Degradation of Thifensulfuron Methyl in Soil: Role of Microbial Carboxyesterase Activity

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Thifensulfuron methyl [methyl 3-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]amino]-sulfonyl]-2-thiophenecarboxylate] degrades rapidly in diverse nonsterile agricultural soils. The initial degradation product of thifensulfuron methyl in nonsterile soils is its deesterified derivative, thifensulfuron acid, which is herbicidally inactive. Rapid deesterification of thifensulfuron methyl is eliminated by heat sterilization of soil but only moderately reduced by sterilization with ethylene oxide. Deesterification is inhibited in both nonsterile and ethylene oxide-sterilized soils by iodoacetamide and specific organophosphorus insecticides. Several actinomycetes and bacteria were isolated from soils which could readily deesterify thifensulfuron methyl in pure culture. Cell-free (sterile) culture filtrates of two actinomycetes also catalyzed the deesterification of this herbicide. We conclude that the rapid deesterification of thifensulfuron methyl results, at least in part, from the activity of microbial extracellular carboxyesterase activity.

Keywords: *Thifensulfuron methyl; sulfonylurea herbicide; soil enzymes; soil carboxyesterase; biodegradation*

INTRODUCTION

Since 1982, 20 sulfonylurea herbicides have been commercialized for use under a wide variety of agronomic conditions in numerous crops (Brown and Cotterman, 1994). These new herbicides collectively span a wide range of soil residual properties which are designed to meet specific agricultural needs. Thifensulfuron methyl [methyl 3-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]amino]sulfonyl]-2-thiophenecarboxylate, formerly DPX-M6316] is a sulfonylurea herbicide widely registered for postemergence broadleaf weed control in wheat, barley, maize, and soybeans. As with other sulfonylurea herbicides, it is used at low application rates, ranging from 4.3 g of ai/ha in soybeans (DuPont, 1989) to 40 g of ai/ha in wheat and barley (DuPont, 1985). Thifensulfuron methyl is one of several short soil residual sulfonylurea herbicides used in agronomic systems requiring rotational cropping flexibility, and fields treated with this herbicide may be replanted to any rotational crop 45 days after treatment or sooner (depending on specific label instructions). Previous work has shown that the short residual characteristic of thifensulfuron methyl is due to its high susceptibility to microbial degradation in soil (Beyer *et al.*, 1987a,b; Brown *et al.*, 1987; Cambon and Bastide, 1992; Smith *et al.*, 1990), and the initial degradation product in soil has been chromatographically identified as thifensulfuron acid, which is herbicidally inactive (Cambon and Bastide, 1992; Smith *et al.*, 1990). Steric and electronic features of thifensulfuron methyl which may account for its rapid deesterification in soil and water have been described (Bastide *et al.*, 1994).

The goal of these studies was to confirm the identity of the primary degradation product of thifensulfuron methyl in soil and to characterize the role of soil microbes and extracellular carboxyesterases in its rapid deesterification.

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MATERIALS AND METHODS

Chemicals. [*thiopheneyl*-^{14}C]Thifensulfuron methyl (8.3 mCi/mmol) and [*triazinyl*-^{14}C]thifensulfuron methyl (12.1 mCi/mmol) were synthesized at DuPont NEN Products to a final radiochemical purity of >98%. Chromatographic and mass spectral standards of thifensulfuron methyl, thifensulfuron acid [3-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]amino]sulfonyl]-2-thiophenecarboxylic acid], *O*-demethyltriazinyl thifensulfuron methyl [methyl 3-[[[[(4-hydroxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]amino]sulfonyl]-2-thiophenecarboxylate], appropriate thiophenesulfonamides and substituted amino triazines were synthesized at E. I. du Pont de Nemours & Co. using published procedures (Levitt, 1991; Gee and Hay, 1994; Cuomo *et al.*, 1991). All solvents were HPLC grade (EM Sciences, Gibbstown, NJ), and other reagents were reagent grade or better. Scinti-Verse 1 (Fisher Scientific, Philadelphia, PA) was used for all liquid scintillation counting (LSC).

Soils. All soils used in this study were freshly collected from the top 6−10 cm of fallow agricultural fields not previously exposed to thifensulfuron methyl. The soils were stored at 5 °C at natural field moisture levels and screened through a 2-mm sieve immediately before use. Soil properties were analyzed by the University of Delaware Soil Testing Laboratory (Newark, DE).

Soil Sterilization. Aliquots (50−200 g) of soils to be sterilized were placed in glass jars of 100−400-mL capacity. Soils to be sterilized with ethylene oxide were covered with Bio-Shield wrap (American Scientific Products, Evanston, IL) held in place with masking tape. The covering prevented microbial contamination of the soil following sterilization while allowing for gas exchange. The covered jars were placed in the sterilization chamber and treated for 16 h with a gas mixture consisting of 12% ethylene oxide (plus 88% inert carrier gas) at 5 psig at room temperature. The chamber was purged and taken through 10 cycles of aeration and vacuum evacuation, and the jars were then removed to a ventilated hood for 96 h of passive aeration.

Soils to be heat-sterilized were placed in glass jars with loosely fitted lids and taken through three cycles of autoclaving for 30 min at 15 psi, 121 °C, with a 24-h pause between each cycle. Lids were tightened following the last cycle.

Sterility of the soils was confirmed by plating these soils

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on separate media designed to favor the growth of bacteria (nutrient agar B1), fungi (potato dextrose agar B13), and actinomycetes (dextrose−nitrate agar) (Difco, 1965). Sterilized soils were adjusted to the desired moisture content with sterile deionized water prior to use.

