Vol. 155, No. 1, 1988
August 30, 1988

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 493-502

EFFECT OF INDUCERS ON METABOLISM OF BENZO(a)PYRENE IN VIVO AND IN VITRO : ANALYSIS BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

Debjani Datta and Timir B. Samanta*

Department of Microbiology, Bose Institute, 93/1, A.P.C. Road, Calcutta 700009, India

Received June 3, 1988

SUMMARY : The characterisation of metabolites formed from benzo(a)pyrene (BP) by Aspergillus ochraceus TS and effect of inducers on BP metabolism are reported. The high pressure liquid chromatographic profile of BP metabolites was similar to that of mammalian microsomes furnishing diols, quinones and phenols. The production of BP-4,5-dihydrodiol (K-region diol) by Aspergillus ochraceus TS seems to be novel and provides first report on BP metabolism by eukaryotic fungi. In control, phenols and quinones were produced in excess over dihydrodiols while the induced preparation showed the reverse order. Presumably the induction effecting production of excess dihydrodiols influenced the synthesis of epoxide hydrolase. In addition, a differential increase in BP metabolism was observed with inducers of narrow and broad specificity.

INTRODUCTION : Metabolism of polynuclear aromatic hydrocarbon particularly benzo(a)pyrene (BP) is vitally important just because this is a ubiquitous environmental pollutant which is cytotoxic and induce cancer. The characterization of BP-metabolites appears to be equally important since some of them being cytotoxic and mutagenic in nature is believed to cause mammalian cell transformation and toxicity (1,2). A prerequisite for understanding the mechanism of polycyclic aromatic hydrocarbon carcinogenesis is a detailed knowledge of the profile of metabolites formed and factors regulating their formation. In addition, use of suitable reference compounds for product characterization should lead to the identification of bioactivated carcinogen(s).

Presumably difficulties inherent in the synthesis of reference standards coupled with poor yield, the characterization of BP metabolites

*To whom correspondence should be addressed.

0006-291X/88 $1.50

had become questionable. However, present availability of synthetic derivatives and high pressure liquid chromatography (HPLC) have helped appreciably the resolution and characterization of BP metabolites formed during oxidative transformation.

In mammals benzo(a)pyrene is believed to be metabolised by a cytochrome P450 (Cyt P450) linked monoxygenase and epoxide hydrolase while in contrast very little is known about degradation of this hydrocarbon in microbial kingdom (3-7). In our previous communications (8-10) it was reported that Aspergillus ochraceus TS oxidises progesterone exclusively to its 11α-hydroxy derivative. Later the same organism was found to transform BP both under in vivo and in vitro conditions (11,12). The metabolism of BP by C. bainieri (13,14) was reported to be apparently initiated by a cyt P450-monoxygenase. The involvement of cyt P450 in oxidation of BP had later been established by our laboratory and others in Aspergillus chraceus TS (11,12), S. cerevisiae (15) and C. elegans (16) respectively. The multiplicity of Cyt P450 in Aspergillus ochraceus TS was also settled. With a view to differentiate the inducers in terms of metabolic profile, BP-metabolism was tried with Aspergillus ochraceus TS induced by agents of diverse chemical structure using HPLC as a tool. However, the first structural information on BP-metabolites was furnished by Cerniglia and Gibson from a transformation of BP by C. elegans (16). The present study provides the first report on the formation of BP-4,5-dihydrodiol (K-region diol) and the effect of inducers on oxidative metabolism of BP by a soil fungus Aspergillus ochraceus TS.

MATERIALS AND METHODS : Benzo(a)pyrene (BP) was purchased from SIGMA, USA, trans-BP-9, 10-dihydrodiol, trans-BP-7,8-dihydrodiol, trans-BP-4,5-dihydrodiols, 3-hydroxy benzo(a)pyrene, 9-hydroxy benzo(a)pyrene, BP-1, 6-quinone and BP-3,6-quinone were provided as gift by Dr. David Longfellow at the National Cancer Institute, Chemical Repository at the IIT Research Institute, Chicago, Illinois, USA. Solvents for high pressure liquid chromatography (HPLC) purchased from BDH, India were processed and used. All other chemicals were of analytical grade and used without further purification.

