Biochemical Pharmacology, Vol. 45, No. 2, pp. 439–447, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

MOLECULAR MECHANISMS OF DIBROMOALKANE CYTOTOXICITY IN ISOLATED RAT HEPATOCYTES*

SUMSULLAH KHAN, CHRISTIAN SOOD and PETER J. O'BRIEN†
Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada M5S 2S2

(*Received* 10 *June* 1992; *accepted* 14 *September* 1992)

Abstract—The cytotoxicity of dibromoalkanes to isolated hepatocytes was proportional to the dibromoalkane concentration and increasing chain length of the dibromoalkane (C_2–C_6). The rapid hepatocyte glutathione (GSH) depletion which occurred upon addition of the dibromoalkanes was also dependent on the concentration and chain length of the dibromoalkane. When added to hepatocytes, dibromoalkanes also caused a loss in protein sulfhydryl groups. After a lag period, lipid peroxidation occurred before the onset of cytotoxicity. Antioxidants or removing the oxygen from the medium markedly delayed dibromoalkane cytotoxicity. Bromoaldehydic metabolites formed by cytochrome P450-dependent mixed-function oxidases were probably responsible for lipid peroxidation as deuterated 1,2-dibromoethane (d4-DBE) induced less lipid peroxidation and was less cytotoxic even though GSH was depleted as rapidly and as effectively. Hepatocytes were also more resistant to dibromoalkanes if cytochrome P450 isoenzymes were inactivated with SKF 525A or methyl pyrazole. Furthermore, hepatocyte susceptibility to dibromoalkanes was increased markedly if aldehyde dehydrogenase was inactivated with disulfiram, cyanamide or chloral hydrate. Cytochrome P450-induced hepatocytes isolated from pyrazole-, phenobarbital- or 3-methylcholanthrene-pretreated rats were also more susceptible to dibromoalkanes. These results suggest that dibromoalkane-induced cell lysis is due to lipid peroxidation as well as cytochrome P450-dependent formation of toxic bromoaldehydic metabolites which can bind with cellular macromolecules. Dibromoethane GSH conjugates also contribute to DBE cytotoxicity as depleting hepatocyte GSH beforehand increased hepatocyte resistance to DBE but not other dibromoalkanes.

Halogenated aliphatic hydrocarbons have important applications in agriculture and industry as pesticides, chemical intermediates as well as solvents, and are potential environmental pollutants. It has been shown that some (particularly the brominated and chlorinated) hydrocarbons are readily metabolized to reactive, electrophilic products that can interact with critical cellular molecules such as DNA and membrane proteins [1–3]. 1,2-Dibromoethane (DBE)‡ is used as an insecticide, nematocide, fumigicide and gasoline additive, and its commercial use has been restricted [4]. DBE is one of the more potentially carcinogenic halogenated aliphatic hydrocarbons to which humans are exposed.

A number of adverse effects of DBE have been reported, notably its mutagenicity towards bacteria [5] and carcinogenicity towards rats and mice [6, 7]. Its toxic effects include liver necrosis in fasted rats [8]. The adverse effects described after humans were exposed to DBE were of acute toxicity resulting from inhalation, skin absorption, and ingestion [9]. The most commonly observed tissue lesions were changes in the liver and kidney. DBE has also been shown to be a reproductive toxin [4].

DBE can be bioactivated in the liver by both the cytochrome P450-dependent monooxygenases and the cytosolic glutathione-*S*-transferases. Microsomal oxidation of DBE produces 2-bromoacetaldehyde which can covalently bind to protein sulfhydryl groups [10, 11]. Conjugation of DBE with glutathione (GSH) produces a reactive episulfonium ion intermediate which covalently binds to DNA and may be mutagenic and genotoxic [12–14]. The goal of this investigation was to determine the molecular mechanism of the cytotoxicity of DBE and other dibromoalkanes using isolated rat hepatocytes as a cell model. Cytotoxicity can be attributed to lipid peroxidation initiated by bromoaldehydic metabolites and/or binding to cellular macromolecules. However, with DBE but not longer dibromoalkanes the GSH conjugate can contribute to cytotoxicity.

MATERIALS AND METHODS

Chemicals. Fluoro-2,4-dinitrobenzene, iodoacetic acid, GSH, disulfiram, methyl pyrazole, pyrazole and trypan blue were purchased from the Sigma Chemical Co. (St. Louis, MO). Dibromoalkanes were obtained from the Aldrich Chemical Co. (Milwaukee, WI). Deuterated 1,2-dibromoethane (d4-DBE, 99.8 atom% D) was obtained from MSD Isotopes, a division of Merck Frost Canada Inc. (Montreal). Collagenase (from *Clostridium histolyticum*), Hepes and bovine serum albumin were obtained from Boehringer-Mannheim (Montreal). All other chemicals used were of analytical grade. HPLC grade solvents were purchased from Calden

* Part of this study was presented at the 31st Annual Meeting of the Society of Toxicology, February 23–27, 1992, Seattle, WA.

† Corresponding author: Dr. Peter J. O'Brien, Faculty of Pharmacy, University of Toronto, 19 Russell St., Toronto, Ontario, Canada M5S 2S2. Tel. (416) 978-2716; FAX (416) 978-8511.

‡ Abbreviations: DBE, 1-2-dibromoethane; d4-DBE, deuterated 1,2-dibromoethane; BHA, butylated hydroxyanisole; DMSO, dimethyl sulfoxide; GSH, glutathione and MDA, malondialdehyde.

(Georgetown, Ontario). SKF 525A was a gift from SmithKline Beecham Pharmaceuticals Inc. (Oakville, Ontario).

Animals and treatments. Hepatocytes were isolated from adult male Sprague–Dawley rats (280–300 g) fed *ad lib*. Pyrazole (200 mg/kg body weight) dissolved in distilled water was administered i.p. for 2 consecutive days to induce cytochrome P450 2E1 [15]. Similarly, sodium phenobarbital (80 mg/kg body weight) dissolved in distilled water or 3-methylcholanthrene (20 mg/kg body weight) dissolved in corn oil were administered i.p. for 3 consecutive days to induce cytochrome P-450 2B and cytochrome P-450 1A, respectively [16].

