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

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Biodegradation 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 in pond water containing unknown microorganisms. A time plot of recovery of (2*E*)-[10-^{3}H]methoprene from pond water showed a half-life of approximately 30 hr at 0.001 ppm and 40 hr at 0.01 ppm. Incubation of (2*E*)-[10-^{3}H]methoprene for 3 days at 0.42 ppm generated three primary metabolites, the result of ester hydrolysis and/or O-demethylation. These metabolites and recovered methoprene were photoequilibrium mixtures of 2-ene double bond isomers. In another incubation experiment with (2*E*)-[5-^{14}C]methoprene at 0.66 ppm in a pond water sample with presumably different microflora, a completely different metabolic profile was observed, the sole identifiable metabolite resulting from oxidative scission of the 4-ene double bond. The principal metabolite in the latter experiment was 7-methoxycitronellic acid (29% of applied dose).

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 (Schaefer and Wilder, 1973; Harris *et al.*, 1973) by disruption of metamorphosis.

Use of methoprene as a commercial mosquito larvicide has been approved under the auspices of an experimental permit granted by the Environmental Protection Agency, the first approval of any sort for commercial usage of an IGR. Since the use pattern of a mosquito larvicide requires aquatic application, we have investigated the biodegradation of methoprene by aquatic microorganisms. This report is part of a comprehensive study of the environmental fate of methoprene (for part I, see Quistad *et al.*, 1974a).

MATERIALS AND METHODS

Synthetic Procedures. A precursor for radiosynthesis of [10-^{3}H]methoprene was prepared by subjecting a mixture of 2*E* and 2*Z* isomers of isopropyl (4*E*)-3,7,11-trimethyl-2,4,10-dodecatrienoate (compound 9 in Henrick *et al.*, 1973) to methoxymercuration conditions (0.35 *M* ester and 0.4 *M* mercuric acetate in absolute methanol, 25°, 1 hr) (*cf.* Henrick *et al.*, 1973), followed by conversion of the resultant organomercurial acetate to the chloride (treatment with slightly alkaline brine). The organomercurial chloride was purified by preparative tlc (silica gel GF, 20 × 20 × 0.1 cm plates, 85% hexane–15% ethyl acetate), and the oily material was reduced with alkaline sodium [^{3}H]borohydride. [A 0.4 *M* solution of substrate in 2-propanol was treated with 2 equiv of KOH (from 1 *M* aqueous solution) followed by solid NaB^3H_4 at 25° for 1 hr. The tritiation was performed by NEN Corp.] The resultant (4*E*)-[10-^{3}H]methoprene isomers were separated from polar radioactive by-products by preparative tlc. Subsequently, the 2*E* and 2*Z* isomers were separated by preparative high-resolution liquid chromatography (hrlc) (3 m × 2.4 mm i.d. Corasil II, 4% ether in pentane). Analysis of the 2*E* isomer by radio-glc showed radiochemical and chemical purity >99%, and a specific activity of 1960 mCi/mmol.

A precursor for radiosynthesis of [5-^{14}C]methoprene was prepared starting with a sample of 2,6-dimethyl-5-hepten-1-ol (spinning band distilled, 99% pure by glc analysis). This alcohol was converted to the bromide *via* its tosylate ester. A sample of 1-bromo-2,6-dimethyl-5-heptene was isolated in 99.4% purity (glc analysis). The bromide was converted to the Grignard reagent in ether, and then carbonated with $^{14}CO_2$ (this and subsequent radiosynthetic reactions were performed by Dr. John C. Leak, ICN Corp.). The resultant acid was reduced with lithium aluminum hydride to the alcohol, and subsequently converted to [1-^{14}C]-3,7-dimethyl-6-octenal ([1-^{14}C]citronellal) by a modified Collins oxidation (Ratcliffe and Rodehorst, 1970). The [1-^{14}C]citronellal was condensed with diisopropyl 3-isopropoxycarbonyl-2-methyl-2-propenyl phosphonate (Henrick *et al.*, 1973) to yield a mixture of 2*E* and 2*Z* isomers of [5-^{14}C]isopropyl (4*E*)-3,7,11-trimethyl-2,4,10-dodecatrienoate. This intermediate was then subjected to the previously mentioned methoxymercuration–borohydride reduction, generating [5-^{14}C]methoprene (mixture of isomers). After preliminary tlc separation of polar impurities, the approximately 80:20 2*E*:2*Z* mixture was separated and purified by preparative hrlc (1 m × 7.8 mm i.d. LiChrosorb SI 60, 10 μ (E. Merck), 6.5% ether in pentane) to give [5-^{14}C]methoprene [58 mCi/mmol; 97.9% 2*E*,4*E*; 1.5% 2*Z*,4*E*]. Radio-glc analysis revealed 0.6% of an unknown radioactive impurity which was chromatographically inseparable from [5-^{14}C]methoprene by hrlc (or tlc) using ether–pentane mixtures.

