Microbial Metabolism and Further Fate of Bentazon in Soil

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The transformation of the herbicide bentazon in soil was investigated with radiolabeled bentazon and its derivatives 8-hydroxybentazon, 8-methoxybentazon, and *N*-methylbentazon. Mineralization of bentazon was a microbial activity which required the presence of oxygen and was impeded by additional carbon substrates such as powdered alfalfa (*Medicago sativa*) residues. The formation of bound residues of bentazon in humic substance depended on microbial activity, too, and was stimulated by the presence of oxygen; however, there was also a significant immobilization of bentazon in the absence of oxygen. A culture of the fungus *Phanerochaete chrysosporium* converted 8-hydroxybentazon to a dimeric derivative. The same dimer was formed by the action of laccase. This dimer and the monomeric 8-OH-bentazon acted as key metabolites in the formation of bound residues from bentazon. The further mineralization of bound bentazon residues was a slow process, with 9.3% mineralization within 141 d.

Introduction

Our knowledge on the fate of the herbicide bentazon in soils is scarce and partly contradictory, especially with respect to the time scales of mineralization and immobilization in soil. Half-lives of bentazon in soils, based on substrate disappearance measurements, have been estimated to be in the range of 5–10 days (1–3), but studies based on measurement of CO₂ release from radiolabeled tracers reported mineralization rates of only 3–9% in 105 days (4). Concerning the degradation mechanisms involved, photooxidative dimerization is only of minor importance in soils (5). Instead, the main transformation of bentazon in soil appears to be caused by microbial activity. Nonetheless, the biochemical pathway of bentazon degradation has not been elucidated in detail. Partial mineralization was detected in liquid laboratory growth media inoculated with fungi, and 8-OH bentazon was identified as a major reaction product (6). The main degradation intermediate in soil was assumed to be 2-amino-*N*-isopropyl benzamide (7) which was detected only at trace concentrations and would easily be hydrolyzed to anthranilate plus isopropylamine. A recent review on the environmental behavior of bentazon confirms that our knowledge on this matter is still fragmentary (8).

A major problem in degradation of xenobiotics in soil is their tendency to attach to humic soil constituents and to

FIGURE 1. Chemical structures of bentazon and its derivatives used in the present study. The asterisk indicates the part labeled for tracer experiments. R₁ = H, R₂ = H: bentazon; R₁ = OH, R₂ = H: 8-hydroxybentazon; R₁ = OCH₃, R₂ = H: 8-methoxybentazon; R₁ = H, R₂ = CH₃: *N*-methylbentazon.

form bound residues which are comparably stable toward microbial attack (9, 10). Also bentazon appears to attach to the soil humic substance as indicated by a negative correlation observed between carbon content of soils and leaching of bentazon into lower soil and groundwater layers (11). A recent study with bentazon and aromatic cosubstrates in the presence of laccase or peroxidase enzymes showed that bentazon and its derivatives were transformed by these enzymes, preferentially under acidic conditions, and that especially the 6-OH and 8-OH derivatives were most reactive (12).

The present study was initiated in order to explain the discrepancy between the reported rates of bentazon disappearance in soil and those of bentazon mineralization. A detailed analysis of the influence of the organic matter content of soil, of oxygen availability, of fungal and bacterial activity, and of possible effects by free enzymes on bentazon transformation was performed to provide a better understanding of bentazon metabolism in soil, including the formation and degradation of intermediates and their interaction with the soil humic substance.

Experimental Section

Radiochemicals. U-¹⁴C-phenyl labeled bentazon (3-isopropyl-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one-2,2-dioxide, 1616 kBq·mg⁻¹) and its metabolites (U-¹⁴C-phenyl) *N*-methylbentazon (1527 kBq·mg⁻¹) and (U-¹⁴C-phenyl) 8-OH-bentazon (1177 kBq·mg⁻¹) were synthesized by BASF AG, Ludwigshafen, Germany (structures see Figure 1). U-¹⁴C-phenyl-labeled anthranilic acid (2-amino benzoic acid; Amersham/Buchler GmbH, Braunschweig, Germany; 3086 kBq·mg⁻¹) was used in transformation experiments for comparison.

