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Metabolism of a Herbicide, Isouron [3-(5-*tert*-Butyl-3-isoxazolyl)-1,1-dimethylurea], in Bean Seedlings

Masakatsu Ishizuka,* Yutaka Kondo, and Yasuyoshi Takeuchi

The matabolism of isouron [3-(5-*tert*-butyl-3-isoxazolyl)-1,1-dimethylurea] was studied in bean plants. Two major degradation pathways, N-demethylation and hydroxylation of the *tert*-butyl group, seemed to be followed. The following four metabolites were identified by cochromatography and mass spectrometry: a monomethylurea derivative [3-(5-*tert*-butyl-3-isoxazolyl)-1-methylurea], a urea derivative [3-(5-*tert*-butyl-3-isoxazolyl)urea], a hydroxy-*tert*-butyl dimethylurea derivative [3-[5-(1,1-dimethyl-2-hydroxyethyl)-3-isoxazolyl]-1,1-dimethylurea], and a hydroxy-*tert*-butyl monomethylurea derivative [3-[5-(1,1-dimethyl-2-hydroxyethyl)-3-isoxazolyl]-1-methylurea]. In addition, three polar metabolites were tentatively identified, which were β-D-glucosides of 3-(5-*tert*-butyl-3-isoxazolyl)-1-(hydroxymethyl)-1-methylurea and hydroxy-*tert*-butyl dimethylurea and hydroxy-*tert*-butyl monomethylurea derivatives.

Isouron is a newly synthesized isoxazole–urea derivative that shows a potent herbicidal activity against many species of annual broadleaf and grassy weeds and some perennial weeds (Yukinaga et al., 1979a). It has been used for control of the total vegetation in noncropland (Ito et al., 1979) and has shown promising selective herbicidal activity in sugarcane field (Yukinaga et al., 1979b). Isouron was reported to inhibit the Hill reaction in isolated spinach chloroplasts; thus the primary site of its action was suggested to be in the photosynthetic electron transport system (Yukinaga et al., 1979c).

Differences among plant species in metabolic rates and pathways of herbicides often contribute to the selectivity of their weed control, and identification of their metabolites is very important in analyzing residues left in crops. The present investigation was conducted to determine the metabolic fate of isouron in bean plants.

MATERIALS AND METHODS

Chemicals. Isouron and its related compounds were synthesized at Shionogi Research Laboratories, Fukushima-ku, Osaka, Japan. [^{14}C]Isouron labeled at the 5 position of the isoxazole ring was also prepared at the laboratories and had a specific activity of 5.03 mCi/mmol. Its radiochemical purity as determined by thin-layer chromatography (TLC) was greater than 99.3%.

Plant Material. Seeds of the kidney bean (*Phaseolus vulgaris* L. cv. "Black Valentine") were surface sterilized with a 0.1% (w/v) sodium hypochlorite solution for 15 min and soaked in running tap water for 3 days at 25 °C. The germinated seeds were planted in moist sand and grown under greenhouse conditions with a 28 °C daytime and 15 °C nighttime temperature, without artificial light. A few days before the primary leaves fully expanded, the cotyledons were removed from the plants. Seedlings were then transferred to water culture in a Hoagland's nutrient solution (Hoagland and Arnon, 1938) and allowed to grow for 3 days prior to root treatment. The nutrient solution was aerated for 30 min at 6-h intervals during growth.

Application of [^{14}C]Isouron. All experiments were carried out under greenhouse conditions. Lots of five uniform seedlings were selected, and each lot was supplied with [^{14}C]isouron through the roots by being placed in 30 mL of a 10^{-4} M solution in a test tube. After 4 h the plants were removed from the test tube, and their roots were rinsed 3 times with 20 mL of distilled water. The plants then were cultured in 100 mL of a fresh nutrient solution

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and grown under aeration until harvest at 0, 24, 48, 96, or 168 h after the treatment. Tap water was added daily to maintain the 100-mL volume. The treatment and rinse solutions were combined, and the radioactivity was determined. The uptake of [^{14}C]isouron by the plants was estimated by determining the radioactivity of the treatment solution before and after application to the roots.

Extraction of Radioactive Metabolites. At each harvest time the plants were removed from the nutrient solution, and their roots were rinsed thoroughly with distilled water. The rinsings were combined with the nutrient solution in order to determine the radioactivity released into the nutrient solution from the plants. The plants were chopped into small pieces and homogenized with an Ultra-Turrax (Janke & Kunkel KG.) in 10 mL of distilled water. The homogenate was successively extracted 3 times each with *n*-hexane, ethyl acetate, and 1-butanol. The three lots of each type of solvent fraction were combined and made up to 100 mL, and the radioactivity was determined in each aliquot. The aqueous phase was filtered through a glass filter, and the tissue debris on the glass filter was washed several times with methanol and distilled water. The filtrate was made to 100 mL, and the aliquot was counted for the total radioactivity in the aqueous fraction. The tissue debris was dried and combusted for the determination of its radioactivity.

