Metabolic Fate of 3,4,5- and 2,3,5-Trimethylphenyl Methylcarbamates, the Major Constituents in Landrin Insecticide

Michael Slade[1] and John E. Casida

In the metabolism of 3,4,5- and 2,3,5-trimethylphenyl methylcarbamates by living bean plants, mice, and houseflies, and by the mixed-function oxidase systems prepared from mouse liver or housefly abdomens, the major attack involves oxidation of one of the ring-methyl groups, while hydroxylation of the *N*-methyl group, hydroxylation of the ring carbons, and hydrolysis of the ester link occur only to a minor extent. The ring-methyl oxidations proceed only to the hydroxymethyl stage in living bean plants and houseflies probably because of rapid conjugation of the hydroxymethyl derivatives. In living mice and in the liver microsome-NADPH system, the oxidations proceed to the benzoic acid derivatives with and without intact ester linkages. Methods of synthesis are given for six of the 13 identified metabolites. Exposure of the Landrin constituents to sunlight on bean foliage results in extensive degradation by oxidation.

Landrin (Shell Chemical Co.) is a new insecticidal product which contains approximately 75% of 3,4,5-trimethylphenyl methylcarbamate and 18% of the 2,3,5-isomer. Since it is useful in the control of certain soil insects, among other pests, it is important to understand the metabolic fate of these two Landrin constituents in plants as well as in insects and mammals.

The present knowledge about other aryl methylcarbamates suggests that metabolism of the two major Landrin constituents in plants, insects, and mammals possibly will involve some hydrolysis, particularly in mammals, but mostly oxidation of the methyl groups and conjugation or further oxidation of the hydroxymethyl derivatives. Also, it is known that mixed-function oxidase enzymes of mammalian liver and houseflies are sometimes useful as model systems in investigating the oxidative reactions involved in the metabolism of methylcarbamates (Lykken and Casida, 1969).

This paper gives the degradation fate of 3,4,5- and 2,3,5-trimethylphenyl methylcarbamates when each of the two isomers is individually injected into bean plants, applied to bean foliage, incubated with mixed-function oxidases of mouse liver or housefly abdomens, or administered to living mice or houseflies, and develops a proposed scheme for the respective metabolic reactions.

MATERIALS AND METHODS

Chemicals. The following ^{14}C-labeled preparations of trimethylphenyl methylcarbamates (Me_3PMC) were provided by the Biological Sciences Research Center, Shell Development Co. (Modesto, Calif.): carbonyl-labeled 3,4,5-isomer (SD 8530; 3,4,5-Me_3PMC-^{14}C=O, 2 mc per mmole), *N*-methyl-labeled 3,4,5-isomer (SD 8530; 3,4,5-Me_3PMC-*N*-$^{14}CH_3$, 1 mc per mmole), 4-methyl-labeled 3,4,5-isomer (SD 8530; 3,4,5-Me_3PMC-4-$^{14}CH_3$, 0.96 mc per mmole), carbonyl-labeled 2,3,5-isomer (SD 8786; 2,3,5-Me_3PMC-^{14}C=O, 1.2 mc per mmole). The radiochemical purity of each labeled sample, as established by thin-layer chromatography (tlc), was greater than 99%. Shell also provided the following nonradioactive compounds: 2-hydroxy-3,4,5-trimethylphenyl methylcarbamate (2-OH-3,4,5-Me_3PMC); 3-carboxy-4,5-dimethylphenyl methylcarbamate (3-COOH-4,5-Me_2PMC); 2-formyl-3,5-dimethylphenyl methylcarbamate (2-CHO-3,5-Me_2PMC); 5-hydroxymethyl-2,3-dimethylphenyl methylcarbamate (5-CH_2OH-2,3-Me_2PMC); 3,4,5-trimethylphenyl carbamate (3,4,5-Me_3PC); 3,4,5-trimethylphenol (3,4,5-Me_3P); 2,3,5-trimethylphenol (2,3,5-Me_3P); 2-hydroxy-3,4,5-trimethylphenol (2-OH-3,4,5-Me_3P); 2-hydroxymethyl-3,5-dimethylphenol (2-CH_2OH-3,5-Me_2P); 5-hydroxymethyl-2,3-dimethylphenol (5-CH_2OH-2,3-Me_2P). Balba *et al.* (1968) describe the synthesis of 3,4,5-trimethylphenyl *N*-hydroxymethylcarbamate (3,4,5-Me_3PHMC) and 2,3,5-trimethylphenyl *N*-hydroxymethylcarbamate (2,3,5-Me_3PHMC).

4-Hydroxymethyl-3,5-dimethylphenyl methylcarbamate (4-CH_2OH-3,5-Me_2PMC) was prepared by hydroxymethylation of 3,5-xylenol in the presence of alkali to obtain 4-hydroxymethyl-3,5-dimethylphenol (4-CH_2OH-3,5-Me_2P) (Auwers, 1907), which was then reacted with methyl isocyanate. A solution of 3,5-xylenol (29 mmoles) and formaldehyde (37% aqueous, 82 mmoles) in 5% sodium hydroxide (33 ml) was held for 5 days at 25° C, and filtered to remove a small amount of precipitate. The filtrate was diluted with an equal volume of water, cooled, and acidified with dilute acetic acid to obtain a mixture of white solid and brown gum which was removed, triturated with cold ether, and crystallized from ethyl acetate, producing yellowish needles of 4-CH_2OH-3,5-Me_2P (mp 186–8° C, reported mp 174–5° C; 7.3%). Reaction of 4-CH_2OH-3,5-Me_2P (2 mmoles) with methyl isocyanate (2 mmoles) in dry ether (50 ml) containing triethylamine (50 mg) by holding for 7 days at 25° C in a stoppered flask, evaporation of solvents, and crystallization of the residue from ether-hexane mixture gave 4-CH_2OH-3,5-Me_2PMC, a white crystalline material (mp 155–6° C; 53%; calculated C = 63.17%, H = 7.18%, N = 6.70%; found C = 63.45%, H = 7.21%, N = 6.93%).

