Mutagenicity and Cytotoxicity of Benzo(a)pyrene Benzo-Ring Epoxides


Department of Biochemistry and Drug Metabolism, Hoffmann-LaRoche Inc., Nutley, New Jersey 07110 [A. W. W., P. G. W., R. L. C., W. L., A. Y. H. L., A. H. C.], and Section on Oxidation Mechanisms, Laboratory of Chemistry, National Institute of Arthritis, Metabolism, and Digestive Diseases, NIH, Bethesda, Maryland 20014 [H. Y., O. H., D. M. J.]

SUMMARY

Four benzo-ring epoxides of the environmental carcinogen benzo(a)pyrene (BP) were tested for mutagenic and cytotoxic activity in 3 strains of Salmonella typhimurium (TA1538, TA98, and TA100) and in Chinese hamster V79 cells. Although very unstable in aqueous solution, 7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (diol epoxide 1), with the 7-hydroxy group on the same face of the molecule as the epoxide oxygen, was 1.5 to 4 times as mutagenic in the bacterial strains as was its more stable stereoisomer 7β,8α-dihydroxy-9α,10β-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (diol epoxide 2). In V79 cells, diol epoxide 1 had one-third the mutagenic activity of diol epoxide 2 but was at least 10 times more labile than diol epoxide 2 in the tissue culture medium. The half-life of diol epoxide 1 in tissue culture medium was about 30 sec, whereas the half-life of diol epoxide 2 was between 6 and 12 min. 9,10-Epox-7,8,9,10-tetrahydrobenzo(a)pyrene, which is saturated in the benzo ring, is also very unstable and has mutagenic activity equal to or greater than diol epoxide 1 in the bacterial and mammalian cells. 7,8-Epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene was more stable in aqueous solution than any of the 9,10-epoxides of BP but was much less mutagenic in both the bacterial and mammalian cells. In V79 cells, diol epoxides 1 and 2 and 9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene were more than 40 times more cytotoxic than 7,8-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene. The mutagenicity of the 2 tetrahydro epoxides toward strain TA98 of S. typhimurium was readily abolished by purified epoxide hydrase, whereas the mutagenic activity of the 2 diol epoxides was relatively unaffected by coinoculation with the enzyme.

INTRODUCTION

The biological activity of the carcinogenic environmental pollutant BP2 (Chart 1) appears to be attributable to metabolic products rather than to the parent compound (3, 6, 7, 9, 12–18, 20, 24, 26, 27, 29–32). Of the very large number of metabolites that can occur from the oxidative metabolism of BP, recent attention has focused on the benzo-ring diol epoxide, BP 7,8-diol-9,10-epoxide (27). Formed by the epoxidation of BP 7,8-dihydriodiol, the diol epoxide can exist as 2 stereochemical isomers (13, 16, 17, 33). Diol epoxide 1 (Chart 1) is distinguished from its stereoisomer diol epoxide 2 (Chart 1) in that the 7-hydroxyl group is on the same face of the molecule as the epoxide ring. This configuration of the 7-hydroxyl group relative to the epoxide ring renders diol epoxide 1 about 150 times more reactive toward the thiol nucleophile p-nitrothiophenolate in t-butyl alcohol when compared to the stereoisomer diol epoxide 2 and 500 times more reactive than H4-9,10-epoxide (Chart 1, Compound 3), which lacks hydroxyl groups altogether (17, 33). Intra- and intermolecular hydrogen bonding between the 7-hydroxyl group and the epoxide oxygen, when these groups are on the same side of the ring, apparently assists the attack of nucleophiles on the epoxide oxygen. Evidence for the intramolecular hydrogen bond is found in the proton magnetic resonance spectra of diol epoxides 1 and 2 (17, 33).

We have recently reported that diol epoxide 1 is one of the most potent mutagens ever tested in histidine auxotrophs of Salmonella typhimurium and in cultured Chinese hamster cells (6, 29). These studies have shown that diol epoxide 1 is 5, 20, and 40 times more mutagenic than benzo(a)pyrene 4,5-oxide in strains TA98 and TA100 of S. typhimurium and in V79 cells, respectively. Thus, despite the high chemical reactivity of diol epoxide 1 (33), it appears to reach and react readily with DNA under physiological conditions. We undertook the present study, utilizing several substituted and unsubstituted benzo-ring epoxides of BP, in an attempt to elucidate the critical molecular and stereochemical requirements for mutagenic activity. In addition to the diol epoxide stereoisomers 1 and 2, we examined the mutagenic activity of H4-9,10-epoxide, H4-7,8-epoxide (Chart 1, Compound 4), the bromohydron precursor of diol epoxide 1 (Chart 1, Compound 5), and the bis(trimethylsilyl) ether of diol epoxide 1 (Chart 1, Compound 6). The tetrahydro epoxides (Compounds 3 and 4) were used to examine the effect of hydroxyl groups on mutagenic activity, whereas the bromo and silyl derivatives might be expected to form diol epoxide 1 in alkaline or acidic solution, respectively. Since somatic cell mutation may be involved in the chemical initi-
form bis(tнимethylsilyl) ethers and are somewhat more stable in this form (33). Diol epoxides 1 and 2 are extremely reactive with water and must be handled with great caution to prevent decomposition. Procedures for storage, handling, and stability assessment have been described (29).

