Mechanism of *S*-Nitrosation of Cysteine Derivatives in the pH range 6–12 using *N*-Methyl-*N*-nitrosotoluene-*p*-sulphonamide

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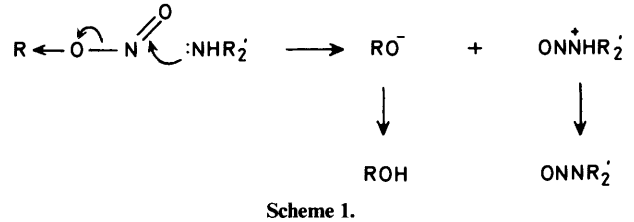
N-Methyl-*N*-nitrosotoluene-*p*-sulphonamide (MNTS) reacts readily with the thiolate anion of cysteine and some of its derivatives, in aqueous ethanol, to give the corresponding *S*-nitroso species. The measured second order rate constant k_2 increases with pH, and the experimental points lie close to the S-shaped curve calculated for reaction *via* the thiolate anion. The pK_a values for SH ionisation were calculated from the experimental results, and gave figures generally in good agreement with the literature. There is no evidence of reaction *via* the thiol RSH, or of any reaction, under these conditions, with less powerful nucleophiles including amines, alcohols and an enol. There was no evidence of *S*-nitrosation of cysteine using dimethylnitrosamine, nitrosoproline or nitrososarcosine. The results are consistent with a direct transfer of the nitroso group to the sulphur atom of the thiolate anion. The results are compared with similar reactions of alkyl nitrites.

Many nitroso compounds (both inorganic and organic) can themselves act as nitrosating agents, *i.e.*, they can transfer the nitroso group in an electrophilic sense to a suitable nucleophilic site.[1] This usually occurs intermolecularly but can, under certain circumstances, take place within the same molecule. In most cases the transfer occurs indirectly; another nucleophile (sometimes the solvent or for example a halide ion) first reacts with the nitroso compound to give a species which is the true nitrosating agent. An example is provided by the nitrosation reactions brought about by alkyl nitrites in aqueous acid. Here, it has been shown[2] that hydrolysis of the alkyl nitrite occurs first (rapidly and reversibly) liberating nitrous acid (*i.e.* an *O*-nitrosation of water) which generates the true nitrosating agent NO^+ or $H_2NO_2^+$. In this case O–N bond fission is greatly assisted by protonation of the oxygen atom (bound to the alkyl group) in the alkyl nitrite. Nitrosamines behave similarly. However in basic and neutral solution (where hydrolysis is much slower, and in any case yields nitrite ion which is not normally a nitrosating agent) alkyl nitrites act directly as nitrosating agents towards a number of substrates including amines; secondary amines yield nitrosamines.[3] This reaction is greatly facilitated by the presence of electron-withdrawing substituents within the alkyl nitrite (see Scheme 1), as expected



Scheme 1.

for an electrophilic process.[4] It is not known with certainty whether corresponding *N*-nitroso compounds can act in a similar way, *i.e.* in a direct reaction, but there are many examples in the literature[5] of the indirect reaction where hydrolysis (or reaction with a nucleophile such as halide ion) of nitrosamines in acid solution releases a reactive nitrosating species. There are two reports[6,7] of a somewhat unusual reaction which occurs at high acidity, where the *protonated* forms of some aromatic nitrosamines react with the *protonated* form of some aniline derivatives.

In this paper we are seeking to establish whether *N*-nitroso compounds can act as direct transfer nitrosating agents under neutral or slightly basic conditions. By analogy with the observations using alkyl nitrites, this end is more likely to be achieved with *N*-nitroso compounds containing electron-withdrawing substituents, and also with the more powerfully nucleophilic substrates. We report the results of a study with *N*-methyl-*N*-nitrosotoluene-*p*-sulphonamide (MNTS) and some other *N*-nitroso compounds, principally with cysteine derivatives and also less reactive species. It is known that MNTS and cysteine react readily at pH 7 in 25% aqueous ethanol to yield finally the disulphide cystine and *N*-methyl-2-toluenesulphonamide, both quantitatively.[8] The reaction mixture initially turned light red due to the formation of *S*-nitrosocysteine which decomposed on standing to give cystine and nitric oxide.

