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# FURTHER STUDY ON S-NITROSATION BY NITRITE

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## 1. ABSTRACT

At neutral pH, S-nitrosoglutathione was formed by the reaction of reduced glutathione and sodium nitrite. The degradation of S-nitrosoglutathione, presumably by transnitrosation/denitrosation, was catalyzed by L-cysteine, or CoA-SH. Additionally, from the crude extract of rat brain, one protein with a large molecular mass was nitrosolated with nitrite, and was split into duplet peptides noted in Western blot. Furthermore, the incubation of nitrite with IgG may generate the intermediates of active nitrogen/oxygen species and lead to significant production of gas bubbles.

## 2. INTRODUCTION

Nitric oxide (NO) is a multifunctional signaling molecule (1-5). However, the excessive production of nitric oxide and its derivatives, such as peroxynitrite (PN) (6, 7) and nitrite (1, 8), may cause oxidative stress in which the native activities of various biological molecules may be unfavorably altered through chemical modifications (9-14). Moreover, in severe cases, these changes may ultimately lead to a myriad of diseases (1, 9, 11). Fortunately, to a certain extent, reduced glutathione (GSH) and L-cysteine may be able to reduce such oxidative stress by converting into S-nitrosoglutathione (GSNO) or S-nitrosocysteine (CySNO), subsequently releasing NO to relay signals *in vivo* at appropriate timings and sites (14-17).

Previous studies indicate that under acidic conditions (pH < 3) nitrite can be protonated to form nitrous acid, thereby nitrosolating thiols (1, 15). Conversely, in this investigation we attempted to nitrosolate thiols and intracellular proteins under physiological pH. In addition, we further explored another possible function of nitrite with emphasis on its interaction with IgG.

## 3. MATERIALS AND METHODS

### 3.1. Materials

L-Cysteine (HCl), reduced glutathione (GSH), hemoglobin (Hb, from pig), bovine serum albumin, histone type III-S, mouse IgG, sodium nitrite, sodium dodecyl sulfate (SDS), cysteine-agarose, coenzyme A-agarose, EDTA, EGTA, pepstatin-A, bestatin, leupeptin and transepoxysuccinyl-L-leucylamido (4-guanido)-butane, all were supplied by Sigma Chemical Company, St. Louis, MO, USA. Western blotting reagents, nitrocellulose membranes, anti-rabbit IgG alkaline phosphatase conjugate were obtained from Bio-Rad Laboratories, Hercules, CA, USA. Peroxynitrite and protein G-agarose were purchased from Upstate Biotechnology, Lake Placid, NY, USA. Rabbit polyclonal anti-S-nitrosocysteine was provided by Calbiochem-Novabiochem Corporation, San Diego, CA, USA. Male CD rats (approximately 200g) Crl:CD(SD) were obtained from Charles River Laboratories, Wilmington, USA. Fischerbrand culture tubes and disposable pipette tips were supplied by Fischer Scientific, Pittsburgh, PA, USA.

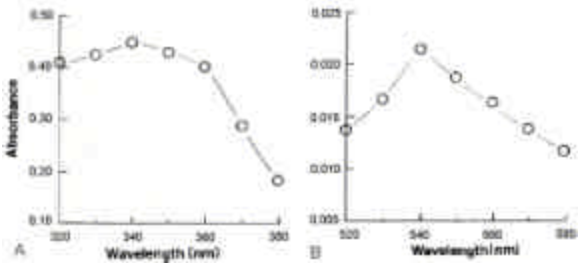


**Figure 1.** Absorption spectra of S-nitrosoglutathione (GSNO) formed in nitrite solution. Incubation mixtures were in a final volume of 300 µl of 75 mM potassium phosphate buffer (pH 7.2) containing 20 mM sodium nitrite and 30 mM GSH. The reactions were carried out at 25°C for 5 min and absorbance was monitored around 340 (A) and 540 nm (B). The values presented had been corrected for the basal (control) values in the presence of nitrite alone.

