PERKIN 2

Formation of peroxynitrite at high pH and the generation of *S*-nitrosothiols from thiols and peroxynitrous acid† in acid solution

Paul J. Coupe and D. Lyn H. Williams*

Department of Chemistry, Durham University, South Road, Durham, UK DH1 3LE.
E-mail: D.L.H.Williams@durham.ac.uk

Received (in Cambridge, UK) 5th April 2001, Accepted 10th May 2001
First published as an Advance Article on the web 5th June 2001

The formation of peroxynitrite from hydrogen peroxide and *S*-nitrosothiols at high pH is shown to involve reaction of the hydroperoxide anion. Kinetic measurements over a pH range 10–13 yield a value close to the literature value for the pK_a of hydrogen peroxide and give a quantitative measure of the reactivity of the hydroperoxide ion. *S*-Nitrosothiols are formed in high yield when peroxynitrous acid reacts with thiols when the thiol is in very large excess, within the pH range 2.7–3.7. Kinetic measurements have shown that reaction occurs by rapid oxidation of the thiol to the disulfide, with release of nitrite ion, which at these pH values is sufficiently protonated to effect nitrosation of the thiol, which is in large excess. Quantitative yields of RSNO can be obtained when there is a very large excess [thiol], when the spontaneous decomposition of peroxynitrous acid cannot compete. The individual kinetic results are fully first order and analysis of the results yields values for the third-order rate constants k (in rate = k [RSH][HNO_2][H^+]) which are in acceptable agreement with the values in the literature for the direct nitrosation of thiols with nitrous acid. Results were obtained for three thiols [glutathione (GSH), cysteine (Cys) and cysteine ethyl ester], confirming the generality of the reaction. At higher pH values the yield of RSNO drops off sharply as nitrous acid becomes deprotonated. A separate experiment with stoichiometric concentration ratios of thiol and peroxynitrous acid in acid solution gave high yields of nitrous acid. At higher acidities (>0.3 mol dm^{-3}) *S*-nitrosothiols are still formed, but now there are clearly two sequential first-order processes which can be fitted to a two exponential model. One reaction is believed to be nitrous acid nitrosation, following oxidation of the thiol as before, whilst the other reaction, which is also acid catalysed, is interpreted as nitrosation *via* a protonated form of peroxynitrous acid as recently reported in the literature, which can compete with the isomerisation of peroxynitrous acid to nitrate anion, when the thiol is at high concentration.

It is widely believed[1] that peroxynitrite anion ($ONOO^-$) is generated *in vivo* from nitric oxide and the superoxide anion in an encounter-controlled process. It is known to be a powerful and toxic oxidant, reacting with a number of biological targets. There has therefore been much interest in establishing the chemistry of peroxynitrite anion and its conjugate acid peroxynitrous acid (ONOOH), both *in vitro* and *in vivo.* A large amount of published work in this area has been generated in recent years, mainly in the biological domain.[2,3] There is an excellent review of the known chemistry of peroxynitrites (sometimes called peroxonitrites) up to 1994 by Edwards and Plumb.[4] It is known that in acid solution isomerisation takes place to nitrate ion [eqn. (1)] *via* the conjugate acid form,[5] (pK_a

$$\mathrm{ONOO^- + H^+ \rightleftharpoons ONOOH \longrightarrow NO_3^- + H^+} \quad (1)$$

6.5) with a rate constant of ~1 s^{-1} which is constant over the pH range 1–6. The observed first-order rate constant (k_o) is given by eqn. (2), where K_a is the acid dissociation constant of

$$k_o = 1.0[H^+]/(K_a + [H^+]) \quad (2)$$

peroxynitrous acid. The anion is much more stable, but does breakdown slowly to give nitrite ion and oxygen[4] in a reaction which is also Cu^{2+} catalysed; the mechanism of this reaction has not been fully established.

