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Metabolism of Benzo[a]anthracene to Its Tumorigenic 3,4-Dihydrodiol

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SUMMARY

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The weak carcinogenicity of benzo[a]anthracene may be due to either low amounts of the tumorigenic 3,4-dihydrodiol formed or poor conversion of this diol to the bay-region diol epoxides, i.e., benzo[a]anthracene 3,4-diol-1,2-epoxides. We have investigated the metabolism of benzo[a]anthracene with rat liver microsomes and a highly purified monooxygenase system reconstituted with cytochrome P-448 to determine the relative amounts of the 3,4-dihydrodiol formed. With liver microsomes from induced and uninduced rats, as well as with the purified and reconstituted system in the presence of epoxide hydrase, benzo[a]anthracene was metabolized predominantly to its 5,6- and 8,9-dihydrodiols. Small but significant amounts of the 3,4- and 10,11-dihydrodiols were also detected by chromatographic methods and fluorescence spectrometry. Since only trace amounts of phenols were detected, the arene oxides of benzo[a]anthracene must be good substrates of epoxide hydrase. With the purified and reconstituted system in the absence of epoxide hydrase, only phenols and the K-region 5,6-oxide were found to be metabolites of benzo[a]anthracene. Moreover, the extent of metabolism of benzo[a]anthracene was substantially reduced in the absence of epoxide hydrase, suggesting that phenolic metabolites are potent inhibitors. Strong inhibition of the metabolism of benzo[a]anthracene by synthetic 5- and 6-hydroxybenzo[a]anthracenes and by a mixture of phenolic metabolites was observed.

INTRODUCTION

Polycyclic aromatic hydrocarbons are among those chemical carcinogens which require metabolic transformation to highly reactive products before they covalently bind to tissue constituents and cause cancer (1-4). Extensive studies on the metabolic activation of the carcinogen benzo[a]pyrene (BP)[1] have indicated that this hydrocarbon is metabolized to an ultimate carcinogen (+)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydro BP through a proximate carcinogen (-)-7β,8α-dihydroxy-7,8-dihy-

[1] Abbreviations used are: BP, benzo[a]pyrene; BA, benzo[a]anthracene; BA 3,4-dihydrodiol, *trans*-3,4-dihydroxy-3,4-dihydrobenzo[a]anthracene; BA 1,2-, 5,6-, 8,9- and 10,11-dihydrodiols, other trans dihydrodiols of BA; 8-HOBA, 8-hydroxybenzo[a]anthracene; other phenols of BA are abbreviated similarly; BA diol epoxides, either or both of the diastereomerically related diol epoxides of benzo-ring trans dihydrodiol in which the benzylic hydroxyl group and the epoxide oxygen are either cis or trans;[2] HPLC, high pressure liquid chromatography.

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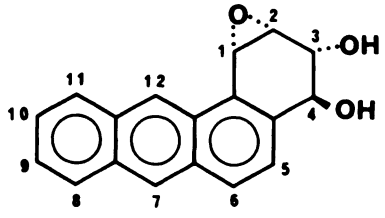
dro BP (5–20). Based on these studies, on existing carcinogenicity data for alkyl and fluorine substituted polycyclic aromatic hydrocarbons, and on quantum mechanical calculations, a hypothesis termed the "bay-region" theory was formulated in an attempt to explain and predict the carcinogenicity of polycyclic aromatic hydrocarbons (21–24). This theory postulates that a diol epoxide on a saturated, angular benzo-ring in which the epoxide forms part of a bay-region of the polycyclic hydrocarbon should be a highly reactive metabolite which may also be an ultimate carcinogen. The ability of the theory to make successful predictions of relative carcinogenicity requires that the hydrocarbon be metabolized to a dihydrodiol with a bay region double bond which is further metabolized to a diol epoxide.

Biological studies of the polycyclic hydrocarbons benzo[a]anthracene (BA)[2] (25–29), dibenzo[a,h]anthracene (30), 7-methylbenzo[a]anthracene (31, 32), 7,12-dimethylbenzo[a]anthracene (33, 34), 3-methylcholanthrene (34–36), and chrysene (37, 38) have provided support for the predictions of the "bay-region" theory. Extensive studies of BA and its derivatives have shown (i) that BA 3,4-dihydrodiol, which contains a bay-region double bond, is metabolically activated to potent bacterial mutagens (25), and is highly tumorigenic on mouse skin (28) and in the newborn mouse (27) when compared with BA and its four other isomeric dihydrodiols; (ii) that the bay-region BA 3,4-diol-1,2-epoxides are 15–125 times more mutagenic to *S. typhimurium* strain TA 100 and Chinese hamster V79 cells than are the other non-bay-region diol epoxides of BA (26); and (iii) that these bay-region diol epoxides show high tumorigenicity on mouse skin (29) and in the newborn mouse.[3] The parent hydrocarbon is, however, a weak carcinogen, possibly due to low metabolic conversion of BA to BA 3,4-dihydrodiol.

Qualitative analyses of the metabolism of BA by thin-layer chromatography (39–45), have unequivocally identified BA 5,6- and 8,9-dihydrodiols as major metabolites. With rat liver homogenates (41) and microsomes (45), small amounts of BA 1,2-dihydrodiol, 3- and 4-HOBA and BA 7,12-quinone were also detected. *In vivo* rats, rabbits and mice produce a mercapturic acid at the 5,6-position of BA as the major metabolite (40). Minor metabolites consisted of sulfates or glucuronides of 3-, 4-, 8- and 9-HOBA as well as BA 3,4-, 5,6-, 8,9- and 10,11-dihydrodiols. Since these earlier studies often lacked appropriate synthetic reference standards, the identification of BA 3,4-dihydrodiol must be considered as tentative. We have studied the metabolism of BA by rat liver microsomes and by a highly purified and reconstituted monooxygenase system and have analyzed the products by high pressure liquid chromatography. The principal aim of the present investigation has been to establish the extent to which BA is converted to BA 3,4-dihydrodiol, a compound which tumor studies (27, 28) indicate is a proximate carcinogen of BA if formed. Although a recent report (46) claimed that rat liver microsomes do not metabolize BA to its 3,4-dihydrodiol, our results indicate that the 3,4-dihydrodiol is indeed formed and that the small quantity produced, together with the lower biological activity of the BA 3,4-diol-1,2-epoxides compared to the BP 7,8-diol-9,10-epoxides (26), could account for the weak carcinogenic activity of BA relative to BP.

