0026-895X/87/050710-13$02.00/0

MOLECULAR PHARMACOLOGY, 32:710-722

Regio- and Stereoselective Metabolism of Dibenz[*a,h*]anthracene: Identification of 12 New Microsomal Metabolites

KARL L. PLATT and I. REISCHMANN

Institute of Toxicology, University of Mainz, D-6500 Mainz, Federal Republic of Germany

Received March 5, 1987; Accepted July 21, 1987

SUMMARY

Incubation of the carcinogenic polycyclic aromatic hydrocarbon dibenz[*a,h*]anthracene (DBA) with liver microsomes of Sprague-Dawley rats, pretreated with Aroclor 1254, yielded more than 30 metabolites. Fifteen of these could be identified, and they account for 95% of the ethyl acetate-extractable metabolites of DBA. Twelve metabolites were identified for the first time, by chromatographic and spectroscopic methods: these were DBA-5,6-oxide, 1-, 2-, 3-, 4-, 5-, 6-phenols, 3,4:12,13-bis-dihydrodiol, 1,4/2,3-tetrol, 1,3/2,4-tetrol, 3,4-catechol, and a phenol dihydrodiol derived from the 2-phenol. Quantitative determination revealed that the attack of cytochrome P-450 dependent monooxygenases occurs at the 1,2-, 3,4- and 5,6-positions of the DBA molecule in the ratio 1.7:1.9:1.0. Evidence is presented which indicates that the phenols of DBA are formed by aromatization of the initially generated arene oxides, rather than by direct hydroxylation. The index N_i obtained by refined perturbational molecular orbital calculations was found to be superior to the reactivity number N_t in predicting the predominant phenols, i.e., 2-, 4-, and 5-phenols, formed by aromatization of the corresponding arene oxides. Their enzymatic hydrolysis leads to the formation of *trans*-dihydrodiols, of which the 3,4-isomer dominates the microsomal metabolites of DBA accounting for more than 22% of the total metabolic conversion, compared to the 1,2-dihydrodiol with 11–16% and the 5,6-dihydrodiol with 2%. These metabolites were obtained as enantiomeric-enriched mixtures in which the *R,R* enantiomer of the 1,2-dihydrodiol prevailed with 84%, of the 3,4-dihydrodiol with 79% and of the 5,6-dihydrodiol with 96%. The metabolic pathway via the 1,2-dihydrodiol proceeds to the vicinal diol epoxides, as indicated by the products of hydrolysis the 1,4/2,3- and 1,3/2,4-tetrols. No evidence for the formation of vicinal dihydrodiol epoxides from the 3,4-dihydrodiol, one of the most mutagenic and carcinogenic metabolite of DBA, could be found. In this case, tetrol epoxides have been proposed as ultimate reactive metabolites. Tetrol epoxides can also be formed from DBA-5,6-dihydrodiol via the identified 3,4:12,13-bis-dihydrodiol. This unprecedented metabolic behavior of a carcinogenic polycyclic aromatic hydrocarbon could have its cause in the high molecular symmetry of DBA which permits subsequent metabolic attacks at discrete, but structurally equivalent sites of the molecule.

PAHs constitute a class with some of the most potent carcinogenic compounds. However, they elicit their biological activity only after metabolic conversion to chemically reactive intermediates. Among them, dihydrodiol bay-region epoxides are thought to be the most significant ultimate carcinogenic metabolites of PAHs (1).

DBA, a member of the class of PAHs, was the first pure compound shown to be carcinogenic in laboratory animals (2). Soon after that report, investigators on the metabolism of this carcinogen began (3). They were performed with experimental animals *in vivo* (3–5) as well as *in vitro* (6–9) and resulted in the identification of 2-OH-DBA (10), 2,9-OH-DBA (10), 4,11-OH-DBA (11), DBA-5,6-quinone, DBA-7,14-quinone and its 4,11-diphenol (5), DBA-1,2-dihydrodiol (8, 9), DBA-3,4-dihydrodiol (8, 9), and DBA-5,6-dihydrodiol (6–9) as metabolites of DBA. These findings, together with the results of studies on bacterial mutagenicity (12) and carcinogenicity (13) of DBA and its *trans*-dihydrodiols, were taken as strong indications for

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 302).

ABBREVIATIONS: PAH, polycyclic aromatic hydrocarbon; DBA, dibenz[*a,h*]anthracene; 2-OH-DBA, 2-hydroxydibenz[*a,h*]anthracene (other phenols are similarly designated); 2,9-OH-DBA, 2,9-dihydroxydibenz[*a,h*]anthracene (other diphenols are similarly designated); DBA-1,2-dihydrodiol, *trans*-1,2-dihydroxy-1,2-dihydrodibenz[*a,h*]anthracene (other dihydrodiols are similarly designated); THF, tetrahydrofuran; DMSO, dimethyl sulfoxide; HPLC, high performance liquid chromatography; DBA-*anti*-3,4-diol-1,2-oxide, *c*-3,*t*-4-dihydroxy-*r*-1,2-epoxy-1,2,3,4-tetrahydrodibenz[*a,h*]anthracene; DBA-*syn*-3,4-diol-1,2-oxide, *t*-3,*c*-4-dihydroxy-*r*-1,2-epoxy-1,2,3,4-tetrahydrodibenz[*a,h*]anthracene; DBA-1,2-oxide, 1,2-epoxy-1,2-dihydrodibenz[*a,h*]anthracene (other arene oxides are similarly designated); DBA-1,2-catechol, 1,2-dihydroxydibenz[*a,h*]anthracene; DBA-3,4:12,13-bisdiol, *trans,trans*-3,4:12,13-tetrahydroxy-3,4,12,13-tetrahydrodibenz[*a,h*]anthracene; 1,2,4/3-tetrol, *r*-1,*c*-2,*t*-3,*c*-4-tetrahydroxy- 1,2,3,4-tetrahydrodibenz[*a,h*]anthracene; 1,3/2,4-tetrol, *r*-1,*t*-2,*c*-3,*t*-4-tetrahydroxy-1,2,3,4-tetrahydrodibenz[*a,h*]anthracene; 1,4/2,3-tetrol, *r*-1,*t*-2,*t*-3,*c*-4-tetrahydroxy-1,2,3,4-tetrahydrodibenz[*a,h*]anthracene; TCPO, 1,1,1-trichloro-2-propene oxide; E_1/E_2, first/second eluting enantiomer; MO, molecular orbital.

the transformation of DBA-3,4-dihydrodiol to the vicinal dihydrodiol bay-region epoxide constituting the ultimate carcinogenic metabolite of DBA.

We investigated the microsomal metabolism of DBA in order to prove this point and to determine whether we could verify our assumption that the high molecular symmetry of DBA would lead to subsequent metabolic attacks at distant but structurally equivalent positions of the molecule.

Since the elucidation of the structure of secondary[1] metabolites can lead directly to the identification of the ultimate mutagenic and carcinogenic metabolites of DBA, conditions were chosen that resulted in extensive metabolic conversion of the primarily formed metabolites.

The main objective of the present study was not only to identify presently unknown metabolites of DBA but to elucidate the metabolic pathways and mechanisms that lead to their formation.

Materials and Methods

Chemicals. DBA was supplied by Fluka (Neu-Ulm, FRG) and purified by column chromatography (silica gel; chloroform/*n*-hexane, 1:1, v/v). [7(14)-^{14}C]DBA with a specific activity of 330 MBq/mmol and a radiochemical purity of >99% as determined by HPLC was obtained from Amersham Buchler (Braunschweig, FRG).

The seven phenols of DBA (14), DBA-3,4-quinone (15), DBA-5,6-quinone (16), DBA-7,14-quinone (11), DBA-1,2-dihydrodiol (17), DBA-3,4-dihydrodiol (18), DBA-5,6-dihydrodiol (16), DBA-*cis*-5,6-dihydrodiol (10), and DBA-*anti*-3,4-diol-1,2-oxide (19) were prepared as previously reported. DBA-1,2-oxide [^{1}H-NMR: 400 MHz, THF-d_8; δ 4.28–4.32 (1, m, H-2), 5.48 (1, d, H-1, $J_{1,2}$ = 3.88 Hz), 6.61–6.64 (1, dd, H-3, $J_{3,4}$ = 9.48 Hz, $J_{2,3}$ = 3.71 Hz), 6.93–6.96 (1, dd, H-4, $J_{3,4}$ = 9.48 Hz, $J_{2,4}$ = 1.65 Hz), 7.53 (1, d, H-5, $J_{5,6}$ = 8.58 Hz), 7.66–7.75 (3, m, H-6,9,10), 7.89–7.96 (2, m, H-11,12), 8.17 (1, d, H-13, $J_{12,13}$ = 8.53 Hz), 8.92–8.97 (1, m, H-8), 9.08 (1, s, H-14), 9.33 (1, s, H-7)], DBA-3,4-oxide [^{1}H-NMR: 400 MHz, THF-d_8; δ 4.26–4.28 (1, m, H-3), 4.69 (1, d, H-4, $J_{3,4}$ = 3.73 Hz), 6.72–6.75 (1, dd, H-2, $J_{1,2}$ = 9.83 Hz, $J_{2,3}$ = 3.69 Hz), 7.59–7.63 (1, m, H-1), 7.66–7.70 (2, m, H-9,10), 7.82–7.94 (4, m, H-5,6,11,12), 8.81 (1, d, H-13, $J_{12,13}$ = 8.51 Hz), 8.92 (1, d, H-8, $J_{8,9}$ = 6.97 Hz), 8.93 (1, s, H-7), 9.33 (1, s, H-14)], DBA-5,6-oxide [^{1}H-NMR: 400 MHz, $CDCl_3$; δ 4.53 (1, d, H-5, $J_{5,6}$ = 4.1 Hz), 4.91 (1, d, H-6, $J_{5,6}$ = 4.1 Hz), 7.42 (1, dt, H-2 or H-3, $J_{2,3}$ = 7.5 Hz, $J_{1,3}$ or $J_{2,4}$ = 1.2 Hz), 7.55 (1, dt, H-2 or H-3, $J_{2,3}$ = 7.5 Hz, $J_{1,3}$ or $J_{2,4}$ = 1.2 Hz), 7.62 (1, dt, H-10, $J_{9,10}$ = 7.5 Hz, $J_{8,10}$ = 1.1 Hz), 7.66–7.72 (2, m, H-9,11), 7.76 (1, d, H-12, $J_{12,13}$ = 8.9 Hz), 7.81 (1, d, H-13, $J_{12,13}$ = 8.9 Hz), 7.89 (1, dd, H-4, $J_{3,4}$ = 8.0 Hz, $J_{2,4}$ = 0.9 Hz), 8.30 (1, d, H-1, $J_{1,2}$ = 8.0 Hz), 8.56 (1, s, H-14), 8.72 (1, d, H-8, $J_{8,9}$ = 7.5 Hz), 8.91 (1, s, H-7)], and DBA-*syn*-3,4-diol-1,2-oxide [^{1}H-NMR: 400 MHz, acetone-d_6/DMSO-d_6, 3:2 (v/v), D_2O; δ 3.83–3.84 (1, m, H-2), 4.00 (1, d, H-3, $J_{3,4}$ = 5.91 Hz), 4.73 (1, d, H-4, $J_{3,4}$ = 5.91 Hz), 4.84 (1, d, H-1, $J_{1,2}$ = 4.16 Hz), 7.61–7.70 (3, m, H-6,9,10), 7.73 (1, d, H-5, $J_{5,6}$ = 9.26 Hz), 7.89–7.96 (2, m, H-11,12), 8.24 (1, d, H-13, $J_{12,13}$ = 8.67 Hz), 8.93 (1, d, H-8, $J_{8,9}$ = 7.74 Hz), 8.95 (1, s, H-14), 9.39 (1, s, H-7)] were obtained by application of methods (20–22) described for other PAHs. The regiospecific synthesis of 2,10-OH-DBA [^{1}H-NMR: 60 MHz, DMSO-d_6, D_2O; δ 7.20–7.87 (8, m, H-3,4,5,6,9,11,12,13), 8.20 (1, d, H-1, $J_{1,3}$ = 2 Hz), 8.80 (1, d, H-8, $J_{8,9}$ = 7 Hz), 9.13 (2, s, H-7,14)], DBA-1,2-catechol [^{1}H-NMR: 60 MHz, DMSO-d_6; δ 7.67–8.23 (9, m, H-3,4,5,6,9,10,11,12,13), 9.03–9.27 (1, m, H-8), 9.47 (1, s, H-7), 9.60 (1, s, —OH), 10.27 (1, s, —OH), 10.53 (1, s, H-14)], DBA-3,4-catechol [^{1}H-NMR: 60 MHz, THF-d_8; δ 7.17 (1, d, H-2, $J_{1,2}$ = 9 Hz), 7.53–8.27 (7, m, H-5,6,9,10,11,12,13), 8.00 (1, s, —OH), 8.43 (1, s, —OH), 8.73–8.93 (2, m, H-1,8), 8.97 (1, s, H-7), 9.13 (1, s, H-14)], DBA-1,2-quinone [^{1}H-NMR: 400 MHz, $CDCl_3$; δ 6.51 (1, d, H-3, $J_{3,4}$ = 10.0 Hz), 7.45 (1, d, H-13, J_{12-13} = 8.4 Hz), 7.54 (1, d, H-4, $J_{3,4}$ = 10.0 Hz), 7.65–7.73 (3, m, H-9,10,11), 7.85–7.89 (2, m, H-5,6), 8.42 (1, d, H-12, $J_{12,13}$ = 8.4 Hz), 8.77 (1, d, H-8, $J_{8,9}$ = 8.1 Hz), 9.10 (1, s, H-7), 9.99 (1, s, H-14)], and DBA-3,4:12,13-bisdiol [^{1}H-NMR: 400 MHz, acetone-d_6/DMSO-d_6, 3:2 (v/v), D_2O δ 4.36–4.39 (1, dt, H-3, $J_{3,4}$ = 11.40 Hz), 4.51–4.55 (1, m, H-13, $J_{12,13}$ = 9.68 Hz), 4.61–4.65 (1, m, H-12, $J_{12,13}$ = 9.68 Hz), 4.73–4.76 (1, dd, H-4, $J_{3,4}$ = 11.40 Hz), 6.14–6.17 (1, dd, H-2, $J_{1,2}$ = 10.08 Hz, $J_{2,3}$ = 2.35 Hz), 7.18–7.21 (1, dd, H-1, $J_{1,2}$ = 10.08 Hz, $J_{1,3}$ = 2.24 Hz), 7.30–7.38 (2, m, H-9, 10), 7.63 (1, d, H-11, $J_{10,11}$ = 7.48 Hz), 7.72 (1, d, H-6, $J_{5,6}$ = 8.49 Hz), 7.84 (1, d, H-5, $J_{5,6}$ = 8.49 Hz), 7.92–7.95 (1, m, H-8), 8.23 (1, d, H-14), 8.34 (1, s, H-7)] will be published elsewhere.[2]

