Stereochemical Aspects in the 4-Vinylcyclohexene Biotransformation with Rat Liver Microsomes and Purified P450s. Monoepoxides and Diols

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The stereochemical course of the biotransformation of 4-vinylcyclohexene (VCH, **1**) by liver microsomes from male and female control and induced rats and purified rat P450 2B1 and 2E1 has been determined. The epoxidation of **1**, catalyzed by male microsomes, occurs on both the endo- and exocyclic double bond to give four isomeric epoxides, *cis*-4-vinylcyclohexene 1,2-epoxide (**2**), *trans*-4-vinylcyclohexene 1,2-epoxide (**3**), (4*R**,7*S**)-4-vinylcyclohexene 7,8-epoxide (**4**), and (4*R**,7*R**)-4-vinylcyclohexene 7,8-epoxide (**5**). On the other hand, microsomes from female rats catalyzed primarily the endocyclic epoxidation. The stereoselectivity of this process was strongly dependent on gender and P450 induction. Only the phenobarbital and pyrazole, at lower levels, were able to enhance the epoxidation of **1** and mostly on the endocyclic double bond. Also, P450 2E1 and 2B1 in a reconstituted system were able to perform the epoxidation of **1** primarily on its endocyclic double bond. The metabolites, *cis*- and *trans*-4-vinylcyclohexene 1,2-epoxide (**2** and **3**, respectively) and the isomeric 4-vinylcyclohexene 7,8-epoxides (**4** and **5**), were rapidly biotransformed into the corresponding vicinal diols by mEH-catalyzed hydrolysis. The reaction of the endocyclic epoxides occurred with good substrate diastereo- and enantioselectivity favoring the hydrolysis of epoxides (1*S*,2*R*,4*S*)-**3** and (1*R*,2*S*,4*S*)-**2** to give, before 50% conversion, selectively (1*R*,2*R*,4*S*)-diol (**6**). At variance, the hydrolysis of the exocyclic epoxides was characterized by a high level of substrate enantioselection associated with a very low, if any, level of substrate diastereoselection, the two epoxides, (4*R*,7*S*)-**4** and (4*R*,7*R*)-**5**, being hydrolyzed practically with the same rate. On the basis of the major resistance to mEH hydrolysis, the endocyclic epoxides, (1*R*,2*S*,4*R*)-**3** and (1*S*,2*R*,4*R*)-**2**, are expected to be further oxidized, in a stereochemical manner, to the specific mutagenic diepoxides which are thought to play a crucial role in VCH ovotoxicity. Thus, VCH ovotoxicity may be markedly affected by the reactivity of the diepoxidic stereoisomers formed and detoxicated.

Introduction

4-Vinylcyclohexene (VCH, **1**) is an industrial compound used as an intermediate in rubber industry and in chemical manifactures and produced as a byproduct in butadiene processing (*1*). Exposure to this compound is of toxicological significance, as rodent studies have pointed out that its toxic effects specifically target the ovary (*2*, *3*). It is noteworthy that ovarian neoplasms, as well as follicular loss, are species dependent (*3*), they occur in B6C3F$_1$ mice but not in rats, and these variations are most likely related to differences in the biotransformation of **1** to ovotoxic monoepoxides and to the diepoxide. The latter compound, which has been characterized as a direct mutagen and carcinogen (*1*), is thought to be the ultimate ovitoxic metabolite of VCH (*2*).

4-Vinylcyclohexene (**1**) is oxidized by P450 systems to the isomeric monoepoxides 4-vinylcyclohexene 1,2-epoxide (1,2-VCHE) and 4-vinylcyclohexene 7,8-epoxide (7,8-VCHE) (Scheme 1). Both epoxides are further oxidized to the diepoxide (VCD) in competition with the hydrolysis to the corresponding vicinal diols (*4*−*6*). In mouse, VCH was found to be metabolized to 1,2-VCHE much faster than in rat both in vivo (*3*) and in vitro (*7*) and that the hydrolysis of both the monoepoxides and diepoxide is less efficient in mice than in rats (*7*). These data taken together have suggested that the resistance of the rat to ovarian tumor induction by VCH is due to the inability of the rat to produce and mantain in the blood a sufficient amount of diepoxide to cause oocyte destruction and cancerogeneity. The differences in the metabolic patterns of VCH in the two species are related to a different substrate specificity and expression of the orthologous enzymes implied and in particular to the P450 2A and 2B enzymes, indicated to be the major enzymes involved in the epoxidation(s) of VCH (*8*, *9*).

It is noteworthy that the metabolic picture related to **1** biotransformation is much more complicated than that reported in Scheme 1. The metabolism studies, carried out using **1** as a substrate, have not ever considered the stereochemistry of the products arising from oxidation and/or hydrolysis, but the stereochemical aspects in the biotransformation processes of these compounds may be important in determining their toxicity. In fact, it has

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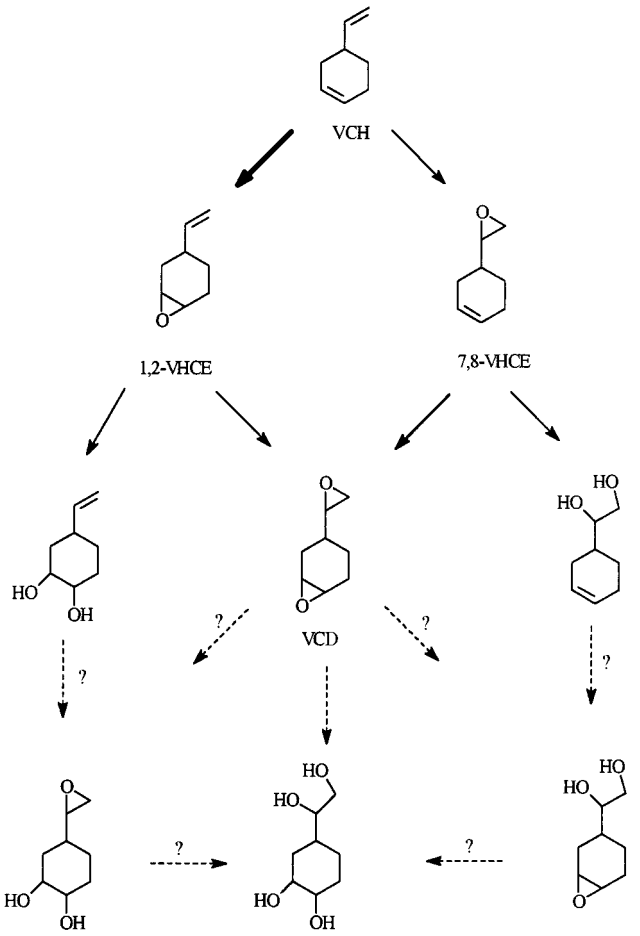
Scheme 1



been shown (*10*, *11*) that the epoxidation of alkyl-substituted olefins, catalyzed by P450s, may occur with a stereo- and enantioselectivity which strongly depend on the specificity and composition of the P450 enzymes, while induction may favor the microsomal epoxide hydrolase (mEH)[1]-catalyzed hydrolysis of epoxides, a process which generally occurs with substrate and product enantioselection.

