Effects of a 6-Fluoro Substituent on the Metabolism of Benzo(a)pyrene 7,8-Dihydrodiol to Bay-region Diol Epoxides by Rat Liver Enzymes*

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Metabolism of trans-7,8-dihydroxy-7,8-dihydro-6-fluorobenzo(a)pyrene by liver microsomes from 3-methylcholanthrene-treated rats and by a highly purified mono-oxygenase system, reconstituted with cytochrome P-450c, has been examined. Although both the fluorinated and unfluorinated 7,8-dihydrodiol formed from benzo(a)pyrene by liver microsomes share (R,R)-absolute configuration, the fluorinated dihydrodiol prefer the conformation in which the hydroxyl groups are pseudodiallial due to the proximate fluoro. The fluorinated 4,5- and 9,10-dihydrodiols are also >97% the (R,R)-enantiomers. For benzo(a)pyrene, metabolism of the (7R,8R)-dihydrodiol to a bay-region 7,8-diol-9,10-epoxide in which the benzylic hydroxyl group and epoxide oxygen are cis are constitutes the only known pathway to an ultimate carcinogen. With the microsomal and the purified mono-oxygenase system, this pathway accounts for 76–82% of the total metabolites from the 7,8-dihydrodiol. In contrast, only 32–49% of the corresponding diol epoxide is obtained from the fluorinated dihydrodiol and this fluorinated diol epoxide has altered conformation in that its hydroxyl groups prefer to be pseudodiallial. Much smaller amounts of the diastereomeric 7,8-diol-9,10-epoxides in which the benzylic hydroxyl groups and the epoxide oxygen are cis are formed from both dihydrodiols. As the fluorinated diol epoxides are weaker mutagen toward bacteria and mammalian cells relative to the unfluorinated dial epoxides, conformation appears to be an important determinant in modulating the biological activity of diol epoxides. One of the more interesting metabolites of 6-fluorinated 7,8-dihydrodiol was a relatively stable arene oxide, probably the 4,5-oxide, which is resistant to the action of epoxide hydrolase.

To date, the metabolic formation of bay-region dial epoxides is the only known pathway by which polycyclic aromatic hydrocarbons are activated to ultimate carcinogens (for recent reviews cf. Refs. 1 and 2). For benzo(a)pyrene (3), benzo(a)anthracene (4), and chrysene (5), the (+)-(R,S)-diol-(S,R)-epoxides+(+)-dihol epoxide-2 have been shown to be the isomers responsible for the tumorigenic activity. In the case of BP, alternate mechanisms of metabolic activation which involve the 6-position have been proposed (6, 7). Although substitution of BP at the 6-position generally reduces tumorigenic activity on mouse skin (8–11), the actual basis for the reduction in activity is presently unknown. Comparison of results obtained with BP and with 6-fluorobenzo(a)pyrene provides an interesting case. In terms of bay-region activation via the diol epoxide pathway, both hydrocarbons are metabolized by hepatic microsomal cytochrome P-450 at about the same rate, and nearly twice as much 7,8-dihydrodiol, as a percentage of total metabolites, is formed from FBP (11). Although this latter result is suggestive that fluorination at the 6-position of FBP might enhance tumorigenic activity via the bay-region pathway, metabolic activation of the 7,8-dihydrodiol from FBP produced a far weaker mutagenic response toward Chinese hamster V79 cells than did the 7,8-dihydrodiol from BP (11). The present study evaluates two possible causes for this decrease in mutagenic response from the fluorinated 7,8-dihydrodiol: that the fluorinated 7,8-diol-9,10-epoxides are metabolically formed at a lower rate and that the fluorinated 7,8-diol-9,10-epoxides have lower inherent mutagenic activity. Both of these factors could contribute to altered tumorigenicity of FBP relative to BP.

MATERIALS AND METHODS

General

HPLC analyses were performed on a Spectra-Physics Model 3500B liquid chromatograph equipped with a Schoeffel Model 770 variable wavelength detector and a HP 3390A integrator. Radioactivity was measured in Aquasol with an Intertechnique SL 4200 liquid scintillation counter. DuPont Zorbax SIL and ODS columns were used as indicated. UV spectra were recorded with a Hewlett-Packard Model 8450A UV/VIS spectrophotometer either in conventional cuvettes in the static mode or in a flow cell for HPLC effluents. NMR spectra were recorded with a JEOL FX-100 spectrometer (100 MHz) or with a Nicolet spectrometer (500 MHz). Chemical ionization mass spectra were measured with a Finnegan Model 1015D combined gas chromatograph–mass spectrometer. Circular dichroism spectra were measured with a JASCO J500A circular dichroism spectrophotometer.

