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Degradation of the Insecticide Azinphos-methyl in Soil and by Isolated Soil Bacteria

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The insecticide azinphos-methyl (I) is degraded in soil to a great extent after a 197-day incubation period. Liberation of two benzoic acid derivatives (benzamide, XV, and salicylic acid, XVI) and of $^{14}CO_2$ from [*carbonyl*-^{14}C]- and [*ring*-U-^{14}C]azinphos-methyl indicates that the 1,2,3-benzotriazinone ring is cleaved in soil. Of the 17 metabolites identified, 3-(mercaptomethyl)-1,2,3-benzotriazin-4(3*H*)-one (VI), 3-[(methylthio)methyl]-1,2,3-benzotriazin-4(3*H*)-one (XI), and the corresponding sulfinyl (V) and sulfonyl (IV) derivatives represent key intermediates in azinphos-methyl degradation in soil. Compounds XI, V, and IV are cleaved by *Pseudomonas fluorescens* DSM 1976, forming anthranilic acid as the main metabolite, thus demonstrating that the insecticide can be metabolized in soil via easily decomposable benzoic acid derivatives.

Although there is some information available concerning the degradation of the insecticide azinphos-methyl [*O*,*O*-dimethyl *S*-[[4-oxo-1,2,3-benzotriazin-3(4*H*)-yl]methyl] phosphorodithioate] both in soil and by isolated soil bacteria, the fate and exact degradation pathway of this compound in soil systems still remain unclear. This fact is mainly due to the polar character of the metabolites, thus forming nonextractable residues, as well as to problems arising during structure elucidation of such degradation products that often show significant thermal lability.

The main metabolites already described during degradation studies of azinphos-methyl in various soils are benzazimide, (thiomethyl)benzazimide, *N*-methylbenzazimide sulfide, and bis(benzazimidyl-methyl) disulfide. They were identified, however, only by chromatographic methods (Schulz et al., 1970). More recently we demonstrated that the soil bacterium *Pseudomonas fluorescens* DSM 1976 degrades azinphos-methyl to bis(benzazimidylmethyl)disulfide [bis[3-(thiomethyl)-1,2,3-benzotriazin-4(3*H*)-one] and benzazimide [1,2,3-benzotriazin-4(3*H*)-one] via hydrolysis and enzymatic oxidation as well as to anthranilic acid by cleavage of the heterocyclic ring (Engelhardt et al., 1981). A great number of other soil bacteria of the genera *Pseudomonas* and *Arthrobacter* also proved capable of cleaving the 1,2,3-triazinone ring of this insecticide, forming anthranilic acid (Wallnöfer et al., 1982). Since anthranilic acid was not formed via benzazimide (or benzazimidylmethanethiol) as an intermediate, the reactions leading to anthranilic acid remained to be elucidated.

In the present study the metabolites formed during degradation of [*carbonyl*-^{14}C]- and [*ring*-U-^{14}C]azinphos-methyl in different soil types were isolated and identified by means of nuclear magnetic resonance and mass spectroscopy and determined quantiatively by measuring the distribution of ^{14}C activity. In addition, some of the major transformation products of azinphos-methyl in soil, which represent structures related to the starting compound, were subjected to further degradation by *P. fluorescens* DSM 1976 to obtain more information on the biodegradability of these substances and on the pathway by which anthranilic acid is formed from the insecticide in this bacterium.

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EXPERIMENTAL SECTION

Chemicals. [*carbonyl*-^{14}C]Azinphos-methyl (specific activity 2.7 mCi/mM) and [*ring*-U-^{14}C]azinphos-methyl (specific activity 25 mCi/mM) were synthesized by Mobay Chemical Corp., Agricultural Division, Kansas City, MO. The synthetic reference compounds of intermediates were prepared by Bayer AG, Leverkusen, West Germany, Abteilung Pflanzenschutz-Chemische Forschung II (Dr. Cölln) and Abteilung Pflanzenschutz-Anwendungstechnik (Dr. Wagner).

Organisms and Culture Conditions. *P. fluorescens* DSM 1976 was cultured in 100 mL of Hegeman's mineral base (Hegeman, 1966) with additional 0.02% yeast extract, 160 mg of the respective substrate/L and 4 g/L disodium fumarate as the carbon source as described (Engelhardt et al., 1981).

Soil Types Used. Soil degradation studies were performed according to the BBA (1980) (Biologische Bundesanstalt Braunschweig, West Germany). The two soil types used were native soil from Laacherhof Experimental Station (organic matter 0.8%; clay 19.8%; silt <20 μm 34.9%; moisture content 11.2%; pH 6.0) and standard soil no. 1 (organic matter 2.66%; silt 14.9%; particle sizes <0.002 mm 5.8%, 0.002–0.02 mm 9.1%, 0.02–0.2 mm 41.8%, and >0.2 mm 43.3%; pH 6.1). Prior to insecticide addition, the standard soil sample (about 2 kg) was adjusted to 40% maximum water capacity by using distilled water (11 g of H_2O/100 g of soil) and incubated for 14 days at 22 °C in the dark. The water that evaporated was replenished at a 3-day interval.

