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Metabolic Activation of 2,6-Dichlorobenzonitrile, an Olfactory-Specific Toxicant, by Rat, Rabbit, and Human Cytochromes P450

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SUMMARY

The herbicide 2,6-dichlorobenzonitrile (DCBN) is known to cause tissue-specific toxicity at very low doses in the olfactory mucosa of rodents. The toxicity of DCBN is reportedly cytochrome P450 (P450) dependent, but the isoforms involved have not been identified, and the effects of this agent on humans are not known. In the present study, DCBN metabolism was examined with microsomes and with purified P450s in a reconstituted system. Rat and rabbit olfactory microsomes act on DCBN to form DCBN-protein adducts as well as two metabolite peaks, designated M1 and M2, identified through high performance liquid chromatography with radiometric detection. The activity of rat olfactory microsomes in DCBN metabolism is much higher than that of liver or lung microsomes. Of seven purified rabbit P450s known to be expressed in the olfactory mucosa, including 1A2, 2A10/11, 2B4, 2E1, 2G1, and 3A6, the 2A10/11 preparation is the most active, producing M2 as well as DCBN-protein adducts; P450 2E1 is the only other active isoform. The addition of purified epoxide hydrolase (EC 4.2.1.63) to the reconstituted enzyme system leads to the formation of M1 and decreased formation of M2. It seems that M1 and M2 are derived from an epoxide intermediate that also forms covalent protein adducts. Gas chromatography- and liquid chromatography-mass spectrometry analyses of nasal microsomal DCBN metabolites and DCBN-glutathione conjugates indicated that the major reactive intermediate may be 2,3-oxo-DCBN and that M1 may be 2,3-dihydroxy-6-chlorobenzonitrile, whereas M2 may correspond to a monohydroxy-DCBN. Interestingly, heterologously expressed human P450s 2A6 and 2E1, but not 1A2, are active in the metabolism of DCBN, forming protein adducts as well as M2. Thus, the preferential expression of P450s of the 2A subfamily in olfactory tissue suggests a molecular basis for the tissue-specific toxicity of the herbicide and may have important implications for risk assessment in humans.

The herbicide DCBN, also called dichlobenil, is a potent, olfactory-specific toxicant in rats and mice (1, 2). After a single intraperitoneal injection of DCBN at a level as low as 12 mg/kg body weight to C57B1 mice or Sprague-Dawley rats, extensive destruction of Bowman's glands and the olfactory neuroepithelium was observed, with no morphological changes found in other parts of the nasal passages or in the liver (3). The amount of DCBN needed to necrotize Bowman's glands was even less than the dose of *N*-nitrosodiethylamine, a known nasal procarcinogen, needed to induce a focal necrosis of these glands in rats (3–5). Autoradiograms of mice given a single intravenous injection of ^{14}C-labeled DCBN showed an extensive and tissue-specific irreversible binding of radioactivity in Bowman's glands but not in the olfactory neuroepithelium (3). *In vitro* and *in vivo* studies have indicated that the toxicity of DCBN in the olfactory mucosa is related to a tissue-specific, possibly P450-dependent metabolic activation of DCBN to a reactive species capable of reacting with GSH and presumably with protein sulfhydryls. The rapid formation of this reactive intermediate in Bowman's glands, which leads to a cell-specific depletion of GSH and pronounced covalent tissue binding, induces a primary lesion in the lamina propria and a delayed, secondary toxicity in the olfactory epithelium (1, 3, 5–7). It has been suggested that the highly efficient metabolic activation of DCBN in the olfactory mucosa may involve a P450 species that is present in higher amounts in this tissue than in others examined (5). However, the P450 isoform or isoforms active in the formation of the reactive DCBN intermediate in olfactory mucosa have not been identified (8).

In the current study, DCBN metabolism was examined

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ABBREVIATIONS: DCBN, 2,6-dichlorobenzonitrile; P450, cytochrome P450; TMS, trimethylsilyl; SIM, selected ion monitoring; BSTFA, bis(trimethylsilyl)-trifluoroacetamide; GSH, glutathione; EH, epoxide hydrolase; GC, gas chromatography; LC, liquid chromatography; MS, mass spectrometry; HPLC, high performance liquid chromatography.

with microsomes and with purified P450s in a reconstituted system. Metabolites and protein adducts were detected and quantified with use of the ^{14}C-labeled compound as a substrate, and the nature of the reactive intermediate was probed with the use of EH. In addition, the microsomal DCBN metabolites and GSH adducts was analyzed through GC-MS and LC-MS. The tissue profile of DCBN metabolism was examined with microsomes from rat olfactory mucosa, liver, and lung, and the activity of recombinant human P450s in DCBN metabolism was also determined.

Materials and Methods

Determination of catalytic activities. DCBN metabolism was assayed with the use of 2,6-[ring-^{14}C]DCBN (16.7 Ci/mol, >99% pure; Sigma Chemical Co.) as a substrate. The radiolabeled compound showed on radiometric HPLC analysis a contaminant peak representing <0.5% of the total radioactivity. The contents of various incubation mixtures are indicated in the tables and figure legends. For quantification of DCBN metabolites, the reactions were stopped by the addition of an ice-cold solution of methanol/perchloric acid at final concentrations of 26% and 1% (v/v), respectively. Each mixture was centrifuged, and an aliquot of the supernatant layer was analyzed through HPLC as described below. The recovery of radiolabeled DCBN and metabolites in the supernatant fraction was essentially complete.

HPLC analysis of DCBN metabolites. DCBN metabolites were analyzed through HPLC with the use of a Waters 4-μm Nova-Pak C8 column (150 × 3.9 mm i.d.) preceded by a Nova-Pak C8 precolumn cartridge. All analyses were performed at room temperature with a Waters HPLC system consisting of a model 626 pump, a model 996 photodiode array detector, a model 600S controller, and a model 717+ autosampler. Radiometric detection of metabolites in the HPLC effluent was accomplished with a Radiomatic model A-500 Radio-Chromatography detector (Packard) with a 0.25-ml flow cell. The column was equilibrated with water/acetonitrile (90:10 v/v, solvent A). Metabolites were eluted at a flow rate of 1.0 ml/min with solvent A for 1 min, followed by a linear gradient of methanol (0–40%) in solvent A in 10 min, and then by 40% methanol in solvent A for 5 min before reequilibration of the column with solvent A. Ecolite(+) liquid scintillation solution (ICN Biomedicals, Costa Mesa, CA) was added to the flow detector at a level of 2.0 ml/min. The optical spectra of individual peaks were obtained with the on-line photodiode array detector with a spectral resolution of 2.4 nm. Spectral data were processed with the use of Millennium Spectrum Review software and corrected for the absorbance of the mobile phase. A two-dimensional chromatogram was obtained with the processing channel set at 210 nm, and the spectrum at the apex of each individual peak was analyzed.

