Nitrosation products from *S*-nitrosothiols *via* preliminary nitric oxide formation

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High yields of *N*-nitroso-*N*-methylaniline were obtained from *S*-nitrosothiols (RSNO) and *N*-methylaniline in water at pH 7.4. Reactions were completely inhibited by the presence of EDTA and also when oxygen was removed from the solutions. Lower yields of 4-nitrosophenol were obtained from phenol under similar conditions and there was strong evidence of the rapid formation of a nitroso product (absorbance maximum at 390 nm) from uric acid which decomposed more slowly under the reaction conditions and could not be isolated. The results are consistent with prior nitric oxide formation, by the well-known Cu^{2+}-catalysed (in which the active reagent is Cu^{+}) decomposition of the *S*-nitrosothiol, subsequent oxidation of NO yielding NO_2, which reacts further with NO to give N_2O_3, which then effects conventional electrophilic nitrosation in direct competition with its hydrolysis to nitrite. With phenol as the reactant, higher yields of 4-nitrosophenol were only possible when there was a very large excess of phenol over RSNO, probably due to the more effective relative competition of the hydrolysis reaction, given the lower reactivity in nitrosation of phenol compared with *N*-methylaniline. Nitrosation of uric acid is unknown, but we were able to observe the fairly rapid build-up of the same absorbance at 390 nm, from uric acid and nitrous acid only at around pH 4, which disappeared more slowly. The results suggest that uric acid behaves as do amides generally in that a nitroso compound is formed, which decomposes by an acid-catalysed route.

The chemistry of *S*-nitrosothiols (RSNO generally) is very much of current interest[1] because of their probable involvement in the biological chemistry of nitric oxide *in vivo*. A number of such species (including *S*-nitrosoglutathione and *S*-nitrosoproteins) have been detected in the body[2] and there is a growing body of opinion which believes that nitric oxide is stored and transferred around the body in the form of RSNO compounds.[3] There is also a current debate as to the role of *S*-nitrosohaemoglobin in the control of blood pressure,[4] and of the involvement of *S*-nitrosoproteins in the regulation of breathing[5] and in many other areas of body chemistry. In addition, *S*-nitrosothiols are being actively examined as potential NO-donors for a variety of medical uses.[6]

Homolysis of the S–N bond occurs both thermally and photochemically[7] generating the corresponding disulfide and initially nitric oxide. However at ambient temperatures and in the absence of incident radiation of the appropriate wavelength, this mode of decomposition is very slow (typically the half-life of *S*-nitrosocysteine at 25 °C at pH 7.4 is about 2 d[8]), and therefore unlikely to be important *in vivo*. In the presence of even trace amounts of Cu^{2+} however (often at the impurity level in buffer components in distilled water) and including Cu^{2+} bound to peptides and proteins,[9] decomposition is much faster, also leading to disulfide and nitric oxide formation. The effective reagent[10] has been shown to be Cu^{+}, which is formed by Cu^{2+} reduction by thiolate ion, always present[11] in solutions of RSNO due to the reversibility of its formation reaction from nitrous acid and a thiol (eqns. (1)–(3)), although the

$$RSH + HNO_2 \rightleftarrows RSNO + H_2O \quad (1)$$

$$2Cu^{2+} + 2RS^{-} \longrightarrow 2Cu^{+} + RSSR \quad (2)$$

$$Cu^{+} + RSNO \longrightarrow Cu^{2+} + RS^{-} + NO \quad (3)$$

equilibrium lies well over to the right with equilibrium constants of ~10^5 dm^3 mol^{-1}.

In addition, RSNO compounds can act as electrophilic nitrosating agents, effectively transferring NO^{+} directly to a large range of nucleophiles[12,13] (eqn. (4)), without the formation of any free intermediate nitrosating species.

$$RSNO + Nu^{-} \longrightarrow RS^{-} + NuNO \quad (4)$$

In general these reactions are not very rapid. Reaction *via* the pathway in eqns. (2) and (3) can be suppressed by the presence of a metal ion chelator *e.g.* EDTA.

