APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1997, p. 4511–4515 Vol. 63, No. 11
0099-2240/97/$04.00+0

Biodegradation of [^{14}C]Benzo[*a*]pyrene Added in Crude Oil to Uncontaminated Soil

ROBERT KANALY,[1] RICHARD BARTHA,[1] SAMUEL FOGEL,[2] AND MARGARET FINDLAY[2]*

Department of Biochemistry and Microbiology, Cook College, Rutgers University, New Brunswick, New Jersey 08903,[1] *and Bioremediation Consulting Incorporated, Newton, Massachusetts 02159*[2]

Received 17 July 1997/Accepted 28 August 1997

To investigate the possible cometabolic biodegradation of benzo[*a*]pyrene (BaP), crude oil spiked with [7-^{14}C]BaP and unlabeled BaP was added to soil with no known pollution history, to give 34 g of oil and 67 mg of BaP/kg of dry soil. The oil-soil mixture was amended with mineral nutrients and incubated in an airtight container with continuous forced aeration. Total CO_2 and $^{14}CO_2$ in the off-gas were trapped and quantified. Soil samples were Soxhlet extracted with dichloromethane at seven time points during the 150-day incubation period, and the extracted soil was subjected to further fractionation in order to recover reversibly and irreversibly bound radiocarbon. Radiocarbon recovery was 100% ± 3% for each time point. During the first 50 days of incubation, no $^{14}CO_2$ was evolved, but over the next 100 days, 50% of the BaP radiocarbon was evolved as $^{14}CO_2$. At 150 days, only 5% of the intact BaP and 23% of the crude oil remained. Of the remaining radiolabel, 20% was found in solvent-extractable metabolites and 25% was incorporated into soil organic matter. Only 1/10 of this could be solubilized by chemical hydrolysis. An abiotic control experiment exhibited binding of only 2% of the BaP, indicating the microbial nature of the BaP transformations. We report that in soil containing suitable cosubstrates, BaP can be completely degraded.

Benzo[*a*]pyrene (BaP), a 5-ring polycyclic aromatic hydrocarbon (PAH), is a carcinogen which can be highly persistent in the environment (4). In pure cultures, bacteria can hydroxylate BaP to *cis* dihydrodiols at the 4,5, 7,8, or 9,10 positions, and fungi hydroxylate BaP to *trans* dihydrodiols at the same positions. These oxidation products generally are not further metabolized and do not result in ring cleavage (21, 25). White-rot fungi, however, can also oxidize BaP to quinones at the 1,6, 3,6, and 6,12 positions, and these are further metabolized to CO_2 (15). Recently, Schneider et al. (21) identified ring cleavage products of BaP produced by a *Mycobacterium* sp. isolated from a coal gasification site. Reports of biodegradation of BaP in field soil samples by naturally occurring soil microbes are few. Fogel (12) reported that during bioremediation of 60,000 μg of no. 6 fuel, including 60 μg of BaP per g of soil, the BaP was biodegraded 83% in 33 months. Grosser et al. (14) applied ^{14}C[BaP] to soils from several contaminated sites and found three soils that were able to mineralize it 4 to 25% in 225 days. The environmental persistence of BaP appears to be connected to its inability to support microbial growth. Our investigation was designed to explore the fate of BaP in the presence of other hydrocarbon substrates. We discuss our results in terms of biotransformation and ring cleavage products relative to mineralization, incorporation into biomass, and binding to soil humus.

MATERIALS AND METHODS

Properties of crude oil and soil. Crude oil was a Texas crude provided by a petroleum company. The oil had an API (American Petroleum Institute) gravity of 38, had a total organic carbon content of 87.1%, was 94% analyzable by gas chromatography, and contained 74, 24, and 2% saturates, aromatics, and polars, respectively, as determined by column chromatography. By gas chromatography, 71% eluted as diesel range organics (nC_8 to nC_{25}) and 15% eluted as gasoline range organics (nC_6 to nC_{10}). Normal alkanes represented 22%, and analyzable PAH represented 1.8%, of the total. Of these, 97% were 2- and 3-ring PAHs. The content of BaP was less than 1 μg/g.

The soil was from an active cattle pasture in the Gulf region of Texas, in use for this purpose for more than 18 years, with no recorded chemical contamination. It had a sand-silt-clay composition of 33:51:16%. Per gram, the soil contained about 1 mg of total nitrogen measured by the Kjeldahl method, about 12.1 mg of total organic carbon, 0.3 mg of solvent-extractable polar organics, and 0.2 mg of hydrocarbons, of which total normal alkanes constituted <3 μg and total priority-pollutant PAHs (detected by gas chromatography-mass spectrometry) constituted only 0.008 μg. All quantities are expressed on a dry-soil basis. The soil was sieved through 2-mm openings and mixed thoroughly before use.

