Effect of a 6-Fluoro Substituent on the Metabolism and Biological Activity of Benzo(a)pyrene


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ABSTRACT

Cytochrome P-450-catalyzed epoxidation of (-)-(7R,8R)-dihydroxy-7,8-dihydrobenzo(a)pyrene to (+)-(7R,8S)-dihydroxy-9S,10R-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene is now well recognized as the primary pathway by which benzo(a)pyrene is converted to an ultimate carcinogen. The present metabolism studies of 6-fluorobenzo(a)pyrene indicate (a) that the 6-fluoro substituent does not dramatically block the formation of quinones involving position 6 and (b) that substantially more (~2-fold) of the 7,8-dihydriodiol is formed from 6-fluorobenzo(a)pyrene than is formed from benzo(a)pyrene. As is the case for benzo(a)pyrene, the 6-fluoro-7,8-dihydriodiol was found to be of high optical purity and has (7R,8R)-absolute configuration. Electrostatic repulsion appears to cause the fluorinated 4,5- as well as 7,8-dihydriodiol to prefer the pseudodiaxial rather than the pseudodiequatorial conformation observed for these dihydriodils when formed from benzo(a)pyrene. Comparison of the tumor-initiating activities of benzo(a)pyrene and 6-fluorobenzo(a)pyrene on the skin of female Sencar mice indicates that the fluorinated hydrocarbon is far less active in the initiation of tumors. In addition, the metabolites of the fluorinated 7,8-dihydriodiol do not display strong mutagenic activity toward Chinese hamster V79 cells. Altered conformation of the fluorinated 7,8-dihydriodiol and the resultant 7,8-diol-9,10-epi diastereomer in which the benzylic 7-hydroxyl group and the epoxide oxygen are trans provides a plausible explanation for the weak tumorigenicity of 6-fluorobenzo(a)pyrene on mouse skin, since all benzo-ring dihydriodils and diol-epoxides in which the hydroxyl groups prefer the pseudodiaxial conformation are weak or inactive as carcinogens. In formulating the bay-region theory, we had pointed out that perfluorine substituents such as at position 6 in benzo(a)pyrene have a marked inhibitory effect on the tumorigenicity of the hydrocarbon. The present results provide a basis for this "pericffect."

INTRODUCTION

At present, the only known pathway for the metabolic conversion of BP1 to an ultimate carcinogen consists of cytochrome P-450-catalyzed oxidation of BP to (+)-benzo(a)pyrene (7R,8S)-oxide, hydration of this arene oxide to (-)-trans-(7R,8R)-dihydroxy-7,8-dihydrobenzo(a)pyrene by microsomal epoxide hydrolase, and subsequent epoxidation of this dihydrodiol to (+)-7,8,9,10-di-oxy-7,8,9,10-tetrahydrobenzo(a)pyrene, the (7R,8S,9S,10R)-enantiomer, by the cytochrome P-450 system (6, 31, 38, 41). Related bay-region diol-epoxides have been identified or implicated as ultimate carcinogens of a number of other polycyclic aromatic hydrocarbons (reviewed in Refs. 48 and 57; see also Refs. 9, 12, 18, and 40), in accord with predictions of the bay-region theory (22, 24-26). 2-Hydroxybenzo(a)pyrene is the only other potential metabolite of BP known to be highly tumorigenic (10, 11, 58, 70), but its contribution to the overall tumorigenicity of BP appears minimal, since evidence for metabolic formation of 2-hydroxybenzo(a)pyrene is presently lacking.

Several lines of evidence have been invoked to implicate a role for position 6 of BP in its overall tumorigenic response. Position 6 of BP is known to be one of the chemically most reactive sites on the hydrocarbon (3, 7, 14). Both chemical and metabolism studies have suggested that position 6 may be involved in the binding of BP to DNA (3), whereas other studies have concluded that the binding of BP to DNA as catalyzed by liver microsomes or in cultured cells does not involve position 6 (34). Part of the confusion was resolved by a subsequent study (51) which demonstrated that the extent of binding of BP to position 6 in DNA was highly dependent on the nature of the test system utilized. 6-Hydroxybenzo(a)pyrene, a probable metabolic precursor of the 1,6-, 3,6-, and 6,12-quinone metabolites of BP (19, 37, 42, 54, 55), is known to bind covalently to DNA (35, 66), presumably via the readily formed 6-oxo free radical (42). Although 6-hydroxybenzo(a)pyrene transforms Syrian hamster cells in culture (52), it is much less active than BP in the induction of sarcomas (36, 44) and in the initiation of skin and pulmonary tumors in mice (10, 30, 58). If position 6 of BP plays a significant role in its bioactivation, substitution at this position might be expected to inhibit the tumorigenic activity of the hydrocarbon. The present report examines the effects of a 6-fluoro substituent on the metabolism and tumorigenicity of BP.

