Formation of Soil-Bound Residues of Cyprodinil and Their Plant Uptake

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The fungicide cyprodinil [4-cyclopropyl-6-methyl-2-(phenylamino)pyrimidine] labeled with ^{14}C in either the phenyl or the pyrimidyl ring was incubated with four different soils under various conditions to evaluate the formation of bound residues and their subsequent plant uptake. About 60% of the initially applied radioactivity was bound to nonsterile soils within 90−180 days, whereas negligible binding was observed under sterile and anaerobic conditions. More binding was observed at higher soil pH, cation exchange capacity, and organic carbon and nitrogen contents. When spring barley was grown in the methanol-extracted soil, the plant uptake of bound residues amounted to about 0.2% for the phenyl label and 1.2% for the pyrimidyl label. The difference indicated that the pyrimidyl moiety was detached from the cyprodinil molecule and taken up more readily.

Keywords: *Bound residues; plant uptake; cyprodinil; bioavailability; barley; fungicide*

INTRODUCTION

The formation of unextractable (bound) residues in soil is one of the key factors that must be considered in the evaluation of the impact of pesticides and other toxic chemicals on the environment (Katan et al., 1976; Roberts, 1984). The ability of soil to retain xenobiotics is attributed to adsorption phenomena and chemical reactions occurring on the active surfaces of mineral particles and humus. Due to binding, the mobility of organic compounds in the terrestrial system is greatly restricted and, consequently, xenobiotics exhibit reduced toxicity and bioavailability; they are also less likely to contaminate groundwater. Discussion continues regarding whether bound xenobiotics are retained permanently or can be released and become a long-term threat to the environment (Führ and Mittelstaedt, 1980; Khan, 1982; Dec and Bollag, 1988; Calderbank, 1989).

Use of ^{14}C-labeled chemicals combined with radiocounting has allowed quantification of xenobiotic binding under laboratory conditions and determination of the distribution of bound residues in the soil matrix. Through application of other analytical techniques, such as isothermal heating, supercritical fluid extraction, microwave sonication, and mass spectrometry (Helling and Krivonak, 1978a; Khan and Hamilton, 1980; Capriel et al., 1986; Scheunert et al., 1992; Koskinen et al., 1995; Nicollier and Donzel, 1994; Bollag, 1991), and recently by using ^{13}C- or ^{15}N- enriched compounds in combination with diverse NMR spectrometrical techniques (Thorn et al., 1996; Haider et al., 1993; Hatcher et al., 1993; Dec et al., 1997), essential progress has been made in obtaining structural information. Unfortunately, none of the above-mentioned methods was suitable for systematic monitoring of bound xenobiotics under field conditions.

Since the concentration of bound residues in cultivated soil could not be determined directly, it had to be estimated on the basis of the results of laboratory tests in which ^{14}C-labeled xenobiotics were incubated with soil under controlled conditions (Klein and Scheunert, 1982). These incubation studies were followed by tests for the release and plant uptake of bound residues (Khan, 1980; Führ and Mittelstaedt, 1980; Racke and Lichtenstein, 1985; MacRae, 1986; Dec et al., 1986; Dec and Bollag, 1988; Lee et al., 1991). Data obtained from such experiments were used for assessment of the risk related to binding.

In the present study, testing was carried out with cyprodinil [4-cyclopropyl-6-methyl-2-(phenylamino)pyrimidine], a new fungicide registered for the protection of a variety of crops against a broad spectrum of plant diseases. The objectives of this investigation were (1) to determine the effect of soil properties and incubation conditions on the rate of cyprodinil binding, (2) to characterize the extractable transformation products and to determine the distribution of bound residues in soil, and (3) to determine the bioavailability of the bound material in different soils using barley as a test plant.

MATERIALS AND METHODS

Chemicals. Unlabeled cyprodinil and ^{14}C-labeled cyprodinil were provided by Ciba-Geigy Ltd. The labeled carbons were located either in the phenyl ring (uniformly labeled) or at the 2-position of the pyrimidyl moiety (Figure 1). The specific radioactivity of both the U-phenyl- and 2-pyrimidyl-^{14}C-labeled cyprodinil was 1.85 MBq/mg.

Soils. The incubation experiments were carried out using four soils designated Hagerstown, California, Collombey, and Les Evouettes soils. Their physical and chemical properties are summarized in Table 1. The soils were stored in a greenhouse, overgrown with turf grass, and watered as needed. Before treatment and incubation, the soils were sieved through a 2-mm screen, adjusted to 40% of maximum water holding capacity (WHC), and equilibrated at room temperature for 10 days.

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S0021-8561(96)00532-8 CCC: $14.00 © 1997 American Chemical Society

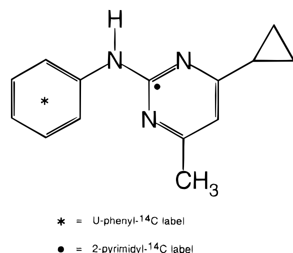

Figure 1. Chemical structure of cyprodinil labeled with ^{14}C either uniformly in the phenyl ring or at the C-2 position of the pyrimidyl ring.

