APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1988, p. 929–936
0099-2240/88/040929-08$02.00/0

Vol. 54, No. 4

Two-Stage Mineralization of Phenanthrene by Estuarine Enrichment Cultures

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Received 14 August 1987/Accepted 29 January 1988

The polycyclic aromatic hydrocarbon phenanthrene was mineralized in two stages by soil, estuarine water, and sediment microbial populations. At high concentrations, phenanthrene was degraded, with the concomitant production of biomass and accumulation of Folin-Ciocalteau-reactive aromatic intermediates. Subsequent consumption of these intermediates resulted in a secondary increase in biomass. Analysis of intermediates by high-performance liquid chromatography, thin-layer chromatography, and UV absorption spectrometry showed 1-hydroxy-2-naphthoic acid (1H2NA) to be the predominant product. A less pronounced two-stage mineralization pattern was also observed by monitoring $^{14}CO_2$ production from low concentrations (0.5 mg liter^{-1}) of radiolabeled phenanthrene. Here, mineralization of ^{14}C-labeled 1H2NA could explain the incremental $^{14}CO_2$ produced during the later part of the incubations. Accumulation of 1H2NA by isolates obtained from enrichments was dependent on the initial phenanthrene concentration. The production of metabolites during polycyclic aromatic hydrocarbon biodegradation is discussed with regard to its possible adaptive significance and its methodological implications.

Polycyclic aromatic hydrocarbons (PAHs) are a class of fused-ring aromatic compounds which are of great concern because of their toxicity, carcinogenicity, potential for trophic biomagnification, and resistance to biodegradation (40, 45). As products of combustion and as components of fossil fuels, PAHs are ubiquitous environmental pollutants, and they are abundant near urban and industrial centers (37, 55). Estuaries are prime repositories for PAHs due to urbanization and the flocculation of river-borne and humic- and particle-associated PAHs induced by seawater mixing (29, 43). Total PAH concentrations in urban estuarine sediments may exceed 100 μg g^{-1} (55). The return to coal combustion as a major energy source in the future promises to reverse the recent trend of decreasing environmental PAH burdens brought on by the conversion from coal to oil and gas in the 1950s.

Phenanthrene ($C_{14}H_{10}$), although not particularly hazardous itself (45), is a good model compound for studying PAH biogeochemistry because it is intermediate in solubility and physical behavior (41, 46). As a result of detailed biochemical studies (3, 15, 30, 34), the degradative pathways for phenanthrene are well known. Common to all published reports is the initial conversion of phenanthrene to 1-hydroxy-2-naphthoic acid (1H2NA), which often accumulates in the growth medium and turns it an orange color (13, 15, 19, 32–34, 48, 56). In comparison with the analogous accumulation of salicylate during naphthalene degradation (26, 28, 36, 42, 52, 56), however, virtually nothing is known about the factors regulating the accumulation or subsequent utilization of 1H2NA during phenanthrene degradation.

Although 1H2NA accumulation is characteristic of most pure cultures that degrade phenanthrene, in a companion paper (21) we describe a *Mycobacterium* sp. which mineralizes phenanthrene via 1H2NA but that does so without accumulating the metabolite. We undertook the present study to determine whether (or which) results with pure cultures could be applied to processes of phenanthrene degradation by mixed cultures. The purpose was to identify and assess the quantitative significance of metabolic intermediates produced by consortia of estuarine microorganisms that degrade phenanthrene. With this information, it might then be possible to evaluate the efficacy of biodegradation methods which measure substrate disappearance or $^{14}CO_2$ evolution from labeled phenanthrene, but which do not consider the potentially important compartment of partially oxidized, soluble transformation products.

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MATERIALS AND METHODS

Media. Two types of phenanthrene growth media were employed in this study. These differed fundamentally in their phenanthrene concentrations and, consequently, in the physical states of the substrate. Solutions with low phenanthrene concentrations were prepared by pumping 75% artificial seawater (31) through a column (4.6 by 250 mm; stainless steel) packed with phenanthrene-coated glass beads (60/80 mesh) at a flow rate of 0.5 ml min^{-1}. The glass beads were prepared as described by May et al. (41). A column of this size generated phenanthrene solutions ranging from about 70 to 90% saturation. After dilution with 75% artificial seawater to give a half-saturated phenanthrene solution (0.5 mg liter^{-1}, as determined by measuring the A_{250}), 1.5 mg of NH_4NO_3, 1.5 mg of Tween 80, and 1 ml of 0.05 M phosphate buffer (pH 7.5) per liter were added. The pH was adjusted to 7.5, and the solution was sterilized by filtration (pore size 0.2 μm; Nuclepore Corp., Pleasanton, Calif.). Portions of 190 ml were aseptically dispensed into sterile 500-ml Erlenmeyer flasks modified by the addition of 8-mm (outer diameter) Pyrex (Corning Glass Works, Corning, N.Y.) side arms fitted with serum stoppers (16). Flasks were inoculated with 10 ml of untreated estuarine water or a 10^{-2} dilution of sediment and spiked with 10 μl (~1 μCi) of [9-^{14}C]phenanthrene in acetone solution (specific activity, 19.3 mCi mmol^{-1}; 98% radiochemical purity by thin-layer chromatography [TLC] and high-performance liquid chromatography [HPLC]; Amersham Corp., Arlington Heights, Ill.) and stoppered. Alternatively, 200-ml water samples were spiked with 10 μl of acetone in which the labeled phenan-

threne (~1 μCi) was diluted with unlabeled phenanthrene to result in a final sample concentration of 0.5 μg ml^{-1}. The total initial activity in each flask was determined from duplicate 1-ml portions taken immediately after inoculation. Enrichments were incubated at 18°C in the dark on a rotary shaker (140 rpm).

