Comparative Metabolism of Methoxychlor, Methiochlor, and DDT in Mouse, Insects, and in a Model Ecosystem

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Following oral administration of radiolabeled compounds to the mouse, 98.3% of methoxychlor, 47.11% of methiochlor, and 1.02% of DDT were eliminated after 24 hr. In both mouse and insects, methoxychlor was metabolized by O-demethylation to form 2-(*p*-hydroxyphenyl)-2-(*p*-methoxyphenyl)-1,1,1-trichloroethane and 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane, which are eliminated largely in conjugated form. In a model ecosystem, methoxychlor was found in fish at the top of the food chain at a level of 1500 times that of the water, as compared to 90,000 times for DDT. The elimination of methoxychlor was in dynamic equilibrium as compared with the storage of DDT, DDE, and DDD. Methiochlor was metabolized in both mouse and insects by oxidation to 2-(*p*-methylsulfinylphenyl) -2-(*p* - methylthiophenyl) - 1,1,1 - trichloroethane and by further oxidation to the corresponding bis-sulfoxide and bis-sulfone. In the model ecosystem, methiochlor was found in fish only in traces together with an eight-fold concentration of its polar metabolite 2,2-bis(*p*-methylsulfinylphenyl)-1,1,1-trichloroethane. The very pronounced selective insecticidal action of methoxychlor is the result of the much greater efficiency of O-dealkylation in the mammalian liver than in insects.

DDT is one of the most stable and water insoluble of widely used organic molecules, and it is probable that a substantial portion of the several billion pounds distributed for insect control since 1943 still remains in circulation in the global ecosystem. There is abundant evidence of its accumulation and biological magnification in the tissues of a great variety of wildlife (Dustman and Stickel, 1969). Moreover, the primary metabolite of DDT, DDE [2,2-bis-(*p*-chlorophenyl)-1,1-dichloroethylene] produced by enzymatic dehydrochlorination and DDD or TDE [2,2-bis-(*p*-chlorophenyl)-1,1-dichloroethane] formed by reductive dechlorination (Datta *et al.*, 1964) are very similar to the parent material in physical properties and are found together with DDT as ubiquitous ppm contaminants of human fat (Hayes, 1966). DDT, DDE, and DDD are all efficient inducers of microsomal oxidases in vertebrate livers (Street, 1969) and enzymes thus induced have been suggested as the cause of abnormal calcium metabolism in the eggs of raptorial birds (Bitman *et al.*, 1969). Thus, there are many reasons to regard DDT as an undesirable environmental pollutant. At the same time, DDT has saved more lives and averted more illness and misery than perhaps any other substance invented by man—through its properties as a persistent insecticide in the control of the insect vectors of malaria, typhus, plague, and other diseases, and of other noxious insects. There is need, therefore, for the prompt replacement of DDT in many insect control programs by a persistent but biodegradable substitute.

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Methoxychlor [2,2-bis(*p*-methoxyphenyl)-1,1,1-trichloroethane] is a very likely candidate as this biodegradable replacement. First synthesized by Elbs (1893), its insecticidal properties were described by Lauger *et al.* (1944) together with DDT, and it has been a commercial insecticide for about 25 years. However, as methoxychlor is somewhat less effective than DDT to many insects and costs about 66¢ per lb *vs.* 18¢ for DDT (USDA, 1967) its usage has been limited. In the United States in 1961 it was registered for 81 agricultural applications, as compared to 334 registrations for DDT (USDA, 1961). Recently, methoxychlor has replaced DDT for several predominantly environmental uses, such as the control of blackfly larvae in streams and of the elm bark beetle vectors of Dutch Elm disease. However, despite a quarter of a century of use and the employment of an estimated 100,000,000 pounds in the United States, little specific information is available about the pathways of metabolic detoxication of methoxychlor and about such critical factors as its elimination from the animal body and its fate in the ecosystem.

Methoxychlor, rat oral LD_{50} >6000 mg per kg, is much less acutely toxic than DDT, oral LD_{50} 118 mg per kg (Hayes, 1963). Von Oettingen and Sharpless (1946) found that methoxychlor did not produce the typical tremors and other nervous manifestations characteristic of DDT intoxication. They conjectured that 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane might be produced as a methoxychlor metabolite. Kunze *et al.* (1950) found that methoxychlor fed to rats at 25 ppm was not stored in adipose tissue, and even at 100 ppm was stored only at 1 ppm. When fed at 500 ppm, storage in 4 weeks reached 36 ppm in the male and 17 ppm in the female, but rapidly declined and no methoxychlor could be detected in the fat at 2 weeks after feeding stopped. The

Table I. Properties of Methoxychlor and Its Model Metabolites

Compound	M.P. °C	Thin-Layer Chromatography (RF)[a] EH1	EH2	SCM	Partition Coefficient	Water Solubility (ppm)	Detection[b] U.V.	D–Z
$CH_3OC_6H_4HCCCl_3C_6H_4OCH_3$	92.0	0.25	0.61	0.78	4.8×10^3	0.62	None	Steel gray
$CH_3OC_6H_4HCCHCl_2C_6H_4OCH_3$	113.0	0.20	0.61	0.78	...	...	None	Bluish green
$CH_3OC_6H_4CCCl_2C_6H_4OCH_3$	109.0	0.32	0.61	0.78	...	...	None	Pink
$CH_3OC_6H_4HCCCl_3C_6H_4OH$	112–114.0	0.07	0.58	0.56	...	0.80	Light yellow	Black
$HOC_6H_4HCCCl_3C_6H_4OH$	194.0	...	0.35	0.41	...	76.0	Yellow	Black
$HOC_6H_4CCCl_2C_6H_4OH$	212.0	...	0.40	0.37	...	...	Orange	Pink
$CH_3OC_6H_4HCCOOHC_6H_4OCH_3$	110.0	...	0.16	0.20	...	...	None	None
$HOC_6H_4HCCOOHC_6H_4OH$	145–150.0	...	0.16	0.20	...	...	Light brown	None
$HOC_6H_4COC_6H_4OH$	213–215.0	...	0.22	0.27	...	...	Yellow	None

[a] Tlc development systems: EH1 = Ether-1 to Pet. ether (60–68° C)-9; EH2 = Ether-3 to Hexane-1; SCM = Pet. ether (60–68° C)-3 to chloroform-2 to methanol-1. [b] Color detection: U.V. = Exposure to U.V. light for 5 to 10 min; D–Z = Spraying with 0.5% diphenyl amine + zinc chloride (in acetone) heating at 110° C for 10 min and exposure to U.V. light for 5 min.

rapid elimination of methoxychlor is in marked contrast to DDT which, when fed to rats at 1 ppm under identical experimental conditions, was stored at 13 ppm in the male and 18 ppm in the female, and when fed at 50 ppm was stored at 284 ppm and 588 ppm, respectively (Laug *et al.*, 1950).