Authentic dienoic metabolite standards were synthesized by the Chemical Department, Zoecon Research, by methods similar to those published (Henrick *et al.*, 1973, 1975a). The standard of 7-methoxycitronellic acid was prepared by air oxidation of 7-methoxycitronellal.

Chromatography. Thin-layer chromatography (tlc) plates (preparative and analytical) were precoated silica gel GF_{254} purchased from Analtech, Inc. Plates were prewashed with methanol and allowed to air dry before use.

All gas–liquid chromatography (glc) data were determined with Hewlett-Packard Model 402 chromatographs using glass columns (generally 2 m × 2 mm i.d. packed with 3% OV-101 on Chromosorb W-AW-DMCS) and utilizing flame ionization detection. For coupled gas chromatographic–mass spectrometric (glc–ms) experiments, a Hewlett-Packard chromatograph was interfaced to a Varian-MAT Model CH-7 mass spectrometer with a single-stage silicone membrane separator designed in these laboratories. Gas–liquid radiochromatography (radio-glc) was performed on another chromatograph equipped with an effluent stream splitter and a Barber-Coleman Model

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A9242 radiomonitor (in addition to the flame ionization detector).

High-resolution liquid chromatographic separations were performed on a custom-made instrument employing a Haskel pneumatic amplifier pump (Haskel Engineering), a Waters Associates septum injector, and a Chromatronix Model 230 ultraviolet absorbance detector. A 1 m × 2.4 mm i.d. column of Corasil/C18 (Waters Associates) was utilized for reversed-phase chromatography, and a 0.5 m × 2.4 mm i.d. column of LiChrosorb SI 60, 10 μ (E. Merck), was generally employed for normal-phase adsorption chromatography.

Radioassay Procedures. The same instruments and procedures previously described (Quistad *et al.*, 1974a) were followed.

EXPERIMENTAL SECTION

Hydrolytic Stability. Separate 1-l. samples of distilled water were buffered to pH 5.0 (0.05 *M* acetate), pH 7.0 (0.05 *M* phosphate), and pH 9.0 (0.05 *M* borate) and were then autoclaved in erlenmeyer flasks stoppered with cotton plugs. After thorough cooling, to each flask was added 250 μl of an acetone stock solution containing 0.5 mg of [5-^{14}C]methoprene (sp act. 0.5 mCi/mmol). The resulting solutions each contained 0.5 ppm of methoprene, well below the water solubility of 1.39 ppm. All flasks were wrapped completely with aluminum foil and maintained under sterile conditions at an incubator temperature of 20°. Aliquots (80 ml) of each solution were withdrawn for analysis at 1, 3, 7, 14, 21, and 30 days. All aliquots were acidified with 2 *M* HCl (to facilitate extraction of possible acidic metabolites) and then extracted with chloroform. The concentrated extracts were then examined for possible breakdown products by tlc with development in hexane–ethyl acetate (100:20). In this tlc system, R_f values for 1 and its *least* polar known metabolite **2** are 0.36 and 0.10, respectively, thus allowing a very clearcut differentiation between 1 and degradation products (see also Results and Discussion).

Photolytic Stability and Rate of Photoisomerization in Aqueous Solution. Two cotton-stoppered Pyrex erlenmeyer flasks, each containing 1-l. samples of 0.02 *M* phosphate buffer solution of pH 7, were autoclaved. After cooling, the solutions were resaturated with air under sterile conditions. Each flask was then dosed with [10-^{3}H]methoprene (0.5 mg, 0.8 μCi, 3:97 2*Z*:2*E* isomer ratio) in 200 μl of acetone, under sterile conditions. The flasks were then placed in broad, shallow pans containing water maintained at 20°, and exposed to full sunlight on the roof. Aliquots were withdrawn for glc and/or tlc analysis at 0.5, 2, 9, 25, and 48 hr. Chloroform extractions gave quantitative recovery of the radiolabel for all samples; the 0.5-, 2-, and 9-hr samples had not undergone significant decomposition according to tlc analysis (development as above). Determination of 2*Z*:2*E* ratios by glc gave values of 25.5:74.5 at 0.5 hr, 39.5:60.5 at 2.0 hr, and 54:46 at 9 hr (see Figure 2). Radioscanning of a tlc plate indicated about 15% breakdown of 1 after 48 hr, and 33% after 72 hr. The weather for the duration of the experiment was warm and sunny with little smog, and in particular, it was virtually cloudless from midmorning to late afternoon everyday. Duration of daylight was slightly over 13 hr. Prior experiments have shown that the methodology used reliably gives sterile solutions, as judged by total plate counts on standard agar.