Epoxide Hydrase. The solubilization, purification to apparent homogeneity, and characterization of epoxide hydrase from liver microsomes of phenobarbital-treat-pretreated rats has been described (19). Units of epoxide hydrase activity are defined as nmoles of styrene glycol formed from styrene oxide per 15 min. Enzyme solutions were sterilized by filtration through 0.20-μm membrane filters.

Bacterial Mutagenesis Assays. Strains TA1538, TA98, and TA100 of S. typhimurium were developed by Ames et al. (1, 2, 23) and kindly provided by his laboratory at the University of California, Berkeley, Calif. Strain TA1538, derived from S. typhimurium LT-2, contains the histidine mutation his-D3052, which renders the bacteria completely dependent on histidine for growth. Frame-shift mutagens that interact with the appropriate DNA sequences at or near the histidine operon will correct the original mutation and revert the bacteria to histidine independence. Two additional mutations, one eliminating excision repair of DNA and the other producing an outer cell membrane deficient in lipopolysaccharide, increase the sensitivity of the strain to mutagen-induced reversion. Strains TA98 and TA100 were developed by introducing an error-prone recombinational repair system into strains TA1538 and TA1535, respectively, and are much more sensitive to mutation by many chemicals than are the parental strains. Mutations (reversions) are quantified in the bacteria by counting the number of colonies that grow on a histidine-free medium. Growth and maintenance of the bacteria and the pour-plate procedure for mutagen testing were essentially as have been described in detail elsewhere (29, 32). Briefly, 2 × 10⁸ bacteria, suspended in 0.1 ml of phosphate-buffered saline (5 mM potassium phosphate-150 mM sodium chloride, final pH 7.0), were added to 2.0 ml of molten top agar (45°) consisting of sodium chloride and agar, 12 mg each, and L-histidine and biotin, 0.1 μmole each. Immediately after addition of the BP derivatives in 0.1 ml of anhydrous DMSO to the bacteria and top agar, the contents of the culture tube (13 × 100 mm) were mixed and poured into a Petri dish containing 15 ml Vogel-Bonner medium with a 2% agar base. An alternative procedure was to incubate the derivative (added in 12.5 μl of solvent) and the bacteria in a final volume of 0.5 ml of phosphate-buffered saline (pH 7.0) at 37° for 5 min before the addition of the top agar. With either procedure, experiments were done in triplicate, and coefficients of variation did not exceed 15% of the mean values shown.

Mutagenicity of BP Epoxides

Materials. Bacterial media and plastic ware for bacterial and mammalian cell culture were obtained from the Bioquest Division of Becton, Dickinson and Co., Cockeysville, Md., and Fisher Scientific Inc., Springfield, N. J. Fetal calf serum was obtained from Reheis Chemical Co., Kankakee, Ill., and all other cell culture medium was obtained from Grand Island Biological Co., Grand Island, N. Y. Commercially available biochemicals were obtained from Sigma Chemical Co., St. Louis, Mo., or Calbiochem, La Jolla, Calif. DMSO was vacuum distilled over calcium hydride and stored under an atmosphere of argon before addition of solutions of the various compounds to the cells.

Synthesis of BP Derivatives. The procedures utilized in the synthesis of H₄-7,8-epoxide and of H₄-9,10-epoxide consisted of cyclization of trans-halohydrin precursors at the indicated positions with base (34). Synthetic BP 7,8-dihydridiol (8) was used to synthesize diol epoxides 1 and 2. The more reactive diol epoxide 1 has been prepared by conversion of BP 7,8-dihydridiol to a bromohydrin at the 9,10-position (7,8,8a,10β-trihydroxy-9-bromo-7,8,9,10-tetrahydrobenzo(a)pyrene, followed by cyclization with base, whereas the less reactive diol epoxide 2 forms on direct epoxidation of BP 7,8-dihydridiol with peroxy acid (18, 33). These procedures provide the diol epoxides free of detectable impurities and free of stereoisomeric cross-contamination, as determined by nuclear magnetic resonance spectroscopy (16, 17, 33). Both diol epoxides readily form bis(trimethylsilyl) ethers and are somewhat more.

---

As indicated in Ref. 33, we are particularly grateful to Dr. D. J. McCaustland and Dr. J. F. Engle of Midwest Research Institute, Kansas City, Mo., for providing us with an unpublished experimental procedure by which diol epoxide 2 can be obtained in pure form.
and the conditions used were described previously (29-31). Derivatives were added in 20 μl of anhydrous DMSO to the cell cultures growing in 5 ml of Eagle's minimal essential medium, which contained a 1.5-fold higher than normal concentration of glutamine and nonessential amino acids and 10% (v/v) dialyzed and heat-inactivated fetal calf serum. For each determination, 4 replicate cultures were used to evaluate toxicity, and 16 replicate cultures were used to score 8-azaguanine-resistant colonies. Neither the S. typhimurium strains nor the Chinese hamster cells contain detectable monooxygenase activity, and BP, without metabolic activation, is inactive as a mutagen in both systems (31).

