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The mechanism of nitric oxide formation from *S*-nitrosothiols (thionitrites)

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S-Nitrosothiols (RSNO) are easily made by electrophilic nitrosation of thiols and are a convenient source of nitric oxide. Reaction occurs readily (in many cases) in aqueous buffer at pH 7.4 to give in addition the corresponding disulfide RSSR. If oxygen is not rigorously excluded from the solution, then the nitric oxide is converted quantitatively to nitrite ion, whereas in the absence of oxygen nitric oxide can be detected using a commercial NO-probe. Reaction, however, only occurs (apart from the photochemical pathway) if Cu^{2+} is present. There is often enough Cu^{2+} in the distilled water–buffer components to bring about reaction, but decomposition is halted if Cu^{2+} is complexed with EDTA. Experiments with the specific Cu^{+} chelator neocuproine however show that the true effective reagent is Cu^{+}, formed by reduction of Cu^{2+} with thiolate ion. Kinetic experiments show that the most reactive nitrosothiols are those which can coordinate *bidentately* with Cu^{+}, and there is a wide range of reactivity amongst the structures studied. Reactivity is crucially dependent on the concentrations of Cu^{2+} and RS^{-}.

Reaction also occurs, although somewhat more slowly, if the source of copper is the Cu^{II} complex with the tripeptide diglycyl-L-histidine (GGH) or as the Cu^{II} complex with human serum albumin (HSA). This allows the possibility that nitrosothiols could in principle generate nitric oxide *in vivo* using the naturally occurring sources of Cu^{II}.

Rapid exchange of the NO-group in RSNO with thiols occurs, again in aqueous buffer at pH 7.4. This reaction has been established as a nucleophilic substitution reaction by the thiolate ion at the nitroso nitrogen atom.

The implications of these results with regard to possible involvement of nitrosothiols *in vivo* are discussed.

Introduction

One of the major biological discoveries made in the last decade surely must be that nitric oxide is synthesised *in vivo* and plays such a range of major roles in human physiology. There were in excess of 3500 papers published in 1995 which referred to NO. The evidence is overwhelming that it is involved in vasodilation and may even control blood pressure. In addition NO is believed to act as a secondary messenger in the peripheral nervous system, is involved in the immune system and probably has a major brain function, possibly involving memory and learning. The subject has been frequently reviewed in the last five years, usually from the biological viewpoint, but some articles have commented on the probable chemistry involved.[1] This article is not the place to expand on these issues. It is now fairly certain that NO is synthesised *in vivo* from L-arginine by way of an enzyme, nitric oxide synthase, which although not yet structurally defined, appears to be present in at least three forms. One approach for the medical treatment of disorders now believed to arise by some natural deficiency of NO is to treat patients with drugs which generate nitric oxide *in vivo*. The most well-known and by far the most widely used example of this approach is the use of glyceryl trinitrate (GTN) to treat angina and other cardiovascular problems. Although GTN has been used clinically for well over a hundred years, the realisation that it generates NO *in vivo* did not come until 1986. However, in spite of its widespread use there are problems associated with its use, notably that it has a relatively short acting period and in many patients a tolerance to it is developed. Surprisingly, the full mechanistic details of NO formation from GTN have not been established, but there is the suggestion that thiols are involved in some way.[2] There is therefore an ongoing search for alternatives to GTN. Other nitrates, notably isosorbide di- and tri-nitrates, are better in some cases. Alkyl nitrites also induce vasodilation (the use of isoamyl nitrite is reported in one of the Sherlock Holmes stories[3]), but there are bigger problems associated with their widespread use. Sodium nitroprusside has been used routinely, particularly to induce blood pressure lowering during medical operations. The search for an alternative NO donor led us in part to this study of *S*-nitrosothiols (thionitries). There have also been suggestions that such compounds may be involved in the storage and transport of NO *in vivo*.

RSNO Synthesis

The obvious route to nitrosothiols is by electrophilic nitrosation of thiols [eqn. (1)], where XNO is in principle any carrier of

$$RSH + XNO = RSNO + H^{+} + X^{-} \quad (1)$$

NO^{+} such as, $H_2NO_2^{+}$, ClNO, BrNO, N_2O_3, N_2O_4, RONO or R_2NNO.[4] If the thiol is water soluble then the simplest treatment is with sodium nitrite in dilute acid solution. Some workers have used *tert*-butyl nitrite in aqueous acid solution. For thiols which are not appreciably soluble in water, *S*-nitrosation can successfully be achieved using the sodium nitrite–hydrochloric acid–methanol system,[5] or sodium nitrite in acetic acid[6] or *tert*-butyl nitrite in toluene, acetone[6,7] or chloroform.[8] There is much confusion in the biological literature concerning the reaction of thiols with NO. Unless oxygen is very rigorously removed, nitrosothiols will be formed when NO is bubbled into a thiol solution, almost certainly after oxidation of NO to NO_2 and consequent formation of N_2O_3. If the dissolved oxygen level is very low then there is, as expected, no reaction between NO and thiols.

Until fairly recently relatively few examples of nitrosothiols were known, partly because many are quite unstable. Early stable examples[10] included, *S*-nitroso-*tert*-butyl thiol **1** and the corresponding triphenyl derivative **2**. Following the interest in nitrosothiols generated by the nitric oxide discoveries, many more stable examples have been prepared and characterised. The list **3–12** is not a comprehensive one, but does include dinitroso compounds (**7** and **8**) and a *S*–nitroso glucose derivative (**12**). In many cases, *e.g.* *S*–nitrosocysteine, it is extremely difficult, if not impossible, to obtain a pure sample at room temperature. Nevertheless it is possible in such cases to carry out experiments with solutions prepared from nitrous acid and the corresponding thiol.

