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Dynamics of interaction of vitamin C with some potent nitrovasodilators, *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) and *S*-nitrosocaptopril (SNOCap), in aqueous solution

Danielle V. Aquart, Tara P. Dasgupta*

Department of Chemistry, University of the West Indies, Mona Campus, Kingston 7, Jamaica

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Abstract

The reductive decomposition of both SNAP and SNOCap by ascorbate in aqueous solution (in the presence of EDTA) was thoroughly investigated. Nitric oxide (NO) release from the reaction occurs in an ascorbate concentration and pH dependent manner. Rates and hence NO release increased drastically with increasing pH, signifying that the most highly ionized form of ascorbate is the more reactive species. The experiments were monitored spectrophotometrically, and second-order rate constants calculated at 37 °C for the reduction of SNAP are $k_b = 9.81 \pm 1.39 \times 10^{-3}$ M^{-1} s^{-1} and $k_c = 662 \pm 38$ M^{-1} s^{-1} and for SNOCap are $k_b = 2.57 \pm 1.29 \times 10^{-2}$ M^{-1} s^{-1} and $k_c = 49.7 \pm 1.3$ M^{-1} s^{-1}. k_b and k_c are the second-order rate constants via the ascorbate monoanion (HA^-) and dianion (A^{2-}) pathways, respectively. Activation parameters were also calculated and are $\Delta H_b^{\ddagger} = 93 \pm 7$ kJ mol^{-1}, $\Delta S_b^{\ddagger} = 15 \pm 2$ J K^{-1} mol^{-1} and $\Delta H_c^{\ddagger} = 51 \pm 5$ kJ mol^{-1}, $\Delta S_c^{\ddagger} = -28 \pm 3$ J K^{-1} mol^{-1} with respect to the reactions involving SNAP. Those for the reaction between SNOCap and ascorbate were calculated to be $\Delta H_b^{\ddagger} = 63 \pm 11$ kJ mol^{-1}, $\Delta S_b^{\ddagger} = -71 \pm 20$ J K^{-1} mol^{-1} and $\Delta H_c^{\ddagger} = 103 \pm 7$ kJ mol^{-1}, $\Delta S_c^{\ddagger} = 118 \pm 8$ J K^{-1} mol^{-1}. The effect of Cu^{2+}/Cu^{+} ions on the reductive decompositions of these *S*-nitrosothiols was also investigated in absence of EDTA. SNOCap exhibits relatively high stability at near physiological conditions (37 °C and pH 7.55) even in the presence of micromolar concentrations of Cu^{2+}, with decomposition rate constant being 0.011 M^{-1} s^{-1} in comparison to SNAP which is known to be more susceptible to catalytic decomposition by Cu^{2+} (second-order rate constant of 20 M^{-1} s^{-1} at pH 7.4 and 25 °C). It was also observed that the reductive decomposition of SNAP is not catalyzed by alkali metal ions, however, there was an increase in rate as the ionic strength increases from 0.2 to 0.5 mol dm^{-3} NaCl.

Keywords: Nitric oxide (NO); *S*-nitrosothiols (RSNO); *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP); *S*-nitrosocaptopril (SNOCap); L-ascorbic acid (H_2A)

1. Introduction

S-nitrosothiols (RSNO) continue to be the subject of vigorous research during the last decade due to their abilities to release the powerful biological messenger, nitric oxide (NO), in a controlled manner in the living system either spontaneously or by the interaction with various biological reductants [1–10]. Specifically, *S*-nitro-

*Corresponding author. Tel.: +876-927-1910; fax: +876-977-1835.

E-mail address: tara@uwimona.edu.jm (T.P. Dasgupta).

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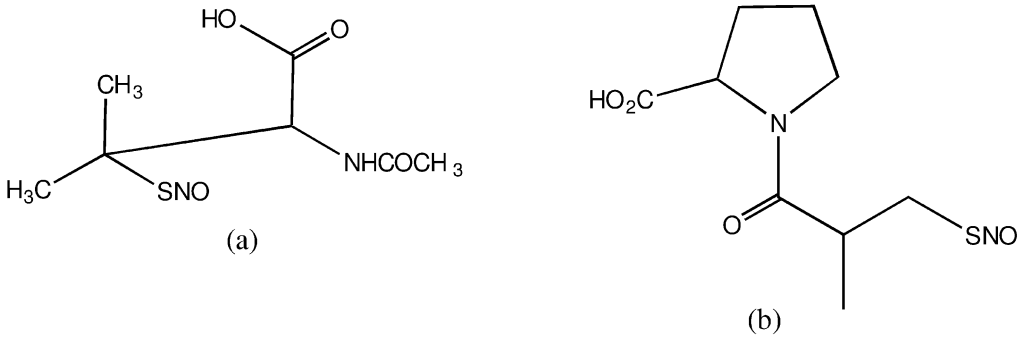


Fig. 1. Chemical Structures of (a) SNAP, (b) SNOCap.

so-*N*-acetyl-D,L-penicillamine (SNAP) and *S*-nitrosocaptopril (SNOCap) are known to be good •NO donors and have shown excellent biological diversity at appropriate concentrations [11–18].