Media containing phenanthrene at concentrations in excess of its solubility were prepared in two parts. Part A was prepared by adding 150 ml of distilled water (Milli Q system; Millipore Corp., Bedford, Mass.), 80 mg of NH_4NO_3, and 1 mg of $FeCl_3 \cdot 6H_2O$ to 750 ml of 100% artificial seawater. The solution was filtered through 0.4-μm-pore-size filters (Nuclepore), and the pH was adjusted to 7.5. Portions (90 ml) were dispensed into 250-ml Erlenmeyer flasks and autoclaved. Part B was prepared by blending 200 mg of dry heat-sterilized (75 to 85°C, 24 h) phenanthrene (94.4% C) with 100 ml of sterile distilled water containing 80 mg of Tween 80 (59% C) and 300 μl of 0.05 M phosphate buffer (pH 7.5). Two 2.5-min blending periods with a 5-min interval to allow reintroduction of the bubble-suspended phenanthrene into the aqueous phase resulted in a fine dispersion. Portions (10 ml) of part B were added to flasks containing part A to produce a phenanthrene–Tween 80 broth with a phenanthrene to Tween 80 carbon ratio of 4:1 and an approximate C:N:P ratio of 100:10:2. The latter ratio varied, as did the initial phenanthrene concentration, due to evaporative losses during autoclaving, volatilization of hydrocarbon during sterilization, and errors inherent in taking portions from particulate suspensions. Volumes were sometimes doubled for incubations in 500-ml Erlenmeyer flasks, and phenanthrene concentrations were varied, as indicated, while maintaining a constant phenanthrene to Tween 80 carbon ratio.

Analyses. Degradation of radiolabeled phenanthrene at 0.5 mg $liter^{-1}$ was followed by periodic analysis of the $^{14}CO_2$ produced from respiration. Duplicate 1% portions of the cultures (2 ml of water, 4 ml of headspace) were collected with a syringe and injected into evacuated test tubes (15 by 125 mm) capped with butyl rubber serum stoppers and containing 1 ml of 2 N HCl. The CO_2 liberated was trapped on a strip (6 by 1 cm) of pleated Whatman no. 1 filter paper (Whatman, Inc., Clifton, N.J.) soaked with 0.2 ml of phenethylamine (National Diagnostics) and suspended from the serum stopper in a plastic cup. After 24 h of degassing with shaking, the filter papers were removed and swirled in 9 ml of Scintiverse E (Fisher Scientific Co., Pittsburgh, Pa.) and 5 ml of 95% ethanol (used to rinse the plastic cup) in 20-ml scintillation vials. Samples were counted (2-min preset time, 2% preset error) on a liquid scintillation counter (LS7000; Beckman Instruments, Inc., Fullerton, Calif.). Counts were converted to disintegrations per minute by use of the automatic quench compensation feature of the instrument. H number versus percent efficiency curves were constructed by using [^{14}C]toluene internal standards, with acetone used as a quenching agent. Careful attention to flask sampling details and scrupulous cleaning of test tubes (combustion at 475°C for 6 h) and stopper cup assemblies between uses resulted in extremely precise measures of $^{14}CO_2$ production. Precision among triplicate tubes averaged ±0.48% (±1 standard deviation) of the total initial activity (n = 87; range, 0.03 to 2.19). To conserve supplies, samples were routinely run in duplicate. Control tubes yielded apparent $^{14}CO_2$ activities amounting to 2 to 3% of the total initial activity due to trapping of volatilized [^{14}C]phenanthrene. At 4 to 5 days after spiking with radiolabeled phenanthrene, the radioactivity in control tubes dropped to background levels due to adsorption. Samples, uncorrected for controls, sometimes manifested this volatilization artifact early in the incubations.

Radioactivity in the flasks described above was separated into polar (including 1H2NA) and nonpolar (phenanthrene) fractions by sequential extractions with 5 ml of CH_2Cl_2 of a 5-ml culture fraction that was made alkaline by the addition of 1 drop of 2 N NaOH in 15-ml glass-stoppered centrifuge tubes. Tubes were centrifuged (10 min, 2,500 × g) after they were shaken for 1 min to give an upper aqueous layer, a lower organic layer, and an intervening pellicle of cells and debris. The first 5 ml of CH_2Cl_2 was removed with a Pasteur pipette and placed in a scintillation vial. After the second extraction, 4 ml of upper aqueous layer was removed with a glass syringe and transferred to a separate scintillation vial containing 1 ml of 2 N HCl. The second 5 ml of CH_2Cl_2 was then withdrawn and combined with the first extract, and the vials were left to evaporate (or degas) in the hood overnight. The following day, 1 ml of 100% ethanol was used to dissolve the organic soluble residue and 9 ml of scintillation cocktail was added to each set of vials. Radioactive counts in the vials were determined as described above, to give measures of undegraded and partially degraded phenanthrene.

The nature of the water-soluble activity was also assessed by HPLC analysis of culture supernatant fluids after centrifugation (12,100 × g, 10 min, 4°C). Elution fractions of 1 ml corresponding to the retention times of known phenanthrene metabolites were collected in scintillation vials containing 9 ml of cocktail. Further details of the HPLC procedure are given below.

