Methemoglobin Formation by Hydroxylamine Metabolites of Sulfamethoxazole and Dapsone: Implications for Differences in Adverse Drug Reactions

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ABSTRACT

Differences in the incidence of adverse drug reactions to trimethoprim-sulfamethoxazole and dapsone may result from differences in the formation, disposition, toxicity, and/or detoxification of their hydroxylamine metabolites. In this study, we examine whether differences in the biochemical processing of sulfamethoxazole hydroxylamine (SMX-NOH) and dapsone hydroxylamine (DDS-NOH) by erythrocytes (red blood cells [RBCs]) contribute to this differential incidence. The methemoglobin (MetHgb)-forming capacity of both metabolites was compared after a 60-min incubation with washed RBCs from four healthy human volunteers. DDS-NOH was significantly more potent (P = .004) but equally efficacious with SMX-NOH in its ability to form MetHgb. The elimination of potential differences in disposition by lysing RBCs did not change the MetHgb-forming potency of either hydroxylamine. At pharmacologically relevant concentrations, greater reduction to the parent amine occurred with DDS-NOH. Maintenance of MetHgb-forming potency was dependent on recycling with glutathione, but no difference in cycling efficiency was observed between DDS-NOH and SMX-NOH. In contrast, the pharmacodynamics of hydroxylamine-induced MetHgb formation were not changed by pretreatment with the glucose 6-phosphate dehydrogenase inhibitor epiprostosterone or by compounds that alter normal antioxidant enzyme activity. Methylene blue, which stimulates NADPH-dependent MetHgb reductase activity, decreased MetHgb levels but did not alter the differential potency of these hydroxylamines. DDS-NOH was also significantly more potent when incubated with washed RBCs from four healthy human volunteers. DDS-NOH was significantly more potent (P = .004) but equally efficacious with SMX-NOH in its ability to form MetHgb. The elimination of potential differences in disposition by lysing RBCs did not change the MetHgb-forming potency of either hydroxylamine. At pharmacologically relevant concentrations, greater reduction to the parent amine occurred with DDS-NOH. Maintenance of MetHgb-forming potency was dependent on recycling with glutathione, but no difference in cycling efficiency was observed between DDS-NOH and SMX-NOH. In contrast, the pharmacodynamics of hydroxylamine-induced MetHgb formation were not changed by pretreatment with the glucose 6-phosphate dehydrogenase inhibitor epiprostosterone or by compounds that alter normal antioxidant enzyme activity. Methylene blue, which stimulates NADPH-dependent MetHgb reductase activity, decreased MetHgb levels but did not alter the differential potency of these hydroxylamines. DDS-NOH was also significantly more potent when incubated with washed RBCs from four healthy human volunteers. DDS-NOH was significantly more potent (P = .004) but equally efficacious with SMX-NOH in its ability to form MetHgb. The elimination of potential differences in disposition by lysing RBCs did not change the MetHgb-forming potency of either hydroxylamine. At pharmacologically relevant concentrations, greater reduction to the parent amine occurred with DDS-NOH. Maintenance of MetHgb-forming potency was dependent on recycling with glutathione, but no difference in cycling efficiency was observed between DDS-NOH and SMX-NOH. In contrast, the pharmacodynamics of hydroxylamine-induced MetHgb formation were not changed by pretreatment with the glucose 6-phosphate dehydrogenase inhibitor epiprostosterone or by compounds that alter normal antioxidant enzyme activity. Methylene blue, which stimulates NADPH-dependent MetHgb reductase activity, decreased MetHgb levels but did not alter the differential potency of these hydroxylamines. DDS-NOH was also significantly more potent when incubated with washed RBCs from four healthy human volunteers.

Tricloprin-sulfamethoxazole and dapsone are viewed by many clinicians as the primary and secondary choice, respectively, for prophylaxis and treatment of Pneumocystis carinii pneumonia in AIDS patients (Gallant et al., 1994). An association with a high incidence of adverse drug reactions (ADRs), however, hampers their clinical usefulness. Limitations in the use of trimethoprim-sulfamethoxazole can be largely attributed to the development of dose-independent hypersensitivity reactions that generally manifest as fever and cutaneous reactions within 7 to 14 days of initiating treatment (Rieder et al., 1989; Cribb et al., 1996a). In certain patients, more severe systemic reactions involving liver, kidney, bone marrow, and heart have also been observed (Rieder et al., 1989; Cribb et al., 1996a). Although dapsone causes similar hypersensitivity reactions, dose-limiting hematologic toxicity characterized by methemoglobinemia and hemolysis also occurs at an equal or greater frequency in patients with AIDS (Lee et al., 1989; Medina et al., 1990; Torres et al., 1993; Beumont et al., 1996) and is the most common dapsone-induced ADR in individuals not infected with human immunodeficiency virus (Jollow et al., 1995).

