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Fate of Diisopropyl Methylphosphonate (DIMP) in a Lactating Cow

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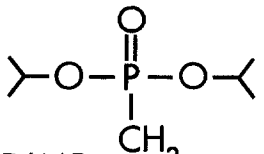
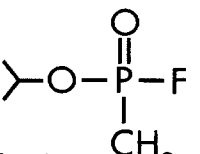
Between 1943 and 1957, a variety of chemical wastes were stored in unlined ponds at the Rocky Mountain Arsenal in Colorado by the U. S. Department of Defense. This has resulted in some groundwater contamination by certain of these chemicals (ROSENBLATT et al., 1975). One of the contaminants is diisopropyl methylphosphonate (DIMP), a byproduct of the production of the nerve gas isopropyl methylphosphonofluoridate (Sarin).



DIMP



Sarin

Because livestock in the affected areas may be exposed to DIMP through the drinking of well water, it is important that the metabolic and residual fate of DIMP be thoroughly defined in these animals. The studies reported here were designed to determine the metabolism of DIMP in the lactating cow.

MATERIALS AND METHODS

DIMP. Both nonradioactive and radioactive DIMP (59 mg/mCi, labeled with ^{14}C in the P-CH_3 group) were provided for these studies by the U.S. Army Medical Bioengineering Research and Development Laboratory, Fort Detrick, MD. The radiolabeled preparation as supplied required further purification, which was accomplished by thin-layer chromatography (TLC) in solvent system 1 (vide infra), giving a sample of >99% radiochemical purity. Both the unlabeled and labeled samples were confirmed as DIMP by gas-liquid chromatography (GLC)/mass spectrometry.

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Treatment of Cow. A 357-kg lactating Jersey cow was obtained from the milking herd of a local dairy. For 5 consecutive days, the animal was orally given at 24-h intervals a single gelatin capsule containing 3.57 g of unlabeled DIMP, a dose equivalent to 10 mg of DIMP/kg body weight/day. During that time, the animal was kept in a small pen, fed coastal bermuda grass hay ad libitum, and given ~ 2 kg of crushed grain concentrate at each milking (twice daily at 12-h intervals). Twenty-four h after the fifth and final dosing with unlabeled DIMP, the animal was moved to a stanchion, catheterized, and given a single oral dose of radioactive DIMP, to which had been added sufficient unlabeled DIMP to make the total dose equivalent to 3.57 g DIMP and thus 10.0 mg/kg of body weight. The total radiocarbon given to the cow was 6.45×10^8 dpm. The specific activity of the administered ^{14}C-labeled DIMP was 181 dpm/μg.

Radiocarbon Quantitation. After treatment, whole blood samples were taken at frequent intervals by intravenous puncture into the jugular vein; total urine and feces samples were collected less frequently. The animal was milked every 12 h. Ninety-six h after the radioactive dose, the cow was sacrificed and several tissues were taken for radiocarbon analysis.

Upon collection of milk and urine samples, 0.5-mL aliquots were assayed directly by liquid scintillation counting (LSC). Large aliquots of the remaining samples were then frozen for later analysis. Fecal samples were mixed thoroughly, 0.5-1.0 g samples were removed for combustion analysis, and portions of the remainder were kept frozen for analysis. Whole blood (1.0 g), tissue samples (0.2-1.0 g), and feces were air dried, and the radiocarbon present in these samples was quantitated by LSC after combustion in an oxygen atmosphere and bubbling the combustion gases through CO_2 trapping solution (IVIE, 1978).

Resolution and Characterization of Metabolites. The radioactive components in the excreta of the ^{14}C-DIMP-treated cow were resolved with TLC. In all cases, the separations were made on 20- x 20-cm, 0.25-mm-thick silica-gel TLC plates (Brinkman Silplate F-22, with fluorescent indicator), developed in either one or two dimensions. Seven solvent systems were used as specified in Table I.

