substituents on the *s*-triazine ring were Cl_3C, *i*-C_3H_7, and 2-pyridyl groups. In general, the mosquito was less susceptible than the housefly to the larvicidal effects of this series, but the most active compounds in *M. domestica* (A13-22641; AI3-60103) were also active in *A. aegypti*. In *T. confusum*, only AI3-61080 and AI3-60138 were active at the highest concentration; the same compounds were ineffective in *A. aegypti* and moderately effective in *M. domestica*. None of the compounds, except AI3-22641 at the highest concentration, were effective larvicides in *P. interpunctella*.

In the series of triamino-*s*-triazines (Table I), the susceptibility of the two dipterous species was again the highest. If the activity of the previously reported larvicide AI3-70670 (Herzog and Brechbuehler, 1976; Miller et al., 1977; Christensen and Knapp, 1976) is considered as a model, AI3-22641, AI3-60103, AI3-60008, AI3-51014, and AI3-60352 surpass its activity in *M. domestica* and AI3-51014 equals its activity in *A. aegypti*. Two of the most active triamino-*s*-triazines (AI3-60008; AI3-51014) are structurally similar to the most active diamino-*s*-triazines (AI3-22641; AI3-60103) by the presence of two free amino groups. Although AI3-70670 and AI3-60352 do not have free amino groups, it is conceivable that they could be dealkylated in the insect to more active compounds (Chang et al., 1968). There is a general similarity between the larvicidal and chemosterilant activities of amino-*s*-triazines in *M. domestica*. The compounds effective as sterilants in females, but ineffective in males, have either two free amino groups (Borkovec et al., 1972) or single substituents on the amino groups (LaBrecque et al., 1968); all the more effective larvicides in Table I belong to this group. On the other hand, compounds containing dimethylamino groups, which are necessary for sterilizing males, appear ineffective as larvicides. Because the detailed physiological effects of amino-*s*-triazines in insects are not known, further studies are required to determine whether the sterilizing and larvicidal activities of these compounds have a common basis.

Like the diamino-*s*-triazines, the triamino-*s*-triazines had low and erratic activity in *T. confusum*: AI3-51146, AI3-61205, and AI3-70670 were larvicidal only at the highest concentration. None was active in *P. interpunctella*.

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Metabolism of *cis*- and *trans*-Chlordane by a Soil Microorganism

Richard W. Beeman*[1] and Fumio Matsumura

Chlordane is an environmentally persistent soil insecticde, particularly useful in the protection of wooden structures from termite damage. A slow change was found to occur in the composition of soil residues of technical chlordane, suggestive of chemical or biological transformation of certain components. An actinomycete (*Nocardiopsis* sp.) isolated from soil was capable of extensively degrading chlordane in pure culture. Growing broth cultures of *Nocardiopsis* metabolized pure *cis*- or *trans*-chlordane to at least eight solvent-soluble substances including dichlorochlordene, oxychlordane, heptachlor, heptachlor *endo*-epoxide, chlordene chlorohydrin, and 3-hydroxy-*trans*-chlordane. Identifications were based on gas chromatographic or mass spectroscopic analysis. Oxychlordane was metabolically inert, and accumulated in the mycelium as a terminal residue. Patterns of metabolic activity in microorganisms were compared to the residue patterns in chlordane-treated soil.

Chlordane is an environmentally persistent, all-purpose soil insecticide which has been used extensively in this country since about 1950. The agricultural uses of chlordane have been restricted in recent years because of a suspicion that this insecticide may be an environmental carcinogen (Environment Protection Agency, 1976; Epstein, 1976).

Any analysis of the environmental fate and impact of chlordane has been hindered by the complexity of the technical mixture. The two major components, *cis*- and *trans*-chlordane, together constitute only about half of the total weight of the technical material. The remainder is

Pesticide Research Center, Michigan State University, East Lansing, Michigan 48824.
[1]Present address: U.S. Grain Marketing Research Laboratory, Manhattan, KS 66502.

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a mixture of various isomers of heptachlor, nonachlor, chlordene, and several dozen related substances (Sovocool et al., 1977).

