Stereochemical Course of the Biotransformation of Isoprene Monoepoxides and of the Corresponding Diols with Liver Microsomes from Control and Induced Rats

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The stereochemical course of the biotransformation of isoprene by liver enzymes from control and induced rats has been determined. Between the two primarily formed metabolites, 2-methyl-2-vinyloxirane (**2**) and isopropenyloxirane (**3**), epoxide **2** is rapidly transformed into the corresponding vicinal racemic diol **4**, predominantly through a nonenzymatic hydrolysis reaction. At variance, epoxide **3** is mainly biotransformed into the diol **5** by microsomal epoxide hydrolase (mEH) to give, before 50% conversion, selectively (*R*)-3-methyl-3-butene-1,2-diol, **5**. The hydrolysis competes with the oxidation of the monoepoxide **3** to the corresponding diepoxides **6**. Epoxidation of **3** catalyzed by P450 is characterized by a moderate stereoselectivity which, however, was strongly dependent on P450 induction. Treatment of rats with phenobarbital (PB) (an inducer of P450 2B1 and 3A) leads to *threo*-(2*R*,2′*R*)-**6** with a high selectivity, while with pyrazole (Pyr) (an inducer of P450 2E1), the formation of both *erythro*-(2*S*,2′*R*)- and *threo*-(2*R*,2′*R*)-**6** is favored. The mEH-catalyzed hydrolysis of diepoxides **6** proceeds, although with a moderate turnover rate, with substrate and product diastereo- and enantioselection by nucleophilic attack on the more substituted oxirane ring to give selectively (2*R*,3*S*)-3,4-epoxy-2-methyl-1,2-diol (**7**). Both diols **4** and **5** may be further oxidized on their double bond by P450. These reactions, which occur at a slow rate and are dependent on P450 induction with PB and Pyr, may be negligible in the overall isoprene biotransformation. On the other hand, the epoxydiol **7**, which is formed by hydrolysis of diepoxides **6** but it is itself not hydrolyzable, may play an important role in the isoprene toxicity.

Introduction

Isoprene, the monomeric unit of natural rubber and naturally occurring terpenes and steroids, is obtained primarily as a byproduct of nafta cracking and is emitted from both plants and animals at significant rates (*1*, *2*). Isoprene is of particular interest in atmospheric chemistry because it participates in the tropospheric reactions that produce ozone; furthermore, its oxidation by the hydroxyl radical reduces the oxidative capacity of the atmosphere (*3*). The high-level diffusion in nature of this chemical and its structural similarity to 1,3-butadiene, a potent carcinogen, explain the relevance of the studies focused on mammalian toxicology of isoprene and its metabolites (*4*−*6*). Recent studies have indicated that isoprene is carcinogenic to mice but much less if any to rats (*4*, *5*) and that among its metabolites only diepoxide but not monoepoxides has been shown to be mutagenic (*7*). Isoprene (**1**) is epoxidized by P450 to the isomeric monoepoxides 2-methyl-2-vinyloxirane (**2**) and 2-isopropenyloxirane (**3**) (Scheme 1). Both epoxides are further epoxidized to 2-methyl-2,2-bioxirane (**6**) in competition with the hydrolysis to the corresponding vicinal diols **4** and **5** (*4*, *8*−*10*). The diepoxide is thought to account for the isoprene carcinogenicity, but it is not known if an important contribution to this toxicity is due to other reactive metabolites such as epoxydiols **7** and **8**, eventually formed by either further epoxidation of **4** and **5** or diepoxide hydrolysis. That may be the case as for the butadiene the 3,4-epoxy-1,2 butanediol was found to exhibit a mutagenic activity (*11*) and to represent the most abundant component of DNA adducts derived from the butadiene epoxides (*12*).

The stereochemical aspects in the biotransformation process of these compounds are also important in determining their toxicity. Studies carried out using liver microsomes from uninduced rats and mice have shown that the oxidation to **6** occurs with substrate enantioselectivity, product diastereoselectivity, and product enantioselectivity (*8*). Furthermore, it has been shown (*8*) that the microsomal epoxide hydrolase catalyzes the in vivo hydrolysis of both monoepoxides **2** and **3**. The hydrolysis of racemic 2-isopropenyloxirane **3** with mouse and rat liver microsomes occurs with substrate enantioselection; the (*R*)-enantiomer is preferentially hydrolyzed. A much lower-level enantioselection was instead observed in the hydrolysis of the more reactive 2-methyl-2-vinyloxirane (**2**) which was attributed (*8*) to a competition between the enzymatic hydrolysis and the spontaneous process. It is, however, also noteworthy that, in the case of **3**, the presence of the double bond may favor the

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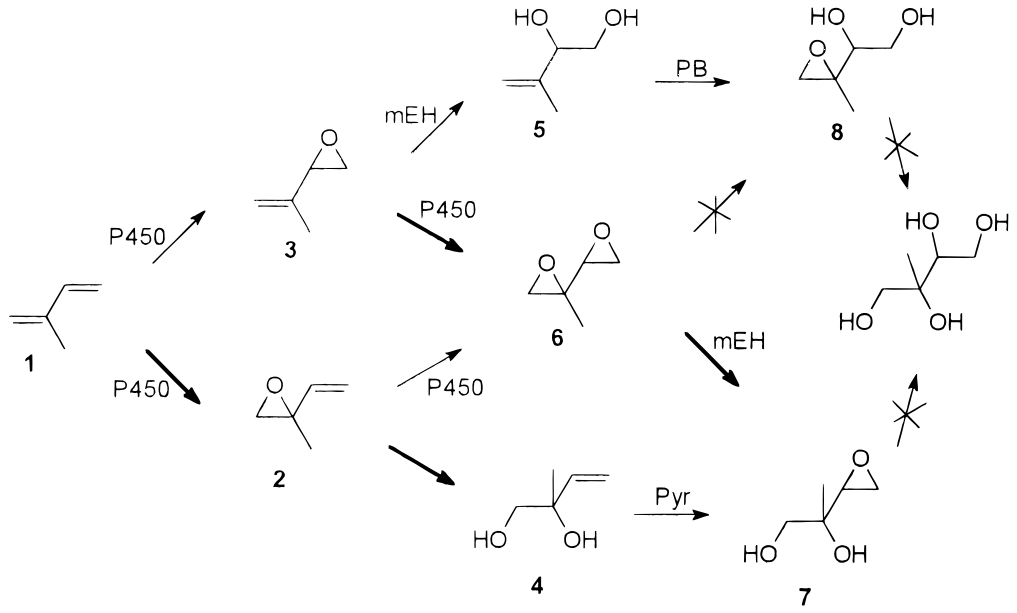
Scheme 1



