Degradation of Demeton S-Methyl Sulfoxide (Metasystox R) by Soil Microorganisms

Walter Ziegler, Gabriele Engelhardt, Peter R. Wallnöfer,* Linthard Oehlmann, and Klaus Wagner

The insecticide demeton S-methyl sulfoxide (I) is degraded by *Pseudomonas putida* 1453 by cleavage of the thioester bond to form three major compounds: 2-(ethylsulfinyl)ethanethiol (II), 2-(ethylsulfonyl)ethanethiol (III), and bis[2-(ethylsulfinyl)ethyl]disulfide (IV), an oxidation product of II. *Nocardia sp.* DSM 43252 converts the insecticide into another three metabolites: bis[2-(ethylsulfonyl)ethyl]disulfide (V), bis[2-(ethylsulfinyl)ethyl]sulfide (VI), and bis[2-(ethylsulfonyl)ethyl]sulfide (VII), via oxidation, β elimination, and hydrolysis. O,O-Dimethylthiophosphoric acid had been detected in the aqueous layer of the medium.

Demeton S-methyl sulfoxide [O,O-dimethyl S-(2-ethylsulfinylethyl) phosphorothioate] is used for the control of plant-sucking insect pests. Its application to large crop areas results in direct or indirect contact with soil. Therefore it is important to know its susceptibility to biological breakdown by soil microorganisms.

Numerous studies have been conducted to investigate the fate of aromatic organophosphorus insecticides in agricultural soil (Matsumura and Benezet 1978). Very little is known, however, about the microbial degradation of aliphatic organophosphorus compounds, the only information available being for the insecticide phorate [O,O-diethyl S-(ethylthio) methyl phosphorodithioate] which chemically is closely related to demeton S-methyl sulfoxide. Phorate is transformed in soils to the corresponding sulfoxide and sulfone. No other products of degradation could be observed (Schulz et al. 1973). More recently, Walter-Echols and Lichtenstein (1978) reported that phorate was converted in flooded soils to the corresponding sulfoxide and sulfone, respectively, whereas up to 10% of the ^{14}C-labeled phorate was released as $^{14}CO_2$ due to microbial activity. However, no intermediate metabolite could be detected.

The present report describes the metabolism of demeton S-methyl sulfoxide by selected soil bacteria with particular emphasis on the structure of intermediates and endmetabolites formed.

MATERIALS AND METHODS

Chemicals. Ethylene-1-^{14}C-labeled O,O-dimethyl S-(2-ethylsulfinyl)ethyl phosphorothioate, specific activity 88 μCi/mg, was kindly provided by Mobay Chemical Corp., Kansas City, MO. All other chemicals used were commercially available compounds.

Microorganisms used included *Nocardia sp.* DSM 43252, *Arthrobacter sp.* DSM 20389, *Arthrobacter roseoparaffineus*, *Corynebacterium petrophilum* ATCC 19080, *Brevibacterium ammoniagenes* ATCC 13745, *Bacillus sphaericus* ATCC 12123, *Bacillus sphaericus* ATCC 12300, *Pseudomonas fragi* 1233, *Pseudomonas putida* 1453, *Pseudomonas fluorescens* 1542.

Culture Conditions. Maintenance of the bacteria was performed as described (Engelhardt et al. 1976). Degradation studies were performed using Hegeman's mineral base (Hegeman, 1966) modified by addition of 0.02% yeast extract. Cultures were incubated on a rotary shaker (New Brunswick G 10) at 28 °C and 220 rpm. Liquid cultures were grown in 100-mL Erlenmeyer flasks containing 25 mL of mineral base with the addition of 1 mmol/L of ethylene-1-^{14}C-labeled demeton S-methyl sulfoxide, specific activity 0.018 μCi/mg, and 2 g/L of disodium fumarate as carbon source. Yielding of metabolites in sufficiently large amounts for structure elucidation was achieved by culturing in 2-L Erlenmeyer flasks each containing 500 mL of medium to which 500 mg/L of the unlabeled insecticide was added.

