Biochemical Pharmacology, Vol. 19, pp. 91–112. Pergamon Press. 1970. Printed in Great Britain

IMPORTANCE OF DEMETHYLENATION IN THE METABOLISM *IN VIVO* AND *IN VITRO* OF METHYLENEDIOXYPHENYL SYNERGISTS AND RELATED COMPOUNDS IN MAMMALS*

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(*Received* 19 *March* 1969; *accepted* 23 *May* 1969)

Abstract—The major metabolic pathway for piperonyl butoxide, the sulfoxide diastereoisomers, dihydrosafrole, safrole and myristicin in mice, after oral administration, involves cleavage of the methylenedioxyphenyl (MDP) moiety and expiration of the methylene carbon as carbon dioxide. In contrast, oxidation or conjugation (or both) of the side chain is the major metabolic pathway for Tropital, piperonal, piperonyl alcohol and piperonylic acid. Products in the urine, after piperonyl butoxide administration, include many compounds lacking the MDP moiety along with small amounts of 6-propylpiperonylic acid and its glycine conjugate, and those from Tropital consist almost entirely of the glycine and glucuronic acid conjugates of piperonylic acid. Mixed-function oxidases of liver microsomes demethylenate several MDP compounds to yield formate and the corresponding catechol; with most MDP compounds, other products also form because of additional oxidation reactions at other functional groups. The action of MDP synergists and related compounds in increasing drug and insecticide potency possibly is the result of combination of the MDP compound with an active site on the mixed-function oxidases resulting in inhibition of normal detoxification mechanisms.

METHYLENEDIOXYPHENYL (MDP) compounds probably act as insecticide synergists by reducing the rate of toxicant metabolism when this detoxication is mediated by the mixed-function oxidases of insect microsomes.[1–6] It is likely that the liver microsomal mixed-function oxidase system is the site of interaction of MDP compounds and certain drugs or insecticide chemicals in mammals, resulting in enhanced potency, toxicity or carcinogenicity of the drug or insecticide chemical.[7–13] The mode of action of MDP compounds possibly is related to the manner in which they are metabolized, based on studies in both this and other laboratories. Intravenous administration to rats of safrole, isosafrole, dihydrosafrole, piperonyl butoxide or Tropital yields a number of metabolites in the bile and urine.[14–17] The liver microsome–reduced nicotinamide adenine dinucleotide phosphate (NADPH) system metabolizes 4-nitro- and 3,4,5,6-tetrachloromethylenedioxybenzenes to the corresponding catechols,[3,18] and liberates formate-^{14}C from certain methylene-^{14}C-dioxyphenyl (M-^{14}C-

* Supported in part by grants from: the United States Public Health Service, National Institutes of Health (Grant No. ESGM-00049); the United States Atomic Energy Commission (Contract AT(11-1)-34, Project Agreement No. 113); S. C. Johnson and Son, Inc., Racine, Wis.; and the McLaughlin Gormley King Co., Minneapolis, Minn.

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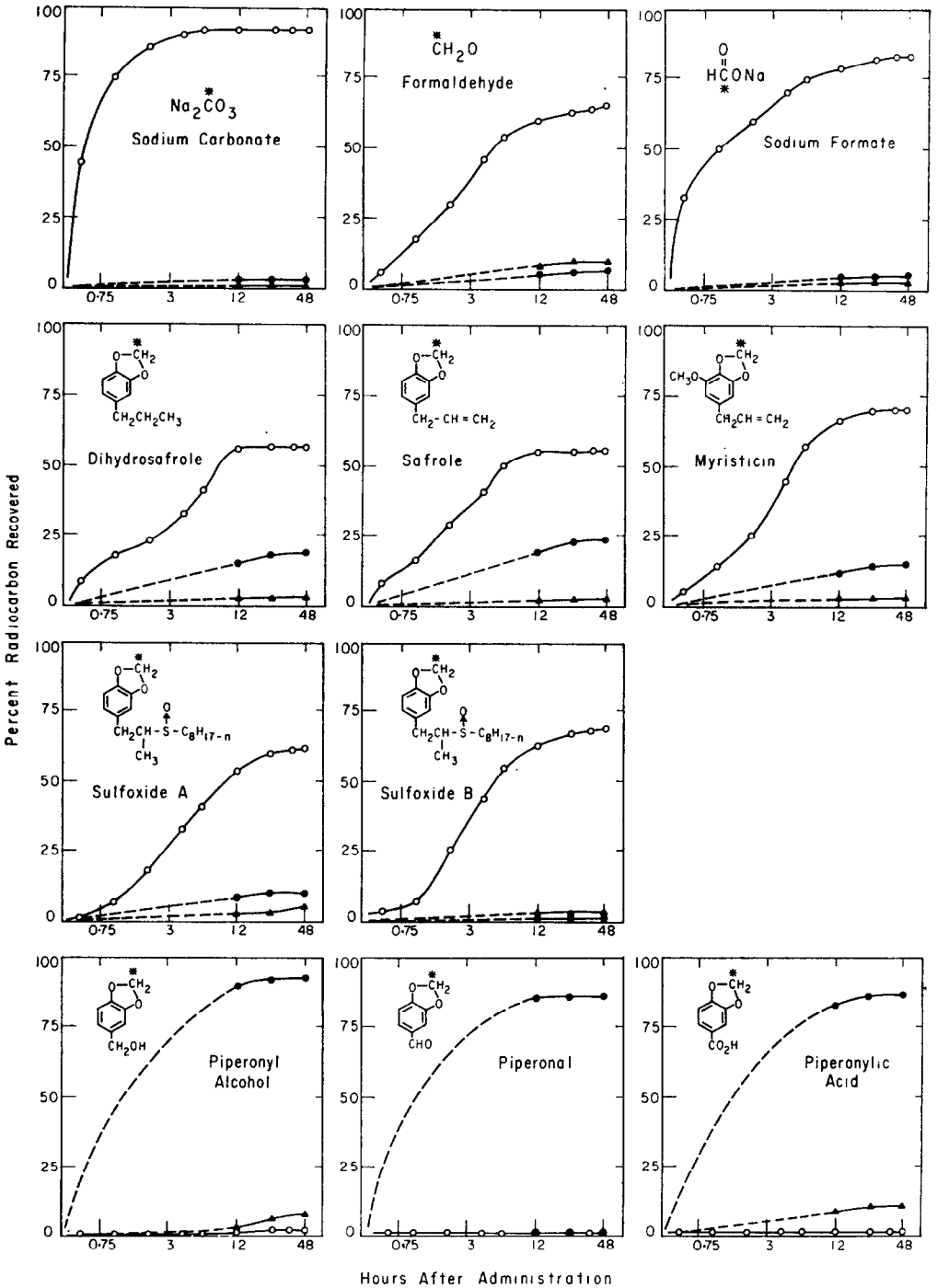

FIG. 1. Rate of elimination of radiocarbon from mice receiving oral doses of sodium carbonate-^{14}C, formaldehyde-^{14}C, sodium formate-^{14}C and each of eight methylene-^{14}C-dioxyphenyl compounds. Legend: CO_2, ○; urine, ●; feces, ▲. Note: the dotted lines for radiocarbon excreted in urine and feces during the first 12 hr after administration indicate that the exact elimination rate is not known, the first sample being taken at 12 hr.

DP) compounds.[3] Cleavage of the MDP moiety leads to extensive expiration of the the methylene carbon as $^{14}CO_2$ from mice and houseflies injected with six different M-^{14}C-DP compounds.[3] These findings, in combination with other studies on the fate of MDP compounds in houseflies,[19–21] support the hypothesis that MDP compounds inhibit oxidative detoxication reactions by serving as alternative substrates for the mixed-function oxidase system.[3]

This report presents additional evidence on the mechanism and significance of demethylenation of MDP compounds in living mammals and by liver mixed-function oxidases in relation to the metabolism and mode of action of the commercial synergist chemicals, piperonyl butoxide, sulfoxide and Tropital.

