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METABOLISM OF DIHALOMETHANES TO CARBON MONOXIDE—III

STUDIES ON THE MECHANISM OF THE REACTION*

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Abstract—The mechanism of reaction for microsomal metabolism of dihalomethanes to carbon monoxide has been investigated. Mass spectral studies using $^{18}O_2$ showed that $C^{18}O$ was produced indicating that molecular oxygen was incorporated during the reaction. Furthermore, cumene hydroperoxide could replace NADPH and oxygen in the reaction. A primary deuterium isotope effect was observed for conversion of dichloromethane to carbon monoxide both by hepatic microsomal fractions and by a stannous phosphate model system. These results suggest an initial oxygen insertion reaction to yield a hydroxydihalomethane intermediate which would be expected to rearrange to yield a formyl halide intermediate. Formyl halides are known to decompose spontaneously to carbon monoxide. Incubation of dibromomethane in the stannous phosphate model system in the presence of 3,4-dimethylaniline resulted in the formation of 3',4'-formoxylidide, thus supporting the intermediacy of a formyl halide. On the basis of these studies, the following mechanism for the metabolism of dihalomethanes to carbon monoxide is proposed: $CH_2X_2 \rightarrow$ (cytochrome P-450) $\rightarrow H\text{–}O\text{–}CHX_2 \rightarrow HC(=O)X + H^+ + X^- \rightarrow : C{=}O + H^+ + X^-$.

Dihalomethanes are widely employed in industry and commerce as solvents, degreasers and chemical intermediates, and in the manufacture of photographic film, fire extinguishers, pressurized spray products and Christmas tree bubble lights. Dichloromethane, the most widely used dihalomethane, possesses relatively low systemic toxicity. However, Stewart *et al.*[1, 2] reported that exposure of human subjects to dichloromethane vapors resulted in elevated blood carboxyhemoglobin levels. Similar findings were reported by Fodor *et al.*[3,4] after inhalation of dihalomethanes by rats.

Previous studies conducted in this laboratory showed that administration of [^{13}C]-dichloromethane to rats resulted in the production of [^{13}C]-carboxyhemoglobin, thereby demonstrating conclusively that dihalomethanes were metabolized *in vivo* to carbon monoxide[5]. Similar results were later reported by Zorn[6], DiVincenzo and Hamilton[7] and Rodkey and Collison[8,9], who showed that the administration of [^{14}C]-dichloromethane to rats resulted in the production of [^{14}C]-carbon monoxide.

Characterization of the biotransformation *in vitro* of dihalomethanes demonstrated that these compounds were converted to carbon monoxide by the hepatic microsomal mixed-function oxidase system[10]. The present study was conducted to elucidate the reaction mechanism for the hepatic microsomal cytochrome P-450-dependent metabolism of dihalomethanes to carbon monoxide. In addition, experiments were undertaken to determine if dihalomethanes were converted to 1-carbon oxidative metabolites other than carbon monoxide by this system.

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MATERIALS AND METHODS

Dichloromethane (99%) was obtained from Fisher Scientific Co., Chicago, IL. Dibromomethane (98%), 3,4-dimethylaniline and 3',4'-formoxylidide were purchased from Aldrich Chemical Co., Milwaukee, WI. [^{14}C]-Dichloromethane (2–3 mCi/m-mole) and [^{14}C]-sodium carbonate (4.99 mCi/m-mole) were obtained from New England Nuclear, Boston, MA. The [^{14}C]-sodium carbonate was diluted to a specific activity of 0.0042 mCi/m-mole with 5 mM sodium carbonate. d_2-Dichloromethane (99%) was purchased from Koch Isotopes, Inc., Cambridge, MA., and $^{18}O_2$ (99% ^{18}O) was obtained from Bio-Rad Laboratories, Richmond, CA. Cumene hydroperoxide was supplied by Pfaltz & Bauer, Inc., Flushing, NY and was assayed according to the method of Thurman *et al.*[11]; bis-(*p*-nitrophenyl)phosphoric acid was obtained from Cal-Biochem, San Diego, CA. All other chemicals were of analytical quality. Male Long–Evans rats weighing 250–400 g were employed.

