TOXICOKINETICS AND METABOLISM OF 1,2-DIETHYLBENZENE IN MALE SPRAGUE DAWLEY RATS—PART 2: EVIDENCE FOR IN VITRO AND IN VIVO STEREOSELECTIVITY OF 1,2-DIETHYLBENZENE METABOLISM

JEAN PAUL PAYAN, BENOIT COSSEC, DOMINIQUE BEYDON, JEAN PAUL FABRY, AND ELISABETH FERRARI

Institut National de Recherche et de Sécurité, Vandoeuvre, France

(Received September 27, 2000; accepted February 28, 2001)

This paper is available online at http://dmd.aspetjournals.org

ABSTRACT:

In a previous study, it was shown that the neurotoxic compound 1,2-diethylbenzene (1,2-DEB) is mainly hydroxylated in the alkyl chain to give 1-(2’-ethylphenyl)ethanol (1,2-EPE) and excreted in urine of rats as two glucuronide compounds (GA1 and GA2). Some findings have suggested that the two enantiomers of 1,2-EPE are formed in vivo. In the present study, a chiral high-performance liquid chromatography method was developed to separate the two enantiomers of 1,2-EPE from a synthesized racemic mixture. Absolute configuration of both enantiomers was determined after esterification with (R)-(+) α-methoxy-α-(trifluoromethyl)phenylacetic acid and analysis of their 1H NMR spectra in CCl 4 added with Eu (fod) 3. The two main urinary metabolites, GA1 and GA2, from [14C]1,2-DEB-treated Sprague-Dawley rats (80 mg/kg, i.p.) were identified, after hydrolysis with β-glucuronidase from Escherichia coli, as (R) and (S) glucuronide conjugates of 1,2-EPE, respectively. In vitro hydroxylation of 1,2-DEB and glucuron conjugation of 1,2-EPE were under stereoselective control in S9 fraction or microsomes from male Sprague-Dawley rat liver. The V max and K m values for (R) and (S) conjugation were determined in S9 fraction greater than those for the (S) enantiomer. In the plasma of bile duct-cannulated rats, the ratio was 1.2 ± 0.02 over the 1- to 4-h period after oral administration of [14C]1,2-DEB (100 mg/kg). In contrast, the glucuron conjugation rate of (S)1,2-DEB enantiomer was 4 times that of (R)1,2-DEB glucuron conjugation. A similar ratio of (R) to (S)1,2-EPE glucuronide conjugates was obtained in the plasma of bile duct-cannulated rats.

Commercial grade diethylbenzene (DEB) is a colorless liquid used as an intermediate in the production of divinylbenzene (Sandmeyer, 1981). It was reported that 1,2-DEB, which is present at a concentration of 7% in commercial grade DEB, was responsible for its neurotoxicity in rats (Gagnaire et al., 1990). It was also reported that 1-(2’-ethylphenyl)ethanol (1,2-EPE) and 1,2-diacetylbenzene, which were identified in the urine of 1,2-DEB-poisoned rats, demonstrated a neurotoxicity pattern similar to that of the parent compound (Gagnaire et al., 1991, 1992). There was no evidence of embryotoxic or teratogenic effects after gavage on gestational days 6 through 20 at dose levels of 0.5 to 35 mg/kg of 1,2-DEB (Saillenfait et al., 1999).

The main metabolic pathway of 1,2-DEB is direct oxidation of the side chain (Payan et al., 1999) as previously reported for the metabolism of ethylbenzene in humans (Angerer and Lehnert, 1979; Drummond et al., 1984; Engström, 1984; Engström et al., 1985) and rabbits (Kiese and Lenk, 1974). After intravenous or oral administration of 1,2[14C]DEB to male Sprague-Dawley rats, radioactivity was mainly excreted in the urine (68–75% of the dose), and to a lesser extent in the feces (15–24% of the dose), or via exhaled air (3–5% of the dose) (Payan et al., 1999). However, experiments with rats fitted with a biliary cannula demonstrated that about 66 to 75% of the administered doses (1 or 100 mg kg⁻1) were initially excrated in the bile, extensively reabsorbed from the gut, and finally excreted in the urine after several enterohepatic recirculations. Insignificant amounts of unchanged 1,2-DEB were recovered in the different excreta (urine, bile, and feces). The two main [14C]1,2-DEB metabolites accounted for about 60 and 80% of urinary and biliary radioactivity, respectively, and have been shown to be the glucuronide conjugates of the two potential enantiomers of 1,2-EPE. The presence of two glucuronide conjugates of 1,2 EPE in the urine in a ratio different from one suggested that the metabolic conversion of 1,2-DEB was under stereochemical control. Thus, the present study was carried out to obtain qualitative and quantitative information on the in vitro and in vivo stereoselectivity of [14C]1,2-DEB metabolism in male Sprague-Dawley rats.