Transformation of BP in vivo : Aspergillus ochraceus TS was cultivated in liquid medium described previously (11,12). The organism was allowed to grow at 28-30°C on a rotary shaker for 48 h. The vegetative mycelium was filtered and transferred to fresh medium followed by the addition of BP (0.4 mM) dissolved in dimethylformamide (DMF) for transformation to continue for another 48 h. The control experiments were run in parallel both in absence of the organism and substrate. The flasks receiving substrate (BP) were covered with black paper during transformation.

Transformation of BP in vitro : The transformation in vitro was tried with individual microsome (12) obtained from cells induced by different agents. The inducers employed for the purpose were 3-methyl cholanthrene 3-MC, benzo(a)pyrene (BP), phenobarbital (PB) and progesterone. The typical incubation mixture contained 0.4μ moles of BP dissolved in dimethylformamide, 1.2μ moles of NADPH and protein (1 mg) in 0.1 M phosphate buffer, pH 7.5 in a total volume of 1 ml. The reaction was initiated by the addition of NADPH and the incubation was carried out at 28°C on a rotary shaker for 1 h under red light. The enzyme activity was frozen by the addition of a mixture of n-hexane:acetone (2:1, 1 ml) followed by the isolation of metabolites by extraction. Parallel controls were maintained.

Isolation of BP-metabolites : After the transformation in vivo the mycelium was filtered, washed successively with 0.5% (w/v) NaCl solution and water to make them free from adhering substrate. The washings pooled in the filtrate were then extracted with half the volume of ethyl acetate three times. The solvent extract washed with water, dried over anhydrous Na_2SO_4 and was finally evaporated to dryness under reduced pressure and temperature. The metabolites from in vitro experiments were isolated exactly in the same way.

Characterisation of BP-metabolites : The crude residue obtained from both in vivo and in vitro experiments was initially resolved on silica gel G plates (TLC) using benzene:methanol (95:5) as solvent system. The chromatogram was developed in an iodine chamber. The synthetic BP derivatives were used as reference.

High pressure liquid chromatography (HPLC) of BP metabolites : To prepare samples for analysis by high pressure liquid chromatography (HPLC), in each set the ethyl acetate extract from five flasks were combined together in order to get sufficient quantity. The crude residue (metabolites and unreacted BP) dissolved in minimum volume of spectral grade methanol was stored in a freezer at -20°C until HPLC analysis. High pressure liquid chromatography (HPLC) of BP metabolites was done with a Waters model 440 chromatograph fitted with a μ Bondapak C_{18} analytical column (reverse phase, 0.7 x 25 cm) in conjunction with a programmed solvent gradient. The initial and final solvent compositions were methanol:water (50:50) and (90:10) respectively. A convex gradient (curvature setting 5) was employed with a flow rate of 2 ml/min at 3000 p.s.i. The column temperature was maintained at 25°C. The elution was monitored at 280 nm with a uv detector. The retention times of BP metabolites were determined using synthetic BP derivatives as reference. Further identity was ascertained by coinjection of the metabolites with synthetic standards one at a time.

Acid catalysed dehydration of BP-9,10-dihydrodiol formed from BP by A. ochraceus TS : The major component of BP metabolite BP-9,10-dihydrodiol was dissolved in methanol to which 0.05 ml of 3N HCl was added. Phenol formation was followed by measuring the increase in absorbance at 375 nm (5,16). Synthetic trans-BP-9,10-dihydrodiol was treated similarly. In another experiment methanolic solution of 9,10-dihydrodiol formed from BP by A. ochraceus TS was heated with 0.1 ml of 6N HCl at 80°C for 15 min and the spectrum was recorded in situ in a Beckman dual beam spectrophotometer (Model 24).

