Dieldrin-^{14}C Metabolism in Sheep

Distribution and Isolation of Urinary Metabolites

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Seven sheep were given 165 to 230 microcuries of dieldrin-^{14}C. Three to 14% of the radioactive dose was recovered in the urine over 3- to 6-day periods. This radioactivity was contained in six metabolites, four of which were extracted from urine with hexane and two of which were not. The extraction and isolation of these metabolites are described.

That chlorinated hydrocarbon insecticides, such as dieldrin, are accumulated in body fat is well known. However, only recently has the metabolism of dieldrin and related insecticides been reported in animals. Korte and Arent (1965) reported the excretion of dieldrin metabolites in urine and feces of rabbits and rats. They isolated six metabolites from rabbit urine, with one comprising 86% of the total. This metabolite was identified as a trans-diol formed by the addition of water across the epoxide ring. Later, Street and Chadwick (1967) presented evidence suggesting that the principal dieldrin metabolite in rat urine differed from that in rabbit urine. However, they have not identified their metabolites, and those in rat urine may be the same metabolites as those in rabbit urine. Most recently, Matthews and Matsumura (1969) isolated two fecal and two urinary metabolites of dieldrin from rats, and they proposed a metabolic scheme whereby these metabolites might be formed in the liver.

In the meantime, studies at our laboratory have also indicated that sheep excrete metabolites of dieldrin in urine. To our knowledge, this is the first definitive study of the metabolism of a chlorinated hydrocarbon insecticide by ruminant animals. The objectives of our experiments were to study the distribution of dieldrin and its metabolites in sheep and to identify these metabolites. We report herein the distribution and separation of metabolites excreted in the urine.

MATERIALS AND METHODS

Animal Treatment. Seven yearling wethers weighing 27 to 85 kg. were used. Three sheep were penned individually and fed 2 mg. dieldrin per kg. body weight per day for 5 weeks prior to dieldrin-^{14}C metabolism studies. Four sheep were given 20 mg. of dieldrin per kg. of body weight in a single dose at the time of the dieldrin-^{14}C metabolism studies. This dose was given orally in gelatin capsules.

After the sheep were accustomed to the metabolism unit (Robbins and Bakke, 1967), a human female urinal was put on them to collect urine uncontaminated with feces. The urinal was drained into a covered stainless steel pan through latex tubing. Dieldrin-^{14}C, specific activity 184 μCi. per mg., was given orally in gelatin capsules. Sheep No. 222 received four successive doses of 57.7 μCi. at 24-hr. intervals. All other sheep were given a single dose as shown in Table I.

Carbon dioxide was collected from sheep No. 176 for 24 hours following the radioactive dose. Urine was collected at 8- to 24-hr. intervals and feces were collected at 24-hr. intervals. Three to 6 days after the radioactive dose, collections were terminated, and three of the sheep were killed. Their entire carcasses were ground and sampled, and the samples freeze dried.

Freeze-dried feces were stored in sealed jars. Urine was refrigerated when stored and never stored longer than a few days. Toluene was layered on the urine collected from sheep 176 and 239 to serve as a bacteriostat. The toluene extracted some of the radioactivity from the urine, however, and caused other problems in later extractions and cleanup of the urinary metabolites, so this practice was discontinued.

Dieldrin-^{14}C Analysis. Quantitation of dieldrin-^{14}C was accomplished with a liquid scintillation spectrometer employing an external standard. Dioxane and toluene scintillator solutions were used for counting the radioactivity (Hayes, 1963).

Analysis for $^{14}CO_2$ in respired gases was accomplished by methods described by Jeffay and Alvarez (1961). Urinary ^{14}C was determined by counting 1 ml. of a 1 to 10 dilution of urine with water in 15 ml. of dioxane solution. Fecal and tissue radioactivity were determined by combustion and assay of the resultant $^{14}CO_2$ (Kelley *et al.*, 1961).

Urinary Metabolite Isolation. Solvents of technical to analytical grade were used for extracting dieldrin metabolites from urine. Solvents were removed from the metabolites by evaporation at temperatures below 40° C.

