Reaction between *S*-Nitrosothiols and Thiols: Generation of Nitroxyl (HNO) and Subsequent Chemistry

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ABSTRACT: *S*-Nitrosothiols have been implicated to play key roles in a variety of physiological processes. The potential physiological importance of *S*-nitrosothiols prompted us to examine their reaction with thiols. We find that *S*-nitrosothiols can react with thiols to generate nitroxyl (HNO) and the corresponding disulfide. Further reaction of HNO with the remaining *S*-nitrosothiol and thiol results in the generation of other species including NO, sulfinamide, and hydroxylamine. Mechanisms are proposed that rationalize the observed products.

S-Nitrosothiols have been proposed to be involved in a variety of biological functions(for a review, see ref *1*). For example, *S*-nitroso species may serve as a nitric oxide (NO)[1] "pool" (*2*), as an intermediate in the bioactivation of organic nitrates and nitrites (*3*), and as a means of altering protein function or activity (*4*). As a result of the emerging physiological importance of *S*-nitrosothiols, an understanding of their biological chemistry and fate becomes vital to understanding the mechanism(s) by which they act. Previous work on the physiological chemistry of *S*-nitrosothiols indicates that they can (a) undergo transnitrosation reactions (*5*−*8*), (b) release NO in the presence of cuprous ion (*9*−*12*), ascorbate (*13*, *14*) or thiols (*14*), and (c) serve as a possible source of nitrosonium (NO^+) or nitroxyl (NO^-) ions (*15*, *16*).

The reaction between *S*-nitrosothiols and other thiols may be an important physiological event due to the typically high levels of thiols in cells [i.e., glutathione may be present at 0.5−10 mM concentrations in mammalian cells (*17*)]. As alluded to above, one possible reaction between *S*-nitrosothiols and thiols is a simple transnitrosation where the NO moiety is directly transferred from one thiol to another. However, this is not the only possible chemical outcome, and indeed a recent report by Singh and co-workers (*18*) indicates that the reaction of *S*-nitrosothiols with thiols is potentially complex and leads to the generation of a variety of species including ammonia (NH_3), NO, nitrous oxide (N_2O), and nitrite (NO_2^-). We have independently examined the reaction between *S*-nitrosothiols and thiols and find that, as indicated by Singh and co-workers (*18*), the chemistry is complex and may involve a series of sequential reactions leading to a multitude of products. Herein, we propose that many of the observed products may result from an initial generation of nitroxyl (HNO), a species previously postulated to be formed from the reaction of *S*-nitrosoglutathione (GSNO) and glutathione (GSH) (*19*).

MATERIALS AND METHODS

Chemicals and Solutions

Angeli's Salt ($Na_2N_2O_3$) was synthesized according to the method of Smith and Hein (*20*). GSNO was synthesized according to the method described by Hart (*21*). GSH, glutathione disulfide (GSSG), and superoxide dismutase (SOD) were purchased from Sigma Chemical Co. (St. Louis, MO). Cyclohexyl mercaptan, sodium cyanoborohydride, *N*-acetyl-L-cysteine, acetic anhydride, diethylenetriaminepentaacetic acid (DTPA), ammonium chloride, and hydroxylamine hydrochloride were purchased from Aldrich Co. (Milwaukee, WI). Dicyclohexyl disulfide was purchased from Lancaster Synthesis Inc. (Windham, NH). All other chemicals, solvents, and reagents were obtained from commercial sources and were of the highest purity available. Authentic NO and N_2O gas was purchased from Matheson Gas Products (Cucamunga, CA). NO was passed through aqueous base before use in order to trap any contaminating NO_2. Unless otherwise noted, all solutions (except those involving SOD) in this study were prepared in 1M pH 7.4 potassium phosphate buffer containing 50 mM DTPA and were adjusted back to pH 7.4 with concentrated KOH after the addition of GSH or GSNO. In experiments containing SOD, DTPA was not used. All GSH and GSNO solutions were prepared and used immediately. Efforts were made to protect GSNO solutions from light during the duration of an experiment. After the completion of reactions, the pH of the resultant solutions was again analyzed and found not to differ by more than 0.2 pH units from the starting 7.4 in all cases.

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[1] Abbreviations: nitric oxide, NO; glutathione, GSH; *S*-nitrosoglutathione, GSNO; nitroxyl, HNO; glutathione disulfide, GSSG; diethylenetriaminetetraacetic acid, DTPA; superoxide dismutase, SOD.

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Reaction Conditions and Procedures

Decomposition of GSNO with GSH. For anaerobic reactions, septum-sealed 3 mL cuvettes containing 1 mL of 2 mM GSNO were degassed by sparging with argon for 5 min. Amounts of 1 mL of sparged GSH solutions (20−200 mM) were then added to the cuvettes via gastight syringe, and the absorbance at 334 nm was monitored. Aerobic reactions were run similarly without argon sparging.

Reaction of GSNO with Angeli's Salt. GSNO decomposition. Angeli's salt is a well-established HNO-donor compound (*22*, and refs therein) and was therefore used to examine the chemistry of HNO with thiols and *S*-nitrosothiols. One milliliter of a 20 mM solution of GSNO was placed in a 3 mL cuvette. To this was then added 1 mL of a freshly prepared solution of 10−200 mM Angeli's salt (total volume 2 mL). GSNO concentration was determined by monitoring the absorbance at 334 nm at 5 min intervals. Since Angeli's salt releases both HNO and NO_2^-, as a control, 1 mL of a 200 mM NO_2^- solution was also added to the GSNO solution.

Reaction of GSNO with Angeli's Salt. NO evolution. To a 25 mL, three-neck round-bottom flask was added 2.5 mg (40 μmol) of the Angeli's salt. The reaction flask was connected to an NO-chemiluminescence detector (described below). Then 3 mL of a 10 mM GSNO solution was injected into the foil-covered flask and NO evolution was monitored (see below). The experiment was repeated using 5 mg (80 μmol) of Angeli's salt.

Reaction of GSNO with GSH. NO Evolution. One milliliter of a 20 mM GSNO solution was injected into a foil-covered 25 mL, three-neck round-bottom reaction flask attached to a chemiluminescence NO detector (described below). Argon was then bubbled into the solution, and NO production from GSNO alone was measured for 5 min. Amounts of 1 mL of 5−200 mM GSH solutions were then injected into the system, and the NO evolution was monitored over time.

NO Evolution from the Reaction between GSH and GSNO. The effect of SOD. A 1 mL solution of 2 mM GSNO was injected into a foil-covered 25 mL, three-neck round-bottom reaction flask connected to an NO chemiluminescence detector (described below). NO evolution from the GSNO solution was monitored for 2 min, after which a 1 mL solution of either 20 mM GSH or 5 mg SOD or both in pH 7.4, 1 M phosphate buffer (without DTPA) was injected into the system. NO evolution was then monitored over time.

