Mutagenicity of Cyclopenta-fused Isomers of Benz(a)anthracene in Bacterial and Rodent Cells and Identification of the Major Rat Liver Micromsomal Metabolites

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ABSTRACT

The microsomal metabolites and mutagenic activity of four cyclopenta-fused benz(a)anthracenes, benz/(aceanthrylene [B(j)A], benz(e)aceanthrylene [B(e)A], benz(j)aceanthrylene [B(j)A], and benz(k)acephenanthrylene [B(k)A], have been studied. Aroclor 1254-induced rat liver microsomes metabolized B(j)A to B(j)A-1,2-dihydrodiol, B(j)A-9,10-dihydrodiol, B(j)A-11,12-dihydrodiol, and 10-hydroxy-B(j)A; B(e)A to B(e)A-1,2-dihydrodiol, B(e)A-3,4-dihydrodiol, and B(e)A-5,6-dihydrodiol; B(k)A to B(k)A-1,2-dihydrodiol, B(k)A-4,5-dihydrodiol, and B(k)A-7,8-dihydrodiol; and B(l)A to B(l)A-4,5-dihydrodiol and B(l)A-8,9-dihydrodiol. With each polycyclic aromatic hydrocarbon, metabolism occurred on the cyclopenta ring. All four isomers were active as gene mutants in Salmonella typhimurium and in Chinese hamster V79 cells. In the S. typhimurium mutation studies, using Aroclor 1254-induced rat liver s9, B(j)A, B(e)A, and B(l)A required significantly less microsomal protein for maximal mutation response than B(k)A and B(a)P, suggesting a one-step activation mechanism, presumably on the cyclopenta-fused ring. B(j)A, B(e)A, and B(l)A were significantly more mutagenic than B(k)A and B(a)P in S. typhimurium. In the Aroclor 1254-induced rat liver S9-mediated V79 mutagenesis system, all four isomers were active, with B(l)A the most active. When Syrian hamster embryo cells were used as the metabolic activation component for V79 cells, only B(l)A produced a significant response and was equivalent in activity to B(a)P. A helical configuration for B(l)A is inferred from the identification of two trans-(B(l)A)-1,2-dihydrodiols, syn and anti, which have been synthesized, separated, and characterized. The metabolically formed dihydrodiol is anti-trans-(B(l)A)-1,2-dihydrodiol, and experimental evidence suggests that the metabolically formed B(l)A-1,2-oxide is the anti-isomer. Synthetic B(l)A-1,2-oxide was found to be a direct-acting mutagen in S. typhimurium and Chinese hamster V79 cells and is estimated to account for up to 40% of the mutagenic activity of the parent hydrocarbon. Therefore, certain cyclopenta-ring fusions on benz(a)anthracene appear to markedly increase its genotoxic and carcinogenic activities.

INTRODUCTION

PAHs are ubiquitous environmental pollutants often formed by the incomplete combustion of fossil fuels. It is estimated that they make a large contribution to the overall carcinogenic activity of extracts of automotive emissions and other environmental contaminants to which humans are exposed; therefore, they are implicated in the etiology of human cancer (18). A unique class of PAH are the cyclopenta-fused PAH of which cyclopenta(cd)pyrene represents a widely studied example. Cyclopenta(cd)pyrene has been identified in automobile exhaust emissions, carbon black, ambient air, and cigarette smoke, is mutagenic in bacteria and mammalian cells (10, 15, 16, 35, 41, 44), induces morphological cell transformation (16), and is a mouse skin tumor initiator, cocarcinogen, and complete carcinogen (5, 6, 44). Other cyclopenta-fused PAH which have been identified in the environment, but have not yet received much attention are acenaphthylene (4, 14) and acephenanthrylene (26).

We have recently synthesized a novel subgroup of this class, the 4 isomeric cyclopenta-fused benz(a)anthracenes [B(j)A, B(e)A, B(k)A, and B(l)A] (36, 39). Our interest in these compounds stems from the likelihood of their occurrence in the environment [B(l)A has recently been identified as a component of wood smoke emissions] and the opportunity to study the activity of the nonalterant systems generated by cyclopenta ring fusion on the benz(a)anthracene ring system (Chart 1). The ∆E_{add}/β values for carboxations on the cyclopenta rings of B(j)A, B(e)A, and B(l)A exceeded E_{add}/β of the mutagenic and carcinogenic bay region C_{9,10} carboxation of B(a)P-7,8-dihydrodiol-9,10-epoxide (Table 1). Since carboxations of PAH with high ∆E_{add}/β values have been implicated as ultimate reactive species in mutagenesis and carcinogenesis (12, 24), it was of interest to correlate genetic activity with cyclopenta-ring metabolism in both bacteria and mammalian cells (38). Also, of interest was the extent to which the unique structure of B(l)A (Chart 1) might contribute to biological activity. This compound has a sterically crowded 4-sided peripheral indentation (gulf region) similar to the fjord region (2, 30) of benzo(c)phenanthrene and, like benzo(c)phenanthrene (22), B(l)A appears to assume a helical configuration. Two sets of isomeric 1,2-oxides and trans-cyclopenta-ring dihydrodiols are identified.
theoretically possible, and both the dihydrials have been identified for B(I)A.