Properties of S-*nitrosothiols*

There is a comprehensive review of the known chemistry of nitrosohiols up to 1983.[11] The stable compounds are either red

or green liquids or solids in their pure state. Tertiary structures are usually green (*e.g.* **1, 2**) and primary structures (*e.g.* **9, 11**) reddish. Visible absorptions occur at around 540 or 590 nm with a low extinction coefficient (10–20 $dm^3\ mol^{-1}\ cm^{-1}$) for the $n_N \rightarrow \pi^*$ transition and there is a UV absorption centred at around 340 nm ($n_o \rightarrow \pi^*$) with $\varepsilon \sim 1000\ dm^3\ mol^{-1}\ cm^{-1}$. The latter has been used to monitor reaction in kinetic studies both for the formation and reaction of nitrosothiols. In the IR there is a N=O stretching vibration in the region 1480–1530 cm^{-1} and another (C–S vibration) at 600–730 cm^{-1}. Both ^1H and ^{13}C NMR spectra have been obtained[7] and the X-ray structure of **5** established.

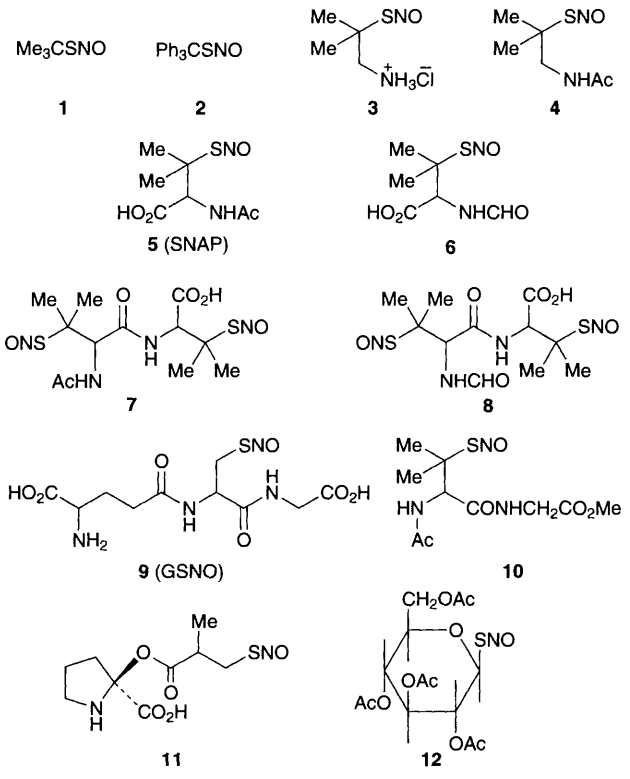
A number of reports[5,12,13] state that nitrosothiols break down down thermally and photochemically to give the disulfide and nitric oxide [eqn. (2)]. In the gas phase NO will oxidise

$$2RSNO \xrightarrow{\Delta \text{ or } h\nu} RSSR + 2NO \qquad (2)$$

readily to give NO_2 in the presence of oxygen whereas the final product in aqueous solution (aerated) is nitrite ion. A recent detailed photochemical study[14] with **9** (*S*-nitrosoglutathione, GSNO) showed that nitric oxide is released when samples are irradiated at either 340 or 545 nm and that the cytotoxic effect of GSNO on leukemia cells is enhanced upon radiation, raising the possibility that GSNO and maybe other nitrosothiols might have a future as photochemotherapeutic agents.

Some literature reports that nitrosothiols can effect nitrosation of amines,[5,15] but a proper mechanistic study had not been carried out, so it is not known whether NO^+ transfer occurs directly (as in some RONO reactions) or as seems more likely, that reaction involves the prior formation of some nitrosating species *e.g.* NO release followed by oxidation.

The biological properties of nitrosothiols are more significant. Many have been shown to effect vasodilation and also to inhibit platelet aggregation *i.e.* they have the same properties as NO itself. It is an intriguing question as to whether NO formation from nitrosothiols accounts for their biological properties. The following (*inter alia*) have been shown in proper test experiments[16,17] to have these biological properties: **5** (SNAP), **7**, **9** (GSNO), **10**, **11** (*S*-nitrosocaptopril), **12** and *S*-nitrosocysteine, but it is likely that all RSNO compounds behave likewise in varying degrees.



Nitric oxide release from *S*-nitrosothiols

(1) Product studies

It has been known for some time that in addition to their thermal and photochemical reactions, nitrosothiols decompose in solution to give the same products *i.e.* the disulfide and nitric oxide [eqn. (2)]. For reactions carried out in aqueous solution at pH around 7.4, we[18] have shown that the final product is the nitrite anion which is formed quantitatively. Further, the formation of nitrite ion follows at least qualitatively the disappearance of RSNO for the reaction of SNAP **5**, as shown in Fig. 1 which gives the relevant absorbance time data.

Initial expectations were that in aerated water nitric oxide would yield an equimolar mixture of nitrite and nitrate ions, following oxidation to NO_2 and subsequent hydrolysis. However the facts require that NO_2 reacts much more repidly with NO than it can hydrolyse, and so quantitative NO_2^- formation can be explained [eqns. (3–5)]. Examination of the relevant rate

$$2NO + O_2 = 2NO_2 \qquad (3)$$

$$NO_2 + NO = N_2O_3 \qquad (4)$$

$$N_2O_3 + 2OH^- = 2NO_2^- + H_2O \qquad (5)$$

constants show that this is likely. It has been shown independently[19] that solutions of nitric oxide in aerated water do in fact yield nitrite ion quantitatively. The explanation that N_2O_3 is a key intermediate has been criticised[19] on the basis of comparison with other competitive kinetic studies, but no other explanation has been forthcoming.

