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Structural Requirements for the Metabolic Activation of Benzo[a]pyrene to Mutagenic Products: Effects of Modifications in the 4,5-, 7,8-, and 9,10-Positions

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SUMMARY

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The metabolism of benzo[a]pyrene and eight benzo[a]pyrene derivatives to products mutagenic to *Salmonella typhimurium* strain TA98 was evaluated with a highly purified, cytochrome P-448-dependent monooxygenase system free of epoxide hydrase. Metabolic activation of benzo[a]pyrene derivatives saturated in the 7,8-position of the molecule either by reduction (7,8-dihydrobenzo[a]pyrene) or by the *cis* or *trans* addition of hydroxyl groups (7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene) resulted in 10-20 times more mutations than occurred after metabolic activation of benzo[a]pyrene. Benzo[a]pyrene 7,8-quinone was not intrinsically mutagenic to the bacteria and could not be metabolically activated. 9,10-Dihydrobenzo[a]pyrene was metabolized to mutagenic products to the same extent as benzo[a]pyrene, but derivatives that were totally saturated in the benzo ring (7,8,9,10-tetrahydrobenzo[a]pyrene and *trans*-7,8-dihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene) could not be activated. 4,5-Dihydrobenzo[a]pyrene, which cannot be epoxidized in the 4,5-position (K-region), was also nonmutagenic in the presence of the purified monooxygenase system. Epoxide hydrase, incubated together with the purified monooxygenase system reconstituted with 0.08 nmole of cytochrome P-448, almost completely deactivated the mutagenic products formed from 9,10-dihydrobenzo[a]pyrene, but reduced the mutation frequency induced by the metabolism of benzo[a]pyrene by only 30%. Evaluation of the intrinsic mutagenic activity of 7,8,9,10-tetrahydrobenzo[a]pyrene 4,5-oxide, benzo[a]pyrene 4,5-oxide, and pyrene 4,5-oxide indicated that saturation or removal of the benzo ring of benzo[a]pyrene 4,5-oxide markedly reduces the intrinsic mutagenicity of this K-region arene oxide.

INTRODUCTION

We have recently described the use of a highly purified monooxygenase system, consisting of cytochrome P-448 from 3-methylcholanthrene-treated rats, NADPH-cytochrome *c* reductase, phosphatidylcholine, and NADPH, to metabolically activate benzo[a]pyrene and some of its derivatives to products that are mutagenic to histidine-dependent strains of

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Salmonella typhimurium (1, 2). In the absence of epoxide hydrase, most of the mutations induced during metabolic activation of benzo[*a*]pyrene appeared to be due to the K-region oxide, BP 4,5-oxide.[3] In addition to benzo[*a*]pyrene, 12 phenols and four dihydrodiols of benzo[*a*]pyrene were utilized as substrates for the monooxygenase system. Of these 16 derivatives, only *trans*-BP 7,8-dihydrodiol was metabolically activated to a greater extent than was benzo[*a*]pyrene. This observation is consistent with the high carcinogenicity of *trans*-BP 7,8-dihydrodiol (3, 4) and its metabolic precursor, BP 7,8-oxide (5), and with studies showing that metabolites of *trans*-BP 7,8-dihydrodiol bind extensively to DNA (6). The high chemical reactivity (7, 8), ability to bind to nucleic acid (9–13), and cytotoxic and mutagenic activity (14–17) of the diastereometic 7,8-diol-9,10-epoxides of benzo[*a*]pyrene indicate that metabolic activation of the 7,8-dihydrodiol proceeds via epoxidation at the adjacent double bond in the 9,10-position (15, 18–20).

Since the BP 7,8-diol-9,10-epoxides and BP 4,5-oxide are highly mutagenic metabolites of benzo[*a*]pyrene, we have now selectively modified the benzo[*a*]pyrene molecule at the critical 4,5-, 7,8-, and 9,10-positions and studied the metabolic activation of these derivatives by the purified monooxygenase system. In addition to elucidating the structural and stereoisomeric requirements for the metabolic activation of benzo[*a*]pyrene, we have attempted to define further the role of epoxide hydrase in the activation and detoxification of this carcinogen.