For analysis of GSH adducts, the column was equilibrated with water, and the metabolites were eluted at a flow rate of 1.0 ml/min with water for 1 min, followed by a linear gradient of both acetonitrile (0–10%, v/v) and methanol (0–40%) in water for 10 min, and then by 40% methanol in solvent A for 5 min before reequilibration of the column with water. The metabolites were quantified by determination of the radioactivity of the individual peaks, and the amounts were calculated based on percent recovery of total radioactivity.

GC-MS analysis of DCBN metabolites. Microsomal reactions were carried out as described above, but with unlabeled DCBN as substrate, and the reactions were terminated by extraction with 3 volumes of ethyl acetate. The organic phase was evaporated to dryness under N_2, and TMS derivatives of the DCBN metabolites were prepared by reaction with 50 μl of BSTFA (Pierce) containing 10% (v/v) pyridine for 30 min at 60°. Aliquots (4 μl) of the reaction mixtures containing the TMS derivatives were analyzed through GC-MS on a Hewlett-Packard 5890 gas chromatograph interfaced to a VG Quattro (Fisons Instruments) mass spectrometer. A 30-m × 0.25-mm DB-1 GC column (J & W Scientific) with 0.25-μm film thickness was used, and the GC temperature program was as follows: initially 75° for 4 min and then a gradient of 10°/min to 300°. The mass spectrometer was operated in the full-scan mode, scanning from m/z 50 to 550 in 1-sec intervals, or in SIM mode, in which case the samples were diluted 1:10 with BSTFA. During SIM acquisition, m/z values corresponding to the $M^{+\cdot}$, $[M + 2]^{+\cdot}$, $[M + 4]^{+\cdot}$, $[M\text{-}CH_3]^{+\cdot}$, $[M + 2\text{-}CH_3]^{+\cdot}$, and $[M + 4\text{-}CH_3]^{+\cdot}$ ions of the DCBN metabolites were monitored, each with a dwell time of 100 msec.

LC-MS analysis of GSH-DCBN adduct. Microsomal reactions were carried out as described above with use of unlabeled DCBN as substrate, and GSH was added to the reaction mixture at a final concentration of 0.6 mM. The reaction was carried out for 30 min and terminated as described above for radiometric HPLC analysis. HPLC separation was performed with a Waters 4-μm Nova-Pak C8 column (150 × 3.9 mm i.d.) preceded by a Nova-Pak C8 precolumn cartridge. The column was equilibrated with water/acetonitrile/trifluoroacetic acid at 96.94:3:0.06 (v/v/v). Metabolites were eluted at a flow rate of 1.0 ml/min with a linear gradient of acetonitrile (3–60%) in water containing 0.06% trifluoroacetic acid in 10 min. For MS analysis, the HPLC effluent was split 9:1 to introduce 100 μl/min into the Fisons VG megaflow electrospray interface, with nitrogen as the nebulizing and bath gas at flow rates of 80 and 350 liter/hr, respectively. The source was maintained at 80°, and the cone bias potential was 45 V. The spectrum was scanned from m/z 100 to 600 in 1-sec intervals, and the photomultiplier was maintained at 650 V.

Protein adduct analysis. To determine the rate of formation of covalent protein adducts, the enzymatic reaction was terminated by the addition of 5 volumes of ice-cold acetone. The mixtures were agitated with a Vortex instrument and then centrifuged at 2000 × g for 10 min. Mixtures to which cold acetone was added at the beginning of the incubation were used as controls. The resulting precipitates were dissolved in 1.0 ml of 1% sodium dodecyl sulfate and then precipitated with 5 ml of acetone as described by Brittebo et al. (5). The sodium dodecyl sulfate/acetone cycle was repeated two additional times, after which no more radioactivity could be extracted and the precipitate from the control incubations exhibited no remaining radioactivity. The proteins were then dissolved in 1 N NaOH. Aliquots of the solution were used for liquid scintillation counting, and the values (expressed as cpm) were compared with those of the total reaction mixture for calculation of the rate of adduct formation. The rates were not corrected for recovery of protein in this procedure, which was >80%.

Other methods and materials. Microsomes were prepared from liver, lung, and olfactory mucosa of male Sprague-Dawley rats (~200 g body weight) obtained from Charles River Co. or from olfactory mucosa of male New Zealand White rabbits (~2.5 kg body weight) as described previously (9). Protein concentrations were determined according to the bicinchoninic acid method (Pierce, Rockford, IL) with bovine serum albumin as a standard. Microsomal P450 was determined according to the procedure of Omura and Sato (10). Rabbit nasal P450s 2A10/11 and 2G1 were purified from olfactory microsomes as described previously (11). Purification of other P450 isoforms, NADPH-P450 reductase, EH, and cytochrome b_5 from rabbit liver microsomes has been described previously (11–13).

Results

Microsomal metabolism of DCBN. The HPLC radiochromatogram of DCBN metabolites generated in reaction mixtures with rabbit olfactory microsomes is shown in Fig. 1A. Two metabolite peaks, designated M1 and M2, were detected, both with retention times shorter than that of DCBN. No product formation was detected in the absence of NADPH. The rates of DCBN metabolite and protein adduct formation with microsomes from rat liver, lung, and olfactory