[2] The numbering system used for benzo[a]anthracene is illustrated below for (±)-3α,4β-dihydroxy-1α,2α-epoxy-1,2,3,4-tetrahydrobenzo[a]anthracene in which the benzylic 4-hydroxyl group and the epoxide oxygen are trans:



MATERIALS AND METHODS

Chemicals. [^{14}C]BA (20 μCi/μmol), [^{3}H]BA (129.8 μCi/μmol), and [^{14}C]BP (8.1

[3] In parallel with the tumor studies on the diastereomeric BP 7,8-diol-9,10-epoxide, the trans diastereomer of the BA 3,4-diol-1,2-epoxides[2] was much more tumorigenic than the cis diastereomer; W. Levin, et al., manuscript in preparation.

μCi/μmol) were obtained from New England Nuclear. [^{14}C]BA was found to be 98.8% pure when analyzed by HPLC. [^{3}H]-BA was purified by silica column chromatography on elution with hexane:benzene (19:1), and its purity was checked by HPLC. BA 1,2-, 3,4-, 8,9- and 10,11-dihydrodiols were prepared as previously described (47) as were diol epoxides of BA (48). BA 5,6-dihydrodiol (49), BA 5,6-oxide (50), BA 5,6-quinone (49), 1- and 4-HOBA (51), 5- and 6-HOBA (52), 8-HOBA (53, 54), and 10-HOBA (55, 56) were prepared by established procedures as were 2- and 9-HOBA. BA 7,12-quinone was obtained from Eastman-Kodak Chemical Co.

Enzyme preparations: Liver microsomes were prepared from immature (50–60 g), male rats of the Long-Evans strain as described previously (57). Cytochrome P-450 content for microsomes from control rats (0.81 nmol/mg of protein) or from phenobarbital-(2.30 nmol/mg of protein) or 3-methylcholanthrene-(1.32 nmol/mg of protein) induced animals were determined as described (58). Highly purified cytochrome P-448 (59), NADPH-cytochrome c reductase (60) and homogeneous epoxide hydrase (61) were prepared by established procedures which define units of activity.

Incubation mixtures with liver microsomes contained 200–1000 μg of microsomal protein, 200 μmol of potassium phosphate buffer (pH 7.4), 6 μmol of $MgCl_2$, 1 μmol of NADPH, and 100 nmol of [^{14}C]BA (in 100 μl of acetone) in a total volume of 2.0 ml. For incubations with the reconstituted system (6, 59) microsomes were replaced by 0.005 to 0.20 nmol of cytochrome P-448, 1500 units of cytochrome c reductase and 60 μg of dilauroyl phosphatidylcholine, and the pH of the incubation mixture was adjusted to 7.0 instead of 7.4 since pH 7.0 is optimal for the reconstituted system. All incubations were at 37° for 10 min. After this period, BA and its metabolites were extracted into ethyl acetate:acetone (2:1), dried (anhydrous Na_2SO_4), and concentrated as described for BP and its metabolites (6, 57, 62). This procedure extracts 94 to 99 percent of the metabolites depending upon the extent of conversion. Dried samples were dissolved in 150 μl of methanol and an aliquot was injected onto the column for analysis. Metabolism of BP was analyzed as previously described (62).

Analysis of BA metabolites by high pressure liquid chromatography. Known and potential metabolites of BA were chromatographed with a Spectra Physics model 3500 B liquid chromatograph on a Du Pont Zorbax octadecyltrimethoxysilane (ODS) column (6.2 mm × 25 cm) with a linear gradient (1.2 ml/min) of 70–85% methanol in water at a rate of gradient change of 1%/min after an initial delay of 1 min. The effluent was monitored at 280 nm (Fig. 1). Dihydrodiols, phenols, and quinones were all readily separated from each other. For reasons which we have detailed elsewhere (63), the 1,2- and 5,6-dihydrodiols emerge from the column as separate peaks while the 3,4-, 8,9-, and 10,11-dihydrodiols are cochromatographic and cannot easily be separated on ODS columns. For metabolized samples of radioactive BA, fractions were collected every 0.3 min throughout the metabolite profile except for the peak containing BA 3,4-, 8,9- and 10,11-dihydrodiols and for the substrate peak. Both of these peaks were collected as single fractions, and aliquots were used for quantitation. Radioactivity of the fractions was determined by scintillation spectrometry.

Determination of BA 3,4-dihydrodiol by fluorescence spectroscopy. An aliquot of the metabolic fraction containing the 3,4-, 8,9- and 10,11-dihydrodiols in 85% CH_3OH/H_2O was used to obtain fluorescence emission and excitation spectra. The emission spectra were measured by exciting the samples at 260 nm, whereas the excitation spectra were measured by monitoring the emission at 439 nm which represents an emission maximum of BA 3,4-dihydrodiol. Thus, the characteristic vinyl anthracene chromophore of the 3,4-dihydrodiol can be readily detected by either fluorescence emission or excitation spectra in the presence of the 8,9- and 10,11-dihydrodiols. Quantitation was achieved by addition of a known amount of BA 3,4-dihydrodiol followed by a second determination of the emission spectrum. All fluorescence spectra were obtained with a Perkin-Elmer Spectro-fluorimeter model MPF-3L.

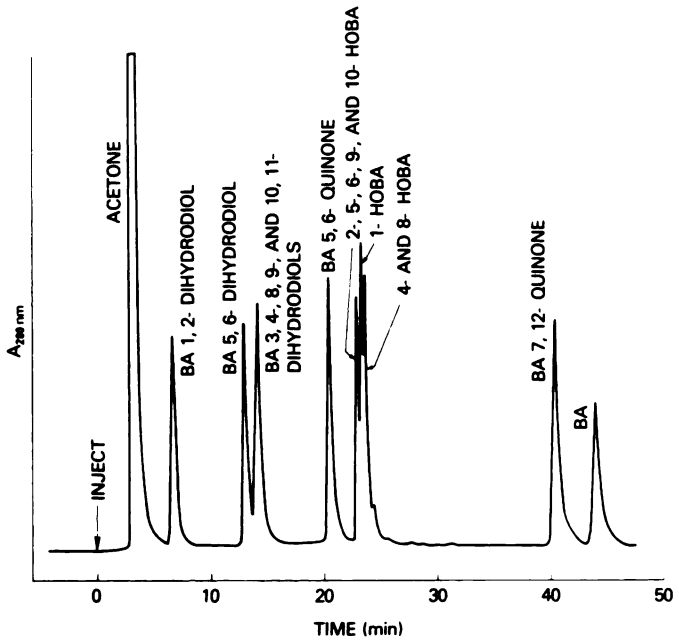


FIG. 1. *Chromatographic mobilities of synthetic derivatives of BA on analysis by HPLC* Chromatographic conditions are described in MATERIALS AND METHODS.

