Mechanism of Thifensulfuron-methyl Transformation in Soil

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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 a diverse range of agricultural soils. The transformation rates of thifensulfuron-methyl in two different soils were found to increase with temperature, however, no linear relationship (Arrhenius relation) was found in the range 20−65 °C. Thifensulfuron was the sole transformation product formed at 20 °C during the first week of this study. Different transformation products were formed at the upper levels of the temperature range, corresponding to both chemical and biological processes. Thifensulfuron-methyl was not transformed in a soil suspension with a water/soil ratio of 10. A linear relationship between the half-life and this water/soil ratio was found. The transformation rate increased with thifensulfuron-methyl concentration, and the relation between the initial velocity and the thifensulfuron-methyl concentration was well fitted with the Michaelis−Menten equation. These different results were in accordance with an enzymatic deesterification of thifensulfuron-methyl in soils.

Keywords: *Sulfonylurea herbicide; soil; transformation; metabolite; thifensulfuron-methyl*

INTRODUCTION

Chemical hydrolysis and microbial degradation are the primary mechanisms of degradation of sulfonylureas in the soil (Beyer et al., 1988). Thifensulfuron methyl [methyl 3-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)-amino]carbonyl]amino]sulfonyl]-2 thiophenecarboxylate] is a sulfonylurea herbicide that has been shown to be susceptible to microbial degradation in soil (Beyer et al., 1987; Smith et al., 1990; Cambon and Bastide, 1992; Brown, 1990), with the first step of degradation being the hydrolysis of the ester function.

Deesterification is the first step in the transformation of some pesticides in soils. However, only a few examples of isolated microorganisms capable of performing this hydrolysis have been reported. Smith-Grenier and Adkins (1996) isolated six bacterial strains that hydrolyze the ester function of diclofop-methyl. The mechanism of action of isolated microorganisms able to transform sulfonylureas concerns the hydroxylation of methyl or phenyl groups (Romesser and Keefe, 1986; Dietrich et al., 1995; Kulowski et al., 1997). The hydrolysis of the sulfonylurea bridge by *Aspergillus niger* and *Penicillium* sp. (Joshi et al., 1985) was probably due to a change in pH rather than to a direct transformation by microorganisms (Stevens and Duxbury, 1992). Recently, several actinomycetes and bacteria that could deesterify thifensulfuron-methyl in pure culture were isolated from soils (Brown et al., 1997). Cell-free culture filtrates of two actinomycetes catalyzed this reaction.

The chemical hydrolysis of thifensulfuron-methyl (**1**) in basic medium also corresponds to a deesterification reaction. In acidic medium, the transformation pathway is more complicated, consisting of the breakdown of the sulfonylurea bridge and hydrolysis of the methoxy group. The same pathway has been reported for thifensulfuron (**2**) (Cambon and Bastide, 1996). Different possible metabolites of thifensulfuron-methyl (Figure 1) are obtained by chemical hydrolysis, and the soil extraction conditions of these different compounds have been optimized.

The goal of these studies was to determine the mechanism of the first step of thifensulfuron-methyl soil transformation and the transformation pathway of thifensulfuron. The influence of different parameters (temperature, moisture, concentration) on this transformation was studied (qualitatively and quantitatively).

MATERIALS AND METHODS

Chemicals. Thifensulfuron-methyl (**1**) was a gift of Procida (Marseille, France). Thifensulfuron [3-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]amino]sulfonyl]-2 thiophenecarboxylic acid] (**2**), methyl 3-sulfamoylthiophene-2-carboxylate (**3**), 3-sulfamoylthiophene-2-carboxylic acid (**4**), 3-[[[[(4-hydroxy-6-methyl-1,3,5-triazin-2yl)amino]carbonyl]-amino]sulfonyl]-2 thiophenecarboxylic acid (**5**), and 1-(methylthiophene-2-carboxylate-3-sulfonyl)-7-acetyltriuret (**6**) were prepared according to the methods of Bastide et al. (1994).

All solvents were of HPLC grade (Carlo Erba reagents).

Soil Properties. Soil samples were collected from a depth of 0−20 cm at Saint-Nazaire and Salanque, two sites in the south of France. All samples were air-dried, sieved (2 mm), and stored in plastic bags at 5 °C. Water content was determined by drying soil aliquots for 24 h at 105 °C. Soils properties are listed in Table 1.