MATERIALS AND METHODS

General. HPLC was done on a Spectra-Physics Model 3500B liquid chromatograph equipped with a Schoeffel Model 770 variable wavelength detector. Columns and conditions were as described. Radioactivity was measured in Aquasol with an Intertechnique SL 4000 liquid scintillation counter. UV spectra were recorded in methanol with a Hewlett-Packard Model 8450A UV/VIS spectrophotometer. NMR spectra were recorded with a JEOL FX-100 spectrometer in deuteriochloroform.
with tetramethysilane as internal standard. Samples with exchangeable hydrogen were first treated with deuteriomethanol. Mass spectra were measured with a Finnigan-MAT 0110 high-resolution gas chromatograph-mass spectrometer by CI. Circular dichroism spectra were measured with a JASCO J 500A circular dichroism spectrophotometer.

**Substrates.** BP was purchased from Sigma Chemical Co., St. Louis, Mo. \( ^{14}C \)BP (8.1 mCi/mmol) was obtained from Cal-Bionuclear Co., Sun Valley, Calif. Chromatography indicated the material was >97% radiochemically pure under the HPLC conditions used for analysis of metabolites. EnantiomERICally pure (+)-BP (7S,8S)- and (−)-BP (7R,8R)-dihydrodiols were synthesized as described (74). Reference standards of other BP metabolites were obtained as indicated in the text.

FBP was prepared by the reaction of \( F_2 \) with BP and was purified as described (1). Analysis by HPLC of material so obtained indicated the presence of a number of impurities. Preparative HPLC on a Whatman Partisol column (9.4 mm x 50 cm) eluted with 5% methylene chloride in hexane followed by further purification on a Whatman Partisol ODS column (9.4 mm x 50 cm) eluted with acetonitrile, and recrystallization from hexane provided material with \( \lambda_{max} = 171.9-172.2 \mu\) (corrected). Material so obtained was chromatographically free of BP and gave a single line in its \( ^{19}F \) NMR spectrum (30.07 ppm relative to internal \( CF_3 \)), suggestive of the absence of other fluorinated isomers in the sample. Combustion analysis indicated 7.18% fluorine (7.03% fluorine calculated for \( CF_2 OH \), and the material had major UV absorptions in methanol at \( \lambda_{max} (log \varepsilon) \) of 254 nm (4.66), 265 nm (4.69), 285 nm (4.66), 297 nm (4.75), 369 nm (4.30), and 390 nm (4.31). Eluates from the preparative HPLC columns were monitored by both absorbance at 254 nm and change in refractive index.

**Incubation Conditions.** Liver microsomes were prepared from immature (50 to 60 g) male rats of the Long-Evans strain as described previously (43). The cytosolme P-450 contents (nmol of cytochrome P-450 per mg of protein) of the microsomes from control (0.84), phenobarbital-treated (1.98), and 3-methylcholanthrene-treated (1.33) rats were determined spectrophotometrically (49).

Incubation mixtures contained 0.2 to 2.0 mg of microsomal protein, 200 \( \mu\)mol of potassium phosphate buffer (pH 7.4), 6 \( \mu\)mol of MgCl\(_2\), 2 \( \mu\)mol of NADPH, and 100 \( \mu\)mol of substrate in a final volume of 2.0 ml. Substrates were added in acetonitrile such that the final aceton concentration was 5%. Reaction mixtures were incubated for 10 min at 37°. Controls consisted of zero time incubations and incubations to which microsomes heated at 60° for 15 min were added. These blanks were comparable to results obtained by direct injection of substrate solutions onto the column. Preparative scale incubations (5 replicates) of FBP to substrate.

**Isolation of Metabolites.** Preparative separation of FBP metabolites was achieved by HPLC on a Du Pont Zorbax SIL column (6.2 mm x 25 cm) eluted with cyclohexane:dioxane:ethanol (87:8:5) at a flow rate of 2 ml/min. Under these conditions, the observed elution times were 3.0 min for FBP, 6.8 min for the phenol fraction, 22.7 min for the 4,5-dihydrodiol, 28.9 min for the 9,10-dihydrodiol, and 36.9 min for the 7,8-dihydrodiol. Individual metabolites were rechromatographed under the same conditions until they were >95% chemically pure based on monitoring the column eluate by both refractive index and absorbance at 254 nm. Stop-flow determinations of the UV spectrum at several points throughout the peak further supported the view that these metabolite fractions were homogeneous when analyzed by analytical HPLC on the Du Pont ODS column used to separate metabolites.

