Metabolic Fate of Pyrethrin I, Pyrethrin II, and Allethrin Administered Orally to Rats

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The oral administration of radio-labeled pyrethrin I, pyrethrin II, and allethrin to rats produces several urinary metabolites, identified by chromatographic and spectroscopic analyses. Each isolated metabolite contains a *trans*-2-carboxyprop-1-enyl side chain resulting from oxidation of the chrysanthemate isobutenyl group or hydrolysis of the pyrethrate methoxycarbonyl group. Also, the *cis*-2′,4′-pentadienyl side chain of pyrethrin I and pyrethrin II is modified to give a *cis*-4′,5′-dihydroxypent-2′-enyl group, a 4′ conjugate of this diol, or a *trans*-2′,5′-dihydroxypent-3′-enyl group. Allethrin is oxidized not only at the chrysanthemate isobutenyl moiety but also at the allyl group to the 1′-hydroxyprop-2′-enyl and 2′,3′-dihydroxypropyl derivatives, or at a methyl on the cyclopropyl moiety to a hydroxymethyl derivative. Allethrin gives some chrysanthemum dicarboxylic acid and allethrolone. Rapid detoxification in mammals by these metabolic pathways is probably an important factor in the selective toxicity of pyrethroids.

Pyrethrum is an extremely effective insecticide and, used normally, is not hazardous to mammals. The insecticidal activity of pyrethrum extract is due mainly to pyrethrin I (1b, Figure 1) and pyrethrin II (2b, Figure 1) (Crombie and Elliott, 1961). These two natural products are more complex, particularly in their stereochemistry, than most other compounds used to control insects, and so their metabolic fate in mammals has not been studied critically until recently. The urine of people treated with pyrethrum as an anthelmintic gave a positive Denigès reaction (Audiffren, 1934), assumed to indicate the presence of chrysanthemic acid (1a, Figure 1). When administered to mammals, (±)-*cis,trans*- or (±)-*trans*-chrysanthemates are known to be hydrolyzed at the cyclopropane ester link, with or without oxidation of the alcohol moiety, from studies with other chrysanthemates such as 2,4-dimethylbenzyl (dimethrin) and 6-chloropiperonyl (Barthrin) (Ambrose, 1963, 1964; Masri *et al.*, 1964) and 3,4,5,6-tetrahydrophthalimidomethyl (phthalthrin) derivatives (Miyamoto *et al.*, 1968). This contrasts with results in houseflies with (+)-*trans*-chrysanthemates (pyrethrin I, allethrin, dimethrin, and phthalthrin) in which the mixed function oxidase system attacks the *trans*-methyl group of the isobutenyl side chain to yield *O*-demethyl pyrethrin II analogs (Yamamoto and Casida, 1966). These products are also produced *in vivo via* the corresponding alcohol and aldehyde intermediates (Yamamoto *et al.*, 1969). Such precedents suggest that pyrethrins I and II may be metabolized in mammals by oxidative or hydrolytic routes, or both.

The only feasible method for establishing the metabolic fate of pyrethroids in mammals is by using radiotracers, which requires pyrethrin I and pyrethrin II with ^{14}C or ^{3}H at known positions in stereochemically pure esters of natural configuration. Products labeled in both acid and alcohol moieties help to determine the relative contribution of cyclopropane ester hydrolysis to metabolic degradation. Methods to synthesize such radio esters have been developed (Elliott *et al.*, 1969; Elliott and Casida, 1972; Nishizawa and Casida, 1965; Yamamoto and Casida, 1968). The metabolism of pyrethrin I and allethrin (1c, Figure 1) was compared because they differ only in the alcohol side chain (*cis*-2,4-pentadienyl or allyl).

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In our work, pyrethrin I, pyrethrin II, and allethrin were administered to rats; the metabolites excreted in the urine were isolated by column chromatography and characterized by derivatives and particularly by nuclear magnetic resonance (nmr) and mass spectroscopy (ms). A brief preliminary account of the work is given elsewhere (Casida *et al.*, 1971).

MATERIALS AND METHODS

Unlabeled Compounds. Pure pyrethrin I (1R, 3R, 4′S) and pyrethrin II (1′E, 1R, 3R, 4′S) (natural configurations of 1b and 2b, respectively, Figure 1) were made by reconstitution from the acid and alcohol moieties isolated from the natural esters (Elliott and Janes, 1969). Allethrin was "bioallethrin" (mixture of two isomers of 1c, Figure 1; 1R, 3R, 4′RS) from McLaughlin Gormley King Co. (Minneapolis, Minn.).

Labeled Compounds. The radio-labeled preparations are designated by abbreviations incorporating the compounds (PyI or pyrethrin I, PyII or pyrethrin II, AI or allethrin, CA or chrysanthemic acid, CDCA or chrysanthemum dicarboxylic acid), the radioisotope present (^{3}H or ^{14}C), the moiety labeled (alcohol or alc and acid), and sometimes the actual positions of labeling [$^{14}C(O)O$, $^{14}CH_3O$, $(^{14}CH_3)_2C=$, $HO(O)^{14}C(CH_3)C=$, and N-$^{14}CH_2$].

[5-CH_2,3-CH_3-^{3}H]-Pyrethrin I (designated PyI-^{3}H-alc), [5-CH_2,3-CH_3-^{3}H]-pyrethrin II (designated PyII-^{3}H-alc), and [5-CH_2,3-CH_3-^{3}H]-allethrin (designated AI-^{3}H-alc) had specific activities of 675, 626, and 317 mCi per mmol, respectively (Elliott *et al.*, 1969; Elliott and Casida, 1972). Pyrethrin I labeled in the cyclopropane carboxy group [designated PyI-$^{14}C(O)O$-acid] and allethrin labeled in the cyclopropane carboxy group [designated AI-$^{14}C(O)O$-acid] had specific activities of 1.3 mCi per mmol (Yamamoto and Casida, 1968). [4-CH,3-CH_3-^{14}C] Allethrin (designated AI-^{14}C-alc) had a specific activity of 0.16 mCi per mmol (Yamamoto and Casida, 1968). Immediately before use, the esters were purified by removal of minor decomposition products; bands were spotted on thin-layer chromatoplates (silica gel F_{254}), developed with ether–hexane (2:1) mixture, and esters recovered by extraction of the appropriate gel region (detected by uv visualization) with ether. The purity of each radiolabeled pyrethroid as used was greater than 98%, determined by thin-layer chromatography (tlc) and radioautography.

Pyrethrin II ^{14}C-labeled in the methoxy position (designated PyII-$^{14}CH_3O$-acid) was prepared (with Loretta Gaughan) from chrysanthemum dicarboxylic acid dichloride, (+)-pyrethrolone and ^{14}C-methanol. Preliminary work

showed that the acid chloride reacted with pyrethrolone preferentially at the cyclopropane acyl chloride and that, after reaction with methanol, the mixture of isomeric esters [shown by nmr comparison of the peaks at τ 6.29 (pyrethrin II) and 6.24 (isomer; 2c, Figure 1) to contain more pyrethrin II than isomeric ester] could be separated by tlc with benzene–nitromethane. Chrysanthemum dicarboxylic acid (CDCA, 2a, Figure 1; 100 mg, 0.5 mmol) was refluxed in benzene (2 ml) with thionyl chloride (150 μl, 2.1 mmol) for 4 hr. Excess reagent was removed by evaporation in a stream of dry nitrogen, and final traces by repeating this procedure after adding a second portion (2 ml) of benzene. Anhydrous (+)-pyrethrolone, obtained by dehydration of the hydrate (100 mg, 0.5 mmol) *in vacuo*, was added in benzene (2 ml), then pyridine (150 μl, 1.9 mmol) was added, and the mixture was set aside in the dark for 15 hr at room temperature. ^{14}C-Methanol (2.5 mg, 0.078 mmol, 12.8 mCi/mmol = 1 mCi) was then transferred to the reaction mixture with benzene (2 × 250 μl); further portions of methanol (unlabeled) were added after 3 hr (15 μl) and 6 hr (15 μl), and then the whole reaction mixture was set aside in the dark at room temperature for 3 days. The mixture (50 mg) containing PyII-$^{14}CH_3O$-acid and the isomeric ester was divided into two equal portions, each of which was processed on a column of Florisil (50 g) by elution with hexane–ether mixtures as described for ^{3}H-pyrethrin II (Elliott and Casida, 1972). PyII-$^{14}CH_3O$-acid and the isomeric ester were located and separated from other products by adding PyII-^{3}H-alc (2 mg, *ca.* 4 mCi) to one portion of the reaction mixture. The ^{14}C-labeled esters, without added PyII-^{3}H-alc, were isolated by monitoring the ^{14}C content of fractions at equivalent positions on a similar column. An early fraction eluted with 25–35% ether in hexane contained ($^{14}CH_3O$) dimethyl chrysanthemum dicarboxylate (5 mg, 0.1 mCi, λ_{max} 238 nm, ϵ 15,000) and the mixture of ^{14}C-labeled isomers (2b and 2c, Figure 1) eluted in 45–65% ether in hexane. Immediately before use, the mixture of ^{14}C-labeled isomers (2 mg) was applied as a band to a thin-layer plate and developed three times in succession with a mixture of benzene–nitromethane (4:1). PyII-$^{14}CH_3O$-acid (located in uv light, R_f 0.54) separated adequately from the isomeric ester (R_f 0.45) and it was extracted from the upper band with ether, the extract was concentrated in nitrogen, and the residue was dissolved in hexane.

Chrysanthemic acid labeled in the cyclopropane carboxy group [designated CA-^{14}C(O)O] had a specific activity of 1.3 mCi per mmol (Nishizawa and Casida, 1965). Chrysanthemic acid labeled in the isobutenyl methyl groups [designated CA-$(^{14}CH_3)_2C$=] with a specific activity of 0.055 mCi per mmol and chrysanthemum dicarboxylic acid labeled in the side chain carboxy group [designated CDCA-HO(O)-^{14}C(CH_3)C=] with a specific activity of 0.009 mCi per mmol were provided by Leslie Crombie (Crombie *et al.*, 1970). The radiochemical purities of these three samples of labeled acids all exceeded 98%, as determined by tlc with the BFE1 solvent system described later and, in the chrysanthemic acid samples, also with the isopropyl acetate–ammonia–methanol system of Nishizawa and Casida (1965).

All the radio-labeled compounds described above were synthesized with the same configurations as those in the natural esters except for AI-^{14}C-alc in which the alcohol center 4′ was RS.

The synthesis and specific activities of the other labeled pyrethroids and hydrolysis products used were as reported (Yamamoto and Casida, 1968).

Preparation of Methyl Esters. Samples dried by evaporation were treated with excess diazomethane in ether–ethanol solution at 25°C for 15 min, and then evaporated to dryness; this procedure was repeated three times to obtain complete methylation.

