Metabolism in Relation to Mode of Action of Methylenedioxyphenyl Synergists in Houseflies

Esaac G. Esaac[1] and John E. Casida

Each of the 10 C^{14}-labeled methylenedioxyphenyl (MDP) compounds studied is metabolized in living houseflies (*Musca domestica* L.) by one or more of the following mechanisms: oxidative attack at the methylene carbon resulting in scission of the MDP moiety; oxidative attack at the aliphatic side chain; conjugation of aromatic acid metabolites, resulting from side chain oxidation, as β-D-glucosides or as derivatives of various amino acids. Side chain oxidation occurs at the alcohol group of piperonyl alcohol, the aldehyde group of piperonal, the acetal group of tropital, each of the three aliphatic ether groups of piperonyl butoxide, the unsaturated side chain of safrole or isosafrole, and the sulfinyl group of sulfoxide-A and sulfoxide-B. Evidence from in vivo and in vitro investigations is presented for the hypothesis that the synergistic activity of MDP compounds results from competitive inhibition of the microsomal mixed-function oxidases responsible for insecticide detoxification.

Synergists are necessary adjuvants for pyrethrins to achieve efficient and economical insect control. They not only enhance and extend the toxicity of pyrethrins, but also increase the toxicity of many other types of insecticides, including carbamates, some organophosphates, and certain chlorinated hydrocarbons. Although many groups of organic compounds are represented among the synergists for insecticides, the most important synergists are methylenedioxyphenyl (MDP) or 1,3-benzodioxole compounds, including piperonyl butoxide, sulfoxide, and tropital, which are in commercial use as synergists for pyrethrins. Less complex natural MDP compounds and their derivatives, including dihydrosafrole, isosafrole, myristicin, and safrole, are also active synergists with some insecticides. Recently, there has been a resurgence of interest in the mode of action of synergists, including many studies on structure-activity relationships, effects on insecticide metabolism in living insects, and inhibition of the microsome-reduced nicotinamide adenine dinucleotide phosphate (NADPH) enzyme system.

The general effect of administering a MDP compound along with an insecticide (pyrethrins or carbamate) is similar to that of increasing the dose of the insecticide; for example, the signs of poisoning are intensified and the mortality is increased. At present, the most widely accepted hypothesis to explain the mode of action of MDP synergists is that they act by inhibiting the detoxication of the insecticide within the insect body (Casida, 1963, 1968; Casida *et al.*, 1966; Hewlett, 1968; Metcalf, 1967). MDP compounds apparently serve as alternative substrates for the mixed-function oxidase system of microsomes, and thereby reduce the rate of metabolism and prolong the action of certain drugs and insecticides (Casida *et al.*, 1966). This hypothesis is based on the finding that a large variety of insecticides are metabolized, in both insects and mammals, by enzymes similar to those involved in metabolism of MDP synergists. Alternative hypotheses involve inhibition of insect tyrosinases responsible for detoxification of carbamate insecticides (Metcalf *et al.*, 1966), and reaction of the electrophilic benzodioxolium ion, resulting from transfer of hydride from the MDP group during oxidative metabolism, with a nucleophilic group in the active site of the pesticide-metabolizing enzyme (Hennessy, 1965).

The mode of action of the MDP synergists possibly is related to the manner in which these compounds are metabolized in insects. Some information exists concerning the metabolism of synergists by insects as determined, among other ways, by gas chromatographic (Wilkinson, 1967) and radiotracer techniques (Schmidt and Dahm, 1956). The MDP group is important for high synergistic activity (Hewlett, 1960; Metcalf, 1967); studies with a radiolabel on the methylene position in the MDP moiety are indicated. On the basis of such studies, made with a series of methylene-C^{14}-dioxyphenyl compounds prepared for use in metabolism studies (Kuwatsuka and Casida, 1965), it is known that the benzodioxole ring is cleaved in houseflies to liberate $C^{14}O_2$ (Casida *et al.*, 1966), that a portion of the injected dose of dihydrosafrole, myristicin, and safrole is volatilized from flies in an unmetabolized form (Casida *et al.*, 1968), and that piperonal, piperonyl alcohol, piperonylic acid, safrole, and tropital are excreted as piperonylic acid conjugates with alanine, glutamine, glutamic acid, glycine, and serine (Esaac and Casida, 1968).

This paper gives the details of work done on the metabolism of synergists in houseflies, utilizing 10 C^{14}-labeled MDP compounds and involving balance studies on expired $C^{14}O_2$, other volatile products, and excreted metabolites. It includes tentative characterization of certain of the excreted metabolites, with particular emphasis on piperonyl butoxide, and deals with the effect of chemicals other than MDP compounds, in unlabeled form, on the metabolism of the MDP synergist, in labeled form, using several combinations of such materials, and utilizing both resistant and susceptible strains of houseflies. Finally, it relates the metabolism results to the possible mode-of-action of synergists in houseflies.

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

[1] Permanent address, Central Agricultural Pesticides Laboratory, Plant Protection Department, Ministry of Agriculture, Dokki, Gizza, Egypt, United Arab Republic

Chromatography. Techniques used for thin-layer chromatography (TLC) and detection of labeled and unlabeled products in cochromatography studies were generally those previously reported (Esaac and Casida, 1968; Kuwatsuka and Casida, 1965). The following chromogenic agents were used to detect the indicated types of compounds: chromotropic acid for MDP compounds (Beroza, 1963); *p*-anisidine hydrochloride for glucoside derivatives (Hough *et al.*, 1950); aqueous ferric chloride (1%) followed by aqueous potassium ferricyanide (1%) solutions for certain catechol (XI) and alcohol (XV-XVII) derivatives, as discussed below. Unless specifically stated otherwise, the solvent systems used were as follows: *a* = benzene (saturated with formic acid)–ether mixture (1 to 3); *b* = ether–hexane (saturated with ammonium hydroxide) mixture (1 to 1); *c* = ether–hexane mixture (3 to 1); *d* = benzene–methanol mixture (5 to 1); *e* = *n*-butanol–acetic acid–water mixture (3:1:1); *f* = chloroform–methanol mixture (100 to 1).

Chemicals. Certain of the MDP compounds used are listed in the following table.

The methylene-C^{14}-dioxyphenyl (M-C^{14}-DP) compounds were obtained from three sources: Shozo Kuwatsuka for I, II, V, and VII-IX (Kuwatsuka and Casida, 1965); McLaughlin Gormley King Co., Minneapolis, Minn., for III and X; F. X. Kamienski, Division of Entomology, University of California, Berkeley, for IV and VI. An α-methylene-C^{14} sample of piperonyl butoxide (piperonyl butoxide-α-C^{14}) (V) was provided by New England Nuclear Corp., Boston, Mass. Each labeled compound, with the exception of tropital, had a radiochemical purity of 99% or better and a specific activity of 1 mc. per mmole or higher; where required, the specific activity was adjusted to 1 mc. per mmole with pure, unlabeled compound. Tropital-C^{14}, used for injection into houseflies, had a radiochemical purity of 95%; the major impurity was piperonal-C^{14}, which constituted 3 to 4%. For in vitro studies, the tropital-C^{14} used was 91% pure, containing 6% piperonal-C^{14}. Radiochemical purity was determined using TLC and scintillation counting of radioactive gel regions scraped from the plates.

Other chemicals used and their sources were as follows: natural myristicin (II) from E. P. Lichtenstein (Lichtenstein and Casida, 1963); piperonyl butoxide catechol (XI), 1,2-methylenedioxy-4-[2-(octylsulfonyl)-propyl]benzene, and 4,5-methylenedioxy-2-propylacetophenone from Shozo Kuwatsuka; XII from F. X. Kamienski; MGK 264 (*N*-octyl bicycloheptene dicarboximide) and pyrethrum from McLaughlin Gormley King Co.; β-glucosidase (almond emulsin, B-grade, 500 EU per mg.) and bovine serum albumin (Fraction V, B-grade) (BSA) from Calbiochem, Los Angeles, Calif.

Synthesis of Potential Metabolites of Piperonyl Butoxide and Their Derivatives. Several derivatives were prepared from 6-propylpiperonylic acid (XIII), and 4,5-methylenedioxy-2-propylbenzyl chloride for use in characterization studies. Each of the products was a single material, based on TLC. Melting points were determined by observing single crystals under a microscope, the specimen being heated between two microscope cover slips on a hot block; the block was checked by reference compounds with known melting points. Infrared spectra were determined with a Beckman IR-4 spectrophotometer. Elemental analyses and mass spectral determinations were performed by the Department of Chemistry, University of California, Berkeley.