Addition of BaP to soil in a crude oil matrix. [7-^{14}C]BaP (58.78 mCi/mmol; 0.97 mCi/ml in toluene) was obtained from Chemsyn Science Laboratories (Lenexa, Kans.), which reported a radiochemical purity by thin-layer chromatography of >98%. In our own thin-layer chromatographic determination, 99% of the total radioactivity traveled with the unlabeled BaP standard. [^{14}C]BaP (242 μl; total, 5.6×10^7 dpm) was added to 203 mg of unlabeled BaP and 122 g of crude oil in a vial, sealed with a Teflon-lined crimped cap, and shaken on a reciprocal shaker for 3 h at 300 rpm at 37°C. The homogeneous distribution of label in the oil was demonstrated by triplicate analysis by scintillation counting, exhibiting a standard deviation of 1.5%. Crude oil containing BaP was added to soil that had a moisture content of 9.5 g of water/100 g of soil. Six batches of 20 g of oil with 500 g (dry weight) of soil were prepared and mixed thoroughly, with repeated sieving through 5-mm openings. The homogeneity of the combined batches (3,000 g) was demonstrated by analysis of triplicate samples for ^{14}C by combustion, obtaining a standard deviation of 1.5%. The soil thus contained, on a dry-weight basis, 19,000 dpm/g and 67 μg of BaP/g. Since the oil contained, by weight, 15% compounds lighter than decane, significant volatilization occurred during mixing, resulting in the mixture containing only 34 mg of crude oil per g of soil.

Addition of fertilizer to soil-oil mixture. Twenty milliliters of 1% KOH and 10 ml of each of the following aqueous solutions were added separately to each kilogram of dry soil: (i) 217 g of KNO_3/liter; (ii) 5.6 g of NH_4Cl; (iii) 32 g of KH_2PO_4 and 56 g of K_2HPO_4/liter; (iv) 3.7 g of $CaSO_4 \cdot 7H_2O$, 9.7 g of $MgSO_4 \cdot 7H_2O$, and 4.2 g of NaCl/liter; (v) 1.6 g of $FeSO_4 \cdot 7H_2O$; and (vi) the trace-element solution of Fogel et al. (11), diluted 5.5-fold. The amended soil contained 315 μg of N/g and 175 μg of P/g and had a pH of 6.8. The moisture content was 17.5 g of water/100 g of dry soil, corresponding to 28% of the water-holding capacity of the soil. On day 42 of treatment, fertilizer was again applied; phosphorus was added at 180 μg/g of soil, and the other elements were added at 1/3 of the previous levels.

Abiotic control soil preparation. Soil (340 g) was placed in a fine-mesh sieve in a desiccator above 200 ml of chloroform, and a vacuum was applied, causing the chloroform to volatilize and fill the soil pore spaces, after which the chloroform atmosphere was maintained for 36 h. Subsequently, the [^{14}C]BaP-crude oil was added by mixing and sieving, the pH was adjusted to 6.9, and the moisture

* Corresponding author. Mailing address: Bioremediation Consulting Incorporated, 55 Halcyon Rd., Newton, MA 02159. Phone: (617) 969-5913.

