

## Bacterial Oxidation of Chemical Carcinogens: Formation of Polycyclic Aromatic Acids from Benz[*a*]anthracene

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A *Beijerinckia* strain designated strain B1 was shown to oxidize benz[*a*]anthracene after induction with biphenyl, *m*-xylene, and salicylate. Biotransformation experiments showed that after 14 h a maximum of 56% of the benz[*a*]anthracene was converted to an isomeric mixture of three *o*-hydroxypolyaromatic acids. Nuclear magnetic resonance and mass spectral analyses led to the identification of the major metabolite as 1-hydroxy-2-anthranic acid. Two minor metabolites were also isolated and identified as 2-hydroxy-3-phenanthroic acid and 3-hydroxy-2-phenanthroic acid. Mineralization experiments with [12-<sup>14</sup>C]benz[*a*]anthracene led to the formation of <sup>14</sup>CO<sub>2</sub>. These results show that the hydroxy acids can be further oxidized and that at least two rings of the benz[*a*]anthracene molecule can be degraded.

Polycyclic aromatic hydrocarbons are ubiquitous environmental contaminants. They are formed by the thermal alteration of organic compounds, and the types of molecules produced depend on the pyrolysis temperatures (4, 5). This class of compounds is interesting because many of them are chemical carcinogens. Extensive studies have shown that mammals oxidize polycyclic aromatic hydrocarbons to reactive electrophilic intermediates (23). The absolute stereochemistry of the ultimate carcinogens formed from several different polycyclic aromatic hydrocarbons has been determined, and it appears that molecules with the bay region are preferentially oxidized to bay region diol epoxides (17). Although four optically active bay region diol epoxides can be formed from a single bay region polycyclic hydrocarbon, the most tumorigenic enantiomer is almost always the (*R,S*)-diol-(*S,R*)-oxide, as shown in Fig. 1 for benz[*a*]anthracene (15).

Certain species of fungi also show similar enantiomeric specificity with respect to the oxidation of polycyclic aromatic hydrocarbons (6, 14). In contrast, bacteria utilize an entirely different mechanism to initiate the degradation of this class of compounds. The enzymes involved incorporate both atoms of molecular oxygen into the aromatic nucleus, and the first detectable metabolites are dihydrodiols in which the hydroxyl groups have a *cis*-relative stereochemistry (14). For benz[*a*]anthracene, the bay region 1,2 position is the preferred site of attack, although oxidation at the 8,9 and 10,11 positions can occur (12). The absolute stereochemistry of the *cis*-1,2-, *cis*-8,9-, and *cis*-10,11-dihydrodiols formed from benz[*a*]anthracene by a mutant strain of a *Beijerinckia* sp. has been reported and is shown in Fig. 1 (16). However, the further metabolism of these compounds was not investigated. Such studies are complicated by the fact that organisms capable of growth with benz[*a*]anthracene have not been isolated. The formation of the *cis*-dihydrodiols mentioned above was achieved by using biphenyl-induced cells of a mutant *Beijerinckia* strain (strain B8/36). We now report the metabolism of benz[*a*]anthracene to an isomeric mixture of *o*-hydroxypolyaromatic acids by the parental strain, *Beijerinckia* strain B1, after induction with biphenyl, *m*-xylene,

or salicylate. Evidence is also presented for the production of radiolabeled carbon dioxide from [12-<sup>14</sup>C]benz[*a*]anthracene. The results represent the first direct demonstration of ring fission in a polycyclic aromatic hydrocarbon that contains more than three aromatic rings.

(A partial summary of these results was presented at the 82nd Annual Meeting of the American Society for Microbiology [W.R. Mahaffey, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, K166, p. 199].)

### MATERIALS AND METHODS

**Organism and growth conditions.** A *Beijerinckia* strain designated strain B1 and originally isolated for its ability to grow with biphenyl as a carbon and energy source was used throughout this study (13). The organism was grown in mineral salts medium (22) containing 0.01% (wt/vol) yeast extract and the carbon sources indicated below. Cultures were grown in 2.8-liter Fernbach flasks with 400 ml of mineral salts medium and 0.2% sodium succinate. When the mid-exponential phase of growth (turbidity as  $A_{600} = 0.4$ ) was attained, biphenyl (0.1%), salicylate (0.05%), or *m*-xylene (vapor) was supplied to the individual cultures. Cells were harvested when the culture attained the early stationary phase of growth ( $A_{600} = 1.0$ ). Large-scale cultures (12 liters) were grown in a fermentor (model M14 Microferm; New Brunswick Scientific Co., Inc., Edison, N.J.) at 30°C with air supplied at a rate of 12 liters/min and agitation maintained at 400 rpm. Substrates used as inducers were added as indicated above. Cultures were initially filtered to remove solid materials, and the cells (60 g) were harvested in an air-driven Sharples continuous-flow centrifuge. The cells were washed twice with 50 mM KH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.5) (phosphate buffer). Biotransformations were performed by resuspending cells to a final concentration of 4.0 g (wet weight) per 100 ml of phosphate buffer in a 2.8-liter Fernbach flask.

**Mineralization of [12-<sup>14</sup>C]benz[*a*]anthracene.** Mineralization experiments were performed with 50 ml of induced cell suspensions in 125-ml Erlenmeyer flasks fitted with Teflon-lined serum septum stoppers. Each flask was supplemented with 1.0 ml of solution containing 1.7 μCi of [12-<sup>14</sup>C]benz[*a*]anthracene plus 10 mg of unlabeled substrate in dimethylformamide. Flasks were incubated in a 30°C water bath and

