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Metabolism of *trans*-9,10-Dihydroxy-9,10-dihydrobenzo[*a*]pyrene Occurs Primarily by Arylhydroxylation Rather than Formation of a Diol Epoxide

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SUMMARY

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Benzo[*a*]pyrene (BP) 7,8-dihydrodiol is metabolized by the rat liver monooxygenase system to diastereomeric benzo[*a*]pyrene 7,8-diol 9,10-epoxides that are highly reactive, toxic, and mutagenic. One of the isomers of these diol epoxides is highly carcinogenic to newborn mice. Oxidative metabolism of (−)-benzo[*a*]pyrene 9,10-dihydrodiol has been investigated to see whether 9,10-diol 7,8-epoxides are formed in analogy to the metabolism of BP 7,8-dihydrodiol, and mutagenic activities of synthetic BP 9,10-diol 7,8-epoxides have been evaluated. Unlike BP 7,8-dihydrodiol, the (−)-9,10-dihydrodiol is metabolized primarily to a phenolic derivative rather than a diol epoxide. Chemical and spectral studies established that the phenolic hydroxyl group had been introduced at either position 1 or 3 of the 9,10-dihydrodiol. Weak inherent mutagenic activity of the synthetic 9,10-diol 7,8-epoxides and the very low degree of their metabolic formation explains why metabolic activation of BP 9,10-dihydrodiol in the presence of *Salmonella typhimurium* results in a weak mutagenic response compared with studies with the 7,8-dihydrodiol as substrate. Chronic application of 0.15 μmole of BP 9,10-dihydrodiol to the backs of C57BL/6J mice once every 2 weeks for 60 weeks failed to produce tumors, whereas the same treatment with BP resulted in a 97% incidence of tumors.

INTRODUCTION

Most of the metabolism-induced mutagenicity and carcinogenicity of the widespread environmental contaminant benzo[*a*]pyrene is thought to be the result of secondary oxidative metabolism of BP[3] 7,8-dihydrodiol to a pair of diastereomerically related 7,8-diol 9,10-epoxides (see refs. 1 and 2 for reviews). One of the isomers of

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[3] The abbreviations used are: BP, benzo[*a*]pyrene; BP 7,8-dihydrodiol, *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene; BP 4,5- and 9,10-dihydrodiol, other dihydrodiols of BP; BP 7,8-diol 9,10-epoxide 1, (±)-7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydro-BP (isomer in which the benzylic 7-hydroxyl group and epoxide oxygen are *cis*); BP 7,8-diol 9,10-epoxide 2, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydro-BP (isomer in which the benzylic 7-hydroxyl group and the epoxide oxygen are *trans*); BP 7,8-diol 9,10-epoxide, either or both of the above diol epoxides; BP 9,10-diol 7,8-epoxide 1, diol epoxide in which the benzylic 10-hydroxyl group and epoxide oxygen are *cis*

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these diol epoxides is an ultimate carcinogen in the newborn mouse (3). Metabolic conversion of BP to its 7,8-diol 9,10-epoxides has been extensively studied (4–7) and found to be subject to a high degree of stereochemical specificity. When racemic BP 7,8-dihydrodiol is metabolized by liver microsomes or a highly purified, reconstituted monooxygenase system, the ratio of the two diol epoxides formed (diol epoxide 2:diol epoxide 1) varied from 1.7 to 0.4, depending upon the prior treatment of the animals and the incubation conditions (4). With the (−) enantiomer of the dihydrodiol as substrate, the ratio of BP 7,8-diol 9,10-epoxide isomers 2 and 1 was 6:1, while with the (+) enantiomer the ratio was 1:22 (5). The 4,5-, 7,8-, and 9,10-dihydrodiols formed from BP by liver microsomes are all highly enriched (more than 95%) in the (−) enantiomer (5, 8), which, in the case of the 7,8-dihydrodiol, has [7*R*,8*R*] absolute stereochemistry (9, 10). Thus the BP 7,8-diol 9,10-epoxide formed from metabolically produced BP 7,8-dihydrodiol by rat liver enzymes is mainly isomer 2 (4–7). Hydroxylation of the aromatic ring system to form a phenolic derivative of BP 7,8-dihydrodiol represents a second major pathway of metabolism (4, 5), which accounts for as much as 40% of the total extractable metabolites produced by liver microsomes from untreated animals.

In contrast to results obtained with BP 7,8-dihydrodiol, the monooxygenase enzymes do not seem to produce highly mutagenic metabolites from BP 9,10-dihydrodiol (11, 12) despite the fact that both dihydrodiols have nonaromatic double bonds that could be epoxidized. Presumably, the BP 9,10-diol 7,8-epoxide isomers from BP 9,10-dihydrodiol either are not formed or are not strongly mutagenic. The present study explores this question through examination of the metabolism of BP 9,10-dihydrodiol and of the mutagenicity of the BP 9,10-diol 7,8-epoxides, and provides a basis for the observed inactivity of BP 9,10-dihydrodiol as a carcinogen on mouse skin.

(stereochemically related to BP 7,8-diol 9,10-epoxide 1 above); BP 9,10-diol 7,8-epoxide 2, other diastereoisomer of BP 9,10-diol 7,8-epoxide; BP 7,10-diol 8,9-epoxide, (±)-7α,10β-dihydroxy-8α,9α-epoxy-7,8,9,10-tetrahydro-BP; H_4-BP 7,8-epoxide, 7,8-epoxy-7,8,9,10-tetrahydro-BP. Where optical enantiomers are possible, racemic compounds were used unless otherwise specified.

