Identification of Cu^+ as the effective reagent in nitric oxide formation from *S*-nitrosothiols (RSNO)

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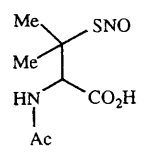
Decomposition of *S*-nitrosothiols (RSNO) in aqueous solution at pH 7.4 is brought about by copper ions, either present as an impurity or specifically added. The primary products are nitric oxide and the disulfide. In the presence of the specific Cu^+ chelator, neocuproine, reaction is progressively inhibited as the [neocuproine] is increased, the reaction eventually stopping completely. The characteristic UV–VIS spectrum of the Cu^+ adduct can be obtained from the reaction solutions. This shows clearly that Cu^+ and not Cu^{2+} is the effective catalyst. Two limiting kinetic conditions can be identified for a range of *S*-nitrosothiols at specific copper ion concentrations (*a*) a first-order dependence and (*b*) a zero-order dependence upon [RSNO]. Normally both situations also have a short induction period. This induction period can be removed by the addition of the corresponding thiol RSH. A mechanism is proposed in which Cu^+ is formed by reduction of Cu^{2+} by thiolate anion *via* an intermediate, possibly $RSCu^+$. Loss of nitric oxide from RSNO is then brought about by Cu^+, probably *via* another intermediate in which Cu^+ is bound to the nitrogen atom of the NO group and another electron-rich atom (such as nitrogen from an amino group, or oxygen from a carboxylate group) involving a six-membered ring. As well as NO this produces both RS^- and Cu^{2+} which then are part of the cycle regenerating Cu^+. Thiolate ion is oxidised to RS$^\cdot$ which dimerizes to give the disulfide. Depending on the structure (and hence reactivity) of RSNO either Cu^+ formation or its reaction with RSNO can be rate-limiting. Computer modelling of the reaction scheme allows the generation of absorbance time plots of the same forms as those generated experimentally, *i.e.* first- or zero-order, both with or without induction periods. We suggest that the thiolate ion necessary to bring about Cu^{2+} reduction is either present as a thiol impurity or is generated in small quantities by partial hydrolysis of the nitrosothiol, which results in an induction period. Addition of small quantities of thiol removes the induction period and leads to catalysis but larger quantities bring about a rate reduction by, it is suggested, complexation of the Cu^{2+}. For two very unreactive substrates, *S*-nitrosoglutathione and *S*-nitroso-*N*-acetylcysteine very large induction periods were observed, typically three hours. This results, we suggest, from competitive re-oxidation of Cu^+ to Cu^{2+} by the dissolved oxygen. Experiments carried out anaerobically confirm this, since there is then no induction period. Addition of hydrogen peroxide extends the induction period ever further. The results are discussed in terms of the biological properties of *S*-nitrosothiols which are related to nitric oxide release.

There is currently much interest in the chemistry of nitric oxide following the discoveries that it is involved in a range of human physiological processes.[1-3] Within the area some attention has been focussed on the release of nitric oxide from *S*-nitrosothiols (or thionitrites) RSNO, not only from the point of view of their potential use therapeutically as alternative NO-releasing drugs, but also with regard to their possible involvement *in vivo* as potential NO-storage and transport vehicles. There is a very real case for the generation of alternative drugs given the tolerance problem associated in many cases with the widespread use of glyceryl trinitrate for the treatment of angina and other circulatory problems.

S-Nitrosothiols are very easily generated in solution from thiols by electrophilic nitrosation,[4] *e.g.* in aqueous solution using acidified sodium nitrite. These reactions have been examined mechanistically[4,5] and show all the characteristics of electrophilic nitrosation reactions. Many *S*-nitrosothiols are too unstable in their pure form to be isolated (contrasting with their oxygen counterparts the alkyl nitrites) and this has contributed to the lack of knowledge of their chemistry relative to that of the alkyl nitrites, until comparatively recently. A number of *S*-nitrosothiols however are sufficiently stable to allow a full structural characterization. In particular *N*-acetyl-*S*-nitrosopenicillamine (SNAP) **1** and *S*-nitrosoglutathione (GSNO) **2** appear to be indefinitely stable as solids at room temperature. It has been known for some time[6,7] that RSNO



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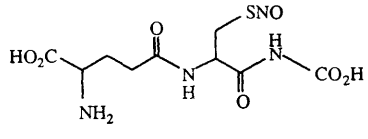

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species decompose photochemically and thermally to give nitric oxide and the corresponding disulfide [eqn. (1)]. Recently the

$$2RSNO \xrightarrow[\text{or } \Delta]{h\nu} RSSR + 2NO \qquad (1)$$

photochemical reaction of GSNO has been examined in more detail and the quantum yield and a first-order rate constant have been determined.[8] In both reactions it is likely that homolysis of the S–N bond is the primary process.