Incubation conditions

Microsomal studies. Microsomal incubation mixtures contained 50 μmoles Tris–HCl buffer (pH 7.4), 15 μmoles magnesium chloride, a NADPH-generating system consisting of 10μmoles DL-isocitric acid, 1 μmole $NADP^+$, 1 enzyme unit of pig heart isocitrate dehydrogenase (Sigma Chemical Co., St. Louis, MO), varying amounts of substrate and 2.4 to 3.0 mg of rat hepatic microsomal protein in a final volume of 3 ml. Liver microsomal fractions were isolated as described previously[12]. In some experiments,

microsomal fractions were isolated from rats that had received phenobarbital sodium (50 mg/kg in 0.9% NaCl) intraperitoneally once daily for 4 days prior to sacrifice. The incubation mixtures were prepared in 25-ml Erlenmeyer flasks capped with sleeve-type stoppers to prevent loss of carbon monoxide. Incubations were carried out at 37° for 15 min in a metabolic shaker. Carbon monoxide analyses were conducted as described by Kubic and Anders[10]; in some experiments the formation of inorganic bromide was quantified using the method of Goodwin[13]. Formic acid was determined by the method of Makar *et al.*[14]. Production of [^{14}C]-carbon dioxide was measured according to the method of Seawright and McLean[15] using microsomal fractions derived from phenobarbital-treated animals; in these experiments, [^{14}C]-dichloromethane (sp. act. 0.025 mCi/m-mole) was used as the substrate at a final concentration of 48 mM. Protein was measured as described by Lowry *et al.*[16]. Enzyme kinetic constants were calculated according to the method of Wilkinson[17] using BASIC programs written in this laboratory.

Cumene hydroperoxide studies. Cumene hydroperoxide was also studied as an oxygen donor for the reaction. In these studies, incubations were conducted as described above except that both the NADPH-generating system and air were omitted and varying amounts of cumene hydroperoxide were added. Carbon monoxide was determined as described above.

Stannous phosphate model system studies. The metabolism of dibromomethane to carbon monoxide was also studied in a stannous phosphate model system[18]. The incubation mixtures contained 40 μmoles stannous chloride, 400 μmoles potassium hydrogen phosphate and 78 μmoles dibromomethane in a total volume of 2 ml. Incubations were carried out at 25° for 40 min with vigorous shaking. Carbon monoxide was measured as mentioned above.

$^{18}O_2$ *experiments*

Experiments designed to measure ^{18}O incorporation into carbon monoxide were conducted essentially as described by Ptashne *et al.*[19] except that the microsomal incubation mixture minus substrate was frozen in a dry ice–acetone bath a second time to facilitate the removal of residual air. The reaction was initiated by the addition of dibromomethane and the incubation was continued for 15 min at 37°.

A Finnigan 3200E gas chromatograph–mass spectrometer fitted with a $\frac{1}{8}$ in. o.d. by 9 ft stainless steel column containing molecular sieve 5A, 60–80 mesh, was employed to separate the $^{18}O_2$ and C^{18}O present in the headspace gas of the incubation mixture. Helium was used as the carrier gas at a flow rate of 17 ml/min. The mass spectrometer was adjusted to scan from m/e 22 to 42. Spectra were recorded at background and at the maximum peak heights for both carbon monoxide and oxygen.

The column temperature was initially set at 60° and a sample of the head-space gas from the incubation mixture was introduced by means of a 0.25-ml gas sampling loop. At this temperature, oxygen eluted from the column with a retention time of 51 sec followed by nitrogen with a retention time of 109 sec. After nitrogen was eluted, the column temperature was increased to 100°. Under these conditions, carbon monoxide eluted as the next peak at about 3.5 min after the nitrogen peak. Mass spectral recordings were used to determine the relative amounts of $^{16}O_2$, $^{18}O_2$, C^{16}O, and C^{18}O after subtraction of the background contribution at m/e 32, 36, 28 and 30 respectively.

