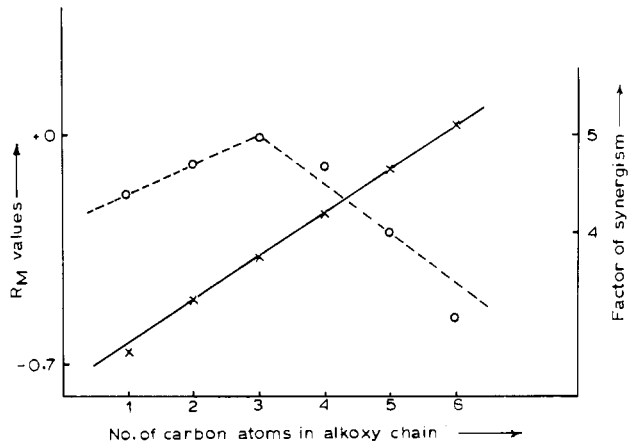

Figure 1. Relationship among the length of alkoxy substituent of dillapiole side chain, R_M values, and factors of synergism. R_M value (x–x); factor of synergism (O--O).

The plot of R_M values against the number of carbon atoms of the alkoxyl substituent in the dillapiole side chain in Figure 1 reveals a linear relationship, implying that lipophilicity of these molecules increases with increasing chain length, but Table II reveals that lipophilicity alone is not the criterion for enhanced synergistic activity. Thus compound IX whose R_M value is less than that of dihydrodillapiole is a better synergist. The same is also true in the case of compound XI, having a methoxyl group more than that of dillapiole. In a homologous series as in compounds XI–XVI, the synergistic activity increased with increasing lipohilicity up to a maximum of three carbon atoms in the side chain. Similar results have been observed recently in other homologous series (Vaidyanathaswamy et al., 1977). Further increase in chain length actually leads to a decrease in synergism.

Supplementary Material Available: A listing of data from NMR spectrometry and elemental analyses of ethers X–XVII (2 pages). Ordering information is given on any current masthead page.

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Metabolism of [^{14}C]Fosamine Ammonium in the Rat

Robert L. Chrzanowski,* Jerry C-Y. Han, and Colin L. McIntosh

The active ingredient in DuPont Krenite Brush Control Agent is ammonium ethyl carbamoylphosphonate (fosamine ammonium salt, formerly known as DPX-1108). When carbonyl-labeled [^{14}C]fosamine ammonium was administered as a single oral dose to preconditioned rats by intragastric intubation, the radioactivity was rapidly eliminated in the feces (87%) and urine (13%). Trace amounts of radioactivity were found in the gastrointestinal tract, hide, and exhaled air (0.1–0.2%). Less than 0.05% radioactivity was found in the body tissues after 72 h. Total recovery of applied radioactivity was nearly 100%. The eliminated carbon-14 in both urine and feces was 87% intact fosamine and about 13% carbamoylphosphonic acid. The synthesis of these compounds is described.

Fosamine ammonium salt (formerly known as DPX-1108) is the active ingredient in DuPont Krenite Brush Control Agent. This water soluble, nonflammable, nonvolatile compound is diluted with water and applied as a foliar spray for control and/or growth suppression of many woody species. When applied in late summer or early fall, fosamine ammonium acts as a bud break inhibitor and has minimum effect on existing foliage except for certain pines which may show a response soon after application (Weed et al., 1974). Susceptible plants fail to refoliate in the spring and subsequently die or their growth is severely retarded.

Fosamine ammonium is relatively nontoxic to mammals, e.g., LD_{50} = 24000 mg/kg in male rats (Weed et al., 1974), and no cumulative toxicity or adverse effects in offspring has been noted.

This paper describes the synthesis of ammonium ethyl [^{14}C]carbamoylphosphonate ([^{14}C]fosamine ammonium) and its metabolism in the rat.

Biochemicals Department, Research Division, Experimental Station, E. I. du Pont de Nemours & Co., Inc., Wilmington, Delaware 19898.

