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NADPH-Supported and arachidonic acid-supported metabolism of the enantiomers of *trans*-7,8-dihydrobenzo[*a*]pyrene-7,8-diol by human liver microsomal samples

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Using a new sensitive reverse-phase HPLC assay relying on UV detection at 344 nm, the capacity of 18 human liver microsomal samples to support NADPH-dependent, cytochrome P450-mediated oxidation and arachidonic acid-dependent oxidation of the enantiomers of *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene (B[*a*]P-7,8-DHD) was determined. The (−)-7*R*,8*R*-enantiomer, the preferred substrate of cytochrome P450, formed 94% diolepoxide 2 (*anti*-isomer; 7*R*,8*S*-dihydroxy-9*S*,10*R*-epoxy-7,8,9,10-tetrahydrobenzo[*a*]-pyrene) measured as derived alcohols, and the (+)-7*S*,8*S*-enantiomer formed 67% diolepoxide 1 (*syn*-isomer; 7*S*,8*R*-dihydroxy-9*S*,10*R*-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene). Arachidonic acid-supported oxidations gave ~70% diol-epoxide 2 from each enantiomer. The involvement of different sets of cytochrome P450 isozymes was supported by incubations in the presence of α-naphthoflavone (α-NF) (50 μM) and correlation studies. In the absence of α-NF, a positive correlation was found between the metabolism of the (−)-enantiomer but not the (+)-isomer of B[*a*]P-7,8-DHD and the relative content of P450IA2. In the presence of α-NF, the P450IIIA3/4 content correlated positively with the metabolism of both the (+)-enantiomer and the (−)-enantiomer. Gestodene (100 μM) inhibited the α-NF-stimulated metabolism, confirming the involvement of cytochrome P450IIIA3/4. No difference was found between the extent of arachidonic acid-supported, peroxyl radical-mediated metabolism of the (+)- and (−)-enantiomers of B[*a*]P-7,8-DHD. The metabolism was almost completely abolished by 2 μM butylatedhydroxyanisole and 100 μM nordihydroguaiaretic acid, confirming the free radical nature of the reaction.

Introduction

Trans-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene (B[*a*]P-7,8-DHD*) can undergo oxidative metabolism catalyzed by rodent liver microsomes to form the B[*a*]P-7,8-diol-9,10-epoxides (1). These exist as a pair of diastereomers in which the 7-hydroxyl group is either *cis* (*syn*-isomer or diolepoxide 1) or *trans* (*anti*-isomer or diolepoxide 2) to the 9,10-epoxide group (2). The formation of two optical enantiomers of each diastereomeric diolepoxide is possible (1). They spontaneously hydrate to stereoisomeric pairs of tetraols by *cis* and *trans* addition of water in reactions not catalyzed by epoxide hydrase (3).

*Abbreviations: B[*a*]P-7,8-DHD, *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]-pyrene; B[*a*]P, benzo[*a*]pyrene; B[*a*]P-tetraols and B[*a*]P-triols, see Materials and methods; BHA, butylated hydroxyanisole; NDGA, nordihydroguaiaretic acid; α-NF, α-naphthoflavone; DMF, dimethylformamide.

The stereochemistry of epoxidation of B[*a*]P-7,8-DHD is of importance since the different diolepoxide isomers differ in their reactivity toward nucleic acids and in their mutagenicity and carcinogenicity. In this respect, the (+)-enantiomer of the *anti* diolepoxide, derived from (−)-B[*a*]P-7,8-DHD, has been found to possess greater biological activity in mammalian cells than the other three stereoisomers of B[*a*]P-7,8-diol-9,10-epoxide (1,4). Due to this high activity, diolepoxide 2 is now regarded as an ultimate carcinogen derived from benzo[*a*]pyrene (B[*a*]P).

There are two possible mechanisms by which the epoxidation of B[*a*]P-7,8-DHD can take place: oxidation may be mediated by the mixed-function oxidase system involving cytochrome P450 (3,5–7), or may proceed through a peroxyl radical-mediated mechanism (8). Peroxyl radicals are generated in a variety of biochemical situations and co-oxidation of B[*a*]P-7,8-DHD by epoxidation at the 9,10-double bond occurs. This diolepoxide formation occurs during microsomal lipid peroxidation in liver microsomes triggered by the addition of ascorbate or NADPH in the presence of iron(III) and ADP (9), and in mouse keratinocytes (10). Enzymatic generation of peroxyl radicals occurs during the lipid hydroperoxide-dependent co-oxygenations of B[*a*]P-7,8-DHD involving prostaglandin H synthase (8,10–15) and lipoxygenases (13,16,17). B[*a*]P-7,8-DHD epoxidation also occurs during hematin-catalyzed decomposition of fatty acid hydroperoxides (18,19) and bisulfite peroxidation (20) or autoxidation (21). In addition the cytochrome P450 enzymes have peroxygenase activity and can oxidize toluene and methylaniline supported by hydroperoxides (22), can convert lipid hydroperoxides to lipid peroxidation products (23), and can reductively cleave hydroperoxides in the presence of NADPH (24).

It has been reported from animal studies that the stereoselectivity of epoxidation of B[*a*]P-7,8-DHD by peroxyl radicals is distinct from that exhibited by the cytochromes P450. Peroxyl radicals epoxidize (+)-B[*a*]P-7,8-DHD-mainly to the (−)-enantiomer of the diolepoxide 2 and the cytochromes P450 epoxidize it mainly to the (+)-enantiomer of the diolepoxide 1. (−)-B[*a*]P-7,8-DHD is epoxidized by both pathways to the (+)-enantiomer of the diolepoxide 2 (9,19,25,26).

The aim of the present study was to determine the relative contribution of the NADPH-dependent, cytochrome P450-mediated oxidation and the arachidonic acid-dependent, peroxyl radical-mediated oxidation of the (+)- and (−)-enantiomers of B[*a*]P-7,8-DHD in human liver microsomes. The stereoselectivities of the reactions and the effects of various additives were also examined.

