Kinetics and Mechanism of *S*-Nitrosation of Some Thiol-containing Amino Acids and Other Thiols

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Rate constants for *S*-nitrosation of cysteine, cysteine methyl ester, *N*-acetylcysteine, penicillamine, *N*-acetylpenicillamine, glutathione, thioglycolic acid, and mercaptosuccinic acid have been determined in water at 25 °C. All are very reactive and show acid and nucleophilic (Cl^-, Br^-, and SCN^-) catalysis. The similarity of the rate constants for reaction *via* $H_2NO_2^+$ and also for reaction *via* ClNO for the more reactive thiols suggests that these reactions are encounter-controlled. The limiting rate constant for ClNO reaction (*ca.* 1×10^7 dm^3 s^{-1}) is very similar to that for nitrosation of a range of aliphatic amines. The high reactivity of *N*-acetyl derivatives and also of glutathione can be explained in terms of an internal stabilisation of the developing positive charge on sulphur by the oxygen atom of the carbonyl group, which involves a six-membered-ring structure. For the more reactive thiols at higher [RSH] the rate of formation of ClNO, BrNO, or ONSCN tends to become rate-limiting. The derived rate constants for XNO formation and also for XNO hydrolysis agree reasonably, and also give values of the equilibrium constants for XNO formation which agree with the literature values which were measured directly.

In recent years nitrosation at sulphur has been increasingly studied,[1] but neither the synthetic aspects nor the mechanistic ones are as well understood as are those for the more familiar *N*-nitrosation. In part this is due to the relative instability of *S*-nitroso compounds and also because many *S*-nitrosations are very rapid processes. The nitrosation of thiourea and its derivatives[2,3] has been the most studied mechanistically. Other known reactions include the nitrosation of sulphinic acids[4] and the inorganic anions thiocyanate[5] and thiosulphate.[6] In principle the simplest example is thiol nitrosation. A number of examples are known, but the product thionitrites are stable isolatable species in only some cases.[7] Thiol nitrosation [equation (1)] is thus akin to alkyl nitrite formation from

$$RSH + HNO_2 \xrightarrow{H^+} RSNO + H_2O \qquad (1)$$

nitrous acid and alcohols. Apart from the difference in stability of the products, alkyl nitrite formation appears generally to be much more a reversible process than is thionitrite formation. Kinetic studies have been reported for cysteine,[2,8,9] 2,2-dimethylethanethiol,[10] *N*-acetylpenicillamine,[11] and some thiol-containing carboxylic acids.[8] All agree with the familiar rate equation (2) for reaction with nitrous acid in aqueous

$$\text{rate} = k_3[RSH][HNO_2][H^+] \qquad (2)$$

mineral acid, and it appears that reaction occurs by rate-limiting attack of the nitrous acidium ion $H_2NO_2^+$. There is no report of a second-order term in $[HNO_2]$ which would result from N_2O_3 attack.

In this paper we have attempted to obtain further information about *S*-nitrosation of thiols so that a more complete mechanistic picture can be built up. In particular we need information on the effect of structure of the thiol on its reactivity, with particular reference to the question of encounter-controlled reactions,[12] with such reactive species. Further, there is very little available information about nucleophilic catalysis in these reactions, other than that it is known to occur in some cases. Again with such reactive substrates it might in principle be possible to achieve rate-limiting formation of the various XNO species under appropriate conditions. Another point of interest concerns the likely *in vivo* formation of thionitrites from free –SH groups in derivatives of the important naturally occurring amino acids cysteine and glutathione. Such thionitrites should be formed readily under gastric (*i.e.* acidic) conditions, and might act *in vivo* as nitrosating agents, generating carcinogenic nitrosamines and nitrosamides in the lower digestive tract.

We have examined the nitrosation in aqueous acid solution of the following eight thiols which include some of biological importance: cysteine (Cys), cysteine methyl ester (Cys-OMe), *N*-acetylcysteine (Ac-Cys), penicillamine (PEN), *N*-acetylpenicillamine (Ac-PEN), glutathione (GSH), thioglycolic acid (TGA), and mercaptosuccinic acid (MSA).

Experimental

All the thiols were commercial samples of the highest purity grade available, and were further purified by recrystallisation where necessary. The kinetic experiments were carried out in a stopped-flow spectrophotometer, in aqueous solution at 25 °C. Reactions were followed by noting the appearance of the product *S*-nitroso thiol at 330 nm. All experiments were carried out under first-order conditions with $[RSH]_0 \gg [HNO_2]_0$, and good first-order behaviour was found in each individual kinetic run. Typical conditions were $[HClO_4]$ 0.1M, [RSH] 1×10^{-2}M, $[NaNO_2]$ 1×10^{-4}M. The rate constants were obtained from the integrated first-order rate equation, using a measured infinity value. The quoted values of the rate constants are the means of at least five determinations; the standard error was generally less than 3%.