Dissipation Rate of Tritium-Labeled 1 in Pond Water. Pond water was collected in Palo Alto Foothills Park in February, 1972. This pond is a waterfowl sanctuary, but it also has limited recreational usage (rowboats and small sailboats); the water is moderately fresh and moderately turbid. Erlenmeyer flasks were used to contain a 1000-ml and a 100-ml sample of water, and each flask was dosed with 10 μl of an acetone solution containing 1 μg of [10-^{3}H]methoprene (6.4 μCi). Thus, the 1000-ml sample received a 0.001-ppm dose and the 100-ml sample a 0.010-ppm dose. The flasks were loosely stoppered and placed outdoors where they would remain fully exposed to natural sunlight during the experiments. Aliquots (10%) of each sample were withdrawn at 0, 12, 24, 48, and 96 hr. Each aliquot was extracted (3×) with half its volume of $CHCl_3$, and the combined organic layers and remaining aqueous phase were radioassayed by liquid scintillation counting to determine the amount of unextractable radioactivity. The $CHCl_3$ extract, after concentration, was chromatographed on tlc to determine the distribution of products. The results are presented in Figure 3.

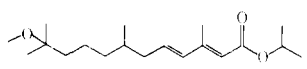
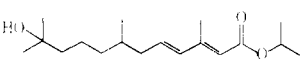
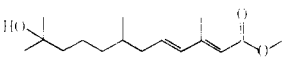
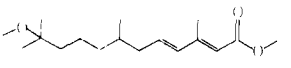
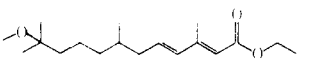
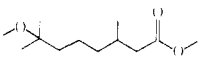
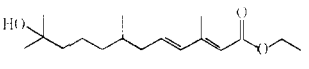
Incubation of Tritium-Labeled 1 in Pond Water and Isolation of Metabolites. Two fresh 6-l. samples of pond water were collected from the previous source 3 weeks later. A 10-μCi sample of [10-^{3}H]methoprene was diluted with 5.3 mg of unlabeled 1 [94.1% 2*E*, 4.3% 2*Z*] to a specific activity of 0.61 mCi/mmol and dissolved in 10.0 ml of 95% ethanol. An equal volume of the solution was administered to each flask, so that the final concentrations were 0.42 ppm of (2*E*,4*E*)-[10-^{3}H]methoprene and 0.08% (v/v) ethanol. The unstoppered pyrex flasks were allowed to stand outdoors in full sunshine at ambient temperature for 66 hr. At the end of this time the water was nearly saturated with sodium chloride and extracted (3×) with chloroform. The combined chloroform layers were washed with brine and then dried (sodium sulfate).

The brownish oil remaining after solvent removal was dissolved in CH_2Cl_2 and subjected to preparative tlc [20 × 20 cm plate, developed once in benzene–ethyl acetate–acetic acid (100:50:5)]; radioactive bands were located by radioscanning an 8% aliquot on a 5 × 20 cm plate. Only *three* radioactive bands were observed, and these zones were isolated, further purified, and analyzed (following sections).

Tlc Band 1. The least polar radioactive zone showed approximate tlc coincidence with **1**. The material was rechromatographed on 5 × 20 cm tlc plates (developed with ethyl acetate–hexane, 20:80). The single radioactive band observed was collected, and then further purified on hrlc (3 m × 2.4 mm i.d. Corasil II eluted with 4% ether in pentane, 1 ml/min). The chromatogram showed resolution of a number of fast eluting impurities from two main peaks corresponding to 1 and its 2*Z* isomer; a rather broad band of eluate (containing both main peaks, *ca.* 250 μg) was collected and submitted to glc–ms analysis. The sample was shown to contain roughly equal amounts of 1 and its 2*Z* isomer (glc retention times and mass spectra identical with standards; see Table I). In addition, a minor component (7% of the amount of 1) showed identical glc retention time and mass spectrum (Table I) with the 2*E* isomer of ethyl ester **5** (Figure 1). Two minor peaks were also observed, one peak (phthalate ester, base ion *m/e* 149) co-eluting with the presumed 2*Z* isomer of **5**.

Tlc Band 2. After elution and concentration, the radioactive zone of intermediate polarity was rechromatographed on tlc (developed 3× with ethyl acetate–hexane, 20:80). The zone now exhibited two radioactive bands, the less polar approximately coincident with a synthetic standard of hydroxy ester **2** and the more polar approximately coincident with a synthetic standard of methoxy acid **4** (Figure 1). The less polar of these two bands (presumed hydroxy ester **2**) was subjected to reversed-phase hrlc (1 m × 2.4 mm i.d. Corasil/C18, 65% methanol in water, 0.5 ml/min). Three radioactive hrlc bands were then observed, collected, and identified (glc–ms) by comparison of retention times and mass spectra (Table I) with those of synthetic standards; the fastest eluting peak (hrlc) was predominantly the 2*Z* isomer of **7**, the second peak contained the 2*Z* isomer of **2** and the 2*E* isomer of **7**, while the third hrlc peak contained the 2*E* isomer of hydroxy

Table I. Mass Spectral Data of Methoprene and Metabolites[a]