Degradation of [^{14}C]Thifensulfuron Methyl in Soil. Nonsterilized and sterilized samples (100−300 g, dry wt basis) of each soil were treated with [*thiopheneyl*-^{14}C]thifensulfuron methyl (23.1 μCi/mg) or [*triazinyl*-^{14}C]thifensulfuron methyl (33.6 μCi/mg) in sterile distilled water to yield a final soil concentration of 100 ppb [^{14}C]thifensulfuron methyl (dry wt basis). (Note that 100 ppb is the approximate expected initial concentration following application of 30 g of ai/ha and assuming distribution in the top 3 cm of soil.) The soil moisture was adjusted to 70% of field-holding capacity (by wt) with sterile distilled water. After the contents of each jar were thoroughly mixed, time zero aliquots (20−30 g) were removed and frozen at −20 °C until analysis. Treated soils were incubated in sealed jars in the dark at 25−30 °C (depending on the study) and sampled aseptically at designated intervals. Samples were stored at −20 °C until analysis. Sterility of the soil was confirmed periodically by the plating procedures described above.

The frozen soil samples were thawed, 10-g subsamples were mixed with 30 mL of 2 M $(NH_4)_2CO_3$ (pH 9)/methanol (1:3, v/v), and the mixture was shaken for 1 h on a wrist-action shaker. The sample was centrifuged (3000*g*, 10 min), the supernatant fluid reserved, and the soil pellet reextracted two more times. The combined extracts were dried by rotary evaporation at 40 °C using water aspiration vacuum. The dry residue was carefully dissolved in 5 mL of deionized water, filtered through a 0.2-μm Acrodisc filter (Gelman Sciences, Ann Arbor, MI), and analyzed by liquid scintillation counting (LSC) and reverse-phase HPLC (described below). This method routinely extracted >90% of the applied radioactivity (as thifensulfuron methyl and its degradates) from diverse soils incubated for up to 30 days.

Chromatography Conditions. HPLC separations were performed on a Hewlett-Packard HP1090 liquid chromatograph (Hewlett-Packard Co., Palo Alto, CA) equipped with a photodiode array detector. Column eluant was monitored at 210 and 230 nm. The binary mobile phase was A = H_2O + 0.1% formic acid (or phosphoric acid), and B = acetonitrile + 0.1% formic acid (or phosphoric acid). The stationary, reverse-phase column was Zorbax ODS (4.6 mm × 250 mm) (Macmod, Chadds Ford, PA) fitted with a 4.6 mm (i.d.) × 50 mm ODS guard column operated at 40 °C and a flow rate of 1.4 mL/min; 0.5- or 1-min fractions were collected and analyzed for radioactivity by LSC. The separation method was as follows: step 1, 5% B to 20% B, 10-min linear gradient; step 2, 20% B to 25% B, 5-min linear gradient; step 3, 25% B to 35% B, 5-min linear gradient; step 4, 35% B to 50% B, 5-min linear gradient; step 5, 50% B to 70% B, 5-min linear gradient; step 6, 70% B to 100% B, 5-min linear gradient.

Hydrolysis of *thiopheneyl*-^{14}C Degradation Product. [*thiopheneyl*-^{14}C]Thifensulfuron methyl and its *thiopheneyl*-^{14}C degradation product were partially purified by high-pressure liquid chromatography of five 500-μL aliquots of an extract from treated nonsterile Gardena silt loam (treatment, extraction, and HPLC methods as described above). This soil extract contained approximately equal amounts of [*thiopheneyl*-^{14}C]thifensulfuron methyl and its radiolabeled degradation product. One-minute fractions were collected during chromatography of each of the aliquots, and radiolabeled fractions corresponding to thifensulfuron methyl and its degradation product were separately pooled. The acetonitrile mobile phase from the pooled fractions was evaporated under a stream of nitrogen at 50 °C, and the remaining acidic (approximately pH 3) aqueous solution of each radiolabeled compound (containing residual phosphoric acid from the mobile phases) was incubated for an additional 2 h at 50 °C. The partially hydrolyzed samples were then rechromatographed (as above) and fractions collected and analyzed by LSC. Using the chromatography conditions and column described above, separately injected authentic thifensulfuron methyl, 2-carboxymethyl ester thiophenesulfonamide, and 2-carboxythiophenesulfonamide eluted in fractions 25, 14, and 6, respectively. Radiolabeled peaks from partially hydrolyzed thifensulfuron methyl eluted in fractions 25 and 14, while radiolabeled peaks from partially hydrolyzed degradation product eluted in fractions 17 (unhydrolyzed degradation product) and 6.