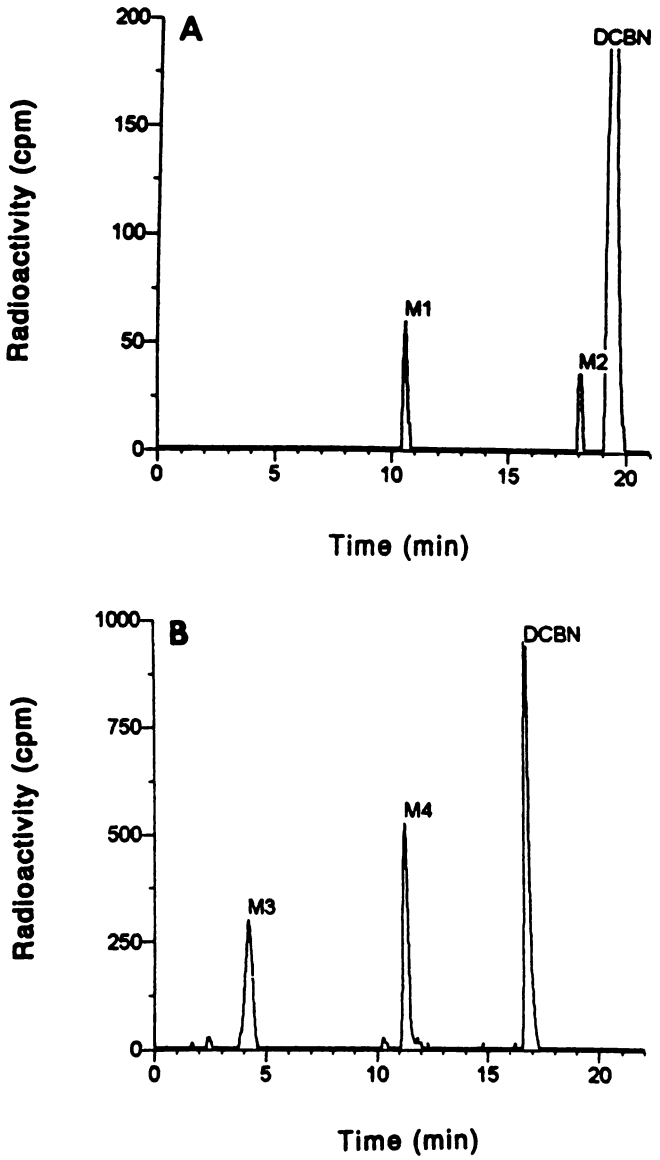

Fig. 1. HPLC radiochromatogram of nasal microsomal DCBN metabolites. A, Metabolites generated from DCBN by microsomal preparations from rabbit olfactory mucosa. The reaction mixture, which contained 50 mM phosphate buffer, pH 7.4, 30 μM 2,6-[ring-^{14}C]DCBN added in 10 μl of methanol, 0.3 mg/ml microsomal protein, and 1 mM NADPH in a final volume of 1.0 ml, was incubated at 37° for 30 min. The reaction was stopped by the addition of 0.4 ml of a solution containing methanol/perchloric acid/water (90:3.5:6.5 v/v/v), and a 20-μl aliquot of the supernatant fraction was analyzed through HPLC as described in Materials and Methods. The retention time of the two metabolite peaks, M1 and M2, and of DCBN was 10.5, 18.0, and 19.3 min, respectively. B, Metabolites generated from DCBN by rabbit olfactory microsomes in the presence of GSH. The microsomal experiment was carried out as described above, except that reduced GSH was added at a final concentration of 3.0 mM. HPLC analysis was performed using slightly different conditions from those in A, as described in Materials and Methods. The retention time of the two new metabolite peaks, M3 and M4, and of DCBN was 4.2, 11.3, and 16.8 min, respectively; the retention time of M1 and M2, which were not detected in these experiments, would be 13.1 and 16.2 min, respectively, under these conditions.

mucosa as well as from rabbit olfactory mucosa were determined with DCBN at 30 μM, as shown in Table 1. Product formation was essentially linear with time for ≥15 min for the microsomal reactions (data not shown). Although both M1 and M2 were formed by olfactory microsomes, only M2 was produced by rat hepatic microsomes. Neither metabolite was detected with rat lung microsomes, with a detection limit of ~5 pmol/min/mg of microsomal protein in these experiments. The rate of M2 formation by olfactory microsomes was much higher than that by liver microsomes, even though the total P450 content was much higher in the latter. Furthermore, the rate of protein adduct formation with olfactory microsomes was >20-fold higher than that observed with liver or lung microsomes, indicating tissue-specific activation of the olfactory toxicant. Interestingly, higher specific activity in DCBN-adduct formation (expressed as pmol product/min/mg of protein) was observed with rat olfactory microsomes when 0.5 mg/ml protein was used rather than 0.1 mg/ml. The rate of DCBN metabolism was even higher in rabbit than in rat olfactory microsomes. In other experiments with rabbit olfactory microsomes, formation of M1, M2, and DCBN-protein adducts was also detected with DCBN at 3 μM (data not shown).

DCBN metabolism by purified rabbit P450 isoforms. The ability of a series of purified rabbit P450 isoforms, including 2B4, 2E1, 3A6, 1A2, 2A10/11, and 2G1 (see Ref. 14 for nomenclature), to metabolize DCBN was examined in a reconstituted system containing 0.1 μM purified P450, 0.4 μM NADPH-P450 reductase, and 30 μg/ml phospholipid, with substrate at 30 μM. These isoforms have all been detected in olfactory microsomes (15, 16) and therefore might contribute to DCBN metabolism in the nasal mucosa. When cytochrome b_5 was not present, the formation of M2 was detected only with 2A10/11, with a rate of 1.1 nmol product/nmol P450/30 min. However, when purified cytochrome b_5 was added to the reaction mixture at a final concentration of 0.4 μM, 2A10/11 was found to be approximately seven times as active in the formation of M2, with a rate of 8.0 nmol product/nmol P450/30 min, and 2E1 was also active, albeit at a much lower rate (1.6 nmol product/nmol P450/30 min). Nevertheless, M1 formation was not detected in reactions with any of the purified P450s, suggesting that M1 was formed by a different P450 isoform or that some other microsomal component, such as EH, was required for its formation. The detection limit for either metabolite was ~0.2 nmol/nmol P450/30 min, and linearity of the rate of product formation with time was not established in these experiments. In other experiments, the formation of protein adducts was also examined in reaction mixtures containing cytochrome b_5; with 2A10/11, the rate was 0.72 nmol product/nmol P450/30 min, but 2E1 was inactive. However, adduct formation was detected with 2E1 (0.38 nmol product/nmol P450/30 min) when 0.5 mg/ml bovine serum albumin (as a donor of protein sulfhydryls) was included in the reaction mixture.