MATERIALS AND METHODS

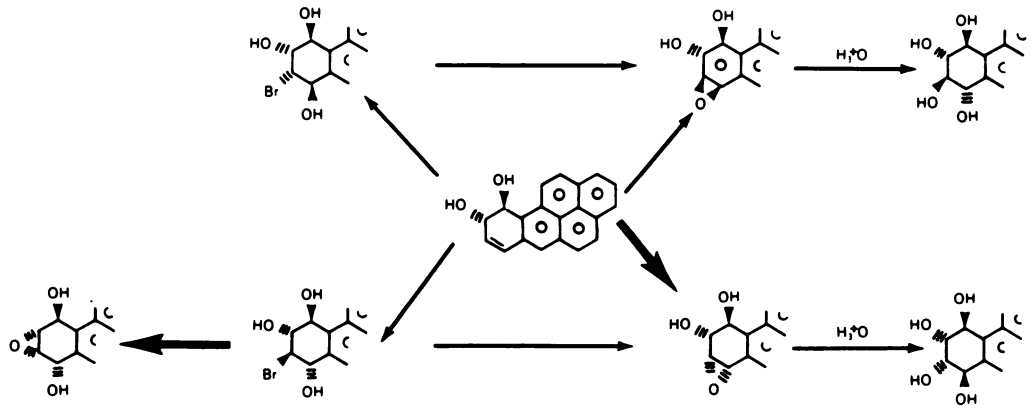
Chemicals

Synthetic (±)-*trans*-9,10-dihydroxy-9,10-dihydro-BP (BP 9,10-dihydrodiol) was prepared[4] by a procedure analogous to that used for the synthesis of *cis*-BP 9,10-dihydrodiol (13). [^{14}C]BP 9,10-dihydrodiol and [^{14}C]BP 4,5-dihydrodiol were prepared biosynthetically by incubation of 46 μmoles of [^{14}C]BP (Amersham/Searle; 19.1 mCi/mmole) with microsomes from 3-methylcholanthrene-treated Long-Evans rats as described previously (5). The biosynthetic BP 4,5- and 9,10-dihydrodiols were more than 95% radiochemically pure when analyzed by high-pressure liquid chromatography under conditions (Zorbax ODS) utilized to analyze their metabolites. The optical purity of both dihydrodiols was 92% [96% (−) enantiomer] as determined by a radiochemical method (5, 8).

Synthesis of 9,10-Diol 7,8-Epoxides from BP 9,10-Dihydrodiol (Scheme 1)

Oxidation with m-chloroperoxybenzoic acid. To a solution of (±)-BP 9,10-dihydrodiol (67 mg) in freshly distilled tetrahydrofuran (15 ml) was added *m*-chloroperoxybenzoic acid (1.0 g). After the mixture had been stirred at room temperature for 2 hr, thin-layer chromatography on silica gel (ethyl acetate–hexane, 1:1) indicated complete conversion of the dihydrodiol to two products, with R_F values slightly above and below 0.2. The reaction mixture was diluted with ethyl acetate (250 ml), and acidic components were removed by extraction with 15-ml portions of 10% aqueous sodium hydroxide. The ethyl acetate was dried (Na_2SO_4) and concentrated to a light yellow solid, from which 40 mg of the major diol epoxide, with the lower R_F, were isolated by trituration with 2–3 ml of tetrahydrofuran; NMR spectrum at 100 MHz in deuterated dimethyl sulfoxide: H_7 4.44, H_8 3.90, H_9 4.16, H_{10} 5.17, OH_9 5.64, and OH_{10} 5.82δ

[4] H. Yagi, V. Mahadevan, D. T. Gibson, and D. M. Jerina, manuscript in preparation.



SCHEME 1

with $J_{7,8}$ = 4.4, $J_{8,9}$ = 1.8, $J_{9,10}$ = 7.3, $J_{9,OH}$ = 5.5, and $J_{10,OH}$ = 7.6 Hz. Successive crops of crystals that contained both products were removed from the tetrahydrofuran solution used in the trituration until the mother liquor contained predominantly (more than 90%) the minor, high-R_F product (7 mg); NMR spectrum as above: H_7 4.48, H_8 4.0, H_9 4.6, H_{10} 5.45, OH_9 5.42, and OH_{10} 4.36δ with $J_{7,8}$ = 4.2, $J_{8,9}$ = 1.8, $J_{9,10}$ = 7.3, $J_{9,OH}$ = 5.5, and $J_{10,OH}$ = 9.0 Hz.

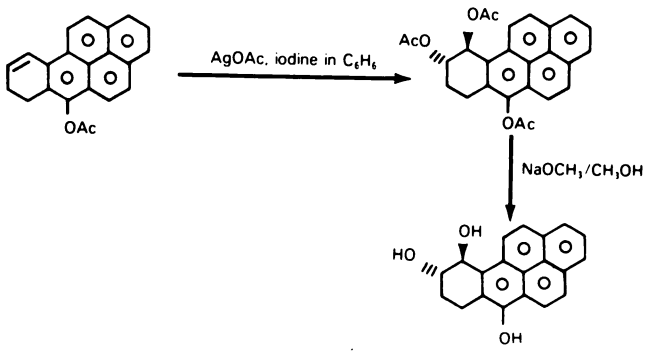
The major, lower-R_F product was assigned as (±)-9α,10β-dihydroxy-7α,8α-epoxy-7,8,9,10-tetrahydro-BP (isomer 2), in which the benzylic hydroxyl group at C-10 and the epoxide ring are *trans*, by comparison of its NMR spectrum with those of other diol epoxides (14–16). The relative stereochemistry was confirmed by the observation that on acid hydrolysis a *trans-cis-trans* tetraol (*trans*-2; cf. ref. 4) is formed. The minor amount of high-R_F product is probably the diastereomeric 9,10-diol 7,8-epoxide isomer 1, in which the benzylic hydroxyl group at C-10 and the epoxide ring are *cis*. Because of the small amount of somewhat impure material, unequivocal structure assignment was not possible. The major basis for assignment of its relative stereochemistry resides in its aqueous hydrolysis to a *trans-trans-trans* tetraol (*trans*-1; cf. ref. 4). Both isomers showed the expected M^+ (*m/e*) 302 on mass spectrometry.

Bromotriol method. Since a pure sample of (±)-9α,10β-dihydroxy-7β,8β-epoxy-7,8,-9,10-tetrahydro-BP (9,10-diol 7,8-epoxide isomer 1) could not be isolated from the above oxidation, the standard procedure (14–16) of forming an intermediate bromotriol was attempted. A 66% yield of a mixture of two bromotriols was obtained. Treatment with the hydroxide form of Amberlite resin by the usual procedure (14–16) resulted in two products (R_F ~ 0.2 on thin-layer chromatography as above). A sample of the pure major product, with the lower R_F value, was isolated in 40% yield by crystallization from ethyl acetate. The NMR spectrum of this material as its diacetate allowed assignment as (±)-7α,10β-dihydroxy-8α,9α-epoxy-7,8,9,10-tetrahydro-BP; NMR spectrum at 100 MHz in deuterochloroform: H_7 6.86, H_8 and H_9 3.85–4.00, H_{10} 7.49, and acetates at 2.08 and 2.42δ with $J_{9,10}$ = 2.2 and $J_{7,8}$ < 1.0 Hz. The minor, high-R_F product is probably the desired 9,10-diol 7,8-epoxide isomer 1, but again could not be isolated in pure form.

