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METABOLISM OF HALOFORMS TO CARBON MONOXIDE—III

STUDIES ON THE MECHANISM OF THE REACTION*

JAMES L. STEVENS and M. W. ANDERS

Department of Pharmacology, University of Minnesota, Minneapolis, MN 55455, U.S.A.

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Abstract—Haloforms are metabolized to carbon monoxide by hepatic microsomal mixed function oxidases and this reaction is markedly stimulated by sulfhydryl compounds. Maximal stimulation occurred at 0.5 mM glutathione (GSH). Formation of 1 mole of carbon monoxide (CO) resulted in the disappearance of 2 moles of GSH and the production of 1 mole of oxidized glutathione (GSSG). Incubation of $^{13}CHBr_3$, or $^{12}CHBr_3$ in the presence of $^{18}O_2$, resulted in the formation of similarly enriched ^{13}CO or $C^{18}O$ respectively. Furthermore, a primary isotope effect was observed when $CDBr_3$ served as the substrate. Dibromocarbonyl is an intermediate in the reaction since 2-oxothiazolidine-4-carboxylic acid (OTZ) was detected when $CHBr_3$ was incubated in the presence of cysteine. In addition, when $^{13}CHBr_3$ was used, a similarly enriched [^{13}C]OTZ was formed. Based on these observations, the following mechanism for the conversion of haloforms to CO is proposed: $CHX_3 \rightarrow COHX_3 \rightarrow X_2CO$; $X_2CO + GSH \rightarrow GS(C{=}O)X$; $GS(C{=}O)X + GSH \rightarrow GSSG + :C{=}O$.

Haloforms have been widely used in medicine and industry for many years. The use of chloroform as an anesthetic has been discontinued, but iodoform is still used occasionally as an antiseptic. Chloroform has also been included in pharmaceutical preparations but this use has not been associated with toxicity [1]. The recent reports that chloroform and bromoform are suspected carcinogens [2, 3] are of environmental importance since they, as well as other haloforms, have been identified as contaminants of finished municipal drinking water supplies [4]. This contamination apparently results from the chlorination process, since these compounds are not found in the raw water supply.

The metabolism and toxicity of the haloforms have been studied extensively, but the relationship between these two processes remains unclear. Zeller [5] showed, in 1883, that animals given chloroform excreted increased amounts of inorganic chloride. Muller [6] suggested in 1911 that the hepatotoxicity of chloroform might be caused by conversion to phosgene; Graham [7] later attempted to correlate the toxicity of the haloforms with the strength of the mineral acid produced upon dehalogenation. More recent *in vitro* and *in vivo* metabolic studies have shown that chloroform is metabolized to carbon dioxide [8, 9]. Lavigne and Marchand [10], as well as others [11], produced parallel alterations in both the hepatotoxicity and the metabolism of chloroform by treatment of rats with phenobarbital and 3-methylcholanthrene, thus suggesting a relationship between metabolism and toxicity. Uehleke and Werner [12] demonstrated that oxygen increased both the metabolism and the covalent binding of chloroform to hepatic microsomal protein using microsomal fractions from phenobarbital-treated animals. Toxic doses of chloroform cause marked depletion of hepatic GSH in phenobarbital-treated animals [13, 14]. Pohl *et al.* [15] and Mansuy *et al.* [16] have shown that phosgene is an intermediate in chloroform metabolism and have suggested that it may be involved in the production of liver damage. Work done in our laboratory showed that bromoform ($CHBr_3$) and other haloforms were metabolized to carbon monoxide both *in vivo* and *in vitro* [17, 18]. It was also shown that GSH stimulates the production of carbon monoxide from bromoform *in vitro*. Thus, it appears that the haloforms are metabolized by the hepatic mixed function oxidase system to both carbon monoxide and carbon dioxide.

The objective of this study was to investigate the mechanism of carbon monoxide production from $CHBr_3$ and the role of GSH in this process. The source of the carbon and oxygen appearing in carbon monoxide was determined as well as the effect of deuterium substitution. The information gained from these studies was used to propose a scheme for the metabolism of bromoform to carbon dioxide and carbon monoxide.

