

Nitric Oxide: Probably the *in Vivo* Mediator of the Bisulfite's Effects

Loris Grossi

Dipartimento di Scienze per la Qualità della Vita, Università di Bologna, Campus di Rimini, Rimini, Italy
Email: loris.grossi@unibo.it

Received 3 July 2014; revised 20 August 2014; accepted 3 September 2014

Copyright © 2014 by author and Scientific Research Publishing Inc.
This work is licensed under the Creative Commons Attribution International License (CC BY).
<http://creativecommons.org/licenses/by/4.0/>



Open Access

Abstract

The use of bisulfite in food and beverage preservation, as well as in commercial goods and pharmaceuticals as antimicrobial agents is well known, but not very much is reported on its action *in vivo*. It has been stressed that its action is connected to the presence of NO, and the only reported/hypothesized evidence concerns the possible interaction with GSNO (S-nitrosoglutathione), an NO releaser. In this light, we investigated the interaction between GSNO and the bisulfite in an aqueous medium at pH = 6.4; actually, a positive effect of the sulfite was evidenced. *i.e.*, the S-nitrosoglutathione becomes a more efficient NO-releaser. But, the nitrite is the real pool of NO *in vivo*, therefore we investigate its interaction with the bisulfite in an aqueous acidic solution at pH = 6.4; this time, a definitely efficient and abundant NO release, 3.61 times higher compared to the GSNO, has been evidenced. Therefore, these results allow hypothesizing a fundamental role of NO in the bisulfite's action *in vivo*, or most probably the bisulfite acts simply as cofactor of NO-releasers.

Keywords

Nitrite, Sulfite, Nitric Oxide, Antioxidant, EPR Spectroscopy

1. Introduction

The generally accepted term “sulfite” identifies sulfiting agents such as sulphur dioxide gas, metabisulfite, bisulfite and sulfite. These are all widely used in food and beverage preservation for their antioxidant properties [1] [2], in commercial goods and pharmaceuticals as antimicrobial agents, and also present as pollutants in the atmosphere. Endogenously generated during the normal metabolic processing of sulfur-containing amino acids or drugs, bisulfite level in the body is tightly regulated by the sulfite-oxidase that acts as detoxifier for endogenous and exogenous sulfite [3]. An extreme example of the necessity of having the concentration of sulfite under tigh-

tened control is a congenital disease, the sulfite-oxidase deficiency, which causes an increase in sulfite concentration, and individuals suffering from this genetic disorder develop severe neurological abnormalities leading to an early death [3]. Sulfite has also been suggested as an endogenous mediator in host defense and/or inflammation, as it activates human neutrophils, *in vitro*, which is one of the key events in the pathogenesis of endotoxemia and, indeed, more generally of the inflammatory response. In healthy individuals beside enzymatic transformation other processes to control the level of sulfite must be involved: for instance, chemical pathways. In fact, the sulfite reacts with disulfide proteins bond such as oxidized glutathione and cystine leading to the formation of the corresponding sulfidryl derivative, or is oxidized to sulfate [4] [5]. But, the mechanism of both the negative and positive actions in which the sulfite could be involved is not clear; for example, it has been suggested that the sulfite might affect some mediators such as the nitric oxide and/or its carriers [6]-[8]. Actually, the sulfite might act as a cofactor of NO releasers [6]-[12]. However, the only evidence reported in favor of this possible synergy has been the highlighting of a decrease in concentration of the S-nitrosogluthathione in the interaction with the bisulfite, as detected by UV spectroscopy [6], and the potential depletion of the extracellular pools of S-nitrosothiol compounds [7] is never fully supported [13] [14]. Furthermore, as for other gasotransmitters, this hypothesized interaction seems to take place mainly in acidic condition [15] (Scheme 1).

2. Experimental

2.1. Materials

All experiments were conducted at room temperature with commercial products except GSNO, S-nitrosogluthathione, which was synthesized as reported in the literature [16], and the $\text{Fe}^{2+}(\text{DETC})_2$, iron (II) N, N-diethyldithiocarbamate, which was synthesized as follows: 25 mL of a water solution of diethyldithiocarbamate (DETC), 20 mmol/L, were added to 100 mL of a water solution of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mmol/L, over a 60-min period. The mixture was stirred for two hours and the precipitate, $\text{Fe}^{2+}(\text{DETC})_2$, collected by filtration, washed several times with water, and dried under vacuum; all processes were conducted in a rigorous nitrogen atmosphere.

