plant. Radioactivity did not move from treated leaves to stalk or to newer leaves emerging from the spindle.

The translocation behavior compares closely with that observed for the *s*-triazine herbicides and contrasts with picloram, which appears to move in the phloem from older to younger leaves. Movement of surface residues of metribuzin is probably that of diffusion in the extracellular spaces. Foliar residues of 2,4-D remained largely at the treated site, with small amounts apparently translocated to higher as well as lower leaves and other plant parts (Ashton, 1958).

ACKNOWLEDGMENT

Metribuzin is being developed as Sencor herbicide by Chemagro, a division of Baychem Corp., Kansas City, Mo. We are indebted to that firm for the radioactive compound and samples of metabolite products. A. Maretski of this Experiment Station prepared the beef liver enzyme extract.

LITERATURE CITED

Ashton, F. M., *Weeds* **6**, 257 (1958).
Eue, L., Westphal, K., Dichoré, K., Meiser, W., in "3rd Symposium on New Herbicides," Vol. 1, European Weed Research Council, Versailles, France, 1969, pp 125–132.
Hilton, H. W., Kameda, S. S., Nomura, N. S., *J. Agr. Food Chem.* **21**, 124 (1973).
Hilton, H. W., Nomura, N. S., Kameda, S. S., *Anal. Biochem.* **49**, 285 (1972).
Hilton, H. W., Yuen, Q. H., Nomura, N. S., *J. Agr. Food Chem.* **18**, 217 (1970).
Pape, B. E., Zabik, M. J., *J. Agr. Food Chem.* **20**, 72 (1972).
Stanley, C. W., Thornton, J. S., "A Gas Chromatographic Method for the Determination of Sencor and Metabolites in Sugarcane and Products," Chemagro Division of Baychem Corp., Kansas City, Mo., Report No. 35115, Dec 1972, private communication.
Thornton, J. S., Stanley, C. W., Schumann, S. A., 164th National Meeting of the American Chemical Society, New York, N. Y., Aug 1972, Abstract PEST-6.

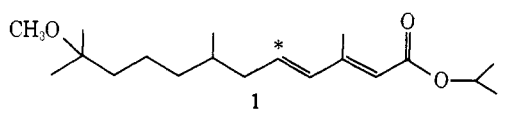
Received for review July 16, 1973. Accepted February 11, 1974. Published with the approval of the Director as Journal Series Paper 353.

Environmental Degradation of the Insect Growth Regulator Methoprene (Isopropyl (2*E*,4*E*)-11-Methoxy-3,7,11-trimethyl-2,4-dodecadienoate). I. Metabolism by Alfalfa and Rice

Gary B. Quistad,* Luana E. Staiger, and David A. Schooley

The metabolic fate 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 alfalfa and rice as a function of time. The major metabolic pathways involved ester hydrolysis, O-demethylation, and oxidative scission of the 4-ene double bond. The principal nonpolar metabolite was 7-methoxycitronellal which was isolated from vapors evolved from the plants. Chromatographic evidence strongly suggests the incorporation of radiolabel from the extensively degraded (2*E*)-[5-^{14}C]methoprene molecule into carotenoids, chlorophylls, and other higher molecular weight plant constituents. Yields of primary metabolites were grossly inflated (10× too high) unless thin layer chromatography was accompanied by prior purification by gel permeation chromatography because much radioactivity was attributed to co-eluting natural products. Rapid metabolism of methoprene to biologically innocuous derivatives was characteristic of both rice and alfalfa.

The possibility of using insect growth regulators (IGR's) to control insect pests by disrupting metamorphosis has received considerable attention (Menn and Beroza, 1972). A new class of potent IGR's (alkyl 3,7,11-trimethyl-2,4-dodecadienoates) has shown efficacy in large-scale field tests (Henrick *et al.*, 1973). A slow-release formulation of methoprene IGR (**1**, isopropyl (2*E*,4*E*)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate; trademark, Altosid) was effective in controlling mosquito larvae (Schaefer and Wilder, 1973), but produced no adverse biological effects on most nontarget aquatic organisms (Miura and Takahashi, 1973) and was rapidly degraded under natural field conditions (Schaefer and Dupras, 1973).



1

Methoprene is the first IGR for which the Environmental Protection Agency has granted an experimental permit. In order to assess the biodegradability of methoprene when used as a mosquito larvicide, we report its metabolic fate in alfalfa and rice; this report represents part of a comprehensive study of the environmental fate of methoprene.

Zoecon Corporation Research Laboratory, Palo Alto, California 94304.

MATERIALS AND METHODS

(2*E*,4*E*)-[5-^{14}C]Methoprene was radiosynthesized (by Dr. John C. Leak, ICN) by condensing [1-^{14}C]citronellal with diisopropyl 3-isopropoxycarbonyl-2-methyl-2-propenyl phosphonate (following the methods of Henrick *et al.*, 1973) to yield [5-^{14}C]isopropyl (4*E*)-3,7,11-trimethyl-2,4,10-dodecatrienoate. This substance was then subjected to methoxymercuration–borohydride reduction, generating [5-^{14}C]methoprene. The approximately 80:20 2*E*:2*Z* mixture was separated and purified by preparative high-resolution liquid chromatography (hrlc) to give (2*E*,4*E*)-[5-^{14}C]methoprene (58 mCi/mmol; 97.9% 2*E*,4*E*; 1.5% 2*Z*,4*E*). While tlc indicated 100% radiochemical purity as [5-^{14}C]methoprene, radio glc analysis revealed 0.6% of an unknown radioactive impurity which was chromatographically inseparable from methoprene by hrlc. The [5-^{14}C]methoprene was diluted with 98% (2*E*,4*E*)-methoprene to a specific activity of 5.0 mCi/mmol for these studies. Authentic metabolite standards were synthesized

by the Chemical Research Department, Zoecon Corporation.

Plant Treatment. An emulsifiable concentrate of methoprene IGR was prepared with the following composition: [5-^{14}C]methoprene, 77.45% (7 μCi); alkyl arylsulfonates, 8.00%; and aromatic solvent, 14.55%. The emulsifiable concentrate (645 μg) was diluted with water (1 ml) containing X-77 (45 mg %, Colloidal Products) as a surfactant.

