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SHORT COMMUNICATIONS

Interaction of haloacetonitriles with glutathione and glutathione-*S*-transferase*

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Haloacetonitriles (HAN) are disinfection by-products produced during the chlorination of drinking water [1, 2]. Dichloroacetonitrile, the most prevalent among the haloacetonitriles, is detected routinely in the 0.3 to 8.1 ppb range [2]. Mink *et al.* [3] have demonstrated the formation of di- and trichloroacetonitrile in the stomach of rats following oral administration of sodium hypochlorite.

Haloacetonitriles have been shown to be direct-acting alkylating agents [4] and mutagens [5, 6], to produce tumors in the Sencar mouse skin initiation–promotion assay [6], and to induce DNA strand breaks and bind to DNA *in vitro* [4]. Dichloroacetonitrile is absorbed rapidly in rats and mice after oral administration, and the molecule is metabolized in rodents to as yet unidentified metabolites and carbon dioxide [7]. Pereira *et al.* [8] reported that haloacetonitriles are metabolized in rats to cyanide and subsequently excreted as thiocyanate in urine. HAN also inhibit dimethylnitrosamine-demethylase activities, and it was suggested that highly toxic metabolites are formed [8]. These findings suggest that haloacetonitriles may exert their biological activities both by direct action and by conversion to toxic metabolites.

Conjugation of electrophiles with glutathione (GSH) and subsequent excretion are important pathways for the detoxification of xenobiotics. Glutathione-*S*-transferases (GSH-T) provide protection not only by catalyzing the conjugation of a potential toxicant with GSH, but also by preferentially binding that toxicant [9]. In this study we examined the interaction of glutathione and glutathione-*S*-transferase with haloacetonitriles and their metabolites generated by microsomal metabolism in order to discern their possible role in the detoxification of these compounds.

Materials and Methods

Dibromoacetonitrile (DBAN) and monochloroacetonitrile (MCAN) were purchased from Aldrich (Milwaukee, WI); dichloroacetonitrile (DCAN) and trichloroacetonitrile (TCAN) were from Pfaltz & Bauer, Inc. (Stamford, CT). The purity of these chemicals was determined by GC/MS to be 96% or higher. Bromochloroacetonitrile (BCAN) was synthesized in our laboratory by Dr. Paul Ringhand [6] with a purity of 93%. Glutathione, 1-chloro-2,4-dinitrobenzene (CDNB), 5,5′-dithiobis(2-nitrobenzoic acid), NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were products of the Sigma Chemical Co. (St. Louis, MO).

Male Fisher 344 rats (Charles River, Portage, MI), weighing 170–220 g, received Purina Rodent Chow (Ralston Purina Co., St. Louis, MO) and distilled water *ad lib.* and were fasted for 24 hr before being decapitated. Haloacetonitriles (0.75 mmol/kg) dissolved in tricaprylin (1,2,3-trioctanoylglycerol) were administered by oral gavage.

The whole liver was homogenized with 4 vol. (w/v) of 0.05 M potassium phosphate, pH 7.4, containing 1.15% potassium chloride and 3 mM EDTA. The homogenate was used for the determination of glutathione concentration, and the 105,000 *g* cytosol was used for the measurement and for the source of GSH-T activities. Microsomes and cytosol were prepared by sequential centrifugation at 12,000 *g* for 20 min and at 105,000 *g* for 1 hr. The microsomes and the cytosol used in the *in vitro* studies were from untreated animals.

Protein concentration was measured by the method of Lowry *et al.* [10]. The reaction of HAN with GSH was estimated by the depletion of GSH after 30 min of incubation at 37° in 0.1 M potassium phosphate buffer, pH 7.4. GSH concentration was estimated by measuring the non-protein sulfhydryl group using Ellman's reagent [11]. GSH-T activities were measured by the method of Habig *et al.* [12] using CDNB as substrate.

Results and Discussion

The direct reaction of haloacetonitriles with GSH is shown in Fig. 1. The reactivities of the HAN were DBAN > BCAN ≫ TCAN > MCAN > DCAN = 0. The amount of GSH consumed by the two brominated HAN in the presence of excess GSH is presented in Table 1. The ratio of GSH consumed to the concentration of HAN in the reaction mixture were 2.5–2.7 for DBAN and 1.5–1.9 for BCAN. When equimolar concentrations of DBAN and GSH (2 mM) were incubated at room temperature and the depletion of the GSH was measured at various times, the time for the depletion of half the concentration of GSH was 2.0 min (data not shown).

