Nitrosation by Alkyl Nitrites. Part 6.[1] Thiolate Nitrosation

Hanif M. S. Patel and D. Lyn H. Williams*
Chemistry Department, University Science Laboratories, South Road, Durham DH1 3LE

Each of the following thiols react with a range of alkyl nitrites in water at 25 °C in the pH range 6–13 to give the corresponding thionitrites in solution: L-cysteine, *N*-acetyl-L-cysteine, L-cysteine methyl ester, L-cysteine ethyl ester, glutathione and thioglycolic acid. The pH-dependence of the rate constant clearly shows that reaction occurs only *via* the thiolate anions RS^-. For *N*-acetyl-L-cysteine and thioglycolic acid only one RS^- species is possible and a quantitative kinetic analysis yields pK_a values for RSH ionisation in good agreement with the literature values. For each of the remaining thiols two thiolate ions (NH_2RS^- and $\overset{+}{N}H_3RS^-$) are possible and the measured rate constants for all alkyl nitrites generally followed the total thiolate ion concentration (as a function of pH) obtained from the published microscopic pK_a values. An alternative approach of fitting the kinetic results to the concentration curve by computer yields microscopic pK_a values generally in good agreement with the literature values. One exception is L-cysteine where the measured $(pK_a)_D$ value (for $NH_2RSH \longrightarrow NH_2RS^-$) differs significantly from the literature value. With simple alkyl nitrites (ethyl, isopentyl, isopropyl and t-butyl) steric effects appear to be the major influences in reactivity, whereas electron-withdrawing substituents in the 2-position greatly increase the rate constants. The reactions of 2,2,2-trichloroethyl nitrite were too fast to measure at all pH values, whereas the reaction of 2,2-dichloroethyl nitrite could only be followed kinetically in the pH range 6–8.25, even by stopped-flow spectrophotometry. The possible relevance of these reactions to the vasodilatory action of alkyl nitrites is discussed. The kinetic results for thiolate nitrosation by *N*-methyl-*N*-nitrosotoluene-*p*-sulphonamide also correlate well with the total concentration of the thiolate ion, with the interesting exception of the reactions of the carboxylic esters of L-cysteine, where there appears to be a preference for reaction *via* the NH_2RS^- form.

Alkyl nitrites have been widely used as nitrosating agents under a variety of experimental conditions. In aqueous acid solution it has recently been shown[2] that hydrolysis occurs and the actual nitrosating agent is one derived from nitrous acid. Under basic conditions it is believed that alkyl nitrites transfer the NO^+ group directly to the nucleophiles (usually amines).[3,4] In a recent paper[5] we have shown that simple alkyl nitrites react directly with the thiolate ion form of L-cysteine to yield the thionitrite in solution. In this paper we present the results of a more detailed and widespread investigation, using a range of thiol species and also an increased range of alkyl nitrite structures, in an attempt to establish the generality of the reaction. In particular we were anxious to establish (in the case of cysteine derivatives) which of the possible thiolate species are reactive, and to link the reactivity with the concentration of such species using where possible the published microscopic pK_a values of the various ionisations. Such an analysis was not attempted in the earlier publication[5] which merely established that the thiolate was the reactive species and treated the data in terms of a single global ionisation. These reactions are of some interest in connection with the well-known vasodilatory properties of alkyl nitrites, since it has been suggested[6] that alkyl nitrites may act in this way by first effecting (*in vivo*) *S*-nitrosation of tissue-bound thiol groups. Subsequent reactions are then believed to involve enzyme activation by the thionitrite leading to smooth muscle relaxation.

Results and Discussion

In this work we have examined the kinetics of thionitrite (sometimes called nitrosothiols) formation [equation (1)] from

