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A chemist's view of the nitric oxide story

D. Lyn H. Williams
Department of Chemistry, University of Durham, South Road, Durham, UK DH1 3LE.
E-mail: D.L.H.Williams@durham.ac.uk

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1 Introduction

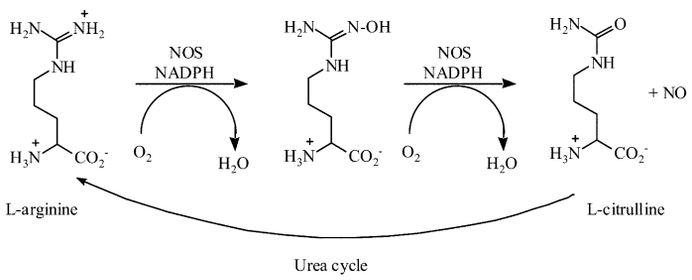
Most chemists are now aware of the series of spectacular developments which occurred from 1988 onwards when it was discovered that nitric oxide is synthesised *in vivo* from L-arginine and that it is involved in a wide range of physiological functions as diverse as vasodilation, inhibition of platelet aggregation, neurotransmission and penile erection together with having a major role to play in the operation of the immune system. The important parts played by Murad,[1] Furchgott[2] and Ignarro[3] in establishing some of these amazing facts were recognised by the award jointly of the Nobel Prize in Physiology or Medicine in 1998. These major discoveries have sparked off massive research efforts in the biology and chemistry of nitric oxide (it has its own Journal) and new developments regarding further involvement of nitric oxide *in vivo* are being made continually. A very large number of papers has been published in the area. There are also many review articles,[4–6] several written by, and aimed at, biologists, biochemists, medical scientists and pharmacologists: it seems an appropriate time to bring together the known chemistry of nitric oxide, within the context of its range of biological activities.

The laboratory preparations of nitric oxide NO, either by the reduction of nitric acid with, for example, copper, or of nitrous acid with, for example, ascorbic acid or iodide ion are very well-known. Equally familiar is the industrial synthesis involving the oxidation of ammonia as the first stage of the manufacture of nitric acid. Nitric oxide is generated in air from nitrogen and oxygen in any high temperature situation, and is a major contributor along with the product of its oxidation nitrogen dioxide, to air pollution in industrial societies. Reduction of NO to nitrogen is one of the main reactions in catalytic converters in cars. It has been estimated that a vast amount of NO is generated annually in electrical storms in the atmosphere, but this is dissipated over a very large volume and is not seen as a major problem. Since nitric oxide is a relatively stable free radical with little tendency to dimerise or to effect hydrogen abstraction reactions, it has also been of much interest to theoretical chemists in terms of establishing its detailed structure. It has also been used to test for the transient appearance particularly of carbon-centred radical intermediates in gas phase reactions.

It came as something of a major surprise, therefore, to learn that *in vivo* nitric oxide is generated in a biosynthetic pathway from the amino acid L-arginine.[7] It has been established by isotopic labelling that the nitrogen atom in nitric oxide derives from the guanidino nitrogen atom of the amino acid and the oxygen atom originates from molecular oxygen. Several studies have shown that *N*-hydroxy-L-arginine is an intermediate, and that the final organic product is L-citrulline, which regenerates L-arginine as part of the urea cycle (Scheme 1). These oxidation processes which require calcium ions, NADPH and tetrahydrobiopterin as co-factors for the enzyme nitric oxide synthase, NOS, have no equivalents in simple non-enzymatic chemistry. Three forms of NOS have been identified:—endothelial NOS (eNOS) which generates NO in the endothelial lining to blood vessels, inducible NOS (iNOS) expressed in macrophages as a response to bacterial and viral infections and neuronal NOS (nNOS) which is present in neurons in the brain and generates NO which acts as a neurotransmitter. Each has a similar structure. Overproduction of NO leads to the serious condition of septic shock and much research has been directed at generating enzyme inhibitors to treat this condition. A number which are effective are modelled on the arginine structure, *e.g.* *N*-methyl- and *N*-nitro-arginine, amongst others. These inhibitors do *not* however show substantial selectivity between the three forms of the enzyme. Overproduction of NO is also increasingly being associated with a range of diseases, although a detailed understanding of the various mechanisms is still at the speculative stage. It is generally believed that brain damage after a blockage of a cerebral artery (a stroke) is at least in part due to the action of NO and there is a suggestion that senility results from NO-induced brain damage. High levels of nitrite ion (the final product of NO oxidation in water) have been found in the joints of sufferers from rheumatoid arthritis, suggesting again overproduction of NO in response to inflammation. More recent work has linked schizophrenia with a malfunction of the biosynthesis of NO. Increased NO levels have also been observed in patients with urinary disorders and multiple sclerosis. The need for a specific enzyme inhibitor is clear. There is also a current debate as to the possible role NO plays in the control of blood pressure. A number of recent reviews are in the extensive literature which discuss in detail the physiological aspects of the action of NO. A useful book, *Methods in Nitric Oxide Research*, was published[8] in 1996 and a series of review articles appeared in *Chemical Reviews* in 2002, particularly highlighting interactions of NO with metal centres together with the chemistry of metal-NO complexes.[9]

2 Reaction with oxygen

Oxidation of NO by oxygen to give nitrogen dioxide is the most well-known and most studied reaction of NO, particularly in



Scheme 1 The biosynthesis of nitric oxide.