Materials and methods

Chemicals

The following chemicals were purchased from the NCI Chemical Carcinogen Repository through Chemsyn Science Laboratories, Lexena, KS, USA: (±)-, (+)- and (−)-*trans*-B[*a*]P-7,8-DHD, racemic 7β,8α,9α,10β-tetrahydro-7,8,9,10-tetrahydroxybenzo[*a*]pyrene (B[*a*]P-7,10/8,9-tetraol), 7β,8α,9α,10α-tetrahydro-7,8,9,10-tetrahydroxybenzo[*a*]pyrene (B[*a*]P-7/8,9,10-tetraol), 7β,8α,9β,10β-tetrahydro-7,8,9,10-tetrahydroxybenzo[*a*]pyrene (B[*a*]P-7,9,10/8-tetraol), 7β,8α,9β,10α-tetrahydro-7,8,9,10-tetrahydroxybenzo[*a*]pyrene (B[*a*]P-7,9/8,10-tetraol), 7β,8α,9α,10-tetrahydro-7,8,9-trihydroxybenzo[*a*]pyrene (B[*a*]P-7/8,

9-triol), 7β,8α,9β,10-tetrahydro-7,8,9-trihydroxybenzo[*a*]pyrene (B[*a*]P-7,9/8-triol), *anti*-B[*a*]P-7,8-diol-9,10-epoxide. B[*a*]P-7,8-diol-9,10-epoxide and *trans*-dihydro-4,5-dihydroxybenzo[*a*]pyrene (B[*a*]P-4,5-DHD).

Biochemicals

NADPH was purchased from Boehringer Mannheim Corp., Sydney, Australia. Butylated hydroxyanisole (BHA), nordihydroguaiaretic acid (NDGA), α-naphthoflavone (α-NF), arachidonic acid, indomethacin and phenylbutazone were obtained from the Sigma Chemical Company, St Louis, MO, USA. SKF525A was purchased from Smith Kline and French Laboratories, Sydney, Australia. *bis*-1,1-Naphthol (Fluka Chemicals, Darmstadt, FRG) and HPLC-grade methanol (Mallinckrodt Speciality Chemicals Co., KY, USA) were obtained from the sources listed. Gestodene (13-ethyl-17β-hydroxy-18,19-di-nor-17α-pregna-4,15-diene-20-yn-3-one) was a generous gift of Dr H.Kuhl (University of Frankfurt, Germany).

Liver microsomes

Rat liver tissue. Control and phenobarbital-induced rat liver microsomal samples were prepared as described (27).

Human liver tissue. All the human liver microsomal samples except SV2 were samples from the liver bank held in the Department of Clinical Pharmacology Flinders University, Bedford Park, SA and were prepared by the method previously described (28). The SV2 liver tissue was obtained from St Vincents Hospital, Sydney. Three of the human liver samples were obtained by liver resection from patients suffering from liver tumour (subjects H12, H13 and SV2). In these cases, an apparently normal part of the tissue was taken. All other samples were removed from organ transplant donors 30–60 min after death (29). The clinical details of the subjects are shown in Table I. The use of human liver tissue in this study has received the approval of both Hospital and University Human Ethics Committees.

Methods

NADPH-Supported metabolism of (+)- and (−)-B[a]P-7,8-DHD by human liver microsomes. Metabolic studies were carried out using 1 ml incubations in 0.05 M Tris–HCl buffer, pH 7.4, containing 0.5 mg human liver microsomal protein and 4.5 mM $MgCl_2$. Various additives were preincubated with the reaction mixture for 2 min at 37°C, the final concentrations being 2 μM for BHA (added in 4 μl methanol), and 50 μM for α-NF (added in 20 μl acetone). The substrate, either (+)- or (−)-B[*a*]P-7,8-DHD (20 μM final concentration) was then added in acetone (20 μl) and then NADPH in buffer (0.1 ml, 0.5 mg, final concentration 0.6 mM) was added. After incubation at 37°C in a shaking water bath for 30 min, the reaction was terminated by rapid cooling in ice and 1.0 M phosphate buffer, pH 3.5 (0.1 ml) was added. One hour later, the internal standard (0.25 μg 1,1′-*bis*-naphthol) in ethyl acetate (1.0 ml) was added to the cold mixtures, which were then vortexed for 20 s. After further extraction with ethyl acetate (2 × 1 ml), the pooled organic layers were evaporated under vacuum at 30°C, and the residue was transferred to an autosampler vial using ethyl acetate (2 × 0.1 ml). The ethyl acetate was evaporated under nitrogen, the residue was dissolved in dimethylformamide (DMF) (30 μl) and an aliquot (20 μl) was analysed by HPLC. Blanks contained no NADPH. All manipulations were performed under yellow light to minimize the decomposition of the compounds.

Inhibition studies using gestodene. Gestodene inhibition of (+)- and (−)-B[*a*]P-7,8-DHD oxidation was determined using three human liver microsomal samples (H5, H11 and H12) in the presence and absence of α-NF. Reaction mixtures were prepared as described. Gestodene (100 μM), in 20 μl of acetone and NADPH (0.5 mg, in 50 μl buffer) were added and preincubated for 30 min in a shaking water bath at 37°C. BHA (2 μM), in 4 μl acetone and α-NF (50 μM) in 10 μl acetone were then added and the mixtures were preincubated for a further 2 min before the substrate (20 μM) was added in 10 μl acetone, followed by a further 0.5 mg NADPH in buffer (50 μl, total 1.2 mM). The oxidation of (+)- and (−)-B[*a*]P-7,8-DHD was then assayed as described above.

Arachidonic acid-supported metabolism of (+)- and (−)-B[a]P-7,8-DHD by human liver microsomes. Incubations (1.0 ml) in 0.05 M Tris–HCl buffer, pH 7.4, contained human liver microsomal protein (0.5 mg) and various additives, which were preincubated for 2 min at 37°C. The concentrations of these additives in the final incubation were BHA (2 μM, added in 4 μl methanol), NDGA (100 μM, added in 100 μl Tris–HCl buffer), indomethacin (100 μM, added in 30 μl acetone) and phenylbutazone (100 μM, added in 30 μl acetone). Substrate, (+)- or (−)-B[*a*]P-7,8-DHD (20 μmol in 20 μl acetone) was added to the cold incubation mixture, and the reaction was started by the addition of arachidonic acid (0.1 μmol in 5 μl Tris–HCl) and transferred to a shaking water bath at 37°C. After 30 min the reaction was terminated by rapid cooling on ice and 1 M phosphate buffer, pH 3.5 (0.1 ml), was added. After vortexing, the incubation mixtures were allowed by stand in ice for 1 h, and worked up as described above for NADPH-supported metabolism. Blanks contained no arachidonic acid.

