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METABOLISM OF (-)-*TRANS*-(3R,4R)-DIHYDROXY-3,4-DIHYDROCHRYSENE TO DIOL EPOXIDES BY LIVER MICROSOMES

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SUMMARY

Metabolism of biosynthetic (-)-*trans*-(3R,4R)-dihydroxy-3,4-dihydrochrysene by liver microsomes from control, phenobarbital-treated and 3-methylcholanthrene-treated rats was investigated. Although previous studies of the metabolism of related benzo[a]pyrene and benzo[e]pyrene dihydrodiols which also prefer the diaxial conformation had indicated that diol epoxides were minor metabolites, the diastereomeric chrysene 3,4-diol-1,2-epoxides-1 and -2 were major metabolites (66-90%). All three types of microsomes metabolized the chrysene 3,4-dihydrodiol at low but essentially similar rates (0.5-0.7 nmol substrate/nmol cytochrome P-450/min).

INTRODUCTION

Metabolism of trans dihydrodiols on benzo-rings of polycyclic aromatic hydrocarbons to diol epoxides represents the only known pathway to ultimately carcinogenic products (1). Quantitative studies of the metabolism of the quasi-diequatorial benzo[a]pyrene 7,8-dihydrodiol (2,3), as well as phenanthrene and chrysene 1,2-dihydrodiols (4), have indicated that formation of benzo-ring diol epoxides is a major pathway. When the diol group forms part of a sterically hindered bay-region, the hydroxyl groups greatly prefer the quasi-diaxial conformation (5). For two such dihydrodiols studied, rat liver enzymes formed very little diol epoxide. Metabolism of the quasi-diaxial B[a]P 9,10-dihydrodiol occurred primarily by aromatic ring hydroxylation at sites remote from the olefinic 7,8-double bond (6). Less than 5% of the total metabolites could be accounted for as 9,10-diol-7,8-epoxides. Similarly,

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metabolism of the quasi-diaxial benzo[e]pyrene 9,10-dihydrodiol failed to provide evidence for the formation of 9,10-diol-11,12-epoxides (7). Instead, a bis dihydrodiol at the 4,5- and 9,10-positions, as well as ring hydroxylated derivatives of the 9,10-dihydrodiol, were identified. Subsequent studies have shown that small amounts of the 9,10-diol-11,12-epoxides can be detected with rat liver microsomes and that liver microsomes from hamsters are even more effective in catalyzing this metabolism (8). The presence of 7,8-benzoflavone in the incubation medium stimulated formation of the 9,10-diol-11,12-epoxides, particularly with microsomes from humans and rabbits (8). Recently, the bay region benzo[a]pyrene 9,10-dihydrodiol (9) and benzo[a]anthracene 1,2-dihydrodiol (10) were shown to be metabolized to diol epoxides by fungus C. elegans and rat liver microsomes respectively, although no quantitative data were presented. In order to gain further insight into the specificity of the cytochromes P-450, the present study examines the metabolism of the quasi-diaxial chrysene 3,4-dihydrodiol by liver microsomes from control and induced rats.

MATERIALS AND METHODS

[^{3}H]-*trans*-3,4-Dihydroxy-3,4-dihydrochrysene: [^{3}H]-Chrysene 3,4-dihydrodiol (13.9 μCi/μmol, 98.5% (-)-enantiomer) was obtained by large scale microsomal incubation and purified by reverse phase, as well as silica HPLC, as described previously (4). Radiochemical purity of the dihydrodiol was >99% when analyzed by HPLC under conditions utilized for analysis of metabolites.

Absolute Stereochemistry of Biosynthetic (-)-Chrysene (3R,4R)-Dihydrodiol: Since the specificity of the cytochromes P-450 can be dependent on the enantiomeric composition of the substrate (2,11), absolute stereochemistry of the (-)-chrysene 3,4-dihydrodiol was determined. Assignment was achieved through chemical correlation of the dihydrodiol with the corresponding tetrahydrodiol of known absolute stereochemistry (see below).