MATERIALS AND METHODS

Chemicals. Structures, names and labeling positions for the MDP-^{14}C preparations are given in Figs. 1, 2 and 3. Sources for most of the MDP compounds used in both labeled and unlabeled form were those described by Esaac and Casida.[21] In addition to the α-methylene-^{14}C sample of piperonyl butoxide (piperonyl butoxide-α-^{14}C) referred to by Esaac, an α-methylene-^{14}C sample of Tropital (Tropital-α-^{14}C) was provided by McLaughlin Gormley King Co., Minneapolis, Minn. Each radiolabeled

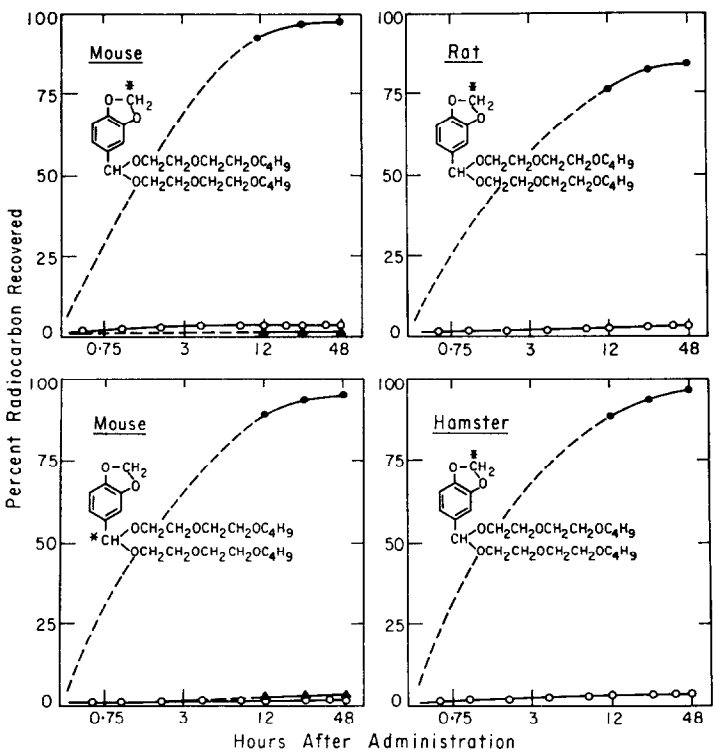

FIG. 2. Rate of elimination of radiocarbon from mice, rats and hamsters receiving oral doses of Tropital-M-^{14}C-DP or Tropital-α-^{14}C. Legend: CO_2, ○; urine, ●; feces, ▲. Note: the dotted lines for radiocarbon excreted in urine and feces during the first 12 hr after administration indicate that the exact elimination rate is not known, the first sample being taken at 12 hr.

MDP compound was of 1·0 mc per m-mole and, with the exception of Tropital-^{14}C, had a radiochemical purity of at least 99 per cent as determined by thin-layer chromatography (TLC).[21,22] Tropital-^{14}C was periodically purified, to remove piperonal and decomposition products, by chromatography on a column of alkaline silica gel, and the purified samples were stored under nitrogen at −4° to minimize decomposition. Although the radiochemical purity of the products recovered from the column was 100 per cent for piperonal-M-^{14}C-DP, 100 per cent for Tropital-M-^{14}C-DP, and 99

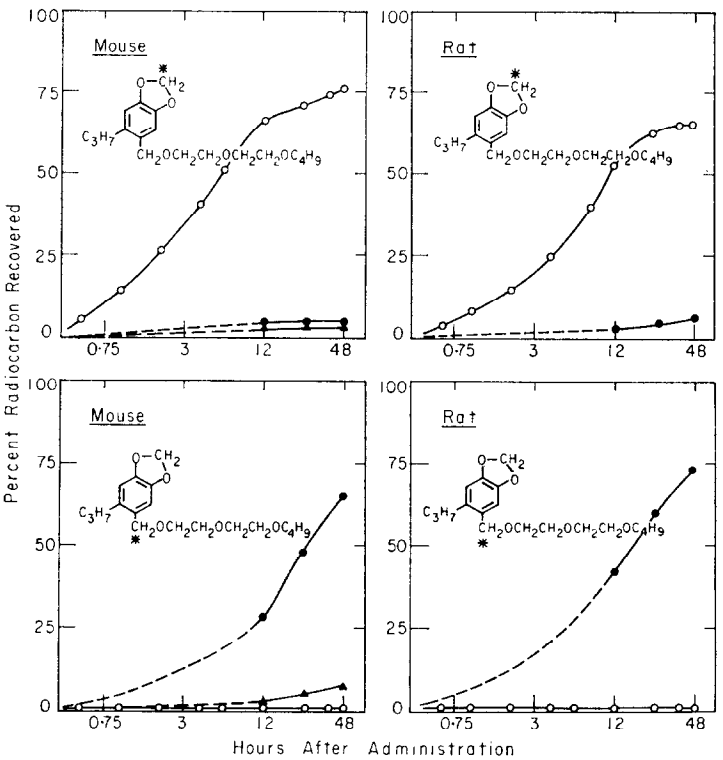

FIG. 3. Rate of elimination of radiocarbon from mice and rats receiving oral doses of piperonyl butoxide-M-^{14}C-DP or piperonyl butoxide-α-^{14}C. Legend: CO_2, ○; urine, ●; feces, ▲. Note: the dotted lines for radiocarbon excreted in urine and feces during the first 12 hr after administration indicate that the exact elimination rate is not known, the first sample being taken at 12 hr.

per cent for Tropital-α-^{14}C, some decomposition of Tropital-^{14}C to piperonal-^{14}C occurred prior to use; so, the purities of the Tropital-^{14}C samples, as used in these studies, were as follows: 88–99 per cent for oral administration to mice, rats and hamsters, and 94–99 per cent for the studies *in vitro*.

Piperonal-M-^{14}C-DP was oxidized to piperonylic acid-M-^{14}C-DP according to Shriner and Kleiderer,[23] but on a semi-microscale, and the acid was recovered by basifying the aqueous solution, extracting with ether to remove unreacted piperonal, acidifying, extracting with ether, drying the ether solution over sodium sulfate, evaporation of the ether, and subliming the crude product (yield 51 %; m.p. 222–224°,

reported 224–225°; appropriate infrared spectrum and TLC characteristics). Piperonyl alcohol-M-^{14}C-DP, prepared in 95 per cent yield by sodium borohydride reduction of piperonal-M-^{14}C-DP at 25°, was pure after chromatography on a silicic acid column using ether–hexane mixture (1:1) for elution (m.p. 57–58°, reported 58°;[24] appropriate infrared spectrum and TLC characteristics).

5-Hydroxy- and 6-hydroxypiperonylic acids were prepared and characterized as described by Kamienski.[25] Piperonylglycine (m.p. 182–183°, reported 178°[26]) was prepared in 86 per cent yield from piperonyl chloride (m.p. 80°, reported 80°[27]) and glycine according to a described procedure,[26] followed by dissolving the material recovered from recrystallization from water in 0·1 N sodium hydroxide, adjusting the pH to 4·7 to precipitate mainly piperonylic acid which was removed by filtration, and adjusting the filtrate to pH 1·0 to yield the pure compound after three additional recrystallizations from water. The infrared spectrum and analyses were appropriate.

Anal. Calc. for: C, 53·82; H, 4·06; N, 6·28. Found: C, 53·36; H, 4·41; N, 6·05.

6-Propylpiperonylglycine was prepared from 6-propylpiperonylic acid[21] by the procedure described above for conversion of piperonylic acid to piperonylglycine, including the step to remove trace amounts of the free acid from the conjugate, except that the final product was washed with hot hexane to remove the final traces of 6-propylpiperonylic acid. Three recrystallizations from water gave white needles, m.p. 160–161°.

Anal. Calc. for: C, 58·86; H, 5·70; N, 5·28. Found: C, 58·03; H, 5·92; N, 5·46.

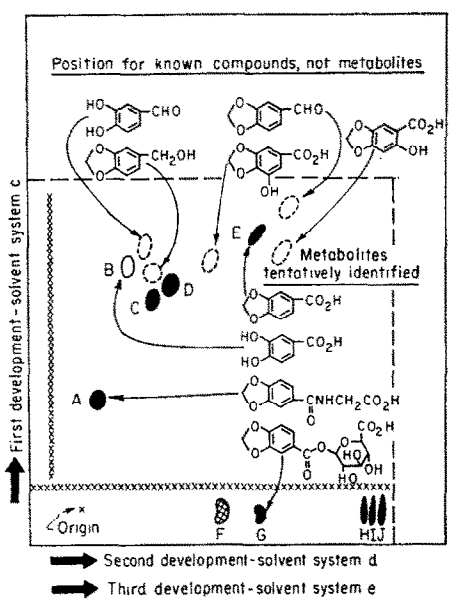

FIG. 4. Thin-layer chromatographic pattern of Tropital metabolites present in accumulated 0–12 hr urine samples from mice. Legend: metabolites present after administration of both radiolabeled preparations of Tropital, ●; metabolite present only after administration of Tropital-M-^{14}C-DP, ⊗; metabolites present only after administration of Tropital-α-^{14}C, ○. (Positions shown for metabolites A to E are those after development with the first two solvent systems only; positions for metabolites F to J, which remain at the origin during the first two developments, are those after development with the third solvent system.)