Transformation of 1 and 5 by Soils. Flasks containing soil equivalent to 20 g of oven-dried weight of soil were treated with a filtered (0.2 μm) aqueous buffer solution (pH 7) (Na_2HPO_4, KH_2PO_4 1/15 M) of product **1** (1294 mg L^{-1}) or product **5** (616 mg L^{-1}). These applications rates correspond to concentrations of product **1** in the soil of 40 mg kg^{-1} and of product **5** of 30 mg kg^{-1}. Water was added to give a moisture content of 25% (w/w of dry weight of soil). After thorough mixing with a spatula, the flasks containing the soil/herbicide

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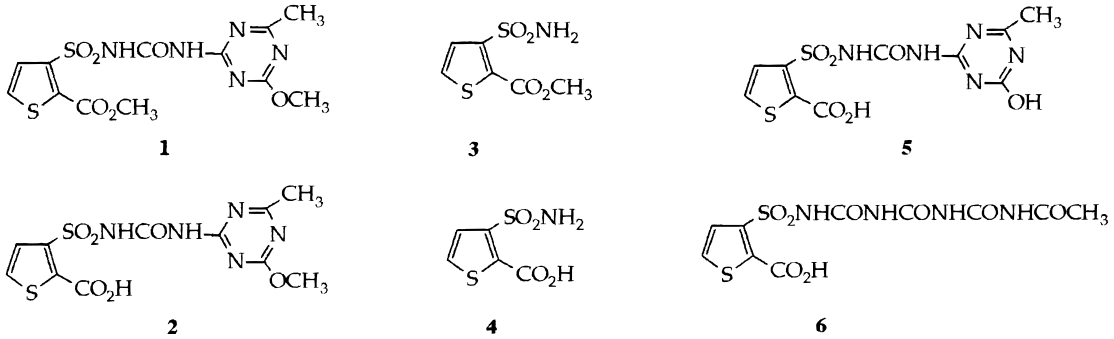

Figure 1. Structures of thifensulfuron-methyl and products.

Table 1. Soil Characteristics

soil	clay (%)	silt (%)	sand (%)	OM (%)	pH	water holding capacity (%)
Saint-Nazaire	22	49.3	24.3	2	6.3	29
Salanque	19.6	55.6	19	1.5	7.8	26

mixture were sealed with Parafilm and placed in an incubator at 28 ± 1 °C. Duplicate samples were periodically removed and frozen (−20 °C) until extraction (method B) and analysis (system B) as described below.

Temperature Dependence Study. The effect of temperature on the rate of thifensulfuron-methyl hydrolysis was determined in each soil at 20, 35, 43, 53, and 65 °C. Each flask of soil was tightly covered and incubated in a water bath at 35, 43, 53, and 65 °C, for 1 h before addition of herbicide solution. The application rate corresponded to a concentration of 40 mg kg^{-1}. Each sample was thoroughly mixed, capped, and incubated at the temperature of the experiment. At the time of application and after different times (from 1 to 24 h), triplicate samples were extracted (method A) and analyzed (system A).

Soil Moisture Dependence Study. Water contents of soils were adjusted to 50, 75, 100, 200, 500, and 1000% in flasks containing 20 g of equivalent dry soil. An aqueous buffer solution of thifensulfuron-methyl was incorporated into each flask to obtain a concentration of 10 mg kg^{-1} of water/soil mixture. All samples were closed and incubated at 28 °C in a rotary shaker at 200 rpm.

Soil Concentration Dependence Study. Different aliquots of an aqueous buffer solution of thifensulfuron-methyl (1294 or 2558 mg L^{-1}) were added to soil (20 g equivalent of dry soil) in 125 mL flasks to obtain different final concentrations (1, 2, 5, 10, 20, 40, 80, 120, 160, and 200 mg kg^{-1} of dry soil). Water was added to give a moisture content of 25% (w/w of dry weight of soil). After thorough mixing with a spatula, the flasks containing the soil/herbicide mixture were sealed with Parafilm and incubated at 28 ± 1 °C

Soil Extraction Methods. Two extraction methods were used.

Method A. Fifty milliliters of a cool mixture of methanol/water/acetic acid (40:10:0.5, v/v/v) was added to the soil (20 g) in a 125 mL flask and shaken for 1 h.

Method B. Each soil sample was extracted with 50 mL of a solution of 44 mL of water and 6 mL of 0.2 M NaOH (Saint-Nazaire soil) or 45 mL of water and 5 mL of 0.2 M NaOH (Salanque soil). After homogenization, the pH of the mixtures was 10 ± 0.1 in the two soils. After decanting (20 min), two aliquots (980 μL) of supernatant were removed from each sample. Twenty microliters of 10 N HCl was added in each vial to precipitate humic acid and to acidify the solution to pH 3. After centrifugation (5 min at 5000*g*), the clean supernatant was directly analyzed by HPLC.