nonenzymatic oxirane ring opening, a racemic process which can affect both the substrate and product enantioselectivity. The stereochemical behavior of the isoprene biotransformation is therefore affected by the stereo- and enantioselection of the oxidation processes as well as of the hydrolysis reactions. With regard to oxidation processes, it has been evidenced (*13*) that the epoxidation of alkyl-substituted olefins, catalyzed by P450s, may occur with a product stereoselectivity which strongly depends on the specificity and composition of the P450 enzymes. Furthermore, induction may also favor the mEH[1] (microsomal epoxide hydrolase)-catalyzed hydrolysis of epoxides with respect to the nonenzymatic process. In the oxidation of isoprene and its monoepoxides, the prominent role of P450 2E1 has been demonstrated (*9*), but the importance of this isoform in the stereochemical course of these biotransformations has not been investigated.

Therefore, to gain further insight into the stereochemical and mechanistic aspect in the in vivo metabolism of isoprene, we investigated (i) the effect of the pretreatment of rats with two P450 inducers, phenobarbital (PB) and pyrazole (Pyr), on the stereochemistry of the oxidation of racemic epoxides **2** and **3** to the isomeric diepoxides *erythro* and *threo*-**6**; (ii) the biotransformation of diols **4** and **5**, arising from the chemical and/or mEH-catalyzed hydrolysis of the metabolites **2** and **3**, to the corresponding *erythro* and *threo* epoxydiols **7** and **8**; (iii) the mEH-catalyzed hydrolysis of diepoxides **6** to epoxydiols **7** and/or **8**; and (iv) the mEH-catalyzed hydrolysis of both **7** and **8**.

Materials and Methods

Caution: *The following chemicals are hazardous and should be handled carefully: 2-isopropenyloxirane, 2-methyl-2-vinyloxirane, and 2-methyl-2,2′-bioxirane.*

Materials. PB and Pyr were obtained from common commercial sources. Isoprene (**1**) and (±)-2-methyl-2-vinyloxirane (**2**) were purchased from Aldrich Chemical Co. (Milwaukee, WI). (±)-2-Isopropenyloxirane (**3**) and a mixture of *erythro*- and *threo*-2-methyl-2,2′-bioxirane (**6**) were prepared as previously reported (*8*).

(±)-2-Methyl-3-butene-1,2-diol (4) and (±)-3-methyl-3-butene-1,2-diol (5) were prepared by acid-catalyzed hydrolysis (1 N $HClO_4$) from the corresponding oxiranes (**2** and **3**).

***erythro*- and *threo*-(±)-3,4-Epoxy-2-methyl-1,2-diol (7).** To a solution of *m*-chloroperoxybenzoic acid (350 mg, 1.35 mmol) in CH_2Cl_2 (25 mL), previously dried over $MgCl_2$, was added diol **4** (69 mg, 0.67 mmol). The reaction mixture was allowed to stand for 3 days at room temperature and 4 h at 4 °C. The solution was filtered, and 80 mg (1.35 mmol) of KF, previously activated by heating at 120 °C in vacuo (0.1 mmHg) for 2 h, was added. The mixture was stirred at room temperature for 1 h; then the insoluble complexes were filtered off, and the solvent was removed under reduced pressure to give a 6:4 mixture of epoxy diols *erythro*- and *threo*-**7**. ^{1}H NMR ($CDCl_3$): δ 1.21 (s, 3H), 1.25 (s, 3H), 2.70 (dd, J = 5.3 and 4 Hz, 1H), 2.78 (dd, 1H), 2.79 (dd, 1H), 2.85 (dd, J = 4 and 2.8 Hz, 1H), 3.50 (d, AB system, J = 11.5 Hz, 1H), 3.55 (d, AB system, J = 11 Hz, 1H), 3.80 (d, AB system, J = 11.5 Hz, 1H), 3.65 (d, AB system, J = 11 Hz, 1H). ^{13}C NMR ($CDCl_3$): δ 20.4, 22.0, 42.7, 44.6, 55.8, 56.5, 69.7, 70.5.

***erythro*- and *threo*-(±)-3,4-Epoxy-3-methyl-1,2-diol (8).** A 6:4 mixture of *erythro*- and *threo*-**8** was prepared from **5** like **7**. ^{1}H NMR ($CDCl_3$): δ 1.37 (s, 3H), 1.39 (s, 3H), 2.66 (d, J = 4.5 Hz, 1H), 2.68 (d, J = 4.3 Hz, 1H), 2.94 (d, J = 4.5 Hz, 1H), 2.99 (d, J = 4.3 Hz, 1H), 3.78 (m, 6H). ^{13}C NMR ($CDCl_3$): δ 17.2, 18.3, 50.45, 51.5, 63.2, 63.5, 72.1, 73.6.

2(*S*)-3-Methyl-3-butene-1,2-diol (5), 2(*S*)-2-Methyl-3-butene-1,2-diol, and 2(*R*)-2-Methyl-3-butene-1,2-diol (4). A 3:1.3:0.7 mixture of diols 2(*S*)-3-methyl-3-butene-1,2-diol (**5**), 2(*S*)-2-methyl-3-butene-1,2-diol (**4**), and 2(*R*)-2-methyl-3-butene-1,2-diol (**4**) was prepared from isoprene using AD-mix α, according to the standard procedure reported by the Sharpless group (*14*).