A sample of N-¹⁴C-methyl labeled *N*-methylbentazon (8032 kBq·mg⁻¹) was synthesized by reaction of nonlabeled bentazon with ¹⁴C-methyl iodide in 100 mL of dichloromethane as solvent. Fifty milliliters of 0.1 M tetrabutylammonium hydrogensulfate in 0.5 M NaOH was added, and the solution was shaken at 80 rpm for 12 h. The product was purified and checked for purity by preparative and analytical thin-layer chromatography. A sample of ¹⁴C-8-methoxy-*N*-methylbentazon was synthesized by reacting 10 mg of nonlabeled 8-OH-bentazon in 10 mL of methanol with ¹⁴C-diazomethane which was added continuously under shaking until a yellow color remained in the solution for more than 2 h. Excess diazomethane was destroyed by addition of formic acid, nonreacted 8-OH-bentazon was removed by phase separation after addition of 0.1 M NaOH, and the product was purified and checked for purity by preparative and analytical thin-layer chromatography [silica gel plates 60 F-254, 200 × 200 × 0.25 mm (Merck, Darmstadt, Germany), separation with 65% cyclohexane, 35% 2-propanol, 0.3% glacial acetic acid for 90 min, scanning either under UV light

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(254 nm) or by radioactivity scanning (LKB 2842, Berthold, Wildbad, Germany)].

Labeled humic substance was synthesized from ^{14}C anthranilic acid following the procedure of Martin and Haider (13). $\text{U-}^{14}\text{C}$ anthranilic acid (6.05 mg) dissolved in 0.25 mL of methanol was added to a 0.9% (w/v) KH_2PO_4 solution, pH 5.0, containing 4500 units of peroxidase (Sigma, Deisenhofen, Germany). One gram of humic substance from soil and 2 mL of H_2O_2 30% (v/v) were added, and the mixture was stirred under air at 25 °C for 12 h. The enzyme was precipitated with acetone under sonication and removed by centrifugation (15 min at 20 000g). The product was fractionated by thin-layer chromatography and binding on Amberlite XAD-8 (Serva, Heidelberg; binding at pH 2.0, release at pH 8.0) to remove nonbound anthranilic acid.

Soil Incubation Experiments. Experiments were carried out with A_p soil samples taken from a barley field to which bentazon had been applied for 5 years. Air-dried soil was sieved (<2 mm) and stored at 4 °C. It was classified as a sandy clay loam (USDA scheme) of the Alfisol type with a maximum waterholding capacity of 36 g of H_2O ·100 g of dry soil $^{-1}$. The pH was 7.5, the total carbon content 1.2% (w/w), and the total nitrogen content 0.13% (w/w) (C/N-ratio 9.2). The microbial biomass was measured indirectly with a Sapro-mat (Type B 12, Voith, Germany) as oxygen consumption after glucose addition (14). It was 36.9 mg of C·100 g of dry soil $^{-1}$. For incubation experiments, 100 g of soil material adjusted to 60% maximum water holding capacity was used per incubation flask, and five replicate flasks were run per experiment. To check for possible effects of the soil organic matter content, alfalfa (*Medicago sativa*) plant residues were added as a dry powder to the soil as indicated. ^{14}C -labeled tracer compounds (0.3 mg per 100 g of soil), actidione (1.6 g per 100 g of soil), and streptomycin (1.0 g per 100 g of soil) were dissolved in aliquots of 3 mL of methanol and mixed into the soil; the methanol was removed afterward by evaporation at 20 °C under a continuous air stream. Actidione and streptomycin served as inhibitors of possible fungal or bacterial activities. Nitrate (5, 10, 20 mg N per 100 g of soil) was added as an alternative electron acceptor in some anoxic incubation experiments. It was supplied as an aqueous solution that was adjusted to give the same final humidity as with the other soil samples. For sterile control assays, soil was sterilized by three cycles of autoclaving for 1 h each on 3 subsequent days. The flasks were incubated in a special airtight steel tube (Memmert, Germany) at 20 °C for a minimum of 21 days in the dark. Anoxic incubation conditions were accomplished by gassing the steel tubes with N_2 for at least 5 min; flushing with air restored oxic conditions.

