Synthesis of C^{14}-Labeled Siduron and Its Fate in Soil

I. J. Belasco and W. P. Langsdorf

Radioactive siduron, 1-(2-methylcyclohexyl)-3-phenyl-2-C^{14}-urea, was synthesized with an overall yield of approximately 80% by reacting 2-methylcyclohexylamine with C^{14}-carbonyl-labeled phenylisocyanate made from the correspondingly labeled benzoic acid *via* its azide. Labeled siduron was used in a study to measure its rate of disappearance, penetration, and mode of decomposition in soil. The results confirmed previous field data, showing that siduron resists leaching, remains in the top layer of the soil, and degrades at a moderate rate. The R_f values for the major siduron metabolites in soil were in good agreement with 1-(4-hydroxy-2-methylcyclohexyl)-3-(*p*-hydroxyphenyl)urea, 1-(4-hydroxy-2-methylcyclohexyl)-3-phenylurea, and 1-(*p*-hydroxyphenyl)-3-(2-methylcyclohexyl)urea, the three major metabolites isolated from the urine of a dog fed siduron. A fungus and two bacterial species (*Pseudomonas* sp.), capable of metabolizing siduron, have been isolated from soil.

Siduron, 1-(2-methylcyclohexyl)-3-phenylurea, is a selective pre-emergence herbicide used for the elimination of several annual seedling grasses from other grasses. In particular, it is useful in the control of crabgrass (*Digitaria* sp.), foxtail (*Setaria* sp.), and barnyard grass (*Echinochloa crusgalli* L.) in newly seeded and in established blue grass (*Poa* sp.), bentgrass (*Agrostis* sp.), and fine fescue (*Festuca* sp.) turf without causing injury to turf species (Varner *et al.*, 1964; Varner *et al.*, 1965; Weed *et al.*, 1966). Siduron has been shown to be resistant to leaching, volatilization, and degradation effected by sunlight irradiation (Weed *et al.*, 1966). The present study was designed to supplement the above findings on siduron with information as to its rate of disappearance, penetration, and mode of decomposition in soil.

Synthesis of 1-(2-Methylcyclohexyl)-3-phenyl-2-C^{14}-urea. The synthesis route involved the conversion of C^{14}-carbonyl-labeled benzoic acid to its acid chloride with thionyl chloride; the acid chloride was then treated with sodium azide. The resultant azide was converted to the C^{14}-carbonyl-labeled phenylisocyanate, which was reacted with 2-methylcyclohexylamine to yield C^{14}-labeled siduron.

One ml. of purified thionyl chloride was added to a small round-bottomed flask containing 272 mg. of carbonyl-labeled (C^{14}) benzoic acid, having an activity of 6 mc. (Nuclear Research Chemicals, Inc.), and 461 mg. of cold benzoic acid. The flask was covered with a calcium chloride tube and the reaction permitted to take place at room temperature for 91 hours. Five ml. of cold (0° C.) dioxane was then added, followed by the addition of an aqueous solution of sodium azide (2 grams in 6 ml. of water). The reaction mixture was extracted twice with 100 ml. of benzene. The combined extracts were washed with 5% aqueous sodium bicarbonate, dried with powdered magnesium sulfate, and filtered. The filtrate was transferred to a completely dry reflux system which had been blanketed with dry nitrogen and capped with a calcium chloride tube. To ensure against water contamination, approximately 100 ml. of the solvent was removed under vacuum with the flask and condenser temperatures maintained at 30° and 55° C., respectively. After release of vacuum with dry nitrogen, the solution was refluxed 2 hours and cooled. Two ml. of 2-methylcyclohexylamine were added, and the solution was again refluxed 30 minutes. This solution was cooled and washed with 10 ml. of 1 *N* hydrochloric acid followed by 5 ml. of distilled water. Solvent was removed by blowing nitrogen over the washed sample.

