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Microbial Transformations of 7,12-Dimethylbenz[a]anthracene

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Microbial transformations of 7,12-dimethylbenz[a]anthracene, a carcinogenic polycyclic aromatic hydrocarbon, in cultures of *Pseudomonas aeruginosa* and *Penicillium notatum* were studied by high performance liquid chromatographic separation of metabolic fractions followed by gas chromatographic-mass spectrometric analysis of the metabolites. Two methyl-hydroxylated metabolites were identified in each of the incubations. The metabolic activation of the polycyclic aromatic hydrocarbon suggests a possible involvement of microorganisms in environmental carcinogenesis.

The ability of microorganisms to accomplish structural modifications of many types of organic compounds has been well documented (4, 7, 9, 16). Selected microorganisms have been advantageously used to perform specific chemical transformations on structurally complex compounds such as the alkaloids and steroids (4, 9). This technique has found widespread use, especially in the preparation of therapeutically important agents (7, 16). Microbial transformation has been developed as a general means for providing quantities of potentially active metabolites of complex antitumor compounds from nature (1, 6, 11); for preparing metabolites difficult to synthesize as analytical standards to facilitate mammalian drug metabolism studies; and for determining potentially important pathways of bioactivation, bioinactivation, and cytotoxicity which may also occur in mammalian species (12). This report deals with the microbial transformation of 7,12-dimethylbenz[a]anthracene (DMBA), a carcinogenic polycyclic aromatic hydrocarbon. DMBA induces cancer in experimental animals (2, 8, 13, 15). Since the polycyclic aromatic hydrocarbon is rather inert chemically, the carcinogenicity of DMBA has been attributed to reactive metabolic intermediates. In this report, we describe the identification of two methyl-hydroxylated metabolites of DMBA from the cultures of *Pseudomonas aeruginosa* and *Penicillium notatum*. The confirmation of their structures was achieved by using high performance liquid chromatography (HPLC) and combined gas chromatography (GC)-mass spectrometry (MS).

Cultures used in this study were maintained on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) slants and were stored in a refrigerator at 4°C before use. *Pseudomonas aeruginosa* and *Penicillium notatum* were obtained from the culture collection of the Department of Medical Microbiology, College of Medicine, The Ohio State University, Columbus. These two microorganisms were chosen because of their documented capabilities to carry out epoxidation (7). Epoxidation is a commonly observed metabolic pathway for polycyclic aromatic hydrocarbons. The cultures were manipulated according to published procedures (1, 6, 11). The microorganisms grown on Trypticase soy agar slants were transferred to other agar slants and allowed to grow for several days at room temperature. Then they were transferred to a liquid medium that contained the following ingredients: 3 g of yeast extract (Difco, Detroit, Mich.), 3 g of malt extract (Difco), 5 g of peptone (Difco), 10 g of dextrose, and distilled water to make up to 1,000 ml. A 100-ml portion was transferred to a 500-ml Erlenmeyer flask (cotton plugged) for each incubation. The flasks were placed in a shaker (Eberbach Corp., Ann Arbor, Mich.) set at low speed and at room temperature. Twenty-four hours later, 10 mg of DMBA in 2 ml of acetone was added to each medium containing the microbes. Control experiments were performed on the substrate in the same medium but without the microorganisms under identical incubation conditions. The flasks were harvested at 2-day, 4-day, and 1-week intervals. Each was extracted three times with 200 ml of chloroform. The chloroform extracts were pooled, dried over anhydrous sodium sulfate, and evaporated to dryness in vaccuo. The residue was reconstituted in 300 μl of methanol. A 50-μl sample was chromatographed on a octadecylsilane column of an HPLC system (Altex, Berkeley, Calif.), using a water-methanol gradient (from 25 to 100% of methanol) at a flow rate of 1 ml/min and at an average pressure of 1,500 lb/in^2. The eluate was monitored by an

ultraviolet detector at 254 nm. The HPLC peaks were collected and evaporated to dryness by a stream of nitrogen. The residue was timethylsilylated (TMS) by reacting with 30 μl of N-O-bis-(trimethylsilyl)trifluoroacetamide in 10 μl of methylene chloride at 60°C for 30 min. The reaction mixture was evaporated to dryness and then reconstituted with 10 μl of methylene chloride. Three microliters of the TMS derivatives were subjected to GC-MS analysis on a Hewlett-Packard (Palo Alto, Calif.) model 5985 GC-MS instrument. The gas chromatograph was equipped with a glass column (6 ft by 1/8 in. [ca. 1.83 m by 3.18 mm]) of 3% OV-17 on Gas-Chrom Q (100 to 120 mesh). The carrier gas was helium at a flow rate of 30 ml/min. The injector temperature was 220°C, and the oven was temperature programmed from 220 to 310°C at 6°C/min. The jet interface to the mass spectrometer was maintained at a temperature of 250°C. The mass spectrometer was operated on the electron impact ionization mode at an electron energy of 70 eV. The ion source temperature was at 200°C. Reference standards of methyl-hydroxylated derivatives of DMBA were obtained from the Chemical Repository of the National Cancer Institute. These compounds were analyzed by HPLC and GC-MS in similar manners.

