cleavage from the product studies or $^{14}CO_2$ studies. Ishizuka et al. (1975) detected the phenol (II), methoxy (IV), and an acid derivative of the phenol (II) with the carbonyl group on the *tert*-butyl group of oxadiazon. Oxadiazon ring cleavage has been confirmed in rice plants, since Hirata and Ishizuka (1975) isolated and identified the 1-(2,4-dichloro-5-isopropoxyphenyl)-1-methoxycarbonyl-2-trimethylenehydrazine.

Oxadiazon was slowly degraded in both moist and flooded soil, with a major amount of the nonrecoverable ^{14}C accounted for in the bound residues. Degradation and binding was more extensive in the high organic matter soil (Matapeake loam, 1.5% organic matter), with most of the bound ^{14}C appearing in the fulvic acid fraction. Oxadiazon was metabolized to a carboxylic acid, a phenol, a dealkylated derivative, and several polar products in concentrations too small for identification purposes.

LITERATURE CITED

Ambrosi, D., Desmoras, J., Symposium Herbicides–Soils, Proceedings of the European Weed Research Council, 163 (1973).

Burgaud, L., Deloraine, J., Desmoras, J., Guillot, M., Petrinko, P., Riottot, M., 3rd Symposium on New Herbicides, Proceedings of the European Weed Research Council, 1, 219 (1969).

Carringer, R. D., Webber, J. B., Monaco, T. J., *J. Agric. Food Chem.* **23**, 568 (1975).

Hirata, H., Ishizuka, K., *Agric. Biol. Chem.* **39**, 1447 (1975).

Ishizuka, K., Hirata, H., Fukunaga, K., *Agric. Biol. Chem.* **39**, 1431 (1975).

Ishizuka, K., Hosaka, H., Takase, I., Tan, K. E., Hirata, H., "Comparative Studies of Food and Environmental Contamination," FAO, WHO, IAEA, IAEA Vinna, 1974, p 363.

Kearney, P. C., Kontson, A., *J. Agric. Food Chem.* **24**, 424 (1976).

Stevenson, F. J., *J. Environ. Qual.* **1**, 333 (1972).

U.S. Environmental Protection Agency, "Guidelines for Registering Pesticides in the United States", Part II; *Fed. Regist.* **40**, 123, 26802 (1975).

Received for review October 26, 1976. Accepted January 31, 1977. Mention of a trademark or a proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

Metabolic Routes of *cis*- and *trans*-Chlordane in Rats

Shigeki Tashiro and Fumio Matsumura*

The major route of metabolism for both *cis*- and *trans*-chlordane is via dichlorochlordene and oxychlordane. These metabolic intermediates are further converted to two key metabolites, 1-*exo*-hydroxy-2-chlorochlordene and 1-*exo*-hydroxy-2-*endo*-chloro-2,3-*exo*-epoxychlordene, which are not readily degraded further. *trans*-Chlordane is more readily metabolized through this route. There is yet another major metabolic route for *cis*-chlordane that involves more direct hydroxylation reactions to form 1-*exo*-hydroxydihydrochlordenes and 1,2-*trans*-dihydroxydihydrochlordene. *cis*-Chlordane is more readily degraded through this route. As judged by a toxicity test on mosquito larvae, none of these newly identified metabolites appear to be more toxic than the original chlordanes.

In terms of the quantities involved, chlordane is currently the second most important chlorinated hydrocarbon insecticide behind toxaphene in the U.S. Its estimated annual production is 20 million pounds. Furthermore, it holds a very unique position among all insecticides in that its domestic agricultural use amounts to only 20% of the total, while industrial (43.3%) and home garden uses (33.3%) constitute the bulk of its consumption (Von Rumker et al., 1975). This could be one of the reasons why the Environmental Protection Agency is in the process of holding a hearing on its potential hazards.

Despite such a background, its metabolic fate has received little attention in the past. One of the reasons could be that chlordane itself is a mixture of several components. However, the recent development is such that now a highly purified chlordane mixture consisting of a 3:1 mixture of *cis*- and *trans*-chlordane and small amounts of other components such as heptachlor and nonachlor is available.

In terms of metabolic fates of chlordane in animals, so far, the center of attention has been the formation of oxychlordane, the acknowledged toxic metabolic product both from *cis*- and *trans*-chlordane (Schwemmer et al., 1970; Lawrence et al., 1970; Polen et al., 1971; Street and Blau, 1972; Dorough and Hemken, 1973). An exception is the paper by Barnett and Dorough (1974), who recognized at least eight metabolic products from a highly purified chlordane preparation. In essence, they tentatively identified mono-, di-, and trihydroxylated products of chlordane in addition to oxychlordane in the rat excreta and concluded that the metabolism of chlordane takes place via a series of oxidative enzyme reactions.

We have made an attempt to isolate and positively identify the metabolic products from both *cis*- and *trans*-chlordane to establish the route of their metabolism and now report the results.

EXPERIMENTAL SECTION

Materials. *cis*-[^{14}C]Chlordane, sp act. 10.9 mCi/mmol, and *trans*-[^{14}C]chlordane, sp act. 6.26 mCi/mmol, were synthesized by New England Nuclear and provided to us by Velsicol Chemical Corp., Chicago, Ill. *cis*-Chlordane (1-*exo*,2-*exo*,4,5,6,7,8,8-octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene), *trans*-chlordane (1-*exo*,2-*endo*,4,5,6,7,8,8-octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene), oxychlordane (1-*exo*,2-*endo*,4,5,6,7,8,8-octachloro-2,3-*exo*-epoxy-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene, heptachlor (1-*exo*,4,5,6,7,8,8-heptachloro-3a,4,7,7a-tetrahydro-4,7-methanoindene), 1,2-dichlorochlordene (1-*exo*,2,4,5,6,7,8,8-octachloro-3a,4,7,7a-tetrahydro-4,7-methanoindene); chlordene chlorohydrin,

Department of Entomology, University of Wisconsin, Madison, Wisconsin 53706.

1,2-dihydroxydihydrochlordene (1,2-dihydroxychlordene), chlordene epoxide, 1-hydroxychlordene, and 1-hydroxychlordene epoxide used in this research were also obtained from Velsicol Chemical Corp. Tri-Sil "Z", a reagent for trimethylsilyl (Me_3Si) derivatization of hydroxylated compound was obtained from Pierce Chemical Co. All cofactors and biochemicals were obtained from Sigma Chemical Co. Silica gel G and aluminum oxide G came from Brinkmann Instruments, Inc., and Florisil (60–100 mesh) was from Fisher Scientific Co. Adult male, albino rats, weighting 200 to 300 g per individual, were supplied by Rolfsmeyer Farm Madison, Wis. The medical x-ray film (NS-541, 8 × 10 in.) used in detecting the radioactivity on chromatograms was purchased from Eastman Kodak Co., N.Y.

Administration of [^{14}C]Chlordane Isomers. Two rats were administered a single oral dose of either *cis*-[^{14}C]-chlordane (5.4 mg/kg) or *trans*-[^{14}C]chlordane (9.7 mg/kg) which was dissolved in 250 μL of corn oil using a stomach tube technique. The animals were held individually in metabolism cages, and feces and urine were collected separately. The radioactivity was analyzed for ^{14}C over a period of 7 days, using a liquid scintillation counting method. Every day each sample of feces (17 to 21 g) was transferred into a 100-mL flask containing 50 mL of acetone. The feces in acetone were crushed into a powder using a glass bar, shaken, and extracted three times with 50 mL of acetone each. The three acetone extracts were combined, and the solvent was removed under a gentle stream of air. The residue was quantitatively taken up in 15 mL of ethanol, and a 0.3-mL aliquot was transferred to 10 mL of liquid scintillation counting solution for radioassay.