In solution, particularly aqueous solution, and in the absence of heat and light many investigations have reported that the same overall reaction occurs. In the presence of oxygen the final product is nitrite anion, as expected from the known reaction[9] of nitric oxide [eqn. (2)], but in the absence of oxygen, nitric

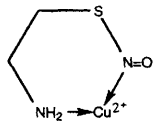
$$4NO + O_2 + 4OH^- = 4NO_2^- + 2H_2O \quad (2)$$

oxide has been detected using an electrode system.[10] At first quantitative rate measurements of RSNO decomposition reactions in aqueous solution have yielded erratic results, with reported half-lives of reaction varying considerably in different reports using the same substrate, usually SNAP. Further, the rate-form reported varied widely, zero-, first-, second- and various intermediate orders having been reported at some stage. This picture was resolved recently when it was realized that reaction occurred by a Cu^{2+}-catalysed reaction pathway, and that for some reactants there can be enough Cu^{2+} in the distilled water/buffer components used, to bring about reaction.[10] The $[Cu^{2+}]$ varied from source to source and often daily within the same source, which goes some way to explaining the erratic nature of the reported results. When Cu^{2+} is removed by complexation with EDTA, virtually no reaction takes place. There was no catalysis for a range of other metal ions investigated, including Zn^{2+}, Ca^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Cr^{3+} and Fe^{3+}.

In the earlier paper[10] we reported that for many RSNO compounds over a given $[Cu^{2+}]$ range (which varied with the substrate) the second-order rate equation, eqn. (3), applied,

$$\text{Rate} = k[\text{RSNO}][Cu^{2+}] \quad (3)$$

often with a small autocatalytic component, which we ignored. The copper is fully regenerated and so is truly catalytic. That work concentrated on the structure–reactivity dependence using nitrosothiol species which were of biological interest, mainly derivatives of cysteine and glutathione. Values of k [eqn. (3)] varied considerably with structure and clearly led to the conclusion that high reactivity was associated with RSNO structures in which the copper could bind with two sites within the molecule *via* six-membered ring intermediates such as those shown in structures **3** and **4**. These two binding sites we wrote as



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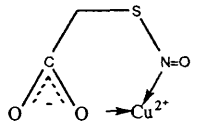

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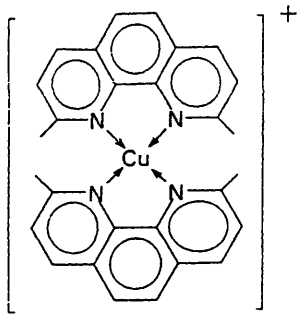
the nitroso–nitrogen atom in each case and either an amine or carboxylate group. In the absence of either of these features reaction was very slow indeed.

Although we did establish a qualitative structure–reactivity pattern it was clear that a full mechanistic picture of this reaction had not been described, since outside a given $[Cu^{2+}]$ (which differed for different substrates) other kinetic patterns emerged, in particular at low $[Cu^{2+}]$ there was an increasing tendency for autocatalysis to be observed and at high $[Cu^{2+}]$ there was a move towards a zero-order dependence upon [RSNO] and also upon $[Cu^{2+}]$. These rate forms together with a whole range of intermediate situations clearly led to quite a complex set of data. A further unusual feature occurred in the reaction of *N*-acetyl-*S*-nitrosocysteine in that there was a very long induction period (many hours) before reaction set in, with approximately a first-order dependence. This paper describes in more detail the more unusual kinetics and proposes a mechanism which is consistent with all of the experimental results.

Results and discussion

The unusual kinetic forms found for a number of RSNO species under different conditions of $[Cu^{2+}]$, particularly the tendency in some cases towards zero-order behaviour, led us to consider the possibility that the effective reagent in these reactions is in fact Cu^+ and not Cu^{2+}. Zero-order dependence upon [RSNO] might then be interpreted in terms of a rate-limiting $Cu^{2+} \longrightarrow Cu^+$ reduction. Earlier[10] we had considered this possibility and had rejected it on the basis that we had failed to find evidence for it from preliminary EPR experiments with SNAP. In an alternative approach, we have now made use of the specific Cu^+-chelator neocuproine[11] shown in the complexed form in **5**. In aqueous solution the stability constant of **5** is



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about 1×10^{19} dm^6 mol^{-2}. When a Cu^{2+} solution (2×10^{-5} mol dm^{-3}) was added to one of neocuproine hydrochloride (4×10^{-5} mol dm^{-3}) there was no detectable change in the UV–VIS spectrum. However upon addition of sodium dithionite (a well-known reducing agent for $Cu^{2+} \longrightarrow Cu^+$) an immediate yellow colour was noted and an absorbance maximum at 453 nm was found, as reported in the literature[11] for the spectrum of **5**. We then examined the decomposition of SNAP (1×10^{-3} mol dm^{-3}) at pH 7.4 containing added Cu^{2+} (2×10^{-5} mol dm^{-3}) in the presence of increasing concentrations of neocuproine in the range 4×10^{-5}–1×10^{-3} mol dm^{-3}. The resulting absorbance–time plots taken at 340 nm (the absorbance maximum for SNAP) are shown in Fig. 1. It is immediately clear that the presence of neocuproine reduces the reaction rate progressively and at 1×10^{-3} neocuproine the reaction is completely suppressed. The full spectra showed the increasing absorbance at 453 nm as expected for the formation of **5**. Similar experiments over a slightly smaller range of [added neocuproine] yielded reasonably good first-order plots and the data are given in Table 1 and Fig. 2 showing clearly the inhibiting effect of neocuproine.