The source or method of preparation of other potential metabolites of Tropital, piperonyl butoxide and sulfoxide, as used in this study, is given by Esaac and Casida.[21]

Chromatography and radioactivity measurements. The techniques used for TLC and detection of labeled and unlabeled products in cochromatography studies were generally those previously reported.[21,22] Unless stated otherwise, TLC was accomplished by using silica gel H plates (20 × 20 cm), having a coating of 0·25 mm thickness, and prepared by using a 1:2 (w/v) slurry of the gel in distilled water. Because it is unstable in acidic solvents, Tropital was chromatographed using ether–hexane mixtures and "basic plates" were prepared as above, except that the water was replaced with 0·5 N sodium hydroxide. The solvent systems used were: (a) ether; (b) ether–hexane mixture (3:1); (c) benzene (saturated with formic acid)–ether mixture (1:3); (d) hexane–ether–formic acid mixture (25:25:1); (e) butanol–acetic acid–water mixture (3:1:1). In certain cases, three different solvent systems were used for resolution of products on the same TLC plate. This development sequence was accomplished by scraping a selected gel region (shown as a cross-hatched band in Figs. 4 and 5) free from the glass so that the third development solvent contacted only those products remaining at the origin with the first two solvent systems. The chromogenic agents used were: chromotropic acid for MDP compounds;[28] aqueous

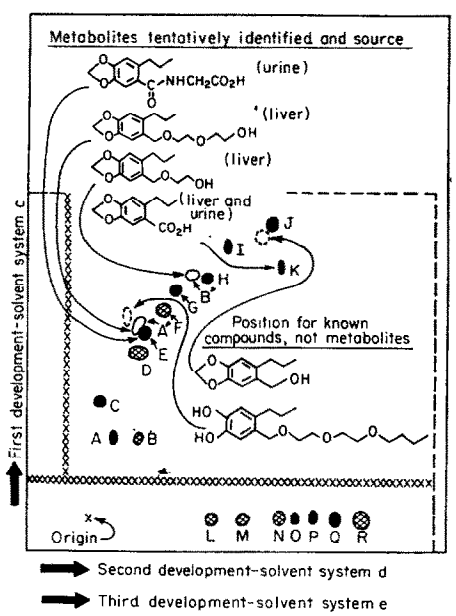

FIG. 5. Thin-layer chromatographic pattern of piperonyl butoxide metabolites present in accumulated 0–12 hr urine samples from mice. Legend: metabolites present after administration of both radiolabeled preparations of piperonyl butoxide, ●; metabolites present only after administration of piperonyl butoxide-α-^{14}C, ⊗; metabolites (A′ and B′) present only when incubated with mouse liver enzymes fortified with NADPH, ○. (Positions shown for metabolites A to K are those after development with the first two solvent systems only; positions for metabolites L to R, which remain at the origin during the first two developments, are those after development with the third solvent system.)

ferric chloride (2%) to detect phenolic hydroxyl groups; aqueous ferric chloride (2%) followed by aqueous potassium ferricyanide (1%) solutions for catechols and piperonyl butoxide derivatives with free alcoholic groups in the side chain;[21] sulfuric acid (50%) and heating at 110° for nonspecific detection of organic compounds.

Tropital-^{14}C was purified on a column of alkaline silica gel. The gel was prepared by suspending 1 part of silica gel H in 2 parts of 0·5 N sodium hydroxide (w/w) and drying the mixture at 120° for 6 hr, grinding it to a fine powder, and passing through a 100-mesh sieve. The column was packed, from slurries in nanograde hexane, with 2 g sodium sulfate at the bottom and 19 g alkaline silica gel at the top, and was covered with aluminum foil to minimize exposure to light during development. The Tropital sample was introduced in hexane and the column was developed with 50 ml hexane followed by 800 ml hexane–ether mixture (5:2) to completely separate piperonal from the more slowly eluting Tropital.

Column chromatography of Tropital urine metabolites utilized a column (3·4 × 51 cm) containing a silicic acid–Celite (Johns Manville's Hyflo Super Cel) mixture (100 g: 37 g), and the column was developed with 200 ml benzene (saturated with formic acid) followed by 1500 ml benzene (saturated with formic acid)–ether mixture (10:1). Six-ml fractions were collected and the metabolites in each fraction were determined by TLC, using chromotropic acid for detection of MDP compounds.

Animals and their treatment. The experimental animals used were: male, white Swiss–Webster mice (18–20 g) and male, white, Sprague–Dawley rats (150–170 g) from Bioscience Animal Labs., Oakland, Calif., reared on Purina laboratory chow; male hamsters (180–200 g) from Simonsens Animal Supply House, Gilroy, Calif., used immediately after delivery; male rabbits (0·8–1·2 kg) from Nitabell Rabbitry, Hayward, Calif., fed commercial rabbit pellets.

The radiolabeled compound was administered orally by stomach tube as a dimethyl sulfoxide (DMSO) solution. The dose and the volume of DMSO used for the dosing and as an additional wash of the stomach tube were as follows: mice, 5 μmoles/kg dose in 50 μl DMSO followed by 100 μl DMSO wash; rats and hamsters, 10 μmoles/kg dose in 150 μl DMSO followed by 200 μl DMSO wash. Each treated animal was held for 48 hr in an individual metabolism cage, a smaller version of one described by Krishna and Casida,[29] and was supplied with food and water *ad lib.* Total radiocarbon determinations were made on expired $^{14}CO_2$ at intervals of 0·5, 1, 2, 4 and 6 hr after treatment and at each 6-hr interval thereafter; urine and feces samples at 12, 24 and 48 hr after treatment; selected organs removed from the animals and the remaining carcass 48 hr after treatment. The analytical procedures used for determination of total radiocarbon and balance sheets for the radiocarbon were similar to those described by Krishna and Casida.[29]

Urinalysis. Unless stated otherwise, only the 12-hr urine samples were used for separation and characterization of the metabolites. Three cleanup procedures were used for the urine samples. For urinalysis after treatment with safrole, dihydrosafrole, sulfoxide B, piperonyl alcohol, piperonal and piperonylic acid, the urine was saturated with sodium chloride, acidified to pH 1·0 with hydrochloric acid, and extracted three times with 2 vol. of ether, recovering 92–97 per cent of the radiocarbon in the ether fraction. With urine containing metabolites of Tropital-M-^{14}C-DP, Tropital-α-^{14}C and piperonyl butoxide-M-^{14}C-DP, use was made of Bio-Rad SM-2 beads (nonionic, crosslinked polystyrene) from Bio-Rad Labs., Richmond, Calif. Approximately

2 g of hydrated beads for each milliliter of urine to be chromatographed were poured into a small glass column (1·9 × 10 cm) and the bed of beads was soaked for 20 min in methanol and washed with 20 bed-volumes of distilled water. The urine sample was acidified to pH 1 with hydrochloric acid, added to the column, and the eluate was recycled six times to insure maximum absorption of the radiolabeled metabolites to the beads. (The unretained fraction is referred to as the "recycle fraction.") After the absorption procedure, the column was washed with 30 ml of distilled water and the desired substances were eluted with methanol. Any precipitate that formed on evaporating the methanol eluate to 1/20 of the original volume was removed by centrifugation prior to TLC spotting. None of the radiocarbon was retained on the column in any case, and the recoveries in the methanol were 100 per cent for Tropital samples and 70–73 per cent for piperonyl butoxide-M-^{14}C-DP, the remaining radiocarbon appearing in the recycle and water fractions. The third cleanup procedure involved lyophilization, washing the residue with methanol, and treating the methanol solution in the same manner as for the methanol fraction from the Bio-Rad bead cleanup. This procedure resulted in no loss of radiocarbon, but removed little if any of the interfering, lipid-like materials in the urine.

Conjugates in the urine or those recovered as resolved products from TLC plates, by extracting the gel with methanol, were hydrolyzed under one of the following conditions: (1) treatment with either 6 N hydrochloric acid or sodium hydroxide for 6 hr at 100° in a sealed ampule; or (2) dissolving the residue (from evaporation of the sample) in 1·6 ml sodium acetate buffer (0·1 M, pH 4·5), adding 0·4 ml buffer or 0·4 ml buffer containing 3 mg β-glucuronidase (Nutritional Biochemicals Corp., Cleveland, Ohio) and incubation for 3 hr at 38°. After hydrolysis, the mixtures, if not already acid, were acidified and extracted three times with 2·5 vol. of spectrograde ether; the radiocarbon contents of the ether and aqueous fractions were determined and the products in the ether fraction were resolved by TLC analysis. Inter-comparisons of hydrolyzed and unhydrolyzed urine or resolved metabolites were made to detect possible loss of a radioactive product, appearance of a new radioactive product or, when working with mixtures, increase in intensity of one component.