Degradation of particulate phenanthrene was monitored by several methods. Substrate disappearance was determined by extracting duplicate 1- to 5-ml portions of the cultures in acid-washed and solvent-rinsed, 60-ml separatory funnels with two 10-ml portions of CH_2Cl_2 (Photrex grade; J. T. Baker Chemical Co., Phillipsburg, Pa.). Extracts were pooled in similarly cleaned, 25-ml graduated cylinders, and 50 to 100 mg of 100/200 mesh silca gel (Supelcosil; Supelco) was added to clear the extracts of Tween 80–H_2O emulsions. The extract was brought to volume before the A_{250} was measured on a spectrophotometer (DU-8; Beckman) in 1-cm quartz cuvettes. Tween 80 served to stabilize the medium; no losses of phenanthrene from uninoculated controls were detected.

Because the cells attached to phenanthrene particles, the enumeration of cells by plate counts or by direct epifluorescence microscopic counts proved unsuitable. Therefore, biomass was estimated as protein by the method of Lowry et al. (39) on a spectrophotometer (Spectronic 88; Bausch & Lomb, Inc., Rochester, N.Y.) by using bovine serum albumin as a standard. Cells were harvested in acid-washed centrifuge tubes (Corex; 12,100 × g, 10 min, 4°C); supernatant fluids were saved for the analysis of polar metabolites.

Analysis of phenanthrene metabolic intermediates was carried out by two methods. Since many of the known phenanthrene metabolites are hydroxylated aromatic compounds, culture supernatant fluids were analyzed for the presence of phenolic compounds by a modification of the Folin-Ciocalteau reaction outlined by Box (6). The A_{750} was measured on a spectrophotometer (Spectronic 88; Bausch & Lomb) after centrifugation to remove the precipitate that formed in the application of this method to seawater. The salt precipitate did not affect the linearity of the calibration. Resorcinol was used as a standard, and all values were expressed in micrograms of resorcinol equivalents (RE) per milliliter.

Phenanthrene degradation products were also analyzed by

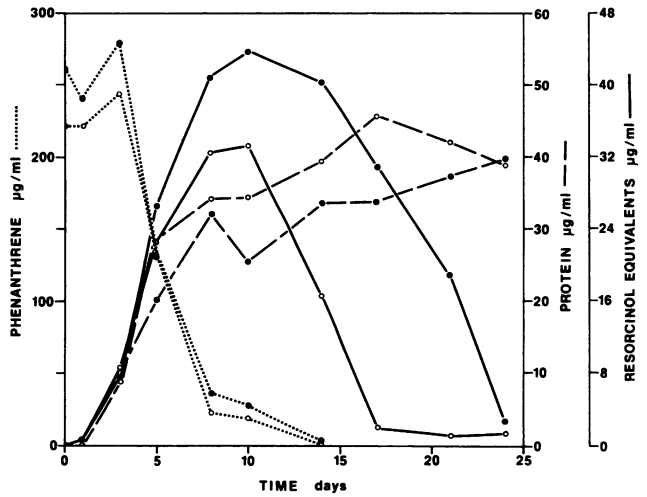

FIG. 1. Time course for phenanthrene degradation by parallel sediment enrichment cultures AP-31 (○) and AP-32 (●).

reverse-phase HPLC. Cold culture supernatants (20 to 250 µl) were injected directly into a gradient liquid chromatograph (model 332; Beckman) with dual pumps (model 110; Beckman) and a microprocessor controller-programmer (model 420; Beckman) and fitted with a 5-µm C-18 Adsorbosphere column (4.6 by 250 mm; Alltech Associates, Inc., Applied Science Div., State College, Pa.). A linear elution gradient (0 to 100% methanol at 5% min^{-1}) was used with a starting solvent of 100% H_2O adjusted to pH 2.75 with H_3PO_4. The solvents and the H_3PO_4 were HPLC grade (Baker). The flow rate was 1 ml min^{-1}, and detection was done by measuring the A_{254} in a spectrophotometer (model 100-40; Hitachi) fitted with a 20-µl flowthrough cell (model 155-00; Altex). Detector sensitivity was set to give full-scale deflection on a recorder (Heath) at an A_{254} of 0.1. Peak retention times were compared with those of authentic standards, which remained constant over the course of an experiment. No adverse effects on column performance were noted when this direct injection procedure was used.

To confirm their identity, metabolites were isolated by ethyl acetate extraction (2 times, 50 ml) of acidified culture supernatants (100 ml). Extracts were dried over anhydrous Na_2SO_4 and concentrated under high-purity N_2 gas. Concentrates and standards were applied to preparative TLC plates (2 mm by 20 cm by 20 cm) and developed in a solvent system of petroleum ether-toluene-acetone-acetic acid (80:20:10:4). Fluorescent spots were scraped from the plates and eluted in acetone, and the UV absorption spectra was recorded on a spectrophotometer (DU-8; Beckman) in quartz cuvettes.

Inocula. As inocula, samples of sediment, beach tar, surface microlayer, and bulk water were collected from locations around the Great Bay Estuary, N.H. A petroleum-contaminated soil sample collected from the University of New Hampshire bus depot was also used in these studies. Several phenanthrene-degrading isolates were obtained from these enrichments on the basis of their ability to form clearing zones in a crystalline phenanthrene overlay on agar plates (35). These isolates were subsequently tested by the methods described above to depict their patterns of phenanthrene degradation and metabolite formation.