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the gas phase in connection with the major air pollution caused by these nitrogen oxides. The same reaction, with the same rate law (eqn. 1), has been established in water, with a third-order rate constant of ~5 × 10^6 dm^6 mol^{-2} s^{-1} at 25 °C, which is unaffected by pH in the range 1–13.[10–12] This does mean that for example if [O_2] is ~1 × 10^{-3} mol dm^{-3}, and [NO] is 1 × 10^{-9} mol dm^{-3} (a typical *in vivo* situation) then the half-life for NO oxidation is ~50 hours, *i.e.* the oxidation reaction is negligibly slow under these conditions. The final product in water is, somewhat unexpectedly, exclusively nitrite ion, with very little if any nitrate ion. This has been rationalised in terms of oxidation of NO to NO_2, then a further reaction of NO_2 with NO to give N_2O_3, which must compete very effectively with the hydrolysis of NO_2 (eqns. 2–5). A numerical analysis using individual rate constants obtained by pulse radiolysis, however, does predict very low nitrate levels.[13]

$$\text{Rate} = k[\text{NO}]^2[\text{O}_2] \quad (1)$$

$$2\text{NO} + \text{O}_2 = 2\text{NO}_2\ (\text{N}_2\text{O}_4) \quad (2)$$

$$\text{NO} + \text{NO}_2 = \text{N}_2\text{O}_3 \quad (3)$$

$$\text{N}_2\text{O}_3 + \text{H}_2\text{O} = 2\text{NO}_2^- + 2\text{H}^+ \quad (4)$$

$$\text{N}_2\text{O}_4 + \text{H}_2\text{O} = \text{NO}_2^- + \text{NO}_3^- + 2\text{H}^+ \quad (5)$$

The interpretation is supported by the fact that these aerated solutions of nitric oxide can effect nitrosation of a number of standard substrates such as amines and thiols: N_2O_3 is of course a very well-known nitrosating agent. Failure to remove all traces of oxygen can result in the formation of nitrosation products, and this has led, particularly in the early biological literature, to erroneous statements that NO itself is an electrophilic nitrosating species. Interestingly, the final 'nitrogen' products of the oxidation of NO *in vivo*, are claimed to be a mixture of nitrite and nitrate ions. It is conceivable that at much lower NO concentrations such as those encountered *in vivo*, the competition between N_2O_4 hydrolysis and bimolecular reaction between NO and NO_2 now favours the former reaction leading to the formation of both nitrite and nitrate ions.

3 Peroxynitrite and the reaction of NO with superoxide

Peroxynitrite ($ONOO^-$) is a very powerful oxidising agent whose chemistry *in vitro* is reasonably well established.[14] The anion is quite stable at high pH, but on protonation (pK_a 6.5) there is a fairly rapid isomerisation reaction to give nitrate ion (eqn. 6) with a first-order rate constant of ~1 s^{-1}.[15] Decomposition also occurs, albeit more slowly, at higher pH, when the products are mainly nitrite ion and oxygen. A free radical mechanism had been proposed for this decomposition, but a more recent study over a wider range of concentration argues in favour of a radical-free mechanism in which an adduct is formed from peroxynitrite and peroxynitrous acid, which breaks down in a rate-limiting step to give the observed products.[16] Nitrosation of hydrogen peroxide or its anion by nitrous acid,[17] alkyl nitrites[18] or *S*-nitrosothiols[19] readily generates peroxynitrous acid/peroxynitrite (eqns 7 and 8). If the nitrous acid route is used then the pH of the solution must be raised immediately after reaction, otherwise nitrate ion will be a significant contaminant. The use of alkyl nitrites or of *S*-nitrosothiols has the advantage that the synthesis itself can be effected in alkaline solution and so no nitrate ion will be formed.

$$\text{ONOOH} = \text{NO}_3^- + \text{H}^+ \quad (6)$$

$$\text{H}_2\text{O}_2 + \text{HNO}_2 = \text{ONOOH} + \text{H}_2\text{O} \quad (7)$$

$$\text{HO}_2^- + \text{RONO (or RSNO)} = \text{ONOO}^- + \text{ROH (or RSH)} \quad (8)$$

It is widely believed that peroxynitrite (or peroxynitrous acid) reacts with a variety of biological targets generating cell damage.[20] These include the nitration of tyrosine, the rupture of DNA strands and the oxidation of haem proteins. There has been a controversy (not yet resolved) regarding the mechanism of the first of these reactions (which is associated with a range of diseases), as to whether nitration here is a free radical process involving NO_2 or a heterolytic process involving the nitronium ion NO_2^+. Given the known chemistry of aromatic nitration, it does seem extremely unlikely that the latter can be involved at physiological pH values. *In vivo* there is a rapid reaction (as expected) between NO and the superoxide radical anion which is believed to occur at a rate close to the diffusion controlled limit.[21] This reaction is claimed to be one of the ways in which the body gets rid of excess NO. There has, given these facts, been a considerable interest in the role of peroxynitrite *in vivo*, and a large literature has built up in the biological domain.

As expected for such a powerful oxidising agent, peroxynitrite converts thiols rapidly to disulfides even more efficiently than hydrogen peroxide.[22] This is an important reaction *in vivo*, for low molecular weight thiols such as cysteine and glutathione and also for protein-bound thiols. This is believed to provide an efficient route for the removal (detoxification) of peroxynitrite in the body. Interestingly there are reports[23] of the formation of low yields (1–2%) of *S*-nitrosothiols RSNO (believed to be carriers of NO *in vivo*—see later) during this reaction with excess thiol. A direct nitrosation reaction by peroxynitrite or its protonated form is chemically very unlikely. We have recently shown[24] that these side-products arise from the nitrous acid generated during the thiol oxidation (eqn. 9). This then effects conventional electrophilic nitrosation with excess thiol (eqn. 10). We showed that nitrous acid is formed in this reaction, by the appearance of its characteristic UV spectrum, when reaction was carried out without a large excess of thiol. Many other oxidations by peroxynitrite (*e.g.* of hydroxylamine and iodide ion) are known to generate nitrous acid (or nitrite anion) quantitatively. Thiol oxidation has only previously been studied in mildly alkaline solution, when it has been demonstrated that reaction occurs *via* peroxynitrous acid rather than its anion. There is no reason therefore to expect that this will also not take place in mildly acidic solution (in competition with the isomerisation to nitrate ion). Using quite a large excess of thiol, we found high conversion to RSNO in the pH range 3–4 The kinetic analysis fitted the proposed reaction pathway (eqns 9 and 10), where the rate-limiting stage is the nitrosation of the thiol, yielding values of third-order rate constants (eqn. 11) for the thiol nitrosation which were in excellent agreement with those in the literature[25] for the direct *S*-nitrosation of thiols, for a number of thiols, including glutathione and cysteine. As the pH is increased the yield and rate of formation of RSNO decreased as expected, as nitrous acid (pK_a 3.1) is deprotonated. We detected 5% RSNO in this reaction carried out at pH 6. This mechanistic interpretation readily accounts for the 1–2% yield of RSNO detected at physiological pH of 7.4. This reaction could be important *in vivo*, since, as will be discussed later, NO can be readily obtained from RSNO species. Interestingly, though probably without significance *in vivo*, at higher acidities (> ~0.3 mol dm^{-3} H^+), there is kinetic evidence (based on two consecutive exponential processes) that a *protonated* form of peroxynitrous acid generates RSNO in a direct nitrosation process. The evidence is not yet conclusive, but the suggestion makes chemical sense in that electrophilic nitrosation can occur from this protonated form, with hydrogen peroxide as the leaving group (eqn. 12). There is of course a