[3] The abbreviations used are: BP, benzo[*a*]pyrene; BP 4,5-oxide, benzo[*a*]pyrene 4,5-oxide; *trans*-BP 7,8-dihydrodiol, *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene; *cis*-BP 7,8-dihydrodiol, *cis*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene; BP 7,8-diol-9,10-epoxide, either or both diastereomeric 9,10-epoxides of *trans*-BP 7,8-dihydrodiol in which the epoxide oxygen is either *cis* or *trans* to the benzylic 7-hydroxyl group; 7,8-H_2BP, 7,8-dihydrobenzo[*a*]pyrene; 9,10-H_2BP, 9,10-dihydrobenzo[*a*]pyrene; 4,5-H_2BP, 4,5-dihydrobenzo[*a*]pyrene; BP 7,8-quinone, benzo[*a*]pyrene 7,8-quinone; H_4-7,8-diol, *trans*-7,8-dihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; 7,8,-9,10-H_4BP, 7,8,9,10-tetrahydrobenzo[*a*]pyrene; 7,8,9,10-H_4BP 4,5-oxide, 7,8,9,10-tetrahydrobenzo[*a*]pyrene 4,5-oxide; 3-HOBP, 3-hydroxybenzo[*a*]pyrene; DMSO, dimethyl sulfoxide. All compounds referred to and used in this study are racemic where different enantiomers are possible.

MATERIALS AND METHODS

Materials. Strain TA98 of histidine-dependent *Salmonella typhimurium* (21) was obtained from Dr. B. Ames, University of California, Berkeley. Bacterial media were obtained from the Bioquest Division of Becton Dickinson, Cockeysville, Md. Petri dishes, reagent-grade inorganic chemicals, and acetone were obtained from Fisher Scientific; dilauroylphosphatidylcholine, from Serdary Research Laboratories, Ontario, Canada; and other biochemicals, from Sigma. DMSO, vacuum-distilled from calcium hydride, was stored under argon.

Synthesis of benzo[a]pyrene derivatives. All derivatives were stored in the solid state at −90° and dissolved just prior to use in either acetone, acetone–NH_4OH (1000:1), or DMSO, as indicated in the figure and table legends. Analytically pure samples of the *cis* (see synthetic route for *cis*-9,10-dihydrodiol in ref. 22)[4] and *trans* (22)[4] isomers of BP 7,8-dihydrodiol, *trans*-H_4-7,8-diol (22), 9,10-H_2BP (23), 7,8-H_2BP (23), 7,8,9,10-H_4BP (23), 4,5-H_2BP (24), BP 4,5-oxide (25), pyrene 4,5-oxide (25), and BP 7,8-quinone (20) were synthesized as described in the references cited.

Synthesis of 7,8,9,10-H_4BP 4,5-oxide was achieved by allowing 2 g of 7,8,9,10-H_4BP to react with 2 g of osmium tetroxide in 25 ml of benzene containing 1.3 ml of pyridine. After 1 week at room temperature, the dark precipitate of osmate ester was hydrolyzed with sodium bisulfite by the procedure of Baran (26) to provide 0.6 g of pale gray solid, which was homogeneous on thin-layer chromatography. This material was identified as *cis*-4,5-dihydroxy-4,5,7,8,9,10-hexahydro-BP after acetylation of a small portion with pyridine–acetic anhydride: mass spectrum, M^+ 374; NMR (100 MHz, $CDCl_3$), acetates at 2.01 and 2.05 Hz, H_4 and H_5 at 6.51 Hz, and 2 H_7 at 2.9 Hz and 2 H_{10} at 3.1 Hz as complex

[4] H. Yagi, V. Mahadevan, H. J. C. Yeh, D. T. Gibson, and D. M. Jerina, manuscript in preparation.

multiplets. The *ortho* ester route (25) was used to convert 200 mg of the diol to 160 mg of crude arene oxide, which was purified by thin-layer chromatography on Florisil (cycohexane–dioxane–triethylamine, 90:10:1) to provide 35 mg of 7,8,9,10-H_4BP 4,5-oxide: NMR (100 MHz, $CDCl_3$), oxirane hydrogens at 4.62 and 4.70 Hz with $J \approx 4$ Hz. The material was free of detecable impurities as determined by thin-layer chromatography, mass spectroscopy, and NMR spectroscopy.