2.2. Trapping of NO

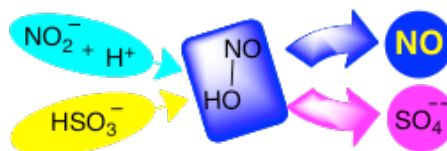
To compare the efficiency of the processes a quantitation of the NO released, via quantitative measurements of $\text{NO-Fe}(\text{DETC})_2$, was conducted according to the following procedure.

After running the EPR-analysis of the samples, the measurement of an internal paramagnetic standard, a synthetic ruby-crystal not removable located inside the spectrometer's cavity, was carried out; in particular, the measurement was conducted without removing the sample-tube (the same for all the experiments), and using the same instrumental setup. Afterwards, the spectrum of a 10^{-5} M benzene solution of DPPH (1,1-diphenyl-2-picrylhydrazyl radical), a spin-concentration reference, was recorded, followed by the measurement of the internal paramagnetic standard. All signals, before to be analyzed, were computing double integrated. Since the intensity of the ruby signal cannot change, the ratio of the double integrals provides a correction factor for the different sensitivity of the cavity for each sample. Thus, the comparison of the intensity of the signals of the NO samples with that obtained from the DPPH, after the sensitivity correction, allows determining the radical concentration in the examined samples. In particular, the absolute concentration of NO for the experiments with $\text{NaNO}_2/\text{NaHSO}_3$ and $\text{GSNO}/\text{NaHSO}_3$ resulted 5.07×10^{-6} M and 1.41×10^{-6} M, respectively. For the blank GSNO experiment the absolute concentration of NO was determined to be 2.19×10^{-7} M. The amount of NO detected comes from an experiment of the duration of 10 minutes, and therefore do not represent the total amount that in principle could be obtained by collecting NO up to depletion of the reaction. The purpose was just to obtain quantitative values of NO, thus to be able to compare of efficiency in nitric oxide releasing from two different NO-releasers present *in vivo*.

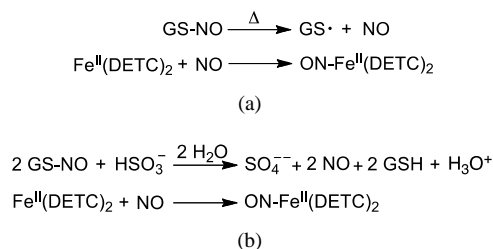
3. Results and Discussion

Since the GSNO/bisulfite interaction was hypothesized [7], to prove or disclaim this as a way of production of NO, a blank experiment with GS-NO, in buffer solution (pH = 6.4), was carried out, (Scheme 2(a)).

The EPR analysis of the resulting trap-solution highlights the formation of the paramagnetic adduct $\text{NO-Fe}(\text{DETC})_2$, in low amount (Figure 1 red trace), due to the thermo-induced release of NO [13], as well as the fading of the characteristic pink color of the solution due to the decrease of the GS-NO concentration, which



Scheme 1. Schematic illustration reflecting the interaction between bisulfite and nitrite.



Scheme 2. Spin-trapping of NO released by GSNO in buffer solution at pH = 6.4: (a) via homolytic cleavage; (b) induced by bisulfite.

