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Fate of Dicyclopentadiene in a Lactating Cow

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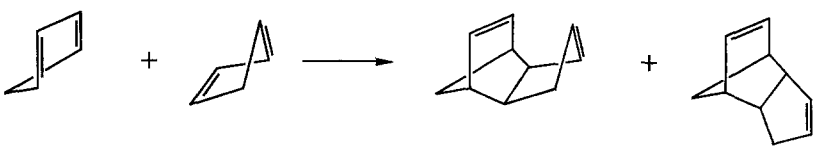
Dicyclopentadiene (DCPD) is a waste-product generated in the synthesis of certain chlorinated cyclodiene insecticides. The compound is formed by the diels-alder condensation of two molecules of cyclopentadiene:



Cyclopentadiene — endo (major) — exo (v. minor)

Dicyclopentadiene (DCPD)

Generally, the endo configuration is almost quantitatively favored over the exo.

DCPD is a contaminant of certain groundwater supplies in Colorado (ROSENBLATT et al. 1975), which resulted from the disposal of pesticide wastes in unlined ponds or from deep-well injection at the Rocky Mountain Arsenal under the auspices of the U. S. Department of Defense. Although the disposal of these wastes ended no later than 1966, DCPD residues in the sub- to low-ppm range continue to be detectable in some well-water supplies. It is possible that DCPD may constitute a hazard both to livestock and other animals that drink contaminated water and to humans through the consumption of contaminated meat or milk supplies. The current investigation was undertaken to evaluate the metabolic and residual behavior of DCPD in cattle, and to determine if this compound or its metabolites are retained by edible tissues or secreted into milk of animals exposed to DCPD.

MATERIALS AND METHODS

DCPD. Both unlabeled and radiocarbon-labeled (uniform [^{14}C], 62.6 mg/mCi) samples of DCPD were supplied by the U.S. Army Medical Bioengineering Research and Development Laboratory, Fort Detrick, MD.

0007-4861/80/0024-0662 $01.80

DCPD is quite volatile, and TLC was inappropriate for purification of the radiochemical. Thus, the [^{14}C]DCPD was purified by column chromatography. Silica gel (25 g, Activity III, ICN Pharmaceuticals, Cleveland, OH) was poured (dry) into a 2.5 x 20 cm glass chromatography column, the [^{14}C]DCPD was added in 3.0 ml of 20% acetone in hexane, then the column was eluted with hexane. The first 20 ml of the column eluate contained the DCPD. Two such column runs were required to purify the entire [^{14}C]sample. The sample was radiochemically pure on the basis of TLC, and GLC/mass spectral analysis (vide infra) showed it to contain only one peak, whose mass spectrum indicated it to be DCPD. The unlabeled DCPD also gave a single peak by GLC (vide infra) whose mass spectrum confirmed it was DCPD.

Treatment of Cow. A 293-kg lactating Jersey cow was obtained directly from the milking herd of a local dairy. The treatment consisted of first orally administering to the animal a single gelatin capsule containing 2.93 g of unlabeled DCPD, daily for 5 consecutive days. This dose was equivalent to 10 mg of DCPD/kg of body weight/day. During this time, the cow was held in a small pen and was fed coastal bermuda grass hay ad libitum, and was given 2 kg of crushed grain concentrate at each milking (twice daily, at 8 a.m. and 8 p.m.). Twenty-four h after the fifth and final dosing with unlabeled DCPD, the animal was moved to a stanchion, catheterized, and given a single oral dose of [^{14}C]DCPD, to which had been added sufficient unlabeled DCPD to make the total dose equivalent to 2.93 g of DCPD and thus 10.0 mg/kg. The total radiocarbon given the cow was 4.00×10^8 dpm. The specific activity was 137 dpm/μg. In preparation of the [^{14}C]DCPD dose, the appropriate amount of unlabeled DCPD (∿2.9 g) was added to the column purified [^{14}C]DCPD before removal of solvent in order to suppress volatility of the labeled compound. Care was also taken not to completely remove the solvent during the concentration process, again to suppress volatility. Thus, the [^{14}C]DCPD given the cow contained about 5-10 ml of solvent in addition to the DCPD.

Radiocarbon Quantitation. After treatment, whole blood samples were taken at frequent intervals by intravenous puncture into the jugular vein, and total urine and feces samples were collected less frequently. The animal was milked every 12 h. Ninety-six h after dosing with [^{14}C]DCPD, the cow was killed 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). A considerable part of the rest of the samples was then frozen for later study. 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 combustion in an oxygen atmosphere followed by bubbling the combustion gases through carbon dioxide trapping solution and counting by LSC (IVIE, 1978).

