Metabolism of Endrin by Certain Soil Microorganisms

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About 150 isolates from various soil samples were screened to investigate the role of these microorganisms in degrading endrin. Of the total cultures tested, 25 were active in degrading endrin. At least seven metabolites of endrin have been isolated from the mass culture of *Pseudomonas* sp. (#103). The metabolites III, IV, and V were the major ones, while metabolites II and VI were minor. The conversion of endrin into metabolite IV (ketoendrin) was common throughout.

As a result of their extensive use, coupled with relatively high stability, residues of chlorinated cyclodiene insecticides remain in the soil for long periods (*e.g.*, Lichtenstein and Schulz, 1965). In addition, Lichtenstein and Schulz (1960) and Wheeler *et al.* (1967) have shown that these materials may be absorbed by plants and distributed throughout the plant. Edwards (1966), from his pesticide-residual studies, indicated that it takes from 5 to 20 years for 95% of dieldrin to disappear from the soil. Evidence has been accumulating, however, to indicate that certain microorganisms are capable of degrading even such highly persistent insecticides. Matsumura and Boush (1967) have demonstrated that dieldrin can be degraded by soil microorganisms and Matsumura *et al.* (1968) have identified some of the metabolites of dieldrin degradation by soil microorganisms.

Studies on microbial degradation of endrin are scarce, except for the work by Korte and his associates (1967).

The principal objective of the present study was to investigate the insecticide-degrading abilities of several microorganisms isolated from soil.

EXPERIMENTAL

Microorganisms for the present study were isolated from soil samples, as described previously by Matsumura and Boush (1966).

For studying the degradation abilities of these microorganisms, 10 μl of 10^{-3} M C^{14}-labeled endrin (2 mCi/mM) solution in acetone was added to yeast-mannitol cultural media (Fred and Waksman, 1928) in a screw-capped 20 ml test tube with a microsyringe. The labeled endrin was obtained from Shell Chemical Co., Modesto, Calif. The extraction and cleanup method used was similar to that described by Matsumura and Boush (1967).

The solvent extract of the culture to be analyzed was evaporated to dryness over a gentle stream of air. The residue was taken up in 0.25 ml chloroform and approximately $^1/_{10}$ of the sample was spotted, using a glass micropipette, on preactivated silica gel thin-layer plates.

In all preliminary experiments, thin-layer chromatograms were developed until 15 cm from the origin with ether-hexane (9:1) as the mobile phase. Different solvent systems were, however, used for comparing the R_f values of the metabolites with known standard metabolites (Table III). Detection of resolved and radioactive compounds on the chromatogram was accomplished by radioautography using "No screen Medical X-ray Safety-Film" (Eastman Kodak Company, Rochester 4, N.Y.). An exposure period of 30 days was given in all cases except for the cochromatographic comparison tests of condensed samples against reference compounds, where 12 days' exposure was sufficient because of their high radioactivities.

The radioactive regions on the chromatogram corresponding to darkened areas on the film were marked and scraped into 20 ml scintillation vials for direct measurement of resolved radioactivity using a liquid scintillation spectrometer.

The chromatogenic agent for detection of the nonradioactive reference compounds was prepared by dissolving 1 g of silver nitrate in 5 ml of distilled water, adding 10 ml of 2-phenoxyethanol, and making up the volume to 200 ml with acetone. A small drop of 30% hydrogen peroxide was added to the mixture as a preservative. The silver nitrate reagent was stored in a dark brown glass bottle.

For the mass production of endrin metabolites, 1 l. of yeast-mannitol broth (Fred and Waksman, 1928) was prepared and divided into four Erlenmeyer flasks (500 ml) for each of the two cultures. These were then inoculated with microorganisms (#93, #103) under sterile conditions. After 48 hr, when the colony of the organism was established, each of the flasks was inoculated with 10 mg of purified endrin in 1 ml acetone. All the flasks were incubated on a mechanical shaker maintained at 30° C for 30 days.

