Catalysis by Cu^{2+} of nitric oxide release from *S*-nitrosothiols (RSNO)

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The decomposition of a range of *S*-nitrosothiols (thionitrites) RSNO, based on cysteine derivatives, yields in water at pH 7.4 nitrite ion quantitatively. If oxygen is rigorously excluded then no nitrite ion is formed and nitric oxide can be detected using an NO-probe. The reaction is catalysed by trace quantities of Cu^{2+} (there is often enough present in distilled water samples) and also to a lesser extent by Fe^{2+}, but not by Zn^{2+}, Cu^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Cr^{3+} or Fe^{3+}. The rate equation (measuring the disappearance of the absorption at *ca.* 350 nm due to RSNO) was established as $v = k[RSNO]\cdot[Cu^{2+}] + k'$ over a range of $[Cu^{2+}]$ typically 5–50 μmol dm^{-3}. The constant term k' represents the component of the rate due to residual Cu^{2+} in the solvent and buffer components, together with the spontaneous thermal reaction. Decomposition can be virtually halted by the addition of EDTA. Reactions carried out in the presence of *N*-methylaniline gave a quantitative yield of *N*-methyl-*N*-nitrosoaniline, but a negligible yield when oxygen was rigorously excluded. Values of the second-order rate constant k were obtained for a range of *S*-nitrosothiols. Reactivity is highest for the *S*-nitrosothiols derived from cysteamine and penicillamine, when Cu^{2+} can be complexed both with the nitrogen atom of the nitroso group and the nitrogen atom of the amino group, *via* a six-membered ring intermediate. If there is no amino (or other electron donating group) present, reaction is very slow (as for RSNO derived from *tert*-butyl sulfide). *N*-Acetylation of the amino group reduces the reactivity drastically as does the introduction of another CH_2 group in the chain. There is evidence of a significant *gem*-dimethyl effect. Kinetic results using the *S*-nitrosothiols derived from mercaptoacetic, thiolactic and thiomalic acids suggests that coordination can also occur *via* one of the oxygen atoms of the carboxylate group. EPR experiments which examined the Cu^{2+} signal showed no spectral change during the reaction suggesting that the mechanism does not involve oxidation and reduction with $Cu^{2+} \rightleftharpoons Cu^{+}$ interconversion.

S-Nitrosothiols, or thionitrites, RSNO are the sulfur analogues of the much more well known alkyl nitrites. Relatively few are stable in the solid or liquid states, but these include those containing bulky substituents as in *S*-nitroso-*tert*-butyl thiol,†[,1] *S*-nitrosotriphenylmethylthiol‡[,2] and *N*-acetyl-*S*-nitroso-penicillamine§[,3] (SNAP). All can be made by conventional electrophilic nitrosation of the corresponding thiol[4] and if not isolated, dilute solutions thus prepared *in situ* can be used for further experiments. *S*-Nitrosation of thiols is essentially an irreversible process, contrasting with alkyl nitrite formation. This results from the different basicities and nucleophilicities of O and S sites.[5]

Decomposition of *S*-nitrosothiols occurs thermally and photochemically to give the disulfide and initially nitric oxide [eqn. (1)]. The photochemical decomposition has been studied

$$2RSNO \longrightarrow RSSR + 2NO \quad (1)$$

$$RSNO \xrightarrow{h\nu} RS^{\cdot} + NO \quad (2)$$

$$RS^{\cdot} + RSNO \longrightarrow RSSR + NO$$

in organic solvents by Barrett *et al.*[6] who proposed the reaction sequence outlined in eqn. (2). Many *S*-nitrosothiols (*e.g.* EtSNO)[7] decompose at room temperature to give again the disulfides, whilst others do so upon heating.[3,8] There is a good review of the chemistry of *S*-nitrosothiols covering the literature up to 1983.[9]

