chain of the acid moiety of pyrethrin I, allethrin, phthalthrin, and dimethrin is oxidized to a carboxyl group.

CHEMICALS

The structures and numbering system of the various acids and esters are shown in Figure 1. All labeled compounds were of greater than 99% radiochemical and stereochemical purity.

***t*-Butyl (±)-ω_t-Oxochrysanthemumate (XII).** (±)-*trans*-Chrysanthemumic acid (I) [from fractional crystallization of (±)-*cis*, *trans*-chrysanthemumic acid or from isomerization of the (±)-*cis*, *trans*-mixture followed by recrystallization; Berteau and Casida, 1969] was converted to *t*-butyl chrysanthemumate (X) (m.p. 27° C.) (Matsui and Yamada, 1963). Oxidation (selenium dioxide) of this acid gave *t*-butyl ω_t-oxochrysanthemumate (XII) [m.p. 81° C., from pentane; single thin-layer chromatography (TLC) spot, R_f 0.54 in hexane–ethyl acetate mixture (10 to 1)], shown to be stereochemically pure by oxidation in air to compound XIII (m.p. 130° C.) and pyrolysis (in diphenyl, 15 minutes, 260 to 270° C.) to a product (m.p. 207° C.) identical (IR spectrum, NMR spectrum) with natural chrysanthemum dicarboxylic acid (IV) (Matsui and Yamada, 1963).

(±)-ω_t-Oxochrysanthemumic Acid (III). Pyrolysis (condition described above) of the *t*-butyl ester (XII) gave this acid (m.p. 85° C., from ether: C% calcd. 65.91, found 66.34; H% calcd. 7.74, found 7.99); 2,4-dinitrophenylhydrazone, m.p. 244° C. (C% calcd. 53.03, found 52.83; H% calcd. 5.01, found 5.36; N% calcd. 15.46, found 15.34).

(±)-ω_t-Hydroxychrysanthemumic Acid (II). The *t*-butyl ester (XII) (5 grams) was reduced in methanol (20 ml.) with sodium borohydride (1.5 grams) at 25° C. for 18 hours. Chloroform (100 ml.) was added and the solution washed with 5% hydrochloric acid, aqueous bicarbonate solution, and water. Evaporation of the chloroform gave *t*-butyl ω_t-hydroxychrysanthemumate (XI) (6 grams) [b.p. 110° C. at 1 mm.: single TLC spot, R_f 0.11 in hexane–ethyl acetate mixture (10 to 1): C% calcd. 69.96, found 69.72; H% calcd. 10.07, found 9.84]. The *t*-butyl ester (XI) gave comparable yields of the acid (II) by pyrolysis (condition described above) or hydrolysis. Compound XI (5 grams) was hydrolyzed by refluxing with potassium hydroxide (3 grams) in ethanol (30 ml.) for three hours. Workup by concentrating under reduced pressure, concentrating again after addition of water, diluting the residue with water, washing with ether, acidifying, extracting the acidic product (2.2 grams) into ether, and recrystallization from ethyl acetate gave compound II (0.9 gram) (m.p. 85° C.: C% calcd. 65.19, found 65.05; H% calcd. 8.75, found 8.55).

Other Chrysanthemumic Acid Derivatives (V to IX, Figure 1). Compound VI was prepared by hydration of (±)-*trans*-chrysanthemumic acid (I) (Harper and Thompson, 1952). Compounds VII, VIII, and IX were from Masanao Matsui and compound V was from Minoru Ohno.

C^{14}-Labeled Pyrethroids. Four acid-labeled pyrethroids, with C^{14} in the carboxyl position, were used: (+)-*trans*-(+)-allethrin (XIV), (+)-*trans*-pyrethrin I, natural stereochemical configuration (XXII), (+)-*trans*-phthalthrin (XXV), and (+)-*trans*-dimethrin (XXVIII) (Yamamoto and Casida, 1968). Two alcohol-labeled pyrethroids were used: (+)-*trans*-(±)-allethrin (XIV) (4-C^{14} and $C^{14}H_3$) and (+)-*trans*-phthalthrin (XXV) (*N*-$C^{14}H_2$) (Yamamoto and Casida, 1968). In metabolic studies where acid- and alcohol-labeled preparations of allethrin were compared, the acid-labeled sample was

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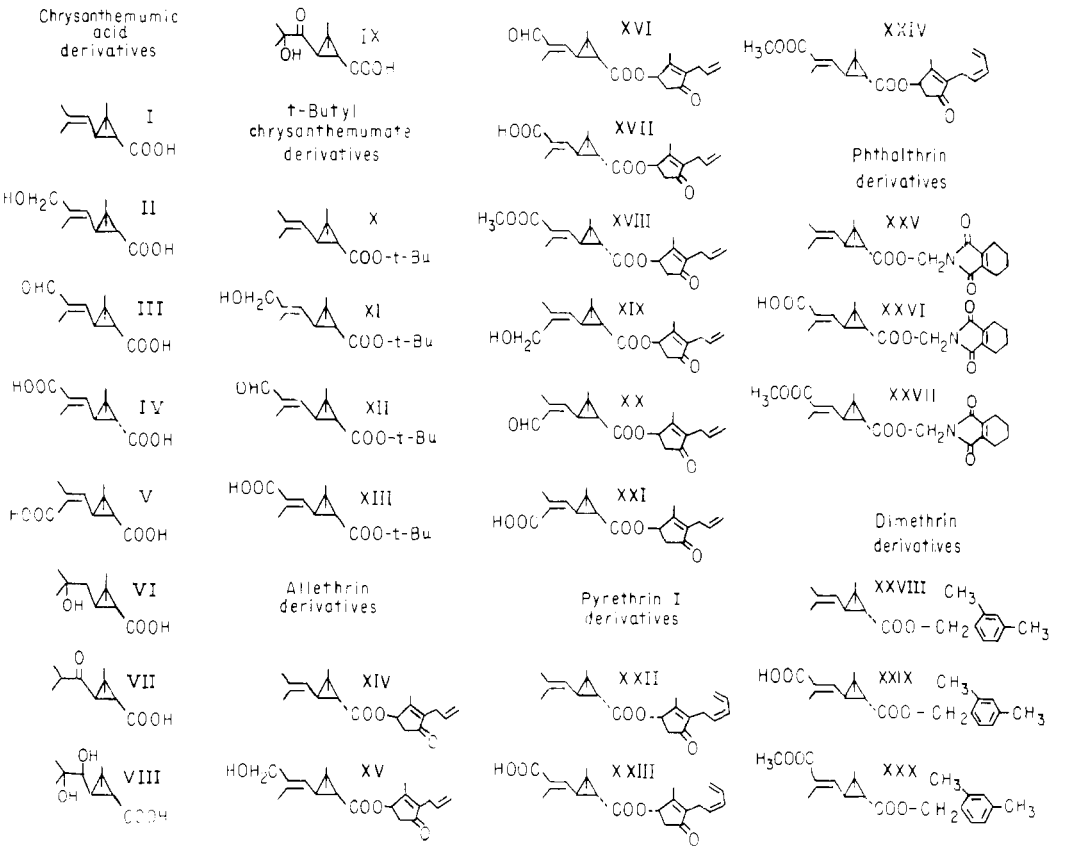

Figure 1. Structures and numbering system for chrysanthemumic acid, *t*-butyl chrysanthemumate, and certain pyrethroids, and their various derivatives of interest

diluted with unlabeled (+)-*trans*-(+)-allethrin to the specific activity of the corresponding alcohol-labeled sample.

