Reactivity of nitrogen nucleophiles towards *S*-nitrosopenicillamine

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We report the results of a kinetic study of the reactions of a number of nitrogen nucleophiles with the nitrosothiol *S*-nitrosopenicillamine (SPEN). The range of nucleophiles includes primary, secondary and tertiary aliphatic amines, together with hydrazine, hydroxylamine, azide ion, ammonia, semicarbazide, thiomorpholine and *S*-methylcysteine. Secondary amines form *N*-nitrosamines quantitatively. As expected, reaction occurs *via* the free base forms of the nucleophiles and consequently most of the reactions take place readily only at relatively high pH. Experiments were carried out with [nucleophile] ⩾ [RSNO], and for many reactions, plots of the first order rate constant *vs*. [nucleophile] were linear. For ammonia and the primary amines, however, this plot tended to level off at high [nucleophile] and an explanation is offered involving the reversible formation of an inactive RSNO–amine complex, for which there is spectral evidence, in parallel with the main reaction. For the secondary amines there is a reasonably good Brönsted plot with a β value of ~0.2. The much greater reactivities of *S*-methylcysteine and thiomorpholine, compared to those of primary amines and morpholine respectively are consistent with initial attack at the sulfur atom, followed by an internal rearrangement. Over the whole range of nucleophiles studied there is a reasonable correlation with the Ritchie N^+ parameter, and not with the Pearson *n* scale. Comparisons are made with the corresponding reactions of alkyl nitrites and *N*-methyl-*N*-nitrosotoluene-*p*-sulfonamide (MNTS).

There is currently much interest in the chemistry of *S*-nitrosothiols (or thionitrites) RSNO, in connection with aspects of the now well-known[1] 'nitric oxide story'. They are under active consideration as nitric oxide donors for possible medical use to make up for any deficiencies in the natural *in vivo* synthesis of NO from L-arginine, and they are currently believed to be involved in the storage and transport of NO within the body.[2] *S*-Nitrosoglutathione (GSNO) is presently used medically to inhibit platelet aggregation during some operations,[3] and both GSNO and *S*-nitroso-*N*-acetylpenicillamine (SNAP), are stable solids which have been, and continue to be, much used in a range of *in vivo* and *in vitro* experiments as NO generators. Less stable (generally non-isolable in the pure state) RSNOs are sometimes used for the same purpose, when they are generated in solution by thiol nitrosation[4] and used as such *in situ.*

Clearly the mechanism of NO release is important. We have shown[5] that apart from the thermal reaction (which is usually very slow at room temperature) and the photochemical pathway, reaction can occur in aqueous solution at pH 7.4, generating the disulfide and initially nitric oxide. This occurs in a Cu^+-catalysed process, in which the catalyst is generated from Cu^{2+} and RS^-, which are often present in buffered solutions of RSNOs at trace impurity levels. In aerated aqueous solutions the nitric oxide is rapidly and quantitatively converted to nitrite anion, at the concentrations used. There is a large structure–reactivity dependence, which has been rationalised. It has been further shown that ascorbate (at low concentration) will also act as a reducing agent, and that Cu^+ can also be generated, by thiolate reduction from Cu^{2+} bound to peptides and proteins.[6]

Another important reaction of RSNOs is that with thiolate ion (eqn. (1)), which leads to a transnitrosation, forming a new

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

S-nitrosothiol if the R groups are different. This exchange process has been known qualitatively for some time,[7] but more recent work has measured the equilibrium and rate constants for the process.[8] Kinetic studies over a range of pH values have shown, as expected, that the reactive species is the thiolate ion, and that mechanistically the reaction can be regarded as a nucleophilic substitution at the nitroso nitrogen atom,[9] or alternatively as an electrophilic *S*-nitrosation by RSNO. At this stage there is no evidence in favour of (or against) the involvement of a reaction intermediate. Since there is a wide range of reactivity of RSNOs towards NO generation,[10] it is possible that NO can be stored *in vivo* as a 'stable' RSNO, which could release NO by first undergoing a transnitrosation to give a more unstable R′SNO. It is known for example that *S*-nitrosoproteins are much more stable than is *S*-nitrosocysteine itself to loss of NO.[11]