The accumulated daily sample of urine (5 to 8 mL/day) was quantitatively transferred to a graduated cylinder by using distilled water to make the final volume 15 mL. A 0.3-mL portion from each sample was transferred to a scintillation vial with 10 mL of counting solution for radioassay.

Isolation of Metabolites. Four male rats were fed on a diet of ground Purina Laboratory Chow, containing 100 ppm of either *cis*-chlordane or *trans*-chlordane. Feces and urine were separately collected daily for 3 to 4 weeks during which period the rats were observed to have consumed 2 kg of the food corresponding to the total intake of 200 mg/rat for each chlordane isomer. Feces were dried in the air at room temperature. The quantities of feces thus collected were 687 g from *cis*-chlordane and 625 g from *trans*-chlordane treated rats, respectively. Dried feces were eventually ground into a fine powder. The general scheme of isolation of the metabolites is summarized in Figure 1. Feces were first extracted with 3 L of acetone. The acetone extracts were concentrated to 100 mL of oily residues through evaporation of the solvent and were partitioned three times with 300 mL of acetonitrile against *n*-hexane. The acetonitrile phase was saved and was concentrated to an oily residue. The residue (35 g from *cis*- or 30 g from *trans*-chlordane) was transferred into a Florisil column (10 × 18 cm) and eluted with 3 L of diethyl ether, and then with 2 L of acetone. The ether fraction was concentrated to 15 mL and further purified on a silicic acid column (4 × 15 cm), by using 4 L each of *n*-hexane, *n*-hexane–ether (4:1 and 2:1), and acetone.

Purification of metabolites was carried out by means of thin-layer chromatography (TLC) on silica gel G or aluminum oxide G (type E) by using solvent systems indicated in the Results section. The spots containing chlordanes and their metabolites were made visible by spraying the

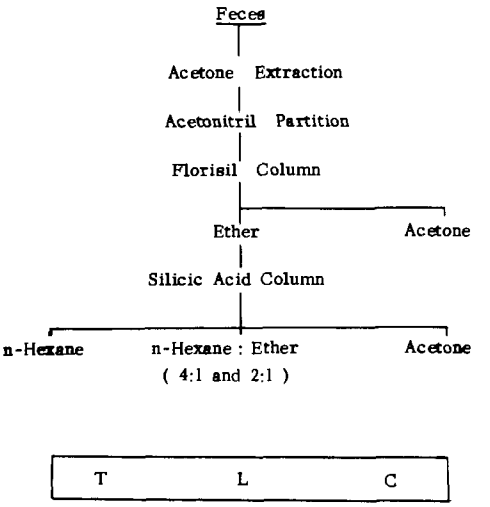

Figure 1. Schematic representation of the general isolation and purification procedures for chlordane metabolites from the feces.

plates with 5% of diphenylamine in acetone solution as a chromogenic agent. Chlorinated hydrocarbons were detected as blue and/or gray spots on TLC plates upon exposure to a strong ultraviolet light. For purification of metabolite, only a portion, usually one edge, was sprayed with the chromogenic agent and the rest of the plate was covered with a sheet of aluminum foil.

Urine samples were collected daily in a bottle with a small amount of toluene over a period of 3 to 4 weeks. They were pooled together, saturated with sodium chloride, adjusted to pH 3 with hydrochloric acid, and extracted with ethyl acetate (600 mL). The extract was concentrated to 5 mL of an oily residue. The procedures for isolation and purification of metabolites were identical with the ones used for fecal metabolites.

Hydrolysis of Polar Metabolite by β-Glucuronidase. The acetone eluates from Florisil and silicic acid columns from feces and urine were combined and concentrated. The condensate containing approximately 1 mg equiv of chlordane metabolites was dissolved in 5 mL of 0.1 M sodium acetate–acetic acid buffer at pH 4.8 and incubated with 50 mL (3000 units) of β-glucuronidase for 24 h at 37 °C in a shaking incubator. Five milliliters of ether was added to the incubation mixture to stop the reaction, and the aqueous phase was extracted three times with 5 mL each of ether. The extracts were combined, and the solvent was concentrated by using a rotary evaporator to 2 mL for further analysis on GLC.

Analyses of Metabolites. Mass spectra of the metabolites were taken by using a Finnigan 1015 Laboratory mass spectrometer at 70 eV at analyzer temperature 200 °C. Infrared (IR) spectra of the metabolites were obtained by using a Beckman IR-33 with KBr pellets. A Varian Aerograph Series 2400 and a F & M Model 5750 Research gas liquid chromatography (GLC) equipped with electron-capture detectors were used throughout the studies. The glass columns (1.2 m) for the F & M system were packed with either 3% SE-30 (220 °C) or 3% QF-1 (180 °C) on Chromosorb P 60/80. The metal column (1.8 m) for the Varian Aerograph system was packed with OV-101 (190 °C). Temperatures for both systems were: injection temperature at 250 °C and detector temperature at 280 °C. The carrier gases employed were argon–methane (95–5%) carrier gas for the F & M system and nitrogen for the Varian system at the carrier flow rate of 15 mL/min. All nuclear magnetic resonance spectra were taken by using a 90-MHz Bruker NMR spectrometer with a Fourier transformer in C_6D_6.

Table I. Relative Abundance of Metabolic Products from *cis*- and *trans*-Chlordane Found in the Feces[a]

cis-[^{14}C]Chlordane		*trans*-[^{14}C]Chlordane	
Metabolites	%	Metabolites	%
C-1	0.1	T-1	0.1
C-2	2.5	T-2	0.5
C-3	0.5	T-3	1.5
cis-Chlordane	13.0	*trans*-Chlordane	19.0
C-4	19.0	T-4	29.5
C-5	7.5	T-5	14.0
C-6	3.0	T-6-a	9.5
C-7	2.0	T-6	3.5
C-8	2.0	T-7	1.0
C-9	1.0	T-8	1.5
C-10[b]	15.5	T-9	1.5
C-11	26.5	T-10[b]	2.5
C-12	3.0	T-11	9.0
C-13	4.0	T-12	1.5
		T-13	5.0

[a] Data expressed in percentage of total of either *cis*-chlordane or *trans*-chlordane recovered in acetone extracts. [b] Includes at least three metabolites.

Preparation of Microsomal Fraction. Rats were stunned by cerebral concussion and livers were dissected out immediately. They were rinsed in ice-cold distilled water, then in ice-cold 0.2 M potassium phosphate buffer at pH 7.4, and blotted of excess liquid on filter paper, weighed, and homogenized at 30% (weight by volume) in fresh 0.2 M phosphate buffer at 0 °C by using a Potter-Elvehjem type Teflon–glass homogenizer for approximately 1 min at 1000 rpm.

Centrifugation of the homogenate at 2 °C for 20 min at 10000*g* resulted in sedimentation of cell wall, nuclei, mitochondria, and other tissue debris. The supernatant contained the microsomal plus soluble portion, i.e., the microsomal fraction.