Analytical HPLC. Extracts from the incubations of [\( ^{3}H \)FBP, the 3 dihydrodiol fractions were isolated as above. The desired [\( ^{3}H \)FBP 7,8-dihydrodiol fraction, which was a major metabolite, was rechromatographed on a Du Pont Zorbax ODS column (6.2 mm x 25 cm) eluted with 66% methanol in water at 3 ml/min. The desired dihydrodiol emerged from the column in 4.6 min (530 \( \mu\)g, 5% of the incubated substrate) and was >95% pure when analyzed by the HPLC conditions used to study its subsequent metabolism. Only 3% of these impurities emerged from the column prior to the dihydrodiol. The remaining 4% constituted unavoidable breakdown products which emerged after the substrate.

**Tumor Studies.** Female Sencar mice, originally obtained from Dr. R. K. Bouthwell at the University of Wisconsin, Madison, Wis., are presently raised at Oak Ridge, Tenn. Mice 7 to 9 weeks of age were shaved with surgical clippers 2 days before treatment, and only those animals in the resting stage of the hair cycle were used. Groups of 30 mice received a single topical application of 200 to 800 nmol of BP or FBP dissolved in 0.2 ml of spectroquality acetone under subidated light, followed 1 week later by twice-weekly applications of 3.2 mmol of TPA in 0.2 ml of acetone. TPA was obtained from the Chemical Carcinogenesis Institute, Eden Prairie, Minn. The incidence of papillomas was recorded weekly. Mice were selected at random for histological verification of the tumors.

**Mutagenesis Assays with Mammalian Cells.** The Chinese hamster cell line V79-6 was the generous gift of Dr. E. H. Y. Chu, University of Michigan, Ann Arbor, Mich. The cells, not known to metabolize polyyclic aromatic hydrocarbons or their derivatives, were cultured as described previously (72). Metabolic activation of the (+) and (−)-BP 7,8-dihydrodiols and of metabolically formed FBP 7,8-dihydrodiol to products causing cytotoxicity or mutagenicity (8-azaguanine resistance) in the V79 cells was determined as a function of the amount of cytochrome P-450 added. The cells were incubated for 30 min at 37° with microsomal protein from the livers of 3-methylcholanthrene-treated rats and a NADPH-generating system. Details for the metabolic activation and continued manipulation of the cells were essentially as described (10). Four replicate culture dishes (60 mm) were each seeded with 10⁴ cells to evaluate toxicity, and 16 replicate culture dishes (also 60 mm) were each seeded with 10⁴ cells to assess induction of 8-azaguanine-resistant colonies.
RESULTS

Identification of FBP Metabolites. The HPLC profile of radioactive metabolites formed from FBP by liver microsomes from 3-methylcholanthrene-treated rats is shown in Chart 1. The first 3 radioactive peaks to emerge from the column were characterized as dihydrodiols based on their mass spectra (CI, NO-N₂ gas). All 3 of the compounds had m/e = 304 (M⁺) corresponding to dihydrodiols of FBP. The remarkable similarity of the UV spectra of these dihydrodiols (Chart 2) to the positionally related 4,5-, 7,8-, and 9,10-dihydrodiols of BP allowed their immediate structural elucidation. In general, the 6-fluorinated dihydrodiols had identical λ_max values, or the values of λ_max were shifted a few nm to shorter wavelengths. The relative extinction coefficients for the 3 fluorinated dihydrodiols, calculated from the specific activity of the substrate, were quite comparable to those of BP.

Assignment of structure to the 3 dihydrodiols from FBP was not apparent from their elution times on the ODS column. Initially, we had anticipated that the FBP dihydrodiols would individually elute after their BP analogues on the reverse-phase column, since fluorination of hydrocarbons often results in decreased polarity (56). Thus, FBP elutes (retention time, 48 min; Chart 1) after BP (retention time, 43.5 min; Chart 1) as anticipated. We had explained previously the elution order of dihydrodiols from reverse-phase columns based on a combination of polarity due to position of the dihydrodiol group on the hydrocarbon skeleton and on the conformation of the dihydrodiol group (63). Thus, dihydrodiols located near an end of an elongated hydrocarbon should be more polar (earlier elution) than those centrally located (later elution). Furthermore, dihydrodiols which preferred a pseudodiaxial conformation for sterical reasons (29) (BP 9,10-dihydrodiol in a bay-region) were more polar than dihydrodiols which preferred a pseudodiequatorial conformation (BP 7,8-dihydrodiol). Thus, BP 9,10-dihydrodiol elutes well before BP 7,8-dihydrodiol (19, 55, 63).