Experimental data support the hypothesis that bioactivation is critical in the pathogenesis of dapsone- and sulfamethoxazole-induced ADRs. Oxidative metabolism produces reactive hydroxylamine metabolites that are cytotoxic and
capable of binding cellular macromolecules (Rieder et al., 1988; Coleman et al., 1989; Cribb et al., 1991; Cribb et al., 1996b). In the case of sulfamethoxazole, sulfamethoxazole hydroxylamine (SMX-NOH)-induced in vitro cytotoxicity toward peripheral blood mononuclear cells correlates with the development of sulfonamide-induced hypersensitivity reactions (Rieder et al., 1989; Carr et al., 1993). Studies with dapsone similarly link hydroxylamine formation to the development of hemotoxicity (Hjelm and Deverdier, 1965; Glader and Condrad, 1973; Grossman and Jollow, 1988) and idiosyncratic blood dyscrasias (Coleman et al., 1994b; Ahmadsi et al., 1996). Taken together, this evidence suggests that hydroxylamine metabolites mediate ADR development, possibly through spontaneous autoxidation to more reactive nitroso byproducts (Cribb et al., 1991). Although a number of other factors are likely to play an important role, this relationship between hydroxylamine toxicity and ADR development also suggests that arylamines giving rise to the largest amount of hydroxylamine should yield the highest incidence of adverse effects.

Comparison of reports on the in vivo metabolism of these arylamines indicate that 20 to 35% of dapsone is excreted as dapsone hydroxylamine (DDS-NOH) and an unknown fraction is excreted as monoacetyldapsone hydroxylamine (MADDS-NOH) (Coleman et al., 1990). In contrast, less than 3% of sulfamethoxazole is excreted as SMX-NOH (Cribb and Spielberg, 1992; van der Ven et al., 1994). Taking into account differences in normal dosages administered for pneumocystis prophylaxis/treatment, comparable levels of hydroxylamine might be observed, although hydroxylamine metabolite levels of dapsone may still be higher, depending on the formation of MADDS-NOH. Importantly, however, dapsone therapy causes a significantly lower incidence of hypersensitivity reactions than trimethoprim-sulfamethoxazole (Lee et al., 1989; Medina et al., 1990; Pertel and Hirschglick, 1994). One study in particular reported a 57% incidence of hypersensitivity to trimethoprim-sulfamethoxazole but only a 30% incidence to trimethoprim-dapsone (Medina et al., 1990). Regardless of whether sulfamethoxazole forms a comparable or lower amount of hydroxylamine, it produces a significantly higher frequency of idiosyncratic ADRs.

One mechanism that may contribute to this differential sensitivity between sulfamethoxazole and dapsone is a difference in the detoxification of reactive metabolites. Erythrocytes have been shown to be an important site for detoxification of hydroxylamine metabolites (Tingle and Park, 1993) but not for other hydroxylamines (Tingle and Park, 1993). Thus, differential detoxification of hydroxylamine metabolites by erythrocytes [red blood cells (RBCs)] might contribute to the differential incidence of hypersensitivity reactions between dapsone and sulfamethoxazole. The studies described herein were designed to further probe the biochemical processing and detoxification of hydroxylamine metabolites by human RBCs.

Materials and Methods

**Chemicals.** Hydroxylamine metabolites of dapsone and sulfamethoxazole were synthesized as described previously (Rieder et al., 1988; Vage et al., 1994). Product identity was confirmed for each hydroxylamine by NMR and IR spectroscopy. Purity, determined by high performance liquid chromatography (HPLC), was found to be greater than 97%. Sulfamethoxazole, dapsone, epiandrosterone (EPI), NADP, glucose 6-phosphate, 1-chloro-2,4-dinitrobenzene (CDNB), diethyl maleate (DEM), 3-amino-1,2,4-triazole (AT), HEPES, DMSO, and purified human hemoglobin A(2), (ferrous) were obtained from Sigma Chemical Co. (St. Louis, MO). Methylene blue was obtained from Aldrich Chemical Co. (Milwaukee, WI), and the remaining chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), Fisher Scientific (Fair Lawn, NJ), and J.T. Baker Chemical Co. (Phillipsburg, NJ). Solvents obtained from Fisher Scientific (Fair Lawn, NJ) and Curtin Matheson Scientific (Houston, TX) were of HPLC grade.