Quantitation of the B[a]P-7,8-DHD metabolites

Preparation of standard curves. Seven standard solutions of the four B[*a*]P-7,8,9,10-tetraols and the two B[*a*]P-7,8,9-triols derived from the *anti*- and *syn*-isomers of B[*a*]P-7,8-diol-9,10-epoxide by hydrolysis and reduction were prepared in DMF so that 50 μl contained 7.5, 15, 30, 60, 120, 180 and 240 ng of each compound respectively. In separate test tubes, an aliquot (50 μl) of each standard solution was added to liver microsomal protein (0.5 mg) prepared from phenobarbitone-pretreated rats in 0.05 Tris–HCl buffer, pH 7.4 (1 ml). The test tubes were vortexed for 10 s and 1 M phosphate buffer, pH 3.5 (0.1 ml), was then added to each. A period of further vortexing (10 s) followed by 1 h in ice was allowed before the internal standard 1,1′-*bis*-naphthol (0.25 μg) in ethyl acetate (1 ml) was added and each was further extracted with ethyl acetate as described for NADPH-supported metabolism. HPCL of the extracted standards allowed the peak area ratios of each analyte to that of the internal standard to be calculated. These values were plotted against the metabolite amount (ng) and linear regression equations for the six compounds were obtained. In every case the value of the correlation coefficient exceeded 0.999. Recoveries were determined for the four tetraols across the whole concentration range by direct injection onto the HPLC; a mean of 60% was obtained.

Table I. Clinical data for liver donors

Subject	Age	Sex	Smoking history	Alcohol consumption	Drug treatment	P450 (nmol/mg)
H5	62	M	unknown	unknown	phenytoin, dexamethasone, gentamycin, ranitidine, cotrimoxazole	0.39
H6	18	M	unknown	social	penicillin, cotrimoxazole	0.28
H7	44	F	none	social	dexamethasone	0.32
H8	24	M	20 cig/day	social	dopamine	0.34
H9	19	M	none	social	dopamine	0.25
H10	67	F	none	social	dopamine, morphine, frusemide, midazolam	0.22
H11	23	M	none	moderate	phenytoin, carbamazepine, fluphenazine	0.28
H12	66	M	none	minimal	insulin	0.48
H13	61	F	none for 3 years	minimal	metoprolol, methylchothiazide	0.26
H14[a]	48	F	unknown	unknown	unknown	0.19
H15	22	M	unknown	unknown	phenobarbitone, phenytoin, dexamethasone, methylprednisolone, morphine, dopamine frusemide, atenolol, hydralazine, amoxil, gentamycin, vit. K, folic acid	0.43
H18	no information available					0.32
H19	11	F	none	none	none	0.36
H20	46	F	unknown	unknown	unknown	0.25
H21	52	M	unknown	unknown	unknown	0.17
H22	24	F	none	none	phenytoin, carbamazepine, haloperidol, benztropine	0.40
H23	no information available					0.39
SV2	66	M	smoker	unknown	prazosin, pindolol	0.41

[a]All liver donors were Caucasians except subject H14 who was of Asian origin.

HPLC analysis of B[a]P-7,8-DHD metabolites. The separation of the hydrolysis products of B[a]P-7,8-DHD metabolites was carried out on a 10 μm reverse-phase Hibar RP-8 column (250 × 4 mm, Merck, Darmstadt, Germany) fitted with a 5 μm RP-8 precolumn (4 × 4 mm) using a multistage methanol/water 40–100% gradient at 40°C and a flow rate of 1 ml/min. A Beckman system comprising two 110B pumps and a 421 gradient controller delivered 40–47.2% methanol over 35 min, 47.2–60% methanol over 1 min, 60% methanol for 20 min and 60–100% methanol over 1 min. The system was kept at 100% methanol for 20 min to wash the column before returning to initial conditions for the next injection. Using a Jasco Uvidec 100 V variable-wavelength detector, the analysis was performed at 344 nm. At 344 nm, the B[a]P-tetraols, the B[a]P-triols and B[a]P-7,8-DHD have a strong absorption peak, and this wavelength is close to a peak at 340 nm in the internal standard. The wavelength 344 nm was chosen because BHA and its metabolites were barely detectable at this wavelength. Retention times (in min) were as follows: B[a]P-7,10/8,90-tetraol, 16.5; B[a]P-7,9,10/8-tetraol, 26.5; B[a]P-7,9/8,10-tetraol, 19.5; B[a]P-7/8,9,10-tetraol, 22.0; B[a]P-7/8,9-triol, 28.5; B[a]P-7,9/8-triol, 40.0; 1,1′-*bis*-naphthol, 44.0; B[a]P-7,8-DHD, 47.2 (Figure 2A). The following compounds and their metabolites did not co-elute with the B[a]P-7,8-DHD metabolites or the internal standard: SKF525A, α-NF (with the exception of one minor metabolite), NDGA, indomethancin and phenylbutazone. The predominant tetraols were B[a]P-7,9,10/8-tetraol from the *syn*-diolepoxide and B[a]P-7,10/8,9-tetraol from the *anti*-diolepoxide (3,30).

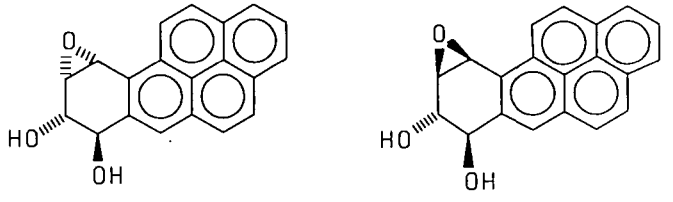

Fig. 1. The structures of the 7,8-diol-9,10-epoxides of 7,8,9,10-tetrahydrobenzo[a]pyrene.

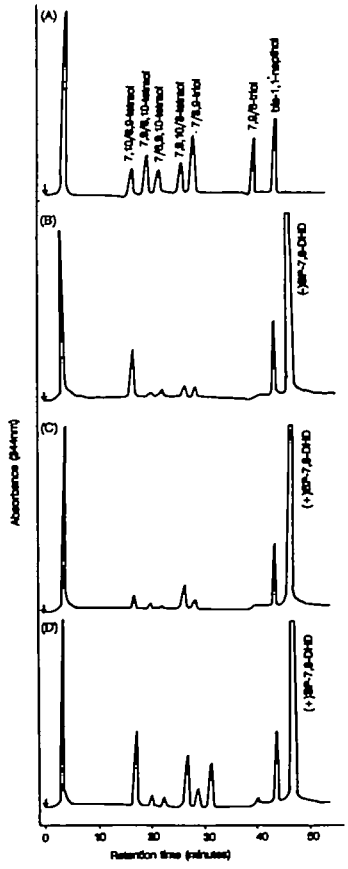

Fig. 2. Reverse-phase HPLC of (A) tetraols and triols derived from the two diastereomers of 7,8,9,10-tetrahydrobenzo[a]pyrene-7,8-diol-9,10-epoxides (30 ng standards); (B) human H23 microsomal metabolites of (−)-B[a]P-7,8-DHD in the absence of α-NF; (C) human H23 microsomal metabolites of (+)-B[a]P-7,8-DHD in the absence of α-NF; (D) human H23 microsomal metabolites of (+)-B[a]P-7,8-DHD formed in the presence of 50 μM α-NF; the peak emerging at ~32 min is a metabolite of α-NF.

