Mutagenicity of Benzo(e)pyrene and Triphenylene Tetrahydroepoxides and Diol-Epoxides in Bacterial and Mammalian Cells

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ABSTRACT

The mutagenic activities of the diol-epoxide and tetrahydroepoxide derivatives of benzo(e)pyrene and triphenylene were assessed in bacterial and mammalian cells to help determine why these two structurally related polycyclic hydrocarbons have little if any carcinogenic activity. In strains TA98 and TA100 of Salmonella typhimurium, a 400-fold range in mutagenic activities of the epoxide derivatives was observed under conditions in which all of the compounds induced significant dose-dependent mutation frequencies. The diastereomeric pair of diol-epoxides of benzo(e)pyrene were 4- to 8-fold more mutagenic than the diastereomeric pair of diol-epoxides of triphenylene. The corresponding tetrahydroepoxide derivatives of both hydrocarbons, in which the hydroxyl groups were absent from the saturated benzo ring, were from 10 to 400 times more mutagenic than were the corresponding diol-epoxides. Qualitatively similar results were observed with cultured Chinese hamster V79 cells except that the diastereomeric pair of diol-epoxides of triphenylene had insignificant mutagenic and cytotoxic activity toward the mammalian cells. A plausible explanation for the relatively low mutagenic activities of the diol-epoxides relative to their tetrahydroepoxides is that the hydroxyl groups interfere with the accessibility to or the critical interaction with cellular DNA. Inability of trans-1,2-dihydroxy-1,2-dihydrotriphenylene, the potential metabolic precursor of the triphenylene diol-epoxides, to be metabolically activated by rat liver microsomes to bacterial mutagens is probably a consequence of the quasidiaxial conformation of the hydroxyl groups in this dihydrodiol.

INTRODUCTION

The carcinogenic polycyclic aromatic hydrocarbons owe most of their biological activity to their ability to be metabolized to highly reactive benzo-ring diol-epoxides in which the epoxide moiety forms part of the bay region of the hydrocarbon (5, 8, 9, 17, 26). The metabolic pathway of diol-epoxide formation, first demonstrated for the potent carcinogen benzo(a)pyrene, consists of oxidation of a terminal benzo ring of the hydrocarbon to an arene oxide, hydration of the arene oxide to form a trans-dihydrodiol, and epoxidation of the olefinic double bond of the dihydrodiol (14, 20, 21, 27). Inability to complete one or more of the steps in this metabolic pathway provides a plausible and experimentally verifiable explanation for the lack of carcinogenicity of a noncarcinogenic polycyclic aromatic hydrocarbon. Recent studies have shown that little if any B(e)P³ (Chart 1) is metabolized to B(e)P 9,10-dihydrodiol by cultured hamster embryo cells (15) and rat liver microsomes, and little if any B(e)P dihydrodiol is metabolized to its bay-region diol-epoxide diastereomers by rat liver microsomes (12, 28). While the reason for the negligible amount of B(e)P 9,10-dihydrodiol formation has not been established, the lack of diol-epoxide formation from B(e)P 9,10-dihydrodiol has been explained, at least in part, by steric and polar factors. It is important to note that both the diol group and the benzo-ring double bond of the 9,10-dihydrodiol form parts of bay regions of the B(e)P molecule. Constraints on the hydroxyl groups of the dihydrodiol by their presence in the bay region of the hydrocarbon force them to adopt a quasidiaxial conformation (10-13). This conformation results in a relatively bulky and polar molecule (22) the further oxidative metabolism of which is directed away from the isolated olefinic double bond, and diol-epoxide formation does not occur to any appreciable extent (12, 28). Similar results have been previously observed for the metabolism of benzo(a)pyrene 9,10-dihydrodiol, which also possesses quasidiaxial hydroxyl groups in the bay region (23).

