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Stereoselective Formations of K-Region and Non-K-Region Epoxides in the Metabolism of Chrysene by Rat Liver Microsomal Cytochrome P-450 Isozymes

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SUMMARY

The K-region 5,6-epoxide and non-K-region 1,2- and 3,4-epoxides of chrysene were isolated by normal phase high performance liquid chromatography (HPLC) from a mixture of products formed in the metabolism of chrysene by liver microsomes from untreated (control), phenobarbital-treated, or 3-methylcholanthrene-treated rats in the presence of an epoxide hydrolase inhibitor, 3,3,3-trichloropropylene 1,2-oxide. Epoxides were characterized by ultraviolet, mass, and circular dichroism spectral and chiral stationary phase HPLC analyses. Each of the metabolically formed epoxides was hydrated by rat liver microsomal epoxide hydrolase to a *trans*-dihydrodiol. The metabolically formed chrysene 5,6-epoxides were determined by chiral stationary phase HPLC and were found to contain (5*S*,6*R*):(5*R*,6*S*) enantiomer ratios of 68:32 (control), 71:29 (phenobarbital), and 5:95 (3-methylcholanthrene), respectively. The enantiomers of chrysene 1,2-epoxide and 3,4-epoxide were also resolved by chiral stationary phase HPLC. However, the enantiomeric compositions of the metabolically formed chrysene 1,2- and 3,4-epoxides, which racemized rapidly at room temperature, could not be directly determined. By using molecular oxygen-18 in the *in vitro* incubation of chrysene and by mass spectral analyses of the resulting oxygen-18-containing dihydrodiol metabolites and their acid-catalyzed dehydration (phenolic) products, both 1,2-epoxide and 3,4-epoxide were found to be converted by microsomal epoxide hydrolase-catalyzed water attack at predominantly (≥97%) the allylic carbons.

Epoxides (arene oxides) are the initial products formed in the metabolism of PAHs by cytochrome P-450 isozymes of drug-metabolizing enzyme systems and are converted to *trans*-dihydrodiols by microsomal epoxide hydrolase (1, 2). Some non-K-region *trans*-dihydrodiols are further metabolized by cytochrome P-450 isozymes to vicinal dihydrodiol-epoxides, some of which are the ultimate carcinogenic metabolites of the parent hydrocarbons (3, 4).

Due to stereoheterotopic interactions between the substrate and the cytochrome P-450 isozymes, some epoxides formed in the metabolism of PAHs are optically active. The optically active epoxide intermediates can be demonstrated by deduction via the formation of optically active dihydrodiols or glutathione conjugates (5, 6), by hydration of enantiomeric epoxides to dihydrodiols (7, 8), or by directly resolving the metabolically formed epoxides by CSP-HPLC (7–10). The latter method was made possible due to two analytical methods recently developed in our laboratory: (i) direct resolution of enantiomeric epoxides of a variety of PAH structures (7) and (ii) stabilization and normal phase HPLC isolation of metabolically formed K-region and non-K-region epoxide intermediates (8–10). Microsomal epoxide hydrolase-catalyzed hydration of metabolically formed epoxides can be blocked by using an epoxide hydrolase inhibitor such as TCPO. If the metabolically formed epoxides are sufficiently stable or can be stabilized by using an organic base such as triethylamine (9), subsequent analyses by CSP-HPLC will reveal the extent of stereoheterotopic interaction between the cytochrome P-450 isozyme and a particular double bond of the parent PAH substrate (7–10). This report describes the elucidation of the mechanisms of epoxidation reactions catalyzed by rat liver cytochrome P-450 isozymes at the K- and non-K regions of CR. The effects of prior treatments of rats with enzyme inducers PB and MC reveal some unique aspects of CR metabolism not commonly known in the metabolism of other PAHs.

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ABBREVIATIONS: PAH, polycyclic aromatic hydrocarbon; CR, chrysene; CR 1,2-epoxide, 1,2-epoxy-1,2-dihydrochrysene; CR 1,2-dihydrodiol, trans-1,2-dihydroxy-1,2-dihydrochrysene; other epoxides and dihydrodiols are similarly abbreviated; MC, 3-methylcholanthrene; PB, phenobarbital; BA, benz[a]anthracene; BaP, benzo[a]pyrene; TCPO, 3,3,3-trichloropropylene 1,2-oxide; (*R*)-DNBPG, (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine; HPLC, high performance liquid chromatography; CSP, chiral stationary phase.

Experimental Procedures

Materials. CR, triethylamine, TCPO, and 4-hydroxy-1,2,3,4-tetrahydrophenanthrene were purchased from Aldrich Chemical Co. (Milwaukee, WI). CR was purified by recrystallization from methanol. Racemic 5,6-epoxide and 1,2-, 3,4-, and 5,6-dihydrodiols of CR and five monohydroxychrysenes (1-, 2-, 3-, 4-, and 6-) were obtained from the Chemical Repository of the National Cancer Institute. $NADP^+$, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase (type XII) were obtained from Sigma Chemical Co. (St. Louis, MO). HPLC grade solvents were purchased from Mallinckrodt, Inc. (Paris, KY).

Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) weighing 100–120 g were treated intraperitoneally with PB (75 mg/kg body weight, injected in 0.5 ml of water) once daily on each of three consecutive days, or with MC (25 mg/kg body weight, injected in 0.5 ml of corn oil) once daily for each of four consecutive days. The rats were sacrificed the next day after the last injection of the drug, liver microsomes were prepared, and the microsomal protein was determined as previously described (9). Cytochrome P-450 contents of liver microsomes were determined by the method of Omura and Sato (11), using an extinction coefficient of 91 mM^{-1} cm^{-1}. Cytochrome P-450 contents of liver microsomes from untreated, PB-treated, and MC-treated rats were 0.543, 1.12, and 1.35 nmol/mg of protein, respectively.

