

# Malignant Transformation of Cells Derived from Mouse Prostate by Epoxides and Other Derivatives of Polycyclic Hydrocarbons<sup>1</sup>

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## SUMMARY

K-region epoxides of benz[a]anthracene, dibenz[a,h]anthracene, and 3-methylcholanthrene have been found to be toxic and more active in producing malignant transformation of cells derived from mouse prostate than their respective parent hydrocarbons and K-region dihydrodiols and phenols. The data support the view that metabolism of these polycyclic hydrocarbons is a prerequisite for their biological activity. 7-Bromomethylbenz[a]anthracene, the K-region epoxide of 7-methylbenz[a]anthracene, and 7-bromomethyl-12-methylbenz[a]anthracene were either inactive or less active in producing malignant transformation than the parent compounds, 7-methylbenz[a]anthracene and 7,12-dimethylbenz[a]anthracene.

The 8,9-epoxide (non-K-region) of benz[a]anthracene was much less active than the K-region epoxide of this hydrocarbon; and the K-region epoxides of the noncarcinogenic hydrocarbons, phenanthrene and chrysene did not transform the cells.

## INTRODUCTION

Metabolic activation of polycyclic aromatic hydrocarbons is believed to be essential for the biological and especially the carcinogenic activities of these compounds (27). It has been demonstrated that epoxides are metabolites of various aromatic hydrocarbons (13, 19, 20, 33) and that such epoxides react with DNA, RNA, and proteins *in vitro* (14) and

in cells in culture (12, 21). We have previously reported (15) that K-region (32) epoxides of BA,<sup>7</sup> DBA, and MCA are more active in the production of malignant transformation in the systems involving hamster embryo cells (1, 2, 8, 9, 17, 22) and in cells derived from C3H mouse ventral prostate (4-6, 28, 29) than the corresponding hydrocarbons. Consequently, we proposed that epoxides are metabolically activated intermediates such as are required for transformation *in vitro* (15). Since we assume that transformation *in vitro* is a model for carcinogenesis *in vivo*, we propose that epoxides are similarly responsible for carcinogenesis induced by those hydrocarbons.

We now report on experiments that extend our earlier work on the transformation by epoxides of cells derived from mouse prostate (15) and include tests on additional compounds.

## MATERIALS AND METHODS

**Chemicals.** MNNG and DMSO were purchased from Aldrich Chemical Co. Inc., Milwaukee, Wis.; and acetone (reagent grade) was purchased from Merck & Co., Inc., Rahway, N. J. Polycyclic hydrocarbons were obtained from Fluka A. G., Buchs, Switzerland, and the K-region derivatives (*cis*- and *trans*-dihydrodiols, epoxides, and phenols) were freshly prepared as described previously (3, 7, 34). The purity of the compounds was checked by thin-layer chromatography. They were found to be pure, with the exception of BA-8,9-epoxide, for which 2 minor spots were seen by thin-layer chromatography, in addition to the epoxide. 7-Bromomethyl-12-methyl-BA and 7-bromomethyl-BA were kindly provided by Dr. P. Brookes (Chester Beatty Research Institute, London, United Kingdom).

**Culture Media.** Eagle's basal medium was used with antibiotics and 10% fetal calf serum. Media were purchased from Grand Island Biological Co., Grand Island, N. Y..

**Transformation Assay.** The G23 clone of cells derived from C3H mouse ventral prostate was used as described (15). For transformation, 10<sup>3</sup> cells (passages 10 to 13) were plated in 60-mm dishes; for estimation of plating efficiency, 200 cells

<sup>1</sup>Aided in part by Grant CA 7175 from the National Cancer Institute, NIH, and by Grant E 556 from the American Cancer Society.

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Received October 12, 1971; accepted December 29, 1971.

