Environmental Degradation of the Insect Growth Regulator Isopropyl (2*E*,4*E*)-11-Methoxy-3,7,11-trimethyl-2,4-dodecadienoate (Methoprene). IV. Soil Metabolism

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Metabolism of isopropyl (2*E*,4*E*)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate, a new insect growth regulator (common name, methoprene; trademark, Altosid), was studied on soils as a function of time, under various conditions. On aerobic sandy loam, [5-^{14}C]methoprene showed an initial half-life of about 10 days at a surface treatment rate of 1 kg/ha; decomposition was much slower on autoclaved soil. Only small amounts of nonpolar metabolites were isolated, including the hydroxy ester resulting from O-demethylation (0.7% of the applied dose). Over 50% of applied dose was converted to $^{14}CO_2$. Radioactivity from [5-^{14}C]methoprene incorporated into humic acid, fulvic acid, and humin fractions of sandy loam. These data indicate rapid and extensive breakdown of methoprene in soils.

Methoprene (1, isopropyl (2*E*,4*E*)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate; trademark, Altosid) is one of a potent new class of insect growth regulators (IGR's) with juvenile hormone activity (Henrick et al., 1973), and is highly effective in controlling dipterous larvae by disruption of pupal–adult metamorphosis (Schaefer and Wilder, 1973; Harris et al., 1973). Methoprene is a commercial mosquito larvicide, and it also shows promise for control of horn and stable flies in cattle manure when administered orally from mineral blocks "feed-through", (Harris et al., 1974). Whatever the use pattern of methoprene, it is probable that soil will be exposed to the material through direct or inadvertent means. Therefore, we have investigated the biodegradability of methoprene in soil, as part of a comprehensive study of the environmental fate of methoprene (for part III, see Quistad et al., 1975b).

MATERIALS AND METHODS

Isotopic Sample. Preparation and purification of [5-^{14}C]methoprene have been described previously (Schooley et al., 1975). The undiluted (58 mCi/mmol) sample of [5-^{14}C]methoprene had a purity of 97.9% 2*E* isomer, 1.5% 2*Z* isomer, and 0.6% of an unknown radioactive impurity. For use in these studies, it was diluted to specific activities of 5.0 and 0.5 mCi/mmol using cold methoprene containing 96.5% 2*E* isomer.

Radioassay and Chromatographic Procedures. Methodology and apparatus were essentially as described previously for radioassay and chromatography (Quistad et al., 1974a) and for high-resolution liquid chromatography (HRLC) (Schooley et al., 1975).

Soils. Sandy loam and silt loam soils were collected in Palo Alto by W. W. Miller. All soil samples (except those in the $^{14}CO_2$ collection experiment, vide infra) were placed in 12-oz Mason jars filled to within 1 cm of the lip (capacity at this level = 360 ml of soil, surface area = 44.2 cm^2). All soils were moistened to their approximate water holding capacity and kept moist for 30 days prior to treatment. Samples of sandy loam and silt loam, maintained under normal aerobic conditions, were watered daily throughout the study (~5–10 ml of water/jar per day) so that the soil was kept moist but not flooded. Sandy loam samples for study under anaerobic conditions were thoroughly purged with CO_2 by placing them in a desiccator, which was then repetitively evacuated and purged with CO_2 (three times), and finally sealed tightly with conventional Mason jar lids. Sandy loam samples for study under aseptic conditions were autoclaved for 1 hr to reduce microbial activity, and were sealed as above after cooling in the autoclave.

Treatment and Maintenance of Soil Samples. For application to soils, emulsifiable concentrates of the [5-^{14}C]-1 dilutions were prepared as described previously (Quistad et al., 1974a). Both emulsifiable concentrates were diluted with distilled water (0.5 ml) and applied to soil as drops (from a Hamilton microsyringe) as evenly as possible; sealed jars (autoclaved and anaerobic studies) were unsealed, treated quickly, and resealed (for anaerobic jars, this procedure was performed under a CO_2 blanket in a deep dish). Amount of radioactivity applied to soil was determined by radioassay of aliquots of the two emulsions; 1.59×10^7 dpm of 5.0 mCi/mmol of 1 was administered to the "standard rate" jars (0.444 mg; 1.0 kg/ha), and 1.61×10^7 dpm of 0.5 mCi/mmol of 1 was administered to the "10× rate" jars (4.5 mg, 10 kg/ha).

