An Enantiomeric Interaction in the Metabolism and Tumorogenicity of (±)- and (−)-Benzo[α]pyrene 7,8-Oxide*

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(Received for publication, April 1, 1980)

The (±)- and (−)-enantiomers of benzo[α]pyrene 7,8-oxide are hydrated stereospecifically at C-8 to (−)- and (±)-trans-7,8-dihydroxy-7,8-dihydrobenzo[α]pyrene, respectively, by rat hepatic epoxide hydrolase. The (−)-enantiomer of benzo[α]pyrene 7,8-oxide is metabolized by microsomal epoxide hydrolase at a rate 5- to 4-fold greater than the (±)-enantiomer. At low conversion of racemic substrate, however, benzo[α]pyrene 7,8-oxide is metabolized to the dihydrodiol at a rate equal to that of the (−)-enantiomer. An analysis of the enantiomeric composition of the dihydrodiol formed from the racemic substrate revealed preferential formation of (−)-trans-7,8-dihydroxy-7,8-dihydrobenzo[α]pyrene. At low substrate conversion (<20% metabolism), the enantiomeric purity of the dihydrodiol was much higher than at high substrate conversion (>50% metabolism). Similar results were obtained with microsomes from hamster, rabbit, guinea pig, mouse, and human liver. These results indicate that epoxide hydrolase has a higher affinity for (+)-benzo[α]pyrene 7,8-oxide than for the (−)-enantiomer. The kinetics of hydration of (+)- and (−)-benzo[α]pyrene 7,8-oxide by purified epoxide hydrolase in detergent solution showed that (+)- and (−)-enantiomers to have apparent $K_v$ values of 1.7 and ±20 μM, respectively. Tumorigenicity studies with benzo[α]pyrene 7,8-oxide on mouse skin and in newborn mice revealed that (−)-benzo[α]pyrene 7,8-oxide, the metabolic precursor of the more tumorigenic (−)-7,8-dihydrodiol, is significantly more tumorigenic than the (−)-enantiomer. However, racemic benzo[α]pyrene 7,8-oxide was more tumorigenic than either enantiomer alone, indicating an enantiomeric synergism in the carcinogenicity of benzo[α]pyrene 7,8-oxide. The data are discussed in relation to the complete sequence of metabolic pathways leading to an ultimate carcinogen from benzo[α]pyrene.

The influence of stereochemical factors in the manifestation of biological activity of isomeric molecules is well known. When optical enantiomers interact with chiral macromolecules, complexes with different physical and chemical properties are formed. Thus, stereoselective interaction of chemicals with chiral enzymes, nucleic acids, receptors, etc. may be expected to influence biological responses in a number of ways. Recent studies have demonstrated marked differences

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1 As suggested by Jenner and Testa (2), we use the term "stereoselectivity" to indicate the preferential but not complete predominance of one stereoisomer over the others in metabolic processes; "stereospecificity" is used to indicate an apparently complete stereoselectivity.

2 Percent enantiomeric purity is defined as (nanomoles of major enantiomer minus nanomoles of minor enantiomer) divided by total nanomoles times 100.

MATERIALS AND METHODS

Chemicals—Racemic trans-BP 7,8-dihydrodiol (13), its (+)- and (−)-enantiomers (7), racemic BP 7,8-oxide (14), and its (+)- and (−)-
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(+)- BENZO[a]PYRENE [7R,8S]-OXIDE

(-)- BENZO[a]PYRENE [7S,8R]-OXIDE

FIG. 1. Structures and absolute stereochemistry of (+)- and (-)-BP 7,8-oxide.