Effects of GSH. The addition of reduced GSH (at 0.3 mM) to nasal microsomal reaction mixture resulted in an 80% decrease in the amount of protein adduct formed and nearly complete inhibition of the formation of M1 and M2 (not shown), with the formation of two additional metabolite peaks detected through radiometric HPLC analysis (designated M3 and M4) (Fig. 1B). Formation of M3 and M4 was not dependent on GSH transferase as both metabolites were also formed in a reconstituted system containing purified 2A10/11 or 2E1 (not shown).

Effects of EH on DCBN metabolite profile. To determine whether the reactive intermediate might be an epoxide,

TABLE 1

Metabolism of DCBN by rat and rabbit microsomal preparations

Reaction mixtures contained 50 mM phosphate buffer, pH 7.4, 30 μM 2,6[ring-^{14}C]DCBN added in 10 μl methanol, 0.1 or 0.5 mg/ml microsomal protein as indicated in parentheses, and 1 mM NADPH in a final volume of 1.0 ml. The reaction was carried out in duplicate at 37°C for 10–15 min.

Source of microsomes	P450 content	Rate of product formation		
		M1	M2	Protein adducts
	pmol/mg protein	*pmol/min/mg microsomal protein*		
Rat[a]				
Liver (0.5 mg/ml)	950	<5[b]	60	8
Lung (0.5 mg/ml)	ND[c]	<5	<5	9
Olfactory mucosa (0.5 mg/ml)	325	124	132	236
Olfactory mucosa (0.1 mg/ml)	325	115	92	138
Rabbit[d]				
Olfactory mucosa (0.1 mg/ml)	563 ± 193	420 ± 204	213 ± 48	287 ± 66

[a] The values are an average of two independent experiments.
[b] <5 indicates below detection limit.
[c] ND, not determined.
[d] The values reported are the mean ± standard deviation of three experiments.

purified EH was included in the reaction mixtures with purified P450 or with olfactory microsomes. Although M2 was the only metabolite detected in reactions with purified 2A10/11 in the absence of EH (Fig. 2A), M1 was the predominant metabolite in the presence of EH, with a concomitant reduction in the amount of M2 formed (Fig. 2B). The addition of EH to rat olfactory microsomal reaction mixtures, as shown in Table 2 for two independent experiments, also resulted in increases in the ratio of M1 to M2 from ~1 to ~2.5. Similar observations were made with rabbit olfactory microsomes and purified rabbit P450 2E1 (data not shown). However, in experiments not presented, neither M1 nor M2 was found to form significant amounts of protein adduct when incubated with bovine serum albumin or with boiled rabbit olfactory microsomes, and M2 was not converted to M1 when incubated with purified EH. Thus, both M1 and M2 may be derived from a common epoxide intermediate, which presumably is also highly reactive in forming protein adducts.

DCBN metabolism by human P450. The ability of human P450s 1A2, 2A6, and 2E1 to metabolize DCBN was also examined. The latter two were studied because their rabbit orthologs are active toward DCBN, and 1A2 was included as a control. The recombinant cytochromes contained in lymphoblastoid microsomal preparations were previously shown to be catalytically active toward other substrates (17, 18). As indicated in Fig. 3, 2A6 and 2E1 were active in the formation of both M2 (Fig. 3A) and protein adducts (Fig. 3, B and C). Metabolite formation was not detected with 1A2, however, and the amount of protein adduct formed by 1A2-containing microsomes was not significantly higher than the low level found with microsomes from control cells, which do not contain any exogenous P450 (Fig. 3C). The level of EH in these microsomes is not known. However, formation of M1 was not detected in reactions with 2A6 or 2E1, even when exogenous EH was added (not shown). It remains to be determined whether M1 is formed when enzyme preparations with higher P450 contents are used.

Identity of microsomal metabolites and reactive intermediate of DCBN. The UV spectra obtained at the apex of HPLC peaks corresponding to M1, M2, and DCBN were obtained (not shown). The absorbance maxima for M1, M2, and DCBN were at ~205, ~218, and ~209 nm, respectively. The spectra recorded for the M1 metabolite formed by rat and rabbit olfactory mucosa microsomes were identical, as were the spectra of M2 produced by rabbit and rat olfactory and rat hepatic microsomes, suggesting that the same metabolites were formed by microsomes from the two species.

The structure of DCBN metabolites generated with rabbit olfactory microsomes was subsequently determined through GC-MS. Analysis of the ethyl acetate extracts of microsomal reaction mixtures without derivatization resulted in the identification of a monohydroxy-DCBN metabolite. The mass spectrum recorded at a GC retention time of 14.8 min showed a molecular ion peak at m/z 187 with isotopic peaks at m/z 189 and 191 (results not given). The spectrum also showed peaks at m/z 159, 161, and 163, consistent with the loss of CO from the phenolic metabolite, from a ring carbon bearing a hydroxyl group.

When the microsomal extracts were derivatized with BSTFA to form TMS derivatives of the metabolites, three DCBN metabolites were identified through full-scan MS: the TMS derivative of the monohydroxy-DCBN metabolite was detected as well as dihydroxy-DCBN and a dihydroxy-monochloro metabolite. Each showed relative intensities of the $M^{+\cdot}$, $[M + 2]^{+\cdot}$, and $[M + 4]^{+\cdot}$ ions consistent with the contributions of ^{37}Cl and ^{30}Si present in the metabolite derivatives (Table 3) and a higher intensity for the peaks representing the loss of a methyl group, $[M\text{-}CH_3]^{+\cdot}$, $[M + 2\text{-}CH_3]^{+\cdot}$, and $[M + 4\text{-}CH_3]^{+\cdot}$, than those for the molecular ions. The catechol nature of the dihydroxy-monochloro metabolite was indicated by the loss of m/z 88 from the molecular ion (19), the mass equivalent of $Si(CH_3)_4$ (Fig. 4). The mass spectrum also showed the sequential loss of the two remaining methyl groups, giving rise to the peaks at m/z 210 and 195. The $[M\text{-}CH_3\text{-TMS}]^{+\cdot}$ ion was also found in the mass spectrum of the dihydroxy-DCBN metabolite, but not of the monohydroxy-dichloro metabolite (not shown). In other experiments not presented, the monohydroxy metabolite was also detected in HPLC-isolated M2 fraction.