Compound	Significant and diagnostic ions, *m/e* (% intensity of base ion)
Methoprene (**1**)  mol wt 310.5	43 (10), 55 (7), 57 (5), 69 (15), 73 (100), 81 (17), 95 (12), 109 (15), 111 (24), 137 (10), 153 (10), 221 (4), 251 (1), 278 (1), no M^+
2  mol wt 296.4	43 (25), 55 (25), 57 (12), 59 (54), 69 (60), 81 (44), 95 (36), 109 (45), 111 (100), 153 (25), 191 (16), 203 (5), 221 (14), 236 (5), 278 (3), $M^+ = 296$ (<1)
Methyl ester of **3**  mol wt 268.4	43 (30), 55 (32), 57 (30), 59 (42), 69 (68), 81 (61), 95 (30), 109 (34), 125 (100), 147 (19), 175 (7), 203 (6), 221 (6), no M^+
Methyl ester of **4**  mol wt 282.4	55 (9), 57 (5), 69 (17), 73 (100), 81 (14), 95 (7), 109 (12), 125 (20), 175 (5), 191 (4), 207 (3), 218 (1), 235 (1), 250 (2), 267 (2), no M^+
5  mol wt 296.4	55 (11), 57 (15), 69 (23), 73 (100), 81 (18), 95 (12), 109 (14), 111 (16), 139 (35), 221 (3), 251 (1), 264 (1), no M^+
Methyl ester of **6**  mol wt 216.3	43 (8), 55 (9), 57 (5), 69 (25), 73 (100), 81 (7), 95 (19), 109 (42), 153 (27), 169 (11), 185 (6), 201 (29), no M^+
7  mol wt 282.4	43 (15), 55 (20), 57 (5), 59 (32), 69 (36), 81 (40), 95 (20), 109 (26), 111 (41), 139 (100), 175 (4), 203 (5), 221 (7), 249 (1), 264 (2), M^+ 282 (1)

[a] Varian-MAT CH-7, 20 eV. 2*E* and 2*Z* isomers of all 2,4-dienoates listed yield qualitatively identical mass spectra, but significantly different retention times on gas–liquid chromatography.

ester **2**. The 2*Z*:2*E* isomer ratios of **2** and **7** were not measured precisely, but were close to 1:1 for each, while the amount of **7** isomers was about 15% of the amount of **2** isomers. The more polar tlc band showing approximate coincidence with synthetic methoxy acid **4** was further purified by reversed-phase hrlc (Corasil/C18, 45% methanol in water, 1 ml/min), yielding apparently a single component (uv monitor). The concentrated eluate was methylated with diazomethane and identified (glc–ms data compared with standards) as an equimolar mixture of 2*E* and 2*Z* isomers of **4**.

Tlc Band 3. The most polar zone from the preliminary tlc separation was chromatographed on tlc using a first development with benzene–ethyl acetate–acetic acid (100:25:4), and subsequent development with ethyl acetate–hexane (20:80). The single radioactive zone was eluted and further purified by reversed-phase hrlc (Corasil/C18, 40% methanol in water, 0.5 ml/min). Only one peak

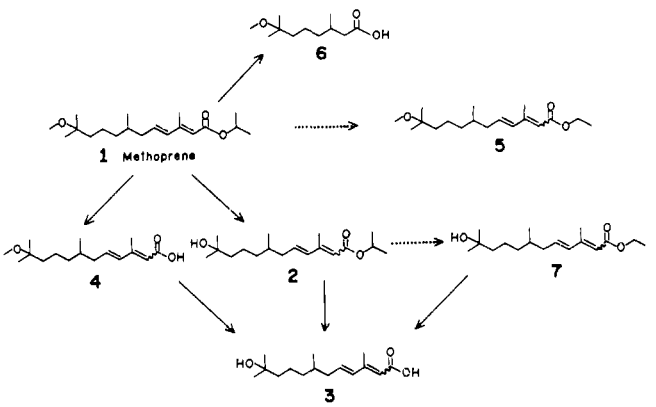


Figure 1. The metabolism of methoprene by aquatic microorganisms. A wavy line in structural formulas indicates an isomeric mixture about the 2-ene bond. Ethyl esters **5** and **7** are artifacts arising from transesterification (indicated by dotted arrow; see Discussion).

containing radioactivity was observed; it was collected, methylated (diazomethane), and shown (glc–ms) to consist of equal amounts of the 2*Z* and 2*E* isomers of the methyl ester of hydroxy acid **3**.

Material Balance in Preceding Incubation. Almost 25% of the administered radioactivity was unextractable. Recovered **1** (plus ethyl ester **5**) accounted for 60% of the applied radiolabel, while the metabolites **2** (plus ethyl ester **7**), **3**, and **4** constituted 7.0, 5.7, and 2.6% (respectively) of the administered radioactivity. *All* extractable radioactivity was identified.