In studies with unlabeled synergist, pure Tropital was administered orally to mice, rats, and rabbits at doses of 0·05, 0·5 and 5·0 ml respectively. The 0–24 hr urine samples were saturated with sodium chloride, acidified with hydrochloric acid, and extracted with ether. Each of these samples was subjected to TLC analysis in solvent systems c and d, using chromotropic acid for detection of MDP compounds. In addition, to isolate some of the metabolites, the ether extract of the acidified urine from four rats was dried over sodium sulfate, evaporated to about 1·0 g of yellow oil, dissolved in benzene and chromatographed on the silicic acid–Celite column (see above).

Enzyme studies. Mouse liver was homogenized at a 20% (w/v) level in 0·05 M sodium phosphate, pH 7·4, and the homogenate was fractionated into nuclear, mitochondrial, microsomal, and soluble fractions according to the procedure of Hogeboom.[30] The substrate, dissolved in 10–30 μl of ethanol, was added to 1·4 ml of the enzyme preparation (equivalent to 280 mg of mouse liver) in pH 7·4 phosphate buffer, and 0·6 ml of buffer or 0·6 ml of buffer containing 1·0 mg of cofactor was added to give a final volume of 2 ml in a 25-Erlenmeyer flask. This reaction mixture was incubated for 2 hr at 38°

in air with shaking. The substrate was either 5 μg of radiolabeled compound or 100 μg 3, 4-methylenedioxynitrobenzene (MDNB).

When MDNB was used as the substrate, the reaction was terminated by addition of 2 ml of cold acetone and, after centrifugation, the optical density of the soluble portion was determined at 432 mμ against a blank containing enzyme and substrate but no cofactor. (The reaction kinetics were followed only with the microsome–NADPH system, in which case the formation of the yellow product continued in a linear manner for about 15 min.) The absorption spectrum of the reaction product in the ultraviolet and visible regions was compared at pH 1·0, 7·4 and 11·0 to that of 4-nitrocatechol. In studies with potential inhibitors, the enzyme and cofactor medium was prepared as above, the test compound was introduced in 10 μl of ethanol and finally the MDNB was added. Analyses of the yellow product were made by the previously described method.

With radiolabeled substrates, the incubated reaction mixture was transferred to a 15-ml centrifuge tube along with a 1-ml distilled water rinse and extracted in one of two ways, depending on the substrate used. For Tropital-M-^{14}C-DP and piperonyl butoxide-α-^{14}C, the combined reaction mixture and rinse at pH 7·4 was extracted three times with 5-ml portions of spectrograde ether to give the "neutral fraction." The aqueous fraction was then acidified to pH 1·0 with hydrochloric acid and again extracted three times with 5-ml portions of spectrograde ether to yield the 'acidic fraction" and the remaining "aqueous fraction". With all other radiolabeled substrates, the combined reaction mixture and rinse was directly acidified and extracted three times with 5-ml portions of ether. In each case, the radiocarbon contents of the aqueous and ether fractions were determined. The ether fractions were dried over anhydrous sodium sulfate and subjected to TLC analysis for quantitation and cochromatography with known compounds, as previously described in the section on urinalysis.

The presence of formic acid-^{14}C and formaldehyde-^{14}C in reaction mixtures containing microsomes, in the presence or in the absence of NADPH, was determined by derivative formation with *p*-bromophenacyl bromide and dimedone respectively. Each incubation mixture was cooled on ice, transferred to a 15-ml centrifuge tube with a 1-ml distilled water rinse of the flask, the aqueous solution was extracted three times with ether, and the radiocarbon contents of the ether and the water phases were determined.

To determine the formic acid-^{14}C content, a 1·0 ml aliquot of the aqueous fraction was added to a 5-ml ampule along with 1·0 m-mole of unlabeled sodium formate, 1 drop of 0·1 N hydrochloric acid, 1·0 m-mole *p*-bromophenacyl bromide, 2 ml ethanol and a magnetic stirrer, in the order mentioned. The ampule was sealed and heated at 85° with stirring for 30 min; the contents were transferred to a 10-ml beaker along with a 2-ml ethanol rinse of the ampule and water was added to make a total volume of 10 ml. The precipitate was collected and thoroughly dried; TLC analysis, using solvent system b, revealed the presence of three products corresponding to the starting material, *p*-bromophenacyl formate and *p*-bromophenacyl alcohol, at R_f values of 0·92, 0·80 and 0·68 respectively. The formate ester was isolated by dissolving the entire precipitate in acetone, adding the solution to 2 g silicic acid, evaporating the acetone, and placing the silicic acid residue on a silicic acid–Celite column (10 g: 5 g) for chromatography. Elution with hexane–ether mixture (3 : 1) gave starting material in the first 90 ml of eluate, only *p*-bromophenacyl formate in the next 100 ml of eluate,

while *p*-bromophenacyl alcohol remained on the column. The *p*-bromophenacyl formate fraction was evaporated to dryness and the ester was recrystallized from ethanol to give 20–30 mg of white platelets (m.p. 99–100°, reported 99–100°[31,32]). A 10-mg sample was counted by liquid scintillation to determine the radiocarbon content; quenching was neglible with this sample size.

Formaldehyde-^{14}C content was determined by taking another 1-ml aliquot of the aqueous fraction, adding 50 μl of 7·4% formaldehyde solution (0·123 m-mole) and 10 ml of 0·5% aqueous dimedone solution (0·360 m-mole), and allowing the formaldehyde derivative to form during an 8-hr period at 25°. The resulting solid was dissolved in hot hexane, filtered, the hexane solution evaporated to dryness, and the residue recrystallized from methanol to yield white needles (m.p. 188–194°, reported 188°[33]). After drying and weighing, a 10-mg portion was counted by liquid scintillation, quenching was negligible.

RESULTS

Distribution of radiocarbon after oral administration of ^{14}C-labeled MDP and one-carbon compounds. As shown in Table 1 and Fig. 1, the radiocarbon from sodium carbonate-^{14}C, sodium formate-^{14}C and, to a lesser extent, from formaldehyde-^{14}C is rapidly and almost completely expired as $^{14}CO_2$. Although not in large amount, the radiocarbon content of urine, feces and the body increases with the decreasing rate and extent of expiration as $^{14}CO_2$, being least with carbonate-^{14}C, greatest with formaldehyde-^{14}C and intermediate with formate-^{14}C.

TABLE 1. FATE OF RADIOCARBON IN VARIOUS MAMMALS RECEIVING ORAL DOSES OF METHYLENEDIOXYPHENYL-^{14}C COMPOUNDS AND CERTAIN METABOLICALLY RELATED ONE-CARBON COMPOUNDS

^{14}C-labeled compound	Species	Radiocarbon recovered after 48 hr (%)*						
		CO_2	Urine	Feces	Intestine	Liver	Carcass	Total
One-carbon compounds								
Sodium carbonate	mouse	90·6	4·4	0·6	0·6	0·3	2·3	98·8
Formaldehyde	mouse	65·1	7·7	11·0	4·9	2·0	10·4	101·1
Sodium formate	mouse	83·1	4·5	3·1	4·6	1·0	7·5	103·8
Methylene-^{14}C-dioxyphenyl compounds								
Dihydrosafrole	mouse	64·4	18·2	4·0	2·2	2·1	5·7	96·6
Safrole	mouse	61·2	23·4	3·1	2·1	2·5	9·1	101·4
Myristicin	mouse	72·8	15·0	3·0	3·1	1·5	6·1	101·5
Sulfoxide A	mouse	63·6	11·0	5·8	2·3	2·7	5·7	91·1
Sulfoxide B	mouse	69·1	7·7	4·9	2·3	1·6	5·0	90·6
Piperonyl alcohol	mouse	3·0	93·3	8·5	0·2	0·1	0·3	105·4
Piperonal	mouse	1·1	89·0	3·2	0·4	0·4	1·2	95·3
Piperonylic acid	mouse	0·9	86·9	9·9	0·2	0·2	2·5	100·6
Tropital†	mouse	3·7	97·9	0·9	0·0	0·1	1·4	104·0
	rat	2·3	84·1					86·9
	hamster	4·2	97·0					101·2
Piperonyl butoxide	mouse	75·5	6·1	4·0	2·3	2·5	6·8	97·2
	rat	65·6	6·3					71·9
Methylenedioxyphenyl-α-^{14}C compounds								
Tropital‡	mouse	0·2	94·8	2·4	0·5	0·1	0·4	98·4
Piperonyl butoxide	mouse	0·3	65·4	8·0	0·8	0·1	0·4	75·0
	rat	0·3	73·3					73·6

* Average of two to four experiments.
† Radiochemical purity, 99%.
‡ Radiochemical purity, 92–95%.