Trapping experiments

These experiments were designed to trap and identify reaction intermediates. Preliminary experiments using ethanol as a solvent for 3,4-dimethylaniline showed that this solvent profoundly inhibited the metabolism by microsomal suspensions of dihalomethanes to carbon monoxide. Preparation of the hydrochloride salt was undertaken to convert the trapping agent to a water soluble compound. Approximately 20 g of 3,4-dimethylaniline was dissolved in 500 ml benzene in a 1000-ml round bottom flask. Pure hydrogen chloride gas was bubbled through the 3,4-dimethylaniline solution for a period of 1 hr and the resulting white precipitate was collected and dried. The dried crystals were dissolved in a minimal volume of methanol, and anhydrous ether was added until 3,4-dimethylaniline hydrochloride began to precipitate. The methanol–ether solution was then stored at 4° overnight and the resulting crystals were collected and dried. Mass spectra of 3,4-dimethylaniline HCl prepared in this manner were identical with those obtained from authentic 3,4-dimethylaniline.

Microsomal incubation mixtures were prepared as described above except that 50 μmoles of 0.1 M sodium phosphate buffer (pH 7.4) was substituted for the Tris–HCl buffer, since preliminary experiments showed that the Tris–HCl was extracted along with 3,4-dimethylaniline and 3',4'-formoxylidide and produced interfering peaks during mass spectral analysis. Dibromomethane was used as the substrate at a final concentration of 26 mM and 3,4-dimethylaniline HCl was added at a final concentration of 1.0 mM. Preliminary experiments also demonstrated that 3',4'-formoxylidide was converted to 3,4-dimethylaniline by microsomal suspensions. To prevent this conversion, bis-(*p*-nitrophenyl)-phosphoric acid, which functions as a nonspecific amidase inhibitor[20], was added to incubation mixtures at a final concentration of 0.01 mM. This concentration prevented the hydrolysis of 3',4'-formoxylidide to 3,4-dimethylaniline without inhibiting the metabolism of dibromomethane to carbon monoxide.

Reactions were initiated by the addition of hepatic microsomal fractions derived from phenobarbital-treated animals and the incubations were continued for 15 min at 37°. One set of incubation mixtures was assayed for its carbon monoxide and inorganic bromide contents while the second set was extracted with chloroform for determination of the 3',4'-formoxylidide content by mass fragmentography. 3',4'-Formoxylidide was extracted by transferring the incubation mixtures

to chilled 40-ml glass centrifuge tubes containing 12 ml chloroform. The tubes were stoppered and shaken vigorously for 5 min and then centrifuged at 2000 *g* for 5 min to separate the aqueous and organic phases. After removal of the chloroform layer, the incubation mixtures were extracted a second time with 12 ml chloroform and the organic phases were pooled. The extracts were evaporated to dryness in a 60° water bath under a stream of nitrogen gas. Absolute ethanol (0.2 ml) was added to the dried residues and the contents were mixed thoroughly. The extracts were stored at 0° until analyzed for 3',4'-formoxylidide content by mass fragmentography. A Finnigan 3200E gas chromatography–mass spectrometer fitted with a $\frac{1}{4}$ in. o.d. by 5 ft glass column containing 1% Versamid 900 on Gas Chrom Q, 80–100 mesh, was employed. The injector and column temperatures were 240 and 200° respectively. Helium was used as the carrier gas at a flow rate of 20 ml/min. Under these conditions, 3',4'-formoxylidide eluted from the column with a retention time of 168 sec. Mass spectral analysis of 3',4'-formoxylidide showed major fragmentation peaks at m/e 77, 106, 120 and 149; the fragmentation peaks at m/e 120 and 149 were monitored to analyze for the 3',4'-formoxylidide content in the extracts. 3',4'-Formoxylidide present in the extracts was identified by comparison with the authentic compound which was routinely analyzed as a control.

Stannous phosphate model system reaction mixtures and incubations were conducted as described above. 3,4-Dimethylaniline was added at a final concentration of 1.5 mM and dibromomethane was used as the substrate at a concentration of 39 mM. The incubation mixtures were extracted and analyzed for 3',4'-formoxylidide by mass fragmentography as described above.

