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METABOLISM OF DIHALOMETHANES TO FORMALDEHYDE AND INORGANIC HALIDE—II

STUDIES ON THE MECHANISM OF THE REACTION*

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Abstract—Metabolism of dihalomethanes by rat liver cytosol fractions yielded formaldehyde and inorganic halide as products. Glutathione is a known cofactor in this reaction. The first step in the reaction appears to be an enzyme catalyzed nucleophilic attack by glutathione on the dihalomethane. The expected product of this reaction would be an *S*-halomethyl glutathione intermediate which should undergo a rapid hydrolysis to yield *S*-hydroxymethyl glutathione. This compound is the hemimercaptal of formaldehyde and glutathione and provides a mechanism for the formation of formaldehyde from dihalomethanes. Glutathione is required by this mechanism but is not consumed, and no primary isotope effect was seen when d_2-dibromomethane was used. *S*-hydroxymethyl glutathione is known to serve as a substrate for hepatic NAD^+-dependent formaldehyde dehydrogenase. This enzyme converts the hemimercaptal of glutathione and formaldehyde (*S*-hydroxymethyl glutathione) to *S*-formyl glutathione which is enzymatically hydrolyzed to yield formic acid and glutathione. Thus, addition of NAD^+ decreased formaldehyde yields with a concomitant increase in formic acid production. Enzyme fractionation studies further substantiate the role of formaldehyde dehydrogenase in this biotransformation.

Dihalomethanes are widely employed in industry and commerce as solvents, degreasers and chemical intermediates. Dichloromethane, the most widely used dihalomethane, is considered to be a compound of a low order of toxicity. Halogenated methanes have long been known to be metabolized via dehalogenation reactions[1–3]. Previous studies in this laboratory have shown that dihalomethanes are metabolized by cytochrome P-450-dependent enzymes to carbon monoxide and inorganic halide[4, 5] and by hepatic cytosol fractions to formaldehyde and inorganic halide[6]. The latter study established glutathione as a cofactor in this reaction and suggested the involvement of a glutathione transferase. Furthermore, the reaction was inhibited by known substrates for these enzymes and by reagents which react with sulfhydryl groups.

The studies reported here were designed to elucidate some of the processes involved in the metabolism of dihalomethanes to formaldehyde and inorganic halide.

MATERIALS AND METHODS

Dibromomethane (Aldrich, Milwaukee, WI) or d_2-dibromomethane (Merck, St. Louis, MO) were used as substrates. Reduced glutathione, used as a 0.1 M solution adjusted to pH 7.4, NAD^+ and ammonium sulfate were obtained from the Sigma Chemical Co., St. Louis, MO.

Male Sprague–Dawley rats weighing 250–350 g were used. Hepatic cytosol fractions were prepared and dialyzed as described previously[6]. Ammonium sulfate fractionation was carried out as described by Dixon and Webb[7]. Ammonium sulfate was added to 45, 65 and 90% of saturation. The protein precipitates obtained at the different ammonium sulfate concentrations were collected by centrifugation at 9000 *g* for 20 min at 4°. The pellets were dissolved in a 1.15% KCl solution. These protein fractions were stored at 0° for up to 8 weeks with no apparent loss in activity.

Unless otherwise stated, incubation mixtures contained 50 μmoles of phosphate buffer (pH 7.4), 30 μmoles glutathione, 72 μmoles substrate and 7–10 mg of dialyzed cytosol or fractionated cytosol protein in a total volume of 3 ml. Incubations were carried out with shaking at 37° in an atmosphere of air for 15 min during which product formation was linear with time[6]. Formaldehyde was determined by the method of Nash[8]; this procedure would be expected to measure both free formaldehyde and that present as *S*-hydroxymethyl glutathione since this hemimercaptal is the equilibrium product of formaldehyde and glutathione[9]. Formic acid was estimated as described by Makar *et al.*[10] and inorganic bromide was quantified by the method of Goodwin[11]. Protein concentrations were measured as described by Lowry *et al.*[12]. Glutathione concentrations were measured by the method described by Sedlak and Lindsay[13].