M-^{14}C-DP compounds fall into two groups in relation to the major route of elimination of radiocarbon from the body (see Table 1 and Figs. 1–3). Dihydrosafrole, safrole, myristicin, sulfoxide A, sulfoxide B and piperonyl butoxide are largely metabolized by oxidation of the methylene group of the MDP moiety to yield $^{14}CO_2$ (61–76%), the radiocarbon residue in the carcass (5–9%) approximating that expected if the portion of radiocarbon ultimately appearing as $^{14}CO_2$ was first liberated as formate-^{14}C. Piperonyl alcohol, piperonal, piperonylic acid and Tropital are not importantly oxidized to $^{14}CO_2$ (1–4%) or retained as labeled compounds in the carcass (0·3–3%) probably because the radiolabeled metabolites are rapidly and almost quantitatively excreted, particularly in urine. No marked species difference is noted with mice, rats and hamsters in the radiocarbon distribution after administration of Tropital-M-^{14}C-DP, and with mice and rats treated with piperonyl butoxide-M-^{14}C-DP. The dose is not critical to the results because the data, obtained with Tropital-M-^{14}C-DP and mice, varied little, if at all, with doses of 5, 50, 500 or 5000 μg/mouse.

The radiocarbon of α-^{14}C preparations of piperonyl butoxide and Tropital is almost entirely eliminated from the body, mostly in the urine (see Table 1 and Figs. 2 and 3), with little difference between mice and rats (Table 1 and Fig. 3) or between a 5 and 5000 μg/mouse dose for the former compound. The M-^{14}C-DP and α-^{14}C samples of Tropital result in similar rates and extents of radiocarbon elimination in urine, suggesting that the metabolic attack occurs at the side chain rather than at the MDP moiety. In contrast, the M-^{14}C-DP label in piperonyl butoxide is rapidly expired as $^{14}CO_2$ while the α-^{14}C label is eliminated more slowly and appears in urine as demethylenated products.

The total recoveries of administered radiocarbon are better than 90 per cent for the studies with mice, the one exception being piperonyl butoxide-α-^{14}C, where the low value of 75 per cent remains unexplained. Totals for the rat and hamster are sometimes lower than 90 per cent because the radiocarbon content of feces and the residual carcass are not included. Most of the variation in radiocarbon distribution with different labeled compounds probably results from the manner in which they are absorbed, distributed and metabolized; some of the variation possibly results from incomplete separation of urine and feces.

Urinary metabolites of Tropital, piperonal, piperonyl alcohol and piperonylic acid. As shown in Table 2 and Fig. 4, Tropital is not excreted in urine, but a series of metabolites are present which do not vary appreciably in their proportion with accumulated 0–12 hr urine samples from mice, rats and hamsters. With two exceptions, all of the metabolites (A to J) are detected with both labeled preparations; metabolite B occurs only with the α-^{14}C compound and metabolite F is detected only with the M-^{14}C-DP compound (Fig. 4). Metabolites C and D appear only with impure samples of Tropital.

The major Tropital-^{14}C metabolite in urine (A) cochromatographs with piperonylglycine. It is not attacked by β-glucuronidase, but it is hydrolyzed by alkali to a labeled product cochromatographing with piperonylic acid. Metabolite B, which lacks the MDP-moiety because it appears only with the α-^{14}C preparation, cochromatographs with 3, 4-dihydroxybenzoic acid. Metabolites C and D comprise up to 1 per cent of the radiocarbon in mouse urine, but only when Tropital-^{14}C of less than 90 per cent purity is used; they probably are derived from piperonal metabolism because piperonal is the major impurity in Tropital and because piperonal appears to give these same compounds (see below). Metabolite E cochromatographs with piperonylic acid, both

before and after treatment with base or β-glucuronidase; no explanation is available for the finding that this metabolite appears in greater amount with the M-^{14}C-DP preparation than with the α-^{14}C preparation. Although metabolite F is detected only with the M-^{14}C-DP sample, it is hydrolyzed by base, but not by β-glucuronidase, to a product cochromatographing with piperonylic acid, suggesting that it is a conjugate of piperonylic acid. Metabolite G is possibly the β-D-glucuronide of piperonylic acid because it is hydrolyzed by either alkali or β-glucuronidase to liberate piperonylic acid. Metabolites H, I and J are poorly resolved under the chromatography conditions used; each is detected with the M-^{14}C-DP preparation; and at least two of the three

TABLE 2. METABOLITES OF TROPITAL IN URINE OF MICE, RATS AND HAMSTERS ACCUMULATED 0–12 hr AFTER ORAL ADMINISTRATION OF M-^{14}C-DP OR α-^{14}C PREPARATIONS

Metabolite designation	Tentative identity of metabolite	Radiocarbon recovered after 12 hr (%)*			
		Mice		Rats	Hamster
		α-^{14}C†	M-^{14}C-DP‡	M-^{14}C-DP	M-^{14}C-DP
A	Piperonylglycine	75·6	82·4	83·0	77·9
B	3,4-Dihydroxybenzoic acid	trace	0·0	0·0	0·0
C, D	Unknown	0·0	trace	0·0	0·0
E	Piperonylic acid	0·1	1·3	1·4	1·1
F	Conjugate of piperonylic acid	0·0	0·9	0·1	0·1
G	β-D-Glucuronide of piperonylic acid	3·0	6·7	3·7	2·0
H, I, J	Unknown	10·4	8·2	10·4	9·1

* Average of two to four experiments.
† Radiochemical purity, 92–96%.
‡ Radiochemical purity, 99%.

appear with the α-^{14}C label, but their composition is not as yet known. None of the Tropital metabolites cochromatographs with piperonyl alcohol, piperonal, 5-hydroxypiperonylic acid, 6-hydroxypiperonylic acid or 3, 4-dihydroxybenzaldehyde (see Fig. 4).

There are limited data on the effect of dose and time on the nature of Tropital-M-^{14}C-DP metabolites in mouse urine. Urine excreted between 12 and 48 hr after Tropital administration contains less than 5 per cent of the administered radiocarbon and consists mostly of piperonylic acid and the glycine conjugate, the former metabolite predominating. As the administered Tropital dose is increased from 5 μg/mouse to 50, 500 and 5000 μg/mouse, piperonylic acid or other metabolites become predominant over piperonylglycine as the major excreted product.

Studies with unlabeled Tropital, utilizing detection of metabolites on TLC plates with chromotropic acid reagent, show the presence of metabolites A, C, D and E, material(s) remaining at the origin, and a material chromatographing in the region of piperonal in the ether extract of urine of mice, of rats and of rabbits. Column chromatography of the metabolites in rat urine yields three major regions for elution of MDP compounds: fractions 32–49 contain piperonylic acid (m.p. 220–226°, reported 229°;[23] infrared spectrum and TLC properties identical with authentic compound) and piperonal (appropriate TLC R_f value; not acidic); fractions 54–120 contain metabolites C and D, along with some piperonal and other impurities in the first fractions (but

the metabolites were not isolated in a pure state); fractions 186–263 yield only piperonylglycine [m.p. 182–183°, mixed m.p. 181°; found: C, 53·94, H, 3·93; N, 6·38; infrared spectrum and TLC properties identical to authentic compound; basic hydrolysis yields piperonylic acid (m.p. 220–224°; appropriate TLC and infrared spectral properties)].[25]

M-^{14}C-DP preparations of piperonal, of piperonyl alcohol and of piperonylic acid give, in the 12-hr accumulated mouse urine, piperonylglycine as the major metabolite along with some piperonylic acid. Piperonyl alcohol and piperonal, but not piperonylic acid, give metabolites C and D, and the individual amounts of these metabolites obtained from piperonyl alcohol and piperonal administration approximate the amount of piperonylic acid found. The accumulated 12–48 hr urine samples contain piperonylic acid and piperonylglycine after piperonal and piperonylic acid administration, but only piperonylglycine is present after piperonyl alcohol administration.

