Metabolism of Benzo(a)pyrene and (−)-trans-7,8-Dihydroxy-7,8-dihydrobenzo(a)pyrene by Rat Liver Nuclei and Microsomes

John M. Pezzuto,1 Chung S. Yang,2 Shen K. Yang,3 David W. McCourt, and Harry V. Gelboin

Department of Biochemistry, New Jersey Medical School, College of Medicine and Dentistry of New Jersey, Newark, New Jersey 07103 [J. M. P., C. S. Y.], and Chemistry Branch, National Cancer Institute, Bethesda, Maryland 20014 [S. K. Y., D. W. M., H. V. G.]

ABSTRACT

The metabolism of benzo(a)pyrene and (−)-trans-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene was examined by high-pressure liquid chromatography. Liver nuclei or microsomes from control, 3-methylcholanthrene-treated, or phenobarbital-treated rats were used. An improved method of separating the metabolites is described. Upon treatment with 3-methylcholanthrene, metabolism of benzo(a)pyrene was enhanced and again the metabolic pattern was similar. Each of the nuclear samples produced less diol-epoxides than did the corresponding microsomes. Treatment with 3-methylcholanthrene caused an 8-fold increase in r-7,8-dihydroxy-9,10-oxy-7,8,9,10-tetrahydrobenzo(a)pyrene (diol-epoxide I) formation with microsomes but only a 2-fold increase with nuclei. Inducible, r-7,8-dihydroxy-c-9,10-oxy-7,8,9,10-tetrahydrobenzo(a)pyrene (diol-epoxide II) formation was also detected. Phenobarbital treatment did not greatly increase diol-epoxide formation. Since both the microsomes and the nuclei can produce diol-epoxides, both organelles may be considered as potentially important sites of carcinogen activation.

INTRODUCTION

Chemicals play a prominent role in the initiation of human cancers (13). As a product of incomplete combustion, carcinogenic PAH are ubiquitous environmental contaminants (9) and components of cigarette smoke (8, 40). As a prototype of PAH carcinogenesis, the chemistry and biological interactions of BP and its derivatives have been intensively investigated. It is generally agreed that metabolic activation is required to exert the biological effects of BP. This leads to covalent binding with critical cellular macromolecules (13, 24), possibly in the cell nucleus. Metabolism by the cytochrome P-450-linked AHH has been shown to yield a host of oxygenated BP derivatives (10, 15, 33, 35, 45, 50).

trans-7,8-Diol has been reported to bind more readily than BP to DNA in the presence of microsomes (3) and to the DNA of cultured human bronchi (46). When further metabolized, the trans-7,8-diol is more mutagenic than is BP (15, 22, 43). Binding to the DNA of transformable embryonic cells (36) and mouse skin (11) has been shown to arise from the 7,8-diol-9,10-epoxide of BP. Rat liver microsomes metabolize BP to an optically pure (−)-trans-7,8-diol (49), which is subsequently metabolized predominantly to diol-epoxide I (15, 39, 50). This metabolite is also produced by cultured human bronchi and is presumably bound to the DNA (46). The mutagenicity of diol-epoxide I exceeds that of any other known BP metabolites to mammalian cells (15, 26, 35, 43), providing additional evidence that this is an ultimate carcinogenic form of BP.

It is well established that AHH is a component of the nuclear envelope (18, 20, 34) and is inducible by treatment with MC (6, 16, 20, 27, 28, 32). It has been reported that incubation of isolated nuclei with BP and necessary cofactors results in covalent binding to DNA, RNA, histones, and nonhistone proteins (2, 6, 27, 28, 41). Treatment of the animals with MC increased the level of AHH and binding, consistent with several other reports (1, 16, 31, 32, 41). Considering the relatively short lifetime of active BP metabolites (43, 48), the cytoplasmic detoxification mechanism (37), and the proximity of the nuclear enzyme to possible target molecules, the nuclear envelope may play an important role in carcinogen activation. Both increases (1, 16, 27, 28) and decreases (31, 41) in BP binding have been reported when microsomes are added to incubation systems containing nuclei. As recently suggested (28), the endoplasmic reticulum may play a more important role in carcinogen activation when BP is present in high concentrations. At lower concentrations, the endoplasmic reticulum should still contribute to the binding, although it also lowers the effective BP concentration available for nuclear metabolism.

In assessing the importance of the nuclear envelope in carcinogen activation, an obvious and important question is the nature of the metabolites that are formed. Total metabolism of BP by nuclei from MC-treated rats has been reported by Jernström et al. (16), Alexandrov et al. (1), and...
Bresnick et al. (6), indicating a pattern similar to that obtained with microsomes. We now report that nuclei are capable of metabolizing BP to the highly mutagenic and possibly ultimate carcinogenic 7,8-diol-9,10-epoxides.