Sample Analysis. Thifensulfuron-methyl (**1**) and metabolites were analyzed using high-performance liquid chromatography (HPLC): Kontron Analytic (LC 410) pump equipped with a UV detector [Laboratory Data Control Division of Milton Roy (Monitor III)]; flow rate, 1 mL min^{-1}; column C_{18} (Ultrabase UB 235, 4.6 × 250 mm, 5 μm); detector, 254 nm in two separate systems.

*System A: Analysis of Products **1**−**4**.* Operating parameters were as follows: injection volume, 20 μL; mobile phase, water/acetonitrile/methanol/acetic acid 60:26:14:1, v/v/v/v. Retention times: **4**, 4.39 min; **3**, 5.70 min; **2**, 6.66 min; **1**, 15.57 min.

*System B: Analysis of Products **2** and **4**−**6**.* Operating parameters were as follows: injection volume, 20 μL; mobile phase, water/acetonitrile/trifluoroacetic acid 80:20:0.3, v/v/v. Retention times: **4**, 7.6 min; **5**, 9.6 min; **6**, 21.35 min; **2**, 23.9 min.

Thifensulfuron-methyl Degradation in Sterilized Soils. Soil sterilization was achieved by autoclaving or by addition of sodium azide. In the first case soil samples were autoclavated according to the method of Cambon and Bastide (1992), and in the second case the inhibitor was mixed into soil at 1 g kg^{-1} soil and the soil was incubated for 24 h at 28 °C prior addition of herbicide as described above.

Thifensulfuron Degradation by Isolated Bacteria. *Bacterial Strains. Chryseomonas luteola, Shingomonas paucimobilis,* and *Acinetobacter baumanii* degrading diclofop-methyl were kindly provided by Smith-Grenier and Adkins (1996). Each strain was incubated in nutrient broth (BioMerieux) before storage at 4 °C.

Streptomyces griseolus ATCC 11796 was obtained from DSM (Deutsche Sammlung von Mikroorganismen, Gottingen, Germany) and stored on yeast extract−glucose−agar slants (in L^{-1}: yeast extract, 4 g; malt extract, 10 g; glucose, 4 g; agar, 15 g).

Degradation Studies. Diclofop-methyl-degrading strains were grown at 28 °C on agar plates containing minimal medium (Smith-Grenier and Adkins, 1996) and amended with thifensulfuron-methyl (10 mg L^{-1}). After several days of incubation, cells were harvested from plates, dispersed in 3 mL of sterile minimal medium, and then inoculated into 30 mL amended with thifensulfuron-methyl (1.5 mg L^{-1}). Cultures were incubated at 28 °C with shaking at 200 rpm. To account for chemical degradation, a control flask was left uninoculated. Samples of all cultures (0.5 mL) were removed at regular intervals, mixed with 0.5 mL of acetonitrile, and centrifuged before analysis by HPLC as described above.

Thifensulfuron-methyl transformation by *S. griseolus* was carried out as previously described by Romesser and O'Keefe (1996).

RESULTS AND DISCUSSION

Extraction Methods. Sulfonylureas may be extracted from soils in either acidic medium (Smith et al., 1990; Cambon and Bastide, 1992; Walker et al., 1989) or alkaline medium (Thirunarayanan et al., 1985; Brown et al., 1997). In some cases, the extraction of sulfonylureas and metabolites requires two different solvent mixtures. As thifensulfuron-methyl is easily transformed in alkaline medium, it was preferable to use an acidic medium for extraction of this product. Two successive extractions with different solvent mixtures

Table 2. Extraction Yields of Thifensulfuron-methyl and Its Degradation Products[a]

soil	product	method A (%)	method B (%)
Saint-Nazaire	1	87	
	2	77	87
	3	86	
	4	75	98
	5		97
Salanque	1	83	
	2	68	75
	3	87	
	4	60	98
	5		87

[a] Method A: methanol/water/acetic acid (40:10:0.5 v/v/v). Method B: water/0.2 M NaOH (44:6 v/v, Saint-Nazaire, or 45:5 v/v Salanque).

