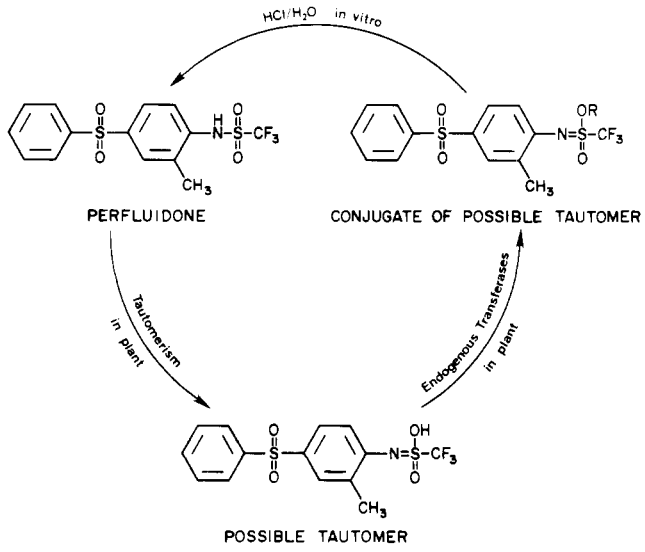

Figure 4. Possible explanation for water-soluble metabolites that liberate perfluidone on hydrolysis.

small amounts in intact shoots, but it accounted for 35% of the ^{14}C in the water-soluble fractions recovered from Bio-Gel P-2 chromatography of the extracts from excised leaves. The chemical nature of the water-soluble metabolites that released perfluidone during mild acid treatment was not established. Salt solutions of perfluidone were readily extracted into chloroform at pH 2, and perfluidone also readily partitioned into chloroform from tissue that was spiked with perfluidone immediately before homogenation and extraction. Attempts to form chelates of perfluidone with Mg^{2+} and Ca^{2+} were not successful. These results indicated that the water-soluble products in question were not simple salts or metal chelates.

Molecular models of perfluidone indicated that the sulfonamide nitrogen was too hindered to form conjugates readily with large molecules such as glucose. However, a tautomeric form of perfluidone, with a more exposed reactive site, might be expected to form an acid-labile conjugate. The trifluoromethylsulfonamide group of perfluidone appears to be an ideal system for tautomerism, and a possible tautomeric form of perfluidone and its corresponding conjugate(s) are shown in Figure 4. This tautomeric form of perfluidone was previously suggested by Lamoureux and Stafford (1974) and by Trepka et al. (1974). A conjugate of this nature would explain the presence of the acid-labile water-soluble products detected in high concentration in the excised leaves. These acid-labile products might also arise via conjugation of the *N*-hydroxy analogue of perfluidone. Although no evidence for this moiety was observed in this study, strong evidence for its existence in animals has been presented by Paulson et al. (1977).

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Metabolism and Degradation of Glyphosate in Soil and Water

Melvin L. Rueppel,* Blanche B. Brightwell, Jacob Schaefer, and John T. Marvel

Complete and rapid degradation of glyphosate [*N*-phosphonomethylglycine (**1**)] occurs in soil and/or water microbiologically and not by chemical action. Using soil/water shake flasks, up to 50% of each carbon of **1**-^{14}C was evolved as $^{14}CO_2$ in 28 days. In two of the three soils examined, **1** was 90% dissipated in less than 12 weeks. Aminomethylphosphonic acid (**2**), the only significant soil metabolite of **1**, also undergoes rapid degradation in soil. Short-term shake flask metabolism experiments with both ^{13}C- and ^{14}C-labeled **1** were carried out in order to permit facile, unequivocal spectral identification of **1** and its transient metabolite aminomethylphosphonic acid (**2**). Comparison of the metabolic samples to both reference standards and the spiked controls by means of ^{1}H, ^{31}P, and ^{13}C NMR, mass spectral analysis, ion-exchange chromatography, and thin-layer chromatography has unequivocally characterized both bound and unbound **1** and **2** in soil. The parent herbicide **1** has also been shown to be stable to sunlight, nonleachable in soil, to have a low propensity for runoff, and to have a minimal effect on microflora.

Glyphosate [*N*-phosphonomethylglycine (**1**), Figure 1] formulated (Roundup is a registered trademark of Monsanto Company, St. Louis, Mo.) as the isopropylamine salt is a new broad spectrum herbicide characterized by high unit activity, effective destruction of both annual and perennial herbaceous plants, inactivation by soil components, and favorable toxicology (Baird et al., 1971). This herbicide will be applied either preplant (foliar application

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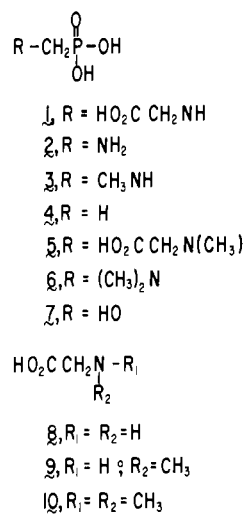

Figure 1. Structures of *N*-phosphonomethylglycine (1) and its potential metabolites.

to target weeds prior to emergence of the crop) and/or by directed spraying at use rates up to 4.48 kg/ha. The anticipated widespread use of this compound has prompted studies of its environmental impact. The research reported in this paper describes the rate of metabolism and dissipation of the parent and its metabolites in soil, the characterization of these metabolites in soil by ^{1}H, ^{31}P, and ^{13}C NMR, and mass spectrometry, and other aspects of its environmental fate. Other papers in this series have discussed in detail (Rueppel and Marvel, 1976; Rueppel et al., 1976) the methods developed and utilized or have summarized in preliminary accounts (Rueppel et al., 1975a,b,c,d; Marvel et al., 1974) the metabolism of the herbicide in soil and plants as well as the spectral and chromatographic characterization of metabolites. Since completion of the studies summarized in this paper, other investigators have reported on some areas covered in this paper (Sprankle et al., 1975a,b; Sprankle et al., 1974; Rieck et al., 1974; Hance, 1976).

EXPERIMENTAL SECTION

Chemicals. *N*-Phosphono-^{14}C-methylglycine (8.03 mCi/mmol), *N*-phosphonomethylglycine-*1*-^{14}C (10.02 mCi/mmol), and *N*-phosphonomethylglycine-*2*-^{14}C (9.40 mCi/mmol) were prepared by Freeman and Moran (1971). The phosphonomethyl-^{14}C labeled 1 was 94.8% pure with 4.3, 0.8, and 0.1% of aminomethylphosphonic (**2**), *N*-methylaminomethylphosphonic (**3**), and methylphosphonic (**4**) acids present, respectively, on the basis of TLC with beta camera (Baird Atomic Model No. 6000) detection. The glycine-*1*-^{14}C (carbonyl) labeled 1 was 99.5% pure with the only detectable ^{14}C-labeled impurity being glycine (0.5%). The glycine-*2*-^{14}C (methylene adjacent to carbonyl) labeled 1 was 98.1% pure containing 0.4 and 1.0% of **3** and glycine-^{14}C, respectively. TLC/beta camera analysis of these three ^{14}C labels in 0.1 M NH_4HCO_3 showed no chemical or microbial decomposition after storage for 9 months at 4 °C.

Aminomethyl-^{14}C-phosphonic acid (**2**, 0.241 mCi/mmol) was obtained by microbial degradation of diluted phosphonomethyl-^{14}C 1, followed by purification as described subsequently in this paper. *N*,*N*-Dimethylglycine-*1*-^{14}C and sucrose-^{14}C were obtained from New England Nuclear Corp. The aforementioned radioactive compounds and all other samples, unless specified, were assayed using Insta-Gel (Hewlett-Packard, Inc.) counting cocktail using Nuclear-Chicago Mark I counters.

N-Phosphonomethylglycines individually ^{13}C labeled at each carbon were prepared by Freeman and Moran (1974). Based on the starting materials, mass spectral analysis, and proton magnetic resonance, the *N*-phosphono-^{13}C-methyl, glycine-*1*-^{13}C, and glycine-*2*-^{13}C labels were each 90% enriched. The ^{13}C labels possessed purities of 97.7, 95.0, and 95.8%, respectively, based on gas chromatographic analysis with internal standardization.

Unlabeled samples of 1–4, *N*-methyl-*N*-phosphonomethylglycine (**5**), *N*,*N*-dimethylaminomethylphosphonic acid (**6**), and hydroxymethylphosphonic acid (**7**) were obtained from our stocks at Monsanto; their identity and purity were confirmed on the basis of NMR, derivatization, GC–MS–COM analysis (COM, computer), and TLC. Samples of glycine (**8**), sarcosine (**9**), and *N*,*N*-dimethylglycine (**10**) were purchased from Sigma Chemical Co.

Soils. The four diverse soil types utilized in these studies along with their percent organic matter, percent clay, percent silt, percent sand, and pH, respectively, were Ray silt loam (1.0, 0.6, 82.3, 6.0, 6.5), Drummer silty clay loam (6.0, 36.8, 55.4, 2.0, 7.0), Lintonia sandy loam (1.1, 2.3, 11.1, 86.6, 6.5), and Norfolk sandy loam (1.0, 2.3, 11.0, 86.0, 5.7).

