Identification of Metabolites of Endrin. Metabolism in Endrin Susceptible and Resistant Strains of Pine Mice

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Oral dosage of endrin resistant and susceptible pine mice with [^{14}C]endrin resulted in the production of one fecal (F-1) and up to three urinary (U-1, U-2, and U-3) metabolites. A single product (L-1) was detected in in vitro metabolism of endrin by hepatic microsomes. Metabolites F-1, U-2, and L-1 were identified as *anti*-12-hydroxyendrin, a compound produced and excreted to a greater extent by the resistant as compared to the susceptible strain. Tentative structural assignments for U-1 and U-3 were necessitated because of limited sample availability. It is suggested that U-1 is one of four possible tertiary alcohols of endrin, and U-3 is one of two possible cyclic hemiketals.

Resistance of pine mice, *Microtus pitymus pinetorum*, to the lethal effects of endrin (LD_{50}: 18.97 vs. 2.56 mg/kg) was the first reported development of resistance to a chlorinated cyclodiene pesticide by mammals in their natural habitat (Webb and Horsfall, 1967). These animals, therefore, represented a unique population well suited for investigation of mechanisms of mammalian resistance to cyclodienes. Studies designed to characterize this resistance have been carried out by this laboratory. Inheritable resistance characteristics as well as cross-resistance to dieldrin have been reported (Webb et al., 1973). Microsomal preparations from the resistant strain hydroxylate benzopyrene in vitro more rapidly than susceptible hepatic microsomal preparations (Webb et al., 1972). A more rapid development of this capacity in R vs. S neonates has also been observed (Hartgrove and Webb, 1973). Most recently, both quantitative and qualitative differences in the in vivo metabolism of endrin by these two strains of pine mice have been reported (Petrella et al., 1975; Petrella and Webb, 1973). The latter studies reported that a major fecal and urinary metabolite, F-1 and U-2, respectively, is produced and excreted by the resistant strain to a twofold greater extent than by the susceptible strain. Differences in rates of in vitro conversion of endrin to a major metabolite, L-1, by hepatic microsomes prepared from resistant and susceptible animals were also observed. Gas–liquid chromatography of the metabolites (F-1, U-2, and L-1) confirmed that these three compounds were identical. One minor urinary metabolite was excreted by both strains in equal amount, while a third urinary metabolite (U-3) was unique to the susceptible strain. Identities of metabolic products of endrin recovered from rat have recently been established (Baldwin et al., 1970; Bedford and Harrod, 1973; Bedford et al., 1975). Endrin metabolites isolated from resistant and susceptible strains of pine mice were structurally analyzed so that the relationship of structure to observed toxicity/excretion differentials for the two strains might be compared.

MATERIALS AND METHODS

[^{14}C]Endrin (>98%) was a gift of Shell Oil Co., San Ramon, Calif. Animal procurement, housing, and LD_{50} determinations followed previously reported protocols (Webb et al., 1973). Fecal and urinary analysis of endrin and metabolites followed published procedures (Baldwin et al., 1970).

Gas Chromatographic–Mass Spectral Analysis. Samples of metabolites were subjected to gas–liquid chromatography on 3.8% SE-30 (80/100 mesh acid-washed Chromosorb W) and 1% OV-225 (80/100 mesh Supelcoport) using a Microtek MT220 gas chromatograph under conditions listed in the footnotes of Table I.

Mass spectra of metabolites were obtained by GC–MS analysis on a Finnegan 9500 gas chromatograph interfaced with a Finnegan Model 1015C Quadrupole mass spectrometer using chemical ionization techniques. Either methane or isobutane was used as the carrier and reagent gas. A System Industries 150 data acquisition system was utilized for GC–MS data collection and analysis.

Oxidation Studies. A saturated solution of chromium trioxide in pyridine was prepared with spectral grade pyridine dried over potassium hydroxide. Samples, 0.1 ml in either benzene or acetone, were added to 0.7 ml of CrO_3 in pyridine. The mixture was heated for 60 min at 100 °C in a 50-ml glass-stoppered centrifuge tube. After cooling to room temperature 2.0 ml of H_2O was added to the mixture which was extracted 3 times with 5 ml of anhydrous diethyl ether. The ether extract was washed with 1 vol of dilute (20%) HCl and 1 vol of Na_2CO_3-saturated H_2O. The ether extract was dried with Na_2SO_4 and evaporated to dryness under N_2. The residue was dissolved in hexane for GC analysis.

Infrared Analysis. Infrared spectra of the purified major metabolite and its oxidation product were obtained by preparation of 1 × 7 mm KBr pellets of approximately 200-μg samples ground into 10 mg of KBr powder and recorded using a Beckman IR-5A. KBr was dried at 110 °C. Samples were stored over $CaSO_4$ in vacuo.