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Insecticide Application. [*carbonyl*-^{14}C]Azinphos-methyl was added to 300 g of native soil (Laacherhof) by using a solution of 3.57 mg in 1.5 mL of acetone (11.9 ppm, total radioactivity 30.4 μCi) (test 1) and to 111 g of reactivated standard soil no. 1 with a solution of 498 μg of azinphos-methyl in 1 mL of acetone (4.5 ppm, total radioactivity 4.25 μCi) (test 2). [*ring*-U-^{14}C]Azinphos-methyl was introduced into standard soil no. 1 by adding 1 mL of a solution or 66 μg of azinphos-methyl (0.6 ppm, total radioactivity 5.24 μCi) to 111 g of soil (test 3). After the insecticide was mixed with the soil, the samples were attached to an apparatus for detection of $^{14}CO_2$ and incubated for 222 (test 1), 552 (test 2), and 365 days (test 3), respectively. The air taken in was prewashed in NaOH to reduce its natural CO_2 content, moistened by passing through water, and washed with 50% sulfuric acid to remove possible volatile basic compounds. In all studies, no radioactivity was detected in the vessels filled with sulfuric acid. The $^{14}CO_2$ evolved was absorbed by passing it successively through three flasks filled with 1 N NaOH and analyzed by liquid scintillation counting.

For large-scale preparation of metaboites, some parallel experiments were run with unlabeled insecticide that was added to the soil to a final concentration of 1000 ppm of air-dried soil. TLC of these tests was performed analogous to that of ^{14}C-labeled compounds.

Extraction Procedures. From the soil degradation studies portions of 30 g each were investigated after a 37-, 67-, 100-, 136-, 164-, and 197-day incubation period as follows: The soil was stirred successively with three portions of 150 mL of chloroform with a magnetic stirrer for 1 h at room temperature, filtered, and combined. The residues were washed with chloroform, this solvent was added to the combined extracts, and the total chloroform extract was concentrated by Büchi evaporation for TLC analysis.

The soil samples were then treated analogously 3 times with three portions of 150 mL of acetone for 1 h at room temperature. Finally the soil samples were stirred 3 times with 120 mL of deionized water for 3 h at room temperature and filtered, the filtrates concentrated by freeze-drying and dissolved in aqueous methanol for TLC analysis. For estimation of residual ^{14}C activity the extracted soil samples were air-dried.

Microbial cultures were extracted and further treated as described recently (Engelhardt et al., 1981).

Thin-Layer Chromatography (TLC). TLC was performed on (a) silica gel plates (150 G/LS 254; 0.1-mm thickness), (b) TLC alumina sheets silica gel (150 G/LS 254), and (c) TLC alumina sheets cellulose [(a–c) purchased from Merck AG, Darmstadt, West Germany]. For special separation problems, precoated silgur-25 UV_{254} plates (Machery-Nagel Co, Düren, West Germany) were also used. All TLC plates were predeveloped in methanol. The following solvent systems were used for TLC: ethyl acetate–2-propanol–water (65:24:11 v/v/v) (A), *n*-hexane–chloroform–2-propanol–water (53:32:24:1 v/v/v/v) (B), ethyl acetate–2-propanol–water (60:27:13 v/v/v) (C), *n*-hexane–acetone (60:40 v/v) (D), *n*-hexane–acetone (50:50 v/v) (E), *n*-butanol–acetone–water (60:15:15 v/v/v) (F), chloroform–methanol–water (60:30:3 v/v/v) (G), chloroform–2-propanol (95:5 v/v) (H), toluene–acetone/triethylamine (8:2:1 v/v/v) (I), toluene–dioxane–acetic acid (90:25:4 v/v/v) (K). Aromatic and heteroaromatic structures were visualized on TLC plates using a Camag UV lamp (254 nm). Compounds containing sulfur were detected on TLC plates by spraying with 0.2% $PdCl_2$ in 4 N HCl. Sulfide compounds formed a yellow color; sulfoxide and sulfone metabolites showed no reaction. For detection of phosphorus-containing substances, plates are first sprayed with 2% 4-(*p*-nitrobenzyl)pyridine in acetone and, after heating (100 °C, 4 min), with 10% tetraethylpentamine in acetone. A dark blue color was then formed by alkylating phosphorus compounds. Additionally plates were sometimes treated with 0.5% *N*-(1-naphthyl)-ethylenediammonium dihydrochloride in 20% acetic acid which with benzazimide derivatives produces deep violet areas on a white background by a coupling reaction formed after hydrolysis of the compounds.

Radioactive areas on TLC plates were recorded in the microbial degradation studies with a Frieseke chromatogram scanner type LB 2733 (Karlsruhe-Durlach, West Germany) and in soil degradation tests by exposing the plates to Agfa-Gaevert Curix RP 1Cb X-ray films. Radioactive areas on TLC plates were scraped from the plates or cut off from the alumina sheets and extracted with acetone or methanol, respectively, depending on their polarity, for analysis by liquid scintillation counting after addition of Insta-Gel (Packard Instrument Co., Illinois). Chloroform and acetone extracts of soil samples were chromatographed repeatedly in solvent systems D, E, and H (18-cm height); methanol eluates of the freeze-dried water extracts of soil were developed in solvent systems A–C, F, and G (18 cm); TLC of extracts of microbial cultures was performed successively in solvent systems I and K.

Analytical Procedures. Radioactivities of culture media, extracts, aqueous layers, the trapped $^{14}CO_2$ (NaOH), and eluates of radioactive TLC areas were measured by counting in a Beckman liquid scintillation counter, type LS 200 (Beckman Instruments, Munich, West Germany). Degradation rates of nonlabeled reference compounds of soil degradation products in growing bacterial cultures were calculated by quantitative UV analysis of the starting compounds and their metabolites after extraction from the media as described (Engelhardt et al., 1976).

Growth of the bacteria was assayed by measuring the turbidity of the cultures at 578 nm with a Zeiss Model DM 4 spectrophotometer.