Synthesis of 6,9,10-Trihydroxy-7,8,9,10-tetrahydro-BP

A mixture of 6-acetoxy-7,8-dihydro-BP (283 mg) (17), silver acetate (668 mg), and iodine (350 mg) in 20 ml of benzene was stirred under nitrogen for 1 hr and then refluxed for 6 hr. The reaction mixture was filtered to remove inorganic materials, and the filtrate was evaporated to leave an oil, which when purified by preparative thin-layer chromatography (silica gel; benzene R_F = 0.1) gave 120 mg of viscous oil. This oil was further purified by high-pressure liquid chromatography to afford approxi-

mately 50 mg of pure 6,9,10-triacetoxy-7,-8,9,10-tetrahydro-BP as an amorphous powder (Scheme 2); NMR (100 MHz, $CDCl_3$): 1.98, 2.09 (aliphatic acetate), 2.58 (aromatic acetate), 2.20 ~ 2.40 ($2H_8$, *m*), 2.80 ~ 3.20 ($2H_7$, *m*), 5.46 (H_9, $J_{9,10} = J_{9,8} = J_{9,8'} = 3.17$ Hz), and 7.9 ~ 8.30δ (7 aromatic protons); mass (CI, N_2—NO), M^+ (*m/e*) 430.



SCHEME 2

The triacetate was dissolved in methanol containing excess sodium methoxide and heated at 60° for 1 hr. The hydrolyzed product was extracted into ethyl acetate after the methanolic solution had been acidified with1 N HCl. The organic layer was washed with water, dried ($MgSO_4$), and evaporated to give 6,9,10-trihydroxy-7,8,-9,10-tetrahydro-BP (Scheme 2); mass (CI, N_2—NO), M^+ (*m/e*) 304. The product was judged pure by analysis on high-pressure-liquid chromatography under conditions described for the separation of dihydrodiol metabolites.

Enzyme Preparations

Immature (50–60-g) male rats of the Long-Evans strain were treated with phenobarbital (75 mg/kg/day) or 3-methylcholanthrene (25 mg/kg/day) for 4 days. Microsomes were prepared and stored at −90° prior to use (see ref. 18). The cytochrome P-450 contents of microsomes from control rats (0.78 nmole/mg of protein) or from phenobarbital (2.35 nmoles/mg of protein) or 3-methylcholanthrene (1.55 nmoles/mg of protein)-induced animals were determined as described previously (19). Highly purified cytochrome P-448 (20), NADPH–cytochrome *c* reductase (21), and homogeneous epoxide hydrase (22) were prepared as described.

Metabolism of Biosynthetic (−)-[^{14}C]BP 9,10-Dihydrodiol and Biosynthetic (−)-[^{14}C]BP 4,5-Dihydrodiol

Incubations were performed either with rat liver microsomes or with a highly purified, reconstituted monooxygenase system. With microsomes, a protein concentration of 500 μg/ml and a substrate concentration of 40 nmoles/ml were used for all incubations. The reconstituted system contained 0.4 nmole of cytochrome P-448 per milliliter. Incubations with reconstituted systems were performed in the absence and presence of 50 units of epoxide hydrase. All incubations were conducted for 10 min at 37°. Details of the incubation conditions and sample preparation are described in preceding papers (4, 5). The metabolism of (−)-[^{14}C]BP 4,5-dihydrodiol was studied only with liver microsomes from 3-methylcholanthrene-treated rats.

Metabolism of (±)-BP 9,10-Dihydrodiol

(±)-BP 9,10-dihydrodiol was metabolized on a large scale to isolate metabolites in quantities sufficient to study their spectral properties. Five flasks containing 4 μmoles of the substrate, 50 mg of microsomal protein prepared from 3-methylcholanthrene-treated rats, 5 mmoles of potassium phosphate (pH 7.4), 0.15 mmole of $MgCl_2$, and 25 μmoles of NADPH in a total volume of 50 ml were incubated at 37° for 20 min with gentle shaking. The contents of each 50-ml

flask were extracted with 150 ml of ethyl acetate–acetone (2:1), and the organic phase was separated, dried with anhydrous Na_2SO_4, and evaporated to dryness.

Analysis of Metabolites by High-Pressure Liquid Chromatography

Chromatographic analysis of radioactive metabolites of (−)-[^{14}C]BP 9,10-dihydrodiol was performed on a du Pont Zorbax ODS column (6.2 mm × 25 cm), using a linear gradient of 60–98% methanol in water for 38 min after a 1-min delay, at a constant rate of 1.2 ml/min. The effluent was monitored at 280 nm, and fractions were collected every 0.5 min over the entire chromatographic profile except in the substrate peak, where 0.2-min fractions were collected. Metabolites of [^{14}C]BP 4,5-dihydrodiol were analyzed by a similar chromatographic system that utilized a linear gradient of 40–99% methanol in water for 59 min. The effluent was monitored at 254 nm, and fractions were collected every 0.5 min. All high-pressure liquid chromatographic analyses were performed on a Spectra-Physics chromatograph, model 3500B.

Mutagenesis Assay

The ability of the synthetic 9,10-diol 7,8-epoxides of BP to induce mutations in bacterial and mammalian cells was evaluated as described previously (23). The bacterial mutagenesis assays were performed with strains TA 98 and TA 100 of *Salmonella typhimurium* (24). Compounds were added in 15 μl of anhydrous dimethyl sulfoxide to 2×10^8 bacteria suspended in 0.5 ml of 5 mM potassium phosphate–150 mM NaCl, pH 7.0. The mixture was incubated for 5 min at 37° before plating on minimal agar medium. Mutation frequency was assayed 48 hr after plating by counting macroscopic colonies of bacteria on the Petri dishes.

The Chinese hamster cell line V79-6, kindly provided by Dr. E. H. Y. Chu of The University of Michigan, was used for mutagenesis assays with mammalian cells. Resistance of the cells to the lethal effects of the purine analogue 8-azaguanine was used as the mutagenic marker in these studies. Conditions of the test were those previously described (23), except that 3 instead of 2 days elapsed between treatment with mutagen and the first addition of 8-azaguanine. Diol epoxides were added in 20 μl of anhydrous dimethyl sulfoxide to cells that were growing in 5 ml of culture medium.

Carcinogenic Activity

Female C57BL/6J mice (4–5 weeks old) were obtained from Jackson Laboratories, Bar Harbor, and were equilibrated and shaved as previously described (25–27). The compounds tested, at a concentration of 0.15 μmole/25 μl of acetone–NH_4OH (1000:1, v/v), were applied topically to the backs of the mice once every 2 weeks for 60 weeks. The control group received 25 μl of solvent alone once every 2 weeks for 60 weeks. Each treatment group consisted of 30 mice.