RESULTS

1-*Carbon metabolites of dihalomethanes*

Previous studies from this laboratory demonstrated that dihalomethanes were metabolized to carbon monoxide, but not to formaldehyde, by the hepatic microsomal mixed-function oxidase system [10]. In the present study, no metabolism of dibromomethane to formic acid by hepatic mocrosomal fractions was detected (data not shown). The rate of conversion of [^{14}C]-dichloromethane to [^{14}C]-carbon dioxide and carbon monoxide by rat liver microsomal preparations was found to be 0.2 ± 0.0 ($n = 3$) and 16.7 ± 1.2 ($n = 3$) nmoles/mg of protein/min respectively.

$^{18}O_2$ *studies*

The microsomal metabolism of dihalomethanes to carbon monoxide has been shown to require molecular oxygen for maximal activity [10]. The source of the oxygen incorporated into carbon monoxide was established by incubating dibromomethane with microsomal fractions derived from phenobarbital-treated animals in an atmosphere enriched in $^{18}O_2$. The data in Table 1 show that about 97 per cent of the oxygen gas was present in the headspace of the incubation flask as $^{18}O_2$. Similarly, C^{18}O accounted for about 97 per cent of the carbon monoxide resulting from the metabolism of dibromomethane.

Table 1. Source of oxygen incorporated into carbon monoxide during the metabolism of dibromomethane*

Isotope	Mass	% ^{16}O	% ^{18}O
$^{16}O_2$	32	2.99	
$^{18}O_2$	36		97.01
C^{16}O	28	2.61	
C^{18}O	30		97.39

* Rats received phenobarbital sodium intraperitoneally (50 mg/kg in 0.9% saline) once daily for 4 days prior to sacrifice. The hepatic microsomal fraction was isolated and incubations and analyses were conducted as described in Materials and Methods. Dibromomethane was used as the substrate at a final concentration of 26 mM.

Cumene hydroperoxide studies

The data in Table 2 show the results of the microsomal metabolism of dibromomethane to carbon monoxide in the presence of cumene hydroperoxide. Addition of increasing concentrations of cumene hydroperoxide resulted in increasing rates of carbon monoxide formation. Cumene hydroperoxide (300 μM) was found to support the conversion of dibromomethane to carbon monoxide at about 40 per cent of the rate observed with NADPH and molecular oxygen.

Table 2. Effect of cumene hydroperoxide on the microsomal metabolism of dibromomethane to carbon monoxide*

Cumene hydroperoxide (μM)	Carbon monoxide (nmoles/mg protein/min)
20	0.23 ± 0.15
40	0.35 ± 0.15
60	0.48 ± 0.15
100	0.64 ± 0.18
150	0.83 ± 0.23
200	0.90 ± 0.25
300	1.06 ± 0.35

* Microsomal fractions were isolated from rat liver, and incubations and analyses were conducted as described in Materials and Methods. Cumene hydroperoxide was diluted with methanol and added at concentrations ranging from 20 to 300 μM, in a volume of 2.5 to 5.0 μl. Concentrations of cumene hydroperoxide were determined by the ferrithiocyanate method described by Thurman *et al.* [11] using hydrogen peroxide as a standard. Anaerobic conditions were produced by evacuating and refilling the capped incubation flasks with pure nitrogen gas ten times prior to the addition of substrate. Carbon monoxide production in the absence of dibromomethane was always less than 0.02 nmoles carbon monoxide/mg of protein/min. The NADPH- and oxygen-dependent metabolism of dibromomethane was found to be 2.54 ± 0.66 nmoles carbon monoxide/mg of microsomal protein/min. Data represent the mean ± S. D. for three experiments.

Deuterium isotope effects

The effect of deuterium substitution on the microsomal metabolism of dichloromethane to carbon monoxide was also studied (Table 3). The apparent Michaelis constant (K_m) decreased from 50.1 mM with dichloromethane to 10.5 mM with d_2-dichloromethane. The maximal velocity (V_{max}) was reduced from 5.4 nmoles carbon monoxide/mg of microsomal protein/min with dichloromethane to 0.7 nmole carbon monoxide/mg of microsomal protein/min with d_2-dichloromethane.