$$RSH + R'ONO \longrightarrow RSNO + R'OH \qquad (1)$$

six thiols *viz.*, L-cysteine, *N*-acetyl-L-cysteine, L-cysteine methyl ester, L-cysteine ethyl ester, glutathione and thioglycolic acid, and ten alkyl nitrites, ethyl nitrite, isopentyl nitrite, isopropyl nitrite, t-butyl nitrite, 2-chloroethyl nitrite, 2-bromoethyl nitrite, 2-iodoethyl nitrite, 2-ethoxyethyl nitrite, 2,2-dichloroethyl nitrite, and 2,2,2-trichloroethyl nitrite. Attempts to synthesise 2,2,2-trifluoro-ethyl nitrite were not successful. Some of the results for L-cysteine have previously been reported[5] but were not fully analysed. In no case have we isolated the thionitrites in this work, since they are generally very unstable in the pure form.[7] A number have been characterised, some with difficulty, including the thionitrite derived from L-cysteine.[8] However thionitrites are easily recognised in solution by their pale yellow colour (in dilute solution) with a broad adsorption of low extinction coefficient centred at around 330 nm. All our reactions were carried out in aqueous buffer solutions at 25 °C over the pH range 6–13. Reactions were sufficiently fast (measured by stopped-flow spectrophotometry) to ensure that product decomposition in solution did not interfere quantitatively with the kinetics of *S*-nitrosation. Rate equation (2)

$$\text{Rate} = k_2[RSH][R'ONO] \qquad (2)$$

was established in all cases and the second-order rate constants k_2 were obtained as a function of the pH of the solution. In every case values increased markedly with pH, particularly in the range 7.5–10, in a broadly S-shaped curve, and levelled off at high pH (*ca.* 12) to a limiting value k_2(lim).

We have attempted to correlate the k_2 values with the concentration of the reactive species in each case. This analysis is complicated in the case of L-cysteine, its carboxylic esters and glutathione by the overlapping ionisation of the

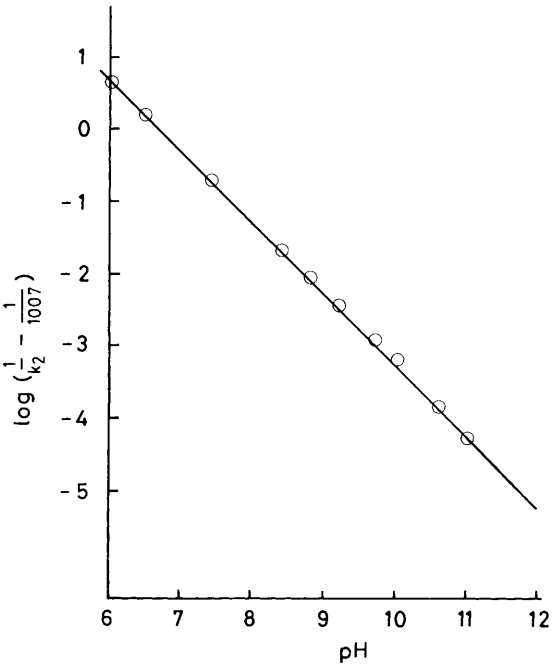


Figure 1. Plot of $\log(k_2^{-1} - k_2(\text{lim})^{-1})]$ *vs.* pH for the reaction of *N*-acetyl-L-cysteine with 2-chloroethyl nitrite.

Table 1. Values of k_2(lim)/dm^3 mol^{-1} s^{-1}, slopes of $\log[k_2^{-1} - k_2(\text{lim})^{-1}]$ *vs.* pH, and the derived pK_a values for the reactions of *N*-acetyl-L-cysteine with R′ONO.

R′	Slope	k_2(lim)	pK_a
CH_3CH_2	−0.98	31	9.55
$(CH_3)_2CH(CH_2)_2$	−0.97	30	9.45
$(CH_3)_2CH$	−0.95	12.1	9.30
$(CH_3)_3C$	−1.01	1.78	9.74
$C_2H_5OCH_2CH_2$	−0.99	169	9.66
$ClCH_2CH_2$	−0.98	1010	9.55
$BrCH_2CH_2$	−1.00	1030	9.75
ICH_2CH_2	−1.00	1020	9.79

Average pK_a = 9.60 ± 0.17

Table 2. Values of k_2(lim)/dm^3 mol^{-1} s^{-1}, slopes of $\log[k_2^{-1} - k_2(\text{lim})^{-1}]$ *vs.* pH, and the derived pK_a values for the reactions of thioglycolic acid with R′ONO.