The residual solid was dissolved in 50 ml. of hot acetone, and the solution was decolorized with activated carbon. After concentration of this solution to 75 ml. by heating on a steam bath, distilled water was added until turbidity was evident. Slow evaporation of the acetone yielded white needles which were filtered, washed with distilled water, and dried to constant weight at 50° C. under aspirator vacuum. Total weight of product obtained was 1112 mg. with a melting point of 134° to 138° C. The overall yield was approximately 80%.

Radio- and Chemical Purity of Labeled Siduron. The labeled preparation of 1-(2-methylcyclohexyl)-3-phenylurea had a specific activity of 1.15 mc. per mmole as assayed in a Model 6801 Nuclear Chicago scintillation spectrometer, using a toluene/ethanol-based scintillation solution having a counting efficiency of between 40 and 50%.

The radiopurity of labeled siduron was determined by thin-layer chromatography (TLC) of a solution of this material on Kieselgel-coated plates. Separate plates were developed in chloroform/ethyl acetate (35/40) and in petroleum ether/2% ammonium hydroxide in acetone (2/1). The R_f values of siduron are 0.58 to 0.60 and 0.67 to 0.69, respectively, in these two developing solvent mixtures. Autoradiographs of the plates showed a very low level of impurities estimated to be between 0.1 and 0.5%.

Increments of silica gel from the origin to the 11-cm. mark were removed from the plate and their activity assayed in the scintillation spectrometer, using a dioxane-based scintillation solution, having a counting efficiency of 70 to 80%. The

Research Division, Industrial and Biochemicals Department, E. I. duPont de Nemours and Company, Wilmington, Del. 19898

Table I. Distribution of C^{14} Activity in Soil Samples

Sample Soil Depth, Inches	Total C^{14} Activity of Soil Samples after-											
	1 Month			3 Months			6 Months			12 Months		
	μc	Original, %	Residual, %	μc	Original, %	Residual, %	μc	Original, %	Residual, %	μc	Original, %	Residual, %
0–1	7.76	78.3	87.2	6.07	61.3	86.4	2.62	26.4	71.3	1.79	18.1	66.8
1–2	0.53	5.3	6.0	0.71	7.2	10.1	0.31	3.1	8.4	0.47	4.7	17.5
2–4	0.29	2.9	3.2	0.23	2.3	3.2	0.32	3.2	8.7	0.20	2.0	7.5
4–6	0.32	3.2	3.6	0.02	0.2	0.3	0.18	1.8	4.9	0.12	1.2	4.5
6–8	<0.01	<0.1	<0.1	<0.01	<0.1	<0.1	0.13	1.3	3.5	0.06	0.6	2.2
8–10	<0.01	<0.1	<0.1	<0.01	<0.1	<0.1	0.10	1.0	2.7	0.03	0.3	1.1
10–11½	<0.01	<0.1	<0.1	0	0	0	0.02	0.2	0.5	0.01	0.1	0.4
Total	8.90	90.0	100.0	7.03	71.0	100.0	3.68	37.0	100.0	2.68	27.0	100.0

radiopurity, based on duplicate sets of samples, was 99.6 and 99.7%.

The chemical purity (99.6%) was determined spectrophotometrically at 241 mμ, on labeled siduron which had been isolated from its impurities by thin-layer chromatography. Mass spectral data also indicated the absence of impurities.

Soil Application of Labeled Siduron and Sampling. Four 1-foot sections of stainless-steel tubing (4-inch O.D.) were driven into the soil (Keyport silt loam) at the test site, allowing ¼ to ½ inch of each cylinder to protrude above the soil surface as a means of minimizing run-off of radiolabeled material. In addition, a wire screen was suspended above the sunken cylinders to fragmentize large raindrops, which otherwise might have incurred loss of labeled siduron through their splashing action. Radiolabeled siduron dissolved in methanol was applied to the soil surfaces within the cylinders at a rate of 10 pounds per acre. The total radioactivity of the siduron applied amounted to 9.91 μc. per cylinder. After evaporation of the methanol from the soil, approximately 50 ml. of water (equivalent to ¼ inch of rainfall) was carefully applied to the soil surface within the confines of each cylinder to facilitate incorporation of siduron into the soil.