In dilute aqueous acid solution it is known[9] that MNTS undergoes hydrolysis (or *O*-nitrosation of water) to give nitrous acid in an essentially irreversible reaction. This reaction is also quite general for nitrosamides,[10] although deamination is often a competing reaction. The denitrosation of both is interesting from the mechanistic viewpoint, in that reaction involves a rate-limiting proton transfer to the *N*-nitroso compound.

Experimental

All of the *N*-nitroso compounds used were purified by recrystallisation or distillation and malononitrile by sublimation. All other materials, including the cysteine derivatives, were of the highest purity grade available. Buffer solutions were made by using suitable aliquots of the following: 0.1 mol dm^{-3} KH_2PO_4 and 0.1 mol dm^{-3} NaOH (pH 5–9), 0.025 mol dm^{-3} borax and 0.1 mol dm^{-3} NaOH (pH 9–11), 0.05 mol dm^{-3} Na_2HPO_4 and 0.1 mol dm^{-3} NaOH (pH 11–12) and 0.2 mol dm^{-3} KCl and 0.2 mol dm^{-3} NaOH (pH 12–13). On mixing solutions of cysteine and MNTS a yellow coloured solution was formed, typical of *S*-nitroso species in solution.[11] After some hours a white precipitate formed, which was identified by elemental analysis and by its i.r. spectrum, as cystine.

Kinetic measurements were carried out at 25 °C by noting the increasing absorbance at 330 nm due to *S*-nitrosocysteine. Most of the faster reactions were carried out by stopped-flow spectrophotometry and the remainder (typically at pH < 6) by conventional recording spectrophotometry. The solvent was

Table 1. Typical run with $[MNTS]_0$ 2 × 10^{-4} mol dm^{-3}, [Cysteine] 4.05 × 10^{-3} mol dm^{-3} at pH 5.86.

t/s	0	600	1 200	1 800	2 400	3 000	3 600	4 200	4 800	5 400	6 000	6 600	7 200	
Absorbance	0.094	0.115	0.135	0.150	0.161	0.172	0.183	0.188	0.194	0.199	0.203	0.206	0.209	0.225
$10^4 k_0/s^{-1}$		2.91	3.13	3.10	2.98	3.02	3.16	3.01	3.00	2.99	2.97	2.93	2.92	

Average value of k_0 3.01 × 10^{-4} s^{-1}

Table 2. Values of k_0 obtained at different [cysteine], all at pH 5.86.

10^3 [Cysteine]/mol dm^{-3}	$10^4 k_0/s^{-1}$
4.05	3.01
6.08	4.51
8.11	6.18
10.1	7.83
12.1	9.37

Table 3. Values of k_2 as a function of pH for the reaction of MNTS with cysteine.

pH	k_2/dm^3 mol^{-1} s^{-1}
5.12	0.012
5.67	0.045
5.86	0.077
5.92	0.092
7.43	2.60
7.82	5.59
7.95	6.82
8.18	9.72
8.26	11.0
8.74	17.7
9.12	21.0
10.5	23.9
12.6	25.0

25% ethanol–water (v/v) and the cysteine concentration was always in a greater than twentyfold excess over that of MNTS. Good first-order behaviour was found and all first order rate constants (k_0) were mean values of four separate measurements, with a standard error of ±4%. A typical run is shown in Table 1.

Results and Discussion

Although *S*-nitrosocysteine and other thionitrites have been isolated,[12] most are very unstable in their pure forms and also decompose in solution. All of the *S*-nitroso species examined in the present work were, however, sufficiently stable to allow the kinetic studies to be carried out without interference from the decomposition. Thionitrites generally have broad absorptions in the u.v.–vis. region centred at around 330 nm, and ^{15}N n.m.r. measurements have characterised such species in solution.[13]

The measured first order rate constants were determined at each of five different cysteine concentrations at each pH value. Plots of k_0 *vs.* [cysteine] were all linear, passing through the origin, thus establishing rate equation (1), where k_2 is the

$$\text{Rate} = k_2[\text{RSH}][\text{MNTS}] \quad (1)$$

derived second order rate constant. One set of results for reactions at pH 5.86 are shown in Table 2, and values of k_2 at different pH values in Table 3. A plot of k_2 *vs.* pH is shown in Figure 1 and is the typical S-shaped curve expected for a system involving reaction with an anionic form of the substrate. The most likely acid–base equilibrium involved here (given that we are observing *S*-nitrosation) is the ionisation of the thiol group to give the thiolate anion. The outline mechanism is given in