In the laboratory, peroxynitrite is conveniently prepared by rapid nitrosation of hydrogen peroxide with nitrous acid under mildly acid conditions, followed by immediate quenching in base.[6] An alternative procedure is to use an alkyl nitrite as the nitrosating agent, so that the reaction can be carried out under basic conditions,[7] thus avoiding the need to raise the pH rapidly at the end of the reaction and consequently generate peroxynitrite which is free from nitrate impurity. This difficulty can also be avoided by using nitric oxide in oxygenated solutions, again in non-acidic solution, when the reagent is probably N_2O_3.[8] We have also shown, in a mechanistic study,[9] that nitrosation of hydrogen peroxide by *S*-nitrosothiols (RSNO) will generate peroxynitrite in high yield fairly rapidly at high pH.

Apart from the question of the cytotoxicity of peroxynitrite, there has been much biological interest in the question as to whether peroxynitrite can act as an electrophilic nitrosating agent particularly towards thiols, since this would provide a route for RSNO synthesis *in vivo* from NO. It is now widely believed that NO is stored and circulates in the body as RSNO rather than as free NO. We have argued[10] that both peroxynitrite and peroxynitrous acid are unlikely to provide vehicles for the direct transfer of NO^+, since O_2^{2-} and HO_2^- (respectively) are very poor leaving groups. Nevertheless, there are reports in the biological literature which describe the finding of very low yields (typically 1–2%) of RSNO from thiols and peroxynitrite at physiological pH,[11,12] and claims made that, even with these very low conversions, peroxynitrite can in some way effect nitrosation. It is, however, conceivable that these very low yields could arise as by-products, rather than from a direct nitrosation. For example, it is likely that nitrite ion is formed as a result of thiol oxidation by peroxynitrite and also as a product of the spontaneous decomposition of $ONOO^-$. This nitrite ion could, in principle (a) generate low concentrations of NO/NO_2, which could then effect thiol nitrosation, or, more likely,

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† The IUPAC name for peroxynitrous acid is hydrogen oxoperoxonitrite.

DOI: 10.1039/b103100g

Table 1 First-order rate constants k_o for the reaction of *S*-nitrosocysteine (5×10^{-3} mol dm^{-3}) with hydrogen peroxide (0.1 mol dm^{-3}) as a function of pH, measuring the disappearance of the absorbance at 545 nm

pH	10^3k_o/s^{-1}
9.8	1.68
10.8	2.55
11.2	3.61
11.4	5.64
11.5	5.81
11.7	7.11
11.9	8.60
12.0	9.49
12.2	11.7
12.5	14.5
12.9	15.9
13.5	17.5

(b) generate sufficient nitrous acid (even at pH 7) also to bring about thiol nitrosation to a very small extent. Very recently a paper has appeared[13] which shows that substantial yields of *S*-nitrosothiols can be obtained from peroxynitrous acid and thiols at quite high acidity, typically 1 mol dm^{-3}. We set out in this work to examine the reactions of peroxynitrite with thiols over a wide pH range so that a clear mechanistic picture might be obtained.