RESULTS

Chemical Ionization–Mass Spectrometry. Chemical ionization mass spectrometry, CI–MS, results in formation of ion fragments which are more stable than produced by electron impact mass spectrometry, EI–MS. CI–MS is less disruptive if gases of increased molecular weight and lower ionization potential than methane are used as the carrier and reagent gas. Fragmentation patterns of the major endrin metabolites using isobutane and methane are compared in Figure 1. In the top spectrum, methane was used as carrier/reagent gas; in the bottom spectrum isobutane was employed. Fragmentation of unstable ions produced by endrin derivatives is inhibited by use of isobutane. Chemical ionization mass spectra of endrin (1) (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-*endo*,*endo*-5,8-dimethanonaphthalene),

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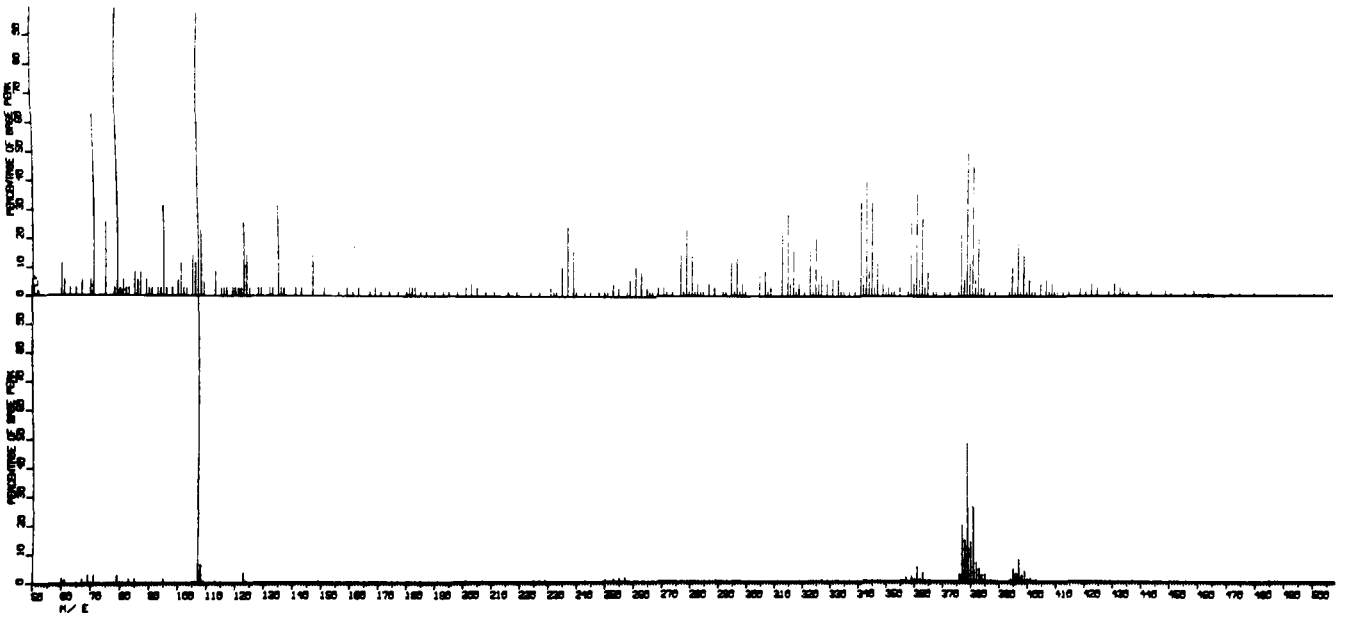

Figure 1. CI–MS spectra of the major metabolite of endrin (F-1, U-2, L-1), with methane (top) and isobutane (bottom) as the carrier and reagent gas.