MATERIALS AND METHODS

Materials

$CDBr_3$, 99 atom % (Stohler Isotopes, Rutherford, NJ), [^{18}O] water, 99.12 atom % (Bio-Rad Laboratories, Richmond, CA), [^{18}O]oxygen, 99 atom % (Prochem, Summit, NJ) and [^{13}C]bromoform, 90 atom % (Kor Isotopes, Cambridge, MA) were used. Isocitrate dehydrogenase, *N*-ethyl maleimide, dithionitro-bisbenzoic acid, GSSG and GSH were obtained from the Sigma Chemical Co., St. Louis, MO. *o*-Phthalaldehyde was purchased from the Aldrich Chemical Co., Mil-

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waukee, WI. All other chemicals were of reagent grade and were used as received.

Male Sprague–Dawley rats, weighing 220–250 g were purchased from Bio-Lab Inc., White Bear Lake, MN.

Methods

Preparation of incubation mixtures. Incubation mixtures, unless otherwise indicated, contained 50 μmoles of potassium phosphate buffer (pH 7.4), 15 μmoles of $MgCl_2$, an NADPH-generating system consisting of 10 μmoles of DL-isocitric acid, 1 μmole of $NADP^+$, 1 enzyme unit of pig heart isocitrate dehydrogenase and 2.9 ± 0.4 mg of microsomal protein in a volume of 0.5 ml, isolated as described previously unless specified otherwise [19], in a final volume of 3 ml. Bromoform was added without solvent and appeared to be solubilized in the incubation mixture. Incubations were conducted in 25-ml Erlenmeyer flasks, fitted with sleeve type serum stoppers to prevent loss of carbon monoxide, for 15 min unless specified otherwise. Reactions were carried out at 37° with shaking and, with the exception of the $^{18}O_2$ studies, were initiated by addition of enzyme and terminated by placing on ice. Protein was determined by the method of Lowry *et al.* [20].

Glutathione assays. GSSG was measured according to the method of Hissin and Hilf [21]. Since there was fluorescent interference from the GSH contained in the incubation mixture, the concentration of GSH in the control flasks was adjusted to equal that in the sample flasks following incubation. Disappearance of GSH was quantified using the method of Sedlak and Lindsay [22].

Preparation of gel filtered microsomes (GFM). To remove glutathione reductase from microsomal fractions, gel filtered microsomes were prepared from rat liver homogenates by a modification of the method of Tangen *et al.* [23]. Microsomal fractions were suspended in buffer to a volume equal to 0.8 ml/g of liver, layered on a 30 × 1.5 cm column of Sepharose 2B-300 (Sigma Chemical Co.) and eluted with 0.15 M KCl/0.05 M potassium phosphate buffer (pH 7.5). Fractions were assayed for cytochrome P-450 by the method of Omura and Sato [24]. Glutathione reductase activity was determined by monitoring the appearance of sulfhydryl groups when eluted fractions were incubated with GSSG. Pooled fractions of GFM contained 5.6 ± 0.4 mg protein/ml.

2-Oxothiazolidine-4-carboxylic acid (OTZ) assay. Incubation mixtures consisted of four times the amounts of enzyme, $MgCl_2$, phosphate buffer and isocitrate dehydrogenase specified above in a volume of 6 ml plus 120 μmoles $CHBr_3$ and 48 mg cysteine HCl; no GSH was included. Both heat-inactivated microsomal fractions and incubation mixtures without $CHBr_3$ served as controls. Incubation mixtures were extracted with 2 volumes of ethyl acetate, the phases were separated by centrifugation, and the ethyl acetate layer was discarded. The aqueous phase was adjusted to pH 1.0 with concentrated HCl and was extracted with 2 volumes of ethyl acetate. The ethyl acetate layer was separated by centrifugation and evaporated to dryness under vacuum at 40°. The residue was dissolved in 1 ml methanol, and the OTZ methyl ester was formed by treatment with diazomethane. The resulting solution was concentrated under nitrogen and the OTZ methyl ester was taken up in no more than 100 μl ethyl acetate. Authentic OTZ and OTZ methyl ester were prepared according to the method of MacClaren [25]; OTZ m.p., obs. 170–173°, lit. 171–173°. The mass spectrum of the methyl OTZ corresponded to published spectra [16]. Spectra were obtained using a Finnigan model 3200 GC-MS operating in the electron impact mode. A 2 ft glass column packed with 3% Dexil on Gas Chrom Q and operated at 150° was used. Injector and detector temperatures were 150° and 175° respectively. Temperatures in excess of 200° were avoided due to pyrolysis of the sample. ^{13}C-Enrichments were determined by comparing the peaks at *m/e* 102 and 103 as well as at *m/e* 161 and 162 following subtraction of appropriate background.

