Biochemical Pharmacology, Vol. 53, pp. 581–585, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(96)00825-8

Inhibition of S-Nitrosation of Reduced Glutathione in Aerobic Solutions of Nitric Oxide by Phosphate and Other Inorganic Anions

Eugene G. DeMaster,† Barry J. Quast* and Robert A. Mitchell‡*

*Medical Research Laboratories, VA Medical Center, Minneapolis, MN 55417; and ‡Department of Biochemistry, Wayne State University, Detroit, MI 48201, U.S.A.

ABSTRACT. Reduced glutathione is nitrosated in aerobic solutions of nitric oxide under physiological conditions; however, the extent of S-nitrosation was found to be dependent on the inorganic anions present. Of nine anions tested, the bifunctional anions, arsenate, phosphate, and pyrophosphate (40 mM), inhibited the S-nitrosation reaction from 20 to 40%, whereas SO_4^{2-}, H_3BO_3, SCN^-, NO_3^-, Cl^-, and acetate inhibited this reaction ≤15%. A mechanism of inhibition is presented that involves the catalytic hydrolysis of N_2O_3 by the bifunctional anions; however, using [^{18}O]phosphate as inhibitor, only 10% of the theoretically produced N_2O_3 was found to be hydrolyzed to nitrite via this mechanism as calculated from the loss of ^{18}O from phosphate. We conclude that this mechanism accounts for only a minor part of the increased inhibition of S-nitrosation by these bifunctional anions. BIOCHEM PHARMACOL 53:4:581–585, 1997. © 1996 Elsevier Science Inc.

KEY WORDS. nitric oxide; glutathione; nitrosation

NO§ readily nitrosates thiols and secondary amines under physiological conditions. The nitrosating species involved in these reactions is not NO, but rather an oxidized form of NO [1, 2]. Although N_2O_3 has generally been considered to be the nitrosating species present in aerobic solutions of NO, recent kinetic studies on the N-nitrosation of sodium azide suggest that N_2O_3 is not the primary nitrosating species produced in oxygenated NO solutions [3]. An alternative oxide of nitrogen to serve as this nitrosation intermediate, however, was not identified. Based on a detailed analysis of the kinetics of N-nitrosation of morpholine, others have subsequently concluded that N_2O_3 is indeed the intermediate nitrosating species in aerobic solutions of NO [4].

The intermediacy of N_2O_3 in nitrosation reactions by aqueous aerobic NO solutions was questioned because the observed rate of azide nitrosation relative to the rate of N_2O_3 hydrolysis at physiological pH was reduced significantly compared with the ratio of these reaction rates determined under acidic conditions [3]. An alternative explanation for these results has been offered [4], which attributes this difference in rate constants to an inhibition of the N-nitrosation reaction by phosphate anion at physiologic pH rather than to different nitrosating species. A mechanism for this inhibition was proposed [4] involving phosphate-catalyzed hydrolysis of N_2O_3, i.e. nitrosation of phosphate followed by rapid hydrolysis of the nitrosated phosphate intermediate to NO_2^- and phosphate anion. In the remainder of this report, we are assuming that the nitrosating agent formed in aerobic NO solutions is, in fact, one of the isomeric forms of N_2O_3.

The discovery that phosphate anion inhibits N-nitrosation reactions at physiological pH [4] suggests that phosphate as well as other anions should also inhibit S-nitrosation reactions under similar conditions. Moreover, there are numerous examples in the literature where inorganic anions have been shown to catalyze hydrolytic and other reactions [5–8]. We now report that phosphate and certain other bifunctional inorganic anions decrease S-nitrosation of GSH under physiological conditions *in vitro*, presumably by catalyzing the hydrolysis of N_2O_3. The mechanism for the catalytic hydrolysis of N_2O_3 was investigated using [^{18}O]phosphate.

MATERIALS AND METHODS

Materials

DEA/NO was obtained from Cayman Chemical (Ann Arbor, MI), and stock solutions of DEA/NO were prepared in deoxygenated 0.1 N KOH. Potassium chloride, potassium thiocyanate, potassium phosphate, and sodium arsenate were from the Sigma Chemical Co. (St. Louis, MO). GSH

† Corresponding author: Eugene G. DeMaster, Ph.D., Medical Research Laboratories (151), VA Medical Center, One Veterans Drive, Minneapolis, MN 55417. Tel. (612) 725-2000, Ext. 2828; FAX (612) 725-2093.