Determination of Degradation Rates. The bacteria were separated from the culture medium by centrifugation and the medium was extracted three times with 10-mL portions of dichloromethane. The combined extracts were dried (Na_2SO_4) and concentrated by evaporation, and the compounds were separated by thin-layer chromatography (TLC) on silica gel 60 F_{254} (Merck, Darmstadt, Germany) in chloroform/2-propanol (17:3, v/v) as developing solvent. Radioactivity on TLC plates was recorded with a Frieseke chromatogram scanner, type LB 2733 (Karlsruhe-Durlach, Germany). Radioactive areas were removed from the plates, and the compounds were eluted with 10 mL of acetone.

The aqueous layers were concentrated by evaporation (Büchi). The resultant solid residue was eluted three times with 15-mL portions of methanol; the extract was reduced to a small volume by evaporation, streaked on TLC plates, and developed in chloroform/methanol (3:2, v/v). The TLC plates were scanned as described above.

Estimations. Radioactivity in the culture media, dichloromethane extracts, aqueous layers, and eluates of radioactive TLC areas was measured by counting in a Beckman liquid scintillation counter, type LS 200. Growth of the bacteria was assayed by measuring the absorbancy of the cultures at 578 nm with a Zeiss Model DM 4 spectrophotometer. Melting points were determined using a Kofler hot stage (Reichert, Austria). Infrared spectra were obtained using a Perkin-Elmer 521 instrument.

Electron-impact mass spectra (EI MS) were recorded on a Varian MAT CH 7 under the condition of automatic evaporation of the sample material by electronic control of the total ion current (Hillig et al. 1979). Field-desorption mass spectra (FD MS) were measured on a Varian MAT CH 5. The field-desorption data were obtained with 10-μm tungsten wires activated at 1200 °C with benzonitrile by using the syringe technique (Beckey et al. 1970).

The 60-MHz proton NMR spectra were obtained on a Bruker WP 60 Fourier-transform spectrometer after 400 to 15 800 transients.

Bayerische Landesanstalt für Bodenkultur und Pflanzenbau, Abt. Pflanzenschutz, 8000 München 19, West Germany (W.Z., G.E., and P.R.W.), and Forschung und Entwicklung, Bayer AG, Leverkusen and Abt. Pflanzenschutz, Anwendungstechnik CE, Metabolismus u. Rückstände, Bayer AG, Leverkusen, West Germany (L.O. and K.W.).

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Table I. Rate of Degradation of Demeton S-Methyl Sulfoxide by Various Soil Microorganisms

microorganisms	% degradation of demeton S-methyl sulfoxide after 14 days of incubation
Nocardia sp. DSM 43252	98
Arthrobacter sp. DSM 20389	70
Arthrobacter roseoparaffineus	65
Corynebacterium petrophilum ATCC 19080	90
Brevibacterium ammoniagenes ATCC 13745	80
Bacillus sphaericus ATCC 12123	85
Bacillus sphaericus ATCC 12300	90
Pseudomonas fragi 1233	94
Pseudomonas putida 1453	99
Pseudomonas fluorescens 1542	90

Detection Reagents. Demeton S-methyl sulfoxide and some of its metabolites could be visualized on the TLC plates by spraying with 0.2% $PdCl_2$ in 4 N HCl. Sulfide and sulfoxide compounds formed a yellow color; sulfone metabolites showed no reaction.

Isolation of Metabolites. Metabolites were each isolated from a 500-mL culture grown with 2 mmol/L demeton S-methyl sulfoxide and 2 g/L disodium fumarate as carbon source. The cultures were harvested after a 13-day incubation period. The cells were separated by centrifugation at 5000*g* and the products isolated by extraction with dichloromethane and separated by TLC in chloroform/2-propanol (17:3, v/v).