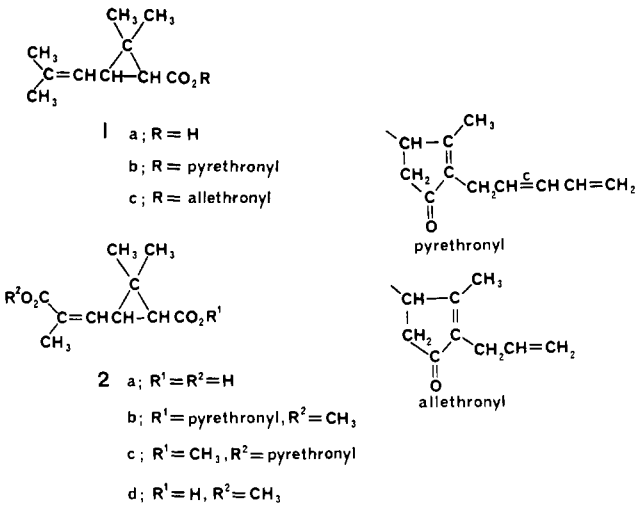


Figure 1. Formulae for compounds investigated

Chromatography. Metabolites were analyzed by tlc and purified and isolated by column chromatography. Thin-layer chromatoplates of silica gel F_{254} (20 × 20 cm, 0.25 mm gel thickness; Merck, Darmstadt, Germany) were developed with chloroform–methanol (39:1) mixture (CM), benzene saturated with formic acid–ether (10:3) mixture (BFE1), benzene saturated with formic acid–ether (5:3) mixture (BFE2), ether–benzene–methanol–formic acid (173:12:11:4) mixture (EBMF), or ether–methanol–benzene–formic acid (147:37:12:4) mixture (EMBF). Labeled compounds were detected on the chromatoplates by radioautography following a reported procedure (Elliott and Casida, 1972). For cochromatography, or to determine metabolite purity, the shape and position of zones detected under uv light (254 nm) and/or with the phosphomolybdic acid reagent (Yamamoto *et al.*, 1969) were compared with the radioactive spots.

Columns (bed volume 2.0 × 30 cm) of silicic acid–Celite (2:1 w/w) or silica gel (silicic acid–water, 2:1, w/v; mixed well to obtain a free flowing powder), packed in hexane, were developed in sequence with hexane, hexane–ether mixtures, ether, ether-acetone mixtures, acetone, and methanol. The eluate fractions were monitored for radioactive content by scintillation counting to indicate when to change to more polar elutriants. After examining the radioactive fractions by tlc, fractions containing single radioactive components were combined, evaporated, and analyzed by nmr and ms.

Treatment of Mammals and Extraction of Excreta. Male albino Sprague–Dawley rats (150 to 200 g) from Bioscience Animal Laboratories, Oakland, Calif., were treated orally by stomach tube with the pyrethroid in dimethyl sulfoxide (DMSO) (100 μl), followed by a DMSO rinse (100 μl) of the stomach tube. PyI-^{3}H-alc and PyII-^{3}H-alc were administered at 3 mg per kg for quantitative studies on metabolite excretion within 100 hr of administration. The ^{14}C-labeled pyrethroids and the related labeled compounds were administered at 1 to 5 mg per kg to determine the ^{14}C content of urine and expired air in the first 48 hr after treatment.

Male albino Swiss–Webster mice (18 to 20 g) (Bioscience Animal Laboratories) were also treated orally by stomach tube with the ^{14}C-pyrethroid or related compound (1 to 5 mg per kg) in DMSO (50 μl) followed by a DMSO rinse (100 μl),

and the ^{14}C content of the urine and expired air were monitored similarly.

When metabolites were to be isolated in milligram quantities, larger doses were administered to rats and the treatments repeated at intervals of 6 to 12 hr using pyrethrin I [PyI-^{3}H-alc plus PyI-^{14}C(O)O-acid], allethrin [AI-^{3}H-alc plus AI-^{14}C-(O)O-acid] and pyrethrin II (PyII-^{3}H-alc, only), each sample previously mixed with the corresponding unlabeled compound (400 to 800 parts). Four to eight individual doses of pyrethrin I (220 to 420 mg per kg) were administered to each rat, depending on its tolerance, within 24 to 72 hr, to a total of 1.10 g. Similarly, pyrethrin II and allethrin were administered to rats, in eight individual doses (280 to 600 and 480 mg per kg, respectively) to totals within 72 hr of 2.40 and 2.30 g, respectively. Pyrethrin II and allethrin did not produce any symptoms of poisoning during the treatments, and so the LD_{50} values are well above 480 and 600 mg per kg for individual doses, or 3840 and 2900 mg per kg, respectively, for the total amount administered. On the same basis, the LD_{50} for pyrethrin I is 260 to 420 mg per kg as a single dose and 450 to 2000 mg per kg as a cumulative dose within 72 hr. The total urine excreted until 24 hr after the last treatment was collected.

Urine samples or homogenates of feces samples (20% w/v, in water) were extracted (Miyamoto *et al.*, 1968) by adding ammonium sulfate (0.2 g per ml) then ethanol (1 vol) and ether (2 vol). After extracting and separating the layers by centrifugation, the organic phase was removed, the aqueous layer acidified with hydrochloric acid to pH 2, and the extraction was repeated with a mixture of ethanol (1 vol) and ether (2 vol). The ether–ethanol extracts were combined, unless stated otherwise, and then dried (sodium sulfate) and evaporated to an oily residue which was examined by tlc or column chromatography. With all the metabolite mixtures, 90 to 95% of the ^{14}C was extracted from the urine into the organic phase by this procedure. The recoveries of radioactivity in the urine and in the extracts relative to the amount administered were as follows: 17.8% in the urine and 16.3% in the neutral-plus-acid extract with PyI-^{3}H-alc; 19.2% in the urine and 13.4% in the neutral extract with PyII-^{3}H-alc; 20.7% in the urine and 13.1% in the neutral extract with AI-^{3}H-alc; 30.0% in the urine and 20.7% in the neutral extract with AI-^{14}C(O)O-acid. These extracts were used directly for metabolite isolation.

Isolation of Urinary Metabolites. The large quantities of material with which the metabolites were contaminated are evident from the weight of extract prepared for column chromatography: 8.8 g with pyrethrin I; 2.6 g with pyrethrin II; and 3.4 g with allethrin. Preliminary chromatography on a silicic acid–Celite column gave two major peaks from each pyrethroid, eluted in turn by ether and by ether–acetone (5:1), and three to four minor peaks, eluted by acetone or methanol. The fractions corresponding to each major peak were evaporated to dryness, methylated (diazomethane), and rechromatographed separately on a column of the same type to obtain three radioactive fractions from each major peak. When these fractions were pure (tlc), they were examined immediately by spectroscopy; others were purified on silica gel before spectroscopic examination. The purity of the isolated metabolites was estimated by tlc (uv visualization and radioautography), by the specific activity relative to the compound administered, and by nmr spectroscopy. The purified metabolites were dissolved in deuterochloroform and evaporated in subdued light under nitrogen; this process was repeated twice to remove traces of other volatile solvents and, finally,

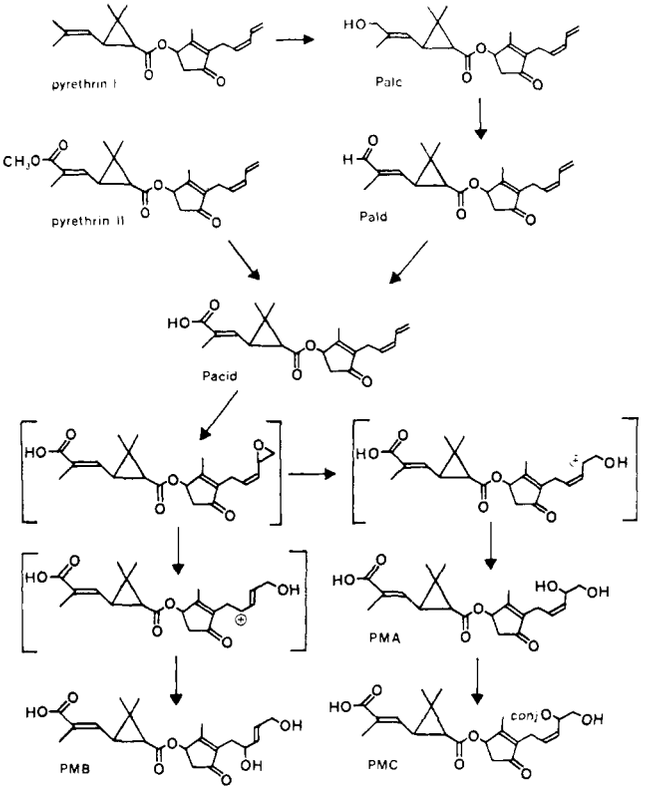


Figure 2. Tentative metabolic pathway for pyrethrins I and II in rats

the products were dissolved in deuterochloroform and filtered through a cottonwool plug into a nmr tube. After determining the nmr spectrum, the sample was subdivided for further analysis, particularly by ms, as described below.

Enzymatic Cleavage of Conjugates. The possibility that some of the metabolites of pyrethrin I occur in the urine as conjugates was tested by attempting to cleave them enzymatically with glusulase. An aliquot of the urine from rats given pyrethrin I [PyI-^{14}C(O)O-acid] was evaporated to dryness, the residue dissolved in 0.05 *M* acetate–0.1 *M* glycine buffer, pH 5, and the solution was subdivided for incubation in 2.0 ml volume at 37°C for 4 hr with or without added glusulase (100 μl, 10,000 and 5000 enzyme units of glucuronidase and aryl sulfatase, respectively; Endo Laboratories, Inc., Garden City, N.Y.). The ether–ethanol extracts were analyzed by tlc using the BFE1 solvent system.

Enzyme Studies. The microsome or microsome-plus-soluble fraction from rat or mouse liver homogenate prepared in phosphate buffer (0.1 *M*, pH 7.4) was incubated for 30 min at 37°C in a 25-ml Erlenmeyer flask with the following constituents: liver fraction at 8 to 200 mg fresh weight equivalent; 0 or 3 μmol NADPH; 2 ml of phosphate buffer; substrate, added last by injecting in ethanol (10 μl) just below the surface of the aqueous medium. With houseflies, enzyme preparations of abdomens from flies of the R_{Baygon}-resistant strain were incubated for 2 hr at 30°C (Yamamoto *et al.*, 1969). The amount of substrate used in each case was as follows: PyI-^{3}H-alc, PyII-^{3}H-alc, and AI-^{3}H-alc—1 μg; PyI-^{14}C(O)O-acid and AI-^{14}C(O)O-acid—7 μg; AI-^{14}C-alc—70 μg. For analysis, the incubation mixtures were extracted with ether–ethanol–ammonium sulfate, as for urine extraction, and the organic phase was examined by tlc. Of the ^{14}C in the metabolite mixture, 95 to 100% was recovered in the organic phase from each compound. For one-dimen-

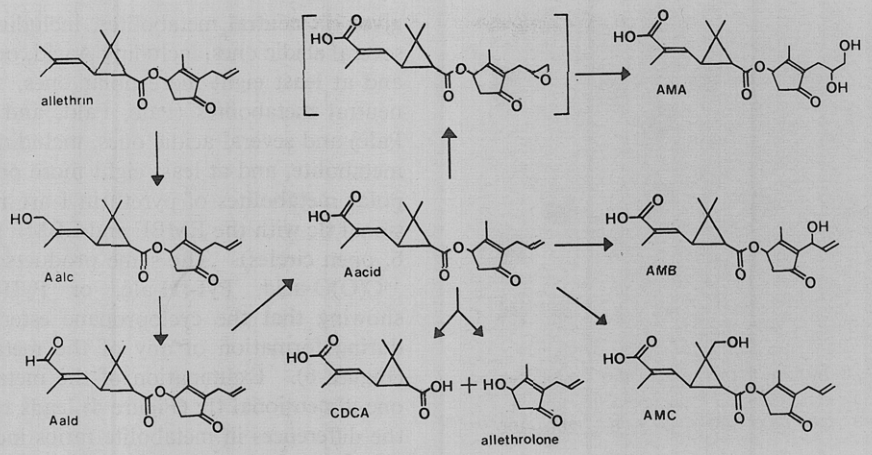


Figure 3. Tentative metabolic pathway for allethrin in rats

sional tlc, the chromatoplates were developed with benzene, air-dried, and then developed with the BFE1 solvent system. For two-dimensional tlc, the chromatoplates were developed with benzene and then with the EMBF solvent system in the first direction, followed by the EBMF solvent system in the second direction. All products obtained from various enzyme preparations were compared by one-dimensional tlc. In addition, two-dimensional cochromatography was used to compare the metabolites of pyrethrins I and II from the rat liver microsome–NADPH enzyme system with those in the urine of rats treated orally with pyrethrins I or II.

