ISOLATION AND CHARACTERIZATION OF AN ACTIVE DNA-BINDING METABOLITE OF BENZO(a)PYRENE FROM HAMSTER LIVER MICROSOMAL INCUBATION SYSTEMS

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SUMMARY: An active metabolite of benzo(a)pyrene (BaP) produced by hamster liver microsomes has been detected and isolated. This active metabolite (A.M.) bound covalently to DNA upon incubation together at 37°C. The presence of cyclohexene oxide increased the yield of A.M. to about 10 fold, while the yield of another major metabolite 4,5-dihydro-4,5-dihydroxy-BaP (4,5-diol) was greatly decreased. The Rf values were 0.65 for BaP, 0.43 for A.M. and less than 0.2 for the other metabolites when chromatographed on thin-layer silica gel with benzene. The molecular formula of A.M. was determined to be $C_{20}H_{12}O$. This active metabolite is preliminarily identified as the 4,5-epoxide of BaP.

INTRODUCTION: Benzo(a)pyrene (BaP) is a polycyclic hydrocarbon (PCH) which has long been known to be a potent carcinogen, and is present widely in our environment. The metabolism of BaP *in vivo* and *in vitro* has been studied by many workers, and was extensively studied by Falk *et al* (1), and by Sims (2,3). Epoxides were postulated as the intermediary metabolites of PCH by hepatic microsomes, which were further metabolized or rearranged to dihydrodiols, phenols or conjugated with glutathione (4). Arene oxides were suggested as the obligate intermediates in aromatic hydroxylation (5,6). A coupled aryl monooxygenase-epoxide hydrase system, which readily converted the epoxide intermediates to dihydrodiols, was shown to be present in hepatic microsomes (7). Many chemically synthesized epoxides inhibited epoxide hydrase activity toward styrene oxide *in vitro* (8).

Epoxide intermediates have been shown in the microsomal metabolism of dibenz(a,h)anthracene (9,10), phenanthrene and benz(a)anthracene (10). K-region epoxides of some of the PCH have been shown to bind cell components (11,12), and produce transformation in certain cell culture systems (13). However, the K-region epoxides or dihydrodiols of BaP as metabolites were not detected (2,3,14). Only very recently, evidence was reported for the presence

of 4,5-epoxide of BaP in a rat liver microosmal incubation system (15).

In our laboratory, we have been studying the metabolism of BaP by hamster tissues, and have consistently found 4,5-diol as one of the major metabolites of BaP with either hamster liver microsomes or homogenates in our incubation systems. In attempts to detect 4,5-epoxide of BaP, we have found and isolated an active metabolite of BaP, which binds to DNA in vitro without the need of further enzymatic activation.

METHODS: Liver microsomes in Tris-sucrose buffer (16) were prepared from 3-month old Golden Syrian male hamsters, which were not pretreated with inducers of mixed-function oxidases (17). Microsomal suspensions were kept frozen at -70°C in a Revco freezer, and thawed just before use. Protein content was determined by Lowry's method (18).

The incubation mixture for ^{3}H-BaP metabolism contained 10 mμmole ^{3}H-BaP (specific activity 4.8 ci/mM) in 100 μl acetone, microsomes containing 3.75 mg protein in 0.5 ml Tris-sucrose buffer, 4.5 μmole NADPH, in a total volume of 3.75 ml in citrate buffer at pH 7.4 (16). For large-scale incubations, 10 flasks containing the above proportions of ingredients in a total volume of 30 ml each were used and pooled after incubation. Incubations were carried out under yellow light at 37°C for 10 minutes, unless otherwise specified. When used, cyclohexene oxide [7-oxabicyclo(4.1.0)heptane] was added in 40 μl acetone prior to the addition of microsomes to the incubation mixture. Following incubation, the mixture was extracted with 1.5 volume ethyl acetate. The organic layer was removed and evaporated to dryness under vacuum or nitrogen. Thin-layer chromatography (TLC) was done immediately on silica gel coated plates (Eastman Chromagram 6061). Solvent systems are described in figure legends, Unlabeled standards of BaP, BaP-3,6-quinone, 3-hydroxy-BaP (3-OH-BaP) and trans-4,5-dihydro-4,5-dihydroxy-BaP (4,5-diol) were co-chromatographed with samples. The TLC plates were then cut up into pieces according to the locations of UV fluorescent spots and radioacticity peaks detected by radioscanning, and counted in 10 ml scintillation solution (16). The incu-