Results and Discussion

All the eight thiols reacted rapidly with aqueous acidic nitrous acid to give yellow solutions, characteristic of *S*-nitroso compounds, with a broad absorbance in the u.v., centred at about 330 nm. In some cases, notably for Ac-PEN[13] the thionitrite has been isolated as a stable solid. Other thionitrites have also been obtained *e.g.* from Cys and Ac-Cys,[14] but they are rather unstable. In some cases ^{15}N n.m.r. studies[15] on the reaction solutions have been interpreted (*e.g.* for Ac-PEN and Ac-Cys) in terms of thionitrite formation. On the synthetic side, under rather different experimental conditions and over a longer timescale, products derived from the reactions of Cys-OMe and PEN are explained only in terms of *N*-nitrosation,[16] but it is likely that the initial product is that of *S*-nitrosation and

Table 1. Values of k_3/dm^6 mol^{-2} s^{-1} [equation (2)] and k_2/dm^3 mol^{-1} s^{-1} [equation (3)] for the nitrosation of thiols[a]

RSH	k_3	k_2(ClNO)	k_2(BrNO)	k_2(ONSCN)
Cysteine (Cys)	3.5×10^2	1.2×10^6	5.4×10^4	7.2×10^2
	3.4×10^2	1.3×10^6	5.6×10^4	6.5×10^2
Cysteine methyl ester (Cys-OMe)	2.2×10^2	1.1×10^6	4.6×10^4	7.5×10^2
N-Acetylcysteine (Ac-Cys)	1.6×10^3	1.0×10^7	4.6×10^5	1.7×10^3
	1.5×10^3			
Penicillamine (PEN)	90			
N-Acetylpenicillamine (Ac-PEN)	7.9×10^2			
Glutathione (GSH)	1.0×10^3	1.2×10^7	5.6×10^5	3.9×10^3
	1.1×10^3	1.2×10^7	5.4×10^5	
Thioglycolic acid (TGA)	2.7×10^3	1.4×10^7	9.2×10^5	2.4×10^4
	2.6×10^3			
Mercaptosuccinic acid (MSA)	1.0×10^3		8.5×10^4	5.8×10^3

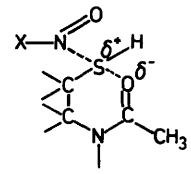
[a] Values of k_3 were obtained from the variation of k_0 with either [H$^+$] or [RSH], in water at 25 °C. Typical reaction conditions were: [$HClO_4$] (1—20) $\times 10^{-2}$M, [RSH] (0.5—2) $\times 10^{-2}$M, [$NaNO_2$] 1×10^{-4}M. Similarly k_2 values were obtained from the variation of k_0 with [nucleophile]. Typical reaction conditions were: [$HClO_4$] 0.2M, [RSH] 1×10^{-2}M, [Cl$^-$] 0—0.5M, [Br$^-$] 0—0.5M, [SCN$^-$] 0—4×10^{-2}M.

that *S*-to-*N* rearrangement occurs later. Such rearrangements are now well documented.[17]

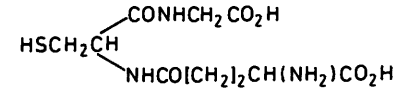
(a) *Acid-catalysed Reactions.*—All reactions were followed spectrophotometrically by a stopped-flow procedure, noting the increasing absorbance at *ca.* 330 nm due to the thionitrite. The measured first-order rate constant k_0 is defined by $-d[HNO_2]/dt = k_0[HNO_2]$. Experiments at different concentrations of both [RSH] and [H$^+$] showed that all the reactions were also first-order in both [RSH] and [H$^+$], thus verifying rate equation (2). Values of k_3 for the eight thiols are given in Table 1. Most were obtained in two ways, from the variation of [RSH] and also of [H$^+$], and both values are given in Table 1, showing an acceptable level of agreement.