Melting points were determined by using a Kofler hot stage (Reichert, Austria).

Transformation products formed were identified by nuclear magnetic resonance (NMR) and different mass spectroscopic (MS) techniques commonly by additional comparison with synthetic reference compounds. High polar compounds like (4-oxo-3*H*-1,2,3-benzotriazinyl)-3-methanesulfonic acid (XIV) or *S*-methyl *S*-[(4-oxo-1*H*-1,2,3-benzotriazin-3-yl)methyl] dithiophosphate (XII) could only be identified by means of fast atom bombardment mass spectroscopy (FAB-MS) and Fourier transform nuclear magnetic resonance spectroscopy (FT-NMR).

Electron impact mass spectra (EI-MS) were recorded on a Varian MAT CH 7 under the conditions of automatic evaporation of the sample material by electronic control of the total ion current (Hillig et al., 1979). Chemical ionization mass spectra (CI-MS) were measured on a Finnigan MAT 112 S and a Finnigan MAT 212 S, field desorption mass spectra (FD-MS) on a Varian MAT CH 5 after application of a solution of the compound on wires with carbon whiskers, and FAB-MS on a Finnigan MAT 312S. ^{1}H nuclear magnetic resonance (^{1}H NMR) spectra were obtained on a Bruker WP 80 Fourier transform spectrometer.

The amount of radioactivity remaining in the extracted soils was estimated by combustion of dried soil residues in an Oxymat combustion apparatus (Intertechnique,

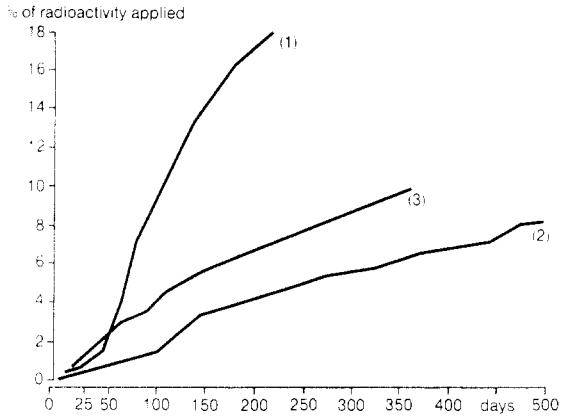

Figure 1. Liberation of $^{14}CO_2$ from carbonyl-^{14}C-labeled insecticide in the native soil (1) and standard soil no. 1 (2) and from the U-ring-^{14}C-labeled compound in standard soil no. 1 (3).

Paris, France) and subsequent scintillation counting.

RESULTS

Elimination of $^{14}CO_2$ from Labeled Insecticide in Different Soils. Mineralization of [*carbonyl*-^{14}C]- and [*ring*-U-^{14}C]azinphos-methyl to $^{14}CO_2$ during a 500-day incubation period in the native soil and in the standard soil no. 1 is depicted in Figure 1. In the native soil (test 1), 222 days after application of the carbonyl-^{14}C-labeled insecticide, 18.6% of the compound was decomposed to $^{14}CO_2$, whereas in the standard soil no. 1 after a 552-day incubation period only 9% of the radioactivity added was eliminated as $^{14}CO_2$ (test 2), which is suggested to be due to minor microbial activity of this stored soil. Finally, 365 days after application of the ring-U-^{14}C-labeled insecticide to the standard soil no. 1, again 10% of the radioactivity applied was identified as $^{14}CO_2$ (test 3), which indicates that the aromatic moiety of the starting molecule can also be mineralized in soil.

Formation of Metabolites from [*carbonyl*-^{14}C]Azinphos-methyl in the Native Soil. Within a 197-day incubation period, about 93% of the carbonyl-^{14}C-labeled insecticide applied was decomposed in the native soil. While $^{14}CO_2$ liberation increased, the total amount of metabolites detectable was nearly constant during the incubation period. Soil-bound residues reached a maximum of approximately 62% of the amount of [^{14}C]azinphos-methyl applied (Figure 2). The distribution of radiocarbon in the chloroform plus acetone extracts and water phases is summarized in Table I. The metabolites II, III, IV, and V of the solvent extracts and XII and XIV of water phases were found in amounts exceeding more than 1%, whereas the concentrations of all other metabolites identified were below 1%, in some cases even below 0.5% of the radioactivity applied.

Identification of Metabolites. Since electron impact mass spectra (EI-MS) of the various metabolites were very similar and the molecular ion could only be detected in few cases, elucidation of their respective structures proved to be difficult: e.g., in the EI-MS of the starting insecticide azinphos-methyl the peak of the highest mass is at *m/e* 160, which is simultaneously the base peak. Further fragments are *m/e* 132 (160 *m/e* − N_2), *m/e* 105 (132 *m/e* − HCN), *m/e* 104 ($C_6H_4CO^+$), *m/e* 93 [$P(OCH_3)_2^+$], and *m/e* 77 ($C_6H_5^+$). The peak at *m/e* 125 corresponds to the thiophosphoric acid fragment. Because the molecular ion is commonly necessary for an exact structure elucidation, careful ionization techniques of field desorption (FD) and chemical ionization (CI) were applied. In the FD-MS of azinphos-methyl, the molecular ion at *m/e* 317 is detected as the base peak as well as the fragments *m/e* 160 and *m/e*