§ *Abbreviations:* DEA/NO, diethylnonoate; GSH, reduced glutathione; GSOH, sulfenic acid of glutathione; GSNO, S-nitrosoglutathione; GSSG, oxidized glutathione; MOPS, 4-morpholinopropanesulfonic acid; and NO, nitric oxide.

Received 1 April 1996; accepted 20 August 1996.

was from ICN (Costa Mesa, CA), and stock solutions of GSH were prepared in cold 0.01 N HCl. ^{18}O-Enriched potassium phosphate was purchased from Miles Laboratories, while potassium sulfate and boric acid were from Fisher Scientific (Springfield, NJ); potassium nitrate was from Mallinckrodt (St. Louis, MO). Diazomethane was generated using a kit from the Aldrich Chemical Co. (Milwaukee, WI).

S-Nitrosation Reactions

These reactions were carried out using mixtures composed of potassium phosphate buffer, pH 7.4, 100 μM GSH, and 0.5 mM DEA/NO in a total volume of 1.0 mL. The final anion concentrations are given in the figure legends. Following the addition of GSH and DEA/NO, the mixtures were incubated for 20 min in a water bath maintained at 37°. The amount of S-nitrosoglutathione formed was determined spectrophotometrically at 336 nm (ϵ = 902 M^{-1} cm^{-1}) [9]. A reaction blank, minus GSH, was subtracted from all samples.

[^{18}O]Phosphate Experiment

Reaction mixtures containing 50 mM potassium phosphate (87.2 atom percent ^{18}O; pH 7.4) and either 0 (control) or 500 nmol DEA/NO (added in two 5-μL aliquots 30 min apart) in a final volume of 0.11 mL were incubated in an air atmosphere in a closed glass reaction vessel for a total of 60 min at 37°. The [^{18}O]phosphate solution was prepared in 100 mM MOPS to increase buffering capacity. Since MOPS is a quaternary amine, it does not interfere with nitrosation.

Following the reaction, the phosphate was converted to the free acid by ion exchange chromatography (AG-50 W-X2 cation exchange resin, H^+ form), and the eluate was taken to dryness under vacuum. The phosphoric acid was derivatized to trimethyl phosphate using an ether/diazomethane distillate produced from Diazald® (Aldrich). A JEOL model AX-505 mass spectrometer was used for electron impact ionization GC–MS analysis of trimethyl phosphate using selective ion monitoring, i.e. m/z 140, 142, 144, 146, and 148 [10].

Statistical Analysis

Results are expressed as means ± SEM of triplicate samples. Statistical analyses of variance were determined using the Dunnett's test; P values of < 0.05 were accepted as significant.

RESULTS

Inorganic phosphate inhibited the S-nitrosation of GSH by N_2O_3 produced from the NO donor, DEA/NO,* in aqueous solution at pH 7.4 (Fig. 1). The degree of inhibition was dependent on the phosphate concentration. Identical results were obtained with Chelex-treated and untreated phosphate buffers. Raising the GSH concentration to 1.0 and 2.0 mM in the presence of 10 mM phosphate increased GSNO production to a level of 25% of the total NO generated from DEA/NO (Fig. 2). Since half of the generated NO forms nitrite as a result of the nitrosating reaction by N_2O_3, only 50% of the generated NO can be theoretically converted to GSNO.

Since both phosphate and chloride ions have been shown to inhibit N-nitrosation [4], these two anions along with seven other anions were compared for their ability to inhibit S-nitrosation of GSH by N_2O_3 produced as described above (Fig. 3). Phosphate and other bifunctional inorganic anions, namely, pyrophosphate and arsenate, were found to be the most inhibitory of the nine anions tested and inhibited the formation of GSNO by 20–40%. None of the remaining six anions reduced GSNO production more than 15%.