Another example of reactions where RSNOs can act as electrophilic nitrosating species is to be found in their reactions with ascorbate. As previously mentioned, it has been demonstrated that ascorbate can act as a reducing agent (at quite low concentration, typically 1×10^{-4} mol dm^{-3}) to generate Cu^+, which brings about RSNO decomposition. However, in the presence of EDTA, which cuts out any metal ion promoted reactions, and at higher concentrations, ascorbate brings about the decomposition of RSNOs generating NO and the corresponding thiol[12] (eqn. (2)). As yet the exact form of

$$2RSNO + Ascorbate \longrightarrow 2RS^- + 2NO + Dehydroascorbic\ acid + 2H^+ \qquad (2)$$

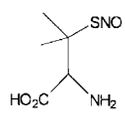
the nucleophile has not been properly established, but since the reactions were carried out at pH 7.4, it is likely that the nucleophile is the monoanion of ascorbic acid. Similarly it has been shown recently that *S*-nitrosothiols react with hydrogen peroxide (*via* the anion) generating initially the peroxynitrite anion, stable at high pH, but which isomerises to nitrate anion at lower pH.[13]

We thought it to be of some interest to determine the range of nucleophiles which would undergo electrophilic nitrosation by RSNOs, thus establishing the possible generality of the reaction. There are literature reports[14] that amines can be nitrosated in this way, but there has not been a proper mechanistic study. It is possible that amine nitrosation could occur by RSNO decomposition (by the copper catalysed route) to give NO,

which by oxidation and further reaction with NO would give N_2O_3, which could readily nitrosate any amine group present. Indeed we showed this to be the case[10] with the reaction of SNAP with *N*-methylaniline, where in the presence of air, the *N*-nitrosamine was formed essentially quantitatively, whereas when oxygen was rigorously excluded, very little *N*-nitrosamine was formed. The copper catalysed pathway is readily blocked by EDTA addition.

Electrophilic nitrosation by other compounds containing the -NO group such as alkyl nitrites and a range of N–NO compounds is well known, and has significant synthetic advantages over the more usual nitrous acid nitrosation, in that generally it can be carried out under basic conditions, and also in a wider range of solvents. Detailed mechanistic studies of reactions of alkyl nitrites[15] and of *N*-nitrososulfonamides[16] have been published, and there are some results on the reactions of *N*-nitrosamines.[17] We hoped to compare the results of RSNO reactions with nitrogen nucleophiles with these literature data.

We found generally that the reactions of amines with RSNO species were quite slow, so drawing on our experience with reactions of several RSNOs[18] with thiolate ion, we elected to work initially with one substrate, *S*-nitrosopenicillamine (SPEN) **1**.



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This compound was significantly more reactive than the other nitrosothiols used, towards thiolate ion at high [thiolate ion], when a different reaction, leading principally to ammonia formation, occurs, but where the rate limiting step is believed to involve thiolate attack at a nitrosothiol. Although SPEN has never been isolated in the pure state, it is easy to work with a solution generated by *S*-nitrosation of penicillamine, which is sufficiently stable as a stock solution in mildly acidic solution for the purpose.

Results and discussion

(a) Reactions of secondary aliphatic amines

A range of secondary aliphatic amines reacted with SPEN (generated *in situ* from equimolar quantities of penicillamine and nitrous acid) in water at pH values near to the pK_a of the protonated amine. Reactions were followed by noting the disappearance of the absorbance at 340 nm due to SPEN. Final spectral scans all showed the complete disappearance of the 340 nm band and the appearance of absorbances in the 230–240 nm region consistent with *N*-nitrosamine formation. Spectral measurements in this range showed that nitrosamine formation is essentially quantitative. All kinetic experiments were carried out with [amine] ⩾ [SPEN], and in the presence of EDTA to prevent SPEN decomposition to NO *via* a copper-catalysed reaction. Good first-order kinetics were always found and the first-order rate constant (k_O) was linearly dependent on the total stoichiometric amine concentration, $[Amine]_T$, as shown in Fig. 1 for both the reactions of pyrrolidine (a) and piperidine (b) at a constant pH. This establishes experimentally the rate eqn. (3). For both of these amines, values of k_O were also