Results and discussion

Metabolic assay methods

Assays for B[a]P-7,8-DHD metabolism have generally relied on the use of radioactive substrates and subsequent radiochemical determination of metabolic products (3,15,17) or changes in UV absorbance due to consumption of substrate (48). The current study employed a reverse-phase HPLC of compounds derived from the diastereoisomers of B[a]P-7,8-diol-9,10-epoxide by hydrolysis and NADPH-mediated reduction and an internal standard. Products were B[a]P-7,10/8,9-tetraol, B[a]P-7/8,9,10-tetraol and B[a]P-7/8,9-triol from the *anti*-diolepoxide diastereoisomer, and B[a]P-7,9,10/8-tetraol, B[a]P-7,9/8,10-tetraol and B[a]P-7,9/8-triol from the *syn*-diolepoxide diastereoisomer. Quantitation was carried out using 1,1′-*bis*-naphthol as the internal standard, and peak area ratios were determined after incubation mixtures were adjusted to pH 3.5 with phosphate buffer and allowed to stand for a sufficient period of time to guarantee complete hydrolysis. Using the *anti*-diolepoxide, which is the isomer of lesser reactivity towards hydrolysis under neutral conditions (30), experiments involving addition of the epoxide to incubation buffer at pH 7.4 containing 0.5 mg/ml rat liver microsomal protein followed by immediate extraction gave incomplete recoveries of the diolepoxide (as hydrolysis products) in the organic extracts. When solutions of the *anti*-diolepoxide in the presence of protein were adjusted from pH 7.4 to pH 3.5 by addition of phosphate buffer, and allowed to stand for periods of up to 1 h, hydrolysis increased. After 15 min the reaction was 85% complete and by 1 h total hydrolysis had occurred. Therefore a 1 h hydrolysis period at pH 3.5 was included in the assay. Problems with the analysis of these diolepoxides have been recognized by others. Thus during hematin-catalyzed oxidation of B[a]P-7,8-DHD, initiated by unsaturated fatty acid hydroperoxides, poor recovery of radioactivity was noted (18), and during methanol/water gradient HPLC of the diolepoxides themselves (3) or of prostaglandin synthase co-oxidation products 10-*O*-methylated B[a]P-tetraols were formed (31). The limit of detection of the assay was ~2 ng for each analyte, which under the conditions of the incubation and HPLC translates to ~1 pmol metabolites/mg protein/min.

Incubations designed to determine NADPH-supported cytochrome P450-catalyzed oxidation of B[a]P-7,8-DHD were effected in the presence of 2 μM BHA. Preliminary experiments with human liver microsomes in the absence of BHA gave high blank values (no NADPH), which made measurements of the NADPH-supported reaction unreliable. Using rat liver microsomal protein to effect the NADPH-supported oxidation of B[a]P-7,8-DHD even higher blanks were obtained, but in the presence of 1, 2, 5 and 10 μM BHA blanks were very substantially reduced. The chosen concentration of 2 μM gave minimal inhibition of the rat liver microsomal catalysed oxidation. The oxidation of B[a]P-7,8-DHD seen even in unincubated blanks in the absence of BHA is probably mediated by peroxides both present in the microsomal preparation and formed during incubations (8,23,24), in part perhaps by lipoxygenase (13,16,17,47) (see later discussion). The very substantial reduction of these blank values in the presence of a low concentration of BHA supports this proposal.

Table II. NADPH-supported metabolism of (+)-B[*a*]P-7*S*,8*S*-DHD by human liver microsomes

Sample no.	Sp. act.[a]		DE1 (%)[b]	DE2 (%)	Fold change[c]	α-NF	
	mg protein	nmol P450				DE1 (%)[b]	DE2 (%)
H5	12.3	31.4	61	39	4.24	36	64
H6	15.7	57.2	82	18	1.26	40	60
H7	10.7	33.5	61	39	3.41	37	63
H8	10.1	29.6	72	28	2.57	35	65
H9	4.3	17.2	92	8	1.78	31	69
H10	11.2	51.0	73	27	2.21	34	66
H11	10.7[d]	39.0[d]	57[d]	43[d]	2.66	42	58
H12[e]	27.8[d]	57.9[d]	70[d]	30[d]	1.73	39	61
H13	9.1	34.3	55	45	2.52	31	69
H14	10.4[d]	54.9[d]	61[d]	39[d]	3.14	35	65
H15[e]	14.0[d]	32.5[d]	59[d]	41[d]	2.22	43	57
H18	11.4	35.5	69	31	1.41	35	65
H19	12.6	35.0	65	35	2.14	35	65
H20	13.3	52.7	66	34	1.45	34	66
H21	14.4	87.5	65	35	1.34	37	63
H22	26.3	65.9	77	23	1.28	57	43
H23	11.7	30.5	56	44	3.34	35	65
SV2	15.7	38.3	71	29	2.08	39	61
Mean	13.4	43.6	67	33	2.27	37	63
SD	5.6	16.8	10	10	0.85	6	6

DE1 and DE2, diolepoxide 1 and 2.
[a]Specific enzyme activities are expressed as pmol metabolites/mg protein/min or pmol metabolites/nmol P450/min, determined in the presence of 0.6 mM NADPH and 2 μM BHA. Data were corrected using incubations containing either 2 μM BHA only or 2 μM BHA and 50 μM α-NF and no NADPH.
[b]Both triols were combined and expressed as a percentage of total metabolites; the proportions of DE-1 and DE-2 were normalized to 100%.
[c]Factor showing an increase in metabolism (>1.00), or inhibition of metabolism (<1.00) when incubations were conducted in the presence of 50 μM α-NF.
[d]Means of duplicate determinations.
[e]Substrate concentration was 40 μM. For all other human liver microsomal samples the substrate concentration was 20 μM.