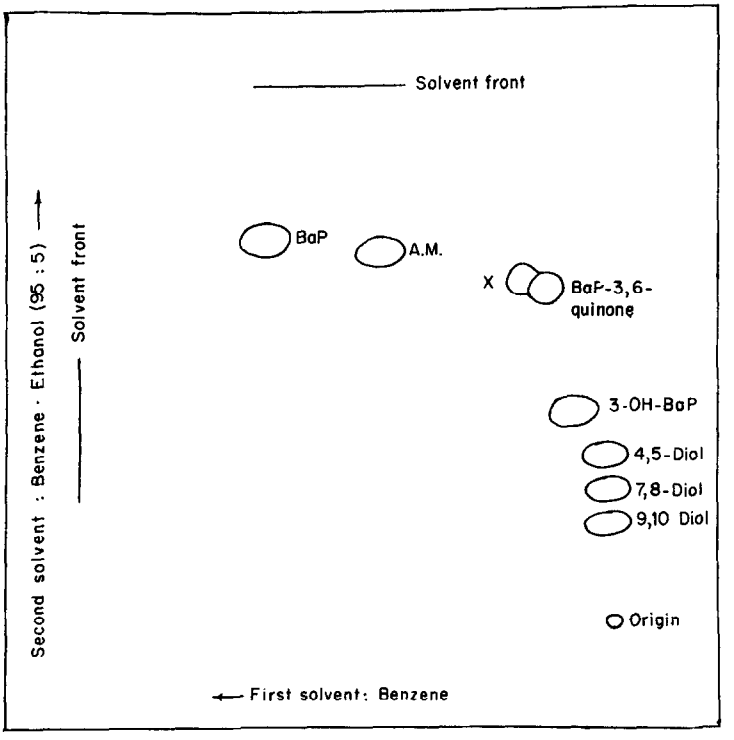

Fig.1. Two-dimensional TLC of BaP and its metabolites produced by hamster liver microsomes during 10 min. incubation. Spots shown were detected by UV fluorescence and by radioscanning. Identification of metabolites is as described in the text.

bation system for DNA-binding consisted of 200 µg salmon testis DNA in 1 ml citrate buffer plus the metabolite being tested in 25 µl acetone. After incubation, the mixture was extracted three times with an equal volume of $CHCl_3$: isoamyl alcohol (24:1), then dialyzed and centrifuged in CsCl (initial density 1.700 gm/cc) at 100,000g for 48 hours. The resulting gradient was collected at 25 drops per fraction, whose optical density at 260 nm and refractive index were measured. 100 µl aliquots were spotted on filter paper, dried, washed and counted in scintillation solution (16). Preparation of metabolites, TLC, and DNA-binding were done on the same day, except for the dialysis and CsCl gradient centrifugation of DNA solutions.

RESULTS AND DISCUSSION: The active metabolite (A.M.) of BaP produced by hamster liver microsomes was well separated from BaP and the other metabolites by two-dimensional TLC as is shown in Fig.1. Identification of metabo-

