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Effect of Glutathione on *In Vitro* Metabolism of Unsaturated Aliphatic Nitriles to Cyanide

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Aliphatic nitriles are widely used as important solvents and intermediates in polymer, plastic, synthetic fibers, resins, dyestuffs, pharmaceuticals and vitamin industries. Occupational exposure to these chemicals is principally through inhalation and dermal routes (NIOSH, 1978; OSHA, 1978). Many reports have described fatalities and signs of poisoning in humans by several of these nitriles (NIOSH, 1978; Willhite, 1979). Human and animal toxicity studies have suggested that the toxicity of aliphatic nitriles is due to their cyanide (CN^-) liberating capacity under biological conditions. Despite the common property of CN^- liberation, their are marked differences among nitriles, in the amounts of CN^- released to cause poisoning, in the duration of exposure and toxicity signs. Additional signs of aliphatic nitrile poisoning including development of duodenal ulcers (Szabo and Seyle, 1972), adrenal apoplexy (Szabo *et al.*, 1980), nuclear changes (Van Breeman and Hirakoa, 1961), thyroid hyperemia and hyperplasia (Spence and Marine, 1932), neurologic bladder dysfunction (Kriess *et al.*, 1980; Farooqui and Ahmed, 1984) and dyskinesia (Tanii *et al.*, 1989) have also been reported.

The extent of metabolism of unsaturated aliphatic nitrile is influenced by the structural properties of the molecule. The purpose of this study is to investigate the effects of functional groups, the type of unsaturated moiety and availability of sulfhydryls especially glutathione (GSH) on the *in vitro* rat liver postmitochondrial metabolism of unsaturated aliphatic nitriles. The postmitochondrial fraction (microsomes + cytosol) is used to provide cytosolic GSH transferase, an essential enzyme of GSH metabolism.

MATERIALS AND METHODS

Methacrylonitrile, allylnitrile and crotononitrile were obtained from Aldrich Chemical Co. (Milwaukee, WI). Glutathione and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade.

Male Sprague-Dawley rats from Harlan Sprague-Dawley, Inc. Houston, TX weighing 200-250 g were acclimatized in our animal facility for one week prior to experiments at a constant temperature of 20-22^{o}C in a room with 12 h light and dark cycles; fed Purina Lab Chow and water *ad libitum*. Normal male healthy rats were anesthetized with diethyl ether, the livers excised and washed with cold normal saline. The livers were combined and homogenized in 0.25 *M* sucrose using motor driven Potter Elvehjem tissue homogenizer. Postmitochondrial fractions were obtained by centrifugation of the liver homogenates at 8900 rpm for 20 min. The pellet containing nuclei and mitochondria were discarded and supernatant containing microsomes and cytosol were used for this study. All experiments were completed on the same day of collection of livers.

Incubation mixtures contained 100 *u*mol of potassium phosphate buffer, pH 7.5, 30 *u*mol of $MgCl_2$, 6 *u*mol NADPH, 6 mg of postmitochondrial protein and 300 *u*mol of the nitriles in a final volume of 6 ml contained in a tightly capped 30 ml glass serum vial. In order to determine the effect of GSH, in appropriate incubation mixtures 10 *mM* GSH was added. Reactions were begun by addition of nitriles and stopped by immersing the vials in ice. Incubations were carried out at 37°C with constant shaking. Cyanide content of the incubation mixtures was determined by a modification of the method outlined in the manual accompanying the Orion silver sulfide electrode (Abreu and Ahmed, 1980). An aliquot (1.5 ml) of the incubation mixture was added to 1.5 ml of 4*N* H_2SO_4 contained in the outer chamber of a Conway microdiffusion cell. The inner chamber contained 1.5 ml of 0.1*N* NaOH. The cells were sealed with a glass cover using silicon grease and rotated at 70 rpm for 2 hours. Electrodes (Orion silver sulfide No. 90-16 and Orion double junction electrode No. 90-02) were placed in the center well of the Conway microdiffusion cell to which has been added 0.05 ml of $KAg(CN)_2$ (ICN Pharmaceuticals, Plainview, N.Y.) indicator solution. The indicator solution contained 17.7 g of Na_2HPO_4, 2.2 g of NaOH and 10 mg of $KAg(CN)_2$ in a final volume of 100 ml of distilled water. The cyanide content of the mixtures was determined from the standard curve developed using KCN standard solutions. Protein was determined with BioRad reagent using globulin as the standard.