Isolation and incubation of hepatocytes. Hepatocytes were isolated by collagenase perfusion of the liver as previously described by Moldéus *et al.* [17]. Cells (10^6 cells/mL) were suspended in round-bottomed flasks continuously rotating in a water bath at 37° in Krebs–Henseleit buffer (pH 7.4), supplemented with 12.5 mM Hepes under an atmosphere of 95% O_2/5% CO_2 or under a hypoxic atmosphere of 95% N_2/5% CO_2. Cell viability was determined by measuring the exclusion of trypan blue (final concentration: 0.16%, w/v). Hepatocytes were preincubated for 30 min before addition of chemicals. Stock solutions of dibromoalkanes in dimethyl sulfoxide (DMSO) were prepared immediately prior to use. GSH-depleted hepatocytes were obtained by preincubating hepatocytes with bromoheptane (100 μM) as described [18]. Inhibitor/modulator/antioxidants were either preincubated with isolated hepatocytes for 5 min or added at time points mentioned in the text. The concentration of DMSO used was too low to be toxic or to compete with the metabolism of dibromoalkanes.

Glutathione assay. The total amount of GSH in isolated hepatocytes was measured on deproteinized samples (5% metaphosphoric acid) after derivatization with iodoacetic acid and fluro-2,4-dinitrobenzene as described by Reed *et al.* [19].

Determination of protein sulfhydryl groups. Protein sulfhydryl groups were determined using Ellman's reagent as described by Sedlak and Lindsay [20] and modified by Albano *et al.* [21]. Total protein was assayed with a modified Lowry procedure [22].

Measurement of lipid peroxidation. Lipid peroxidation in hepatocytes was measured by the thiobarbituric acid test as described by Ottolenghi [23] and expressed as the amount of malondialdehyde (MDA) formed using an absorption coefficient of 1.56×10^6 M/cm at 532 nm. To monitor lipid peroxidation in hepatocytes, the thiobarbituric acid assay method correlates extremely well with the measurement of ethane production, chemiluminescence and fluorescent products [24].

RESULTS

The addition of dibromoalkanes to the hepatocyte suspension resulted in cell death as monitored by trypan blue uptake (Table 1). Cytotoxicity increased with chain length (C_2–C_6) with 1,6-dibromohexane being the most toxic. Dibromoalkanes longer than C_6 were less toxic (Table 1). Also, the cytotoxicity of dibromoalkanes was dependent on dibromoalkane concentration (Fig. 1).

When hepatocytes were incubated with dibromoalkanes, intracellular GSH was also depleted much more rapidly than cytotoxicity ensued. It can be seen that the rate of GSH depletion increased with chain length C_2–C_6 and decreased with C_7 and C_8 dibromoalkanes (Table 2). Most of the hepatocyte GSH was depleted by a toxic dose of dibromoalkanes, indicating that the mitochondrial GSH pool was also depleted. A similar dependence of the rate of GSH depletion and dibromoalkane chain length was also found for a purified cytosolic GSH-*S*-transferase preparation. An enzyme kinetic analysis showed that dibromohexane had the lowest K_m (0.6 mM) of all the dibromoalkanes and DBE had the highest K_m (3.5 mM). Addition of DBE to hepatocytes also decreased total protein thiols which was prevented if hepatic GSH was depleted beforehand (Fig. 2).

To further evaluate the cytotoxic mechanisms of dibromoalkanes, lipid peroxidation (MDA formation) was measured. Lipid peroxidation increased with incubation time after dibromoalkane addition. Toxic doses of all dibromoalkanes induced lipid peroxidation and their effectiveness increased with chain length up to C6 (Table 3).

As DBE is oxidized by a cytochrome P450-dependent reaction [10, 11], the effects of various cytochrome P450 inducers on DBE cytotoxicity were studied (Table 4). Hepatocytes isolated from pyrazole-pretreated rats were much more susceptible to DBE which indicates that cytochrome P450 2E1 is involved in DBE-induced cytotoxicity. Furthermore, as shown in Table 5 hepatocytes were more resistant to DBE if cytochrome P450 was inhibited with SKF 525A or if the cytochrome P450 2E1 isozyme was inhibited by methyl pyrazole, ethanol (not shown) or carbon tetrachloride (not shown).

The deuterated form of DBE (d4-DBE) was only half as toxic to hepatocytes as DBE (Fig. 1C) and further implicates cytochrome P450 in the cytotoxic mechanism. The amount of lipid peroxidation was also less (Fig. 3) However, intracellular GSH was depleted by d4-DBE in a similar fashion and to the same extent as DBE (Fig. 4).

The effects of aldehyde dehydrogenase inhibitors on DBE-induced cytotoxicity were also studied (Table 5). The inhibitors disulfiram, cyanamide and chloral hydrate markedly increased DBE-induced cytotoxicity which further implicates 2-bromoacetaldehyde in the cytotoxic mechanism.

In DBE-treated hepatocytes, lipid peroxidation (as determined by MDA formation) increased in a time-dependent manner after a 30-min lag period (Fig. 3). MDA formation was more rapid if aldehyde dehydrogenase was inhibited with cyanamide. Furthermore, the antioxidant butylated hydroxyanisole (BHA) or the iron chelator desferoxamine prevented DBE-induced cytotoxicity over a 3-hr period. Also, as shown in Fig. 1, hepatocytes were much more resistant to DBE in the absence of oxygen. Lipid peroxidation (MDA formation) was also decreased markedly under hypoxic conditions (results not shown).