Allethrin-ω_t-oic Acid (XVII) and Allethrin II (XVIII). (+)-*trans*-Chrysanthemum dicarboxylic acid (naturally derived) (IV) (1 gram) was refluxed for two hours in acetic anhydride (5 ml.); excess acetic anhydride was removed (reduced pressure), benzene was added and then evaporated to leave a dry residue. This anhydride in benzene (5 ml.) was set aside with (±)-allethrolone (1 gram) and pyridine (1 ml.) for four days at 25° C.; the mixture was then poured into ice, and the product extracted into ether. The ether extract was washed with 4% hydrochloric acid (20 ml.) and saline water to give four principal products [R_f 0.6, 0.5, 0.4, and 0.2 after TLC development with benzene and solvent system A (see below), in sequence and in the same direction]: two materials (R_f 0.4 and 0.2) chromatographed in the regions of chrysanthemum dicarboxylic acid and allethrolone, respectively; the product (R_f 0.5) cochromatographed with the major *in vitro* metabolite of allethrin (see below). Allethrin-ω_t-oic acid (XVII) was isolated by chromatography on a column of Silica gel H (70 grams) using the following developers: hexane (50 ml.), 50% ether in hexane (200 ml., fractions 1 to 20), and 60% ether in hexane (1000 ml., fractions 21 to 100). Fractions 34 to 55 contained allethrin-ω_t-oic acid (0.88 gram, 52%) (TLC control, R_f 0.5 in solvent system A, see below); amorphous solid (C% calcd. 68.65, found 68.41; H% calcd. 7.28, found 7.05); semicarbazone (m.p. 170° C., from methanol) (C% calcd. 61.68, found 61.73; H% calcd. 6.99, found 7.03; N% calcd. 10.79, found 10.79). Methylation of compound XVII with diazomethane in ether solution gave a product identical (IR spectrum) with allethrin II (XVIII) (Matsui and Meguro, 1964); also, cochromatography showed coincidence for the methyl ester of a major allethrin metabolite with allethrin II (XVIII) obtained from compound XVII, developing with hexane–ethyl acetate mixture (5 to 1) and solvent system B (see below). The amorphous product (compound XVII) was a mixture of two materials, with very similar R_f values; only the upper one cochromatographed with the major metabolite. The minor product (lower R_f) was probably the isomeric monoester of allethrolone and chrysanthemum dicarboxylic acid which would give the same analytical data and, when methylated, would give an infrared spectrum similar to allethrin II made by an unequivocal procedure (Matsui and Meguro, 1964).

Phthalthrin-ω_t-oic Acid (XXVI), Phthalthrin II (XXVII), Dimethrin-ω_t-oic Acid (XXIX), and Dimethrin II (XXX). The acids were prepared by reacting the anhydride made from chrysanthemum dicarboxylic acid with *N*-hydroxymethyl-3,4,5,6-tetrahydrophthalimide or 2,4-dimethylbenzyl alcohol, respectively, in the presence of benzene and pyridine. The product from reaction with the phthalimide was chromatographed on a column of Silica gel H and developed with benzene, 20% ether in benzene, and 30% ether in benzene; the product (compound XXVI), along with a minor component which possibly was the isomer, was in the fraction eluted with 20% ether in benzene. A major phthalthrin metabolite cochromatographed with phthalthrin-ω_t-oic acid (XXVI) [TLC development with benzene in the first direction and benzene (saturated with formic acid)-ether mixture (3 to 1) in the second direction], before methylation, and with phthalthrin II (XXVII) [TLC development with hexane–ethyl acetate mixture (9 to 1)], after methylation. The product from the benzyl alcohol reaction was chromatographed on a Silica gel H column with hexane and ether in hexane (20, 40, 60, and 80%) to give the desired material (compound XXIX) (0.33 gram) along with a minor impurity, possibly the isomer,

in the 60 and 80% ether in hexane fractions. As described below, a major dimethrin metabolite cochromatographed with dimethrin-ω_t-oic acid (XXIX) [TLC development with benzene in the first direction and benzene (saturated with formic acid)-ether mixture (5 to 1) in the second direction], before methylation, and with dimethrin II (XXX) [TLC development with hexane-ethyl acetate mixture (9 to 1)], after methylation.

Allethrin-ω_t-al (XVI). (±)-ω_t-Oxochrysanthemumic acid (III) (0.5 gram) in benzene (2.5 ml.) was treated with thionyl chloride (300 μl., 1.5 mol. equiv.) in hexane (1.5 ml.) at 25° C. for 18 hours. After removing volatile material, the acid chloride was reacted with (±)-allethrolone (0.5 gram, 1.2 mol. equiv.) in benzene (5 ml.) containing pyridine (0.5 ml.) at 25° C. for 18 hours in a sealed flask. The reaction mixture was poured onto a Florisil column (110 grams, packed with hexane), and eluted with portions of hexane containing increasing amounts of ether (10, 20, 30, 35, 40, 45, and 50%, each 200 ml.; 50 and 60%, each 250 ml.; methanol, 250 ml.). The eluate fractions were monitored by TLC (solvent systems A and C, see below) to locate the desired material (about 50 mg.) in the fractions eluting with 45 and 50% ether in hexane. The material obtained was a mixture of two principal compounds (TLC analysis); the upper one was isolated in pure form (single TLC spot) by developing the materials as a band with solvent system C (see below), removing the appropriate band by scraping, and eluting with 20% methanol in ether. The recovered material cochromatographed (TLC, solvent system C, see below) with a radioactive metabolite of allethrin. It probably was allethrin-ω_t-al (XVI) because hydrolysis yielded a single acid, with the R_f value of ω_t-oxochrysanthemumic acid (III), and treatment with 2,4-dinitrophenylhydrazine in methanol, as described later, gave the 2,4-dinitrophenylhydrazone of allethrolone methyl ether (yellow spot, appropriate TLC R_f value).

2,4-Dinitrophenylhydrazones (DNP) of Allethrolone and of Allethrolone Methyl Ether. By the general procedure for the hydrazone of pyrethrolone methyl ether (Crowley *et al.*, 1962), technical allethrin (2 grams) and 2,4-dinitrophenylhydrazine (1.2 grams) in 12 ml. of methanol–sulfuric acid mixture (9 to 1) were warmed at 60° C. for 30 minutes; the crystals were collected, dissolved in dichloromethane, and chromatographed on an alumina column using benzene and chloroform for elution. The major fraction, eluted with chloroform, gave the DNP of allethrolone methyl ether (m.p. 145° C., from chloroform-methanol: $\lambda_{max}^{C_2H_5OH}$ 382 mμ (log e 4.45): C% calcd. 55.48, found 55.56; H% calcd. 5.24, found 5.23; N% calcd. 16.18, found 16.46). (±)-Allethrolone (0.5 gram) in methanol (1.0 ml.) was added to a solution of 2,4-dinitrophenylhydrazine (1.3 grams) in 50 ml. of methanol–sulfuric acid mixture (99 to 1) to obtain, after three hours at 25° C., the DNP of allethrolone (0.9 gram) (m.p. 206° C., from chloroform or benzene; $\lambda_{max}^{C_2H_5OH}$ 382 mμ (log e 4.42); N% calcd. 16.86, found 16.99). Reaction of the DNP of allethrolone with methanol–sulfuric acid mixture (9 to 1) for 30 minutes at 50° C., adding a large volume of water, extracting with chloroform, washing the chloroform with water, concentrating the chloroform, discarding the first solid precipitating, and recovery of the crystals obtained on further concentration of the mother liquor gave the DNP of allethrolone methyl ether [m.p. and mixed m.p. 145° C.; TLC R_f value in benzene-ether mixture (30 to 1) identical to that for the product prepared from allethrin as described above].