Radioactivity Balance Studies. The metabolism cages, the methods for collecting the urine, feces, and expired CO_2, and for combusting feces, to determine their respective ^{14}C and ^{3}H contents, were as described by Krishna and Casida (1966). The same procedures were used in ^{3}H studies except that ethanolamine was omitted from the trapping solutions.

Spectroscopy. Nmr spectra were determined for dilute solutions in deuterochloroform (containing less than 0.5% $CHCl_3$, τ 2.73) using tetramethylsilane as internal standard, on a Perkin-Elmer R10 spectrometer at 60 MHz. When the sensitivity available was insufficient, signal strengths were enhanced by a NS-544 spectrum-accumulating computer using up to 303 runs; the signal:noise ratio then improved *ca.* 17-fold.

Mass spectra were recorded on a Perkin-Elmer Hitachi RMU 6E spectrometer. An aliquot of the solution after nmr examination (2 μl) and a solution of *N,O*-bis(trimethylsilyl)acetamide (5 μl of 10% in chloroform) (Pierce, 1968) were mixed in a quartz dipper, evaporated, and the residue was introduced into the ion source at 70°C on the direct-insertion probe, after final removal of volatile impurities.

Examination of Metabolite Hydrolyzates. The metabolite (*ca.* 0.5 mg) was boiled with 2% potassium hydroxide in methanol (10 ml) for 1 hr, then evaporated at reduced pressure, shaken with aqueous sulfuric acid (5 ml, 10% v/v) and chloroform (5 ml), and the chloroform layer dried (Na_2SO_4) and evaporated (nitrogen stream). The residue was dissolved in chloroform (50 μl) containing *N,O*-bis(trimethylsilyl)-acetamide (5 mg) then injected onto a gas–liquid chromatography (glc) column (5 ft × $^1/_8$-in. stainless steel, 5% QF1 on Chromosorb W; Varian Aerograph 1200 apparatus; injector at 250°C, detector at 250°C).

Designation of Metabolites. The intermediates previously established (Yamamoto *et al.*, 1969) involving oxidation of the *trans*-methyl group of the isobutenyl moiety without other modification are designated Palc, Pald, and Pacid for the pyrethrin I series, and Aalc, Aald, and Aacid for the allethrin series (as in Figures 2 and 3). New metabolites of pyrethrins and allethrin are designated PMA, PMB, PMC . . . .; AMA, AMB, AMC . . . .; respectively. When they are methyl esters this is indicated by a suffix, thus: PMA_{Me}, AMA_{Me}, etc.

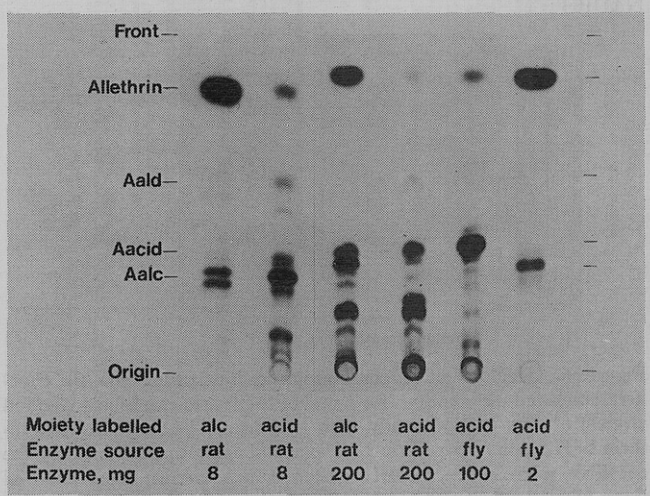


Figure 4. Tlc radioautogram showing the metabolites of allethrin-^{14}C labeled in the alcohol or in the acid moiety formed by varying amounts of a microsome preparation of rat liver or a homogenate of housefly abdomens fortified with NADPH

RESULTS

Products of Enzymatic Metabolism of Pyrethroids. Some of the products of allethrin metabolism by the rat liver microsome–NADPH system are identical to those formed in the fly abdomen–NADPH system, including Aalc, Aald, Aacid, and a series of more polar esters (detected with both AI-^{14}C(O)O-acid and AI-^{14}C-alc) (Figure 4). In Figures 4 and 5, the enzyme weight refers to the fresh weight equivalent of liver, from which the microsomes were prepared, or, in the case of houseflies, to the total fresh abdomen weight. Development was with the BFE1 solvent system. The same allethrin metabolites are also formed by mouse liver microsomes and microsome-plus-soluble fractions of mouse or rat liver. Similarly using pyrethrin I [PyI-^{14}C(O)O-acid and PyI-^{3}H-alc] as substrate, both rat liver and fly enzyme preparations gave some of the same products, especially Palc, Pald, and Pacid, as well as more polar metabolites. With both allethrin and pyrethrin I in the rat liver enzyme system, the alcohols (Aalc and Palc) are the predominant products under the milder

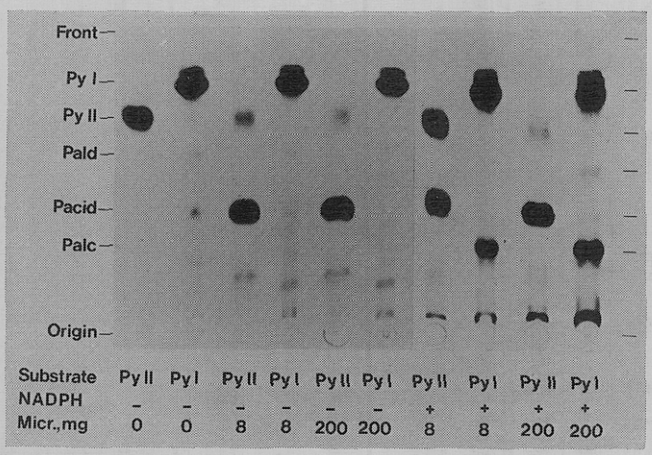


Figure 5. Tlc radioautogram showing metabolites of pyrethrin I-^{3}H-alc and pyrethrin II-^{3}H-alc formed by varying amounts of a microsome preparation of rat liver with or without fortification with NADPH

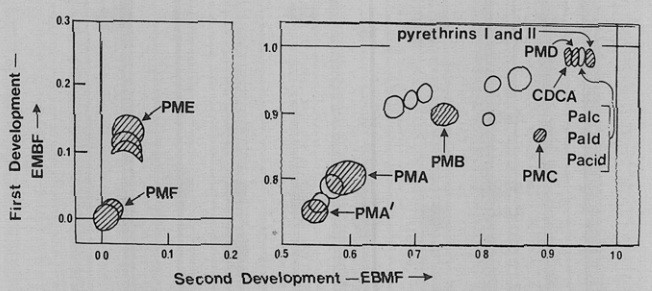


Figure 6. Regions of a chromatogram illustrating the tlc characteristics and designations for metabolites in urine and feces (shaded circles) of rats treated orally with pyrethrin I-^{14}C(O)O-acid, pyrethrin I-^{3}H-alc, and pyrethrin II-^{3}H-alc and of products formed by the rat liver microsome–NADPH system (open circles) from each of these labeled substrates

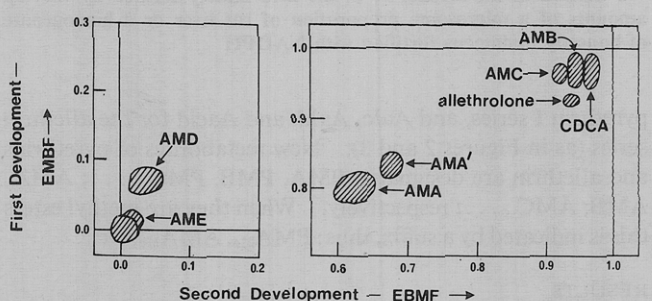


Figure 7. Regions of a chromatogram illustrating the tlc characteristics and designations for metabolites in the urine of rats treated orally with allethrin-^{14}C(O)O-acid or allethrin-^{3}H-alc

metabolic conditions of low microsome level with NADPH fortification (Figures 4 and 5) or higher enzyme level without NADPH fortification; the aldehydes (Aald and Pald) never accumulate appreciably; the acids (Aacid and Pacid) are more prominent on fortification of the microsomes with both NADPH and soluble fraction; the more polar products become the major metabolites with high levels of microsomes and soluble fraction in the presence of NADPH. The neutral and acidic metabolites of allethrin and pyrethrin I formed by the rat liver microsome–NADPH system were differentiated by two-dimensional tlc, first in an acidic solvent and then in an ammoniacal one (general procedure illustrated in Figure 5 of the publication of Yamamoto *et al.*, 1969). Allethrin gives six neutral metabolites, including Aalc and Aald, and several acidic ones, including Aacid, one less polar metabolite, and at least eight more polar ones. Pyrethrin I gives four neutral metabolites (Palc, Pald, and two more polar than Palc) and several acidic ones, including Pacid, one less polar metabolite, and at least eight more polar ones. These more polar metabolites of pyrethrin I are resolved by two-dimensional tlc with the EMBF and EBMF solvent systems (Figure 6, open circles). The same products are detected with PyI-^{14}C(O)O-acid, PyI-^{3}H-alc, or PyII-^{3}H-alc as substrates showing that the cyclopropane ester group is not cleaved during formation of any of the metabolites of pyrethrin I (Figure 6). Examination of the metabolites of allethrin by one-dimensional tlc (Figure 4) leads to a similar conclusion; the differences in metabolite ratios indicated by the chromatograms from AI-^{14}C(O)O-acid and AI-^{14}C-alc may be due to the larger amount of substrate (70 μg) used with AI-^{14}C-alc than with AI-^{14}C(O)O-acid (7 μg). All these results suggest that allethrin and pyrethrin I are metabolized by enzymes to the respective acids, Aacid and Pacid, *via* the corresponding alcohols and aldehydes, and then to a series of more polar products possibly by attack on Aacid and Pacid (or Aalc and Palc) to give products hydroxylated elsewhere in the molecule.

Conversion of pyrethrin II to Pacid, a hydrolysis reaction, occurs with small amounts of rat liver microsomes (8 mg equivalents) and does not depend on NADPH fortification; in fact, NADPH may indeed suppress the enzymatic hydrolysis (Figure 5). However, further attack after formation of Pacid does depend on NADPH and then more polar derivatives are formed, especially at the higher enzyme level; these metabolites seem to be the same as those formed from pyrethrin I under comparable conditions. Thus, an esterase rapidly cleaves the methoxycarbonyl group and the mixed-function oxidase system then converts Pacid to more polar derivatives.