No.	Designation	Chemical Name
	C^{14}-Labeled MDP Compounds	
I	Dihydrosafrole	1,2-Methylenedioxy-4-propylbenzene
II	Myristicin	5-Allyl-1-methoxy-2,3-methylenedioxybenzene
III	Piperonal	3,4-Methylenedioxybenzaldehyde
IV	Piperonyl alcohol	3,4-Methylenedioxybenzyl alcohol
V	Piperonyl butoxide	α-[2-(2-Butoxyethoxy)ethoxy]-4,5-methylenedioxy-2-propyltoluene
VI	Piperonylic acid	3,4-Methylenedioxybenzoic acid
VII	Safrole	4-Allyl-1,2-methylenedioxybenzene
VIII	Sulfoxide-A (isomer)	1,2-Methylenedioxy-4-[2-(octylsulfinyl)propyl]-benzene
IX	Sulfoxide-B (isomer)	Ditto: sulfoxide-B is a diastereoisomer of sulfoxide-A about the sulfoxide grouping, and the asymmetric carbon of the propyl grouping
X	Tropital	Piperonal bis-[2-(2-butoxyethoxy)ethyl]-acetal
	Potential Metabolites of Piperonyl Butoxide and Their Derivatives	
XI	Piperonyl butoxide catechol	α-[2-(2-Butoxyethoxy)ethoxy]-4,5-dihydroxy-2-propyltoluene
XII	Glycine conjugate of 6-propylpiperonylic acid	*N*-(4,5-Methylenedioxy-2-propylbenzoyl)glycine
XIII	6-Propylpiperonylic acid	4,5-Methylenedioxy-2-propylbenzoic acid
XIV	Glucoside of 6-propylpiperonylic acid	1-*O*-(4,5-Methylenedioxy-2-propylbenzoyl)-β-D-glucopyranose
XV	6-Propylpiperonyl alcohol	4,5-Methylenedioxy-2-propylbenzyl alcohol
XVI		α-(2-Hydroxyethoxy)-4,5-methylenedioxy-2-propyltoluene
XVII		α-[2-(2-Hydroxyethoxy)-ethoxy]-4,5-methylenedioxy-2-propyltoluene
XVIII	Methylcarbamate of XV	4,5-Methylenedioxy-2-propylbenzyl methylcarbamate
XIX	Methylcarbamate of XVI	α-(2-Methylcarbamoyloxyethoxy)-4,5-methylenedioxy-2-propyltoluene
XX	Methylcarbamate of XVII	α-[2-(2-Methylcarbamoyloxyethoxy)ethoxy]-4,5-methylenedioxy-2-propyltoluene

6-Propylpiperonylic acid (XIII) was prepared, in 98% yield, by potassium hypobromide oxidation of 4,5-methylenedioxy-2-propylacetophenone, according to the procedure used by Cram and Allinger (1955) for oxidation of 4-acetyl[2.2]paracyclophane. Recrystallization of the product (XIII) from 30% acetic acid gave white needles, m.p. 140–2° C. [reported, 139–41°

C., Koelsch (1946)]. Elemental analysis: calculated, C 63.45, H 5.81; found, C 63.29, H 5.88.

6-Propylpiperonylic acid (XIII) was converted, via the silver salt and 2,3,4,6-tetra-acetyl-1-*O*-(4,5-methylenedioxy-2-propylbenzoyl)-β-D-glucopyranose, to the glucoside of 6-propylpiperonylic acid (XIV). Aqueous 0.1*M* silver nitrate was added to 1 gram of XIII dissolved in dilute aqueous ammonium hydroxide solution until no further precipitate formed, and the mixture was stirred at 25° C. in the dark for 1 hour. The precipitate was filtered off and washed, successively, with water, acetone, and ether, and dried over phosphorus pentoxide. A suspension of this silver salt in 100 ml. of dry benzene containing 2.2 grams of 2,3,4,6-tetra-acetyl-α-D-glucosyl bromide (prepared according to Hickinbottom, 1962; m.p. 86° C., reported 88° C.) was refluxed, with stirring, for 2 hours. After filtering off the silver bromide, the filtrate was evaporated to 25 ml. Ten milliliters of hexane were added to obtain the crystalline tetra-acetyl glucoside (m.p. 110-2° C., R_f = 0.8 in benzene-methanol mixture (7 to 1) and 0.95 in solvent system *a*, 33% yield from XIII). Elemental analysis: calculated, C 55.76, H 5.62; found, C 55.61, H 5.82. The tetra-acetyl glucoside (200 mg.) in 25 ml. of dry methanol was cleaved to the glucoside (XIV) by saturating the methanol with dry ammonia gas and holding at 5° C. for 3 hours, followed by evaporation of the clear, colorless solution to dryness and crystallization of the residue from ethyl acetate [m.p. 162-5° C. (decomposed 159-60° C.), 21% yield from XIII]; the product appeared to be the hydrate of the β-D-glucoside. Elemental analysis: calculated for $C_{17}H_{22}O_9 \cdot H_2O$, C 52.58, H 6.23; found, C 52.86, H 6.30.

4,5-Methylenedioxy-2-propylbenzyl chloride (b.p. 96° C. at 0.04 mm., FMC Corp., New York, N. Y.) was converted in 95 to 98% yield to each of three alcohols (XV, XVI, and XVII). Compound XV was made by the procedure of Barthel and Alexander (1957), involving reaction of the benzyl chloride with potassium acetate in acetic acid, followed by saponification of the acetate with sodium hydroxide in methanol, and distillation (b.p. 114-5° C. at 0.03 mm.). Elemental analysis: calculated, C 68.02, H 7.27; found, C 68.40, H 7.66. Compounds XVI and XVII were prepared by reacting 0.1 gram atom of sodium with 0.4 mole of the glycol (ethyleneglycol or diethyleneglycol), addition of 0.1 mole of the benzyl chloride at 25° C., and stirring at 25° C. for 2 hours. The products were recovered and isolated by adding 50 ml. of water, extracting with ether, washing the ether extract, in sequence, with 5% sodium hydroxide, 2% hydrochloric acid, and water, drying the ether over sodium sulfate, evaporating the ether, and distilling the residue (b.p. 138° C. at 0.03 mm. for XVI, and 164° C. at 0.04 mm. for XVII). Elemental analysis calculated for XVI: C 65.53, H 7.61; found, C 65.31, H 7.70. Elemental analysis calculated for XVII: C 63.81, H 7.85; found, C 63.34, H 8.00. Each of the three alcohols showed appropriate infrared absorption bands at 927 cm.$^{-1}$ (MDP group), 1040 and 1250 cm.$^{-1}$ (ether linkages), and 3400 cm.$^{-1}$ (hydroxyl stretching); molecular ion peaks in the mass spectra are 238 for compound XVI and 282 for compound XVII, as compared to calculated molecular weights of 238 and 282, respectively.

Each of the three alcohols (XV, XVI, and XVII), in an amount of 0.5 gram, was dissolved in 2 ml. of methyl isocyanate containing 20 mg. of triethylamine. The reaction mixture was held at 25° C. for 24 hours, and volatile materials (mostly residual methyl isocyanate) were evaporated. Methylcarbamates XVIII (m.p. 63-5° C.) and XIX (m.p. 49-51° C.) were recrystallized from ether-hexane mixture and methylcarbamate XX was purified on a Florisil column, using ether-hexane mixtures for elution. The yields ranged from 75 to 88%. Elemental analysis calculated for XVIII, C 62.14, H 6.82, N 5.57; found, C 61.91, H 6.89, N 5.79. Elemental analysis calculated for XIX, C 61.00, H 7.17, N 4.74; found, C 60.94, H 7.24, N 4.62. Elemental analysis calculated for XX, C 60.16, H 7.42, N 4.13; found, C 59.84, H 7.41, N 4.55. The R_f values for the methylcarbamates are almost the same as for the alcohols from which they are derived, when solvent systems *c* and *d* are used. However, they are resolved from the alcohols in solvent system *f*, in which case the methylcarbamates give R_f values as follows: 0.87, 0.63, and 0.48 for compounds XVIII, XIX and XX, respectively.

Houseflies and Metabolism Unit. Five housefly (*Musca domestica* L.) strains were used. Two of these strains, the R_{Hokota} and R_{Baygon}, are resistant to carbamate insecticide chemicals and are described by Shrivastava (1967), Tsukamoto *et al.* (1968), and Shrivastava *et al.* (1969), along with the SCR and SCRS susceptible strains. The $SCRS_{DHS}$ strain resulted from larval selection of the SCRS strain with dihydrosafrole. Conditions for rearing and holding the flies during the experimental period were those described by Shrivastava (1967), Tsukamoto *et al.* (1968), and Shrivastava *et al.* (1969). The metabolism unit was that described by Casida *et al.* (1968).

Treatment of Living Houseflies, and Analysis of Flies and Excreta. Female houseflies were individually injected with 1 μg. of C^{14}-labeled synergist in 0.5 μl. of water-acetone mixture (3 to 2), with an equivalent amount of formaldehyde-C^{14}, sodium formate-C^{14}, or sodium carbonate-C^{14} in 1.0 μl. of aqueous solution, or with 2.3 μg. of unlabeled safrole, isosafrole, piperonylic acid, or 6-propylpiperonylic acid in 0.5 μl. of water-acetone mixture (Casida *et al.*, 1968; Esaac and Casida, 1968). In certain studies on the metabolism of M-C^{14}-DP-labeled tropital and piperonyl butoxide, the flies were pretreated with a nonradioactive test chemical [MGK 264 (5 μg.), dimethyl *p*-nitrophenyl carbamate (DpNC) (5 μg.), or carbaryl (1 μg.)] by topical application of the test compound in acetone solution to the pronotum 1.5 hours before proceeding with injection of the radioactive compound and subsequent analyses.

Fly excreta were fractionated into water-soluble and ether-soluble fractions, and the metabolites in the ether-soluble fractions were analyzed by TLC as previously reported (Esaac and Casida, 1968). The conditions for alkaline- and β-glucosidase-hydrolysis of the water-soluble and ether-soluble metabolite fractions prior to the use of TLC for tentative identification of labeled products were generally the same as those used before (Esaac and Casida, 1968; Kuhr and Casida, 1967). The enzymatic hydrolysis involved 4 hours'

incubation, with shaking, at 37° C., with or without 10 mg. of β-glucosidase in 5 ml. of citrate-phosphate buffer at pH 4.8.