When oxygen is carefully removed from solution[17] then (*a*) no nitrite ion is formed and (*b*) NO can be readily detected using the NO-probe electrode system. In the presence of oxygen and an added amine such as *N*-methylaniline, the nitrosation product *N*-methyl-*N*-nitrosoaniline is formed in *ca.* 80% yield, which suggests that nitrosation by N_2O_3 (believed to be close to an encounter controlled process) competes effectively with N_2O_3 hydrolysis. Wink *et al.*[19] have also reported that nitric oxide solution in aerated water will generate nitrite ion quantitatively, and that the solutions will also nitrosate (diazotise) sulfanilamide and oxidise ferrocyanide. In a separate study[7] NO has been trapped from nitrosothiol decomposition reactions as the nitrosyl ferrohaemoglobin complex which was observed by its EPR spectrum. The same group have made quantitative measurements based on the reduction of oxyhaemoglobin to methaemoglobin, and an analysis of the consequent spectral changes. Quantitative NO production has been demonstrated for a number of RSNO compounds. Disulfide formation has also

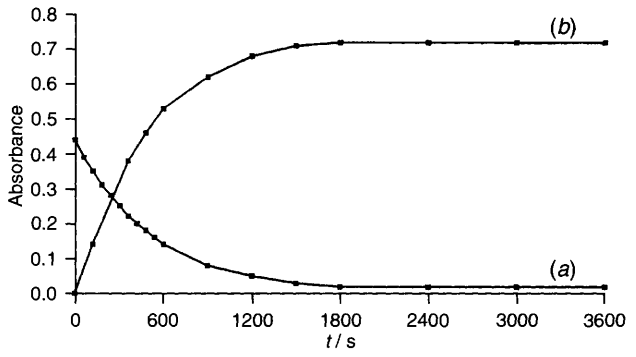


Fig. 1 Absorbance–time plots (*a*) for the decomposition of SNAP measured at 340 nm and (*b*) for the appearance of nitrite ion determined by the Griess method measured at 540 nm

been shown by ^{1}H NMR and by HPLC to be quantitative. In all of those nitrosation experiments no reaction occurs if oxygen is removed from the system.

(2) Kinetic studies and the importance of Cu^{2+}

Our kinetic measurements[17] on the decomposition of nitrosothiols were carried out in aqueous buffer solutions (mostly at pH 7.4) at 25 °C, by monitoring the disappearance of the absorbance due to the nitrosothiol at *ca.* 340 nm. Initially the results were extremely disappointing in that we found a variety of rate forms which corresponded to zero-, half-, first- and intermediate-order kinetics. There was also often a relatively short induction period. The reproducibility of the experiments was also very poor, as were comparisons with some literature results. One literature report even claimed a second order dependence upon [RSNO]. Our collaborators in St Andrews University found the same. Finally a suggestion was made that traces of metal ion impurities might be playing a part. This was confirmed when addition of EDTA brought about major rate reductions, eventually stopping reaction altogether. This was found to be true for all RSNO compounds studied, even the most reactive *e.g. S*-nitrosocysteine. In fact in the absence of light all nitrosothiols appear to be totally stable in solution when metal ions are eliminated. Subsequent addition of specific metal ions (in a slight excess over the EDTA) revealed that reaction is brought about by Cu^{2+} even in concentrations as low as 10^{-5} to 10^{-6} mol dm^{-3}. Often there is enough Cu^{2+} in the distilled water–buffer components used to effect reaction. This observation goes a long way to providing an explanation for the erratic kinetic behaviour found by us and others. We found a smaller effect by added Fe^{2+} (but this has not yet been studied further) and no measureable effect by added Mg^{2+}, Zn^{2+}, Ca^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Cr^{3+} or Fe^{3+}. Quite a different reaction is brought about by added Hg^{2+} and Ag^+ which will be discussed later.

Further kinetic experiments[17] revealed that for many RSNO compounds there was a range of [Cu^{2+}] (different for different nitrosothiols) for which rate eqn. (6) held. In many cases there

$$\text{Rate} = k[Cu^{2+}][\text{RSNO}] \quad (6)$$

was additionally a small induction period (discussed later), which at this stage was ignored. Since Cu^{2+} is catalytic in these reactions we measured a first order observed rate constant k_o which is $k[Cu^{2+}]$, and within certain limits plots of k_o *vs* [Cu^{2+}] were linear. There was always a small positive intercept at [Cu^{2+}] = 0, which can reasonably be attributed to the component of the reaction brought about by the adventitious Cu^{2+} present in the water–buffer components.

Most of our work was carried out at pH 7.4 to relate to possible *in vivo* reactions but we did note that RSNO solutions are much more stable in acid solutions (and also at high pH) even in the presence of added Cu^{2+}. An explanation will be put forward in the next section.

(3) Structure–reactivity

Values of k were obtained for a large range of RSNO structures, many based on the cysteine structure, because of possible biological implications. A few of the nitrosothiols were stable solids and were used as such to prepare standard solutions, but many were prepared in acid solution by nitrosation of the corresponding thiol and the solutions used after pH adjustment. Some of the results are given in Table 1. Inspection of the results reveals (a) that there is a very wide range of reactivity, and that on the time scale of our experiments many are effectively stable in solution and (b) that the very reactive compounds are those which contain a substituent able to complex with Cu^{2+} (*i.e.* electron donating substituents), in addition to coordination to the nitroso group, *i.e.* when the copper can be bidentately bound. Such compounds are **13** and **14**. Acetylation of **13** and esterification of **14** reduces reactivity drastically. Similarly when the proposed intermediate would have an unfavourable seven-membered ring configuration, as in *S*-nitrosohomocysteine, again reactivity is reduced to almost zero. In more strongly acid solutions both $-NH_2$ and $-CO_2^-$ groups will be extensively protonated, thus reducing the possibility of bidentate copper coordination.