R′	Slope	k_2(lim)	pK_a
CH_3CH_2	−0.96	75	9.89
$(CH_3)_2CH(CH_2)_2$	−0.97	75	9.93
$(CH_3)_2CH$	−0.97	30	9.89
$(CH_3)_3C$	−0.93	4.9	9.47
$C_2H_5OCH_2CH_2$	−0.97	417	9.94
$ClCH_2CH_2$	−0.98	2 260	9.99
$BrCH_2CH_2$	−0.99	2 240	10.12
ICH_2CH_2	−1.00	2 260	10.18

Average pK_a = 9.93 ± 0.21.

$-\overset{+}{N}H_3$ and −SH groups. This is not a problem for *N*-acetyl-L-cysteine and thioglycolic acid, which will be discussed first. Throughout, of course, any carboxylic acid group present is effectively fully ionised in our buffer solutions.

(*a*) *Reactions of* N-*acetyl*-L-*cysteine and thioglycolic acid.* All of the kinetic results here are consistent with a reaction which occurs exclusively *via* the thiolate anion RS^-, which in these cases involves only one ionisation. The reaction is outlined in Scheme 1. The expected expression for k_2

$$RSH \overset{K_a}{\rightleftharpoons} RS^- + H^+$$

$$RS^- + R'ONO \xrightarrow{k} RSNO + R'O^- \quad (\downarrow\uparrow\ R'OH)$$

Scheme 1.

[equation (2)] is given by equation (3), and the limiting form at

$$k_2 = kK_a/(K_a + [H^+]) \quad (3)$$

high pH gives k_2(lim) = k, the second order rate constant for reaction of the alkyl nitrite with the thiolate anion. This relationship can be tested either by constructing the k_2–pH profile using a measured k_2(lim) and the literature value for the dissociation constant K_a, and comparison with the experimental k_2 values, or by rewriting equation (3) in the form given in equation (4); a plot of the left-hand side [again using a measured

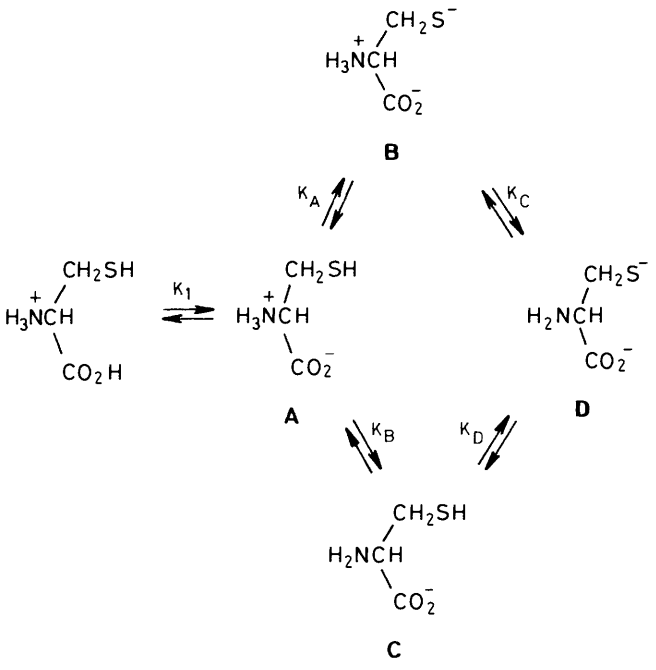
$$\log\left(\frac{1}{k_2} - \frac{1}{k}\right) = -\text{pH} - \log kK_a \quad (4)$$

k_2(lim) = k] against pH should then be linear of slope −1 and from the intercept K_a may be obtained and compared with the literature values. We have chosen to analyse our results for these two substrates by the latter procedure. A typical plot is shown in Figure 1 for the reaction of 2-chloroethyl nitrite with *N*-acetyl-L-cysteine. Similar plots were obtained with all of the alkyl nitrites and also for their reactions with thioglycolic acid. The results are summarised in Table 1 (for *N*-acetyl-L-cysteine) and in Table 2 (for thioglycolic acid). In every case good lines were obtained with slopes very close to −1. The average pK_a values for RSH ionisations are 9.60 ± 0.2 for *N*-acetyl-L-cysteine, which agrees reasonably with the literature value[9] of 9.76, and 9.93 ± 0.2 for thioglycolic acid which again is in good agreement with the range of values reported in the literature, 9.82,[10] 10.01,[11] 10.22,[12] and 10.32.[13]

