Metabolites of 3-Phenyl-5-methyl-1,2,4-oxadiazole (PMO) in Rats, Dogs, and Mice

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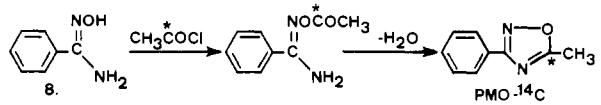
Metabolites of 3-phenyl-5-methyl-1,2,4-oxadiazole (PMO), a potential tobacco additive, were isolated from the physiological fluids of rats, dogs, and mice. PMO is metabolized by hydroxylation at the para phenyl and the methyl positions. Also, the methyl position was the site of attachment of *N*-acetylcysteine to form the mercapturic acid derivative. Urine was found to contain the glucuronide and sulfate conjugates of the hydroxylated materials in addition to the hydroxylated materials themselves. The glutathionyl precursor of the mercapturic acid was the major metabolite in rat bile. Metabolites of PMO-5-^{14}C were distributed as follows in the rat: urine, 48%; feces, 23%; CO_2 in breath, 13%; intestines, 3%; and other body tissues, 5% of the original PMO dose.

During studies on the effect of antitussive agents added to tobacco, Dalhamn (1969) found that the smoke of cigarettes treated with oxolamine citrate (1) (see Chart I for structures) was rendered less ciliotoxic to tracheal cilia of live cats than the smoke of untreated cigarettes. Testing of other 1,2,4-oxadiazoles revealed that 3-phenyl-5-vinyl-1,2,4-oxadiazole (2), a pyrolysis product of oxolamine citrate, and 3-phenyl-5-methyl-1,2,4-oxadiazole (PMO) (3) were also physiologically active (Dalhamn and Rylander, 1971; Dahlgren and Dalhamn, 1972). Our studies were undertaken to determine the metabolic fate of PMO in animals. Other pharmacological data will be published elsewhere.

PMO was administered orally to rats, dogs, and mice. The 24-h urine was examined for metabolites. Bile was collected from a cannulated rat over a 7-h period after dosing. In most instances, after physical data on a purified metabolite indicated a probable structure, the suspected metabolite was prepared synthetically for verification. The identified PMO metabolites are either products formed by hydroxylation on the phenyl ring, 4, and on the methyl group, 5, or the mercapturic acid conjugate, 6, and the glutathionyl conjugate, 7, formed by substitution on the methyl group. In urine, the hydroxylated materials are additionally present as glucuronide or sulfate conjugates. Finally, the material balance was determined in rats using PMO labeled with radiocarbon in the 5 position of the oxadiazole ring, PMO-^{14}C.

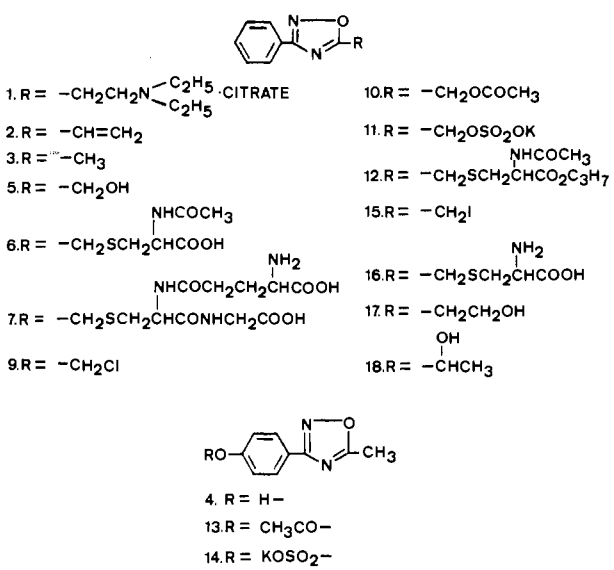


MATERIALS AND METHODS

Synthesis of Metabolites. With the exception of the glucuronides, compounds which were suspected of being present as PMO urinary metabolites were synthesized and characterized. Infrared spectra were run as KBr pellets on a Perkin-Elmer Model 621 infrared spectrophotometer. Ultraviolet spectra were determined on a Beckman DK-2 spectrophotometer. Mass spectra were obtained either on a CEC Type 21-104 mass spectrometer with accelerating voltage scans using the direct probe inlet system, or commercially with a Hitachi Perkin-Elmer RMU-6D mass spectrometer on samples submitted to Morgan-Schaffer Corp., Montreal, Quebec, Canada. Elemental analyses were obtained from Galbraith Laboratories, Knoxville, Tenn. Samples of most compounds have been forwarded to Sadtler Research Laboratories, Inc., Philadelphia, Pa.

Lorillard Research Center, 420 English Street, Greensboro, North Carolina 27420.