Metabolites in Urine and Feces Following Oral Administration of Variously Labeled Preparations of Pyrethrin I, Pyrethrin II, and Allethrin. The designations used for several of the metabolites excreted in the urine of rats treated orally with either pyrethrin I or pyrethrin II are shown in Figure 6 (shaded circles) and those for allethrin are shown in Figure 7 (shaded circles). Metabolites do not appear in the regions other than those illustrated in Figure 6 (for pyrethrins I and II) and Figure 7 (for allethrin). Pyrethrin I and pyrethrin II are not detected in urine but are excreted unchanged in feces. Excreted metabolites PMA, PMB, PMC, PME, and PMF are present in both urine and feces from pyrethrin I and pyrethrin II. A minor metabolite, PMA′, appears with each labeled preparation but only in urine. CDCA is detected only in urine following pyrethrin I-^{14}C(O)O-acid administration. Pyrethrin I-^{3}H-alc or pyrethrin II-^{3}H-alc give metabolite PMD only in feces; this metabolite is not detected with pyrethrin I-^{14}C(O)O-acid, possibly because of the lower specific activity. Thus, the metabolites detected in urine are the same after administering PyI-^{14}C(O)O-acid, PyI-^{3}H-alc, or PyII-^{3}H-alc and so these metabolites are all esters derived from the common intermediate Pacid, except that a trace of CDCA is formed from pyrethrin I. Diazomethane with metabolites PMA, PMB, PMC, and possibly several of the others yields methylated derivatives of higher R_f value, indicating that these metabolites contain at least one carboxylic acid group, probably at the same position as that present in Pacid. The methyl ester of PMA (PMA_{Me}) is converted completely on reaction with acetyl chloride and pyridine in ether to a product of even higher R_f, so PMA contains one or more hydroxyl groups as well as the carboxylic acid

group. With the possible exception of PMA or PMA′, the metabolites in urine differ (tlc) from those formed by the rat liver microsome–NADPH system. In addition to the four metabolites with high R_f value in the EMBF and EBMF solvent systems (from pyrethrins I and II), there are two other metabolite spots (PME and PMF) with low R_f, each a mixture not adequately resolved with the solvent systems used. Pyrethrolone and Pacid are not detected as metabolites of pyrethrins I and II by direct tlc analysis of the organic extracts of the urine; however, Pacid is detected as a very minor metabolite of pyrethrin I only when the urine extract is purified and concentrated by column chromatography before tlc analysis, as discussed later.

Metabolites cochromatographing with each of the major metabolites in urine occurred in the feces of rats treated with pyrethrins I and II; moreover, a portion of the pyrethrins administered is not absorbed, but is excreted unmodified in the feces. Also, a minor ester metabolite, PMD, appears in the feces but not in the urine after administration of pyrethrins I and II. Table I gives quantitative results on the major metabolites in urine and feces following oral administration of pyrethrins I and II (PyI-^{3}H-alc and PyII-^{3}H-alc). At least one of the more polar metabolites of pyrethrin I in both urine and feces seems to be a conjugate because the metabolite mixture, when incubated with glusulase but not without it, gives a product of higher R_f value in the BFE1 solvent system than any of the major metabolites (PMA, PMB, or PMC).

Most of the metabolites of allethrin excreted in the urine following oral administration are detected with both AI-^{14}C(O)O-acid and AI-^{3}H-alc, and so they are esters. However, there are two exceptions: CDCA [detected from AI-^{14}C(O)-O-acid only] and allethrolone (detected from AI-^{3}H-alc only). Diazomethane converts several of the allethrin metabolites (AMA, AMB, AMC, CDCA, and possibly others) to products with higher R_f values, indicating that the metabolites with the cyclopropane ester group intact have at least one carboxylic acid group, probably at the same position as that present in Aacid.

For allethrin the feces were not examined chromatographically. However, it is known that within 48 hr of oral administration, AI-^{14}C(O)O-acid yields 29% and AI-^{3}H-alc 27% of the administered radioactivity in the feces. Allethrin (AI-^{3}H-alc) was administered to rats and the water in the expired air was monitored for ^3H_2O by trapping in 2-methoxyethanol, but no radioactivity was found.

Table I. ^{3}H-Compounds Present in the Urine and Feces of Male Rats 100 Hr after Receiving 3 mg per kg of Pyrethrin I-^{3}H-alc or Pyrethrin II-^{3}H-alc, Orally

^{3}H-Compounds	Administered ^{3}H Recovered, %[a]		
	Urine	Feces	Total
	Pyrethrin I administered		
Pyrethrin I	0.0	18.0	18.0
PMA[b]	9.5	4.8	14.3
PMB	2.9	1.5	4.4
PMC	1.8	2.1	3.9
Unknowns			
Less polar (PMD)	0.0	1.4	1.4
More polar (PME and PMF)	16.0	13.2	29.2
Total	30.2[c]	41.0[c]	71.2
	Pyrethrin II administered		
Pyrethrin II	0.0	4.0	4.0
PMA[b]	15.8	5.3	21.1
PMB	2.1	1.2	3.3
PMC	2.8	3.4	6.2
Unknowns			
Less polar (PMD)	0.0	3.0	3.0
More polar (PME and PMF)	12.3	13.8	26.1
Total	33.0[c]	30.7[c]	63.7

[a] Analyses of the proportion of individual metabolites in urine samples at 6-hr intervals during the first 24 hr after treatment and in feces samples at 0–20, 20–28, and 28–48 hr after treatment showed no significant deviations from the overall average values presented. [b] Includes a small amount of PMA′ not adequately resolved for separate analysis. [c] The percentages of the administered ^{3}H excreted in the 0–20, 20–48, and 48–100 hr intervals were as follows: pyrethrin I, urine 23.2, 5.5 and 1.5; feces 14.8, 23.3, and 2.9; pyrethrin II, urine 24.6, 6.7 and 1.7; feces 16.6, 11.4, and 2.7.

Isolation of Urinary Metabolites of Pyrethrin I, Pyrethrin II, and Allethrin. The metabolites were separated on three columns in succession. Much interfering material was first removed by a preliminary separation on a column of silicic acid–Celite. Next the metabolites were methylated with diazomethane to improve their chromatographic behavior and alter their polarity relative to that of impurities still present. This step involved loss of material, but permitted greater purity to be eventually attained. Table II gives the solvents and packings for the column purification of metabolites and the tlc systems with R_f values used to assess their purity.

While isolating the metabolites, a nonradioactive crystalline material was obtained from each urine sample. This compound, eluted from the silicic acid–Celite column with ether–

Table II. Behavior on Chromatographic Columns and Thin-Layer Systems of Urinary Metabolites of Pyrethrin I, Pyrethrin II, and Allethrin Before and After Methylation

Metabolite name or designation	Elutriant for column chromatography			Tlc R_f with indicated solvent system						Amount isolated, mg[b]
	Before methylation	After methylation		Before methylation	After methylation					
	Silicic acid–Celite	Silicic acid–Celite	Silica gel	BFE1	EBMF[a]	CM	BFE1	BFE2	EBMF	
	Pyrethrin I and Pyrethrin II									
PMA	Ether–acetone (5:1)	Ether–acetone (9:1)	Hexane–ether (5:1)		0.43	0.45	0.15	0.25	0.57	17 (52)
PMB	Ether	Hexane–ether (1:5)	Hexane–ether (6:1)		0.64	0.67			0.77	5 (19)
PMC	Ether	Hexane–ether (7:1)			0.77	0.93	0.55	0.68	0.93	3 (7)
	Allethrin									
AMA	Ether–acetone (5:1)	Ether		0.00	0.44	0.14	0.04	0.04	0.50	15
AMB	Ether	Hexane–ether (1:2)		0.25	0.92	0.43	0.30	0.44	0.94	44
AMC	Ether	Hexane–ether (3:7)	Hexane–ether (9:1)	0.13	0.87	0.40	0.25	0.32	0.93	14
Allethrolone	Ether	Hexane–ether (3:7)	Ether		0.90	0.20	0.18	0.23	0.90	5
CDCA	Ether	Hexane–ether (9:1)		0.37	0.93	0.52	0.72	0.80	0.97	25

[a] The R_f values differ slightly from those shown in Figures 6 and 7 because in this table the compounds were chromatographed in one dimension only and they were free of contaminants that might alter their chromatographic behavior. [b] The numbers in parentheses refer to pyrethrin II.

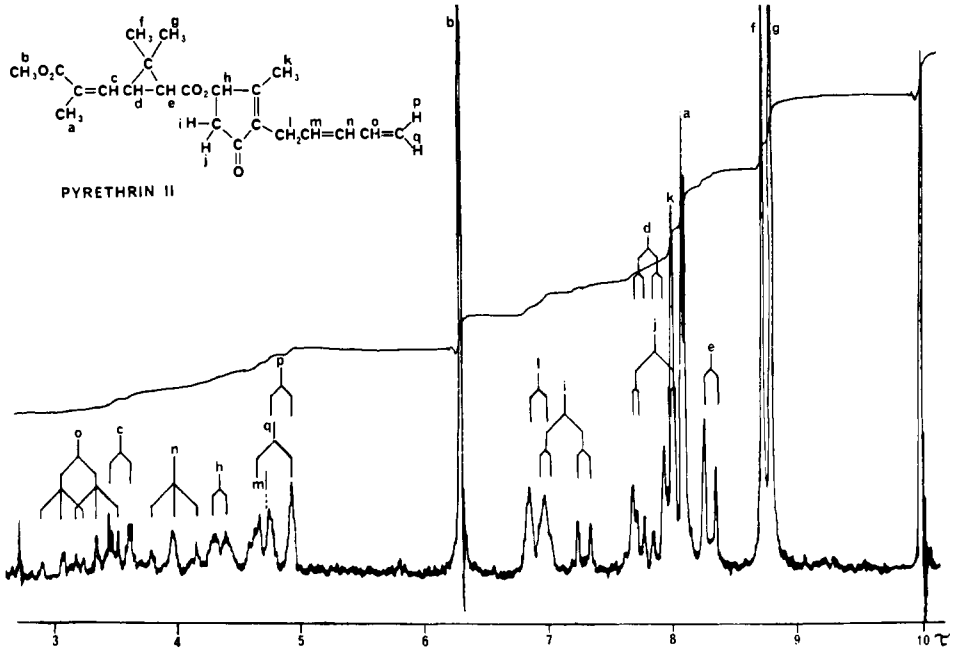


Figure 8. Nmr spectrum of pyrethrin II

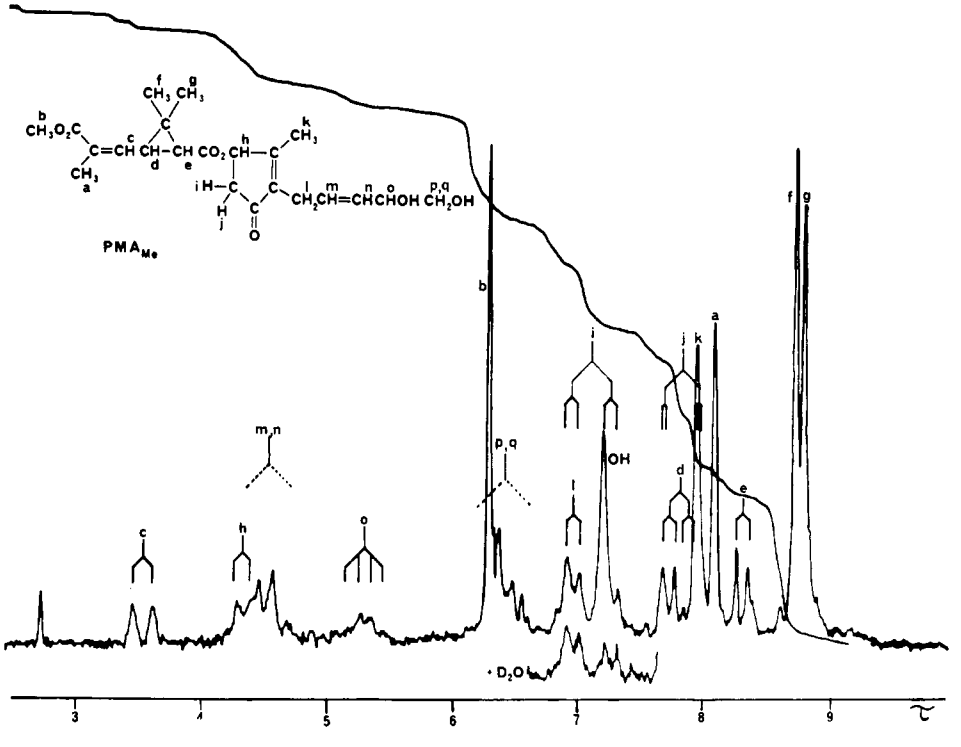


Figure 9. Nmr spectrum of the pyrethrins metabolite PMA after methylation, *i.e.*, PMA_{Me} (16 scans)