At pH values < 6 the reaction rate constant is negligibly small for both thiols with all the alkyl nitrites, so reaction effectively only occurs *via* the thiolate ion. This reveals that the alkyl nitrites generally are less effective as nitrosating species, by many orders of magnitude, than the nitrite species derived from acidic solutions of nitrous acid *i.e.* $H_2NO_2^+$ or NO^+. This arises because the latter react with cysteine and the other thiols in the unionised RSH form with rate constants not far removed from those expected for diffusion-controlled reactions.[14]

The relative reactivities of the alkyl nitrites [given by the k_2(lim) values] are comparable for both thiols and will be discussed after section (*b*).

(*b*) *Reactions of* L-*cysteine, L-cysteine methyl and ethyl esters and glutathione.* As for section (*a*) each of these substrates reacted with each alkyl nitrite to give the corresponding thionitrites with sharply increasing rate constants in the pH region 7.5–10. The analysis is now complicated by simultaneous overlapping ionisations of $-\overset{+}{N}H_3$ and −SH. The various equilibria are laid out in Scheme 2 for L-cysteine,[15] and similar schemes can be drawn up for the other thiols in this group. We can ignore K_1 in our work since the carboxylic acid group is fully ionised. The microscopic dissociation constants are K_A, K_B, K_C, and K_D with their associated pK_a values $(pK_a)_A$, $(pK_a)_B$, $(pK_a)_C$, and $(pK_a)_D$. The *macroscopic* second and third dissociation constants K_2 and K_3 are given by equations (5) and (6). If reaction occurs at S^- then we must consider the



Scheme 2

Table 3. Comparison of literature and our derived microscopic pK_a values for RSH ionisations

Substrate	pK_A	pK_B	pK_C	pK_D	Ref.
L-Cysteine	8.21	8.65	10.0	9.56	10
	8.53	8.86	10.36	10.03	13
	8.50	8.85	10.35	10.00	11
	8.64	8.62	10.47	10.49	15
	8.21	8.65	8.96	8.52	This work
L-Cysteine ethyl ester	7.3	6.76	8.33	8.87	10
	7.45	6.77	8.41	9.09	13
	7.45	6.77	8.41	9.09	This work
L-Cysteine methyl ester	7.45	6.77	8.41	9.09	This work
Glutathione	8.72	9.47	9.47	8.72	10
	8.93	9.13	9.28	9.08	*a*
	8.72	9.28	9.28	8.72	This work
N-Acetyl-L-cysteine	9.76				9
	9.60				This work
Thioglycolic acid	9.82				10
	10.32				13
	10.01				11
	10.22				12
	9.93				This work

[a] D. L. Rabenstein, *J. Am. Chem. Soc.*, 1973, **95**, 2792.

$$K_2 = K_A + K_B \quad (5)$$

$$K_3^{-1} = K_C^{-1} + K_D^{-1} \quad (6)$$

involvement of both forms **B** and **D**. The fraction of each form **A**, **B**, **C**, and **D** can be expressed in terms of the various microscopic K values and $[H^+]$. It is then possible to deduce the fraction present as **B** + **D** (*i.e.* total RS^-) given in equation (7).