Copper coordination is well known at nitrogen, sulfur and oxygen sites and many complexes have been synthesised and characterised. We prefer to write the coordination with the –SNO group at nitrogen rather than at sulfur for the following reason. Nitrosothiols are very readily decomposed by the addition of mercuric ion (well-known for its coordinating ability to sulfur). This reaction however results in NO^+ expulsion leading to nitrous acid formation in acid solution and nitrite anion formation at pH > *ca.* 3 [eqn. (7)]. This reaction is quite

$$\text{RSNO} + Hg^{2+} \rightleftarrows \underset{\displaystyle |\atop \displaystyle Hg}{[\text{RSNO}]^{2+}} \longrightarrow \text{RSHg}^+ + NO^+ \quad (7)$$

well known and has been adapted as an analytical procedure for thiol determination.[20] Even when carried out anaerobically there is no NO formation and this reaction shows very little of the structural dependence so pronounced in the copper reactions.[21] It is also easy to formulate a reaction mechanism following coordination at sulfur when water can attack the nitrogen atom of the nitroso group. This reaction is related to the denitrosation of *N*–nitrosamines brought about in acid solution[22] and also the acid catalysed hydrolysis of RSNO compounds.[23] Silver ion also brings about the decomposition of nitrosothiols and the reaction shows the same characteristics (but a lower reactivity) as that induced by mercuric ion.[21]

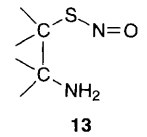
(4) Identification of Cu^+ as the true reagent

Whilst many of our observations were consistent with a reaction mechanism involving bidentate chelation by Cu^{2+}, some experiments could not be accommodated by such a mechanism. The key observations were (*a*) the finding of zero-order kinetics under some circumstances and (*b*) the observation of an induction effect in many experiments. The obvious explanation which would account for (*a*) is that the true reagent is Cu^+ and that under certain circumstances reduction of Cu^{2+} is rate limiting. In our initial communication[24] we had indeed suggested that reaction was brought about by Cu^+, but at that time we had no evidence to support the suggestion. Experiments with the specific Cu^+ chelator neocuproine[25] **15** provided the

Table 1 Values of k [eqn. (6)] for the reactions of some nitrosothiols in water at pH 7.4 and 25 °C

RSNO	k^a (dm^3 mol^{-1} s^{-1})
S-nitrosopenicillamine	67 000
S-nitrosocysteamine	65 000
S-nitrosocysteine	24 500
S-nitroso-*N*-acetylpenicillamine	20
S-nitroso-*N*-acetylcysteamine	*ca.* 0
S-nitrosothiolactic acid	1100
S-nitrosomercaptoacetic acid	300
Methyl *S*-nitrosomercaptoacetate	*ca.* 0
S-nitrosohomocysteine	*ca.* 0
S-nitrosoglutathione	*ca.* 0
S-nitroso-*tert*-butylthiol	*ca.* 0

[a] These values of k depend crucially on the level of RS^- present (see later), and so may not be reproducible with different RSNO samples.



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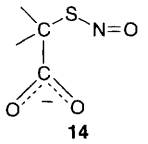

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evidence.[26] Fig. 2 shows the absorbance–time plots (at 340 nm) for the decomposition of SNAP in the presence of added Cu^{2+} (2×10^{-5} mol dm^{-3}) and increasing concentrations of neocuproine. Clearly reaction is inhibited by addition of the chelator and is virtually halted at the higher [neocuproine]. In addition we observe in these experiments the characteristic visible absorbance spectrum of the Cu^{+}–neocuproine complex with a maximum at 453 nm.

The question then arises as to how Cu^{2+} reduction occurs. A real possibility in these systems is reduction by thiolate ion [eqn. (8)]. This reaction is well-known and has been studied by

$$Cu^{2+} + RS^{-} = Cu^{+} + RS^{\cdot} \quad (8)$$

EPR.[27] It is the first step of the oxidation of thiols to disulfides catalysed by Cu^{2+}; an oxidising agent such as hydrogen peroxide then oxidises Cu^{+} back to Cu^{2+}. In our systems there are two possible sources of thiolate ion. For the solid RSNO samples, contamination by thiols is likely and we have demonstrated that this is the case by the use of Ellman's reagent.[28] Alternatively it is conceivable that small amounts of thiolate can be generated by hydrolysis of RSNO [eqn. (9)].

$$RSNO + 2OH^{-} = RS^{-} + NO_2^{-} + H_2O \quad (9)$$

Thiolate ion is only required in catalytic quantities (see later), and it is possible that the hydrolysis is Cu^{2+} catalysed. We have independent evidence for such a hydrolysis. This would account for, at least qualitatively the requirement for an induction period as observed. This was tested experimentally by the specific addition of a small amount of thiol, which dramatically removed the induction period, both for the first-order and zero-order reactions. This is shown graphically in Fig. 3 for the reaction of $Me_2NCH_2CH_2SNO$ demonstrating the effect of the addition of $Me_2NCH_2CH_2SH$.

This effect is quite general (and can also be brought about by ascorbic acid). Fig. 4 shows the effect of the addition of *N*-acetylpenicillamine (NAP) on the first observed rate constant for the decomposition of SNAP in the presence of added Cu^{2+}. Addition of the thiol initially brings about a very sharp increase in k_o until [RSH] $\simeq$ [Cu^{2+}], and thereafter a progressive decrease as [RSH] is increased further. The initial increase is easily associated with the increase in rate of Cu^{+} formation and the later decrease occurs as a result of the chelation of Cu^{2+} by NAP, thus effectively removing it from solution. Here NAP is having the same effects as the addition of EDTA. Indeed the closely related penicillamine is used clinically to complex copper in the treatment of Wilson's disease. The results demonstrated in Fig. 4 account for the confusion in the biological literature[17] where there are some claims that added RSH increases the rate of RSNO decomposition whereas others claim a retarding effect. Clearly both are possible and the important factors are the concentrations of RSH and Cu^{2+}.

Our combined results allow the outline mechanism given in

$$[RSNO + 2OH^{-} \overset{Cu^{2+}\ ?}{=\!=} RS^{-} + NO_2^{-} + H_2O]$$

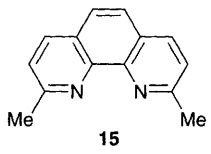
$$Cu^{2+} + RS^{-} \rightleftarrows X \rightarrow Cu^{+} + RS^{\cdot}$$

$$Cu^{+} + RSNO \rightleftarrows Y \rightarrow Cu^{2+} + RS^{-} + NO$$

$$2RS^{\cdot} = RSSR$$

Scheme 1

Scheme 1 to be proposed. Intermediate X is probably $RSCu^{+}$ and possible structures for Y are **16** and **17**. For the very reactive RSNO compounds (such as $NH_2CH_2CMe_2SNO$) Cu^{2+} reduction to Cu^{+} is the rate limiting step whereas for the less reactive