The metabolites of DBE formed following oxidation by cytochrome P450-dependent mixed-function oxidase include 2-bromoethanol and

Table 1. Cytotoxicity of dibromoalkanes towards isolated rat hepatocytes

Addition	Concn (mM)	Cytotoxicity (% trypan blue uptake at time indicated) 0.5 hr	1.0 hr	2.0 hr	3.0 hr
None		18 ± 3	20 ± 2	21 ± 3	23 ± 3
Dibromomethane	10.0	31 ± 3	36 ± 3	35 ± 4	42 ± 4
1,2-Dibromoethane	0.8	23 ± 2	37 ± 3	52 ± 3	72 ± 5
1,3-Dibromopropane	0.5	25 ± 3	37 ± 3	57 ± 4	92 ± 6
1,4-Dibromobutane	0.2	25 ± 2	30 ± 3	53 ± 4	83 ± 5
1,5-Dibromopentane	0.16	26 ± 3	35 ± 3	51 ± 4	74 ± 6
1,6-Dibromohexane	0.16	31 ± 3	43 ± 4	57 ± 4	81 ± 6
1,7-Dibromoheptane	0.3	29 ± 3	40 ± 3	54 ± 5	90 ± 6
1,8-Dibromooctane	0.6	21 ± 2	33 ± 3	53 ± 4	83 ± 5
1,9-Dibromononane	1.0	26 ± 4	34 ± 4	51 ± 5	76 ± 5

Values are means ± SD from three separate experiments.

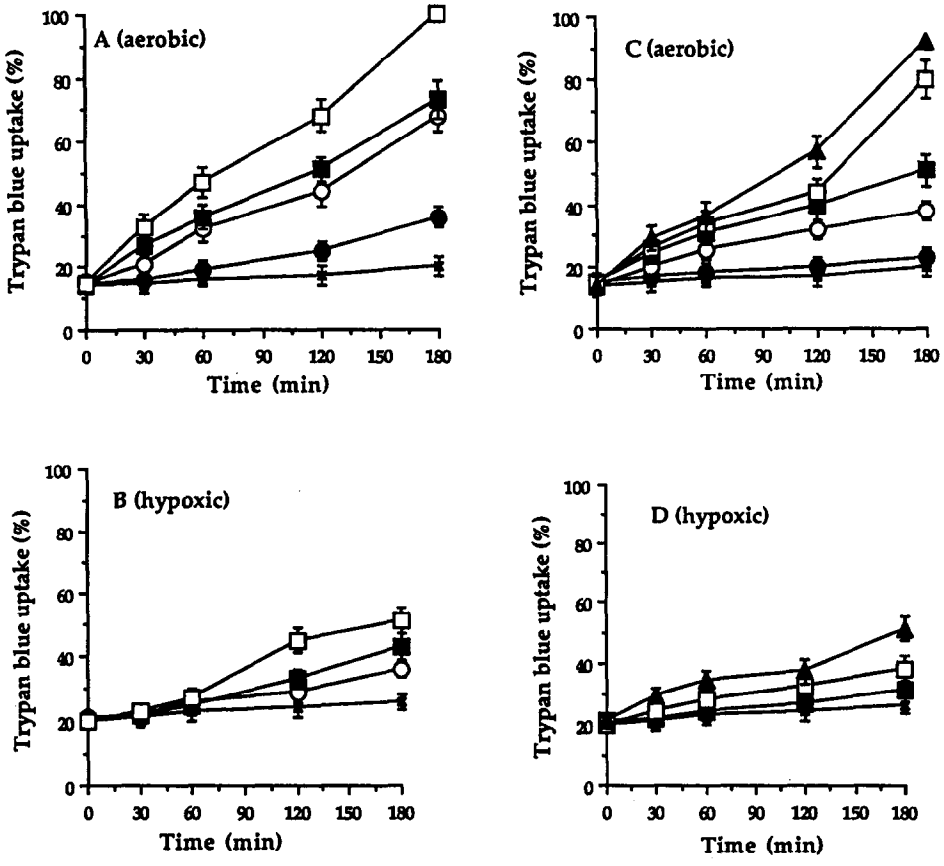

Fig. 1. 1,2-Dibromoethane (DBE)- and deuterated 1,2-dibromoethane (d4-DBE)- induced cytoxicity towards isolated hepatocytes (10^6 cells/mL) under aerobic and hypoxic conditions. Cell viability was determined by trypan blue uptake at various time points. Key: (A) and (B) (×) control, (●) DBE (0.2 mM), (○) DBE (0.5 mM), (■) DBE (0.8 mM), and (□) DBE (1.0 mM); and (C) and (D): (×) control, (●) d4-DBE (0.2 mM), (○) d4-DBE (0.5 mM), (■) d4-DBE (1.0 mM), (□) d4-DBE (1.5 mM), and (▲) d4-DBE (2.0 mM). Values are means ± SD of three separate experiments.

bromoacetic acid. Table 6 compares the cytotoxicity of these metabolites towards isolated rat hepatocytes. The order of cytotoxicity expressed as the concentration required to cause 50% cell death in 2 hr (EC_{50}) was 2-bromoacetaldehyde > 2-bromoethanol > bromoacetic acid ≫ DBE. 2-Bromoacetaldehyde was therefore at least 13-fold more cytotoxic than DBE.

Cytotoxicity induced by 1,6-dibromohexane was also modulated by various agents in a similar way to DBE. Thus, cytotoxicity was prevented or delayed by the cytochrome P450 inhibitor SKF 525A, the antioxidant BHA, the ferrous ion chelator desferoxamine, and by removing oxygen. Furthermore, cytotoxicity was also enhanced by the aldehyde dehydrogenase inhibitors chloral hydrate or

Table 2. GSH depletion by dibromoalkanes in isolated rat hepatocytes

Addition	GSH (nmol/10^6 cells)				
	1 min	10 min	20 min	40 min	60 min
None	52	52	51	51	50
1,2-Dibromoethane (200 μM)	50	46	37	32	25
1,3-Dibromopropane (100 μM)	49	32	26	15	9
1,4-Dibromobutane (100 μM)	44	15	6	5	4
1,5-Dibromopentane (100 μM)	38	8	4	3	3
1,6-Dibromohexane (100 μM)	34	7	3	2	0
1,7-Dibromoheptane (100 μM)	44	15	6	4	3
1,8-Dibromooctane (100 μM)	47	31	21	10	8

Values are means from three separate experiments.