Woodard *et al.* (1948) fed methoxychlor to rats and, finding neither methoxychlor nor bis(*p*-methoxyphenyl)-acetic acid in the urine, suggested that the metabolism of this compound involved more profound changes in the molecule than were found with DDT. Weikel (1957) used ^{14}C-ring-labeled methoxychlor to study liver and biliary excretion in the rat following intravenous administration. The radioactivity was rapidly eliminated from the animals, largely in the feces through secretion from liver into the bile. Methoxychlor was not excreted and the metabolites were found to be much more polar and water soluble, and were suggested to be strongly acidic anionic compounds. With this evident paucity of information regarding the biological fate of a valuable "soft" insecticide, we report here the detailed metabolic pathways of methoxychlor in mammals and insects, and its biodegradability in a small model ecosystem.

In the search for biodegradable analogues of DDT with suitable insecticidal properties, we have also studied "methiochlor" or 2,2-bis(*p*-methylthiophenyl)-1,1,1-trichloroethane. This almost totally neglected relative of methoxychlor was first synthesized by Caccia-Bava and Vitali (1950). Its insecticidal properties resemble those of methoxychlor, but it appears to be generally less active (Metcalf and Fukuto, 1968). However, the presence of the *p,p'*-methylthio groups with their well known capacity for *in vivo* oxidation to the CH_3SO and CH_3SO_2 moieties (Benjamini *et al.*, 1959) suggests that methiochlor might be substantially biodegradable. Thus, the rate of dehydrochlorination of DDT-type compounds to the corresponding ethylenes is controlled by the availability of electrons at the α-hydrogen and is determined by the electron withdrawing capacity of the *p,p'* substituents as measured by summation of σ values. The data of Metcalf and Fukuto (1968) suggest that methiochlor, which should be more stable than DDT because of the electronically neutral CH_3S moiety, would dehydrochlorinate 4×10^3 times more rapidly if oxidized to the bis-CH_3SO_2 compound. No information is available on the metabolic pathways for methiochlor, and we have made studies of its biological behavior in parallel with those of methoxychlor.

MATERIALS AND METHODS

Radiolabeled Compounds. The investigation was conducted with ^{3}H-ring substituted methoxychlor synthesized by the method of Hilton and O'Brien (1964), and purified by column chromatography on silica gel by elution with 6% ether in petroleum ether b.p. 60–68° C (Skellysolve B). The product had a purity of 99.9+% evaluated by thin-layer chromatography (tlc), using two systems of solvents (Table I) and a specific activity of 4.52 mCi per mM. A small sample of ^{14}C-ring labeled methoxychlor with a purity of 94.3% and a specific activity of 0.133 mCi per mM was supplied by E. I. DuPont. The principal impurity was 2,2-bis(*p*-methoxyphenyl)-1,1-dichloroethylene. ^{14}C-ring labeled DDT was supplied by the World Health Organization, Geneva, Switzerland, and was purified by column chromatography to give a chromatographic purity of 99.9+% on tlc by two systems (Table I) with a specific activity of 5.48 mCi per mM.

^{3}H-Methiochlor with a specific activity of 5.81 mCi per mM was tritiated by a procedure identical to that described for methoxychlor. It had a chromatographic purity by tlc of 99.9+% using the solvents systems listed in Table II.

Model Metabolites. The presence of the *p,p'*-methoxy groups of methoxychlor together with Weikel's (1957) suggestion that the liver is the primary site of methoxychlor metabolism suggest that the primary metabolites are likely to be the mono- and dihydroxyphenols produced by O-dealkylation. Therefore, in analogy with the normal dehydrochlorination and reductive dechlorination pathways of DDT metabolism (Datta *et al.*, 1964), we obtained the following model metabolites.

Methoxychlor or 2,2-bis(*p*-methoxyphenyl)-1,1,1-trichloroethane (I) m.p. 92° C (Schneller and Smith, 1949) was produced through condensation of 10.8 g of anisole with 7.4 g of anhydrous chloral in 250 ml of chloroform at 4° C to which was added 7.4 g of anhydrous aluminum chloride. Stirring was continued at 20° C for 30 min and for a further 8 hr at room temperature.

The 2,2-bis(*p*-methoxyphenyl)-1,1-dichloroethylene (II) m.p. 109° C (Schneller and Smith, 1949; give m.p. 109° C) was obtained from I by refluxing 3.45 g in 100 ml of ethanol, containing 0.75 g KOH, and was recrystallized from ethanol.

2-*p*-Methoxyphenyl-2-*p*-hydroxyphenyl-1,1,1-trichloroethane (III) was prepared from *p*-methoxyphenyltrichloromethyl carbinol produced by the dropwise addition of a

Table II. Properties of Methiochlor and its Model Metabolites

Compound	M.P. °C	Thin-Layer Chromatography (RF)[a] ES	AS	AC	Partition Coefficient	Water Solubility (ppm)	Detection[b] D–Z	Q
$CH_3SC_6H_4HCCCl_3C_6H_4SCH_3$	115–117	0.51	0.77	0.76	1.1×10^5	0.57	Gray	Reddish brown
$CH_3SC_6H_4CCCl_2C_6H_4SCH_3$	119.0	0.48	0.77	0.76	...	...	Pink	-do-
$CH_3SOC_6H_4HCCCl_3C_6H_4SCH_3$	133–136	...	0.46	0.50	...	1.25	Bluish green	Orange
$CH_3SOC_6H_4HCCCl_3C_6H_4OSCH_3$	150–153	...	0.09	0.13	...	29.0	None	None
$CH_3SO_2C_6H_4HCCCl_3C_6H_4OSCH_3$	182–183	...	0.33	0.43	...	...	None	None
$CH_3SO_2C_6H_4HCCCl_3C_6H_4O_2SCH_3$	236.0	...	0.67	0.65	...	1.5	None	None
$CH_3SO_2C_6H_4CCCl_2C_6H_4O_2SCH_3$	225.0	...	0.68	0.66	...	...	None	None

