Reactivity of sulfur nucleophiles towards *S*-nitrosothiols

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Rate constants have been measured for the reactions of a range of *S*-nitrosothiols with the following sulfur-centred nucleophiles: sulfite ion, thiourea, thiocyanate ion, thiosulfate ion, thiomethoxide ion and sulfide ion. Many of the reactions were very fast and were followed in a stopped-flow spectrophotometer. For the sulfite reaction the reactive species over the pH range 4–8 was shown to be exclusively SO_3^{2-}. For two RSNO species the reactivity sequence was established as: $SO_3^{2-} > MeS^- > S_2O_3^{2-} \gg SC(NH_2)_2 > SCN^-$. The reaction with sulfide ion was also rapid and generated a fairly stable yellow species (λ_{max} 410 nm), which was probably the nitrosodisulfide ion $ONSS^-$, but the absorbance–time data were too complex for a simple kinetic analysis. This reaction could have some potential as an analytical procedure for the determination of RSNO species. The kinetic results are discussed in terms of the factors affecting nucleophilicity and are compared with the corresponding reactions of other nitrosating species.

Currently there is much interest in the chemistry of *S*-nitrosothiols, RSNO, formerly described as thionitrites, with regard to their ability either to generate nitric oxide under *in vivo* conditions or to transfer the NO group to nucleophilic sites generally.[1] There has built up a strong body of evidence suggesting that NO might be stored in the body in the form of *S*-nitrosothiols where nitrosation of a thiol group (or groups) in a protein such as serum albumin has occurred.[2] Very recently a debate has evolved regarding the role of *S*-nitrosohaemoglobin in the regulation of blood pressure.[3,4]

It is now established that at least *in vitro*, NO is released from RSNO species in solution at pH 7.4 by a Cu^{2+}-catalysed process, where the effective reagent is Cu^+, generated by reduction.[5] Whether this occurs *in vivo* is a matter of conjecture at this point, although it has been shown that Cu^+ can be obtained from Cu^{2+} bound to proteins and peptides, promoting RSNO decomposition[6] and it has also been shown that chelation of Cu^+ does indeed reduce the biological activity of nitrosothiols.[7] The spontaneous thermal decomposition of nitrosothiols is very slow in aqueous solution and generally does not compete effectively with the Cu^{2+} catalysed reaction.

The transfer of the NO group from RSNO to R′SH (sometimes called a transnitrosation reaction) is also well established [eqn. (1)] and occurs quite rapidly at pH 7.4, without the prior

$$RSNO + R'S^- \longrightarrow RS^- + R'SNO \qquad (1)$$

release of NO,[8,9] and takes place by nucleophilic attack by $R'S^-$ at the nitroso nitrogen atom. Reactions also occur with other nucleophiles. Recently we published the kinetic details of the reaction of a number of nitrogen-centred nucleophiles [*e.g.* eqn. (2)] with a nitrosothiol.[10] Similar reactions with ascorbate[11] [eqn. (3)] and hydroperoxide[12] [eqn. (4)] have also been

$$RSNO + R'_2NH \longrightarrow RSH + R'_2NNO \qquad (2)$$

$$RSNO + Asc^{2-} + H^+ \longrightarrow RSH + NO + \text{dehydroascorbic acid} \qquad (3)$$

$$RSNO + HOO^- \longrightarrow RSH + ONOO^- \qquad (4)$$

described. In the present paper we similarly give the kinetic details of the corresponding reactions with the following sulfur-centred nucleophiles: sulfite ion, thiourea, thiocyanate ion, thiosulfate ion, thiomethoxide ion and sulfide ion.

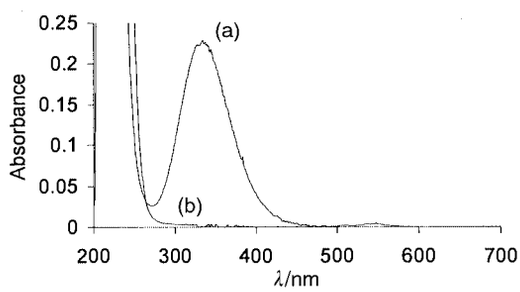

Fig. 1 Spectra of (a) GSNO (2.5×10^{-4} mol dm^{-3}) in buffer pH 7.4 containing EDTA (2.5×10^{-4} mol dm^{-3}), and (b) 30 s after the addition of $NaHSO_3$ (0.01 mol dm^{-3}).