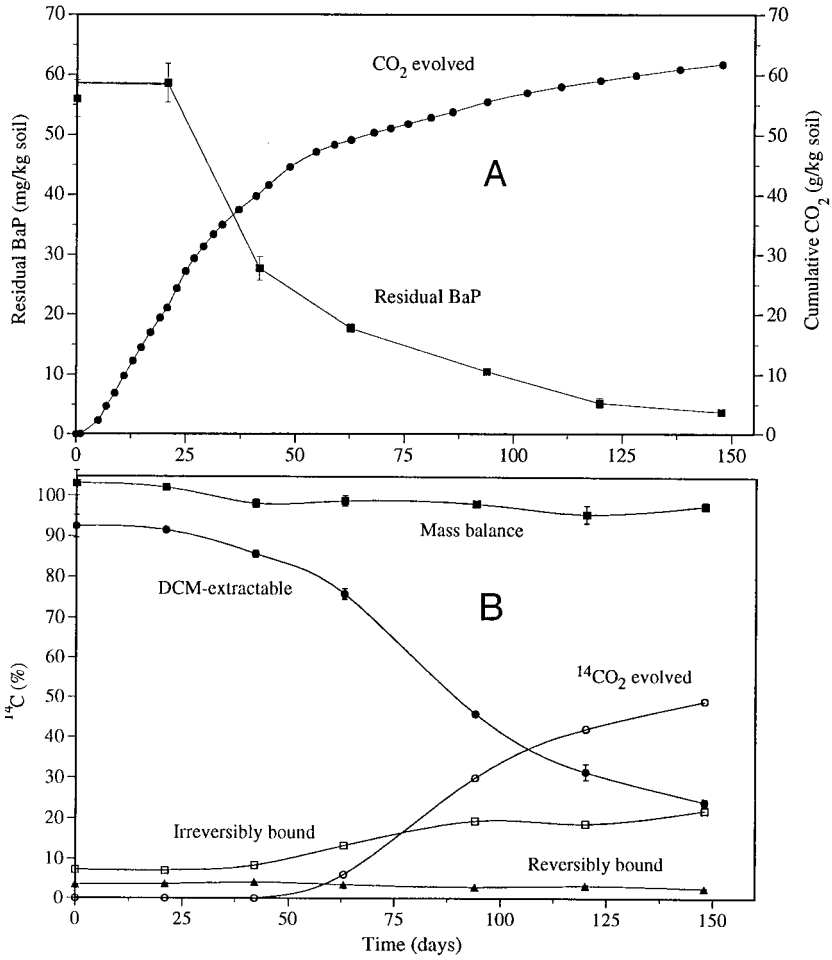

FIG. 1. (A) Time course of cumulative CO_2 evolution and BaP depletion, during biodegradation, in soil containing 34 g of crude oil and 67 mg of BaP per kg. Each error bar for BaP represents the standard deviation of triplicate determinations from the same solvent extract. (B) Recovery of ^{14}C from [7-^{14}C]BaP in the same experiment. Analytical procedures for $^{14}CO_2$ and for bound and extractable residues are given in the text. $^{14}CO_2$ data are the cumulative amounts. Irreversibly bound radiocarbon was liberated only by wet combustion of the soil organic matter. Except for $^{14}CO_2$ evolution, all analyses were performed in duplicate. Error bars represent the coefficients of variation and are omitted when smaller than the symbol.

was adjusted to 160 mg/g of soil. The contaminated soil was then incubated in Whatman extraction thimbles in a desiccator filled with chloroform vapor.

Incubation and sampling. The soil containing live microbes was placed in a 6-liter aluminum reactor (a modified pressure cooker with a diameter of 25 cm and a height of 15 cm) fitted with a stainless-steel wire screen for soil support and provided with bottom air input so that air passed through the soil before exiting from the top. CO_2-free air flowed continuously at 25 cc/min through a rotameter and was humidified by bubbling through water in a gas washing bottle. The air passed through the soil, then through two KOH traps consecutively in order to collect CO_2, and finally through a second rotameter. The system was checked frequently for leaks by comparison of the flow rates on the two rotameters, as well as by testing of each connection. The temperature was maintained at 22°C by immersion of the reactor in a temperature-regulated water bath. In order to obtain soil samples, the reactor was opened briefly on five occasions during the 150-day treatment period; at these times, all of the soil was removed and mixed thoroughly prior to sampling.

Quantitation of CO_2 and $^{14}CO_2$ evolution. Off-gas flowed continuously through two 250-ml gas washing bottles with fritted spargers, each containing 240 ml of 1 N KOH. Phenolphthalein in trap 1 was used to indicate exhaustion of KOH, whereupon the second KOH bottle was moved to position 1 and a new KOH bottle was put in position 2. For ^{14}C analysis, two 1-ml samples of KOH were added to 10 ml of Scintiverse BD counting fluid (Fisher Scientific, Pittsburgh, Pa.) and analyzed by liquid scintillation counting in a Beta-Trac model 6895 counter (TM Analytic, Elk Grove Village, Ill.). For total CO_2 analysis, two 10-ml samples were treated with $BaCl_2$ to precipitate carbonate, and the supernatant liquid was titrated with 0.5 M HCl. CO_2 measurements were made daily until day 42 and then were spaced at greater intervals as respiration decreased.

Bacteria, available minerals, pH, and total extractable organics. Viable bacteria were extracted by shaking with 4 volumes of buffer (1.25 g of NH_4Cl, 0.73 g of KH_2PO_4, and 1.25 g of K_2HPO_4/liter) for 30 min on a reciprocal shaker and were enumerated by spread plate counting with plates containing 4.5 g of nutrient agar and 10.5 g of Bacto agar/liter. Available nitrogen and phosphate were determined by extraction of soil by shaking with 5 volumes of distilled water for 20 min on a reciprocal shaker, centrifugation for 30 min, and analysis of the supernatant liquid with LaMotte (Chestertown, Md.) kits for phosphate (no. 4408), ammonia-N (no. 4795), and nitrate-N (no. 3110). pH measurements were carried out on the centrifuged water extract. Dichloromethane (DCM)-extractable organics were determined gravimetrically in triplicate for each Soxhlet DCM extract, prepared as described below, by evaporation of 20-ml samples of the DCM extract with a warm-air flow. This method significantly underestimates extractable organics for samples with high fractions of light hydrocarbons below C_{15}.