[a] Tlc development systems: ES = Ether-1 to Pet. ether (60–68° C)-9; AS = Acetone-3 to Pet. ether (60–68° C)-1; AC = Acetone-3 to cyclohexane-1. [b] Color detection: D–Z = Spraying with 0.5% diphenyl amine + zinc chloride (in acetone), heating at 110° C for 10 min and exposure to U.V. light for 5 min; Q = spraying with 0.5% 2,6-dibromo-*N*-chloro-*p*-benzoquinoneimine.

mixture of 7.4 g chloral and 5.4 g anisole into 100 ml anhydrous ether cooled to 5° C and containing 6.7 g anhydrous $AlCl_3$. The reaction mixture was stirred for 20 hr at room temperature and poured over ice-HCl mixture and the organic layer washed with Na_2CO_3 and water. The product distilled at 150–2° C/0.6 mm and was recrystallized from petroleum ether to m.p. 45–7° C. Compound III was obtained by reacting 2.7 g of the carbinol with 0.9 g of phenol in 100 ml of ethanol-free chloroform, cooled to 4° C with the addition of 1.3 g anhydrous $AlCl_3$. The mixture was stirred for 12 hr at room temperature, diluted with 100 ml of water, the chloroform removed under reduced pressure and the excess phenol by distillation at 1.0 mm. The residue was extracted with ether, dried over anhydrous Na_2SO_4, and recrystallized from a mixture of benzene and cyclohexane, m.p. 112–14° C. Schneller and Smith (1949) reported m.p. 113–115° C for a compound believed to be the above. Nmr spectrometry showed CH_3O-aryl-protons at τ 6.23, α-H at τ 5.05, OH-aryl at τ 4.71, and two aryl rings.

The 2-*p*-methoxyphenyl-2-*p*-hydroxyphenyl-1,1-dichloroethylene (IV) was prepared by dehydrochlorination of III as described for II (above). The product crystallized from cyclohexane, m.p. 110-115° C, and gave a pink color with diphenylamine·$ZnCl_2$ reagent, indicating the presence of the ethylene. Nmr spectrometry showed CH_3O protons at τ 6.2, aryl OH at τ5.02, and absence of α-H.

The 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (ter Meer, 1874) was prepared by condensing phenol with chloral as for methoxychlor (I) above. The product was crystallized from methylene chloride with a trace of ethanol to give m.p. 194° C (literature 202° C). The 2,2-bis-(*p*-hydroxyphenyl)-1,1-dichloroethylene (VI) was prepared from 3.2 g of V by refluxing in 100 ml of 50% ethanol containing 2.2 g KOH, for 4 hr. The mixture was diluted with 25 ml water and acidified to pH 2.0. The product crystallized upon standing overnight, and was recrystallized from ethanol to m.p. 212° C (literature 214–15° C).

Bis(*p*-methoxyphenyl)acetic acid (VII) was prepared essentially as described by Brault and Kerfanto (1964) from α,α-dimorpholinoacetic acid morpholine salt and anisole, and recrystallized from benzene and cyclohexane to m.p. 106–7° C (literature 110° C). By infrared spectrometry, the compound showed C=O (s) at 1700 cm^{-1}, and CH_3O aryl at 1250 cm^{-1}. Nmr spectrometry showed CH_3O protons at τ 6.30 and OH at τ −2.07.

Bis(*p*-hydroxyphenyl)acetic acid (VIII) was prepared by the method of Prey (1945), and recrystallized from benzene to m.p. 145–50° C (literature m.p. 150° C). Bis-(*p*-hydroxy)-benzophenone (IX), m.p. 212° C, was recrystallized from a commercial sample (Aldrich Chemical Co.). The 2,2-bis-(*p*-methoxyphenyl)-1,1-dichloroethane (X), m.p. 113° C was obtained from E. I. DuPont. Weichell (1879) gives m.p. 113° C.

Methiochlor (XI). M.p. 117° C was synthesized from chloral and thioanisole by the method of Caccia-Bava and Vitali (1950). Nmr spectrum showed the α-H at τ 6.03 and CH_3S protons at τ 7.57. The 2,2-bis(*p*-methylthiophenyl)-1,1-dichloroethylene m.p. 119° C (literature m.p. 119° C) was prepared by dehydrochlorination in ethanolic KOH. The bis-sulfone 2,2-bis(*p*-methylsulfonylphenyl)-1,1,1-trichloroethane (XII) m.p. 236° C (literature m.p. 247.5° C) was prepared by oxidizing the parent compound with excess performic acid. The product showed the characteristic SO_2 absorption in infrared at 1140 and 1300 cm^{-1}. Nmr spectrometry showed α-H at τ 4.68 and CH_3SO_2 protons at τ 6.92. The product was dehydrochlorinated to 2,2-bis(*p*-methylsulfonylphenyl)-1,1-dichloroethylene (XIII) m.p. 225°C by refluxing in alcoholic KOH. Nmr spectrometry showed CH_3SO_2 protons at τ 6.93 and the absence of α-H.

The mixed oxidation products of methiochlor were prepared by oxidation in glacial acetic acid with performic acid. The residue was evaporated to dryness and passed through a silica gel column, followed by elution with various solvents. The 2-(*p*-methylthiophenyl)-2-(*p*-methylsulfinylphenyl)-1,1,1-trichloroethane (XIV) m.p. 133–6° C was eluted with 1% diethyl ether in petroleum ether. Infrared spectrometry showed SO absorption at 1040 cm^{-1} and nmr spectrometry showed the α-H at τ 4.88, CH_3S protons at τ 7.70, and CH_3SO protons at 7.28. The compound gave a single spot with tlc (Table II).

The 2,2-bis(*p*-methylsulfinylphenyl)-1,1,1-trichloroethane (XIV) was eluted with 1, 5, and 10% acetone in petroleum ether, and recrystallized from 5% benzene in cyclohexane, m.p. 150–3° C. The product was identified by infrared spectrometry, showing the characteristic SO absorption at 1040 cm^{-1} and by nmr spectrometry showing α-H τ 4.72 and CH_3SO protons at τ 7.25. The product gave a single spot with tlc (Table II).

