Differences in the toxicities observed for dithiocarbamates have been proposed to result from the influence of nitrogen substitution, oxidation state, and route of exposure. To better characterize the fate of dithiocarbamates in vivo as a function of structure and route of exposure, rats were administered equimolar doses of carbon disulfide (CS$_2$), N-methyldithiocarbamate, pyrrolidine dithiocarbamate, N,N-diethyldithiocarbamate, or disulfiram daily for five days, either po or ip, and sequential blood samples obtained. Protein dithiocarbamates formed by the in vivo release of CS$_2$, parent dithiocarbamate, and protein-bound mixed disulfides were assessed in plasma and hemolysate by measuring toluene thio-carbonate generated upon treatment with toluene-3, 4-dithiol (TdT). To aid in determining the bioavailability of CS$_2$ from the administered dithiocarbamates, the urinary CS$_2$ metabolites, 2-thiothiazolidine-4-carboxylic acid (TTCA) and 2-thiothiazolidine-4-ylcarboxylglycine (TTCG), were also determined. The levels of TdT-reactive moieties detected depended upon both the compound administered and the route of exposure. Parent dithiocarbamates, with the exception of disulfiram, were eliminated from blood within 24 h; but protein associated TdT-reactive moieties persisted and accumulated with repeated exposure, regardless of the route of exposure. N-Methyldithiocarbamate demonstrated the greatest potential to produce intracellular globin modifications, presumably through its unique ability to generate a methylisothiocyanate metabolite. Urinary excretion of TTCA and TTCG was more sensitive than TdT analysis for detecting dithiocarbamate exposure, but TdT analysis appeared to be a better indicator of in vivo release of CS$_2$ by dithiocarbamates than were urinary CS$_2$ metabolites. These data suggest that CS$_2$ is a more important metabolite, following oral exposure, than are other routes of exposure, e.g., inhalation or dermal. In addition, data also suggest that acid stability, nitrogen substitution, and route of exposure are important factors governing the toxicity observed for a particular dithiocarbamate.

Key Words: N,N-diethyldithiocarbamate; N-methyldithiocarbamate; pyrrolidine dithiocarbamate; disulfiram; carbon disulfide; neurotoxicity; hepatotoxicity; toluene-3,4-dithiol; 2-thiothiazolidine-4-carboxylic acid; 2-thiothiazolidin-4-ylcarboxylglycine.

Dithiocarbamates and their disulfides have a broad spectrum of applications in agriculture, industry, and medicine (Haley, 1979; Eneanya et al., 1981; WHO, 1988). Fungicidal, herbicidal, and insecticidal applications can result in high-level acute exposures in either the workplace or home garden, and chronic exposures may occur occupationally or through consumption of residues on food crops. Due to the extensive worldwide use and high potential to be found on food commodities moving through international trade, dithiocarbamates have maintained a priority classification by the FAO/WHO meeting on pesticide residues since 1963 (Vettorazzi et al., 1995). The accelerator and metal-chelating properties of dithiocarbamates are also used in analytical chemistry; rubber production; wastewater treatment; and the production of sugar, pulp, and paper. Intentional human exposures result from medical applications that include alcohol aversion therapy and chelation treatment for nickel intoxication (Brewer, 1993; Jones and Jones, 1984). Additional medical applications are also currently under investigation, including attenuating the dose-limiting toxicity of some antineoplastic agents and increasing the efficacy of 5-fluorouracil for the treatment of colorectal cancer (Bach et al., 2000; Chinery et al., 1997; DeWoskin and Riviere, 1991).