$$\text{Rate} = k[\text{SPEN}][\text{Amine}]_T = k_O[\text{SPEN}] \qquad (3)$$

obtained over a range of pH values in the region of the pK_a value of the protonated amine. The experimental results for piperidine are given in Table 1. In both cases, plots of k_O *vs.* pH are S-shaped with an inflexion point in the vicinity of the pK_a value, confirming that reaction occurs *via* the free base form of the amine as expected. This means that k_O and the acidity

Table 1 Values of k_O for the reaction of SPEN with piperidine (0.2 mol dm^{-3}) as a function of pH

pH	$k_O/10^{-5}$ s^{-1}
10.58	5.8
10.73	7.2
10.87	9.1
11.00	10.6
11.21	13.9
11.39	16.6
11.56	19.8
11.74	22.3
11.92	23.0
12.02	24.2
12.10	25.3

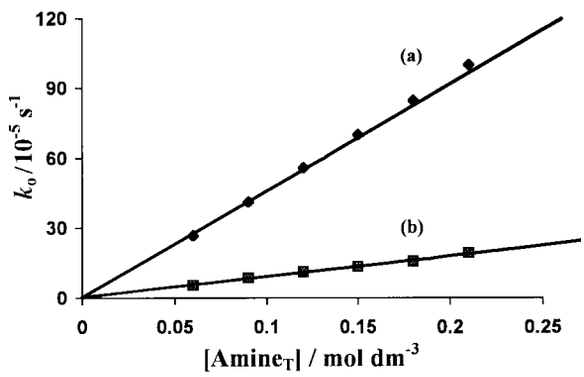

Fig. 1 Plots of k_O *vs.* total amine concentration for the reaction of SPEN with (a) pyrrolidine at pH 11.57 and (b) piperidine at pH 11.21.

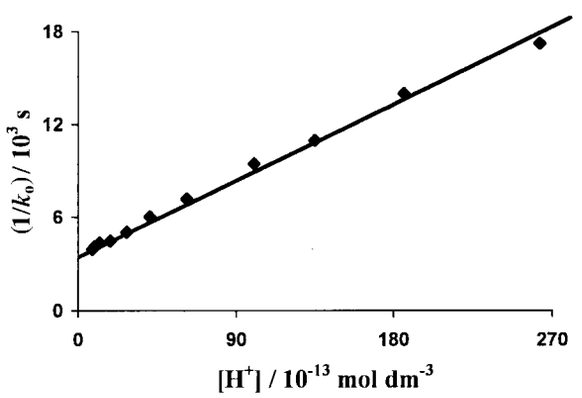

Fig. 2 Plot of $(k_O)^{-1}$ *vs.* $[H^+]$ for the reaction of SPEN with piperidine.

should be related by eqn. (4), where k_2 is the bimolecular rate

$$k_O = k_2K_a[\text{Amine}]_T/(K_a + [H^+]) \qquad (4)$$

constant for reaction between SPEN and the free base form of the amine. Plots of $(k_O)^{-1}$ against $[H^+]$ should then be linear with a positive slope and intercept. A typical plot is shown in Fig. 2 for the reaction of piperidine. The data for pyrrolidine gave a similar straight line plot. Values of the pK_a of the protonated form of the amines found from the slopes and intercepts of these lines were determined as 11.15 (piperidine) and 11.55 (pyrrolidine), which agree well with the respective literature values[19] of 11.12 and 11.30. These plots also gave values of 1.34×10^{-3} dm^3 mol^{-1} s^{-1} (piperidine) and 7.00×10^{-3} dm^3 mol^{-1} s^{-1} (pyrrolidine) for k_2, the bimolecular rate constant for reaction of the free amine with SPEN.