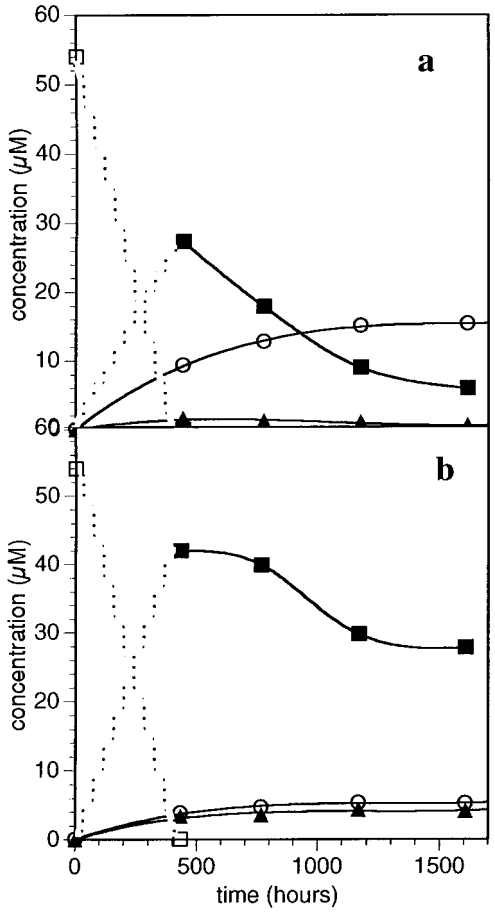

Figure 2. Thifensulfuron-methyl transformation in Saint-Nazaire (a) and Salanque (b) soils. Products: **1** (□); **2** (■); **4** (○); **5** (▲). Each point represents the mean of triplicate results, and the average coefficients are 8.6% (a) and 7.5% (b).

were necessary to obtain >75% extraction of different compounds. (Table 2).

Transformation Products in Soils. We have previously shown that thifensulfuron-methyl is rapidly transformed into thifensulfuron in soils (Cambon and Bastide, 1992), with compound **2** also transformed in soils, albeit slowly (Figure 2). In the present study, sulfonamide acid **4** was found to be the major transformation product in Saint-Nazaire soil, while only a small quantity of compound **5** was found. To determine whether the formation of **4** occurred directly from **2** or from the intermediate **5**, we examined the transformation of **5** in soil (Figure 3a). A rapid transformation of **5** was observed ($t_{1/2}$ = 5.4−6.6 days) but without the

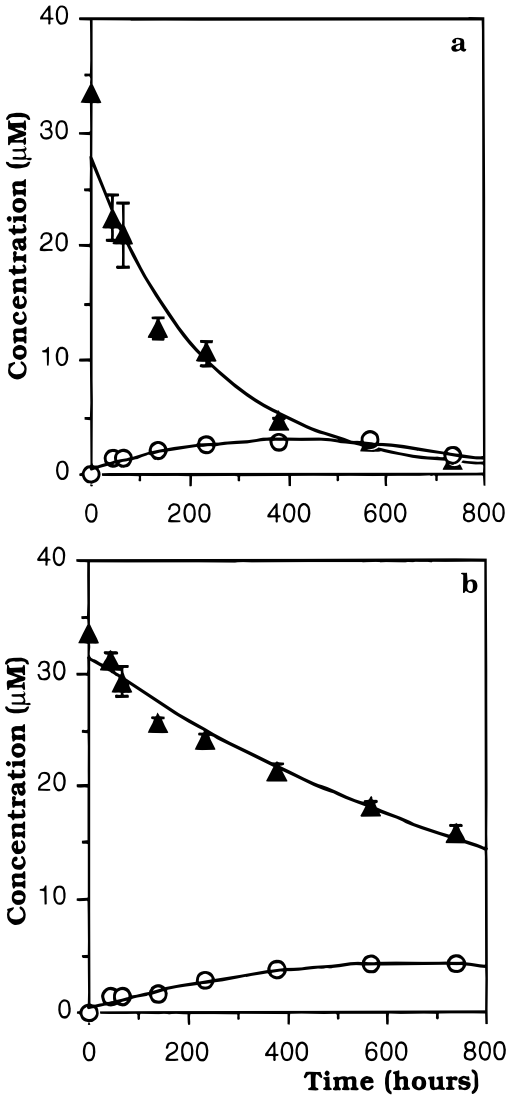

Figure 3. Transformation of product **5** [3-[[[[(4-hydroxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]amino]sulfonyl]-2-thiophenecarboxylic acid] in Saint-Nazaire (a) and Salanque (b) soils. Products: **4** (○); **5** (▲). Vertical bars represent standard error of each mean value.

formation of a significant quantity of **4.** Thus, the formation of **5** from thifensulfuron (**2**) might have been considerable, but this compound disappeared rapidly without the formation of a known metabolite. The transformation of thifensulfuron (**2**) in Salanque soil was slow, with compounds **4** and **5** being obtained in equivalent amounts (Figure 2b). The increase in the relative concentration of **5** compared to that found in Saint-Nazaire soil may be explained by the heightened stability of this compound in this soil ($t_{1/2}$ = 29.7 days) (Figure 3b).