Intense interest in the chemistry of *S*-nitrosothiols has been generated recently in connection with the newly discovered remarkable physiological roles of nitric oxide,[10] including particularly vasodilation and cytotoxic action of macrophages. Thiols and *S*-nitrosothiols are believed to be involved in the *in vivo* processes, possibly in the mechanism of NO transfer reactions. One view[11] suggests that an *S*-nitrosothiol is a more likely candidate than is free nitric oxide as the so-called endothelium-derived relaxing factor (EDRF), although this is not the majority view. Certain *S*-nitrosothiols have been detected *in vivo*[12] and a number have been shown to have vasodilatory activity[13] and also produce inhibition of platelet aggregation.[14] A further potential application for *S*-nitrosothiols lies in their possible use as therapeutic drugs for treatment of angina and other circulation problems, which relies on their ability to generate NO *in vivo*. Presently organic nitrates particularly glyceryl trinitrate (GTN) are much used but suffer from the disadvantage of induced tolerance after a period in many patients. Very recently[15] a number of *S*-nitrosothiols derived from penicillamine dipeptides have been synthesized and one dinitrosothiol has been shown to have biological activity comparable to that of GTN.

A number of studies have been carried out, particularly within biological laboratories, on the decomposition of *S*-nitrosothiols in solution, but the results have been erratic and generally irreproducible so that no clear mechanistic picture has

† 1,1-Dimethyl-*S*-nitrosoethanethiol.
‡ *S*-Nitrosotriphenylmethanethiol.
§ *N*-Acetyl-3-(*S*-nitrosomercapto)valine [$ONSCMe_2CH(NHAc)CO_2H$].

emerged. We set out to examine these reactions mechanistically, using a range of structures likely to provide reasonable models for the *in vivo* situation. A preliminary account of our results has been presented.[16]

Results and discussion

Product studies

We determined nitrite ion or nitrous acid using the well known Griess method. Table 1 shows that within the experimental error nitrite ion is formed quantitatively from SNAP over the range of pH studied. Fig. 1 shows the formation of nitrite ion as a function of time, using a sampling method and determination of the absorbance at 540 nm due to the azo dye formed after coupling of the diazonium ion. The figure also shows the simultaneous disappearance of the absorption at 340 nm due to the *S*-nitrosothiol. Acid-catalysed hydrolysis of *S*-nitrosothiols can take place[17] only if the nitrous acid is removed (as the reaction lies well over towards the *S*-nitrosothiol) and also only at very high acidity, typically 2–3 mol dm^{-3} H_2SO_4. This is again different from the behaviour of alkyl nitrites, where hydrolysis is very facile, probably due to the greater basicity of the oxygen atom than the sulfur atom. Clearly acid catalysed hydrolysis is not a viable pathway under the conditions of our experiments quoted in Table 1 and Fig. 1.

The disulfide has been characterized for a number of reactions of RSNO species, for example, cystine often precipitates out of solution and has been identified by the melting point and IR spectrum, from the reaction of *S*-nitrosocysteine.

When SNAP was allowed to react in the presence of *N*-methylaniline a virtually quantitative yield of *N*-methyl-*N*-nitrosoaniline was obtained as measured by the UV absorbance peak maximum at 275 nm. However when oxygen was rigorously excluded very little nitrosoamine formed, probably arising from trace amounts of oxygen still present in the solution. These results can be rationalized in terms of release of nitric oxide which in the presence of oxygen produces a reagent capable of effecting electrophilic nitrosation. A possible scenario is outlined in eqn. (3) involving the formation of N_2O_3 which

$$2NO + O_2 \longrightarrow 2NO_2$$
$$NO_2 + NO \longrightarrow N_2O_3 \qquad (3)$$

yields nitrous acid or nitrite anion (depending on the pH) by hydrolysis. In the presence of a reactive amine such as *N*-methylaniline, *N*-nitrosation can occur to give the nitrosamine. A difficulty with this interpretation is that the hydrolysis of NO_2 to yield a mixture of nitrite and nitrate anions might be expected to compete to some extent with its reaction with nitric oxide, and yet nitrite formation is quantitative. Yet recent studies[18,19] of the reaction of NO with O_2 show that nitrite ion is the sole product and it has been argued[19] that the nitrosating species derived from NO and O_2 is not N_2O_3 (nor NO_2 or NO^+) but a new and as yet uncharacterized species $N_xO_y^{n-}$, which is also capable of oxidation of $[Fe(CN)_6]^{4-}$ and of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and of effecting nitrosation of sulfanilamide.