The treated jars were maintained in an indoor plant growth chamber: 22° day (16 hr), 19° night (8 hr), relative humidity ~50%, 4000 lx luminescence (fluorescent lamps). The previously mentioned watering regimen (for unsealed jars) was carefully maintained.

Measurement of Evolution of $^{14}CO_2$. The apparatus for measuring rate of evolution of $^{14}CO_2$ from soil consisted of a cylindrical filter tube (Kontes part no. K-956250) with glass frit for supporting a moistened soil sample. A controlled stream of house compressed air was passed through soda lime (to remove CO_2) and then through two water bubblers (to humidify the air to prevent drying of the soil). The humidified air passed vertically through the moistened soil, then through two 5% aqueous KOH traps in series. Undiluted (58 mCi/mmol) [5-^{14}C]methoprene was applied as an aqueous emulsion (vide supra); assay of aliquots revealed that 3.74×10^7 dpm (90 μg) was applied to the soil surface (~13 cm^2), corresponding to a 0.7 kg/ha treatment. The total contents (200 ml) of each KOH trap were changed and radioassayed at 1, 3, 7, 10, 14, 21, 28, 35, 49, 56, and 63 days. Total volatile radioactivity was determined by counting an aliquot in Insta-Gel (Packard), while verification that volatile radioactivity was $^{14}CO_2$ was achieved by: (1) acidification of an aliquot with 2 *N* HCl, saturation with nonradioactive CO_2 (Dry Ice chips), and radioassay of the remaining solution (Insta-Gel); (2) precipitation as $Ba^{14}CO_3$ (a solution of 0.4 *N* $BaCl_2$ + 0.5 *N* NH_4Cl was mixed with KOH trapping solution in a ratio of 3:5), radioassay of the $Ba^{14}CO_3$ (10 ml of Insta-Gel + 3 ml of H_2O), and radioassay of the soluble filtrate after precipitation (Insta-Gel). The volatile radioactivity was found to be ≥95% $^{14}CO_2$.

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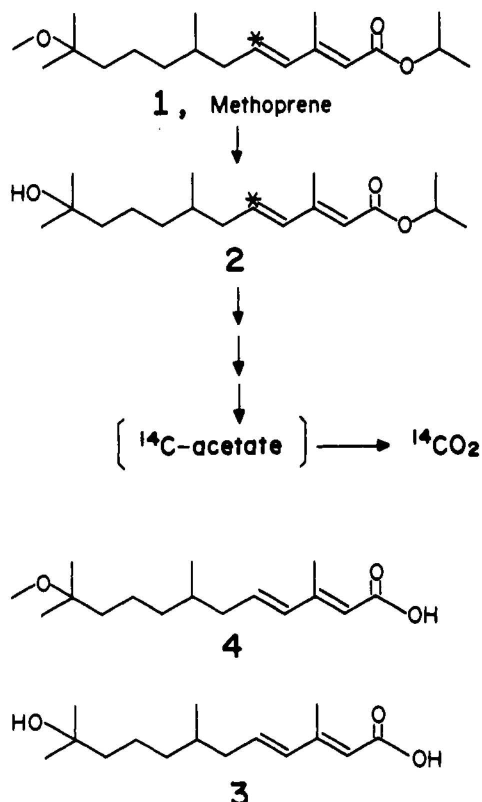

Figure 1. Methoprene (**1**) and three known (Schooley et al., 1975; Quistad et al., 1974a) primary metabolites, **2**, **3**, and **4**. Only metabolite **2** could be isolated in these soil metabolism studies, and in low yield (0.7% of applied dose). An asterisk indicates location of the carbon-14 tracer. The major soil metabolite from [5-^{14}C]methoprene is $^{14}CO_2$. [^{14}C]Acetate has been shown to be a catabolite of [5-^{14}C]methoprene in a steer (Quistad et al., 1974b).