Nonsterile Soil Degradation Studies with Shake Flasks. Soil/water shake flasks were carried out according to the method recently developed and authenticated (Marvel et al., 1976; Marvel et al., 1977). Each flask contained 4.5 g (dry weight) of the appropriate soil, 1 mg of the desired radioactive compound, and distilled water (100 mL). For studies in which the effect of the herbicide on the degradation of sucrose-^{14}C was examined, 1 mg of unlabeled 1 was also added along with sucrose-^{14}C as appropriate.

For aerobic experiments, 250-mL Erlenmeyer flasks containing a side arm sealed with a rubber septum and a trapping tower of two layers of ascarite sandwiched between drierite were used. After the desired period (normally 7 days) of metabolism on an incubator shaker at 30 °C, flasks were flushed with air for 1.25 h to collect the evolved CO_2. $^{14}CO_2$ trapped on the ascarite was assayed as described (Marvel et al., 1976; Marvel et al., 1977). After removing the collectors, aliquots of the flask supernatants were removed periodically for analysis by TLC/beta camera. Collectors containing fresh ascarite and drierite were added to each flask and the metabolism continued as desired.

In the case of anaerobic studies, the 250-mL side-armed Erlenmeyer flask was sealed with two rubber septums and flushed with nitrogen immediately after addition of the soil, water, and radioactive compounds. For $^{14}CO_2$ collection, the flasks were flushed with nitrogen into a 20-mL disposable syringe filled with ascarite and fitted with an 18 gauge needle; the flushing period was 1.25 h. Samples of supernatant were also periodically removed for the TLC/beta camera analysis as described above; additional flushing with N_2 was carried out after sampling to maintain the anaerobic condition.

The aerobic and anaerobic shake flasks were terminated by first taking a final $^{14}CO_2$ collection. The flask contents were then transferred to 250-mL centrifuge bottles, centrifuged at 8000 rpm for 15 min, and the supernatant transferred to a 100-mL volumetric flask for analysis by liquid scintillation counting and TLC/beta camera. The soil was washed once with 25 mL of H_2O, centrifuged, and the supernatant removed for analysis for radioactivity. The ^{14}C content of the soil was analyzed by combustion (Peterson et al., 1969; Peterson, 1969) of triplicate 25–

50-mg aliquots of each sample in 200 mg of cellulose. The soils were lyophilized prior to the combustion, and the calculation of ^{14}C content of the soils was related to the calculated dry weight of the soil sample added to the flask initially.

Bound, nonwater extractable soil residues were analyzed by extraction with 0.5 N NH_4OH, followed by TLC/beta camera analysis. One-half of the lyophilized soil sample (2–2.5 g) was extracted in a 250-mL centrifuge bottle three times with 40 mL of 0.5 N NH_4OH. Control extractions of 25 g of zero time soil samples of Ray, Norfolk, and Drummer soils to which 102 μg of the phosphonomethyl-^{14}C-labeled 1 had been added were carried out with 100 mL of 0.5 N NH_4OH three times each. Of the applied ^{14}C-labeled material, 95.9, 100.0, and 76.3% of the radioactivity were extractable from Ray, Norfolk, and Drummer soils, respectively.

Sterile Soil Degradation Studies with Aerobic Shake Flasks. Each flask contained 4.5 g (dry weight) Ray silt loam soil and 100 mL of distilled H_2O with the side arm closed with septums and the top joint fitted with the lower part of CO_2 collecting apparatus. The flasks were autoclaved four times for 20 min at 15 psi and 120 °C with a period of 30 min at room temperature between autoclavings. The radioactive compounds were passed through Swinnex-13 filters (0.22 μ), collected in sterile vials, and placed in the cooled flasks with sterile pipettes. The CO_2 collection apparatus was added as described above. Sampling, termination, and quantitation of radioactivity were carried out as discussed above.

Soil Dissipation Studies. The dry weight of each of the three soils, Ray, Norfolk, and Drummer, was determined by lyophilization of aliquots of each. All soils were made to contain 11% H_2O just prior to treatment. Unlabeled and phosphonomethyl-^{14}C labeled 1, each at a concentration of 1.0 mg/mL, were added in a 1.1/1 ratio to 2100 g of each soil. By adding the appropriate amount of the aforementioned compounds, each soil was treated so that two samples of 2100 g each contained 4 and 8 ppm of the herbicide by weight of soil with 11% moisture. The treated soils were then thoroughly blended in a Hobart mixer for 30 min. Two thousand grams of each treated soil sample as well as 2000 g of each untreated soil were placed into Pyrex dishes (12.7 × 22.9 × 6.3 cm) blackened on the outside and placed in the greenhouse at 26–32 °C. Four DeKalb XL-45 corn seeds (*Zea mays* L.) were planted in each pan; two of the germinating seeds were allowed to grow in each pan.

The soils were watered daily such that optimum greenhouse plant growth conditions were maintained. Full-strength Hoagland's solution (Hoagland and Arnon, 1950) was added periodically as a nutrient supplement.

The treated soils were sampled at zero time and periodically thereafter as desired. One hundred grams of each treated soil was retained initially as a zero time sample. Periodically, four samples of each treated soil were taken with a No. 9 cork bore (12 mm i.d.), the sample composited, and the resulting hole filled with a glass rod.

For extraction, the soil samples (approximately 25 g of each) were placed in a tared 250-mL centrifuge bottle and suspended with vigorous shaking in 100 mL of 0.5 N NH_4OH. The sample was centrifuged and the supernatant removed. The soils were resuspended and extracted in like manner two more times for Ray and Norfolk soils and four more times for Drummer soil samples. The composited extracts were assayed for total ^{14}C radioactivity by liquid scintillation counting before analysis by TLC/beta camera.

Ammoniacal extracts (40 mL) of Ray and Drummer soils were taken to dryness in vacuo on a rotary evaporator. Extracts (6 mL) from Norfolk soil were chromatographed on columns (1 × 30 cm) containing 16 mL of AG-50W-X4 (H+ form, 20–50 mesh, Bio-Rad) resin. The columns were washed with 0.5 N HN_4OH to give a total effluent of 50 mL; based on ^{14}C content, between 91.1 and 96.7% of the applied ^{14}C activity was eluted from the column. The latter sample was taken to dryness, and the dried extracts of each soil were taken up in 200–300 μL of 0.5 M NH_4OH and 20–50-μL aliquots analyzed by TLC/beta camera. After beta camera quantitation, the validity of the TLC was assessed by spraying sequentially with ninhydrin and then Hanes reagent (Stahl, 1969) as described below.

The extracted soils were further analyzed for their dry weight and ^{14}C content. The extracted soils contained in the tared centrifuge bottles were frozen, lyophilized, and dry weight measured in order to calculate the maximum theoretical extractable ^{14}C activity. The dried soil was thoroughly mixed and combusted as described above to determine the residual ^{14}C content.

The soil dissipations were terminated in the following manner. The corn plants were cut off 2.5 cm above the soil, the wet weights measured, frozen, lyophilized, the dry weight determined, ground to 40 mesh in a Wiley mill, and combusted. The maximum ^{14}C content was 0.37% of the applied ^{14}C; control plants grown in untreated soil and located within 15 cm of treated soils contained up to 0.11% of the applied ^{14}C due to incorporation of $^{14}CO_2$ evolved into the open greenhouse. The soil was sampled and analyzed as described previously. The remainder of the soils were stored in the cold for subsequent large-scale extraction, characterization, and bound residue studies.

Assay for Total Microorganism Population. Soil samples from the dissipation studies were taken 33 days after the initiation of the 0 and 4 ppm treatments and 32 days after the initiation of the 8 ppm treatments. A single sample from each treatment was taken using a No. 9 cork borer. The soil was suspended in 100 mL of sterile, autoclaved water in a tared, sterile 250-mL centrifuge bottle. After settling for 20 min, 1-mL aliquots were removed and added to 100 mL of sterile water. A 1-mL aliquot of this latter dilution was taken and diluted 100-fold. Triplicate 1-mL aliquots were taken from the serial dilutions and placed in sterile disposable Petri dishes. Sabourand dextrose agar (20 mL, Fisher Scientific, Inc.) was added to each dish and gently swirled to mix. The agar had been previously prepared by dissolving 60 g in 1000 mL of H_2O, boiling for 1 min, sterilizing at 15 psi (120 °C) for 20 min, and maintaining the sample at 45–50 °C until needed. The Petri dishes were incubated at 30 °C for 18 h, and the total number of colonies were recorded using a Quebec colony counter. The 10^5 dilution was read after incubation for 42 h.

After all dilutions were made, the bottles containing the soil samples were centrifuged at 8000 rpm for 20 min. The supernatant was removed, and the dry weight of each soil was determined so that the number of microorganisms per gram of soil could be calculated.