Radioactivity Measurement. The treatment solution and the nutrient solution were radioassayed directly in Monophase-40 scintillation solution (Packard Instruments Co.). The radioactivity of the plant extracts and tissue debris was determined after combusting them in a Packard Model 306 sample oxidizer; an aliquot of each extract was spotted onto filter paper, dried, and combusted. The $^{14}CO_2$ was trapped in Carbo-sorb (Packard) and radioassayed in Permafluor V scintillation solution (Packard). All samples were counted with an Aloka Model 673 scintillation counter.

Time-Course Study. After determination of the total radioactivity, 5 mL each of the hexane, ethyl acetate, and butanol extracts from each time of harvest was dried under reduced pressure at 40 °C. The residue was redissolved in 250 μL of hexane, ethyl acetate, or methanol, and 50 μL was spotted onto a precoated 0.25 mm thick silica gel 60 F_{254} TLC plate (Merck). The plates were developed with chloroform–methanol (9:1 v/v). The radioactive areas were located by autoradiography using Fuji X-ray film, IX-150. The radioactive compounds were designated A, B, C, D, E, F, G, and H according to the R_f values on the TLC (Figure 1). Each was scraped separately from the plate and combusted in the sample oxidizer for radioactivity analysis.

Isolation and Identification of Radioactive Metabolites. As above, [^{14}C]isouron and its metabolites were separated on a 0.25 mm thick precoated silica gel 60 TLC plate with chloroform–methanol (9:1 v/v); the plate was scanned with a radioscanner (Berthold LB 2723). Each radioactive area was separately removed from the plate, and its radioactive compounds were eluted from the silica gel with chloroform–methanol (1:1 v/v). The compounds were further purified by TLC using the same solvent. Portions of the isolated compounds A–D were dried and treated overnight with acetic anhydride–pyridine at room temperature. The isolated compounds and their acetyl derivatives were cochromatographed with the authentic samples and their acetates on a high-performance thin-layer chromatography (HPTLC) plate of silica gel 60 F_{254} or RP-8 F_{254S} (Merck) in several solvent systems. The

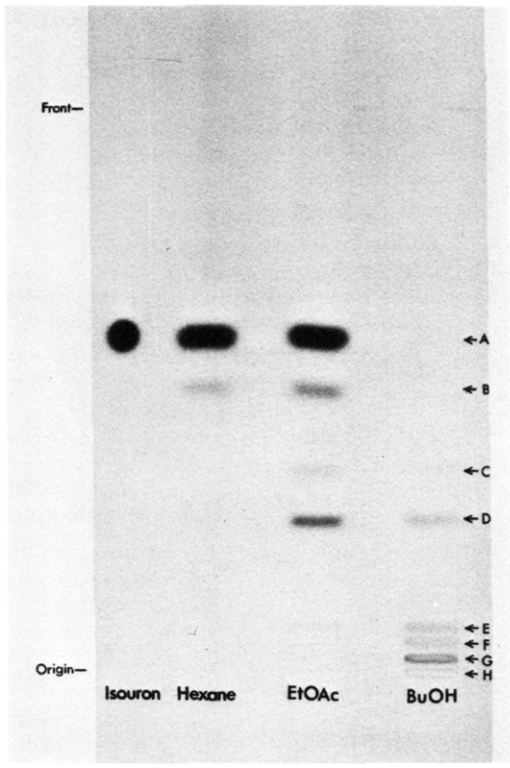

Figure 1. Autoradiography of [^{14}C]isouron and its metabolites. Hexane, ethyl acetate, and butanol fractions from the plants grown for 168 h after a 4-h root application of [^{14}C]isouron were chromatographed by TLC with $CHCl_3$–MeOH (9:1 v/v). The dried TLC plate was exposed to X-ray film at −20 °C for 10 days. Authentic [^{14}C]isouron was cochromatographed as a standard (left lane).

radioactive compounds were detected by autoradiography and the authentic samples by *p*-(dimethylamino)cinnamaldehyde reagent diluted with ethanol to one-tenth the strength originally used (Harley-Mason and Archer, 1958).

Enzymatic Hydrolysis of Butanol-Soluble Metabolites. The isolated butanol-soluble compounds (E–G) were hydrolyzed with 125 μg/mL β-glucosidase (Boehringer) at 30 °C for 2 h in 0.4 mL of 0.01 M acetate buffer (pH 5.0) containing 0.06 M $(NH_4)_2SO_4$ and 0.5% methanol. The reaction was stopped by adding 1.6 mL of cold ethanol; then the reaction mixture was left overnight at −20 °C. The compounds was also treated with α-glucosidase, α-galactosidase, and β-galactosidase in essentially the same manner. The reaction mixture was centrifuged at 1000*g* for 15 min, and the supernatant was collected. The precipitate was washed twice with cold ethanol. The supernatant and the washings were combined, evaporated to dryness, and dissolved in 0.1–0.2 mL of methanol for TLC separation with chroloform–methanol (9:1). The separated hydrolysate detected by autoradiography was identified by cochromatography with authentic samples as described above.