Radioactivity in eluates from column chromatography was monitored by a flow detector equipped with a 1-ml. flow-through cell containing a scintillator of insoluble cerium-activated silicate glass beads (0.2 to 0.4 mm. diam.) with 2.5% natural lithium (Picker Nuclear).

Thin-layer and paper chromatograms were monitored for radioactivity on a radiochromatogram scanner.

Gel Filtration Columns. Gels used were Sephadex G-10 (40- to 120-micron particle size) and Sephadex LH-20 (25- to 100-micron particle size; Pharmacia Fine Chemicals Inc., Piscataway, N.J.). These gels were poured as a slurry into columns (1 to 2 cm. i.d.) to depths of 50 to 100 cm. Slurries were prepared by mixing the gel in the solvent desired and allowing adequate time for solvent regain before pouring.

Trioctylamine-Porapak Column. A column (2.5 cm. i.d.) was filled to approximately 10 cm. with Porapak (Robbins *et al.*, 1969). Five milliliters of trioctylamine in 15 ml. of ethanol was slowly applied to dry Porapak in the column. This column was then washed with water to remove the ethanol.

Anion-Exchange Column. Aminoethyl cellulose (Cellex-AE; Bio-Rad Laboratories, Richmond, Calif.) was swollen in methanol, poured into a column (2.5 cm. i.d.) to a depth of 50 cm. with a constant release of solvent from the column, and washed with 400 ml. of water to remove the methanol. It was then washed successively with 40 ml. of 1*N* hydrochloric acid, 200 ml. of water, 400 ml. of 1*N* ammonium

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Table I. Radioactive Dosages and Recovery of Dieldrin-^{14}C Given to Sheep

Sheep No.	Sheep Wt., kg.	Dieldrin, mg./kg. BW	Dieldrin-^{14}C, μCi	Collection Period, Days	Recovery of ^{14}C, % Carcass	G.I. Tract	Feces	Urine	Total	Hexane Extractable from Urine, %
176	62	2[a]	166.1	4	79.2	1.2	9.5	5.3	95.2	1.5
239	60	2[a]	182.5	3	74.0	2.0	10.2	9.0	95.2	3.4
222	51	2[a]	230.8[b]	4	61.6	12.9	8.7	8.1	91.3	35.0
180	77	20[c]	199.2	5	...	...	37.8	3.7		<1.0
192	85	20[c]	199.2	5	...	...	49.1	2.9		<1.0
265	27	20[c]	165.5	6	...	...	19.6	13.9		65.0
266	27	20[c]	165.5	6	...	...	18.8	13.1		65.0

[a] Given daily for 5 weeks prior to the metabolism study, and throughout the collection.
[b] Given in four successive doses of 57.7 μCi.
[c] Given as a single dose at the beginning of the metabolism study.

