Metabolism of *trans*-Chlordane-^{14}C and Isolation and Identification of Its Metabolites from the Urine of Rabbits

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Every day male rabbits were fed 14.3 mg *trans*-chlordane-^{14}C for 10 weeks. Toward the end of the feeding period 70% of the daily administered amount was excreted in urine as metabolites. Within a total period of 12 weeks, 70% of the total administered amount was excreted. Only in subcutaneous fat were hydrophilic metabolites not present. It is shown that *trans*-chlordane has a high rate of metabolism and excretion in mammals. Its storage in fatty tissue is relatively very small. One of the metabolites of *trans*-chlordane was isolated in a crystalline form from the urine and analyzed. The two components of chlorohydrin of chlordene were separated, crystallized, and analyzed. The analyses of one of these two were the same as those of the isolated metabolite. The exact position of the hydroxyl group was also determined. Thus the structural formula of the isolated metabolite was ascertained, and a formula of the other metabolite of *trans*-chlordane was proposed.

It is known that chlordane is metabolized by a number of organisms, *e.g.*, German roaches (U.S. Pub. Health Service, 1953) and rats (Poonawalla and Korte, 1964). In previous studies with rats, hydrophilic metabolites of *trans*-chlordane were chromatographically detected. Recent studies using ^{14}C-labeling techniques have shown that "drin" insecticides are metabolized to more hydrophilic compounds (Cueto and Hayes, 1962; Korte *et al.*, 1963; Mörsdorf *et al.*, 1963; Ludwig *et al.*, 1964; Datta *et al.*, 1965). In the following study we investigated the metabolism of *trans*-chlordane-^{14}C in rabbits after multiple doses. ^{14}C-labeled *trans*-chlordane was synthesized by Rechmeier (1962).

During the experiment, carrots, cabbages, and green leaves were fed to rabbits. The animals had no previous history of insecticidal use. The animals received orally daily doses of 14.3 mg of *trans*-chlordane-^{14}C for 10 weeks. Urine and feces were collected separately every day for 12 weeks. Then the animals were sacrificed; their viscera, carcass, and other tissues were separated and handled individually. All tissues were homogenized and extracted with methanol. Feces and urine were extracted with ether. After 5 days of continuous extraction of urine, only 65% of the total radioactivity present in the urine was extracted. After acidification with sulfuric acid, extraction was complete within 24 hr.

The amount of radioactivity present in each extract was determined in a proportional flow counter. Thin-layer chromatography with silica gel G (Stahl) was carried out for qualitative analysis.

By thin-layer chromatography a metabolite was isolated from the concentrated extract of urine. After repeated chromatography and extraction of silica gel with ether, the colorless extract was evaporated to dryness. Yellowish-white crystals were obtained. After repeated recrystallization from cyclohexane, colorless crystals were obtained and analyzed, and the structure elucidated. Other compounds structurally related to *trans*-chlordane were also analyzed by thin-layer chromatography. In a mixture of two synthetic compounds, one was found to have the same R_f value as that of the isolated metabolite. This was verified by cochromatography in various solvents. This compound was isolated in a similar way and compared with the metabolite by several analytical methods.

RESULTS AND DISCUSSION

Visible signs of sickness or disability were not observed during the entire feeding period.

Excretion. After the end of the fifth week the excretion of radioactive substances increased rapidly. Toward the end of the experiment, the daily excreted radioactivity was roughly 70% of the daily administered amount. As soon as the administration of *trans*-chlordane-^{14}C was discontinued, the excretion of radioactivity in urine decreased rapidly. The results are shown in Figure 1. During the feeding period (10 weeks) and the following 2 weeks, 70% of the total administered amount was excreted; 22.7% of the administered amount was excreted in feces, 30% of which was detected as unchanged *trans*-chlordane. The entire radioactivity