MATERIALS AND METHODS

Chemicals. $[^{14}C]BP$ (38 mCi/mmol) was purchased from Amersham/Searle Corp., Arlington Heights, Ill., and was purified prior to use by HPLC with a 6.2-mm (i.d.) x 25-cm DuPont ODS column (47). $[^{14}C](-)-trans-7,8$-dial-BP (53.9 mCi/mmol) was resolved by HPLC from $[^{14}C]$(±)-trans-7,8-diol-BP as previously described (47). The $[^{14}C]$(±)-trans-7,8-diol-BP was obtained through the Chemical Repository of the National Cancer Institute and was synthesized by McCausland et al. (23). Due to its instability upon storage, $[^{14}C]$(±)-trans-7,8-diol-BP was further purified prior to use by HPLC (51). NADPH was purchased from Calbiochem, San Diego, Calif. Glass-distilled acetone, methanol, and ethyl acetate were purchased from Burdick & Jackson Laboratories, Muskegon, Mich. MC was purchased from Schwarz/Mann, New York, N. Y., and PB was from Eli Lilly & Co., Indianapolis, Ind.

Treatment of Animals. Male Long-Evans rats with a body weight of about 100 g were obtained from Marland Farms, Hewitt, N. J. Prior to use, the rats were given (a) a daily i.p. injection of MC (25 mg/kg, in corn oil) for 4 days, (b) a daily i.p. injection of PB (75 mg/kg, in H$_2$O) for 4 days, or (c) no treatment. The rats were given a commercial laboratory chow and water ad libitum and kept in air-conditioned quarters with a 12-hr light-dark cycle.

Isolation of Nuclei and Microsomes. The rats were decapitated. The livers were rapidly excised and rinsed 4 to 6 times with ice-cold 0.14 M NaCl. Microsomes were isolated by differential centrifugation as previously described (44). Nuclei were isolated by the method of Kasper (19). The liver was minced, homogenized in 2 volumes (v/w) of 0.25 M sucrose-TKM, and filtered through cheesecloth. Twenty ml of 2.3 M sucrose-TKM were added to 10 ml of the homogenate and thoroughly mixed. This was underlaid with 5 ml of 4.3 M sucrose-TKM and centrifuged. The resulting nuclear pellets were washed with 1.0 M sucrose-TKM and 0.25 M sucrose-TKM and finally suspended in 0.25 M sucrose-TKM (28). Examination by electron microscopy indicated that the nuclei were intact, contained inner and outer membranes, and were visually free of cytoplasmic contaminants. Centrifugation through a second discontinuous sucrose gradient did not reduce the level of cytochrome P-450 or AHH activity. Protein was measured by the method of Lowry et al. (21). The preparations were stored frozen at −90° and sonically disassembled and aliquoted on ice. This mixture was then extracted with 2 × 2 ml of ethyl acetate, aliquots of both the aqueous and organic phases were counted, and it was found that greater than 99.8% of radioactivity was in the organic phase in all samples. The organic phase was then dried over MgSO$_4$ and evaporated to dryness under nitrogen for HPLC analysis (51). Similar incubations and extractions were performed with the blank, which contained no nuclei or microsomes.

HPLC Analysis of BP Metabolites. A Spectra-Physics Model 3500 high-pressure liquid chromatograph was used. Metabolites of BP were analyzed on a 4.6-mm (i.d.) x 25-cm Whatman Partisil ODS-2 column (Whatman Inc., Clifton, N. J.). The column was eluted at ambient temperature (24 ± 1°) with a 20-min linear gradient from 60% methanol in water to 100% methanol at a flow rate of 0.8 ml/min. Metabolites of (-)-trans-7,8-diol were analyzed on a 6.2-mm (i.d.) x 25-cm DuPont Zorbax ODS column which was eluted at ambient temperature with a 100-min linear gradient from 60% methanol in water to 100% methanol at a flow rate of 0.8 ml/min (51). The elution order of tetrahydroxytetrahydrobenzo(a)pyrene in which the 9- and 10-OH are cis and the 8-OH is trans to the reference 7-OH and trihydroxytrihydrobenzo(a)pyrene in which 8- and 9-OH are trans to the reference 7-OH was reversed from those reported earlier (50) due to the variable performance of the purchased DuPont Zorbax prepacked columns.

RESULTS AND DISCUSSION

The metabolite pattern of $[^{14}C]BP$ incubated with MC nuclei is shown in Chart 1. With the column and elution conditions described in this report, the elution orders of phenols and quinones are reversed as compared to those reported earlier (14, 33) and are similar to those reported recently (51) with a different prepacked column from the manufacturer. Under our HPLC conditions (Chart 1), there was no significant radioactivity eluted prior to 15 min. Under the HPLC conditions reported earlier (14, 33), there was always a peak eluted prior to the elution of trans-9,10-diol. However, with low substrate concentrations (≤0.01 mm) or prolonged incubation, significant amounts of radioactivity were detected prior to the elution of trans-9,10-diol.
which was due to further metabolism of phenols and trans-diols (38, 46).