Mass Spectral Analysis. Aliquots (300 μL) of a 1000 ppm solution of nonradiolabeled thifensulfuron methyl in HPLC grade acetonitrile were added to separate 10-g (fresh wt) samples of fresh Fargo silt loam (see Table 1) and thoroughly stirred. The samples were incubated in the dark for 0, 3, and 6 days at 28 °C and then extracted with 20 mL of 90:10 acetonitrile/water (v/v) for 16 h using a wrist-action shaker. The samples were centrifuged (3000*g*, 10 min), the supernatant fluids reserved, and the pellets washed twice with additional 20-mL aliquots of 90:10 acetonitrile/water. The respective supernatant fluids were combined, a 10-mL aliquot was evaporated to dryness under a stream of air at room temperature, and the residue was suspended in 10:90 acetonitrile/water (v/v). The concentrated extracts were filtered (Acrodisc, 45 μm; Gelman Sciences, Ann Arbor, MI) prior to chromatography. A 5-μL aliquot of the 6-day soil extract containing the primary degradation product of thifensulfuron methyl was chromatographed on a 3 × 150 mm, 3.5 μm Zorbax (Macmod, Chadds Ford, PA) SB-C_{18} HPLC column equilibrated with 5:95 acetonitrile/water (both solvents containing 0.5% formic acid) using a 10-min linear gradient to 95:5 acetonitrile/water (acidified). The postcolumn flow of 400 μL/min was split delivering 50 μL/min to the megaflow electrospray source (source temperature = 120 °C, cone voltage = 20 V) of a Micromass Quattro II triple quadrupole mass spectrometer (Micromass UK Ltd., Altrincham, U.K.). Daughter ion scans [gas cell pressure = 3.4×10^{-3} mBar, collision energy ramps of 20^{-5} Volts (ES+) and 15^{-1} V (ES−), scan range = m/z 80−500/0.75 s] of the singly charged quasi-molecular ion (m/z = 374 for positive ion and 372 for negative ion) eluting at 6.78 min were averaged and background subtracted. The remainder of the postcolumn flow was monitored at 230 nm using a UV−vis photodiode array detector.

Treatment of Soils with Carboxyesterase Inhibitors. Suspensions (nominally 0.5 M) of the following compounds were prepared by vigorous shaking in sterile deionized water: iodoacetamide, paraoxon (*O*,*O*-diethyl *O*-*p*-nitrophenyl phosphate), fenchlorphos (*O*,*O*-dimethyl *O*-2,4,5-trichlorophenyl phosphorothioate), chlorpyrifos (*O*,*O*-diethyl *O*-3,4,6-trichloropyridin-2-yl phosphorothioate), dichlorvos (DDVP, 2,2-dichlorovinyl dimethyl phosphate). Five milliliters of each suspension was added to 100 g of fresh (nonsterile) or ethylene oxide-sterilized Gardena silt loam, stirred thoroughly, and incubated for 1 h at 30 °C. Control soils received 5 mL of sterile deionized water. Following this incubation, 2.5 mL of 4 ppm [*thiopheneyl*-^{14}C]thifensulfuron methyl was added to each treated soil and thoroughly stirred to yield a final concentration of 93 ppb (fresh wt basis). Duplicate 20-g aliquots were immediately removed from each treated soil and frozen at −20 °C for subsequent analysis. The treated soils were returned to the 30 °C incubator, and additional samples were taken after 60 h (2.5 days) and frozen. All operations with the ethylene oxide-sterilized soils were conducted under sterile conditions. The treated soils were extracted and analyzed by reverse-phase HPLC as described above.

Assay of Soil Carboxyesterase Activity. General carboxyesterase activity in soil was assayed using the chromogenic esterase substrate *p*-nitrophenyl butyrate (Sigma Chemical, St. Louis, MO). A 1.2 mM solution of *p*-nitrophenyl butyrate was prepared in 20 mM potassium phosphate buffer, pH 8.0, and frozen until use. To 2.5 mL of 1.2 mM *p*-nitrophenyl butyrate was added 0.1 g of nonsterile, ethylene oxide-sterilized or autoclaved Gardena silt loam (fresh wt), and the mixture was shaken for 2−10 min. At specific times, aliquots were centrifuged (3000*g* for 10 min), and the absorbency of the supernatant fluid was measured at 402 nm. Moisture levels (for correction of the data to dry wt soil basis) for the nonsterile, ethylene oxide-sterilized, and autoclaved soils were 10.3%, 1.4%, and 8.3%, respectively.

Isolation and Screening of Soil Microbes. One gram of each fresh soil was separately shaken vigorously in 99 mL

Table 1. Degradation of [^{14}C]Thifensulfuron Methyl in Several Soils at Constant Temperature and Moisture

soil series	USDA taxonomy	textural class[d]	pH	clay (%)	OM (%)	DT_{50}[a] (days)	DT_{90}[a] (days)
Cape Fear[b]	typic umbraquult; fine-loamy, mixed, thermic (j)	sl	4.8	6	11.2	2.5	>14.0
Hyde[b]	typic umbraquult; fine-loamy, mixed, thermic (j)	sl	5.6	11	3.3	2.0	9.0
Drummer[b]	typic haplaquoll; silty, mixed, mesic (j)	sil	5.7	18	4.9	3.5	15
Cape Fear[b]	typic umbraquult; fine-loamy mixed, thermic (j)	l	6.6	17	6.4	2.5	>14.0
Meggett[b]	typic albaquale; fine, mixed, thermic (j)	sl	6.3	18	1.3	1.2	5.0
Fargo[c]	vertic haplaquoll; fine, montmorillinitic, frigid	sc	7.5	50	5.7	1.8	5.5
portneuf[c]	durixerollic calciorthids; coarse-silty, mixed, mesic	sil	7.6	21	1.6	1.5	5.0
Gardena[b]	vertic haplaquoll; fine, montmorillinitic, frigid	sil	7.8	12	5.0	0.75	4.1

[a] Time in days to degrade 50% (DT_{50}) and 90% (DT_{90}). [b] 25 °C and 70% field-holding moisture capacity. [c] 30 °C and 70% field-holding moisture capacity. [d] s, sand; si, silt; c, clay; l, loam.

of sterile deionized water for 5 min, and 0.1 mL of each soil suspension was spread evenly on the surface of nutrient agar, potato dextrose agar, and dextrose−nitrate agar plates (Difco, 1965) to isolate bacteria, fungi, and actinomycetes, respectively. The agar plates were incubated at 30 °C for 2−6 days. Individual bacterial and fungal colonies were picked off, purified by repeated subculturing, and maintained on the appropriate agar medium. The actinomycetes were maintained on a liquid sporulation broth (0.1% yeast extract, 0.1% beef extract, 0.2% tryptose, 1% glucose, and 1.5% $CaCO_3$). Approximately 180 distinct colonies were chosen and subcultured to purity from five soils on the basis of colony morphology (color, texture, size), Gram stain response, and degree of filamentous branching.