2(*S*)-3-Methyl-1-[*p*-(toluenesulfonyl)oxy]-3-butene-1,2-diol (10), 2(*S*)-2-methyl-1-[*p*-(toluenesulfonyl)oxy]-3-butene-1,2-diol, and 2(*R*)-2-methyl-1-[*p*-(toluenesulfonyl)oxy]-3-butene-1,2-diol (9) were prepared from the mixture of diols, arising from the asymmetric Sharpless hydroxylation, as previously described (*8*). Compound **9** and **10** were separated by HLPC (Spherisorb S5 CN column, 96:4 hexane/2-propanol). Compound **9** (ee = 33%, determined by GC). ^{1}H NMR ($CDCl_3$): δ 1.27 (s, 3H), 2.45 (s, 3H), 3.88 (s, 2H), 5.15 (dd, J = 0.8 and 10.5 Hz, 1H), 5.30 (dd, J = 0.8 and 17.5 Hz, 1H), 5.80 (dd, J = 10.5 and 17.5 Hz, 1H), 7.35 (d, AB system, 2H), 7.80 (d, AB system, 2H). ^{13}C NMR ($CDCl_3$): δ 21.6, 24.1, 75.7, 115.0, 127.9,

[1] Abbreviations: mEH, microsomal epoxide hydrolase; PB, phenobarbital; Py, pyrazole; TCPO, trichloropropene oxide; GC, gas chromatography.

129.9, 139.9, 145.0. Compound **10** (ee = 95%, determined by GC). ^{1}H NMR ($CDCl_3$): δ 1.68 (s, 3H), 2.45 (s, 3H), 3.95 (dd, J = 7.5 and 10.2 Hz, 1H), 4.10 (dd, J = 3.4 and 10.2 Hz, 1H), 4.30 (dd, J = 3.4 and 7.5 Hz, 1H), 4.95 (s, 1H), 5.05 (s, 1H), 7.35 (d, AB system, 2H), 7.80 (d, AB system, 2H). ^{13}C NMR ($CDCl_3$): δ 18.5, 21.6, 72.34, 72.78, 113.58, 127.9, 129.9, 132.5, 141.9, 145.0.

2(*S*)-Isopropenyloxirane (3). To a solution of **10** (50 mg) in CH_2Cl_2 (2 mL) were added 50 mg of KOH and crown-18-ether (5 mg). The mixture, stirred at room temperature for 18 h, was filtered, and the solvent was evaporated to give (*S*)-**3** (ee = 95%, determined by GC).

2(*R*)-Methyl-2-vinyloxirane and 2(*S*)-Methyl-2-vinyloxirane (2). The 2:1 mixture of the two enantiomers of **2** was prepared from the corresponding 2:1 mixture of 2(*S*)-2-methyl-1-[*p*-(toluenesulfonyl)oxy]-3-butene-1,2-diol and 2(*R*)-2-methyl-1-[*p*-(toluenesulfonyl)oxy]-3-butene-1,2-diol like **3**. The crude product was analyzed by GC.

(2*S*,2′*R*)- and (2*R*,2′*R*)-2-Methyl-2,2′-bioxirane (6). Epoxidation of 2(*S*)-isopropenyloxirane with *m*-chloroperbenzoic acid in CH_2Cl_2 (2 h) gave a 6:4 mixture of (2*S*,2′*R*)- and (2*R*,2′*R*)-2-methyl-2,2′-bioxirane (**6**). The crude product was analyzed by GC.

Mixture of Diepoxides 6 Enriched in (2*R*,2′*S*)- and (2*R*,2′*R*)-2-Methyl-2,2′-bioxirane (6). Epoxidation of the 2:1 mixture of 2(*R*)-methyl-2-vinyloxirane and 2(*S*)-methyl-2-vinyloxirane with *m*-chloroperbenzoic acid in CH_2Cl_2 gave a mixture of the four epoxides enriched in the (2*R*,2′*S*)- and (2*R*,2′*R*)-diastereoisomers. The crude product was analyzed by GC.

(2*R*,3*R*)- and (2*R*,3*S*)-3,4-Epoxy-3-methyl-1,2-diol (8). The mixture of (2*R*,3*R*)- and (2*R*,3*S*)-**8** was prepared from 2(*S*)-3-methyl-3-butene-1,2-diol (**5**) like **8**.

Mixture of Epoxydiols 7 Enriched in (2*R*,3*S*)- and (2*R*,3*R*)-3,4-Epoxy-2-methyl-1,2-diol (7). The mixture of epoxydiols **7** enriched in the diastereoisomers (2*R*,3*S*)- and (2*R*,3*R*)-**7** was prepared from 2(*S*)-2-methyl-3-butene-1,2-diol (**4**) like **7**.

Animals and Microsomal Preparations. Male Sprague-Dawley rats were purchased from Charles River. They were treated for 3 days with PB ip (80 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*).

Enzymatic Incubations. (1) Determination of the Cytochrome P450 Activity and of the Enantiomeric Excesses of the Products in the Oxidation of Monoepoxides 2 and 3. Incubation mixtures (2 mL) containing 100 mM potassium phosphate buffer (pH 7.4), 4 mg of hepatic microsomal proteins, a 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 initiated by the addition of a proper amount of **2** or **3**. 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 (Carbowax column, 90 °C) after addition of appropriate amounts of cycloheptanone as an internal standard. The substrate concentrations ranged from 0.1 to 20 mM.

The enantiomeric composition of **6** was determined by GC using a 30 m Chiraldex G-TA (ASTEC) column, with a helium flow of 50 KPa, and with an evaporator and detector set at 200 °C, under the following conditions: 32 °C for 12 min, at a rate of 10 °C/min, 55 °C for 4 min, at a rate of 10 °C/min, and 75 °C for 22 min.

The absolute configurations of the excess enantiomers of **6** were determined by comparison of the retention times of the two enantiomers of each isomer with those of (2*S*,2′*R*)-, (2*R*,2′*R*)-, and (2*R*,2′*S*)-**6** obtained as reported above.