Extraction of 1 and Metabolites from Soil. Samples of soil were extracted with methanol for 1 min using a Polytron PT 20 ST (Brinkmann Instruments) (after Johnsen and Starr, 1972) or a Polytron rotor-stator head in a stainless steel Waring Blendor (Matheson Scientific). Extracted soil was filtered (Büchner funnel) and washed (3×) with methanol. The methanolic filtrate was radioassayed, concentrated, and analyzed by thin-layer chromatography (TLC). After development of the TLC plate (benzene-ethyl acetate-acetic acid, 100:30:3), radioactive bands were located by radioscanning and quantitated by scraping and counting as previously described (Quistad et al., 1974a). Using this procedure, the amount of extractable 1 and metabolites was determined for all samples.

For a few selected soil samples, an additional methanol extraction (after the "ultrasonic" Polytron extraction) was performed using a Soxhlet apparatus for 22 hr. However, the amount of radioactivity recovered was only 1% of that in the previous step, so this additional procedure was not generally employed.

A control sample of [5-^{14}C]-1 in methanol (50 ml) was "sonicated" for 1 min with the Polytron PT 20 ST at maximum intensity. Analysis of the solution revealed no decomposition of 1 due to ultrasonic action. Moreover, quantitative recovery of radiolabel from the zero-time soil sample was observed. However, with our soils the rotor teeth were quickly worn down, thereby decreasing the shear forces and the ultrasonic action. We were able to process only $\frac{1}{10}$ as many samples as the 150–200 reported by Johnsen and Starr, between replacements of the costly rotor and/or stator. We found the Polytron Waring blendor rotor-stator head to provide somewhat greater durability and economy, but this technique (unparalleled for speed and convenience of extraction) remains rather expensive.

Isolation and Identification of Metabolites from Two-Week Sandy Loam Sample. The methanol extract from a sandy loam sample (aerobically incubated for 2 weeks after 1 kg/ha treatment) was separated on TLC (five 5 × 20 cm plates, developed 2× as above). A zone corresponding to unmetabolized 1 was collected, eluted, and further purified by HRLC (0.5 m × 2.4 mm i.d. LiChrosorb SI-60, 10 μ, eluted with 6.5% ether in pentane). The radioactive band of eluate was analyzed by coupled gas–liquid chromatography/mass spectroscopy (GLC/MS), revealing the presence of parent 1 and its 2*Z*,4*E* isomer in a 79:21 ratio (for mass spectral data on **1**, see Dunham and Leibrand, 1974). Radioactive material from a second (TLC) zone reacted with diazomethane to yield a substance whose TLC mobility (developed with hexane–ethyl acetate, 100:20) was similar to the methyl ester of 3 (Figure 1); however, HRLC analysis (LiChrosorb column eluted with 25% ether in pentane) did not reveal coelution of radioactivity with the methyl ester of standard 3 (Henrick et al., 1975), but rather a broad, nondistinct radioactive band; the material (ca. 0.5% of the applied dose of radioactivity) was therefore not **3**. Radioactive material in a third (TLC) zone was unreactive to diazomethane and showed TLC and HRLC mobility identical with hydroxy ester 2 (Figure 1). Analysis by GLC–MS of the metabolite (purified by HRLC) revealed unambiguously the presence of the 2*E*,4*E* isomer of 2 (Schooley et al., 1975); abundance of 2 after TLC purification was 0.7% of the applied dose.

Extraction of Bound Residues. Radioactivity in soil after methanol extraction was considered as bound residue. Various solubilization techniques were attempted on a 2-week, anaerobically maintained sample of sandy loam, with the most effective treatment found for release of bound residues being prolonged (48 hr) shaking of the soil sample with 0.5 *N* aqueous NaOH (>7% of the administered radioactivity was recovered). The following alternative techniques yielded 1% or less of the applied radioactivity from the same sample (before prolonged base extraction): "sonication" (Polytron) for 1 min with 0.1 *N* HCl or 0.1 *N* NaOH, 48-hr agitation with 0.5 *N* aqueous HCl-1,4-dioxane (1:1), and 24-hr agitation with 3 *N* NaCl.