We conclude from these studies that oxidation on the cyclopenta-ring is a primary pathway for metabolic activation of B(I)A, B(e)A, and B(l)A to forms mutagenic to Salmonella typhimurium respectively. Molar fractions of both oxidations were determined using an NADPH-generating system according to the previously published procedures describing the syntheses of related cyclopenta-fused PAH epoxides (29). A single compound was present in the reaction mixture. B(I)A-1,2-oxide was characterized by the following physicochemical data: UV (methanol) \( \lambda_{	ext{max}} \) 394 (0.140); 372 (0.166); 364 (0.200); 355 (0.233); 290 (2.17); and 269 (1.73); accurate mass of molecular ion, 268.0868; calculated for \( 
{\text{C}}_{18}\text{H}_{12}\text{O} \), 268.0866 (\( \Delta m = -0.3 \text{ ppm} \)); fragmentation pattern, Table 2. \(^{1}H\) NMR (250 MHz, acetone-\( d_6 \), \( \delta_{5.13} \) and \( \delta_{5.85} (d; each 1H; J \text{ calculated for } \text{CaHsO} \), 268.0886 (A = -0.3 ppm); fragmentation pattern, \( \delta_{5.13} \) and \( \delta_{5.85} \) (d; each 1H; \( J \text{ calculated for } \text{CaHsO} \). \( \delta_{5.13} \) and \( \delta_{5.85} \) (d; each 1H; \( J \text{ calculated for } \text{CaHsO} \)). The extremely-low-field oxirane proton resonance (\( \delta 5.85 \)) is consistent with its location in the highly deshielded gulf region as expected for an anti-epoxide.\(^{6}\)

Syn-trans-B(I)A-1,2-dihydrodiol(B(l)A\(_{10}\)) was obtained by KCl hydrolysis of B(I)A-1,2-oxide in dimethyl sulfoxide (3) and also by the sodium borohydride reduction of B(I)A-1,2-dione obtained from the sodium borohydride reduction of B(I)A (20) of 1-oxo-B(I)A. A mixture of B(I)A and anti-trans-B(I)A-1,2-dihydrodiol(B(l)A\(_{10}\)) was generated by sodium borohydride reduction of 1-hydroxy-2-oxo-B(I)A obtained from 2,3-dichloro-5,6-dicyano-1,4-benzobenzene oxidation (36) of a mixture of the cis-B(I)A-1,2-dihydrodiols (19). Dihydradiols B(l)A and B(I)A were separated by preparative HPLC (Dupont Model 850; Dupont Instruments, Wilmington, DE) on a 9.1 mm x 25 cm DuPont Zorbax ODS reverse-phase column with a methanol-water gradient programmed as described in Chart 2C. (inside diameter) x 25 cm Zorbax-ODS column using methanol:water or trifluoroacetic acid:water as the eluant. Fractional crystallization of the fras-diacetate isomers were separated from the trans-diacetate isomers by column chromatography on deactivated alumina using hexane:methylene chloride (3:2) as the eluant. Fractional crystallization of the trans-diacetate mixture from hexane separated the trans-diacetate of B(I)A-1,2-dihydrodiol from the trans-diacetate of B(e)A-5,6-dihydrodiol. Each trans-diacetate was then hydrolyzed with dry ammonia in methanol:tetrahydrofuran (9:1), and the crude dihydrodiol recrystallized from benzene. The purity and yield of each compound was confirmed by \(^{1}H\) NMR, HPLC, UV, and mass spectroscopy. The syn- and anti-B(I)A-7,8-dihydrodiol-9,10-epoxides were obtained from the National Cancer Institute Repository.

Generation and Collection of Metabolites. The PAH were incubated with Aroclor 1254-induced male Charles River rat liver microsomes and a NAD(P)H-generating system according to the previously published procedures (38). A 5-mL incubation mixture containing 5 \( \mu l \) of NADP+, 22.5 \( \mu l \) of glucuronic acid, 9.0 units of glucose-6-phosphate dehydrogenase (Sigma Chemical Co., St. Louis, MO), 15 \( \mu l \) of magnesium chloride, 250 \( \mu l \) of potassium phosphate buffer (pH 7.5), 300 nmol of PAH, and 10.0 mg of microsomal protein was used and incubated 15 min at 37° with agitation. The incubation mixture was extracted with 15 ml of ethyl acetate:acetone (2:1). The ethyl acetate:acetone metabolite extracts were filtered (0.5-μm Teflon filter; Millipore Corp., Bedford, MA) and chromatographed by HPLC on a 6.2 mm (inside diameter) x 25 cm Zorbax-ODS column using methanol:water and methanol:water:tetrahydrofuran (3:1) gradient mixtures. Gradient programs are illustrated in Chart 2.

