Metabolites Isolated from Urine of Rats Fed ^{14}C-Photodieldrin

Alfred K. Klein, Robert E. Dailey, Mae S. Walton, Vivian Beck, and James D. Link

Analysis of urine from rats receiving a subacute level of ^{14}C-photodieldrin (1,9,10,10,11-*exo*-12-hexachloro - 4,5 - *exo* - epoxy - 8,3 - 7,6 - *endo* - 8,9 - 7,11-*exo*-pentacyclo[7.3.0.0^{2,6}.0^{3,8}.0^{7,11}]dodecane over a period of 12 weeks yielded the following facts. The principal metabolite of the male rat urine proved to be the ketodieldrin known to the literature of pesticide chemistry by the trivial name of "Klein's Metabolite." The manner of photodieldrin metabolism in the female rat is apparently different. The female rat urine showed only 40% of the ^{14}C radioactivity found in the male urine. No ketodieldrin was detected; instead, at least four different metabolites of undetermined identity were observed. Compared to the ketodieldrin metabolite, they are very polar and nonvolatile. No photodieldrin, the compound administered, could be detected in the urine of either sex.

The results of photoirradiation studies of dieldrin (1,2,3,4,10,10-hexachloro-*exo*-6,7-epoxy-1,4,4a,5,6,7,8,-8a-octahydro-1,4-*endo*,*exo*-5,8-dimethanonaphthalene) are of major interest to pesticide chemists. Harrison *et al.* (1967), Robinson *et al.* (1966), Roburn (1963), and Rosen *et al.* (1966) have agreed that an isolated product derived from sunlight irradiation is identical with a material formed by ultraviolet irradiation of dieldrin in the laboratory. These workers have also shown that the irradiated product, called photodieldrin, has the same molecular formula and chemical constitution as dieldrin. The two substances differ, however, in chemical structure (Harrison *et al.*, 1967; Rosen *et al.*, 1966).

Comparative toxicity data have also been reported. Harrison *et al.* (1967) state that mammalian toxicity of dieldrin and photodieldrin are the same. However, the mammalian toxicity data reported by the Food and Agriculture Organization of the United Nations (1968) list the photoisomerization product of dieldrin as 5 to 10 times more toxic to rats, mice, and guinea pigs than dieldrin itself. Rosen *et al.* (1966) reported that photodieldrin is about twice as toxic to houseflies and mosquitoes as dieldrin, and that the onset of toxic symptoms given by photodieldrin is much faster.

These foregoing findings prompted the writers to study the fate of photodieldrin fed to mammals. Rats were chosen as representative subjects. Both male and female rats were used to learn whether sex was a factor in the manner of metabolism. The subject of urinary excretion is part of a general study.

Division of Pesticide Chemistry and Toxicology, and Division of Toxicology, Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D.C. 20204

MATERIALS AND METHODS

^{14}C-photodieldrin was prepared by ultraviolet irradiation of ^{14}C-dieldrin purchased from Nuclear Chicago Corp. The final purified product (specific activity 4.3 mCi per mmole) was essentially free of interfering compounds as tested by electron capture gas chromatography and thin-layer chromatography. One microgram dissolved in 15.0 ml of scintillant solution gave a count of 25,300 disintegrations per minute (dpm) in a Packard Tri-Carb Model 3375 liquid scintillation counter.

The Packard Instrument Co., Downers Grove, Ill., distributes two of the ingredients of the scintillant mixture used for measuring the radioactive count. They are 2,5-diphenyloxazole (PPO) and 1,4-bis[2-(4-methyl-5-phenyloxolyl)] benzene (DMPOPOP). The scintillant mixture was made by dissolving 4.0 g of PPO and 0.100 g of DMPOPOP in a solution containing 700 ml of toluene and 300 ml of methanol.

Photodieldrin was prepared by ultraviolet irradiation of recrystallized dieldrin (mp 175–176° C). The final crystallized product melted at 191.5–193.5° C. The structural formula (Figure 1) was deduced from infrared, mass spectrometric, and nmr measurements.

Spectroline Model C-3, Ultraviolet cabinet, Black Lamp Eastern Corp., 4 Manhasset Ave., Port Washington, Long Island, N.Y., was used for ultraviolet irradiation.

Ketodieldrin was isolated and purified as described by Klein *et al.* (1968). The structural formula of the compound is shown in Figure 1.

Dieldrin was the Shell Co. product labeled as recrystallized HEOD 99.1 ± 0.3%.