Incubation of [^{14}C]Chlordane Isomers with Microsomal Fraction. One milliliter of the microsomal fraction was incubated for 2 h with either 0.27 μmol of *cis*-[^{14}C]chlordane (5 μCi) or 0.48 μmol of *trans*-[^{14}C]-chlordane (5 μCi) plus various combinations of cofactors in a 18-mL test tube. The cofactors such as ATP–Tris (3.6 μmol), glucose 6-phosphate (6.5 μmol), reduced glutathione (0.7 μmol), NAD (3.6 μmol), NADPH (3.6 μmol), or UDPGA (0.7 μmol) were added in 0.2 M, pH 7.4 phosphate buffer. The tubes, which were open to the atmosphere, were shaken at approximately 60 cycles/min at 37 °C for 2 h. The reactions were stopped by extracting the system with ether. Each 5-mL incubation mixture was extracted three times with 10 mL each of ether by vigorous shaking on a Vortex mixer. The extracts were pooled, and the solvent was removed under a gentle stream of air. The residue was taken up in ether and spotted on TLC plates (silica gel G) to make a radioautogram. All spots which had radioactivities were scraped off from the plate and assayed for their radioactivities.

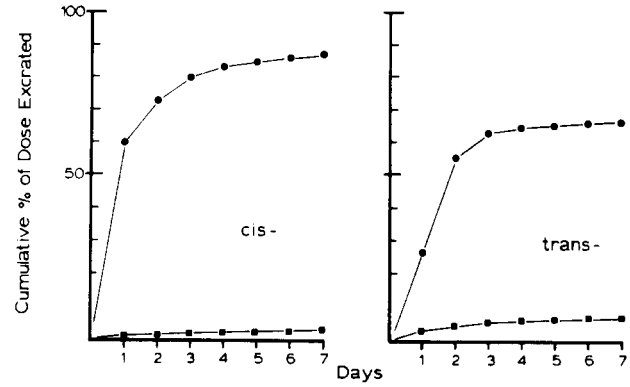

Figure 2. Excretion of radioactivity from the rats on [^{14}C]-chlordane isomers in feces (●) and in urine (■).

Toxicities (LC_{50}) of Chlordane Metabolites and Analogues. The metabolites of chlordane isomers and analogues were tested for toxicity on fourth instar *Aedes aegypti* mosquito larvae (CSMA Strain, WARF Institute, Inc., Wis.). Each testing vial contained ten larvae in 10 mL of distilled water. Each compound for the toxicity test was dissolved in 95% ethanol, and a 4-μL aliquot was added to each vial. The test was carried out for 24 h at room temperature.

RESULTS

Excretion Studies on [^{14}C]Chlordane Isomers. The results of the excretion studies are summarized in Figure 2. The total elimination of *cis*-chlordane and *trans*-chlordane reached 86 and 66% of the originally added doses by 7 days after treatment, respectively. Only 3% of ^{14}C from *cis*- and 6% from *trans*-chlordane was excreted in the urine in this period. The rate of excretion of radioactive compounds was more rapid with *cis*-chlordane treated rats (59% after 24 h) than those treated with *trans* isomer (27%).

Recognition of Metabolites. The relative abundance of metabolites in the feces is shown in Table I. Thirteen metabolites from *cis*- and 14 metabolites from *trans*-chlordane were recognized by TLC–autoradiographic and GLC analyses. The R_f values of several fecal metabolites of nonradioactive *cis*-chlordane or *trans*-chlordane, in hexane and hexane–ether eluates on silicic acid columns, were determined by using various TLC systems (Table II). The acetone eluates from both Florisil and silicic acid columns were combined and also analyzed on TLC. The

Table II. R_f Values of Metabolites of *cis*- and *trans*-Chlordane on Thin-Layer Chromatographic Systems

	Metabolites from *cis*- and *trans*-chlordane[a]													
Mobile phase	C-1	C-2	C-3	C-4	C-5	C-6	T-6-a	C-7[b]	C-8[b]	C-9	C-10	C-11	C-12	C-13
Benzene-EtOAC (2:1)	0.90	0.90	0.90	0.80	0.80	0.70	0.80	0.60	0.45	0.38	0.37	0.20	0.07	0.00
n-Hexane-Et_2O (4:1)	0.88	0.85	0.80	0.35	0.35	0.18	0.22	0.10-0.20		0.12	0.05	0.02	0.00	0.00
n-Hexane-acetone (2:1)	0.86	0.87	0.84	0.75	0.75	0.62					0.40	0.22	0.08	0.00
n-Hexane-$CHCl_3$ (4:1)	0.82	0.85	0.55	0.05	0.05	0.05						0.00		
n-Hexane-CH_2Cl_2 (4:1)	0.62	0.64	0.45	0.15	0.13	0.03						0.00		

[a] All *trans*-chlordane metabolites (T-1 through T-13) have identical R_f values as the corresponding *cis*-chlordane metabolites (e.g., C-4 vs. T-4), except T-6a which is listed here. [b] These R_f values were obtained by radioautographic analyses only. Other values were determined by both radioautographic and conventional thin-layer chromatograph (silica gel G) with diphenylamine as a chromogenic agent.

Table III. R_f Values of Known Reference Compounds on Silica Gel TLC

Mobile phase	Hepta-chlor	1,2-Di-chloro-chlordene	Oxy-chlordane	*cis*-Chlordane	*trans*-Chlordane	Chlordene-chloro-hydrin	1,2-Di-hydroxy-chlordene
Benzene-EtOAc (2:1)	0.90	0.90	0.90	0.90	0.88	0.70	0.20
n-Hexane-Et_2O (4:1)	0.88	0.85	0.80	0.79	0.80	0.18	0.02
n-Hexane-$CHCl_3$ (4:1)	0.82	0.85	0.52	0.65	0.60	0.05	0.00
n-Hexane-CH_2Cl_2 (4:1)	0.62	0.64	0.45	0.50	0.48	0.03	0.00
n-Hexane-acetone (2:1)	0.87	0.87	0.87	0.79	0.79	0.62	0.22

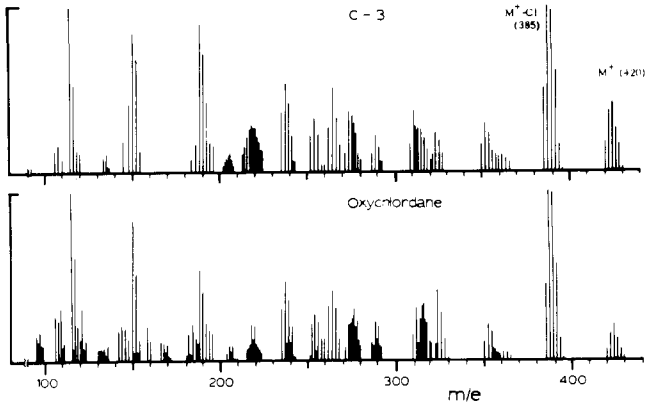

Figure 3. Comparison of mass spectra of metabolite C-3 from *cis*-chlordane and oxychlordane.