acetone (5:1) mixture, was identified as dimethyl sulfone by mp and mixed mp (109°C), ms (peaks at *m/e* 94, 79), nmr (single peak at τ 7.0), and tlc (cochromatography in EBMF solvent system, R_f 0.40, detected with iodine vapor). This sulfone undoubtedly arises from metabolic oxidation of the large dose of administered DMSO.

CDCA was isolated as its dimethyl ester following allethrin administration and methylation. Although it was detected as a trace metabolite when PyI-^{14}C(O)-acid was administered (above), the high ^{3}H activity and the elution position relative to PMC obscured the previously detected CDCA spot when a mixture of PyI-^{14}C(O)O-acid and PyI-^{3}H-alc was administered. Pacid was detected as a minor metabolite of PyI-^{3}H-alc (cochromatographed as methyl ester with pyrethrin II on tlc with chloroform as developer, R_f 0.25). It was eluted with ether–acetone (5:1) from the silicic acid–Celite column both as the free acid and as its methyl ester. Because PMA_{Me} and AMA_{Me} were finally obtained pure for spectroscopic examination, metabolites PMA′ and AMA′ (Figures 6 and 7) which had similar polarities must have been removed during the process of isolation.

Three pyrethrin metabolites (PMA, PMB, and PMC) were isolated as their methyl esters (PMA_{Me}, PMB_{Me}, and PMC_{Me}) from both pyrethrin I and pyrethrin II. Identical properties (tlc, nmr, ms) proved that either precursor gave these same three metabolites. Three metabolites of allethrin also were isolated as their methyl esters (AMA_{Me}, AMB_{Me}, AMC_{Me}) by column chromatography. In preliminary work,

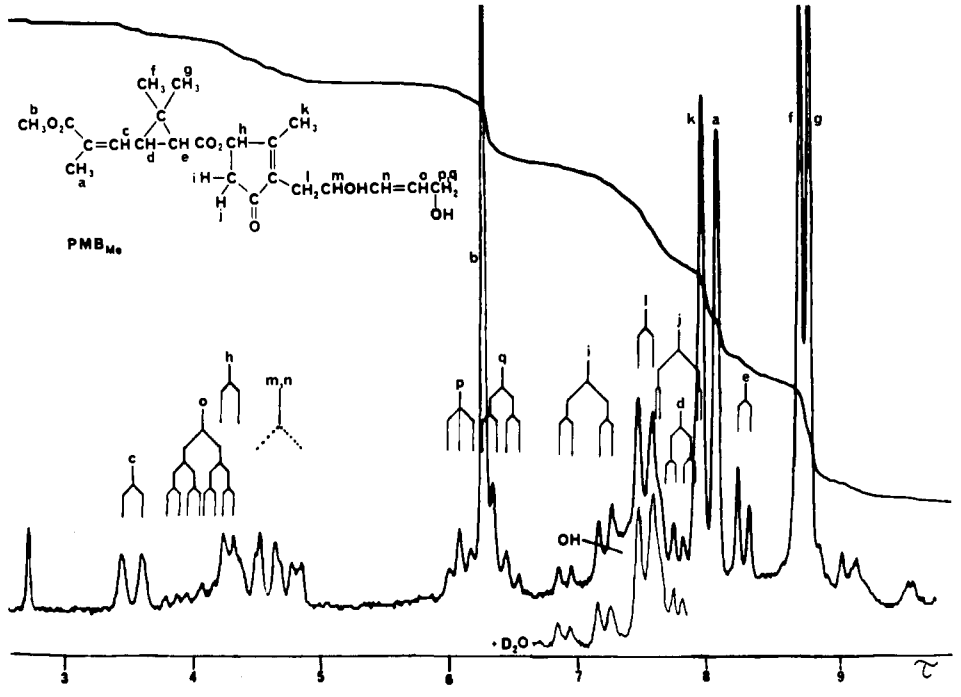


Figure 10. Nmr spectrum of the pyrethrins metabolite PMB after methylation, *i.e.*, PMB_{Me} (302 scans)

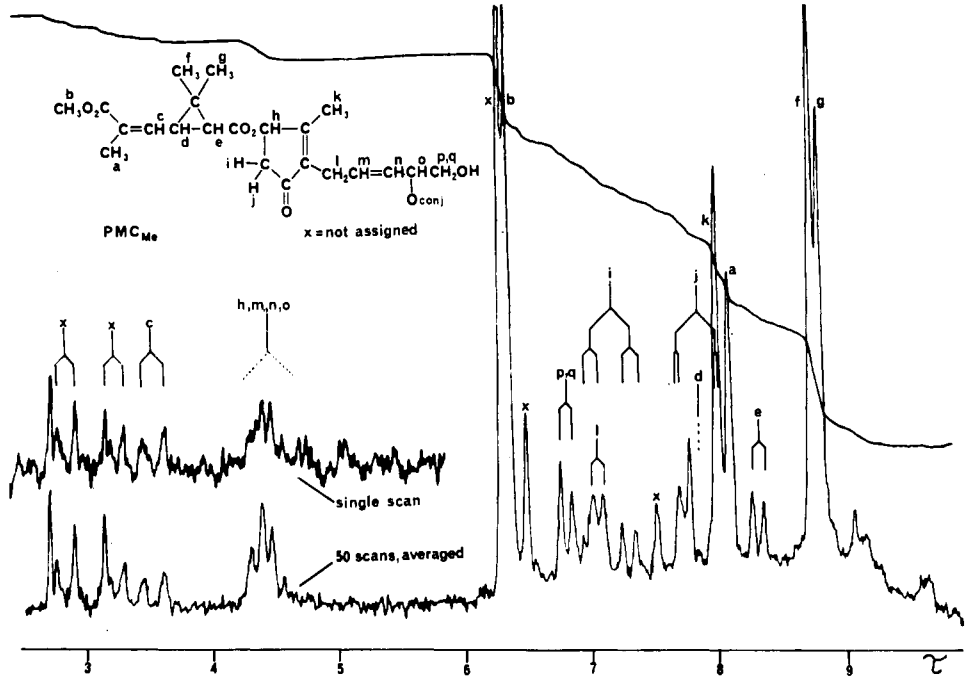


Figure 11. Nmr spectrum of the pyrethrins metabolite PMC after methylation, *i.e.*, PMC_{Me} (1 and 50 scans)

AMA_{Me} and AMB_{Me} were isolated by preparative scale tlc; their characteristics (tlc, nmr) were identical with the products later obtained by column chromatography.

Photodecomposition, rearrangements, and other nonmetabolic reactions during processing of small amounts of pyrethroids and metabolites were minimized by working, where possible, under neutral conditions and in dim light in an inert atmosphere. The isolated metabolites (used for nmr and ms) from each labeled pyrethroid preparation were found to cochromatograph in the CM and EBMF tlc solvent systems with those obtained by direct methylation (diazomethane) of urine (15 μl); thus the products isolated as methyl esters represent the metabolites present in the urine, without alteration during processing.

Nmr Spectra of the Metabolites. Nmr spectra of the natural pyrethrins sufficiently detailed for full interpretation (Bramwell *et al.*, 1969) are obtained from 50-mg samples on the spectrometer used in this work as illustrated in Figure 8 for pyrethrin II. Little more than 3 mg of some metabolites were isolated; then spectrum accumulation was used to obtain spectra comparable to those recorded directly with larger samples (*cf.* the two traces in Figure 11). The nmr spectra of six insecticidal constituents of pyrethrum extract and related compounds have been analyzed and discussed (Bramwell *et al.*, 1969), so the spectra of pyrethroid metabolites can be compared with those of their precursors to indicate directly the sites modified.

Figures 9 to 14 show the spectra of six of the metabolites and

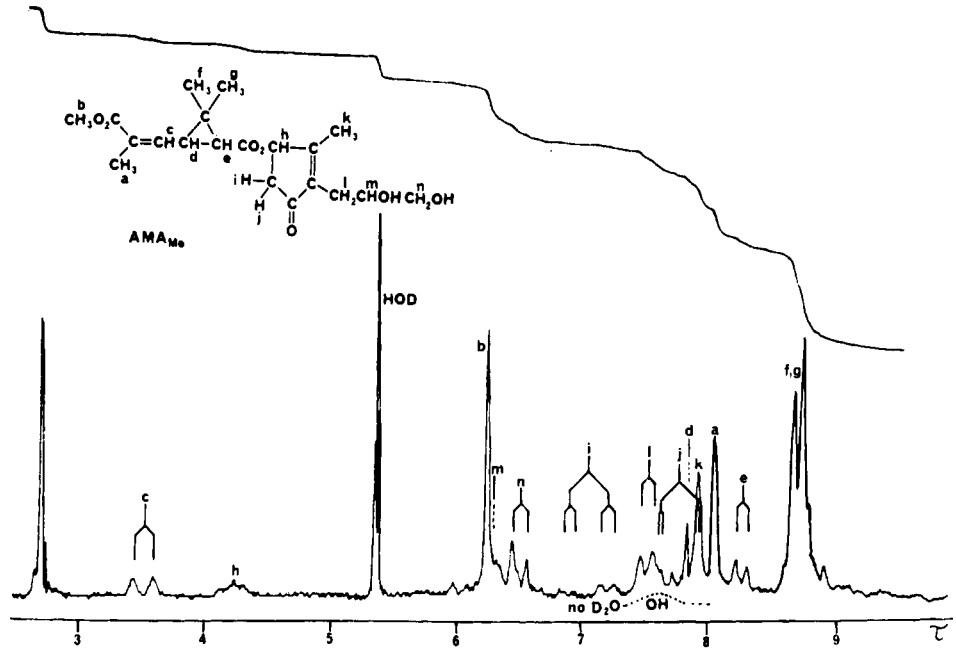
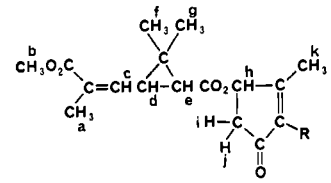


Figure 12. Nmr spectrum of the allethrin metabolite AMA after methylation, *i.e.*, AMA$_{Me}$ (303 scans)

Table III. τ Values of Protons Designated in Structural Diagram as They Appear in Pyrethrin II and in Metabolites of Pyrethrins I and II and of Allethrin



	a	b	c	d	e	f, g		h	i	j	k
Pyrethrin II	8.07	6.25	3.52	7.79	8.27	8.70	8.77	4.31	7.10	7.81	7.97
PMA$_{Me}$	8.07	6.28	3.53	7.8*[a]	8.26	8.70	8.77	4.3*	7.1*	7.8*	7.92
PMB$_{Me}$	8.05	6.27	3.53	7.8*	8.26	8.70	8.77	4.3*	7.10	7.8*	7.94
PMC$_{Me}$	8.06	6.27	3.52	7.8*	8.27	8.71	8.77	4.3*	7.1*	7.8*	7.97
AMA$_{Me}$	8.05	6.26	3.53	7.8*	8.26	8.70	8.76	4.24	7.10	7.8*	7.93
AMB$_{Me}$	8.03	6.25	3.52	7.8*	8.26	8.70	8.76	4.3*	7.10	7.8*	7.92
AMC$_{Me}$	8.03	6.26	3.54					4.3	7.12	7.8*	7.99

[a] Asterisks refer to peaks that are obscured (see text).