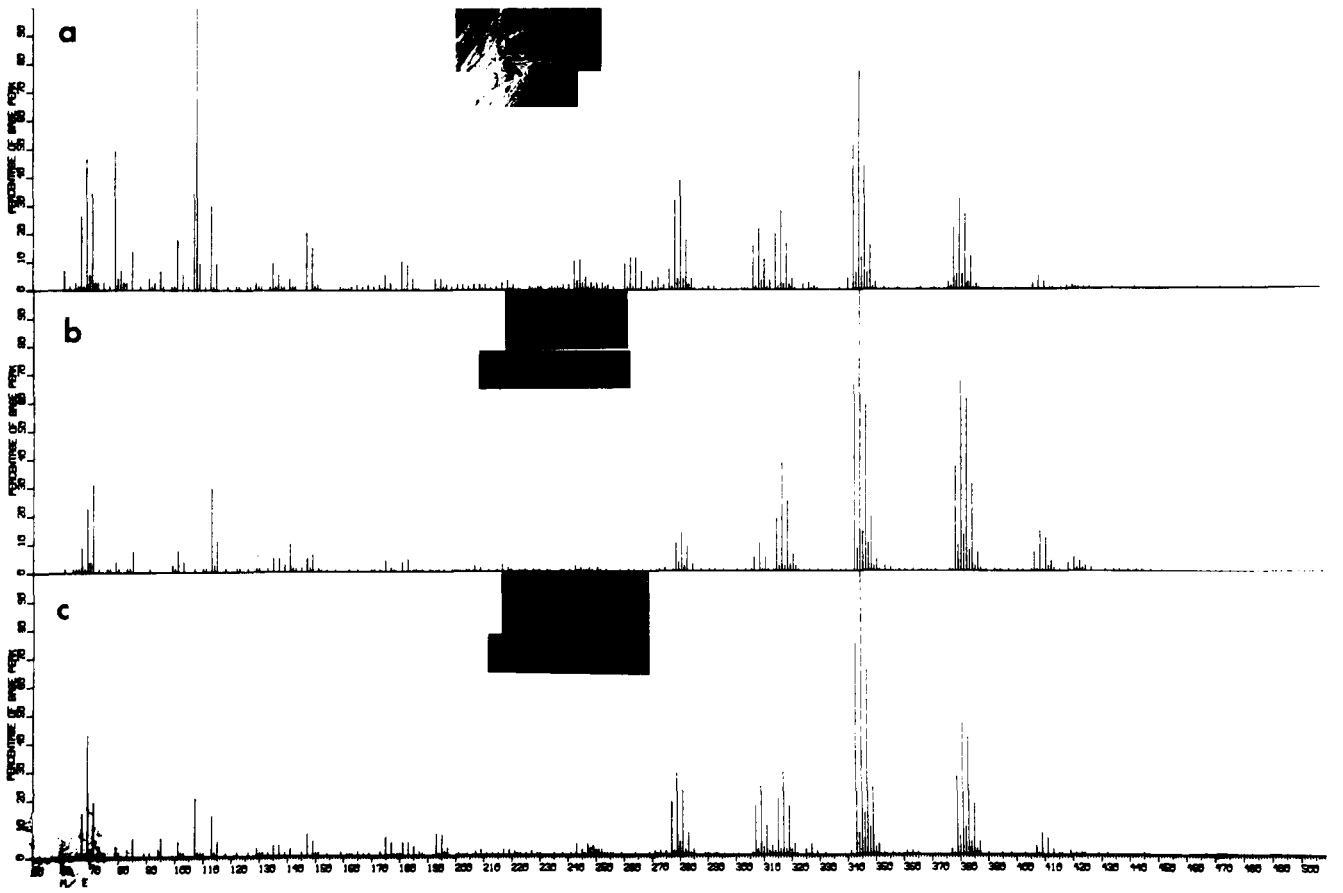

Figure 2. CI–MS spectra using methane as the carrier and reagent gas for endrin (1) (a), Δ-ketoendrin (3) (b), and endrin aldehyde (2) (c).

endrin aldehyde (**2**) (2,3,3,4,5,6-hexachloropentacyclo[6.-3.0.$0^{2,6}$.$0^{5,7}$.$0^{4,11}$]undecanyl-10-carboxaldehyde), and Δ-ketoendrin (**3**) (1,8-*exo*-9,10,11,11-hexachloropentacyclo-[6.2.1.$1^{3,6}$.$0^{2,7}$.$0^{4,10}$]dodecan-5-one) using methane as a carrier gas are shown in Figure 2.

Significant features of the methane CI–MS spectrum of the major metabolite were a base peak (BP) of 107 which may be attributed to a retro-Diels–Alder (RDA) process (Figure 3), resulting in a fragment or its equivalent, **4**, as shown in Figure 4. RDA decomposition indicates that the molecule is "uncaged" in structure (a caged structure is common to several cyclodiene breakdown products). The quasi-molecular ion $(M + 1)^+$ of m/e 395 corresponds to a molecule containing six chlorine atoms which has a mass of 394 based on ^{35}Cl. This is consistent with a molecular formula of $C_{12}H_8O_2Cl_6$. Also present in the spectrum are

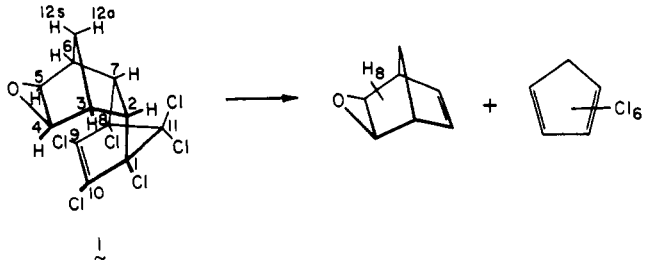

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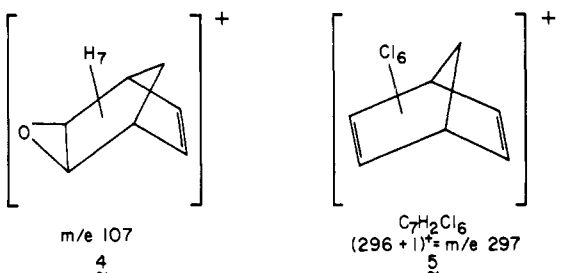
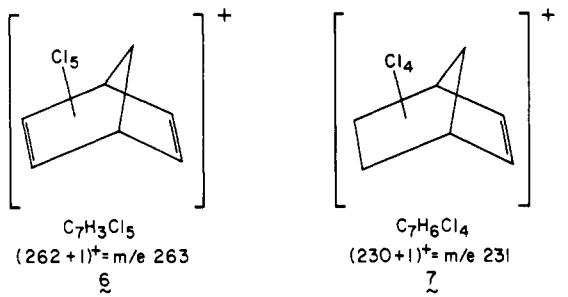
Figure 3. Chemical structures of products of the retro-Diels–Alder decomposition of endrin (1).



