In Vivo and in Vitro Hepatotoxicity and Glutathione Interactions of \(N\)-Methyldithiocarbamate and \(N,N\)-Dimethyldithiocarbamate in the Rat

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The ability of \(N\)-methyldithiocarbamate (NMDC) to generate methylisothiocyanate and HS\(^{-}\) together with its greater acid stability suggest that NMDC may exert greater acute toxicity following oral exposure than its dialkyl analog, \(N,N\)-dimethyldithiocarbamate (DMDC). To assess this possibility, cell culture, perfused liver, and in vivo studies were performed to delineate differences in the hepatotoxicity and thiol interactions of NMDC and DMDC in the rat. The role of methylisothiocyanate and HS\(^{-}\) in NMDC-induced hepatotoxicity was evaluated and glutathione interactions characterized through analysis of reduced glutathione (GSH), glutathione disulfide (GSSG), and \(S\)-methylthiocarbamoylglutathione (GSMITC) using HPLC and liquid chromatography tandem mass spectrometry (LC/MS/MS). Following oral administration, centrilobular hepatocyte necrosis and enzyme leakage was observed for NMDC but not for DMDC. Dose dependent decreases of intracellular GSH were produced by both dithiocarbamates in primary hepatocytes but DMDC appeared to deplete GSH through the generation of GSSG whereas NMDC produced GSMITC consistent with the generation of a methylisothiocyanate intermediate. In primary hepatocytes, both NMDC and DMDC cytotoxicity was increased by prior depletion of intracellular GSH and diminished by prior supplementation of GSH. The results obtained using perfused livers were similar for NMDC in that elevated levels of GSMITC were detected in the bile; however, DMDC produced only a modest increase of GSSG over controls that was not significant different to that produced by NMDC. Results obtained from isolated liver mitochondria and primary hepatocytes were not consistent with NMDC producing HS\(^{-}\)-mediated inhibition of mitochondrial respiration. These data support a greater potential for hepatotoxicity to result following oral exposure to NMDC relative to DMDC and that glutathione may play a role in cytoprotection for NMDC, presumably through detoxification of a methylisothiocyanate metabolite.

Key Words: \(N\)-methyldithiocarbamate; \(N,N\)-dimethyldithiocarbamate; metam-sodium; methyl isothiocyanate; hepatotoxicity; glutathione.

Populations that are most susceptible to high level exposures include those utilizing dithiocarbamates in the occupational setting, e.g., orchard workers, and individuals administered dithiocarbamates therapeutically (Brewer, 1993; Shinobu et al., 1983). In addition to human exposure, plant and animal wildlife are also exposed to dithiocarbamates through their use as pesticides and release into the environment as occurred with the 1991 spill of metam-sodium into the Sacramento River in California (CEPA, 1992). Two classes of commonly used dithiocarbamates include the monoalkyl and dialkyl dithiocarbamates that are distinguished by the number of alkyl substituents on the nitrogen. The sodium salt of \(N\)-methyldithiocarbamate (NMDC), metam-sodium, is a prevalent monoalkyl dithiocarbamate used principally as a soil fumigant (Keil et al., 1996). \(N,N\)-diethylthiocarbamate (DEDCC) and \(N,N\)-dimethyldithiocarbamate (DMDC), prepared as salts of various metals or as bis disulfides, are common dialkyl dithiocarbamates used in a variety of agricultural, medical, and industrial applications.

Nitrogen substitution influences the rates of decomposition, the decomposition products generated, and the metabolic pathways observed for dithiocarbamates. Both monoalkyl and dialkyl dithiocarbamates share the ability to undergo acid-promoted decomposition to parent amine and CS\(_2\), as well as biotransformation through methylation and subsequent oxidation to monothiocarbamate sulfone and sulfoxide metabolites (Joris et al., 1970; Staub et al., 1995). An additional pathway unique to monoalkyl dithiocarbamates is generation of an alkyl isothiocyanate under physiological conditions, presumably through release of sulphydryl ion (HS\(^{-}\)) (Drobnica et al., 1977; Joris et al., 1970). Both the isothiocyanate and the HS\(^{-}\) generated are biologically active species with the potential to acylate nucleophiles and inhibit mitochondrial respiration, respectively (Lam et al., 1993). Generation of the potent broad-spectrum cytotoxicant, methyl isothiocyanate, is generally given credit for the effectiveness of NMDC as a soil sterilant (Pruett et al., 2001). Thus the unique ability of monoalkyl dithiocarbamates to generate the HS\(^{-}\) and isothiocyanate suggests that monoalkyl dithiocarbamates may present hazards not associated with the dialkyl dithiocarbamates.

Dialkyl dithiocarbamates have been demonstrated in vitro to...
modulate apoptosis, enzyme function, transcription, and oxidation status depending upon the experimental conditions and cells used (Chinery et al., 1997; Hosni et al., 1992; Nobel et al., 1995, 1997). Although the biological consequences and target organs of diithiocarbamates are still being delineated in vivo, neurotoxicity has been associated with subchronic administration of DEDC in rabbits, rats, sheep, and hens while the use of disulfiram, the disulfide of DEDC, in alcohol aversion therapy in humans has produced two well recognized sequelae, neurotoxicity and hepatotoxicity (Edington and Howell, 1969; Forns et al., 1994; Johnson et al., 1998). Whereas hepatotoxicity appears to be an idiosyncratic reaction possibly mediated through an auto-immune mechanism, neurotoxicity appears to be dose dependent. Thus the potential for acute toxicity following common dialkyl(diithiocarbamate) exposures appears to be low with adverse effects requiring repeated exposures. In contrast, there is considerably less toxicological data available for NMDC in mammals, with the data available consisting primarily of reports on workers and wildlife following the metam-sodium spill in California and immune system effects produced in rodents (CEPA, 1992; Kell et al., 1996).

