METABOLISM AND MUTAGENICITY OF SOURCE WATER CONTAMINANTS 1,3-DICHLOROPROPANE AND 2,2-DICHLOROPROPANE

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ABSTRACT:
Cytochrome P450-dependent oxidation and glutathione (GSH)-dependent conjugation are the primary routes of metabolism of haloalkanes. Using rat liver microsomes and cytosol, we investigated the metabolism of two haloalkanes found on the U.S. Environmental Protection Agency Contaminant Candidate List, 1,3-dichloropropane (1,3-DCP) and 2,2-dichloropropane (2,2-DCP). An automated headspace technique using gas chromatography was developed to determine rates of metabolism. Additional dihaloalkanes (1,2-dichloroethane, 1,2-dichloropropane, 1,4-dichlorobutane, 1,2-dibromoethane, 1,2-dibromopropane, 1,4-dibromobutane) were evaluated to assess structure-activity relationships. In general, brominated dihaloalkanes were eliminated from rat cytosol faster than chlorinated dihaloalkanes, reflecting the expected halide order of reactivity (Br > Cl). Furthermore, the rate of GSH conjugation was proportional to α,ω-haloalkane chain length. The clearance of 1,3-DCP via the GSH conjugation pathway (1.6 × 10^{-4} l/h/mg cytosol protein) was minor relative to the P450 pathway (2.8 × 10^{-2} l/h/mg microsomal protein). In contrast, we did not observe metabolism of 2,2-DCP via the GSH-dependent conjugation pathway and observed only a minor level of clearance via the P450 pathway (7 × 10^{-4} l/h/mg microsomal protein). Neither compound was mutagenic in various strains of Salmonella, including those containing GSTT1-1, indicating that GSTT1-1 does not metabolize 1,3-DCP or 2,2-DCP to mutagens. Analysis of the reaction products of 1,3-DCP and GSH in cytosol by liquid chromatography/mass spectrometry revealed significant production of S-(3-chloropropyl) glutathione conjugate, indicating that the conjugate halomustard does not rearrange to form a sulfonium ion, as typically occurs with vicinal dihaloalkanes.

In 1996, amendments to the Safe Drinking Water Act required that the U.S. Environmental Protection Agency (EPA)¹ create a list of unregulated water contaminants that could eventually require regulation.

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¹ Abbreviations used are: EPA, Environmental Protection Agency; 1,3-DCP, 1,3-dichloropropane; 2,2-DCP, 2,2-dichloropropane; P450, cytochrome P450; GSTs, glutathione S-transferases; GSH, glutathione; 1,2-DCE, 1,2-dichloroethane; 1,2-DBE, 1,2-dibromoethane; 1,3-DBP, 1,3-dibromopropane; 1,4-DCB, 1,4-dichlorobutane; PRR, predicted relative rate; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography/mass spectrometry.

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these agents are the cytochromes P450 (Guengerich et al., 1980; Guengerich and Shimada, 1991; Yin et., 1995) and glutathione S-transferases (GSTs) (Wheeler et al., 2001a,b). P450-catalyzed oxidative dehalogenation results in an aldehyde or acid halide that may react with cell macromolecules (Sipes and Gandolfi, 1991). GSTs catalyze the conjugation of glutathione (GSH) with numerous haloalkanes to form potentially genotoxic compounds (Thier et al., 1993; Hallier et al., 1994; Wheeler et al., 2001a,b). With respect to 1,2-dihaloethanes, the GST pathway is regarded as the principal mechanism of activation and mutagenesis (Wheeler et al., 2001b). The mutagenicity of 1,2-dichloroethane has been demonstrated in Salmonella TA1535 supplemented with rat liver cytosol and GSH, but not with rat liver microsomes and NADPH (Guengerich et al., 1980).

Alkylation species are produced when the sulfur atom of the GSH conjugate reacts intramolecularly, eliminating the halogen from the neighboring carbon to form an episulfonium ion (Rannug et al., 1978; van Bladeren et al., 1980). P450 oxidation of 1,2-dihaloethanes result in 2-haloacetaldehydes that are capable of reacting with DNA to form adducts (Guengerich et al., 1981; Guengerich, 1992); however, their rates of reaction with DNA are slow in comparison with reactive species derived from GSH conjugation (Rannug et al., 1978; van Bladeren et al., 1980). Both cytochrome P450- and GST-dependent pathways are operative in the metabolic activation and mutagenicity of 1,2-dibromo-3-chloropropane and other haloalkanes (Omichinski et al., 1987; Pearson et al., 1990a,b).

Because of the importance of P450 and GST pathways in the clearance and activation of haloalkanes, we have applied in vitro methods to quantify the flux of these substrates through these pathways. An automated recirculating gas-liquid system, based on the equilibrium vial method (Sato and Nakajima, 1979), was developed and used to determine the rates of metabolism of the chemicals 1,3-DCP and 2,2-DCP. To give some insight into the mechanistic basis of P450 and GST metabolism, several other haloalkanes also were examined in the same system. To assess the potential for mutagenicity, 1,3-DCP and 2,2-DCP were evaluated in the Salmonella plate-incorporation assay in the presence or absence of rat liver homogenate (S9). Various strains were used, including RSJ100 and GSTT1, which are transfected with cDNA of the rat and human GST71-1, respectively, thereby aiding in distinguishing bioactivation by GST from P450 mechanisms.