Application of Unlabeled Isouron. Bean seedlings were also treated with unlabeled isouron to isolate its metabolites for mass spectrometric analysis. Five uniform seedlings were water cultured for 6 h in 100 mL of 10^{-3} M isouron under greenhouse conditions; then the culture medium was changed to 100 mL of fresh Hoagland's nutrient solution and allowed to grow for 7 days. About 3000 seedlings in total were treated in this manner.

Purification and Identification of Nonradioactive Metabolites. The nonradioactive metabolites were extracted with methanol, and the methanol solubles were successively fractionated into *n*-hexane, ethyl acetate,

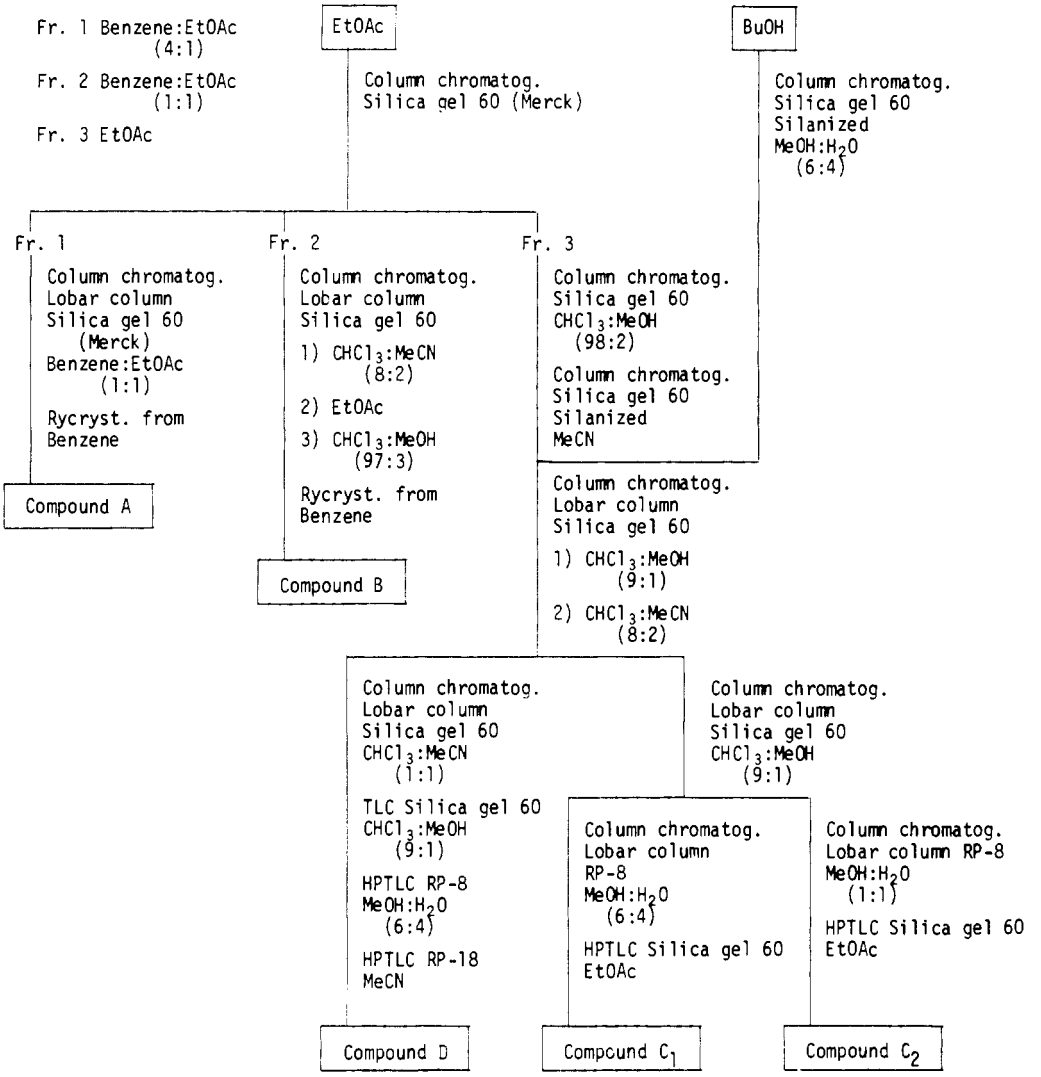

Figure 2. Outline of the procedures for isolation of isouron and its metabolites from bean seedlings.