RESULTS AND DISCUSSION : BP-metabolites obtained from both in vivo and in vitro transformations showed similar pattern. The mixture of the metabolites exhibited five bands on silica gel G plate (TLC) including the unreacted substrate (BP). These bands corresponded to synthetic BP-quinones, phenols and dihydrodiols used as reference. The major band below the diol region was tentatively labelled as pre-diol area. The individual band on TLC plate was scraped and extracted with methanol. Resolution of the diol region revealed the presence of at least three components having retention time 6.26, 9.9 and 11.0 minutes corresponding to that of trans-BP-9,10-dihydrodiol, trans-BP-4,5-dihydrodiol and trans-BP-7,8-dihydrodiol (Fig. 1.1) (Table 1). The trans-configuration was ascertained by comparing the relative rates of dehydration of the fungal metabolite with synthetic trans-dihydrodiol (Fig. 1.2). The dehydration experiment was done with BP-9,10-dihydrodiol since this metabolite was obtained in appreciable amount

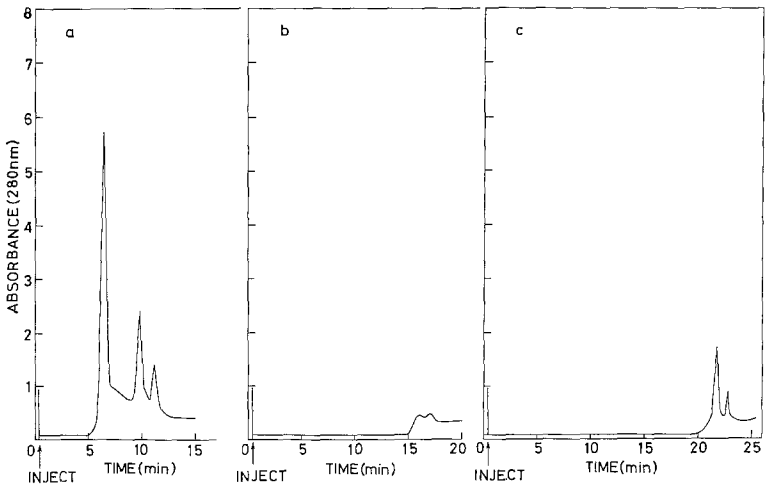


Fig. 1.1 HPLC Elution profile of BP-metabolites a)diol; b)quinone; & c) phenol region.

Table 1 : Retention time of benzo(a)pyrene (BP) metabolites separated by High Pressure Liquid Chromatography (HPLC)

Metabolite	Retention time (min)
BP-9,10-Dihydrodiol	6.26
BP-4,5-Dihydrodiol	9.9
BP-7,8-Dihydrodiol	11.0
BP-1,6-Quinone	15.8
BP-3,6-Quinone	16.75
9-OH-BP	21.25
3-OH-BP	22.0
BP	32.0

only. BP-9,10-dihydrodiol (BP-metabolite) had a similar rate of dehydration to that observed with synthetic trans-BP-9,10-dihydrodiol. Moreover, BP-9,10 -dihydrodiol formed from BP by A. ochraceus TS on treatment with acid furnished a product whose absorption spectrum was identical with that of an authentic sample of 9-hydroxybenzo(a)pyrene (Fig. 1.2a).

Analysis of the quinone band by HPLC exhibited the presence of two peaks identical with that of synthetic 1,6- and 3,6-quinones (Fig. 1.1) while the phenol region disclosed two peaks having retention time identical with that of synthetic 3-hydroxy and 9-hydroxy benzo(a)pyrene (Fig. 1.1). Figure 1.3 and 1.4 exhibit the resolution of BP-metabolites and synthetic standards by HPLC while Fig. 1.5 represents their co-elution

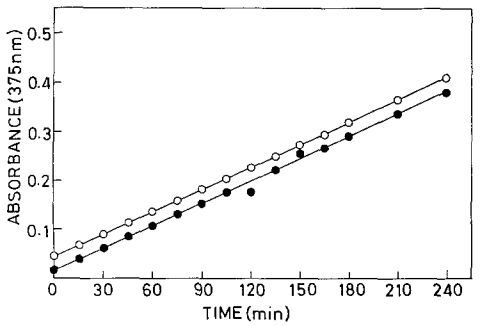


Fig. 1.2 Relative rate of dehydration of dihydrodiol formed from BP by A. ochraceus TS; (o-o) synthetic trans BP-9,10-dihydrodiol, (●—●) BP-9,10-dihydrodiol formed by A. ochraceus TS.