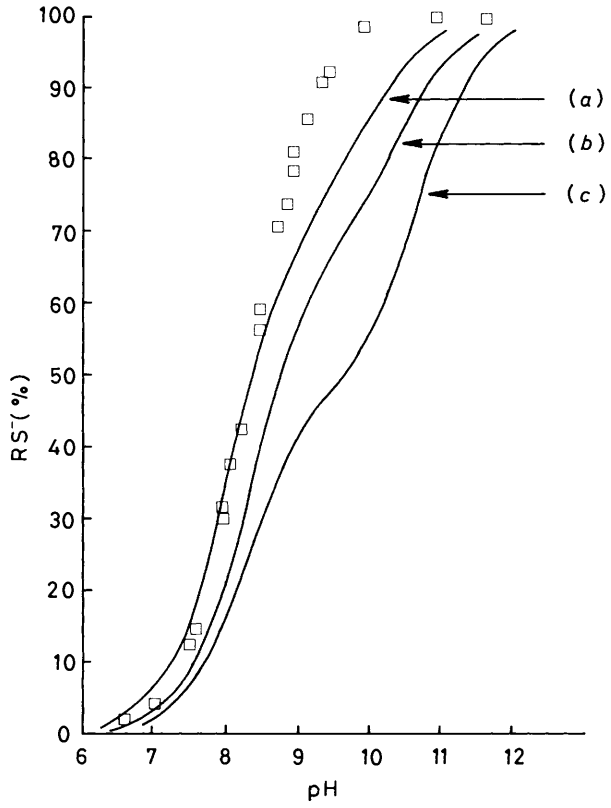


Figure 2. Rate constant and $\%RS^-$ profile *vs.* pH for L-cysteine from (*a*) ref. 10; (*b*) refs. 11 and 13; (*c*) ref. 15. □, $100\,k_2/k_2(\text{lim.})$

$$\frac{[\mathbf{B}] + [\mathbf{D}]}{[RS^-]_{max}} = \frac{K_A/K_B + K_D/[H^+]}{[H^+]/K_B + K_A/K_B + 1 + K_D/[H^+]} \quad (7)$$

This was first deduced in a classical paper by Benesch and Benesch[13] and was also used later by Reuben and Bruice[10] in a study of the reaction of benzene oxide with thiols. For L-cysteine there are four sets of published microscopic pK_a values, see Table 3. Figure 2 shows the percentage total thiolate ion *i.e.* [**B**] + [**D**] as a function of pH derived from these values. Curve (*a*) is deduced from the values reported by Reuben and Bruice,[10] curve (*b*) from the data of Benesch and Benesch[13] and also Elson and Edsall[11] (which are very close together) and curve (*c*) from the results of Splittgerber and Chinander.[15] The experimental points are the k_2 values expressed as a percentage of the limiting values for a range of alkyl nitrites in reaction with L-cysteine. For clarity we have not included all the points. It is obvious that our results follow closely curve (*a*) up to pH *ca.* 8.5 and thereafter departs from it. The departure from curves (*b*) and (*c*) is even more marked. The conclusion is that (in common with all the other thiols, see later) reaction does indeed take place *via* both thiolate species, but that there is not an exact correlation with the total thiolate ion concentration (*i.e.* [**B**] + [**D**]) over the whole pH range, in contrast with the behaviour (see later) of the carboxylic acid esters, where there is an excellent correlation between the kinetic rate constants and the total thiolate ion concentration. There are three possible reasons for the discrepancy in the L-cysteine results. (*a*) The reactivity of the two forms **B** and **D** may be different. We can in fact 'correct' the concentration curve (*a*), to match more closely with the kinetic points if we assume that **B** is *ca.* 20% more reactive than **D**. This does not really make a lot of sense, since we would expect any reactivity difference between **B** and **D** to be

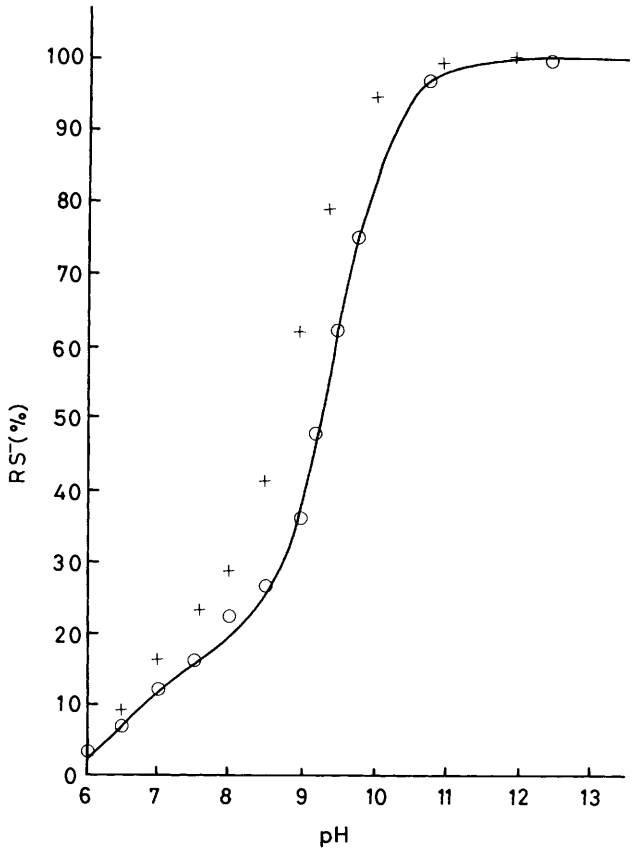


Figure 3. Rate constant and % RS^- profile *vs.* pH for L-cysteine ethyl ester: ○, 100 k_2/k_2(lim); +, % RS^- calculated from ref. 10.