Resolution of Metabolites. TLC and high performance liquid chromatography (HPLC) were used to resolve the radioactive components in the excreta of the [^{14}C]DCPD-treated cow. In all analyses involving TLC, the separations were made on 20- x 20-cm, 0.25-mm-thick TLC plates, developed either single dimensionally or in two dimensions. The solvent systems used were as follows: (1) chloroform-methanol-acetic acid (10-5-1); and (2) ethyl acetate-methanol-acetone (1-1-1).

Radioactive components in whole urine were also studied by HPLC. The instrument was a Waters Model ALC 202 liquid chromatograph, equipped with dual pumps and solvent programmer. The reverse phase column was a μBondapak C_{18}, 3.9-mm i.d. x 300 mm. The initial solvent was 1% acetic acid in water, at a flow rate of 1.0 ml/min. Immediately after injection of whole urine (0.2 ml), acetonitrile was programmed into the solvent at a linear rate up to 50% over a period of 60 min, with the flow rate maintained at 1.0 ml/min. The column effluent was collected as 0.5-ml samples (1 every 30 sec) directly into scintillation vials, and the radiocarbon in these samples was quantitated by LSC.

Whole urine was spotted directly on TLC for resolution of the radiocarbon present whereas feces samples (5 g) were extracted by blending thoroughly with 10 ml methanol with a Willems polytron homogenizer. The slurry was then centrifuged to precipitate the residue, and the methanol was pipetted off. The residue was extracted 3 more times with methanol as before, and the radiocarbon in the combined methanol extracts was quantitated by LSC and that in the extracted residue by oxygen combustion.

Studies were also conducted to determine to what extent the DCPD cow urine metabolites were in the form of glucuronide conjugates. In these studies, whole urine (2.5 ml) was added to 0.2 M, pH 4.5, sodium acetate buffer (2.5 ml), and the mixture was readjusted to pH 4.5 by the addition of hydrochloric acid. A solution of β-glucuronidase (from Helix pomatia,

Calbiochem, San Diego, CA) was then added. The enzyme activity in each sample was equivalent to about 50,000 Fishman units. The samples were then incubated with shaking for 24 h at 37-38°C, then the solution was applied directly to TLC for resolution of the radiolabeled products. Samples were also studied in which the activity of the added enzyme had first been destroyed by heat (100°C , 30 min).

Whole milk (50 g) from the 12 h sampling period was acidified to pH 2.0 with hydrochloric acid, then partitioned 3 times with equal volumes of ethyl acetate. The combined ethyl acetate extracts were dried over sodium sulfate, the solvent was removed under reduced pressure, and the oily residue was partitioned between acetonitrile and hexane to remove lipid material. Radiocarbon in aqueous, acetonitrile, and hexane phases was quantitated by LSC, and the radioactive components in the acetonitrile phase were studied by TLC.

GLC/mass spectroscopy. Samples of both the radiolabeled and unlabeld DCPD used in these studies were analyzed for confirmation of identity by GLC/mass spectroscopy. The instrument used was a Varian/MAT CH-7 magnetic scan spectrometer, coupled with a Varian 2700 gas chromatograph. The glass column (1.8 m x 2 mm) was packed with 3% SE 30 on Varaport 30. The column temperature was 100°C; injector, detector, and separator temperatures were slightly higher. Helium carrier gas was maintained at a flow rate of 50 ml/min. The spectra were recorded at 70 eV. The retention time of DCPD under these parameters was 2.1 min. The mass spectrum of DCPD exhibited diagnostic ions at m/e 132 (M^+) and m/e 66 (base peak, cyclopentadiene ion from retro diels-alder fragmentation).

RESULTS AND DISCUSSION

Excretion and Tissue Retention. Radiocarbon was quite rapidly excreted after oral administration of [^{14}C]DCPD to the cow (Table I). About 81% of the administered [^{14}C] was eliminated in the urine, about 4% in the feces, and <0.1% was secreted into the milk. Only exceedingly low levels of radiocarbon appeared in milk, and residues were not detected in milk samples collected later than 48 h posttreatment.

Radiocarbon in whole blood had reached maximum levels (290 dpm/g) within 2 h after dosing. Blood radiocarbon levels then declined rapidly, and residues were not detectable (<20 dpm/g) in blood samples taken later than 24 h after treatment. None of the tissue

samples collected contained detectable radiocarbon residues. Tissues included brain, fat, gall bladder, heart, kidney, liver, muscle, ovary, lung, adrenal, skin, spleen, urinary bladder, and udder.