METHODS

Thin-Layer Chromatography (TLC) and Radioactivity Measurements. Unless stated otherwise, pyrethroid metabolites, derivatives, and degradation products were resolved on 20 × 20 cm. chromatoplates, coated with silica gel G (0.25 mm.), by developing with the following solvent systems: A, benzene (saturated with formic acid by shaking 10 parts of benzene with 1 part of 88% formic acid)-ether mixture (10 to 3); B, ether-hexane mixture (1 to 1) saturated with ammonia by shaking 10 parts of the mixture with 1 part of 30% aqueous ammonia; C, ether-hexane mixture (2 to 1). In a few cases, specified later, Silica gel F_{254} chromatoplates (precoated, abrasion-resistant, Merck) were used. Unlabeled pyrethroids and related compounds were detected by spraying the plates with 20% (w./v.) phosphomolybdic acid in ethanol and heating at 110° C. for several minutes. 2,4-Dinitrophenylhydrazones were detected by their intense yellow color.

Radioactive materials on TLC plates were detected by autoradiography, at 5° C. when some of the products were to be recovered for further chemical studies or at 25° C. in other cases, or with an automatic radiochromatogram scanner (Tracerlab 4π Scanner, Model 525A). For quantitation, the radioactive regions of the TLC gel were scraped from the plates into scintillation vials containing 10 ml. of 0.55% (w./v.) 2,5-diphenyloxazole in toluene-methylcellosolve mixture (2 to 1) for direct counting with a Packard Tri-Carb liquid scintillation spectrometer (Model 3003). Samples for counting included: 10 μl. to 100 μl. of organic solvent extracts; the residue from evaporation of larger volumes of organic solvent extracts; up to 0.2 ml. of aqueous solution; gel regions scraped from TLC plates.

C^{14}-Labeled Derivatives for Cochromatography Studies. Metabolites of acid-labeled pyrethroids were scraped from TLC plates and recovered by extracting the gel with ether-methanol mixture (9 to 1) and concentration under nitrogen. Part or all of the extract was heated with 0.1N sodium hydroxide in methanol (1 ml.) in a sealed ampoule for 30 minutes (boiling water bath) to hydrolyze the esters. After adding a non-labeled reference compound (for example, chrysanthemum dicarboxylic acid), the mixture was concentrated under nitrogen, 5% sodium hydroxide solution (0.5 ml.) was added, and the aqueous phase was washed with ether (2 × 2 ml.). (Loss of radiocarbon in the ether, at this stage, was always negligible.) The aqueous phase was acidified with 20% hydrochloric acid (2 ml.) and extracted with ether (4 × 2 ml.), the ether was concentrated and spotted for TLC analysis, and the plate was developed with benzene in the first direction, to move interfering materials but not the acids away from the origin and toward the front, and then with solvent system A (see above) in the second direction to resolve the acids. The labeled chrysanthemumic acid derivatives were tentatively identified by TLC cochromatography, using solvent system A, with authentic compounds at the following R_f values: I, 0.76; II, 0.34; III, 0.52; IV, 0.48; V, 0.41; VI, 0.30; VII, 0.58; VIII, 0.12; and IX, 0.38.

Metabolites of alcohol-labeled allethrin, recovered by extraction (see above) of the appropriate gel regions, were deposited as a dry residue in the bottom of a small conical centrifuge tube, to which was added a small crystal of 2,4-dinitrophenylhydrazine (about 100 μg.) and 40 μl. of methanol-sulfuric acid mixture (9 to 1). The solution was heated at 50° C. for 30 minutes and, while still warm, was spotted for TLC development with benzene-ether mixture (30 to 1), in two dimensions. The unlabeled DNP of allethrolone methyl ether, spotted on the origin over the spot for the labeled material(s) from the reaction mixture, appeared as a yellow spot near the center of the plate (R_f 0.6) and cochromatographed with the labeled derivative prepared from alcohol-

labeled allethrin. Two additional products were often found: allethrolone DNP (R_f 0.1) and an unidentified product (R_f 0.17); the presence of these two products verified the identity of the allethrolone moiety because they also cochromatographed, when present, with yellow spots, on a TLC plate, derived from reaction of unlabeled allethrin under the same conditions. Samples containing low radiocarbon levels (a few hundred counts per minute) were chromatographed in one dimension, only, with benzene-ether mixture (30 to 1), and the plate was scanned with an automatic scanner; otherwise, radioautograms were utilized to detect the DNP of allethrolone methyl ether.

The DNP procedure is not ideal for conversion in high yield of a few micrograms of alcohol-labeled allethrin to an identified labeled product derived only from the alcohol moiety. However, it was found to be more satisfactory than direct alkaline hydrolysis, reduction with sodium borohydride followed by alkaline hydrolysis, reaction with ethylenediamine (Hogsett *et al.*, 1953), and treatment with liquid ammonia.

***In Vitro* and *In Vivo* Metabolism Studies.** Adult houseflies of six strains (SRS, SCR, S_{2356}, S_{2456}, R_{Hokota}, and R_{Baygon}), described by Tsukamoto *et al.* (1968) and Shrivastava *et al.* (1969), were fed only on sugar and water. Only female flies were employed for *in vivo* studies but abdomens of both male and female flies were used for *in vitro* studies. Unless stated otherwise, the R_{Baygon} or R_{Hokota} strain was used; the metabolites were completely the same with either strain.

Living houseflies, lightly anesthetized with carbon dioxide, were treated by injection through the thorax with 0.4 to 0.6 μg. of the test compound as a uniform emulsion in 1.0 μl. of acetone-water mixture (2 to 3). The flies were sacrificed for analysis in groups of 10 by freezing with dry ice at 0 or 90 minutes after injection. (The injected flies showed typical and intense symptoms of pyrethroid poisoning but none died during the 90-minute period after treatment.) Each group of flies was extracted by homogenizing in acetone (2 ml.) in an all-glass homogenizer, followed by sedimentation of insoluble materials by centrifugation. This procedure was repeated on the insoluble fraction twice more so that the acetone-soluble materials ended up in 6 ml. of total extract. Combustion analysis was used to determine the total radiocarbon content of the acetone-insoluble residue. The total organosoluble radiocarbon was determined on a small aliquot of the extract, and after 18 hours at −10° C., insoluble products were removed from the remainder by centrifugation (with a loss of less than 0.2% of the radiocarbon), and the supernatant was concentrated, under nitrogen, to a small volume preparatory to clean-up by descending paper chromatography on a 15-cm. strip of Whatman No. 1 paper, using acetonitrile for complete elution of soluble products from the paper, over a period of eight hours in the dark, and leaving interfering materials but no radiocarbon on the strip. A portion of the acetonitrile-soluble material was used for radiocarbon determination; the remaining portion was evaporated, under nitrogen, and the components were resolved on a TLC plate (Silica gel G, 0.30-mm. thickness) using solvent system A (see above) after an initial clean-up, on the plate, by development with benzene to move interfering materials but not any of the radioactive compounds from the origin and toward the front. The radioactive regions were located by autoradiography and their radioactivity levels were measured. Recovery analyses on unmetabolized compound in flies frozen immediately after injection and subsequently extracted for TLC analysis establish that recoveries are high and are independent of the labeling position; the values found were: allethrin, 92%; pyrethrin I, 87%; phthalthrin, 92%; and dimethrin, 97%. In one study involving living flies 90 minutes after injection, the acetone-insoluble residue (after extraction as described above) was suspended by homogenization in 0.2*M* phosphate–0.1*M* citrate buffer (pH 4.4) and divided into two equal parts; one was used directly and β-glucosidase (10 mg.) was added to the other. After incubation for two hours at 37° C., both reaction mixtures were extracted with ether and analyzed, as above, for total radioactivity and TLC-resolvable compounds.