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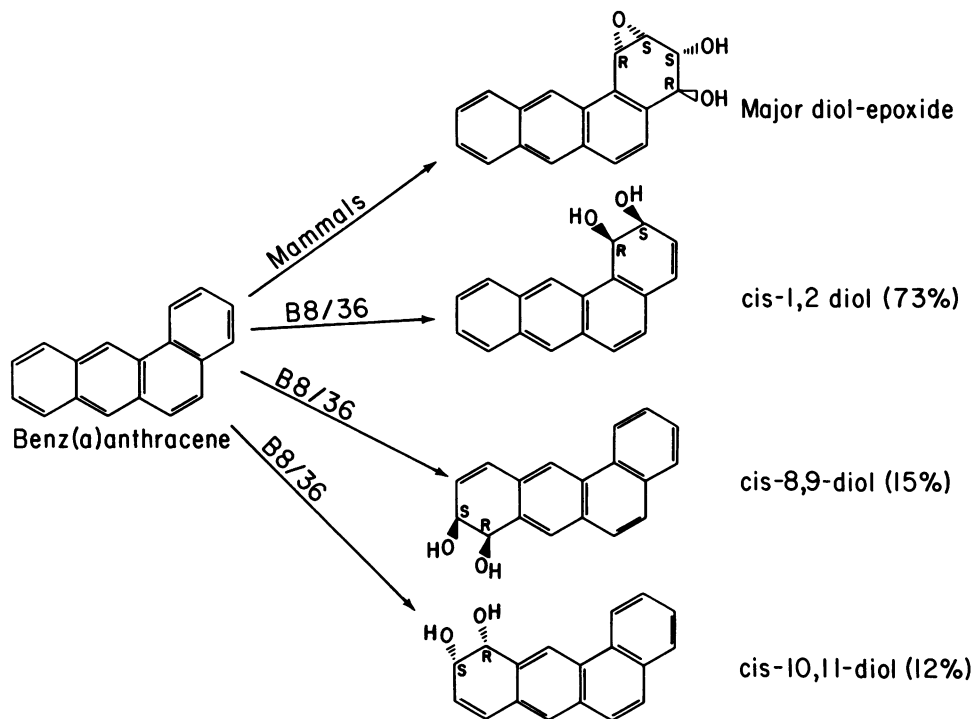


FIG. 1. Metabolites formed from benz[a]anthracene by mammals and *Beijerinckia* strain B8/36 (16).

aerated with humidified air. Exhaust air was filtered through an XAD-4 resin filter before  $^{14}\text{CO}_2$  was collected in a solution of 2 N sodium hydroxide (5.0 ml). The XAD-4 resin filter served as a column to trap volatile organics. Volatile  $^{14}\text{C}$  residues were extracted from the resin with 5.0 ml of ethyl acetate. Quantitation was achieved by liquid scintillation counting (LSC) of 1.0-ml samples of the extract in 14 ml of scintillation cocktail (MP; Beckman Instruments, Inc., Fullerton, Calif.). At various times, the  $\text{CO}_2$  traps were removed and a fresh solution of sodium hydroxide was added. Samples (0.5 ml) of the trapping solution were mixed with 15 ml of scintillation cocktail (Beckman MP), and radioactivity was determined by LSC.

**Detection of benz[a]anthracene metabolites.** Upon termination of mineralization experiments, each culture was centrifuged at  $23,300 \times g$ . Cell pellets were extracted with three 30-ml aliquots of acetone, and this extract was designated as the neutral extract. Culture supernatants were acidified to pH 2.5 with 6 N sulfuric acid and extracted with 3 volumes of ethyl acetate (acid extract). Samples (1.0 ml) of the extracted aqueous phase were mixed with 15 ml of scintillation cocktail (Beckman MP) and assayed for nonextractable radioactivity by LSC. Extracts were dried over anhydrous sodium sulfate, and the solvent was removed in vacuo at  $40^\circ\text{C}$ . Residues were dissolved in 5.0 ml of methanol, filtered to remove amorphous material, and analyzed by thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC).

TLC was performed on silica gel 60 (F254) plastic plates which were developed in either solvent A (benzene-acetic acid-water, 125:74:1, vol/vol/vol) or solvent B (chloroform-acetone, 80:20, vol/vol). Metabolites were visualized by observing quenching of fluorescence at 254 nm and by spraying with a 2% solution (wt/vol) of 2,6-dichloroquinone-4-chloroimide in methanol (Gibbs reagent). HPLC was per-

formed with a Beckman system consisting of two model 114 pumps and a model 160 absorbance detector with a 254-nm filter. System A consisted of a  $5.0\text{-}\mu\text{m}$  Zorbax ODS column (4.6 mm by 25 cm) with a solvent program composed of an acetonitrile-water linear gradient (35 to 95%, vol/vol, 30 min) containing 1.0% acetic acid. The flow rate was 1.0 ml/min. System B consisted of a  $5.0\text{-}\mu\text{m}$  Spherisorb ODS column (4.25 mm by 25 cm) with an isocratic solvent composed of acetonitrile-water (40%, vol/vol) containing 1.0% acetic acid. The flow rate was 1.5 ml/min.

**Kinetics of benz[a]anthracene metabolite formation.** Large-scale biotransformations were performed in 2.8-liter Fernbach flasks with biphenyl-induced cells. Induced cell suspensions (400 ml) were prepared as described above. Benz[a]anthracene (75 mg) was added in 6.0 ml of *N,N*-dimethylformamide, and each flask was incubated on a rotary shaker (150 rpm) at  $30^\circ\text{C}$ . Quantitative measurement of product accumulation in the culture medium was conducted at various time intervals. A 20-ml portion of the culture was adjusted to pH 2.5 and extracted with 3 volumes of ethyl acetate. The organic extract was dried over anhydrous sodium sulfate, and the solvent was removed in vacuo at  $40^\circ\text{C}$ .

Duplicate samples of culture extract were examined by analytical TLC with system A as described above. After development of chromatograms, compounds were located with UV light. Regions of the plate containing benz[a]anthracene and suspected metabolites were removed and extracted with 5.0 ml of methanol-acetic acid (10:1). Using this procedure, 90 to 95% of the total radioactivity applied to the chromatogram could be recovered.