enantiomers (11, 12) were synthesized as described. Briefly, the (+)- and (-)-dihydriodiol were obtained by chromatographic resolution of their noncarcinogenic precursor, (±)-trans-7,8-dihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene, as its diastereomeric bis-esters with MTPA (Aldrich Chemical Co., Milwaukee, Wis.). Chromatographic resolution of (±)-trans-7,8-dihydroxy-8-bromo-7,8,9,10-tetrahydrobenzo[a]pyrene as its diastereomeric esters with (−)-menthol-oxycetic acid (Aldrich Chemical Co., Milwaukee, Wis.) allowed preparation of the (+)- and (−)-enantiomers. The samples of BP 7,8-dihydriodiol and BP 7,8-oxide used in this study were free of impurities as judged by thin and chromatography, and the (+)- and (−)-enantiomers of each compound were greater than 98% enantiomerically pure. Syntheses of [(3-H]-(+)-phenanthrene 9,10-oxide and [(3-H]-(-)-BP 7,8-oxide have been reported (15). 12-O-Tetradecanoylphorbol-13-acetate was purchased from Dr. Peter Borchert, Chemical Carcinogenesis, Eden Prairie, Minn. Me80 was vacuum-distilled from calcium hydride and stored under an atmosphere of argon. The detergent Brij 35 was obtained from Sigma Chemical Co., St. Louis, Mo.

Enzyme Assays—Immature male rats (50 to 60 g) of the Long-Evans strain (Blue Spruce Farms, Altonam, N. Y.) were treated with phenobarbital (75 mg/kg/day, intraperitoneally) or 3-methylcholanthrene (25 mg/kg/day, intraperitoneally) for 4 days prior to killing. Microsomes from control, 3-methylcholanthrene- and phenobarbital (75 mg/kg/day, intraperitoneally) or 3-methylcholanthrene-pretreated rats were prepared as previously described (16) and were stored at −90°C. Epoxide hydrolase was purified to apparent homogeneity as described (17).

Metabolism of BP 7,8-oxide—BP 7,8-oxide (8.5 µCi/µmol) and [3-H]-BP 7,8-oxide (6.3 µCi/µmol) was measured as previ-ously described (18). Rates of hydration of unlabeled racemic BP 7,8-oxide to BP 7,8-dihydrodiol by microsomal epoxide hydrolase were followed at 381 nm on a Beckman Acta V spectrophotometer thermostated at 25°C. Reactions were carried out in 3.0 ml of 50 mM Tris, 100 mM NaCl (pH 8.5 at 25°C) containing 20 mM Brij 35. Substrates were added in 10 µl of tetrahydrofuran:NH4OH (1000:1) to a final concentration of 6 to 8 µM. Reactions were initiated by addition of 25 to 50 µl of purified epoxide hydrolase solution. The catalytic rate constant, kcat, and apparent KM (Kmapp) were obtained by fitting the time course data to the appropriate integrated rate expression.

Determination of the Enantiomeric Purity of Metabolically Formed BP 7,8-Dihydrodiol—BP 7,8-oxide, 800 nmol in 40 µl of tetrahydrofuran:NH4OH (1000:1), was incubated with 2.5 mg of microsomal protein from phenobarbital-pretreated rats and 0.30 mmol of Tris-HCl buffer (pH 8.9 at 37°C) in a final volume of 2.0 ml for 1 or 10 min at 37°C. Reactions were terminated by the addition of 6 ml of ethyl acetate. The samples were blended on a Vortex mixer for 1 min and centrifuged. The organic phase was removed, dried with 5 g of sodium sulfate, and evaporated to dryness under N2 after addition of an ethyl acetate wash of the sodium sulfate. Separation of BP 7,8-dihydriodiol from the incubation mixture, as well as formation and analytical separation of its bis-MTPA diesters by high pressure liquid chromatography were as previously described (17, 18). Extent of dihydriodiol formation was quantified by absorbance at 367 nm in methanol (ε = 50,500 M−1 cm−1) or radiochemically, and enantiomeric purity was established from the areas of the diastereomeric bis-MTPA peaks upon high pressure liquid chromatography as well as radiochemically when possible.

Tumor-initiating Activity of BP 7,8-Oxide on Mouse Skin—Female CD-1 mice, 7- to 8-weeks-old, were obtained from Charles River Mouse Farms, North Wilmington, Mass. After an equilibration period of 1 week, the mice (30 animals/group) were anesthetized with ether and shaved, and on the dorsal surface with electric clippers 3 days prior to treatment. BP 7,8-oxide was dissolved in acetone:NH4OH (1000:1), and 200 µl of the solution was applied once to the dorsal surface of the mice. Control mice received only solvent. All manipulations of BP 7,8-oxide were performed under subdued light, and the animals were kept in the dark for 24 h following application of the compound. Mice received twice weekly applications of 12-0-tetradecanoylphorbol-13-acetate (16 nmol/200 µl of acetone) beginning 7 days after treatment with BP 7,8-oxide or solvent. The development of skin papillomas was recorded once every 2 to 3 weeks, and papillomas greater than 2 mm in diameter were included in the cumulative total if they persisted for 2 weeks or longer.