Large-Scale Shake Flask Metabolism of *N*-Phosphono-^{14}C-methylglycine. In a 2000-mL flask equipped with an ascarite–drierite trapping tower was placed 100 g of Ray silt loam soil and 1000 mL of water containing 97 mg of unlabeled and 3 mg of *N*-phosphono-^{14}C-methyl labeled 1, respectively. The container was shaken at 30 °C for a total of 93 h. The progress of the degradation was monitored by removing 4-mL aliquots at 14, 24, 45, and 93 h. These aliquots were centrifuged,

assayed for ^{14}C content by liquid scintillation counting, and for metabolic distribution by TLC/beta camera analysis. After 93 h, the solution contained 39.2% of the applied radioactivity and was separated from the soil by filtration. After freeze drying, combustion analysis of the soil indicated that 16.7% of applied activity was bound to the soil. Extraction of 70 g of soil three times with 400 mL of 0.5 M NH_4OH as described above gave sequentially 10.6, 4.2, and 1.1% (total of 15.9%) of the starting activity. The supernatant and ammoniacal extracts were stored frozen until ready for purification and spectral identification.

Isolation and Purification of ^{14}C-Labeled Metabolites from the Large-Scale Shake Flask. One-fourth (250 mL) of the supernatant was fractionated on a 250-mL column of AG-50W-X4 (20–50 mesh, H^+ form, Bio-Rad Co.). The sample was eluted with 150 mL of H_2O, followed by a linear gradient of 1000 mL of 1.0 M NH_4OH into 1000 mL of H_2O. Collection of fractions of 20 mL volume with liquid scintillation counting assay gave two radioactive peaks (I and II) eluting at fractions 2–19 (58%) and 32–63 (41%), respectively. Control experiments indicated that ^{14}C-labeled 1 normally eluted at the former volume. Individually, both fractions were taken to dryness, dissolved in 10 mL of water and separately chromatographed on 1.5 × 39 cm columns of DEAE-cellulose (Whatman DE-22) using a two-stage gradient of 200 mL of 0.06 M NH_4HCO_3 into 200 mL of H_2O, followed by 200 mL of 0.2 M NH_4HCO_3 into 200 mL of 0.06 M NH_4HCO_3. Collection of 10-mL fractions afforded AG-50W-X4 fractions I and II eluting at 490–570 mL (81%) and 280–370 mL (99%), respectively. Based on the chromatography of ^{14}C-labeled 1 and unlabeled 2 on DEAE-cellulose, 1 and 2 normally elute at 490–520 and 255–295 mL, respectively, under these conditions. TLC/beta camera analysis of the metabolic fractions indicated that the earlier eluting DEAE-cellulose fraction was metabolite 2 and the late eluting fraction 1. Both DEAE-cellulose metabolic fractions were evaporated to dryness in vacuo and exchanged twice with D_2O before being taken up in 150 μL of D_2O for proton magnetic resonance analysis in a Wilmad 502PP tube. Preliminary NMR analysis of the fraction eluting at 280–370 mL indicated the presence of cellulosic materials, necessitating further purification on a 25-mL column of AG-50W-X4 using 50 mL of water, followed by a linear gradient of 100 mL of 1.0 M NH_4OH into 100 mL of H_2O. The radioactivity (93.6% applied) eluted from this latter column at 70–190 mL. Evaporation and exchange with D_2O as mentioned above was then carried out for analysis by proton magnetic resonance with measurement of the ^{31}P chemical shift by optimum heteronuclear decoupling (Rueppel and Marvel, 1976).

Using the chromatographic methods described above, the ammoniacal soil extract and reference samples of 1 and 2 were prepared in an identical manner. One-half of the ammoniacal extract of the ^{14}C-activity bound to soil was concentrated to 250 mL and purified as described above to give two fractions corresponding to 1 and 2 based on TLC/beta camera analysis. Similarly, a control sample of standard 1 and 2 was prepared by adding a small aliquot of the shake flask supernatant (250 000 dpm; 77 μg total) and 5 mg each of standards 1 and 2 to the 250 mL of water extract of 25 g of untreated soil. Subsequent purification as above afforded a control sample of standards 1 and 2 for use as proton magnetic resonance and mass spectral reference standards. Both 1 and 2 from the bound soil extract and the control experiment were prepared for NMR analysis as described above.

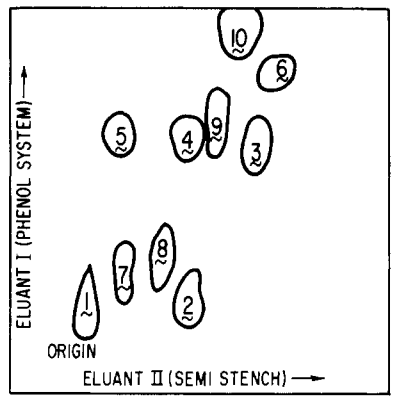

Figure 2. Two-dimensional cellulose thin-layer chromatogram of *N*-phosphonomethylglycine (1) and its potential metabolites as designated in experimental section.

Large-Scale Shake Flask Metabolism of *N*-Phosphono-^{13}C-methylglycine. Into each of two 500-mL shake flask apparatuses containing 25 g of Ray silt loam soil and 250 mL of distilled water was added 180 μg and 25 mg of *N*-phosphono-^{14}C-methyl and -^{13}C-methyl labeled 1, respectively. After shaking at 30 °C for 3 days, TLC/beta camera analysis indicated that the supernatant ^{14}C activity (30.5% of starting total) consisted of 53% of labeled 1 and 47% of labeled 2. The soil slurry was centrifuged and the supernatant concentrated to 53 mL, recentrifuged, and then evaporated to dryness. The residue was dissolved in 0.9 mL of H_2O, filtered with three 0.1-mL washes, and 0.1 mL of dimethyl sulfoxide added as internal standard. The sample was recentrifuged and the supernatant placed in 10-mm Wilmad 529-E tube for analysis by ^{13}C NMR.

Thin-Layer Chromatography Method. Standard compounds, shake flask supernatants, and ammoniacal soil extracts were analyzed routinely by two-dimensional TLC on microcrystalline cellulose plates [20 × 20 cm, 250-μm layer, Quantum Industries (No. 1021)]. Aqueous samples of 5–100 μL were spotted such that a minimum of 10 000 dpm was spotted in most cases. Some samples dilute in radioactivity were lyophilized and redissolved in 100 μL of H_2O; several control experiments established that no loss of radioactivity occurred in this procedure. Fifty micrograms each of the nonradioactive standards 1–9 were spotted coincident with the ^{14}C sample in a total volume of 5 μL. Thin-layer plates were developed two dimensionally, first with a phenol system [90% aqueous phenol–H_2O–acetic acid (84:16:1 v/v), containing 37.2 mg of disodium ethylenediaminetetraacetic acid] and, after drying overnight in a hood, with semistench [isobutyric acid–H_2O–1-propanol–concentrated NH_4OH–2-propanol–1-butanol (500:95:70:20:15:15 v/v), containing 0.24 g disodium ethylenediaminetetraacetic acid] prepared at least 24 h previous to use.

The analysis of the developed TLC plates were carried out sequentially. The air-dried plates were examined by beta camera to locate radioactive spots and quantitate by zoning. The TLC was then sprayed with ninhydrin reagent and heated at 100 °C in order to detect primary and secondary amines. Subsequently, the TLC was sprayed with Hanes reagent (Stahl, 1969) in order to detect phosphorus containing compounds after exposure to UV light. Comparisons between the R_f of a radioactive spot on a beta camera picture and a mass spot on the corresponding TLC were made using a Gerber variable scale (Model TPOO 7100B). A representative TLC separation of the aforementioned compounds and *N*,*N*-dimethyl-

glycine has been shown in Figure 2.

Due to salt effects, it is most important that the TLC behavior of a metabolite fraction be compared with the TLC behavior of standards using cochromatography and subsequent analysis/mass detection. In the presence of excessive quantities of soil extracts, the TLC behavior of these standards can be grossly altered.

Nuclear Magnetic Resonance. High-resolution proton spectra (60 mHz) were run on a JEOL JNM C-60-HL spectrometer equipped with a JNM-AS-1 resolution stabilizer, JRA-1 spectrum accumulator, Monsanto 100 A frequency counter, an external Hewlett Packard Model 200 CD wide-range audio oscillator, a Hewlett Packard Model 5245L electronic counter, a heterospin decoupler (JNM-5DOHC), and an RF oscillator adapter (JNM-OA-1). For a 1% (1 mg) sample in a Wilmad 502PP tube, the R.F. level, A.F. gain, response, and scan time settings for 9 ppm were 32, 1 × 10, 6, and 120 min, respectively. ^{31}P chemical shifts relative to H_3PO_4 were obtained from the ^1H NMR spectra of standard and metabolic samples of phosphonates using optimum heteronuclear decoupling techniques as described in detail elsewhere (Rueppel and Marvel, 1976).