### 3.2. Methods

Distilled/deionized water, which may still contain trace metal cations, was used to prepare all solutions. The stock solutions of reduced glutathione (GSH), L-cysteine and nitrite were all prepared in 1 M potassium phosphate buffer (pH 7.0 and 7.2). Prior to incubation, the buffer was not "deaired" (e.g., to drive off dissolved air from the solution via saturation with an inert gas such as argon). The products of the S-nitroso compound were monitored around 340 and 540 nm using a Bio-Tek µQuant (MQX200) microplate spectrophotometer.

### 3.3. Degradation of nitrosoglutathione (GSNO)

Approximately 6 ml suspension of agarose, agarose-CoASH or agarose-cysteine were pre-washed and re-suspended in the same volume of 50 mM potassium phosphate buffer (pH 7.2). Reactions were then performed by incubating the above mentioned suspended agarose samples with 5 mM nitrosoglutathione for 2 hours at 25°C. The agarose gels were removed by filtration after incubation, and the absorbances of filtrates were measured.

### 3.4. Cytosolic crude extract from rat brains

The cerebral cortex from two rat brains (approximately 0.8 g) was homogenized using a teflon-glass homogenizer in 2.4 ml of 50 mM potassium phosphate buffer (pH 7.2) containing 1 mM various protease inhibitors [pepstatin-A, bestatin, leupeptin and transepoxysuccinyl-L-leucylamido (4-guanido)-butane]. Homogenates were then centrifuged at 25,500g for 5 min at 4°C. The supernatants were collected as crude extracts. Then, the crude extract was incubated in 5 ml of protein G-agarose for 2 hours at 4°C, followed by the retention of protein G (on filter paper) by filtration. The resulting filtrate was incubated with 30 mM L-cysteine (HCl) for 2 hours at 25°C, followed by dialysis (10 kDa-cut off) overnight against 50 mM potassium phosphate buffer (pH 7.2) containing 1 mM EDTA and EGTA. Finally, the dialyzed sample containing cytosolic protein was used for nitrosation and subsequent Western blot analysis.

### 3.5. Nitrosation of cytosolic proteins

Incubation was in 100 µl of 50 mM potassium phosphate solution (pH 7.2) containing 120 µg of brain cytosolic crude extract (from the above mentioned procedure) and varied amounts of sodium nitrite or peroxynitrite (PN). The incubation was performed for 45 min at 30ºC with gentle and constant shaking, followed by subjecting 25 µl of each incubated sample to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (12%).

### 3.6. Western blot analysis of nitrosolated cytosolic protein

With minor modifications, the experimental procedures of Western blot basically followed those from past studies (18, 19). Bio-Rad's MiniPROTEIN 3 Cell was employed for both SDS-PAGE (20) and protein blotting (to the nitrocellulose membrane). Experiments required pre-cooled tank buffer and prefrozen Towbin transfer buffer. Anti-S-nitrosocysteine was used as the primary antibody (0.05 µg/ml) (20), and anti-rabbit IgG-AP conjugate (21) as the secondary antibody.

### 3.7. Gas production by the incubation of nitrite with proteins

Incubation mixtures were in 2 ml of 250 mM potassium phosphate buffer (pH 7.1) containing 100 µg protein and 250 mM sodium nitrite solution. The reaction was initiated by gentle and brief agitation, followed by incubation without shaking for up to 3 hours at 25°C. Additionally, each reaction was in a Fischerbrand culture tube (13 X 100 mm) with a 100 µl pipette tip left inside. Finally, the amount of resulting gas bubbles, which adhered around the exterior wall of pipette tips, was visually compared among incubated samples.

## 4. RESULTS AND DISCUSSION

The cytosol of most cells may exhibit a pH at about 7.2 (22). At this cytosolic pH, a pink-orange product was generated when nearly saturated nitrite in 1 M potassium phosphate buffer was mixed with nearly saturated GSH in the same buffer. After this colored compound was precipitated by the addition of acetone and redissolved in 75 mM potassium phosphate buffer (pH 7.2), it displayed peak absorbance around 340 and 540 nm (data not shown). These characteristics indeed indicate the product was S-nitrosoglutathione (GSNO). Moreover, a similar pink-orange product was also observed when GSH was replaced by L-cysteine. These initial findings provided a basis for the following detailed experiments.