Preparation of 3-hydroxymethyl-4,5-dimethylphenyl methylcarbamate (3-CH_2OH-4,5-Me_2PMC) required a more complicated synthesis route to insure proper isomeric configuration of the product; this route involved 3-carboxy-4,5-dimethylphenol (3-COOH-4,5-Me_2P) as a critical intermediate. 2,3-Dimethylbenzoic acid, obtained by oxidation of 1,2,3-trimethylbenzene (Charlesworth and Levene, 1963) or from Aldrich Chemical Co. (Milwaukee, Wis.), was sulfonated with sulfuric acid, and the sulfonate was converted to 3-COOH-4,5-Me_2P (mp 185–6° C, reported 179–9.5° C; 37%) on fusion with potassium hydroxide (Charlesworth and Levene,

Division of Entomology, University of California, Berkeley, Calif. 94720

[1] Present address, Zoecon Corp., Palo Alto, Calif. 94304

1963). Esterification of 3-COOH-4,5-Me_2P by reaction with equivalent diazomethane in ether at 25° C for 0.5 hr (a procedure which prevented formation of any methyl ether) and recrystallization from ether-hexane mixture yielded the methyl ester (mp 101–2° C, reported to be 104–5° C as prepared by a different synthetic route by Divekar and Vining, 1964; 84%). Reduction of the methyl ester (3-COOMe-4,5-Me_2P) with a molar equivalent amount of lithium aluminum hydride in ether, under reflux, yielded 3-hydroxymethyl-4,5-dimethylphenol (3-CH_2OH-4,5-Me_2P), which was crystallized from benzene (mp 144–6° C; 43%) with or without preliminary purification on a florisil column (Balba and Casida, 1968). Reaction of the phenol with methyl isocyanate, under the conditions described above, yielded 3-CH_2OH-4,5-Me_2PMC (mp 104–6.5° C; calculated C = 63.17%, H = 7.18%, N = 6.70%; found C = 63.48%, H = 7.20%, N = 6.84%).

Similar procedures were used to prepare 3-hydroxymethyl-2,5-dimethylphenyl methylcarbamate (3-CH_2OH-2,5-Me_2-PMC; mp 105–6° C, from ether-hexane; 60%; calculated C = 63.17%, H = 7.18%, N = 6.70%; found C = 63.22%, H = 7.18%, N = 6.86%) *via* the following intermediates, in sequence: 2,5-dimethylbenzoic acid (Eastman Organic Chemicals, Rochester, N.Y.); 3-carboxy-2,5-dimethylphenol (3-COOH-2,5-Me_2P; mp 169–70° C, from water; reported mp 163–5° C; 57%) (Charlesworth and Levene, 1963); the methyl ester (3-COOMe-2,5-Me_2P; mp 174–5° C, from hexane; 84%); 3-hydroxymethyl-2,5-dimethylphenol (3-CH_2OH-2,5-Me_2P; mp 139–41° C, from benzene-hexane; 28%).

Preparation of 4-hydroxy-2,3,5-trimethylphenyl methylcarbamate (4-OH-2,3,5-Me_3PMC) involved reaction of 2,3,5-trimethylhydroquinone (K and K Laboratories, Rahway, N.J.; 10 mmoles) with methyl isocyanate (10 mmoles) in anhydrous ether (150 ml) containing triethylamine (75 mg) for 5 days at 25° C. The desired isomer was isolated by filtering, evaporating the filtrate, and crystallizing from ether-hexane (mp 132–3° C; 26%; calculated C = 63.17%, H = 7.18%, N = 6.70%; found C = 63.22%, H = 7.13%, N = 6.90%).

Purification and Analysis. Resolution of reaction products and metabolites was accomplished by two-dimensional tlc using silica gel F_{254} precoated plates with a gel thickness of 0.25 mm (Brinkmann Instruments Inc., Westbury, N.Y.). The neutral solvent system, used in the first direction, involved development first with benzene and then twice in the same direction with ether-hexane mixture (4:1). (The initial development with benzene moves interfering material, but not the desired products, away from the origin and towards the front.) The second direction was with the acidic solvent system consisting of benzene (saturated with formic acid)–ether mixture (2:1). Chromogenic reagents used were: ninhydrin to detect methylcarbamates (Krishna *et al.*, 1962); chromotropic acid for visualization of N-hydroxymethylcarbamates (Balba *et al.*, 1968); phosphomolybdic acid (10% w/v in ethanol) and heating for 10 min at 110° C as a general chromogenic reagent. Radioactive materials were detected by radioautography. For quantitation, the radioactive gel regions were scraped into scintillation vials for direct radiocarbon assay with a liquid scintillation spectrometer. Melting points were determined on a Fisher-Johns micro hot stage.

Treatment of Bean Plants and Determination of Metabolites or Photodecomposition Products. The materials and test conditions used for growing the snapbean plants (Contender variety, 11 days old, trifoliate stage, 5-g average fresh weight) and extraction of the metabolites or photodecomposition products were the same as those described by Abdel-Wahab *et al.* (1966), with certain exceptions and changes as noted. Thus, 15 μg of test compound, dissolved in 40 μl of acetone-water mixture (2:1), were injected into the stem of each growing bean plant. These studies were made under greenhouse conditions during June and July of 1968 and 1969. Three treated plants were frozen at the following times after injection for subsequent analyses: 0, 1, 3, and 6 days for the 1968 study; 0, 6, and 12 days for the 1969 study. The reported extraction procedure (Abdel-Wahab *et al.*, 1966; Kuhr and Casida, 1967) divided the labeled components into three fractions: the acetone-chloroform or organic phase, the acetone-water or aqueous phase, and the insoluble or unextractable residue. Components of the organic phase were analyzed by tlc after evaporation of the solvent, dissolving in hexane, and spotting the hexane solution. The insoluble or unextractable residue was not further analyzed, other than to determine the total radiocarbon content by combustion (Krishna and Casida, 1966). It is possible that extraction of the insoluble residue with a more polar solvent would recover further metabolites from this fraction, but this point was not examined.

Constituents of the aqueous fractions were analyzed by a modification of the method of Kuhr and Casida (1967). After removal of organic solvents from the aqueous fraction with the rotary evaporator, the remaining solution was added directly to a column of Bio-Beads SM-2 (Bio-Rad Laboratories, Richmond, Calif.), made up on the basis of 1.0 ml of solution to 1.0 g of beads. Ninety-eight percent or greater of the radiocarbon was retained on the column by recycling the aqueous solution three times, and all of the retained radiocarbon was removed by subsequent elution with methanol. The residue remaining after evaporation of the methanol at 25° C was incubated in air with β-glucosidase (0 or 3 mg, Calbiochem, Los Angeles, Calif.) in phosphate buffer (4.0 ml, 0.05 M, pH 4.8) at 37° C for 4 hr. The incubation mixture was washed into a separatory funnel with 0.01 N sulfuric acid (15 ml) and extracted three times with 15-ml portions of chloroform. The chloroform was evaporated and the residue was dissolved in hexane for tlc spotting and analysis. The radiocarbon recovery at each step in the extractions and analyses was 95% or higher.

Leaves on growing bean plants (Pinto variety, size the same as with Contender variety) were individually treated, at approximately 1.0 μg per sq cm, with each labeled compound in 50 μl of ethanol solution and exposed to sunlight for periods of up to 4 days prior to surface rinsing with acetone and analysis of the rinse by tlc. This method is similar to that employed by Abdel-Wahab *et al.* (1966).