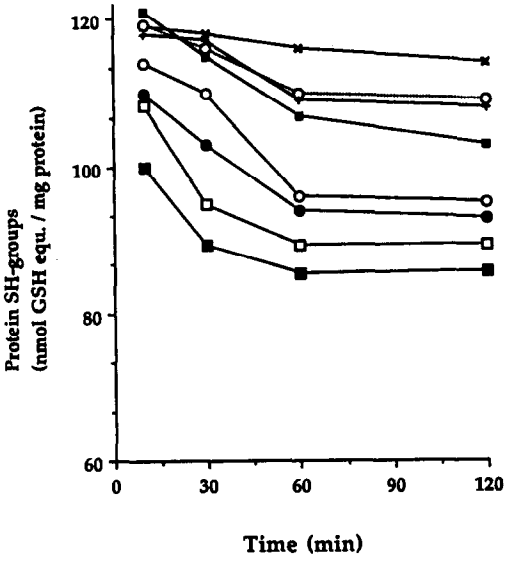

Fig. 2. Dibromoalkane-induced changes in protein thiols. Key: (×) control, (⊡) GSH depleted, (+) GSH depleted + DBE (0.8 mM), (○) DBE (0.8 mM), (●) DBE (0.8 mM) + cynamide, (--○--) d4-DBE (1.0 mM), (□) 1,6-dibromohexane (0.16 mM), and (■) 1,6-dibromohexane (0.16 mM) + cynamide. Cynamide (0.2 mM) did not affect protein thiols. Values are the means from three separate experiments.

cyanamide (Table 7). However, in contrast to DBE, cytotoxicity was not affected by prior GSH depletion.

DISCUSSION

Cytotoxicity induced by dibromoalkanes towards aerobic isolated hepatocytes, as determined by loss of membrane integrity (trypan blue uptake), increased markedly as the concentration of dibromoalkanes and their chain length increased (C_2–C_6). Dibromoalkanes with longer chain lengths, C_7–C_9, were less effective. This is the first study comparing the cytotoxicity of dibromoalkanes. The rate and extent of GSH depletion showed a similar dependence on chain length and dibromoalkane concentration. Similar results were obtained for the rate of GSH depletion with mitochondrial, microsomal and cytosolic GSH-*S*-transferases, and an enzyme kinetic analysis indicated that 1,6-dibromohexane had a 6-fold higher affinity for cytosolic GSH-*S*-transferase than DBE. Inskeep and Guengerich [25] also reported that rat liver GSH-*S*-transferases catalyzed the conjugation of 1,3-dibromopropane and 1,4-dibromobutane with GSH more readily than did DBE. This suggests that GSH level and enzymes related to glutathione metabolism may be important variables for dibromoalkane-induced cytotoxicity.

GSH depletion could also be attributed to the reaction of GSH with 2-bromoacetaldehyde formed by the cytochrome P450-dependent mixed-function oxidase pathway [11, 26]. However, d4-DBE is much

Table 3. MDA (lipid peroxidation) formation during cytotoxicity induced by dibromoalkanes in isolated rat hepatocytes

Addition	Concn (mM)	MDA formation (nmol/10^6 cells)		
		0.5 hr	1.0 hr	2.0 hr
None		1.08 ± 0.02	1.19 ± 0.03	1.23 ± 0.03
1,2-Dibromoethane	0.8	1.15 ± 0.05	1.63 ± 0.08	3.88 ± 0.3
1,3-Dibromopropane	0.5	1.09 ± 0.03	1.73 ± 0.10	4.96 ± 0.4
1,4-Dibromobutane	0.2	1.67 ± 0.05	2.23 ± 0.12	6.71 ± 0.52
1,5-Dibromopentane	0.16	1.48 ± 0.04	2.63 ± 0.18	6.70 ± 0.48
1,6-Dibromohexane	0.16	1.44 ± 0.05	2.25 ± 0.14	6.19 ± 0.32
1,7-Dibromoheptane	0.3	1.32 ± 0.03	1.79 ± 0.11	3.52 ± 0.27

Experimental details are described in Materials and Methods.
Values are means ± SD from at least three separate experiments.

Table 4. Cytotoxicity of 1,2-dibromoethane (0.8 mM) towards isolated rat hepatocytes pretreated with cytochrome P450 inducing agents

Conditions	Cytotoxicity (% trypan blue uptake at time indicated)			
	0.5 hr	1.0 hr	2.0 hr	3.0 hr
Untreated rats				
Control	18 ± 3	20 ± 2	20 ± 3	21 ± 3
1,2-Dibromoethane	23 ± 3	36 ± 3	51 ± 4	72 ± 6
Pyrazole-pretreated rats				
Control	21 ± 3	23 ± 2	24 ± 3	25 ± 3
1,2-Dibromoethane	43 ± 4	78 ± 3	89 ± 6	100
Phenobarbital-pretreated rats				
Control	21 ± 2	23 ± 3	25 ± 2	26 ± 3
1,2-Dibromoethane	27 ± 3	39 ± 4	57 ± 5	84 ± 6
3-MC-pretreated rats				
Control	20 ± 2	22 ± 3	23 ± 3	25 ± 3
1,2-Dibromoethane	26 ± 3	45 ± 5	65 ± 6	86 ± 6

Pretreatment of rats with various inducing agents was carried out as described in Materials and Methods. Values are means ± SD from three separate experiments.