Although these observations on the metabolism of B(e)P provide an explanation for the lack of, or very weak, carcinogenicity of B(e)P (3), several considerations suggested that it would be of particular interest to examine the intrinsic biological activity of the authentic diol-epoxides of B(e)P: (a) quantum mechanical calculations indicate that the diastereomeric B(e)P 9,10-diol-11,12-epoxides should have moderate to high reactivity based on their predicted ease of triol carbonium ion formation (9); (b) B(e)P H4-9,10-epoxide, which is structurally related to the bay-region diol-epoxides of B(e)P (see Chart 1), is highly mutagenic in bacterial and mammalian cells (28); (c) the conformational restraints which force the hydroxyl groups of the B(e)P 9,10-dihydrodiol to become diaxial are also present in the diol-epoxides (11, 28, 29). Thus, evaluation of the mutagenicity of the B(e)P diol-epoxides provides the first opportunity to ascertain how such a conformational factor could play a role in the biological activity of benzo(e)pyrene (28, 29).

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2. The abbreviations used are: B(e)P, benzo(e)pyrene; B(e)P 9,10-dihydrodiol, trans-9,10-dihydroxy-9,10-dioxydibenzo(e)pyrene; B(e)P H4-9,10-epoxide, 9,10-epoxy-9,10,11,12-tetrahydrobenzo(e)pyrene; TP, triphenylene; H2B(e)P, 9,10-dihydrobenzo(e)pyrene; 1,2-H2, TP 1,2-dihydrotriphenylene; TP H4, 1,2-diol, trans-1,2-dihydroxy-1,2,3,4-tetrahydrotriphenylene; TP H4-9,10-epoxide, 1,2-epoxy-1,2,3,4-tetrahydrotriphenylene; B(e)P diol-epoxide 1, (±)-9,10-dihydroxy-9,10,11,12-tetrahydrobenzo(e)pyrene; B(e)P diol-epoxide 2, (±)-9,10a-dihydroxy-9,10,11,12-tetrahydrobenzo(e)pyrene; B(e)P diol-epoxide 3, (±)-9,10a-dihydroxy-9,10,11,12-tetrahydrobenzo(e)pyrene; TP diol-epoxide 1, (±)-9,10a-dihydroxy-9,10,11,12-tetrahydrobenzo(e)pyrene; TP diol-epoxide 2, (±)-9,10a-dihydroxy-9,10,11,12-tetrahydrobenzo(e)pyrene; TP diol-epoxide 3, (±)-9,10a-dihydroxy-9,10,11,12-tetrahydrobenzo(e)pyrene. All compounds are racemic mixtures where enantiomers are possible.

influence biological activity. Since the above considerations also apply to the polycyclic hydrocarbon triphenylene (Chart 1), the biological activity of the diol-epoxides and tetrahydro-epoxide of TP has also been examined.

MATERIALS AND METHODS

Materials. Aroclor 1254 (Lot KC 12-638) was obtained from Monsanto Co., St. Louis, Mo. Media for culturing the bacteria and mammalian cells were obtained from Becton Dickinson and Co., Cockeysville, Md., and Grand Island Biological Co., Grand Island, N. Y., respectively. Fetal calf serum was obtained from Reheis Chemical Co., Kankakee, Ill. Other commercially available biochemicals were obtained from Sigma Chemical Co., St. Louis, Mo.

Polycyclic Hydrocarbons and Their Derivatives. B(e)P (99% pure) and TP (98% pure) were purchased from Aldrich Chemical Co., Milwaukee, Wis., and were free of detectable impurities as determined by nuclear magnetic resonance spectrometry and reverse-phase high-pressure liquid chromatography. 9,10-H2 B(e)P (13), B(e)P H4-9,10-epoxide (13), 1,2-H2 TP (28), and the diol epoxides of B(e)P and TP (31) were synthesized as described in the indicated references. TP H4-1,2-epoxide, TP 1,2-dihydrodiol, and TP H4-1,2-diol were synthesized in a manner analogous to that for the corresponding B(e)P derivatives (13). All of the compounds were of analytical purity. In accordance with our predictions and prior observations (23, 30, 31), the quasi-diaxial nature of the hydroxyl groups in the B(e)P and TP dihydrodiols results in a loss of stereochemical control upon direct epoxidation. Thus, under a wide variety of experimental conditions, ~1:1 mixtures of diol-epoxide diastereomers 1 and 2 are produced upon epoxidation of either dihydrodiol. In each case, the mixture of diastereomers is readily separated by high-pressure liquid chromatography (31). Although Harvey et al. (6) claimed to have prepared the diol-epoxide 2 diastereomer of B(e)P and TP, these authors failed to recognize that mixtures of both diastereomers were formed upon direct epoxidation of the dihydrodiols; they have used such mixtures in at least one subsequent biological study (7).