For MSA there is good agreement with a previously measured[8] value of 1.33×10^3 dm^6 mol^{-3} s^{-1}, and also for Ac-PEN where k_3 at 31 °C was determined[11] as 8.40×10^2 dm^6 mol^{-2} s^{-1}. For cysteine, literature values of 456 (ref. 2) and 443 dm^6 mol^{-2} s^{-1} (ref. 8) have been reported. However a more detailed study[9] has shown that Cys reacts *via* both forms, $HSCH_2CH(\overset{+}{N}H_3)CO_2H$ and $HSCH_2CH(\overset{+}{N}H_3)CO_2^-$, in a proportion which depends on the acidity. The k_3 values measured for Cys are thus composite values which will depend on the [H$^+$] of the experimental measurements. In our case for reaction at 0.19M-$HClO_4$ the contribution from $HSCH_2CH(\overset{+}{N}H_3)CO_2^-$ is probably negligible. Even so our k_3 value is somewhat lower than 514 dm^6 mol^{-2} s^{-1} found in the more detailed study.[9] The discrepancy may arise from a salt effect. The evidence in favour of reaction *via* both forms of a conjugate acid–base pair is that significant positive intercepts (on the k_0 axis) are found[8,9] for plots of k_0 *vs.* [H$^+$]. This is confirmed in the present work, and as expected the intercept disappears for the reaction of Cys-OMe where the zwitterionic form cannot exist.

Significantly Ac-Cys is more reactive than Cys. This is also true for the nucleophile-catalysed reactions [see (b)]. The same effect is apparent from a comparison of the k_3 values for PEN and Ac-PEN. In both examples, under the experimental conditions used, the primary amines will exist predominantly in the protonated form ($-\overset{+}{N}H_3$), whereas the *N*-acetyl compounds will not be significantly protonated. If these different electron-attracting properties can be transmitted in a through-bond sense to the sulphur atom, this would account for the greater reactivity of the *N*-acetyl derivatives. However the nitrogen and sulphur atoms are separated by two carbon atoms and this effect may not be transmitted sufficiently to effect such a reactivity difference. We suggest an alternative explanation. The developing positive charge on sulphur in the transition state, for



(1)



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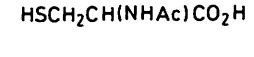

(3)

reaction with XNO generally, may be stabilised to some extent by interaction with the oxygen atom of the carbonyl group in the *N*-acetyl derivatives. This involves a sterically favourable six-membered-ring structure for the transition state (**1**). Such a stabilisation would be in addition to any arising from the carboxy group present in all four thiols under consideration. The effect is greater for the penicillamine system than for the cysteine system, perhaps as expected for a more sterically crowded molecule. This effect may also account for the reactivity of GSH (**2**), which is greater than that of Cys for all the nitrosating agents considered; indeed GSH is structurally quite similar to Ac-Cys (**3**) from the point of view of such an interaction.

One point of interest is that for the four most reactive thiols studied (Ac-Cys, GSH, TGA, and MSA) the k_3 values all lie within a very small range indeed, *i.e.* 1.0—2.7×10^3 dm^6 mol^{-2} s^{-1}. It has been argued[12] that such a small range of rate constants over a wide range of structures indicates that the reactions are encounter-controlled. This range is quite close to the range 4—7×10^3 dm^6 mol^{-2} s^{-1}, which has been noted for the acid-catalysed nitrosation of a wide variety of reactants, varying from aniline to thiourea. This adds further weight to the idea that these *S*-nitrosation reactions are so favourable that the rate is limited only by the encounter rate.

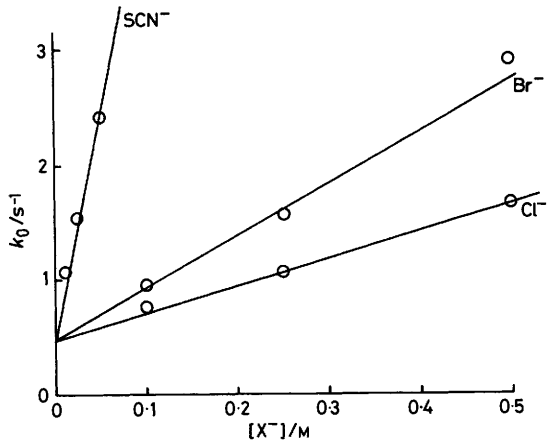

Figure 1. Catalysis by Cl^-, Br^-, and SCN^- in the nitrosation of cysteine methyl ester (Cys-OMe) in water at 25 °C, with [$HClO_4$] 0.198M, [Cys-OMe] 1×10^{-2}M, and [$NaNO_2$] 1×10^{-4}M