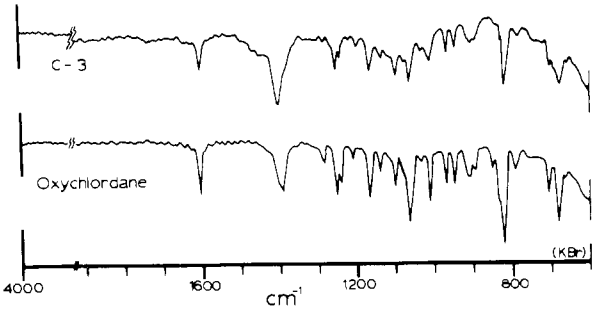

Figure 4. Comparison of infrared spectra of C-3 and oxychlordane.

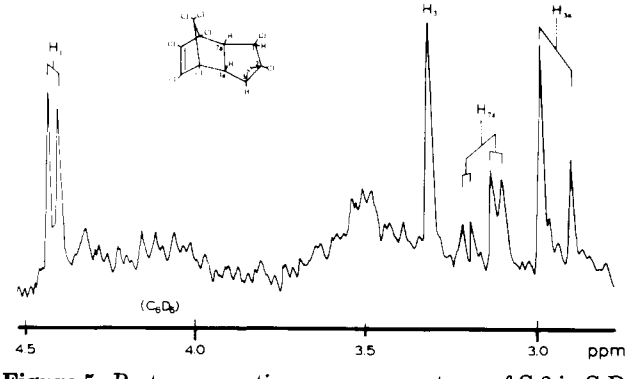

Figure 5. Proton magnetic resonance spectrum of C-3 in C_6D_6.

R_f values of known reference compounds on equivalent TLC systems are also shown in Table III.

Metabolites of *cis*-Chlordane. *C-1 and C-2.* Metabolite C-1 and C-2, minor components of the fecal metabolite, exhibited the same R_f values on various TLC systems (Table II) and retention times as the known samples of heptachlor and 1,2-dichlorochlordene, respectively, on three GLC analyses (SE-30, 220 °C, 3.1 and 4.0 min; QF-1, 180 °C, 1.8 and 2.3 min; OV-101, 190 °C, 2.6 and 3.6 min, for C-1 and C-2, respectively). Metabolite C-2 was isolated on TLC systems in a small quantity (approximately 50 μg), but no spectroscopic data could be obtained.

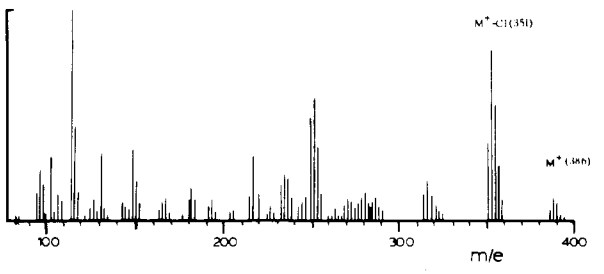

Figure 6. Mass spectrum of metabolite C-4 from *cis*-chlordane. The pattern of chlorine cluster at m/e 386 (as Cl = 35) indicates the presence of seven chlorines in the molecule.

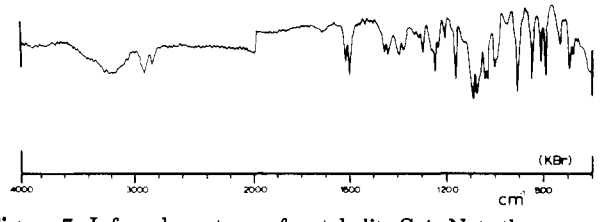

Figure 7. Infrared spectrum of metabolite C-4. Note the presence of OH (at 3300 cm^{-1}) and two double bond (around 1600 cm^{-1}) peaks.

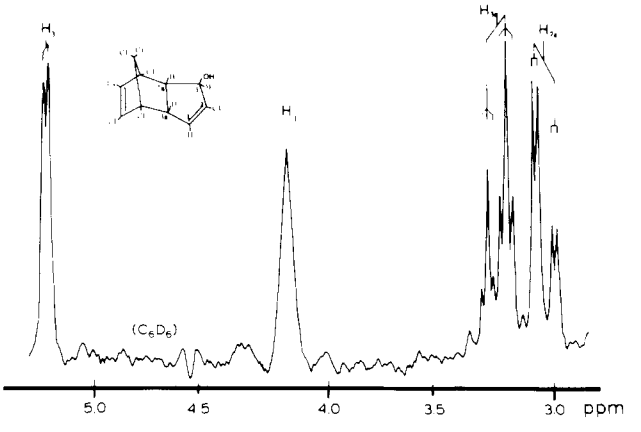

Figure 8. Proton magnetic resonance spectrum of C-4; taken in C_6D_6.

C-3. Metabolite C-3 was purified by means of aluminum oxide G (AO-G) TLC (R_f 0.50, *n*-hexane–ether, 95:5). The metabolite exhibited the same retention time on GLC (SE-30, QF-1, and OV-101; 4.6, 3.2, and 4.4 min, respectively), mass (Figure 3), infrared (Figure 4), and nuclear magnetic resonance (Figure 5) spectra as did an authentic oxychlordane.

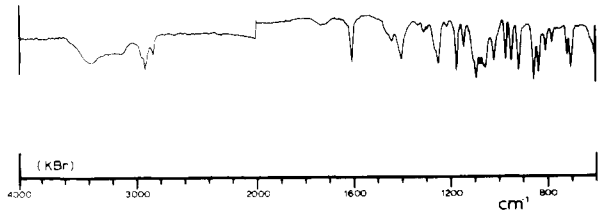

Figure 9. Infrared spectrum of metabolite C-5. Note the presence of OH (3400 cm^{-1}) and epoxy (1250 and 850 cm^{-1}) peaks.

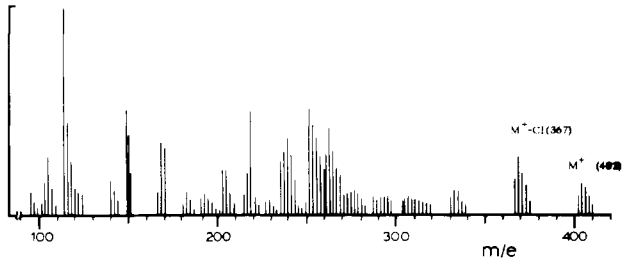

Figure 10. Mass spectrum of C-5. The pattern of chlorine cluster at m/e 402 indicates the presence of seven chlorines.

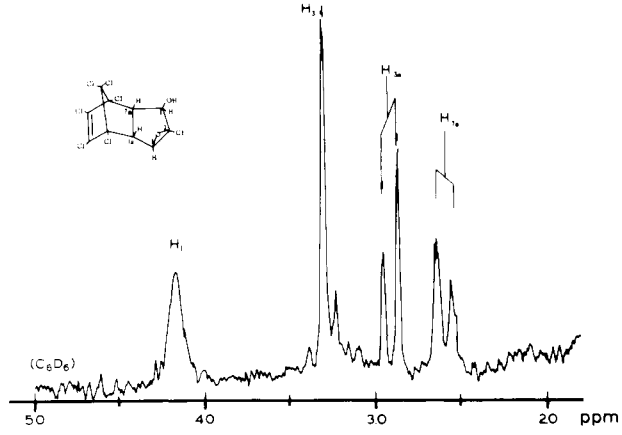

Figure 11. Proton magnetic resonance spectrum of C-5.