Recently it has been demonstrated[14,15] that although nitric oxide itself is not a conventional electrophilic nitrosating species, it can readily bring about nitrosation in aerated solution. The likely explanation is that oxidation of NO occurs generating NO_2 which reacts further with NO to give the powerful nitrosating species N_2O_3. This has been the source of much confusion in the biological literature when oxygen has not been sufficiently rigorously excluded from the reacting solutions, and there have been many claims that NO itself is an electrophilic nitrosating species, although no convincing mechanistic scheme has ever been advanced. In this paper we have investigated whether NO generated from RSNO by the Cu^{2+}-catalysed process (in normal aerated solution) can also be instrumental in bringing about nitrosation at around pH 7. There is one example in the literature from the results of a study of the reactions of RSNO compounds with ascorbic acid.[16] At low ascorbic acid concentrations (below ~1×10^{-4} mol dm^{-3}) decomposition proceeds smoothly to give nitric oxide. The reaction does not occur in the presence of EDTA and is stopped when EDTA is added during reaction. At higher ascorbic acid concentrations (above ~1×10^{-3} mol dm^{-3}) however, a different reaction occurs, also leading to nitric oxide formation, when ascorbate acts as a nucleophile and the reaction given in eqn. (4) occurs, quite independently of the concentration of added Cu^{2+} or of EDTA. The other difference between the two reactions is that the 'organic' product at low [ascorbate] is the disulfide, whereas at the higher [ascorbate]

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the corresponding product is the thiol. We have looked at the possible generality of nitrosation by *S*-nitrosothiols *via* prior NO formation and subsequent oxidation by examining the reactions with *N*-methylaniline (as a typical amine) and phenol (as an example of aromatic *C*-nitrosation), and also have examined a possible reaction with uric acid.

Results

(a) Reactions with *N*-methylaniline

We chose to look at the possible nitrosation reaction of *N*-methylaniline (NMA) as a typical amine nitrosation where the expected product *N*-nitroso-*N*-methylaniline (NNMA) is stable under our experimental conditions and which has a well-known absorbance band with λ_{max} around 265 nm. We found that the characteristic absorption spectrum of NNMA built up quickly when a solution of *S*-nitrosocysteine (SNCys) at 1.0×10^{-3} mol dm^{-3} was allowed to react with NMA over a range of concentration 1.7–10.3 $\times 10^{-5}$ mol dm^{-3}, in a phosphate buffer at pH 7.4. In these experiments there was no added Cu^{2+} or metal ion chelator EDTA. At each of five different [NMA] within the range, where [NMA] is in considerable deficit compared to [SNCys], the yield of NNMA is > 90% (see Fig. 1).

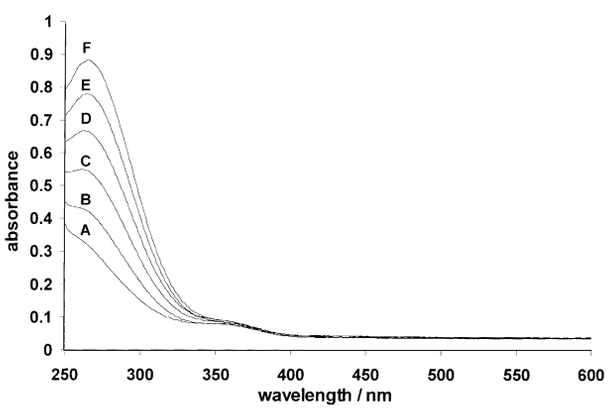

Fig. 1 Wavelength scans after the reaction of *S*-nitrosocysteine (1×10^{-3} mol dm^{-3}) with a range of [*N*-methylaniline] at pH 7.4. A, 1.7×10^{-5}, B, 3.4×10^{-5}, C, 5.2×10^{-5}, D, 6.9×10^{-5}, E, 8.6×10^{-5} and F, 10.3×10^{-5} mol dm^{-3}.