Carbon monoxide assays. CO was determined by gas chromatography as described previously [26]. [^{13}C]carbon monoxide (^{13}CO) was measured using a Finnigan model 1015 GC-MS operating in the electron impact mode. The effluent gases from a 6 ft stainless steel column packed with molecular sieve 5Å, and maintained at 160°, were passed through a reactor containing Hopcalite® (Mine Safety & Appliances Co., Pittsburgh, PA) which converted the carbon monoxide to carbon dioxide. A complete description of this method will appear elsewhere. Samples were introduced onto the column via a gas sampling valve and *m/e* 44 and 45 were monitored; the enrichment was determined by comparing the two peaks after subtraction of appropriate background. $C^{18}O$ and $^{18}O_2$ were measured using a Finnigan model 3200 GC-MS using the same column and conditions described above except that the Hopcalite® reactor was removed and *m/e* 28 and 30 as well as 32 and 36 were monitored to determine the isotopic enrichment of $C^{18}O$ and $^{18}O_2$ respectively. $^{18}O_2$ was introduced into incubation flasks as described

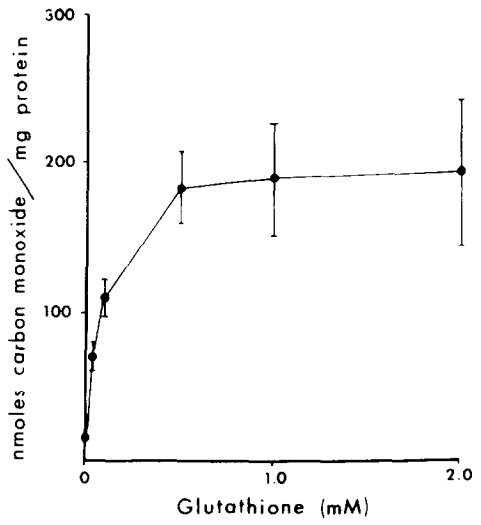

Fig. 1. Effects of GSH on carbon monoxide production from $CHBr_3$. Incubation mixtures containing 0.5 ml of microsomal protein, cofactors, various concentrations of GSH and 60 μmoles of substrate were prepared and analyzed for CO by gas chromatography. Values are means ± S.D. ($n = 3$).

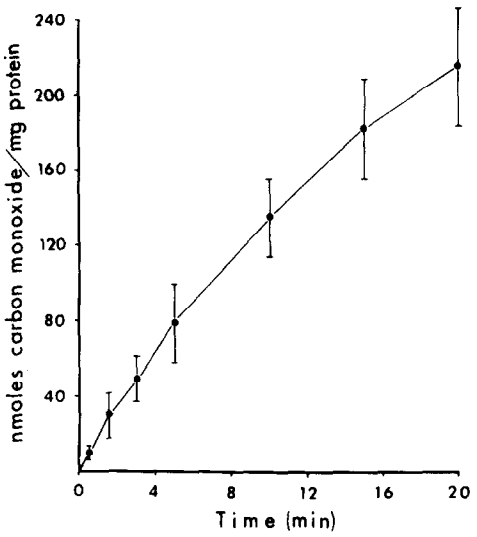

Fig. 2. Time dependence of bromoform metabolism to CO in the presence of GSH. Reaction mixtures, prepared as described in Fig. 1 and containing 10 mM GSH, were incubated for various time periods and analyzed for carbon monoxide by gas chromatography. Values are means ± S.D. ($n = 3$).

previously [27], and reactions were initiated by addition of substrate. [^{18}O]water was added prior to capping the flask and the resulting enrichment determined according to volume added and purity of $H_2{}^{18}O$.