TCPO was supplied by EGA (Steinheim, FRG). Aroclor 1254 was obtained from Bayer (Leverkusen, FRG), and trioctanoin was from Serva (Heidelberg, FRG). Biochemicals were from Boehringer (Mannheim, FRG), solvents for HPLC were from Baker (Gross-Gerau, FRG); all other chemicals of analytical grade were purchased from Merck (Darmstadt, FRG).

Metabolism studies. Liver microsomes of adult male Sprague-Dawley rats (190–240 g; Interfauna Süddeutsche Versuchstierfarm, Tuttlingen, FRG) were prepared as previously described (23) 6 days after the animals had received a single intraperitoneal injection of Aroclor 1254 (500 mg/kg body weight) in trioctanoin (2.5 mg/kg body weight).

Microsomal incubations contained a 1- to 2-mg protein equivalent of liver microsomes, 0.6 mM NADP, 8 mM glucose 6-phosphate, 1.2 units of glucose-6-phosphate dehydrogenase, and 5 mM $MgCl_2$ in a final volume of 2 ml 50 mM isotonic (150 mM KCl) sodium phosphate buffer (pH 7.4). This mixture was preincubated for 5 min at 37°. The incubation was started by the addition of 100 μM [^{14}C]DBA (specific activity 90 MBq/mmol) dissolved in 50 μl acetone and performed with shaking (80 min^{-1}) at 37°. The incubation was stopped by the addition of 1.5 ml of ice-cold ethyl acetate followed by vortexing for 1 min and separation of the phases by centrifugation at 1000 × *g*. The extraction was repeated twice more with 1 ml of ethyl acetate. The organic phases were combined, dried over anhydrous magnesium sulfate, and brought to dryness with a stream of nitrogen; then, the residue was stored at −70° until later HPLC separation, for which the sample was dissolved in 30 μl of acetone.

All steps were performed under subdued light. The recovered radioactivity in the organic and aqueous phase was usually >97% of that applied. When the metabolites were photometrically quantified (see below), 100 μl of a solution of DBA-*cis*-5,6-dihydrodiol in dimethylformamide (4.66 μg/ml) were added after termination of the incubation.

HPLC analysis. Chromatographic separations were performed with a system consisting of two high-pressure pumps (model 740; Spectra-Physics, Darmstadt, FRG), a sample injection valve (model C6U; Valco, Untertannberg, Switzerland) with a 10-μl sample loop, UV detector (model 230; Spectra-Physics) connected to a recorder (model LS 438880; Linseis, Selb, FRG), and an integrator (Autolab System I, Spectra-Physics).

For the reverse phase separation of metabolites, LiChrosorb RP-18 (5 μm, 4 × 250 mm; Merck, Darmstadt, FRG) was used as the stationary phase. The mobile phase consisted of a mixture of methanol and 10 mM Tris-HCl buffer (pH 8.0), with the methanol content changing from 60 to 100% (v/v) in 50 min (0.8%/min) at a flow rate of 0.8 ml/min. The slightly alkaline pH of the mobile phase was essential for high chromatographic recovery of the DBA-5,6-oxide (24), since the use of twice-distilled water due to its acidic pH reduced the recovery of the arene oxide to just 30%. For the separation of 2-,5-,7-OH-DBA and 1-,3-,6-OH-DBA, respectively, which cochromatographed (cf. Fig. 6) under the conditions described above, the mobile phase had to be replaced by 70% (v/v) methanol in 1% (v/v) aqueous *n*-butylamine (25).

The separation of chiral metabolites into enantiomers was accom-

[1] The term secondary metabolite refers to the two monooxygenase attacks necessary to generate this metabolite.

[2] K. L. Platt, H. Frank, and F. Oesch, manuscript in preparation.

plished by chromatography on chiral stationary phases prepared by covalent binding of (−)-(*R*)-2-(2,4,5,7-tetranitro-9-fluorenylideneaminooxy)carboxylic acids to silica gel (LiChrosorb Si 100, 5 μm, 4 × 250 mm; Merck) over γ-aminopropyl groups as described (26). A mixture of dichloromethane and methanol (for composition see Table 4) was used as the mobile phase at a flow rate of 1.5 ml/min.

Quantification of metabolites. Metabolites were photometrically quantified with DBA-*cis*-5,6-dihydrodiol as an internal standard as previously described (27). This method was used because of its simplicity and satisfactory sensitivity (27). Calibration curves were obtained with synthetic derivatives of DBA or, in the case of metabolites of unknown structure, from the radiometrically determined amount of metabolites formed from [^{14}C]DBA. Calibration curves of metabolites of known structure obtained with both synthetic compounds and radioactive metabolites were indistinguishable.

Total metabolic conversion was radiometrically determined; it was calculated from the combined radioactivity eluting prior to DBA upon HPLC and the amount of radioactivity remaining in the aqueous phase after ethyl acetate extraction. The latter fraction, termed "water-soluble metabolites," contains hydrophilic and protein-bound metabolites.

Metabolism of *trans*-dihydrodiols. Synthetic racemic DBA-1,2-, -3,4-, or -5,6-dihydrodiols (75 μM each) were incubated with liver microsomes of Sprague-Dawley rats pretreated with Aroclor 1254 (1.2 mg of protein/ml incubation volume), 0.6 mM NADP, 8 mM glucose 6-phosphate, 1.2 units of glucose-6-phosphate dehydrogenase, and 5 mM $MgCl_2$ in a final volume of 2 ml 50 mM isotonic (150 mM KCl) sodium phosphate buffer (pH 7.4) for 15 min at 37°. The isolation of metabolites and their chromatographic separation proceeded exactly as described above for DBA.

Spontaneous isomerization of DBA arene oxides. The arene oxides (20 μM) were incubated for 10 min at 37° in 50 mM isotonic (150 mM KCl) sodium phosphate buffer (pH 7.4) containing 1.2 mg of microsomal protein and 1 mM TCPO, but no NADPH, in order to prevent enzymatic hydrolysis and monooxygenation and yet provide conditions similar to those during metabolic formation of arene oxides. Work-up yielded a mixture of isomeric phenols that was quantified as described above: DBA-1,2-oxide was accordingly transformed to 20.4 ± 2.1% (mean ± SD, n = 3) 1-OH-DBA and 79.6 ± 3.5% 2-OH-DBA, DBA-3,4-oxide to 13.7 ± 0.9% 3-OH-DBA and 86.3 ± 3.7% 4-OH-DBA, and DBA-5,6-oxide to 69.4 ± 2.6% 5-OH-DBA and 30.6 ± 1.5% 6-OH-DBA.

Hydrolysis of diastereomeric DBA-3,4-diol-1,2-oxides. Diol epoxides (4.6 mg; 14 μmol) were dissolved in dioxane (3 ml) and stirred after the addition of water (2 ml) for 24 hr at room temperature under argon in the dark. Removal of the solvent yielded the tetrols as a white powder (4.5 mg; 93%); MS (70 eV) *m/z* (relative intensity): 346 (100, $M^{+\cdot}$), 328 (9.5, M^+-H_2O), 310 (49.3, M^+-2 H_2O); HPLC analysis (cf. Table 1) revealed the formation of two tetrols (tetrol-1 and -2; weight ratio, 41:59) in the case of DBA-*syn*-3,4-diol-1,2-oxide, and only one tetrol (tetrol-3) in the case of DBA-*anti*-3,4-diol-1,2-oxide. Tetrol-1 and -2 were separated by preparative HPLC [stationary phase: Polygosil 60-5 C_{18}; 5 μm, 8 × 250 mm; mobile phase: 60% (v/v) methanol in water; flow rate: 3 ml/min; t_R (tetrol-1) 13.1 min, t_R (tetrol-2) 20.7 min]. Acetylation (Ac_2O/pyridine) transformed the tetrols to their tetraacetates whose relevant ^{1}H-NMR data are summarized in Table 1. Interpretation of these data, compared with earlier results (28, 29), reveals that DBA-*syn*-3,4-diol-1,2-oxide is hydrolyzed via *trans*- and *cis*-opening of the oxirane ring to 1,3/2,4-tetrol (tetrol-1) and 1,2,4/3-tetrol (tetrol-2), respectively, whereas DBA-*anti*-3,4-diol-1,2-oxide is hydrolyzed via exclusive *trans*-opening of the oxirane ring to 1,4/2,3-tetrol (tetrol-3).

TABLE 1

Chromatographic and ^{1}H-NMR spectroscopic properties of tetrols obtained by spontaneous hydrolysis from diastereomeric diol epoxides of *trans*-3,4-dihydroxy-3,4-dihydrodibenz[*a,h*]anthracene

Compound	t_R[a]	^{1}H-NMR data of tetraacetates[b]						
		Chemical shift, δ				Coupling constants (Hz)		
		H_1	H_2	H_3	H_4	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$
	min	ppm				Hz		
Tetrol-1[c]	14.5	6.95	5.64	5.42	6.48	4.0	7.5	7.5
Tetrol-2[c]	18.3	7.28	5.45	5.93	6.47	3.5	11.5	7.7
Tetrol-3[d]	12.8	6.84	5.74	5.67	6.57	3.7	2.7	9.0

[a] t_R, retention time; stationary phase: LiChrosorb RP-18; 5 μm, 4 × 250 mm; mobile phase: linear gradient from 60 to 100% (v/v) methanol in water in 50 min; flow rate, 0.8 ml/min.
[b] Measured in $CDCl_3$ at 400 MHz.
[c] Tetrol derived from DBA-*syn*-3,4-diol-1,2-oxide.
[d] Tetrol derived from DBA-*anti*-3,4-diol-1,2-oxide.

Spectral methods. UV absorption spectra of the metabolites in ethanol were obtained on a Beckman model 25 spectrophotometer. Mass spectra were recorded with a Varian CH7A spectrometer at 70 eV. Proton NMR spectra were measured on a Bruker AM 400 spectrometer at 400 MHz or on a Varian EM 360 spectrometer at 60 MHz. Chemical shifts (in ppm) are relative to tetramethylsilane.

Results

Chromatographic and spectral characterization of the microsomal metabolites of DBA. Incubation of [^{14}C]DBA (100 μM) with liver microsomes (1 mg of protein/ml) of male Sprague-Dawley rats after pretreatment with Aroclor 1254 yielded more than 30 peaks upon HPLC (Fig. 1). Since all peaks contained radioactivity and are not formed in the absence of NADPH (data not shown), they represent microsomal metabolites of DBA. Metabolites that could be identified in the course of this work have been designated with roman numerals (cf. Fig. 1 and Table 2). Incubation in the presence of 1 mM TCPO, a competitive inhibitor of epoxide hydrolase (30, 31), suppressed formation of all metabolites (marked with asterisks in Fig. 1) that elute prior to metabolite IX. The characterization

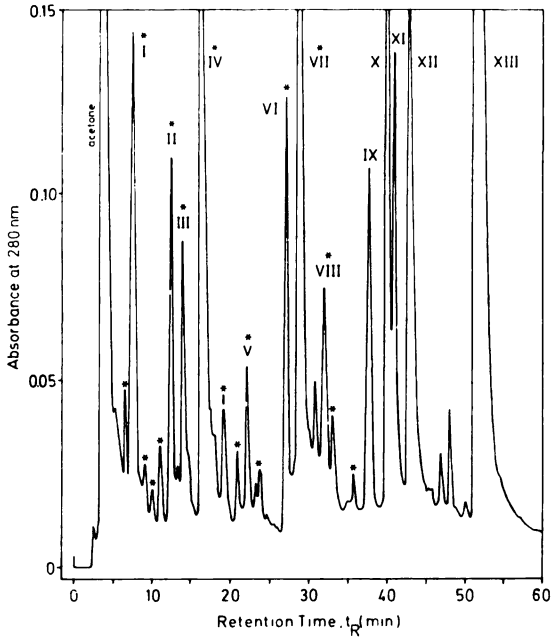

Fig. 1. Reverse phase HPLC chromatogram of the organic ethyl acetate-extractable metabolites obtained from incubation of DBA with liver microsomes of Sprague-Dawley rats pretreated with Aroclor 1254. Roman numerals denote structurally identified metabolites and unmetabolized DBA (XIII). *, metabolites that were suppressed when the incubation was performed in the presence of 1 mM TCPO. For experimental conditions, see Materials and Methods.