Table III. NADPH-supported metabolism of (−)-B[*a*]P-7*R*,8*R*-DHD by human liver microsomes

Sample no.	Sp. act.[a]		DE1 (%)[b]	DE2 (%)	Fold change[c]	α-NF	
	mg protein	nmol P450				DE1 (%)[b]	DE2 (%)
H5	26.7	68.4	3	97	2.25	31	69
H6	51.6	187.7	1	99	0.51	25	75
H7	32.5	101.4	9	91	1.08	38	62
H8	35.3	103.9	2	98	0.72	30	70
H9	21.7	86.9	3	97	0.47	27	73
H10	32.2	146.3	7	93	0.65	39	61
H11	25.4[d]	92.4[d]	2[d]	98[d]	1.20	47	53
H12[e]	51.0[d]	105.9[d]	5[d]	95[d]	0.59	36	64
H13	31.1	117.7	6	94	0.59	24	76
H14	22.5[d]	134.2[d]	9[d]	91[d]	1.11	37	63
H15[e]	22.8[d]	53.0[d]	11[d]	89[d]	1.06	45	55
H18	32.7	102.3	3	97	0.52	34	66
H19	56.2	156.1	5	95	0.50	26	74
H20	30.1	118.8	6	94	0.64	27	73
H21	34.5	209.3	6	94	0.52	35	65
H22	77.2	192.9	3	97	0.47	38	62
H23	18.3	47.5	14	86	1.79	32	68
SV2	32.3	78.9	8	92	0.76	39	61
Mean	35.2	116.3	6	94	0.86	34	66
SD	14.8	47.0	4	4	0.49	7	7

DE1 and DE2, diolepoxide 1 and 2.
[a]Specific enzyme activities are expressed as pmol metabolites/mg protein/min or pmol metabolites/nmol P450/min, determined in the presence of 0.6 mM NADPH and 2 μM BHA. Data were corrected using incubations containing either 2 μM BHA only or 2 μM BHA and 50 μM α-NF and no added NADPH.
[b]Both triols were combined and expressed as a percentage of total metabolites; the proportions of DE-1 and DE-2 were normalized to 100%.
[c]Factor showing an increase in metabolism (>1.00), or inhibition of metabolism (<1.00) when incubations were conducted in the presence of 50 μM α-NF.
[d]Means of duplicate determinations.
[e]Substrate concentration was 40 μM. For all other human liver microsomal samples the substrate concentration was 20 μM.

Data collected in the Tables show the capacity of 18 samples of human liver microsomes to catalyze the NADPH-supported oxidation (Tables II and III), and arachidonic acid-supported oxidation (Tables IV and V) of the enantiomers of B[*a*]P-7,8-DHD to B[*a*]P-diolepoxides 1 and 2. The total metabolic capacity and the relative amounts of the two diolepoxides derived from the sums of their hydrolysis and reduction products are given. The proportion of this found as triols ranged from 4 to 30%. Protein-bound products from the diolepoxides could not be determined but in other work the proportion was low (~5%) (14) or moderate (>30%) (3).

NADPH-dependent metabolism of (+)- and (−)-B[a]P-7,8-DHD

Tables II and III display the extents of NADPH-supported metabolism of (+)- and (−)-B[*a*]P-7,8-DHD by human liver microsomal samples in the presence and absence of α-NF. The activity towards the (−)-enantiomer of B[*a*]P-7,8-DHD was about twice that seen towards the (+)-enantiomer (Figures 2B and C for sample H23) and the product ratios were characteristic for each enantiomer of the dihydrodiol. The (+)-enantiomer was metabolized to a mean 67% diolepoxide 1, while the (−)-enantiomer was converted to a mean 94% diolepoxide 2. With cytochromes P450LM$_2$ and P450LM$_4$ purified from rabbit liver, LM$_4$ showed a much greater activity towards these B[*a*]P derivatives than LM$_2$ (5,25), and LM$_4$ preferentially metabolizes the (−)-isomer. In a similar fashion, the (−)-isomer was converted predominantly to diolepoxide 2. Thakker *et al.* (1) reported the high stereoselectivity in the formation of diolepoxide 2 relative to diolepoxide 1 from the 7*R*,8*R*-enantiomer by liver microsomes from 3-methylcholanthrene-pretreated rats and with purified cytochrome P448 (IA1). Microsomes from control and phenobarbitone-treated rats metabolized (+)- and (−)-B[*a*]P-7,8-DHD with less stereoselectivity. These differences in stereoselectivity in the metabolism of the B[*a*]P-7,8-dihydrodiols reflect the differences in the proportion of various cytochrome P450s present in liver microsomes from control, MC-induced and phenobarbitone-induced rats.

In the present work, differences between individuals in the proportions of diolepoxide 1 and diolepoxide 2 derived metabolites formed from (+)- and (−)-B[*a*]P-7,8-DHD also probably reflect differences in the content of particular cytochrome P450s in the liver microsomes. Drug pretreatment, smoking habit and genetic factors have been shown to influence the expression of specific P450s in human liver. α-NF has been reported to be capable of stimulating some P450-mediated reactions in human liver microsomes and inhibiting others (4,6,33−36). In the present work, 50 μM α-NF increased the metabolism of (+)-B[*a*]P-7,8-DHD by a mean 2.27 fold ($P <$

Table IV. Arachidonic acid-supported oxidation of (+)-B[a]P-7S,8S-DHD by human liver microsomes