RESULTS AND DISCUSSION

Degradation of Demeton S-Methyl Sulfoxide by Isolated Soil Bacteria. For investigation of the potential toxicity of the insecticide to microorganisms, the compound was added to the culture medium in concentrations varying from 0 to 2 mmol/L. It was demonstrated that all the organisms tested were able to grow in the presence of insecticide concentrations of up to 2 mmol/L. This concentration was used in all degradation tests in order to obtain sufficient quantities of transformation products of the insecticide. All strains transformed up to 99% (Table I) of the insecticide to several different metabolites. Since *Pseudomonas putida* 1453 and *Nocardia sp.* DSM 43252 exhibited the highest rates of insecticide transformation, these organisms, which represent predominant strains of Gram-negative and Gram-positive soil bacteria, were selected for further studies.

Degradation by *Pseudomonas putida* 1453. During growth on 2 g/L disodium fumarate, 2 mmol/L demeton S-methyl sulfoxide was almost completely metabolized within 13 days; 21.5% of the ^{14}C activity remained in the aqueous layer after extraction with dichloromethane. The ^{14}C activity of the dichloromethane fraction (78% of the starting activity) consisted of the three major metabolites II, III, and IV (Figure 1). The time course of the formation of these compounds is shown in Figure 2. Bis-[2-(ethylsulfinyl)ethyl]disulfide (IV), the main transformation product, accumulated after 13 days of incubation. It most probably is formed by oxidative dimerization of 2-(ethylsulfinyl)ethanethiol (II) which could be identified as a transient intermediate (Figure 2). Compound II is a hydrolysis product of the parent compound, I. 2-(Ethylsulfonyl)ethanethiol (III) presumably is formed by hydrolysis of demeton S-methyl sulfone, an oxidation product of the parent insecticide.

Degradation by *Nocardia sp.* DSM 43252. During growth in mineral base, 2 mmol/L of ^{14}C-labeled demeton S-methyl sulfoxide underwent 98% transformation within 13 days. About 53% of the initially applied ^{14}C activity was not extractable from the aqueous layer, thus indicating an increasing formation of polar degradation products. In the dichloromethane fraction three metabolites, V, VI, and

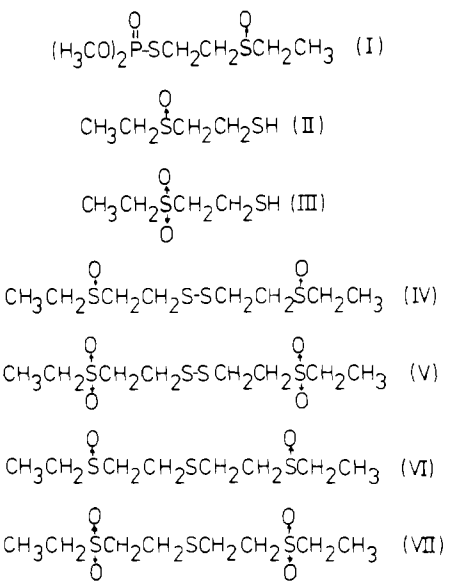


Figure 1. Metabolites of demeton S-methyl sulfoxide (I) formed during degradation by *Pseudomonas putida* 1453 and *Nocardia sp.* DSM 43252: 2-(ethylsulfinyl)ethanethiole (II), 2-(ethylsulfonyl)ethanethiol (III), bis[2-(ethylsulfinyl)ethyl]disulfide (IV), bis[2-(ethylsulfonyl)ethyl] disulfide (V), bis[2-(ethylsulfinyl)ethyl] sulfide (VI), bis[2-(ethylsulfonyl)ethyl] sulfide (VII).