SNAP and SNOCap (Fig. 1) have been isolated to give a deep green solid (with red reflections), and feather-like red crystals, respectively. Tertiary *S*-nitrosothiols, such as SNAP, are usually green compounds, whereas primary (e.g. SNOCap) and secondary *S*-nitrosothiols are red. Their crystal structures have already been determined [19,20]. Both are very stable, but what is even more interesting is that of the two, SNOCap by itself shows greater stability in aqueous buffered medium than SNAP whose half-life ($t_{1/2}$) in aqueous buffered medium (pH 7.4) and in the presence of EDTA is approximately 16 h [21]. However, SNOCap has been documented to possess a half-life of 2.8 h in the presence of physiological buffer and plasma (specifically physiologic salt solution or platelet-poor plasma) [22] and at 37 °C. We assume the plasma must have some effect on the mode of decomposition, owing to the fact that various reactions can occur in biological systems to facilitate the breakdown of *S*-nitrosothiols [23]. It has been documented that trace amounts of metal ions can effect decomposition of SNAP [24,25] and hence, much shorter half lives (5 h, 10 h, etc.) quoted [21,26] for spontaneous decomposition of SNAP in absence of EDTA are erroneous. The effect of Cu^{2+} on the reactions of SNAP (generated in situ) with ascorbic acid has been studied under limited conditions and dual mechanistic pathways have been proposed [27].

SNOCap, the *S*-nitrosylated derivative of captopril has been shown to inhibit vascular reactivity through activation of soluble guanylate cyclase and inhibition of angiotensin converting enzyme [22,28,29]. Also, SNOCap and not captopril (CapSH) has shown to be able to effect inhibition of neovascularization [30], establishing itself as an essential and effective NO donor. SNOCap has become a very popular RSNO owing to its dual role, i.e. it possesses the capacity of both an angiotensin converting enzyme inhibitor as well as an NO donor [31–33].

Thus, the modes of decomposition of RSNO to afford NO is of great importance and as such we ventured to investigate the manner in which this decomposition may occur in the presence of one of the more important biological reductants, L-ascorbic acid (vitamin C). The kinetics and mechanism of this redox process could account for the metabolism of RSNOs when taken in vivo as therapeutic drugs, since it is known that ascorbate exists in cellular systems at relatively high concentrations (millimolar) and is a very essential antioxidant [34]. It has also been reported [35] that the release of NO from GSNO and *S*-nitrosoalbumin in human blood plasma was enhanced in the presence of ascorbate. We now report here a detailed kinetic study on the reductive degradation of two important RSNOs—SNAP and SNOCap,

which have potential to become first choice therapeutic drugs.

2. Experimental procedures

2.1. Materials and solutions

SNAP was prepared according to published procedures [20]. Yield = 70%, (Lit. 68%). The purity of the compound was checked by UV–visible, infrared spectroscopy and melting point determination; λ_{max} (H_2O) = 340 and 590 nm; $\varepsilon_{340} = 1042$; $\varepsilon_{590} = 13$ dm^3 mol^{-1} cm^{-1}; (Lit. [36] $\varepsilon_{340} = 815$ dm^3 mol^{-1} cm^{-1}; $\varepsilon_{590} = 12$ dm^3 mol^{-1} cm^{-1}); Exp. $\nu_{NO} = 1480$ cm^{-1}, Lit. [26] $\nu_{NO} = 1483$ cm^{-1}; Exp. mp = 152–154 °C, (Lit. [20] mp = 152–154 °C).

SNOCap was prepared in situ by nitrosation of captopril with $NaNO_2$ in 2 M HCl (1:1 thiol to NO_2^- mixture). Complete nitrosation was ensured by checking the maximum absorbance (ε determination) at the end of the nitrosating reaction. λ_{max} (H_2O) = 332 and 547 nm; $\varepsilon_{332} = 1003$ dm^3 mol^{-1} cm^{-1}and $\varepsilon_{547} = 14$ dm^3 mol^{-1} cm^{-1}, respectively; (Lit. [37] $\varepsilon_{332} = 940$ dm^3 mol^{-1} cm^{-1}; $\varepsilon_{546} = 16$ dm^3 mol^{-1} cm^{-1}). All other chemicals used were of analytical grade, obtained in at least 99% purity from Aldrich or Sigma Chemical companies. Fresh solutions of L-ascorbic acid (sodium salt) were prepared prior to each spectrophotometric run, and immediately used owing to the ease of aerial oxidation. Deionized water from Labconco water processor was used in all experiments along with Na_2EDTA so as to eliminate catalysis by trace quantities of metal ions, namely copper.