**Study Subjects.** Healthy human volunteers were recruited from the staff and students of the Department of Pharmaceutical Sciences at Wayne State University. Written informed consent was obtained from all volunteers before collecting 10 to 20 ml of whole blood into heparin-containing Vacutainer (Becton Dickinson; Rutherford, NJ) tubes. The study was approved by the Human Investigation Committee at Wayne State University.

**Preparation of Intact RBCs and RBC Lysate.** Human RBCs were isolated from heparinized whole blood by centrifuging samples at 2200g for 10 min at 4°C. After removal of the plasma and buffy coat layer, RBCs were washed once or twice with phosphate-buffered saline (PBS), pH 7.4. The final RBC pellet was then diluted with HEPES medium (15 mM HEPES, pH 7.4, 125 mM NaCl, 6 mM KCl, 1.2 mM MgSO₄, 1 mM NaH₂PO₄, 1 mM CaCl₂, 10 mM glucose) to yield a 35 to 50% hematocrit. RBC lysate was prepared by diluting isolated RBCs 1:1 with deionized water and then sonicating and vortexing samples to ensure complete cell lysis.

**Methemoglobin Assay.** DDS-NOH and SMX-NOH solutions were prepared in acetone to yield concentrations ranging from 1 mM to 20 mM. Then, 75-μl aliquots were evaporated under a gentle stream of N₂ and samples were cooled to 4°C. Isolated RBCs or RBC lysate was distributed to each tube (75 μl) on ice to ensure a synchronous start time, and incubations were continued for 1 h in a 37°C shaking water bath. The percent of total hemoglobin converted to methemoglobin (MetHgb) was then analyzed using 50 μl from each sample, as described below. All incubations used in generating pharmacodynamic data from intact cells or lysate of human volunteers were performed in triplicate. Incubations in the presence and absence of the various chemical agents described below were performed in duplicate.

Each sample (50 μl) was hemolyzed with 3.95 ml of deionized water and vortexed, and pH was maintained by adding 4 ml of PBS (pH 7.4). MetHgb levels, expressed as a percentage of total hemoglobin in each sample, were then determined according to the method of Fairbanks and Klee (1987). Vehicle-treated control incubations were performed for each subject for proper pharmacodynamic analysis.

**Equilibration of Parent Amines Across RBC Membranes.** Because the instability of hydroxylamine metabolites prevents accurate determination of their movement across biological membranes, equilibration of the parent arylamines was used as an indirect indicator of metabolite flux. RBCs were isolated as described above and then diluted to 50% hematocrit with human plasma or PBS, pH 7.4. Concentrated aliquots of dapsone and sulfamethoxazole in DMSO (1%) were added to yield 200 μM final concentrations. After mixing for 30 sec, samples were placed in a 37°C shaking water bath. At various time points during a 1-h incubation period, 500-μl aliquots were removed for analysis. RBCs were pelleted by centrifugation, and 200 μl of the supernatant was extracted with 3 ml of water-saturated ethyl acetate. After discarding the remaining supernatant, RBCs were lysed with deionized water, sonication, and vortexing, and 200 μl was extracted as described above. The organic layers of all samples were evaporated under a gentle stream of N₂, and the residue was reconstituted in either 200 μl or 100 μl mobile phase, so as to account for sample dilution during the lysis procedure. HPLC analysis was performed according to the method of Coleman et al. (1989). Data was used to calculate ratios of intracellular (RBCs) to extracellular arylamine concentration in each sample. Because steady-state concentrations were observed within 5 min (data not presented)
shown), data shown represent the mean ± S.D. of all time points during the 1-h incubation period.

**RBC-Mediated Formation of Parent Amines.** DDS-NOH and SMX-NOH solutions were prepared in acetone, and 75-µl aliquots were added to dry culture tubes and evaporated under a gentle stream of N2. A 75-µl aliquot of RBCs (50% hematocrit) was added to each tube on ice to ensure a synchronous start to all incubations. After a brief 30-sec equilibration period at 37°C, incubations were continued for 1 h in a shaking water bath and then terminated by placing all samples on ice. RBCs were subsequently lysed with 125 µl of deionized water, sonication, and vortexing and extracted with water-saturated ethyl acetate, and parent amine formation was analyzed by HPLC as described previously (Coleman et al., 1989).