The reaction conditions of Tsukamoto and Casida (1967b) were used for *in vitro* studies with fly abdomen homogenates. The labeled compound (10 μg.) was transferred with hexane to a 25-ml. Erlenmeyer flask, and the hexane was evaporated while tilting the flask to obtain a uniform coating on the bottom of the flask. The following constituents were added in sequence: not any or 3.5 mg. of reduced nicotinamide–adenine dinucleotide phosphate (NADPH); 2 ml. of fly abdomen homogenate, from 10 abdomens (unless stated otherwise) in 0.25*M* sucrose–0.15*M* sodium phosphate solution (pH 7.4). On occasion, the enzyme level, the subcellular fraction, the housefly strain used, the nature of the cofactor, the composition of the enzyme preparation and incubation mixtures, and the incubation time were varied as described below (Tsukamoto and Casida, 1967a, 1967b). After 0.5, 1.0, or 2.0 hours incubation at 30° C., the flask constituents were extracted with anhydrous ether (5 × 8 ml.), the combined ether extracts were dried with sodium sulfate, an aliquot was counted for radiocarbon content, the remainder was evaporated under nitrogen, and the components analyzed by TLC and autoradiography. In one series of experiments, individual flies were treated topically on the thorax with piperonyl butoxide (5 μg.) in 1.0 μl. of acetone, at varying periods of time before removing the abdomens for enzyme activity assay. In another series of experiments, involving 30 minutes incubation in the absence of NADPH fortification, the recovery values of unmetabolized compound were found to be independent of labeling position and were as follows: allethrin, 95%; pyrethrin I, 79%; phthalthrin, 79%; and dimethrin, 97%.

Labeled metabolites from *in vivo* and *in vitro* studies were compared, by cochromatography, with each other and with authentic unlabeled compounds from synthesis. The former type of intercomparison involved appropriate combinations of the *in vivo* and *in vitro* samples so that materials that might uniquely interfere with R_f values from either type of extract were represented in both extracts; this resulted in comparable samples in relation to non-radioactive interfering materials, differing only in the origin of radioactive compounds.

RESULTS

Preliminary Studies with Mixed-Function Oxidase System. Neither abdomen homogenates nor the microsomal fraction from these homogenates metabolize allethrin, pyrethrin I, phthalthrin, or dimethrin unless fortified with a pyridine nucleotide cofactor; the activity for the cofactor decreases in the order of NADPH, NADH, NADP, NAD, and not any. Using unfractionated homogenates of either the SCR or R_{Hokota} strains as the enzyme source, the activity with allethrin as the substrate is nearly the same with either male or female abdomens; accordingly, in subsequent studies, both sexes were used. The activity for allethrin metabolism of the abdomen homogenates, fortified with NADPH and incubated for two hours, decreases with strain in the order of R_{Baygon}, R_{Hokota},

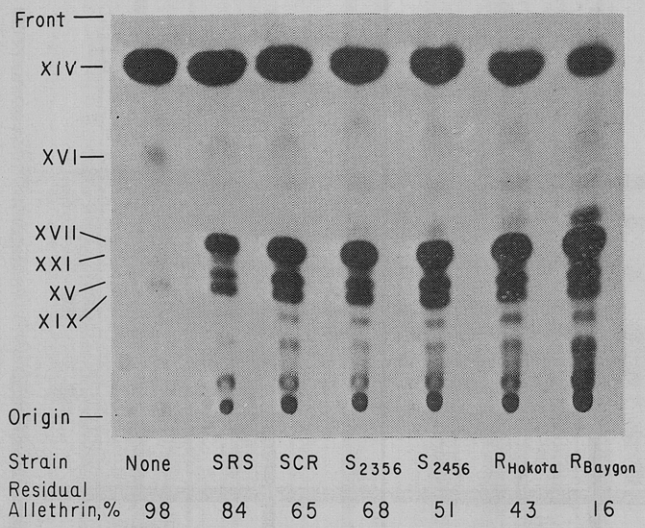

Figure 2. TLC radioautogram showing metabolites of allethrin-acid-C^{14} formed by the mixed-function oxidase system of various housefly strains

Roman numerals refer to chemical structures tentatively assigned to metabolites (see Figure 1). Development sequence (same direction): 1. benzene; 2. solvent system A

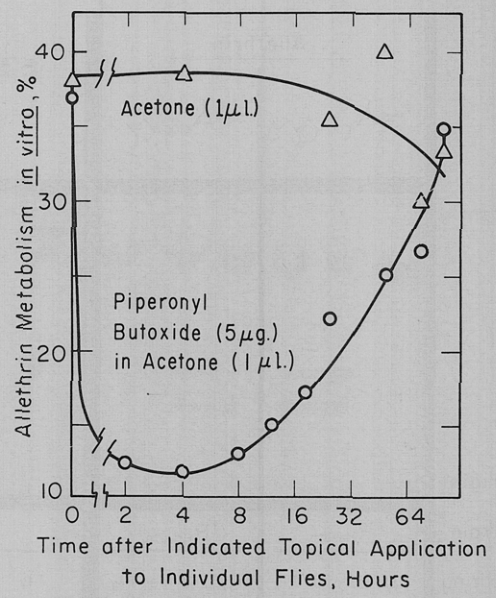

Figure 3. Extent of metabolism of allethrin-acid-C^{14} by mixed-function oxidase system prepared from abdomens of SCR female houseflies at various times after topical application of piperonyl butoxide to the thorax

S_{2456}, S_{2356} = SCR, and SRS (Figure 2); interestingly, the strains also become more susceptible to allethrin in the same general sequence, although the topical LD_{50} values vary only 2.5-fold from the most resistant to the most susceptible strain. Most of the studies utilize the R_{Baygon} and R_{Hokota} strains because they have high enzyme activity and resistance to poisoning; however, the metabolic products produced do not appear to vary with strain. Adding bovine serum albumin (BSA) to the abdomen homogenization and incubation mixtures increases enzyme activity, assayed with allethrin, confirming the findings of Tsukamoto and Casida (1967a) with carbamate substrates. In routine studies, BSA was not added to the incubations to minimize the number of constituents.

