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1,2-Dichloroethane (DCE), a solvent and byproduct of the manufacture of polymers, and 1,2-dibromoethane, a soil fumigant, are known to be metabolized by conjugation with glutathione (GSH) to yield mercapturic acid derivatives. An alternate route of metabolism of the GSH conjugate involves β -elimination of halide ion to form an

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1,2-Dichloroethane (DCE), a solvent and byproduct of the manufacture of polymers, and 1,2-dibromoethane, a soil fumigant, are known to be metabolized by conjugation with glutathione (GSH) to yield mercapturic acid derivatives. An alternate route of metabolism of the GSH conjugate involves β -elimination of halide ion to form an olefin. In the present study, ethylene production from DCE was measured by gas chromatography in rat tissues as an index of this latter route of metabolism. The rate of enzymic ethylene production was linear over a 1-hr incubation time and from 1 to 8 mg of protein

per ml reaction volume. The temperature optimum for ethylene formation from DCE was 55°C and no distinct pH optimum was observed. DCE metabolism was highly dependent on the presence of reduced GSH. Metabolic activity was limited to hepatic and renal cytosolic fractions. The reaction was inhibited only by p-chloromercuribenzoic acid and by diethyl maleate and methyl iodide, which are substrates for GSH S-transferases. S-(2-Chloroethyl)-DL-cysteine-HCl, an analog of the conjugate formed from DCE and GSH, was nonenzymically converted to ethylene.

Vicinally substituted halogenated hydrocarbons are widely used in a variety of industrial, commercial, and agricultural applications. The annual U. S. production of 1,2-dichloroethane (DCE)¹ in 1977 was 7×10^9 kg, ranking it fifteenth among chemicals produced in this country (1). DCE has been identified in drinking water at concentrations up to 0.8 μ g/liter (2). The use of 1,2-dibromoethane (DBE) as a soil fumigant and gasoline additive has led to widespread exposure of the general population to this chemical. The chemical reactivity of these and other 1,2-dihalo-substituted hydrocarbons has made them important intermediates in many synthetic pathways.

Previous metabolic studies on vic-dihalides have shown the formation of a variety of products. Early studies by Heppel and Porterfield (3) showed that both DBE and DCE were dehalogenated by rat liver cytosol. The conversion of DBE to ethylene by soil cultures has been described (4). Subsequent work has shown that DCE is converted to chloroacetic acid and S-carboxymethylcysteine (5), and urinary metabolites of DCE include mercapturic acid derivatives (6). The involvement of glutathione (GSH) in the detoxification of these compounds was investigated by Nachتمي (7) who showed that S-(2-hydroxyethyl)glutathione and S,S'-ethylenedisglutathione, the presumed conjugation products of DBE with one and two molecules of GSH, respectively, were present in hepatic and renal tissues of rats dosed with DBE. Hepatic glutathione levels have been shown to be reduced follow-

ing exposure of rats to DCE (8). Recently, bromoacetaldehyde has been identified as a metabolite of DBE in rat liver microsomes (9). Both DCE and DBE have been shown to be mutagenic in *Salmonella typhimurium* (10-12) and DBE has also been shown to produce mutagenic effects in eukaryotic systems (13-15). Rannug *et al.* (12) have shown that the mutagenic potential of DCE is enhanced in the presence of glutathione and hepatic cytosol. Furthermore, both DCE and DBE have been classified as carcinogens by the National Cancer Institute.

Our interest in the metabolism of vic-dihalides stems from chemical considerations concerning the stability of GSH conjugates possessing a good leaving group *beta* to the sulfur atom. Such conjugates may undergo elimination reactions to yield olefinic metabolites. Results of investigations on the metabolism of both DBE and DCE to ethylene *in vitro* are presented herein.

Materials and Methods

Chemicals. 1,2-Dibromoethane (99%), 1,2-dichloroethane (99+ %), and D-(-)-penicillamine (99+ %) were obtained from Aldrich Chemical Co., Milwaukee, Wis. Reduced glutathione, L-cysteine-HCl, and cysteamine were obtained from Sigma Chemical Co., St. Louis, Mo. d₄-Dibromoethane was purchased from Merck and Co./Isotopes, Los Angeles, Cal. and contained 99 atom % deuterium. S-(2-Chloroethyl)-DL-cysteine-HCl was prepared by the method of Carson and Wong (16). The prepared material had a mass spectrum consistent with the expected structure. All other reagents and chemicals were of reagent grade and were obtained from commercial sources.