Each sample was extracted separately to study the specific degrading activity of each microbial isolate. The four flasks of each sample (total 1000 ml) were pooled and extracted twice with equal amounts of chloroform in a 600 ml separatory funnel by shaking vigorously. Sometimes an emulsion was formed during shaking which prevented appropriate separation. This problem was solved by keeping the sample overnight at 0° C, or by dividing the sample into a number of smaller separatory funnels and then extracting them separately. The pooled chloroform phase was dried over anhydrous sodium sulfate (granular) and filtered through Whatman filter paper #2. This was then evaporated to dryness under reduced pressure in a rotary evaporator. A small amount of distilled water was added to the residue, and after shaking with an equal amount of ether in a separatory funnel, the ether phase was saved. The water phase was washed with a second aliquot of ether. The combined ether phase was again dried on anhydrous sodium sulfate, and evaporated to dryness in the manner described above. The residue was picked up in 50 ml of hexane and extracted three times with equal amounts of acetonitrile. The acetonitrile phase was retained and evaporated again to dryness in a rotary evaporator. The residue in the round bottom flask was quantitatively transferred to a small beaker by thoroughly rinsing with 15% ether in *n*-hexane.

Florisil column chromatography was used for further purification and isolation of the metabolites produced. Approximately 75 g of activated (180° C, 1 hr) Florisil (60/100

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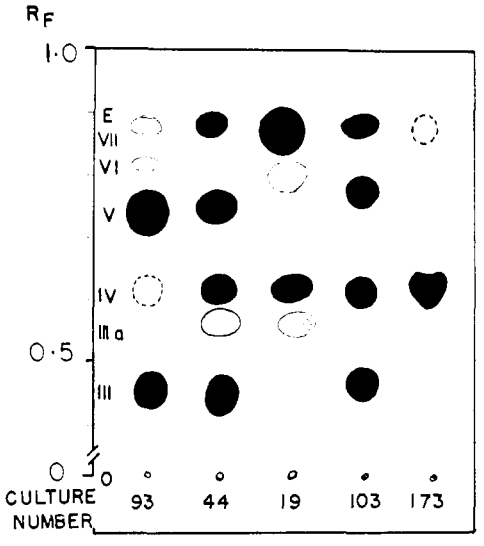

Figure 1. Radioautographic presentation of thin-layer chromatograms of C^{14} endrin and its metabolites produced by cultures of certain microorganisms isolated from soil. Figures on the left 0 to VII designate the spots of the metabolites on the chromatogram, while E is the parent compound endrin remaining unaltered. Spots showing strongest radioactivity are represented by black; medium radioactivity, shaded; weak radioactivity, open circles with solid line; weakest, open circles with dotted lines

Table I. Distribution of Radioactivities Between the Water and Solvent Phase after Incubation of C^{14}-Endrin with Soil Microorganisms

Data are expressed in percentages of applied insecticide actually recovered in each phase

Culture No.	Soil Source	Micro-organisms	% Distribution of Radioactivity: Water phase	Solvent phase
17	Snyder (Wooster, Ohio)	unidentified	76.4	21.6
18	Snyder (Wooster, Ohio)	unidentified	40.5	36.1
19	Snyder (Wooster, Ohio)	*Bacillus* sp.	54.6	72.5
20	Snyder (Wooster, Ohio)	unidentified	32.7	26.3
26	Tope (Fredricksburg, Ohio)	unidentified	11.5	22.9
44	Snyder (Wooster, Ohio)	unidentified	78.6	15.7
48	Snyder (Wooster, Ohio)	unidentified	13.6	63.2
49	Snyder (Wooster, Ohio)	unidentified	22.1	78.2
52	Snyder (Wooster, Ohio)	unidentified	40.5	4.0
53	Snyder (Wooster, Ohio)	unidentified	35.7	31.0
83	Snyder (Wooster, Ohio)	unidentified	19.4	39.7
93	Snyder (Wooster, Ohio)	unidentified	0.2	79.8
95	Snyder (Wooster, Ohio)	*Micrococcus* sp.	36.0	31.1
97	Snyder (Wooster, Ohio)	unidentified	40.0	37.1
98	Snyder (Wooster, Ohio)	unidentified	15.2	82.8
100	Snyder (Wooster, Ohio)	unidentified	0	29.1
103	Shell Chemicals (Denver, Colo.)	*Pseudomonas* sp.	28.3	47.6
115	Farm soil (Lexington, Ky.)	unidentified	3.4	56.5
117	Farm soil (Lexington, Ky.)	*Pseudomonas* sp.	31.1	18.0
118	Farm soil (Lexington, Ky.)	unidentified	43.4	21.4
119	Farm soil (Lexington, Ky.)	unidentified	36.6	22.1
120	Farm soil (Lexington, Ky.)	unidentified	1.4	29.7
136	Farm soil (Lexington, Ky.)	*Pseudomonas* sp.	29.7	22.1
137	Farm soil (Lexington, Ky.)	unidentified	23.4	44.7
173	Farm soil (Norfolk, Va.)	yeast	1.2	19.8