Materials and Methods

Chemicals. 1,2-Dichloroethane (99%), 1,2-dichloropropane (99%), 2,2-dichloropropene (98%), 1,3-dichloropropane (99%), 1,4-dichlorobutane (99%), 1,2-dibromoethane (99%), 1,3-dibromopropane (99%), and 1,4-dibromobutane (99%) were purchased from Sigma-Aldrich. D-Glucose-6-phosphate dehydrogenase (99%), 1,2-dibromoethane (99%), 1,3-dibromopropane (99%), and 1,4-dichlorobutane (98%), 1,3-dichloropropane (99%), 1,4-dichlorobutane (99%) were purchased from Bio-Rad (Hercules, CA). 2-Nitrofluorase (99%, type VII, 372 units/mg protein) was purchased from Fluka (Buchs, Switzerland). 1,2-Dichloroethane (99%), 1,2-dibromoethane, and methyl glyoxal were purchased from Sigma-Aldrich and were used as positive controls in the Ames assay. All chemicals were diluted in dimethyl sulfoxide, and its final concentration in the genotoxicity assays was <1% w/v.

Animals. Male Fischer-344 rats (217–243 g) were obtained from Charles River Laboratories, Inc. (Raleigh, NC) and were housed in polycarbonate cages on bedding of hardwood shavings maintained at 25°C and 50% relative humidity with a 12-h light/dark cycle. Animals were supplied with Purina Rat Chow 5001 (St. Louis, MO) and tap water ad libitum for 5 days before euthanization.

Enzyme Preparations. Rats were euthanized with CO2 and bled by cardiac puncture. Livers were excised, minced, and washed four times with 10- to 15-ml aliquots of ice-cold 0.1 M sodium phosphate buffer (pH 7.4). A 13.3% (w/v) crude homogenate was prepared in a glass Potter-Elvehjem homogenizer. The homogenization buffer consisted of 10% glycerol (w/v), 250 mM sucrose, 1 mM dithiothreitol, 0.5 mM EDTA, and 35 mM KCl (pH 7.4). After homogenization, the preparation was centrifuged at 15,000g for 15 min, and the supernatant (S9) was stored at −80°C in individual vials. On the day of the experiment, S9 was thawed under running tap water and centrifuged at 105,000g for 60 min at 4°C. The resulting pellet was suspended in 5 ml of 0.1 M sodium phosphate buffer (pH 7.4) and designated “microsomes.” The supernatant from this second centrifugation was designated “cytosol.” Total protein was estimated by the Bradford method (1976), and microsomal oxidation activity was determined according to Reinke and Meyer (1985). Glutathione S-transferase activity in the cytosol preparation was assessed by the method of Habig and Jakoby (1981).

Incubation of Subcellular Fractions. Incubations were carried out at 37°C using rat hepatic microsomes or cytosol. The incubation medium consisted of 1 ml of subcellular suspension and 4 ml of 0.1 M sodium phosphate buffer (pH 7.4). Final protein concentrations in each incubation were 0.72 and 2.0 mg/ml for microsomal and cytosolic media, respectively. Incubations were performed in a cylindrical vessel (118 ml) partially immersed in a constant-temperature water bath and plumbed to a gas chromatograph via stainless steel tubing (Fig. 1). All internal surfaces consisted of glass, stainless steel, or Teflon (DuPont, Wilmington, DE), and the total volume of the system was 135 ml. To achieve a specific concentration in the system, chemical-laden air was transferred from a Tedlar bag through the system via a metal bellows pump operating at 300 ml/min (Fig. 1A). The volume of zero grade air in all bags was determined with a dry gas meter (American Meter Company, Horsham, PA) that maintained an accuracy of ± 1%. After 2 min, the system was closed off (Fig. 1B), and the contents of the headspace were sampled at regularly spaced time points. The air was circulated within the closed system while the incubation mixture was stirred. At the end of the equilibration period (~30–50 min), the incubation medium was supplemented with cofactor or cosubstrate solution via a septum, which resulted in a 7% change in the volume of medium. To investigate microsomal elimination of substrate, reactions were initiated by the addition of NADPH (0.4 mM, final concentration), glucose 6-phosphate (3.8 mM), and glucose-6-phosphate dehydrogenase (1.76 units/ml). Glutathione S-transferase catalyzed substrate elimination was achieved by the addition of glutathione (15 mM) (Kreuzer et al., 1991). Run times of 2 h were possible without evident departure from linearity.

Standard curves were prepared from Tedlar bags of varying substrate concentration. The atmosphere within each bag was transferred to the dry system (without medium) and the system was sampled after a 2-h min loading period.