Enzyme Preparation. Male Sprague-Dawley-derived rats weighing 225-250 g were employed. Animals were killed by decapitation and livers were perfused *in situ* with 1.15% (w/v) KCl. The tissue was minced, homogenized in a Dounce homogenizer in 1.15% KCl, and centrifuged at 9,000g for 20 min. The resulting supernatant fraction was centrifuged at 105,000g for 60 min to yield the cytosol fraction which, unless otherwise stated, was dialyzed overnight against 0.1 M phosphate buffer, pH 7.4, at 4°C. Cytosol fractions of kidney, lung, brain, and muscle were similarly prepared but were not dialyzed. Preparation of subcellular fractions was accomplished by centrifuging the liver homogenate at 600g for 20 min. The pellet, when resuspended, centrifuged again at 600g, and resuspended, constituted the nuclear fraction. The 600g supernatant fraction was centrifuged at 9,000g for 20 min, and the pellet, when resuspended, centrifuged again at 9,000g, and resuspended, constituted the mitochondrial fraction. The 9,000g su-

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¹ Abbreviations used are: DCE, 1,2-dichloroethane (also known as ethylene dichloride or EDC); DBE, 1,2-dibromoethane (also known as ethylene dibromide or EDB); GSH, reduced glutathione; CEC, S-(2-chloroethyl)-DL-cysteine hydrochloride.

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pernatant fraction was centrifuged at 105,000g for 60 min to prepare microsomal (pellet) and cytosolic (supernatant) fractions. Volumes of the prepared fractions were adjusted to yield the equivalent of 250 mg of liver (wet weight) when used in incubation mixtures.

Incubation Mixtures. Unless otherwise stated, incubation mixtures contained 50 μ mol of phosphate buffer (pH 7.4), 30 μ mol of GSH, 255 μ mol of substrate, and 6 mg of cytosol protein in a total volume of 3 ml. When the pH dependency of the reaction was studied, 50 μ mol of phosphate buffer were used from pH 6.5–8.0 and 50 μ mol of Tris-HCl from pH 8.0–9.0. Incubations were carried out in flasks closed with sleeve-type stoppers with shaking at 37°C for 30 min in an atmosphere of air. The reaction was stopped by placing the flasks on ice.

Analytical Methods. Ethylene was quantified gas chromatographically by injecting water into the incubation flasks, thereby displacing a 1-ml sample of the flask headspace gas into the sample loop of a gas-sampling valve. Ethylene concentrations were not corrected for partitioning of the gas between the medium and the headspace. However, inasmuch as the blood/gas partition coefficient of ethylene is 0.15 (17), only a small underestimation of ethylene concentrations would have been incurred. A Varian series 1400 gas chromatograph equipped with a flame-ionization detector was used. Ethylene was separated from other components of the headspace gas on a 2-mm i.d. $\times$ 1.8-m glass column packed with Carbo-sieve-B (Supelco, Bellefonte, Pa.), 60/80 mesh, and maintained at 140°C. The injector and detector temperatures were 193 and 220°C, respectively. A 100-ppm calibration standard of ethylene in N_2 was eluted in 1.5 min. Protein was measured by the method of Lowry *et al.* (18) with bovine serum albumin as the reference standard.

Gas Chromatography-Mass Spectrometry. A Finnigan model 3200E gas chromatograph/mass spectrometer was fitted with a 2-mm i.d. $\times$ 1.5-m glass column packed with Carbo-sieve-B, 120/140 mesh. The column was operated at 180°C with He as the carrier gas. A 1-ml sample of the headspace gas was introduced onto the column by use of a gas-sampling valve. A Finnigan PROMIM multiple-ion monitoring device was used to monitor the intensities of ions at m/e 26 and 30. All ion-source parameters were adjusted to yield maximum intensity peaks at the analyzed masses consistent with instrument stability.