**Effect of Glucose-6-phosphate Dehydrogenase Inhibition.** To investigate the role of NAPDH in the formation of MetHgb by hydroxylamine metabolites, intact RBCs (50% hematocrit) were incubated with 1 mM EPI for 1 h at 37°C. Crude analysis of glucose-6-phosphate dehydrogenase (G6PD) activity, the enzyme responsible for maintenance of intracellular NAPDH levels, was performed in the presence and absence of EPI as described previously (Glock and McLean, 1953). Concentration-MetHgb response relationships were subsequently generated for both DDS-NOH and SMX-NOH after pretreatment with 1 mM EPI or vehicle control (acetone, 0.5%).

**Effect of Reduced Glutathione Depletion.** For experiments aimed at investigating the effect of reduced glutathione (GSH) depletion on hydroxylamine-induced MetHgb formation, GSH was depleted using both CDNB and DEM. Initially, GSH depletion was accomplished by incubating RBCs (50% hematocrit) with 5 mM CDNB for 1 h at 37°C before continued incubation with SMX-NOH or DDS-NOH. Due to the significant baseline level of MetHgb formed under these conditions (~40%), however, incubation time with 5 mM CDNB was reduced to 15 min, followed by washing RBCs twice with PBS to remove all CDNB. Subsequent results were confirmed by depleting GSH with 50 mM DEM for 1 h at 37°C. The extent of GSH depletion relative to vehicle controls was determined by measuring GSH levels using an ion-exchange HPLC assay described previously (Farass and Reed, 1987). For each hydroxylamine, concentration-MetHgb response relationships were generated in both normal and GSH-depleted RBCs.

The effect of hydroxylamine metabolites on RBC GSH and glutathione disulfide levels was also determined by incubating RBCs (50% hematocrit) with 50 µM or 500 µM DDS-NOH or SMX-NOH. These concentrations correspond with the approximate EC50 values for MetHgb formation by DDS-NOH and SMX-NOH, respectively. After 15 min or 1 h at 37°C, 500-µl aliquots were mixed with 100 µl of perchloric acid (70%) and assayed as described above. Simultaneous aliquots were also taken for analysis of MetHgb formation as described above. All incubations were performed in duplicate.

**Effect of Catalase Inhibition.** To examine whether production of reactive oxygen species, particularly hydrogen peroxide, might play some role in the differential MetHgb-forming potency of hydroxylamine metabolites, human RBCs (35% hematocrit) were incubated with 50 mM AT (dissolved in PBS) for 30 min at 37°C. Previous studies have demonstrated that AT significantly inhibits catalase activity, even at lower concentrations (Margoliash and Novogrodsky, 1958; Margoliash et al., 1960). After incubation with or without AT, RBCs were exposed to SMX-NOH or DDS-NOH, and MetHgb levels were analyzed as described above.

**Effect of N-[2-(2-oxo-1-imidazolindinyl)-Ethyl]-N'-Pheny lurea.** The heterocyclic compound N-[2-(2-oxo-1-imidazolindinyl)-ethyl]-N'-phenylurea (EDU) has previously been shown to increase the activity of superoxide dismutase and catalase in lung, liver, and heart tissue (Stevens et al., 1988). EDU also significantly decreases the rate of hydroxyl radical and superoxide anion production from a xanthine-xanthine oxidase system with and without chelated iron, respectively, suggesting that EDU might directly quench reactive oxygen species (Leanderson et al., 1994). Therefore, to further explore the potential role of reactive oxygen species in hydroxylamine-induced MetHgb formation, RBCs (50% hematocrit) were pretreated with 20 mM EDU or vehicle control (1% DMSO) for 5 min at 37°C. Hydroxylamine-induced MetHgb formation was then determined after 1 h as described above.

**Effect of Stimulation of NADPH MetHgb Reductase with Methylene Blue.** Previous studies failed to show an inhibitory effect of DDS-NOH on NADH MetHgb reductase, quantitatively the most important MetHgb reducing enzyme in vivo (Kramer et al., 1972). Whether hydroxylamines affect methylene blue-induced stimulation of NADPH MetHgb reductase activity is unknown. Therefore, RBCs (50% hematocrit) were incubated with or without 15 ng/ml methylene blue for 30 min in a 37°C shaking water bath. Subsequently, hydroxylamine-induced MetHgb formation was assessed after an additional 1-h incubation at 37°C as described above.

**Effect of Hydroxylamines on Purified Hemoglobin.** To compare the inherent reactivity of DDS-NOH and SMX-NOH, purified human hemoglobin A0 (ferrous) was suspended in PBS, pH 7.4, to yield a 50 mg/ml final concentration (corresponds, approximately, with a 15% hematocrit). After incubation with DDS-NOH or SMX NOH, MetHgb formation was analyzed as described above. All incubations were performed in duplicate.