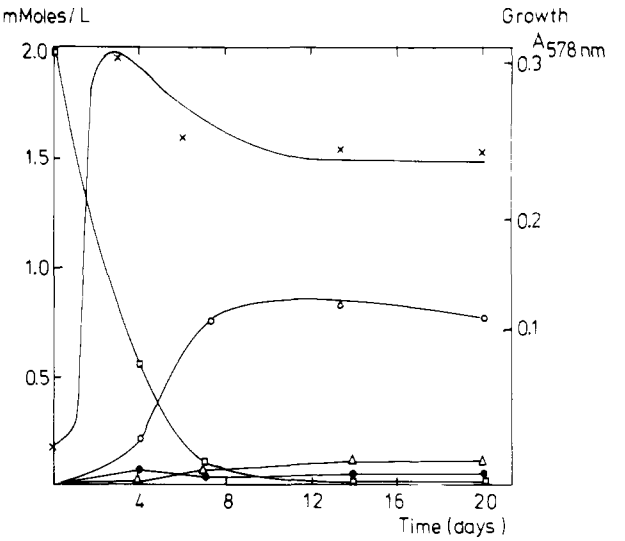


Figure 2. Degradation of demeton S-methyl sulfoxide by *Pseudomonas putida* 1453. (×) Growth (A_{578}) and (□) degradation of demeton S-methyl sulfoxide (I), (●) formation of compound II, (△) formation of compound III, (○) formation of compound IV.

VII, were detected and identified by means of their physical properties (Table II). The formation of bis[2-(ethylsulfonyl)ethyl]disulfide (V), the major metabolite, results from an oxidative dimerization of 2-(ethyl-

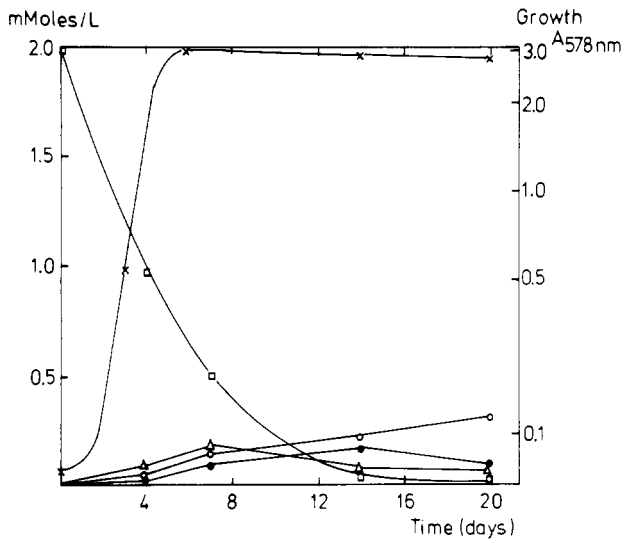


Figure 3. Degradation of demeton *S*-methyl sulfoxide (I) by *Nocardia sp.* DSM 43252. (×) Growth (A_{578}) and (□) degradation of demeton *S*-methyl sulfoxide (I), (○) formation of compound V, (△) formation of compound VI, (●) formation of compound VII.

sulfonyl)ethanethiol (III). This compound, however, could not be detected in cultures incubated with *Nocardia sp.* DSM 43252. The concentration of bis[2-(ethylsulfinyl)-ethyl] sulfide (VI) peaked after 7 days of incubation with the bacteria and then decreased significantly (Figure 3). The structure suggests that this compound is formed by elimination of *O,O*-dimethyl thiophosphate from demeton *S*-methyl sulfoxide, thus producing ethyl vinyl sulfoxide, which then reacts with 2-(ethylsulfinyl)ethanethiol (II), a further product of degradation, to form compound VI. It is conceivable that the metabolite bis[2-(ethylsulfonyl)-ethyl] sulfide (VII) results from the reaction of ethyl vinyl sulfone with compound III.