Results

The dependence of the enzymic metabolism of DCE to ethylene on incubation time, protein concentration, temperature, and pH are shown in fig. 1. The reaction was linear with time for at least 1 hr and with protein concentrations up to at least 8 mg of protein per ml. The temperature dependency of the enzymic production of ethylene from DCE showed a maximum at 55°C. No distinct pH optimum for ethylene production from DCE was observed; rather, the reaction rate increased as the pH was increased from 6.5 to 9.0. Based on these studies, the following conditions were employed for subsequent experiments: 30-min incubation time at 37°C with 2 mg of protein per ml; pH 7.4.

The cofactor requirements for DCE metabolism to ethylene are shown in table 1. Of the several cofactors tested, only GSH supported appreciable generation of ethylene and the presence of a NADPH-generating system did not alter the rate of DCE metabolism. Other thiols, including D-penicillamine and L-cysteine, could not substitute for GSH.

Table 2 shows the distribution of the enzymes catalyzing the conversion of DCE to ethylene in various rat tissues. Liver cytosol exhibited the highest activity of the tissues studied; kidney also showed an appreciable rate of ethylene formation. Negligible reaction rates were detected with cytosol prepared from lung, brain, or muscle.

Subcellular fractions of liver tissue were compared for their ability to convert DCE to ethylene (table 3). Cytosol exhibited the highest rate of ethylene formation, whereas mitochondrial and microsomal preparations formed only low levels of ethylene. A

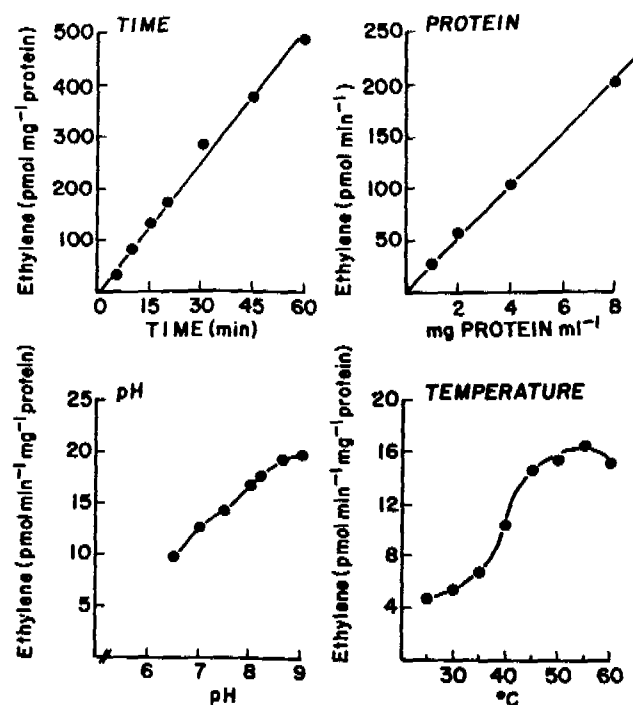


FIG. 1. Dependence of the metabolism of DCE to ethylene on time, protein concentration, pH, and temperature.

Standard conditions of 30-min incubation time at 37°C with 2 mg of protein per ml at pH 7.4 were employed, except that the parameters were varied as indicated on the abscissa of each panel. Substrate and GSH concentrations were 86 and 10 mM, respectively. Values are the averages of two experiments and are corrected for nonenzymic reaction.

TABLE 1

Cofactor requirements for the metabolism of 1,2-dichloroethane to ethylene by rat liver cytosol

Substrate and cofactors were present at final concentrations of 86 and 10 mM, respectively. The NADPH-generating system consisted of isocitric acid (3.3 mM), NADP⁺ (0.33 mM), and isocitrate dehydrogenase (1.0 unit). Values are the average of two experiments and are corrected for nonenzymic reaction.

Cofactor	Ethylene pmol/min/mg
GSH	10.5
NADPH-generating system + GSH	9.8
NADPH-generating system - GSH	0.1
D-Penicillamine · HCl	0.0
L-Cysteine · HCl	0.3

TABLE 2

Metabolism of 1,2-dichloroethane to ethylene in various rat tissues

The final concentrations of DCE and GSH were 86 and 10 mM, respectively. Cytosol prepared from the various tissues was used without dialysis. Data represent the means $\pm$ SD of two or three animals, as indicated by the numbers in parentheses, and are corrected for nonenzymic reaction.