**Statistical Analysis.** Results are presented as mean ± S.D. where appropriate. Concentration-MetHgb response data from each subject were analyzed by fitting mean data for sample replicates to the $E_{\text{max}}$ model. Where appropriate, concentration-MetHgb response relationships were generated in both normal and GSH-depleted RBCs.

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**Statistical Analysis.** Results are presented as mean ± S.D. where appropriate. Concentration-MetHgb response data from each subject were analyzed by fitting mean data for sample replicates to the $E_{\text{max}}$ model (Table 1). Statistical significance was determined using either paired t-tests or one-way analysis of variance, with pairwise comparisons being performed according to the Student-Newman-Keuls method. P < .05 was considered statistically significant in every case.

**Results**

**Comparison of MetHgb Formation and Conversion to Parent Amines.** Comparison of the relative MetHgb-forming capacity of SMX-NOH and DDS-NOH revealed significant
differences between these two hydroxylamine metabolites. Using washed human RBCs from four normal volunteers (all Caucasian men; age range, 24–42 years), a concentration-dependent increase in MetHgb formation was observed with both hydroxylamines (data not shown). EC₅₀ and Eₘₐₓ values were generated for each subject by fitting data according to the Eₘₐₓ model (Table 1). A within-subjects pairwise comparison (Student-Newman-Keuls method) demonstrated a significant difference between EC₅₀ values for SMX-NOH and DDS-NOH (P = 0.004) but not between Eₘₐₓ values. This difference in potency was maintained at all time points from 3 min to 23 h (data not shown). Thus, DDS-NOH is significantly more potent than but equally efficacious as SMX-NOH using intact RBCs.

Differences in the ability of hydroxylamines to cross RBC membranes would obviously affect their ability to induce MetHgb formation. Unfortunately, the instability of hydroxylamine metabolites in aqueous media prevented accurate experimental determination of their flux across biological membranes. Thus, we investigated the ability of the parent amines to equilibrate into RBCs as an indirect indicator of compound flux. Dapsone and sulfamethoxazole concentrations within RBCs and in the extracellular environment were analyzed by HPLC during 1 h. Regardless of whether RBCs were diluted with human plasma or PBS, dapsone concentrations within RBCs were significantly higher than intracellular sulfamethoxazole concentrations (Fig. 1; P < .05). RBC/PBS ratios indicate that unbound sulfamethoxazole equilibrates freely across RBC membranes, whereas dapsone becomes concentrated within RBCs.

Disruption of the membrane barrier, however, should eliminate differences in accessibility to intracellular hemoglobin. Therefore, MetHgb formation induced by SMX-NOH and DDS-NOH was evaluated using RBC lysate from two subjects. A concentration-dependent increase in MetHgb formation was observed in each case (data not shown). Pharmacodynamic parameters derived from data fit according to the Eₘₐₓ model indicate that DDS-NOH is still a significantly more potent, although equally efficacious MetHgb-forming agent, than SMX-NOH (Table 2).

To evaluate the possibility that differences in conversion to the parent amine might contribute to the difference in potency, dapsone and sulfamethoxazole formation were determined after RBC exposure to DDS-NOH or SMX-NOH, respectively. At initial hydroxylamine concentrations resembling that which might be achieved in vivo (10 and 50 µM), dapsone formation in RBC incubations was nearly 20% greater than sulfamethoxazole formation (Fig. 2). Conversion of SMX-NOH to sulfamethoxazole was significantly greater only at the highest concentration (10 mM).

Role of Redox Cycling in Hydroxylamine-Induced MetHgb Formation. Hydroxylamines are regenerated when nitroso species produced during co-oxidation with hemoglobin react with NADPH (Kiese, 1966; Kramer et al., 1972) or GSH (Kramer et al., 1972; Coleman et al., 1994a). We hypothesized that increased efficiency in the regeneration of DDS-NOH might be a key component in its increased MetHgb-forming potency. To test this hypothesis, we determined whether inhibition of G6PD, which normally converts NADP⁺ to NADPH, has any effect on hydroxylamine-induced MetHgb formation. Although significant inhibition of G6PD activity was observed (>90%), only a very minor rightward shift in the concentration-MetHgb response curves for both DDS-NOH and SMX-NOH is apparent (Fig. 3). Prevention of NADPH regeneration did not significantly affect the MetHgb-forming potency of either hydroxylamine metabolite.