Therefore, to gain insight into the stereochemical and mechanistic aspect of the metabolism of 4-vinylcyclohexene, as a first step, we investigated (i) the effect of the pretreatement of rats with three P450 inducers, phenobarbital (PB), pyrazole (Pyr), and β-naphthoflavone (β-NF), and the role of two purified P450s (2B1 and 2E1) in the stereochemistry of **1** oxidation to the isomeric endocyclic epoxides *cis*- and *trans*-4-vinylcyclohexene 1,2-epoxide (**2** and **3**, respectively), as well as the isomeric esocyclic epoxides (4*R**,7*S**)- and (4*R**,7*R**)-4-vinylcyclohexene 7,8-epoxide (**4** and **5**, respectively), and (ii) the mEH-catalyzed hydrolysis of epoxides **2** and **3**, and **4** and **5**, to the corresponding diols **6**−**8**.

Materials and Methods

Caution: *The following chemicals are hazardous and should be handled carefully: 4-vinylcyclohexene, 4-vinylcyclohexene 1,2-epoxide, and 4-vinylcyclohexene 7,8-epoxide.*

[1] Abbreviations: mEH, microsomal epoxide hydrolase; PB, phenobarbital; Pyr, pyrazole; β-NF, β-naphthoflavone; TCPO, trichloropropene oxide; GC, gas chromatography.

Materials. PB, Pyr, and β-NF were obtained from common commercial sources. 4-Vinylcyclohexene (**1**) and (±)-4-vinylcyclohexene 1,2-epoxide (1:1 mixture of the *cis* and *trans* isomers) (**2** and **3**, respectively) were purchased from Aldrich Chemical Co. (Milwaukee, WI). TCPO was obtained from EGA-Chemie (Steinheim-Albuch, Germany). (*S*)-4-Vinylcyclohexene (**1**) was prepared as previously reported (*12*).

Separation of the 1:1 Mixture of Epoxides 2 and 3. The 1:1 mixture of epoxides **2** and **3** (3 g, 24 mmol) was dissolved in 20 mL of chloroform, and 5.8 g (29 mmol) of *p*-nitrobenzoyl chloride (recrystallized from petroleum ether) was added. With magnetic stirring, a stream of anhydrous HCl was slowly passed into the solution over a period of 20 min. After 6 h at room temperature, the solution was washed with water to remove the excess acid and evaporated to give a residue which was taken up in pyridine. A few chips of ice were added, after which the mixture was extracted with ethyl ether and washed with 5% HCl, and then with a diluted aqueous solution of $NaHCO_3$ and water. The combined organic solution was evaporated to give a crude product (3.1 g) which was purified by column chromatography using hexanes and ethyl acetate (8:2) as the eluent. The fractions containing practically a sole product (*trans*-2-chloro-*cis*-4-vinylcyclohexyl-*p*-nitrobenzoate) were collected and evaporated: ^{1}H NMR ($CDCl_3$) δ 0.8−2.7 (m, 7H), 4.35 (m, 2H), 5.10 (m, 2H), 5.25 (m, 1H), 5.85 (m, 1H), 8.25 (AA′BB′ system, 4 aromatic H); ^{13}C NMR ($CDCl_3$) δ 25.1 (CH_2), 26.55 (CH_2), 34.7 (CH), 56.5 (CH), 62.2 (CH), 114 (CH_2), 123.5 (2CH), 130.7 (2CH), 141 (CH).

To a solution of *trans*-2-chloro-*cis*-4-vinylcyclohexyl-*p*-nitrobenzoate (150 mg) in methanol (10 mL) was added 150 mg of Na_2CO_3 dissolved in 0.1 mL of water. The mixture was refluxed with stirring for 1 h, then diluted with water, and extracted with pentane. The organic phase was dried and evaporated to give a residue containing epoxide **2**: ^{1}H NMR ($CDCl_3$) δ 0.8−2.0 (m, 7H), 3.05 (m, 2H), 4.85 (m, 2H), 5.5 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 23.0 (CH_2), 26.5 (CH_2), 31.0 (CH_2), 33.8 (CH), 51.2 (CH), 52.7 (CH), 112.7 (CH_2), 141.7 (CH).

The *cis*-epoxide **2** was identified by lithium aluminum hydride reduction (*13*), which gives as the major product *cis*-4-vinylcyclohexanol: ^{1}H NMR ($CDCl_3$) δ 1.1−1.8 (m, 8H), 2.4 (m, 1H), 3.95 (m, 1H), 5.05 (m, 2H), 5.75 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 26.9 (CH_2), 32.5 (CH_2), 40.8 (CH), 67.5 (CH), 112.9 (CH_2), 143.6 (CH).

(±)-4-Vinylcyclohexene 7,8-Epoxide (4) and (±)-4-Vinylcyclohexene 7,8-Epoxide (5). 3-Cyclohexene-1-carboxaldehyde (3.3 g, 30 mmol) was slowly added to a stirred mixture of trimethylsulfonium methyl sulfate (6.5 g, 34 mmol) and NaOH (7.5 g) in anhydrous dichloromethane (50 mL). After 5 h at room temperature, water (25 mL) was added. The organic phase was separated, washed with water (2 × 15 mL), dried ($MgSO_4$), and concentrated in vacuo. The residue was distilled to give a 1:1 mixture of epoxides **4** and **5**: yield 3.25 g (86%); ^{1}H NMR ($CDCl_3$) δ 1.3−1.6 (m, 2H), 1.7−2.2 (m, 12H), 2.50 (dd, J = 2.9 and 4.9 Hz, 2H), 2.78 (m, 4H), 5.6 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 23.9 (CH_2), 24.3 (CH_2), 24.6 (CH_2), 25.2 (CH_2), 26.9 (CH_2), 27.8 (CH_2), 35.8 (CH), 36.2 (CH), 45.4 (CH_2), 46.0 (CH_2), 55.5 (CH), 55.9 (CH), 125.1 (CH), 125.4 (CH), 126.7 (CH), 127.1 (CH).

(±)-4-Vinylcyclohexene 1,2-diol (6) was prepared by acid-catalyzed hydrolysis (1 N $HClO_4$) of the 1:1 mixture of epoxides **2** and **3:** yield 90%; ^{1}H NMR ($CDCl_3$) δ 1.4−2.0 (m, 6H), 2.5 (m, 1H), 3.4 (m, 1H), 3.6 (m, 1H), 5.05 (d, J = 16.5 Hz, 1H), 5.05 (d, J = 10.5 Hz, 1H), 5.85 (ddd, J = 16.5, 10.5, and 5.5 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 27.4 (CH_2), 27.9 (CH_2), 35.7 (CH_2), 35.8 (CH), 70.7 (CH), 74.3 (CH), 113.7 (CH_2), 127.1 (CH).