The nature of radioactive components in urine of the DIMP-treated cow was studied in samples collected 4-, 8-, and 12-h after treatment, since these samples contained the highest levels of radiocarbon. Only the fecal sample collected 24 h after treatment was

TABLE I

TLC Rf Values of DIMP, IMPA, and MIMP in Several Solvent Systems[a,b]

TLC solvent system[c]	Rf of indicated product		
	DIMP	IMPA	MIMP
1	0.57	0.04	0.48
2	.60	.41	.49
3	.64	.38	.60
4	.32	.00	.22
5	.31	.01	.20
6	.25	.00	.16
7	.69	.33	.64

[a]Abbreviations: DIMP - diisopropyl methylphosphonate, IMPA - isopropyl methylphosphonic acid, MIMP - methyl isopropyl methylphosphonate. [b]TLC on Brinkman Silplate F-22, with fluorescent indicator, 0.25 mm gel thickness. [c]Solvent systems as follows: 1 (hexane-ethyl acetate-methanol, 2-2-1); 2 (methanol-ethyl acetate, 7-3); 3 (methanol- n-propanol-acetic acid, 10-10-1); 4 (benzene-chloroform-acetone, 1-1-1); 5 (ethyl acetate-acetic acid, 49-1); 6 (benzene-ethyl acetate-methanol, 15-15-1); 7 (chloroform-methanol, 1-1).

analyzed due to the very low levels of radiocarbon present in samples collected after this time.

Whole urine was spotted directly on TLC for resolution of the radiocarbon present, while fecal samples (5 g) were extracted by blending thoroughly with 20 mL of methanol with a Willems polytron homogenizer. The slurry was then centrifuged to precipitate the residue, and the methanol extract was pipetted off. The residue was extracted an additional 3 times with methanol as before; radiocarbon in the combined methanol extracts was quantitated by LSC and then analyzed by TLC. Radiocarbon in the extracted residue was determined by oxygen combustion.

Chemical characterizations were made by TLC comparisons of products isolated from the excreta, or their derivatives, with compounds of known structure in each of the seven solvent systems used. Where possible, identifications were made or confirmed with GLC/mass spectrometry, using a Varian/MAT CH-7 magnetic scan spectrometer coupled with a Varian 2700 gas chromatograph. The column was a 1.8- m x 2-mm (I.D.) glass column packed with 3% SE 30 on Varaport 30. The

column temperature was 110°C.; injector and detector ovens and all transfer lines were maintained at a slightly higher temperature than the column. Helium carrier gas was maintained at a flow rate of 50 mL/min. All spectra were recorded at 70 eV.

RESULTS

Excretion and Tissue Retention. Carbon-14 was rapidly excreted after administration of a single oral dose of ^{14}C-DIMP to the cow (Table II). About 84% of the dose was eliminated in the urine, about 7% in the feces, and <0.1% in the milk. The data in Table II indicate that the radiocarbon was rapidly absorbed and excreted by the cow, because samples collected later than 24 h posttreatment contained only very low amounts of radiocarbon.

Combustion analysis of whole blood samples confirmed that both absorption and excretion were very rapid. The sample collected 2 h after treatment with the radioactive dose contained the highest blood residues observed (1485 dpm/g) but these residues rapidly declined to undetectable levels (<20 dpm/g) within 24 h after treatment.

Although only about 91% of the administered radiocarbon was accounted for in the urine, feces, and milk, combustion analysis of numerous tissue samples showed that none contained detectable radiocarbon residues. The tissues analyzed included brain, fat, gallbladder, heart, kidney, liver, muscle, ovary, skin, spleen, urinary bladder, and udder.

Metabolite characterization -- Urine. Two-dimensional TLC (systems 1 and 2) of whole urine from samples collected 4, 8, or 12 h after treatment revealed that in each case, the urine radiocarbon consisted of a single product that, based upon TLC behavior, was clearly not DIMP. Attempts were made to obtain the product in quantities sufficient for spectral analysis by extracting the acidified urine (pH ~1.0) with ethyl acetate. Even though the product partitioned poorly into the organic phase, repeated extraction with ethyl acetate recovered the metabolite in good yield.