TABLE I

Elimination of Radiocarbon After Treatment of a Lactating Cow With[^{14}C]DCPD at 10 mg/kg of Body Weight

Hours After Treatment	Excretion (Cumulative % of Dose) Milk	Urine	Feces
4	-[a]	20.1	-[a]
8	-[a]	39.0	-[a]
12	0.03	55.0	-[a]
24	.04	73.5	2.2
36	.06	78.3	-[a]
48	.07[b]	80.1	3.7
72	.07	81.0	4.1
96[c]	.07	81.4	4.2
Total Excretion 85.7%			

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

Metabolite Resolution--Urine. Two-dimensional TLC analysis of whole urine collected 4, 8, or 12 h after the [^{14}C]DCPD dose showed several radiocarbon labeled compounds in the urine, but most of these products were poorly resolved and quite polar. There were no major differences in metabolite distribution among the samples analyzed. Examination of whole urine by HPLC confirmed numerous DCPD metabolites in the urine. At least 14 radioactive constituents were resolved or partly resolved by HPLC analysis of whole urine collected 8 h after administration of the [^{14}C]DCPD dose (Figure 1).

Attempts by TLC to obtain sufficient amounts of individual DCPD urine metabolites for analysis by spectrometric, chemical degradation, or by derivatization techniques were hampered for several reasons. The relatively low concentration of individual metabolites in urine made these products difficult to obtain in appreciable amounts free of normal urine constituents. More limiting, however, was the fact that the metabolites were extractable with great difficulty from silica gel and, invariably, the isolated compounds suffered considerable breakdown, usually to several products, during the isolation

process. Attempts to use HPLC to isolate the metabolites were also unsuccessful, primarily because analysis of whole urine resulted in rapid, rather severe column deterioration, and seriously affected reproducability. Also, metabolites that were isolated by HPLC were unstable during the extraction and lyophilization process necessary to remove the aqueous-based eluting solvent.

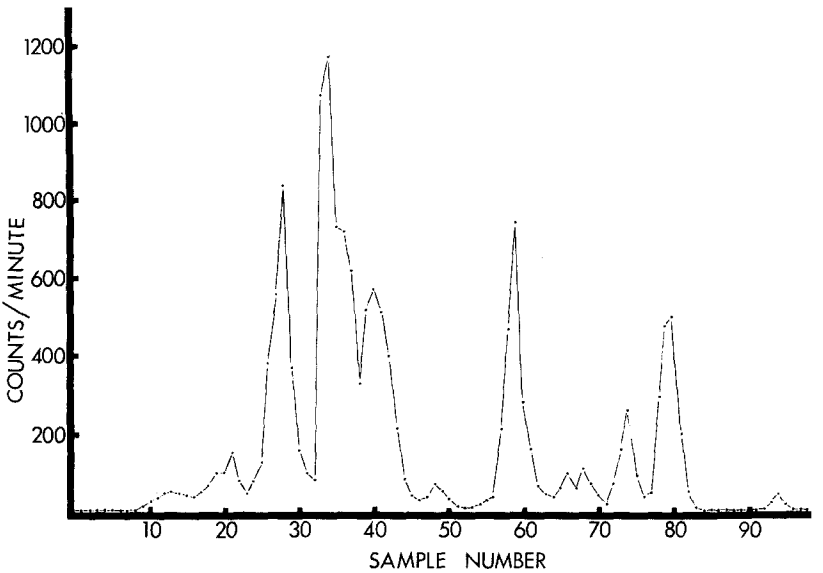


Figure 1. HPLC resolution of metabolites in whole urine of a cow treated with [^{14}C]DCPD at 10 mg/kg of body weight. The urine sample analyzed was collected 8 h after treatment. Parameters for the HPLC analysis were as specified in text. Samples collected after the 95th sample did not contain detectable radiocarbon residues.

Attempts to obtain [^{14}C]labeled urine metabolites in more concentrated form by solvent extraction were unsuccessful. Direct extraction of whole urine with ethyl acetate gave <3% partitioning of the radiocarbon into the organic phase. Acidification of the urine to pH 1-2, followed by extraction with ethyl acetate, likewise was only marginally effective because 3 ethyl acetate extractions recovered <20% of the radioactivity present. In the latter case, high levels of interfering materials in the extracts made subsequent metabolite isolation attempts unsuccessful. Other procedures attempted were also unsuccessful.

Chemical and Enzymatic Hydrolysis of Urine Metabolites. Incubation of whole urine with

β-glucuronidase clearly showed that at least 65% of the total urinary radiocarbon was in the form of glucuronide conjugates (Table II). Incubation of whole urine from the DCPD-treated cow in buffer (no enzyme) or with enzyme that had been heat deactivated (boiled enzyme) resulted in little change in TLC behavior of the radiocarbon present. Only about 7% of the radiocarbon in these samples was associated with products of "high" TLC R_f (Table II). However, in those samples that were incubated with active β-glucuronidase, more than 70% of the radiocarbon migrated to a higher TLC R_f; thus, the glucuronides had been cleaved to yield aglycones of reduced polarity. On the basis of TLC, at least 3 distinct aglycones were formed as a result of β-glucuronidase hydrolysis. Acid hydrolysis of the components in whole urine gave results similar to those obtained upon β-glucuronidase incubation (Table II) except that at least 5 products of higher TLC R_f appeared to be generated. It may well be and in fact seems likely that some of these products resulted from additional degradations in the acid media.