Chart I. Structures of Synthesized Compounds[a]



[a] Compounds are: oxolamine citrate (1); 3-phenyl-5-vinyl-1,2,4-oxadiazole (2); 3-phenyl-5-methyl-1,2,4-oxadiazole (PMO) (3); 3-(*p*-hydroxyphenyl)-5-methyl-1,2,4-oxadiazole (4); 3-phenyl-5-hydroxymethyl-1,2,4-oxadiazole (5); *N*-acetyl-*S*-(3-phenyl-1,2,4-oxadiazol-5-yl)methyl-L-cysteine (6); 3-phenyl-5-(*S*-glutathionyl)methyl-1,2,4-oxadiazole (7); 3-phenyl-5-chloromethyl-1,2,4-oxadiazole (9); 3-phenyl-5-acetoxymethyl-1,2,4-oxadiazole (10); 3-phenyl-5-sulfatoxymethyl-1,2,4-oxadiazole, K^+ salt (11); *N*-acetyl-*S*-(3-phenyl-1,2,4-oxadiazol-5-yl)methyl-L-cysteine, isopropyl ester (12); 3-(*p*-acetoxyphenyl)-5-methyl-1,2,4-oxadiazole (13); 3-(*p*-sulfatoxyphenyl)-5-methyl-1,2,4-oxadiazole, K^+ salt (14); 3-phenyl-5-iodomethyl-1,2,4-oxadiazole (15); *S*-(3-phenyl-1,2,4-oxadiazol-5-yl)methyl-L-cysteine (16); 3-phenyl-5-(β-hydroxyethyl)-1,2,4-oxadiazole (17); and 3-phenyl-5-(α-hydroxyethyl)-1,2,4-oxadiazole (18).

for inclusion in their collection of spectra.

3-Phenyl-5-methyl-1,2,4-oxadiazole (PMO) was prepared by the reaction of benzamidoxime (8) and acetic anhydride as first described by Tieman and Krüger (1884) and later by Schulz (1885): mp 38–39 °C; uv_{max} (EtOH) 238 nm (ε 12970).

3-Phenyl-5-chloromethyl-1,2,4-oxadiazole (9) was obtained from benzamidoxime and chloroacetyl chloride in refluxing toluene in 82% yield: bp 103 °C (0.01 mm); mp 36–38 °C (lit. (Buyle et al., unpublished results) mp 38–39 °C). Anal. Calcd for $C_9H_7N_2OCl$: C, 55.54; H, 3.63; N, 14.40. Found: C, 55.74; H, 3.59; N, 14.45.

3-Phenyl-5-acetoxymethyl-1,2,4-oxadiazole (10) was obtained from 9 by the method of Palazzo (1966): bp 129 °C (1.0 mm); uv_{max} (EtOH) 238 nm (ε 12600).

3-Phenyl-5-hydroxymethyl-1,2,4-oxadiazole (**5**) was obtained by the alkaline hydrolysis of **10** (Palazzo, 1966): mp 57–60 °C; uv_{max} (EtOH) 238 nm (ϵ 12 000).

3-Phenyl-5-sulfatoxymethyl-1,2,4-oxadiazole, potassium salt (**11**) was prepared by a procedure described by Feigenbaum and Neuberg (1941). A stirred solution of 7.04 g (0.040 mol) of **5** in 250 ml of dry pyridine under nitrogen was maintained at 0 °C while 7.0 g (0.06 mol) of chlorosulfonic acid was added during 1 h. After 15 h at room temperature, 24 ml of 50% aqueous KOH was added. The precipitate was collected by centrifugation, washed with ether, and extracted with hot ethanol (EtOH). The crude product which crystallized upon cooling was recrystallized from 18 ml of hot water to give 3.5 g (30%) of analytically pure product: mp 245–260 °C dec; uv_{max} (EtOH) 238 nm (ϵ 12 600). Anal. Calcd for $C_9H_7N_2O_5SK$: C, 36.73; H, 2.40; S, 10.90; K, 13.28. Found: C, 36.86; H, 2.43; S, 11.05; K, 13.01.

*N-Acetyl-S-(3-phenyl-1,2,4-oxadiazol-5-yl)methyl-*L*-cysteine* (**6**). A solution prepared from 1.04 g (0.045 g-atom) of sodium in 40 ml of absolute methanol (MeOH) was added to a solution of 3.98 g (0.02 mol) of *N*-acetyl-L-cysteine in 15 ml of MeOH. The MeOH was removed under vacuum to afford the disodium salt of *N*-acetyl-L-cysteine.

A solution of the disodium salt and 3.88 g (0.020 mol) of **9** in 50 ml of *N,N*-dimethylformamide was heated at reflux for 1.5 h. The solvent was rotary evaporated and the residue was dissolved in water. The solution was acidified and extracted with ether. Evaporation of the ether gave 2.6 g (42%) of white solid, mp 154–156 °C. Recrystallization of 2.1 g from ethyl acetate (EtOAc) gave 1.5 g of pure **6**: mp 155–156 °C; uv_{max} (EtOH) 238 nm (ϵ 12 820). Anal. Calcd for $C_{14}H_{15}N_3O_4S$: C, 52.32; H, 4.71; N, 13.08. Found: C, 52.20; H, 4.70; N, 12.92.