strong analogy here with the suggestion that the protonated form of nitrous acid $H_2NO_2^+$ is the nitrosating agent in acidic solutions of nitrous acid, when the leaving group is the water molecule. Peroxynitrous acid can also effect hydroxylation as well as nitration, and homolytic fission (eqn. 13) is an attractive possibility, but thus far does not have the necessary experimental support.

$$2RSH + ONOOH = RSSR + HNO_2 + H_2O \quad (9)$$

$$RSH + HNO_2 = RSNO + H_2O \quad (10)$$

$$Rate = k\,[RSH]\,[HNO_2]\,[H^+] \quad (11)$$

$$ONO^+(H)OH + RSH = RSNO + H_2O_2 + H^+ \quad (12)$$

$$HOONO = HO^{\bullet} + NO_2^{\bullet} \quad (13)$$

4 Nitrosation products from aerated NO aqueous solutions

The solubility of NO in water is ~1.7×10^{-3} mol dm^{-3} at 25 °C, *i.e.* it is similar to that of oxygen and nitrogen. It is now accepted that when oxygen is rigorously excluded, aqueous NO solutions do not generate nitrosation products. There are many reports in the literature of the formation of nitrosation products from aerated NO solutions, notably from amines and thiols. Indeed one assay[26] for NO depends on generating a diazonium ion from 2,3-diaminonaphthalene, which cyclises to give a naphthotriazole, which is highly fluorescent, and so can easily be quantified. Additionally there is, at quite high pH, a reaction between NO and thiolate ions, which leads to disulfide formation and is not a conventional nitrosation reaction.[27] and probably not an important reaction *in vivo.*

Recently, two papers[28,29] have independently presented results of a mechanistic study of the nitrosation of thiols and amines by aerated aqueous NO solutions. Both establish the rate law, which is the same as that found for the oxidation of NO in water (eqn. 1), and both sets of results show good agreement with the measured third-order rate constant. This reveals that the rate limiting step is one of the steps involved in the oxidation of NO. This has been confirmed by showing that the measured rate of reaction is independent of the nature or concentration of the amine or thiols involved. In each case high yields of nitrosation products were observed when NO was bubbled into a solution containing the substrate. However, when the thiols were added to a pre-prepared NO solution no RSNO was formed. The nitrosating property is lost by hydrolysis of the intermediate N_2O_3, except in acidic solution when nitrite ion is protonated. This must mean that the nitrosation reaction (eqn. 14) competes very effectively with the hydrolysis of N_2O_3 (eqn. 15). One study interprets the results entirely in terms of N_2O_3 reactions, whilst the other allows for the possibility of reaction *via* N_2O_4 as well. Interestingly, one group concludes that this series of reactions with thiols cannot provide a route for the biosynthesis of RSNO species, since the reactions are considered to be too slow: the other study argues, particularly for glutathione, that this *is* a feasible pathway for the formation of *S*-nitrosoglutathione *in vivo.*

$$R_2NH + N_2O_3 = R_2NNO + NO_2^- + H^+ \quad (14)$$

$$N_2O_3 + H_2O = 2NO_2^- + 2H^+ \quad (15)$$

We have recently shown[30] that NO generated from *S*-nitrosothiols by the copper catalysed route (discussed later) in aerated aqueous solution at pH 7.4 will also generate nitroso compounds. Thus we obtained almost quantitative yields of *N*-nitroso-*N*-methylaniline from *N*-methylaniline, but much lower yields of 4-nitrosophenol from phenol. Here, the rate limiting step is the release of NO from RSNO, and as expected reactions were completely inhibited when Cu^{2+} or oxygen were rigorously excluded. Phenol is orders of magnitude less reactive in nitrosation than is *N*-methylaniline, and so the nitrosation of phenol here by N_2O_3 competes much less well with N_2O_3 hydrolysis. Increasing the phenol excess concentration does lead to higher yields of 4-nitrosophenol as predicted. We also find nitrite ion quantitatively as the inorganic nitrogen product, which supports the proposal that the intermediate involved is N_2O_3.

Unexpectedly the formation of what is probably a nitroso derivative of uric acid was noted (never previously reported), from the reaction of aerated NO (generated *in situ*) with uric acid at pH 7.4. The product was unstable and not isolated. This procedure using NO in aerated aqueous solution should allow a ready synthetic route to nitrosoamides and nitrososulfonamides in a non-acidic medium, which thus prevents their ready acid-catalysed decomposition.

5 Nitric oxide donors

As mentioned earlier, considerable effort has been/is being directed at developing enzyme inhibitors to counteract overproduction of NO. Similarly, there are medical conditions where due to some malfunction of the arginine–NOS cycle, there are deficiencies of NO. To this end much effort has been directed at the development of NO-donors for clinical use. In addition, there are clinical situations (*e.g.* in some operations) where benefits can be achieved by making use of the biological properties of NO generated from NO-donors, such as the lowering of blood pressure following vasodilation and also the inhibition of platelet aggregation. Testing for other clinical uses is under way.