TABLE 2

Chromatographic behavior of derivatives and microsomal metabolites of DBA as well as mass spectral data and biochemical properties of the metabolites

DBA derivative	Metabolite[a]	TCPO sensitivity[b]	t_R[c]	M+·[d]
			min	
3,4:12,13-Bisdiol	I	+	7.9	346
1,4/2,3-Tetrol	II	+	12.9	346
1,3/2,4-Tetrol	III	+	14.6	346
1,2-Dihydrodiol	IV	+	16.9	312
1,2,4/3-Tetrol			18.3	
	V	+	22.6	328
cis-5,6-Dihydrodiol[e]			25.8	
5,6-Dihydrodiol	VI	+	27.4	312
3,4-Dihydrodiol	VII	+	29.0	312
3,4-Catechol	VIII	+	32.5	310
5,6-Quinone			33.4	
5,6-Oxide	IX	–	37.8	294
7-Phenol			39.6	
2-Phenol	Xb	–	39.9	294
5-Phenol	Xa	–	40.1	294
1-Phenol	XIc	–	40.9	294
6-Phenol	XIa	–	41.0	294
3-Phenol	XIb	–	41.3	294
4-Phenol	XII	–	42.7	294
DBA	(XIII)		51.6	
7,14-Quinone			54.3	

[a] For meaning of numerals see Fig. 1 and 6 legends.
[b] Metabolite was not formed (+) or was formed (–) in the presence of 1 mM TCPO.
[c] t_R, retention time; stationary phase: LiChrosorb RP-18, 5 μm, 4 × 250 mm; mobile phase: linear gradient from 60 to 100% (v/v) methanol in Tris-HCl buffer (pH 8.0) in 50 min; flow rate: 0.8 ml/min.
[d] M+·, molecular ion of metabolite in EI-mass spectrum at 70 eV.
[e] Internal standard.

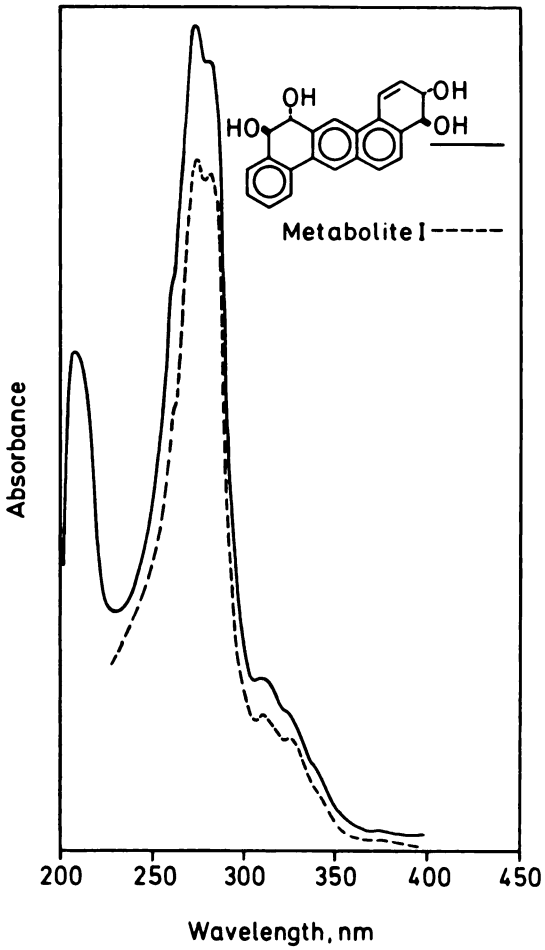

Fig. 2. UV-visible spectra of DBA-3,4:12,13-bisdiol (—) and of metabolite I (– – –) (cf. Table 1 and Fig. 1).

of these metabolites (I–VIII), which should contain alcoholic hydroxyl groups, as the consequence of epoxide hydrolase-catalyzed reactions, will be summarized first.

Metabolites IV, VI, and VII were identified as DBA-1,2-, -5,6-, and -3,4-dihydrodiol, respectively, based upon comparison of their UV (not shown) and mass spectra (Table 2), as well as HPLC retention times (Table 2) with those of the authentic compounds. Metabolites IV, VI, and VII could also be isolated from microsomal incubations lacking NADPH of DBA-1,2-, -5,6-, and -3,4-oxide, respectively (data not shown).

Metabolites II and III were identical with DBA-1,4/2,3- and 1,3/2,4-tetrol, respectively, on the basis of their UV (not shown), mass spectral (Table 2), and chromatographic (Table 2) properties.

The molecule ion at 346 in the mass spectrum of metabolite I (Table 2) is suggestive of a tetrahydroxytetrahydro derivative of DBA; metabolite I is metabolically formed not only from DBA but also from DBA-5,6-dihydrodiol (cf. Fig. 7); finally, the fact that the chromatographic (Table 2) and UV spectral properties (Fig. 2) of metabolite I and DBA-3,4:12,13-bisdiol are indistinguishable, is strongly indicative that the two compounds are identical.

The chemical structure of metabolite V has not been fully elucidated. The mass spectral data: m/z (relative intensity) 328 ($M^{+\cdot}$, 67.7), 310 ($M^{+\cdot}$-H_2O, 27.4), 281 ($M^{+\cdot}$-H_2O-CHO, 17.1%), are in agreement with a phenoldihydrodiol of DBA. This assumption is further supported by the fact that metabolite V is metabolically formed from 2-OH-DBA (data not shown), whereas it could be detected neither as a metabolite of DBA-3,4-dihydrodiol (cf. Fig. 7) nor as a product of hydrolysis of the diastereomeric DBA-3,4-diol-1,2-oxides (see Material and Methods). The UV spectrum of metabolite V (Fig. 3) shows resemblance to the 1,2,3,4-tetrahydro-DBA chromophore. Thus, metabolite V is tentatively identified as the 2-keto tautomer of 2,3,4-trihydroxy-3,4-dihydro-DBA.

Metabolite VIII behaves chromatographically (Table 2) and UV spectroscopically (Fig. 4) as DBA-3,4-catechol, whose UV-visible spectrum is strikingly different from that of DBA-3,4-quinone (Fig. 4) (32). Metabolite VIII is metabolically formed from 4-OH-DBA and to a minor extent from DBA-3,4-dihydrodiol (data not shown). The molecular ion at m/z = 310 in the mass spectrum of VIII is not unequivocal evidence for a catechol, since *o*-quinones can also give rise to a ion at m/z = 310, besides their molecular ion at m/z = 308 (Table 3), due to traces of moisture in the mass spectrometer which lead to M+2 ions (33, 34). However, the absence of fragment ions at m/z = 308 and at m/z = 280, typical for *o*-quinones (Table 3), in the mass spectrum of metabolite VIII strongly indicates its identity with a catechol. Although it can be expected that a catechol, once metabolically formed, should be autoxidized to the corresponding *o*-quinone during work-up and chromatographic separation, the combined metabolic, chromatographic, and even more, the UV and mass spectral data nevertheless support the assumption that metabolite VIII is identical to DBA-3,4-catechol.

The formation of metabolites IX-XII (cf. Fig. 1) from DBA

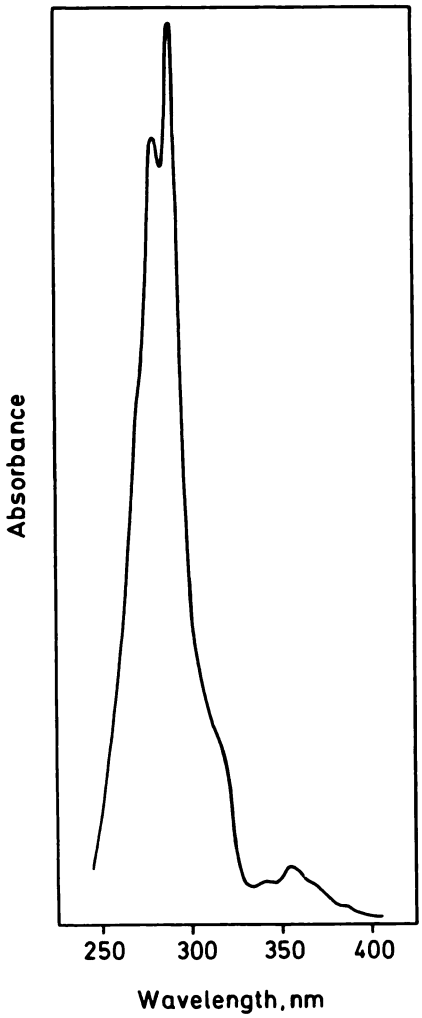

Fig. 3. UV-visible spectrum of metabolite V (cf. Table 1 and Fig. 1).

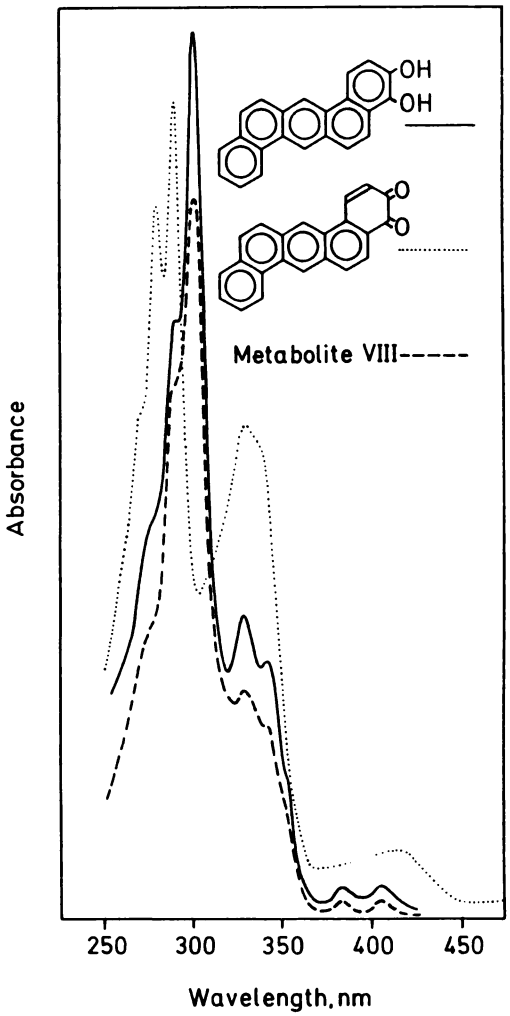

Fig. 4. UV-visible spectra of 3,4-OH-DBA (—), DBA-3,4-quinone (· · · ·) (32), and of metabolite VIII (– – –) (cf. Table 1 and Fig. 1).

in the presence of 1 mM TCPO (Table 2) implies that epoxide hydrolase is not involved in their generation. Metabolite IX is indistinguishable from DBA-5,6-oxide on the basis of its chromatographic (Table 2) and UV spectroscopic (Fig. 5) properties. The mass spectrum of metabolite IX (Table 3), showing the same molecular ion as DBA phenols, i.e., $m/z = 294$, is typical for arene oxides because of the mass fragment at $m/z = 278$ which is absent in the mass spectrum of DBA phenols (Table 3), and because of the high intensity of the fragment ion at $m/z = 265$ characteristic for K-region oxides (35). Metabolite IX is therefore identified as DBA-5,6-oxide.

Since 2-,5-,7-OH-DBA and 1-,3-,6-OH-DBA, respectively, elute as two peaks under standard HPLC conditions (see Materials and Methods) (X and XI in Figs. 1 and 6), further separation was needed and was achieved by using methanol/1% (v/v) aqueous *n*-butylamine (25) as mobile phase (Fig. 6). Chromatography of peak X isolated from an incubation of [^{14}C] DBA reveals metabolites Xa and Xb coeluting with 5- and 2-OH-DBA, respectively, whereas a metabolite with the chromatographic properties of 7-OH-DBA could not be detected. The identity of metabolites Xa and Xb with 5-OH-DBA and 2-OH-DBA, respectively, was finally confirmed by comparison of their UV and mass spectra with those of the synthetic compounds (Table 3) (14), as well as by their spontaneous formation from DBA-5,6-oxide and DBA-1,2-oxide, respectively (see Materials and Methods, and Table 6).