Freundlich adsorption isotherms were determined under sterile conditions with artificial clay-humic complexes synthesized according to ref 15 by mixing 1 g of freeze-dried humic substance with 10 g of amino silicagel (Nucleosil 5 NH_2 , Macherey and Nagel, Düren, Germany) in 100 mL of double distilled H_2O . This material was washed with 20 mL of 0.9% (w/v) KH_2PO_4 , 20 mL of H_2O , and 20 mL of methanol with intermediate centrifugation steps (12 000g, 10 min) to remove nonbound humic material. For tests, 100 μg of $\text{U-}^{14}\text{C}$ -phenylbentazon, $\text{U-}^{14}\text{C}$ -phenyl-8-OH-bentazon, or $\text{N-}^{14}\text{C}$ -methylbentazon was added to 250 mg of clay-humic complexes in 6 mL of sterile distilled H_2O . After stirring for 72 h, the complexes were centrifuged again, and 250 mL aliquots of supernatant were counted by liquid scintillation. A control experiment was run with aminosilicate alone.

Binding of bentazon and its derivatives to artificial clay-humic complexes by the action of enzymes was studied under similar conditions. An aliquot of 15 mg of labeled bentazon, 8-OH-bentazon, or a dimeric 8-OH-bentazon was incubated with 450 U of laccase, peroxidase, or tyrosinase (Sigma, Deisenhofen; definitions of enzyme units according

to the manufacturer's catalog) in 10 mL of 0.1 M $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 7.2, under air with stirring (80·min $^{-1}$) for 24 h at 22 °C. The assay with peroxidase contained in addition 45 mL of H_2O_2 (30% v/v). The reactions were stopped by precipitation of the enzymes with acetone under sonication and subsequent centrifugation (15 min at 20 000g).

Enzymatic Dimerization of 8-OH-Bentazon. Two hundred micrograms of nonlabeled 8-OH-bentazon dissolved in 10 mL of 0.1 M $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 6.5, was reacted with laccase (450 U·mL $^{-1}$; Sigma, Deisenhofen) for 24 h at 25 °C. The deep-red product was purified by precipitation with 4 M HCl and subsequent dialysis against distilled H_2O . It was characterized by mass spectrometry on an HPLC-MS system (PE-SCIEX API III; Perkin-Elmer, Überlingen, Germany).

Incubation Experiments with a Pure Fungal Culture. The white-rot fungus *Phanerochaete chrysosporium* (DSM 1556) was obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, and was cultivated in mineral medium (16). For incubation experiments, about 200 mg of washed wet mycelium was mixed with 100 g of natural or sterilized soil, and incubations with bentazon were run analogous to those with natural soil as described above.

Analyses. The released radioactive compounds were trapped in 25 mL of ethylene glycol/methanol (1:1) and 25 mL of 1 N H_2SO_4 ; $^{14}\text{CO}_2$ was absorbed in a further flask with 25 mL of 1 M NaOH. After incubation, the soil material was stored at -20 °C until further extraction. Parent compounds and metabolites were extracted twice with a 10-fold volume of methanol. Aliquots of these extracts were transferred into 20 mL of scintillation cocktail (Ultima Gold XR, Packard, Meridan, CT) and counted in a liquid scintillation counter (1219 RackBeta, LKB, Wallac Oy, Turku/Finland). Extracts were also separated by thin layer chromatography (silica gel plates 60F-254, 20·20·0.25 cm, Merck, Darmstadt, Germany; solvent: 65% (v/v) cyclohexane/35% (v/v) 2-propanol/0.3% (v/v) acetic acid) for 90 min. The following R_f values were obtained: bentazon 0.41, 8-OH-bentazon 0.26, 8-methoxybentazon 0.34, *N*-methylbentazon 0.62.

Soil humic material was extracted under N_2 atmosphere with 0.1 M sodium pyrophosphate and 0.5 M NaOH, according to refs 17 and 18. Extracts were centrifuged (10 000g, 15 min) and filtered through a folded filter. Radioactivity in this alkaline extract was quantified by scintillation counting. Radioactivity bound to fulvic acids was separated after adjustment to pH 1.0 with 4 N HCl, freezing, thawing, and centrifugation (10 000g, 15 min). Radioactivity in the supernatant was counted again, and the differences in radioactivity were attributed to labeled compounds in humic acids. The amount of radioactivity bound to humin and the inorganic soil fraction was calculated by subtraction of the total amount of alkali-extracted radioactivity from the data gained by combustion of soil material after methanol extraction.

For determination of soil-bound radioactivity, 0.2 g of samples of methanol-extracted soils were incinerated in an analytic oxidizer (Ox 300, Zinsser, Frankfurt/Main, Germany), and $^{14}\text{CO}_2$ was absorbed in Oxosol ^{14}C (National Diagnostics).