^{13}C NMR spectra were obtained at 22.6 mHz and 35 °C by standard pulsed techniques (Farrar and Becker, 1971) using a Bruker HFX spectrometer, details of which have been described (Schaefer, 1971a,b; Schaefer, 1972). Repetitive intense rf pulses excited the ^{13}C spin system, and the resulting NMR transient responses, or free induction decays, were digitized and accumulated in a Nicolet 1074 time-averaging computer. After a suitably strong signal had been accumulated, a Fourier transform was performed by a Digital Equipment Corporation PDP-8/I, a small laboratory computer which was interfaced to the Nicolet computer. Newer modifications have recently been described (Stejskal and Schaefer, 1974a,b).

Derivatization of 1 and 2. Conversion of metabolic and standard samples of **1** and **2** to the corresponding *n*-butyl *N*-trifluoroacetyl derivatives was effected by trifluoroacetylation with a mixture of trifluoroacetic acid and the corresponding anhydride, followed by esterification with ethereal diazo-*n*-butane. The derivatization procedure has been described in detail previously (Rueppel et al., 1976).

Gas Chromatography–Mass Spectrometry. Analysis of derivatized 1 and 2 by both gas chromatography with flame ionization detection and combined gas chromatography–mass spectrometry methods have been described in detail elsewhere (Rueppel et al., 1976). Mass spectra were acquired and processed using a Varian MAT SS-100MS data system.

Reaction of Ninhydrin with 2. With a slow nitrogen sweep, 111 mg of **2** and 500 mg of ninhydrin in 15 mL of H_2O was heated at 150 °C in an oil bath until the flask was distilled almost to dryness. The distillate was collected in a flask containing 400 mg of 5,5-dimethylcyclohexane-1,3-dione in 50 mL of 50% aqueous ethanol. After cooling the solution, the precipitate was filtered and the resulting white solid air-dried to 140 mg (49%) of the formaldehyde dimedone derivative: mp 189–191 °C [lit. (Liebman et al., 1967) 192 °C]. Repetition of the above experimental procedure without ninhydrin or with 1 mmol of 85% phosphoric acid instead of **2** gave no formaldehyde dimedone derivative in either case.

Runoff. Soil beds were contained in stainless steel trays (91.5 cm long × 30.5 cm wide × 15.3 cm deep). The lower end of the 30.5 cm side was adjustable and contained a funnel across the entire width of the tray to collect runoff water and sediment. *N*-Phosphono-^{14}C-methylglycine (**1**) was applied with a DeVilbis atomizer at a 1.12 kg/ha rate to the upper square third of soil. After 1 day, the soil trays were inclined at an angle of 7.5°, and an artificial rainfall of 1.9 cm per hour was applied using a sprayer (Bouse and Bovey, 1965). Rainfall was applied until the soil became saturated and then two sequential 50-mL runoffs were collected. The sediment was separated by centrifugation and the ^{14}C activity of both soil and water measured. The rainfall runoff procedure was repeated at 3 and 7 days. The temperature was 26–32 °C during this study.

Table I. $^{14}CO_2$ Evolution from Sterile Soil for 7 Days

^{14}C-Label Position[a]	% ^{14}C released
Methane-^{14}C	0.1
Glycine-*1*-^{14}C	0.6
Glycine-*2*-^{14}C	0.3
Sucrose-^{14}C control	0.0

[a] In Phosphonomethylglycine.

Table II. TLC Analysis of *N*-Phosphonomethylglycine Labels at Time Zero and after 7 Days Exposure in Sterile Soil and Aqueous Supernatant

^{14}C label	Exposure (days)	% activity 1	2	3	8
N-Phosphono-^{14}C-methyl	0	94.8	4.3	0.8	
	7	95.3	4.7		
Glycine-*1*-^{14}C	0	99.5			0.5
	7	100.0			
Glycine-*2*-^{14}C	0	98.6		0.4	1.0
	7	99.4			0.6

Photolysis. Forty milligrams of unlabeled **1** and 2 mg of *N*-phosphono-^{14}C-methyl labeled **1** in 250 mL of water were photolyzed using the procedure of Crosby (Crosby and Leitus, 1969; Crosby and Tang, 1969a,b). At 0, 12, 24, and 48 h, aliquots were removed, assayed for ^{14}C content, and analyzed by TLC/beta camera and NMR.

Leaching. Leachability studies were carried out as described by Helling and Turner (1968). Ray, Norfolk, and Drummer soils were seived to 500 μm, slurried with water, and 20 × 20 cm soil TLC plates of 750-μm thickness prepared. After air drying, 10 μg of *N*-phosphono-^{14}C-methylglycine was spotted in a band 2 cm long and then developed with water. After drying, each plate was evaluated by TLC/beta camera analysis. The plates were developed with water again and analyzed by TLC/beta camera.

RESULTS AND DISCUSSION

Soil Degradation Studies. The propensity for chemical degradation of *N*-phosphonomethylglycine in soil and water was examined using sterile soil shake flasks. Sterility was obtained by repetitive autoclaving of slurries of Ray silt loam soil and water. Table I summarizes the $^{14}CO_2$ evolved in a 7-day period, and Table II summarizes the composition of the aqueous supernatants. In line with the minimal $^{14}CO_2$ evolution, TLC/beta camera analysis failed to show any significant change in composition as a result of this exposure. The lack of chemical degradation with any of the three possible ^{14}C labels of **1** indicates that chemical degradation is not a major pathway of degradation of this compound. Similar conclusions have been reached by Sprankle et al. (1975a,b).

N-Phosphonomethylglycine is clearly a biodegradable compound in soil in the presence of soil microflora. The rate and extent of metabolism of **1** in soil is clearly rapid and complete as evidenced by both aerobic and anaerobic

Table III. Summary of $^{14}CO_2$ Evolution from Shake Flasks as a Function of Time and ^{14}C Label for Ray Silt Loam Soil

^{14}C label	Type of metabolism[b]	Percent ^{14}C released as $^{14}CO_2$ at 30 °C at day analyzed					Total ^{14}C as $^{14}CO_2$
		3	7	14	21	28	
N-Phosphono-^{14}C-methyl[a]	A	13.2	16.7	10.1	4.6	2.2	46.8
	An	6.2	8.2	17.8	3.7	1.4	37.3
Glycine-*1*-^{14}C[a]	A	28.1	10.1	13.8	1.9	1.4	55.3
	An	12.6	14.8	18.9	4.8	1.3	51.4
Glycine-*2*-^{14}C[a]	A	19.0	16.2	12.8	4.0	3.3	55.3
	An	3.8	6.8	9.9	5.9	7.1	33.5
Sucrose-^{14}C	A	41.5	5.3	4.9	3.0	2.4	57.9
	An	34.3	10.1	5.3	3.1	2.5	55.3
Sucrose-^{14}C	A	26.7	13.0	11.7	2.2	2.0	55.6
Plus unlabeled 1	An	22.3	9.5	7.4	11.5	10.8	61.5

[a] Labeled *N,N*-phosphonomethylglycine. [b] Aerobic (A); anaerobic (An).

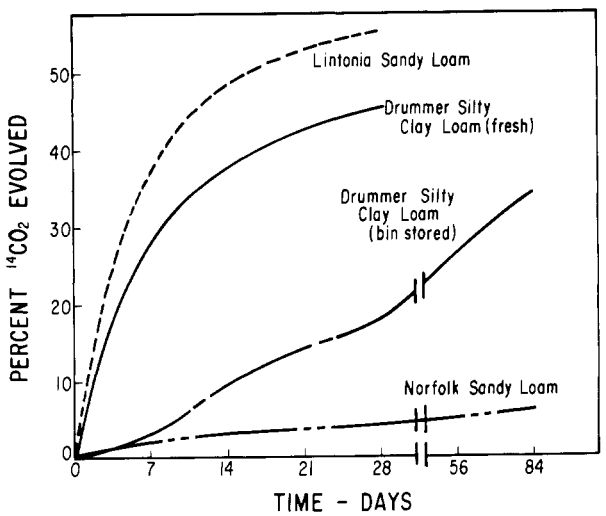

Figure 3. $^{14}CO_2$ evolution under aerobic conditions from *N*-phosphono-^{14}C-methylglycine on Lintonia, Drummer, and Norfolk soils.

shake flask metabolism studies. As summarized in Table III, all three carbons of 1 were rapidly degraded to $^{14}CO_2$ at comparable rates in the presence of Ray silt loam soil. In addition, all three ^{14}C-labeled compounds were degraded to nearly the same extent and rate as the natural and general metabolite sucrose-^{14}C. From the three ^{14}C labels, 47–55% of 1-^{14}C was given off as $^{14}CO_2$ in 4 weeks on Ray silt loam soil; sucrose-^{14}C gave off 57.9% of its label as $^{14}CO_2$ under the same conditions. These data clearly illustrate the significant effect of microflora on 1 in contrast to the insignificant contribution of nonbiological chemical degradation. Clearly, the chemical bonds of 1 can be cleaved easily by microflora.