No metabolite was found in the controls and in the 2-day-old incubation. Metabolites were detected in the 4-day- and 1-week-old incubations. The HPLC chromatogram of each microbial incubation of DMBA revealed two metabolite fractions at retention times of 46 and 47 min. From peak area measurements, the metabolites amounted to about 2.5 mg, whereas unmetabolized DMBA amounted to about 6.5 mg. The proportions of the two metabolites, however, were different between the *Pseudomonas aeruginosa* incubation and the *Penicillium notatum* incubation (Fig. 1). GC-MS analysis of the earlier eluting HPLC fraction indicated a peak at 13.3 min on the gas chromatogram. The mass spectrum (Fig. 2) of this component displayed ions at m/z values of 344 (molecular ion), 329 (loss of CH_3), 255 (loss of OTMS), and 73 (TMS group). These features are indicative of a methyl-hydroxylated metabolite of DMBA (17). Finally, by comparing the HPLC, GC, and MS data with those of authentic synthetic samples, we confirmed this compound to be 7-hydroxymethyl-12-methylbenz[a]anthracene (7-OHM-12MBA). GC-MS analysis of the later HPLC fraction revealed a peak at 13 min on the gas chromatogram. This compound exhibited very similar MS features (Fig. 3) as those of 7-OHM-12MBA except with differences in the relative intensities of some ions. On the basis of the GC and MS data and by comparison with reference standards, this metabolite was characterized as 12-hydroxymethyl-7-methylbenz[a]anthracene (12-OHM-7MBA).

Thus, these data demonstrated that both *Penicillium notatum* and *Pseudomonas aeruginosa* possess DMBA metabolizing capabilities. It should be noted, however, that neither phenolic nor dihydrodiol metabolites were detected. Such metabolites would have arisen via an epoxida-

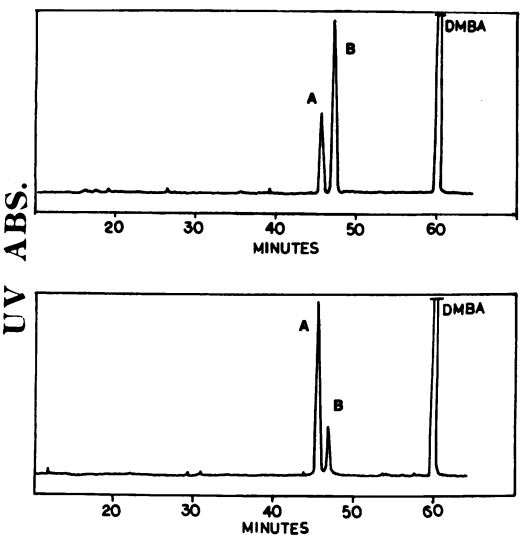

FIG. 1. *HPLC chromatograms from the incubations of Pseudomonas aeruginosa (upper trace) and Penicillium notatum (lower trace). The metabolite peak A is 7-OHM-12MBA and peak B is 12-OHM-7MBA. UV ABS., Ultraviolet absorption.*

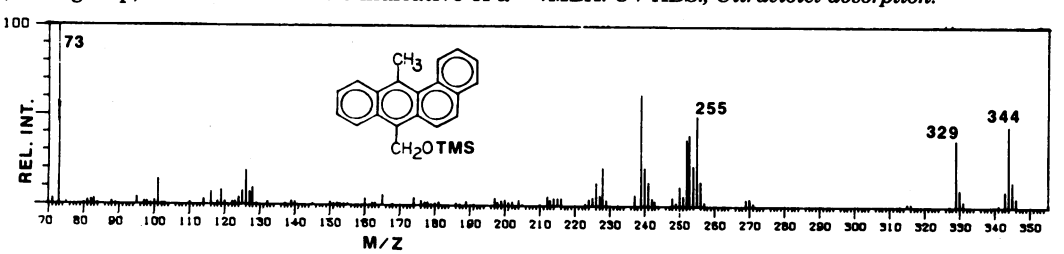

FIG. 2. *Mass spectrum of the 7-OHM-12MBA metabolite analyzed as the TMS derivative. REL. INT., Relative intensity.*

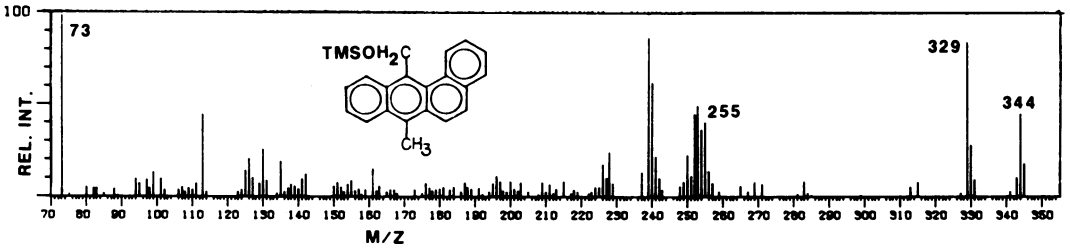

FIG. 3. *Mass spectrum of the 12-OHM-7MBA metabolite analyzed as the TMS derivative. REL. INT., Relative intensity.*

tion pathway. Instead of an epoxidation, the microbes performed hydroxylations of the methyl side chains. Whereas methyl-hydroxylated metabolites of DMBA are commonly detected in liver microsomal incubation of the parent hydrocarbon in vitro (17, 14), our experiments represent the first report of such metabolites derived from microorganisms. The current concept of polycyclic aromatic hydrocarbon carcinogenesis emphasizes the role of arene oxides, particularly the bay region diol epoxides as ultimate carcinogens (5, 10). Nonetheless, the possible formation of reactive carbonium ions from alkyl side chains of polycyclic aromatic hydrocarbons has not been ruled out as alternative electrophilic intermediates involved in chemical carcinogenesis (3). Thus, hydroxylation of a suitable alkyl substituent followed by conjugation may generate a good leaving group which enables the hydrocarbon to undergo reaction with cellular nucleophiles. Methyl-hydroxylated derivatives of DMBA represent compounds of this class. Our findings may bear significance in terms of environmental carcinogenesis. *Penicillium notatum* is one of the common soil microorganisms. It may metabolize polycyclic aromatic hydrocarbons in our environment. Further investigations are deemed necessary to assess the impact of microbial transformation in chemical carcinogenesis.

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