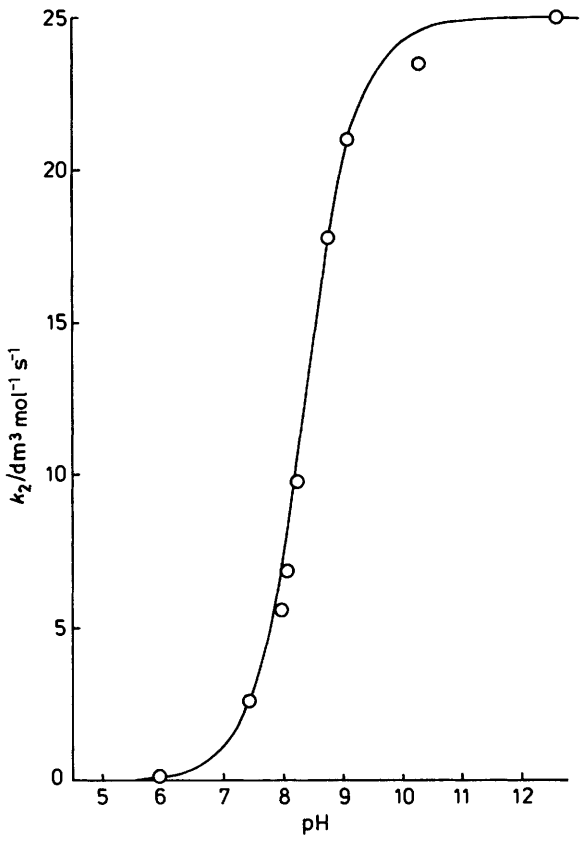
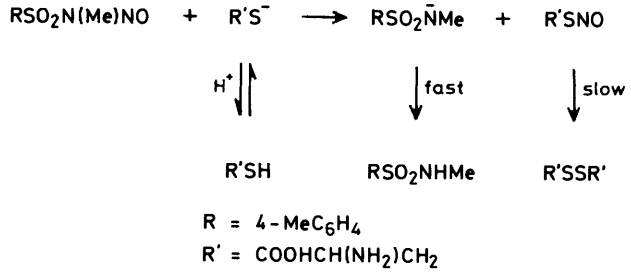


Figure 1. Plot of k_2 [equation (1)] against pH for the nitrosation of cysteine by MNTS. The solid line is the calculated curve and the points are the experimental ones.

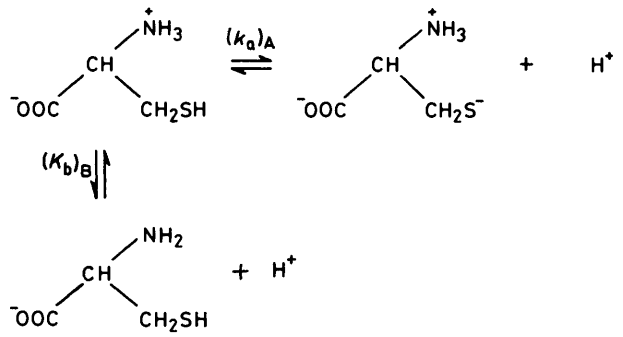


Scheme 2.

Scheme 2. Similar reactions occur with cysteine and other nitrosating agents *e.g.* alkyl nitrites[14] where reaction occurs *via* the thiolate anion, and also with nitrous acid[15] in acid solution, where the reactive species is the thiol.

The expression for k_2 expected from Scheme 2 is given in equation (2), where k is the second order rate constant for reaction between MNTS and $R'S^-$, and is given by the limiting

$$k_2 = kK_a/(K_a + [H^+]) \quad (2)$$



Scheme 3.

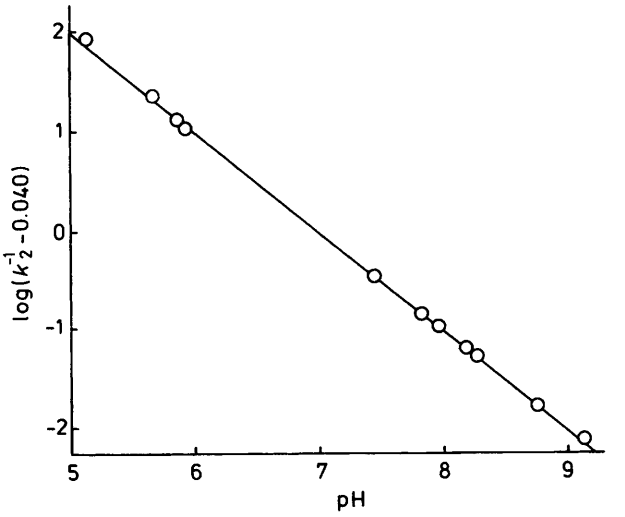


Figure 2. Plot of $\log(k_2^{-1} - k^{-1})$ against pH [equation (3)] for the nitrosation of cysteine by MNTS.