Reaction of GSNO + GSH. To 5 mL pear-shaped flasks equipped with septum-capped glass stopcocks and magnetic stirrers was added 1.5 mL of 10, 20, 30, 40, 50, 100, and 200 mM GSH solutions in buffer. For anaerobic reactions, the solutions were degassed by sparging with argon for 10 min via a needle protruding through the rubber septa which capped the stopcocks. A 20 mM solution of GSNO was prepared and degassed by sparging with argon in a septum-capped 50 mL round-bottom flask. Each of the pear-shaped flasks was then covered in foil, and 1.5 mL of the GSNO solution was added via a syringe needle through the stopcock septum. The solutions were then sealed and allowed to react. After 24 h, headspace analysis was conducted for NO and N_2O using procedures mentioned below. The solutions were then purged of NO and N_2O by sparging with argon for 10 min. The sparged solutions were then analyzed for nitrite, hydroxylamine, ammonia, and GSNO using the methods mentioned and described below. For aerobic reactions, the procedures above were repeated with the degassing steps omitted.

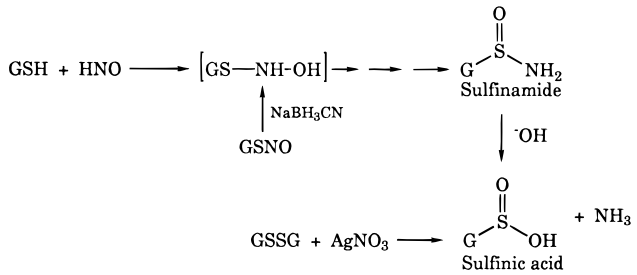
Reaction of NO, Angeli's Salt, HNO_2, and NH_2OH with GSH. To duplicate 5 mL pear-shaped flasks equipped with septum-capped stopcocks were added 3.7 mg Angeli's salt and 2.1 mg $NaNO_2$ or 2.1 mg of hydroxylamine hydrochloride (30 μmol in each case). The flasks were then degassed by sparging with argon for 5 min. Additionally, two pear-shaped flasks were sparged with argon and injected with 0.67 mL (30 μmol) of pure NO gas. For each sample, 3 mL of either degassed pH 7.4 phosphate buffer or degassed 100 mM GSH solution was injected into the reaction flasks. The solutions were mixed for 24 h and analyzed for nitrite, hydroxylamine, and ammonia, using the methods described below.

Reaction between Cyclohexyl Mercaptan and HNO (*Angeli's Salt*). Reactions between cyclohexyl mercaptan and Angeli's salt were carried out in 50 mM Tris buffer, pH 7.4. Typically, an aliquot of a freshly made stock solution of Angeli's salt was added to a buffered solution of the mercaptan to give final concentrations of 1 mM mercaptan and 0.5 mM Angeli's salt. The reaction was allowed to proceed for 30 min at 37 °C. Due to the basic nature of Angeli's salt, the pH of the solution was checked after completion of the reaction and was found not to change. Products were then analyzed by HPLC and GC. Prior to GC analysis, the organic products were extracted with methylene chloride. HPLC analysis was performed directly on the reaction solution.

Reaction of GSH with Angeli's Salt (*HNO*). *Generation of a GSH−HNO Adduct*. Angeli's salt and GSH were taken up in 3 mL of 50 mM Tris buffer, pH 7.4, to give final concentrations of 0.5 and 1 mM, respectively. The solution was then incubated for 30 min at 37 °C. Direct analysis of the reaction mixture by HPLC was performed as described below.

In a similar experiment, GSH and Angeli's salt were reacted with each other following a procedure patterned after Eyer and Schneller (*23*). Thus, 100 mL of deionized water was purged and continuously bubbled with nitrogen. Then 244 mg of Angeli's salt (2 mmol) was added, and the mixture was brought to pH 7.4 using 1 N H_3PO_4. A solution of GSH (305 mg in 100 mL of nitrogen-purged deionized water) was then added to the Angeli's salt solution with the aid of a peristaltic pump. After the addition of the GSH solution was complete, 114 mg of ammonium sulfamate was added to remove nitrite. The pH of the final solution was determined to be 5.1. The mixture was agitated for an additional 45 min and lyophilized. The reaction products were then separated by ion exchange chromatography using a 2.4 cm × 13 cm column of Dowex 2-X8 (acetate form). Five hundred milligrams of the lyophilized reaction mixture in 15 mL of water was applied to the column. A gradient of 500 mL of 1 M formate/pyridine, pH 3, to 500 mL of water at a flow rate of 4 mL/min was used to elute the column. Ninhydrin-positive fractions were pooled and lyophilized. One hundred twenty milligram of the lyophilizate was then suspended in 2 mL of water and centrifuged. Then, 6 mL of acetone was added to the

Scheme 1



supernatant, and the mixture placed in the freezer overnight. The resulting white precipitate was collected and dried in vacuo.

Reduction of GSNO with Sodium Cyanoborohydride. Millimolar quantities of GSNO were taken up in nitrogen-purged water and heated on a steam bath. Small portions of sodium cyanoborohydride were then added until the red color disappeared. The product of this reaction was then isolated by ion exchange chromatography using the procedure described above for the GSH/Angeli's salt reaction. This product had an R_f of 0.3 in the thin-layer chromatographic system described below.

Synthesis of Glutathione Sulfinic Acid. Glutathione sulfinic acid (GS(O)OH) was prepared by oxidation of GSSG with $AgNO_3$ (Scheme 1) (*24*). On paper electrophoresis (vida infra) this compound migrated (toward the cathode) 12.3 cm from the origin [reported 13.5 cm (*28*)].

Hydrolysis of the GSH−HNO Adduct. The product from the reaction of GSH and Angeli's salt (HNO) was hydrolyzed in 0.5 N NaOH at room temperature for 10 min. Analysis of the resulting hydrolysis product was then carried out as described below.

Analytical Procedures

Detection and Quantitation of NO. Possible evolution of NO from reaction mixtures was monitored using a chemiluminescence detection method described previously (*25*). Briefly, argon was passed through a 25 mL, three-neck round-bottom flask, equipped with a gas inlet adapter, a gas outlet adapter, and a septum-sealed inlet, directly into an Antek 720 chemiluminescence NO detector (Houston, TX). For the analysis of liquid samples, reaction mixtures were injected directly into the flask through the septum-sealed inlet, and the NO was sparged from the solution with argon using a glass pipet attached to the gas inlet adapter and bubbling through the solution. Gas samples were simply injected into the headspace of the three-neck flask through one of the septum-sealed inlets and the gases swept directly into the detector.

Detection and Quantitation of N_2O. Headspace analysis for N_2O was performed as previously described (*25*). Briefly, N_2O detection was accomplished using a 5710 Hewlett-Packard gas chromatograph equipped with a thermal conductivity detector, 6 ft × 1/8 in. Porapak Q column, operating at 60 °C and a flow rate of 20 mL/min of helium gas. A standard curve for N_2O was generated from the injection of various quantities of authentic N_2O gas. Quantitation was then accomplished by comparing the detector response (peak area) of the N_2O in the sample to the standard curve. Retention time of N_2O was 2.0 min.