Using a different RSNO, *S*-nitroso-*N*-acetylpenicillamine, (SNAP), it was found that the rate of formation of NNMA (measured at 280 nm), was qualitatively the same order of magnitude as the rate of disappearance of SNAP, (as measured by the disappearance of the absorbance at 340 nm due to SNAP). It was not possible to get precise rate constants as many of the kinetic runs were not simple first-order. The scans are shown in Figs. 2 and 3 respectively. These experiments were carried out with SNAP in a slight excess over NMA. Reactions were accelerated in the presence of added Cu^{2+} in the range 0–5 $\times 10^{-5}$ mol dm^{-3}, and were *completely inhibited* when EDTA was added. Further, when all of the solutions were purged with nitrogen before reaction was initiated, the yield of NNMA was drastically reduced to ~10% compared with ~100% obtained with aerated solutions in the same time. The rate of reaction was also much reduced and probably represents the small amount of reaction taking place as a result of leakage of air into the system.

No NNMA was generated from *S*-nitrosoglutathione (at millimolar concentrations) and NMA, consistent with the fact that at these concentrations this RSNO does not decompose to a significant extent, since the disulfide product (GSSG) is a very powerful complexing agent for Cu^{2+}, and so has the same effect as does the addition of EDTA.[17]

When the initial RSNO and NMA concentrations are equal, only ~50% NNMA is formed together with ~50% free nitrite

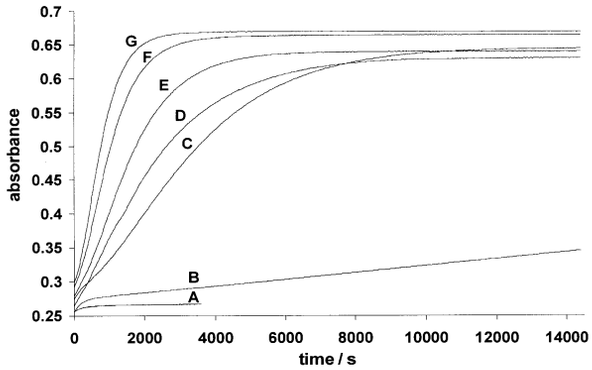

Fig. 2 Absorbance measurements at 280 nm for the reaction of *N*-methylaniline (1×10^{-4} mol dm^{-3}) with *S*-nitroso-*N*-acetylpenicillamine (5.1×10^{-4} mol dm^{-3}), A, EDTA (1×10^{-4} mol dm^{-3}) added, B, nothing added, C,1×10^{-5} mol dm^{-3} added Cu^{2+}, D, 2×10^{-5} mol dm^{-3} added Cu^{2+}, E, 3×10^{-5} mol dm^{-3} added Cu^{2+}, F, 4×10^{-5} mol dm^{-3} added Cu^{2+} and G, 5×10^{-5} mol dm^{-3} added Cu^{2+}.

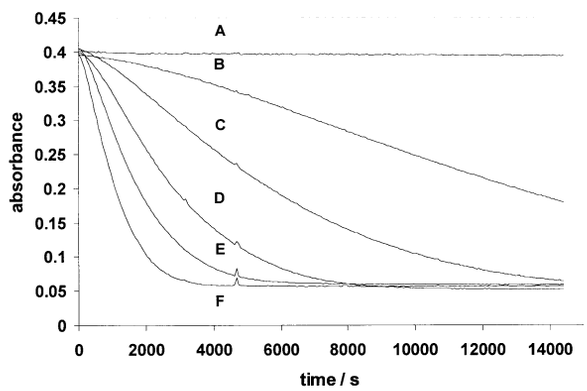

Fig. 3 Absorbance measurements at 340 nm following the disappearance of *S*-nitroso-*N*-acetylpenicillamine (5.1×10^{-4} mol dm^{-3}) in reaction with *N*-methylaniline (1×10^{-4} mol dm^{-3}) at pH 7.4 as a function of added Cu^{2+}, A, no added Cu^{2+}, B, 1×10^{-5}, C, 2×10^{-5}, D, 3×10^{-5}, E, 4×10^{-5}. F, 5×10^{-5} mol dm^{-3} added Cu^{2+}.

anion, which suggests (as in the case of the reactions of NO itself in aerated solutions[14,15]) that reaction involves N_2O_3 as the true nitrosating species. As expected (given that the pK_a of nitrous acid is 3.1) control experiments showed that NMA and NO_2^- at pH 7.4 generated no measurable increase in the absorbance at 265 nm over the time-scale of our experiments.