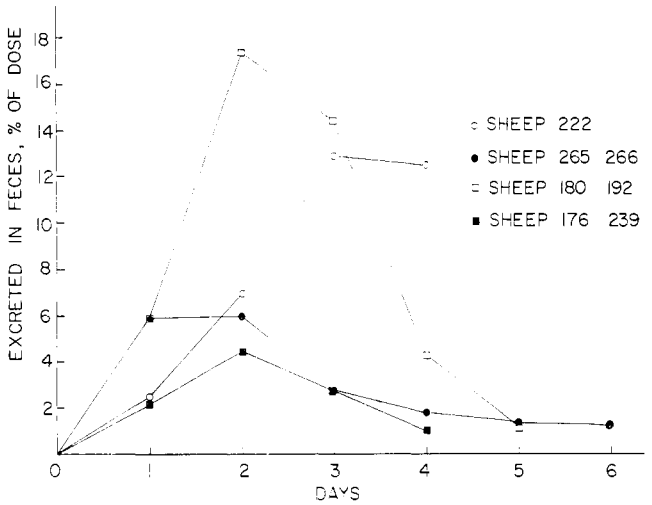

Figure 1. Excretion of radioactivity in feces

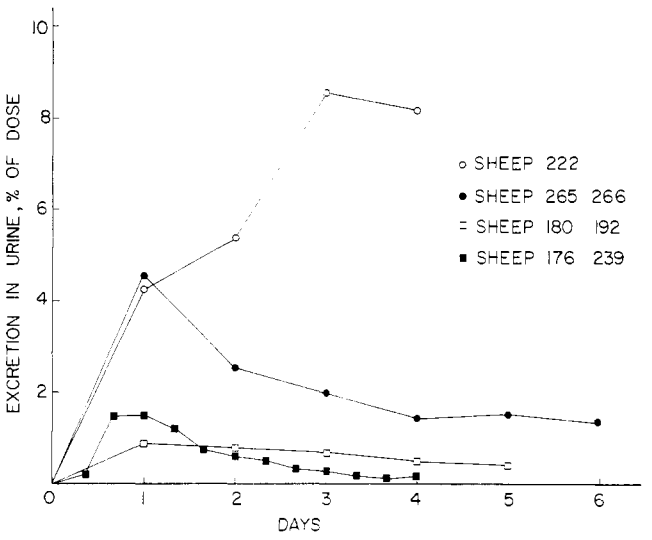

Figure 2. Excretion of radioactivity in urine

hydroxide, and finally water until the eluate was neutral to litmus paper.

RESULTS AND DISCUSSION

During the 5-week feeding period, no manifestation of acute or chronic toxicity occurred in the three sheep fed dieldrin at the 2-mg. level, and autopsy at the end of the experiment revealed no deleterious effects. Tremors occurred intermittently the day following dosing in sheep given the single 20-mg. dose.

Distribution and Recovery of Dieldrin-^{14}C. The amount of ^{14}C expired represented only 0.25% of the total dose, indicating that dieldrin is not metabolized to carbon dioxide.

Distribution of radioactivity among urine, feces, gastro-intestinal contents, and carcasses is shown in Table I. Recovery of ^{14}C from the three sheep slaughtered averaged 93.9%.

Radioactivity excreted in the feces and urine is plotted in Figures 1 and 2, respectively. The excretion curves of sheep 222 appear different from the others because this sheep was dosed continuously with dieldrin-^{14}C throughout the collection. In all cases, urinary excretion of ^{14}C by each sheep was lower than fecal excretion.

Dieldrin Metabolites in Urine. Only radioactive compounds excreted in the urine were analyzed qualitatively. Pilot extractions were made on separate 10-ml. samples of untreated urine with 10-ml. volumes of solvent. The radioactive compounds in the urine were poorly extracted with benzene, hexane, chloroform, diethyl ether, and ethyl acetate. They were extracted with butanol, but this solvent was not desirable because of its miscibility with the urine. Dieldrin added to urine was easily extracted with the above solvents.

Acidification of the urine to pH 2.5 with hydrochloric acid and extraction with ethyl acetate gave nearly 100% extraction (Figure 3). But only 80% of the radioactivity was extracted from acidified urine by diethyl ether, and smaller amounts were extracted by hexane.

An ethyl acetate extract from acidified urine was reduced to near dryness with a flash evaporator. The residue was dissolved in methanol and chromatographed in a 2- × 20-cm. column of Sephadex LH-20. The column was eluted with methanol by gravity flow and the eluate collected in 10-ml. fractions. The radioactive materials eluted in the 60- to 90-ml. fraction. This fraction was reduced to near dryness. A portion of it was applied to silica gel thin-layer chromatography plates, and the chromatograms were developed in a solvent containing two parts chloroform to one part methanol. Two radioactive areas resulted with R_f values of 0.3 and 0.85. Dieldrin gave an R_f of 1.0 when developed in this system. This evidence suggested that at least two groups of dieldrin metabolites occur in sheep urine.

Essentially none of the radioactivity would extract into hexane from freshly collected urine. However, after urine from some of the sheep had been standing at room temperature for a few hours, variable amounts of radioactivity would extract into hexane. This phenomenon never occurred with urine collected from sheep 176, 239, 180, and 192. But with sheep 222, 265, and 266 extraction increased to a maximum by the end of 24 hours. This observation suggests that some of the radioactivity was contained in labile conjugates such as esters of sulfuric acid.

