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Differences in Cytochrome P450-Mediated Biotransformation of 1,2-Dichlorobenzene by Rat and Man: Implications for Human Risk Assessment

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The oxidative biotransformation of 1,2-dichlorobenzene (1,2-DCB) was investigated using hepatic microsomes from male Wistar, Fischer-344 and Sprague-Dawley (SD) rats, phenobarbital (PB)- and isoniazid (ISO) pretreated male Wistar rats and from man. In addition, microsomes from cell lines selectively expressing one cytochrome P450 (P4502E1, 1A1, 1A2, 2B6, 2C9, 2D6, 2A6 and 3A4) were used. The rate of conversion was 0.09 nmol/min/mg. protein for both Wistar and Fischer-344 rat microsomes, 0.04 for SD-microsomes and 0.14 for human microsomes. Induction of Wistar rats with isoniazid (ISO, a P4502E1 inducer) or phenobarbital (PB, a P4502B1/2 inducer) resulted in an increased conversion rate of 0.20 and 0.42 nmol/min/mg. protein, respectively. Covalent binding of radioactivity to microsomal protein was similar for Wistar, Fischer and ISO-pretreated rats (16−17% of total metabolites), whereas induction with PB resulted in an increased covalent binding of 23% of total metabolites. Covalent binding was 31% for SD-microsomes and only 4.6% for human microsomes. Ascorbic acid notably reduced the amount of covalently bound metabolites for the SD-microsomes only, indicating that for these microsomes quinones were likely to be involved in this part of the covalent binding. Conjugation of epoxides with glutathione (GSH) inhibited most of the covalent binding for all microsomes. In the absence of GSH, the epoxides were hydrolyzed by epoxide hydrolase, resulting in the formation of dihydrodiols. Inhibition of epoxide hydrolase resulted in a decreased conversion and an increased covalent binding for all microsomes tested, indicating a role of epoxides in the covalent binding. Fischer-344 rat liver microsomes showed a lower epoxide hydrolase activity than microsomes from Wistar and Sprague-Dawley rats, which may explain the higher sensitivity to 1,2-DCB induced hepatotoxicity of Fischer rats *in vivo*. Conjugation of the epoxides with GSH was predominantly non-enzymatic for the rat, whereas for man, conjugation was nearly exclusively catalyzed by glutathione-*S*-transferases. This difference may be explained by the formation of a 'non-reactive' 3,4-epoxide by P4502E1 in human microsomes: incubations with microsomes selectively expressing human P4502E1 as well as human liver microsomes, resulted in the formation of similar amounts of 2,3- and 3,4-dichlorophenol (DCP), as well as two GSH-epoxide conjugates in equal amounts. For rat microsomes, one major GSH-epoxide conjugate was found, and a much higher covalent binding, particularly for the PB-microsomes. Therefore, we postulate that rat P4502B1/2 preferentially oxidizes the 4,5-site of 1,2-DCB, resulting in a reactive epoxide. Postulating these epoxides to be involved in the mechanism(s) of toxicity, human risk after exposure to 1,2-DCB will be overestimated when risk assessment is solely based on toxicity studies conducted in rat.

Introduction

1,2-Dichlorobenzene (1,2-DCB)[1] is used as a chemical intermediate in the synthesis of pesticides and other chlorinated compounds, as a solvent, and as deodorizer. Environmental contamination may lead to human exposure via inhalation and drinking water (*1*, *2*).

1,2-DCB is a potent hepatotoxicant (*3*−*6*). The hepatotoxicity of halogenated benzenes is thought to be mediated by the generation of reactive metabolites as a result of their oxidation by cytochrome P450 (P450) (*7*−*9*). Induction of these P450 enzymes enhances toxicity (*6*, *10*, *11*). The ability of 1,2-DCB to deplete glutathione (GSH) and the enhanced toxicity of 1,2-DCB after GSH depletion have been reported by several investigators (*6*, *12*, *13*).

Halogenobenzene-induced toxicity and covalent binding to macromolecules have been ascribed mainly to its primary oxidation products, the epoxides (*14*). However, in a recent study, in which the microsomal biotransformation of 1,4-DCB was investigated, we showed that quinones, secondary oxidation products, are the principal metabolites involved in covalent binding.[2] In addition, studies on bromobenzene-induced hepatotoxicity indicated that secondary quinone metabolites are involved in the alkylation of hepatic proteins (*15*, *16*). The

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[1] Abbreviations: 1,2-DCB, 1,2-dichlorobenzene; 2,3-DCP, 2,3-dichlorophenol; 3,4-DCP, 3,4-dichlorophenol; DHD, dihydrodiol; GSH, glutathione; GST, glutathione *S*-transferase; AA, ascorbic acid; PB, phenobarbital; ISO, isoniazid; P450, cytochrome P450; EH, epoxide hydrolase; CO, cyclohexene oxide; WIS, Wistar rats; FIS, Fischer-344 rats; SD, Sprague-Dawley rats; BSA, bovine serum albumine.

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majority of the protein-bound residues which could be structurally identified were quinone-derived (10−15%), whereas epoxides only accounted for less than 0.5% of total covalent binding. For 1,2-DCB, it has also been proposed that quinones play a more important role in covalent binding and liver toxicity than epoxides (*17*). However, recently we observed that *in vivo* 1,2-DCB is mainly metabolized to epoxides, which are conjugated with GSH. No quinone-derived metabolites were observed (*18*).

The present study was designed to further evaluate the individual roles of epoxides and quinones in covalent binding *in vitro*, and the roles of P450 enzymes in the formation of these metabolites. This was performed for rat as well as for man.