Duplicate samples of 68 single alfalfa leaves for each time interval were painted with the diluted methoprene emulsive concentrate by camel hair brush (11 μg of methoprene/cm^2 of leaf, *ca.* 1 lb/acre). Duplicate samples of eight rice leaf blades were also painted with emulsive concentrate (11 μg/cm^2 of leaf). The potted alfalfa (3–4 months old) and rice (1 month old) plants were placed in an indoor growth chamber: 19.5° night (8 hr), 24.5° day (16 hr), relative humidity 40–50%, 4000-lx luminescence.

Radioassay Procedures. Radioactivity was quantitatively determined on either Packard Instruments Model 3380/544 or Model 2425 liquid scintillation spectrometers. The extent of quenching in samples was determined by the automatic external standardization (AES) method. The scintillation fluid generally used for organic extracts or silica gel scrapings was a 0.55% solution of PPO in a 2:1 mixture of toluene-2-methoxyethanol, although Insta-Gel (Packard Instruments) was used for some aqueous samples. Unextractable radioactivity in biological samples was determined following total combustion using a Packard Model 305 sample oxidizer. The carbon-14 reagents program used in our oxidizer yielded a solution of ethanolamine (5 ml), methanol (9 ml), and scintillator (5 ml) (toluene containing 15 g/l. of PPO and 1 g/l. of bis-MSB). Location of radioactive bands on tlc plates was determined using a Packard Model 7201 radiochromatogram scanner. Side markers of reference standards of methoprene and dienoic metabolites were readily determined by their very strong quenching of fluorescence on silica gel GF_{254}. Other reference standards were visualized by spraying side markers with ceric ammonium sulfate. Quantitation of radioactivity in bands was achieved by scraping the silica gel into a scintillation vial with use of the aforementioned fluid.

Chromatography. Thin layer chromatography (tlc) plates used were precoated silica gel GF_{254} purchased from Analtech, Inc.

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, acid washed, DMCS treated) and a flame ionization detector. For coupled gas chromatographic–mass spectrometric 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 (L. L. Dunham).

All gel permeation chromatography (gpc) separations were performed with an instrument utilizing a Waters Associates M-6000 solvent pump, a Waters loop injector (with 1.1-ml loop), a Chromatronix Model 230 ultraviolet absorbance detector, and a 1 m × 12.7 mm i.d. column slurry packed with Bio-Beads S-X2 (Bio-Rad Laboratories), eluted with distilled methylene chloride at a flow rate of 1.5 ml/min.

High-resolution liquid chromatographic (hrlc) purification of hydroxycitronellic acid (**7**) (as its methyl ester) was achieved by use of the Waters M-6000 pump, a Waters septum injector, a Waters R-401 differential refractometer, and a 0.5 m × 7.8 mm i.d. column of LiChrosorb SI 60, 20 μ, eluted with 50% diethyl ether–50% pentane at a flow rate of 5.0 ml/min. Methoxycitronellal (**5**) was purified by hrlc utilizing a DuPont Instruments Model 830 chromatograph with a uv photometer and employing a 0.5 m × 2.4 mm i.d. column of LiChrosorb SI 60, 10 μ, eluted with 10% diethyl ether–90% pentane at a flow rate of 0.3 ml/min.

Metabolite Extraction, Separation, and Characterization. At various times treated rice leaves were homogenized in a Broeck tissue grinder with chloroform (3 × 40 ml) and then with methanol (3 × 40 ml). The chloroform fraction was concentrated *in vacuo* and examined by silica gel GF tlc with development in hexane–ethyl acetate (100:15) followed by a second development in benzene–ethyl acetate–acetic acid (100:30:3). Metabolites were tentatively characterized by comparative tlc behavior with known standards. The positional distribution of metabolites was determined by radioscanning, and distinct radioactive bands were quantified by scraping the silica gel from the plate for liquid scintillation counting.

The nonpolar metabolites were poorly resolved by tlc because of a multitude of products. The chloroform extract from four rice plants (two treated 1 day and two treated 3 days) was submitted to preliminary tlc after extraction in order to separate methoprene (R_f 0.72) from metabolites. The methoprene was eluted from silica gel with freshly distilled THF and further separated from contaminating plant constituents by gel permeation chromatography (gpc). The nonpolar metabolite band (R_f on silica gel 0.16–0.55) was also separated from contaminating plant constituents by gpc and, as before, aliquots (10 μl) from 1-min fractions were radioassayed to determine a histogram of radioactivity *vs.* uv absorbance (and molecular weight). However, rather than mere separation of metabolites from natural plant extractives, four distinct molecular weight fractions of radioactivity were found. The two lower molecular weight (<600) fractions were examined by tlc. Distinct metabolite bands were scraped and eluted with freshly distilled THF. Structural identity with reference standards of metabolites (*i.e.*, **2**–**7**) was further verified by glc analysis (after diazomethane treatment of acidic metabolites). More rigorous criteria were applied for identification of metabolites **5** and **7**.

The sample of hydroxycitronellic acid (**7**) for structure proof was prepared by extracting four treated rice plants with methanol. Treatment of the methanol extract with sulfatase–cellulase followed by methylation with diazomethane and preparative tlc gave 4.5 × 10^5 dpm in the 4-hydroxycitronellic acid methyl ester region. There were at least three distinct radioactive impurities and a maximum of 1.1 × 10^5 dpm (2.0 μg) of 7-hydroxycitronellic acid methyl ester. The radiolabeled metabolite was preparatively chromatographed on hrlc with radioassay of timed fractions of the total effluent; coincidence of radiolabel with the RI detector signal for a separate injection of standard was observed. The purified sample (0.80 μg) of methyl ester of the metabolite was analyzed by gc–mass spectra: *m/e* (relative intensity) 59 (76), 69 (28), 101 (100), 109 (29), 110 (15), 128 (10), 152 (7), 153 (5), 155 (12), 170 (2), 187 (2), and no M$^+$. The mass spectrum ob-

Table I. Enzymatic Cleavage of Alfalfa Conjugates

Enzyme (source)	% cleavage
Peptidase (hog mucosa)	1
β-Glucuronidase (*Escherichia coli*)	2
β-Glucosidase (almonds)	7
Phosphatase (wheat germ with lipase)	10
NaOH (0.1 *M*), 37°, 20 hr	24
HCl (0.1 *M*), 37°, 20 hr	25
β-Glucuronidase (*Helix pomatia* contains sulfatase)	43
Sulfatase (*H. pomatia*)	52
Cellulase (*Aspergillus niger*)	56
Cellulase and sulfatase	57