Table III summarizes some of the chemical shifts indicated by the attached analyses. In Table III and throughout this section, peaks that are obscured, partially or completely, in a complex area so that their individual shapes cannot be observed but are detected by their contribution to the integram are marked with an asterisk. Resonances from the side chain [$(CH_3)_2C{=}CH{-}$] of the parent chrysanthemates (pyrethrin I and allethrin) at τ 8.3 and 5.1 are replaced in the spectra of all the methylated metabolites (and in that of pyrethrin II, Figure 8) by signals at 8.1, 6.3, and 3.5 from a modified side chain [$CH_3O_2C{-}C(CH_3){=}CH{-}$]. In all but one of the metabolites, peaks at 8.77, 8.70, 8.26, and 7.8* indicate that oxidation of the *trans*-methyl group in the isobutenyl moiety to a carboxyl group is the only change on the acid side of the molecule, so they are esters of pyrethric acid (2d, Figure 1), *i.e.*, protons a to g occur in the same place as in a typical pyrethrate, pyrethrin II (Figure 8).

Metabolite PMA$_{Me}$ (Figure 9) shows resonances a to g, so it is a pyrethrate ester. Protons h, i, j, and k are all unchanged, so the cyclopentenone ring and its attached methyl are present. The changes observed are in the part of the spectrum from the side chain (see Figure 15): the olefinic region (five protons in pyrethrin II) now shows only two protons; the peak from CH_2(l) has moved marginally upfield; the three other olefinic protons in pyrethrin II now appear as a peak (1 H) at 5.29 and another (2 H) at *ca.* 6.3; a new 2 H signal is at 7.2, removed on shaking with D_2O. The CH_2(l) is at 6.93 (6.88 in pyrethrin II) so it is still flanked by two olefinic bonds. The side chain therefore contains units $-CH_2CH{=}CH-$ and $-CHOH-CH_2OH$. Since the peak from protons l is a doublet (so the CH_2 has only one CH neighbor) the side chain can only be $-CH_2-CH{=}CH-CHOH-CH_2OH$. The shapes and positions of the peaks at 5.29 (a quartet, J = 6 Hz) and *ca.* 6.3 (a multiplet) support this interpretation. The chemical shifts are close to those expected for an allylic hydroxylic methine group (the change $RCH_2OH \rightarrow R{-}CHOH$, when R is alkyl, shifts the peak 0.30 ppm, so $CH_2{=}CH{-}CH_2OH$ (τ 5.87) $\rightarrow$ $=CH{-}CHOH$ gives a predicted value of 5.57) and a methylene group of an α,β-diol (that in ethylene glycol has τ 6.35), respectively.

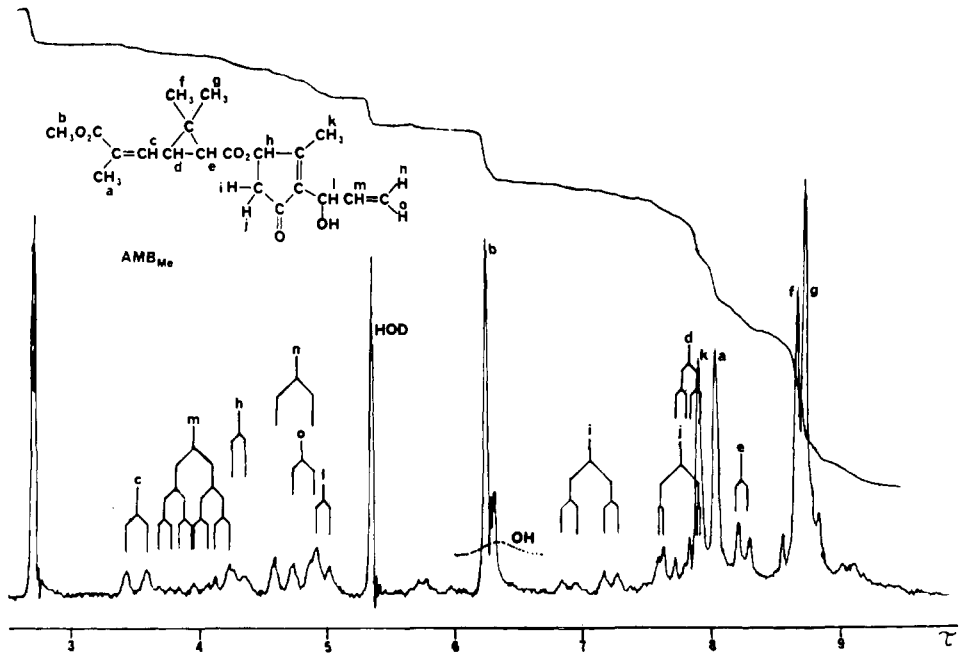


Figure 13. Nmr spectrum of the allethrin metabolite AMB after methylation, *i.e.*, AMB$_{Me}$ (261 scans)

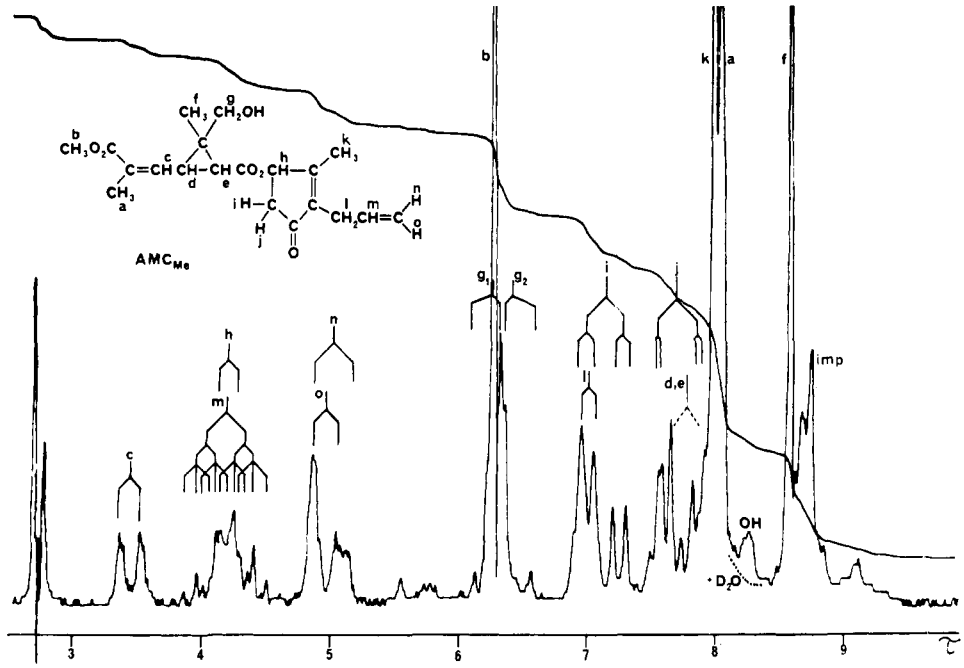


Figure 14. Nmr spectrum of the allethrin metabolite AMC after methylation, *i.e.*, AMC$_{Me}$ (298 scans)

Proton o is flanked by a —CH— and a —CH_2—, each splitting it approximately equally (J_{no} = J_{op} = 6 Hz); protons p and q are nonequivalent because they are near the asymmetric centre at C_4'.

Metabolite PMB$_{Me}$ (Figure 10) shows peaks from the same acid and cyclopentenonyl units (protons a to k) but the side chain is modified (Figure 15). Again, there are two olefinic protons, three —C*H*O— protons, a two-proton hydroxyl peak, and a doublet (protons l). This last peak has moved from 6.88 to 7.51, indicating one adjacent double bond instead of two, so the structure is —CH_2—CHOH—CH=CH—CH_2OH which agrees completely with the rest of the spectrum. Thus protons m and n appear as a complex area at 4.3 to 4.9, chemical shifts expected for a deshielded allylic alcoholic methine proton (see later), and an olefinic proton. The signal from proton o appears downfield as a double double doublet with splittings (6, 10, 17 Hz) due to coupling with protons n, p, and q. The high value for J_{no} (17 Hz) shows that the double bond is trans (Jackman and Sternhell, 1969a). Protons p and q are sufficiently nonequivalent for them to appear as separate peaks, with appropriate shapes for J_{oq} = 10, J_{op} = J_{pq} = 6 Hz. The large differences, not only in the chemical shifts for protons p and q, but also in J_{op} and J_{oq}, suggest hindrance to free rotation about the C_4'–C_5' bond. The small value for the geminal coupling, J_{pq}, is also more typical of a cyclic than an open-chain system (Sternhell, 1969). Indeed, models indicate that the cyclopentenone carbonyl is situated to form a hydrogen bond easily, particularly with the 5′-hydroxyl; protons p and q can then be regarded as axial and equatorial substituents on a cyclic system. In such a cyclic conformation, proton m is in the region where the diamagnetic anisotropy of the carbonyl group deshields it