To screen for thifensulfuron methyl degraders, fungi were grown on potato dextrose agar, bacteria on nutrient agar, and actinomycetes on sporulation agar. One loopfull of cells from each culture was separately added to 50 mL of the appropriate sterile liquid media, calcium carbonate (1.5% w/v) was added to the broth to maintain the pH above 7.2, and the inoculated broths were incubated in an incubator−shaker at 30 °C and 200 rpm. After 1−2 days, 2 mL of each culture was separately transferred to 50 mL of fresh broth containing 1 ppm [*thiopheneyl*-^{14}C]thifensulfuron methyl (23.1 μCi/mg) and the cultures were incubated at 30 °C on a rotary shaker (200 rpm). Samples were withdrawn at designated intervals for analysis by HPLC and LSC.

Preparation of Sterile Microbial Culture Filtrates. Pure cultures of actinomycetes previously shown to be able to metabolize thifensulfuron methyl were started as described above. However, at the end of the first 2-days of growth in sporulation broth, 2 mL of each culture was separately transferred into 50 mL of fresh sporulation broth lacking thifensulfuron methyl and incubated at 30 °C for 2 more days. The fresh cultures were then centrifuged at 5000*g* for 10 min, and the resultant cell pellets were discarded. The supernatant fluids were filtered through a 0.2-μm sterile filter unit, and sterility of the filtrates was confirmed by plating 0.1-mL samples on sporulation agar. One-half of each sterile culture filtrate was autoclaved (121 °C, 15 min) to denature cell-free enzyme(s) produced by the actinomycete cultures. Three treatments, each containing 1 ppm [*thiopheneyl*-^{14}C]thifensulfuron methyl (51 720 dpm/mL; prepared in 5 mM potassium phosphate buffer, pH 7.2, and filter-sterilized prior to addition), were established as follows: (1) unautoclaved culture filtrate plus [*thiopheneyl*-^{14}C]thifensulfuron methyl, (2) autoclaved culture filtrate plus [*thiopheneyl*-^{14}C]thifensulfuron methyl, and (3) sterile sporulation broth plus [*thiopheneyl*-^{14}C]thifensulfuron methyl. All three treatments were incubated at 30 °C on a rotary shaker (200 rpm), and 1-mL aliquots were withdrawn at 0, 5, and 8 days and frozen at −20 °C until analysis. Reverse-phase HPLC analysis and LSC were performed as described above.

RESULTS AND DISCUSSION

Thifensulfuron methyl degrades rapidly in multiple soil types. Table 1 shows the times required for 50% (DT_{50}) and 90% (DT_{90}) of the applied thifensulfuron methyl to degrade in eight soils under conditions of constant moisture and temperature. In these fresh, nonsterile soils which include a wide range of pH, organic matter, and textural classes, DT_{50}s for thifensulfuron range from 0.75−3.5 days. These results are consistent with those of Smith *et al.* (1990) who showed that thifensulfuron methyl degraded with a DT_{50} of considerably less than 1 week in three different soils under constant temperature conditions of 10, 20, and 30 °C. Cambon and Bastide (1992) found that thifensulfuron methyl degraded with a DT_{50} of 1.6 and 1.7 days at 28 °C in two soils of pH 6.3 and 7.8, respectively.

Thifensulfuron methyl degrades more rapidly in soils than some other sulfonylurea herbicides. For example, the DT_{50} values for thifensulfuron are approximately 10−50 times shorter than those for chlorsulfuron and metsulfuron methyl, depending on the soil type and incubation conditions (Bastide *et al.*, 1994; Beyer *et al.*, 1987a,b; Brown, 1990). This result is not readily explained by the physical properties of thifensulfuron methyl since its pK_a, octanol−water partition coefficient, and soil thin layer chromatography mobility are similar to those of chlorsulfuron and metsulfuron methyl (Beyer *et al.*, 1987b). In addition, abiotic hydrolysis of thifensulfuron methyl is too slow to account for its rapid degradation in soils. Bastide *et al.* (1994) measured hydrolytic half-lives of 81.25 and 30.8 days in sterile aqueous buffer at pH 6.0 and 8.0 (25 °C), respectively, and we have obtained similar results (data not shown). This paper will address the basis for this surprisingly rapid degradation.