Incubation of Carbon-14 Labeled 1 in Pond Water. Five 6-l. erlenmeyer flasks were each filled with pond water containing some sediment (Palo Alto Foothills Park pond, August, 1972). The pH of the water was 8.3, the biochemical oxygen demand (BOD) was 1.2 mg/l., the chemical oxygen demand (COD) was 8.0 mg/l., and the average weight of sediment per flask was 450 mg (the BOD and COD values were determined by Pacific Environmental Laboratories, San Francisco, Calif., using APHA standard methods). Each flask was treated with 2.0 ml of an acetone solution of 6.5 μCi of [5-^{14}C]methoprene, diluted with 4.0 mg of unlabeled **1** [97% 2*E*] to give a specific activity of 0.5 mCi/mmol. The resultant 0.65-ppm solutions were placed outdoors in full sunlight unstoppered, and allowed to stand at ambient temperature (the continuously recorded water temperatures varied from 16° at night to as high as 42° during the continuously sunny late August days). The flasks were sampled and analyzed by tlc at 66, 136, and 312 hr; at 312 hr (13 days) no **1** was present; consequently the incubation was ended. Scintillation counting revealed that only 48% of the radiolabel remained in solution and sediment. However, of that amount remaining in solution, 61% (or 29% of the applied dose of radioactivity) was present in a single, sharp tlc band with tlc mobility between that of standards of metabolites **3** and **4**. All remaining radioactivity appeared as a broad smear as determined by radioscanning the tlc plate (Figure 4).

A 1.8-l. sample of the same pond water was autoclaved to serve as a sterile control, stoppered with sterile cotton, treated with [5-^{14}C]methoprene (same concentration as nonsterile sample), and exposed (concurrently) to conditions identical with the nonsterile material. At the end of the 13-day exposure, scintillation counting revealed that 98% of the administered radioactivity was still present in solution in the sterile control, and tlc analysis of a 5% aliquot revealed a completely different radioprofile of products (Figure 4) than in the nonsterile water. In addition to the indication of the probable presence of 10–20% undegraded **1**, the radioprofile of the sterile control appeared similar to the profile of photooxidative breakdown prod-

ucts of 1 in sterile solution in buffered distilled water (Quistad *et al.*, 1975).

Identification of Major Metabolite from 13-Day Incubation. In order to facilitate handling of the large (30 l.) volume of the nonsterilized water sample, radioactive compounds were extracted with Amberlite XAD-2 neutral resin. The commercial resin (500 g) was thoroughly washed with water followed by methanol and carefully reequilibrated with water prior to use.

Following filtration to remove sediment, the contents of the separate flasks were made acidic with dilute HCl and then percolated (30 ml/min) through the XAD-2 column. The aqueous effluent was monitored every 2000 ml for radioactivity not retained by the support (less than 20 cpm/ml). After all water had been passed through, the column was eluted with six 500-ml portions of methanol, followed by four 500-ml portions of acetone. All organic wash fractions were sampled for radioactivity, and then examined on tlc to determine metabolite distribution. About 95% of the radioactivity present in water was recovered from elution of the XAD-2 column with methanol and acetone. Following evaluation of label distribution and respective tlc radioscans, fractions 2 and 3 of the methanol wash (containing 82.5% of the eluted radioactivity) were combined and prepared for further purification. These combined fractions were preparatively chromatographed three times on reversed-phase hrlc in the following order: (1) Poragel PN (1 m × 7.8 mm i.d., gradient elution from 100% water to 100% methanol); (2) Corasil/C18 (1 m × 4.9 mm i.d., 40% methanol in water); and (3) Corasil/C18 (as before, except 30% methanol in water). In the last system a radioactive peak showed a k' value of 2.4 while metabolite 3 showed $k' = 7.5$, clearly showing nonidentity. In the first hrlc separation (gradient elution), *only one major, well-resolved radioactive peak was observed* (similar to the tlc behavior seen in Figure 4). The second and third hrlc purifications were therefore of this substance only.

During purification, the metabolite did not cause an appreciable response from the uv detector (254 nm), and a uv spectrum of the purified metabolite showed only end absorption at 210 nm (no characteristic dienoate maximum). Based on the specific activity and actual disintegrations per minute isolated, the final hrlc purification yielded approximately 5.6 μmol of material.

Methylation (diazomethane) of the metabolite yielded a substance which migrated slightly behind 1 on tlc. Radioglc analysis of the (presumed) methyl ester after preparative tlc indicated about 90% chemical purity and 99% radiochemical purity. A single scan 100-MHz pmr spectrum (Varian HA-100) of the methyl ester in CCl_4 revealed characteristic resonances despite a low signal-to-noise ratio: δ 0.88 (d, 3, J = 6 Hz, $CHCH_3$), 1.02 (s, 6, $OC(CH_3)_2$), 3.04 (s, 3, OCH_3), and 3.57 (s, 3, CO_2CH_3). Glc–ms analysis of the single radioactive component (together with pmr data) allowed tentative assignment of the metabolite structure as 6 (see Table I for mass spectral data of the methyl ester of 6). A sample of 7-methoxycitronellic acid was prepared and the free acid and methyl ester showed chromatographic mobility and spectral data identical with metabolite 6 and its methyl ester.