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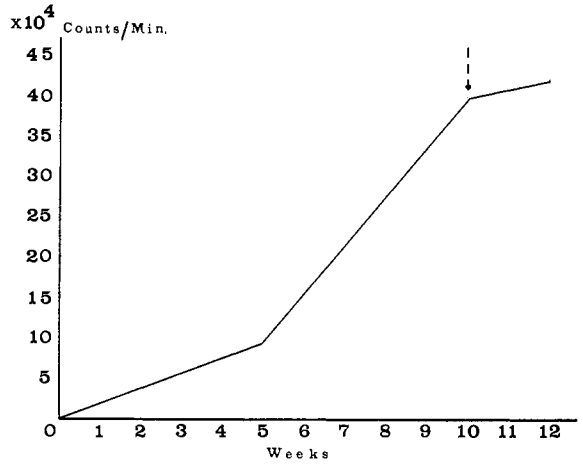


Figure 1. Excretion of *trans*-chlordane-^{14}C as metabolite in the urine of rabbits. Time is indicated when the administration of *trans*-chlordane was discontinued

excreted in urine (47% in 12 weeks) consisted of metabolites only. This high rate of excretion, as compared to the high doses (1 g in 10 weeks), indicated that the compound does not show a tendency to accumulate in mammals.

Distribution. Figure 2 shows the amount of radioactivity present in different tissues and excreta. The relative distribution of radioactivity in the form of unchanged *trans*-chlordane and hydrophilic metabolites is also shown. As can be seen, only in subcutaneous fat metabolites could not be detected. All other tissues contained hydrophilic metabolites. Two weeks after the application of the last dose of *trans*-chlordane, its concentration in subcutaneous fat was found to be 231.8 ppm. Among other organs, liver, which is most probably the site of detoxification for this group of insecticides, was found to contain the highest percentage of radioactivity. Thirty percent of this was in the form of metabolites. The high percentage of the metabolite in excreta (100% in urine and 70% in feces) shows that this compound can be easily metabolized to more hydrophilic products and eliminated from the body. Table I shows the analysis of the presence of radioactivity in different tissues (concentration in ppm) and the relative presence of the metabolic products to unchanged *trans*-chlordane.

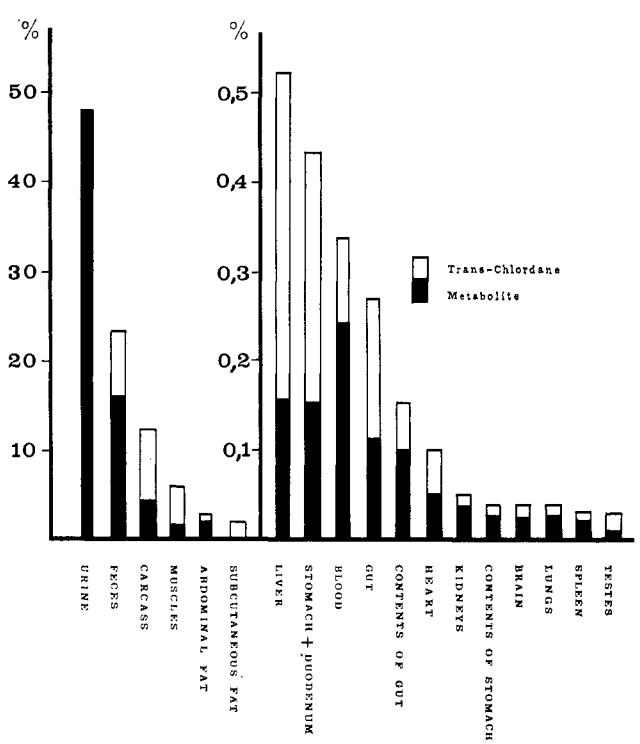


Figure 2. Relative distribution of *trans*-chlordane and its metabolites in different tissues and excreta after multiple oral doses to rabbits

Table I. Distribution and Concentration in ppm of Radioactivity in Tissues and Excreta of Rabbits after Multiple Oral Doses

	% Radioactivity Detected	ppm	Results of Analyses: % *trans*-Chlordane[a]	Results of Analyses: % Metabolites[a]
Stomach plus duodenum	0.43	13.6	65	35
Contents of stomach	0.04	3.5	40	60
Gut	0.27	20.4	60	40
Contents of gut	0.14	5.6	30	70
Abdominal fat	2.59	240.9	30	70
Subcutaneous fat	1.53	231.8	100	0
Kidneys	0.05	35.2	20	80
Liver	0.52	44.3	70	30
Heart	0.09	90.9	40	60
Lungs	0.04	29.5	25	75
Spleen	0.03	75.0	20	80
Testes	0.03	26.1	70	30
Brain	0.04	25.0	30	70
Muscles	5.68	38.6	75	25
Carcass	11.25	86.3	65	35
Blood	0.34	40.9	30	70
Feces	22.73	...	30	70
Urine	47.20	...	0	100
Total recovery	93.00			

[a] Percentage based on radioactivity measured by tlc scanning.