Table I. Distribution of Radioactivity of Residual Azinphos-methyl and Metabolites Formed in the Native Soil (Test No. 1) (Percent of ^{14}C Radioactivity Applied)

	chloroform plus acetone extracts										water extract					
day	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIV	XIII	XV	XVI	sum of metabolites
37	55.4	5.06	0.43	0.42	3.22	0.61	0.44	0.36	0.28	n.d.	0.23	0.54	0.15	0.57	0.29	0.03	13.0
67	33.1	6.67	1.33	0.27	1.79	0.86	0.22	0.26	0.17	0.15	0.23	0.53	0.47	0.53	0.42	0.06	14.8
100	18.9	5.74	1.12	0.64	1.27	0.20	0.23	0.30	0.40	0.05	0.18		1.60	0.30	0.57	0.07	13.0
136	10.7	5.44	n.d.	0.55	1.66	0.25	0.40	0.29	0.41	0.24	0.11		1.83	0.14	0.13	0.04	11.7
164	8.1	4.24	0.23	1.43	1.64	0.24	0.27	0.25	0.33	0.12	0.08		2.10	0.08	0.13	0.04	11.3
197	6.9	3.86	0.63	1.93	1.57	0.33	0.36	0.26	0.35	0.19	0.11		2.06	0.07	0.20	0.04	11.8

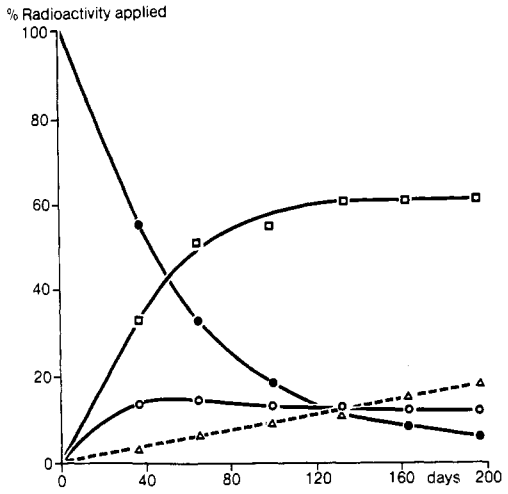

Figure 2. Fate of [*carbonyl*-^{14}C]azinphos-methyl in the native soil (test 1). Symbols: (●—●) degradation of azinphos-methyl; (○—○) formation of metabolites; (Δ- - -Δ) liberation of $^{14}CO_2$; (□—□) soil-bound radioactivity.

125, found also in the EI-MS, and *m/e* 147, which reflects a cleavage of the molecular ion forming benzazimide. The CI-MS contains two peaks, the MH^+ ion at *m/e* 318 and *m/e* 160.

The metabolites listed in Table II were identified by comparing their mass spectra as well as their ^{1}H Fourier transform nuclear magnetic resonance spectra (^{1}H FT-NMR) with those recorded for the reference substances.

Bis[3-(thiomethyl)-1,2,3-benzotriazin-4(3*H*)-one] (II). Besides the peaks at *m/e* 125 and *m/e* 93 corresponding to the fragments of the phosphoric acid residue of the starting compound, the EI-MS of this disulfide are identical with those of I, making a structure determination impossible. In the FD-MS, in addition to the known fragment at *m/e* 160, the molecular ion at *m/e* 384 was detected. The ^{1}H FT-NMR spectrum in acetone consists of a singlet at 5.60 ppm (methylene group) and a multiplet for the aromatic protons at 8.1 ppm.

3,3′-Thiobis[methylene-1,2,3-benzotriazin-4(3*H*)-one](III). Since the EI-MS of this monosulfide is identical with that discussed above, the FD-MS was prepared that shows the molecular ion at *m/e* 352 and the known fragment at *m/e* 160. A further fragment ion at *m/e* 192 can be explained by loss of benzazimide from the molecule ion. The ^{1}H FT-NMR spectrum in dimethyl sulfoxide shows a singlet at 5.91 ppm (methylene groups) and a multiplet centered at 8.2 ppm for the aromatic protons.

3-[(Methylsulfonyl)methyl]-1,2,3-benzotriazin-4-(3*H*)-one (IV). The FD-MS of this compound contains the molecular ion at *m/e* 239 and the ion at *m/e* 160. The CI-MS with isobutane as reagent gas shows the MH^+ ion at *m/e* 240. With ammonia as the reagent gas only a small MH^+ peak was detected; the base peak, however, was found at *m/e* 257, which is attributed to the $M + NH_4^+$ ion. The ^{1}H FT-NMR spectrum in deuteriochloroform as the solvent contains singlets at 3.13 ppm (methylsulfonyl group) and 5.73 ppm (methylene group) and the multiplet centered at 8.1 ppm (aromatic protons).

3-[(Methylsulfinyl)methyl]-1,2,3-benzotriazin-4-(3*H*)-one (V). For this compound a molecular ion could not be detected either in the EI-MS or in the CI-MS (isobutane). In the FD-MS, a MH^+ ion at *m/e* 224 was obtained, *m/e* 160 being the base peak. Since an MH^+ ion was not previously observed for this type of compound in the FD-MS, this suggestion was confirmed by the CI-MS (ammonia), which showed the "quasi"-molecular ion MH^+ at *m/e* 224 in addition to $M + NH_4^+$ at *m/e* 241 and the