TABLE II

TLC Behavior of Urine Metabolites From a [^{14}C]DCPD-Treated Cow After Incubation With β-Glucuronidase or Hydrolysis With 1 N Hydrochloric Acid[a]

	Products at Indicated R_f Range (%)[d]	
Hydrolysis Procedure	0.0-0.5	0.5-1.0
None, whole urine	97.2	2.8
β-Glucuronidase[b]		
no enzyme	92.7	7.3
boiled enzyme	92.6	7.4
active enzyme	25.2	74.8
1 N Hydrochloric acid[c]	26.4	73.6

[a]Urine collected 8 h after dosing. [b]See text for incubation conditions. [c]Whole urine adjusted to 1 N with HCl, then held for 30 min at 100°C. [d]As determined by direct single dimension TLC analysis of the hydrolysate, developed in chloroform-methanol-acetic acid (10-5-1).

Feces. Analysis of the feces sample collected 24 h after the [^{14}C]DCPD treatment revealed that 84% of the very low amounts of radiocarbon present was extractable with methanol. TLC analysis of the extract

followed by radioautography indicated that in solvent system 1, all of the extracted feces radiocarbon was associated with a single component of R_f ~0.5. The product was not DCPD, it was available in only very limited quantity, and levels of interfering materials were too high to permit further studies of its nature.

Milk. Although <0.1% of the administered radiocarbon was secreted into the milk of the DCPD-treated cow, attempts were made to determine something of its nature. Extraction of acidified whole milk with ethyl acetate, with subsequent cleanup by hexane-acetonitrile partitioning, resulted in 43.0% of the milk radiocarbon residing in the acetonitrile and 2.8% in hexane; 54.2% remained unextracted from the aqueous phase. TLC analysis of the acetonitrile phase indicated that it apparently contained only a single radioactive product that was not DCPD. The product had an R_f of 1.00 in solvent system 1 and 0.56 in solvent system 2. The availability of the product in only exceedingly low quantities prevented definitive studies of its chemical nature.

CONCLUSIONS

DCPD is subjected to rapid and extensive metabolism after oral exposure to a lactating cow. Of the total radiolabeled dose administered to the animal, about 86% was recovered in the urine and feces, and only trace amounts were secreted into milk. The portion of the dose not accounted for in the excreta may reflect, in part, experimental error; but quite probably, because of the relatively high volatility of DCPD, some of the administered material may have been eliminated through gases eructated from the rumen or perhaps even through respiration. The fact that more than 80% of the administered dose was ultimately excreted in the urine and only about 4% in feces indicates that the orally adminstered compound was extensively absorbed from the gastrointestinal tract. The extent of radiocarbon elimination through the feces was possibly greater than that indicated in Table I because any intact DCPD that might have been eliminated in the feces would almost certainly have suffered some volatility loss before collection and possibly more extensive loss during analysis. Also, even though radiocarbon was not detected in any tissue samples analyzed, the possibility that such residues were indeed present cannot be ruled out because of the volatility of DCPD.

Little was learned about the chemical nature of DCPD metabolites during this study except that, in urine, they are primarily in the form of glucuronide

conjugates. It may well be that these metabolites in the cow arose, at least in part, through epoxidation of one or both of the DCPD double bonds, followed by hydrolysis of the epoxides to diols (or possibly epoxy diols or tetraols), then ultimately conjugation with glucuronic acid.

The current studies suggest that low-level exposure of ruminants to DCPD will not result in appreciable retention of residues by edible tissues or their secretion into milk. Although the retention of some DCPD residues by tissues cannot be absolutely ruled out, the relatively high volatility of the compound makes it almost certain that normal food preparation procedures will prevent residues from reaching the human food supply. Recent studies in this laboratory have shown that DCPD is not appreciably toxic to cattle (PALMER 1979), thus the exposure of these animals to low levels of DCPD in the environment will not likely result in any significant toxicological hazard.

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REFERENCES

IVIE, G. W.: J. Agric. Food Chem. 26, 81 (1978).

PALMER, J. S.: U. S. Department of Agriculture, College Station, TX, private communication (1979).

ROSENBLATT, D. H., T. A. MILLER, J. C. DACRE, I. MUUL, AND D. R. COGLEY: U. S. Army Med. Bioeng. Res. Dev. Tech. Rep. No. 7508, December 1975.