The urine metabolite was more polar than DIMP, based on its TLC behavior and partitioning characteristics, and it appeared logical to assume that the product might be isopropyl methylphosphonic acid (IMPA), formed by hydrolysis of 1 of the 2 DIMP isopropyl groups. Subsequent derivatization studies showed that this was indeed the case. A solution of

TABLE II

Elimination of Radiocarbon After Treatment of a Lactating Cow with ^{14}C-DIMP as a Single Oral Dose at 10 mg/kg of Body Weight

Hours after treatment	Excretion (cumulative % of dose)		
	Milk	Urine	Feces
4	-[a]	29.7	-[a]
8	-[a]	64.9	-[a]
12	0.06	74.5	-[a]
24	0.08[b]	82.3	6.6
36	0.08	82.9	-[a]
48	0.08	83.3	7.4[c]
72	0.08	83.8	7.4
96[d]	0.08	84.0	7.4

[a]Sample not collected. [b]Radiocarbon not detected in milk samples collected after 24 h. [c]Radiocarbon not detected in feces samples collected after 48 h. [d]Cow sacrificed 96 h after treatment.

the metabolite in ethyl acetate was added to a 5-ml glass ampoule, the solvent was evaporated, and the metabolite was then dissolved in 1.0 ml acetone. A small stirring bar was added, as were isopropyl bromide (0.1 ml) and anhydrous potassium carbonate (100 mg). The ampoule was then heat sealed and placed on a hot plate (with stirring) with sufficient heat to cause the solution within the ampoule to reflux. After 3 h the sample, upon examination by TLC, was converted (56% yield) to a product having identical TLC behavior to that of authentic DIMP. The remaining radiocarbon was as unreacted starting product. Analysis of the derivatized metabolite by GLC/mass spectrometry confirmed that it was indeed DIMP (Table III).

It was possible (though unlikely) that the metabolite had arisen through hydrolysis of both of the DIMP isopropyl groups to give methylphosphonic acid (MPA), in which case isopropyl bromide esterification could conceivably convert the product back to DIMP through a transient monoester. This possibility was ruled out by preparing the methyl ester of the metabolite by its reaction with methyl iodide by use of essentially the same procedure described above for preparation of the isopropyl derivative. Analysis of the methyl derivative by GLC/mass spectrometry (Table III) confirmed that it was methyl isopropyl methylphosphonate (MIMP), and thus that the single radioactive product in urine was IMPA. MIMP was also generated from the urine metabolite by its reaction with an ether

TABLE III

Summary of Mass Spectral Data of DIMP and MIMP[a,b]

DIMP			MIMP		
Ion Fragment	m/e	RI[c]	Ion Fragment	m/e	RI[c]
$CH_3PO(OiPr)_2$	180	<0.1[d]	$CH_3PO(OiPr)(OCH_3)$	152	<0.1[d]
$CH_3PO(OiPr)(OC_2H_4)$	165	2.9	$CH_3PO(OC_2H_4)(OCH_3)$	137	26.1
$CH_3P(OiPr)(OH)_3$	139	7.1	$CH_3P(OCH_3)(OH)_2$	111	73.7
$CH_3PO(OiPr)(OH)$	138	2.1	$CH_3PO(OCH_3)(OH)$	110	3.0
$CH_3PO(OC_2H_4)(OH)$	123	59.0	$CH_3PO(OCH_3)$	93	100.0
$CH_3PO(OiPr)$	121	7.0	$CH_3P(OH)_2$	80	12.6
$CH_3P(OH)_3$	97	100.0	$CH_3PO(OH)$	79	29.7
$CH_3PO(OH)$	79	22.4			

[a]Abbreviations as follows: DIMP - diisopropyl methylphosphonate, MIMP - methyl isopropyl methylphosphonate. [b]See OCCOLOWITZ and WHITE (1963) for mass spectrum of DIMP. [c]RI - relative intensity, base peak = 100. [d]Parent ion not observed.

solution of diazomethane, and its structure was confirmed by GLC/mass spectrometry.

Feces. Methanol extraction of the feces sample collected 24 h after treatment resulted in >99% extractability of the radiocarbon present, based upon LSC of the extract and combustion analysis of the extracted residue. TLC analysis of the radiocarbon in the feces extract in solvent systems 1, 2, and 6 revealed that in each case 2 radioactive components were present. One of these, containing 97.1% of the total radioactivity present, was apparently identical to the metabolite seen in urine. The second product, comprising 2.9% of the total, was found in subsequent experiments to co-chromatograph on TLC with authentic DIMP in each of the solvent systems used. The DIMP isolated from the fecal extracts was not obtained in sufficient quantity for GLC/mass spectral confirmation

of its structure. The major fecal metabolite was, however, confirmed as IMPA by its reaction with diazomethane. The methylated feces metabolite exhibited the same TLC behavior as did the methylated urine metabolite. GLC/mass spectral analysis confirmed the methyl derivative as MIMP, and thus that the feces metabolite was IMPA.