Tumorigenesis of BP 7,8-Oxide in Neoborn Mice—Pregnant mice of the Swiss-Webster (BLU:Ha (ICR)) strain were obtained from Blue Spruce Farms, Altonam, N. Y., 1 to 7 days before parturition. Within 24 h of birth, 10 pups in each litter were injected intraperitoneally with the first dose of compound. BP 7,8-oxide was injected in Me80:NH4OH (1000:1) at a concentration of 100 nmol/5 µl. The mice were given a total dose of 700 nmol of BP 7,8-oxide divided into three injections of 100, 200, and 400 nmol administered on the 1st, 8th, and 15th day of life, respectively. Control mice were injected with solvent. The mice were weighed weekly at 25 days of age, and the experiment was terminated by killing the animals when they were 31 to 35 weeks of age. At autopsy, the major organs of each animal were examined grossly, tumors were counted, and tissues were fixed in 10% buffered formalin. A representative number of pulmonary tumors, all hepatic tumors, and all other tissues with suspected pathology were examined histologically. Pathology of the lung tumors was the same as has been previously described (19). Liver tumors were characterized as neoplastic nodules (20).

RESULTS

Metabolism of (+)- and (-)-BP 7,8-Oxide by Microsomal Epoxide Hydrolase—Formation of BP 7,8-dihydriodiol from (+)- and (-)-BP 7,8-oxide by rat hepatic microsomal epoxide hydrolase was proportional to enzyme concentration (Fig. 2). The (+)-enantiomer was metabolized at a rate approximately 4 times greater than the (+)-enantiomer. When racemic 7,8-oxide was incubated with hepatic microsomes however, metabolism occurred at a rate equal to the (+)-enantiomer when less than 20% of the substrate was converted to product. The rate of metabolism of unlabeled racemic BP 7,8-oxide (6.3 nmol/min/mg of protein) with the newly developed fluoro-metric assay was comparable to values previously obtained with a radiometric assay (18). Table I summarizes the results of studies with hepatic microsomes from control and phenobarbital-pretreated rats.

Since racemic BP 7,8-oxide was metabolized at a rate equal...
to the (+)-enantiomer, a large difference in the affinity of the enzyme for the (+)- and (−)-enantiomers was suggested, i.e., a higher affinity for the (+)-enantiomer. This was verified in experiments where (+)- and (−)-BP 7,8-oxide were used as inhibitors of the hydration of [3-3H]phenanthrene 9,10-oxide (Fig. 3). At a concentration of 375 μM phenanthrene 9,10-oxide, 37 μM (+)-BP 7,8-oxide resulted in a 50% inhibition in the formation of phenanthrene 9,10-dihydrodiol while (−)-BP 7,8-oxide had no detectable inhibitory activity at a final concentration up to 190 μM. When the concentration of [3-3H]phenanthrene 9,10-oxide was reduced to 125 μM, (−)-BP 7,8-oxide caused a 35% inhibition of enzymatic activity at a 190 μM concentration. This same percentage inhibition of phenanthrene 9,10-oxide hydration was obtained with 15 μM (+)-BP 7,8-oxide, indicating at least a 12-fold difference in the ability of the two enantiomers to inhibit the hydration of phenanthrene 9,10-oxide.

Hydration of BP 7,8-oxide by purified epoxide hydrolase can be followed spectrophotometrically at 381 nm (Δε = −13,000 M⁻¹ cm⁻¹) as shown in Fig. 4. Complications from the competing spontaneous isomerization of the substrate were avoided by monitoring the reaction at an isosbestic point for the isomerization (381 nm) and by the use of high enzyme concentrations. BP 7,8-oxide isomerizes spontaneously to phenol (monitored at 344 nm) with a half-life of 52.7 min under the conditions employed. Thus, the spontaneous isomerization occurs 16 times more slowly than the slowest enzyme-catalyzed reaction, resulting in ≥95% recovery of BP 7,8-dihydrodiol based on starting oxide. The enzyme-catalyzed hydration of the (+)-enantiomer in detergent solution followed Michaelis-Menten kinetics with a $K_{mapp} = 1.7$ μM and $k_2 = 0.10$ s⁻¹ (Fig. 4A). In contrast, the hydration of the (−)-enantiomer followed first order kinetics under the conditions employed, suggesting that $K_{mapp} > [S]$ (Fig. 4B). This kinetic behavior allows a lower limit of $K_{mapp}$ for the (−)-enantiomer to be set at ≥20 μM. Furthermore, a ratio of the second order