The effect of deuterium substitution on the conversion of dichloromethane to carbon monoxide was tested in three separate model systems. Neither the trifluoroperacetic acid nor the iron–EDTA–ascorbate model systems[18] converted dichloromethane to carbon monoxide (data not shown). The rate of formation of carbon monoxide from dichloromethane and d_2-dichloromethane by the stannous phosphate model system was found to be 6.0 ± 0.6 and 2.7 ± 0.4 nmoles carbon monoxide/min (n = 10) respectively. A deuterium isotope effect (v_H/v_D) of 2.3 was observed.

Trapping experiments

A consideration of the possible reaction mechanisms for the conversion of dihalomethanes to carbon monoxide led to the suggestion that formyl halides might be formed as intermediates in this reaction. Krauskopf and Rollefson[21] reported that formyl chloride decomposed spontaneously to carbon monoxide and hydrogen chloride at room temperature. These investigators employed dimethylaniline (xylidine) to trap formyl chloride which resulted in the production of formoxylidide. In order to determine if formyl halides were formed as intermediates in the metabolism of dihalomethanes to carbon monoxide dibromomethane was incubated with hepatic microsomal fractions in the presence of 1.0 mM 3,4-dimethylaniline; this concentration of 3,4-dimethylaniline inhibited carbon monoxide production by about 95 per cent without appreciably altering bromide formation (data not shown). However, 3',4'-formoxylidide was not detected under these conditions.

3,4-Dimethylaniline (1.5 mM) was observed to produce about 30 per cent inhibition of the conversion of dibromomethane to carbon monoxide by the stannous phosphate model system; the rate of formation of carbon monoxide in the absence and presence of 3,4-dimethylaniline was found to be 25.8 ± 2.2 and 18.8 ± 2.1 (n = 9) respectively.

Mass fragmentograms of extracts of these stannous phosphate model system incubation mixtures are shown in Fig. 1. It can be seen that 3',4'-formoxylidide was formed only when the incubation mixtures contained both dibromomethane and 3,4-dimethylaniline (Fig. 1E). This suggests that, in the stannous phosphate model system, formyl bromide was formed as an intermediate in the conversion of dibromomethane to carbon monoxide and that it was trapped by 3,4-dimethylaniline to yield 3',4'-formoxylidide.

DISCUSSION

Dihalomethanes have been shown to be metabolized to carbon monoxide and inorganic bromide by rat hepatic microsomal fractions[10] and to formaldehyde and inorganic bromide by hepatic cytosol fractions[10,22]. Incubation of dihalomethanes with hepatic microsomal fractions failed to yield formaldehyde as a metabolic product[10]. In the present study, formic acid was not detected as a metabolite after incubation of dibromomethane with hepatic microsomal fractions. In addition, the microsomal metabolism of [^{14}C]-dichloromethane to [^{14}C]-carbon dioxide was found to be minor compared with its metabolism to carbon monoxide. Thus, carbon monoxide appears to be the principal 1-carbon metabolite formed during the microsomal metabolism of dihalomethanes.

Previous studies have shown that the microsomal metabolism of dihalomethanes to carbon monoxide requires molecular oxygen for maximal activity[10]. Incubation of dibromomethane with microsomal preparations in an atmosphere of $^{18}O_2$ resulted in the formation of ^{18}O–carbon monoxide, labeled in an isotopic ratio similar to the $^{18}O_2$ employed in the experiment. These results demonstrate conclusively that the oxygen incorporated into carbon monoxide during the metabolism of dihalomethanes was derived from molecular oxygen.