Hepatic microsomal enzymes. NADPH–cytochrome *c* reductase (27) and epoxide hydrase (28) from phenobarbital-treated rats and cytochrome P-448 from 3-methylcholanthrene-treated rats (29) were purified and assayed as described in the indicated references. Reconstitution of the reductase and hemoprotein in the presence of appropriate amounts of phosphatidylcholine and NADPH results in a monooxygenase system capable of oxidatively metabolizing a number of lipophilic compounds such as steroids, drugs, and carcinogens (1, 2, 19, 20, 27, 29). Units of epoxide hydrase are defined as nanomoles of styrene glycol formed from styrene oxide per minute at 37°.[5]

Metabolic activation assay. Complete procedures for the metabolic activation of benzo[*a*]pyrene and a number of benzo[*a*]pyrene derivatives by the purified monooxygenase system and the detection of the mutagenic metabolites with histidine-dependent strains of *S. typhimurium* have been described in detail elsewhere (1, 2). Briefly, reaction mixtures of 0.5 ml contained 2.5 μmoles of sodium phosphate, 75 μmoles of sodium chloride, 0.08 μmole of phosphatidylcholine, 150 units of NADPH–cytochrome *c* reductase, various amounts of cytochrome P-448, 12.5 nmoles of benzo[*a*]pyrene or benzo[*a*]pyrene derivative, 2×10^8 bacteria of *S. typhimurium* strain TA98, and 0.1 μmole of NADPH. The final pH was 6.8, and the incubations were carried out for 5 min at 37°. The reaction was stopped by the addition of 9 nmoles of menadione, then 2 ml of molten top agar (45°), consisting of sodium chloride and agar (12 mg each), and 0.1 μmole each of L-histidine and biotin were added to the incubation mixture, and the entire contents of the culture tube (13 × 100 mm) were mixed and pured into a Petri dish containing 15 ml of Vogel-Bonner minimal medium with a 2% agar base. The Petri dishes were incubated in the dark at 37° for 2 days. During this time only those bacteria which were mutated from histidine dependence to histidine independence were capable of growth, and thus mutation frequency was assessed by counting the macroscopic colonies of bacteria on the Petri dishes. All experiments were performed in triplicate, and coefficients of variation of the colony counts rarely exceeded 15%. Comparisons of absolute mutation frequencies among experiments performed during different weeks showed somewhat larger variations, but the same relative activities among the derivatives were always observed, and all the data presented in a single figure or table were obtained from 1 day's experiment. Strain TA98 of *S. typhimurium* was selected as the tester strain for these studies because of its high sensitivity to mutation by polycyclic hydrocarbon metabolites (1, 21).

[5] In a previous publication (1) units of epoxide hydrase activity were defined as nanomoles of glycol formed per 15 min.

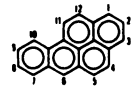
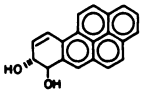
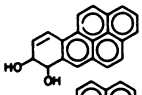
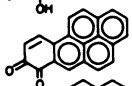
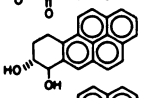
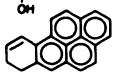
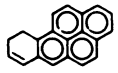
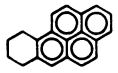
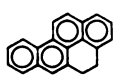
RESULTS

Metabolic activation of benzo[a]pyrene derivatives. Results of experiments that evaluated the metabolic activation of benzo[*a*]pyrene and eight benzo[*a*]pyrene derivatives with a highly purified, cytochrome P-448-dependent monooxygenase system in the absence of epoxide hydrase are summarized in Table 1. The derivatives that are metabolized to the most mutagenic metabolites express their activity at lower amounts of added cytochrome P-448, since this component of the monooxygenase system is rate-limiting under the assay conditions. The number of histidine revertants induced per plate without added hemoprotein is an assessment of the intrinsic mutagenic activity of the derivatives, because the monooxygenase system is inactive in the absence of the hemoprotein. Since the spontaneous muta-

TABLE 1

Metabolic activation of benzo[a]pyrene derivatives to products mutagenic to Salmonella typhimurium strain TA98

The monooxygenase system was reconstituted with the indicated amount of cytochrome P-448 from 3-methylcholanthrene-treated rats, 150 units of NADPH–cytochrome *c* reductase, and 0.08 μmole of phosphatidylcholine as described in MATERIALS AND METHODS. *trans*-and *cis*-BP 7,8-dihydrodiol and *trans*-H_4-7,8-diol were dissolved in acetone–NH_4OH, (1000:1) while the other derivatives and benzo[*a*]pyrene were dissolved in acetone. Separate experiments showed that background mutation frequencies and the extent of benzo[*a*]pyrene activation were the same with either solvent. The final concentration of all substrates was 25 μM. Reaction mixtures that contained no hemoprotein or substrate induced 20 revertants/plate, and this value has been subtracted from the data. Dashes indicate that assays were not performed.

