An Epoxide is an Intermediate in the Microsomal Metabolism of the Chemical Carcinogen, Dibenz(a,h)anthracene[1]

James K. Selkirk, Eliezer Huberman[2], and Charles Heidelberger[3]

McArdle Laboratory for Cancer Research
University of Wisconsin
Madison, Wisconsin 53706

Received April 16, 1971

Summary: Washed rat liver microsomes that had been heated for 5 minutes at 50°C were incubated at room temperature for 30 seconds with ^{3}H-dibenz(a,h)-anthracene, TPNH, $MgCl_2$, and unlabeled 5,6-epoxy-dibenz(a,h)anthracene. Analysis of the incubation mixture by TLC revealed the presence of an epoxide. This epoxide was characterized by its behavior on TLC and by its conversion under acidic conditions to the corresponding phenol. The possible role of epoxides in hydrocarbon carcinogenesis is discussed.

In 1950 it was suggested by Boyland (1) that epoxides are the intermediates in the metabolism of carcinogenic hydrocarbons to dihydrodiols, phenols, and glutathione conjugates. This metabolism may occur at the K-region (2) or elsewhere in the molecule (3). This work, largely done by Sims and his colleagues, has been carried out mainly in rat liver microsomes (2) and in cultured mouse embryo cells (3). It has also been demonstrated that K-region epoxides of dibenz(a,h)anthracene and related compounds are converted by rat liver microsomes to the corresponding phenols (2) and dihydrodiols (4). It has further been shown by Jerina et al that epoxides are probably obligatory intermediates in the microsomal oxidation of benzene (5) and naphthalene (6).

---

[1]This work was supported in parts by grants CA 7175 and CRTY 5002 from the National Cancer Institute, National Institutes of Health.

[2]Holder of a Cancer Research Fellowship from the W.H.O. International Agency for Research on Cancer.

[3]American Cancer Society Professor of Oncology.

It is well known that carcinogenic hydrocarbons become covalently bound to the DNA (7,8), RNA (7), and proteins (9) of mouse skin following topical application. It is considered by many (cf. 10) that carcinogens which are not chemically reactive must be metabolically activated to electrophilic reagents in order to bind to macromolecules and exert their carcinogenic effects. The covalent binding of carcinogenic hydrocarbons to exogenous DNA in liver microsomes has been demonstrated (11,12), as has the binding of K-region epoxides to DNA and histones (13). Although chemically reactive metabolites are ordinarily more carcinogenic than their precursors (10), this was not so in the case of epoxides of polycyclic hydrocarbons, which were less carcinogenic than the corresponding hydrocarbons (14). However, in collaboration with Grover and Sims, it has been found in this laboratory that K-region epoxides are more active at producing in vitro malignant transformation than the corresponding hydrocarbons, dihydrodiols, and phenols (15).

Thus, there is a strong probability that epoxides are produced from carcinogenic hydrocarbons in the course of metabolism and that (at least in the case of hydrocarbons that lack methyl groups) epoxides are the chemically reactive derivatives responsible for binding to macromolecules and the initiation of carcinogenesis. However, because of their chemical reactivity, until now epoxides had never been isolated as metabolic products of carcinogenic hydrocarbons. By making use of the observation of Yamamoto and Bloch (16) that heating the appropriate enzyme permitted the isolation of squalene-2,3-oxide, we have now succeeded in demonstrating that an epoxide is produced in the microsomal metabolism of dibenz(a,h)anthracene (DBA). We have used heated microsomes from livers of rats that had been pretreated with methylcholanthrene to induce the hydroxylating enzymes, the soluble fraction was removed, the incubations were carried out for very short times with labeled DBA and a large amount of unlabeled DBA-5,6-epoxide to trap any labeled epoxide that might be formed.

Materials and Methods

DBA-^{3}H (Sp. act. 500 mCi/mMole) was obtained from Amersham-Searle, and unlabeled DBA from Eastman Kodak. The following compounds were synthesized as described in the references: 5,6-dihydro-5,6-dihydroxy-DBA (17), DBA-5,6-quinone (18), DBA-7,12-quinone (19), 5-hydroxy-DBA and 5,6-epoxy-DBA (20). The purity of all compounds was checked by TLC prior to use, using freshly redistilled solvents.