From *in vitro* assays of enzymes from synergist-treated insects, with allethrin as substrate, it appears that the enzymatic system is inhibited *in vivo* by pyrethroid synergists. Thus, the enzyme activity of SCR female flies treated topically with piperonyl butoxide (5 μg.) decreases to a minimum at four hours after treatment but returns nearly to normal after 72 and 96 hours (Figure 3); the same metabolities are produced in the absence of the synergist and at different times after treatment with the synergist.

Comparison of Metabolites in *In Vivo* and *In Vitro* Studies with Each of Four Pyrethroids. The metabolites of allethrin, pyrethrin I, phthalthrin, and dimethrin generally are the same whether these metabolities are formed by living flies or by the *in vitro* enzyme system (Figure 4). Based on a separate study, it is known that the products are completely the same as those illustrated with all labeled preparations, when using either the R_{Baygon} or R_{Hokota} strains and when TLC development involves Silica gel G or F_{254} chromatoplates. Allethrin gives the same metabolites from the alcohol- and acid-labeled preparations and in approximately the same ratio, indicating that ester hydrolysis does not occur and that the different stereochemistry of the acid-labeled preparation [(+)-*trans*-(+)] and the alcohol-labeled preparation [(+)-*trans*-(±)] does not alter the metabolism rate or pathway. Most of the phthalthrin metabolites are also the same, both *in vivo* and *in vitro*, from either the acid- or alcohol-labeled samples.

Identification of Allethrin Metabolites. Metabolites were most easily characterized from allethrin, of which acid- and alcohol-labeled samples were available; the products from the abdomen homogenate system were examined because metabolites which cochromatograph with those formed in living houseflies were easily produced with minimum interfering materials.

Each of the ten or more metabolites of allethrin is an ester (Figures 4 and 5), is more polar than allethrin, and is formed by the mixed-function oxidase system, suggesting that they are oxidation products. After removing allethrin by chromatography, the remaining metabolite mixture gives almost all of the radiocarbon from the alcohol-labeled preparation as allethrolone methyl ether dinitrophenylhydrazone, based on the DNP test, and little, if any, of the radiocarbon from the acid-labeled preparation occurs as chrysanthemumic acid (I). No free chrysanthemumic acid or allethrolone is produced by the fly abdomen enzyme–NADPH system. Thus, the major modifications occur on the acid and not on the alcohol moiety of the intact esters.

The major allethrin metabolites remain at or near the origin when the TLC plate is developed with a neutral and relatively apolar solvent system [ether-hexane mixture (1:1) or benzene-ether mixture (10:3)]. A benzene-ether system containing formic acid (solvent system A) moves the major metabolites from the origin, giving good resolution (Figures 2, 4, 5, and 6). When the ether-hexane mixture is saturated with aqueous ammonia (solvent system B), the major metabolite does not move from the origin, suggesting that it contains an acidic grouping, even though it is an ester as indicated above. Development in two-dimensions, first with the formic acid-containing solvent (system A) and then with the ammonia-containing solvent (system B), leaves the major metabolite at the origin in the second direction of development; so, the major metabolite is acidic, but a few metabolites are neutral because they move from the origin in both solvent systems;

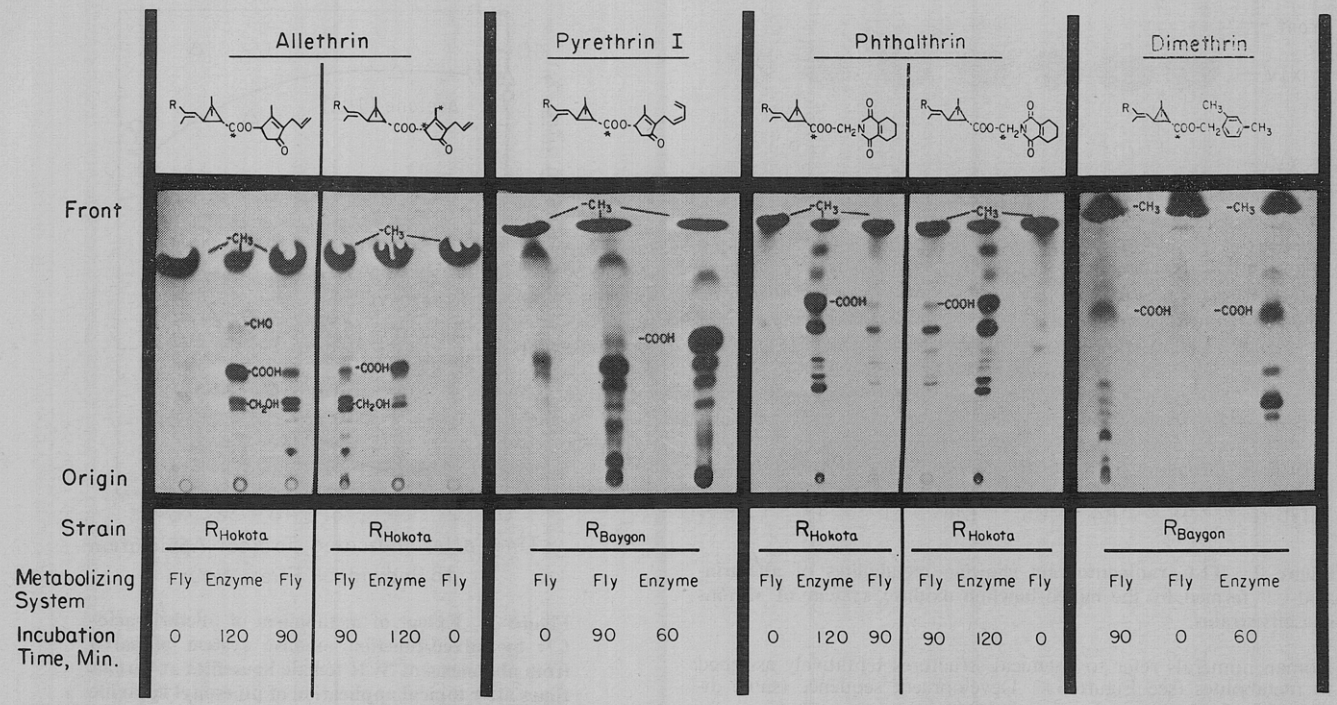

Figure 4. TLC radioautograms showing metabolites of various C^{14}-labeled preparations of allethrin, pyrethrin I, phthalthrin, and dimethrin formed by living houseflies and by their mixed-function oxidase system

Metabolites from oxidation of the *trans*-methyl group of the isobutenyl moiety are indicated. Conditions for TLC development: Silica gel G for allethrin and phthalthrin, silica gel F_{254} for pyrethrin I and dimethrin; benzene as the first developer followed by a second development in the same direction with solvent system A except in the case of phthalthrin where benzene (saturated with formic acid)—ether mixture (5 to 2) was used

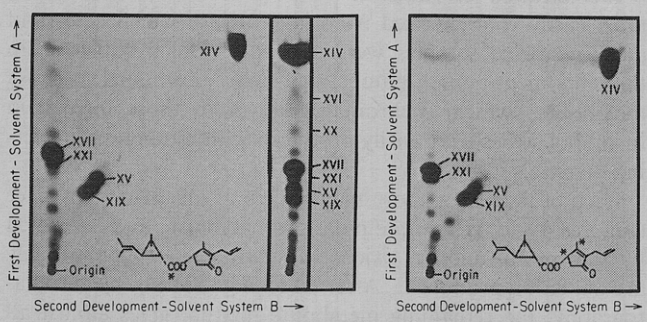

Figure 5. TLC radioautograms showing metabolites of acid- and alcohol-labeled preparations of allethrin formed by mixed-function oxidase system and resolved with an acidic solvent (A) which moves neutral and acidic products and an ammoniacal one (B) which moves only neutral products

Roman numerals refer to chemical structures tentatively assigned to metabolites (see Figure 1)

the same results are obtained with acid- and alcohol-labeled preparations (Figure 5).