Because the bifunctional anions, namely phosphate, pyrophosphate, and arsenate, showed greater inhibition of the S-nitrosation reaction than the monofunctional anions, we postulated that their nitrosated anion intermediates may hydrolyze via an intramolecular mechanism. For example, nitrosated phosphate would decompose to nitrite and a *meta*-phosphate intermediate (Eq. 1) followed by rapid hy-

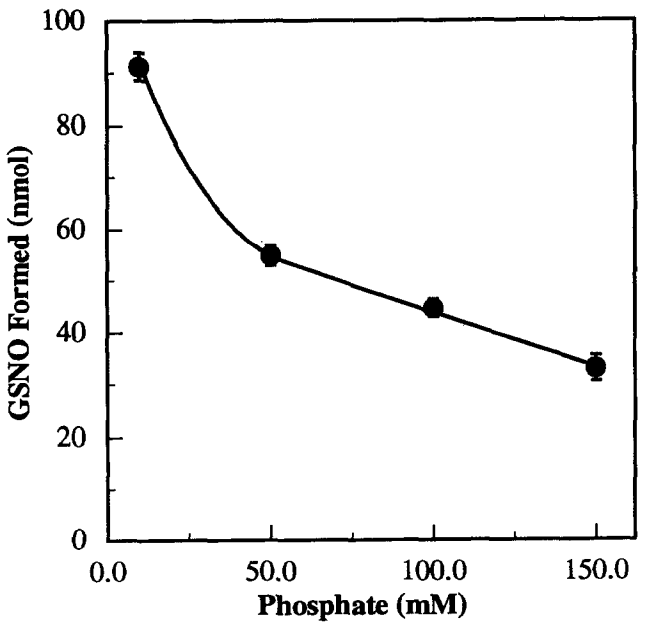


FIG. 1. Phosphate inhibition of S-nitrosation of GSH by N_2O_3 produced from DEA/NO under physiological conditions. The reaction mixtures (1.0 mL) composed of potassium phosphate buffer (pH 7.4), 100 μM GSH, and 0.5 mM DEA/NO were incubated for 20 min at 37°. The S-nitrosoglutathione produced was quantified by spectrophotometric analysis [9]. Other experimental details were as given under Materials and Methods. Results are means ± SEM of triplicate samples.

* DEA/NO decomposes at pH 7.4 and 37° according to a first order process with a half-life of 2.1 min [11]. Two moles of NO are produced per mole of DEA/NO.

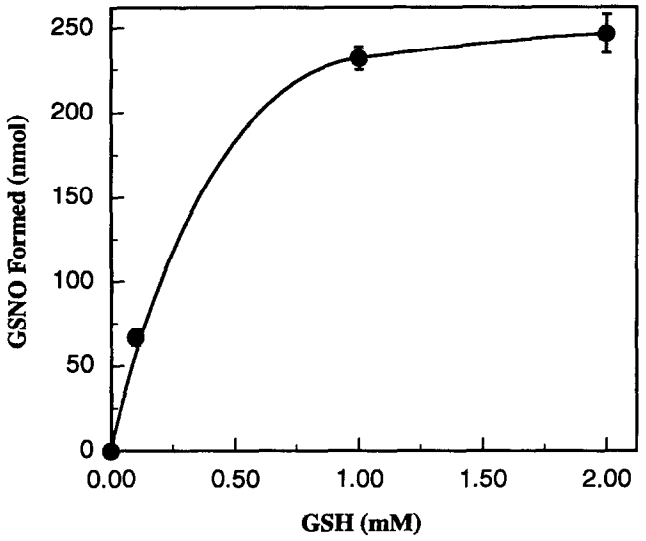
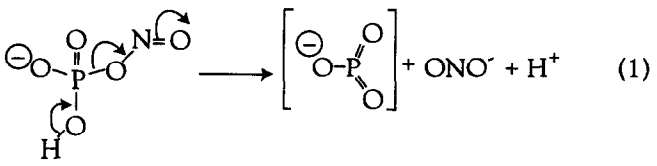


FIG. 2. Effect of GSH concentration on the formation of GSNO by N_2O_3 produced from DEA/NO under physiological conditions. The experimental details were as in Fig. 1 except that the phosphate buffer concentration was 10 mM.

dration of the latter back to *ortho*-phosphate. This mechanism was



(1)

tested by measuring the loss of ^{18}O from phosphate [12].

The incubation mixtures contained 5.0 μmol phosphate (or 20 μmol exchangeable oxygen) and potentially 500 nmol N_2O_3. After incubation, the mean ^{18}O-contents of control and DEA/NO-treated phosphate were 87.46 (N = 2) and 87.22 atom percent (N = 2), respectively.* The difference of 0.24 atom percent corresponds to an oxygen exchange of 54 nmol. This suggests that at most only 10% of the N_2O_3 formed was hydrolyzed to nitrite by a phosphate-catalyzed reaction involving P–O bond cleavage.