A Packard gas chromatograph, Model #7520, was used for glc. The operating conditions were the same as described by Klein *et al.* (1968).

Twelve Osborne-Mendel rats of both sexes, each weighing

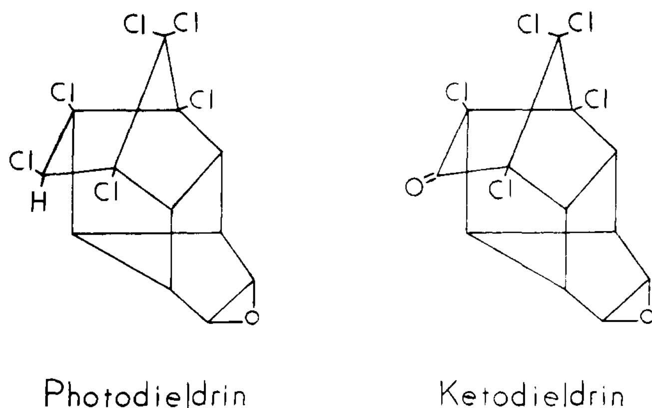

Figure 1. Structures of photodieldrin and ketodieldrin

approximately 100 g at the beginning of the study, were housed individually in metabolism cages and fed Purina rat pellet chow and water *ad libitum*. The 24 animals were divided into four groups. Six rats of each sex were given ^{14}C-photodieldrin intraperitoneally, and the other two groups of each sex received the compound by stomach tube. Each rat received a daily dose of 5 μg of toxicant, dissolved in 0.25 ml of corn oil, 5 days a week for a period of 12 weeks (a total of 300 μg of toxicant). The 5-μg level was correctly judged as subacute; no abnormalities were noted in animal behavior during the study.

Quantitative collections of urine were made every day and pooled each week for each of the four groups. Each weekly urine pool was diluted to 1 l. with distilled water, and samples were stored at −20° C until processed for analysis. Aliquots of each weekly pool were counted. The average count for the male urine pool of six rats was 1,100,000 dpm per week, corresponding to 43.5 μg of metabolite calculated as ^{14}C-photodieldrin. The average count for the female urine pool of six rats was 440,000 dpm per week, corresponding to 17.4 μg of metabolite or about 40% of the male urine count. At the end of the 12-week dosing schedule, the male rats had excreted into the urine a total of 33.0% of the cumulative ^{14}C-activity given intraperitoneally and 26.8% of that given orally.

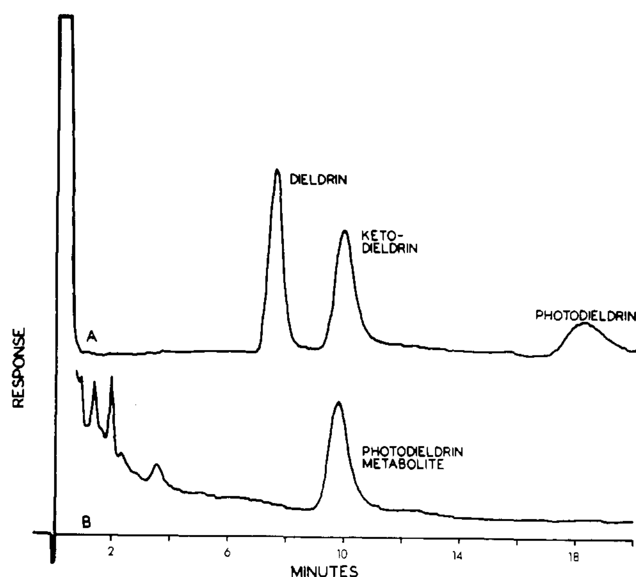

Figure 2. (A) Standard dieldrin, ketodieldrin, and photodieldrin; 0.2 μg/ml each; 5 μl injected. (B) Photodieldrin male metabolite. 1 ml of solution corresponds to 1.0 ml of urine; 30 μl injected

By contrast, only 9.4% and 10.7% of the total activity given to the female rats by the respective routes of administration were found in the urine.

To isolate the metabolites, the urine was extracted in 100-ml portions with ethyl ether continuously for 48 hr in a liquid-liquid extractor. For the male urine an average of 85.7% (73.3 to 94.2%) of the ^{14}C-activity was extracted. Only 48.9% (39.1 to 58.3%) was recovered from the female urine. Additional time of extraction did not enhance this yield commensurately. When the extraction time was increased from 48 to 180 hr for a sample of female urine, the count was increased by only 17%, illustrating that the metabolism pattern of photodieldrin in the female rat is different from that in the male. Lesser amounts are excreted in the urine and the metabolites are more water-soluble than the metabolite excreted by the male rat.