The reported biological effects and metabolites of dithiocarbamates and their disulfides are quite varied. At the cellular level, using in vitro models, dithiocarbamates have been shown to act as pro-oxidants or antioxidants, inhibitors or inducers of apoptosis, enzyme inhibitors, or modulators of transcription (Gessener and Gessner, 1992; Nobel et al., 1995; Tsai et al., 1996). Data acquired from humans and experimental animals have identified neurotoxicity and hepatotoxicity as sequelae to dithiocarbamate exposure (Forns et al., 1994; Frisoni and Di Monda, 1989; Rasul and Howell, 1973). The nitrogen substituents and oxidation state of a dithiocarbamate influence its rate of decomposition, decomposition products, and metabolic pathways. Similarly, due to differences in acid stability and the potential for acid hydrolysis to occur following oral exposure,
the route of exposure may also influence the disposition and biological effects of a dithiocarbamate in vivo. For example, oral administration of the acid labile N,N-diethylthiocarbamate (DEDC) can produce biologically significant amounts of carbon disulfide (CS₂) that manifest in CS₂-mediated protein cross-linking (Johnson et al., 1998). In contrast, parenteral administration of DEDC or oral administration of the more acid-stable dimer of DEDC, disulfiram, is characterized by the generation of S-(diethylaminocarbonyl)cysteine adducts in the absence of CS₂-mediated protein cross-linking (Tonkin et al., 2000, 2003). Additionally, a single alkyl substituent on nitrogen bestows enhanced acid stability and provides for the generation of an alkyl isothiocyanate capable of acylating nucleophilic sites within biological systems (Thompson et al., 2002). Consistent with the diversity of protein modifications observed for the individual dithiocarbamates has been the corresponding identification of separate toxicological targets and lesions. Oral administration of DEDC has been associated with the production of an axonopathy, whereas parental DEDC or po disulfiram produces a segmental demyelination. Relative to its dialkyl analog, N-methylthiocarbamate has been demonstrated to be a more potent hepatotoxicant. Thus, characteristics such as acid stability and generation of an isothiocyanate appear biologically relevant and suggest that both the chemical structure and type of exposure must be taken into consideration when assessing hazards associated with exposure to a particular dithiocarbamate.

The potential for human exposure and toxicity to occur from dithiocarbamates supports a need for a method to evaluate internal exposure and better characterize the fate of dithiocarbamates within biological systems. Because CS₂ is released in vivo by dithiocarbamates, biomarkers for CS₂ including urinary 2-thioxothiazolidin-4-ylcarbonylglycine (TTCG) were also measured. A limitation of these three indices is that they only reflect free CS₂ that has been released from a dithiocarbamate. Carbon disulfide is eliminated rapidly from biological systems and the quantity of CS₂ released can vary considerably for equimolar doses of different dithiocarbamates, thereby hindering its utility as a common dosimeter. Alternative methods have used alkylaion of the free dithiocarbamate in plasma followed by extraction and analysis by gas chromatography (GC) or gas chromatography mass spectrometry (GC/MS) (Cobby et al., 1978). Similarly, a method based upon high-performance liquid chromatography (HPLC) of parent dithiocarbamate has also been reported (Frank et al., 1995). Methods measuring parent or alkylated dithiocarbamate have been useful for determining the pharmacokinetics of certain dithiocarbamates administered intravenously but are not well suited for evaluating occupational exposures and do not integrate cumulative exposures due to the relatively short half-lives of dithiocarbamates in plasma. A method for quantifying CS₂-mediated protein modifications in blood based upon the reaction of thiol carbonyl moieties with toluene-3,4-dithiol (TdT) to generate toluene trithiocarbonate (TTC) has been demonstrated to exhibit a linear response following inhalation and ip exposure to CS₂ (Valentine et al., 1999). Because dithiocarbamates, dithiocarbamate disulfides, as well as CS₂-mediated protein modifications are expected to produce TTC, this method may provide information that is complementary to existing methods for assessing exposures to dithiocarbamates. The biological life observed for TdT-reactive protein modifications also suggests that this assay may be able to integrate cumulative exposures.

To determine if TdT analysis can be used to detect and quantify internal exposure to dithiocarbamates, rats were exposed by oral or ip administration to representative dithiocarbamate compounds. Serial blood samples were then obtained and the plasma and hemolysate components analyzed for free and protein-associated TdT-reactive moieties. For comparison and to aid in evaluating the release of CS₂ by the administered dithiocarbamates, the urinary CS₂ metabolites TTC and 2-thiothiazolidin-4-ylcarbonylglycine (TTCG) were also measured. The dithiocarbamates examined differed regarding their nitrogen substitution, oxidation state, and acid stability in order to assess the effect of these properties on the disposition of dithiocarbamates. The results obtained provide further insight into how the route of exposure and chemical structure of a dithiocarbamate may influence its toxicity. The results also support the utility of the TdT analysis as a complementary method to urinary CS₂ metabolites for analyzing dithiocarbamates within biological systems.