Urinary metabolites of safrole, dihydrosafrole, sulfoxide B and piperonyl butoxide. Metabolites recovered in ether on extraction of the acidified accumulated 0–12 hr urine samples from mice treated with M-^{14}C-DP preparations of safrole, dihydrosafrole and sulfoxide B were separated by TLC using solvent systems c and d in two dimensions. The ether extracts contain 15, 19 and 4 per cent, respectively, of the administered radiocarbon, and contain the following compounds: dihydrosafrole and safrole, 6 and 5 metabolites, respectively, including in each case one cochromatographing with piperonylic acid but none cochromatographing with piperonyl alcohol or piperonylglycine; sulfoxide b, 3 unidentified metabolites.

Piperonyl butoxide-M-^{14}C-DP results in a level of radiocarbon in the urine (6%) approximating that amount arising from sodium formate-^{14}C administration (5%) (see Table 1). Therefore, it is possible that the metabolites in the urine from piperonyl butoxide-M-^{14}C-DP administration are the same as those from formate-^{14}C administration, the synergist being converted initially and completely to formate-^{14}C, which is then metabolized. However, this is not the case, because the piperonyl butoxide-M-^{14}C-DP and formate-^{14}C metabolites fractionate differently on the Bio-Rad bead column, 80 per cent of the former metabolites appearing in the methanol fraction but none of the metabolites of formate-^{14}C appearing in this fraction. No more than 1 per cent of the administered dose of piperonyl butoxide-M-^{14}C-DP exists in urine as formate metabolites and at least 5 per cent are MDP compounds.

Metabolites of both M-^{14}C-DP- and α-^{14}-C-piperonyl butoxide also were separated by TLC analysis, utilizing the Bio-Rad bead cleanup for the urine from animals receiving the M-^{14}C-DP preparation and utilizing lyophilization and methanol extraction with the urine from those receiving the α -^{14}C preparation. The recoveries of administered radiocarbon for TLC analyses are 5·2 and 65·4 per cent for the M-^{14}C-DP and α-^{14}C preparations respectively. The metabolite pattern found is shown in Fig. 5. At least 18 metabolites appear with the α-^{14}C preparation, only 12 being evident with the M-^{14}C-DP preparation. Metabolites A to K, collectively, represent the following percentages of the administered radiocarbon: 1·6 per cent for the M-^{14}C-DP preparation; 8·3 per cent for the α-^{14}C preparation. The only metabolites tentatively identified are 6-propylpiperonylic acid and 6-propylpiperonylglycine, each representing less than 0·5 per cent of the administered radiocarbon. Several other compounds suspected of being metabolites chromatographing in the region of A to K do not cochromatograph with the minor metabolites in this region (see Fig. 5). The major piperonyl butoxide

metabolites appear in the region of metabolites L to R, comprising 2 per cent of the M-^{14}C-DP radiocarbon and 50 per cent of the α-^{14}C radiocarbon; this difference in radiocarbon content strongly suggests that the piperonyl butoxide metabolites in this chromatographic region, for the most part, lack the MDP moiety.

Metabolism of MDP compounds by mixed-function oxidases of mouse liver microsomes. Extensive demethylenation of MDNB to the corresponding catechol occurs

TABLE 3. DISTRIBUTION OF RADIOCARBON AFTER INCUBATION OF VARIOUS METHYLENEDIOXYPHENYL-^{14}C COMPOUNDS WITH MOUSE LIVER MICROSOMES AND TWO COFACTORS

^{14}C-labeled substrate	Radiocarbon fraction*	Radiocarbon recovered (%) with indicated cofactor addition†		
		None	NADH	NADPH
Methylene-^{14}C-dioxyphenyl compounds				
Dihydrosafrole	ether-neutral	1	3	12
	water	0	5	24
	loss	99	92	64
Safrole	ether-neutral	2	3	7
	water	1	1	5
	loss	97	96	88
Myristicin	ether-neutral	70	53	47
	water	2	6	23
	loss	28	41	30
Sulfoxide A	ether-neutral	94	89	78
	water	6	11	22
Sulfoxide B	ether-neutral	96	94	80
	water	4	6	20
Piperonyl alcohol	ether-neutral	92	96	76
	water	1	2	10
	loss	7	2	14
Piperonal	ether-neutral	98	94	50
	water	0	2	6
	loss	2	4	44
Piperonylic acid	ether-neutral	100	100	100
Tropital	ether-neutral	82	49	34
	ether-acid	8	19	23
	water	0	4	6
	loss	10	28	37
Piperonyl butoxide	ether-neutral	90	87	50
	water	10	13	50
Methylenedioxyphenyl-α^{14}-C compound				
Piperonyl butoxide	ether-neutral	79	22	8
	ether-acid	2	5	13
	water	0	31	31
	loss	19	42	48

* Values for loss and water soluble products are tabulated only when they constitute more than 4% of the radiocarbon in the presence of one of the cofactors.

† Average of at least two experiments.

only with preparations containing liver microsomes fortified with NADPH, but not with NAD, NADH or NADP. The reaction product is identical to 4-nitrocatechol in respect to its absorption spectrum in the visible region at pH 1·0, 7·4 and 11·0, and it does not further degrade in the enzymatic system. Addition of the soluble fraction of the liver homogenate to the microsomal fraction increases the extent of nitrocatechol liberation, but only in the presence of added NADPH; the effect is progressive when

the amount of soluble fraction ranges from that equivalent to 10 mg liver to that equivalent to 220 mg liver, the addition of the higher levels of soluble fraction more than doubling the nitrocatechol liberation. When added to the incubation mixture at the 100 μg level, the following compounds, which are known to be substrates for or inhibitors of microsomal mixed-function oxidases, reduce the nitrocatechol liberation from 100 μg of MDNB: allethrin; pyrethrin I; from *N*-octyl bicycloheptene dicarboximide (MGK 264); β-diethylaminoethyl 2, 2-diphenyl propylacetate hydrochloride (SKF-525A); and piperonyl butoxide. These findings suggest that the microsomal mixed-function oxidases are involved in the demethylenation of MDNB.[3, 25]

Based on the data from radiolabeled MDP studies given in Table 3, there is no doubt that the microsome–NADPH system is involved in metabolism of MDP compounds, but the results obtained depend of the substrate used, because of the varying volatility of the substrates and of the nature and partitioning characteristics of the products formed. (Table 3 contains the most important findings only; therefore, it gives only a portion of the available data.)[25] Not tabulated in Table 3 are data supporting the following conclusions: NAD and NADP are less effective or ineffective as cofactors with the microsomal fraction; the nuclear, mitochondrial and soluble fractions are of low metabolizing activity even with NADPH fortification; NADPH enhances metabolism more than NADP with whole homogenates; when fortified with NADPH, the activity of the microsome-plus-soluble fraction is equal to or greater than that of the microsomal fraction alone.[25]

As shown in Table 3, metabolism by microsomes is enhanced to a greater extent by NADPH fortification than by NADH addition, with most of the labeled substrates. Metabolism of the volatile substrates (dihydrosafrole, safrole and myristicin) results in formation of less volatile products and products remaining in the aqueous phase on extraction with ether. Sulfoxide A and sulfoxide B are converted to water-soluble products. Piperonyl alcohol and piperonal are converted to water-soluble products in addition to products of greater volatility than the original compound or products not recovered for radioanalysis, while no change occurs with the extraction or recovery characteristics of piperonylic acid. Tropital is converted to acidic products, water-soluble products, and volatile materials or materials which are not recovered for radioanalysis. Piperonyl butoxide-M-^{14}C-DP is converted to water-soluble products and the α-^{14}C preparation forms acidic products, water-soluble products, and products not recovered for radioactivity counting.