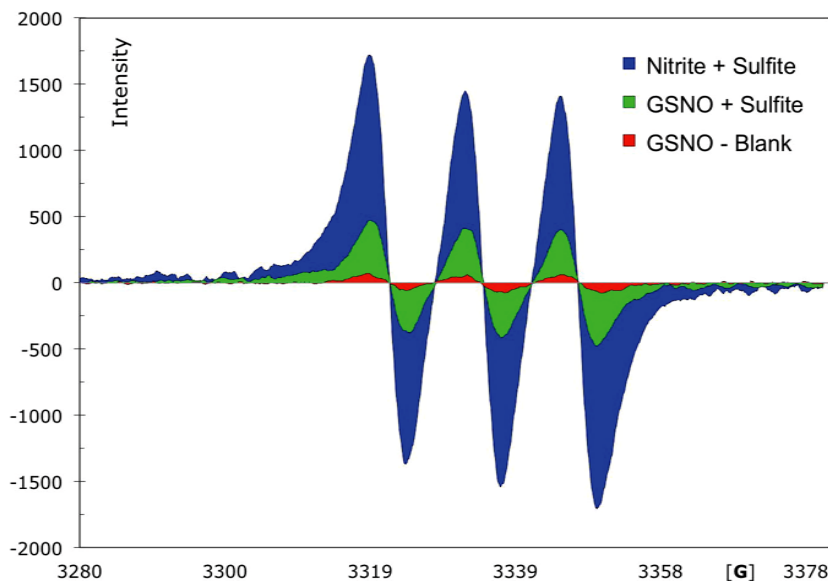


Figure 1. EPR spectrum of $\text{NO-Fe}(\text{DETC})_2$, $a_N = 1.28 \text{ mT}$ and $g = 2.039$ red trace—NO released via the thermal induced homolysis of GSNO; green trace—NO released in the reaction between GSNO and the bisulfite; blue trace—NO released in the reaction between NaNO_2 and the bisulfite. It must be noted that in the experimental spectra the Magnetic Field is listed in Gauss (G), whilst the value reported in the text is in mT, ($1 \text{ mT} = 10 \text{ G}$), as requested by SI. Finally, the spectra are experimental and worked (color and double integration) using Excel.

even if qualitative can be considered a positive supporting proof. However, since the possible role of the bisulfite as a cofactor of NO-derivatives was not clearly demonstrated, a test with GSNO and NaHSO_3 , in buffer solution (pH = 6.4), was conducted.

This time, the spin-trap solution showed an intense EPR signal of the trapped radical, $\text{NO-Fe}(\text{DETC})_2$ (Figure 1 green trace), and a rapid, intense bleaching of the pink-colored solution. These proofs led to hypothesize the possibility to induce nitric oxide release via interaction with the bisulfite also from other NO releasers. In this light, the nitrite, claimed as the most important reservoir of NO *in vivo* [17], was tested. The experiment, conducted in acidic buffer solution (pH = 6.4), allowed the detection of a very intense EPR signal due to the

NO-Fe(DETC)₂, (Figure 1 blue trace), which proved the abundant release of NO, and the high efficiency of the process [12].

Although this was a strong evidence of the positive interaction bisulfite/nitrite, frequently the dismutation of the nitrous acid is invoked as responsible of NO production in acid medium, so a blank test with nitrite in acidic buffer solution, pH = 6.4, was conducted. The possible NO formed was collected for more than 40 minutes (four times the collecting time used for the experiments with the bisulfite!), and the spin trap solution analyzed. The EPR spectrum showed a very feeble signal of the NO-Fe(DETC)₂ adduct, fully negligible compared to that obtained in the experiment conducted in the presence of the bisulfite, leading to conclude that the hypothesized production of NO via dismutation is totally irrelevant.

Among the physiological roles of the sulfite, the interaction with NO gas was also hypothesized [8], but this possibility has to be excluded since the chemistry of NO allows reactions only with radical species, or the coordination to metal ions. Most probably, to account for the results reported in the literature [8] [19], different intermediates, or reactions, leading to a sulfite-derived radical must be hypothesized.

The evidences obtained in the GSNO blank experiment could support the thermal homolysis of S-nitrosothiols (S-N bond), as responsible for the NO release [13], and then the involvement of the bisulfite in principal unnecessary, Scheme 1(a); on the contrary, those obtained from the reaction between GSNO and the bisulfite were unquestionably opposite. In fact, the fast and marked fading of the solution (pink color), and the intense EPR-signal of the NO-Fe(DETC)₂, proved the involvement of the bisulfite, Scheme 2(b). As a matter of fact, the very low rate of the S-N bond homolysis in these experimental conditions would not be able to account for this result [13], Scheme 2(a). Furthermore, the quantitation of NO, Figure 2, shows that the amount of NO-Fe(DETC)₂ radical detectable is 6.44 times higher when GSNO is reacted with the bisulfite compared to the blank experiment.