5.1 Glyceryl trinitrate GTN (nitroglycerine)[31]

The most widely used NO-donor (which has been used for over a century) is, rather unexpectedly, the high explosive glyceryl trinitrate (structure **1**). This was first used by an English doctor William Murrell around 1879 to treat angina pectoris. It has proved very effective in that it is quick acting in producing relief from the acute chest pain, using a variety of delivery procedures, including transdermal, sublingual and spray methods. It does suffer from being short-acting and in a number of patients a tolerance to it is generated. Nevertheless it continues to be included in the top 100 prescribed drugs. Other alkyl nitrates, including isosorbide dinitrate and isosorbide 5-mononitrate have also been developed and used clinically, but appear to have no major advantages over the use of GTN.

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It is generally assumed that GTN develops an endothelium derived relaxant factor *in vivo*, which then brings about vasodilation. This suggestion was first made by Murad in 1977,[1] and has subsequently been demonstrated experimentally, when this factor was shown to be NO. However, there have been conflicting reports regarding NO release from GTN *in vitro.* Many claim that thiols are necessary for this reaction to occur: some workers report that NO can be detected by trapping it with Fe(II)-oxyhaemoglobin, whilst others fail to detect NO from the reaction of GTN with cysteine using the very sensitive NO-specific electrode method. There is no doubt, however, about the detection of NO *in vivo* following GTN administration. This has been noted in exhaled air, cells, blood, the liver and in other organs. Considering the importance of GTN as a prescribed drug it is very surprising that more is not known about its mode of action. Only a few studies have been reported for

the situation *in vitro*, and their conclusions have been far from satisfactory in terms of establishing a reaction mechanism, which in any case probably has no relevance *in vivo.* Organic nitrates generally are quite stable in aqueous solution at physiological pH, but there is a reaction in the presence of thiols to give (eqn. 16) an *S*-nitrothiol (a thionitrate), which decomposes in the presence of excess thiol to give the disulfide and nitrite ion.[32] Perplexingly it is claimed that only for *some* thiols (including cysteine) the reaction goes on to give some NO! The yields of NO are often quite low (typically 5%) and in some cases studies have failed to find any NO at all. One proposed rationalisation includes, for some thiols, a rearrangement of the *S*-nitrothiol to an unstable sulfinyl nitrite R′SONO, from which NO could be generated by homolytic fission. Other schemes have also been advocated, but almost nothing is known with any certainty, and this remains a major challenge for physical organic chemists.

$$RONO_2 + R'SH = ROH + R'SNO_2 \qquad (16)$$

In contrast to the scant information for the situation *in vitro*, there have been a large number of studies carried out *in vivo.* However, it is much more difficult to establish a mechanism under these conditions and many of the conclusions are not helpful and in some cases are contradictory. It is virtually certain however that NO is generated,[33] together with 1,2-, and 1,3-glyceryl dinitrates. Clearly this reaction needs enzyme activation, given the absence of a clear pathway to NO *in vitro*. Two enzyme systems had been proposed for this biosynthetic transformation of GTN to NO. One is an NADPH-dependent cytochrome P450,[34] and the other some isozymes of the glutathione-*S*-transferase family.[35] Other studies have often questioned the involvement of these systems in GTN bioactivation. Recently however Stamler and co-workers[33] have isolated an enzyme in the mitochondria of cells (mitochondrial aldehyde dehydrogenase) which generates NO from GTN. The enzyme becomes used up on repeated dosage which accounts for the tolerance problem in some patients. This work, it is claimed represents a major advance in the understanding of the mode of action of GTN *in vivo*.

5.2 Alkyl nitrites RONO

In common with alkyl nitrates the antianginal properties of alkyl nitrites, usually amyl nitrite, have also been known for over a hundred years. They were used clinically for this purpose in the early years, by inhalation, possibly because of their higher volatility. There are however a number of unwelcome side-effects compared with the use of GTN, *e.g.* headaches and dizziness, and so are not currently used routinely for controlling angina. They are however widely used or rather abused as recreational drugs, particularly in the gay community. Amyl nitrite is the constituent of 'poppers'. They act by making use of their vasodilatory properties. *In vitro* it is easy to see a non-enzymic pathway to nitric oxide formation. There is a fairly rapid reaction with thiolate ion[36] (eqn. 17) at physiological pH (the pK_a values of most thiols are ~8) generating an *S*-nitrosothiol, from which there is a ready pathway to NO formation by a Cu^{2+} catalysed reaction (see later). It has been also been shown that NO is released from alkyl nitrites *in vivo*, but it is not known if this is an enzyme-catalysed process.

$$RONO + R'S^- = RO^- + R'SNO \qquad (17)$$

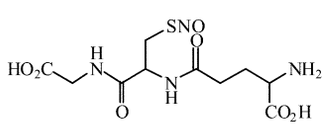
5.3 *S*-Nitrosothiols RSNO

In the last few years there has been an explosion of interest in the chemistry, biochemistry and physiology of the sulfur analogues of alkyl nitrites, *S*-nitrosothiols, sometimes called thionitrites. The reason for this interest arises because as a class they have been found to have much the same physiological properties as NO itself, particularly of vasodilation[37] and of the inhibition of platelet aggregation.[38] They have also been identified in bodily fluids, notably as *S*-nitrosoglutathione (GSNO)[39] and *S*-nitrosoalbumins.[40] Indeed, the current belief[41] is that NO is transported around the body as RSNO (mostly as the nitrosoalbumins), from which NO can be released under certain conditions. This belief derives mostly from the fact that the measured half-life of NO itself *in vivo* is very short (estimates range from a few seconds to a few minutes), whereas RSNO species are generally much more stable in solution.