The above considerations only partially account for the elution order and retention times of the 3 dihydrodiols from FBP relative to those from BP (Chart 3). Whether 6-fluorinated or not, BP 9,10-dihydrodiol must remain pseudodiaxial because of the steric constraint of the bay-region (29). This was confirmed from the NMR spectrum of FBP 9,10-dihydrodiol: H₉, δ 4.62; and H₁₀, δ 5.74; with J₉₁₀, ≤2 Hz. For comparison, J₉₁₀ = 1.9 Hz for BP

![Chart 1](image1.png)
![Chart 2](image2.png)
![Chart 3](image3.png)
9,10-dihydrodiol. Decreased polarity due to fluorine substitution causes the FBP 9,10-dihydrodiol to elute later than the BP 9,10-dihydrodiol (19 min compared to 14 min, Chart 3). Thus, the above predictions account for the elution order of the BP and FBP 9,10-dihydrodiols. However, the 4,5- and 7,8-dihydrodiols of FBP elute much earlier and are far more polar than might have been expected (Chart 3). In both cases, we suspected that a marked increase in preference for the pseudodiadixial conformation accounted for this increased polarity. This was confirmed by the NMR spectra of these dihydrodiols. For FBP 4,5-dihydrodiol with J 4.5 = 3.8 Hz (H 4, δ 5.26, and H 5, δ 5.80) and for FBP 7,8-dihydrodiol with J 7.8 = 3.5 Hz (H 7, δ 5.66; and H 8, δ 4.64), the pseudodiadixial conformation is clearly preferred based on the small coupling constants. By comparison, the value of J 7.8 for BP 7,8-dihydrodiol is ~10 Hz (15). In the crystalline state, the hydroxyl groups of BP 7,8-dihydrodiol are known to occupy the pseudodiadixial conformation as well (46). Thus, when fluorine is adjacent to the dihydrodiol function, adverse polarity interactions between the fluorine substituent and the proximal hydroxyl group are relieved by these dihydrodiols adopting conformations in which the hydroxyl groups become pseudodiadixial.

Other metabolites of FBP consisted of a quinone fraction and a phenol fraction (Chart 1). The quinone fraction consisted of at least 2 components (retention times, 32.7 and 33.2 min) which were cochromatographic with BP 1,6- and 3,6-quinones, respectively. Mass spectrometry (Cl, NH3 gas) confirmed their assigned mass as quinones of BP by the presence of signals at m/e = 283 for (M* + 1), and their UV spectra (broad maxima in the 220- to 500-nm region) were identical to synthetic standards of 3- and 9-hydroxybenzo(a)pyrene. All of the λmax observed for 3-hydroxybenzo(a)pyrene were present and differed in position by no more than a few nm. Relative extinction coefficients were also very similar. The spectrum displayed a marked bathochromic shift when recorded in ethanol containing 0.1 N NaOH. Although the phenol fraction probably consisted mainly of 3-hydroxy-6-fluorobenzo(a)pyrene, stop-flow UV spectra taken throughout the peak indicated the presence of lesser amounts of other phenols. When the phenol fraction from FBP was rechromatographed on a Du Pont Zorbax SIL column eluted with 5% tetrahydrofuran in cyclohexane, a single peak was observed. These conditions readily separate 3- and 9-hydroxybenzo(a)pyrene. When the phenol fraction was chromatographed on the ODS column with an acetonitrile:water gradient, a substantial amount of a second phenol with a slightly longer retention time and a different UV spectrum was apparent. Decreased polarity (longer retention) of these phenols relative to 3-hydroxybenzo(a)pyrene is again consistent with fluorine substitution.

Quantitation of Metabolites from FBP. For purposes of comparison, the same microsomal preparations from control and treated rats were used to study the metabolism of BP and FBP (Table 1). Metabolism of FBP was linear with protein up to 2 mg of microsomal protein from control rats, 1 mg for phenobarbital-treated rats, and 0.4 mg for 3-methylcholanthrene-treated rats in the 2.0-ml incubations. As had been noted previously for BP (19), treatment of rats with phenobarbital caused little or no increase in the rate (nmol products per nmol cytochrome P-450 per min) of metabolism of FBP, whereas treatment with 3-methylcholanthrene caused a severalfold increase in rate. FBP was metabolized at about half the rate observed for BP with liver microsomes from control and phenobarbital-treated rats, whereas both substrates were metabolized at a high and comparable rate (4 to 5 nmol per nmol cytochrome P-450 per min) with liver microsomes from 3-methylcholanthrene-treated rats. The distributions of metabolites for the 2 substrates and the 3 microsomal preparations were quite similar, the major difference being that the 7,8-dihydrodiol as a percentage of total metabolites was increased by 2- to 3-fold with FBP compared to BP as substrate. Relative to BP, total dihydrodiols increased 35 to 50%. Notably, total quinones involving position 6 were not dramatically reduced for FBP compared to BP, particularly when microsomes from phenobarbital-treated rats were used (Table 1).