Results and discussion

(a) Reactions with sulfite ion

Reaction of sulfite ion with nitrosothiols was very rapid. Fig. 1 shows the spectrum of *S*-nitrosoglutathione (GSNO), before and 30 s after the addition of excess sulfite, when the reaction is essentially complete. Rate measurements were thus only possible using the stopped-flow procedure. Each reaction solution contained EDTA at 2.5×10^{-4} mol dm^{-3}, well in excess of the likely impurity level of Cu^{2+}. This ensures that we can rule out any reactions which follow from the formation of free NO by the well-established Cu^{2+}-catalysed process. All reactions gave very good first-order behaviour in the individual kinetic experiments, and there was an excellent first-order dependence on $[SO_3^{2-}]_T$, the total stoichiometric sulfite concentration, when reactions were carried out under pseudo first-order conditions, with $[SO_3^{2-}] \gg [RSNO]$. Results for five *S*-nitrosothiols are shown in Fig. 2. Similar results were obtained for a number of other *S*-nitrosothiols.

Values of the measured pseudo first-order rate constant, k_{obs}, were obtained as a function of pH in the range 4–8 for two *S*-nitrosothiols. The results are shown in Fig. 3 for *S*-nitrosopenicillamine and *S*-nitrosocysteine and strongly suggest that reaction occurs *via* the free sulfite ion, since the pK_a of HSO_3^- is ~7. If this is the case, then eqn. (5) should apply,

$$k_{obs} = k_2K_a[SO_3^{2-}]_T/(K_a + [H^+]) \qquad (5)$$

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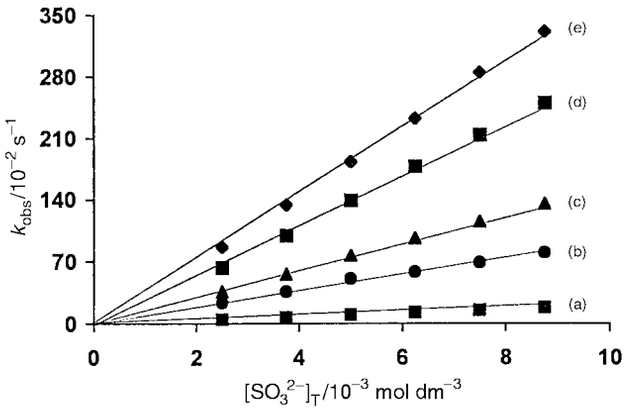

Fig. 2 Plots of the measured first-order rate constant, k_{obs}, against [added SO_3^{2-}] for five *S*-nitrosothiols (a) *S*-nitroso-*N*-acetylcysteine, (b) *S*-nitrosoglutathione, (c) *S*-nitrosotiopronin, (d) *S*-nitroso-2-aminoethanethiol and (e) *S*-nitrosopenicillamine.

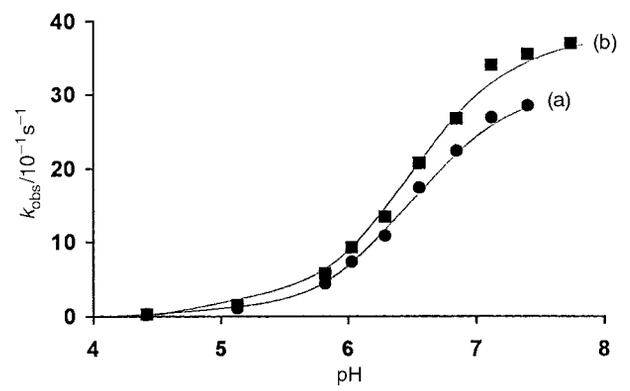

Fig. 3 Values of k_{obs} as a function of pH for the reaction of (a) *S*-nitrosopenicillamine and (b) *S*-nitrosocysteine (both 2.5×10^{-4} mol dm^{-3}) with sulfite (7.5×10^{-3} mol dm^{-3}).