The chemical properties of its unique decomposition products combined with its greater acid stability suggest that NMDC may exert greater acute toxicity following oral exposure relative to its dialkyl analog, DMDC. In the study presented here the potential for NMDC to produce hepatotoxicity in the rat following acute oral exposure was examined. The role of methylisothiocyanate and HS in NMDC induced hepatotoxicity was evaluated and the major interactions of NMDC with intracellular thiols were characterized through the analysis of reduced glutathione (GSH), glutathione disulfide (GSSG), and glutathione conjugates in primary rat hepatocytes and intact rat liver perfusion systems. Reactions and biological effects unique to the mono substituted NMDC were identified through comparison to results obtained for its dialkyl analog, DMDC.

MATERIALS AND METHODS

Caution: Carbon disulfide is volatile, flammable, toxic, and a skin irritant, and methylisothiocyanate is a skin irritant; gloves and a fume hood should be used when handling these compounds.

Chemicals. Reduced glutathione (GSH), glutathione disulfide (GSSG), Collagenase Type IV, bathophenanthroline disulfonic acid (BDPS), dansyl chloride, diethylmaleate (DEM), γ-GluGlu, and the salts used for buffer preparation were obtained from Sigma (St. Louis, MO). Sodium N,N-dimethylthiodiocarbamate hydrate, methyl amine, and methylisothiocyanate were obtained from Fluka (Milwaukee, WI). Ethyl isothiocyanate was obtained from Aldrich (Milwaukee, WI); carbon disulfide was purchased from EM Scientific (Gibbstown, NJ). Pyrrolidine diithiocarbamate (PyDTC) was a gift from Mark M. Jones, Department of Chemistry, Vanderbilt University.

NMDC synthesis. All steps were performed in a chemical fume hood. NMDC was generated by taking methyl amine (20 mmol) into 20 ml each of double distilled water (ddH2O) and 95% ethanol. Carbon disulfide (30 mmol) was added with constant stirring at room temperature. The pH was monitored and when the pH dropped below 10.0, 4 N NaOH was added to maintain the pH in the range of 9.5 to 10.0. When the pH remained steady at 9.5, the solution was concentrated to 5 ml and 10 ml water was added. The solution was again concentrated to 5 ml, the UV spectra checked, and the concentration of the NMDC solution was determined (UV max 282 nm, ε 13,500). The yield of NMDC was 19.4 mmol, indicating 97% of the methyl amine was converted to NMDC. The product was further purified under vacuum to remove the excess volatile, unreacted carbon disulfide, and methyl amine. The NMDC solution was stored at 4°C.

Preparation of glutathione compounds. The monomethyl ester of glutathione (GSE) was prepared using the sulfuric acid catalyzed reaction of Anderson (Anderson and Meister, 1989). The purity of the dry GSE crystals was determined to be >99% by HPLC.

Synthetic S-(methylaminothiocarbonyl) glutathione (GSMITC) was prepared using the method of Lam and was purified by HPLC (Lam et al., 1993). GSH (4.9 mmol) was dissolved in 2 ml ddH2O and the pH raised to 7.0 with NaOH. MITC (15 mmol) was added and the mixture was stirred overnight at room temperature. The internal standard used for mass spectral analysis, S-(ethylaminothiocarbonyl) glutathione (GSEEITC), was prepared in an identical manner except ethyl isothiocyanate was substituted for MITC.

In vivo exposure of rats to NMDC and DMDC. All animal experiments were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (250 g) were fasted for 18 h prior to being administered a single dose of either NMDC or DMDC (2 mmol/kg) po using approximately 0.5 ml PBS, pH 7.4 as the vehicle. Control rats were given the vehicle alone; all dosing groups consisted of three rats. Food was withheld from the rats for 5 h after po dosing. At time 24 h, the rats were anesthetized and exsanguinated via cardiac puncture. Serum was prepared from the blood and stored in the dark at 4°C until analysis of alanine aminotransferase (Sigma, D1G159-K), aspartate aminotransferase (analysis performed by Vanderbilt University Medical Center Clinical Chemistry Laboratory on a Vitros 950, Johnson and Johnson), γ-glutamyl transferase (Sigma, G419-10), and total bilirubin (Sigma, 550-A). The abdominal cavity was opened and the medial portion of the left lobe and the right lobe of the liver were placed into 10% buffered formalin. The formalin-fixed liver was processed routinely, embedded in paraffin, and 5 μm sections stained with H&E were examined using light microscopy.

Additional in vivo experiments were performed with lower concentrations of NMDC (0.5, 1.0, and 1.5 mmol/kg). One ml of blood was drawn via the tail vein from each rat 24 h before dosing and was used to obtain baseline ALT and AST levels. Rats were fasted for 18 h before oral dosing and 5 h after dosing with the appropriate dose of NMDC with three rats dosed in each group. After 24 h, another 1 ml of blood was drawn from each rat and levels of ALT and AST were measured.