The identity of the major allethrin metabolites and the basis for the structural assignments are given in Figure 6.

The major allethrin metabolite is allethrin-ω_t-oic acid (*O*-demethyl allethrin II) (XVII) (Yamamoto and Casida, 1966). The acid-labeled metabolite yields chrysanthemum dicarboxylic acid (IV) on saponification (TLC cochromatography) and allethrin II (XVIII) on methylation with diazomethane (TLC cochromatography). In the DNP test, the alcohol-labeled metabolite yields, as expected for esters of allethrolone, mostly the DNP of allethrolone methyl ether with small amounts of allethrolone DNP and the unknown substance normally encountered in this type of reaction. Thus, one methyl group (ω_t position) in the isobutenyl moiety is oxidized to a carboxylic acid group while the ester linkage remains intact and the alcohol moiety is not modified.

It is probable that the metabolic formation of allethrin-ω_t-oic acid involves allethrin-ω_t-ol (XV) and allethrin-ω_t-al (XVI) as intermediates because a few neutral metabolites are found. When a neutral solvent (system C) is used for development of the metabolites on TLC plates, allethrin-ω_t-oic acid and other acidic metabolites remain near the origin, allethrin moves near the front, and the neutral metabolites occur in an intermediate position (Figure 7). Two neutral metabolites (XV and XIX) appear with both the acid- and alcohol-labeled preparations and a third (XVI) clearly appears in the radioautogram for the acid-labeled preparation but does not show on the film for the alcohol-labeled preparation. (These same neutral metabolites are also evident in Figures 4 and 6.) Both metabolites XIX (discussed below) and XV from the alcohol-labeled preparation are converted to the DNP of allethrolone methyl ether (Figure 6); this confirms that the modifications are on the acid moiety, probably by oxidation. When saponified, acid-labeled metabolite XV gives ω_t-hydroxychrysanthemumic acid (II) (TLC cochromatography) (Figure 6); so, metabolite XV is probably allethrin-ω_t-ol. Saponification of an acid-labeled sample of metabolite XVI gives ω_t-oxochrysanthemumic acid (III) (TLC cochromatography) (Figure 6); metabolite XVI is identical with synthetic allethrin-ω_t-al (TLC cochromatography).

There is some evidence of the structure of two additional metabolites of allethrin: XIX, which chromatographs just below allethrin-ω_t-ol (XV) and the acidic metabolite (XXI) which chromatographs just below allethrin-ω_t-oic acid (XVII). Each metabolite is an ester, appearing with both acid- and alcohol-labeled preparations. The acidic metabolite (XXI), with the radiolabel in the alcohol, contains unmodified allethrolone, as determined by the DNP test (Figure 6). In TLC separation with solvent system A (which suppresses ionization of the carboxyl group), the acidic metabolite

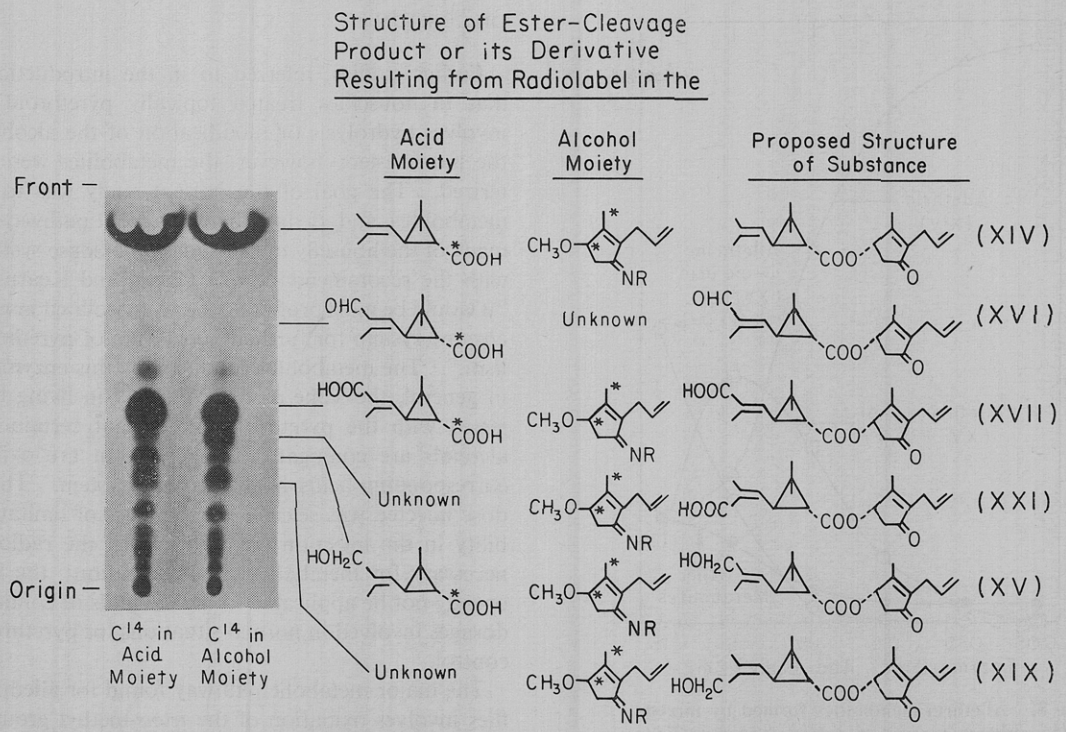

Figure 6. TLC radioautogram showing metabolites of acid- and alcohol-labeled preparations of allethrin-C^{14} formed by the mixed-function oxidase system

Structures are those proposed for products derived from degradation of labeled metabolites and for the metabolites themselves. Developing sequence (same direction): 1. benzene; 2. solvent system A. NR refers to 2,4-dinitrophenylhydrazone derivative. Roman numerals refer to structures given in Figure 1

(XXI) appears just below allethrin-ω_t-oic acid in the same manner as ω_c-chrysanthemum dicarboxylic acid (V) appears just below (+)-ω_t-chrysanthemum dicarboxylic acid (IV). This circumstantial evidence is the basis for designating metabolite XXI allethrin-ω-oic acid. Although poorly resolved, it is known that allethrin-ω_t-oic acid appears in twofold or greater amounts than allethrin-ω_c-oic acid. Metabolite XIX, with the alcohol label, also has the unmodified allethrolone moiety (Figure 6). Hydrolysis of the acid-labeled sample of metabolite XIX yields an uncharacterized acid different from any available for study; the known chrysanthemumic acid derivatives (II to IX, except V) involve oxidation of the ω_t-methyl group or oxidation or hydration of the double bond. This unknown acidic product chromatographs just below ω_t-hydroxychrysanthemumic acid (II), the position anticipated for ω_c-hydroxychrysanthemumic acid. The R_f value, relative to allethrin-ω_t-ol, indicates that metabolite XIX is allethrin-ω_c-ol.