The identity of the labeled products in treated flies was determined only in the case of sulfoxide-A and sulfoxide-B, where 0.2 μl. of acetone was the injection vehicle. After an appropriate time interval following the injection, each group of 10 flies was homogenized in 5 ml. of acetone at 5° C., and the insoluble residue was reextracted twice with 5-ml. portions of acetone. The acetone-soluble materials were analyzed by TLC.

The radiocarbon content of each sample from the studies with living flies was determined as previously reported (Casida *et al.*, 1968; Esaac and Casida, 1968). Three replicates of three flies each were combusted immediately after injection, and the average of these results was used to calculate the radiocarbon content of the 10 flies originally in the group. In a similar manner, flies were combusted 24 hours after injection to determine the residual radiocarbon in the flies at the termination of the experiment. In one series of experiments, determinations were made of the radiocarbon distribution into volatile substances, excreted products, and residual materials in the fly. In other studies, the excreta were homogenized in water, the radiocarbon content was determined, using two 0.1-ml. aliquots, and the remainder of the homogenate was used for extraction with ether and resolution of metabolites by TLC. The results, for all fractions, including volatile, excreted, and residual products, were calculated in terms of per cent of the injected radiocarbon, and the average of two or more separate experiments was used in the reported results.

Metabolism by Housefly Enzymes. Studies on the metabolism of M-C^{14}-DP compounds by fly abdomen homogenates or the microsome fraction from fly abdomen homogenates were made according to the procedure of Tsukamoto and Casida (1967a, 1967b). Each incubation mixture consisted of the whole homogenate of 10 fly abdomens or the microsome fraction from 20 fly abdomens, approximately 0.1 μmole of labeled synergist, 5 μmoles of NADPH, and 2 ml. of 0.25*M* sucrose–0.15*M* phosphate solution, pH 7.5. After incubation for 2 hours at 30° C. in air with shaking, each reaction mixture was extracted three times with 7-ml. portions of ether and the C^{14}-labeled compounds recovered in the ether were analyzed by TLC. In one study, involving tropital-C^{14} as the substrate, BSA was used at 1% (w./v.) concentration in enzyme preparation and incubation; other reaction constituents and the analysis procedure were not altered. In another study, the protein-bound radiocarbon was determined by precipitation with trichloroacetic acid and washing the precipitate with acetone prior to combustion analysis by the procedure of Oonnithan and Casida (1968). Results for the different fractions were calculated in terms of the per cent of the total radiocarbon found in the reaction mixture prior to incubation by scintillation counting of two 0.1-ml. aliquots. The loss was calculated as the amount of radiocarbon unaccounted for after the following steps: extraction step with tropital; incubation, extraction, and analysis steps with sulfoxide-A, sulfoxide-B, and piperonyl butoxide; incubation step only with the other labeled compounds.

RESULTS

Relative Activity of MDP Compounds as Pyrethrum and Carbaryl Synergists. Kuwatsuka and Casida (1965) give the synergistic activity of many of the MDP compounds studied on carbaryl and pyrethrum toxicity to female SCR flies following topical application of 10 μg. of synergist per fly. Subsequently, the studies of Esaac (1967) show that, under the same conditions of topical synergist application, tropital is equal to piperonyl butoxide as a synergist for carbaryl, and is even more potent than piperonyl butoxide for pyrethrum synergism. In the following six cases, a 1.0-μg. dose of injected synergist is equally effective or more effective than a 10-μg. dose of topically-applied synergist: each of dihydrosafrole, isosafrole, piperonyl butoxide, and safrole with carbaryl; each of piperonyl butoxide and sulfoxide-B with pyrethrum. For this reason and for greater precision in quantitation of metabolism rates, the metabolism studies reported here utilize a 1.0-μg. injected synergist dose. The duration of the synergist metabolism studies is based, partially, on the finding that the synergistic activity of injected safrole for carbaryl toxicity and of injected sulfoxide-B or piperonyl butoxide for pyrethrum toxicity does not diminish with periods of up to 4 hours between synergist injection and topical application of the insecticide; however, the synergist is much less effective when the delay between synergist injection and topical application of the insecticide is 24 hours. Thus, there is evidence that the MDP compound persists, as such or as an effective metabolite, to yield maximum synergism for at least 4 hours after injection (Esaac, 1967).

Elimination Rate of Radiocarbon from Houseflies Injected with C^{14}-Labeled Methylenedioxyphenyl and One-Carbon Compounds. Table I and Figures 1 and 2 show the extent and rate, respectively, of radiocarbon elimination, by various routes, following injection of

Table I. Distribution of Radiocarbon 24 Hours After Injection of Female SCR Flies with Various C^{14}-Labeled Methylenedioxyphenyl and One-Carbon Compounds

C^{14}-Labeled Compound	Amount of Radiocarbon Recovered,[a] % Volatile Products: $C^{14}O_2$	Volatile Products: Other Products	Excreta	Residue in fly	Total
Methylene-C^{14}-dioxyphenyl Compounds					
Dihydrosafrole	12	51	6	16	85
Myristicin	13	21	8	50	92
Piperonal	1	0	83	4	88
Piperonyl alcohol	4	0	76	22	102
Piperonyl butoxide	11	0	69	15	95
Piperonylic acid	1	0	91	5	97
Safrole	7	65	8	12	92
Sulfoxide-A	19	0	55	25	99
Sulfoxide-B	20	0	35	41	96
Tropital	19	0	62	18	99
Other C^{14}-Labeled Preparations					
Piperonyl butoxide-α-C^{14}	1	0	81	19	101
Formaldehyde	43	0	15	19	77
Sodium carbonate	87	0	2	1	90
Sodium formate	47	0	7	26	80

[a] Average of three to six experiments.

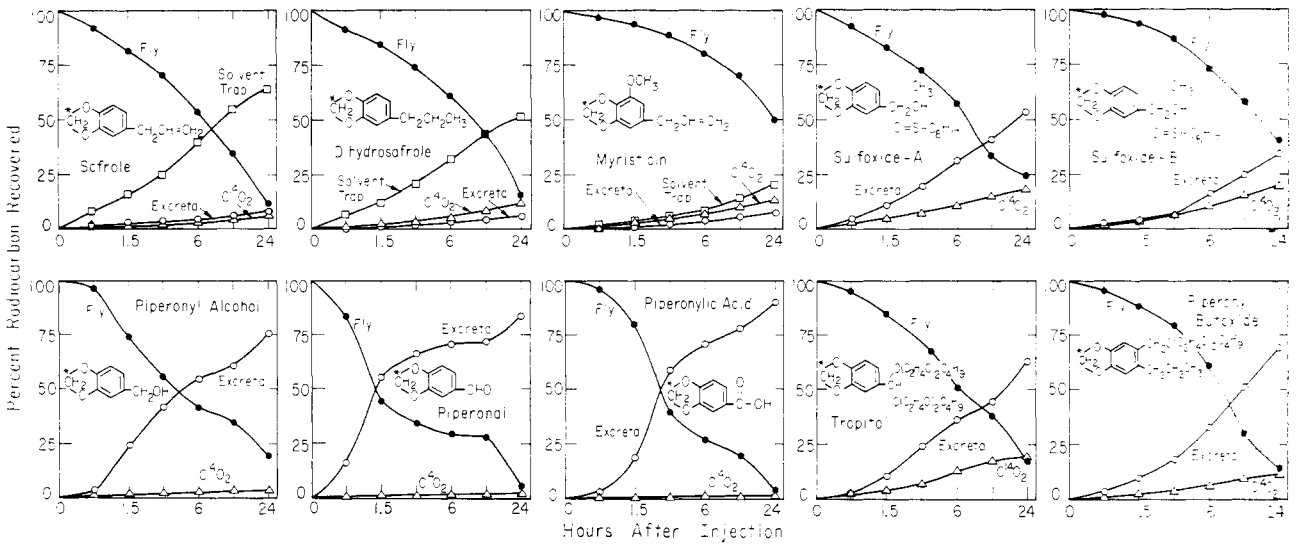

Figure 1. Rate of elimination of radiocarbon from female SCR houseflies injected with each of 10 methylene-C^{14}-dioxyphenyl compounds

female SCR flies with each of 10 M-C^{14}-DP compounds and with piperonyl butoxide-α-C^{14}, sodium carbonate-C^{14}, sodium formate-C^{14}, and formaldehyde-C^{14}. The data for the three latter compounds are important because these compounds are possible intermediates in the metabolism of the M-C^{14}-DP synergists to yield $C^{14}O_2$.

In general, the radiocarbon from injection of M-C^{14}-DP synergists is eliminated less rapidly from flies than that from some of the one-carbon compounds (carbonate and formaldehyde) and from certain potential intermediate metabolites (piperonylic acid, piperonal, and piperonyl alcohol). The one-carbon compounds are primarily metabolized to and expired as $C^{14}O_2$. In the case of sodium formate-C^{14} and formaldehyde-C^{14}, the greater part of the radiocarbon which does not appear as $C^{14}O_2$ persists in the fly. Appreciable amounts of $C^{14}O_2$ are also produced from several M-C^{14}-DP compounds, but not from piperonal, piperonyl alcohol, or piperonylic acid, which are rapidly converted to conjugates and excreted (Esaac and Casida, 1968). Piperonyl butoxide-α-C^{14} yields essentially no $C^{14}O_2$. Loss of safrole, dihydrosafrole, and myristicin by direct volatilization from flies as the unmetabolized compounds (Figure 1—"solvent trap" curve; Casida *et al.*, 1968) results in smaller amounts persisting in the fly for potential excretion or conversion to $C^{14}O_2$. While high retention of radiocarbon in the body occurs with sulfoxide-B and myristicin, the excreta contains the highest proportion of the injected dose with sulfoxide-A, piperonyl butoxide (M-C^{14}-DP and α-C^{14}), and tropital. The elimination route differs with each compound because the mechanisms involve varying proportions of direct volatilization in an unmetabolized form and formation of a variety of metabolites of different chemical types (Casida *et al.*, 1968; Esaac and Casida, 1968).