In contrast, depletion of GSH with CDNB (5 mM for 15 min; >99% depletion) before incubation with hydroxylamine metabolites produced profound effects (Fig. 4). Although CDNB pretreatment alone caused a significant baseline level of MetHgb formation (Fig. 4A), hydroxylamine potency was significantly decreased in GSH-depleted RBCs compared with cells pretreated with vehicle control. An analysis of percent MetHgb levels minus the appropriate controls (normal or GSH-depleted RBCs, respectively, without hydroxylamine exposure) revealed a significant rightward shift of the concentration-MetHgb response relationship of DDS-NOH and SMX-NOH (Fig. 4B). Importantly, the relative pharmacodynamics of these two hydroxylamines, with DDS-NOH being significantly more potent, remained unchanged. GSH depletion by pretreatment with DEM (50 mM for 60 min; >95% depletion), although not causing a significant increase

![Fig. 1. Distribution of dapsone and sulfamethoxazole into human RBCs.](image)

**Table 2**
Pharmacodynamics of hydroxylamine metabolite-induced MetHgb formation in RBC lysate

Washed human RBCs were lysed with deionized water (1:1 dilution), samples were vortexed and sonicated to ensure complete lysis, and MetHgb formation was determined after 1-h incubation at 37°C. Pharmacodynamic modeling was performed as described in Table 1.

<table>
<thead>
<tr>
<th>Subject</th>
<th>SMX-NOH</th>
<th>DDS-NOH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Eₘₐₓ</td>
<td>EC₅₀</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>µM</td>
</tr>
<tr>
<td>1</td>
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</tr>
<tr>
<td>Mean</td>
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</table>
in MetHgb alone, also yielded similar changes in the pharmacodynamics of both hydroxylamines (data not shown).

Although the incubation of intact human RBCs with DDS-NOH or SMX-NOH caused concentration-dependent increases in MetHgb formation, thiol concentrations were not significantly altered from control levels. After a 60-min exposure, neither hydroxylamine caused a substantial decrease in GSH levels or an increase in GSSG levels relative to controls (Table 3). Similar results were obtained with a limited 15-min exposure, although MetHgb levels were significantly lower at each concentration with this shorter incubation period (data not shown).

Investigation of Potential Role of Reactive Oxygen Species in MetHgb Formation. Although reactive oxygen species, including hydroxyl radicals and hydrogen peroxide, are known to be produced within RBCs exposed to hemotoxic agents, studies suggest varied responses on exposure to similar chemical entities (Jollow et al., 1995). In light of the fact that hydrogen peroxide is a potent MetHgb-forming agent, we hypothesized that differential production of hydrogen peroxide or other reactive oxygen species may play some role in the observed difference in MetHgb formation. To examine the possible role of hydrogen peroxide, human RBCs were pretreated with the catalase inhibitor AT before incubation with DDS-NOH or SMX-NOH for 1 h. AT has previously been shown to produce an immediate reversible inhibition of catalase at high concentrations (Margoliash et al., 1960) and a more slowly developing irreversible inhibition in the presence of low concentrations of hydrogen peroxide (Margoliash and Novogrodsky, 1958). The pretreatment of human RBCs
with high concentrations of AT (50 mM for 30 min) had no effect on the pharmacodynamics of hydroxylamine-induced MetHgb formation (data not shown).

To further test the hypothesis that hydroxylamine-induced production of reactive oxygen species affects MetHgb formation, human RBCs were pretreated with the antioxidant compound EDU. As shown in Fig. 5, EDU also had no effect on MetHgb formation caused by DDS-NOH and SMX-NOH.

**Effect of NADPH MetHgb Reductase Stimulation on MetHgb Formation.** Inhibition of MetHgb reduction is likely to be deleterious toward RBCs exposed to hemotoxic agents or those under oxidant stress. Although one study failed to show an inhibitory effect of DDS-NOH on NADH-dependent MetHgb reductase (Kramer et al., 1972), we are unaware of previous studies investigating the potential effect of hydroxylamines on the NADPH-dependent system, which can be stimulated with methylene blue. To address this issue, human RBCs were pretreated with or without methylene blue (15 ng/ml) before continued incubation with DDS-NOH or SMX-NOH, respectively. As demonstrated in Fig. 6, hydroxylamine-induced MetHgb formation was significantly decreased in methylene blue-pretreated RBCs. Hydroxylamine concentration-MetHgb response curves are shifted to the right of those pretreated with vehicle control (Fig. 6).

This dampening effect of methylene blue treatment, however, was no different in cells that were subsequently exposed to DDS-NOH or SMX-NOH. Thus, neither hydroxylamine demonstrated an inhibitory effect on stimulation of NADPH MetHgb reductase activity.