![Fig. 2. Effect of microsomal protein concentration on the rate of (+)-, (−)-, and (±)-BP 7,8-oxide hydration. Hepatic microsomes from untreated rats were incubated with 190 μM BP 7,8-oxide; ▲, (+)-BP 7,8-oxide; □, (±)-BP 7,8-oxide.

![Fig. 3. Metabolism of [3-3H]phenanthrene 9,10-oxide in the presence of various concentrations of (+)- and (−)-BP 7,8-oxide. Hepatic microsomes from untreated rats (10 μg of protein) were incubated with 375 μM [3-3H]phenanthrene 9,10-oxide (left) or 125 μM [3-3H]phenanthrene 9,10-oxide (right) in the presence of the indicated concentrations of BP 7,8-oxide for 2 min. Formation of [3-3H]phenanthrene 9,10-dihydrodiol was quantitated as described under “Materials and Methods.” ▲, (−)-BP 7,8-oxide; □, (±)-BP 7,8-oxide.]
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Fig. 4. Time courses of the hydration of the (+)- and (-)-benzo[a]pyrene BP 7,8-oxide catalyzed by purified epoxide hydrolase. A, (+)-benzo[a]pyrene 7,8-oxide (7.25 μM) was incubated with 0.25 μM enzyme. The trace was started 25 s after start of the reaction. The inset shows a fit of the data to a linear form of the integrated Michaelis-Menten equation, \[ \frac{[P]_t}{[P]_0} = \frac{[S]_0/[S]/t}{t+V_{max}} \] Linear regression analysis gave a slope of -1.7 μM = -K_a and an intercept of 0.042 s⁻¹ or k = 0.10 s with a correlation coefficient of 0.990. B, (-)-benzo[a]pyrene 7,8-oxide (6.29 μM) was incubated with 0.49 μM enzyme. The trace was started after a 50 s delay. The inset shows a fit of the data to the integrated first order rate expression, \[ \ln([S]) = -k_{cat} + \ln([S]) \] Linear regression analysis gave a slope of -0.0033 s⁻¹, an intercept of 1.74 and a correlation coefficient of 0.997. A value of (k_{cat}/K_m) = 6.7 x 10⁻³ s⁻¹ μM⁻¹ can be calculated from k_{cat}.

rate constants (k_{cat}/K_m) for the two enantiomers (k_{cat}/K_m)⁺/ (k_{cat}/K_m)⁻ = 15 can be calculated. Since the catalytic rate constant for the hydration of (-)-BP 7,8-oxide is greater than that for (+)-BP 7,8-oxide (Fig. 2), the K_m for the (+)-enantiomer must be >15 times the K_m for the (-)-enantiomer. Thus, the kinetic results obtained with purified epoxide hydrolase in detergent solution, which have previously been proposed to mimic microsomal kinetic conditions (21), are consistent with the above results obtained with microsomal suspensions.