Mutagenesis Assays with Bacteria. Strains TA98 and TA100 of histidine-dependent S. typhimurium (16) were obtained from Dr. B. Ames, University of California, Berkeley, Calif., and were cultured as described (24). Intrinsic mutagenicity was assessed by incubating the epoxides (added in 15 µl of dimethyl sulfoxide) with 2 x 10⁷ bacteria suspended in 0.5 ml of phosphate-buffered saline (5 mM potassium phosphate: 150 mM sodium chloride, final pH 7.0) for 5 min at 37° before addition of top agar. Mutagenesis experiments with rat liver microsomes as the source of the monoxygenase activity were based on the procedure described by Ames et al. (1). NADP (2.0 µmol), glucose 6-phosphate (2.5 µmol), and glucose-6-phosphate dehydrogenase (1 unit) were added to a 13 x 100-mm culture tube in 0.25 ml of pH 7.4 buffer which contained 50 µmol sodium phosphate, 4 µmol MgCl2, and 16.5 nmol KCl. Hepatic microsomes, obtained from immature male Long Evans rats pretreated for 4 days with the polychlorinated biphenyl mixture Aroclor 1254 (18, 19), were sterilized by filtration through a 0.45-µm filter and were added in 0.25 ml of 0.15 M KCl. The bacteria (2 x 10⁹) were added in 0.1 ml of phosphate-buffered saline. Reactions were started by addition of the compound in 15 µl of acetone:dimethyl sulfoxide (9:1), and the complete mixtures were incubated at 37° for 5 min before the addition of top agar. After the 5-min incubation of the histidine-dependent bacteria with or without the metabolic activation system, 2 ml of top agar were added, and the entire mixture was poured onto histidine-deficient agar in a Petri dish. Mutations from histidine auxotrophy to histidine prototrophy were assessed by counting the macroscopic colonies of bacteria after a 2-day incubation of the plates at 37°. All experiments were done in triplicate.

Mutagenesis Assays with Mammalian Cells. Line V79-6 of Chinese hamster cells (4) was the generous gift of Dr. E. H. Y. Chu, University of Michigan, Ann Arbor, Mich. The cells, which appeared to be devoid of the enzymes that metabolize polycyclic hydrocarbons, were cultured as previously described (24). Four replicate 60-mm culture dishes were seeded with 10² cells to evaluate toxicity, and 16 replicate 60-mm culture dishes were each seeded with 1 x 10⁴ cells to assess induction of 8-azaguanine-resistant colonies.

RESULTS

Intrinsic Mutagenicity of the Epoxides of B(e)P and TP. The intrinsic mutagenic activities of the diastereomeric pairs of bay-region diol-epoxides of B(e)P and TP toward strains TA98 and TA100 of S. typhimurium are shown in Chart 2. Also shown are the dose-response curves for the bay-region tetrahydro-epoxide derivatives of the 2 hydrocarbons. All of the compounds induced a linear or near-linear dose-dependent increase in histidine-independent colonies in both strains of bacteria. In strain TA98, the diol-epoxide 1 isomers of both polycyclic hydrocarbons had the same mutagenic activity as did their respective diol-epoxide 2 isomers. The diol-epoxides of B(e)P induced approximately 200 histidine revertants/nmol
and were about 8 times more mutagenic than were the 2 diol-epoxide diastereomers of TP. The mutation frequencies induced by B(e)P H4-9,10-epoxide and TP H4-1,2-epoxide differed by only 1.6-fold but were 30- and 150-fold higher than the mutation frequencies induced by their respective diol-epoxides.