Table 5. Effects of modulators on 1,2-dibromoethane-induced cytotoxicity towards isolated rat hepatocytes under aerobic conditions

Addition	Cytotoxicity (% trypan blue uptake at time indicated)			
	0.5 hr	1.0 hr	2.0 hr	3.0 hr
Control hepatocytes	17 ± 3	16 ± 2	20 ± 3	21 ± 3
+ 1,2-Dibromoethane (0.8 mM)	29 ± 3	36 ± 4	51 ± 5	73 ± 6
+ SKF 525A (0.1 mM)	27 ± 3	30 ± 4	39 ± 4	61 ± 5
+ Methyl pyrazole (0.5 mM)	28 ± 3	30 ± 3	47 ± 4	63 ± 5
+ Disulfiram (0.05 mM)	33 ± 3	45 ± 5	68 ± 6	100
+ Cyanamide (0.2 mM)	34 ± 4	47 ± 4	69 ± 7	100
+ Chloral hydrate (1.0 mM)	29 ± 3	42 ± 4	63 ± 6	88 ± 6
+ Prior GSH depletion	25 ± 3	31 ± 4	41 ± 4	59 ± 5
+ BHA (0.05 mM)	25 ± 2	29 ± 3	36 ± 4	43 ± 4
+ Desferoxamine (0.2 mM)	24 ± 3	30 ± 3	33 ± 4	36 ± 3
Deuterated 1,2-dibromoethane (1.5 mM)	26 ± 4	34 ± 4	54 ± 5	80 ± 7
+ Cyanamide (0.2 mM)	25 ± 3	32 ± 4	50 ± 4	81 ± 6

Modulators were added 5–10 min before the addition of 1,2-dibromoethane or as otherwise specified. Values are means ± SD from three separate experiments.

less readily oxidized by cytochrome P450 [27, 28] and yet depleted hepatocyte GSH at the same rate and to the same extent as DBE, indicating that GSH depletion could be attributed to direct catalysis by GSH-*S*-transferase. The mixed-function oxidase inhibitors SKF 525A and ethanol also did not affect the rate and extent of GSH depletion (data not shown). Other investigators also showed that d4-DBE decreased hepatic nonprotein sulfhydryls *in vivo* to the same extent as DBE [27].

Because of the central role that mitochondria play in the respiration of the cell, the GSH status of this organelle is of particular interest. Approximately 10–15% of total intracellular GSH is located in the mitochondria with 85–90% present in the cytosol [29]. The depletion of mitochondrial GSH in addition to cytosolic GSH may be more likely to contribute to cell injury or death caused by toxins such as ethacrynic acid or Adriamycin® [30]. Mitochondrial GSH has been reported to be depleted by DBE [31]. Depletion of mitochondrial GSH by DBE correlated with the biochemical and morphological parameters of hepatotoxicity *in vivo* [32] and with the activation of a selective Ca^{2+} release pathway from mitochondria *in vitro* [33]. Hepatocyte GSH depletion was dependent on dibromoalkane chain length and concentration and was complete with 1,6-dibromohexane, indicating that both cytosolic and mitochondrial GSH depletion occur in dibromoalkane-induced cytotoxicity. Also, our studies with isolated mitochondria showed that dibromohexane was much more effective than DBE in depleting GSH in mitochondria and inhibiting mitochondrial respiration and oxidative phosphorylation (results not shown).

Several studies have focused on the depletion by DBE of GSH levels *in vivo* [34, 35] and *in vitro* [36]. In the present study, DBE conjugated GSH in

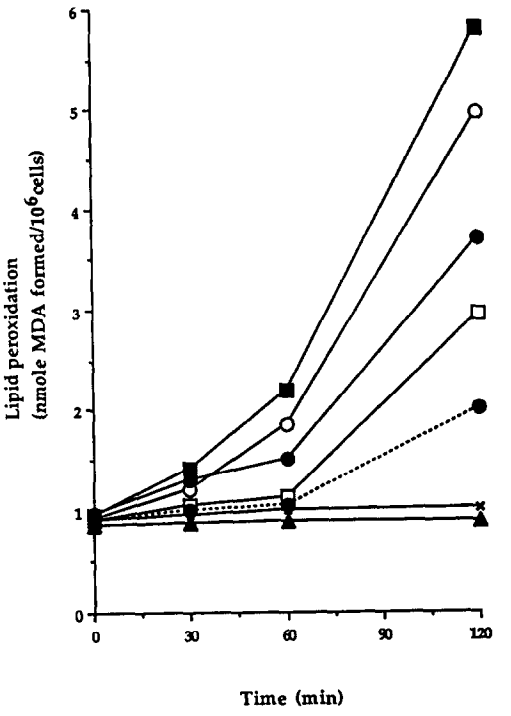

Fig. 3. Lipid peroxidation (MDA) formation. Key: (×) control, (●) DBE (0.8 mM), (■) DBE (0.8 mM) + cyanamide (0.2 mM), (○) DBE (0.8 mM) + prior GSH depletion, (□) DBE (0.8 mM) + ethanol (20.0 mM), (▲) DBE (0.8 mM) + BHA (0.05 mM) and (--●--) d4-DBE (1.0 mM). Values are the means of three separate experiments.

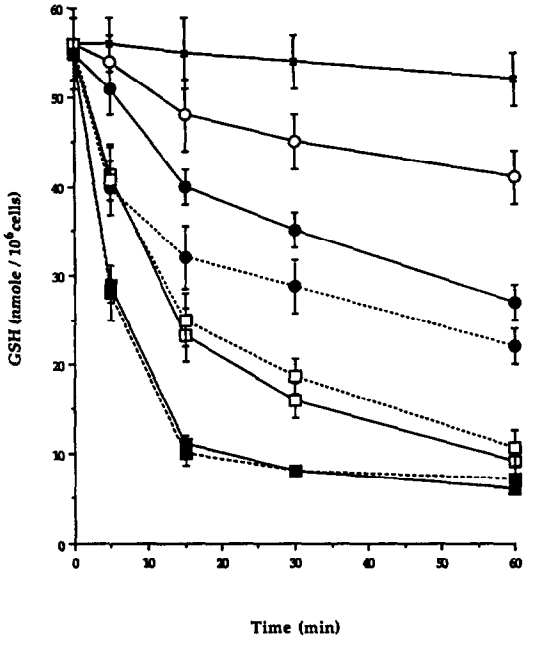

Fig. 4. GSH depletion by DBE and d4-DBE in isolated hepatocytes. Key: (×) control, (○) DBE (0.05 mM), (●) DBE (0.2 mM), (□) DBE 0.5 mM), (■) DBE (1.0 mM), (--●--) d4-DBE (0.2 mM), (--□--) d4-DBE (0.5 mM), and (--■--) d4-DBE (1.0 mM). GSH was determined by HPLC as described in Materials and Methods. Values are the means ± SD of three separate experiments.