The potential of chlordane as a serious environmental contaminant was realized in 1970 when a highly toxic and persistent residue, oxychlordane, was found in the milk of cows which had grazed on chlordane-treated alfalfa (Lawrence et al., 1970). Since that time several research groups have examined the metabolic fate of pure *cis*- and *trans*-chlordane in animal systems (Poonawalla and Korte, 1971; Barnett and Dorough, 1974; Tashiro and Matsumura, 1977; Brimfield et al., 1978; Feroz and Khan, 1979). However, no work has been published on the metabolic fate of chlordane in microorganisms or in soil or aquatic environments. Iyengar and Rao (1973) claim to have studied "metabolism" of chlordane by *Aspergillus niger*. However, these workers measured only the disappearance of chlordane from the medium and made no attempt to differentiate between metabolism, uptake, adsorption onto the glass containers, or volatilization of chlordane from the medium. In our studies *A. niger* was inactive. To determine whether the transformation of chlordane occurs in nature, we analyzed residues in chlordane-treated soil from Mississippi. To test whether such transformation of chlordane could be catalyzed by soil microflora, we isolated microorganisms from two soil sources and screened them for chlordane metabolizing ability.

MATERIALS AND METHODS

Chemicals. All authentic standards were gifts of the Velsicol Chemical Corp., Chicago, IL. These included the following: cis-rich [^{14}C]chlordane, sp act. 10.8 mCi/mmol (70% cis); *trans*-[^{14}C]chlordane, sp act. 6.27 mCi/mmol; [^{14}C]oxychlordane, 99+% pure, sp act. 12.1 mCi/mmol; *cis*-chlordane; *trans*-chlordane; oxychlordane; dichlorochlordene; heptachlor; heptachlor *exo*-epoxide; chlordene chlorohydrin. The radiolabeled chlordanes were purified on thin-layer chromatography (TLC) in hexane–methylene chloride (4:1) (5× development).

Analysis of Chlordane Residues in Soil. In 1958, as part of an efficacy study conducted jointly by the U.S. Department of Agriculture, Shell Oil Co., and Velsicol Chemical Co., soil plots (Rumford sandy loam) in Mississippi were treated with 1 pt/ft^2 of 0.03% or 1% formulations of technical chlordane. In 1978 soil samples were collected and extracted with *n*-hexane. We analyzed the hexane extracts directly by gas chromatography (GLC).

Isolation and Screening of Soil Microflora. Topsoil from a local vegetable garden was spiked with 2000 ppm of *cis*-chlordane and incubated for 1 month at 100% relative humidity to precondition the microbial population. Another soil sample was obtained from a Hooker Chemical Co. dump site in Montague, MI. Microorganisms from both soil samples were then isolated in pure culture by the dilution plate method, as described by Matsumura and Boush (1967). The liquid medium used in subsequent experiments consisted of the following (in G/L): mannitol, 10; Bacto yeast extract (Difco Laboratories), 10; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; $CaCl_2 \cdot 2H_2O$, 0.001. The final pH was 6.9–7.0.

Forty random soil isolates, including bacteria, antinomycetes, and fungi, were grown in standing broth cultures in the presence of 0.75 ppm of cis-rich [^{14}C]chlordane (70% cis, sp act. 10.8 mCi/mmol) for 1 month. Culture were extracted with chloroform and were monitored for the appearance of metabolites by liquid scintillation spectroscopy (water phase) or electron-capture gas chromatography (solvent phase). In subsequent experiments involving the active isolate, *Nocardiopsis*, culture flasks were shaken continuously on a linear shaker at 100 strokes/min, since chlordane metabolism was more extensive under these conditions than in standing cultures.

Isolation of Metabolites. To study metabolic pathways for chlordane in cultures of *Nocardiopsis*, we inoculated 20 mL of mannitol–yeast medium with an aliquot of a growing culture and incubated the flasks with shaking for 2–4 days. Cultures were then spiked with varying concentrations of *trans*-[^{14}C]chlordane, sp act. 6.27 mCi/mmol, or with nonradioactive *cis*- or *trans*-chlordane, added with ethanol (final concentration of ethanol, ≤0.5%), and shaking was continued for 1–4 weeks. All incubations were carried out at room temperature in the dark.

After the addition of 2 mL of methanol, cultures were homogenized in a glass–Teflon homogenizer, and aliquots were extracted 3 times with equal volumes of chloroform. In other cases the mycelium was separated from the medium by filtration and homogenized in 10% methanol before extraction and analysis. Combined solvent extracts were dried over Na_2SO_4, the solvent was evaporated under nitrogen, the residue was redissolved in acetone, and the resulting solutions were subjected to gas chromatographic analysis either directly or after TLC fractionation. Radioactive metabolites separated on TLC plates were visualized by autoradiography and quantified by liquid scintillation spectroscopy.