However, since S-nitrosothiols do not represent the main reservoir of NO *in vivo* but, as widely accepted is the nitrite [17] [18], its possible interaction with the sulfite would be truly significant and could disclose a new scenario [20] [21]. For example, because the level of the sulfite must be closely regulated in the body, its interaction with the nitrite could contribute to keep the sulfite under control (lowering the concentration!) through a chemical pathway, as well as to support the hypothesized function of the nitrite as the mediator of the sulfite action [6] [12], Scheme 3.

The result obtained in the presence of the nitrite, analogously to that reported for the hydrogen sulfide [15], show a process under pH control, *i.e.*, the NO release is more efficient in acidic medium. Actually, these conditions might correspond, *in vivo*, to those in which the synergy between the sulfite and NO transporters is invoked;

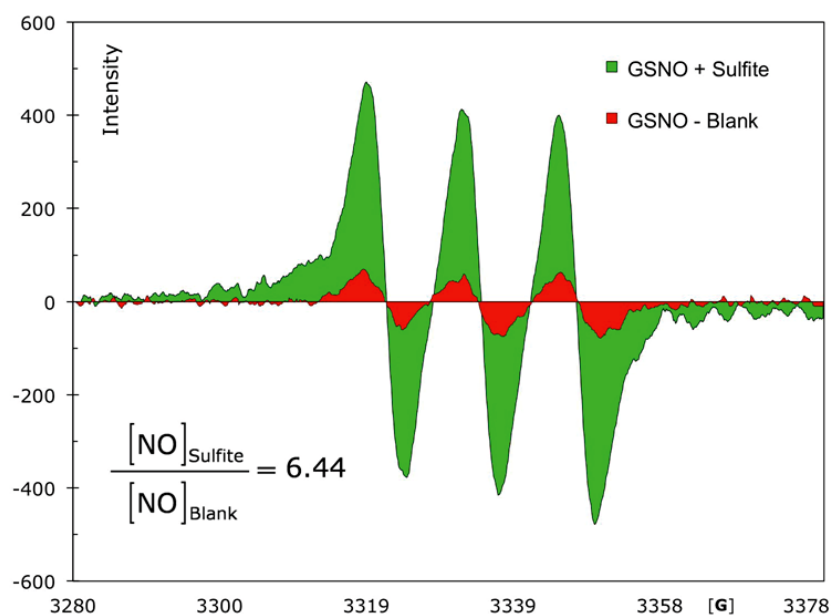
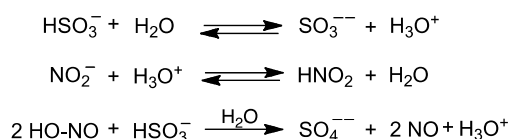


Figure 2. Quantitative comparison (NO) of the EPR spectra obtained from the reaction between the GSNO and the bisulfite, and the GSNO alone.



Scheme 3. NO release induced by the bisulfite on the nitrous acid at pH = 6.40.

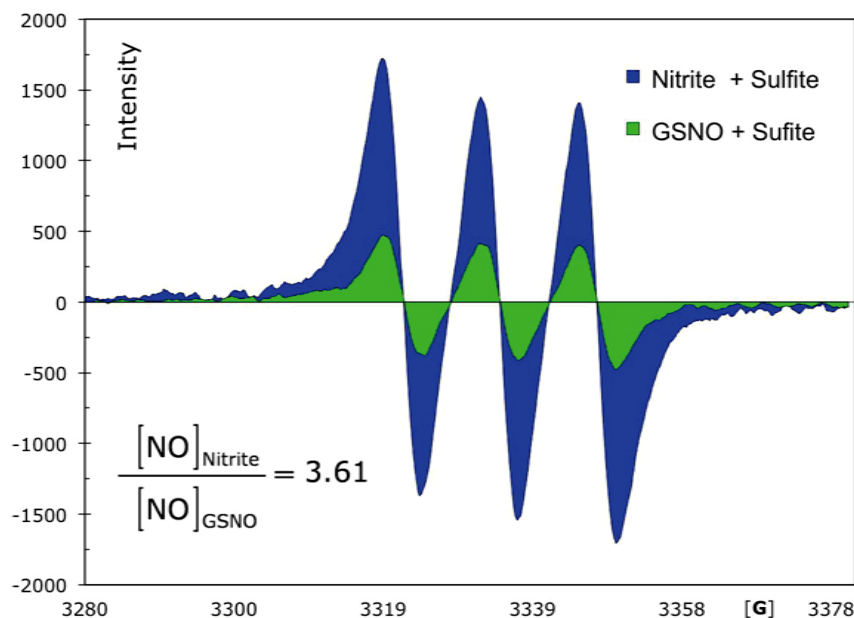


Figure 3. Quantitative comparison (NO) of the EPR spectra obtained from the reaction between the Nitrite and the bisulfite (blue), and GSNO and the bisulfite (green).