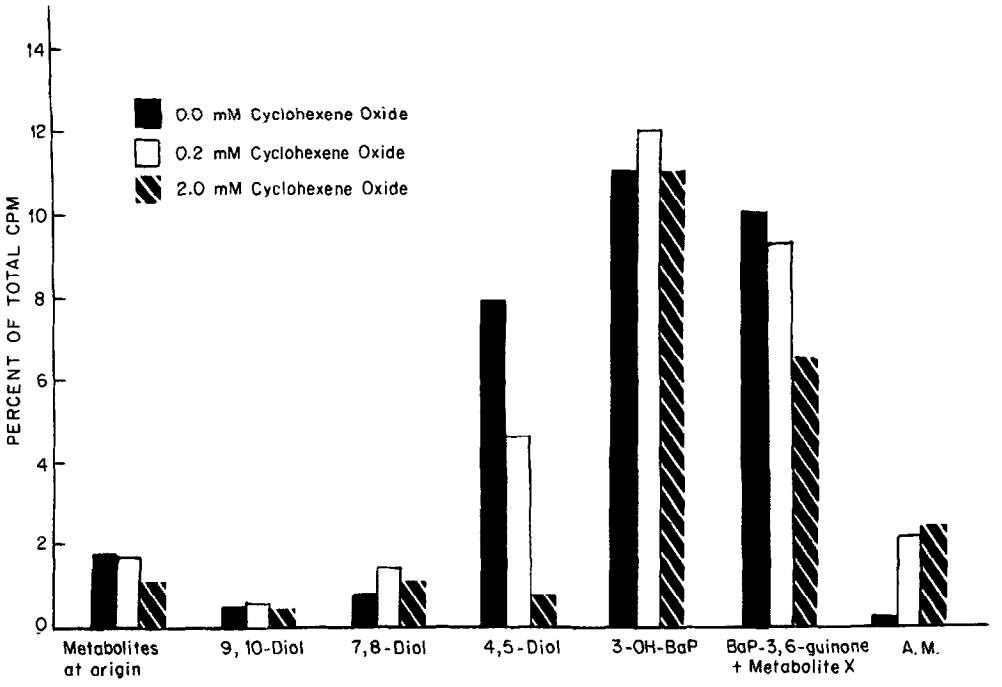

Fig.2. Effect of cyclohexene oxide on the metabolism of ^{3}H-BaP. Metabolites were separated by two-dimensional TLC of ethyl acetate extracts of incubation mixtures as shown in Fig.1. Metabolites were calculated as per cent of total radioactivity extracted into the ethyl acetate layer.

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lites was made by co-chromatography with standard compounds (BaP, BaP-3, 6-quinone, 3-OH-BaP, and 4,5-diol), as well as by comparison of UV spectra of the metabolites (isolated and purified from large-scale incubation of BaP) with those of the standard compounds or with published data (19). The details of the identification of metabolites of BaP will be published elsewhere. Since A.M. was located between BaP and BaP-3,6-quinone on TLC, the possibility of its being an epoxide was explored. An AEI high resolution mass spectrometer (equipped with a chemical ionization source, using 10% H_2O in CH_4 at 0.7 torr and 270°C source temperature) gave a measured peak mass for A.M. (as a metabolite of unlabeled BaP) of MH^+ = 269.095250; expected MH^+ = 269.0966 for a molecular formula of $C_{20}H_{12}O$. Binding to DNA was tested by using ^{3}H-BaP, A.M., and the mixture of other metabolites eluted from the TLC plate