Hepatocyte isolation and culture. Primary rat hepatocytes were isolated from male Sprague-Dawley rats (250–300 g) fed standard rat chow and water ad libitum. The livers of anesthetized rats were perfused using a single step isolation protocol (Berry et al., 1991). The liver was removed, minced with scissors, incubated at 37°C/5% CO2 for 10 min and then passed through 210 μm nylon mesh. The resulting cells were washed three times with ice cold, modified KH buffer and pelleted by centrifugation at 40 × g for 2 min. After the final wash, viability was assessed by trypan blue exclusion and was routinely greater than 90%. Suspension cultures (4 ml cultures of 1 × 10⁶ cells/ml) were established in 10 ml Erlenmeyer flasks and equilibrated with gentle shaking for 30 min in an incubator at 37°C/5% CO2 prior to dosing with test compounds. Aliquots were taken at predetermined intervals for assessment of viability by LDH analysis and collection of intracellular and extracellular fractions for glutathione analysis. LDH analysis was performed using the Sigma Diagnostics LD-L test kit adjusted to run in a 96-well microplate assay format. The LDH assay results obtained using this kit were calculated by subtracting the LDH activity in the media of a time-matched control (1 × 10⁶ cells) from the LDH activity in the media of exposed cells (1 × 10⁶ cells) and dividing by the total LDH activity (intracellular and extracellular) obtained after lysing control cells (1 × 10⁶ cells). Glutathione samples were collected...
by layering an aliquot of hepatocyte suspension over dibutyl phthalate and spinning the viable cells into a solution of 10% perchloric acid (PCA) containing 1 mM BPDS (Fariss et al., 1985). To the extracellular fractions, BPDS and PCA were added to give a final concentration of 10% PCA and 1 mM BPDS.

**Intact rat liver perfusion.** Sprague-Dawley rats (300–350 g) were anesthetized and the liver was perfused using the method of Krieter (Krieter et al., 1985). Time zero was taken to be the point at which the caval tubing was connected and collection of bile commenced. Bile was collected into preweighed tubes containing 10% PCA with 1 mM BPDS in 5-min intervals. At the end of each interval, approximately 1 ml of the perfusate was collected from the caval catheter. A portion of the caval perfusate was acidified with PCA containing BPDS to bring the final concentration of PCA and BPDS to 10% and 1 mM, respectively. The remaining caval perfusate was analyzed for the presence of LDH content. Four collection intervals were performed (20 min) to achieve baseline conditions prior to perfusing either NMDC (80, 200, 500 μM), DMDC (200, 2000, 2000 μM), or water (control) into the perfusion buffer stream. NMDC or DMDC were infused for 30 min followed by another 20-min period of perfusion with buffer alone. After perfusion the liver was removed and weighed. Both the acidified and caval fractions were stored at −80°C until analysis using either the HPLC or mass spectrum methods listed below.

**HPLC analysis.** Levels of GSH and GSSG were measured by derivatization with dansyl chloride followed by HPLC with fluorescence detection (Jones et al., 1998) with several modifications. To a 90 μl aliquot of cell extract, 10 μl of 0.25 mM γ-GluGlu internal standard, 100 μl of 200 mM boric acid/50 mM potassium tetraborate, and 40 μl of 40 mM iodoacetic acid were added, in that order. The pH was adjusted to 9.0 with 1 M KOH solution saturated with potassium tetraborohydrate and the samples were mixed and added and the samples were vortexed and stored in the dark at 4°C. The pH was adjusted to 9.0 with 1 M KOH solution saturated with potassium tetraborohydrate and the samples were mixed and allowed to set at room temperature for 30 min. A volume of 300 μl of a saturated dansyl chloride solution (10 mg dansyl chloride/mI acetone) was added and the samples were vortexed and stored in the dark at 4°C for 24 h. Acetone and excess dansyl chloride were then extracted by addition of 500 μl chloroform. The samples were stored at 4°C with the salt precipitate and chloroform until analysis was performed.

HPLC analysis was performed using a Waters 2690 and 474 fluorescence detector with a LiChropher 100 NH₄, 5 μm column. Sample temperature was maintained at 4°C and the column temperature was 25°C. Solvent A was 80% methanol, 20% water and solvent B was 80% solvent A and 20% of an acetate solution (272 g sodium acetate, 378 ml glacial acetic acid, 122 ml water). The flow rate was 1 ml/min and the initial solvent conditions were 80% A:20% B for 10 min. The final composition was held for 7 min before returning to initial conditions. For the first 3 min of each run the flow was diverted to waste by an inline switch valve. The electrospary voltage of the positive ion source was 4.0 kV and capillary temperature was 250°C. Nebulizer pressure was 74 psi. Collision induced dissociation occurred in Q2 with argon as the collision gas (2.3 mT) at a collision energy of ~17 eV. SRM experiments to detect GSMITC were conducted by monitoring the m/z 381 to 252 transition that arose from the standard loss of mass 129 from glutathione containing compounds (m/z 395 to 266 for the GSEITC standard) (Baillie and Davis, 1993) using a scan time of 1.0 s. An increase in sensitivity was achieved through degrading resolution on Q1 and Q3 by raising the voltages by 5 V on each quad. Semi-quantitative analysis was performed by generating a standard curve of response ratios using known concentrations of GSMITC to which a fixed amount of GSEITC was added. The same quantity of GSEITC used for the standard curve samples was also added to each of the cell extracts prior to analysis. The standard curve for GSMITC analysis was y = 0.087 + 0.0021 x, r² = 0.99. No samples analyzed were outside the 0.26–26 pmol range used to generate the standard curve.