**Isolation of benz[a]anthracene metabolites.** Biotransformations for the isolation and characterization of metabolites were performed as described above but without the addition of  $^{14}\text{C}$ -labeled substrate. After cultures were incubated for

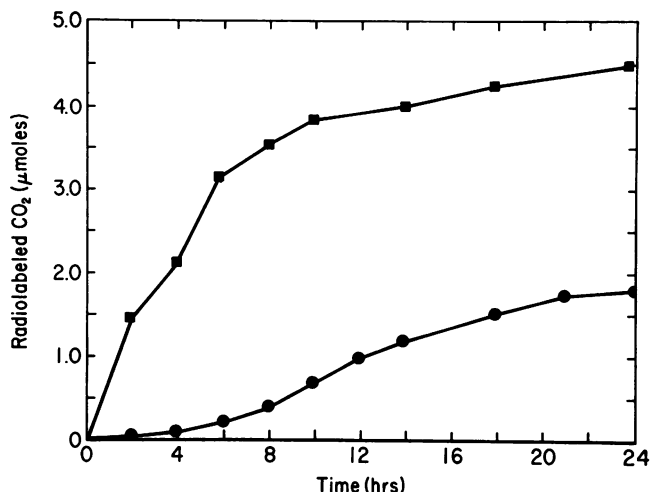


FIG. 2. Carbon dioxide formation from [12-<sup>14</sup>C]benz[a]anthracene by *Beijerinckia* strain B1 (●). Cells were induced with biphenyl and suspended (2.0 g/50 ml) in 50 mM KH<sub>2</sub>PO<sub>4</sub> buffer, pH 7.5. Labeled substrate (1.7 μCi, 35 nmol) and 10 mg (43.86 μmol) of unlabeled substrate in 1.0 ml of *N,N*-dimethylformamide were added to the reaction vessel. Carbon dioxide evolution was not observed with uninduced cells. A positive control with [U-<sup>14</sup>C]biphenyl is presented (■). Labeled biphenyl (0.93 μCi, 400 nmol) and 10 mg (65 μmol) of unlabeled substrate in 1.0 ml of dimethylformamide were added to the reaction vessel.

14 to 16 h, they were centrifuged at 23,300 × *g* for 15 min to remove cell material. Cell pellets were washed twice with 100 ml of phosphate buffer, and the supernatant solutions were pooled. The combined supernatant solutions (600 ml) were acidified to pH 2.5 and extracted as described above.

**Physicochemical analysis of metabolites.** The absorption spectra of metabolites were determined in methanol on a Cary model 14 spectrophotometer. Electron impact mass spectra (EIMS) were obtained with a mass spectrometer (model 4023; Finnegan Corp., Sunnyvale, Calif.) at an ionizing voltage of 70 eV and a source temperature of 270°C. Direct probe mass spectrometry was performed by recording the spectra as the probe was heated ballistically from 30 to 300°C while scanning the mass/charge region of *m/z* 35 to 750 AMU. Chemical ionization mass spectra were obtained as described above except that the reagent gas was methane with a source pressure of 0.25 torr. An Incos (Finnegan Corp.) data system was used to scan the magnetic sector from 50 to 750 AMU in 2 s.

<sup>1</sup>H nuclear magnetic resonance (NMR) spectra were recorded on a Bruker WM 500 or a Nicolet NT 200 spectrometer as indicated. Spectra were recorded in anhydrous

acetone-*d*<sub>6</sub> or chloroform-*d*<sub>1</sub>, and chemical shifts are reported in parts per million (ppm) downfield from the internal standard, tetramethylsilane. Assignments were made through consideration of shielding effects, coupling patterns, deuterium exchange, and homonuclear decoupling experiments (3).

Metabolites were esterified with diazomethane generated with a Diazald kit (Aldrich Chemical Co., Inc., Milwaukee, Wis.). Esterifications were performed at 4°C in diethyl ether. When the reaction was complete, esterified products were isolated by preparative TLC in solvent B.

**Materials.** Benz[a]anthracene was purchased from the Eastman Kodak Co. (Rochester, N.Y.). Radiolabeled substrates were obtained from Amersham Corp. (Arlington Heights, Ill.). [12-<sup>14</sup>C]benz[a]anthracene (specific activity, 49 mCi/mmol) was shown to have a radiochemical purity of 99.46% as determined by reverse-phase HPLC and LSC. [U-<sup>14</sup>C]biphenyl (specific activity, 19 mCi/mmol) was shown to have a radiochemical purity of 98.8% as determined by reverse-phase HPLC and LSC. Analytical TLC was performed with Kieselgel 60 (F254) 0.2-mm coated plastic sheets (20 by 20 cm) from E. Merck AG (Darmstadt, Federal Republic of Germany). A 5.0-μm Spherisorb ODS column (3.9 by 30 cm) was prepared by slurry packing 3.0 g of Spherisorb ODS (Phase Sep., Hauppauge, N.Y.) in isopropanol at a constant flow rate of 1.5 ml/min. A Du Pont 5.0-μm Zorbax ODS column (4.6 mm by 25 cm) was also used for HPLC analysis. Solvents for HPLC were from Fisher Scientific Co. (Springfield, N.J.) and were filtered through a 0.45-μm-pore-size filter before use. All other materials were of the highest purity commercially available and were used without further purification.

## RESULTS

**Evidence for ring fission during metabolism of benz[a]anthracene by *Beijerinckia* strain B1.** Cells of *Beijerinckia* strain B1 grown with succinate did not metabolize benz[a]anthracene. However, cells grown with succinate in the presence of biphenyl were able to oxidize [12-<sup>14</sup>C]benz[a]anthracene to <sup>14</sup>CO<sub>2</sub> (Fig. 2). Over a 24-h period, biphenyl-induced cells produced 1.90 μmol of <sup>14</sup>CO<sub>2</sub>, which corresponds to an overall conversion of 4.2%. In addition, 16.6% of the <sup>14</sup>C label was present as acid-extractable metabolites (Table 1). Relatively little benz[a]anthracene was converted to nonextractable products as compared with the biphenyl control. The radioactivity associated with the neutral extracts was found to be unmetabolized substrate.

An analysis of the acid extracts by TLC (solvent A) indicated the presence of two acidic metabolites (compounds 1 and 2). Both compound 1 (*R*<sub>f</sub> = 0.52) and compound 2 (*R*<sub>f</sub> = 0.65) absorbed UV light (254 nm) and gave positive

TABLE 1. Distribution of radioactivity after oxidation of [<sup>14</sup>C]biphenyl<sup>a</sup> and [<sup>14</sup>C]benz[a]anthracene<sup>b</sup> by biphenyl-induced cells of the *Beijerinckia* sp.