Detection and Quantitation of ammonia. The concentrations of NH_3 were measured with a commercial ammonia diagnostic kit purchased from Sigma (St. Louis, MO). The assay involves measuring the consumption of NADPH during the reductive amination of 2-oxoglutamate by glutamate dehydrogenase.

Detection and Quantitation of nitrite. The concentration of inorganic nitrite was measured using the Griess reaction as described previously (*26*) and was performed using a Beckman DU-30 UV/vis spectrophotometer.

Detection and Quantitation of hydroxylamine. The concentration of hydroxylamine in samples was determined using an indooxine assay described elsewhere (*27*) and was performed using a Beckman DU-30 UV/vis spectrophotometer. Standard curves were prepared using authentic hydroxylamine solutions. Correction for possible decrease color yields due to thiols were accomplished by running samples of known hydroxylamine concentrations with reaction sample aliquots.

Detection and Quantitation of S-nitrosoglutathione. GSNO concentrations were determined using absorbance at 334 nm using a Spectron UVIKON 810 double-beam spectrophotometer.

Chromatographic Analysis of the Products of the Reaction between GSH and Angeli's Salt. Direct analysis of reaction mixtures was accomplished using a Rainin HPX liquid chromatograph equipped with a Beckman Ultrasphere ODS 5 μm, 4.6 mm × 25 cm column and Spectra Physics Spectra 100 variable wavelength UV−vis detector operating at 210 nm. The products were eluted using a 20 mM potassium phosphate, pH 2.7 buffer mobile phase at a flow rate of 1 mL/min. Under these conditions, GSH, GSSG, Angeli's salt, and nitrite eluted at 5.9, 20, 2.4, and 3.45 min, respectively, based on authentic standards. Analysis of the reaction mixture indicated that another major product was formed with a retention time of 2.8 min. Attempted isolation of this product failed due to apparent decomposition resulting from the isolation procedure.

Paper electrophoresis of the reaction product was also performed as described by Cliffe and Waley (*28*), except that Beckman filter paper strips were used and electrophoresis was carried out for 6 h at 7 v/cm. Glutathione derivatives were visualized with ninhydrin (*29*). Reaction mixtures were also analyzed using silica gel thin layer plates developed in butanol/acetic acid/water (8:2:2) as described by Brenner et al. (*30*).

HPLC Analysis of the Products of the Reaction of HNO with Cyclohexyl Mercaptan. The products of the reaction of cyclohexyl mercaptan and HNO were analyzed by HPLC. Thus, an aliquot of the reaction mixture was injected directly into a Rainin HPLC system equipped with a 4.6 mm × 25 cm 5 μM Beckman Ultrasphere reverse phase column, Spectra Physics 100 UV−visible detector operating at 210 nm. The column was eluted using the following gradient: 0−10 min-50% H_2O/50% acetonitrile to 100% acetonitrile, 10−20 min-100% acetonitrile at a flow rate of 1.5 mL/min. Three primary peaks were observed in the chromatogram. Comparison with authentic standards indicated that one peak was the starting material (cyclohexyl mercaptan, 7.3 min) and one peak was the oxidized disulfide product (dicyclohexyl disulfide, 13.5 min). A third peak could not be identified by comparison with standards and had a retention

time of 2.16 min. Note that the attempted isolation of this product resulted in decomposition.

HPLC-MS Analysis of the Products of the Reaction of HNO with Cyclohexylmercaptan. Using a Sciex APCI triple quadrapole API 300 mass spectrometer coupled to a Shimadzu LC-10AD binary liquid chromatograph utilizing a 200 mm × 2.1 mm 5 μM Hewlett-Packard Hypersil column, characterization of the unidentified product of the reaction of cyclohexyl mercaptan and HNO was attempted. Thus, using the mobile phase described above, mass spectral analysis of the unknown chromatographic peak (retention time approximately 2 min) was performed. This product generated the following major ions; m/z = 269, 170, 148, and 122.

GC-MS Analysis of the Products of the Reaction of HNO with Cyclohexylmercaptan. The product from the reaction of HNO with cyclohexyl mercaptan was also examined by GC−MS. Thus, utilizing a Hewlett-Packard 5971A mass selective detector coupled to a Hewlett-Packard 5890A gas chromatograph equipped with a 12 m × 0.2 mm (i.d.) HP1 methyl silicon (0.33 μm liquid phase) column operating at 40 °C for 1 min followed by an increase in temperature (20 °C/min) to a final temperature of 200 °C, the mass spectra of all the reaction components were obtained. Cyclohexyl mercaptan, dicyclohexyl disulfide, and the unknown product had retention times of 2.75, 9.39, and 6.68 min, respectively. The unknown product at 6.68 min generated a spectrum whose major ions were m/z = 147 (6.7%), 83 (99%), 65 (38.5%), 55 (100%), and 41 (27%). Mass spectral analysis of peaks at 2.75 and 9.39 min confirmed their identity as the mercaptan and disulfide on the basis of comparison with published spectra.

RESULTS AND DISCUSSION

Reaction of GSNO with GSH. Previous reports indicate that *S*-nitrosothiols react with thiols in a second-order process with rate constants ranging from 3×10^{-2} M^{-1} s^{-1} for *S*-nitroso-L-cysteine/cysteine (*31*) to 8×10^{-3} M^{-1} s^{-1} for GSNO/GSH (*32*). We also examined the kinetics of the reaction between GSNO and GSH by monitoring, over time, the decrease in absorbance at 334 nm of a 1 mM GSNO solution in the presence of varying concentrations of GSH (10−100 mM). Our results corroborate the previous reports as we find that GSNO reacts with GSH under aerobic conditions with a second-order rate constant of approximately 7×10^{-3} M^{-1} s^{-1}. Under anaerobic conditions, the rate constant was slightly smaller, 5×10^{-3} M^{-1} s^{-1}. Interestingly, the lower rate for the reaction under anaerobic conditions has also been observed to an even greater extent by Singh and co-workers (*18*).

GSNO + GSH; *Generation of HNO*. Thus, it is clear that GSNO reacts with GSH by pathways in addition to simple transnitrosation since, in these experiments, transnitrosation reactions would be nonproductive and simply lead to the regeneration of GSNO. It is conceivable that thiols can react with *S*-nitrosothiols by either nucleophilic attack on the nitrogen of the *S*-nitrosothiol (reaction 1), i.e., the initial step in the transnitrosation process, or via nucleophilic attack on the sulfur atom of the *S*-nitrosothiol (reaction 2). Sulfur atom attack on *S*-nitrosothiols has been postulated previously (for example, ref *16*) and should lead to the generation of the corresponding disulfide and nitroxyl (HNO).[2] HNO is a metastable species that can react with itself at near diffusion controlled rates to generate hyponitrous acid, which then decomposes to nitrous oxide (N_2O) and water (reaction 3) (for a recent review of HNO and related species, see ref *22*).