Clearly Cu^+ is being generated and the question arises as to the nature of the reducing agent. Previously we have suggested that the reduction could be achieved by thiolate ion present from a small quantity of thiol impurity in the *S*-nitrosothiol sample. However we have been careful to avoid any thiol impurity by carrying out reactions on solution samples of RSNO generated from thiols and nitrous acid with the nitrous acid present in a slight excess, and reaction still occurs. An alternative suggestion is that thiolate is generated by hydrolysis of the *S*-nitrosothiol [eqn. (4)]. This is not expected to be a

$$\text{RSNO} + 2OH^- \longrightarrow RS^- + NO_2^- + H_2O \quad (4)$$

rapid process given our earlier studies of the hydrolysis in acid solution,[12] but even a few percent reaction could be enough to initiate the reduction. When the corresponding thiol is added to the reaction mixtures initially a rapid increase in the rate constant is found. We worked with SNAP with the addition of the corresponding thiol *N*-acetylpenicillamine NAP. Use of a different thiol would complicate the situation by rapid NO

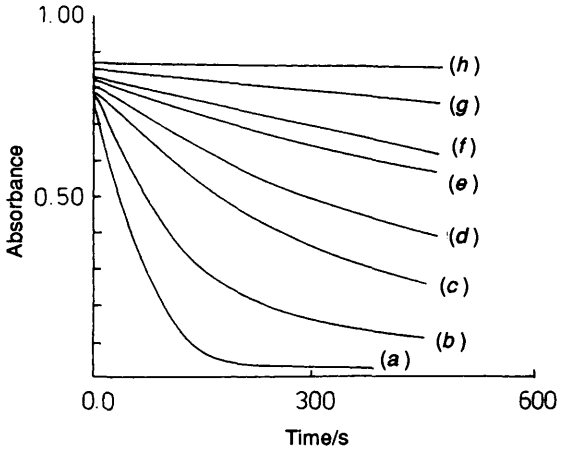


Fig. 1 Reaction of SNAP (1×10^{-3} mol dm^{-3}) in the presence of Cu^{2+} (2×10^{-5} mol dm^{-3}) and varying concentrations of neocuproine: (*a*) no added neocuproine; (*b*) 4×10^{-5} mol dm^{-3} neocuproine; (*c*) 5×10^{-5} mol dm^{-3} neocuproine; (*d*) 6×10^{-5} mol dm^{-3} neocuproine; (*e*) 8×10^{-5} mol dm^{-3} neocuproine; (*f*) 1×10^{-4} mol dm^{-3} neocuproine; (*g*) 2×10^{-4} mol dm^{-3} neocuproine; (*h*) 1×10^{-3} mol dm^{-3} neocuproine

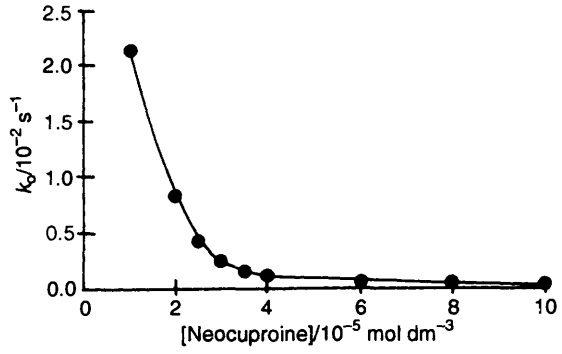


Fig. 2 First-order rate constants (k_o) for the reaction of SNAP (1×10^{-3} mol dm^{-3}) in the presence of Cu^{2+} (2×10^{-5} mol dm^{-3}) as a function of [neocuproine]

Table 1 Values of k_o for the decomposition of SNAP in the presence of added Cu^{2+} (2×10^{-5} mol dm^{-3}) and increasing amounts of neocuproine

Neocuproine/10^{-5} mol dm^{-3}	$k_o/10^{-2}$ s^{-1}
1.0	2.14
2.0	0.83
2.5	0.43
3.0	0.26
3.5	0.16
4.0	0.12
6.0	0.06
8.0	0.04
10.0	0.003

group transfer from RSNO to R'SH leading to R'SNO formation.[13] With [SNAP] of 1×10^{-3} mol dm^{-3}, added [Cu^{2+}] of 1×10^{-5} mol dm^{-3} and [NAP] in the range of 1×10^{-6}–1×10^{-3} mol dm^{-3}, reactions are kinetically first-order. The results are given in Table 2 and are also shown more dramatically in Fig. 3. As expected at low added NAP there is a very sharp linear increase in the rate constant until [NAP] $\approx 1 \times 10^{-5}$ mol dm^{-3}. The reduction of Cu^{2+} by thiolate is a well-known process[14,15] and has been studied mechanistically as the Cu^{2+} catalysed oxidation of thiols to give disulfides [eqn. (5)].

$$2Cu^{2+} + 2RSH = 2Cu^{+} + 2H^{+} + RSSR \quad (5)$$

At higher [NAP] there is initially a sharp drop in k_o followed

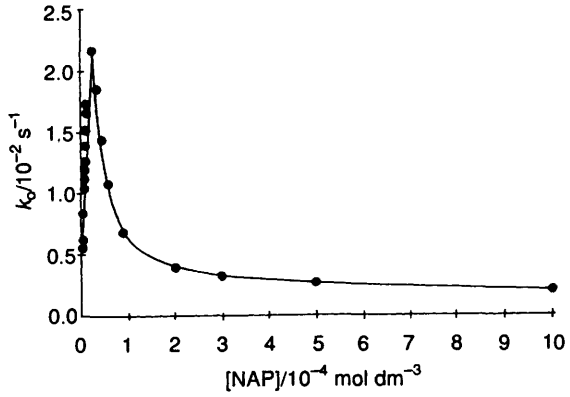
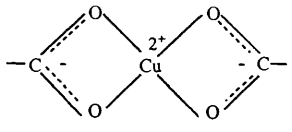