Compound	Radioactivity (Mcpm)						<sup>14</sup> CO <sub>2</sub>	Recovery (%) <sup>c</sup>
	Total added	Volatile residue	Neutral extract	Acid extract	Nonextractable			
Biphenyl	2.06	0.10	0.22	0.47	0.83	0.14	1.7 (87.5)	
Benz[a]anthracene	3.79	ND <sup>d</sup>	2.52	0.63	0.02	0.16	3.3 (87.6)	

<sup>a</sup> [U-<sup>14</sup>C]biphenyl.

<sup>b</sup> [12-<sup>14</sup>C]benz[a]anthracene.

<sup>c</sup> Percentage of the total radioactivity added.

<sup>d</sup> ND, Not detected.

TABLE 2. Metabolism of [<sup>14</sup>C]benz[a]anthracene by intact cells of the *Beijerinckia* sp. after induction with biphenyl, *m*-xylene, and salicylate<sup>a</sup>

Inducer	Metabolite (μmol)		
	CO <sub>2</sub>	Compound 1	Compound 2
Biphenyl	1.90	1.80	2.40
<i>m</i> -Xylene	0.50	0.92	1.06
Salicylate	0.15	0.70	0.75

<sup>a</sup> Experiments were done as described in Materials and Methods.

reactions with Gibbs reagent, suggesting the presence of a phenolic functional group. These results suggested that *Beijerinckia* sp. metabolizes benz[a]anthracene through ring fission with the production of two phenolic acids and carbon dioxide. Cells grown with succinate in the presence of salicylate or *m*-xylene also oxidized benz[a]anthracene through ring fission with the production of the same metabolites and carbon dioxide (Table 2). Since the levels of product formation from *m*-xylene- and salicylate-induced cells were at least 50% lower than those observed for biphenyl-induced cultures, the latter were used for subsequent studies.

**Kinetics of benz[a]anthracene metabolite formation.** The formation of compounds 1 and 2 was monitored by TLC and LSC. Both metabolites appeared to be produced in equimolar amounts during the initial phase of transformation, after which compound 2 was produced in excess of compound 1 (Fig. 3). Maximum accumulation of both metabolites occurred approximately 14 h after the addition of substrate. After this time, the concentration of both compounds began to decline. A 56% conversion of benz[a]anthracene to the two phenolic acids was observed after 14 h.

**Isolation of benz[a]anthracene metabolites.** Isolation of compounds 1 and 2 was achieved by reverse-phase HPLC. Analysis of the acid extract in system A resulted in the elution of compound 1 at 17 min and compound 2 at 22 min (Fig. 4). Preliminary NMR analysis indicated that the fraction designated compound 1 was a mixture. Further chromatography of this fraction by HPLC in system B resulted in

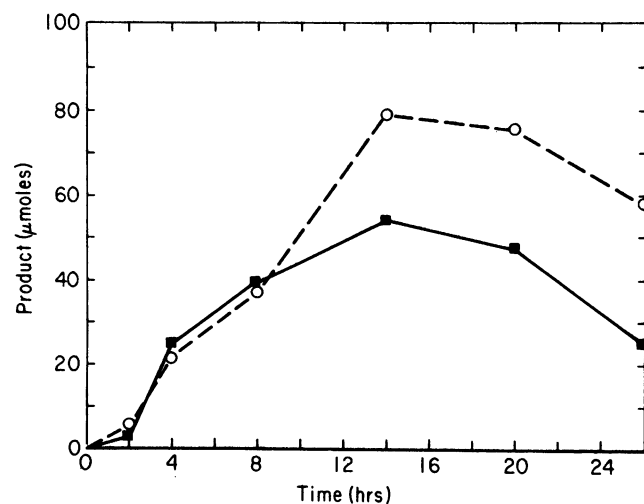


FIG. 3. Formation of acidic metabolites from benz[a]anthracene by biphenyl-induced cells of *Beijerinckia* strain B1. Compounds 1 (■) and 2 (○) were quantitated as described in Materials and Methods.

the isolation of two components which were designated compound 1A and 1B (Fig. 5). After chromatography of the entire extract, compounds 1A, 1B, and 2 were extracted from the HPLC solvent into ethyl acetate. Removal of the solvent gave pure samples of compounds 1A (4.8 mg), 1B (4.2 mg), and 2 (18.8 mg).

**Characterization of the major metabolite: compound 2.** The absorption spectrum of compound 2 (methanol) showed a maximum at 260 nm ( $\epsilon = 5,600 \text{ M}^{-1} \text{ cm}^{-1}$ ) and minor absorptions at 365, 285 (shoulder), and 228 nm (Fig. 6). EIMS gave a parent ion ( $M^+$ ) at  $m/z$  238 (40%) and fragment ions at  $m/z$  220 ( $M^+ - H_2O$ ) and  $m/z$  195 ( $M^+ - CO_2$ ). To confirm the presence of a carboxylic acid functional group, we derivatized 3.2 mg of compound 2 with diazomethane. Purification of the product by TLC in solvent B yielded 2.4 mg of the methyl ester of compound 2. The EIMS of this product gave a parent ion ( $M^+$ ) at  $m/z$  252 (100%) and fragment ions at  $m/z$  220 ( $M^+ - CH_3OH$ ) and  $m/z$  192 ( $M^+ - HCOOCH_3$ ).

An analysis of the proton magnetic resonance spectrum (Fig. 7) of compound 2 is presented in Table 3. These data suggest that compound 2 is 1-hydroxy-2-anthraic acid. A characteristic of an anthracene molecule is the resonance at 8.85 ppm (2H) of the two periprotons, H-9 and H-10. In compound 2, these two protons have different chemical shifts owing to substituent effects. The signal at 9.05 ppm (1H) is assigned to H-9 and is shifted downfield owing to the electron-withdrawing effect of the carboxyl group at C-2.