Figure 3 summarizes the $^{14}CO_2$ evolutions observed for three other soils under aerobic conditions using the *N*-phosphono-^{14}C-methyl labeled 1 as the substrate. As can be seen, rapid $^{14}CO_2$ evolution from and degradation of 1 occurred with both Lintonia sandy loam and fresh Drummer silty clay loam soils. Significant, rapid degradation of 1-^{14}C to $^{14}CO_2$ occurred even on aged, dry, bin-stored Drummer soil, although at an expected slower rate, due to reduced microbial population. Although the data have not been included, the rate and extent of $^{14}CO_2$ evolution from these latter two soils were comparable with the rate of $^{14}CO_2$ evolution from the reference standard sucrose-^{14}C; in addition, the rate of $^{14}CO_2$ evolution was also independent of ^{14}C-label position in 1 as mentioned previously for Ray silt loam. The rate of evolution of $^{14}CO_2$ from 1-^{14}C on Norfolk sandy loam was slower than the other soils but still significant. The decreased rate of degradation of 1 with Norfolk soil is not understood, but may reflect the microbial composition and population as well as the extent of soil binding. As a result of the studies summarized in this paper and due to the recently published studies of Sprankle et al. (1975a,b), the total rapid biodegradability of *N*-phosphonomethylglycine is, in general, indicated conclusively.

When degradation of *N*-phosphonomethylglycine occurs rapidly in soil, a single pattern is followed for fresh soil samples. This pattern is characterized by an initial rapid phase of degradation, followed by a slower rate of degradation after approximately 7 days. No lag phase was observed with fresh soil samples. The decline in rate of degradation with time reflects the decrease in soluble pesticide by the adsorption of 1 and metabolites to soil. This pattern (Table III and Figure 3) probably indicates that a high percentage of the total microflora population are actively capable of degrading 1. Similar patterns have been observed upon recharging of a soil with the same biodegradable substrate (Tiedje and Mason, 1974); this reflected induction of a large microflora population capable of degrading the substrate nitrilotriacetic acid (NTA). Only with a Drummer soil sample which had been stored for a prolonged period and allowed to dry out was a lag phase observed in the degradation of 1; plate counts indicated that the microflora population of this latter soil was 100-fold less than normal.

The utility and relevance of soil shake flask metabolism studies is clearly indicated by the aforementioned studies and subsequent studies reported below. The shake flask "biometer" approach with soil/water ratios as desired clearly lends itself to both aerobic and anaerobic metabolism studies. Aerobic conditions are obtained by free air interchange through the ascarite trap, while a closed system nitrogen atmosphere generates anaerobic conditions. The ascarite trapping tower clearly permits quantitation of evolved $^{14}CO_2$ without requiring a closed system (Bartha and Pramer, 1965) or a cumbersome gas train (Parr and Smith, 1969). The 44 soil shake flasks in this study had an average total accountability of 91.3%. Although we have used in this study a 20:1 ratio of water to soil, we have found the rate of degradation to be very comparable with the rate of dissipation in moist soil alone as discussed below. The use of the soil shake flask "biometer" approach to quantitative soil dissipation/degradation studies should prove to be advantageous for future pesticide metabolism studies (Marvel et al., 1976; Marvel et al., 1977).

TLC/beta camera analyses of the supernatants from both aerobic and anaerobic shake flask experiments have established the metabolite distribution from the three ^{14}C labels of 1 and provided a chromatographic identification of metabolites as summarized in Table IV. The use of

Table IV. Analysis of Terminal Aqueous Supernatants from Soil Shake Flask Experiments

Label position in 1	Soil[a]	Type of metabolism[b]	% ^{14}C activity in supernatant	% starting activity		
				1	2	Others[c]
N-Phosphono-^{14}C-methyl	Ray	A	5.4		4.4	1.0 (1)
		AN	2.6		1.9	0.6 (2)
	Drummer	A	18.1	7.6	8.3	2.1 (3)
		AN	18.8	1.0	15.0	2.3 (3)
Glycine-*1*-^{14}C	Ray	A	0.3			
		AN	0.2			
	Drummer	A	15.9	15.7		0.2 (1)
		AN	8.1	8.1		
Glycine-*2*-^{14}C	Ray	A	1.4			
		AN	4.8			
	Drummer	A	8.4	8.3		0.1 (1)
		AN	16.2	15.2		1.0 (1)

[a] Ray silt loam and Drummer silty clay loam soil experiments had durations of 28 and 84 days, respectively. [b] Aerobic (A); anaerobic (AN). [c] The number of components comprising the percentage is in parentheses.

Table V. Extraction and Analysis of Terminal Soils from Soil Shake Flask Experiments

Lable position in 1	Soil[a]	Type of metabolism[b]	% starting activity of 1					
				NH_4OH		Extractable as		
			Bound[c]	Unextractable[c]	Extractable	1	2	Others[d]
N-Phosphono-^{14}C-methyl	Ray	A	31.4	8.5	22.9	1.5	21.2	0.2 (1)
		AN	46.3	12.8	33.5	3.0	29.7	0.9 (1)
	Drummer	A	36.3	16.7	19.6	12.0	7.1	0.5 (1)
		AN	30.4	15.1	15.3	3.3	12.0	
Glycine-*1*-^{14}C	Ray	A	12.4	9.7	2.7	1.7		1.0 (1)
		AN	18.2	14.1	4.1	2.4		1.7 (1)
	Drummer	A	31.0	18.0	13.0	12.7		0.3 (1)
		AN	18.9	15.6	3.3	2.8		0.5 (1)
Glycine-*2*-^{14}C	Ray	A	46.7	40.3	6.4	1.1		5.3 (8)
		AN	47.8	40.3	7.5	1.7		5.8 (5)
	Drummer	A	46.0	33.9	12.1	9.9		2.2 (4)
		AN	47.1	31.6	15.5	11.8		3.7 (3)

[a] All Ray and Drummer soil samples had metabolized 1-^{14}C for 28 and 84 days, respectively. [b] Aerobic (A); anaerobic (AN). [c] Determined by combustion analysis. [d] The number of components comprising the percentage is in parentheses.

all three ^{14}C labels provided confirmatory characterization data. The metabolite distribution was clearly similar for both the aerobic and anaerobic shake flask for a given soil. The same major metabolites were observed, in general, regardless of the soil type. The principal soil metabolite observed from the *N*-phosphono-^{14}C-methyl label of **1** was aminomethylphosphonic acid (**2**); as expected due to the ^{14}C-label position, **2** was not observed radioactively from the two glycine-^{14}C labels. The maximum amount of **2** detected in the supernatant was 15% of the starting ^{14}C activity. Several other minor metabolites were also detected chromatographically in some cases. These minor metabolites included *N*-methylaminomethylphosphonic acid (**3**), glycine (**8**), *N*,*N*-dimethylaminomethylphosphonic acid (**6**), hydroxymethylphosphonic acid (**7**), and two unknown metabolites; none of these minor metabolites were normally present to an extent greater than 1% of the applied radioactivity. No metabolic products containing an intact *N*-phosphonomethylglycine grouping were detected in these studies.

Analysis of the shake flask soils was also carried out by TLC/beta camera analysis after extraction of the soil with 0.5 M NH_4OH in each case (Table V). The extractable bound residue consists mainly of the parent **1** and aminomethylphosphonic acid (**2**), the major metabolite in agreement with the analysis of the supernatants discussed above. As in the case with the shake flask supernatants, several minor metabolites were observed, and the same general distribution was observed on both the aerobic and anaerobic soils.

After mild, room temperature extraction of the aforementioned soils with 0.5 M NH_4OH, combustion analysis of the soils indicated that from 8.5 to 40.3% of the applied ^{14}C activity was bound to soil. As can be seen in Table V, the amount of NH_4OH nonextractable residue is highly dependent on the ^{14}C-label position. Although present technology permits only a qualitative characterization of soil-bound residue, a reasonable insight can be gained into their nature as a result of these studies. The demonstrated efficient extractability of **1** from soils with NH_4OH rules out the presence of the intact parent as well as the metabolite **2** which would be expected to possess similar extraction characteristics. In lieu of the extensive $^{14}CO_2$, extensive metabolism of **1** occurs in soil, and it seems reasonable to assume the NH_4OH nonextractable residue represents extensively metabolized products of **1** and **2** (see below). The fact that **2** is the principal metabolite of **1** indicates that the glycine methylene and carboxyl groups may be lost as glyoxylate. Glyoxylate can be readily metabolized (Mahler and Cordes, 1966) via the glyoxylate and citric acid cycles. Examination of the cycles show that the aldehydic carbon of glyoxylate (equivalent to the glycine-2 position of **1**) should be especially well incorporated into a variety of natural products by microflora of the soil. Simple natural products are, of course, known to be precursors of complex, insoluble, nonextractable natural constituents in soil organic matter. In fact, the amount of NH_4OH unextractable ^{14}C activity was higher in the glycine-2-^{14}C label experiments by a factor of two–five; furthermore, the detection of several unknown minor metabolites only in the glycine-2-^{14}C case (Table V) is supporting evidence. Biosynthetic utilization of gly-

oxylate without decarboxylation or reincorporation of $^{14}CO_2$ from the glycine-*1*-^{14}C label could also lead to bound residue. The latter process as well as the biochemical utilization of formaldehyde formed from transamination of **2** easily accounts for bound residue formation from the *N*-phosphono-^{14}C-methyl position. Previous work (Roberts et al., 1968) and our studies with ninhydrin have established the biochemical and chemical bases, respectively, for converting **2** to formaldehyde via formylphosphonic acid.