(±)-4-Vinylcyclohexene 7,8-diol (7) and (±)-4-vinylcyclohexene 7,8-diol (8) were prepared by acid-catalyzed hydrolysis (1 N $HClO_4$) of the ca. 1:1 mixture of epoxides **4** and **5**: yield 90%; ^{1}H NMR ($CDCl_3$) δ 1.5−2.1 (m, 14H), 3.4−4.0 (m, 6H), 5.65 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 24.4 (CH_2), 24.8 ($2CH_2$), 25.0 (CH_2), 26.9 (CH_2), 27.6 (CH_2), 36.4 (CH), 36.5 (CH), 64.2 (CH_2), 64.6 (CH_2), 75.7 (CH), 75.8 (CH), 125.5 (CH), 125.9 (CH), 126.7 (CH), 127.2 (CH).

(1*R*,2*S*,4*S*)- and (1*S*,2*R*,4*S*)-4-Vinylcyclohexene 1,2-Epoxide. Epoxidation of (*S*)-4-vinyl-1-cyclohexene with *m*-chloroperbenzoic acid in dichloromethane (reaction for 2 days) yielded a mixture of (1*R*,2*S*,4*S*)- and (1*S*,2*R*,4*S*)-4-vinyl-1-cyclohexene 1,2-epoxide. The crude product was analyzed by GC on a 50 m chiral CP-Cyclodex B (CHROMPACK) column at 85 °C (helium flow of 50 kPa, with an evaporator and detector set at 200 °C).

(1*R*,2*R*,4*S*)-4-Vinylcyclohexene 1,2-Diol (6). Acid-catalyzed (1 N $HClO_4$) hydrolysis of the 1:1 mixture of (1*R*,2*S*,4*S*)- and (1*S*,2*R*,4*S*)-4-vinyl-1-cyclohexene 1,2-epoxide gave (1*R*,2*R*,4*S*)-4-vinylcyclohexene 1,2-diol (**6**). The crude product was analyzed by GC, after transformation into the corresponding bis-trifluoroacetyl derivatives, on a chiral 30 m Chiraldex G-TA (ASTEC) column at 80 °C (helium flow of 50 kPa, with an evaporator and detector set at 200 °C).

Preparation of (4*S*,7*S*)- and (4*R*,7*S*)-4-Vinylcyclohexene 7,8-Diol. (1) Protection of the Endo Cyclic Double Bond. To a solution of **1** (330 mg, 3 mmol) in 1,2-dichloroethane (15 mL) was added a solution of Br_2 (480 mg, 3 mmol) in the same solvent (15 mL), and the reaction mixture was stored in the dark at room temperature until it was colorless. Then the reaction mixture was washed with water, and the organic phase, dried ($MgSO_4$), was evaporated to give a crude mixture containing dibromide **9** (800 mg).

(2) Asymmetric Dihydroxylation. The crude product arising from bromine addition to the endocyclic double bond (800 mg) was dihydroxylated using AD-mix α, according to the standard procedure reported by the Sharpless group (*14*).

(3) Deprotection of the Endocyclic Double Bond. To an ethanolic solution (8 mL) of diols **10** (300 mg) arising from asymmetric dihydroxylation were added 300 mg of Zn power and 20 mg of $ZnCl_2$, and the reaction mixture was stirred at room temperature for 2 days. Then the reaction mixture was filtered and diluted with water, and the reaction products were extracted three times with ethyl acetate. The organic layer was dried ($MgSO_4$), and the solvent was removed under reduced pressure to give a practically 1:1 mixture of (4*R*,7*S*)- and (4*S*,7*S*)-4-vinylcyclohexene 7,8-diol (**7** and **8**). The crude product was analyzed by NMR and GC, after transformation into the corresponding bis-trifluoroacetyl derivatives, on a chiral 30 m Chiraldex G-TA (ASTEC) column at 90 °C (helium flow of 50 kPa, with an evaporator and detector set at 200 °C).

Preparation of (4*S*,7*S*)-4-Vinylcyclohexene 7,8-Diol (8). (4*S*,7*S*)-**8** was synthesized from (*S*)-4-vinylcyclohexene (**1**) according to the procedure reported above for the preparation of the diastereoisomeric mixture of (4*R*,7*S*)-**7** and (4*S*,7*S*)-**8**. The crude product was analyzed by GC, after transformation into the corresponding bis-trifluoroacetyl derivatives, under the conditions reported above.

Preparation of (4*S*,7*R*)-4-Vinylcyclohexene 7,8-Diol (7). (4*S*,7*R*)-**7** was synthesized from (*S*)-4-vinylcyclohexene (**1**) according to the procedure reported above for the preparation of the diastereoisomeric mixture of (4*R*,7*S*)-**7** and (4*S*,7*S*)-**8** using AD-mix *β*. The crude product was analyzed by GC after transformation into the corresponding bis-trifluoroacetyl derivatives, under the conditions reported above.

Preparation of (4*S*,7*S*)-4-Vinylcyclohexene 7,8-Epoxide (5) and (4*R*,7*S*)-4-Vinylcyclohexene 7,8-Epoxide (4). To 300 mg (2 mmol) of a 1:1 mixture of (4*R*,7*S*)-**7** and (4*S*,7*S*)-**8** in 4 mL of dry pyridine was added a solution of *p*-toluenesulfonyl chloride (420 mg, 2.2 mmol) in the same solvent (4 mL) at 0 °C. The mixture was stored in the refrigerator for 12 h and then diluted with 10 mL of an ice/water mixture. The aqueous layer was extracted four times with 5 mL of CH_2Cl_2. The combined organic solution was washed five times with 5 mL of H_2SO_4 (2 N), followed by a saturated $NaHCO_3$ solution, dried ($MgSO_4$), and evaporated in vacuo to give a mixture of (4*S*,7*S*)-4-vinylcyclohexene-8-[(*p*-toluenesulfonyl)oxy] 7,8-diol (**11**) and (4*R*,7*S*)-4-vinylcyclohexene-8-[(*p*-toluenesulfonyl)oxy] 7,8-diol (**12**). To the crude reaction mixture (400 mg), dissolved in CH_2Cl_2 (20 mL), were added 600 mg of KOH and 18-crown-6 (50 mg). The mixture, stirred at room temperature for 18 h, was filtered, and the solvent was evaporated to give a 1:1 mixture of (4*R*,7*S*)-**4** and (4*S*,7*S*)-**5**. The crude product was analyzed by GC, on a chiral 30 m Chiraldex G-TA (ASTEC) column at 70 °C (helium flow of 50 kPa, with an evaporator and detector set at 200 °C).