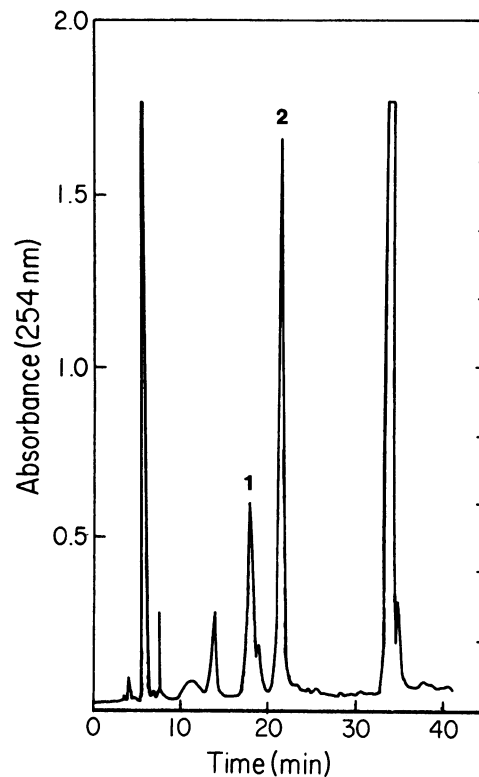


FIG. 4. HPLC elution profile of benz[a]anthracene metabolites produced by biphenyl-induced cell suspension of *Beijerinckia* strain B1. A 50-μl sample of a 5.0-ml extract was analyzed. The separation was obtained on a 5.0-μm Zorbax ODS column (6.25 mm by 25 cm) with a linear gradient of acetonitrile-water (35 to 95%, vol/vol, 30 min) containing 1% acetic acid. The flow rate was 1.5 ml/min. The large peak eluting at 34 min is benz[a]anthracene.

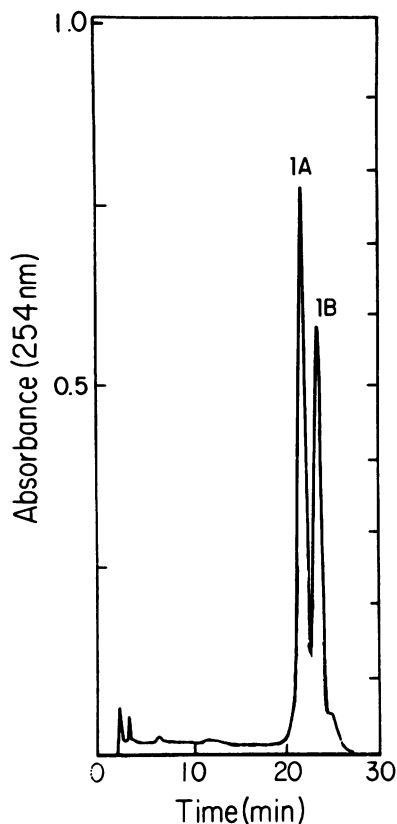


FIG. 5. HPLC resolution of the fraction designated compound 1 (Fig. 4) to yield compounds 1A and 1B. Separation was achieved on a 5.0- $\mu$ m Spherisorb ODS column (3.9 mm by 30 cm) with a solvent composed of acetonitrile-water (40%, vol/vol) and 1% acetic acid. The flow rate was 1.5 ml/min.

Proton H-3 resonates as a downfield doublet centered at 7.78 ppm owing to the deshielding effects of an *ortho*-carboxyl group. A signal (not shown in Fig. 7) at 12.9 ppm (1H) was assigned to the phenolic proton. This is consistent with the observation of a proton signal at 12.4 ppm (1H) in the methyl ester derivative (data not shown). A methyl group singlet for the ester was located at 4.03 ppm (3H) and was associated with the loss of a proton signal in the aromatic region. This suggests that the signal for the carboxyl proton of compound 2 is located in the region of the aromatic signals at 7.50 to 7.65 ppm. A deuterium exchange experiment was performed with the methyl ester of compound 2. The result of this experiment indicated that the signal at 12.9 ppm was suppressed and thus that the phenolic proton had exchanged with deuterium, lending further evidence to support this assignment.

**Characterization of minor metabolites 1A and 1B.** The absorption spectra of compounds 1A and 1B are shown in Fig. 6. Both compounds gave similar spectra with absorption maxima at 259 and 290 nm. They each gave broad absorption peaks in the 360- to 375-nm range. Compound 1B showed additional absorption peaks at 266 and 272 nm which are detectable only as shoulders in the spectrum of compound 1A. Extinction coefficients were calculated at 259 nm and found to be  $3,000 \text{ M}^{-1} \text{ cm}^{-1}$  for 1A and  $3,200 \text{ M}^{-1} \text{ cm}^{-1}$  for 1B. Mass spectral analyses of compounds 1A and 2B indicated that they were structurally similar to 1-hydroxy-2-anthranic acid. EIMS of both compounds gave molecular ions at  $m/z$  238 with fragment ions at  $m/z$  220 ( $\text{M}^+ - \text{H}_2\text{O}$ ),  $m/z$  192 ( $\text{M}^+ - \text{HCOOH}$ ), and  $m/z$  164 ( $\text{M}^+ - \text{HCOOH}, -\text{CO}$ ). Derivatization of 1A and 1B with diazomethane generated the methyl esters of both compounds. EIMS of each methyl ester gave molecular ions at  $m/z$  252 and fragment ions at  $m/z$  220 ( $\text{M}^+ - \text{CH}_3\text{OH}$ ),  $m/z$  192 ( $\text{M}^+ - \text{HCOOCH}_3$ ), and  $m/z$  163 ( $\text{M}^+ - \text{HCOOCH}_3, -\text{HCO}$ ).