When oxygen is excluded we have been able to detect nitric oxide as the primary product from a range of *S*-nitrosothiols from reaction in water at pH 7.4, using an NO-probe electrode. For the faster reacting *S*-nitrosothiols (*e.g.* those that are derived from cysteamine and penicillamine) the yield of NO detected was >70%, but for the lower reacting species, *e.g.* SNAP and GSNO, detected yields were lower, no doubt reflecting the loss of NO by other pathways.

Table 1 Nitrite measured (%) from decomposition of SNAP in phosphate buffer pH 6–8; all results are averages of five runs

pH	NO_2^- (%)
6.0	91
6.5	88
6.9	95
7.0	95
7.4	93
7.9	92

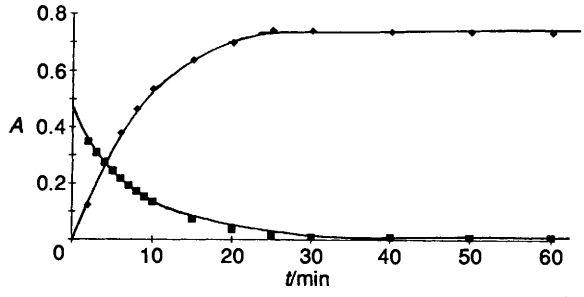

Fig. 1 Absorbance–time plots for the decomposition of SNAP (0.5 mmol dm^{-3}) at 340 nm at pH 5 and for concomitant nitrite ion determined by the Griess method at 540 nm

Kinetic studies

These were carried out spectrophotometrically noting the disappearance of the absorbance due to the *S*-nitrosothiols at 340 nm. Initially working mainly with SNAP, results were quite erratic and not reproducible. Different half-lives were found for experiments conducted in our two laboratories. The kinetic order was not clear, occasionally reasonable first-order fits were obtained and at other times good half-order plots occurred. This pattern has been noted in the literature; one report[20] quotes a second-order dependence. We investigated the possibility that this pattern of behaviour arises from catalysis by low concentrations of adventitious metal ions. We found that addition of EDTA completely suppressed the decomposition of SNAP. Metal ion catalysis was examined by the addition of specific salts in slight excess over EDTA. We found substantial catalysis by Cu^{2+} (and to a lesser extent by Fe^{2+}) but no effect from added Zn^{2+}, Ca^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Cr^{3+} or Fe^{3+}. There was[16] a qualitative relation between the naturally occurring Cu^{2+} (as measured by atomic absorption) in tap water and two sources of distilled water and the rate of decomposition of SNAP. Fig. 2 shows first-order plots for the disappearance of the absorption at 339 nm of SNAP (0.4 mmol dm^{-3}) at pH 7.4 in the presence of different $[Cu^{2+}]$. There is clearly Cu^{2+} catalysis and good first-order behaviour, except for the experiment without added Cu^{2+}, which is more complex. The effect of EDTA addition is shown in Fig. 3. Without any added Cu^{2+} the addition of EDTA virtually stops the reaction, which also is the case when there is a two-fold excess of EDTA over Cu^{2+}. Increasing the $[Cu^{2+}]$ further allows the reaction to take place, and again Cu^{2+} catalysis is evident. More quantitatively we have followed the decomposition as a function of $[Cu^{2+}]$ generally within the range 5–50 μmol dm^{-3} $[Cu^{2+}]$. The range of $[Cu^{2+}]$ differed for different substrates because of the range of reactivity encountered. Individual kinetic runs showed good first-order behaviour and plots of the observed rate constant k_o *vs.* $[Cu^{2+}]$ are linear, as shown in Fig. 4 for the reaction of *S*-nitrosocysteine. The intercept (k') probably represents the component of the reaction catalysed by the residual Cu^{2+} present in the reaction solution, deriving from the solvent and buffer components, together with any spontaneous (thermal) decomposition. At higher $[Cu^{2+}]$ the