The reactions of eight secondary amines with SPEN were followed kinetically. Each individual experiment gave good first-order behaviour, and plots of k_O against $[Amine]_T$ were all linear. Typical experimental plots (for piperidine and pyrrolidine) are shown in Fig. 1. The other amines behaved similarly

Table 2 Values of k_2 from eqn. (4) for the reaction of SPEN with a range of secondary amines

Amine	k_2/dm^3 mol^{-1} s^{-1}
Pyrrolidine	7.2×10^{-3}
	7.0×10^{-3} [a]
	7.9×10^{-3} [b]
Piperidine	1.7×10^{-3}
	1.4×10^{-3} [a]
D,L-Proline	5.8×10^{-4}
Sarcosine	1.3×10^{-3}
Piperazine	3.5×10^{-4} [c]
4-Hydroxyproline	6.1×10^{-4}
Morpholine	3.8×10^{-4}
N-Methylaniline	Too slow to measure

[a] From the reciprocal plot $(k_O)^{-1}$ *vs.* [H$^+$]. [b] From a reaction carried out anaerobically. [c] Corrected for the statistical factor.

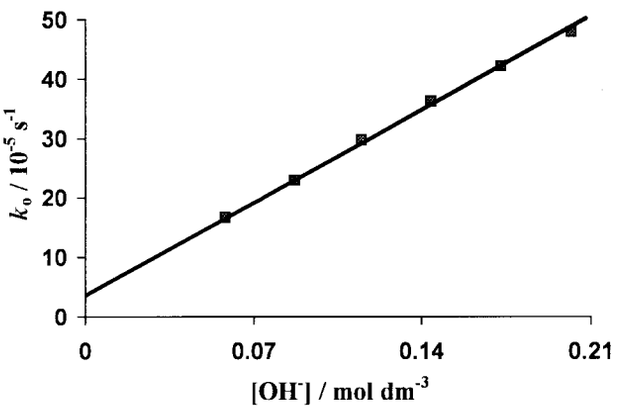

Fig. 3 Plot of k_O *vs.* [OH$^-$] for the alkaline hydrolysis of SPEN.

except that in some cases there was a small positive intercept at $[\text{Amine}]_T = 0$, which will be discussed later. Values of k_2 obtained from the slopes and from eqn. (4) are listed in Table 2. The value for pyrrolidine is duplicated, within the experimental error, when reaction is carried out anaerobically, demonstrating that dissolved oxygen plays no part in these reactions.

Small positive intercepts for the k_O against $[\text{Amine}]_T$ plots are evident for the slower reacting amines, notably for proline, piperazine and morpholine, and can reasonably be ascribed to the alkaline hydrolysis pathway, where the nucleophile is the hydroxide ion. This explanation was suggested[20] to account for similar small intercepts observed in the nitrosation of amines brought about in alkaline solution by *N*-methyl-*N*-nitroso-toluene-*p*-sulfonamide (MNTS). To check this possibility, we examined the kinetics of the alkaline hydrolysis of SPEN independently. Over the range of [OH$^-$] 0.06–0.20 mol dm^{-3}, first order behaviour occurred and the plot of k_O against [OH$^-$] is linear with a very small intercept, which could arise from the spontaneous thermal decomposition of SPEN (see Fig. 3). The expected 'nitrogen' product in the hydrolysis reaction is the nitrite anion, which was determined in the final reaction solution only at ~50% yield. This suggests that in addition to attack of OH$^-$ at the nitrogen atom, some reaction also occurs possibly by way of initial attack at the sulfur atom, which could lead to nitrous oxide formation, notionally by release of NO$^-$. Reactions of nucleophiles with MNTS show this ambident feature,[21] for while amines and other nitrogen nucleophiles attack the nitroso-nitrogen atom (leading to amine nitrosation), the 'harder' nucleophiles OH$^-$ and OEt$^-$ attack at the sulfur atom leading to diazomethane formation.

