Vol. 94, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 14, 1980 Pages 226-232

FUNGAL OXIDATION OF BENZO[a]PYRENE: FORMATION OF (-)-*trans*-7,8-DIHYDROXY-7,8-DIHYDROBENZO[a]PYRENE BY *Cunninghamella elegans*

Carl E. Cerniglia[1], William Mahaffey and David T. Gibson

Department of Microbiology, The University of Texas at Austin 78712

Received March 24,1980

SUMMARY. *Cunninghamella elegans* oxidizes benzo[a]pyrene to several metabolites that are soluble in ethyl acetate. One of these, *trans*-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene, was converted to a di-(-)menthoxyacetate that had the same retention time on high pressure liquid chromatography as the di-(-)-menthoxyacetate formed from (-)-*trans*-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene. Radioactive *trans*-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene formed by *C. elegans* was mixed with synthetic racemic dihydrodiol prior to reaction with (-)menthoxyacetyl chloride. Resolution of the resulting diastereomers by high pressure liquid chromatography showed that more than ninety-five percent of the radioactivity cochromatographed with the di-(-)menthoxyacetate formed from (-)-*trans*-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene. These results are similar to those reported for mammals which form the more tumorigenic (-)-enantiomer of *trans*-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene.

Benzo[a]pyrene (BP)[2] is a widespread environmental pollutant that exhibits toxic, mutagenic and carcinogenic activity (1-4). Recent evidence (5-10) indicates that microsomal enzymes from mammalian liver oxidize BP almost exclusively to the (-)-enantiomers of *trans*-9,10-dihydroxy-9,10-dihydro BP, *trans*-4,5-dihydroxy-4,5-dihydro BP and *trans*-7,8-dihydroxy-7,8-dihydro BP (BP 7,8-dihydrodiol). In addition, microsomes prepared from rat liver nucleii also form predominately the (-)-enantiomer of BP 7,8-dihydrodiol (11). The enantiomeric specificity of the mammalian monooxygenase and epoxide hydrase enzyme systems has received considerable attention since the (-)-enantiomer of BP 7,8-dihydrodiol is 5- to 10-fold more potent as a tumor initiator than (+)-BP 7,8-dihydrodiol. Furthermore, the metabolic conversion of (-)-BP 7,8-dihydrodiol to (+)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-

[1]Present address: National Center for Toxicological Research, Food and Drug Administration, Jefferson, Arkansas 72079.

[2]Abbreviations; BP, benzo[a]pyrene; BP 7,8-dihydrodiol, *trans*-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene; diol epoxide-2, 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; HPLC, high pressure liquid chromatography.

0006-291X/80/090226-07$01.00/0

tetrahydrobenzo[a]pyrene [(+)-diol epoxide-2] also occurs with a high degree of enantiomeric specificity (4,5). Diol epoxide-2 is highly mutagenic towards bacterial and mammalian cells and has been implicated as the ultimate carcinogen formed from BP (12-14).

In contrast to the extensive investigations on the metabolism of BP by higher organisms relatively little is known about the degradation of this compound by microorganisms. Recently we reported that the filamentous fungus, *Cunninghamella elegans* oxidizes BP to BP 7,8- and 9,10-dihydrodiols in addition to BP quinones, BP phenols and conjugated derivatives (15). This organism also oxidizes BP and (±)-BP 7,8-dihydrodiol to 7β,8α,9α,10β-tetrahydroxy-7,8,9,10-tetrahydro BP indicating the prior formation of the highly mutagenic and tumorigenic diol epoxide-2 (16). These results could be explained by the fungus forming both enantiomers of BP 7,8-dihydrodiol and diol epoxide-2 or by the formation of a single enantiomer of BP 7,8-dihydrodiol followed by stereospecific epoxidation at the 9,10-position. The results show that the BP 7,8-dihydrodiol formed from BP by *C. elegans* is almost exclusively the (-)-enantiomer.