Dihydrodiols (in 0.3 ml of acetone) were converted to phenolic products by treatment with 0.15 ml of concentrated HCl at 37° for 1 hr. Ethyl acetate (5 ml) was added and the resulting solution was washed three times with water (3 ml) to remove the acid. Ethyl acetate was removed by evaporation and the residue was dissolved in methanol for reverse phase HPLC analysis.

High performance liquid chromatography. HPLC was performed on one of the following two systems. (i) a Waters Associates (Milford, MA) liquid chromatograph consisting of a model 6000A solvent delivery system, a model M45 solvent delivery system, a model 660 solvent programmer, and a model 440 absorbance (254 or 280 nm) detector. Samples were injected via a Valco model N60 loop injector (Valco Instruments, Houston, TX). Retention times and ratios of chromatographic peaks, determined by areas under the chromatographic peaks, were recorded with a Hewlett-Packard model 3390A integrator. (ii) A Hewlett-Packard model HP 1090A liquid chromatograph equipped with an HP 85B personal computer, an HP model 1040 diodearray detector, a ternary solvent delivery system, and an automatic sampler/injector.

Reverse phase HPLC. For reverse phase operation, the Waters Associates liquid chromatograph was used. Dihydrodiol and phenolic metabolites, obtained from incubation of CR with rat liver microsomes, were separated on a DuPont Zorbax ODS (monomeric C_{18}) column (4.6 mm i.d. × 25 cm). The column was eluted with a 30-min linear gradient from methanol/water (3:2, v/v) to methanol at 1.2 ml/min. Phenolic derivatives of chrysene were further separated on a Vydac polymeric C_{18} column (201TP54.6, 5-μm particles, 4.6 mm i.d. × 25 cm; The Separations Group, Hesperia, CA) as described previously (12).

Normal phase HPLC. In the normal phase operation, the Hewlett-Packard liquid chromatograph was used. Epoxides and phenolic metabolites formed in the metabolism of CR by rat liver microsomes in the presence of TCPO were analyzed by using a DuPont Zorbax SIL column (6.2 mm i.d. × 25 cm). The column was eluted with ethyl acetate/hexane (1:4, v/v) containing 0.25% (v/v) triethylamine for 10 min, followed by a 10-min linear gradient to ethyl acetate at 2 ml/min.

Chiral stationary phase HPLC. The enantiomers of CR 1,2-, 3,4-, and 5,6-epoxides were separated on the Waters Associates liquid chromatograph using an analytical HPLC column (4.6 mm i.d. × 25 cm; Regis Chemical Co., Morton Grove, IL) packed with spherical 5-μm-diameter particles of γ-aminopropylsilanized silica to which (*R*)-DNBPG was ionically bonded. The elution solvent was 1–1.25% (v/v) of ethanol/acetonitrile (2:1, v/v) in hexane, and the flow rate was 2 ml/min. Enantiomers of dihydrodiols were resolved as previously described (13).

Preparation of biosynthetic epoxides. Enzymatically formed K- and non-K-region epoxides of CR were isolated from a mixture of products formed by incubation of CR with liver microsomes prepared from untreated, PB-treated, and MC-treated rats, respectively. A 100-ml reaction mixture contained 100 mg protein equivalent of rat liver microsomes, 5 mmol of Tris-HCl (pH 7.5), 0.3 mmol of $MgCl_2$, 10 units of glucose-6-phosphate dehydrogenase (type XII, Sigma), 10 mg of $NADP^+$, 48 mg of glucose 6-phosphate, and 0.06 mmol of the microsomal epoxide hydrolase inhibitor, TCPO. This reaction mixture was preincubated at 37° for 2 min in a water shaker bath. CR (8 μmol in 4 ml of acetone) was then added and incubated for 30 min. Residual CR and its metabolites were extracted by sequential additions of 100 ml of acetone/triethylamine (125:1, v/v) and 200 ml of ethyl acetate/triethylamine (250:1, v/v). The resulting aqueous phase was extracted with an additional 200 ml of ethyl acetate/triethylamine (250:1, v/v). The organic solvent extracts were combined and dehydrated with anhydrous $MgSO_4$, filtered, and evaporated to dryness under reduced pressure. The epoxide-containing residue was redissolved in ethyl acetate/hexane/triethylamine (20:79.7:0.3, volume ratio) for normal phase HPLC isolation of epoxides. To estimate the relative amounts of CR epoxides and phenols formed by different rat liver microsomal preparations, an internal standard (4-hydroxy-1,2,3,4-tetrahydrophenanthrene, 50 nmol in 0.1 ml of acetone) was added following the addition of acetone/triethylamine to the reaction mixture.

Enzymatic hydration of CR 1,2- and 3,4-epoxides. Biosynthetic 1,2- and 3,4-epoxides of CR, isolated by normal phase HPLC as described above, were each dissolved in acetone and were converted to *trans*-dihydrodiols by incubation with liver microsomes from PB-treated rats in the absence of NADPH. The reaction mixture (10 ml), which contained 0.05 M Tris-HCl (pH 7.5) and 10 mg protein equivalent of rat liver microsomes per ml of incubation mixture, was incubated at 37° for 30 min in a water shaker bath. Hydration products were extracted with 10 ml of acetone and 20 ml of ethyl acetate. The resulting organic phase was dehydrated with anhydrous $MgSO_4$, filtered, and evaporated to dryness under reduced pressure. The dihydrodiol products were purified by reverse phase HPLC on a DuPont Zorbax ODS column as described above. Purified dihydrodiols were dried and redissolved in methanol for CD spectral measurements.

Preparation of oxygen-18-containing CR *trans*-dihydrodiols. CR was incubated under molecular oxygen-18 (98 atom %; KOR Isotopes, Cambridge, MA) by liver microsomes from MC-treated rats by a method similar to the one described (14). The incubation was carried out under ~0.1 atm of $^{18}O_2$ in the absence of N_2. The ^{18}O-containing dihydrodiol metabolites were purified on a DuPont Zorbax ODS column as described above.