Computations. Enzyme kinetic constants were determined by the method of Wilkinson [28] using BASIC programs written in this laboratory. Significant differences were determined at $P < 0.01$, using Student's t-test. Samples were treated as unpaired observations of equal variance. Equality of variance was determined using the F test.

RESULTS

Ahmed *et al.* [18] demonstrated the dependence of CO production from $CHBr_3$ on GSH and NADPH. The objective of this study was to better understand the role of GSH in this reaction. The data in Fig. 1 show that 0.5 mM GSH produced maximal stimulation of the formation of CO from $CHBr_3$. Figure 2 illustrates the time dependence of CO prodution from $CHBr_3$ under conditions of maximal GSH stimulation. A slight deviation from linearity appeared at about 6 min. Ahmed *et al.* [18] found that the production of CO from $CHBr_3$ was linear for 20 min in the absence of added GSH.

Table 2. ^{18}O-incorporation into carbon monoxide produced from bromoform*

Experiment	C^{18}O % enrichment	^{18}O % enrichment
$^{18}O_2$	75.4 ± 2.6	89.9 ± 2.2
$H_2{}^{18}O$	ND+	24.8

* Reaction mixtures containing 30 μmoles of GSH, 0.5 ml of microsomal protein and 60 μmoles of $CHBr_3$ were prepared, and incubations conducted for 30 min as described in Materials and Methods, with the exception that reactions were initiated by addition of substrate. C^{18}O- and $^{18}O_2$-enrichments were determined by gas chromatography-mass spectrometry. Values are means ± S.D. ($n = 2$). ND = not detectable.

Accordingly, an incubation period of 5 min was used when velocites of CO production were measured in the presence of 10 mM GSH.

In formulating possible reaction mechanisms, the production of GSSG was considered. Initial attempts to measure GSSG in incubation mixtures supplemented with 1 mM GSH were unsuccessful due to substantial glutathione reductase activity in the microsomal fractions. It was found that Sepharose 2B-300 chromatography adequately removed the glutathione reductase contamination. The results of experiments performed using gel-filtered microsomes appear in Table 1. With an initial concentration of 1 mM GSH, 60 μmoles of substrate and a 30-min incubation period, stoichiometries of 2.07:1.0 for (GSH decrease):(GSSG formed) and 1.0:1.04 for (CO formed):(GSSG formed) were observed. Thus, it appears that the disappearance of 2 moles of GSH and the appearance of 1 mole of GSSG is an obligate step in the formation of 1 mole of carbon monoxide from $CHBr_3$.

Another objective was to determine the source of the carbon and oxygen atoms appearing in the CO produced from $CHBr_3$. Incubations were conducted in the presence of both $^{18}O_2$ and $H_2{}^{18}O$ for 30 min following which the per cent of ^{18}O-enrichment in the carbon monoxide was compared to that in the head space as determined by gas chromatography-mass spectometry. The results of this study are shown in Table 2. There was no detectable C^{18}O produced in incubations containing $H_2{}^{18}O$; however, an ^{18}O-enrichment of 75.8 ± 2.6 atom % was seen when $CHBr_3$ was incubated in flasks containing 89.9 ± 2.2 atom % $^{18}O_2$ in the head space.

To determine the source of the carbon atom in the carbon monoxide and OTZ, 90 and 45 atom % [^{13}C]bromoform were introduced into capped incubation flasks and incubations were conducted for 30 min

Table 1. Stoichiometry of GSSG production during bromoform metabolism to carbon monoxide*

CO formed (nmoles/mg protein)	GSSG formed (nmoles/mg protein)	GSH disappearance (nmoles/mg protein)
145.54 ± 17.15	150.80 ± 27.44	311.73 ± 21.02

* Reaction mixtures containing 1 mM GSH were prepared using gel filtered microsomes and analyzed for GSH, GSSG and CO, as described in Materials and Methods. Values are means ± S.D. ($n = 3$).