Headspace Analysis by Gas Chromatography. Volatiles in the headspace compartment were analyzed by gas chromatography with flame ionization detection. Vapor aliquots of 200 µl were injected automatically onto a Hewlett-Packard model 5890 Series II GC (Palo Alto, CA) equipped with 1/8-inch i.d. × 6-ft length stainless steel column packed with 0.1% SP-1000 on 80/100 mesh Carbopack C (Supelco, Bellefonte, PA). Operating conditions were as follows: column/oven temperature, 160°C and flame ionization detection temperature, 250°C. The flow of the carrier gas, helium, was 10.5 ml/min; air, 400 ml/min; and hydrogen, 40 ml/min. The retention times of 1,2-DCE, 2,2-DCP, 1,3-DCP, and 1,2-DBE were 0.69, 0.85, 1.37, and 1.66 min, respectively, which were sufficiently short to permit repeated sampling of the system every 5 min. Longer retention times were observed for 1,4-DCB (3.66 min) and 1,3-DBP (3.4 min), necessitating a sampling interval of 10 min. All peaks were integrated with Hewlett-Packard Chem-Station computer software.

Kinetic Analysis of Concentration-Time Courses. Concentration-time courses were analyzed according to the two-compartment model of Kedderis and Held (1996) modified to account for system losses (Fig. 2). The initial loading of airborne substrate into the system from a Tedlar bag was simulated with a first-order rate constant of Q/V, where Q is the flow rate and V is the system volume.

The rate constant describing uptake of substrate (k1) and the thermodynamic partition coefficient (P) were obtained independently from the enzymatic parameters by solving the differential equations describing the introduction and equilibration of substrate in the system (Fig. 2: S1 = 1 for 2 min, then S1 = 0 for 28 min). Figure 3 shows a typical determination of k1 and P (for 1,3-DCP).
The estimated parameters $k_1$ and $P$ were subsequently used in the model describing metabolic clearance of substrate (Fig. 2; $S_2 = 1$ at 30 min). Uptake of substrate into the medium and evaporation into the headspace were described as first-order processes. Enzyme-mediated elimination of substrate was assumed to follow Michaelis-Menten (P450) or pseudo first-order (GST) kinetics. Kinetic parameters were obtained by fitting the two-compartment model to sets of measured concentration-time courses, determined in experiments having equivalent incubation media yet differing in initial concentration. A first-order term ($k_{loss}$) of 0.0275 h$^{-1}$ (S.E. = 0.027 h$^{-1}$) was used to account for system losses. The coupled differential equations were written in Matlab (Mathworks, Inc., Natick, MA) and solved with a variable-step, variable-order integration routine. The Nelder-Mead simplex optimization algorithm was used to determine the parameter values that minimized a weighted least-squares criterion.

**GST Activity Determined by Loss of GSH.** Rates of glutathione conjugation of several haloalkanes, catalyzed by rat liver cytosol, were determined using the method of Inskeep and Guengerich (1984). The reaction mixture consisted of 750 μl of 0.1 M Tris-HCl buffer (pH 8.0), 250 μl of cytosol (10.1 mg protein/ml), GSH in 200 μl of deionized water (0.74 mM, final), and haloalkane substrate dissolved in 100 μl of ethanol (9.2 mM, final). Samples were incubated at 37°C in sealed conical 1.5-ml polypropylene centrifuge tubes. Reactions were stopped by the addition of 100 μl of 33% (w/v) trichloroacetic acid. Samples were centrifuged for 5 min at 14,000g, and a 100-μl aliquot of supernatant was added to a solution consisting of 1.5 ml of 0.5 mM 5,5'-dithiobis(2-nitrobenzoic acid) in 0.1 M sodium phosphate buffer (pH 6.5). After a 10-min incubation at room temperature, the absorbance (412 nm) of each sample was measured using a Shimadzu UV-2101PC UV-visible spectrophotometer (Kyoto, Japan).

**Computational Model for Predicting Haloalkane Oxidation.** To evaluate structure activity and, in particular, to verify the low rate of oxidative clearance observed for 2,2-DCP, the relative rates of P450-mediated oxidation of haloalkanes were predicted by a semiempirical computational model (Harris et al., 1992; Yin et al., 1995; Jones et al., 2002). Relative rates of aliphatic hydroxylation were approximated with the predicted relative energies for hydrogen atom abstraction, which is postulated to be the product-determining step of the hydroxylation reaction. For slow reactions, such as hydrogen atom abstraction from hydrocarbons, less hydroxylated product and more water are produced when the hydrogen atom is more difficult to abstract (Yin et al., 1995).
Differential equations related to kinetic model: gas phase (1), \( V_1 \text{d}C_1/\text{d}t = S_1Q_{C_1}G + k_1V_1C_1/P - k_1V_1C_1 - k_{\text{met1}}V_1C_1; \) liquid phase (2), \( V_2 \text{d}C_2/\text{d}t = -k_1V_1C_1/P + k_1V_1C_1 - S_2Q_{C_2}G_2. \)

\[ V_1 = \text{volume of gas phase (L)} \]
\[ V_2 = \text{volume of incubation medium (L)} \]
\[ C_1 = \text{substrate concentration in the gas phase (μM)} \]
\[ C_2 = \text{substrate concentration in the medium (μM)} \]
\[ k_1 = \text{rate constant of substrate uptake into medium (h\(^{-1}\))} \]
\[ k_{\text{met1}} = \text{rate constant of substrate loss in headspace compartment (h\(^{-1}\))} \]

**FIG. 2.** Two-compartment model used to describe the kinetics of halogenated hydrocarbon biotransformation in gas uptake system.