Table 4. Values of k_2 as a function of pH for the reaction of MNTS with *N*-acetylcysteine.

pH	k_2/dm^3 mol^{-1} s^{-1}
7.67	0.190
8.71	1.92
9.04	3.81
9.24	5.37
9.49	7.97
9.70	11.0
9.91	13.7
10.04	15.4
10.38	19.0
11.86	23.3

value of k_2 at high pH; K_a is the acid dissociation constant for R'SH ionisation. There has been considerable confusion in the earlier literature regarding the identification and magnitude of the various ionisation modes in cysteine. The situation is now resolved, and is set out in Scheme 3. The measured macroscopic ionisation constant K_2 is the sum of the two microscopic constants $(K_a)_A$ and $(K_a)_B$. The macroscopic pK_a value for the composite ionisations is usually quoted at around 8.37,[16] but more recently it has been shown that the $\overset{+}{N}H_3$ and SH ionisations in cysteine are close together with the thiol group ionisation taking place slightly before the $\overset{+}{N}H_3$ ionisation. Values of $(pK_a)_A$ have been variously determined[17] at 8.21, 8.53, 8.50 and 8.64 and values of $(pK_a)_B$ as 8.65, 8.86, 8.85 and 8.62.

Table 5. Values of k_2 as a function of pH for the reaction of MNTS with cysteine methyl ester.

pH	k_2/dm^3 mol^{-1} s^{-1}
6.40	0.034
7.65	0.708
8.42	3.35
9.00	8.49
9.30	11.8
9.48	13.4
9.89	16.5
10.29	18.0
10.71	18.7
11.10	19.1

Table 6. Values of k_2 as a function of pH for the reaction of MNTS with cysteine ethyl ester.

pH	k_2/dm^3 mol^{-1} s^{-1}
7.15	0.190
7.50	0.410
8.11	1.59
8.30	2.37
8.61	4.60
8.88	6.86
9.08	9.13
9.25	11.2
10.28	18.5
11.46	19.6

Thus the quoted macroscopic value of 8.37 is reasonably close to the microscopic constant for SH ionisation in this case. We have used this value together with a value of 25.0 dm^3 mol^{-1} s^{-1} for k to calculate k_2–pH plot. The solid line in Figure 1 is indeed the calculated curve, and the points are the experimentally determined ones, the agreement is good. Small changes in the value of $(pK_a)_A$ do not change the position of the calculated curve very much. An alternative procedure is to determine $(K_a)_A$ from the experimental results. Rearrangement of equation (2) to equation (3) shows that $(K_a)_A$ can be obtained from the

$$\log(k_2^{-1} - k^{-1}) = -\text{pH} - \log k(K_a)_A \qquad (3)$$

intercept of a plot of the left hand side of equation 3 *vs.* pH. Such a plot is shown in Figure 2; the measured slope is −1.01 and $(pK_a)_A$ determined as 8.47 which is in good agreement with the microscopic $(pK_a)_A$ values for SH ionisation in cysteine.

Figure 1 shows further that k_2 tends to zero at low pH, so that there is no evidence of reaction of MNTS with the thiol form of cysteine. This contrasts markedly with the corresponding reactions of nitrous acid with thiols in acid solution, where often reaction occurs with rate constants within a power of ten or so of the encounter value. This puts in perspective that, as expected MNTS and also alkyl nitrites (both non-protonated) are, by many orders of magnitude less powerful nitrosating species than that generated in acid solutions of nitrous acid.

Other cysteine derivatives behave in a similar way to cysteine itself. Thus we observe the same pattern of kinetic behaviour in the reactions of MNTS with *N*-acetyl cysteine, cysteine methyl carboxylate, and cysteine ethyl carboxylate. The experimental results for these substrates are given in Tables 4, 5 and 6 respectively. As can be seen from the limiting values of k_2 at high pH, the reactivity of the R'S$^-$ species is not significantly affected by *N*-acetyl substitution or by esterification. All the cysteine derivatives give excellent straight line plots for equation (3) with slopes very close to −1. The derived pK_a values are given in

Table 7. Values of $(pK_a)_A$ determined from equation (3).