**Mitochondria isolation and respiration experiments.** Male Sprague-Dawley rats fed ad libitum on Rat Lab Diet were anesthetized and the liver was removed into ice cold isolation buffer (0.25 M sucrose, 10 mM Tris, 0.5 mM potassium EDTA, pH 7.4). The tissue was chopped finely and manually homogenized in isolation buffer using a Dounce homogenizer. Total homogenate was centrifuged at 4°C for 3 min at 2000 x g. The supernatant was retained and centrifuged at 12,500 x g for 8 min. The mitochondrial pellet was resuspended and centrifuged twice more at 12,500 x g for 8 min. After the final wash, the pellet was resuspended in 2 ml of ice cold isolation buffer and the protein concentration determined using the Bio-Rad protein assay with bovine serum albumin as the standard. Freshly isolated mitochondria were stored on ice until use in respiration studies (Picklo et al., 1999).

Respiration of the freshly isolated mitochondria was measured via oxygen consumption using an oxygen electrode (YSI International) and oxygen consumption chamber (Gilson). Assay buffer consisted of 40 mM KCl, 50 mM mannitol, 50 mM sucrose, 0.5 mM disodium EDTA, 10 mM HEPES, 5 mM MgCl₂, and 10 mM KH₂PO₄, pH 7.4. Control liver mitochondria had respiratory control ratios of greater than 5 with glutamate and malate as substrates. Experiments were performed by suspending mitochondria (0.1 mg/ml) in assay buffer (25°C) followed by addition of glutamate (3 mM) followed by NMDC (2 mM), DMDC (2 mM), or NaSH (4.9 μM). After 5 min, 0.4 μmol ADP was added. State 3 respiration was taken as the rate of oxygen consumption for 2 min following ADP addition.

**Dithiocarbamate decomposition rate determination.** Solutions of NMDC, DMDC, and PyDTc (5 mM) were prepared and diluted to 0.1 mM in either 100 mM HCI (pH 1.0), 100 mM formate (pH 4.0), or 100 mM phosphate buffer (pH 8.0). Absorbance was measured immediately and decomposition was monitored by observing the decrease in the UV absorbance at λabs = 266 nm for NMDC, λabs = 272 nm for DMDC, or λabs = 271 nm for PyDTc at pH 1.0. At pH 4.0 and pH 8.0 absorbance was monitored at λabs = 281 nm for NMDC, λabs = 280 nm or DMDC for λabs = 278 nm for PyDTc.

**RESULTS**

**Effect of Dithiocarbamates on the Rat Liver after Oral Administration**

Rats were dosed with NMDC or DMDC (2 mmol/kg) and 24 h later serum and liver sections were collected. Analysis of the serum for markers of liver injury indicated significant elevations for both ALT and AST in NMDC but not DMDC treated animals relative to controls (Table 1). Neither NMDC nor DMDC resulted in increased levels of γ-GT or bilirubin, indicators of bile duct injury in rats. Rats were also dosed with 0.5, 1.0, and 1.5 mmol/kg NMDC with only levels of AST found to be slightly, but significantly, elevated relative to controls (89.3 U/l ± 3.9) at the 1.5 mmol/kg dose of NMDC (142.0 U/l ± 22.6). Upon examining the liver sections using light microscopy no differences were observed for any of the animals administered DMDC relative to controls (Figs. 1A and 1B). In contrast, infiltration of mononuclear inflammatory cells...
into the region surrounding the central hepatic vein (Zone 3) occurred in all three of the animals administered NMDC (2 mmol/kg; Figs. 1C and 1D). The inflammation observed was uniform in all lobules examined and hepatocytes in the centrilobular region also exhibited signs of cell injury and death.

<table>
<thead>
<tr>
<th>Serum marker</th>
<th>Control</th>
<th>NMDC</th>
<th>DMDC</th>
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<tbody>
<tr>
<td>ALT (U/l)</td>
<td>84.3 ± 6.4</td>
<td>409.0 ± 112.6*</td>
<td>76.7 ± 1.8</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>80.0 ± 5.6</td>
<td>1579.0 ± 707.5*</td>
<td>72.7 ± 3.4</td>
</tr>
<tr>
<td>γ-GT (U/l)</td>
<td>4.0 ± 1.3</td>
<td>4.8 ± 0.4</td>
<td>4.4 ± 0.6</td>
</tr>
<tr>
<td>Bilirubin (mg/dl)</td>
<td>0.9 ± 0.2</td>
<td>1.2 ± 0.4</td>
<td>1.2 ± 0.1</td>
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</table>

Note. Measurements performed in duplicate and reported as the mean ± SEM (n = 3). Oral dose of NMDC and DMDC was 2.0 mmol/kg. *Significantly different (p < 0.01) compared to control levels using the ANOVA test with the Dunnett post test.