Sample no.	Sp. act.[a]	Additive[b]							
		BHA[c]		NDGA[c]		Phenbut[c]		Indometh	
		Sp. act.[a]	Fold change[d]	Sp. act.[a]	Fold change[d]	Sp. act.[a]	Fold change[d]	Sp. act.[a]	Fold change[d]
H5	34.3 (79)[e]	5.2	0.15	4.6	0.13	3.2	0.09	62.0	1.81
H6	77.9 (86)	12.1	0.16	18.2	0.23	8.9	0.11	77.7	0.99
H7	23.2 (67)	4.1	0.18	–	–	–	–	–	–
H8	123.1 (84)	11.0	0.09	6.9	0.06	13.8	0.11	172.1	1.40
H9	44.1 (84)	5.4	0.12	8.8	0.20	4.0	0.09	44.4	1.01
H10	20.0 (65)	4.3	0.21	–	–	1.0	0.05	11.7	0.59
H11	41.5 (57)	–	–	–	–	2.5	0.06	22.9	0.55
H12	31.3 (79)	–	–	–	–	14.7	0.47	31.9	1.02
H13	11.0 (68)	1.0	0.09	1.2	0.11	0.1	0.01	28.7	2.62
H14	9.3 (65)	2.4	0.26	1.7	0.19	0.5	0.05	5.8	0.62
H15	17.6 (67)	3.9	0.22	–	–	1.7	0.10	16.5	0.94
H18	21.4 (73)	4.1	0.19	–	–	–	–	–	–
H19	35.1 (88)	4.2	0.12	5.3	0.15	1.6	0.05	48.3	1.38
H20	17.5 (67)	2.3	0.13	0.8	0.04	1.0	0.06	15.9	0.91
H21	23.3 (66)	1.6	0.07	1.9	0.08	1.4	0.06	20.4	0.87
H22	18.0 (78)	1.6	0.09	1.7	0.10	2.6	0.15	41.5	2.31
H23	40.0 (67)	3.3	0.08	1.3	0.03	2.1	0.05	31.4	0.78
SV2	4.2 (74)	1.9	0.46	4.2	1.0	1.0	0.23	9.0	2.14
Mean	32.9 (73)	4.3	0.16	4.7	0.19	3.8	0.11	40.0	1.20
SD	28.1 (9)	3.1	0.10	5.0	0.26	4.6	0.11	40.4	0.60

[a]Specific enzyme activities are expressed as pmol metabolites/mg protein/min using 100 μM arachidonic acid and a substrate concentration of 20 μM.
[b]Additives were BHA (2 μM), NDGA (100 μM), phenylbutazone (100 μM) or indomethacin (100 μM).
[c]These data have been corrected for endogenous oxidation in the presence of the additive and absence of added arachidonic acid.
[d]Fold change shows the inhibition (factor < 1.0) or enhancement (factor > 1.0) of metabolism when incubations were conducted in the presence of the additive BHA, NDGA, phenylbutazone or indomethacin.
[e]The percentage of diolepoxide 2 are formed in the uninhibited reaction is shown in parenthesis.

0.001) (Figure 2D; Table II). This stimulatory effect occurred in all samples; however, the extent of stimulation varied from 1.26-fold to 4.24-fold. The α-NF caused a mean 4.3-fold increase in the amounts of diolepoxide 2 hydrolysis products formed, while the amounts of diolepoxide 1-derived products remained virtually unchanged. (There was a mean 1.3-fold change.) This translated as a decreased proportion of diolepoxide 1 products and an increased proportion of tetraols derived from diolepoxide 2.

The effect of 50 μM α-NF on the metabolism of (−)-B[a]P-7,8-DHD was to decrease it by a mean 0.86-fold ($P < 0.05$) (Table III). This inhibitory effect was not observed in all samples, and six of the 18 samples showed increases in metabolism. α-NF generally increased the amounts of diolepoxide 1 metabolites formed and decreased the amounts of diolepoxide 2 metabolites formed, resulting in the changed proportions shown in Table III.

The liver samples used were those described in McManus *et al.* (29), and the relative P450IA2 and P450IIIA3/4 contents of these samples were available. Those results were used to determine the correlation of the relative content of these P450 isozymes with the rates of metabolism of (+)- and (−)-B[a]P-7,8-DHD. Statistically significant correlations were observed between the P450IIIA3/4 levels and the metabolism (pmol metabolites/mg protein/min) of (+)-B[a]P-7,8-DHD ($r = 0.77$, $r^2 = 0.587$) and (−)-B[a]P-7,8-DHD ($r = 0.82$, $r^2 = 0.667$) in the presence of α-NF, but not in its absence. The human P450IIIA3 IgG used for the quantitation of the P450IIIA3 content of the human liver microsomal samples used in the present work was a polyclonal antibody (29) and would therefore recognize at least P450HLp, P450NF25 and P450NF10 in human liver microsomes. It is therefore possible to conclude that the P450IIIA3/4 proteins play an important role in the metabolism of (+)- and (−)-B[a]P-7,8-DHD after stimulation by α-NF. This conclusion was also supported by the observation that the human liver microsomal samples obtained from patients known to be receiving dexamethasone, a synthetic glucocorticoid, as a medication (patients H5, H7 and H15) were among those that showed the highest fold increases in metabolism of (+)- and (−)-B[a]P-7,8-DHD after stimulation by α-NF. The samples H5, H7 and H15 gave 4.24-, 3.41- and 2.22-fold increases in the metabolism of the (+)-enantiomer, and 2.25-, 1.08- and 1.06-fold increases in the metabolism of the (−)-enantiomer respectively when α-NF was included in the incubation mixture. The specific activities of these three samples towards both the (+)- and (−)-enantiomers of B[a]P-7,8-DHD in the absence of α-NF were not significantly higher than the 15 other human liver microsomal samples. Since dexamethasone is an inducer of the cytochrome P450IIIA enzymes (37), these results support the correlation studies which point to an involvement of the P450IIIA3/4 enzymes in the metabolism of the (+)- and (−)-enantiomers of B[a]P-7,8-DHD only after stimulation by α-NF, but not in the absence of α-NF.

In all human liver microsomes examined, P450IA2 is the dominant P450IA protein present (29). However, due to the difficulty associated with separating P450IA1 and IA2 proteins on SDS−PAGE, the presence on P450IA1 mRNA in 11 of the 23 livers tested in our laboratory (38), and the fact that the polyclonal anti-rabbit P450IA2 IgG used in these studies recognizes both P450IA proteins, the possibility of the P450IA2 band containing P450IA1 protein cannot be totally discounted. In the present study in the absence of α-NF, the metabolism (pmol metabolites formed/mg protein/min) of the (−)-enantiomer of B[a]P-7,8-DHD correlated positively with P450IA2 protein content ($r = 0.72$, $r^2 = 0.518$) of human liver microsomes. No

Table V. Arachidonic acid-supported oxidation of (−)-B[*a*]P-7*R*,8*R*-DHD by human liver microsomes