Chromatographic separation of peak XI shows the presence of three metabolites, XIa, XIb, and XIc, detected by their radioactivity, which coeluted with 6-, 3-, and 1-OH-DBA, respectively. Final confirmation of the identity of metabolites XIa with 6-OH-DBA, XIb with 3-OH-DBA, and XIc with 1-OH-DBA was obtained by comparison of their UV and mass spectra with those of the synthetic compounds (Table 3) (14), as well as by their spontaneous formation from DBA-5,6-oxide, DBA-3,4-oxide, and DBA-1,2-oxide, respectively (see Materials and Methods, and Table 6).

Metabolite XII was identified as 4-OH-DBA on the basis of its UV (data not shown), mass spectral (Tables 2 and 3), and chromatographic (Table 2) properties, as well as by its spontaneous formation from DBA-3,4-oxide (see Materials and Methods, and Table 6).

Enantiomeric purity of chiral metabolites of DBA. For determination of the enantiomeric composition of the chiral metabolites I–VII and IX, chromatographic separation into enantiomers at chiral stationary phases (36, 37) was chosen because of its experimental simplicity. Of the commercially available chiral stationary phases, only one, i.e., (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine, covalently bound to γ-aminopro-

TABLE 3

Relevant mass spectral data of arene oxides, phenols, catechols, and o-quinones as well as of metabolites VIII and IX of DBA

Compound	Sample temperature	Intensity at m/z							
		310	308	294	281	280	278	265	252
	°C				%				
DBA-1,2-oxide	150	–[a]	–	100	–	–	13	20	–
DBA-3,4-oxide	160	–	–	100	–	–	4	34	–
DBA-5,6-oxide	150	–	–	100	–	–	5	99	–
Metabolite IX	150	–	–	100	–	–	4	95	–
1-OH-DBA	135	–	–	100	–	–	–	43	–
2-OH-DBA	185	–	–	100	–	–	–	48	–
3-OH-DBA	180	–	–	100	–	–	–	24	–
4-OH-DBA	165	–	–	100	–	–	–	45	–
5-OH-DBA	195	–	–	100	–	–	–	79	–
6-OH-DBA	180	–	–	100	–	–	–	64	–
7-OH-DBA	185	–	–	100	–	–	–	36	–
DBA-1,2-catechol	250	100	–	–	12	–	–	4	19
DBA-3,4-catechol	200	100	–	–	17	–	–	6	19
2,10-OH-DBA	290	100	–	–	36	–	–	2	16
Metabolite VIII	240	100	–	–	37	–	–	9	16
DBA-1,2-quinone	150	100	5	–	31	45	–	–	31
DBA-1,2-quinone	250	11	29	–	35	100	–	–	32
DBA-3,4-quinone	150	100	6	–	26	29	–	–	40
DBA-3,4-quinone	300	8	23	–	31	100	–	–	55
DBA-5,6-quinone	170	24	18	–	25	100	–	–	23
DBA-5,6-quinone	250	2	40	–	25	100	–	–	20

[a] –, intensity < 2%.

pylsilanized silica, was found to separate DBA-5,6-dihydrodiol into its enantiomers (mobile phase: *n*-hexane/ethanol/acetonitrile/THF, 93:4:2:1, v/v; flow rate: 1.2 ml/min; $\alpha = 1.1$, $R_s = 0.94$, E_1: (−)-5,6-dihydrodiol); other chiral metabolites of DBA were not resolved. Also, we were unable to confirm the successful separation of the enantiomers of DBA-5,6-oxide, as previously reported, using the same chiral ligand as described above, ionically bound to silica (37) (data not shown). This difference has probably been caused by a different selectivity and, less likely, by a lower efficiency of the chiral stationary phase column used in the present study as compared to the one applied in the earlier investigation (37).

Finally, successful enantiomeric separation of most of the chiral metabolites of DBA, i.e., metabolites II, IV, V, VI, VII, and IX (Table 4) was achieved by applying stationary phases, containing (−)-(*R*)-2-(2,4,5,7-tetranitro-9-fluorenylideneaminooxy)carboxylic acids as chiral π-acceptor ligands, that has been prepared according to described procedures (26).

Rechromatography of metabolites II, IV, V, VI, VII, and IX, isolated by reverse phase HPLC (cf. Fig. 1), on these chiral phases revealed that the metabolism of DBA by liver microsomes of rats pretreated with Aroclor 1254 proceeds with remarkable stereoselectivity (Table 4). The highest enantiomeric purity is observed in the case of the K-region dihydrodiol (*ee* = 92%) and of DBA-1,4/2,3-tetrol (*ee* = 86%), whereas DBA-5,6-oxide, as well as the 1,2- and 3,4-dihydrodiols, exhibits an enantiomeric excess of ~60%. Similarly, metabolite V formed from 2-OH-DBA is obtained enantiomerically enriched, although to a lesser extent (*ee* = 38%). Because the absolute configuration of the three *trans*-dihydrodiols of DBA has recently been assigned (38), it could be determined that the metabolically formed principal enantiomers of the 1,2-, 3,4-, and 5,6-dihydrodiols all possess (*R*,*R*) absolute configuration.

The results of Table 4 demonstrate, further, that the enantiomeric composition of the metabolically formed DBA-5,6-

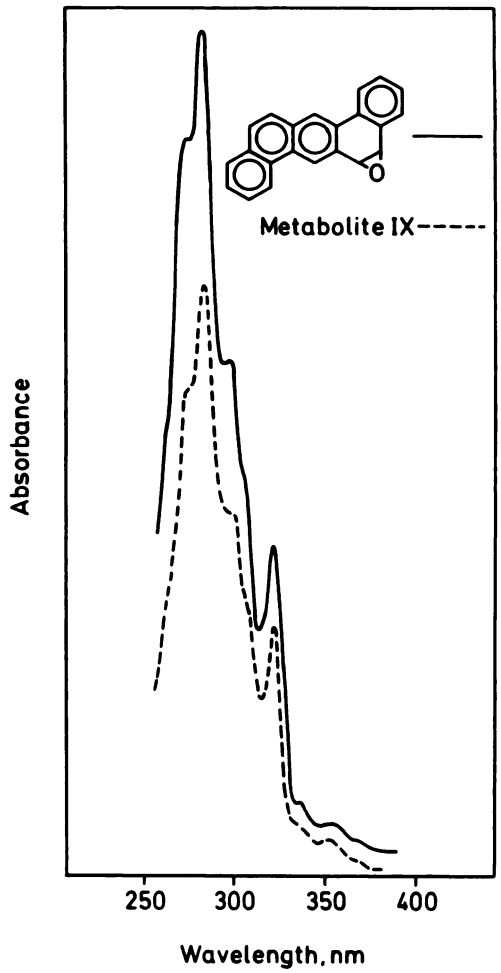

Fig. 5. UV-visible spectra of DBA-5,6-oxide (—) and of metabolite IX (– – –) (cf. Table 1 and Fig. 1).

oxide was not changed in the presence of 1 mM TCPO, which prevents enzymatic hydrolysis of the K-region oxide. Incubation of racemic DBA-5,6-oxide with microsomal protein in the absence of an NADPH-regenerating system—conditions under which enzymatic hydrolysis of the arene oxide proceeds yet no further metabolism by cytochrome P-450-dependent monooxygenases occurs—yielded (+)-(5*R*,6*R*)-dihydrodiol, with enantiomeric purity similar to that isolated from microsomal incubations of DBA (Table 4).

Microsomal metabolism of the *trans*-dihydrodiols of DBA. Incubations of synthetic racemic 1,2-, 3,4-, and 5,6-dihydrodiols (75 μM) with liver microsomes (1.2 mg of protein/ml incubation volume) of rats pretreated with Aroclor 1254 were performed for 15 min at 37° and subsequently extracted with ethyl acetate. Reverse phase HPLC separation of the extracts is depicted in the chromatograms of Fig. 7. The main microsomal metabolites of *trans*-1,2-dihydrodiol eluted prior to the parent compound (Fig. 7A) and were indistinguishable from metabolites II and III, i.e., 1,4/2,3- and 1,3/2,4-tetrol, respectively, based on their UV and mass spectral (data not shown) as well as chromatographic properties. These tetrols, however, were metabolically formed from *trans*-3,4-dihydrodiol only in trace quantities (Fig. 7B). Incubation of biosynthetic 3,4-dihydrodiol, obtained and isolated as microsomal metabolite of ^{14}C-labeled DBA in the same way as described above, resulted in 32% metabolic conversion of the dihydrodiol and revealed that

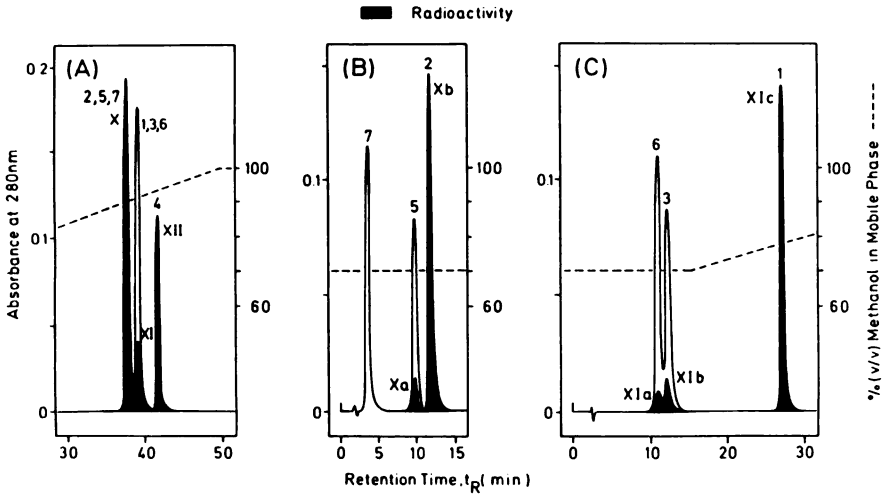

Fig. 6. Reverse phase HPLC chromatograms of the phenols of DBA. A, Mixture of 1-, 2-, 3-, 4-, 5-, 6-, and 7-OH-DBA and of metabolites X, XI, and XII (cf. Fig. 1), obtained by incubation of [^{14}C]DBA in the presence of 1 mM TCPO. B. Mixture of 2-, 5-, and 7-OH-DBA and of peak X isolated from A. C. Mixture of 1-, 3-, and 6-OH-DBA and of peak XI isolated from A. *Arabic numbers* refer to the respective isomeric phenol(s) of DBA. Mobile phase in A was methanol/water and in B and C, methanol/1% aqueous *n*-butylamine. For further experimental conditions, see Materials and Methods.

TABLE 4

Chromatographic separation and enantiomeric compositions of chiral metabolites of DBA

Metabolite[a]	Stationary phase[b]	α[c]	R_s[d]	E_1[e]	E_2[f]
				%	
Metabolite II (1,4/2,3-Tetrol)	TAPA	1.81	2.50	93.0 ± 0.2	7.0 ± 0.05
Metabolite IV (1,2-Dihydrodiol)	TAPA	1.63	3.36	84.0 ± 0.2 (−)-(1*R*,2*R*)	16.0 ± 0.05
Metabolite V	TAPA	1.16	0.89	31.0 ± 0.6	69 ± 1.4
Metabolite VI (5,6-Dihydrodiol)	TABA	1.17	2.50	96.0 ± 0.2 (90.0 ± 0.2 (+)-(5*R*,6*R*)	4.0 ± 0.05 10.0 ± 0.05)[g]
Metabolite VII (3,4-Dihydrodiol)	TAPA	1.10	0.74	79 ± 3 (−)-(3*R*,4*R*)	21 ± 1
Metabolite IX (5,6-Oxide)	TAVA	1.13	0.60	82 ± 5 (81 ± 5	18 ± 1 19 ± 1)[h]

[a] Metabolites were isolated from incubations (30 min) of DBA (102 μM) with the microsomal liver fraction (1.1 mg of protein/ml incubation volume) of Sprague-Dawley rats pretreated with Aroclor 1254 in the presence of an NADPH-generating system.

[b] TAPA, (−)-(*R*)-2-(2,4,5,7-tetranitro-9-fluorenylideneaminooxy)propionic acid; TABA, (−)-(*R*)-2-(2,4,5,7-tetranitro-9-fluorenylideneaminooxy)butyric acid; TAVA, (−)-(*R*)-2-(2,4,5,7-tetranitro-9-fluorenylideneaminooxy)valeric acid. All ligands were covalently linked over γ-aminopropyl spacers to silica gel (LiChrosorb Si 100, 5 μm) as described (26). Mobile phase consisted of dichloromethane/methanol (60:40, v/v; metabolites IV, VII: 73:27, v/v; metabolite VI: 40:60, v/v) with a flow rate of 1.5 ml/min.

[c] α, separation factor.

[d] R_s, chromatographic resolution.

[e] E_1, first eluting enantiomer.

[f] E, second eluting enantiomer.

[g] Metabolite VI was obtained by incubating (10 min) racemic DBA-5,6-oxide (20 μM) with the microsomal liver fraction (1.2 mg of protein/ml incubation volume) of Sprague-Dawley rats pretreated with Aroclor 1254 in the absence of an NADPH-generating system.

[h] Metabolite IX was obtained as described under Footnote *a* in the presence of 1 mM TCPO.

about 60% of its metabolites remained in the aqueous phase after extractive work-up. Hence it can be concluded that most metabolites of the 3,4-dihydrodiol are quite polar and very poorly extractable with ethyl acetate.

