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Rate of Microbial Transformation of Polycyclic Aromatic Hydrocarbons: a Chromatographic Quantification Procedure[1]

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A chromatographic procedure has been developed for isolating and quantifying microbial transformation products of ^{14}C-labeled polycyclic aromatic hydrocarbons. Transformation rates of naphthalene, anthracene, benz(a)anthracene, and benz(a)pyrene by a mixed bacterial population have been measured. With this procedure, extremely slow or incomplete transformations may be quantified that would not be detectable by previously used techniques.

Polycyclic aromatic hydrocarbons (PAH) are fused-ring compounds, some of which are highly carcinogenic, which are found in wastewaters from such high-temperature industrial processes as coking and petroleum refining (1). Some PAH, including the carcinogens benz(a)anthracene and benz(a)pyrene, are susceptible to transformation to oxygenated derivatives by microbial action (6). However, little is known of the rates at which these transformations occur in the environment. Previous studies have used measurement of oxygen utilization (9), disappearance of the initial compound (2, 12), or CO_2 evolution (11) to estimate rates of microbial transformation. None, however, is able both to detect extremely slow transformation processes and to quantitate transformations that do not proceed to complete degradation (i.e., evolution of CO_2). To overcome these limitations, we have developed a chromatographic procedure to separate and quantify transformation products of ^{14}C-labeled PAH compounds. We report tests with known compounds and results of initial studies of microbial transformation products.

Mixed PAH-utilizing cultures were isolated from soil obtained at a desert oil-drilling site (Riverdale, Calif.), which has been in continuous operation for several decades. Additional cultures were obtained from a bench-scale wastewater treatment unit, which requires phenol-degrading bacteria. Microorganisms were grown by inoculation of soil and wastewater samples into autoclaved basal inorganic medium (4) saturated with naphthalene or phenanthrene. By colony morphology on hydrocarbon-enriched agar plates several bacterial strains (mainly opaque and transparent mucous whites and yellows) were isolated; no further attempt was made to characterize or identify the strains.

To determine microbial PAH transformation rates, cells from an exponential culture were centrifuged (12,000 × *g* for 10 min), washed, and suspended in 50 ml of hydrocarbon-free basal inorganic medium. Approximately 10^4 to 10^5 dpm (0.02 to 0.2 μg) of an individual ^{14}C-labeled PAH compound (obtained from ICN, Amersham/Searle, or American Radiochemical) was added in 4 μl of acetone, and the culture was incubated on a rotary shaker (150 rpm) at 23°C for 2 to 3 days. Autoclaved cells incubated similarly served as controls. At intervals, portions were removed and extracted in triplicate (after acidification) with ethyl acetate (10). Prior to chromatographic separation, extracts were combined and gently evaporated to near dryness, and the residue was redissolved in benzene.

Tests with pure compounds (Aldrich; reagent grade) demonstrated that representative PAH compounds and dihydrodiol, diol, and dione derivatives were quantitatively extracted with ethyl acetate after acidification; phenolic compounds, however, were not extractable under basic conditions. Similarly, extraction recovery of ^{14}C after incubation of [9-^{14}C]anthracene with a mixed naphthalene-grown culture increased from 20% at pH 11 to 61% at pH 7, 72% at pH 4.8, and 79% at pH 2, thus suggesting that substantial fractions of metabolic PAH alteration products are acidic. Recoveries of ^{14}C were identical after 30-min and 15-h extractions. Cell digestion in 0.1 N NaOH increased recovery by only 3% and was not deemed necessary.

To separate unaltered PAH from PAH metabolites in extracts, Silica Gel G (EM Labora-

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tories) columns (11 by 30 mm) were prepared in benzene. A 5-ml sample extract and five 1-ml sample beaker rinses were applied to each column and eluted sequentially with 20 ml of benzene and 25 ml of butanol–acetic acid (19:1, vol/vol). Ten-milliliter portions of benzene eluant and 5-ml portions of butanol–acetic acid were collected; ^{14}C activity in each fraction was determined by liquid scintillation counting. Each sample beaker was then rinsed with butanol–acetic acid, which was counted separately. Column packings were used once and discarded. Greater than 85% of the ^{14}C applied to the columns was consistently recovered.