For solvent-extractable organics, duplicate 35-g soil samples were air dried to constant weight and extracted with 250 ml of DCM by Soxhlet extraction for 6 h (60 cycles). Drying with sodium sulfate was not performed because the extracted soil was to be further analyzed. Alumina cleanup of the DCM extract was not performed, since some biotransformed BaP products might be removed by the alumina. Extracts were stored at 4°C in serum bottles with Teflon-lined crimped caps. Extracted soil was air dried and was stored covered at 4°C. Triplicate 1-ml samples of each DCM extract were analyzed by scintillation counting.

Analysis of extractable BaP. All operations were carried out in dim yellow light in order to avoid photodegradation of BaP. DCM extracts of soil samples were concentrated at 22°C in a rotary evaporator, dried with an N_2 stream at 40°C, and dissolved in isooctane. PAHs were separated from other hydrocarbons according to the method of Pancirov et al. (20), as follows. PAHs were partitioned from isooctane into dimethyl sulfoxide preequilibrated with phosphoric acid. After dilution of the dimethyl sulfoxide with water, PAHs were counterextracted back into isooctane and concentrated under N_2 at 40°C. BaP standards

TABLE 1. Biotreatment monitoring data for biodegradation of crude oil with added BaP, on a dry-soil basis

Day	10^6 CFU/g of soil	Available NO_3-N (μg/g)	Available PO_4 (μg/g)	Extractable organics[a] (mg/g [range])
0	28	315[b]	520[b]	34.0[b]
21	1,430	40	20	19.4 (0.6)
42	1,410	3	16	12.2 (0.2)
63	3,700	90	53	10.0 (0.2)
94	2,750	60	60	9.2 (0.4)
120	1,900	90	30	7.7 (1.0)
150	1,280			7.7 (0.2)

[a] Extractable-organic analysis was carried out for samples other than those for day 0 by Soxhlet extraction with DCM, with no alumina cleanup, followed by gravimetric quantitation. Values are the averages of duplicates.

[b] Concentrations are those added on day 0. Minor and trace elements were also added. Additional fertilizer was applied on day 42 after sampling.

processed in this manner were recovered with 85% efficiency, and unknown samples were corrected for a 15% loss due to this partition procedure. Gas chromatographic analysis was performed on the "cleaned-up" sample by using a Hewlett-Packard model 5890 instrument with a model 3392 integrator. A 15-m by 0.53-mm fused silica macrobore column was used with a bonded 2.65-μm-thick polydimethylsiloxane stationary phase (Alltech, Deerfield, Ill.). The temperature program was 100°C for 10 min, followed by an increase of 10°C/min to 250°C, which was held for 30 min. The injector and detector were at 200 and 250°C, respectively. The carrier was helium at 22.5 ml/min. The samples in isooctane were diluted in DCM and spiked with naphthalene as an internal standard. The BaP retention time was 36.8 min, and the detection limit was 1 μg/ml in the solvent extract. The identity of the BaP peak was confirmed by comparison of its retention time to that of an authentic standard, as well as by mass spectrometric identification of the molecular ion (*m/z* 252) and characteristic fragments (*m/z* 126) carried out at the Mass Spectrometry Center, Cook College, Rutgers University.

Analysis of DCM-extracted soil for reversibly and irreversibly bound ^{14}C-labeled residues. To determine reversibly bound ^{14}C-labeled residues, 3 g of DCM-extracted soil was extracted with 2.8 ml of methanol and 0.2 ml of 2 N KOH for 1 h at 98°C (9) and the extract was analyzed by scintillation counting. To determine total bound BaP residues by wet oxidation with chromic acid, 3 g of DCM-extracted soil was added to a 100-ml round-bottom flask with 1 g of $K_2Cr_2O_7$. The reflux condenser was then attached, and 25 ml of concentrated H_2SO_4–H_3PO_4 (60:40) was added. The mixture was boiled for 20 to 30 min and cooled while flushing with CO_2-free air under negative pressure into Oxosol C^{14} (National Diagnostics, Atlanta, Ga.) to trap liberated CO_2 (27). The $^{14}CO_2$ in the Oxosol was quantitated by scintillation counting. To obtain the value for irreversibly bound ^{14}C, the value for loosely bound ^{14}C was subtracted from this total.