Fig. 3 First-order rate constants (k_o) for the reaction of SNAP (1×10^{-3} mol dm^{-3}) as a function of added *N*-acetylpenicillamine (NAP)

Table 2 Values of k_o for the decomposition of SNAP in the presence of added Cu^{2+} (1×10^{-5} mol dm^{-3}) and increasing amounts of NAP

[NAP]/10^{-6} mol dm^{-3}	$k_o/10^{-3}$ s^{-1}
0	4.97
1.0	5.63
2.0	6.25
3.0	8.35
4.0	10.4
5.0	11.9
6.0	12.8
7.0	14.0
8.0	15.3
9.0	16.6
10	17.4
20	21.7
30	18.7
40	14.5
50	10.9
90	6.79
200	4.14
300	3.37
500	2.67
1000	2.12

by a gradual decrease towards zero. We can explain this pattern in terms of the complexing of Cu^{2+} by NAP, thus effectively removing it from solution. These results explain the apparently contradictory reports in the literature,[16] some of which report catalysis of RSNO decomposition by added thiols whilst others find a reduction in rate upon thiol addition. It is now clear that at low added [RSH] there will be catalysis, as this favours the reduction of Cu^{2+}, whereas complexation of Cu^{2+}, probably by the carboxylate group, takes over at higher added [RSH] resulting in inhibition of nitric oxide formation. Such copper-carboxylates are well-known (see structure **6**) and some have



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been isolated and examined structurally.[17] We have previously noted[10] a reduction in reactivity with increasing buffer concentration when the buffer contains a carboxylic acid, an effect we attributed to competitive complexation with the carboxylic acid.

Earlier[10] we concentrated our kinetic analysis on rate forms which gave first-order dependencies upon both RSNO and Cu^{2+} in order to establish structure–reactivity factors. We now

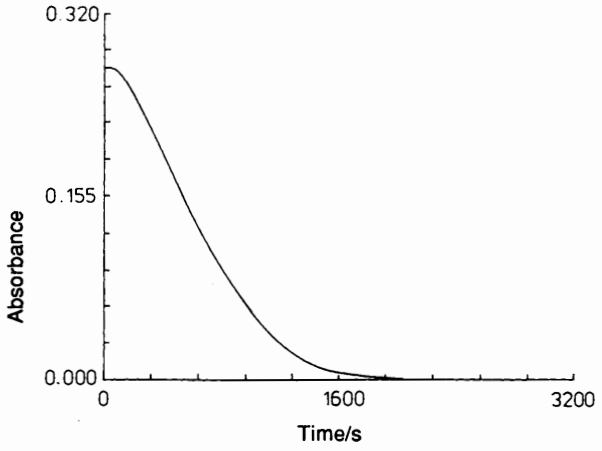


Fig. 4 Absorbance–time plot for the reaction of *S*-nitroso-2-dimethylaminoethanethiol (5×10^{-4} mol dm^{-3}) in the presence of added Cu^{2+} (1×10^{-6} mol dm^{-3})

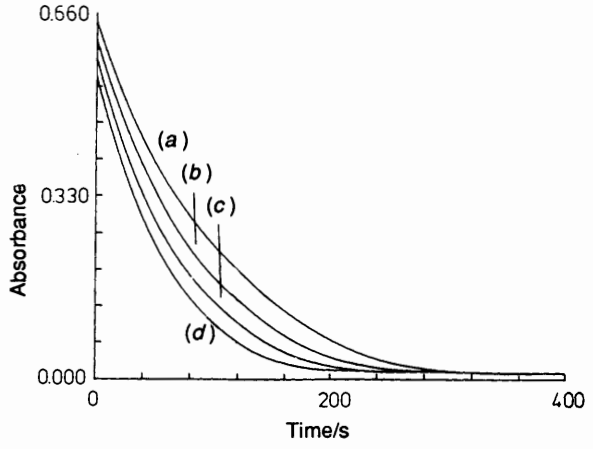


Fig. 5 Absorbance–time plots for the reaction of SNAP (1×10^{-3} mol dm^{-3}) in the presence of Cu^{2+} (1×10^{-5} mol dm^{-3}) and varying concentrations of added *N*-acetylpenicillamine (NAP): (*a*) 4×10^{-6} mol dm^{-3} NAP; (*b*) 6×10^{-6} mol dm^{-3} NAP; (*c*) 8×10^{-6} mol dm^{-3} NAP; (*d*) 1×10^{-5} mol dm^{-3} NAP