- \( S_1 = \text{switch indicating system configuration} \) (0 = closed (Fig. 1B), 1 = open (Fig. 1A))
- \( C_{\text{met}} = \text{substrate concentration in Tedlar bar (Fig. 1)} \)
- \( Q = \text{flow rate of pump (L) } \)
- \( G = \text{G} = \exp(-QV_1/t) \)

**Metabolism**

\[ k_{\text{met}} = k_{\text{met}} = k, \text{ if first-order kinetics; } k_{\text{met}} = V_{\text{max}}/(K_m + C), \text{ if saturable kinetics (L/h)} \]
\[ V_{\text{max}} = \text{maximum rate of metabolism (μmol/h)} \]
\[ K_m = \text{apparent Michaelis-Menten constant (μM)} \]

model predicts the enthalpy of activation (\( \Delta H_{\text{act}} \)) from the ground-state reactants and product for a given reaction by the semiepipirical Austin model 1 (Dewar et al., 1985). The enthalpies of activation were calculated from the following regression equation:

\[ \Delta H_{\text{act}} = 2.60 + 0.22(\Delta H_{\text{red}}) + 2.38(IP) \]

where \( \Delta H_{\text{act}} \) is the predicted enthalpy of activation, \( \Delta H_{\text{red}} \) is the Austin model 1 enthalpy of reaction for hydrogen atom abstraction from the halogenated hydrocarbon by p-nitrosophenoxo radical, and IP is the ionization energy of the halogenated hydrocarbon radical product. Oxidation rates of each halohydrocarbon by P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard activities of P450 and GST were determined with standard
substrates (Table 1). The rate of p-nitrophenol hydroxylization in rat liver microsomes was 1.8-fold the value (0.76 nmol/min/mg microsomal protein) reported by Faller et al. (2001). GST activity toward 1-chloro-2,4-dinitrobenzene in rat liver cytosol was 2.3-fold the value (320 nmol/min/mg protein) reported by Swales and Caldwell (1997) and 0.7-fold the value (1054 nmol/min/mg protein) observed by Faller et al. (2001). Thus, overall activities did not differ by more than a factor of 3 from published values, confirming the quality of the subcellular fractions.

**Histochemical Determination of 1,3-DBP.** At an initial headspace concentration of 20 ppm, the apparent first-order rate constant for clearance of 1,3-DBP in rat cytosol was 20.2 \( \times 10^{-4} \) l/h/mg protein (Table 3); however, for an order-of-magnitude increase in concentration (260 ppm), the rate constant decreased only 5.5% (18.9 \( \times 10^{-4} \) l/h/mg protein). Examples of halogenated alkanes that exhibit nonsaturating GST enzyme kinetics are widespread (Guengerich et al., 1980; Ploemen et al., 1997; Wheeler et al., 2001a).

Brominated dihaloalkanes were cleared from rat cytosol faster than their chlorinated analogs, reflecting the expected halide order of reactivity (Br > Cl) (Table 3): the apparent first-order rate constants for 1,3-DBP and 1,3-DCP were 20.2 \( \times 10^{-4} \) and 1.6 \( \times 10^{-4} \) l/h/mg protein, respectively. The halide order of reactivity was also demonstrated by examining the rate of loss of GST, as described previously by Inskeep and Guengerich, 1984 (Fig. 7). For example, incubation of GSH-fortified cytosol with 1,3-DBP resulted in a significantly faster loss of GSH (11.9 \( \pm 0.85 \) nmol of GSH/min/mg protein) than incubation with 1,3-DCP (0.27 \( \pm 0.01 \) nmol of GSH/min/mg protein). Furthermore, by measuring either the rate of loss of halokalane from the vial headspace or GSH from the reaction medium, the rate of GSH conjugation was proportional to chain-length separation of dihalides (see Fig. 7 and Table 3). In contrast to the other halokalanes, no clearance was observed for 2,2-DCP in incubations of rat cytosol fortified with GSH (<1 \( \times 10^{-4} \) l/h/mg cytosol protein). Furthermore, no loss of glutathione was observed in incubations of rat cytosol fortified with 2,2-DCP (< 0.1 nmol of GSH/min/mg protein). Thus, 2,2-DCP was not metabolized by rat liver cytosol.