Enzyme Studies. Using the general procedure of Oonnithan and Casida (1968), the microsome fraction equivalent to 400 mg of mouse liver was incubated in air for 2 hr at 37° C, with 15 μg of 3,4,5-Me_3PMC-4-$^{14}CH_3$, 3,4,5-Me_3PMC-^{14}C=O or 2,3,5-Me_3PMC-^{14}C=O in 2 ml of 0.25 M sucrose-0.05 M phosphate buffer (pH 7.5), in the presence and in the absence of 2 mg of reduced nicotinamide adenine dinucleotide phosphate (NADPH). After incubation, 1 g of ammonium sulfate was added to each reaction mixture, which was then extracted twice with 6–8 ml of ether-ethanol mixture (2:1). After concentration of the organic extract, the radioactive components were analyzed by tlc.

Using the general procedure of Tsukamoto and Casida (1967), the homogenate prepared from the abdomens of 10 adult houseflies (*Musca domestica* L., R_{Baygon} strain, Shrivastava *et al.*, 1969) was incubated in air for 2 hr at 30° C, with 15 μg of 3,4,5-Me_3PMC-4-$^{14}CH_3$, 3,4,5-Me_3PMC-^{14}C=O, or 2,3,5-Me_3PMC-^{14}C=O in 2 ml of 0.25 M sucrose-0.15 M phosphate buffer (pH 7.4) in the presence or in the absence of 2 mg of NADPH. Following incubation, the reaction mix-

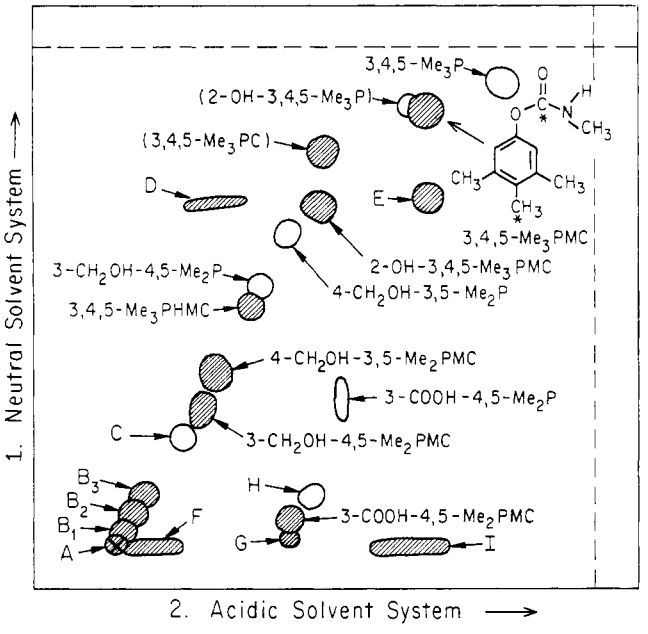

Figure 1. Chromatographic positions for derivatives of 3,4,5-trimethylphenyl methylcarbamate

Esters (shaded circles) and phenols derived from hydrolysis (open circles) include unidentified metabolites (A through I), compounds tentatively identified as metabolites (chemical abbreviations not in parentheses), and compounds which are not metabolites (chemical abbreviations in parentheses). The phenolic hydrolysis products are detected only with the 3,4,5-Me_3PMC-4-$^{14}CH_3$ preparations whereas the esters are detected also with the 3,4,5-Me_3PMC-^{14}C=O and -N-$^{14}CH_3$ preparations

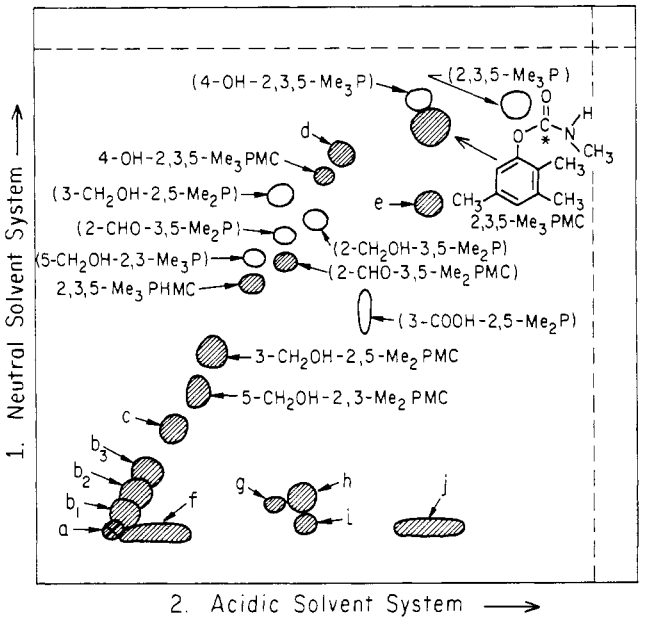

Figure 2. Chromatographic positions for derivatives of 2,3,5-trimethylphenyl methylcarbamate

Esters (shaded circles) include unidentified metabolites (a through j), compounds tentatively identified as metabolites (chemical abbreviations not in parentheses), and compounds which are not metabolites (chemical abbreviations in parentheses). Phenols derived from hydrolysis (open circles) are not detected as metabolites (chemical abbreviations in parentheses) because ony 2,3,5-Me_3PMC-^{14}C=O was investigated

tures were extracted as with the liver microsome preparations and the organosoluble components were analyzed by tlc.

Studies on *In Vivo* Metabolism by Mice and Houseflies. Male albino mice (20 g, Swiss-Webster strain, Bioscience Animal Laboratories, Oakland, Calif.) were treated orally, by stomach tube, with 40 μg of the labeled methylcarbamate (3,4,5-Me_3PMC-4-$^{14}CH_3$, 3,4,5-Me_3PMC-^{14}C=O, 3,4,5-Me_3-PMC-N-$^{14}CH_3$, or 2,3,5-Me_3PMC-^{14}C=O), a dose that did not yield symptoms of poisoning. The administration vehicle was 50 μl of dimethylsulfoxide (DMSO) followed by a 100 μl DMSO rinse of the stomach tube. The treated mice were held for 48 hr while receiving food and water *ad libitum* in individual metabolism chambers designed for the collection of urine and expired $^{14}CO_2$ (Krishna and Casida, 1966). Adult female houseflies (SCR-susceptible strain, 4 days after emergence) were individually injected with 0.5 μg of the labeled compound (3,4,5-Me_3PMC-4-$^{14}CH_3$, 3,4,5-Me_3PMC-^{14}C=O, or 2,3,5-Me_3PMC-^{14}C=O) in 0.1 μl of acetone, a dose which resulted in immediate knockdown and paralysis of all flies and recovery within 24 hr of 40% of the flies treated with 3,4,5-Me_3PMC and 60% of those treated with 2,3,5-Me_3PMC. The treated flies were held for 24 hr while receiving sugar and water, either in metabolism chambers for collection of expired $^{14}CO_2$ (Casida *et al.*, 1968) or in glass beakers for collection of excreta.