The principal microsomal metabolite of *trans*-5,6-dihydrodiol (Fig. 7C) could be identified as the 3,4:12,13-bisdiol, i.e., metabolite I of DBA, on the basis of UV, mass spectral (data not shown), and chromatographic comparison with the synthetic compound.

Quantitative distribution of the metabolites of DBA. The metabolic conversion of DBA by liver microsomes of rats pretreated with Aroclor 1254 was linear with protein concentrations up to 1.2 mg/ml (40). In order to avoid severe limitation of the rate of metabolic conversion by depletion of substrate, DBA was incubated at a concentration of 102 μM; this value ensured saturation of the cytochrome P-450-dependent monooxygenases involved in the metabolism of DBA because of the apparent Michaelis constant, K_m, for DBA of 12 μM (40).

In order to exclude enzymatic hydrolysis by epoxide hydrolase and to obtain a clear indication of the positions of primary metabolic attack of cytochrome P-450-dependent monooxygenases, DBA was incubated with liver microsomes in the presence of 1 mM TCPO, an effective epoxide hydrolase inhibitor (30, 31). The qualitative analysis of isolated metabolites revealed that the phenols at the 1-, 2-, 3-, 4-, 5-, and 6-positions of DBA, together with DBA-5,6-oxide, had been formed. DBA-1,2- or -3,4-oxide could not be detected, probably due to their instability and spontaneous isomerization to phenols. The total metabolic conversion, which is linear up to 6 min (40), amounted to 2 nmol/mg of protein/min, the metabolic attack at the 3,4-position contributing most with 34.2%, followed by that at the 1,2-position with 29.6%, and that at the 5,6-position with 17.8% (Table 5).

Omitting TCPO is without influence on the total metabolic conversion but leads to a complete change in the composition of metabolites due to the enzymatic action of epoxide hydrolase. The level of the 3-, 5-, and 6-phenols falls below detectability, whereas the 3,4-dihydrodiol dominates the metabolites with 23.8%, followed by the 2-phenol with 13.6%, the 5,6-oxide with 12.1%, the 1,2-dihydrodiol with 10.6%, and the 4-phenol with 9.3% (Table 5). The amount of the combined secondary metabolites (I, II, III, V, and VIII; see Footnote 1) exceeded 4%. After an incubation time of 30 min, the situation changed drastically. Although the 3,4-dihydrodiol is still the principal metabolite of DBA with 22.4%, the amount of the phenols (14.3%) and of the 5,6-oxide (3.3%) has decreased considerably, while the tetrols, the bisdiol, and metabolites V and VIII contribute now with 23% to the total metabolic conversion. The 3,4:12,13-bisdiol is then, in fact, the third most prevalent metabolite after the 1,2-dihydrodiol. The nature of the hydrophilic and protein-bound metabolites is presently unknown; it amounted to 13–16% of the total metabolic conversion independent of the incubation time and of the absence or presence of TCPO. In the latter case, protein binding could occur with the primarily

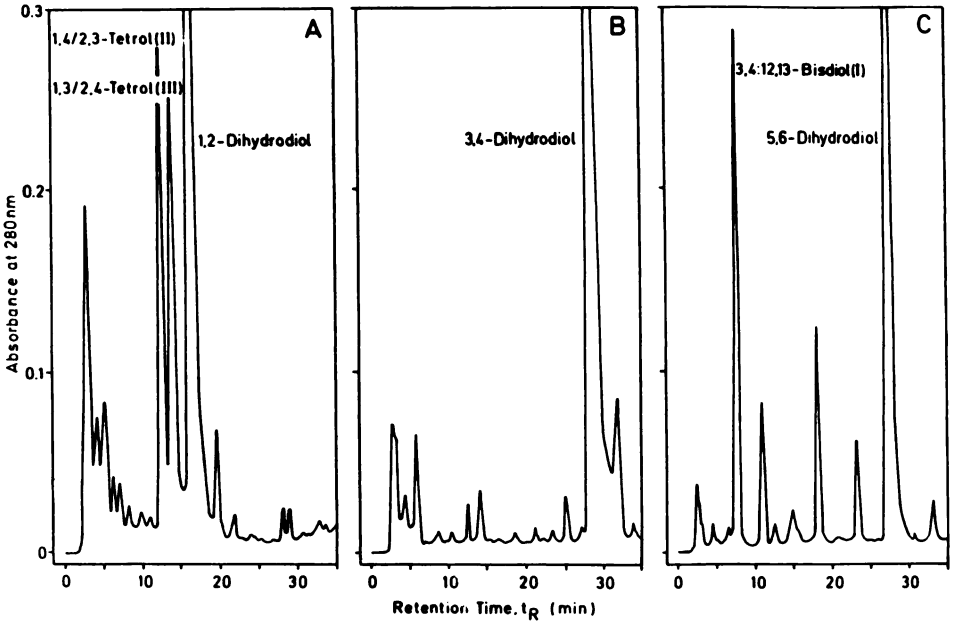

Fig. 7. Reverse phase HPLC chromatograms of the organic ethyl acetate-extractable metabolites obtained from incubations of synthetic *trans*-1,2-dihydrodiol (A), *trans*-3,4-dihydrodiol (B), and *trans*-5,6-dihydrodiol (C) of DBA with liver microsomes of Sprague-Dawley rats pretreated with Aroclor 1254. For experimental conditions, see Materials and Methods.

TABLE 5

Time-dependent distribution of metabolites upon incubation of DBA with the microsomal liver fraction of Sprague-Dawley rats after Aroclor 1254 treatment in the absence and presence of 1 mM TCPO

Values are means ± standard deviations; $n = 3$.

	Metabolite formation[a] (nmol/mg protein)[b] at incubation time		
	6 min (+1 mM TCPO)	6 min	30 min
Total metabolite conversion	12.2 ± 0.9 (100)[c]	12.0 ± 1.2 (100)	28.3 ± 2.0 (100)
1-OH-DBA (XIc)	0.86 ± 0.06 (7.1)	0.52 ± 0.09 (4.3)	0.35 ± 0.04 (1.2)
2-OH-DBA (Xb)	2.74 ± 0.07 (22.5)	1.63 ± 0.14 (13.6)	1.40 ± 0.05 (5.0)
2-OH-DBA-Diol (V)	<0.02	0.05 ± 0.03 (0.4)	0.66 ± 0.05 (2.3)
DBA-1,2-dihydrodiol (IV)	<0.04	1.27 ± 0.11 (10.6)	4.38 ± 0.17 (15.5)
DBA-1,4/2,3-Tetrol (II)	<0.02	0.08 ± 0.03 (0.7)	0.84 ± 0.11 (3.0)
DBA-1,3/2,4-Tetrol (III)	<0.04	0.10 ± 0.02 (0.8)	0.83 ± 0.10 (2.9)
3-OH-DBA (XIb)	0.49 ± 0.05 (4.0)	<0.02	<0.02
4-OH-DBA (XII)	3.68 ± 0.12 (30.2)	1.11 ± 0.15 (9.3)	2.29 ± 0.29 (8.1)
DBA-3,4-catechol (VIII)	<0.06	0.14 ± 0.08 (1.2)	1.21 ± 0.30 (4.3)
DBA-3,4-dihydrodiol (VII)	<0.03	2.85 ± 0.31 (23.8)	6.35 ± 0.44 (22.4)
DBA-5,6-Oxide (IX)	2.02 ± 0.13 (16.6)	1.45 ± 0.12 (12.1)	0.92 ± 0.11 (3.3)
5-OH-DBA (Xa)	0.11 ± 0.03 (0.9)	<0.02	<0.02
6-OH-DBA (XIa)	0.04 ± 0.01 (0.3)	<0.02	<0.02
DBA-5,6-dihydrodiol (VI)	<0.03	0.19 ± 0.03 (1.6)	0.58 ± 0.05 (2.1)
DBA-3,4:12,13-bisdiol (I)	<0.03	0.14 ± 0.05 (1.2)	2.96 ± 0.15 (10.5)
Water-soluble metabolites	1.55 ± 0.32 (12.7)	1.93 ± 0.44 (16.1)	4.44 ± 0.57 (15.7)

[a] From incubation of 102 μM DBA.
[b] Concentration at 1.1 mg of protein/ml incubation volume.
[c] Values in parentheses refer to percentage of total metabolic conversion.

formed arene oxides, as has already been described for DBA-5,6-oxide (41).

Discussion

The results of the present investigation demonstrate that the carcinogenic DBA is transformed by liver microsomes of rats pretreated with Aroclor 1254, both regio- and stereoselectively, via three distinct metabolic pathways (Fig. 8) to produce more than 30 metabolites, 15 of which could be structurally identified. These microsomal metabolites comprise about 95% of the ethyl acetate-extractable metabolites of DBA.

The initial monooxygenase attack at the 1,2-, 3,4-, and 5,6-positions of DBA proceeds in the ratio 1.7:1.9:1.0. From the thus-generated arene oxides, DBA-5,6-oxide could be isolated and positively identified for the first time after several attempts (42, 43), which indicated its existence in microsomal incubations. The metabolic formation of DBA-1,2- and -3,4-oxides could only be indirectly demonstrated by the presence of the products of spontaneous aromatization, the phenols (i.e., metabolites XIc, Xb, XIb, XII), or of enzymatic hydrolysis, the *trans*-dihydrodiols (i.e., metabolites IV and VII). The conversion of the arene oxides of DBA by microsomal epoxide hydrolase obviously proceeds with largely different efficiency. Considering the further metabolism of the *trans*-dihydrodiols, about 40% of the 1,2-oxide, 70% of the 3,4-oxide, and 15% of the 5,6-oxide are hydrolyzed after short incubation times. Thus, it can be concluded that, of the arene oxides of DBA, the 3,4-oxide is

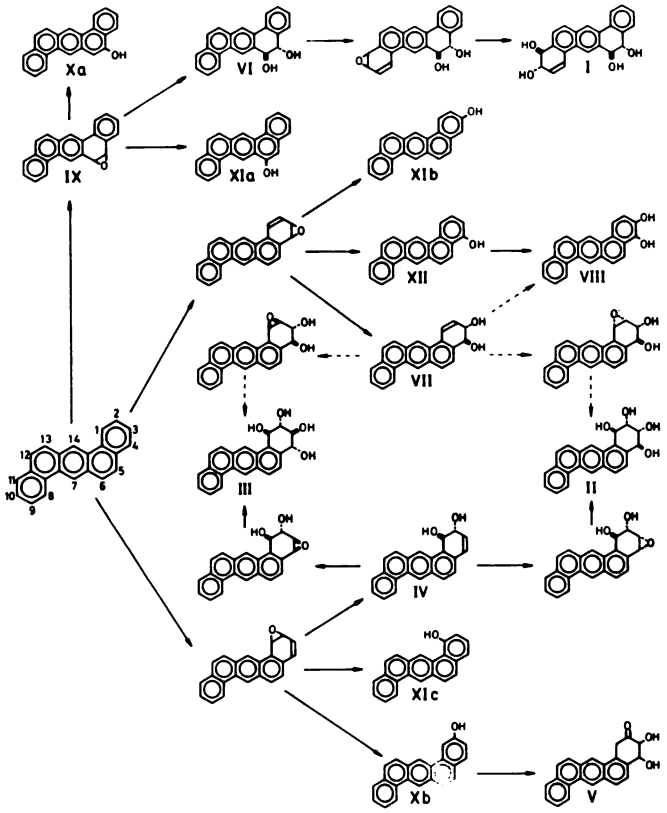

Fig. 8. Metabolic pathways of DBA with liver microsomes of Sprague-Dawley rats pretreated with Aroclor 1254. *Roman numerals* denote structurally identified metabolites. The structure of metabolite V is tentative. In the case of chiral metabolites, only one arbitrary enantiomer is shown.

the best and the 5,6-oxide the poorest substrate of microsomal epoxide hydrolase, a result supported by earlier investigations (8, 44, 45). As a consequence of this enzyme property, DBA-3,4-dihydrodiol not only dominates the mixture of the three *trans*-dihydrodiols (66%) but constitutes also the major microsomal ethyl acetate-extractable metabolite of DBA, with more than 22% of the overall metabolism irrespective of incubation time (present study) or of enzyme induction (8). In contrast to this, DBA-1,2-dihydrodiol is formed at less than half the rate of the 3,4-dihydrodiol, while the 5,6-dihydrodiol is only a minor metabolite of DBA, with about 2% of total metabolic conversion by liver microsomes of rats pretreated with Aroclor 1254. Whereas *trans*-dihydrodiols of DBA have been known for some time (6–9), the identity of the metabolically formed phenols of DBA remained speculative (6–8, 46). By comparison with the synthetic compounds (14), we were able to identify six of the seven structurally possible phenols as metabolites of DBA. 1-, 2-, and 4-OH-DBA are among the six major metabolites of DBA after short incubation times, whereas 3-, 5-, and 6-OH-DBA can only be detected after prevention of the enzymatic hydrolysis of the originally formed arene oxides by inhibition of microsomal epoxide hydrolase with TCPO (30, 31). Thus, the presence of 3-OH-DBA as a major metabolite of DBA (6, 7) could not be confirmed, while the missing evidence for the metabolic conversion at the L-region of DBA that would result in the formation of 7-OH-DBA or DBA-7,14-quinone is in agreement with earlier observations (6, 8).