*N-Acetyl-S-(3-phenyl-1,2,4-oxadiazol-5-yl)methyl-*L*-cysteine, isopropyl ester* (**12**) was prepared by refluxing **6**, 2-propanol, and dry benzene in the presence of a catalytic amount of concentrated H_2SO_4. The water was removed using a Dean-Stark trap. Recrystallization from 2-propanol gave 52% yield of analytically pure **12**: mp 95–96 °C; uv_{max} (EtOH) 238 nm (ϵ 11 800). Anal. Calcd for $C_{17}H_{21}N_3O_4S$: C, 56.19; H, 5.83; N, 11.56. Found: C, 56.17; H, 5.86; N, 11.36.

p-Hydroxybenzamidoxime (Krone, 1891) was prepared from *p*-hydroxybenzonitrile by the method of Clarke (1954).

3-(p-Acetoxyphenyl)-5-methyl-1,2,4-oxadiazole (**13**) was prepared in 41% yield by heating crude *p*-hydroxybenzamidoxime with excess acetic anhydride, and recrystallizing the crude product from EtOH: mp 117–118 °C; uv_{max} (EtOH) 242 nm (ϵ 16 600). Anal. Calcd for $C_{11}H_{10}N_2O_3$: C, 60.54; H, 4.60; N, 12.84. Found: C, 60.80; H, 4.57; N, 12.84.

3-(p-Hydroxyphenyl)-5-methyl-1,2,4-oxadiazole (**4**). A solution of 6.4 g (0.029 mol) of **13** and 4.5 g (0.080 mol) of KOH in 25 ml of EtOH was heated at reflux for 1.5 h. After the solvent was evaporated, the residue was dissolved in water and the solution was acidified with 12 N HCl. The precipitate was collected and recrystallized from 25 ml of EtOH to give 4.0 g of crude **4**, mp 150–193 °C. Two additional recrystallizations gave 3.0 g (58%) of pure **4**: mp 192–193 °C (lit. (Krone, 1891) mp 185 °C); uv_{max} (EtOH) 260 nm (ϵ 16 600). Anal. Calcd for $C_9H_8N_2O_2$: C, 61.36; H, 4.58; N, 15.90. Found: C, 61.22; H, 4.47; N, 15.84.

3-(p-Sulfatoxyphenyl)-5-methyl-1,2,4-oxadiazole, potassium salt (**14**) was prepared from 1.7 g of **4** by the procedure given for **11** to give 0.8 g (28%) of product, mp 186–190 °C dec. Additional recrystallizations from hot water gave an analytical sample: mp 195–196 °C dec; uv_{max} (EtOH) 248 nm (ϵ 16 800). Anal. Calcd for $C_9H_7N_2O_5SK$: C, 36.73; H, 2.40; S, 10.90; K, 13.28. Found: C, 36.79; H, 2.37; S, 11.05; K, 12.97.

3-Phenyl-5-methyl-1,2,4-oxadiazole-5-^{14}C (*PMO-*^{14}C). Unlabeled acetyl chloride (195 mg, 2.49 mmol) was combined with 40.5 mg (0.516 mmol, 3 mCi) of acetyl-*1-*^{14}C chloride (New England Nuclear, Boston, Mass.) and 4 ml of toluene in a 50-ml flask. The magnetically stirred solution was chilled (−20 °C) while a solution of 409 mg (3.0 mmol) of benzamidoxime, 15 ml of toluene, and 0.5 ml of triethylamine was added over a 15-min period. After the reaction mixture was stirred 16 h, it was heated at reflux 6 h. Upon cooling, the mixture was washed with 10 ml of 0.1 N HCl, three 5-ml portions of water, and 5 ml of saturated NaCl solution. The toluene solution was transferred to a sublimation apparatus where the solvent was evaporated under reduced pressure. The product was sublimed to give 383 mg (80%) of PMO-^{14}C of very satisfactory radiochemical purity: mp 39–40 °C (lit. (Schulz, 1885) mp 41 °C). [An autoradiogram is given in Figure 1 of the supplementary material; see paragraph at end of paper.] The specific activity was found to be 5.055 μCi/mg; hence the radiochemical yield was 65%.

3-Phenyl-5-iodomethyl-1,2,4-oxadiazole (**15**). A mixture of 38.9 g (0.20 mol) of **9**, 120 g (0.80 mol) of NaI, and 800 ml of acetone was stirred at reflux in the dark for 16 h. The acetone was evaporated and 400 ml of water was added to the residue. The mixture was extracted with two 300-ml portions of ether. The combined ether extracts were decolorized with a small amount of neutral alumina. Removal of the solvent gave 52 g (91%) of crude **15**: mp 63–65 °C. An analytical sample prepared by recrystallization from hexane–ether (4:1) had mp 65–66 °C. Anal. Calcd for $C_9H_7IN_2O$: C, 37.78; H, 2.47; N, 9.79; I, 44.36. Found: C, 37.82; H, 2.37; N, 9.62; I, 44.42.