Enantiomeric Purity of BP 7,8-Dihydriodiol Formed from BP 7,8-Oxide—The enantiomeric purity of BP 7,8-dihydriodiol formed on hydration of (+)- and (-)-BP 7,8-oxide by liver microsomal epoxide hydrolase is shown in Fig. 5. Enantiomeric purity of the metabolically formed BP 7,8-dihydriodiol was determined by separation of the two diastereomers produced when the dihydriodiol was converted to diesters with (-)-MTPA as described (7, 8). When BP 7,8-oxide was incubated with microsomal epoxide hydrolase, the (-)-[7S,8R]-enantiomer was converted to enantiomerically pure (+)-BP 7,8-dihydriodiol and the (+)-enantiomer was metabolized exclusively to (-)-BP-[7R,8S]-dihydriodiol (Fig. 5). These findings are consistent with attack by solvent water at C-8 as previously determined by [¹⁸O]water studies (8, 10) and are similar to the enzymatic hydration of naphthalene 1,2-oxide (22). Thus, racemic BP 7,8-oxide should have been converted to racemic BP 7,8-dihydriodiol if both enantiomers of BP 7,8-oxide were equally good substrates for the enzyme. However, the results presented in Figs. 2 and 3 indicate that there was a marked enantiomeric preference in the metabolism of BP 7,8-oxide. Determination of the enantiomeric purity of metabolically formed BP 7,8-dihydriodiol from various mixtures of (+)- and (-)-BP 7,8-oxide confirmed this observation (Fig. 6). When different proportions of (+)- and (-)-BP 7,8-oxide were incubated with rat liver microsomes under conditions of less than 25% metabolic conversion of substrate (1-min incubation), the (+)-enantiomer of BP 7,8-oxide was preferentially metabolized. With these incubation conditions, racemic BP 7,8-oxide was converted to 85% (-)-BP 7,8-dihydriodiol and 15% (+)-BP 7,8-dihydriodiol (70% optically pure (-)-enantiomer). When incubation times were extended to 10 min (>50% metabolic conversion of substrate), the enantiomeric...
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dihydrodiol is from 5- to 20-fold more tumorigenic on mouse skin (3) and in newborn mice (4) than the (+)-enantiomer because of the stereoselective metabolism of (-)-BP 7,8-dihydrodiol to (+)-7,8-8a-dihydroxy-9a,10a-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (7, 27, 28). An examination of the tumorigenic activity of the (+) and (-)-enantiomers of BP 7,8-oxide revealed that (+)-BP 7,8-oxide is significantly more tumorigenic than the (-)-enantiomer on mouse skin and in newborn mice (Tables III and IV). When 100 to 400 nmol of BP 7,8-oxide was applied to the skin of CD-1 mice followed by twice-weekly applications of the tumor promoter 12-O-tetradecanoylphorbol-13-acetate for 25 weeks, (+)-BP 7,8-oxide was at least 2- to 7-fold more active as a tumor initiator than the (-)-enantiomer. In newborn mice which had received three intraperitoneal injections of BP 7,8-oxide during the first 15 days of life, (+)-BP 7,8-oxide was at least 10-fold more active than the (-)-enantiomer in producing lung adenomas when the data were expressed as average number of lung tumors/mouse (Table IV). (+)-BP 7,8-oxide also appeared to induce a higher number of hepatic tumors than the (-)-

![Graphical representation of metabolites formation](image)

**Fig. 6.** Metabolic formation of (+) and (-)-BP 7,8-dihydrodiol from BP 7,8-oxide. Various proportions of (+)- and (-)-BP 7,8-oxide were obtained by mixing the pure enantiomers. Hepatic microsomes (2.5 mg of protein) from phenobarbital-pretreated rats were incubated with 0.33 mmol of Tris-HCl (pH 8.9 at 37°C) and 800 nmol of BP 7,8-oxide in a total volume of 2.0 ml for 1 min (A) or 10 min (B). The solid line represents the theoretical ratio of (+) and (-)-BP 7,8-dihydrodiol formed from various mixtures of (+) and (-)-BP 7,8-oxide, assuming that both enantiomers are metabolized equally by epoxide hydrolase. The enantiomeric purity of BP 7,8-dihydrodiol formed from pure (+)- and (-)-BP 7,8-oxide was determined at both 1 and 10 min of incubation.

purity of (-)-BP 7,8-dihydrodiol decreased, i.e. racemic BP 7,8-oxide was converted to 62% (-)-BP 7,8-dihydrodiol and 38% (+)-BP 7,8-dihydrodiol (Fig. 6). These results are consistent with a higher affinity of epoxide hydrolase for (+)-BP 7,8-oxide compared to the (-)-enantiomer, resulting in the preferential metabolism of the (+)-enantiomer under conditions of saturating substrate. When metabolic conversion of total substrate approaches 50%, the (+)-enantiomer is then metabolized to (+)-BP 7,8-dihydrodiol and the resulting BP 7,8-dihydrodiol has lower enantiomeric purity.

