Reversibility of *S*-nitrosothiol formation

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The nitrosation of thiols is sufficiently reversible to allow, in many cases, the low thiol concentration present at equilibrium to reduce Cu^{2+} and bring about loss of nitric oxide from *S*-nitrosothiols.

It is now clear that the decomposition of *S*-nitrosothiols in an aqueous buffer to give nitric oxide and the disulfide is brought about by Cu^{+}, which in turn is generated by reduction of Cu^{2+} by thiolate ion,[1] or in principle by any other reducing agent. In many cases there is enough free Cu^{2+} present in the aqueous buffer solution for this reaction to take place. Reduction of Cu^{2+} sources where the copper is bound to peptides and proteins also occurs, thus allowing the possibility that these reactions could occur *in vivo*.[2] Currently there is considerable interest in the chemistry/biochemistry of nitrosothiols, since (*a*) they are being examined as possible drugs to effect vasodilation and to reduce platelet aggregation,[3] and (*b*) they are now believed to play an important part in some of the physiological processes involving nitric oxide.[4]

There is a question as to where the thiolate ion comes from, and it has been suggested that enough is present as an impurity, deriving from the nitrosothiol synthesis, whereas it has also been suggested that some partial hydrolysis of the nitrosothiol occurs.[1] We have eliminated the first possibility as a general explanation, by working with solutions of nitrosothiols generated *in situ*, using a very large excess of nitrous acid, when we find that reaction often still occurs.[5] We therefore looked at the possibility that the *S*-nitrosation is a reversible process.

Nitrosation of alcohols is a well-known reversible process in acid solution, [eqn. (1)],[6] and equilibrium constants have been

$$ROH + HNO_2 = RONO + H_2O \quad (1)$$

determined for many alcohols. Some typical values are 3.5 and 1.2 $dm^3\ mol^{-1}$ for methanol and ethanol respectively.[7] A kinetic method for demonstrating this reversibility is by the presence of a positive intercept at [ROH] = 0, when the first-order rate constant (k_{obs}) is plotted against [ROH], when reactions are carried out with [ROH] $\gg$ [HNO_2]. Equilibrium constants determined in this way[8] are in reasonable agreement with those obtained from direct measurements.[7] Such plots for the nitrosation of thiols showed no measurable intercepts;[9] it was generally assumed by us that the reactions are effectively (*i.e.* from a preparative point of view) irreversible, and this has been rationalised in terms of the difference in nucleophilicities (S > O), important for the forward reaction, and the difference in basicities (O > S), important in the reverse reaction, since *O*- or *S*-protonation occurs. We examined this more carefully in the case of the nitrosation of thiomalic acid. The results are shown in Fig. 1, for reaction at two different acidities. It is clear that there is no measurable intercept at [RSH] = 0. Nevertheless we have analysed solutions of nitrosothiols for thiol content, using Ellman's reagent.[10] Calibration experiments revealed that we were able to measure thiol concentrations in the range 8×10^{-6} to 5×10^{-5} mol dm^{-3} very readily, from measurements at 412 nm of the absorbance due to the dianion of 2-nitro-5-thiobenzoic acid. All of our calibration measurements gave values for the extinction coefficient at this wavelength which were within 3% of the literature value[10] (14 150 $dm^3\ mol^{-1}\ cm^{-1}$).

Results were obtained for the product of nitrosation of penicillamine (6.6×10^{-4} mol dm^{-3}), using stoichiometric ratios of [RSH]:[HNO_2] ranging from 1:1 to 1:2. The nitrosation reactions were carried out under mildly acid conditions (*ca*. 0.05 mol dm^3 H^+), where it is known that reaction is very fast. Analysis for thiol content was carried out at pH 7.27 as described in the literature.[10] At this pH no reactions associated with eqn. (2) will occur. The results are

$$RSH + HNO_2 = RSNO + H_2O \quad (2)$$

presented in Table 1 in the form of the % [RSH] present at the equilibrium position. All of these *S*-nitrosations are very rapid processes. It is clear that thiol remains in all of these solutions, in a quite significant concentration for the 1:1 solution, dropping as expected as we move to the 1:2 mixture. These figures (ignoring the final value, where the concentration is very low and therefore subject to a large uncertainty), give an approximate equilibrium constant (*K*) for eqn. (2) of 3×10^5 $dm^3\ mol^{-1}$.