**MATERIALS AND METHODS**

**Chemicals, caution.** Carbon disulfide is volatile, flammable, toxic, and a skin irritant; therefore, gloves and a fume hood should be used when handling this compound. Carbon disulfide was obtained from EM Science, Gibbstown, NJ. Disulfiram [DS; tetraethylthiuram disulfide; bis(diethylthiocarbamoyl)disulfide] and corn oil were obtained from Sigma Chemical Company, St. Louis, MO. N,N-Diethyldithiocarbamic acid, sodium salt, trihydrate (DEDC) was obtained from Fluka Chemical Corporation, Ronkonkoma, NY. Methylamine used for the preparation of N-methylthiocarbamate (NMDC) and 1-pyrrolidinedicarbethioic acid, and ammonium salt (PDTC, NH₄⁺) were purchased from Aldrich Chemicals, Milwaukee, WI. Sodium N-methylthiocarbamate, pyrrolidine dithiocarbamate, sodium salt (PDTC), and TTC were prepared according to published procedures (Kopecky and Smejkal, 1984; Valentine et al., 1995). 2-Thioxothiazolidin-4-ylcarbonylglycine (TCA) was synthesized according to previously published methods (Johnson et al., 1996). Commercial toluene-3,4-dithiol was purified by distillation at 0.5 Torr and stored at −20°C under argon. Ketamine hydrochloride (Ketaset; 100 mg/ml) and xylazine (Sedazine; 100 mg/ml) were obtained from Fort Dodge Laboratories, Incorporated, Fort Dodge, IA. The Bio-Rad Protein Assay kit was purchased from Bio-Rad Laboratories (Hercules, CA).

**Animals and exposures.** All exposures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats, 250–300 g, obtained from Harlan Bioproducts for Science (Indianapolis, IN) were used in this study. Prior to dosing, each animal was weighed and baseline blood samples from tail veins were obtained so that each animal could serve as its own control. The rats were given 1.5 mmol/kg body weight (bw) doses of CS₂, DS, or the sodium salts of DEDC, NMDC, or PDTC (n = 4 per compound). The dithiocarbamate compounds were prepared in 0.1 M phosphate buffer, pH 7.5, and given at 24-h intervals for 5 days, either by oral gavage or intraperitoneal injection at a volume of 1 ml/100 g bw. Carbon disulfide was administered in corn oil such that 0.1 ml was given for each 100 g of bw. Because of low solubility, DS was...
administered as a suspension in 0.1 M phosphate buffer, pH 7.5 containing 1,2-propanediol:buffer (1:2, v:v) that was sonicated and passed through a 19-gauge needle to break up any large particles. This enabled the DS to be administered either through a 19-gauge needle or a gavage tube. Purity of the test compounds was determined spectrophotometrically to be approximately 99%. Freshly prepared solutions were diluted to 0.1 mM using 3 mM phosphate buffer, pH 7.4, the UV spectra of the diluted solutions were compared to published spectra, and the concentrations were determined using the appropriate extinction coefficients (Okiavac et al., 1979). The animals were dosed within one h of preparing the dosing solutions. For urine analysis, rats were housed individually in metabolic cages immediately after dosing and urine was collected for 24 h. Control urine samples were collected at 24 h intervals for 1–2 days prior to dosing so that each animal would serve as its own control and urine samples were kept at −20°C until analysis. Blood samples from tail veins were obtained from anesthetized (100 mg/kg bw of ketamine) animals 24 h after administration of the first and third doses, i.e., prior to administration of the second and fourth doses, respectively. Twenty-four h after administration of the fifth dose, deeply anesthetized animals were euthanized by aortic exsanguination.

**Sample preparation for TTC analysis.** Heparinized blood samples were centrifuged at 4000 × g for 5 min. Plasma was removed and 50-μl aliquots were pipetted into 1.5-ml centrifuge tubes for TTC determination. A 10-μl aliquot of plasma was taken for protein determination. The red cells were resuspended in 5 mM phosphate buffer, pH 7.4 containing 150 mM NaCl, and were centrifuged again at 4000 × g for 5 min. The supernatant was withdrawn while carefully removing as much of the buffy coat as possible. This process was repeated two more times. The red cells were then lysed by adding 700 μl of 5 mM phosphate buffer, pH 7.4 for every 300 μl of red blood cells and centrifuged at 13,000 × g for 40 min. The resulting hemolysate was withdrawn and 100-μl aliquots were pipetted into 1.5-ml centrifuge tubes for TTC determination. A 20-μl aliquot of hemolysate was used for protein determination. To each of the 1.5-ml centrifuge tubes was added 100 μl water, 150 μl 0.2 M phosphate buffer (pH 8.5), 270 μl methanol, and 30 μl of 100 mM TdT in methanol. Samples were incubated for 1 h in a 65°C water bath with occasional mixing. Samples were then cooled overnight at −20°C, followed by centrifugation at 10,000 × g for 10 min, and TTC was analyzed by HPLC as described above.