Since only prolonged agitation with base was effective in solubilizing significant amounts of bound residues, samples of sandy loam (aerobically incubated for 7, 14, 30, and 60 days after treatment with 1 at 1.0 kg/ha) were subjected to a humic–fulvic acid fractionation scheme (cf. Stevenson, 1965). Methanol-extracted soil samples were washed with 0.1 *N* HCl (1000 ml) by continuous shaking for 48 hr and then sedimented by centrifuging at 1000*g* for 15 min. The soil sediments were extracted with 0.5 *N* NaOH (1000 ml) by continuous agitation for 48 hr. The alkaline suspension was mixed with Celite (100 g) and then filtered using Whatman GF/A glass fiber paper. Aliquots (1.0 ml) of the basic extract were radioassayed in Insta-Gel.

For precipitation of humic acids an aliquot (100 ml) of the basic extract was acidified to pH 1 with concentrated HCl. The precipitate was separated by centrifugation and washed by dissolution in 0.5 *N* NaOH followed by reprecipitation with HCl. This washing process was performed twice. The precipitated humic acids were quantitated by dissolving in aqueous NaOH and radioassay of an aliquot in Insta-Gel. The supernatant containing fulvic acids, after precipitation of humic acids, was also assayed for radioactivity.

Determination of Unextractable Radioactivity (Humin Fraction) in Soil. The residual soil, after exhaustive extraction with 0.5 *N* NaOH, was dried in a desiccator. Samples of soil (0.3 g) were mixed with an equal

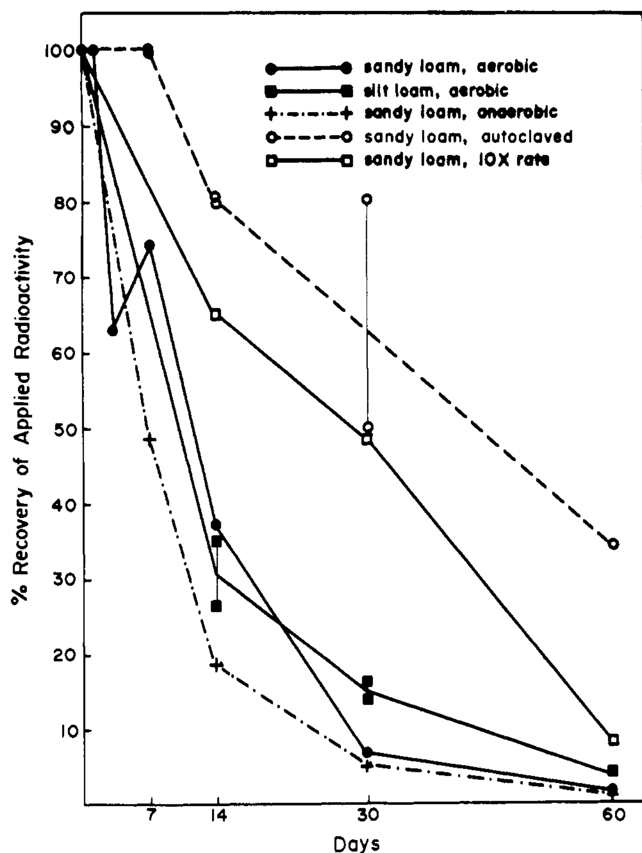

Figure 2. Time plot of radiolabel recovered by methanol extraction of soils treated under varying conditions with [5-^{14}C]methoprene. Certain samples were run in duplicate, with results indicated by connecting those data points with a thin vertical line.