Compound	Cytochrome P-448 (pmol) 0	5	10	20	80
	His$^+$ Revertants/plate ± S.E.				
 Benzo[a]pyrene (BP)	1 ± 0.6	34 ± 2	78 ± 6	144 ± 9	695 ± 21
 trans-BP 7,8-dihydrodiol	3 ± 1.5	518 ± 30	588 ± 6	294 ± 22	-
 cis-BP 7,8-dihydrodiol	10 ± 4	562 ± 17	824 ± 63	844 ± 35	-
 BP 7,8-quinone	0	3 ± 1	0	7 ± 2	5 ± 3
 H_4-7,8-diol	2 ± 1	6 ± 4	10 ± 3	9 ± 2	29 ± 6
 7,8-H_2BP	34 ± 4	358 ± 7	756 ± 11	862 ± 36	-
 9,10-H_2BP	17 ± 3	39 ± 2	64 ± 8	125 ± 16	640 ± 19
 7,8,9,10-H_4BP	6 ± 2	2 ± 1	1 ± 0.6	5 ± 3	18 ± 7
 4,5-H_2BP	2 ± 1	12 ± 4	24 ± 8	23 ± 4	33 ± 4

tion frequency, which has been subtracted from the data, was 20 revertants/plate, none of the derivatives was inherently capable of inducing a significant amount of histidine autotrophy in strain TA98 of *S. typhimurium*.

There was about a 1000-fold difference in the extent of metabolic activation of the nine compounds in the presence of the complete monooxygenase system. *cis*- and *trans*-BP 7,8-dihydrodiol and 7,8-H_2BP were activated to the greatest extent. A 5-min incubation with 5 pmoles of cytochrome P-448 from 3-methylcholanthrene-treated rats caused an 18–25-fold increase in mutation frequency over the spontaneous background rate. All three of these compounds have a nonaromatic double bond at the 9,10-position. Benzo[*a*]pyrene and 9,10-H_2BP, the next most active compounds, required about 8–10 times as much cytochrome P-448 to induce the same increase in mutation frequency. Neither H_4-7,8-diol or 7,8,9,10-H_4BP was metabolically activated to a mutagenic product, even in the presence of 80 pmoles of cytochrome P-448. Both compounds are completely saturated in the benzo ring. Oxi-

dation of the diol groups of BP 7,8-dihydrodiol to form BP 7,8-quinone, or saturation of the K-region 4,5-double bond of benzo[*a*]pyrene to form 4,5-H_2BP, resulted in markedly decreased metabolic activation.

In separate experiments, addition of highly purified epoxide hydrase to the monooxygenase system failed to activate any of the inactive derivatives. Thus we were unable to obtain any evidence for the activation of these inactive compounds via diol epoxides.[6]

Metabolic activation of highly active benzo ring derivatives. Reconstitution of the monooxygenase system with 5 pmoles of cytochrome P-448 resulted in the formation of mutagenic products at or near the top of the dose-response curve when *cis*- or *trans*-BP 7,8-dihydrodiol or 7,8-H_2BP was metabolized (Table 1). Under these conditions cell toxicity or death may obscure the absolute or relative mutagenic activity of a compound. In order to obtain better dose-response curves relating the number of mutations to the amount of hemoprotein in the monooxygenase system, these highly active benzo ring derivatives were metabolically activated with lower concentrations of cytochrome P-448 (Fig. 1). In a 5-min incubation the number of revertants induced by the metabolites of all the compounds increased in proportion to the cytochrome P-448 concentration. The products of *trans*-BP 7,8-dihydrodiol and 7,8-H_2BP were both 19 times as mutagenic as the products of BP, and the metabolites of *cis*-BP 7,8-dihydrodiol were 12 times as mutagenic as the BP metabolites. As little as 0.75 pmole of hemoprotein induced significant activation of all three benzo ring derivatives.