Incubation of Cyprodinil in Soil. Soil samples (200 g dry weight) were distributed in 1-L Erlenmeyer flasks and thoroughly mixed with ^{14}C-labeled cyprodinil dissolved in 0.6 mL of acetone. Control samples were amended with the same amount of acetone, but no [^{14}C]cyprodinil was added. The initial concentration of the fungicide in treated samples was 3.0 ppm for Hagerstown and California soils and 1.5 ppm for Collombey and Les Evouettes soils. The contributions of the ^{14}C-labeled compound to the total concentrations were 2.7 and 1.2 ppm, respectively. The remainder was contributed by nonlabeled cyprodinil. The initial radioactivity per incubation flask was 1.0 MBq (Hagerstown soil) and 0.44 MBq in the other soils, respectively. After readjustment of soil moisture to 40% WHC, the incubation flasks were wrapped in aluminum foil and inserted into air-flow systems operating under slight vacuum. The systems were incubated at 25 °C.

Two traps with 4 N NaOH to remove CO_2 from the incoming air and one trap with water for moistening the soil were placed on the inlet of the incubation flask. The outlet was followed by one 1 N H_2SO_4 trap and one ethylene glycol trap to adsorb organic volatiles, one empty trap, and three 0.5 N NaOH traps for adsorption of respiratory CO_2 and $^{14}CO_2$ resulting from mineralization of ^{14}C-labeled cyprodinil.

Some incubations with [*2-pyrimidyl*-^{14}C]cyprodinil in Les Evouettes soil were carried out under sterile or anaerobic conditions. For sterile conditions, the acetone solution of the fungicide to be mixed with soil was passed through an autoclaved 0.45-μm pore size membrane (Millipore Corp., Bedford, MA). Both the soil and the entire air-flow systems were sterilized by autoclaving, and the incoming air was passed through autoclaved 0.45-μm membranes.

Anaerobic conditions were created after 16 days of aerobic incubation by flooding the soil with 200 mL of water and replacing the air with nitrogen. The incubations for different labels and incubation conditions were carried out in duplicate for 197 days (Hagerstown soil), 239 days (California soil), 154–180 days (Collombey soil), and 363–366 days (Les Evouettes soil). Trapping solutions were analyzed periodically by radiocounting and titration with HCl according to the procedure of Stotzky (1965). Soil samples (1 g dry weight) taken after the specific incubation periods were extracted six times by 1-min vortexing with 3 mL of methanol or acetonitrile, centrifuged, and analyzed for extractable and bound radioactivity.

Fractionation of Soil-Bound Residues. Samples of Hagerstown and Les Evouettes soils (10 g dry weight) from the 197- or 363-day incubations with 3.0 and 1.5 ppm, respectively, of [U-*phenyl*-^{14}C]cyprodinil or [*2-pyrimidyl*-^{14}C]cyprodinil were extracted four times by shaking for 1 h with 30-mL portions of methanol. The methanol extract was analyzed by thin-layer chromatography (TLC), radioscanning, and mass spectrometry (MS). After air-drying overnight, the soil was extracted for 24 h by shaking with 50 mL of 0.5 N NaOH under nitrogen. The NaOH extract was separated by centrifugation, and the soil was washed three times with 0.1 N NaOH. The combined solution (extract plus washings) was acidified to pH <1 with 5 N HCl, stored overnight at 4 °C for complete precipitation of humic acid, and centrifuged. The supernatant with unprecipitated fulvic acid was extracted three times with methylene chloride, and the extract was analyzed by TLC and radioscanning. The humic acid precipitate was washed three times with acidified water (pH <1; the washings were combined with fulvic acid for extraction with methylene chloride as described above) and redissolved in NaOH solution for radiocounting.

Plant Uptake Experiments. Plant uptake of bound and/or free residues of [^{14}C]cyprodinil was studied using Hagerstown, Les Evouettes, and Collombey soils. The investigation was performed in plastic pots (5.0–7.5-cm diameter; 7.0-cm height) filled with a 1:4 mixture of soil (25 g) and clean silica sand (100 g). After 3 days of seed germination, four plants of spring barley (cultivar Roder) were transferred into each pot. The pots were amended with fertilizer equivalent to 200 mg of N, 70 mg of P, and 100 mg of K, Hoagland trace element solution (5 mL), and 15 mL of soil extract. The experiments were carried out in triplicate for each of three soil variants.

Variant I: Soil was incubated for 154, 197, and 366 days (Collombey, Hagerstown, and Les Evouettes, respectively) with 1.5 ppm (Collombey and Les Evouettes) or 3.0 ppm (Hagerstown) of cyprodinil labeled with ^{14}C at the phenyl or pyrimidyl ring. Afterward, it was exhaustively extracted with methanol (six times for 1 h by shaking and for 24 h by Soxhlet extraction) before use in the plant uptake study.

Variant II: Soil was incubated as in variant I but was not extracted before use in the plant uptake study.

Variant III: Soil was treated (fortified) with 3 ppm of cyprodinil labeled with ^{14}C at the phenyl or pyrimidyl ring immediately before use in the plant uptake study.