Materials and Methods

Materials. Microsomes of human liver (a pool of 5 individuals) were obtained from Human Biologics, Inc. (Phoenix, AZ). Microsomes from cell lines transfected with cDNAs expressing human P4502E1, 1A1, 1A2, 2B6, 2C9, 2D6, 2A6, or 3A4 or human epoxide hydrolase were obtained from the Gentest Corp. (Woburn, MA). 1,2-Dichloro[^{14}C]benzene with a radiochemical purity of >98% and a specific activity of 244 MBq/mmol was obtained from Sigma Chemical Co., St. Louis, MO. [^{35}S]Glutathione with a radiochemical purity of >90% and a specific activity of 3075 GBq/mmol was purchased from DuPont NEN, Dordrecht, The Netherlands. The unlabeled 1,2-DCB (purity >99%) was from Merck, Darmstadt, Germany. 2,3- and 3,4-dichlorophenol were from Aldrich, Steinheim, Germany. Isoniazid (isonicotinic acid) was from Janssen Chimica (Beerse, Belgium), and phenobarbital was from CAV, Utrecht, The Netherlands. Glutathione (GSH) and NADPH were obtained from Boehringer Mannheim GmbH, Germany. Cyclohexene oxide was from Sigma Chemical Co., St. Louis, MO. All other chemicals used in this study were of reagent grade.

Animals. Adult male Wistar rats (strain Crl: (WI)WUBR), Fischer-344 rats (strain CDF(F-344)/CrlBR), and Sprague-Dawley rats (strain Crl: CD(SD)BR), weighing 250−300 g and 9−10 weeks of age, were obtained from Charles River Wiga GmbH, Sulzfeld, Germany. The animals were provided ad libitum with the Institute's cereal-based rodent diet (SDS Special Diet Services, Witham, England) and tap water. The phenobarbital (PB) pretreated rats (Wistar) received drinking water with 0.1% (w/v) PB during 4 days prior to sacrifice. The isoniazid pretreated rats (Wistar) received drinking water with 0.1% (w/v) isoniazid (isonicotinic acid) during 10 days prior to sacrifice. The animal room was air-conditioned and light controlled, with a light/dark cycle of 12 h.

Preparation of Microsomes. Microsomes were isolated from male Wistar rats (untreated, phenobarbital and isoniazid pretreated) and from male Sprague-Dawley and Fischer-344 rats. The animals were sacrificed by abdominal bleeding and the livers were removed, washed, and homogenized in ice-cold 20 mM KCl-Tris buffer (pH 7.4). Livers of at least 3 animals were pooled. The liver homogenates were centrifuged for 30 min at 10000*g* and 2 °C. The supernatants were then centrifuged for 90 min at 105000*g* and 2 °C. The resulting supernatants (cytosol) were stored at −30 °C, and the pellets (microsomes) were washed with KCl-Tris buffer and centrifuged again. The pellets were then resuspended in 0.1 mM potassium phosphate buffer (pH 7.4) and stored at −80 °C.

Biochemical Assays. Total P450 content was determined by the method of Omura and Sato (*19*), modified by Rutten *et al.* (*20*). The protein concentration of the microsomes was determined according to the method of Lowry *et al.* (*21*) using bovine serum albumin (BSA) as standard.

Microsomal Incubations. All incubations were performed in duplicate. Incubation mixtures contained 0.1 M potassium phosphate buffer (pH 7.4), 3 mM $MgCl_2$, 3 mM NADPH, and hepatic microsomes from rat (pool of at least 3 animals) or man (pool of 5 individuals) at a protein concentration of 0.5 mg/mL, in a final volume of 200 μL. P450 contents of human and rat (control Wistar, ISO, PB, SD, and Fischer) microsomes were 0.49, 0.63, 0.82, 2.21, 0.74, and 0.62 nmol/mg of protein, respectively. Where indicated, additions were 5 mM GSH, 1 mM ascorbic acid (AA), 0.6 mM cyclohexene oxide (CO), 2.5% acetone (v/v), or rat liver cytosol as a source of glutathione *S*-transferases (GST), with a final protein concentration of 2 mg/mL. Substrate concentrations were ca. 15 μM, but exact concentrations were calculated for all incubation mixtures. A stock solution of [1,2-^{14}C]DCB was prepared in acetone and BSA, with a final concentration of acetone in the incubation mixtures of 0.05%, which was shown not to inhibit P4502E1 to a detectable amount (*22*). Incubation mixtures contained ca. 0.85 kBq. The reactions were started by the addition of the substrate and were performed using glass vials with Teflonized caps in a shaking water bath at 37 °C. For each experiment, control incubations were performed without NADPH. After 15 min, the reactions were stopped by adding 20 μL of 6 M HCl through the cap using a Hamilton needle. The vials were put on ice immediately and then frozen at −20 °C. After thawing on ice, the vials were centrifuged for 10 min at 1560*g* and 4 °C to precipitate the protein. The caps were removed and the supernatants were transferred to HPLC vials with Teflonized caps and immediately analyzed by HPLC. The protein pellets were resuspended in 0.5 mL of water and stored at −20 °C until further processing, i.e., measuring covalent protein binding.

Covalent Binding to Protein. Total covalent binding of radioactivity to microsomal protein was measured for all protein pellets obtained with the various experiments. Blank corrections were made for incubations without NADPH. The vials containing protein suspensions were thawed on ice and centrifuged for 15 min at 1560*g* and 4 °C. Supernatant was removed, and 1 mL of methanol was added to the pellets. Vials were extensively vortexed and centrifuged for 10 min at 1560*g*. The same procedure was followed with acetone and then *n*-hexane, until the supernatant contained background radioactivity. The pellets were dried at room temperature, dissolved in 1 mL of Soluene-350 from Packard, and mixed with 20 mL of Hionic Fluor from Packard. This mixture was analyzed for radioactivity.