For large-scale preparation of metabolities, mycelium was accumulated from a total of 1 L of *Nocardiopsis* culture which had been incubated for 1 month with 20 ppm of *trans*-chlordane. The mycelium was extracted with acetone, the acetone evaporated on a rotary evaporator, and the residue taken up in water and extracted 5 times with equal volumes of *n*-hexane. Combined hexane extracts were dried over Na_2SO_4, concentrated to 5 mL, and applied to a 1 × 10 in. silica gel 100 column, 70–230 mesh (EM Laboratories, Inc.). Metabolites were successively eluted from the column with 200 mL each of *n*-hexane, *n*-hexane–diethyl ether (93:7, 85:15, and 50:50), diethyl ether, and acetone. Column fractions were monitored for chlordane metabolites by gas chromatography. Nonpolar fractions from the silica gel column were further purified on a 1 × 9 cm acidic aluminum oxide column, activity grade 1 (Alupharm Chemicals). Metabolites were eluted with 20 mL of *n*-hexane, followed by 40 mL of *n*-hexane–diethyl ether (94:6). One of the polar metabolites (compound 7) was further purified by TLC and preparative GLC prior to mass spectral analysis.

Analysis of Metabolites. An Varian 1400 series GLC equipped with an electron-capture detector (tritium source) was used throughout the study. Stainless steel columns (10 ft) were packed with 3% SE-30, 3% QF-1, or 3% OV-17 on Gas-Chrom Q, 80–100 mesh, and were run isothermally at 220, 210, and 230 °C, respectively. For preparative GLC, a Varian 1700 series GLC was equipped with a $^1/_4$ in. × 6 ft metal column packed with 10% SE-30 on Gas-Chrom Q, a 10:1 splitter, and a flame ionization detector. Mass spectral analyses were performed on a Hewlitt-Packard Model 5985 GC–MS–computer system. The mass spectrometer was operated at 70 eV. The column used was a 6 ft × 2 mm glass column packed with 3% SE-30 on Gas-Chrom Q, 80–100 mesh. A jet separator connected the GC to the mass spectrometer source. The mass spectrometer was run on the chemical ionization mode, using methane or isobutane as the reactant gas.

RESULTS

Analysis of Soil Residues. Several qualitative and quantitative differences were consistently recognized be-

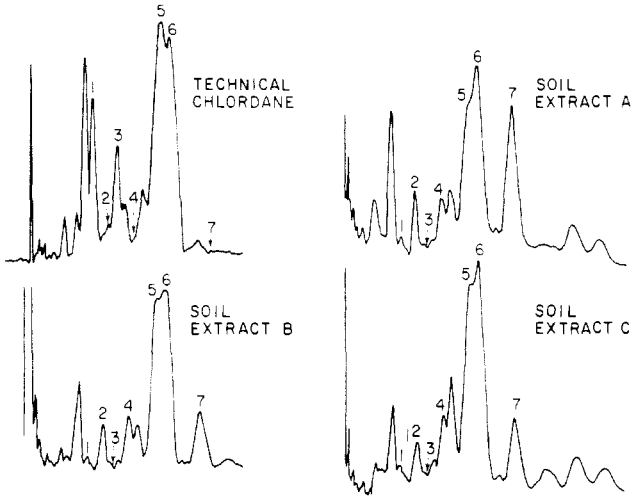

Figure 1. Gas chromatograms of hexane extracts of chlordane-treated soil from Mississippi. Soil plots A–C were treated with a 0.03% formulation of technical chlordane and extracted with *n*-hexane. The hexane extracts were adjusted to the appropriate concentration and analyzed directly on GLC with electron-capture detection. A chromatogram of a hexane solution of technical chlordane is shown for comparison. GLC conditions: 2 mm × 10 ft metal column packed with 3% OV-17; 230 °C, isothermal; 30 mL/min N_2.

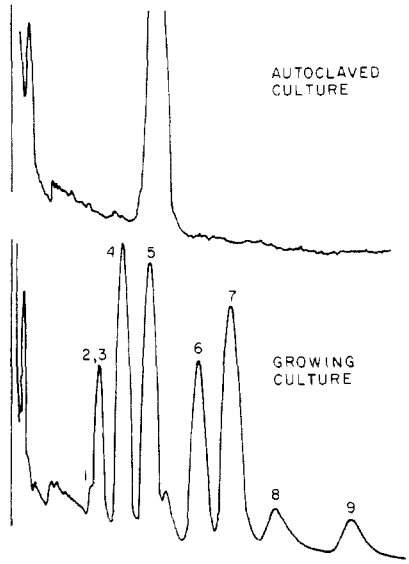

Figure 2. GLC resolution of metabolites of *trans*-chlordane in *Nocardiopsis* cultures. Three-day-old cultures were incubated for 1 week with 2 ppm of *trans*-chlordane. Chloroform extracts of the mycelium were evaporated to dryness under N_2, and the residue was taken up in acetone prior to GLC analysis. See the legend of Figure 1 for GLC conditions.