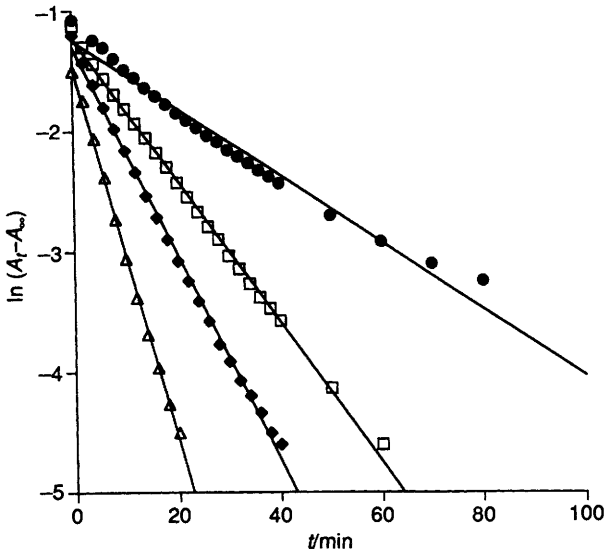

Fig. 2 First-order plots for the decomposition of SNAP (0.4 mmol dm^{-3}) with increasing $[Cu^{2+}]$: (●) no Cu^{2+}; (□) 5 μmol dm^{-3} Cu^{2+}; (◆) 10 μmol dm^{-3} Cu^{2+}; (△) 50 μmol dm^{-3} Cu^{2+} added

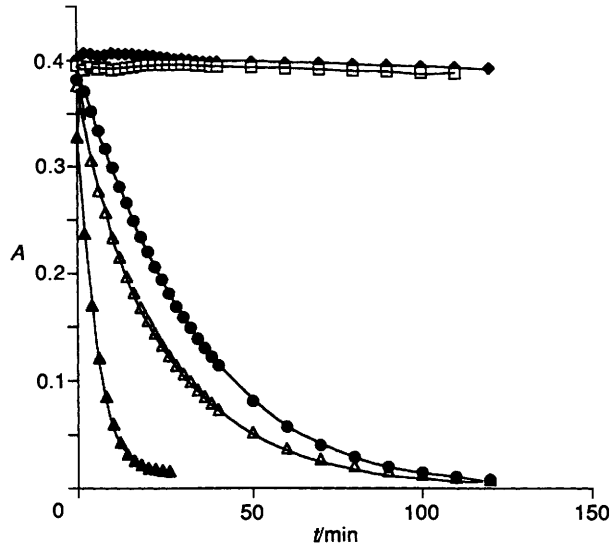

Fig. 3 Absorbance–time plots for the decomposition of SNAP (0.4 mmol dm^{-3}) with increasing $[Cu^{2+}]$ in the presence of EDTA (10 μmol dm^{-3}): (●) no added Cu^{2+}, no EDTA; (□) no added Cu^{2+} with EDTA; (◆) 5 μmol dm^{-3} added Cu^{2+} with EDTA; (△) 10 μmol dm^{-3} added Cu^{2+} with EDTA; (▲) 100 μmol dm^{-3} added Cu^{2+} with EDTA

plots of k_o *vs.* $[Cu^{2+}]$ lost linearity and erratic kinetic behaviour occurred. This is probably due to complex formation involving Cu^{2+} and the phosphate component of the buffer solution. This establishes the rate eqn. (4), which has been shown to hold for a

$$\text{Rate} = k[\text{RSNO}][Cu^{2+}] + k' \qquad (4)$$

large range of *S*-nitrosothiol structures. Table 2 gives values of the second-order rate constant k for all of the substrates studied. It is clear that a large range of reactivity is represented and a number of RSNO species are in effect quite stable in solution at pH 7.4 even in the presence of added Cu^{2+}. Three of the most reactive substrates studied are *S*-nitrosopenicillamine, *S*-nitrosocysteamine and *S*-nitrosocysteine. An explanation of this reactivity is that Cu^{2+} becomes bidentately complexed with the nitrogen atoms of the nitroso group and the amino group, *via* a six-membered ring as shown in Fig. 5. Without such a structural feature, as in 1,1-dimethyl-*S*-nitrosoethanethiol (Bu^{t}SNO) there