Quantitation of BA 10,11-dihydrodiol. An aliquot of the fraction containing BA 3,4-, 8,9- and 10,11-dihydrodiols was injected onto a Du Pont Zorbax SIL column (6.2 mm × 25 cm) with the synthetic dihydrodiols of BA added as carriers. The 10,11-dihydrodiol separated from the mixture of the 3,4- and 8,9-dihydrodiols when the column was eluted with 1.2% ethanol and 12% dioxane in cyclohexane at a flow rate of 5 ml/min. Effluent was monitored at 254 nm (Fig. 2A). Fractions were collected every 10 sec in the metabolite region and every 30 sec elsewhere. Radioactivity was quantitated by scintillation spectrometry.

Although the above SIL column readily separated the 10,11-dihydrodiol from the other two dihydrodiols, separation of the 3,4- and 8,9-dihydrodiols remained as an extremely difficult chromatographic problem. An attempt to separate the synthetic 3,4- and 8,9-dihydrodiols by continuous recycle of the peak through the column failed to show a significant separation after 25 recycles.

Separation of BA 3,4- and 8,9-dihydrodiols as their diacetates. An aliquot of the metabolite fraction containing BA 3,4-, 8,9- and 10,11-dihydrodiols with added carrier of each dihydrodiol was chromatographed on a Zorbax SIL column as described above to separate BA 10,11-dihydrodiol from the mixture. The fraction containing BA 3,4- and 8,9-dihydrodiols was acetylated (acetic anhydride/pyridine) and the diacetates were injected onto the Zorbax ODS column (6.2 mm × 25 cm) after removal of pyridine. The diacetates were completely separated by elution with 55% CH_3CN/H_2O at a flow rate of 2.4 ml/min (Fig. 2B). The effluent was monitored at 260 nm, and fractions were collected every 0.3 min in the metabolite region and every 2 min elsewhere. From the radioactivity in the peaks containing BA 8,9-dihydrodiol diacetate and BA 3,4-dihydrodiol diacetate (retention times 30.0 min and 32.0 min, respectively), the presence of BA 3,4-dihydrodiol in the mixture was confirmed.

Inhibition of BA metabolism by phenolic metabolites. [^{3}H]BA (50 nmol/ml) was incubated with cytochrome P-448 (0.05 nmol/ml), cytochrome c reductase (750 units/ml), potassium phosphate buffer (100 μmol/ml, pH 7.0), $MgCl_2$ (3 μmol/ml), NADPH (0.5 nmol/ml), and dilauroyl phos-

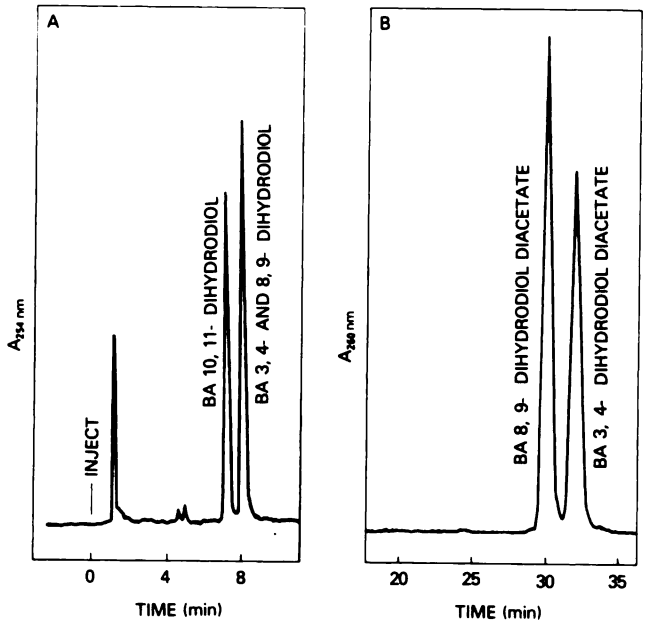


FIG. 2. *Separation of dihydrodiols of BA*

A. Separation of BA 10,11-dihydrodiol from BA 3,4- and 8,9-dihydrodiols by HPLC on a Zorbax SIL column. The chromatographic conditions are described in Materials and Methods. Metabolites are quantitated from the radioactivity associated with each peak. BA 5,6- and 1,2-dihydrodiols chromatograph on this system with retention times of 4.6 and 12.4 min, respectively. B. Separation of BA 3,4- and 8,9-dihydrodiol diacetates by HPLC on a Zorbax ODS column. The chromatographic conditions are described in MATERIALS AND METHODS. BA 3,4- and 8,9-dihydrodiol metabolites were quantitated by the radioactivity associated with their respective diacetates.

phatidylcholine (30 μg/ml) for 10 min at 37°. The reaction was stopped by further incubation with a cytochrome P-448 specific antibody (20 mg of protein IgG/nmol P-448) for 5 min (64). BA 5,6-oxide in the reaction mixture was then converted to its corresponding dihydrodiol by addition of purified epoxide hydrase (17 units/ml), and the reaction mixture was incubated for an additional 10 min. The products were extracted, dried and the phenol-1 and -2 peaks (see Results) from several incubations were isolated by HPLC as described for [^{14}C]BA. Incubation of [^{14}C]BA and known amounts of tritium labeled phenols from phenol-1 peak (1 nmol/ml) and phenol-2 peak (1 and 2.5 nmol/ml), followed by HPLC analysis of percent metabolism of the [^{14}C]BA allowed measurement of the amount of inhibition caused by the phenolic metabolites.