Table II. The R_f Values of the Metabolites A and B and Their Products C and D after Treatment with Sulfuric Acid

		1[a]	2	3	4	5
Metabolites present in urine	A	0.35	0.76	0.85	0.72	0.82
	B	0.00	0.00	0.18	0.00	...
Compounds obtained after acidification	C	0.45	0.83	0.87	0.77	...
	D	0.00	0.33	0.57	...	...

[a] 1 = benzene; 2 = benzene:acetic acid ester (8:2); 3 = benzene:acetic acid ester (5:5); 4 = benzene:acetonitrile (9:1); and 5 = methanol:water (97:3), paper chromatography, Whatman No. 1 impregnated with vaseline.

Purification and Isolation of the Metabolites. By thin-layer chromatography the two metabolites were separated from the concentrated ether extract of urine. The two radioactive zones were collected separately and extracted with ether. This extract was concentrated and rechromatographed until the ether extract was colorless. It was then evaporated to dryness. Yellowish-white crystals were obtained which were recrystallized from freshly distilled cyclohexane.

The two metabolites A and B of the natural urine differed from metabolites C and D of the acidified urine. The R_f values of these compounds are shown in Table II. It was found that metabolites A and B were converted to substances C and D, respectively, when they were treated with sulfuric acid. These, upon treatment with potassium hydroxide, were changed to compounds X and Y, respectively. Compounds X and Y show the same R_f values as metabolites A and B, respectively. As can be seen from Table II, metabolite B is more hydrophilic than A.

$$\text{Metabolite A} \xrightarrow{H_2SO_4} \text{Metabolite C} \xrightarrow{KOH} \text{Metabolite A (or comp. X)}$$

$$\text{Metabolite B} \xrightarrow{H_2SO_4} \text{Metabolite D} \xrightarrow{KOH} \text{Metabolite B (or comp. Y)}$$

Identification of the Metabolite A. The crystals of metabolite A were analyzed and the results of two chemical analyses were:

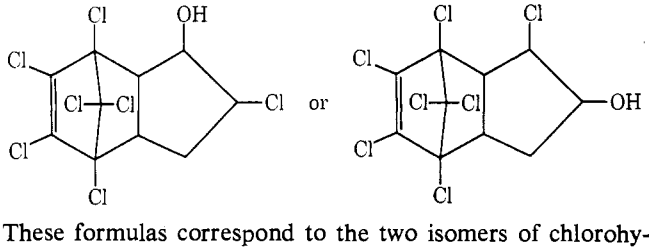
	I	II
C	30.7%	30.7%
H	1.8%	1.5%
Cl	63.6%	...

Assuming that in the metabolite the number of carbon atoms remains unchanged (C_{10}), the molecular weight which corresponds to 30.7% carbon and 10 carbon atoms in the molecule is 391.25.

The infrared spectrum of metabolite A indicated the presence of a hydroxyl group, and its mass spectrum indicated the presence of seven chlorine atoms. The latter also confirmed the molecular weight to be 391. Chlordane does not contain any hydroxyl group and its mass spectrum indicates the presence of eight chlorine atoms. The molecular weight of chlordane, as is also confirmed by its mass spectrum, is 410. Hence, it is clear that during metabolism one chlorine atom was replaced by a hydroxyl group. Therefore the molecular formula of metabolite A is $C_{10}H_7Cl_7O$.

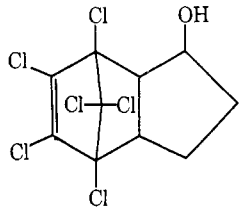
Isolation, Identification, and Cochromatography of the Synthetic Metabolite A. A number of substances with molecular formulas similar to that of *trans*-chlordane were analyzed by thin-layer chromatography with blancophore (Tschesche *et al.*, 1963). It was found that one of the two chlorohydrins which were obtained from perbenzoic acid epoxide of chlordene had the same R_f value as that of metabolite A. These two components of chlorohydrin were separated by thin-layer chromatography and recrystallized from cyclohexane. Upon cochromatography with metabolite A -^{14}C, it showed the same R_f value. Its mass spectrum and infrared spectrum were also found to be identical with those of metabolite A. The R_f values of these two components of chlorohydrin are shown in Table III.