(b) Reactions with phenol

It is well known that phenol readily forms 4-nitrosophenol by reaction with acidified nitrous acid solutions. Substituted phenols behave similarly. Nitrosation can also be brought about using alkyl nitrites and other sources of NO^+. 4-Nitrosophenol is readily characterised by an absorbance maximum at 397 nm with an extinction coefficient of 26400 dm^3 mol^{-1} cm^{-1}. When SNCys (1×10^{-3} mol dm^{-3}) was reacted with phenol (2.6×10^{-4} mol dm^{-3}) the absorbance at ~350 nm due to SNCys disappeared with a half-life of ~2.5 min. At the same time there was a build up of the absorbance at 397 nm. The estimated yield of 4-nitrosophenol however was only ~3%. (See Fig. 4). In contrast, at much higher [phenol], increased yields of 4-nitrosophenol were obtained. Table 1 shows the trend more clearly over a range of phenol concentrations where the % 4-nitrosophenol increased from 4 to 13%. Similarly ~23% 4-nitrosophenol was obtained in 15 minutes from *S*-nitrosopenicillamine (SNP) (2.5×10^{-4} mol dm^{-3}) and phenol (0.1 mol dm^{-3} mol dm^{-3}). Again reaction was virtually halted by the addition of EDTA, although a very small absorbance was noted at 395 nm probably arising from the thermal decomposition of SNP. In the absence of EDTA the kinetic form of the appearance of

Table 1 Yield and % conversion of *S*-nitrosocysteine (4.9×10^{-5} mol dm^{-3}) to 4-nitrosophenol as a function of the phenol concentration, for reaction at pH 7.4

[Phenol]/10^{-3} mol dm^{-3}	[4-nitrosophenol]/10^{-6}mol dm^{-3}	% conversion
2.03	1.89	3.8
4.06	3.44	7.0
6.08	4.55	9.3
8.11	5.52	11.2
10.1	6.29	12.8

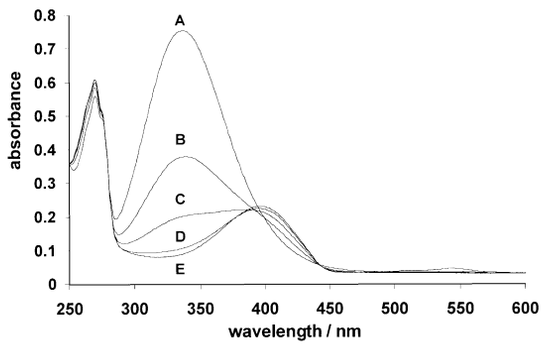

Fig. 4 Absorbance measurements for the reaction of phenol (2.6×10^{-4} mol dm^{-3}) with *S*-nitrosocysteine (1×10^{-3} mol dm^{-3}) at pH 7.4 as a function of time, A, initial measurement, B, after 2.5 min, C, after 5 min, D, after 10 min, E, after 20 min.

the 395 nm was unusual in that it showed autocatalysis with a sudden ending to the reaction when all of the nitrosating species is consumed. Similar autocatalytic behaviour occurs when measuring the disappearance of the 350 nm absorbance (due to SNP) in the Cu^{2+} catalysed decomposition of SNP,[18] which arises for this *S*-nitrosothiol when the reduction to Cu^{+} is partly rate-limiting.

(c) Reactions with uric acid

We chose to look for any reaction of *S*-nitrosothiols with uric acid, because of its presence and importance *in vivo*, even though, as far as we are aware, no nitrosation reaction of uric acid by any nitrosating agent has ever been reported. We were surprised to see the rapid build up of an absorbance centred at 390 nm, when SNCys (1.0×10^{-3} mol dm^{-3}) was allowed to react with uric acid ($1.0–4.8 \times 10^{-4}$ mol dm^{-3}) at pH 7.4. This absorbance disappeared more slowly on standing, as shown in Figure 5, for experiments with five different [uric acid].