Identification of Major Acidic Metabolite of Pyrethrin I, Phthalthrin, and Dimethrin. *Each major metabolite produced* by housefly *in vitro* attack on pyrethrin I-acid-C^{14}, phthalthrin-acid-C^{14}, phthalthrin-alcohol-C^{14}, and dimethrin-acid-C^{14} is acidic. Phthalthrin is metabolized, *in vitro* and *in vivo*, without significant hydrolysis of the ester grouping because the same major products are detected with both the acid- and alcohol-labeled preparations (Figure 4); saponification of the major acid-labeled metabolite yields chrysanthemum dicarboxylic acid (IV) (TLC cochromatography). A major metabolite from the acid-labeled preparation of phthalthrin cochromatographs, under TLC conditions, with phthalthrin-ω_t-oic acid (XXVI) and that from dimethrin cochromatographs with dimethrin-ω_t-oic acid (XXIX); to support this, methylation of the two metabolites gives derivatives which cochromatograph with phthalthrin II (XXVII) and dimethrin II (XXX), respectively. Similarly, a material

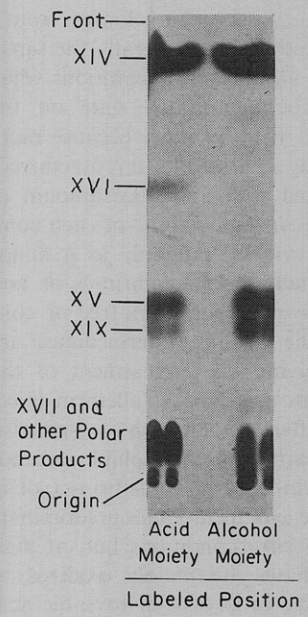

Figure 7. TLC radioautogram showing metabolites of acid- and alcohol-labeled preparations of allethrin-C^{14} formed by the mixed-function oxidase system and resolved with a neutral solvent system (C) that moves only neutral products from the region of the origin

Roman numerals refer to chemical structures tentatively assigned to metabolites (see Figure 1)

cochromatographing with natural pyrethrin II (XXIV), in hexane–ethyl acetate mixture (5:1), is formed on methylation of the major metabolite of pyrethrin I, indicating that the metabolite is pyrethrin-ω_t-oic acid (*O*-demethyl pyrethrin II) (XXIII). These findings establish that oxidation of the ω_t-methyl group of the isobutenyl moiety is important in the metabolism of these three pyrethroids (Yamamoto and Casida, 1966), but the TLC positions of the intermediate compounds in formation of the ω_t-oic acids are unknown.

Quantitative Considerations on Pyrethroid Metabolism. Allethrin and phthalthrin are more rapidly metabolized than

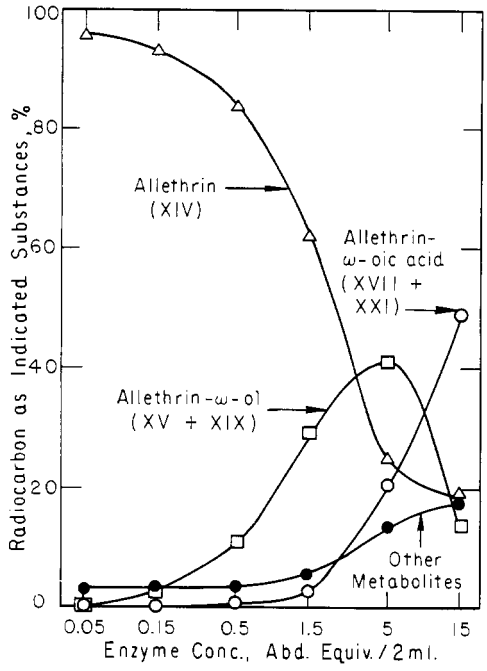

Figure 8. Allethrin metabolites formed by mixed-function oxidase system at various concentrations of housefly abdomen homogenate

pyrethrin I or dimethrin by the isolated housefly abdomen enzyme system but not by living flies (Table I). Although the results presented are with the R_{Baygon} strain, it is known from a separate experiment that the findings are generally the same, both *in vivo* and *in vitro*, with all labeled preparations when the R_{Hokota} strain is used. The quantitative data on the products are most meaningful with allethrin because more metabolites have been tentatively identified for this pyrethroid. The ω_t-oic acid (possibly mixed with a smaller amount of the ω_c-oic acid) is the major *in vitro* metabolite of each compound studied and it is also evident, but only as a minor metabolite, under *in vivo* conditions. Allethrin-ω_t-ol and some -ω_c-ol are the major *in vivo* metabolites, in free or conjugated form. Apparently, the alcohol intermediates are conjugated as glucosides in living flies; treatment of the acetone-insoluble fraction, from acid-labeled allethrin injection, by homogenization in buffer and addition of β-glucosidase, releases 85% of the radiocarbon as ether-soluble metabolites, consisting only of allethrin-ω_t-ol and allethrin-ω_c-ol in an approximate 3:1 ratio. The ω-ol metabolites predominate at low enzyme levels under *in vitro* conditions but, at high enzyme levels, these intermediates are mainly oxidized to the ω-oic acids (Figure 8). The ratio of ω-ol to ω-oic acid metabolites appears to depend on the relative ratio of the oxidative enzymes present and, in any case, there is little accumulation of the ω-al derivative. When 5×10^{-3} *M* sodium cyanide is added to the *in vitro* enzymatic system, the oxidation of allethrin to the ω-ol metabolites is not inhibited but the alcohol compounds accumulate rather than being converted to the ω-oic acids, suggesting that the enzyme active in initial hydroxylation is less sensitive to cyanide than the enzyme(s) involved in subsequent oxidation of the alcohol intermediate(s). The quantitative differences between the *in vitro* data obtained with the alcohol- and acid-labeled preparations shown in Table I probably result from the slightly higher substrate levels used with the alcohol-labeled preparations giving a lower rate of oxidation.

DISCUSSION

Earlier studies, referred to in the introduction, indicated that in houseflies treated topically pyrethroid metabolism involves hydrolysis or modification of the alcohol moiety of the intact ester; however, the metabolites were not characterized. The goal of the present study was to identify the metabolites and define the metabolic pathways. Use was made of the housefly mixed-function oxidase system in accord with the recommendation of Chang and Kearns (1964) that "it would be most profitable to use an isolated insect enzyme or enzyme system for further elucidation of pyrethroid metabolism." The metabolites produced by this enzyme system are, in general, the same as those formed in living houseflies injected with the pyrethroid except that certain intermediate alcohols are conjugated in the fly but are oxidized to the corresponding acids in the enzyme system. The pyrethroid dose injected was selected on the basis of limitations of solubility in the injection medium and of the radiocarbon level necessary for metabolite characterization; the findings may or may not be applicable to the methods of contact and lower dosages involved in normal situations for pyrethroid use in fly control.