The initial radioactivities determined in the extracted, unextracted, and freshly fortified soils are listed in Table 2. In control experiments, barley plants were grown in pots filled with clean silica sand or with mixtures of sand and methanol-extracted or unextracted soil that were not treated with cyprodinil.

All pots were put in Petri dishes to collect leachate after watering, placed in a growth chamber with controlled temperature (20 °C) and humidity (60%) for a period of 5 weeks, and watered daily as necessary. During barley growth, 12-h intervals of light and dark were applied. The plants were harvested at the shooting stage, 35 days after germination. At harvest, the shoots of barley were cut close to the soil surface, and the roots and seed shells were carefully separated from soil by shaking and washing on a sieve with tap water. After 24 h of drying at 60 °C, the plant materials were weighed and, after milling with a 20-mesh screen (Arthur H. Thomas Co., Scientific Apparatus, Philadelphia, PA), were analyzed for radioactivity.

After the experiment, Hagerstown soils from the three variants were analyzed for remaining radioactivity and then extracted with methanol to determine extractable and bound radioactivity. The methanol extracts were analyzed by TLC and radioscanning.

TLC, Mass Spectrometry, and Radiocounting. TLC analysis was carried out on 0.25-mm silica gel $60F_{254}$ precoated plates (E. Merck, Darmstadt, Germany) using a 90:10 toluene/methanol or a 90:10:1 chloroform/ethanol/acetic acid solvent system (Tables 3 and 7). Chemical ionization (CI) mass spectra were obtained with a Finnigan 4500 mass spectrometer using either a direct exposure probe or a gas chromatograph.

Radioactivity in liquid samples (e.g., methanol extracts from soil, NaOH solutions with adsorbed $^{14}CO_2$, aqueous solutions of humic and fulvic acids, CH_2Cl_2 extracts from unprecipitated fulvic acids, humic acid pellets redissolved in NaOH solution) was analyzed by liquid scintillation counting on a Beta Trac 6895 liquid scintillation counter (Tracor Analytic, Elk Grove, IL). Soil samples before and after methanol extraction (0.3 g) as well as plant materials from the plant uptake study (0.03–0.15 g) were combusted in a Harvey Biological Oxidizer OX 600 (Hillsdale, NJ) using Harvey Carbon 14 Cocktail for adsorption and counting of $^{14}CO_2$. Radioscanning of TLC plates was carried out on a System 200 Imaging Scanner (Bioscan, Washington, DC).

RESULTS

Formation of Bound Residues in Various Soils. The disappearance, binding, and mineralization of cyprodinil differed with the type of soil, location of the ^{14}C label, and incubation conditions. Under aerobic conditions, the rates of bound residue formation in Les

Table 1. Physicochemical Properties of Soils

characteristic	soil			
	Hagerstown	California	Collombey	Les Evouettes
sand (%)	27.60	74.00	81.40	26.90
silt (%)	43.00	19.00	13.50	59.70
clay (%)	29.40	7.00	5.10	13.40
pH	5.70	5.00	7.20	7.20
organic C (%)	1.59	1.00	2.20	1.95
N (%)	0.13	0.06	0.27	0.25
CEC[a] (mequiv/100 g)	11.10	7.00	14.95	13.70
textural class	clay loam	sandy loam	loamy sand	silt loam
soil classification[b]	Typic Hapludalf	Noncalcic brown Ferrosol	Mollic/Aquic Udifluvent	Mollic/Aquic Udifluvent

[a] Cation exchange capacity. [b] Soil Survey staff, U.S.A.

Table 2. Initial Radioactivities in Mixtures of Three Different Soils with Silica Sand Used in the Plant Uptake Study

soil	^{14}C label	radioactivity, MBq/pot		
		extracted	unextracted	fortified
Hagerstown	U-phenyl	0.043	0.111	0.070
	2-pyrimidyl	0.044	0.112	0.072
Collombey[a]	U-phenyl	0.093	0.098	0.070
	2-pyrimidyl	0.089	0.098	0.072
Les Evouettes[a]	U-phenyl	0.043	0.040	0.070
	2-pyrimidyl	0.040	0.034	0.072

[a] Samples of the extracted and unextracted soil originated from different incubation experiments; therefore, radioactivities in the extracted soils could be approximately the same as or even greater than those in the unextracted soils.

Table 3. TLC Analysis: Distribution of Radioactivity of Acetonitrile Extracts from Collombey Soil Incubated with 1.5 ppm of [*2-pyrimidyl*-^{14}C]Cyprodinil[a]

days	R_f[b]				
	0.01 unk[c]	0.15 2-hydroxy-4-cyclopropyl-6-methyl-pyrimidine	0.31 2-amino-4-cyclopropyl-6-methyl-pyrimidine	0.50 di-4-cyclopropyl-6-methyl-pyrimidine ether	0.78 cyprodinil
0					99.3
3	0.6		2.1		84.5
6	1.0		3.5		77.2
10	1.7		5.9		62.2
14	1.5		6.2		64.3
20	2.0	0.4	7.3	0.4	55.8
28	2.1	0.5	7.9	0.3	43.6
45	2.1	1.2	9.4	0.5	31.6
61	2.2	2.0	9.0	0.7	23.0
90	2.1	3.7	7.6	0.4	11.5
122	2.1	4.3	5.0	0.5	7.1
180	2.1	4.1	2.9	0.3	4.8

[a] Values represent the percent of the initially applied radioactivity. [b] Plates were developed in a 90:10 toluene/methanol solvent system. [c] Unknown.