Absolute Configuration of FBP 7,8-Dihydrodiol. In an attempt to establish the absolute configuration of FBP 7,8-dihydrodiol, its circular dichroism spectrum was determined (Chart 4). For purposes of comparison, the spectrum of the (−)-BP (7R,8R)-dihydrodiol (74), the major enantiomer formed from BP (62, 78), is also shown. Although the close mirror image relationship of these spectra is suggestive that the FBP 7,8-dihydrodiol has (7S,8S)-absolute configuration, this may not be the case. Since

### Table 1

**Metabolism of [14C]BP and [3H]FBP by rat liver microsomes from control and treated rats**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Protein mg</th>
<th>4,5-diol</th>
<th>7,8-diol</th>
<th>9,10-diol</th>
<th>Quinones</th>
<th>Phenols</th>
<th>Total conversion</th>
<th>Recovery (%)</th>
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</thead>
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<tr>
<td>[14C]BP</td>
<td>Control</td>
<td>2.0</td>
<td>9.2</td>
<td>6.0</td>
<td>11</td>
<td>28</td>
<td>45</td>
<td>12 (0.74)</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>1.0</td>
<td>16</td>
<td>4.0</td>
<td>5.6</td>
<td>36</td>
<td>39</td>
<td>16 (0.80)</td>
</tr>
<tr>
<td></td>
<td>3MC</td>
<td>0.2</td>
<td>12</td>
<td>10</td>
<td>19</td>
<td>24</td>
<td>35</td>
<td>13 (4.8)</td>
</tr>
<tr>
<td>[3H]FBP</td>
<td>Control</td>
<td>2.0</td>
<td>9.8</td>
<td>19</td>
<td>12</td>
<td>16</td>
<td>43</td>
<td>6.9 (0.41)</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>1.0</td>
<td>23</td>
<td>7.9</td>
<td>5.5</td>
<td>32</td>
<td>32</td>
<td>5.6 (0.28)</td>
</tr>
<tr>
<td></td>
<td>3MC</td>
<td>0.2</td>
<td>7.6</td>
<td>28</td>
<td>20</td>
<td>13</td>
<td>31</td>
<td>11 (4.1)</td>
</tr>
</tbody>
</table>

4. Percentage of each metabolite is based on total radioactivity in identified metabolite fractions.

5. In the case of BP, the entry for phenols represents the sum of 2 phenolic fractions of which 3- and 9-hydroxybenzo(a)pyrene are predominant components (20). For FBP, the single phenol fraction is thought to be mainly the 3-hydroxy isomer.

6. Percentage of total radioactivity emerging before BP or FBP from the column.

7. Percentage of radioactivity emerging before BP or FBP in defined metabolite peaks.

8. Numbers in parentheses, nmol of product formed per nmol of cytochrome P-450 per min.

9. PB, phenobarbital; 3MC, 3-methylcholanthrene.
Metabolism and Tumorigenicity of BP

Chart 4. Circular dichroism spectra (methanol) of synthetic (−)-BP (7R,8R)-dihydrodiol (−−−−) and of FBP 7,8-dihydrodiol (------) obtained with liver microsomes from 3-methylcholanthrene-treated rats. Data for the synthetic (−)-dihydrodiol are taken from Ref. 62.