Measurement of Radioactivity. Aliquots of incubation mixtures (20 μL) were mixed with 4.5 mL of Ultima Gold scintillation cocktail (Packard Instrument Co., Reading, U.K.), and radioactivity was measured in a Pharmacia Wallac S1409 liquid scintillation counter (Pharmacia, Uppsala, Sweden) using automatic external standard quench correction and was counted at efficiencies >85%.

HPLC Analysis. HPLC was carried out with a Pharmacia LKB HPLC 2248 Pump (Pharmacia, Sweden), fitted with a Pharmacia VWM 2141 UV detector operating at 254 nm. Radioactivity was measured on line with a Canberra Packard Radiomatic Detector (type A500, Flo-one Beta). A liquid flow cell of 500 or 2000 μL was used, and radioactivity was measured using Flo Scint A (Packard) with a scintillator flow of 3 mL/min. Injections of 100−160 μL were done automatically by a Pharmacia LKB-Autosampler 2157 at 4 °C. Separations were carried out using a reversed phase column, 250 × 4.6 mm, C18 hypersil ODS 5 μM (Chrompack). Eluting solvents were methanol and 20 mM ammonium acetate (pH 6.7), used with the following gradient: 0−5 min: 15% methanol; 5−10 min: 15 → 30% methanol; 10−30 min: 30 → 50% methanol; 30−45 min: 50 → 95% methanol. The flow rate was 1 mL/min.

LC-MS Measurements. Incubation mixtures with microsomes from phenobarbital pretreated rats were directly measured using LC-MS (liquid chromatography mass spectrometry) to identify the major metabolites in the absence or presence

[2] Hissink, A. M., Oudshoorn, M. J., Van Ommen, B., and Van Bladeren, P. J. Species and strain differences in the cytochrome P450-mediated biotransformation of 1,4-dichlorobenzene; implications for human risk assessment. Submitted for publication.

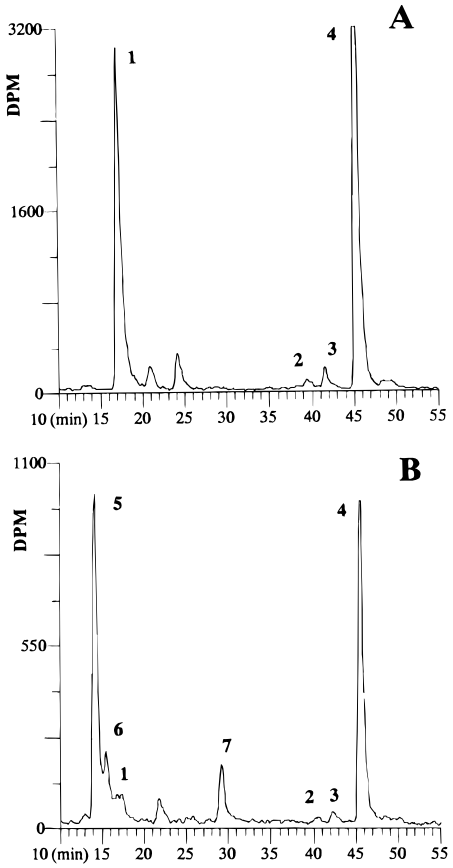

Figure 1. ^{14}C-HPLC chromatograms of incubation mixtures with microsomes from male Wistar rats pretreated with phenobarbital in the absence (A) or presence (B) of GSH. Peak 1: Dihydrodiol. Peaks 2 and 3: 2,3- and 3,4-dichlorophenol. Peak 4: 1,2-Dichlorobenzene. Peaks 5, 6, and 7: GSH conjugates of the epoxides.

of GSH. A C18 hypersil ODS 5 μM (250 × 4.6 mm) column was used. The mobile phase A was 20 mM ammonium acetate (pH 6.7), and mobile phase B was 100% methanol. The solvent gradient used was the same as described in the HPLC analysis section. The flow was 1 mL/min. Mass spectrometric analyses were performed using electrospray ionization (Finnigan ESP2) on a Finnigan MAT TSQ 700.

Results

Identification of the Major Metabolites. In Figure 1, ^{14}C-HPLC chromatograms are shown of incubation mixtures with microsomes from male Wistar rats pretreated with phenobarbital in the absence (A) or presence (B) of glutathione (GSH). Peak 1 is identified as a dihydrodiol, a hydrolyzed epoxide, since in the presence of cyclohexene oxide, an inhibitor of epoxide hydrolase, this metabolite completely disappeared. In addition, in Figure 2A a mass spectrum of this metabolite is shown. The mass at m/z = 215 arises from the molecular ion (m/z = 180) clustered with a chlorine atom, with a mass of 35. The mass at m/z = 239 arises from the dihydrodiol with acetate, which was present in the mobile phase. The mass at m/z = 179 originates from a cluster of three acetate molecules. Peaks 2 and 3 are assigned to 2,3- and 3,4-dichlorophenol (DCP) respectively, by coelution with the reference compounds. Peak 4 is 1,2-dichlorobenzene (1,2-DCB). Peaks 5 and 6 in Figure 1B, which appeared in the presence of GSH, are GSH conjugates of the epoxides of 1,2-DCB. The mass spectrum of these