Figure 1 shows a typical time course of thifensulfuron methyl degradation in fresh Gardena silt loam (see Table 1 for soil properties) at 28 °C and 70% field-holding capacity moisture level. Under these conditions, the DT_{50} for thifensulfuron methyl is 0.65 days and the DT_{90} is 3 days (slightly faster than the experiment reported in Table 1 due to the slightly higher temperature in this experiment). Figure 1 also shows the kinetic and mass balance relationships between disappearance of thifensulfuron methyl and the appearance of its primary initial degradation product (determined by HPLC). These results show that this degradation product (shown below to be thifensulfuron acid) is formed in nearly stoichiometric proportion to the parent, comprising 91−99% of the degradation products during the 3-day incubation period. Identical results were obtained with [^{14}C]thiophene- and [^{14}C]triazine-labeled thifensulfuron methyl, each of which produces the same single early degradation product, showing that this more polar degradation product is comprised of both the thiophene and triazine moieties.

Previous workers have presented good evidence that thifensulfuron methyl initially degrades in soil to the deesterified thifensulfuron acid. Identification of the degradation product was based on comigration of the soil degradate with authentic thifensulfuron acid in one or more thin layer chromatography systems (Smith *et al.*, 1990) and high-performance liquid chromatography

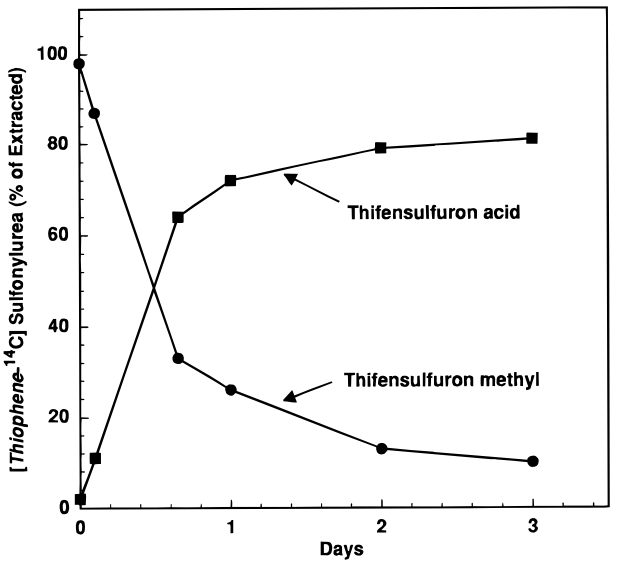


Figure 1. Precursor−product relationship between degradation of [*thiopheneyl*-^{14}C]thifensulfuron methyl and production of a primary degradation product (thifensulfuron acid) in nonsterile Gardena silt loam at 28 °C and 70% moisture-holding capacity. Extraction efficiency of total radiolabel was 90% for each sample.

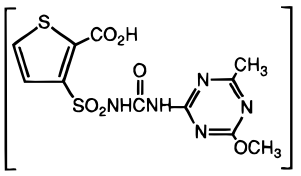
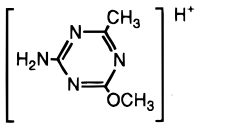
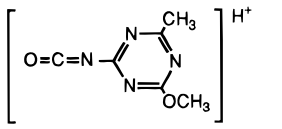
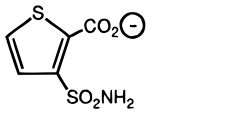
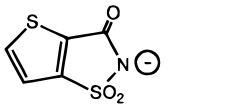
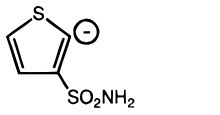
systems (Cambon and Bastide, 1992). We have also found that thifensulfuron methyl is rapidly and nearly quantitatively converted to thifensulfuron acid in nonsterile soils. This identification was based on four independent approaches to prevent confusion with another potential metabolite, *O*-demethyltriazinyl thifensulfuron methyl [methyl 3-[[[[(4-hydroxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]amino]sulfonyl]-2-thiophenecarboxylate], which can be difficult to resolve chromatographically from thifensulfuron acid (Brown *et al.*, 1990). These approaches, described below, include cochromatography with authentic standards, comparison of UV spectra, acid hydrolysis studies, and mass spectroscopy.

Identification of the soil degradation product was based on comigration with authentic thifensulfuron acid in reverse-phase HPLC systems and comparison of the UV spectra of the degradation product with authentic thifensulfuron acid acquired by diode array detection during chromatography. The UV spectra of thifensulfuron acid and thifensulfuron methyl are nearly identical and quite distinct from that of *O*-demethyltriazinyl thifensulfuron methyl (data not shown).

A third line of evidence was based on acid hydrolysis studies. Acid hydrolysis of the sulfonylurea bridge of the *thiopheneyl*-^{14}C degradation product purified from soil and [*thiopheneyl*-^{14}C]thifensulfuron methyl, followed by reverse-phase HPLC, showed that the radiolabeled thiophenesulfonamide released from the purified degradation product coeluted with authentic 2-carboxythiophenesulfonamide (see Materials and Methods). Hydrolysis of thifensulfuron methyl under the same conditions produced the intact carboxymethyl ester thiophene sulfonamide. These results are consistent with the identification of the degradation product as thifensulfuron acid.

The degradation product was confirmed as thifensulfuron acid using HPLC electrospray ionization mass spectroscopy (Table 2). Strong ion signals in electrospray-positive mode are seen at m/z = 374 (quasi-molecular ion, M + 1), 141, and 167. Strong ion signals in electrospray-negative mode include m/z 372 (quasi-molecular ion, M − 1), 206, 188, and 162 (see Table 2 for proposed fragment structures). The quasi-molecular ion peaks at m/z 372 and 374 (negative and positive modes, respectively) indicate loss of a methyl group from thifensulfuron methyl (FW = 387). The strong m/z signals at 141, 167, and 206 show unambiguously that the loss of this methyl group has occurred *via* deesterification rather than O-demethylation from the triazine methoxy substituent.