The formation of all three metabolites was NADPH dependent. The absence of the metabolite in the control reactions is not due to losses during sample preparation because the internal standard, 4,6-dichlororesorcinol, was detected with or without NADPH addition (data not given). In experiments not presented, the integrated peak intensity at m/z values corresponding to $[M\text{-}CH_3]^{+\cdot}$ ions, which were the most intense peaks in the high mass region for all DCBN metabo-

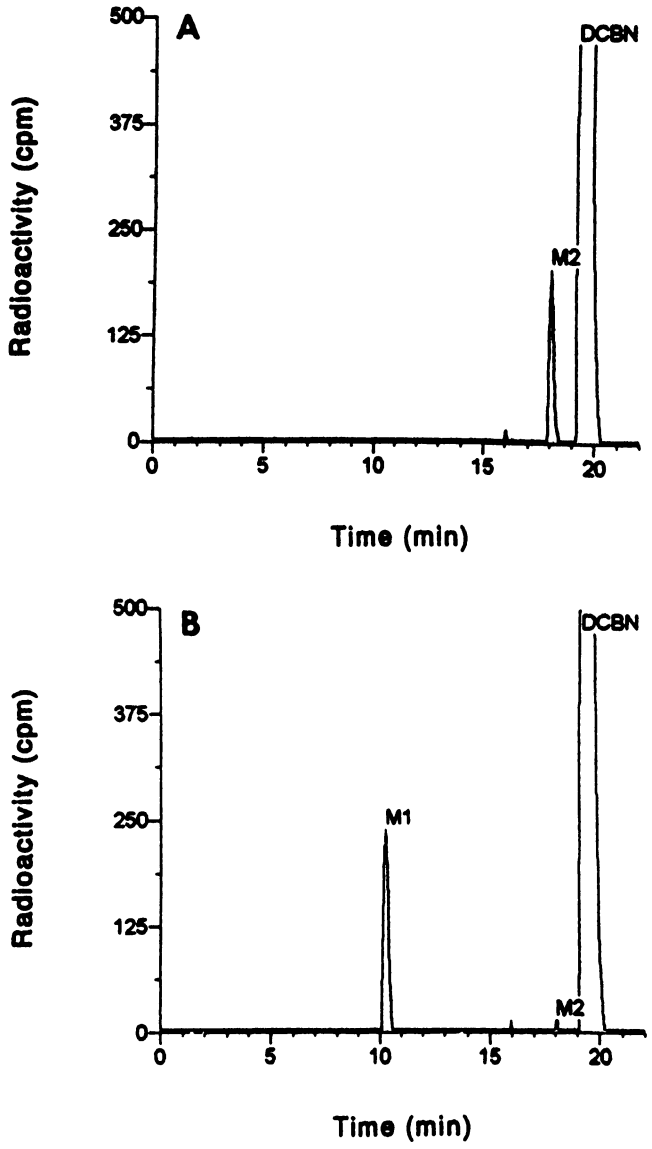

Fig. 2. Effects of EH on DCBN metabolite formation by purified P450 2A10/11. The conditions were as described in the legend to Fig. 1A, except that a reconstituted system containing 0.1 μM purified 2A10/11, 0.4 μM NADPH-P450 reductase, 30 μg/ml phospholipid, and 0.4 μM cytochrome b_5 was used in place of microsomes. Purified EH was not included in the experiment in A but was added at a final concentration of 50 μg/ml in the experiment in B.

lites, was 26 times as high for the dihydroxy-monochlorobenzonitrile metabolite as for the dihydroxy-dichlorobenzonitrile metabolite. Thus, the amount of dihydroxy-DCBN metabolite was probably below detection limit in radiometric HPLC analysis (Fig. 1), even though it was consistently detected through GC-MS after formation of the TMS derivative. The integrated peak intensity for the dihydroxy-monochloro metabolite detected through GC-MS was approximately seven times lower than that for the monohydroxy-dichloro metabolite (not shown), even though comparable amounts were detected through radiometric HPLC analysis (Table 1).

The structure of DCBN metabolites formed in the presence of GSH was subsequently determined through electrospray LC-MS analysis in the positive ion mode. As shown in Fig. 5A, one metabolite was identified as an adduct of GSH with a monohydroxy-monochlorobenzonitrile, presumably a conjugation product of GSH with 2,3-oxo-DCBN. The presence of a chlorine is indicated by the relative abundance of the $[M + H + 2]^+$ peak (m/z 461). The loss of the γ-glutamyl moiety (129 Da) as indicated by the peak at m/z 330 and the isotopic peak at m/z 332 is typical of GSH adducts (20). Formation of this adduct, which was also detected through LC-MS in HPLC fractions corresponding to M4, but not M3 (data not given), was not observed when NADPH was omitted from the microsomal reaction mixture (Fig. 5B).

TABLE 2

Effects of EH on DCBN metabolite formation with rat olfactory microsomes

The contents of the reaction mixtures were the same as described in Table 1 with olfactory microsomes at 0.5 mg protein/ml. Purified EH was added to a final concentration of 45 μg/ml where indicated. Reactions were carried out at 37° for 10 min. The results are from two independent experiments.

Experiment	Addition	Rate of metabolite formation			M1/M2 ratio
		M1	M2	M1 + M2	
		pmol/min/nmol P450			
1	None	390	410	800	0.95
	EH	630	240	870	2.62
2	None	460	470	930	0.98
	EH	820	340	1160	2.41

Discussion

The metabolic fate of DCBN in reactions with microsomal P450s is summarized in Fig. 6. Our data suggest that DCBN is metabolized by P450 to 2,3-oxo-DCBN and, to a lesser extent, 3,4-oxo-DCBN, which could be converted to the respective dihydroxy metabolite after hydrolysis by EH, could rearrange to form 3- or 4-hydroxy-DCBN, or could form covalent adducts with microsomal proteins. In the presence of GSH, its adduct formed with 2,3-oxo-DCBN was detected. Previous studies by others have established that both the benzene ring and the nitrile group are retained in the covalent DCBN-protein adducts (3, 19), and both 3- and 4-hydroxy-DCBN were detected in the reaction of DCBN with mouse olfactory microsomes as reported by Genter and Deamer (21). Metabolites identified in the urine or bile after a single oral dose of this compound to rats, chickens, and goats included 3-hydroxy-DCBN and its sulfur-containing dechlorinated derivatives as the major components (19, 22–25). This is consistent with the present finding that 2,3-oxo-DCBN may be the major reactive intermediate in olfactory microsomes, although it remains to be determined to what extent the nasal mucosa contributes to systemic clearance of the olfactory toxicant. Both 2,3-epoxy-DCBN (25) and 3,4-epoxy-DCBN (22) have been proposed as intermediates in previous studies.