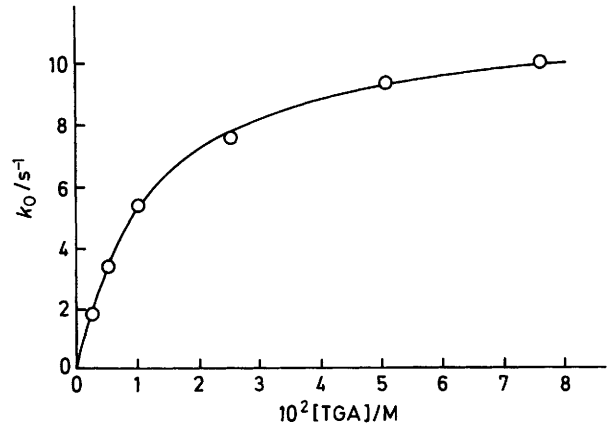

Figure 2. Variation of k_0 with [TGA] in the nitrosation of TGA in the presence of SCN^- (4×10^{-2}M) and $HClO_4$ (1.9×10^{-2}M); [$NaNO_2$] 1×10^{-4}M

(b) *Nucleophilic Catalysis.*—The kinetic effect of added Cl^-, Br^-, and SCN^- on the reactions of Cys, Cys-OMe, Ac-Cys, GSH, TGA, and MSA was investigated. All showed the usual catalytic features observed for *N*-nitrosation of amines in the sequence $SCN^- > Br^- > Cl^-$. The effect is shown graphically in Figure 1 for the reactions of Cys-OMe, which is typical of the series. The positive intercept represents reaction *via* $H_2NO_2^+$ and catalysis arises by reaction of the corresponding XNO species. Under the experimental conditions (where there is no significant ionisation to nitrite ion and no substantial conversion of HNO_2 into XNO) the expression for k_0 is now that given in equation (3), where k_2 is the second-order rate constant

$$k_0 = k_3[RSH][H^+] + k_2K_{XNO}[RSH][H^+][X^-] \quad (3)$$

for reaction by XNO and K_{XNO} is the equilibrium constant for XNO formation. The derived k_2 values (see Table 1) all show the well known trend ClNO > BrNO > ONSCN expected from electronegativity arguments, and also predicted by theoretical calculations.[18] For any one XNO reagent the reactivity closely parallels that found for the $H_2NO_2^+$ reactions. Cys-OMe is as expected very similar in reactivity to Cys, whereas Ac-Cys is significantly more reactive. Again for the ClNO reactions the k_2 values for Ac-Cys, GSH, and TGA, the most reactive thiols, lie in a very small range, 1.0—1.4 $\times 10^7$ dm^6 mol^{-2} s^{-1}, again suggesting that these reactions are also encounter-controlled. This is in spite of the facts (*a*) that these numbers are *ca.* 10^2 times smaller than that calculated for such a process,[12] and (*b*) that the values are also *ca.* 10^2 times smaller than those determined for diazotisation of aniline derivatives.[19] However there is a striking similarity between these values for RSH and those determined recently[20] for BrNO reactions of aliphatic amines over a wide range of basicity (pK_a 8.0—11.5). It was suggested[20] that the encounter limit for aliphatic amines is *ca.* 2×10^7 dm^3 mol^{-1} s^{-1} and that the extra reactivity of the anilines arises in some other way. These results with *S*-nitrosation of thiols support such a suggestion.

The experiments with added nucleophiles were all performed at relatively low [RSH] (typically 1×10^{-2}M) when reaction is strictly first-order in [RSH]. However upon increasing [RSH] the k_0 *vs.* [RSH] plots develop a pronounced downward curvature. This occurs for all three nucleophiles, but is more pronounced for thiocyanate ion (see Figure 2). The effect is also more evident for the more reactive thiols, and was quite clear for Ac-Cys, GSH, TGA, and MSA. Under these conditions the nitrosation of [RSH] by XNO becomes so rapid as to allow the formation of XNO to be rate-limiting. Until now we have assumed that the equilibrium formation of XNO is rapid and that the equilibrium in the Scheme is always maintained; at

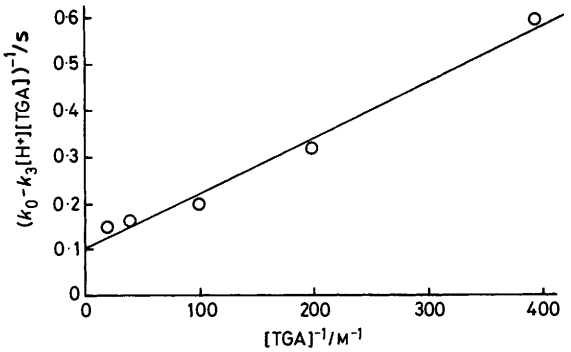

Figure 3. Double reciprocal plot [derived from equation (4)] for the nitrosation of TGA, using the data from Figure 2

$$HNO_2 + H^+ + X^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} XNO + H_2O$$

$$XNO + RSH \xrightarrow{k_2} RSNO + X^- + H^+$$

Scheme.