<sup>7</sup>The abbreviations used are: BA, benz[a]anthracene; DBA, dibenz[a,h]anthracene; MCA, 3-methylcholanthrene; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; DMSO, dimethyl sulfoxide; DMBA, 7,12-dimethylbenz[a]anthracene.

were plated in 60-mm dishes. (In preliminary experiments, it was established that the toxicity of hydrocarbons and their derivatives did not depend on cell number treated, if this is between 100 and 10,000 cells per 60-mm dish.) The cells were treated with test compounds that were added to the culture media as solutions in DMSO (0.5%) or acetone (0.5%) for a period of 24 hr beginning 24 hr after plating. Following treatment, the compounds were removed by media changes; subsequently, media were changed twice weekly. After 7 to 14 days, dishes plated with 200 cells were fixed in methanol, and stained with Giemsa, and colonies were counted to determine the plating efficiency. After 56 days, the dishes plated with  $10^3$  cells were fixed, stained, and scored for piled-up foci (6).

**Transplantation Experiments.** Piled-up foci of morphologically transformed cells and non-piled-up areas of the same dish as controls were ring isolated (31), and cells thus obtained were passaged 2 times and inoculated s.c. into male C3H mice (Gibco). Each mouse was inoculated with  $10^6$ ,  $10^5$ , or  $10^4$  cells/0.2 ml. The mice were observed until death. Most of the tumors appeared between 2 and 3 months after inoculation and were fibrosarcomas.

## RESULTS

The results on the toxicity and transforming activity of BA, DBA, and MCA and their K-region derivatives (*cis*- and *trans*-dihydrodiols, epoxides, and phenols) in G23 cells are

Table 1

### Transformation and toxicity produced by BA and its derivatives

In Tables 1 to 6, the data represent the composite of 2 or more experiments.

Derivative	Concentration ( $\mu\text{g/ml}$ )	Plating efficiency (%)	No. of transformed foci/no. of dishes
Control	DMSO 0.5%	25	0/30
MNNG	0.1	17	16/18
	0.2	11	25/17
BA <sup>a</sup>	1.0	18	0/13
	5.0	15	0/18
BA-5,6-epoxide (K-region) <sup>a</sup>	0.5	10	9/19
	1.0	3.0	23/25
BA-8,9-epoxide (non-K-region)	0.1	19	0/14
	0.5	17	0/18
	1.0	13	0/14
	5.0	5.0	2/16
	10.0	1.0	0/18
BA- <i>cis</i> -5,6-dihydrodiol (K-region)	1.0	22	0/10
	10.0	20	0/10
BA- <i>trans</i> -5,6-dihydrodiol (K-region)	5.0	27	0/10
	10.0	26	0/8
5-OH-BA (K-region phenol)	1.0	11	0/12
	3.0	3.0	0/18
	10.0	0	

<sup>a</sup> Data already published (15).

Table 2

### Transformation and toxicity produced by DBA and its K-region derivatives

For controls (DMSO, 0.5%), see Table 1.

Derivative	Concentration ( $\mu\text{g/ml}$ )	Plating efficiency (%)	No. of transformed foci/no. of dishes
DBA <sup>a</sup>	1.0	22	0/15
	10.0	20	0/18
DBA-5,6-epoxide <sup>a</sup>	0.5	22	4/20
	1.0	17	9/17
	10.0	6.5	12/15
DBA- <i>cis</i> -5,6-dihydrodiol	1.0	22	0/12
	10.0	20	0/15
DBA- <i>trans</i> -5,6-dihydrodiol	5.0	25	0/10
	10.0	5.5	0/12
5-OH-DBA (K-region phenol)	1.0	19	0/6
	5.0	10	0/10
	10.0	0	

<sup>a</sup> Data already published (15).

Table 3

### Transformation and toxicity produced by MCA and its K-region derivatives

For controls (DMSO, 0.5%), see Table 1.

Derivative	Concentration ( $\mu\text{g/ml}$ )	Plating efficiency (%)	No. of transformed foci/no. of dishes
MCA <sup>a</sup>	1.5	17	1/13
	10.0	17	10/33
MCA-11,12-epoxide <sup>a</sup>	0.75	17	38/27
	1.5	9.5	58/26
MCA- <i>cis</i> -11,12-dihydrodiol	1.5	23	0/7
	5.0	26	0/10
	10.0	23	0/18
MCA- <i>trans</i> -11,12-dihydrodiol	5.0	27	0/10
	10.0	26	0/12
11-Hydroxy-MCA (K-region phenol)	1.0	21	0/8
	2.5	13	8/16
	5.0	2.5	0/10