The monohydroxy metabolite apparently corresponds to M2 detected through radiometric HPLC analysis, as indicated by the mass spectrum of HPLC-isolated M2, whereas the dihydroxy-monochlorobenzonitrile most likely corresponds to M1, as judged by the relative abundance of the two dihydroxy metabolites. GC-MS analysis of HPLC-isolated M1, which is predicted to be a dihydrodiol or a more stable dihydroxy derivative based on the experiments with EH, has been unsuccessful to date due to the low yield of organic extraction and the apparent instability of the metabolite during sample preparation. In addition, the proposed DCBN

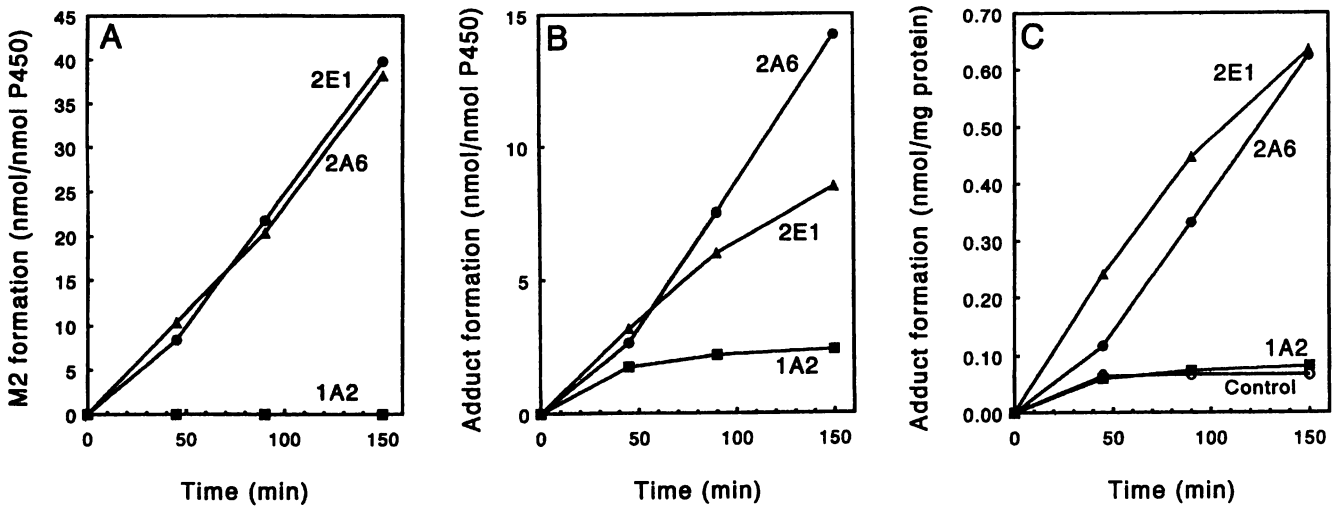

Fig. 3. DCBN metabolite and protein-adduct formation by human P450. Reaction mixtures contained 30 μM 2,6-[ring-^{14}C]DCBN; microsomes (0.5 mg protein/ml) from control human B lymphoblastoid cells (○) or cells with heterologously expressed human P450 1A2 (■), 2A6 (●), or 2E1-reductase (▲); and an NADPH-generating system containing 100 mM phosphate buffer, pH 7.4, 1.3 mM NADP, 3.3 mM $MgCl_2$, 3.3 mM glucose-6-phosphate, and 0.4 unit/ml glucose-6-phosphate dehydrogenase. The P450 contents of the microsomal preparations were 34, 44, and 75 pmol/mg of protein for 1A2, 2A6, and 2E1-reductase, respectively, and were <0.1 pmol/mg of protein in control cells. The reaction was initiated by the addition of microsomal proteins and carried out at 37° for 150 min, as indicated. Aliquots were removed at various times for metabolite analysis (A) and adduct determination (B and C) as described in Materials and Methods. The rates of adduct formation are expressed as nmol/nmol P450 (B) or nmol/mg microsomal protein (C). No metabolite formation was detected in reactions with control microsomes or in microsomes containing P450 1A2. The data reported are the average of duplicate determinations.

TABLE 3

GC-MS analysis of TMS derivative of DCBN metabolites formed in olfactory microsomal reactions

The contents of reaction mixtures were the same as described in the legend to Table 1, except that unlabeled DCBN was used as substrate at a final concentration of 128 μM. The reactions were carried out at 37° for 60 min, and the metabolites were extracted with ethyl acetate, derivatized, and analyzed by GC-MS in EI^+ mode as described in Materials and Methods.

Metabolite	*m/z* of $M^{+\cdot}$ ion	Relative abundance					
		$M^{+\cdot}$	$[M + 2]^{+\cdot}$	$[M + 4]^{+\cdot}$	$[M - CH_3]^{+\cdot}$	$[M - CH_3 + 2]^{+\cdot}$	$[M - CH_3 + 4]^{+\cdot}$
		% of $[M - CH_3]^+$ *ion*					
Monohydroxydichlorobenzonitrile	259	17	12	2	100	66	13
Dihydroxymonochlorobenzonitrile	313	25	11	1	100	42	3
Dihydroxydichlorobenzonitrile	347	57	37	7	100	80	18