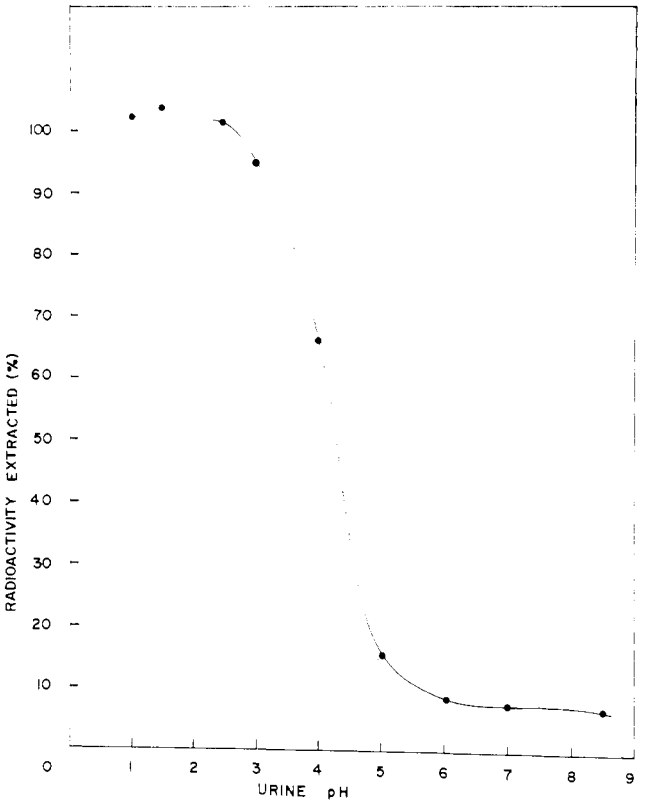

Figure 3. Effect of pH upon extraction of dieldrin-^{14}C metabolites from sheep urine with ethyl acetate

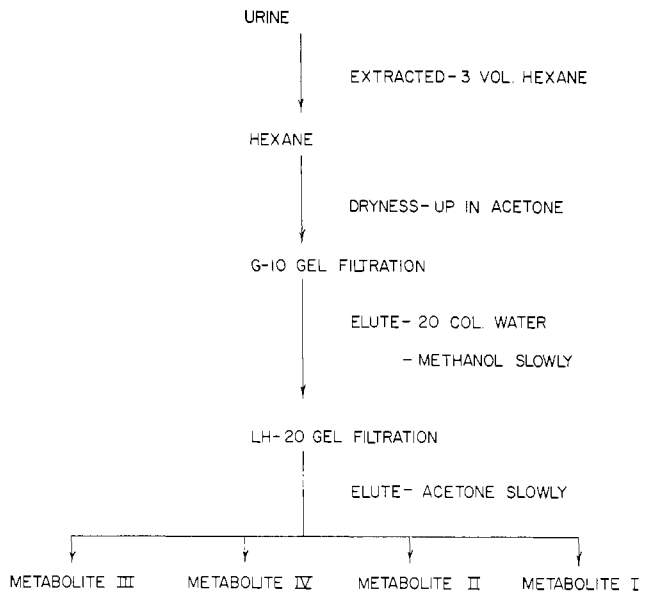

Figure 4. Flow diagram of isolation of hexane-soluble dieldrin metabolites from sheep urine

From the preceding information, an extraction sequence was established. The urine was extracted with hexane (or petroleum ether), acidified to pH 1 with 1*N* HCl, extracted with diethyl ether, and finally extracted with ethyl acetate, which removed essentially all of the remaining radioactivity. Metabolites that extracted into hexane were arbitrarily called hexane-soluble metabolites. Those that remained to be extracted after acidification were arbitrarily called water-soluble metabolites.