Metabolite Characterization. Separated metabolites were characterized by UV-vis spectroscopy in methanol on a Coleman 124 spectrophotometer. Electron impact mass spectra, both high resolution for elemental composition of the molecular ion and low resolution for fragmentation patterns, were obtained on a Micromass V/3707F mass spectrometer equipped with a VG 2035B data-processing system. High-field \(^{1}H\) NMR spectra were recorded on a Bruker WM250 spectrometer. Accurate

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**MATERIALS AND METHODS**

**Synthesis of Cyclopenta-fused PAH.** Compounds were synthesized and purified by published methods (36, 39) and were shown to be pure by HPLC prior to use in metabolism experiments (38). B(I)A-1,2-oxide was synthesized and purified by published procedures describing the syntheses of related cyclopenta-fused PAH epoxides (29). A single compound was present in the reaction mixture. B(I)A-1,2-oxide was characterized by the following physicochemical data: UV (methanol) \( \lambda_{	ext{max}} \) \( \times 10^{-3} \); 394 (0.140); 372 (0.166); 364 (0.200); 355 (0.233); 290 (2.37); 279 (2.29); and 269 (1.73); accurate mass of molecular ion, 268.0868; calculated for \( 
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**Table 1**

<table>
<thead>
<tr>
<th>Epoxyd</th>
<th>Carbonioion</th>
<th>( \Delta E_{\text{act}}(\beta) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>B(( \text{I} ))A-1,2-oxide</td>
<td>( \text{C}_1 )</td>
<td>0.879</td>
</tr>
<tr>
<td>B(e)A-5,6-oxide</td>
<td>( \text{C}_2 )</td>
<td>0.679</td>
</tr>
<tr>
<td>B(( \text{I} ))A-1,2-oxide</td>
<td>( \text{C}_3 )</td>
<td>0.833</td>
</tr>
<tr>
<td>B(( \text{I} ))A-4,5-oxide</td>
<td>( \text{C}_4 )</td>
<td>0.722</td>
</tr>
<tr>
<td>B(a)P-7,8-diol-9,10-oxide</td>
<td>( \text{C}_5 )</td>
<td>0.794</td>
</tr>
</tbody>
</table>

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\(^{6}\) The terms syn and anti refer to the relationship between the C-7-C-8 oxirane oxygen or the C-9-C-10 hydroxyl group and the angular benzo-fused ring of B(I)A.
Table 2

Mass spectral fragmentation patterns of cyclopenta-fused benz(a)anthracene derivatives

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>m/e</th>
</tr>
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<tbody>
<tr>
<td>B[1]A&lt;sub&gt;1&lt;/sub&gt;</td>
<td>288</td>
</tr>
<tr>
<td>B[7]A&lt;sub&gt;7&lt;/sub&gt;</td>
<td>267</td>
</tr>
<tr>
<td>B[8]A&lt;sub&gt;8&lt;/sub&gt;</td>
<td>256</td>
</tr>
<tr>
<td>B[10]A&lt;sub&gt;10&lt;/sub&gt;</td>
<td>252</td>
</tr>
<tr>
<td>B[12]A&lt;sub&gt;12&lt;/sub&gt;</td>
<td>239</td>
</tr>
<tr>
<td>B[13]A&lt;sub&gt;13&lt;/sub&gt;</td>
<td>238</td>
</tr>
<tr>
<td>B[14]A&lt;sub&gt;14&lt;/sub&gt;</td>
<td>237</td>
</tr>
<tr>
<td>B[15]A&lt;sub&gt;15&lt;/sub&gt;</td>
<td>236</td>
</tr>
</tbody>
</table>


Mass determinations and fragmentation patterns were consistent with all dihydriodiol structural assignments and with the single phenol metabolite identified (Table 2). Metabolite yields were approximately ordered by comparison of the signal/noise ratios of the 1H NMR spectra of samples accumulated from an equal number of semipreparative HPLC runs, made up to equivalent volumes in ace tone-d<sub>6</sub> and generated from the same number of transients (4 x 10<sup>4</sup>).

**Bacterial Mutagenesis Assays.** The standard plate incorporation assay of Ames et al. (1) was used with minor modifications (7) using S. typhimurium strains TA1535, TA1537, TA1538, TA98, and TA100. The B[1]A-1,2-oxide and dioxi-epoxides of B[1]A<sub>1</sub> were evaluated using the procedure of Wood et al. (46). The epoxide was added in 12.5 μl of anhydrous dimethyl sulfoxide to 0.5 ml of PBS, pH 7.0, which contained 2.0 x 10<sup>8</sup> bacteria. After 5.0 min at 37°, 2.0 ml of molten-top agar were added above the solution and poured onto Petri dishes as described above.

**Cell Culture Conditions.** V79 cells were provided by Dr. E. Huberman, Argonne National Laboratory, Chicago, IL. The cells were maintained in William’s Medium E supplemented with 10% fetal bovine serum and 2 mM L-glutamine (Grand Island Biological Co., Grand Island, NY). Cultures were incubated in humidified incubators at 37° in 5% CO<sub>2</sub> in air. Cell cultures were routinely selected with hypoxanthine, aminopterin, thymine, and glycine before each experiment.