**Determination of BP Epoxide Stability in Aqueous Media.** Stability of the epoxides was evaluated by determining the decrease in observed mutations that resulted from preincubating the derivatives in either top agar, phosphate-buffered saline, or tissue culture medium for defined periods of time before the addition of the cells. In the bacterial studies, zero time values were determined by adding the *Salmonella* immediately before the epoxides. In the studies with the Chinese hamster V79 cells, the epoxides were preincubated in culture medium and added to the cell cultures in 1.0 ml of medium. Mutations induced by the normal procedure of adding the derivatives in DMSO directly to the cell cultures represented the zero time values. Volumes of medium and DMSO were adjusted so that under all preincubation and culture conditions the DMSO concentration was 0.33%, and the volume of medium was 6.0 ml. The mutagenic activity of all the derivatives in each of the aqueous media decreased in a monoeponential fashion as a function of preincubation time.

**RESULTS**

**Stability of BP Epoxides in Aqueous Medium.** Preliminary studies indicated that diol epoxide 1, diol epoxide 2, H$_4$-7,8-epoxide, and H$_4$-9,10-epoxide were mutagenic in the *Salmonella* tester strains and in Chinese hamster V79 cells. Clearly, the observed mutagenic activity of these derivatives was dependent on both their intrinsic activity as mutagens and their stability in an aqueous environment. A highly mutagenic but unstable compound might decompose at a rate sufficient to preclude its interaction with DNA inside the cell. Therefore, in order better to evaluate the intrinsic and relative mutagenic activities of the epoxides, we determined their stabilities in aqueous solution (Table 1). Diol epoxide 1 was very unstable in all 3 of the media used for mutagen testing; half-lives never exceeded 1 min. The stereoisomeric diol epoxide 2 was also quite unstable but nevertheless was 2 to 20 times more stable than diol epoxide 1. The half-life of H$_4$-9,10-epoxide was 1 min or less when tested in top agar or phosphate-buffered saline with strain TA98 of *S. typhimurium* and was thus comparable to diol epoxide 1. In contrast, H$_4$-9,10-epoxide appeared considerably more stable when tested with the V79 cells in tissue culture medium. The half-life of H$_4$-9,10-epoxide in this medium was 10 to 20 times longer than the half-life of diol epoxide 1 and was about the same as the half-life of diol epoxide 2. H$_4$-7,8-epoxide was the most stable of the 4 compounds, with half-lives ranging from 10 to 90 min in the 3 media.

**Mutagenic Activity in Salmonella Tester Strains.** The number of reversions to histidine independence induced in strains TA98, TA100, and TA1538 of *S. typhimurium* by the 4 BP epoxide derivatives is illustrated in Chart 2. All 4 compounds were mutagenic in all 3 strains, and linear dose responses were observed over finite concentration ranges. At higher concentrations of epoxide, there was generally a decrease in revertant colonies accompanied by an irregular background lawn of cells, characteristic of toxicity and cell death. Experiments with excess histidine (1.0 μmole), which permitted all viable bacteria to grow and form colonies, indicated that few if any bacteria were killed from exposure to the derivatives at concentrations within the linear dose-response range. The 2 error-prone strains, TA98 and TA100, were considerably more sensitive to mutations induced by the 4 epoxides than was strain TA1538. In the linear portion of the dose-response curve, diol epoxide 1 and H$_4$-9,10-epoxide were about equally mutagenic in TA98 and TA100, whereas the latter compound was twice as mutagenic as diol epoxide 1 in TA1538. Diol epoxide 1 was about 4.8, 1.6, and 4.2 times more mutagenic than its stereoisomer, diol epoxide 2, in strains TA98, TA100, and TA1538, respectively. Half-life data in Table 1 indicate that the relative differences in mutagenic activity between the 2 stereoisomers could be increased substantially if the 6-fold difference in stability of the 2 isomers in top agar were considered. H$_4$-7,8-epoxide was one-tenth as mutagenic as H$_4$-9,10-epoxide in strain TA98 and very much less active in TA100 and TA1538.

In the experiments just described, bacteria were exposed to the derivatives in molten top agar at 45° and immediately poured into Petri dishes, as described by Ames et al. (2). To evaluate further the mutagenic activity of the epoxides under more defined and physiological conditions, bacteria of strain TA98 and the epoxide were preincubated in 0.5 ml of phosphate-buffered saline (pH 7.0) for 5 min at 37° before addition of 2 ml of top agar (Chart 3). In the linear region of the dose-response curves, H$_4$-9,10-epoxide was the most mutagenic of the 4 epoxides. Approximately 850 histidine-independent revertants were induced by 0.1 nmole of H$_4$-9,10-epoxide. About 500, 270, and 70 revertants were induced by the same amount of diol epoxide 1, diol epoxide 2, and H$_4$-7,8-epoxide, respectively. The marked shift in the dose-response curve to lower amounts of derivative in Chart 3 results, in part, from the smaller incubation volume and, hence, higher initial concentration of derivative.