m/e 107
4

$C_7H_2Cl_6$
$(296+1)^+$ = m/e 297
5



$C_7H_3Cl_5$
$(262+1)^+$ = m/e 263
6

$C_7H_6Cl_4$
$(230+1)^+$ = m/e 231
7

Figure 4. Chemical structures of ions produced by fragmentation of an uncaged endrin derivative upon ionization, successive chlorine loss, and ring saturation.

fragments $(MH - H_2O)^+$, *m/e* 377, $(MH - HCl)^+$, *m/e* 359, and $(BP + O)^+$, *m/e* 123. A recombinant (ions of reagent gas combined with sample fragments) of *m/e* 405 corresponds to $(M + C_2H_5 - H_2O)^+$.

The bottom of Figure 1 shows the CI–MS spectrum of the major endrin metabolite using isobutane as carrier/reagent gas. The mildness of the ionization process is evident in the simplicity of the spectrum. The salient features are peaks corresponding to BP, *m/e* 107; $(M + 1)^+$, *m/e* 395; $(MH - H_2O)^+$, *m/e* 377; $(MH - HCl)^+$, *m/e* 359; and the $(BP + O)^+$, *m/e* 123. These data confirm the CI–MS methane data.

Due to limited sample quantity, the CI–MS/methane spectrum obtained for U-1 was very weak. A strong *m/e* 107 peak was present. Discernible ions at *m/e* 397 and 399 were confirmed using a computer limited mass search. These could be part of an $(M + 1)^+$ pattern consistent with the loss of chlorine to generate an observed peak at *m/e* 359. Also present was a fragment of *m/e* 123 which may correspond to a fragment of *m/e* 107 + oxygen.

U-1 yielded peaks at *m/e* 297, **5**, 263, **6**, and 231, **7**, which would result from fragmentation producing a hexachloronorbornadiene (Figure 4). The fragment of *m/e* 297, **5**, may lose chlorine successively with ring saturation yielding *m/e* 263, **6**, and 231, **7** (Figure 4). The presence of these fragments is consistent with the chlorinated portion of endrin remaining unchanged in the metabolite

Table I. Relative Retention Volumes of Endrin, Δ-Ketoendrin, Endrin Aldehyde, and Endrin Metabolites

Compound	Rel retention vol for stationary phases	
	SE-30[a]	OV-225[b]
Endrin	1.00[c]	1.00[d]
Δ-Ketoendrin	1.84	5.65
Endrin aldehyde	1.22	3.44
F-1, U-2, L-1	2.57	7.04
U-1	1.76	5.79
U-3	1.49	4.70
Oxidation product of F-1	1.16	2.25

[a] 3.8% SE-30 on 80/100 mesh acid-washed Chromosorb W; glass column 92 cm × 3.2 mm i.d., operated at 190 °C; inlet 225 °C, ^{63}Ni detector 300 °C, N_2 flow rate 120 ml/min at 40 psi. [b] 1% OV-225 on 80/100 mesh Supelcoport; glass column 92 cm × 3.2 mm i.d., operated at 190 °C; inlet 225 °C, ^{63}Ni detector 300 °C, N_2 flow rate 120 ml/min at 40 psi. [c] t_R (endrin) = 5.38 min. [d] t_R (endrin) = 4.50 min.

U-1, implying that if an OH were present, it would have to reside on the nonchlorinated portion of the molecule.

The CI–MS/methane spectrum of U-3 (not shown) was also very weak due to extremely small sample availability. Consequently, interpretation can be considered slightly more than speculation. Several features of the mass spectrum were of value in making a tentative assignment of the structure. Using either isobutane or methane for CI–MS, a peak corresponding to BP, *m/e* 107, was detected. The CI–MS/isobutane spectrum contained a peak at *m/e* 377 which would be consistent with $(MH - H_2O)^+$, *m/e* 394 − 17. It is apparent that the parent ion contains all of its chlorines. The less stable CI–MS/methane spectrum contained few fragments in the higher *m/e* range. However, a prominent peak at *m/e* 167 was detected which may result from a rapid decomposition with successive chlorine loss from the hexachloronorbornadiene moiety. No loss of CO was observed which would have implied the presence of a ketone.

In summary, U-1 is an uncaged endrin system containing all of its chlorines exhibiting *m/e* fragments which would result from loss of chlorine from an endrin–alcohol parent ion. U-3 is an unbridged endrin system, which is not a ketone but may be a very unstable alcohol.