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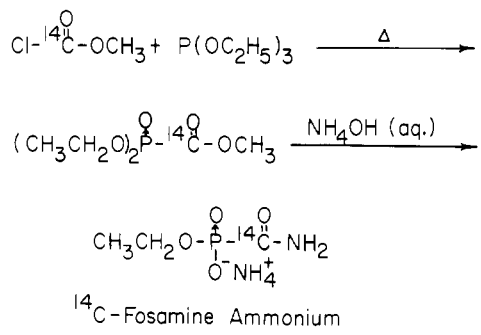

Figure 1. Synthesis of [^{14}C]fosamine ammonium.

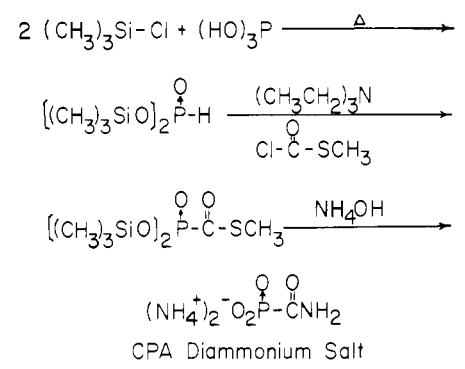

Figure 2. Synthesis of carbamoylphosphonic acid diammonium salt.

EQUIPMENT AND METHODS

All radioactive liquid samples were measured by liquid scintillation counting (LSC) in premixed scintillation cocktail (Formula 947, New England Nuclear) using an Isocap 300 scintillation counter (Searle Analytic). Quenching corrections were made by internal standard procedures employing [^{14}C]toluene. The radioactivity of homogenized solid samples was determined by combustion analysis (CA) in a Model 305 sample oxidizer (Packard Instruments Co.) followed by LSC.

The radiochemical purity of ^{14}C-labeled compounds was determined by thin-layer chromatography (TLC) analysis on 250 μm silica gel G chromatoplates (Brinkmann Instruments Co.) developed for 15 cm in the solvent systems methanol/glacial acetic acid (4:1, v/v) and 0.05 M ammonium carbonate/acetonitrile (5:95, v/v). Radioactive bands were located with a Berthold Model 6000-2 Automatic TLC radioscanner (Varian-Aerograph). Examination of the separated radioactive components was also performed by autoradiography using SB-5 X-ray film (Kodak). The amount of radioactivity in each band was determined by scraping the silica gel from the plate, eluting the radioactivity with distilled water and counting aliquots by LSC. Silica gel was removed from the samples by centrifugation on a Universal Model UV centrifuge (International Equipment Co.).

The ^{14}C-labeled metabolites in the urine and feces were separated and analyzed by TLC on 100 μm cellulose MN-300 chromatoplates using the solvent systems 0.5 M ammonium carbonate/methanol/acetonitrile (5:60:35, v/v/v) and 0.5 M ammonium carbonate/methanol/water (5:60:35, v/v/v). Radioscanning and quantitation was performed as described above.

Gas chromatography of methylated derivatives was performed with a Microtek Model MT-220 Gas Chromatograph equipped with a flame ionization detector and a 10:1 effluent splitter connected to a Packard Model 894 gas proportional counter which permitted ^{14}C monitoring of the column effluent. The GC column was 1.8 m × 4 mm glass packed with 3% OV-17 on 80–100 mesh Chromosorb W HP (Supelco, Inc., Bellefonte, PA). The temperature was programmed from 100–250 °C at 10 °C/min with a flow rate of 60 mL/min helium. All mass spectra were obtained with a Du Pont Model 21-492 mass spectrometer coupled with a Perkin-Elmer Model 990 gas chromatograph.

EXPERIMENTAL SECTION

Synthesis of Radiolabeled Fosamine Ammonium. The synthesis of fosamine ammonium is shown in Figure 1 and consisted of the following steps.

A mixture of methyl chloro[^{14}C]formate (250 mg, 2.6 mmol; 40.7 mCi, New England Nuclear) and nonradioactive methyl chloroformate (1.6 g, 17 mmol) in 10 mL of toluene was prepared and cooled to 0 °C in an ice bath. Triethyl phosphite (3.4 g, 20 mmol) was added to this solution with constant stirring and then heated at reflux for 1.5 h.