report the results of a more systematic kinetic study in which other rate forms appeared. In all cases we have been following the decreasing absorbance at *ca.* 340 nm due to the RSNO reactant, usually at an initial concentration of around 5×10^{-4} mol dm^{-3}. We have been able to isolate four limiting absorbance–time patterns: (*a*) first-order reaction with an induction period, (*b*) first-order reaction with no induction period, (*c*) zero-order reaction with an induction period and (*d*) zero-order reaction with no induction period. In addition we observed many forms which could be regarded as intermediate between any two of the four limiting forms. Examples of each are given in Figs. 4, 5, 6, 7 and 8. Fig. 4 is the reaction of *S*-nitroso-2-dimethylaminoethanethiol (SNDMA) **8**, at low [Cu^{2+}] with no added thiol, Fig. 5 is the reaction of SNAP at 1×10^{-5} mol dm^{-3} Cu^{2+} in the presence of varying [NAP], Fig. 6 is the reaction of *S*-nitroso-2-diethylaminoethanethiol with various concentrations of added Cu^{2+}, Fig. 7 is a similar pattern for the reaction of *N*-acetyl-D,L-2,2-dimethylcysteinyl-glycine methyl ester **7** with different [Cu^{2+}] and Fig. 8 is SNDMA **8** at low [Cu^{2+}] varying the concentration of added thiol. The induction period is quite clear in Figs. 4, 6 and 7 and is completely absent in Figs. 5 and 8 at high [RSH]. Equally clear is the first-order pattern in Figs. 4 and 5 and zero-order dependence in Figs. 6, 7 and 8.

On the basis of these and earlier results we propose the following outline mechanism: Cu^{2+} is reduced by RS^- (generated from RSNO or added as RSH) *via* intermediate X

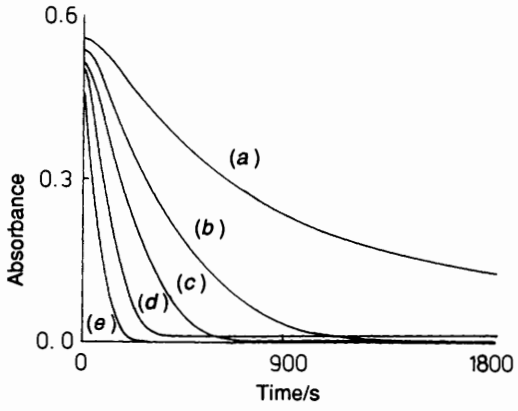


Fig. 6 Absorbance–time plots for the reaction of *S*-nitroso-2-diethylaminoethanethiol (1×10^{-3} mol dm^{-3}) in the presence of added Cu^{2+}: (*a*) 5×10^{-6} mol dm^{-3} Cu^{2+}; (*b*) 7.5×10^{-6} mol dm^{-3} Cu^{2+}; (*c*) 1×10^{-5} mol dm^{-3} Cu^{2+}; (*d*) 3×10^{-5} mol dm^{-3} Cu^{2+}; (*e*) 6×10^{-5} mol dm^{-3} Cu^{2+}

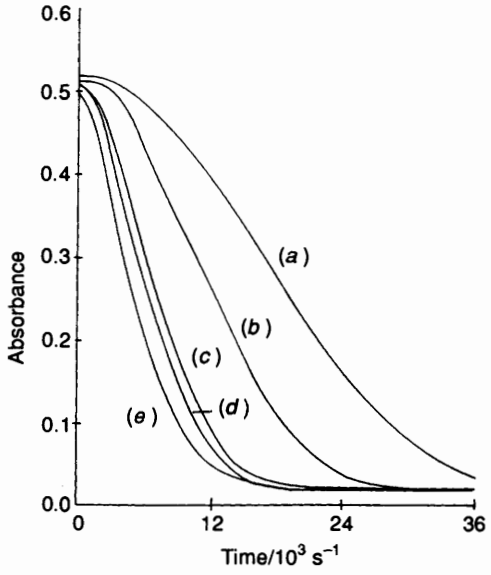


Fig. 7 Absorbance–time plots for the reaction of SNAP–Gly (1×10^{-3} mol dm^{-3}) in the presence of added Cu^{2+}: (*a*) 2.7×10^{-6} mol dm^{-3} Cu^{2+}; (*b*) 5.4×10^{-6} mol dm^{-3} Cu^{2+}; (*c*) 8.1×10^{-6} mol dm^{-3} Cu^{2+}; (*d*) 1.1×10^{-5} mol dm^{-3} Cu^{2+}; (*e*) 1.4×10^{-5} mol dm^{-3} Cu^{2+}

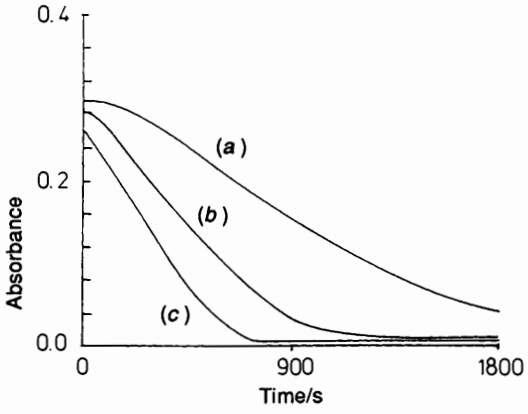


Fig. 8 Absorbance–time plots for the reaction of *S*-nitroso-2-dimethylaminoethanethiol (2×10^{-4} mol dm^{-3}) in the presence of added Cu^{2+} (1×10^{-6} mol dm^{-3}) as a function of added 2-*N*,*N*-dimethylaminoethanethiol: (*a*) No added thiol; (*b*) Added thiol (1×10^{-6} mol dm^{-3}) (*c*) Added thiol (3×10^{-6} mol dm^{-3})

to give Cu^+ and RS$^{\cdot}$. Intermediate X is probably $RSCu^+$. Reaction then occurs between Cu^+ and RSNO *via* intermediate Y releasing Cu^{2+}, RS^- and NO. This is essentially the