Evouettes and Collombey soils were greater than those in Hagerstown and California soils (Figure 2). For example, a maximal binding in Les Evouettes soil treated with [U-*phenyl*-^{14}C]cyprodinil (58.3% of the initially applied radioactivity) occurred by 62 days of incubation. In Hagerstown soil, a somewhat lower percentage of binding (54.1%) was found by 197 days; however, the slope of the time course curve indicated that maximal binding had not yet been achieved. A similar relationship was observed for the 2-pyrimidyl label (data not shown).

Approximately 100 and 150 days were required for the extractable radioactivity to be reduced by 50% in Hagerstown and California soils, respectively, whereas in Les Evouettes and Collombey soils, the 50% reduction was observed by only 40 days. Figure 2 indicates that only 6.3−24.7% of ^{14}C from the applied cyprodinil was converted into CO_2 and more than 50% into nonextractable residues.

The $^{14}CO_2$ production in Les Evouettes and Collombey soils (24.7 and 16.5%, respectively) was 1.5−4-fold greater than that in Hagerstown and California soils. The mineralization of [*2-pyrimidyl*-^{14}C]cyprodinil in Les Evouettes soil (24.4%) and Collombey soil (13.4%) differed only slightly (data not shown) from that of the U-phenyl-labeled fungicide. However, in Hagerstown and California soils, the transformation of [*2-pyrimidyl*-^{14}C]cyprodinil to $^{14}CO_2$ (data not shown) was 3−5-fold less (2.7 and 1.7%, respectively) than that of the U-phenyl-labeled compound.

These variations in the rates of cyprodinil transformation may be attributed to differences in the soil properties (Table 1). For instance, the pH values of Hagerstown and California soils (5.7 and 5.0, respectively) differed notably from that of Les Evouettes and Collombey soils (7.2). Further differences were found in organic carbon contents (1.6 and 1.0% vs 2.0 and 2.2%, respectively), nitrogen contents (0.13 and 0.06% vs 0.25 and 0.27%, respectively), and cation exchange capacity (11.1 and 7.0 mequiv/100 g vs 13.7 and 15.0 mequiv/100 g, respectively).

Limited binding (3.4%) and insignificant evolution of $^{14}CO_2$ (0.02%) were observed in Les Evouettes soil incubated for 90 days with [*2-pyrimidyl*-^{14}C]cyprodinil under sterile conditions (data not shown). Under nonsterile conditions, the 3.4 and 0.02% values were already exceeded after 3 days of incubation. These findings clearly indicated that both binding and mineralization of cyprodinil greatly depended on the activity of soil microorganisms.

The process of binding in Les Evouettes soil treated with [*2-pyrimidyl*-^{14}C]cyprodinil was virtually stopped when, after 16 days of aerobic incubation, anaerobic conditions were created by flooding the soil and replacing air with nitrogen (data not shown). On day 16, the percentage of binding and $^{14}CO_2$ evolution amounted to 27.5 and 1.6%, respectively. Throughout the 104-day period of anaerobic incubation that followed, the amount of bound residue increased to only 33.1%, and no increase in $^{14}CO_2$ evolution was determined. These data suggest that the anaerobic microbial population present could not metabolize cyprodinil.

Analysis of Extractable and Bound Residues. As found by TLC and radioscanning, the methanol or acetonitrile extracts of soil samples taken periodically from the incubation flasks contained mainly unchanged cyprodinil and minor quantities of transformation products (0.3−9.4% of the initial radioactivity). The unchanged cyprodinil was detected at R_f 0.78 (Table 3); its structure was confirmed by CI mass spectrometry (m/z 241). Table 3 presents the distribution of radio-