Effect of Fructose Supplementation on Cytotoxicity of Mitochondrial Respiration Inhibitors and Dithiocarbamates

Hepatocytes were preincubated with and without 5 mM fructose for 30 min prior to dosing. In hepatocytes fructose can serve as an alternate source of ATP via glycolysis. The ATP generated is sufficient to maintain cellular processes in the presence of inhibitors of mitochondrial respiration. ATP levels were measured prior to exposing cells to fructose and just before dosing with test compounds to ensure that ATP levels had returned to normal (data not shown). The media was assayed for the presence of LDH as an indicator of viability every h for 4 h. Incubation of hepatocytes with fructose alone did not elicit a change in LDH release relative to controls (data

![FIG. 1. Histology of rat liver subsequent to po administration of NMDC or DMDC. Livers from rats dosed orally with PBS, pH 7.4 (A), 2 mmol/kg DMDC (B), or 2 mmol/kg NMDC (C, D) were collected 24 h after exposure and fixed in 10% buffered formalin and stained with H&E. Animals dosed with NMDC demonstrate infiltration of mononuclear inflammatory cells and hepatocyte necrosis (arrows) around the central vein. Bar = 20 μm.]
not shown). Hepatocytes dosed with 2.5 mM NaCN in the presence of the alternate energy source, fructose, exhibited reduced cytotoxicity at 4 h compared to hepatocytes exposed to NaCN alone, consistent with previous reports (Table 2) (Nieminen et al., 1994). In contrast, dosing hepatocytes with 1.1 mM NaSH in the presence of fructose resulted in enhanced cytotoxicity relative to NaSH alone. The cytotoxicity of both NMDC and DMDC (3 mM) was decreased by the presence of fructose.

**Effect of NMDC, DMDC, and HS⁻ on Isolated Liver Mitochondria Respiration**

To examine the relative potential of NMDC and DMDC to inhibit mitochondrial respiration, freshly isolated rat liver mitochondria were exposed to NMDC (2 mM), DMDC (2 mM), and HS⁻ (4.9 μM). Relative to control mitochondria (37.1 ± 1.3 nmol O₂/min/mg protein) no effect was noted on state 3 respiration for either NMDC (32.9 ± 3.3 nmol O₂/min/mg protein) or DMDC (33.7 ± 3.1 nmol O₂/min/mg protein). In contrast, state 3 respiration was significantly impaired at the much lower concentration of HS⁻ (4.3 ± 0.5 nmol O₂/min/mg protein).

**Effect of Glutathione Depletion and Supplementation on Dithiocarbamate Mediated Cytotoxicity**

To lower intracellular stores of reduced glutathione, hepatocytes were treated with diethylmalate (2 mM) for 30 min prior to exposure to either 2 mM NMDC or 2 mM DMDC. Three h after the addition of the dithiocarbamate, the media was assayed for the presence of LDH as an indicator of viability. Incubation of hepatocytes with DEM, NMDC, or DMDC alone resulted in no significant change in LDH release relative to controls (Table 3). In contrast, coincubation of DEM and dithiocarbamate resulted in significant increases in LDH release for both NMDC and DMDC.

Intracellular levels of GSH were increased in hepatocytes by preincubation with either 10 mM or 30 mM GSE. Measurements of intracellular GSH from the hepatocytes pretreated with 10 mM GSE indicated that intracellular GSH stores were elevated from three to four-fold over the nonsupplemented controls. After 30 min of incubation with GSE, the hepatocytes were washed with culture buffer to remove excess GSE prior to incubation with the dithiocarbamates. Neither incubation with 10 mM nor 30 mM GSE alone resulted in cytotoxicity when compared to the nonsupplemented controls (Fig. 2). The supplementation of intracellular stores of GSH was protective against NMDC (6 mM) induced toxicity at both of the concentrations of GSE examined (Fig. 2). Similarly, preincubation with 30 mM GSE decreased the levels of LDH released from hepatocytes incubated with DMDC (6 mM).

![FIG. 2. Supplementation of intracellular GSH by pretreatment with GSE. Hepatocytes were pretreated with either 10 mM or 30 mM GSE for 30 min. Excess GSE was washed away prior to dosing with either NMDC (6 mM), DMDC (6 mM), or an equal volume of KH culture buffer. Three h after dosing with dithiocarbamates, viability was assessed using the LDH assay with results being expressed as percentage of control cultures not receiving GSE. Data are presented as mean ± SEM values (n = 3). Significantly different results relative to no added GSE are denoted with *(p < 0.01) using the ANOVA test with the Dunnett post test.](image-url)
Dose Dependency of Dithiocarbamate-Mediated Glutathione Depletion in Isolated Hepatocytes

Samples were taken from hepatocyte cultures incubated for 3 h with either NMDC or DMDC and levels of extracellular and intracellular GSH and GSSG were determined as a function of dithiocarbamate concentration (Fig. 3A). NMDC produced a concentration dependent decrease in intracellular and increase in extracellular GSH. Although significant differences relative to controls occurred at the 2 and 4 mM concentrations, no clear trends regarding intracellular and extracellular GSSG were observed for NMDC (Fig. 3B). A different pattern was noted in the GSH and GSSG distribution in hepatocytes exposed to DMDC. Although a concentration dependent decrease in intracellular GSH was also observed for DMDC, no corresponding increase in extracellular GSH was observed (Fig. 3A). Rather, DMDC also produced a dose dependent decrease in extracellular GSH. Whereas NMDC produced little or no effect on intracellular or extracellular GSSG, DMDC produced a concentration-dependent increase in extracellular GSSG with a correspondingly small but significant increase in intracellular GSSG (Fig. 3B).