There is an acceptable agreement between the k_2 values obtained for piperidine and pyrrolidine from the variation of k_O with $[\text{Amine}]_T$ (Table 2) and those obtained from the reciprocal plot (Fig. 2). Qualitatively there is a clear link between the reactivity of the secondary amines and their basicity, and there is a reasonable Brönsted plot with a β value of ~0.2. This is significantly smaller than the value of 0.6 found for the corresponding reactions of secondary amines with MNTS[21] suggesting that charge transfer in the transition state of the RSNO reactions is less extensive, possibly reflecting the powerful electron withdrawing effect of the sulfonyl group in MNTS.

Less basic amines such as *N*-methylaniline (NMA) react much too slowly for a meaningful rate constant to be obtained. This means that an earlier report of nitrosamine formation[10] from NMA and a nitrosothiol, carried out without removal of all trace amounts of Cu^{2+} ions, was correctly interpreted in terms of prior NO formation followed by oxidation and hydrolysis giving nitrous acid which effects *N*-nitrosation, rather than as a direct nitrosation reaction by the nitrosothiol.

Table 3 Values of k_2 for reaction of SPEN with a range of nitrogen nucleophiles

Nucleophile	k_2/dm^3 mol^{-1} s^{-1}
Propylamine	7.8×10^{-4}
Ethylamine	4.9×10^{-4}
Ethylenediamine	1.8×10^{-4} [a]
Quinuclidine	7.1×10^{-4}
Trimethylamine	3.6×10^{-4}
Hydrazine	3.3×10^{-2} [a]
Azide ion	1.1×10^{-2} [a]
Methoxyamine	1.5×10^{-5}
Hydroxylamine	9.9×10^{-4}
Semicarbazide	4.4×10^{-5}
Thiomorpholine	1.5×10^{-2}
S-Methylcysteine	3.8×10^{-2}

[a] Corrected for the statistical factor.

(b) Reactions of other nitrogen nucleophiles

Primary aliphatic amines behave very much like their secondary counterparts, particularly at relatively low concentrations of amine and k_2 values were obtained in the same way. Values obtained are given in Table 3. Again reaction was shown to occur *via* the free base form by the linearity of a plot of $(k_O)^{-1}$ *vs.* [H$^+$], and satisfactory agreement was found between the derived and literature pK_a values. Reactivity was comparable with that of the secondary amines of similar basicity. Other nitrogen nucleophiles (of lower basicity), azide ion, and hydrazine reacted much more rapidly than did the amines: hydroxylamine also reacted, but at a rate comparable with those of the primary amines. Reaction also occurred with tertiary amines *e.g.* trimethylamine and quinuclidine, as is the case with nitrous acid nitrosation,[22] but reactions were quite slow and were not examined in detail.

We did find one significant difference between the behaviour of the primary and secondary amines, in that for the former the plots of k_O against $[\text{Amine}]_T$ showed a very pronounced tendency to curvature leading to a levelling off at high $[\text{Amine}]_T$, which was not present for any of the secondary amines studied. An example is shown in Fig. 4 for the reaction of propylamine at pH 10.9. All of the primary amines behaved similarly, but there was no obvious curvature in the plots for the reactions of azide ion, hydrazine and hydroxylamine. For the primary amines therefore values of k_2 were obtained at lower concentrations of amine (below ~0.09 mol dm^{-3}) where the inequality $[\text{Amine}]_T \gg [\text{SPEN}]$ still was valid. The products were not characterised; it is very likely that a primary aliphatic diazonium ion is formed, as in the nitrous acid reaction, leading to a range of products of deamination. There was, as expected, no absorbance formation in the 230–240 nm range corresponding to the formation of a stable nitrosamine.

The curvature of the k_2 *vs.* $[\text{Amine}]_T$ plot was even more pronounced in the reaction of ammonia with SPEN, which did proceed readily (with k_O values in the region of 1×10^{-3} s^{-1}),

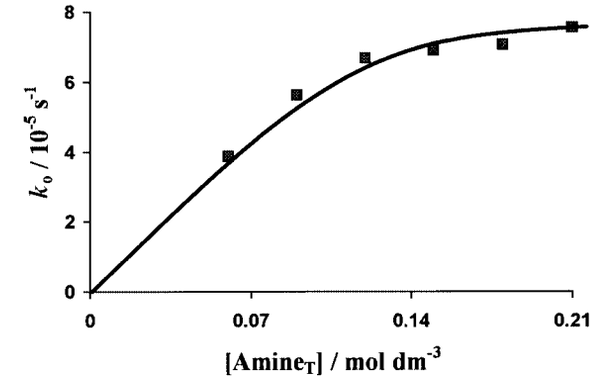

Fig. 4 Plot of k_O *vs.* total amine concentration for the reaction of SPEN with propylamine.