Table II. Metabolites from Alfalfa Metabolism of Methoprene

	Yield, % of applied dose, at time, days					
	0	1	3	7	15	30
Nonpolar metabolites ($CHCl_3$ extract)						
Methoprene (**1**)	97.8	65.0	30.6	10.3	5.8	1.0
Metabolites (at least 10 including **2**, **3**, and **6**)	0.0	3.8	8.8	6.8	6.1	3.6
Origin	0.9	0.8	9.8	11.3	8.8	7.5
Polar metabolites (MeOH extract)						
Conj. methoxy acid (**4**)	0.0	1.9	2.1	2.2	0.9	0.3
Conj. methoxycitronellic acid (**6**)	0.0	0.3	0.7	0.4	0.8	0.4
Conj. hydroxy acid (**3**)	0.0	1.7	3.3	7.4	3.2	5.3
Conj. hydroxycitronellic acid (**7**)	0.0	2.1	2.1	2.2	3.1	2.3
Origin	1.0	2.5	5.8	12.3	17.8	23.3
Untreated plant	0.0	0.3	0.4	0.3	0.6	0.6
Roots						0.0
Unextractable radioact.	0.1	0.4	3.0	5.2	6.3	9.7
Conj. hydroxy acid (**3**)						0.9
[^{14}C]Glucose						0.2
[^{14}C]Cellobiose						0.5
Total recovered radioact. (excluding volatiles)	99.8	78.8	66.6	58.4	53.4	54.0

Table III. Metabolites from Rice Metabolism of Methoprene

	Yield, % of applied dose, at time, days				
	0	1	3	7	15
Nonpolar metabolites ($CHCl_3$ extract)					
Methoprene (**1**)	97.5	33.8	3.3	2.5	0.4
Metabolites (at least 13 including **2**, **4**, **6**, and **7**)	0.0	13.3	12.0	5.4	7.9
Origin	0.0	4.9	4.0	3.9	3.1
Polar metabolites (MeOH extract)					
Conj. methoxycitronellic acid (**6**)	0.0	1.0	0.8	1.1	1.2
Conj. hydroxycitronellic acid (**7**)	0.0	0.1	0.2	0.3	0.3
Conj. unknowns (at least 4)	0.0	0.5	0.8	1.3	1.6
Origin	0.0	2.0	2.3	2.7	4.6
Roots					0.1
Unextractable radioact.	0.0	1.2	3.2	5.6	11.8
Conj. methoxycitronellic acid (**6**)					0.5
[^{14}C]Glucose					0.1
[^{14}C]Cellobiose					0.1
Total recovered radioact. (excluding volatiles)	97.5	56.8	26.6	22.8	31.0

served was identical with that of an authentic synthetic standard of 7-hydroxycitronellic acid methyl ester. Satisfactory mass spectral identification of other metabolites was previously obtained in water and soil metabolism studies (Schooley *et al.*, 1974).

The above section specifically pertains to identification of radioactive constituents in the chloroform extract of rice. However, the chloroform extract of alfalfa was analyzed in a similar manner. Untreated alfalfa leaves and stems were combined and homogenized in methanol (3 × 25 ml) and the amount of radioactivity was determined by liquid scintillation counting. Likewise, the radioactivity in roots was measured.

Enzymatic Cleavage of Conjugates and Identification of Aglycones. The polar metabolites (methanol extract) were concentrated and an aliquot (25% in 100 μl of methanol) was enzymatically cleaved at 37° for 20 hr using citrate–phosphate buffer (5.0 ml, pH 4.5, made by mixing 0.1 *M* citric acid with 0.2 *M* disodium phosphate), sulfatase containing β-glucuronidase (3 mg, *Helix pomatia*, Sigma Chemical Co.), and cellulase (5 mg, *Aspergillus niger*, Sigma Chemical Co.). Studies on the effect of varying time, temperature, and enzyme(s) showed these were the optimum conditions for conjugate cleavage (Table I). After enzymatic hydrolysis, the aglycones were extracted from the aqueous phase with ether (3 × 10 ml). The metabolites in the ether extract were characterized by tlc and glc (after methylation of acids with diazomethane).

Attempted Solubilization of Unextractable Radioactivity with Cellulase. The residual pulp of plant tissue (after exhaustive extraction with chloroform and methanol) was subjected to total combustion to determine the amount of unextractable radioactivity. A 10-mg portion of this plant residue was also treated with cellulase (10 mg) under the following conditions: citrate–phosphate buffer (1.0 ml, pH 4.5), 20 hr, 47°. The aqueous hydrolysate (48% hydrolysis of alfalfa pulp, 22% for rice pulp) was extracted with ether (3 × 4 ml). Products were characterized by tlc. The aqueous phase (46–58% of solubilized radioactivity) was concentrated *in vacuo* and chromatographed (MN 300F cellulose, normal, 250-μ precoated, Analtech) with β-D(+)-cellobiose and β-D(+)-glucose standards. After development in isopropyl alcohol–acetic acid–water (3:1:1), the reducing sugars were detected by

Table IV. Characterization of Alfalfa Metabolites[a]

Isolated compound	Tlc	Tlc of methyl ester	Glc	Isolated mass, μg	% yield[b]	Δ^2 Z:E
Nonpolar metabolites (one 7-day plant)						
1. Methoprene (**1**)	+	NA	+	24	6.3	16:84
2. Hydroxy acid (**3**)	+	+	+	2	0.7	36:64
3. Hydroxy ester (**2**)	+	NA	+	2	0.6	27:73
4. Methoxycitronellic acid (**6**)	+	+	NA	0.4	0.1	NA
Nonpolar metabolites (two 3-day plants)						
1. Methoprene (**1**)	+	NA	+	180	21	7:93
2. Mixture of **2** and **3**	+	+	NA	NA	1:2	NA
3. Methoxycitronellic acid (**6**)	+	+	NA	4	0.6	NA
Conjugates (aglycone) (three plants, 7, 15, and 30 day)						
1. Hydroxy acid (**3**)	+	+	+	19	1.7	13:87
2. Methoxy acid (**4**)	+	+	+	1	0.1	24:76
3. Methoxycitronellic acid (**6**)	+	+	−	2	0.2	NA
4. Hydroxycitronellic acid (**7**)	+	+	+	10	1.2	NA

[a] NA, does not apply or not attempted; +, positive chromatographic behavior; −, negative chromatographic behavior. [b] Expressed as per cent of applied dose.

spraying with a solution of aniline (0.9 ml), oxalic acid (0.45 g), and water (100 ml), and then heating for 15 min at 100°.