Table II. Physical Data of Azinphos-methyl and Its Metabolites

compound	mp, °C	mass spectrum (rel intensity)					
		M^+	*m/e*	*m/e*	*m/e*	*m/e*	*m/e*
O,O-dimethyl *S*-[[4-oxo-1,2,3-benzotriazin-4(3*H*)-yl]methyl] phosphorodithioate (I)	73–74	317[a] (100)	160[a] (70)	147[a] (42)	125[a] (12)		
bis[3-(thiomethyl)-1,2,3-benzotriazin-4(3*H*)-one] (II)		384[a]	160 (100)	132 (78)	105 (25)	104 (22)	77 (97)
3,3′-thiobis[methylene-1,2,3-benzotriazin-4(3*H*)-one] (III)		352[a] (100)	192[a] (13)	160[a] (80)			
3-[(methylsulfonyl)methyl]-1,2,3-benzotriazin-4(3*H*)-one (IV)	170	240[b] (100)	160[b] (56)	134[b] (38)			
3-[(methylsulfinyl)methyl]-1,2,3-benzotriazin-4(3*H*)-one (V)	165	224[c] (73)	241[c] (40)	160[c] (100)			
3-(mercaptomethyl)-1,2,3-benzotriazin-4(3*H*)-one (VI)		193 (4)	160 (100)	132 (35)	105 (18)	104 (19)	77 (73)
3-methyl-1,2,3-benzotriazin-4(3*H*)-one (VII)		161 (65)	133 (22)	132 (15)	105 (63)	104 (100)	77 (78)
3,3′-oxybis(methylene)bis[1,2,3-benzotriazin-4(3*H*)-one] (VIII)		337[c] (100)	354[c] (24)	160[c] (84)			
3-(hydroxymethyl)-1,2,3-benzotriazin-4(3*H*)-one (IX)	216	177 (9)	147 (86)	104 (35)	92 (96)	77 (100)	51 (52)
1,2,3-benzotriazin-4(3*H*)-one (X)	120	147 (100)	119 (12)	104 (31)	92 (96)	91 (32)	90 (14)
3-[(methylthio)methyl]-1,2,3-benzotriazin-4(3*H*)-one (XI)	87	207 (5)	160 (88)	132 (60)	105 (22)	104 (21)	77 (100)
S-methyl *S*-[(4-oxo-3*H*-1,2,3-benzotriazin-3-yl)methyl] dithiophosphate (XII)		348[a] (100)	364[a] (5)	673[a] (12)	Na salt		
O-methyl *S*-[(4-oxo-3*H*-1,2,3-benzotriazin-3-yl)methyl] dithiophosphate (XIII)		348[a] (100)	364[a] (10)	673[a] (48)	160 (13)		
(4-oxo-3*H*-1,2,3-benzotriazin-3-yl)methanesulfonic acid (XIV)		264[d]	356[d]				
benzamide (XV)	133	121 (89)	105 (100)	77 (96)	51 (38)	50 (36)	
salicylic acid (XVI)	159	138 (59)	121 (10)	120 (100)	92 (58)	65 (6)	64 (11)
anthranilic acid[e] (XVII)	146	151 (62)	120 (28)	119 (100)	92 (43)	65 (19)	

[a] FD mass spectrum. [b] CI mass spectrum with isobutane. [c] CI mass spectrum with ammonia. [d] FAB mass spectrum. [e] Compound after methylation with diazomethane.

ion at m/e 160. The ^{1}H FT-NMR spectrum showed, in addition to aromatic protons at 8.1 ppm, a singlet at 2.8 ppm for the methylsulfinyl group and an AB system at 5.57 ppm for the methylene group.

3-(Mercaptomethyl)-1,2,3-benzotriazin-4(3*H*)-one (VI). In the EI-MS of this metabolite, the molecular ion at m/e 193 was observed in addition to the fragment peaks already described. The FD-MS contains only one signal at m/e 193, which confirms the molecular ion in the EI-MS.

3-Methyl-1,2,3-benzotriazin-4(3*H*)-one (VII). The FD-MS of this compound showed only the molecular ion at m/e 161. In the CI-MS (ammonia) the ion MH^+ at m/e 162 and a further peak at m/e 179, which is assigned to $M + NH_4^+$, were observed.

3,3′-Oxybis[methylene-1,2,3-benzotriazin-4(3*H*)-one] (VIII). The CI-MS (ammonia) of this compound contains the MH^+ ion at m/e 337 and a further peak at m/e 354 for $M + NH_4^+$. Two smaller peaks at m/e 673 and m/e 690 are assigned to $2MH^+$ and $2M + NH_4^+$, respectively.

3-(Hydroxymethyl)-1,2,3-benzotriazin-4(3*H*)-one (IX). For this compound, the molecular ion at m/e 177 could be detected in the EI-MS. Its structure was confirmed by comparison of its spectrum with that of the authentic substance.

1,2,3-Benzotriazin-4(3*H*)-one (X). The EI-MS of this metabolite and the synthetic compound are identical; the molecular ion at m/e 147 represents also the base peak.

3-[(Methylthio)methyl]-1,2,3-benzotriazin-4(3*H*)-one (XI). In the EI-MS a small peak at m/e 207 was observed, the further fragment peaks being identical with that of compound II. The assumption that this peak represents the molecular ion was confirmed by an additional FD-MS with just one peak at m/e 207.