The amount of expired $C^{14}O_2$ and the residual radiocarbon in SCR and R_{Hokota} flies following tropital-C^{14} injection decreases, whereas the excreted radiocarbon increases as a result of pretreatment with the pyrethrum synergist, MGK 264, or the carbamate, DpNC (Figure 3). The effect of carbaryl on tropital metabolism in the SCR flies is to reduce excretion and increase $C^{14}O_2$

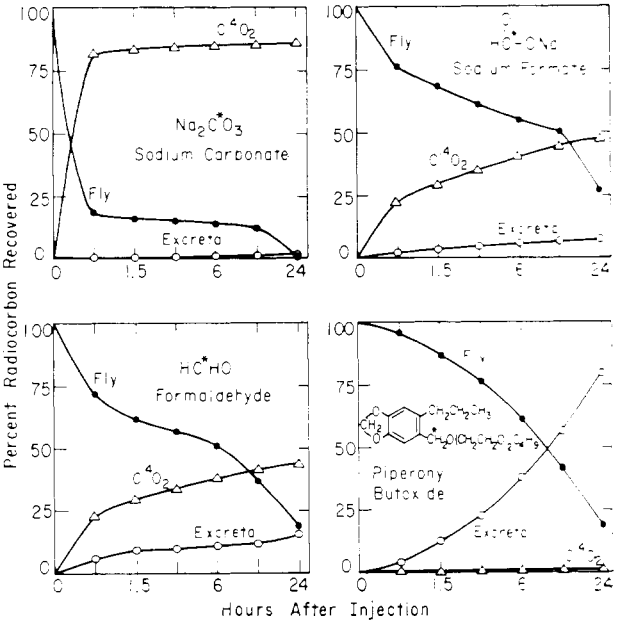

Figure 2. Rate of elimination of radiocarbon from female SCR houseflies injected with sodium carbonate-C^{14}, sodium formate-C^{14}, formaldehyde-C^{14}, or piperonyl butoxide-α-C^{14}

production and C^{14} retention in the body; in considering these results, it is necessary to note that 30 to 70% of the flies suffered initial "knockdown" for 1 to 2 minutes from the toxicant and 50% of the flies were dead at the end of the experiment. In the case of piperonyl butoxide-M-C^{14}-DP, the amount of radioactivity excreted is decreased, the amount of $C^{14}O_2$ expired is not changed and, as a result, residual radiocarbon in the fly is increased; however, combinations of piperonyl butoxide and MGK 264 or DpNC are slightly toxic to the flies.

The elimination rates of tropital are not greatly different between female flies of the SCR and R_{Hokota} strains (Figure 3). Excreta from tropital-treated flies contain the same ether-soluble metabolites when either the SCR or the R_{Hokota} strain is used, after pretreatment with the various unlabeled compounds. In each case,

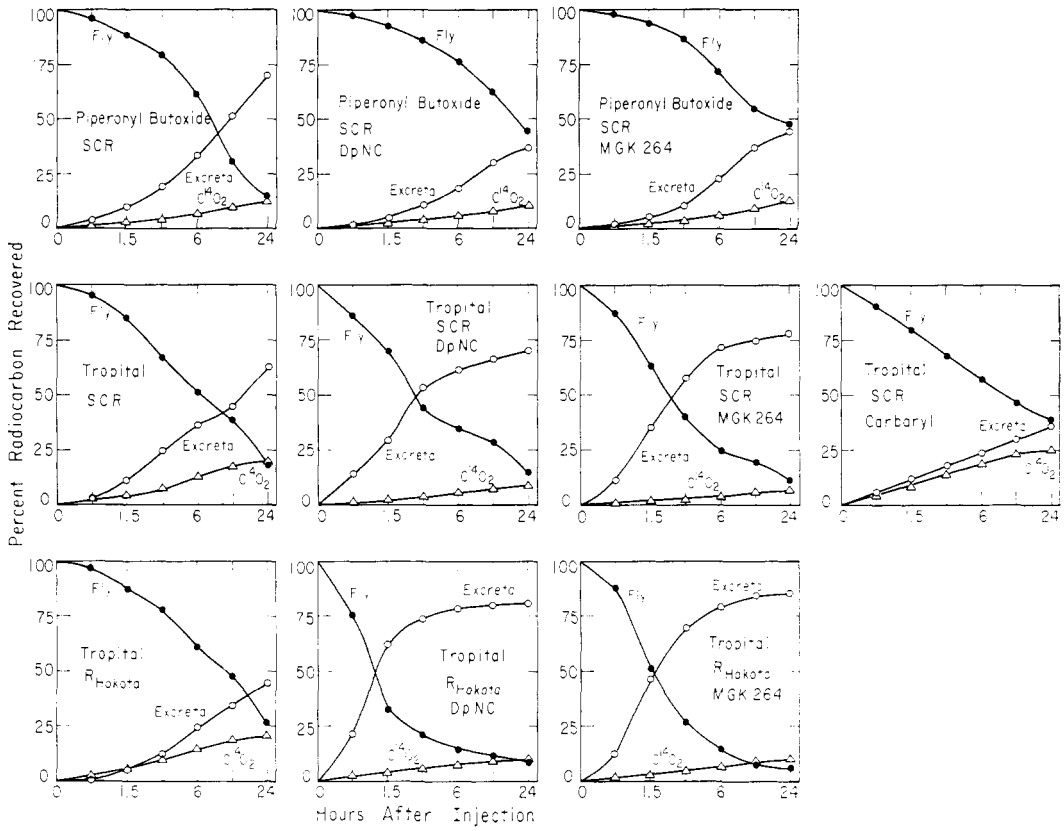

Figure 3. Rate of elimination of radiocarbon from female SCR or R_{Hokota} houseflies injected with tropital-M-C^{14}-DP or piperonyl butoxide-M-C^{14}-DP following pretreatment with other chemicals

5 *N*-piperonyl amino acids form (Esaac and Casida, 1968) and the use of pretreatment compounds increases the amount of each metabolite as a result of an over-all increase in excreted radioactivity.

Chemical Nature of Sulfoxide Metabolites. In houseflies, sulfoxide-A and sulfoxide-B convert to the corresponding sulfone and to two or three unidentified metabolites which appear in the acetone extract of the flies (Table II). With the use of ether as the developer for TLC, the R_f values are as follows: unidentified metabolites, 0.0 to 0.1; sulfoxide-B, 0.45; sulfoxide-A, 0.53; sulfone, 0.95. In this study, the unrecovered radiocarbon appears as volatile or excreted products, or is not removed on acetone extraction of the flies. Although the amount of sulfone found with sulfoxide-A and sulfoxide-B does not vary greatly at various times after injection, the rate of metabolism of sulfoxide-A is greater than that of sulfoxide-B, and the amounts of unidentified acetone-soluble metabolites tend to be greater with sulfoxide-B. Excreted metabolites of sulfoxide-B-C^{14} comprise, in relation to the injected radiocarbon, 6% water-soluble products and 29% ether-soluble products; the sulfone constitutes about half of the radiocarbon amount of the ether-soluble metabolites, as determined with ether as the developer for TLC, and 8 to 11 additional products make up the remainder, as determined with solvent system *a*.

The rate of sulfoxide-A destruction is greater with the R_{Baygon} resistant strain than with "insecticide-susceptible" strains, but the sulfone content formed and accumulated in the flies is greater in the strains other than the R_{Baygon} strain (Table III). The rate of destruction of sulfoxide-A is remarkably less in flies selected with dihydrosafrole ($SCRS_{DHS}$) than in other strains.

Table II. Rate of Metabolism and/or Elimination of Sulfoxide-A-C^{14} (A) and Sulfoxide-B-C^{14} (B) Following Injection into Female SCR Flies

Time after Injection, Hours	Isomer	Amount of Radiocarbon Recovered from Flies as Acetone-Soluble Products,[a] %		
		Original sulfoxide	Sulfone	Unidentified products
0	*A*	97.0	1.0	2.0
	B	96.4	0.8	2.8
0.25	*A*	80.5	4.4	3.5
	B	67.2	5.2	7.1
1	*A*	46.7	3.3	4.3
	B	63.7	3.6	5.4
4	*A*	41.6	6.4	5.3
	B	55.9	6.0	7.3
12	*A*	26.2	4.4	3.4
	B	37.7	6.3	4.6
24	*A*	10.1	5.4	2.5
	B	25.9	5.8	6.8

[a] Average of two experiments.

Chemical Nature of Metabolites of Tropital, Piperonal, Dihydrosafrole, Isosafrole, and Safrole. The chemical nature of the metabolites excreted by houseflies injected with each of piperonal, piperonyl alcohol, piperonylic acid, tropital, and safrole is given in a previous publication (Esaac and Casida, 1968). The present study gives some additional information on certain of these compounds, but most of the further studies concern dihydrosafrole and isosafrole.