DISCUSSION

The reactions involved in the formation of N_2O_3 from DEA/NO-derived NO and subsequent hydrolysis of N_2O_3 and nitrosation of GSH by N_2O_3 are depicted in Eqs. 2–5 [4].

$$2NO + O_2 \rightarrow 2NO_2 \quad (2)$$

$$NO + NO_2 \rightarrow N_2O_3 \quad (3)$$

* The individual values were 87.42 and 87.49 for controls and 87.26 and 87.18 atom percent [^{18}O]phosphate for DEA/NO-containing samples.

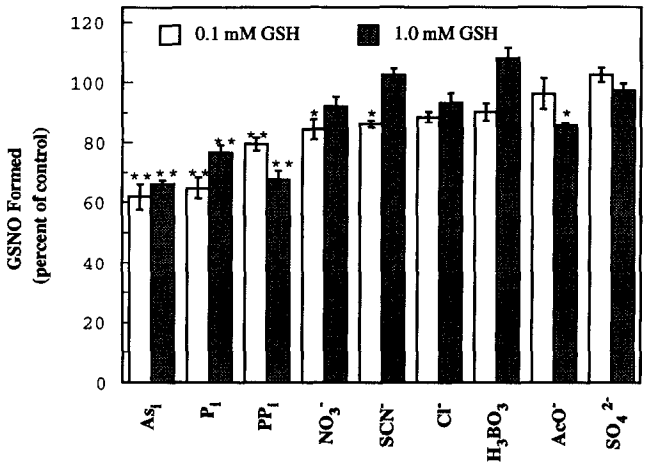


FIG. 3. Evaluation of nine anions for their ability to inhibit S-nitrosation of GSH by N_2O_3. The abbreviations, As_i, P_i and PP_i, denote inorganic arsenate, phosphate, and pyrophosphate, respectively. All incubations contained 10 mM phosphate and GSH as indicated plus a 40 mM concentration of the anion tested with a final pH of 7.4. The control samples contained 10 mM phosphate with no other added anion, whereas the phosphate test samples contained 50 mM phosphate. The control values for 0.1 and 1.0 mM GSH were 75.9 ± 2.2 and 244 ± 12.0 nmol GSNO produced, respectively. Other experimental details are given under Materials and Methods. Results are means ± SEM of triplicate samples. P values of < 0.05 (*) and < 0.01 (**) vs control values are indicated.

$$N_2O_3 + H_2O \rightarrow 2NO_2^- + 2H^+ \quad (4)$$

$$N_2O_3 + GSH \rightarrow GSNO + NO_2^- + H^+ \quad (5)$$

Since half of the NO generated is converted to nitrite (Eq. 5), the theoretical maximum GSNO yield is equal to 50% of the NO produced. However, in the presence of excess GSH, we observed that 25% of the NO generated was converted to GSNO (Fig. 2). Under the conditions used in these experiments, NO can also oxidize GSH to the sulfenic acid, GSOH, which in turn readily reacts with a second GSH molecule to yield the disulfide, GSSG (Eqs. 6 and 7) [9]. Thus,

$$2NO + GSH \rightarrow GSOH + N_2O \quad (6)$$

$$GSOH + GSH \rightarrow GSSG + H_2O \quad (7)$$

in the presence of a thiol, some of the NO is diverted to N_2O. The relative amount of NO converted to N_2O or thiol to sulfenic acid is, in part, dependent on the pK_a of the thiol present [9]. Thus, some of the unaccounted for NO was likely diverted to N_2O, and some may have been lost due to diffusion into the headspace of the reaction vessel [13]. It might also be expected that a finite amount of diethylamine liberated from DEA/NO was also nitrosated. Hydroxylamine, however, is not an expected end-product because GSH (≤ 20 mM) does not reduce either

NO or GSNO to hydroxylamine under physiologic conditions (unpublished results).