Spectral analysis. Mass spectral analysis was performed on a Finnigan model 4000 gas chromatograph-mass spectrometer-data system by electron impact with a solid probe at 70 eV and 250° ionizer temperature. Ultraviolet-visible absorption spectra of samples were determined using a 1-cm path length quartz cuvette with a Varian model Cary 118C spectrophotometer. CD spectra of samples in a quartz cell of 1 cm path length at room temperature were measured using a Jasco model 500A spectropolarimeter equipped with a model DP-500 data processor (13).

Results

Isolation and characterization of metabolically formed epoxides. The epoxides and phenols, formed in the metabolism of CR by liver microsomes from PB-treated rats in the presence of TCPO, were separated by normal phase HPLC (Fig. 1). The identified metabolites are: CR (substrate, peak *1*), CR 5,6-epoxide (peak *2*), CR 3,4-epoxide (peak *3*), CR 1,2-epoxide (peak *4*), 4-OH-CR (peak *6*), 1-OH-CR (peak *7*), 6-OH-CR (peak *8*), 2-OH-CR (peak *9*, a front shoulder of peak *10*), and 3-OH-CR (peak *10*). Peak 5 is the internal standard (4-hydroxy-1,2,3,4-tetrahydrophenanthrene) added for determining the relative amounts of the various metabolites formed by

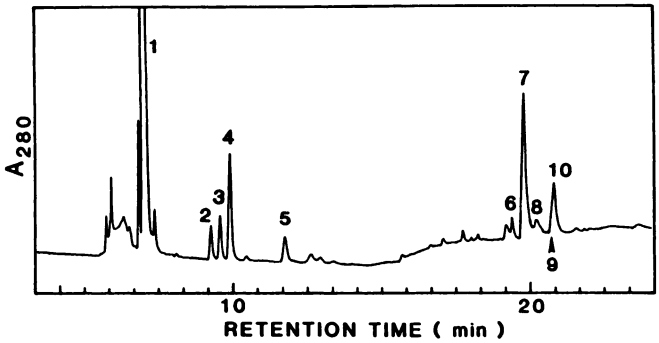

Fig. 1. Normal phase HPLC separation of epoxide and phenol metabolites formed in the metabolism of CR by liver microsomes from PB-treated rats in the presence of TCPO. Chromatography was performed on a Hewlett-Packard model 1090A liquid chromatograph (see Experimental Procedures for chromatographic conditions). Identities of chromatographic peaks are: *1*, CR; *2*, CR 5,6-epoxide; *3*, CR 3,4-epoxide; *4*, CR 1,2-epoxide; *5*, internal standard (4-hydroxy-1,2,3,4-tetrahydrophenanthrene); *6*, 4-OH-CR; *7*, 1-OH-CR; *8*, 6-OH-CR; *9*, 2-OH-CR (front shoulder of peak *10*); *10*, 3-OH-CR. No metabolite was eluted with the same retention time as the internal standard.

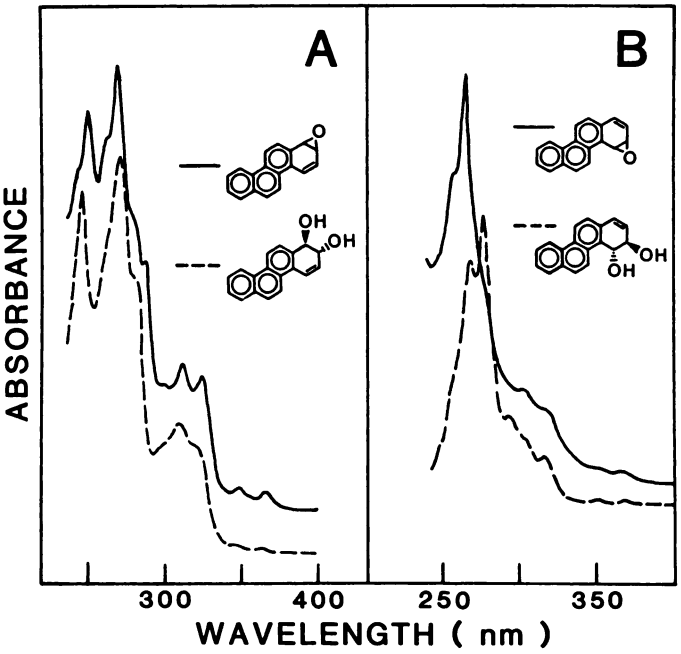

Fig. 2. Ultraviolet absorption spectra of CR 1,2-epoxide (—, A; a metabolite in hexane), CR 1,2-dihydrodiol (– – –, A; a synthetic standard in methanol), CR 3,4-epoxide (—, B; a metabolite in hexane), and CR 3,4-dihydrodiol (– – –, B; a synthetic standard in methanol).

different rat liver microsomal preparations. Retention times of most of the identified metabolites are each identical to those of authentic standards available for this study.

The metabolite contained in peak 2 has identical retention time on normal phase HPLC and ultraviolet absorption and mass spectra to those of the synthetic CR 5,6-epoxide. The metabolites contained in chromatographic peaks 3 and 4 of Fig. 1 are identified as CR 3,4-epoxide and CR 1,2-epoxide, respectively, on the basis of the following evidence: (i) the ultraviolet absorption characteristics of the epoxide metabolites are similar (except for shifts in absorption maxima) to those of CR 3,4-dihydrodiol and CR 1,2-dihydrodiol, respectively (Fig. 2), indicating that the epoxide metabolites have a saturated double bond at 3,4- and 1,2-positions, respectively; (ii) both epoxide metabolites have molecular ions at m/z 244 by mass spectral analyses; and (iii) the epoxide metabolites are hydrated by microsomal epoxide hydrolase to 3,4-dihydrodiol and 1,2-dihydrodiol, respectively. It is apparent, from the results shown in Fig. 1, that a considerable portion of each metabolically formed epoxide was isomerized to phenolic products.