Table 3. Metabolism of [^{13}C]bromoform to ^{13}CO and [^{13}C]OTZ by hepatic microsomes*

	% Enrichment in ^{13}C	
	Exp. 1	Exp. 2
[^{13}C]bromoform	90.0	45.0
[^{13}C]carbon monoxide	88.2	—
[^{13}C]OTZ	—	45.2

* ^{13}CO and [^{13}C]OTZ were measured by gas chromatography-mass spectrometry following incubation for 30 min. Reaction mixtures in which [^{13}C]OTZ was measured contained four times the usual amount of enzyme and cofactors in a volume of 6 ml plus 120 μmoles $^{13}CHBr_3$. ^{13}CO measurements were made using concentrations described in Fig. 1.

to determine the [^{13}C]-incorporation into CO and OTZ respectively. An enrichment of 88.2 atom % ^{13}C was observed in the CO and 45.2 atom % in the OTZ (Table 3).

The effect of deuterium substitution on the kinetics of bromoform metabolism was also studied. The deuterium isotope effect was measured in both the presence and absence of added GSH (Table 4). There was a significant difference between the K_m values for $CDBr_3$ vs $CHBr_3$ in the absence but not in the presence of GSH. There was no significant difference between the V_{max} values in the absence of GSH; these values differed significantly in the presence of GSH.

The final objective was to determine the presence of possible toxic intermediates formed during the metabolism of $CHBr_3$. Phosgene has been shown to react with cysteine to form 2-oxothiazolidine-4-carboxylic acid [15, 16, 25]; thus, if the bromine analogue of phosgene, dibromocarbonyl, is an intermediate in $CHBr_3$ metabolism, a similar reaction would be expected. Figure 3A shows the mass spectrum of the authentic OTZ methyl ester; the peak seen at *m/e* 161 corresponds to the molecular ion. The prominent fragment ions seen at *m/e* 102 and 74 correspond to the loss of formate methyl ester and formate methyl ester plus the carbonyl, respectively. Figure 3B shows the mass spectrum of methyl OTZ from incubation mixtures. When 45 atom % $CHBr_3$ was included in the incubation mixture, the spectrum shown in Figure 3C was obtained. The 13C-enrichment seen in the fragments at *m/e* 102 and 103 is 45.2 atom %. As required, no increase in *m/e* 75 is seen since this fragment does not contain the labeled car-

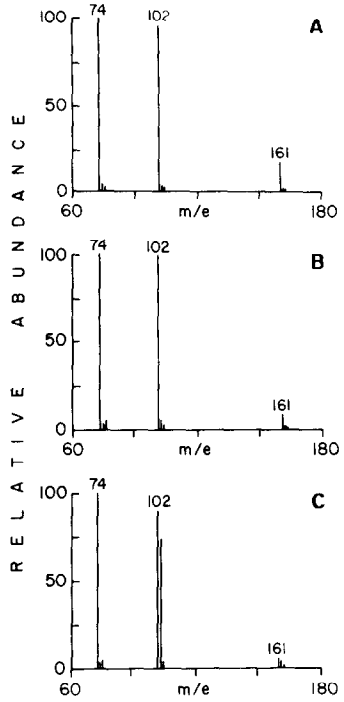

Fig. 3. Mass spectra of OTZ methyl ester. Figures show the mass spectra of authentic OTZ methyl ester (A) that formed following isolation from reaction mixtures containing $^{12}CHBr_3$ (B) and from reaction mixtures containing $^{13}CHBr_3$ (C).

Table 4. Effect of deuterium substitution on the kinetics of bromoform metabolism in the presence and absence of glutathione*

Substrate	GSH (10 mM)	K_m (mM)	V_{max} (nmoles CO/mg protein/min)
$CHBr_3$	–	1.07 ± 0.29†	1.32 ± 0.17
$CDBr_3$	–	1.81 ± 0.38†	1.06 + 0.15
$CHBr_3$	+	2.04 ± 0.27	21.89 ± 1.25‡
$CDBr_3$	+	1.98 ± 0.79	11.63 ± 1.14‡
$V_H/V_D = 1.25 \pm 0.19$§		$(V_H/K_H)/(V_D/K_D) = 2.11 \pm 0.82$	
$(V_H/V_D)_{GSH} = 1.88 + 0.21$§		$(V_H/K_H)/(V_D/K_D)_{GSH} = 1.83 \pm 0.80$	

*Reaction mixtures containing varying amounts of $CDBr_3$ and $CHBr_3$ were prepared as described in Materials and Methods. Incubation times in the presence and absence of GSH (10 mM) were 5 and 15 min, respectively (see text for explanation). Values subtitled GSH were determined in the presence of 10 mM GSH. Values are means ± S.D. ($n = 3$).