For analysis of excreted metabolites, the excreta from 25 flies or the residue from evaporation of the urine of individual mice was incubated in air with glusulase (0 or 100 μl, 100,000 units of glucuronidase and 50,000 units of sulfatase per ml, Endo Laboratories, Inc., Garden City, N.Y.) in acetate buffer (4.0 ml, 0.05 M, pH 4.8) at 37° C for 4 hr. Following incubation, the reaction mixtures were extracted and analyzed as with the microsome preparations.

RESULTS

Tlc Characteristics of Analogs and Derivatives of 3,4,5- and 2,3,5-Trimethylphenyl Methylcarbamates. The two-dimensional tlc patterns found for known compounds and for unidentified metabolites or degradation products derived from 3,4,5-Me_3PMC are given in Figure 1, and those derived from 2,3,5-Me_3PMC are given in Figure 2. Similar tlc characteristics are evident for comparable compounds in the two different isomeric series (3,4,5- and 2,3,5-Me_3PMC). The methylcarbamates are of lower R_f values than the corresponding phenols in those cases where both compounds are available for comparison. There is good resolution of the carbamates resulting from introduction of a single hydroxyl group into various positions on the insecticide chemical. The carboxylic acid derivatives, in contrast to other compounds, characteristically are of higher R_f value in the acidic than in the neutral solvent system.

Photodecomposition Products on Treated Bean Leaves. 3,4,5-Me_3PMC and 2,3,5-Me_3PMC are decomposed more rapidly on bean foliage when exposed to sunlight as compared with normal laboratory light. The percentage values for radiocarbon recovery on acetone rinsing of the treated leaves are similar for each of the labeled compounds (3,4,5-Me_3PMC-4-$^{14}CH_3$, 3,4,5-Me_3PMC-^{14}C=O, and 2,3,5-Me_3PMC-^{14}C=O); the recovery ranges for the three preparations at various times after treatment are: 0 hour—100%; 1 hr—52 to 79%; 4 hr—37 to 52%; 24 hr—20 to 28%; 96 hr—11 to 20%. The tlc chromatographic patterns for the recovered products are also similar with each of the labeled compounds. Hydrolysis of the ester group of 3,4,5-Me_3PMC probably is not a major photochemical reaction because, as indicated above, the two labeled preparations of this compound gave similar recovery values and tlc characteristics for the products. The com-

pound applied was also the major material recovered during the first 4 hr of exposure, but it was a minor product after 1 day and was almost absent after 4 days. Many products were formed and those of intermediate R_f values, using the solvent systems shown in Figures 1 and 2, were transient and converted to products remaining at the origin. The transient intermediates included compounds cochromatographing with: 4-CH_2OH-3,5-Me_2PMC (major) and 3,4,5-Me_3PHMC (trace) as derived from 3,4,5-Me_3PMC; 3-CH_2OH-2,5-Me_2PMC (major), 5-CH_2OH-2,3-Me_2PMC (trace), and 2,3,5-Me_3PHMC (trace) as derived from 2,3,5-Me_3PMC. The only significant photodecomposition products of each labeled preparation after 4 days are those remaining at the origin. Separation of these materials by two-dimensional tlc using solvent systems of greater polarity [methanol in the first direction, and *n*-butanol-glacial acetic acid-water (3:1:1) mixture in the second direction] yielded only two resolved products, present in about equal amounts, which did not vary in chromatographic region (R_f 0.6 to 0.8) with the different labeled preparations. These polar products are esters probably formed by oxidation of two or more methyl groups.

Metabolites in Bean Plants After Stem Injection. Metabolism of 3,4,5- and 2,3,5-Me_3PMC-^{14}C=O in bean plants results in a rapid loss of radiocarbon from the organosoluble fraction, an almost equivalent increase of radiocarbon in the water-soluble fraction, and a gradual increase in the unextractable radiocarbon over a period of 6 days (Table I). The chemical nature of the metabolites in the unextractable residue remains unknown. Direct comparison of the metabolism rates for the two isomers is not appropriate from this data because the experiments with the two isomers were not made at the same time.

Metabolites in the organosoluble fractions from plants 1 and 6 days after injection of 3,4,5-Me_3PMC-^{14}C=O were identified by cochromatography, and those present 6 days after injection of 3,4,5-Me_3PMC-4-$^{14}CH_3$ and 2,3,5-Me_3PMC-^{14}C=O were tentatively identified by their chromatographic positions. The products derived from 3,4,5-Me_3PMC-^{14}C=O, as percentages of the total radiocarbon in the organosoluble fraction, were: 3,4,5-Me_3PMC—97.6% at 1 day and 58.3% at 6 days; 3,4,5-Me_3PHMC—0.4% at 1 day and 10.9% at 6 days; 4-CH_2OH-3,5-Me_2PMC—1.7% at 1 day and 9.8% at 6 days; 3 CH_2OH-4,5-Me_2PMC—0.3% at 1 day and 8.0% at 6 days; unknown A (origin of tlc plate)—less than 0.1% at 1 day and 13.0% at 6 days. 3,4,5-Me_3PMC-4-$^{14}CH_3$ at 6 days yielded the same products and in addition a small amount of unknown C. 2,3,5-Me_3PMC-^{14}C=O at 6 days gave 16.8% of the organosoluble radiocarbon as the injected compound, 6.7% as 2,3,5-Me_3PHMC, 52.3% as 3-CH_2OH-2,5-Me_2PMC, 20.6% as 5-CH_2OH-2,3-Me_2PMC, and 3.6% as unknown a (origin of tlc plate). Thus, the compound administered is the

Table I. Fractionation of Radiocarbon from Carbonyl-Labeled Preparations of 3,4,5- and 2,3,5-Trimethylphenyl Methylcarbamates Following Injection into Growing Snapbean Seedlings

Fraction Designation	% Radiocarbon Recovered as Indicated Fraction at Various Days After Injection[a]			
	0	1	3	6
3,4,5-Trimethylphenyl Methylcarbamate				
Organosoluble	97.9	55.5	13.5	2.0
Water-soluble	0.5	39.5	75.3	85.0
Unextractable Residue	1.6	5.0	11.2	13.0
2,3,5-Trimethylphenyl Methylcarbamate				
Organosoluble	99.4	22.9	7.0	0.8
Water-soluble	0.1	69.9	83.3	87.0
Unextractable residue	0.5	7.2	9.7	12.2

[a] The radiocarbon recovered at each time interval was essentially the same as that initially injected, indicating little or no loss as $^{14}CO_2$ or other fractions which are not tabulated.