1 The abbreviations used are: BP, benzo(a)pyrene; FBP, 6-fluorobenz(a)pyrene; BP, 6-fluoro 7,8-dihydroxy-7,8-dihydro-6-fluoro-7,8-diol epoxide-1, (-7,8-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydro BP; BP diol epoxide-2, (-7,8-8,9,10-epoxy-7,8,9,10-tetrahydro BP; BP diol epoxide of FBP are similarly abbreviated. FBP, 7,8-dihydrodiol, trans-7,8-dihydroxy-7,8-dihydro-6-fluoro BP; other dihydrodiols of FBP and diols of BP are similarly abbreviated: FBP, 7,8-dihydriodiol, trans-7,8-dihydroxy-7,8,9,10-tetrahydro-6-fluoro BP; FBP H4, 9,10-diol, trans-9,10-dihydroxy-7,8,9,10-tetrahydro-6-fluoro BP; FBP H4, 7,8-diol is similarly abbreviated; MAA, (−)-mentholoxacyclic acid; MTPA, (−)-α-methoxy–α-trifluoromethylbenzylcetic acid; HPLC, high-performance liquid chromatography.
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**Chemicals**

BP, glucose 6-phosphate, NADP*, and NADPH were purchased from Sigma Chemical Co. Preparative HPLC was performed with a Waters Associates HPLC pump equipped with a Waters Associates 484 variable wavelength detector and a Waters Associates Radial Pak silica column (0.8 em, 5 μm) on methylene chloride. Retention times of FBP and its metabolites were as follows: FBP (4.4 min), phenols (6.0 and 6.5 min), quinones (9.0 and 10.6 min), 4,5-, 7,8-, and 9,10-dihydrodiols (11.9, 13.2, and 15.4 min, respectively). The dihydrodiols were rechromatographed on the same column eluted with methylene chloride: retention time 1.3 min.

**Enzymes**

Glucose-6-phosphate dehydrogenase (Type XII, sulfate-free) was purchased from Sigma. Liver microsomes were prepared from male Sprague-Dawley rats (60-70 g) as described previously (11). The cytochrome P-450 content was 1.61 nmol/mg of protein as determined spectrophotometrically (18). The highly purified monooxygenase system was reconstituted with lipid, cytochrome P-450c (19), and NADPH-cytochrome P-450 reductase (20). The reconstituted and reconstituted system were performed in the presence of homogenous microsomal epoxide hydrolase (EC 3.3.2.1) (21).

**Incubations**

Metabolism of FBP—Preparative incubations were carried out in 500-ml Erlenmeyer flasks. Each flask contained 5 mmol of potassium phosphate buffer (pH 7.4), 0.15 mmol of magnesium chloride, 82.5 μmol of NADP*, 340 μmol of glucose 6-phosphate, 375 units of glucose-6-phosphate dehydrogenase, 43-132 mg of microsomal protein, and 8 μmol of FBP (added in 2.5 ml of acetone) in a total volume of 50 ml. Incubations were at 37°C for 20 min, at which time reaction was stopped by addition of 2.5 ml of acetone and 100 ml of ethyl acetate to each flask. After extraction of the products and unreacted substrate, the organic phase was separated, dried (anhydrous Na2SO4), and evaporated. The residue was subjected to preparative HPLC for isolation of individual metabolites.

Metabolites of FBP were isolated by HPLC on a DuPont Zorbax Sil column (2.1 × 25 cm) eluted with cyclohexane/dioxane/ethanol (80:15:5) at a flow rate of 20 ml/min. Retention times of FBP and its metabolites were as follows: FBP (4.4 min), phenols (6.0 and 6.5 min), quinones (9.0 and 10.6 min), 4,5-, 7,8-, and 9,10-dihydrodiols (11.9, 13.2, and 15.4 min, respectively). The dihydrodiols were rechromatographed on the same column eluted with methylene chloride: ethyl acetate (1:1) at a flow rate of 22 ml/min. Retention times of FBP 4.5-, 7,8-, and 9,10-dihydrodiols were 6.0 min, 8.0 min, and 9.0 min, respectively.

**Tetraols from FBP 7,8-Diol-9,10-epoxides**

Metabolically formed (–)-FBP (7R,8R)-dihydrodiol (>98% enantiomerically pure) isolated in the present study was epoxidized to its diastereomeric pair of 7,8- and 9,10-epoxides (isomer-1 in which the benzylc 7-hydroxyl and 9-hydroxyl groups are cis) as described separately. 2 The pure diol epoxides were used in the mutagenesis experiments described here and were hydrolyzed to tetraols by both cis and trans hydration of the epoxides at the benzylc 10-position. Relative stereochemistry of the tetraols was deduced from the NMR spectra of their tetaacetates. 2

—H. Yagi, D. R. Thakker, J. M. Sayer, W. Levin, and D. M. Jerina, unpublished observations.
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which were growing in 5 ml of culture medium. In each of at least 2 separate experiments for each compound, 4 and 16 replicate dishes were used to assess cell survival and 8-azaapamine resistance, respectively. The spontaneous mutation frequency for 3 experiments averaged 0.13 ± 0.08 (S.E.) 8-azaapamine-resistant colonies/10^6 surviving cells and never exceeded a value of 0.26. The absolute plating efficiencies of the cells averaged 90 ± 4 (S.E.) per cent for the 3 experiments and was never less than 82%.