Tropital and piperonal are metabolized to and excreted as a series of amino acid conjugates of piperonylic acid, which appear in the ether fraction on extraction of excreta and which amount to 41 and 51%, respectively, of the injected doses. Tropital yields 12% and piperonal 17% of the injected radiocarbon as excreted products which stay in the water fraction on extraction with ether (Esaac and Casida, 1968). In each case, heating of the water fraction with sodium hydroxide results in cleavage of 85 to 95% of the labeled products to release only piperonylic acid and piperonyl alcohol, the former product being the major one. The β-glucoside of piperonyl alcohol appears to be one of the constituents of the water fraction because incubation of the latter with β-glucosidase, but not without, releases 6% of the radiocarbon as piperonyl alcohol. The β-glucoside of piperonylic acid apparently is not a major constituent in the water fraction because incubation of this fraction with β-glucosidase does not release any piperonylic acid; the nature of the piperonylic acid conjugate(s) in the aqueous fraction is not known.

The isosafrole and safrole metabolism studies involve injection of unlabeled materials into houseflies and detection of excreted metabolites with the chromotropic acid reagent, only. In each case, houseflies excrete three major metabolites having R_f values corresponding to those of *N*-piperonyl glycine, *N*-piperonyl serine, and *N*-piperonyl glutamate; these unlabeled metabolites cochromatograph, in solvent system *a*, with the labeled metabolites excreted from flies injected with piperonal-C^{14} (see also Esaac and Casida, 1968). Thus, the allyl and propenyl groups are oxidized to the carboxylic acid group which, prior to excretion, is conjugated with amino acids.

Dihydrosafrole-C^{14} yields 3% of the injected radiocarbon in the water fraction and 2% in the ether fraction of the excreta; the ether fraction consists of two radioactive products which have R_f values, in solvent system *a*, of 0.00 and 0.85. The radioactive product(s) at the origin are of unknown nature, while that occurring at R_f 0.85 cochromatographs with unlabeled sesamol: the identity of this product with sesamol is not definitely established [although it also cochromatographs with sesamol, R_f 0.88, in hexane–ether–formic acid mixture (25:25:1)] because of the small amount present and the limited number of analogs available for use in cochromatographic comparisons.

Identity of Piperonyl Butoxide Metabolites. Piperonyl butoxide is metabolized to products which are excreted to the extent of 69 and 81% with the M-C^{14}-DP and α-C^{14} labels, respectively (Table IV). The difference between the two C^{14}-labeled preparations in the amount of radiocarbon excreted (12%) approximates the loss from the M-C^{14}-DP sample as $C^{14}O_2$ (11%), suggesting that most of the excreted products are MDP compounds, but a portion consists of a catechol derivative(s). Of the excreted radiocarbon, the major part is ether-soluble, accounting for 65% of the dose with the M-C^{14}-DP and 72% of the dose with the α-C^{14} samples (Table IV).

Resolution of the ether-soluble metabolites excreted from flies injected with piperonyl butoxide is illustrated in Figure 4. Product I appears to be a small amount of the original compound excreted in unmetabolized form. Metabolite F is detected only with the α-C^{14} preparation and, thus, probably is a catechol derivative, whereas all other metabolites (A-E, G, and H) appear with both labeled samples and, therefore, are MDP compounds. Metabolite C is a mixture of three compounds (Figure 4, Table IV), and metabolite A resolves in solvent system *e* into one major (R_f 0.46) and two minor products (R_f 0.55 and 0.65); both the major and minor components of metabolites A and C are detected with both labeled preparations. This means that the excreted metabolites consist of 11 MDP compounds and one catechol derivative: each of these metabolites constitute greater than 1% of the injected dose. When

Table III. Rate of Metabolism and/or Elimination of Sulfoxide-A-C^{14} Following Injection into Female Flies of Four Different Strains

Fly Strain	Time after Injection, Hours	Amount of Radiocarbon Recovered from Flies as Acetone-Soluble Products,[a] %: Original sulfoxide	Sulfone	Unidentified products
SCR	0	97.0	1.0	2.0
	1	51.3	4.5	4.5
	24	13.5	6.4	3.0
$SCRS_{DHS}$	0	97.0	1.0	2.0
	1	82.2	3.6	5.5
	24	55.0	6.7	5.4
SCRS	0	97.0	1.0	2.0
	1	80.2	5.5	6.5
	24	11.3	1.2	2.0
R_{Baygon}	0	97.0	1.0	2.0
	1	47.7	1.8	10.1
	24	6.9	1.6	3.2

[a] Average of two experiments.

Table IV. Labeled Metabolites Excreted from Female SCR Houseflies Injected with Piperonyl Butoxide-M-C^{14}-DP and Piperonyl Butoxide-α-C^{14}

Metabolite or Fraction	Amount of Radiocarbon Recovered,[a] %: M-C^{14}-DP	α-C^{14}	TLC R_f Values for Indicated Solvent System: *a*	*b*	*c*	*d*	*e*	*f*
Excreta-ether fraction[b]								
A	13	14	0.00	0.00	0.00	0.00	0.46[c]	0.00
B	14	15	0.08	0.00	0.05	0.25	0.83	0.04
C	4	4	0.15[d]	0.00	0.15[d]	0.32[e]	0.98	0.00
D	5	3	0.37	0.00	0.30	0.29	0.98	0.10
E			0.47	0.05	0.33	0.50	0.98	0.35
F	0	9	0.63	0.00	0.43	0.29	0.98	0.48
G	17	14	0.66	0.15	0.59	0.57	0.98	0.54
H	10	11	0.85	0.22	0.77	0.52	0.98	0.76
I	2	2	0.98	0.85	0.93	0.86	0.98	0.94
Subtotal	65	72						
Excreta-water fraction	4	9						
$C^{14}O_2$	11	1						
Residue in fly	15	19						
Total recovery	95	101						

[a] Average of four experiments.
[b] See Figure 4 for tentative identification of certain metabolites.
[c] Two minor products of R_f 0.55 and 0.65 also occur.
[d] Two minor products of R_f 0.10 and 0.20 also occur.
[e] Two minor products of R_f 0.25 and 0.39 also occur.

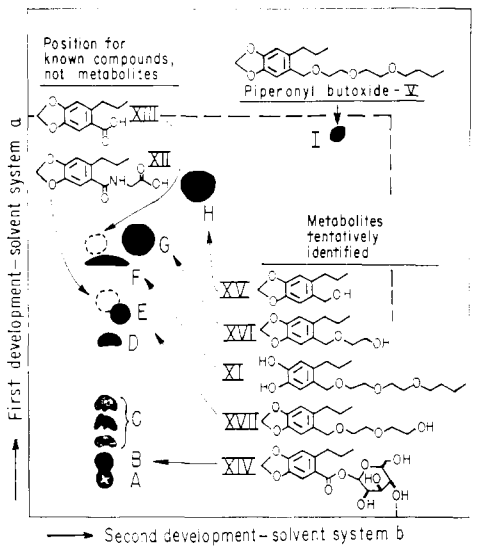

Figure 4. Thin-layer chromatographic pattern of piperonyl butoxide metabolites present in the ether-soluble fraction of housefly excreta

metabolites are considered which constitute 0.1 to 1.0% of the injected dose, 12 additional α-C^{14}-metabolites are found with two-dimensional development in solvent systems *c* and *f*. No other information is available on the nature of metabolites C and D, except that they are not modified by treatment with sodium hydroxide or β-glucosidase. The evidence given below serves to tentatively identify metabolites A, B, E, F, G, and H.

Labeled products cochromatographing with 6-propylpiperonylic acid (XIII) and its glycine conjugate (XII) are not excreted by flies injected with labeled piperonyl butoxide, based on two-dimensional TLC with solvent systems *a* and *b* (Figure 4) or *a* and *e*. When flies are injected with an unlabeled preparation of 6-propylpiperonylic acid, the injected compound is not excreted and only compounds with R_f values comparable to metabolites A and B of piperonyl butoxide are present in the ether-soluble fraction, based on TLC analysis with solvent systems *a* and *e*, and using chromotropic acid and *p*-anisidine hydrochloride for detection; each of the metabolites of 6-propylpiperonylic acid is cleaved by sodium hydroxide or β-glucosidase to liberate 6-propylpiperonylic acid. Metabolites A and B from piperonyl butoxide-α-C^{14} are also cleaved by either sodium hydroxide or β-glucosidase to yield 6-propylpiperonylic acid. Prior to hydrolysis, the unlabeled metabolites of 6-propylpiperonylic acid cochromatograph with labeled metabolites A and B derived from piperonyl butoxide-α-C^{14}. These facts suggest that metabolites A and B of piperonyl butoxide and the metabolites of 6-propylpiperonylic acid are sugar conjugates of this acid. Metabolite B cochromatographs with the synthetic glucoside of 6-propylpiperonylic acid (XIV) in each of solvent systems *a* through *e* (Figure 4, Table IV). It is possible that the major component of metabolite A (R_f = 0.46 using solvent system *c*) is a glucoside-6-phosphate of 6-propylpiperonylic acid—i.e., the 6-phosphate of metabolite B—based on analogy with the findings of Heenan and Smith (1967).

Metabolite F of piperonyl butoxide appears to be a catechol, or a catechol derivative, because the carbon in the benzodioxole ring is removed during its formation, and it is probably acidic inasmuch as it is retained at the origin in solvent system *b*, which contains ammonia (Figure 4). The chromatographic characteristics of metabolite F resemble those reported by Kuwatsuka and Casida (1965) for the catechol formed on demethylenation of piperonyl butoxide. Actually, metabolite F cochromatographs with this catechol in solvent systems *a*, *c*, *d*, and *f*.