The relative acid stabilities of the protein modifications Nα-acetylslysine dithiocarbamate and S-(methylaminothiocarbonyl) N-acetylcysteine were evaluated by adding 40 μl of 1N HCl to 4 ml aqueous solutions (100 μM) of each compound and incubated at room temperature. Following incubation periods of 1.5 h and 19 h, duplicate 1-ml aliquots of each sample were neutralized by adding 10 μl of 1 N NaOH. Aliquots (50 μl) of the neutralized solutions were added to 1.5-ml centrifuge tubes, derivatized with TdT, and TTC analyzed by HPLC as described above.

**Urine TTC and TTCG analysis.** Urine samples were centrifuged for 5 min. at 4000 × g and frozen at −20°C prior to TTC and TTCG analysis. The solid-phase extraction steps prior to the chromatographic analysis were performed on multiple samples by passing urine through Waters Oasis extraction cartridge (HLB 1 cc) using a LiChrolut sample preparation unit with drying attachment (EM Science). A Waters 2690 LC with 996 diode array detector and Millennium software were used for the chromatographic analysis. Two columns (a Lichrophere 100RP-8 10 μm 4 × 250 mm column connected to a Whatman Partisil 5 ODS-3 4.6 × 250 mm column) were used and the solvent systems were aqueous 2.5–3% acetonitrile with 1% acetic acid (A) and 95:4:1 methanol-water-acetic acid (B). The elution profile consisted of running 100% A isocratically for 18 min, 100% B for 6 min, and then 100% A again for 10 min before the next injection. To generate a standard curve for the estimation of TTC and TTCG in rat urine samples, varying amounts of TTC and TTCG were added to 0.5-ml aliquots of control rat urine containing a constant amount of T-C. Peak area ratios of TTCG and TTC were plotted against their mole ratios to measure TTCG and TTCG in nmol/0.5 ml urine.

**Determination of creatinine.** The determination of creatinine in the urine samples was performed using the Sigma Diagnostics creatinine kit 555-A (Sigma, St. Louis, MO). This kit uses a modified Jaffe reaction and was scaled down for use as a microplate assay on the Spectramax 250 Plate Reader (Molecular Devices). A standard curve and 3-mg/dl creatinine standard were run with each sample plate. From the nmol of TTC or TTCG in a 0.5 ml urine sample and the creatinine values, TTCA or TTCG was expressed as nmol per mg of creatinine in urine.

**Protein determination of plasma and hemolysate.** The protein concentration of plasma was determined using the Bio-Rad Protein Assay, with bovine serum albumin (BSA) for the standard curve. Hemolysate concentrations were measured by taking the absorbance at 540 nm (λmax of heme) and using the equation Absorbancecondition × 1.14 x dilution = mg/ml protein where 1.14 is a constant. Normally 5–7 mg of protein was present in the 100 μl of hemolysate used for derivatization with TdT.

**Statistical analyses.** Descriptive statistics, F-tests, and Student’s t-test were performed for both unequal and equal variances, using Microsoft Excel software. ANOVA, inclusive of Dunnett’s multiple comparison test for measuring differences between control and treatment groups and Bonferroni’s multiple comparison test (Kapinski, 1991) to measure differences between the CS; treatment group and dithiocarbamate-treated group, were performed using GraphPad Prism software. The level of significance was taken to be p < 0.05, unless otherwise noted.