(4*S*,7*S*)-4-Vinylcyclohexene 7,8-Epoxide (5). (4*S*,7*S*)-**5** was synthesized from (4*S*,7*S*)-**8** according to the procedure reported above for the preparation of the diastereoisomeric mixture of (4*R*,7*S*)-**4** and (4*S*,7*S*)-**5**. ee = 95% (determined by GC under the conditions reported above).

(4*S*,7*R*)-4-Vinylcyclohexene 7,8-Epoxide (4). (4*S*,7*R*)-**4** was synthesized from (4*S*,7*R*)-**7** according to the procedure reported above for the preparation of the diastereoisomeric mixture of (4*R*,7*S*)-**4** and (4*S*,7*S*)-**5**. ee = 95% (determined by GC under the conditions reported above).

Animal Microsomal Preparations and Enzymes. Male and female Sprague-Dawley rats were purchased from Charles River. Male rats were treated for 3 days with PB ip (80 mg/kg daily), *β*-NF (40 mg/kg daily), or Pyr (200 mg/kg daily), and microsomes were obtained from the liver as previously described (*15*). Microsomal protein concentrations were assayed by using the method of Lowry et al. (*16*); the total P450 concentration was measured according to the method of Omura and Sato (*17*). P450 2B1, 2E1, and NADPH−cytochrome P450 reductase were purified from rat liver as previously described (*10*).

Enzymatic Incubations. (1) Determination of the P450 Activity and of the Enantiomeric Excesses of the Products. Incubation mixtures (2 mL) containing 100 mM potassium phosphate buffer (pH 7.4), 5 mg of hepatic microsomal proteins, the NADPH-generating system, consisting of 0.5 mM $NADP^+$, 5 mM glucose 6-phosphate, and 0.5 unit/mL glucose-6-phosphate dehydrogenase, and trichloropropene oxide (TCPO, 1 mM), to inhibit the mEH, were preincubated at 37 °C for 5 min, and the reactions were initiated by the addition of a proper amount of **1**. The substrate concentration was 10 mM to saturate the P450 enzymes (*7*). After 60 min, a saturating amount of NaCl was added to precipitate the microsomal proteins. The reaction products were extracted with ethyl acetate (2 × 5 mL) and analyzed by GC after addition of appropriate amounts of cyclohexanol as an internal standard. The enantiomeric composition of monoepoxides was determined by GC on a 50 m chiral CP-Cyclodex B (CHROMPACK) column (helium flow of 50 kPa, with an evaporator and detector set at 200 °C, at 85 °C) for **2** and **3** and on a chiral 30 m Chiraldex G-TA (ASTEC) column (helium flow of 50 KPa, with an evaporator and detector set at 200 °C, at 70 °C) for **4** and **5**.

The absolute configurations of the excess enantiomers of **2**−**5** were determined by comparison of the retention times of the two enantiomers of each isomer with those of enantiomerically pure compounds obtained as reported above.

The epoxidations of **1** were also assessed in a reconstituted system (0.5 mL) containing 0.1 nmol of purified rat 2B1, or 2E1, 0.3 nmol of P450 reductase, 30 *μ*g of dilauroylphosphatidylcholine, and the substrate at a concentration of 10 mM. The reactions were carried out at 37 °C for 30 min after the addition of 1 mM NADPH, and the products were extracted and analyzed as described above.

(2) Determination of the Product Enantioselectivity of the mEH-Catalyzed Hydrolysis of Monoepoxides 2 and 3. Aliquots (10 *μ*L) of an ethanolic stock solution of the mixture of (±)-**2** and (±)-**3** were added to 1 mL of a microsomal preparation (control) containing 3 or 5 mg of protein/mL in a such way to obtain a 20 mM substrate concentration, and the reaction mixtures were incubated at 37 °C. At prefixed times (between 1 and 5 h), a saturating amount of NaCl was added to precipitate the microsomal proteins and the incubation mixtures were extracted with hexane (3 × 1 mL) to remove the unreacted epoxides followed by a brief centrifugation to separate the emulsion. Cyclohexanol was added as a standard to the combined extracts, which were analyzed directly by GC on a 50 m chiral CP-Cyclodex B (CHROMPACK) column (helium flow of 50 KPa, with an evaporator and detector set at 200 °C, at 85

°C). The enantiomer ratios and the absolute configurations were determined for each couple of diastereoisomers by comparison of the retention times with those of samples of enantiomerically pure epoxides and diols obtained as reported above.

The incubation mixtures were then extracted with ethyl acetate (5 × 1 mL), and the enantiomer ratios and the absolute configurations of the formed diols were determined by GC on a chiral 30 m Chiraldex G-TA (ASTEC) column (helium flow of 50 KPa, with an evaporator and detector set at 200 °C, at 80 °C), after transformation into the corresponding bis-trifluoroacetyl derivatives, by comparison of the retention times with those of a sample of pure (1*R*,2*R*,4*S*)-4-vinylcyclohexene 1,2-diol (**6**) obtained as reported above. Blank experiments carried out by boiling deactivated microsomal preparations show that spontaneous hydrolysis does not contribute to diol formation under the incubation conditions.

(3) Determination of the Product Enantioselectivity of the mEH-Catalyzed Hydrolysis of Monoepoxides 4 and 5. Aliquots (10 μL) of an ethanolic stock solution of a 46:54 mixture of (±)-**4** and (±)-**5** were added to 1 mL of a microsomal preparation (control) containing 2 or 3 mg of protein/mL in a such way to obtain a 20 mM substrate concentration, and the reaction mixtures were incubated at 37 °C and pH 7.4. At prefixed times (1, 2, 3, and 4 h), a saturating amount of NaCl was added to precipitate the microsomal proteins and the incubation mixtures were extracted with hexane (3 × 1 mL) to remove the unreacted epoxides followed by a brief centrifugation to separate the emulsion. Cyclohexanol was added as a standard to the combined extracts, which were analyzed directly by GC on a 30 m Chiraldex G-TA (ASTEC) column (helium flow of 50 KPa, with an evaporator and detector set at 200 °C, at 70 °C). The enantiomer ratios and the absolute configurations were determined for each couple of diastereoisomers by comparison of the retention times with those of samples of pure (4*S*,7*S*)-4-vinylcyclohexene 7,8-epoxide (**5**), (4*R*,7*S*)-4-vinylcyclohexene 7,8-epoxide (**4**), and (4*S*,7*R*)-4-vinylcyclohexene 7,8-epoxide (**4**) obtained as reported above.