Sample no.	Sp. act.[a]	Additive[b]							
		BHA[c]		NDGA[c]		Phenbut[c]		Indometh	
		Sp. act.[a]	Fold change[d]	Sp. act.[a]	Fold change[d]	Sp. act.[a]	Fold change[d]	Sp. act.[a]	Fold change[d]
H5	53.0 (63)[e]	7.0	0.13	3.5	0.07	1.1	0.02	56.7	1.07
H6	52.5 (79)	12.5	0.24	12.9	0.25	12.4	0.24	85.6	1.63
H7	13.1 (67)	4.0	0.30	–	–	–	–	–	–
H8	64.5 (82)	19.6	0.30	26.1	0.40	6.9	0.11	99.1	1.54
H9	20.7 (72)	3.0	0.15	5.6	0.27	0.4	0.02	41.9	2.03
H10	16.9 (64)	4.7	0.28	–	–	3.9	0.23	8.9	0.53
H11	41.8 (65)	–	–	–	–	1.2	0.03	37.2	0.89
H12	66.0 (73)	–	–	–	–	17.0	0.26	54.2	0.82
H13	14.0 (68)	1.0	0.07	0	0	6.3	0.45	30.6	2.19
H14	8.6 (82)	5.7	0.67	2.3	0.26	2.3	0.26	12.1	1.41
H15	26.5 (62)	4.3	0.16	–	–	3.5	0.13	28.9	1.09
H18	16.8 (73)	1.1	0.06	–	–	–	–	–	–
H19	24.7 (76)	0.9	0.03	3.3	0.13	1.0	0.04	28.7	1.16
H20	23.5 (66)	0.8	0.03	0.8	0.03	1.8	0.08	43.4	0.15
H21	13.8 (63)	3.5	0.26	2.1	0.15	1.1	0.08	18.0	1.30
H22	31.2 (57)	1.2	0.04	1.0	0.03	0.3	0.01	153.5	4.92
H23	25.2 (62)	1.5	0.06	1.0	0.04	0.5	0.02	104.2	4.14
SV2	2.5 (64)	2.5	1.0	4.0	1.60	1.2	0.48	5.3	2.15
Mean	28.6 (69)	4.6	0.23	5.2	0.27	3.8	0.15	50.5	1.69
SD	19.1 (7)	5.0	0.26	7.4	0.44	4.8	0.15	40.9	1.25

[a]Specific enzyme activities are expressed as pmol metabolites/mg protein/min using 100 μM arachidonic acid and a substrate concentration of 20 μM.
[b]Additives were BHA (2 μM), NDGA (100 μM), phenylbutazone (100 μM) or indomethacin (100 μM).
[c]These data have been corrected for endogenous oxidation in the presence of the additive and absence of added arachidonic acid.
[d]Fold change shows the inhibition (factor < 1.0) or enhancement (factor > 1.0) of metabolism when incubations were conducted in the presence of the additive BHA, NDGA, phenylbutazone or indomethacin.
[e]The percentage of diolepoxide 2 are formed in the uninhibited reaction is shown in parenthesis.

such positive correlation between P450IA2 levels and the metabolism of the (+)-enantiomer of the substrate was evident. These correlative studies suggest that in the absence of α-NF P450IA2 may play some role in the metabolism of the (−)-enantiomer of B[*a*]P-7,8-DHD by human liver microsomes, but not of the (+)-enantiomer.

α-NF must stimulate P450IIIA3/4 and inhibit P450IA1 and P450IA2 since the overall effect of 50 μM α-NF on the metabolism of (−)-B[*a*]P-7,8-DHD was to inhibit it by a mean 0.86-fold. Inhibition of P450IA protein activity is supported by the poor correlation of their specific P450 content with the metabolism of (−)-B[*a*]P-7,8-DHD in the presence of α-NF. Stimulation of P450IIIA3/4 and inhibition of P450IA1 and P450IA2 by α-NF is consistent with previous observations reported in the literature. Shimada and co-workers (6,39) have previously provided evidence that human $P450_{NF}$ (IIIA4) shows a stimulatory response to α-NF. McManus *et al.* (29) observed that the ability of expressed human P450IA1 and P450IA2 to *N*-hydroxylate AAF (2-acetylaminofluorene) was strongly inhibited by α-NF. In addition, α-NF at low concentrations also inhibited the capacity of the P450IA1 and P450IA2 proteins to activate IQ (2-amino-3-methylimidazo[4,5-*f*]quinoline) and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine) in the Ames test (29), and at high concentrations stimulated the ability of P450IIIA proteins to metabolize these compounds to mutagens (34). Shimada *et al.* (6) also observed strong inhibition of $P450_{PA}$ (P450IA2) catalytic activities by α-NF.

The differential effects of α-NF and the correlative studies suggest that different P450 isozymes are involved in the metabolism of the (+)- and (−)-enantiomers of B[*a*]P-7,8-DHD. Cytochrome P450IA2 appears to have an affinity for the (−)-enantiomer of B[*a*]P-7,8-DHD but not for the (+)-enantiomer. Cytochrome P450IIIA3/4 is able to activate both enantiomers of the substrate and favours diolepoxide 2 formation from both enantiomers of the dihydrodiol after stimulation by α-NF.

The observations made in the present work do not totally agree with those of Shimada and co-workers (7). By measuring the *umu* gene response, they found that α-NF stimulated the activation of (±)-, (+)- and (−)-B[*a*]P-7,8-DHD in incubations with human liver microsomes and with a reconstituted system containing $P450_{NF}$ (P450IIIA4). Polyclonal antibodies to $P450_{NF}$ inhibited the microsomal *umu* gene response with (±)-, (+)- and (−)-B[*a*]P-7,8-DHD, suggesting that $P450_{NF}$ is the major enzyme in the activation of B[*a*]P-7,8-DHD in human liver. Correlation studies supported this conclusion. In the present work α-NF stimulated the metabolism of (+)-B[*a*]P-7,8-DHD and inhibited the metabolism of (−)-B[*a*]P-7,8-DHD. According to the correlation studies, cytochrome P450IIIA3/4 was important in the metabolism of both enantiomers of the substrate only after stimulation by α-NF. This conclusion was drawn by Shimada *et al.* (6), but contrary to the present findings, they found no correlation of either B[*a*]P-7,8-DHD isomer *umu* gene responses with the activities catalyzed by $P450_{PA}$ (P450IA2). We examined the effect of gestodene, an ethynylic steroid with progestational properties and which was shown to be an effective inactivator of the cytochromes P450IIIA (40−43). Using three microsomal preparations (H5, H11 and H12), preincubation of the microsomes with NADPH and gestodene in experiments conducted in the absence of α-NF had only a relatively small inhibitory effect on the metabolism of (+)- and (−)-B[*a*]P-

7,8-DHD (~22% decrease) compared to experiments in which α-NF was added to the preincubation. Here metabolism of (−)-B[a]P-7,8-DHD was inhibited by 78.3 ± 3.0% while that of (+)-B[a]P-7,8-DHD was inhibited by 66.3 ± 9.1% ($n = 3$). These results further show the major role of the IIIA3/4 proteins in the metabolism of both enantiomers of the substrate after α-NF pretreatment, while illustrating the minor role these enzymes play when there is no activation by α-NF. The extent of the inhibition by gestodene was not dependent on the prior activation of the IIIA protein by α-NF since results were independent of the order of preincubation.