tween gas chromatograms of technical chlordane and those of hexane extracts of soil which had been treated with 0.03% technical chlordane (Figure 1). Components 1 and 3 of technical chlordane (heptachlor and chlordene, respectively) were not present in the soil extracts. Furthermore, component 5 of technical chlordane disappeared in soil faster than component 6. Components 5 and 6 correspond to *trans*- and *cis*-chlordane, respectively. Extraction efficiencies of technical chlordane components were not calculated. Thus, disappearance of the components could have resulted from binding to soil. However, extracts of soil which had been treated with 1% technical chlordane always gave GLC traces identical with those of technical chlordane itself. In addition, three major peaks (2, 4, and 7, Figure 1) appear in the soil, which were not present in technical chlordane. One of these (component

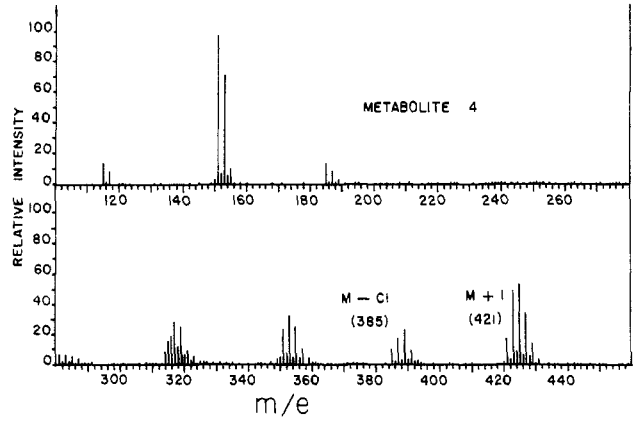

Figure 3. Chemical ionization GC–MS spectrum of *trans*-chlordane metabolite 4. The reactant gas was isobutane.

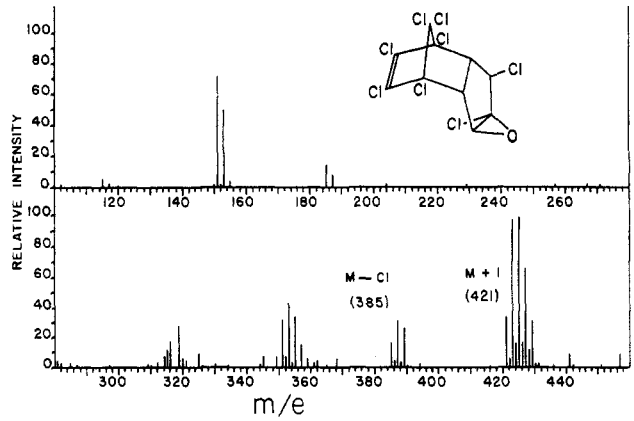

Figure 4. Chemical ionization GC–MS spectrum of authentic oxychlordane. The reactant gas was isobutane.

4) corresponds to oxychlordane in GLC retention time.

Screening of Microbial Isolates. Most of the 40 isolates screened were incapable of catalyzing chlordane breakdown. None of the chemical dump isolates were active. However, two actinomycetes from garden soil metabolized chlordane to several solvent-soluble products with different retention times on GLC. The more active of the two strains was identified as a species of *Nocardiopsis* on the basis of morphologic and biochemical criteria (Meyer, 1976). This species was chosen for further study.

Figure 2 shows a typical gas chromatogram of a solvent extract of *Nocardiopsis* mycelium, in which *trans*-chlordane and eight of its metabolites are resolved. Metabolites 2 and 3 were poorly resolved on GLC, but could be separated on silca gel or aluminum oxide columns. The GLC retention times of these eight metabolites were compared to those of authentic samples of candidate metabolites on SE-30, QF-1, and OV-17 stainless steel columns. On this basis compounds 1, 2, 4, and 6 were tentatively identified as heptachlor, dichlorochlordene, oxychlordane, and chlordene chlorohydrin, respectively.

Spectroscopic Analysis. The major metabolite of *trans*-chlordane (compound 4 in Figure 2) was isolated on TLC plates. Its identity as oxychlordane was confirmed by mass spectroscopy (Figures 3 and 4). Compound 2 in Figure 2 was also analyzed by mass spectroscopy, which confirmed its identity as dichlorochlordene (Figures 5 and 6). Mass spectroscopic confirmations of compounds 1 (heptachlor) and 6 (chlordene chlorohydrin) were not performed.