a substantial portion of the circular dichroism response of an optically active benzo-ring dihydrodiol is probably due to the skew sense of the double bond relative to the remainder of the x-aromatic system (68), a change in the conformation of the hydroxyl groups of the dihydrodiol which results in a change in this skew sense could markedly alter the spectrum. Such a change has been noted (2) when the circular dichroism spectrum of trans-1,2-dihydroxy-1,2-dihydroanthracene was compared to that of its more pseudodial (29) diacetate. The free dihydrodiol (J1,2 = 10.5 Hz) has a marked preference for the pseudodiseudatiorial conformation compared to the diacetate (J1,2 = 5.6 Hz) based on their NMR spectra (29). Since the NMR spectrum of the FBP 7,8-dihydrodiol established that it prefers the pseudodialixial conformation (this study) while the BP 7,8-dihydrodiol prefers the pseudodiequatorial conformation, the direct circular dichroism spectra of the 2 dihydrodiols can only be taken as suggestive evidence that configurational inversion to the (7S,8S)-dihydrodiol, in the FBP case relative to the BP case, has taken place. The very strong circular dichroism response of the FBP 7,8-dihydrodiol (Δε295 = −51.3; Chart 4) compared to the 7,8- and 9,10-dihydrodiols of BP (62) is indicative, however, that the FBP 7,8-dihydrodiol is either largely or entirely a single enantiomer.

Definitive assignment of absolute configuration to the FBP 7,8-dihydrodiol was achieved through an exciton chirality experiment (16). Numerous applications of this approach have been successful in assigning absolute configuration to dihydrodiols and their derivatives (cf. Refs. 33, 45, 47, 61, and 74). The (−)-BP (7R,8R)-dihydrodiol (3 mg) was dissolved in freshly distilled tetrahydrofuran (15 ml) and cooled to 0°. An excess of sodium hydride was added, and the resulting dark green solution was stirred for 30 min before addition of solid p-N,N-dimethylamino- benzoyl chloride. Within a few min, the color faded, and the mixture was brought to room temperature and stirred overnight. Usual work-up followed by HPLC purification (6.2-mm x 25-cm Du Pont Zorbax SIL column eluted with 0.5% tetrahydrofuran in methylene chloride at a flow rate of 3 ml/min; retention time, 4.5 min) provided the desired bis-ester in near quantitative yield. The mass spectrum (Cl, NH3 gas) indicated the expected molecular weight at m/e = 581 (M+ + 1). The NMR spectrum of the ester, with δ7,8 = 8 Hz (H7, δ 7.04; and H8, δ 6.16), indicated that the pseudodiexial conformations was present to a large extent. A similar approach provided the desired bis-ester of the fluorodihydrodiol. Due to instability, this ester was purified by direct injection of the crude reaction mixture onto the Du Pont Zorbax ODS column initially at 60% methanol in water. After eluting the column (1.2 ml/min) with a gradient (1%/min) to pure methanol, the desired bis-ester eluted at 60 min. The addition of a trace of ammonia and storage of the solution at −70° were effective in preventing decomposition prior to spectral determinations. The high degree of similarity between the circular dichroism spectra (Chart 5) of the bis-dimethylaminobenzoates derived from FBP 7,8-dihydrodiol and from (−)-BP (7R,8R)-dihydrodiol (74) establishes that the fluorodihydrodiol also has (7R,8R)-absolute configuration. Thus, direct comparison of the circular dichroism spectra of free dihydrodiols is not a valid means of assigning absolute configuration when substituent-induced conformational changes have occurred.

Metabolism of FBP 7,8-Dihydrodiol. Incubations identical to those which assessed metabolism of FBP were done for [3H] FBP 7,8-dihydrodiol with the exception that microsomal protein was used from either control (4.0 mg) or 3-methylcholanthrene-treated (0.5 mg) rats. These studies established that FBP 7,8-dihydrodiol was metabolized at a rate of 0.31 for microsomes from control rats and 1.24 for microsomes from 3-methylcholanthrene-treated rats, when expressed as nmol total metabolites per nmol cytochrome P-450 per min. Although considerable induction is observed on treatment of rats with 3-methylcholanthrene, the dihydrodiol is only about one third as good as substrate as FBP with these microsomes.

Chart 5. Circular dichroism spectra (methanol) of the bis-dimethylaminobenzoates of (−)-BP (7R,8R)-dihydrodiol (−−−−) and FBP 7,8-dihydrodiol (------). The spectrum of the BP 7,8-dihydrodiol ester is very similar to that reported previously in chloroform (45). Due to instability, both spectra were recorded with a relatively rapid scan time (4 min) in order to avoid decomposition during the course of the spectral determinations.
Metabolic Activation of FBP 7,8-Dihydrodiol to Mutagens.