RESULTS

Metabolism of FBP—The FBP 7,8-dihydrodiol required for the synthesis of the bay-region FBP 7,8-diol-9,10-epoxides was obtained by preparative scale incubation of FBP with hepatic microsomes from 3-methylcholanthrene-treated rats. In order to optimize the yield of the dihydrodiol, both the total metabolism of FBP and the amount of FBP 7,8-dihydrodiol formed were examined as a function of protein concentration (Fig. 1). As the protein concentration was increased from 0.9 mg/ml to 1.4 mg/ml, the percentage conversion of FBP went from 76% (6.1 μmol) to 92% (7.4 μmol); however, the yield of FBP 7,8-dihydrodiol increased by more than 2-fold from 0.5 μmol to 1.23 μmol per 50-ml incubation containing 8 μmol of FBP (8–17% of total metabolites). Further increase in protein concentration to 1.8 mg/ml did not significantly alter the yield of the 7,8-dihydrodiol. At an even higher protein concentration of 2.6 mg/ml, the yield of FBP 7,8-dihydrodiol dropped to 0.6 μmol per incubation (8% of total metabolites). At protein concentrations below 1.4 mg/ml, the percentage of 7,8-dihydrodiol formed together with a concomitant increase in the phenols formed (for phenols not shown) appears to be caused by an insufficient amount of epoxide hydrolase relative to cytochromes P-450. At protein concentrations above 1.8 mg/ml, the lower yield of the 7,8-dihydrodiol presumably reflects secondary metabolism of the dihydrodiol by the monooxygenase systems present. Interestingly, when the incubation was carried out in a 250-ml flask instead of a 500-ml flask, the conversion of FBP dropped from 76% (6.1 μmol) to 46% (3.7 μmol) at 0.9 mg/ml microsomal protein with no change in the yield of FBP 7,8-dihydrodiol. An insufficient supply of oxygen in the incubation medium in the 250-ml flask appears to be the cause for reduced conversion. Even in the 500-ml flask, the amount of oxygen must be somewhat limited since aeration of the incubation medium periodically during the 20-min incubation period increased the overall conversion of FBP by about 15% (Fig. 1). These experiments led to the conditions of incubation which gave optimum yield of FBP 7,8-dihydrodiol. Thus, 28 mg (93 μmol) of FBP 7,8-dihydrodiol was obtained by incubation of 800 μmol of FBP in 100 Erlenmeyer flasks (500-ml capacity) with 7 g of hepatic microsomal protein from 3-methylcholanthrene-treated rats for 20 min. In addition, 12 mg of the 4,5-dihydrodiol and 15 mg of the 9,10-dihydrodiol were obtained.

Enantiomeric Purity and Absolute Configuration of the 4,5-, 7,8-, and 9,10-Dihydrodiols Metabolically Formed from FBP—Assignment of enantiomeric purity to the dihydrodiols was based on the HPLC separation of derived diastereomeric bis-esters with optically active acids (Table I). Since none of the three dihydrodiols is presently available in racemic form by synthesis, the 4,5- and 7,8-dihydrodiols were prepared in racemic form by (i) oxidation to the achiral quinones with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone followed by (ii) reduction of the quinones back to the racemic trans dihydrodiols (cf. Refs. 26 and 27). The cyclic sequence was affected with excellent yields at both steps. Diastereomer separation as the bis-MAA ester was achieved directly on the racemic 4,5-dihydrodiol with an 80% valley between the peaks. For the metabolically formed dihydrodiol, 98% chromatographed as the (R,R)-diastereomer (three recycles). No attempt was made to establish that the 2% which chromatographed as the (S,S)-diastereomer peak was actually this compound. For reasons of stability, the racemic and metabolically formed 7,8-dihydrodiol was first reduced to the H4-7,8-diol before conversion to the racemic mixture of enantiomers; 80% valley for racemic. In this case, the minor peak (<2%) which corresponded to the (S,S)-diastereomer was found to have the expected UV spectrum. The diastereomeric bis-MAA esters of the semisynthetic H4,9,10-diol (enriched in (S,S)-enantiomer) were base-line-separated. None of the (S,S)-diastereomer of the reduced and esterified

| TABLE I |
| --- |
| **Position of Dihydrodiol** | **Per cent enantiomer composition** | **mg/ml tetrahydrofuran** |
| | (R,R) | (S,S) |
| 4,5* | 98 (7.8 min) | 2 (8.2 min) | ~44 (4.0) |
| 7,8* | >98 (9.0 min) | <2 (6.7 min) | ~62 (3.5) |
| 9,10* | >99 (2.9 min) | <1 (3.4 min) | ~214 (6.0) |

a Assignment of absolute configuration is as described under “Results.” Enantiomer compositions of the metabolites are based on the HPLC separations of pairs of diastereomeric bis-esters of the racemic 4,5-dihydrodiol (254 nm), H4,7,8-diol (345 nm), and H4,9,10-diol (254 nm) of FBP. Retention times under the chromatography conditions described below are given in the parentheses.