**RESULTS**

**Animals**

The only treatment group that experienced morbidity was that receiving DS ip, in which the animals demonstrated severe physical depression and were not administered additional doses. Parenteral DS was repeated with a second group of animals with similar results.
Levels of TTC in Plasma from Repeated Dosing

TTC was detected in plasma as a single peak eluting between 5 to 6 min (Fig. 1). Levels of TdT-reactive species in plasma, following administration of CS₂, DS, and DEDC, appeared to increase over the period of ip administration (Fig. 2A). In contrast, no TdT-reactive moieties were observed following ip administration of NMDC or PDTC. TTC could be generated from the plasma obtained following po administration of all compounds tested (Fig. 2B). Significant increases in TTC levels from day 1 to day 5 were observed for all the compounds administered orally and for CS₂ and DEDC administered ip, consistent with accumulation of TdT-reactive moieties over the exposure period. This trend was also observed for DS administration ip, which showed a significant increase in TTC levels from day 1 to day 3.

Levels of TTC in Hemolysate from Repeated Dosing

TTC was detected in the hemolysates for all compounds administered and by both routes of exposure, with the exception of PDTC ip (Fig. 3). There were significant increases in TTC levels from day 1 to day 5 for ip- and po-administered CS₂, NMDC, DEDC, and po PDTC and DS consistent with the accumulation of TdT-reactive moieties with continued exposure. This trend was also observed for DS ip administration, which showed a significant increase in TTC levels from day 1 to day 3.
Nonprotein-Bound vs. Protein-Bound TdT-Reactive Moieties

TTC was absent in the filtrates of treated plasma for all compounds tested with the exception of DS, and to a much lesser extent, of CS₂ (Table 1). TTC was detected in DS-treated plasma filtrates from both routes of administration, consistent with the presence of free parent compound. For oral NMDC and ip DS, a substantial amount of the TdT-reactive moieties detected before filtration of plasma could not be accounted for after filtration. In the hemolysate, protein-bound TdT-reactive moieties represented the entire amount of TTC detected from exposure to the test compounds, except for ip-administered NMDC and DS, for which a substantial amount was lost from filtration.

Evaluation of TTC Recoveries and Acid Stabilities

Percent molar recoveries (SE) of TTC from the TdT analyses of N-acetyllysine dithiocarbamate, S-(diethylaminothiocarbonyl)cysteine, and CS₂ were 100 (6.9), 99.9 (3.5), and 44.1 (0.8), respectively. Percent molar recoveries (SE) of TTC from N-acetyllysine dithiocarbamate and S-(diethylaminothiocarbonyl)cysteine after incubation at lowered pH for 1.5 h were 23.3 (0.5) and 83.0 (1.4), respectively, and after 19 h, incubations were 8.3 (0.9) and 92.2 (2.8), respectively.

Dependence of Urinary TTCA and TTCG on Route of Exposure

The amounts of TTCA and TTCG, excreted in the urine over a 24-h period following a single dose of each test compound, are presented in Figure 4. Significantly greater amounts of TTCA were excreted following oral exposure, relative to ip administration, for each compound. The relative amounts of TTCA excreted in the urine for each of the dithiocarbamates, following oral administration, increased in the order DEDC ≈ DS < NMDC < PDTC; and following ip administration, urinary TTCA increased in the order PDTC < NMDC < DS < DEDC. Smaller quantities of TTCG were excreted relative to TTCA, but a similar relationship was observed regarding the relative amounts of TTCG excreted following oral vs. ip administration. Also, similar to TTCA, significantly greater

<table>
<thead>
<tr>
<th>Compound</th>
<th>Route of admin.</th>
<th>Plasma Filtrate</th>
<th>Plasma Retentate</th>
<th>Hemolysate Filtrate</th>
<th>Hemolysate Retentate</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS₂</td>
<td>ip</td>
<td>3</td>
<td>97</td>
<td>N/D^a</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>po</td>
<td>N/D^a</td>
<td>90</td>
<td>N/D^a</td>
<td>100</td>
</tr>
<tr>
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<td>ip</td>
<td>N/D^a</td>
<td>N/D^a</td>
<td>N/D^a</td>
<td>68</td>
</tr>
<tr>
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<td>po</td>
<td>N/D^a</td>
<td>56</td>
<td>N/D^a</td>
<td>100</td>
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<tr>
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<td>N/D^a</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
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<td>46</td>
<td>N/D^a</td>
<td>37</td>
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<td>po</td>
<td>29</td>
<td>70</td>
<td>N/D^a</td>
<td>100</td>
</tr>
<tr>
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<td>N/D^a</td>
<td>N/D^a</td>
<td>N/D^a</td>
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<tr>
<td></td>
<td>po</td>
<td>N/D^a</td>
<td>84</td>
<td>Trace^b</td>
<td>99</td>
</tr>
</tbody>
</table>

Note. ip, intraperitoneal; po, oral.