Microorganisms in soil were counted in extracts obtained from 20 g of dry soil with 180 mL of 0.18% (w/v) aqueous sodium pyrophosphate solution. Aliquots were used for counting of colony-forming units (CFU) by plating on nutrient broth agar (incubation for 48 h at 25 °C) or by the most-probable-number method with liquid nutrient broth medium (incubation for 3 weeks at 25 °C). To check for possible side effects of cosubstrates, the carbon content of the soils was increased in parallel experiments by addition of a powder of *Medicago sativa*.

TABLE 1: Conversion of Bentazon (3 mg per kg of soil) to CO₂, Counts of Heterotrophic Bacteria, and Specific Bacterial Bentazon-Degrading Activity in Unamended Soil and in Soil Amended with Plant Material (*Medicago sativa* Powder)^a

	unamended	+ 1% <i>M. sativa</i>	+ 3% <i>M. sativa</i>
μg ¹⁴ C-bentazon mineralized × kg dry soil ⁻¹	379 ± 44	315 ± 36	268 ± 31
= % of total label applied	12.3	10.3	8.6
CFU·g dry soil ⁻¹	6.6 ± 2.4 · 10 ⁷	2.04 ± 0.8 · 10 ⁸	8.6 ± 2.9 · 10 ⁸
μg ¹⁴ CO ₂ released × kg dry soil ⁻¹	63.6	56.6	47.3
fg ¹⁴ CO ₂ released × CFU ⁻¹	0.96	0.28	0.055

^a Assays were performed after 35 days of incubation. The ¹⁴CO₂ released was measured for every set of experiments (five replicas in each set). Bacterial counts were determined in three separate runs each. CFU = colony-forming units, fg = 10⁻¹⁵ g.

Results and Discussion

Influence of Organic Cosubstrates on Bentazon Mineralization. Previous experiments had indicated that bentazon metabolism in different soils was influenced by the soil carbon content. Therefore, we studied bentazon degradation in the presence and absence of various amounts of alfalfa (*Medicago sativa*) powder added to the soil. Alfalfa powder was chosen because of its high content of easily available carbon and nitrogen (C/N-ratio 13) to prevent nitrogen limitation of microbial growth. In four parallel runs, ¹⁴CO₂ was released from labeled bentazon at a constant rate after an initial adaptation period of 7–10 days (results not shown). As shown in Table 1, the total amount of ¹⁴CO₂ released after 35 days decreased with increasing total carbon content in the soil although the number of microorganisms increased, as assayed by plate counts on complex medium. This effect became even more pronounced when the amounts of mineralized bentazon per colony-forming unit were calculated (Table 1). In the presence of 3% (w/w) alfalfa powder per kg of soil, the bentazon mineralization activity per colony-forming unit was only 5% of that in unamended soil.

Obviously, bentazon mineralization was impeded by the presence of easily degradable carbon. This effect may be due in part to sorption of bentazon to the added organic material. Nonetheless, bentazon degradation appears not to be a strictly cometabolic process that would be expected to be enhanced by addition of carbon cosubstrates as described for several other xenobiotic compounds (19, 20). On the other hand, the degradative activity did not adapt to bentazon utilization since soils treated with bentazon for several years showed only slightly greater mineralization activities than unamended soils (unpublished data, BASF AG). A different study reported that bentazon-treated soils develop a higher bentazon degradation capacity than untreated soils (21), indicating that bentazon-degrading microorganisms may be enriched upon long-term soil treatment. Efforts in our laboratory to enrich for bentazon-degrading microorganisms in liquid cultures were unsuccessful, indicating that at least one step in this process was catalyzed by an organism which did not profit sufficiently from this activity to allow specific cell multiplication under the enrichment conditions applied.

Influence of Microbial Activities and Oxygen on Bentazon Mineralization and on Formation of Bound Residues. Release of ¹⁴CO₂ from U-¹⁴C-phenyl labeled bentazon was due to microbial activity. The highest release of ¹⁴CO₂ was found with untreated soil (Figure 2). In sterilized soil, no mineralization could be detected. The influence of bacteria or fungi on bentazon mineralization was checked in experiments with soil amended with streptomycin to inhibit

FIGURE 2. Recovery of radioactive derivatives from ring-labeled bentazon after incubation in soil for 28 d under various conditions as indicated. Three milligrams of labeled bentazon was applied per kg of soil.

bacteria or with actidione to inhibit fungi. In both cases, the release of ¹⁴CO₂ was only 20% of that of untreated soil (Figure 2) indicating that both bacteria and fungi contributed to bentazon mineralization.