Degradation of Demeton *S*-Methyl Sulfoxide under Abiotic Conditions. In noninoculated (sterile) controls, about 65% of the total ^{14}C activity applied was extractable with dichloromethane after 20 days of incubation. The extract contained about 48% of the parent compound and 12% of compound IV. In the aqueous phases of the noninoculated controls as well as in those from the tests with the two bacterial strains, one–three distinct radioactive areas could be separated by TLC, some of which were visualized by spraying with the $PdCl_2$ reagent after TLC (methanol/dichloromethane, 2:3, v/v). The polar metabolites could not be identified by mass spectrometry because of their high polarity and low vapor pressure. Nuclear magnetic resonance studies could not be performed because the amounts of the purified products of the aqueous layers were too small.

Table II. Physical Data of the Metabolites of Demeton *S*-Methyl Sulfoxide

compd	R_f[a]	mp, °C	formation of compd mmol/L[b]
O,O-dimethyl *S*-(2-ethylsulfinylethyl) phosphorothioate (I)	0.68	oil	
2-(ethylsulfinyl)ethanethiol (II)	0.75	oil	0.02
2-(ethylsulfonyl)ethanethiol (III)	0.85	116-120	0.07
bis[2-(ethylsulfinyl)-ethyl] disulfide (IV)	0.47	76-88	1.05
bis[2-(ethylsulfonyl)-ethyl] disulfide (V)	0.96	102-107	0.22
bis[2-(ethylsulfinyl)-ethyl] sulfide (VI)	0.79	66-67	0.11
bis[2-(ethylsulfonyl-ethyl] sulfide (VII)	0.90	111-112	0.15

[a] Chloroform/2-propanol (17:3 v/v). [b] Concentration of starting compound (I) 2.0 mmol/L.

Identification of Metabolites. The metabolites III to VII shown in Table II were identified by comparing their EI mass spectra with those recorded for the synthesized reference substances. The mass spectra presented in

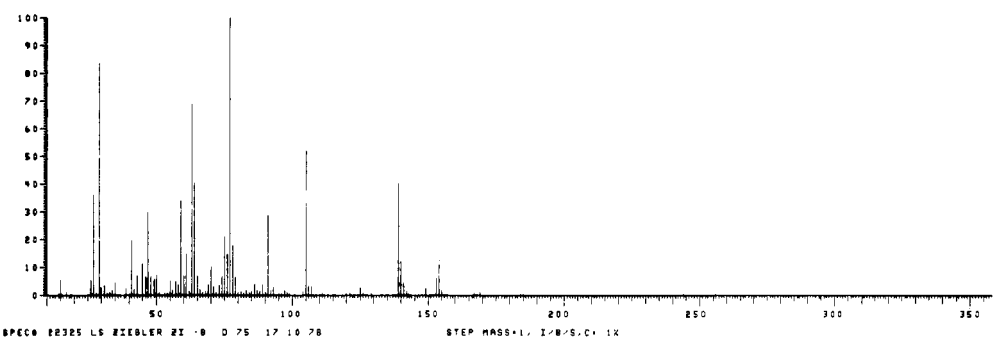


Figure 4. Mass spectrum of 2-(ethylsulfonyl)ethanethiol (III).

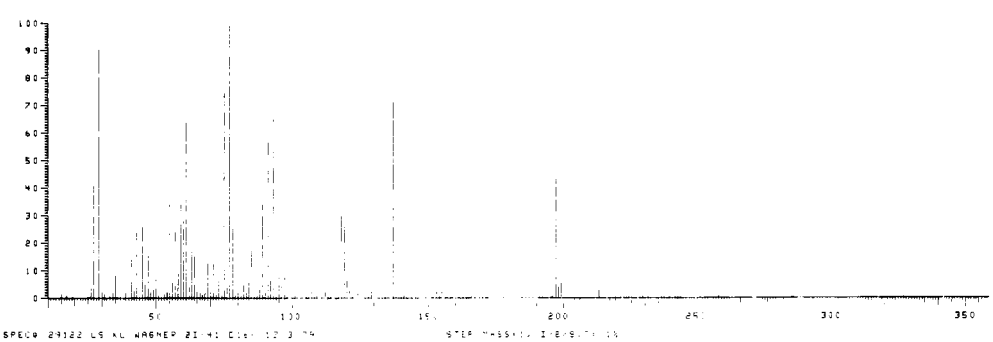


Figure 5. Mass spectrum of bis[2-(ethylsulfinyl)ethyl] disulfide (IV).