3-Phenyl-5-(S-glutathionyl)methyl-1,2,4-oxadiazole (**7**). Fifty milliliters of ammonia was dried by distilling from sodium into a dry 100-ml flask containing reduced glutathione (1.5 g, 4.39 mmol) and sodium (75 mg, 3.26 mg-atom). This mixture was stirred with a glass-coated magnetic stirring bar while a solution of 0.93 g (3.26 mmol) of **15** in 5 ml of dry ether was added over a period of 1 min. The ammonia was allowed to evaporate and the residue was dissolved in MeOH–EtOH (85:15, v/v). The volume was reduced and the solution allowed to crystallize. The resulting white semiamorphous crystals of **7** weighed 1 g: mp 163–165 °C. Recrystallization from MeOH–H_2O (85:15) gave an analytical sample (mp 192–193 °C) which was homogeneous by TLC, but which analyzed low for carbon. Anal. Calcd for $C_{19}H_{23}N_5O_7S$: C, 49.03; H, 4.98; N, 15.05. Found: C, 47.87; H, 5.06; N, 14.49.

Although both chloro compound **9** and iodo compound **15** reacted with cysteine in carbonate or phosphate buffered aqueous dimethylformamide to give **16** in high yield, neither glutathione nor *S*-benzyloxycarbonylglutathione (Sokolovsky et al., 1964) under the same conditions gave the desired adduct. Attempts to construct the glutathione moiety from **16** by procedures similar to those employed by Hooper et al. (1956), Goldschmidt and Jutz (1953), and Zervas et al. (1963) to prepare *S*-benzylglutathione were unsuccessful. Selective removal of the protective groups was not possible without causing side reactions involving the oxadiazole ring.

Dosage of Animals. Ten male Long-Evans rats weighing about 600 g each were fasted overnight and 100 mg (167 mg/kg) of PMO was administered to each as a

suspension in 2 ml of 2% Tween-80 by stomach intubation. This dose was followed with 8 ml of 0.9% aqueous NaCl solution. The rats, and 10 controls, were placed in individual stainless steel cages equipped to collect urine and feces separately. Food was withheld but water was provided freely as the urine was collected over a 24-h period in containers cooled with dry ice. When pooled and filtered the urine from the treated group amounted to 179 ml.

Twenty-four hour urine samples from two purebred male Beagle dogs in the 35th week of a 52-week chronic oral administration experiment were supplied by Hazleton Laboratories, Vienna, Va. The animals had been dosed daily, 6 days per week, with 100 mg/kg of PMO administered orally in gelatin capsules.

Mice were dosed either by skin painting with PMO in acetone (25 mg of PMO/mouse) or by intraperitoneal (ip) injection at the rate of 20-mg/kg PMO suspended in 0.1% carboxymethylcellulose. The 24-h urine from each group of mice (20 25-g mice for skin painting, 15 35-g mice for ip injection) and controls were collected in containers cooled in dry ice.

For the study of metabolites from PMO-^{14}C, one female Long-Evans rat (245 g) was fasted overnight and dosed by stomach intubation with 82.1-mg/kg PMO containing 14.96 μCi of ^{14}C suspended in 0.5% carboxymethylcellulose. Then, 3.4 ml of 0.9% NaCl was administered ip and the animal was placed in a stainless steel cage. The 24-h urine and feces were collected as described previously. The animal was autopsied and the organs were frozen pending analysis.

Two male Long-Evans rats were used in experiments designed to collect expired $^{14}CO_2$ in addition to urine and feces. One rat (199 g) was dosed by stomach intubation with 85.0 mg/kg, 12.63 μCi, of PMO-^{14}C in 0.5% carboxymethylcellulose and 1.8 ml of 0.9% NaCl. Later, another rat (247 g) received 80.6 mg/kg, 14.90 μCi, of PMO-^{14}C in 0.5% carboxymethylcellulose by stomach intubation and 2.2 ml of 0.9% NaCl ip. Each animal was tightly confined in a 1.5-l. animal chamber (Nuclear Associates, Model 18-350) and the air flow through the apparatus was adjusted to ~0.5 l./min.

Bile containing PMO-^{14}C metabolites was obtained from the cannulated bile duct of a fasted 400-g male Long-Evans rat. The rat was anesthetized with pentabarbital sodium (62 mg/ml per kg, ip) and then hourly with 0.05 ml given subcutaneously. A dose of 73.9-mg/kg PMO-^{14}C containing 22.9 μCi of radiocarbon suspended in 1.9 ml of 0.5% carboxymethylcellulose was administered by stomach intubation. The bile (7.3 ml) was collected over a 7-h period and frozen.