Compound 3 (nonpolar) and compounds 7, 8, and 9 (polar) did not match any of the authentic standards in

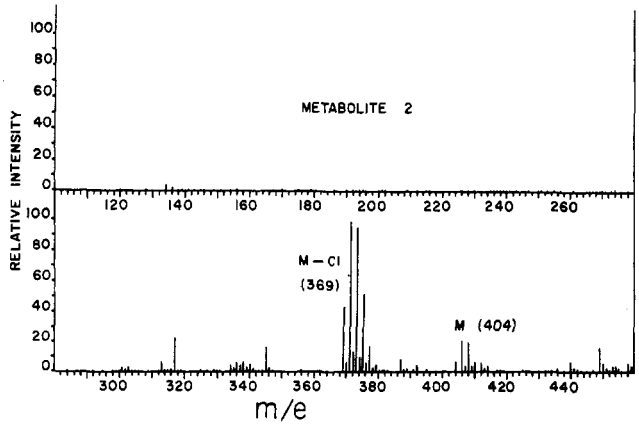

Figure 5. Chemical ionization GC–MS spectrum of *trans*-chlordane metabolite 2. The reactant gas was isobutane.

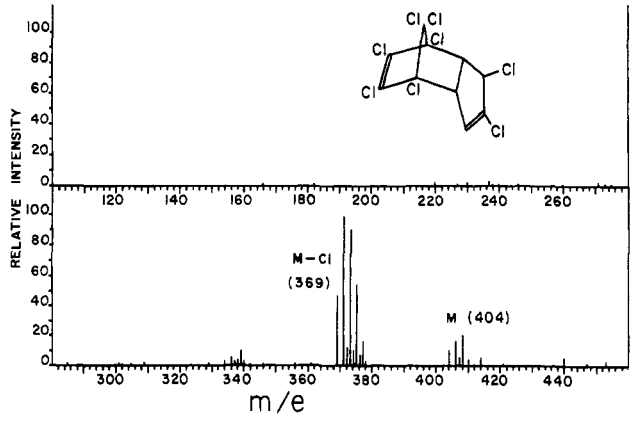

Figure 6. Chemical ionization GC–MS spectrum of dichlorochlordene. The reactant gas was isobutane.

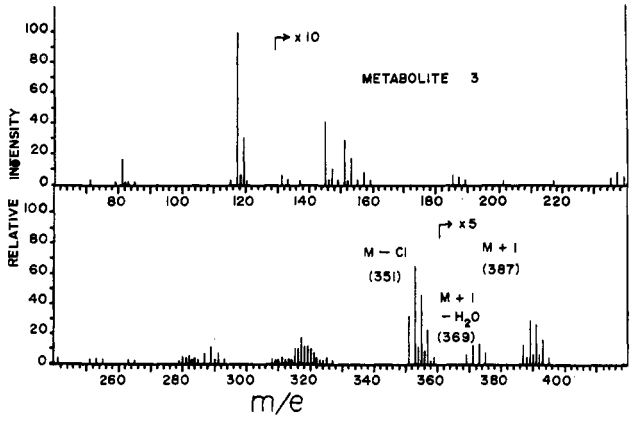

Figure 7. Chemical ionization GC–MS spectrum of *trans*-chlordane metabolite 3. The reactant gas was methane.

GLC retention time. Compound 3 is not an alcohol, since it was readily eluted from from silica gel or aluminum oxide with hexane. Mass spectral analysis of compound 3 revealed a molecular weight of 386 and the presence of seven chlorine atoms and one oxygen atom in the molecule (Figure 7). In all these respects compound 3 resembled heptachlor *exo*-epoxide (Figure 8). Reverse Diels-Alder fragments at m/e 81 (no chlorines) and m/e 117 (one chlorine) were also characteristic of heptachlor *exo*-epoxide. However, the relatively fast elution time of compound 3 on GLC indicated a compound with considerably lower boiling point than heptachlor *exo*-epoxide. On this basis compound 3 was tentatively identified as the unusual endo isomer of heptachlor epoxide. The melting points of the *exo*- and *endo*-epoxides of heptachlor have been reported as 160 and 90 °C, respectively (Brooks and

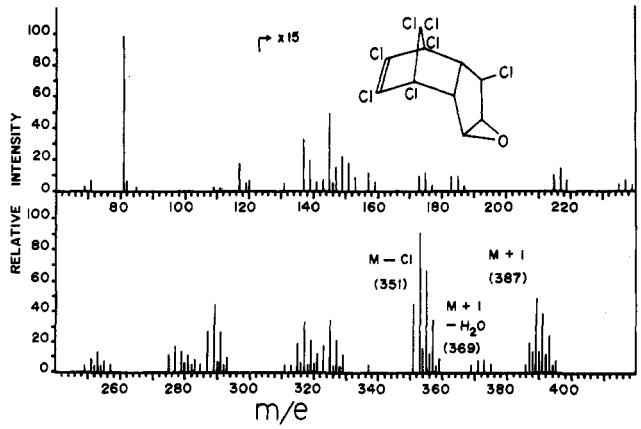

Figure 8. Chemical ionization GC–MS spectrum of heptachlor *exo*-epoxide. The reactant gas was methane.