**Cytotoxicity and Mutagenicity of the Benzo-Ring Epoxides in Mammalian Cell Culture.** The cytotoxicity of the epoxide derivatives, inferred from the decreased number of his$^+$ revertants and the poor background lawn in the bacterial studies, is demonstrated quantitatively in the clonal survival of the V79 cells (Chart 4). All 4 BP epoxides were toxic to the cells in a dose-dependent manner. Diol epoxide 2 was the most toxic of the 4 compounds, whereas H$_4$-7,8-epoxide was 40 to 80 times less toxic than the three 9,10-epoxide derivatives. Concentrations of diol epoxide 1, diol epoxide 2, H$_4$-9,10-epoxide, and H$_4$-7,8-epoxide, which killed 50% of the V79 cells, were 0.87, 0.46, 0.56, and 37 μM, respectively. To illustrate the effect of stability of the BP
The half-lives of the epoxides were determined by measuring the decrease in mutagenic activity or cytotoxicity that occurred after the compounds were preincubated in the various media before the addition of the cells. Each value represents a single experiment.

<table>
<thead>
<tr>
<th>Aqueous medium</th>
<th>Temperature (°C)</th>
<th>Tester strain</th>
<th>Diol epoxide 1 (min)</th>
<th>Diol epoxide 2 (min)</th>
<th>( H_4)-7,8-epoxide (min)</th>
<th>( H_4)-9,10-epoxide (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top agar</td>
<td>45</td>
<td>TA98</td>
<td>0.3, 0.4</td>
<td>2.1, 2.2</td>
<td>40, 93</td>
<td>0.4, 0.7</td>
</tr>
<tr>
<td>Phosphate-buffered saline</td>
<td>37</td>
<td>TA98</td>
<td>0.4, 0.8</td>
<td>1.8, 2.0</td>
<td>10, 13</td>
<td>0.7, 1.0</td>
</tr>
<tr>
<td>Tissue culture medium</td>
<td>37</td>
<td>V79</td>
<td>0.3, 0.6</td>
<td>6, 13</td>
<td>15, 18</td>
<td>7, 12</td>
</tr>
</tbody>
</table>

* Mutagenicity was determined.

† Cytotoxicity was determined.

**Table 1**

**Half-lives of benzo-ring epoxides of BP in aqueous medium**

Epoxides on biological activity, direct addition to the cultures of diol epoxides 1 and 2, 1.5 and 0.75 nmole/ml, respectively, killed over 90% of the cells. Preincubating the same amount of diol epoxide 1 in tissue culture medium for 1 min before addition to the cells resulted in only 29% cell death, and a 4-min preincubation resulted in no observable toxicity. In contrast, preincubating diol epoxide 2 for 10 min still led to 33% cell death. Thus, when stability of the derivatives in tissue culture medium is considered (Table 1), diol epoxide 1 probably has the highest intrinsic cytotoxicity, since it is at least 10 times more labile than diol epoxide 2 in tissue culture medium.

The mutagenic activity of the benzo-ring epoxides in V79 cells is illustrated in Chart 5. All 4 derivatives induced 8-azaguanine-resistant colonies in proportion to the epoxide concentration in the tissue culture medium. Diol epoxide 1 and \( H_4\)-9,10-epoxide had similar activities and were about 200 times more mutagenic than \( H_4\)-7,8-epoxide. At the concentrations indicated in Chart 5, all the derivatives exhibited comparable and low toxicity. Consideration of the differences in stability of the compounds (Table 1) suggests that diol epoxide 1 may have more intrinsic mutagenic activity than diol epoxide 2 in the mammalian cells under the assay conditions used.

Chart 2. Mutagenicity of benzo-ring epoxides of BP in strains TA98, TA100, and TA1538 of *S. typhimurium*. Compounds in the amounts indicated were added in 100 μl of anhydrous DMSO to 2 ml of molten top agar which contained 2 × 10⁶ bacteria. Each value represents the average of 3 replicate determinations, and the spontaneous mutation frequency (ordinate intercept) has not been subtracted.