**Salmonella Mutagenicity Assay.** 1,3-DCP and 2,2-DCP were tested in Salmonella strains TA98, TA100, TA104, TA1535, RS100, and GSTT1–1. Cytotoxicity, assessed visually by a thinning of the background lawn of cells or by a reduction in revertants below the background level, was achieved at 1.0 to 2.0 mg/plate with 1,3-DCP and 2,2-DCP. There was not a consistent difference when tested in the presence or absence of S9, nor was there a notable difference in the

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**TABLE 1**

<table>
<thead>
<tr>
<th>Specific activities of rat liver enzymes</th>
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<tbody>
<tr>
<td>Body weight (g)*</td>
</tr>
<tr>
<td>Liver weight (g)*</td>
</tr>
<tr>
<td>Protein content in microsomal fraction (mg/ml)*</td>
</tr>
<tr>
<td>Protein content in cytosolic fraction (mg/ml)*</td>
</tr>
<tr>
<td>P450 activity (nmol/min/mg microsomal protein)</td>
</tr>
<tr>
<td>GST activity (nmol/min/mg cytosol protein)</td>
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* All values are expressed as mean ± S.D. for ten Fischer-344 rats.

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**FIG. 4.** Concentration-time courses of 1,3-DCP in the gas phase of the closed system (Fig. 1B) with incubation medium containing rat liver microsomes (0.72 mg of protein/ml, pH 7.4, 37°C). The apparent \( V_{max} = 30.3 \) nmol/h/mg microsomal protein. The apparent \( K_m \) in the incubation medium, and the corresponding \( K_m \) in the gas phase was 3.2 ppm [calculation: 1.09 \( \mu M \times 24.45 M^{-1} (P = 8.4) \).]

**FIG. 5** shows the relationship between the apparent first-order rate constant and the PRR for the halokalanes examined. A reasonable correlation (\( R^2 = 0.79 \), log-log space) was observed between the predicted rates of P450 metabolism and the experimentally determined rates. The predicted rate of oxidation of 2,2-DCP was 1.85% that of 1,3-DCP, which was reasonably consistent with the experimental determination (2.5% of 1,3-DCP).

**Cytoxosol-Mediated Elimination of 1,3-DCP, 2,2-DCP, and Related Haloalkanes.** We observed nonsaturating first-order kinetics in the clearance of halokalanes in rat liver cytosol that was supplemented with GSH. Figure 6 shows a representative time course for clearance of 1,3-DBP. At an initial headspace concentration of 20 ppm, the apparent first-order rate constant for clearance of 1,3-DBP in rat cytosol was 20.2 \( \times 10^{-4} \) l/h/mg protein (Table 3); however, for an order-of-magnitude increase in concentration (260 ppm), the rate constant decreased only 5.5% (18.9 \( \times 10^{-4} \) l/h/mg protein). Examples of halogenated alkanes that exhibit nonsaturating GST enzyme kinetics are widespread (Guengerich et al., 1980; Ploemen et al., 1997; Wheeler et al., 2001a).
cytotoxic dose between the strains tested here. Neither compound was mutagenic in any strain in the absence or presence of S9 mix (Table 4).

GSH Conjugation Reactions of 1,3-DBP and 1,3-DCP. The reaction mixture of 1,3-dihalopropanes and cytosol was analyzed by LC-MS to characterize the GSH products. Mouse liver cytosol-catalyzed GSH conjugation of 1,3-DBP and 1,3-DCP produced significant levels of \( S-(3\text{-bromopropyl})\text{GSH} \) and \( S-(3\text{-chloropropyl})\text{GSH} \), respectively (Fig. 8A). No other GSH conjugate peaks were produced after a 1-h reaction. In keeping with the halide order of reactivity, 1,3-DBP produced more GSH conjugate than did 1,3-DCP. The mass spectra of the GSH conjugates (Fig. 8, B and C) were consistent with the formation of a half-mustard (GSCH₂CH₂CH₂X) (Baillie and Davis, 1993). Furthermore, the abundance ratios of the halogen-containing compounds were also consistent with the structure of a half-mustard. The detection of a single GSH conjugate produced in relative abundant amounts from both 1,3-dihalopropanes suggested that the conjugates were relatively stable species.

Discussion

We have investigated the kinetics of 1,3-DCP and 2,2-DCP in rat liver microsomes and cytosol because these cellular fractions contain the enzymes known to be most important in mammalian metabolism of haloalkanes: membrane bound P450 in microsomes and soluble GST in cytosol. The bulk of the metabolic clearance of 1,3-DCP occurred by P450-dependent oxidation with a much smaller clearance mediated by GST (Tables 2 and 3). For concentrations of 1,3-DCP below the \( K_m \) of oxidative metabolism, we estimate that the GST pathway accounted for 1.3% of the combined flux of P450 and GST, assuming 40 mg of microsomal protein per gram of rat liver tissue and 89 mg of cytosol protein per gram of rat liver tissue (Ploemen et al., 1997). In contrast, we estimate that 10 and 39% of the total metabolism of 1,2-DBE and 1,3-DBP, respectively, would occur through the GST pathway, reflecting the expected halide order of reactivity and chain length. van Bladeren et al. (1981) previously estimated about 20% of the metabolism of 1,2-DBE occurred via the GST route in rats.