Temperature Effect. The biological degradation of thifensulfuron-methyl was shown by the difference of rates after soil sterilization (Cambon and Bastide, 1992). The dependence upon temperature of biological degradation was different from that of the dependence of chemical transformation on temperature. The dependence upon temperature of the chemical transformation rates of different sulfonylureas demonstrates a linear relationship between the inverse of temperature and the logarithm of rate (Arrhenius relation), with the calculated activation energy varying from 20.5 to 35 kJ mol^{-1} according to Thirunarayanan et al. (1985),

Table 3. Transformation Rates of Thifensulfuron-methyl in Soils at Different Temperatures

soil	temp (°C)	$k \times 10^3$ h^{-1}	soil	temp (°C)	$k \times 10^3$ h^{-1}
Saint-Nazaire	20	8 ± 0.4	Salanque	20	5.4 ± 0.4
	35	21.6 ± 0.4		35	15.4 ± 0.8
	43	22.4 ± 0.72		43	25.4 ± 0.9
	53	33 ± 1.5		53	31.7 ± 1.8
	65	111 ± 13.9		65	77.6 ± 2.0

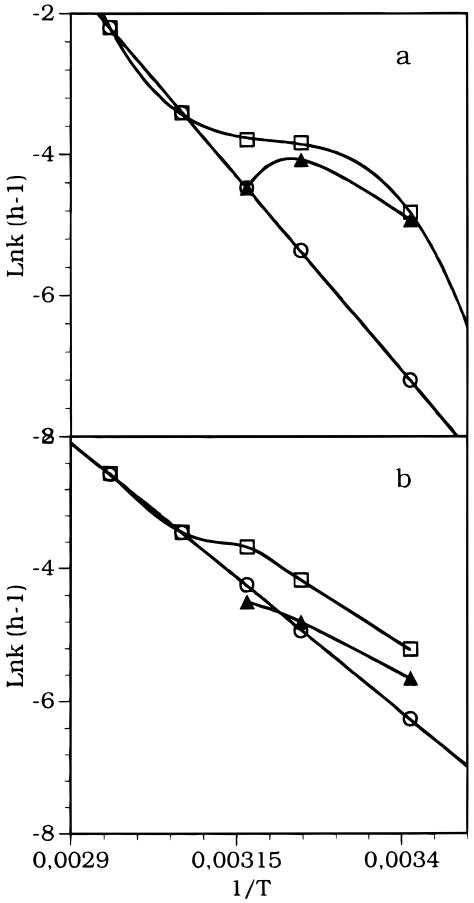

Figure 4. Ln k vs $1/T$ in Saint-Nazaire (a) and Salanque (b) soils: experimental results (□); theoretical chemical rates (○); theoretical biological results (▲).

to 67 kJ mol^{-1} according to Walker and Brown (1983), and to 25−60 kJ mol^{-1} according to Smith and Aubin (1992).

In the temperature range studied, 20−65 °C, the rates of transformation in soils increased with temperature (Table 3). This rate increase was high between 20 and 35 °C and between 53 and 65 °C, but low between 35 and 53 °C. There was no linear relationship between the inverse of the temperature and the logarithm of the rate, indicating a change in the transformation mechanism in the range 20−65 °C (Figure 4). At 28 °C the thifensulfuron-methyl transformation was biological (Smith et al., 1990; Cambon and Bastide, 1992). The biological transformation rates increase with temperature up to 40−45 °C and decrease rapidly at higher temperature, corresponding to the degradation of biological factor. The relation log k versus $1/T$ may be explained by a different effect of temperature on biological and chemical transformation rates

For temperatures above 53 °C, the main process of transformation was chemical, while for temperatures below 53 °C both chemical and biological processes of transformation may have occurred. Under these conditions, we calculated the activation energy of chemical transformation from the data obtained for temperatures of 53 and 65 °C, with values of 67.8 and 91.2 kJ mol^{-1} obtained for the Salanque and Saint-Nazaire soils, respectively. These values are in agreement with values calculated for the chemical hydrolysis of different sulfonylureas (Smith and Aubin, 1993). Using the activation energies of chemical transformation, the chemical rates of transformation were calculated for the different temperatures studied; the biological rates were then determined by subtraction from the total rates.

The results shown in Figures 4 indicate that, for Saint-Nazaire soil, the transformation at 20 °C was primarily biological, whereas both chemical and biological processes occurred in the Salanque soil at the same temperature. However, for the two soils, the sole transformation product formed at 20 °C was thifensulfuron (**2**) (Figure 5). This compound may be formed by biological transformation or alkaline hydrolysis.

At 63 °C, the transformation products identified were different in the two soils. Compound **3**, corresponding to acidic hydrolysis, was the major product in the Saint-Nazaire soil. A mixture of compounds **2**−**4**, corresponding to the hydrolysis of ester in alkaline medium and a breakdown of the sulfonylurea bridge at this high temperature, were the major products in the Salanque soil. At an intermediate temperature (43 °C) the products obtained corresponded to a mixture of chemical and biological transformations (Figure 5).