Oxidation Studies. Oxidation of the three isolated metabolites of endrin was carried out as described in the Materials and Methods section. Oxidation of the major metabolite (F-1, U-2, L-1) in CrO_3/pyridine resulted in complete conversion to a single product as determined by gas chromatography utilizing two different column packings with either electron capture or flame ionization detectors (Table I). Recovery of ^{14}C from the reaction mixture using the extraction procedure described was greater than 77%. Gas chromatography of the sample recovered from CrO_3/pyridine oxidation of U-1 indicated that only U-1 was present in the extract.

Oxidation of U-3 resulted in only 40% recovery of ^{14}C label from the reaction mixture in the ether phase. Oxidation products of U-3 were soluble in the alkali wash (20.3%). Acidification of the CrO_3/pyridine mixture followed by extraction with ether resulted in recovery of only 1% ^{14}C. Upon acidification of the Na_2CO_3 wash and extraction with ether, 12.5% of ^{14}C was recovered in the ether phase.

Infrared Analysis. Shown in Figures 5 and 6 are infrared spectra of the major endrin metabolite U-2, its CrO_3/pyridine oxidation product, endrin (1), endrin "birdcage" alcohol (8) (3,4,4,6,9,10-hexachlorohexacyclo-

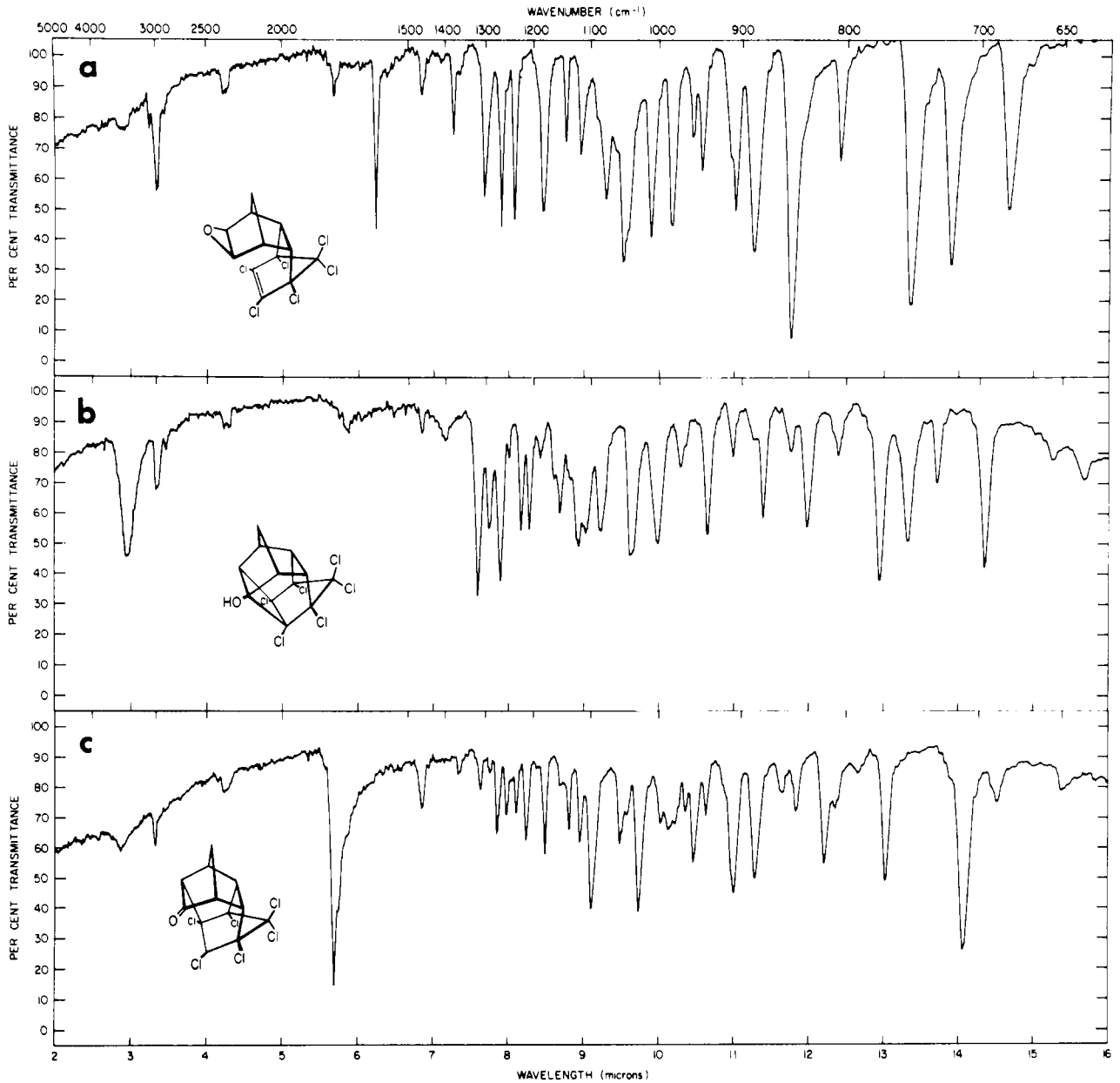


Figure 5. Infrared spectra of endrin (1) (a), endrin "birdcage" alcohol (8) (b), and Δ-ketoendrin (3) (c).