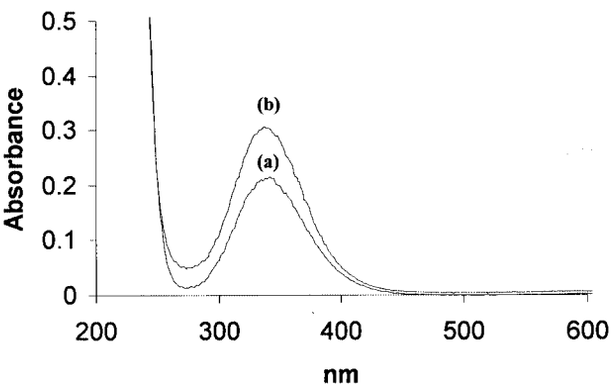

Fig. 5 Spectra of (a) SPEN (2.5×10^{-4} mol dm^{-3}) alone and (b) with added ammonia (0.2 mol dm^{-3}) at pH 9.55.

which precluded the determination of a k_2 value. Curved plots of this type could arise if an inactive complex between the amine and the nitrosothiol is formed rapidly as indicated in Scheme 1. The general expression for k_O from such a scheme is

$$\mathrm{RSNO} + \mathrm{R'NH_2} \rightleftharpoons [\mathrm{RSNO{\cdot}R'NH_2}] \quad \text{rapid} \quad K$$
$$\mathrm{RSNO} + \mathrm{R'NH_2} \longrightarrow \mathrm{RS^-} + \mathrm{R'N_2^+} \quad \text{slow} \quad k_2$$

Scheme 1

given by eqn. (5), which predicts a constant value for k_O at high

$$k_O = k_2K_a[\mathrm{R'NH_2}]_T/(K_a + [\mathrm{H^+}] + KK_a[\mathrm{R'NH_2}]_T) \quad (5)$$

$[\mathrm{R'NH_2}]_T$ if $K[\mathrm{R'NH_2}]_T \gg 1$. Support for this idea comes from the observation of a significant change in the initial spectrum of SPEN in the 340 nm range at high $[\mathrm{R'NH_2}]_T$. This is shown in Fig. 5 for the interaction of ammonia with SPEN at pH 9.55. A possible structure for this inactive complex could be that shown in Fig. 6, where there are two H-bonding interactions involving the O- and S-atoms of RSNO in a six-membered ring structure. This is possible for primary amines and ammonia, but not for secondary amines, in keeping with the experimental findings.

(c) Ambident nucleophiles

There are two examples in Table 2 where there is evidence that SPEN effects nitrosation initially at a sulfur site, which is then followed by an internal rearrangement of the nitroso group to the nitrogen atom. The rate constant for the reaction with thiomorpholine is 40 times greater than that for morpholine, and the value for *S*-methylcysteine is ~100 times larger than the values for simple primary amines: a direct comparison with *e.g.* alanine is not possible since, unusually, the kinetics for that reaction were not straightforward. It is quite clear however that

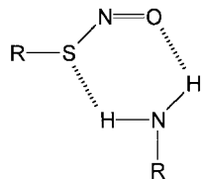

Fig. 6 Possible structure of a complex between RSNO and a secondary amine or ammonia (R = H).