S-Nitrosothiols are very readily generated in solution by conventional nitrosation of thiols, and examples have been known for about a hundred years.[42] The simplest procedure if the thiol is reasonably water soluble, is to use sodium nitrite in mildly acidic solution (eqn. 18). In contrast with the corresponding

$$RSH + HNO_2 \rightleftarrows RSNO + H_2O \qquad (18)$$

reactions of alcohols to give alkyl nitrites, the equilibrium position lies well over to the right with equilibrium constants of ~10^5–10^6 dm^3 mol^{-1}.[43] This difference between ROH and RSH has been rationalised in terms of the different nucleophilicities of the O- and S-atoms in the alcohols and thiols, and the different basicities of the same atoms in the alkyl nitrites and *S*-nitrosothiols. Nitrosation of thiols has been examined mechanistically and follows the pattern of amine nitrosation, in which both acid- and halide ion-catalysis occurs. The reactive species are $H_2NO_2^+$ (or NO^+), N_2O_3 or the nitrosyl halide, depending on the reaction conditions. Most of the reactions are very rapid, and solutions of the *S*-nitrosothiol can be prepared often in under a minute. In principle any other nitrosating agent (such as an alkyl nitrite) will also bring about reaction in non-aqueous solution or in basic media if necessary. However, in contrast to the alkyl nitrites, relatively few *S*-nitrosothiols are stable in the pure form. Two well-known examples, which are indefinitely stable as solids are *S*-nitrosoglutathione (GSNO) **2**[44] and *S*-nitroso-*N*-acetylpenicillamine (SNAP) **3**.[45] More recently the number of reported stable examples (in the pure form) has increased significantly. These include certain thiosugar derivatives[46,47] (*e.g.* **4** and **5**) and derivatives containing large bulky groups, which seem to confer stability.[48] Solutions generated in aqueous acid solution are however generally sufficiently stable to allow kinetic and other experiments to be carried out.



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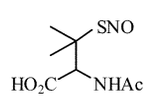

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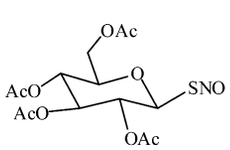

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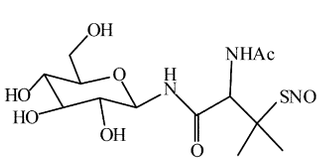

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S-Nitrosothiols decompose thermally and photochemically to give the disulfide and initially nitric oxide (eqn. 19). For some, the thermal reaction is clearly very slow at room temperature—solid SNAP has to be heated to about 150 °C before significant decomposition occurs,[49] but it decomposes much more readily in refluxing methanol.[45] In many other cases *e.g.* *S*-nitrosocysteine, the decomposition rate in the pure solid state is much higher, not allowing isolation. In aqueous solution however, in the presence of metal ion chelators, the thermal decomposition rate is too slow to be significant at room temperature. The photochemical reaction occurs readily when irradiation at ~340 or 540 nm (maximum absorbances in the

UV/visible spectrum) takes place.[50,51] The likely sequence of reactions for GSNO decomposition is shown in eqns 20 and 21. In the presence of oxygen the peroxy radical GSOO$^{\bullet}$ was observed (eqns 22 and 23), giving an alternative route to nitric oxide formation. The liberated nitric oxide was trapped with oxyhaemoglobin (to give nitrate ion) or allowed to oxidise and yield nitrite ion in aqueous solution. Thiyl radicals have also been detected by EPR during photolysis,[52] as have more stable nitroxides generated on addition of radical generators during both photolysis and thermolysis.[53] Photolysis of GSNO results in an enhanced cytotoxic effect on some leukaemia cells, and there is considerable potential in this form of visible light phototherapy, which is not (in contrast with photodynamic therapy) entirely dependent on the presence of oxygen.

$$RSNO = RSSR + NO \quad (19)$$

$$GSNO = GS^{\bullet} + NO \quad (20)$$

$$GS^{\bullet} + GSNO = GSSG + NO \quad (21)$$

$$GS^{\bullet} + O_2 = GSOO^{\bullet} \quad (22)$$

$$GSOO^{\bullet} + GSNO = GSSG + O_2 + NO \quad (23)$$

In addition to the above reactions, *S*-nitrosothiols also decompose at ambient temperatures in aqueous solution at around pH 7.4, by a copper ion-catalysed process.[54] No other metal ions appear to effect catalysis, although there are claims that Fe^{2+} may do so, but this has not been satisfactorily demonstrated experimentally. There is another well-known reaction with mercuric and silver ion,[55,56] which generates nitrous acid and not nitric oxide, and which has been adapted for the analytical determination of thiols and also *S*-nitrosothiols. The copper ion catalysed-reaction has been much confused with the spontaneous thermal decomposition, particularly in the earlier biological literature, since Cu^{2+} present at even trace impurity levels are sufficient to bring about decomposition. Copper ion catalysis was first observed by us in 1993[54] when we found that decomposition was completely halted in the presence of the metal ion chelator EDTA; there was a first-order kinetic dependence on added Cu^{2+}. Later,[57] we showed (making use of the specific Cu^{+} complexing agent neocuproine) that the effective reagent is in fact Cu^{+}, which is generated by thiolate reduction. Again only trace catalytic quantities of thiolate are needed, which are supplied by the (slight) reversibility of the formation reaction of RSNO from thiol and nitrous acid.[43] The effect of changing the [RS^{-}] is shown graphically in Fig. 1. Trace (a) shows very little decomposition of SNAP, when [RS^{-}] is much reduced by generating SNAP with a two-fold excess of nitrous acid. Trace (b) is generated when equimolar concentrations of thiol and nitrous acid are used to produce SNAP and trace (c) results when a twofold excess of thiol is used to generate the *S*-nitrosothiol. The range of rate constants is very large.

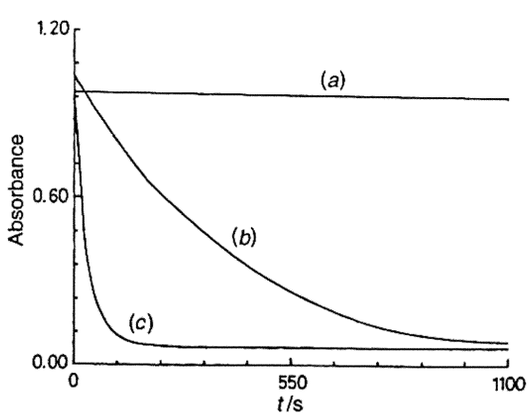


Fig. 1 Absorbance–time plots for the decomposition of SNAP (1×10^{-3} mol dm^{-3}) in the presence of added Cu^{2+} (1×10^{-5} mol dm^{-3}), prepared *in situ* with (a) excess HNO_2 (2×10^{-3} mol dm^{-3}), (b) equimolar thiol and HNO_2 and (c) excess thiol (1×10^{-3} mol dm^{-3}).