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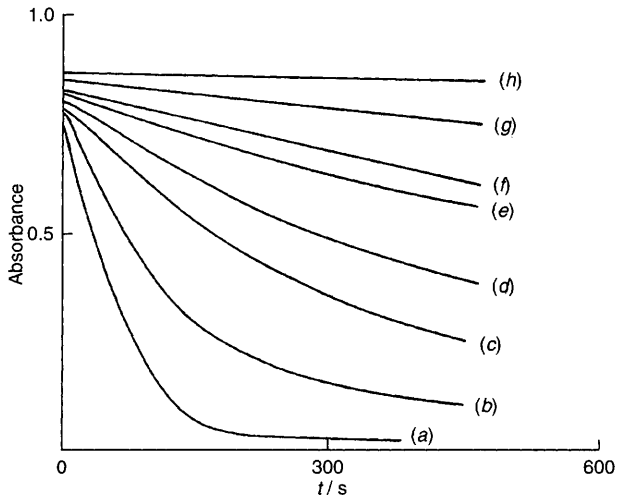


Fig. 2 Absorbance–time plots for the decomposition of SNAP (1×10^{-3} mol dm^{-3}) at 340 nm, in the presence of Cu^{2+} (2×10^{-5} mol dm^{-3}) and varying concentrations of neocuproine; (*a*) no added neocuproine, (*b*) 4×10^{-5}, (*c*) 5×10^{-5}, (*d*) 6×10^{-5}, (*e*) 8×10^{-5}, (*f*) 1×10^{-4}, (*g*) 2×10^{-4} and (*h*) 1×10^{-3} mol dm^{-3} neocuproine

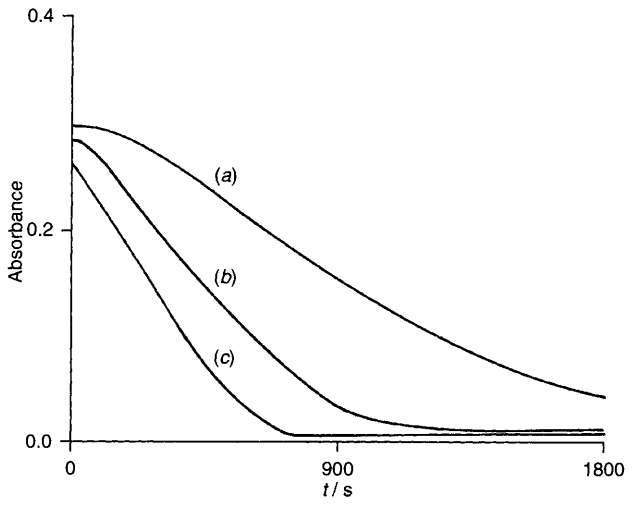


Fig. 3 Absorbance–time plots for the reaction of $Me_2NCH_2CH_2SNO$ (5×10^{-4} mol dm^{-3}) in the presence of Cu^{2+} (1×10^{-6} mol dm^{-3}) as a function of added $Me_2NCH_2CH_2SH$; (*a*) no added thiol, (*b*) added thiol (1×10^{-6} mol dm^{-3}) and (*c*) added thiol (3×10^{-6} mol dm^{-3})

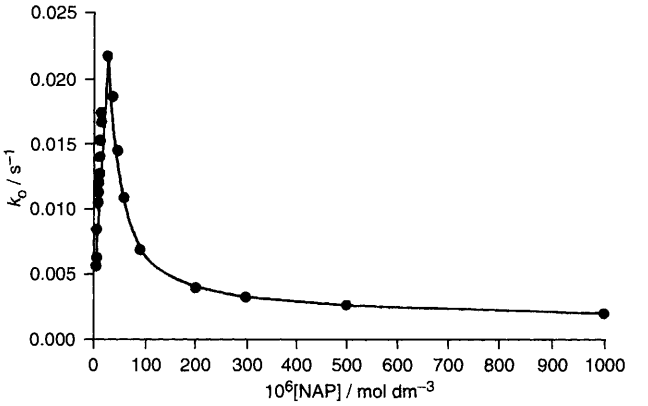


Fig. 4 First-order rate constants k_o for the reaction of SNAP (1×10^{-3} mol dm^{-3}) as a function of added *N*-acetylpenicillamine (NAP)

species then their reaction with Cu^+ is rate limiting. We have been able to model Scheme 1 by computer generating absorbance–time plots which match well those four limiting cases found experimentally[26] *i.e.* (*a*) a first-order process with an induction period, (*b*) a first-order process with no induction period, (*c*) a zero-order process with an induction period and (*d*) a zero-order process with no induction period. In all of the experiments described thus far there was no significant change to the kinetic patterns when oxygen was removed from the system. This means that for these reactants the re-oxidation of Cu^+ by oxygen is not a competing process with the reaction of Cu^+ with RSNO.

Having established the importance of the $[Cu^{2+}]$ and $[RS^-]$ in establishing the overall reactivity of a nitrosothiol, it is now clear that any one measured first-order rate constant does not give the intrinsic reactivity of that nitrosothiol. It is possible to account for the $[Cu^{2+}]$ dependency by plotting k_o *vs* [added Cu^{2+}] and the slope will be a true measure of reactivity. The problem of the $[RS^-]$ is altogether more complicated. Fig. 4 shows how dependent k_o is upon $[RS^-]$. We ourselves have obtained k values [eqn. (6)] for the reactions of SNAP which have varied by up to a factor of 100 for different preparations of SNAP (including the use of the solution prepared *in situ*), which necessarily contain different concentrations of thiol impurities. Rate constants must therefore be treated with some caution. Very large differences, however, such as in Table 1 between *S*-nitrosopenicillamine and *S*-nitroso-*N*-acetylpenicillamine, are certainly indicative of a large reactivity difference.

No details are yet available for the nature of the breakdown of intermediates Y such as **16** and **17**, so that it is not known whether RS^-, Cu^{2+} and NO are released in a one-stage process, or whether other intermediates possibly including some Cu^+–NO complexes are involved.