Materials and Methods

Chemicals. Radiolabeled 1,2-diyethyl[U-14C]benzene ([14C]1,2-DEB) was supplied by Amersham Pharmacia Biotech UK Ltd. (Buckinghamshire, England). It had a radiochemical purity exceeding 98.5% and a specific activity of 614 MBq mmol⁻¹ (16 mCi/mmol). Unlabeled 97% pure 1,2-DEB was purchased from Merck (Darmstadt, Germany). Mosher’s acid R (+), Eu (fod) 3,
β-glucuronidase from *Escherichia coli*, β-NADPH, and uridine 5′-diphospho-glucuronic acid (UDPGA) were provided by Sigma Chemical Co. (St. Louis, MO). All other reagents and chemicals were obtained from commercial sources at the highest purity available. The racemic mixture of 1-(2-ethylphenyl) ethanol [(rac)-1,2-EPE] was synthesized as previously described (Payan et al., 1999).

**Apparatus.** IR spectra were recorded using a Nicolet 710 FT-IR (Madison, WI). NMR data on a Bruker AC 250 (Karsruhe, Germany), and MS data on a Nermag R 10-10 T (Rueil-Malmaison, France). The HPLC system used was composed of a Waters 717 autosampler (Waters Associates, Milford, MA), a Waters 501 isocratic pump, a Waters 490E UV detector set at 263 nm, and a fraction collector (Gilson FC 224, Villier Le Bel, France). Two columns were used to analyze 1,2-DEB and its main metabolites, a Spherisorb C8 (4.6 × 250 mm, 5 μm) from Phase Sep (Pessac, France) and a reverse phase Chiralcel-OJ column (4.6 × 250 mm, 5 μm) from Daicel Chemical Industries distributed by J. T. Baker Inc. (Phillipsburg, NJ). The radioactivity contained in HPLC eluants was measured on-line with a flow scintillation analyzer (Flow-One 500Tr, Packard) or in fractions with a Tricarb (model 1900, Packard, Saint Louis, MO).

**Incubation Procedures.** Incubation reagents. The S9 fraction was prepared from livers of control male rats (Sprague-Dawley, 200–250 g) purchased from Ifla Creo (Saint Germain sur l’Arbresle, France). The livers were homogenized in an ice-cold mixture (1:2) of phosphate buffer (0.01 M, pH 7.4) and KCl (0.15 M) and immediately centrifuged at 9000g for 15 min at 5°C. The S9 fraction was stored at −80°C for up to 8 weeks. Preliminary experiments showed no reduction in enzymatic activity for 1,2-DEB hydroxylation or 1,2-EPE glucuroconjugation during this period. Protein concentration (Bradford’s method) was 33 ± 2 mg·ml⁻¹ (n = 6). S9 fraction was used for in vitro hydroxylation of 1,2-DEB or for (R) and (S)-1,2-EPE glucuroconjugation experiments.

Microsomes from livers of male rats (Sprague-Dawley) were obtained from Ifla Creo (cytochrome P450 = 0.85 nmol/mg of protein and total protein = 23 mg/ml). They were used for additional experiments on 1,2-DEB hydroxylation.