RESULTS

Metabolism of [^{14}C]BA by Purified Cytochrome P-448. A saturating amount of [^{14}C]BA (50 nmol/ml) was incubated with cytochrome P-448 (0.0025 to 0.1 nmol/ml) in the presence and absence of epoxide hydrase. The total percent of BA metabolized is plotted vs. nmols of added cytochrome P-448 in Figure 3. In the presence of epoxide hydrase, metabolism is linear up to 0.025 nmol of added cytochrome P-448/ml (40% metabolism), whereas in the absence of epoxide hydrase, metabolism is at best linear only up to 0.005 nmol of added cytochrome P-448/ml (9% metabolism). Below 9% metabolism, conversion is independent of the presence of epoxide hydrase. Hence, 0.005 nmol/ml of cytochrome P-448 was used for the comparison of metabolite profiles of BA obtained in the presence and absence of epoxide hydrase (Table 1). The apparent stimulation of the metabolism of [^{14}C]BA in the presence of epoxide hydrase (0.05 nmol/ml of cytochrome P-448, Fig. 3) is not due to added protein since replacement of the epoxide hydrase by an equivalent amount of either bovine serum albumin

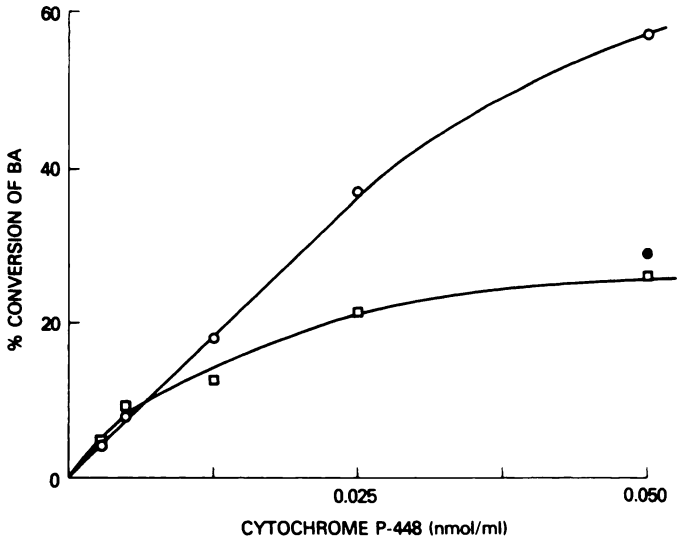


FIG. 3. *Total metabolism of [^{14}C]BA by the reconstituted system as a function of added cytochrome P-448 in the presence* (○) *and absence* (□) *of epoxide hydrase or in the presence* (●) *of epoxide hydrase which had been inactivated completely by the site directed inhibitor p-nitrophenacyl bromide*

Cytochrome P-448 is the rate-limiting component in the incubation medium. Total metabolism was determined by HPLC. Incubation conditions are described in MATERIALS AND METHODS.

or by epoxide hydrase which had been specifically inactivated with p-nitrophenacyl bromide (65, cf. Fig. 3) failed to enhance the metabolism.

The HPLC profiles of the metabolites of [^{14}C]BA obtained with cytochrome P-448 in the presence and absence of epoxide hydrase are shown in Figure 4. The uv traces were obtained by monitoring the effluent at 280 nm without added standards, and the radioactivity profiles were obtained by plotting cpm vs. fraction numbers. Both the uv and the radioactivity profiles show that dihydrodiols are the preponderant metabolites in the presence of the epoxide hydrase with only trace amounts of phenols formed (Fig. 4A). This is in contrast to results obtained with BP as substrate where large amounts of epoxide hydrase failed to completely prevent the formation of phenols (6). The two dihydrodiol peaks in Figure 4A correspond to BA 5,6-dihydrodiol and the mixture of BA 3,4-, 8,9- and 10,11-dihydrodiols, respectively. Neither BA 1,2-dihydrodiol nor quinones were detected (<1%) under these experimental conditions.

In absence of epoxide hydrase, two major radioactive peaks (phenol-1 and -2, Fig. 4B) were obtained. Analysis of the phenol-1 fraction by uv indicated that it contained mainly a mixture of BA 5,6-oxide along with 5- and 6-HOBA. When BA 5,6-oxide was chromatographed in this system, most of the oxide emerged from the column in the phenol-1 region along with 5- and 6-HOBA which result by isomerization on analysis. Addition of ammonia to the solvent blocks this isomerization but also shortens the lifetime of the column. Minor metabolites, unknowns -1 (probably BA 5,6-dihydrodiol), -2, and -3 (unknowns -2 and -3 are probably other methanolysis and hydrolysis products of BA 5,6-oxide), are solvolysis products of BA 5,6-oxide (Fig. 4B) which arise even when the pure oxide is injected on the column. Analysis of phenol-2 fraction by uv indicated that it contained mainly 8-HOBA. Although no further attempts were made to identify minor phenols in the two metabolite fractions, synthetic 2-, 5-, 6-, 9-, and 10-HOBA chromatograph in the region of phenol-1 while synthetic 1-, 4-, and 8-HOBA chromatograph in the region of phenol-2.

BA 5,6-oxide (K-region) was found to survive incubation with the reconstituted system in the absence of NADPH and epoxide hydrase. That BA 5,6-oxide was predominantly responsible for the phenol-1 peak as well as unknowns -1, -2, and -3 was

TABLE 1

Metabolites of BA obtained with rat liver microsomes and a purified and reconstituted system containing cytochrome P-448

Protein	Metabolites[a]				Total conversion	Recovery
	BA 5,6-dihydrodiol	BA 3,4-, 8,9- and 10,11-dihydrodiols	Phenol-1	Phenol-2		
Microsomes (Control)	44.4 0.58	49.4 0.65	4.6 0.06	1.6 0.02	2.4 1.50	88
Microsomes[b] (Phenobarbital)	43.9 0.52	41.7 0.51	3.0 0.04	3.9 0.04	6.5 1.42	85
Microsomes[c] (3-methylcholanthrene)	41.6 2.72	53.7 3.52	3.9 0.27	0.9 0.06	18.3 6.94	91
Cytochrome P-448[c] + Epoxide Hydrase	48.7 34.4	49.9 35.2	1.4 1.00	0.0 0.00	8.7 86.80	81
Cytochrome P-448[d]	—	—	28.8 21.07	38.6 28.30	9.4 94.43	78

[a] The upper row of numbers represents the percent of each metabolite formed and the lower row of numbers represents nmols of product formed per nmol of hemeprotein per min. Total conversion was calculated from the total radioactivity above blank which emerges from the column prior to BA. Recovery is the percentage of radioactivity emerging before BA in discrete metabolite peaks. All microsomal incubations contained 0.1 mg protein/ml and were linear to this protein concentration. Incubations with the reconstituted system used 0.005 nmol hemeprotein/ml.