for example, patients in acute phase of inflammation that show high acidity and high levels of serum sulfite [10] [11]. Thus, pH-sensitive NO derivatives must be taken into consideration: the nitrite, whose aci-form (HO-NO) increases in acidic condition, fulfills this request, since able to undergo a reductive NO release, **Scheme 2**.

The focal role of the interaction between the nitrite and the bisulfite, as a source of NO, is definitely supported by the quantitation of nitric oxide released: it results 3.61 times higher than that released in the interaction with GSNO, **Figure 3**.

4. Conclusion

In the light of these results, as for molecules hypothesized to have a gasotransmitter role [14], the bisulfite also requires synergy with NO releasers to account for its actions, and such behavior leads to underline the role of the bisulfite as a cofactor. Moreover, it is worth noting that the nitrite plays a crucial role regarding all species considered gasotransmitters, and therefore a more careful study of its “chemistry” *in vivo* would be fundamental also for understanding the positive and/or negative effects of molecules such as the sulfite.

Acknowledgements

This work was financially supported by MIUR, Rome, Fund PRIN 2010-2011 2010PFLRJR (PROxi project). I thank L.A. Winter for the helpful discussion.

References

- [1] Winkler, C., Frick, B., Schroecksadel, K., Schennach, H. and Fuchs, D. (2006) Food Preservatives Sodium Sulfite and Sorbic Acid Suppress Mitogen-Stimulated Peripheral Blood Mononuclear Cells. *Food and Chemical Toxicology*, **44**, 2003-2007. <http://dx.doi.org/10.1016/j.fct.2006.06.019>