**Mutagenicity of the Bromohydrin Precursor and the Tri-**
methyisily Ether Derivative of Diol Epoxide 1 in S. typhi-
murium and V79 Cells. The bromohydrin precursor of diol
epoxide 1 (Chart 1, Compound 5) and the trimethyisilyl
ether of diol epoxide 1 (Chart 1, Compound 6) were tested
for mutagenic activity on the hypothesis that they would be
converted to diol epoxide 1 in alkaline and acidic environ-
ments, respectively. Both Compound 5 and Compound 6
are considerably more stable in neutral aqueous solution
than is diol epoxide 1 (33). Chart 6 illustrates the mutagenic-
ity of the bromohydrin precursor in S. typhi-
murium and V79
cells as a function of the pH of the incubation medium.
In
strain TA98 of S. typhi-
murium, the number of reversions to histidine independence
induced by 4.0 nmoles of the bro-
mothyrin precursor increased from 2 times background at
pH 7 to almost 20 times background at pH 8.6. Similarly,
incubation of 0.8 nmole of the bromohydrin derivative
with the bacteria resulted in a 10-fold increase in mutagenicity
when the pH was increased from pH 7.0 to 8.6 (data not
shown). There was no difference in the spontaneous muta-
tion frequency or in the mutagenic activity of diol epoxide 1
over the pH range examined. In V79 cells, the mutagenicity
of the bromohydrin precursor, incubated at a concentration
of 0.5 nmole/ml, increased 7-fold by raising the pH from 7.0
to 8.0. The trimethyisilyl ether derivative was very weakly
mutagenic from pH 4.7 to 7.1 in strain TA98 and from pH 6.0
to 7.5 in V79 cells. Although a more acidic environment is
probably necessary to effectively cleave the ether bonds
and generate diol epoxide 1, cell death and a marked de-
crease in the stability of diol epoxide 1 precluded studies at
lower pH values.

Effect of Epoxide Hydrase on the Mutagenic Activity of
the Benzo-ring Epoxides. Microsomal epoxide hydrase cat-
alysts the hydration of a large number of arene and alkene
oxides to trans-dihydriodiolis. It has recently been shown
that mutations induced by BP 4,5-oxide in strains TA98 and
TA1538 of S. typhi-
murium are completely quenched by 75
units of epoxide hy-
hydrase, whereas mutations induced by diol epoxide 1 are unaf-
fected (32). The effect of epoxide
hydrase on the mutagenicity of all 4 benzo-ring epoxides in
strain TA98 is shown in Chart 7. The mutagenic activities
of H4-9,10-epoxide and H4-7,8-epoxide were readily quenched
by epoxide hydrase. Approximately 12 and 30 units of the
enzyme halved the number of mutations induced by H4-7,8-
Growth of normal and 8-azaguanine-resistant cells in culture medium containing 8-azaguanine or aminopterin

<table>
<thead>
<tr>
<th>Clone</th>
<th>Control</th>
<th>8-AG HATG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-1</td>
<td>89</td>
<td>91</td>
</tr>
<tr>
<td>Control-2</td>
<td>74</td>
<td>79</td>
</tr>
<tr>
<td>AGR-1</td>
<td>42</td>
<td>51</td>
</tr>
<tr>
<td>AGR-2</td>
<td>83</td>
<td>77</td>
</tr>
<tr>
<td>AGR-3</td>
<td>83</td>
<td>85</td>
</tr>
<tr>
<td>AGR-4</td>
<td>82</td>
<td>97</td>
</tr>
<tr>
<td>AGR-5</td>
<td>78</td>
<td>79</td>
</tr>
<tr>
<td>AGR-6</td>
<td>100</td>
<td>97</td>
</tr>
<tr>
<td>AGR-7</td>
<td>56</td>
<td>57</td>
</tr>
<tr>
<td>AGR-8</td>
<td>50</td>
<td>58</td>
</tr>
<tr>
<td>AGR-9</td>
<td>96</td>
<td>97</td>
</tr>
</tbody>
</table>

a 8-Azaguanine-resistant clones were isolated from 2 different experiments by ring isolation 14 days after treatment with diol epoxide 1. Control clones were not exposed to mutagens or 8-azaguanine. After cloning, the cells were grown for 2 weeks in medium free of any purine analogs. Cells from all clones were passed twice weekly and underwent a minimum of 15 cell divisions.

b Values represent the average number of cell colonies observed 7 days after seeding 100 cells in each of 4 tissue culture dishes containing 5 ml of culture medium. 8-Azaguanine (100 μM) or 10 μM hypoxanthine, 10 μM thymidine, 10 μM glycine, and 3.3 μM aminopterin was added 18 hr after seeding the cells.

Mutagenicity of BP Epoxides

Chart 5. Mutagenicity of the benzo-ring epoxides of BP in Chinese hamster V79 cells. Monolayer cultures of 10⁶ cells/culture dish were initiated and treated with the epoxides as described in the legend to Chart 4. Mutagenicity was determined 14 days after treatment by counting the number of viable colonies of 50 or more cells in 16 replicate cultures that had been treated with 8-azaguanine (10 μg/ml) beginning 2 days after treatment with the BP derivatives. Cell survival at all of the concentrations of derivatives indicated was 80% or more of solvent-treated controls.