Comparison of Total GSH, GSSG, and GSMITC Levels in Intact Hepatocytes

After 4 h of incubation, samples were taken from hepatocyte cultures exposed to noncytotoxic doses, as evaluated by LDH release, of either NMDC (2 mM) or DMDC (2 mM) and intracellular and extracellular fractions were analyzed. When compared to time matched controls, the intracellular levels of GSH were significantly decreased by both NMDC and DMDC (Fig. 4). Extracellular levels of GSH were significantly greater for hepatocytes treated with NMDC but no extracellular GSH was detected in samples from the DMDC treated cells. Measurements of GSSG levels indicated that the cells treated with DMDC did have significantly greater levels of both intracellular and extracellular GSSG compared to controls (Fig. 4). No significant differences in GSSG levels were measured in the extracellular fractions of the cells treated with NMDC. Analysis of cellular fractions by SRM indicated that only the cells treated with NMDC contained significant levels of either intracellular or extracellular GSMITC (Fig. 4). The amount of extracellular GSMITC (104.7 ± 3.0) detected using the SRM method was not significantly different from the levels of GSH in extracellular GSH. Whereas NMDC produced little or no effect on intracellular or extracellular GSSG, DMDC produced a concentration-dependent increase in extracellular GSSG with a correspondingly small but significant increase in intracellular GSSG (Fig. 3B).

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(107.3 ± 1.4 pmol) detected by HPLC analysis of the extracellular fractions obtained from NMDC treated cells.

**Effect of Dithiocarbamates on GSH, GSSG, and GSMITC Levels in Perfused Rat Liver**

Rat liver was perfused in situ with the addition of either NMDC or DMDC. Bile and caval fractions were collected at 5-min intervals for analysis of GSH, GSSG, and GSMITC levels. Analysis of the bile fractions as a function of time demonstrated that livers perfused with NMDC (500 μM) secreted elevated levels of both GSH and GSSG relative to controls (Figs. 5A and 5B). In contrast, DMDC (500 μM) exposure did not result in increased levels of GSH being secreted into bile but GSSG was increased to an extent similar to that observed for NMDC. The increases observed in GSH and GSSG for NMDC and GSSG for DMDC coincided with the duration of dithiocarbamate administration and gradually returned to control levels upon cessation of dithiocarbamate perfusion. Analysis of the caval fractions indicated that perfusion of either NMDC or DMDC resulted in decreased levels of GSH and increased levels of GSSG relative to controls (Figs. 5C and 5D). Levels of LDH in the caval effluent were not elevated by either NMDC or DMDC relative to controls indicating that general cellular injury was not occurring.

The dose dependency of GSH and GSSG transport were determined using perfusions of NMDC (80, 200, 500 μM) or DMDC (200, 500, 2000 μM). Biliary GSH increased in a dose dependent manner during NMDC perfusion whereas only at 2000 μM DMDC did the level of GSH present in the bile increase slightly but significantly relative to controls (Fig. 6A). NMDC and DMDC displayed a similar dose response for increased GSSG release into the bile (Fig. 6B). Caval GSH decreased upon perfusion of both NMDC and DMDC with suppression of GSH release greater in the case of NMDC than for DMDC (Fig. 6C). DMDC resulted in increased release of GSSG into the caval fraction in a dose dependent manner (Fig. 6D).
Generation of GSMITC in the bile fractions of NMDC perfused livers increased in a dose dependent manner and corresponded closely to the levels of GSH measured by HPLC (Fig. 7). GSMITC was not detected in the bile fractions collected from control livers nor those perfused with DMDC.

Decomposition Rates of NMDC, DMDC, and PyDTC under Acidic Conditions

NMDC, DMDC, and PyDTC were incubated in buffers at pH 1.0, 4.0, or 8.0 and the loss of absorbance over time was monitored using UV spectroscopy. NMDC and PyDTC exhibited similar rates of decomposition at pH 1.0 and 4.0 (Table 4). DMDC decomposed too rapidly at pH 1.0 to accurately obtain a rate constant but at pH 4.0 the decomposition rate of DMDC was approximately 200 times more rapid than that observed for NMDC. In contrast, at pH 8.0 the rate of decomposition observed for NMDC and DMDC were similar, whereas the decomposition rate of PyDTC was two orders of magnitude less.