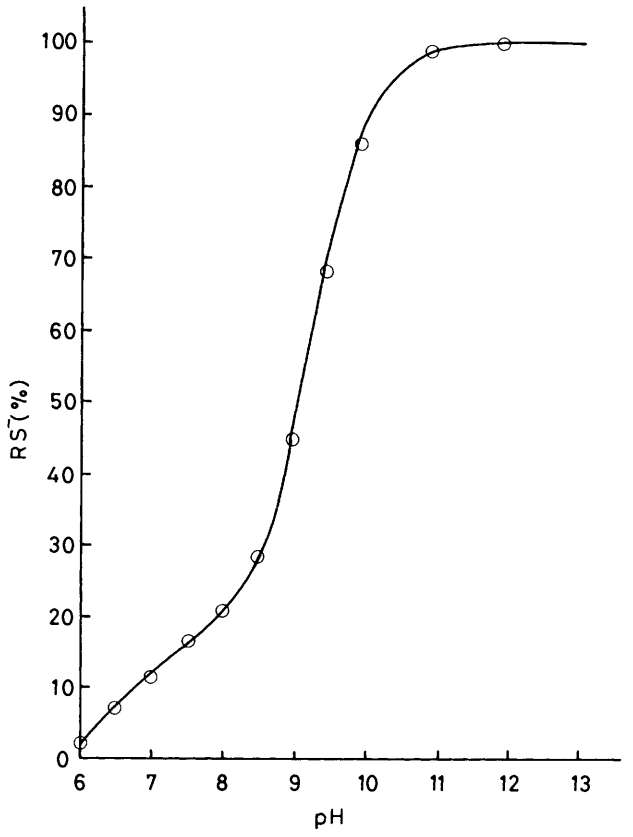


Figure 4. Rate constant and % RS^- profile *vs.* pH for glutathione: ○, 100 k_2/k_2(lim).

in the opposite sense *i.e.* **D** more reactive than **B**. Also, it is difficult to see why the thiolate ions from L-cysteine have a different reactivity if those from the carboxylate esters do not. (*b*) It is possible here that we are neglecting thiolate ions of the type $CO_2HNH_2S^-$ and/or $CO_2\overset{+}{H}NH_3S^-$, although we would have expected these forms containing the unionised carboxylic acid groups to be present in very low concentration at the pH values used. This explanation however would account for the difference observed between L-cysteine and the esters. (*c*) Another possibility of course is that the published microscopic pK_a values are in error. Indeed the range of values in the literature [leading to the significantly different curves (*a*), (*b*), and (*c*)] is an indication of the difficulty in obtaining the true values. We have produced a smooth curve which best fits the experimental kinetic points using pK_a values of $(pK_a)_A$ 8.21, $(pK_a)_B$ 8.65, $(pK_a)_C$ 8.96, and $(pK_a)_D$ 8.52. This means that these values of $(pK_a)_C$ and $(pK_a)_D$ are approximately 1 pK_a unit smaller than those deduced spectrophotometrically by Reuben and Bruice,[10] in a treatment which does require some assumptions to be made. There is even less of a correspondence between k_2 and %([**C**] + [**D**]) *i.e.* with the concentration of species containing the free NH_2 group, so a mechanism involving *N*-nitrosation followed by *N*- to *S*-migration of the NO group is not likely.

Both the methyl and ethyl carboxylic esters of L-cysteine behave in a similar fashion with all the alkyl nitrites, leading to a broadly S-shaped curve for the k_2–pH profile. Figure 3 shows the experimental results for the reaction of the ethyl ester with ethyl nitrite, but all the nitrites behave similarly. The smooth curve is computed as the best fit to the experimental points using $(pK_a)_A$ 7.45, $(pK_a)_B$ 6.77, $(pK_a)_C$ 8.41, and $(pK_a)_D$ 9.09, which agree well with the two reported sets of data (see Table 3).