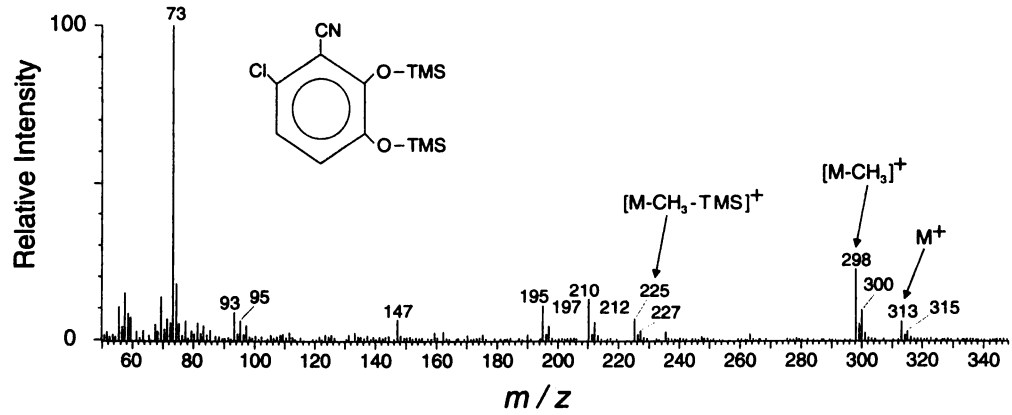

Fig. 4. Electron-ionization mass spectrum of the TMS derivative of dihydroxy-monochlorobenzonitrile. Microsomal reactions were carried out as described in the legend to Table 3. The structural assignment and the *m/z* value of the major peaks are indicated, and the predicted structure of the TMS derivative of the metabolite is shown. The spectrum was obtained at a GC retention time of 17.4 min.

epoxide intermediate was not detected through SIM GC-MS, with or without acid addition to the reaction mixture before extraction of the metabolites, although the detection of both the monochloro-dihydroxy and dichloro-dihydroxy metabolites of DCBN suggested that both 2,3- and 3,4-oxo-DCBN were formed.

The adduct of GSH with a monohydroxy-monochlorobenzonitrile probably corresponds to M4 because it was detected through LC-MS in HPLC fractions corresponding to M4 but not M3. The identification of M3 has so far been unsuccessful, although preliminary experiments with radiolabeled GSH indicate that M3 may also be an adduct with GSH or its

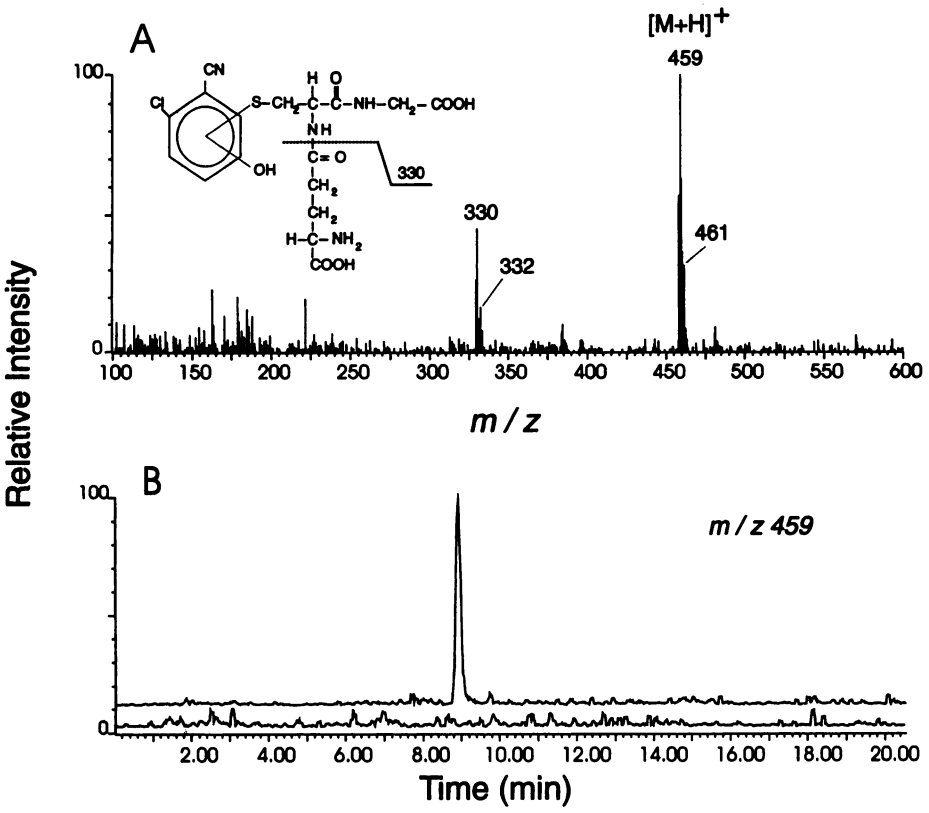

Fig. 5. LC-MS analysis of DCBN-GSH adducts formed in olfactory microsomal reactions. The experiments were carried out as described in the legend to Table 3. A, Electrospray positive ion detection mass spectrum of DCBN-GSH adduct, with fragmentation pattern of the predicted structure indicated. The position of the OH and GSH groups cannot be distinguished through MS analysis. B, The reconstructed ion (*m/z* 459) chromatogram indicates the detection of a peak with an HPLC retention time of 8.94 min in the complete reaction mixture (*top*) but not when NADPH was omitted (*bottom*). The mass spectrum in A was obtained at the same retention time.

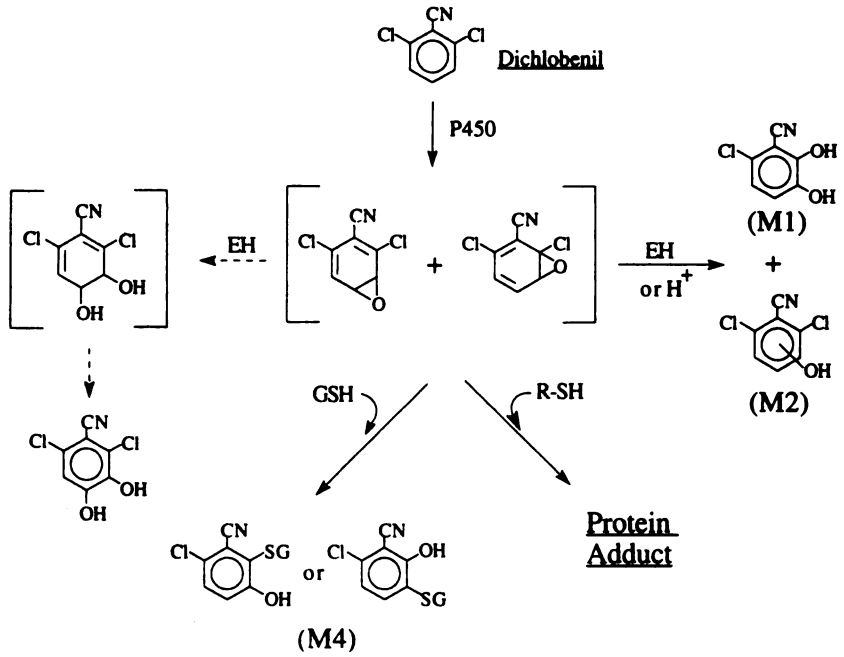

Fig. 6. Proposed scheme for the metabolic activation of DCBN in olfactory mucosa microsomes. The identified metabolites and the proposed reactive intermediates are shown. The corresponding metabolite peaks on HPLC radiochromatogram are indicated in *parentheses*. *R-SH*, protein sulfhydryl groups.