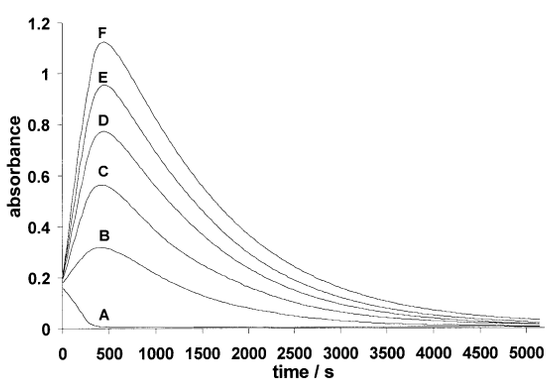

Fig. 5 Absorbance measurements at 390 nm for the reaction between *S*-nitrosocysteine (1.0×10^{-3} mol dm^{-3}) and uric acid at pH 7.4, A, no uric acid, B, 1.0×10^{-4}, C, 1.9×10^{-4}, D, 2.9×10^{-4}, E, 3.8×10^{-4} and F, 4.8×10^{-4} mol dm^{-3} uric acid.

Qualitative measurements using the NO probe showed that nitric oxide is generated during these experiments. Again there was no discernible reaction when EDTA was present. The build-up and decay of the 390 nm absorbance also occurred

Table 2 Initial rates for the reaction of sodium nitrite (0.010 mol dm^{-3}) at pH 4.3 with uric acid

[Uric acid]/10^{-5} mol dm^{-3}	Initial rate/10^{-4} absorbance s^{-1}
5.28	0.98 ± 0.01
10.6	2.10 ± 0.01
15.9	3.08 ± 0.02
21.1	4.40 ± 0.01
26.4	5.00 ± 0.01
31.7	7.08 ± 0.02

when uric acid was in large excess over SNCys. Reaction was measurable even when [SNCys] was as low as 1×10^{-6} mol dm^{-3}.

As a result of these unusual results found for the reaction of uric acid with SNCys, we decided to look at the conventional nitrous acid nitrosation of uric acid, which appears never to have been reported in the literature. In moderately acid solution (0.4 mol dm^{-3}), there was no evidence of any build-up of absorbance in the 390 nm region (or in any other region). However at pH 4.3 using a phthalate buffer, we find a rapid build-up of the absorbance at 390 nm followed by a more slow decay. Fig. 6 shows some absorbance–time plots for the

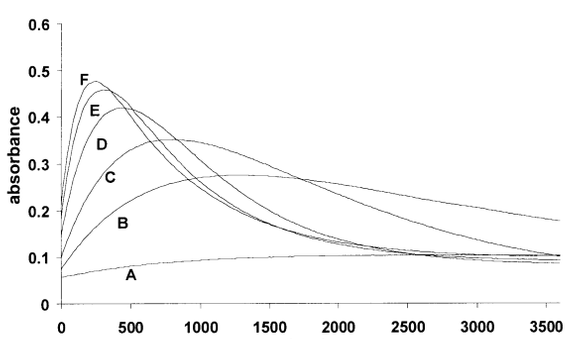

Fig. 6 Absorbance–time plots monitored at 400 nm, for the reaction at pH 4.3 of uric acid (2.5×10^{-4} mol dm^{-3}) with added sodium nitrite, A, 1.0×10^{-3}, B, 5.8×10^{-3}, C, 1.1×10^{-2}, D, 2.1×10^{-2}, E, 3.2×10^{-2}, and F, 4.2×10^{-2} mol dm^{-3}.

reaction of uric acid (2.5×10^{-4} mol dm^{-3}) at pH 4.3 with added sodium nitrite over the range $1–40 \times 10^{-3}$ mol dm^{-3}. It is clear that at the higher [nitrite], initial reaction (formation of 390 nm absorbance) is both faster and proceeds to a greater extent than at the lower [nitrite], consistent with an equilibrium formation of the product absorbing at 390 nm. Initial rate measurements indicate a first order dependence on nitrite. Other results, given in Table 2, with a range of [uric acid] at constant nitrite showed a first-order dependence on uric acid, again from initial rate measurements. Experiments were carried out over the pH range 2.18–5 80. At the high pH end there was only little conversion to the 390 nm absorbance, and reaction was quite slow. As the pH was decreased the reaction rate increased, reaching a maximum at about pH 4. Thereafter, the maximum absorbance decreased, showing that the decomposition of the probable nitroso compound (390 nm absorbance), is accelerated as the acidity is increased. Crude initial rate measurements showed that the initial rate increased as the pH

decreased until there was a levelling off below pH ~3. Below about pH 1 there is no evidence of any reaction at all.