Microsomes were prepared from the livers of 250 g female rats (Charles River) that had been treated with 1 mg of methylcholanthrene 3 days prior to sacrifice, by homogenization in 0.1 M phosphate buffer, pH 7.5, removal of large particulates by centrifugation at 9800 x g for 10 minutes, and sedimentation at 105,000 x g for 60 min. The microsomes were washed once by centrifugation as before. They were then suspended in the above buffer so that a 5 ml aliquot was equivalent to 6 g of liver, and were heated in a 50° water bath for 5 min after the temperature of the suspension reached 50°C.

Incubations were carried out in 50 ml plastic centrifuge tubes (Falcon) for 30 seconds at room temperature. All ingredients except microsomes were combined in the tube, and the reaction was started by the addition of the microsomes. Each tube contained: 100 µg of ^{3}H-DBA in 0.1 ml of dimethyl sulfoxide; 250 µg of DBA-5,6-epoxide, 2 mg of TPNH (Sigma), and 2 µMol of $MgCl_2$ in dry form; then 2.5 ml of microsomal suspension was added and mixed vigorously. At the end of 30 sec, 20 ml of ethyl acetate was added to terminate the incubation, the tube was vigorosuly shaken and then centrifuged rapidly to separate the phases. The upper organic layer was removed and chromatographed immediately on TLC silica-gel plates with fluorescent indicator (Eastman 6060) and developed in cyclohexane: dioxane (9:1 v/v)(CD), in which the epoxide does not tail. Benzene (B) was also used for confirmation of identity. Authentic samples of the compounds were chromatographed as standards in each series of plates, the spots were

located with an ultraviolet lamp, and radioactivity was measured on a Packard radiochromatogram scanner. The R_f values in the systems were: DBA, CD = 0.30, B = 0.70; DBA-5,6-epoxide, CD = 0.22, B = 0.0-0.5 (streak); 5-hydroxy-DBA, CD = 0.00, B = 0.30; DBA-5,6-dihydrodiol, CD = 0.00, B = 0.00; DBA-5,6-quinone, CD = 0.00, B = 0.50; DBA-7,12-quinone, CD = 0.27, B = 0.65.

DBA-5,6-epoxide was converted to 5-hydroxy-DBA according to the method of Newman and Blum (20). One ml of the ethyl acetate extract of the microsomal incubation or of an extract from a TLC was evaporated to dryness in vacuo, and the residue was dissolved in 1 ml of acetone containing two drops of conc. HCl. The solution was refluxed for 30 min, cooled, and chromatographed on TLC.

Results

Fig. 1A shows the radioactivity scan of a 30 second incubation of ^{3}H-DBA with unheated microsomes; the location of the unlabeled epoxide is shown at the bottom. The main radioactive peak was non-metabolized DBA, and phenols and/or dihydrodiols that were produced metabolically remained at the origin. A comparable scan of the ^{3}H-DBA revealed no radioactivity at the origin. There was no labeled epoxide detectable in this incubation. However, when the microsomes were heated for 5 min at 50°C prior to incubation, an additional radioactive peak with an R_f of 0.2 was found (Fig. 1B), which corresponds to the location of the carrier epoxide. None of the other known metabolites of DBA has an R_f similar to this. When an aliquot of the ethyl acetate extract of the incubation used in Fig. 1B was heated with acidic acetone and chromatographed, the result is shown in Fig. 1C. The labeled epoxide peak has disappeared, and an increased amount of radioactivity at the origin, corresponding to the phenol, was found. When the radioactive area corresponding to the epoxide in Fig. 1B was eluted and heated in acidic acetone, a single radioactive peak with the R_f of 5-hydroxy-DBA was found after chromatography in cyclohexane-dioxane (R_f = 0.0) and

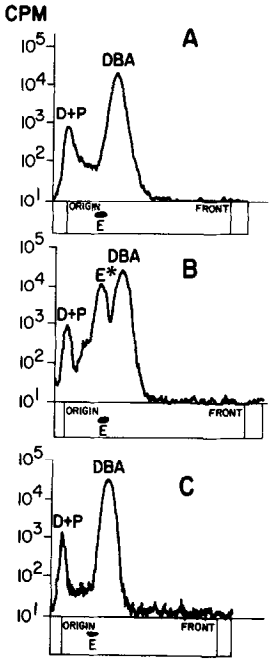

Fig. 1.