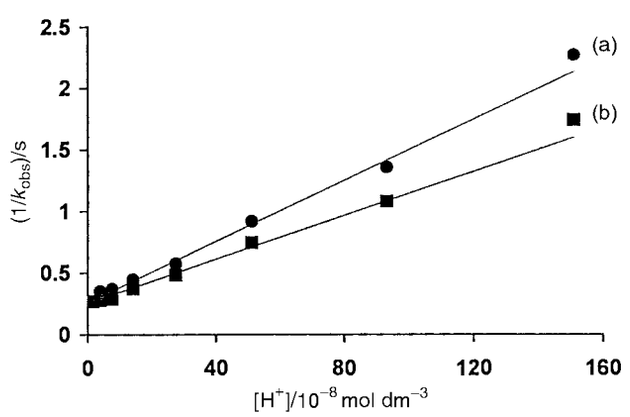

Fig. 4 Reciprocal plot of $1/k_{obs}$ against [H^+] for the reactions of (a) *S*-nitrosopenicillamine and (b) *S*-nitrosocysteine with sulfite.

where k_2 is the bimolecular rate constant for reaction of SO_3^{2-} with RSNO, and K_a is the acid dissociation constant of HSO_3^-. Plots of $(k_{obs})^{-1}$ against [H^+] should then be linear with a positive slope and intercept. The plots in Fig. 4 show that this is the case for two nitrosothiols, confirming beyond any doubt that the reactive entity is in fact the sulfite ion SO_3^{2-}. The results from the reciprocal plots yield values of k_2 and also of K_a. We find values of 6.7 and 6.6 respectively for the pK_a value of HSO_3^- from the experiments using *S*-nitrosopenicillamine and *S*-nitrosocysteine, which are in reasonable agreement with the literature value of 6.91.[13] The reaction is quite general for all nitrosothiols. We report some k_2 values [obtained from eqn. (5)] for a range of structures in Table 1. Structures were chosen to represent the nitrosothiols currently being used as NO-donors in both *in vivo* and *in vitro* experiments, and the choice was not made in order to use the structure–reactivity pattern to establish or to confirm mechanistic ideas. One feature, however, does stand out from Table 1, in that the two sugar derivatives, GPSNO[14] and *S*-nitrosothioglucose, are particularly reactive. This probably arises from the electron-attracting properties of the ring oxygen atoms.

Table 1 Values of k_2 [eqn. (5)] for reaction of a number of *S*-nitrosothiols with sulfite ion at pH 7.4 and in the presence of EDTA (2.5×10^{-4} mol dm^{-3})

RSNO	k_2/dm^3 mol^{-1} s^{-1}
2-Acetamido-2-deoxy-*S*-nitroso-1-thio-β-D-glucopyranose 3,4,6-triacetate (GPSNO)	12100 ± 40
S-Nitroso-1-thio-β-D-glucose	3400 ± 50
S-Nitrosocysteine (SNCys)	650 ± 10
S-Nitrosopenicillamine (SPEN)	527 ± 4
S-Nitrosoglutathione (GSNO)	134 ± 3
S-Nitroso-*N*-acetylcysteine	28.4 ± 0.2
S-Nitroso-*N*-acetylpenicillamine	7.6 ± 0.1
S-Nitrosocaptopril	7.1 ± 0.1

We have established in the case of the reaction of GSNO at two different sulfite concentrations that one of the products of the reaction is the thiol, which is formed essentially quantitatively (94 and 96%). In addition we have shown that no significant amounts of either NO (using the NO electrochemical probe) or nitrite ion (using the Griess test) are formed. We have not established the nature of the 'nitrogen' product in this study. Normally the main product of the nitrosation of bisulfite in acid solution by nitrous acid[15] is hydroxylamine disulfonate. This is an important reaction in the industrial production of hydroxylamine (the Raschig Process), and is the probable product in the reaction of sulfite ion with alkyl nitrites and nitrososulfonamides. All of the results are consistent with rate-limiting formation of nitrosyl sulfonate $ONSO_3^-$,[16] which then nitrosates another bisulfite ion to give the hydroxylamine derivative [eqns. (6) and (7)]. Nitrosation by nitrous acid can also give a

$$RSNO + SO_3^{2-} + H^+ \longrightarrow RSH + ONSO_3^- \quad (6)$$

$$ONSO_3^- + SO_3^{2-} + H^+ \longrightarrow HON(SO_3^-)_2 \quad (7)$$

variety of other products, under other experimental conditions. A very similar sequence of reactions occurs in the nitrous acid nitrosation of benzene sulfinic acids.[17] In a parallel study[18] it has been shown that the product of reaction of sulfite with *S*-nitrosothiols is that expected from an electrophilic nitrosation process.