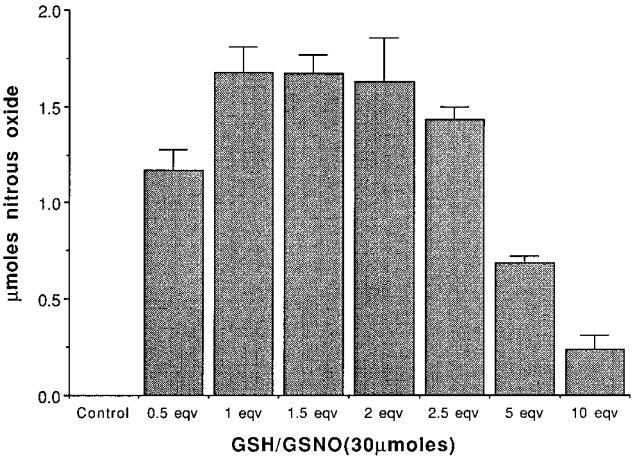


FIGURE 1: N_2O release from the reaction of GSNO (30 μmol) and GSH (15−300 μmoles) in 3 mL of 1M, pH 7.4 phosphate buffer. N_2O was measured after 24 h. Each value represents the mean of two experiments, with error bars representing standard errors of the mean.

$$RSNO + R^*SH \Rightarrow RSH + R^*SNO \quad (1)$$

$$RSNO + R^*SH \Rightarrow RSSR^* + HNO \quad (2)$$

$$HNO + HNO \Rightarrow HONNOH \Rightarrow N_2O + H_2O \quad (3)$$

Thus, the existence of HNO in a reaction is suggested by the detection of N_2O. Indeed, when GSNO is reacted with GSH under anaerobic conditions, we are able to detect the formation of N_2O. The generation of N_2O increased as the concentration of GSH was increased relative to a fixed concentration of GSNO. At a 1:2 GSH/GSNO ratio, maximum levels of N_2O were observed. However, as the GSH/GSNO ratio increased above 2, the amount of N_2O detected decreased significantly (Figure 1). This phenomenon was not due to a reaction between N_2O and GSH since we find that under the conditions of our experiments, they do not react (data not shown). These experiments indicate that the reaction of GSH with GSNO generates a reduced nitrogen species (very likely HNO) which then decomposes to generate N_2O via reaction 3. Moreover, this reduced nitrogen intermediate appears to react with excess thiol,

[2] Nitroxyl can exist in protonated (HNO) and unprotonated forms ($^-$NO). HNO generated by pulse radiolysis was found to have a pK_a of 4.7 (*33*). Thus, it may be expected that HNO would exist primarily in the anionic form at physiological pH. However, $^-$NO can exist either in the singlet or triplet spin state, and these may have different pK_a values. The HNO formed from the reactions described herein is likely to be in the singlet spin state which is expected to have a different pK_a than that of the triplet. We will not make this distinction or address this issue at this time and will generally refer to nitroxyl as HNO with the understanding that it can exist as the deprotonated form as well. Also, nitroxyl has been written as either HNO or NOH. Considering the mechanisms in which it is likely formed herein, we have chosen to depict nitroxyl as HNO with the understanding that it can also exist as NOH.

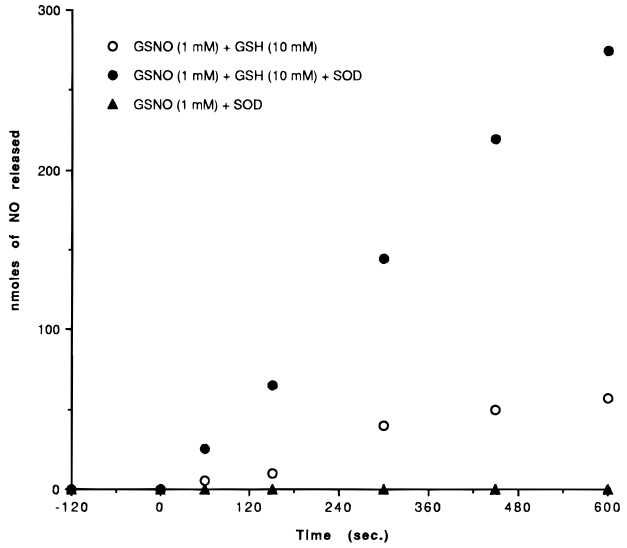


FIGURE 2: NO release from the reaction of 1 mM GSNO with 10 mM GSH (○), 5 mg of SOD (▲) or both GSH and SOD (●). Total volume 1 mL.

thereby shunting this species away from N_2O formation (this process will be discussed further below).

We are aware that the detection of N_2O may not always be indicative of HNO intermediacy since mechanisms for N_2O formation from reduced *S*-nitroso intermediates have been proposed (*34*, *35*). It has been established that HNO is capable of rapidly reducing a variety of one-electron oxidants to give NO (for example, refs *36*, *37*). Therefore, if HNO were in fact being generated in the reaction between GSH and GSNO, the addition of a simple one-electron oxidant should lead to the release of NO via HNO oxidation. We have previously demonstrated that the metalloenzyme CuZn superoxide dismutase (SOD) is capable of reacting with HNO to generate NO (*38*). Thus, when SOD was added to the reaction of GSH and GSNO, a dramatic increase in the release of NO was observed (Figure 2). Similar results were also obtained using methylene blue as the oxidizing agent (data not shown). SOD was chosen as an ideal oxidizing agent for this experiment since the reactive center, a Cu^{2+} ion, is buried deep in a cationic pocket and is likely to be accessible only to small, negatively charged species such as deprotonated HNO, $^{-}$NO.

These results, along with the detection of N_2O, are consistent with the generation of HNO from the reaction of GSH with GSNO (reaction 2). It is likely that reaction 3 (the dimerization of HNO to generate N_2O) is not the only fate of HNO in the presence of GSH and GSNO, since it has been reported that HNO can react with thiols (*35*, *36*, *39*). Accordingly, we examined the reaction of HNO with GSH, one of the predominant species in solution.

Reaction of HNO with GSH. It has already been established that the reaction of HNO with thiols can generate the corresponding disulfide and hydroxylamine (NH_2OH) (for example, see refs *37*, *39*) (reactions 4 and 5).

$$RSH + HNO \Longrightarrow RSNHOH \qquad (4)$$

$$RSNHOH + RSH \Longrightarrow RSSR + NH_2OH \qquad (5)$$

Thus, if HNO were produced in the reaction of GSH with GSNO (reaction 2), it may be expected that the HNO would

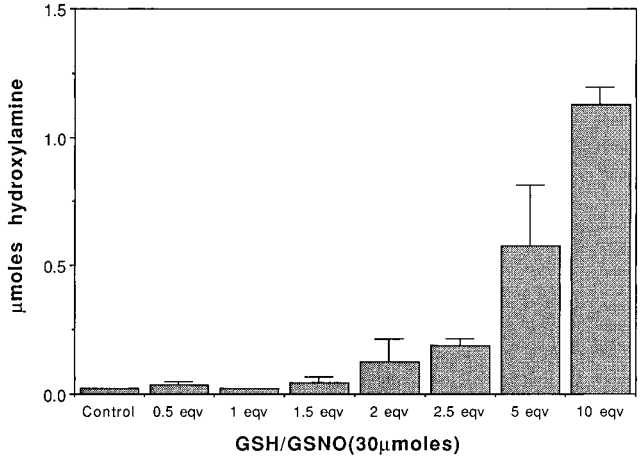


FIGURE 3: NH_2OH production from the reaction of GSNO (30 μmol) and GSH (15−300 μmol) in 3 mL of 1 M, pH 7.4 potassium phosphate buffer. NH_2OH measured after 24 h. Each value represents the mean of two experiments, with error bars representing standard errors of the mean.