Haloacetonitriles possess two reactive centers with which a nucleophilic agent can react, i.e. displacement of a halogen atom or by the addition at the sp carbon of the cyano group which has a partial positive charge. HAN have been reported to react with 4-(*p*-nitrobenzyl)pyridine (DNBP) by the displacement of a halogen atom [13], with the reactivities of DBAN > BCAN > MCAN > DCAN > TCAN [4]. In the reaction of HAN with GSH, the same order of reactivity was observed with the exception of TCAN. An electron-withdrawing substituent on the α-carbon increases the reaction rate of nucleophilic addition on the cyano group carbon [14], and TCAN would be the most reactive among the HAN toward the nucleophilic addition on the cyano group in contrast to the nucleophilic replacement of halogen. Therefore, GSH may react with DBAN, BCAN and MCAN by the displacement of halogen atom on the α-carbon (scheme 1) and react with TCAN at the cyano carbon (scheme 2). DCAN apparently did not react with GSH at either reactive center. Since more than an equimolar amount of GSH was depleted in the reaction with DBAN or BCAN, more than one of the halogen atoms could be displaced by GSH. A third molecule of GSH may have reacted by attacking the GSH–HAN conjugate forming oxidized glutathione (GSSG), thus resulting in depletion of more GSH than twice the concentration of DBAN. Indeed, HPLC of the reaction mixture showed the presence of a peak which was not present in control GSH incubation and co-eluted with GSSG (data not shown).

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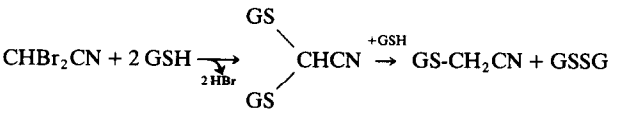

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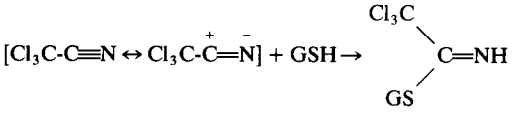

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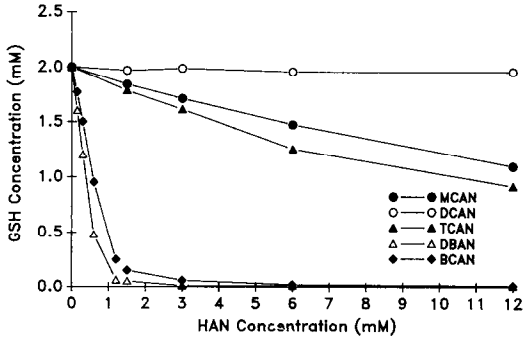

Fig. 1. Direct reaction of GSH with HAN. GSH (2 mM) was incubated with various concentrations of HAN at 37° for 30 min, and the remaining GSH was assayed. Each point is the mean of five determinations. The standard errors were all within 10% of the mean values.

To test the selectivity of HAN toward GSH relative to thiol groups and other nucleophilic centers of other proteins, the reaction of GSH with TCAN and DBAN was carried out in the presence or absence of bovine serum albumin (BSA). In the presence of BSA, the depletion of GSH by TCAN was decreased, whereas no difference was observed in the consumption of GSH by DBAN. The lower reactivity in the presence of BSA showed that TCAN probably will react with other thiol groups in other proteins and possibly with other nucleophilic centers. On the other hand, DBAN was more selective toward GSH.

Figure 2 presents the conjugation of GSH with MCAN, TCAN and DBAN under various conditions. The presence of cytosolic protein increased the depletion of GSH by MCAN, but not by TCAN or DBAN. Thus, GSH-T catalyzes the conjugation of GSH with MCAN, but not with TCAN or DBAN. The addition of microsomes in the incubation mixture increased the depletion of GSH by MCAN and decreased that by TCAN and DBAN, indicating metabolism to metabolites that are either more (MCAN) or less (TCAN and DBAN) reactive toward GSH than the parent compound. DCAN failed to react with GSH either in the presence or absence of GSH-T. A small amount of GSH was consumed when microsomes were included in the incubation mixture (data not shown), indicating metabolism of DCAN by microsomal enzymes.

Table 1. Reaction of GSH with DBAN and BCAN

HAN (mM)	GSH consumed (mM)	GSH/HAN
DBAN		
0.15	0.40	2.7
0.30	0.80	2.7
0.60	1.52	2.5
BCAN		
0.15	0.23	1.5
0.30	0.50	1.7
0.60	1.15	1.9

GSH (2 mM) was incubated with various concentrations of DBAN or BCAN at 37° for 30 min, and the GSH remaining was assayed. Each value is the average of two determinations.