Table 2. Positively and Negatively Charged Ions Obtained by Single-Sector HPLC Electrospray Ionization Mass Spectroscopy of the Soil Degradation Product of Thifensulfuron Methyl

Proposed Ion Structure	Ionization Mode	Observed m/z
	Positive Negative	374 (M+1) 372 (M-1)
	Positive	141
	Positive	167
	Negative	206
	Negative	188
	Negative	162

These lines of evidence conclusively show that thifensulfuron methyl is readily deesterified to thifensulfuron acid in this soil. Thifensulfuron acid was also found to be the primary initial degradation product in each of the eight soils used in the study shown in Table 1. The initial degradation pathway of thifensulfuron methyl in soil and the respective *in vitro* activities of these compounds against acetolactate synthase are shown in Figure 2.

Thifensulfuron acid is herbicidally inactive when applied pre- or postemergence to numerous plant species in greenhouse studies (E. I. du Pont de Nemours & Co., Smith *et al.*, 1990) and is inactive against acetolactate synthase, the target site of action of the sulfonylurea herbicides (Cotterman and Saari, 1989). These results confirm that the short soil residual property of thifensulfuron methyl results from its rapid deesterification in diverse field soils to the herbicidally inactive thifen-

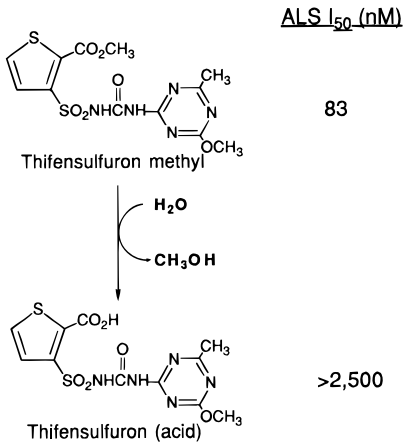


Figure 2. Initial deesterification reaction of thifensulfuron methyl in soil and *in vitro* activity of thifensulfuron methyl and thifensulfuron acid against acetolactate synthase from wheat (Cotterman and Saari, 1989; ALS assays according to Ray, 1984).

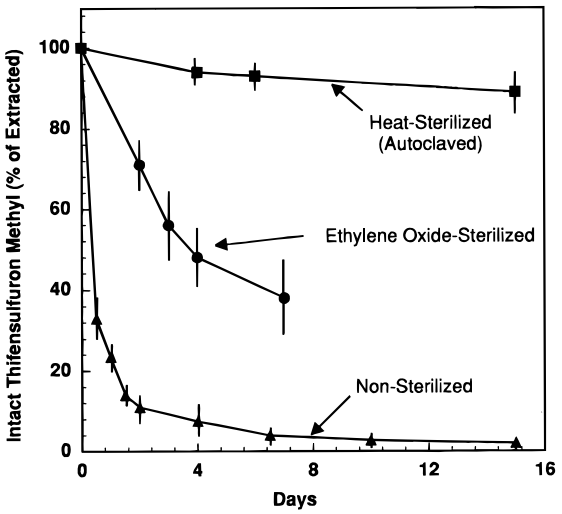


Figure 3. Degradation of [*thiopheneyl*-^{14}C]thifensulfuron methyl in nonsterilized (fresh), ethylene oxide-sterilized, and autoclaved Gardena silt loam at 28 °C and 70% moisture-holding capacity. Error bars represent the extremes of duplicate extractions and analyses. Extraction efficiency of total radiolabel was >90% for each sample.

sulfuron acid. Longer term laboratory and field studies have shown that thifensulfuron acid subsequently degrades via several pathways including evolution as CO_2 (E. I. du Pont de Nemours & Co., manuscript in preparation).

The role of soil microbial and chemical processes in the rapid deesterification of thifensulfuron methyl was probed by comparing degradation rates in sterilized and nonsterilized soils. Figure 3 shows the typically rapid degradation of thifensulfuron methyl in fresh, nonsterile Gardena silt loam ($DT_{50} \sim 1$ day). As previously described, the primary degradation product formed in this nonsterile soil over this time period was thifensulfuron acid. By comparison, thifensulfuron methyl degrades in heat-sterilized (autoclaved) soil much more slowly, with an estimated $DT_{50} = 50-70$ days. Within the limits of this 15-day time resolution, this is a degradation rate comparable to its hydrolysis rate in sterile water at this pH and temperature (Bastide *et al.*, 1994). In addition, the degradation products formed in autoclaved soil were identified, by cochromatography with separately injected standards, as the 2-carboxy-

Table 3. Hydrolysis of *p*-Nitrophenyl Butyrate in Sterilized and Nonsterilized Gardena Silt Loam

soil treatment	$A_{402nm/min/0.1gdwsoil}$[a]	percent[b]
nonsterile (Fresh)	0.243	100
ETOX-sterilized	0.170	70
autoclaved	0.016	6.6

[a] 2-min reaction time of 0.1 g of soil (dry wt basis) in 1.2 mM *p*-nitrophenyl butyrate in 20 mM potassium phosphate buffer, pH 8.0 (25 °C). [b] Percent of nonsterile rate.

methyl ester thiophenesulfonamide and the 2-amino-4-methoxy-6-methyltriazine fragments, which are typical of the hydrolysis of the sulfonylurea bridge of these herbicides (Beyer *et al.*, 1987a,b) with almost no production of thifensulfuron acid (data not shown). These results show that the rapid deesterification of thifensulfuron methyl in nonsterile soil is eliminated by heat sterilization, providing strong evidence that microbes are primarily responsible for the process.