The 2-(*p*-methylsulfinylphenyl)-2-(*p*-methylsulfonylphenyl)-1,1,1-trichloroethane (XIV) was prepared from the bis-sulfoxide (XV) by oxidation in glacial acetic acid with one equivalent of performic acid and purified on a silica gel column by elution with 5% ether in petroleum ether. The desired product, m.p. 182–3° C, was identified by infrared spectrometry, showing SO at 1040 cm^{-1} and SO_2 at 1140 and 1300 cm^{-1}, and by nmr spectrometry, showing α-H at τ 4.67,

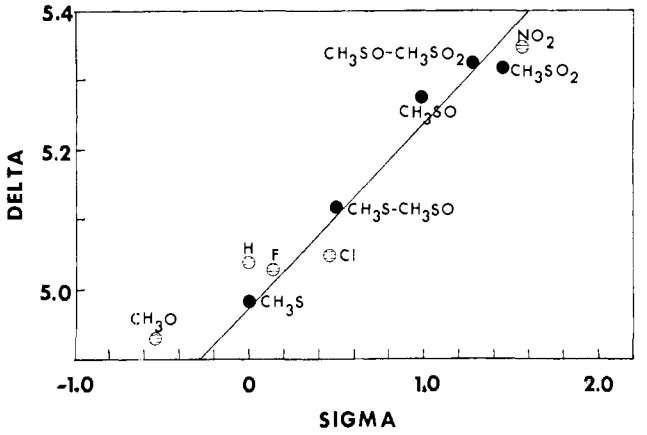

Figure 1. Relationship of chemical shift δ (ppm) of α-H of various DDT analogues to summation of Hammett σ values for p,p'-substituents. Solid circles are original values for methiochlor and oxidation products

CH_3SO protons at τ 7.27, and CH_3SO_2 protons at τ 6.93. The compound gave a single spot on tlc (Table II).

The nmr chemical shifts of the α-H of the various oxidation products of 2,2-bis(*p*-methylthiophenyl)-1,1,1-trichloroethane have been a valuable guide to structural identity. As shown by Sharpless and Bradley (1965), the values for the α-H of various *p,p*-substituted DDT analogues give a straight line relationship when plotted against the summation of Hammett σ values. We have extended this series to a number of additional compounds, as shown in Figure 1. The correlation of observed with calculated σ values is excellent. The line computed by the method of least squares has the equation $y = 0.267\,x + 4.72$.

Chromatographic and Chromogenic Techniques. Thin-layer chromatography was performed in the usual manner using 0.25 mm silica gel coated, glass plates with solvent systems as shown in Tables I and II. For DDT and its metabolites, Skellysolve B alone was used.

The R_f values and other properties of methoxychlor, methiochlor, and their metabolites are presented in Tables I and II as averages of three replicate determinations.

The model compounds were followed on the tlc plates by the quenching of the ultraviolet fluorescence of silica gel containing fluorescein, and by two useful chromogenic reactions. Diphenylmethylene moieties containing one or two free hydroxy groups produced bright yellow, orange, or brownish colors (Table I) when exposed to ultraviolet radiation of less than 265 mμ. These color reactions have a limit of detection of about 1 μg, and are due to rearrangement to form quinoid derivatives. The bis-hydroxy compounds formed the color more rapidly than the monohydroxy compounds, and the rate of appearance of the color with irradiation was trichloroethane > dichloroethylene > acetic acid. When sprayed with diphenylamine 0.5% and $ZnCl_2$ 0.5% in acetone, heated to 110° C for 10 min, and exposed to ultraviolet, the dichloroethylene derivatives II and VI produced characteristic bright pink colors with a limit of detection of about 1 μg, as compared to dark colors for the trichloroethanes (Table I).

Radioautographs of the ^{14}C-labeled metabolites from the various systems investigated were made by exposing the developed chromatograms on Eastman No Screen X-ray film. Model metabolites were incorporated as internal standards for cochromatography wherever animal extracts were investigated, and all such extracts were "cleaned up" before chromatography by partitioning in acetonitrile petroleum ether 2:1. The acetonitrile fraction was evaporated and the radiolabeled metabolites taken up in acetone or ether for application to the tlc plates.

RADIOASSAY

Tritium labeled compounds in biological fluids were assayed by the Schöniger Oxygen Flask combustion technique (Kelly *et al.*, 1961) using an infrared igniter (Thomas-Ogg, Arthur Thomas & Co., Philadelphia) to overcome color quenching. Samples of 0.5 ml urine or 15 mg ground feces were spotted on black Whatman No. 29 paper, dried overnight at room temperature, and ignited in 500 ml flasks. After cooling the flask in dry ice for 1 hr, 10 ml absolute methanol was added, and after thorough mixing, 2 ml aliquots of the methanol extract were counted in duplicate in 10 ml of "^3H cocktail" (200 g naphthalene, 10 g PPO, and 0.25 g POPOP in dioxane to make 1 l.) using a Beckman S-250 scintillation counter. The vials were left in the dark overnight before counting to quench extraneous fluorescence.

The ^{14}C labeled metabolites in urine were counted as 0.5 ml aliquots of 25-fold dilution, in 10 ml "cocktail D" (100 g naphthalene and 5 g PPO in dioxane to make 1 l.). The samples were allowed to stand overnight in the dark and counted by liquid scintillation. The feces samples were dried at 50° C, finely ground, and extracted with 10-fold amounts (by weight) of 1:1 water-acetone. Duplicate 1 ml aliquots were evaporated to dryness in scintillation vials and the residue counted in "cocktail D."

Insect homogenates in acetonitrile and feces extracts in hot methanol were assayed as 0.5 ml aliquots evaporated to dryness in scintillation vials, and the residues counted in "cocktail D."

TOXICOLOGICAL METHODS

Metabolic studies in female Swiss mice were carried out by the oral administration of ^{14}C-DDT at 12.5 mg per kg or ^{14}C and ^3H-methoxychlor or ^3H-methiochlor at 50 mg per kg, in a 4:1 mixture of olive oil and acetone. The dosage was accurately controlled by micrometer driven syringe and from 25 to 50 μl was given to each mouse. At least two mice were used with each radiotracer and were kept after treatment in metabolism cages supplied with laboratory chow and water. Urine and feces were collected every 24 hr, and with methoxychlor and methiochlor the total samples over 2 days were pooled for studying the metabolic pathways.