Discussion

It is clear from the results with NMA that nitrosation here occurs by initial formation of nitric oxide, *via* the Cu^{2+}-catalysed route (eqns. (1)–(3)), since reactions are completely inhibited in the presence of EDTA. Subsequent oxidation to nitrogen dioxide and formation of dinitrogen trioxide N_2O_3, allows the possibility that either or both of these nitrogen oxides can bring about *N*-nitrosation of NMA; both are well-known nitrosating species.[19] Under these conditions we favour the pathway *via* N_2O_3, since nitrite ion is generated as the inorganic product and not a mixture of nitrite and nitrate ions which would be expected from the hydrolysis of NO_2/N_2O_4. Reaction rates are drastically reduced in the absence of oxygen. Thus we can eliminate, under these reaction conditions, the slower pathway involving direct NO^+ transfer from RSNO to the amine.

Recent publications[14,15] have shown that oxygenated solutions of nitric oxide in water readily nitrosate both amines (typically morpholine) and thiols (typically cysteine). Further, the rate law is identical with that obtained earlier for the auto-oxidation of NO, and the reaction rate is independent of the nature and concentration of the thiol or amine present.

In our case the rate limiting step is the release of NO from RSNO. This is clear from the data on the Cu^{2+} catalysis of the reactions of SNAP with NMA (Figs. 2 and 3) where the dependence upon $[Cu^{2+}]$ is evident.

Reactions with phenol gave the expected 4-nitrosophenol product but in much lower yield than for the corresponding reactions with NMA. This is readily explained in terms of the expected competition between N_2O_3 nitrosation and its hydrolysis to nitrite (eqn. (5)).

$$N_2O_3 + H_2O \longrightarrow 2HNO_2 \rightleftarrows 2NO_2^- + 2H^+ \qquad (5)$$

It is well-known that NO in aerated water generates a quantitative yield of nitrite ion. For the NMA reactions under our experimental conditions the competition very much favoured the nitrosation reaction, perhaps as expected since the reaction of N_2O_3 with NMA is known to occur at or close to the diffusion controlled limit.[20] There appears to be no corresponding literature value for the phenol, but it is known that phenol is orders of magnitude less reactive than NMA towards other nitrosating species, such as the nitrosyl halides.[21] Competition with hydrolysis is then expected to be less effective for phenol. As expected, increasing [phenol] does increase the percentage of 4-nitrosophenol (see Table 1).

The situation with uric acid is rather unusual. As far as we are aware there is no reference to the formation of a nitroso derivative of uric acid in the literature. We were somewhat surprised therefore to see a fairly rapid build-up of an absorbance at 390 nm from the reaction of *S*-nitrosothiols with uric acid at pH 7.4. This product is quite likely to be a nitroso derivative, but this was quite unstable under the reaction conditions and so no product could be isolated. The maximum yield of this unknown product increased with [uric acid] when SNCys was in a large excess. Again there was no discernible reaction when EDTA was present demonstrating that as for the reactions of NMA and phenol, Cu^{2+} is essential, even at the low level impurity from the distilled water and buffer components, to generate NO from SNCys as the first step. In order to probe this further we carried out some preliminary experiments on the attempted nitrosation of uric acid using the more usual HNO_2/H^+ method. In moderately acidic solution (0.4 mol dm^{-3}) there was no evidence of any reaction whatsoever and we could only see the fairly rapid build-up of the 390 nm absorbance when the acidity was reduced to pH ~ 4. Here the behaviour was very similar to that encountered earlier using SNCys as the source of the nitrosating species, *ie* a fairly rapid build-up followed by a slower decay. Fig. 6 suggests that we are looking at an equilibrium process here, generating the highest product yield at the highest $[HNO_2]$ in very large excess over uric acid. The pH dependence experiments gave complicated results, but it was clear that at the higher pH, reaction is much slower as expected when $[HNO_2]$ is much reduced (pK_a 3.1). At the lower pH, more product is generated (and more rapidly), but the rate of decomposition is also increased. The result of the balance here is that no product is seen at pH values <~1. The crude initial rate measurements showed as expected that the rate increased from pH ~5, as the concentration of HNO_2 increased, and appeared to level off above pH ~3. This suggests that when all the nitrous acid has been released, reaction occurs either between $H_2NO_2^+/NO^+$ and an ionised form of uric acid, or more likely, given the low acidity and relatively high $[NaNO_2]$, that reaction occurs between N_2O_3 and a neutral form of uric acid. Clearly more experimental work is necessary to get a full mechanistic picture.