Synthetic (+)-chrysene 3,4-dihydrodiol (4) ($[\alpha]_D$ + 313°, c = 0.66 mg/ml in THF) was converted to *trans*-3,4-dihydroxy-1,2,3,4-tetrahydrochrysene by catalytic reduction of the 1,2-double bond; 0.25 mg of dihydrodiol and 0.5 mg of 5% Pd on carbon in 1 ml of THF was agitated for 10 min under 1 atm of hydrogen. The resultant tetrahydrodiol was converted into its bis-ester with (-)-α-methoxy-α-trifluoromethylphenylacetic acid (Aldrich Chemical Co.) by established procedures (4). This bis-ester was cochromatographic on reverse phase HPLC with the bis-ester of (+)-*trans*-(3S,4S)-dihydroxy-1,2,3,4-tetrahydrochrysene (retention time 12.6 min) and was clearly separated from the bis-ester of the (-)-tetrahydrodiol (retention time 13.2 min). Chromatography conditions consisted of elution from a Du Pont Zorbax ODS column (0.62 x 25 cm) with a linear gradient of water versus 30% THF in acetonitrile. The water content varied from 55% to 30% over a period of 25 min at a flow rate of 1.2 ml/min. Thus, the (+)-dihydrodiol has (3S,4S)-absolute stereochemistry, and the (-)-dihydrodiol (predominant metabolic enantiomer) has (3R,4R)-absolute stereochemistry (cf.4).

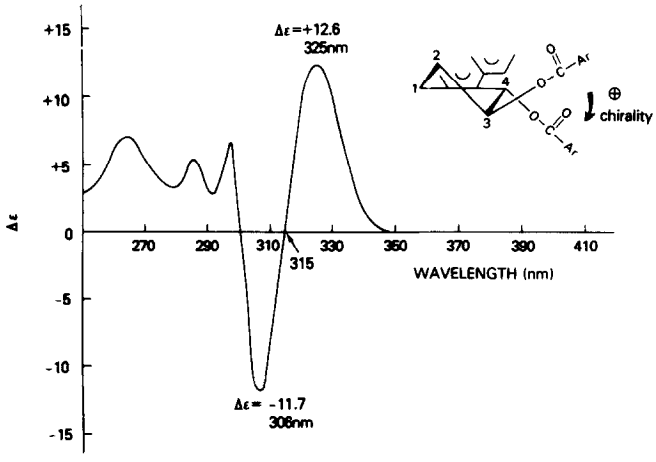

Fig. 1 Circular dichroism spectrum of the bis p-N,N-dimethylaminobenzoate of (+)-*trans*-3,4-dihydroxy-1,2,3,4-tetrahydrochrysene in methanol. Values of Δε are based on the assumption that the bis ester and the free diol have the same extinction coefficients.

(+)-*trans*-(3S,4S)-Dihydroxy-1,2,3,4-tetrahydrochrysene: Optically pure (+)- and (-)-3,4-epoxy-1,2,3,4-tetrahydrochrysene were prepared by application of the general procedure of Boyd et al. (12). Previously, the racemic tetrahydro epoxide was shown to undergo facile acid-catalyzed hydrolysis to a mixture of the cis and trans tetrahydrodiols by attack of water at the benzylic 4-position of the epoxide (13).

A solution of (+)-3,4-epoxy-1,2,3,4-tetrahydrochrysene ($[\alpha]_D$ + 63°, c = 4 mg/ml in THF) in 10% THF in water containing 0.1 N $NaClO_4$ was adjusted to pH 3 and stored at 25°C for 1.5 hrs. Standard workup followed by purification of the products on HPLC allowed isolation of (+)-*trans*-3,4-dihydroxy-1,2,3,4-tetrahydrochrysene ($[\alpha]_D$ + 8.5°, c = 11 mg/ml in THF) as well as the (-)-cis-isomer ($[\alpha]_D$ - 23.7, c = 3 mg/ml in THF); retention times of 12.5 min and 22.5 min, respectively, on elution from a Du Pont Zorbax ODS column (0.94 x 25 cm) with 70% methanol in water at a flow rate of 2.6 ml/min.

Assignment of absolute stereochemistry to the (+)-*trans*-tetrahydrodiol was achieved by an application of the exciton chirality rule (14). The diol (5 mg), p-(N,N-dimethylamino)benzoyl chloride (14 mg), and a catalytic amount of p-N,N-dimethylaminopyridine in two drops of dry pyridine were warmed at 75°C for 16 hrs. Standard workup followed by purification by TLC (silica gel eluted with chloroform) and then by HPLC (Du Pont Zorbax SIL eluted with 5% THF in methylene chloride, k' = 4.3) provided the requisite bis-ester; chemical ionization mass spectrum (NH_3 gas), $M^+ + 1$ = 559. The circular dichroism spectrum of the bis-ester (Fig. 1) shows a pair of nearly symmetric curves which pass through zero at 315 nm. The presence of a positive long wavelength band ($\Delta\varepsilon_{325nm}$ = +12.6) and a negative shorter wavelength band ($\Delta\varepsilon_{306nm}$ = -11.7) are indicative of (3S,4S)-absolute stereochemistry.