IDENTITY OF THE METABOLITES

Male Metabolites. The gas chromatographic (glc) tracing illustrated in Figure 2 gives evidence of the presence of ketodieldrin. The retention times of the urinary photodieldrin metabolite and standard ketodieldrin are the same. No dieldrin is observed. More important is the fact that no photodieldrin is observed, indicating that the compound is either stored in the animal or metabolized. The sample solution used to obtain the data in Figure 2 (B) was prepared by blending 100 g of the urine successively with ethyl alcohol, ethyl ether, and petroleum ether in the manner described by Klein *et al.* (1968). The organic solvent layer was separated, washed with water, and dried over Na_2SO_4. No further purification was needed for this test. Any dieldrin, photodieldrin, or other common chlorinated pesticide present would have been detected and determined quantitatively.

The urine used for the test was part of a large batch that contained 47.5 μg/liter of metabolite, based on the ^{14}C activity. One liter was used to isolate and purify sufficient metabolite for further confirmatory identification. It was blended as described before. The organic solvent layer was washed with water, dried over Na_2SO_4, and concentrated to about 25 ml. The metabolite was separated and purified by acetonitrile extraction, elution through activated Florisil, and collection on glass beads or on KBr after glc separation as outlined by Klein *et al.* (1968). The yield of purified metabolite was 34 μg. Mass spectral examination gave the following data: molecular weight, 358 (Cl = 35); 5 chlorine atoms; a base peak, *m/e* 81, indicating an epoxide group. These constants are in accord with those determined by Damico *et al.* (1968) in establishing the structure of a ketodieldrin metabolite found in the urine of male rats fed aldrin and dieldrin. Infrared analysis lent additional evidence; the spectrum of the photodieldrin metabolite is identical with that of standard ketodieldrin (Figure 3). Analysis of the isolated and purified metabolite by glc agreed very well with that calculated from the ^{14}C activity. Thus, duplicate amounts of 1.065 μg as determined by glc gave counts that corresponded to 1.099 and 1.102 μg as photodieldrin, averaging 1.101 μg (1.043 μg of ketodieldrin, based on molecular weights of 380.5 for photodieldrin and 360.5 for ketodieldrin) or within 3% of the glc values. This close agreement is not obtained with sample extracts that have not been purified. The dpm value for unpurified extracts is always significantly higher. As an example, an ether extract of a male urine sample gave a ^{14}C value equivalent to 2.67 μg metabolite; gas chromatography gave a value of 2.27 μg. This variation of 16% is beyond the error of analysis. Radioactive metabolites other than keto-