All five haloacetonitriles decreased the rate of GSH-T catalyzed conjugation of GSH with 1-chloro-2,4-dinitrobenzene. TCAN was the most reactive and DCAN was the least reactive (Fig. 3). The difference in the reactivities was small, with a factor of only 4 between the most and the least reactive ones. Haloacetonitriles can decrease the GSH-T catalyzed conjugation of GSH with CDNB by three possible mechanisms: (1) by depleting GSH through a direct reaction which may be important for DBAN and possibly BCAN due to their high reactivity toward GSH, but probably not important for the other HAN; (b) by competing with CDNB as a substrate for conjugation with GSH which is possible only for MCAN, since the other HAN do not undergo GSH-T catalyzed conjugation; and (3) by binding to GSH-T, thus inactivating the enzyme. The HAN may inactivate GSH-T by interaction with thiol groups, amino groups or other nucleophilic centers in the protein through replacement of a halogen atom (MCAN, DBAN, BCAN) or reaction at a cyano group (TCAN and DCAN) [15, 16]. Varied modes of inhibition mechanisms and HAN–enzyme interaction may account for the small difference in the inhibitory activities of the HAN.

Following a single dose of HAN administered orally to rats, GSH concentration and GSH-T activities in liver were determined at 1, 3 and 18 hr post treatment. With the exception of MCAN, the haloacetonitriles increased liver to body weight ratio at 18 hr to the same extent (14%). None of the haloacetonitriles had any significant effect on the homogenate protein concentration. Cytosolic protein was decreased significantly only by DBAN at 3 hr. The GSH-T activity was decreased slightly by TCAN and DBAN at 3 hr and significantly by DBAN at 18 hr. The inhibition of GSH-T activity following oral administration of DBAN to rats has also been observed by Ahmed and Hussein [17].

The effect of HAN on the concentration of the GSH in the rat liver is presented in Fig. 4. MCAN depleted GSH concentration at 1 and 3 hr. By 18 hr GSH concentration was recovered to the control level. GSH concentration was depleted by the dihalogenated HAN at 1 hr, rebounded to control level at 3 hr, and became elevated at 18 hr. GSH level was not affected significantly by TCAN at 1 hr, but became elevated at both 3 and 18 hr.

The reaction of GSH with DBAN *in vitro* was rapid; however, the depletion of GSH 1 hr after oral administration of DBAN was only 34%. The fast reaction of GSH with DBAN probably caused depletion of GSH soon after dosing, and by 1 hr the GSH level may already have been replenished. Ahmed and Hussein [17] reported maximum depletion of GSH at 0.5 hr (the earliest time point of measurement) and recovery at 4 hr following oral administration of DBAN to rats. It is possible that the maximum

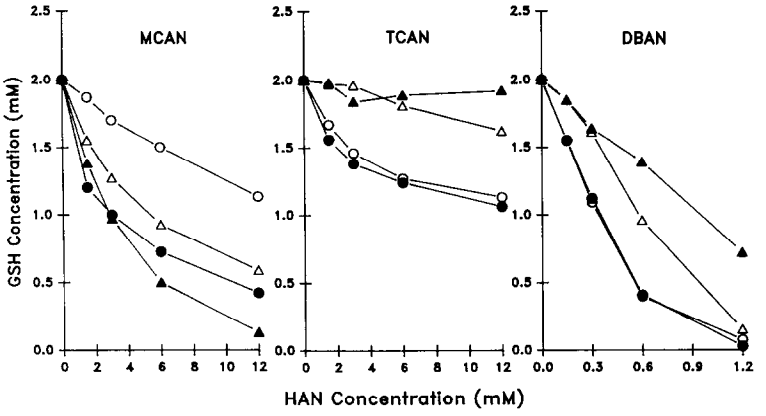

Fig. 2. Reaction of GSH with MCAN, TCAN and DBAN under various conditions. GSH (2 mM) was incubated with various concentrations of HAN at 37° for 30 min, and the remaining GSH concentration was determined. Key: (○) GSH and HAN alone; (●) in the presence of 0.8 mg of cytosolic protein; (△) in the presence of 0.9 mg of microsomal protein and an NADPH-regenerating system; (▲) in the presence of 0.8 mg of cytosolic protein, 0.9 mg of microsomal protein and an NADPH-regenerating system (5 mM glucose-6-phosphate, 0.4 mM NADP, 0.5 units/ml glucose-6-phosphate dehydrogenase, 10 mM magnesium chloride). Each value is the average of five determinations. The standard errors were all within 15% of the mean values.