Table I. Recovery of Radioactivity of [^{14}C]Isouron Taken up by Bean Plants[a]

h after end of treatment	total recovery in the extracts, % of uptake
0	99.8 ± 0.7
24	101.0 ± 0.7
48	101.0 ± 0.6
96	101.0 ± 0.7
168	96.9 ± 0.6

[a] Experiments were done in triplicate, and the data represent mean values with standard errors.

1-butanol, and aqueous fractions. The metabolites in the ethyl acetate and butanol fractions were further purified by column chromatography and TLC (Figure 2). During this experiment isouron and its metabolites were detected by *p*-(dimethylamino)cinnamaldehyde reagent as described above. Mass spectra of the purified isouron and its metabolites were obtained by a direct inlet probe by using a Hitachi mass spectrometer, M-68, in the electron ionization (70 eV) mode. The data output from the mass spectrometry was monitored with a Hitachi datalyzer, Model 002B.

RESULTS AND DISCUSSION

Distribution of Radioactivity in the Extracted Fractions. About 3% of the absorbed radioactivity was lost after 168 h, but the radioactivity absorbed by the

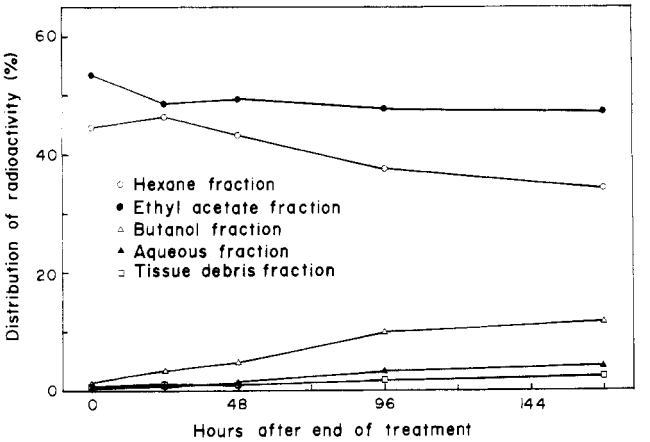

Figure 3. Changes in the distribution of radioactivity in the separated fractions of bean seedlings treated with [^{14}C]isouron. The percentages are based on the total radioactivity found in the plants.

plants was recovered almost completely in the extracts even after 96 h of metabolism (Table I). This suggests that the 5-carbon atom of the isoxazole ring was scarcely converted to $^{14}CO_2$ or other volatile compounds. The radioactivity levels in the hexane and ethyl acetate fractions decreased very slowly with time, while a corresponding increase occurred in the butanol, aqueous, and tissue debris fractions (Figure 3). After 168 h of metabolism, more than

Table II. HPTLC R_f Values for Isouron and Its Metabolites in Various Solvent Systems

compound	R_f values in solvent system[a]						
	I[b]	II[b]	III[b]	IV[c]	V[b]	VI[b]	VII[b]
A, isouron	0.64	0.23	0.60	0.44			
B, monomethylurea derivative	0.54	0.14	0.55	0.43			
C_1, urea derivative	0.34	0.06	0.49	0.53			
C_2, hydroxy-*tert*-butyl dimethylurea derivative	0.36	0.03	0.49	0.67			
D, hydroxy-*tert*-butyl monomethylurea derivative	0.26	0.02	0.50	0.68			
E	0.12				0.38	0.50	0.70
F	0.08				0.39	0.38	0.67
G	0.04				0.38	0.46	0.68
H	0.00						

[a] Solvent system I = $CHCl_3$–MeOH (9:1); solvent system II = ethyl ether; solvent system III = acetone; solvent system IV = MeOH–H_2O (7:3); solvent system V = EtOH–NH_4OH–H_2O (2:1:1); solvent system VI = *n*-BuOH–AcOH–H_2O (4:1:5); solvent system VII = *n*-BuOH–AcOH–pyridine–H_2O (15:12:3:10). [b] HPTLC plate silica gel 60 F_{254}. [c] HPTLC plate RP-8 F_{254S}.

Table III. Enzymatic Hydrolysis of Compounds E, F, and G

treatment	separated reaction products, %					
	compound E		compound F		compound G	
	glycoside	monomethylurea	glycoside	hydroxy-*tert*-butyl dimethylurea	glycoside	hydroxy-*tert*-butyl monomethylurea
0.01 M acetate buffer (pH 5.0)	98.6	1.4	99.2	0.8	99.9	0.1
β-glucosidase in 0.01 M acetate buffer (pH 5.0)	11.2	88.8	3.3	96.7	4.0	96.0
α-glucosidase in 0.01 M Mes buffer (pH 6.5)	98.6	1.4	100.0	0.0	99.7	0.3
β-galactosidase in 0.01 M Hepes buffer (pH 7.0)	98.9	1.1	100.0	0.0	100.0	0.0
α-galactosidase in 0.01 M Mes buffer (pH 6.5)	99.4	0.6	100.0	0.0	99.8	0.2

80% of the total radioactivity was extracted in the hexane and ethyl acetate fractions and less than 16% of the radioactivity was found in the butanol and aqueous fractions. This suggests that isouron very slowly changed to polar metabolites in bean plants.