Table II. Metabolites of ^{14}C-Labeled Preparations of 3,4,5- and 2,3,5-Trimethylphenyl Methylcarbamates Following Stem Injection into Growing Snapbean Seedlings

Product Designation	% Radiocarbon Recovered as Indicated Products at Various Days After Injection[a]				
	0	6		12	
3,4,5-Trimethylphenyl Methylcarbamate					
	^{14}C=O and 4-$^{14}CH_3$	^{14}C=O	4-$^{14}CH_3$	^{14}C=O	4-$^{14}CH_3$
Organosoluble and Water-soluble Fractions					
3,4,5-Me_3PMC[b,c]	99.1	1.7	2.2	0.7	0.7
3,4,5-Me_3PHMC[b,d,e]	0	18.5	6.4	13.4	5.5
4-CH_2OH-3,5-Me_2PMC[b,d,f]	0	53.8	57.0	63.8	52.5
Unknown C[d]	0	0	19.2	0	22.8
Unknown A[d,g]	0	2.5	3.5	2.9	2.1
Unextractable Residue	0.9	23.5	11.7	19.2	16.4
2,3,5-Trimethylphenyl Methylcarbamate					
	^{14}C=O	^{14}C=O		^{14}C=O	
Organosoluble and Water-soluble Fractions					
2,3,5-Me_3PMC[b,c]	99.4	1.1		0.7	
2,3,5-Me_3PHMC[b,d,e]	0	7.2		5.3	
3-CH_2OH-2,5-Me_2PMC[b,d]	0	53.4		62.2	
5-CH_2OH-2,3-Me_2PMC[b,d]	0	25.0		22.2	
Unknown a[d,g]	0	1.9		1.7	
Unextractable Residue	0.6	11.4		7.9	

[a] The radiocarbon recovered relative to that initially injected with the ^{14}C=O preparations averaged 90.7% at 0 days, 88.4% at 6 days, and 74.8% at 12 days. [b] Identification by cochromatography with authentic unlabeled compound. [c] Present only in organosoluble fraction. [d] Present to the extent of 95 to 100% in the water-soluble fraction as conjugates cleaved by β-glucosidase. [e] Includes small amounts of the unsubstituted carbamates, or compounds chromatographing in the position anticipated for these carbamates, resulting from degradation of the hydroxymethylcarbamates during analysis. [f] Contains some 3-CH_2OH-4,5-Me_2PMC as a minor component, although resolution is usually not adequate for separate analysis. However, in one case where resolution was adequate, 4-CH_2OH-3,5-Me_2PMC and 3-CH_2OH-4,5-Me_2PMC were present as aglycones in the ratio of 6.4:1 after injection of 3,4,5-Me_3PMC-^{14}C=O. [g] Products remaining at origin of tlc plate.

Table III. Metabolites of Various ^{14}C-Labeled Preparations of 3,4,5-Trimethylphenyl Methylcarbamate and of Carbonyl-^{14}C-Labeled 2,3,5-Trimethylphenyl Methylcarbamate Following Incubation with Mixed-Function Oxidase Enzymes or Administration to Living Houseflies or Mice

Product Designation	Radiocarbon Recovered as Indicated Products, %[a]			
	Mixed Function Oxidase Enzymes		Living Animals	
	Housefly Abdomen Homogenate	Mouse Liver Microsomes	Housefly Excreta Cleaved by Glusulase	Mouse Urine Cleaved by Glusulase
3,4,5-Trimethylphenyl Methylcarbamate[b]				
Organosoluble[c]				
3,4,5-Me_3PMC	74.9[d]	0.9	0.9	0.8
3,4,5-Me_3P	...	...	...	1.2[e]
2-OH-3,4,5-Me_3PMC	0.1	1.0	0.5[e]	...
4-CH_2OH-3,5-Me_2P	...	1.5	...	4.6[e]
3-CH_2OH-4,5-Me_2P	...	1.1	...	...
3,4,5-Me_3PHMC	0.3	0.3[d]	1.1[e]	...
4-CH_2OH-3,5-Me_2PMC	8.0[d]	32.8[d]	58.9[f]	1.4[e]
3-CH_2OH-4,5-Me_2PMC	5.5[d]	3.5[d]	10.9[f]	0.5[e]
3-COOH-4,5-Me_2PMC	1.0	13.1[d]	1.5[f]	9.6[f]
3-COOH-4,5-Me_2P	...	0.8	...	0.4[e]
Unknown A	...	3.3	10.6	10.2
Unknown B_{1-3}	1.7	33.8	6.2[f]	2.1[f]
Unknown C	3.2	1.4	...	...
Unknown D	...	0.5	...	...
Unknown E	0.7	...	...	...
Unknown F	1.6	1.8	6.7[f]	4.8[f]
Unknown G	...	0.1	...	1.5[f]
Unknown H	...	2.9	...	2.2[f]
Unknown I	2.1	...	...	...
Minor unknowns	0.6	0.7	1.2	1.3
Water-soluble	0.3	0.5	1.5	11.7
Total	100.0	100.0	100.0	52.3
2,3,5-Trimethylphenyl Methylcarbamate				
Organosoluble[g]				
2,3,5-Me_3PMC	74.6[d]	0.1	...	...
4-OH-2,3,5-Me_3PMC	...	0.3	...	...
2,3,5-Me_3PHMC	0.3	0.3[d]	...	...
3-CH_2OH-2,5-Me_2PMC	9.6[d]	25.9[d]	31.2[f]	2.5[e]
5-CH_2OH-2,3-Me_2PMC	9.9[d]	8.4[d]	34.7[f]	...
Unknown a	...	5.1	7.9	7.3
Unknown b_{1-3}	...	37.9	1.9[f]	1.1[e]
Unknown c	...	2.2	14.3[f]	...
Unknown d	...	1.0	...	...
Unknown e	2.5	...	...	...
Unknown f	0.6	2.7	7.3[e]	6.5[f]
Unknown g	...	2.3	...	2.5[f]
Unknown h	0.2	11.6	...	4.2[f]
Unknown i	...	0.2	...	0.2[f]
Unknown j	1.0	...	...	...
Minor unknowns	0.9	1.7	1.3	0.8
Water-soluble	0.4	0.3	1.4	11.2
Total	100.0	100.0	100.0	36.3

[a] The amount of radiocarbon is relative to the total recovered with the enzyme preparations and housefly excreta, but with urine it is relative to the total administered to the mice. The radiocarbon recovered was in all cases greater than 95% of the initial level with the enzyme preparations, but with houseflies the amount excreted, although high, is not meaningful because some persisting paralysis and mortality occurred during the 24-hour period. [b] The results are with the 4-$^{14}CH_3$ preparation unless indicated otherwise. [c] The positions for the products identified and for the unknowns designated by letters are given in Figure 1, which also indicates which products are esters (essentially the same results with each labeled preparation) and which are phenols (detected only with the 4-$^{14}CH_3$ preparation). [d] Identification by cochromatography with authentic unlabeled compounds. [e] Present almost entirely as conjugates at origin or in water-soluble fraction prior to glusulase cleavage. [f] Present partially as conjugates at origin or in water-soluble fraction prior to glusulase cleavage. [g] The positions for the products identified and for the unknowns designated by letters are given in Figure 2.

major component of the organosoluble fraction shortly after injection, but within a few days, when little organosoluble fraction remains, there is a significant proportion of hydroxylation products.