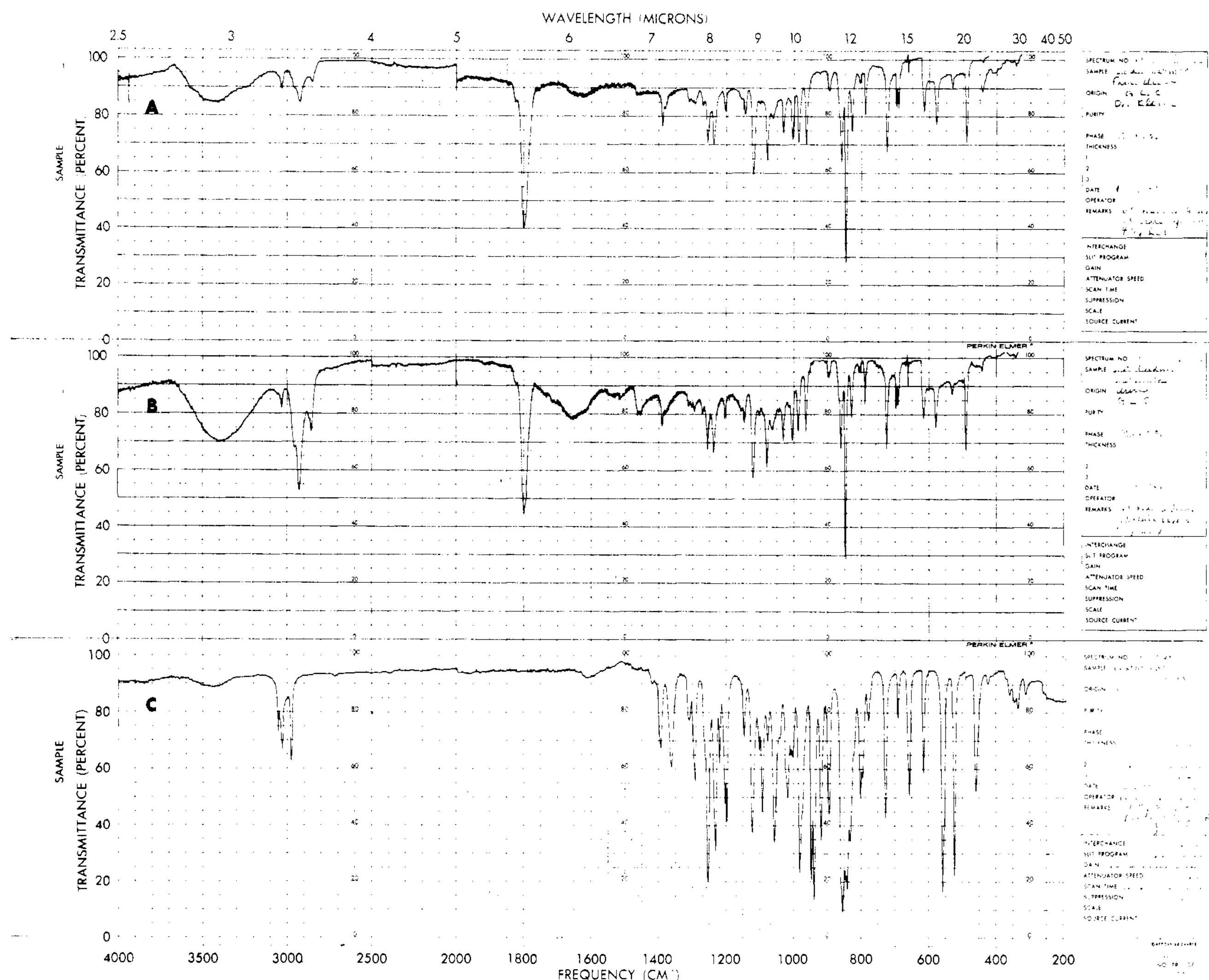

Figure 3. (A) Ketodieldrin standard; about 15 μg. (B) Photodieldrin male metabolite; about 20 μg. In (A) and (B) 4 mg of KBr powder 6X beam condenser, and 3X scale expansion were used. (C) Photodieldrin standard; 1 mg in 100 mg of KBr powder

dieldrin are believed to be present in the male urine and are apparently removed during sample purification in the isolation of the ketodieldrin.

In summary, about 30% of the photodieldrin fed to male rats at subacute levels is excreted into the urine as metabolites. About 85% of the metabolites are extractable and about 85% of the extract is ketodieldrin. Figure 1 suggests that ketodieldrin may be regarded as a ketone derivative of photodieldrin. The male rat is able to effect the oxidation.

Female Rat Urine. The resolution of the identity of the metabolites present in the experimental female rat urine is more complicated. The blending and extraction procedures which were used in isolating the metabolite in the male urine consistently yielded very low ^{14}C activity. Furthermore, the metabolites are so polar that they could not be isolated or purified by solvent elution through activated Florisil, although elution solvents of comparatively high polarity were tried. Moreover, the metabolites are not volatile enough to be purified or isolated by glc. When injected into the instrument they remain on the column, and at least a week of gas flow is required to remove them. (This fact was proved by monitoring the column effluent for ^{14}C.)

The metabolites were partially separated and purified by elution through silica gel. Benzene and various concentrations of ethyl acetate in benzene were used as elution solvents. The column consisted of 20 g of silica gel (Stahl) packed in a borosilicate glass tube, 300 × 20 mm (o.d.), prewetted with benzene. A benzene solution (approximately 3 ml) of 0.55 μg of metabolite was put on the column. The value 0.55 μg was calculated by referring the dpm of 13,900 to that of 1 μg of ^{14}C standard photodieldrin, 25,300 dpm. Eluate fractions of 50 ml were uniformly collected. The counts (dpm) and other pertinent data are listed in Table I.