Results and discussion

Formation of $ONOO^-$ from RSNO and H_2O_2

We have recently shown[9] that *S*-nitrosocysteine (CysNO) and other RSNO species react with hydrogen peroxide to give peroxynitrite. At pH 13.1 it is possible to observe the decreasing absorbance of RSNO at 340 nm and the simultaneous build up of $ONOO^-$, which is stable under these conditions, at 302 nm. However at pH 7.4 we observe only the decay at 340 nm, since decomposition of $ONOO^-$ occurs readily at this pH (*via* the protonated form). At intermediate pH values it is possible to see, spectrophotometrically, the disappearance of RSNO, the formation and later the decomposition of $ONOO^-$. The rate of decay of RSNO increases qualitatively with pH, suggesting that the reactive species is the hydroperoxide anion. To establish this more quantitatively we have determined the rate constant as a function of pH by measurement of the disappearance of the smaller absorbance due to RSNO at 545 nm, because of problems resulting from the overlapping absorbances in the 300–340 nm region. Rate constants were obtained over the pH range 9.8–13.5 for the reaction of CysNO (5×10^{-3} mol dm^{-3}) with H_2O_2 (0.1 mol dm^{-3}). Good first-order behaviour was found throughout and the pseudo first-order rate constant k_o obtained from the computer package. The results are shown in Table 1. If we assume that reaction occurs exclusively *via* the hydroperoxide anion then the expression for k_o is that given in eqn. (3). Here, $[S]_T$ is the total stoichiometric concentration of H_2O_2, k_2 the bimolecular rate constant for reaction between CysNO and HOO^- and K_a the acid dissociation constant of H_2O_2. The data were analysed in two ways (i) by a curve fitting procedure using the Scientist computer package[14] and (ii) by the reciprocal plot of $1/k_o$ against $[H^+]$. The two plots are shown as Figs. 1 and 2, respectively. Values of k_2 and the pK_a of H_2O_2 were obtained from the two approaches as 0.17 and 0.15 dm^3 mol^{-1} s^{-1} and 11.7 and 11.6, respectively. The validity of eqn. (3) is clearly demonstrated by these results. The quantitative agreement between the two procedures is excellent, and the pK_a values are in line with the literature value[15] of 11.6. There is some evidence that below pH ~11 some other factor comes in to play. This is further demonstrated by direct measurement of the disappearance of a number of other RSNO species (including GSNO and *S*-nitrosopenicillamine) at pH 7.4, where the measured rate constant is about a factor of 10 larger than that predicted by eqn. (3). In this region reaction is very slow and it is likely that the spontaneous thermal decomposition of RSNO and photochemical decomposition (resulting from long-term exposure to radiation at 340 nm) are playing significant roles. The Cu^{2+}-catalysed decomposition of RSNO[16] is eliminated here by the addition of EDTA.

$$k_o = k_2[S]_TK_a/(K_a + [H^+]) \qquad (3)$$

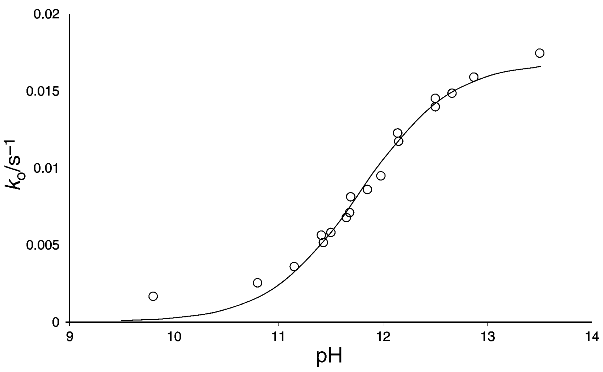


Fig. 1 Plot of k_o *vs.* pH for the reaction of CysNO (5×10^{-3} mol dm^{-3}) with hydrogen peroxide (0.1 mol dm^{-3}). The solid line is that calculated from eqn. (3) with $k_2 = 0.17$ dm^3 mol^{-1} s^{-1} and $K_a = 2.04 \times 10^{-12}$.

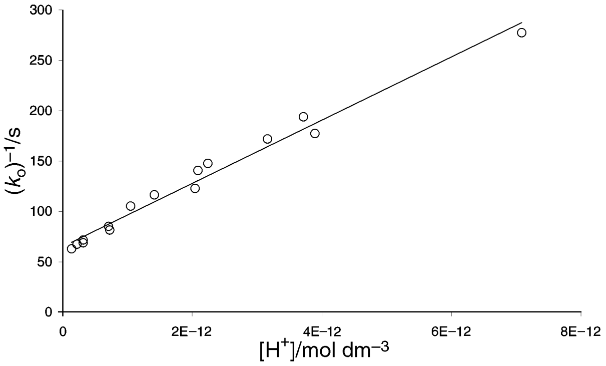


Fig. 2 Plot of $1/k_o$ *vs.* $[H^+]$ for the data in Table 1.