In animal liver microsomes, 2-diethylaminoethyl 2,3-diphenylvalerate (SKF525A) has been shown to be a potent inhibitor of the cytochrome P450 system (44,45). In the present work, further confirmation of the involvement of the cytochrome P450 enzymes in the NADPH-dependent metabolism of (+)- and (−)-B[a]P-7,8-DHD is provided through the significant inhibition effected by 500 μM SKF525A. In the presence of 2 μM BHA, the metabolism of the (+)-enantiomer was inhibited by a mean 35% and the metabolism of the (−)-enantiomer was inhibited by a mean 42%. Similar extents of inhibition were observed in the absence of BHA. The presence of SKF525A in the incubation mixtures generally had little effect on the proportions of diolepoxide 1- and diolepoxide 2-derived products produced (data not shown).

Arachidonic acid-supported conversion of (+)- and (−)-B[a]P-7,8-DHD to diolepoxides

Tables IV and V display the extents of arachidonic acid-supported conversion of (+)- and (−)-B[a]P-7,8-DHD by human liver microsomal samples in the presence and absence of various additives. The activity towards the (+)-isomer was about twice that seen in the NADPH-supported reaction (Table II), while that towards the (−)-B[a]P-7,8-DHD was approximately equal to the NADPH-supported oxidation (Table III). In the presence of both arachidonic acid and either BHA, NDGA or phenylbutazone, the activities were reduced to <27% of the conversion in the presence of added arachidonic acid only. The (+)-enantiomer of the substrate gave a mean 73% diolepoxide 2-derived products and the (−)-enantiomer afforded a mean 69% diolepoxide 2-derived metabolites. This stereochemistry is in agreement with that of the peroxyl radical-mediated oxidations (8−10,16−18, 21,26).

There was no significant difference between the mean extent of arachidonic acid-supported oxidation of the (+)- and (−)-enantiomers of B[a]P-7,8-DHD but there was a wide variation in activities within the human liver microsomal samples. Sample SV2, for example, had a very low activity towards both of these substrates, whereas sample H8 showed a higher than average activity. The SV2 liver sample was obtained by liver resection from a patient (a smoker) with a liver tumor, while H8 liver sample was obtained from a renal transplant donor who was also a smoker.

Confirmation of the free radical nature of the arachidonic acid-dependent metabolism of (+)- and (−)-B[a]P-7,8-DHD is provided by the substantial inhibition effected by the antioxidants BHA and NDGA. Prostaglandin synthase co-oxidations are inhibited at the hydroperoxidase step by both BHA and NDGA, but this enzyme was not thought to be involved in these samples in a peroxide radical-mediated process because 100 μM indomethacin, an inhibitor of cyclooxygenase, did not inhibit the metabolism consistently and in many samples increases occurred. The ratio of diolepoxide 2 to diolepoxide 1 is also inconsistent with a major role for prostaglandin synthase oxidation of the B[a]P-7,8-DHDs because the latter enzyme forms little or no diolepoxide 1 (11,15).

Alternatively, lipoxygenase may be responsible for this activity. Such an involvement in the arachidonic acid-dependent metabolism of (+)- and (−)-B[a]P-7,8-DHD was consistent with the substantial inhibition by 100 μM NDGA, a lipoxygenase inhibitor (46) as well as an antioxidant. This proposal is supported by observations by Nemato and Takayama (47) who suggested a role for lipoxygenase in the arachidonic acid-dependent activation of B[a]P to protein-binding species with cytosolic and microsomal fractions from rat liver and lung. The observation that phenylbutazone (100 μM) inhibited the arachidonic acid-supported metabolism of (+)- and (−)-B[a]P-7,8-DHD generally by >80% appears to discount this pathway in the present work. If lipoxygenase was involved in the metabolism of these compounds an increase in 7,8-dihydrodiol oxidation would have been seen. Reed *et al.* (48) found that 100 μM phenylbutazone markedly stimulated the prostaglandin H synthase-catalyzed oxidations of B[a]P-7,8-DHD, and we found that phenylbutazone (100 μM) markedly augmented the metabolism of (±)-B[a]P-7,8-DHD by soyabean lipoxygenase (data not shown). The augmentation was higher in the absence of added arachidonic acid.

It appears more likely that oxidation of the enantiomers of B[a]P-7,8-DHD occurs by a mechanism in which the peroxidase activity of cytochrome P450 is involved together with lipid peroxides or hydroperoxide present in the microsomal preparations (9,18,24). In such a mechanism phenylbutazone may act as an alternative substrate to the B[a]P-7,8-DHD and undergo preferential oxidation (48). However, a contribution from active peroxyl radicals produced by lipoxygenase via arachidonic acid cannot be completely discounted.

The ability of these microsomal preparations to convert B[a]P-7,8-DHD enantiomers to diolepoxides via hydroperoxide-mediated or oxene-mediated reactions represent significant bioactivation pathways. The formation of the (+)-enantiomer of diolepoxide 2 from B[a]P-7*R*,8*R*-DHD is a particularly significant pathway since this 7,8-diol-9,10-epoxide is highly tumorigenic in rodents in contrast to its enantiomer and the two diolepoxide 1 enantiomers (1,4). This ultimate rodent carcinogen is virtually the only detectable product of the NADPH-supported human hepatic microsomal metabolism of (−)-B[a]P-7,8-DHD and is the major product of the pathway supported by arachidonic acid. While rodent hepatic metabolism of B[a]P is known to favour the formation of the (−)-7*R*,8*R*-dihydrodiol, the stereoselectivity of polycyclic aromatic hydrocarbon oxidation catalysed by human cytochromes P450 has not to our knowledge been reported. If pathways similar to those of rodents are followed, the oxidation of the (+)-7*S*,8*S*-dihydrodiol is unimportant toxicologically, firstly because it is either not formed in liver or formed in exceptionally low amounts compared to the (−)-isomer, and secondly, although its metabolic products are genotoxic, they have minimal activity in rodent bioassays for tumorigens (1). The use of the presently described assay showing the stereoselectivity of metabolism of B[a]P-7,8-DHD is therefore more pertinent than conversion to products which mutate the *umu*, *his* G and *his* D genes of *Salmonella typhimurium* (1,6,7).

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