Volatilization of Methoprene and Metabolites from Glass Plates and Leaf Surfaces. [5-^{14}C]Methoprene (500 μg, 7 μCi, 5.3 mCi/mmol) in 1 ml of benzene was applied to 43-cm^2 areas on 20 × 20 cm Pyrex plates by a camel hair brush. This application rate (11 μg/cm^2) approximates the treatment rate of alfalfa and rice leaves (1 lb/acre). The plates were placed under the same indoor growth chamber conditions as the plants. At various time intervals the radioactivity was rinsed from the plate with methanol (50 ml). The amount of radioactivity was determined by liquid scintillation counting and product identity by tlc.

[5-^{14}C]Methoprene (1.4 μg) was applied in benzene to a 0.13-cm^2 area on a Pyrex plate. This surface treatment rate approximates 1 lb/acre. The disappearance of ^{14}C-labeled volatiles was monitored by averaging three radiochromatogram scans as a function of time. Two other spots corresponding to 0.6 and 50 lb/acre were also monitored (0.8 and 71 μg/0.13 cm^2, respectively).

The evaporation of methoprene and volatile metabolites was studied by painting four rice leaves with an emulsive concentrate of [5-^{14}C]methoprene (250 μg, 1 lb/acre). The two potted rice plants were enclosed in an inverted erlenmeyer flask (1000 ml) with an exit to traps at the top. Air (400 ml/min) was drawn through the flask by applying a slight vacuum. Volatilized methoprene and metabolites were collected in a gas washing bottle (250 ml) containing a glass wool plug and immersed in a Dry Ice–acetone bath. Radioactive carbon dioxide was collected in two 5% potassium hydroxide traps (200 ml). Structures of volatile products were verified by tlc and glc. The structure of 7-methoxycitronellal (**5**) was confirmed by gc–mass spectra following rigorous purification by hrlc: *m/e* (relative intensity) 43 (4), 55 (3), 69 (6), 73 (100), 81 (8), 95 (3), 121 (3), 137 (2), 171 (7), and no M^+. A negligible amount (<3%) of the ^{14}C label trapped by Dry Ice–acetone was attributable to $^{14}CO_2$. The radioactivity trapped in the gas washing bottle containing potassium hydroxide was verified as $^{14}CO_2$ by precipitation as barium carbonate and quantitative loss of ^{14}C label after acidification followed by purging with carbon dioxide (Dry Ice). The only distinct volatile compounds found were methoprene (14% of applied dose) and 7-methoxycitronellal (**5**, 13% of applied dose) which together represented greater than 80–90% of the trapped radioactivity. Similarly, monitoring of volatiles from 68 alfalfa leaflets treated with [5-^{14}C]methoprene (500 μg, 1 lb/acre) gave only methoprene (3%) and **5** (2%) after 7 days.

RESULTS AND DISCUSSION

The recovery of unmetabolized methoprene IGR (Tables II and III) approximated a first-order decay plot with a half-life ($t_{1/2}$) of *ca.* 2 days for alfalfa and 0.5 day for rice. The rate of metabolism in rice was about four–eight times faster than the rate of alfalfa. After 30 days in alfalfa, 1% of the applied methoprene was retrieved with retention of chemical integrity, but only 0.4% of the applied methoprene was found from rice metabolism after 15 days. Since photochemical decomposition was minimized by the relatively low light intensity employed (4000 lx, *ca.* 20× less than sunlight), the residual methoprene should be considerably less under field conditions after comparable times. In addition, a high surface treatment rate (1 lb/acre, *ca.* 40× the maximum field treatment rate of methoprene as a mosquito larvicide) was used to pressure the plant's metabolic system. The metabolic rate of plants under actual field conditions would probably be much higher. The rather rapid initial disappearance of methoprene is attributed to volatility which will be discussed later.

Five primary nonpolar metabolites were detected in the plants (Tables II and III). Oxidative demethylation of methoprene gave isopropyl 11-hydroxy-3,7,11-trimethyl-2,4-dodecadienoate (**2**) which subsequently was hydrolytically cleaved to 11-hydroxy-3,7,11-trimethyl-2,4-dodecadienoic acid (**3**). Initial hydrolytic cleavage of methoprene gave 11-methoxy-3,7,11-trimethyl-2,4-dodecadienoic acid (**4**) which could also give rise to **3** by oxidative O-demethylation. Oxidative scission of the 4-ene double bond of methoprene to 7-methoxycitronellal (**5**) was also a major metabolic pathway. The methoxycitronellal either evaporated or was oxidized to 7-methoxycitronellic acid (**6**). O-Demethylation of **6** resulted in 7-hydroxycitronellic acid (**7**). The primary metabolites (Tables II and IV) in alfalfa after 7 days were hydroxy acid **3** (0.7%), hydroxy ester **2** (0.6%), and methoxycitronellic acid (**6**, 0.1%). The primary nonpolar rice metabolites (Tables III and V) after 3 days were methoxy acid **4** (0.1%), methoxycitronellic acid (**6**, 0.2%), hydroxy ester **2** (0.9%), and hydroxycitronellic acid (**7**, 0.9%). Differences in the metabolic pathways in rice and alfalfa were evident. The major unconjugated alfalfa metabolite (**3**) was completely absent in rice. A trace amount (0.1%) of **4** was characterized in rice, but not alfalfa. The abundance of hydroxy ester **2** in rice suggests a deficient esterase mechanism for hydrolytic cleavage of **2** to hydroxy acid **3**. Oxidative scission of the 4-ene double

Table V. Characterization of Rice Metabolites[a]

Isolated compound	Tlc	Tlc of methyl ester	Glc	Isolated mass, μg	% yield[b]	Δ^2 Z:E
Nonpolar metabolites (four plants; two 1 day and two 3 day)						
1. Methoprene (**1**)	+	NA	+	307	16.4	6:94
2. Methoxy acid (**4**)	+	+	−	1	0.1	NA
3. Methoxycitronellic acid (**6**)	+	+	−	2	0.2	NA
4. Hydroxycitronellic acid (**7**)	+	+	+	7	0.9	NA
5. Hydroxy ester (**2**)	+	NA	+	9	0.9	7:93
Conjugates (aglycone) (4 plants; 1, 3, 7, and 15 day)						
1. Methoxycitronellic acid (**6**)	+	+	+	5	0.3	NA
2. Hydroxycitronellic acid (**7**)	+	+	+	2	0.1	NA

[a] NA, does not apply or not attempted; +, positive chromatographic behavior; −, negative chromatographic behavior. [b] Expressed as per cent of applied dose.