[b] Under these conditions, BA 7,12-quinone constitutes 7–8% of the metabolites.

[c] At high conversions microsomes (0.5 mg/ml) from rats induced with 3-methylcholanthrene and the purified and reconstituted system (0.1 nmol cytochrome P-448/ml) give more polar metabolites which chromatograph before BA 5,6-dihydrodiol (see Fig. 4). These metabolites are formed to the extent of 11 to 13% of the total metabolism when 75–77% of the substrate was metabolized.

[d] On chromatography BA 5,6-oxide is partially decomposed into 3 broad peaks labeled as unknowns 1, 2 and 3 as well as to 5- and 6-HOBA which chromatograph in phenol-1 peak (cf. Fig. 4). Unknowns 1, 2, and 3 constitute 32.6% of the total metabolites.

shown by the following experiment: BA was incubated with the reconstituted system for 10 min in the absence of epoxide hydrase, oxidative metabolism was stopped by addition of specific antibody to cytochrome P-448 (64) and incubation continued for 5 min, and the mixture was further incubated with added epoxide hydrase for 10 min. Analysis of the products by HPLC indicated complete loss of unknowns -1, -2, and -3 and almost complete loss (85%) of the phenol-1 fraction along with concomitant formation of BA 5,6-dihydrodiol. The phenol-2 fraction remained unchanged, and no dihydrodiols other than BA 5,6-dihydrodiol were formed. Thus, the non-K-region arene oxides of BA were unstable and had isomerized to phenols prior to the addition of epoxide hydrase. A small portion of these combined with residual BA 5,6-oxide could account for the radioactivity which epoxide hydrase failed to remove from the phenol-1 fraction.

The extent to which BA is converted into metabolites by 0.005 nmol/ml of cytochrome P-448 is unaffected by addition of epoxide hydrase (Table 1). In the presence of epoxide hydrase, >98% of the metabolites chromatograph as two equal peaks which correspond to BA 5,6-dihydrodiol and the mixture of the BA 3,4-, 8,9-, and 10,11-dihydrodiols. In the absence of epoxide hydrase, 29% and 38% of the metabolites appear in phenol-1 and -2 peaks, respectively. The unknown peaks (33%, Fig. 4B), which end up as BA 5,6-dihydrodiol when

epoxide hydrase is present, account for the balance of the metabolism.

Metabolism of [^{14}C]BA by liver microsomes. [^{14}C]BA was incubated with 100–500 μg/ml of liver microsomes from untreated, phenobarbital-treated and 3-methylcholanthrene-treated rats at a saturating concentration (50 nmol/ml). The HPLC profile of the metabolites was qualitatively similar to that obtained with cytochrome P-448 and epoxide hydrase (Fig. 4A). Results obtained with various microsomal prepara-

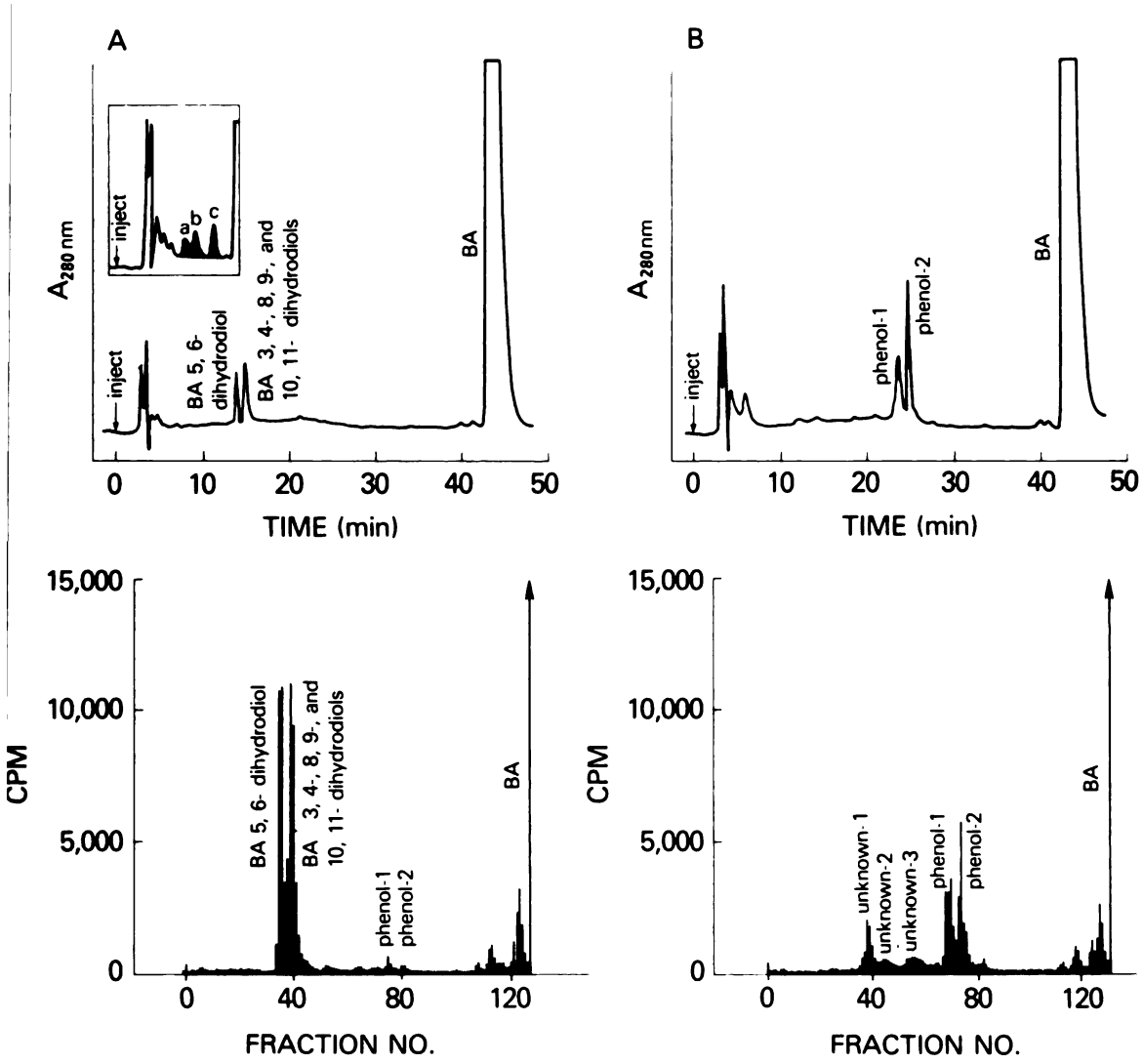


FIG. 4. *High pressure liquid chromatographic profiles of BA metabolites obtained with the purified and reconstituted system containing 0.005 nmol/ml of cytochrome P-448 in the presence (A) and absence (B) of epoxide hydrase*