The major metabolite of **1** has also been established to be highly biodegradeable. Aminomethyl-^{14}C-phosphonic acid shake flask studies with Ray silt loam and Drummer silty clay loam soils gave 34.8 and 16.1%, respectively, of the applied ^{14}C as $^{14}CO_2$ in 63 days. As discussed below, the rapid metabolism of **2** has also been established by the soil dissipation studies. The slower degradation of **2** than **1** may reflect tighter binding to soil and/or lower permeability of **2** through the cell walls of microflora.

The rapid and total environmental degradation of both **1** and **2** is not surprising in lieu of previous work on other aminoalkylphosphonates. Many natural aminoalkylphosphonates have been isolated from living organisms (Korn et al., 1973; Kittredge and Hughes, 1964; Kittredge et al., 1967). The metabolism of some of these phosphonates and several unnatural analogues has been reported by Marvel et al. (1975), Roberts et al. (1968), and Harkness (1966). Marvel et al. (1975) has reported on the total biodegradability of glyphosine [*N*,*N*-bis(phosphonomethyl)glycine] and Harkness (1966) found that six of ten organisms examined could grow on **2** as the sole phosphorus source.

Soil Dissipation. The rate of dissipation of **1** was measured in three general and representative soil types so that a thorough understanding of its fate in different soil types could be obtained. Soil samples of Ray silt loam, Norfolk sandy loam, and Drummer silty clay loam soil were treated with the *N*-phosphono-^{14}C-methyl labeled **1** at the rate of 4 and 8 ppm (approximately equivalent to 4.48 and 8.96 kg/ha) and homogeneously mixed. In order to provide a more complete and normal ecological environment for this greenhouse study, corn was planted immediately after treatment. The crop also provided a means of monitoring the desired soil moisture. At selected times, quadruplicate core samples were taken, composited, and extracted thoroughly with 0.5 M NH_4OH. The extracts were assayed for ^{14}C activity by liquid scintillation counting and for composition by TLC/beta camera. The extracted soils were frozen, lyophilized, the dry weight determined in order to calculate the maximum ^{14}C activity to be expected, and combusted to measure the ^{14}C bound to the soil.

As expected as a result of the nonsterile shake flask study, the rate of dissipation of **1** was clearly rapid on two of the three soil types as shown in Figure 4. In particular, **1** was 90% dissipated after 14 and 80 days in Ray silt loam and Drummer silty clay loam soils, respectively. The half-lives of **1** in Ray and Drummer soils at 4 ppm were 3 and 27 days, respectively, and the ^{14}C accountability decreased to 23.4 and 35.8%, respectively. The half-lives of **1** in Ray and Drummer soils were 3 and 25 days, respectively, at 8 ppm, indicating that the rate of degradation is reproducible and independent of concentration. Clearly complete biodegradation of **1** to $^{14}CO_2$ (64–76% of the total by difference) occurred with Drummer and Ray soils in 112 days as observed in the aforementioned shake flask studies. As seen in Figure 4 in agreement with the shake flask studies, the main metabolite **2** was also rapidly degraded. As a consequence, neither the parent **1** or its

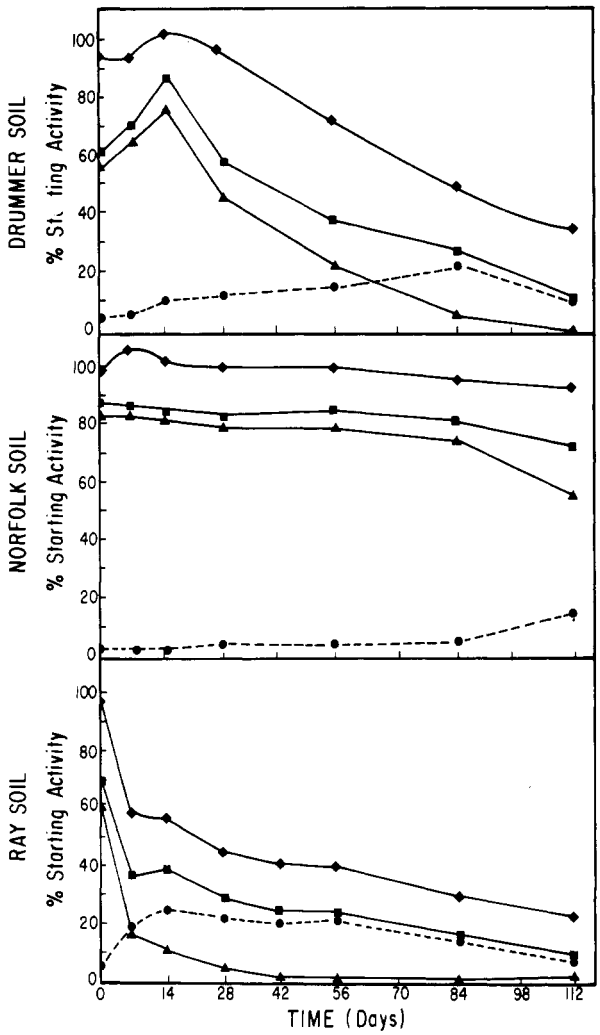

Figure 4. Dissipation of *N*-phosphono-^{14}C-methylglycine and its metabolite in soil showing total ^{14}C-accountability (♦), extractability (■), *N*-phosphono-^{14}C-methylglycine (▲), and aminomethyl-^{14}C-phosphonic acid (●).

metabolite should persist in the environment.

Norfolk soil alone degraded **1** more slowly with 43% dissipation in 112 days; the data indicate that the mode and extent of metabolism is proceeding in a similar, albeit slower (half-life of 130 days) manner than with the other two soils. This slower metabolism of **1** is not a general property of this soil type in lieu of studies with Lintonia sandy loam and the work of Sprankle et al. (1975) using Conover and Spinks sandy loam soils. Actual field studies (Sharp, 1974) on eleven different soils covering a full range of soil types and geographical areas indicated an average half-life of 2 months further substantiating the rapid degradation of **1**.

Effect on Microorganisms. The available data are all consistent with the general interpretation that the effect of **1** on the soil microflora is minimal and not pronounced. First, the rate of degradation of sucrose-^{14}C to $^{14}CO_2$ (Table III) was comparable in both the presence and absence of **1**; in the three soils examined, the rate $^{14}CO_2$ evolution was similar in each case both anaerobically and aerobically and in both the presence and absence of added **1**. Secondly, the rapid rate of degradation of **1** indicates that a large microflora population can degrade and, therefore, detoxify **1**. Finally, microbiological plate counts of both treated and untreated soils indicate that glyphosate has no adverse effect on the overall microflora population (Table VI).

Table VI. Total Microorganisms in *N*-Phosphonomethylglycine Treated and Untreated Soils

Soil	Rate of treatment, ppm	Number of microorganisms per gram of soil[a] ($\times 10^{-5}$)
Ray	0	6.1
Silt	4	3.6
Loam	8	4.5
Norfolk	0	3.8
Sandy	4	24.0
Loam	8	62.0
Drummer	0	4.8
Silty	4	4.0
Loam	8	2.3

[a] Average of 10^3 and 10^5 dilutions.

Independently, Quilty and Geoghegan (1976) have found that glyphosate has minimal effect on microflora in peat. In fact, in the case of Norfolk soil, the total population is actually increased in the presence of *N*-phosphonomethylglycine.

Runoff. Runoff of *N*-phosphono-^{14}C-methylglycine from Ray, Drummer, and Norfolk inclined soil beds at 7.5° was examined using a rate of 1.12 kg/ha applied uniformly to the upper third (1 sq ft) of the soil surface. The entire soil surface was then subjected to three artificial rainfalls at 1, 3, and 7-day intervals after treatment. Each time rainfall was continued through collection of two consecutive 50-mL samples of runoff water and sediment. The water was separated from the sediment by centrifugation and the ^{14}C content of each determined.

In both the sediment and runoff water, the amount of ^{14}C activity collected was extremely low ranging from 6.5×10^{-3} down to 1×10^{-4} % of that applied for the water and 3×10^{-3} to 1×10^{-5} % for the sediment. These data correspond to a maximum runoff of less than 2×10^{-4} kg/ha. The lack of runoff is not surprising in lieu of the tight binding of 1 to soil [Sprankle et al. (1975a) and data presented below].

