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INHIBITORY ACTION OF CHLORAMINE ON FORMATE-METABOLIZING SYSTEM

STUDIES SUGGESTED BY AN UNUSUAL CASE RECORD

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Abstract—We previously reported on a patient exposed simultaneously to methyl chloride and chloramine gas who developed metabolic acidosis and permanent blindness [M. Minami *et al.*, *Hum Exp Toxicol* **11**: 27–34, 1992]. The case report suggested the possibility of potentiation of methyl chloride toxicity by chloramine. The potentiating mechanism was investigated by exposing mice to methyl chloride followed by ammonia chloramine, and then the level of formate in urine samples was measured with an enzyme coupling method to detect disturbance of formate metabolism. Mice dosed with 0.05 mL 1.0 mM chloramine after methyl chloride exposure excreted a significantly larger amount of urinary formate than mice treated with only methyl chloride. There was no difference in urinary formate levels between mice treated with only 0.05 mL 1.0 mM chloramine and those given only the vehicle (0.1 M phosphate buffer pH 6.0) for chloramine. The underlying biochemical mechanism of deterioration of formate metabolism was found to be the inhibition of the enzyme, N^{10}-formyl tetrahydrofolate (N^{10}-f-THF) dehydrogenase by 0.56–3.35 μM chloramine in the *in vitro* experiment using the purified enzyme. Positive control mice, given orally 0.1 mL 10% methanol in 0.1 M phosphate buffer (pH 6.0) excreted the same amount of urinary formate as those receiving 0.05 mL 1.0 mM chloramine after methanol administration. This was ascribed to the inhibitory effect of chloramine on formaldehyde dehydrogenase and depletion of substrate for further metabolism. The inhibition of the enzyme by chloramine (2.7–100.8 μM) was confirmed by *in vitro* experiments, using the purified enzyme, formaldehyde dehydrogenase.

Chloramines generated during inflammatory processes seem to have destructive effects on foreign biological bodies and invading microbes [1]. Some studies have examined the biological effects of chloramines in the infectious focus [2], but the systemic effect of chloramine remains to be elucidated, with only one reported by Maier *et al.* [3] related to this subject.

We recently examined a case which provided information on the systemic effect of ammonia chloramine [4]. The patient ingested organic substances generated in a sewage tank while cleaning it with a mixture of detergents, dilute HCl and NaOCl [4]. She eventually developed blindness caused by methyl chloride (CH_3Cl), the toxicity of which was potentiated by co-generated chloramine. CH_3Cl was generated by the reaction of hydrocarbons in the tank and active choline developed when HCl and NaOCl reacted in the mixed detergent solution. Chloramine was synthesized from NH_4OH in the toilet tank and NaOCl in the detergent.

The sequence of chemical reactions was simulated experimentally and reported by Minami *et al.* [4], who also confirmed chloramine's enzyme inhibitory actions on carbonic anhydrase and aldehyde dehydrogenase and its enzyme-activating effect on cytosolic superoxide dismutase. These results could explain the metabolic acidosis in the present case, however the cause of amaurosis requires further clarification. The ocular toxicity of halogenated methane, is ascribed to one of its metabolites, formate [5–7], and is aggravated by co-existing infectious processes [8, 9], during which chloramine is generated through biochemical myeloperoxidase reactions in activated granulocytes [10–12]. According to these reports [5–12], the causal factors of the patient's amaurosis can be considered to be not only formate, the metabolite of CH_3Cl, but co-generated chloramine which disturbs formate metabolism and precipitates the disease process.

The key metabolite having ocular toxicity may have been formic acid, which is common to methanol poisoning. Co-generated ammonia chloramine had an aggravating effect on the blindness in the present case. It is well established that blindness due to methanol is caused by one of its metabolites, formic acid [13–15], and is aggravated by a N^{10}-formyl-tetrahydrofolate (N^{10}-f-THF§) dehydrogenase inhibitor, N_2O [16]. Chloramine seems to have a similar effect on N^{10}-THF dehydrogenase. Primates are sensitive to methanol toxicity because of their low activity of formate metabolism in the liver [14–16].