All data were calculated as the mean values $\pm$ SD of at least 4 replicate determinations of a pooled liver homogenate preparation. Statistical analysis was accomplished with Student's *t-test.*

RESULTS AND DISCUSSION

Assay conditions for the hepatic postmitochondrial conversion of the nitriles were characterized with respect to time (Figure 1). The reactions were linear with the time of incubation. In view of the decrease in tissue GSH observed *in vivo* after acute nitrile administration, GSH was added to incubation mixtures to test its effect upon microsomal metabolism of nitriles (Figure 2). As seen in the Figure 2, GSH had varying degree of effects on the rate of cyanide release. MeAN demonstrated a decrease in cyanide liberation of 43% versus the control. ALN exhibited a similar trend with GSH causing a decline in cyanide formation by over 58% of the control. In contrast, CRN when incubated with GSH, produced significantly higher amounts of cyanide (139% of the control).

GSH plays a critical role in the biochemical and physiological functions of the cells. Conjugation of xenobiotics or their metabolites with GSH is one of the primary mechanisms of detoxication (Kosower, 1976). Reactions of electrophiles with GSH are of a 1,4 Michael addition type which are typical for conjugated carbonyls (Esterbauer *et al.*, 1975), hydroxyhexanals (Winter *et al.*, 1987), alpha and beta unsaturated aldehydes (Esterbauer *et al.*, 1975) and unsaturated nitriles (Kosower, 1976). The Michael addition reactions with GSH do not lead to the cleavage of the parent molecule (Silver *et al*, 1982) and result in the formation of a polar product which could be excreted in the urine. However, formation of epoxides prior to transfer of GSH results in cleavage of the molecule and in case of aliphatic nitriles, releases cyanide (Abreu and Ahmed, 1980; Farooqui *et al.*, 1990). Thus the significant reduction in cyanide liberation from MeAN and ALN in this study indicates a detoxication mechanism involving either the nonenzymic conjugation of the nitriles with GSH or GSH-S-transferase mediated mechanism. It is interesting to note that these findings are in contrast to what we have reported for the microsomal metabolism of MeAN in the absence of cytosolic fraction that provides the enzyme GSH-S-transferase. In the absence of GSH-S-transferase the microsomal conversion of MeAN to cyanide was actually enhanced to approximately 150% of the control (Farooqui *et al.*, 1990). CRN, on the other hand, showed an actual increase in the cyanide formation implicating that GSH potentiated the formation of cyanide. The clarification of the mechanism of this potentiation in case of CRN needs further studies. The

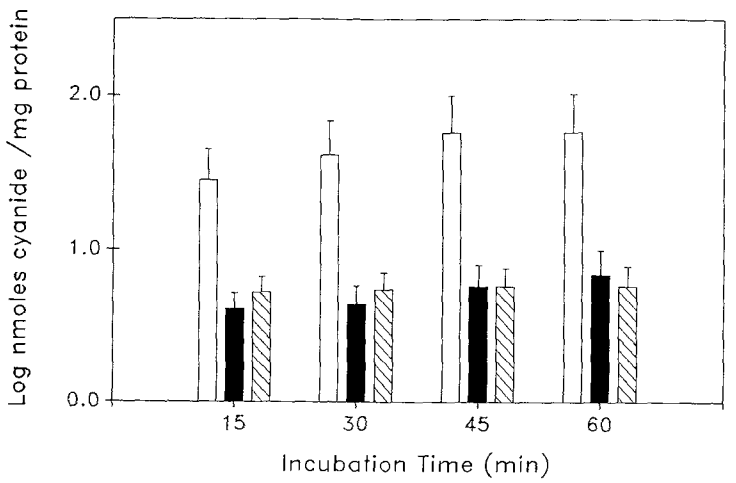

Figure 1. Postmitochondrial metabolism of three unsaturated aliphatic nitriles MeAN (methacrylonitrile; open bars), ALN (allylnitrile; filled bars) and CRN (crotononitrile; crossed bars). Each bar represents mean $\pm$ S.D. of 4 replications. Incubation mixtures contained 6 mg of postmitochondrial protein, 300 μmol of nitriles, 30 μmol of $MgCl_2$ and 6 μmol of NADPH in 100 μmol phosphate buffer, pH 7.5. Reactions were carried out at 37°C for 1 hr.