A cylinder of soil was removed after 1, 3, 6, and 12 months from the test site. The soil within each cylinder was removed in the following fractions: 0–1, 1–2, 2-3, 4–6, 6 8, 8–10, and 10–11½ inches. The soil fractions were weighed before and after air-drying in order to assess the moisture content of the soil. They were ball-milled, air-dried, and passed through a 20-mesh screen two times to ensure representative soil sampling.

Analysis of Soil and Soil Extracts. Triplicate 2-gram samples of each soil increment were analyzed directly for total C^{14}-content by the wet combustion technique of Smith *et al.* (1964). Briefly, this technique involves the oxidation of the sample with Van Slyke combustion reagents and the trapping of liberated $C^{14}O_2$ in ethanolamine. Aliquots were counted by conventional LSC procedures, using the toluene/ethanol-based liquid scintillation mixture.

Samples (25 grams) of the soil fractions showing radioactivity were extracted with a 1-to-1 mixture of methanol-acetone in Soxhlet extraction equipment over a 72-hour period. The radioactivity of each extracted soil sample and each soil extract was determined by the wet combustion and LSC techniques as described above to determine extraction efficiencies.

All of the soil extracts were chromatographed on TLC plates with a 250-micron-thick layer of Kieselgel and developed in a 9-to-1 mixture of chloroform and methanol. The plates were subjected to a conventional autoradiographic procedure, as a means of locating the position of the residual C^{14}-labeled siduron and its radiolabeled metabolites. Incremental areas of Kieselgel at 1-cm. intervals beginning at the origin and ending at the 10-cm. mark were then removed from the plates and placed in scintillation vials containing the dioxane-based scintillation solution for counting. This provided information about the residual siduron and the relative concentration of siduron metabolites in the soil fractions.

Microbiological Procedures. A modified technique of Whiteside and Alexander (1960) was used as a preliminary means of culturing soil microorganisms capable of metabolizing siduron. This technique involved the inoculation of an inorganic nutrient medium containing 0.5 gram NH_4NO_3, 0.8 gram of KH_2PO_4, 0.2 gram of $MgSO_4 \cdot 7H_2O$, 0.03 gram of $FeSO_4$, 0.1 gram of $CaCl_2 \cdot 2H_2O$ per liter of distilled water, and the herbicide as the sole organic carbon source with a sample of enriched greenhouse soil. In view of the low water solubility of siduron (17 p.p.m.), it was found efficacious to make up this medium in a saturated water solution of siduron. In a typical experiment, approximately 200–250 ml. of inorganic medium, with and without siduron, were placed in separate 500-ml. flasks, inoculated with 1 gram of enriched greenhouse soil, stoppered, placed on a shaker and incubated at room temperature (24° to 27° C.). No attempt was made to sterilize the nutrient medium in the flasks prior to inoculation, since the prime objective at this point was to ascertain microbial degradability of siduron, regardless of bacterial source. Aliquots of the culture medium were removed at regular intervals for siduron extraction and analysis.

The analysis for siduron entailed the extraction of the aliquot with dichloromethane. The residue of the dichloromethane extract after evaporation was taken up in a known volume of methanol and the residual siduron was calculated from the ultraviolet absorbance of the methanol solution at a wavelength of 241 mμ. Values for residual siduron probably included ring-hydroxylated metabolites of siduron with the ring intact, since these ring-hydroxylated metabolites isolated from dog urine (Belasco and Reiser, 1969) all had absorption maxima at 240–241 mμ. Therefore, decreases in absorbance was indicative of further biological degradation.

Standard dilution and plating techniques were used to isolate the predominant bacterial species for further assessment. Sterile inorganic nutrient medium containing siduron in separate flasks was then inoculated with each of the predominant microbial species to determine the capability of each microorganism of metabolizing siduron. Those found capable were then tentatively identified on the basis of their morphological and biochemical characteristics.