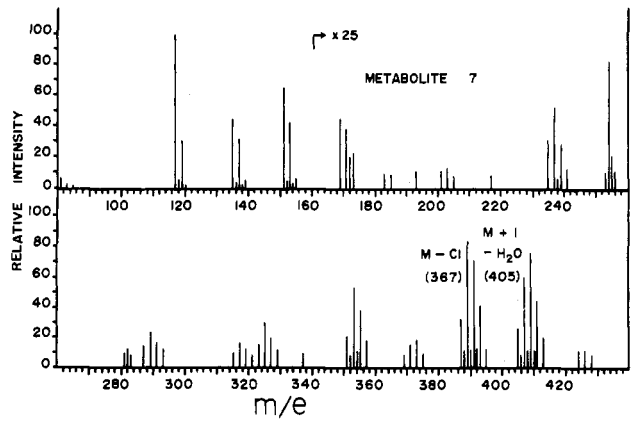

Figure 9. Chemical ionization GC–MS spectrum of *trans*-chlordane metabolite 7. The reactant gas was methane.

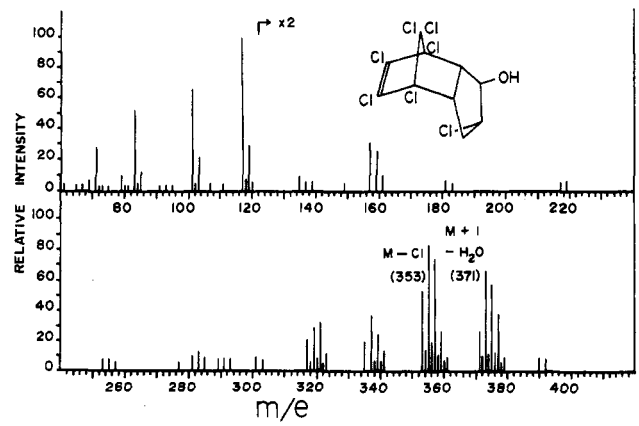

Figure 10. Chemical ionization GC–MS spectrum of chlordene chlorohydrin. The reactant gas was methane.

Harrison, 1965). This is the first evidence of the formation of heptachlor *endo*-epoxide in a biological system.

Mass spectral analysis of compound 7 (Figure 9) revealed a molecular weight of 422 and the presence of eight chlorine atoms and one hydroxyl group, suggesting that this substance was formed by direct hydroxylation of chlordane. Since compound 7 formed only from *trans*-chlordane and not from the cis isomer, it was assumed that the two chlorines on the cyclopentane ring were in the trans configuration. examination of the reverse Diels-Alder fragmentation pattern in the mass spectrum strongly suggested that the hydroxyl group was on the 3-carbon of the cyclopentane ring, probably in the sterically preferred exo configuration. Thus, compound 7 is identified as 3-*exo*-hydroxy-*trans*-chlordane. The mass spectrum of an

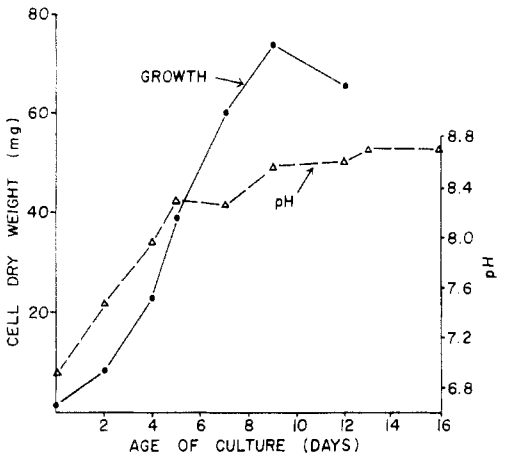

Figure 11. Kinetics of *Nocardiopsis* growth in broth culture. 20-mL portions of sterile broth in 125-mL flasks were inoculated with 0.5-mL aliquots of a 3-day-old culture. After 4 days of growth, 1 ppm of *trans*-chlordane was added to each culture for metabolism studies (see Figure 12). At appropriate time intervals, 13-mL aliquots were removed, the mycelium was separated by filtration and oven-dried to constant weight, and the pH of the filtrate was measured. Data are expressed as mg dry wt/20 mL.