The known facts are fully accounted for by the sequence of eqns 24–26. Details of the mechanism of the actual reaction of Cu^{+} with RSNO remain speculative at this stage, but it is likely that coordination at the sulfur atom occurs, possibly by way of an intermediate. Both Cu^{2+} and RS^{-} are regenerated and so are truly catalytic. Often the rate-limiting stage is reaction 26, when there is a first-order dependence on RSNO and Cu^{2+}, but for some reactants, under certain conditions, the reduction of Cu^{2+} is rate limiting which results in a zero-order kinetic dependence on RSNO. The involvement of thiolate was clearly demonstrated[58] by reducing its concentration, by increasing the nitrous acid concentration in RSNO synthesis (eqn. 24). This significantly reduced the rate of RSNO decomposition, eventually to a negligible level. This accounts for the stability of RSNO solutions in acid media, where thiolate is protonated. One consequence of these findings is that rate constants for RSNO decomposition reported in the literature have very little if any value, so that there are no reliable structure–reactivity data. It is possible to account for the copper problem by working over a range of added [Cu^{2+}], but to take into account the probable variation in [RS^{-}] in separate experiments with different RSNO compounds is much more difficult.

$$RSH + HNO_2 \rightleftarrows RSNO + H_2O \quad (24)$$

$$2Cu^{2+} + 2RS^{-} = 2Cu^{+} + RSSR \quad (25)$$

$$Cu^{+} + RSNO = Cu^{2+} + RS^{-} + NO \quad (26)$$

The situation is more complicated still if there are higher concentrations of thiol present or added, since many thiols (notably penicillamine,[58] which is used clinically for this purpose in the treatment of Wilson's disease) will complex Cu^{2+}, acting in effect as does EDTA, thus stopping the reaction. In some cases the resulting effect is that low thiolate concentrations promote reaction by increasing the rate of Cu^{+} formation, whereas at higher thiolate levels reaction is inhibited by Cu^{2+} complexation.[57] This is shown in Fig. 2, where the first-order rate constants for SNAP decomposition are plotted against the concentration of added *N*-acetylpenicillamine (NAP).

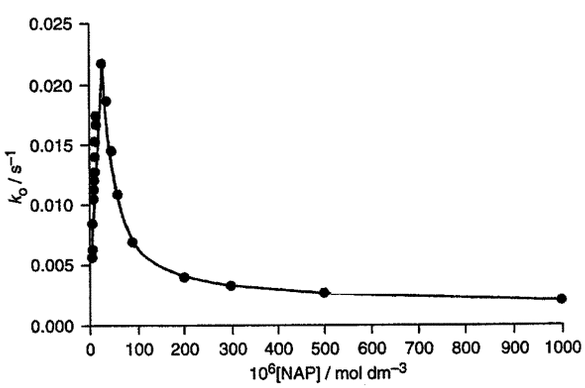


Fig. 2 First order rate constants for the decomposition of SNAP as a function of added *N*-acetylpenicillamine (NAP).

Another complication arises if the RSNO contains the glutamate residue (as does GSNO and the *S*-nitroso derivative of the dipeptide Glu-Cys), since the two glutamate residues in the product disulfide complex Cu^{2+} bringing reaction to a rapid conclusion.[59] This is evident experimentally in the reaction of GSNO at millimolar concentrations (which is easily followed spectrophotometrically noting the disappearance of the absorbance due to GSNO), and disappears at micromolar levels (when

NO release can be directly monitored using the specific NO electrode), since the concentration of the disulfide is now very much reduced. Explanations of the 'stability' of GSNO based only on observations at millimolar concentrations are not correct.[60] This stability is lost not only by the action of the enzyme γ-glutamyltranspeptidase, but also by simple dilution to micromolar concentration levels—a more likely state of affairs *in vivo*. Under these conditions GSNO decomposition occurs at approximately the same rate as does that of *S*-nitrosocysteine. Similar behaviour was noted for the copper-catalysed decomposition of the *S*-nitroso sugar derivative **4**.[46]

No radical intermediates have been detected[61] during the copper catalysed process, in contrast to the photochemical process. Many authors however continue to write the mechanism in terms of 'spontaneous homolytic bond fission', which is clearly not the case.

There has been a significant reluctance by biologists to accept the possibility that NO can be generated from RSNO species *in vivo*, since any copper ion in the body is largely complexed with proteins and peptides. However it has been shown that Cu^{+} *can* readily be accessed from Cu^{2+} bound in this way, by thiolate reduction at physiological pH, and such complexes can act catalytically in the decomposition of *S*-nitrosothiols.[62] Thus the neocuproine–Cu^{+} complex can readily be generated from Cu^{2+} when it is bound to the tripeptide Gly-Gly-His, or to histidine itself or even to human serum albumin, when each is treated with a thiol. Each of these systems catalysed the decomposition of SNAP. Similarly GSNO will decompose completely[62,63] and deliver nitric oxide at millimolar concentrations in the presence of added glutathione (GSH), no doubt since the thiolate can generate Cu^{+} from Cu^{2+} bound to GSSG.