RESULTS AND DISCUSSIONS

The distribution of C^{14}-activity in the various soil fractions of the samples taken after 1, 3, 6, and 12 months is shown in Table I. These data indicate a moderate rate of siduron

Table II. Distribution of C^{14} Activity in Extracts of Soil Samples

Sample Soil Depth, Inches	Siduron, %		Metabolite 1, %		Metabolite 2, %		Metabolite 3, %		Other, %	
	6 Mo.	12 Mo.	6 Mo.	12 Mo.	6 Mo.	12 Mo.	6 Mo.	12 Mo.	6 Mo.	12 Mo.
0–1	84.4	72.8	29.9	46.5	46.5	65.2	56.7	80.0	52.3	35.4
1–2	4.8	13.2	9.4	17.9	12.8	13.0	7.2	11.4	9.1	29.4
2–4	3.7	6.1	12.8	10.7	14.0	8.7	20.5	2.9	15.9	17.6
4–6	3.0	4.4	13.7	7.1	12.8	6.5	10.8	...	11.4	17.6
6–8	2.2	2.6	17.9	7.1	8.1	4.4	3.6	5.7	6.8	...
8–10	1.9	0.9	16.3	10.7	5.8	2.2	1.2	...	4.5	...
10–11½	...	...	...	...	...	...	...	...	...	...
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Total extractable radioactivity, μc	0.270	0.114	0.117	0.028	0.086	0.046	0.083	0.035	0.044	0.017

degradation in the soil. By extrapolation, 50% of the original radioactivity had disappeared within 4 to 5 months. The results also show that the major portion of the radioactivity was confined to the top inch of the soil, even after 12 months (Figure 1). The total radioactivity in the soil beneath the top inch, at all four sampling periods, approximated 10% of the radioactivity originally applied as labeled siduron. Negligible amounts of C^{14} activity were found beyond the 6-inch soil depth in the samples taken after 1 and 3 months' exposure. Only in the 6- and 12-month samples was there C^{14} activity beyond this soil depth, amounting to only 2.5 and 1.0%, respectively, of the original siduron radioactivity applied. This slow downward movement of siduron in soil under field conditions is due in part to its low water solubility (17 p.p.m.).

These data support the field experiences reported by Weed *et al.* (1966), where they noted that siduron is resistant to leaching and that crabgrass roots fail to penetrate a layer of siduron positioned one-half inch below the surface level. They also reported on the favorable disappearance pattern of siduron under field conditions. Although active against crabgrass throughout the growing season, there is no evidence that siduron accumulates in soil from repeated annual applications.

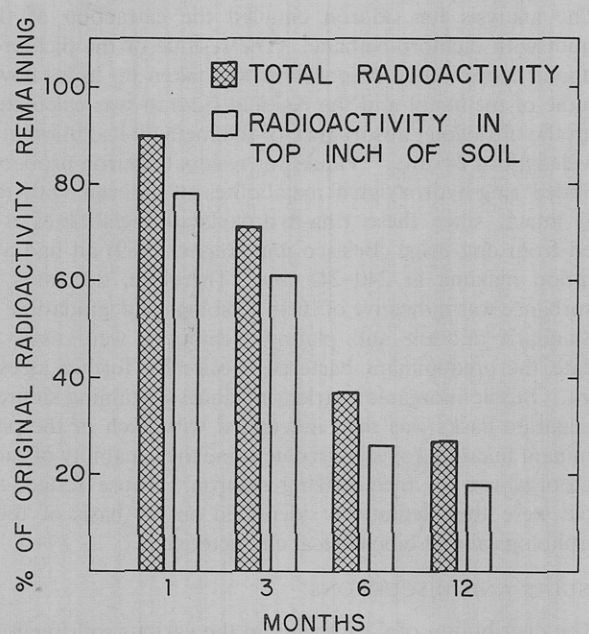

Figure 1. Radioactivity in top inch of soil and in whole soil sample at various time intervals after application

It was also evident from data in Table II that siduron, comprising the major portion of the radioactivity in the extractable portion from the top inch of soil, also decreased with passage of time. After 12 months of field exposure, siduron accounted for 52% of the extractable activity as opposed to 95% after 1 month. In each instance, the relative siduron content of the soil diminished with increasing depth. Conversely, the relative concentration of siduron metabolites, as a whole, increased with increasing depth of soil.