Some experiments were carried out using sodium sulfite, Na_2SO_3, instead of sodium bisulfite, $NaHSO_3$. Not surprisingly the k_2 values obtained for both salts were close, within the experimental error of measurement, *e.g.* 527 ± 8 and 510 ± 9 dm^3 mol^{-1} s^{-1}, respectively, for the reactions with *S*-nitrosopenicillamine. Reaction occurred also when the sulfite source was sodium metabisulfite ($Na_2S_2O_5$). Here the pseudo first-order rate constant was approximately twice that obtained from sodium sulfite, implying that, as expected, two sulfite ions are generated in the former case.

Activation parameters were measured for four nitrosothiols. Values of the activation energy were in the range 35 to 40 kJ mol^{-1} and the activation entropy in the range −60 to −80 J K^{-1} mol^{-1}. The high reactivity of sulfite ion is thus in large part due to relatively low values of the activation energy. The moderate negative values of the entropy of activation are consistent with a bimolecular process generating a more ordered transition state; values are approximately in the same range as for S_N2 reactions at saturated carbon atoms.

To our knowledge this is the first observation of this reaction, apart from a reference in the biological literature[19] to an

observed increase in rate of decomposition of GSNO and also of *S*-nitroso serum albumin when sulfite is added. These authors raised the possibility that the toxicity of sulfite might be connected with the destruction of RSNO compounds and of NO itself *in vivo* by reaction with sulfite.

In the absence of a metal ion chelator the reaction mechanism could, in principle, involve reduction of residual Cu^{2+} by sulfite, leading to NO and disulfide (RSSR) as the products. This is the pathway for reaction of nitrosothiols with ascorbate at low [ascorbate]. At higher [ascorbate] the reaction of ascorbate as a nucleophile takes over.[10] This duality of reaction pathways has not been looked for in the case of the sulfite reaction, but it is a real possibility. However the overall reactivity of sulfite ion is orders of magnitude greater than that of ascorbic acid, so it is possible that the copper reaction would be completely masked. Nevertheless in all our work, experiments were always carried out in the presence of EDTA, so we can be sure that no pathway *via* copper ion catalysis occurs. The very high reactivity of sulfite ion here can be used to support the earlier suggestion[19] that the toxicity of sulfite could be related to its reactivity *in vivo* with *S*-nitrosothiols or even with NO itself.

Apart from the nitrosation of bisulfite by nitrous acid in acid solution mentioned earlier, nitrosation of sulfite/bisulfite has been effected by an alkyl nitrite[20] and a nitrososulfonamide (MNTS)[20] as well as by iron nitrosyl complexes[21] and by dissolved aerated NO[22] and possibly by dissolved NO itself.[19] The MNTS reaction gave *N*-methyltoluene-*p*-sulfonamide quantitatively, consistent with an *N*-nitrosation process, although the other likely product, hydroxylamine disulfonate, was not identified.

In the corresponding reactions of nitrosothiols with nitrogen nucleophiles,[9] we found that reactivity correlated better with the Ritchie N_+ function than with the Pearson *n* parameter. This was also the earlier conclusion from the reactions of MNTS and an alkyl nitrite,[20] and a case was made that the reactions are frontier orbital controlled. Unfortunately the paucity of N_+ values does not enable us to extend that analysis to the reactions of the sulfur based nucleophiles discussed in this paper. It is abundantly clear, however, that SO_3^{2-} (for which there is a N_+ value) is much more reactive than predicted by the N_+ equation. Reaction is so rapid generally, for a large range of nitrosothiols, that reaction with sulfite ion constitutes the most efficient way of rapid removal of nitrosothiols from solution, should this be necessary. It is not immediately obvious why this should be so, particularly given the earlier results when the reactivity of sulfite ion with MNTS did correlate reasonably well with N_+.[20]

(b) Reactions with thiosulfate ion, thiourea, thiomethoxide ion and thiocyanate ion

All four nucleophiles reacted with *S*-nitrosopenicillamine and *S*-nitrosocysteine and the reactions were monitored at 340 nm as before. The reactions of thiosulfate ion were sufficiently rapid to require the stopped-flow method, whereas the other nucleophiles were conveniently measured by conventional spectrophotometry. With [nucleophile] ⩾ [RSNO], as for the sulfite reactions, good first-order behaviour occurred, and there was also a good first-order dependence on [nucleophile] for reactions at pH 7.4. For $S_2O_3^{2-}$, $SC(NH_2)_2$ and SCN^- the appropriate pK_a values are such that at this pH the nucleophiles are overwhelmingly in the forms given, so no correction for any protonation is required. For CH_3S^- however this correction [eqn. (5)] is necessary, since the pK_a value of CH_3SH is ~10.5 and we are assuming that the reactive species is the anion.