Diol Epoxides and Tetraols: Since direct epoxidation of dihydrodiols which prefer the quasi-diaxial conformation is known to produce a diastereomeric mixture of diol epoxides in which the benzylic hydroxyl group is either cis (isomer-1) or trans (isomer-2) to the epoxide oxygen (15), chrysene 3,4-dihydrodiol was oxidized with m-chloroperoxy-benzoic acid. The resulting mixture of 3,4-diol-1,2-epoxides-1 (k' = 3.6) and -2 (k' = 4.3) were separated by elution through a Du Pont Zorbax SIL column (0.62 x 25 cm) with 40% THF in hexane. Both diol epoxides undergo facile acid catalyzed hydrolysis (pH 3,

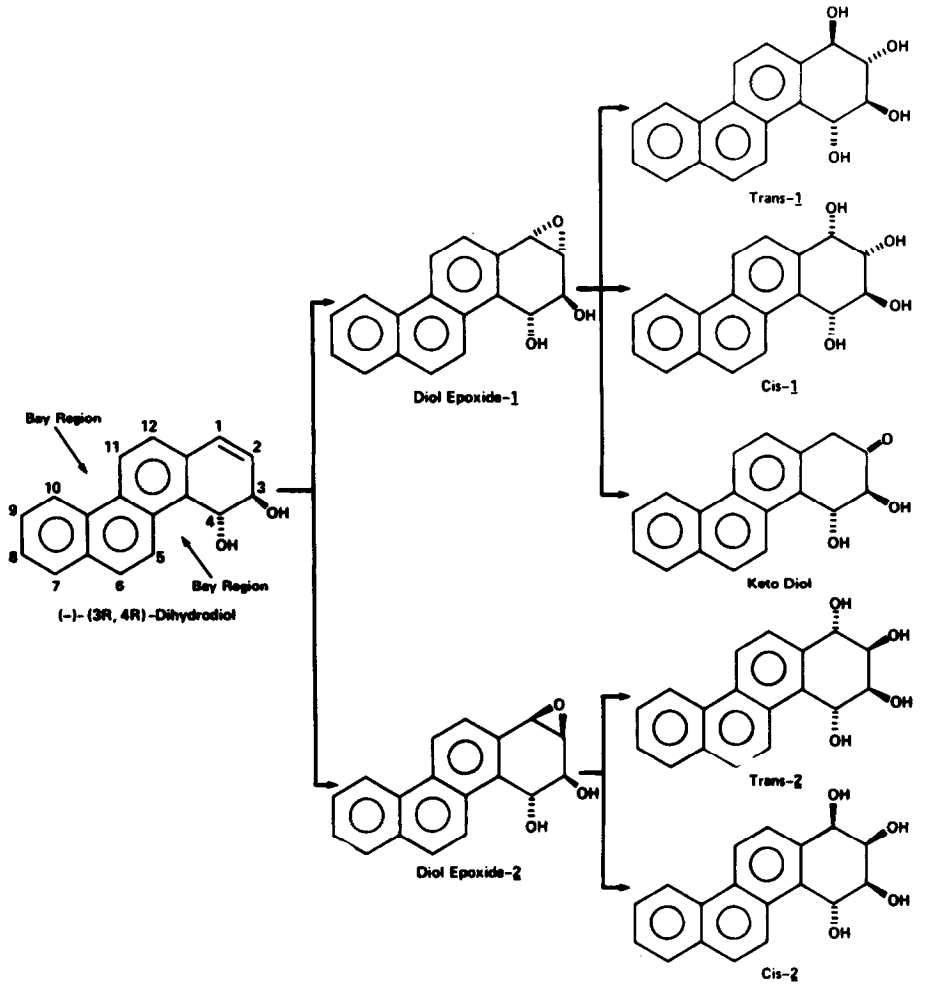

Fig. 2 Metabolism of (-)-(3R,4R)-dihydroxy-3,4-dihydrochrysene to 3,4-diol-1,2-epoxides. All of the solvolysis products of the diol epoxides except the very minor cis-2 were detected as metabolites.