***S*-Methyl *S*-[(4-Oxo-3*H*-1,2,3-benzotriazin-3-yl)-methyl] Dithiophosphate (XII).** This polar metabolite was obtained as an alkali salt. Since EI-MS usually are not possible from salts, FD-MS were performed, which seemed more suitable for structure elucidation of such substances. The FD-MS contained peaks at m/e 23 (Na^+), m/e 348 ($M + Na^+$), m/e 364 ($M + K^+$), and m/e 674 ($2M + 1 + Na^+$). Methoxy and thiomethyl isomers were distinguished by ^{1}H FT-NMR, which contained a doublet at 2.10 ppm (coupling constant J_{PSCH_3} = 16 Hz) assigned exactly to a thiomethyl group attached to the phosphorus atom. Furthermore, a doublet at 5.77 ppm (J_{PSCH_2} = 15 Hz) and a multiplet at 8.1 ppm could be detected. The ratio of the peak areas is 3:2:4, corresponding to the number of protons.

***O*-Methyl *S*-[(4-Oxo-3*H*-1,2,3-benzotriazin-3-yl)-methyl] Dithiophosphate (XIII).** The highly polar compound XIII could only be separated in small concentrations. In analogy to metabolite XII, the chemical structure of this substance was elucidated from its FD-MS and ^{1}H FT-NMR spectra. The FD-MS contained, in addition to Na^+, K^+, and the known fragment at m/e 160, the base peak at m/e 348, which represents the sodium salt of compound XIII with a further addition of Na^+. Furthermore, peaks at m/e 364 ($M + K^+$) and m/e 673 ($2 M + Na^+$) were observed.

The isomer compound of XII is explained by its ^{1}H FT-NMR spectrum since, in contrast to the proton NMR spectrum of XII, a doublet at 3.33 ppm (J = 16 Hz) is detected that is assigned to a methoxy group on the basis of its chemical shift and peak areas (three protons). For the methylene group, an AB system centered at 5.33 ppm is observed, which is further splitted by the coupling with the phosphorus atom. Finally, the spectrum contains a multiplet centered at 8.2 ppm assigned to the four aromatic protons.

(4-Oxo-3*H*-1,2,3-benzotriazin-3-yl)methanesulfonic Acid (XIV). The highly polar, water-soluble compounds XII and XIV could not always be separated exactly by TLC. From this sulfonic acid no suitable mass spectra were obtained by the methods applied; however, from the ^{1}H FT-NMR spectrum the structure of this metabolite was proved by the existence of a singlet at 5.63 ppm besides the aromatic protons for the methylene groups. The positive FAB mass spectrum confirms this finding with peaks at m/e 264 (free sulfonic acid + Na^+), m/e 286 (sodium salt + Na^+), m/e 356 (264 + glycerine), and m/e 378 (286 + glycerine).

Benzamide (XV), Salicylic Acid (XVI), and Anthranilic Acid (XVII). The structures of these compounds (XVI and XVII as the methyl ester) were confirmed by EI-MS by comparison with those of authentic material.

Further Metabolites. Two polar metabolites from the aqueous extracts of soil with concentrations <0.35% of the radioactivity applied were obtained that could not yet be identified.

Microbial Degradation of Azinphos-methyl and Some of Its Transformation Products. The degradation products of azinphos-methyl in soil, 3-methyl-1,2,3-benzotriazin-4(3*H*)-one (VII), 3-(hydroxymethyl)-1,2,3-benzotriazin-4(3*H*)-one (IX), 3-[(methylthio)methyl]-1,2,3-benzotriazin-4(3*H*)-one (XI), 3-[(methylsulfinyl)-methyl]-1,2,3-benzotriazin-4(3*H*)-one (V), and 3-[(methylsulfonyl)methyl]-1,2,3-benzotriazin-4(3*H*)-one (IV), were subjected to degradation by *P. fluorescens* DSM 1976 to ascertain whether these benzazimide derivatives are also degraded to anthranilic acid by this bacterium as azinphos-methyl and to isolate intermediates formed before or during cleavage of these heteroaromatic compounds. No degradation was observed with 3-methyl- and 3-(hydroxymethyl)-1,2,3-benzotriazin-4(3*H*)-one whereas 3-[(methylthio)methyl]-1,2,3-benzotriazin-4(3*H*)-one (XI) and its sulfinyl and sulfonyl analogues are cleaved by this soil bacterium, thus forming anthranilic acid. From about 1 μmol/mL applied after a 9-day incubation period, 10% of compound XI, 65% of IV, and 75% of V were decomposed, respectively, forming anthranilic acid. No intermediates in the formation of anthranilic acid from these substrates were detected on TLC plates. From compound V a second metabolite was isolated and identified as its reduction product, 3-[(methylthio)methyl]-1,2,3-benzotriazin-4-(3*H*)-one (XI), by comparison of its TLC data, melting point, and mass spectrum with the reference substance. The formation of this metabolite and anthranilic acid from compound V during growth of a culture of *P. fluorescens* DSM 1976 is depicted in Figure 3.

DISCUSSION

The results obtained from $^{14}CO_2$ measurements and from the elucidation of metabolite structures clearly show that azinphos-methyl is mineralized in soil to a great extent, 3-(mercaptomethyl)-1,2,3-benzotriazin-4(3*H*)-one (VI) representing a key intermediate. Since mercaptan compounds are very reactive, this metabolite as well as the thioether 3-[(methylthio)methyl]-1,2,3-benzotriazin-4-(3*H*)-one (XI) could be isolated only in amounts below 1.0% of the starting radioactivity.