Investigations with model systems showed that neither the iron–EDTA–ascorbate model system nor the trifluoroperacetic acid model system[18] catalyzed the conversion of dihalomethanes to carbon monoxide; however, the stannous phosphate "oxene" model system was found to convert dihalomethanes to carbon monoxide. The oxidizing agent in the stannous phosphate model system is thought to be either a free triplet oxygen atom or a triplet oxygen atom transfer reagent[23]. Regardless of the actual form of the active oxygen species generated, the results of these studies suggest that hydroxylation of dihalomethanes oc-

Table 3. Effect of deuterium substitution on the microsomal metabolism of dichloromethane to carbon monoxide*

Substrate	K_m (mM)	V_{max} (nmoles CO/mg protein/min)	V_{max}/K_m
CH_2Cl_2	50.1 ± 2.3	5.4 ± 0.1	0.11
CD_2Cl_2	10.5 ± 1.2	0.7 ± 0.1	0.07

* Incubations and analyses were conducted as described in Materials and Methods. Substrates were added in concentrations ranging from 10 to 50 mM. Data are shown as the mean ± S. E. for five experiments. For the above data, $V_{max(H)}/V_{max(D)} = 7.7$ and $(V_{max}/K_m)_H/(V_{max}/K_m)_D = 1.6$.

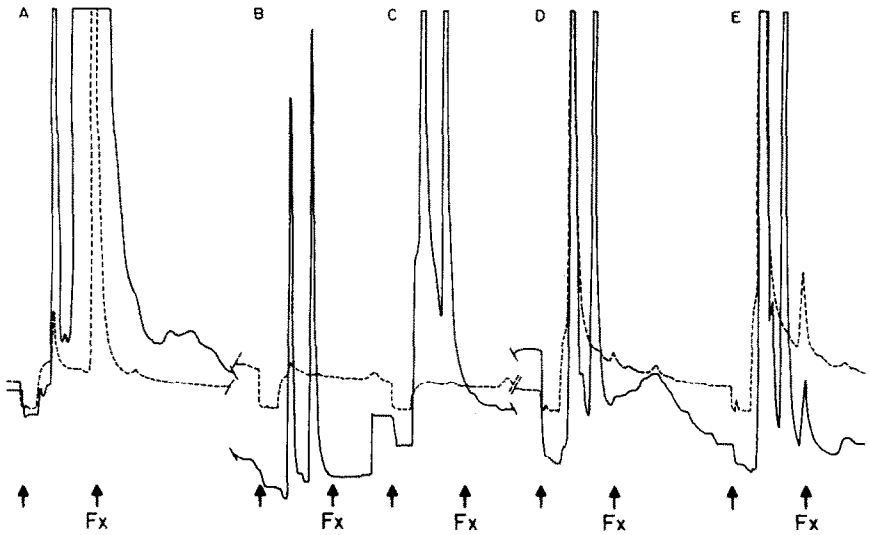

Fig. 1. Mass fragmentograms of incubation extracts from trapping experiments utilizing the stannous phosphate model system. Incubations and analyses were conducted as described in Materials and Methods. Injection times are indicated by small arrows at the lower margin of the figure. The broken and solid lines represent m/e 120 and 149 respectively. The figure shows mass fragmentograms of extracts of incubations containing (A) authentic 3',4'-formoxylidide, (B) neither dibromomethane nor 3,4-dimethylaniline, (C) dibromomethane but no 3,4-dimethylaniline, (D) 3,4-dimethylaniline but not dibromomethane and (E) both dibromomethane and 3,4-dimethylaniline. Note that the peaks at both m/e 120 and 149 (indicated by the arrows labeled "Fx") are present in E at a retention time identical to that authentic 3',4'-formoxylidide (A).

curs by a direct oxygen insertion reaction rather than by an ionic or a radical mechanism.