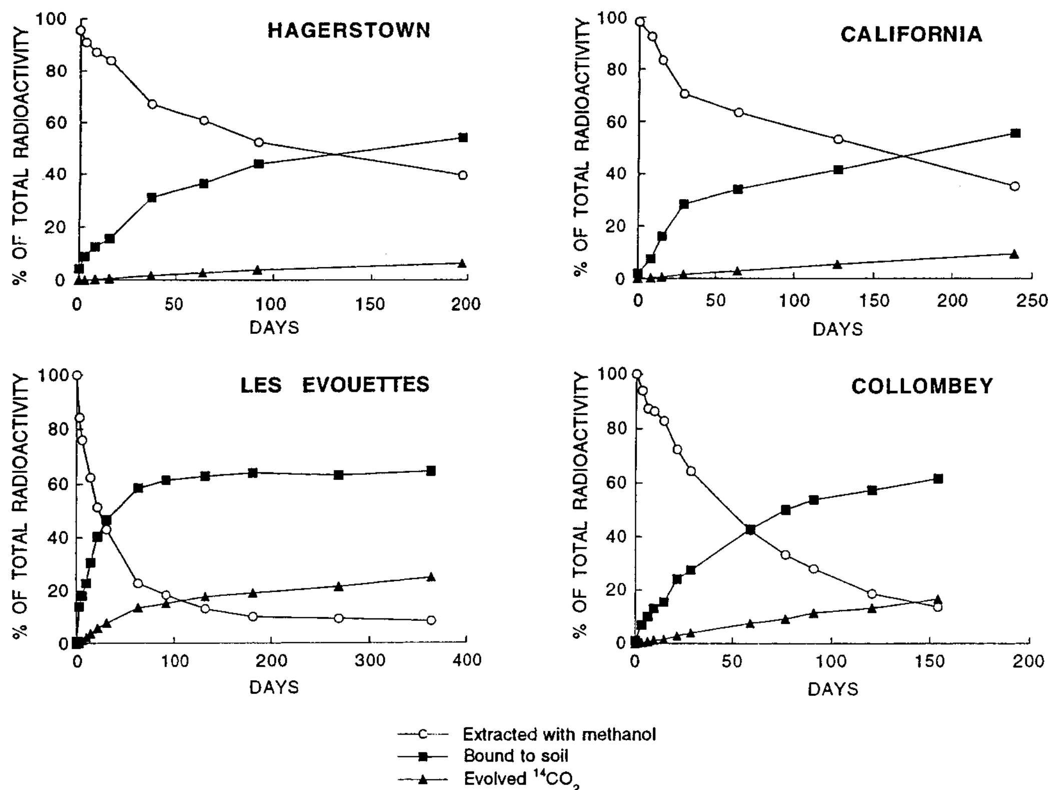

Figure 2. Binding of [U-*phenyl*-^{14}C]cyprodinil in four different soils. The soils were incubated with 1.5 ppm (Hagerstown and California) or 3 ppm (Les Evouettes and Collombey) of the fungicide. The standard deviation for the radioactivity extracted with methanol ranged between 0.3 and 4.8%, bound to soil between 0.3 and 5.0%, and evolved as $^{14}CO_2$ between 0.01 and 0.11%.

activity (2-pyrimidyl label) during TLC analysis of the organic solvent extracts from Collombey soil sampled after specific incubation times.

Cochromatography with known standards indicated that the radioactive spots (2-pyrimidyl label) at R_f 0.15 and 0.31 represented two cleavage products of the cyprodinil molecule: 2-hydroxy- and 2-amino-4-cyclopropyl-6-methylpyrimidine. Chemical structures of both metabolites were confirmed by CI mass spectrometry. The mass spectrum for the amino derivative showed the protonated molecular ion *m*/*z* 150. For the hydroxy derivative, the protonated molecular ion *m*/*z* 151 was detected in the positive registration mode with CH_4 and NH_3 as reactant gases. The corresponding spectrum in the negative mode showed the molecular ion *m*/*z* 150.

On the basis of the mass spectrum showing the molecular ion *m*/*z* 283 (positive CI mode with CH_4 and NH_3), the spot at R_f 0.50 appeared to represent a dimer with an oxygen bridge between two pyrimidine rings. The spot at R_f 0.01 seemed to represent a mixture of various transformation products, and the MS analysis was inconclusive. Also, due to low concentrations of the U-phenyl-^{14}C-labeled transformation products, no chemical structures could be proposed on the basis of MS analysis.

After extraction with methanol or acetonitrile, Hagerstown and Les Evouettes soils containing only bound residues of cyprodinil (labeled either in the phenyl or pyrimidyl ring) were extracted with 0.5 NaOH, which removed extractable humic material together with humus-bound radioactivity. In the case of the 2-pyrimidyl label, approximately half of the bound radioactivity (50.4 and 42.2%, respectively) remained unextracted in the humin fraction (Table 4). Upon acidification with 5 N HCl, 13.9 and 24.1% of the bound radioactivity was found in the humic acid precipitates. The remainder was found in the unprecipitated fulvic acid (20.0 and 22.4%) and in the methylene extract from fulvic acid

Table 4. Percentage Distribution of Radioactivity upon Fractionation of Methanol- or Acetonitrile-Extracted Soils Containing Bound Residues of [*2-pyrimidyl*-^{14}C]Cyprodinil

fraction	Hagerstown soil	Les Evouettes soil
extracted soil	100.0	100.0
humin	50.4	42.2
humic acid	13.9	24.1
fulvic acid (FA)	20.0	22.4
CH_2Cl_2 extract from FA	12.9	2.0

(12.9 and 2.0%). The results of fractionation for the phenyl label were very similar (data not shown). The fractionation patterns differed with the type of soil. As shown in Table 4, the percentage of radioactivity bound to humic acid from Les Evouettes soil was almost twice as great as that found in humic acid from Hagerstown soil. On the other hand, about 6 times more radioactivity was extracted from the Hagerstown fulvic acid than from the Les Evouettes fulvic acid.