The inset to part A (both uv and radioactivity) shows polar metabolites formed at high conversion of the substrate by cytochrome P-448 (0.1 nmol/ml) in the presence of epoxide hydrase. For the inset, peak *a* co-chromatographs with BA 1,2-dihydrodiol, peak *b* co-chromatographs with 8β,9α-dihydroxy-10β,11β-epoxy-8,9,10,11-tetrahydro BA, and peak *c* co-chromatographs with 8β,9α-dihydroxy-10α,11α-epoxy-8,9,10,11-tetrahydro BA. In the absence of epoxide hydrase, 0.1 nmol/ml of cytochrome P-448 gives a metabolite profile similar to the one shown in B. The incubation conditions and the chromatographic conditions are described in MATERIALS AND METHODS.

tions are given in Table 1. A comparison of the total conversion of substrate indicates that pretreatment of the animals with 3-methylcholanthrene results in about a 4½ fold increase in the rate at which BA is metabolized per nmol of cytochrome P-450. Pretreatment with phenobarbital increases total conversion of the substrate per mg of microsomal protein by 2½ fold. However, no increase is observed in the specific activity of the hemeprotein (nmol product formed/nmol cytochrome P-450/min) compared to microsomes from control animals. With the purified and reconstituted monooxygenase system, BA is metabolized at a much higher rate (87 and 94 nmol product formed per nmol hemeprotein per min, respectively, in the absence and presence of epoxide hydrase) than with microsomes from 3-methyl-cholanthrene-treated rats. For metabolism of BA, all microsomal preparations form two major dihydrodiol peaks; one consisting of the 5,6-dihydrodiol (~43%) and the other corresponding to the BA 3,4-, 8,9- and 10,11-dihydrodiols (42–54%). Phenol-1 and -2 fractions are minor metabolites, each constituting no more than 5% of the total metabolites. In addition, microsomes from phenobarbital-induced animals gave small amounts (7–8%) of BA 7,12-quinone. Microsomes from 3-methylcholanthrene-induced rats as well as the purified and reconstituted monooxygenase system in the presence of epoxide hydrase gave polar metabolites at high conversion (77%) of BA which emerged from the column early in the HPLC profile (insert of Fig. 4A). The radioactivity in peaks *b* and *c* co-chromatograph with 8β,9α-dihydroxy-10β,11β-epoxy-8,9,10,11-tetrahydro BA, and with 8β,9α-dihydroxy-10α,11α-epoxy-8,9,10,11-tetrahydro BA, respectively, when the synthetic diol epoxides were carried through the incubation and isolation procedure. The radioactivity in peak *a* co-chromatographs with BA 1,2-dihydrodiol. The co-chromatography of the above reference compounds with the radioactive metabolites in peaks *a, b,* and *c* does not in itself constitute identification of the metabolites. At 30% conversion of BA with liver microsomes from phenobarbital-treated rats, HPLC of the metabolites on the SIL column (Fig. 2A, legend) gave a small radioactive peak (<2%) which co-chromatographed with the BA 1,2-dihydrodiol. The identity of this metabolite peak was confirmed by fluorescence studies (Fig. 5, legend).

Quantitation of BA 3,4-, 8,9- and 10,11-dihydrodiols. Quantitative fluorescence spectroscopy (Fig. 5) was used to measure the amount of the BA 3,4-dihydrodiol present in the dihydrodiol fraction from the ODS column which contained the 3,4-, 8,9-, and 10,11-dihydrodiols. Further chromatography of this fraction (Fig. 2A) on the SIL column permitted radiochemical quantitation of the separated 10,11-dihydrodiol. After acetylation of the mixture of the 3,4- and 8,9-dihydrodiols from the SIL column, this isomeric pair of diacetates was found to separate on the ODS column (Fig. 2B).

The percent of each of the three dihydrodiols present in the composite peak from the original ODS column are given in Table 2. BA 8,9-dihydrodiol constitutes 83–91% of the total dihydrodiols in this fraction for all enzyme preparations examined. The highest percent of 3,4-dihydrodiol in the composite peak was found with microsomes from control (8%) and phenobarbital-treated (6%) animals. Only 3% of the peak corresponds to the 3,4-dihydrodiol with microsomes from 3-methylcholanthrene-treated animals. Since the composite peak represents about half of the total metabolism with the various enzyme preparations (Table 1), actual conversion of BA to its 3,4-dihydrodiol with the bay-region 1,2-double bond ranges from 1.5–4%.

When the metabolism of the extremely weak carcinogen BA and the potent carcinogen BP were compared in the same experiment, the rates of metabolism of the two substrates were similar. With microsomes from 3-methylcholanthrene-treated rats, 6.9 nmols of BA and 5.2 nmols of BP are metabolized per nmol of hemeprotein per min. With microsomes from untreated rats, BA and BP are metabolized at rates of 1.5 and 1.4 nmol of products per nmol of hemeprotein per min. In this experiment, the amount of the BP 7,8-dihydrodiol with the bay-region 9,10-double bond was quantitated. With microsomes from control and