The reaction mixture was cooled to 10 °C, made basic with 7 mL of 30% ammonium hydroxide, and stirred at room temperature for 1.5 h. The solvent was evaporated at 45 °C in a rotary evaporator and the resulting residue triturated with 10 mL of acetone to give 3.0 g of a granular product. This product was purified by repeatedly washing with 10-mL portions each of chloroform, benzene, and acetone and was then recrystallized from 95% ethanol to yield 2.4 g of white crystalline needles.

This product was further purified by dissolving in methanol, followed by chromatography on a 2 × 30 cm, 28–300 mesh silica gel (Fisher Scientific Co.) column. The column was eluted with 200 mL of methanol followed by 150 mL of methanol/water (95:5, v/v) and finally with 150 mL of methanol/water (50:50, v/v). Ten 50-mL fractions of eluant were collected. Fractions 4, 5, and 6 containing the bulk of the radioactivity were combined and evaporated to dryness. Trituration of the resulting residue with acetone gave a 65% recovery of radiochemically pure (>99%) [^{14}C]fosamine ammonium (mp 175–176 °C; sp act., 1.6 mCi/mmol).

Unlabeled fosamine ammonium was prepared in the same manner using unlabeled methyl chloroformate.

Synthesis of Carbamoylphosphonic Acid Diammonium Salt. Carbamoylphosphonic acid (CPA) was prepared as the diammonium salt as shown in Figure 2 and consisted of the following sequence of reactions:

A. Bis(trimethylsilyl) Phosphite. Chlorotriethylsilane (500 g, 5.0 mol) was added to a solution of phosphorus acid (164 g, 2.0 mol) in 250 mL of benzene and then heated at reflux for 6 h. The mixture was cooled to room temperature and the solvent was evaporated under vacuum. The product was distilled at 78–80 °C/3 mmHg to yield bis(trimethylsilyl)phosphite (226 g).

B. Methyl Bis(trimethylsilyl) Thiocarbamoylphosphonate. Triethylamine (100 g) was added dropwise to a solution of bis(trimethylsilyl) phosphite (226 g, 1.0 mol) in 1 L of anhydrous ether maintained at <20 °C in an ice bath. Methyl chlorothioformate (100 g, 0.91 mol) was then added dropwise to this solution at <10 °C and the resulting mixture was stirred for 16 h at room temperature. Triethylamine hydrochloride was removed by filtration under a nitrogen atmosphere and the ether was evaporated under vacuum. The product was distilled at 100–103 °C (0.25 mmHg) to give methyl bis(trimethylsilyl) thiocarboxyphosphonate (182 g).

C. CPA Diammonium Salt. Methyl bis(trimethylsilyl) thiocarboxyphosphonate (182 g, 0.61 mol) was added dropwise to a mixture of 175 mL of concentrated ammonium hydroxide and 200 g of ice. The mixture was

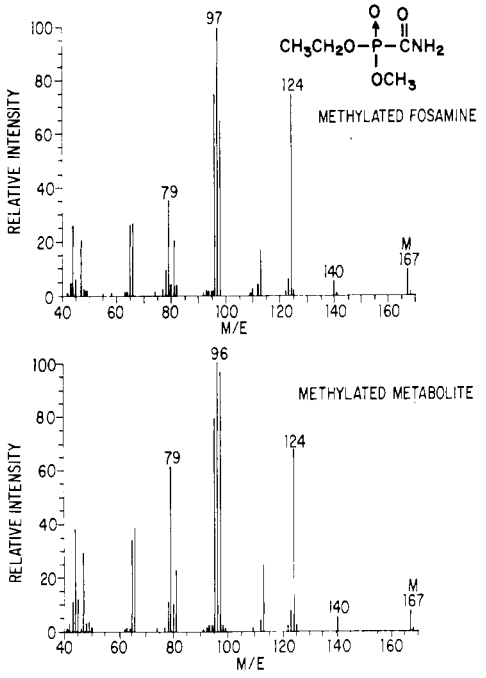

Figure 3. Mass spectra of *O*-ethyl-*O*-methyl carbamoylphosphonate.