Effects of enzyme induction. The relative amounts of epoxide and phenolic metabolites formed in the metabolism of CR by three rat liver microsomal preparations in the presence of TCPO were studied using an internal standard for chromatography. An identical amount of the internal standard was added to each ml of incubation mixture. When the areas under the chromatographic peaks are normalized against that of the internal standard, the relative amounts of metabolites formed by various enzyme preparations can be compared. The highest amounts of K- and non-K-region epoxides were formed by liver microsomes from PB-treated rats (Table 1). Although liver microsomes from MC-treated rats have the highest activity in catalyzing the metabolism of CR (15), a smaller portion of the metabolically formed epoxides survived the experimental conditions for incubation, extraction, and normal phase HPLC analyses. Regardless of which rat liver microsomes were used, most of the metabolically formed epoxides were isomerized nonenzymatically to phenolic products (Table 1). The results of Table 1 were obtained by four to six separate determinations. In the absence of the epoxide hydrolase inhibitor, phenolic products constitute 13–17% of all the products formed in the metabolism of CR by liver microsomes from untreated, PB-treated, and MC-treated rats (15).

Optical properties of metabolically formed epoxides. The enantiomeric compositions of CR 5,6-epoxides (Table 2) formed by liver microsomes from untreated, PB-treated, and MC-treated rats, isolated as shown in Fig. 1, were determined by CSP-HPLC as previously described (7, 13). The (5*R*,6*S*):(5*S*,6*R*) enantiomer ratios were 32:68 (control), 27:73 (PB), and 95:5 (MC), respectively (Fig. 3). Rechromatography of each enantiomeric 5,6-epoxide by CSP-HPLC resulted in a single chromatographic peak, indicating that the enantiomeric CR 5,6-epoxides do not undergo racemization. Elucidation of the absolute configurations of enantiomeric CR 5,6-epoxides was recently reported (13).

Purified CR 1,2-epoxide and CR 3,4-epoxide, when stored in hexane containing 0.4% triethylamine at 4°, were stable as epoxides for at least 36 days. The enantiomers of metabolically formed CR 1,2-epoxide and CR 3,4-epoxide were resolved by CSP-HPLC (Fig. 4). However, when each of the resolved enantiomers of either CR-1,2-epoxide or CR 3,4-epoxide was rechromatographed, a pair of chromatographic peaks with nearly equal areas under the chromatographic peaks was observed. Identical results were obtained when each of the resolved enantiomers was rechromatographed immediately (within 5 min) following its isolation. It is thus apparent that the enantiomers of CR 1,2-epoxide and of CR 3,4-epoxide racemize spontaneously with a half-life of less than 10 min at room temperature in the solvent [1–1.25% of ethanol/acetonitrile (2:1, v/v) in hexane] employed for chromatography. Interestingly, each resolved enantiomer does not seem to racemize while it is still in the column; perhaps it is stabilized due to the formation of a diastereomeric complex with the CSP. A single and broad peak without enantiomeric resolution would be expected if the resolved enantiomers were spontaneously race-

TABLE 1

Effects of enzyme induction on the metabolism of CR by rat liver microsomes in the presence of TCPO

Peak no.[a]	Identity of metabolite	Area ratio[b] Control	PB	MC
2	CR 5,6-epoxide	0.17 ± 0.03 (1.2) 0.31 ± 0.05 (3.1)	0.66 ± 0.28 (4.7) 0.59 ± 0.25 (5.9)	0.14 ± 0.03 (1.0) 0.10 ± 0.02 (1.0)
3	CR 3,4-epoxide	0.68 ± 0.15 (4.5) 1.25 ± 0.28 (11.4)	0.96 ± 0.38 (6.4) 0.86 ± 0.34 (7.8)	0.15 ± 0.04 (1.0) 0.11 ± 0.03 (1.0)
4	CR 1,2-epoxide	1.28 ± 0.19 (9.8) 2.36 ± 0.35 (23.6)	3.16 ± 0.03 (24.3) 2.82 ± 0.03 (28.2)	0.13 ± 0.01 (1.0) 0.10 ± 0.01 (1.0)
6	4-OH-CR	1.36 ± 0.04 (0.42) 2.50 ± 0.07 (1.0)	0.66 ± 0.15 (0.20) 0.59 ± 0.13 (0.25)	3.24 ± 0.22 (1.0) 2.40 ± 0.16 (1.0)
7	1-OH-CR	4.74 ± 0.16 (0.25) 8.73 ± 0.29 (1.0)	6.51 ± 0.21 (0.34) 5.81 ± 0.19 (0.41)	19.0 ± 1.49 (1.0) 14.1 ± 1.10 (1.0)
8	6-OH-CR	0.56 ± 0.05 (1.8) 1.03 ± 0.09 (4.3)	1.05 ± 0.07 (3.3) 0.94 ± 0.06 (3.9)	0.32 ± 0.03 (1.0) 0.24 ± 0.02 (1.0)
9 & 10	2-OH-CR & 3-OH-CR[c]	3.37 ± 0.18 (0.71) 6.21 ± 0.33 (1.8)	2.49 ± 0.16 (0.52) 2.22 ± 0.14 (0.63)	4.74 ± 0.31 (1.0) 3.51 ± 0.23 (1.0)

[a] Chromatographic peak numbers are as shown in Fig. 1.

[b] Area ratios of metabolite peak and internal standard (4-hydroxy-1,2,3,4-tetrahydrophenanthrene, peak 5 in Fig. 1) were detected at 280 nm. All reaction mixtures had 1 mg protein equivalent of liver microsomes per ml of incubation mixture and an identical amount of internal standard was added after the reaction had been stopped by the addition of acetone. The relative amounts of various metabolites formed (shown in parentheses) were calculated both on a per mg protein basis (numbers in first row) and on a per nmol P-450 basis (numbers in second row) and were normalized against those formed by liver microsomes from MC-treated rats. Values are averages of four to six separate determinations.

[c] 3-OH-CR is the major component (70–90% of total areas under peaks 9 and 10).