Isolation of Soil Microorganisms and Relation of Transformation Rate to Moisture Contents in Soils. A soil suspension (1 g of soil in 50 mL of mineral medium amended with thifensulfuron-methyl) was performed to isolate degrading microbes from the soil. The transformation rates of thifensulfuron-methyl in this suspension were very low. It was found that the soil suspension was not able to degrade the thifensulfuron-methyl as the increase in the water/soil ratio significantly decreased the transformation rate of thifensulfuron-methyl (Figure 6). Under the conditions used (constant concentration in the mixture soil/water), the relative concentration of thifensulfuron-methyl in soil decreases with the increase of the water/soil ratio. However, the rate variation according to concentration (see below) was very low against the rate variation obtained in this experiment. A linear relationship was obtained between the half-life (hours) of the transformation and the water/soil ratio

$$t_{1/2} = 95R + 11.9 \qquad (r^2 = 0.999 \text{ for Saint-Nazaire soil})$$

$$t_{1/2} = 73R + 8.2 \qquad (r^2 = 0.951 \text{ for Salanque soil})$$

where R is the water/soil ratio.

In addition to these findings, several microorganisms were isolated from soil suspensions and screened for their ability to metabolize thifensulfuron-methyl in a pure culture with thifensulfuron-methyl as the sole nutrient source. None of the isolated microorganisms was able to degrade thifensulfuron-methyl in pure culture.

Recently, Brown et al. (1997) isolated 7 microorganisms (from 180 isolated microorganisms) capable of metabolizing thifensulfuron-methyl over a period of 3−8 days. However, these microorganisms were able to