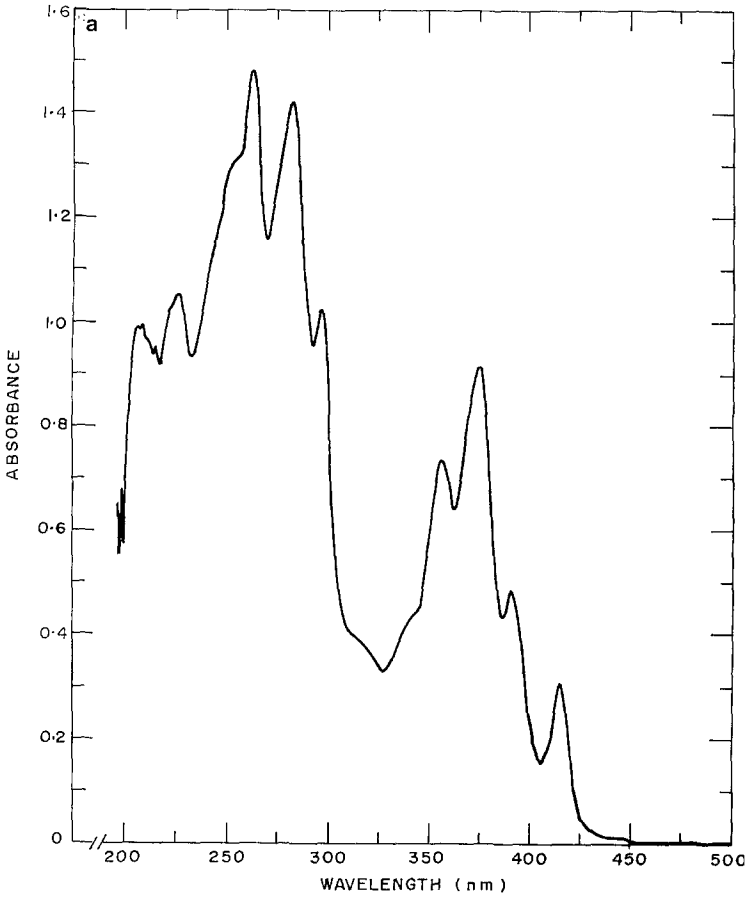
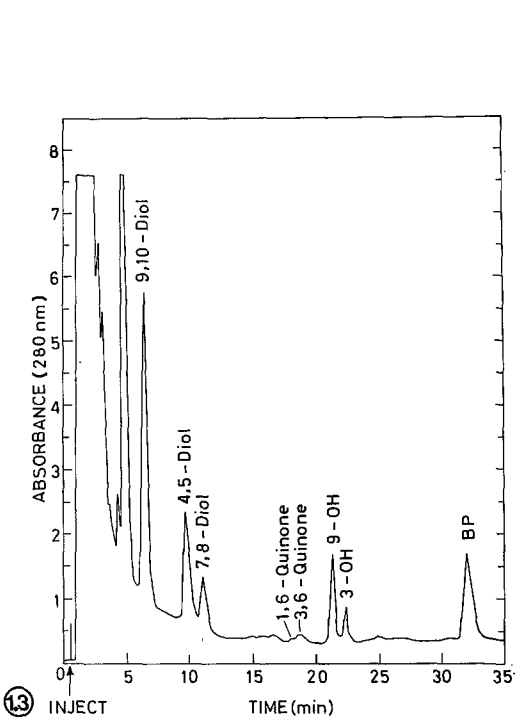


Fig. 1.2(a) Absorption spectrum of acid-catalyzed dehydration product of trans BP-9,10-dihydrodiol formed from BP by A. ochraceus TS.

profile. The low intense profile of the unreacted substrate in the chromatogram (Fig. 1.3) was due to its partial removal from the crude metabolite mixture by treatment with petroleum ether (60-80°C).

In addition to thin layer (TLC) and high pressure liquid chromatography (HPLC) BP-metabolites were further characterized with the help of their fluorescent and ultra-violet spectra. The products formed from BP by A. ochraceus TS are presented in Fig. 1.6. The control experiments in which BP- was incubated simply in the medium in absence of the organism showed no degradation of the substrate and in absence of the substrate (BP) the organism did not produce anything.