RESULTS AND DISCUSSION

Radiosynthesis of 1. Initially we prepared a sample of methoprene, nominally labeled at C-10 with tritium, due to the relative simplicity, economy, and ease of preparation. Preparation of precursor was simple (selective methoxymercuration of the suitable trienoic ester), and only one radiochemical step was involved, a borotritide reduction of the organomercurial chloride. Although the latter reaction gives variable yields (40–70%), specificity of insertion of label was expected, but not verified.

For synthesis of carbon-14 labeled 1, we chose C-5 of the methoprene skeleton for location of isotope, since it appeared to present the easiest preparation of material with a (hopefully) biologically nonlabile position of radiolabel. A precursor was prepared which on carbonation ($^{14}CO_2$) and reduction afforded [1-^{14}C]citronellol, a common intermediate for radiosynthesis of [5-^{14}C]hydroprene, another dienoate IGR (Henrick *et al.*, 1973). Conversion of this intermediate to [1-^{14}C]citronellal, followed by condensation with a phosphonate reagent, gave a substance convertible to [5-^{14}C]methoprene by the methoxymercuration–borohydride reduction sequence. Had a sample of *only* [5-^{14}C]methoprene been desired, a preparation utilizing "cold" precursor already bearing the terminal methyl ether substituent would have been preferable, since insertion of this functionality in the last step of the radiosynthesis gave only 40–50% yield. Purification of both [10-^{3}H]- and [5-^{14}C]methoprene involved tlc removal of the usual polar by-products of the methoxymercuration–reduction sequence, followed by hrlc separation of 2*E* and 2*Z* isomers. For maximum stability, these samples are stored in dilute benzene solution; the high specific activity (58 mCi/mmol) sample of [5-^{14}C]-1 appears to undergo slow radiodecomposition (*ca.* 5%/year) in benzene, while [10-^{3}H]-1 seems more stable, despite higher specific activity.

Hydrolytic Stability. Sterile aqueous 0.5-ppm solutions of 98% pure [5-^{14}C]methoprene (1), buffered at various pH values, were found to be extremely stable to hydrolysis over 4 weeks at 20° in the dark. Quantitative recovery of radioactivity from the aqueous phase following chloroform extraction was observed for all samples. No degradation (detectable limit ~1%) was seen for the duration of the experiment in sterile water, buffered at pH 7 and 9, and similar stability was noted in the pH 5 buffer through the 3-week sampling time. However, the sterility of the pH 5 buffer was accidently lost between the 21- and 30-day observations, and analysis revealed 59% degradation of methoprene had occurred in that 9-day interval. Such degradation of methoprene was undoubtedly due to microbial action.

Photolytic Stability and Rate of Photoisomerization in Aqueous Solution. In order to ascertain the relative importance of microbial *vs.* photoinitiated breakdown of 1, studies were conducted on the breakdown of methoprene specifically labeled with tritium at C-10. An autoclaved 0.02 *M* phosphate buffer was resaturated with air and treated with an acetone solution of 1 to give a 0.5-ppm aqueous solution, with appropriate steps being taken to maintain sterility throughout. The apparatus was continuously exposed at 20° controlled temperature, to direct sunlight, but the treated solution was subject to the filtering effects of 2–4 mm of Pyrex and several centimeters of 20° cooling water. Methoprene (1) was rapidly photoisomerized ($t_{1/2}$ ~ 30 min; see Figure 2) to a final 2*E*:2*Z* isomeric mixture of 44:56. Previous observations (Henrick *et al.*, 1975b) have shown the same photoequilibrium mixture for hydroprene, a similar dienoate IGR. The 2*Z* isomer of 1 has much lower biological activity (Henrick *et al.*, 1973) than 1 itself, hence photoisomerization of 1 in the field should quickly result in a nondegradative loss of about half the biological activity. However, slow release formulations of 1 maintain biological potency over many days (Graves, 1972).

The 0.5-ppm solutions of 1 were found to undergo photoinitiated decomposition to more polar products, to the extent of 15% in 2 days and 33% in 3 days at 20°. Earlier experiments at 0.1 ppm at two temperatures (24 and 40°) had shown no breakdown at 24° and only 5% breakdown at 40°, in 1 day. Studies on the structure of photoproducts of 1 (Quistad *et al.*, 1975) indicate a considerably shorter half-life for 1 as a 0.5-ppm solution than the 4–5 days at 20° estimated in this work; however, the apparent shorter half-life in the other study may be due in part to higher

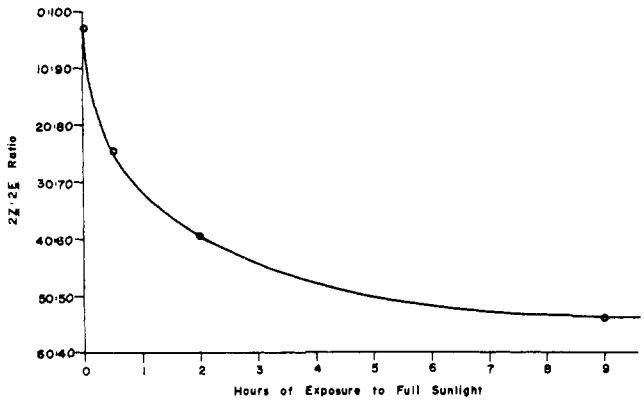


Figure 2. Photoisomerization rate of 97% pure (2*E*)-methoprene as a 0.5-ppm aqueous solution.