Strong-acid or -alkali extraction of DCM-extracted soil. DCM-extracted soil, 3 g, was placed in a Hungate tube, 8 ml of 1 M H_2SO_4 was added, the tube was flushed with N_2, and the mixture was digested at 97°C for 6 h. Before opening, 1 ml of DCM was injected and the tube was shaken for 30 s, vortexed for 30 s, and then centrifuged at 3,000 rpm for 15 min. The supernatant was decanted into a 30-ml separatory funnel, the residue was reextracted with 3 ml of DCM and 3 ml of H_2SO_4, and the supernatants were combined. The DCM was separated from the aqueous phase and transferred to a vial. The aqueous phase was reextracted twice with 1 ml of DCM, which was added to the vial. The DCM extract was concentrated to 1 ml, added to Scintiverse BD, and ^{14}C quantitated by scintillation counting. The extracted soil was treated by wet combustion, and the CO_2 produced was analyzed by scintillation counting. The alkali procedure was the same as the acid procedure, except that 1 M NaOH was added initially, and after the digestion and the first DCM extraction, the mixture was acidified to pH 2 with H_2SO_4 to precipitate humic acids.

RESULTS

Biotreatment monitoring. Evolution of CO_2 from crude oil started after a lag of only 1 day and reached a peak of 1.3 g of CO_2/day/kg of soil by day 10, maintaining this rate through day 25; then it decreased gradually to 0.1 g of CO_2/day/kg by day 150 (Fig. 1A). Microbial numbers increased from an initial 2.8×10^7 to 3.7×10^9 CFU/g of dry soil by day 63, then decreased gradually to 1/3 of this number by day 150 (Table 1). Monitoring for available N and P showed that these elements were low on day 42, and additional mineral nutrients were added. The pH remained between 7.4 and 7.6, and the water content was 130 to 180 mg/g of dry soil.

During treatment, the extractable organics (mainly petroleum hydrocarbons) decreased from an initial 34 mg/g to 7.7 mg/g, a reduction of about 77%. Analysis of the DCM extract by gas chromatography for intact BaP (Fig. 1A) indicated that only about 60 μg/g of the original 67 μg/g added could be detected on day 0, consistent with the finding that 10% of the initial ^{14}C was bound on day 0. BaP concentration did not change by day 21, but it dropped rapidly by day 42 and more gradually thereafter, decreasing to 5.5% of the starting value by day 150.

Fate of ^{14}C from BaP. In contrast to the 1-day lag observed for total CO_2 production, $^{14}CO_2$ was not detected until day 52, after which it increased rapidly, reaching 50% of the maximum rate on day 57, and slowed by day 80. By day 150, 50% of the total applied ^{14}C had been converted to $^{14}CO_2$ (Fig. 1B). Radiocarbon recovery in $^{14}CO_2$, DCM-extractable, reversibly bound, and irreversibly bound fractions ranged from 103% on day 0 to 96% on day 120. DCM-extractable ^{14}C represented 90% of the added ^{14}C on day 0 and remained relatively unchanged through day 42. A significant decrease was observed on day 63, and more rapid decreases were observed through day 150. Reversibly bound ^{14}C contained only 2.5 to 4% of the originally added radiolabel and did not change throughout the entire 150-day experiment. Irreversibly bound ^{14}C, calculated by subtracting the reversibly bound ^{14}C from the total ^{14}C in the DCM-extracted soil, initially contained 7% of the added ^{14}C, did not change significantly until after day 42, and then increased to 23% of the total by day 150. Strong-acid or -alkali hydrolysis of DCM-extracted soil failed to release more of the bound radiocarbon than the methanolic KOH treatment (data not shown). In summary, by 150 days, 50% of the ^{14}C was in CO_2, 25% was DCM extractable, 22.5% was irreversibly bound, and 2.5% was reversibly bound. In the DCM-extractable portion, BaP represented 5% of the original ^{14}C. Thus, 95% of the added BaP was either mineralized, biotransformed, or irreversibly bound.

Day-0 binding of ^{14}C in the biotic soil (which occurred during the 8 h between the initial contact of the oil with the soil and the removal of the day-0 samples) consisted of 7% strong binding and 3% reversible binding. In contrast, the abiotic control, which was treated with chloroform prior to the addition of oil, showed only 1.2% strong binding and 0.8% reversible binding of ^{14}C on day 0. These values did not change significantly during the next 28 days (Table 2).