RESULTS

Synthesis of Diastereomeric BP 9,10-Diol 7,8-Epoxides

Synthesis of the two diastereomeric BP 9,10-diol 7,8-epoxide isomers 1 and 2 (Scheme 1) was attempted for the purpose of obtaining synthetic standards to be utilized in the study of metabolism of BP 9,10-dihydrodiol and to determine their mutagenic activities. BP 9,10-diol 7,8-epoxide 2 was synthesized by peroxy acid oxidation of BP 9,10-dihydrodiol; however, a small but significant amount of the other diastereomeric diol epoxide was also formed (Scheme 1). We have previously pointed out the importance of *trans* diequatorial conformation of dihydrodiols in directing the attack of peroxy acid (15). The quasi-axial conformation of the hydroxy groups in BP 9,10-dihydrodiol may be the reason for the lack of high stereoselectivity in the attack of peroxy acid in this dihydrodiol (Scheme 1). The small amount of BP 9,10-diol 7,8-epoxide 1 formed in this reaction could not be isolated in completely pure form. Thus the bromotriol method used in the synthesis of BP 7,8-diol 9,10-epoxide 1 (14, 15) was attempted for BP 9,10-diol 7,8-epoxide 1, but the major product isolated from this reaction was found to be BP 7,10-diol 8,9-epoxide (Scheme 1).

Mutagenic Activity of BP 9,10-Diol 7,8-Epoxides and BP 7,10-Diol 8,9-Epoxide

The BP 9,10-diol 7,8-epoxides were tested for mutagenic activity toward *S. typhimurium* as well as toward Chinese hamster V79 cells. BP 7,10-diol 8,9-epoxide was also tested to gain an insight into the importance of the relative positions of the oxirane ring and hydroxyl groups in determining the mutagenic activity of isomeric diol epoxides on the benzo ring. The relative mutagenic activities of BP 9,10-diol 7,8-epoxides 1 and 2, BP 7,8-diol 9,10-epoxides 1 and 2, BP 7,10-diol 8,9-epoxide, and H_4-BP 7,8-epoxide toward *S. typhimurium* strains TA 98 and TA 100 are compared in Table 1. Mutagenic activities of BP 7,8-diol 9,10-epoxides have been reported previously (6, 23, 28, 29) but are included in Table 1 for comparison. As reported previously (23), BP 7,8-diol 9,10-epoxide 1 is a more potent mutagen than BP 7,8-diol 9,10-epoxide 2 toward both *S. typhimurium* strains TA 98 and TA 100. BP 9,10-diol 7,8-epoxides 1 and 2 and H_4-BP 7,8-epoxides have 1–10% of the activity of BP 7,8-diol 9,10-epoxide 1 toward strain TA 98 and show even less mutagenic activity toward strain TA 100. BP 7,10-diol 8,9-epoxide is practically inactive.

Table 2 compares the mutagenic activities of these diol epoxides toward V79 cells. As reported previously (6, 23, 29), BP 7,8-diol 9,10-epoxide 2 is more potent than BP 7,8-diol 9,10-epoxide 1 as a mutagen in this system. BP 9,10-diol 7,8-epoxide 2 has only 8% of the activity of the BP 7,8-diol 9,10-epoxide 2 at 10 times the dose. BP 7,10-diol 8,9-epoxide and BP 9,10-diol 7,8-epoxide 1 are practically inactive as mutagens in this system.

TABLE 1

Mutagenic activities of BP diol epoxides toward S. typhimurium TA 98 and TA 100

The experimental procedure for determining mutagenicity is described in MATERIALS AND METHODS.

Compound	Revertants		Relative activity	
	TA 98	TA 100	TA 98	TA 100
	No./nmole		%	%
BP 7,8-diol 9,10-epoxide 1	3460	9700	100	100
BP 7,8-diol 9,10-epoxide 2	1330	4070	38	42
BP 9,10-diol 7,8-epoxide 1[a]	51	38	1.5	0.4
BP 9,10-diol 7,8-epoxide 2	380	110	11	1
BP 7,10-diol 8,9-epoxide	2	40	<0.1	0.4
H_4-BP 7,8-epoxide	320	260	9	3

[a] As discussed in the text, the material assigned to this structure was somewhat impure.

TABLE 2

Mutagenic activities of BP diol epoxides toward V79 cells

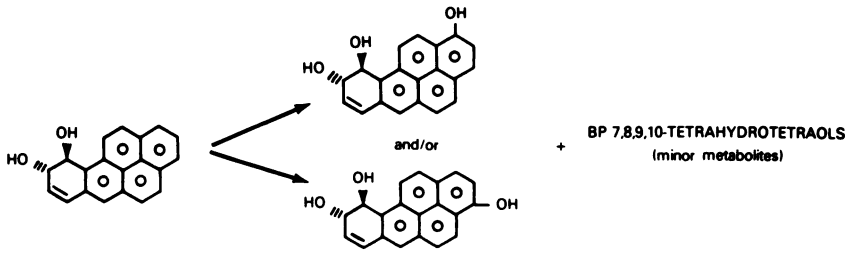
The experimental procedure for determining mutagenicity is described in MATERIALS AND METHODS.

Compound	Concentration	Survival	Mutation frequency[a]
	μM	%	
BP 7,8-diol 9,10-epoxide 1	0.5	56	106
BP 7,8-diol 9,10-epoxide 2	0.15	84	283
BP 9,10-diol 7,8-epoxide 1	0.3	94	0.8
	2.0	83	2.7
	15.0	83	4.0
BP 9,10-diol 7,8-epoxide 2	0.3	88	7.8
	1.0	88	3.8
	2.0	88	18
	15.0	79	21
BP 7,10-diol 8,9-epoxide	0.3	96	0.3
	2.0	100	1.8
	15.0	96	3.2

[a] 8-Azaguanine-resistant colonies per 10^5 survivors. Each value is the average of two determinations and is corrected for solvent.