**In vitro hydroxylation of 1,2-DEB.** The incubation conditions were optimized for two concentration levels of [14C]1,2-DEB (20 and 400 μM) and by adjusting the S9 fraction and the incubation time successively. The hydroxylation rate appeared linearly related with time and protein concentration up to an incubation period of 1 h, and up to a S9 fraction dilution of 1:50, respectively. The hydroxylation step, therefore, used a mixture consisting of S9 fraction (40 μl, 1.3 mg of protein) or microsomal fraction (40 μl, 0.92 mg of protein), phosphate buffer (0.2 M, pH 7.4), MgCl₂ (1.6 mM), glucose 6-phosphaté (1 mM), and NADPH (0.8 mM) in a final volume of 2 ml. The reactions were initiated by adding 100 μl of polyethylene glycol 600 (PEG)/H₂O (1:1, v/v) containing [14C]1,2-DEB. The tubes were closed with screw plugs. After vortex mixing for 5 s, the mixture was incubated at 37°C for 60 min with constant shaking. Enzymatic reactions were stopped by immersion of the tubes in liquid nitrogen.

**In vitro glucuroconjugation of 1,2-EPE.** The 1,2-EPE glucuroconjugation step used a mixture consisting of S9 (40 μl, 1.3 mg of protein), phosphate buffer (0.2 M, pH 7.4), MgCl₂ (10 mM), glucose 6-phosphaté (1 mM), and β-NADPH (0.8 mM) in a final volume of 2 ml. After 5 min of preincubation, the substrates (R) or (S) [14C]1,2-EPE were added in PEG 50% (100 μl). The reactions were initiated by adding UDPGA (1 mM). After vortex mixing for 5 s, the mixture was incubated at 37°C for 60 min with constant shaking. Enzymatic reactions were stopped by immersing the tubes in liquid nitrogen. Prior to use, the glucuroconjugation activities of the mixture were evaluated on an aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentration was evaluated using a aliquot of S9 fraction over a 30-min period using phenolphthalein (50 mM) as substrate. The residual phenolphthalein concentratio
fraction with UDPGA as cofactor, the samples were purified with a Sep-Pak C18 cartridge. Fractions 1 and 2 were concentrated and analyzed with a chiral and reverse HPLC method, respectively.

In Vitro 1,2-DEB Stereochemical Metabolism. Rate of 1,2-DEB hydroxylation. The enzymatic constants of the [14C]1,2-DEB hydroxylation reaction were studied in S9 fraction as described above. Different [14C]1,2-DEB concentrations were tested in the 0.5 to 405 μM range. The mixtures of [14C]1,2-DEB in 50% PEG/H2O were not homogenous for concentrations of 1,2-DEB > 500 μM. The [14C]1,2-DEB and (R) and (S)-[14C]1,2-DEB contents in the incubates were analyzed with the chiral HPLC method. Enzymatic rates were expressed in nanomoles of product per minute and per milligram of total proteins. The enzymatic constants were calculated from the Lineweaver-Burk curve, which correlates the inverse ratio of enzymatic synthesis speed to the inverse ratio of incubated substrate concentrations.

Rate of (R) and (S)-1,2-EPE glucuroconjugation. The enzymatic rates of the [14C]1,2-EPE glucuroconjugation were achieved in S9 fraction with different (S) or (R)-[14C]1,2-EPE concentrations (1.7–400 μM). At the end of the incubation period (60 min), the radiolabeled metabolites were purified from the incubate medium on a C18 cartridge and analyzed by the chiral HPLC method (fraction 1) and the reverse HPLC method (fraction 2).

Analysis of 1,2-DEB and Its Metabolites in the Plasma of Bile Duct-Cannulated Rats. The animals (Sprague-Dawley rats) were acclimatized to laboratory conditions for at least 4 days prior to initiating the studies in rooms with a 12-h light/dark cycle, designed to control relative humidity to 50 ± 5% and temperature to 22 ± 1°C. Commercial food pellets (UAR Alimentation-Villeminopon, Epinay sur Orge, France) and tap water, which was complemented with 0.9% w/v NaCl and 1.5% w/v glucose, were available ad libitum (Tse et al., 1982). One day before the experiment, a catheter was introduced into the common bile duct and into the carotid artery to collect bile and blood on heparin, as previously described (Payan et al., 1999), over a period of 50 h after oral dosing of 100 mg of [14C]1,2-DEB/kg in mineral oil (2 ml/kg). Sampled blood volume was replaced by saline solution (200–250 μl). The radiolabeled metabolites in plasma samples (1, 2, and 4 h) were analyzed by reverse (14C)GA1 and (14C)GA2 or chiral ([14C]1,2-DEB and (S) and (R) [14C]1,2-EPE) HPLC methods.