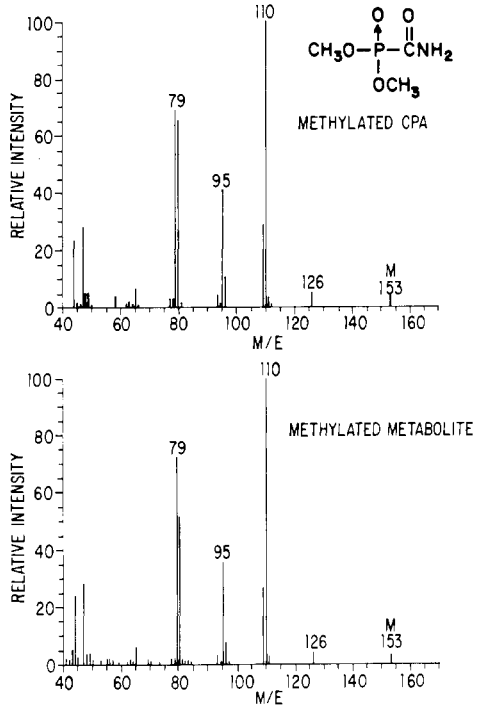

Figure 4. Mass spectra of *O,O*-dimethyl carbamoylphosphonate.

stirred for 16 h at room temperature and then an additional 200 mL of water was added. The resulting solution was extracted three times with 100-mL portions of methylene chloride and the water phase was evaporated in a rotary evaporator to give CPA diammonium salt (99.2 g, 0.62 mol, mp 164 °C). Calcd for $CH_{10}N_3O_4P$: C, 7.55; H, 6.34. Found: C, 7.83; H, 6.07. The pK_a's for CPA were determined to be 2.14 and 5.50.

For purposes of this investigation, a small amount (ca. 200 μCi) of [^{14}C]CPA diammonium salt (>99% radiochemically pure; sp act., 1.6 mCi/mmol) was obtained as a hydrolysis product (Han, 1979) of [^{14}C]fosamine ammonium by preparative TLC on cellulose plates.

Methylation of [^{14}C]Fosamine and [^{14}C]CPA. Fifty milligrams of [^{14}C]fosamine ammonium and 10 mg of [^{14}C]CPA diammonium salt were eluted separately through a 4.5 × 50 cm column packed with 50–100 mesh activated 50W-X8 cation-exchange resin in the H^+ form (Bio-Rad Laboratories) with 625 mL of distilled–deionized water. The eluants were immediately evaporated to dryness under vacuum at 35 °C and redissolved in 5 mL of methanol. Diazomethane (1.5 g) in ether at 0 °C was added and allowed to stand at ambient temperature for 2 h. Concentration under dry nitrogen gave a 50% yield of radiochemically pure (>99%) *O*-ethyl-*O*-methyl [^{14}C]carbamoylphosphonate and *O,O*-dimethyl [^{14}C]carbamoylphosphonate from [^{14}C]fosamine ammonium and [^{14}C]CPA diammonium, respectively. Combination gas chromatography/mass spectrometry (GC/MS) produced mass patterns consistent with the proposed compounds. Methylated fosamine gave m/e^+ 167 (7.8), 140 (5.5), 124 (68), 96 (100) (Figure 3), and methylated CPA gave m/e^+ 153 (3.9), 126 (5.5), 110 (100), 79 (68) (Figure 4).

Animal Study. Two male rats (Charles River-CD, rats A and B) were preconditioned for 18 days on a diet of ground Purina Laboratory Chow spiked with 1000 ppm unlabeled fosamine ammonium. The rats, weighing about 350 g each, were then dosed by gastric intubation with 57 mg/kg, 191 μCi, of [^{14}C]fosamine ammonium in 2 mL of water. After treatment, each rat was placed in a glass metabolism unit (Stanford Glass Blowing Labs, Inc.) for 72 h, through which 500 mL/min of dried and CO_2 free air was drawn. The effluent air from the metabolism unit was first scrubbed with 4 N sodium hydroxide to remove respired $^{14}CO_2$, then passed through a cupric oxide hot tube (700 °C) to oxidize other respiratory ^{14}C organic compounds to $^{14}CO_2$, and finally through another 4 N caustic trap to absorb any $^{14}CO_2$ produced by combustion. The caustic traps were changed daily, frozen, and retained for total ^{14}C analysis. Three 24-h samples of urine and feces were collected and frozen for analysis.