† $P < 0.01$.

‡ $P < 0.01$.

§ $P < 0.01$.

bonyl group. These results prove that the dibromocarbonyl is an intermediate in $CHBr_3$ metabolism and that the source of the carbonyl carbon is $CHBr_3$.

DISCUSSION

It is well known that haloforms are metabolized to carbon dioxide [8, 9]. Following the observation of Fodor and Roscovanu [29] that haloforms are metabolized to carbon monoxide, experiments were performed in this laboratory to further characterize this biotransformation [17, 18]. These studies demonstrated that this reaction is catalyzed by a cytochrome P-450-dependent mixed function oxidase requiring NADPH and molecular oxygen for maximal activity. Furthermore, it was observed that several sulfhydryl compounds markedly stimulated the production of CO from haloforms. These observations have been extended in the current investigation to better understand the detailed reaction mechanism involved in haloform biotransformation.

The observation that GSH stimulates the conversion of $CHBr_3$ to CO confirms the earlier work of Ahmed *et al.* [18]. The 0.5 mM GSH required for maximal stimulation is far below the 5–8 mM concentration normally found in rat liver. Thus, it would be expected that $CHBr_3$ biotransformation to CO proceeds maximally *in vivo*. Furthermore, stoichiometric studies showed that 2 moles of GSH are consumed and 1 mole of GSSG formed/mole of CO produced.

Kirsch [30] states that primary kinetic isotope effects (KIE) lie in the range of 2–10, while secondary KIE are approximately 1.2–1.4. In the absence of added GSH, the V_H/V_D term, which approximates the isotope effect on the reaction rate constant at high substrate concentration, is in the range of a secondary KIE, namely 1.25 ± 0.19. In contrast, $(V/K)_H/(V/K)_D$, which approximates the isotope effect on the reaction rate constant at very low substrate concentration, shows a primary KIE. When 10 mM GSH is added, the V_H/V_D value increases to 1.88 ± 0.21, while the $(V/K)_H/(V/K)_D$ value of 1.83 ± 0.80 does not show a significant change. The lack of a primary KIE on V_H/V_D when no GSH is added appears to be a function of sulfhydryl availability which suggests that, at very high substrate concentrations, sulfhydryl group availability becomes rate-limiting for CO production. At low substrate concentrations the available sulfhydryl groups can keep pace with the rate-limiting step, thereby yielding a primary KIE. As expected, the V_H/V_D value increases in the presence of 10 mM GSH while the $(V/K)_H/(V/K)_D$ shows no change. Thus, it appears that C—H bond cleavage is the rate-limiting step in the biotransformation of $CHBr_3$ to CO *in vitro*. The observation of Anders *et al.* [17], that animals given $CDBr_3$ show lower carboxyhemoglobin levels than those given $CHBr_3$, suggests that C—H bond cleavage is rate-limiting *in vivo* as well. The work of Pohl *et al.* [31, 32] indicates that C—H bond cleavage is also rate-limiting in the production of hepatotoxicity.

The observation that the CO produced in the presence of $^{18}O_2$ was similarly enriched in $C^{18}O$ proves that the CO produced from $CHBr_3$ is derived from molecular oxygen; in contrast, no $C^{18}O$ was seen when $H_2{}^{18}O$ was included in incubation mixtures. The difference between the headspace $^{18}O_2$ and $C^{18}O$ enrichments presumably is due to $^{16}O_2$ trapped in the incubation medium and, thus, not reflected in the headspace $^{18}O_2$-enrichment. The difference is not due to participation of a hydrolytic pathway since no incorporation of ^{18}O from $H_2{}^{18}O$ into carbon monoxide occurs. These data support the suggestion of Ahmed *et al.* [18] that the biotransformation of $CHBr_3$ is catalyzed by a cytochrome P-450-dependent oxygenase.

In two separate experiments using $^{13}CHBr_3$, it was observed that the ^{13}C-label was incorporated into both ^{13}CO and [^{13}C]OTZ with an enrichment similar to the $^{13}CHBr_3$ used. Thus, in agreement with the work of Pohl *et al.* [15] and Mansuy *et al.* [16], who identified phosgene as an intermediate in the biotransformation of chloroform, dibromocarbonyl is an intermediate in $CHBr_3$ metabolism to CO.