Swine also seem to be sensitive to methanol, because their formate-metabolizing system in liver shows low activity [17, 18]. The primary structure of N^{10}-f-THF dehydrogenase is reported to be similar among species [19]. Therefore, the murine exper-

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§ Abbreviations: THF, tetrahydrofolate; f-THF, formyl tetrahydrofolate.

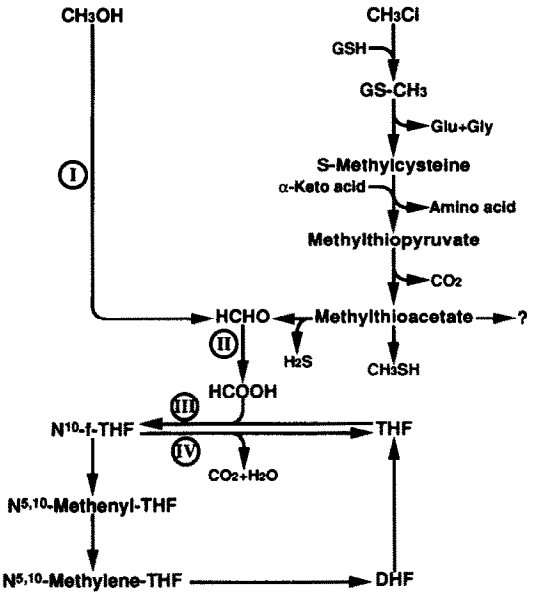

Fig. 1. Metabolism of methyl chloride and methanol and their relation to THF-metabolizing system. The roman numbers indicate the relevant enzymes to the pathway: (I) alcohol dehydrogenase; (II) formaldehyde dehydrogenase; (III) N^{10}-f-THF synthetase; (IV) N^{10}-f-THF dehydrogenase (N^{10}-f-THF hydrolase also participates in N^{10}-f-THF decomposition).

imental model is applicable for the investigation of the mechanism of toxicity. A similar mechanism of toxicity to that of methanol poisoning may have operated in the present case, since methyl chloride is metabolized to formic acid with reduction of glutathione and NADP [20].

The common metabolite to methanol, formate, is taken into the active one-carbon pool as N^{10}-f-THF, and then degraded to CO_2 and H_2O by NADP-dependent N^{10}-f-THF dehydrogenase in the cytosol [20, 21]. The main substances responsible for the disease process of the reported case are considered to be (1) formate generating from the CH_3Cl metabolism and (2) ammonia chloramine which potentiates CH_3Cl toxicity through enzyme inhibitory actions on carbonic anhydrase [4] and possibly on formate-metabolizing system similar to N_2O [16] and on formaldehyde dehydrogenase.

Experiments based on the above hypothesis regarding chloramine's potentiation of CH_3Cl toxicity were conducted by administering CH_3Cl and/or ammonia chloramine to mice in order to evaluate the deterioration of formate metabolism. The results of *in vivo* experiments were confirmed by *in vitro* experiments using partially purified enzymes from rat liver, namely N^{10}-f-THF dehydrogenase, N^{10}-f-THF synthetase, N^{10}-f-THF hydrolase and formaldehyde dehydrogenase. Figure 1 illustrates the metabolism of CH_3Cl and methanol and the tetrahydrofolate-metabolizing system related to these two substances.

MATERIALS AND METHODS

Reagents. N^5-f-THF and THF were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). N^{10}-f-THF was prepared from N^5-f-THF by a method described by Kutzbach and Stokstad [21]. A 1 M CH_3Cl solution in ethyl ether was purchased from Aldrich Chemicals (Milwaukee, WI, U.S.A.). Ammonia chloramine was synthesized by a method published previously [1, 4]. Ammonia chloramine was used for the experiment, because the patient seemed to have been exposed to this substance generated in the tank containing a large amount of ammonia.

DOWEX 50W x 2 resins were purchased from Dow Chemicals (Midland, MI, U.S.A.). DEAE-cellulose (DE52) was from Whatman (Maidstone, U.K.). All chemicals were of analytical reagent grade unless otherwise indicated.

Animals. Six-week-old ICR-(SPF) mice were supplied by Saitama Experimental Animal Laboratory (Saitama, Japan). After a week's observation of their general condition, they were housed in individual metabolic cages without diet supply for 9 hr (8.00 a.m. to 5.00 p.m.), and then were administered the substances shown in Table 1, but tap water was given to them *ad lib.* all through the experimental period. Control animals were administered the corresponding vehicle.