The incubation mixtures were then extracted with ethyl acetate (5 × 1 mL), and the enantiomer ratios and the absolute configurations of the formed diols were determined by GC on a GC 30 m Chiraldex G-TA (ASTEC) column (helium flow of 50 KPa, with an evaporator and detector set at 200 °C, at 90 °C), after transformation into the corresponding bis-trifluoroacetyl derivatives, by comparison of the retention times with those of samples of (4*R*,7*S*)-4-vinylcyclohexene 7,8-diol (**7**), (4*S*,7*S*)-4-vinylcyclohexene 7,8-diol (**8**), and (4*S*,7*R*)-4-vinylcyclohexene 7,8-diol (**7**) obtained as reported above. Blank experiments carried out by boiling deactivated microsomal preparations show that spontaneous hydrolysis does not contribute to diol formation under the incubation conditions.

Results and Discussion

The stereochemical course of epoxidation of the endo and exocyclic double bond of 4-vinylcyclohexene and of the hydrolysis of the corresponding formed monoepoxides **2** and **3**, and **4** and **5**, was investigated using various microsomal preparations from male and female rats and two purified P450s (2B1 and 2E1). This investigation has also been carried out with male rats since the level of VCH oxidation on the exocyclic double bond with microsomes from female rats was found, in keeping with previous authors (*7*), to be too low to allow a stereochemistry study. As a convenient analytical tool, inclusion gas chromatography, which enables a time-dependent enantiomer screening of epoxides and diols in the nanomole range, was employed for the determination of the enantiomeric ratios (Figure 1) and for the absolute configurations. To use this method to determine the absolute configuration of the metabolites, the synthesis of reference substances with unequivocal stereochemistries was necessary.

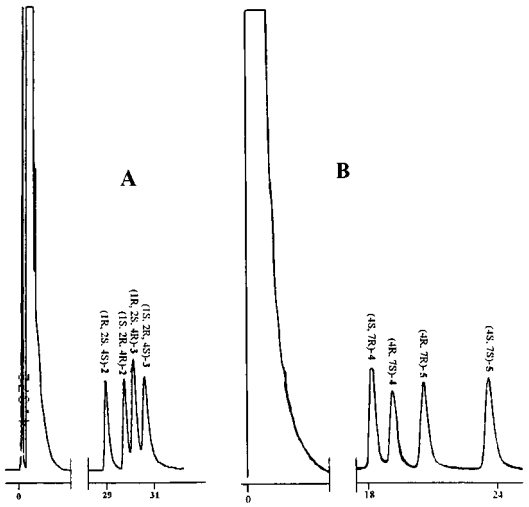
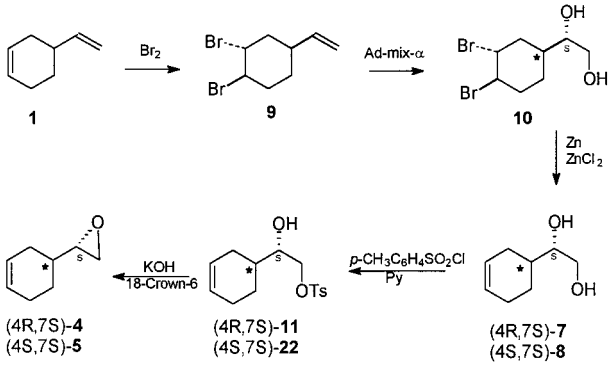


Figure 1. (A) Gas chromatographic enantiomer separation of (1*R*,2*S*,4*S*)-**2**, (1*S*,2*R*,4*R*)-**2**, (1*R*,2*S*,4*R*)-**3**, and (1*S*,2*R*,4*S*)-**3**. (B) Gas chromatographic enantiomer separation of (4*R*,7*S*)-**4**, (4*S*,7*R*)-**4**, (4*R*,7*R*)-**5**, and (4*S*,7*S*)-**5**. Analysis conditions are outlined in Materials and Methods.

Scheme 2



Synthesis of Reference VCH Metabolites. The separation of the 1:1 mixture of epoxides **2** and **3** was achieved by transformation into the regioisomeric mixture of the two corresponding chloro-*p*-nitrobenzoates, which were purified by column chromatography, and the isolated *trans*-2-chloro-*cis*-4-vinylcyclohexyl-*p*-nitrobenzoate was again transformed into the epoxides **2** by treatment with potassium carbonate in methanol. (*S*)-4-Vinylcyclohexene was prepared from *d*-pantolacton via an asymmetric Diels−Alder reaction, as previously reported (*12*). The epoxidation of (*S*)-4-vinylcyclohexene with *m*-chloroperbenzoic acid led to the diastereoisomeric (1*R*,2*S*,4*S*)-**2** and (1*S*,2*R*,4*S*)-**3**. Since the retention times of the *cis* and *trans* isomers have been known, it has been possible to attribute at each GC peak the absolute stereochemistry. The diastereoisomeric mixture of epoxides (1*R*,2*S*,4*S*)-**2** and (1*S*,2*R*,4*S*)-**3** was transformed into the corresponding diol (1*R*,2*R*,4*S*)-**6** by $HClO_4$-catalyzed hydrolysis. A ca. 1:1 mixture of (4*R*,7*S*)-4-vinylcyclohexene 7,8-epoxide (**4**) and (4*S*,7*S*)-4-vinylcyclohexene 7,8-epoxide (**5**) was prepared in five steps via a monotosyl derivative from the corresponding diol mixture [(4*R*,7*S*)-**7** and (4*S*,7*S*)-**8**], the latter being synthesized by asymmetric Sharpless dihydroxylation using AD-mix α of olefin **1**, previously protected on the endocyclic double bond by bromination (*14*) (see Scheme 2).

Table 1. Stereochemical Course for the Oxidation on the Endocyclic Double Bond of 1 by Microsomes from Male and Female Control and Treated Rats and Purified P450s (2B1 and 2E1)[a]

	(1*R*,2*S*,4*S*)-**2** (*b*)[c] [nmol (%)]	(1*S*,2*R*,4*R*)-**2** (*d*)[c] [nmol (%)]	(1*R*,2*S*,4*R*)-**3** (*c*)[c] [nmol (%)]	(1*S*,2*R*,4*S*)-**3** (*a*)[c] [nmol (%)]
CTR	22 (31)	(<1)	26 (37)	23 (32)
CTR[b]	8 (17)	3 (6)	27 (56)	10 (21)
PB	85 (15)	232 (41)	125 (22)	125 (22)
Pyr	29 (23)	21 (17)	52 (41)	23 (19)
β-NF	12 (15)	21 (25)	27 (32)	23 (28)
2E1	16 (37)	10 (23)	12 (28)	5 (12)
2B1	6 (7)	32 (41)	30 (39)	10 (13)

[a] Each value represents the mean of at least two determinations. [b] From female rats. [c] *a*−*d* refer to the pathways reported in Scheme 3.