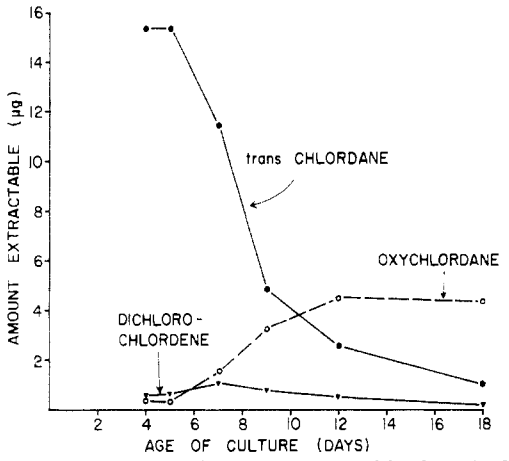

Figure 12. Rate of metabolism of *trans*-chlordane in *Nocardiopsis* cultures. Cultures were treated as described in the legend of Figure 11. At appropriate time intervals, 7-mL aliquots were extracted and analyzed by quantitative GLC.

authentic sample of the closely related alcohol, chlordene chlorohydrin, obtained under identical conditions, is shown in Figure 10. It is evident from a comparison of the chlorine numbers in homologous clusters in Figures 9 and 10 that compound 7 and chlordene chlorohydrin differ by a single chlorine atom on the cyclopentane ring.

Compound 8 was produced in large amounts in cultures treated with chlordene chlorohydrin and in lesser amounts from both *cis*- and *trans*-chlordane. It was more polar than chlordene chlorohydrin and may be a direct hydroxylation product of that substance. No attempt was made to characterize either compound 8 or 9.

Kinetics of Growth and Metabolism. The growth of *Nocardiopsis* was slow (as is typical for actinomycetes) and continued for up to 9 days. The pH of the growth medium gradually increased from 6.9 to 8.7 over a period of 2 weeks (Figure 11).

When chlordane was added to 4-day-old growing cultures of *Nocardiopsis*, a lag period of ~1 day occurred, during which time no metabolic activity was detected. This was followed by the exponential disappearance of chlordane from the mycelium, with a half-time of ~3 days. The disappearance of chlordane from the mycelium was paralleled by the accumulation of oxychlordane. Evidence

Table I. Transformation of *trans*-[^{14}C]Chlordane in Autoclaved and Nonautoclaved *Nocardiopsis* Cultures[a]

TLC band	R_f value	% of recovered ^{14}C	
		autoclaved	nonautoclaved
1	0.0	0.1	18.5
2	0.04	0.1	23.2
3	0.12	0.1	0.3
4	0.24	0.3	0.3
5	0.42	0.3	0.2
6A[b]	0.57	98.5	2.4
6B[b]	0.57	<0.2	49.3
7	0.68	0.2	5.7
8	0.86	0.2	0.1

[a] Four-day-old cultures were incubated for 2 weeks with 0.75 ppm of *trans*-[^{14}C]chlordane. The mycelium was collected, homogenized in 10% methanol, and extracted 3 times with chloroform, the extracts were combined and concentrated, and a portion was spotted on a silica gel TLC plate. The plate was developed 5 times with hexane-chloroform (4:1) and autoradiographed, and the radioactive zones were scraped and quantified by liquid scintillation spectroscopy. [b] Band 6 contained both *trans*-chlordane (6A) and oxychlordane (6B), which were not resolved. The two were separated and quantified by electron-capture gas chromatography using a standard curve.

Table II. Comparison of Rates and Routes of Metabolism of *cis*- and *trans*-Chlordane in *Nocardiopsis*[a]

metabolite	rel amount produced[b]	
	from cis	from trans
1 + 2 + 3	4.4 ± 2.0	7.2 ± 1.7
4	6.3 ± 3.9	17.6 ± 4.2
trans-chlordane		20.2 ± 1.7
cis-chlordane	30.0 ± 4.5	
6	13.8 ± 3.6	13.5 ± 2.2
7	<0.1	20.6 ± 2.6

[a] Four-day-old cultures were incubated for 1 week with 2 ppm of pure *cis*-chlordane or pure *trans*-chlordane. Mycelium was extracted and analyzed by GLC as described in the text. GLC peaks were quantified by area measurement. [b] Values are means ± SD of three determinations each (arbitrary units). See the text for identifications of metabolites.