Table VI. Tlc Systems for Metabolites[a]

Compound	SS-1	SS-1 then SS-2	SS-3
Methoprene (**1**)	0.36	0.72	1.0
Hydroxy ester (**2**)	0.10	0.41	1.0
Hydroxy acid (**3**)	0.00	0.25	1.0
Methoxy acid (**4**)	0.02	0.41	1.0
Methoxycitronellic acid (**6**)	0.01	0.35	1.0
Hydroxycitronellic acid (**7**)	0.00	0.17	1.0
(**3**) Methyl ester		0.36	1.0
(**4**) Methyl ester		0.70	1.0
(**6**) Methyl ester		0.61	1.0
Hydroxycitronellic acid methyl ester		0.29	
β-D(+)-Glucose			0.48
β-D(+)-Cellobiose			0.28
Methoxycitronellal (**5**)	0.22	0.62	1.0

[a] SS-1, hexane–EtOAc (100:15); SS-2, benzene–EtOAc–HOAc (100:30:3); SS-3, isopropyl alcohol–HOAc–H_2O (3:1:1).

bond of methoprene (or **4**) to generate **5** (and subsequently **6** and **7**) was much more prevalent in rice than alfalfa. These low yields (based on applied dose) of primary methoprene metabolites suggest that the initially formed products are subsequently degraded or conjugated.

The concentration of nonpolar "metabolites" maximized after 1 day in rice and after 3 days in alfalfa. After 1 week a plateau (Tables II and III) in the concentration of nonpolar "metabolites" was reached, which would suggest either a steady state for product formation-degradation (unlikely due to the rapid depletion of methoprene) or that the nonpolar "metabolites" are relatively stable to further catabolism. Subsequent gel permeation chromatographic (gpc) separation of these extracts showed considerable radiolabel (56–70% of nonpolar metabolites) present in high molecular weight bands corresponding to natural plant constituents, *i.e.*, nonmetabolite residues (Rosenblum *et al.*, 1971). It is suggested that the peak in nonpolar "metabolite" concentration (Tables II and III, 3 days for alfalfa and 1 day for rice) represents a maximum for primary metabolites (*i.e.*, **2**–**7**). The plateau after 1 week probably reflects much more extensive degradation to secondary lipophilic nonmetabolite residues which occur naturally within the plants and therefore have a slower turnover rate.

A variety of enzymes (Table I) were employed to cleave the polar conjugates of the methanol extract in order to release characterizable aglycones. Optimal enzymatic hydrolysis was achieved with a mixture of cellulase and sulfatase containing β-glucuronidase (57% cleavage). The conditions for enzymatic hydrolysis of conjugates were optimized with regard to time and pH. Combinations of various enzymes were utilized in an attempt to enhance deconjugation.

The most abundant aglycone in alfalfa (Table II) after enzymic cleavage was the hydroxy acid **3**. It was most prevalent at 7 days (7.4%), but some remained at 30 days (5.3%), indicating considerable conjugate stability toward further breakdown. Other metabolite conjugates in alfalfa were methoxy acid **4** (2.2%), methoxycitronellic acid (**6**, 0.8%), and hydroxycitronellic acid (**7**, 3.1%). After 7 days there was ten times more conjugated hydroxy acid **3** than free **3**, which substantiates the transient nature of primary metabolites. The absence of conjugated hydroxy ester **2** is not surprising since it must conjugate through its tertiary alcohol functionality. Glycosides of tertiary alcohols are less common but certainly known [*e.g.*, the glucoside of mevalonic acid lactone (Tschesche *et al.*, 1971) and many carotenoid glycosides (Isler, 1971)]. A separation of intact conjugates was attempted by Ecteola-cellulose tlc (methanol–water–acetic acid, 100:20:3). A broadly diffuse band of radioactivity was found indicating nonhomogeneity of conjugate composition (at least three partially separable conjugates which were not characterized further).

The predominant aglycones in rice (Table III) were methoxycitronellic acid (**6**, 1.2%) and hydroxycitronellic acid (**7**, 0.3%). At least four unknown aglycones represented a total of 1.6% after enzymatic cleavage. Conjugates were apparently fairly stable toward further metabolism.

Structural assignments for metabolites were based on preliminary tlc (Table VI), gpc, and glc with comparison to authentic standards. Acids were converted to their methyl esters prior to glc. All dienoate standards possessed double markers since 2*Z* isomers and 2*E* isomers were easily separable by glc and metabolites were always present as an isomeric mixture. The characterization of metabolites is summarized in Tables IV and V.

The 2,4-dodecadienoate structure of methoprene IGR is quite susceptible to photoisomerization of the 2-ene double bond. The 2*E* isomer is readily converted to the biologically less active (Henrick *et al.*, 1973) 2*Z* isomer in aqueous solution. A photoequilibrium mixture (approximately 1:1 isomeric ratio of 2*Z* to 2*E*) is recovered after exposure of aqueous methoprene to full sunlight for 5 hr and a 1:1 ratio was always observed for field samples of aqueous methoprene (Schaefer and Dupras, 1973). The 4-ene double bond is photochemically stable. Therefore, it was desirable to observe cis–trans differences in methoprene and 2,4-dienoic metabolites in order to assess the importance of 2-ene isomerization on or within the leaf. The 2-ene cis–trans ratios are given in Tables IV and V. The (2*E*)-methoprene isomerized slowly on the leaf surface to (2*Z*)-methoprene. After 3 days on alfalfa there was 5% isomerization (*Z*:*E* from 2:98 to 7:93). After 7 days the recovered methoprene had a *Z*:*E* ratio of 16:84 (14%

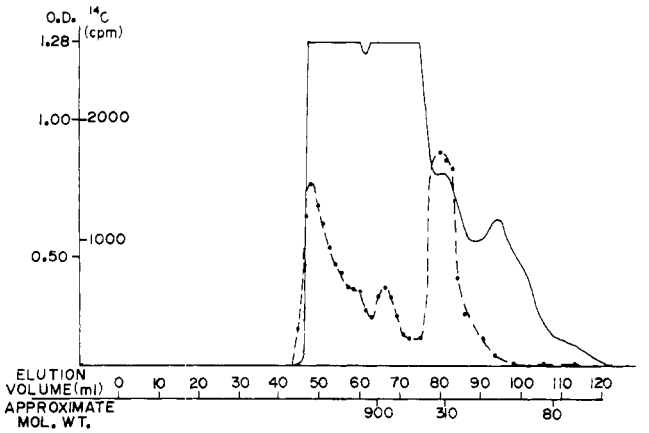


Figure 1. Gel permeation chromatography of metabolite band from methoprene IGR metabolism by alfalfa. Radioactivity (counts per minute) indicated by dashed line (- -); ultraviolet absorbance at 254 nm (1.28 OD units full scale) indicated by solid line (—).