Table 2. Stereochemical Course for the Oxidation on the Exocyclic Double Bond of 1 by Microsomes from Male Control and Treated Rats[a]

	(4*R*,7*S*)-**4** (*d*)[b] [nmol (%)]	(4*S*,7*R*)-**4** (*b*)[b] [nmol (%)]	(4*R*,7*R*)-**5** (*c*)[b] [nmol (%)]	(4*S*,7*S*)-**5** (*a*)[b] [nmol (%)]
CTR	41 (40)	30 (30)	15 (15)	15 (15)
PB	27 (21)	32 (25)	19 (15)	51 (39)
Pyr	17 (23)	(<1)	40 (55)	16 (22)
β-NF	(<5)	(<5)	19 (42)	18 (40)

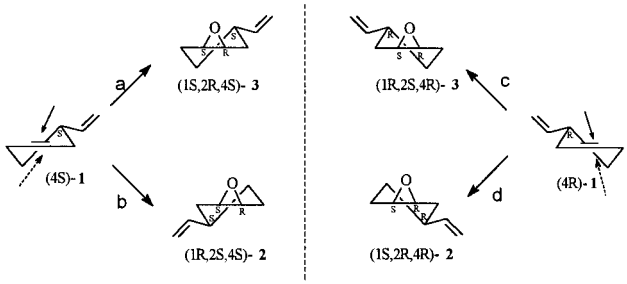
[a] Each value represents the mean of at least two determinations. [b] *a*−*d* refer to the pathways reported in Scheme 4.

The pure (4*S*,7*S*)-**4** and the pure (4*S*,7*R*)-**5**, and the corresponding diols (4*S*,7*S*)-**8** and (4*S*,7*R*)-**7**, were prepared following the same synthetic pathway starting from pure (4*S*)-**1** and using AD-mix α and AD-mix *β* in the dihydroxylation step, respectively.

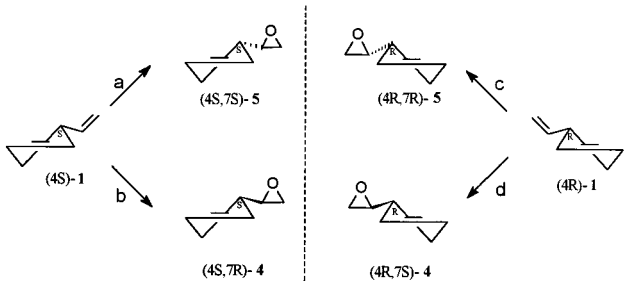
Epoxidation of 4-Vinylcyclohexene 1 by Rat Liver Microsomes. 4-Vinylcyclohexene possesses two prochiral carbon−carbon double bonds and a chiral center. The enzymatic epoxidation of the chiral diene represents a competitive process between the enantiomers which can occur with regioselectivity, and substrate and/or product enantio- and diastereoselectivity. The correct determination of regio- and stereoselectivity in the epoxidation reactions requires the complete inhibition of epoxide hydrolase, since the enzymatic hydrolysis of the epoxides may represent an efficient competing enantioselective process. The stereochemical course of the double bond oxidation of **1** in the corresponding endo- and exocyclic epoxides **2** and **3**, and **4** and **5**, was investigated by incubating **1** (10 mM) in the presence of TCPO (1 mM), an efficient inhibitor of epoxide hydrolase (*18*), by using liver microsomes obtained from untreated rats or rats pretreated with PB, Pyr, and *β*-NF, classical inducers of 2B1/2-3A, 2E1, and IA1/2, respectively (*15*). At prefixed times (60 min), the reactions were stopped by adding a saturating amount of NaCl, and the incubation mixtures were extracted with ethyl acetate and analyzed by GC on a chiral column, after addition of cyclohexanol as an internal standard, to evaluate the chemical yields. The use of the chiral column allowed evaluation of not only the diastereoisomeric ratio of the formed epoxides but also the enantiomeric ratio for each diastereoisomer and the absolute configuration of the enantiomer in excess (see Figure 1). The latter was evaluated by the comparison of the retention time of the formed products with those of samples of known configuration prepared as reported above.

Related to the stereochemical behavior of the epoxidation of **1**, the data reported in Tables 1 and 2 show that both double bonds are oxidized by liver microsomes from male rats, whereas microsomes from female rats

Scheme 3



Scheme 4



oxidized primarily the endocyclic double bond. With PB-induced microsomes which give the highest yield in oxidation products, the reaction is characterized by a remarked regioselectivity favoring the formation of the endocyclic epoxides. It is noteworthy that, while the PB-induced microsomes and, to a lower extent, the Pyr-induced microsomes, increase the total amount of endocyclic double bond oxidation products, these inducers, as well the *β*-NF-induced microsomes, practically do not enhance the chemical yield of the exocyclic double bond oxidation products. The stereochemical behavior strongly depends on the specificity and composition of the P450 isoforms. It must be remarked that for each double bond the stereochemical results, arising from the competition among processes *a*−*d* (Schemes 3 and 4), may be rationalized considering three stereoselective processes: substrate enantioselectivity, product diastereoselectivity, and product enantioselectivity.

The endocyclic double bond oxidation proceeds with control male microsomes with a moderate substrate enantioselectivity (63:37), given by *a* + *b* versus *c* + *d*, in favor of the (4*S*)-enantiomer, which is however inverted when induced microsomes are used. On the other hand, the substrate enantioselectivity in the exocyclic double bond oxidation with control male microsomes is very low, 45:55, in favor of the (4*R*)-enantiomer, but increases to 22:78 using Pyr-induced microsomes, while a moderate selection 64:36 in favor of the (4*S*)-enantiomer was found using PB-induced microsomes. Also, the

product diastereoselectivity, given by *a* versus *b* and *c* versus *d*, shows a behavior that is inducer- and gender-dependent. The epoxidation of the endocyclic double bond of (4*R*)-**1** with male control microsomes occurs with an extremely high face selectively to give exclusively *trans*-epoxide **3**, while that of the (4*S*)-enantiomer is practically nonselective. With female control microsomes, the enantiofacial selectivity in the endocyclic double bond oxidation of both enantiomers (4*R* and 4*S*) is similar to that observed with male microsomes but less pronounced. The induction generally reduces the product diastereoselectivity characterizing the epoxidation of the (*4R*)-enantiomer, while with PB-induced microsomes, a moderate enantioselection in favor of the *cis*-epoxide **2** has been observed. In the oxidation of (4*S*)-**1**, instead a moderate effect has been found only in the case of *β*-NF-induced microsomes. Related to the exocyclic double bond oxidation, the use of control microsomes gives the four diastereoisomeric epoxides with a moderate diastereoselectivity in favor of (4*S*,7*R*)-**4** and (4*R*,7*S*)-**4** which however is reversed, favoring epoxides (4*S*,7*S*)-**5** or (4*R*,7*R*)-**5**, with induced microsomes.

