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IDENTIFICATION OF THE 11,12-DIHYDRO-11,12-DIHYDROXYBENZO(A)PYRENE AS A MAJOR METABOLITE PRODUCED BY THE GREEN ALGA, SELENASTRUM CAPRICORNUTUM

Bernadette Lindquist and David Warshawsky

Department of Environmental Health, University of Cincinnati Medical College, Cincinnati, Ohio, 45267-0056

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SUMMARY: Benzo(a)pyrene metabolites were isolated after incubation of [^{14}C]-benzo(a)pyrene with the green alga, Selenastrum capricornutum. A significant amount of radioactivity chromatographed in the dihydrodiol region which did not coelute with any of the previously identified dihydrodiol metabolites isolated from this system. Following characterization by mass spectrometry, fluorescence spectroscopy, and high pressure liquid chromatography, this metabolite was identified as the cis-11,12-dihydro-11,12-dihydroxybenzo(a)-pyrene. This metabolite has not been identified previously as a metabolite formed in a plant system.

INTRODUCTION: The polycyclic aromatic hydrocarbon, BP is metabolized by various organisms. In mammals, BP is metabolized to epoxides by a cytochrome P-450 monooxygenase. These epoxides can then be enzymatically hydrated to trans-dihydrodiols, nonenzymatically rearranged to phenols, or conjugated to polar molecules, such as glutathione. Trans-dihydrodiols may be further oxidized to dihydrodiol-epoxides. The 7,8-diol-9,10-oxide-BP is considered to be the ultimate carcinogenic metabolite of BP (1).

In bacteria, polycyclic aromatic hydrocarbons are metabolized to cis-dihydrodiols (2). We have shown in this laboratory that BP is metabolized to cis-4,5- and cis-7,8-diols of BP by the green alga, Selenastrum capricornutum (3). The 9,10-diol of BP has also been identified as a metabolite in this system, though its stereochemistry has not been established. In the present

Abbreviations: BP, Benzo(a)pyrene; 7,8-diol-9,10-oxide-BP, trans-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene; cis-4,5-diol-BP, cis-4,5-dihydro-4,5-dihydroxybenzo(a)pyrene; cis-7,8-diol-BP, cis-7,8-dihydro-7,8-dihydroxybenzo(a)pyrene; 9,10-diol-BP, 9,10-dihydro-9,10-dihydroxybenzo(a)pyrene; cis- or trans-11,12-diol-BP, cis- or trans-11,12-dihydro-11,12-dihydroxybenzo(a)pyrene; HPLC, high pressure liquid chromatography; DPM, desintegrations per minute.

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study, a previously unidentified metabolite has been characterized by mass spectrometry, HPLC, and fluorescence spectroscopy and identified as the cis-11,12-diol-BP.

MATERIALS AND METHODS: [^{14}C]-BP was obtained from Amersham. Unlabeled BP was obtained from Aldrich Chemical Company. Both were checked for chemical purity by HPLC and were found to be 99% pure. Unlabeled BaP was further purified by use of neutral alumina column chromatography with benzene or toluene as the eluant followed by recrystallization in a benzene-isopropanol mixture. The trans-11,12-diol of BP was the generous gift of Shen K. Yang (Uniformed Services University of the Health Sciences, Bethesda, Maryland). The cis-4,5-diol, cis-7,8-diol, and trans-9,10-diol of BP were obtained from the NCI Chemical Carcinogen Reference Standard Repository. Benz(a)anthracene was obtained from Pfaltz & Bauer and purified as described above for unlabeled BP.