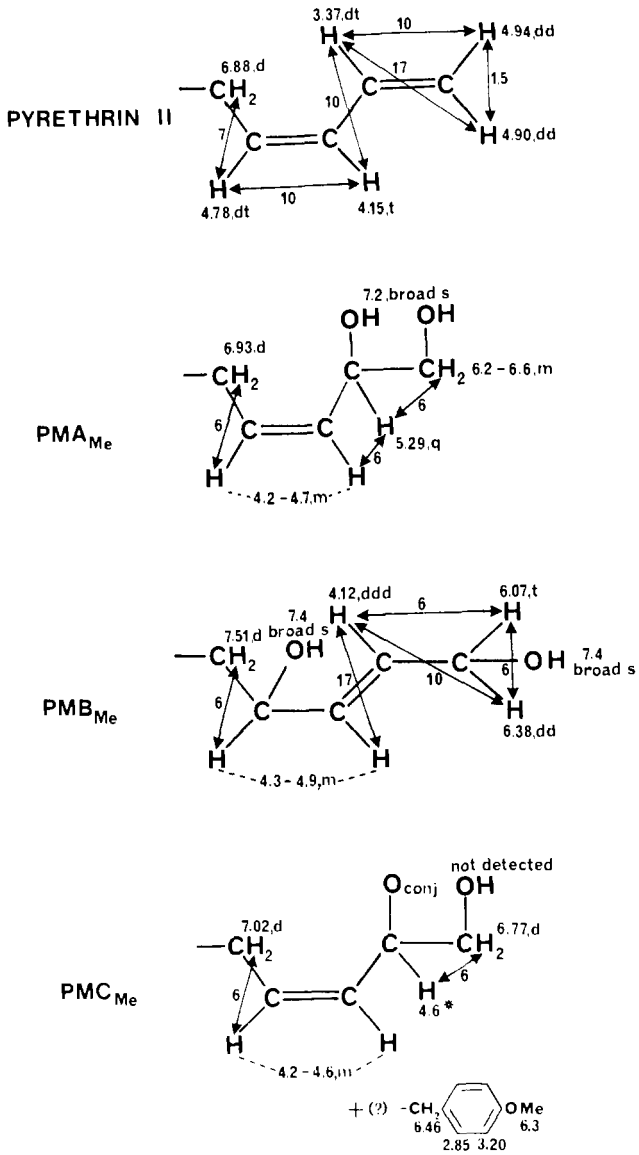


Figure 15. Comparison of nmr data for alcohol side chains of pyrethrin II, PMA_{Me}, PMB_{Me}, and PMC_{Me}

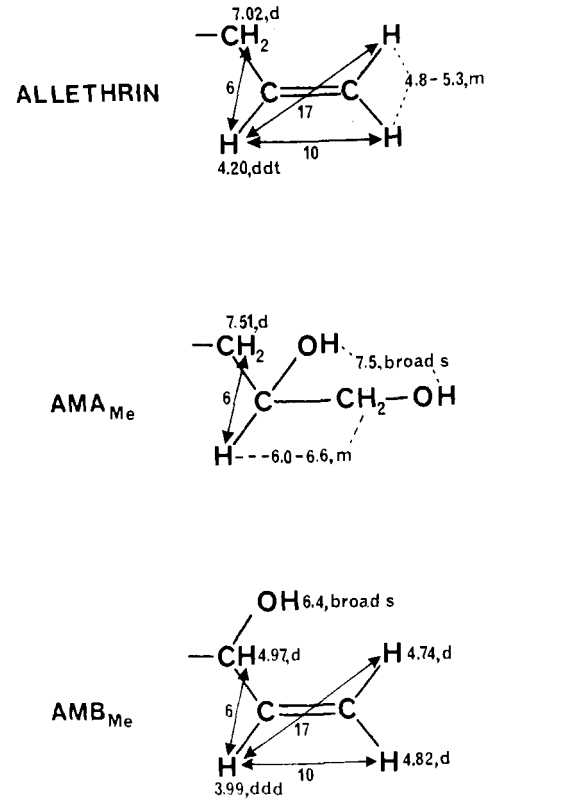


Figure 16. Comparison of nmr data for alcohol side chains of allethrin, AMA_{Me} and AMB_{Me}

significantly, so it is reasonable that its signal (at 4.3 to 4.9) is further downfield than that from the similar proton (o) in PMA_{Me}. Even if the suggested cyclic structure is favored only by a small energy difference, this conformation would nonetheless dominate the appearance of the spectrum.

Metabolite PMC_{Me} (Figure 11) shows peaks from protons a to k all in their normal positions, and protons l, m, and n are as in PMA_{Me}, so PMC_{Me} is a derivative of PMA_{Me} (see Figure 15). The spectrum differs from that of PMA_{Me} as follows: (A) a prominent four-proton area, approximating to two doublets at τ 2.9, 3.2 with splittings of J = 9 Hz, values characteristic of a *p*-disubstituted aromatic nucleus, when the four aromatic protons give such an AA'BB' spectrum; (B) the peak at 5.29 (1 H) has moved to 4.6*; (C) a new singlet (2 H) has appeared at 6.46; (D) the area for protons p, q has moved from *ca.* 6.3 to 6.77 and become a doublet (J = 6 Hz) (loss of nonequivalence); (E) there is no obvious hydroxyl peak. Thus, the aromatic residue must be attached to the side chain, and the large shift observed for proton o [~0.7 ppm, of the same order as the "acetylation shift" (Jackman and Sternhell, 1969b) for methine protons] suggests that the hydroxyl on C_4' is esterified. The esterifying acid is not yet identified, but the large anisotropy of an aromatic nucleus could explain the small shifts in protons l, m, and n, and the large shifts for protons p, q. The τ values for the additional peaks (2.9, 3.2, 6.46) are close to those in homoanisic esters, suggesting that the acid with which the metabolite is conjugated through an ester link at C_4' contains the p-CH_3O—C_6H_4—CH_2— unit. Further interpretation of the spectrum is complicated by the suspected presence of impurities containing methoxyl. Examination (glc) of the trimethylsilylated hydrolysate of PMC_{Me} proved the presence of CDCA; no other glc volatile products were observed, however, even when the oven was temperature programmed, 50 to 200°C.

Metabolite AMA_{Me} (Figure 12) shows all the peaks from protons a to k, in their normal positions, but those from protons l to n indicate that the side chain in this metabolite is —CH_2—CHOH—CH_2OH (Figure 16). Thus proton l is at 7.51 instead of 7.02 (consistent with saturation of the side chain double bond, *cf.* 6.88 → 7.51 for pyrethrin II → PMB_{Me}), but is still a doublet; a complex area (3 H) appears at 5.8 to 6.6 for protons m, n; two hydroxyl groups give an area at *ca.* 7.6 (2 H), removed by D_2O.

Metabolite AMB_{Me} (Figure 13) shows protons a to k, but there is not the usual doublet for protons l at *ca.* 7.0 or 7.5, so C_1' has been modified (see Figure 16). The region τ 3.8 to 5.5 compared with that of allethrin shows that protons n, o are present in both, but that in AMB_{Me} proton m is now a double double doublet, and proton l (still a doublet) appears at 4.96 (1 H). In addition, a broad hydroxyl peak at 6.4 is observed. Thus, the structure of the side chain is —CHOH—CH=CH_2. The analyses for the side chains in AMB_{Me} and in allethronyl derivatives are very similar (Figure 16).

Metabolite AMC_{Me} (Figure 14) shows peaks in normal positions from protons a, b, c, and h to o so this is the only

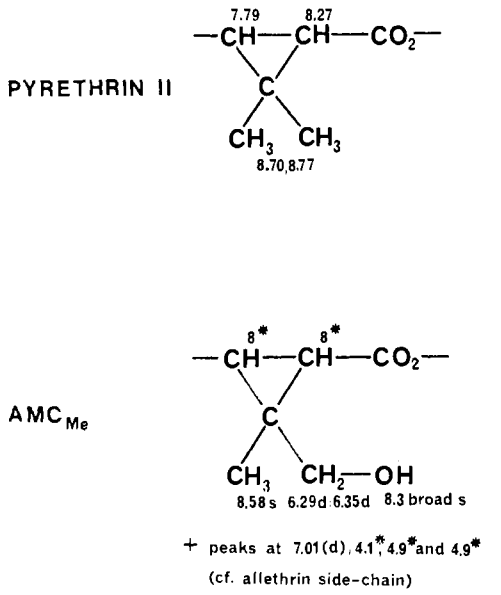


Figure 17. Comparison of nmr data for acid portions of pyrethrin II and AMC_{Me}

Table IV. Radiocarbon Distribution in the Urine and Expired Carbon Dioxide of Male Mice and Rats 48 Hr after Receiving 1 to 5 mg per kg of Various ^{14}C-Labeled Preparations of Pyrethroids or Their Hydrolysis Products

Name	Designation including labeling position	Administered ^{14}C recovered, % Urine	$^{14}CO_2$
	Mice		
Pyrethroids			
Pyrethrin I	PyI-^{14}C(O)O-acid	52	1.0
Allethrin	AI-^{14}C(O)O-acid	51	1.5
	AI-^{14}C-alc	46	0.1
Dimethrin	Dim-^{14}C(O)O-acid	44	0.4
Phthalthrin	Phth-^{14}C(O)O-acid	64	3.1
	Phth-*N*-$^{14}CH_2$-alc	26	39
Hydrolysis products			
Chrysanthemic acid	CA-^{14}C(O)O	82	0.4
N-Hydroxymethyl-3,4,5,6-tetrahydrophthalimide	HTP-*N*-$^{14}CH_2$	37	34
	Rats		
Pyrethroids			
Pyrethrin I	PyI-^{14}C(O)O-acid	46	0.3
Allethrin	AI-^{14}C(O)O-acid	47	0.8
	AI-^{14}C-alc	49	0.0
Pyrethrin II	PyII-$^{14}CH_3$O-acid	7	53
Hydrolysis products			
Chrysanthemic acid	CA-^{14}C(O)O	50	0.1
	CA-$(^{14}CH_3)_2C=$	54	0.2
Chrysanthemum dicarboxylic acid	CDCA-HO(O)^{14}C(CH_3)C=	86	0.9
Methanol	$^{14}CH_3OH$	8	67

metabolite isolated with the side chain on the cyclopentenone ring unchanged, but a second modification is detected on a cyclopropane substituent (Figure 17). One of the methyl groups (f or g) is absent, and the other has moved to 8.58; an OH peak is at 8.3, and there are doublets at 6.29, 6.35 (J = 12 Hz) from two protons in a methylene group, non-

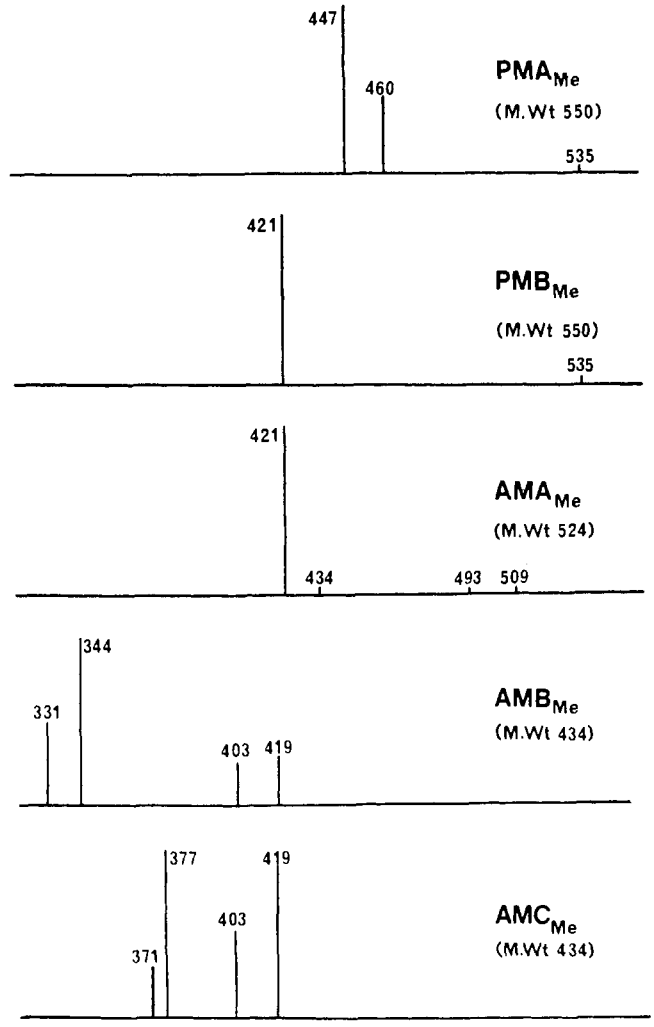


Figure 18. Mass spectra (high mass end only) of trimethylsilyl ethers of PMA_{Me}, PMB_{Me}, AMA_{Me}, AMB_{Me}, and AMC_{Me}

equivalent because they are close to the asymmetric ring system. The complex 7.8 to 8.3 area now conceals the peaks from protons d and e. These facts lead directly to a structure in which one of the cyclopropyl methyls has been hydroxylated (Figure 17).