**V79 Cell Mutagenesis.** The V79 cells were plated at a density of 1 x 10<sup>4</sup> cells/25-cm<sup>2</sup> T-flask (2 flasks/treatment group) and incubated overnight. The media were removed, and the cells were incubated in 2 ml of reaction mixture, containing an Arador 1254-induced rat liver S9 mix, and glycine before each experiment.

**Metabolite Generation, Collection, and Identification.** To obtain metabolite mixtures for characterization, multiple 5-ml incubations were performed under conditions similar to those used in the Salmonella and V79 mutation studies and the extracts combined for HPLC analysis and collection. Higher product yields were obtained by this method than by large-scale incubations.

**RESULTS**

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**RESULTS**

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been assigned structures and their relative yields are estimated to be B(j)A \text{II} > B(j)A \text{I} > B(j)A \text{III}. The principal metabolite B(j)A \text{IV} has been identified as trans-B(j)A-1,2-dihydrodiol. Its UV absorption spectrum resembles that of benz(a)anthracene (Chart 3). 1H NMR resonances (H6, H7) characteristic of aromatic bay-region protons are present, while absence of vinylic resonances rules out partial saturation of the terminal or angular benzo rings and absence of a cyclopenta-ring AX quartet precludes a K-region dihydrodiol formulation (Table 3). Absolute confirmation of this structure is provided by comparison with a synthetic standard. All spectral and chromatographic data from B(j)A \text{IV} and synthetic trans-B(j)A-1,2-dihydrodiol were identical. Metabolite B(j)A \text{I} was assigned the K-region dihydrodiol structure [B(j)A-11,12-dihydrodiol] and is distinguished from B(j)A \text{IV} by its UV absorption spectrum (Chart 3), which does not arise from the benz(a)anthracene chromophore. Proton resonances from solvent and HPLC column impurities, which appear as major signals because of the low concentration of B(j)A \text{I}, obscure the region where AX quartet of the intact cyclopenta-ring should be observed. However, other features of the 1H NMR spectrum are consistent with a K-region dihydrodiol, i.e., a low-field singlet and doublet (H6, H7), indicating an aromatic bay-region periphery, and absence of vinylic resonances precluding terminal or angular benzene-ring metabolism (Table 3). Characterization of B(j)A \text{II} as B(j)A-9,10-dihydrodiol rests partly upon the similarity of its UV absorption spectrum to that of anthracene (Chart 3), consistent with disruption of angular benzo-ring aromaticity. A crucial feature of the 1H NMR spectrum is the presence of vinyl resonances (H7, H8), since a possible 4,5-dihydrodiol would have only a single vinylic signal (Table 3). The marked downfield position of one of the vinyl signals (H7) is consistent with location of a vinyl proton in the bay indentation, deshielded by the ring current of the
Chart 3. UV absorption spectra (methanol) of the major metabolites of cyclopenta-fused benz(a)anthracenes. A (top to bottom), B(j)A4, B(j)A3, B(j)A2, B(j)A1, B(j)A6; B(e)A6, B(e)A5, B(e)A4, B(e)A3, C, B(e)A2, B(e)A1; D, B(k)A6, B(k)A5. Ordinate, absorbance.

adjacent residual anthracene nucleus. B(j)A6 was recognized as a phenol by its relative HPLC retention time, molecular weight, elemental composition, and the long-wavelength band in the UV absorption spectrum indicating preservation of the B(j)A nucleus (Chart 3). Positional isomerism was established by analysis of the first-order couplings of the 1H NMR spectrum (Table 3). Absence of a downfield aromatic AB or AX quartet rules out substitution of the terminal ring. The presence of the 2 highly deshielded bay-region signals is inconsistent with a C6- or C7-phenol, while substitution of the K-region would have resulted in...
Table 3

Cyclopana-fused benz(a)anthracene metabolite proton shifts and coupling constants

For data shown, 1H NMR is at 250 MHz, 25° (acetone-d6).