Quantitative difference in BP metabolism by induced and uninduced cells of Aspergillus ochraceus TS was observed. The results presented in Table 2 indicated that in control phenols and quinones were formed in excess over dihydrodiols while in induced preparation reverse order was noticed. In



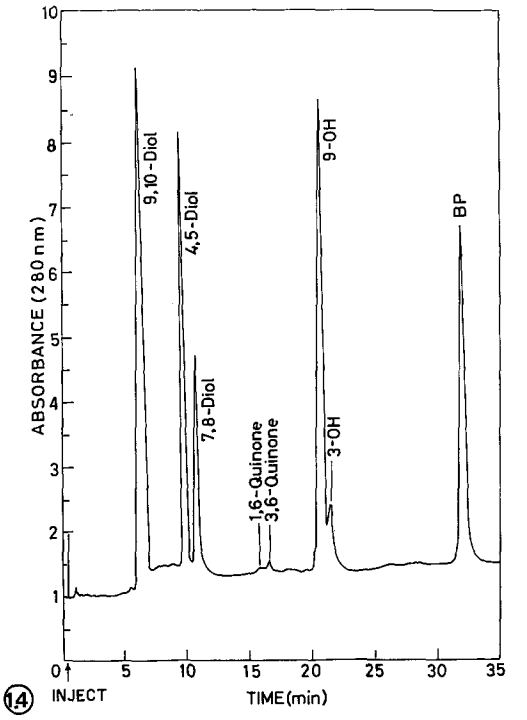


Fig. 1.3 HPLC Elution profile of BP metabolites by Aspergillus ochraceus TS.

Fig. 1.4 Resolution of a mixture of synthetic BP derivatives by HPLC.

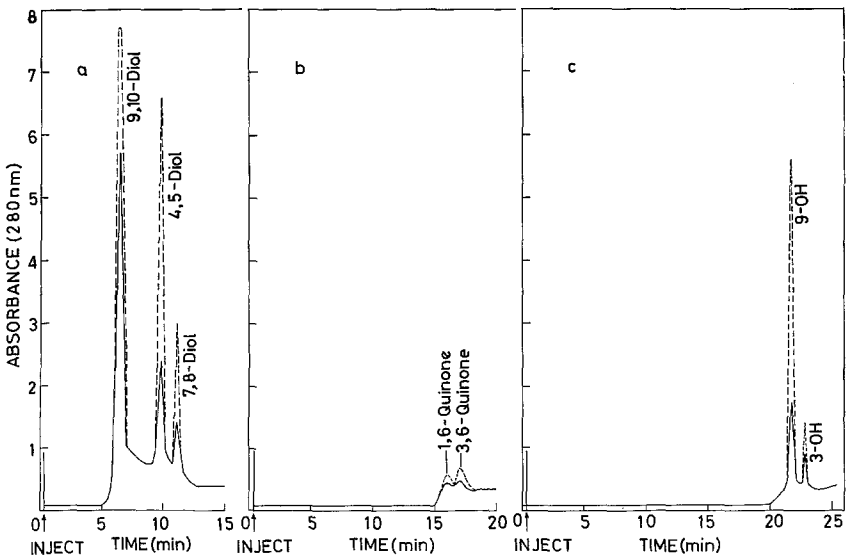


Fig. 1.5 HPLC Co-elution profile of BP metabolites (—) with synthetic standards (--); a) diols, b) quinones, & c) phenols.