The average value of k_2 (0.16 dm^3 mol^{-1} s^{-1}) gives the reactivity of the hydroperoxide ion towards CysNO. Comparison can be made with other nucleophiles: amines are about 10^3 times less reactive[17] as is perhaps expected for neutral species, whereas among the sulfur nucleophiles[18] sulfite, thiolate and thiosulfate anions are significantly more reactive, whereas thiocyanate ion and thiourea are less so. Here reactivity does not follow the Swain–Scott nucleophilicity parameter *n* and it has been argued[18] that the Ritchie N^+ scale is more appropriate, although we do not have enough data to test this quantitatively.

Our measured yields of peroxynitrite at high pH are a little less than quantitative—typically 80% when hydrogen peroxide is in large excess over the *S*-nitrosothiol, whereas yields from alkyl nitrites and hydrogen peroxide[7] are ~100%. This is almost certainly due to loss of some peroxynitrite in effecting oxidation of the thiol product, which does not occur in the alkyl nitrite reaction, when the alcohol product is stable towards oxidation. This is confirmed by the results of an experiment shown in Fig. 3. Here we are monitoring the slow decomposition (by the decreasing absorbance at 302 nm) of peroxynitrite generated from CysNO and a ten-fold excess of hydrogen peroxide at pH 13. There is a slow decay over ~8000 s after which Cys is added, resulting in a sharp decrease of the absorbance, not attributable to a dilution. Oxidation is believed to occur by reaction of the thiol rather than the thiolate form. Our results show that any

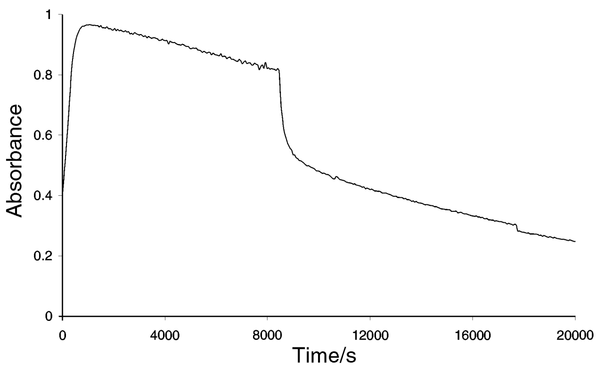


Fig. 3 The absorbance at 302 nm due to peroxynitrite generated from CysNO and a ten-fold excess of H_2O_2 at pH 13, as a function of time. Cys is added (in 0.1 cm^3 water) after ~8000 s and water (0.1 cm^3) added after ~18 000 s.

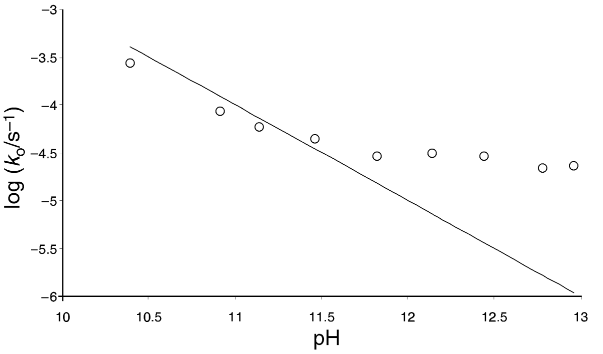


Fig. 4 The spontaneous decay of peroxynitrite as a function of pH. The line is that predicted by eqn. (2) and the points are the experimental ones.

thiolate formed in the peroxynitrite synthesis has already been oxidised by the excess hydrogen peroxide, but any added thiolate will result in reaction with peroxynitrite in competition with hydrogen peroxide oxidation. Peroxynitrite is known to be a more powerful oxidant (from rate constant measurements) than hydrogen peroxide (by a factor of $\sim 10^3$[19]).