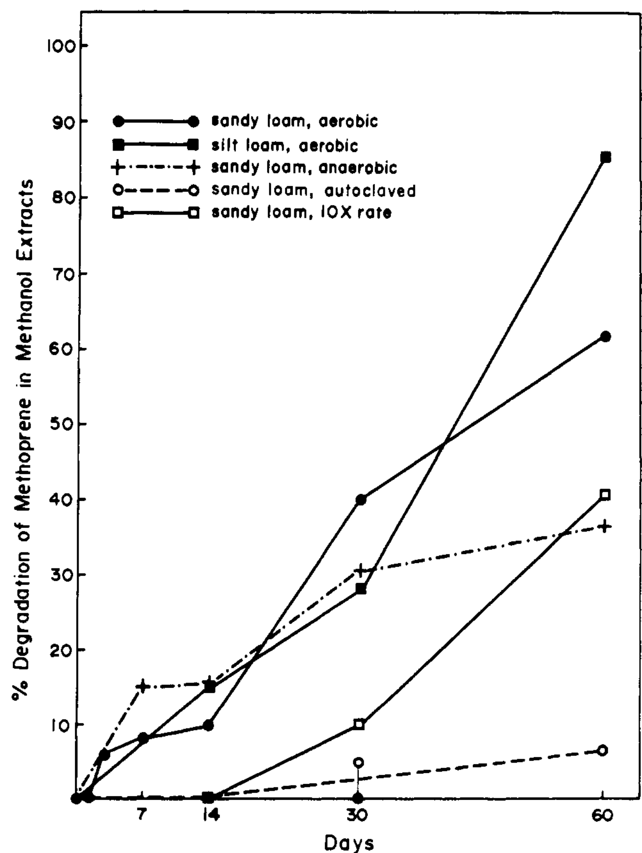

Figure 3. Time plot of extent of degradation of methoprene in methanol extracts (see Figure 2) of various soils.

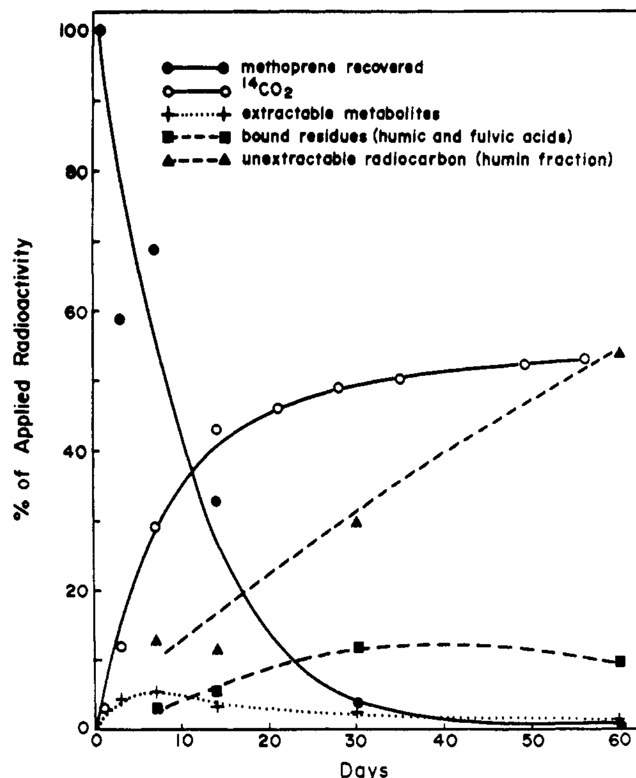

Figure 4. Radioactive products characterized from aerobic sandy loam after treatment at 1 kg/ha with [5-^{14}C]methoprene.

volume of cellulose powder, pelletized, and combusted in an oxygen flask sample oxidizer (Packard Model 305).

RESULTS AND DISCUSSION

The main emphasis in this work was to study very thoroughly the metabolic fate of methoprene applied to sandy loam soil and maintained under normal, aerobic conditions. In addition, degradation was studied concurrently under controlled environmental conditions on silt loam soil and on sandy loam soil at an exaggerated dose rate, with reduced microbial activity (autoclaved soil), and with reduced oxygen supply (nominally anaerobic).