Expression of Data. Results are given as mean ± S.E.M. One-way or two-way analysis of variance tests were used to determine the significance of the data. The level of significance was set at p < 0.05.

The biliary excretion algorithm was based on the Sigma minus method (Ritschel, 1980). The urinary clearance (Clu) of 1,2-EPE glucuroconjugates and the hepatic extraction of unchanged 1,2-DEB (Hse) in the plasma were calculated from eqs. 1 and 2, respectively:

\[ Cl_u = \frac{\Delta U/\Delta t}{C_p} \]  

with (ΔU/Δt) the urinary excretion rate of the metabolite over the 0- to 4-h period and Cp the mean plasma concentration (1–4 h) in the plasma after [14C]1,2-DEB administration.

\[ H_{se} = \text{unchanged 1,2-DEB concentration in plasma} \times \text{hepatic flow} \]  

with plasma hepatic flow = 1.57 ml/min/× body weight (g) 0.75/2 × 0.16 (Dallas et al., 1994).

Results

Determination of (R) and (S)-1,2-EPE Absolute Configuration. Separation of the racemic mixture of 1,2-EPE by chiral HPLC method with hexane-isopropanol as eluent gave two peaks, 1,2-EPE1 and 1,2-EPE2, with elution times of Et = 6.1 min and Et = 7.1 min, respectively. The optical purity of 1,2-EPE1 and 1,2-EPE2 was higher than 96%. The infrared and 1H NMR spectra of each peak were similar to (rac)-1,2-EPE compound (Payan et al., 1999).

The derivatizations of 1,2-EPE1 and 1,2-EPE2 by (R)MTPA were monitored and optimized using a chiral HPLC method with hexane-ethanol eluent (9.75:0.25, v/v). Elution times were as follows: MTPA ester 1, Et = 6.83; MTPA ester 2: Et = 9.38; 1,2-EPE1, Et = 10.95; and 1,2-EPE2, Et = 12.75 min at a flow rate of 0.8 ml/min.

The reaction parameters (reaction time and reagent reactant ratio) were modified to obtain a quantitative derivatization. With these optimized conditions, it was verified that no racemization occurred during the synthesis and that no by-products were formed. Quantification was confirmed using purification, and the absence of racemization was confirmed by using optical purity comparison of final and initial products. The characteristics of the two 1,2-EPE esters were as follows. IR (neat): v = 3066 (Ar), 1746 (C = O), 1451 (OMe), 1268 and 1172 (C-O-C), 762 and 716 cm−1; MS (m/z, relative intensities): (189, 24.8%), (133, 100%), (117, 12.8%), (115, 6.7%), (105, 29.5%), (91, 24.8%), (77, 19.5%).

1H NMR (in CDCl3): δ (MTPA ester 1): 1.26 (t, J = 7.5 Hz, 3H), 1.55 (d, J = 6.5 Hz, 3H), 2.73 to 2.81 (p, J = 7.4 Hz, 2H), 3.47 (d, J = 0.9 Hz, 3H), 6.39 (q, J = 6.5 Hz, 1H), 7.17 to 7.45 (m, 5 + 4H) ppm. δ (MTPA ester 2): 1.26 (t, J = 7.5 Hz, 3H), 1.61 (d, J = 6.6 Hz, 3H), 2.7 to 2.82 (p, J = 7.4 Hz, 2H), 3.56 (d, J = 0.9 Hz, 3H), 6.32 (q, J = 6.5 Hz, 1H), 7.08 to 7.42 (m, 5 + 4H) ppm.

MTCA 1,2-EPE1, which had an OMe signal at a higher field than MTPA 1,2-EPE1, had a R absolute configuration (S for 1,2-EPE2, respectively) as explained by Dale and Mosher (1973). These configurations were confirmed using the Yamaguchi’s protocol (Yamaguchi et al., 1976). The OMe signal, which originally appeared at a higher field in the absence of Eu(fod)3, shifted further downfield after each addition, passing over the OMe signal. The lanthanide-induced shift (LIS) was calculated for the OMe signals of each diastereomer at each added Europium concentration (Fig. 1). As expected, the slope of the plot LIS OMe values versus molar ratio [Eu(fod)/MTPA ester] was steeper for the diastereomer that was synthesized on the basis of 1,2-EPE1. This last result confirmed that MTPA 1,2-EPE1 was the (R,R) diastereomer, and MTPA 1,2-EPE2 the (R,S) diastereomer, respectively.