mesh) (Fisher Scientific Co.) was slurried in *n*-hexane and packed in a (3.5 cm × 30 cm) column. The volume of the sample in 15% ether in *n*-hexane was reduced to about 2 ml over nitrogen and was quantitatively transferred to the column by using the same solvent mixture for rinsing. The elution was accomplished by the following solvent sequence: 500 ml of *n*-hexane, 500 ml of 15% ether in *n*-hexane, 600 ml of 100% ether, and 600 ml of acetone. Each of the fractions containing the majority of the metabolites (*i.e.*, 15% ether in *n*-hexane and ether fraction) was evaporated to dryness in a rotary evaporator. Following this, the residue was dissolved in a small amount of ether and further purified by three successive series of thin-layer chromatography by using solvent mixtures of ether and *n*-hexane (2:1), and methylene chloride and CCl_4 (1:1) as mobile phases. All mass spectra were obtained by using a Hitachi-Perkin-Elmer mass spectrometer (RMU-6) at 70 ev of energy under a vacuum of 2×10^{-7} Torr at 200° C.

RESULTS AND DISCUSSION

About 150 isolates from various soil samples were screened to investigate the role of these microorganisms in degrading endrin. The results of extraction of the metabolites from the cultural media represented in Table I for soil microorganisms. In most cases the recovery of added radioactivity (*i.e.*, the combined figures for the solvent and the water phases, Table I) was satisfactory.

Of the total cultures tested, 25 appeared to be active in degrading endrin. The most promising ones were #44, 93, 95, 97, 98, 103, 115, and 173. In addition, all dieldrin-degrading microbes earlier reported to be highly active in degrading dieldrin by Matsumura and Boush (1967) were found to be also active in degrading endrin (Patil *et al.*, 1970).

The percentage distribution of radioactivity among endrin and its solvent-extractable metabolites has been presented in Table II. The corresponding R_f values for those metabolites to various tlc systems have been summarized in Table III. It can be seen from the tables that metabolites III, IV, and V were the major breakdown products of endrin in most cases. This is also evident from the various autoradiographs presented here (Figure 1). Cultures #44, 93, and 95 yielded as high as 20.6, 36.5, and 32.3% of metabolite III alone. Cultures #20, 44, 53, and 103 produced 19.3, 19.5, 29.4, and 46.3% of metabolite IV, while cultures #26, 44, 93, 95, and 97 yielded 44.9, 32.2, 38.0, 40.3, and 35.1% as metabolite V, respectively. Cultures #20 and 53 appeared to be active in producing metabolite IIIa in addition to others. In some cases the endrin remaining unaltered was below 50% (*e.g.*, 5.8% for #93).

The pattern of degradation, as evidenced from the autoradiographic presentation (Fig. 1), appeared to be fairly uniform throughout. Culture #173 (yeast) exclusively produced metabolite IV only.

Two of the most promising cultures as selected from the radioautographs were utilized for the mass production and subsequent purification of the endrin metabolites. These were #93 and #103.

According to the data shown in Table II, metabolites IIIa, VI, and VII were the minor compounds observed. These did not match with any of the known metabolites of endrin. All the metabolites were, however, produced by culture #103 (*Pseudomonas* sp.).

The metabolites I and VII isolated from #103 could not be purified completely due to interfering fluorescent impurities. The fluorescent contaminant actually did not respond to the chromogenic reagent used for detecting the metabolites.

In none of the cases could complete crystallization be

achieved, and hence chemical characteristics such as melting point, etc., could not be measured.

At least seven breakdown products of endrin have been isolated in sufficiently clean form (Figure 2) from mass culture experiment of culture #103, mainly by thin-layer chromatography and recrystallization. Culture #93 yielded qualitatively similar metabolites, but the amounts of these metabolites were relatively small. Metabolites III, IV, and V were the major metabolic products. Metabolite IV was most abundant and consistent throughout (Table II). This metabolite, which was also abundant in the metabolic product from culture #173, was found to behave chromatographically identical to "keto-endrin" (or sometimes referred to as δ-keto-1,5,3) thermal isomerization product of endrin (Phillips *et al.*, 1962) in all the solvent systems tested. The R_f value of this compound was 0.64 in ether/hexane (9:1) solvent system.