Since hepatic epoxide hydrolase from rabbit, hamster, guinea pig, and human have different antigenic properties than the rat enzyme (23), we investigated the metabolism of racemic BP 7,8-oxide to BP 7,8-dihydrodiol by various species. Mouse liver epoxide hydrolase, which is antigenically identical to the rat enzyme (23), as well as enzyme from hamster, rabbit, guinea pig, and human, all formed (-)-BP 7,8-dihydrodiol of high enantiomeric purity when less than 30% of the substrate was consumed in the reaction (Table II). However, in all cases investigated, high conversion of racemic BP 7,8-oxide (>50% metabolism) resulted in formation of (-)-BP 7,8-dihydrodiol of significantly lower optical purity. These results suggest that various enzyme forms of epoxide hydrolase have a similar enantiomeric preference for metabolism of (+)-BP 7,8-oxide compared to the (-)-enantiomer.

### Table II

Metabolism of (+)BP 7,8-oxide by different species

| Source of microsomes | Per cent metabolism of substrate | BP 7,8-dihydrodiol formed |
|----------------------|---------------------------------|--------------------------|
| Rat liver            | 19                              | 92                       |
| Mouse liver          | 19                              | 96                       |
| Hamster liver        | 17                              | 91                       |
| Rabbit liver         | 26                              | 91                       |
| Guinea pig liver     | 26                              | 91                       |
| Human liver          | 10                              | 90                       |
| Human lung           | 55                              | 79                       |

### Table III

Tumor initiating activity of BP 7,8-oxide on mouse skin

The indicated dose of BP 7,8-oxide was applied once in 200 µl of acetone-NH₄OH (1000:1). Seven days later, 12-O-tetradecanoylphorbol-13-acetate was applied twice weekly (16 nmol/200 µl of acetone) for 25 weeks. Control mice were treated with solvent. Each treatment group consisted of 30 mice.

| Experiment | Compound | Dose (nmol) | Per cent of mice with tumors | Average number of tumors/mouse |
|------------|----------|-------------|------------------------------|-------------------------------|
| 1          | None     | 0           | 7                           | 0.07                          |
|            | (+)-BP 7,8-oxide | 100        | 11                          | 0.11                          |
|            | (+)-BP 7,8-oxide | 100        | 38                          | 0.76                          |
|            | (+)-BP 7,8-oxide | 100        | 67                          | 1.48                          |
| 2          | None     | 0           | 0                           | 0.11                          |
|            | (+)-BP 7,8-oxide | 100        | 11                          | 0.54                          |
|            | (+)-BP 7,8-oxide | 100        | 18                          | 0.54                          |
|            | (+)-BP 7,8-oxide | 100        | 55                          | 1.03                          |
|            | (+)-BP 7,8-oxide | 100        | 50                          | 0.83                          |
|            | (+)-BP 7,8-oxide | 100        | 60                          | 1.67                          |
Swiss-Webster mice (80 animals/group) were given intraperitoneal injections of 100 nmol, 200 nmol, and 400 nmol of BP 7,8-oxide on the 1st, 8th, and 15th day of life, respectively. The animals were weaned at 25 days of age and the experiment was terminated when the animals were 31 to 35 weeks of age.

| Compound          | Per cent survival (31-35 wks) | Sex | Per cent of mice with pulmonary tumors | Average number of tumors/mouse | Per cent of male mice with hepatic tumors | Average number of tumors/mouse |
|-------------------|-------------------------------|-----|----------------------------------------|--------------------------------|------------------------------------------|-----------------------------|
| None              | F                             | 12  | 0.16                                   |                                | 0                                         |                             |
|                   | M                             | 14  | 0.15                                   |                                |                                          |                             |
|                   | Total                         | 13  | 0.14                                   |                                |                                          |                             |
| (-)-BP 7,8-oxide  | F                             | 21  | 0.33                                   |                                | 3                                         | 0.03                        |
|                   | M                             | 20  | 0.20                                   |                                |                                          |                             |
|                   | Total                         | 20  | 0.25                                   |                                |                                          |                             |
| (+)-BP 7,8-oxide  | F                             | 86  | 1.82                                   |                                | 7                                         | 0.15                        |
|                   | M                             | 83  | 2.57                                   |                                |                                          |                             |
|                   | Total                         | 84  | 2.28                                   |                                |                                          |                             |
| (±)-BP 7,8-oxide  | F                             | 93  | 3.89                                   |                                | 14                                        | 0.19                        |
|                   | M                             | 86  | 3.39                                   |                                |                                          |                             |
|                   | Total                         | 89  | 3.60                                   |                                |                                          |                             |