Biochemical assay procedures. Formic acid concentration in mouse urine was determined by the enzyme coupling method of Makar *et al.* [22].

The assay method of formaldehyde in the environment was applied to the urine sample [23]. First, 1 mL 2,4-dinitrophenylhydrazine solution [dissolved in 1 N hydrochloric acid solution (0.2 g/L) and then washed with toluene] was added to 1 mL of urine sample which had been passed through a DOWEX 50W x 2 column (0.4 × 5.0 cm) to remove the contaminants disturbing the formate condensation reaction with 2,4-dinitrophenylhydrazine. The DOWEX column had previously been washed and conditioned by 0.1 N HCl.

Procedures hereafter were as reported previously [23], to which the following concentration processes for improving the sensitivity was added to the original method, i.e. the reacted formaldehyde phenylhydrazole was transferred to toluene, the toluene extract was dried under nitrogen, methanol was added to the dried sample to dissolve the hydrazone and 10 μL of the methanol phase was injected into the HPLC apparatus. The detection limit was 30 nmol/mL.

N^{10}-f-THF dehydrogenase and N^{10}-f-THF hydrolase were prepared from rat liver by the method of Case *et al.* [24], but DE52 was used instead of DEAE-Sephadex and purification steps ended at DEAE-cellulose chromatography. The activity assay procedures for both enzymes were as reported previously by Case *et al.* [24]. The enzyme sample, N^{10}-f-THF synthetase, was obtained from crude N^{10}-f-THF dehydrogenase extract, before DEAE-cellulose chromatography by the method of Case *et al.* [24], and the activity was assayed by the method of Ho and Jones [25].

Formaldehyde dehydrogenase being highly specific

Table 1. Dosing schedule of substances investigated

Substance	Preparation	Dose	Route of administration
(1) Methyl chloride (CH_3Cl)	1 M solution in diethyl ether	0.05 mL	Intraperitoneal
(2) Methanol (MeOH)	10% MeOH in 0.1 M phosphate buffer pH 6.0	0.05 mL	Oral
(3) Ammonium chloramine	1.0 mM solution in 0.1 M phosphate buffer pH 6.0	0.05 mL	Intraperitoneal
(4) CH_3Cl and chloramine	1.11 mM chloramine solution in 0.1 M phosphate buffer pH 6.0	Dose and route of administration were the same as in 1 and 3.	
(5) MeOH and chloramine	1.02 mM chloramine solution in the phosphate buffer pH 6.0	Dose and route of administration were the same as in 2 and 3.	

Substances were administered after 9 hr of fasting (8.00 a.m. to 5.00 p.m.), with water supplied *ad lib.*

Urine was collected for 15 hr after dosing. Formate in the urine sample was determined by the enzyme coupling method [21].

Control animals were administered the vehicle.

only for formaldehyde was purified by the method of Tsuboi *et al.* [26], but the purification process ended at the step of DEAE-cellulose chromatography where the non-specific enzymes oxidizing substances other than formaldehyde were separated from the specific enzyme. Formaldehyde dehydrogenase activity was assayed by the method of Tsuboi *et al.* [26].

Protein concentrations of enzyme samples were determined by the method of Bradford [27]. Statistical significance of difference between two mean values was evaluated by Student's *t*-test.

RESULTS

The statistical distribution of urinary formate levels in the resting state before treatment had a skewed distribution. Therefore, the following calculation for evaluation of *t*-value concerning difference between means was conducted with logarithmically converted data. The calculated geometric mean was converted to the anti-logarithmic value, and the range (95% confidence interval) was estimated from the logarithmic mean (m) and the standard logarithmic deviation (d); [$\log x_1 = \log (m/d)$, $\log x_2 = \log (m \cdot d)$], and then the anti-logarithmic values of $\log x_1$ and $\log x_2$ were read from the logarithmic table. Accordingly, data are shown as mean x_0 (range; x_1 x_2).