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Proton, shift (ppm), (multiplicity), coupling constants</th>
</tr>
</thead>
<tbody>
<tr>
<td>B(e)A1</td>
<td>H9, 7.95 (d); J9A = 8.3 Hz; H10, 8.37 (d); J10A = 7.5 Hz; H11, 9.20 (s); H12, 8.96 (d); J12A = 7.5 Hz; H13, 5.48 (bs); H14, 5.75 (bs); remaining aromatic H, 7.4-7.8 (m)</td>
</tr>
<tr>
<td>B(e)A2</td>
<td>H9, 5.68 (bs); H10, 5.56 (bs); H11, 8.05 (d); J11A = 8.3 Hz; H12, 8.35 (d); J12A = 8.3 Hz; H13, 9.33 (s); H14, 9.00 (d); J14A = 7.3 Hz; remaining aromatic H, 7.5-8.0 (m)</td>
</tr>
<tr>
<td>B(e)A3</td>
<td>H9, 7.73 (d); J9A = 5.5 Hz; H10, 8.10 (d); J10A = 8.7 Hz; H11, 8.35 (d); J11A = 8.3 Hz; H12, 8.35 (d); J12A = 8.3 Hz; H13, 9.34 (s); H14, 9.00 (d); J14A = 7.3 Hz; remaining aromatic H, 7.5-8.0 (m)</td>
</tr>
<tr>
<td>B(e)A4</td>
<td>H9, 6.85 (d); J9A = 6.2 Hz; J9B = 4.2 Hz; H10, 8.28 (dd); J10A = 6.3 Hz; J10B = 5.2 Hz; H11, 5.47 (bs); H12, 7.65 (d); H13, 7.97 (dd); J13A = 7.3 Hz; J13B = 2.1 Hz; H14, 8.66 (dd); J14A = 7.3 Hz; J14B = 2.3 Hz; remaining aromatic H, 7.58-7.70 (m)</td>
</tr>
<tr>
<td>B(e)A5</td>
<td>H9, 5.15 (d); J9A = 9.4 Hz; H10, 4.54 (bd); J10A = J10B = 9.4 Hz; H11, 6.07 (dd); J11A = 10.4 Hz; J11B = 2.5 Hz; H12, 6.97 (dd); J12A = J12B = 10.4 Hz; J12C = 2.5 Hz; H13, 7.12 (d); J13A = J13B = 5.3 Hz; H14, 8.06 (d); H15, 8.08 (d); H16, 8.72 (dd); J16A = J16B = 7.3 Hz; H17, 8.28 (d); H18, 8.38 (d); H19, two dd, 7.70 and 7.12; J19A = J19B = 5.3 Hz; J19C = 7.5 Hz; remaining aromatic H, 7.58-7.70 (m)</td>
</tr>
<tr>
<td>B(e)A6</td>
<td>H9, 6.12 (bs); H10, 5.56 (bs); H11, 8.47 (s); H12, 9.40 (d); J12A = J12B = J12C = 7.5 Hz; remaining aromatic H, 7.60-8.07 (m)</td>
</tr>
<tr>
<td>B(e)A7</td>
<td>H9, 8.21 (d); J9A = 5.3 Hz; H10, 8.66 (d); J10A = J10B = 5.3 Hz; H11, 6.71 (d); J11A = J11B = 3.0 Hz; J11C = 4.96 (dd); J11D = 6.4 Hz; H12, 5.15 (d); J12A = J12B = 8.4 Hz; H13, 7.90 (s); H14, 9.03 (d); J14A = J14B = J14C = 7.8 Hz; H15, 7.97 (dd); J15A = J15B = 5.2 Hz; H16, 7.17 (d); J16A = J16B = 5.2 Hz; H17, 8.22 (s); H18, 8.20 (d); H19, 8.36 (d); J19A = J19B = 8.3 Hz; H20, 8.88 (s); H21, 7.46-7.67 (m)</td>
</tr>
<tr>
<td>B(e)A8</td>
<td>H9, 6.69 (d); J9A = 7.5 Hz; H10, 5.36 (bs); H11, 5.90 (bs); H12, 7.80 (s); H13, 8.53 (s); H14, 9.30 (s); remaining aromatic H, 7.50-7.70 (m)</td>
</tr>
<tr>
<td>B(e)A9</td>
<td>H9, 8.54 (d); H10, 7.20 (d); J10A = 5.0 Hz; H11, 7.29 (d); J11A = J11B = J11C = 5.0 Hz; H12, 4.82 (s); H13, 8.17 (s); H14, 8.30 (d); H15, 4.91 (d); J15A = 10.0 Hz; H16, 4.55 (bd); J16A = J16B = 10.0 Hz; H17, 6.17 (dd); J17A = J17B = J17C = 10.0 Hz; J17D = 2.5 Hz; H18, 8.53 (d); remaining aromatic H, 7.88-7.90 (m)</td>
</tr>
</tbody>
</table>

Multiplets are designated as follows: s, singlet; bs, broadened singlet; d, doublet; bd, broadened doublet; dd, doublet of doublets; m, complex multiplet.

the presence of a second aromatic singlet. Substitution at C9 or C10 would result in an upfield shift of 2 adjacent aromatic protons by the "ortho effect" (31). Integration of the aromatic resonances shows that only one aromatic proton can be shifted upfield (H8, obscured by the impurity at 7 to 7.2 ppm); hence, substitution at C10 [10-hydroxy-B(j)A] is compatible with the 1H NMR spectrum.