In Table 4, a comparison is given of the water-soluble products formed from M-^{14}C-DP compounds by the liver microsome system, with or without NADPH fortification, with those formed, under the same conditions, from sodium carbonate-^{14}C, formaldehyde-^{14}C and sodium formate-^{14}C. The one-carbon compounds remain largely unmetabolized, even with NADPH fortification, as indicated by the following observations: the radiocarbon of sodium carbonate-^{14}C is lost and does not become incorporated during incubation; formaldehyde-^{14}C suffers little loss and only partial conversion to formate-^{14}C and other labeled compounds; formate-^{14}C remains mostly unchanged and is not converted to other labeled materials. With the M-^{14}C-DP preparations of dihydrosafrole and safrole, the large loss without NADPH fortification probably results from volatilization of the unmetabolized compound; the metabolites formed on NADPH fortification are less volatile, so they persist and are recovered as ether—

or water-soluble products. With the four less volatile M-^{14}C-DP compounds (myristicin, sulfoxide A, sulfoxide B and piperonyl butoxide), the ether-soluble products are markedly reduced by fortification with NADPH, the cofactor greatly increasing the radiocarbon in the aqueous fraction; this radiocarbon consists almost entirely of formate-^{14}C with no significant amount of formaldehyde-^{14}C and only small amounts of other labeled compounds. Thus, NADPH fortification is necessary for extensive formation of water-soluble products and for the release of formate-^{14}C from the MDP moiety. Formaldehyde is not an intermediate in the formation of formate because it is sufficiently stable under the incubation conditions to be detected in the incubated mixture if it were formed, but the fact is that none is found.

TLC analysis of the radiolabeled metabolites recovered on extraction of enzyme

TABLE 4. DISTRIBUTION OF RADIOCARBON AFTER INCUBATION OF METHYLENE-^{14}C-DIOXYPHENYL COMPOUNDS AND CERTAIN METABOLICALLY RELATED ONE-CARBON COMPOUNDS WITH MOUSE LIVER MICROSOMES IN THE PRESENCE AND IN THE ABSENCE OF NADPH

		Radiocarbon recovered in indicated fraction (%)				
				Aqueous fraction		
^{14}C-labeled substrate	NADPH (mg)	Loss during incubation and extraction	Ether extract	Formate-^{14}C	Formalde-hyde-^{14}C	Other labeled compounds
One-carbon compounds						
Sodium carbonate	0	100	0	0	0	0
	1	100	0	0	0	0
Formaldehyde	0	12	1	15	61	12
	1	9	1	15	48	27
Sodium formate	0	5	0	114	0	−19
	1	4	0	120	0	−24
Methylene-^{14}C-dioxyphenyl compounds						
Dihydrosafrole	0	95	3	1	0	1
	1	47	23	31	2	−3
Safrole	0	87	11	0	0	2
	1	61	26	15	0	−2
Myristicin	0	24	73	1	1	1
	1	29	38	32	0	1
Sulfoxide A	0	1	92	1	0	6
	1	3	75	20	1	1
Sulfoxide B	0	−2	100	0	0	2
	1	6	74	15	0	5
Piperonyl butoxide	0	3	86	2	0	8
	1	6	46	50	0	−3

incubation mixtures with ether serves to identify tentatively only a few of the many products formed (Table 5). In each case, degradation of the original compound occurs to give one or a greater number of more polar metabolites, the number of such metabolites being unusually high with the sulfoxide diastereoisomers and piperonyl butoxide. Dihydrosafrole, safrole and myristicin are converted, in NADPH dependent reactions, to one or two metabolites of unknown nature. Sulfoxide A and sulfoxide B are converted to 8 and 9 metabolites, respectively, two of these being tentatively identified as the corresponding catechols and the sulfone resulting from oxidation of the sulfoxide group. Each of these conversions is dependent on NADPH fortification except for the formation of the sulfone, which appears in an amount of 11 and 8 per cent for the A and B isomers respectively. Only a single metabolite forms with piperonyl

TABLE 5. TLC R_f VALUES FOR METABOLITES FORMED ON INCUBATION OF METHYLENEDIOXYPHENYL-^{14}C COMPOUNDS WITH THE MOUSE LIVER MICROSOME–NADPH SYSTEM

^{14}C-labeled substrate	Solvent system	R_f values: Substrate	Unidentified metabolites	Tentatively identified metabolites*
Dihydrosafrole	b	0·96	0·21, 0·38	none
Safrole	b	0·90	0·21	none
Myristicin	b	0·90	0·10. 0·20	none
Sulfoxide A	c	0·58	0·00, 0·22, 0·32, 0·37, 0·49, 0·55	0·53(C), 0·88(S)
	d	0·24	0·00, 0·03, 0·05, 0·08, 0·09, 0·23	0·15(C), 0·43(S)
Sulfoxide B	c	0·54	0·00, 0·13, 0·21, 0·26, 0·41, 0·54, 0·70	0·46(C), 0·86(S)
	d	0·30	0·00, 0·02, 0·04, 0·07, 0·26, 0·30, 0·42	0·19(C), 0·46(S)
Piperonyl alcohol	c	0·76	0·70	none
Piperonal-M-^{14}C-DP	c	0·90	0·69	0·34†(PG), 0·87(PA)
	d	0·90	0·60	0·12†(PG), 0·81(PA)
Piperonal-α-^{14}C	c	0·90	0·69	0·34†(PG), 0·63(DHB), 0·87(PA)
	d	0·90	0·60	0·12†(PG), 0·27(DHB), 0·81(PA)
Piperonylic acid	c	0·87	none	0·34†(PG)
	d	0·81	none	0·12†(PG)
Tropital-M-^{14}C-DP	c	‡	0·67	0·34†(PG), 0·87(PA)
	d		0·61	0·12†(PG), 0·81(PA)
Tropital-α-^{14}C	c	‡	0·67	0·34†(PG), 0·63(DHB), 0·87(PA)
	d		0·61	0·13†(PG), 0·27(DHB), 0·81(PA)
Piperonyl butoxide-M-^{14}C-DP	a	0·86	0·00, 0·03, 0·06, 0·13, 0·36, 0·44, 0·68, 0·79, 0·84, 0·90	none
Piperonyl butoxide-α-^{14}C	c	0·67	0·00, 0·12, 0·19, 0·30, 0·50, 0·52, 0·57, 0·67	0·59(PPA)
	d	0·49	0·00, 0·01, 0·04, 0·08, 0·18, 0·27, 0·36, 0·49	0·60(PPA)

* C = sulfoxide catechol; S = sulfone of the sulfoxide synergist; PA = piperonylic acid; PG = piperonylglycine; DHB = 3,4-dihydroxybenzoic acid; PPA = 6-propylpiperonylic acid.

† Present only in the whole homogenate but not in microsomal fractions.

‡ Tropital degrades to piperonal under the extraction conditions and by development in acidic solvent systems c and d.

alcohol and this product remains unidentified except that NADPH is required for its formation. The M-^{14}C-DP preparations of piperonal and Tropital yield the same three metabolites, two of these being formed by the microsomes in NADPH-dependent reactions, including piperonylic acid and an unidentified metabolite, and the third being piperonylglycine, which appears only from the incubations with the whole homogenate. Similarly, the MDP-α-^{14}C preparations of these two compounds yield the same three metabolites mentioned above plus small amounts of 3,4-dihydroxybenzoic acid resulting from demethylenation, a reaction dependent on NADPH. Incubation of piperonyl butoxide-M-^{14}C-DP with microsomes and TLC analysis of the products in a single solvent system reveal the presence of at least 9 unidentified metabolites in addition to material remaining at the origin. The α-^{14}C preparation yields 8 metabolites, most of which are dependent on fortifying the microsomes with NADPH (TLC analysis accomplished on Silica gel F_{254} "precoated" plates from Brinkmann Instruments, in two dimensions, using solvent systems c and d). 6-Propylpiperonylic acid and two alcohols resulting from cleavage of the polyether side chain (metabolites A′ and B′, Fig. 5), but not 6-propylpiperonyl alcohol, are tentatively identified as minor metabolites with the α-^{14}C preparation. It is not known which metabolite of the M-^{14}C-DP sample corresponds to any particular product of the α-^{14}C sample because different solvent systems were used for TLC analysis.

DISCUSSION

Demethylenation of the MDP moiety is the major metabolic pathway in mammals with piperonyl butoxide, the sulfoxide diastereoisomers, safrole, dihydrosafrole and myristicin, while side chain oxidation or conjugation (or both) predominates with Tropital, piperonal, piperonyl alcohol and piperonylic acid. The microsome–NADPH enzyme system is involved in the demethylenation and in certain of the side chain modification reactions. Cleavage of the MDP group by the microsome–NADPH system results in liberation of the corresponding catechol (as shown with the sulfoxide diastereoisomers, Tropital, piperonal and MDNB) and production of formate, which forms directly and not from oxidation of formaldehyde. These results indicate that the intermediate is possibly a hydroxymethylenedioxyphenyl compound which is unstable and undergoes hydrolysis to a monoformate ester and then to formate and the catechol.[3] An alternative possibility, suggested by physicochemical considerations,[34–36] is that the oxidation results in formation of a benzodioxolium ion which acylates a protein, yielding formate on hydrolysis of the acylated protein. The M-^{14}C-DP compounds that are extensively converted to formate or to water-soluble metabolites in the microsome–NADPH system are the same ones that are extensively metabolized to $^{14}CO_2$ *in vivo*, and the ones giving small amounts of water-soluble products *in vitro* give little $^{14}CO_2$ *in vivo*. Thus, the nature of attack on the substrate by the microsome–NADPH system apparently governs the fragments liberated for deposition or excretion by the intact animal and this in turn relates to the nature of the groupings in the side chain and the ease with which they are oxidized, hydrolyzed or conjugated.