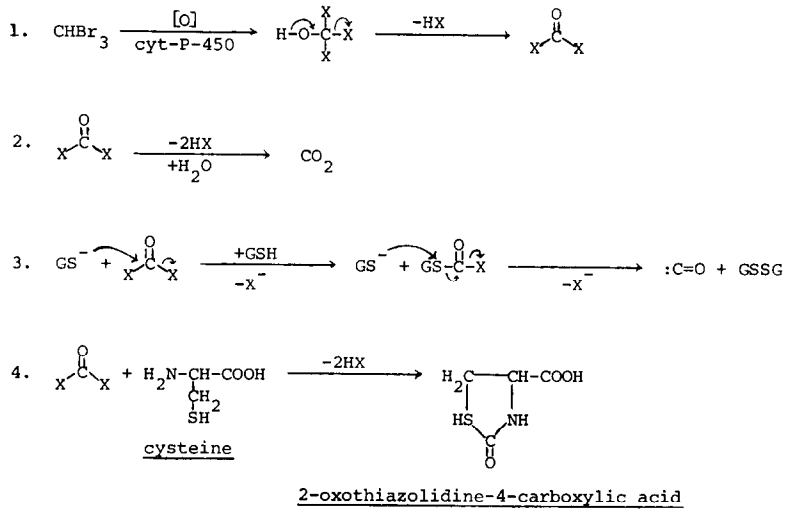

Fig. 4. Pathways of haloform metabolism.

These results suggest the reaction mechanism for haloform metabolism shown in fig. 4. In the first step, a microsomal mixed function oxidase catalyzes an oxygen insertion reaction at the C—H bond, yielding the trihalo-methanol [CX_3OH]. Nonenzymatic loss of HX results in the formation of dihalocarbonyl [CX_2O] which can be hydrolyzed to carbon dioxide. Alternatively the attack of GSH on CX_2O would yield glutathione-*S*-formyl halide [GS—C(=O)X] and subsequent attack by a second GSH on the sulfur of the first would result in the formation of CO and GSSG. There is substantial precedent for the attack of sulfur on sulfur in disulfide exchange reactions [33] as well as in the reactions of α-carbonyl sulfides with thiolates [34]. Initial attack by cysteine rather than GSH on dihalocarbonyl would result in the formation of OTZ, as described previously [15, 16, 25], or of carbon monoxide, as described here. The factors governing the ratio of OTZ to carbon monoxide are poorly understood.

Several alternative mechanisms for haloform metabolism are possible. Were oxygen inserted at a carbon—halogen bond, the resulting dihalomethyl ester of a hypohalous acid (CHX_2—O—X) would be expected to yield formyl halide (CHXO) via a free radical mechanism analogous to the Hunsdeicker reaction of acyl hypohalites [35]. The formyl halide could yield CO, as proposed by Kubic and Anders [27]; however, this mechanism does not account for the formation of dihalocarbonyl or the stimulation of CO production by GSH. The production of ^{13}CO from $^{13}CHBr_3$ eliminates the possibility that haloforms might also stimulate CO production through processes such as heme degradation. Wolf *et al.* [36] have suggested that some halogenated hydrocarbons may form CO as a result of carbene formation at cytochrome P-450 under anaerobic conditions. The failure to detect $C^{18}O$ in incubations performed with $H_2{}^{18}O$ excludes this pathway in our system.

Reynolds and Yee [37] have reported that $CHCl_3$, as well as other halogenated methanes, bind covalently to hepatic protein and lipid. The highly reactive dihalocarbonyl could acylate tissue nucleophiles resulting in covalent binding of the form R—(C=O)—X. Attack by a second nucleophile could result in the formation of crosslinked macromolecules. Indeed, the work of Nash and Prattle [38] indicates that phosgene readily reacts with macromolecules in aqueous solution. Thus, the covalent binding of chloroform may be attributed to the formation and subsequent reaction of the dihalocarbonyl intermediate, phosgene. This covalent binding may be involved in the production of hepatotoxicity seen with chloroform.

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