be trapped by excess GSH to generate NH_2OH (reactions 4 and 5). Indeed, we find that under anaerobic conditions, NH_2OH is generated in the reaction of GSH with GSNO. Moreover, the yield of NH_2OH increases as the GSH/GSNO ratio increases (Figure 3). However, the yield of NH_2OH is very small (typically <4% based on GSNO, even at a GSH/GSNO ratio of 10). This may partly be due to the known reaction of NH_2OH with HNO to give N_2 and H_2O (*22* and references therein). However, we also considered another mechanistic alternative for the fate of HNO under the conditions of our experiment. On the basis of previous work with analogous nitrosoarenes (*40*−*44*), we surmised that HNO reacts with GSH to form an *N*-hydroxysulfenamide (reaction 4), which can rearrange to generate a sulfinamide (reaction 6, also Scheme 1)

$$RSNHOH \Longrightarrow\Longrightarrow RS(O)NH_2 \text{ (sulfinamide)} \qquad (6)$$

Analysis of the products from the reaction of GSH with HNO indicated that the major sulfur-containing product was not the disulfide but was an unidentified species which we postulated to be the sulfinamide. Independent synthesis of this sulfinamide by sodium cyanoborohydride reduction of GSNO generated a product whose chromatographic properties, using both paper-electrophoresis and silica gel thin-layer chromatography, were identical to that generated in the GSH−Angeli's salt reaction. Hydrolysis of the putative sulfinamide product in aqueous base generated a product with identical chromatographic properties (by paper-electrophoresis and silica gel thin-layer chromatography) as that of the independently synthesized glutathione−sulfinic acid species (data not shown). These results are all consistent with the generation of a sulfinamide from the reaction of GSH and HNO (Scheme 1).

Due to an apparent inherent instability, all attempts to isolate and further chemically characterize the GSH−HNO adduct were unsuccessful. This prompted us to utilize a simpler model thiol compound, viz., cyclohexyl mercaptan, to further explore this chemistry. It was surmised that its reaction with HNO would generate structurally analogous products which would be easier to characterize and would be amenable to GC−MS analysis. Thus, reaction of HNO,

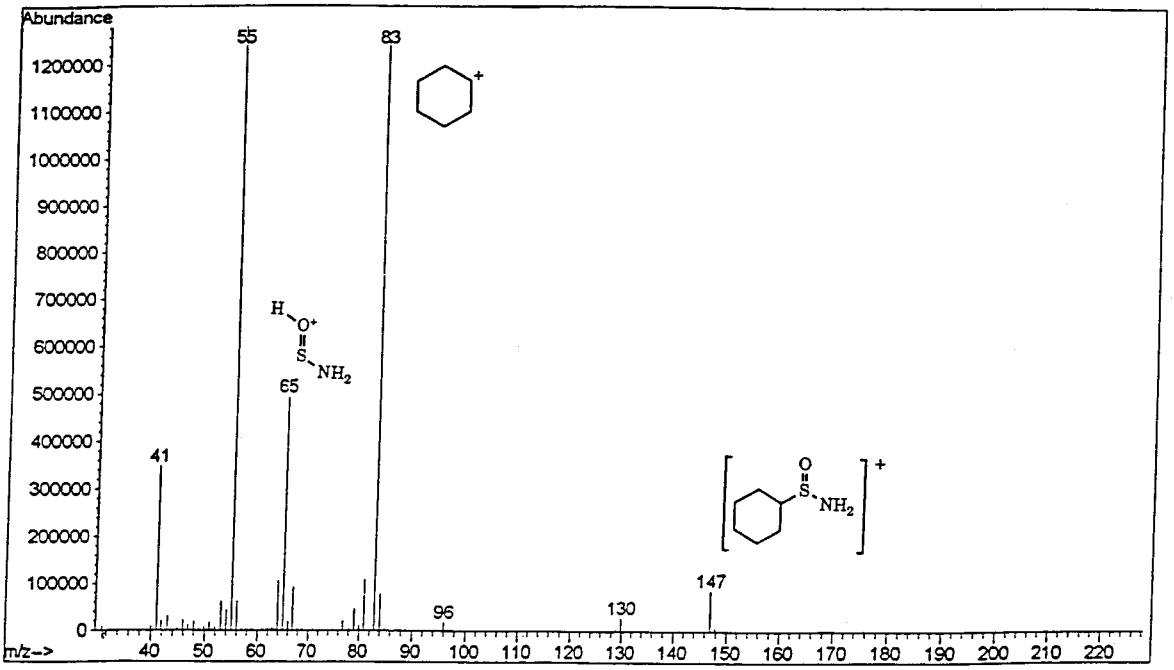
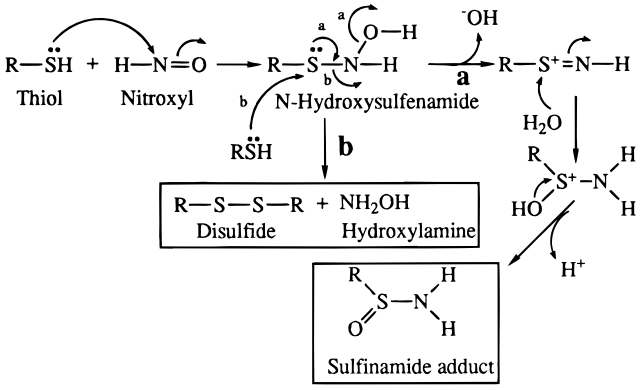


FIGURE 4: Results of the GCMS analysis of the unknown product from the reaction of cyclohexyl mercaptan with HNO.

generated indirectly from Angeli's salt (0.5 mM), with cyclohexyl mercaptan (1 mM) at neutral pH generates two organic products. The minor product had chromatographic properties identical to authentic dicyclohexyl disulfide by both gas and liquid chromatography. Its identity was then confirmed by mass spectral analysis and comparison of the mass spectrum with an authentic standard, the following major ions being observed for both reaction product and standard; m/z = 230 (26%), 148 (60%), 83 (100%), 55 (29.5%), and 41 (8%). The major product was also subjected to GC−MS analysis. The major ions observed were m/z = 147 (6.7%), 83 (99%), 65 (38.5%), 55 (100%), and 41 (27%) (Figure 4). Examination of these data indicates that the unknown product is an adduct between HNO and cyclohexyl mercaptan since a molecular ion at 147 was observed corresponding to 31 amu (HNO) + 116 amu (cyclohexyl mercaptan). The fragment ions (m/z = 83, 55, and 41) are all found in the mass spectrum of authentic cyclohexyl mercaptan indicating that the adduct of HNO and cyclohexyl mercaptan contains a sulfur−nitrogen bond. As was the case with the GSH−HNO adduct, attempts to isolate this adduct were unsuccessful as the compound appears to decompose when it is concentrated.