Differences in the extractability of the metabolites between sheep are not attributed to the method of dosing, but are

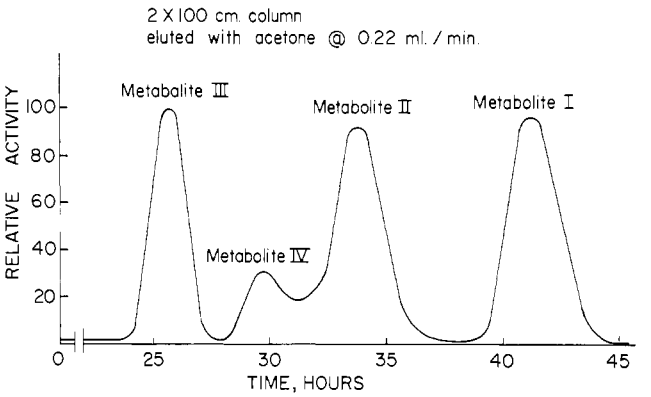

Figure 5. LH-20 chromatography of hexane-soluble dieldrin metabolites

probably related to differences within the sheep. Of the first three sheep dosed, sheep 222 excreted more hexane-soluble metabolites. The freeze-dried carcass of this sheep contained 41% fat, whereas the carcasses of sheep 176 and 239 contained 72 and 65% fat, respectively. Two very fat sheep (180 and 192) and two thin sheep (265 and 266) were selected to see if body condition of the sheep influenced the metabolism of dieldrin to hexane-soluble products. More hexane-soluble products were produced by thin sheep, indicating that there may be a relationship. However, this relationship was not proved conclusively.

Separation of Hexane-Soluble Dieldrin-^{14}C Metabolites. A flow diagram of this separation is shown in Figure 4. Urine from sheep 222, 265, and 266 was used because it contained a greater percentage of hexane-soluble metabolites. Since these metabolites partitioned readily from urine into hexane, a Sephadex G-10 gel filtration column was developed to utilize these hydrophobic properties. The G-10 column was poured in water. The urine extract was reduced to dryness by flash evaporation. The resultant residue was dissolved in a minimal amount of acetone and applied on the G-10 column. The column was eluted with about 20 column volumes of water followed by methanol at the rate of 0.1 ml. per minute. The metabolites remained on the column during water elution, while many urinary pigments and salts were removed. The metabolites were removed in a broad band by the methanol.

After the methanol was evaporated, the metabolites were dissolved in acetone (<0.5 ml.) and applied to a 2- × 100-cm. Sephadex LH-20 column which was poured in acetone. This column, when eluted with acetone at a rate of 0.1 to 0.2 ml. per minute, separated the hexane-soluble metabolites into four radioactive fractions (Figure 5). Three of these fractions were present in approximately equal concentrations, whereas the fourth comprised only about 10% of the total. Further cleanup, followed by infrared, mass, and nuclear magnetic resonance spectroscopy proved that these radioactive fractions were separate metabolites. These details and the identification will be published in companion and subsequent manuscripts.

Isolation of Water-Soluble Dieldrin Metabolites. Identical cleanup procedures were used for metabolites in the diethyl ether and ethyl acetate extracts (Figure 6). The water-soluble metabolites were partitioned from these solvents into an aqueous solution of 0.5*N* ammonium hydroxide. Ammonia was removed by flash evaporation, and this ammonia-free solution was slowly percolated through a trioctylamine–Porapak column. When all of the sample had been applied,