Cell culture conditions and BP metabolism: Cultures of S. capricornutum (strain no. UTEX 1648) obtained from the University of Texas in Austin were maintained in a synthetic nutrient medium (4). Algal cultures were aseptically dispensed into 125 ml Delong flasks at a density of 5 x 10^5 cells/ml in 25 ml of medium per flask. A mixture containing [^{14}C]-labeled (0.5 μCi) and unlabeled BP (a total of 30 μg) dissolved in 100 μl ethylene glycol monomethyl ether was added to each flask. Neither the solvent nor the dose of BP administered had a significant effect on growth (5). Algal cultures were incubated on a New Brunswick Scientific gyrotory shaker at 100 rpm in a Percival incubator at 23°C. Cultures were illuminated during incubation with gold fluorescent lamps (G.E. F20T12/GO) on a 24-hour light/dark cycle (16 hours light, 8 hours dark). Photooxidation of BP is negligible and algal growth is sustained under these conditions (5). Cultures were routinely monitored for bacterial, fungal, or yeast contamination (5) and no contamination was detected in the cultures used in this study. After a four-day incubation period, the algal suspension was centrifuged at 1500 rpm for five minutes at 5°C. BP and its metabolites, 90% of which were found in the medium, were recovered by extraction of the medium with ethyl acetate (3 x 12.5 ml). The extracts were pooled, evaporated to dryness under nitrogen, and stored at -20°C. Samples were brought to room temperature just prior to HPLC analysis.

HPLC analysis of dihydrodiols: Samples were reconstituted in chloroform prior to injection onto the HPLC. Separations were performed at room temperature on a Waters liquid chromatographic system equipped with a Whatman Partisil 10 ODS-2 reversed-phase column with a flow of 1 ml/min. Eluant was monitored at 254 nm with a Waters Model 440 ultraviolet absorbance detector. Fractions were collected with an ISCO Model 2111 automatic fraction collector and radioactivity was quantitated by scintillation counting using a Packard Tri Carb Liquid Scintillation Counter Model 460 CD.

Analysis by fluorescence spectroscopy and mass spectrometry: Mass spectra were run on a Kratos MS/80 high resolution mass spectrometer with resolution capabilities in excess of 20,000. Samples were introduced via direct probe insertion. Fluorescence spectra were recorded on collected fractions with an Aminco-Bowman spectrophotofluorometer that corrects for instrumentation artifacts including lamp output, photomultiplier response and monochromator grating.

RESULTS AND DISCUSSION: Following exposure of the green alga, Selenastrum capricornutum, to BP, the culture medium was extracted and analyzed by HPLC.

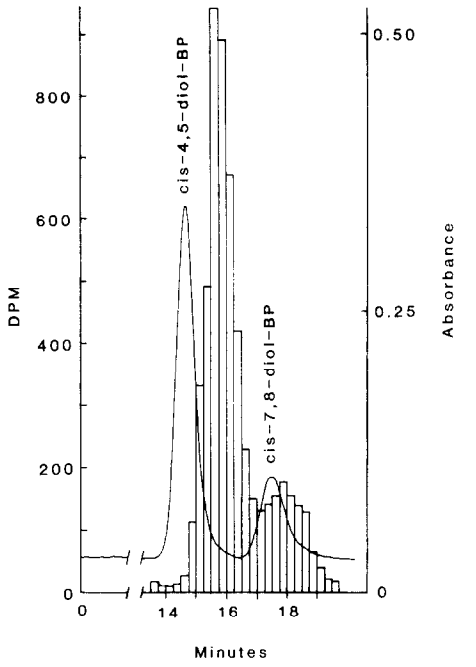

Figure 1. HPLC chromatograph of standard cis-4,5- and cis-7,8-diols of BP cochromatographed with metabolites isolated from algal media after incubation of S. capricornutum with [^{14}C]-BP. Bars represent radioactivity extracted from algal media. Solid line represents ultraviolet absorbance of synthetic dihydrodiol metabolites.

Radioactive products were present which coeluted with 4,5-, 7,8-, and 9,10-diol standards. In addition, a radioactive product was present which eluted in the diol region, but did not cochromatograph with any of the known standards (Figure 1, peak at 16 min.). This new metabolite was subjected to mass spectrometry; the resulting mass spectrum had a molecular ion with an m/e value of 286 (relative abundance of 12.6), with an elemental analysis of 20 carbon, 14 hydrogen, and two oxygen atoms. This corresponds to the molecular weight and elemental composition of BP dihydrodiols. There was a peak present in the mass spectrum at m/e 268 (relative abundance 26.9), representing loss of the neutral fragment, H_2O, from the metabolite. The base peak of m/e 252 (relative abundance 100) corresponds to the molecular weight of the parent compound, BP.