Four excreted products from piperonyl butoxide appear to be neutral because they are not greatly affected in their chromatographic characteristics by the inclusion of ammonia in solvent system *b* (Figure 4). One of them, I, is probably piperonyl butoxide. The others, E, G, and H, are not modified by β-glucosidase or sodium hydroxide, suggesting the fact that they are more polar than piperonyl butoxide is not the result of conjugate formation. On the other hand, it appears likely that they are a series of related alcohols of increasing polarity, such as might be formed on cleavage of the various ether groups in the butyl carbityl side chain of piperonyl butoxide. Three such alcohols (XV, XVI, and XVII) were synthesized for cochromatographic comparisons. As shown in each of two replicates and in each of solvent systems *a* through *f* (Table IV), metabolites E, G, and H cochromatograph with compounds XVII, XVI, and XV, respectively. Further evidence confirming the identity of metabolites E, G, and H comes from the finding that, after their conversion to their respective methylcarbamate derivatives (with methyl isocyanate under the reaction conditions previously described), successful cochromatography occurs with compounds XX, XIX, and XVIII, respectively, using two-dimensional development with solvent systems *c* and *f*, and *d* and *f*. The unlabeled methylcarbamates (compounds XX, XIX, and XVIII) do not cochromatograph with metabolites E, G, and H (prior to methylcarbamoylation) in solvent systems *c* and *f*, on two-dimensional development.

Metabolism of M-C^{14}-DP Compounds, Carbonate-C^{14}, Formaldehyde-C^{14}, and Formate-C^{14} by the Fly Abdomen Homogenate or Fly Abdomen Microsome-NADPH System. Incubation of sodium carbonate-C^{14}, sodium formate-C^{14}, or formaldehyde-C^{14} with the fly abdomen enzyme system of SCR flies results in radiocarbon distribution among the fractions which is not dependent on NADPH fortification; complete radiocarbon loss occurs in 2 hours with carbonate-C^{14}, while 66 to 80% is retained in the water-soluble fraction with the other two compounds and none of the three compounds yield ether-soluble products (Table V). Analysis of the water fraction by a described procedure (Casida *et al.*, 1966) involving derivative formation shows that more than 95% of the remaining radiocarbon, following incubation of SCR fly abdomen homogenates with formaldehyde-C^{14} or formate-C^{14}, is not the original compound. Apparently, these compounds are unstable in the system, giving $C^{14}O_2$ and unidentified metabolites.

Cofactor fortification generally increases the radiocarbon in the water fraction on incubation of fly abdomen or fly microsome preparations with various M-C^{14}-DP compounds; the only exceptions are tropital incubated with the microsome or abdomen homogenate system, and myristicin incubated with the abdomen

Table V. Metabolism of Various C^{14}-Labeled Methylenedioxyphenyl and One-Carbon Compounds by Abdomen Homogenate or Microsome Enzyme-NADPH System from Female Flies

C^{14}-Labeled Compound	Amount of Radiocarbon Recovered,[a] %: Without Cofactor: Water fraction	Without Cofactor: Ether fraction	Without Cofactor: Loss	With NADPH: Water fraction	With NADPH: Ether fraction	With NADPH: Loss
Methylene-C^{14}-dioxyphenyl Compounds						
SCR Abdomen Homogenates						
Dihydrosafrole	9	55	34	17	51	32
Myristicin	15	82	3	18	80	2
Piperonyl butoxide	9	57	34	18	57	25
Safrole	9	55	36	18	65	17
Sulfoxide-A	20	64	16	34	52	14
Sulfoxide-B	11	62	27	40	50	10
R_{Hokota} Abdomen Homogenates						
Sulfoxide-A	3	73	24	33	60	7
Sulfoxide-B	3	44	53	34	30	36
Piperonyl butoxide	2	75	23	10	58	32
$SCRS_{DHS}$ Abdomen Homogenates						
Sulfoxide-A	11	89	1	23	77	10
Piperonyl butoxide	4	86	10	24	76	0
SCR Abdomen Microsomes						
Sulfoxide-B	29	62	9	64	30	6
Piperonyl butoxide	42	47	11	50	48	2
Tropital	2	65	33	2	59	39
Other Labeled Preparations						
SCR Abdomen Homogenates						
Formaldehyde	78	0	22	80	0	20
Sodium carbonate	0	0	100	0	0	100
Sodium formate	66	0	34	67	0	33

[a] Average of two to four experiments.

Table VI. Metabolism of Tropital-M-C^{14}-DP by Abdomen Homogenate or Microsome Enzyme-NADPH System of Female SCR Flies with and without BSA

Metabolite or Fraction	Amount of Radiocarbon Recovered, %: Without BSA: Without cofactor	Without BSA: With NADPH	With BSA: Without cofactor	With BSA: With NADPH
Abdomen Homogenate[a]				
Ether fraction[b]				
Tropital	49	43	47	31
Piperonylic acid	14	10	15	19
Water fraction	3	3	3	4
Loss during extraction	34	44	35	46
Abdomen Microsome[c]				
Ether fraction[b]				
Tropital	28	30	45	41
Piperonal	4	5	5	3
Piperonylic acid	29	37	20	23
Unknown metabolite	0	3	0	7
Water fraction	4	7	5	7
C^{14}-bound to protein	0.2	0.2	0.4	0.8
Loss during extraction	35	17	25	18

[a] Average of three experiments.
[b] Analysis by TLC.
[c] Average of two experiments.

homogenate system from SCR flies (Tables V and VI). The microsome system results in a higher incorporation of label into water-soluble compounds than the abdomen homogenate system with piperonyl butoxide and sulfoxide-B. Different strains do not vary greatly in metabolism of the sulfoxide diastereoisomers and piperonyl butoxide by the abdomen homogenate–NADPH system (Table V).

Incorporation of BSA into the enzyme preparation and incubation mixtures has no marked effect on metabolism of tropital by abdomen homogenate or microsome preparations (Table VI). In all cases, radiocarbon in the water fraction is small; with the microsome preparation, only a small amount of the radiocarbon is protein-bound and some of this may be bound to BSA. In contrast to the situation with fly abdomen preparations, the radiocarbon loss with the abdomen microsome preparations during extraction and analysis is greater in the absence than in the presence of NADPH fortification. Losses are generally greater with the abdomen homogenates than with the microsome preparations. (In control experiments in which fly-abdomen incubation mixtures were extracted immediately after preparation rather than after incubation, to determine the loss experienced in the ether extraction, tropital, piperonal, and formate gave radiocarbon losses of 13, 25, and 68%, respectively.) The addition of NADPH to the microsome incubation mixtures results in the formation of a larger amount of piperonylic acid and in the formation of an unidentified ether-soluble metabolite (R_f 0.05, solvent system *c*); these degradations do not take place or are not dependent on NADPH-addition in the abdomen homogenate system.

DISCUSSION

Metabolism of MDP synergists in living houseflies involves oxidation at the benzodioxole moiety, resulting ultimately in carbon dioxide liberation, and at the aliphatic side chain, yielding metabolites which retain the MDP structure but have a modified side chain. There is no evidence that ring hydroxylation takes place. Some of the metabolites are conjugated prior to excretion and others are not.

Studies with mouse liver enzymes show that MDP compounds are most readily degraded by the microsome-NADPH system, and that formate is the major metabolite released from the MDP moiety during oxidation to the catechol; this suggests that hydroxymethylenedioxyphenyl compounds and monoformate esters are involved as intermediates (Casida *et al.*, 1966). MDP compounds are also metabolized by the fly abdomen homogenates and microsomes, under in vitro conditions; in certain cases, this metabolism is stimulated by NADPH. The ether fractions were not subjected to TLC analyses in these studies, and so possible ether-soluble metabolites were not determined. Housefly abdomen microsomes are significantly more active than the fly abdomen homogenate system in degrading sulfoxide-B and piperonyl butoxide, converting them to water-soluble metabolites. It is not known whether formate or formaldehyde is released as a result of metabolic attack on the MDP moiety by the fly abdomen-NADPH system; since formate and formaldehyde

are unstable in this system, their role as intermediates in metabolism of the MDP moiety is not definitely established. The nature of the water-soluble metabolites is not known, although they presumably arise from nonenzymatic reactions with tissue constituents when the incubations are made in the absence of NADPH.

The distribution of radioactivity is practically identical following injection of labeled sodium formate or formaldehyde into houseflies. Injection of formate-C^{14} yields, among other radiolabeled compounds, proline, serine, glutamic acid, uric acid, and allantoin; eventually these compounds are excreted or become incorporated into fly tissues (Cline and Pearce, 1963; Gilmour, 1961). Less than 50% of the sodium formate-C^{14} injected into houseflies is expired as $C^{14}O_2$; therefore, if formate-C^{14} is one of the intermediates in metabolism of M-C^{14}-DP compounds in flies, the amount of $C^{14}O_2$ expired from flies probably represents only a portion of the attack at the MDP moiety. The amount of radioactivity expired as $C^{14}O_2$ from flies treated with M-C^{14}-DP compounds ranges from 1 to 20% of the initial dose in the 24-hour period following injection; however, in view of the fact that only up to 50% of the formate oxidizes to carbon dioxide, it is possible that the amount of metabolic cleavage of the benzodioxole moiety may, in certain instances, be as high as 40% of the initial injected dose. However, a portion of the dose remaining in the fly may not be derived from formate but instead may contain the intact MDP ring, because M-C^{14}-DP and α-C^{14} samples of piperonyl butoxide give somewhat similar retention values.