Identification of the Relationships between 1,2-EPE Enantiomers and Their Respective Glucuronide Conjugates. Enzymatic hydrolysis of [14C]GA1 and [14C]GA2. The two main urinary radioactive metabolites, [14C]GA1 and [14C]GA2, excreted in the urine of [14C]1,2-DEB-treated rats were purified and separated by the reverse HPLC method (Fig. 2A). Each metabolite was hydrolyzed by a β-glucuronidase from E. coli and analyzed by HPLC. On the reverse phase column, [14C]GA1 and [14C]GA2 enzymatic hydrolysis residues gave one distinct radioactive peak with the same elution time as (rac)-1,2-EPE standard. On the chiral column, [14C]GA1 and [14C]GA2 enzymatic hydrolysis residues gave one radioactive peak with the same elution time as (R)1,2-EPE and (S)1,2-EPE standard, respectively (Fig. 2B).

Glucuron conjugation of (R) and (S)-14C1,2-EPE. The purified (R)(14C) or (S)(14C)1,2-EPE obtained after in vitro hydroxylation of pure [14C]1,2-DEB in S9 fraction with UDPGA as glucuron conjugation cofactor. On the reverse phase column, the incubated residue of (R)(14C)1,2-EPE and of (S)(14C)1,2-EPE gave one radioactive peak that had the same retention time as [14C]GA1 and [14C]GA2, respectively. In addition, the radioactivity recovered in these peaks corresponded to the decrease in the initial radioactivity of the corresponding [14C]1,2-EPE enantiomers used as substrate. After incubation of (R)(14C)1,2-EPE and (S)(14C)1,2-EPE in S9 fraction without UDPGA as a cofactor, all the radioactivity was recovered at the same elution time as (R)1,2-EPE and (S)1,2-EPE standard, respectively. GA1 and GA2 metabolites were therefore (R) and (S)1,2-EPE glucuronide forms, respectively (Fig. 2C).
In vitro 1,2-DEB Stereochemical Metabolism. Rate of 1,2-DEB hydroxylation into \((R)\) and \((S)\)1,2-EPE. The rate of \((R)\) or \((S)\)[\(^{14}\)C]1,2-EPE formation was determined by incubation of [\(^{14}\)C]1,2-DEB as substrate (0.5–405 mM) in S9 fraction from rat liver. After incubation, more than 95% of the radioactivity in the incubate corresponded to [\(^{14}\)C]1,2-DEB, \((R)\), and/or \((S)\)[\(^{14}\)C]1,2-EPE. For concentrations of [\(^{14}\)C]1,2-DEB lower than 27 mM, the ratio of \((R)\) versus \((S)\) enantiomeric forms was about 1.2. In contrast, for concentrations between 27 and 405 mM, the ratio of \((R)\) versus \((S)\) enantiomeric forms increased steadily to 1.6. In this concentration range, the Lineweaver-Burk plots were linear for both enantiomers (Fig. 3). The Michaelis-Menten constants \(K_m\) and \(V_{max}\) for \((R)\)1,2-EPE formation were 2.6 and 3.2 times that for \((S)\) enantiomer formation, respectively (Table 1). Incubation of 1,2-DEB (12 mM) was conducted in microsomal fraction from rat liver with three protein concentrations (0.125–0.5 mg/ml) and at three incubation times (5 min to 1 h). For each incubation time, the formation of the two enantiomers was proportional to the quantity of protein contained in the incubates. In every case, the ratio of \((R)\)1,2-EPE form was higher than \((S)\)1,2-EPE form (1.3–2.3) (results not shown).