Compound XI may be oxidized rapidly by soil oxidases to the corresponding sulfinyl and sulfonyl derivatives V and IV, which amounted to 1.6 and 1.9% of the starting radioactivity, respectively, after a 197-day incubation period, the sulfinyl compound having a maximum of 3.2%

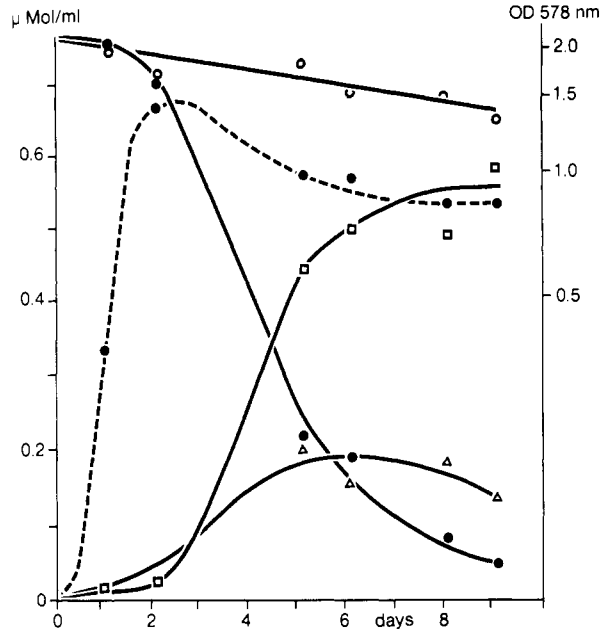

Figure 3. Degradation of 3-[(methylsulfinyl)methyl]-1,2,3-benzotriazin-4(3*H*)-one (V) by *P. fluorescens* DSM 1976. Symbols: (●---●) growth (OD_{578}); (○—○) degradation of compound V under sterile conditions; (●—●) bacterial degradation of compound V; (□—□) formation of anthranilic acid; (Δ—Δ) formation of 3-[(methylthio)methyl]-1,2,3-benzotriazin-4(3*H*)-one (XI).

of the starting radioactivity after 37 days. Both compounds were also found during soil degradation of azinphos-methyl, demonstrating that the thioether compound XI as well as the methylated sulfinyl and sulfonyl derivatives V and IV is derived via microbial transmethylation from the mercaptan VI.

Degradation studies of compounds XI, V, and IV with *P. fluorescens* DSM 1976 revealed that these main soil degradation products of azinphos-methyl are easily cleaved to anthranilic acid analogously to the parent insecticide. Since intermediates formed before or during cleavage of these heterocyclic compounds could never be detected, it is suggested that all four substrates applied for microbial degradation studies are cleaved directly by hydrolysis at the bond between the carbonyl group and N1 leading to anthranilic acid (Figure 4).

Since two benzoic acid derivatives, benzamide (XV) and salicylic acid (XVI), are isolated during decomposition of azinphos-methyl in soil, it is proved that the 1,2,3-benzotriazinone ring is cleaved in the soil. Therefore, these metabolites XV and XVI represent key intermediates of azinphos-methyl degradation in the soil matrix. Both compounds could be detected in the soil degradation studies with ^{14}C-labeled insecticide only in concentrations below 0.6% of the initial radioactivity and were isolated for identification by applying higher substrate concentrations. Anthranilic acid, the main bacterial cleavage product of azinphos-methyl and its intermediates isolated in soil (XI, V, and IV), was never found during soil degradation studies. This is presumably due to the strong polar character of this compound, which can be bound to the soil matrix, and to the fact that it is easily metabolized via catechol or gentisic acid by various soil bacteria (Kieslich, 1976). Salicylic acid is supposed to be formed in soil from anthranilic acid by hydroxyl replacement as was also shown for the microbial conversion of 5-hydroxyanthranilic acid to gentisic acid (Ladd, 1962). Additionally, low amounts of salicylic acid have also been detected on TLC plates during degradation of azinphos-methyl and the metabolites V and IV by *P. fluorescens*

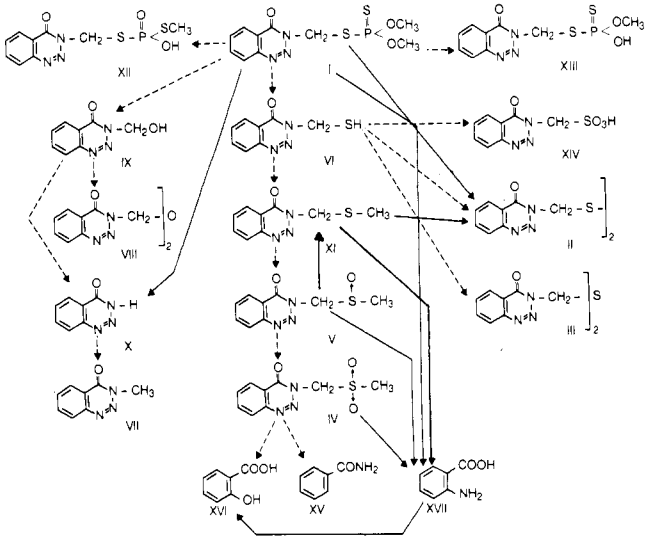

Figure 4. Metabolism of azinphos-methyl in soil and by *P. fluorescens* DSM 1976. Solid-line arrows: proposed pathway by *P. fluorescens*; broken-line arrows: proposed pathway in soil.

DSM 1976. Finally, the $^{14}CO_2$ elimination from [*ring*-U-^{14}C]azinphos-methyl demonstrates that the aromatic moiety of the insecticide molecule can be mineralized in soil (Figure 1).