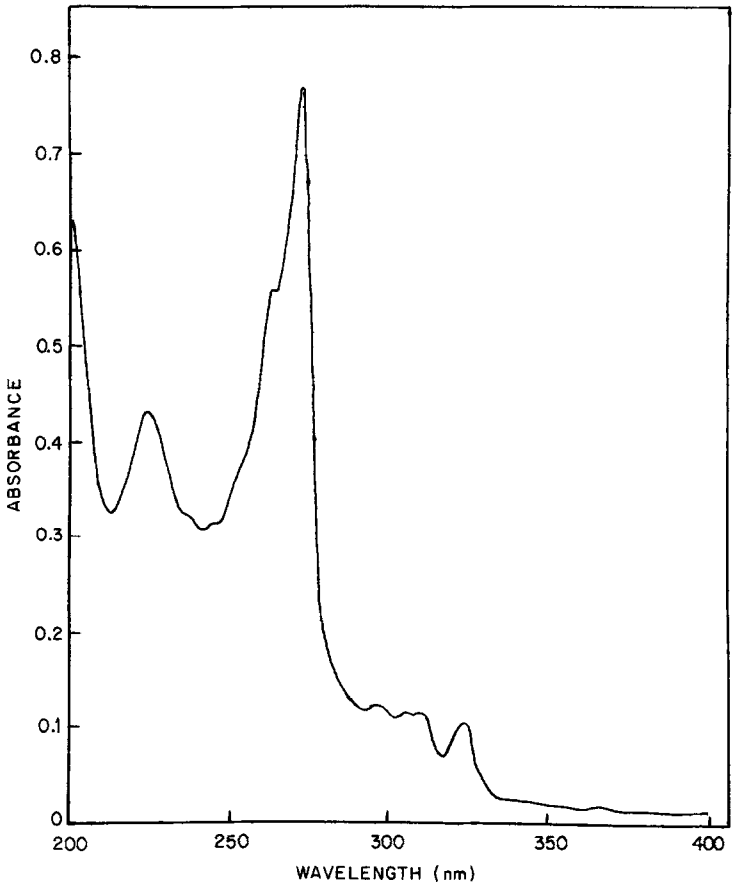

Fig.3. UV absorption spectrum in absolute ethanol of metabolite identified as 4,5-diol-BaP.

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of a large-scale incubation of ^{3}H-BaP. Each of the three fractions was incubated with DNA at 37°C for 10 minutes (input radioactivity was 1 x 10^{6} dpm each). The amounts bound to DNA were found to be 4, 20 and 6 cpm/O.D.$_{260\ nm}$ for ^{3}H-BaP, A.M., and the mixture of the other metabolites respectively.

In the absence of inhibitors of epoxide hydrase (8), the yield of A.M. was about 0.2 - 0.3% of the total radioactivity extracted into the ethyl acetate layer. Fig.2 shows that the potent inhibitor cyclohexene oxide increased the yield of A.M. about 10 fold and the 4,5-diol yield was drastically reduced. At higher concentration of cyclohexene oxide, the combined metabolic yield of BaP-3,6-quinone and metabolite X was reduced also. Metabolite X might be a