A reasonable rationalisation for this unexpected reaction with uric acid, given our results using SNCys and also with HNO_2, is that we are looking here at the nitrosation of an amide (albeit a cyclic one). We and others[22,23] have found that there are in general significant differences between the nitrosation of amines and of amides. For the latter, there appears to be no nucleophilic catalysis, but there is base catalysis and a substantial kinetic solvent isotope effect, and for secondary amides the reaction is significantly reversible. We proposed a mechanism which involves rapid and reversible *N*-nitrosation followed by rate-limiting proton transfer to the solvent. The change of rate-limiting step (when compared with amine nitrosation) is as a result of the powerful electron-attracting effect of the carbonyl group. Sulfonamides are believed to react in a similar fashion as do amines containing powerful electron attracting substituents, *e.g.* 2,4-dinitroaniline. The mechanism is borne out by measurements on the reverse reaction, *i.e.*, the denitrosation of nitrosamides.[22] With this mechanism it is not possible to make any deductions about the nature of the nitrosating species, since the nitrosation step is reversible (and the next stage is rate-limiting), reaction will always be first-order in $[HNO_2]$ regardless of whether the reagent is $H_2NO_2^+/NO^+$, N_2O_3, or ClNO etc. It has been argued that initial nitrosation takes place at the carbonyl oxygen atom of amides, which is followed by a *O*- to *N*- rearrangement.[23] Such rearrangements are not uncommon in nitrosation reactions.

We propose that uric acid undergoes *N*-nitrosation at one of the amide nitrogen atoms, by N_2O_3, derived either from RSNO species *via* the intermediate formation of NO, or from relatively high nitrite ion concentrations in the pH region around 4. The position of nitrosation is purely speculative at this stage. The *N*-nitrosamide then undergoes acid-catalysed denitrosation, to such an extent that at pH < ~1, no *N*-nitrosamide can be detected. For this nitrosouric acid derivative, there may also be some homolytic decomposition, since some nitric oxide was detected, but was not quantified. This fits in with the fact that *N*-nitrosamides generally are more usually synthesised, not from the more conventional nitrous acid in acidic media (as are *N*-nitrosamines), but by using N_2O_3, N_2O_4 or NOCl in, *e.g.*, carbon tetrachloride under non-acidic conditions, alkyl nitrites in organic solvents or nitrosonium salts in acetonitrile. It was possible to measure the formation of *N*-methyl-*N*-nitrosourea at low acidities (pH ~2), and to examine the reverse reaction at much higher acidities.[22]

Experimental

The *S*-nitrosothiols were all synthesised by conventional nitrous acid nitrosation of the corresponding thiol. The pH of the solution was adjusted and the solution used without further treatment. All other materials were commercial samples of the

highest purity grade available. Kinetic traces were obtained using conventional spectrophotometry (Perkin Elmer λ12 or λ2) sometimes scanning a wavelength range and sometimes using the time drive at fixed wavelength as appropriate. Nitric oxide levels were measured in solution using the World Precision Instruments ISO-NO MK II commercial NO electrode system calibrated with ascorbic acid and sodium nitrite.

Conclusion

We have established in this work the generality of the nitrosation ability of *S*-nitrosothiols by a pathway involving prior nitric oxide formation *via* the Cu^{2+}-catalysed process. Subsequent oxidation etc leads to the probable nitrosating species, N_2O_3, which reacts in competition with its hydrolysis to NO_2^-/HNO_2 (depending on the pH). High yields of nitroso compound can only be achieved for very reactive substrates, or when the substrate concentration is in large excess. In passing we discovered that it is possible to nitrosate uric acid, but the nitroso compound is extremely sensitive to acid-catalysed decomposition.

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