(2) Determination of the Cytochrome P450 Activity in the Oxidation of Diols 4 and 5. Incubation mixtures (10 mL) containing 100 mM potassium phosphate buffer (pH 7.4), 2 mg of hepatic microsomal proteins, a NADPH-generating system (consisting of 0.5 mM $NADP^+$, 5 mM glucose 6-phosphate, and 0.5 unit/mL glucose-6-phosphate dehydrogenase), and TCPO (1 mM) to inhibit the mEH were initiated by the addition of **4** or **5** (30 mM). After 60 min, a saturating amount of NaCl was added to precipitate the microsomal proteins and the reaction mixtures were lyophilized. The solid phase was then dissolved in ethyl acetate (2 mL), filtered, and analyzed by GC (NPGS column, 120 °C) after addition of appropriate amounts of an ethyl acetate stock solution of cycloheptanediol as an internal standard.

(3) Determination of the Product Enantioselectivity of the mEH-Catalyzed Hydrolysis of Monoepoxides 2 and 3. Aliquots (10 μL) of an ethanolic stock solution of (±)-**2** or (±)-**3** were added to 1 mL of a microsomal preparation (control or PB-induced) containing 2 or 4 mg of protein/mL in a such way to obtain a substrate concentration of 5, 10, or 20 mM, and the reaction mixtures were incubated at 37 °C. At prefixed times (10, 25, 50, and 80 min), a saturating amount of NaCl was added to precipitate the microsomal proteins, and the incubation mixtures, after addition of a proper amount of cycloheptanol as an internal standard, were analyzed directly by GC (Carbowax column, 120 °C) to determine the diol yields.

The enantiomer ratios and the absolute configurations of the formed diols were determined, after extraction with ethyl acetate (5 × 1 mL) by GC on a chiral 30 m Chiraldex G-TA (ASTEC) column (helium flow of 50 KPa, and with an evaporator and detector set at 200 °C, at 85 °C) by comparison of the retention times with those of samples of 2(*S*)-3-methyl-3-butene-1,2-diol [(*S*)-**5**], 2(*S*)-2-methyl-3-butene-1,2-diol [(*S*)-**4**], and 2(*R*)-2-methyl-3-butene-1,2-diol [(*R*)-**4**] obtained as reported above.

(4) Determination of the Product Enantioselectivity of the mEH-Catalyzed Hydrolysis of Diepoxides 6. Aliquots (10 μL) of an ethanolic stock solution of a 60:40 mixture of (±)-*erythro*-**6** and (±)-*threo*-**6** were added to 1 mL of a microsomal preparation (control or PB-induced) containing 2.5 or 5 mg of protein/mL in a such way to obtain a substrate concentration of 10, 25, or 50 mM, and the reaction mixtures were incubated at 37 °C and pH 7.4. At prefixed times (30 min and 1, 2, 4, and 6 h), a saturating amount of NaCl was added to precipitate the microsomal proteins, and the incubation mixtures, after addition of a proper amount of cycloheptanediol as an internal standard, were analyzed directly by GC (NPGS column, 120 °C) to determine the epoxydiol yields.

The enantiomer ratio and the absolute configurations of the formed epoxydiols **7** and **8** at 20% conversion (6 h) were determined, after dehydration and extraction with ethyl acetate, by GC on a chiral 30 m Chiraldex G-TA (ASTEC) column (helium flow of 50 KPa, and with an evaporator and detector set at 200 °C, at 90 °C) by comparison of the retention times with those of samples of *erythro*-(2*R*,3*S*)-3,4-epoxy-2-methyl-1,2-diol and *threo*-(2*R*,3*R*)-3,4-epoxy-2-methyl-1,2-diol obtained in excess by epoxidation of a 2:1 mixture of (*S*)-2-methyl-3-butene-1,2-diol and (*R*)-2-methyl-3-butene-1,2-diol, as reported above. The *erythro* and *threo* isomers have been identified, on the same column, on the basis of the ratio (around 6:4) between the two pairs of enantiomers corresponding to the ratio determined by NMR.

(5) Evaluation of the mEH-Catalyzed Hydrolysis of Epoxydiols 7 and 8. Aliquots (10 μL) of an ethanolic stock solution of mixtures of (±)-*erythro*-**7** and (±)-*threo*-**7** or (±)-*erythro*-**8** and (±)-*threo*-**8** were added to 2 mL of microsomal preparation (control or PB-induced) containing 2.5 or 5 mg of protein/mL in a such way to obtain a substrate concentration of 50 mM, and the reaction mixtures were incubated at 37 °C and pH 7.4. At prefixed times (at 30 min and 1, 2, 4, and 6 h), a saturating amount of NaCl was added to precipitate the microsomal proteins, and the incubation mixtures, after addition of a proper amount of cycloheptanediol as an internal standard, were analyzed directly by GC (NPGS column, 120 °C) to determine the epoxydiol yields. The epoxydiols **7** or **8** were always quantitatively recovered, showing that these compounds were not substrates for the mEH.

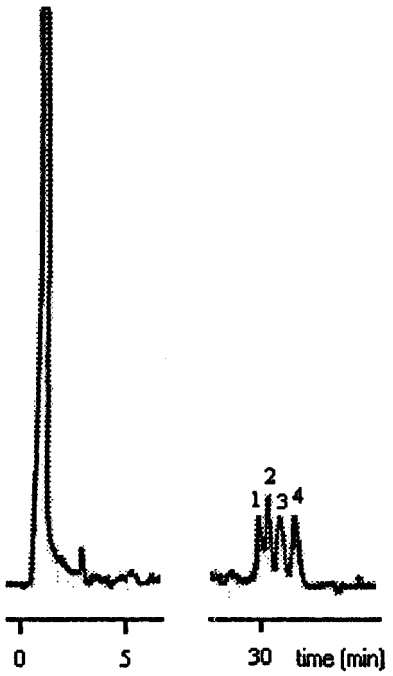


Figure 1. Gas chromatographic enantiomer separation of (2*S*,2′*S*)-**6** (1), (2*S*,2′*R*)-**6** (2), (2*R*,2′*R*)-**6** (3), and (2*R*,2′*S*)-**6** (4). Analysis conditions are outlined in Materials and Methods.