hepatocytes well before cytotoxicity ensued (Fig. 3). Prior depletion of GSH in hepatocytes also made them more resistant to DBE but did not affect 1,6-dibromohexane cytotoxicity. This could be attributed to the particular reactivity of the DBE:GSH conjugate in covalently binding to critical protein thiols [37] and/or nucleic acids [13, 37]. Protein thiols were decreased in the hepatocytes after the addition of DBE, an effect that was prevented by prior GSH depletion (Fig. 2). Recently, Jean and Read [38] confirmed that the *S*-(2-bromoethyl) glutathione conjugate is responsible for the extensive covalent binding to cellular protein during [^{14}C]DBE metabolism in rat hepatocytes. The cysteine-*S* conjugate of 1,2-dibromoethane has also been shown to cause GSH depletion, lipid peroxidation and cytotoxicity in isolated rat hepatocytes [39]. The reactivity of the DBE:GSH conjugate with nucleophiles has been attributed to its sulfur half mustard nature enabling it to form a reactive 3-membered ring episulfonium ion [40]. The lack of an effect of GSH depletion on 1,6-dibromohexane-induced hepatocyte cytotoxicity could therefore be attributed to the inability of its GSH conjugate to form an episulfonium ion. For instance, the formation of a 3-membered ring episulfonium ion is kinetically much more favoured than a 4-membered ring episulfonium ion [41].

The formation of 2-bromoacetaldehyde via a cytochrome P450-dependent mixed-function oxidase pathway may also be important. In the present study, the major route of DBE-induced cytotoxicity in hepatocyte seems to involve 2-bromoacetaldehyde. The evidence found for this was as follows: (a) d4-DBE was much less cytotoxic even though the rate and extent of GSH depletion were the same, (b) hepatocytes in which cytochrome P450 was induced by pyrazole, phenobarbital or 3-methylcholanthrene were more susceptible to DBE, (c) SKF 525A, disulfiram or methyl pyrazole, inhibitors of mixed-function oxidase [42], prevented or delayed DBE cytotoxicity, (d) cyanamide, chloral hydrate and disulfiram, mitochondrial aldehyde dehydrogenase inhibitors [43], markedly increased DBE-induced cytotoxicity presumably by inhibiting the oxidative detoxification of 2-bromoacetaldehyde, (e) increasing mitochondrial NADH levels with the respiratory inhibitors cyanide or rotenone also increased DBE cytotoxicity presumably because NAD^+ levels were then too low for aldehyde dehydrogenase to function, and (f) protein thiol depletion was lower with d4-DBE. Using [^{14}C]DBE, Wiersma *et al.* [44] also reported that most of the protein binding by DBE could be attributed to bromoacetaldehyde formed by mixed-function oxidases. All of these results suggest that 2-bromoacetaldehyde formed by mixed-function oxidase contributes to DBE cytotoxicity [26, 45].

Lipid peroxidation as measured by MDA formation occurred in the hepatocytes 30–60 min after DBE or other dibromoalkanes were added to hepatocytes. Cytotoxicity and lipid peroxidation were prevented in the presence of the antioxidant

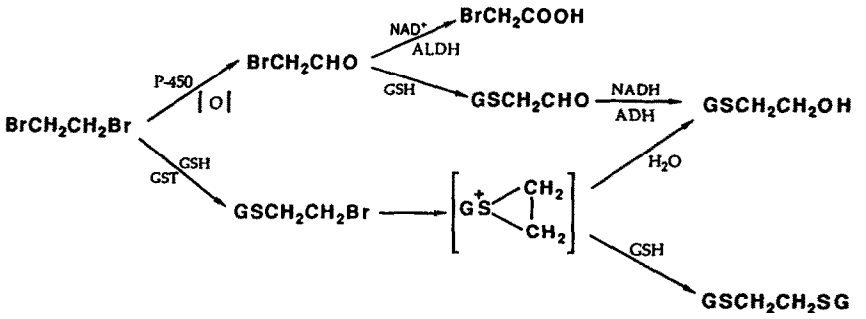

Fig. 5. Proposed mechanism of dibromoethane cytotoxicity. Abbreviations: ADH, alcohol dehydrogenase; ALDH, aldehydr dehydrogenase; and GST, glutathione-*S*-transferase.

Table 6. Lipid peroxidation and cytotoxicity (EC_{50}) induced by 1,2-dibromoethane metabolites in isolated rat hepatocytes

Metabolite	EC_{50}* (mM)	Lipid peroxidation at 2 hr (nmol MDA/10^6 cells)
1,2-Dibromoethane	0.80	3.82
2-Bromoacetaldehyde	0.06	5.23
2-Bromoethanol	0.10	4.35
Bromoacetic acid	0.15	2.09

* EC_{50} is defined as the concentration required to cause 50% cell death in 2 hr.

Table 7. Modulation of 1,6-dibromohexane cytotoxicity in isolated rat hepatocytes

Addition	Cytoxicity (% trypan blue uptake at time indicated)			
	0.5 hr	1.0 hr	2.0 hr	3.0 hr
Control hepatocytes	17 ± 4	16 ± 5	20 ± 4	19 ± 5
+ 1,6-Dibromohexane (0.16 mM)	27 ± 5	38 ± 5	51 ± 6	80 ± 7
+ SKF 525A (0.1 mM)	26 ± 3	32 ± 4	38 ± 5	61 ± 5
+ Ethanol (20.0 mM)	24 ± 3	34 ± 4	44 ± 5	64 ± 6
+ Cyanamide (0.2 mM)	28 ± 4	37 ± 5	66 ± 6	92 ± 7
+ Chloral hydrate (1.0 mM)	30 ± 4	41 ± 4	63 ± 5	83 ± 7
+ Prior GSH depletion	27 ± 3	36 ± 4	54 ± 4	75 ± 5
+ BHA (0.05 mM)	24 ± 3	27 ± 4	31 ± 4	32 ± 5
+ Desferoxamine (0.2 mM)	23 ± 4	28 ± 4	30 ± 5	31 ± 4

Values are means ± SD from three separate experiments.