Dihydrosafrole, safrole, myristicin, sulfoxide A and sulfoxide B are extensively demethylenated in living mice and are converted to more polar and yet ether-soluble metabolites in the liver microsome–NADPH system. The first two compounds listed give small amounts of piperonylic acid in the urine along with unidentified metabolites,

while sulfoxide B gives several unidentified MDP compounds in urine. Liver microsomes oxidize sulfoxide to the corresponding sulfone and demethylenate it to the corresponding catechol, the latter reaction requiring NADPH fortification.

Piperonyl alcohol, piperonal and piperonylic acid are excreted largely as MDP compounds, with small and decreasing amounts of demethylenation in the order given. It is reasonable to expect the following conversion sequence: alcohol to aldehyde to acid to conjugates of the acid with glycine or glucuronic acid. The acid and glycine conjugates are tentatively identified from urine in each case, but other unknown metabolites are also present. However, in enzyme studies, the mouse liver fractions degrade piperonyl alcohol to an unidentified metabolite which is not piperonylic acid; no piperonylic acid is produced by any of the reaction mixtures studied and no piperonylglycine is formed in the whole homogenate incubations. In the case of piperonal, some is converted in the enzyme systems to piperonyl alcohol, but most goes to piperonylic acid. In the whole homogenate, both piperonal and piperonylic acid are converted to piperonylglycine. Piperonal is demethylenated and oxidized, in very small part, to 3, 4-dihydroxybenzoic acid in the microsome–NADPH system. The polar nature of these compounds or their ease of conversion to polar products probably minimizes their entrance into the lipid components of the microsomal enzymes so that extensive demethylenation is not involved.

A tentative metabolic pathway for Tropital, based on the present studies, is given in Fig. 6. These results support and extend those of Fishbein *et al.*[16, 17] While not a

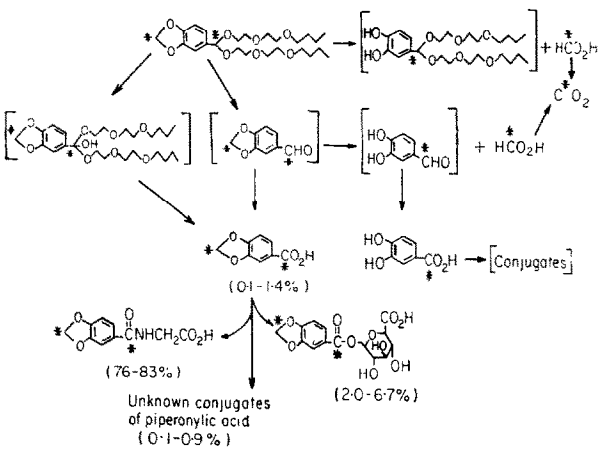

FIG. 6. Tentative metabolic fate of Tropital in mammals.

major mechanism, Tropital or an intermediate in its metabolism is demethylenated at the MDP moiety to yield $^{14}CO_2$, which is expired. Products arising in equivalent amounts from the liberated catechol probably are excreted, either as such or as a conjugate of sulfuric acid or glucuronic acid. In the mouse liver microsome–NADPH system, the demethylenation product is 3,4-dihydroxybenzoic acid. The major detoxification mechanism for Tropital *in vivo*, accounting for more than 76 per cent of the dose, is oxidation of the side chain to form piperonylic acid and excretion as the free acid or conjugates. Tropital is converted to piperonylic acid either by initial

acid-catalyzed hydrolysis in the stomach to give piperonal or by enzymatic hydroxylation at the α-carbon to yield piperonylic acid without going through piperonal; unfortunately, the available evidence is not adequate to differentiate these alternatives in living animals. Tropital conversion to piperonylic acid by the mouse liver enzyme system is enhanced by NADPH; this suggests either that the α-methinyl group of the side chain is hydroxylated to yield an unstable intermediate, which decomposes to yield piperonylic acid, or that the cofactor enhances the oxidation of piperonal to piperonylic acid. Among other enzymes, aldehyde dehydrogenase of liver is known to oxidize aromatic aldehydes to aromatic acids.[37]

Tropital is metabolized in living mice, rats and hamsters to the same metabolites which vary only in their relative amounts; so, the metabolic pathway is not species-dependent among the species studied. The ratio of the Tropital metabolites excreted by mice is partially dose-dependent. The system for formation of piperonylglycine apparently does not efficiently conjugate large doses of piperonylic acid; so, when large amounts are present due to high Tropital doses, the free acid and glucuronic acid ester are excreted. Studies elsewhere indicate that piperonylic acid is excreted as the free acid, glycine conjugate and glucuronic acid conjugate[38, 39] and that, with benzoic acid, this conjugation with glycine accounts for almost all of the amount with low doses, but at high doses the glucuronic acid conjugation also occurs.[39, 40]

A tentative, partial metabolic pathway for piperonyl butoxide is given in Fig. 7.

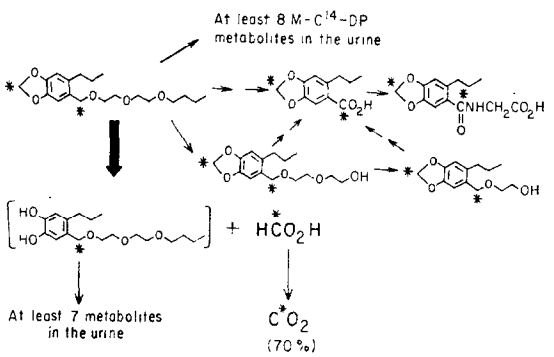

FIG. 7. Tentative metabolic fate of piperonyl butoxide in mammals.

Many sites on the molecule are attacked, as evidenced by the large number of metabolites detected in the urine, in confirmation of the results of Fishbein *et al.*[17] Demethylenation is the primary mechanism of metabolism *in vivo* and *in vitro*, the microsome–NADPH system being responsible for this cleavage. Side chain oxidation also occurs because 6-propylpiperonylic acid and 6-propylpiperonylglycine appear in the urine and two of the three possible alcohols from cleavage of ether groups in the butylcarbityl moiety are detected in the enzyme studies. The catechol derived on demethylenation of piperonyl butoxide does not occur in urine or enzyme systems, suggesting that the catechol is unstable or that side chain modification also occurs; the sequence of side chain oxidation and demethylenation is unknown. The propyl and butylcarbityl groups possibly suffer additional hydroxylation and degradation, based on

analogy with the metabolism of piperonyl butoxide in houseflies and of other compounds in mammals.[21, 39, 41]

Jaffe *et al.*[9] found strong inhibition of liver microsomal metabolism of dimethylaminopyrine 1 hr after intraperitoneal administration to mice of piperonyl butoxide, dihydrosafrole and safrole, but not after injection of sulfoxide, Tropital, piperonal, piperonyl alcohol or piperonylic acid; the present study establishes that the first three compounds, but not the last five, are undergoing extensive demethylenation of the MDP moiety at this interval after oral administration. These findings suggest a possible correlation, at any given time after administration, between the extent of demethylenation (or perhaps, as well, of other microsomal hydroxylation reactions acting on the MDP compound) and the inhibition *in vivo* of microsomal hydroxylations of other substrates. Thus, the MDP compounds possibly serve as competitive inhibitors or alternative substrates for the detoxifying enzymes.[3] In utilizing this hypothesis, consideration must be given to the relative biological stability of the MDP compound and the alternative substrate, and to the inhibitor and substrate specificities of the liver mixed-function oxidase system(s).

Acknowledgements—We wish to thank the following for their suggestions, assistance or contribution of materials: Judith L. Engel, Esaac G. Essac, Shozo Kuwatsuka and Louis Lykken, Division of Entomology, University of California, Berkeley; Joseph B. Moore and Griffin J. Baker, McLaughlin Gormley King Co., Minneapolis, Minn.; Donald E. Whyte, Robert V. Smith and Clayton W. Yoho, S. C. Johnson & Son, Inc., Racine, Wis.; Thomas F. Sullivan, New England Nuclear Corp., Boston, Mass.

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