The three metabolites which were isolated from the urine of dogs fed high levels (2500 p.p.m.) of siduron in the diet and identified as 1-(4-hydroxy-2-methylcyclohexyl)-3-(*p*-hydroxyphenyl)urea, 1-(4-hydroxy-2-methylcyclohexyl)-3-phenylurea, and 1-(*p*-hydroxyphenyl)-3-(2-methylcyclohexyl)urea (Belasco and Reiser, 1969) were co-chromatographed with the extracts of the 0–1 and the 1–2-inch soil fractions of the 6-month soil. The resulting autoradiograph is shown in Figure 2. The R_f values of the three labeled metabolites in the soil extract were in excellent agreement with those isolated from urine (Table III). Thus, it was concluded that the urine and soil metabolites are very similar, and could very well be identical. The concentrations of the radioactive metabolites in the soil were not sufficient to permit mass spectrometric characterization.

Soil Microbiological Degradation. The disappearance of siduron from soil appeared to implicate the soil micro-

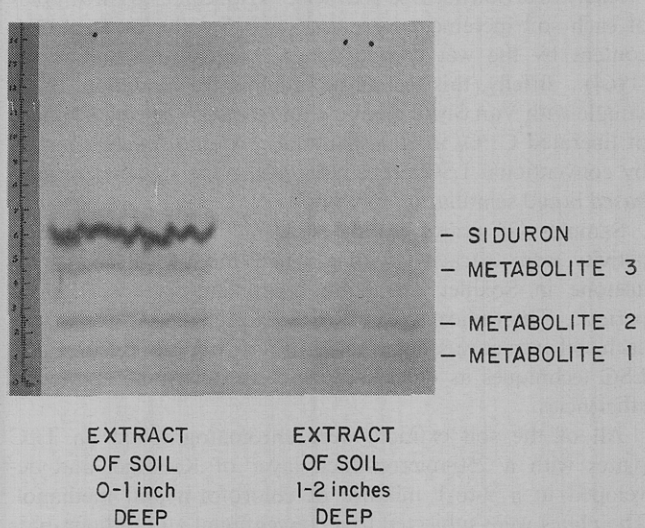

Figure 2. Autoradiograph of thin-layer chromatogram of soil extracts after 6 months of field exposure

Table III. Comparative R_f Values (×100) for Siduron Metabolites Isolated from Soil and Urine

Soil		Urine	
Metabolites[a]	R_f (×100)	Metabolites[a]	R_f (× 100)
1	10–12	1	11–13
2	23–25	2	24–25
3	43–45	3	43

[a] Siduron metabolites characterized in urine (Belasco and Reiser, 1969):

Metabolite 1. 1-(4-hydroxy-2-methylcyclohexyl)-3-(*p*-hydroxyphenyl)-urea.
Metabolite 2. 1-(4-hydroxy-2-methylcyclohexyl)-3-phenylurea.
Metabolite 3. 1-(*p*-hydroxyphenyl)-3-(2-methylcyclohexyl)-urea.

Table IV. Microbial Degradation of Siduron *In Vitro*

Test No.	Days of Incubation	Siduron Concentration, Initial p.p.m.	Siduron Concentration, Final p.p.m.	Siduron Metabolized,[a] %	Inoculum Source
1	70	17.4	12.2	30	Garden soil
2	70	17.4	13.6	22	Garden soil
3	93	17.1	13.3	22	Garden soil
4	84	17.2	9.3	46	Siduron test plot[b]

[a] Based on spectrophotometric estimation.
[b] Rate of siduron application, 10 lbs/A.

organisms in view of the stability of siduron in the presence of sunlight and its resistance to leaching (Weed *et al.*, 1966). The results of Fields and Hemphill (1968) indicate that siduron, and the reported degradation products of siduron, *i.e.*, aniline and 2-methylcyclohexylamine, have either a growth-suppressing or no effect on selected soil microflora. In contrast, the results reported here, demonstrating that certain soil microorganisms can utilize siduron as their sole carbon source, substantiate the conclusion that microbial degradation is the major route of siduron disappearance in the soil.