b Chromatographed as the bis-MAA esters of the metabolically formed FBP 4,5-dihydrodiol on a DuPont Zorbax SIL column (0.62 × 25 cm) eluted with 85% ether in cyclohexane at a flow rate of 6.5 ml/min.

c Chromatographed as the bis-MTPA esters of the H4,9,10-diol obtained by reduction of the metabolically formed FBP 7,8-dihydrodiol; DuPont Zorbax ODS column (0.46 × 25 cm) eluted with a water-acetonitrile gradient from 90% acetonitrile in water to 100% acetonitrile (1% min) at a flow rate of 2.0 ml/min.

d Chromatographed as the bis-MTPA esters of the H4,9,10-diol obtained by reduction of the metabolically formed FBP 9,10-dihydrodiol; DuPont Golden SIL column (0.62 × 10 cm, 3 μ) eluted with 10% ether in cyclohexane at a flow rate of 2.2 ml/min.

Fig. 1. Effect of protein concentration on the metabolism of FBP (C—C) and on the metabolic formation of FBP 7,8-dihydrodiol (C—C). The solid circle and square indicate results of experiments performed in 250-ml flasks as opposed to other experiments performed in 500-ml flasks. Lower percentage conversion of the substrate in 250-ml flasks compared to that in 500-ml flasks appears to be due to the limited supply of oxygen. The arrow indicates increase in total conversion of the substrate upon aeration of the incubation medium.
9,10-dihydrodiol metabolite was detected. Thus, all three of
the metabolically formed fluoro dihydrodiols consist of at
least 98% of the (R,R)-enantiomer. Details of the chromatog-
raphy are described in the footnotes to Table I.

Assignment of (7R,8R)-absolute configuration to the met-
abolically formed (−)-FBP 7,8-dihydrodiol was achieved in
an earlier study (11). Assignment of absolute configuration
to the other two dihydrodiols is described in the present study.

As previously noted (11), circular dichroism spectra of
closely related dihydrodiols may be used to infer absolute
configuration provided their preferred conformations are iden-
tical. Evidence based on HPLC retention times suggests that
BP 4,5-dihydrodiol is mainly pseudodiequatorial while FBP
4,5-dihydrodiol is mainly pseudodiaxial (11). Although the
NMR spectrum of FBP 4,5-dihydrodiol confirms that its
hydroxyl groups prefer the pseudodiaxial conformation (11),
magnetic equivalence of the hydrogens at C-4 and C-5 in BP
4,5-dihydrodiol precludes the use of NMR to assign its con-
formation. However, the 500-MHz NMR spectra (CDCl3) of
the diacetates of BP 4,5-dihydrodiol and FBP 4,5-dihydrodiol
revealed coupling constants of 4.0 Hz and 2.0 Hz, respectively,
between the hydrogens at C4 and C5, indicating that the ace-
toxy groups prefer the pseudodiaxial conformation in both
esters. The marked similarity of the CD spectra (Fig. 2A) of
the diacetates of the (−)-FBP 4,5-dihydrodiol metabolite and
of (+)-BP (4R,5R)-dihydrodiol (28) indicates that (4R,5R)-
absolute configuration is common to both dihydrodiols despite
the fact that they have opposite signs for their values of [α]D
(tetrahydrofuran). Although the CD spectra (methanol) and
NMR spectra (chloroform) were run in different solvents, this
should not significantly affect the conformations of the di-
hydrodiols as their diacetates. Because the hydroxyl groups of
both BP and FBP 9,10-dihydrodiol form parts of bay-
regions, steric hindrance causes them to prefer the pseudo-
diaxial conformation (11, 29). Thus, their CD spectra can be
directly compared. The marked similarity of the CD spectra
(Fig. 2B) of (−)-BP 9R,10R-dihydrodiol (30) and the (−)-
FBP 9,10-dihydrodiol indicates that both have (R,R)-absolute
configuration.

An alternate method of assigning absolute configuration to
trans dihydro- and tetrahydrodiols of polycyclic aromatic hy-
drocarbons is based on the NMR spectra (CDCl3) of their bis-
MAA esters. For the (S,S)-diastereomers, the hydrogens of the
−OCH2CO− groups are magnetically nonequivalent whereas these hydrogens are magnetically equivalent or very
nearly so in the (R,R)-diastereomer (31 and references therein).
For the bis-MAA ester of the metabolically formed
FBP 4,5-dihydrodiol, the hydrogens of each −OCH2CO−
group appear as singlets (δ 3.50 and 3.61) while in the more
polar diastereomer (Table I) each of these groups appears as
an AB-quartet centered at δ 3.50 and 3.62 with δm n = 16 Hz.
A similar result pertains to the enantiomers of the FBP Hg
9,10-diol as its bis-MAA esters. Thus, both the CD and NMR
methods lead to the same assignments as designated in Table I.