The formation of the disulfide bis[3-(thiomethyl)-1,2,3-benzotriazin-4(3*H*)]one (II) in soil reached a maximum 67 days after insecticide application with 6.7% of the radioactivity added, which decreased to about 3.9% at day 197. Since compound II was also found as a main degradation product of azinphos-methyl in the microbial degradation studies (Engelhardt et al., 1981), its formation is assumed to result from enzymatic oxidative dimerization of the mercaptan derivative VI. The amounts of the monosulfide compound III isolated were about 0.6% after 197 days of incubation.

The concentrations of the highly polar compound *S*-methyl *S*-[(4-oxo-3*H*-1,2,3-benzotriazin-3-yl)methyl] dithiophosphate (XII) and 4-oxo-3*H*-1,2,3-benzotriazine-3-methanesulfonic acid (XIV) reached a maximum of 2.1% (added together) of the applied radioactivity after 164 days. Compound XII is assumed to be formed by transmethylation of the *S*-[(4-oxo-3*H*-1,2,3-benzotriazin-3-yl)-methyl] dithiophosphate, which could not be isolated, and of the sulfonic acid via microbial oxidation of the mercaptan VI and/or the disulfide II. Compound XIII, the "monodesmethylazinphos-methyl", was found only in very limited amounts (<1% of the applied radioactivity).

The alcohol, 3-(hydroxymethyl)-1,2,3-benzotriazin-4-(3*H*)-one (IX), identified in concentrations below 0.5% of the starting radioactivity, represents a product of hydrolysis of azinphos-methyl and is undoubtedly a precursor of 1,2,3-benzotriazin-4(3*H*)-one (X), which is formed from IX by cleavage of formaldehyde. The latter compound was found in soil extracts in concentrations below 0.2% of the starting radioactivity; it represents, however, one of the main degradation products by *P. fluorescens* DSM 1976 (Engelhardt et al., 1981).

The unpolar ether 3,3'-oxybis[methylene-1,2,3-benzotriazin-4(3*H*)-one] (VIII) is released from azinphos-methyl by hydrolysis and represents only a minor metabolite of this insecticide. 3-Methyl-1,2,3-benzotriazin-4(3*H*)-one (VII) is a relatively apolar heterocyclic compound found in soil in concentrations below 0.5% of the applied radioactivity and is certainly formed via microbial transmethylation from 1,2,3-benzotriazin-4(3H)-one (X, benzazimide).

On the basis of these findings, the pathway shown in Figure 4 is proposed for the degradation of azinphos-methyl in soil and by soil bacteria.

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Evaluation of Analytical Methods for the Determination of Residues of the Bee Repellent, Phenol, in Honey and Beeswax

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Reverse-phase high-performance liquid chromatographic (HPLC), fluorometric, and colorimetric methods (based on either the Gibbs or aminoantipyrine reaction) were evaluated for detection of phenol residues in honey and beeswax. All methods required an initial steam distillation prior to analysis. The most suitable method for phenol analysis in honey was found to be the reverse-phase HPLC method using 4-chlorophenol as an internal standard. Both colorimetric methods could also be used for phenol analysis in honey and were the methods of choice for analysis of phenol in beeswax.

The use of phenol, or carbolic acid, as a bee repellent began in the 1930s (*Bee World*, 1968). A concentrated solution (up to 90%) of pure crystalline phenol in water is applied to the adsorbent cloth on the underside of a special hive cover called a phenol board. On the upper surface of the phenol board is a black sheet of metal, which is in contact with the cloth.

The phenol is placed on the top of the hive and the heat from the sun vaporizes the phenol, which then has a repelling effect on the bees, therefore enabling the beekeeper to collect honey and/or beeswax with a minimum of interference. It has been reported that phenol and other bee repellents (*Bee World*, 1968) have been widely used, especially by commercial beekeepers, since honey can be rapidly collected with the assistance of relatively unskilled labor.

Careless use of phenol in collecting honey results in contamination with significantly high residues of phenol that impart a disagreeable medicinal taste to the honey. Since the application of phenol as a bee repellent causes contamination not only in the honey but also in the surrounding wax cells, it may be necessary to monitor phenol residues in beeswax. High levels of phenol in wax frames can be passed on to honey being collected. The potential risk of accumulation of phenol residues is acute because of the normal procedure for beekeepers to reuse empty wax frames. Many countries are considering greater regulation of chemicals, such as phenol, used in beekeeping. Increased regulation will require reliable analytical techniques for the detection of phenol residues in honey.

Sporns (1981) first introduced a procedure using high-performance liquid chromatography (HPLC) for the analysis of phenol in honey. In this paper, we will discuss and compare an alternate HPLC procedure based on reverse-phase separation, colorimetric (Gibbs, aminoantipyrine), and fluorometric techniques for the analysis of phenol in honey and beeswax. These methods are all capable of determining phenol at parts per million levels.

MATERIALS AND METHODS

Honey for recovery studies was obtained from a local beekeeper, who collected the honey without the use of a phenol board. The honey was checked by all methods presented in this paper and an earlier work (Sporns, 1981) and found to be free of detectable phenol.

Beeswax (laboratory grade) was obtained from Fisher Scientific Co. All water used in the analysis was purified by a Millipore Milli-Q system.

Steam Distillation. A honey or wax sample weighing 10 g was transferred to a Kjeldahl flask with 30 mL of water; for the HPLC procedure, 25 mL of distilled water and 5 mL of internal standard solution were used. The contents of the flask were steam distilled by using a Buchi Model 320 Kjeldahl distillation apparatus and the first 10 mL of the steam distillate was collected for analysis.

Cross contamination due to residual phenol in the distillation apparatus was prevented by steam distilling 40 mL of water in a clean Kjeldahl flask, until about 100 mL

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