^aThe toluene trithiocarbonate product was determined in triplicate as pmol/mg protein for samples obtained after the fifth dose (n = 4).

^bToluene trithiocarbonate in filtrate expressed as percent of total TTC present before filtration.

^cToluene trithiocarbonate in retentate expressed as percent of total TTC present before filtration.

^dN/D, TTC was not detected by assay method used.

^eAmount of TTC found was barely detectable.
amounts of TTCG excretion resulted from oral exposure relative to ip administration, with the exception of CS₂ and DEDC for which there was no difference between oral and ip routes of exposure. Smaller quantities of TTC were generated from hemolysate samples. Similar to plasma, no significant difference was observed in hemolysate for DS administration via the oral or ip routes; and DEDC and PDTC both produced more TTC via the oral route. But unlike plasma, both CS₂ and NMDC produced significantly greater amounts of TTC in hemolysate from ip administration relative to po administration.

**DISCUSSION**

Generation of TTC by toluene-3,4-dithiol utilizes the facile reaction between a thiocarbonyl group and the vicinal sulhydral...
dryl groups of toluene-3,4-dithiol. Previously a similar reaction was used to measure isothiocyanates present in plants (Zhang et al., 1992, 1996). The TdT-reactive structures anticipated following administration of the compounds examined in the present study are represented in Figure 6. As shown, multiple structures can be detected by this assay, including the parent dithiocarbamates and dithiocarbamate disulfides, isothiocyanates, CS₂-derived protein dithiocarbamates, isothiocyanate-derived protein dithiocarbamate esters, and protein-associated mixed disulfides. In contrast to a previous report that used benzenedithiol, incubation of CS₂ with TdT did not result in the quantitative formation of TTC (Zhang et al., 1996). Considering the relatively low yield of TTC from CS₂ and the rapid elimination of CS₂ 

\[ CS_2 \rightarrow CS_2 \] 

in vivo, the major TdT-reactive moiety not associated with protein, i.e., in the filtrate, is expected to be the parent compound administered or possibly mixed disulfides formed with glutathione in hemolysate. Because the number of sulphydryl groups available to participate in mixed disulfides is low relative to protein amino functions and isothiocyanate formation is available only for NMDC, the major protein-associated, TdT-reactive moiety for compounds other than NMDC is expected to be CS₂-derived protein dithiocarbamates.

The reported rates of clearance from plasma for dithiocarbamates predict that parent dithiocarbamate would not be detected at the 24-h sampling time employed in this study (Awni et al., 1994; Cobby et al., 1978; Frank et al., 1995). Results obtained using TdT analysis were consistent with these previous studies; and negligible or no free NMDC, DEDC, PDTC, or glutathione mixed disulfide was detected for either route of exposure, as evidenced by the lack of TTC generated from the filtrates. In contrast, filtrates obtained from rats administered the dithiocarbamate disulfide, disulfiram, contained considerable amounts of TdT-reactive moieties in plasma, consistent with the presence of parent compound in plasma. Delayed clearance of DS relative to DEDC, NMDC, and CS₂ has also been suggested by increasing levels of TTCA excreted in the urine of rats administered sequential oral doses of DS (Johnson et al., 1996).