TABLE 2

Enantiomeric compositions of dihydrodiols and 5,6-epoxide formed in the metabolism of CR by rat liver microsomes

Enzyme inducer	Enantiomeric composition (%) 1,2-Dihydrodiol		3,4-Dihydrodiol		5,6-Dihydrodiol		5,6-Epoxide[a]	
	1S, 2S	*1R, 2R*	*3S, 4S*	*3R, 4R*	*5S, 6S*	*5R, 6R*	*5R, 6S*	*5S, 6R*
Control[b]	49	51	1	99	14	86	32	68
PB[b]	59	41	1	99	13	87	27	73
MC[b]	4	96	1	99	8	92	95	5
MC[c]	14	86	8	92				

[a] Epoxides formed by rat liver microsomes in the presence of TCPO.

[b] These results are taken from an earlier report (13) for ready reference and comparison.

[c] Incubation was carried out under ~0.1 atm of $^{18}O_2$ (see Experimental Procedures).

mized on the CSP column. Because of spontaneous racemization of the enantiomeric CR 1,2-epoxide and CR 3,4-epoxide, it is not possible to determine the enantiomeric compositions of the metabolically formed non-K-region epoxides of CR.

^{18}O incorporation experiments. The dihydrodiol and phenolic products formed in the metabolism of CR by liver microsomes from MC-treated rats under $^{18}O_2$ were isolated by reverse phase HPLC using a DuPont Zorbax monomeric ODS column; the elution pattern was similar to that reported by Nordqvist *et al.* (15). The phenolic metabolites were further separated on a Vydac polymeric C_{18} column (12). Mass spectral analyses of phenolic metabolites indicated an ^{18}O incorporation of ~96% (Table 3). Under the experimental conditions, the metabolically formed 1,2- and 3,4-dihydrodiols had (*S,S*):(*R,R*) enantiomer ratios of 14:86 and 8:92, respectively. The enantiomeric ratios were 4:96 and 1:99, respectively, when incubation was carried out under atmospheric oxygen (13, 15). Acid-catalyzed dehydration of the ^{18}O-containing 1,2-dihydrodiol gave 1-OH-CR (~66%) and 2-OH-CR (~34%). Approximately 97% of the ^{18}O label was associated with 1-OH-CR, indicating that the epoxide hydrolase-catalyzed water attack occurred predominantly, if not exclusively, at the allylic C_2 positions of both 1,2-epoxide

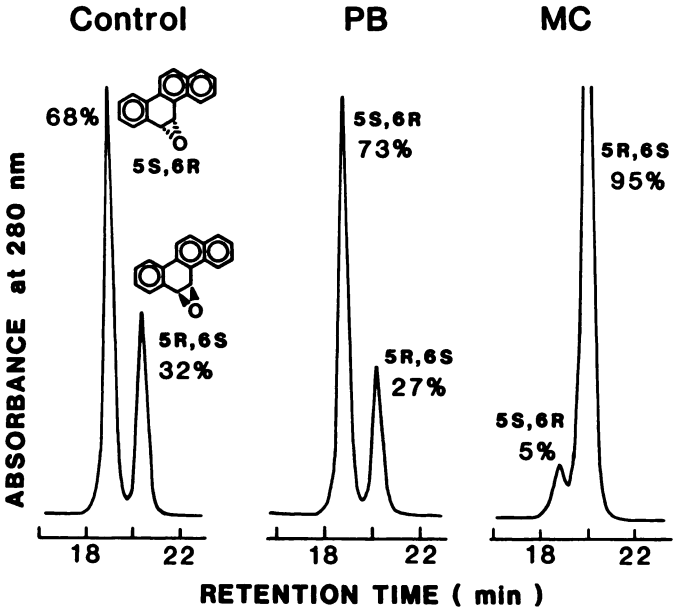

Fig. 3. CSP-HPLC analyses of CR 5,6-epoxides formed in the metabolism of CR by liver microsomes from untreated, PB-treated, and MC-treated rats, respectively. An ionically bonded (*R*)-DNBPG column was used and the elution solvent was 1.25% (v/v) of ethanol/acetonitrile (2:1, v/v) in hexane at 2 ml/min.

enantiomers. Acid-catalyzed dehydration of the ^{18}O-containing 3,4-dihydrodiol gave 3-OH-CR (~59%) and 4-OH-CR (~41%). Greater than 98% of the ^{18}O label was associated with 4-OH-CR (Table 3), indicating that the epoxide hydrolase-catalyzed water attack occurred predominantly, if not exclusively, at the allylic C_3 positions of both 3,4-epoxide enantiomers.

Discussion

The relative amounts of non-K-region dihydrodiols formed, on a per nmol P-450 basis, in the metabolism of CR by liver microsomes from untreated and treated rats of the Long-Evans

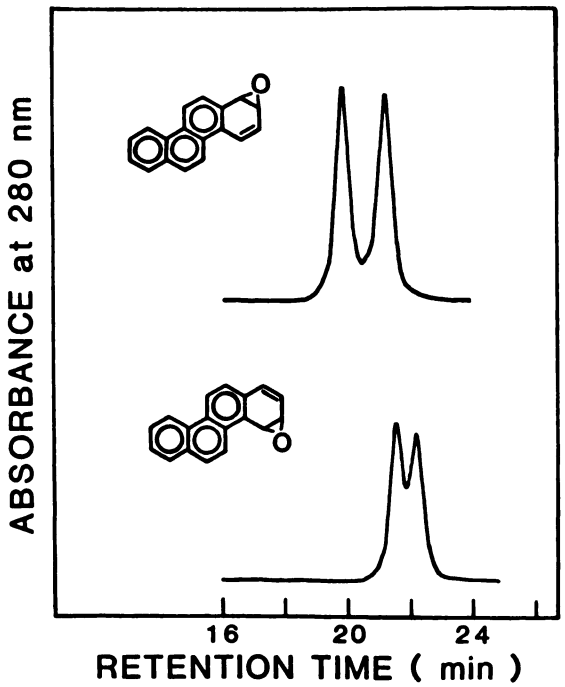

Fig. 4. CSP-HPLC separation of the enantiomers of CR 1,2-epoxide and of CR 3,4-epoxide. An ionically bonded (*R*)-DNBPG column was used. Elution solvent was 1.25% (for CR 1,2-epoxide) and 1% (for CR 3,4-epoxide) of ethanol/acetonitrile (2:1, v/v) in hexane at 2 ml/min.

strain are: 1,2-dihydrodiol, MC (7.6) > control (2.6) > PB (1.0) and 3,4-dihydrodiol, MC (13.3) > control (2.4) > PB (1.0) (15). Similar results were obtained when rats of the Sprague-Dawley strain were used (data not shown). As shown in Table 1, the relative amounts of non-K-region epoxides formed in the metabolism of CR in the presence of TCPO by liver microsomes from untreated and treated rats of the Sprague-Dawley strain are: 1,2-epoxide, PB (28.2) > control (23.6) > MC (1.0) and 3,4-epoxide, control (11.4) > PB (7.8) > MC (1.0).