The collected values of k_2 [eqn. (5)] are shown in Table 2 for reaction with the two *S*-nitrosothiols, together with the earlier results for SO_3^{2-} and the literature values[10] for the ambident nucleophiles *S*-methylcysteine and thiomorpholine

Table 2 Values of k_2 [eqn. (5)] for reaction of SPEN and SNCys with a range of sulfur nucleophiles

	k_2/dm^3 mol^{-1} s^{-1}	
Nucleophile	SPEN	SNCys
SO_3^{2-}	527 ± 4	650 ± 10
MeS^-	40 ± 6	39 ± 3
$S_2O_3^{2-}$	2.3 ± 0.1	1.9 ± 0.05
S-Methylcysteine	3.8×10^{-2}	—
Thiomorpholine	1.5×10^{-2}	—
$SC(NH_2)_2$	$(1.3 \pm 0.1) \times 10^{-3}$	$(6.3 \pm 0.2) \times 10^{-4}$
SCN^-	$(2.5 \pm 0.06) \times 10^{-4}$	$(2.11 \pm 0.09) \times 10^{-4}$

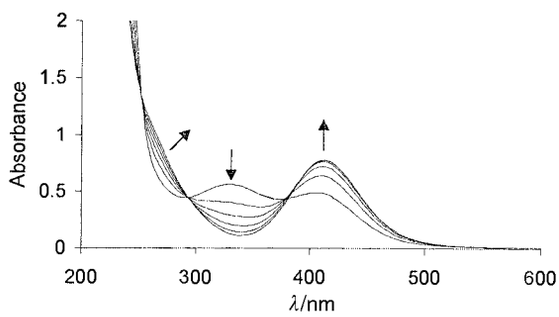

Fig. 5 Repeat scans (every 25 s) for the reaction of GSNO (1×10^{-3} mol dm^{-3}) with sodium sulfide (1×10^{-3} mol dm^{-3}) in water (pH ~9.8) containing EDTA (1×10^{-4} mol dm^{-3}).

for comparison. Because of the lack of literature values of N_+ for most of these nucleophiles it is not possible to generate quantitative comparisons. However there is an approximate qualitative link between reactivity here ($SO_3^{2-} > MeS^- > S_2O_3^{2-} > SC(NH_2)_2 > SCN^-$) and the Pearson nucleophilicity parameter, *n*, ($S_2O_3^{2-} > SO_3^{2-} > SC(NH_2)_2 > SCN^-$) with the exception of the interchanging of $S_2O_3^{2-}$ and SO_3^{2-}. We can read nothing into this other than a confirmation that, in general, the more powerful nucleophiles in other reactions are also the more powerful nucleophiles in reaction with *S*-nitrosothiols. The kinetic evidence suggests[10] that both ambident nucleophiles *S*-methylcysteine and thiomorpholine react initially at their sulfur atoms (which cannot lead to a stable *S*-nitroso product), before a rearrangement to the nitrogen atom occurs, which can lead to a stable product. This behaviour has been noted on previous occasions.[23]

(c) Reaction with sulfide ion

Reaction of a number of *S*-nitrosothiols (GSNO, SPEN and SNCys) with a large excess of sodium sulfide (added as the nanohydrate $Na_2S{\cdot}9H_2O$) at pH ~ 11 occurred very rapidly as measured by the disappearance of the characteristic absorbance peak at 350 nm, to be replaced by one of slightly higher absorbance at 410 nm (Fig. 5). Reaction was complete in under one minute and the peak at 410 nm was relatively stable, decomposing slowly after about 30 min. The absorbance at 410 nm was also generated, more slowly, when the reagents were added in equimolar quantities. Measurement at fixed wavelength (410 nm) at lower [sulfide] gave complex absorbance–time plots which were not easily interpreted, and were too complicated for a simple kinetic analysis, but indicated that several rapid processes were occurring. The extinction coefficient was determined at 410 nm for two nitrosothiols, SPEN and SNCys, assuming that reaction was quantitative. Values of 772 ± 17 and 774 ± 29 dm^3 mol^{-1} cm^{-1} were obtained, confirming that this assumption is probably justified.