Most of the added labeled bentazon was not extractable with methanol after incubation with untreated soil (Figure 2). Also this transformation of bentazon to a bound (non-extractable) residue was mainly due to microbial activity. A minor part of the added label was found to be bound also in sterile soil, perhaps as a consequence of abiotic partial oxidation by metal oxides (10, 22). Nonetheless, the majority of the label was bound by microbial activity in the nonsterile soil, and both prokaryotes and eukaryotes contributed to this binding activity. Fungi appeared to be involved to a greater extent than bacteria: in soils treated with actidione, 10.7% less radioactivity was bound to soil material, compared to soils treated with streptomycin; however, the small number of experiments reported here does not allow a generalization of these findings at present. Anderson and Domsch (23) regarded fungi as the most important microorganisms active in metabolism of xenobiotics in soil.

Significant release of ¹⁴CO₂ from U-¹⁴C-phenyl labeled bentazon was observed only in the presence of oxygen. Under anoxic conditions, the mineralization activity was less than 5% of the aerobic activity (Figure 3a). Addition of nitrate as an alternative electron acceptor to anoxic incubation experiments did not increase the ¹⁴CO₂ release. If the atmosphere was changed from anoxic to oxic and back to anoxic again, ¹⁴CO₂ release was observed nearly exclusively during the periods of oxygen access (Figure 3a).

Also the formation of bound residues was stimulated by oxygen (Figure 3b). Even small amounts of oxygen (0.05% (v/v) O₂) allowed formation of bound residues to nearly full extent; higher oxygen contents did not increase the rate of formation of bound residues further (results not shown). Anoxic conditions did not prevent further formation of bound residues after an initial oxic incubation, indicating that a reactive derivative had been formed that could bind also under anoxic conditions.

Formation of Bound Residues from Bentazon Derivatives. To characterize the type of binding to the humic substance into more depth, ¹⁴C-labeled bentazon and its derivatives 8-OH-bentazon, N-methylbentazon, and anthranilate were incubated with soil in experiments similar to those described above. 8-OH-bentazon was used because it had been detected as a metabolite in cultures of bentazon-degrading fungi (6). N-methylbentazon has been detected in soil amended with bentazon ((23) also unpublished results, BASF AG); methylation of bentazon and subsequent demethylation have been reported to be unspecific side activities of several microorganisms (24), and such a meth-

FIGURE 3. Fate of radioactivity of ring-labeled bentazon (3 mg per kg of soil) in soil during incubation under changing gas atmospheres (anoxic/oxic/anoxic): (a) release of $^{14}\text{CO}_2$ and (b) formation of bound residues. $^{14}\text{CO}_2$ released was measured for every separate set of experiments (five replicas in each set).

ylation–demethylation cycle was described also for the fungicide Chloroneb (25). Anthranilate was applied as a putative intermediate in bentazon mineralization and as an important precursor of humic substance formation. Of these compounds, 8-OH-bentazon was bound most efficiently to humic substance. This binding was equally effective in native and in sterile soil, indicating that it was a purely chemical process (Figure 4a). Also anthranilate exhibited a high tendency to bind to humic material, independent of microbial activity. Bentazon and *N*-methylbentazon were bound to equal but far lower extent than the hydroxy compound. Both substances formed more than twice as much bound residues in native soils as under sterile conditions.

Whereas radioactive 8-OH-bentazon or anthranilate after incubation was associated with humic acids or humin, most of the bound bentazon or *N*-methylbentazon was found in the fulvic acid fraction (Figure 4b). If it is true that humic substances age during humification from fulvic acids via humic acids to humins, our observations indicate that 8-OH-bentazon and anthranilate are transformed faster into the more stable fraction of soil humics, perhaps through a further intermediate (see below), than bentazon or *N*-methylbentazon.