Metabolism of BP 9,10-Dihydrodiol

When (−)-[^{14}C]BP 9,10-dihydrodiol (biosynthetic) was incubated with liver microsomes from control, phenobarbital-treated, or 3-methylcholanthrene-treated rats or with a highly purified, reconstituted system containing cytochrome P-448, one major metabolite was formed in all cases, with varying amounts of four to six minor metabolites. The major metabolite appears to be 1(3),9,10-trihydroxy-9,10-dihydro-BP (Scheme 3). The observations that led to the structure assignment are as follows. The major metabolite could be completely (more than 99%) extracted into 1 ml of 1 N NaOH from 1 ml of CH_2Cl_2, whereas only 22% of the metabolite could be extracted



SCHEME 3

into 1 ml of H_2O from 1 ml of CH_2Cl_2. These results indicate the phenolic nature of the metabolite(s). The ultraviolet spectra of an acidic and an alkaline methanolic solution of the metabolite are shown in Fig. 1. The bathochromic shift of the 360 nm band to 468 nm in alkaline solution further confirms the phenolic nature of the metabolite. In methanol solution that had been acidified with a trace of ammonium chloride, two fluorescence bands, at 435 nm and 458 nm, were present (excitation at 260 nm). However, in methanol containing a trace of sodium methoxide, an additional fluorescence band at 500 nm was observed, which disappeared when the solution was acidified. Mass spectral analysis of the metabolite showed a molecular ion at *m/e* 302. These data clearly indicate that the metabolite is a phenolic derivative of BP 9,10-dihydrodiol (Scheme 3). To obtain more definitive information about the position of the phenolic hydroxyl group, the metabolite was reduced with H_2/PtO_2 in 30% aqueous methanol at 1 atm of H_2. The reduced metabolite showed the expected molecular ion at *m/e* 304 and had an ultraviolet spectrum in methanol that was remarkably similar to that of 6,9,10-trihydroxy-7,8,9,10-tetrahydro-BP (Fig. 2). These two compounds, however, were not identical, based on their different retention times on reverse-phase chromatography (du Pont Zorbax ODS column eluted with a linear gradient of 60–98% methanol in water in 38 min after a 1-min delay, at a flow rate of 1.2 ml/min). The reduced metabolite emerged from the column at 10.5 min, whereas 6,9,10-trihydroxy-7,8,9,10-tetrahydro-BP emerged at 13 min. Comparison of the ultraviolet spectrum of the reduced metabolite with those of the known pyrene phenols (30) revealed that the spectrum of the reduced metabolite re-

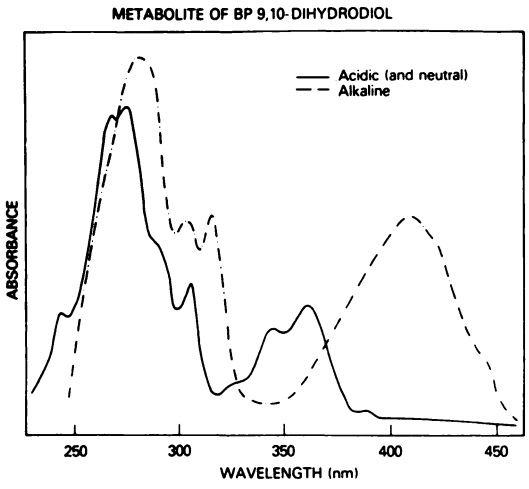


FIG. 1. *Ultraviolet-visible spectra of major metabolite of BP 9,10-dihydrodiol in acidic* (——) *and alkaline* (–·–·) *methanol*

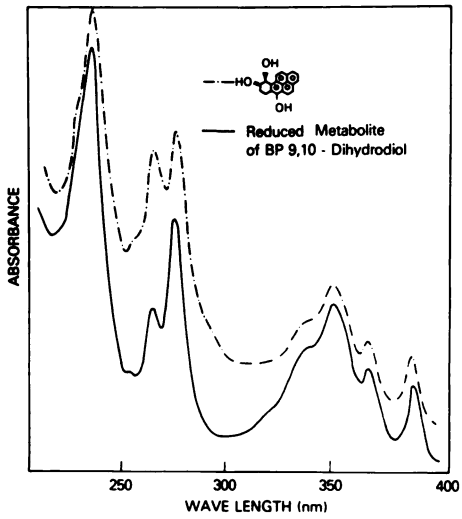


FIG. 2. *Ultraviolet-visible spectra of reduced metabolite of BP 9,10-dihydrodiol* (——) *and 6,9,10-trihydroxy-7,8,9,10-tetrahydro-BP* (–·–·) *in methanol*

sembles very closely the spectrum of 1-hydroxypyrene. Because of the symmetry of the pyrene chromophore, a phenolic hydroxyl group at position 1, 3, or 6 of the reduced metabolite would produce molecules with similar ultraviolet spectra. Since the hydroxyl group cannot be at position 6, based on the high-pressure liquid chromatographic evidence above, the metabolite formed from BP 9,10-dihydrodiol must be 1(3),9,10-trihydroxy-9,10-dihydro-BP. Although the minor metabolites were not extensively investigated, they all had ultraviolet spectra similar to that of tetrahydro-BP, a result which suggests that they are probably derived from BP 9,10-diol 7,8-epoxides.

A typical chromatographic trace of metabolites obtained by monitoring the effluent at 280 nm and the radioactivity profile of the metabolites is shown in Fig. 3. Quantitative results obtained with different microsomal preparations and the reconstituted system are summarized in Table 3. In all cases the phenolic metabolite constituted 60–75% of the total metabolism. Four other radioactive peaks, which chromatographed prior to, and two radioactive peaks that chromatographed after, BP 9,10-dihydrodiol constituted the balance of the metabolism. The major phenolic metabolite is formed to the extent of 75% and 69% in the absence and presence of epoxide hydrase, respectively, indicating the lack of effect of this enzyme on its formation by the reconstituted cytochrome P-448 system. 3-Methylcholanthrene treatment results in an increased rate of metabolism of BP 9,10-dihydrodiol, although to a lesser extent (1.5-fold) compared with BP (31) or BP 7,8-dihydrodiol (4,5), which are induced 4.5-fold and 2.6-fold, respectively. The over-all metabolism profile remains approximately the same with microsomes from control rats as well as from phenobarbital- or 3-methylcholanthrene-treated rats and with purified P-448.

Metabolism of BP 4,5-Dihydrodiol

Both BP 7,8-dihydrodiol and BP 9,10-dihydrodiol are good substrates for the cytochrome P-450-dependent monooxygenase system from rat liver. Similarly, (−)-[^{14}C]BP 4,5-dihydrodiol (biosynthetic) is also a good substrate for the monooxygenase system and is metabolized extensively (53%, Fig. 4) under experimental conditions that metabolize BP 9,10-dihydrodiol to the extent of 31% (Table 3). At least six chromatographically distinct products (see MA-

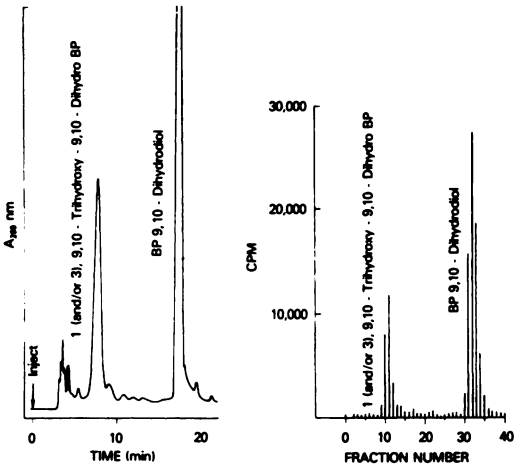


FIG. 3. *Chromatographic ultraviolet trace and radioactivity profile for metabolism of biosynthetic [^{14}C]BP 9,10-dihydrodiol*

The chromatographic conditions are described in MATERIALS AND METHODS. [^{14}C]BP 9,10-dihydrodiol was obtained by metabolic conversion of [^{14}C]BP with liver microsomes from 3-methylcholanthrene-induced rats and was found to be almost exclusively (94%) the (−) enantiomer (5).