The major metabolic pathway found for allethrin in houseflies involves oxidation of the *trans*-methyl group of the isobutenyl moiety, by microsomal mixed-function oxidases, to the hydroxymethyl compound which, by the action of other enzymes, forms a conjugate or is oxidized to the corresponding aldehyde and acid compounds (Figure 9). A comparable, but less prominent, pathway appears to involve a similar series of reactions at the *cis*-methyl group. Little if any hydrolysis or modification of the alcohol moiety is detected. Pyrethrin I, phthalthrin, and dimethrin are probably similarly metabolized but the products are not so well characterized. Three points should be considered in evaluating which metabolism step is the significant detoxification reaction: the ω_t-oic acids of chrysanthemumates are of greatly reduced toxicity (Yamamoto and Casida, 1966); the isobutenyl group is not essential for insecticidal activity (Berteau and Casida, 1969; Berteau *et al.*, 1968; Matsui and Kitahara, 1967); it is unlikely that very polar or ionized metabolites will penetrate the nerve and show activity. The microsomal mixed-function oxidases first form the alcohols and therefore their presence and action are critical in initiating the reactions leading to detoxification; however, the actual detoxification steps may be the subsequent conjugation of the alcohols and the oxidation of the aldehydes to the acids.

High activity for oxidative metabolism of allethrin is conferred primarily by the second chromosome in one strain of houseflies (R_{Baygon}) and by the fifth chromosome in another strain (F_c); with each of these strains, the major resistance factor(s) is conferred by the same chromosome which confers high mixed-function oxidase activity, suggesting a cause-and-effect relationship (Plapp and Casida, 1969).

Synergists in general, and piperonyl butoxide in particular, probably act by blocking the initial oxidation of pyrethroids, at the isobutenyl-methyl groups, to the alcohol intermediates. The methylenedioxyphenyl synergists possibly substitute for the pyrethroid at the active site of the detoxifying enzyme, undergoing demethylenation in the process but sparing the pyrethroid from detoxification (Casida *et al.*, 1966). However, compounds lacking the isobutenyl grouping are also synergized (Berteau and Casida, 1969) indicating that there are other sites, in addition to the isobutenyl group, which are attacked by mixed-function oxidases and limit stability *in vivo*.

Three types of structure-activity studies have been used in

Table I. Metabolic Fate of Variously-labeled Pyrethroids in Living Houseflies and after Incubation with Abdomen Microsome-NADPH Enzyme System

Fraction/Compound[a]	Compound Number[b]	Radiocarbon Recovery, %: Enzyme System, 30 Min. SRS acid-C^{14}	SRS alc-C^{14}	R_{Baygon} acid-C^{14}	R_{Baygon} alc-C^{14}	Living Flies, 60 Min. R_{Baygon} acid-C^{14}	R_{Baygon} alc-C^{14}
Allethrin							
Organosoluble[c]							
Allethrin	XIV	63.0	67.7	16.2	28.4	54.9	42.0
-ω_t-al	XVI	1.6	0.9	0.8	1.1	2.1	1.8
Unknown (1)		0.0	0.0	1.3	0.9	0.0	0.0
-ω_t- and -ω_c-oic acids	XVII, XXI	9.6	4.2	49.1	39.9	2.9	3.0
-ω_t-ol	XV	8.3	4.0	2.9	0.0	7.8	6.0
-ω_c-ol	XIX	2.8	3.6	2.7	4.2	4.2	6.8
Unknowns (4)		3.3	2.5	5.4	6.6	6.0	8.2
Origin		2.1	1.7	4.1	5.6	3.9	6.4
Water-soluble[d]		2.7	1.8	9.9	8.7	16.0	23.7
Loss[e]		6.6	13.6	7.6	4.6	2.2	2.1
Pyrethrin I							
Organosoluble[c]							
Pyrethrin I	XXII	62.5		45.2		55.7	
Unknown (1)		4.0		4.0		3.8	
-ω_t-oic acid	XXIII	2.3		7.0		2.0	
Unknowns (6)		11.7		13.8		19.5	
Origin		4.9		9.4		4.5	
Water-soluble[d]		6.9		14.3		14.5	
Loss[e]		7.7		6.3		0.0	
Phthalthrin							
Organosoluble[c]							
Phthalthrin	XXV	43.4	52.1	3.8	21.8	54.0	42.3
Unknowns (2)		4.2	1.3	2.0	1.2	3.8	3.0
-ω_t-oic acid	XXVI	11.7	9.7	40.0	36.7	2.4	2.4
Unknowns (6)		20.2	19.1	8.2	6.0	18.0	24.5
Origin		2.3	1.8	0.8	0.7	3.9	1.8
Water-soluble[d]		8.1	9.6	31.0	25.6	16.2	19.1
Loss[e]		10.1	6.4	14.2	8.0	1.7	6.9
Dimethrin							
Organosoluble[c]							
Dimethrin	XXVIII	69.8		49.4		69.4	
Unknown (1)		0.3		0.0		1.3	
-ω_t-oic acid	XXIX	5.3		13.2		3.5	
Unknowns (4)		3.6		12.4		12.0	
Origin		0.4		0.6		1.9	
Water-soluble[d]		1.8		9.7		8.7	
Loss[e]		18.8		14.7		3.2	

[a] Compounds listed in order of decreasing R_f values. Number in parenthesis is the number of "unknown" compounds.
[b] See Figures 1 and 4.
[c] Ether extract of incubated enzyme mixture or acetone-soluble fraction from living flies.
[d] Aqueous phase after ether extraction of incubated enzyme mixture or acetone-insoluble residue from living flies.
[e] Includes excretions from living flies.

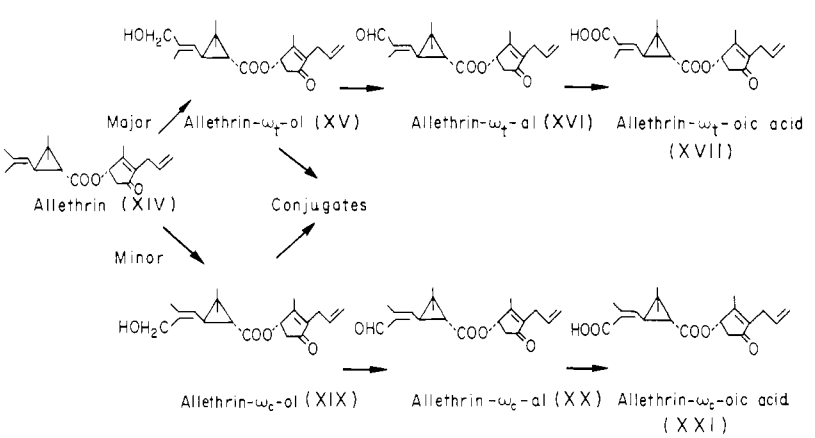

Figure 9. Tentative metabolic pathway for allethrin in houseflies

understanding the insect toxicology of pyrethroid insecticide chemicals; structure-toxicity experiments, based on insect knockdown and kill, which indicate desirable structural features for insecticidal activity; structure-neurotoxicity studies, to determine the structures necessary for optimal fit at the site of physiological disruption at the nerve membrane (Berteau *et al.*, 1968); structure-toxicity studies in the presence and in the absence of synergists. The present investigation indicates that a fourth type of structure-activity study involving the substrate-specificity of insect mixed-function oxidases for metabolism of pyrethroids might be a useful supplement to determine groupings on the pyrethroid molecule which limit their biological stability and effect.

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