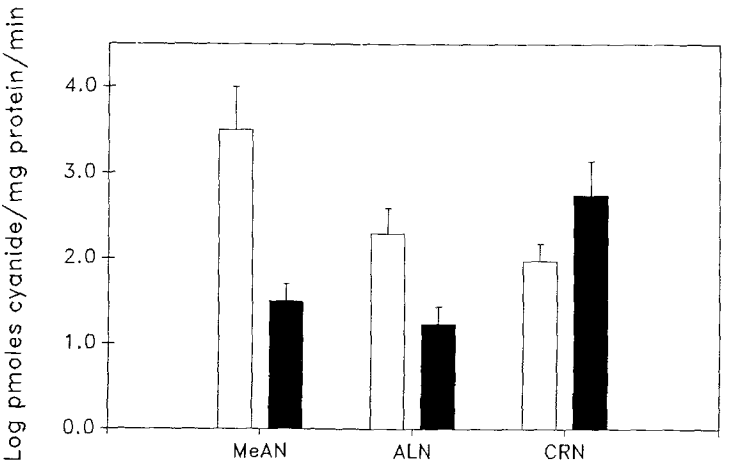

Figure 2. Effect of glutathione (GSH) on the postmitochondrial metabolism of three unsaturated aliphatic nitriles to cyanide. MeAN = methacrylonitrile, ALN = allylnitrile and CRN = crotononitrile). Open bars represent controls incubations (without GSH) and filled bars represent incubations with GSH (10 mM). Each bar represents mean $\pm$ S.D. of 4 replications. All values for cyanide are significantly different from controls at $p < 0.05$. Incubation conditions are the same as in Figure 1.

position of double bond in CRN closer to cyanide moiety may be involved in the differential effect of GSH on its conversion to cyanide. Metabolism of MeAN and CRN may proceed via an epoxide which upon conjugation with GSH may allow cyanide formation. According to the position of the double bond, the molecule of ALN is structurally different from those of MeAN and CRN and does not behave as the alpha beta unsaturated nitriles. The lack of formation of a cyanohydrin from ALN via an epoxide (Silver *et al.*, 1982) suggests a possible hydroxylation reaction to release cyanide. Silver *et al.*, (1982) have postulated that cyanide release from ALN may be via hydroxylation in the gastrointestinal tract. Proposed metabolic pathways for these nitriles involving GSH are shown in Figure 3.

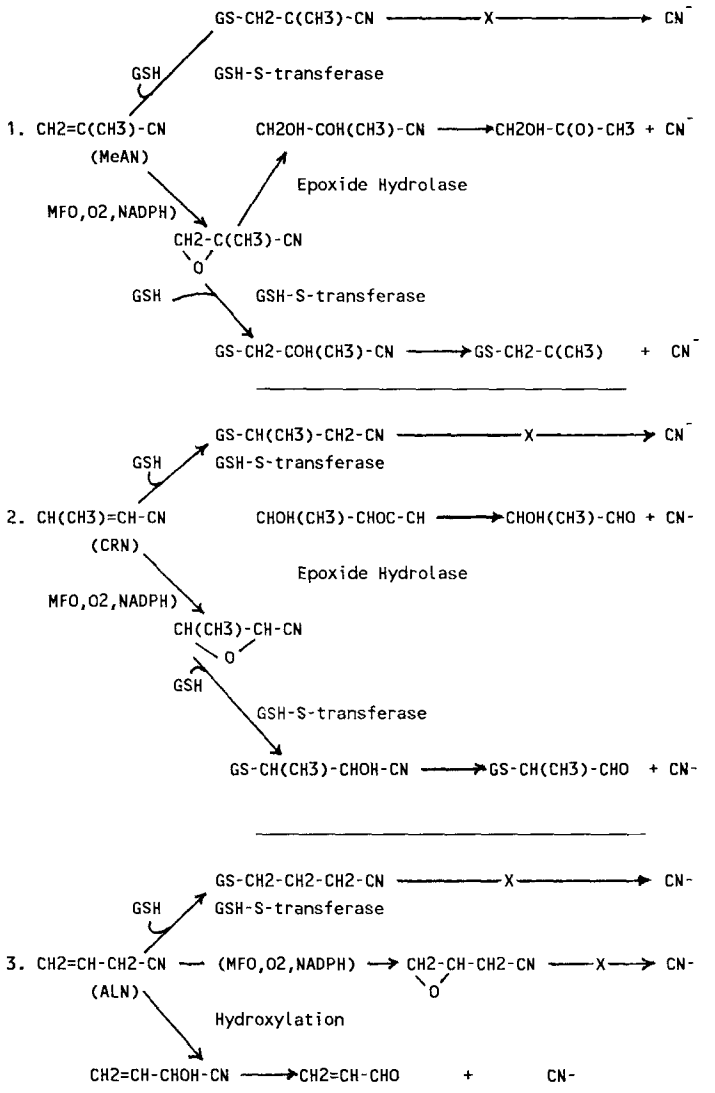

Figure 3. Proposed pathways for the cyanide release from three unsaturated aliphatic nitriles MeAN (methacrylonitrile), CRN (crotononitrile) and ALN (allylnitrile). See text for explanation.

The nitriles studied in this work all belong to the family of aliphatic nitriles which exhibit a varying range of biochemical effects, despite the fact that, in general, all are potent neurotoxins. However their organ specific toxicity is remarkably different (Ahmed and Farooqui, 1982; Ahmed *et al.*, 1985). Additional extensive investigations are needed to understand the molecular events underlying the toxicity of unsaturated nitriles in order to assess an acceptable regimen of exposure to these chemicals in work places.

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