For 2,2-DCP we observed only a minor level of clearance via the P450 pathway in comparison with 1,3-DCP. We had anticipated a lower rate of oxidation for 2,2-DCP because the halogen-bearing carbon contains no carbon-hydrogen bond, which is the site of oxygen insertion in the oxidative dehalogenation mechanism (Sipes and Gandolfi, 1991). The observed difference in oxidative rates for 1,3-DCP and 2,2-DCP was investigated by a computational method (Harris et

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc. Range (ppm)</th>
<th>( k_1 ) (liquid:air)</th>
<th>( P ) (liquid:air)</th>
<th>( V_{max} ) (nmol/h/mg protein)</th>
<th>( K_m ) (( \mu M ))</th>
<th>( V_{max}/K_m ) (l/h/mg) ( \times 10^4 )</th>
<th>PRR*</th>
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</thead>
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<td>1,3-DCP</td>
<td>0.5–220</td>
<td>4.0</td>
<td>8.3</td>
<td>30.3</td>
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<td>276</td>
<td>108.2</td>
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<tr>
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<td>5–75</td>
<td>4.9</td>
<td>8.5</td>
<td>33.9</td>
<td>1.67</td>
<td>203</td>
<td>158.8</td>
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<td>8.8</td>
<td>26.2</td>
<td>2.47</td>
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<td>31.6</td>
</tr>
<tr>
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<td>37.3</td>
<td>5.25</td>
<td>71</td>
<td>109.8</td>
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<tr>
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<td>14.1</td>
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</tr>
<tr>
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<td>0.8</td>
<td>N.D.</td>
<td>N.D.</td>
<td>7</td>
<td>2.0</td>
</tr>
</tbody>
</table>

N.D., not determined.

* Relative to 2,2-DCP at 310K, see under Materials and Methods.

**TABLE 2**

Kinetic parameters of P450-dependent metabolism of haloalkanes catalyzed by rat liver microsomes

Kinetic parameters were obtained by fitting the kinetic model (see Fig. 2) to the data.

**FIG. 5.** Apparent first-order rate constant \( (V_{max}/K_m; \text{l/h/mg protein}) \) versus predicted relative rate of metabolism by P450 (relative to 2,2-dichloropropane).

**FIG. 6.** Concentration-time courses of 1,3-DBP in the gas phase of the closed system with incubation medium containing rat liver cytosol (protein content 2.0 mg/ml, pH 7.4, 37°C); GSH was added to incubation medium at 40 min.
TABLE 3

Kinetic parameters of GSH-dependent metabolism of haloalkanes in rat liver cytosol

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc.</th>
<th>( V_{\text{rel}} ) (( \mu \text{mol/min/mg} ))</th>
<th>Relative Flux GST/GST (+ P450)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,3-DBP</td>
<td>20</td>
<td>20.2 ± 1</td>
<td>39</td>
</tr>
<tr>
<td>1,3-DBP</td>
<td>260</td>
<td>18.9 ± 1</td>
<td>37</td>
</tr>
<tr>
<td>1,2-DBE</td>
<td>30</td>
<td>5.3 ± 1</td>
<td>10</td>
</tr>
<tr>
<td>1,4-DCB</td>
<td>30</td>
<td>1.9 ± 1</td>
<td>2.0</td>
</tr>
<tr>
<td>1,3-DCP</td>
<td>30</td>
<td>1.6 ± 1</td>
<td>1.3</td>
</tr>
<tr>
<td>1,2-DCE</td>
<td>30</td>
<td>&lt;1</td>
<td>N.D.</td>
</tr>
<tr>
<td>2,2-DCP</td>
<td>3</td>
<td>&lt;1</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D., not determined.

Fig. 7. Specific GST activities (nmol/min/mg) of \( \alpha, \omega \)-haloalkanes in rat liver cytosol determined by loss of GSH in presence of haloalkane substrate (Inskeep and Guengerich, 1984).

1,4-DBB, 1,4-dibromobutane.

al., 1992; Yin et al., 1995; Jones et al., 2002) based on the predicted relative energies of hydrogen atom abstraction. Both predicted and experimental rates indicated that 1,3-DCP was cleared ~40 to 50 times faster than 2,2-DCP via the P450 pathway.

We also evaluated GST activity for a series of related \( \alpha, \omega \)-haloalkanes to assess structure activity and to validate the observed metabolism rates for 2,2-DCP and 1,3-DCP by GST. Using an assay for glutathione depletion to infer conjugation via loss of GSH, we reproduced previous findings (Inskeep and Guengerich, 1984). These results demonstrated that the rate of GSH conjugation of \( \alpha, \omega \)-bromoalkanes increased with increasing chain length. The relative rates of GSH conjugation of 1,3-DBP and 1,4-dibromobutane were ~3- and 10-fold greater, respectively, than for 1,2-DBE. We also observed a similar trend with a series of \( \alpha, \omega \)-chloroalkanes that included 1,3-DCP, both with the headspace system and with the method of Inskeep and Guengerich (1984).