The incubation of 30 mM GSH with 20 mM nitrite solution yielded unique product(s), which exhibited peak absorbance around 340 and 540 nm (Figures 1 A and B, respectively). Absorbance around 340 nm (Figure 1A) was much more intense than that of 540 nm (Figure 1B). The pH of the incubation mixture was maintained at 7.2 by using 75 mM potassium phosphate buffer throughout this experiment. Therefore, GSNO was indeed generated at physiological pH in comparison with experiments under acidic conditions (4). Moreover, the formation of nitrosocysteine (CySNO) and GSNO was achieved in the

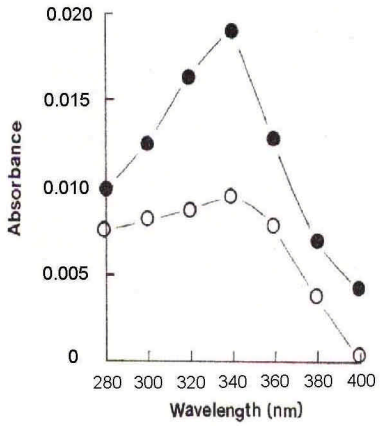


**Figure 2.** Absorption spectra of S-nitrosocysteine (CySNO, •) and S-nitrosoglutathione (GSNO, o) formed in nitrite solution. Incubation mixtures were in a final volume of 300 µl of 75 mM potassium phosphate buffer (pH 7.0) containing 1 mM sodium nitrite and 10 mM GSH or L-cysteine. The reactions were carried out at 25°C for 5 min, and absorbance was monitored around 340 nm. The values presented had been corrected for the basal (control) values in the presence of nitrite alone.

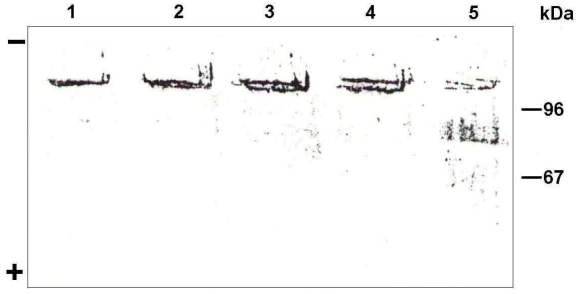


**Figure 3.** Nitrosation of cytosolic protein detected by Western blot. Experimental conditions were described under "Materials and Methods". Lane 1, cytosolic crude extract (alone); Lane 2, crude extract incubated with 0.7 mM sodium nitrite; Lane 3, crude extract incubated with 1.4 mM nitrite; Lane 4, crude extract incubated with 5 mM nitrite; Lane 5, crude extract incubated with 70 µM PN.

incubation using much less concentrations of L-cysteine, or GSH (10 mM) and nitrite (1 mM) at pH 7.0 (Figure 2). Both products exhibited characteristic maximal absorbance around 340nm. The absorbance of CySNO was greater than that of GSNO. However, much less absorbance was noted at pH 7.2 (data not shown), indicating that nitrosothiol formation may be preferable at pH 7.0.

It has been documented that the removal of S-nitroso group from GSNO leads to the decrease in absorbance around 340 nm (14, 15). Incubation of GSNO with agarose-CoASH or agarose-Cys resulted in a decrease in absorbance at 23 and 46%, respecitively (Table 1). Both agarose-CoA an agarose-Cys contain free –SH groups, which are reactive for S-transnitrosation/S-denitrosation during the incubation, thereby resulting in a decrease of absorbance (14). However, it remains to be determined the biological significance of these processes.