C-4. Metabolite C-4 was a major metabolite, isolated by TLC (AO-G, R_f 0.40, *n*-hexane–ether, 4:1) purification attempts. The compound reacted with Tri-Sil-Z to form a Me_3Si-derivatized product (C-4-Z) with a different retention time on GLC (QF-1: C-4, 3.1 min; C-4-Z, 2.4 min) from C-4, indicating that the metabolite was a hydroxylated compound. Mass spectral analysis (Figure 6) indicated a parent ion at m/e 386 (7 Cl). The IR (Figure 7) and the NMR spectra (Figure 8) indicated that the identity of C-4 to be 1-*exo*-hydroxy-2-chlorochlordene.

C-5. The metabolite was purified in a white powder form on TLC (AO-G; R_f 0.38, hexane–ether, 4:1, Table II). It gave a single peak in each GLC analyses (SE-30, 5.0; QF-1, 3.4; OV-1, 5.2 min). The compound (C-5-Z) exhibited a different retention time on GLC systems (SE-30, QF-1, and OV–101; 6.0, 2.9, and 5.6 min, respectively) from C-5, indicating the presence of at least one hydroxy group. The IR (Figure 9) and the mass spectra (Figure 10) showed the presence of COH (3400 cm^{-1}), an epoxy ring (1250 and 850 cm^{-1}), and CCl (700 cm^{-1}) moieties and molecular ions at m/e 402 (7 Cl) for Me_3Si derivative and at m/e 375 (6 Cl). Along with the result of NMR analysis (Figure 11), C-5 was identified as 1-*exo*-hydroxy-2-*endo*-chloro-2,3-*exo*-epoxychlordene.

C-6. Metabolite C-6 was a minor component in fecal metabolite. It showed an identical behavior in three different GLC systems (SE-30, 5.8; QF-1, 5.9; OV-101, 5.8 min) as the authentic sample of chlordene chlorohydrin

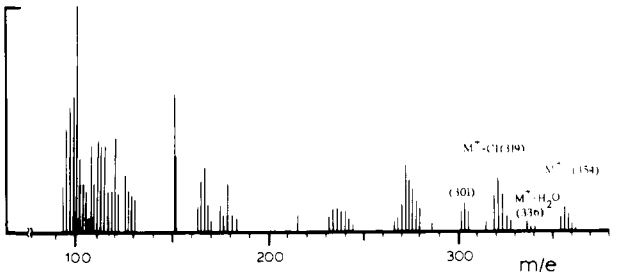

Figure 12. Mass spectrum of metabolite C-10-a. Note the presence of $M^+ - H_2O$ (336) and $M^+ - Cl$ (319) peaks.

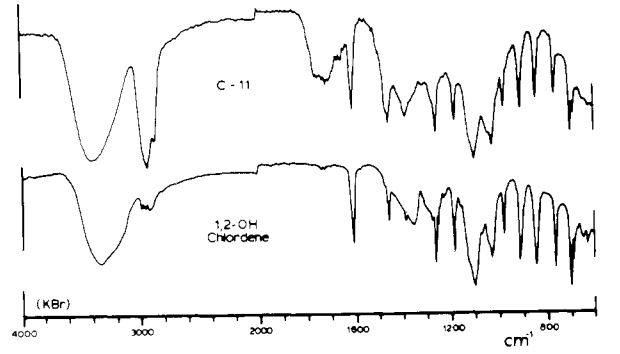

Figure 13. Comparison of infrared spectra of metabolite C-11 and 1,2-dihydroxydihydrochlordene.

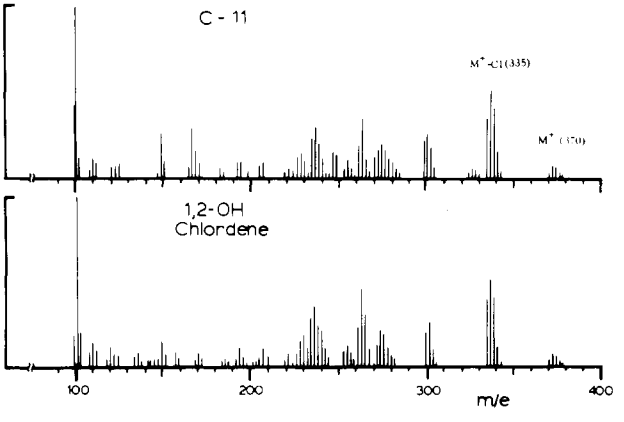

Figure 14. Comparison of mass spectra of C-11 and 1,2-dihydroxydihydrochlordene.

on TLC (Table II and IV). The Me_3Si derivative (C-6-Z) also exhibited the same retention times (SE-30, QF-1, and OV-101; 6.7, 3.8, and 7.3 min, respectively). The metabolite was identified as chlordene chlorohydrin (1-*exo*-hydroxy-2-*endo*-chlorodihydrochlordene).

C-7, C-8, and C-9. These compounds were detected by radiotracer analysis, but were not present in sufficient quantities in the fecal products from nonlabeled *cis*-chlordane (Table I).

C-10. It was found that C-10 consisted of at least three compounds by GLC analysis. One of them (C-10-a, <100 μg) which was the most abundant component as judged by GLC was isolated on TLC (AO-G; R_f 0.40, hexane–acetone, 2:1). C-10-a gave a single peak on each GLC analysis (Table V). The Me_3Si derivatized product exhibited a peak with different retention times on GLC (QF-1, 3.0 min) from C-10-a. The mass spectrum (Figure 12) of C-10-a showed a molecular ion m/e at 354 (6 Cl). The appearance of trace peaks at 336 and 301 (expressed as Cl = 35) indicates the loss of H_2O and Cl, respectively, suggesting the presence of an OH group. The evidence, along with the above GLC behavior of the Me_3Si-deriv-

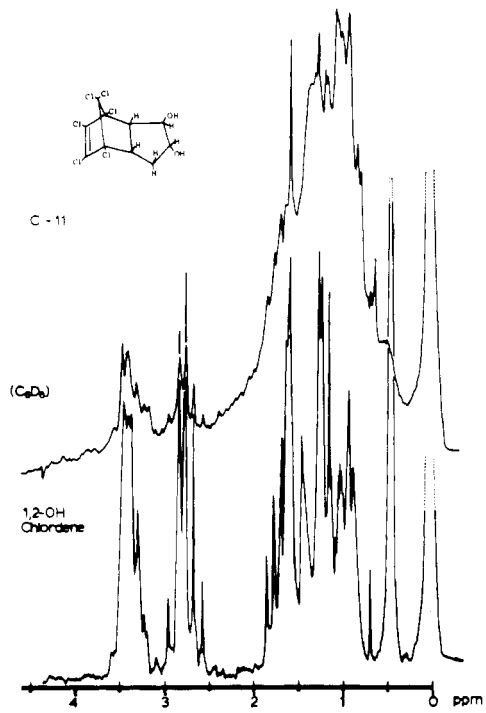

Figure 15. Proton magnetic resonance spectra of metabolite C-11 and reference compound 1,2-dihydroxydihydrochlordene.

atized product, suggests that C-10-a is a monohydroxylated dihydrochlordene.

C-11. C-11 was isolated by chromatography on silica gel G and aluminum oxide G TLC. The metabolite had the same infrared absorption (Figure 13), mass (Figure 14), and NMR spectra (Figure 15) as authentic 1,2-dihydroxychlordene.