For the carboxylic esters the $(pK_a)_A$ and $(pK_a)_B$ values are (*a*) smaller than the L-cysteine values and (*b*) are reversed; this is a consequence of changing the $-CO_2^-$ group to $-CO_2R$. The methyl ester as expected behaves in a very similar fashion leading to computed pK_a values of $(pK_a)_A$ 7.45, $(pK_a)_B$ 6.77, $(pK_a)_C$ 8.41 and $(pK_a)_D$ 9.09, in very close agreement to the ethyl ester values. There appears to be no literature report of the microscopic pK_a values for the methyl ester.

Glutathione also reacts in the same way. Figure 4 shows the results for the reaction with 2-chloroethyl nitrite, but as for all the thiols, all the alkyl nitrites behave in a similar way. Again the curve is the best computed one to fit the experimental points leading to average (for all the alkyl nitrites) values of $(pK_a)_A$ 8.72, $(pK_a)_B$ 9.28, $(pK_a)_C$ 9.28, and $(pK_a)_D$ 8.72, which agree reasonably well with the literature values of Reuben and Bruice[10] and also of Rabenstein (see Table 3).

In summary, it is clear that alkyl nitrites generally react in mildly basic aqueous solution with the thiolate anion of thiols. With the exception of some of the results for L-cysteine there is an excellent correspondence between the experimental rate constants and the percentage total [RS^-].

Reactivity of the Thiols and of the Alkyl Nitrites.—The combined results for k_2(lim) values for each of the reactions studied are shown in Table 4. As expected, since there are no major structural changes in the thiols, their reactivities are very much the same with the exception of thioglycolic acid which is consistently more reactive than the others by a factor of between two and three.

It is clear in the alkyl nitrites that the presence of β-electron-withdrawing groups have a significant enhancing effect on the reactivity. This is to be expected for an electrophilic nitrosation

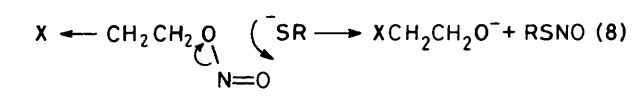
Table 4. Values of k_2(lim)/dm^3 mol^{-1} s^{-1} for the reactions of alkyl nitrites with six thiols.[a]

RONO	1	2	3	4	5	6
$(CH_3)_3CONO$	1.7	1.6	1.5	1.8	1.8	4.9
$(CH_3)_2CHONO$	11	12	12	12	11	30
CH_3CH_2ONO	28	24	25	31	28	75
$(CH_3)_2CH(CH_2)_2ONO$	27	25	26	30	27	75
$C_2H_5O(CH_2)_2ONO$	169	150	165	169	159	417
$Cl(CH_2)_2ONO$	1 045	1 050	1 100	1 010	1 070	2 260
$Br(CH_2)_2ONO$	1 055	1 055	1 085	1 030	1 055	2 240
$I(CH_2)_2ONO$	1 060	1 057	1 080	1 020	1 060	2 260
Cl_2CHCH_2ONO[b]	1.2×10^4	—	—	—	—	—
Cl_3CCH_2ONO	Too fast to measure					

[a] 1, L-cysteine; 2, L-cysteine methyl ester; 3, L-cysteine ethyl ester; 4, *N*-acetyl-L-cysteine; 5, Glutathione; 6, Thioglycolic acid. [b] Measured only in the pH range 6–8.25.

Table 5. Rate constants for the nitrosation of L-cysteine by 2-ethoxyethyl nitrite (5×10^{-4} mol dm^{-3}) at pH 12.85.