An analysis of the proton magnetic resonance spectra (Fig. 7) of compounds 1A and 1B is presented in Table 3. The data suggest that compound 1A be identified as 2-hydroxy-

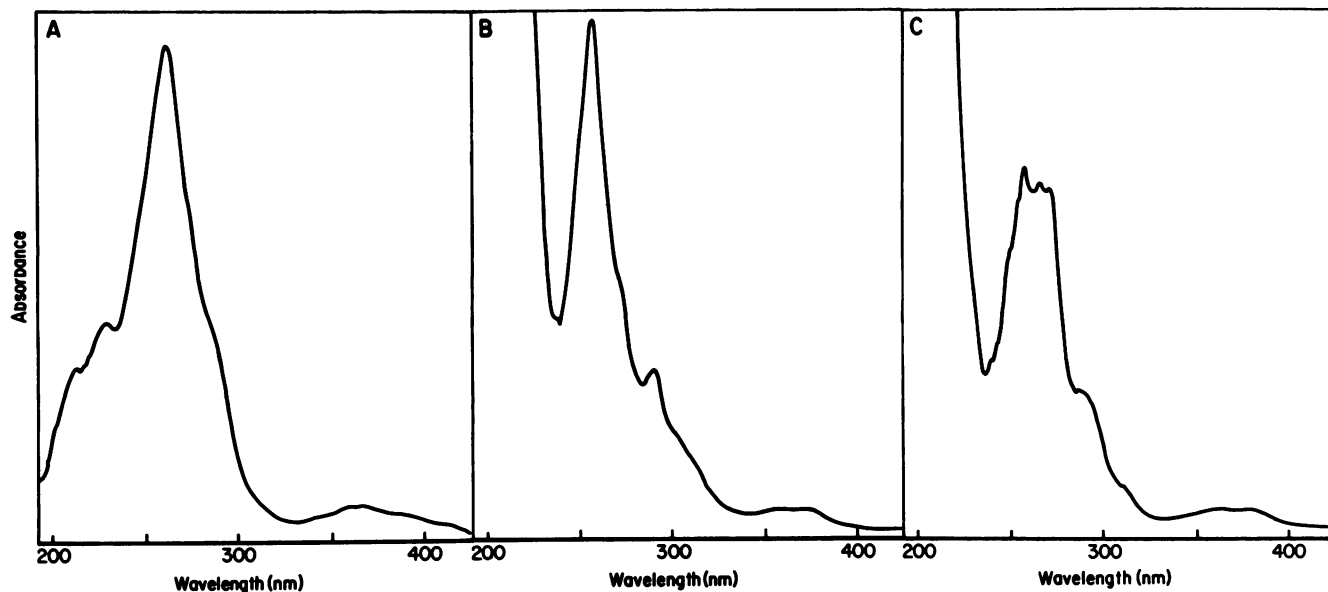


FIG. 6. UV absorption spectra of compound 2 (A), compound 1A (B), and compound 1B (C). Samples were dissolved in methanol to yield the following concentrations: 2 (0.04 mg/ml), 1A (0.08 mg/ml), and 1B (0.05 mg/ml). The spectra shown are for 1.0-ml samples and were recorded with a Cary model 14 spectrophotometer at 1.0 absorbance unit full scale.