Metabolism of (−)-FBP (7R,8R)-Dihydrodiol—The semisyn-
thetic FBP 7,8-diol-9,10-epoxide-1 and -2 of BP and FBP
were found to hydrolyze completely to tetraols (Fig. 3) upon incuba-
tion with microsomes in the absence of NADPH, work-up,
and chromatographic analysis. This is consistent with the
observed spectrophotometric half-life (0.10 m phosphate, pH
7.4, 37 °C) of 88 s for diol epoxide-2 under the conditions of
incubation. Notably, under the solvolysis conditions used to
study the chemical hydrolysis of diol epoxide-2 (pH ≥ 7, 1
mM buffer containing 0.1 m NaClO4 and 10% dioxane, 25 °C),
~40% of the products consists of the 9-keto 7,8-diol under
these spontaneous hydrolysis conditions. Although this keto
diol is not detected chromatographically, it is readily identi-
fiable after reduction with sodium borohydride to a diaster-
eoemeric pair of 7,8,9-triols. Application of this technique to
FBP diol epoxide-2 which had been subjected to the incuba-
tion conditions failed to produce detectable triol. The absence
of detectable keto diol which forms under the conditions of
spontaneous hydrolysis is probably due to the fact that about
half of the diol epoxide hydrolysis which occurs in the incu-

![Fig. 2. Circular dichroism spectra of (A) the diacetate of FBP 4,5-
dihydrodiol (−) and (B) FBP 9,10-dihydrodiol (−). The dihydro-
diols were formed by liver microsomes from 3-methylcholanthrene-treated rats. For comparison, circular dichroism spectra of (A) the diacetate of (−)-(4R,5R)-BP 4,5-dihydrodiol (−) and of (B) (−)-(9R,10R) -BP 9,10-dihydrodiol (−) are also shown. All spectra were measured in methanol.](image)

![Fig. 3. Hydrolysis products of the diastereomeric 7,8-diol 9,10-epoxides-1 and -2 of BP and FBP.](image)
Metabolism of 6-Fluorobenz(a)pyrene 7,8-Dihydrodiol

TABLE II

Metabolism of (−)-FBP (7R,8R)-dihydrodiol by liver microsomes from 3-methylcholanthrene-treated rats and by a highly purified and reconstituted system containing cytochrome P-450c with or without epoxide hydrolase.

Experimental conditions were as described under "Materials and Methods." The substrate concentration was 100 nmol/2.0-ml incubation. Data are expressed as the percentage of each metabolite compared with total metabolites eluting in the defined peaks.

| Protein (amount/2.0-ml incubation) | Per cent of total metabolites |
|-----------------------------------|------------------------------|
|                                   | Tetrosols from diol epoxide-1 | Tetrosols from diol epoxide-2 | Phenolic dihydrodiols | 7,8-Diol-4,5-oxide | % metabolism* (rate) |
|                                   | trans-1 + cis-1 | trans-2 | cis-2 | 1 + 2 | 3 | |
| Microsomes (0.5 mg)               | 6              | 38      | 11    | 23   | 11 | 11 | 10 |
| Cytochrome P-450c (0.1 nmol)      | 6              | 25      | 7     | 29   | 23 | 10 | 10 |
| Cytochrome P-450c (0.2 nmol)      | 4              | 25      | 11    | 28   | 22 | 10 | 11 |
| Cytochrome P-450c (0.4 nmol)      | 6              | 26      | 10    | 25   | 23 | 10 | 28 |
| Cytochrome P-450c (0.4 nmol + 32 μg epoxide hydrolase) | 7 | 27 | 9 | 25 | 21 | 11 | 19 |

* Per cent metabolism denotes the percentage conversion of substrate; i.e. total radioactivity above blank which emerges from the column before the substrate. Total metabolism does not take into account traces of unextracted metabolites, highly polar and nonextracted metabolites, or covalently bound metabolites which remain in the aqueous phase after extraction. Recovery (the percentage of the total radioactivity due to metabolism emerging from the column before the substrate in defined metabolite peaks as compared with total radioactivity due to metabolism) was 64-77% for the above incubation conditions. Rate of metabolism given in parentheses is expressed as nanomoles of extracted metabolites/nmol of cytochrome P-450c/min.