Inorganic anions can react with N_2O_3 to form nitrosated intermediates as given in Equation 8 for the chloride anion [4]. Like N_2O_3, ClNO can be hydrolyzed yielding nitrite and

$$Cl^- + N_2O_3 \rightarrow ClNO + NO_2^- \quad (8)$$

$$ClNO + H_2O \rightarrow NO_2^- + Cl^- + 2H^+ \quad (9)$$

$$ClNO + GSH \rightarrow GSNO + Cl^- + H^+ \quad (10)$$

chloride (Eq. 9) or can nitrosate GSH to product GSNO and the chloride anion (Eq. 10). Consequently, chloride will exhibit an inhibition of S-nitrosation if the rate of ClNO hydrolysis (Eq. 9) relative to the rate of nitrosation by ClNO (Eq. 10) is greater than the rate of N_2O_3 hydrolysis versus the rate of nitrosation by N_2O_3. This type of mechanism may account for the low level of inhibition of S-nitrosation by several of the monofunctional anions (Fig. 3).

The bifunctional anions, namely phosphate, pyrophosphate, and arsenate, showed greater inhibition of the S-nitrosation reaction than the monofunctional anions (Fig. 3). Since the monovalent and divalent forms of these anions are bifunctional, both forms would be expected to participate in this inhibition. We postulated that this inhibition could be due to an intramolecular decomposition of the nitrosated bifunctional anion intermediates to nitrite and an anhydrous form of their anions (e.g. *meta*-phosphate) followed by rapid hydration of the latter back to its original anionic species (e.g. *ortho*-phosphate). This type of catalytic participation by these bifunctional anions could greatly enhance the overall rate of N_2O_3 hydrolysis compared with a bimolecular reaction for the monofunctional anions, as shown in Eq. 9. However, using highly enriched]^{18}O]phosphate and measuring the loss of ^{18}O from phosphate, we determined that at most only 10% of the theoretically produced N_2O_3 was hydrolyzed to nitrite via this mechanism. Based on these results, we conclude that this mechanism could only account for a minor part of the increased inhibition of S-nitrosation by these bifunctional anions.

The inhibition of N-nitrosation of azide by phosphate as reported in a previous study [4] may not totally account for the disparity between the relative rates of hydrolysis and of nitrosation at acid and neutral pH. The finding of equivalent rate constants calculated for the N-nitrosation of morpholine, a strong base, from reactions carried out in low phosphate buffer concentrations at neutral pH and at acidic pH was considered strong evidence for a single nitrosating species under these conditions [4]. However, the kinetics of N-nitrosation carried out under acidic and neutral pH with other amines, viz. weaker bases than morpholine, did not show this agreement [14]. This difference in reactivity toward amines by gaseous N_2O_3 dissolved in water at neutral pH and by N_2O_3 generated *in situ* from aqueous acidified nitrite was attributed to structural isomerism of N_2O_3 [15].

N_2O_3 can potentially exist in two isomeric forms, i.e. as a typical anhydride (O=N=O=N=O, structure I) and as a nitroso-nitrite (O=N–NO_2, structure II). The studies related to the structural identity of N_2O_3 invariably have been conducted using N_2O_3 preparations based on the combination of NO and NO_2 radicals (Eq. 3). The N_2O_3 in these preparations is a planar molecule and contains an N–N bond consistent with structure II [16–18]. Structure II is an unlikely form for a product derived from the bimolecular reaction between nitrosonium ion (NO^+) and nitrous acid [19], and, indeed, based on mechanistic considerations, structure I would be a more probable candidate.

We conclude that phosphate and certain other anions inhibit S-nitrosation as well as N-nitrosation by gaseous NO under aerobic conditions, and that the proposed phosphate-catalyzed hydrolysis of N_2O_3 does not proceed primarily through a *meta*-phosphate intermediate. We suggest that the isomeric structures of N_2O_3 generated from nitrous acid under acidic conditions and from NO under aerobic conditions at neutral pH are likely different, and if so, this difference would contribute to the reported disparity in rates of nitrosation of azide and of hydrolysis of N_2O_3 at neutral and acid pH.

This research was supported by the Department of Veterans Affairs. We thank Dr. H. T. Nagasawa for helpful discussions. Mass spectral data were obtained at the Michigan State Mass Spectrometry Facility which is supported, in part, from a grant (RR00480) from the Biotechnology Research Program, National Center for Research Sources, National Institute of Health.