The spontaneous decay of peroxynitrite in the alkaline region is a first-order process and depends on pH as shown in Fig. 4. The solid line is that predicted by eqn. (2), based only on the isomerisation to nitrate. The experimental points for the reaction of peroxynitrite generated from CysNO and hydrogen peroxide follow this line reasonably at pH values up to ~11.5, but above this, deviate in a way consistent with the incursion of another mechanism. Similar results have been noted by a number of authors, and these earlier results have been brought together in ref. 4. This reaction, much slower than isomerisation to nitrate in the acid region, is believed to be that leading to nitrite anion and oxygen [eqn. (4)]. The detailed reaction

$$2\,ONOO^- \longrightarrow 2\,NO_2^- + O_2 \qquad (4)$$

mechanism is still only speculative, but there are reports that the reaction is catalysed by Cu^{2+}.

Reaction of peroxynitrous acid with thiols in acid solution

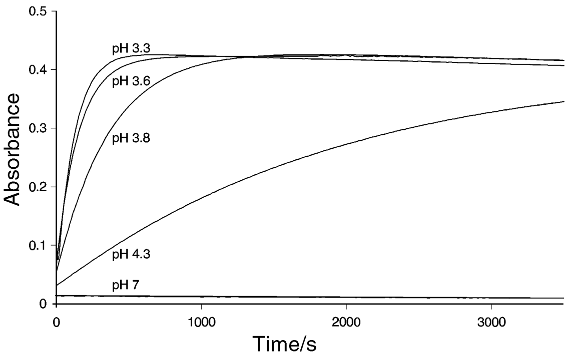


Fig. 5 Absorbance–time plots (at 340 nm) for the formation of GSNO from GSH (0.025 mol dm^{-3}) and peroxynitrite (0.5 × 10^{-3} mol dm^{-3}) at different pH values.

In the pH range 2–4, we find, from spectrophotometric measurements, that peroxynitrous acid reacts with thiols present in large excess to generate the corresponding *S*-nitrosothiol. This was detected by the characteristic UV-Visible absorbances at 350 and 545 nm (for *S*-nitrosoglutathione GSNO), and also, after pH adjustment and addition of Cu^{2+}, observation and identification of the absorbance at 620 nm due to the known Cu^{2+} complex of the corresponding disulfide (GSSG) [eqns. (5) and (6)]. A possible explanation for RSNO formation, given

$$2RSNO \longrightarrow RSSR + 2NO\ (Cu^{2+}\ \text{catalysed}) \qquad (5)$$

$$RSSR + Cu^{2+} \longrightarrow RSSR : Cu^{2+} \qquad (6)$$

that direct nitrosation by peroxynitrous acid is unlikely, is that oxidation of the thiol occurs (probably rapidly), almost certainly releasing nitrite anion [eqn. (7)], although this has not been established, as far as we are aware. However many other oxidation reactions by peroxynitrite, including that of hydroxylamine and iodide ion, do generate nitrite ion. Reaction (7) has

$$2RSH + ONOOH \longrightarrow RSSR + HNO_2 + H_2O \qquad (7)$$

been studied in the alkaline region[19] and been shown to involve the thiol (and not the thiolate anion, which is the effective reagent in the corresponding hydrogen peroxide oxidation). If thiol oxidation occurs in mildly acid solution releasing nitrite, then it is reasonable to expect that nitrite ion will be sufficiently protonated (pK_a 3.1), to generate enough nitrous acid to effect conventional electrophilic nitrosation of the thiol [eqn. (8)], always present in large excess.