Radioactive peak counts indicate the presence of at least four metabolites. The benzene eluates 1–4 (Table I) show a peak dpm value in eluate 2 which is indicative of a metabolite. Eluates 1–4 were combined, taken to dryness, and spotted on a thin-layer glass plate. A solution of 25% ethyl acetate in benzene was used as mobile solvent to develop the chromatogram. The plate was then examined by the radiochromatogram scanner (Packard Model 7201). A radioactive compound was observed which peaks 70 mm from the origin. The calculated R_f value is 0.47 (70/155) by this system of elution. This value was checked in additional runs. The combined dpm of elutions of 1–4 is 3450, or 24.8% of 13,900, the dpm of the material added to the column. The average value of subsequent runs was 24.2%. Metabolite A is the least polar of the four metabolites (see Figure 4).

A second metabolite (B) is indicated in the 5% ethyl acetate eluate. A peak dpm value is shown in fraction 7 (Table I).

Table I. Elution of the Female Urine ^{14}C-Photodieldrin Metabolites (Silica Gel) in 50 ml Eluate			
Eluate No.	Solvent	Metabolite	Count, dpm[a]
1	benzene		0
2			2400
3		A	880
4			170
5	5% ethyl acetate[b]		50
6			375
7		B	1435
8			205
9	10% ethyl acetate[b]		75
10		B	105
11			110
12			105
13	25% ethyl acetate[b]		95
14		C	220
15			60
16			65
17			25
18			20
19		D	235
20			635
21			1045
	Total Volume 1050 ml	Total Count	8310

[a] Dpm are calculated values. The counts were made on 10.0 ml eluates. [b] The acetate solutions were prepared in benzene.

A composite of fractions 5–8 (2065 dpm) was treated and examined as before. The calculated R_f value of the metabolite is 0.34 (54/155, Figure 4). It accounts for about 15% (2065/13,900) of the ^{14}C activity.

No peak dpm value is noted in the 10% ethyl acetate elutions. The combined count, 395, accounts for only 2.8% of the added material. Five additional runs yielded a total of about 2000 dpm. Only the metabolite that peaks at 54 mm on the tlc plate was observed.

Two metabolites are noted in the 25% ethyl acetate eluates. One (C) was indicated in eluates 13–15, Table I. The combined dpm of the three fractions, 375, represents only 2.8% or 375/13,900 of the total metabolites. Five runs were needed to yield enough metabolite for isolation and examination. The average dpm per run was 528, which accounts for 3.6% of the total metabolite added. The calculated R_f of metabolite C is 0.21 (32/155), Figure 4.

The other metabolite (D) is indicated in fractions 19–21, Table I. The R_f of this metabolite is 0.06 (9/155). It will be noted in Table I that although 21 fractions or a total of 1050 ml of eluates were used, only 8310/13,900 or 60% of the material was eluted. Increasing the number of fractions by 10 increased the radioactive count of the eluate by only 5%. The residual ^{14}C material in the column could be eluted with 150 ml of a solvent prepared by adding 50 ml of methyl alcohol to 950 ml of the 25% ethyl acetate reagent. This material was the same as the metabolite eluted in fractions 19 through 31. It is the most polar compound of the metabolites isolated and accounts for about 54% of the total.

In summary, four metabolites were isolated from the urine of female rats fed ^{14}C photodieldrin at the subacute level of 5 μg per rat per day. These metabolites vary in polarity and the amounts present. The procedures employed in this work do not yield pure products; urinary pigments are not completely removed. It has been mentioned that a pool of six female rats at the dosage now fed excretes an average of 17.4 μg of metabolite per week into the urine. Of this amount, 50% or only about 9 μg/l. is extractable by ethyl ether, and this amount is divided among four metabolites. To obtain the amount of metabolites needed to separate, purify, and establish their chemical structures would require large volumes of urine if the present feeding level is maintained. The authors are currently experimenting with a higher dosage level which should yield larger amounts of metabolites and yet prove to be subacute. We have as yet done no work with the fraction not extractable by ethyl ether nor the water-soluble fraction of the female urine which represents 50% of urine ^{14}C activity. That portion of the problem will be studied separately.

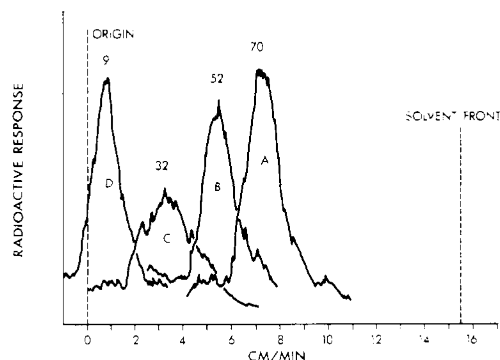

Figure 4. Radioactive peak counts (R_f values) of the four metabolites of ^{14}C-labeled photodieldrin obtained from urine of female rats

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