A more detailed analysis of the metabolites was made 6 and 12 days after injection of ^{14}C-labeled preparations of 3,4,5- and 2,3,5-Me_3PMC (Table II). A small amount of the injected compounds still remains in the organosoluble fraction, but the major metabolites are water-soluble products which are cleaved, almost quantitatively, by the action of β-glucosidase to yield organosoluble products (aglycones). This finding establishes that the water-soluble fraction consists of conjugates that are glycosides and possibly are β-D-glucosides. The pattern of metabolites is very similar at 6 and 12 days, indicating that the rapid steps in the turnover of metabolites are nearly complete by this time. In fact, more than 95% of the extractable radiocarbon is in the form of conjugated metabolites by 6 days. The carbamate aglycones derived from 3,4,5-Me_3PMC are 4-CH_2OH-3,5-Me_2PMC (major), 3,4,5-Me_3PHMC, and 3-CH_2OH-4,5-Me_2PMC. In addition, a hydrolysis product, unknown C, is detected as an aglycone with the 4-$^{14}CH_3$ preparation but not with the $^{14}C{=}O$ prep-

aration of 3,4,5-Me_3PMC. 2,3,5-Me_3PMC-^{14}C=O yields the following aglycones: 3-CH_2OH-2,5-Me_2PMC (major), 5-CH_2OH-2,3-Me_2PMC, and 2,3,5-Me_3PHMC.

In a separate study, the aglycones derived from 3,4,5-Me_3PMC-4-$^{14}CH_3$ were recovered as a mixture, with or without tlc cleanup, and subjected to the following sequence of treatments: hydrolysis in 5% sodium hydroxide (1 hr, 30° C), neutralization, extraction of the labeled phenolic products into ether, and subsequent tlc analysis. As expected, the saponification products of the metabolites presumed to be 3-CH_2OH-4,5-Me_2PMC and 4-CH_2OH-3,5-Me_2PMC cochromatographed with 3-CH_2OH-4,5-Me_2P and 4-CH_2OH-3,5-Me_2P.

Ester hydrolysis does not appear to be a major metabolic pathway because there is only a small loss of radiocarbon from beans treated with carbonyl-^{14}C-labeled preparations; however, the detection of the metabolite designated as unknown C with 3,4,5-Me_3PMC-4-$^{14}CH_3$ and not with 3,4,5-Me_3PMC-^{14}C=O establishes that some hydrolysis takes place.

Metabolites Formed by Mixed-Function Oxidases of Mouse Liver and Housefly Abdomens. Metabolism of 3,4,5-Me_3PMC and 2,3,5-Me_3PMC by housefly abdomen homogenates or mouse liver microsomes is dependent on fortification with NADPH. Little if any hydrolysis takes place because there is less than 5% loss of radiocarbon from the carbonyl-^{14}C-labeled preparations during incubation and extraction; carbonate-^{14}C from hydrolysis is lost from solution during incubation (Oonnithan and Casida, 1968). Instead, the metabolism primarily involves hydroxylation or oxidation of the N-methyl and ring-methyl groups, mainly to the alcohols in insect preparations but, in part, to acid derivatives which probably are substituted-benzoic acids with mammalian preparations (Table III). Thus, 3-COOH-4,5-Me_2PMC is a metabolite produced by liver microsomal action on 3,4,5-Me_3PMC. There is a small amount of ring hydroxylation because products chromatographing in the region of 2-OH-3,4,5-Me_3PMC and 4-OH-2,3,5-Me_3PMC are formed from 3,4,5-Me_3PMC and 2,3,5-Me_3PMC, respectively. The individual 4-$^{14}CH_3$-labeled hydroxymethyl-dimethylphenyl methylcarbamate metabolites (3-CH_2OH-4,5-Me_2PMC and 4-CH_2OH-3,5-Me_2PMC) recovered from each of the mouse liver and housefly preparations were saponified and chromatographed with the anticipated phenolic products; cochromatography confirmed the identity of the products.

Metabolites Formed by Living Mice and Houseflies. As shown in Table IV, the radiocarbon expired from mice as $^{14}CO_2$ decreases and that excreted in urine increases in the order of ^{14}C=O, N-$^{14}CH_3$, and 4-$^{14}CH_3$-preparations of 3,4,5-Me_3PMC. Based on the results obtained with 3,4,5-Me_3PMC-^{14}C=O, the metabolism appears to involve almost equal amounts of hydrolysis, yielding $^{14}CO_2$, and nonhydrolytic reactions, yielding labeled products excreted in the urine. Limited studies involving administration of 2,3,5-Me_3PMC-^{14}C=O to mice indicate a similar extent of elimination in urine but less $^{14}CO_2$ than that derived from 3,4,5-Me_3PMC-^{14}C=O. However, only a portion of the products found in the urine are tentatively identified (Table III). Hydroxymethyl-dimethylphenyl methylcarbamates are liberated on incubation with glusulase, indicating that these compounds are present as conjugates and probably as β-D-glucuronides. Certain of the metabolites possibly are glucuronic acid- or amino acid conjugates of 3-COOH-4,5-Me_2PMC or other carboxylic acids resulting from oxidation of ring-methyl groups.

Houseflies expire less than 4% of the injected dose of 3,4,5-Me_3PMC-^{14}C=O and 2,3,5-Me_3PMC-^{14}C=O as $^{14}CO_2$ within 24 hr, indicating that hydrolysis is a minor reaction in metabolism of these carbamates. Analysis of the excreta (Table III) confirmed this finding because the same products in the same proportion were encountered with 3,4,5-Me_3PMC-4-$^{14}CH_3$ and 3,4,5-Me_3PMC-^{14}C=O. The principal excreted metabolites are the hydroxymethyl-dimethylphenyl methylcarbamates encountered with the fly enzyme preparations and they appear almost entirely as conjugates at the origin of the tlc plate unless cleaved by glusulase prior to chromatography. The *N*-hydroxymethylcarbamate derivatives, also excreted primarily as conjugates, are minor metabolites.