RESULTS

Previous studies in this laboratory[6] have shown that the enzymes catalyzing the biotransformation of dihalomethanes to formaldehyde and inorganic halide require glutathione for maximal activity. It was important to determine further whether glutathione played a catalytic role or was consumed in the reaction. The data shown in Table 1 indicate that the glutathione concentration in the incubation mixtures did not change during the

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Table 1. Glutathione concentrations during dibromomethane metabolism*

Incubation time (min)	Glutathione (μmoles/3 ml)	Formaldehyde (μmoles formed)
0	2.90 ± 0.14	ND†
15	2.92 ± 0.02	0.54 ± 0.02

*Incubation mixtures containing 7–10 mg of dialyzed hepatic cytosol protein were prepared as described in Materials and Methods except that glutathione was added to a final concentration of 1 mM (3 μmoles/3 ml). The data are shown as the mean ±S.D. of three experiments.
†ND = not detected.

15-min incubation period even though formaldehyde was formed.

It was previously observed that the addition of NAD^+ to incubation flasks decreased the rate of formaldehyde formation but did not alter bromide production during dibromomethane metabolism [6]. As can be seen from the data in Table 2, addition of NAD^+ to the unfractionated cytosol resulted in a decrease in formaldehyde formation with a concomitant increase in formic acid production. Three fractions, designated fraction A (45% of saturation), B (65% of saturation) and C (90% of saturation), were obtained by ammonium sulfate fractionation (Table 2). As further shown in Table 2, fractions A and B were similar to the unfractionated cytosol in that both metabolized dibromomethane to formaldehyde, formic acid and inorganic bromide when NAD^+ was added. Furthermore, the expected stoichiometric ratio of 2 bromide/formic acid plus formaldehyde was not observed. Fraction C did not metabolize dibromomethane to formic acid in the presence or absence of NAD^+. Using fraction C, a stoichiometric ratio of 2 bromide/formaldehyde was observed. A combination of fractions B and C yielded results similar to those obtained with the unfractionated cytosol.

Studies were also carried out to determine the metabolism of formaldehyde to formic acid by the isolated protein fractions in the presence of glutathione. As can be seen from the data in Table 3, unfractionated cytosol and fraction B metabolized formaldehyde to formic acid only in the presence of NAD^+. In contrast, fraction C did not catalyze the metabolism of formaldehyde to formic acid in the presence or absence of NAD^+; this fraction also metabolized dibromomethane only to formaldehyde and inorganic bromide in the presence or absence of NAD^+ (Table 2).

The data in Table 4 show that no detectable deuterium isotope effect was observed for formaldehyde production in the presence or absence of NAD^+, although a slight increase in v_H/v_D

Table 2. Metabolism of dibromomethane to formaldehyde, formic acid and inorganic bromide by dialyzed hepatic cytosol and ammonium sulfate fractionated hepatic cytosol*

Fraction (% ammonium sulfate saturation)	Cofactors	Formaldehyde (nmoles product/mg protein/min)	Bromide Formic acid (nmoles product/mg protein/min)	Bromide	Bromide/Formaldehyde plus formic acid
Dialyzed cytosol	−NAD^+	7.5 ± 0.6	ND†	20.9 ± 0.8	2.78
	+NAD^+	1.7 ± 0.5	6.1 ± 1.2	19.4 ± 3.0	2.49
A (45)	−NAD^+	9.2 ± 2.2	ND	23.2 ± 1.9	2.52
	+NAD^+	1.1 ± 0.4	5.4 ± 0.8	15.8 ± 3.4	2.43
B (65)	−NAD^+	7.5 ± 0.9	ND	20.6 ± 1.5	2.75
	+NAD^+	0.9 ± 0.3	7.9 ± 1.2	20.8 ± 4.7	2.36
C (90)	−NAD^+	12.4 ± 1.6	ND	26.0 ± 2.7	2.09
	+NAD^+	11.9 ± 1.6	ND	24.5 ± 3.0	2.06
B + C	−NAD^+	8.5 ± 0.7	ND	24.8 ± 2.7	2.87
	+NAD^+	2.4 ± 0.5	5.5 ± 1.2	19.0 ± 0.8	2.41

*Incubation conditions and analyses were carried out as described in Materials and Methods; incubation mixtures contained 7–10 mg of protein/3 ml. NAD^+ was added to a concentration of 1 mM. The data are shown as the mean ± S. D. of three experiments.
†ND = not detected.