Table 2

Growth of normal and 8-azaguanine-resistant cells in culture medium containing 8-azaguanine or aminopterin

<table>
<thead>
<tr>
<th>Clone</th>
<th>Control</th>
<th>8-AG</th>
<th>HATG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-1</td>
<td>89</td>
<td>0</td>
<td>91</td>
</tr>
<tr>
<td>Control-2</td>
<td>74</td>
<td>0</td>
<td>79</td>
</tr>
<tr>
<td>AGR-1</td>
<td>42</td>
<td>51</td>
<td>0</td>
</tr>
<tr>
<td>AGR-2</td>
<td>83</td>
<td>77</td>
<td>0</td>
</tr>
<tr>
<td>AGR-3</td>
<td>83</td>
<td>85</td>
<td>0</td>
</tr>
<tr>
<td>AGR-4</td>
<td>82</td>
<td>97</td>
<td>0</td>
</tr>
<tr>
<td>AGR-5</td>
<td>78</td>
<td>79</td>
<td>0</td>
</tr>
<tr>
<td>AGR-6</td>
<td>100</td>
<td>97</td>
<td>0</td>
</tr>
<tr>
<td>AGR-7</td>
<td>56</td>
<td>57</td>
<td>0</td>
</tr>
<tr>
<td>AGR-8</td>
<td>50</td>
<td>58</td>
<td>0</td>
</tr>
<tr>
<td>AGR-9</td>
<td>96</td>
<td>97</td>
<td>0</td>
</tr>
</tbody>
</table>

a 8-Azaguanine-resistant clones were isolated from 2 different experiments by ring isolation 14 days after treatment with diol epoxide 1. Control clones were not exposed to mutagens or 8-azaguanine. After cloning, the cells were grown for 2 weeks in medium free of any purine analogs. Cells from all clones were passed twice weekly and underwent a minimum of 15 cell divisions.

b Values represent the average number of cell colonies observed 7 days after seeding 100 cells in each of 4 tissue culture dishes containing 5 ml of culture medium. 8-Azaguanine (100 μM) or 10 μM hypoxanthine, 10 μM thymidine, 10 μM glycine, and 3.3 μM aminopterin was added 18 hr after seeding the cells.

The abbreviations used are: 8-AG, 8-azaguanine; HATG, 10 μM hypoxanthine, 10 μM thymidine, 10 μM thymidine, and 3.3 μM aminopterin; AGR, 8-azaguanine-resistant clone.

Discussion

Diol epoxide 1 is a highly mutagenic derivative of BP, both in bacterial and mammalian cells (6, 29). In the present study, we have explored the molecular and stereochemical requirements of 9,10-epoxides of 7,8,9,10-tetrahydrobenzo(a)pyrene that are needed for high chemical reactivity, cytotoxicity, and mutagenicity. The results with the highly active H₄-9,10-epoxide and its derivatives (Chart 1, Compounds 1, 2, 3, and 6) contrast with the results obtained with the relatively inactive H₄-7,8-epoxide (Compound 4). The stereoisomeric diol epoxide 1 and diol epoxide 2 differ by the configuration of the 7- and 8-hydroxyl groups relative to the 9,10-epoxide ring (Chart 1). In diol epoxide 1, the 7-hydroxyl group is on the same face of the plane of the molecule as the epoxide ring and can thus form an intramolecular hydrogen bond with the epoxide oxygen. When this stereochemical situation exists in sterol epoxides or in the antileukemic agent triptolide, a marked increase in reactivity toward nucleophiles is observed (cf. Refs. 13 and 33). Although anchimeric assistance by a proximate hydroxyl group markedly increases the reactivity of the diol epoxides of BP and naphthalene toward p-nitrothiophenolate (16, 33), its importance in explaining the high mutagenic activity of diol epoxide 1 is uncertain. Addition of diol epoxide 1 to strains TA98, TA100, and TA1538 of S. typhimurium in top agar causes, respectively, about 4.8, 1.6, and 4.2 times more mutations than diol epoxide 2 which cannot form the intramolecular hydrogen bond (Chart 2). Consideration of the 3-fold-longer half-life of diol epoxide 2 in top agar increases the relative difference in mutagenic activity 5- to 11-fold, and, thus, anchimeric assistance may be a factor that enhances mutagenic activity. However, in all 3 strains, H₄-9,10-epoxide, which contains no hydroxyl epoxide (epoxide hydrolase sensitive) both have half-lives of less than 1 min in phosphate-buffered saline at 37° (Table 1).
groups, has mutagenic activity equal to or greater than diol epoxide 1, and both compounds possess similar stabilities. The same pattern of mutagenic activity is also seen when the epoxides and bacteria of strain TA98 are preincubated in phosphate-buffered saline at 37°, before the addition of agar.