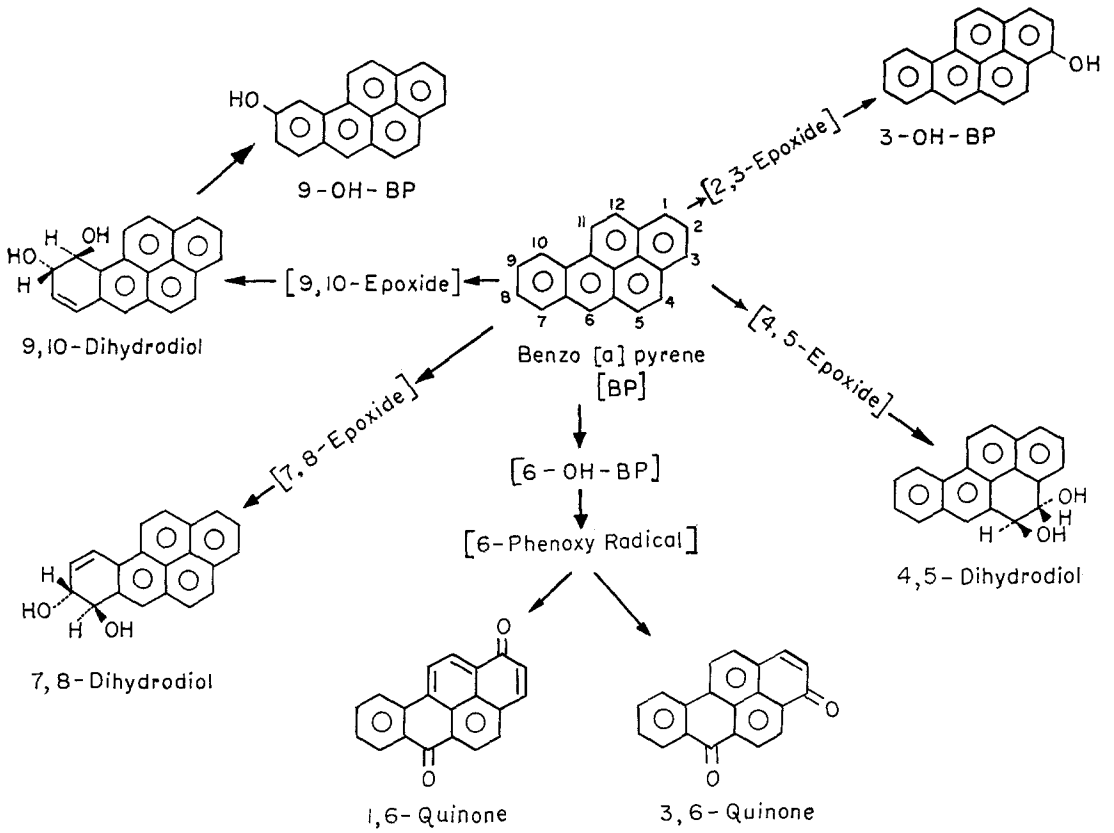


Fig. 1.6 Metabolic profile of BP by Aspergillus ochraceus TS.

Table 2 : Microsomal metabolism of benzo(a)pyrene by Aspergillus ochraceus TS

Experiment	Total area of metabolites	Diol*			Quinone*		Phenol*	
		9,10	4,5	7,8	1,6	3,6	9	3
Control	7.73	6.08	5.04	1.94	7.50	34.15	12.90	32.34
3-methyl cholanthrene pretreated	22.447	56.44	7.57	17.77	5.21	2.08	6.81	4.09
Benzo(a)pyrene pretreated	20.14	52.33	16.23	14.74	1.98	1.48	6.45	6.75
Phenobarbital pretreated	10.30	50.77	10.87	13.10	2.90	3.88	9.70	8.73
Progesterone pretreated	10.40	48.55	11.53	8.65	3.84	4.80	8.65	13.94

* Percent of each metabolite expressed as proportion of total area of metabolites eluting before BP

mammals induction by 3-MC effected increased production of dihydrodiols with concomitant decrease in phenols and quinones. Thus it may be suggested that induction somehow influences the enhanced synthesis of epoxide hydrolase (EC 3.3.2.3) in A. ochraceus TS since this enzyme had been shown to be necessary for the production of dihydrodiols during BP-metabolism in mammalian microsomes (17). Again, BP-metabolism was enhanced by inducers of narrow specificity, i.e., by BP and 3-MC over others like PB and progesterone. Similar results were obtained in respect of induction and kinetics of BP-hydroxylation by the test organism (11,12).