Intraperitoneal methyl chloride administration (0.05 mL 1 M CH_3Cl in ether) increased formate level in urine (mean, 5.96; range, 2.00–17.69 μmol/15 hr (Table 2) compared to control mice (0.05 mL i.p. injection of ethyl ether; 2.47 (1.69–4.27) μmol/15 hr). The following i.p. chloramine treatment (0.05 mL of 1.11 mM ammonia chloramine), enhanced formate excretion in urine [9.10 (6.36–13.00) μmol/15 hr]. This value far surpassed the value of controls dosed with the buffer, with the vehicle for chloramine [0.23 (0.12–0.42) μmol/15 hr] or with ether. Positive control mice dosed with methanol (0.05 mL of methanol in buffer) excreted a higher level of formate in urine [5.84 (5.35–10.18) μmol/15 hr] than corresponding control mice, but the successive doses of chloramine did not enhance formate excretion [4.50 (1.22–16.79) μmol/15 hr]. Single treatment with only chloramine did not enhance formate excretion [0.19 (0.09–3.77) μmol/15 hr]. Chloramine enhancement of formaldehyde excretion in urine was detectable only in mice given methanol (Table 3).

Enzymological study

(i) Chloramine (13.3–66.5 μM) had no inhibitory effect on N^{10}-f-THF synthetase (protein, 17.1 μg/1 mL of reaction medium) activity, nor had 5.1–26.3 μM chloramine on N^{10}-f-THF hydrolase (protein, 2.13 μg/1 mL of reaction medium).

(ii) N^{10}-f-THF dehydrogenase (protein, 1.79 μg/1 mL of reaction medium) activity was inhibited by 0.56–3.35 μM chloramine, as shown in Fig. 2.

(iii) Formaldehyde dehydrogenase (protein, 3.03 μg/1 mL of reaction medium) activity was inhibited by 2.7–100.8 μM chloramine as shown in Fig. 3.

DISCUSSION

Minami *et al.* [4] reported a case of intoxication with complex chemical substances, generated while cleaning an old-style toilet chamber directly connected to a sewage storage tank, when the patient, a housewife, used a mixture of detergents, NaOCl and HCl. The toxins considered responsible for the acute symptoms consisting of metabolic acidosis and ensuing blindness 2 days after the exposure were methyl chloride and chloramine. Methyl chloride can be synthesized by the reaction of (1) chlorine from the mixture of detergents, hypochlorite and hydrochloric acid and (2) hydrocarbons in the storage tank. Chloramine is produced by the reaction of hypochlorite and ammonia and/or urea in the tank. We proposed that metabolic acidosis was due to the inhibition of carbonic anhydrase by the chloramine and the ocular toxicity was due to methyl chloride. The latter toxicity was potentiated by chloramine and lead to permanent blindness. Halogenated methyl often leads to amaurosis, especially when they are complicated by infectious diseases [8, 9, 28]. Granulocytes are

Table 2. Results of formate assay in mouse urine after single and combined administration of toxic substances as listed in Table 1

Substance	Number of mice treated	Formate in urine (μmol/15 hr urine) Mean	Range	
(1) Methyl chloride (CH_3Cl)	15	5.96	2.00–17.69	with (ii)†
(2) Methanol (MeOH)	14	5.84	3.35–10.18	with (iii)‡
(3) Ammonia chloramine	10	0.19	0.09–3.77	
(4) CH_3Cl and chloramine	7	9.10	6.36–13.00	with (ii)‡
(5) MeOH and chloramine	10	4.50	1.22–16.79	with (iii)‡
(6) Control				
(i) No administration	16	0.22	0.12–0.42	
(ii) Diethyl ether	4	2.47	1.69–4.27	
(iii) 1 M Phosphate buffer (pH 6.0)	11	0.23	0.12–0.43	
(7) Before treatment*	104	0.31	0.13–0.75	

The statistical distribution of formate values in the histogram was skewed, therefore, values were adjusted by converting the value into logarithms resulting in a normal distribution. All data were converted into the logarithmic values for statistical analysis, and the range (95% confidence interval) was calculated from the mean and SD.

* Formate value (μmol/9 hr urine) in the fasting period.

† $P < 0.05$; ‡ $P < 0.01$: mean value significantly higher than the corresponding control.