Isolation of Metabolites. *Extraction of Urine and Bile.* The procedure used for the isolation of the neutral and acidic urinary PMO metabolites was adapted from methods described by Olson (1970) and by Dalgliesh et al. (1966). The urine was made 4 M in respect to NaCl and acidified to pH 1–2 with 12 N HCl. The aqueous layer was extracted with redistilled EtOAc. The combined extracts were evaporated just to dryness and the residue was redissolved in MeOH to a definite volume. The basic metabolites were removed from the aqueous raffinate by adjusting to pH 12 with 40% aqueous KOH and extracting with EtOAc. The biliary metabolites were not extracted; instead, aliquots of whole bile were subjected to TLC.

Paper and Thin-Layer Chromatography. Aliquots of the urinary extracts, and appropriate synthetic metabolites, were chromatographed on Whatman No. 3MM paper overnight in ascending 1-butanol–concentrated NH_4OH (4:1, v/v). The chromatograms were visualized under short-wave uv light. Paper chromatograms from experiments with animals receiving labeled PMO were also examined by autoradiography using Kodak RP Royal X-Omat x-ray film (10 × 12 in.).

The PMO urinary metabolites were purified further by elution of the spots from the paper with MeOH and rechromatography of the concentrated extract on 20 × 20 cm Q1F silica gel thin-layer plates (Quantum Industries, Fairfield, N.J.). These were visualized under short-wave uv light. Solvent system A was benzene–acetonitrile (17:3, v/v) and solvent system B was benzene–acetonitrile–acetic acid–water (48:50:2:1, v/v). By combining similar TLC fractions, sufficient amounts of the pure metabolites were gathered to perform qualitative spot tests and to obtain uv, ir, and mass spectra for comparison with the spectra of the synthetic suspected metabolites (see Supplementary Material I).

Qualitative Spot Tests. Purified materials from chromatograms were partially characterized by qualitative spray reagents and spot tests. The most useful reagents were diazotized *p*-nitroaniline (buffered) described by Stahl (1969, reagent No. 182) and potassium ferricyanide–ferric chloride (Stahl, 1969, No. 111) for phenols, ninhydrin spray reagent (Stahl, 1969, No. 230) for peptide conjugates in the bile, and potassium chromate–silver nitrate spray (Knight and Young, 1958) for peptide conjugates containing divalent sulfur. Sulfur containing compounds were verified by a sodium formate fusion method (Feigl, 1960). Glucuronides gave a purple color when warmed with carbazole in boric–sulfuric acid (Bitter and Muir, 1962). The isolated glucuronides were hydrolyzed enzymatically with β-glucuronidase (bovine liver, Calbiochem) using the method described by Knaak et al. (1970). The aglycones were removed by acidification to pH 1 with 12 N HCl, followed by extraction with EtOAc. Simultaneous TLC or paper chromatography of the EtOAc extracts and the synthetic compounds **4** and **5** verified the original structures of the glucuronides. Sulfate conjugates were hydrolyzed under the same conditions using β-glucuronidase/arylsulfatase (*Helix pomatia*, Calbiochem).

Radioactivity Measurements. The radioactivities of samples obtained following administration of PMO-^{14}C were determined using a Model 702A liquid scintillation counter (Nuclear Chicago). The counts were made in the L1-∞ channel (0.6–9.9 V) at a data tube setting of 950 V and gate tube setting of 1475 V. Counting efficiency was determined by the internal standard technique using toluene-^{14}C (4.17×10^5 dpm/ml, New England Nuclear).

The stream of purge air (0.5 l./min) leaving the metabolism chamber was first scrubbed by passing it through two gas washing bottles containing 200 ml of toluene to absorb unmetabolized PMO-^{14}C. A third gas washing bottle containing 400 ml of 1.5 N NaOH solution removed $^{14}CO_2$. After 24 h the animal was sacrificed and the chamber was rinsed with 100 ml of hexane. The radioactivities of the hexane cage washings, hexane washings of the clipped hair, and the toluene air scrubbing solutions were determined using aliquots which were transferred to liquid scintillation vials containing 10 ml of a 0.4% solution of Omnifluor (New England Nuclear) in toluene.

Radioactive samples from urine, urine extracts, chromatographic eluates, and the NaOH solution containing $^{14}CO_2$ from breath were dissolved in Aquasol (New England Nuclear) universal liquid scintillation counting (lsc) solution. Samples of feces, blood, and tissue or tissue homogenates in 1% aqueous NaCl were digested in Protosol tissue solubilizer (New England Nuclear) for lsc in Aquasol.

Radioactive spots, separated from bile on LQDF TLC plates, were assayed by scraping the spot into a counting vial. The radioactivity was eluted with 2 ml of methanol–concentrated NH_4OH (20:1, v/v) and 15 ml of Aquasol added to the vial. After the silica had settled, the radioactivity was measured by lsc.