DISCUSSION

Oral administration of a single dose of NMDC produced centrilobular hepatocyte necrosis accompanied by inflammation and leakage of AST and ALT. In contrast, hepatotoxicity was not observed following oral administration of an equimolar dose of DMDC in the present study nor from oral administration of a four-fold greater dose of another dialkyldithio-
Methyl dithiocarbamate, and Pyrrolidine Dithiocarbamate at dosing (Johnson being released from NMDC relative to DEDC following oral carbamate, DEDC, in a previous study (Ishiyama et al., 1990). Alternatively, when hepatocytes were exposed directly to each dithiocarbamate in cell culture, NMDC and DMDC exhibited similar cytotoxicities. These results suggest that hepatotoxicity is dependent upon parent dithiocarbamate reaching the liver and that the difference in potency regarding hepatotoxicity for NMDC and DMDC may not be as great when exposure occurs via routes other than per os. Consistent with this interpretation is the ability of DEDC to produce similar hepatic lesions to those observed for NMDC in the rat following sc injection (Ishiyama et al., 1990). The relative stabilities of NMDC and DMDC at acidic pH also appear consonant with this interpretation. Due to the greater stability of NMDC at low pH, more intact NMDC is expected to be absorbed and reach the liver than would occur for DMDC that undergoes acid-promoted decomposition to dimethylamine and carbon disulfide to a greater extent in the stomach. Evidence that this is occurring can be derived from previous studies measuring the levels of the carbon disulfide metabolite, 2-thiothiazolidine-4-carboxylic acid. That study showed lower levels of carbon disulfide being released from NMDC relative to DEDC following oral dosing (Johnson et al., 1996). Although carbon disulfide can also be hepatotoxic, its hepatotoxicity appears dependent upon the induction of hepatic mixed function oxidases and the ip administration route; therefore hepatotoxicity is not expected to occur from the oral doses of DMDC used here (de Matteis and Seawright, 1973; Torres et al., 1980).

Sulfhydryl ion is known to be a potent inhibitor of state 3 mitochondrial respiration due to its ability to inhibit cytochrome c oxidase (Petersen, 1977). Interestingly, NMDC-mediated liver lesions occurred primarily in Zone 3, an area marked by low oxygen levels and thus potentially more sensitive to inhibitors of mitochondrial respiration. However, in the isolated rat liver mitochondria, neither NMDC nor DMDC exerted an effect on mitochondrial respiration although sodium hydrogen sulfide did at much lower concentrations. This may have resulted from the relatively short incubation times used but a similar conclusion is also obtained when the influence of fructose on cytotoxicity was examined using longer incubation times. In hepatocytes, fructose is readily phosphorylated and enters glycolysis where a net gain of ATP is achieved that is sufficient to maintain the cellular processes required for hepatocyte viability (Anundi et al., 1987). As a result, hepatocytes supplemented with fructose are protected against the toxic effects of the cyanide ion, another potent inhibitor of cytochrome c oxidase (Nieminen et al., 1994). Interestingly, although fructose was expected to be cytoprotective for HS− in a manner similar to that observed for cyanide ion, fructose instead increased the toxicity of HS−. This suggests HS− may exert biological effects in addition to inhibition of cytochrome c oxidase in hepatocytes. In comparison, fructose slightly decreased the cytotoxicity of NMDC and DMDC, suggesting that generation of HS− from NMDC is not contributing to hepatocyte cytotoxicity.

Both dialkyl and monoalkyl dithiocarbamates can potentially alter the oxidative state of intracellular thiol pools through direct and indirect mechanisms. Because GSH is the main source of intracellular thiol groups within hepatocytes, the effect of NMDC and DMDC exposure on GSH levels was used to examine potential differences between monoalkyl and dialkyl dithiocarbamates. Incubation of hepatocytes with either NMDC or DMDC resulted in a decrease in the levels of intracellular GSH in a dose-dependent manner. For NMDC, the depletion of intracellular GSH was accompanied by a concomitant rise in extracellular GSH as measured by HPLC. The data support NMDC depletion of intracellular GSH occurring through formation of the GSMITC conjugate and subsequent transport out of the cell (Scheme 1). The dansyl chloride derivatization method employed for HPLC analysis requires a pH of 9.0, a condition that effectively regenerates GSH from GS-MITC (data not shown). Thus, the equivalent quantities of extracellular GSMITC and extracellular GSH detected following incubation with NMDC are consistent with the majority of extracellular GSH measured by HPLC being GSMITC. Formation of the conjugate may be facile or enzyme-catalyzed given the long incubation times in the primary cultures; although the similar results obtained for the perfused livers indicate that GSMITC is formed rapidly in the intact liver.

DMDC exposure also effected a decrease in intracellular GSH but appeared to do so through a different mechanism as the extracellular fraction did not contain elevated levels of GSH. In contrast to NMDC, the levels of GSH in the extracellular fractions obtained from DMDC exposed cells were decreased significantly at the lowest exposure level examined. DMDC appeared to shift the balance of the glutathione pool to the oxidized state as witnessed by elevated levels of GSSG in both the intracellular and extracellular fractions. Based upon the quantity of extracellular GSSG the oxidation of GSH to

**TABLE 4**
Rate of Decomposition of N-Methyldithiocarbamate, N,N-Dimethyldithiocarbamate, and Pyrrolidine Dithiocarbamate at Three pH Conditions

<table>
<thead>
<tr>
<th>Compound</th>
<th>pH</th>
<th>k (1/min)</th>
<th>Half-life (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMDC</td>
<td>1.0</td>
<td>$2.7 \times 10^{-2}$</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>$2.1 \times 10^{-3}$</td>
<td>$3.3 \times 10^{1}$</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>$3.0 \times 10^{-4}$</td>
<td>$2.3 \times 10^{1}$</td>
</tr>
<tr>
<td>DMDC</td>
<td>1.0</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>$4.7 \times 10^{-4}$</td>
<td>1.49</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>$1.7 \times 10^{-4}$</td>
<td>$4.1 \times 10^{1}$</td>
</tr>
<tr>
<td>PyDTC</td>
<td>1.0</td>
<td>$3.2 \times 10^{-2}$</td>
<td>21.7</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>$3.1 \times 10^{-3}$</td>
<td>$2.2 \times 10^{1}$</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>$1.6 \times 10^{-6}$</td>
<td>$4.4 \times 10^{1}$</td>
</tr>
</tbody>
</table>