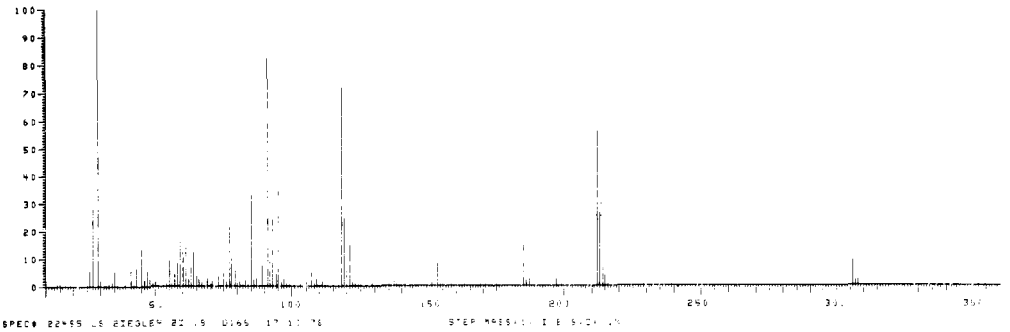


Figure 6. Mass spectrum of bis[2-(ethylsulfonyl)ethyl] disulfide (V).

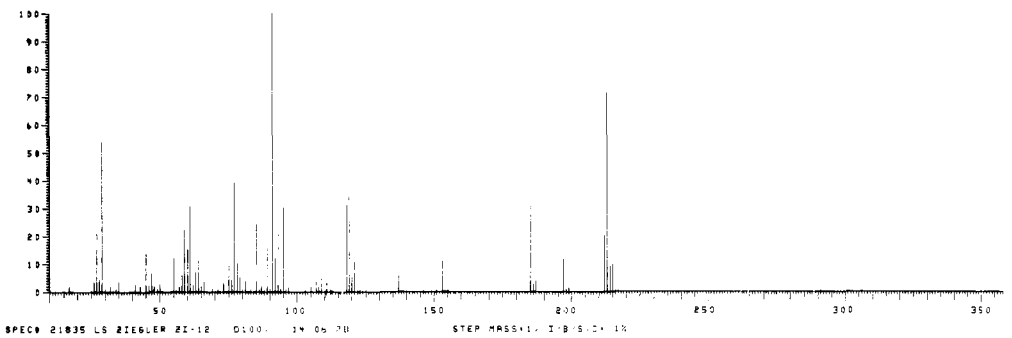


Figure 7. Mass spectrum of bis[2-(ethylsulfinyl)ethyl] sulfide (VI).

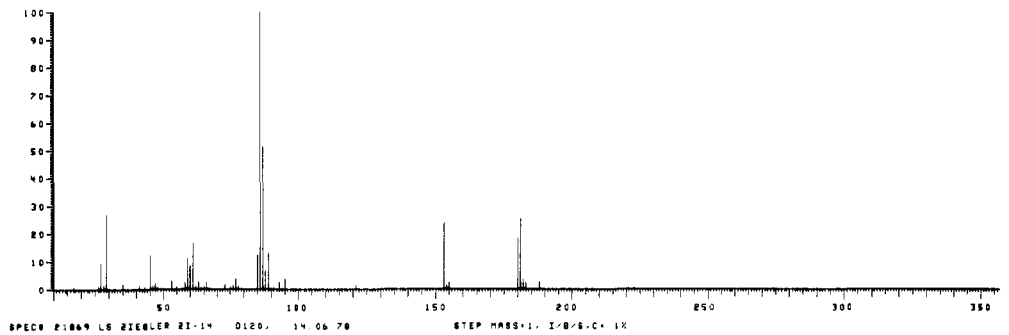
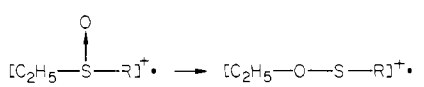


Figure 8. Mass spectrum of bis[2-(ethylsulfonyl)ethyl] sulfide (VII).