25% dioxane in water, <2 hrs at 25°) by cis and trans addition of water at the benzylic 4-position to form pairs of tetraols (Fig. 2) in which the trans adducts are greatly favored (80-90%). Trans-1 and Trans-2 were identical (HPLC retention time, nmr of acetates) to the trans tetraols formed on hydrolysis of the chrysene 1,2,-diol-3,4-epoxides-1 and -2 (13), as is required for the structural assignments to be correct. The nmr and mass spectra of the acetylated cis tetraols confirmed their structures. Under neutral conditions, diol epoxide-1 isomerizes to a keto diol (Fig. 2) in addition to forming tetraols. The keto diol was characterized from its mass and UV spectra as well as by its facile reduction to a pair of triols by sodium borohydride.

Incubation: Liver microsomes were prepared from control, phenobarbital-treated and 3-methylcholanthrene-treated, immature (50-60 g) male rats of the Long-Evans strain and were assayed as previously described (16); specific contents of cytochromes P-450 (nmol/mg protein) were 0.76 for control, 1.82 for phenobarbital-treated and 1.27 for 3-methylcholanthrene-treated rats.

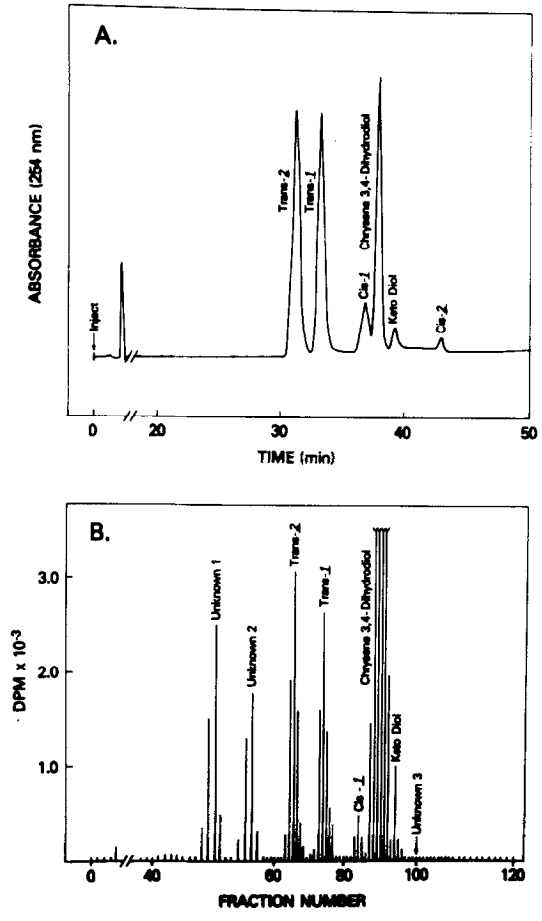

Fig. 3 Chromatographic separation of synthetic standards (A) and radioactive metabolites (B) formed by liver microsomes from 3-methylcholanthrene-treated rats.

Incubations (1.0 ml) were for 10 min at pH 7.4 and 37°C as described (4) except where noted otherwise. Microsomal protein concentration ranged from 0.5 mg to 2.0 mg protein/ml and a substrate concentration of 50 nmol/ml was used. Substrate was added in acetone such that the final concentration of acetone was 5%. Substrate and metabolites were extracted as described (4). Controls consisted of zero time incubations.

Analysis of Dihydrodiol Metabolites by HPLC: Concentrated extracts of incubations were dissolved in 100 µl of methanol, tetraol markers were added, and the samples were analyzed on a Du Pont Zorbax ODS column (0.62 mm x 25 cm) eluted with a linear gradient of 30% methanol in water to 80% methanol in water (1%/min) at a flow rate of 1.2 ml/min after an initial 1 min delay. The eluent was monitored at 254 nm, and the fractions were collected every 0.5 min over the entire chromatographic profile except in the metabolites and substrate region, where 0.3 min fractions were collected. Separation of synthetic standards and radioactive metabolites are shown in Fig. 3.