Piperonal, piperonyl alcohol, and piperonylic acid do not metabolize extensively to $C^{14}O_2$, probably because the side chain in these molecules is very susceptible to metabolism, including conjugation. MDP compounds with side chains that are less susceptible to metabolic attack are more likely to undergo oxidative hydroxylation at the methylene group of the benzodioxole moiety, to ultimately yield $C^{14}O_2$.

Flies treated with M-C^{14}-DP preparations of dihydrosafrole, myristicin, or safrole excrete 6 to 8% of the administered radiocarbon. Also, each of these compounds gives the same amount of radiocarbon (17 to 18%) in the water fraction after incubation with the fly abdomen enzyme–NADPH system. These results suggest that the rates of metabolism for dihydrosafrole, myristicin, and safrole in flies are approximately the same. That portion of the safrole dose which is excreted and recovered on ether extraction of excreta is in the form of piperonylic acid and its five amino acid conjugates (Esaac and Casida, 1968). Isosafrole, studied in unlabeled form, gives rise to the same amino acid conjugates of piperonylic acid. Thus, safrole and isosafrole are oxidized at the side chain to yield piperonylic acid; this reaction is analogous with that which occurs in the oxidation of allyl- and propenylbenzenes in mammals to yield benzoic acid (Williams, 1959). Dihydrosafrole does not give rise to piperonylic acid but, instead, results in the excretion of a radioactive metabolite which cochromatographs with sesamol. It is possible that this metabolite (which was not examined further because authentic comparison materials were not available) is not sesamol, but instead is formed by hydroxylation of the propyl group to give α-ethylpiperonyl alcohol because hydroxylation of alkylbenzenes (containing 2 to 4 carbons in the side chain), by locust fat bodies and housefly abdomens, occurs easily at the α-methylene group and less readily at penultimate and terminal methylene groups (Chakraborty and Smith, 1967). Enzymatic oxidation of the MDP moiety takes place to a similar extent with dihydrosafrole and myristicin ($C^{14}O_2$, 12 to 13%), but it occurs to a much lesser extent with safrole ($C^{14}O_2$, 7%), possibly because the latter is more volatile than the other two. The amount of residual radiocarbon in the fly (50%) is much higher with myristicin than with any other M-C^{14}-DP compound studied, but the identity of the C^{14}-labeled materials remaining in the flies is unknown.

M-C^{14}-DP preparations of sulfoxide-A and sulfoxide-B are metabolized by flies to yield some $C^{14}O_2$ and considerable amounts of radiocarbon in the excreta and the body residues. The sulfone is the major metabolite excreted by flies and, based on studies with sulfoxide-B only, eight to 11 other ether-soluble metabolites occur in the excreta. The rate of metabolism and elimination of sulfoxide-A is much greater than that of sulfoxide-B in female SCR flies; it is possible that this phenomenon accounts, to some extent, for the slightly higher synergistic activity of the sulfoxide-B isomer (Kuwatsuka and Casida, 1965).

The fate of tropital in living houseflies is, in part, given in Figure 5. Although tropital is probably hydroxylated at the MDP moiety (ultimately yielding $C^{14}O_2$), the major detoxication mechanism for tropital, accounting for more than 60% of the dose, is oxidation of the side chain to yield piperonylic acid, which is excreted as amino acid conjugates, apparently with little or no formation of the glucoside. Conversion of tropital to piperonylic acid by the fly microsomes is enhanced by NADPH; this suggests that the α-methylene group of the side chain is hydroxylated to yield an unstable intermediate which decomposes to give piperonylic acid; however, it is also possible that this conversion results from initial nonenzymatic hydrolysis at the labile acetal group to give piperonal, which is subsequently oxidized to piperonylic acid. The radiocarbon loss on incubation

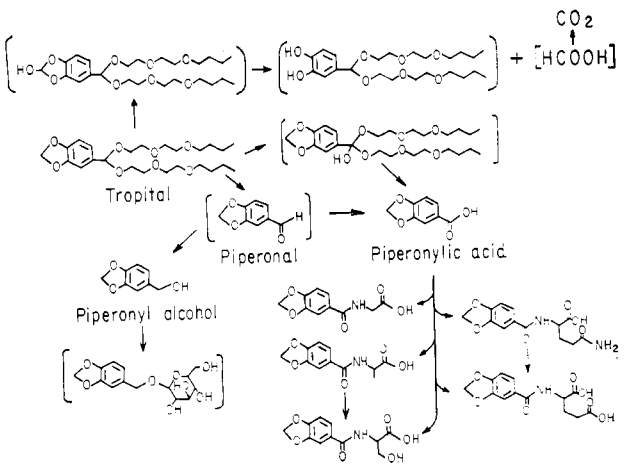

Figure 5. Tentative, partial metabolic pathways for tropital in houseflies

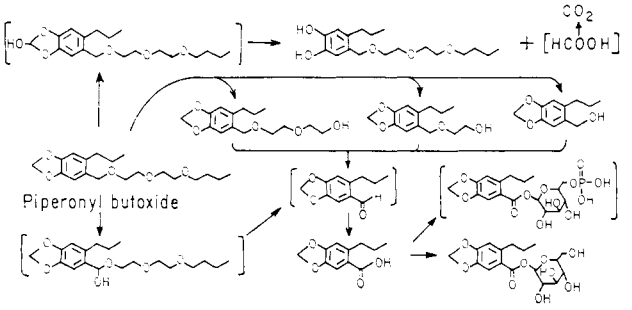

Figure 6. Tentative, partial metabolic pathways for piperonyl butoxide in houseflies

of tropital-M-C^{14}-DP with the fly enzyme system is possibly, at least in part, the result of the release of formate on hydroxylation and cleavage of the benzodioxole moiety. Bovine serum albumin (BSA) does not enhance the activity of the fly microsomal enzyme in metabolizing tropital, even though it remarkably enhances the activity of the same enzyme system for Baygon and Matacil metabolism (Tsukamoto and Casida, 1967a). The presence of BSA in the enzyme incubation mixture containing tropital slows down the degradation of tropital, possibly by binding a portion of the tropital, thus preventing it from reaching the active site of the enzyme.

The metabolic pathways for piperonyl butoxide in living houseflies are somewhat similar to those for tropital (Figure 6). However, enzymatic oxidation at the benzodioxole moiety takes place to a much smaller degree with piperonyl butoxide than with tropital, based on the amount of expired $C^{14}O_2$ from M-C^{14}-DP preparations. A catechol, formed as a result of cleavage of the MDP moiety, is excreted without conjugation. Another major pathway involves oxidation at the α-methylene position to give, ultimately, 6-propylpiperonylic acid, which is conjugated completely, prior to excretion, as the glucoside and, possibly, as a glucoside-6-phosphate; it does not form amino acid conjugates (in contrast to the situation with piperonylic acid). Each ether group in the polyether side-chain is cleaved, probably as a result of hydroxylation at one of the carbons adjacent to the three ether linkages. (If this occurs on the carbon towards the ring, MDP compounds which are aldehydes result, whereas, if it occurs on the carbon towards the butyl group, MDP compounds which are alcohols are formed, on cleavage of the resulting hemiacetals. Since certain of the resulting aldehydes and/or alcohols probably oxidize to the corresponding acid, it is not actually known whether only one or both of these sites of attack is involved.) Some of the unidentified metabolites possibly are aliphatic aldehyde derivatives, or products modified on the propyl group, or are compounds formed by combinations of two or more sites of attack. Formation of each of the three alcohols derived from cleavage of the ether linkages in the side chain of piperonyl butoxide, and of the catechol and of the glucoside of 6-propylpiperonylic acid, appears to be a detoxification mechanism because each of the compounds (bioassayed as respective synthetic compounds) is much less active, as a synergist, than piperonyl butoxide when injected into houseflies at a dose of 1 μg. per fly followed by topical application of allethrin.

Resistant strains of houseflies metabolize some MDP synergists, such as tropital-C^{14}, at the same rate and by the same pathway as susceptible strains. Little or no difference exists in the extent and rate of hydroxylation of the MDP moiety in tropital by SCR and R_{Hokota} flies, based on the amount of $C^{14}O_2$ expired from the flies. Although the amount of radioactivity excreted by SCR flies (62%) is much larger than that excreted by R_{Hokota} flies (44%), the same amino acid conjugates are detected in the excreta of both fly strains and, in each case, *N*-piperonyl glycine is the major conjugate excreted. The conjugation system probably is more efficient in susceptible (SCR) flies than in resistant (R_{Hokota}) flies. These results agree with the data obtained by Shrivastava (1967) and Shrivastava *et al.* (1969) for Baygon-C^{14}, using several susceptible and resistant strains of houseflies. Of four fly strains, R_{Baygon} flies most rapidly metabolize sulfoxide-A, but the level of sulfone production does not vary greatly with the strain used. The strain $SCRS_{DHS}$ (selected with dihydrosafrole), metabolizes sulfoxide-A at the lowest rate, possibly as a result of inhibition of sulfoxide-A metabolism by the residual dihydrosafrole or metabolites of dihydrosafrole carried through into the adult flies.