RESULTS

All estuarine and soil enrichment cultures followed the same general pattern of degradation when phenanthrene was supplied in particulate suspension. The time courses for duplicate incubations designated AP-31 and AP-32 with initial phenanthrene concentrations of 220 and 260 mg $liter^{-1}$, respectively, are shown in Fig. 1. Sediment from a boat ramp at Adam's Point, N.H., was used as the initial inoculum. Time zero in Fig. 1 represents the time of the second transfer of the mixed culture to fresh phenanthrene–Tween 80 broth cultures. The preadaptation of this culture to growth on phenanthrene led to the rapid disappearance of the hydrocarbon during the first stage of growth. Phenanthrene disappearance was accompanied by an increase in biomass and the production of Folin-Ciocalteau reactive intermediates (in RE), resulting in an orange coloration of the growth medium. At 10 days, Folin-Ciocalteau-reactive product concentrations were maximal, and protein concentrations plateaued or decreased slightly. Further increases in protein concentrations occurred during the second stage of degradation during which metabolites were consumed. Culture AP-32 lagged 7 days behind culture AP-31 during this stage. After the disappearance of metabolites from the medium, protein concentrations began to decrease.

The time course for phenanthrene degradation in primary enrichments, with surface microlayer and bulk water microbial populations from a marina in Prescott Park, Portsmouth, N.H., used as inocula, is shown in Fig. 2. Initial phenanthrene concentrations were about 40 mg $liter^{-1}$. The microlayer sample (mean thickness of 45 µm collected with a glass plate) was enriched threefold in total viable heterotrophs relative to the underlying bulk water and showed a shorter lag period prior to phenanthrene degradation. Phenanthrene and metabolites disappeared from the microlayer sample by 23 days, with a maximum protein yield of about 15 µg ml^{-1}. Degradation by the bulk water population occurred more slowly, with metabolites just beginning to disappear at 33 days and maximum protein concentrations of about 10 µg ml^{-1}. Secondary increases in protein concentrations fol-

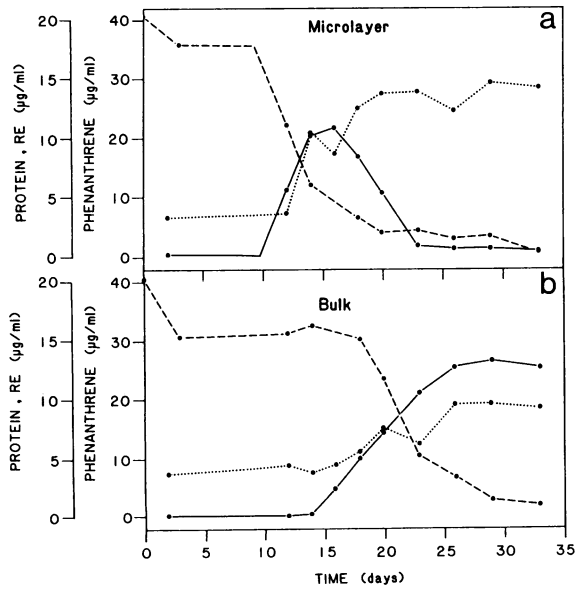

FIG. 2. Time course for phenanthrene degradation by surface microlayer (a) and bulk water (b) microbial populations from Prescott Park. Breaks in the phenanthrene (dashed line) and RE (solid line) curves at 10 days in the microlayer incubation are extrapolations based on subsequent data points. Protein is denoted by the dotted lines.

lowed temporary decreases that coincided with the depletion of phenanthrene (<10 μg ml^{-1} at 16 days) and Folin-Ciocalteau-reactive intermediates (26 days) during the microlayer incubation and the depletion of phenanthrene (23 days) during the bulk water incubation.

Analysis of culture supernatant fluids by HPLC during the course of these incubations revealed that the onset and disappearance of Folin-Ciocalteau reactivity was due to the production and subsequent consumption of a single component whose retention time matched that of 1H2NA. Other UV-absorbing intermediates were present only in minor amounts. The HPLC chromatogram for the Prescott Park bulk water sample corresponding to day 34 of the incubation shown in Fig. 2 is shown in Fig. 3a. The retention time of the major peak in this sample matched that of 1H2NA. Likewise, on TLC plates, acidified culture extracts gave a blue fluorescent spot with an R_f value that matched that of 1H2NA. The identity of the major metabolite was confirmed by matching the UV absorption spectrum of authentic 1H2NA with that of the TLC-isolated product (Fig. 3b).

Since only a single compound was responsible for Folin-Ciocalteau reactivity in these enrichments, RE could be used to provide a quantitative measure of metabolite production after intercalibration with 1H2NA. A maximum of 33 and 36 mol% of the initial phenanthrene could be accounted for as 1H2NA in sediment enrichment cultures AP-31 and AP-32, respectively; and a maximum of 59 and 72 mol% was present as 1H2NA in the Prescott Park microlayer and bulk water enrichments, respectively.

During incubations with [9-^{14}C]phenanthrene, degradation beyond 1H2NA was necessary before $^{14}CO_2$ was evolved. A degradation pattern in which some of the phenanthrene carbon accumulated as 1H2NA (labeled at C-3 or C-4) and was subsequently mineralized would then be expected to show $^{14}CO_2$ evolution curves similar to the biphasic growth (protein) curves in Fig. 1 and 2. Such a two-stage pattern of mineralization was evidenced in the stepped $^{14}CO_2$ evolution curves shown in Fig. 4 for several water and sediment samples collected from the estuary over a 1-year period. In general, this stepped pattern was more pronounced in the more active samples, i.e., those with a short lag period, a high initial rate of mineralization, and a high final percent mineralization. Because of the precision of the $^{14}CO_2$ measurement, however, two stage patterns were also discerned in samples with low activities.