Identification of Metabolites. The TLC analysis of the hexane, ethyl acetate, and butanol extracts revealed that eight radioactive compounds (A–H) were formed (Figure 1). The R_f values of the radioactive compounds A, B, and D coincided with those of isouron, a monomethylurea derivative, and a hydroxy-*tert*-butyl monomethylurea derivative, respectively, in four different solvent systems (Table II). But compound C separated into two radioactive spots in solvent systems II and IV; they were designated C_1 (R_f 0.53 in solvent system IV) and C_2 (R_f 0.67). Compounds C_1 and C_2 behaved the same as the urea and hydroxy-*tert*-butyl dimethylurea derivatives, respectively, in the first four solvent systems.

Compounds C_2 and D, but not compounds A, B, and C_1, could be acetylated with acetic anhydride–pyridine. Compounds C_2 and D each yielded only one acetyl derivative, which were identical with the acetates of hydroxy-*tert*-butyl dimethylurea and the hydroxy-*tert*-butyl monomethylurea derivatives, respectively, on TLC. Thus, the radioactive compounds A, B, C_1, C_2, and D were identified with unaltered isouron and its monomethylurea, urea, hydroxy-*tert*-butyl dimethylurea, and hydroxy-*tert*-butyl monomethylurea derivatives. These results were confirmed by mass spectrometric analysis of nonradioactive metabolites. The mass spectra of these nonradioactive compounds, which behaved identically with isouron, and its monomethylurea, urea, hydroxy-*tert*-butyl dimethylurea, and hydroxy-*tert*-butyl monomethylurea derivatives on TLC, were same as those of the corresponding authentic reference compounds (Figure 4).

The butanol soluble compounds E–G, which were each purified as a single radioactive spot by TLC in solvent systems I and V–VII, were individually treated with several kinds of glycosidases (Table III). All of the compounds could be hydrolyzed with β-glucosidase but not with α-glucosidase or α- or β-galactosidase. During a 2-h incubation period at 30 °C, about 88% of compound E and about 96% of compounds F and G were hydrolyzed with β-glucosidase (125 μg/mL). The TLC analysis of each hydrolysate showed the presence of a single radioactive product. The radioactive products of compounds E, F, and G were designated products a, b, and c. The hydrolytic products b and c and their acetates were identical with hydroxy-*tert*-butyl dimethylurea and hydroxy-*tert*-butyl monomethylurea derivatives and their acetates in solvent systems I, II, III, and IV. These data show that compounds F and G are tentatively identified with β-D-glucosides of hydroxy-*tert*-butyl dimethylurea and hydroxy-*tert*-butyl monomethylurea derivatives, respectively. Product a was not acetylated and was identical with a monomethylurea derivative in its R_f values on TLC in the first four solvent systems.

Frear and Swanson (1972) reported that acid hydrolysis of a monuron metabolite yielded monomethylmonuron, formaldehyde, and glucose in a 1:1:1 molar ratio and hydrolysis by β-glucosidase gave monomethylmonuron. They concluded that the metabolite was a β-glucoside of a *N*-hydroxymethyl intermediate, 3-(4-chlorophenyl)-1-(hydroxymethyl)-1-methylurea, and that the intermediate was highly unstable and gave rise to monomethylmonuron after acid or enzyme hydrolysis. Therefore, product a may be