DISCUSSION

Recovery of BaP radiocarbon. The approximately 100% recovery of the originally added [^{14}C]BaP in both the biotic and abiotic experiments, together with excellent agreement of duplicates for extractable and bound fractions, indicates the general validity of the approach. Data for the abiotic-control soil

TABLE 2. Distribution of BaP radiocarbon[a] in the abiotic control

Day	DCM extractable	Irreversibly bound	Reversibly bound	% Recovery
0	98.3 (1.4)	1.2 (0.2)	0.8 (<0.2)	100 (1.4)
7	93.9 (7.4)	1.6 (0.2)	0.7 (<0.2)	96 (7.8)
14	96.2	2.6 (0.4)	1.2	100
28	95.3 (1.0)	2.1 (0.2)	0.7 (<0.2)	98 (1.0)

[a] Values are percentages of the total radiocarbon added and represent the averages of duplicate measurements. The ranges between the high and low values are given in parentheses. Where no range appears, the duplicate was lost.

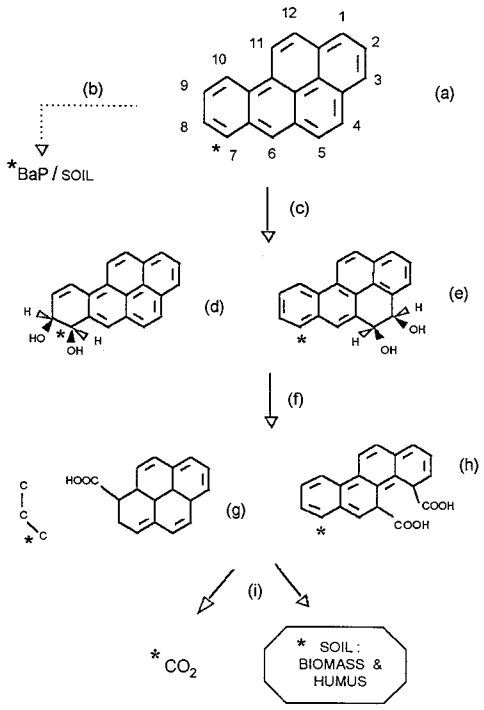

FIG. 2. Proposed mechanisms for achieving both $^{14}CO_2$ release and incorporation of radiolabel from [7-^{14}C]BaP (a) into soil organic matter. The asterisk indicates the labeled carbon. On day 0, 10% of the BaP radiocarbon was bound to soil by an unknown mechanism (b) that was present in live soil but not in killed control soil. Proposed cometabolic oxidation (c) would convert BaP to *cis*-7,8-BaP-dihydrodiol (d) and *cis*-4,5-BaP-dihydrodiol (e). Further oxidation steps and ring cleavage would result in 7,8-dihydro-pyrene-8-carboxylic acid (g) and 4,5-chrysene dicarboxylic acid (h). The metabolites shown in structures d, g, and h were isolated by Schneider et al. (21) from a *Mycobacterium* culture exposed to BaP. The proposed metabolism (i) of compounds g and h by soil microbes results in the release of $^{14}CO_2$ (50% of ^{14}C) and irreversible incorporation of ^{14}C into soil organic matter (15% of ^{14}C).

showing lack of change in DCM-extractable ^{14}C or bound ^{14}C residues further enhance the validity of the results.

Biodegradation of BaP. BaP can be cometabolically oxidized by a number of microorganisms growing on other substrates (5–7, 13). Recently, Schneider et al. (21) reported on the isolation of ring cleavage products of BaP produced by resting cells of a *Mycobacterium* sp. but not metabolized further. One of the products, 7,8-dihydro-pyrene-carboxylic acid, is proposed to be derived from initial hydroxylation of the BaP at carbons 7 and 8, resulting in the removal of carbon 7 of BaP. The other product isolated, 4,5-chrysene dicarboxylic acid, would result from initial hydroxylation of BaP carbons 4 and 5, without any changes at BaP carbon 7 (Fig. 2). Whereas in laboratory cultures of single species, oxidized products of BaP are generally not reported to be mineralized, it is reasonable to expect that such ring cleavage intermediates, produced cometabolically in soil in the presence of other petroleum hydrocarbons, would be metabolized further in a soil ecosystem containing a variety of microbes acting as a consortium. This idea is supported by the fact that pyrene itself can be mineralized as a sole carbon and energy source by microbial isolates in culture (17, 25). Thus, the observed mineralization of BaP would be explained as the result of cometabolic oxidation and continued metabolism by a consortium of hydrocarbon degraders (1). The organisms responsible for the biotransformations reported here have not been investigated. However, phospholipid fatty-acid analysis indicated that bacteria, actinomycetes, and fungi increase extensively in a mixture of this oil and soil during biotreatment (10).