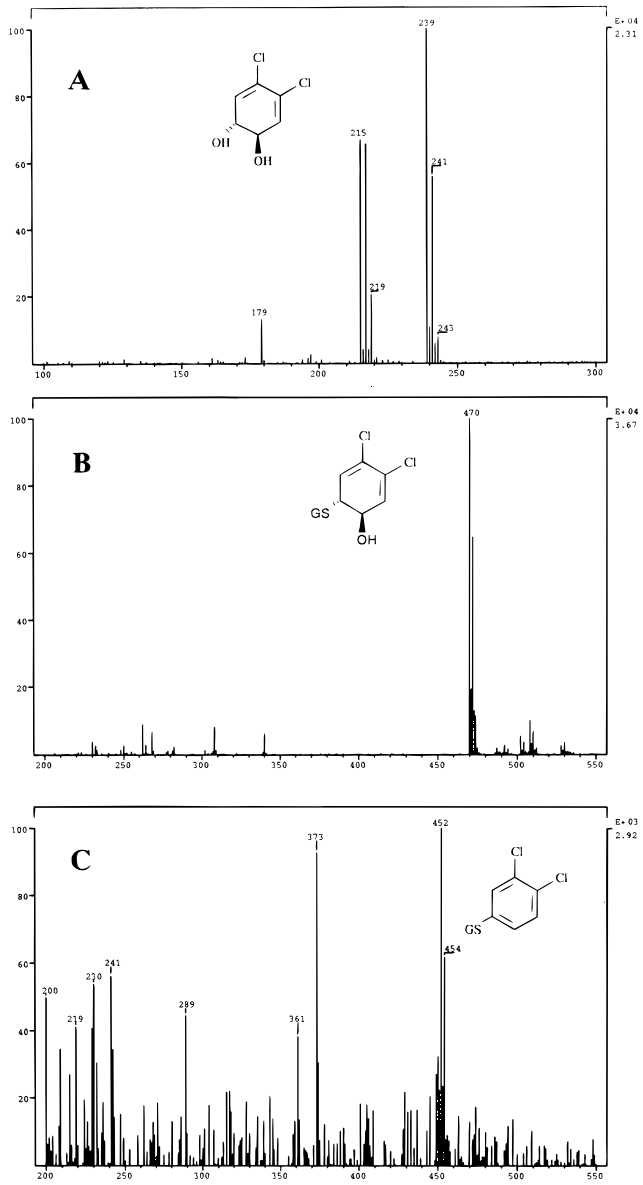

Figure 2. Mass spectra of peak 1, the dihydrodiol (A), peaks 5 and 6, the GSH conjugates of the epoxides (B, m/z = 470), and peak 7, the GSH conjugate of an epoxide with loss of a water molecule (C, m/z = 452). For the chiral metabolites in panels A and B, two alternative diastereoisomeric structures are possible; the ones with trans vicinal bonds are shown.

metabolites is shown in Figure 2B: the conjugates have a molecular ion at m/z = 470. The metabolite represented by peak 6 disappeared in time, in favor of peak 7 (results not shown). Peak 7 appeared to be formed from peak 6 by loss of a water molecule, since analysis by LC-MS revealed a molecular ion at m/z = 452 for peak 7 (Figure 2C).

In Figure 3 ^{14}C-HPLC chromatograms are shown of incubation mixtures with human liver microsomes in the absence of GSH (A), in the presence of GSH (B), and in the presence of both GSH and rat liver GST (C). Peak numbers correspond with the numbers in Figure 1. Peak 1 and peak 8 both disappeared in the presence of cyclohexene oxide, an inhibitor of epoxide hydrolase, so both metabolites were assigned to dihydrodiol (-derived) metabolites. Peak 2, 3, 4, 5, 6, and 7 constitute the same compounds as in Figure 1.

Rate of Conversion of 1,2-Dichlorobenzene by the Various Microsomes. In Table 1, the rate of conversion