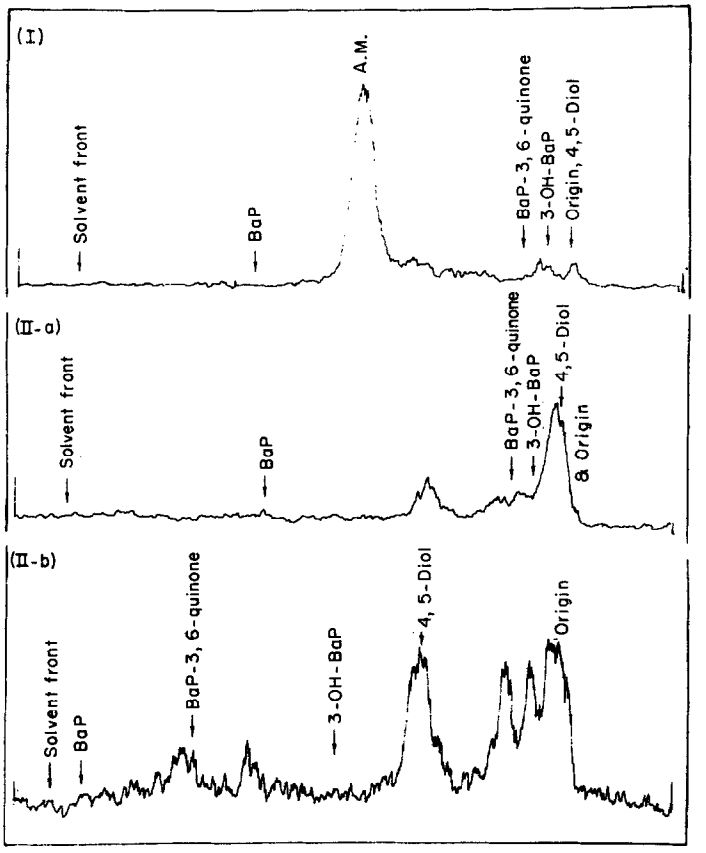

Fig.4. Radioscans of TLC of purified A.M. and its conversion products. Unlabeled standard compounds were co-chromatographed. Arrows indicate the locations of standards after TLC.
(I) A.M. chromatographed with benzene. (II-a) Conversion products of A.M. after exposure to air for 5 days. Solvent: benzene. (II-b) TLC plate of (II-a) developed again in the same direction as (II-a). Solvent: benzene:ethanol(95:5).

quinone of BaP and be related to A.M. also, e.g., BaP-4,5-quinone. The other metabolites were not much affected. Thus A.M. was definitely related to the metabolite identified here as 4,5-diol, whose UV spectrum is shown in Fig.3. This spectrum was very similar to that of the synthetic 4,5-diol which was a gift from Dr. P. Sims. In our incubation system, 4,5-diol was also a metabolite of BaP when hamster liver homogenate was used (unpublished data). Since 4,5-diol has consistently been detected as one of the major metabolites of BaP by hamster liver preparations, at least a quantitative difference in the

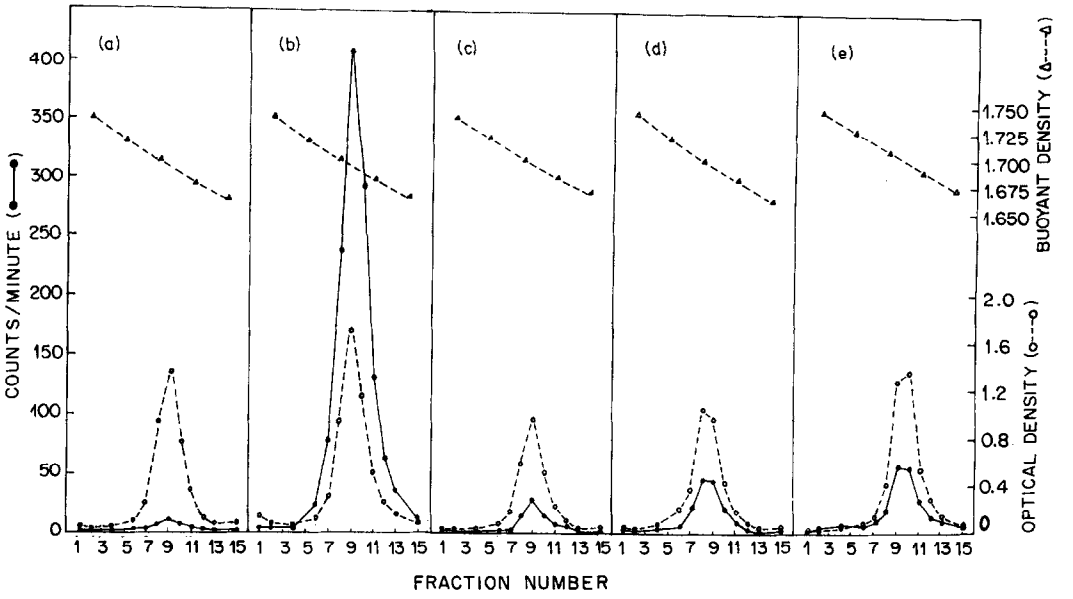

Fig.5. CsCl gradient containing DNA which was incubated with metabolic fractions of ^{3}H-BaP. Input radioactivity 6 x 10^{6} dpm each. Incubation was at 37°C for 30 minutes. Optical densities were measured at 260 nm; refractive index at 25°C. Other details were as described in Methods. DNA incubated with (a) BaP; (b) A.M.; (c) BaP-3,6-quinone and metabolite X; (d) 3-OH-BaP; (e) mixture of diols and more polar metabolites.

profiles of metabolites of BaP by hamsters and by rats (3) is clearly indicated.