Effect of epoxide hydrase on metabolic activation of 9,10-H_2BP. We have previously reported (1, 2) that addition of various amounts of epoxide hydrase to the purified monooxygenase system resulted in a maximum 30–35% decrease in the number of mutations induced in strain TA98 of *S. typhimurium* when benzo[*a*]pyrene was the substrate. The stereoisomeric 7,8-diol-9,10-epoxides of benzo[*a*]pyrene are poor substrates for epoxide hydrase (1, 2, 17, 19), and formation of these highly mutagenic compounds may account for the incomplete deactivation of benzo[*a*]pyrene by epoxide hydrase. To examine this hypothesis, we utilized 9,10-H_2BP, which cannot form a 7,8-diol 9,10-epoxide, as a substrate for the monooxygenase system in the presence of various amounts of purified epoxide hydrase. In

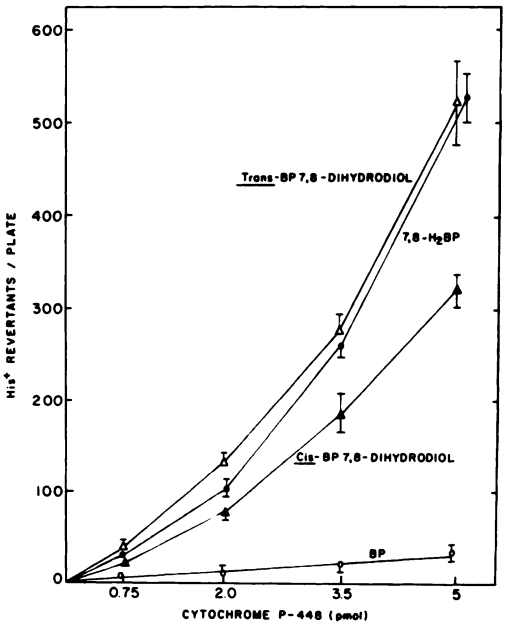


FIG. 1. *Metabolic activation of benzo ring derivatives of benzo[a]pyrene as a function of cytochrome P-448 concentration*

The monooxygenase system was reconstituted with the indicated amounts of cytochrome P-448 from 3-methylcholanthracene-treated rats and the other components as described under MATERIALS AND METHODS. Derivatives were dissolved in the solvents indicated 1. Background mutation frequencies, obtained by omitting the cytochrome from the otherwise complete incubation mixtures, did not exceed 29 revertants/plate and have been subtracted from the data. Values represent the means and standard errors of three replicate determinations.

[6] When the inactive derivatives were metabolized by rat liver microsomes or 9000 × g supernatant according to the pour plate procedure of Ames (21), 4,5-H_2BP, but not any of the other derivatives, was activated to mutagenic products. Although other explanations are possible we believe that the prolonged incubation time of the Ames procedure, as opposed to the 5 min. incubation time with the purified system, may have facilitated the formation of an active metabolite.

the absence of epoxide hydrase, benzo[a]pyrene and 9,10-H_2BP were nearly equally activated to mutagenic products by the purified monooxygenase system reconstituted with 80 pmoles of cytochrome P-448 (Fig. 2). However, when 9,10-H_2BP was the substrate, addition of 3.5 units of epoxide hydrase halved the mutation frequency, and addition of 10 units of enzyme resulted in greater than 85% inactivation of the mutagenic metabolites. In contrast to these results, addition of 10 units of epoxide hydrase to the monooxygenase system resulted in the deactivation of only 30% of the mutagenic products of benzo[a]-pyrene.