After 72 h the animals were lightly anesthetized with chloroform and blood was removed from the heart by cardiac puncture. The animals were then sacrificed and all organs and tissues were surgically removed, frozen immediately, and stored frozen until analysis.

Aliquots of the gas trap samples, urine and blood were analyzed for total ^{14}C by direct LSC. Tissue and feces samples were homogenized with water in a blender and lyophilized. Aliquots of the lyophilized tissue and feces samples were analyzed for ^{14}C residues by CA.

Metabolite Identification. Feces (5 g) were extracted twice with 150 mL of 0.5 M ammonium carbonate at 100 °C for 1 h to remove water-soluble radioactive compounds. Two extractions removed most of the radioactivity (90–98%). The residue was separated by centrifugation, dried, and assayed by CA. Total ^{14}C in the extract was determined by LSC, and the extract was then evaporated under vacuum at 65 °C to dryness. An aliquot of the residue in methanol was cochromatographed with radioactive standards of fosamine ammonium and CPA diammonium salt. Radiolabeled compounds from the feces extract corresponding to the retention time of the standards were removed by scraping, extraction with water, and were then analyzed by LSC. For confirmation of the identities of the compounds found, additional purification and derivatization procedures were performed as follows.

Anionic fractions in the dried feces extract were isolated by ion exchange chromatography with a 16–50 mesh Rexyn 201 (Fisher Scientific Co.) column. The 2 × 50 cm column was prepared in the carbonate form by washing with excess 5% aqueous ammonium carbonate/5% ammonium bi-

Table I. Distribution of Radioactivity after Treatment of Rats with [^{14}C]Fosamine Ammonium

treatment	percent of original treatment rat A	rat B
external fractions		
prefurnace gas trap	0.05	0.00
postfurnace gas trap	0.00	0.00
subtotal	(0.05)	(0.00)
urine, 0-24 h	9.84	11.5
urine, 24-48 h	1.67	2.20
urine, 48-72 h	0.21	0.28
subtotal	(11.7)	(14.0)
feces, 0-24 h	73.5	14.5
feces, 24-48 h	13.7	69.9
feces, 48-72 h	1.16	1.35
subtotal	(88.4)	(85.8)
total eliminated	100	99.8
body fractions		
hide	0.00	0.11
carcass	0.01	0.04
g.i. tract	0.02	0.05
liver	0.00	0.00
blood	0.00	0.00
kidneys	0.00	0.00
testes	0.00	0.00
lungs	0.01	0.00
heart	0.00	0.00
spleen	0.00	0.00
brain	0.00	0.00
fat	0.00	0.00
subtotal	(0.04)	(0.20)
total recovery	100	100

carbonate solution, followed by distilled water. The residue was applied to the column with a minimum of water, and nonpolar materials were eluted with 1 L of distilled water. Anionic radioactive compounds were eluted with 800 mL of 0.2 M ammonium carbonate which was concentrated to near dryness at 65 °C in a rotary evaporator.

The ^{14}C-containing compounds in the concentrate were separated by preparative TLC on cellulose plates and were derivatized with diazomethane as previously described. These derivatives were then purified by preparative TLC on silica gel plates developed in water/acetonitrile (5:95, v/v). Separated fractions were extracted, quantitated by LSC, and confirmed by GC/MS.

Urine was analyzed in the same manner as the feces except that ammonium carbonate extraction was unnecessary, and the samples were applied directly to TLC plates and ion-exchange columns for quantitation and metabolite identification.

RESULTS AND DISCUSSION

When male rats which had been preconditioned on a diet containing 1000 ppm fosamine ammonium were treated by intragastric intubation with 57 mg/kg (191 μCi) of radiolabeled fosamine ammonium, all of the dose was eliminated within 72 h. Approximately 87% of the administered dose was recovered from the feces and 13% from the urine (Table I). Trace amounts of radioactivity were also found in the gastrointestinal tract, hide, and expired air (0.1–0.2% total). No radioactivity (<0.05%) was detected in the body tissues after 72 h.