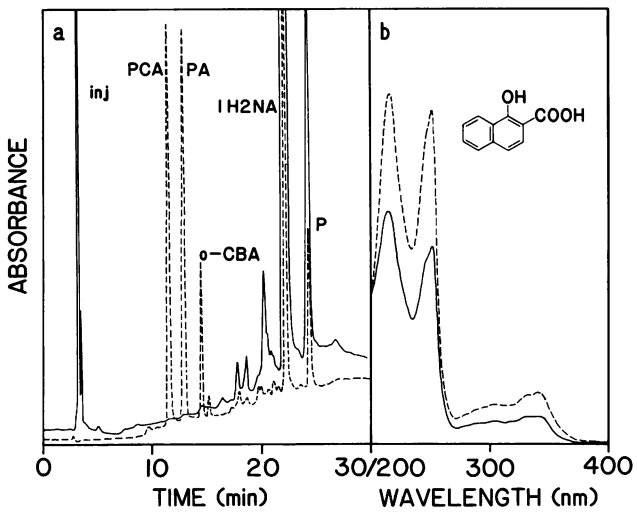

FIG. 3. Analysis of metabolites in a Prescott Park bulk water sample (see Fig. 2b) after 34 days of incubation. (a) High-performance liquid chromatograms for 34-day culture supernatant fluid (solid line) and a standard mixture made up of authentic compounds dissolved in distilled water (dashed line). Abbreviations: PCA, protocatechuic acid; PA, phthalic acid; o-CBA, *o*-carboxybenzaldehyde; 1H2NA, 1-hydroxy-2-naphthoic acid; P, phenanthrene; inj, injection peak. (b) UV absorption spectra of authentic 1H2NA (dashed line) and the TLC-isolated product after ethyl acetate extraction of the acidified bulk water sample at 34 days (solid line).

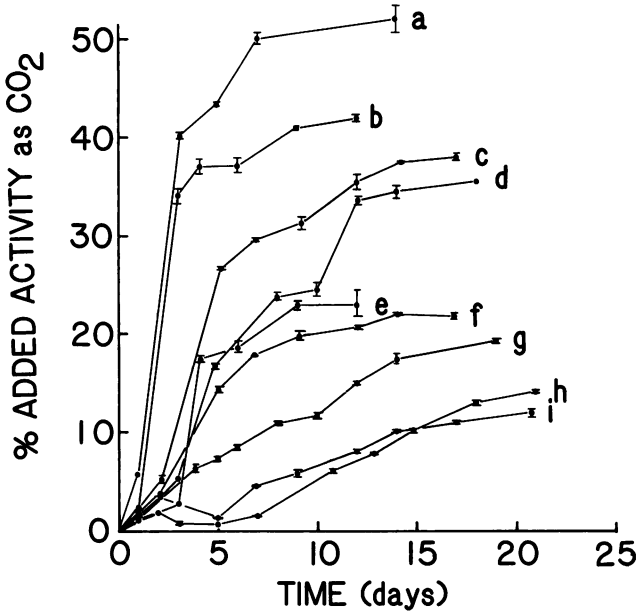

FIG. 4. Two-stage mineralization curves showing evolution of $^{14}CO_2$ during low-concentration (0.5 mg $liter^{-1}$) incubations with [9-^{14}C]phenanthrene for several surface microlayer (curves d and h), bulk water (curves a, c, f, g, and i), and sediment (curves b and e) samples. Error bars for curves d, g, and h are ±1 standard deviation around the mean of triplicate determinations. All other bars represent the ranges of duplicate determinations.

At the low phenanthrene concentrations (0.5 mg $liter^{-1}$) used in the radiolabel experiments, water-soluble products did not account for more than about 10% of the total initial activity. In some cases, the water-soluble radioactivity showed production and subsequent consumption curves similar to the RE curves in Fig. 1 and 2, while in others the activity increased slowly over time. Approximately one half (0.46 ± 0.12; mean ± 1 standard deviation) of the water-soluble radioactivity eluted in the HPLC fraction corresponding in retention time to 1H2NA after 7 days of incubation of eight water samples. In accordance with these values, the incremental $^{14}CO_2$ produced during the second stage of mineralization ranged from approximately 1 to 11% of the total initial activity (Fig. 4).

Isolates were obtained from enrichments on the basis of their ability to form clearing zones in a crystalline phenanthrene overlay after colony development on phenanthrene (0.02%) agar plates. Usually, clearing of the phenanthrene overlay caused a brown or orange coloration of the agar below due to diffusion of soluble intermediates. When grown in phenanthrene–Tween 80 broth, these isolates accumulated 1H2NA (up to 60 μg ml^{-1}) without a parallel increase in biomass. Eventually, 1H2NA production stopped, even though abundant particulate phenanthrene remained. This was not attributable to acid inhibition since the pH in the naturally buffered seawater medium never dropped below 7.

The HPLC chromatograms of phenanthrene–Tween 80 culture supernatants for four isolates obtained from enrichments inoculated with petroleum-contaminated soil (BD3-E and BD2-Y), estuarine sediment (APBS61-B), or beach tar (TBB-C) are shown in Fig. 5. The organisms, all of which were gram-negative rods, showed the production of high concentrations of 1H2NA as the sole or major metabolite while phenanthrene was degraded.