Methanol extraction of sandy loam (aerobic and anaerobic) and silt loam soils treated at 1.0 kg/ha followed by radioassay of the extract showed very similar time plots for recovery of radioactivity (see Figure 2). Aerobically maintained sandy loam treated at a tenfold higher (10×) rate showed higher recoveries of radioactivity (slower metabolism) than the above three samples. Recovery of radioactivity from autoclaved samples was higher still (Figure 2). It is notoriously difficult to sterilize soils by autoclaving, and the gradual decrease in extractable radioactivity in autoclaved soil after the 7-day samples may be due to biodegradation. Thus, the decrease in recovery of radioactivity from nonautoclaved samples most likely reflects microbial, rather than chemical, degradation. In contrast to autoclaved samples, the results from "anaerobic" incubation were not significantly different from aerobic incubation. However, the soil dosing technique could have compromised the anaerobic conditions so that it can only be said that the oxygen supply was reduced with these samples.

Analysis of methanol extracts by TLC allowed determination of the extent of degradation of parent compound in the extract (Figure 3). Using these data and recovery percentages, a time plot for recovery of parent 1 and methanol-soluble degradation products was constructed (see Figure 4, sandy loam under aerobic conditions only). The maximum abundance of degradation products was only

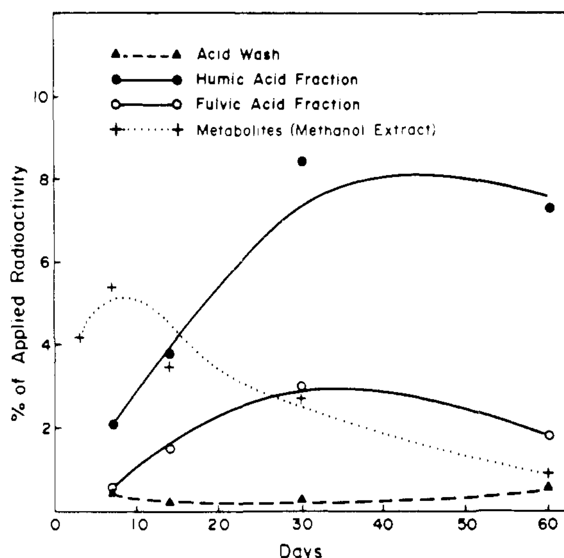

Figure 5. Time plot of radioactivity associated with bound residues (humic and fulvic acid fractions) and methanol extractables (same aerobic sandy loam soil as in Figure 4).

5.4% at 7 days. At early times (1–14 days), most of the radioactive degradation products migrated on TLC in the region of known primary metabolites of **1** (such as **2**, **3**, and **4**, Figure 1). However, at 60 days, almost half of the radioactive degradation products were so polar as to remain at the origin. The 14-day aerobic sandy loam extract was analyzed carefully to determine metabolite identity. The only metabolite positively identified (TLC, HRLC, and GLC–MS data) was the hydroxy ester **2** resulting from O-demethylation of **1** (present in only 0.7% of the applied dose). Another radioactive substance migrated like hydroxy acid **3** on TLC but HRLC analysis showed the methyl ester to be a rather broad band (possibly several components) noncoincident with the authentic methyl ester of **3**. Due to the low abundance of this material (0.5% of applied dose) and small mass isolated ($\sim$2 μg), positive identification was not pursued. No attempt was made to isolate and identify the polar unknowns, due also to their low abundance.

The most abundant metabolite was $^{14}CO_2$ (greater than 50% of the applied dose on sandy loam, 0.7 kg/ha; Figure 4). Analysis indicated that for a few samples, as much as 5% of the volatile radioactivity could have been due to volatile acids other than $^{14}CO_2$. The $^{14}CO_2$ evolution rate, the rate of disappearance of **1**, and the low abundance of metabolites (Figure 4) suggest that degradation of primary metabolites of **1** in soil may be as rapid as degradation of parent compound.