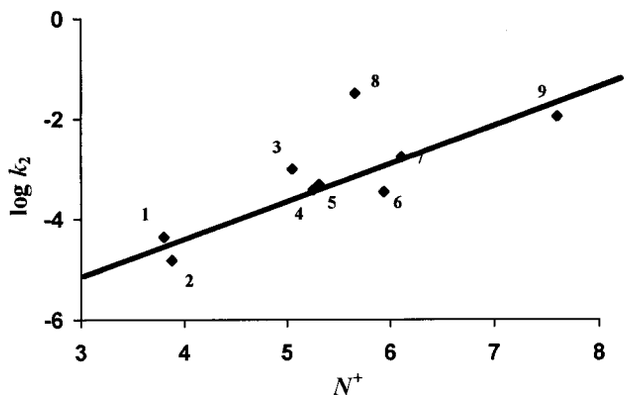

Fig. 7 Plot of log k_2 *vs.* the Ritchie N^+ parameter for the reactions of SPEN with a range of nucleophiles. (1) Semicarbazide, (2) methoxyamine, (3) hydroxylamine, (4) morpholine, (5) ethylamine, (6) piperazine, (7) piperidine, (8) hydrazine and (9) azide.

the presence of the sulfur atom results in an increased reactivity, suggesting that reaction occurs initially at sulfur, a more nucleophilic site. This contrasts with the results obtained for the reactions of MNTS,[16] where the kinetic results indicate that the nitrogen site is more nucleophilic than the sulfur atom. This suggests that MNTS and SPEN possess different discriminating properties. Details of the reaction of *S*-nitrosothiols with a range of sulfur nucleophiles will be published shortly,[23] when this point will be discussed further. Comparable 'additional reactivity' has been described for the nitrosation of *S*-methylcysteine,[24] thioproline[25] and thiomorpholine[26] by nitrous acid itself, and the results were also interpreted in terms of initial attack at the sulfur atom followed by internal rearrangement of the NO group to the amino nitrogen atom, but these results refer to reaction in an acid medium, where the amino group is substantially protonated, and so provides no direct comparison for the relative nucleophilicities of *N*- and *S*- sites.

Correlation of the kinetic results

Although there is a reasonable correlation between log k_2 and the pK_a values for the family of secondary amines, there is no discernible link when all of the nitrogen nucleophiles are included. Similarly there is no correlation with the Pearson nucleophilicity parameter n[27] for the nucleophiles in this work for which values are available (for example both hydrazine and azide ion are substantially more reactive than are pyrrolidine and piperidine, even though their n values would predict otherwise). There is however a reasonable correlation between log k_2 and the Ritchie N^+ parameter[28] as shown in Fig. 7. The slope of the drawn line is 0.8, a little lower than the predicted value of 1.0 and the one point for hydrazine lies well above the line.

These results parallel almost exactly those reported in an extensive study of nucleophilic reactivity for attack at the nitrogen atom of the nitroso group in the nitrososulfonamide MNTS.[16,20,21] Here also there was a correlation with N^+ but not with n. This was interpreted using the ideas of Hoz[29] and Shaik[30] in terms of a Klopman frontier-controlled reaction[31] in which the transition state has substantial diradical nature. From the general parallel between the MNTS (and an alkyl nitrite) and SPEN reactions it appears that direct nitrosation

reactions effected by *S*-nitrosothiols can be characterised in the same way.

Experimental

SPEN was generated in mildly acid solution by the reaction of equimolar quantities (around 1×10^{-2} mol dm^{-3}) of sodium nitrite and penicillamine. This stock solution was stable for many hours and a fresh batch was made up daily. Reaction solutions were prepared with [SPEN] typically 1×10^{-3} mol dm^{-3} and the nucleophile typically in the range 0.002–0.200 mol dm^{-3}. The pH was adjusted by the addition of perchloric acid or sodium hydroxide solution, and the amine/amine salt acted as a buffer. All other materials used were commercial samples of the highest purity available.

Kinetic measurements were carried out in aqueous solution containing EDTA (1×10^{-4} mol dm^{-3}) at 25 °C at 340 nm in a conventional spectrophotometer following the disappearance of the absorbance due to the nitrosothiol. Good first order behaviour was found throughout and rate constants were obtained using the Enzfitter program and were generally reproducible to ±3%. Nitrite analysis was done by the conventional diazotisation–azo dye procedure, following pH adjustment to 7.

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