RESULTS

Fractionation. Examination of the paper chromatograms of the EtOAc extracts of PMO rat urine under uv light revealed eight fractions in the acidified urine and one fraction in the alkaline urine not present in control urine extracts. These fractions had approximately the following R_f values on Whatman No. 3MM paper developed with 1-butanol–concentrated NH_4OH (4:1, v/v) (fraction, R_f range): U1, 0.82–0.84; U2, 0.67–0.68; U3, 0.48–0.57; U4, 0.39–0.44; U5, 0.31–0.39; U7, 0.10–0.18; U8, 0.08–0.13; U9, 0.02–0.03; U10, 0.45–0.49. A brief description of each fraction is given below. The yield of metabolite in each experiment is presented in Table I. (See Supplementary Material I for comparison of the ir and mass spectral data of the natural and synthetic metabolites, and Figures 2 and 3 for paper and TLC.)

U1. MeOH elution of the paper and evaporation of the MeOH gave a residue which was purified on Q1F silica gel plates with solvent A. The spot at R_f 0.35, after elution with MeOH, was identical with **5**.

U2. This minor metabolite was suggestive of a phenol, but was not investigated further.

U3. The MeOH extract of the paper was purified on Q1F silica gel plates, solvent A. The major component (R_f 0.35) gave a positive test for a phenol. The uv_{max} (MeOH) at 259 nm (acidic or neutral) shifted to 290 nm at pH 14, identical with synthetic **4**.

U4. This was first observed with PMO-^{14}C treated animals. The MeOH extract of the paper was purified on Brinkman silica gel sheets (SIL-N-HR/UV_{254}), solvent B. The major component at R_f 0.17 was hydrolyzed to **5** by β-glucuronidase/arylsulfatase, but not by glucuronidase. It was identified as the acid of **11** by comparison with synthetic material.

U5. The MeOH extract of the paper was purified on Q1F silica gel, solvent B. The major component at R_f 0.15 had sulfur, and spectra identical with synthetic **6**. When heated 40 min (95 °C) in a sealed tube with 10% anhydrous HCl in 2-propanol, it gave a compound identical with ester **12**.

U7. This was an acid with a uv unchanged by acid or base. It gave a positive carbazole test for glucuronides, and was cleaved by β-glucuronidase to **5**. The metabolite is indicated to be 3-phenyl-5-hydroxymethyl-1,2,4-oxadiazole glucuronide.

U8. This material gave a positive test for a glucuronide and had a uv spectrum characteristic of **4** in neutral solution. It was hydrolyzed to **4** by glucuronidase, and is considered to be 3-(*p*-hydroxyphenyl)-5-methyl-1,2,4-oxadiazole glucuronide.

U9. Although this material was observed frequently, the low yields demonstrated with PMO-^{14}C precluded further investigation.

U10. The material from the alkaline urine extracts was purified on preparative Q1F plates. The unidentified major component (uv_{max} 242 nm) had a parent peak *m/e* 176, suggestive of an oxygenated PMO, or a fragment of a more complex compound.

Quantitation of Urinary Metabolites. Areas of the developed paper chromatograms representing the fractions U1–U10 were cut out and eluted with methanol. The percentage of the original PMO dose represented by each

Table I. Urinary Metabolites of PMO, Percentage of Dose Recovered in 24 h

Animal	Route of admin.	Dose, mg/kg [μCi of ^{14}C]	Anal. method	Urinary fraction and metabolite										Total[f]
				PMO[a]	U1 5	U3 4	U4 11	U5 6	U7[c] 5 gl	U8[d] 4 gl	U9 Unk	U10[e] Unk		
10 male rats	po	167	uv	0.66	7.16	3.28		11.6	2.3			4.5		32.5
1 female rat	po	82	uv		1.19	0.75		9.1	6.37	0.66				18.1
		[14.96]	lsc		1.19	1.70		11.2	7.45	1.20		0.46		23.2
1 male rat	po	85												
		[12.63]	lsc		7.41	2.93	25.1	12.3	1.85		0.25	8.6		58.4
1 male rat	po	81												
		[14.90]	lsc		1.53	1.51	7.96	9.48	1.71		0.37	3.94		26.5
1 male dog[b]	po	100	uv	3.7	0.0	0.50		8.5	3.7	0.80				17.2
1 male dog[b]	po	100	uv	1.68	0.0	1.05		6.5	0.73	0.0				10.0
15 mice	ip	20	uv			1.4		4.1	10.3	3.0		0.5		19.3
20 mice	Skin	~1000	uv		0.17			0.40	3.14	0.37		0.02		4.6

[a] PMO determined by GLC (for method, see Supplementary Materials II). [b] Beagle dogs in 35th week of a 52-week chronic oral feeding experiment; the urine not collected quantitatively. [c] Uv method assumed extinction coefficient for glucuronide (gl) of 5 at 239 nm was 12 000. [d] Uv method assumed extinction coefficient for glucuronide (gl) of 4 at 252 nm was 16 600. [e] Uv method assumed extinction coefficient for basic metabolite of 12 000 at 238 nm. [f] Total of all analyzed urinary fractions.