Fractionation positions on silica gel columns of representative PAH compounds and potential metabolites are shown in Table 1. Column elution with up to 5 column volumes (40 ml) of benzene resulted in quantitative elution of PAH but negligible movement of dihydrodiols, diols, diones, or carboxylic acids. The three aldehydes tested were partially eluted in the benzene fraction; however, all known microbial transformation products of PAH are diols, dihydrodiols, diones, or carboxylic acids (3–8), none of which were eluted in the benzene fraction.

In tests with soil-derived microorganisms several naphthalene-grown isolates rapidly converted [^{14}C]anthracene to compounds that eluted in the butanol–acetic acid column fraction. Tests with two strains show a fivefold difference in the transformation rate per cell. Rates were proportional to cell density over the range tested (10^2 to 10^4/ml). To compare alteration rates of other PAH compounds, portions of a mixed culture were added to flasks containing 2×10^4 dpm of either ^{14}C-labeled naphthalene, anthracene, benz(a)anthracene, or benz(a)pyrene; at intervals samples were removed, extracted, and fractionated. Naphthalene and anthracene were completely converted (Fig. 1) to polar compounds within 1 h, whereas alteration of benz(a)anthracene and benz(a)pyrene proceeded 10^3 to 10^4 times more slowly. Although naphthalene volatilized during the experiment, precluding a mass balance, nearly all (88 to 105%) the ^{14}C added initially as [9-^{14}C]anthracene was recovered in the column

TABLE 1. *Recovery of PAH compounds and oxygenated derivatives in silica gel column chromatographic fractions*[a]

Compound	% Recovered in each fraction	
	Benzene	Butanol–acetic acid
[1-^{14}C]naphthalene	98	2
[9-^{14}C]anthracene	100	0
[9-^{14}C]phenanthrene	99	1
[5,6-^{14}C]benz(a)anthracene	100	0
[7,10-^{14}C]benz(a)pyrene	100	0
2,3-Dihydroxynaphthalene	0	100
1-Naphthalenecarboxylic acid	0	100
1-Naphthaldehyde	92	8
(1-Naphthyl)acetic acid	0	100
9-Phenanthrenecarboxaldehyde	91	9
9-Anthracenecarboxaldehyde	44	56
9-Anthracenecarboxylic acid	0	100
trans-4,5-Benz(a)pyrene dihydrodiol	0	100
trans-9,10-Benz(a)anthracene dihydrodiol	1	99
Benz(a)pyrene-6,12-dione	0	100
Benz(a)pyrene-3,6-dione	0	100
Benz(a)pyrene-4,5-dione	0	100

[a] Labeled compounds were quantified by liquid scintillation; unlabeled compounds were quantified by ultraviolet spectrophotometry after fraction evaporation and redissolution in methanol.