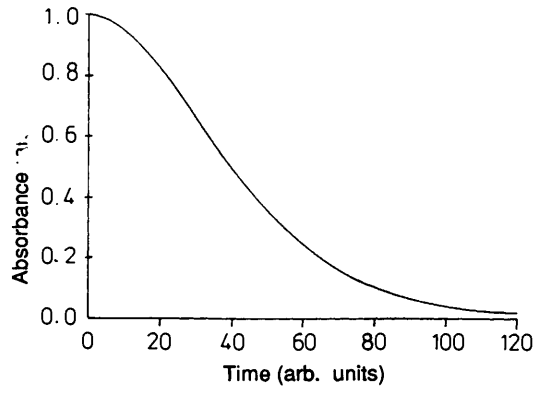


Fig. 9 Computed absorbance–time plot with no added RSH for rate-limiting RSNO reaction with Cu^+

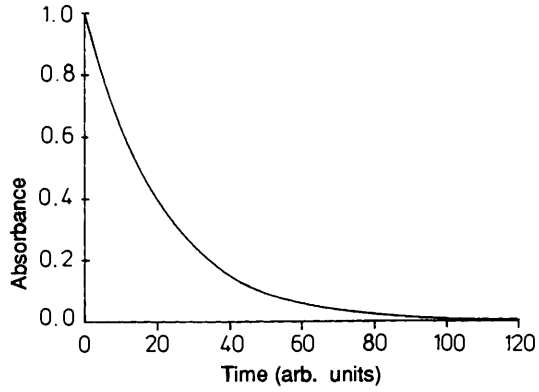


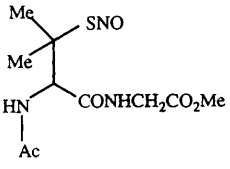
Fig. 10 Computed absorbance–time plot with added RSH for rate-limiting RSNO reaction with Cu^+

$$Cu^{2+} + RS^- \rightleftharpoons X \longrightarrow Cu^+ + RS^\cdot$$

$$Cu^+ + RSNO \rightleftharpoons Y \longrightarrow Cu^{2+} + RS^- + NO$$

$$2RS^\cdot \longrightarrow RSSR$$

Scheme 1



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mechanism we suggested earlier [18] as a possibility, but without at that time sufficient evidence to support it. Intermediates Y we believe are akin to structures **3** and **4**, now with Cu^+ and not Cu^{2+}, probably also co-ordinated to two water molecules.

Qualitatively we can account for the induction period as the time required for the generation of RS^- and Cu^+. A first-order dependence occurs if the reaction of RSNO with Cu^+ is rate-limiting and a zero-order dependence occurs (for the more reactive RSNO species) when Cu^+ formation is rate limiting. The length of the induction period can be reduced by the addition of either RSH or Cu^{2+} which will increase the rate of formation of Cu^+.

We have attempted to explain the various rate patterns encountered experimentally more quantitatively by use of a computer model of Scheme 1, and to extract the limiting forms which this model predicts. We have not aimed at a full treatment optimizing all of the parameters because of the uncertainties of the values of the initial $[Cu^{2+}]$ and $[RS^-]$ particularly when an induction period occurs. Our hope was that we would be able to reproduce qualitatively the absorbance–time experimental data with those predicted from Scheme 1, particularly under four limiting conditions.

(1) Rapid formation and regeneration of Cu^+

Scheme 1 reduces (for kinetic purposes) to eqns. (6) and (7). If RSNO, Cu^{2+} and RS^- are present at the start then the result

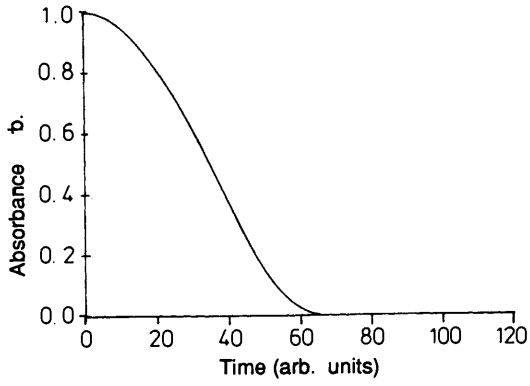

Fig. 11 Computed absorbance–time plot with no added RSH for rate-limiting Cu^+ formation

$$RSNO + Cu^+ \xrightarrow{slow} RS^- + NO + Cu^{2+} \quad (6)$$

$$RS^- + Cu^{2+} \xrightarrow{fast} \tfrac{1}{2}RSSR + Cu^+ \quad (7)$$

will be a simple first order rate equation [eqn. (8)] where the

$$-d[RSNO]/dt = d[NO]/dt = k_o[RSNO] \quad (8)$$

observed rate constant k_o will be given by $k[Cu^{2+}]$ when $[Cu^{2+}] \ll [RS^-]$ (the normal state of affairs with added RS^-) and by $k[RS^-]$ when $[Cu^{2+}] \gg [RS^-]$. The computer simulation is given in Fig. 10. We have observed many examples of such behaviour experimentally (as in Fig. 5 as [NAP] is increased). In effect we now have rapid formation and regeneration of Cu^+, the concentration of which remains constant in any one experiment.