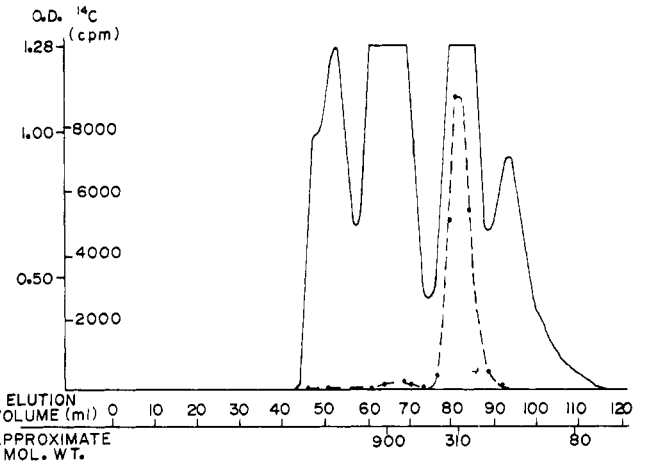


Figure 2. Gel permeation chromatography of methoprene band from methoprene metabolism by alfalfa. Radioactivity (counts per minute) is indicated by dashed line (- -); ultraviolet absorbances at 254 nm (1.28 OD units full scale) are indicated by a solid line (—).

isomerization). Metabolites from alfalfa were substantially isomerized. The 2*E* isomer always predominated by at least two-three times over the 2*Z* isomer. Since the intensity of light in this experiment was *ca.* 20× less than sunlight, considerably more cis-trans isomerization would be expected for methoprene and dienoic metabolites under field conditions. Similar photoisomerization (50%) of the 2-ene double bond and stability of the 4-ene double bond has been observed for the (2*Z*,4*E*)-dienoate chromophore in abscisic acid (Bonnafous *et al.*, 1973).

Preliminary tlc of the presumed nonpolar metabolites gave nondescript peaks of radioactivity suggestive of a multitude of products. In order to separate the metabolites from substantial amounts of contaminating natural plant components, the metabolite mixture was separated from methoprene and highly polar products by tlc. The crude metabolites were collectively removed from higher molecular weight lipids by gel permeation chromatography on Bio-Beads S-X2 (Stalling *et al.*, 1972; Dark and Limpert, 1973) (Figures 1 and 2). Although gpc separates molecules primarily on the basis of steric factors, a rather accurate linear correlation exists between elution volume and log molecular weight in the molecular weight range 150–900 (Schmit *et al.*, 1973) with Bio-Beads S-X2.

Gpc of the 7-day nonpolar rice metabolites showed four distinct regions of radioactivity. The majority of the ^{14}C label (70% of the chloroform extract) was associated with products of high molecular weight (>600) as shown by gpc. These labeled high molecular weight products are attributed to naturally occurring plant constituents [*e.g.*, polymeric decomposition products of carotenoids (Pokorny *et al.*, 1973)]. Subsequent tlc of each fraction of radioactivity from gpc revealed a total of at least 13 nonpolar metabolites from the chloroform extract (including **2**, **4**, **6**, **7**, and nonmetabolic residues). There were indications from tlc and gpc of incorporation of ^{14}C label into chlorophyll (0.8% of applied dose) and a yellow carotenoid (1.0%). Confirmation of such incorporation was not pursued because of the usual pigment instability. No products of low molecular weight (<500) other than those listed in Table V were detectable in greater than 1% of the applied dose.

A similar gpc and tlc purification of the chloroform extract of alfalfa showed at least ten nonpolar "metabolites" (including **2**, **3**, **6**, and nonmetabolite residues). Again a large amount (56%) of the radioactivity in this fraction was associated with high molecular weight products (mol wt > 600). Further analysis of gpc fractions by tlc revealed seven yellow and green bands of radioactivity, again implicating the incorporation of ^{14}C label into naturally occurring plant pigments. No low molecular weight product other than methoprene or its known primary metabolites (Table IV) contained more than 1% of the applied dose. It is especially noteworthy that determination of primary metabolite yields without prior purification by gpc resulted in grossly inflated yields (ten times too high) caused by cochromatography of compounds with similar polarities. Gpc was absolutely essential for the rapid removal of high molecular weight impurities from metabolites. Contaminating radioactive natural plant constituents were of minor importance for the gpc purification of the less polar methoprene band after preliminary tlc (Figure 2). However, a small amount of radioactivity was associated with the chlorophyll band by tlc.

Combustion of the plant tissue remaining after exhaustive extraction with chloroform and methanol revealed residual radioactivity (10–12% of applied dose). The residual plant pulp was enzymatically degraded with cellulase. Hydrolysis of alfalfa pulp gave hydroxy acid **3** (0.9%), [^{14}C]glucose (0.2%), and [^{14}C]cellobiose (0.5%). Enzymatic cleavage of rice pulp gave methoxycitronellic acid (**6**, 0.5%), [^{14}C]glucose (0.1%), and [^{14}C]cellobiose (0.1%). It appears that conjugation of **3** and **6** to cellulose accounts for a substantial amount of the residual radioactivity in plant pulp. However, incorporation of ^{14}C label into cellulose (perhaps *via* $^{14}CO_2$) is less important. This bound radioactivity is attributed to extensive decomposition products which became incorporated into natural cellular products. The bulk of the residual radioactivity is probably associated with unextractable lignins and tannins. The preponderance of an immobile radioactive origin (Tables II and III) after enzymatic cleavage of conjugates is also an indication of extensive biodegradation to polar metabolites.

Extraction of a combination of all new alfalfa leaves (which emerged after dosing) and untreated stems revealed a maximal accumulation of 0.6% radioactivity after 15 days. This radioactivity is more likely the result of transport of extensively degraded polar metabolites rather than the translocation of methoprene. The roots of both alfalfa and rice contained negligible radioactivity.