Further analysis of the unknown product by LC−MS generated the following major ions: m/z = 269, 170, 148, and 122. The APCI mass spectrum confirms that the unknown product is an adduct between HNO and cyclohexyl mercaptan. Under these chromatographic conditions the unknown compound appeared in the elution tail of the void volume components. The void volume should contain all the highly polar components of the reaction mixture including Na^+ (a component of Angeli's salt, 23 amu) and the Tris buffer (tris(hydroxymethyl)aminomethane, 122 amu). It may be expected, therefore, that ions resulting from the combination of Na^+ with the reaction product (m/z = 170) and Tris with the reaction product (m/z = 269) could be observed. Indeed, these ions were seen as well as the Tris ion (122 amu). Thus, the APCI LC−MS analysis corroborates the results of the GC−MS analysis, and the unknown product is clearly an adduct between HNO and cyclohexyl mercaptan.

Scheme 2



On the basis of previous work examining the reaction between aromatic nitroso compounds and thiols (*40*−*43*), it is likely that the nucleophilic thiol function adds to the electrophilic nitrogen of HNO (Scheme 2). The initial addition product, an *N*-hydroxysulfenamide, is then subject to two possible reactions. It can react with another equivalent of thiol to generate the corresponding disulfide and hydroxylamine (Scheme 2, pathway b) or it can spontaneously eliminate hydroxide ion followed by addition of water to the sulfiminium intermediate to give a sulfinamide (Scheme 2, pathway a). It appears that pathway b also occurs since disulfide products are detected.

Thus, HNO appears to be electrophilic and susceptible to nucleophilic attack by GSH (or RSH) to give, presumably, an *N*-hydroxysulfenamide intermediate (reaction 4). This intermediate is then capable of either rearranging to generate the sulfinamide or reacting with another equivalent of thiol to give NH_2OH and disulfide. Clearly, the predominance of one reaction over the other will be a function of the relative concentrations of the species involved as well as the chemical nature of the thiols and *S*-nitrosothiols. Since GSH

concentrations can be in the low millimolar range in cells (*17*) whereas the *S*-nitrosothiol concentrations are in the low micromolar range (*44*), it is likely that much of the HNO formed from the GSH−GSNO reaction will end up being further reduced to NH_2OH. It is worth noting that sulfinamide formation from the thiol/*S*-nitrosothiol reaction has not yet been demonstrated and is only presented as a possible end product if HNO is generated in significant quantities.

Reaction of HNO with GSNO. Another potential fate of HNO generated under the conditions of our experiments (i.e., from GSH + GSNO) is its reaction with excess GSNO. We therefore investigated the reaction of HNO (generated from Angeli's salt) with GSNO. To our knowledge, this reaction has not been extensively studied. There are at least two possible reactions: HNO can react as a nucleophile (probably as ^-NO)[3] and attack either the sulfur or nitrogen atom of GSNO. Attack at the sulfur atom would be nonproductive and simply regenerate the *S*-nitrosothiol (reaction 7). However, attack at the nitrogen atom would lead to an intermediate which could conceivably decompose to generate two NO molecules and the corresponding thiol, GSH (reactions 8 and 9).

$$GSNO + HN^*O \Rightarrow GSN^*O + HNO \quad (7)$$

$$GSNO + HNO \Rightarrow GSN(OH)NO \quad (8)$$

$$GSN(OH)NO \Rightarrow GSH + 2NO \quad (9)$$

If reactions 8 and 9 were occurring, it would be expected that the addition of HNO to a solution of GSNO should result in the loss of GSNO and the generation of NO. Indeed, HNO (generated in situ from Angeli's salt) causes a rapid decrease in GSNO levels (data not shown). Moreover, NO is generated under these conditions and this rate of NO production is dependent on the amount of HNO added (Figure 5). The release of NO from the intermediate formed in reaction 8 is not surprising since this intermediate is structurally similar to a well-known class of NO donors commonly referred to as "NONO-ates" (*46*). It should be noted that reactions 7−9 are speculative and other possible reactions are possible.

The reaction of GSH with GSNO can lead to the generation of HNO. HNO can then react with either excess GSH or GSNO. The reaction of HNO with GSH results in the formation of either disulfide/NH_2OH or sulfinamide. On the other hand, the reaction of HNO with GSNO results in the generation of NO and, indeed, we are able to detect NO from this reaction (Figure 6). This observation is consistent with previous reports (*14*). Thus, HNO possesses amphoteric reactivity in that it is capable of reacting as an electrophile, as evidenced by its reaction with GSH, or as a nucleophile or reducing agent, as evidenced by its reaction with GSNO. The reactivity of HNO in these regards will surely be a function of its protonation state and the species with which it is reacting. Further characterization of the reactivity of HNO is currently being pursued in our laboratories.

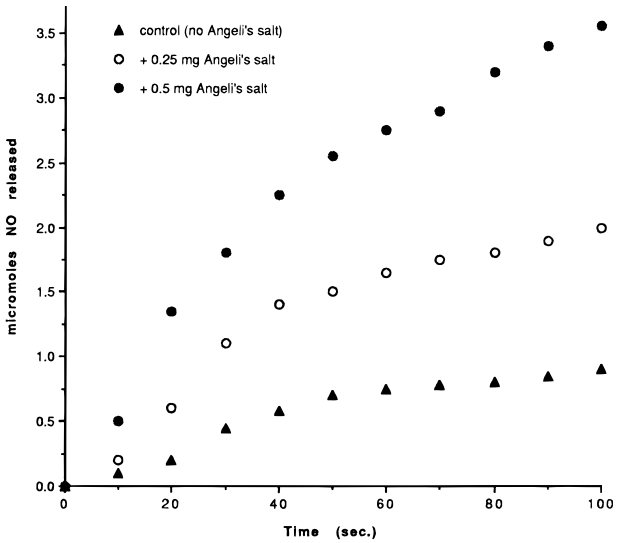


FIGURE 5: NO evolution from the reaction of GSNO with the HNO donor, Angeli's salt. Three millilters of a 10 mM GSNO was injected into a flask containing 0 (▲), 2.5 mg (20.5 μmol) (○), or 5 mg (41 μmol) (●) of Angeli's salt. NO was then sparged out of the reaction mixture and swept into the NO chemiluminescence detector.