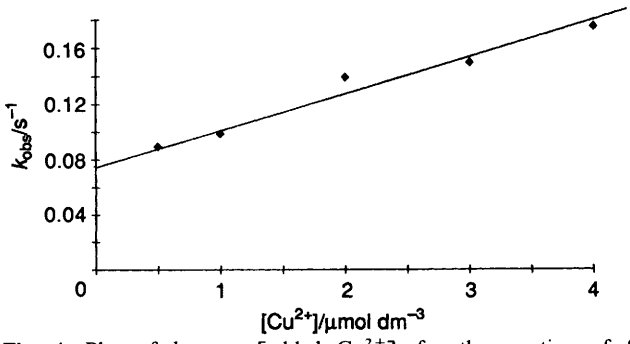

Fig. 4 Plot of k_{obs} *vs.* [added Cu^{2+}], for the reaction of *S*-nitrosocysteine

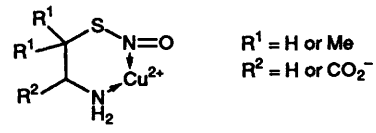

Fig. 5 Proposed intermediate in the Cu^{2+}-catalysed decomposition of amino-*S*-nitrosothiols

is no measurable reactivity. The coordination chemistry of Cu^{II} is dominated by coordination to N and O sites,[21] although coordination to sulfur and other elements is also well known. Much of the bioinorganic chemistry of Cu^{II} involves coordination compounds of this type, *e.g.* with proteins. We have written the proposed structure in Fig. 5 as a two-coordinate species. It is very likely that four- or six-coordination involving water molecules (or even the OH group at pH 7.4), but we have not included these for simplicity of presentation. The kinetic evidence of the first-order dependence upon [RSNO] argues against two molecules of RSNO being involved in a four-coordinate system. Coordination of Cu^{2+} to sulfur sites is also well known, though it is more difficult to envisage reaction pathways for NO release *via* such species. This contrasts with the decomposition of RSNO compounds brought about by Hg^{2+} where it is believed[22] that co-ordination to sulfur occurs resulting in loss of NO^+.

Increasing the chain length by one methylene group (as in *S*-nitrosohomocysteine) results in a massive rate reduction, but reaction still does occur, probably *via* the more unfavourable seven-membered ring intermediate.

N-Acetylation of the amino group has a drastic effect on the reaction, reducing reactivity to a virtually unmeasurable rate for both *S*-nitrosothiols derived from cysteine and cysteamine. This is consistent with the expected large reduction in electron density on the amino nitrogen atom upon acetylation thus making coordination with copper much less strong. Using this model it is perhaps a little surprising to see any reactivity for SNAP. Two explanations are possible (*i*) that the *gem*-dimethyl effect, evident in the reactions of *S*-nitrosocysteine and *S*-nitrosopenicillamine, is sufficient to allow the reaction to occur or (*ii*) that an alternative pathway exists involving a seven-membered ring intermediate where coordination occurs with the nitroso nitrogen atom and one of the oxygen atoms of the carboxylate group.