Milk. Only exceedingly low levels of radiocarbon were secreted into the milk of the DIMP-treated cow (Table II), but attempts were made to determine something of its nature. Milk from the 12-h collection period was separated into cream and skim-milk phases, and LSC indicated that all of the milk radioactivity was associated with the skim milk. Direct extraction of the skim milk with ethyl acetate did not result in partitioning of any radiocarbon into the organic phase, but when the skim milk was adjusted to pH ~ 1.0, ethyl acetate extraction (twice) partitioned 15-20% of the radiocarbon present into the organic solvent. However, acidification of the milk also resulted in large amounts of interfering material partitioning into the ethyl acetate. This problem, and the exceedingly low levels of radiocarbon present in the extract, did not permit successful TLC analysis of the milk radiocarbon.

Volatility of DIMP. Several indications were seen in these studies that DIMP, as might be expected, is quite volatile. In TLC purification of the radioactive sample before its administration to the cow, as much as 25% of the radiolabeled sample was lost from volatility during sample application to TLC, plate development, and drying. So that additional loss would be minimal, the rather large amount (~3.5 g) of unlabeled DIMP was added to the ^{14}C-DIMP solution extracted from the gel before concentration, and the solvent was not completely removed before administration of the compound to the cow.

A brief, simple study confirmed the volatility of DIMP. A solution of radioactive DIMP (15 µg in 100 µL acetone) was added to the bottom of scintillation vials and the samples were held uncapped in a hood for periods up to 4 h. Residual radiocarbon was quantitated simply by adding cocktail to the vials and counting by LSC. Under these conditions, DIMP rapidly volatilized from the glass surfaces. About one-fourth of the deposit was lost simply during the evaporation of the 100 µL acetone carrier, and after only 1 h, >95% of the ^{14}C-DIMP had volatilized.

The high volatility of DIMP *per se* contrasted sharply with the very low volatility of its only

metabolite in the cow, IMPA. Studies indicated that whole urine from the DIMP-treated cow could be lyophilized, at pressures of <10μHg, with no detectable volatility loss of the IMPA metabolite.

DISCUSSION

These studies have shown that DIMP was readily absorbed, metabolized, and excreted by a cow after oral exposure, and that DIMP showed little potential for secretion into milk or accumulation in tissues. The metabolism of DIMP in the cow proceeded through a single mechanism--hydrolysis of one of the isopropyl ester linkages. The resulting metabolite, isopropyl methylphosphonic acid (IMPA), was the only radioactive component in urine and the only DIMP metabolite in feces. Although the chemical nature of the very minor amount of radiocarbon secreted into the milk of the DIMP-treated cow was not determined, that it was not DIMP was almost certain, on the basis of partitioning characteristics. Because the analyses of urine and feces revealed that DIMP was almost quantitatively metabolized to IMPA in the cow, it may well be and, in fact, seems likely that IMPA was the major if not the only radioactive constituent in milk. It must be emphasized, however, that there are no direct experimental data to support the assignment of the milk radiocarbon as IMPA.

Although only about 91% of the calculated ^{14}C-DIMP dose was accounted for in the excreta of the treated cow, none of several tissues analyzed contained detectable radiocarbon residues, suggesting that elimination of the ^{14}C-DIMP dose was essentially quantitative. However, the tissue analysis procedure used was such that any intact DIMP residues present might well have volatilized during the analysis and thus were not detected. It may also be that all or part of the about 9% of the dose not recovered in the excreta represents experimental error, but it is possible that at least some of this loss reflects volatilization by any or a combination of several mechanisms. These include loss of radiocarbon during final sample preparation before administration to the cow, volatilization in gases eructated from the rumen, and volatilization from the excreta, either before it was collected or during the storage and analysis steps.

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