After TLC analysis of the methylene chloride extracts from the Hagerstown fulvic acid, four major radioactive spots (2-pyrimidyl label) were detected, at R_f 0.05, 0.43, 0.56, and 0.80 (Figure 3). According to cochromatography with the cyprodinil standard, the spot at R_f 0.80 represented the unchanged fungicide. Only three spots, at R_f 0.05, 0.56, and 0.80, were detected for the U-phenyl label (Figure 3), indicating that the spot at R_f 0.43 represented a cleavage product, probably 2-amino-4-cyclopropyl-6-methylpyrimidine as suggested by cochromatography with the known standard. The products detected at R_f 0.05 and 0.56 for both the phenyl and pyrimidyl label appeared to be derivatives of the intact cyprodinil molecule.

Plant Uptake. Results of the investigation into plant uptake of bound and/or free residues of ^{14}C-labeled cyprodinil are summarized in Table 5. The total uptake by plants grown in soil extracted with organic solvents

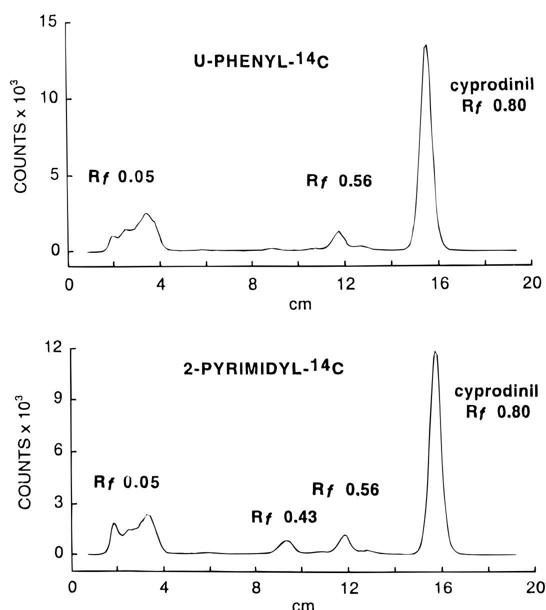

Figure 3. Radioscanning of TLC plates after analysis of the methylene chloride extract of fulvic acid from Hagerstown soil incubated with 3 ppm of [U-*phenyl*- or *2-pyrimidyl*-^{14}C]cyprodinil. Plates were developed in a 90:10:1 chloroform/ethanol/acetic acid solvent system.

(variant I) was 0.21−0.24% of the initially bound radioactivity for the U-phenyl label and 0.61−1.15% for the 2-pyrimidyl label. From 1.5 to 4 times more radioactivity was taken up by plants grown in the unextracted soils (variant II), and from 3 to 9 times more radioactivity was taken up from the soils freshly treated with ^{14}C-labeled cyprodinil (variant III). In each variant, the uptake for the pyrimidyl label was 2−5-fold times greater than that for the phenyl label.

Among the extracted soils, most of the originally bound U-phenyl radioactivity was found in barley shoots (0.16−0.19%), about 3 times less was found in the roots (0.05−0.06%), and only traces were found in the seed shells (0.004−0.01%). The respective values for the pyrimidyl label were 0.57−1.06, 0.03−0.08, and 0.004−0.01%. Similar relationships were observed for the unextracted and freshly fortified soils (Table 5). The average weights (dry weight per pot) of the shoots, roots, and seed shells were 0.30, 0.11, and 0.04 g, respectively.

After the plant uptake study, the average concentration of [^{14}C]cyprodinil residues (for phenyl and pyrimidyl label) in Hagerstown soil (Table 6) was reduced by about 16% (methanol-extracted soil), 35% (unextracted soil), and 25% (fortified soil) relative to the radioactivity determined before the experiment. The percentage reduction exceeded the plant uptake, indicating that during watering significant amounts of radioactivity (13.0−35.6%) were lost due to leaching from the pots into the Petri dishes underneath.

Apparently, mobilization of the initially bound radioactivity occurred due to growth of the plants, because after the experiment, about 0.002 MBq (5% of the initial radioactivity) could be further extracted with methanol from Hagerstown soil used in variant I. On the other hand, a significant part (about 40%) of the free radioactivity added to soil immediately before the experiment (variant III) became bound to soil (0.03 MBq). In variant II soil (unextracted), mobilization of the bound residues and binding of the free radioactive chemicals must have simultaneously occurred since bound radioactivity determined after plant uptake (0.047 MBq) was approximately equal to that which was bound before plant uptake in the variant I experiment (0.043 MBq).

In the case of the 2-pyrimidyl label, four major radioactive spots were detected on TLC plates for each of the three experimental variants upon analysis of the methanol extracts (Table 7). According to cochromatography with known standards, three of the spots represented unchanged cyprodinil (R_f 0.74) and its cleavage products, 2-hydroxy- (R_f 0.31) and 2-amino-4-cyclopropyl-6-methylpyrimidine (R_f 0.44). Only two spots, at R_f 0.02 (unknown) and 0.74 (cyprodinil), were detected for the U-phenyl label, confirming that the spots at R_f 0.31 and 0.44 represented cleavage products containing the pyrimidyl moiety.