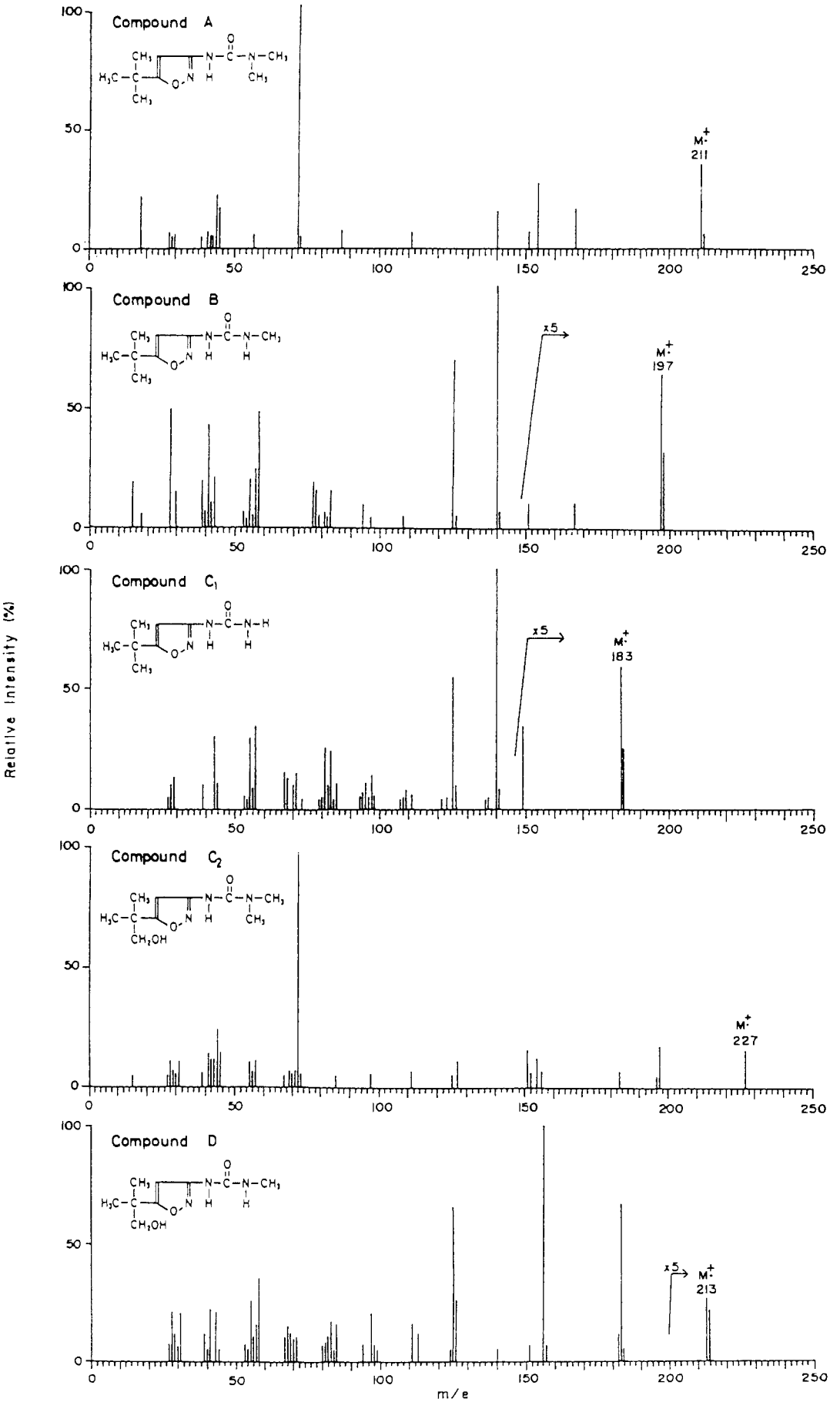

Figure 4. Mass spectra of the isouron and its metabolites isolated from bean seedlings.

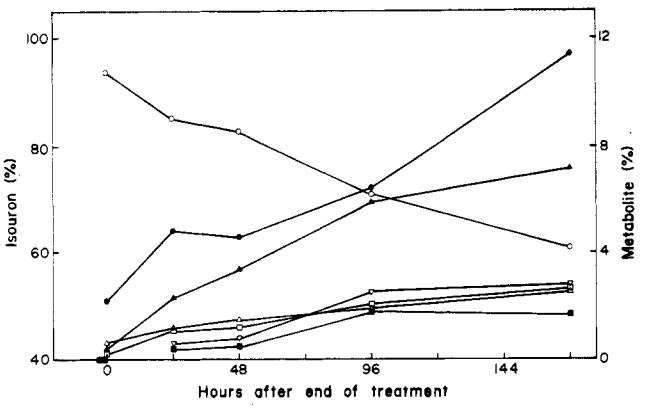

Figure 5. Time course of conversion of [^{14}C]isouron to its metabolites during growth after a 4-h treatment with [^{14}C]isouron medium. The solid bar denotes the period of [^{14}C]isouron treatment, and at 0 h, the plants were transferred to Hoagland's culture medium without isouron. The percentages are based on the total radioactivity found in plants: isouron (○), the monomethylurea derivative (●); the urea and hydroxy-*tert*-butyl dimethylurea derivatives (△); the hydroxy-*tert*-butyl monomethylurea derivative (▲); the glucoside of the hydroxymethyl methylurea derivative (□); the glucoside of the hydroxy-*tert*-butyl dimethylurea derivative (■); the glucoside of the hydroxy-*tert*-butyl monomethylurea derivative (▽).

derived from an unstable hydroxymethyl methylurea derivative, 3-(5-*tert*-butyl-3-isoxazolyl)-1-(hydroxymethyl)-1-methylurea, by elimination of formaldehyde. Thus compound E is probably a β-D-glucoside of the hydroxymethyl methylurea derivative.