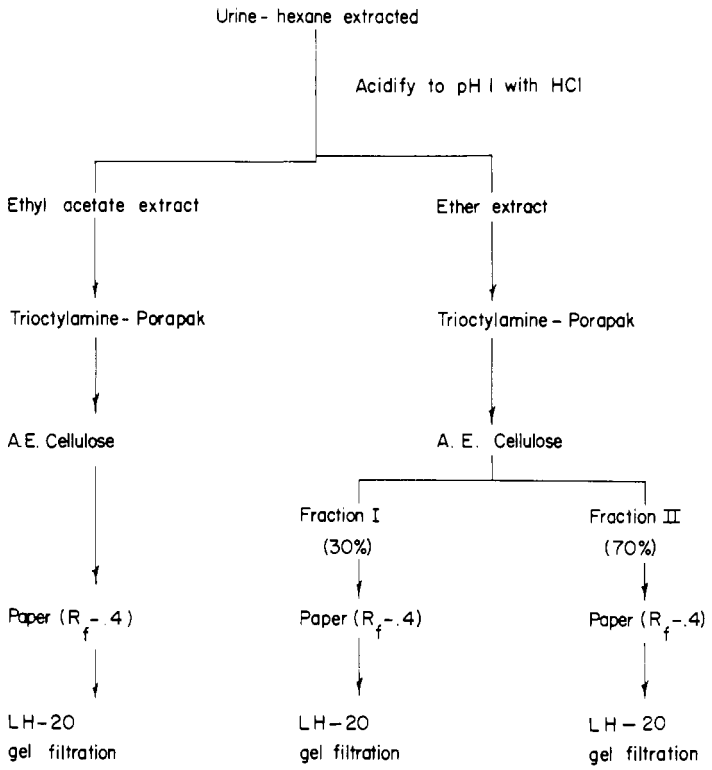

Figure 6. Flow diagram of the isolation of water-soluble dieldrin metabolites from sheep urine

the column was washed with water, followed by 1*N* ammonium hydroxide. The metabolites were eluted from the column with methanol. The trioctylamine–metabolite mixture was dried and the mixture was dissolved in chloroform. Aqueous 1*N* ammonium hydroxide was added. The metabolites partitioned into the aqueous phase and trioctylamine partitioned into the chloroform phase.

Ammonia was again removed by flash evaporation and the aqueous sample was applied to a 2.5- × 50-cm. AE-Cellulose column. The sample remained on the column while it was washed at 0.5 ml. per minute with 200 ml. of water. The metabolites were eluted with aqueous 1*N* formic acid. Metabolites from the ethyl acetate extract eluted as one fraction with about 50 ml. of the formic acid solution. Metabolites from the diethyl ether extract eluted as two fractions. Fraction I eluted with about 50 ml. of eluant, as for the ethyl acetate extract, and Fraction II eluted with about 600 ml. of eluant. This column was regenerated by washing it with 1*N* ammonium hydroxide and then with water.

At this point, the metabolites appeared to be weak acids, since they eluted from the column with formic acid. They were then partitioned into diethyl ether from the formic acid eluate. The ether solution was concentrated on a flash evaporator, washed with water, and finally flash evaporated to remove the ether and formic acid. The sample was basified with a small amount of 1*N* ammonium hydroxide, again taken to dryness, and the residue dissolved in a small amount of methanol.

Further purification involved descending paper chromatography. The developing system was prepared as follows: A mixture containing 40% butanol, 10% concentrated ammonium hydroxide, and 50% water was allowed to separate into two phases; the butanolic phase placed in a tray at the top of the tank was the migrating solvent, and the aqueous phase placed in a tray on the bottom of the tank was the stationary solvent. The radioactive areas on the paper chromatograms were dissected from the sheet and eluted with methanol. This eluate was dried and the residue dissolved in 1*N* hydrochloric acid. The metabolites were extracted from the acid solution with ether. The ether was evaporated, and the metabolites, presumably now in their free acid form, were dissolved in nanograde methanol.

Final purification was by gel filtration on a 1- × 50-cm. Sephadex LH-20 column which was poured in nanograde methanol. The column was eluted with nanograde methanol at 0.1 ml. per min. A portion of the eluates was incorporated into micro-KBr pellets for infrared spectroscopy. The infrared spectra of the metabolites present in the ethyl acetate extract and in Fraction I of the diethyl ether extract were identical, whereas that of Fraction II was different. Therefore, it appears that two water-soluble metabolites of dieldrin are excreted in sheep urine. We are seeking positive identification of these metabolites. Preliminary evidence suggests that one of these is a glucuronic acid conjugate of the transdiol and that the other is a conjugate containing glucuronic acid and possibly glycine.

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