A major concern in this study was to quantitatively relate formation of products with the applied dose of radioactive methoprene IGR. There was a progressive decrease in the amount of recoverable radioactivity with a plateau reached after 2 weeks (Tables II and III). The rapid loss of isolable ^{14}C label within the first 3 days (73%

Table VII. Volatile Products from Methoprene Metabolism on Rice and Alfalfa[a]

Day	% 1 collected as vapor	% 5 collected as vapor	% $^{14}CO_2$	% radioact. collected as vapor
		Rice		
1	5.3	0.2	0.2	6.4
3	12.9	2.2	0.3	16.1
4	13.2	9.6	0.5	26.2
6	13.6	11.5	0.6	28.5
8	13.8	12.7	0.7	30.5
		Alfalfa		
1	1.9	0.7		3.0
3	2.7	1.5	0.2	4.8
4	2.9	1.7		5.3
6	3.2	1.7	0.2	5.6
7	3.3	1.7	0.3	5.8

[a] Expressed as per cent of applied dose.

for rice and 34% for alfalfa) suggested both loss by volatization of methoprene or facile metabolism to volatile products. Both of these mechanisms were shown to contribute to substantial losses of radioactivity.

In order to separate metabolic effects from the physical property of volatility, initial efforts were directed at investigating volatilization of methoprene from an inert surface (glass plates). Two different techniques were employed for assaying the evaporation rate of radioactivity from [5-^{14}C]methoprene on glass plates. In the first method, a relatively large area of glass plates was treated with [5-^{14}C]methoprene and at the end of a given interval the residue was washed off the plate and analyzed by tlc. When [5-^{14}C]methoprene was applied to a glass plate at the same rate as for alfalfa and rice leaves (*ca.* 1 lb/acre) and placed under the same environmental conditions, initially a first-order type loss curve was observed. The half-life for methoprene on the plate was about 6 hr. However, the loss rate curve rather quickly reached a plateau; this nonlinear evaporation curve (*cf.* Gückel *et al.*, 1973) presumably resulted from the photodecomposition of methoprene on the glass surface. After 7 hr, the extracted residue contained only 60% methoprene. After 29 hr of indoor greenhouse exposure, only 53% of the residual radioactivity was methoprene.

To verify this finding using a different radioassay technique, small spots of methoprene (0.6, 1.0, and 50 lb/acre) were applied to glass plates by identical techniques and then repeatedly radioscanned (Geiger-type counter). The loss rate of radioactivity determined by this method gave a ten times longer half-life ($t_{1/2}$ = 65 hr) for the 1.0 lb/acre rate than the previous study (also $t_{1/2}$ = 55 hr for 0.6 lb/acre and $t_{1/2}$ = >200 hr for 50 lb/acre). The calculated film thickness of methoprene at 1 lb/acre is about 0.1 μ, much too thin to cause erroneous results from β-ray self-absorption and much too thick (>100 monolayers) to run into chemisorption problems. We are unable to explain this discrepancy in half-lives, but the number of variables in evaporation studies is large and relatively difficult to control.

In order to check the identity of the evaporated components a cold-temperature trapping system was devised. Evolved methoprene and primary metabolites from rice were trapped at −78° on a glass wool plug and radioactive carbon dioxide by aqueous potassium hydroxide. Volatilization of methoprene was most important during the first 3 days after foliar application (Table VII). After a week, 14% of the applied dose was collected as unmetabolized methoprene. A single major volatile metabolite characterized as 7-methoxycitronellal (**5**) began to appear after 3 days and maximized at 4 days. It was recoverable in 13% yield relative to the applied dose and represented by far the most abundant metabolite of methoprene from rice.

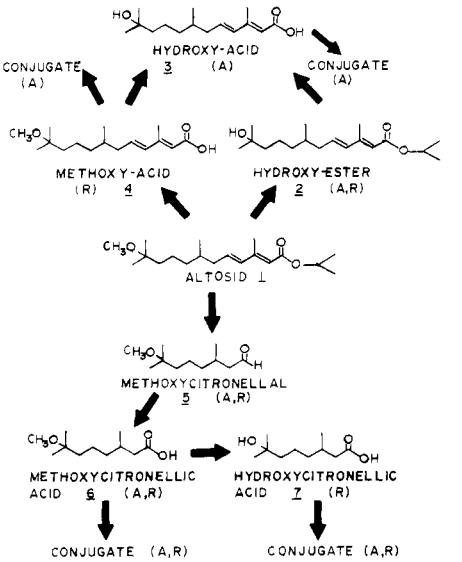


Figure 3. Metabolic fate of methoprene on rice (R) and alfalfa (A).

The evolution of $^{14}CO_2$ was a relatively minor pathway to loss of recoverable radioactivity although 0.7% was found after 1 week. The recovery of 30% of the applied dose of methoprene on rice as condensed vapors after 1 week proves that volatility contributes substantially to the loss of recoverable radioactivity from the plant. Furthermore, these yields of evaporated methoprene and 7-methoxycitronellal should be regarded as minimums since a possibility exists of adsorption from the vapor phase by the glass apparatus or the interconnecting rubber tubing, or adsorption from aqueous solution onto glass in the cold trap. An aqueous solution of methoprene was necessarily formed on thawing the cold trap because much water vapor was condensed from the atmosphere. We have encountered phenomena occasionally that suggest methoprene may adsorb to certain surfaces from aqueous solution.

Methoprene and methoxycitronellal (**5**) were also collected as volatiles from alfalfa plants although in lesser amounts (3 and 2%, respectively). Again small amounts (0.4%) of $^{14}CO_2$ were released and a total of 7% of the applied dose was isolated as condensed vapors. The progressive evolution of volatiles from alfalfa is shown in Table VII.

The metabolic fate of methoprene on rice and alfalfa is summarized in Figure 3. However, some additional unknown processes clearly operate by which ^{14}C label from the C-5 atom of methoprene (or the C-1 atom of metabolites **5**, **6**, or **7**) can incorporate into such natural products as cellulose and (presumably) chlorophylls and carotenoids. It may well be that this unknown process involved degradation to a natural intermediary metabolite (*e.g.*, [^{14}C]acetate) which is anabolically converted to a host of natural products.

An important revelation from this work was the potential for greatly overestimating metabolite quantities from tlc alone in cases of extensive degradation of the parent pesticide. Many workers quantify metabolites by simply scraping zones of radioactivity from tlc plates for scintillation counting. We found that radiolabel arising from methoprene catabolism was converted into a multitude of natural products which co-eluted with primary metabolites on tlc. A tenfold overcalculation of metabolite yields resulted when tlc was not preceded by purification by gel permeation chromatography. Thus, separation of high molecular weight natural products by gpc was essential for accurate determination of metabolite quantities. Like-

wise, structure proof by tlc based on cochromatography with standards can be inaccurate and misleading if precautions are not taken to remove radioactive natural products resulting from extensive biodegradability.