Metabolic studies in the female SP (DDT resistant) housefly were conducted by the topical application of 1.0 μl droplets of 0.1% w/v ^{14}C-DDT, ^{14}C-methoxychlor, and ^3H-methiochlor. Two to four-day-old flies were treated in groups of 25 and held in 125 ml Erlenmeyer flasks for 24 hr. The flies were killed by freezing, and the bodies washed three times with acetone to a total volume of 25 ml to determine percent penetration of the radiotracers. The bodies were then homogenized in acetonitrile and the feces extracted with methanol.

Three fifth instar larvae of the salt marsh caterpillar, *Estigmene acrea*, were allowed to feed on 0.5 mg of ^{14}C-DDT or ^{14}C-methoxychlor or ^3H-methiochlor contained in 3.0 g of synthetic larval diet. After 24 hr, the larvae were homogenized in anhydrous sodium sulfate and extracted with acetonitrile, and the feces were extracted with methanol.

The relative proportions of various radiolabeled metabolites were determined by scraping 1 cm or other appropriate sections of silica gel from developed tlc plates into "cocktail D."

MICROSOMAL AND ECOSYSTEM TECHNIQUES

Microsomal Oxidations. The *in vitro* technique of Wilkinson and Hicks (1969) was used for preparation of microsomal pellets from fresh Swiss mouse liver precipitated at 114,000–140,000 × g. The microsomal pellet was resuspended using 20 mg per ml of 1.15% KCl. One ml of this suspension was made up to 25 ml final incubation mixture containing $5 \times 10^{-2}M$ Tris-HCl buffer at pH 7.4, G-6-P ($2.4 \times 10^{-3}M$), nicotinamide ($2.45 \times 10^{-3}M$), NADP ($5.2 \times 10^{-5}M$) and G-6-P dehydrogenase (5 units). Either 100 μg of radiolabeled DDT or methoxychlor, or 20 μg of methiochlor in acetone was evaporated on the bottom of 125 ml Erlenmeyer flasks, and 10 ml of the incubation mixture added. After 60 min shaking of the open flasks at 37.5° C, the labeled compounds were extracted with diethyl ether, concentrated, and chromatographed by tlc using the appropriate solvent mixtures shown in Tables I and II. After development, the tlc plates were placed on No-Screen x-ray film to produce radioautographs. Appropriate sections of the developed plates were scraped into scintillation vials for quantitative determination of the radiolabeled products produced.

Model Ecosystem. A model ecosystem for evaluating pesticide biodegradability has been developed in this laboratory. It consists of a 10 × 12 × 18 in. glass aquarium containing a shelf of 15 kg of washed white quartz sand which is molded into a sloping soil-air-water interface. The lower portion is covered by 12 l. of "standard reference water" which provides satisfactory mineral nutrition for the growth of *Sorghum halpense* on the aerial portion, and the algae *Oedogonium cardiacum* in the aquatic portion. The latter is seeded with a compliment of plankton, and contains *Daphnia magna* and *Physa* snails. The aquarium is provided with aeration and is kept in an environmental plant growth chamber at 80° F (26° C) with 12 hr daylight exposure to 5000 ft candles.

In operation, the *Sorghum* seeds were planted and the aquarium allowed to equilibrate for 20 days until the *Sorghum* plants are about 6 in. high. The leaves were then treated with 5.0 mg of radiolabeled compounds in acetone applied through a micropipette so that only the plant surface was contaminated. Ten large *Estigmene acrea* larvae were placed in the chamber and allowed to feed until the plants were consumed. The radiolabeled fecal materials thoroughly contaminate the aqueous portion and are taken up into the several food chains. After 26 days, 300 *Culex quinquefasciatus* mosquito larvae were added to the chamber, and after 30 days three *Gambusia affinis* fish were added. The experiment was terminated after 33 days, when weighed samples of fish, snails, mosquito larvae, algae, and water were removed and assayed to total radioactivity. These samples were homogenized and extracted with diethyl ether, and both water and ether layers examined by tlc to determine the qualitative and quantitative nature of the degradative products present, using radioautography and serial scintillation counting of the areas containing radioactivity. The results of the total examination of the model system provide evidence of the relative biodegradability of the pesticide, as shown in Table VI. A full account of the model ecosystem technique will be published elsewhere.

RESULTS AND DISCUSSION

Toxicity and Metabolism in Houseflies. The comparative metabolism of DDT, methoxychlor, and methiochlor was investigated in the susceptible S_{NAIDM} strain and the DDT-resistant R_{SP} strain whose toxicological responses were described by Metcalf and Fukuto (1968). The pretreatment of the houseflies with 50 μg of piperonyl butoxide, applied to the abdomen for 1 hr prior to determination of the LD_{50} values for the DDT analogues by topical application to the prothorax, indicates the extent of metabolism of the compounds by the multi-function oxidases which are inhibited by the piperonyl butoxide (Metcalf, 1967). The values in Table III show that methoxychlor is substantially more effective against the DDT resistant R_{SP} flies than is DDT, and this can be related to its much lower capacity for dehydrochlorination by DDTase (Metcalf and Fukuto, 1968). The high synergistic ratios (SR values) for methoxychlor and methiochlor compared to DDT show that both methoxychlor and methiochlor are detoxified in the S_{NAIDM} housefly by multi-function oxidase activity rather than by dehydrochlorination. In the R_{SP} strain there is some evidence for additional detoxication of DDT by multi-function oxidase activity. Methoxychlor behaves about the same as in the S_{NAIDM} strain, but methiochlor is much less toxic and has a lower SR value. This suggests that both multi-function oxidase activity to form the sulfoxide and sulfone derivatives, and DDTase activity which is particularly effective because of the enhanced ease of dehydrochlorination of the oxidized derivatives play important roles in the detoxication of methiochlor in the R_{SP} strain.