The phenols of DBA could be formed by spontaneous aromatization of the precursor arene oxides (47) or by cytochrome P-450-catalyzed direct hydroxylation via oxygen insertion (48). Comparison of the product ratio of phenols formed by spontaneous aromatization of synthetic arene oxides of DBA with the ratio found upon metabolism of DBA under conditions where further enzymatic conversion is inhibited could indicate which mechanism takes place.

The product ratio of phenols formed by spontaneous isomerization of synthetic arene oxides of DBA is summarized in Table 6. In all mixtures of phenols one isomer prevails, i.e., 2-OH-DBA in the case of DBA-1,2-oxide, 4-OH-DBA in the case of DBA-3,4-oxide, and 5-OH-DBA in the case of DBA-5,6-oxide. In the latter case the product ratio obtained in this study differed somewhat from that reported earlier (49) (5-/6-OH-DBA = 85:15), probably due to different experimental conditions.

According to Fu *et al.* (50), the structures of the phenols formed by isomerization of arene oxides can be predicted by application of simplified MO theoretical calculations based on the perturbational method of Dewar and Doughtery (51) by calculating the reactivity numbers, N_t, of the ionic intermediate of the isomerization reaction.

N_t is calculated from the formula

$$N_t = 2\,(a_r + a_s)$$

where a_r and a_s are the non-bonding MO coefficients for adjacent positions (i.e., C-1 and C-3 in the case of 2-OH-DBA). In all cases investigated (50) the predominant isomer was the one for which the calculated value of N_t was the minimum. The results of Table 6 show that predictions based on the reactivity number could be confirmed for DBA-3,4- and -5,6-oxide but not for DBA-1,2-oxide, where an equal amount of both phenols was expected. Simplified MO calculations obviously are not sufficient to predict correctly the products of isomerization of arene oxides, since they do not distinguish between the formation of a radical and that of a carbocation.

This difference was recently taken into consideration by Szentpály (52), who proposed the index

$$N_i = \sum_r c_{or}^4$$

TABLE 6

Product ratios of phenols formed by spontaneous isomerization of synthetic arene oxides or metabolic conversion of DBA in the presence of liver microsomes of Sprague-Dawley rats pretreated with Aroclor 1254

Values are means ± standard deviations; $n = 3$.

Arene oxide	Phenol	N_t[a]	N_i[b]	Percentage of phenols after: Spontaneous isomerization of arene oxides[c]	Percentage of phenols after: Metabolic conversion of DBA[c,d]
DBA-1,2-Oxide	1-OH-DBA	1.97	0.254	20.4 ± 2.1	23.9 ± 1.7
	2-OH-DBA	1.97	0.201	79.6 ± 3.5	76.1 ± 2.0
DBA-3,4-Oxide	3-OH-DBA	2.16	0.246	13.7 ± 0.9	11.8 ± 1.2
	4-OH-DBA	1.83	0.209	86.3 ± 3.7	88.2 ± 2.9
DBA-5,6-Oxide	5-OH-DBA	1.66	0.281	69.4 ± 2.6	73 ± 20
	6-OH-DBA	1.71	0.312	30.6 ± 1.5	27 ± 7

[a] N_t, reactivity number (49,50).
[b] Index proposed by Szentpály (51).
[c] Incubation was performed with 1.1 mg of microsomal protein/ml and 1 mM TCPO at pH 7.4 and 37°.
[d] Incubation was performed with 0.6 mM NADPH. Values were taken from Table 5.

where c_{or} is the non-binding MO coefficient for a given atom, r. The results in Table 6 demonstrate that index N_i is more appropriate for the prediction of preferentially formed phenols of DBA, and probably of other PAHs,[3] than the reactivity number N_t.

The product ratios of phenols formed by spontaneous isomerization of synthetic arene oxides as compared to metabolic conversion of DBA by liver microsomes in the presence of TCPO show no significant differences (Table 6). This result supports the hypothesis that phenols of DBA are formed by spontaneous isomerization of the corresponding arene oxide and less likely by direct hydroxylation.

A striking feature of the metabolism of DBA as compared to other carcinogenic PAHs is the large number (i.e., more than 30) of metabolites; this has already been noticed by earlier investigators (8). After an incubation time of 30 min, almost one-fourth of the total metabolic conversion consists of secondary metabolites (see Footnote 1), i.e., I, II, III, V, and VIII (cf. Fig. 8), that originate from phenols and *trans*-dihydrodiols.

Metabolite V is derived from 2-OH-DBA and could be an indication of the formation of a phenol oxide explaining the bacterial mutagenicity of 2-OH-DBA[4] after metabolic activation. Metabolite VIII was identified as DBA-3,4-catechol arising from 4-OH-DBA. This is, to our knowledge, the first example of a catechol as a microsomal metabolite of a carcinogenic PAH, while the formation of the 9,10-catechol in the case of benzo[*a*]pyrene (53) could not be confirmed later (54). Recently, however, a metabolite of the *trans*-3,4-dihydrodiol of benz[*a*]anthracene which resembled the corresponding catechol was identified as *o*-quinone (29). The observation that the formation of metabolite VIII is suppressed by TCPO does not exclude the cytochrome P-450-catalyzed *o*-hydroxylation of 4-OH-DBA since TCPO is also known as an inhibitor of monooxygenases (55, 56). There also exists the possibility that the 3,4-catechol originates, at least in part, by dehydrogenation of DBA-3,4-dihydrodiol.

Metabolites I, II, and III are derived from *trans*-dihydrodiols. The metabolic conversion of DBA-5,6-dihydrodiol to the 3,4:12,13-bisdiol (I) proceeds very likely via epoxidation in the 10,11-position and enzymatic hydrolysis to I (cf. Fig. 8). Indications for the formation of the intermediate dihydrodiol arene oxide were obtained earlier, when a trihydroxy glutathionyl-adduct could be isolated from microsomal incubations of the 5,6-dihydrodiol in the presence of glutathione (57).

Bisdiol I is to our knowledge the first structurally identified bis-dihydrodiol metabolically formed from a K-region dihydrodiol. Extensive metabolism by subcellular preparations of rat liver has been observed with the K-region dihydrodiols of benz[*a*]anthracene (58) and benzo[*a*]pyrene (54), although identification of the products was not achieved.

DBA-3,4:12,13-bisdiol can also be given as an explanation for the surprisingly high bacterial mutagenicity of DBA-5,6-dihydrodiol when incubated with liver microsomes of Long-Evans rats pretreated with Aroclor 1254 (12) or of C3H mice pretreated with phenobarbital (59), since the resulting tetrol epoxide, 1,2-epoxy-3,4,12,13-tetrahydrotetrol, is expected to be highly reactive (12).

Since DBA-5,6-oxide is a rather poor substrate of microsomal epoxide hydrolase (this study, and Refs. 8, 44, and 45), DBA-5,6-dihydrodiol should be metabolically formed at a slow rate. Therefore, it is difficult to conceive that DBA-3,4:12,13-bisdiol, which constitutes one of the major metabolites of DBA after longer incubation times, originates entirely from DBA-5,6-oxide via the 5,6-dihydrodiol. The fact that DBA-3,4:12,13-bisdiol cannot be detected in incubations of DBA-3,4-dihydrodiol (cf. Fig. 7B) supports the assumption of its sole formation from the rather stable 5,6-oxide. In this case the generation of DBA-5,6:10,11-bisoxide, followed by the more facile enzymatic hydrolysis of the oxiranyl ring at the K-region of this intermediate as compared to 5,6-(mono)oxide, could explain the abundance of DBA-3,4:12,13-bisdiol.

Tetrols II and III are likely to be formed by hydrolysis of diol epoxides at the 1,2,3,4-benzo ring of DBA. The origin of these metabolites, i.e., 1,4/2,3- and 1,3/2,4-tetrol, is not unequivocal since both can arise by *trans*-opening of the oxirane ring from the diastereomeric pairs of diol epoxides of DBA-1,2- as well as -3,4-dihydrodiol (Fig. 9).

The main microsomal ethyl acetate-extractable metabolites of the 1,2-dihydrodiol are tetrols II and III. This is in close agreement with results by Chou *et al.* (60), who demonstrated that DBA-1,2-dihydrodiol is metabolically converted via vicinal diol epoxide(s) yielding two tetrols, D1 and D2, which very likely are identical with tetrols II and III, respectively.

Although a high metabolic conversion of DBA-3,4-dihydrodiol to vicinal diol epoxides has been assumed (61), we could not find indications for extensive epoxidation of the olefinic double bond of DBA-3,4-dihydrodiol by liver microsomes of Sprague-Dawley rats pretreated with Aroclor 1254: neither diastereomeric 3,4-dihydrodiol-1,2-epoxides nor the products of their spontaneous hydrolysis (see Materials and Methods and Fig. 9), i.e., DBA-1,4/2,3-tetrol (II), DBA-1,2,4/3-tetrol, or DBA-1,3/2,4-tetrol (III), could be detected in microsomal incubations of DBA-3,4-dihydrodiol. Since about 60% of the

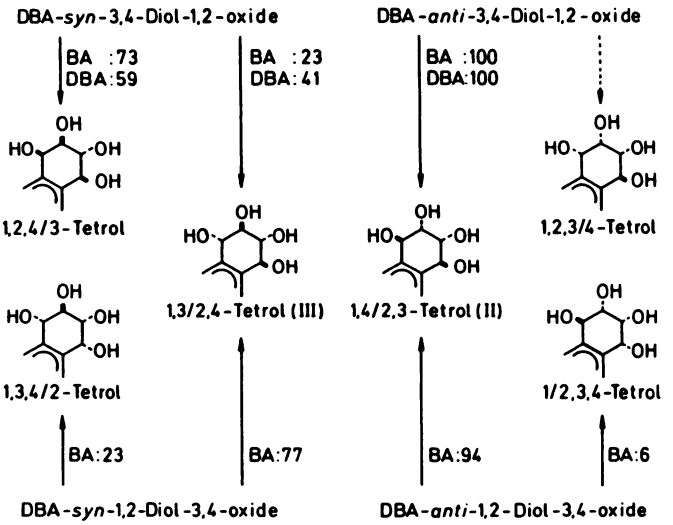

Fig. 9. Possible structures of tetrahydrotetrols formed from the diastereomeric dihydrodiol epoxides of *trans*-1,2- and -3,4-dihydrodiols of DBA. The *numbers* at the *arrows* give the product ratios upon hydronium ion-catalyzed hydrolysis in the case of benz[*a*]anthracene (*BA*) (39) and DBA. *Roman numerals* denote identified metabolites of DBA.

[3] Index N_i also turned out to be superior to reactivity number N_t in predicting the ratio of 1- and 2-phenols of picene formed by aromatization of the corresponding arene oxide (K. L. Platt, P. Petrovic, A. Seidel, D. Beermann, and F. Oesch, submitted for publication).

[4] K. L. Platt and F. Oesch, manuscript in preparation.

microsomal metabolites of DBA-3,4-diol remain in the aqueous phase after extractive work-up, it could be the case that vicinal diol epoxides are indeed formed but immediately bind covalently to microsomal protein or phosphate in the incubation mixture and, therefore, cannot be extracted with ethyl acetate. This possibility could positively be excluded because of the virtually equal recovery of DBA-1,4/2,3-tetrol regardless of whether synthetic DBA-*anti*-3,4-diol-1,2-oxide was incubated in water, phosphate buffer, or a solution containing microsomal protein (1 mg/ml).

Our results seem to be in contrast to work by Nordqvist *et al.* (8), who reported the formation of metabolites, possibly tetrols, possessing the benz[*a*]anthracene chromophore from DBA-3,4-dihydrodiol. These metabolites behave differently upon chromatography under similar conditions, compared to tetrols II and III, i.e., tetrols II and III elute *earlier* and the metabolites of DBA-3,4-dihydrodiol, observed by Nordqvist *et al.* (8), *later* than DBA-1,2-dihydrodiol. The latter metabolites, therefore, cannot be identical with tetrols II and III and, hence, cannot be taken as evidence for the formation of vicinal diol epoxides from DBA-3,4-dihydrodiol.

A further indication that metabolically formed 1,3/2,4-tetrol (III) is derived from the *syn*-diol epoxide of DBA-1,2-dihydrodiol and not of DBA-3,4-dihydrodiol can be obtained from the fact that 1,2,4/3-tetrol (cf. Fig. 9) could not be detected as a metabolite of DBA; this tetrol should be the main product of hydrolysis of DBA-*syn*-3,4-diol-1,2-oxide (see Materials and Methods), whereas 1,3/2,4-tetrol (III) would be expected to be formed preferentially from DBA-*syn*-1,2-diol-3,4-oxide, in a manner similar to the situation of benz[*a*]anthracene (39).