[5.4.1.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$.$0^{8,11}$]dodecan-8-ol), and Δ-ketoendrin (3). The spectrum of U-2 shows a broad absorbance band with its center at 2.9 μm, strongly suggesting the presence of an OH group. Also present are absorption bands at 3.4 μm (C–H stretch), 6.22 μm (C=C stretch), and 11.85 μm (1,2-epoxide). The band at 2.9 μm corresponding to OH stretching is absent in the spectrum of the oxidation product while a new absorption at 5.57 μm corresponding to a C=O moiety is present. Absorption bands at 3.4 μm (C–H stretch) and 6.25 μm (C=C stretch) are also apparent.

Gas Chromatography. Retention volumes of metabolites relative to endrin are summarized in Table I.

DISCUSSION

Structures of endrin metabolites may be assigned from oxidation studies and the IR and mass spectra. A molecular ion of m/e 394 based on ^{35}Cl present in the CI–MS spectrum of the major metabolite, U-2, using either methane or isobutane as carrier/reagent gas is consistent with a molecular formula of $C_{12}H_8O_2Cl$, i.e., endrin plus one oxygen. The major metabolite undergoes a retro-Diels–Alder decomposition during CI–MS eliminating the possibility of a caged rearrangement derivative. The presence of an OH moiety is apparent in the IR spectrum along with the C=C and 1,2-epoxide functional groups. Based on infrared and mass spectra there are only seven carbon atoms upon which an OH group can reside. If the hydroxyl group were substituted at C-2, -3, -6, or -7 the metabolite would be a tertiary alcohol. If the hydroxyl group were substituted at C-12, the metabolite would be a secondary alcohol. If the hydroxyl group were substituted at C-4 or -5, the metabolite would be a cyclic hemiketal, 9 (Figure 7). Chromium trioxide saturated pyridine is a mild oxidizing reagent which reacts with primary and secondary alcohols but not with tertiary alcohols. Oxidation of a cyclic hemiketal would form a mixture of products. A single product, 12-ketoendrin (10) (Figure 7), was isolated from the reaction mixture upon oxidation of U-2. IR analysis of this product indicated the disappearance of the hydroxyl group and appearance of a carbonyl function. Accordingly, the major metabolite (F-1, U-2, L-1) of endrin in both strains of pine mice is 12-hydroxyendrin. Two orientations of the hydroxyl group