Tissue	Ethylene pmol/min/mg
Liver	10.3 $\pm$ 3.5 (3)
Kidney	5.2 $\pm$ 1.9 (3)
Lung	0.4, 1.2 (2)
Brain	0.2, 0.4 (2)
Muscle	0, 0 (2)

low but consistent activity was detected in the nuclear fraction, which may be attributable to cytosolic contamination.

The substrate specificity of the enzymes catalyzing this reaction was investigated by use of several vicinally substituted ethanes (table 4). Ethylene bis(methanesulfonate) and 2-chloroethyl methanesulfonate showed low rates of ethylene production, and nearly as much nonenzymic as enzymic reaction was observed. 2-Bromoethyl acetate did not yield an appreciable amount of ethylene even in the presence of paraoxon, an inhibitor of cellular esterases (19). Reactivity within the series of *vic*-dihaloethanes followed the halide order with C—Br bond breakage occurring at a faster rate than C—Cl bond cleavage.

The effect of potential inhibitors of the metabolism of DCE to ethylene is shown in table 5. Metabolic inhibitors, such as cyanide, fluoride, and EDTA, showed no inhibitory effect; in contrast, the sulfhydryl reagent, *p*-chloromercuribenzoic acid, produced a pronounced inhibition. The microsomal mixed-function oxidase inhibitor, SKF 525-A, had no effect on DCE metabolism. Among the known substrates for glutathione S-transferases tested, both methyl iodide and diethyl maleate inhibited DCE conversion to ethylene.

The identity and source of the ethylene formed was investigated by GC/MS with deuterium-labeled DBE as the substrate (fig. 2). Single ions corresponding to known fragments of ethylene and *d*₄-ethylene were monitored; thus, *m/e* 26, corresponding to C₂H₂⁺ or C₂D₂⁺, and *m/e* 30, representing only C₂D₃⁺, were measured. The fragmentogram of the ethylene produced from unlabeled DBE was identical to that of authentic ethylene; i.e., a peak was seen at *m/e* 26 but not at *m/e* 30. When *d*₄-DBE served as the substrate, the fragmentogram showed signals at both *m/e* 26 and 30, indicating the formation of *d*₄-ethylene. The literature values for the fragmentation pattern of authentic *d*₄-ethylene have been reported (20). The ratio of ion abundances at *m/e* 30/26 is approximately

4. This ratio corresponds well with our experimentally determined value of 3.75.

A presumed intermediate in the conversion of DCE to ethylene is the GSH conjugate of DCE, S-(2-chloroethyl)glutathione. An analog of this intermediate, S-(2-chloroethyl)-DL-cysteine-HCl (CEC), was tested for its ability to form ethylene. These results are shown in table 6. Ethylene formation from CEC was not dependent on the presence of enzyme but was highly dependent

TABLE 5

Effect of various enzyme inhibitors on the metabolism of 1,2-dichloroethane to ethylene

Incubations were carried out with final concentrations of DCE and GSH of 43 and 10 mM, respectively. Inhibitors were added at final concentrations of 5 mM, except as noted, before addition of DCE. Data represent the means $\pm$ SD.

Addition	N	Ethylene Formation ^a % of control
KCN	4	99 $\pm$ 8
NaF	4	102 $\pm$ 9
Na ₂ EDTA	4	107 $\pm$ 8
<i>p</i> -Chloromercuribenzoic acid	4	22 $\pm$ 2
Diethyl maleate	4	67 $\pm$ 8
Methyl iodide	4	14 $\pm$ 3
1,2-Dichloro-4-nitrobenzene	3	97 $\pm$ 15
1,2-Epoxy- <i>p</i> -nitrophenoxyp propane	3	92 $\pm$ 9
SKF 525-A ^b	4	107 $\pm$ 19
Halothane	4	105 $\pm$ 16
Methoxyflurane	4	93 $\pm$ 7
Dichloromethane	4	89 $\pm$ 4

^a Control rate of ethylene formation was 6.6 $\pm$ 0.7 pmol/min/mg of protein (*N* = 4).

^b Final concentration was 0.1 mM.