Finally, the results reported in Tables 1 and 2 may be discussed in terms of product enantioselectivity, given by *a* versus *c* and by *b* versus *d*, arising from the differentiation between the two enantiotopic faces of the double bond of the two enantiomers. When the formation of *cis*-epoxide **2** is taken into account, male control microsomes and female ones, although to a lesser extent, showed a high product enantioselectivity in favor of enantiomer (1*R*,2*S*,4*S*)-**2**. This selectivity markedly decreases with Pyr-induced microsomes and increases again in favor of the *cis*-(1*S*,*2R*,4*R*)-**2** enantiomer using PB- and *β*-NF-induced microsomes. On the contrary, *trans*-epoxide **3** is formed in a practically racemic way when the incubations were carried out using male control microsomes but not female ones. When Pyr-induced microsomes were used, the reaction is characterized by a moderate selectivity in favor of (1*R*,2*S*,4*R*)-**3**. Related to the exocyclic double bond oxidation, the product enantioselectivity is low or completely absent when control or *β*-NF-induced microsomes are used but increases with all the other inducers. The oxidation with Pyr-induced microsomes gives exclusively epoxide (4*R*,7*S*)-**4** and selectively (46% ee) epoxide (4*R*,7*R*)-**5**. At variance, PB-induced microsomes give epoxide **4** without any product enantioselection, while in the case of **5**, the formation of the (4*S*,7*S*)-enantiomer is favored.

The stereoselectivity of the oxidation of **1** was also evaluated by reconstituting the monooxygenase reaction in the presence of DLPC, NADPH−cytochrome P450 reductase, purified rat liver 2B1 or 2E1, and NADPH. Only epoxides **2** and **3**, arising from the regioselective oxidation of the endocyclic double bond, were extracted from the reconstituted system of both P450 2B1 and 2E1 and analyzed by GC on the chiral column. It is of interest to note also that 2E1, although to a lesser extent than 2B1, is able to perform the oxidation on the endocyclic double bond, in agreement with the results obtained with Pyr-induced microsomes. The data, reported in Table 1, show a high substrate enantioselectivity for the 2B1 but not for the 2E1, in favor of the (4*R*)-enantiomer which is associated with a high product enantioselectivity, at least concerning the formation of *cis*-epoxide **2**. It is noteworthy that although the results obtained using purified 2B1 and 2E1 are similar to those obtained using PB- and Pyr-induced microsomes, respectively, there are some differences which may be attributed to the involvement in the oxidation process of other isoforms present in the microsomal preparations.

In conclusion, the oxidation data for **1** seem to indicate that, as previously observed (*10, 11*) for the stereoselectivity in the P450 oxidation of prochiral and chiral olefins, besides 2B1 and 2E1, other isoforms including the major ones expressed in male and female rats are involved in the epoxidation process and the single enzymes catalyze the reaction with a different stereoselectivity, substrate enantioselectivity, product enantioselectivity, and product diastereoselectivity. The different stereoselectivity may imply a different chemical recognition which can be determined by the active site architecture of the P450 isoenzymes able to give distinct interactions of the two enantiomers of the substrate in the fundamental state (affecting the binding) and/or in the active complex (affecting k_{cat}). However, at least concerning the product diastereoselectivity, the possibility that the different behavior may arise from the involvement of different iron species (peroxo, hydroperoxo, or oxenoid), or the two reactive states of the same active species, which can perform the epoxidation also through different mechanisms as recently suggested for alkyl hydroxylation (*19*), cannot be excluded.

Hydrolysis of Monoepoxides 2 and 3, and 4 and 5. The P450-catalyzed oxidation of monoepoxides **2** and **3**, as well as **4** and **5**, to the corresponding diepoxides competed with the hydrolysis of the monoepoxides to the corresponding vicinal diols (see Scheme 1), the latter process of which is exclusively due to the enzymatic reaction. Blank experiments carried out using heat-deactivated microsomal preparations showed indeed that the spontaneous hydrolysis can always be neglected. To evaluate the stereoselectivity of the mEH-catalyzed hydrolysis of the monoepoxides, therefore, the experiments were carried out by incubating the 50:50 mixture of the two distereoisomeric endocyclic epoxides **2** and **3** (20 mM), or the 46:54 mixture of exocyclic epoxides **4** and **5**, with a microsomal preparation from control rats, containing 2−3 mg of protein/mL, at 37 °C and pH 7.4. The incubations were stopped at different times, and the residual epoxides and the formed diols (after derivatization) were identified by GC, after addition of cyclohexanol as an internal standard, on a chiral column. The results are reported in Tables 3 and 4.

It is noteworthy that all the monoepoxides were substrates for mEH but the hydrolysis rate of epoxides **4** and **5** was 3−4 times higher than that of epoxides **2** and **3**. Furthermore, the stereochemical results reported in Table 3 show that the hydrolysis of the 1:1 mixture of epoxides **2** and **3** proceeds with a high substrate diastereo- and enantioselectivity, as well as with a high product enantioselectivity. In agreement with the previuosly obtained results about the mEH-catalyzed hydrolysis of epoxy derivatives of cyclohexenes (*20*), the hydrolysis takes place exclusively by diaxial opening of the oxirane ring, and the two diaxial diol enantiomers, (1*R*,2*R*,4*S*)-**6** and (1*S*,2*S*,4*R*)-**6**, were the sole products (Scheme 5). The four diastereoisomeric epoxides are transformed into the corresponding diols with a different rate, showing that, as observed in the mEH-catalyzed hydrolysis of the stereoisomeric 4-*tert*-butyl-1,2-epoxycyclohexanes (*20*), the helicity of the cyclohexane ring and

Table 3. Time and Stereochemical Course of the mEH-Catalyzed Hydrolysis of a 50:50 Mixture of Endocyclic Epoxides 2 and 3[a]

		residual epoxides (%)				formed diols (%)	
time (h)	hydrolysis (%)	(1*R*,2*S*,4*S*)-**2**	(1*S*,2*R*,4*R*)-**2**	(1*R*,2*S*,4*R*)-**3**	(1*S*,2*R*,4*S*)-**3**	(1*R*,2*R*,4*S*)-**6**	(1*S*,2*S*,4*R*)-**6**
0	0	25	25	25	25	–	–
0.5	6.5	25	25	25	17.5	100	0
1	17.5	19	25	25	12.5	100	0
3	63	3	11	25	0	86	14
12	95	0	0	5	0	50	50

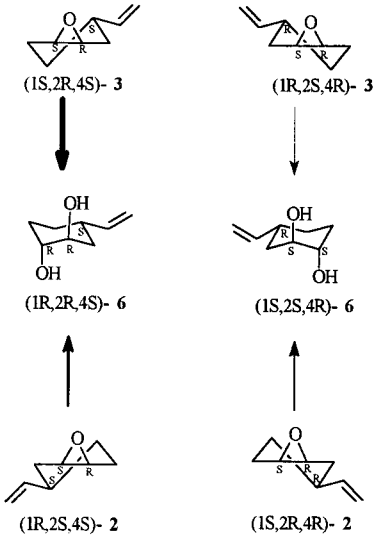
[a] Each value represents the mean of at least two determinations.