FIG. 4. Metabolites of radioactive (−)-FBP (7R,8R)-dihydrodiol formed by a highly purified monooxygenase system reconstituted with cytochrome P-450c (0.1 nmol/ml). Metabolites were separated by HPLC on DuPont Zorbax ODS column (0.62 × 25 cm) eluted with a linear gradient of 50% methanol in water to 100% methanol at a flow rate of 1.2 ml/min. Column effluent was monitored at 254 nm and fractions were collected every 0.3 min for scintillation spectrometry. Tetrosols from unlabeled FBP 7,8-diol-9,10-epoxides were used as chromatographic standards. See Figs. 3 and 6 for structures of the metabolites.

bation medium is the result of general acid catalysis (32) by the phosphate present. A longer half-life of 160 s was observed in 1 mM N,N-bis-(2-hydroxyethyl)-2-aminethanesulfonic acid buffer, pH 7.0, containing 0.1 M sodium perchlorate, 37 °C. Thus, the extent to which diol epoxides are formed on metabolism of the dihydrodiol will be reflected by the amount of tetrosols detected. Metabolites formed on incubation of
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**Fig. 6.** Metabolites of (−)-FBP (7R,8R)-dihydrodiol (DHD) formed by liver microsomes from 3-methylcholanthrene-treated rats and by the purified system reconstituted with cytochrome P-450c.

| Table III |
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Effect of a 6-fluoro substituent on the mutagenicity of bay-region epoxides of BPC in bacterial and mammalian cells

| Epoxide | Relative mutagenic activitya |
| --- | --- |
| (−)-BP (R,S)-diol(S,R)-epoxide-2 | 100 |
| FBP (R,S)-diol-(S,R)-epoxide-2 | 100 |
| (−)-BP (R,S)-diol-(R,S)-epoxide-I | 100 |
| FBP (R,S)-diol-(R,S)-epoxide-I | 100 |
| (±)-BP H2,9,10-epoxide | 100 |
| FBP H2,9,10-epoxideb | 100 |

*Within each of the 3 pairs of epoxides, the mutagenic activity of the unsubstituted BP epoxide was assigned a relative activity of 100.

*Reversion of histidine-dependent growth to histidine-independent growth in strains TA 98 and TA 100 of *Salmonella typhimurium* was assessed as previously described (34) utilizing 4 concentrations of each epoxide.

Induction of 8-azaguanine resistance in cultured Chinese hamster V79 cells was assessed as previously described (25), utilizing from 3-4 concentrations of each epoxide.

The enantiomeric composition of FBP H2,9,10-epoxideb is 68% (S,R,10)-enantiotmer and 32% (R,S,10)-enantiotmer. For purposes of comparison, the racemic activity of the (±) and (−)-enantiomers of BP H2,9,10-epoxide did not differ by more than a factor of 1.3 in either strain of *Salmonella typhimurium.*

radioactive (−)-FBP (7R,8R)-dihydrodiol with liver microsomes from 3-methylcholanthrene-treated rats and with the highly purified monooxygenase system reconstituted with cytochrome P-450c are given in Table II. A typical metabolism profile and the HPLC conditions are shown in Fig. 4. Since the tetraols which arise from diol epoxide-1 were minor metabolites (<7%), chromatographic conditions were selected to optimize the separation of other metabolites. Tetraols, identified by their UV spectra and retention times, ranged from 38% to 55% of the total metabolites. The next most significant group of metabolites consisted of three phenolic dihydrodiols, and the phenolic nature of these metabolites was apparent from the chromatographic shift of their absorption bands in alkaline solutions (Fig. 5A, inset) and by an increase of 16 mass units over that of the dihydrodiol as apparent from their molecular ions (Cl, NO/N2) at m/z = 318. The metabolite that elutes just after phenolic dihydrodiol-1 and -2 has a UV spectrum similar to that of chrysene 1,2-dihydrodiol (33) (Fig. 5B) indicating that it is probably an arene oxide at the 4,5-position of FBP 7,8-dihydrodiol. From experiments with the reconstituted system in the presence of epoxide hydrolase, it is clear that the arene oxide is highly resistant to the action of this enzyme. The arene oxide is labile and readily isomerizes in the presence of a trace amount of acid to form the phenolic dihydrodiol-3. Although the total amount of the arene oxide and resultant phenolic dihydrodiol remained constant for a given incubation, their ratio varied depending upon the particular ODS column utilized for the analysis. Structures of the various metabolites are shown in Fig. 6.

**Mutagenicity of the FBP 7,8-Diol-8,10-epoxides—Mutagenicity of FBP 7,8-diol-8,10-epoxides-1 and -2 toward S. typhimurium strains TA 98 and TA 100 as well as toward Chinese hamster V79 cells was compared with that of their unfluorinated counterparts (Table III). The fluoro diol epoxides had only 13-36% of the mutagenic activity of the unfluorinated bay-region diol epoxides in bacterial and mammalian cells. In contrast, FBP H2,9,10-epoxide showed 75-104% of the mutagenic activity of its unfluorinated counterpart toward both bacterial and mammalian cells (Table III).