The results of topical administration of 1 μg each of radiolabeled DDT, methoxychlor, or methiochlor to R_{SP} houseflies, followed by tlc separation and determination of the relative amounts of various metabolites in body homogenates and in feces, are shown in Table IV. The percent penetration was

Table III. Toxicity and Synergism by Piperonyl Butoxide for DDT, Methoxychlor, and Methiochlor to S_{NAIDM} and R_{SP} Housefly

LD_{50} μg per female

	S_{NAIDM}			R_{SP}		
	A (alone)	PB[a]	(SR)[b]	A (alone)	PB	(SR)
DDT	0.28	0.11	(2.5)	3.4	0.8	(4.3)
Methoxychlor	0.90	0.07	(12.8)	0.96	0.092	(10.4)
Methiochlor	4.5	0.34	(13.2)	56	10	(5.6)

[a] PB = pretreated for 1 hr with 50 μg piperonyl butoxide. [b] SR = synergistic ratio A/PB.

Table IV. Metabolism of DDT, Methoxychlor, and Methiochlor in R_{SP} Housefly

	Percent Total Radioactivity	
	Homogenate	Excreta
DDT treatment (9.1% wash, 24.8% excreta, 60.0% homogenate = 93.9% recovery)		
DDT	20.69	4.00
DDE	21.07	35.26
Kelthane	19.80	30.88
conjugates	38.44	30.46
methoxychlor treatment (9.7% wash, 48.8% excreta, 19.9% homogenate = 78.4% recovery)		
methoxychlor	42.34	24.63
$HOC_6H_4CHCCl_3C_6H_4OCH_3$	17.77	...
conjugates	29.47	63.35
methiochlor treatment (50.3% wash, 8.9% excreta, 9.9% homogenate = 69.1% recovery)		
methiochlor	36.90	55.37
$CH_3SC_6H_4C{=}CCl_2C_6H_4SCH_3$	14.23	2.35
$CH_3SOC_6H_4HCCCl_3C_6H_4SCH_3$	26.57	8.81
$CH_3SOC_6H_4HCCCl_3C_6H_4SOCH_3$	5.78	1.96
$CH_3SOC_6H_4HCCl_3C_6H_4SO_2CH_3$	trace	3.14
$CH_3SO_2C_6H_4HCCl_3C_6H_4SO_2CH_3$	9.67	26.70

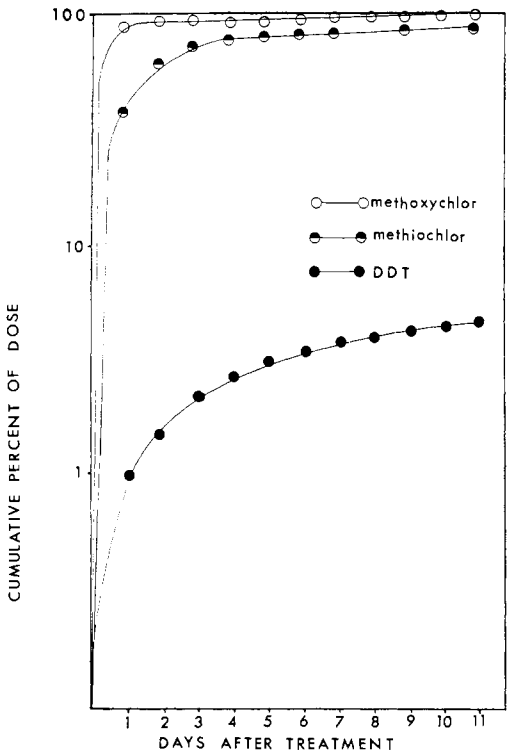

Figure 2. Accumulative rates of elimination of ^{14}C-DDT, ^{3}H-methoxychlor, and ^{3}H-methiochlor in urine and feces of mice following oral administration

very high for methoxychlor (89.9%) and substantially lower for methiochlor (49.7%). Examination of the composition of the metabolites shows clear evidence of the metabolic pathways. DDT is converted by dehydrochlorination to DDE, but also undergoes conversion to what appears, by chromatography, to be 2,2-bis(*p*-chlorophenyl)-2-hydroxy-1,1,1-trichloroethane, or Kelthane, presumably by multi-function oxidase action. There is some dehydrochlorination of methoxychlor which is converted to the monophenol by *O*-dealkylation and excreted as conjugates. Methiochlor is oxidized to sulfoxides and sulfones, and also dehydrochlorinated. Further study of these pathways by the efficient tlc systems available is indicated.

Metabolism by Salt Marsh Caterpillar. This insect is a voracious feeder and readily devoured 0.5 mg samples of ^{14}C-DDT and methoxychlor, and ^{3}H-methiochlor spread on thin 3.0 g blocks of moist "moth media" (Vail *et al.*, 1967). Excreta collected over 24 hr were homogenized in acetonitrile for scintillation counting and tlc analysis. The bodies of the caterpillars were homogenized with 20 g of anhydrous Na_2SO_4 and the fine powder extracted with acetonitrile and assayed as above. Most of the radioactivity fed the caterpillars was recovered in the excreta (DDT 93.5%, methoxychlor 93.5%, and methiochlor 92.1%). With DDT the excreta contained almost entirely DDT with a trace of DDE, and the homogenate contained 46.8% DDT and 44.9% DDE. With methoxychlor, the excreta contained 96% methoxychlor and traces of its ethylene and conjugates. The homogenate contained only methoxychlor. It is evident that this insect has little capacity for *O*-dealkylation, as compared to the mouse. With methiochlor, the excreta contained 92.7% of the parent compound, 0.68% of its ethylene, and 3.64% of the monosulfoxide. The homogenate contained 78.8% methiochlor, 0.9% ethylene, 13.82% monosulfoxide, and 1.35% bis-sulfoxide. Thus with methiochlor, metabolism by