MATERIALS AND METHODS:

Organism and Growth Conditions: *C. elegans* was maintained on Difco Sabouraud dextrose agar medium (17). BP biotransformation experiments were conducted as previously described (15). BP was added at 2 mg/ml in dimethylformamide. Experiments with [^{14}C]-BP (53.10 μmole, 1.0 μCi in 1.0 ml of dimethylformamide) were conducted as described earlier (15). After 24 hours incubation, organisms were removed by filtration through cheesecloth and the filtrate was extracted with three equal volumes of ethyl acetate. The organic extract was dried over Na_2SO_4 and the solvent was removed *in vacuo*. The BP 7,8-dihydrodiol formed was isolated as described previously (15).

Determination of Enantiomeric Purity: Metabolically-formed [^{14}C]-BP 7,8-dihydrodiol (16,500 dpm) was mixed with 1 mg of non-radioactive synthetic (±)-BP 7,8-dihydrodiol in 0.2 ml of pyridine and reacted with 10 mg (-)menthoxyacetyl chloride for 16 hours at room temperature (9). The (-)menthoxyacetyl chloride was synthesized as described previously (18). Water (2 ml) was added to the mixture which was then extracted with three 2.0 ml portions of diethyl ether and benzene (1:1). The organic extract was evaporated to dryness with a gentle stream of nitrogen. The sample was dissolved in 200 μl of methylene chloride and the resulting di-(-)menthoxyacetates were separated by high pressure liquid chromatography (HPLC). A Waters Associates instrument equipped with a M6000A pump, a M440 UV detector with a 280 nm filter, a U6K loop injector, and a normal phase μPorasil column (30 cm x 3.9 mm) was used for all HPLC analyses. The mobile phase was 0.25%

ethyl acetate (V/v) in methylene chloride with a flow rate of 2.0 ml/min (11). As the compounds eluted from the column they were collected at 0.5 min intervals into scintillation vials containing 5.0 ml of Aquasol-2. The radioactivity in each fraction was determined in a Beckman LS-250 liquid scintillation counter. Experiments with unlabelled BP were conducted as described above. Ultraviolet and visible spectra were recorded on a Beckman model 25 recording spectrophotometer.

Chemicals: BP was purchased from Eastman Organic Chemical Co. [7,10-^{14}C]-BP was obtained from California Bionuclear Corp. BP 7,8-dihydrodiol was kindly provided by Dr. David Longfellow of The National Cancer Institute Carcinogenesis Research Program. (-)Menthoxyacetic acid was purchased from Aldrich Chemical Co. Solvents for high pressure liquid chromatography analysis were purchased from Burdick and Jackson Laboratories, Inc. All other chemicals were of reagent grade or the highest available purity.

RESULTS AND DISCUSSION:

When intact cells of *C. elegans* were incubated with BP for 24 hours 3.2 percent of the added substrate was converted to ethyl acetate-soluble metabolites. BP 7,8-dihydrodiol, which had been identified previously (15), accounted for 25 percent of the total metabolic products. Fig. 1 shows that the BP 7,8-dihydrodiol produced by *C. elegans* formed a diester with (-)men-