Table IV. Fate of Radiocarbon in Male Mice 48 Hours after Oral Administration of Variously-Labeled Samples of Trimethylphenyl Methylcarbamates

Compound and Labeling Position	Administered Radiocarbon Recovered, %		
	Urine	Expired $^{14}CO_2$	Total[a]
3,4,5-Me_3PMC			
4-$^{14}CH_3$	81	0.2	81
^{14}C=O	42	35	77
N-$^{14}CH_3$	62	12	74
2,3,5-Me_3PMC			
^{14}C=O	45	17	62

[a] A major portion of the radiocarbon which is not accounted for was probably excreted in the feces which were not analyzed.

DISCUSSION

Tentative metabolic pathways for 3,4,5-Me_3PMC and 2,3,5-Me_3PMC are given in Figures 3 and 4, respectively. Hydrolysis is not a major metabolic pathway in the systems examined other than in the living mice. The rate of hydroxylation appears, in general, to decrease in the order of the *meta*- or *para*-methyl groups, the N-methyl group, and the unsubstituted ring positions. The ease of oxidation of the hydroxymethyl-dimethylphenyl methylcarbamates to the carboxy-dimethylphenyl methylcarbamates seems to be greatest, in living mice and in the liver microsomes, at the 3-position of 3,4,5-Me_3PMC and at the 5-position of 2,3,5-Me_3PMC, *i.e.*, at the positions which are less sterically hindered.

In bean plants, houseflies, and in the housefly mixed-function oxidase system, the methyl oxidation mostly stops at the hydroxymethyl stage because conjugates form in the living organisms or the dehydrogenases involved in the enzyme systems are of low activity. The initial sites of metabolic attack are the same in beans as in houseflies or mice, but once these metabolites are formed they are probably converted in beans to the glycosides of the hydroxymethyl-dimethylphenyl methylcarbamate isomers, whereas in mammals they are more extensively degraded. The aldehyde intermediates appear to be transient because unidentified metabolites are minor or absent in the chromatographic region anticipated for these materials; accordingly, these intermediates are designated with brackets in Figures 3 and 4. These findings are consistent with previous studies on the metabolism of related substituted-phenyl methylcarbamates (Abdel-Wahab *et al.*, 1966; Baron and Doherty, 1967; Casida and Lykken, 1969; Dorough, 1968; Friedman and Lemin, 1967; Hook and Smith, 1967; Krishna *et al.*, 1962; Krishna and Casida, 1966; Kuhr and Casida, 1967; Metcalf *et al.*, 1968; Miyamoto *et al.*, 1969; Oonnithan and Casida, 1968; Shrivastava

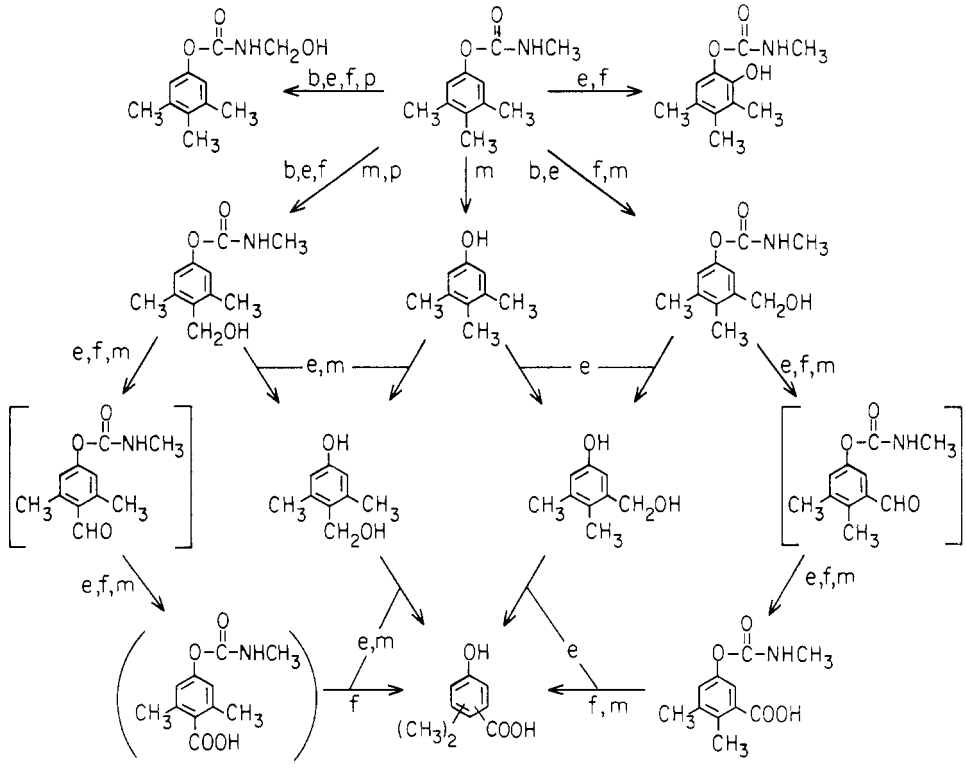

Figure 3. Tentative metabolic and degradation pathways for 3,4,5-trimethylphenyl methylcarbamate in (b) growing bean plants, (e) enzyme systems of mouse liver microsomes and housefly abdomens, (f) living houseflies, (m) living mice, and (p) on photodecomposition on bean foliage. The compounds containing phenolic or hydroxymethyl groupings appear predominantly as conjugates in (b), (f), and (m)

Structures are designated as follows: no bracket or parentheses—compound identified by cochromatography; bracket—intermediate consistent with reaction series; parentheses—compound not available as authentic chemical but possibly is represented among the unidentified metabolites based on its anticipated chromatographic position

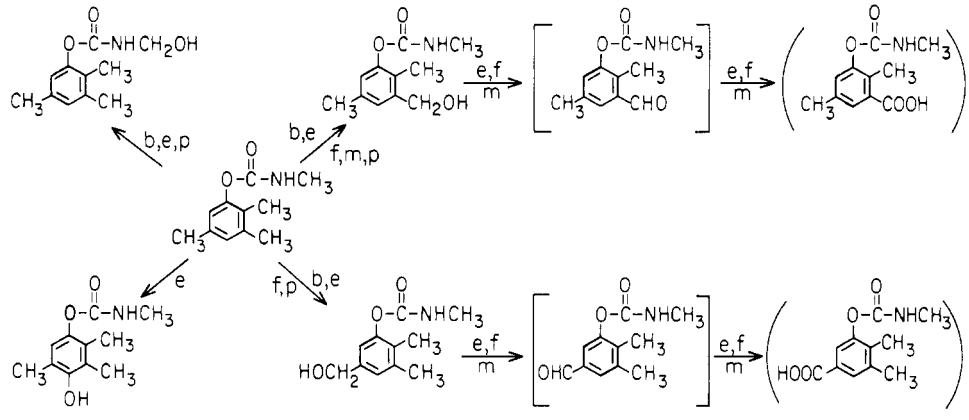

Figure 4. Tentative metabolic and degradation pathways for 2,3,5-trimethylphenyl methylcarbamate in (b) growing bean plants, (e) enzyme systems of mouse liver microsomes and housefly abdomens, (f) living houseflies, (m) living mice, and (p) on photodecomposition on bean foliage. The compounds containing hydroxymethyl groups appear predominantly as conjugates in (b), (f), and (m)

Products from hydrolysis of the carbamate grouping are not shown because the studies were made with a carbonyl-^{14}C preparation. Structures are designated as follows: no bracket or parentheses—compound identified by cochromatography; bracket—intermediate consistent with reaction series; parentheses—compound not available as authentic chemical but possibly is represented among the unidentified metabolites based on its anticipated chromatographic position

et al., 1969). One factor in the more extensive metabolism of the carbamates in the mouse liver than in the housefly mixed-function oxidase systems may be that the cytochrome P-450 concentration used was 20 times greater in the former than in the latter case.