FIG. 7. Formation and absolute stereochemistry of benzo[a]pyrene metabolites responsible for the carcinogenicity of the parent hydrocarbon. Solid arrows indicate major metabolic pathways. (+)-BP 7,8-oxide [7R,8S]; (-)-BP 7,8-dihydriodiol [7R,8R]; (+)-BP 7,8-diol-9,10-epoxide-2 [7R,8S,9S,10R]; (-)-BP 7,8-diol-9,10-epoxide-1 [7R,8S,9R,10S]. Corresponding enantiomeric forms of these compounds are also shown.

enantiomer although these values are probably too low for any meaningful comparisons. Since (+)-BP 7,8-oxide is converted exclusively to the more tumorigenic (-)-enantiomer of BP 7,8-dihydriodiol (Figs. 5 and 6), these results were expected. However, in both tumor models, racemic BP 7,8-oxide produced a higher tumor incidence than either optical enantiomer. Without any enantiomeric interaction, the tumorigenic activity of racemic BP 7,8-oxide should have been intermediate to the activity of the (+)- and (-)-enantiomers. This is not the case, and the above results indicate that there is a synergistic interaction in the tumorigenicity of the optical enantiomers of BP 7,8-oxide.

**DISCUSSION**

Although stereochemical factors have long been known to be involved in the metabolism and expression of biological activity of isomeric molecules, only recently has the importance of these factors been demonstrated in the expression of mutagenicity and carcinogenicity. For the well studied polycyclic aromatic hydrocarbon BP, marked differences in the metabolism, mutagenicity and carcinogenicity of the biologically active metabolites has been demonstrated (1). Previous studies (7-10, 27-28), together with the results presented here, have now established the stereochemical factors involved in the sequence of reactions for the metabolism of BP to an ultimate carcinogen (Fig. 7). The results presented here demonstrate that (+-) and (-)-BP 7,8-oxide are stereospecifically metabolized to (+-) and (+-)BP 7,8-dihydriodiol, respectively, by hepatic microsomal epoxide hydrolase. While the rat enzyme metabolizes the (-)-enantiomer at a rate 3 to 4 times greater than the (+)-enantiomer, epoxide hydrolase has a higher affinity for the (+)-enantiomer compared to the (-)-enantiomer. Thus, when various ratios of the two enantiomers are incubated, the (+)-enantiomer is preferentially metabolized. At low conversion of substrate, this results in the formation of (+)-BP 7,8-dihydriodiol of high enantiomeric purity. Extensive metabolism of racemic BP 7,8-oxide results in the formation of large amounts of both enantiomers of the dihy-
dihydrodiol. These results probably explain the apparent discrepancy in the reported enantiomeric purity of BP 7,8-dihydrodiol formed from racemic BP 7,8-oxide. Thakker et al. (7, 8) reported low enantiomeric purity at high substrate conversion, while Yang et al. (9, 10) reported high enantiomeric purity at low conversion (≤20%) of substrate.