for the role of dichlorochlordene as an intermediate was provided by the observation that the levels of this substance in the mycelium increased to a maximum after 3 days and declined thereafter (Figure 12). In addition, large amounts of oxychlordane were recovered from cultures incubated for 2 weeks with dichlorochlordene. In cultures incubated for 2 weeks with 0.75 ppm of *trans*-[^{14}C]chlordane, oxychlordane accounted for almost half of the total recovered radioactivity (Table I). Oxychlordane could not be further metabolized by the actinomycete: cultures incubated with 0.15 ppm of [^{14}C]oxychlordane (sp act. 12.1 mCi/mmol) for 2 weeks yielded only unchanged oxychlordane, as determined by TLC–autoradiography of the culture extracts. Chlordane was almost completely metabolized under these conditions, accounting for only 2.4% of the recovered radioactivity. No breakdown of chlordane occurred in autoclaved cultures or in stationary phase cultures similarly incubated (Table I).

There were several differences between *cis*-chlordane and *trans*-chlordane in their rates and routes of metabolism. The overall metabolism of *trans*-chlordane was significantly more rapid than that of the cis isomer (Table II). In particular, oxychlordane formation proceeded from *trans*-chlordane almost 3 times as rapidly as from *cis*-chlordane. 3-Hydroxy-*trans*-chlordane was a major metabolite of *trans*-chlordane, but no evidence of the corre-

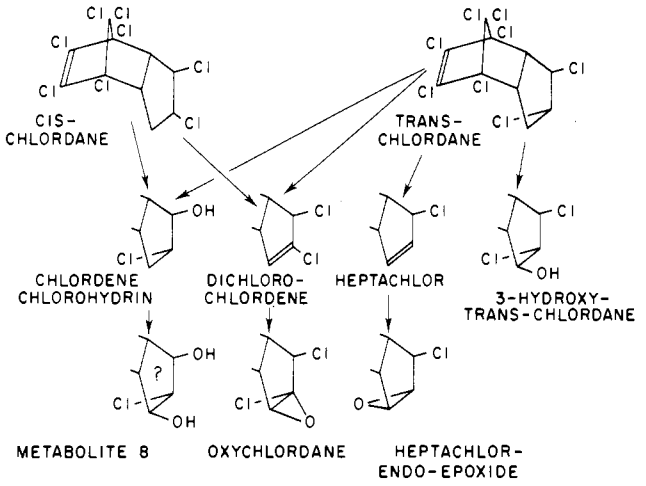

Figure 13. Pathways of chlordane metabolism in *Nocardiopsis*. Identifications were based on GLC coelution with authentic standards (chlordene chlorohydrin and heptachlor), mass spectroscopy (3-hydroxy-*trans*-chlordane and heptachlor *endo*-epoxide), or a combination of both methods (dichlorochlordene and oxychlordane). Metabolite 8 was not identified. Pathways to metabolite 8 and oxychlordane were based on partial reactions.

sponding cis isomer was found in cultures treated with *cis*-chlordane. In contrast, chlordene chlorohydrin formed in equal yield from *cis*- and *trans*-chlordane (Table II). The conversions of *cis*-chlordane to heptachlor and heptachor *endo*-epoxide were not assessed. Figure 13 summarizes the proposed pathways of chlordane metabolism in *Nocardiopsis*.

DISCUSSION

Certain comparisons can be made between pathways of chlordane metabolism in animals and microorganisms. In both mammals (Tashiro and Matsumura, 1977) and insects (Beeman and Matsumura, 1978), it has been shown that oxychlordane is slowly converted to 1-hydroxy-2-chlorochlordene. In contrast, the present work demonstrates the recalcitrance of oxychlordane in a microbial isolate which actively degrades chlordane. *trans*-Chlordane is metabolically more labile than the cis isomer in the microorganism described here. This generalization also holds true for mammals, at least with regard to the pathway to oxychlordane (Street and Blau, 1972).

The brief analysis of chlordane residues in soil presented here represents the first published information on the transformation of technical chlordane in a soil environment. Photolytic, microbial, chemical, and physical forces all contribute to pesticide degradation in the environment. It is unclear how each of these forces contributes to the total "weathering" of chlordane in soil over a period of years. However, the two observations reported here, the appearance of oxychlordane and the more rapid disappearance of *trans*-chlordane compared to the cis isomer, are both consistent with the metabolic capabilities of the soil microorganism *Nocardiopsis*, as determined by the present work.

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