In V79 cells, diol epoxide 2 is the most cytotoxic and mutagenic of the 9,10-epoxide derivatives. However, it is at least 10 times more stable than diol epoxide 1 toward solvolytic reactions. The very short half-life of about 30 sec in tissue culture medium for diol epoxide 1 makes comparison of its intrinsic mutagenic activity with other more stable compounds very difficult. Given the lability of diol epoxide 1, it is indeed remarkable that it is such a potent mutagen in bacteria and in the mammalian cell-culture system. Malaville et al. (20) recently reported that a BP 7,8-diol-9,10-epoxide has mutagenic activity in strain TA100 of S. typhimurium. Although the purity and relative stereochemistry of the compound studied were not defined, the method of synthesis (27) would be expected (33) to yield diol epoxide 2. The mutagenic activity of the diol epoxide they used had less than 10% of the activity of diol epoxide 2 reported in the present study. During the preparation of this manuscript, Huberman et al.* reported on the mutagenic and cytotoxic activity of diol epoxide 1 and diol epoxide 2 in V79 cells (12). A direct comparison of the mutagenic and cytotoxic activities of diol epoxide 2 obtained in our study and in the study by Huberman et al. is difficult, since 2 different clones of V79 cells were used in the 2 studies and because the studies by Huberman et al. were done with concentrations of diol epoxides that were not within a linear dose-response range. Nevertheless, diol epoxide 2 had similar high mutagenic and cytotoxic activities in both studies. In contrast, diol epoxide 1 was many times more mutagenic and cytotoxic in the present study and in earlier reports from our laboratory (6, 29) than was reported by Huberman et al. (12).

* The authors express their appreciation to Dr. E. Huberman for providing us with a copy of his manuscript (12) prior to publication.

** We have previously shown that diol epoxide 1 is at least 40 times more...
The presence of an epoxide group in the benzo ring does not in itself confer the BP molecule with significant mutagenic activity, since BP 7,8-oxide and BP 9,10-oxide are very weak mutagens in several strains of S. typhimurium (30, 31) and in Chinese hamster V79 cells (31). Diol epoxides 1 and 2 differ chemically from BP 9,10-oxide in that the diol epoxides should be much more sensitive to attack by nucleophiles and water compared to BP 9,10-oxide, for which spontaneous isomerization to a phenol is expected to be a more facile process (4). We utilized H8-9,10-epoxide, although it is not a potential metabolite of BP, to determine whether the diol group is a requirement for the mutagenicity of the diol epoxides. The tetrahydro epoxides, like the diol epoxides, lack a formal double bond in the benzo ring and thus undergo reactions characteristic of epoxides rather than of arene oxides. The results with both the bacterial (Charts 2 and 3) and mammalian cells (Charts 4 and 5) indicate that H8-9,10-epoxide is a very potent mutagen and that the hydroxyl groups are not essential for mutagenic or cytotoxic activity. Although diol epoxide 1 is more than 450 times as reactive toward β-nitrothiophenolate in t-butyl alcohol as compared to H8-9,10-epoxide (17, 33), both the medium and the attacking nucleophile are clearly important in assessing relative reactivity.

The high mutagenicity and cytotoxicity of H8-9,10-epoxide relative to the poor activity observed for H8-7,8-epoxide indicates that the position of the epoxide group on the benzo ring has a tremendous effect on the biological activity of the molecule. H8-9,10-epoxide is 7 to 37 times more mutagenic than H8-7,8-epoxide in the 3 strains of Salmomorella, 200 times more mutagenic than H8-7,8-epoxide in V79 cells, and 65 times more cytotoxic than H8-7,8-epoxide in V79 cells. Consideration of the relative stabilities of the 2 compounds in the bacterial system (Table 1) would magnify these differences 10- to 100-fold. The 9,10-diol-7,8-epoxides of BP have yet to be synthesized, and it was thus not possible to examine their mutagenic activity. Metabolic activation studies (20, 32) with trans-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene and trans-9,10-dihydroxy-9,10-dihydrobenzo(a)pyrene, however, are consistent with the idea that BP dihydrodiols with an epoxide in the 9,10-position are much more active than the corresponding derivatives with an epoxide in the 7,8-position.