There is a further complication for the reaction of GSNO, (probably the most interesting nitrosothiol from the biologists' viewpoint given that it has been detected *in vivo*) and possibly for other slow reacting compounds. On its own GSNO at pH 7.4 does not break down even when the added $[Cu^{2+}]$ is relatively high, but decomposition can be brought about by addition of glutathione GSH, presumably following its reduction of Cu^{2+} to Cu^+. Even so it appears that the reaction of GSNO with Cu^+ is so slow that it competes with the re-oxidation of Cu^+ to Cu^{2+} by dissolved oxygen. As a result quite long induction periods appear, up to a few hours, during which time all the oxygen is used up and reaction can proceed. These induction periods are much longer than those encountered earlier which we ascribe to the time required to generate RS^- and which disappear on addition of RS^-. The long induction periods disappear if oxygen is carefully removed from the solution. Conversely the addition of another oxidising agent, hydrogen peroxide, results in an extension of the induction period, so that on the time scale of our experiments decomposition is minimal. Further work is in progress in an attempt to quantify the behaviour of GSNO.

(5) *Reaction with Cu^{2+}-protein complexes*

We have established that nitrosothiols generate nitric oxide in aqueous solution at pH 7.4 in a reaction with Cu^+ which is generated by reduction of Cu^{2+} with thiolate ion, ascorbate ion, or in principle with any reducing agent. Throughout we have used solid copper(II) sulfate as our source of Cu^{2+}. The human body contains the equivalent of *ca.* 0.1 g copper per 75 kg body weight, distributed in the blood, bone and muscle. The metal is mostly complexed to amino acids and proteins (particularly serum albumin) and very little indeed exists as free (hydrated) Cu^{2+}. If we are to propose that nitrosothiol decomposition can occur *in vivo* then we must establish whether reaction can occur when Cu^{2+} is bound in these ways.

Experiments have been carried out[29] (*a*) with Cu^{2+} bound to the tripeptide glycine-glycine-histidine (GGH) which it is claimed [30] is a good model for Cu^{2+} binding *in vivo* in human serum albumin (HSA), and (*b*) with Cu^{2+} bound to HSA itself. We find that the visible absorbance due to GGH–Cu^{2+} at 527 nm disappears quite rapidly on addition of the thiol *N*-acetylpenicillamine. Further if the Cu^+ chelator neocuproine **15** is present then we see the rapid build up of the characteristic absorbance due to the Cu^+ chelate at 453 nm (Fig. 5). Approximately 90% of the calculated theoretical maximum amount of available Cu^+ was trapped in this way. Similar results were obtained with HDA–Cu^{2+}. These experiments show that Cu^+ may be obtained from the complexed forms of Cu^{2+} by thiolate reduction. We found that decomposition of SNAP (and other nitrosothiols) occurs when copper is added as either GGH–Cu^{2+} or HSA–Cu^{2+}, although significantly slower than when copper is added as the Cu^{II} salt. Fig. 6 shows the absorbance (at 340 nm)–time plots for these experiments when the same concentrations (1×10^{-5} mol dm^{-3}) of Cu^{2+}, GGH–Cu^{2+} and HSA–Cu^{2+} are added to a SNAP solution. Clearly reaction occurs in all cases. Addition of HSA alone effectively stops the reaction, presumably by complexation of the very small concentration of free Cu^{2+}.

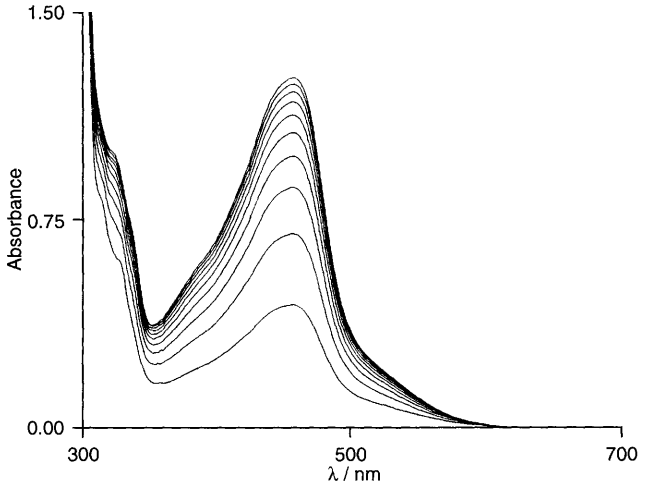


Fig. 5 Increasing absorbance at 453 nm with time for the formation of the neocuproine–Cu^+ complex from the reaction of GGH–Cu^{2+} (2×10^{-4} mol dm^{-3}) with *N*-acetylpenicillamine NAP (2×10^{-4} mol dm^{-3})

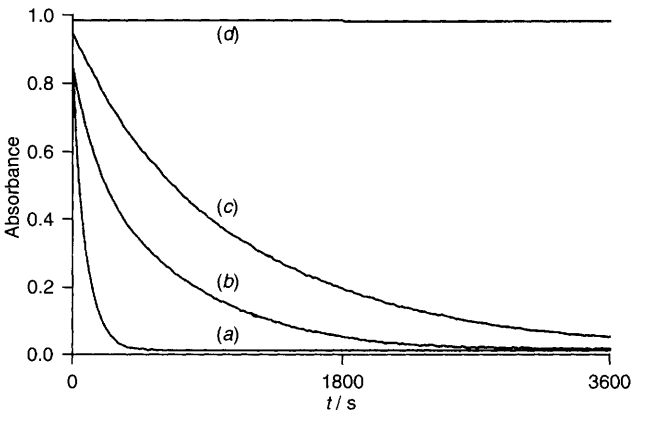
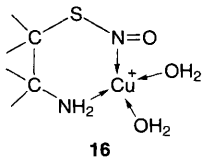


Fig. 6 Absorbance–time plots measured at 340 nm for the reaction of SNAP (1×10^{-3} mol dm^{-3}) in the presence of (*a*) 1×10^{-5} mol dm^{-3} Cu^{2+}; (*b*) 1×10^{-5} mol dm^{-3} GGH–Cu^{2+}; (*c*) 1×10^{-5} mol dm^{-3} HSA–Cu^{2+}; (*d*) 1×10^{-5} mol dm^{-3} HSA