In strain TA100 of S. typhimurium, as in strain TA98, the diol-epoxides of B(e)P were more mutagenic than those of TP (approximately a 4-fold difference). The tetrahydroepoxides of B(e)P and TP differed only slightly in their mutagenic activities, and the tetrahydroepoxides of both hydrocarbons were manyfold more active than were their respective diol-epoxides. In strain TA100, however, the diol-epoxide 2 diastereomers of B(e)P and TP, in which the benzylic hydroxyl group and the epoxide oxygen are trans, were about 2-fold more mutagenic than their respective diol-epoxide 1 diastereomers in which the benzylic hydroxyl group and the epoxide oxygen are cis. B(e)P diol-epoxide 2 was the most active of the 4 diol-epoxides, but it still had less than 10% of the mutagenic activity of B(e)P H4-9,10-epoxide.

Examination of the cytotoxic and mutagenic activities of the epoxides of B(e)P and TP in Chinese hamster V79 cells (Chart 3) indicates that qualitatively similar biological activities exist in bacterial and mammalian cells. B(e)P H4-9,10-epoxide, the most active of the 6 compounds tested, induced 8-azaguanine-resistant colonies in 10^5 surviving cells at a frequency of 21 variants/nmol and killed one-half of the exposed cells at a concentration of 1.1 nmol/ml. TP H4-1,2-epoxide had about 65 and 40% of the cytotoxic and mutagenic activity, respectively, of B(e)P H4-9,10-epoxide. B(e)P diol-epoxide 2 had 5 to 7% of the cytotoxic and mutagenic activity of B(e)P H4-9,10-epoxide but was 1.5 times more cytotoxic and 4.5 times more mutagenic than was B(e)P diol-epoxide 1. At concentrations of up to 20 nmol/ml of medium, neither of the diastereomeric diol-epoxides of TP exhibited appreciable mutagenic or cytotoxic activity toward Chinese hamster V79 cells.

**Metabolic Activation of Potential Precursors of TP Epoxides.** The mutagenic activities of the metabolic products which formed when TP or 3 TP derivatives were incubated with varying amounts of hepatic microsomes from Aroclor-pretreated rats are shown in Chart 4. 1,2-H2 TP and 1,2-dihydrodiol are the expected metabolic precursors of TP H4-1,2-epoxide and the diastereomeric TP diol-epoxides, respectively. Consistent with this expectation and the data in Chart 2, 1,2-H2-TP was metabolized to highly mutagenic products, while TP 1,2-dihydrodiol was not metabolized to products mutagenic to strains TA98 or TA100 of S. typhimurium. The data in Chart 4 also indicate that the parent hydrocarbon, TP, was only poorly, if at all, activated to mutagenic products. TP H4-1,2-diol, which is structurally analogous to TP 1,2-dihydrodiol (except that it lacks an adjacent olefinic double bond in the benzo ring), was also inactive. While the lack of activation of TP H4-1,2-diol was expected, tetrahydrodiols are useful analogs when their corresponding dihydrodiols are metabolized to highly mutagenic products because they illustrate the impor-
with the ring opening of diol-epoxides or tetrahydroepoxides to carbonyl ions (9). Values of $\Delta E_{\text{elec}}/\beta$ are identical for diol-epoxides and their structurally analogous tetrahydroepoxides, since the effects of the hydroxyl groups cannot be taken into account in this model. Higher values of $\Delta E_{\text{elec}}/\beta$ predict higher chemical reactivities. For benzo(a)pyrene, B(e)P, triphenylene, and chrysene, the values are 0.794, 0.714, 0.664, and 0.639, respectively. Thus, one would expect from these calculations that the diol-epoxides of B(e)P and TP would have chemical reactivities between those observed for the corresponding diol-epoxides of chrysene and benzo(a)pyrene. In strains TA98 and TA100 of S. typhimurium and in Chinese hamster V79 cells, the bay-region diol-epoxide 2 isomer of benzo(a)pyrene was 13, 30, and 80 times more mutagenic, respectively, than was B(e)P diol-epoxide 2 and over 100 times more mutagenic than was TP diol-epoxide 2 in all 3 systems [data for the diol-epoxides of benzo(a)pyrene not shown]. The diol-epoxide 2 isomer of the bay-region diol-epoxide of chrysene induced essentially the same mutation frequency as did B(e)P diol-epoxide 2 in strain TA98 and Chinese hamster V79 cells but was twice as active as B(e)P diol-epoxide 2 in strain TA100. Relative to TP diol-epoxide 2, the chrysene diol-epoxide isomer was at least 10 times more mutagenic in the 3 systems (25). In contrast to these results, the tetrahydroepoxide of B(e)P, which lacks these hydroxyl groups, has the high mutagenicity expected from the quantum mechanical calculations, and in general the mutagenic activity of the polycyclic hydrocarbon tetrahydroepoxides in strain TA100 shows a good correlation with the quantum mechanical calculations (26). The relatively rigid quasidiaxial conformation of the B(e)P and TP diol-epoxides may be the reason for the divergence of the observed biological activity from the predicted chemical reactivity for these compounds.