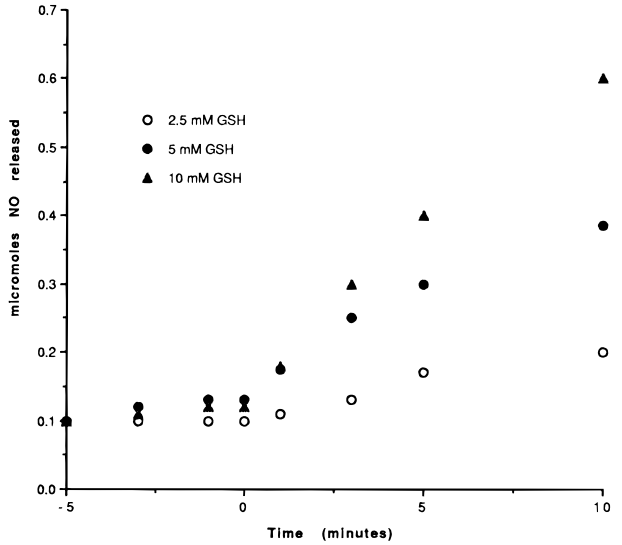


FIGURE 6: NO release from the reaction of GSNO (final concentration = 10 μM) with 2.5 (○), 5 (●), and 10 μM (▲) GSH. One millilter of the GSH solution was injected into the NO detector reaction flask 5 min after the addition of the 1 mL GSNO solution.

Reaction of NO with GSH. As indicated above, NO can be generated from the reaction of GSH with GSNO. NO generation is likely a result of initial HNO formation followed by reaction with GSNO. Thus, the fate of NO under the conditions of our reactions must also be considered. Although it would not be expected that NO will react with GSNO, it has been reported that it can react with thiols. Under aerobic conditions, NO will react with thiols to generate *S*-nitrosothiols (*47*−*49*). Much of this chemistry is oxygen-dependent and involves oxidized nitrogen species such as NO_2 and N_2O_3. Also a recent report by Gow and co-workers indicates that NO may react directly with thiol to produce an intermediate which is oxidized to a *S*-nitrosothiol via reduction of dioxygen to superoxide (O_2^-)

[3] For the sake of discussion, we are viewing ^-NO as a nucleophile. However, based on the lack of nucleophilicity of a similar negatively charged diatomic species, superoxide (O_2^-), in aqueous media (*45*), the apparent nucleophilic reactions of ^-NO may instead involve electron-transfer mechanisms. The proposed products, however, would be the same.

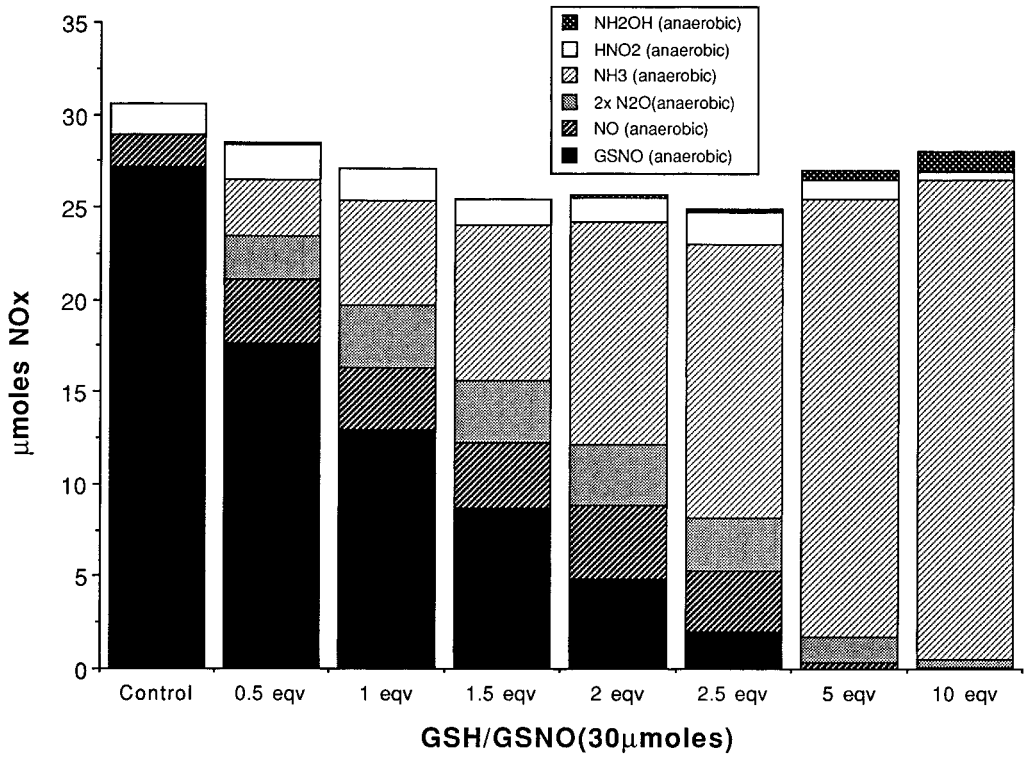
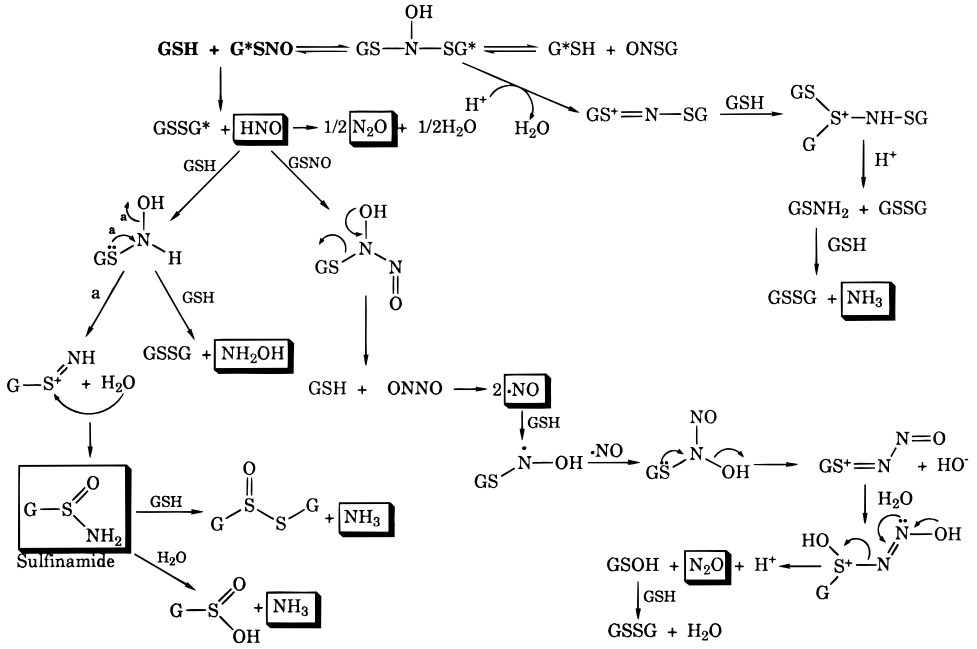


FIGURE 7: Total nitrogen products formed in the anaerobic reaction of GSNO (30 μmoles) with 15−300 μmol of GSH in 3 mL of 1M pH 7.4 potassium phosphate buffer. Each value represents the mean of two experiments.