In a reconstituted enzyme system containing highly purified cytochrome P-450c, phenolic metabolites constitute 68% of all the products formed in the metabolism of CR (15). The percentage of phenolic metabolites is reduced to 4% when epoxide hydrolase is included in the incubation mixture (15). It is thus apparent that phenolic metabolites are predominantly (>94%) derived from isomerization of metabolically formed epoxide intermediates. However, the possibility that a small portion (<6%) of the phenolic metabolites was derived from direct hydroxylation of CR could not be excluded. The results in Table 1 indicate that a large percentage of the metabolically formed non-K-region epoxides are spontaneously rearranged to phenolic products; the relative amounts of epoxides detected in our experiments do not reflect the relative amounts of epoxides actually formed metabolically. When phenols are included for comparisons, the relative extent of epoxidation at the 1,2-double bond, estimated from the sum of 1-OH-CR, 2-OH-CR, and 1,2-epoxide, is MC > control > PB, which is the same relative order as that found when TCPO was not added to the incubation mixture. The extent of epoxidation at the 3,4-double bond, estimated from the sum of 3-OH-CR, 4-OH-CR, and 3,4-epoxide, is also higher for liver microsomes from MC-treated rats than the other two rat liver microsomal preparations (Table 1). The relative extent of K-region epoxidation by different rat liver microsomal preparations is PB (5.9) > control (3.1) > MC (1.0), with or without inclusion of 6-OH-CR (Table 1). This differs from the relative amounts of 5,6-dihydrodiol formed (MC >> PB ~ control) in the metabolism of CR by liver microsomes prepared from rats of either the Long-Evans strain (15) or the Sprague-Dawley strain.

Dihydrodiols were not detected in the product mixture formed by incubation of CR with any one of the three rat liver microsomal preparations in the presence of TCPO. Hence the microsomal epoxide hydrolase was completely inhibited by the concentration of TCPO used. A metabolically formed epoxide should have similar stability in all incubation mixtures containing different rat liver microsomal preparations. One also expects that a similar percentage of a metabolically formed epoxide is converted to phenols, regardless of which rat liver microsomal preparation is used. However, this is not what was observed (Table 1); a considerably higher proportion of the metabolically formed non-K-region epoxides was rearranged to phenols when liver microsomes from MC-treated rats were used (Table 1). The exact reason(s) for this observation is (are) not known. Apparently TCPO not only inhibits the microsomal epoxide hydrolase, but also hastens the rearrangement (isom-

TABLE 3

Mass spectral analyses of phenolic and dihydrodiol metabolites formed in the metabolism of CR under $^{18}O_2$ and the acid-catalyzed dehydration products of the ^{18}O-containing dihydrodiol metabolites[a]

Metabolite[b] or dehydration product	*m/z* of major M^+	Isotope content (%)[c]	
		^{16}O	^{18}O
CR 1,2-dihydrodiol[d]	264	9.3 (5.7)	90.7 (94.3)[e]
1-OH-CR (~66%)[f] from CR 1,2-dihydrodiol	246	7.0 (4.3)	93.0 (96.7)
2-OH-CR (~34%)[f] from CR 1,2-dihydrodiol	244	96.8 (96.7)	3.2 (3.3)
CR 3,4-dihydrodiol[g]	264	11.0 (7.5)	89.0 (92.5)[e]
3-OH-CR (~59%)[f] from CR 3,4-dihydrodiol	244	99.7 (99.7)	0.3 (0.3)
4-OH-CR (~41%)[f] from CR 3,4-dihydrodiol	246	5.5 (3.8)	94.5 (98.2)
1-OH-CR	246	5.2 (1.5)	94.8 (98.5)
3-OH-CR	246	4.2 (0.4)	95.8 (99.6)
4-OH-CR	246	3.8 (0.0)	96.2 (100.0)

[a] Liver microsomes from MC-treated rats were used.
[b] The amount of CR 5,6-dihydrodiol was insufficient for analysis.
[c] The isotope content is calculated by comparing the mass fragmentation pattern of each ^{18}O-containing compound with that of the unlabeled compound. The isotope contents shown in parentheses are normalized values by using 96.2 atom % as the extent of ^{18}O incorporation as 100%.
[d] This dihydrodiol has a (1*R*, 2*R*):(1*S*, 2*S*) enantiomer ratio of 86:14, determined both by CSP-HPLC and CD spectral data (13).
[e] Estimated minimal ^{18}O contents due to background ions in mass spectral analyses.
[f] Estimated by areas under the chromatographic peaks, detected at 280 nm, by reverse phase HPLC analysis using a Vydac C_{18} column (12).
[g] This dihydrodiol has a (3*R*, 4*R*):(3*S*, 4*S*) enantiomer ratio of 92:8, determined both by CSP-HPLC and CD spectral data (13).

erization) of the metabolically formed epoxides in liver microsomes from MC-treated rats.