C-12. This compound was not isolated in a sufficient quantity in a pure form for spectroscopic analyses, though it showed one spot on various TLC systems (Table II) and a single peak on GLC (SE-30, 10.2 min). The Me_3Si-derivatized product exhibited a different retention time on GLC (SE-30, 5.4 min) from C-12. As judged by the R_f values (i.e., C-12 is much more polar than C-11) on TLC systems, C-12 is expected to have more than two OH groups. It is likely a trihydroxydihydrochlordene, but its spacial arrangement of OH groups could not be established.

Metabolites of *trans*-Chlordane. *T-1 and T-2.* Both compounds were minor components in fecal metabolites as judged by TLC analyses, as shown in Table I. T-1 and T-2 exhibited the identical behavior as the authentic samples of heptachlor and 1,2-dichlorochlordene on several TLC and GLC (Table IV and V).

T-3. The metabolite T-3 showed the same properties and characteristics on TLC (Table II and III) and GLC systems (Table IV and V) as the known reference compound of oxychlordane.

T-4. T-4 was isolated from *trans*-chlordane by TLC and GLC analyses. It had the same retention times as C-4 on GLC (Table IV). Also, IR, mass, and NMR spectra were identical with C-4 spectra (Figure 7, 8, 9). Metabolite T-4 was thus identified as 1-*exo*-hydroxy-2-chlorochlordene.

T-5. The mass spectrum of C-5 (not shown) exhibited an identical fragmentation pattern to C-5. The Me_3Si-derivatized product of T-5 had the same retention times on GLC systems (Table IV and V) as C-5-Z. On the basis of TLC and GLC analyses, the metabolite T-5 was identified as 1-*exo*-hydroxy-2-*endo*-chloro-2,3-*exo*-epoxychlordene.

Table IV. Retention Times (t_R) of *cis*- and *trans*-Chlordane[a] Metabolites on Gas Chromatographic Systems

	t_R, min		
	SE-30 (220 °C)	QF-1 (180 °C)	OV-101 (190 °C)
C-1	3.1	1.8	2.6
C-2	4.0	2.3	3.6
C-3	4.6	3.2	4.4
C-4	4.5	3.0-3.1	4.4
C-4-Z[b]	5.1	2.4	5.0
C-5	5.0	3.4	5.2
C-5-Z[b]	6.0	2.9	5.6
C-6	5.8	5.9	5.8
C-6-Z	6.7	3.8	7.3
C-10-a	4.6	5.7	4.5
C-10-a-Z[b]	5.0	3.0	5.3
C-11	7.2	*c*	*c*
C-11-Z	8.9	4.4	10.9
C-12	10.2	*c*	*c*
C-12-Z[b]	5.4	5.3	4.9

[a] All *trans*-chlordane metabolites (T-1 through T-12) have identical t_R values as the corresponding *cis*-chlordane metabolites (e.g., C-4 vs. T-4, C-5 vs. T-5). [b] Z refers to silanized (i.e., trimethylsilane ether derivative) product. [c] Sensitivity on electron-capture detector was too low to ascertain the t_R's.

Table V. Retention Times t_R of Known Reference Compounds on GLC Systems

	t_R, min		
Compd	SE-30 (220 °C)	QF-1 (180 °C)	OV-101 (190 °C)
Heptachlor	3.1	1.8	2.6
1,2-Dihydroxychlordane	4.0	2.3	3.6
Oxychlordane	4.6	3.2	4.4
cis-Chlordane	5.6	4.1	5.5
trans-Chlordane	5.1	3.8	4.9
Chlordene Chlorohydrin (CH)	5.8	5.9	5.8
CH-Z[a]	6.7	3.8	7.3
1,2-Dihydroxychlordene	7.2	*b*	*b*
1,2-Dihydroxychlordene-Z[a]	8.9	4.4	10.9

[a,b] See Table IV.

T-6. The results of chromatographic analyses on T-6, a rather minor metabolite (Table I), indicated R_f values on TLC (Tables II and III) and retention times on GLC (Tables IV and V) identical with those of chlordene chlorohydrin.

T-6-a, T-7, T-8, and T-9. Metabolites T-6-a, T-7, T-8, and T-9 were observed on autoradiograms of radiotracer analyses. However, the identical metabolites from nonlabeled *trans*-chlordane were not found on TLC, indicating that they are present in rather small quantities.

T-10. Metabolite T-10 was isolated from *trans*-chlordane fed rats as a minor metabolite. It showed the same properties on TLC and GLC (Tables IV and V) as C-10. Both C-10 and T-10 were identified as monohydroxylated dihydrochlordenes.

T-11. Metabolite T-11 was isolated from the feces of *trans*-chlordane fed rats as a major metabolite and showed the same properties as did the authentic compound of 1,2-dihydroxychlordene. The Me_3Si-derivatized product also exhibited identical R_f values and retention times with TLC and GLC systems (Tables II, III, IV, and V) as the Me_3Si derivative of dihydroxydihydrochlordene.

T-12. This very minor metabolite showed the same behavior as C-12 on TLC and GLC analyses. The Me_3Si-derivatized product also had a different peak on

Table VI. Radioactive Components of Ether-Soluble Metabolites of *cis*-[^{14}C]Chlordane and *trans*-[^{14}C]Chlordane in Vitro[a]

Tube no.[b]	Added cofactors	% of total ^{14}C compounds from *cis*-chlordane						
		C-1~3	C-4~5	C-6	C-10	C-11	C-12	C-13[c]
		cis-Chlordane						
1	NADPH, ATP, G-6-P	93.5	2.2	0.6	0.5	0.1	0.1	2.5
2	NADPH	95.3	1.4	0.6	0.5	0.1	0.1	1.5
3	ATP, G-6-P	98.0	0.1	0	0	0.1	0.1	0.8
4	GSH	97.7	0.7	0.1	0	0	0.1	0.7
5	UDPGA	96.8	0.3	0.5	0.1	0.1	0.1	2.8
6	NAD, UDPGA	97.0	0.1	0.1	0	0	0.1	2.0
7	NADPH, NAD, UDPGA	92.5	0.8	0.4	0.3	0.1	0.1	5.2
Tube no.[b]	Added cofactors	% of total ^{14}C compounds from *trans*-chlordane						
		T-1~3	T-4~6-a	T-6	T-10	T-11	T-12	T-13[c]
		trans-Chlordane						
1	NADPH, ATP, G-6-P	86.2	2.9	1.3	0.6	0.6	0.6	8.1
2	NADPH	89.7	1.0	1.1	0.6	0.1	0.1	6.2
3	ATP, G-6-P	93.3	1.7	0.6	0.1	0	0	2.7
4	GSH	95.5	1.5	0.1	0	0	0	2.4
5	UDPGA	99.1	0	0	0	0	0	0.5
6	NAD, UDPGA	96.0	2.1	0	0	0	0	1.9
7	NADPH, NAD, UDPGA	92.0	1.5	0.6	0.5	0	0	4.4

[a] Data expressed in percentages of the totally recovered chlordane from each chromatogram. [b] All incubation tube containing 5 μCi of either *cis*- (0.27 μmol) or *trans*-[^{14}C]chlordane (0.48 μmol) and microsonial fraction along with cofactors, and made up to 50 mL with 0.2 M phosphate buffer (pH 7.4). Incubated at 37 °C for 2 h. The cofactor quantities were 3.6 μmol for NADPH, NAD, and ATP, 6.5 μmol for G-6-P and 0.7 μmol for UDPGA and GSH. [c] Including other polar metabolites such as glucuronides.