Rate of \((R)\) and \((S)\)1,2-EPE glucuron conjugation. A preliminary experiment showed that \((R)\) and \((S)\)[\(^{14}\)C]1,2-EPE glucuron conjugates were not hydrolyzed in S9 fraction. The rate of \((R)\) or \((S)\)[\(^{14}\)C]1,2-EPE glucuron conjugation was determined by incubation of \((R)\) or \((S)\)[\(^{14}\)C]1,2-EPE as substrate in the range 1.7 to 400 \(\mu\)M in S9 fraction. The Lineweaver-Burk plots were linear in a concentration range from 17 to 400 \(\mu\)M and from 1.7 to 40 \(\mu\)M for \((R)\) and \((S)\) of 1,2-EPE glucuron conjugation, respectively (Fig. 4). The \(K_m\) for glucuron conjugation of \((S)\)1,2-EPE was slightly higher than that for its antipode formation. In contrast, the \(V_{max}\) value for \((S)\)1,2-EPE glucuron conjugation was about 4 times that for \((R)\)1,2-EPE glucuron conjugation (Table 2).

Analysis of 1,2-DEB and Its Metabolites in the Plasma of Bile Duct-Cannulated Rats. The peak of total radioactivity in the plasma occurred between 1 and 2 h after oral administration of [\(^{14}\)C]1,2-DEB (100 mg/kg) and subsequently decreased slowly (Fig. 5). Because radioactivity levels were low (<0.03% of the administered dose/ml) and to reduce the total volume of collected blood, analysis of metabolites was carried out on a limited number of samples (between 1 and 4 h after administration). During this period, the mean concentrations of unchanged 1,2-DEB were 1.0 ± 0.1 \(\mu\)M \((n = 9)\) (Table 3). The mean ratio of \((R)\)1,2-EPE versus \((S)\) enantiomer was 1.2 ± 0.1 \((n = 9)\) and ratio of \((R)\) versus \((S)\)1,2-EPE glucuronide conjugate was 0.28 ± 0.02 \((n = 4)\). The kinetic bile rate excretion profiles were almost identical to a previous report (Payan et al., 1999) with an apparent plateau between 29 and 34 h. The maximum excretion rate of radioactivity in bile occurred between 2 and 3 h after dosing and accounted for 4.0 ± 0.6\% \((n = 5)\) of the administered dose/h. This maximum corresponded to about 15 mmol-Eq/min/g of liver. The ratio of \((R)\) versus \((S)\)1,2-EPE glucuronide conjugate in the bile over the period of 0 to 4 h was 0.57 ± 0.07 \((n = 20)\). The sum of the two glucuronide conjugates and (rac)-1,2-EPE accounted for 83 ± 3 and 1.1 ± 1% of the radioactivity excreted in the bile. The urinary
A. GA1 and GA2 were purified from a pool of urines (n = 3) of [14C]1,2-DEB-treated rats (80 mg/kg, i.p.) by a reverse HPLC method. The ratio of GA1/GA2 peak radioactivity was 0.54. B. GA1 and GA2 were hydrolyzed separately by a β-glucuronidase from E. coli. The radioactivity contained in hydrolysate of GA1 (○) or GA2 (□) was analyzed by a chiral HPLC method. The elution time of the radioactivity peaks was compared with authentic 1,2-DEB and (R) and (S)1,2-EPE standards. C. reverse HPLC chromatogram after in vitro glucuronidation of (R) and (S)[14C]1,2-EPE (50 μM) carried out in S9 fraction from male rat liver (2 ml, 1.3 mg of protein) containing UDPGA (1 mM) as cofactor.

Fig. 2. Relationship between urinary glucuronides conjugates and their respective aglycones, enantiomers (R) and (S)1,2-EPE.
excretion rate of total radioactivity over the periods of 0 to 4 and 4 to 8 h was 3 times lower than the biliary excretion rate (1.36 ± 0.15% of administered dose/h). The ratio of \((R)\) versus \((S)\)1,2-EPE glucuronide conjugate in the urine over the period of 0 to 8 h was 0.85 ± 0.26 \((n = 4)\). The sum of the two glucuronide conjugates and \((rac)-1,2\)-EPE accounted for 74 ± 8 and 1.1 ± 1% of the radioactivity excreted in the urine, respectively.

**Discussion**

The results presented in this report confirm that the two main metabolites of 1,2-DEB in urine of treated rats are the glucuronide conjugates of the two enantiomers of 1,2-EPE and give their absolute configuration. Moreover, the experiments conducted in vitro and in vivo show that the two metabolic steps, which lead to the two glucuronide conjugates, are under stereoselective controls.