BHA or the iron chelator desferoxamine. DBE-induced lipid peroxidation in isolated hepatocytes has also been reported by others [36, 46], and recently Warren and Reed [47] showed that vitamin E (a natural antioxidant) in rats becomes depleted following treatment with DBE.

In conclusion, our studies have demonstrated that the cytotoxicity of dibromoalkanes is maximal with 1,6-dibromohexane and is associated with GSH depletion, lipid peroxidation, ATP depletion and mitochondrial toxicity. The cytotoxic mechanisms for dibromoalkanes (Fig. 5) can probably be attributed to lipid peroxidation and/or protein binding induced by bromoaldehydic metabolites. With DBE, however, the glutathione conjugate formed also contributes to DBE cytotoxicity.

REFERENCES

1. Cox PJ, King LJ and Parke DV, The binding of trichlorofluoromethane and other haloalkanes to cytochrome P-450 under aerobic and anaerobic conditions. *Xenobiotica* **6**: 363–375, 1976.
2. Sipes IG, Krishna G and Gillette JR, Bioactivation of carbon tetrachloride, chloroform and bromotrichloroethane: Role of cytochrome P-450. *Life Sci* **20**: 1541–1548, 1977.
3. Buijs W, van der Gen A, Mohn GR and Breimer DD, The direct-mutagenic activity of α,ω-dihalogenoalkanes in *Salmonalla typhimurium*. Strong correlation between chemical properties and mutagenic activity. *Mutat Res* **141**: 11–14, 1984.
4. Alexeeff GV, Kilgore WW and Li M-Y, Ethylene dibromide toxicology and risk assessment. In: *Reviews of Environmental Contamination and Toxicology* (Ed.