Rates of the Spontaneous Hydrolysis of 2 and 3. Aliquots (10 μL) of an ethanolic stock solution of (±)-**2** (0.5 or 1 M) or (±)-**3** (1 or 2 M) were added to 1 mL of Tris-HCl buffer (pH 7.4), and the reaction mixtures were thermostated at 37 °C. Samples were withdrawn at 10 min intervals, neutralized with $CaCO_3$, and subjected to GC analysis (Carbowax column, 120 °C). The reactions were carried out in duplicate. The diol concentration versus time data were fitted to the integrated first-order rate law ($\ln[\text{diol}] = kt + \ln[\text{diol}]_0$), and the rate constants k were obtained with the usual least-squares procedure.

Results and Discussion

Epoxidation of Monoepoxides 2 and 3 Catalyzed by Rat Liver Microsomes. The stereochemical course of the double bond oxidation of the epoxides **2** and **3** in the corresponding diepoxides **6** was investigated by incubating **2** and **3** (20 mM) by using liver microsomes obtained from untreated rats or rats pretreated with PB and Pyr, classical inducers of 2B1/2, 3A, and 2E1, 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 the chiral column. The use of the chiral column allowed evaluation of not only the diastereoisomeric ratio of the formed diepoxides **6** 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 with known configurations. A 6:4 mixture of (2*S*,2′*R*)- and (2*R*,2′*R*)-2-methyl-2,2′-bioxirane (**6**)[2] was obtained by epoxidation of the optically active 2(*S*)-isopropenyloxirane, prepared via a monotosyl derivative from the corresponding diol, the latter synthesized by asymmetric Sharpless dihydroxylation (*14*). Similarly, a mixture of four diepoxides **6**, enriched in the isomers (2*R*,2′*S*)- and (2*R*,2′*R*)-2-methyl-2,2′-bioxirane (**6**), was prepared by epoxidation of 2(*S*)-methyl-2-vinyloxirane (ee = 33%) obtained from the corresponding diol arising from the asymmetric Sharpless dihydroxylation (*14*).

Table 1. Stereochemical Course for the Oxidation of 3 by Microsomes from Control and Treated Rats[a]

microsomes	(2*R*,2′*S*)-**6** (*a*)	(2*S*,2′*S*)-**6** (*b*)	(2*S*,2′*R*)-**6** (*c*)	(2*R*,2′*R*)-**6** (*d*)
CTR	26	19	36	19
PB	17	13	17	53
Pyr	12	16	34	38

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

Table 2. Effect of PB and Pyr Pretreatment of Rats on the Microsomal Oxidation of Monoepoxides 2 and 3[a]

microsomes	**2** (nmol)	**3** (nmol)
CTR	15 ± 4	275 ± 8
PB	26 ± 7	260 ± 19
Pyr	70 ± 15	475 ± 26

[a] Values are means ± SD of three experiments performed with different preparations of hepatic microsomes. The protein concentration was 2 mg/mL, and the incubation time was 60 min.

Only for epoxide **3** was it possible to obtain data related to the stereochemical course of the oxidation of the remaining double bonds (Table 1). Attempts to study the substrate and product enantioselectivity of the epoxidation of **2** failed due to the low biotransformation rate of this substrate. Since conflicting results have been reported about the oxidation rate of this epoxide by P450s (*4*, *8*), to obtain further information about the microsomal biotransformation of **2** and **3**, the reaction mixtures were analyzed by GC using a Carbowax glass column. In this case, all the stereochemical information was lost, but it was possible to quantify the very low amounts of the product.

In agreement with the data previously reported by Wistuba et al. (*8*), the epoxide **2** may also be oxidized by the P450 system, although at a rate lower than that obtained using epoxide **3** as the substrate (Table 2). Furthermore, an increase in the oxidation rates of both monoepoxides was observed in the Pyr-induced microsomes compared to the control microsomes according to the high turnover toward these substrates shown by the recombinant P450 2E1 among the P450 human isoforms (*9*). The oxidation of **2** performed with control or induced microsomes followed simple Michaelis−Menten kinetics, which were linear up to 45 min and 1.5 mg of microsomal protein. From the Lineweaver−Burk plots of **6** formation data, we determined the apparent kinetic parameters V_{max} and K_M illustrated in Table 3. Pyr microsomes exhibited a V_{max} that was higher (about 3-fold) than that obtained with control microsomes, although also with a higher K_M. It is worth noting that the apparent K_Ms for the oxidation of **2** shown in Table 3 are similar to the K_M observed for the oxidation of **3** by liver microsomes from PB-induced rats (*7*).

Related to the stereochemical behavior of the epoxidation of **3**, in accord with the results obtained by

[2] The carbon atom C-2 of isopropenyloxirane becomes C-2′ in 2-methyl-2,2′-bioxirane because of the formal change of the carbon atom numbering caused by the IUPAC rule. Furthermore, as a consequence of the formal change in the descriptor caused by the priority rule of Chan, Ingold, and Prelog, the 2*R* (or 2*S*) stereochemistry of the isopropenyloxirane becomes 2′*S* (or 2′*R*) in 2-methyl-2,2′-bioxirane and the 2*R* (or 2*S*) stereochemistry of the 2-methyl-2-vinyloxirane becomes 2*S* (or 2*R*) in 2-methyl-2,2′-bioxirane.