The metabolites IIIa and VI were minor metabolites in most cases, but did not match chromatographically with any of the thermal products of endrin tested. The thermal products of endrin for this comparison were obtained by thermal isomerization of pure endrin in gas-liquid chromatogram at 230° C using a Beckman GC-4 gas chromatograph system with a 6 ft column of 5% SE52 on Chromosorb W.

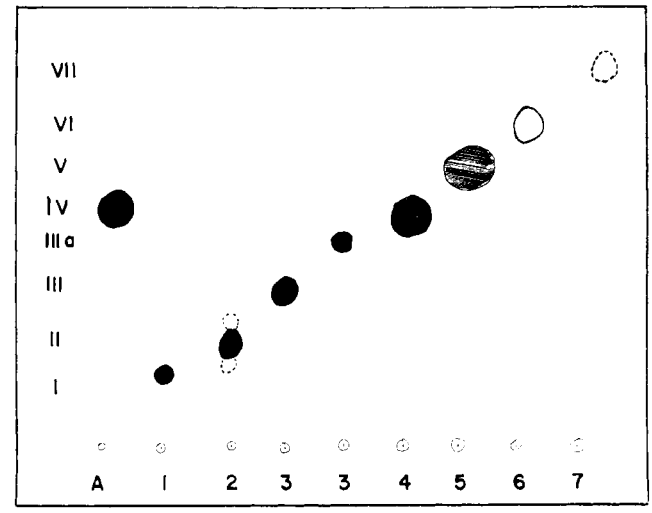

Figure 2. Purification of endrin metabolites. The chromatogram represents the final purification of endrin metabolites (#103) on a thin-layer chromatogram. From left to right: keto-endrin, metabolite 0, 1, 2, 3, 4, 5, 6, and 7, respectively. The broken lines indicate visible impurities by the silver nitrate detection technique. Mobile phase: ether-hexane (2 : 1)

Table II. Percent Distribution of Endrin and Its Solvent-Extractable Metabolites Due to Microorganisms

Based on Radioactivity

Culture No.	Metabolite						
	0	III	IIIa	IV	V	VI	Endrin
17	2.0	...	2.7	3.4	...	...	91.9
18	4.6	...	4.2	3.2	6.7	...	81.3
19	2.0	...	2.1	4.6	...	...	91.3
20	1.4	10.4	27.9	19.3	...	...	41.0
26	4.9	1.9	...	3.4	44.9	...	44.9
44	2.2	20.6	5.9	19.5	32.2	...	19.6
48	1.1	...	2.5	3.3	...	...	93.1
49	3.2	...	2.7	3.3	...	...	90.8
52	2.1	...	...	16.0	...	...	81.9
53	0.5	13.3	19.0	29.4	...	...	37.8
83	1.2	...	...	...	...	18.5	80.3
93	0.7	36.5	...	9.5	38.0	9.5	5.8
95	1.1	32.3	...	7.6	40.3	...	18.7
97	0.6	12.5	...	10.8	35.1	...	41.0
98	3.6	6.8	...	9.4	15.3	...	64.9
100	0.6	...	...	2.3	...	...	97.1
103	0.5	13.0	trace	46.3	16.2	9.0	15.0
115	0.6	...	...	3.1	...	...	96.3
117	1.4	...	...	16.1	...	...	82.5
118	1.4	...	...	11.0	...	...	87.6
119	0.8	...	...	5.8	...	...	93.4
120	2.1	...	...	9.6	...	...	88.3
136	1.9	...	...	9.8	10.1	...	78.2
137	1.3	5.8	...	20.2	14.8	...	57.9
173	4.1	...	...	80.6	...	...	15.3

Table III. R_f Values[a] of Endrin and Its Metabolites as Compared with Standards in Different Solvents

Mobile Phase	Metabolite and R_f Values						Reference Compounds		
	III	IIIa	IV	V	VI	VII	Endrin	Keto-endrin	Photo-dieldrin
Ether/Hexane (1:1)	0.19	...	0.36	0.45	0.56	0.71	0.91	0.36	0.46
Hexane/Acetone (4:1)	0.33	0.39	0.46	...	0.59	0.65	0.83	0.46	0.46
Benzene/Ethyl Acetone (3:1)	0.63	...	0.76	...	0.85	...	0.93	0.80	0.83
Cyclohexanol Acetone (3:1)	0.25	0.35	0.39	...	0.54	0.62	0.78	0.40	0.50
Methylene chloride/Carbon-tetrachloride (1:1)	0.39	...	0.53	...	0.61	...	0.77	0.52	0.49

[a] The stationary phase in all cases was a thin-layer (0.25 mm) of silica gel HF.