GLC (QF-1, 180 °C, 5.3 min) from the original metabolite.

C-13, T-13, and the Major Urinary Metabolite from *cis*- and *trans*-Chlordane. Metabolites in urine from either *cis*- or *trans*-chlordane were eluted with acetone from Florisil and silicic acid columns. No metabolite was detected in the ether fraction from the Florisil column and in the *n*-hexane or *n*-hexane–ether (4:1) fraction from the silicic acid column. The urinary metabolite from both chlordane isomers behaved in a very similar manner as C-13 and T-13 on several TLC systems (Tables II and IV). C-13 and urinary metabolite from *cis*-chlordane or T-13 and the urinary metabolite from *trans*-chlordane were combined and incubated with β-glucuronidase in acetate buffer (pH 4.8), and the product was extracted with ether for GLC analysis. It was found that, in all cases, the β-glucuronidase hydrolysis yielded only one product, 1-*exo*-hydroxydihydrochlordene, based upon the result of the chromatographic matching tests against an authentic sample: its t_R being 4.2 min on SE-30 and 3.2 min on QF-1, respectively.

In Vitro Studies. The effects of NADPH and other cofactors on in vitro metabolism of chlordane isomers were studied by using microsomal fraction from the rat liver. The results and the concentration of each cofactor used were summarized in Table VI and VIII. NADPH had the most significant stimulatory effect among cofactors tested. Analysis of ether extractable metabolites were accomplished by using TLC (silica gel G) with benzene–ethyl acetate (2:1). All the in vitro metabolites have been compared with each of the in vivo metabolites on a TLC system (benene–ethyl ether).

Toxicities (LC_{50}) of the Chlordane Metabolites and Analogues on Mosquito Larvae. The results of the toxicity tests on mosquito larvae are shown in Table VII. Heptachlor (C-1, T-1), heptachlor epoxide, 1,2-dichlorochlordene (C-2, T-2), and oxychlordane (C-3, T-3) were more toxic to the larvae than *cis*- and *trans*-chlordane. C-4, T-4, C-5, and T-5 showed less toxicity than the original compounds; however, C-5 and T-5 indicated slightly higher toxicity than C-4 and T-4. C-10 and T-10 failed to show any toxicity to the larvae even at the highest concentration tested (100 ppm). C-11 and T-11 (1,2-dihydroxychlordene) did not show any symptoms of poisoning at 100 ppm.

Table VII. Toxicities (LC_{50}) in Vivo of the Metabolites and Chlordane Analogues toward Mosquito Larvae, *Aedes aegypti* L.

Compounds	Metabolite designation	LC_{50}/24 h (ppm)
Heptachlor	C-1	2.0×10^{-2}
	T-1	
1,2-Dichlorochlordene	C-2	
	T-2	3.8×10^{-2}
Oxychlordane	C-3	
	T-3	2.5×10^{-2}
cis-Chlordane		5.0×10^{-2}
trans-Chlordane		5.0×10^{-2}
Nonachlor		3.5×10^{-2}
Chlordene epoxide		5.0×10^{-1}
	C-4	
	T-4	2.2
	C-5	
	T-5	2.0
2-Chlorochlordene		1.4
Chlordene chlorohydrin	C-6	
	T-6	9.0
1-Hydroxychlordene		15.0
1-Hydroxychlordene epoxide		20.0
1,2-Dihydroxydihydrochlordene	C-11	
	T-11	<100

Determination of the Route of Metabolism. To study the relationship among the metabolites, an in vitro study was conducted with the microsomal fraction. In this study, the individual pure metabolite was incubated in vitro, and the resulting metabolic products were extracted with ether and analyzed on GLC with an electron-capture detector and on TLC. The results are summarized in Table VIII.

DISCUSSION

Figure 16 summarizes our current thought on the general scheme of chlordane metabolism. The routes that were active are shown by solid lines and the ones which are weak are indicated by broken lines.

Table VIII. Formation of Further Metabolic Products from Each Intermediate in Vitro

Starting material	In vitro incubation products[a]									
	C-1 (T-1)	C-2 (T-2)	C-3 (T-3)	C-4 (T-4)	C-5 (T-5)	C-6 (T-6)	1-OH[b]	C-10 (T-10)	C-11 (T-11)	C-12 (T-12)
Heptachlor (C-1, T-1)		–	–	–	–	–	–	–	±	–
1,2-Dichlorochlordene (C-2, T-2)	–		+	++	+	±	–	–	±	–
Oxychlordane (C-3, T-3)	–	–		++	+	±	–	–	±	–
C-4, (T-4)	–	–	–		±	–	–	–	–	–
C-5, (T-5)	–	–	–	–		–	–	–	–	–
Chlordene chlorohydrin (C-6, T-6)	–	–	–	+	–		–	–	±	–
1-OH[b]	–	–	–	–	–	–		–	+	–
C-10 (T-10)	–	–	–	–	–	–	–		–	–
1,2-*trans*-Dihydroxy-dihydrochlordene (C-11, T-11)	–	–	–	–	–	–	–	–		++

[a] ++ products detected in abundance, + products in small quantities, ± trace, but positively recognized product. [b] 1-OH; 1-*exo*-hydroxydihydrochlordene.