Rat liver microsomes and cultured hamster cells metabolize negligible amounts of B(e)P to B(e)P 9,10-dihydrodiol, and rat liver microsomes convert little B(e)P 9,10-dihydrodiol to B(e)P diol-epoxides (28). These metabolism studies provide an explanation for recent experiments which showed that B(e)P 9,10-dihydrodiol as well as B(e)P had no tumor-initiating activity on mouse skin and failed to induce lung adenomas when injected into neonatal mice (3). However, the question remains as to whether B(e)P would be tumorigenic in a species capable of carrying out the appropriate metabolic activation steps leading to diol-epoxide formation. Tumorigenicity studies with the diol-epoxides of B(e)P are in progress as are metabolism studies with B(e)P and B(e)P 9,10-dihydrodiol in a number of species. Given the comparable mutagenic activity of the bay-region diol-epoxide 2 isomers of B(e)P and the bay-region diol-epoxide 2 isomer of chrysene, the recent demonstration of the carcinogeticity of the latter derivative (2) is of interest.

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DISCUSSION

The results of the present study indicate that the diol-epoxides of B(e)P and TP have significant dose-dependent mutagenic activity in S. typhimurium and that the B(e)P diol-epoxides are also mutagenic to Chinese hamster V79 cells. However, the corresponding tetrahydroepoxides of the 2 polycyclic hydrocarbons are from 15 to over 200 times more mutagenic in both bacterial and mammalian cells and are among the more potent polycyclic hydrocarbon epoxides which have been tested to date in these mutagenesis systems. Thus, B(e)P H$_2$-9,10-epoxide has from 0.4 to 1.8 times the mutagenic activity of the bay-region tetrahydroepoxide of the potent carcinogen benzo(a)pyrene. The marked difference in mutagenic activities between the diol-epoxides and tetrahydroepoxides of B(e)P and TP appears attributable, at least in part, to the unique diastereomeric conformation of the hydroxyl groups which result from steric crowding in the bay region (10). The mutagenic activity differences between the bay-region diol-epoxides and the respective bay-region tetrahydroepoxides of benzo(a)pyrene (29), benzo(a)anthracene (24), chrysene (25), and phenanthrene (25) are all smaller than the differences observed in the present study. Indeed, for the potent carcinogen benzo(a)pyrene, ratios of mutagenic activity of the bay-region diol-epoxide to the bay-region tetrahydroepoxide range from 0.4 to about 5 in several mutagenesis test systems.

The marked attenuating effect of the hydroxyl groups of B(e)P and TP diol-epoxides is also illustrated by comparing the predicted chemical reactivity of the diol-epoxides of several polycyclic hydrocarbons with their observed mutagenic activity. Perturbational molecular orbital calculations can be used to predict the $\pi$-electron energy change ($\Delta E_{\text{elec}}/\beta$) associated

Chart 4. Metabolic activation of TP and B(e)P derivatives to products mutagenic to strains TA98 and TA100 of S. typhimurium catalyzed by liver microsomes from Aroclor-pretreated rats. The amount of substrate incubated in the 0.6-mi reaction mixture was 20 nmol. Background mutation frequencies of 17 and 87


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