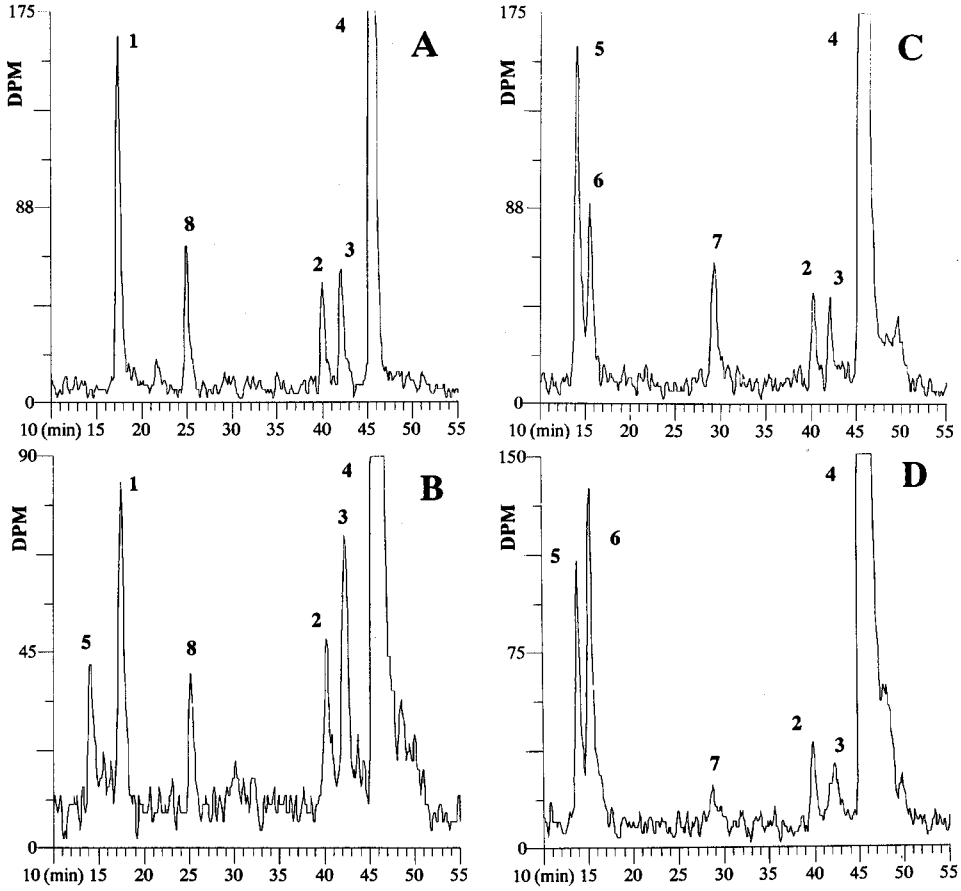

Figure 3. ^{14}C-HPLC chromatograms of incubation mixtures with human liver microsomes in the absence of GSH (A), in the presence of GSH (B), and in the presence of GSH and rat liver GST (C). Peaks 1 and 8: Dihydrodiols. Peaks 2 and 3: 2,3- and 3,4-dichlorophenol. Peak 4: 1,2-Dichlorobenzene. Peaks 5, 6, and 7: GSH conjugates of the epoxides (peak 7 = -H_2O). (D) ^{14}C-HPLC chomatogram of an incubation mixture with microsomes derived from a cell line selectively expressing human P4502E1, in the presence of GSH and rat liver GST.

Table 1. Total Conversion of 1,2-DCB and Formation of the Three Major Metabolites by the Various Microsomes

			identified metabolites (% of total conversion)[c]			
			GSH-epoxide[d]			
microsome species[a]		conversion[b]	−GST	+GST	dihydrodiol	2,3- + 3,4-DCP
Wistar	−GSH	0.090	ND	ND	49.87 (67)	21.85 (29)
	+GSH	0.075	72.04 (81)	79.59% (90)	BLQ	14.97 (17)
Wistar-ISO	−GSH	0.196	ND	ND	48.58 (143)	25.97 (76)
	+GSH	0.254	65.60 (250)	76.92 (293)	2.59 (10)	16.90 (64)
Wistar-PB	−GSH	0.424	ND	ND	42.87 (273)	4.92 (31)
	+GSH	0.320	58.42 (280)	74.82 (359)	15.31 (73)	4.20 (20)
Fischer-344	−GSH	0.099	ND	ND	23.84 (35)	21.05 (31)
	+GSH	0.11	56.34 (93)	76.91 (127)	BLQ	8.87 (15)
Sprague-Dawley	−GSH	0.038	ND	ND	69.31 (40)	BLQ
	+GSH	0.031	58.66 (27)	68.55 (32)	14.70 (7)	BLQ
human	−GSH	0.141	ND	ND	56.78 (120)	38.71 (82)
	+GSH	0.116	8.48 (15)	78.0 (136)	38.87 (68)	40.07 (70)

[a] Liver microsomes from male Wistar rats were isolated from control, isoniazid (ISO), or phenobarbital (PB) pretreated rats (pools of at least 3 animals). For Fischer and SD rats, pooled microsomes of 3 animals were used. For human, microsomes of 5 individuals were pooled. [b] Conversion is expressed as nmol of product formed per min per mg of protein. All incubations were performed in duplicate, as described under Materials and Methods. Variation in conversion of 2 incubations ranged from 0−10%. Presented data are the means of two incubations. [c] Expressed as percentage of total conversion. To determine total conversion, metabolites (including a small (<15%) percentage of unidentified peaks) quantified with HPLC and covalently bound radioactivity (1,4-DCB equivalents) were summed. Of all metabolites eluting from the column, at least 85% was accounted for stucturally. Between parentheses, the absolute amounts (pmol) of metabolites formed in 15 min by 0.1 mg of protein (assay conditions) are given. [d] Effect of addition of rat liver GST is represented. ND = not determined. BLQ = below limit of quantification.

of 1,2-DCB into metabolites (including covalent binding) by the various microsomes is represented expressed as nmol of product per minute per mg of protein. Preliminary experiments indicated that the assay conditions were linear with respect to time and protein concentration. No conversion was observed in incubation mixtures without NADPH. Rate of conversion by the control Wistar and Fischer rat was similar, whereas conversion by the Sprague-Dawley rat was lower. For the Wistar rat, pretreatment with both ISO and PB increased the rate of conversion, namely, 2 and 4 times, respectively. The rate of conversion of 1,2-DCB by human microsomes was higher compared to control rat microsomes, for all strains.

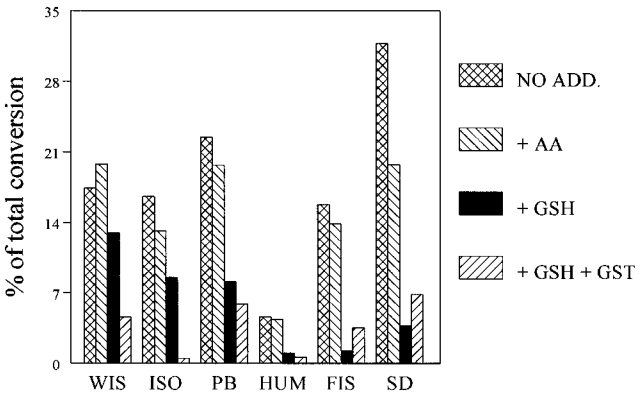

Figure 4. Covalent binding of radioactivity to microsomal protein, expressed as percentage of the total amount of metabolites formed. The effect of 1 mM ascorbic acid (AA), 5 mM GSH, or 5 mM GSH + rat liver GST (2 mg of protein/mL) is represented in separate bars. Presented data are the means of two incubations; variation between duplicates was 0−10%.