Production of TTC from the retentate of plasma and hemolysate is consistent with the generation of protein-associated TdT-reactive moieties. As mentioned previously, CS₂-derived protein dithiocarbamates are expected to be the predominant protein associated, TdT-reactive moiety and the data appears consistent with this interpretation. Based on the pKₐ's of the test compounds (Fig. 7), oral administration is expected to favor acid-promoted decomposition with generation of CS₂. Accordingly, oral administration resulted in detectable levels of protein-associated dithiocarbamates for all the compounds. In contrast to the results obtained for oral administration, not all compounds produced protein-associated TdT-reactive moieties after ip administration. Intraperitoneal administration of either NMDC or PDTC did not result in TdT-reactive protein modifications in plasma, and PDTC produced no TdT-reactive moieties on hemolysate proteins as well. These observations are consistent with minimal mixed disulfide formation or decomposition of NMDC and PDTC occurring when exposure to either of these compounds occurs parenterally. Indeed, both NMDC and PDTC are less susceptible to acid-promoted decomposition relative to straight-chain dialkyl dithiocarbamates; and this has been the basis for using PDTC in in vitro biological systems when activities mediated by parent dithiocarbamate are desired (Nobel et al., 1995). The much greater stability of PDTC relative to straight-chain dialkyl dithiocarbamates, and therefore decreased generation of CS₂ at neutral pH, may account for the inability to detect TdT-reactive species following ip administration of PDTC (Thompson et al., 2002; Tonkin et al., 2003). Interestingly though, ip administration of NMDC produced the greatest level of reactive protein modifications in hemolysate, suggesting either intracellular decomposition to amine and CS₂ or, more likely, that decomposition or metabolism to methylisothiocyanate oc-

curred accompanied by dithiocarbamate ester formation, as has been observed for hepatic glutathione (Lam et al., 1993; Thompson et al., 2002). Relative to the other compounds in this study, NMDC is more base-labile and has been shown to undergo facile generation of isothiocyanate at physiological pH, resulting in covalent modification of nucleophilic sites of proteins (Valentine et al., 1995). Although methylisothiocyanate can also react with protein amino functions to produce methylthiourea protein adducts, this covalent modification would not have been detected by the TdT analysis. The data suggest that entrance of NMDC into the red cell favors generation of methylisothiocyanate that is trapped as dithiocarbamate ester, due to the high concentration of globin sulfhydryl functions within the red cell (Rossi et al., 1998).

The present study argues against the dithiocarbamate disulfide, DS, acting only as a source of DEDC in vivo. DS did not produce twice the level of TdT-reactive moieties as DEDC except in plasma following ip administration. Even then, unlike DEDC, a substantial contribution resulted from nonprotein-associated TdT-reactive species. Also, for DS, regarding the protein-associated, TdT-reactive species, it cannot be determined whether the TTC generated resulted from CS$_2$-derived protein dithiocarbamates or from mixed disulfides formed from the reduction of DS by cysteine residues, as has been observed previously (Gessner and Gessner, 1992). Generation of mixed disulfides through this manner would yield only one free DEDC per DS molecule and may contribute to the lower than anticipated amount of CS$_2$ metabolites observed in urine. As mentioned previously, a unique finding for DS was the detection of nonprotein-associated TdT-reactive moieties in the plasma after both oral and ip administration, suggesting the presence of parent compound. The presence of parent compound for DS may have resulted from the greater stability of the disulfide to acid- or base-promoted decomposition, or alternatively, from slower absorption due to its low solubility and administration in a suspension. Similarly, incomplete or delayed absorption may have also contributed to the lower quantities of TdT-reactive moieties and urinary CS$_2$ metabolites observed for DS than would be predicted from the molar content of CS$_2$ in DS.

TTCA is an established urinary metabolite of CS$_2$ and is currently used to monitor exposure in the workplace. Generation of TTCA is thought to proceed through the addition of CS$_2$ to glutathione to generate a trithiocarbonate, followed by removal of glutamic acid and glycine in the mercapturic acid metabolic pathway (Fig. 8) (Bus, 1985). Support for this metabolic pathway has been provided by the identification of TTCG, the cyclic metabolite formed prior to removal of glycine (Amarnath et al., 2001). Although TTCA may also be produced from the addition of CS$_2$ to either cysteinyl glycine or cysteine, the lower concentrations of these two sulfhydryl donors in biological systems suggests their contribution will be considerably less than that of glutathione. In any of these possibilities though, the release of CS$_2$ from parent dithiocarbamate is thought to be required for production of TTCA or TTCG, and thus, these two metabolites are expected to reflect the bioavailability of CS$_2$ from each compound and route of exposure.

The estimated amount of the total dose administered that was recovered in urine within 24 h as TTCA and TTCG ranged from a low of 0.05% for ip PDTC to a high of 1.2% for oral PDTC, indicating that the majority of a dose was processed.