<sup>a</sup> Data already published (15).

shown in Tables 1 to 3. Treatment of the cells with DMSO or acetone did not give rise to any transformed foci. As was done previously (15), we used as a positive control MNNG, which does not require prior metabolism for its biological activity (23). In the transformation assay, MCA was weakly active in this clone of prostate cells. However, BA and DBA, the K-region *cis*- and *trans*-dihydrodiols of BA, DBA, and MCA, and the K-region phenols of BA and DBA were completely inactive. The K-region phenol of MCA was moderately active. However, as previously reported (15), the K-region epoxides of BA, DBA, and MCA possessed a marked activity in producing

Table 4

Toxicity and lack of transformation produced by phenanthrene and chrysene and their K-region epoxides

Derivative	Concentration (µg/ml)	Plating efficiency (%)	No. of transformed foci/no. of dishes
Control	Acetone 0.5%	24	0/20
	MNNG 0.2	13	12/13
Phenanthrene	1.0	24	0/12
	5.0	20	0/12
	10.0	11	0/12
Phenanthrene-9,10-epoxide	1.0	13	0/12
	5.0	1.5	0/15
	10.0	0	
Chrysene	1.0	22	0/10
	5.0	22	0/12
	10.0	22	0/12
Chrysene-5,6-epoxide	1.0	17	0/15
	5.0	1.0	0/18
	10.0	0	

Table 5

Transformation and toxicity produced by 7-methyl-BA, DMBA the K-region epoxide of 7-methyl-BA, 7-bromomethyl-BA, and 7-bromomethyl-12-methyl-BA

Derivative	Concentration (µg/ml)	Plating efficiency (%)	No. of transformed foci/no. of dishes
Control	Acetone 0.5%	23	0/18
MNNG	0.2	13	12/16
7-Methyl-BA	0.1	21	5/20
	10.0	13	9/17
7-Methyl-BA-5,6-epoxide (K-region)	0.01	20	0/10
	0.05	17	4/18
	0.1	14	1/10
	0.2	4.0	0/10
7-Bromomethyl-BA	0.1	13	0/18
	0.2	5.5	0/20
DMBA	0.1	18	3/10
	1.5	17	6/10
	10.0	10	20/19
7-Bromomethyl-12-methyl-BA	0.1	18	0/18
	0.2	15	1/19

transformation *in vitro*. The only non-K-region epoxide tested, the 8,9-epoxide of BA, was very weakly active.

Table 4 shows that the noncarcinogenic hydrocarbons, phenanthrene and chrysene, and their K-region epoxides did not transform these prostate cells.

Table 5 gives our results with 7-methyl-BA, its K-region epoxide, 7-bromomethyl-BA, 7-bromomethyl-12-methyl-BA, and DMBA. In contrast to the results described above, the K-region epoxide of 7-methyl-BA was slightly less active in

Table 6

Induction of sarcomas in C3H mice after s.c. injection of cells from morphologically transformed clones

Clone	No. with sarcoma/no. injected with cells per mouse			
	5 × 10 <sup>6</sup>	10 <sup>6</sup>	10 <sup>5</sup>	10 <sup>4</sup>
Control cells	0/5	0/4	0/4	0/4
MNNG-transformed				
Clone 1		4/4	2/4	0/4
Clone 2		4/4	3/4	1/4
MCA-11,12-epoxide-transformed				
Clone 1		4/4	1/4	1/4
Clone 2		3/3	0/4	0/4
Clone 3		1/4	4/4	1/4
Clone 4		3/3	4/4	4/4
Clone 5		3/4	4/4	0/4

producing transformation than the parent hydrocarbon. Both bromo-substituted compounds were almost inactive. DMBA, however, was very active in transforming G23 cells.

The toxicity of all compounds tested increased with increasing concentrations. The K-region epoxides of BA and MCA were more toxic than the other derivatives; the epoxide of DBA was as toxic as the DBA-phenol. The bromo-substituted hydrocarbons and the *trans*-dihydrodiol of DBA were also very toxic.