derivative.[1] Nevertheless, the formation of a dichloromonohydroxy-DCBN adduct with GSH, a predicted conjugation product with 3,4-oxo-DCBN, was not detected in these experiments. This is consistent with the low level of dihydroxy-DCBN detected in the GC-MS experiments and suggests that 2,3-epoxy-DCBN is the major reactive intermediate formed in the olfactory microsomal reactions.

It is not clear why M1 was not formed with rat hepatic microsomes, even when purified EH was added and the reaction was carried out for 2 hr with the use of an NADPH-regenerating system. It is likely that because the rate of formation of the epoxide intermediate was much lower with liver than with nasal microsomes and because EH has to compete with other microsomal proteins for the reactive epoxide, the amount of substrate available to EH may be insufficient to form detectable amounts of M1. Attempts to isolate the proposed epoxy intermediate have so far been unsuccessful, and therefore its reactivity with EH has not been exam-

[1] C. Liu and X. Ding, unpublished observations.

ined. The contribution of M1 to the tissue-specific toxicity of DCBN and the potential role of this relatively stable metabolite in the neurodegenerative effects of DCBN remain to be determined. The potential importance of EH, which is required for the formation of M1, in the detoxification of DCBN epoxide has been suggested in a recent study by Deamer *et al.* (2).

Of rabbit P450s known to be expressed in the olfactory mucosa, both 2A10/11 and 2E1 are active in the metabolic activation of DCBN. However, 2A10/11 is most abundant in the nasal tissue, accounting for ~25% of the total P450 (26), whereas 2E1 is a minor form (27). Therefore, 2A10/11 is probably responsible for most of the DCBN metabolism in this tissue. On the other hand, induction of 2E1 by ethanol and acetone has been demonstrated in the olfactory mucosa (27–29), and it remains to be determined whether 2E1 plays an important role in the metabolic activation of DCBN after induction, particularly in human alcoholics. In rats, evidence has been obtained that 2E1 may not play a major role in olfactory metabolism of DCBN (8). However, recent studies (30) indicate that heterologously expressed rat 2A3 is active in the metabolic activation of DCBN and that this isoform is predominantly expressed in rat olfactory epithelium (31). Thus, it is likely that 2A3, like 2A10/11, plays a major role in DCBN activation in rodent olfactory tissue, where toxicity occurs.

The activity of human 2A6 and 2E1 in the metabolic activation of DCBN suggests that this compound may also cause olfactory toxicity in humans. Recent immunohistochemical studies have indicated the abundance of 2A-related P450s in human olfactory and respiratory mucosa (32). However, the expression of 2E1 in human nasal tissues has not been examined. A quantitative comparison of the activity of 2A6 and 2E1 toward DCBN could not be made in the current study because although the 2E1-containing microsomes also contained recombinant human P450 reductase, the 2A6-containing microsomes contained only endogenous P450 reductase. Nevertheless, these results indicate that, similar to the findings with rabbit P450, human 2A and 2E isoforms are active in the metabolic activation of DCBN. It remains to be determined whether additional human nasal P450s are active toward this compound.

The kinetics of DCBN metabolism by P450 2A and 2E isoforms was not examined. A substrate concentration of 30 μM was used in the current study based on a previous report (6) that the apparent K_m value for the formation of DCBN-protein adducts with rat nasal and liver microsomal reactions was 1.8 and 4.0 μM, respectively. However, the present findings on the metabolic fate of DCBN in nasal microsomes suggest that kinetic parameters for this substrate will be significantly affected by the amount of total protein or sulfhydryl groups in the reaction mixture and, in experiments with microsomes, by the level and activity of EH. This is exemplified by the experiment in which a higher rate of protein adduct formation was observed with higher levels of protein.

The lowest toxic dose of DCBN (for a single intraperitoneal injection in mice) was reported to be in the range of 6–12 mg/kg (7). However, the effective *in vivo* concentration of DCBN available to olfactory P450 has not been determined. In a recent study with explants of mouse olfactory mucosa, cell-specific metabolic activation in the Bowman's gland was observed with DCBN at 27 μM in the culture media (6). Interestingly, in the current study, the formation of M1, M2, and protein adducts was detected in olfactory microsomal reaction with DCBN at either 30 or 3 μM, suggesting that the same reactive intermediate was formed at the two different substrate concentrations and, furthermore, that the same metabolites are likely to be produced *in vivo* on exposure to DCBN at a toxicologically relevant dose.

The possibility that DCBN is a mechanism-based inhibitor of P450 2A or 2E isoforms is under investigation. Although the rates of product formation were essentially linear with time for ≥15 min in experiments with nasal microsomes, a significant decrease in the rate of total product formation was observed after 30 min. Furthermore, DCBN-protein adducts were formed in reactions with purified 2A10/11 in a reconstituted system, suggesting that P450 and/or NADPH-P450 reductase may form covalent adducts with the reactive intermediate. The rate of total metabolite formation (including M1, M2, and protein adducts) was increased with higher concentrations of microsomal protein in the reaction mixture. This was confirmed by other experiments not presented in which the addition of GSH, bovine serum albumin, or normal sheep IgG as donors of protein sulfhydryl groups resulted in increased rates of total DCBN metabolite formation by nasal microsomes. These observations are consistent with DCBN being a suicide inhibitor of the P450 enzyme system as higher concentrations of sulfhydryl groups would lead to greater quenching of the reactive intermediate and thus give increased protection of the enzyme from inactivation by the epoxide.

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