The degradation of siduron was apparent in the initial tests, following soil inoculation and incubation of the inorganic nutrient medium containing the unlabeled herbicide. It was evident after one week of incubation that small but significant amounts of siduron had been metabolized. The summary data in Table IV show that the extent of degradation after incubation of from 70 to 93 days ranged from 22 to 46%. The largest value (46%) was obtained in the test using a soil inoculum from a siduron test plot.

By standard dilution and plating techniques, two bacterial and a single fungal species were isolated. These microbial forms, when assayed in sterile inorganic nutrient broth, were found capable of metabolizing siduron. The data (Table V) showed that the bacterial species metabolized 49 and 33% of the siduron, respectively, while the fungus metabolized 88% in 127–132 days. No siduron breakdown was detected in a sterile control system. The bacterial species

Table V. Siduron Degradation of Microbial Isolates from Siduron Test Field Plot[a]

Test No.	Days of Incubation	Siduron Concentration, Initial p.p.m.	Siduron Concentration, Final p.p.m.	Siduron Metabolized, %	Organism
8	132	17.0	8.6	49	Bacteria
9	132	17.0	11.4	33	Bacteria
11	127	17.0	2.1	88	Fungus

[a] Rate of siduron application 10 lbs/A.

Table VI. Morphological and Biochemical Characteristics of Siduron Bacterial Cultures

	Culture No. 1	Culture No. 2
Form	Rod	Rod
Gram Stain	Neg.	Neg.
Size (μ)	1.2–1.5 × 0.5	0.7–0.9 × 0.3
Motility	Motile	Motile
Growth on agar slant	Rhizoid	Filiform
Color of colonies	Yellow	Ivory to tan
Pigment in agar	Yellow	Brown
Relation to free oxygen	Aerobic	Aerobic
Indole production	Neg. after 24 & 48 hours	Neg. after 24 & 48 hours
Nitrate reduction	Neg. after 48 hrs.	Neg. after 48 hrs.
Hydrolysis of starch	Slight	Neg.
Methyl red test	Neg.	Neg.
Voges-Proskauer test	Neg. after 4 days	Neg. after 4 days
Litmus milk test	Neg.	Neg.
H_2S production	V. slight	Slight
Gelatin liquefaction	Neg.	Neg.

	Gas Production	Acid Formation	Gas Production	Acid Formation
Reaction to lactose	Neg.	Neg.	Neg.	Neg.
Reaction to maltose	Neg.	V. slight to neg.	Neg.	Neg.
Reaction to dextrose	Neg.	Neg.	Neg.	Neg.
Reaction to sucrose	Neg.	Neg.	Neg.	Neg.

were tentatively identified on the basis of morphological and biochemical characteristics, (Table VI), as belonging to genus *Pseudomonas* sp. while the fungus was identified as belonging to the group of *fungi imperfecti*.

LITERATURE CITED

Belasco, I. J., Reiser, R. W., J. Agr. Food Chem., 17, 1000 (1969).
Fields, M. L., Hemphill, D. D., *Abstracts*, Meeting of Weed Society of America, p. 73, 1968.
Smith, G. N., Ludwig, P. D., Wright, K. C., Bauriedel, W. R., J. Agr. Food Chem. **12,** 172 (1964).
Varner, R. W., Weed, M. B., Ploeg, H. L., *Proc. Brit. Weed Control Conf.* **7,** 38 (1964).
Varner, R. W., Weed, M. B., Ploeg, H. L., *Proc. Northeast Weed Control Conf.* **19,** 520 (1965).
Weed, M. B., Belasco, I. J., Ploeg, H. L., Meeting of Weed Society of America, St. Louis, Mo., February 1966.
Whiteside, J. S., Alexander, M., *Weeds* **8,** 204 (1960).

Received for review November 7, 1968. Accepted April 28, 1969