Two minor metabolites of allethrin were confirmed as allethrolone and CDCA, as the methyl ester, by comparison of their nmr spectra with those (Bramwell *et al.*, 1969) of authentic samples.

Mass Spectra of the Metabolites. The structures of PMA_{Me}, PMB_{Me}, AMA_{Me}, AMB_{Me}, and AMC_{Me}, deduced from their nmr spectra, were confirmed by ms. To increase volatility and to simplify spectra the metabolites (hydroxy compounds) were examined as their trimethylsilyl ethers. All five metabolites then gave mass spectra (Figure 18) with peaks at values corresponding to M-15 (Pierce, 1968), *i.e.* PMA_{Me}, PMB_{Me}, and AMA_{Me} are confirmed as dihydroxy-, and AMB_{Me} and AMC_{Me} as monohydroxy compounds. The allethrin metabolites also lost methoxyl to give M-31 peaks, like pyrethrin II (King and Paisley, 1969). It is well established (Eglinton *et al.*, 1968; Capella and Zorzut, 1968) that in ethers from vicinal diols, fragments from cleavage of the bond between the ether groups dominate the spectrum; the two terminal vicinal diols, PMA_{Me} and AMA_{Me}, as ethers, behaved in this way, giving prominent M − 103 peaks corresponding to loss of a $—CH_2OSi(CH_3)_3$ fragment. In PMB_{Me},

the two hydroxyl functions are separated vinylogously, and the fragment lost (129) corresponds to —CH=CH—$CH_2OSi(CH_3)_3$, confirming the 2′,5′-diol structure. Three of the metabolites, PMA_{Me}, AMA_{Me}, and AMB_{Me}, gave significant M − 90 peaks [loss of $HOSi(CH_3)_3$] as well, and, unexpectedly, AMB_{Me} gave a definite (though much smaller) M − 103 peak. PMC_{Me} was not sufficiently volatile, even after trimethylsilylation, so it gave no further evidence on the structure of the conjugating acid.

Radiocarbon Distribution in the Urine and Expired Carbon Dioxide of Mice and Rats Treated Orally with Various ^{14}C-Labeled Preparations of Pyrethroids and Their Hydrolysis Products. Table IV gives the percentages of the administered radiocarbon appearing in urine and as expired $^{14}CO_2$ following oral treatment with several labeled compounds. Pyrethrin II (PyII-$^{14}CH_3O$-acid) is metabolized in rats to give a large amount of $^{14}CO_2$ although less than from direct administration of an equivalent amount of ^{14}C-methanol. The times at which half the total expired ^{14}C appeared as $^{14}CO_2$ were *ca.* 2.5 hr with both substances, and the urine contained little ^{14}C. Thus the methoxycarbonyl group of pyrethrin II is rapidly hydrolyzed *in vivo* and the liberated methanol oxidized to carbon dioxide. Pyrethroids labeled with ^{14}C in the carboxyl group attached to C_1 of the cyclopropane ring [PyI-$^{14}C(O)O$-acid, AI-$^{14}C(O)O$-acid, Dim-$^{14}C(O)O$-acid, and Phth-$^{14}C(O)O$-acid] give a small but significant amount of $^{14}CO_2$, possibly by hydroxylation at C_1, followed by ring cleavage and release of the carboxyl carbon as $^{14}CO_2$. In contrast, the absence of $^{14}CO_2$ from AI-^{14}C-alc (Table IV) and of ^3H_2O from AI-^3H-alc (above) indicate that the cyclopentenone ring is not destroyed during metabolism.

Chrysanthemic acid [CA-$^{14}C(O)O$ and CA-$(^{14}CH_3)_2C$=] and CDCA [—$HO(O)^{14}C(CH_3)C$=] all give some $^{14}CO_2$, indicating that a small proportion of the acid is extensively degraded. The urine of rats receiving CA-$^{14}C(O)O$ contains little or no chrysanthemic acid, but there are large amounts of CDCA and more polar unidentified compounds (tlc with the BFE1 solvent system).

Under conditions where formate and formaldehyde are converted to CO_2 in yields of 83 and 65%, respectively (Casida *et al.*, 1966), Phth-*N*-$^{14}CH_2$-alc and its alcohol fragment (HTP-*N*-$^{14}CH_2$) (Yamamoto and Casida, 1968) give $^{14}CO_2$ in 39 and 34% yields, respectively. The extensive cleavage possibly occurs after hydroxylation of the *N*-methylene group in the ester rather than by direct attack by an esterase.

DISCUSSION

Figures 2 and 3 show some of the products of metabolism of pyrethrin I, pyrethrin II, and allethrin, and the pathways by which they may be formed. The sites most susceptible to metabolic attack are on the acid portions, *i.e.*, the methoxycarbonyl group of pyrethrin II and the *trans*-methyl group of the isobutenyl side chains of pyrethrin I and allethrin. Oxidases able to oxidize this methyl group (Yamamoto *et al.*, 1969) and esterases able to hydrolyze methyl esters are probably distributed widely in mammals. The compounds Pacid and Aacid seem to be the central intermediates in the metabolic pathways. No more than trace amounts of metabolites with the alcohol side chain unmodified were recovered in urine, indicating that the pentadienyl side chain of the pyrethrins is also very susceptible to attack. Probably a terminal epoxide is first formed that is then degraded (Figure 2), with or without rearrangement, to give two diols and a conjugate of one of them with an unidentified aromatic acid. The double bond in the allyl side chain of allethrin is less susceptible to attack; consequently, besides the diol corresponding to the product from pyrethrins, metabolites are formed by hydroxylation at the methylene group of the side chain and at one of the methyl groups on the cyclopropane ring. The side chain methylene group is also one of the positions attacked during metabolism of allethrone, a related compound, by *Aspergillus niger* (LeMahieu *et al.*, 1970). Hydroxylation of a methyl group on the cyclopropane ring of pyrethroids is new and may be important in the metabolism of insecticidal tetramethylcyclopropane carboxylates.

These metabolic pathways indicate neither the nature of the less polar and more polar metabolites that remain to be identified nor the minor pathway by which the carboxyl group in the acid moiety is converted to carbon dioxide. It seems probable that at least some of the more polar metabolites, difficult to separate from interfering materials, are formed by combined attacks at the sites already identified, or by conjugation of some of the hydroxy derivatives with compounds such as glucuronic acid. Conjugates of Palc, probably with glucose, have been reported in living houseflies (Yamamoto *et al.*, 1969).

The low toxicity of pyrethrates to mammals noted in this paper, by Martel and Buendia (1970) and Elliott (1971) probably results in part from the ease with which the methoxycarbonyl group is hydrolyzed. The susceptibility to oxidation of the *trans*-methyl group of the isobutenyl moiety in (+)-*trans*-chrysanthemates is also probably an important factor in their low toxicity to mammals. The methyl ester, PMB_{Me} (synthesis: Elliott and Janes, 1971) was less than one-fourth as toxic as pyrethrin II when injected intraperitoneally into mice. The other metabolites are probably also of low mammalian toxicity because even small structural modifications in the compounds usually drastically diminish their potency, at least to insects. Another factor contributing to the low mammalian toxicity of ingested pyrethroids is excretion of a portion of the dose unmetabolized in the feces, both with pyrethrins I and II (this work) and with phthalthrin (Miyamoto *et al.*, 1968). The fact that pyrethrin I, pyrethrin II, and allethrin have multiple sites in their structures readily attacked in biological systems makes it improbable that they will be concentrated when passing through food chains, or that they will persist unmetabolized in the mammalian body.

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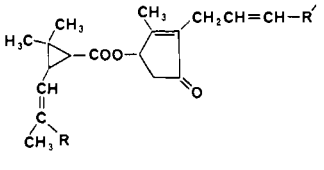
Stabilization of Thin Films of Pyrethrins and Allethrin

Raymond P. Miskus* and Theresa L. Andrews

The combination of an antioxidant and an ultraviolet screening agent in a mineral oil formulation can significantly stabilize pyrethroids for at least 4 hr. Nonstabilized formulations show almost complete destruction in 4 hr.

Pyrethrins and analogous compounds have long been known as powerful insecticides, and are effective against a wide variety of arthropods. The term *pyrethroids* in this paper includes the natural insecticidal constituents of pyrethrin flowers, the pyrethrin, and the synthetic compound allethrin. For a review of the pyrethroids and their composition see Metcalf (1955) or Elliot (1969).



Most pyrethroids have the important practical aspect of a low order of toxicity to warm-blooded animals, and the advantage of a very low persistence of less than 4 hr. Low persistence is also disadvantageous—pyrethroids are highly unstable in the field; they rapidly convert to products having little insecticidal activity (Crowe *et al.*, 1961).

Their high instability when exposed to air and light (Stahl, 1960; Chen and Casida, 1969), as well as their high cost, have kept pyrethroids from finding much use in agriculture. Their primary use had been in the control of household pests, such as housefly, mosquito, and cockroach.

Many attempts have been made to stabilize pyrethroids and to prolong their effective life. The results evidently were not satisfactory, since they have not been commercially used. A few examples are the addition of trialkylphenols (Smith and Hill, 1947), the addition of 4-aminoazobenzene (Smith and Templin, 1956), and the addition of Food Yellow 10 (2,4-dihydroxyazobenzene) (Warner, 1963). Many other patents on stabilization are available, but none have been effective in the field, and are not used. The addition of Food Yellow 10 provides about 50% stabilization after exposure to sunlight for 4 hr, but has the undesirable feature of being a staining dye.

We have eliminated the use of staining dyes, and have discovered that the precise range of ultraviolet radiation that induces pyrethroid destruction is 290 to 320 nm. This range of photoreactivity in the near ultraviolet was found by measuring the transmittance of borosilicate glass and that of soft or window glass. Soft glass transmits radiation only above 300 nm, while borosilicate glass transmits radiation beginning at 290 nm. Pyrethroids were not destroyed when sunlight was filtered through soft glass, but were rapidly destroyed when exposed to sunlight filtered through borosilicate glass. Pyrethroids were not destroyed when exposed to the atmosphere in the dark, nor did any loss occur through evaporation. This difference explains the increased stability of pyrethrins in homes and greenhouses, and also corresponds to the erythermal spectra of sunlight (Das Gupta, 1962). Once this was realized we needed to find materials compatible with pyrethroids that would also be photostable and absorb in the 290 to 320 nm range.

The use of ultraviolet absorbers alone did not provide complete stability (Table II). Even more stabilization was provided by the use of certain oil-soluble antioxidants, although previous trials were not completely successful (Eddy, 1951; Head and Jones-Glynne, 1965; Bell and Kido, 1965).

METHODS AND MATERIALS

The primary materials tested were 20% purified pyrethrins (Fairfield Chemical Co., and the McLaughlin Gormley King Co.) and allethrin (City Chemical Co.). The stock solutions were refrigerated in dark brown bottles; they remained stable

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