**Effect of Hydroxylamines on Purified Hemoglobin.** Differences in RBC processing of hydroxylamines do not appear to account for the observed difference in potency between DDS-NOH and SMX-NOH. If this is indeed true, then DDS-NOH should display greater hemotoxicity than SMX-NOH in a cell-free system. To test this hypothesis, purified ferrous hemoglobin Aₐ was incubated with hydroxylamine metabolites for 1 h before determining the percent of hemoglobin converted to MetHgb. Despite their decreased potency in the absence of intact RBC or cell lysate, as demonstrated by the rightward shift of both concentration-MetHgb response curves, DDS-NOH still caused significantly greater levels of MetHgb formation than SMX-NOH (Fig. 7).

**Discussion**

Trimethoprim-sulfamethoxazole causes a high incidence of hypersensitivity reactions that complicate its use for combatting opportunistic infections in patients with AIDS (Gallant et al., 1994; Cribb et al., 1996a). Dapsone causes fewer idiosyncratic reactions (Lee et al., 1989; Medina et al., 1990) but is associated with frequent dose-dependent hemotoxic effects (Lee et al., 1989; Medina et al., 1990; Torres et al., 1993; Beumont et al., 1996). Although the pathogenesis of these ADRs remains unclear, evidence suggests that bioactivation to reactive hydroxylamine metabolites occurs before the manifestation of adverse effects. Despite similar metabolic fates, however, differences in the incidence of ADRs suggest that important differences in the production, disposition, detoxification, and/or reactivity of hydroxylamine metabolites derived from these substrates may occur in vivo. These stud-

### TABLE 3

<table>
<thead>
<tr>
<th>Hydroxylamine</th>
<th>GSH (mM)</th>
<th>GSSG (mM)</th>
<th>MetHgb (%)</th>
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<tr>
<td>Control</td>
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<td>183</td>
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<tr>
<td>DDS-NOH 50</td>
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<td>SMX-NOH 500</td>
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</table>

**Fig. 5.** Effect of EDU on DDS-NOH- and SMX-NOH-induced MetHgb formation. Washed human RBCs (50% hematocrit in HEPES media) were preincubated for 5 min at 37°C with the heterocyclic compound EDU (20 mM) or vehicle control (1% DMSO). EDU has previously been shown to have important antioxidant effects (Stevens et al., 1988; Leanderson et al., 1994). MetHgb formation due to addition of SMX-NOH or DDS-NOH was then determined after an additional 60 min at 37°C. Data represent average of duplicate samples.

**Fig. 6.** Effect of methylene blue on hydroxylamine-induced MetHgb formation. Washed human RBCs (50% hematocrit in HEPES media) were pretreated for 30 min at 37°C with methylene blue (15 ng/ml) or vehicle control. Incubation with SMX-NOH or DDS-NOH was then continued for an additional 60 min at 37°C before the determination of MetHgb formation as a function of hydroxylamine concentration. Data represent average of duplicate samples.
DDS-NOH remained approximately 20-fold lower than EC50 MetHgb-forming potency of DDS-NOH and SMX-NOH. Red differences in distribution could significantly affect the membranes. If hydroxylamine metabolites behave similarly, sulfamethoxazole concentrations were nearly identical in the significantly concentrate within RBCs (Fig. 1). In contrast, rect indicator of hydroxylamine flux, dapsone was shown to and dapsone movement across RBC membranes as an indicator attributed to a potential difference in the entry of DDS-NOH and MADDS-NOH (Table 3) (Kramer et al., 1972, 1994) and be detoxified in a GSH-dependent mechanism that yields the parent arylamine (Cribb et al., 1991; Coleman and Jacobus, 1993). Increased efficiency in the regeneration of DDS-NOH would increase MetHgb formation, whereas increased shunting of the nitroso product of sulfamethoxazole toward the detoxifying pathway would decrease the MetHgb potency of SMX-NOH. Our results support the role of GSH, not NADPH, as the key reducing species responsible for regenerating hydroxylamine metabolites. As has been observed previously, hydroxylamines did not significantly deplete GSH from human RBCs (Table 3) (Kramer et al., 1972; Glader and Conrad, 1973; Scott and Rasbridge, 1973). Any GSH that was consumed in the recycling of hydroxylamines must have been rapidly regenerated. The minor shift in the concentration-MetHgb response curves caused by inhibition of G6PD (Fig. 3) is, therefore, believed to result from the secondary requirement of NADPH for regeneration of GSH. Interestingly, hydroxylamines have been shown to significantly deplete GSH from rat RBCs (Grossman et al., 1992, 1995), which may account for the species difference in sensitivity to hydroxylamine-induced MetHgb formation (Vage et al., 1994; Tingle et al., 1997). The inability of rat RBCs to maintain GSH levels would decrease their ability to efficiently recycle nitroso derivatives back to the hydroxylamine. Although depletion of GSH levels did decrease the MetHgb-forming capacity of DDS-NOH and SMX-NOH, presumably by eliminating the recycling process, it did not eliminate the relative difference in potency (Fig. 4). The lack of difference in cycling efficiency.
therefore precludes this as a contributing factor toward the difference in potency.