Table V. Metabolism of Methoxychlor and Methiochlor in Mouse

	Percent Total Radioactivity			
	Urine		Feces	
	Hexane	Polar	Hexane	Polar
methoxychlor treatment (feces 90%, urine 10%)	36.2	63.8	14.4	85.6
methoxychlor	17.23	...	53.50	...
$HOC_6H_4HCCCl_3C_6H_4OCH_3$	62.02	9.66	42.0	28.30
$HOC_6H_4HCCCl_3C_6H_4OH$	5.36	22.42	...	28.03
$HOC_6H_4CCCl_2C_6H_4OH$	...	17.07	...	...
$HOC_6H_4COC_6H_4OH$	...	13.54	...	12.11
$HOC_6H_4HCCOOHC_6H_4OH$	5.09	6.85	...	traces
methiochlor treatment (feces 83%, urine 17%)	87.9	22.1	35.5	64.5
methiochlor	2.67	6.41	86.65	6.63
$CH_3SOC_6H_4HCCl_3C_6H_4SCH_3$	13.65	...	4.41	4.20
$CH_3SOC_6H_4HCCCl_3C_6H_4SOCH_3$	43.53	37.00	...	10.18
$CH_3SO_2C_6H_4HCCCl_3C_6H_4SOCH_3$	6.04	16.67	...	6.08
$CH_3SO_2C_6H_4HCCCl_3C_6H_4SO_2CH_3$	32.94	28.75	8.79	60.30

S-oxidation is substantially lower in the caterpillar than in the mouse. It seems clear that both methoxychlor and methiochlor are selectively insecticidal because they are much less rapidly degraded by mixed function oxidase activity in the insect than in the mammal.

Rates of Excretion in the Mouse. The data of Figure 2 show the comparative rates of excretion of radiolabeled DDT, methoxychlor, and methiochlor following oral administration of single doses to mice. DDT is eliminated very slowly (1.02% in the first 24 hr), while methiochlor (47.11%) and methoxychlor (98.3%) are eliminated rapidly. The degree of polarity of the excretory metabolites is indicated by the ratios of radioactivity in urine/feces which is 0.67 for DDT, 0.13 for methoxychlor, and 0.19 for methiochlor. Over a period of 11 days all of the methoxychlor and 86.69% of the methiochlor were eliminated, as compared to only 4.3% of the DDT. The data for methoxychlor can be compared with that of Weikel (1957), who recovered 50% of an intravenous dosage from the rat after 96 hr and also found that most of the activity was excreted from the liver into the bile, ending up in the feces. The substantial differences in the rates of excretion of DDT as compared to methoxychlor and methiochlor demonstrate the biodegradability of the latter two compounds.

Methoxychlor and Methiochlor Metabolism in the Mouse. Urine and feces were collected over a 48 hr period from mice fed ^{14}C and ^{3}H methoxychlor and ^{3}H methiochlor, and were extracted with petroleum ether (60–68° C) to give the "hexane fractions" and with diethyl ether to give the polar fractions. With methoxychlor, 90% of the recovered radioactivity was in the feces and 10% in the urine, and with methiochlor, the values were 83% in feces and 17% in urine. After analysis of the fractions by tlc, the qualitative and quantitative distributions of the metabolites were determined as shown in Table V.

The primary metabolites of DDT in the rat are DDE and DDD, and the latter is converted to DDA (Peterson and Robison, 1964) which is slowly excreted as conjugates in the feces, along with traces of DDE or in urine (Jensen *et al.*, 1957). As shown in Table V, however, methoxychlor is *O*-demethylated to phenolic products, the major metabolites being 2-(*p*-methoxyphenyl)-2-(*p*-hydroxyphenyl)-1,1,1-trichloroethane and 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane and its ethylene. The other significant metabolite

Table VI. Distribution of ^{14}C-DDT, ^{3}H-methoxychlor, ^{3}H-methiochlor and Their Metabolites in a Model Ecosystem

		Concentration (ppm)		
	H_2O	*Physa* (snail)	*Culex* (mosquito)	*Gambusia* (fish)
DDT				
Total ^{14}C	0.004	22.9	8.9	54.2
p-p'-DDT	0.00022	7.6	1.8	18.6
p-p'-DDE	0.00026	12.0	5.2	29.2
p-p'-DDD	0.00012	1.6	0.4	5.3
polar metabolites	0.0032	0.98	1.5	0.8
Methoxychlor				
Total ^{3}H	0.0016	15.7	0.48	0.33
p-p'-methoxychlor	0.00011	13.2	...	0.17
p-p'-methoxychlor ethylene		0.7	...	...
$HOC_6H_4HCCCl_3C_6H_4OCH_3$	0.00013	1.0	...	...
$HOC_6H_4CCCl_2C_6H_4OH$	0.00003	...	...	...
$HOC_6H_4HCCCl_3C_6H_4OH$	0.00003	...	...	...
unknowns	0.00009	...	...	...
polar metabolites	0.00125	0.8	...	0.16
Methiochlor				
Total ^{3}H	0.073	1.3	0.39	0.48
p-p'-methiochlor	0.0018	0.539	...	...
p-p'-methiochlor ethylene	0.0006	0.199	...	...
$CH_3SC_6H_4HCCCl_3C_6H_4SOCH_3$	0.0029	...	...	...
$CH_3SOC_6H_4HCCCl_3C_6H_4SOCH_3$	0.0207	0.186	...	0.162
$CH_3SOC_6H_4HCCCl_3C_6H_4SO_2CH_3$	0.0092	...	...	...
$CH_3SO_2C_6H_4HCCCl_3C_6H_4SO_2CH_3$	0.0093	...	...	0.059
polar metabolites	0.019	...	...	0.058

present in the polar fraction appears from cochromatography and color reaction to be 4,4'-dihydroxybenzophenone. The bis-phenolic metabolites of methoxychlor are much more water soluble than the mono-phenolic metabolites and, as expected, were found largely in the polar fractions of urine and feces (Table V).

Methiochlor is also metabolized by oxidative degradation and is converted to the corresponding sulfoxides and sulfones. However, substantial quantities of the parent material are excreted unchanged. The major metabolite in urine is the more polar 2,2-bis(*p*-methylsulfinylphenyl)-1,1,1-trichloroethane (H_2O solubility, 29 ppm) and that in the feces is 2,2-bis(*p*-methylsulfonylphenyl)-1,1,1-trichloroethane. The presence of strongly electron-withdrawing groups suggests that these compounds should be readily dehydrochlorinated to their ethylene derivative. However, we have not been able to find a tlc system which would resolve the ethylene of the major products, the bis-sulfoxide and bis-sulfone, and its presence in urine and feces has not been established.