low [RSH] this is so. But when (see Scheme) k_2[RSH] is comparable with k_{-1} ([H_2O] is included in k_{-1}) this will no longer be the case. Now, the general expression for k_0 is that given by equation (4). The first term represents reaction *via*

$$k_0 = \frac{k_1k_2[H^+][X^-][RSH]}{k_{-1} + k_2[RSH]} + k_3[H^+][RSH] \quad (4)$$

XNO and the second, *via* $H_2NO_2^+$. At sufficiently high [RSH], for a sufficiently reactive thiol we can envisage the first term being reduced to $k_1[H^+][X^-]$, when the formation of XNO is rate-limiting. We have written a third-order constant k_1 for XNO formation; the reaction clearly involves two steps, the second probably being the reaction of $H_2NO_2^+$ with X^-. This Scheme explains the observed curvature; the high [RSH] limit is only achieved in some cases for the SCN^- reactions. For a quantitative test of equation (4), we plot $(k_0 - k_3[H^+][RSH])^{-1}$ *vs.* $[RSH]^{-1}$. Such plots are linear (see Figure 3) with a positive slope and intercept, which allows the calculation of k_1 and k_2/k_{-1}. With our data, the curvature was sufficient to allow

Table 2. Values of k_1/dm^6 mol^{-2} s^{-1} and k_{-1}/s^{-1} (from the Scheme) and derived K_{XNO} values (dm^6 mol^{-2})

	N-Acetylcysteine	Thioglycolic acid	Mercaptosuccinic acid
$k_1(Cl^-)$	2.3×10^3	2.9×10^3	
$k_1(Br^-)$	4.6×10^3	4.0×10^3	
$k_1(SCN^-)$	6.0×10^3	1.2×10^4	1.1×10^4
k_{-1}(ClNO)	1.8×10^6	2.7×10^6	
k_{-1}(BrNO)	7.8×10^4	9.6×10^4	
k_{-1}(ONSCN)	1.8×10^2	2.8×10^2	5.1×10^2
K_{ClNO}	1.3×10^{-3}	1.1×10^{-3}	
K_{BrNO}	5.9×10^{-2}	4.2×10^{-2}	
K_{ONSCN}	34	43	21

a sufficiently accurate plot only for Ac-Cys (for Cl^-, Br^-, and SCN^-), TGA (for Cl^-, Br^-, and SCN^-), and MSA (for SCN^- only). Table 2 gives the collected results for k_1 and also for k_{-1} (using the k_2/k_{-1} ratios and k_2 values from Table 1). These calculations also enable values of K_{XNO} to be deduced from k_1/k_{-1}. We stress the inherent inaccuracy in a double reciprocal plot treatment, particularly when the intercept is very small, as in many cases here. Bearing this in mind the agreement between the three substrates is quite reasonable. The third-order k_1 values for Cl^-, Br^-, and SCN^- increase only a little from Cl^- to SCN^-, as expected for reactions which must be close to the encounter limit. Values of k_1 are given in the literature (see ref. 12) for the reactions of these (and other) anions at 0 °C; these also show a trend $Cl^- < Br^- < SCN^-$ but the differences are very small as we note here for the reactions at 25 °C. For SCN^- there is a literature value [21] for k_1 of 1.17×10^4 dm^6 mol^{-2} s^{-1} at 25 °C, derived from the nitrosation of hydrazoic acid at high [HN_3]. This agrees very well with two of the three currently determined values given in Table 2.

The k_{-1} values for the hydrolysis of XNO, or of the *O*-nitrosation of water, show a much bigger change with changing X, and further demonstrate the well established reactivity sequence ClNO > BrNO > ONSCN.

Finally, the K_{XNO} values deduced from our kinetic analysis ($K_{XNO} = k_1/k_{-1}$) are in remarkably good agreement with the established literature values, which were obtained by direct spectrophotometric measurements, *i.e.* 1.1×10^{-3} for ClNO,[22] 5.1×10^{-2} for BrNO[23] and 32 dm^6 mol^{-2}, for ONSCN.[5] This confirms that our mechanistic ideas and detailed kinetic analysis are substantially correct.

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