The results of the present study, therefore, imply that DBA-3,4-dihydrodiol is not converted via epoxidation of its olefinic double bond to vicinal diol epoxides which yield tetrols upon spontaneous hydrolysis but, rather, metabolizes to more polar products remaining in the aqueous phase after ethyl acetate extraction. This behavior of an M-region[5] dihydrodiol with the hydroxyl groups preferentially adopting a quasidiequatorial conformation, although unexpected, has its percedent in the metabolism of the racemic *trans*-3,4-dihydrodiols of benz[*a*]anthracene (29) and benzo[*c*]phenanthrene (65). When these M-region dihydrodiols were incubated with liver microsomes from rats pretreated with 3-methylcholanthrene, only 3–4% of the metabolites consisted of diol epoxides, whereas 87–90% were bis-dihydrodiols (29, 65) and phenolic dihydrodiols originating from dihydrodiol arene oxides. Apart from this similarity in the metabolism of benz[*a*]anthracene and benzo[*c*]phenanthrene with that of DBA, there exists a decisive difference: the weakly tumorigenic benz[*a*]anthracene (66, 67) and benzo[*c*]phenanthrene (68) are very inefficiently converted by the cytochrome P-450 system at the angular benzo-ring while, in the case of the carcinogenic DBA, the main routes of metabolism start with epoxidation of the 1,2- and 3,4-position.

Since DBA-3,4-dihydrodiol probably makes the largest contribution to the bacterial mutagenicity of DBA due to its high specific mutagenicity (12) and extensive metabolic formation, the question remains which metabolites of the 3,4-dihydrodiol, if not the vicinal diol epoxide, are responsible for its biological activity. Considering the predominant formation of bis-dihydrodiols in the case of M-region dihydrodiols of benz[*a*]anthracene (29) and benzo[*c*]phenanthrene (65) and the observed high polarity of the metabolites of DBA-3,4-dihydrodiol, tetrol epoxides could be reasonable candidates. Quantum mechanical calculations used to predict chemical reactivity of diol epoxides (69) indicate that the 10,11-diol-8,9-epoxide derivative of DBA-3,4-dihydrodiol would be expected to be highly reactive ($\Delta E_{deloc}/\beta = 0.780$ compared to 0.738 for DBA-3,4-diol-1,2-oxide) and thus can be proposed as an ultimate mutagenic metabolite of DBA-3,4-dihydrodiol.

Six of the eight isolated chiral metabolites of DBA could successfully be separated on chiral stationary phases which opened the way to investigate the metabolic stereoselectivity. Investigations with liver microsomal fractions of rats pretreated with different inducers of cytochrome P-450-dependent monooxygenases revealed that the enantiomeric purity of the metabolites in the case of induction with Aroclor 1254 (this study) did not differ from that obtained with 3-methylcholanthrene (data not shown).

The enantiomeric purity of DBA-5,6-oxide was decisively lower as compared to the corresponding K-region oxide of benzo[*a*]pyrene (70), although the geometry of the DBA molecule should fulfill the spatial requirement for the postulated catalytic binding site of cytochrome P-450$_c$ (71, 72) and thus be transformed highly selectively to the (5*S*,6*R*)-oxide. The enantiomeric composition of DBA-5,6-oxide cannot be a consequence of preferential enzymatic hydrolysis of one of its enantiomers since inhibition of microsomal epoxide hydrolase is without influence on the enantiomeric purity of the K-region oxide (cf. Table 4).

Metabolic conversion of DBA-5,6-oxide to the corresponding *trans*-dihydrodiol by epoxide hydrolase is governed by stereoselective rather than by regioselective enzymatic attack since racemic 5,6-oxide is converted to a high extent to (+)-(5*R*,6*R*)-dihydrodiol.

Thus, microsomal epoxide hydrolase seems to add water with the same stereoselectivity as in the case of the K-region oxide of benzo[*a*]pyrene (73) to the *S*-configurated carbon atom of the oxirane ring of DBA-5,6-oxide, leading to the 5,6-dihydrodiol with *R*,*R* absolute configuration and with much higher enantiomeric purity than that of the precursor arene oxide.

Both non-K-region *trans*-dihydrodiols of DBA were obtained preferentially as *R*,*R* enantiomers, as observed with other PAHs (1), but in poorer enantiomeric excess, as already determined by Nordqvist *et al.* (8) in the case of DBA-3,4-dihydrodiol. This could be the result of the geometry of the DBA molecule which does not fit well into the proposed catalytic binding site of cytochrome P-450$_c$ (71, 72) for epoxidation in the 1,2- and 3,4-positions.

The present study has added much new information to the knowledge of the regio- and stereoselectivity of the microsomal metabolism of DBA. The most interesting result of our investigation is the proposed formation of tetrol epoxides as ultimate reactive metabolites of the M- and the K-region dihydrodiols. This has no precedent in the literature on the metabolism of PAHs. It can be reasoned that the high molecular symmetry of DBA permits subsequent metabolic attacks at discrete but structurally equivalent sites of the molecule. Indications for this unique behavior were first obtained almost half a century

[5] The term M-region (52, 62) is used here in a structural sense, i.e., denoting the 3,4-positions in DBA, benz[*a*]anthracene, and benzo[*c*]phenanthrene, the 1,2-positions in phenanthrene and chrysene, or the 7,8-position in benzo[*a*]pyrene. In this respect the M-region corresponds with the A-region of Smith *et al.* (63) or the prebay region of Glatt and Oesch (64).

ago when the 2,9- and the 4,11-diphenols were identified as metabolites of DBA (10, 11).

Acknowledgments

The authors express their appreciation to S. Kuthan for excellent technical assistance, Dr. H. Frank for providing chiral stationary phases, Dr. F. Setiabudi for performing NMR measurements, C. Böhres for running the mass spectra, and S. Pollok and I. Böhm for skillful help in the preparation of the manuscript.

References

1. Thakker, D. R., H. Yagi, W. Levin, A. W. Wood, A. H. Conney, and D. M. Jerina. Polycyclic aromatic hydrocarbons: metabolic activation to ultimate carcinogens, in *Bioactivation of Foreign Compounds* (M. W. Anders, ed.). Academic Press, Orlando, 177–242 (1985).
2. Kennaway, E. L. LVII. Further experiments on cancer producing substances. *Biochem. J.* **24**:497–504 (1930).
3. Levi, A. A., and E. Boyland. The production of dihydroxy-1:2:5:6-dibenzanthracene from 1:2:5:6-dibenzanthracene by rabbits. *Chem. Ind. (Lond.)* **15**:446 (1937).
4. Dobriner, K., C. P. Rhoads, and G. I. Lavin. Conversion of 1,2,5,6-dibenzanthracene by rabbits, rats, and mice. Significance in carcinogenesis of this conversion. *Proc. Soc. Exp. Biol. Med.* **41**:67–69 (1939).
5. Heidelberger, C., H. I. Hadler, and G. Wolf. The metabolic degradation in the mouse of 1,2,5,6-dibenzanthracene-9,10-C^{14}. III. Some quinone metabolites retaining the intact ring system. *J. Am. Chem. Soc.* **75**:1303–1308 (1953).
6. Boyland, E., and P. Sims. The metabolism of benz[*a*]anthracene and dibenz[*a,h*]anthracene and their 5,6-epoxy-5,6-dihydro derivatives by rat-liver homogenates. *Biochem. J.* **97**:7–16 (1965).
7. Sims, P. Qualitative and quantitative studies on the metabolism of a series of aromatic hydrocarbons by rat-liver preparations. *Biochem. Pharmacol.* **19**:795–818 (1970).
8. Nordqvist, M., D. R. Thakker, W. Levin, H. Yagi, D. E. Ryan, P. E. Thomas, A. H. Conney, and D. M. Jerina. The highly tumorigenic 3,4-dihydrodiol is a principal metabolite formed from dibenzo[*a,h*]anthracene by liver enzymes. *Mol. Pharmacol.* **16**:643–655 (1979).
9. MacNicoll, A. D., P. M. Burden, H. Rattle, P. L. Grover, and P. Sims. The formation of dihydrodiols in the chemical or enzymic oxidation of dibenz[*a,c*] anthracene, dibenz[*a,h*]anthracene and chrysene. *Chem. Biol. Interact.* **27**:365–379 (1979).
10. LaBudde, J. A., and C. Heidelberger. The synthesis of the mono- and dihydroxy derivatives of 1,2,5,6-dibenzanthracene excreted by the rabbit and of other hydroxylated dibenzanthracene derivatives. *J. Am. Chem. Soc.* **80**:1225–1236 (1958).
11. Cason, J., and L. F. Fieser. Synthesis of 4′,8′-dihydroxy-1,2,5,6-dibenzanthracene and its relation to products of metabolism of the hydrocarbon. *J. Am. Chem. Soc.* **62**:2681–2687 (1940).
12. Wood, A. W., W. Levin, P. E. Thomas, D. Ryan, J. M. Karle, H. Yagi, D. M. Jerina, and A. H. Conney. Metabolic activation of dibenzo[*a,h*]anthracene and its dihydrodiols to bacterial mutagens. *Cancer Res.* **38**:1967–1973 (1978).
13. Buening, M, K., W. Levin, A. W. Wood, R. L. Chang, H. Yagi, J. M. Karle, D. M. Jerina, and A. H. Conney. Tumorigenicity of the dihydrodiols of dibenzo[*a,h*]anthracene on mouse skin and in newborn mice. *Cancer Res.* **39**:1310–1314 (1979).
14. Platt, K. L., and F. Oesch. Synthesis and properties of the seven isomeric phenols of dibenz[*a,h*]anthracene. *J. Org. Chem.* **47**:5321–5326 (1982).
15. Platt, K. L., and F. Oesch. Synthesis of non-K-region orthoquinones of polycyclic aromatic hydrocarbons from cyclic ketones. *Tetrahedron Lett.* **23**:163–166 (1982).
16. Platt, K. L., and F. Oesch. K-Region trans-dihydrodiols of polycyclic arenes; an efficient and convenient preparation from *o*-quinones or *o*-diphenols by reduction with sodium borohydride in the presence of oxygen. *Synthesis* 459–461 (1982).
17. Oesch, F., G. Stillger, H. Frank, and K. L. Platt. Improved synthesis of (±)-*trans*-9,10-dihydroxy-9,10-dihydrobenzo[*a*]pyrene and of (±)-*trans*-1,2-dihydroxy-1,2-dihydrodibenz[*a,h*]anthracene. *J. Org. Chem.* **47**:568–571 (1982).
18. Platt, K. L., and F. Oesch. Efficient synthesis of non-K-region *trans*-dihydrodiols of polycyclic aromatic hydrocarbons from *o*-quinones and catechols. *J. Org. Chem.* **48**:265–268 (1983).
19. Lee, H. M., and R. G. Harvey. Synthesis of biologically active metabolites of dibenz[*a,h*]anthracene. *J. Org. Chem.* **45**:588–592 (1980).
20. Yagi, H., and D. M. Jerina. A general synthetic method for non-K-region arene oxides. *J. Am. Chem. Soc.* **97**:3185–3192 (1975).
21. Oesch, F., A. J. Sparrow, and K. L. Platt. Radioactively labelled epoxides. Part II. Tritum labelled cyclohexene oxide, *trans*-stilbene oxide and phenathrene 9,10-oxide. *J. Labelled Compds. Radiopharm.* **17**:93–101 (1980).
22. Yagi, H., and D. R. Thakker, O. Hernandez, M. Koreeda, and D. M. Jerina Synthesis and reactions of the highly mutagenic 7,8-diol 9,10-epoxides of the carcinogen benzo[*a*]pyrene. *J. Am. Chem. Soc.* **99**:1604–1611 (1977).
23. Schmassmann, H., A. Sparrow, K. Platt, and F. Oesch. Epoxide hydratase and benzo[*a*]pyrene monooxygenase activities in liver, kidney and lung after treatment of rats with epoxides of widely varying structures. *Biochem. Pharmacol.* **27**:2237–2245 (1978).
24. Selkirk, J. K., R. G. Croy, and H. V. Gelboin. Isolation by high pressure liquid chromatography and characterization of benzo[*a*]pyrene-4,5-epoxide as a metabolite of benzo[*a*]pyrene. *Arch. Biochem. Biophys.* **168**:322–326 (1975).
25. Tjessum, K., and J. J. Stegeman. Improvement of reverse-phase high pressure liquid chromatographic resolution of benzo[*a*]pyrene metabolites using organic amines: application to metabolites produced by fish. *Anal. Biochem.* **99**:129–135 (1979).
26. Mikes, F., G. Boshart, and E. Gil-Av. Resolution of optical isomers by high-performance liquid chromatography, using coated and bonded chiral charge-transfer complexing agents as stationary phases. *J. Chromatogr.* **122**:205–221 (1976).
27. Platt, K. L., I. Reischmann, C. Flenner, and F. Oesch. Isolation, separation and quantification of metabolites of the carcinogenic polycyclic aromatic hydrocarbon dibenz[*a,h*]anthracene. *Fresenius Z. Anal. Chem.* **324**:357 (1986).
28. Lee, H., and R. G. Harvey. Stereoselective *syn* epoxidation of the dihydrodiols of dibenz[*a,h*]anthracene and 7-methylbenz[*a*]anthracene. *Tetrahedron Lett.* **22**:1657–1660 (1981).
29. Thakker, D. R., W. Levin, H. Yagi, M. Tada, D. E. Ryan, P. E. Thomas, A. H. Conney, and D. M. Jerina. Stereoselective metabolism of the (+)- and (−)-enantiomers of *trans*-3,4-dihydroxy-3,4-dihydrobenz[*a*]anthracene by rat liver microsomes and by a purified and reconstituted cytochrome P-450 system. *J. Biol. Chem.* **257**:5103–5110 (1982).
30. Oesch, F., N. Kaubisch, D. M. Jerina, and J. W. Daly. Hepatic epoxide hydrase. Structure-activity relationships of substrates and inhibitors. *Biochemistry* **10**:4858–4866 (1971).
31. Oesch, F. Mammalian epoxide hydrases: inducible enzymes catalysing the inactivation of carcinogenic and cytotoxic metabolites derived from aromatic and olefinic compounds. *Xenobiotica* **3**:305–340 (1973).
32. Kundu, N. G. Synthesis of some non-K-region derivatives of dibenz[*a,h*] anthracene. *J. Org. Chem.* **44**:3086–3088 (1979).
33. Ukai, S., K. Hirose, A. Tatematsu, and T. Goto. Organic mass spectrometry. IX. The reductive reaction of 1,2-quinones in the mass spectrometer. *Tetrahedron Lett.* 4999–5002 (1967).
34. Oliver, R. W. A., and R. M. Rashman. Mass spectrometry of quinones. Part II. A study of the distinguishing features found in the mass spectra of 1,2,- and 1,4-naphthoquinones. *J. Chem. Soc. (B)* 341–344 (1971).
35. Patel, J. R., I. R. Politzer, G. W. Griffin, and J. L. Laseter. Mass spectra of the oxygenated products generated from phenanthrene under simulated environmental conditions. *Biomed. Mass Spectrom.* **5**:664–670 (1978).
36. Weems, H. B., M. Mushtaq, P. P. Fu, and S. K. Yang. Direct separation of non-K-region mono-ol and diol enantiomers of phenanthrene, benz[*a*]anthracene, and chrysene by high-performance liquid chromatography with chiral stationary phases. *J. Chromatogr.* **371**:211–225 (1986).
37. Weems, H. B., M. Mushtaq, and S. K. Yang. Resolution of epoxide enantiomers of polycyclic aromatic hydrocarbons by chiral stationary-phase high-performance liquid chromatography. *Anal. Biochem.* **148**:328–338 (1985).
38. Schollmeier, M., H. Frank, F. Oesch, and K. L. Platt. Assignment of absolute configuration to metabolically formed *trans*-dihydrodiols of dibenz[*a,h*]anthracene by two distinct spectroscopic methods. *J. Org. Chem.* **51**:5368–5372 (1986).
39. Sayer, J. M., D. L. Whalen, S. L. Friedman, A. Paik, H. Yagi, K. P. Vyas, and D. M. Jerina. Conformational effects in the hydrolyses of benzo-ring diol epoxides that have bay-region diol groups. *J. Am. Chem. Soc.* **106**:226–233 (1984).
40. Reischmann, I. Investigation of the microsomal metabolism of dibenz[*a,h*] anthracene. Ph.D. thesis, University of Mainz, Mainz, FRG (1983).
41. Kuroki, T., E. Huberman, H. Marquardt, J. K. Selkirk, C. Heidelberger, P. L. Grover, and P. Sims. Binding of K-region epoxides and other derivatives of benz[*a*]anthracene and dibenz[*a,h*]anthracene to DNA, RNA, and proteins of transformable cells. *Chem. Biol. Interact* **4**:389–397 (1972).
42. Selkirk, J. K., E. Huberman, and C. Heidelberger. An epoxide is an intermediate in the microsomal metabolism of the chemical carcinogen, dibenz[*a,h*]anthracene. *Biochem. Biophys. Res. Commun.* **43**:1010–1016 (1971).
43. Grover, P. L., A. Hewer, and P. Sims. Epoxides as microsomal metabolites of polycyclic hydrocarbons. *FEBS Lett.* **18**:76–80 (1971).
44. Bentley, P., H. Schmassmann, P. Sims, and F. Oesch. Epoxides derived from various polycyclic hydrocarbons as substrates of homogeneous and microsome-bound epoxide hydratase. *Eur. J. Biochem.* **69**:97–103 (1976).
45. Jerina, D. M., P. M. Dansette, A. Y. H. Lu, and W. Levin. Hepatic microsomal epoxide hydrase: a sensitive radiometric assay for hydration of arene oxides of carcinogenic aromatic hydrocarbons. *Mol. Pharmacol.* **13**:342–351 (1977).
46. Lubet, R. A., G. M. Connolly, D. W. Nebert, and R. E. Kouri. Dibenz[*a,h*] anthracene-induced subcutaneous tumors in mice. Strain sensitivity and the role of carcinogen metabolism. *Carcinogenesis* **4**:513–517 (1983).
47. Jerina, D. M., and J. W. Daly. Arene oxides: a new aspect of drug metabolism. *Science Wash. D. C.* **185**:573–582 (1974).
48. Gelboin, H. V. Benzo[*a*]pyrene metabolism, activation, and carcinogenesis: role and regulation of mixed-function oxidases and related enzymes. *Physiol. Rev.* **60**:1107–1166 (1980).
49. Keller, J. W., and C. Heidelberger. Polycyclic K-region arene oxides. Products and kinetics of solvolysis. *J. Am. Chem. Soc.* **98**:2328–2336 (1976).
50. Fu, P. P., R. G. Harvey, and F. A. Beland. Molecular orbital theoretical