For a demonstration that cells morphologically transformed by epoxides will grow as tumors in isologous mice after s.c. inoculation, such transformed cells were injected into mice and found to be malignant (Table 6), as had been shown for prostate cells transformed by hydrocarbons (6). Two of 2 MNNG-transformed clones and 5 of 5 MCA-epoxide-transformed clones induced anaplastic soft-tissue sarcomas in isologous C3H mice (microscopic slides were kindly read by Dr. H. Pitot, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wis.). Cells isolated from non-piled-up areas of carcinogen-treated dishes and cells isolated from control dishes did not give rise to any tumors.

## DISCUSSION

The data obtained in this study support our previous conclusion that epoxides are the metabolically activated intermediates of BA, DBA, and MCA required for the production of malignant transformation *in vitro* (15). Although epoxides are less active carcinogens *in vivo* than the corresponding hydrocarbons, we believe that because of their high chemical reactivity they are used up by reaction with extracellular keratin and other molecules before they can reach their intracellular targets; this occurs to a much lesser extent in culture. Moreover, the epoxides were either more toxic than (BA and MCA) or as toxic (DBA) as the other derivatives tested. The exact mechanisms whereby they produce toxicity and transformation are presently unknown. We have studied the binding of the epoxides and other derivatives to the DNA, RNA, and proteins of various

transformable and transformed cells in culture but could find no clear-cut relationship between such binding and either toxicity or transformation (21). Nevertheless, the high yield of malignant transformation induced by K-region epoxides makes them very useful tools for further investigations of the biochemical events involved in chemical carcinogenesis *in vitro*, because they bypass the metabolic activation step. Such studies of the effects of epoxides on the synthesis of macromolecules in transformable cells are currently under way. Preliminary results show that the K-region epoxide of BA stimulates DNA synthesis in logarithmically growing hamster embryo cells (26).

Epoxides, however, may not account for the metabolic activation of all polycyclic hydrocarbons. Dipple *et al.* (10) have proposed that compounds such as DMBA may be metabolically activated on the methyl groups by formation of a carbonium ion. Consequently, bromomethyl derivatives of BA and DMBA have been prepared as model compounds capable of giving rise to such carbonium ions (11). The latter was found to be carcinogenic (11). We found (Table 5) that neither 7-bromomethyl-BA nor 7-bromomethyl-12-methyl-BA was significantly active at producing transformation in our system. Moreover, the epoxide of 7-methyl-BA was less active at producing malignant transformation than was the parent hydrocarbon. This raises at least the possibility that metabolic activation may not be necessary for the transforming activity of methylated hydrocarbons such as DMBA, in accord with an earlier suggestion (24). Furthermore, we have found that, while the treatment of G23 cells with inducers of the microsomal hydroxylating enzymes (30) increased the frequency of transformation produced by BA and MCA, the transformation produced by DMBA was not enhanced (25). Such treatment increases the metabolism of carcinogenic hydrocarbons in the G23 cells, which normally have a very low rate of metabolism (18, 25).

There are, however, other explanations for our findings. The K-region epoxide of 7-methyl-BA and the bromomethyl compounds may be so highly reactive that they are inactivated before they can reach their intracellular target. However, we found these latter 3 compounds to be highly mutagenic to Chinese hamster cells in culture (16); thus they must have entered those cells. It must also be borne in mind that the bromomethyl compounds are only models of metabolically activated compounds and not, like epoxides, actual metabolic intermediates. In view of the lack of transforming activity of the highly toxic epoxides of phenanthrene and chrysene, which however, do transform hamster embryo cells (E. Huberman, T. Kuroki, H. Marquardt, J. K. Selkirk, C. Heidelberger, P. L. Grover, and P. Sims, manuscript in preparation), it is possible that an epoxide other than the K-region epoxide of 7-methyl-BA might be more active. The situation is further complicated by the fact that the 8,9-epoxide of BA, which must be the precursor of the 8,9-dihydrodiols found by Sims (35) to be the major metabolite of BA, was considerably less active than the K-region epoxide in producing transformation in our system. Clearly, further work is needed to elucidate the structural characteristics of epoxides required for malignant transformation and the manner in which these requirements relate to the Pullman (32) K-region hypothesis.

## ACKNOWLEDGMENTS

We are grateful to Mr. David Brankow for skilled technical assistance.

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