Chloramine administration after methyl chloride significantly increased formate in urine ($P < 0.05$), but chloramine after methanol did not.

Table 3. Formaldehyde levels in mouse urine collected for 15 hr after single methanol, or methyl chloride administration and after chloramine following methanol or methyl chloride administration

Treatment	N	Formaldehyde in urine (μmol/15 hr urine)
Methanol only	7	0.68 ± 0.83
Methanol and chloramine	10	6.36 ± 4.48*
Methyl chloride only	7	4.48 ± 2.51
Methyl chloride and chloramine	7	6.04 ± 10.46

Data are expressed as mean ± SD.

All of the urinary formaldehyde levels of animals given only methanol exceeded those of animals given chloramine after methanol. Student's *t*-test showed a statistically significant difference between the groups dosed with methyl chloride or methanol.

* $P < 0.01$. Statistically significant difference was detected between the mean values of the methanol only and methanol plus chloramine treatment groups.

activated by foreign bodies and microbes, and then produce active oxygen species and hypochlorite ions, which produce chloramines by their reaction with amino acids and peptides [1–3, 10–12]. Chloramines may be toxic to the host during infectious processes.

The reported case had also contracted a cold, which worsened the symptoms caused by the toxins [8, 9, 28]. The experiments reported in this paper were performed to explain the disease mechanism with the hypothesis that chloramine potentiates the ocular toxicity of formate, which is the metabolite of methyl chloride. The results of the experiments also add some other systemic effects of chloramine to those reported by Maier *et al.* [3].

Increased urinary excretion of formate indicates the disturbance of formate metabolism and suggests the exposure to ocular toxic substances [13–16, 18, 19].

The *in vivo* experiments of methyl chloride exposure of mice, followed by chloramine exposure were conducted to detect the enhancement by chloramine of formate excretion in urine. The latter may indicate that disturbed formate metabolism occurs. Methyl chloride increased urinary excretion of formate which originated mainly from methyl chloride biotransformation [19], because methyl chloride is conjugated with sulfhydryl compounds in its first metabolic step, and is degraded to methane thiol (CH_3SH) [20, 29], which is then oxidized to formaldehyde and formic acid [29]. A formate-metabolizing enzyme, N^{10}-f-THF-dehydrogenase, was strongly inhibited by chloramine added to the reaction medium for the assay of enzyme activity. Chloramine did not inhibit reactions of other formate-metabolizing enzymes tested, N^{10}-f-THF synthetase and N^{10}-f-THF hydrolase. Species with inherently low formate metabolism (e.g. humans, monkeys and swine [13–19]) are considered to be vulnerable to methanol toxicity.

In mice, folate metabolism disturbance by nitrous oxide (N_2O) causing reduction of the formate oxidation rate, or by chronic methotrexate adminis-

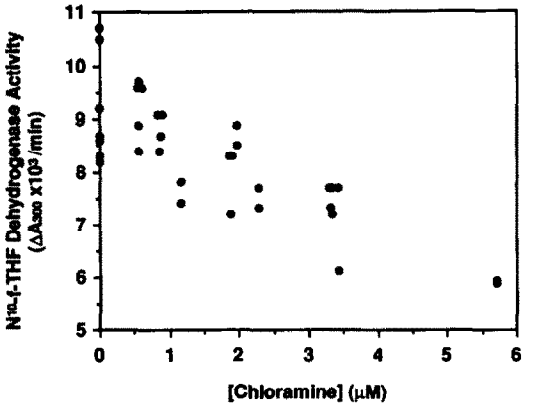

Fig. 2. Inhibitory effect of ammonia chloramine on activity of N^{10}-f-THF dehydrogenase. Chloramine was prepared by a previously reported method using gas absorbing equipment [4]. The enzyme, N^{10}-f-THF dehydrogenase, was purified from the cytosolic fraction of rat liver as described by Case *et al.* [24], ending the purification process of DEAE-cellulose chromatography. N^{10}-f-THF dehydrogenase activity was assayed spectrophotometrically using a Beckman DU68 spectrophotometer equipped with a kinetic module. The reaction medium contained 2.5 mL 0.2 M Tris–HCl buffer pH 7.0, 0.1 mL 20% (v/v) Triton X-100, 0–200 μL chloramine solution, 5 μL NADP Na_2 (10 mg/100 μL of water) and 50 μL enzyme solution. The total volume was adjusted to 3.055 mL with the Tris–HCl buffer. The reaction was initiated by addition of enzyme. Absorbance change at 300 nm was traced for 3 min and absorbance/min was calculated.