TABLE 3

Metabolism of [^{14}C]BP 9,10-dihydrodiol by rat liver microsomes and purified P-448

Incubation mixtures contained 500 μg/ml of microsomal protein or 0.4 nmole/ml of purified P-448 hemoprotein. Recovery is the percentage of radioactivity that emerged from the column in distinct metabolite peaks. The numbers in the parentheses are the specific activities expressed as nmoles of products formed per nmole of P-450 or P-448 per min.

Protein	Major metabolite	Conversion	Recovery
	%	%	%
Control	61 (0.56)	11 (0.97)	79
Phenobarbital	64 (0.33)	17 (0.51)	85
3-Methylcholanthrene	75 (1.06)	31 (1.42)	87
P-448	75 (2.49)	40 (3.33)	89
P-448 + epoxide hydrase	69 (2.04)	41 (2.97)	72

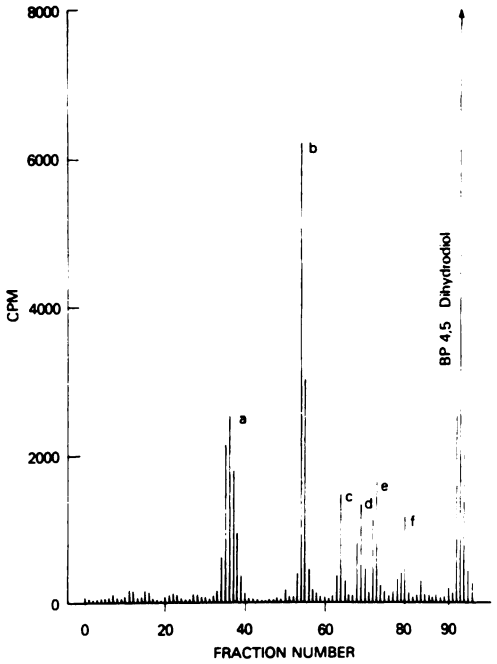


FIG. 4. *Chromatographic radioactivity profile for metabolism of biosynthetic [^{14}C]BP 4,5-dihydrodiol*

The chromatographic conditions are described in MATERIALS AND METHODS. [^{14}C]BP 4,5-dihydrodiol was obtained by metabolic conversion of [^{14}C]BP with liver microsomes from 3-methylcholanthrene-induced rats and was found to be almost exclusively (94%) the (−) enantiomer (5). Structures of the metabolites in peaks a–f were not determined.

TERIALS AND METHODS for the chromatographic system) are formed from the BP 4,5-dihydrodiol. Since requisite synthetic standards were unavailable and since these metabolites do not appear to possess high mutagenic activity (11), their structures were not investigated.

Carcinogenicity of (±)-BP 9,10-Dihydrodiol

Application of 0.15 μmole of the synthetic BP 9,10-dihydrodiol to the dorsal region of female C57BL/6J mice once every 2 weeks for 60 weeks (Table 4) failed to produce any tumors. Although 11 of the 30 animals died during the 60 weeks of treatment, there were no indications that this was due to toxicity of the dihydrodiol. Under identical experimental conditions, 0.15 μmole of BP caused a 97% incidence of tumors. The dose of 0.15 μmole was selected because it represents the approximate top of the dose-response curve for BP under these conditions (25–27). No tumors were observed in animals treated with the solvent alone.

TABLE 4

Carcinogenic activity of BP and (±)-BP 9,10-dihydrodiol on chronic application to mouse skin

Each treatment group consisted of 30 female C57BL/6J mice. The mice were treated with 0.15 μmole of the indicated compounds in 25 μl of acetone-NH_4OH (1000:1) or with the solvent alone once every 2 weeks for 60 weeks. The percentage of mice with tumors was computed from the number of surviving animals with and without tumors and the tumor-bearing mice that died during the course of the treatment.

Treatment	Tumor-bearing animals/animals at risk	Mice with tumors
		%
Solvent only	0/25	0
BP	29/30	97
BP 9,10-dihydrodiol	0/19	0

DISCUSSION

Since metabolism of BP 7,8-dihydrodiol by the cytochrome P-450-dependent monooxygenase system in liver is known to produce 7,8-diol 9,10-epoxides (4–7) as major metabolites, a related pathway in which BP 9,10-dihydrodiol is epoxidized to BP 9,10-diol 7,8-epoxides might be expected. From a thermodynamic standpoint, epoxidation of the 9,10-double bond in BP 7,8-dihydrodiol or of the 7,8-double bond in BP 9,10-dihydrodiol is favored over formation of an arene oxide from the pyrene aromatic nucleus. Nonetheless, as much as 40% of the total extractable metabolites of BP 7,8-dihydrodiol formed by microsomes from untreated rats consists of a phenolic metabolite (5) rather than a diol epoxide. The phenolic hydroxyl group was tentatively assigned to the 6-position of the dihydrodiol.

When BP 9,10-dihydrodiol was incubated with liver microsomes from control, phenobarbital-treated, or 3-methylcholanthrene-treated rats in the present study, the major metabolite (60–75%) was found to be 1(3),9,10-trihydroxy-9,10-dihydro-BP, with some minor metabolites containing a 7,8,9,10-tetrahydro-BP chromophore. With

purified, reconstituted cytochrome P-448, the metabolic pattern remained the same in the presence and absence of epoxide hydrase. The data indicate that an epoxide is not involved or that the epoxide isomerized to the phenol before epoxide hydrase could hydrate it to a dihydrodiol. The major metabolite was identified as a phenolic metabolite of BP 9,10-dihydrodiol, based on its mass spectrum (*m/e* 302), its ultraviolet and fluorescence spectra in acidic and alkaline solutions, and its extractability into alkali. This conclusion was further supported by the observation that the metabolite could be reduced by PtO_2/H_2 to give a compound with molecular ion *m/e* 304. This reduced metabolite has an ultraviolet spectrum identical with that of 6,9,10-trihydroxy-7,8,9,10-tetrahydro-BP. The two compounds were found to be different, however, based on the difference in their retention times on a du Pont Zorbax ODS column. Symmetry of the pyrene moiety of the 7,8,9,10-tetrahydro-BP predicts that 1-, 3-, or 6-hydroxy-7,8,9,10-tetrahydro-BP will have similar ultraviolet spectra. These observations fixed the position of the phenolic hydroxyl group at either position 1 or 3 of the metabolite.