Several recent reports[24–27] have demonstrated that certain organic hydroperoxides can replace NADPH and molecular oxygen in supporting the cytochrome P-450-dependent hydroxylation of various drugs and fatty acids. Hrycay *et al.*[28] have also shown that sodium periodate and sodium chlorite, as well as various organic hydroperoxides, can support the hydroxylation of androstenedione by a partially purified cytochrome P-450 preparation from rat liver at reaction rates comparable to the NADPH-dependent hydroxylation. In these systems, cytochrome P-450 was proposed to function as a peroxidase resulting in clevage of the hydroperoxide and the formation of a cytochrome P-450–ferryl ion complex. The ferryl ion complex was suggested by Hrycay *et al.*[28] to be the species of active oxygen responsible for the hydroxylation of drugs and other substrates by cytochrome P-450. However, more recent studies[29] using iodosobenzene as an oxygen donor have suggested that cytochrome P-450 may function as an oxene transferase utilizing $[FeO]^{3+}$ as the active oxygen intermediate.

Cumene hydroperoxide was found to support the cytochrome P-450-dependent conversion of dihalomethanes to carbon monoxide at approximately 40 per cent of the rate observed with NADPH and oxygen. These results also support the proposal that the hydroxylation of dihalomethanes, with the resulting production of carbon monoxide, probably occurs through an oxygen insertion reaction similar to that proposed for the hydroxylation of drug substrates by cytochrome P-450[28,29].

In order to define further the mechanism for the conversion of dihalomethanes to carbon monoxide, the effect of deuterium substitution on this metabolic pathway was investigated. Deuterium substitution substantially reduced the rate of conversion of dichloromethane to carbon monoxide by hepatic microsomal preparations, resulting in a deuterium isotope effect ($V_{max(H)}/V_{max(D)}$) of 7.7 on the maximal velocity of the reaction. This marked effect of deuterium substitution on the maximal velocity suggests that C–H bond breakage is the limiting step in the reaction. These results were confirmed in the stannous phosphate model system where a deuterium isotope effect of 2.3 was observed for the rate of conversion of dichloromethane to carbon monoxide. Deuterium isotope effects greater than about 1.8 to 2.0 suggest that carbon–hydrogen bond cleavage is at least, partly rate limiting[30]. Furthermore deuterium substitution produced an effect on V_{max}/K_m; $(V_{max}/K_m)_H/(V_{max}/K_m)_D = 1.6$. Cleland[31] has discussed the significance of V_{max}/K_m in the interpretation of isotope effects. The observation of effects on both V_{max} and V_{max}/K_m allows some analysis of intermediate steps in the mechanism. For the simple model:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} X \xrightarrow{k_3} E + P$$

and defining

$$K_m = (k_2 + k_3)/k_1 \text{ and } V_{max} = k_3 E_t,$$

one obtains $V_{max}/K_m = (k_1 k_3 E_t)/(k_2 + k_3)$.

Considering the effect of deuterium substitution,

the relationship:

$$(V_{max}/K_m)_H/(V_{max}/K_m)_D = (k_1k_{3_H}E_t/(k_2 + k_{3_H}))/k_1k_{3_D}E_t/(k_2 + k_{3_D}))$$

simplies to give

$$(V_{max}/K_m)_H/(V_{max}/K_m)_D = (k_{3_H}/k_{3_D} + k_{3_H}/k_2)/(1 + k_{3_H}/k_2).$$

From the data in Table 3, $k_{3_H}/k_{3_D} = 7.7$ and $k_{3_H}/k_2 = 10$.

Thus, it appears that C–H bond breakage is a limiting factor in the metabolism of dichloromethane to carbon monoxide and that a high proportion of the collision complexes between the enzyme and dichloromethane yields product, rather than dissociating to give free enzyme and substrate.

A proposed reaction mechanism for the cytochrome P-450-dependent metabolism of dihalomethanes to carbon monoxide is shown in Fig. 2. The hydroxydihalomethane intermediate (I) could be formed by a cytochrome P-450-dependent oxygen insertion reaction; rearrangement would then yield a formyl halide intermediate (II). Formyl halides are known to decompose spontaneously at room temperature, producing carbon monoxide, hydrogen ion and inorganic halide[21].