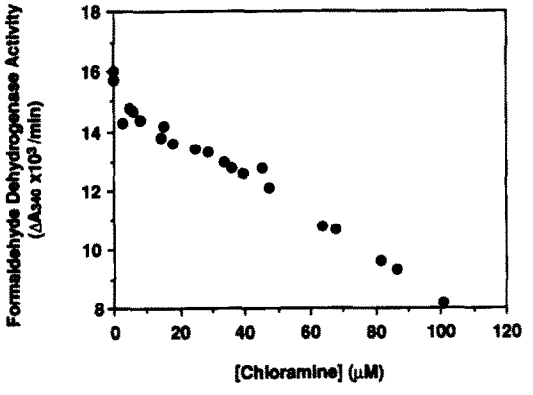

Fig. 3. Inhibitory effect of ammonia chloramine on formaldehyde dehydrogenase activity. Chloramine was prepared by a method described previously using gas absorbing equipment [4]. The enzyme, formaldehyde dehydrogenase, was purified from the cytosolic fraction of rat liver as described by Tsuboi *et al.* [26]. Formaldehyde dehydrogenase activity was assayed spectrophotometrically with a Beckman DU68 spectrophotometer equipped with a kinetic module. One milliliter of reaction medium contained 1 mM NAD, 1 mM glutathione, 0.12 mM formaldehyde and enzyme in 50 mM glycine-NaOH buffer (pH 9.5). Absorbance change at 340 nm was traced for 3 min and absorbance/min was calculated.

tration lowering hepatic THF levels, aggravates methanol or methyl chloride toxicity [16, 20]. This is because murine species have comparatively higher N^{10}-f-THF dehydrogenase levels than primates [15, 30, 31]. Although the activity of this enzyme varies between species, the primary enzyme structure is similar in humans and murine species [19]. This suggests that the mouse is a suitable model for studies of folate metabolism in such a limited occasion as in the study of chloramine, which inhibits this enzyme as strongly in murine species as the reduced activity level of the enzyme of primates. Therefore, mice and rats were used as models in our experiment. Chloramine was considered to exacerbate toxic symptoms after methyl chloride exposure, by reducing formate oxidation catalysed by N^{10}-f-THF dehydrogenase.

Kornbrust and Bus [20, 31] extensively studied methyl chloride metabolism in rats and proposed a mechanism for methyl chloride metabolism involving formate oxidation through the folate enzyme system, which was inhibited by N_2O and methotrexate [20]. This inhibition of the metabolic route of methanol and methyl chloride, occurs at the same site (folate-dependent formate oxidation) [20]. Chloramine inhibits the same site in this route, but urinary formate levels after chloramine doses followed by methanol administration did not surpass that level after administration of only methanol. Also, chloramine has a different inhibitory site in methanol metabolism from the folate-related formate metabolism, and caused reduction of formate production due to its inhibition of formaldehyde dehydrogenase (Table 3 and Fig. 3). Thus chloramine, by decreasing the activity of formaldehyde dehydrogenase after methanol administration, did not significantly increase formate levels in urine because of substrate, formate, depletion for one-carbon pool (Fig. 1). Chloramine treatment following methanol administration, therefore, did not show such a significant increase of formate in urine as observed with administration of methanol only.

Chloramine treatment following methyl chloride administration did not increase the urinary formaldehyde excretion compared to administration of only methyl chloride, because methyl chloride metabolism also proceeds through two different alternate routes; one is through methane thiol oxidation to H_2S; and the other through methylthiopyruvate oxidation to CO_2 and methylthioacetic acid [20, 31], as shown in Fig. 1. Methyl chloride could be metabolized through an alternative route with no relation to formaldehyde dehydrogenase [20]. This alternative pathway cannot increase formaldehyde excretion in urine, and methyl chloride might be metabolized to formate without involving formaldehyde dehydrogenase. This possibility requires further investigation.

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