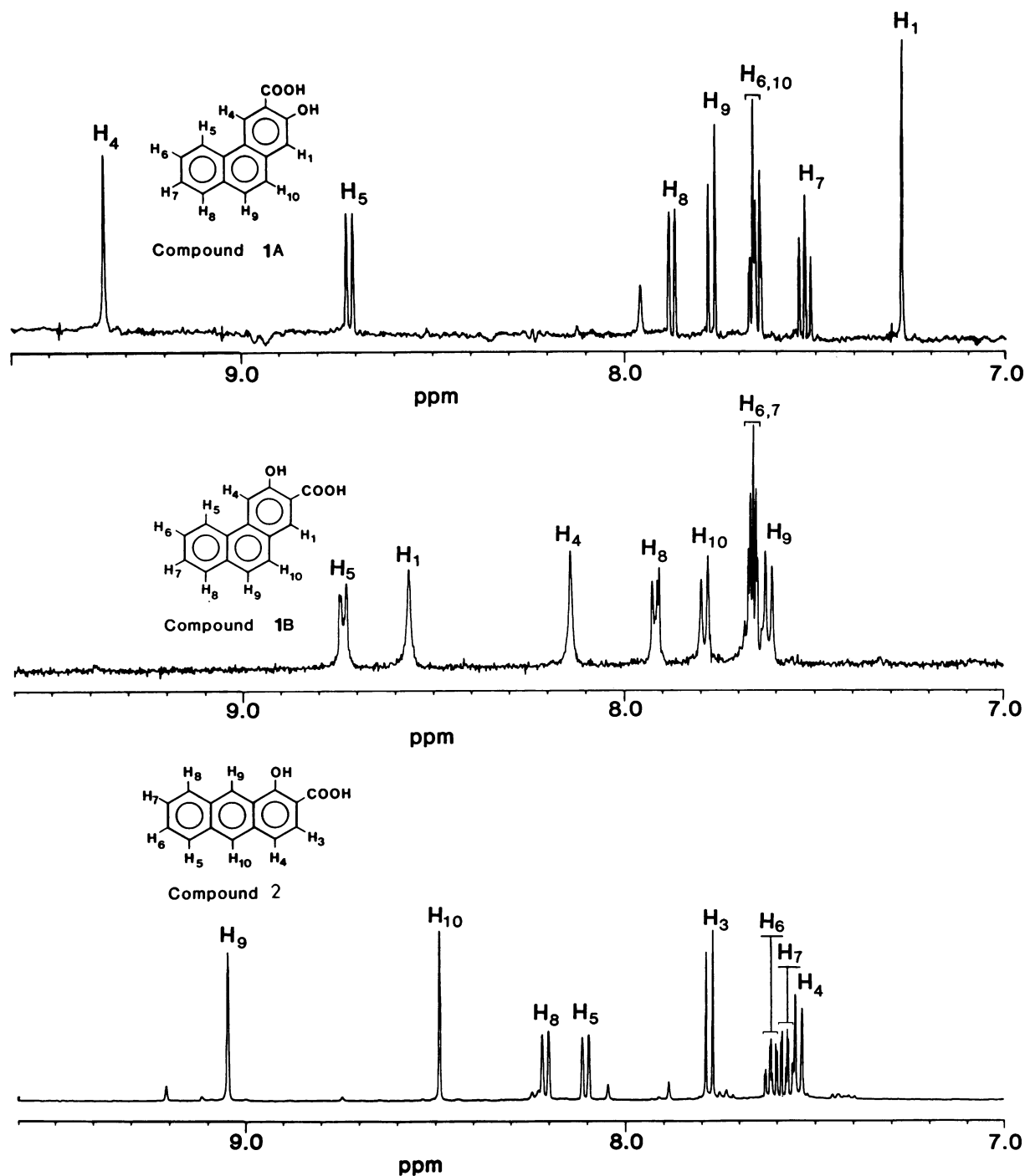


FIG. 7. 500-MHz proton magnetic resonance spectra of compounds 1A, 1B, and 2. Samples were dissolved in anhydrous acetone- $d_6$ , and spectra were recorded on a Bruker WM 500 spectrometer. Chemical shifts are referenced in parts per million (ppm) downfield from the internal standard tetramethylsilane.

3-phenanthroic acid. A deuterium-exchangeable proton with a broad signal (not shown in Fig. 7) centered at 11.2 ppm (1H) was assigned to the phenolic proton by analogy to compound 2. A second deuterium-exchangeable proton (not shown in Fig. 7) was observed at 6.55 ppm (1H) and was assigned to the carboxylic proton. Assignment of the phenolic group to C-2 is based on the shielding effect this group has on an *ortho*-proton and is evidenced by the upfield shift

of proton H-1. In addition, carboxyl groups have a deshielding effect on *ortho*-protons, and this is seen in the downfield shift of proton H-4. Using the same rationale, compound 1B was identified as 3-hydroxy-2-phenanthroic acid. It is important to note that proton H-1 is now shifted downfield owing to the influence of the *ortho*-carboxyl group, while proton H-4 is shifted upfield owing to the *ortho*-hydroxyl substituent. This compound also exhibited deuterium-exchangeable

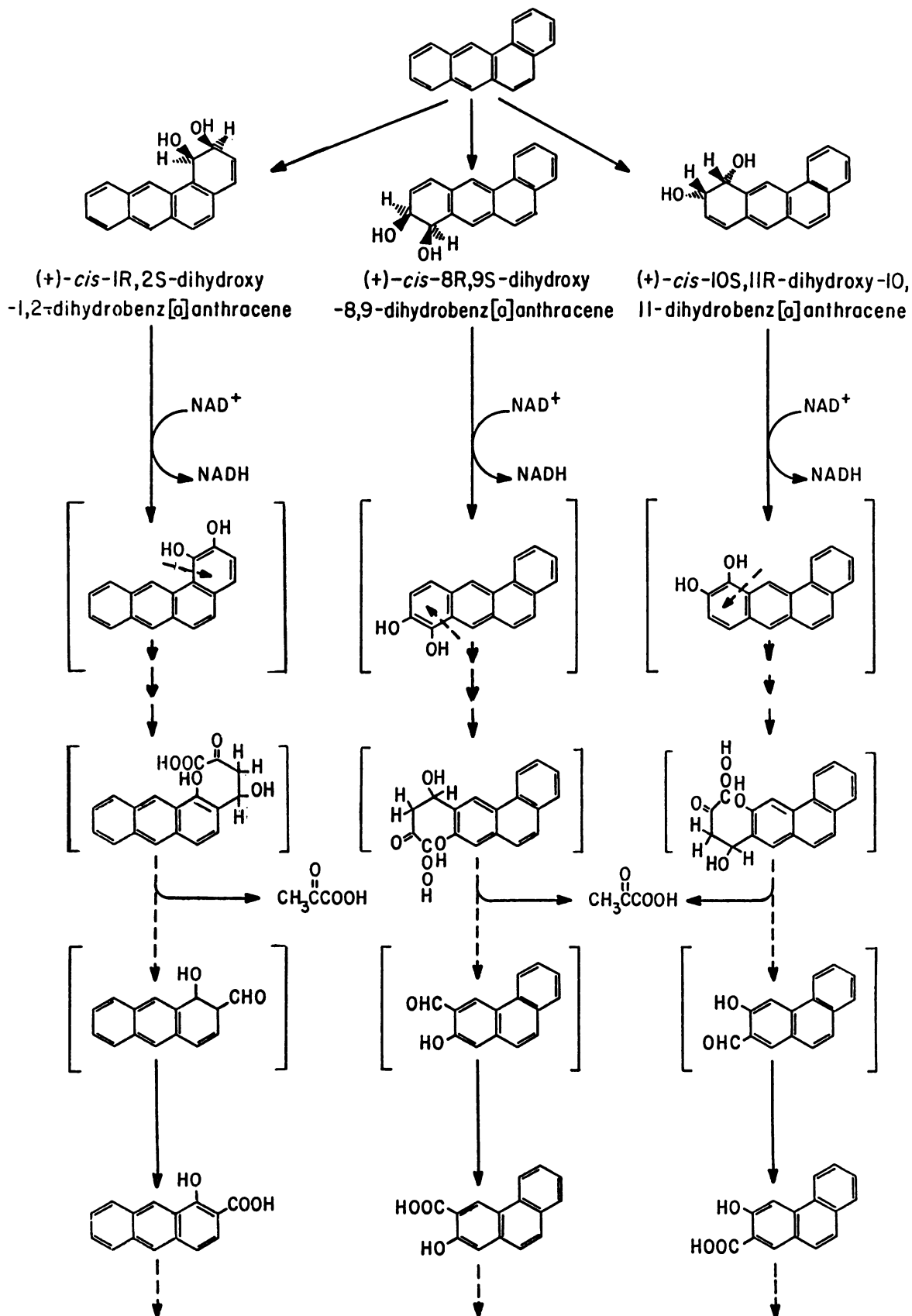


FIG. 8. Proposed pathways for the metabolism of benz[a]anthracene by *Beijerinckia* strain B1. The major pathway on the left is initiated by oxidation at the 1,2 position. The structures shown in brackets are proposed intermediates and have not been characterized.