Table 4. Time and Stereochemical Course of the mEH-Catalyzed Hydrolysis of a 46:54 Mixture of Exocyclic Epoxides 4 and 5[a]

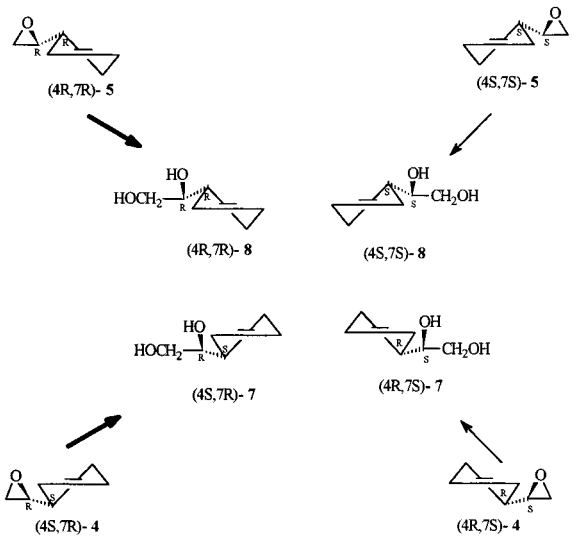
		residual epoxides (%)				formed diols (%)			
time (h)	hydrolysis (%)	(4*R*,7*S*)-**4**	(4*S*,7*R*)-**4**	(4*R*,7*R*)-**5**	(4*S*,7*S*)-**5**	(4*R*,7*S*)-**7**	(4*S*,7*R*)-**7**	(4*R*,7*R*)-**8**	(4*S*,7*S*)-**8**
0	0	23	23	27	27	–	–	–	–
1	9	23	19	22	27	–	44	55	–
2	41	20	7	9	22	9	34	43	14
6	99	1	0	0	0	23	23	27	27

[a] Each value represents the mean of at least two determinations.

Scheme 5



Scheme 6



the orientation of the substituent (vinyl group) with respect to the oxirane ring play an important role in determining the substrate diastereoselectivity. In accordance with the hydrolysis of 4-*tert*-butyl-1,2-epoxycyclohexanes, the first metabolized epoxide is *trans*-(1*S*,2*R*,4*S*)-**3** having the six-membered ring held in the conformation of (3,4 M) elicity, which generally is preferentially hydrolyzed (*20*, *21*), the substituent on the right when the epoxide is oriented with the oxygen upward, and attack takes place on the (*S*)-carbon. However, in the case of *cis*-epoxide **2**, at variance with the results mentioned above for 4-*tert*-butyl-1,2-epoxycyclohexanes, nucleophilic attack preferentially occurs on the (*S*)-carbon of the enantiomer having the (3,4 M) conformation, showing that for this substrate these features are more important than the disposition of the substituent with respect the oxirane ring. This aspect seems to determine the relative reactivities of the two enantiomers of *cis*-4-*tert*-butyl-1,2-epoxycyclohexane. It is however possible that the two substituents, which have different steric requirements and different abilities to interact with the mEH active site, may affect in a different way the balance of the factors which determine the substrate enantioselectivity.

Finally, related to the hydrolysis of the exoepoxides **4** and **5**, the data reported in Table 4 show that, as generally observed in the mEH-catalyzed hydrolysis of monosubstituted epoxides (*22*), the nucleophilic attack occurs regioselectively on the less substituted carbon to give, with uncomplete hydrolysis, the corresponding diols having at C(7) the same configuration of the transformed epoxides. Furthermore, the reaction occurs with a high level of substrate enantioselection, which previously was observed exclusively in the mEH-catalyzed hydrolysis of monosubstituted epoxides bearing branched bulky alkyl groups, suggesting that the cyclohexane ring is able to satisfy this requirement (Scheme 6). For both diastereoisomeric epoxides, the enantiomer which is preferentially hydrolyzed is that having the (*R*)-configuration at the oxirane carbon, i.e., the enantiomer bearing the cyclohexenyl group in the proper position to interact with the hypothesized mEH hydrophobic pocket (*22*) situated to the right backside of the epoxide binding site when the epoxide is oriented with the oxygen upward. This should be the enantiomer which gives the more stable enzyme–substrate complex, and acts as a competitive inhibitor toward the other enantiomer. The mEH-catalyzed hydrolysis of the ca. 1:1 mixture of the two epoxides **4** and

5 proceeds however without substrate diastereoselection; the two epoxides, (4*S*,7*R*)-**4** and (4*R*,7*R*)-**5**, are hydrolyzed practically with the same rate, showing that for these substrates the stereochemistry at the carbon adjacent to the oxirane ring does not affect the reaction selectivity.

Conclusions

In conclusion, these results show that the stereochemical course of the biotransformation of 4-vinylcyclohexene formulates a composite picture markedly affected by the composition of different P450s present in the male and female control or induced rat liver.

The stereochemical composition of the primarily formed metabolites, endo- and exocyclic monoepoxides **2** and **3**, and **4** and **5**, strongly depends on the specificity and composition of the P450 isoforms, but it also affected by the substrate stereo- and enantioselectivity characterizing the mEH-catalyzed hydrolysis of these substrates. The hydrolysis of the endocyclic epoxides occurs with a good substrate diastereo- and enantioselectivity favoring the hydrolysis of epoxides (1*S*,2*R*,4*S*)-**3** and (1*R*,2*S*,4*S*)-**2** to give, before 50% conversion, selectively (1*R*,2*R*,4*S*)-diol **6**. At variance, the hydrolysis of the exocyclic epoxides is characterized by a high level of substrate enantioselection associated with very little, if any, substrate distereoselection, and the two epoxides, (4*S*,7*R*)-**4** and (4*R*,7*R*)-**5**, are hydrolyzed practically with the same rate.

The higher resistance to mEH-catalyzed hydrolysis of (1*R*,2*S*,4*R*)-**3** and (1*S*,2*R*,4*R*)-**2** allows them to be the major epoxides to be further oxidated to diepoxides. The latter may be formed by the P450 system, hydrolyzed by mEH, and conjugated with GSH by GSTs in a stereoselective manner. Thus, on the basis of the balance between the formation and detoxication pathway and their reactivity, specific diepoxidic stereoisomers may account for the VCH ovotoxicity in a given species.

Work is underway to investigate the stereochemical aspects of the second part of VCH biotransformation in the rat. Next, the availability of the reference stereoisomers will allow a stereochemical study in mice and humans, and by comparison, it will be possible to reveal the toxicological significance deriving from the VCH whole metabolism.

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