Scheme 2

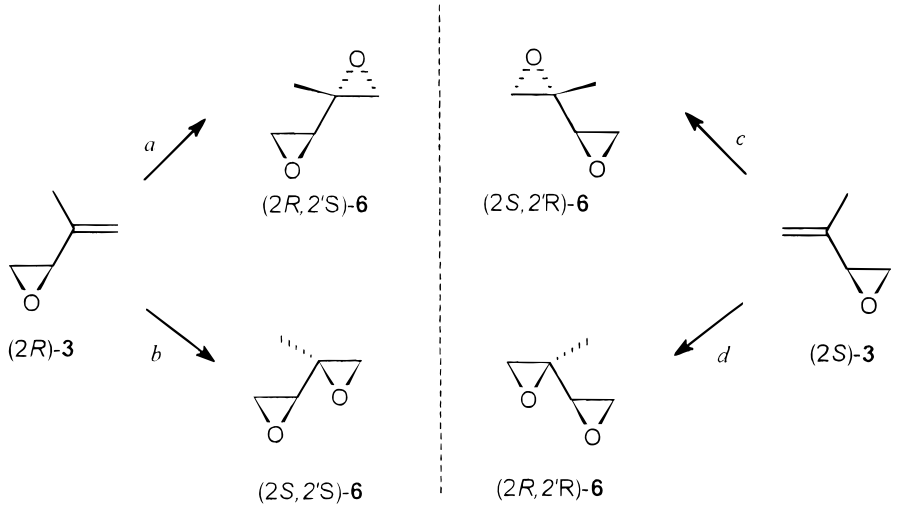


Table 3. Apparent Kinetic Constants for the Oxidation of Monoepoxide 2 by Microsomes from Control and Treated Rats[a]

microsomes	K_M (mM)	V_{max} [nmol min^{-1} (mg of protein)$^{-1}$]
CTR	0.13 ± 0.06	0.14 ± 0.03
PB	0.28 ± 0.11	0.20 ± 0.04
Pyr	0.83 ± 0.32[b]	0.37 ± 0.06[b]

[a] Values are means ± SD of three experiments performed with different preparations of hepatic microsomes. [b] Significantly different from control microsomes as determined by a Student's *t* test ($p < 0.05$).

Wistuba et al. (*8*) using untreated rat liver microsomes, the data reported in Table 1 show that the product distribution, at least with control microsomes, is characterized by a moderate selectivity. It must be mentioned that the stereochemical results, arising from the competition among processes *a*−*d* (Scheme 2), may be rationalized considering three stereoselective processes: substrate enantioselectivity, product diastereoselectivity, and product enantioselectivity.

The substrate enantioselectivity, given by *a* and *b* versus *c* and *d*, is extremely low (45:55) and in favor of the (2*S*)-configured oxirane carbon. A moderate substrate enantioselectivity in favor of the (2*R*)-enantiomer has been reported by Wistuba (*8*). However, as it has been recently shown (*13*), the stereoselectivity of the P450-catalyzed double bond epoxidation may depend on substrate concentration, and therefore, reactions carried out under different conditions may be difficult to compare. The main result is, however, the effect of the type of P450 induction on stereoselectivity. The substrate enantioselectivity increases indeed to 70:30, always in favor of the (2*S*)-configured oxirane carbon, using PB- or Pyr-induced microsomes. It is notheworthy that, while the PB-induced microsomes do not modify the total amount of products **6**, the Pyr-induced microsomal preparations give a larger amount of **6**. This result is in agreement with the fact that P450 2E1 is considered to be the isozyme principally responsible for the biotransformation of isoprene and the two isoprene monoepoxides (*9*). Also, the product diastereoselectivity, given by *a* versus *b* and *c* versus *d*, shows a behavior that is inducer-dependent. The epoxidation of (2*R*)-**3** with control microsomes occurs preferentially at the *re* face, while that of (2*S*)-enantiomer shows a selectivity toward the *si* face. The induction, with both PB and Pyr, reverses the enantioface discrimination on the (2*S*)-enantiomer, whereas the level becomes extremely low on (2*R*)-**3**. Finally, the results reported in Table 1 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 control microsomes are used, the enantiomeric pair (2*R*,2′*S*)/(2*S*,2′*R*) was formed by epoxidation of the *re* face of (2*R*)-**3** and the *si* face of the (2*S*)-enantiomer with an enantiomeric excess (ee) of 16% in favor of the (2*S*,2′*R*)-enantiomer. This ratio decreases to practically zero with PB-induced microsomes and increases again to 48% in favor of the (2*S*,2′*R*)-enantiomer using Pyr-induced microsomes. On the contrary, the enantiomeric pair (2*S*,2′*S*)/(2*R*,2′*R*) was formed in a practically racemic way when the incubations were carried out using control microsomes, and it was characterized by enantiomeric excesses of 60 and 40%, respectively, in favor of the (2*R*,2′*R*)-enantiomer when the PB- or Pyr-induced microsomes were used.

These results therefore seem to indicate that, as previously observed (*13*) for the product enantioselectivity in the P450 oxidation of prochiral olefins, more than one isoform is involved in the epoxidation process and the single enzymes catalyze the reaction with a different stereoselectivity, substrate enantioselectivity, product enantioselectivity, and product diastereoselectivity. The substrate and product enantioselectivity imply a different interaction of the two enantiomers of the substrate with the active site of the distinct P450 enzymes in the fundamental state (affecting the binding) and/or in the activate complex (affecting k_{cat}). On the other hand, the product diastereoselectivity may arise, if the epoxidation occurs through a concerted mechanism (*13*), exclusively from a different face recognition which can be determined by the site active architecture or by the ability of these enzymes to use different iron species (peroxo, hydroperoxo, or oxenoid) as the active oxidant.

Epoxidation of Diols 4 and 5 Catalyzed by Rat Liver Microsomes. The ability of diols **4** and **5**, arising from the hydrolysis of epoxides **2** and **3**, to be the substrate of the P450s was checked by incubating **4** and **5** (30 mM) at 37 °C in 10 mM phosphate buffer (pH 7.4), in the presence of a NADPH-regenerating system and TCPO (1 mM), using liver microsomal preparations (10

mL, 2 mg of protein/mL) obtained from untreated rats or rats pretreated with PB and Pyr. At prefixed times, the incubation mixtures were stopped and lyophilized. The residue was suspended in ethyl acetate, and the organic phase was analyzed by GC. Dehydration was necessary to avoid the loss of the eventually formed epoxydiols **8** and **9** as they are hardly recovered by ethyl acetate extraction of their water solutions. Control experiments ensured a recovery of the epoxydiols **7** or **8** of >85% using this procedure. No measurable amount of epoxydiols **7** or **8** was, however, found when control microsomes were used. On the contrary, when PB- or Pyr-induced microsomes were used, very small amounts [around 10 nmol in both cases, i.e., at a rate of 18 pmol min^{-1} (mg of protein)$^{-1}$] of the corresponding epoxydiols were detected. In particular, it was found that only diol **5** was oxidized to epoxydiol **8** by using PB-induced microsomes, whereas with Pyr-induced microsomes, only diol **4** was transformed in the corresponding epoxydiol **7**. Unfortunately, in this case, the amounts of formed products were also too small to allow a stereochemical study.