Table 3. Metabolism of formaldehyde to formic acid by dialyzed hepatic cytosol and ammonium sulfate fractionated hepatic cytosol*

Fraction (% ammonium sulfate saturation)	Cofactors	Formaldehyde (μmoles recovered/3 ml)	Formic acid (μmoles formed)	Formic acid (moles/mg protein/min)
Dialyzed cytosol	−NAD^+	1.67	ND†	ND
	+NAD^+	0.26	1.42	13.6
B (65)	−NAD^+	1.70	ND	ND
	+NAD^+	0.14	1.56	14.2
C (90)	−NAD^+	1.80	ND	ND
	+NAD^+	1.73	ND	ND

*Incubation conditions and analyses were carried out as described in Materials and Methods. Formaldehyde was added to a concentration of 2 μmoles/3 ml, NAD^+ was added to a concentration of 1 mM and no dibromomethane was added. The data are shown as the mean of two determinations.
†ND = not detected.

Table 4. Metabolism of dibromomethane and d_2-dibromomethane to formaldehyde, formic acid and bromide*

Cofactor	Substrate	Bromide (nmoles product/mg protein/min)	Formaldehyde (nmoles product/mg protein/min)	Formic acid (nmoles product/mg protein/min)	Bromide	Formaldehyde (v_H/v_D)	Formic acid
$-NAD^+$	CH_2Br_2	28.3 ± 4.1	11.8 ± 1.6	ND†	1.3	1.06	ND
	CD_2Br_2	22.1 ± 1.5	11.1 ± 1.5	ND			
$+NAD^+$	CH_2Br_2	29.7 ± 3.8	3.5 ± 0.9	9.1 ± 0.7	1.3	1.02	2.1
	CD_2Br_2	22.6 ± 2.9	3.4 ± 0.4	4.1 ± 0.3			

*Incubation and analyses were carried out as described in Materials and Methods; incubation mixtures contained 7–10 mg of dialyzed cytosol protein/3 ml; NAD^+ was added to a concentration of 1 mM. The data are shown as the mean ± S. D. of four experiments.
†ND = not detected.

was observed for bromide formation. A primary isotope effect ($v_H/v_D = 2.1$) was observed in the formation of formic acid from dibromomethane in the presence of NAD^+.

DISCUSSION

Previous studies have suggested the involvement of a glutathione transferase in the metabolism of dihalomethanes to formaldehyde[6]. The presumed mechanism of glutathione transferases involves a nucleophilic attack by glutathione on the alpha carbon of the haloalkane[14]. The previous observation[6] that both dibromomethane and bromochloromethane have identical kinetic constants suggests that nucleophilic displacement of halide, bromide in this case, is the first and rate-limiting step in the reaction. The product of this reaction is likely to be an *S*-halomethyl glutathione conjugate ($GS-CH_2-X$). By analogy to *S*-halomethyl alkyl sulfides[15], *S*-halomethyl glutathione may be expected to undergo a rapid nonenzymic hydrolysis to yield *S*-hydroxymethyl glutathione ($GS-CH_2-OH$). *S*-hydroxymethyl glutathione is a hemimercaptal which is in equilibrium with formaldehyde and glutathione[9] and is, therefore, not an isolable compound. This reaction sequence would account for the formation of formaldehyde as a product of dihalomethane metabolism. This reaction sequence is analogous to the base catalyzed hydrolysis of *gem*-dihalides which is known to yield aldehydes or ketones as products[16].

This conclusion is supported by the observation that glutathione was not consumed during the metabolism of dibromomethane to formaldehyde (Table 1). As required by this suggested mechanism, glutathione is regenerated during the reaction. Further evidence supporting this view is available. Johnson[17] reported that bromochloromethane did not serve as a substrate for glutathione *S*-alkyltransferase; however, in that study, reaction rates were determined by measuring glutathione disappearance. According to the above mechanism, this would be the expected result since glutathione plays only a catalytic role in the reaction.