Methoprene and all characterized metabolites are nontoxic to mammals (LD_{50} >5000 mg/kg, rat). In fact, hydroxycitronellic acid (7) is an air oxidation product of one of the most widely used perfume bases (hydroxycitronellal) and methoxycitronellal (5) itself is widely used as a floral fragrance (Arctander, 1969). Since methoprene IGR is rapidly biodegraded by both alfalfa and rice to innocuous metabolites, it should be considered an ecologically acceptable alternative for pest control.

ACKNOWLEDGMENT

We thank L. L. Dunham, R. Veit, and P. J. McFall for assistance with gas-liquid chromatography and gc-mass spectrometry. We also thank J. B. Siddall for valuable suggestions.

LITERATURE CITED

Arctander, S., "Perfume and Flavor Chemicals," Steffen Arctander, Elizabeth, N. J., 1969, pp 1728, 1870.
Bonnafous, J., Mani, J., Olivé, J., Mousseron-Canet, M., *Tetrahedron Lett.*, 1119 (1973).
Dark, W. A., Limpert, R. J., *J. Chromatogr. Sci.* **11**, 114 (1973).
Gückel, W., Synnatschke, G., Rittig, R., *Pestic. Sci.* **4**, 137 (1973).
Henrick, C. A., Staal, G. B., Siddall, J. B., *J. Agr. Food Chem.* **21**, 354 (1973).
Isler, O., Ed., "Carotenoids," Birkhäuser Verlag, Basel, 1971, p 117.
Menn, J. J., Beroza, M., Ed., "Insect Juvenile Hormones—Chemistry and Action," Academic Press, New York, N. Y., 1972, pp 3–341.
Miura, T., Takahashi, R. M., *J. Econ. Entomol.* **66**, 913 (1973).
Pokorny, S., Coupek, J., Luan, N. T., Pokorny, J., *J. Chromatogr.* **84**, 319 (1973).
Rosenblum, C., Trenner, N. R., Wolf, D. E., *J. Label. Compounds* **7**, 225 (1971).
Schaefer, C. H., Dupras, M. F., *J. Econ. Entomol.* **66**, 923 (1973).
Schaefer, C. H., Wilder, W. H., *J. Econ. Entomol.* **66**, 913 (1973).
Schmit, J. A., Williams, R. C., Henry, R. A., *J. Agr. Food Chem.* **21**, 551 (1973).
Schooley, D. A., Bergot, B. J., Creswell, K. M., Quistad, G. B., Staiger, L. E., Siddall, J. B., manuscript in preparation, 1974.
Stalling, D. L., Tindle, R. C., Johnson, J. L., *J. Ass. Offic. Anal. Chem.* **55**, 32 (1972).
Tschesche, R., Struckmeyer, K., Wulff, G., *Chem. Ber.* **104**, 3567 (1971).

Received for review January 28, 1974. Accepted March 6, 1974. Contribution No. 19 from Zoecon Research Laboratory.

Fate of Neodecanoic Acid in Onion and Soil

Mason D. Gilbert, Ann Pendergrass, Francis M. Isenberg, and Donald J. Lisk*

The metabolism of ^{14}C-carboxy-labeled neodecanoic acid (NDA) was studied in onions on which the compound is used for drying tops. It appeared that the compound was immobile in the plant and did not decompose appreciably. In muck soil about 10% of the added NDA decomposed with the release of $^{14}CO_2$ during the first 12 days after which $^{14}CO_2$ evolution was negligible. When onion foliage was added to muck soil containing labeled NDA, $^{14}CO_2$ was continuously evolved over a period of 30 days with little change in rate of evolution.

Neodecanoic acid (NDA) is a mixture of di-α-branched decanoic acids which have been shown to be useful for drying onion tops to facilitate harvesting (Pendergrass *et al.*, 1969; Isenberg and Abdel-Rahman, 1972). It is manufactured by the Enjay Chemical Co., Linden, N. J., and marketed by Agway, Inc., under the trade name Topper 5-E. Using thin-layer chromatography (Pendergrass *et al.*, 1969) harvest residues of NDA in onion bulbs treated at the rate of 55 kg/ha were estimated to be about 1 ppm. In the work reported, ^{14}C-carboxy-labeled NDA was used to determine its possible translocation from onion tops to bulbs and its stability in onions and soil.

EXPERIMENTAL SECTION

Plant Studies. A preliminary study was performed to determine the general pattern of NDA translocation in intact onion plants. A series of onions, cultivar Elba Globe, grown to maturity under greenhouse conditions of an 18-hr 27° day and 22° night was treated with 5-μl droplets of NDA containing 0.5 μCi of ^{14}C in xylene as the carrier. (Xylene is the solvent used in the commercial formulation of NDA.) This material was applied to bulb scales, softening neck tissue, green leaf, and the foliage at the point where the youngest leaf emerged through the pore in the neck. Plants were held intact for 15 days for translocation to occur and then dried and exposed for 1 week to Kodak Royal X-Omat medical X-ray film before development.

In subsequent greenhouse experiments, mature Elba Globe onion plants were sprayed with a radioactive NDA formulation in a plastic enclosure using spray conditions that were designed to simulate field application (Isenberg and Abdel-Rahman, 1972). Some bulbs were completely covered by the growing medium, while others were partially exposed. The sprayer was operated at 2.11 kg/cm^2 pressure and a formulation consisting of NDA (66.4%, 28.4 μCi of ^{14}C), xylene (28.6%), Atlox 3404 (1%), and Atlox 3403F (4%) was diluted with water equivalent to rates of 33.6 and 44.8 kg of NDA/ha. Agway Booster +E, marketed by Agway, Inc., Syracuse, N. Y., was used as a surfactant at a rate of 8.1 kg/ha. Following treatment, the plants were held in a greenhouse for 11 days without irrigation. Several control plants were treated in a similar fashion with NDA omitted. At harvest, each onion plant was subdivided into foliage, bulb, and outer two loose, dry bulb scales. All tissues were stored in a freezer at −20° up to, but not exceeding, 10 weeks.

In preparation for analysis of radioactivity, the tissues were freeze-dried and ground in a Wiley mill. The powdered samples were Soxhlet extracted with 150 ml of di-

Departments of Food Science and Vegetable Crops, Cornell University, Ithaca, New York 14850.