Chinese hamster V79 cells were incubated for 30 min at 37°C with 25 nmol of (+)-BP (7S,8S)-dihydrodiol, (-)-BP (7R,8R)-dihydrodiol, or FBP 7,8-dihydrodiol; varying amounts of microsomal cytochrome P-450 from 3-methylcholanthrene-treated rats; and an NADPH-generating system (Chart 6). In accord with previous results, the (-)-dihydrodiol of BP was much more mutagenic and cytotoxic than the (+)-dihydrodiol (21), which is consistent with the mutagenic potency of the 4 metabolically possible diol-epoxides (73). The FBP 7,8-dihydrodiol was practically inactive by either criterion, e.g., less than thirteen 8-azaguanine-resistant colonies per 10⁶ survivors for the fluorodiol compared to 253 for the (-)-BP 7,8-dihydrodiol with 0.05 nmol of cytochrome P-450. Halving or doubling the concentrations of the dihydrodiols had practically no effect (<15%) on the mutation frequencies with 0.05 nmol of cytochrome P-450. Thus, FBP 7,8-dihydrodiol is an ineffective substrate for the formation of mutagens when compared to (-)-BP 7,8-dihydrodiol in this system.

Tumor Studies. The relative tumorigenic activity of BP and FBP by initiation and promotion on mouse skin is shown in Table 2. At 3 initiating doses from 200 to 800 nmol, BP was found to be at least 10-fold more active than FBP when papillomas per mouse were compared. The top of the dose-response curve was reached above 400 nmol of BP, whereas little dose-response was observed with FBP.

DISCUSSION

Comparative metabolism and tumor studies have been undertaken with BP and FBP in an attempt to evaluate the role played by position 6 in the expression of biological activity by the parent hydrocarbon. The high strength of the carbon-fluorine bond relative to the carbon-hydrogen bond was expected to inhibit metabolism and/or binding involving position 6 in FBP relative to BP. If position 6 is involved primarily in detoxification of the hydrocarbon via metabolic formation of inactive quinones (60), bay-region 7,8-diol-9,10-epoxides could constitute a larger percentage of the total metabolites of FBP relative to BP, thereby resulting in enhanced tumorigenicity on fluorination. On the other hand, tumorigenicity of BP could decrease if 6-fluorination inhibited metabolic activation and binding involving this position. The present initiation-promotion experiments on mouse skin indicate that FBP is far less active than BP (Table 2). At a 400-nmol initiating dose, BP caused 6 papillomas per mouse, whereas FBP caused only 0.4 papillomas per mouse under conditions where TPA alone caused 0.2 papillomas per mouse. A separate identical experiment gave comparable results, 5.5 papillomas per mouse with BP and 0.23 papillomas per mouse with FBP. In parallel with the present study, 6-methylbenzo(a)pyrene (59, 60) and 6-hydroxymethylbenzo(a)pyrene (59) were also found to be much weaker tumor initiators than BP on mouse skin.

Examination of the metabolism of FBP relative to BP by liver microsomes has provided an alternative explanation for the decreased activity of FBP relative to BP. Liver microsomes from control and treated rats metabolize BP and FBP at very similar rates (Table 1). Two features of these comparative metabolism studies are particularly noteworthy: (a) Total quinones involving position 6 of BP (24 to 36% of total metabolites from BP) are only reduced by a modest amount (13 to 32% of total metabolites) when FBP is the substrate. Other monoxygenase-catalyzed losses of aromatic fluorine substituents are known (13, 32). With microsomes from phenobarbital-treated rats, the reduction in quinones is so small (Table 1) that it cannot be considered significant. Furthermore, the amount of quinones from [3H]FBP is probably an underestimate, since tritium would be lost from FBP at the substituted positions. Catalytic tritiation of BP, for example, produces [3H]BP which has 28% of the tritium at position 1 (3). At least part of these BP quinones formed from FBP probably arises by cytochrome P-450-catalyzed oxidation at position 6 of FBP. Notably, the phenol fraction from FBP was found to be stable to the complete incubation system minus NADPH. In the presence of NADPH, the phenol fraction was extensively metabolized by liver microsomes from 3-methylcholanthrene-treated rats (data not shown), but BP 3,6-quinone was at best only a minor metabolite. Regardless of the...
origin of these quinones, fluorine substitution cannot be considered an effective means of blocking metabolism at position 6 of BP. (b) Total dihydrodiols, mainly the 7,8-dihydrodiol, are increased substantially with FBP as the substrate. Under the preparative incubation conditions, FBP 7,8-dihydrodiol was actually the major metabolite from the hydrocarbon. Despite the decreased tumorigenicity of FBP relative to BP, more of the potential proximate carcinogen, the 7,8-dihydrodiol of FBP, is formed. Spectral and mutagenesis studies, however, provide a basis for expecting decreased biological activity of the 6-fluoro-7,8-dihydrodiol relative to BP 7,8-dihydrodiol.