There are, however, a few references in the biological literature which suggest that the copper-catalysed decomposition pathway for RSNO compounds may have some importance *in vivo.* For example, it has been demonstrated that both the vasodilation properties and the anti-platelet aggregation effects of GSNO and SNAP are significantly reduced in the presence of a specific Cu^{+} chelator.[64,65] More recently, again by using the same chelator, it has been found that Cu^{+} has a major role to play in the decomposition of GSNO in endothelial cells.[66]

Another reaction of *S*-nitrosothiols which may also have a role to play *in vivo*, is the so-called transnitrosation reaction (eqn. 27) whereby the NO group is transferred directly to a different thiol thus generating another *S*-nitrosothiol.[67] This reaction is not affected by the presence of copper ions or of EDTA and experiments have usually been carried out in the presence of a chelator to avoid any complication from the Cu^{2+}-catalysed decomposition. As expected the reaction is reversible and follows reversible second-order kinetics.[68] The forward reaction can be studied independently by working under conditions where $[R'S^{-}] \gg [RSNO]$, although the reaction is not easy to follow spectrophotometrically, since the change in extinction coefficient is usually quite small during reaction. It has been shown[69] from rate–pH studies that the reactive species is the thiolate anion and not the thiol, and kinetic substituent effects confirm that the reaction is one of nucleophilic attack by the thiolate anion. This reaction is of course a specific example of a more general range of reactions where nucleophiles react at the nitroso nitrogen atom in a nucleophilic substitution reaction. Thus far there is no evidence for the existence of an intermediate. The following nucleophiles have been studied and their reactivities have been established:—amines, hydrazine, hydroxylamine, azide ion, ammonia, semicarbazide, thiomorpholine, ascorbate, hydroperoxide anion, sulfite ion, thiourea, thiocyanate ion, thiosulfate ion, sulfide ion as well as a range of thiolate ions.[70,71,19] Chemists will not be surprised at these reactions, since *S*-nitrosothiols are merely behaving in a similar way to their oxygen counterparts, the alkyl nitrites, in which they act as electrophilic nitrosating agents. The reaction of *S*-nitrosothiols with ascorbate is interesting in that there are two reactions, both generating NO.[72] At low [ascorbate] at pH 7.4, reaction occurs by the Cu^{2+}-catalysed route, which is cut out by the addition of EDTA, whilst at higher [ascorbate], ascorbate acts as a nucleophile, which is unaffected by Cu^{2+} or EDTA. Note that the 'organic products' here are different from the two reactions, *i.e.* the disulfide and the thiol respectively.

$$RSNO + R'S^{-} \rightleftarrows RS^{-} + R'SNO \qquad (27)$$

There has been much work directed at the observation and detection of RSNO compounds *in vivo*. There is no doubt that GSNO and *S*-nitrosoproteins are present in a variety of body organs, although in some cases their magnitudes may have been exaggerated, and there is a lot of evidence which implicates *S*-nitrosothiols as intermediates in signalling processes. *S*-Nitroso-haemoglobin has been detected in the bloodstream.[73] An *S*-nitroso protein derivative of serum albumin (nitrosation taking place at the free -SH group of Cys-34) has been isolated and characterised.[74] It is quite stable in aqueous solution at pH 7.4, no doubt since the rest of the protein molecule effectively complexes Cu^{2+}, but like GSNO, will release NO in a Cu^{2+}-catalysed reaction when it is present at the micromolar concentration level.[75] Transnitrosation to cysteine occurs quite readily generating *S*-nitrosocysteine which decomposes to give NO at a much faster rate than does the protein derivative. *S*-Nitrosoalbumins have also been generated by transnitrosation from a number of low molecular weight *S*-nitrosothiols.[76] There is currently much debate regarding the possible role of *S*-nitrosohaemoglobin (HbSNO) in the control of vascular tone. The following are known facts:

(i) HbSNO has been detected in red blood cells—the S-nitrosation is believed to occur at the β-93 cysteine residue:

(ii) Oxygenated haemoglobin (oxyHb) reacts rapidly with nitric oxide to give nitrate ion and metHb—a reaction which is believed to be a major route to the destruction of excess NO:

(iii) blood plasma contains ~28 nM RSNO, largely as the serum albumin derivative:

(iv) NO reacts with deoxygenated haemoglobin to form a stable nitrosyl Hb.

A hypothesis has been put forward which in outline suggests that when HbSNO is deoxygenated, the NO group is transferred (possibly by transnitrosation) to GSH and the GSNO formed is eliminated from the red cells, generating vasodilation.[77] The haemoglobin molecule can sense the oxygen content around it, and if it is too low, remedy this by effecting vasodilation. This hypothesis has not been fully tested and is currently being hotly disputed by certain groups. The mode of formation of HbSNO is also a contentious issue. Initially a transnitrosation process (eqn. 27) from GSNO was advocated. However, a recent communication,[78] where FTIR is used to probe intermediates, supports the formation of NO *via* the Cu^{+} reaction (eqn. 26), and further proposes that HbSNO is formed by Cu^{2+} catalysis of the reaction of NO with thiolate *i.e.* the reverse of reaction 26. There is at least one earlier interesting report that NO in the presence of Cu^{2+} brings about rapid *S*-nitrosation of both bovine- and human-serum albumin.[79] This highlights one of the great unsolved problems—how are *S*-nitrosothiols formed *in vivo*? All sensible estimates involving reaction *via* N_2O_3 suggest that at the prevailing concentrations of both NO and O_2, the oxidation of NO is much too slow to be significant. Some have argued[80] that in membranes the reactant concentrations are both higher than they are in the bulk, which will increase the reaction rate. Transnitrosation reactions cannot be totally ruled out in-spite of the evidence presented above with HbSNO. There are many reports which show *in vitro* that some metal nitrosyls (readily generated from NO) can effect conventional electrophilic nitrosation of a number of substrates including thiols. There is also the possibility of some other (unknown) oxidant which will generate N_2O_3 more rapidly—this might be the situation in the recently reported formation of

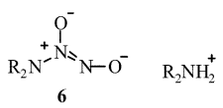
RSNO from RSH and NO in the presence of ferrimyoglobin.[81] There, a reasonable rationale would be that NO is oxidised by Cu^{2+} to N(III), but as yet there have been no mechanistic studies.