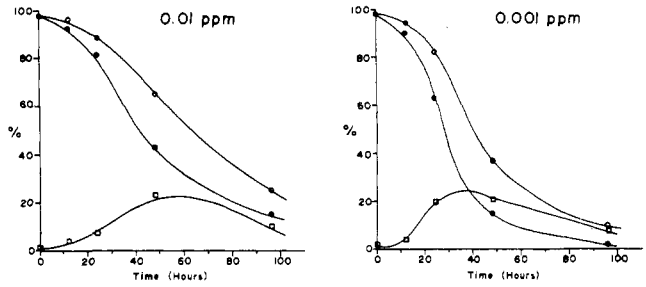


Figure 3. Dissipation rate of [10-^{3}H]methoprene in pond water, incubated outdoors. Conditions were identical except for concentration. Lines indicate percentages of applied dose remaining as: 1 (●), metabolites (□), and total extractable radioactivity (○).

temperatures (39° maximum) or different sunlight conditions.

Metabolism of Tritium-Labeled 1 in Pond Water. These studies (the first metabolism studies on methoprene) were conducted with material specifically labeled at C-10 with tritium, since carbon-14 labeled material was not yet available. Subsequent work has utilized [5-^{14}C]methoprene.

The dissipation rate of pure [10-^{3}H]methoprene was measured at 0.001 and 0.01 ppm in pond water. The dose rates approximate anticipated use rates for mosquito control; incubations were conducted outdoors in full sunlight at ambient temperature to approximate the natural environment. In this experiment, the amount of chloroform-extractable radioactivity and the percentage of methoprene remaining in the extract (tlc analysis) at the various time points were determined. Any radioactive material not coincident with 1 on tlc was considered as "metabolites," although identification was not attempted (due to the extremely small mass).

A sharp and steady decrease in both the amount of extractable radioactivity and the remaining [10-^{3}H]methoprene was observed (see Figure 3), accompanied by an initial increase in the concentration of metabolites, followed by a gradual decrease in extractable metabolites. There was a concomitant steady rise in unextractable radioactivity, apparently present as tritiated water (distillation of extracted sample showed equal amounts of tritium in each volume fraction of distillate). The half-lives observed for 1 in pond water were about 30 hr at 0.001 ppm and 40 hr at 0.010 ppm.

In order to accumulate a sufficient mass of metabolites for isolation and identification, a larger scale incubation of [10-^{3}H]methoprene was undertaken. Pond water (12 l.) was collected at the same site as previously, but 21 days later. The water was treated with an ethanolic solution of 1 and incubated outdoors (full sunlight, ambient temperature) for 66 hr. Extraction of the incubation gave 75% recovery of administered tritium; *all* radioactivity in the chloroform extract was identified as methoprene (60.% of applied dose), hydroxy ester **2** (7.0%), methoxy acid **4** (5.7%), and hydroxy acid **3** (2.6%). Recovered 1 and metabolites were separated and highly purified by tlc followed by hrlc.

Rigorous proof of structural identity was achieved by glc–ms analysis (Table I; acids were converted to methyl esters prior to glc–ms). The most intriguing observation in this experiment was the isolation, along with parent 1 and hydroxy ester metabolite **2**, of corresponding ethyl esters **5** and **7**, also as photoequilibrium mixtures. These ethyl esters contributed 7 and 15%, respectively, of the co-isolated (tlc) mass of the corresponding "parent" isopropyl ester. Since starting 1 was known to be free of any ethyl ester **5**, we are forced to conclude that **5** and **7** arose by transesterification with the ethanol used in dosing the medium (ethanol concentration in the incubation was only 0.08% v/v). Acetone (when permissible) is therefore clearly preferred as a dosing solvent for **1**. Recovered parent 1 and all metabolites were found to be approximately 1:1 photoequilibrium mixtures, as would be expected (*cf.* Figure 2).

In this experiment two independent metabolic pathways were operating: O-demethylation and ester hydrolysis. Bacterial O-demethylation has been studied extensively (Ribbons and Harrison, 1972); similarly, carboxylic esterase cleavage is a well-precedented, unremarkable process in all animals and bacteria (Parke, 1968). O-Demethylation of methoprene to **2** results in a substance with about tenfold lower activity on *Aedes aegypti* (Staal, 1972) than methoprene (Henrick *et al.*, 1973), while ester hydrolysis of 1 or **2** to **4** and **3**, respectively, yields biologically inactive products (Staal, 1972; Henrick *et al.*, 1973).