The trend in the \( \alpha, \omega \)-chloroalkanes and the corresponding \( \alpha, \omega \)-bromoalkanes may be explained by recognizing that \( \beta \)-halogens on substituted \( \alpha, \beta \)-dihaloethanes are deactivating with respect to mono-haloethanes when both sets of compounds undergo \( S_n^2 \) reactions at the C-\( \omega \) position (Hine and Brader, 1953). The deactivating effect of the C-\( \beta \) halogen is apparently due to its interference with both the incoming nucleophile and leaving group, resulting in a transition-state intermediate with an increased potential energy (due to compressions of the covalent bonds) and to a reduced entropy (due to the restricted motion of the atoms) (Lowry and Richardson, 1987). We postulate that intervening methylene groups between carbon-bearing halogens tend to lessen this interference, resulting in faster rates of conjugation. This explains why 1,3-DCP undergoes GSH conjugation faster than 1,2-DCE but not as fast as 1,4-DCB. For 2,2-DCP, a similar explanation may also hold, since \( \alpha \)-halogens on halogenated methanes decrease the reactivity of other halogens attached to the same carbon atom (Hine et al., 1955). It was found for the methylene halide \( \text{XCH}_2\text{Br} \) that the \( S_n^2 \) reactivity varied with the nature of the \( \alpha \)-substituent X as: \( H > F > Cl > Br \), I (Hine et al., 1955). The deactivating influence of the second chlorine atom in 2,2-DCP likely explains the lack of GST-catalyzed metabolism.

Analysis of the reaction mixture of 1,3-DCP and GSH by LC-MS revealed significant production of \( \text{GSHCH}_2\text{CH}_2\text{Cl} \), suggesting that the conjugate half-mustard did not form a reactive sulfonium ion as occurs with 1,2-dichloroethane, 1,2-dibromoethane, and 1,2-dibromo-3-chloropropane.

The production of \( S \)-\( (\text{3-halopropyl}) \text{GSH} \) was clearly catalyzed by the mixture of GST isoforms present in mouse liver cytosol. Interestingly, neither \( S,S' \)-propyl-bis(GSH) nor \( S \)-\( (\text{3-hydroxypropyl}) \text{GSH} \) was detected in reaction mixtures containing 1,3-DBP or 1,3-DCP following a 1-h reaction. This was in marked contrast to GSH reactions with 1,2-dihaloethanes, which produced abundant amounts of both \( S,S' \)-ethylene-bis(GSH) and \( S \)-\( (\text{2-hydroxyethyl}) \text{GSH} \) (Cmarik et al., 1990; Wheeler et al., 2001b). The half-mustards (\( \text{GSHCH}_2\text{CH}_2\text{X} \)) were not detectable because they rearrange to a highly reactive episulfonium ion that is rapidly hydrolyzed or, alternatively, can react with cellular nucleophiles such as GSH or DNA (Peterson et al., 1988). These secondary reactions are not catalyzed by GST enzyme (Wheeler et al., 2001b). The half-lives of \( S \)-\( (\text{2-chloroethyl}) \text{GSH} \) and \( S \)-\( (\text{2-bromoethyl}) \text{GSH} \) in aqueous buffer were previously estimated to be 5.3 and 0.44 min, respectively (Wheeler et al., 2001b), and this gave direct evidence of the instabilities of these half-mustards. Furthermore, formation of a sulfonium ion intermediate derived from GSH conjugation of 1,3-dihaloethanes is unlikely to be an energetically favored pathway (Smit et al., 1979). Thus, \( S \)-\( (\text{3-halopropyl}) \text{GSH} \) conjugates are more stable, and as a result subsequent reactions with nucleophiles (e.g., water, GSH, or DNA) are likely to proceed much more slowly than \( S \)-\( (\text{2-haloethyl}) \text{GSH} \) conjugates. Thus, this GST pathway may be considered a detoxication mechanism for 1,3-DCP. This reduced reactivity may account for the much lower mutagenic activity of 1,3-dihaloalcohol, compared with 1,2-dihaloethanes, in \( S \).\text{typhimurium} that express rat or human glutathione transferase T1–1 (Thier et al., 1996; Wheeler et al., 2001b).