$$RSH + HNO_2 \longrightarrow RSNO + H_2O \qquad (8)$$

We have followed the rate of formation of GSNO from peroxynitrous acid and a large excess of GSH in the pH range 3–5, and find good first-order behaviour in individual experiments, and also a first-order dependence on the thiol. Some of the results are shown as absorbance–time plots in Fig. 5. There was close to 100% conversion to RSNO. Reactions were also acid catalysed, establishing the rate eqn. (9). Values of k_3 were

$$\text{Rate} = k_3[RSH][ONOOH][H^+] \qquad (9)$$

obtained for three thiols from the variation of the observed first-order rate constant with acidity. For GSH, Cys and cysteine ethyl ester we found values of k_3 of 1077, 529, and 215 dm^6 mol^{-2} s^{-1}, respectively. These values bear a very close resemblance to those obtained for the third-order rate constants for the direct nitrosation of these thiols by nitrous acid in acid solution [eqn. (10)], *i.e.* 1080,[20] 456[21] (514[22]) and 213[20] dm^6

$$\text{Rate} = k_3[RSH][HNO_2][H^+] \qquad (10)$$

mol^{-2} s^{-1}, respectively. This shows clearly that formation of RSNO species from RSH and peroxynitrous acid in this acidity range occurs by rapid oxidation of two equivalents of thiol (present in large excess), generating one equivalent of nitrite/nitrous acid, which then nitrosates the thiol in a conventional electrophilic *S*-nitrosation. To test this further we reacted GSH with peroxynitrous acid in a 2 : 1 molar ratio (so that there will be no excess thiol) in acid solution. There was a very rapid formation of nitrous acid, detected by its characteristic UV

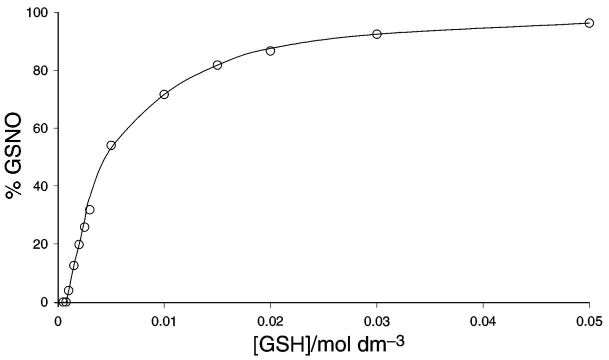


Fig. 6 The yield of GSNO as a function of [GSH] for the reaction of peroxynitrous acid (0.5×10^{-3} mol dm^{-3}) at [H$^+$] 0.5 mol dm^{-3}.

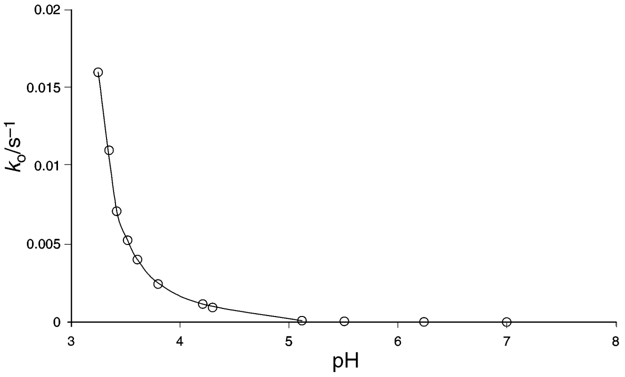


Fig. 7 Decreasing values of k_o with increasing pH for the reaction of GSH (0.025 mol dm^{-3}) with peroxynitrous acid (0.5×10^{-3} mol dm^{-3}).

spectrum. Spectra were obtained after 1.3×10^{-3}, 9×10^{-3} and 0.02 s, which showed that reaction was complete in an extremely short time. Quantitative analysis using the known extinction coefficient at 370 nm revealed that nitrous acid is formed in >90% yield. As far as we are aware, this is the first time that thiol oxidation by peroxynitrous acid has been demonstrated in acid solution, and it is clear that this is a very rapid process.