TABLE 3

1,2-Dichloroethane metabolism by subcellular fractions of rat liver

DCE and GSH final concentrations were 43 and 10 mM, respectively. Each reaction mixture contained a volume of the subcellular fraction equivalent to 250 mg of liver. Data represent the means $\pm$ SD of three experiments and are corrected for nonenzymic reaction.

Fraction	Ethylene pmol/min
Nuclear	4.7 $\pm$ 0.9
Mitochondrial	0.4 $\pm$ 0.4
Microsomal	2.3 $\pm$ 0.8
Cytosolic	85.9 $\pm$ 7.2

TABLE 4

The metabolism of vicinally-substituted ethanes to ethylene by rat liver cytosol

Substrates and GSH were added to final concentrations of 25 and 10 mM, respectively. Paraaxon was added to a final concentration of 10⁻⁶ M. Nonenzymic reaction was determined by performing incubations of substrate with buffer in place of cytosol. Enzymic reaction is the difference between total reaction and nonenzymic reaction. Data represent the means $\pm$ SD of three experiments

Substrate	Ethylene	
	Nonenzymic	Enzymic
	pmol/min	
Ethylene bis(methanesulfonate)	11.9 $\pm$ 1.0	8.3 $\pm$ 1.8
2-Chloroethyl methanesulfonate	6.2 $\pm$ 1.1	13.6 $\pm$ 4.7
Bromoethyl acetate	0.4 $\pm$ 0.2	0
Bromoethyl acetate + paraoxon	0.2 $\pm$ 0.1	0.4 $\pm$ 0.3
1,2-Dichloroethane	0.6 $\pm$ 0.2	26.8 $\pm$ 8.5
1-Bromo-2-chloroethane	5.9 $\pm$ 4.9	135.7 $\pm$ 55.6
1,2-Dibromoethane	323.5 $\pm$ 161.2	305.1 $\pm$ 87.6

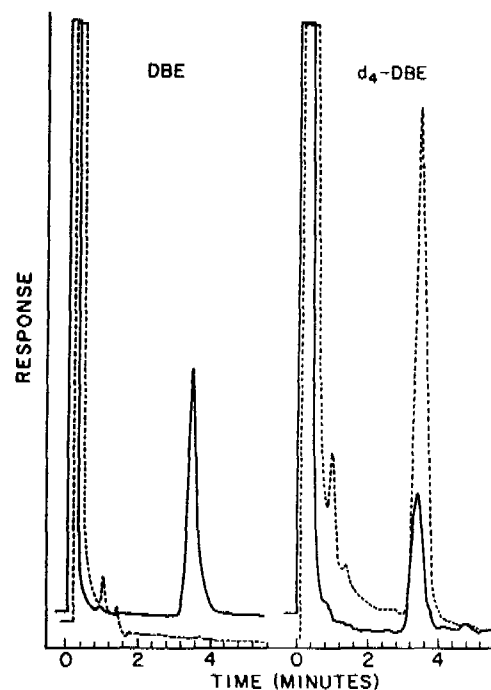


FIG. 2. GC/MS analysis of ethylene produced by the metabolism of 1,2-dibromoethane and *d*₄-1,2-dibromoethane.

---, *m/e* 30 (C₂D₃); —, *m/e* 26 (C₂H₂ or C₂D).

TABLE 6

The conversion of *S*-(2-chloroethyl)-DL-cysteine-HCl to ethylene by rat liver cytosol

The final concentration of substrate in the reaction flasks was 10 mM. Thiol compounds, when present, were added to a final concentration of 10 mM. Data represent the means $\pm$ SD.

Additions	N	Ethylene	
		Cytosol absent	Cytosol present
		pmol/min	
None	4	114 $\pm$ 4	130 $\pm$ 3 ^a
GSH	4	619 $\pm$ 48	810 $\pm$ 39
L-Cysteine-HCl	3	729 $\pm$ 24	782 $\pm$ 18
D-Penicillamine-HCl	3	219 $\pm$ 7	242 $\pm$ 10
Cysteamine-HCl	2	585 $\pm$ 40	594 $\pm$ 64

^a Cytosol was denatured by heating for 5 min at 100°C.

on the presence of sulfhydryl compounds, such as glutathione, cysteine, penicillamine, or cysteamine.