The biggest surprise is the reactivity of the ethyl ester of *S*-nitrosocysteine. It is to be expected that esterification will increase reactivity since there is now no competition with the well known[23] coordination of Cu^{2+} with carboxylic acid anions as outlined in Fig. 6. A number of copper(II) carboxylates have been isolated and examined structurally.[24] In the solid state they are binuclear with four carboxylate bridges. Indeed we have come across this effect ourselves when using carboxylic acids as buffer components. However it is not easy to see on our model why the reactivity of the ethyl ester of *S*-nitrosocysteine

Table 2 Second-order rate constant, k [eqn. (4)], for a range of *S*-nitrosothiols

S-Nitrosothiol	k/mol^{-1} dm^3 s^{-1}	k'/s^{-1}
S-Nitroso-*N*-acetylpenicillamine (SNAP)	20 ± 1	8×10^{-4}
S-Nitrosopenicillamine	67 000 ± 2 000	0.16
S-Nitrosocysteamine	65 000 ± 1 300	0.02
S-Nitrosocysteine	24 500 ± 500	0.08
S-Nitrosocysteine ethyl ester	270 000 ± 11 000	0.02
1,1-Dimethyl-*S*-nitrosoethanethiol	0	
S-Nitrosohomocysteine	16 ± 0.5	3×10^{-4}
S-Nitroso-*N*-acetylcysteine (SNAC)	0	
S-Nitroso-*N*-acetylcysteamine	0	
S-Nitrosoglutathione (GSNO)	0	
S-Nitrosocaptopril (SNOCAP)	0	
S-Nitrosomercaptoacetic acid	300 ± 5	6×10^{-4}
Methyl *S*-nitrosomercaptoacetate	0	
3-*S*-Nitrosomercaptopropionic acid	16 ± 0.3	2×10^{-4}
Methyl 3-*S*-nitrosomercaptopropionate	0	
S-Nitrosothiolactic acid	900 ± 60	1×10^{-3}
S-Nitrosothiomalic acid	1 100 ± 60	4×10^{-4}
2-Hydroxy-*S*-nitrosoethanethiol	0	

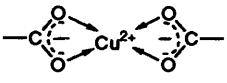

Fig. 6 Cu^{2+}-carboxylates

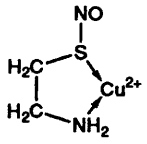

Fig. 7 Alternative five-membered ring structure for the intermediate

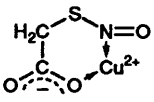

Fig. 8 Proposed intermediate in the Cu^{2+}-catalysed decomposition of carboxy-*S*-nitrosothiols

is significantly greater than that of *S*-nitrosocysteamine, for example, we would have expected the rate constants to have been comparable.

The significant difference in reactivity between SNAC ($k \approx 0$) and 3-*S*-nitrosomercaptopropionic acid ($k \approx 16$ dm^3 mol^{-1} s^{-1}) suggests that the former complexes some of the free Cu^{2+} *via* the CO_2^- and NHAc groups reducing the effective free Cu^{2+}. For SNAP this effect is probably offset by the rate constant enhancing *gem*-dimethyl effect.

As expected *S*-nitrosoglutathione is quite stable in solution in the presence of Cu^{2+}, since the appropriate nitrogen atoms are acetylated. Similarly *S*-nitrosocaptopril is unreactive since there is no suitable coordination site.

An alternative explanation involving coordination of Cu^{2+} to the amino group and the sulfur atom of the nitrosothiol (see Fig. 7) *via* a five-membered ring intermediate is worthy of consideration. D-Penicillamine is a well known complexing agent for free Cu^{2+} and is believed to be more efficient than EDTA. This is likely to involve a five-membered ring species. EPR spectroscopy has been used to characterize a range of Cu^{II} complexes with thiols in aqueous solution at pH *ca.* 10, where it is believed that copper is complexed at the sulfur atom in a bridged structure.[25] The electron-withdrawing NO group however is likely to have an adverse effect on the coordinating ability at sulfur. Additionally, it is believed that the Hg^{2+}-catalysed[22] and also the acid-catalysed[17] pathways for RSNO decomposition involve Hg^{2+}–S coordination and S-protonation respectively, both of which lead to NO^+ expulsion and nitrous acid formation. Even when those reactions are carried out in the absence of oxygen, no nitric oxide is formed as measured with the NO probe, contrasting with the Cu^{2+} reactions.