Inherent mutagenic activity of 4,5-oxides of benzo[a]pyrene, pyrene, and 7,8,9,10-tetrahydrobenzo[a]pyrene. BP 4,5-oxide is a potent mutagen in *S. typhimurium* strain TA98 (1, 2, 30). The inability of 7,8,9,10-H_4BP and H_4-7,8-diol to be metabolically activated to mutagenic products (Table 1) may have been the result of lack of formation of an arene oxide at the 4,5-position of these molecules. Alternatively, the arene oxides of the tetrahydrobenzo[a]pyrene derivatives may be nonmutagenic. In order to evaluate better the structure-activity relationships that occur on saturation of the benzo ring, we synthesized the 4,5-oxide of 7,8,9,10-H_4BP and compared its intrinsic mutagenic activity with that of BP 4,5-oxide and pyrene 4,5-oxide (Fig. 3). While 7,8,9,10-H_4BP 4,5-oxide and pyrene 4,5-oxide induced, respectively, 41 and 70 histidine revertants/nmole of derivative, BP 4,5-oxide induced

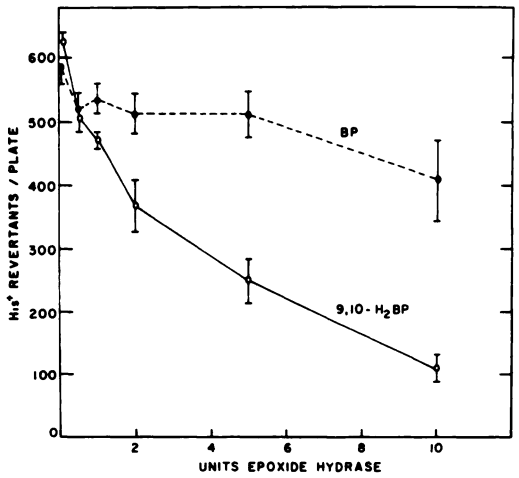


FIG. 2. *Effect of epoxide hydrase on metabolic activation of benzo[a]pyrene and 9,10-dihydrobenzo[a]pyrene by a purified rat cytochrome P-448 monooxygenase system*

The monooxygenase system was reconstituted as described in MATERIALS AND METHODS and the legend to Fig. 1, and the indicated amounts of purified expoxide hydrase were added immediately after the cytochrome P-448 and before the hydrocarbons. Final BP and 9,10-H_2BP concentrations were 25 μM, and both compounds were dissolved in acetone. In the absence of added cytochrome both compounds gave the same mutation frequency (27 revertants/plate), which has been subtracted from the data. Values are the means and standard errors of three replicate determinations.

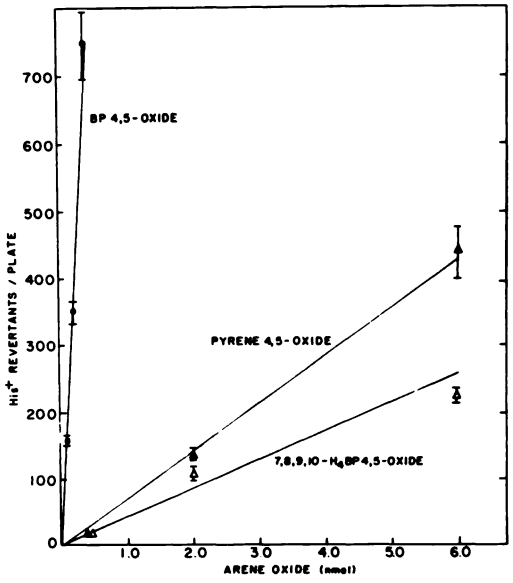


FIG. 3. *Reversion of Salmonella typhimurium strain TA98 to histidine autotrophy by 4,5-K-region arene oxides of benzo[a]pyrene, pyrene, and 7,8,9,10-tetrahydrobenzo[a]pyrene*

Each arene oxide was dissolved in and diluted with a solution of DMSO-NH_4OH (1000:1) so that the indicated amounts of compound were contained in 12.5 μl. Bacteria (2×10^8) were suspended in 0.5 ml of isotonic buffer (pH 7.0) consisting of 2.5 μmoles of sodium phosphate and 75 μmoles of sodium chloride. After addition of the compounds and incubation at 20° for 5 min, 2 ml of top agar were added and the entire contents of the culture tube were poured onto Petri dishes. An average of 20 revertants/plate was observed in incubations receiving solvent alone, and this value has been subtracted from the data, which are expressed as mean revertants per plate ± standard error of three replicates.

1600 revertants/nmole under the same experimental conditions. Thus 7,8,9,10-H_4BP 4,5-oxide is even less mutagenic than the 4,5-oxide of the noncarcinogen pyrene, and both compounds are substantially less mutagenic than BP 4,5-oxide.