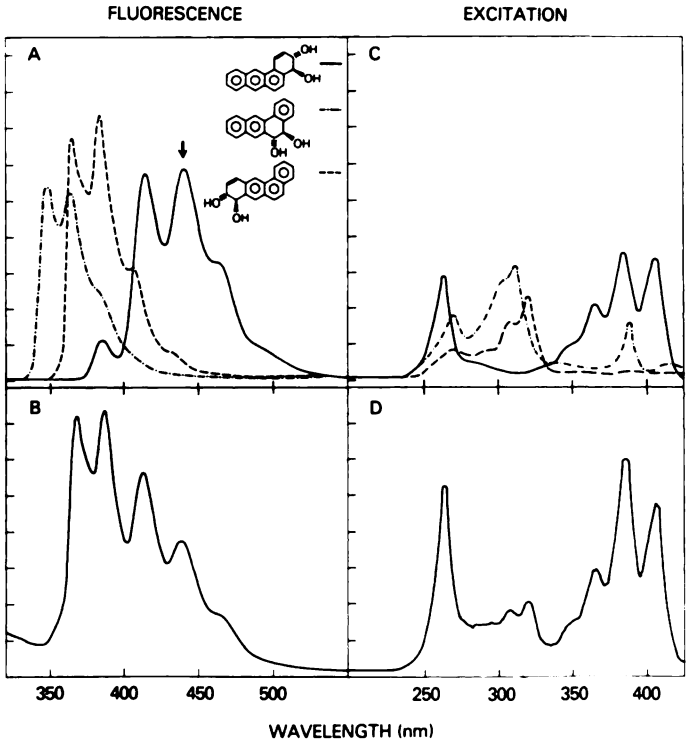


FIG. 5. *Fluorescence (A), and excitation (C) spectra (85% methanol/water) of synthetic BA 3,4-, 5,6- and 8,9-dihydrodiols as well as fluorescence (B) and excitation (D) spectra of the metabolite fraction which contains the 3,4-, 8,9- and 10,11-dihydrodiols produced by liver microsomes from phenobarbital-induced rats*

The fluorescence spectra were obtained by exciting the samples at 260 nm and the excitation spectra were obtained by monitoring the emission at 439 nm, conditions selected to maximize the fluorescence and excitation bands of BA 3,4-dihydrodiol and to supress the signals of the other dihydrodiols. Relative amounts of the three synthetic dihydrodiols in part A and C were selected to produce approximately equal spectrophotometric responses. The amount of BA 3,4-dihydrodiol in a metabolite fraction can be readily quantified by the intensity of emission at 439 nm (arrow). Fluorescence spectra of BA 1,2- and 10,11-dihydrodiols are not shown but are similar to those of BA 3,4- and 8,9-dihydrodiols, respectively. BA 1,2-dihydrodiol gives fluorescence emission bands at 410 and 435 nm, and BA 10,11-dihydrodiol gives fluorescence bands at 370 and 390 nm.

3-methylcholanthrene-treated animals, BP 7,8-dihydrodiol represented 8% and 14% of the total metabolites, respectively. The purified system containing cytochrome P-448 and epoxide hydrase formed 12% of the 7,8-dihydrodiol. Thus, BP is converted more extensively to a proximate carcinogenic dihydrodiol than is BA.

Inhibition of metabolism by phenols of BA. Benzo[a]anthracene could not be metabolized to more than 30% in the absence of epoxide hydrase even at a cytochrome P-448 concentration of 0.1 nmol/ml. In the presence of epoxide hydrase, 77% of the substrate was metabolized under the same conditions (cf. Fig. 3, data not shown). Addition of purified epoxide hydrase which had been rendered catalytically inactive by either thermal denaturation or by an active site directed inhibitor (65) failed to produce the apparent stimulation observed with catalytically active epoxide hydrase. These results suggest that one or more phenolic metabolites of BA, the 5,6-oxide, or any of the non-K-region arene oxides of BA must be highly inhibitory toward cytochrome P-448. Hence, BA 5,6-oxide, 5-, 6-, 8- and 9-HOBA were tested as inhibitors of BA metabolism since these compounds represent major sites of metabolism of the hydrocarbon. Table 3 shows that BA 5,6-oxide is a very poor inhibitor, 8- and 9-HOBA (26%

TABLE 2

Percent of 3,4-, 8,9- and 10,11-dihydrodiols in the composite diol peak containing these three diols

Protein	BA 3,4-dihydrodiol[a] (%)	BA 8,9-dihydrodiol (%)	BA 10,11-dihydrodiol[b] (%)
Microsomes (control)	8	84	8
Microsomes (phenobarbital)	6	83	11
Microsomes (3-methylcholanthrene)	3	91	6
Cytochrome P-448 + epoxide hydrase	5	88	7

[a] Determined by fluorescence as shown in Figure 5. The nature of the fluorescence is such that these values represent the maximum possible amounts.

[b] Determined by radioactivity in the isolated BA 10,11-dihydrodiol fraction (Fig. 2).

TABLE 3

Inhibition of monooxygenases by synthetic phenols of BA, BA 5,6-oxide and by metabolically formed phenols of BA

Inhibitor	Conc. of Inhibitor (nmol/ml)	% Inhibition[a]
BA 5,6-oxide	5	10
5-HOBA + 6-HOBA	5	88
5-HOBA[b]	5	89
	1	71
8-HOBA	5	26
	1	18
9-HOBA	5	35
	1	15
Phenol-1[c]	1	8
Phenol-2[c]	2.5	48

[a] % Inhibition was calculated with respect to the conversion of BA (50 nmol/ml) by cytochrome P-448 (0.05 nmol/ml) in the absence of epoxide hydrase (Fig. 3).

[b] The sample contained predominantly 5-HOBA and a trace amount of 6-HOBA.

[c] These are the mixtures of phenolic metabolites which chromatograph in phenol-1 and phenol-2 peaks (cf. Fig. 4).

and 35% inhibition, respectively, at 5 nmols/ml) are moderate inhibitors, and 5- and 6-HOBA are strong inhibitors of BA metabolism. Both 5-HOBA and a mixture of 5- and 6-HOBA produced 88–89% inhibition at a concentration of 5 nmols/ml, and 5-HOBA inhibited the metabolism of BA by 71% at a concentration of 1 nmol/ml. Since BA 5,6-oxide survives the incubation (boiled microsomes) and work-up conditions, 5- and 6-HOBA should not be present among the metabolites of BA in the incubation medium unless they are formed by direct hydroxylation.

To identify the specific inhibitor(s) among metabolites of BA formed by the reconstituted system in the absence of epoxide hydrase, phenolic products present in phenol-1 and -2 peaks were tested as inhibitors of BA metabolism. After conversion of BA 5,6-oxide to BA 5,6-dihydrodiol by a postincubation with epoxide hydrase, a greatly reduced phenol-1 peak and an unchanged phenol-2 peak were isolated and tested for inhibition. Metabolites in phenol-2 peak inhibited BA metabolism by 48% at a concentration of 2.5 nmol/ml which represents about 65% of the phenol-2 metabolites generated at 20% conversion of BA (cf. Fig. 3). The amount of 8-HOBA in the added phenol-2 fraction is insufficient to account for the observed inhibition. Metabolites in the greatly reduced phenol-1 peak, produced after post-incubation with epoxide hydrase, inhibited metabolism of BA only to the extent of 8% at a concentration of 1 nmol/ml. Even though 5- and 6-HOBA are potent inhibitors, their absence from the incubation medium excludes them from causing the product inhibition. Thus, unidentified phenols or possibly non-K-region arene oxides are responsible for the product inhibition observed. These unidentified phenols are necessarily minor metabolites of the parent hydrocarbon.