Certain compounds modify the rates of metabolism of the synergist. Pretreatment of flies with MGK 264 or DpNC reduces the attack at the MDP moiety of tropital, as indicated by the reduced amount of expired $C^{14}O_2$, and increases the amount of excreted material resulting from attack at the acetal side chain. Each of the excreted products, the various *N*-piperonyl amino acids, is increased in amount, but their ratio is not altered by this treatment. Carbaryl increases the persistence of tropital-C^{14} and its metabolites in the fly body and, therefore, permits more conversion to $C^{14}O_2$; however, it is possible that this effect is largely the result of the increased toxicity of the tropital-carbaryl combination. Piperonyl butoxide-M-C^{14}-DP conversion to $C^{14}O_2$ is unaltered and excretion of metabolites is reduced by MGK 264 and DpNC. Retention of piperonyl butoxide metabolites in the body, in the presence of DpNC and MGK 264, possibly results from the diminution of biochemical reactions because of the toxicity of the combinations used. Each of the aforementioned compounds is metabolized by, or is an inhibitor of, the microsome-NADPH system (Casida *et al.*, 1966; Hodgson and Casida, 1960, 1961; Leeling and Casida, 1966); therefore, it is possible that the interaction noted occurs at the microsome level. It is likely that the synergists are attacked by the microsome-NADPH system at more than one site, such as the side chain and the MDP moiety, in a ratio dependent on the synergist. If this is so, a second chemical acting as a competitive substrate could shift the ratio of attack at the alternative sites on the synergist and thereby alter the elimination pattern.

MDP synergists appear to have a greater structural specificity for synergism of pyrethrum toxicity than of carbaryl toxicity; most pyrethrum synergists are also carbamate synergists, but the converse is not as generally true (Hewlett, 1960; Metcalf, 1967). The most active MDP synergists for pyrethrum are those having

a long side chain with one or more oxygen atoms incorporated into ether, acetal, or sulfoxide groupings (Beroza and Barthel, 1957; Hopkins and Maciver, 1966; Maciver, 1966). In each of the MDP synergists tested, the methylene position of the MDP moiety is oxidatively attacked. In addition, the long side chain of MDP compounds which are particularly good pyrethrum synergists is sometimes easily attacked by oxidative enzyme systems; thus, with tropital and piperonyl butoxide, an active methylene group attached to the aromatic ring is oxidized, during metabolism in flies, to yield piperonylic acids. On the other hand, a simple side chain is, in many structures, adequate for carbamate synergism (Metcalf, 1967; Wilkinson, 1967; Wilkinson *et al.*, 1966) and this also holds for certain ring substituents which probably resist attack by oxidative enzyme systems.

The high efficiency of the MDP synergists possibly is associated with their fit at the active site of the insecticide-detoxifying enzyme, a fit involving both the planar benzodioxole ring and the side chain. If the same enzyme is responsible for pyrethrum and carbamate detoxication, it is possible that only one site of attack on the synergist and a one-site fit on the detoxifying enzyme, involving the MDP moiety, are essential for carbamate synergism, whereas two sites of attack on the synergist and a one- or two-site fit on the enzyme, involving the MDP moiety and the side chain as well, are optimal for pyrethrum synergism. An alternative hypothesis is that different enzymes are responsible for carbamate and pyrethrum detoxification and that these enzymes vary in the nature of their active sites with respect to synergist inhibition. Further insight into the mode of action of MDP compounds at the molecular level requires a better understanding of the nature and kinetics of the microsome-NADPH enzyme systems from insect sources (Casida, 1968).

A large variety of insecticides are oxidatively metabolized by the fly abdomen enzyme-NADPH system (Tsukamoto and Casida, 1967a, 1967b). Biochemical genetic studies with houseflies establish that a gene on the 5th chromosome confers high activity for the microsome-NADPH system, which metabolizes certain carbamate insecticides, and that piperonyl butoxide reduces the impact of this metabolism on the resulting resistance level (Tsukamoto *et al.*, 1968). MDP compounds are also metabolized by the same or by a similar enzyme system, serving as substrates for the hydroxylation enzymes that detoxify many insecticides. Thus, they slow down the detoxication processes of these toxicants in insects and, thereby, exhibit synergistic activity. It seems likely that factors important for high synergistic activity of MDP compounds are: a high efficiency of penetration into the insect and into the endoplasmic reticulum, an adequate fit at the active site of the microsome-NADPH system, and sufficient resistance to detoxification at this site to minimize metabolism of the insecticide long enough for poisoning to occur.

ACKNOWLEDGMENT

Acknowledgment is made to the following persons for suggestions, assistance, and/or materials: Judith Engel, Loretta Gaughan, Irene Jackson, F. X. Kamienski, Ella Kimmel, Shozo Kuwatsuka, Louis Lykken, and Hisayuke Matsuo, each of the Division of Entomology, University of California, Berkeley.

LITERATURE CITED

Barthel, W. F., Alexander, B. H., U.S.D.A., Research Service, Entomol. Research Branch **ARS-33-42,** 24 pp. (1957).
Beroza, M., J. AGR. FOOD CHEM. **11,** 51 (1963).
Beroza, M., Barthel, W. F., J. AGR. FOOD CHEM. **5,** 855 (1957).
Casida, J. E., *Ann. Rev. Entomol.* **8,** 39 (1963).
Casida, J. E., "Insect Microsomes and Insecticide Chemical Oxidations," in "Microsomes and Drug Oxidations," Academic Press, New York, submitted for publication, 1968.
Casida, J. E., Engel, J. L., Esaac, E. G., Kamienski, F. X., Kuwatsuka, S., *Science* **153,** 1130 (1966).
Casida, J. E., Shrivastava, S. P., Esaac, E. G., *J. Econ. Entomol.* **61,** 1339 (1968).
Chakraborty, J., Smith, J. N., *Biochem. J.* **102,** 498 (1967).
Cline, R. E., Pearce, G. W., *Biochemistry* **2,** 657 (1963).
Cram, D. J., Allinger, N. L., *J. Am. Chem. Soc.* **77,** 6289 (1955).
Esaac, E. G., Ph.D. dissertation, University of California, Berkeley, Calif., 1967.
Esaac, E. G., Casida, J. E., *J. Insect Physiol.* **14,** 913 (1968).
Gilmour, D., "The Biochemistry of Insects," Academic Press, New York, 1961.
Heenan, M. P., Smith, J. N., *Life Sci.* **6,** 1753 (1967).
Hennessy, D. J., J. AGR. FOOD CHEM. **13,** 218 (1965).
Hewlett, P. S., *Advan. Pest Control Res.* **3,** 27 (1960).
Hewlett, P. S., *Chem. Ind. (London)* **1968,** 701.
Hickinbottom, W. J., "Reactions of Organic Compounds," 3rd ed., pp. 278–9, Wiley, New York, 1962.
Hodgson, E., Casida, J. E., *Biochim. Biophys. Acta* **42,** 184 (1960).
Hodgson, E., Casida, J. E., *Biochem. Pharmacol.* **8,** 179 (1961).
Hopkins, L. O., Maciver, D. R., *Pyrethrum Post* **8**(4), 10 (1966).
Hough, L., Jones, J. K. N., Wadman, W. H., *J. Chem. Soc.* **1950,** 1702.
Koelsch, C. F., *J. Am. Chem. Soc.* **68,** 148 (1946).
Kuhr, R. J., Casida, J. E., J. AGR. FOOD CHEM. **15,** 814 (1967).
Kuwatsuka, S., Casida, J. E., J. AGR. FOOD CHEM. **13,** 528 (1965).
Leeling, N. C., Casida, J. E., J. AGR. FOOD CHEM. **14,** 281 (1966).
Lichtenstein, E. P., Casida, J. E., J. AGR. FOOD CHEM. **11,** 410 (1963).
Maciver, D. R., *Pyrethrum Post* **8**(3), 3 (1966).
Metcalf, R. L., *Ann. Rev. Entomol.* **12,** 229 (1967).
Metcalf, R. L., Fukuto, T. R., Wilkinson, C., Fahmy, M. H., Abd El-Aziz, S., Metcalf, E. R., J. AGR. FOOD CHEM. **14,** 555 (1966).
Oonnithan, E. S., Casida, J. E., J. AGR. FOOD CHEM. **16,** 28 (1968).
Schmidt, C. H., Dahm, P. A., *J. Econ. Entomol.* **49,** 729 (1956).
Shrivastava, S. P., Ph.D. dissertation, University of California, Berkeley, Calif., 1967.
Shrivastava, S. P., Tsukamoto, M., Casida, J. E., *J. Econ. Entomol.* **62,** in press, 1969.
Tsukamoto, M., Casida, J. E., *J. Econ. Entomol.* **60,** 617 (1967a).
Tsukamoto, M., Casida, J. E., *Nature* **213,** 49 (1967b).
Tsukamoto, M., Shrivastava, S. P., Casida, J. E., *J. Econ. Entomol.* **61,** 50 (1968).
Wilkinson, C. F., J. AGR. FOOD CHEM. **15,** 139 (1967).
Wilkinson, C., Metcalf, R. L., Fukuto, T. R., J. AGR. FOOD CHEM. **14,** 73 (1966).
Williams, R. T., "Detoxication Mechanisms," pp. 202, 371 Wiley, New York, 1959.

Received for review July 22, 1968. Accepted December 23, 1968. From a dissertation submitted by the first author in December 1967 to the Graduate Division, University of California, Berkeley, in partial fulfillment of the requirements for the degree of doctor of philosophy. Supported, in part, by grants from: U.A.R. Cultural and Educational Bureau, Washington, D. C.; U. S. Public Health Service, National Institutes of Health (Grant ESGM-00049); U. S. Atomic Energy Commission, Contract AT(11-1)-34, Project Agreement 113; Forest Service, U. S. D. A. (Grant 4); S. C. Johnson and Son, Inc., Racine, Wis.; McLaughlin Gormley King Co., Minneapolis, Minn.