The present results dramatically emphasize the important role played by the position of a dihydrodiol group in directing further metabolism of a BP metabolite. With liver microsomes from 3-methylcholanthrene-treated rats, the 7,8-dihydrodiol is metabolized almost entirely by epoxidation at the 9,10-double bond (4–8) whereas the 9,10-dihydrodiol undergoes hydroxylation at position 1 (or 3) (approximately 75%). Differences in the chemical reactivity of the double bonds in the two dihydrodiols and changes in the specificity of binding to cytochrome P-450 due to changes in the position of the polar dihydrodiol function on the hydrocarbon are probable factors responsible for these alterations in site of oxidation. Particularly noteworthy is the fact that BP 9,10-dihydrodiol has the diol group in the hindered bay region. This causes the hydroxyl groups to occupy a quasi-axial conformation which greatly increases both bulk and polarity in this region of the molecule. BP 4,5-dihydrodiol is also an excellent substrate for liver microsomes, although the nature of the several metabolites produced from it was not examined. When biosynthetic BP 4,5-, 7,8-, and 9,10-dihydrodiols were metabolized under similar incubation conditions (0.5 mg of microsomal protein from 3-methylcholanthrene-treated rats per milliliter; 40 μM substrate), turnover numbers of 2.7, 0.9, and 1.4 nmoles of products per nanomole of hemoprotein per minute, respectively, were observed (present study and ref. 5). These results indicate that BP 4,5-dihydrodiol is the best substrate of the three dihydrodiols and is at least as good a substrate as BP (see ref. 31).

Previously Booth and Sims (32) had studied the metabolism of BP 9,10-dihydrodiol by rat liver microsomes and had concluded, based on the ultraviolet spectrum of their metabolite and a signal in the mass spectrometer at *m/e* 284, that the metabolite was the catechol-like compound 9,10-dihydroxy-BP. Since the ultraviolet spectrum of their metabolite is identical with that of the 1(3),9,10-trihydroxy-9,10-dihydro-BP reported here, and since the base peak of our metabolite appears at *m/e* 284 because of loss of water ($M^+ - H_2O$), we must conclude that their assignment as 9,10-dihydroxy-BP is in error, possibly as a result of dehydration of the metabolite either before or during the measurement of its mass spectrum. The metabolite is indeed very labile when not stored at low temperature in the absence of traces of acid.

Inability to elicit a strong mutagenic response when BP 9,10-dihydrodiol is metabolically activated in the presence of the *S. typhimurium* tester strains (11, 12) is due to two factors. First, very little of the potentially mutagenic 9,10-diol 7,8-epoxides is actually formed (Fig. 3); second, testing of the synthetic 9,10-diol 7,8-epoxides (isomers 1 and 2) has established that they have very low inherent mutagenic activity (Tables 1 and 2). As previously had been shown for the BP 7,8-diol 9,10-epoxides (4, 33, 34) and for several diol epoxides derived from benz[*a*]anthracene (35), the mutagenic activity of BP 9,10-diol 7,8-epoxide 2 is not blocked by the addition of epoxide hydrase to the test system under conditions that result in the loss of 80% of the mutagenic activity of H_4-BP 7,8-epoxide (data

not shown). Quantum mechanical calculations of the ease of carbonium ion formation (1, 36) had predicted that compounds such as the BP 9,10-diol 7,8-epoxides, H_4-BP 7,8-epoxide, and BP 7,10-diol 8,9-epoxide, which do not have the epoxide function in the "bay region" of the hydrocarbon, will have low chemical reactivity relative to the highly reactive BP 7,8-diol 9,10-epoxides (37). Thus the carcinogenic activity of BP 7,8-dihydrodiol (3, 26, 27) and the lack of carcinogenic activity of BP 9,10-dihydrodiol reported in the present study are consistent with the mutagenicity of the metabolites formed from these dihydrodiols and our prior theoretical considerations (1, 36).

Note added in proof. During the preparation of this manuscript Malaveille *et al.* (*Mutation Research* **44**: 313–326, 1977) reported the mutagenic activity of the diastereomeric BP 9,10-diol-7,8-epoxides in strains TA 98 and TA 100 of *S. typhimurium* without providing evidence for the structure or purity of these diol epoxides. In both strains, the activity of BP 9,10-diol-7,8-epoxide-*1* relative to BP 7,8-diol-9,10-epoxide-*1* was nearly identical to our results. However, in strain TA 98 Malaveille *et al.* (C. Malaveille, J. Kuroki, P. Sims, P. L. Grover, and H. Bartsch (1977), *Mutation Research*, **44**, 313–326) found that BP 9,10-diol-7,8-epoxide-*2* had over *twice* the activity of BP 7,8-diol-9,10-epoxide-*2* while we report here the ratio of the two activities to be *0.3*. The 6-fold difference in relative activities may be due, in part, to the surprisingly low mutagenic activity of BP 7,8-diol-9,10-epoxide-*2* reported in their study (74 his^+ revertants/nmol epoxide in strain TA 98 versus 1330 his^+ revertants/nmol epoxide in the present study). Even when comparisons are made with data obtained under essentially the same assay conditions (Chart 2, reference 22), Malaveille *et al.* reported significantly less mutagenic activity for both diastereomeric 7,8-diol-9,10-epoxides in strain TA 98. In contrast, values obtained by the two laboratories in strain TA 100 agree closely.