The observed binding of bentazon to humic substances was not only physicochemical adsorption. Freundlich adsorption isotherms were determined with artificial clay-humic complexes ($k = 0.058$ for bentazon, 0.13 for 8-OH-bentazon, and 0.022 for *N*-methylbentazon) and were found to be substantially lower than the lowest such value reported in the literature (adsorption of benzene to a static clay-humic phase, $k = 0.23$ (15)). Thus, adsorption might play only a minor role in binding of bentazon and its derivatives to clay-humic complexes.

FIGURE 4. Fate of *N*-methylbentazon, 8-OH-bentazon, bentazon, and anthranilate (3 mg per kg of soil each) during incubation in soil for 32 d. (a) Mineralization and formation of bound residues. (b) Recovery of radioactivity in different fractions of humic material.

FIGURE 5. Formation of bound residues from ring-labeled 8-OH-bentazon, 8-methoxy-*N*-methylbentazon, and *N*- ^{14}C -methylbentazon in comparison to *N*-methyl- ^{14}C -phenylbentazon during incubation in soil for 28 d. 1.75 mg of labeled compound was applied per kg of soil.

To investigate the function of the hydroxyl group in binding of 8-OH-bentazon to humic material, we masked this group by methylation. The methylated derivative was bound to a far lower extent than the parent compound (Figure 5a). *O*-methylation also changed the binding pattern of 8-OH-bentazon in the various fractions of humic substances: whereas 56% of 8-OH-bentazon bound to the humic acid fraction, 98% of the *O*-methylated bentazon bound to the fulvic acid fraction (results not shown). *N*-methylation, on the other hand, did not prevent the formation of bound residues. Labeled *N*-methylbentazon was bound to the same extent as labeled bentazon. The label of *N*- ^{14}C -methylbentazon was bound to humic material to the same extent as did the radioactivity of the phenyl-labeled tracer although the methyl carbon was mineralized three times faster than the phenyl carbon (Figure 5b). Thus, also the methyl carbon was incorporated into the humic substance, but this transforma-

TABLE 2: Binding of Bentazon, 8-OH-Bentazon, and Its Dimeric Derivative to Artificial Clay-Humic Complexes and Aminosilicate by Reaction with Laccase, Peroxidase, and Tyrosinase^a

enzyme	metabolite provided $\mu\text{g}\cdot\text{mL}^{-1}$	metabolite dissolved after incubation $\mu\text{g}\cdot\text{mL}^{-1}$	metabolite bound $\mu\text{g}\cdot\text{mL}^{-1}$	part bound of total metabolite provided %
Bentazon Plus Aminosilicate^b				
laccase	34.2	33.9	0.3	0.9
peroxidase	32.0	35.2	0	0
tyrosinase	33.3	35.6	0	0
Bentazon Plus Artificial Clay-Humic Complex^b				
laccase	33.8	35.8	0	0
peroxidase	33.5	34.9	0	0
tyrosinase	31.7	34.5	0	0
8-OH-Bentazon Plus Aminosilicate^b				
laccase	20.9	24.6	0	0
peroxidase	25.5	26.6	0	0
tyrosinase	24.7	26.9	0	0
8-OH-Bentazon Plus Artificial Clay-Humic Complex^b				
laccase	24.6	6.5	18.1	73.6
peroxidase	25.7	26.3	0	0
tyrosinase	25.4	10.7	14.8	58.1
8-OH-Bentazon Dimer Plus Clay-Humic Complex^b				
	4.6 ± 0.2	1.9 ± 0.01	2.7	58.7

^a Reaction mixtures were incubated for 72 h at 25 °C as detailed in the Experimental Section. Numbers give means of the results of two independent experiments which differed by 12% at maximum.
^b Treatment.

tion proceeded at a different rate and probably by a reaction process different from that of the phenyl carbon.

Oxidative Dimerization of 8-OH-Bentazon. Although 8-OH-bentazon had been detected earlier as an intermediate in cultures of bentazon-degrading fungi (6) we could never detect this metabolite in soils. Instead, we found a polar metabolite in methanol extracts of soil, especially in those soil samples that had been inoculated with the fungus *Phanerochaete chrysosporium*. This compound accumulated to 3–4% (w/w) of the added parent compound. The same metabolite could be produced from 8-OH bentazon enzymatically by treatment with laccase. The deep-red derivative was purified. Mass spectrometry after HPLC separation (not shown) revealed that two molecules of 8-OH-bentazon were linked to each other by an ether linkage, most probably between the carbon atoms 8 and 5. This structure was confirmed by mass spectrometry with secondary fragmentation and subsequent mass spectrometry (MS-MS coupling; results not shown). Tri- or tetramers were not detected; however, these could have been destroyed in the fragmentation process.