The quantitation of BP metabolism by nuclei and microsomes is shown in Table 1. The pattern of metabolites obtained with MC nuclei was similar to that obtained with MC microsomes, as was the case with control or PB samples. This is consistent with the report of Jeršnström et al. (16) obtained with MC nuclei and microsomes. The values are higher than those reported by Alexandrov et al. (1) with MC nuclei and microsomes, probably due to differences in the preparations of microsomes and in incubation conditions. As shown in Table 1, treatment of the animals with MC significantly enhanced production of all metabolites with both nuclei and microsomes. PB treatment had no significant effect on the metabolite pattern, except for production of the trans-4,5-diol with incubations containing microsomes (30). It is known that PB induces microsomal cytochrome P-450, a form less efficient in metabolizing PAH than is the cytochrome P-448 induced by MC treatment. The level of cytochrome P-450 in PB nuclei was 87 pmol/mg protein (average of 2 preparations), similar to that in MC nuclei (28). Control nuclei contained 36 pmol of cytochrome P-450 per mg protein (28). The total metabolism of BP with microsomes and nuclei analyzed by HPLC can be correlated with AHH activity measured by the fluorometric method (25), and the results are similar to those of nuclear preparations reported by Jeršnström et al. (16). Each of the samples yielded trans-7,8-diol, and this is apparently available for further metabolism.

As shown in Table 2, nuclei as well as microsomes were capable of metabolizing [14C](-)-trans-7,8-diol. Incubation of nuclei or microsomes resulted in the formation of tetrals.

### Table 1

<table>
<thead>
<tr>
<th>Metabolism of [14C]BP by nuclei and microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei or microsomes (0.25 mg protein) were incubated with [14C]BP as described in “Materials and Methods.” HPLC analyses were performed, and the data were converted to pmol of BP metabolites formed per min per mg of protein. The lower detection limit of the HPLC fractions is about 0.1 pmol.</td>
</tr>
<tr>
<td><strong>BP metabolite</strong></td>
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<tr>
<td>------------------</td>
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<tr>
<td>Retention time (min)</td>
</tr>
<tr>
<td>trans-9,10-Diol</td>
</tr>
<tr>
<td>trans-4,5-Diol</td>
</tr>
<tr>
<td>trans-7,8-Diol</td>
</tr>
<tr>
<td>9-OH</td>
</tr>
<tr>
<td>1-OH, 3-OH, and 7-OH</td>
</tr>
<tr>
<td>1,8-quinone</td>
</tr>
<tr>
<td>3,6- and 6,12-quinone&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

<sup>a</sup> Elution times of chromatographic peaks.

<sup>b</sup> trans-9,10-Diol, trans-9,10-dihydroxy-9,10-dihydrobenzo(a)pyrene; trans-4,5-Diol, trans-4,5-dihydroxy-4,5-dihydrobenzo(a)pyrene; trans-7,8-Diol, trans-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene; 9-OH, 9-hydroxybenzo(a)pyrene; 1-OH, 1-hydroxybenzo(a)pyrene; 3-OH, 3-hydroxybenzo(a)pyrene; and 7-OH, 7-hydroxybenzo(a)pyrene.

<sup>c</sup> 3-OH-BP is the predominant phenol.

<sup>d</sup> BP 3,6-quinone is the major quinone.

### Table 2

<table>
<thead>
<tr>
<th>Metabolism of <a href="-">14C</a>-trans-7,8-diol by nuclei and microsomes</th>
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</thead>
<tbody>
<tr>
<td>Nuclei or microsomes (0.25 mg protein) were incubated with <a href="-">14C</a>-trans-7,8-diol. Control incubations were run in parallel, and radioactivity eluting prior to the (-)-trans-7,8-diol was subtracted. Except for MC microsomes, no significant triol formation (45) was detected. The identities of the metabolites eluting with retention times of 30.8 and 36.4 min are not known. Numbers in parentheses indicate whether diol-epoxide I or II is the precursor. Other conditions are the same as in Table 1.</td>
</tr>
<tr>
<td><strong>BP metabolite</strong></td>
</tr>
<tr>
<td>------------------</td>
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<tr>
<td>(7,10/8,9)-Tetrol&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(7/8,9,10)-Tetrol&lt;sup&gt;c&lt;/sup&gt; (I)</td>
</tr>
<tr>
<td>(7/8,9)-Triol&lt;sup&gt;c&lt;/sup&gt; (I)</td>
</tr>
<tr>
<td>(7/8,9,10)-Tetrol&lt;sup&gt;c&lt;/sup&gt; (II)</td>
</tr>
<tr>
<td>(7,9,10/8)-Tetrol&lt;sup&gt;c&lt;/sup&gt; (II)</td>
</tr>
<tr>
<td>(7/8,9)-Triol&lt;sup&gt;c&lt;/sup&gt; (II)</td>
</tr>
<tr>
<td>Unknown 1</td>
</tr>
<tr>
<td>Unknown 2</td>
</tr>
<tr>
<td>Total</td>
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</table>