RESULTS AND DISCUSSION

Diol epoxides were found to be major metabolites of biosynthetic chrysene (3R,4R)-dihydrodiol (Table 1). Interestingly, neither phenobarbital nor 3-methylcholanthrene-treatment of rats had a significant effect on the rate at which their liver microsomes metabolized the dihydrodiol compared to microsomes from control animals. To our knowledge, this is the only example in which treatment of rats with 3-methylcholanthrene fails to enhance the rate of metabolism of a polycyclic hydrocarbon. Microsomes from control and phenobarbital-treated animals produced nearly identical profiles of metabolites. Total diol epoxides (sum of tetraols and keto diol) accounted for 83 to 90% of the metabolites. Based on individual tetraols and keto diol, more than twice as much diol epoxide-1 was formed compared to diol epoxide-2. Three unidentified metabolites accounted for the balance of the products. With microsomes from 3-methylcholanthrene-treated rats, only 66% of the total metabolites could could be accounted for as diol epoxides, and the diol epoxide-1 isomer was only slightly favored. Attempted mass spectral analysis of unknowns 1 and 2 failed to establish their molecular weights. The UV spectra of unknowns 1 and 2 (λ_{max} = 276) were quite similar but different from unknown 3 (λ_{max} = 255). Although the spectra of 1 and 2 change on addition of alkali, the change was not deemed sufficient to conclude that they were phenolic dihydrodiols. Unknown 3 was formed mainly by liver microsomes from phenobarbital-treated rats.

Incubations of racemic, unlabeled chrysene 3,4-dihydrodiol had been done in order to obtain sufficient tetraols and keto diol such that their structures could be confirmed by mass spectral and UV analysis. These studies also indicated that products from diol epoxide-1 relative to -2 were favored by 3 to 1 with microsomes from 3-methylcholanthrene-treated rats. Thus, much more diol epoxide-1 relative to -2 is formed from the (+)-dihydrodiol, while similar amounts of each diol epoxide are formed from the (-)-dihydrodiol. Circular dichroism studies have established that the (-)-dihydrodiol has (3R,4R)-

Table 1. Metabolism of biosynthetic [^{3}H]-chrysene (3R,4R)-dihydrodiol by rat liver microsomes[a]

Microsomes (mg protein/ml)	Individual Metabolites as % of Total Metabolites									% Conversion[b]	% Recovery[c]
	Trans-1	Cis-1	Keto Diol	Trans-2	Total Diol Epoxide -1	Total Diol Epoxide -2	Unknowns 1	Unknowns 2	Unknowns 3		
Control											
(1.0)	41.6	0.0	20.9	27.3	62.5	27.3	2.6	7.7	0.0	11.2 (0.74)	86
(2.0)	51.0	0.0	9.5	26.0	60.5	26.0	2.7	6.4	4.3	17.6 (0.58)	90
Phenobarbital											
(1.0)	54.8	2.5	4.2	22.2	61.2	22.2	1.8	4.1	10.3	22.1 (0.61)	93
(2.0)	64.4	1.7	3.8	18.6	69.9	18.6	1.4	3.3	6.8	33.4 (0.46)	92
3-Methylcholanthrene											
(0.5)	22.9	3.0	12.1	28.0	38.0	28.0	19.8	14.1	0.0	8.2[d] (0.65)	73
(1.0)	26.7	4.2	6.3	28.9	37.2	28.9	19.4	14.4	0.0	13.3 (0.52)	81

[a] Incubation conditions are described in MATERIALS AND METHODS.

[b] Percent conversion indicates total radioactivity above blank which emerges from the column before and after the substrate. Numbers in parentheses indicate nmol of dihydrodiol metabolized per nmol of cytochrome P-450 per min.

[c] Recovery represents the percent of the total radioactivity due to metabolism emerging from the column before and after the substrate in discrete metabolite peaks compared to total radioactivity due to metabolism.

[d] Although no induction by 3-methycholanthrene was observed compared to microsomes from control rats, these same microsomes were 30-fold more active toward another polycyclic aromatic hydrocarbon substrate.

absolute stereochemistry. Although previous studies (2,4) of the metabolism of (R,R)-dihydrodiols to bay-region diol epoxides had shown marked preferences for the diol epoxide-2 diastereomer, such is not the case for the (-)-(3R,4R)-dihydrodiol of chrysene.

Nonaromatic double bonds of benzo-ring dihydrodiols should be prime sites of oxidation by cytochrome P-450 from a purely thermodynamic standpoint. When we observed that this was not the case for the quasi-diaxial 9,10-dihydrodiols of benzo[a]pyrene and benzo[e]pyrene, we had speculated that the increased bulk and polarity of the diaxial hydroxyl groups in these two diols retarded metabolism of the proximate double bond. These novel results on extensive diol epoxide formation from the quasi-diaxial 3,4-dihydrodiol of chrysene indicate that further studies will be required to predict the regiospecificity of the cytochromes P-450 toward bay-region dihydrodiols.

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