Laccase and tyrosinase both catalyzed a covalent binding of 8-OH-bentazon to artificial clay-humic complexes. Laccase bound 73.6% and tyrosinase 58.1% of the added parent compound; no binding was found with peroxidase (Table 2). Peroxidase and tyrosinase did not immobilize bentazon at all, and the monomeric 8-OH-bentazon was bound specifically to clay-humic complexes (58–74%), not to aminosilicate. The dimeric 8-OH-bentazon derivative bound to the clay-humic complexes and also to aminosilicate, even without participation of enzymes.

Addition of *Phanerochaete chrysosporium* to native soil caused significantly faster binding of bentazon to humic substances (0.91 ± 0.03 mg per kg of soil) than did noninoculated soil with its autochthonous microorganisms (0.65 ± 0.02 mg per kg of soil). Obviously, the high reactivity of 8-OH-bentazon precluded detection of this intermediate

FIGURE 6. Release of $^{14}\text{CO}_2$ from (^{14}C -phenyl)bentazon and from bound residues previously formed from (^{14}C -phenyl)bentazon during incubation in soil for 141 d. The $^{14}\text{CO}_2$ released was measured for every separate set of experiments (three replicates in each set).

in soil because it was immediately dimerized and/or bound to the humic substance.

We conclude that the formation of bound residues was due to microbial hydroxylation of bentazon to 8-OH-bentazon, and this oxidation was the rate-limiting step in the binding process. Oxidative polymerization by laccase or peroxidase leads to precursors of humic substances also with other phenolic compounds (9, 26). Methylation of the hydroxy group reduced the binding of 8-OH-bentazon to humic substances substantially. Our results suggest that 8-OH-bentazon was dimerized by oxidative microbial enzymes such as laccase and that this dimer bound to clay-humic complexes. Also *Phanerochaete chrysosporium* which does not form laccase but lignin peroxidase or manganese peroxidase accelerated the binding of bentazon to soil humic substances, indicating that the oxidative enzymes produced by this fungus also produce reactive oxidized bentazon derivatives. Whether the dimeric derivative is an obligate intermediate of 8-OH-bentazon binding or only a reactive side product is a matter that needs to be elucidated further.

Degradation of Bound Residues. The rate of mineralization of bound bentazon residues was assayed in an incubation experiment with humic material that was prepared by preincubation with U- ^{14}C -phenyl labeled bentazon, analogous to the experiment described in Figure 2. Nonbound parent compounds were removed by extraction with methanol. After 141 days of incubation, 9.3% of the bound labeled carbon was transformed to $^{14}\text{CO}_2$, equivalent to one-third of the mineralization rate observed with the free parent compound (Figure 6). The bound radioactivity was found in all humic fractions, i.e., fulvic acids, humic acids, and humins. A time-dependent shift of the bound residues from low- to high-molecular humic fractions ("aging") was observed by gradient gel electrophoresis (27).

As shown in our experiments, up to 9.3% (w/w) of the bound residues was mineralized in 141 days. This mineralization rate is similar to that reported earlier (4) and was also comparable to that published for bound residues formed from 2,4-dichlorophenol (28).

Summary

The results of our experiments could be summarized in a flow scheme illustrating the flux of bentazon and its metabolites through various pools in the soil (Figure 7): 12–15% of added bentazon was mineralized immediately, 5% methylated and afterward demethylated, and 65–85% was hydroxylated by microbial activity to 8-OH-bentazon. 8-OH-Bentazon could either bind directly to the humic matrix or could be dimerized by fungal or bacterial activity or by laccase

FIGURE 7. Proposed model of transformation of bentazon and its metabolites in soil. The width of the arrows indicates the relative quantitative importance of the respective reaction path.

action. This dimer binds covalently to humic substances through a nonbiotic, chemical reaction. The bound labeled residues in the humic substance behave in a similar manner as natural humic constituents do; within 141 days, 9.3% of the bound residues was mineralized.

Acknowledgments

The authors want to thank BASF AG, Ludwigshafen, for financial and laboratory support.

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Received for review April 14, 1999. Revised manuscript received November 19, 1999. Accepted November 23, 1999.

ES990426H