The reasons for the 42-day lag time for decrease in extractable intact BaP and for the 52-day lag time for appearance of $^{14}CO_2$ have yet to be understood. To date, BaP has not been reported to support microbial growth (4), and therefore a time-dependent enrichment of BaP degraders is an unlikely explanation. However, naphthalenes have been found to competitively inhibit the biodegradation of higher-molecular-weight PAHs (22, 24). Naphthalene and its alkylated derivatives were present initially at 590 μg/g, and in a parallel study they were 80% biodegraded by day 42 (10). Therefore, it is possible that 2-ring PAHs, present initially, competitively inhibited BaP degradation during the first few weeks.

Binding of radiolabel to soil. When organic residues undergo biodegradation in soil, some of the carbon is released as CO_2, while a portion is converted to microbial biomass. The biomass itself is eventually biodegraded, and some of its organic residues are incorporated into soil organic matter (humus) which is not extractable by DCM. Humic compounds are random polymers containing cross-linked organic residues comprised of aromatic, heterocyclic, and quinoidal rings, linked by carbon-carbon, ether, amino, and azo bonds, and having reactive groups such as carboxyl, phenolic, and carbonyl (23). The incorporation of new material by the formation of covalent bonds with these groups is termed humification. The increase in irreversibly bound ^{14}C between days 42 and 150, amounting to 15% of the total radiolabel, most likely reflects the incorporation of BaP carbon into microbial biomass and/or soil humus. The latter may occur indirectly via humification of ^{14}C-labeled residues from biodegraded biomass or directly by covalent binding of BaP biodegradation intermediates.

The day-0 binding of ^{14}C in the abiotic control, 0.8% reversible and 1.2% irreversible, can be taken as that resulting from abiotic processes (19). For the biotic soil, however, 3% reversible and 7% irreversible binding was observed on day 0, and the difference appears to be due to biotic processes. Since the day-0 binding in the biotic soil was not associated with $^{14}CO_2$ evolution, it seems likely that the process involved the entire BaP residue, possibly after its oxidation to the dihydroxy compounds, which would be candidates for binding to soil organic matter by extracellular enzymes (8). A number of ^{14}C-labeled xenobiotic compounds have been shown to bind irreversibly to soil, becoming nonextractable by organic solvents (2, 26). The incorporation of some xenobiotic compounds into soil organic matter has been shown to be covalent and to be mediated by naturally occurring soil enzymes and microbes. These investigations have been carried out with whole soil (16) as well as with model humic compounds and extracellular fungal enzymes, such as laccases and peroxidases (3, 18).

Significance. This research demonstrates that under carefully controlled conditions, with quantitative recovery of radiolabel, extensive ring cleavage and mineralization of [^{14}C]BaP can be accomplished in soil by naturally occurring microorganisms. No previous exposure to petroleum or xenobiotics was required, but components of simultaneously added crude oil appeared to support BaP cometabolism.

ACKNOWLEDGMENT

This research was supported by the Petroleum Environmental Research Forum (PERF), project 93-04.

REFERENCES

1. **Atlas, R. M., and R. Bartha.** 1993. Microbial ecology: fundamentals and applications, 3rd ed., p. 346–400. Benjamin Cummings, Redwood City, Calif.
2. **Berry, D. F., and S. A. Boyd.** 1985. Decontamination of soil through en-