Metabolism of BP to BP 7,8-dihydrodiol via the sequential action of the mixed function oxidase system and epoxide hydrolase results in the formation of predominantly the (−)-enantiomer (>90%). The high enantiomeric purity of the dihydrodiol could be the result of one of the two following metabolic events: (i) formation of highly enantiomerically pure BP 7,8-oxide or (ii) formation of BP 7,8-oxide of low enantiomeric purity followed by preferred hydration of (+)-BP 7,8-oxide at low substrate conversion (see “Results”). Thus, to simulate the conditions under which BP 7,8-dihydrodiol is formed from the parent hydrocarbon, we have performed experiments in which racemic BP 7,8-oxide was infused, with and without the addition of other metabolically formed BP arene oxides, into an incubation with hepatic microsomes at a rate equal to the formation of the corresponding dihydrodiols from BP (33). During these incubations, these metabolites were quantitatively converted into the corresponding diastereomeric BP 7,8-dihydrodiols (4 to 8% of the total BP 7,8-dihydrodiol formed) are detected when BP is metabolized by rat liver microsomes. In contrast to the conclusion by Yang et al. (9, 10) that a single enantiomer of BP 7,8-oxide is formed by rat liver microsomes, the present results indicate that both enantiomers are formed albeit the (+)-enantiomer is formed in 10- to 20-fold excess. In addition, when BP is applied topically to mouse skin (33) or to cells in culture (34-37), enantiomers of the diastereomeric BP 7,8-diol-9,10-epoxides derived from both (+)- and (−)-BP 7,8-dihydrodiol are detected as adducts bound to macromolecules, although the covalently bound product(s) derived from the (−)-enantiomer predominate.

Fig. 7 summarizes the metabolic pathways involved in the stereoselective metabolism of BP to the bay region 7,8-diol-9,10-epoxides. It is of interest to note that the enzymes made all of the wrong stereochemical choices in their conversion of BP to the BP 7,8-diol-9,10-epoxides as far as the welfare of the animal was concerned. (+)-7,8a-Dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene [(+)-BP 7,8-diol-9,10-epoxide-2] is the most potent carcinogenic diol epoxide of BP (5, 6), and it is formed from BP2 to a considerably greater extent than are the other three diol epoxide isomers (7). If the mixed function oxidase system has metabolized BP exclusively to (−)-BP 7,8-oxide, the resulting diol epoxides would have had very markedly reduced carcinogenicity.

An evaluation of the tumorigenic activity of the enantiomers of BP 7,8-oxide revealed that the (+)-enantiomer was significantly more tumorigenic than the (−)-enantiomer, which is consistent with the formation of the highly tumorigenic (+)-7,8a-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene from (+)-BP 7,8-oxide. Interestingly, an enantiomeric enhancement in the tumorigenicity of racemic BP 7,8-oxide was also observed in these studies. These results are the first demonstration of a synergistic carcinogenic effect for enantiomers. Although the reason(s) for this synergism are not known, metabolic factors described above could contribute to this phenomenon. It should be emphasized, however, that factors other than metabolism may be involved in the higher than expected tumorigenic activity of racemic BP 7,8-oxide.

We recently reported (38) a synergistic interaction in the inherent mutagenicity of (+)- and (−)-BP 4,5-oxide in Chinese hamster V79 cells. These cells have very low or nondetectable levels of the mixed function oxidase system and epoxide hydrolase which are involved in the detoxification of BP 4,5-oxide.

**Acknowledgments:** We thank Dr. Gary Williams, C. Q. Wong, and the staff of the Naylor Dana Institute for Disease Prevention, American Health Foundation, Valhalla, N.Y., for the histological studies. We thank Nelson Montero and Dennis Tighe for their help in caring for the animals and Ann Marie Williams for her assistance in the preparation of this manuscript.

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**TABLE V**

Determination of the optical purity of BP 7,8-dihydrodiol formed from (±)-BP 7,8-oxide

| Sample | Per cent metabolism of BP 7,8-oxide | Per cent (+)-enantiomer | Per cent (-)-enantiomer |
|--------|---------------------------------|------------------------|------------------------|
| A      | 49                              | 57                     | 43                     |
| B      | 51                              | 58                     | 42                     |

Microsomal protein from 3-methylcholanthrene pretreated rats (5 mg of protein) was incubated in 100 mM potassium phosphate buffer (pH 7.4) for 10 min at 37°C in a final volume of 50 ml. A solution of 20 μM [6-3H]-BP 7,8-oxide in the absence (A) or presence (B) of 14 μM (+)-BP 4,5-oxide and 33 μM (+)-BP 9,10-oxide was added at a constant rate of 0.25 ml/min to the incubation vessels during the 10-min incubation. The substrates were dissolved in acetonitrile:NH₄OH (1000:1). Enantiomeric purity of the metabolically formed BP 7,8-dihydrodiol was determined as described under "Materials and Methods."
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Metabolism of (+)- and (-)-Benzo[a]pyrene 7,8-Oxide

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