In order to determine if formyl halides were formed as intermediates in the conversion of dihalomethanes to carbon monoxide, trapping experiments utilizing 3,4-dimethylaniline were conducted. Krauskopf and Rollefson[21] previously employed 3,5-dimethylaniline as a trapping agent for formyl chloride; the reaction of this intermediate with 3,5-dimethylaniline led to the formation of 3′,5′-formoxylidide. In this investigation, 3,4-dimethylaniline was employed as the trapping agent. As noted earlier, 3,4-dimethylaniline, at a concentration of 1.0 mM, inhibited the microsomal metabolism of dibromomethane to carbon monoxide by about 95 per cent without significantly altering the production of inorganic bromide. This observation is important because, if formyl halides are formed as intermediates in the metabolism of dihalomethanes to carbon monoxide, trapping the formyl halides should inhibit carbon monoxide formation but should not alter production of inorganic halide according to the reaction shown in Fig. 3.

Incubation of 3,4-dimethylaniline with microsomal preparations and dibromomethane did not yield the expected 3′,4′-formoxylidide. However, addition of the trapping agent to the stannous

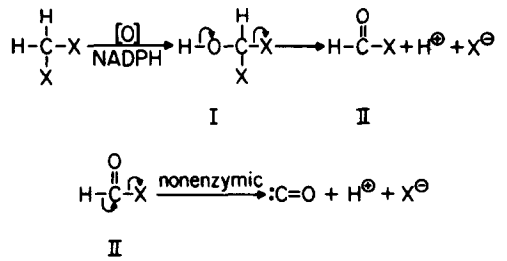

Fig. 2. Reaction mechanism for the metabolism of dihalomethanes to carbon monoxide.

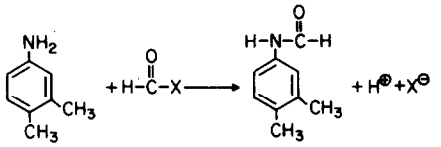

Fig. 3. Reaction of 3,4-dimethylaniline with formyl halide to yield 3′,4′-formoxylidide.

phosphate model system in the presence of dibromomethane resulted in the production of 3′,4′-formoxylidide, suggesting that formyl bromide was formed as an intermediate in the conversion of dihalomethanes to carbon monoxide. The failure to trap the formyl halide intermediate in the microsomal system could be attributed to the small amount of 3′,4′-formoxylidide likely to be formed or to the further metabolism of 3′,4′-formoxylidide to compounds which would not have been detected by mass fragmentography under the conditions employed.

Although the proposed mechanism is consistent with the data presented above, alternative mechanisms should also be considered. Closs and Closs[32] reported that treatment of dichloromethane with butyl lithium resulted in the formation of carbon monoxide via a monochlorocarbene intermediate(:CHCl). In addition, Mansuy *et al.*[33] have suggested that carbenes may be formed as intermediates in the cytochrome P-450-dependent metabolism of halogenated anesthetics. The formation of a carbene as an intermediate in the microsomal metabolism of dihalomethanes to carbon monoxide would necessitate the incorporation of oxygen derived from water into the dihalomethane molecule. However, the results presented above provide evidence that this oxygen is derived from molecular oxygen, thus excluding the intermediacy of a carbene in the conversion of dihalomethanes to carbon monoxide. This conclusion is supported by the finding that dibromo- and dichloromethane fail to produce an absorption band with reduced cytochrome P-450 which has been associated with carbene formation[34].

Another possible mechanism involves an oxygen insertion reaction in the carbon–halogen bond yielding the halomethyl ester of a hypohalous acid (XCH_2–O–X) as an intermediate. This pathway appears to be an unlikely alternative since formaldehyde would be the expected product and formaldehyde has not been detected as a metabolite of dihalomethanes in microsomal systems[10]. Furthermore, such a reaction would not be expected to exhibit a primary deuterium isotope effect.

Reynolds and Yee[35] have shown that administration of [^{14}C]-dichloromethane to rats resulted in the binding of label to tissue constituents. The proposed formyl halide may be responsible for acylating tissue nucleophiles. Preliminary observations in this laboratory indicate that [^{14}C]-dichloromethane irreversibly binds to both microsomal lipid and protein when incubated with rat hepatic microsomal fractions[36]. The objective of future investigations will be further determination of the extent and nature of this binding to cellular macromolecules.

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