The 60% recovery of 1 isomers was more than expected from the dissipation rate studies; however, the latter were conducted at lower concentrations and with a different water sample. Since part of the tritium label of 1 was lost apparently as ^3H_2O during incubations, we sought to explore the nature of metabolites produced on longer incubation of 1 using carbon-14 labeled material.

Metabolism of Carbon-14 Labeled 1 in Pond Water. When [5-^{14}C]methoprene became available, pond water samples were collected from the same source and treated with an acetone solution of [5-^{14}C]-1, with an autoclaved sample of this water at the same treatment rate serving as control. Both control and active incubations were exposed identically outdoors. At 312 hr (13 days), analysis of the active incubation revealed the absence of any 1, so both control and incubation were worked up and analyzed. The single major metabolite was isolated, purified, converted to the methyl ester, and identified by nmr and glc–ms as 7-methoxycitronellic acid (**6**). This metabolite was formed in high yield (29% of the applied dose, or 61% of the radioactivity remaining in solution), with the remaining 39% of recovered radioactivity constituting a nondistinct "smear" [determined by tlc-radioscanning (Figure 4) and gradient elution hrlc with scintillation counting of fractions].

Metabolite **6** was probably formed by a combination of microbial metabolic and photolytic processes; **6** has been observed as a photolytic product of 1 (Quistad *et al.*, 1975), but in much lower quantitative abundance than observed in this work. The profile of radioactive products found in the autoclaved control indicated the presence of 10–20% intact **1**, and was similar to that observed in studies on photooxygenative breakdown of solutions of 1 in sterile aqueous buffers (Quistad *et al.*, 1975).

The low recovery (48%) of administered radioactivity from the microbially active water (*vs.* 98% recovery in autoclaved control) may well reflect formation of $^{14}CO_2$, since subsequent studies have shown $^{14}CO_2$ evolution on

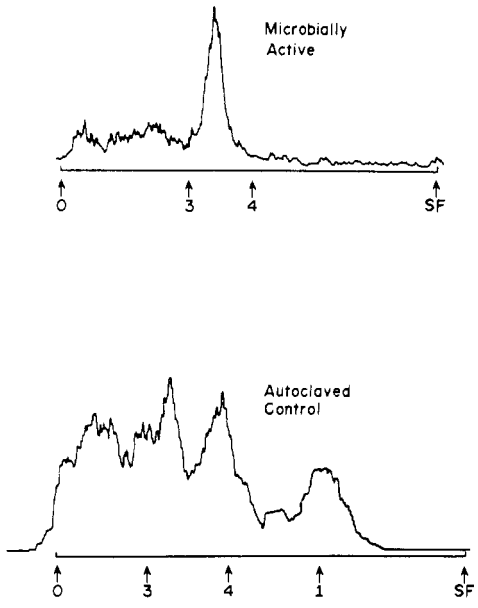


Figure 4. Analysis by tlc–radioscanning of extracts of 13-day incubation of [5-^{14}C]methoprene in pond water (sterilized and nonsterilized). Tlc plates were developed once in benzene–ethyl acetate–acetic acid (100:30:3). Position of origin (O), solvent front (SF), and relative positions of chromatographic side markers of metabolites **3** and **4** and parent **1** are indicated by arrows.

metabolism of [5-^{14}C]methoprene by plants (Quistad *et al.*, 1974a), soils (Schooley *et al.*, 1975), and in a steer and a cow (Chamberlain *et al.*, 1975). In a steer metabolism study, formation of specifically labeled [2-^{14}C]acetate has been demonstrated to be an important metabolic product of [5-^{14}C]methoprene (Quistad *et al.*, 1974b), accounting therefore for evolution of $^{14}CO_2$ as well as incorporation into certain natural products. Further metabolism of **6** (by α oxidation to $^{14}CO_2$ or by β oxidation to [^{14}C]acetate) would thus explain the radioactivity loss. The notable absence of primary metabolites **2**, **3**, and **4** at the three sampling times is likely due to the differing conditions in experiments with [10-^{3}H]-**1** and [5-^{14}C]-**1**. The microbial populations were likely quite different in the two studies (water samples collected in February *vs.* August), and the maximum incubation temperature of 42° was much higher in the study with [5-^{14}C]-**1**.

In summation, the metabolism of **1** by aquatic microorganisms is extensive and apparently more rapid than the competing photoinitiated decomposition of **1** in solution. However, the competing effects are of the same order of magnitude, and the relative importance of these two principal modes of degradation in water solution would be dependent on many environmental factors in a given field situation: weather conditions, temperature, and microbial population. Indeed, in field trials aqueous solutions of methoprene (from an emulsifiable concentrate) degrade so quickly as to be of little use as a mosquito larvicide, but slow release micro-encapsulated formulations (Graves, 1972) give excellent control of mosquito larvae (Schaefer and Wilder, 1973; Schaefer and Dupras, 1973).

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