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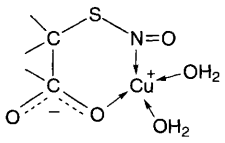

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(6) NO-Group transfer between RSNO and R'SH

There are a number of literature references[15] to the exchange of the NO group between nitrosothiols and thiols [eqn. (10)].

$$RSNO + R'SH \rightleftarrows RSH + R'SNO \quad (10)$$

Usually decomposition of R'SNO (and RSNO) also occurs over the timescale of the experiments and the final observed products are the three disulfides RSSR, R'SSR' and RSSR'. These have been reported for the reaction of GSNO with cysteine.[31] Direct observations of the primary products [eqn. (10)] have been made spectrophotochemically and equilibrium[32] and rate [33] constants have been determined. Variation of the rate constant with pH has shown clearly that the reactive species is the thiolate anion $R'S^-$ and that the reaction can be regarded as a straightforward nucleophilic substitution at the nitroso nitrogen atom. Reaction rates of course depend on the pK_a of R'SH but for many of the reactions studied, reaction is fast enough at pH 7.4 to require the use of stopped-flow facilities to obtain rate constants. The reaction shows the expected electronic (*e.g.* rate acceleration by electron-withdrawing substituents in R) and steric effects, and resembles closely the corresponding reaction of alkyl nitrites with thiolate.[34]

Conclusions

We have shown that nitrosothiols provide a readily accessible source of nitric oxide which can be released in aqueous buffer at pH 7.4. A necesssary requirement is the presence of Cu^{2+}. Even Cu^{2+} present as an impurity in the water–buffer component as low as $\sim 1 \times 10^{-6}$ mol dm^{-3} can in some circumstances be sufficient to generate nitric oxide. The active reagent however has been shown to be Cu^+ formed by reduction with thiolate (or in principle with other reducing agents). Generally there are three possible sources of thiolate; (*a*) from the presence of thiol impurities in solid RSNO samples or even in samples made up in solution and used as such, (*b*) by generation by hydrolysis of RSNO (which is possibly Cu^{2+} catalysed) and (*c*) by the specific addition of RSH. Overall reaction rates are thus crucially dependent upon the concentrations of both Cu^{2+} and RS^-. Reaction rates also vary widely with RSNO structure and analysis of data reveals that Cu^+ is probably bound, in an intermediate, to *two* sites within the molecule. Reaction also occurs, but somewhat less readily, when the copper source is GGH–Cu^{2+} or HSA–Cu^{2+} which does allow for the possibility but these reactions could/do occur *in vivo*.

There is some debate as to whether the biological properties of nitrosothiols necessarily require their breakdown to give NO. Intuitively it would perhaps be expected to be so, given the similarities in the biological properties of RSNO and NO. Gordge *et al.*[35] working with *S*-nitrosocysteine and GSNO, showed that for both compounds the formation of cyclic GMP (a necessary precursor for vasodilation) is inhibited by the presence of the Cu^+ chelator, bathocuproine sulfonate (similar in structure to neocuproine **15**). Also the inhibition of platelet aggregation by GSNO is reduced when the Cu^+ chelator is present. These results suggest that the biological properties of these RSNO compounds requires NO formation. However, on the other hand, Bannenburg *et al.*[36] have concluded that the bronchodilation effects in guinea pigs brought about by GSNO are not attributable to NO formation but to the intact nitrosothiol, since the effects are not inhibited by oxyhemoglobin, which traps out NO. Clearly the situation is not yet resolved.

It has been suggested that nitrosothiols play an important part in the *in vivo* storage of NO. The rapid NO group exchange reaction may play a part here, given the very wide reactivity range of RSNO compounds towards NO release. For example this difference is quite marked between GSNO and nitrosocysteine. Stable GSNO can be readily converted to the much more reactive nitrosocysteine by the presence of cysteine.

Whatever the *in vivo* mode of action of nitrosothiols, there is no doubt that their biological properties may well be widely used clinically in the near future. Indeed it has already been shown in clinical trials that GSNO is very effective at inhibiting platelet aggregation following coronary angioplasty[37] and it has also found a useful role in the treatment of one form of pre-eclampsia,[38] a high blood pressure condition suffered by some pregnant women.

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Lyn Williams was born in Ammanford, Dyfed, and educated in the local grammar school and at University College London. He gained a First Class Honours degree and a PhD working under the supervision of the late Professor Peter de la Mare. After a short spell as a postdoctoral fellow in UCL and UC Swansea he was appointed to the staff at Durham University and has remained there, becoming Professor of Chemistry in 1991 and Head of Department 1992–5. He has led a research group in Physical Organic Chemistry working with mechanistic problems in, electrophilic additions to alkenes, neighbouring group participation, aromatic rearrangements, nucleophilic substitution reactions, nitrosation reactions and most recently the chemistry of nitric oxide with reference to its physiological roles. He is most well-known for his work with nitrosation reactions and the chemistry of nitroso compounds and produced a book 'Nitrosation' in 1988. He has published over 130 articles and was awarded a DSc. by London University in 1976.

References

1 S. Moncada, R. M. J. Palmer and E. A. Higgs, *Pharmacol. Rev.*, 1991, **43**, 109; P. L. Feldman, O. W. Griffith and D. J. Stuehr, *Chem. Eng. News*, 1993, December 20, 26; A. R. Butler and D. L. H. Williams, *Chem. Soc. Rev.*, 1993, **22**, 233; M. Fontecave and J. L. Pierre, *Bull. Soc. Chim. Fr.*, 1994, **131**, 620.

2 M. Feelisch and E. Noack, *Eur. J. Pharmacol.*, 1987, **139**, 19.

3 A. C. Doyle, *The Memoirs of Sherlock Holmes (The Resident Patient)*, Penguin Books, 1950, p.167. (There is an error in this edition, but not in the original, which refers to nitrate of amyl instead of nitrite of amyl).