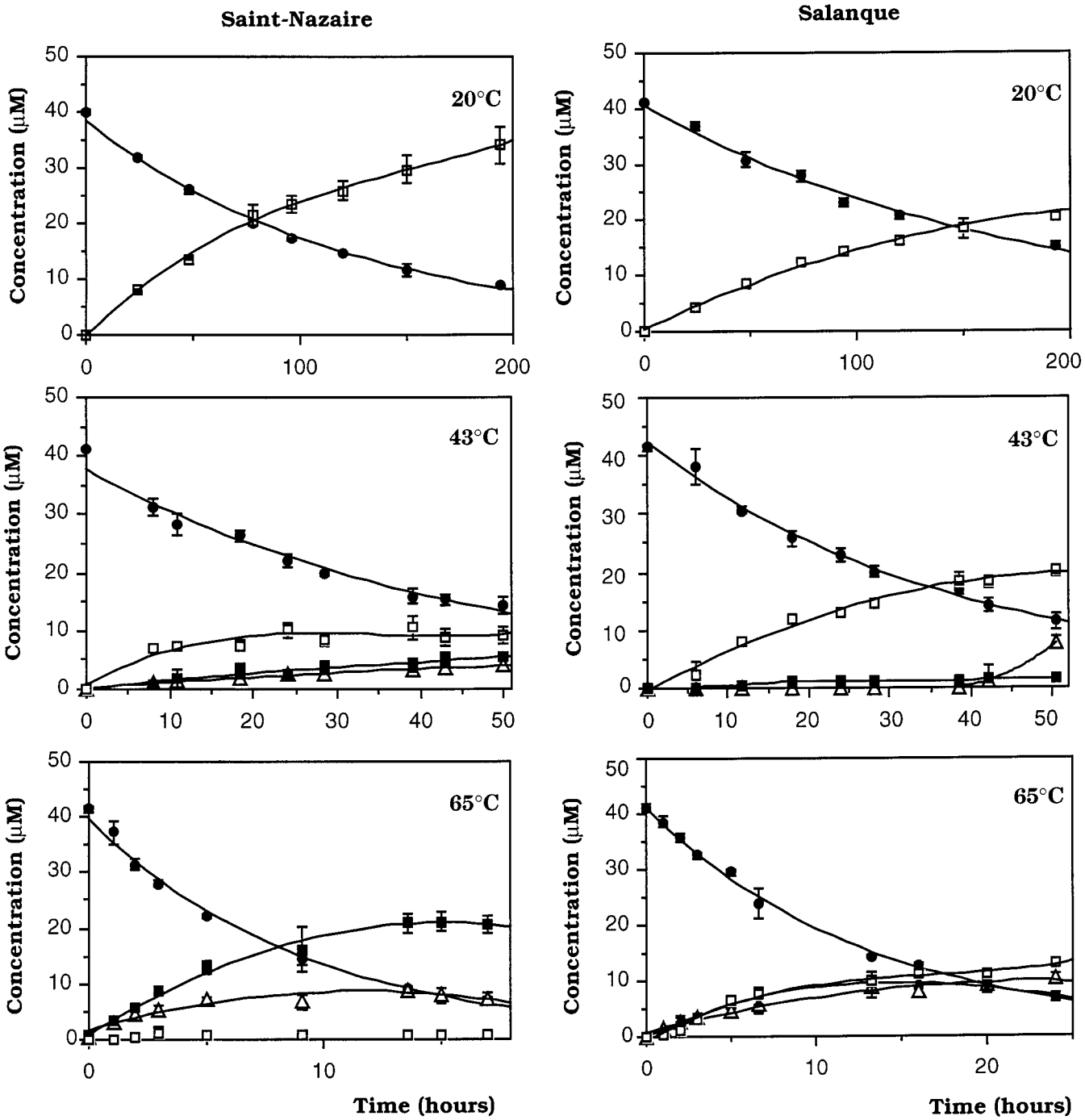

Figure 5. Thifensulfuron-methyl transformation at different temperatures in Saint-Nazaire and Salanque soils. Products: **1** (●); **2** (□); **3** (■); **4** (△). Vertical bars represent standard error of each mean value.

transform thifensulfuron-methyl only in a rich medium in which the final pH was not controlled. This degradation was related to the presence of extracellular carboxyesterase activity in two actinomycetes. Under these conditions, the degradation rates were related to substrate concentration.

Rate of Transformation versus Thifensulfuron-methyl Concentration. The half-lives for the degradation of sulfonylureas are not concentration dependent in the cases of chlorsulfuron (Walker and Brown, 1983), sulfometuron-methyl (Anderson and Dulka, 1985), and metsulfuron-methyl (Vega et al., 1992). With thifensulfuron-methyl, the half-life for degradation increased when the concentration of thifensulfuron-methyl was increased (Table 4). The initial transformation rate does not linearly vary with concentration; therefore, we can analyze the degradation as an enzymatic reaction. In this condition, at very high substrate concentration the velocity should be independent of the substrate concentration. However, in the experiment of thifensulfuron-methyl transformation in soil, the solubility of substrate in water was too low to obtain the substrate saturation. In this condition the use of the Michaelis−Menten equation may be a method to confirm the enzymatic mechanism for transformation. Up to 120 mg kg^{-1} the data fit very well to the Michaelis−Menten equation: $v = V_{max}[S]/(K_S + [S])$ ($r^2 = 0.998$, $n = 8$, $V_{max} = 14$ mg kg^{-1} h^{-1}, $K_S = 64.5$ mg kg^{-1}). For the upper concentration (120−200 mg kg^{-1}) the fit to the model was less good ($r^2 = 0.98$, $n = 10$) (Figure 7). Neverthe-

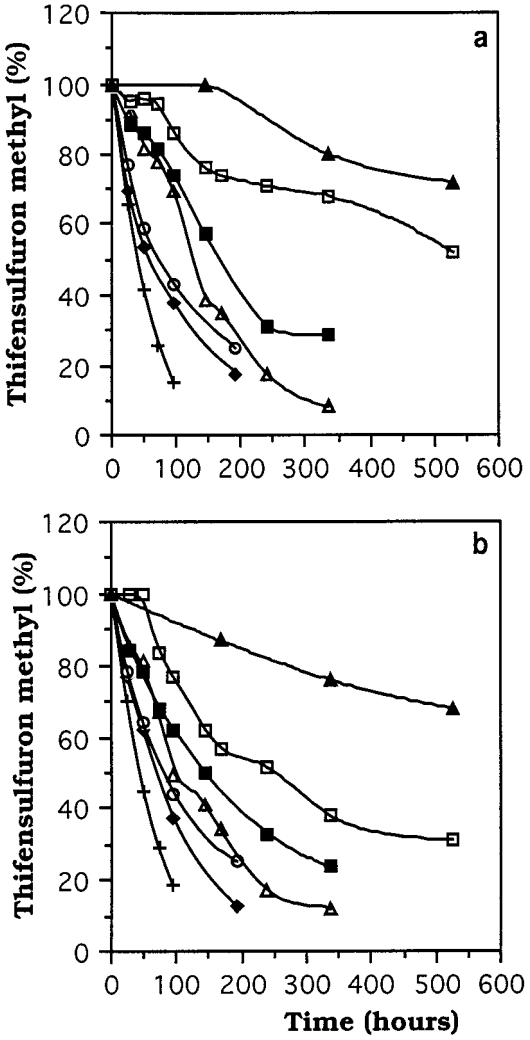

Figure 6. Thifensulfuron-methyl degradation in Saint-Nazaire (a) and Salanque (b) soils at different water/soil ratios: 0.25 (+); 0.5 (◆); 0.75 (○); 1 (△); 2 (■); 5 (□); 10 (▲).