DISCUSSION

The bay-region theory predicts that BA should be a stronger carcinogen than dibenzo[a,h]anthracene since quantum mechanical calculations indicate that the bay region 3,4-diol-1,2-epoxides of BA ($\Delta E_{deloc}/\beta = 0.766$) should be more reactive than the bay region 3,4-diol-1,2-epoxides ($\Delta E_{deloc}/\beta = 0.738$) of dibenzo[a,h]anthracene (23). Benzo[a]anthracene is, however, considered a weak carcinogen, whereas di-

benzo[a,h]anthracene is a fairly strong carcinogen (66). This reversal of predicted relative carcinogenicity could be due to the differences in the extent to which the two hydrocarbons are converted to bay region diol epoxides. Sims has reported semiquantitative data on the metabolism of dibenzo[a,h]anthracene by rat liver homogenates which indicate that the 3,4-dihydrodiol with a bay-region double bond is one of the major metabolites of this hydrocarbon (39). If BA is metabolized to form very low amounts of the 3,4-dihydrodiol, the low carcinogenicity of BA relative to dibenzo[a,h]anthracene might be explained. Hence, we have studied the metabolism of BA with the specific aim of quantitative determination of the amount of BA 3,4-dihydrodiol formed by various rat liver microsomal preparations and by a highly purified and reconstituted monooxygenase system in the presence of epoxide hydrase.

When total metabolism of BA was studied as a function of cytochrome P-448 concentration in the presence and absence of epoxide hydrase, marked product inhibition of the cytochrome P-448 system (Fig. 3) was observed in the absence of epoxide hydrase. This represents the first example of product inhibition by phenols or arene oxides on the cytochrome P-450 system. At 1/50*th* of the substrate concentration 5- and possibly 6-HOBA were potent inhibitors which blocked as much as 70% of the metabolism of BA (Table 3). Since BA 5,6-oxide is stable to the incubation conditions and is inactive as an inhibitor, the K-region arene oxide and its phenolic isomerization product are not responsible for the effect. The next major site of metabolism of BA is at the 8,9-double bond. However, both 8- and 9-HOBA were only moderately inhibitory. Thus, untested non-K-region phenols which chromatograph in phenol-2 fraction or arene oxides must cause the inhibition. Since most of the inhibitory activity can be accounted by the phenol-2 metabolite fraction, 4- and 11-HOBA are possible candidates.

In contrast to the results with BP, rat liver microsomes metabolized BA almost exclusively to dihydrodiols with only trace amounts of phenols and quinones. This is also true for the purified and reconstituted monooxygenase system in the presence of epoxide hydrase. These results suggest that the arene oxides derived from BA are more stable to isomerization than those derived from benzo[a]pyrene or they are more efficiently hydrolyzed by epoxide hydrase than arene oxides of BP. Kinetic studies of epoxide hydrase have indicated that BA 5,6-oxide is a better substrate than four arene oxides of BP which were tested (67, 68).

The possible dihydrodiols of BA chromatograph in three peaks; one which contains BA 1,2-dihydrodiol, a second which contains BA 5,6-dihydrodiol, and a third which contains BA 3,4-, 8,9- and 10,11-dihydrodiols (Fig. 1). The reasons for the cochromatography of these three non-K-region dihydrodiols of BA have been discussed elsewhere (63). BA 5,6- and 8,9-dihydrodiols are the two major metabolites (>80%) of the hydrocarbon. The 1,2-, 3,4-, and 10,11-dihydrodiols are formed to a much lesser extent. To quantitate BA 3,4-dihydrodiol, advantage was taken of the fact that this dihydrodiol has a vinyl anthracene chromophore in contrast to the vinyl phenanthrene chromophore of BA 8,9- and 10,11-dihydrodiols. Hence, the presence of BA 3,4-dihydrodiol could be unequivocally established by the fluorescence emission at 413 nm and 439 nm (Fig. 5) and it could be quantitated by its fluorescence emission at 439 nm with very little interference from BA 8,9- or 10,11-dihydrodiol. Excitation spectra of the metabolites which chromatograph in the region of the BA 3,4-, 8,9- and 10,11-dihydrodiols had absorption bands at 365, 385 and 407 nm characteristic of a vinyl anthracene chromophore, thus providing additional evidence for the presence of BA 3,4-dihydrodiol among the metabolites of BA. Furthermore, BA 8,9- and 3,4-dihydrodiol diacetates could be separated on a Zorbax ODS column, and the presence of BA 3,4-dihydrodiol among the metabolites of BA was confirmed. Thus, the recent report by Yang et al. (46) which concluded that BA 3,4-dihydrodiol was not a liver microsomal metabolite of BA is no longer tenable.

In conclusion, the low carcinogenicity of BA may be explained at least in part by the low percentage of the highly tumorigenic BA 3,4-dihydrodiol formed among the me-

tabolites of BA. Moreover, the skin tumor-initiating ability of the (+)- and (-)-enantiomers of the BA 3,4-dihydrodiol has been tested in CD-1 mice, and the (-)-[3R,4R]-enantiomer is approximately 5- to 15-fold more potent as a tumor initiator than the (+)-[3S,4S]-enantiomer (29). Hence, further studies are in progress to determine the absolute stereochemistry of the metabolically formed dihydrodiols of BA. The present study provides an example of how the "bay-region" theory (21–24) and a quantitative knowledge of metabolism profiles can help to explain the relative carcinogenicity of polycyclic aromatic hydrocarbons.

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Note added in proof. Since the submission of this manuscript we have also tested 1-, 3-, 4- and 11-HOBA as inhibitors of the metabolism of BA (69). Thus, of all the phenols tested, 5- and 6-HOBA are the strongest inhibitors, 1-, 3-, 8-, and 9-HOBA are moderate inhibitors and 4-HOBA is a weak inhibitor of the metabolism of BA.

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