There is a recent comprehensive review of the biochemistry and physiology of *S*-nitrosothiols,[82] and an account of their analysis and biological roles.[83]

5.4 Diazeniumdiolates (NONOates)

Nitric oxide forms reasonably stable 1 : 1 complexes with amines. Formerly these were known as Drago complexes,[84] but more recently have acquired the NONOates terminology, following the developmental work of Keefer and co-workers.[85] They are diazeniumdiolate salts of the type $R_2N^+H_2R_2NN_2O_2^-$ (see structure **6**) and are readily prepared by exposing a solution of the amine in an organic solvent to several atmospheres of NO gas for a few days, and the complex filtered off. They are hygroscopic and oxidise slowly in air, so are stored under nitrogen or argon. In water they decompose spontaneously and photochemically to regenerate the amine and NO (eqn. 28). No detailed mechanistic study of this regeneration reaction appears to have been carried out, but it is likely that this is a free radical process. Not surprisingly, NONOate solutions show the same biological properties as does NO, in particular of vasodilation and inhibition of platelet aggregation, and as such are/have been much used as NO donors in research experiments both *in vivo* and *in vitro*.

$$R_2NN_2O_2^- = 2NO + R_2N^- \; (\longrightarrow R_2NH) \qquad (28)$$



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5.5 Transition metal nitrosyl complexes

There is a book[86] devoted to the synthesis and reactions of metal nitrosyl complexes, which gives an excellent and comprehensive account of the subject. Within the context of the nitric oxide 'story' by far the most well-known nitrosyl complex is the salt sodium nitroprusside $2Na^+ [Fe(CN)_5NO]^{2-}$. Its hypotensive property and ability to reduce platelet aggregation have been known for a long time and it has been used clinically to lower blood pressure in operations and medical emergencies for over forty years. In recent times much research has shown that a vast range of biological effects of nitroprusside parallel those of nitric oxide itself, so that it is reasonable to assume that free NO is generated *in vivo*.[87] Nitroprusside does not however spontaneously release NO in the dark, and so it is likely that some enzymatic metabolism is necessary. Photochemical decomposition is well-known,[88] leading to the aqua complex and NO (eqn. 29), which then leads on to cyanide release. *In vitro* many nucleophilic species, including amines, thiols, hydroxide ion and carbanions, react readily with nitroprusside generating products which have been rationalised in terms of an overall transfer of NO^+—amines forming diazonium ions or nitrosamines for example.[89] The reaction with thiols generates an intense red coloured solution, believed to be an adduct (eqn. 30). This structure has recently been confirmed using FTIR techniques for the first time.[90] The current belief is that the thiol adduct is an intermediate in the generation of NO *in vivo*, which requires additionally some enzyme or protein (recently isolated in membrane-bound form) activation.

$$[Fe(CN)_5NO]^{2-} + H_2O = [Fe(CN)_5H_2O]^{2-} + NO \qquad (29)$$

$$[Fe(CN)_5NO]^{2-} + RS^- = [Fe(CN)_5RSNO]^{3-} \qquad (30)$$

Dinitrosyl iron thiol complexes, DNIC, *e.g.* **7** have been discovered in biological systems.[91] This has prompted much research activity into their chemistry along with the related iron sulfur nitrosyl clusters such as Roussin's red and black salts ($[Fe_2S_2(NO)_4]^{2-}$ and $[Fe_4S_3(NO)_7]^{2-}$ respectively).[92] All show biological properties, such as vasodilation *etc.*, associated with NO release, and it has been shown that a dinitrosyl iron complex will effect *S*-nitrosation of serum albumin.[93] Suggestions have been made that DNICs could act as storage and transport vehicles of NO *in vivo*, but just as for *S*-nitrosothiols, this has not been established with certainty.

7

A large range of other NO donors has been studied, including other metal nitrosyls, *N*-nitrosamines and their derivatives, furoxans (which have potential antiplatelet aggregation clinical use), *C*-nitroso compounds, sydnonimines (one derivative is in current use as an antianginal drug) and oximes. This article has concentrated on the known chemistry of the more well-known NO donors. There is a recent excellent and comprehensive review of the current position of all known NO donors.[94]

6 Mode of action of nitric oxide

As expected, there has been a vast amount of research aimed at establishing the mode of action of NO in the body. It is now generally accepted that vasodilation occurs by activation of the enzyme soluble guanylate cyclase (sGC) by nitric oxide (probably by reaction with an iron atom in a haem structure). This then catalyses the conversion of guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP) which brings about smooth muscle relaxation and so vasodilation.[95] The inhibition of platelet aggregation (very necessary to prevent clotting) is also believed to involve the cGMP pathway. The binding of NO to sGC has been examined spectrally and the results are consistent with the formation of a pentacoordinate derivative in which the bond from the iron to a histidine subunit is lost.[96]

There is a major paradox here which has puzzled workers in this area for some time. It is known[97] that NO reacts rapidly (with a second-order rate constant of $\sim 3 \times 10^7$ dm^3 mol^{-1} s^{-1}) with oxygenated haemoglobin (oxyHb) irreversibly to give metHb and nitrate ion (eqn. 31). Indeed, this reaction has been adapted as one of many analytical procedure for NO determination.[98] Why then is NO generated in the endothelial cells not rapidly oxidised to nitrate when it enters the blood stream? Nitric oxide also binds to the iron atom of both metHb and deoxyHb (Hb–Fe^{II}). Various explanations have been proposed. The effect of blood flow creates a differential gradient of red cells—their concentration being smallest adjacent to the endothelium, from which the NO diffuses. Mathematical models have been used to quantify this situation.[99] Further it appears that the scavenging of NO by red cells is ~3 orders of magnitude less than that by cell-free oxyHb, although it is not clear why this is so. An alternative explanation is that given by Stamler and co-workers, mentioned earlier in section 5.3, in which it is claimed that the NO is transported in the blood flow as the *S*-nitroso derivative HbSNO, making it less available for scavenging by oxyHb. There are problems with this hypothesis, not least of which is the mechanism of formation of HbSNO *in vivo*. This is very much a current issue, and the relative merits of the various theories have recently been presented.[100]

$$Hb\text{–}Fe^{II}(O_2) + NO = Hb\text{-}Fe^{III} + NO_3^- \qquad (31)$$

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