TABLE 3. 500-MHz NMR spectral parameters for the acid products formed from benz[a]anthracene by the *Beijerinckia* sp.

Metabolite	Proton assignment (ppm) <sup>a</sup>									
	H-1	H-2	H-3	H-4	H-5	H-6	H-7	H-8	H-9	H-10
Compound 1A <sup>b</sup>	7.29	11.1	6.55	9.37	8.73	7.67	7.54	7.89	7.78	7.60
Compound 1B <sup>c</sup>	8.54	6.50	11.3	8.12	8.71	7.67–7.66 <sup>d</sup>		8.90	7.60	7.77
Compound 2 <sup>e</sup>	12.9	NO <sup>f</sup>	7.78	7.54	8.10	7.62–7.57 <sup>d</sup>		8.21	9.05	8.49

<sup>a</sup> In acetone-d<sub>6</sub> downfield from tetramethylsilane.

<sup>b</sup> Coupling constants are  $J_{5,6} = 8.6$  Hz,  $J_{7,8} = 8.2$  Hz, and  $J_{9,10} = 9.0$  Hz.

<sup>c</sup> Coupling constants are  $J_{5,6} = 8.5$  Hz and  $J_{9,10} = 8.6$  Hz.

<sup>d</sup> Protons H-6 and H-7 exhibit a complex coupling pattern and the chemical shifts are given as a range.

<sup>e</sup> Coupling constants are  $J_{3,4} = 9.0$  Hz,  $J_{5,6} = 8.6$  Hz, and  $J_{7,8} = 8.6$  Hz.

<sup>f</sup> No, An isolated resonance signal was not observed. Integration of a D<sub>2</sub>O experiment places this proton resonance frequency within the range of 7.50 to 7.65 ppm.

protons with signals centered at 11.3 ppm (1H) and 6.5 ppm (1H) indicative of the carboxyl and phenolic protons, respectively (Table 3).

### DISCUSSION

*Beijerinckia* strain B1 used in this study was unable to utilize benz[a]anthracene as a source of carbon and energy for growth. However, intact cells after growth with succinate in the presence of biphenyl, *m*-xylene, or salicylate as inducing substrates oxidized benz[a]anthracene to carbon dioxide and a mixture of *o*-hydroxypolyaromatic acids. Cells which were induced with biphenyl were most active in producing these metabolites. An interesting observation was that upon addition of the hydrocarbon substrate (e.g., benz[a]anthracene), a fine particulate film formed at the air-water interface of the medium without cells. However, when benz[a]anthracene was added to the cell suspension, a particulate film formed initially but within 1 h of incubation was no longer visible. An explanation for these observations is that the benz[a]anthracene, owing to its hydrophobic character, partitions in the cell surface.

Formation of 1-hydroxy-2-anthranic acid as the major product is consistent with previous results which showed that the preferential oxidation of benz[a]anthracene by *Beijerinckia* strain B1 occurs at the 1,2 position (12, 16). The formation of *o*-hydroxyaromatic acids as intermediates in the bacterial degradation of naphthalene, anthracene, and phenanthrene has been reported (7–9, 18). The major pathway proposed for benz[a]anthracene degradation is shown in Fig. 8 and is analogous to that reported by Evans et al. (8) for the oxidation of phenanthrene to 1-hydroxy-2-naphthoic acid. Furthermore, the formation of 2-hydroxy-3-phenanthroic acid and 3-hydroxy-2-phenanthroic acid presumably occurs by oxidative cleavage of catechol formed at the 10,11 and 8,9 positions of benz[a]anthracene, respectively (11). Both of these acids appear to be formed by a series of reactions analogous to those proposed for the oxidation of anthracene to 2-hydroxy-3-naphthoic acid (8). The dioxygenase that initiates the degradation of benz[a]anthracene is known to have a relaxed stereoheterotopicity (16), and it now appears that subsequent enzymes of the pathway may also exhibit this characteristic. Further studies are needed to clarify this point.

It is interesting that *Beijerinckia* strain B1 is capable of forming <sup>14</sup>CO<sub>2</sub> from [12-<sup>14</sup>C]benz[a]anthracene. The position of the <sup>14</sup>C label is significant, since interior ring labeling means that at least two aromatic rings of the substrate must be cleaved before any CO<sub>2</sub> is released. This is supported by the observation that the isomeric *o*-hydroxypolyaromatic acids are slowly metabolized (Fig. 3). The tendency of the

*o*-hydroxypolyaromatic acids to accumulate during the metabolism of benz[a]anthracene may be due to a rate-limiting reaction similar to that observed in naphthalene metabolism. For example, it is well known that during the metabolism of naphthalene by several *Pseudomonas* species, salicylate accumulates in high yield (7, 21). This is due to the fact that salicylate is metabolized at about 50% of the rate at which naphthalene is oxidized. A similar explanation may also apply to benz[a]anthracene metabolism by *Beijerinckia* strain B1.

The results presented here show that in addition to biphenyl, salicylate and *m*-xylene are also capable of inducing the enzyme system(s) required for the oxidation of benz[a]anthracene. Previous studies have shown that salicylate enhances the degradation of fluoranthene and benzo[a]pyrene by *Pseudomonas putida* NCIB 9816 (1). This is probably due to increased expression of naphthalene-degrading enzymes by salicylate (2). Furukawa et al. (10) have suggested that the catabolism of biphenyl, xylene-toluene, and salicylate is regulated by a common unit in *Pseudomonas paucimobilis* (Q1). These researchers proposed that the metabolism of these compounds is interrelated since benzoate and toluate are common intermediates of biphenyl and xylene-toluene metabolism and salicylate is produced from *o*-phenylphenol. Similar coordinate regulation of biphenyl, *m*-xylene, and salicylate metabolism was observed in *Beijerinckia* strain B1. These findings are of fundamental importance in environmental fate studies and in the treatment of waste contaminated with polycyclic aromatic hydrocarbons. Organisms which utilize benz[a]anthracene and larger molecules as sole sources of carbon and energy have yet to be isolated (19, 20). Since these molecules do not appear to induce their own metabolism cooxidation may represent a significant mode of degradation in situ.

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