**DISCUSSION**

As is the case for the metabolism of BP, the combined action of hepatic microsomal cytochrome P-450 and epoxide hydrolase converts FBP to (R,R)-dihydrodiols at the 4,5-, 7,8-, and 9,10-positions with very high enantiomeric purity. Based on a steric model for the catalytic binding site of cytochrome P-450c (34), the only known cytochrome P-450 isoyme which has a high turnover number for the oxidation of polycyclic aromatic hydrocarbons, this result was to be expected since the two hydrocarbons are isosteric. The site model also accommodates the fact that (−)-BP (7R,8R)-dihydrodiol is converted mainly to the 7,8-diol-9,10-epoxide-2 diastereomer (81-86% of total diol epoxides) relative to the diol epoxide-1 diastereomer. Since the reverse is true (dil epoxide-1 favored 20:1 over diol epoxide-2) for (+)-BP (7S,8S)-dihydrodiol, cytochrome P-450c shows a marked preference for a specific stereoheterotrophic face of BP 7,8-dihydrodiol and ignores the absolute configuration of the trans diol group. Consistent with the model, cytochrome P-450c favors formation of the diol epoxide-2 (83-89% of total diol epoxides) relative to the diol epoxide-1 diastereomer of (−)-FBP (7R,8R)-dihydrodiol. The parameter of altered substrate conformation has been introduced through study of the metabolism of the fluorinated dihydrodiol. Unlike BP 7,8-dihy-
driodiol, the FBP 7,8-dihydrodiol prefers the pseudodiaxial rather than the pseudodiequatorial conformation for its hydroxyl groups due to adverse electrostatic interaction between the proximate fluorene and hydroxyl substituents when in the pseudodiequatorial conformation. Thus, at least in the cases of the pseudodiequatorial conformation of BP and FBP, the predicted facial preference of cytochrome P-450c at the 9,10-position is practically unaffected by either the absolute configuration (compare (+)- and (-)-7,8-dihydrodiols of BP and (-)-dihydrodiol of FBP) or preferred conformation (compare (-)-BP 7,8-dihydrodiol with (+)-FBP 7,8-dihydrodiol).

We had previously observed that two other pseudodiaxial, benzo-ring dihydrodiols, BP 9,10-dihydrodiol (35) and benzo(e)pyrene 9,10-dihydrodiol (36) formed at best only trace amounts of benzo-ring diole epoxides on metabolism by cytochrome P-450c or liver microsomes from 3-methylcholanthrene-treated rats despite the obvious chemical preference for oxidation on the nonaromatic double bond. Hence, we had proposed that the increased bulk and/or polarity of the pseudodiaxial hydroxyl groups inhibited metabolism at the adjacent double bond (35,36). Although several conflicting reports (37–39) have attempted to address this question, a definitive conclusion has not been forthcoming (27). The present study represents the first example where the metabolism of the same regioisomer of a dihydrodiol in either the pseudodiaxial or -diequatorial conformation, depending on the presence or absence of an adjacent perfluorine substituent (40), has been studied. Three features are salient. First, neither the overall rate of metabolism nor the amount of 9,10-dihydrodiol as a per cent of total metabolites is changed by introduction of the 6-fluores substituent into BP when metabolized by liver microsomes from 3-methylcholanthrene-treated rats (11). Thus, fluorination at the 6-position does not affect the regiospecificity of cytochrome P-450c for the 9,10-double bond when the two hydrocarbons are compared. Second, the rates at which cytochrome P-450c oxidizes FBP 7,8-dihydrodiol (10 nmol/nmol of cytochrome P-450c/min) and BP 7,8-dihydrodiol (cf. Ref. 15 and redetermined in the present study as 8 nmol/nmol of cytochrome P-450c/min) are essentially identical. Third, bay-region diole epoxides account for about 95% of the metabolites from (+)-BP 7,8-dihydrodiol but only 38–43% of the metabolites from (-)-FBP 7,8-dihydrodiol with cytochrome P-450c. Regardless of the mechanism, we interpret these results as clear evidence that pseudodiaxial hydroxyl groups inhibit metabolism at an adjacent benzo-ring, double-bond relative to pseudodiequatorial hydroxyl groups. In conclusion, we feel that future comparisons of relative mutagenic activity for reactive metabolites should attempt to take into consideration such differences in solvolytic lifetime.

A particularly interesting aspect of the present study has been the tentative identification of a K-region, arene 45-oxide as a relatively stable metabolite of FBP 7,8-dihydrodiol. Because the metabolite is a relatively minor product with microsomes and because it readily isomerizes to phenolic dihydrodiol-3, attempts were not made to isolate a sufficient quantity for an NMR spectrum. The similarity of its UV spectrum to that of chrysene 1,2-dihydrodiol rather than benzo(a)anthracene 8,9-dihydrodiol along with its relative stability argue for a K-region arene oxide at the 4,5-position of the FBP 7,8-dihydrodiol. The site model for cytochrome P-450c predicts that the FBP 7,8-dihydrodiol as well as BP are attacked from the same face during epoxide formation at the 4,5-position. The main diastereomer produced would thus have the epoxide group (45S,5R-absolute configuration) trans to the benzylic 7-hydroxyl group with FBP 7,8-dihydrodiol as the substrate. This particular stereoisomer would be predicted to bind poorly to the catalytic site of epoxide hydrolase (41) since the diol group would reside in the hydrophobic pocket of the enzyme. This argument perhaps accounts for the fact that an amount of epoxide hydrolase (16 pg/ml) sufficient to hydrate completely 3 times the amount of BP 4,5-oxide formed in the reconstituted system (42) failed to metabolize a detectable amount of the FBP 7,8-diol-4,5-oxide. The low solvolytic reactivity and recalciitrance of the metabolite toward epoxide hydrolase is reminiscent of a recently isolated arene oxide metabolite of β-naphthoflavone (43–45).