BP 7,8-diol-9,10-epoxide is believed to be synthesized from BP via formation of BP 7,8-oxide, hydration of this arene oxide to BP 7,8-dihydriodiol, and then by epoxidation of the dihydriodiol at the 9,10-position. The epoxidation of BP is catalyzed by a microsomal NADPH-dependent monooxygenase system (6, 10), and a monooxygenase system is also required for the metabolism of BP 7,8-dihydriodiol (3, 12, 20, 27, 32). The hydration of BP 7,8-oxide is catalyzed by microsomal epoxide hydrazine (6, 10), and BP 7,8-oxide and BP 7,8-dihydriodiol are known products of BP metabolism. Both BP 7,8-oxide (18) and BP 7,8-dihydriodiol (W. Levin, A. W. Wood, H. Yagi, D. M. Jerina, and A. H. Conney, submitted manuscript) are carcinogenic on mouse skin, and it is possible that 1 or both of the 2 stereoisomers BP 7,8-diol-9,10-epoxides may be an ultimate carcinogen obtained from BP metabolism. The nonmutagenic BP 7,8-dihydriodiol can be metabolized to a potent bacterial mutagen(s) by a 9000 × g liver supernatant fraction (20) or by a purified cytochrome P-450 monooxygenase system (32). The weakly mutagenic BP 7,8-oxide has been metabolically activated to a potent bacterial mutagen by a combination of the purified monooxygenase system and purified epoxide hydras (32). DNA binding profiles of metabolites formed by the metabolis of BP and BP 7,8-dihydriodiol are similar to the profiles obtained with BP 7,8-diol-9,10-epoxide and are consistent with its formation (24, 27). A recent report has provided evidence for the liver microsomal metabolism of BP 7,8-dihydriodiol to a diol epoxide (27), which has subsequently been suggested to be diol epoxide 2 (12). It is of considerable interest that the diol epoxides 1 and 2 are poor substrates for epoxide hydrase (Chart 7), and it is possible that this may have importance for the biological activities of these molecules. We are currently investigating the metabolism of BP 7,8-dihydriodiol and the carcinogenicity of the 2 diol epoxide stereoisomers, the tetrahydro epoxides, the bromohydrin precursor (Compound 5), and the bis(triethylsilyl) ether (Compound 6) derivatives of diol epoxide 1. Both the bromohydrin (Compound 5) and the silyl ether (Compound 6) may act as relatively stable precursors for diol epoxide 1, thus permitting higher intracellular levels of this diol epoxide.

The high mutagenicity of diol epoxides 1 and 2 and of the H8-9,10-epoxide, coupled with the carcinogenicity of BP 7,8-dihydriodiol and BP 7,8-oxide which can metabolically lead to derivatives of the H8-9,10-epoxide provide an intriguing clue to the carcinogenicity of polycyclic aromatic hydrocarbons in general. A unique feature of the diol epoxides and tetrahydro epoxides of BP studied here is that only the 9,10-epoxide derivatives are highly active. The 9,10-region of BP is chemically and structurally distinct from any of the several other regions that are possible sites for hydrocarbon epoxidation in that it is the only place that a bay region tetrahydroepoxide can be formed. The qualitative observation that the bay region tetrahydroepoxide of BP and other hydrocarbons are unusually chemically reactive prompted a reexamination of the available carcinogenicity data on methylated and fluorinated polycyclic aromatic hydrocarbons. The data showed that substituents that would be

---

*Mutagenicity of BP Epoxides*

---

4 Metabolism of BP 7,8-dihydriodiol to both diol epoxide 1 and diol epoxide 2 has been recently demonstrated (Thakker, D. R., Yagi, H., Lu, A. Y. H., Levin, W., Conney, A. H., and Jerina, D. M. Metabolism of Benzo(a)pyrene. V. Conversion of (±)-Trans-7,8-Dihydroxy-7,8-Dihydrobenzo(a)pyrene to the Highly Mutagenic 7,8-Diol-9,10-Epoxides. Proc. Natl. Acad. Sci. U. S. A., in press).

5 A bay region in a polycyclic aromatic hydrocarbon exists when 7 nonadcent benzene rings, 1 of which is a benzo ring, are in close proximity. The prototype for a bay region is the sterically hindered area between positions 4 and 5 of the phenanthrene molecule. Thus, the region between positions 10 and 11 of BP (Chart 1) is a bay region.
expected to block formation of bay region diol epoxides markedly reduced carcinogenicity (15). Thus, we suggest as a working hypothesis that the epoxidation of nonaromatic double bonds in the bay region of a benzo-ring dihydrodiol (e.g., epoxidation at the 9,10-position of BP 7,8-dihydrodiol, the 1,2-position of benzo[a]anthracene 3,4-dihydrodiol and various methylated benzo[a]anthracene dihydrodiols, and the 7,8-position of 3-methylcholanthrene 9,10-dihydrodiol) may be considered as key reactions in the metabolic activation of these and other polycyclic aromatic hydrocarbons.

ACKNOWLEDGMENTS

We thank MaryAnn Augustin for her excellent help in the preparation of this manuscript.

ADDENDUM

Benzo[a]anthracene and 5 trans-dihydrodiols of benzo[a]anthracene have been metabolized, in the presence of S. typhimurium strain TA100, by a highly purified hepatic microsomal monoxygenase system. Our results showed that the metabolic products(s) of benzo[a]anthracene 3,4-dihydrodiol was nearly 10 times as mutagenic to the bacteria as were the metabolites of benzo[a]anthracene and the other 4 dihydrodiols. The marked activation of benzo[a]anthracene 3,4-dihydrodiol, presumably to the 3,4-diol-1,2-epoxide, is consistent with and supports the hypothesis that bay-region epoxides of unsubstituted polycyclic hydrocarbons are ultimately reactive forms of these carcinogenic compounds.

REFERENCES

Mutagenicity and Cytotoxicity of Benzo(a)pyrene Benzo-Ring Epoxides


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/36/9_Part_1/3358

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/36/9_Part_1/3358.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.