Some differences were observed, however, in the conversion of hydroxylamine metabolites to their parent arylamines. At concentrations of DDS-NOH reportedly achieved in vivo (Grossman and Jollow, 1988), substantially greater amounts of dapsone were formed by RBCs (Fig. 2). Again, these data cannot account for the greater MetHgb-forming potency of DDS-NOH, but they might contribute somewhat to the differential incidence of drug-induced hypersensitivity reactions. If under physiologic conditions, an RBC-mediated, GSH-dependent reductive pathway detoxifies more of the reactive metabolites of dapsone, less hydroxylamine would be present to initiate dapsone-induced hypersensitivity reactions.

Indirect evidence also suggested that a free radical mechanism involving hydroxyl radicals and hydrogen peroxide may contribute to MetHgb formation (Kiese, 1966). Reactive oxygen species are produced when hydroxylamines react with hemoglobin in the presence of O2 (Kiese, 1966), although the formation of free radicals may differ with the hydroxylamine metabolite under consideration (Jollow et al., 1995). To investigate whether these differences might contribute to the differential formation of MetHgb, various agents were used to manipulate RBC antioxidant enzyme systems. If reactive oxygen species did play a role, recent studies suggested that catalase should be more important than glutathione peroxidase in protecting RBCs from oxidative stress (Scott et al., 1991a, b; Gaetani et al., 1996). To investigate whether these differences might contribute to the differential formation of MetHgb, various agents were used to manipulate RBC antioxidant enzyme systems. If reactive oxygen species did play a role, recent studies suggested that catalase should be more important than glutathione peroxidase in protecting RBCs from oxidative stress (Scott et al., 1991a, b; Gaetani et al., 1996). However, in our experimental system, pretreatment with the catalase inhibitor AT had no effect on hydroxylamine-induced MetHgb formation. Moreover, although NADPH has been shown to be required as a cofactor for reactivation of catalase complex II (Kirkman and Gaetani, 1984), inhibition of G6PD, which prevents regeneration of NADPH levels, also had no significant effect on MetHgb formation (Fig. 3). Together with previous evidence demonstrating that AT had no effect on DDS-NOH-induced hemolytic anemia (Grossman et al., 1995), our data suggest that catalase does not protect against the hemotoxic effects of DDS-NOH.

To further probe the potential involvement of reactive oxygen intermediates, we also questioned whether stimulation of antioxidant activity might decrease the observed MetHgb formation. The heterocyclic compound EDU, which has been shown to possess several antioxidant properties (see Materials and Methods) (Stevens et al., 1988; Leanderson et al., 1994), had no effect (Fig. 5). Collectively, these data strongly suggest that reactive oxygen species do not play a role in hydroxylamine-induced MetHgb formation. Reactive oxygen species generated during MetHgb formation may instead play a critical role in the development of hemolytic anemia (Rasbridge and Scott, 1973; Scott and Rasbridge, 1973; Jollow et al., 1995), although further comment is beyond the scope of this discussion.

The observed difference in MetHgb-forming potency might also occur if hydroxylamines differentially affect the MetHgb reductase activity that combats oxidative stress and hemotoxic agents (see Fig. 8). Inhibition of the NADH-dependent system that predominates under normal conditions or the NADPH-dependent system stimulated under conditions of oxidative stress might significantly increase MetHgb formation. Our data provide indirect evidence that DDS-NOH and SMX-NOH do not affect stimulation of NADPH-dependent reductase activity with methylene blue (Fig. 6). Together with previous evidence showing that DDS-NOH did not inhibit the NADH-dependent system (Kramer et al., 1972), these data indicate that alterations in MetHgb reductase activity also cannot account for the differences in MetHgb-forming potency.

The inherent reactivity of each metabolite was assessed using a cell-free system. The increased conversion of purified hemoglobin A0 to MetHgb (Fig. 7) suggests that DDS-NOH is inherently more reactive with heme constituents of hemoglobin.


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