Table 4. Half-Life Times in Saint-Nazaire Soil at Different Concentrations of Thifensulfuron-methyl

concn (mg kg^{-1})	half-life[a] (h)	initial rate (mg h^{-1} kg^{-1})	concn (mg kg^{-1})	half-life[a] (h)	initial rate (mg h^{-1} kg^{-1})
1	22	0.032	40	52.8	0.525
2	23.4	0.059	80	73.3	0.756
5	29.2	0.119	120	89	0.934
10	33	0.210	160	90	1.231
20	41.7	0.332	200	91	1.523

[a] Mean from duplicate experiments.

less, these results were in accordance with an enzymatic transformation of thifensulfuron-methyl in soils.

Influence of Sterilization Method. The transformation rates of thifensulfuron-methyl were largely decreased by the heat sterilization of soils (Table 5). The treatment of soils by sodium azide had a different effect on the two soils: a significant decrease in the transformation rate in Saint-Nazaire soil and a slight decrease in Salanque soil. For the two soils, the decrease in rate transformation for azide-treated soil was less than in the heat-sterilized soils. Sodium azide effectively suppresses respiration but does not affect the activity of other enzymes. In contrast, sterilization by autoclaving killed microorganisms and altered the number of enzymes present. Another sterilization method using ethylene oxide leads to a similar result (Brown et al., 1997).

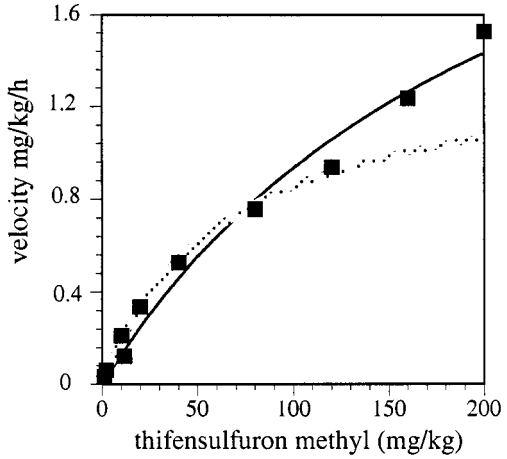

Figure 7. Michaelis−Menten relation of thifensulfuron-methyl transformation rate in Saint-Nazaire soil: (: : :) fit of data up to 120 mg kg^{-1}; (—) fit of data up to 200 mg kg^{-1}.

Table 5. Half-Life Times (Hours) in Sterilized and Nonsterilized Soils

soil	nonsterile	+ sodium azide (1 g kg^{-1})	autoclaved[a]
Saint-Nazaire	38.5	180	680
Salanque	40	66	495

[a] Cambon and Bastide (1992).

Involvement of Isolated Bacteria Strains Able To Deesterify Ester Compounds. Numerous pesticides contain a functional ester, although there have been very few reports in the literature of microorganisms able to transform these functional esters. Smith-Grenier and Adkins (1996) reported on several microorganisms able to deesterify diclofop-methyl. Three of them, *C. luteola, S. paucimobilis*, and *A. baumanii*, have been tested for their ability to degrade thifensulfuron-methyl but without success. The strain *S. griseolus*, able to degrade some sulfonylureas by co-metabolism (Dietrich et al.,1995), was tested with thifensulfuron-methyl. Under the same conditions as those used by Dietrich et al. (1995), thifensulfuron-methyl was rapidly transformed in the medium. However, the measured pH in the medium (pH 3.5) indicated that the observed degradation was by chemical hydrolysis. *S. griseolus* exhibits little activity below pH 5. An analogous conclusion was reached by Steven and Duxburry (1992) for the transformation of chlorsulfuron by *A. niger* and *Penicillium* sp.

The rapid deesterification of thifensulfuron-methyl in soil was related to biological transformation. Results presented in this paper support the hypothesis that the extracellular enzyme activity was responsible for the degradation: The increase of the water/soil ratio results in a dilution of the biological factor associated with soil, and consequently the degradation rate proportionally decreases to this dilution.

The relation between the herbicide transformation velocity and the herbicide concentration is well represented by a Michaelis−Menten equation. The role of an extracellular carboxyesterase in thifensulfuron-methyl degradation was shown by Brown et al. (1997). The degradation rate was slow by ethylene oxide sterilization but was hardly decreased by treatment

with carboxyesterase inhibitors chlorpyrifos and fenchlorphos. An exocellular carboxyesterase activity was found in an actinomycete culture. The results obtained in this work provide more evidence that soil enzymes were responsible of the first step of thifensulfuron-methyl transformation in soils.

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