Alternatively, if only RSNO and Cu^{2+} are initially present the step for the generation of Cu^+ [eqn. (4)] must be included in the simulation. This results in an absorbance–time profile given in Fig. 9, with an autocatalytic feature which reproduces that observed experimentally in Fig. 4.

(2) Rate-limiting Cu^+ formation

Under these circumstances Scheme 1 effectively reduces to eqns. (9) and (10). If RSNO, Cu^{2+} and RS^- are all present

$$RS^- + Cu^{2+} \xrightarrow{slow} \tfrac{1}{2}RSSR + Cu^+ \quad (9)$$

$$Cu^+ + RSNO \xrightarrow{fast} RS^- + NO + Cu^{2+} \quad (10)$$

initially then we get a truly zero-order reaction (simulation in Fig. 12) with RSNO scavenging Cu^+ as it is formed immediately regenerating RS^- and Cu^{2+}. However if only RSNO and Cu^{2+} are present initially, then RS^- has to be generated as before and this results in an induction period

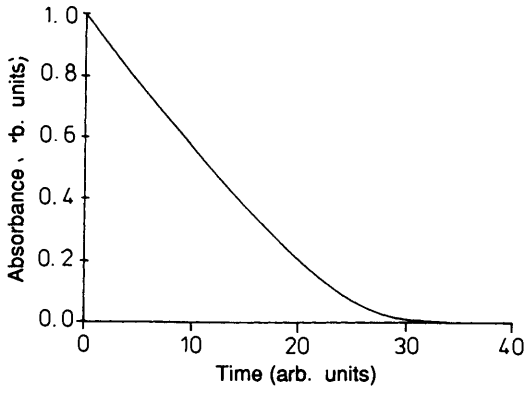

Fig. 12 Computed absorbance–time plot with added RSH for rate-limiting Cu^+ formation

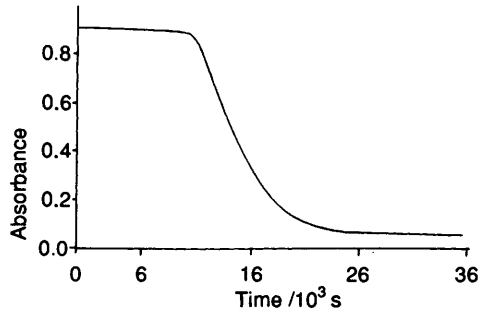

Fig. 13 Absorbance–time plot for the reaction of *N*-acetyl-*S*-nitrosocysteine SNAC (crude sample)

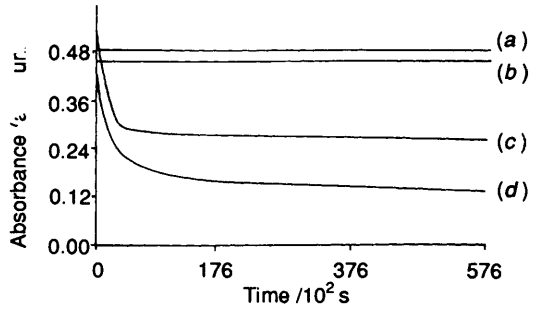

Fig. 14 Absorbance–time plots for the reactions of GSNO and *N*-acetyl-*S*-nitrosocysteine (SNAC) showing the effect of the presence of oxygen: (*a*) GSNO under aerobic conditions; (*b*) SNAC under aerobic conditions; (*c*) GSNO under anaerobic conditions; (*d*) SNAC under anaerobic conditions

followed by a zero-order reaction (Fig. 11). Experimental examples of both types of behaviour have been observed as in Fig. 8, as $[RS^-]$ is increased, Figs. 6 and 7. Figs. 6 and 7 clearly show a saturation effect in Cu^{2+} at high $[Cu^{2+}]$ which requires that in Cu^+ formation we have an equilibrium involving intermediate X.

All of these experiments were conducted at pH 7.4 in normal aerated solvent. When oxygen was rigorously excluded then we found no significant difference in behaviour for any of the limiting rate forms. This implies that any oxidation reaction, such as of Cu^+ by dissolved oxygen, is not a significant reaction under these conditions. However for very slow reacting RSNO compounds, such as *N*-acetyl-*S*-nitrosocysteine (SNAC) we found a very long induction period typically 3 hours as shown in Fig. 13. This result was obtained using a rather impure solid sample of SNAC which was known to contain some of the thiol and was very difficult to purify. Repetition of this experiment with SNAC generated *in situ* in solution gave no reaction whatsoever over a 16 hour period (Fig. 14), so it is likely that the thiol is playing a part here in Cu^+ generation as expected. However when the same reaction was carried out in the absence of oxygen, reaction occurred immediately, with no induction period. Exactly the same pattern was found with *S*-nitrosoglutathione (GSNO), although in both cases it is not clear why complete reaction does not occur. There is however a very dramatic effect due to the presence of oxygen here, which implies that oxidation of Cu^+ by oxygen [eqn. (11)] is a competing reaction with the Cu^+ reaction

$$Cu^+ + O_2 = Cu^{2+} + O_2^- \qquad (11)$$

with RSNO.