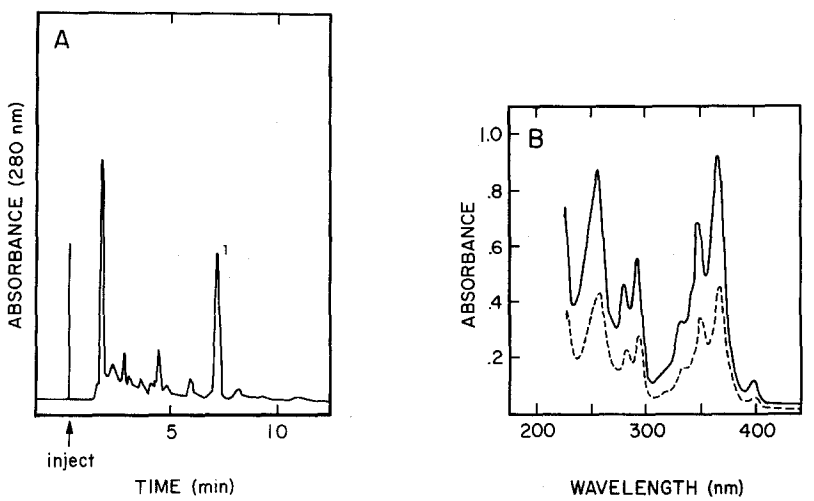

Fig. 1. A. Isolation of the di-(-)menthoxyacetate of BP 7,8-dihydrodiol formed by *C. elegans* by HPLC. Chromatographic conditions are described in Materials and Methods.
B. Absorption spectra of the di-(-)menthoxyacetate (compound I) of BP 7,8-dihydrodiol formed by *C. elegans* (————) and the di-(-)menthoxyacetate of (-)-BP 7,8-dihydrodiol (----------). The latter compound was prepared from (±)-BP 7,8-dihydrodiol as described in Materials and Methods. Compounds were dissolved in methylene chloride and absorption spectra were recorded on a Beckman model 25 recording spectrophotometer.

thoxyacetyl chloride that had a similar retention time (7.5 min., compound I), when analyzed by HPLC, as that reported for the di-(-)menthoxyacetate formed from the (-)-enantiomer of BP 7,8-dihydrodiol (11). In order to confirm the identity of compound I its absorption spectrum was compared to that given by the di-(-)menthoxyacetate formed from (-)-BP 7,8-dihydrodiol (19, Fig. 1B). Although absorption spectra do not differentiate between (+)- and (-)-enantiomers these results together with those in Fig. 1A suggest that *C. elegans* oxidizes BP primarily to (-)-BP 7,8-dihydrodiol.

In order to determine the optical purity of the biologically-formed dihydrodiol several experiments with [^{14}C]-BP were conducted. The [^{14}C]-BP 7,8-dihydrodiol fractions were pooled (16,500 dpm) and mixed with non-radioactive (±)-BP 7,8-dihydrodiol. After reaction with (-)menthoxyacetyl chloride the resulting di-(-)menthoxyacetates were separated by HPLC (Fig. 2). The di-(-)menthoxyacetates formed from (-)- and (+)-BP 7,8-dihydrodiol eluted

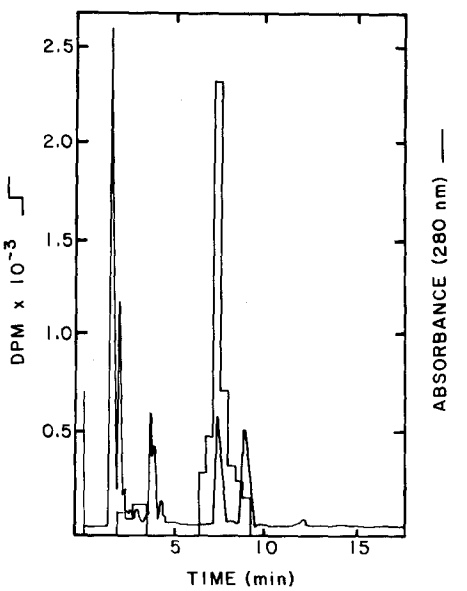

Fig. 2. HPLC separation of the di-(-)menthoxyacetates formed from synthetic (±)-BP 7,8-dihydrodiol and [^{14}C]-BP 7,8-dihydrodiol formed by *C. elegans*. The compound eluting at 7.5 min is the (-)-diester corresponding to (-)-BP 7,8-dihydrodiol. The compound eluting at 9.0 min is the (+)-diester corresponding to (+)-BP 7,8-dihydrodiol. Chromatographic conditions are described in Materials and Methods.

at 7.5 and 9.0 min respectively. The order of elution has been established by several investigators (5,9,11,19,20). Almost all of the radioactivity co-chromatographed with the di-(-)menthoxyacetate formed from the (-)-enantiomer of BP 7,8-dihydrodiol. Thus, *C. elegans* oxidizes BP almost exclusively to (-)-BP 7,8-dihydrodiol (>95% optical purity). Fig. 2 also shows that radioactivity eluted prior to the di-(-)menthoxyacetates. Apparently some of the [^{14}C]-BP 7,8-dihydrodiol decomposes to polar products. The instability of [^{14}C]-(-)-BP 7,8-dihydrodiol has been reported previously (21).