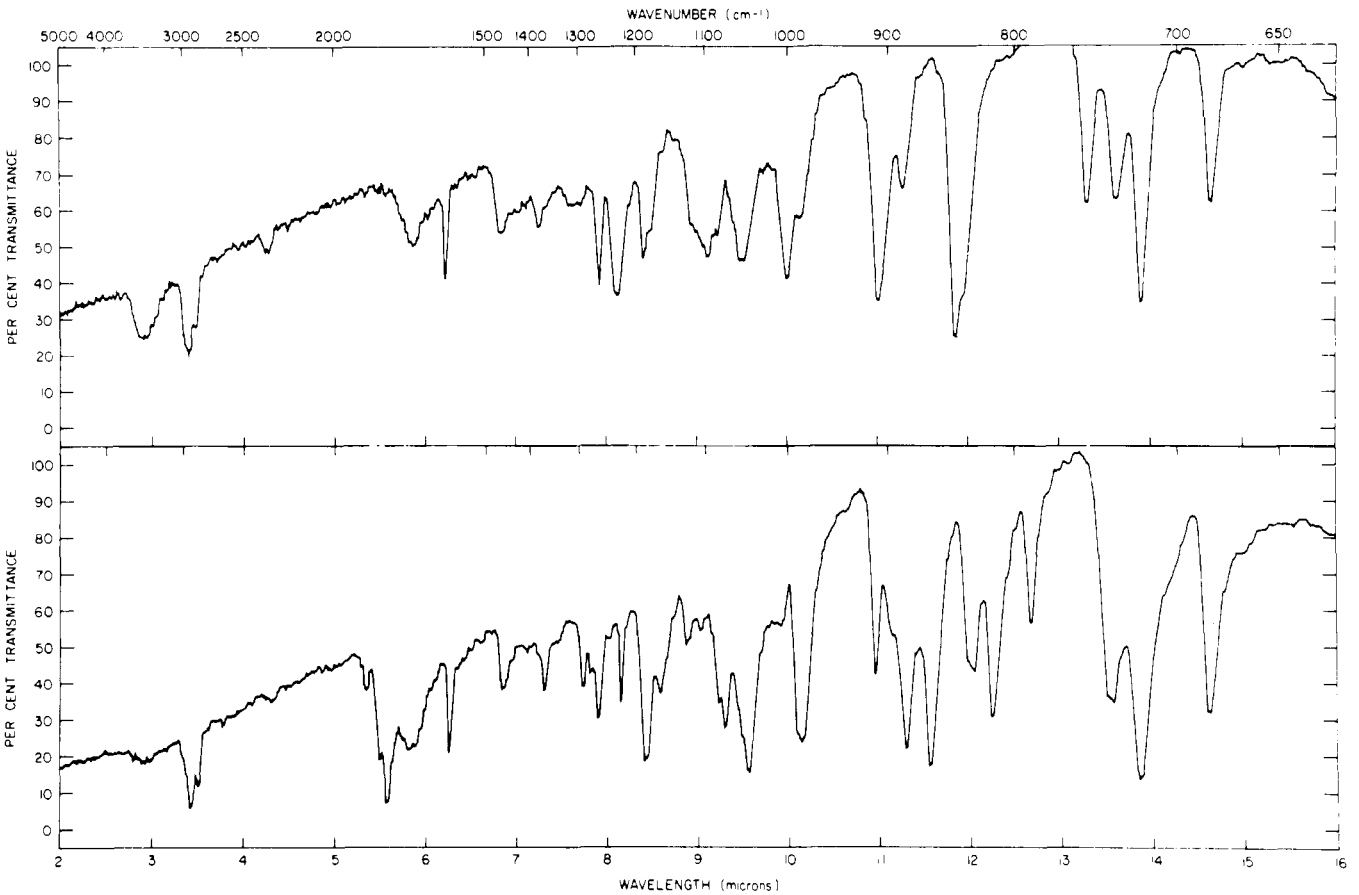


Figure 6. Infrared spectra of the major metabolite of endrin (F-1, U-2, L-1) (top) and its CrO_3/pyridine oxidation product (bottom).

with respect to the epoxy group are possible, positions 12a (anti) or 12s (syn) as shown for endrin (1) (Figure 7). Intramolecular hydrogen bonding between the epoxy oxygen and the hydroxyl hydrogen would be possible in the syn alcohol. This would result in a sharp absorption in the OH stretching region of the IR spectrum. The IR spectrum of the major metabolite did not exhibit a sharp absorption in the OH region (Figure 6). This combined with evidence previously reported in which the relative retention time of this major metabolite was similar to *anti*-12-hydroxyendrin (11) (Figure 7), using identical GC conditions (Petrella and Webb, 1973), suggests that U-2 is *anti*-12-hydroxyendrin (11).

A tentative assignment of structure for U-1 based upon mass spectra is possible. The CI–MS spectrum of U-1 was indicative of an uncaged endrin system containing all of its chlorines and exhibiting peaks at m/e which would be consistent with the loss of chlorine from an endrin alcohol parent ion. If U-1 were a monohydroxy derivative of endrin the OH would have to reside on the nonchlorinated portion of the molecule. CrO_3/pyridine oxidation resulted in no change in U-1 implying that U-1 is a tertiary alcohol (OH residing on C-2, -3, -6, or -7). 3-Hydroxyendrin (12) (Figure 7) has been isolated as a minor metabolite from rats (Baldwin et al., 1970). Chromatographic behavior of U-1 is similar to 3-hydroxyendrin.

CI–MS spectra of U-3 characterized this compound as being very unstable to the ionization process. Isobutane stabilized some of the higher mass fragments, preserving a peak of m/e 377. This would be consistent with the loss of OH from an endrin alcohol parent ion. Description of the *syn*-12-hydroxyendrin as very labile to the ionization process suggested a similar structure for U-3 (Bedford and Harrod, 1973). However, identity of U-3 as *syn*-12-

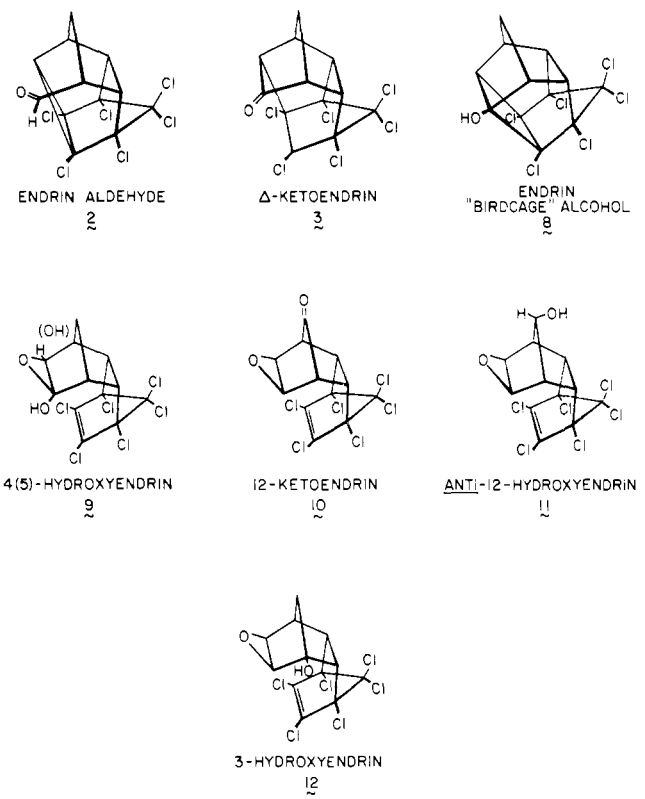


Figure 7. Chemical structures of endrin isomers, monohydroxylated endrins, and 12-ketoendrin.