B(e)A. Metabolites B(e)A2, B(e)A3, and B(e)A4 have been assigned structures (Chart 2), with relative abundances estimated to be B(e)A2 > B(e)A3 > B(e)A4. B(e)A2 was identified as trans-B(e)A-5,6-dihydrodiol by its benz(a)anthracene-like UV absorption spectrum (Chart 3) and by the absence of both vinylic proton resonances and a cyclopana-ring AX quartet in the 1H NMR (Table 3). Absolute confirmation of the structure is provided by comparison with a synthetic standard. All spectral and chromatographic data from B(e)A and synthetic trans-B(e)A-5,6-dihydrodiol were identical. B(e)A2 and B(e)A4 were identified as terminal ring dihydrodiols by the similarity of their UV absorption spectra to that of acephenanthrylene (Chart 3) (11, 26, 27), and the presence of aromatic bay-region signals in the 1H NMR (Table 3). The meso bay-region proton resonance (H12), with the dihydrodiol distal to the bay region is expected to be shifted upfield relative to the resonance of the bay-region proton (H11) on the angular ring (13). On this basis, B(e)A3 is B(e)A-3,4-dihydrodiol, and B(e)A is B(e)A-1,2-dihydrodiol.

B(l)A. Metabolites B(l)A2, B(l)A3, and B(l)A4 have been identified (Chart 2), and their relative amounts have been estimated to be B(l)A2 > B(l)A3 > B(l)A4. B(l)A2 was identified as anti-trans-B(l)A-1,2-dihydrodiol from its benz(a)anthracene-like UV absorption spectrum (Chart 3) and by the absence both of vinylic proton resonances and of a cyclopana-ring AX quartet in the 1H NMR (Table 3). Severe steric crowding within the gulf region requires
The cyclopenta-ring dihydrodiol, B(k)A-4,5-dihydrodiol. The 1H NMR of K-region positions (19). The amount of substrate was 30 µg/plate. The background mutation accounted for the only nonaromatic signals in the spectrum (Table 4). The 1H NMR substantiates the assignment, since vinyl resonances and carbinyl protons appear as a true AB quartet, typical of the same magnetic environments of K-region positions (19).

Metabolite B(l)A is the K-region dihydrodiol, B(l)A-7,8-dihydrodiol. The UV absorption spectrum indicates a chromatophore different from benz(a)anthracene (Chart 3) and the 1H NMR, while having no vinylic signals, exhibits the AX quartet of the unsaturated cyclopana ring (Table 3). The critical feature is the presence of only a single vinyl resonance split by allylic coupling to a carbinyl proton.

Metabolite B(l)A is the K-region dihydrodiol, B(l)A-7,8-dihydrodiol. The UV absorption spectrum indicates a chromatophore different from benz(a)anthracene (Chart 3) and the 1H NMR, while having no vinylic signals, exhibits the AX quartet of the unsaturated cyclopana ring (Table 3). The critical feature is the presence of only a single vinyl resonance split by allylic coupling to a carbinyl proton.

Metabolite B(l)A is the K-region dihydrodiol, B(l)A-7,8-dihydrodiol. The UV absorption spectrum indicates a chromatophore different from benz(a)anthracene (Chart 3) and the 1H NMR, while having no vinylic signals, exhibits the AX quartet of the unsaturated cyclopana ring (Table 3). The critical feature is the presence of only a single vinyl resonance split by allylic coupling to a carbinyl proton.

Mutagenic Activity of Cyclopenta-fused PAH in S. typhimurium. Previous studies with cyclopenta(c,d)/pyrene and B(l)A had shown a sharp dependence of mutagenic response on S9 concentration in bacterial (15, 38) assays. Similar studies were performed with all 4 cyclopenta-fused benzo(a)anthracene isomers and B(a)P in TA98 (Chart 5). The mutagenic activity of 2 of the 4 isomers, B(l)A and B(l)A, exhibited a strong dependence on S9 protein concentration, with optimal activity observed at 0.5- and 0.3-mg/ml S9 protein, respectively. Higher concentrations of S9 resulted in markedly diminished activity. The mutagenic activity of B(l)A exhibited a plateau at 0.5-mg/ml S9 protein and retained that level up to 3.0 mg/ml. Higher concentrations of S9 protein were required for B(k)A and B(a)P activity to reach a plateau; the range was 1.0 to 1.5 mg/ml for B(k)A and 3.0 to 3.5 mg/ml for B(a)P. Comparison of the 5 PAH at their optimum S9 concentrations indicated that B(k)A > B(l)A > B(l)A > B(k)A, B(a)P (Chart 5) based on the maximum number of revertants per plate. The sensitivity of the 4 cyclopenta-fused PAH to S. typhimurium strain differences was also examined. All were active in strains TA1538, TA98, and TA100 and inactive in TA1535 and TA1537, except for B(k)A and B(a)P which were active in TA1537 (data not shown).

Mutagenic Activity of Cyclopenta-fused PAH in V79 Cells. Induced gene mutation in V79 cells was measured at the hypoxanthine-guanine phosphoribosyltransferase locus (6TG-resistance) using an Aroclor 1254-induced liver S9 from male Charles River rats as the metabolic activation system. The dependence of mutagenic activity on S9 protein concentration (0 to 3 mg/ml) was examined at PAH concentrations of 1, 5, 10, 20 µg/ml (Charts 6 and 7). B(e)A, B(l)A, B(2)A, B(l)A, and B(a)P were mutagenic at 5 and 10 µg/ml but had marginal or no activity at 1 µg/ml even at S9 protein concentrations as high as 3.0 mg/ml. Mutagenic activity appeared to plateau at S9 protein concentrations of 0.5 mg/ml or less for B(l)A, B(e)A, B(2)A, and B(a)P. With a 0.5-mg/ml protein concentration, the 5 PAH were examined at a concentration range of 0 to 20 µg/ml (Chart 8). At 20 µg/ml, B(l)A was the most active, and the remaining PAH were equivalent in activity to B(a)P.