In formulating the bay-region theory, we had pointed out that substituents which were peri to the critical benzo-ring (position 6 in BP is peri to the 7,8,9,10-ring) had a marked inhibitory effect on tumorigenic activity (22, 26). The basis of this “perieffect” was unknown. A recent qualitative study of the metabolism of 5-methyl-12-fluorochrysenes (17) concluded that the peri-12-fluoro substituent inhibited formation of the 1,2-dihydrodiol relative to metabolism of 5-methylchrysene. Such is not the case for the metabolism of FBP and may not be the case for 5-methyl-12-fluorochryscene either, since the structural assignment of the 1,2-dihydrodiol was only tentative and was based primarily on relative retention time on HPLC. The present results suggest that the 1,2-dihydrodiol of 5-methyl-12-fluorochryscene would also prefer the more polar pseudodiaxial conformation. Thus, relative retention times could be quite misleading as a means of assigning structure. In summary, there is presently no evidence to suggest that reduced dihydrodiol formation is a basis by which the perieffect of the fluorine substituent can be explained.

The 7,8-dihydrodiol from FBP has markedly increased polarity relative to the 7,8-dihydrodiol from BP, based on its much earlier elution from reverse-phase HPLC columns (Chart 3). NMR spectra (see “Results”) indicate that the 6-fluorinated 7,8- (as well as 4,5-) dihydrodiol(s) prefers the more polar pseudodiaxial conformation rather than the usual pseudodiequatorial conformation when formed from BP (Chart 7). Based on theoretical arguments regarding the shape of the catalytic site of cytochrome P-450 from the livers of 3-methylcholanthrene-treated rats (27, 67, 75) and on the present spectral determinations, the fluorinated 7,8-dihydrodiol was anticipated and was found to have (7R,8R)-absolute configuration as is the case for the 7,8-dihydrodiol from BP (45, 62, 74, 78). The argument presumes that the fluoro-7,8-oxide undergoes attack by water at the nonbenzylic position as is the case for all non-K-region arene oxides for which this point has been examined with epoxide hydrolysis (23, 65, 67, 79). The absolute configuration of the fluorinated 7,8-dihydrodiol is of considerable significance, since the (7S,8S)-dihydrodiol formed from BP (62) is metabolized primarily to the 7,8-diol-9,10-epoxide diastereomer which is nontumorigenic (6).

The fluorinated 7,8-dihydrodiol is metabolized by liver microsomes from control and 3-methylcholanthrene-treated rats at very comparable rates to those observed for the unfluorinated (−)-7,8-dihydrodiol (62). Preliminary analysis has established that the major metabolite is a tetraol [UV spectrum practically identical to that of the tetraols which arise by attack of water at C-10 on the BP 7,8-diol-9,10-epoxides (76) and mass spectrum] derived from a 7,8-diol-9,10-epoxide of the fluoro-7,8-dihydrodiol. Despite the fact that the fluorinated 7,8-dihydrodiol is metabolized to a bay-region diol-epoxide, it is a weak premutagen toward Chinese hamster V79 cells (Chart 6) on metabolic activation. The weak mutagenic response on metabolic activation and presumed low tumorigenicity could, however, be explained if the resultant fluorinated 7,8-diol-9,10-epoxide(s) prefers the conformation in which the hydroxyl groups are pseudodiauxial, as is the case for the fluorinated 7,8-dihydrodiol. To date, all diol-epoxides in which the hydroxyl groups prefer the pseudodiauxial conformation are highly tumorigenic when derived from carcinogenic hydrocarbons (28, 48).

In separate studies utilizing several different tumor models, FBP has been found to have 45 to 80% of the activity of BP.5 The combined results indicate that FBP ranges from comparable to much weaker in activity than BP. If the reduced activity of FBP in certain tumor models is indeed due to a change in conformation of its bay-region 7,8-diol-9,10-epoxide, such a diol-epoxide could provide an extremely powerful probe to explore further the mechanism by which polycyclic aromatic hydrocarbons induce cancer.

ADDITIONAL

Subsequent to submission of the present manuscript, Chin et al. (Chin, P. F.; Fu, P. P.; and Yang, S. K. Effect of a Peri fluor substituent on the conformation of dihydrodiol derivatives of polycyclic aromatic hydrocarbons. Biochem. Biophys. Res. Commun., 106: 1405–1411, 1982) reported that a 7-fluorine substituent on benz[a]anthracene causes the 5,6- and 8,9-dihydrodiols produced from antracene to be weak premutagenic. The basis of this “perieffect” of the fluorine substituent can be explained.

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D. R. Buhler et al.


Effect of a 6-Fluoro Substituent on the Metabolism and Biological Activity of Benzo(α)pyrene


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