prediction of the isomeric products formed from reactions of arene oxides and related metabolites of polycyclic aromatic hydrocarbons. *Tetrahedron* **34**:857–866 (1978).
51. Dewar, M. J. S., and R. C. Dougherty. *The PMO Theory of Organic Chemistry*. Plenum Press, New York (1975).
52. Szentpály, L.v. Carcinogenesis by polycyclic aromatic hydrocarbons: a multilinear regression on new type PMO indices. *J. Am. Chem. Soc.* **106**:6021–6028 (1984).
53. Booth, J., and P. Sims. Different pathways involved in the metabolism of the 7,8- and 9,10-dihydrodiols of benzo[*a*]pyrene. *Biochem. Pharmacol.* **25**:979–980 (1976).
54. Thakker, D. R., H. Yagi, R. E. Lehr, W. Levin, M. Buening, A. Y. H. Lu, R. L. Chang, A. W. Wood, A. H. Conney, and D. M. Jerina. Metabolism of *trans*-9,10-dihydroxy-9,10-dihydrobenzo[*a*]pyrene occurs primarily by arylhydroxylation rather than formation of a diol epoxide. *Mol. Pharmacol.* **14**:502–513 (1978).
55. Yang, C. S., and F. S. Strickhart. Effects of some epoxides on aryl hydrocarbon hydroxylase activity. *Biochem. Pharmacol.* **24**:646–648 (1975).
56. Ivanetich, K. M., M. R. Ziman, and J. J. Bradshaw. 1,1,1-Trichloropropene-2,3-oxide: an alternate mechanism for its inhibition of cytochrome P-450. *Res. Commun. Chem. Pathol. Pharmacol.* **35**:111–120 (1982).
57. Golan, M. D. The metabolism and mutagenicity of the carcinogenic polycyclic aromatic hydrocarbon dibenz[*a,h*]anthracene. Ph.D. thesis, University of Mainz, Mainz, FRG (1979).
58. Keysell, G. R., J. Booth, and P. Sims. Glutathione conjugates as metabolites of benz[*a*]anthracene. *Xenobiotica* **5**:439–448 (1975).
59. Platt, K. L., M. Bücker, M. Golan, and F. Oesch. The mutagenicity of dibenz[*a,h*]anthracene activated by phenobarbital-inducible mouse-liver mono-oxygenase is potentiated by the presence of hydrophilic residues at the K-region of the molecule. *Mutat. Res.* **96**:1–13 (1982).
60. Chou, M. W., P. P. Fu, and S. K. Yang. Metabolic conversion of dibenz[*a,h*]anthracene (±)*trans*-1,2-dihydrodiol and chrysene (±)*trans*-3,4-dihydrodiol to vicinal dihydrodiol epoxides. *Proc. Natl. Acad. Sci. USA* **78**:4270–4273 (1981).
61. Thakker, D. R., W. Levin, H. Yagi, M. Tada, A. H. Conney, and D. M. Jerina. Comparative metabolism of dihydrodiols of polycyclic aromatic hydrocarbons to bay-region diol epoxides, in *Polynuclear Aromatic Hydrocarbons: Chemistry and Biological Effects* (A. Bjørseth and A. J. Dennis, eds.). Battelle Press, Columbus, OH, 267–286 (1980).
62. Pullman, A., and B. Pullman. Electronic structure and carcinogenic activity of aromatic molecules. New developments. *Adv. Cancer Res.* **3**:117–169 (1955).
63. Smith, I. A., G. D. Berger, P. G. Seybold, and M. P. Servé. Relationships between carcinogenicity and theoretical reactivity indices in polycyclic aromatic hydrocarbons. *Cancer Res.* **38**:2968–2977 (1978).
64. Glatt, H., and F. Oesch. Structural and metabolic parameter governing the mutagenicity of polycyclic aromatic hydrocarbons, in *Chemical Mutagens* (F. J. de Serres, ed.), Vol. 10. Plenum Publishing Corp., New York, 73–127 (1986).
65. Thakker, D. R., W. Levin, H. Yagi, H. J. C. Yeh, D. E. Ryan, P. E. Thomas, A. H. Conney, and D. M. Jerina. Stereoselective metabolism of the (+)-(*S,S*)- and (−)-(*R,R*)-enantiomers of *trans*-3,4-dihydroxy-3,4-dihydrobenzo[*c*]phenanthrene by rat and mouse liver microsomes and by a purified and reconstituted cytochrome P-450 system. *J. Biol. Chem.* **261**:5404–5413 (1986).
66. Thakker, D. R., W. Levin, H. Yagi, D. Ryan, P. E. Thomas, J. M. Karle, R. E. Lehr, D. M. Jerina, and A. H. Conney. Metabolism of benzo[*a*]anthracene to its tumorigenic 3,4-dihydrodiol. *Mol. Pharmacol.* **15**:138–153 (1979).
67. MacNicoll, A. D., C. S., Cooper, O. Ribeiro, K. Pal, A. Hewer, P. L. Grover, and P. Sims. The metabolic activation of benz[*a*]anthracene in three biological systems. *Cancer Lett.* **11**:243–249 (1981).
68. Ittah, Y., D. R. Thakker, W. Levin, M. Croisy-Delcey, D. E. Ryan, P. E. Thomas, A. H. Conney, and D. M. Jerina. Metabolism of benzo[*c*]phenanthrene by rat liver microsomes and by a purified monooxygenase system reconstituted with different isozymes of cytochrome P-450. *Chem. Biol. Interact.* **45**:15–28 (1983).
69. Jerina, D. M., R. E. Lehr, H. Yagi, O. Hernandez, P. M. Dansette, P. G. Wislocki, A. W. Wood, R. L. Chang, W. Levin, and A. H. Conney. Mutagenicity of benzo[*a*]pyrene derivatives and the description of a quantum mechanical model which predicts the ease of carbonium ion formation from diol epoxides, in *In Vitro Metabolic Activation in Mutagenesis Testing* (F. J. de Serres, J. R. Fouts, J. R. Bend, and R. M. Philpot, eds.). Elsevier/North-Holland Biomedical Press, Amsterdam, 159–177 (1976).
70. Armstrong, R. N., W. Levin, D. E. Ryan, P. E. Thomas, H. D. Mah, and D. M. Jerina. Stereoselectivity of rat liver cytochrome P-450$_c$ on formation of benzo[*a*]pyrene 4,5-oxide. *Biochem. Biophys Res. Commun.* **100**:1077–1084 (1981).
71. Jerina, D. M., D. P. Michaud, R. J. Feldmann, R. N. Armstrong, K. P. Vyas, D. R. Thakker, H. Yagi, P. E. Thomas, D. E. Ryan, and W. Levin. Stereochemical modeling of the catalytic site of cytochrome P-450$_c$, in *Microsomes, Drug Oxidations, and Drug Toxicity* (R. Sato and R. Kato, eds.). Japan Scientific Societies Press, Tokyo, 195–201 (1982).
72. Jerina, D. M., J. M. Sayer, H. Yagi, P. J. van Bladeren, D. R. Thakker, W. Levin, R. L. Chang, A. W. Wood, and A. H. Conney. Stereoselective metabolism of polycyclic aromatic hydrocarbons to carcinogenic metabolites, in *Microsomes and Drug Oxidations* (A. J. Boobis, J. Caldwell, F. De Matteis, and C. R. Elcombe, eds.). Taylor & Francis, London, 310–319 (1985).
73. Armstrong, R. N., B. Kedzierski, W. Levin, and D. M. Jerina. Enantioselectivity of microsomal epoxide hydrolase toward arene oxide substrates. *J. Biol. Chem.* **256**:4726–4733 (1981).

Send reprint requests to: Karl L. Platt, Institute of Toxicology, University of Mainz, Obere Zahlbacher Str. 67, D-6500 Mainz, FRG.