The results of the \( S \).\text{Salmonella} plate-incorporation assay for 1,3-DCP and 2,2-DCP were negative, including those strains expressing GSTT1–1 activity. These mutagenicity data, which show that GST-theta does not activate 1,3-DCP or 2,2-DCP to mutagens, are consistent with the toxicokinetic data and structural consideration indicating that GST does not activate 1,3-DCP or 2,2-DCP to a mutagen. The negative results obtained in the \( S \).\text{Salmonella} assay conducted with S9 indicate that the P450 pathway did not activate 1,3-DCP or 2,2-DCP. Clearly, 1,3-DCP was metabolized via the P450 pathway. This was deduced based on its increased rate of decline in microsomal incubations in the presence (versus the absence) of an NADPH-generating system. By analogy with the 1,2-dihaloethanes, we expect production of 3-chloropropanaldehyde, the corresponding haloaldehyde. The computational model for hydroxylation predicts that the terminal carbons would be energetically favored 9 to 1 [\( (49 + 49)/11 \) over the center carbon, consistent with the production of 3-chloropropanaldehyde. By analogy, P450 oxidation of 1,2-dichloroethane leads to 2-chloroacetaldehyde (Guengerich et al., 1980), an aldehyde found to be mutagenic in TA1535 (Rannug et al., 1978). However, 1,2-dichloroethane was not found to be mutagenic when tested in \( S \).\text{Salmonella} TA1535 with microsomes supplemented with NADPH-regenerating system (Guengerich et al., 1980), suggesting that quantitatively relevant levels of 2-chloroacetaldehyde were not produced. Our negative
TABLE 4

Mutagenicity (rev/plate ± S.E.) of 1,3-dichloropropane and 2,2-dichloropropane in Salmonella

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<thead>
<tr>
<th>Dose (mg/plate)</th>
<th>TA98 +S9</th>
<th>TA98 −S9</th>
<th>TA100 +S9</th>
<th>TA100 −S9</th>
<th>TA104 +S9</th>
<th>TA104 −S9</th>
<th>TA1535 +S9</th>
<th>TA1535 −S9</th>
<th>GSTT1 +S9</th>
<th>GSTT1 −S9</th>
<th>RSJ100 +S9</th>
<th>RSJ100 −S9</th>
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</thead>
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<tr>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>0.00</td>
<td>31 ± 2</td>
<td>17 ± 3</td>
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<td>89 ± 9</td>
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<td>262 ± 9</td>
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<td>12 ± 2</td>
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<td>123 ± 6</td>
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<td>303 ± 9</td>
<td>258 ± 11</td>
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<td>Toxic</td>
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<td>Toxic</td>
<td>Toxic</td>
<td>Toxic</td>
<td>112 ± 113</td>
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<td>866 ± 20</td>
<td>634 ± 24</td>
<td>1479 ± 39</td>
<td>721 ± 19</td>
<td>879 ± 18</td>
<td>458 ± 33</td>
<td>1132 ± 23</td>
<td>379 ± 22</td>
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</table>

'S Results are the average of two experiments, each in duplicate; dimethyl sulfoxide controls were done in triplicate.

'b n toxicity was observed in one of the two experiments.

'A, LC-MS total ion chromatograms (m/z: 350–460) of the reaction mixture following GSH conjugation of the 1,3-dihalopropanes. Traces 1 and 3, nonenzymatic reactions of GSH with 1,3-DCP and 1,3-DBP, respectively. Traces 2 and 4, enzyme-catalyzed reactions of GSH with 1,3-DCP and 1,3-DBP, respectively. The GSH conjugate peaks are indicated by arrows, along with their molecular weights. Full-scan mass spectra of the S-(3-bromopropyl)GSH (B) and S-(3-chloropropyl)GSH peaks (C) are shown. The parent molecular ions (MH+) are indicated as well as fragment ions. Loss of 129 Da from the parent molecular ion represents the neutral loss of pyroglutamic acid from the GSH moiety (Baillie and Davis, 1993). The abundances of each doublet peak (M, M + 2) for each GSH conjugate are consistent with the isotopic ratios of 35Cl/37Cl (≈3) and 79Br/81Br (≈1) and gives evidence for the presence of a single chlorine or bromine atom in each GSH conjugate molecule (Budde, 2001).
findings with 1,3-DCP in Salmonella TA1535 and rat liver S9 suggest that quantitatively relevant levels of reactive species were not produced or were scavenged by nucleophiles such as GSH. In a study examining renal toxicity of various halogenated propanes, 1,3-DPB and 1-bromo-3-chloropropane showed no evidence of in vivo renal DNA damage (Låg et al., 1991).

In conclusion, we have used in vitro methods and computational approaches to gain insight into the genotoxic potential posed by these drinking water contaminants. For 2,2-DCP, the overall slow rate of metabolic clearance in hepatic subcellular fractions suggested low reactivity, consistent with the findings of the Salmonella assay. For 1,3-DCP, we observed significant clearance via the P450 and GST pathways, consistent with predictions based on its structure. Although 1,3-DCP was readily metabolized in hepatic subcellular fractions, we found no evidence of genotoxicity.

Acknowledgments. We thank Peggy Matthews of the U.S. Environmental Protection Agency for media preparation.

References


Tafazoli M and Kirsch-Volders M (1996) In vitro mutagenicity and genotoxicity study of 1,2-dichloroethylene, 1,1,2-trichloroethane, 1,2,3-dichloropropane, 1,2,3-trichloropropane, 1,1,3-trichloropropene, using the microcumulus test and the alkaline single cell gel electrophoresis technique (comet assay) in human lymphocytes. Mutat Res 373:185–202.