To achieve close to quantitative formation of RSNO it is necessary to maintain the thiol concentration in a high molar excess (~50 fold) over the peroxynitrous acid. With a smaller excess the yield drops off, no doubt due to the increasing competition from the spontaneous decomposition of peroxynitrous acid to nitrate anion. This is shown graphically in Fig. 6 as the % GSNO obtained from peroxynitrous acid (5×10^{-4} mol dm^{-3}) in 0.5 mol dm^{-3} $HClO_4$ as a function of the [GSH]. Similar plots were found at lower acid concentration.

As the pH is increased the yield of RSNO also decreases, as does its rate of formation (see Fig. 7). This is to be expected on our mechanistic interpretation, since nitrous acid becomes progressively more deprotonated as pH is increased and so the rate of *S*-nitrosation is reduced and this pathway now competes much less efficiently with the spontaneous decomposition of peroxynitrous acid. We measured, spectrophotometrically, yields of GSNO which were >90% in the pH range 3–5, when GSH was present in large excess. The yield of GSNO dropped progressively as the pH increased to ~60% at pH 4, 25% at pH 5 and to ~5% at pH 6. This mechanistic interpretation easily accounts for the few % RSNO detected at pH 7 in earlier reports.[11,12]

We have also examined the reaction of peroxynitrous acid with thiols at a much higher acidity (>0.3 mol dm^{-3}). Here we find that *S*-nitrosothiols are still generated when again the thiol is present in a large excess, in a very rapid reaction. In this region, however, the kinetic traces are more complicated, in that they are not simple first-order plots, but appear to show two reactions. We have used the Scientist package to analyse the raw data successfully in terms of two consecutive exponential absorbance increases at 340 nm, *i.e.* in terms of two consecutive first-order processes, from which both first-order rate constants were obtained. Again to obtain high yields of RSNO it is necessary as before to maintain a large excess of thiol over peroxynitrous acid. Some absorbance–time curves (which can all be analysed in terms of a double exponential fit) are shown in Fig. 8 for the reactions with GSH. The Scientist software allows the determination of the first-order rate constants for both processes. Varying the overall acid concentration shows that both reactions are acid catalysed, and so the yield of RSNO decreases with decreasing acidity because of the competition with the spontaneous decomposition of peroxynitrite, as before. Both reactions are also bromide ion catalysed, when presumably nitrosyl bromide is generated in both pathways. We identify one of the reactions as the nitrous acid nitrosation following thiol oxidation, which we described earlier. The other we suggest is an electrophilic nitrosation of the thiol from a *protonated* form of peroxynitrous acid. Such a species has not been characterised, but it has been suggested recently as a possible reagent for this reaction, based on some product studies in acetonitrile solvent.[13] A protonated form could in principle be generated at high acidities, and could reasonably be expected to act as a conventional electrophilic nitrosating agent, since there is now a suitable leaving group in the form of the hydrogen peroxide molecule [eqn. (11)]. Grossi *et al.*[13] suggested that this