Radiochromatogram scans of ethylacetate extracts of 30 second incubations of microsomes with ^{3}H-DBA under various conditions (for details, see text). Solvent system: Cyclohexane:dioxane (9:1 v/v), D + P means dihydrodiols and phenols, E means epoxide. The origins and solvent fronts are indicated.

A, Incubation with unheated microsomes.

B, Incubation with heated microsomes.

C, Acidic treatment of extract from incubation B.

---

in benzene (R_f = 0.30). When TPNH was omitted all metabolic activity was absent, and acetone-precipitated heated microsomes were also inert. The amount of the radioactive epoxide peak was maximal with heated microsomes incubated for 30 seconds, and it could not be detected at the end of a 2 minute incubation.

Discussion

This paper reports the first direct demonstration that an epoxide is produced in the microsomal metabolism of dibenz(a,h)anthracene. We succeeded by the use of heated microsomes to inhibit epoxide hydrase, by the use of short term (30 second) incubations, and by the use of non-radioactive epoxide to trap the labeled epoxide produced during the incubation. The evidence that the radioactive peak is an epoxide is the following: 1) the kinetics of its appearance and disappearance are as expected for a reactive and unstable intermediate; 2) it migrates on TLC with the same R_f as an authentic sample of epoxide, and an R_f different from other known metabolites of DBA; and 3) on refluxing with acidic acetone it is converted into a phenol (as is authentic epoxide), which was characterized in two solvent systems. We do not at present feel justified in identifying the metabolically produced epoxide as DBA-5,6-epoxide, because DBA is primarily metabolized at non K-region positions, because the R_f's of the phenols are very similar (3), and because non K-region epoxides have not yet been synthesized. It is even possible that more than one epoxide was produced in our system. Thus, final characterization of this epoxide(s?) must await further research. Nevertheless, the present finding of epoxide as an intermediate in the microsomal metabolism of DBA strengthens the view that epoxides are the activated metabolic and the ultimately carcinogenic derivatives of carcinogenic hydrocarbons, at least the ones that lack methyl groups. Others have postulated that the methyl groups of 7,12-dibenz(a,h)anthracene may be a site of metabolic activation (21,22).

We gratefully acknowledge the skilled technical assistance of Mr. Raphael Wong.

References

1. Boyland, E. Symp. Biochem. Soc. 5, 40, 1950.

2. Boyland, E., and Sims, P. Biochem. J. 97, 7, 1965.

3. Sims, P. Biochem. Pharmacol. 19, 285, 1970.

4. Pandov, H., and Sims, P. Biochem. Pharmacol. 19, 299, 1970.

5. Jerina, D. M., Daly, J. W., 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. Biochemistry 9, 149, 1970.

7. Brookes, P., and Lawley, P. D. Nature 202, 781, 1964.

8. Goshman, L. M., and Heidelberger, C. Cancer Res. 27, 1678, 1967.

9. Abell, C. W., and Heidelberger, C. Cancer Res. 22, 931, 1962.

10. Miller, J. A. Cancer Res., 30, 559, 1970.

11. Grover, P. L., and Sims, P. Biochem. J. 110, 159, 1968.

12. Gelboin, H. V. Cancer Res. 29, 1272, 1969.

13. Grover, P. L., and Sims, P. Biochem. Pharmacol. 19, 2751, 1970.

14. Miller, E. C., and Miller, J. A. Proc. Soc. Exp. Biol. Med. 124, 915, 1967.

15. Grover, P. L., Sims, P., Huberman, E., Marquardt, H., Kuroki, T., and Heidelberger, C. Proc. Nat. Acad. Sci. U.S., in press.

16. Yamamoto, S., and Bloch, K. J. Biol. Chem. 245, 1670, 1970.

17. Cook, J. W., and Schoental, R. J. Chem. Soc. 9, 1952.

18. Bhargava, P. M., Hadler, H. I., and Heidelberger, C. J. Am. Chem. Soc. 77, 2877, 1955.

19. Cason, J., and Fieser, L. F. J. Am. Chem. Soc. 62, 2681, 1940.

20. Newman, M. S., and Blum, S. J. Am. Chem. Soc. 86, 5559, 1964.

21. Fried, J., and Schumm, D. E. J. Am. Chem. Soc. 89, 5508, 1967.

22. Dipple, A., Lawley, P. D., and Brookes, P. Europ. J. Cancer 4, 493, 1968.