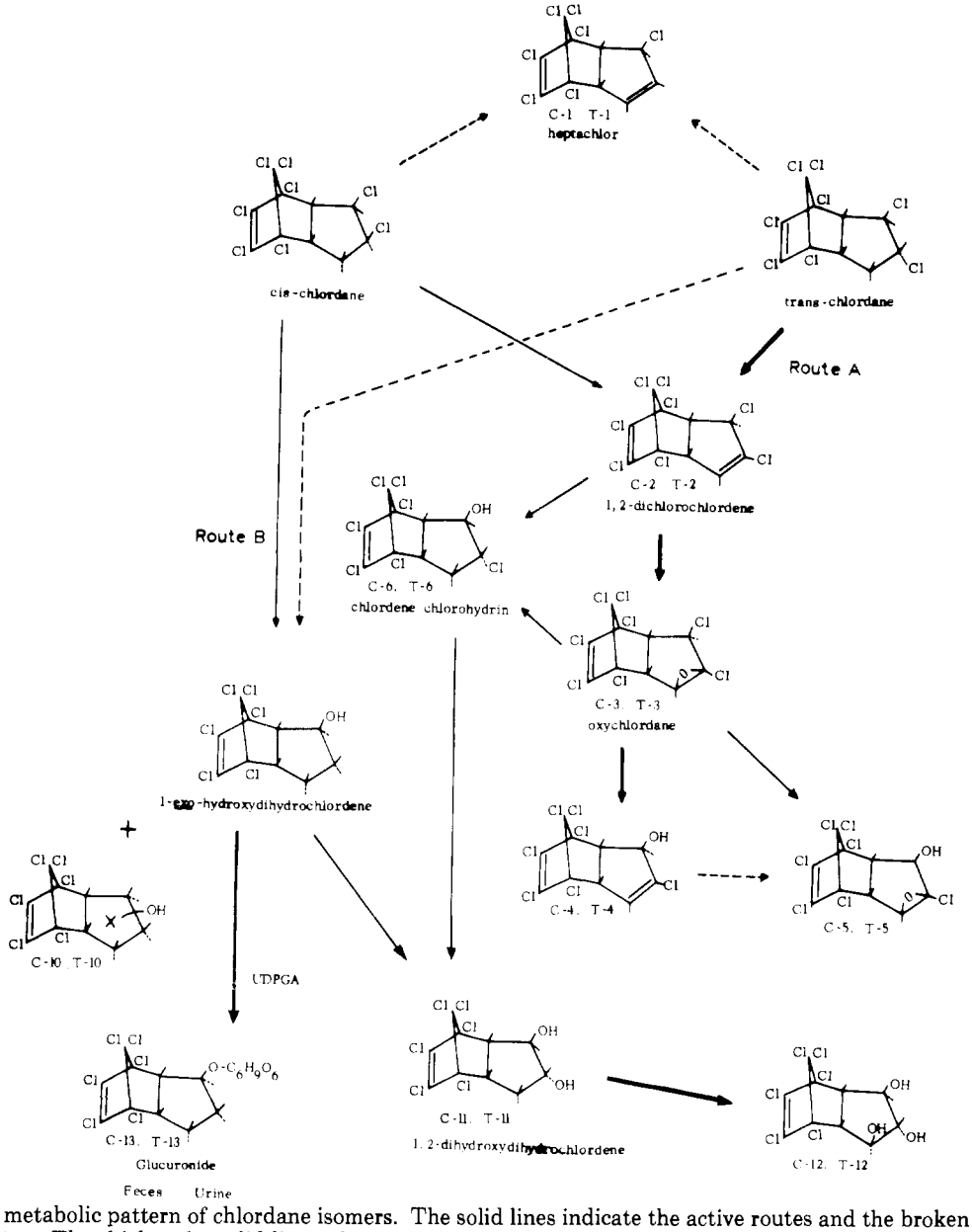

Figure 16. Overall metabolic pattern of chlordane isomers. The solid lines indicate the active routes and the broken lines represent weak metabolic routes. The thicker the solid lines, the more active the metabolic activities.

In the current study, we were able to identify two entirely new major metabolic products from both chlordane isomers which happen to be relatively stable end products in the metabolic route of chlordane. They are 1-*exo*-hydroxy-2-chlorochlordene (C-4, T-4) and 1-*exo*-hydroxy-2-chloro-2,3-*exo*-epoxydihydrochlordene (C-5, T-5). Both of these metabolites can be formed either from dichlorochlordene (C-2, T-2) or from oxychlordane (Table VIII), probably because of the liability of the *exo*-chlorine at the 1 position in analogous situation with heptachlor and heptachlor epoxide. Thus, this route of metabolism (route A, Figure 16) of chlordane is not the direct hydroxylation on chlordane, itself, but formation of dichlorochlordene via dehydrogenation, followed by both epoxidation at the 2–3 carbons and hydroxylation, replacing the chlorine at the 1 position. In addition, there must be another route (route B) involving a direct hydroxylation at the 1 position on both *cis*- and *trans*-chlordane, yielding 1-*exo*-hydroxydihydrochlordene, since C-10 (T-10) is not formed from any of the metabolites belonging to route A.

There are two aspects that warrant some attention: first, the formation of heptachlor from chlordanes has previously not been shown to occur in any biological systems, and second, the qualitative differences between metabolism of *cis* and *trans* isomers in the rat. We were able to show in vitro that the trans isomer is the more degradable member of chlordane (Table VI). In studies in vivo (Table I), the most conspicuous differences in the relative abundance of metabolites between these two isomers were in the levels of C-3 (T-3), C-4 (T-4), and C-5 (T-5). Since all these metabolites come from the same metabolic intermediate, dichlorochlordene (C-2, T-2), it must mean that the formation of the latter is favored for the trans isomer. Indeed, this finding agrees well with the observation by Street and Blau (1972) that a higher level of oxychlordane was observed in the rat treated with *trans*-chlordane as compared with the one treated with *cis*-chlordane.

On the other hand, in terms of excretion, it has been consistently shown that the cis isomer is eliminated faster than the trans isomer in the rat (Barnett and Dorough, 1974; Figure 2 of this paper). Upon examination of the data in Table I, it is apparent that the levels of both C-10 and C-11 are much higher than corresponding metabolites of *trans*-chlordane, T-10 and T-11. These are mono- and dihydroxydihydrochlordenes, and they are expected to play significant roles in increasing excretability of the xenobiotics. Moreover, the major conjugation product in urine and feces is the glucuronide of 1-*exo*-hydroxydihydrochlordene, another hydroxy compound which forms more readily from *cis*-chlordane. As for C-11 (T-11), 1,2-*trans*-dihydroxydihydrochlordene, one route for its formation as shown in Figure 16 appears to be from oxychlordane. Since the level of oxychlordane is expected to be higher in the *trans*-chlordane fed rat than in the *cis*-chlordane treated one, the high rate of C-11 production as opposed to T-11 cannot be adequately explained from route A alone. The most logical explanation would be that at least a part of the formation of 1,2-*trans*-dihydroxydihydrochlordene is carried out via route B, particularly from *cis*-chlordane, which has relative difficulty (against *trans*-chlordane) in being metabolized through the major route A via oxychlordane. Indeed, this assumption is in good agreement with the data shown in Table VIII in that C-10 could not be formed in vitro from any of the intermediates belonging to the metabolic route A.

In conclusion, the basic metabolic pattern of both *cis*- and *trans*-chlordane was established in this work. 1-*exo*-Hydroxy-2-chlorochlordene (C-4, T-4) and 1-*exo*-hydroxy-2-chloro-2,3-epoxychlordene (C-5, T-5) are two important metabolites. It must be noted that unlike oxychlordane, these two metabolites are not easily degraded further in rats (Table VIII), and, therefore, it is possible that they accumulate in the animal body as terminal residues. Their toxicological significance must be carefully examined in the future. As judged by the toxicities to mosquito larvae, however, none of these metabolites appear to be more toxic than the original compounds.

ACKNOWLEDGMENT

We thank Toshiya Ikeda of this research unit for his help in spectroscopic analyses and Velsicol Chemical Corporation for providing radioactive materials and reference standards used in this study.

LITERATURE CITED

Barnett, J. R., Dorough, H. W., *J. Agric. Food Chem.* **22**, 612 (1974).

Dorough, H. W., Hemken, W. R., *Bull. Environ. Contam. Toxicol.* **10**, 208 (1973).

Lawrence, J. H., Barron, R. P., Chen, J.-Y. T., Lombard, P., Benson, W. R., *J. Assoc. Off. Anal. Chem.* **53**, 261 (1970).

Polen, B., Hester, M., Benizer, J., *Bull. Environ. Contam. Toxicol.* **5**, 521 (1971).

Schwemmer, B., Cockran, P. W., Polen, B. P., *Science* **169**, 1087 (1970).

Street, J. C., Blau, S. E., *J. Food Chem.* **20**, 395 (1972).

Von Rumker, R., Matter, M. R., Clement, P. D., Erickson, K. F., "Production, Distribution, Use and Environmental Impact Potential of Selected Pesticides", U.S. Environmental Protection Agency, Washington, D.C., 1975, p 21.

Received for review October 18, 1976. Accepted March 16, 1977. Supported by College of Agriculture and Life Sciences, University of Wisconsin, Madison, and by a research grant (ES-00857) from the National Institute of Environmental Health Sciences, Research Triangle Park, N.C.