To obtain this result, a chiral HPLC method was developed, first, to separate the two enantiomers contained in the synthesized 1,2-EPE compound. The enantiomeric excess was calculated from the area under the HPLC peak to be about 0.05. This result indicates, as expected, that the chemical synthesis of 1,2-EPE is not stereoselective.

Second, the absolute configuration of the two enantiomers was determined after derivatization with \((R)\)MTPA following a modifica-
tion of the protocol described by Dale and Mosher (1973). These modifications avoided a preliminary MTPA chloride synthesis, and in the esterification step the normal used base was replaced by a dehydrating agent to avoid racemization. Based on their comparative chemical shifts and on the respective slopes of the LIS OMe curves (Fig. 1), the absolute configuration of each 1,2-EPE enantiomers has been determined.

Third, the relationship between the two R/S 1,2-EPE enantiomers and their respective glucuronide conjugates was identified by two separate in vitro methods. Moreover, it was determined that the urinary excretion of (R)1,2-EPE glucuronide conjugate was half that of the (S)-derived glucuronide conjugate.

The presence of the two glucuronide conjugates in a ratio different from one in the urine or bile of 1,2-DEB-treated rats suggests that the metabolism of 1,2-DEB is under stereochemical control (Payan et al., 1999). The in vitro experiments conducted in S9 fraction or in microsomes from rat liver confirmed this hypothesis. The $V_{\text{max}}$ and $K_{\text{m}}$ constants for the formation rate of (R)1,2-EPE enantiomer, determined in S9 fraction, were higher than those for its antipode. For 1,2-DEB concentrations lower than the $K_{\text{m}}$ values, the ratio of (R)/(S) 1,2-EPE levels was calculated to be about 1.2. This latter value closely corresponded to the value obtained in the plasma from bile duct-cannulated rats administered orally with a large dose of 1,2-DEB (100 mg/kg). Furthermore, in a group of bile duct-cannulated rats receiving the same treatment, the total $^{14}$C level in liver 1 h after administering the dose was $64 \pm 9 \mu M$ ($n = 5$) (result not shown). This last value

Fig. 4. Lineweaver-Burk curves of in vitro (R) and (S)$^{14}$C 1,2-EPE glucuron conjugation.

(R) and (S)$^{14}$C 1,2-EPE (2–400 nM) were incubated separately for 1 h in S9 fraction from male rat liver containing NADPH and UDPGA as cofactor (1 mM). The radioactivity contained in the incubates was analyzed by a reverse HPLC method. The Lineweaver-Burk curves of the formation of (R)1,2-EPE (○) and (S)1,2-EPE (■) glucuron conjugates were linear over the concentration range of 27 to 400 and 2 to 400 nM, respectively. Equations of the Lineweaver-Burk curves were as follows. For (R)1,2-EPE formation: $1/V = 512 (\pm 38) \times 1/S + 12.0 (\pm 0.4), r = 0.94$; and for (S)1,2-EPE formation: $1/V = 213 (\pm 16) \times 1/S + 2.9 (\pm 0.3), r = 0.99$.

### TABLE 2

<table>
<thead>
<tr>
<th>Enantiomer</th>
<th>$K_{\text{m}}$</th>
<th>$V_{\text{max}}$</th>
<th>$K_{\text{m}}(R)/K_{\text{m}}(S)$</th>
<th>$V_{\text{max}}(R)/V_{\text{max}}(S)$</th>
<th>$V_{\text{max}}(R)/\text{mg/min}$</th>
<th>$V_{\text{max}}(S)/\text{mg/min}$</th>
<th>$V_{\text{max}}(\text{mg/min/g liver})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(R)1,2-EPE</td>
<td>42 ± 1</td>
<td>0.082 ± 0.001</td>
<td>0.79 ± 0.02</td>
<td>0.27 ± 0.004</td>
<td>0.34 ± 0.02</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td>(S)1,2-EPE</td>
<td>53 ± 4*</td>
<td>0.31 ± 0.01*</td>
<td></td>
<td></td>
<td></td>
<td>23.8</td>
<td></td>
</tr>
</tbody>
</table>

* Significantly different from (R)1,2-EPE formation, p < 0.05.

a Ratio of the formation rate of (R) versus (S)1,2-EPE glucuronide conjugates for 1,2-EPE concentrations very high compared with $K_{\text{m}}$ values.

b Ratio of the formation rate of (R) versus (S)1,2-EPE glucuronide conjugates for 1,2-EPE concentrations very low compared with $K_{\text{m}}$ values.

c One milligram of protein in S9 mixture corresponded to 13 mg of fresh liver.
was lower than the $K_m$ value for the formation of $(R)$ and $(S)$1,2-EPE enantiomers.