Irradiated SHE cells were examined as an alternate metabolic activation system for V79 cells. Preliminary SHE cell density experiments suggested 2.0 × 10⁶ SHE cells to be optimum for B(a)P activity. B(l)A, B(e)A, and B(k)A did not induce gene mutation in V79 cells when cocultivated with SHE cells in the concentration range 0 to 2.0 µg/ml, where significant cytotoxicity was evident at the highest concentrations. Experiments performed at lower SHE cell densities (0.25 × 10⁶, 0.5 × 10⁶, or 1.0 × 10⁶) also indicated no genetic activity (data not shown). However, B(l)A was quite active, producing 635 6TG-resistant mutants/10⁶ cells, while B(a)P, under similar conditions and at equimolar concentrations, produced 1178 6TG-resistant mutants/10⁶ survivors (Chart 9).

Mutagenic Activity of B(l)A-1,2-oxide. The mutagenic activity of B(l)A-1,2-oxide was compared to anti-B(a)P-7,8-dihydrodiol-9,10-oxide and syn-B(a)P-7,8-dihydrodiol-9,10-oxide in both S. typhimurium and V79 cells without exogenous metabolic act
Chart 6. Relationship between Aroclor 1254-induced rat liver S9 concentration, cytotoxicity, and mutagenic activity of B(j)A, B(e)A, and B(I)A in Chinese hamster V79 cells. Cells were treated for 4 hr and subsequently scored for cytotoxicity and 6TG-resistance according to "Materials and Methods." Data represent the mean of triplicate determinations. A, B(j)A; B, B(e)A; C, B(I)A.

Chart 7. Relationship between Aroclor 1254-induced rat liver S9 concentration, cytotoxicity, and mutagenic activity of B(k)A and B(a)P in Chinese hamster V79 cells. Cells were treated for 4 hr and subsequently scored for cytotoxicity and 6TG-resistance according to "Materials and Methods." Data points, mean of triplicate determinations. A, B(k)A; B, B(a)P.

Discussion

Large values of \( \Delta E_{aodc} / \beta > 0.79 \) for benzylic carbonium ions derived from ring-opened epoxides appear to be associated with biological activity (12, 24). Consequently, the large delocalization energies of carbonium ions from the cyclopenta-ring epoxides of B(j)A, B(e)A, and B(I)A suggest that if epoxidation of the cyclopenta-ring is an important metabolic pathway, these compounds should be active. On the other hand, application of the same criterion to B(k)A indicates that the 5-membered ring may not be a potential site for activation. Elucidation of the metabolite profiles of the 4 isomers demonstrates that epoxidation of the cyclopenta-fused ring is indeed a major pathway. The mutagenicity assays verify the prediction of biological activity for B(j)A, B(e)A, and B(I)A and support the importance of large \( \Delta E_{aodc} / \beta \).

Additional potentially active metabolites of B(j)A and B(I)A have been identified and may also contribute to total mutagenic response. B(j)A-9,10-dihydrodiol is capable of further metabolic...
transformation to a bay-region diol-epoxide, while the isolation of K-region dihydrodiols of B(j)A and B(l)A imply the presence of the corresponding potentially active K-region oxides. However, the K-region oxide of B(j)A is a minor metabolite and, for reasons discussed below, B(j)A-9,10-dihydrodiol does not appear to be an important promutagen. The K-region dihydrodiol is the major B(l)A metabolite isolated, but the high level of direct-acting mutagenicity of B(l)A-1,2-oxide, which is also a major metabolite, is consistent with a role for the 1,2-oxide in the activity of B(l)A.

In S. typhimurium, the peak mutagenic activity of B(j)A, B(e)A, and B(f)A was not observed at the highest concentration tested. However, a clear dose-response relationship was evident for B(l)A, with increasing mutagenic activity at higher concentrations. The mutagenic activity of B(l)A was also observed in Chinese hamster V79 cells, with a similar dose-response relationship. These results suggest that B(l)A is a more potent mutagen in vitro than in vivo, and that the mutagenic activity of B(l)A is not due to the direct action of the parent compound.
and B(III)A occurred at considerably lower S9 protein concentrations (0.3 to 0.5 mg/ml) than B(k)A and B(a)P (1.0 to 3.5 mg/ml), suggesting activation via different pathways for the 2 groups. A low optimum S9 protein concentration has been reported for cyclopenta(cd)pyrene (13) and interpreted as a one-step cyclopenta-ring epoxide activation pathway in contrast to the multi-step diol-epoxide pathway of B(a)P. The broad curve of the dependence of bacterial mutagenicity on S9 protein concentration for B(III)A is interpreted as the result of a combination of activation and deactivation curves resulting from the formation of the 2 major contributors to the genetic activity; B(a)P-4,5-oxide and B(a)P-diol epoxides. The similarity between S9 dependence curves of cyclopenta(cd)pyrene, B(III)A, B(II)A, and B(I)A strongly support involvement of one-step epoxidation of the cyclopenta-ring as demonstrated in the case of cyclopenta(cd)pyrene. The direct-acting mutagenicity of anti-B(III)A-1,2-oxide, which accounts for up to 40% of the response of B(III)A in S. typhimurium and V79 cells, provides additional support for this contention.