Since Cu^{II} coordination also occurs readily with suitable oxygen donors we have examined the decomposition of a number of *S*-nitrosocarboxylic acids and esters. *S*-Nitrosothioglycolic acid (or *S*-nitrosomercaptoacetic acid) reacts quite readily with Cu^{2+} with a rate constant of 300 dm^3 mol^{-1} s^{-1}, *i.e.* it is not as reactive as species which have a free $-NH_2$ group present. We propose that reaction occurs *via* an intermediate such as that shown in Fig. 8. Esterification results in complete stability of the *S*-nitroso compound, an effect similar to *N*-acetylation of the amine group. Increasing the chain length by one CH_2 group (3-*S*-nitrosomercaptopropionic acid) reduces reactivity considerably, but not to a negligible level, which suggests that reaction can occur *via* a seven-membered ring. Inclusion of a methyl group (*S*-nitrosothiolactic acid) and a further $CH_2CO_2^-$ group (*S*-nitrosothiomalic acid) increases the reactivity slightly, possibly *via* a small steric effect. We looked for a possible reaction involving *O*-coordination from an alcohol by studying the reaction of 2-hydroxy-*S*-nitrosoethanethiol, but found the reaction to be slow, and evidence to suggest that the disulfide product complexes Cu^{2+} more efficiently than does the nitrosothiol, since reaction effectively stops a long way short of complete conversion. It seems that the high electron density available in the carboxylate anion is necessary to allow a sufficient concentration of the cyclic intermediates to allow the reaction to occur.

Having established the structural requirements for rapid NO release from these *S*-nitrosothiols we can say no more in mechanistic detail as to the mode of NO release from the proposed cyclic intermediates. At this stage we suggest that spontaneous break up can occur forming RS$^\bullet$ (yielding RSSR), free Cu^{2+} and NO, although the situation may be more complex than this. We have examined the possibility of there being a redox $Cu^{II} \rightleftharpoons Cu^{I}$ interconversion here. Many of the bioinorganic reactions involving copper proceed this way. We looked at the EPR spectrum of Cu^{2+} during decomposition of SNAP in a static and also in a flow system, but were unable to detect a change in the spectrum during reaction. In the absence of a buffer we attribute the spectrum to $[Cu(H_2O)_6]^{2+}$ which is modified in the presence of a dimethylglutaric acid buffer system, probably to the copper carboxylate species discussed earlier. It is possible from these results to eliminate the quantitative conversion of $Cu^{II} \longrightarrow Cu^{I}$ and its reappearance by oxidation.

Experimental

SNAP[3] and GSNO[26] were prepared by the literature procedures of nitrosation of the thiols with nitrous acid. The *S*-nitroso derivatives of captopril and *N*-acetylcysteine were made by similar methods. The latter was obtained in an impure form. All of the other *S*-nitrosothiols used in this work were prepared in solution by nitrosation of the thiol with nitrous acid and the solution used as such after pH adjustment. All materials used were of the highest purity grade available.

Nitric oxide was detected using a World Precision NO probe which was calibrated using sodium nitrite and ascorbic acid. Nitrous acid (or nitrite ion) was determined by the standard Griess method.[27]

Disulfides were precipitated from solution when reactions were carried out at higher concentrations (typically 1×10^{-2} mol dm^{-3}). These were identified by comparison of mp and IR spectra with authentic samples, from the reactions of the nitrosothiols derived from cysteine, *N*-acetylpenicillamine and penicillamine. For the reaction of nitrosocysteine, the yield of cysteine was shown to be quantitative from the weight of the precipitate and the known solubility of cystine in water.

Kinetic studies were all carried out in water at 25 °C. Appropriate standard buffers were used. Reactions were followed by noting the decreasing absorbance (in the range 330–350 nm) due to the *S*-nitrosothiol, in a recording spectrophotometer. Some of the faster reactions were measured using a stopped-flow spectrophotometer. Reactions were generally first-order throughout and first-order rate constants k_o obtained from standard software packages. In general k_o values were reproducible to within ± 5%.

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