Table II. Distribution of Radioactivity in Rats 24 h after Oral Administration of PMO-^{14}C

Sample	% of PMO-^{14}C recovered[b] Female rat dosed with 14.96 μCi	Male rat dosed with 12.63 μCi	Male rat dosed with 14.90 μCi
Urine, total known metabolites	38.46 (23.2)	66.1 (58.4)	48.3 (26.5)
Feces	4.9	8.7	23.4
Breath, toluene trap		0.41	0.27
Breath, NaOH trap (CO_2)		9.3	13.3
Animal chamber washings		0.02	0.02
Hair			0.41
Blood[a]	0.41		
Brain	0.03		
Fat[a]	0.18		1.00
Heart	0.02		
Intestines	6.7		3.15
Kidney	0.10		0.34
Liver	0.77		0.53
Lung	0.03		
Muscle[a]	1.4		0.95
Spleen	0.02		
Stomach	0.05		
Total recovery	53.07	84.53	91.67

[a] Assumed body composition (percent): blood, 7.8; fat, 15; muscle, 40. [b] Tissues and feces were dissolved in Protosol tissue solubilizer before liquid scintillation counting in Aquasol.

fraction was estimated in the eluates either by lsc, uv absorption, or both. Molar absorptivities for the uv analyses were obtained from pure synthetic compounds, or in the case of the glucuronides, from a reasonable estimate based on the values for the aglycones. The uv absorbances of the methanolic solutions obtained from the test animals were corrected for normal urinary constituents by placing similar extracts from the urine of the control animals in the spectrophotometer reference beam. The results are given in Table I.

The aqueous raffinate remaining from the urine, after the acidic and basic metabolites were extracted with ethyl acetate, was found to contain 7–10% of the administered radioactivity. By all indications, this activity is due to catabolism of the PMO-^{14}C and incorporation of the ^{14}C into body pools of normal tissue components.

Balance Study. The results of the distribution of the radioactivity in three rats 24 h after administration of PMO-^{14}C are given in Table II.

Stability of Oxadiazole Sulfates. Sulfates are common metabolic products. When no sulfate metabolites were observed in the experiment involving the 10 male rats, experiments were performed to examine the stability of the sulfates derived from the salts **11** and **14**. The synthetic compounds were prepared and subjected to the extraction and separation scheme described for the measurement of the other metabolites. The sulfate **11** was shown to be stable to extraction conditions. It hydrolyzed slowly to the alcohol **5** with a half-life of 50 h in EtOAc that was in equilibration with acidified (pH 1, HCl) aqueous 4 M NaCl solution.

The sulfate from **14** was relatively stable in ethanol, and in 0.1 N HCl (pH 1) for 4 days. However, it was rapidly hydrolyzed to the phenol **4** when dissolved in EtOAc equilibrated with acidified 4 M NaCl solution. Under extraction conditions at pH 0.9 the half-life of the compound was 12 min; at pH 0 (1 N HCl) it was 5 min. But, at pH 2.8, no measurable hydrolysis occurred after 3 days. The rapid hydrolysis also took place when 1-butanol or chloroform was used for extraction.

Table III. Biliary Metabolites Obtained from Rats 7 h after Oral Administration of PMO-^{14}C (73.9 mg/kg)

R_f[a]	Metabolite	% of ^{14}C dose
0.03	Unidentified	1
0.11	Unidentified	5.3
0.21	3-Phenyl-5-(*S*-glutathionyl)methyl-1,2,4-oxadiazole (**7**)	7.2
0.30	3-Phenyl-5-hydroxymethyl-1,2,4-oxadiazole glucuronide	3.6
0.39	Same as product from acid hydrolysis of glutathione conjugate	1.1
0.48	*N*-Acetyl-*S*-(3-phenyl-1,2,4-oxadiazol-5-yl)methyl-L-cysteine (**6**)	2.9
0.63	Unidentified	1
0.76	Unidentified	1

[a] R_f by TLC on silica gel (LQDF, Quantum Industries) developed with 1-butanol–1-propanol–2 N ammonium hydroxide (2:1:1, v/v).

Biliary PMO-^{14}C Metabolites. All the biliary metabolites of PMO-^{14}C identified were found to be conjugates containing free carboxylic acid groups. They could be isolated best by TLC of aliquots of whole bile on LQDF hard silica (Quantum Industries) developed with 1-butanol–1-propanol–2 N NH_4OH (2:1:1, v/v) (see Supplementary Material, Figure 4). Eight metabolites were located by autoradiography and characterized by the usual spot and spray reagents. Identifications were made, where possible, by comparison of R_f values on the TLC with synthetic metabolites. Quantitation was by lsc of the scrapings from the TLC plate. These results are summarized in Table III.

Although the biliary PMO metabolites include the *S*-glutathionyl derivative **7**, the mercapturic acid **6**, and the hydroxymethyl glucuronide, neither the free hydroxymethyl metabolite **5**, the phenolic metabolite **4**, nor their corresponding sulfates were found. The material at R_f 0.39 (Table III) could possibly be the cysteinylglycine conjugate since a compound having the same R_f and spot test results was found upon acid hydrolysis of the *S*-glutathionyl derivative. A major biliary metabolite at R_f 0.11 remains unidentified.