Hydrolysis of Monoepoxides 2 and 3 and Diepoxides 6. The P450-catalyzed oxidation of monoepoxides **2** and **3** to diepoxides **6** competes with the hydrolysis of the primarily formed monoepoxides to the corresponding vicinal diols, a process which is a superposition of a spontaneous and an enzymatic reaction. In particular, although the spontaneous process is particularly important for epoxide **2**, it also cannot be neglected in the case of epoxide **3**. The rates of spontaneous hydrolysis of **2** and **3** were measured in Tris-HCl buffer (pH 7.4) at 37 °C, under conditions that were identical to those employed for the enzymatic reactions. Samples were withdrawn at intervals, neutralized by addition of calcium carbonate, and analyzed directly by GC on a Carbowax column, after addition of cyclohexanol as a standard. The determinations of diols **4** and **5**, at several times during the course of triplicate runs for the hydrolysis reactions, were used to evaluate the kinetic constants for the ring opening of epoxides **2** (at concentrations of 5 and 10 mM) and **3** (at concentrations of 10 and 20 mM). In both cases, the diol formation obeyed a first-order rate law with k values of $(1.1 \pm 0.1) \times 10^{-3}$ and $(3.1 \pm 0.1) \times 10^{-4}$ s^{-1} for **2** and **3**, respectively. These data show that both epoxides underwent significant nonenzymatic hydrolysis. However, the rate of hydrolysis of **2** was about 4-fold as large as that of **3**, as expected for a *gem*-disubstituted epoxide as compared to a monosubstituted one. The spontaneous hydrolysis is a racemic process able to compete significantly also in the case of **3** with the enzymatic reaction (diol **5** arising from nonenzymatic hydrolysis represents ca. 35% of the total diol when the incubations were carried out at a substrate concentration of 20 mM and using a control microsomal preparation containing 4 mg of protein/mL). To evaluate the stereoselectivity of the mEH-catalyzed hydrolysis, the incubations of **2** (5 mM) and **3** (10 mM) were carried out using PB microsomes (in which mEH is induced) containing 4 mg of protein/mL. GC analysis on the chiral column of diols **5** and **4**, at 20 and 30% conversion, respectively, arising from hydrolysis of **3** and **2**, showed a 70:30 and a 40:60 ratio of the two peaks corresponding to the (*R*)- and (*S*)-enantiomers of **5** and **4**, respectively. After correction for the racemic diol obtained nonenzymatically, the ee of the enzymatically formed diol (*R*)-**5** was 70% and that of (*S*)-**4** 30% (Table 4). It is noteworthy that, although a substrate enantioselection was observed in the mEH hydrolysis of **3** (*8*), the formation of a practically racemic diol was reported to be independent of the incubation time. Furthermore, when it is considered that with monoalkyl- and *gem*-dialkyl-substituted oxiranes the mEH-catalyzed ring opening occurs at the less hindered oxirane carbon atom, the stereochemistry of the preferentially formed diols is in agreement with the generally observed mEH selectivity (*18*) and with the substrate enantioselectivity previously reported for the hydrolysis of **2** and **3** catalyzed by rat liver microsomes (*8*). In particular using rabbit liver microsomes, it has been shown that the hydrolysis of monosubstituted oxiranes proceeds with a substrate selectivity that is strongly dependent on substituent but always favoring the oxirane ring opening of the (*R*)-enantiomer (*19*). The introduction of a methyl group to give a *gem*-disubstituted oxirane markedly increases the hydrolysis rate, reversing the substrate enantioselection, and the transformation of the (*S*)-enantiomer is generally favored (*20*).

Table 4. Enantiomeric Ratio and Absolute Configuration of Diols 4 and 5 Formed by Hydrolysis of Racemic Monoepoxides 2 and 3 with Microsomes from PB-Induced Rats[a]

		diol		
substrate	hydrolysis (%)		enantiomeric ratio[b]	absolute configuration
2	30	**4**	40:60 (65:35)	*S*
3	20	**5**	70:30 (85:15)	*R*

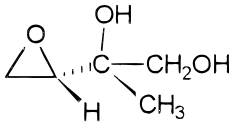
[a] Each value represents the mean of two determinations. The incubations and analyses were performed as described in Materials and Methods. [b] Values in parentheses are corrected for the amount of racemic diol formed by nonenzymatic hydrolysis and give an evaluation of the substrate selectivity of this reaction.

Hydrolysis of Diepoxides 6 and Epoxydiols 7 and 8. To obtain a more complete picture of the biotransformation of isoprene and its metabolites, the mEH-catalyzed hydrolysis of diepoxides **6** and epoxydiols **7** and **8** has been investigated. The experiments were carried out by incubating the mixtures of the diastereoisomeric diepoxides *erythro*-(±)-**6** and *threo*-(±)-**6**, or of the diasteroisomeric epoxydiols *erythro*-(±)-**7** and *threo*-(±)-**7**, and *erythro*-(±)-**8** and *threo*-(±)-**8** (10−50 mM) with a microsomal preparation from control or PB-induced rats at 37 °C and in potassium phosphate buffer (pH 7.4) containing 2.5 or 5 mg of protein/mL. The incubations were stopped at several times (between 30 min and 6 h), and the residual **6** or the formed epoxydiols **7** and **8** were identified and quantified by GC, after addition of cycloheptanediol as an internal standard. No biotransformation was observed in the cases of **7** and **8**. The epoxydiols were also quantitatively recovered after incubation for 6 h using PB-induced microsomes, showing that these compounds were not substrates for the mEH. It is noteworthy that, although lipophilic compounds are generally the best substrates for mEH, epoxides bearing a hydroxyl group may be hydrolyzed by this enzyme. The four stereoisomers of 1,2-epoxyhexan-3-ol are not only substrates for mEH but also biotransformed into the corresponding triols with a high rate (*13*). The results found in this work, related to the hydrolysis of epoxydiols **7** and **8**, therefore suggest that the mEH-catalyzed oxirane ring opening of epoxides bearing hydroxyl groups is strongly dependent on the ability of the alkyl portion

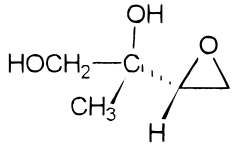
of the substituent to the oxirane ring to attenuate the polar effect due to the OH group(s). This probably affects the formation of the enzyme−substate complex which arises from a delicate balance of entropic and enthalpic factors (*21*).