Following methanol extraction, sandy loam soil samples were subjected to a humic–fulvic acid extraction and fractionation. A time plot of radioactivity incorporated into humic (base soluble, acid insoluble) and fulvic (base and acid soluble) acid fractions is shown in Figure 5. Substantial amounts of radioactivity also incorporate (Figure 4) into the humin fraction (methanol, acid, and base insoluble).

We believe that much of the above data is explained by catabolism of [5-^{14}C]methoprene to intermediary metabolites of normal biochemical pathways. In studies of metabolism of **1** by a steer, it was shown (Quistad et al., 1974b) that C-5 of [5-^{14}C]methoprene is degraded to [2-^{14}C]acetate which incorporates into [^{14}C]cholesterol and other natural products. The mechanism of the conversion of [5-^{14}C]-**1** to [2-^{14}C]acetate by a steer appears to involve an α-oxidation followed by two β-oxidations (Quistad et al., 1974b, 1975a); mammalian oxidation of phytanic acid (possessing the same isoprenoid methyl substitution as **1**) has been shown to involve an initial α-oxidation followed by subsequent β-oxidations (Tsai et al., 1969; Hutton and Steinberg, 1973). However, *Pseudomonas citronellolis* has been shown to degrade isoprenoid acids (citronellic, geranic, farnesoic) solely by a modified β-oxidation in which the methyl "branches" are carboxylated and removed as free acetic acid, rendering the chain suitable for straightforward β-oxidation (Seubert and Remberger, 1963; Seubert and Fass, 1964). Soil microorganisms appear to degrade [5-^{14}C]methoprene to [^{14}C]acetate (whatever the mechanism), since $^{14}CO_2$ (a Krebs cycle combustion product of [^{14}C]acetate) is such an important metabolic product.

In addition, formation of [^{14}C]acetate may explain the incorporation of radioactivity into the humic and fulvic acid fractions. Although the incorporation of acetate into humic acid in soils does not appear to have been studied, [^{14}C]glucose has been shown to incorporate into humic acid (Matschke and Igel, 1971; Freytag, 1969) and also into fulvic acid and humin (Nussbaumer et al., 1970). In addition, certain soil microorganisms grown in defined media devoid of lignin synthesize phenolic substances (presumed humic acid precursors) and/or dark polymers with the characteristics of humic acids [*Aspergillus niger* on "potato-dextrose-mineral salts agar medium" (Kang and Felbeck, 1965), *Epicoccum nigrum* on glucose–asparagine medium (Haider and Martin, 1967), *Stachybotrys atra* and *Stachybotrys chartarum* on glucose–asparagine medium (Martin and Haider, 1969), and *Aspergillus sydowi* on glucose–asparagine medium (Haider and Martin, 1970)]. While the question of whether humic acid is synthesized *predominantly* from plant lignins or synthesized de novo by microorganisms remains somewhat controversial, apparently much humic acid is synthesized microbially from common intermediary metabolites arising from glucose. Three different species of microorganisms have been shown (Martin and Haider, 1969; Haider and Martin, 1970) to produce both orsellinic and 4-hydroxycinnamic acids, representatives of the two major pathways of aromatic biosynthesis. The former acid is generally believed to arise from acetate via the acetogenin pathway, the latter from phosphoenolpyruvate and a tetrose via the shikimic acid pathway. Thus, acetate should incorporate into aromatic humic acid precursors either directly, or indirectly after conversion to phosphoenolpyruvate. Acetate could also incorporate into the hydrolyzable part of humic acids (amino acids and carbohydrates).

However, the possibility also exists that some of the radioactivity in the fulvic acid fraction consists of acidic metabolites of **1** which were bound covalently to humic and/or fulvic acid phenolic hydroxyls and liberated by the prolonged strong base treatment. A similar situation was encountered in studies on the plant metabolism of [5-^{14}C]methoprene (Quistad et al., 1974a), where exhaustive extraction of plant tissue left a radioactive cellulosic residue. Enzymic (cellulase) hydrolysis of this residue gave traces of [^{14}C]glucose and [^{14}C]cellobiose and radioactive acidic primary metabolites of **1**, probably bound to cellulose before hydrolysis.