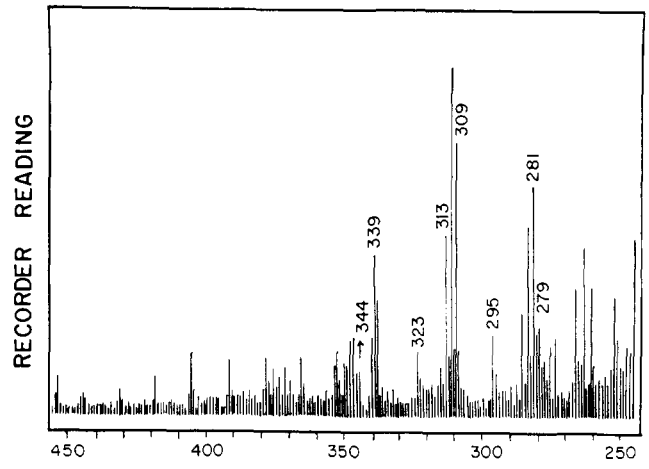

Figure 3. Mass spectrum of metabolite V from endrin

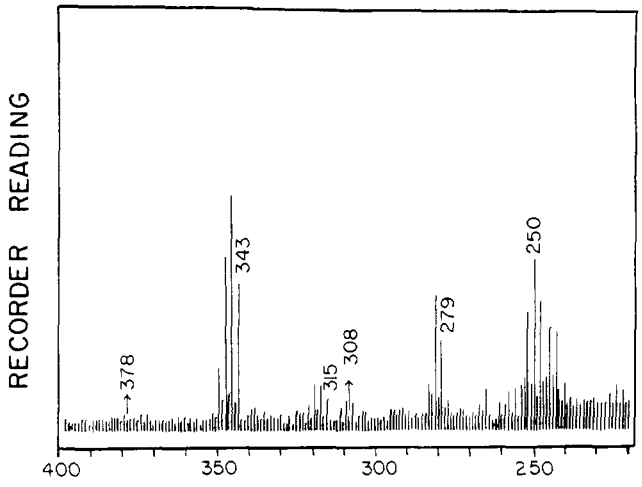

Figure 4. Mass spectrum of metabolite II from endrin

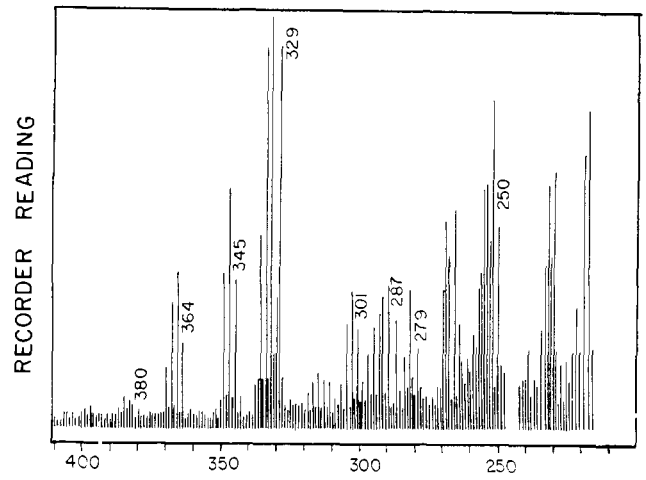

Figure 5. Mass spectrum of metabolite III from endrin

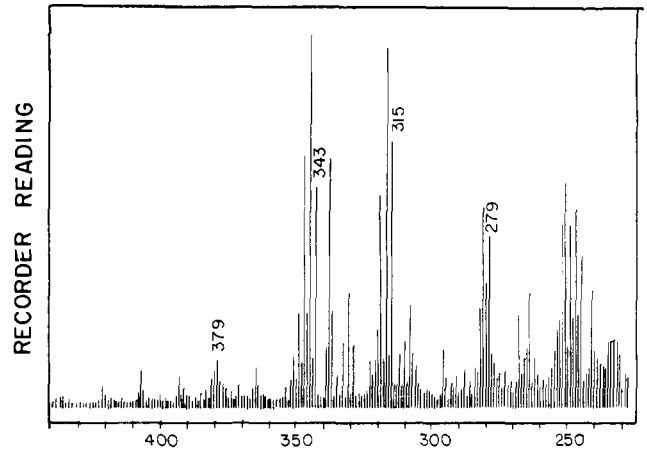

Figure 6. Mass spectrum of metabolite VI from endrin

Spectroscopic Analyses of Endrin Metabolites. The mass spectrum of the metabolite V showed a prominent peak group of m/e = 309 (Figure 3). This corresponds to the mass number of endrin minus two chlorine atoms plus one hydrogen atom (all peaks expressed as Cl = 35, the number of chlorine atoms judged by the isotope ratio). The presence of a trace of a peak group at 344 (Cl = 35) indicates that the actual molecular ion is with five chlorines rather than four chlorines. The infrared spectrum of this compound, though it was insufficient to judge in detail, indicated the presence of carbonyl group at 1720 cm^{-1}. Also noticed was the absence of —ClC=Cl— (6.3 μ) and the epoxy (11.8 μ) peaks. In general, the total spectrum of this compound was almost identical to the one obtained for aldehyde of endrin studied by Phillips *et al.* (1962).

Metabolite II with a prominent peak group at 279 (Figure 4) showed a similar fragmentation pattern as V, except that the apparent parent peak group was at 343 (five chlorines), indicating that it is a similar aldehyde as V with an extra chlorine atom (*i.e.*, actual parent mass for II is 378). The infrared spectrum was again almost identical to the one for the endrin aldehyde (Phillips *et al.*, 1962) with a strong peak representing a carbonyl group at 1720 cm^{-1}.

The mass and infrared spectrum of metabolite IV matched exactly with that of authentic "keto-endrin." Metabolite III (Figure 5) appeared to be another ketone except that it had an apparent parent mass of 364. The infrared spectra of metabolite III showed a peak at 1580 cm^{-1} (6.3 μ), indicating the presence of an intact —ClC=CCl—. In view of the presence of a carbonyl peak at 1745 cm^{-1} and its overall similarity to the infrared spectrum of keto-endrin, this metabolite's chemical structure was estimated to be a nonbridged ketone. No further attempt was made to identify this compound.