Thin-layer cochromatographic analysis of the feces extracts showed two major, polar metabolites which corresponded to standards of [^{14}C]fosamine ammonium (86%) and [^{14}C]CPA diammonium salt (14%). The radioscans shown in Figure 5 represent characteristic TLC behavior of these compounds with several solvent systems.

The TLC radioscans shown in Figure 6 represent the behavior of the methylated radioactive metabolites after

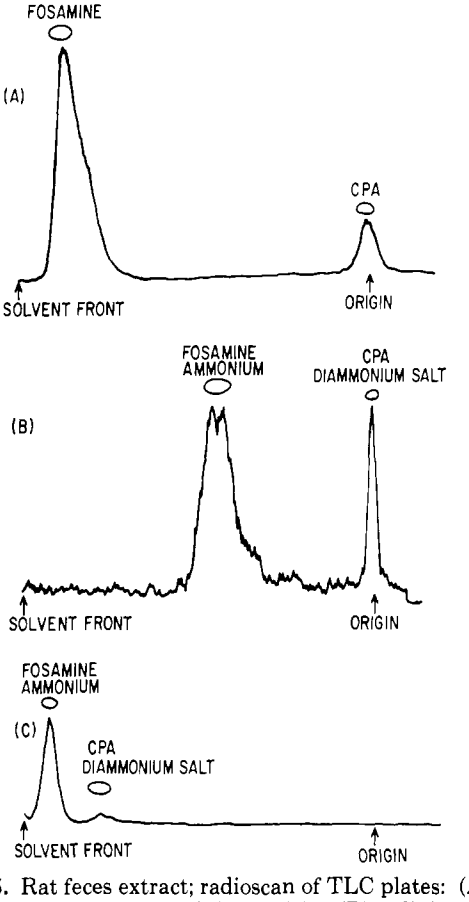

Figure 5. Rat feces extract; radioscan of TLC plates: (A) silica gel G; methanol/acetic acid (4:1, v/v); (B) cellulose; 0.5 M ammonium carbonate/methanol/acetonitrile (5:60:35, v/v/v); (C) cellulose; 0.5 M ammonium carbonate/methanol/water (5:60:35, v/v/v).

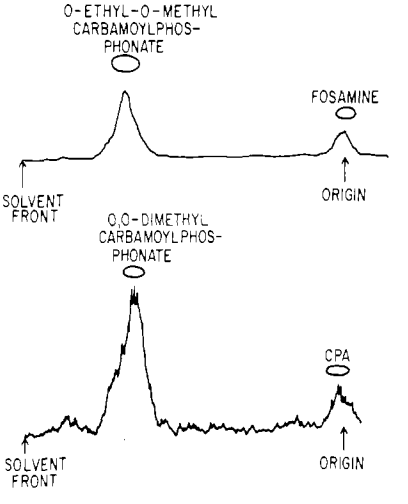

Figure 6. Methylated ^{14}C metabolites from rat feces; radioscan of TLC plates (silica gel G, 0.05 M ammonium carbonate/acetonitrile).

isolation by ion-exchange chromatography and preparative cellulose TLC. The derivatization was incomplete due to the biological matrix which apparently suppressed methylation. Also, TLC autoradiography of both the methylated metabolites and the corresponding standard reference compounds showed that additional methylation products had been formed in this reaction. GC analysis coupled with gas proportional counting of the radioactive material showed a retention time of about 3 min for *O*,*O*-dimethyl carbamoylphosphonate and *O*-ethyl-*O*-methyl

Table II. Radioactivity Identified in Rat Feces and Urine (as percent of original dose)

fraction	rat A		rat B	
	fosamine	CPA	fosamine	CPA
urine, 0-24 h	8.2	1.3	10.3	1.1
feces, 0-24 h	63	6.6	11.6	1.9
subtotal	(71)	(7.9)	(22)	(3.0)
urine, 24-48 h	1.4	0.22	1.9	0.24
feces, 24-48 h	12	1.2	49	12
subtotal	(13)	(1.4)	(51)	(12)
urine, 48-72 h	0.17	0.03	0.26	0.04
feces, 48-72 h	1.0	0.10	0.05	0.85
subtotal	(1.2)	(.13)	(0.31)	(0.9)
total recovery	85	9.4	73	16

carbamoylphosphonate. The other methylation products which apparently form during this reaction are postulated to be due to methylation of an amide proton in the enol tautomeric position.