Scheme 3



(*50*). Under anaerobic conditions, NO also reacts with thiols to give reduced nitrogen species and oxidized thiols (*32*, *34*, *35*, *51*). These observations are fully confirmed, as the reaction of GSH with NO under anaerobic conditions results in the formation of N_2O as the major nitrogen oxide product (data not shown). Several mechanisms for the formation of N_2O from this reaction have been proposed. The mechanism proposed by Pryor and co-workers (*51*) requires a dimerization of an initial GSH−NO radical adduct followed by release of hyponitrous acid (HONNOH), which then decomposes to give N_2O and H_2O (reactions 10−13).

$$GSH + NO \Rightarrow [GSNOH]^{\cdot} \quad (10)$$

$$2\,[GSNOH]^{\cdot} \Rightarrow GSN(OH)N(OH)SG \quad (11)$$

$$GS(OH)N(OH)SG \Rightarrow GSSG + HON{=}NOH \quad (12)$$

$$HON{=}NOH \Rightarrow N_2O + H_2O \quad (13)$$

It has also been proposed that N_2O can be generated from NO trapping of the GSH−NO radical adduct, followed by several rearrangement steps to give N_2O and a sulfenic acid which will react further with GSH to give the GSSG and

H_2O (mechanism detailed later in Scheme 3) (*34*). We favor the latter mechanism since it appears more likely for the GSH−NO radical intermediate to be trapped by NO rather than to react with itself in a second-order process.

Reduction of Nitrogen Oxide Species to Ammonia (*NH_3*). It has been reported that the terminal nitrogen product generated from the reaction of GSH with GSNO is NH_3 (*18*). We have confirmed this finding. That is, NH_3 constitutes one of the major products when GSH is reacted with GSNO and the reaction is analyzed after 24 h. Moreover, the percent of NH_3 generated versus GSNO increases as the relative concentration of GSH increases (Figure 7). Singh and co-workers have proposed that NH_3 is produced through a series of thiol reductions on the intermediate formed initially in the transnitrosation reaction (reactions 14 and 15) (*18*).

$$\mathrm{GSH} + \mathrm{GSNO} \Rightarrow \mathrm{GSN(OH)SG} \quad (14)$$

$$\mathrm{GSN(OH)SG} + 4\,\mathrm{GSH} \Rightarrow\Rightarrow\Rightarrow 3\,\mathrm{GSSG} + \mathrm{H_2O} + \mathrm{NH_3} \quad (15)$$

The NH_3 could not have resulted from the reduction of other reaction products/intermediates since we have found that NO, HNO, NH_2OH, and NO_2^- are not reduced to NH_3 under the conditions of our experiments (i.e., authentic HNO, NO, NH_2OH, or NO_2^- are not readily reduced to NH_3 by GSH, data not shown). However, it is conceivable that NH_3 could have been formed from the sulfinamide product via reactions 16 and 17.

$$\mathrm{GS(O)NH_2} + \mathrm{H_2O} \Rightarrow \mathrm{GS(O)OH} + \mathrm{NH_3} \quad (16)$$

$$\mathrm{GS(O)NH_2} + \mathrm{GSH} \Rightarrow \mathrm{GS(O)SG} + \mathrm{NH_3} \quad (17)$$

Further reduction of the sulfinic acid (GS(O)OH) or the thiosulfinate (GS(O)SG) by excess thiol should result in the generation of disulfides. We are unable to distinguish between NH_3 generation as a result of reaction 14 and 15 versus reactions 16 and 17. This issue is currently under investigation.

Aerobic Conditions. *Nitrite Formation*. It has previously been reported that a significant amount of nitrite (NO_2^-) could be formed from the reaction of GSH with GSNO, even under anaerobic conditions (*18*). In our experiments, we see only very low levels of NO_2^- under anaerobic conditions, likely due to the incomplete deoxygenation of reaction solutions, and NO_2^- formation was consistent regardless of the GSH/GSNO ratios, never exceeding more than about 5% of total nitrogen species. However, under aerobic conditions, NO_2^- formation accounted for as much as 50% of the total nitrogen species (data not shown). Again, NH_3 was a major product, especially at high GSH/GSNO ratios. The generation of NO_2^- likely comes from simple air oxidation of NO since NO was not detected under these conditions.

Reaction of GSH with GSNO. *Material Balance*. Figure 7 shows the material balance for all the nitrogen species for the reaction of GSH with GSNO under anaerobic conditions and at varying GSH/GSNO ratios. In all cases, greater than approximately 85% of all the nitrogen species were accounted for. Notable are the observations that (1) NO is generated under all conditions but its levels are decreased at higher GSH/GSNO ratios, (2) NO_2^- generation is minor, does not vary significantly and is less than or equal to the levels found in control experiments (GSNO without added GSH), (3) NH_3 is a major product, its levels increasing with increasing GSH/GSNO ratios, (4) NH_2OH generation is significant only at the highest GSH/GSNO ratios, and (5) N_2O is formed, possibly through the intermediacy of HNO or via the reaction of NO with excess thiols, and its levels decrease as the GSH/GSNO ratio increases. Possible explanations for these observations are the following. Since HNO is the proposed precursor to NO (reactions 9 and 10), increased GSH levels will result in an increase in the rate of reactions 4−7. This implies that at higher GSH/GSNO ratios, NO levels will decrease and NH_2OH will increase. This is indeed the case. Moreover, if more sulfinamide is formed as a result of increased GSH levels which then releases NH_3 via reactions 16 or 17, it would also be expected that higher GSH levels would give higher levels of NH_3 as well. Again, this is what is observed. The bell-shaped curve for N_2O formation (as a function of the GSH/GSNO ratio) can also be explained by the trapping of HNO by excess thiol. This reaction would shunt the HNO away from N_2O by competing with the HNO dimerization (reaction 3) and by lowering HNO-derived NO levels, thereby decreasing N_2O generation from the reaction of NO with thiols (reactions 10−13). This would lead to an increase in levels of NH_2-OH, sulfinamide, and/or NH_3.

Summary. The chemical reaction between GSH and GSNO is complex due to the multiple secondary reactions that take place and the differing conditions which govern the favorability of each reaction. Scheme 3 summarizes the chemistry that may be occurring.

In biological systems, the GSH/GSNO ratio is one of many factors which must be considered when speculating on the fate of GSNO. It is likely that this GSH/GSNO ratio will be extremely high under physiological conditions, since the intracellular concentration of GSH may be as high as 10 mM (*17*) while the GSNO concentrations would be expected to be much lower. Conditions where thiol/*S*-nitrosothiol ratios may be significantly lower may be found when protein *S*-nitrosothiols are generated and are accessible only to other proximal protein thiols. In these cases it is likely that HNO will be formed and all the subsequent chemistry associated with this species is then possible. Although it has yet to be demonstrated that sulfinamide formation from the reaction of HNO with thiols is physiologically relevant, this does represent an irreversible modification of thiols and may be a mechanism of irreversible inhibition of protein activity. Indeed, the mechanism of irreversible inhibition of aldehyde dehydrogenase by HNO has been postulated to involve the conversion of the active-site cysteine sulfhydryl group to a sulfinamide (*52*).

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