The mass spectrum of metabolite VI (Figure 6) indicated that the apparent parent peak, 343, contained five chlorine atoms. This must mean that the true parent mass is 378 with six chlorine atoms. The pattern of fragmentation of this compound closely resembled that of keto-endrin. The infrared spectrum of this compound indicated a peak due to C = 0 at 1740 cm^{-1}. In view of the scarcity of this compound as the microbial metabolic product, no further test was conducted to identify it.

On the basis of these spectroscopic analyses the conclusion can be made that the majority of the microbial metabolites of endrin are ketones and aldehydes with five to six chlorine atoms. Only metabolite IV is identified positively, since this metabolite matched well with all the descriptions of the ketone derivative of endrin described by Phillips *et al.* (1962). The point of dechlorination to produce metabolite V could not be determined by this method.

In studying the mass spectra of the metabolites only the spectra of high mass regions were examined (*e.g.*, 250 to 400

m/e). Because of the use of the high degrees of amplification to obtain the spectra from small quantities of samples, some of the peaks in the lower spectral regions (*e.g.*, 40–100 m/e) became too high to measure their peak heights accurately. Such treatments also produced somewhat high background noise levels which had to be corrected by the use of background reading. Nevertheless, the use of the high region had an advantage in that peak groups created by the presence of naturally occurring isotopes of chlorine would indicate the number of chlorines present in each fragment (Silverstein and Bassler, 1964). In addition, only the pattern of initial fragmentation should be the important criterion here, since at lower mass fields the majority of cyclodiene insecticides merely show the spectra resulting from a set of retro-Diels-Alder decomposition reactions (Damico *et al.*, 1968) which gives similar patterns for the majority of endrin metabolites with the same carbon-chlorine skeleton.

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