Prior to Western blot analysis, the treatment of crude extract with protein G-agarose may remove nonspecific immunoreactivities that may otherwise interfere with the results. The subsequent preincubation of cysteine also facilitated the removal of the nitroso group from endogenous nitrosolated proteins, which otherwise may interfere with the observations. Interestingly, one protein with a large molecular mass (greater than 96 kDa) was found to be nitrosolated with nitrite and was split into two duplet peptides in a concentration-dependant manner (0.7 mM up to 5 mM) (Figure 3, lanes 2-4). In addition to these large proteins mentioned above (lanes 2-4), other proteins/peptides at 67 and 96 kDa were also noted to be nitrated by PN (lane 5). Nevertheless, the brain extract prepared for this study may still contain denitrosation enzymes/proteins (1, 14, 15); therefore, the removal of these enzymes may enable us to detect more nitrite or PN-mediated nitrosation.

The novel catalytic activities of antibodies on both the formation of hydrogen peroxide from singlet molecular oxygen and water, and the formation of ozone in bacterial killing and inflammation have been recently reported (23, 24). In this study, we explored yet another possible function of antibodies by the incubation in nitrite solution at pH 7.1. In comparison with another extracellular protein, bovine serum albumin, the incubation of mouse IgG with nitrite significantly produced gas bubbles (Table 2). The gas bubbles produced adhered to the walls of the pipette tip that remained inside the incubation mixture, and the gas bubble product increased after 3 hours of incubation. Bovine serum albumin and mouse IgG were freshly prepared, however, when old IgG (stored at −20°C for 4 years) was used, the effect of gas bubble production was lost. This finding implies that the incubation of nitrite with IgG may facilitate the production of reactive nitrogen/oxygen species, such as $NO^{\bullet}$, $NO_2$, $NO_2^+$, $N_2O_3$, $N_2O_4$, $NO_2Cl$, $O_2^{\bullet -}$ and $O_3$ (2, 14), which leads to gas bubble production. Under pathophysiological condition nitrite has been implicated in a variety of diseases (2, 25), for example, the accumulation of nitrite in human immunodeficiency virus infection (25). Nevertheless, sodium nitrite has been used as additives in certain foods to inhibit the growth of *Clostridium botulinum* (2). Owing to this unique nature of nitrite, it would be of great interest to investigate weather the above mentioned process of IgG-catalyzed gas production, varied in timing, site, and intensity, involves either pathogenesis or immunological defense. Moreover, the significant gas bubble production by the incubation of nitrite with hemoglobin (Table II) apparently is in agreement with our previous finding that hemoglobin can be nitrosolated by nitrite (14). These results imply that the production of reactive nitrogen/ oxygen species and gas bubbles may be crucial for the onset of diversified effects.

## 5. ACKNOWLEDGMENTS

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**Table 1.** Degredation of nitrosoglutathione (GSNO) by cysteine and coenzyme A

| Reaction Mixtures | Absorbance (340nm) | Degradation of GSNO (%) |
|---|---|---|
| Agarose + 5mM GSNO | 0.657 | 0 |
| Agarose-CoASH + 5mM GSNO | 0.504 | 23.3 |
| Agarose-Cysteine + 5mM GSNO | 0.355 | 46.0 |

Experimental conditions are described under "Materials and Methods". Each value shown represents the mean of three samples.

**Table 2.** The production of gas bubble from nitrite solution incubated with various proteins

| Protein | Incubation time (minute) | Gas bubble production (0, Traceable to 3+) |
|---|---|---|
| None | 30 | 0 |
| | 180 | 0 |
| Bovine serum albumin | 30 | 0 |
| | 180 | Traceable/1+ |
| Hemoglobin (pig) | 30 | Traceable |
| | 180 | 2+ |
| Histone III-S | 30 | Traceable |
| | 180 | 1+ |
| Mouse IgG | 30 | 1+ |
| | 180 | 3+ |

Experimental conditions are described under "Materials and Methods".

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**Key Words***:* S-Nitrosation; Nitrite; Peroxynitrite; Nitric Oxide; Antibody

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