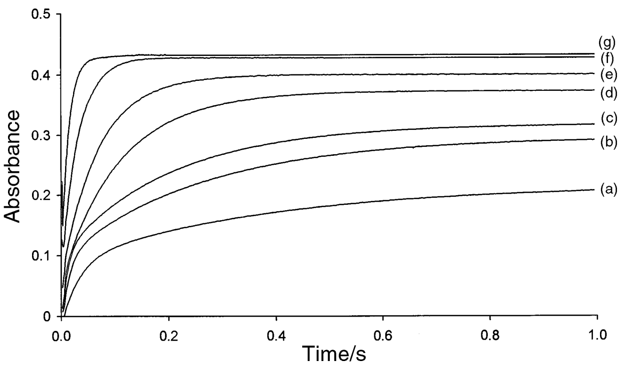


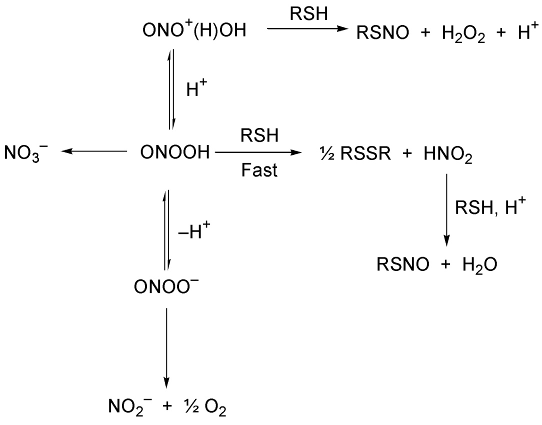
Fig. 8 Absorbance–time plots at 340 nm for the formation of GSNO at high acidity from GSH (0.025 mol dm^{-3}) and peroxynitrous acid (0.5×10^{-3} mol dm^{-3}) as a function of [H$^+$], (a) 0.3, (b) 0.5, (c) 0.6, (d) 0.7, (e) 0.8, (f) 0.9 and (g) 1.0 mol dm^{-3}.

$$\mathrm{ONOOH + H^+ \rightleftharpoons ONO^+(H)OH + RSH \longrightarrow RSNO + H_2O_2 + H^+} \quad (11)$$

protonated species might generate an unspecified nitrosating agent X-NO, but in the absence of any evidence in its favour we prefer to retain protonated peroxynitrous acid as the probable reagent. We have written the protonated form with the proton on the oxygen atom attached to nitrogen, as this allows the hydrogen peroxide molecule to depart as the leaving group (as do Grossi *et al.*) but we have no evidence for this. There is the close analogy, of course, with the proposal that the protonated form of nitrous acid $H_2NO_2^+$ is the direct nitrosating species derived from acidified solutions of nitrous acid,[23] where the leaving group is the water molecule.

Conclusions

We have shown that the hydroperoxide anion is the effective nucleophile in the generation of peroxynitrite anion from *S*-nitrosothiols and hydrogen peroxide and have reported its reactivity compared with other nucleophiles. In slightly acidic solution peroxynitrous acid readily oxidises thiols to the disulfide and nitrous acid, which will readily nitrosate any excess thiol present thus generating *S*-nitrosothiols. The reaction can be made quantitative if the thiol excess is large enough for this pathway to be much faster than the unimolecular decomposition of peroxynitrous acid to nitrate anion. At higher acidities (>0.3 mol dm^{-3}) there is kinetic evidence that there is another pathway to *S*-nitrosothiol formation, which we suggest involves the *protonated* form of peroxynitrous acid. We can accommodate these findings in the reaction summary shown in Scheme 1.



Scheme 1

Experimental

Peroxynitrite was prepared by the conventional procedure involving nitrosation of hydrogen peroxide with a deficiency of nitrous acid, so that no excess nitrite develops in the solution on making the solution alkaline. *S*-Nitrosothiols were generated in solution by reaction of the corresponding thiol with an equimolar amount of nitrous acid under mildly acidic conditions. Solutions were prepared before each set of experiments and aliquots were introduced to the appropriate buffer when required. Spectra were recorded on conventional spectrophotometers using the data acquisition software provided. The slower reactions (where the half-lives were greater than a few seconds) were followed kinetically, usually at one wavelength, using the software to establish the kinetic order and to obtain appropriate rate constant values. The more rapid reactions were followed in a Applied Photophysics SX17MV stopped-flow spectrophotometer. The diode array system associated with the stopped-flow spectrophotometer was used in the experiment recording the formation of nitrous acid from a 2 : 1 molar ratio of thiol and peroxynitrous acid. All reactions were carried out in aqueous solution at 25 °C. Decay and formation of *S*-nitrosothiols were both followed either at 545 or 340 nm and some experiments measured the decay of peroxynitrite at 302 nm. All kinetic experiments (except those at high acidity) showed good first-order behaviour and values of the first-order rate constants were reproducible to better than ±3%.

Acknowledgement

We thank the EPSRC for an Equipment Grant and for a Research Studentship to P. J. C.

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