Covalent Binding of Radioactivity to Microsomal Protein; Effect of GSH and AA. Figure 4 presents the covalent binding of radioactivity to microsomal protein, expressed as percentage of the total amount of metabolites formed. Covalent binding was most extensive for the SD microsomes (31% of total metabolites) and least extensive for human microsomes (4.6% of total metabolites formed). Covalent binding by Wistar and Fischer rat microsomes was similar (ca. 16%), whereas pretreatment with PB (and not with ISO) increased the formation of reactive metabolites, namely, 23% of total metabolites. Addition of ascorbic acid (AA) reduced the amount of covalently bound metabolites only for the SD microsomes. This is because AA scavenged reactive benzoquinones which are produced by oxidation of hydroquinone metabolites. This indicates that quinones were only involved in part of the covalent binding observed in SD microsomes. Nonenzymatic conjugation of epoxides with GSH inhibited covalent binding for all microsomes to a considerable extent. Addition of rat liver GST further decreased covalent binding for all Wistar and human microsomes, in favor of the GSH conjugates of the epoxides. This decrease was larger for the ISO microsomes compared to the PB microsomes. Thus, for all microsomes, covalent binding was predominantly mediated by the epoxides of 1,2-DCB.

Relative Contributions of Different Metabolites. The formation of the dihydrodiol metabolites, the GSH conjugates of the epoxides and the dichlorophenols, relative to the total amount of metabolites formed, is shown in Table 1, for all microsomes. Relative formation of dihydrodiols was rather similar for all microsomes, except for the Fischer microsomes, that produced less of these metabolites. The amount of GSH−epoxide conjugates was similar for all microsomes. The conjugation occurred nonenzymatically for all rat microsomes, whereas for human microsomes conjugation nearly completely depended on catalysis by glutathione *S*-transferases. Formation of dichlorophenols was most extensive for the human microsomes. For the SD microsomes no dichlorophenols could be detected (the lower limit of quantification was ca. 4% of total metabolites). Induction with phenobarbital resulted in a significant relative decrease of the formation of dichlorophenols, compared to the control microsomes.

Incubations with Microsomes Containing Human Cytochrome P450 and Epoxide Hydrolase. Incubations with microsomes derived from different cell lines selectively expressing one human P450 revealed that only P4502E1, and not 1A1, 1A2, 2B6, 2C9, 2D6, 2A6, or 3A4, metabolized 1,2-DCB to detectable amounts of 2,3- and 3,4-dichlorophenol (DCP). The isomers were produced in equal amounts (not shown). Incubation of microsomes containing P4502E1 together with microsomes derived from a cell line selectively expressing human epoxide hydrolase resulted in a single extra metabolite, which corresponded with a metabolite which was also present in the incubation mixtures with human microsomes, peak 1 in Figure 3 (not shown). Figure 3D shows an HPLC chromatogram of an incubation mixture with human P4502E1 microsomes, GSH, and rat liver GST. The metabolites formed were the same as shown in Figure 3C, with peak 6 being converted to peak 7 in time. Without the addition of rat liver GST, no conjugates were produced.

Table 2. Effect of Addition of 2.5% Acetone or 0.6 mM Cyclohexene Oxide (CO) to Microsomal Mixtures on Total Conversion, Covalent Binding, and Amount of Three Metabolites Formed[a]

species/ conditions	conv[b] (%)	cov binding[b,c] (%)	DHD[c] (%)	2,3-DCP[c] (%)	3,4-DCP[c] (%)
Wistar					
+AA	100	100	72.99	6.35	12.11
+acetone	29.9	285	71.95	<LLOQ[d]	10.38
+CO	48.1	144	4.95	35.97	27.08
human					
+AA	100	100	74.34	10.32	13.91
+acetone	31.7	822	62.91	10.69	14.66
+CO	25.2	578	<LLOQ	49.38	42.36
ISO					
+AA	100	100	60.90	4.99	9.78
+acetone	19.8	515	60.15	4.66	7.35
+CO	38.5	247	5.0	28.65	22.78
PB					
+AA	100	100	68.46	1.49	3.09
+acetone	26.0	100	58.95	0.40	4.18
+CO	57.7	167	8.18	18.11	10.09

[a] Concentration of 1,2-DCB was ca. 80 μM. [b] Conversion (% of substrate present) and covalent binding are set at 100% in the presence of ascorbic acid (AA) only, which was present in all mixtures. [c] Expressed as % of total metabolites formed. [d] <LLOQ = below the lower limit of quantification.

Effect of Inhibition by Cyclohexene Oxide and Acetone. In Table 2, the effect of acetone and cyclohexene oxide on total conversion, on covalent binding, and on the formation of three metabolites is given for the different microsomes. SD and Fischer microsomes were not included in this experiment. Both acetone and cyclohexene oxide inhibited total conversion for all microsomes. Addition of acetone increased covalent binding for all microsomes except for PB microsomes. Addition of cyclohexene oxide resulted in increased covalent binding for all microsomes. The dihydrodiol was hardly formed in the presence of cyclohexene oxide, whereas the formation of the DCP's increased, with slightly more 2,3-DCP than 3,4-DCP. Addition of cyclohexene oxide to the incubation mixtures with PB microsomes resulted in the formation of a relatively high amount of unidentified metabolites.