Successful isolation of metabolically formed epoxides and direct CSP-HPLC separation of epoxide enantiomers are important requisites in allowing rapid determination of the enantiomeric compositions of epoxides formed by cytochrome P-450-catalyzed stereoselective epoxidation reactions. Direct CSP-HPLC separation of dihydrodiol enantiomers also allows rapid determination of epoxide hydrolase-catalyzed hydrations of enantiomeric epoxides. The usefulness of these powerful analytical techniques has been demonstrated in the present study (Figs. 1 and 3) and in earlier reports concerning the formation and hydration of enantiomeric K-region epoxides of CR (Ref. 13 and this paper), benz[*a*]anthracene (BA) (8), benzo[*a*]pyrene (BaP) (8), and 12-methyl-BA (10).

In agreement with the theoretical predictions (16), K-region epoxides of CR (this paper), BA (8), BaP (8), 12-methyl-BA (10) do not undergo racemization. Because of their stability and non-racemizing property, the enantiomeric compositions of metabolically formed K-region epoxides can be determined by CSP-HPLC (7, 8, 10), allowing elucidations of the stereoselective epoxidation reactions catalyzed by microsomal cytochrome P-450 isozymes. These exact stereoselective epoxidation reactions at the K-regions of PAHs could not be predicted on the basis of known hydration mechanisms of enantiomeric K-region epoxides (8, 10, 13).

Non-K-region 1,2- and 3,4-epoxides of CR are also sufficiently stable to be isolated as metabolites (Fig. 1). The enantiomeric pairs of both CR 1,2- and 3,4-epoxides can also be directly resolved by CSP-HPLC (Fig. 4). However, because of their rapid and spontaneous racemization, the mechanisms of the formation and hydration of the non-K-region epoxides cannot be elucidated in the same manner as that which has been demonstrated for the K-region 5,6-epoxide of CR (Fig. 3; Ref. 13). Our observations on the racemization of CR 1,2-epoxide and 3,4-epoxide enantiomers are consistent with the results reported by Boyd and co-workers (16–18), who observed the spontaneous racemization of synthetically prepared 1*R*,2*S*-epoxide and 3*S*,4*R*-epoxide of CR.

In order to elucidate the position of epoxide hydrolase-catalyzed water attack of the metabolically formed 1,2- and 3,4-epoxides, an ^{18}O incorporation experiment was carried out. The results (Table 3) indicate that both enantiomers of CR 1,2-epoxide and of CR 3,4-epoxide are hydrated by microsomal epoxide hydrolase-catalyzed water attack predominantly (≥97%) at the allylic carbons. Thus, CR 1*R*,2*R*-dihydrodiol and 3*R*,4*R*-dihydrodiol are formed by microsomal epoxide hydrolase-catalyzed hydrations of the precursor 1*R*,2*S*-epoxide and 3*S*,4*R*-epoxide, respectively. These results are consistent with hydration mechanisms known for other non-K-region PAH epoxides (19). Currently, naphthalene 1*S*,2*R*-epoxide is the only non-K-region epoxide known to be hydrated at both benzylic and allylic carbons to form a 1,2-dihydrodiol with a (1*R*, 2*R*):(1*S*,2*S*) enantiomer ratio of 40:60 (20).

Since CR 1*R*,2*S*-epoxide and 3*S*,4*R*-epoxide are each the predominant epoxide enantiomer and CR 1*R*,2*R*-dihydrodiol and 3*R*,4*R*-dihydrodiol are each the predominant dihydrodiol enantiomer formed in the metabolism of CR by liver microsomes from MC-treated rats, it is apparent that the metabolically formed epoxide enantiomers do not undergo racemization and are converted to dihydrodiols while they are still in the microsomal enzyme complex. If the metabolically formed epoxides can be transported out of the microsomal enzyme complex before they are enzymically hydrated, they are expected to undergo racemization, at least partially, and the subsequent epoxide hydrolase-catalyzed hydration reactions are expected to result in dihydrodiols containing a considerably lower percentage of the *R*,*R*-dihydrodiol enantiomer than what has been observed (Table 2). Epoxide hydrolase in liver microsomes from untreated, PB-treated, and MC-treated rats has essentially the same regioselective properties in the hydration of epoxide enantiomers (8, 21). The 1*S*,2*S*-dihydrodiol formed in the metabolism of CR by liver microsomes from either untreated or PB-treated rats (Table 2) must be derived from the microsomal epoxide hydrolase-catalyzed hydration of metabolically formed 1*S*,2*R*-epoxide. Cytochrome P-450c, the predominant cytochrome P-450 isozyme contained in liver microsomes from MC-treated rats (22), is therefore responsible for the high stereoselective epoxidation reactions at the 1,2- 3,4-, and 5,6-double bonds of CR. Cytochrome P-450 isozymes contained in liver microsomes from untreated and PB-treated rats are also highly stereoselective toward the 3,4-double bond, but are nearly stereochemically nonselective toward the 1,2-double bond of CR (Table 2). Cytochrome P-450c contained in liver microsomes from MC-treated rats stereoselectively catalyzes the formation of 5*R*,6*S*-epoxide, whereas preferential formation of the opposite 5*S*,6*R*-epoxide enantiomer is catalyzed by cytochrome P-450 isozymes other than cytochrome P-450c contained in liver microsomes from untreated and PB-treated rats (Table 2, Fig. 3).

A minimal boundary of a steric model for the catalytic binding site and the position of oxygenation of cytochrome P-450c (Fig. 5A) has been deduced through metabolism studies of BaP (23). The results found in this report on the stereoselective formations of 1*R*,2*S*-epoxide (Fig. 5B), 3*S*,4*R*-epoxide (Fig. 5C), and 5*R*,6*S*-epoxide (Fig. 5D) in the metabolism of CR by cytochrome P-450c contained in liver microsomes from MC-treated rats can be explained by applying the steric model proposed (23). Based on the hydration mechanism of CR 3,4-epoxide and the enantiomeric compositions of metabolically formed 3,4-dihydrodiols, the steric model (Fig. 5A) is used to see if it can be applied to account for the stereoselective formation of 3*S*,4*R*-epoxide by cytochrome P-450 isozymes other than cytochrome P-450c contained in liver microsomes from untreated and PB-treated rats. Jerina *et al.* (23) indicated that their proposed model has a "minimal boundary," implying that the size of the catalytic binding site may be expanded when new experimental evidence calls for it.