Figures 4–8 show that molecular ions were detected in the EI spectrum only for metabolites III and V. For the other metabolites, fragmentation ions only were detected. Fragmentation to the ions with the highest m/e is accounted for by an end group being split off from the molecule (Table III). Cleavage of ethoxy from bis[2-(ethylsulfinyl)ethyl] sulfide (VI) is possible only after rearrangement of



as described by Budziciewicz et al. (1967).

It was also observed that the sulfones V and VII split off ethylsulfinic acid with formation of the olefins CH_2=$CHSSC_2H_4SO_2C_2H_5$ (m/e 212; 58%) and CH_2=$CHSC_2H_4SO_2C_2H_5$ (m/e 180; 26%). Renewed cleavage of ethylsulfinic acid in the mass spectrometer results in fragmentation to the ions at m/e 118, 72%, and at m/e 86, 100%, which are assigned to divinyl disulfide (CH_2=$CHSSCH$=CH_2) and the divinyl sulfide (CH_2=$CHSCH$=CH_2), respectively. In the 60-MHz proton NMR spectrum of *O,O*-dimethyl thiolophosphoric acid only a doublet at 3.59 ppm with a coupling constant J_{HP} = 13 Hz was detected. The field-desorption mass spectrum shows signals at m/e 187 and 203 which represents the Na and K salt. Furthermore the cluster ions m/e 351 and 515 could be observed; these represent $2M^+$ + Na and $3M^+$ + Na. Field-desorption mass spectra were also recorded for the compounds III to VII to detect the molecular ions. In all cases, the molecular ion was seen to be the base peak; other ions observed were M* + 1, M* + 2, and M* + 3. Fragment peaks were not seen in any of the FD spectra.

Table III. First Decompositions from the Molecular Ion

compd	mol wt	M − X, m/e	X (fragment)
III	154	139	CH_3
IV	274	197	C_2H_5SO
V	306	213	$C_2H_5SO_2$
VI	242	213	C_2H_5
		197	C_2H_5O
VII	274	181	$C_2H_5SO_2$

The 60-Hz proton NMR spectra show a triplet for the CH_3 group at 1.3–1.4 ppm. The corresponding quartet of the ethyl groups is observed at 2.8 ppm for the sulfoxides and at 3.1 ppm for the sulfones. This quartet is superimposed by the AA'BB' system of the ethylene group.

Results presented here indicate that demeton *S*-methyl sulfoxide is transformed to different metabolites by various soil microorganisms rather than mineralized. Since in the course of degradation studies of demeton *S*-methyl sulfone in model soils (unpublished data) similar metabolites could be observed, it would be of great interest to prove the

potential biological activity of the different metabolites.

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Received for review February 25, 1980. Accepted June 3, 1980.

Position of the Radiolabel in Glycine Resulting from [2-^{14}C]DPX-3217 (^{14}C-Labeled Curzate Fungicide) Metabolism

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Studies using *Peptococcus glycinophilus*, a glycine-specific bacterium, demonstrated that radiolabeled glycine in plants treated with [2-^{14}C]DPX-3217 (^{14}C-labeled Curzate fungicide) was exclusively labeled in the methylene group, corresponding to the position in the parent compound. These findings provide evidence that glycine is a primary metabolite of DPX-3217 rather than a product of metabolic reincorporation of the radiolabeled carbon.