One organism (isolate PC1), a yellow-pigmented, nonmotile, gram-negative rod, was tested for its ability to degrade phenanthrene beyond 1H2NA by monitoring $^{14}CO_2$ production from labeled phenanthrene over a range of initial concentrations (0.084 to 68.5 mg liter^{-1}) and with KNO_3 or NH_4Cl used as N sources. The results (Table 1) indicated that phenanthrene mineralization by isolate PC1 was concentration dependent. At concentrations below 2 or 3 mg liter^{-1}, 40 to 47% of the added radioactivity was present as $^{14}CO_2$ after 19 days, and intermediates did not accumulate. At 68.5 mg liter^{-1}, $^{14}CO_2$ production was lower (25 to 30% of the initial activity) and intermediate concentrations remained high even after 3 weeks. The 19-day RE estimate of metabolite accumulation was higher than the estimate based on water-soluble radioactivity due to incomplete equilibration between the unlabeled particulate phenanthrene and the [^{14}C]phenanthrene added in acetone. Likewise, solvent-extractable ^{14}C was probably an underestimate of the actual percentage of the initial phenanthrene that was undegraded after 19 days. A slightly higher production of $^{14}CO_2$ and intermediates was noted at high initial phenanthrene concentrations when KNO_3 rather than NH_4Cl was the N source.

Occasionally, isolates were obtained which degraded phenanthrene without significant accumulation of metabolites (including 1H2NA), regardless of the initial phenanthrene concentration. These isolates attained high cell densities and utilized all of the phenanthrene provided to them. The characteristics of one such organism, a *Mycobacterium* sp., are given in a companion paper (21).

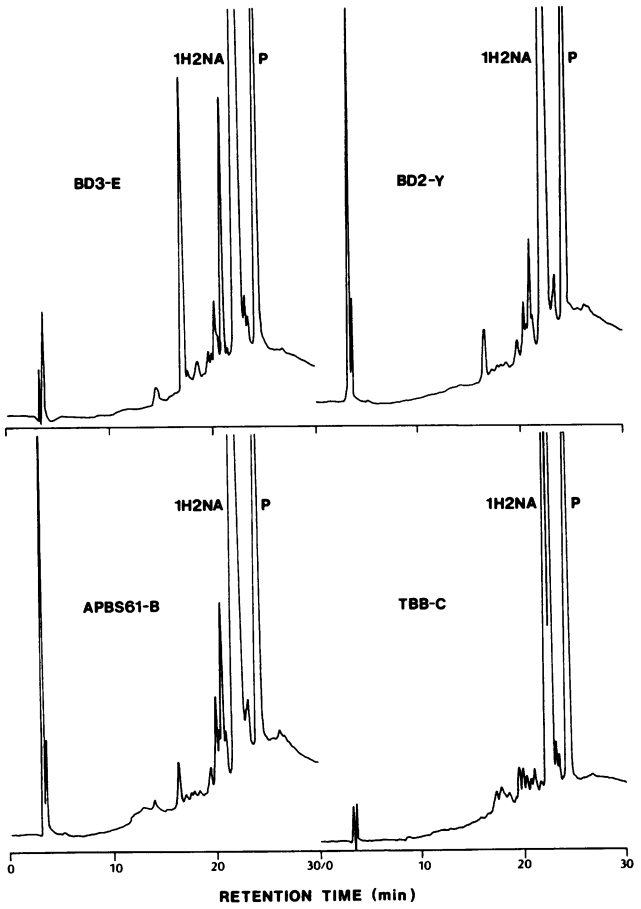

FIG. 5. High-performance liquid chromatograms of four phenanthrene-degrading isolates showing the production of the predominant intermediate 1H2NA. P, Phenanthrene.

TABLE 1. Effects of nitrogen source and phenanthrene concentration on the percent distribution of radioactivity between CO_2, water-soluble, and CH_2Cl_2-extractable fractions[a]

Nitrogen source	Initial phenanthrene concn (μg ml^{-1})[b]	% Initial radioactivity in the following fractions:			RE concn at 19 days (μg ml^{-1})
		CO_2	Water-soluble	CH_2Cl_2-extractable	
NH_4Cl	0.084	42.3	3.9	3.8	<1
	0.38	42.4	4.2	3.2	<1
	2.65	40.0	4.7	4.5	<1
	68.5	25.3	6.6	17.0	4.6
KNO_3	0.084	46.8	3.5	3.5	<1
	0.38	41.9	3.4	3.8	<1
	2.65	44.0	5.9	4.5	<1
	68.5	30.1	7.1	13.0	6.4

[a] Effects were determined after 19 days of growth of isolate PC-1 on [9-^{14}C]phenanthrene.

[b] Initial phenanthrene concentrations were varied by dilution with unlabeled compound.

DISCUSSION

Metabolite accumulation is a common characteristic of PAH (but not benzene) catabolism by bacteria (1). The most common intermediates produced from PAH are the *o*-hydroxy, carboxy compounds which result from cleavage of a terminal ring. The initial dihydroxylation sites and end ring cleavage products of naphthalene, phenanthrene, anthracene, benzanthracene, and benzo(*a*)pyrene are shown in Fig. 6. The well-studied transformations involving naphthalene and phenanthrene have shown salicylate and 1H2NA, respectively, to be the major or sole transformation products. Work with anthracene has been limited, but 2-hydroxy-3-naphthoic acid (2H3NA) has been identified as a major intermediate (15, 49). The recent identification of 1-hydroxy-2-anthranoic acid as the dominant metabolite of benz(*a*)anthracene degradation by a *Beijerinckia* sp. (W. R. Mahaffey, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, K166, p. 199) was the first report of ring cleavage of a PAH with more than three rings. While the 9,10-dihydroxy derivative of benzo(*a*)pyrene is the major early transformation product of this compound (20), the ring cleavage product depicted in Fig. 6 is hypothetical. End ring cleavage, however, may account for reports of the slow production of $^{14}CO_2$ from [7,10-^{14}C]benzo(*a*)pyrene by mixed cultures (14, 18, 46, 50) and, recently, by a fungus (7). The similarities of these reactions suggest a generalized approach by bacteria to PAH transformations.