Note. Decomposition determined by monitoring loss of absorbance at $\lambda_{max}$. N/D, not determined.
GSSG, with subsequent extracellular transport of intracellular GSSH, appeared to account for the majority of the reduction in intracellular GSH observed. Treatment of hepatocytes with DMDC diminished the total amount of glutathione measured in the GSH and GSSG pools (Fig. 4). Possible explanations for this observation include formation of the mixed disulfide of GSH with DMDC or metabolic activation of DMDC followed by subsequent conjugation with GSH, neither of which would have been detected by the analytical methods used (Staub et al., 1995).

Although the exact mechanism of GSSG formation subsequent to DMDC exposure cannot be determined from the experiments presented here, there are at least two potential contributing mechanisms. Typically GSSG is reduced to GSH by GSH reductase in an attempt to maintain and conserve intracellular levels of GSH; however, under conditions of oxidative stress GSSG can be transported extracellularly (Akerboom and Sies, 1989; Oude Elferink et al., 1990). In the present study it is possible that GSSG formation results from an attempt to reduce intracellular disulfides formed between DMDC and GSH or protein thiols in a GSH peroxidase/GSH catalyzed reaction. Another possibility derives from the ability of dithiocarbamates to chelate and then transport redox active metals intracellularly in in vitro systems resulting in the generation of reactive oxygen species (Nobel et al., 1995).

Supplementation with GSE diminished the cytotoxicity of both NMDC and DMDC while depletion of GSH with DEM increased the cytotoxicity of both NMDC and DMDC. Although DEM may exert effects additional to lowering the levels of GSH, the concentration of DEM used in these experiments produced no increase in LDH release relative to controls. Thus, these findings support a role for GSH in protecting hepatocytes from both NMDC and DMDC-mediated cytotoxicity, consistent with a previous report examining DEDC-mediated toxicity in cultured astrocytes (Trombetta et al., 1988). In the case of NMDC, GSH may serve to intercept the reactive methylisothiocyanate intermediate resulting in a less reactive conjugate that can be transported out of the cell before acylation of important protein thiols occurs. It is of interest to note that these glutathione conjugates are reversible and may serve to distribute the isothiocyanate to other locations where it can subsequently acylate other nucleophilic sites in vivo (Bailie and Slatter, 1991; Valentine et al., 1995). In contrast, under the conditions used in the present study, DMDC appeared to exert pro-oxidative effects and thus GSH may be cytoprotective through maintaining the redox status of the cell.

To better assess whether similar differences in the processing of dithiocarbamates occur in vivo, liver perfusion studies were conducted in which the normal architecture of the liver is maintained. Livers perfused with NMDC exhibited a rapid production of GSSG with the GSSG formed being found exclusively in the bile fraction in sufficient quantities to account for the GSH detected in bile by HPLC. This suggests that the export of the GSSG conjugate occurs using transporters located on the biliary surface of the hepatocytes and that the capacity of GSSG generated in the liver to distribute MITC to other organs may be limited (Staub et al., 1995). Another interesting finding during the perfusion of NMDC was that the release of GSH into the caval fraction ceased and then resumed after the cessation of NMDC perfusion. The results obtained for the perfused liver studies using NMDC closely paralleled those obtained for the cultured hepatocyte studies, supporting a similar processing pathway in vivo. For DMDC this was not the case. While DMDC did elicit an increase in the amount of GSSG released into both the caval and bile fractions, the increase of GSSG was not greater than that produced by NMDC at an equimolar dose and was substantially less than the amount of GSSG produced by NMDC. Thus, the greater pro-oxidant effects observed for DMDC in primary cell cultures may have been an artifact of cell culture conditions and similar pro-oxidant effects have been reported for other cell types incubated with DEDC in cell culture (Trombetta et al., 1988; Nobel et al., 1995).

The single alkyl substituent on NMDC increases acid stability and allows for the generation of methylisothiocyanate. These two properties appear to be manifested by enhanced hepatotoxicity following oral exposure for NMDC relative to DMDC or DEDC, both of which are more acid labile and probably decompose to carbon disulfide and parent amine to a greater extent in the stomach. In contrast, differences in the inherent cytotoxicity of NMDC and DMDC toward hepatocytes are not so apparent when presented to hepatocytes directly in primary cultures. Intracellular thiols appear to play a role in the detoxification of dithiocarbamates by hepatocytes.
although different interactions were identified for NMDC and DMDC. The principle pathway observed for NMDC was diminished in the intact liver suggesting that primary cell culture may not accurately reflect in vivo processing and toxicity. The relative role of increased acid stability versus generation of an alkyl isothiocyanate is not clear and needs to be evaluated further to determine if other relatively acid stable dithiocarbamates that do not generate an isothiocyanate, e.g., PyDTC, present a similar enhanced risk of hepatotoxicity. Additionally, determining if the differences observed for NMDC and DMDC are more generally applicable to other mono and dialkyl dithiocarbamates will help formulate structure-activity relationships useful for characterizing the hazards associated with dithiocarbamate exposure.

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