The results indicate the close similarity in the mechanisms used by mammals and a eucaryotic microorganism for the oxidation of BP. Thus rat liver microsomes from control, phenobarbital-treated, or 3-methylcholanthrene-treated Long-Evans rats form 92-96% of the (-)-enantiomer of BP 7,8-dihydrodiol (5,22). The significance of this observation lies in the fact that (-)-BP 7,8-dihydrodiol is 5-10 times more potent than the (+)-enantiomer as a tumor initiator (10,22), 10-20 times more active than the (+)-enantiomer in causing pulmonary adenomas and lymphomas in new born, Swiss-Webster mice (14,22) and 10 times more active than the (+)-enantiomer in causing tumors on the skin of C57/B6 female mice (22). In addition, (-)-BP 7,8-dihydrodiol is oxidized primarily to (+)-diol epoxide-2 by liver microsomes from 3-methylcholanthrene-treated rats and by a highly purified and reconstituted system containing cytochrome P-448 (5,22). (+)-Diol epoxide-2 is considerably more mutagenic and tumorigenic than the other three isomers (22,23). *C. elegans* also forms (-)-BP 7,8-dihydrodiol and diol epoxide-2 (15,16). The physio-

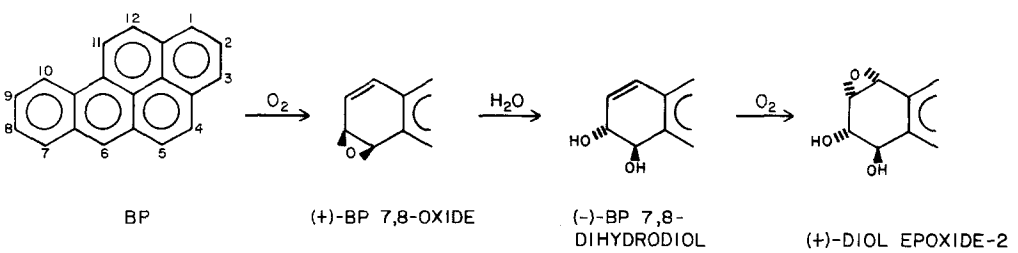

Fig. 3. Proposed pathway for the formation of diol epoxide-2 from benzo[a]pyrene by *C. elegans*.

logical consequences of these reactions in terms of the metabolism of the microorganism have yet to be determined. Also, the results suggest that the fungal monooxygenase and epoxide hydrase systems show high stereospecificity in the formation of diol epoxide-2 (Fig. 3). However, at this time we cannot be certain that the monooxygenase is stereospecific. Thus both enantiomers of BP 7,8-oxide could be formed with only the (+)-enantiomer serving as a substrate for epoxide hydrase. Confirmation of the reaction sequence shown in Figure 3 awaits studies with purified fungal enzymes.

ACKNOWLEDGEMENTS:

This research was supported by grant ROl CA-19078 from the National Cancer Institute, DHEW. C.E. Cerniglia was a postdoctoral trainee supported by grant T32 CA-09182 from the National Cancer Institute, DHEW. We thank Cathy Potter and Roberta DeAngelis for assistance in preparing the manuscript.