Because pyruvate is readily utilized by most organisms, its production during the end ring cleavage reactions depicted in Fig. 6 would violate at least one condition required to call these reactions cometabolic (27). However, the production of intermediates which can not be further metabolized by an

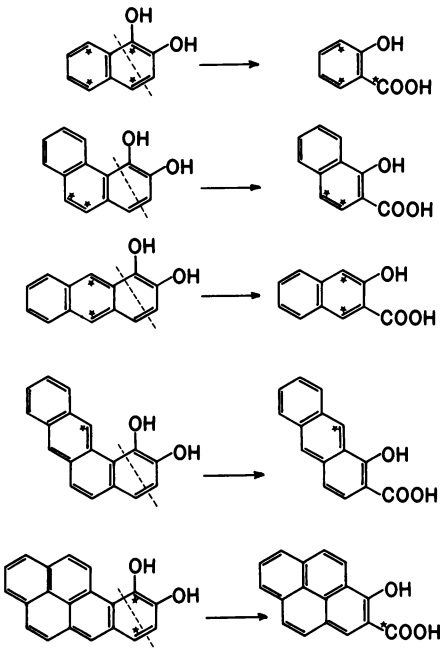

FIG. 6. Initial reactions in the bacterial dissimilation of naphthalene, phenanthrene, anthracene, benzanthracene, and benzo(*a*)pyrene (from top to bottom, respectively). Major sites of dihydroxylation and planes of ring cleavage (dashed lines) are shown on the left. On the right are shown the *o*-hydroxy, carboxy intermediates frequently reported to accumulate during PAH degradations. The ring cleavage product for benzo(*a*)pyrene is hypothetical. Stars indicate the most common positions of ^{14}C labeling in commercially available PAHs and the bacterial metabolites of them.

organism through the coincidental action of low-specificity (dioxygenase) enzymes is common to such (cometabolic) reactions (17, 44). The production of salicylate and 2H3NA from naphthalene and anthracene, respectively, by a *Corynebacterium* sp. growing on hexadecane and glucose, respectively, has been attributed to cometabolism (12). Reports of $^{14}CO_2$ evolution from end ring-labeled benzo(*a*)pyrene may be another example of such a process and not necessarily evidence of complete mineralization (Fig. 6). However, cometabolism does not describe the accumulation of salicylate (28, 53), 1H2NA (33, 34), or 2H3NA (15) by organisms capable of the complete dissimilation of the respective parent PAHs. While it is not clear why an organism with the potential to mineralize a hydrocarbon does not do so in a coordinated manner without accumulating metabolites, the extensive work on naphthalene metabolism may shed some light on this problem.

Much of the interest in naphthalene metabolism is directed at maximizing production of salicylate and other intermediates (10) for chemical and industrial uses. Shamsuzzaman and Barnsley (52), working with a *Pseudomonas* sp., reported that the molar ratio of salicylate produced to naphthalene consumed was 0.53. Others have reported values as high as 0.87 (36). Hosler (26) noted that during the early stages of naphthalene degradation, cells grew efficiently with little salicylate accumulation; as degradation proceeded, stoichiometric salicylate production from naphthalene occured and cell growth at the expense of the ring cleavage product (pyruvate) was poor.

It was recently reported (2) that a *Pseudomonas* sp. accumulated salicylate and grew poorly when NH_4^+ or NH_4NO_3 was supplied as the nitrogen source. When grown with NO_3^-, salicylate was detected in trace amounts only early in the incubation, and the culture attained high cell densities. Oxygen consumption and CO_2 production were also sevenfold higher when NO_3^- was used, suggesting a much more efficient use of the substrate. The phenomenon described by these investigators (2) does not apply to naphthalene degraders in general, however (54), nor to the analogous accumulation of 1H2NA during phenanthrene degradation (Table 1).

With many organisms, the salicylate that is produced during naphthalene dissimilation is later consumed as the primary substrate is exhausted (28, 36, 42, 52, 53). Diauxic growth curves corresponding to the successive utilization of naphthalene and salicylate by *Pseudomonas aeruginosa* (28) and *Pseudomonas putida* (53) have been presented. In the latter study, mutant strains bearing a defective plasmid lost the ability to oxidize the salicylate which accumulated during naphthalene degradation. At the point of maximum salicylate concentration, cell counts decreased preceding a second period of growth, during which the salicylate was utilized (42). Morphological changes associated with this shift in metabolism have been noted (26).

When present in excess, then, naphthalene is not treated by most organisms as a single substrate to be mineralized by consecutive enzymatic reactions. Rather, under these substrate-sufficient conditions, naphthalene is taken to be a readily available source of pyruvate and the salicylate that is produced assumes the role of a second substrate. Once the naphthalene is depleted, and provided that the acidic byproduct does not attain inhibitory concentrations, organisms may then synthesize a new suite of enzymes, or derepress existing ones, for the efficient utilization of salicylate (22).