After exposure to air in the dark for several days, A.M. was converted to a mixture of compounds as is shown in Fig.4; one of the major products had chromatographic properties resembling 4,5-diol, suggesting that A.M. might have been slowly hydrated by moisture from the air to 4,5-diol.

The A.M. was isolated from large-scale incubation of ^{3}H-BaP in the presence of 2 mM cyclohexene oxide. Fig.5 compares the extent of binding to DNA by five fractions of metabolites of ^{3}H-BaP from this incubation which were separated by TLC with benzene. It is evident that A.M. was much more active in binding to DNA than any other metabolic fraction of ^{3}H-BaP. Fractions (d) and (e) in Fig.5 show some binding to DNA, but the extents of binding of these two fractions were much smaller than that of A.M.

Our results show that A.M. had some of the characteristics of an epoxide

of BaP: (a) the molecular formula of A.M. was $C_{20}H_{12}O$; (b) A.M. migrated faster than any other metabolites of BaP (Fig.4-I); (c) A.M. behaved like a precusor of 4,5-diol in that inhibition of epoxide hydrase increased the yield of A.M. and decreased that of 4,5-diol; (d) A.M. was slowly hydrated in air to 4,5-diol; (e) A.M. had higher activity in binding to DNA than BaP and any other metabolites of BaP. Therefore, the A.M. of BaP reported here was very likely one of the K-region epoxides of BaP, i.e., BaP-4,5-epoxide. More detailed studies on this active metabolite are in progress.

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REFERENCES

1. Falk, H.L., Kotin, P., Lee, S.S. and Nathan, A., J.Nat.Cancer Inst., 28, 699 (1962).
2. Sims, P., Biochem. Pharmac., 16, 613 (1967).
3. Sims, P., Biochem. Pharmac., 19, 795 (1970).
4. Boyland, E., Symp. Biochem. Soc., 5, 40 (1950).
5. Jerina, D., Daly, J., Witkop, B., Zaltzman-Nirenberg, P. and Udenfriend, S., Arch. Biochem. Biophys., 128, 176 (1968).
6. Jerina, D.M., Daly, J.W., Witkop, B., Zaltzman-Nirenberg, P., and Udenfriend, S., Biochem., 9, 147 (1970).
7. Oesch, F. and Daly, J., Biochem. Biophys. Res. Commun., 46, 1713 (1972).
8. Oesch, F., Kaubisch, N., Jerina, D.M. and Daly, J.W., Biochem., 10, 4858 (1971).
9. Selkirk, J.K., Huberman, E. and Heidelberger, C., Biochem. Biophys. Red. Commun., 43, 1010 (1971).
10. Grover, P.L., Hewer, A. and Sims, P., FEBS. Letters, 18, 76 (1971).
11. Grover, P.L. and Sims, P., Biochem. Pharmac., 19, 2251 (1970).
12. Kuroki, T., Huberman, E., Marquardt, H., Selkirk, J.K., Heidelberger, C., Grover, P.L. and Sims, P., Chem.-Biol. Interactions, 4, 389 (1971/72).
13. Grover, P.L., Sims, P., Huberman, E., Marquardt, H., Kuroki, T. and Heidelberger, C., Proc.Nat.Acad.Sci. USA, 68, 1098 (1971).
14. Sims, P., Biochem. Pharmac., 19, 285 (1970).
15. Grover, P.L. and Sims, P., Proc.Am.Assoc.Cancer Res., 13, 25 (1972).
16. Wang, I.Y., Marver, H.S., Rasmussen, R.E. and Crocker, T.T., Arch.Intern. Med., 128, 125 (1971).
17. Gelboin, H.V., Advances in Cancer Res., 10, 1 (1967).
18. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., J.Biol. Chem., 193, 265 (1951).
19. Sims, P., Biochem. J., 125, 159 (1971).