Leachability. Using the method of Helling and Turner (1968), the mobility (i.e., leachability) of *N*-phosphonomethylglycine in Ray, Norfolk, and Drummer soils has been examined. Soil thin-layer plates were spotted with *N*-phosphono-^{14}C-methylglycine and developed twice with water; the distribution of ^{14}C activity relative to the origin was determined by beta camera analysis after each development. The parent compound was so strongly adsorbed by all three soils that 97–100% of the ^{14}C activity had an R_f of less than 0.09. Similarly, 95–99% of the starting ^{14}C activity remained at an R_f of less than 0.09 after the second development. In no case was any of the radioactivity at an R_f greater than 0.18. Using the Helling and Turner (1968) classification system, 1 would be categorized as a class I pesticide and thereby possess no propensity for leaching.

Photodecomposition. The propensity for photodecomposition of 1 to occur using a Crosby photoreactor (Crosby and Leitus, 1969; Crosby and Tang, 1969a,b) has been examined. Irradiation of a solution of *N*-phosphono-^{14}C-methyl labeled 1 for 48 h (equivalent to sixteen 8-h days of sunlight) was carried out in the presence of air; the ^{14}C content and its composition was measured periodically by liquid scintillation counting and TLC/beta camera analysis. As shown in Table VII, no loss of ^{14}C content via volatile degradation products occurred, and the composition of the solution was not altered. As a consequence of the above data and the rapid microbial metabolism of 1, photodecomposition will be, at most, a very minor cause of degradation of 1 in the environment.

Table VII. Exposure of an Aqueous Solution of *N*-Phosphono-^{14}C-methylglycine (1) to Light in a Crosby Reactor

Time, h	% ^{14}C in solution	% ^{14}C		
		1	2	3
0	100.0	95.3	4.1	0.4
12	101.3	96.2	3.5	0.1
24	99.2	95.8	4.0	0.2
48	98.4	94.7	5.0	0.4

Table VIII. TLC Analysis of Aqueous Supernatant from Large-Scale Aerobic Shake Flask as a Function of Time

Time, h	% of zero time in solution	% starting activity	
		1	2
0	100.0	96.0	3.3
14	72.4	67.4	5.0
24	68.4	60.6	7.6
45	58.7	44.2	14.6
93	39.2	19.0	19.6

Spectral Characterization of Soil Metabolites. Based on thin-layer chromatography, the major and essentially only soil metabolite of 1 was indicated to be aminomethylphosphonic acid (2). In order to verify 2 as the soil metabolite of 1, characterization has been carried out by ^1H, ^{31}P, and ^{13}C NMR techniques as well as mass spectral methods. The use of ^{31}P and ^{13}C NMR techniques represents the first application of these techniques to the characterization of pesticide metabolism; a preliminary account has appeared previously (Marvel et al., 1974). ^{13}C NMR, in particular, utilizing ^{13}C-enriched pesticides should prove to be a valuable characterization technique with broad applicability to many metabolic problems.

In order to facilitate characterization and overcome a case in which the metabolite is also transient in nature, a large scale (100 mg) metabolism shake flask experiment was carried out, monitored by TLC/beta camera, and terminated before all parent and metabolite had degraded. Table VIII summarizes the results of this experiment with termination carried out at 93 h after initiation. Rapid degradation clearly occurred with only 39% of the ^{14}C activity remaining in solution and 16% bound to the soil. An analogous experiment was separately carried out using *N*-phosphono-^{13}C-methyl labeled 1 with a similar result; it will be discussed subsequently in more detail.

In order to carry out ^{31}P and ^1H NMR and mass spectral characterization of the metabolic supernatant, soil-bound, and control samples, purification was required. The parent and metabolite 2 in each case could be easily separated by chromatography on the cation-exchange resin (AG-50, Bio-Rad Labs). Identical elution volumes were observed in each case for fractions containing parent 1 and metabolite 2, respectively. Separately both 1 and 2 from the AG-50 columns were then purified in each case using a column of DEAE-cellulose and NH_4HCO_3 gradients. The supernatant, soil-bound, and control samples of 1 were then exchanged with deuterium oxide in preparation for analysis by NMR. The aminomethylphosphonate fractions contained cellulosic materials, and purification through a second small AG-50 (H^+) column was required in each case before exchange in preparation for analysis by NMR. In all column chromatographies, the elution volumes of control standard samples of 1 and 2 corresponded well with the volumes observed for the parent and its suspected metabolite 2 in agreement with their respective identities

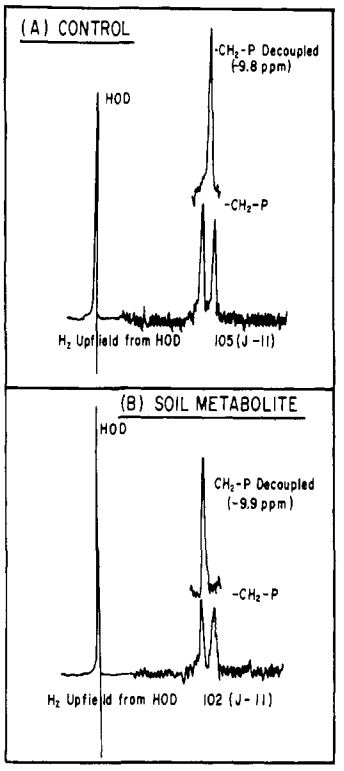

Figure 5. ^{1}H NMR (^{31}P decoupled) spectra of control (A) and supernatant (B) samples of aminomethylphosphonic acid.

based on TLC/beta camera analysis.

As detailed elsewhere (Rueppel and Marvel, 1976), ^{1}H NMR and ^{31}P NMR spectra for **1** and possible metabolites **2**, **3**, **4**, **5**, and **7** permit unique, unequivocal characterization capabilities for these six phosphonates when taken in combination; in addition, the feasibility of accurately measuring the ^{31}P chemical shift indirectly was also established. This latter technique was important due to the minimal sample size and the inherent insensitivity of ^{31}P NMR relative to ^{1}H NMR. Application of the aforementioned techniques to the supernatant, soil-bound, and control samples of **1** and **2** have unequivocally characterized the parent and metabolite **2**. The control samples of **1** and **2** were identical with the metabolic samples as seen in Figure 5, for example, which compares the ^{1}H NMR spectra and ^{31}P chemical shift of the control sample of **2** with the actual supernatant metabolite. Metabolite **2** bound to and extracted from soil had a ^{1}H chemical shift of 103 Hz (doublet, $J = 11$) upfield from water and a ^{31}P chemical shift of -9.4 ppm downfield from H_3PO_4 in excellent agreement with aforementioned control and metabolic samples of **2**. Similarly, parent **1** reisolated from the supernatant showed ^{1}H chemical shifts at 70 Hz (singlet) and 107 Hz (d, $J = 12$) upfield for the glycine and phosphonomethylenes, respectively, while **1** from soil had ^{1}H chemical shifts at 62 Hz (singlet) and 95 Hz (doublet, $J = 12$); the corresponding ^{31}P chemical shifts were both -7.8 ppm from H_3PO_4. The control sample of **1** showed a singlet and a doublet at 64 and 98 Hz ($J = 12$), respectively, and a ^{31}P chemical shift at -7.8 ppm from H_3PO_4. These spectral data clearly confirm parent **1** and its metabolite **2** as essentially the only labeled residues present in the large-scale shake flask supernatant and soil and verify the TLC and column chromatographic characterizations.

The aforementioned characterizations have also been confirmed by combined gas chromatography–mass spectrometry. After back-exchange with water, the four

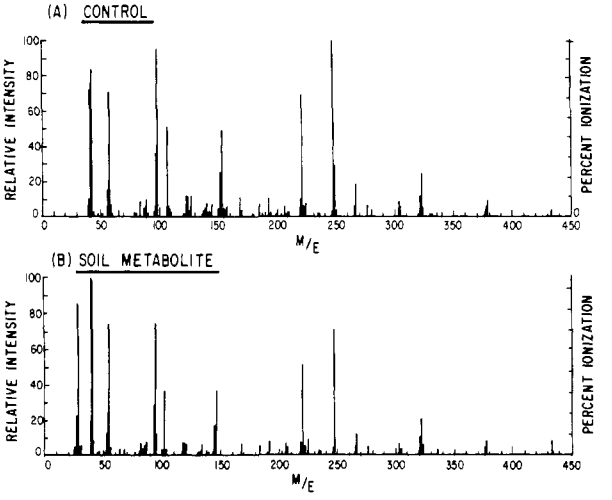

Figure 6. Mass spectra of tri-*n*-butyl-*N*-trifluoroacetyl-*N*-phosphonomethylglycine from control (A) and metabolic (B) supernatants.