Our expectation was that the absorbance at 410 nm would be due to the HSNO species or, more likely at these pH values, its anion ONS^-. We have been unable to find a reference in the

literature to this mono-thio derivative of nitrous acid. However we found a reference [24] to a yellow species with an identical UV-visible spectrum to our product which was generated from NO and solutions of either Na_2S, NaSH or Na_2S_2 under anaerobic conditions. The yellow species was identified as the nitroso-disulfide (or perthionitrite) ion $ONSS^-$. Other compounds containing this grouping have also been identified.[25] Our spectrum is so similar to that in the literature that we are convinced that we too are looking at the formation of the $ONSS^-$ species. It is also likely that the same species is also generated from MNTS and sodium hydrosulfide at pH 7 in a reaction which also showed complex kinetic behaviour and generated an orange-yellow solution.[20] We extended the range of nitroso compounds in this reaction by examining the spectra of the reactions of alkyl nitrites and *N*-nitrosamines. Both isopentyl nitrite and *tert*-butyl nitrite reacted readily with added sodium sulfide at pH 7, again generating the same yellow species as before with an absorption maximum at 410 nm, whereas neither *N*-nitrosopyrrolidine nor *N*-methyl-*N*-nitrosoaniline showed any trace of reaction under the same conditions.

It is not immediately obvious how the yellow species $ONSS^-$ is formed here. Two possible routes are (a) the formation of $ONSH/ONS^-$ by the expected nitrosation reaction [eqn. (8)],

$$RSNO + HS^- \longrightarrow RSH + ONSH/ONS^- \qquad (8)$$

followed by further reaction of $ONSH/ONS^-$ with HS^- to give the final product [eqn. (9)] or (b) the prior formation of HSS^-, which then acts as the nucleophile [eqn. (10)].

$$ONSH/ONS^- + HS^- \longrightarrow ONSS^- \qquad (9)$$

$$RSNO + HSS^- \longrightarrow RSH + ONSS^- \qquad (10)$$

Route (a) is perhaps the more plausible, given that alkyl nitrites and MNTS undergo the same reaction, but pathway (b) is a possibility for RSNO reaction, since it is known [26] that any RSSR present (by RSH oxidation?) will generate HSS^-, and we have found substantial catalysis of product formation from the RSNO reaction by added RSSR. Nitrosamines are generally unreactive as electrophilic nitrosation reagents in the absence of powerful electron attracting substituents (as in MNTS),[27] so it is not surprising that the two nitrosamines examined here do not react.

Given the relative stability of the $ONSS^-$ species, and the generality of the reaction leading to its formation, it is likely that an analytical procedure for the determination of [RSNO] in the range 10^{-4}–10^{-3} mol dm^{-3}, could readily be developed.

Experimental

All materials were obtained at the highest purity grade available. *S*-Nitrosothiols were all synthesised *in situ* from equimolar concentrations of the corresponding thiol and nitrous acid in acid solution, before adjustment of the pH to 7.4 and other pH values. The sugar derivative GPSNO was synthesised, purified and characterised as described earlier.[14] Nitrite analysis was carried out by the Griess test, thiol analysis by using the Ellman reagent and the WPI electrode (calibrated using ascorbic acid–sodium nitrite) used to examine for nitric oxide.

Kinetic measurements were carried out at 25 °C in water, mostly at pH 7.4 (usually with a phosphate buffer), either in a conventional or stopped-flow spectrophotometer, noting the decreasing absorbance at 340 nm due to RSNO. Experiments were carried out in the presence of EDTA (2.5×10^{-4} mol dm^{-3}) and with the nucleophile concentration in large excess (typically 2.5–9 $\times 10^{-3}$ mol dm^{-3}) over the [RSNO] (typically 2×10^{-4} mol dm^{-3}). All reactions, apart from those with sulfide, gave good first-order behaviour (reproducible within ±3%) and reactions were also first-order in the [nucleophile]. The first-order rate constants (k_{obs}) were obtained from the computer correlation program "Enzfitter".

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