DISCUSSION

The formation of bound residues of cyprodinil in Les Evouettes soil under sterile or anaerobic conditions was negligible compared with the extensive binding observed in the nonsterile soil under aerobic conditions (Figure 2). Similar observations were made for many other xenobiotics tested in different soils (Klein and Scheunert, 1982; Khan, 1982; Calderbank, 1989). On the basis of these findings, binding may be attributed to the activity of aerobic microorganisms capable of transforming the xenobiotics to more reactive derivatives that eventually are incorporated into the soil matrix. Bound residues of many urea, anilide, and phenoxy herbicides, for instance, were found to be formed via microbial transformation of the active ingredients to chloroanilines or chlorophenols (Hsu and Bartha, 1976; Stott et al., 1983). The involvement of microbes in binding was demonstrated also by Katan and Lichtenstein (1977) using [^{14}C]parathion. However, there are known instances of abiotic binding, such as immobilization of anilazine in soil through ligand exchange (Haider et al., 1993) or the entrapment of prometryn in the molecular net of humus (Khan, 1982).

Binding of cyprodinil appears to be controlled by a mixed mechanism involving both biotic and physical interactions with soil constituents. According to recent NMR studies (Dec et al., 1997), the formation of soil-bound residues of cyprodinil was mostly due to biotic cleavage of the fungicide molecule between the phenyl and pyrimidyl rings followed by binding of each moiety to humic acid. This type of biotic cleavage is also indicated by the present study. A certain portion of the bound radioactivity, however, consisted of unaltered fungicide, which was retained or sequestered by physical forces and upon fractionation of the soil matrix could be extracted from fulvic acid with methylene chloride (Table 4 and Figure 3). Unaltered cyprodinil (R_f 0.74) was also found in the methanol extract from soil after the plant uptake study (Table 7). In addition to cyprodinil, the sequestration involved minor quantities of its transformation products (Figure 3), including 2-amino-4-cyclopropyl-6-methylpyrimidine (spot at R_f 0.43) and two other metabolites (spots R_f 0.05 and 0.56), which have not been identified.

It should be noted that the abiotic sequestration of the unchanged fungicide accounted for $<$12.9% of the nonextractable radioactivity (Table 4); the majority of the applied radioactivity was either mineralized (especially in Les Evouettes and Collombey soils) or bound in the form of transformation products resulting from the activity of microorganisms. Relatively small amounts of the free metabolites (9.4% at most) were extracted from soil with methanol or acetonitrile at various incubation times, which suggested that the transformation products were bound and mineralized almost immediately after being formed. Characteristically, after

Table 5. Uptake of Radioactivity by Barley Plants from Methanol-Extracted and Unextracted Soils Incubated Previously with ^{14}C-Labeled Cyprodinil and from the Soils Fortified with the Labeled Compound Immediately before the Experiment

		uptake of radioactivity[a] (%)											
		Hagerstown soil				Les Evouettes soil				Collombey soil			
^{14}C label	soil variant	shoots	roots	seed shells	total	shoots	roots	seed shells	total	shoots	roots	seed shells	total
phenyl	extracted (I)	0.16	0.06	0.01	0.23	0.19	0.05	0.004	0.24	0.16	0.05	0.004	0.21
	unextracted (II)	0.79	0.13	0.02	0.94	0.32	0.07	0.004	0.39	0.24	0.07	0.005	0.32
	fortified (III)	0.82	0.37	0.04	1.23	1.02	0.15	0.057	1.23	1.41	0.52	0.063	1.99
pyrimidyl	extracted (I)	0.57	0.03	0.01	0.61	0.66	0.07	0.004	0.73	1.06	0.08	0.009	1.15
	unextracted (II)	1.72	0.18	0.03	1.93	1.28	0.16	0.008	1.45	1.67	0.10	0.010	1.78
	fortified (III)	2.16	0.29	0.05	2.50	2.32	0.22	0.060	2.60	2.73	0.41	0.070	3.21

[a] The SD for shoots ranged from 0.02 to 1.0%; the SD for roots ranged from 0.01 to 0.13%;and the SD for seed shells ranged from 0.001 to 0.009%.

Table 6. Radioactivities in Mixtures of Hagerstown Soil with Silica Sand before and after the Plant Uptake Study

		radioactivity, MBq/pot			
^{14}C label	soil variant	before plant uptake	after plant uptake	methanol extract	bound after plant uptake
U-phenyl	extracted (I)	0.043	0.035	0.002	0.030
	unextracted (II)	0.111	0.075	0.018	0.047
	fortified (III)	0.070	0.051	0.030	0.027
2-pyrimidyl	extracted (I)	0.044	0.038	0.003	0.032
	unextracted (II)	0.112	0.070	0.021	0.047
	fortified (III)	0.072	0.056	0.024	0.024

Table 7. TLC Analysis: Distribution of Radioactivity of Methanol Extracts from the Mixtures of Hagerstown Soil and Silica Sand after the Plant Uptake Study[a]

		R_f[b]			
soil variant	^{14}C label	0.02	0.31	0.44	0.74
extracted (I)	U-phenyl	7.7			89.6
	2-pyrimidyl	1.8	1.2	19.1	77.0
unextracted (II)	U-phenyl	4.2			91.0
	2-pyrimidyl	2.7	2.2	14.9	77.9
fortified (III)	U-phenyl	2.5			95.5
	2-pyrimidyl	1.8	0.1	7.3	90.7

[a] Values represent the percentage of extracted radioactivity. [b] Plates were developed in 90:10:1 chloroform/ethanol/acetic acid solvent system.