Finally, the material balance of recovered radioactivity in the detailed studies on sandy loam (Figure 4) ranges from 97 to >100%. Since $^{14}CO_2$ collection was performed on different samples than those used in extraction–fractionation, such recovery estimates are approximate.

Thus, it appears that not only is methoprene degraded rapidly in soil, but the traces of primary metabolites isolated are rapidly and very extensively metabolized. This finding is not surprising in view of the resemblance of the carbon chain of methoprene to natural products, such as farnesoic and phytanic acids, which are also readily degraded.

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Metabolism of 2,4-Dichlorophenoxyacetic Acid. VII. Comparison of Metabolites from Five Species of Plant Callus Tissue Cultures

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The metabolism of 2,4-dichlorophenoxyacetic acid (2,4-D) was investigated in carrot, jackbean, sunflower, tobacco, and corn callus tissue. Amino acid conjugates and hydroxylated metabolites were identified in all plant species although in varying amounts. After 8 days of incubation with 2,4-D water-soluble metabolites were formed in the callus tissue as follows: carrots (13.2%), jackbean (12.8%), sunflower (18.3%), tobacco (42.2%), and corn (64.6%). Five metabolites were identified as aglycones after treatment of these water-soluble fractions with β-glucosidase, three of which were present in all plants, 4-hydroxy-2,3-dichlorophenoxyacetic acid, 4-hydroxy-2,5-dichlorophenoxyacetic acid, and 2,4-D. Two tentatively identified aglycone metabolites, 3-hydroxy-2,4-dichlorophenoxyacetic acid (major) and 4-hydroxy-2-chlorophenoxyacetic acid (minor), were present only in corn. Glutamic acid was identified conjugated with 2,4-D in all tissues examined. The aspartic acid conjugate was present in corn, tobacco, and jackbean. Corn, the only monocot examined, was somewhat unusual since it possessed a high percentage of hydroxylated metabolites, including two not found in the other plant species.

Previous investigations (Feung et al., 1971, 1972, 1973b) have demonstrated that soybean callus tissue cultures readily metabolize 2,4-dichlorophenoxyacetic acid (2,4-D) to at least seven amino acid conjugates (Asp, Glu, Ala, Val, Leu, Phe, and Trp) and two ring hydroxylated metabolites which accounted for 97% of the soluble metabolites. The amino acid conjugates are biologically active and strongly stimulate plant cell elongation and division (Feung et al., 1974). On the other hand the two identified ring hydroxylated metabolites, 4-hydroxy-2,5-dichlorophenoxyacetic acid (4-OH-2,5-D) and 4-hydroxy-2,3-dichlorophenoxyacetic acid (4-OH-2,3-D), do not possess any growth stimulatory activity (Feung et al., 1974). 2,4-Dichlorophenoxyacetylaspartic acid (2,4-D-Asp) has also been detected in excised pea roots (Andreae and Good, 1957) and wheat coleoptile sections (Klämbt, 1961) and the two ring-hydroxylated metabolites were previously found in bean and other plants by Thomas et al. (1964), Hamilton et al. (1971). Montgomery et al. (1971), and Fleeker and Steen (1971).

Since the amino acid conjugates were major metabolites in soybean callus tissue and possess growth stimulatory activity it is essential to determine the generality of the presence of the amino acid conjugates in other plant species. Therefore, the metabolism of 2,4-D was investigated and compared in five additional callus tissue cultures, four dicots (carrots, jackbean, sunflower, and tobacco) and one monocot (corn).

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EXPERIMENTAL SECTION

The plant callus tissues used in these studies were jackbean (*Canavalia ensiformis*) pod callus, sweet corn (*Zea mays*) endosperm callus, tobacco (*Nicotiana tobacum*) pith callus, carrot (*Daucus carota* var. *sativa*) pith callus, and sunflower (*Helianthus annus*) pith callus. All callus stock cultures were grown on a solidified agar medium (Miller, 1963) under continuous fluorescent light at 25° for