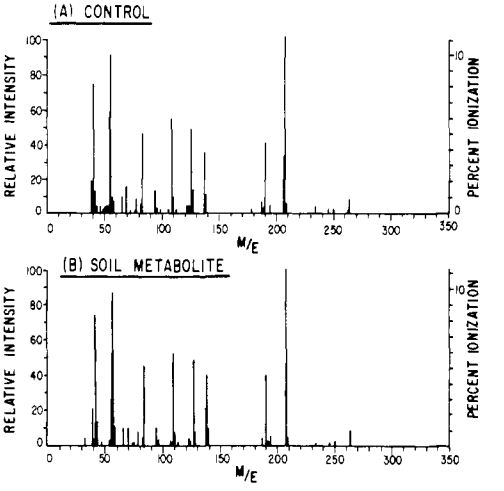

Figure 7. Mass spectra of di-*n*-butyl *N*-trifluoroacetylaminomethylphosphonate from control (A) and metabolic (B) supernatants.

metabolic and two control samples were derivatized to the *n*-butyl *N*-trifluoroacetyl esters with trifluoroacetic acid anhydride, followed by esterification with ethereal diazo-*n*-butane as described previously (Rueppel et al., 1976). Gas chromatography–mass spectrometric analysis was carried out as described in the latter reference. The mass spectra of control and supernatant samples of **1** and **2** can be seen in Figures 6 and 7, respectively. These mass spectra were also identical with previously published (Rueppel et al., 1976) mass spectra of the *n*-butyl *N*-trifluoroacetyl derivatives of pure standards of *N*-phosphonomethylglycine and aminomethylphosphonic acid, respectively. The mass spectra of **1** and **2** extracted from the soil were also identical with mass spectra shown in Figures 6 and 7 and the latter reference. These data further confirm the previously chromatographic and NMR characterizations of **1** and **2**.

We have also carried out a large-scale shake flask experiment using 25 mg of 90% enriched *N*-phosphono-^{13}C-methylglycine. In addition to providing additional spectral characterization of the parent and its soil metabolite(s), the great potential for the use of stable isotopes in metabolic studies prompted us to explore its capabilities in this case since an assessment relative to the other

Table IX. ^{13}C NMR (Proton Decoupled) Chemical Shift Data for *N*-Phosphonomethylglycine and Its Potential Metabolites in Water[a]

Compd	O=*C*-C	C-*CH*$_2$N	C-(*CH*$_3$)N-C	N-*CH*$_2$-P	*CH*$_3$P	O-*CH*$_2$-P
8	176.3	42.7				
5	174.1	60.6	45.3	54.9		
4					13.6	
7						59.4
2				37.4		
3			36.5	47.5		
9	175.4	51.9	34.3			
1	174.7	51.5		44.7		

[a] Parts per million downfield from tetramethylsilane with a minimum accuracy of ±1 ppm and a pH less than 2.5. Me_2SO was used as the internal reference.

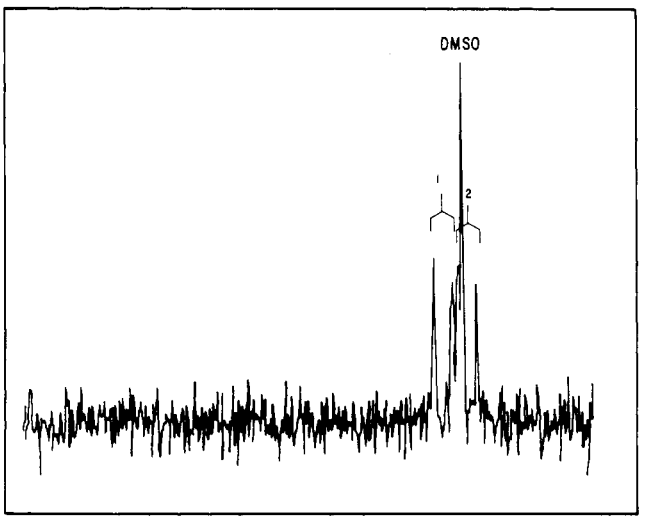

Figure 8. ^{13}C NMR (^1H decoupled) spectrum of filtered, concentrated supernatant from shake flask experiment using *N*-phosphono-^{13}C-methylglycine and Ray silt loam soil.

spectral methods described above was feasible. The use of ^{13}C NMR in conjunction with ^{13}C-enriched *N*-phosphonomethylglycine offered promise of providing substantial structural information more rapidly than other techniques and with minimal purification. The use of a highly (80–90%) ^{13}C-enriched material should improve the sensitivity almost 100-fold over natural abundance and eliminate natural abundance contributions by localizing a large percentage (approximately 50% if 1% pure) of the total sample carbon-13 in just a few resonance frequencies related to the parent and/or metabolites. The structural information would result from the fact that the carbon-13 chemical shift range is broad, spanning approximately 200 ppm and very sensitive to the electronic environment of the given carbon. As we shall discuss below, our predictions have been substantiated.

As a basis for our studies, ^{13}C NMR reference spectra have been obtained in water for the three different ^{13}C-enriched *N*-phosphonomethylglycines and seven natural abundance potential metabolites or metabolite analogues. These data have been summarized in Table IX and establish that substantial structural information and/or identification could be obtained by ^{13}C NMR in this case. The 17 carbons were clearly distinguishable from one another in all cases on the basis of the carbon-13 chemical shifts. Although very closely related structurally and/or electronically, all six of the carbons directly bonded to phosphorus were clearly discernible and possessed distinct carbon-13 chemical shifts characterized by a doublet with a prominent ^{13}C, ^{31}P coupling constant of approximately 140 Hz in each case. These data clearly indicate that with appropriate model compounds, ^{13}C NMR analysis alone would permit an intelligent hypothesis as to the type of substitution (N, O, H, etc.) present on the phosphonomethylene functionality. This latter type of information obviously could have great utility in elucidating the structure of an unknown phosphonate; many applications of the ^{13}C chemical shift as a probe of metabolic alteration can be envisioned for a multitude of metabolic problems involving pesticides and drugs.

The ^{13}C NMR spectrum obtained by analysis of the concentrated supernatant from a shake flask experiment with *N*-phosphono-^{13}C-methylglycine can be seen in Figure 8. The expected two doublets with coupling constants of approximately 140 Hz can be discerned for both the parent compound and its metabolite aminomethylphosphonic acid (2); with respect to tetramethylsilane using Me_2SO as the internal reference, 1 and 2 had ^{13}C chemical shifts of 46.3 and 38.3, respectively, in the proton decoupled spectrum. The 1:1 ratio of 1 to 2 based on TLC analysis was also detected by ^{13}C NMR. The spectrum shown in Figure 8 was obtained without purification; the aqueous shake flask supernatant was simply concentrated and filtered in contrast to the multiple chromatographies required to obtain the analogous ^1H NMR spectra and/or mass spectral analysis. The ^{13}C NMR spectrum (Figure 8) required at least a factor of 5 less time than the previous ^1H and ^{31}P NMR studies to achieve a substantially definitive characterization since the natural abundance carbon-13 contributions of the normal soil constitutents were overwhelmed by the ^{13}C-enriched parent and metabolite (2).

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Studies with Manganese [^{14}C]Ethylenebis(dithiocarbamate) ([^{14}C]Maneb) Fungicide and [^{14}C]Ethylenethiourea ([^{14}C]ETU) in Plants, Soil, and Water

Robert C. Rhodes

Total radiochemical residues on tomato and bean plants decreased rapidly with time following foliar field applications of either [^{14}C]maneb or [^{14}C]ETU (ethylenethiourea). No accumulation or persistence of intact ETU residues was observed from either treatment. In soil treated with [^{14}C]ETU or with [^{14}C]maneb, the half-life of total radiochemical ^{14}C-labeled residues under field conditions was less than 4 weeks for ETU and between 4 and 8 weeks for maneb, whereas the half-life for intact ETU itself was less than 1 week. In plant uptake tests, only trace amounts of total radiochemical residues were taken up by tomato plants grown in field soil treated with [^{14}C]maneb or [^{14}C]ETU. No intact ETU residues were detected in plants from these treatments, and ripe tomatoes from these plants contained no detectable ^{14}C-labeled residues (<0.01 ppm). In water, glycine was confirmed as the major photodegradation product of [^{14}C]ETU. Ethyleneurea, hydantoin, and Jaffe's base were also identified by mass spectrometry. Overall, these ^{14}C data indicate very little likelihood for the appearance of significant amounts of ETU in the environment or on maneb-treated crops.

The ethylenebis(dithiocarbamate) (EBDC) fungicides are used extensively for the control of a variety of fungus diseases of certain vegetable, fruit, and ornamental crops. Ethylenethiourea (ETU), a possible degradation product of the EBDC fungicides, has been reported to be carcinogenic to rats (Graham and Hansen, 1972; Graham et al., 1973). In a more recent paper (Graham et al., 1975), these workers confirmed carcinogenicity at higher dose rates but concluded that ETU was "not biologically deleterious to the rat" at 5- and 25-ppm dietary levels in 2-year studies.

Trace amounts of ETU have been reported on EBDC-sprayed crops (Lyman, 1971; Lyman and LaCoste, 1975; Newsome et al., 1975; Nash, 1974, 1975, 1976; Yip et al., 1971). In general, several of these workers and others have shown that ETU disappears rapidly from treated plants and soil (Blazquez, 1973; Hoagland and Frear, 1976; Kaufman and Fletcher, 1973). Other workers, however,

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