Discussion

These studies show that GSH conjugates may be metabolized by routes other than those leading to water-soluble mercapturic acid derivatives. In particular, the enzymic conversion of DCE to ethylene is characterized by many of the same properties as conjugations of GSH with other electrophilic centers of foreign compounds but leads to the formation of a volatile, unsaturated product. The absence of a pH optimum for ethylene formation from DCE is similar to results obtained by Johnson (21) for iodomethane metabolism and by Ahmed and Anders (22) with dihalomethanes. Alkaline pH values would be expected to increase the ionization of the sulfhydryl group of GSH and, thus, should promote the reactivity of this cofactor. The cofactor requirements, the effect of various inhibitors, as well as the tissue and subcellular distribution of the enzymes catalyzing the conversion of DCE to ethylene, are similar to those of the GSH S-transferases. Ethylene production from other vicinally substituted ethanes shows a marked specificity for halogen leaving groups on the β -carbon; methanesulfonates showed little reactivity. However, neither of the methanesulfonates inhibited DCE metabolism to ethylene (data not shown). Within the series of *vic*-dihaloethanes, initial conjugation with GSH appeared to be the rate-limiting step and β -elimination of the second halide ion was accomplished rapidly. Ethylene production from CEC suggested that this second step is not an enzyme-mediated process, but is a chemical reaction between the GSH conjugate and a thiol.

Based on these findings, at least two reaction mechanisms may be tentatively proposed for olefin formation from *vic*-dihaloethanes (fig. 3). The first step in the pathway shown in fig. 3A is a nucleophilic attack (S_N2) of GSH on the electrophilic carbon of the *vic*-dihalide; this reaction is presumably catalyzed by a GSH S-transferase. The second step of this mechanism involves the attack of a thiol on the sulfur atom of the conjugate, S-(2-haloethyl)glutathione, followed by the β -elimination of a halide ion and the formation of a disulfide. The direct reaction of sulfur with sulfur is thought to be involved in the chemical and enzymic reduction of α -haloketones (23,24) and in the oxidation of GSH (25), and has been proposed as a step in the conversion of haloforms to carbon monoxide (26). The S-(2-haloethyl)glutathione intermediate may rearrange to yield an episulfonium ion. The pathway shown in fig. 3B involves an E2 elimination mechanism and has been proposed by Weygand and Zurnach for the oxidation of thiols to disulfides by 1,2-diiodo-

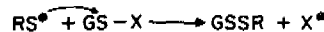
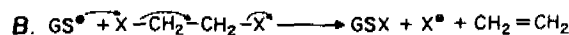
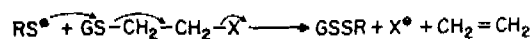
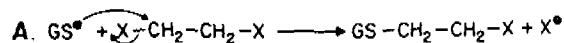


FIG. 3. Possible reaction mechanisms for the conversion of 1,2-dihaloethanes to ethylene.

ethane (27). In this case, a disulfide would be obtained by reaction of the sulfenyl halide with a thiol. Although it is not possible to choose between these proposed mechanisms, that shown in fig. 3A is supported by the identification of S-(2-hydroxyethyl)glutathione and S,S'-ethylenebisglutathione as metabolites of DBE (7); the former compound could be formed by hydrolysis of the episulfonium ion and the latter by attack of GSH on either the episulfonium ion or on S-(2-haloethyl)glutathione. The detailed mechanism of ethylene formation from *vic*-dihaloethanes is currently under investigation.

The conversion of *vic*-dihaloethanes to ethylene may be viewed as a detoxication reaction, inasmuch as ethylene is much less toxic than its precursors. However, the intermediates formed in the reaction, S-(2-haloethyl)glutathione or the episulfonium ion, may be involved in the carcinogenic and mutagenic action of these compounds. Indeed, Rannug *et al.* (12) have shown that S-(2-chloroethyl)cysteine is more mutagenic than DCE and does not require the presence of an activating system. Finally, as was indicated above, this reaction serves a detoxifying role in the case of *vic*-dihaloethanes. However, when other *vic*-polyhalides are involved, this would not necessarily be the result, because products of increased toxicity may be formed. The scope of the reaction is currently being investigated.

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