Different from **7** and **8**, diepoxides **6** were substrates for mEH, although the biotransformation, which was not under saturation conditions even at the high substrate concentration (50 mM), occurred with a moderate rate. The extent of product formation was indeed linear with time, microsomal protein amount, and substrate concentration, and a conversion higher than 20% has never been obtained even after incubation for 6 h.

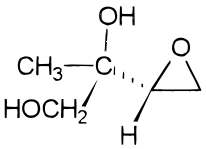
The reaction gave exclusively the epoxydiols **7** arising from the selective opening of the more substituted oxirane ring, and at 20% conversion, all four isomers of **7** were present in a 27:45:15:13 ratio.



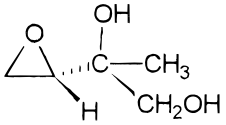
erythro-(2*R*,3*S*)-**7**



erythro-(2*S*,3*R*)-**7**



threo-(2*R*,3*R*)-**7**



threo-(2*S*,3*S*)-**7**

By co-injection of the reaction mixture product with samples with a known configuration, obtained as reported in Materials and Methods, it has been established that the ratio between the diastereoisomeric pairs *erythro* and *threo* was 70:30 and the reaction occurred with a product selectivity favoring the formation of the enantiomer having the (*R*)-configuration at C-2.[3] When the regioselectivity generally observed in the mEH-catalyzed oxirane ring opening of *gem*-dialkyl-substituted oxiranes is considered (the selective attack occurs on the less substituted carbon), the results presented here show that of the two diastereoisomeric pairs of **6**, the *erythro* isomers are preferentially hydrolyzed with a moderate selectivity in favor of the (2*R*,2′*S*)-enantiomer.

Conclusions

In conclusion, these results show that the stereochemical course of the biotransformation of isoprene formulates a composite picture markedly affected by the composition of different P450 present in the control or induced rat liver.

Between the two primarily formed metabolites, 2-methyl-2-vinyloxirane (**2**) and isopropenyloxirane (**3**), the main product (*4*), epoxide **2**, is rapidly transformed into the corresponding vicinal racemic diol **4**, predominantly through a nonenzymatic hydrolysis reaction. On the other hand, epoxide **3**, which is more stable toward the nonenzymatic hydrolysis reaction, is biotransformed into the diol **5** by microsomal epoxide hydrolase. The reaction occurs with a fairly good substrate and product enantioselectivity favoring the hydrolysis of the (*R*)-enantiomer to give, before 50% conversion, selectively (*R*)-3-methyl-3-butene-1,2-diol, **5**.

This hydrolysis reaction competes with the oxidation of the monoepoxide **3** to the corresponding diepoxides **6**. When racemic compound **3** was used as a substrate, the P450-catalyzed oxidation of the remaining double bond is generally characterized by a moderate stereoselectivity (substrate enantioselectivity, product diastereoselctivity, and product enantioselectivity) which, however, strongly depends on the composition of the P450 isoforms. Treatment of rats with PB leads with a higher selectivity to *threo*-(2*R*,2′*R*)-**6**, while with Pyr the formation of both *erythro*-(2*S*,2′*R*)- and *threo*-(2*R*,2′*R*)-**6** was favored.

The mEH-catalyzed hydrolysis of epoxides **6** proceeds, although with a low turnover rate, with a substrate and product diastereo- and enantioselection by nucleophilic attack on the more substituted oxirane ring to give selectively (2*R*,3*S*)-3,4-epoxy-2-methyl-1,2-diol (**7**).

Both diols **4** and **5** may be further oxidized by P450. The epoxidation of the double bond, however, occurs slowly, and the reaction is markedly dependent on P450 induction.

At the present time, no data about the genotoxic activities of the epoxydiols **7** and **8** are available, although their activity is expected as seen for the butadiene epoxydiol (*11*). Contribution to the overall toxicity of isoprene in addition to other reactive intermediates and in particular the diepoxide, a potent mutagen (*7*), may be provided in particular by the epoxydiol **7**. Indeed, only this metabolite [namely, the (2*R*,3*S*)-3,4-epoxy-2-methyl-1,2-diol] and not the epoxydiol **8** may be formed at a consistent rate from the mEH-catalyzed hydrolysis of the diepoxide, and it might represent an important toxic metabolite of the isoprene biotransformation process as in the case of butadiene (*22*). The formation of the epoxydiols **7** and **8** would be possible through the P450-dependent oxidation of the diols **5** and **6**, but a significant contribution of this pathway is not expected because of the low rates observed in these enzymatic reactions.

Since the toxicity of many metabolites depends on their stereochemistry, the results presented here, showing that the stereochemical course of the isoprene biotrasformation is markedly affected by induction, suggest that environmental or diet exposure to certain substances may considerably modify the metabolic pathway of isoprene and consequently the toxicity of this natural compound largely produced by the petrochemical industry.

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[3] As a consequence of the formal change in the descriptor caused by the priority rule of Chan, Ingold, and Prelog, the 2*R* (or 2*S*) stereochemistry of the 2-methyl-3-butene-1,2-diol becomes 2*S* (or 2*R*) in 3,4-epoxy-2-methyl-1,2-diol and the 2*R* (or 2*S*) stereochemistry of the 3-methyl-3-butene-1,2-diol becomes 2*S* (or 2*R*) in 3,4-epoxy-3-methyl-1,2-diol.

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