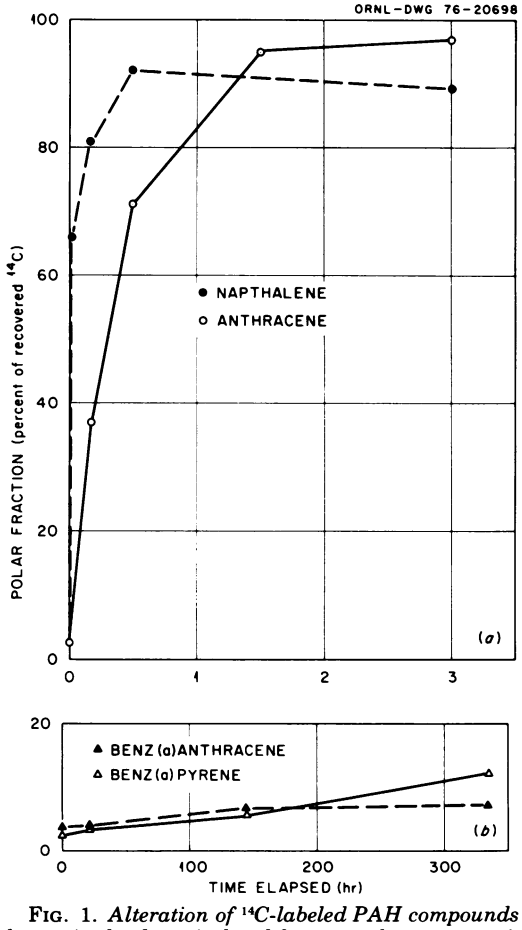

FIG. 1. *Alteration of ^{14}C-labeled PAH compounds by a mixed culture isolated from petroleum-contaminated soil. Cell inocula: approximately 10^4/ml. Polar fractions of autoclaved controls (not shown) for all four compounds ranged from 1.0 to 3.7% of the total recovered ^{14}C over the experimental time. 23°C.*

fractions; losses, which would indicate evolution of gaseous ^{14}C, were not observed. Significantly, use of the three standard methods to determine PAH transformations gave erroneous results: neither oxygen utilization nor disappearance of the initial compound is sufficiently sensitive to detect the minimal (less than 0.03%/h) alterations of benz(a)pyrene and benz(a)anthracene observed in Fig. 1, whereas entrapment of $^{14}CO_2$ would drastically underestimate the alteration rate of anthracene.

The chromatographic method described permits an initial determination of the relative potential importance of microbial transformations of PAH compounds in the environment. Rates of PAH alteration by water-borne microorganisms may be determined by direct application. Rate measurement in sediment samples by an alternate extraction procedure has been optimized also, and application to the determination of PAH transformation rates in petroleum-contaminated and uncontaminated sediments is presently underway.

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LITERATURE CITED

1. **Andelman, J. B., and M. J. Seuss.** 1970. Polynuclear aromatic hydrocarbons in the water environment. Bull. W.H.O. 43:479–508.
2. **Barnsley, E. A.** 1975. The bacterial degradation of fluoranthene and benzo(a)pyrene. Can. J. Microbiol. 21:1004–1008.
3. **Davies, J. I., and W. C. Evans.** 1964. Oxidative metabolism of naphthalene by soil pseudomonads. Biochem. J. 91:251–261.
4. **Evans, W. C., H. N. Fernley, and E. Griffiths.** 1965. Oxidative metabolism of phenanthrene and anthracene by soil pseudomonads. Biochem. J. 95:819–831.
5. **Gibson, D. T.** 1968. Microbial degradation of aromatic compounds. Science 161:1093–1097.
6. **Gibson, D. T., 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.
7. **Gibson, D. T., R. L. Roberts, M. C. Wells, and W. M. Kobal.** 1973. Oxidation of biphenyl by a *Beijerinckia* species. Biochem. Biophys. Res. Commun. 50:211–219.
8. **Jerina, D. M., J. W. Daly, A. M. Jeffrey, and D. T. Gibson.** 1971. *Cis*-1,2-dihydroxy-1,2-dihydronaphthalene: a bacterial metabolite from naphthalene. Arch. Biochem. Biophys. 142:394–396.
9. **Malaney, G. W., P. A. Lutin, J. J. Cibulka, and L. H. Hickerson.** 1967. Resistance of carcinogenic organic compounds to oxidation by activated sludge. Water Pollut. Control 39:2020–2029.
10. **Sims, P.** 1970. Qualitative and quantitative studies on the metabolism of a series of aromatic hydrocarbons by rat-liver preparations. Biochem. Pharmacol. 19:795–818.
11. **Sisler, F. D., and C. E. Zobell.** 1947. Microbial utilization of carcinogenic hydrocarbons. Science 106:521–522.
12. **Walker, J. D., and R. R. Colwell.** 1974. Microbial petroleum degradation: use of mixed hydrocarbon substrates. Appl. Microbiol. 27:1053–1060.