(-)-FBP (7R,8R)-dihydrodiol produces a far weaker mutagenic response than does (+)-BP (7R,8R)-dihydrodiol when metabolically activated by liver microsomes from 3-methylcholanthrene-treated rats in the presence of Chinese hamster V79 cells (11). Results of the present study show that the markedly decreased mutagenic response is not explicable in terms of the decreased conversion of FBP 7,8-dihydrodiol to bay-region diol epoxides. For this reason, the diastereomeric 7,8-diol-9,10-epoxides of (-)-FBP (7R,8R)-dihydrodiol were compared to their BP counterparts for inherent mutagenic activity toward bacterial and mammalian cells. A several-fold decrease in mutagenic activity was observed for the fluorinated diol epoxides relative to the unfluorinated compounds in the three test systems. Although changes in chemical reactivity due to fluorine substitution might be responsible for the decreased mutagenicity, this does not seem to be the case. At neutral to alkaline pH in the absence of buffer catalysis, the FBP 7,8-diol-9,10-epoxide-2 diastereomer undergoes spontaneous hydrolysis to tetraols at about 3 times the rate of its highly tumorigenic (+)-BP (7R,8S)-dihol-9S,10R)-epoxide counterpart. The small increase in rate for the fluorinated molecule has been attributed to competing inductive and stereoelectronic factors. Notably, in the phosphate-buffered saline medium at 37 °C used for the bacterial mutagenesis assays, BP diol epoxide-2 reacts approximately 1.5 times faster than its fluorinated analog which has a half-life of ~2 min. This reversal in reactivity results from differential susceptibility of the two diol epoxides to general acid-catalyzed hydrolysis by the 5 mM phosphate buffer. Thus, the solvolytic lifetime of the fluorinated FBP diol epoxide-2 is actually longer than that of BP diol epoxide-2 under conditions of the bacterial mutagenesis assay. Since the FBP diol epoxide-1 is much more reactive (cf. Table 111) than its BP counterpart 9S,10R)-epoxide-2 diastereomer is 240-fold less reactive (much longer lifetimes) than its BP counterpart toward spontaneous solvolyis, there was no need to check for differential susceptibility to phosphate catalysis with the fluorinated and unfluorinated diol epoxide-1 diastereomers. In conclusion, the several-fold decrease in mutagenic activity on fluorination of the BP diol epoxide-1 and -2 diastereomers is not due to decreased solvolytic lifetime since the fluorinated compounds have comparable to greatly increased solvolytic half-lives. In view of the above findings, we feel that future comparisons of relative mutagenic activity for reactive metabolites should attempt to take into consideration such differences in solvolytic lifetime.

Comparisons of mutagenic activity between the BP and FBP 7,8-diol-9,10-epoxide-2 isomers were made for compounds with the same absolute configurations, (7RS,8S)-dihol-9(S,10R)-epoxide. This point is important since marked differences in mutagenic activity have been noted between enantiomers, especially toward Chinese hamster V79 cells (46–49). In contrast, bay-region tetrahydro epoxides show little difference in mutagenic activity between enantiomers (cf. Table 111 and Refs. 47 and 48). The data in Table 111 indicate that the presence of a 6-fluoro substituent on BP H4-9,10-epoxide has only a minor retarding effect (0–25%) on mutagenic activity toward the bacterial and mammalian cells. In the phosphate-buffered saline medium used with the bacterial cells, BP H4-9,10-epoxide has a solvolytic half-life of 37 s and
FBP, 9,10-epoxide has a half-life of 420 s. The competing effects, 10-fold increased half-life versus decreased chemical reactivity for the FBP H,9,10-epoxide, appear to cause comparable mutagenic activity for the fluorinated and unfluorinated 9,10-epoxides. At present, our best explanation for the 81–87% decrease in mutagenic activity of the fluorinated diol epoxides toward the mammalian cells is that it is a consequence of the markedly enhanced preference of these diol epoxides for the in which their hydroxyl groups are pseudodial..

The decreased tumorigenic activity of FBP relative to BP on mouse skin cannot be explained as a direct consequence of differences in metabolism. In terms of the bay-region diol epoxide-2 stereoisomers is comparable but preferred conformation is 10-fold increased half-life.

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