DISCUSSION

Evaluation of the intrinsic mutagenic activity of more than 30 benzo[*a*]pyrene derivatives has indicated that the stereoisomeric BP 7,8-diol-9,10-epoxides and 9,10-epoxy-7,8,9,10-H_4BP are the most mutagenic compounds of this polycyclic hydrocarbon identified to date (7, 14–17, 30–32). Thus, of the eight benzo[*a*]pyrene derivatives used as substrates for the monooxygenase system in the present study, seven had modifications in the apparently critical 7-, 8-, 9-, and/or 10-positions of the molecule. In agreement with previous studies utilizing the purified monooxygenase system (1, 2), intact cultured cells (15), or a 9000 × *g* supernatant fraction of rat liver homogenate (33), *trans*-BP 7,8-dihydrodiol was metabolized to highly mutagenic products, presumably the diol epoxides. Although the *trans* isomer of BP 7,8-dihydrodiol is formed from hepatic metabolism of benzo[*a*]pyrene, it was of interest from considerations of structure-activity relationships to examine the metabolic activation of *cis*-BP 7,8-dihydrodiol, which is formed by certain microorganisms (22). It is apparent from the high mutagenicity of the products of *cis*-7,8-dihydrodiol metabolism (Table 1 and Fig. 1) that the relative configuration of the hydroxyl groups is of limited importance in activating BP 7,8-dihydrodiol. However, oxidation of the 7- and 8-hydroxyl groups to form BP 7,8-quinone resulted in a compound that was not metabolized to mutagenic products, despite the retention of a double bond in the 9,10-position of the molecule. BP 7,8-quinone is also completely inactive as an initiator of skin tumors on CD-1 mice under conditions that result in 90–100% tumor incidence when benzo[*a*]pyrene or *trans*-BP 7,8-dihydrodiol is the initiator.[7] The products of 7,8-H_2BP metabolism were considerably more mutagenic than the products of 9,10-H_2BP metabolism, and were comparable in mutagenic activity to the metabolic products of the BP 7,8-dihydrodiols. This latter observation is further confirmation that the hydroxyl groups in the positions 7 and 8 are not essential for activation of the hydrocarbon. Dihydrodiol formation or reduction at positions 7 and 8 of BP saturates the 7,8-double bond so that subsequent oxidation at the 9,10-position of the molecule yields an aliphatic epoxide rather than an arene oxide. Aliphatic epoxides and K-region arene oxides are more susceptible to nucleophilic attack compared with other solvolytic reactions than are their non-arene oxide counterparts (34). Thus aliphatic epoxides and K-region arene oxides are anticipated to be more effective alkylating agents toward cellular constituents. The mutagenic activity of 9,10-epoxy-7,8,9,10-H_4BP is comparable to that of the BP 7,8-diol-9,10-epoxides and is at least two orders of magnitude greater than the mutagenic activity of BP 9,10-oxide (17, 31, 32). The unique structural feature of BP 7,8-dihydrodiol and 7,8-H_2BP is the presence of an isolated double bond in the 9,10-position of the benzo ring, which can be oxidized by the monooxygenase system to an aliphatic epoxide. Quantum mechanical calculations predict that epoxides that form part of a bay region[8] on a saturated angular benzo ring of benzo[*a*]pyrene are more reactive than their non-bay region counterparts (32). 9,10-epoxy-7,8,9,10-H_4BP is at least 9 times more mutagenic than 7,8-epoxy-7,8,9,10-H_4BP in *S. typhimurium* strain TA98 (17), a result that agrees well with the present studies on the metabolic activation of 7,8-H_2BP relative to 9,10-H_2BP and the calculated predictions.

In the absence of epoxide hydrase, benzo[*a*]pyrene is metabolized only to ar-

[7] Unpublished observations from these laboratories.