References

1. Butler AR and Williams DLH, The physiological role of nitric oxide. *Chem Soc Rev* **22:** 233–240, 1993.
2. Wink DA, Nims RW, Darbyshire JF, Christodoulou D, Hanbauer I, Cox GW, Laval F, Laval J, Cook JA, Krishna MC, DeGraff WG and Mitchell JB, Reaction kinetics for nitrosation of cysteine and glutathione in aerobic nitric oxide solutions at neutral pH. Insights into the fate and physiological effects of intermediates generated in the NO/O_2 reaction. *Chem Res Toxicol* **7:** 519–525, 1994.
3. Wink DA, Darbyshire JF, Nims RW, Saavedra JE and Ford PC, Reactions of the bioregulatory agent nitric oxide in oxygenated aqueous media. Determination of the kinetics for oxidation and nitrosation by intermediates generated in the NO/O_2 reaction. *Chem Res Toxicol* **6:** 23–27, 1993.
4. Lewis RS, Tannenbaum SR and Deen WM, Kinetics of N-nitrosation in oxygenated nitric oxide solutions at physiological pH. Role of nitrous anhydride and effects of phosphate and chloride. *J Am Chem Soc* **117:** 3933–3939, 1995.
5. Cunningham BA and Schmir GL, Iminolactones. II. Catalytic effects on the nature of the products of hydrolysis. *J Am Chem Soc* **88:** 551–558, 1966.
6. Chaturveldi RK and Schmir GL, The hydrolysis of N-substituted acetimidate esters. *J Am Chem Soc* **90:** 4413–4420, 1968.
7. Lee Y-N and Schmir GL, Concurrent general acid and general base catalysis in the hydrolysis of an imidate ester. 2. Bifunctional catalysis. *J Am Chem Soc* **101:** 3026–3035, 1979.
8. Low JE, Borch RF and Sladek NE, Conversion of 4-hydroperoxycyclophosphamide and 4-hydroxycyclophosphamide to

phosphoramide mustard and acrolein mediated by bifunctional catalysis. *Cancer Res* **42:** 830–837, 1982.
9. DeMaster EG, Quast BJ, Redfern B and Nagasawa HT, Reaction of nitric oxide with the free sulfhydryl group of human serum albumin yields a sulfenic acid and nitrous oxide. *Biochemistry* **34:** 11494–11499, 1995.
10. Hackney DD, Stempel KE and Boyer PD, Oxygen-18 probes of enzymic reactions of phosphate compounds. *Methods Enzymol* **64:** 60–83, 1980.
11. Maragos CM, Morley D, Wink DA, Dunams TM, Saavedra JE, Hoffman A, Bove AA, Isaac L, Hrabie JA and Keefer LK, Complexes of $^{\bullet}$NO with nucleophiles as agents for the controlled biological release of nitric oxide. *J Med Chem* **34:** 3242–3247, 1991.
12. Mitchell RA, Enzyme-catalyzed oxygen exchange reactions and their implications for energy coupling. *Curr Top Bioenerg* **13:** 203–255, 1984.
13. Archer SL, Shultz PJ, Warren JB Hampl V and DeMaster EG, Preparation of standards and measurement of nitric oxide, nitroxyl and related oxidation products. *Methods: Companion Methods Enzymol* **7:** 21–34, 1995.
14. Challis BC and Kyrtopoulos SA, The chemistry of nitrosocompounds. Part 11. Nitrosation of amines by the two-phase interaction of amines in solution with gaseous oxides of nitrogen. *J Chem Soc Perkin I* 299–303, 1979.
15. Challis BC and Kyrtopoulos SA, The chemistry of nitrosocompounds. Part 12. The mechanism of nitrosation and nitration of aqueous peperidine by gaseous dinitrogen tetraoxide and dinitrogen trioxide in aqueous alkaline solutions. Evidence for the existence of molecular isomers of dinitrogen tetraoxide and dinitrogen trioxide. *J Chem Soc Perkin II* 1296–1302, 1978.
16. Ingold CK and Ingold EH, Constituents of dinitrogen tetroxide and trioxide. *Nature* **159:** 743–744, 1947.
17. Beattie IR, Dinitrogen trioxides. *Prog Inorg Chem* **5:** 1–26, 1963.
18. Brittani AH and Cox AP, Microwave spectrum, structure, low frequency vibrations, dipole moment and quadrupole coupling constants of dinitrogen trioxide. *Trans Faraday Soc* **65:** 1963–1974, 1969.
19. Archer MC, Catalysis and inhibition of N-nitrosation reactions. *IARC Sci Publ* **57:** 263–274, 1984.