A similar approach to phenanthrene degradation is the most likely explanation for the accumulation of 1H2NA by pure cultures and for the two-stage mineralization pattern observed here for mixed microbial cultures. At high phenanthrene concentrations (>0.5 mg liter^{-1}), enrichment cultures are dominated by phenanthrene degraders which, although perhaps able to mineralize the hydrocarbon, degrade it only as far as 1H2NA, making the ring cleavage product pyruvate available for growth. In comparison with phenanthrene mineralizers (21), which derive three molecules of pyruvate and one of acetaldehyde from the coordinate mineralization of phenanthrene via *meta* cleavage (11), these phenanthrene degraders must process much more substrate to acquire an equivalent amount of energy. The result is that 1H2NA is produced more rapidly than it is consumed during the first stage of mineralization and thus accumulates. Only on depletion of the phenanthrene is 1H2NA, as the second substrate, degraded, allowing a second stage of protein production to ensue.

At low phenanthrene concentrations, 1H2NA accumulates to a lesser extent. Under these nutrient-limiting conditions, concurrent utilization of phenanthrene and 1H2NA allows phenanthrene degraders to mineralize the substrate more efficiently (Table 1). Alternatively, phenanthrene mineralizers may be more competitive at low substrate concentrations because of their greater inherent efficiency of substrate utilization. The result in either case is that 1H2NA does not accumulate in high molar proportion to the phenanthrene that is degraded, and a more subtle, two-stage pattern of mineralization is sometimes, but not always, discerned.

The two-stage mineralization pattern observed at high phenanthrene concentrations was characteristic of all the samples examined, regardless of the extent of PAH pollution

and, hence, preexposure (preadaptation) of the community to phenanthrene. In comparison with the samples from Prescott Park (Fig. 2), however, the more rapid phenanthrene degradation and lower molar conversion to 1H2NA by the AP cultures (Fig. 1) may have been due to selection for a more efficient phenanthrene-degrading population during repeated transfer of the AP culture to fresh medium.

The rapid transformation of PAHs to polar intermediates may have adaptive significance. In an open system, the advantage of this mode of metabolism is that hydrophobic aromatic compounds which could potentially disrupt membrane functions (1) are converted to water-soluble products which are free to diffuse or be advected away. Incomplete mineralization of PAHs may be the procaryotic equivalent of the eucaryotic detoxification strategy of solubilization of PAHs by conjugation (9). In soil, sedimentary, and aquatic environments, the reactivity of the aromatic acid and phenolic products would favor their interaction or condensation with natural humic materials which contain structural units with similar compositions (5, 51). Thus, PAHs may be rendered innocuous without being completely mineralized.

Of the few studies concerned with PAH biodegradation by environmental samples, most do not consider polar transformation products as a separate compartment. Notable exceptions are the works of Herbes and co-workers (23, 25). They noted the almost complete conversion of [^{14}C]naphthalene and [^{14}C]anthracene to polar products during short-term incubations with mixed cultures of environmental isolates (25). With water samples, polar ^{14}C was the dominant anthracene transformation product, but it accounted for only a small percentage of the added radioactivity (23). From sediments, $^{14}CO_2$ and bound ^{14}C (perhaps a humic acid-2H3NA adduct) were the major products recovered after incubations with labeled anthracene (24). Transformation to polar products may also be implicated by the slow rates of $^{14}CO_2$ production from labeled anthracene, despite its rapid removal from a marine enclosure (38) and from sediment slurries (4). We are unaware of any similar studies in which phenanthrene degradation by natural samples has been examined.

Herbes and Schwall (24) presented $^{14}CO_2$ evolution curves for naphthalene that closely resembled the stepped curves for phenanthrene observed here. Using a procedure similar to the one described here for seawater samples, Reichardt et al. (47) also presented $^{14}CO_2$ evolution data from U-^{14}C-labeled biphenyl and monochlorobiphenyl compounds (labeled uniformly on one ring) which showed stepped curves. Biphenyl (or chlorinated biphenyl) degradation is often accompanied by accumulation of (chloro)benzoic acid or other ring cleavage products (8), and although no significant radioactive pool of water-soluble transformation products was detected here, the analyses were performed at the end of the incubation (14% conversion to CO_2), after the incremental $^{14}CO_2$ was produced.

The transformation of PAHs to polar intermediates has important implications regarding the methodology used to measure PAH biodegradation. The disappearance of parent compounds or the production of $^{14}CO_2$ from exterior ring-labeled PAHs during incubations with natural samples should not be equated with mineralization. Conversely, $^{14}CO_2$ evolution from interior ring-labeled PAHs (Fig. 6) would provide an underestimation of transformation rates at high test concentrations. For phenanthrene at low concentrations (<1 mg liter^{-1}), however, metabolites constitute a minor fraction of the ^{14}C added, and $^{14}CO_2$ production is a reasonable index of degradation. Nevertheless, from the shapes of the mineralization curves depicted here (Fig. 4), the biodegradation rate data derived by connecting a time zero and some later $^{14}CO_2$ evolution datum point may be erroneous. Use of the procedure described here for monitoring mineralization of ^{14}C-labeled PAHs provides information and precision that are unattainable by other methods.

ACKNOWLEDGMENTS

We thank M. Ikawa for allowing us the generous use of the HPLC. We also thank T. Eighmy for carefully reviewing the manuscript. This study benefited from discussions with W. R. Chesbro and R. P. Blakemore. We are grateful to them.

This work is the result of research sponsored by the NOAA National Sea Grant College Program, Department of Commerce, under grant NA-81-AA-D-00035 to the University of New Hampshire.

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