The unknown metabolite was further characterized by fluorescence spectroscopy. Both the excitation and emission fluorescence spectra were characteris-

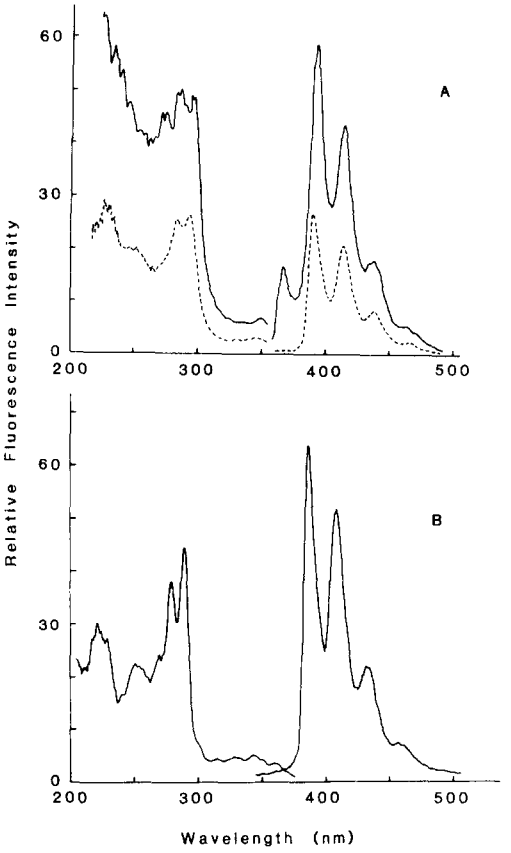

Figure 2. (A) Fluorescence excitation (200-350 nm) and emission (350-500 nm) spectra of synthetic cis-11,12-diol-BP (----) and fluorescence excitation and emission spectra of the previously unidentified metabolite isolated from incubations of algae with [^{14}C]-BP (——). (B) Fluorescence excitation and emission spectra of synthetic benz(a)anthracene.

tic of a benz(a)anthracene chromophore (Figures 2A and 2B). When compared with the trans-11,12-diol-BP standard fluorescence spectra, the spectra of the unknown metabolite was identical (Figure 2B). However, due to the fact that the new metabolite did not coelute with the trans-11,12-diol standard, which elutes between 4 and 5 minutes, yet had identical spectral properties, we are able to conclude that the new metabolite is the cis-11,12-diol-BP. This is consistent with other findings from our laboratory in which we have shown that the 4,5- and 7,8-diols isolated from the algal medium are cis-diols (3).

Four BP-diols have been isolated as metabolites in the green alga, S. capricornutum. The cis-4,5- and cis-7,8-diols represent <1% and 13% of

the total dihydrodiol fraction, respectively. The 9,10-diol, which elutes at 6.6 minutes, is present in much greater quantities, representing 36% of the total diol metabolites. As shown in the present study, the cis-11,12-diol is the major dihydrodiol metabolite, representing 50% of the total diol fraction. This represents an 8.3% conversion of BP to the 11,12-diol.

The significance of metabolism of BP to the 11,12-diol is unknown at the present time. Of the systems in which this metabolite has been looked for, the trans-11,12-diol-BP has been found as a metabolite in rat skin, both in vivo and in short-term organ culture (6,7). It has not been found as a metabolite in mouse skin or in rat liver microsomal preparations (7,8). Presently there is no evidence to implicate metabolism at the 11 and/or 12 positions as having a role in the carcinogenic process (9,10). However, the effect of metabolism of BP to the cis-11,12-diol by a widespread aquatic plant on the algae themselves or on organisms in the food chain is unknown.

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