hanced formation of bound residues. Environ. Sci. Technol. **19:**1132–1133.
3. **Bortiatynski, J. M., P. G. Hatcher, R. D. Minard, J. Dec, and J.-M. Bollag.** 1994. Enzyme-catalyzed binding of ^{13}C-labeled 2,4-dichlorophenol to humic acid using high resolution ^{13}C NMR, p. 1091–1098. *In* N. Senesi and T. M. Miano (ed.), Humic substances in the global environment and implications on human health. Elsevier Science B.V., Amsterdam, The Netherlands.
4. **Cerniglia, C. E.** 1993. Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation **3:**351–368.
5. **Cerniglia, C. E., W. Mahaffey, and D. T. Gibson.** 1980. Fungal oxidation of benzo[*a*]pyrene: formation of (−)-*trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene by *Cunninghamella elegans*. Biochem. Biophys. Res. Commun. **94:**226–232.
6. **Cerniglia, C. E., and D. T. Gibson.** 1979. Oxidation of benzo[*a*]pyrene by the filamentous fungus *Cunninghamella elegans*. J. Biol. Chem. **254:**12174–12180.
7. **Datta, D., and T. B. Samanta.** 1988. Effect of inducers on metabolism of benzo[*a*]pyrene *in vivo* and *in vitro*: analysis by high pressure liquid chromatography. Biochem. Biophys. Res. Commun. **155:**493–502.
8. **Dec, J.** 1997. Personal communication.
9. **Eschenbach, A., R. Kastner, R. Bierl, G. Schaefer, and B. Mahro.** 1994. Evaluation of a new, effective method to extract polycyclic aromatic hydrocarbons from soil samples. Chemosphere **28:**683–692.
10. **Findlay, M.** Unpublished data. 1997.
11. **Fogel, M., A. R. Taddeo, and S. Fogel.** 1986. Biodegradation of chlorinated ethenes by a methane-utilizing culture. Appl. Environ. Microbiol. **51:**720–724.
12. **Fogel, S.** 1994. Full-scale bioremediation of no. 6 fuel oil-contaminated soil, p. 569–579. *In* P. Flathman, D. Jerger, and J. Exner (ed.), Bioremediation: field experience. Lewis Publishing Co., Boca Raton, Fla.
13. **Gibson, D. T., V. Mahadevan, D. M. Jerina, H. Yagi, and H. J. C. Yeh.** 1975. Oxidation of the carcinogens benzo[*a*]pyrene and benzo[*a*]anthracene to dihydrodiols by a bacterium. Science **189:**295–297.
14. **Grosser, R. J., D. Warshawsky, and J. R. Vestal.** 1991. Indigenous and enhanced mineralization of pyrene, benzo[*a*]pyrene, and carbazole in soils. Appl. Environ. Microbiol. **57:**3462–3469.
15. **Haemmerli, S. D., M. S. A. Leisola, D. Sanglard, and A. Fiechter.** 1986. Oxidation of benzo[*a*]pyrene by extracellular ligninases of *Phanerochaete chrysosporium*. J. Biol. Chem. **261:**6900–6903.
16. **Haider, K., M. Spiteller, A. Wais, and M. Fild.** 1993. Evaluation of the binding mechanism of anilazine and its metabolites in soil organic matter. Int. J. Environ. Anal. Chem. **53:**125–137.
17. **Jimenez, I., and R. Bartha.** 1996. Solvent-augmented mineralization of pyrene by a *Mycobacterium* sp. Appl. Environ. Microbiol. **62:**2311–2316.
18. **Liu, S.-Y., A. J. Freyer, R. D. Minard, and J.-M. Bollag.** 1985. Enzyme-catalyzed complex-formation of amino acid esters and phenolic humus constituents. Soil Sci. Soc. Am. J. **49:**337–342.
19. **Pal, S., J.-M. Bollag, and P. M. Huang.** 1994. Role of abiotic and biotic catalysts in the transformation of phenolic compounds through oxidative coupling reactions. Soil Biol. Biochem. **26:**813–820.
20. **Pancirov, R. J., T. D. Searl, and R. A. Brown.** 1980. Methods of analysis for polynuclear aromatic hydrocarbons in environmental samples. Adv. Chem. Ser. **185:**123–142.
21. **Schneider, J., R. Grosser, K. Jayasimhulu, W. Xue, and D. Warshawsky.** 1996. Degradation of pyrene, benzo[*a*]anthracene, and benzo[*a*]pyrene by *Mycobacterium* sp. strain RJGII-135, isolated from a former coal gasification site. Appl. Environ. Microbiol. **62:**13–19.
22. **Shuttleworth, K. L., and C. E. Cerniglia.** 1996. Bacterial degradation of low concentrations of phenanthrene and inhibition by naphthalene. Microb. Ecol. **31:**305–317.
23. **Stevenson, F. J.** 1976. Organic matter reactions involving pesticides in soils. ACS Symp. Ser. **29:**180–207.
24. **Stringfellow, M. T., and M. D. Aitken.** 1995. Competitive metabolism of naphthalene, methylnaphthalenes, and fluorene by phenanthrene-degrading pseudomonads. Appl. Environ. Microbiol. **61:**357–362.
25. **Sutherland, J. B., F. Rafii, A. A. Khan, and C. E. Cerniglia.** 1995. Mechanisms of polycyclic aromatic hydrocarbon degradation, p. 269–306. *In* L. Y. Young and C. E. Cerniglia (ed.), Microbial transformation and degradation of toxic organic chemicals. Wiley-Liss, New York, N.Y.
26. **Weiss, U. M., I. Scheunert, W. Klein, and F. Korte.** 1982. Fate of pentachlorophenol ^{14}C in soil under controlled conditions. J. Agric. Food Chem. **30:**1191–1194.
27. **You, I.-S., and R. Bartha.** 1982. Evaluation of the Bleidner technique for analysis of soil-bound 3,4-dichloroaniline residues. J. Agric. Food Chem. **30:**1143–1147.