Earlier, it was observed that the addition of NAD^+ to incubation mixtures decreased yields of formaldehyde[6]. The postulated *S*-hydroxymethyl glutathione intermediate has been shown to serve as the substrate for the NAD^+-dependent cytosol enzyme, formaldehyde dehydrogenase (EC 1.2.1.1)[9]; formaldehyde dehydrogenases have been identified in both human[9] and rat liver[18]. The product of this reaction, *S*-formyl glutathione, is hydrolyzed by the cytosol enzyme, *S*-formyl glutathione hydrolase, to yield formic acid and glutathione[19]. Thus, it would appear that the role of NAD^+ in the reaction is to divert the *S*-hydroxymethyl glutathione intermediate, formed either by the hydrolysis of the *S*-halomethyl glutathione intermediate or by reaction of glutathione and formaldehyde to formic acid production. This conclusion is supported by the finding that, when formaldehyde dehydrogenase was removed by ammonium sulfate fractionation (Tables 2 and 3), only formaldehyde was detected as a product of

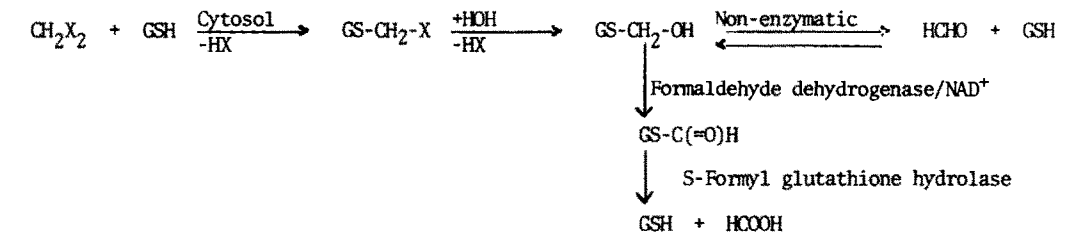

Fig. 1. Proposed reaction mechanism for the metabolism of dihalomethanes to formaldehyde, formic acid and inorganic halide.

dibromomethane metabolism and NAD^+ exerted no effect. Furthermore, the expected stoichiometric ratio of 2 bromide/formaldehyde was observed under these conditions. Finally, it should be pointed out that, although these results suggest the involvement of a formaldehyde dehydrogenase, additional pathways for the conversion of formaldehyde to formic acid have been described[18].

On the basis of these findings, the reaction mechanism shown in Fig. 1 is proposed for the metabolism of dihalomethanes to formaldehyde, formic acid and inorganic halide. Formaldehyde [3, 5, 6] and inorganic halide [1–3, 6] are known products of dihalomethane metabolism. While formic acid has not been identified previously as a metabolite of dihalomethanes, Kuzelova and Vlasak[20] reported elevated urinary formic acid levels in workers exposed to high levels of dichloromethane, suggesting that this compound may be formed as a metabolite.

Further support for this mechanism is available. According to the mechanism shown in Fig. 1, a deuterium isotope effect should not be observed when formaldehyde is measured as the product; the data in Table 4 show that this is the case. A primary deuterium isotope effect was observed when formic acid was measured as the product (Table 4). This is the expected result and may be attributed to deuteride abstraction by NAD^+ during the conversion of *S*-hydroxymethyl glutathione to *S*-formyl glutathione by formaldehyde dehydrogenase.

Although the reaction mechanism proposed above is consistent with the data presented here, alternative mechanisms should be considered. One such mechanism could involve an elimination reaction. This is unlikely since, as shown by Closs and Closs[21], dihalomethanes do not readily undergo elimination reactions and very strong bases, such as butyl lithium, are required. In addition, the intermediate formed would be the monohalocarbene (:CHX) which would be expected to yield carbon monoxide upon hydrolysis. Furthermore, Wolf *et al.*[22] found that dihalomethanes did not form a complex with reduced cytochrome P-450 that is thought to involve carbene formation. Finally, the metabolism of dibromomethane to formaldehyde and inorganic bromide by cytosol fractions in the presence of glutathione is not decreased under anaerobic conditions[6]; this tends to rule out an oxygenation reaction.

This reaction yields formaldehyde and formic acid as metabolic products of dihalomethanes and, thus, may serve to detoxify dihalomethanes. While these metabolites are relatively stable, the *S*-halomethyl glutathione intermediate would be expected to be quite reactive[15] and may be capable of alkylating various tissue nucleophiles. Evidence that such alkylation may take place is suggested by the work of Reynolds and Yee[23] who reported that [^{14}C]-dichloromethane becomes covalently bound to tissue constituents when administered to rats. Indeed, this intermediate, an α-halomethyl thioether, may possess reactivities similar to halomethyl methyl ethers or bis-halomethyl ethers[24], which are suspected carcinogens[25, 26].

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