Metabolites of 3-Phenyl-5-hydroxymethyl-1,2,4-oxadiazole. Long-Evans rats were dosed orally with 100 mg/kg of **5**. The 24-h urine was extracted with ethyl acetate and the extracts after purification by paper chromatography were measured by uv spectrometry. In addition to 3.8% of **5** which was recovered unchanged, the urine contained 21.4% of the dose as the mercapturic acid **6** and 9.5% as the glucuronide of **5**.

DISCUSSION

There are few reports on the metabolism of 1,2,4-oxadiazoles related to PMO. Palazzo and Corsi (1962) studied the amount of oxolamine citrate (**1**) in blood and urine following administration of this drug to cats, rats, and humans. Catanese et al. (1963) found a number of 5-(2-dialkylaminoethyl)-1,2,4-oxadiazoles to be exceptionally unstable, both biologically and chemically, by ready elimination of the dialkylamino group. Silvestrini et al. (1964) examined the urine of rats treated with oxolamine (1000 mg/kg) and found 1–5% of the unchanged drug, 5–15% of the dose as hippuric acid, about 15% as benzoylglucuronic acid, about 10% as diethylamine, and 1–5% as a compound thought to be 3-phenyl-5-hydroxyethyl-

1,2,4-oxadiazole (17 or 18). The only previous study of PMO metabolism was reported by Catanese et al. (1963). He stated that "the urine of rats treated orally with 1000 mg per kilogram of PMO disclosed the presence of small quantities of unaltered product and large quantities of hippuric acid and benzoylglucuronide. Diethylamine was absent as expected." Our investigation showed that urines from guinea pigs, rats, and dogs receiving PMO, or rats dosed with oxolamine citrate, did not show any significantly increased amount of hippuric acid or benzoylglucuronic acid when compared to controls. Also, we were unable to find the hydroxyethyl compounds 17 or 18, even though these were synthesized to aid identification.

We believe that all of the significant PMO metabolites have been identified. Based on the data in Table II, 88% of the radiocarbon in the urine of one of the male rats was accounted for among the identified metabolites. In another of the male rats, the total radioactivity of the breath, feces, urine, and body parts accounted for 91.7% of the PMO-^{14}C administered. The radioactivity not accounted for is due, no doubt, to either: (1) an erroneous determination of the $^{14}CO_2$ in the breath or (2) an erroneous estimate of the weight percents of blood, fat, and muscle in the body. These studies show that PMO appears to be rapidly excreted without persisting in tissues and organs.

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Supplementary Material Available: Figure 1, TLC autoradiogram of PMO-5-^{14}C; Figure 2, paper chromatographic presentation of the urinary metabolites of PMO-^{14}C in the male rat; Figure 3, TLC of the urinary metabolite fractions U1, U3, U4, and U5 of PMO-^{14}C; Figure 4, TLC separation of the biliary metabolites of PMO-^{14}C found in the male rat; I, comparison of the infrared and mass spectral data for natural and synthetic PMO metabolites; and II, GLC method for PMO (8 pages). Ordering information is given on any current masthead page.

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Fate of 2,2'-Dichlorobiphenyl-^{14}C in Carrots, Sugar Beets, and Soil under Outdoor Conditions

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2,2'-Dichlorobiphenyl-^{14}C was applied to soil (38 mg on a 60 × 60 cm area) in a box under outdoor conditions. In 1973 carrots and in 1974 sugar beets were grown. In the first vegetation period, 53.5% of the applied radioactivity was lost by volatilization, and in two vegetation periods 78.7%. In the first year, 49.5% of the radiocarbon in the treated soil layer was unchanged dichlorobiphenyl, 8.8% was soluble metabolites, and 41.7% was unextractable; in the second year, the percentage of unextractable residues increased to 74.3%. The presence of oxygenated metabolites in the extracts of soil and plants was confirmed by mass spectrometry. Carrot roots contained 0.240 ppm of 2,2'-dichlorobiphenyl and 0.012 ppm of metabolites; sugar beet roots contained <0.001 ppm of 2,2'-dichlorobiphenyl and 0.004 ppm of metabolites. The bioconcentration factor of 2,2'-dichlorobiphenyl in carrots was 2; metabolites were not concentrated. No bioconcentration of 2,2'-dichlorobiphenyl or metabolites was observed for sugar beets.

The occurrence of unchanged polychlorinated biphenyls (PCB's) in environmental samples including human food is well documented (summarized in Klein and Weisgerber, 1976). However, information on the possible presence of degradation products in agricultural soils and crops is very limited. PCB's are known to undergo various conversions under environmental conditions and to form phenols, together with other products, by irradiation (Hustert and Korte, 1974) or by metabolism in various organisms (Block

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