hydroxyendrin could not be confirmed by CrO_3/pyridine oxidation to 12-ketoendrin, the oxidation product of U-2. Rather, U-3 was converted to a mixture of products, some

highly polar, one or more of which may have been acidic in nature. Oxidation of both *syn*- and *anti*-12-hydroxyendrin to the same ketone (Bedford and Harrod, 1973) suggests U-3 is not *syn*-12-hydroxyendrin. Since CI–MS data provided evidence that all of the chlorines were intact on an uncaged molecule, it would seem reasonable that U-3 may be a cyclic hemiketal (**9**) (Figure 7), which would produce water-soluble and acidic materials upon CrO_3 oxidation.

The recent report that *anti*-12-hydroxyendrin is the least toxic of the known metabolites of endrin in rats suggests that rapid production and excretion of this metabolite by the resistant strain represent a major contribution to the resistance phenomenon (Bedford et al., 1975). Other contributions to resistance are indicated by an apparent accumulation of endrin in the susceptible strain which retains 25% more of the administered dose of [^{14}C]endrin than the resistant strain (Petrella et al., 1975). Rapid excretion of *anti*-12-hydroxyendrin as a metabolite of endrin by the resistant strain suggests that a major contributing factor to resistance is the rapid conversion of endrin to a less toxic derivative which may be readily excreted. The most toxic metabolite of endrin is 12-ketoendrin (Bedford et al., 1975). Although excretion of this metabolite by either resistant or susceptible strains was not observed, 12-ketoendrin has been described as a predominant tissue metabolite (Baldwin et al., 1970; Bedford et al., 1975). The availability of 12-hydroxyendrin for production of a more toxic metabolite, i.e., 12-ketoendrin, would therefore be greatly reduced in the resistant as compared with the susceptible strains.

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Fate of Methoprene (Isopropyl (2*E*,4*E*)-11-Methoxy-3,7,11-trimethyl-2,4-dodecadienoate) in Rats

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The metabolic fate of methoprene, isopropyl (2*E*,4*E*)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate, a new insect growth regulator (Altosid), has been studied in rats. After oral administration of [5-^{14}C]methoprene, means of 19.6 and 18.0% of the dose were excreted in the urine and feces, respectively, during 5 days. During the same time, 38.8% was excreted in the expired air as $^{14}CO_2$ and, after 5 days, a mean of 17.2% was retained in the body. Most of the residual radioactivity was present in the fat (8.5% equivalents) and muscle (2.2%) and concentrations were highest in the liver (84.5 ppm), kidneys (29 ppm), lungs (26 ppm), and fat (36.5 ppm). Whole-body autoradiographs showed extensive distribution of radioactivity and particularly notable was the high concentration in the adrenal cortex. No methoprene was detected unchanged in bile or urine and about 12 radioactive components could be detected in urine, none of which corresponded to the authentic reference compounds, which would arise by simple ester hydrolysis and/or O-demethylation. The available evidence indicated that the metabolites probably arose from β-oxidation of methoprene.

Methoprene (1, isopropyl (2*E*,4*E*)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate; trademark, Altosid) is a member of a potent new class of insect growth regulators (IGR's) with juvenile hormone activity (Henrick et al., 1973) which is in commercial use as a mosquito and horn fly larvicide. The compound is relatively nontoxic to mammals and no effects have been observed at doses of >34 600 mg/kg to rats. Studies on the environmental degradation of methoprene by plants (Quistad and Staiger, 1974), aquatic microorganisms (Schooley et al., 1975a), photooxidation (Quistad et al., 1975a), and soil (Schooley et al., 1975b) have shown that it is nonpersistent and rapidly degraded. Some animal metabolism studies have been performed in the guinea pig, steer, and cow (Quistad et al., 1975b,c; Chamberlain et al., 1975) and complementary to these investigations we now report the fate of methoprene in rats.

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MATERIALS AND METHODS

(2*E*,4*E*)-[5-^{14}C]Methoprene (**1**), synthesized as described previously (Schooley et al., 1975a), was supplied by the Zoecon Corporation (Palo Alto, Calif.). The specific activity was 3.11 mCi/mmol and the radiochemical purity was greater than 99%. Authentic reference compounds (**2**–**4**) listed below (see Table I) 7-methoxycitronellic acid were synthesized by the Chemical Department, Zoecon Research.