Discussion

In the present study, the biotransformation of 1,2-dichlorobenzene (1,2-DCB), by hepatic microsomes from man, from male Wistar, Fischer-344, and Sprague-

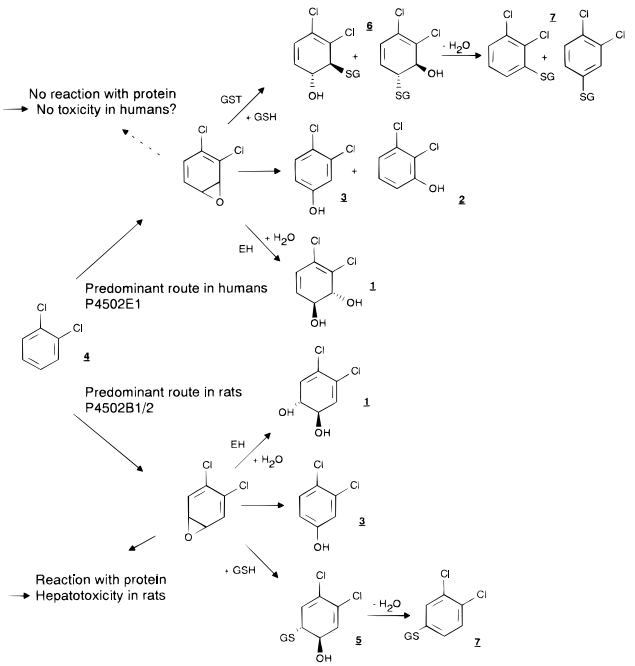

Figure 5. Metabolic scheme, representing the proposed oxidative biotransformation of 1,2-DCB by rat and human liver microsomes. The numbers correspond to the peak numbers in Figure 1. SG = glutathione derivative, EH = epoxide hydrolase, GST = glutathione *S*-transferase. Absolute stereochemistry is not implied in the structures. Trans metabolites with trans vicinal bonds are depicted since they are mechanistically most likely.

Dawley rats, and from male Wistar rats pretreated with phenobarbital (PB) or isoniazid (ISO), has been examined. In Figure 5 a metabolic scheme is presented, depicting the proposed biotransformation pathways of 1,2-DCB. In this scheme, the predominant routes of biotransformation for rat and man are shown. For man, it has been shown that P4502E1 is the major cytochrome P450 enzyme involved in oxidation of 1,2-DCB (*22*). Our results indicated that this isozyme preferentially, or even selectively, oxidizes 1,2-DCB at the 3,4-position of the chlorine atoms, since incubations with microsomes selectively expressing human P4502E1 resulted in the formation of equal amounts of 2,3- and 3,4-DCP. Moreover, in the presence of GSH and glutathione *S*-transferases, two GSH−epoxide conjugate peaks with comparable peak area were observed in the chromatograms. Incubations with human microsomes resulted in a very similar metabolic profile, compared to microsomes selectively expressing human P4502E1, with equal amounts of DCP's and GSH−epoxide conjugates. The covalent binding as percentage of total metabolites formed by human microsomes was much smaller compared to that of each of the rat microsomes. Therefore, we conclude that the metabolite(s) produced by the human microsomes are much less reactive than those produced by the rat microsomes. For rat microsomes, one particular GSH−epoxide conjugate was formed. This conjugate did not need glutathione *S*-transferase catalysis for its formation. The same nonenzymatically formed GSH conjugate was detected only in very small amounts in human microsomes. By addition of GSH, most of the covalent binding was inhibited for both human and rat liver microsomes. Since from the "para"−4,5-epoxide only a single GSH conjugate can be formed (see Figure 5), we speculate that rat microsomal enzymes preferentially oxidize the 4,5-site of the 1,2-DCB molecule, resulting in a reactive epoxide. The relative high formation of this metabolite in rat microsomes compared to human microsomes is responsible for the higher covalent binding to rat microsomal protein. In agreement with these data, we found a selective increase in the formation of 3,4-DCP *in vivo* by male Wistar rats after pretreatment with phenobarbital, an inducer of P4502B1/2 (*18*). In several reports it has been described that 1,2-DCB-induced hepatotoxicity was to a large extent increased by phenobarbital (*6, 8, 10, 11*). Based on the results of the present study, this may be a consequence of the selective formation of the reactive 4,5-epoxide. The speculated difference in reactivity and toxicity of different epoxides of 1,2-DCB has also been demonstrated for bromobenzene (*23*−*25*). Bromobenzene was preferentially metabolized to *p*-bromophenol by the phenobarbital pretreated rabbit via the toxic 3,4-epoxide, whereas the formation of the nontoxic 2,3-epoxide was induced by 3-methylcholanthrene and β-naphthoflavone in rats. Similar to bromobenzene metabolism, the two possible epoxidation pathways of 1,2-DCB appear to require specific P450 enzymes. The 4,5-epoxidation would then preferentially be catalyzed by the phenobarbital inducible P4502B enzymes, whereas human P4502E1 most likely selectively oxidizes the 3,4-site of 1,2-DCB. Conversion by rat liver microsomes was induced by both phenobarbital and isoniazid (a P4502E1 inducer (*26*)), indicating that rat P4502E1 is also involved in 1,2-DCB oxidation. Covalent binding as percentage of total metabolites formed was increased to a small extent for the PB microsomes, but not for the ISO microsomes, confirming the prevalenced formation of reactive metabolites by P4502B1/2. Rat P4502E1 would thus not be explicitly involved in the formation of reactive metabolites. However, it has been shown *in vivo* that 1,2-DCB-induced hepatotoxicity is potentiated by acetone and other low molecular weight ketones, which are associated with induction of P4502E1 (*27*). Possibly rat P4502E1, a significant constitutive enzyme, is involved in the oxidation of both the 3,4- and 4,5-site of the molecule, to the same extent. This is confirmed by the 2:1 ratio of 3,4- and 2,3-DCP, which is shown in Table 2 for the rat.