- [2] Gunnison, A.F. and Jacobsen, D.W. (1987) Sulfite Hypersensitivity. A Critical Review. *CRC Critical Reviews in Toxicology*, **17**, 185-214. <http://dx.doi.org/10.3109/10408448709071208>
- [3] Mitsuhashi, H., Yamashita, S., Ikeuchi, H., Kuroiwa, T., Kaneko, Y., Hiromura, K., Ueki, K. and Nojima, Y. (2005) Oxidative Stress-Dependent Conversion of Hydrogen Sulfide to Sulfite by Activated Neutrophils. *Shock*, **24**, 529-534. <http://dx.doi.org/10.1097/01.shk.0000183393.83272.de>
- [4] Bailey, J.L. and Cole, R.D. (1959) Studies on the Reaction of Sulfite with Proteins. *Journal of Biological Chemistry*, **234**, 1733-1739.
- [5] Neta, P. and Huie, R.E. (1985) Free-Radical Chemistry of Sulfite. *Environmental Health Perspectives*, **64**, 209-217. <http://dx.doi.org/10.1289/ehp.8564209>
- [6] Harvey, S.B. and Nelsestuen, G.L. (1995) Reaction of Nitric Oxide and Its Derivatives with Sulfites: A Possible Role in Sulfite Toxicity. *Biochimica et Biophysica Acta*, **1267**, 41-44. [http://dx.doi.org/10.1016/0167-4889\(95\)00059-2](http://dx.doi.org/10.1016/0167-4889(95)00059-2)
- [7] Harvey, S.B. and Nelsestuen, G.L. (1996) Influence of Sulfites on the Action of Nitric Oxide and S-Nitrosoglutathione *in Vitro* and *in Vivo*. *FASEB Journal*, **10**, A704.
- [8] Li, J. and Meng, Z. (2009) The Role of Sulfur Dioxide as an Endogenous Gaseous Vasoactive Factor in Synergy with Nitric Oxide. *Nitric Oxide*, **20**, 166-174. <http://dx.doi.org/10.1016/j.niox.2008.12.003>
- [9] Meng, Z., Li, Y. and Li, J. (2007) Vasodilatation of Sulfur Dioxide Derivatives and Signal Transduction. *Archives of Biochemistry and Biophysics*, **467**, 291-296. <http://dx.doi.org/10.1016/j.abb.2007.08.028>
- [10] Mitsuhashi, H., Ikeuchi, H., Yamashita, S., Kuroiwa, T., Kaneko, Y., Hiromura, K., Ueki, K. and Nojima, Y. (2004) Increased Levels of Serum Sulfite in Patients with Acute Pneumonia. *Shock*, **21**, 99-102. <http://dx.doi.org/10.1097/01.shk.0000105501.75189.85>
- [11] Kajiyama, H., Nojima, Y., Mitsuhashi, H., Ueki, K., Tamura, S., Sekihara, T., Wakamatsu, R., Yano, S. and Naruse, T. (2000) Elevated Levels of Serum Sulfite in Patients with Chronic Renal Failure. *Journal of the American Society of Nephrology*, **11**, 923-927.
- [12] Takahama, U. and Hirota, S. (2012) Effects of the Food Additive Sulfite on Nitrite-Dependent Nitric Oxide Production under Conditions Simulating the Mixture of Saliva and Gastric Juice. *Journal of Agricultural and Food Chemistry*, **60**, 1102-1112. <http://dx.doi.org/10.1021/jf2049257>
- [13] Grossi, L., Montevecchi, P.C. and Strazzari, S. (2001) Decomposition of S-Nitrosothiols: Unimolecular versus Autocatalytic Mechanism. *Journal of the American Chemical Society*, **123**, 4853-4854. <http://dx.doi.org/10.1021/ja005761g>
- [14] Munro, A.P. and Williams, D.L.H. (2000) Reactivity of Sulfur Nucleophiles towards S-Nitrosothiols. *Journal of the Chemical Society, Perkin Transactions*, **2**, 1794-1797. <http://dx.doi.org/10.1039/b004415f>
- [15] Grossi, L. (2009) Hydrogen Sulfide Induces Nitric Oxide Release from Nitrite. *Bioorganic & Medicinal Chemistry Letters*, **19**, 6092-6094. <http://dx.doi.org/10.1016/j.bmcl.2009.09.030>
- [16] Hart, T.W. (1985) Some Observations Concerning the S-Nitroso and S-Phenylsulphonyl Derivatives of L-Cysteine and Glutathione. *Tetrahedron Letters*, **26**, 2013-2016. [http://dx.doi.org/10.1016/S0040-4039\(00\)98368-0](http://dx.doi.org/10.1016/S0040-4039(00)98368-0)
- [17] Lundberg, J.O., Weitzberg, E. and Gladwin, M.T. (2008) The Nitrate-Nitrite-Nitric Oxide Pathway in Physiology and Therapeutics. *Nature Reviews Drug Discovery*, **7**, 156-167. <http://dx.doi.org/10.1038/nrd2466>
- [18] Butler, A.R. and Feelisch, M. (2008) Therapeutic Uses of Inorganic Nitrite and Nitrate: From the Past to the Future. *Circulation*, **117**, 2151-2159.
- [19] Littlejohn, D., Hu, K.Y. and Chang, S.G. (1986) Kinetics of the Reaction of Nitric Oxide with Sulfite and Bisulfite Ions in Aqueous Solution. *Inorganic Chemistry*, **25**, 3131-3135. <http://dx.doi.org/10.1021/ic00238a007>
- [20] Lagercrantz, C. (1992) Radicals Formed in the Reaction between Some Alkyl Nitrites and Sulfite Ions Studied by EPR Spectroscopy. *Acta Chemica Scandinavica*, **46**, 304-306. <http://dx.doi.org/10.3891/acta.chem.scand.46-0304>
- [21] Shi, X.J. (1994) Generation of SO_3^- and OH Radicals in SO_3^{2-} Reactions with Inorganic Environmental Pollutants and Its Implications to SO_3^{2-} Toxicity. *Journal of Inorganic Biochemistry*, **56**, 155-165. [http://dx.doi.org/10.1016/0162-0134\(94\)85002-X](http://dx.doi.org/10.1016/0162-0134(94)85002-X)