Time-Course Study. Time courses of metabolic conversion of isouron in bean plants are shown in Figure 5. After 168 h, more than 60% of isouron remained unchanged, indicating that isouron was metabolized slowly in these plants. The plants metabolized isouron into two major metabolites, monomethylurea and hydroxy-*tert*-butyl monomethylurea derivatives. The conversion of isouron to the monomethylurea derivative occurred rapidly. About 2% of the isouron converted to the monomethylurea derivative during a 4-h treatment period. After the end of treatment, the level of the monomethylurea derivative increased rapidly for 24 h, stayed steady during the next 24 h, and then started to rise again after 48 h, reaching about 11% of the total radioactivity at 168 h. The monomethylurea derivative continued to form in spite of the damage to the plant which was evident after 96 h. The initital symptom, chlorosis at the margin of leaves, appeared in bean plants by 96 h after the application of isouron to the roots, and the chlorosis developed further with time.

A trace of the hydroxy-*tert*-butyl monomethylurea derivative was detected at 0 h, and conversion to this compound proceeded rapidly until 96 h. The conversion slowed thereafter and reached a level of about 7% after 168 h. The formation of the other metabolites, i.e., the urea derivative, the hydroxy-*tert*-butyl dimethylurea derivative and its glucoside, and the glucosides of hydroxy-*tert*-butyl monomethylurea and hydroxymethyl methylurea derivatives, was significantly less, reaching less than 3% of the total radioactivity found in the plants after 168 h. These results suggest that isouron is mainly metabolized to the monomethylurea derivative and then to the hydroxy-*tert*-butyl monomethylurea derivative and that the *tert*-butyl group of isouron is presumably hydroxylated more slowly than that of the monomethylurea derivative. Although the amount of the glucoside of hydroxymethyl methylurea derivative was small, its formation occurred within 48 h. On the other hand, only traces of the glucosides of the hydroxy-*tert*-butyl dimethylurea and hydroxy-*tert*-butyl monomethylurea derivatives were detected until 48 h, but they slowly increased thereafter.

Metabolic Pathways. The metabolism of isouron can be divided into two major pathways; one through N-de-