Thiol	$(pK_a)_A$	Literature values[a]
Cysteine	8.47	8.21, 8.53, 8.50, 8.64
N-Acetylcysteine	9.76	9.76[b]
Cysteine methyl ester	9.15	8.99,[b] 9.1
Cysteine ethyl ester	9.24	9.17,[b] 9.09, 8.87

[a] All are microscopic values except [b] which are macroscopic, and are taken from reference 17, CRC Handbook of Biochemistry and Molecular Biology 3rd edition, vol. 1, J. Inczedy and J. Marothy, *Acta Chim. Acad. Sci. Hung.*, 1975, **86**, 1, and R. K. Boggess, J. R. Absher, S. Morelen, L. T. Taylor, and J. W. Hughes, *Inorg. Chem.*, 1983, **22**, 1273.

Table 7, together with a selection of literature values. For all there is good agreement between our values and those in the literature.

MNTS is itself decomposed in alkaline solution, in the familiar reaction to generate diazomethane in solution. We have measured the rate constants for this reaction over a range of pH values, by noting the disappearance of the absorbance at 260 nm. This reaction is very much slower than the nitrosation reaction under all our experimental conditions, and there is no need to correct our nitrosation rate constants.

We have further attempted to establish whether MNTS under these mildly alkaline or neutral conditions is able to nitrosate other species. There was no discernable reaction (as a change in the u.v. spectrum at 260 nm over 12 h) for reactions of MNTS at pH 7 and 11 with any of the following: morpholine, isopropylamine, isopropyl alcohol, and dimedone (an enol reactive in nitrosation[18]). There are, however, one or two literature reports[19] where amine nitrosation (in low yield) has been accomplished using MNTS; experimental conditions may have been more forcing than ours. In one study,[20] MNTS nitrosated diethylamine quantitatively after refluxing in CH_2Cl_2 for 16 h. Attempts to nitrosate the anion derived from malononitrile (which can be achieved with nitrous acid in dilute acid solution[21]) were thwarted by the concurrent base-catalysed hydrolysis of malononitrile. It is clear that MNTS will act as a direct-transfer nitrosating agent under mild conditions in aqueous ethanol, only towards the most reactive of substrates *i.e.* thiolate anions.

We have also examined the possibility of thiolate anion nitrosation by other *N*-nitroso species. Each of the following, however, failed to yield any detectable *S*-nitrosocysteine, even at pH 11: dimethylnitrosamine, nitrosoproline, and nitrososarcosine. It is clear that reaction will not occur unless there is a powerful electron-withdrawing group close to the amino nitrogen atom. An obvious family of compounds to test is therefore the nitrosamides, which would be expected to behave in a similar fashion to the nitrososulphonamides. We have confirmed the literature reports[8,22] that *N*-methyl-*N*-nitrosourea yields no cystine when treated with cysteine, and there is also no colour evidence for the formation of *S*-nitrosocysteine. Use of nitrosamides in this way is complicated, particularly at high pH by the concurrent hydrolysis reaction (*i.e.* nucleophilic attack at the carbonyl carbon atom). It is reported[23] that *N*-methyl-*N*-nitrosourethane gives many products when treated with cysteine, none of which can be rationalised in terms of a nitrosation reaction. However, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine does produce some cystine[8] and the reaction is thought to involve concurrent nucleophilic attack at the nitroso group and at the imino carbon atom.

We conclude that for *N*-nitroso compounds to act as direct nitrosating agents under mild conditions the presence of a very powerful electron-withdrawing group (*e.g.* SO_2 in MNTS) is required, even for nitrosation of the most powerful of nucleophiles (*e.g.* $R'S^-$). This contrasts with the behaviour of alkyl nitrites under similar conditions where *S*-nitrosation of thiolate anions occurs readily even with simple primary, secondary, and tertiary nitrites[14] and nitrosation of amines,[3] alcohols,[24] ketones, and nitro compounds[25] is also readily achieved. The presence of electron-withdrawing groups in the alkyl nitrites further adds to their reactivity.[4,26] The reactivity difference between *N*-nitroso and *O*-nitroso compounds in these reactions probably reflects the stronger N–NO bond compared with the O–NO bond.

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