![FIG. 7. Structure and pK$_a$ of the compounds administered: The pK$_a$ values were obtained from Aspila et al., 1973.](https://academic.oup.com/toxsci/article-abstract/76/1/65/1639496)

![FIG. 8. Formation of TTCA and TTCG from CS$_2$.](https://academic.oup.com/toxsci/article-abstract/76/1/65/1639496)
through other pathways, e.g., protein modification, exhaled as CS$_2$ or excreted in urine as other metabolites. All of the compounds in the present study produced more TTCA following their oral administration compared to their ip administration, consistent with a greater portion of a dose undergoing acid hydrolysis within the stomach. Almost contrary to this interpretation, though, is the observation that equal molar oral doses of the more acid-stable dithiocarbamates (i.e., NMDC, PDTC) produced the highest levels of TTCA, and that all of the dithiocarbamates produced more TTCA than CS$_2$ after oral administration. In contrast, examination of TTC yields in plasma after the final oral or ip dose more closely parallel the acid stabilities of the compounds administered with CS$_2$, producing the greatest level of TTC. This suggests that TdT analysis of plasma may be a better parameter for assessing the bioavailability of CS$_2$ from dithiocarbamates than TTCA. In general the quantities were lower, but similar relationships were observed for the T TTCG metabolite among the compounds and routes of exposure, with the exception of DEDC. The results obtained for the urinary metabolites underscore potential limitations for using urinary CS$_2$ metabolites to assess the internal exposure of dithiocarbamates.

Examining urinary TTCA in conjunction with TdT-reactive moieties in plasma and hemolysate demonstrates that the bioavailability of CS$_2$ and distribution of parent dithiocarbamate varies depending on the route of exposure and structure of the dithiocarbamate. The greatest levels of CS$_2$-mediated protein modification resulted from oral exposures consistent with the report that oral administration of DEDC can produce a CS$_2$-mediated distal axonopathy accompanied by covalent protein cross-linking (Erve et al., 1998; Johnson et al., 1998). Examination of the plasma filtrates demonstrated the greatest amounts of free parent dithiocarbamate for disulfiram following oral exposure. It is, therefore, of interest to note that although disulfiram is the dimer of DEDC, when disulfiram is administered orally, it produces a Schwannopathy, as does DEDC administered parenterally, suggesting that dithiocarbamate disulfides can be absorbed intact and then be reduced, serving as a source of systemic free dithiocarbamate after oral exposure (Tonkin et al., 2000). The monoalkyl dithiocarbamate examined demonstrated the ability to produce high levels of intracellular protein modifications in the absence of similar extracellular protein modifications. Thus, the ability of NMDC to generate an isothiocyanate metabolite appears biologically relevant and this property may be responsible for the greater hepatotoxicity of NMDC relative to its dialkyl analog following oral exposure (Thompson et al., 2002).

**Conclusion**

Several limitations and strengths of the two analytical methods are apparent from this investigation. Analysis of urinary TTCA demonstrated greater sensitivity through its ability to detect PDTC exposure following ip administration that, in comparison, produced undetectable levels of TdT-reactive moieties in blood; but, the amount of TTCA excreted in the urine appears to be dependent upon more than just the level of internal CS$_2$ exposure, as demonstrated by the greater levels of TTCA generated from oral administration of dithiocarbamates relative to equimolar doses of CS$_2$. Although the ability of TdT analysis to detect multiple chemical species makes interpretation of the results more complex, this characteristic also provides greater versatility and is more amenable to the detection of all dithiocarbamates within biological systems. Since TdT analysis can detect parent dithiocarbamates, it can potentially also be used to detect dithiocarbamates and their disulfides in other biological and non-biological matrices. To its advantage the analysis of proteins by TdT also appears to reflect cumulative exposures, allows for a longer time period for procuring samples after cessation of exposure, and detects non-CS$_2$-mediated protein modifications produced by dithiocarbamates as compared to urinary TTCA or previous methods used to analyze dithiocarbamates in plasma. Together the two analytical methods, TdT analysis and TTCA excretion, provide complimentary information that has provided further insight into the relative bioavailability of CS$_2$ and parent compound resulting from oral and non-oral exposure to NMDC, DEDC, PDTC and DS that helps to interpret the differences in toxicity observed for these structurally related compounds. The data from this study are also useful for extrapolating data derived from *in vitro* studies on dithiocarbamates to *in vivo* systems.

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**REFERENCES**