However, as seen in Figure 3, thifensulfuron methyl also degrades relatively rapidly in soil sterilized with ethylene oxide, an observation that was made repeatedly with independent batches of ethylene oxide-sterilized soil. The typical results presented in Figure 3 show that thifensulfuron methyl degraded with a DT_{50} of approximately 4−8 days in ethylene oxide-sterilized soil compared to an estimated DT_{50} of 50−70 days in autoclaved soil. Furthermore, as for nonsterile soils, the degradation product formed from thifensulfuron methyl in ethylene oxide-sterilized soil was nearly exclusively thifensulfuron acid. Sterility of this soil was confirmed throughout the course of these experiments by repeated plating on a variety of media designed to culture soil bacteria, actinomycetes, and fungi (see Materials and Methods). These results show that rapid deesterification of thifensulfuron methyl in soil is eliminated by heat sterilization but only modestly decreased in ethylene oxide-sterilized soil.

Repeated heat sterilization (autoclaving) is known to kill living microbes and spores and to inactivate extracellular enzymes of microbial and plant origin. Although ethylene oxide sterilization also kills living microbes (and many spores), as was confirmed in these studies, it can leave extracellular enzymes active in the otherwise sterile soil (Fletcher and Kaufman, 1980). The effects of the sterilization method on thifensulfuron methyl deesterification suggests a role for extracellular enzymes capable of catalyzing deesterification of carboxy esters.

We tested the effects of the sterilization method on general soil carboxyesterase activity by following the deesterification of *p*-nitrophenyl butyrate (a chromogenic carboxyesterase substrate) in nonsterile, autoclaved, and ethylene oxide-sterilized Gardena silt loam. The results of a typical experiment are shown in Table 3. Nonsterile Gardena silt loam catalyzed the rapid deesterification of this substrate to *p*-nitrophenol (readily monitored at its absorption maximum at 402 nm). Soil which had been sterilized by autoclaving and confirmed to be sterile expressed less than 6% of the nonsterile carboxyesterase activity, while carboxyesterase activity in soil sterilized with ethylene oxide (and confirmed sterile) was 70% of the nonsterile rate. These results with this general carboxyesterase substrate are similar to those obtained for the deesterification of thifensulfuron methyl shown in Figure 3, and they support the hypothesis that extracellular carboxyesterase activity is at least partially responsible for the rapid degradation of this herbicide.

Table 4. Effect of Carboxyesterase Inhibitors on [*thiopheneyl*-^{14}C]Thifensulfuron Methyl Deesterification in Fresh (Nonsterile) and Ethylene Oxide-Sterilized Gardena Silt Loam

		thifensulfuron methyl[a]					
		nonsterile soil			ETOX-sterilized soil		
treatment	conctn in soil (ppm)[c]	0 day (%)	2.5 days (%)	half-life[b] (days)	0 day (%)	2.5 days (%)	half-life[b] (days)
none (water)		98	21	1.1	94.4	76.2	8
iodoacetamide	4300	99	73	5.7	100	97.9	>50
chlorpyrifos	8150	98	97	>50	98.2	91.9	29
fenchlorphos	7475	98	97	>50	97.7	91.9	29
paraoxon	575	96	40.5	2			
dichlorvos	465	96	55	3.1			

[a] Intact [^{14}C]thifensulfuron methyl extracted from treated soil after 0 and 2.5 days of incubation at 30 °C. [b] Half-life (days) based on first-order kinetic treatment of day 0 and day 2.5 data points. *c*Nominal compound wt/soil fresh wt.

Table 5. Soil Microorganisms That Degrade Thifensulfuron Methyl in Pure Culture

microorganism	source	products
Streptomyces griseolus	ATCC 11796[a]	thifensulfuron acid
Penicillium sp.	Flanagan silt loam (Illinois)	four polar metabolites (no thifensulfuron acid)
actinomycete 2	Keyport silt loam (Delaware)	thifensulfuron acid
actinomycete 3	Tripp sandy loam (Colorado)	thifensulfuron acid
actinomycete 4	Panoche clay loam (California)	thifensulfuron acid
actinomycete 5	Panoche clay loam (California)	thifensulfuron acid
bacterium 1	Flanagan silt loam (Illinois)	thifensulfuron acid
bacterium 2	Gardena silt loam (North Dakota)	thifensulfuron acid

[a] American Type Culture Collection, Rockland, MD.

Table 6. Deesterification of [*thiopheneyl*-^{14}C]Thifensulfuron Methyl by Sterile Culture Filtrate Derived from Actinomycete 4 (See Table 4)

	thifensulfuron methyl[b] (% of total radiolabel)		thifensulfuron acid (% of total radiolabel)	
sterile medium[a]	day 5	day 8	day 5	day 8
uninoculated broth	94	91.6	<1[c]	<1
autoclaved culture filtrate	90.8	89.6	<1	<1
unautoclaved culture filtrate	66	57	26	37

[a] Each sterile medium was adjusted to pH 7.2, and this pH remained stable through the course of the experiment. [b] Time zero samples showed >98% intact thifensulfuron methyl in each sterile medium. [c] Radiolabel mass balance is accounted for by several minor degradation products (primarily hydrolysis products).