Thus, it appears that Aspergillus ochraceus TS oxidises BP in a manner qualitatively similar to that achieved by mammalian microsomes (17). In the present study the formation of dihydrodiols as major product apparently suggests that a cyt P450 monoxygenase catalyses the prior formation of arene oxide at 9,10 ; 7,8 and 4,5 positions. The production of 4,5-dihydrodiol (K-region diol) by A. ochraceus TS is really interesting and seems to be novel because this is a well known mammalian microsomal metabolite (18) which was not produced earlier (16) by microbial system during oxidative metabolism of BP. In addition, these reactions in fungi are really important due to the fact that newer product(s) may be identified in future which are yet to be detected as mammalian metabolite(s) thereby establishing the role of these reactions for management of PAHS in the ecosystem. Holder et al., had reported that all dihydrodiols formed by liver microsome are (-) enantiomer which have high optical purity. Furthermore, (-) trans-BP-7,8-dihydrodiol forms the highly mutagenic 7β, 8α-dihydroxy -9α, 10α- epoxy- 7,8,9,10-tetrahydro benzo(a)pyrene when it is activated by microsomal aryl hydrocarbon hydroxylase (19,20). The optical purity of trans-7,8,9,10 and 4,5-dihydrodiol furnished by A. ochraceus TS remains to be determined. However, it would be interesting to see if the fungal enzymes exhibit similar enantiomeric specificity to that observed in mammalian system.

ACKNOWLEDGEMENTS : The authors wish to thank Prof. B. B. Biswas, Director and Prof. A. C. Ghosh, Chairman, department of Microbiology, Bose Institute for encouragements. One of the authors (D.D.) is thankful to C.S.I.R., New Delhi for award of a Senior Research Fellowship.

REFERENCES

1. Miller, E.C. and Miller, J.A. (1973) in The Molecular Biology of Cancer, ed. Busch, H. (Academic Press, New York), pp. 377.
2. Miller, J.A..AND Miller, E.C. (1976) in Biology of Radiation Carcinogenesis, Yuhas, J.M. (Raven Press, New York), pp. 147.
3. Sisler, F.D. and ZoBell, C.E. (1947) Science, 106, 521-522.

4. Barnsley, E.A. (1975) Can. J. Microbiol., 21, 1004-1008.
5. Gibson, D.T., Mahadevan, V., Zerina, D.M., Yagi, H. and Yeh, H.J.C. (1975) Science, 189, 295-297.
6. Gibson, D.T. (1977) in Fate and Effects of Petroleum Hydrocarbons in Marine Ecosystems and organisms, ed. Wolfe, D.A. (Pergamon Press, New York) pp. 36-46.
7. Herbes, S.E. and Schwall, L. (1978) Appl. Environ. Microbiol., 35, 306-316.
8. Samanta Timir B., Roy, N. and Chattopadhyay, S. (1978). Biochem. J., 176, 593-594.
9. Ghosh, D.K. and Samanta, Timir B. (1981) J.Steroid Biochem., 14, 1063-1067.
10. Samanta, Timir B. and Ghosh, D.K. (1987) J. Steroid Biochem., 28, 327-32.
11. Ghosh, D.K., Datta, D., Mishra, A.K. and Samanta, Timir B. (1983) Biochem. Biophys. Res. Commun., 113, 497-505.
12. Datta, D., Ghosh, D.K., Mishra, A.K. and Samanta, Timir B. (1983) Biochem. Biophys. Res. Commun., 115, 692-699.
13. Ferris, J.P., Fasco, M.J., Stylianopoulou, F.L., Jerina, D.M., Daly, J.W. and Jeffery, A.M. (1973) Arch. Biochem. Biophys., 156, 97-103.
14. Ferris, J.P., MacDonald, L.H., Patrie, M.A. and Martin, M.A. (1976) Arch. Biochem. Biophys., 175, 443-452.
15. Azari, M.R. and Wiseman, A. (1982) Anal. Biochem., 122, 129-138.
16. Cerniglia, C.E. and Gibson, D.T. (1979) J. Biol. Chem., 254, 12174-12180.
17. Holder, G., Yagi, H., Dansette, P., Jerina, D.M., Levin, W., Lu, A.Y.H. and Conney, A.H. (1974) Proc. Natl. Acad. Sci., (USA) 71, 4356-4360.
18. Grover, P.L., Hewer, A. and Sims, P. (1972) Biochem. Pharmacol., 21, 2713-2726.
19. Thakker, D.R., Yagi, H., Akagi, H., Koreeda, M., Lu, A.Y.H., Levin, W., Wood, A.W., Conney, A.H. and Jerina, D.M. (1977) Chem. Biol. Interact., 16, 281-300.
20. Yang, S.K., McCourt, D.W., Leutz, J.C. and Gelboin, H.V. (1977) Science, 196, 1199-1201.