4 D. L. H. Williams, *Nitrosation*, Cambridge University Press, 1988, ch. 1, pp. 1–32.

5 L. Field, R. V. Dilts, R. Ravichandran, P. G. Lenhert and G. E. Carnahan, *J. Chem. Soc., Chem. Commun.*, 1978, 249.

6 H. A. Moynihan and S. M. Roberts, *J. Chem. Soc., Perkin Trans. 1*, 1994, 797.

7 B. Roy, A. du M. d'Hardemare and M. Fontecave, *J. Org. Chem.*, 1994, **59**, 7019.

8 M. P. Doyle, J. W. Terpstra, R. A. Pickering and D. M. LePoire, *J. Org. Chem.*, 1983, **48**, 3379.

9 D. A. Wink, R. W. Nims, J. F. Darbyshire, D. Christodoulou, I. Hanbauer, G. W. Cox, F. Laval, J. Laval, J. A. Cook, M. C. Krishna, W. G. DeGraff and J. B. Mitchell, *Chem. Res. Toxicol.*, 1994, **7**, 519.

10 G. Kresze and U. Uhlich, *Chem. Ber.*, 1959, **92**, 1048; H. Rheinboldt, *Chem. Ber.*, 1926, **59**, 1311.

11 S. Oae and K. Shinhama, *Org. Prep. Proced. Int.*, 1983, **15**, 165.

12 H. Rheinbolt and F. Mott, *J. Prakt. Chem.*, 1932, **133**, 328.

13 J. Barrett, D. F. Debenham and J. Glauser, *J. Chem. Soc., Chem. Commun.*, 1965, 248; J. Barrett, L. J. Fitygibbones, J. Glauser, R. H. Still and P. N. W. Young, *Nature*, 1966, **211**, 848.

14 D. J. Sexton, A. Muruganandam, D. J. McKenney and B. Mutus, *Photochem. Photobiol.*, 1994, **59**, 463.

15 S. Oae, Y. H. Kim, D. Fukushima and K. Shinhama, *J. Chem. Soc., Perkin Trans. 1*, 1978, 913.
16 M. W. Radomski, D. D. Rees, A. Dutra and S. Moncada, *Br. J. Pharmacol.*, 1992, **107**, 745; W. R. Mathews and S. W. Kerr, *J. Pharmacol. Exp. Ther.*, 1993, **267**, 1529; J. Loscalzo, D. Smick, N. Andon and J. Cooke, *J. Pharmacol. Exp. Ther.*, 1989, **249**, 726.
17 M. Feelisch, M. te Poel, R. Zamora, A. Deussen and S. Moncada, *Nature*, 1994, **368**, 62.
18 S. C. Askew, D. J. Barnett, J. McAninly and D. L. H. Williams, *J. Chem. Soc., Perkin Trans. 2*, 1995, 741.
19 D. A. Wink, J. F. Darbyshire, R. W. Nims, J. E. Saavedra and P. C. Ford, *Chem. Res. Tioxicol.*, 1993, **6**, 23; H. H. Awad and D. M. Stanbury, *Int. J. Chem. Kinet.*, 1993, **25**, 375.
20 B. Saville, *Analyst*, 1958, **83**, 670.
21 H. R. Swift and D. L. H. Williams, to be published.
22 I. D. Biggs and D. L. H. Williams, *J. Chem. Soc., Perkin Trans. 2*, 1975, 107.
23 S. S. Al-Kaabi, D. L. H. Williams, R. Bonnett and S. L. Ooi, *J. Chem. Soc.,Perkin Trans. 2*, 1982, 227.
24 J. McAninly, D. L. H. Williams, S. C. Askew, A. R. Butler and C. Russell, *J. Chem. Soc., Chem. Commun.*, 1993, 1758.
25 Y. Yoshida, J. Tsuchiya and E. Niki, *Biochem. Biophys. Acta*, 1994, **1200**, 85 and references cited therein.
26 A. P. Dicks, H. R. Swift, D. L. H. Williams, A. R. Butler, H. H. Al-Sadoni and B. G. Cox, *J. Chem. Soc., Perkin Trans. 2*, 1996, 481.
27 T. J. Wallace, *J. Org. Chem.*, 1966, **31**, 3071; G. Scrivens, B. C. Gilbert and T. C. P. Lee, *J. Chem. Soc., Perkin Trans. 2*, 1995, 955.
28 P. W. Riddles, R. L. Blakeley and B. Zerner, *Anal. Biochem.*, 1979, **94**, 75.
29 A. P. Dicks and D. L. H. Williams, to be published.
30 S. J. Lau, T. P. A. Kruck and B. Sarkar, *J. Biol. Chem.*, 1974, **249**, 5878.
31 J. W. Park, *Biochem. Biophys. Res. Commun.*, 1988, **152**, 916.
32 D. J. Meyer, H. Kramer, N. Ozer, B. Coles and B. Ketterer, *FEBS Lett.*, 1994, **345**, 177.
33 D. J. Barnett, A. Rios and D. L. H. Williams, *J. Chem. Soc., Perkin Trans. 2*, 1995, 1279.
34 H. M. S. Patel and D. L. H. Williams, *J. Chem. Soc., Perkin Trans. 2*, 1990, 37.
35 M. P. Gordge, D. J. Meyer, J. Hothersall, G. H. Neild, N. N. Payne and A. Noronha-Dutra, *Br. J. Pharmacol.*, 1995, **114**, 1083.
36 G. Bannenberg, J. Xue, L. Engman, I. Cotgreave, P. Moldeus and A. Ryrfeldt, *J. Pharmacol. Exp. Ther.*, 1995, **272**, 1238.
37 E. J. Langford, A. S. Brown, R. J. Wainwright, A. J. de Belder, M. R. Thomas, R. E. A. Smith, M. W. Radomski, J. F. Martin and S. Moncada, *Lancet*, 1994, **344**, 1458.
38 A. de Belder, C. Lees, J. Martin, S. Moncada and S. Campbell, *Lancet*, 1995, **345**, 124.

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