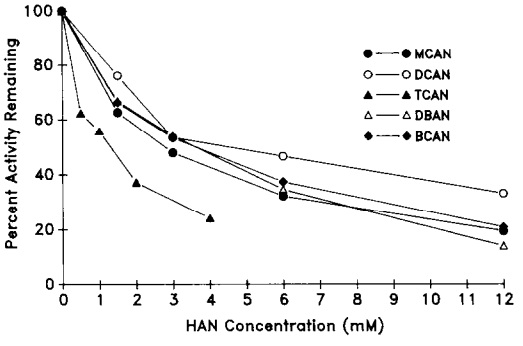

Fig. 3. Inhibition of GSH-*S*-transferase activities by HAN. GSH (1 mM), CDNB (1 mM) and 16.6 μg of cytosolic protein were incubated in the presence of various concentrations of HAN, and the rate of formation of GSH-CDNB adducts was measured. Values are the averages of five determinations and are expressed as the percent of control (no HAN added) activity remaining. The standard errors were all within 15% of the mean values.

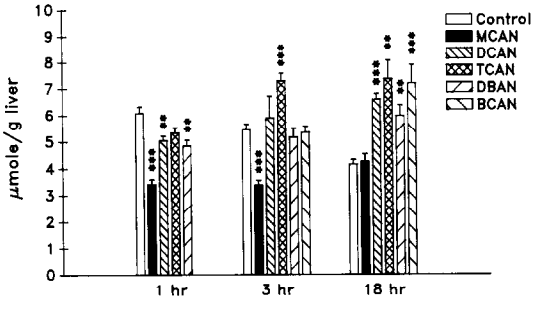

Fig. 4. Effects of HAN on GSH concentration in liver. GSH concentration in liver homogenate was assayed at 1, 3, and 18 hr following oral administration of 0.75 mmol/kg of HAN to rats. Values are means ± SE, N = 3–6. Key: (**) and (***) indicate significant difference from control values by Student's *t*-test at $P < 0.01$ and 0.001 respectively.

depletion of GSH occurred even earlier than 0.5 hr after dosing.

MCAN depleted the most GSH and for a longer time which is consistent with microsomal metabolism to metabolites more reactive toward GSH observed *in vitro*. TCAN depleted less GSH than other HAN, probably due either to biotransformation to metabolites which are less reactive toward GSH in agreement with *in vitro* study or to general lesser reactivity *per se*. Even though DCAN did not react with GSH *in vitro*, it depleted the hepatic GSH level, suggesting metabolism to metabolites which react with GSH.

The toxicity of many organonitriles has been suggested to result largely from the liberation of cyanide in the body [18]. The acute toxicity of HAN in rats was reported to be DBAN > MCAN > DCAN [19]. During the dosing of the rats, we observed that TCAN was as toxic as DBAN as judged from the overt signs of acute toxicity (gasping and salivation) and the observed deaths at 18 hr (2/20 for TCAN, 1/10 for DBAN, and none for the other HAN). In view of the higher toxicity of DBAN and TCAN and less cyanide (as thiocyanate) liberation by DBAN and TCAN (7.7 and 2.3% of dose respectively) than by MCAN (14.2% of dose) reported by Pereira *et al.* [8], cyanide could not be the important factor in the toxicity of HAN. The toxicity of HAN probably results mostly from the direct interaction of these chemicals with cellular macromolecules. Depletion of cytochrome P-450 by pretreatment with carbon tetrachloride results in increased toxicity by MCAN in mice [20], suggesting that MCAN largely exerts its toxicity directly. The high toxicity of TCAN compared to its relative reactivity toward nucleophiles may result from its lower

reactivity toward GSH and consequently less detoxification, giving it more chance to react with macromolecules in the body. Stronger induction of DNA strand breaks [4] and inhibition of GSH-T activity *in vitro* by TCAN than DBAN and less depletion of GSH in the presence of BSA are in agreement with this suggestion. However, metabolism of TCAN to more toxic metabolites cannot be ruled out.

In summary, HAN and/or their metabolites reacted with GSH and inhibited GSH-T *in vitro* and depleted GSH *in vivo*. GSH reacted with TCAN by the addition across the cyano group and reacted with the other HAN by the displacement of a halogen atom. Both GSH and GSH-T appear to play important roles in the detoxification of the haloacetonitriles.

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