Confirmation of the identity of the fecal metabolites was achieved by combination GC/MS of the methylated metabolites. Figures 3 and 4 illustrate the mass spectra of methylated fosamine and CPA, respectively, as recovered from rat feces. Comparison with standard reference compounds confirmed the identity of each metabolite.

Cochromatography of the urinary metabolites with standards again showed only two major metabolites which had behavior identical with those isolated from the feces. In this case, however, the low amount of radioactivity present in the urine precluded the successful isolation and confirmation of the metabolites by GC/MS. Consequently, cochromatography was relied upon for identification of fosamine and CPA in the urine.

Table II summarizes the quantitative excretion pattern obtained for the fecal and urinary metabolites of fosamine ammonium. Overall, an average of 79% of the administered [^{14}C]fosamine ammonium was rapidly eliminated unchanged from the animals while only an average of 13% of the compound was hydrolytically degraded to [^{14}C]CPA. The remaining unextractable radioactivity was bound to the feces residue presumably as fosamine.

This degradation route is consistent with known metabolic pathways of organophosphorus agrichemicals in animals (Menn and McBain, 1974) in that the C–O–P is easily hydrolyzed, whereas the C–P bond resists metabolic cleavage.

This study has shown that fosamine ammonium, the active ingredient in Krenite brush control agent is rapidly eliminated from a mammalian system and the identity of the eliminated ^{14}C in both urine and feces has been established.

ACKNOWLEDGMENT

We wish to thank Henry Sherman and A. M. Kaplan for assistance in the dosing of the animals and collection of samples, Robert W. Reiser for analytical assistance, especially the GC/MS studies, and Arthur G. Jelinek for synthesis of [^{14}C]fosamine ammonium.

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Determination of Pentachlorophenol in Marine Biota and Sea Water by Gas-Liquid Chromatography and High-Pressure Liquid Chromatography

Linda F. Faas* and James C. Moore

A method is described for measuring pentachlorophenol (PCP) in samples from the estuarine environment. Gas–liquid chromatography (GLC) is used to determine PCP residues in tissues as low as 0.01 ppm by formation of the ethyl diazohydrocarbon derivative, followed by Florisil cleanup. Application of the method to exposed organisms indicates that PCP accumulates in mullet (*Mugil cephalus*), grass shrimp (*Palaemonetes pugio*), and eastern oysters (*Crassostrea virginica*). Sea water concentrations as low as 0.002 ppb may be detected by formation of the amyl diazohydrocarbon derivative. Formation of the amyl derivatives of PCP and several related compounds gives GLC separation not possible with the methyl or ethyl derivatives. Parameters are outlined for high-pressure liquid chromatography (LC) determination of the free phenol without cleanup. Ultraviolet detection limits for PCP by LC are 5.0 ppm in tissues and 2.0 ppb in seawater.

Pentachlorophenol (PCP) is a herbicide, fungicide/bactericide, and insecticide that, together with its salts, has a broad spectrum of industrial, agricultural, and domestic applications (Benvenue and Beckman, 1967). U.S. production of PCP in 1977 was expected to be 80 million pounds (Cirelli, 1978), and annual Canadian usage was estimated to be 20 million pounds (Hoos, 1978).

PCP has been found in drinking water (Abrams et al., 1975), rivers, lakes, and streams (Buhler et al., 1973; Fountaine et al., 1976; Pierce and Victor, 1978; Rudling, 1970), sewage effluents (Abrams et al., 1975; Buhler et al., 1973), aquatic biota (Pierce and Victor, 1978; Rudling, 1970; Zitko et al., 1974), and even in man (Barthel et al., 1969; Rivers, 1972).

The toxicity of PCP to aquatic organisms (Adelman et al., 1976; Benvenue and Beckman, 1967; Schimmel et al., 1978) and its effects on settling communities (Tagatz et

U.S. Environmental Protection Agency, Environmental Research Laboratory, Sabine Island, Gulf Breeze, Florida 32561.