When the oxygen is removed decomposition can occur. For both of these substrates reaction can be induced by the addition of the corresponding thiol. An induction period occurs which is reduced as the concentration of added thiol is increased until we get a good first-order plot at very high [thiol] ≈ 0.15 mol dm^{-3}. In the presence of thiol, the effect of increasing the $[Cu^{2+}]$ is also to reduce the induction period, and as before the removal of oxygen also removes the induction period completely. For these slower reacting substrates it does seem that re-oxidation of Cu^+ by dissolved oxygen is an important pathway in their decomposition reactions.

We believe that we have shown that Cu^+ is the effective reagent in bringing about decomposition of *S*-nitrosothiols to yield nitric oxide. Both the experiments with neocuproine and all the kinetic evidence support the outline mechanism given in Scheme 1. Some details remain as yet unresolved, *e.g.* the detailed mechanism for the breakdown of intermediate Y, for which at present we have no experimental evidence.

These findings could well have implications for the reactions of *S*-nitrosothiols *in vivo*. It is known that they have specific biological activity notably, vasodilation,[19] the inhibition of platelet aggregation[20] and the inhibition of neutrophil functions.[21] In the body copper is present not as free Cu^{2+} but in a bound form with amino acids and proteins. Possibly this is reducible to Cu^+ by thiolate. Experiments to test this possibility are in hand. A recent particularly interesting finding[22] shows that the inhibition of platelet aggregation activity shown by GSNO is much reduced in the presence of neocuproine and the closely related bathocuproine, both specific Cu^+-chelators. This strongly suggests (*a*) that nitric oxide is required to effect the activity and (*b*) that it is generated from *S*-nitrosothiols by a process which involves Cu^+. However another recent article[23] claims that bronchodilation induced by GSNO does not require the formation of NO. The medical importance of *S*-nitrosothiols has been highlighted recently by two reports,[24,25] which describe the clinical use of GSNO to inhibit platelet aggregation during coronary angioplasty and also to treat a form of pre-eclampsia, a high blood pressure condition suffered by some pregnant women.

The reactivity pattern of GSNO is particularly interesting. In aerobic solution it is particularly stable, with only a negligibly small amount of decomposition occuring over many hours. However, in the presence of glutathione GSH decomposition occurs much more rapidly but possibly with an induction period, the length of which is dependent on [GSH] and $[Cu^{2+}]$. Further, in the absence of oxygen and without added GSH, reaction occurs quite readily without an induction period. Finally, the presence of another oxidizing agent (*e.g.* hydrogen peroxide) has the effect of stabilizing GSNO, again over a period of many hours, in our experiments no perceptible decomposition occurred overnight. Again the re-oxidation of $Cu^+ \longrightarrow Cu^{2+}$ does not allow the Cu^+ + RSNO to occur.

Experimental

All thiols, buffer components, *etc.* were commercial samples of the highest purity grade available. The *S*-nitrosothiols were mostly synthesized in solution, and not isolated, by nitrosation of the thiols with an equivalent of nitrous acid under mildly acid conditions. All gave the characteristic broad absorption band centred around 340 nm. Aliquots of these freshly prepared solutions were used after pH adjustment, taking care to minimize exposure to light. In the case of SNAP **1** and GSNO **2**, the solid derivatives were prepared as described in the literature.[26,27] An impure sample of *N*-acetyl-*S*-nitrosocysteine was prepared by a modification of the method used for GSNO. The *S*-nitroso dipeptide **7** was a new compound and was prepared as follows.

A solution of glycine methyl ester hydrochloride (6.28 g, 50 mmol) in water (40 cm^3) was treated with a solution of potassium carbonate (72 mmol) in water (20 cm^3) and the mixture extracted with dichloromethane. After drying ($MgSO_4$), the solvent was removed by evaporation. The residue was added to a suspension of *N*-acetyl-D,L-penicillamine (3.28 g, 20 mmol) in purified dichloromethane (100 cm^3) followed by 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulfonate (8.47 g, 20 mmol). The urea derivative started to precipitate immediately and after 2 days was removed by filtration and washed with dichloromethane. The combined filtrate and washings were extracted with a saturated solution of citric acid (60 cm^3), a saturated solution of potassium hydrogen carbonate (60 cm^3) and water (60 cm^3), dried ($MgSO_4$) and the solvent removed by evaporation. The residue was washed with cold ether to give white, flaky crystals of product, mp 142 °C (decomp.). It was used without purification for the next stage [Found: *m/z* (FAB) 263.1061 (MH^+). $C_{10}H_{19}O_4N_2S$ requires 263.1066 (MH^+)]. The *S*-nitroso derivative was prepared by dissolving the dipeptide (0.26 g, 1 mmol) in dichloromethane (8 cm^3) and adding *tert*-butyl nitrite (1 cm^3). After 1 h the solvent from the green solution was removed by evaporation to give a green solid. After washing with water the solid was dried *in vacuo* (0.24 g, 82%), *m/z* (FAB) 292.0967 (M^+, 292.0976) [Found: C, 41.0; H, 6.2; N, 14.2. $C_{10}H_{17}O_5N_3S$ requires C, 41.2; H, 5.9; N, 14.4%].

Products

The disulfide products, nitrite anion and nitric oxide (in the absence of oxygen) were determined as reported earlier.[10]

Kinetics

These were carried out also as reported earlier[10] by monitoring the decreasing absorbance at *ca.* 340 nm.

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