Several products are categorized as unknowns because they remain to be characterized, particularly those in the mouse urine and the liver microsomal systems. Most of these are relatively polar carbamates (of low R_f values) which do not appear to be conjugates inasmuch as they are found in the microsome-NADPH system and are not cleaved by glusulase when products from the urine are examined. Possibly the carbamate metabolites near the origin which move about equally in the neutral and acidic solvent systems (metabolites B_1, B_2, and B_3 from 3,4,5-Me_3PMC and b_1, b_2, and b_3 from 2,3,5-Me_3PMC) contain two or more hydroxymethyl group-

ings; this may also be the case for the phenolic metabolite (C) from 3,4,5-Me_3PMC. Although certain of the possible metabolites shown in Figures 3 and 4 are not available in synthetic form, their chromatographic position can be anticipated, in part, by analogy between derivatives of 3,4,5-Me_3PMC and 2,3,5-Me_3PMC. Thus, the methylcarbamates containing one aldehyde or one carboxyl group should appear, with each isomer, in the general region associated with the authentic 2-CHO-3,5-Me_2PMC and 3-COOH-4,5-Me_2PMC, respectively. On the same basis, the metabolites of 2,3,5-Me_3PMC designated in Figure 2 as c and d chromatograph in the regions anticipated for 2-CH_2OH-3,5-Me_2PMC and 6-OH-2,3,5-Me_3PMC, respectively. Another possibility is that a methyl group may undergo, among other reactions, a shift in position in the ring or be replaced by a hydroxyl group on hydroxylation-induced migration (Udenfriend *et al.*, 1969), but there is no evidence that this actually occurs with the Landrin constituents. Although metabolites in mouse feces were not examined in detail, the patterns of metabolites extractable by water were almost the same as those noted in the urine, after the conjugates in the urine were treated with glusulase.

Identification of the metabolites from the two major constituents of Landrin insecticide has been useful in demonstrating the need for developing appropriate methods for the determination of residues, because the products in plants are not the compounds applied but rather are, for the most part, conjugates of methylcarbamate metabolites. Further, these studies establish that the trimethylphenyl methylcarbamate isomers examined are rapidly metabolized in insects and mammals.

ACKNOWLEDGMENT

The authors thank the following persons for their suggestions, assistance, and/or contribution of materials: J. L. Engel, E. C. Kimmel, L. C. Gaughan, M. H. Balba, P. E. Berteau, and Louis Lykken, Division of Entomology, University of California, Berkeley; A. C. Page, J. E. Loeffler, and J. G. Morales, Biological Sciences Research Center, Shell Development Co., Modesto, Calif.

LITERATURE CITED

Abdel-Wahab, A. M., Kuhr, R. J., Casida, J. E., J. AGR. FOOD CHEM. **14,** 290 (1966).
Auwers, K., *Chem. Ber.* **40,** 2524 (1907).
Balba, M. H., Casida, J. E., J. AGR. FOOD CHEM. **16,** 561 (1968).
Balba, M. H., Singer, M. S., Slade, M., Casida, J. E., J. AGR. FOOD CHEM. **16,** 821 (1968).
Baron, R. L., Doherty, J. D., J. AGR. FOOD CHEM. **15,** 830 (1967).
Casida, J. E., Lykken, L., *Ann. Rev. Plant Physiol.* **20,** 607 (1969).
Casida, J. E., Shrivastava, S. P., Esaac, E. G., *J. Econ. Entomol.* **61,** 1339 (1968).
Charlesworth, E. H., Levene, L., *Can. J. Chem.* **41,** 1071 (1963).
Divekar, P. V., Vining, L. C., *Can. J. Chem.* **42,** 63 (1964).
Dorough, H. W., *Bull. Environ. Contam. Toxicol.* **13,** 164 (1968).
Friedman, A. R., Lemin, A. J., J. AGR. FOOD CHEM. **15,** 642 (1967).
Hook, G. E. R., Smith, J. N., *Biochem. J.* **102,** 504 (1967).
Krishna, J. G., Dorough, H. W., Casida, J. E., J. AGR. FOOD CHEM. **10,** 462 (1962).
Krishna, J. G., Casida, J. E., J. AGR. FOOD CHEM. **14,** 98 (1966).
Kuhr, R. J., Casida, J. E., J. AGR. FOOD CHEM. **15,** 814 (1967).
Lykken, L., Casida, J. E., *Can. Med. Assoc. J.* **100,** 145 (1969).
Metcalf, R. L., Fukuto, T. R., Collins, C., Borck, K., Abd El-Aziz, S., Munoz, R., Cassil, C. C., J. AGR. FOOD CHEM. **16,** 300 (1968).
Miyamoto, J., Yamamoto, K., Matsumoto, T., *Agr. Biol. Chem.* **33,** 1060 (1969).
Oonnithan, E. S., Casida, J. E., J. AGR. FOOD CHEM. **16,** 28 (1968).
Shrivastava, S. P., Tsukamoto, M., Casida, J. E., *J. Econ. Entomol.* **62,** 483 (1969).
Tsukamoto, M., Casida, J. E., *Nature* **213,** 49 (1967).
Udenfriend, S., Daly, J. W., Guroff, G., Jerina, D. M., Zaltzman-Nirenberg, P., Witkop, B., In *Microsomes and Drug Oxidations*, 225–30 (Gillette, J. R., Conney, A. H., Cosmides, G. J., Estabrook, R. W., Fouts, J. R., Mannering, G. J., Ed., Academic Press, N.Y., 547 pp., 1969).

Received for review November 28, 1969. Accepted February 24, 1970. Study supported in part by grants from the following sources: the U.S. Public Health Service, National Institutes of Health (Grant GM-12248); the U.S. Atomic Energy Commission [Contract AT(11-1)-34, Project Agreement 113]; Shell Chemical Co., New York, N.Y.