Values of *K* of this magnitude would not lead to a measureable intercept for plots such as that shown in Fig. 1, so this kinetic method is obviously not sufficiently sensitive to measure equilibrium constants of this magnitude. We have shown that this is the general situation for the nitrosation of thiols, by determining the equilibrium thiol concentration in a number of other cases. For *S*-nitrosocysteine generated *in situ*, we find 7.8% thiol remains at equilibrium from a 1:1 ratio of reactants (corresponding to an approximate *K* value of 6×10^5 $dm^3\ mol^{-1}$). The thiol content drops significantly on standing (to a fifth of its value in 1 h), no doubt because of aerial oxidation. Similarly, the nitrosation of thiomalic acid gives

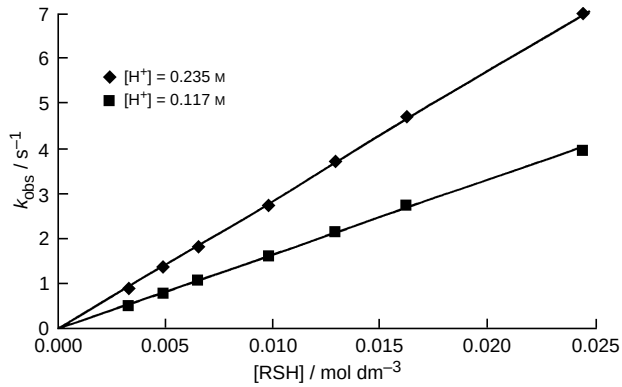

Fig. 1 Plot of the first order rate constant (k_{obs}) *vs* [RSH] for the nitrosation of thiomalic acid by nitrous acid (1×10^{-4} mol dm^{-3}) at two different acidities

Table 1 Percentage thiol remaining at equilibrium in the nitrosation of penicillamine (6.7×10^{-4} mol dm^{-3})

[RSH]:[HNO_2]	% RSH at equilibrium
1:1	5.3
1:1.1	4.5
1:1.2	3.2
1:1.5	0.65
1:2	0.08

2.0% thiol at equilibrium from a 1 : 1 ratio of reactants, dropping to 0.11% for a 1 : 1.5 ratio, giving a K value of *ca.* 3×10^6 $dm^3\ mol^{-1}$. Nitrosation of *N*-acetyl penicillamine gave 0.45% thiol from a 1 : 1 reactants ratio. It is not clear why this is so much smaller than that found for penicillamine. A fuller systematic study of the system is underway.

It follows that if *S*-nitrosation of thiols is significantly reversible in this way, then solutions of *S*-nitrosothiols, initially prepared in acid solution, will always contain a small thiol concentration. This will apply to solutions of nitrosothiols made up in any mildly acidic solution, but not presumably to solutions made up in basic solution. Solutions made up (directly in alkaline buffer) from two stable solid nitrosothiols, *S*-nitroso-*N*-acetyl penicillamine (SNAP) and *S*-nitrosoglutathione GSNO, analysed respectively for 0.78 and 0.80% thiol, probably derived from thiol impurity resulting from the incomplete *S*-nitrosation during synthesis. The difficulty in producing nitrosothiol samples totally free from thiol has been reported[11] in the case of GSNO.

Our results provide an explanation as to why reduction of Cu^{2+} by thiolate (in solutions of *S*-nitrosothiols), eqn. (3),

$$2Cu^{2+} + 2RS^- = 2Cu^+ + RSSR \qquad (3)$$

occurs so readily in solutions where it would be expected that there would be no thiolate present. This applies whenever the *S*-nitrosothiol solution is prepared *in situ* from solutions of the thiol and nitrous acid, or in cases where mildly acidic solutions are first made up from solid samples.

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