REFERENCES

1. Jerina, D. M., Lehr, R. E., Schaefer-Ridder, M., Yagi, H., Karle, J. M., Thakker, D. R., Wood, A. W., Lu, A. Y. H., Ryan, D., West, S., Levin, W. & Conney, A. H. (1977) in *Origins of Human Cancer* (Hiatt, H., Watson, J. D. & Winsten, I., eds.), pp. 639–658, Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
2. Levin, W., Wood, A. W., Lu, A. Y. H., Ryan, D., West, S., Conney, A. H., Thakker, D. R., Yagi, H. & Jerina, D. M. (1977) in *Drug Metabolism Concepts*, ACS Symposium Series, No. 44 (Jerina, D. M., ed), pp. 99–123, American Chemical Society, Washington, D. C.
3. Kapitulnik, J., Levin, W., Conney, A. H., Yagi, H. & Jerina, D. M. (1977) *Nature*, **266**, 378–380.
4. Thakker, D. R., Yagi, H., Lu, A. Y. H., Levin, W., Conney, A. H. & Jerina, D. M. (1976) *Proc. Natl. Acad. Sci. U. S. A.*, **73**, 3381–3385.
5. Thakker, D. R., Yagi, H., Akagi, H., Koreeda, M., Lu, A. Y. H., Levin, W., Wood, A. W., Conney, A. H. & Jerina, D. M. (1977) *Chem.-Biol. Interactions*, **16**, 281–300.
6. Huberman, E., Sachs, L., Yang, S. K. & Gelboin, H. (1976) *Proc. Natl. Acad. Sci. U. S. A.*, **73**, 607–611.
7. Yang, S. K., McCourt, D. W., Roller, P. R. & Gelboin, H. V. (1976) *Proc. Natl. Acad. Sci. U. S. A.*, **73**, 2594–2598.
8. Thakker, D. R., Yagi, H., Levin, W., Lu, A. Y. H., Conney, A. H. & Jerina, D. M. (1977) *J. Biol. Chem.*, **252**, 6328–6334.
9. Yagi, H., Akagi, H., Thakker, D. R., Mah, H. D., Koreeda, M. & Jerina, D. M. (1977) *J. Am. Chem. Soc.*, **99**, 2358–2359.
10. Nakanishi, K., Kasai, H., Cho, H., Harvey, R., Jeffrey, A., Jennette, G. & Weinstein, I. (1977) *J. Am. Chem. Soc.*, **99**, 258–259.
11. Wood, A. W., Levin, W., Lu, A. Y. H., Yagi, H., Hernandez, O., Jerina, D. M. & Conney, A. H. (1976) *J. Biol. Chem.*, **251**, 4882–4890.
12. Malaveille, C., Bartsch, H., Grover, P. L. & Sims, P. (1975) *Biochem. Biophys. Res. Commun.*, **66**, 693–700.
13. Gibson, D. T., Mahadevan, V., Jerina, D. M., Yagi, H. & Yeh, H. J. C. (1975) *Science*, **189**, 296–297.
14. Yagi, H., Thakker, D. R., Hernandez, O., Koreeda, M. & Jerina, D. M. (1977) *J. Am. Chem. Soc.*, **99**, 1604–1611.
15. Yagi, H., Hernandez, O. & Jerina, D. M. (1975) *J. Am. Chem. Soc.*, **97**, 6881–6883.
16. Lehr, R. E., Schaeffer-Ridder, M. & Jerina, D. M. (1977) *Tetrahedron Lett.*, 539–542.
17. Yagi, H. & Jerina, D. M. (1975) *J. Am. Chem. Soc.*, **97**, 3185–3192.
18. Lu, A. Y. H. & Levin, W. (1972) *Biochem. Biophys. Res. Commun.*, **46**, 1334–1339.
19. Omura, T. & Sato, R. (1964) *J. Biol. Chem.*, **239**, 2379–2385.
20. Levin, W., Ryan, D., West, S. & Lu, A. Y. H. (1974) *J. Biol. Chem.*, **249**, 1747–1754.
21. Dignam, J. D. & Strobel, H. W. (1975) *Biochem. Biophys. Res. Commun.*, **63**, 845–852.
22. Lu, A. Y. H., Ryan, D., Jerina, D. M., Daly, J. W. & Levin, W. (1975) *J. Biol. Chem.*, **250**, 8283–8288.
23. Wood, A. W., Wislocki, P. G., Chang, R. L., Levin, W., Lu, A. Y. H., Yagi, H., Hernandez, O., Jerina, D. M. & Conney, A. H. (1976) *Cancer Res.*, **36**, 3358–3366.

24. McCann, J., Spingarn, N. E., Kobori, J. & Ames, B. N. (1975) *Proc. Natl. Acad. Sci. U. S. A.*, **72**, 979–983.
25. Levin, W., Wood, A. W., Yagi, H., Dansette, P. M., Jerina, D. M. & Conney, A. H. (1976) *Proc. Natl. Acad. Sci. U. S. A.*, **73**, 243–247.
26. Levin, W., Wood, A. W., Yagi, H., Jerina, D. M. & Conney, A. H. (1976) *Proc. Natl. Acad. Sci. U. S. A.*, **73**, 3867–3871.
27. Levin, W., Wood, A. W., Wislocki, P. G. Kapitulnik, J., Yagi, H., Jerina, D. M. & Conney, A. H. (1977) *Cancer Res.*, **37**, 3356–3361.
28. Wislocki, P. G., Wood, A. W., Chang, R. L., Levin, W., Yagi, H., Hernandez, O., Jerina, D. M. & Conney, A. H. (1976) *Biochem. Biophys. Res. Commun.*, **68**, 1006–1012.
29. Newbold, R. F. & Brookes, P. (1976) *Nature*, **261**, 52–54.
30. *Elsevier's Encyclopedia of Organic Chemistry* (1951) Ser. 3, Vol. 14 (suppl.) (Radt, F., ed.), pp. 4025–4585, Elsevier, New York.
31. Holder, G., Yagi, H., Dansette, P., Jerina, D. M., Levin, W., Lu, A. Y. H. & Conney, A. H. (1974) *Proc. Natl. Acad. Sci. U. S. A.*, **71**, 4356–4360.
32. Booth, J. & Sims, P. (1976) *Biochem. Pharmacol.*, **25**, 979–980.
33. Wood, A. W., Levin, W., Lu, A. Y. H., Yagi, H., Hernandez, O., Jerina, D. M. & Conney, A. H. (1976) *J. Biol. Chem.*, **251**, 4882–4890.
34. Wood, A. W., Wislocki, P. G., Chang, R. L., Levin, W., Yagi, H., Hernandez, O., Jerina, D. M. & Conney, A. H. (1976) *Cancer Res.*, **36**, 3358–3366.
35. Wood, A. W., Chang, R. L., Levin, W., Lehr, R. E., Schaefer-Ridder, M., Karle, J. M., Jerina, D. M. & Conney, A. H. (1977) *Proc. Natl. Acad. Sci. U. S. A.*, **74**, 2746–2750.
36. Jerina, D. M., Lehr, R. E., Yagi, H., Hernandez, O., Wislocki, P. G., Wood, A. W., Chang, R. L., Levin, W. & Conney, A. H. (1976) in *In Vitro Metabolic Activation in Mutagenesis Testing* (Deserres, F. J., Fouts, J. R., Bend, J. R. & Philpot, R. M., eds.), pp. 159–177, Elsevier/North Holland Biomedical Press, Amsterdam.
37. Whalen, D. L., Montemarano, J. A., Thakker, D. R., Yagi, H. & Jerina, D. M. (1977) *J. Am. Chem. Soc.*, **99**, 5522–5524.