The effect of several carboxyesterase inhibitors on thifensulfuron methyl deesterification in soil was also examined. Iodoacetamide is an inhibitor of carboxyesterases and several other enzymes (Zollner, 1989). Chlorpyrifos, fenclorphos, paraoxon (the oxon analog of parathion), and dichlorphos (DDVP) are organophosphorus insecticides which are potent inhibitors of the carboxyesterase enzyme acetylcholine esterase (Hassal, 1990; Cremlyn, 1978). As seen in Table 4, pretreatment of nonsterile or ethylene oxide-sterilized Gardena silt loam reduced the rate of thifensulfuron methyl deesterification by a factor of 2 to >50. For example, the DT_{50} of thifensulfuron methyl in nonsterile Gardena silt loam was increased from 1.1 days in the untreated soil to >50 days by a 1-h pretreatment of the soil with chlorpyrifos or fenchlorphos. A similar effect of these carboxyesterase inhibitors was seen in ethylene oxide-sterilized soil (Table 4), where the DT_{50} increased from 8 days in the untreated soil to 29 to >50 days in the inhibitor-treated soils. These results affirm the role of carboxyesterase activity in the deesterification of thifensulfuron methyl in both nonsterile and ethylene oxide-sterilized soil and support the concept that extracellular carboxyesterases remain active in this sterile soil.

Approximately 180 distinct microorganisms, isolated from five soils, were screened for their ability to metabolize thifensulfuron methyl in pure culture (see Materials and Methods). Seven distinct microorganisms, which could rapidly degrade thifensulfuron methyl in pure culture, were identified from five soils from geographically separate locations and an eighth obtained from the American Type Culture Collection (ATCC) (see Table 5). Each of the bacterial and actinomycete isolates was distinct by virtue of colony color, growth habit, or cell morphology. Of the eight organisms, five actinomycetes and two bacteria readily and nearly exclusively deesterified thifensulfuron methyl to thifensulfuron acid in pure culture over a period of 3−8 days.

Two actinomycetes were tested for the production of extracellular carboxyesterase activity capable of deesterifying thifensulfuron methyl. Actinomycetes 3 (from Tripp sandy loam) and 4 (from Panoche clay loam) were separately transferred from actively growing liquid cultures into fresh sporulation broth and grown for 2 days at 30 °C. The fresh cultures were centrifuged, and the supernatant fluids were filter-sterilized (see Materials and Methods). One half of each sterile culture filtrate was autoclaved, while the other half was preserved unautoclaved. Table 6 shows the rates of deesterification of 1 ppm [*thiophenylyl*-^{14}C]thifensulfuron methyl by uninoculated sporulation broth, autoclaved sterile culture filtrate, and unautoclaved sterile culture filtrate from actinomycete 4. Unautoclaved sterile filtrates derived from actively growing cultures of these soil actinomyces readily catalyzed the deesterification of thifensulfuron methyl, and this activity was eliminated by autoclaving, presumably through denaturation of the responsible extracellular carboxyesterases. Also, the rate of deesterification in the unautoclaved culture filtrates was nearly the same as the growing microbial cultures, suggesting that much of the responsible enzymatic activity was extracellular (data not shown). Nearly identical results were obtained with actinomycete 3.

A wide variety of extracellular enzymatic activities have been described and characterized (for reviews, see Burns, 1978; Skujins, 1976; Tabatabai and Fu, 1992). These soil enzymatic activities include phosphomono- and diesterases, catalase, various oxidases, cellulase and other saccharide hydrolases, urease, proteinases, and carboxy- and arylesterases. It is generally accepted that

much or most of the enzymatic activities in soil can be attributed to microbially derived extracellular enzymes. Relatively little work has been published describing the role of extracellular carboxyesterases in the degradation of agrichemicals in soil. Getzin and Rosefield (1971) partially purified and characterized a soil carboxyesterase that catalyzed the hydrolysis of malathion to its corresponding monoacid. Satyanarayana and Getzin (1973) extended this work by accomplishing a 550-fold purification of this enzyme from soil and showing that it was a glycoprotein, possibly accounting for its excellent stability. While we have not attempted the direct extraction and purification of the carboxyesterase activity responsible for this reaction, the results presented in this paper suggest that thifensulfuron methyl is readily deesterified by extracellular carboxyesterases in soil. This evidence includes the fact that deesterification continues at a rapid rate in ethylene oxide-sterilized soil (but not in heat-sterilized soil), the activity in nonsterile and ethylene oxide-sterilized soils is inhibited by carboxyesterase inhibitors, and microorganisms isolated from soil produce an extracellular, heat-labile factor which accelerates the deesterification of thifensulfuron methyl in sterile culture filtrates.

The short soil residual properties of thifensulfuron methyl result from its rapid deesterification in nonsterile soils to thifensulfuron acid, which is herbicidally inactive. Soybean tolerance to thifensulfuron methyl is also based on rapid deesterification in that crop to form thifensulfuron acid (Brown *et al.*, 1990). In that case, selectivity between tolerant soybeans and sensitive weed species is based on the selective ability of tolerant soybeans to accomplish this deesterification. The distinct differences in ability to deesterify this herbicide among plant species are in contrast to the apparently universal capability of soils to readily accomplish this reaction. The ability of diverse soils to deesterify thifensulfuron methyl may result from microbial carboxyesterases having relatively broad substrate specificities or, alternatively, the presence of a multitude of narrow specificity carboxyesterases, some of which recognize thifensulfuron methyl as a substrate.

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