Elucidation of the Structure of Metabolite A. Considering the analyses of metabolite A and chlorohydrin, the structural formula with $C_{10}H_7Cl_7O$ should either be

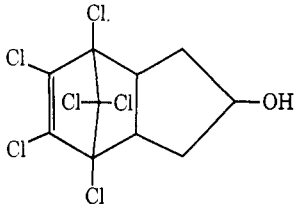


These formulas correspond to the two isomers of chlorohydrin of chlordene. Arbitrarily we have named the compound on the left side as Chlorohydrin A, and the one on the right as Chlorohydrin B.

In order to find the exact position of the hydroxyl group in the metabolite, the following four isomers, which are authentic samples, were subjected to thin-layer chromatography.

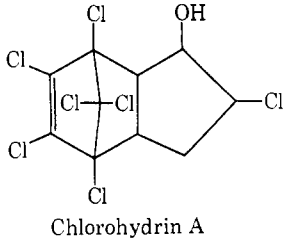


Ia (exo-OH)
Ib (endo-OH)



IIa (exo-OH)
IIb (endo-OH)

Isomers IIa and IIb were found to be more hydrophilic than Ia and Ib. As Chlorohydrin A and B differ from the above compounds by one chlorine atom only, it is not possible that thereby the chromatographic behavior is reversed. Hence it can be concluded that the less hydrophilic chlorohydrin contains the hydroxyl group in position 1 and chlorine in position 2. This component of chlorohydrin (Chlorohydrin A) was identical to metabolite A. This establishes the structure of chlorohydrin A to be the same as that of metabolite A.



Chlorohydrin A
and
Metabolite A

Since metabolite B was found to be more hydrophilic than metabolite A, it is possible that here both the chlorine atoms in the cyclopentane ring have been replaced by hydroxyl groups.

EXPERIMENTAL

Preparation of a Standard Solution of *trans*-Chlordane-^{14}C. Into a 50-ml measuring flask, 2 g of *trans*-chlordane were accurately weighed, a solution of *trans*-chlordane-^{14}C in ethyl alcohol (756 μg; sp. act. 8.6 mCi per mmol) was added and the flask filled with olive oil. Hence 0.35 ml of this solution contained 14.3 mg of *trans*-chlordane-^{14}C with a specific activity of 3.26 μCi per mmol.

Administration of the Standard Solution to Rabbits. To two healthy male rabbits, 0.35 ml per day of the above solution was administered. During the period of administration (10 weeks) and the following 2 weeks, urine and feces were collected every day.

Extraction of Radioactive Substances from Urine. In a universal extractor urine was extracted with ether. The extraction of the radioactivity was not quantitative, even after 5 days of extraction. Fresh and partly extracted urine, when acidified with sulfuric acid and extracted with ether, was found to be free from radioactivity within 24 hr. The concentrated extract was analyzed.

Extraction of Radioactive Substances from Feces. The feces were dried in batches, pulverized, and extracted in a

Table III. The R_f Values of the Two Components of Chlorohydrin of Chlordene and Metabolite A

	X[a]	Y
Chlorohydrin A	0.35	0.76
Chlorohydrin B	0.20	0.50
Metabolite A	0.35	0.76

[a] X = benzene; Y = benzene:acetic acid ester (8:2).

Soxhlet with ether. The extract was concentrated and its radioactive contents were measured.

Extraction of Radioactive Substances from Other Tissues. All tissues were individually homogenized and extracted with methanol. The concentrated extract was subjected to analysis.

Measurement of Radioactivity in Urine and Other Extracts. All measurements were made in a proportional flow counter.

Chromatography. Silica gel G according to Stahl (Merck) was used for thin-layer chromatography of all samples. All extracts were chromatographed and a metabolite was isolated from the concentrated extract of urine. At times blancophore was added with a view to trace the inactive component in ultraviolet light.

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