The maximum glucuronide conjugation rate of $(R)$1,2-EPE enantiomer in a S9 fraction was lower than that for the $(S)$1,2-EPE glucuronide conjugation. However, their $K_m$ values were similar (40–50 µM). Consequently, the ratio of the two diastereomers should vary slightly with the concentration of their aglycones. In the in vitro experiment, the ratio of the formation rate of $(R)$ versus $(S)$1,2-EPE glucuronide conjugate was calculated to be 0.34 and 0.27 for low and high concentrations of $(R)$ and $(S)$1,2-EPE as substrates, respectively. Similar results were obtained in the plasma of rats given a low intravenous dose of 1,2-DEB (1 mg/kg; result not shown) and cannulated rats dosed orally with a large dose of 1,2-DEB (100 mg/kg, present study); the ratios were 0.33 and 0.28, respectively.

This and previous studies have shown that whatever the dose administered (1 or 100 mg/kg) or the administration route (i.v. or oral), unchanged 1,2-DEB was excreted in the urine or bile in minimal quantity. In contrast, about 80 to 90% of the radioactivity present in the urine or bile corresponded to polar metabolites. Additionally, a large part of the 1,2-DEB metabolites was mostly excreted in bile. These findings taken together suggest that metabolism of 1,2-DEB occurs mainly in the liver. The plasma concentrations of unchanged 1,2-DEB were almost constant between 1 and 4 h in bile duct-cannulated rats. Thus, the maximum extraction rate of unchanged 1,2-DEB in plasma by the liver was calculated to be less than 40 nmol/min. This value was lower than the maximum biliary excretion rate of the 1,2-DEB metabolites (150 nmol/min).

Stereochemical factors are important in the metabolism, pharmacology, and toxicology of drugs. Individual enantiomers can interact differently with enzymatic systems or receptors and produce distinct pharmacodynamic responses. In bile duct-cannulated rats, the urinary ratio of $(R)$ versus $(S)$1,2-EPE glucuronide conjugates (0.8) was greater than that in plasma (0.3), indicating that the urinary clearance...
of (R)1,2-EPE glucuronide conjugate was higher than that of its antipode. Stereoselective renal tubular secretion of organic anions has been previously reported (Spahn et al., 1987; Higaki et al., 1994; Laethem et al., 1995).

The oxidation of ethylbenzene into 1-phenylethanol, which is the main metabolic pathway, has also been shown to be under stereoch\-emical control. Notably, the ratio of (R) to (S) formation of 1-phen\-ylethanol in a microsomal preparation was similar to the ratio of maximum velocity rates of (R) to (S) formation of 1,2-EPE in S9 fraction (McMahon and Sullivan, 1969). In contrast, the glucuronide conjugates of 1-phenylethanol were excreted to a lesser extent in urine compared with 1,2-diethylbenzene. After enzymatic hydrolysis, 1-phenylethanol accounted for only 12 to 25% of total urinary metabolites in rats exposed to ethylbenzene by inhalation (Engström et al., 1995).

In conclusion, the present study has shown that in vivo and in vitro hydroxylation of 1,2-DEB give two enantiomers, which are extensively excreted in the urine or bile after glucuroconjugation. These two metabolic steps are under stereoselective control. The (S)1,2-EPE glucuronide conjugate is excreted in the urine and bile to a greater extent than its antipode.

Acknowledgments. We thank P. Mutzenhardt from NANCY 1 University (laboratoire de Méthodologie R.M.N.) in particular for helpful suggestions and stimulating discussions during our configuration study. F. Canel for technical assistance, and C. Cael for secretarial services.

References