Ware GW), Vol. 12, pp. 49–122. Springer, New York, 1990.
5. Rannug U, Genotoxic effects of 1,2-dibromoethane and 1,2-dichloroethane. *Mutat Res* **76**: 269–295, 1980.
6. Olson WA, Habermann RT, Weisburger EK, Ward JM and Weisburger JH, Induction of stomach cancer in rats and mice by halogenated aliphatic fumigants. *J Natl Cancer Inst* **51**: 1993–1995, 1973.
7. Wong LCK, Winston JM, Hong CB and Plotnick H, Carcinogenicity and toxicity of 1,2-dibromoethane in the rat. *Toxicol Appl Pharmacol* **63**: 155–165, 1982.
8. Broda C, Nachtomi E and Alumot E, Differences in liver morphology between rats and chicks treated with ethylene dibromide. *Gen Pharmacol* **7**: 345–348, 1976.
9. Olmstead EV, Pathological changes in ethylene dibromide poisoning. *AMA Arch Ind Health* **21**: 525–529, 1960.
10. Guengerich FP, Mason PS, Stott WT, Fox TR and Watanabe PG, Role of 2-haloethylene oxides and 2-haloacetaldehydes derived from vinyl bromide and vinyl chloride in irreversible binding to protein and DNA. *Cancer Res* **41**: 4391–4398, 1981.
11. Van Bladeren PJ, Hoogeterp JJ, Brimer DD and van der Gen A, The influence of disulfiram and other inhibitors of oxidative metabolism on the formation of 2-hydroxyethyl-mercapturic acid from 1,2-dibromoethane by the rat. *Biochem Pharmacol* **30**: 2983–2987, 1981.
12. van Bladeren PJ, Breimer DD, Rotteveel-Smijs GMT, de Jong RAW, Buijs W, van der Gen A and Mohn GR, The role of glutathione conjugation in the mutagenicity of 1,2-dibromoethane. *Biochem Pharmacol* **29**: 2975–2982.
13. Inskeep PB, Koga N, Cmarik JL and Guengerich FP, Covalent binding of 1,2-dibromoalkanes to DNA and stability of the major DNA adduct. *Cancer Res* **46**: 2839–2844, 1986.
14. Kim D-H and Guengerich FP, Formation of the DNA adduct *S*-[2-(N^7-guanyl)ethyl]glutathione from ethylene dibromide: Effects of modulation of glutathione and glutathione S-transferase levels and lack of a role for sulfation. *Carcinogenesis*. **11**: 419–424, 1990.
15. Palakodety RB, Clejan LA, Krikun G, Feierman DE and Cederbaum AI, Characterization and identification of a pyrazole-inducible form of cytochrome P-450. *J Biol Chem* **263**: 878–884, 1988.
16. Burke MD, Thompson S, Elcombe CR, Halpert J, Haaparanta T and Mayer RT, Ethoxy-, pentoxy- and benzyloxy phenoxazones and homologues: A series of substrates to distinguish between different induced cytochromes P-450. *Biochem Pharmacol* **34**: 3337–3342, 1985.
17. Moldéus P, Hogberg J and Orrenius S, Isolation and use of liver cells. *Methods Enzymol* **52**: 60–71, 1978.
18. Khan S and O'Brien PJ, 1-Bromoalkanes as new potent nontoxic glutathione depleters in isolated rat hepatocytes. *Biochem Biophys Res Commun* **179**: 436–441, 1991.
19. Reed DJ, Babson JR, Beatty BW, Brodie AE, Ellis WW and Potter DW, High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. *Anal Biochem* **106**: 55–62, 1980.
20. Sedlak J and Lindsay RH, Estimation of total, protein bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal Biochem* **25**: 192–205, 1968.
21. Albano E, Rundgren M, Harvison PJ, Nelson SD and Moldéus P, Mechanisms of *N*-acetyl-*p*-benzoquinone imine cytotoxicity. *Mol Pharmacol* **28**: 306–311, 1985.
22. Peterson GL, A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Anal Biochem* **83**: 346–356, 1977.
23. Ottolenghi A, Interaction of ascorbic acid and mitochondrial lipids. *Arch Biochem Biophys* **79**: 355–361, 1959.
24. Smith MT, Thor H, Hartzell P and Orrenius S, The measurement of lipid peroxidation in isolated hepatocytes. *Biochem Pharmacol* **31**: 19–26, 1982.
25. Inskeep PB and Guengerich FP, Glutathione-mediated binding of dibromoalkanes to DNA: Specificity of rat glutathione-S-transferases and dibromoalkane structure. *Carcinogenesis* **5**: 805–808, 1984.
26. Shih T-W and Hill DL, Metabolic activation of 1,2-dibromoethane by glutathione transferase and microsomal mixed function oxidase. Further evidence for formation of two reactive metabolites. *Res Commun Chem Pathol Pharmacol* **33**: 449–461, 1981.
27. White RD, Gandoli AJ, Bowden GT and Sipes IG, Deuterium isotope effect on the metabolism and toxicity of 1,2-dibromoethane. *Toxicol Appl Pharmacol* **69**: 170–178, 1983.
28. White RD, Petry TW and Sipes IG, The bioactivation of 1,2-dibromoethane in rat hepatocytes: Deuterium isotope effect. *Chem Biol Interact* **49**: 225–233, 1984.
29. Deleve LD and Kaplowitz N, Importance and regulation of hepatic glutathione. *Semin Liver Dis* **10**: 251–263, 1990.
30. Meredith M and Reed DJ, Status of the mitochondrial GSH pool in the isolated hepatocyte. *J Biol Chem* **257**: 3747–3753, 1982.
31. Ceccarelli BD, Tomasi A, Vannini V, Muscatello U and Masini A, Biochemical mechanism of GSH depletion induced by 1,2-dibromoethane in isolated rat liver mitochondria. Evidence of a GSH conjugation process. *Biochim Biophys Acta* **992**: 327–332, 1989.
32. Botti B, Bini A, Calligaro A, Meletti E, Tomasi A and Vannini V, Decrease of hepatic mitochondrial glutathione and mitochondrial injury induced by 1,2-dibromoethane in the rat *in vivo*: Effect of diethylmaleate pretreatment. *Toxicol Appl Pharmacol* **83**: 494–505, 1986.
33. Masini A, Botti B, Ceccarelli D, Muscatello U and Vannini V, Induction of calcium efflux from isolated rat liver mitochondria by 1,2-dibromoethane. *Biochim Biophys Acta* **852**: 19–24, 1986.
34. Kluwe WM, McNish R, Hook JB and Smithson K, Depletion by 1,2-dibromoethane, 1,2-dibromo-3-chloropropane, tris(2,3-dibromopropyl)phosphate, and hexachloro-1,3-butadiene of reduced non-protein sulfhydryl groups in target and non-target organs. *Biochem Pharmacol* **30**: 2265–2271, 1981.
35. Mann AM and Darby FJ, Effects of 1,2-dibromoethane on glutathione metabolism in rat liver and kidney. *Biochem Pharmacol* **34**: 2827–2830, 1985.
36. Albano E, Poli G, Tomasi A, Bani A, Vannini V and Dianzani MN, Toxicity of 1,2-dibromoethane in isolated hepatocytes: Role of lipid peroxidation. *Chem Biol Interact* **50**: 255–265, 1984.
37. Cmarik JL, Inskeep PB, Meredith MJ, Meyer DJ, Ketterer B and Guengerich FP, Selectivity of rat and human glutathione-*S*-transferases in activation of ethylene dibromide by glutathione conjugation and DNA binding and induction of unscheduled DNA synthesis in human hepatocytes. *Cancer Res* **50**: 2747–2752, 1990.
38. Jean PA and Reed DJ, Utilization of glutathione during 1,2-dihaloalkane metabolism in rat hepatocytes. *Chem Res Toxicol* **5**: 386–391, 1992.
39. Webb WW, Elfarra AA, Webster KD, Thom RE and Anders MW, Role for an episulfonium ion in *S*-(2-chloroethyl)-DL-cysteine-induced cytotoxicity and its reaction with glutathione. *Biochemistry* **26**: 3017–3023, 1987.
40. Sundheimer DW, White RD, Brendel K and Sipes IG, The bioactivation of 1,2-dibromoethane in rat hepatocytes: Covalent binding to nucleic acids. *Carcinogenesis* **3**: 1129–1133, 1982.

41. Smit WA, Zefirov NS, Bodrikov IV and Krimer MZ, Episulfonium ions: Myth and reality. *Accounts Chem Res* **12**: 282–288, 1979.
42. Brady JF, Xiao F, Wang M-H, Li Y, Ning SM, Gapac JM and Yang CS, Effects of disulfiram on hepatic P450 IIE1, other microsomal enzymes, and hepatotoxicity in rats. *Toxicol Appl Pharmacol* **108**: 366–373, 1991.
43. Cederbaum AI and Dicker E, Effect of cyanamide on the metabolism of ethanol and acetaldehyde and on gluconeogenesis by isolated rat hepatocytes. *Biochem Pharmacol* **30**: 3079–3088, 1981.
44. Wiersma DA, Schnellmann RG and Sipes IG, The *in vitro* metabolism and bioactivation of 1,2-dibromoethane (ethylene dibromide) by human liver. *J Biochem Toxicol* **1**: 1–11, 1986.
45. Banerjee S and Van Duuren BL, Binding of carcinogenic halogenated hydrocarbons with cell macromolecules. *J Natl Cancer Inst* **63**: 707–711, 1979.
46. van Iersel AAJ, van Holsteijn I and Blaauboer BJ, Effect of 1,2-dibromoethane on isolated hepatocytes: Functional alterations and induction of lipid peroxidation. *Xenobiotica* **18**: 675–683, 1988.
47. Warren DL and Reed DJ, Modification of hepatic vitamin E stores *in vivo*. III. Vitamin E depletion by 1,2-dibromoethane may be related to initial conjugation with glutathione. *Arch Biochem Biophys* **288**: 449–455, 1991.