[L-Cysteine]/mol dm^{-3}	k_o/s^{-1}
1.00×10^{-2}	1.72 ± 0.01
2.00×10^{-2}	3.45 ± 0.01
4.01×10^{-2}	6.92 ± 0.01
8.02×10^{-2}	13.9 ± 0.01



process [equation (8)] and has previously been well documented for nitrosation reactions of amines by alkyl nitrites under similar conditions.[3,4,16] We find that a β-OC_2H_5 group activates by a factor of about six, a β-halogen substituent by about 38 and two β-chloro substituents by over 400. The β-trichloro nitrite was synthesised but was found to react with L-cysteine at a rate which was too fast to measure even by our stopped-flow procedure. Given that the rate constant for reaction of the dichloro compound is 1.2×10^4 dm^3 mol^{-1} s^{-1}, it is likely that the trichloro compound reacts at a rate near to the diffusion-controlled limit.

The electron-releasing effects of α-methyl substituents may account for the reactivity sequence primary:secondary:tertiary = 15:6:1. This could also arise from a steric effect (*cf.* alkaline hydrolysis of carboxylic esters), particularly given that the σ_I and σ_R values are not very different for the various alkyl groups.[17]

We have no compelling evidence for or against the existence of an intermediate in these reactions. It has been argued[5] in the case of the amine reactions[3] that the process is a synchronous one, since reactivity correlates with the vertical ionisation potential rather than with the basicity, and there is no solvent kinetic isotope effect. We do not have sufficient data for the thiolate reaction, where the solvent kinetic isotope effect is not diagnostic, but since there is no buffer catalysis of the reactions the indications here are also in favour of a synchronous one-stage process.

The exact parallel between RS^- and RO^- reactivity in these reactions cannot be drawn. Nitroso-group exchange between an alkyl nitrite and alkoxide ion has been demonstrated using the corresponding alcohol as the solvent.[18] It is likely that RS^- is a far more nucleophilic species. We can make an exact comparison with OH^-, in the reaction of 2-ethoxyethyl nitrite, where all of the thiolate ions used in this work are many orders of magnitude more reactive than is the hydroxide ion.[4]

The results described in this paper demonstrate the generality of the reaction between thiolate ions and alkyl nitrites, and establish the feasibility of thionitrite formation *in vivo*, as part of the chain of events occurring during the vasodilatory action of alkyl nitrites. It has been suggested[6] that thionitrites derived from tissue-bound thiol groups activate the enzyme guanylate cyclase leading to smooth muscle relaxation.

Reactions of N-*Methyl*-N-*nitrosotoluene*-p-*sulphonamide* (*MNTS*) *with Alkyl Nitrites.*—We have recently shown that MNTS reacts under mildly alkaline conditions with thiols *via* the thiolate ions to give thionitrites.[19] Clearly the reactions have strong similarities to those described in this paper. The breaking of the stronger N–N (as distinct from the O–N) bond is made possible by the very strongly electron-withdrawing effect of the SO_2 group. We have used the results in the present analysis and find that the reaction with *N*-acetyl-L-cysteine follows the concentration of the thiolate and the reaction with L-cysteine follows the concentration of total thiolate (*i.e.* [**B**] + [**D**]), but surprisingly the reactions of both carboxylic esters follow a profile which is between that of total thiolate and the NH_2RS^- form. This suggests that there is some degree of preference for reaction with the latter, which could arise from some intramolecular interaction in the transition state which favours this pathway.

Experimental

All of the thiols were commercial samples of high grade purity. The alkyl nitrites were all prepared from the corresponding alcohols and nitrous acid in the conventional way,[20] and fractionally distilled before use in the kinetic experiments. The majority of the rate measurements were carried out in a stopped-flow spectrophotometer, measuring the increasing absorbance at 330 nm due to the thionitrite. Some of the slower reactions were followed in a conventional spectrophotometer, also at 330 nm. All kinetic experiments were carried out in aqueous solution at 25 °C using the necessary buffer solutions to cover the pH range 6–13, in all cases with [RSH] ≫ [R′ONO]. Throughout, good first-order behaviour was found; the quoted first-order rate constants (k_o) are mean values of at least five determinations are subject to a standard error of *ca.* ±2%. Typical conditions were [RSH] $1–6 \times 10^{-2}$ mol dm^{-3} and [R′ONO] 5×10^{-4} mol dm^{-3}. All reactions were strictly first order in [RSH]; a typical set of results is given in Table 5, and k_2 values were obtained from the slopes of k_o *vs.* [RSH] plots. There is no buffer catalysis with either of two buffer systems examined, and the ionic strength effect is negligible.

Acknowledgements

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