REFERENCES:

1. Huberman, E., Aspiras, L., Heidelberger, C., Grover, P.L. & Sims, P. (1971) Proc. Natl. Acad. Sci. USA 68, 3195-3199.

2. McCann, J., Choi, E., Yamasaki, E. & Ames, B.N. (1975) Proc. Natl. Acad. Sci. USA 72, 5135-5139.

3. Slaga, T.J., Bracken, W.M., Vioge, A., Levin, W., Yagi, H., Jerina, D.M. & Conney, A.H. (1977) Cancer Res. 37, 4130-4133.

4. Thakker, D.R., Yagi, H., Lu, A.Y.H., Levin, W., Conney, A.H. & Jerina, D.M. (1976) Proc. Natl. Acad. Sci. USA 73, 3381-3385.

5. Thakker, D.R., Yagi, H., Akagi, H., Koreeda, M., Lu, A.Y.H., Levin, W., Wood, A.W., Conney, A.H. & Jerina, D.M. (1977a) Chem. Biol. Interact. 16, 281-300.

6. Yang, S.K., McCourt, D.W., Roller, P.P. & Gelboin, H.V. (1976) Proc. Natl. Acad. Sci. USA 73, 2594-2598.

7. Yang, S.K., McCourt, D.W., Gelboin, H.V., Miller, J.R. & Roller, P.P. (1977a) J. Am. Chem. Soc. 99, 5124-5130.

8. Yang, S.K., Roller, P.P. & Gelboin, H.V. (1977b) Biochemistry 16, 3680-3687.

9. Yang, S.K., McCourt, D.W., Lentz, J.C. & Gelboin, H.V. (1977c) Science 196, 1199-1201.

10. Levin, W., Wood, A.W., Chang, R.L., Slaga, T.J., Yagi, H., Jerina, D.M. & Conney, A.H. (1977) Cancer Res. 37, 2721-2725.

11. Jennette, K.W., Bornstein, W., Chuang, A.H.L. & Bresnick, E. (1979) Biochem. Pharmacol. 28, 338-339.

12. Sims, P., Grover, P.C., Swaisland, A., Pal, K. & Hewer, A. (1974) Nature 252, 326-328.

13. Kapitulnik, J., Levin, W., Conney, A.H., Yagi, H. & Jerina, D.M. (1977) Nature 266, 278-380.

14. Kapitulnik, J., Wislocki, P.G., Levin, W., Yagi, H., Jerina, D.M. & Conney, A.H. (1978) Cancer Res. 38, 354-358.

15. Cerniglia, C.E. & Gibson, D.T. (1979) J. Biol. Chem. 254, 12174-12180.

16. Cerniglia, C.E. & Gibson, D.T. (1980) J. Biol. Chem. (in press).

17. Cerniglia, C.E. & Perry, J.J. (1973) Z. Allg. Mikrobiol. 13, 299-306.

18. Leffler, M.T. & Calkins, A.E. (1943) In: Organic Synthesis (L.I. Smith, ed.) Vol. 23, pp. 55-56, Wiley, New York.

19. Harvey, R.G. and Cho, H. (1977) Anal. Biochem. 80, 540-546.

20. Yang, S.K., Gelboin, H.V., Weber, J.D., Sankaran, V., Fischer, D.L. & Engel, J.F. (1977) Anal. Biochem. 78, 520-526.

21. Pezzuto, J.M., Yang, C.S., Yang, S.K., McCourt, D.W. & Gelboin, H.V. (1978) Cancer Res. 38, 1241-1245.

22. Jerina, D.M., Yagi, H., Thakker, D.R., Karle, J.M., Mah, H.D., Boyd, D.R., Gadaginamath, G., Wood, A.W., Buening, M.K., Chang, R.L., Levin, W. & Conney, A.H. (1979) Adv. Pharmacol. Therapeut. 9, 53-62.

23. Buening, M.K., Wislocki, P.G., Levin, W., Yagi, H., Thakker, D.R., Akagi, H., Koreeda, M., Jerina, D.M. & Conney, A.H. (1978) Proc. Natl. Acad. Sci. USA 75, 5358-5361.