A possible explanation for the contrasting S9 dependence observed for B(III)A is that B(III)A-8,9-dihydrodiol may contribute to activity via further metabolism to a mutagenic B(III)A-8,9-dihydrodiol-10,11-oxide, in a manner analogous to benzo(e)-anthracene (43, 45). However, confirmation of this route awaits the identification of tetrol and triol products of secondary metabolism of the B(III)A-8,10-dihydrodiol. Also, additional as yet unidentified primary metabolites present in the incubation might be involved in mutagenic activity. Nevertheless, multiple pathways appear to be involved in the activity of B(III)A whose cyclopenta-ring oxide does not open to as highly stabilized a carbonium ion as B(a)P-7,8-dihydrodiol-9,10-epoxide or the cyclopenta-epoxides of the benzaceanthracene isomers.

Mutagenesis studies with B(II)A, B(II)A, B(III)A, and B(k)A in V79 cells do not reveal the same sensitivity to S9 protein concentration observed in the mutation response curves of S. typhimurium. In V79 cells, B(II)A proved to be the most active of the 4 isomers, comparable in mutagenicity to B(a)P.

Lack of mutagenicity of B(II)A, B(II)A, and B(k)A in SHE cell-V79 cell-mediated studies is of interest because most PAH are generally activated more efficiently by SHE cells than by rat liver S9 (28). Preliminary comparative studies on the quantititative metabolism of B(III)A by SHE cells and Aroclor 1254-induced rat liver S9 indicate that SHE cells produce twice as much B(III)A-9,10-dihydrodiol as B(III)A-1,2-dihydrodiol, while rat liver rapidly generates predominantly B(III)A-1,2-dihydrodiol (33). These results suggest that SHE cells can display regioselectivity for metabolism at sites other than cyclopenta-rings and that differences in activity obtained with different activating systems may, therefore, be partly ascribed to changes in regioselectivity of mixed-function oxidases.

This report suggests that B(III)A will serve as a valuable model for investigation of the balance between electronic and stereochemical factors in biological activity. Evidence clearly points to B(III)A-1,2-oxide as a major ultimate mutagen in both bacterial and V79 cell systems. The influence of both steric and electronic factors can be invoked to explain some of the behavior observed in this study. In the mammalian assays, B(III)A proved to be the most active cyclopa-hydroPAH despite the fact that the oxirene-derived carbonium ion has a smaller ΔESMIN/β than either B(II)A or B(II)A. The severe steric crowding within the 4-sided gulf, which forces the molecule to adopt a nonplanar configuration, probably plays a role in enhancing activity. Similar steric interactions appear to be associated with enhanced activity in benzo(c)phenanthrene (23, 30) and bay-region PAH in which methyl substituents protrude into bay areas (21, 40).

Cyclopenta(cd)pyrene, like B(III)A, contains a fused cyclopenta-ring, and has been reported to be a strong bacterial mutagen in S. typhimurium (10, 44) but less active than B(a)P as a gene mutagen in V79 cells (35) and less active than B(a)P in mammalian cell morphological transformation (16). In contrast, B(II)A, B(II)A, and B(k)A are more active as gene mutagens than B(a)P in S. typhimurium and are as active or more active than B(a)P in V79 cells. We have recently reported that B(I)A is approximately 4-fold more active than B(a)P, and B(II)A as active as B(a)P as a mouse skin tumor initiator in SENCAR mice (32). These results are in sharp contrast to the mouse skin tumor-initiating activity of cyclopenta(cd)pyrene which is considerably less than B(a)P (6, 35, 44).

Benz(a)anthracene is reported to be weakly active as a gene mutagen in both the SHE cell-V79 cell and rat liver S9-V79 cell mutagenesis systems (25, 42) and weakly active as a mouse skin tumor initiator (42). Fusion of a cyclopenta-ring on benz(a)anthracene to give B(II)A, B(I)A, and B(III)A has produced dramatic increases in the ability of this angular PAH to induce gene mutation in bacteria and mammalian cells and in 2 examples B(I)A and B(II)A to initiate papilloma formation in mouse skin.

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Mutagenicity of Cyclopenta-fused Isomers of Benz(a)anthracene in Bacterial and Rodent Cells and Identification of the Major Rat Liver Microsomal Metabolites

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