Metabolism in Model Ecosystem. The data of Table VI summarize the distribution of radiolabeled material in the model ecosystem units after administration of radiolabeled DDT, methoxychlor, and methiochlor. With DDT, radioactivity concentrated to very high levels in the organisms in the higher positions in the food chains, mosquito larva, snail, and fish and was present, *e.g.*, in fish, as DDE 53.9%, DDT 34.3%, and DDD 9.8%. The overall concentration of DDT from water 0.00022 ppm to fish 18.6 ppm was approximately 90,000-fold and DDE was stored at substantially higher levels. Our unpublished data shows that DDE is much more stable in the model ecosystem than DDT. It is evident that this sort of experimental approach demonstrates the nonbiodegradability of DDT and its ultimate storage as DDE, which is currently appearing in Lake Michigan.

With methoxychlor in the model ecosystem, the concentration of radioactivity in fish was much less, only about 1500-fold, and there is substantial evidence from this and unpublished experiments that the methoxychlor in fish is in a dynamic equilibrium rather than in a storage state as with DDT. However, it is evident from Table VI that methoxychlor is stored in snails in large quantities and this organism appears unable to metabolize it rapidly.

With methiochlor there was no evidence of storage of the parent material in the fish and only moderate levels of bis-sulfoxide and bis-sulfone were found. This compound is also substantially biodegradable in the snail. These results indicate the applicability of the model ecosystem approach in demonstrating the ecological fate and biodegradability of pesticides.

Metabolism by Mouse Liver Microsomes. When incubated with mouse liver microsomes, ^{14}C DDT was recovered after 2 hr as 99.5% of the intact DDT R_f 0.61, with two other trace constituents R_f 0.0 and 0.08 in EH_1 solvent.

^{14}C-methoxychlor under identical conditions was recovered as 97.09% intact methoxychlor R_f 0.66, 2.55% monophenol, 2-(*p*-hydroxyphenyl)-2-(*p*-methoxyphenyl)-1,1,1-trichloroethane R_f 0.58, and a trace of bis-phenol, 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane R_f 0.49 in EH_2 solvent. This data suggests that *O*-dealkylation in the mammalian liver is a major pathway for methoxychlor metabolism.

^{3}H-methiochlor under identical conditions was recovered as 85% intact methiochlor R_f 0.91, 13.3% monosulfoxide, 2-(*p*-methylsulfinylphenyl)-2-(*p*-methylthiophenyl)-1,1,1-trichloroethane, R_f 0.67; and 0.7% bis-sulfoxide, 2,2-bis(*p*-methylsulfinylphenyl)-1,1,1-trichloroethane, R_f 0.27 in AS solvent. This data suggests that oxidation of the methylthio moiety in the mammalian liver is a major pathway for methiochlor metabolism.

LITERATURE CITED

Benjamini, E., Metcalf, R. L., Fukuto, T. R., *J. Econ. Entomol.* **52,** 94 (1959).
Bitman, J., Cecil, H. C., Harris, S. J., Fries, G. F., *Nature* (London) **224,** 44 (1969).
Brault, A., Kerfanto, M., *Compt. Rend. Acad. Sci.* **258,** 5465 (1964).

Caccia-Bava, A., Vitali, T., *Chimica* (Milano) **5,** 173 (1950).
Datta, P. R., Lang, E. P., Klein, A. K., *Science* **145,** 1052 (1964).
Dustman, E. H., Stickel, L. F., *Ann. N.Y. Acad. Sci.* **160,** 162 (1969).
Elbs, K., *J. Prakt. Chem.* **47,** 44 (1893).
Hayes, W. J., Jr., Clinical Handbook on Economic Poisons, U.S. Dept. Health, Educ., Welfare, Comm. Dis. Center, Atlanta, Ga. (1963).
Hayes, W. J., Jr., Monitoring Food and People for Pesticide Content, p. 314, in Scientific Aspects of Pest Control, Nat. Acad. Sci., Pub. 1402, Washington, D.C. (1966).
Hilton, B. D., O'Brien, R. D., J. AGR. FOOD CHEM. **12,** 236 (1964).
Jensen, J. S., Cento, C., Dale, W. E., Rothe, C. F., Pearce, G. W., Matteson, A. M., J. AGR. FOOD CHEM. **5,** 919 (1957).
Kelly, R. G., Peets, E. A., Gordon, S., Buijske, D. A., *Anal. Biochem.* **2,** 267 (1961).
Kunze, F. M., Lang, E. P., Prickett, C. S., *Proc. Soc. Exptl. Biol. Med.* **75,** 415 (1950).
Laug, E. P., Nelson, A., Fitzhugh, O., Kunze, F. M., *J. Pharmacol. Exp. Ther.* **98,** 268 (1950).
Lauger, P., Martin, H., Muller, P., *Helv. Chim. Acta* **27,** 892 (1944).
Metcalf, R. L., Fukuto, T. R., *Bull. World Health Org.* **38,** 633 (1968).
Metcalf, R. L., *Ann. Rev. Entomol.* **12,** 229 (1967).
Peterson, J. E., Robison, W. H., *Toxicol. Appl. Pharmacol.* **6,** 321 (1964).
Prey, V., *Ber.* **75B,** 350 (1945).
Schneller, G. H., Smith, G. B. L., *Ind. Eng. Chem.* **41,** 1027 (1949).
Sharpless, N. E., Bradley, R. B., *Appl. Spectros.* **19,** 150 (1965).
Street, J. C., *Ann. N.Y. Acad. Sci.* **160,** 274 (1969).
ter Meer, E., *Ber.* **7,** 1200 (1874).
U.S. Dept. Agr., Agricultural Handbook. No. 120 "Insecticide Recommendations," (1961).
U.S. Dept. Agr., Agricultural Stabilization and Conservation Service, *Pesticide Review*, Washington, D.C. (1967).
Von Oettingen, W. F., Sharpless, N. W., *J. Pharm. Exp. Ther.* **88,** 400 (1946).
Vail, P. V., Henneberry, T. J., Pengalden, R., *Ann. Entomol. Soc. Amer.* **60,** 134 (1967).
Weikel, J. H., Jr., *Arch. Int. Pharmacodyn.* **CX(4) 423** (1957).
Weichell, H., *Ann.* **279,** 337 (1879).
Wilkinson, C. F., Hicks, L. J., J. AGR. FOOD CHEM. **17,** 829 (1969).
Woodard, G., Davidow, B., Lehman, A. J., *Ind. Eng. Chem.* **40,** 711 (1948).

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