<sup>a</sup> 7,10/8,9-Tetrols, tetrahydroxytetrahydrobenzo(a)pyrene in which the 10-OH is cis and the 8- and 9-OH are trans to the reference 7-OH (other tetrols and triols are similarly designated).

and triols, which are indicative of the metabolic formations of diol-epoxides (15, 39, 50). Diol-epoxide I was the major diol-epoxide formed in the present study. Metabolites with HPLC retention times of 30.8 and 36.4 min were also observed with incubations containing microsomes but not nuclei. Treatment of the animals with PB did not greatly increase the formation of diol-epoxides in incubations with nuclei or microsomes. Treatment of the animals with MC resulted in about an 8-fold increase in diol-epoxide formation over control microsomes, but the induction was only 2-fold with nuclei. The 2-fold increase is much less than the 9-fold increase caused by MC induction in the nuclear metabolism of BP (Table 1). By comparison of the effect of MC induction on the metabolism of BP and (-)-trans-7,8-diol, it appears that the induction would reduce the proportion of diol-epoxide formation in the nuclear metabolism of BP. Alternatively, this result may reflect the loss of a larger portion of free diol-epoxide in the MC nuclear sample through the binding of this reactive metabolite to nuclear components. Diol-epoxides are known to bind to DNA and other macromolecules (5, 36, 42).

After the submission of this manuscript, Bresnick et al. (5) reported the metabolism of BP and (±)-trans-7,8-diol-BP by liver microsomes and nuclei from control and MC-treated rats. Discrepancies exist between the results in their report (5) and those in this report concerning the metabolism of trans-7,8-diol. We have used the optical purity (-)-trans-7,8-diol as the substrate, whereas Bresnick et al. (5) have used the racemic (±)-trans-7,8-diol. BP is metabolized to the (-)-trans-7,8-diol in liver microsomes (49, 50) and in cultured human bronchi (46). The (-)-trans-7,8-diol is me-
tabolized predominantly to diol-epoxide I (38, 46, 49, 50), whereas the (+)-trans-7,8-diol is metabolized predominantly to diol-epoxide II (38, 50). The use of (-)-trans-7,8-diol as the substrate would result in the formation of a higher amount of diol-epoxide II than if the optically pure (-)-trans-7,8-diol were used. This factor appears to be responsible for the higher amount of diol-epoxide II (ref. as diol-epoxide I in Ref. 5) formation reported by Bresnick et al. (5) than that presently observed.

Blank incubations containing no enzyme resulted in significant radioactivity eluting prior to the [14C](-)-trans-7,8-diol on HPLC. Blank incubations that were cochromatographed with the known tetrol and triol standards were used to correct the amount produced by the sample. Under the identical conditions, the [14C]BP was converted to BP quinones which constitute only 0.01% of the original [14C]BP. The instability of the [14C](-)-trans-7,8-diol is not due to the presence of microsomes or nuclei or the incubation conditions, since on storage at -20° the [14C](-)-trans-7,8-diol was much less stable than was [14C]BP or the racemic [14C]trans-7,8-diol.

Numerous studies have shown that carcinogens bind to the components of the cell nucleus in vivo (4–7, 11, 12, 17, 29, 36, 46). The subcellular site of carcinogen activation, however, cannot be assessed in such experiments, and the complexity of cell ultrastructure complicates in vitro studies. The nuclear envelope can metabolize BP; this leads to binding within the nucleus (5, 6, 16, 27, 28, 31, 32, 41). When microsomes are added to nuclei, they increase (1, 16, 27, 28) or at least contribute to the nuclear binding (28), depending upon the incubation conditions. This report has shown that nuclei can metabolize BP to the highly mutagenic and possibly ultimate carcinogenic diol-epoxides, and the overall pattern of the metabolites is not different from that obtained with microsomes. The endoplasmic reticulum has a much higher capability of metabolizing BP, but the nuclear envelope is in the proximity of the genetic apparatus. The relative importance of these organelles in BP metabolism is related to the overall kinetics of BP metabolite formation, as well as the transport and degradation of these compounds in the cell. These remain to be elucidated.

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Metabolism of BP and Diol-BP by Nuclei and Microsomes


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