In order to account for the formation of CR 1*S*,2*R*-epoxide by cytochrome P-450 isozymes other than cytochrome P-450c contained in liver microsomes from untreated and PB-treated rats, it is necessary to orient part of the CR molecule outside of the minimal boundary proposed for cytochrome P-450c (Fig. 5E). Similarly, the 1,2,3,4-ring of CR must be situated outside of the minimal boundary in order to account for the formation of CR 5*S*,6*R*-epoxide by cytochrome P-450 isozymes contained in liver microsomes from untreated, PB-treated, and MC-treated rats (Fig. 5F). The metabolic formation of 8-methyl-BA 1*R*,2*S*-epoxide, detected as the 1*R*,2*R*-dihydrodiol (24), requires that part of the 8,9,10,11-ring of 8-methyl-BA be situated outside of the minimal boundary proposed for cytochrome P-450c (Fig. 5G). The 2,3-double bond of BaP is the

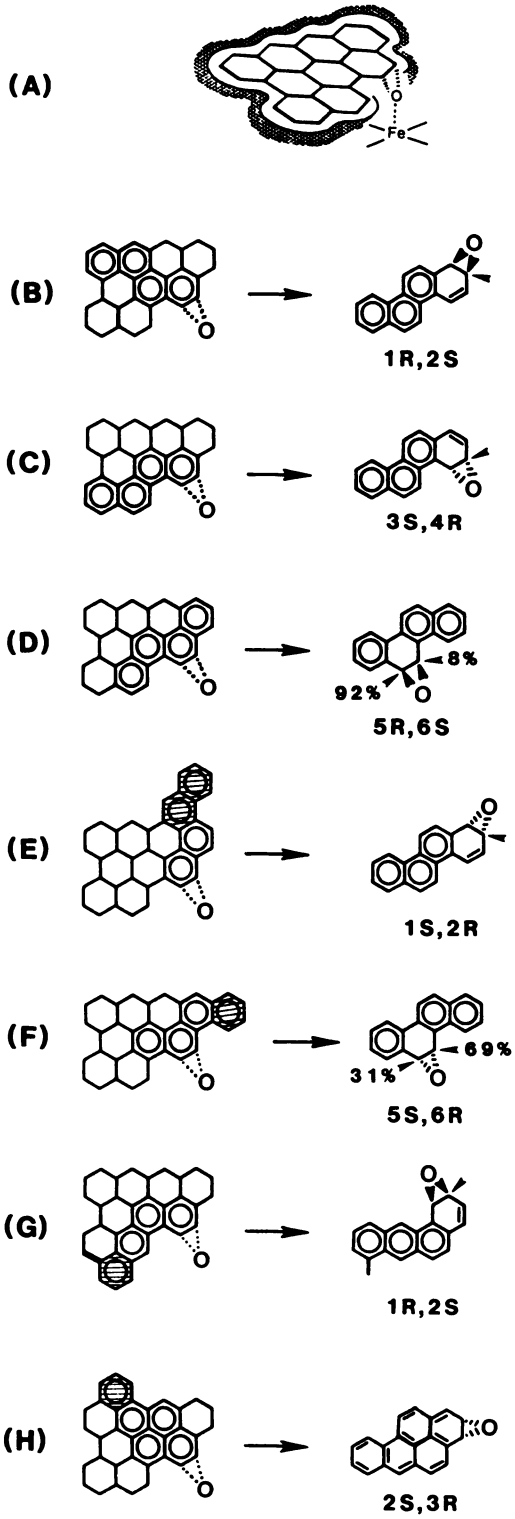

Fig. 5. The steric model for the catalytic binding site and the site of oxygenation of cytochrome P-450c proposed by Jerina *et al.* (23) (A). Modes of binding between CR and cytochrome P-450c that lead to the formation of CR 1*R*,2*S*-epoxide (B), CR 3*S*,4*R*-epoxide (C), CR 5*R*,6*S*-epoxide (D), CR 1*S*,2*R*-epoxide (E), CR 5*S*,6*R*-epoxide (F), 8-methyl-BA 1*R*,2*S*-epoxide (G), and BaP 2*S*,3*R*-epoxide (H), respectively. *Hatched areas* indicate that they are outside of the minimal boundary proposed by Jerina *et al.* (23). *Arrowheads* indicate exclusive or predominant (≥97%) site of microsomal epoxide hydrolase-catalyzed water attack in forming the dihydrodiols. Hydration mechanisms of enantiomeric CR 5,6-epoxides were reported earlier (13). BaP 2,3-epoxide undergoes a spontaneous isomerization to form 3-OH-BaP (25).

most abundant site of oxygenation, leading to the formation of 3-OH-BaP (25). Thus, a binding site model of any cytochrome P-450 isozyme should accommodate the BaP molecule and allow the 2,3-double bond to be the major site of oxygenation. As illustrated in Fig. 5H, part of the 7,8,9,10-ring of BaP has to situate outside of the catalytic binding site model to permit oxygenation at the 2,3-double bond. Although the catalytic binding site model of cytochrome P-450c was developed based on the metabolism results of BaP (23), it failed to consider the occurrence of oxygenation at the 2,3-double bond. Since the metabolically formed 2,3-epoxide (presumably the 2*S*,3*R*-enantiomer) is readily isomerized to form 3-OH-BaP (25), it is not possible to determine, by any presently known techniques, whether the cytochrome P-450-catalyzed epoxidation reaction actually takes place stereoselectively. It is thus clear that, without modification, the steric model proposed for the catalytic binding site of cytochrome P-450c (23) cannot be used to account for some metabolites known to be formed from the parent hydrocarbons, nor can it be used for cytochrome P-450 isozymes other than cytochrome P-450c. Future studies employing highly purified cytochrome P-450 isozymes and/or monoclonal antibodies that inhibit cytochrome P-450 isozyme-specific reactions may provide a clearer understanding of the stereoselective properties of cytochrome P-450 isozymes.

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