Inhibition of P450 enzymes with acetone resulted in a significant decrease in conversion for all microsomes. For control and ISO rat microsomes and human microsomes, relative covalent binding was considerably increased by the addition of acetone. By inhibiting P4502E1 in these microsomes, the activity of P4502B enzymes will become relatively more important, resulting in the formation of a relatively larger amount of reactive metabolites. These data suggest that, for human microsomes, another type of P450 enzyme (possibly 2B6) becomes involved after inhibition of P4502E1. For PB microsomes, no increase in covalent binding was observed, probably because the major isoenzyme involved in 1,2-DCB oxidation in these microsomes is already P4502B1/2 and not P4502E1, since phenobarbital induces P4502B1/2 and significantly decreases the amount of P4502E1 (*30*). Hence, inhibition of P4502E1 will have no effect.

As mentioned earlier, covalent binding was much higher for all rat microsomes compared to human microsomes. In order to elucidate the identity of the metabolites giving rise to covalent binding, GSH and ascorbic acid (AA) were used to scavenge epoxides and prevent hydroquinones from oxidation to benzoquinones, respectively. Ascorbic acid inhibited covalent binding for the SD microsomes to some extent only, indicating that

generally quinones do not play a significant role in covalent binding *in vitro*. The fact that no DCP's could be quantified in the experiment with the SD microsomes is in line with the assumption that these microsomes are more active in secondary oxidation of DCP's into quinones compared to the other microsomes.

The high amount of dihydrodiols in the incubation mixtures, which were formed by epoxide hydrolase, points to the extensive formation of epoxides. Addition of GSH resulted in a shift from the formation of the dihydrodiols to the GSH conjugates of the epoxides and also in a significant decrease of covalent binding, indicating that epoxides were responsible for this part of the covalent binding. To check the involvement of microsomal GST, *S*-hexylglutathione, an inhibitor of (microsomal) glutathione *S*-transferases (*29*), was added and shown to have a negligible effect on GSH conjugation (results not shown), indicating that the large amount of GSH conjugates found for rat liver microsomes were indeed formed non-enzymatically. For human microsomes, GSH conjugation was nearly completely catalyzed by cytosolic GST. However, the small amount of nonenzymatically formed conjugates (8% vs 78% enzymatically formed conjugates) seemed to be responsible for the major part of the decrease in covalent binding. In the absence of GSH, epoxide hydrolases play an important role in the detoxification of epoxides *in vitro*. However, we could not identify dihydrodiols *in vivo* (*18*), most likely due to the favored conjugation of the epoxides with GSH. It is noteworthy that for 1,4-DCB no dihydrodiols were found, *in vitro* as well as *in vivo*.[2,3] Apparently, not all types of epoxides are suitable substrates for epoxide hydrolase. Inhibition of epoxide hydrolase by cyclohexene oxide resulted in a decreased conversion of 1,2-DCB for all microsomes tested. Most likely this decrease is caused by inactivation of the P450 enzymes involved in oxidation, by the epoxides of 1,2-DCB formed. It has been shown *in vivo* that 1,2-DCB reduced the amount of P450 to a large extent (*30*, *31*), and therefore, it has been proposed that 1,2-DCB is a suicide substrate. Covalent binding increased in the presence of cyclohexene oxide for all microsomes, confirming the involvement of epoxides in covalent binding. In addition, for all microsomes the dihydrodiols nearly completely disappeared, in favor of the two isomers of DCP. These isomers appeared in more or less equal amounts, with even slightly more 2,3-DCP, indicating that these DCP's were derived from the 3,4-epoxide. The "para" 4,5-epoxide might thus be responsible for the inactivation of the P450 enzymes, resulting in the decreased conversion, which confirms the higher reactivity of the 4,5-epoxide. Stine and co-workers (*6*) found a dramatic difference in 1,2-DCB-induced hepatotoxicity between F344 rats and SD rats. They proposed a higher susceptibility of the Fischer rat due to a lower epoxide hydrolase activity of the F344 rat compared to the SD rat, which was found by Glatt and Oesch (*32*). We observed indeed relatively less dihydrodiol formation for the Fischer rat, compared to the other two rat strains and human microsomes, whereas the other metabolites were found in similar quantities. The *in vivo* detoxification route via hydrolysis of the epoxides appears not to be relevant (*18*), but at the dose levels used by Stine and co-workers (1.8 and 5.4 mmol/kg) GSH might be depleted, which would result in a more important role for epoxide hydrolases *in vivo* in detoxification. However, the overall lower conversion (and thus toxification) of 1,2-DCB by SD microsomes might also play a role in the lower susceptibility of this rat strain.

The present results have revealed a striking difference in the oxidative biotransformation of 1,2-DCB between rat and human liver microsomes, with a resultant difference in covalent binding. Rat liver microsomes produced much more reactive metabolites, whereas the total rate of conversion by human microsomes was higher. The observed difference in covalent binding seems to arise from different P450 enzymes involved in oxidation of 1,2-DCB, and the subsequent formation of different metabolites with different reactivity. From these data it is tempting to suggest that man would be less susceptible to 1,2-DCB-induced hepatotoxicity compared to the rat, and that human risk assessment of 1,2-DCB solely based on toxicity studies conducted in rats will lead to an overestimation of human risk. However, this speculation would go beyond the scope of the present *in vitro* results. Many other pharmacokinetic and -dynamic factors need to be considered before one can make an accurate risk estimate for man. Nevertheless, we emphasize that elucidating the molecular mechanism of toxicity of xenobiotics is essential in accurate risk assessment, and that the animal models used for assessing the risk to man have to be selected carefully.

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