The active ingredient in Du Pont Curzate fungicide, 2-cyano-*N*-[(ethylamino)carbonyl]-2-(methoxyimino)-acetamide (DPX-3217), is an effective fungicide against grape downy mildew as well as tomato and potato late blight. In studies on tomatoes, potatoes, and grapes reported by Belasco et al. (1980), ^{14}C-labeled glycine was found to be a primary metabolite of [2-^{14}C]DPX-3217. Similarly, Belasco and Baude (1980) found [^{14}C]glycine to be the major metabolite in rats dosed with [2-^{14}C]DPX-3217. The relatively high concentrations of radiolabeled glycine in crops and in the urine of rats treated with DPX-3217 and the structural relationship of DPX-3217 suggested that glycine might be a direct metabolite of DPX-3217. This study was undertaken to demonstrate that the radiolabeled glycine isolated from both potato and tomato plants treated with [2-^{14}C]DPX-3217 was present primarily as [2-^{14}C]glycine, using an anaerobic glycine-specific bacterium, *Peptococcus glycinophilus*.

EXPERIMENTAL SECTION

Radiolabeled Chemicals. [1-^{14}C]-Glycine and [2-^{14}C]glycine were purchased from Amersham/Searle Corp., Arlington Heights, IL 60005. Each compound had a radiochemical purity of 95% and a specific radioactivity of 40 mCi/mmol. [2-^{14}C]DPX-3217 was synthesized according to the procedure described by Belasco and Baude (1980). This preparation had a radiochemical purity of >99% and a specific activity of 1.90 mCi/mmol or 9.57 μCi/mg.

Bacterium. *P. glycinophilus* (ATCC 23195) was obtained from the American Type Culture Collection, Rockville, MD 20852. In the earlier literature, this organism also was known as *Deplococcus glycinophilus*.

Culture Media. Stock cultures of *P. glycinophilus*, an anaerobic bacterium, were carried in Bacto-Thioglycollate medium without dextrose (Difco Laboratories, Detroit, MI). This culture medium was supplemented with 0.3 g of glycine per L of medium to support the growth of this glycine-specific bacterium.

An experimental nutrient medium described by Cardon and Baker (1947) was also used. This medium was supplemented with sterile sodium sulfide (0.01% concentration) after autoclaving as a means of maintaining anaerobic conditions.

In those tests employing washed cell suspensions of *P. glycinophilus*, a minimal medium containing 0.04 M glycine, 0.02 M phosphate buffer, pH 7.0, and 0.02% $Na_2S{\cdot}9H_2O$ was used. Here, also, sterile sodium sulfide was added after the medium containing the other ingredients was autoclaved.

Source of DPX-3217 Metabolites. (1) Potato tubers were lyophilized from plants which had received five weekly foliar applications of [2-^{14}C]DPX-3217 at a rate equivalent to 0.2 kg/(1000 L ha) (Belasco et al., 1980).

(2) Tomato fruit was lyophilized from plants which had received seven weekly foliar treatments of [^{14}C]DPX-3217 at a concentration of 0.15 kg/(1000 L ha) (Belasco and Baude, 1980).

The lyophilized plant tissues were hydrolyzed in 6 N hydrochloric acid under reflux (100 °C) for 24 h. The hydrolysate was filtered and the residue washed with water. The filtrate was reduced to near dryness on a vacuum rotary evaporator and redissolved in 5 mL of water. The pH was adjusted to 7.0, and the solution was again taken to near dryness. This concentrate was then picked up in a small volume of water (0.5–1.0 mL) for microbiological assay.

Microbiological Procedure. Twenty milliliters of the culture medium was introduced into a 50-mL screw-cap Erlenmeyer flask which had a center well containing 1 mL of 10% sodium hydroxide solution to absorb metabolic $^{14}CO_2$ for radioassay. The culture medium was then supplemented with the ^{14}C-labeled test substrate, e.g., ^{14}C-labeled glycine or radiolabeled tissue hydrolysate. The

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