[8] A bay region in a polycyclic aromatic hydrocarbon exists when two nonadjacent benzene rings, one 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 (Table 1) is a bay region.

ene oxides, phenols, and quinones by the purified monooxygenase system. Metabolism of the parent hydrocarbon to products mutagenic to *S. typhimurium* therefore cannot be attributed to the formation of the diol epoxides. Of 21 known and potential primary oxidative metabolites of BP that have been tested for mutagenicity in *S. typhimurium,* only BP 4,5-oxide is a potent mutagen (32). Thus BP 4,5-oxide probably accounts for most of the mutations induced when BP is metabolically activated by the purified monooxygenase system in the absence of epoxide hydrase. A small number of mutations (fewer than 20% of the total mutations observed) may be due to the other primary oxidative metabolites of BP, particularly 6-HOBP. The modest increase in mutation frequency when 4,5-H_2BP, which cannot form an arene oxide in the 4,5-position, was metabolized by the purified monooxygenase system provides further evidence for the importance of BP 4,5-oxide formation with respect to mutagenic activity. Interestingly, BP 4,5-oxide is a poor complete carcinogen (5) or tumor initiator (35) on mouse skin.

Neither H_4-7,8-diol or 7,8,9,10-H_4BP was metabolically activated to a mutagenic product, even in the presence of 80 pmoles of cytochrome P-448. Since both compounds are saturated in the benzo ring, neither can form mutagenic benzo ring epoxides. Both compounds could, however, be metabolized to an arene oxide at the 4,5-position. Such an arene oxide need not be as highly mutagenic as BP 4,5-oxide (Fig. 3). Saturation of the benzo ring produces a molecule more similar in electronic structure to the noncarcinogen pyrene. The low mutagenic activity of 7,8,9,10-H_4BP and its metabolites is consistent with the inability of 7,8,9,10-H_4BP to induce leukemia in DBA/2 mice under conditions in which benzo[a]pyrene is active (36).

We have previously reported (1, 2) that addition of various amounts of epoxide hydrase to a purified monooxygenase system reconstituted with 100 pmoles of cytochrome P-448 resulted in a maximum 30–35% decrease in the number of mutations induced in *S. typhimurium* strain TA98 when BP was the substrate. BP 4,5-oxide, the primary oxidative metabolite most mutagenic to strain TA98, is totally deactivated to nonmutagenic metabolites by relatively small amounts of epoxide hydrase, whereas both of the highly mutagenic diastereomeric BP 7,8-diol-9,10-epoxides, which are dependent on epoxide hydrase for their formation, are resistant to deactivation by epoxide hydrase (1, 2, 17). Therefore we postulated that the partial deactivation of BP metabolites observed when mutation frequency was plotted against the amount of epoxide hydrase added to thz monooxygenase system represented a composite of BP 4,5-oxide deactivation and activation of BP 7,8-oxide to the diol epoxides via BP 7,8-dihydrodiol. This hypothesis is supported by the present studies with 9,10-H_2BP, in which the benzo[a]pyrene molecule was selectively protonated so that a diol epoxide could not form in the benzo ring. If the failure of epoxide hydrase to completely block the metabolic activation of benzo[a]pyrene results from the formation of the stereoisomeric 7,8-diol-9,10-epoxides, a BP derivative that cannot form a 7,8-diol-9,10-epoxide should be deactivated by epoxide hydrase. As shown in Fig. 2, epoxide hydrase was relatively efficient in deactivating the metabolites of 9,10-H_2BP. 9,10-H_2BP presumably is activated via epoxidation at the 4,5- and/or 7,8-positions of the molecule, and both BP 4,5-oxide and 7,8-epoxy-7,8,9,10-H_4BP are readily deactivated by epoxide hydrase (1, 2, 17). Metabolic studies and mutagenesis experiments in the presence of epoxide hydrase indicate that diol epoxides of both benzo[a]pyrene (1, 2, 17, 19) and benz[a]anthracene (37) are very poor substrates for the enzyme. Since the corresponding tetrahydroepoxides of both hydrocarbons are metabolized by epoxide hydrase, one can conclude that the presence of hydroxyl groups in diol epoxides has a marked inhibitory effect on the action of epoxide hydrase.

The present results emphasize the complex interrelationship of the two microsomal enzyme systems, the cytochrome-dependent monooxygenase system and

epoxide hydrase, which are primarily responsible for the metabolic activation and detoxification of benzo[*a*]pyrene. These studies also indicate that specific and apparently subtle changes in the structure of benzo[*a*]pyrene have important effects on the extent to which the molecule is metabolically activated and provide additional information on the pathways of benzo[*a*]pyrene metabolism that result in mutagenic products.

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