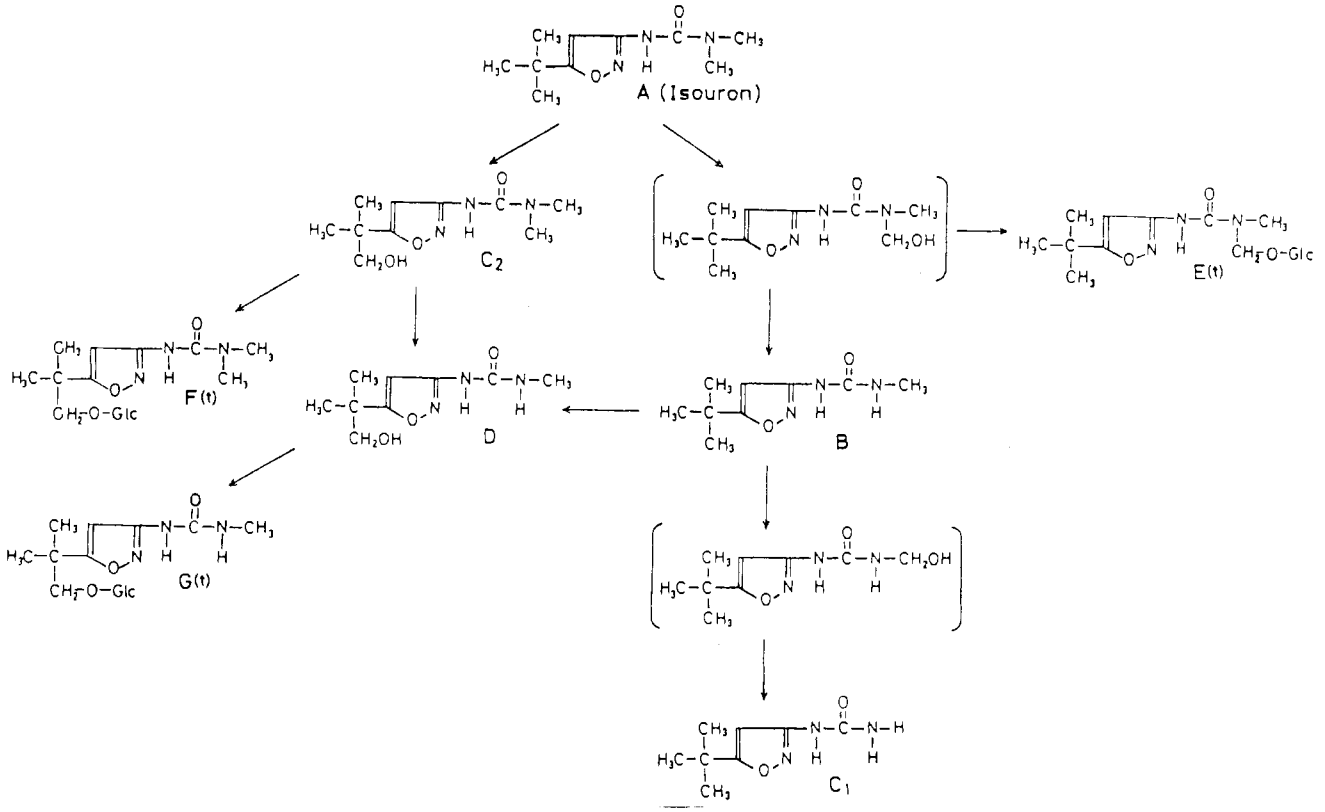

Figure 6. Presumed metabolic pathways of isouron in bean seedlings. (t) Tentative identification. Compounds in brackets are postulated intermediates.

methylation and the other through hydroxylation of the *tert*-butyl group. The metabolism of isouron through N-demethylation is similar to the path reported for phenylurea herbicides (Goren, 1969; Onley ett al., 1968; Swanson and Swanson, 1968). This could occur through the hydroxylation of the *N*-methyl group in a microsome fraction (Frear, 1968; Frear et al., 1969). The formation of the β-glucoside of the hydroxymethyl methylurea derivative indicates that hydroxylation of the *N*-methyl group occurs before N-demethylation.

Further N-demethylation to form the urea derivative occurred at a slower rate. Many investigators have reported that aniline formation from phenylurea herbicides occurred in small quantities in plants (Geissbühler et al., 1975), but the amine derivative, 5-*tert*-butyl-3-aminoisoxazole, has not been detected in this study. As the substituted isoxazole amine is very volatile, it might have been lost during the isolation procedures and its formation in small amounts cannot be ruled out. Hydroxylation of the *tert*-butyl group of isouron also occurred to a limited extent. However, the hydroxylation of the group of the monomethylurea derivative proceeds much more rapidly. Hydroxylation of the *tert*-butyl group was observed in the metabolism of tebuthiuron (Morton and Hoffman, 1976) and buthidazole (Atllah et al., 1980) in animals. It is presumed from this study that the N-demethylation of isouron followed by the hydroxylation of the *tert*-butyl group is the major route of its metabolism in bean plants.

On the basis of the above interpretation, a scheme for the metabolism of isouron in bean plants is proposed in Figure 6.

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Plant Metabolism of Fluvalinate [α-Cyano-3-phenoxybenzyl 2-[2-Chloro-4-(trifluoromethyl)anilino]-3-methylbutanoate]

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The metabolism of [*trifluoromethyl*-^{14}C]fluvalinate (1) was studied at the approximate field use rate (0.1 kg/ha) on cotton (leaves, squares, bolls), on tomatoes (leaves, fruit), and on leaves of tobacco, lettuce, and cabbage. In general, fluvalinate was metabolized predominantly by hydrolysis to the anilino acid 2 that was present in small amounts as the free acid (1–5% of applied dose) or in larger amounts (particularly with longer times posttreatment) as conjugated metabolites. 4′-Hydroxyfluvalinate (4) was detected in cotton and tomato leaves (1–2% of applied dose), and 2-chloro-4-(trifluoromethyl)aniline (3) was found in plant tissues (up to 4% of applied dose) and was also volatilized. Fluvalinate and its metabolites do not appear to translocate in plants. The approximate half-life of fluvalinate at 0.1 kg/ha, under greenhouse conditions, was greater than 6 weeks on lettuce and tomato leaves and fruit and about 4 weeks on cabbage, tobacco, and cotton. The asymmetric center of the alcohol moiety of fluvalinate is prone to partial epimerization on cotton leaves while the asymmetric center of the anilino acid portion seems relatively stable.

Fluvalinate [α-cyano-3-phenoxybenzyl 2-[2-chloro-4-(trifluoromethyl)anilino]-3-methylbutanoate, MAVRIK] is an insecticide with pyrethroid-like activity that has been selected from numerous synthetic analogues for commercialization by Zoecon Corporation (Henrick et al., 1980). As part of our efforts to determine the environmental fate of fluvalinate, we studied its degradation in several plant species and report our results herein.

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EXPERIMENTAL SECTION

Radiosynthesis. The anilino acid (2) was prepared from $Ba^{14}CO_3$ in six steps (Figure 1) under the direction of Dr. Ron Hale (Dynapol, Palo Alto, CA) using methods developed at Zoecon by Dr. R. J. Anderson. The crude

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