60 days of incubation, the percentage of binding in Les Evouettes soil (about 58%) ceased to increase, but evolution of $^{14}CO_2$ continued (Figure 2). It is not clear whether the additional $^{14}CO_2$ originated from the bound radioactivity with the losses being compensated for by further binding of the remaining extractable residues or whether only the latter were mineralized.

In view of the complexity of the binding mechanism, it was imperative to evaluate the bioavailability of the bound substances. As determined in the plant uptake study, no more than 1.15% of the soil-bound residues were absorbed by the barley plants and constituted mostly cleavage products of the original fungicide (Table 5). The plant uptake ranged from 0.2 to 3.2% of the initial radioactivity depending on the type of soil, experimental variant, and location of the ^{14}C label.

These results are in agreement with previous findings. Führ and Mittelstaedt (1980), for instance, determined that the availability of soil-bound residues of [^{14}C]methabenzthiazuron to maize plants amounted to 1.0%, which was about 3 times less than the uptake from soil containing both bound and extractable residues and 5 times less than the uptake from soil freshly treated with the radioactive compound. This proportion of the respective uptakes (1:3:5) was similar to ratios determined in the present research for the phenyl and pyrimidyl labels using Hagerstown soil (1:4:5 and 1:3:4, respectively). In studies of Fuhremann and Lichtenstein (1978), the ratio of the uptake from soil containing only bound residues of [^{14}C]parathion to the uptake from soil freshly treated with the radioactive compound was 1:5. The same relationship was determined by Helling and Krivonak (1978b) in a work with six dinitroaniline herbicides.

As in the present investigation, in studies by Khan (1980), Azam et al. (1986), Fuhremann and Lichtenstein (1978), and Lee et al. (1991) using ^{14}C-labeled prometryn, malathion, parathion, and carbofuran, respectively, most of the absorbed radioactivity was found in the shoots, while the least was detected in the seed shells of the test plants. However, in several other investigations involving ^{14}C-labeled carbaryl, 1-naphthol, carbofuran, and chlorfenvinphos (Murthy and Raghu, 1988; El Zorgani et al., 1986; Dec et al., 1986), slightly more radioactivity remained in the roots or vegetable bulbs than was translocated to the shoots.

Under all three experimental variants, the barley plants removed from soil significantly more 2-pyrimidyl-^{14}C-labeled material than U-phenyl-^{14}C-labeled residues. This led to the conclusion that, after cleavage of cyprodinil, mainly the pyrimidyl moiety was subject to uptake, whereas much of the phenyl moiety was retained in the soil by physical or chemical interactions. The contribution of the pyrimidine cleavage products to uptake was confirmed by TLC analysis of methanol extracts from soils in which barley plants had been grown (Table 7). By means of cochromatography with known standards, two radioactive compounds detected for the pyrimidyl label at R_f 0.31 and 0.44 were identified as 2-hydroxy- and 2-aminopyrimidine, respectively. The presence of the pyrimidine products was also suggested by the fact that no radioactive spots were detected at the above R_f values for the phenyl label.

As shown in Table 6, 4.6 and 6.8% (U-phenyl and 2-pyrimidyl label, respectively) of the originally bound radioactivity was extracted from Hagerstown soil with methanol after the plant uptake experiment (variant I). Apparently, the extractable material was released due to the activity of microorganisms whose growth was stimulated by the development of the root system. It is noteworthy that, although as much as 4.6 and 6.8% of bound radioactivity (U-phenyl and 2-pyrimidyl label, respectively) was released and thus made available for uptake, only 0.2 and 0.6%, respectively, was eventually absorbed by the barley plants. A similar pattern of plant uptake, limited to no more than $^1/_{10}$ of the available residues, was also observed for variants II and III. There is no doubt that the barley plants possessed sufficient capacity to accommodate much more radio-

activity than they did; otherwise, the percentage of uptake would not increase with the increasing amounts of radioactivity available in variants II and III (Tables 5 and 6). This limitation upon uptake may be ascribed to adsorption phenomena in the soil. In addition, in the case of variant III, significant amounts of cyprodinil and its transformation products became firmly bound to soil and could not be extracted even with methanol. The above observations confirm that immobilization processes of xenobiotics in soil greatly diminish their bioavailability to plants and microbes, as was emphasized recently by Alexander (1995).

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Received for review July 17, 1996. Accepted November 20, 1996.[⊗]

JF960532S

[⊗] Abstract published in *Advance ACS Abstracts,* January 15, 1997.