The required pH within the range pH 5–8 was controlled using the phosphate buffer system (Na_2HPO_4/NaH_2PO_4). pHs above 8 were maintained using borate buffer. Neither of the two buffer systems showed any influence on the reaction. NaOH was used to adjust the pH of the SNOCap stock solutions to near neutral before usage. An Orion expandable ion analyzer EA 920 meter fitted with Cole Parmer electrodes was used to measure the actual pH.

2.2. Stoichiometric determination

Different molar ratios of ascorbic acid and *S*-nitrosothiol (i.e. SNAP and SNOCap) were mixed together under an argon enriched atmosphere and left for several hours to facilitate complete reactions. The final absorbance values were then measured at 590 nm (wavelength of maximum absorption change for SNAP in the visible region of the spectrum) and 547 nm (wavelength of maximum absorption change for SNOCap). The absorbances obtained were plotted against the [total ascorbate]/[RSNO] ratio in order to determine the reaction stoichiometry.

2.3. Kinetic measurements

A Hewlett-Packard 8453 Diode Array equipped with a multicell transport system and interfaced with a ChemStation programme was utilized for gathering all spectrophotometric data. All experimental solutions for kinetic measurements were thermostatted at the required temperatures (± 0.1) using HAAKE D8 and GH refrigerated circulator. Data was analyzed using the ChemStation fit programmes. Reactions involving SNAP were monitored at 340 nm, while those for SNOCap were done at 547 nm, as there was a clear overlap of the peaks belonging to SNOCap and ascorbic acid in the 320–360 nm region. Due to the low extinction coefficient of all RSNOs in the visible region, we had to work with high concentrations (at least 0.3 mM) of SNOCap, to ensure good absorbance changes during the reaction. Pseudo-first-order conditions were employed for all kinetics experiments, i.e. at least 10-fold excess of ascorbic acid to *S*-nitrosothiol. Very good first-order behaviour was observed and pseudo-first-order rate constants (k_{obs}) reported are the average of at least three kinetic runs with $<5\%$ S.D. Ionic strength (I) was maintained at 0.5 mol dm^{-3} using NaCl as the supporting electrolyte.

Catalytic decomposition of SNOCap by Cu^{2+} in the presence of and absence of ascorbic acid was investigated, since similar studies on SNAP were reported previously [27]. The reactions were carried out in the absence of EDTA, at constant pH, temperature and ascorbate concentrations, and

incorporating micromolar concentration of Cu^{2+}. The effect of ionic strength variation on the SNAP-ascorbate reaction was studied by varying the concentration of NaCl (the electrolyte) within the range 0.2–0.5 mol dm^{-3}, and maintaining the concentration of ascorbic acid at 20 mM, at pH 7.2 and the temperature 25 °C. The chloride salts of alkali metals (Li, Na, K, Cs) were used for experiments dealing with metal ion catalysis at a constant ionic strength of 0.1 mol dm^{-3}. Both the ionic strength and metal ion variation experiments were done under aerobic conditions and in the presence of EDTA. Tris buffer was used for all kinetic experiments to maintain the required pH.

2.4. Products determination

Dehydroascorbic acid (DHA), the oxidized product of ascorbic acid was determined by standard procedures [38]. Dehydroascorbic acid couples with 2,4-dinitrophenylhydrazine to form the bishydrazone derivative, which on treatment with sulfuric acid produces a red colour, which is measured photometrically. Standard solutions containing 0.01–0.2 mM DHA were used for the calibration curve at 525 nm after adding the appropriate reagents under the given conditions.

Nitric oxide measurements were made using a calibrated World Precision ISO-NOP electrode fitted to a WPI ISO-NO Mark II meter. Calibration of the electrode was accomplished by the chemical generation of NO from the reaction with $NaNO_2$, excess H_2SO_4 and KI. In all cases of measurement, the reactants' solution was saturated with argon gas. A continuous flow of argon was maintained at approximately 250 cm^3 min^{-1} and vessel pressure approximately 1 atm.

Nitric oxide is expected to be converted to nitrite (NO_2^-) under aerobic conditions. The nitrite content is easily detected by the Griess reaction [39]. The colour intensity of the resulting red solution is directly proportional to nitrite concentration and is measured photometrically at 541 nm.

3. Results

3.1. Nature of the reaction

The colourless ascorbic acid solution reacts with the pale green SNAP solution and also with the pale rose–red SNOCap solution to give colourless solutions. The SNAP-ascorbate reaction system showed spectral changes similar to that of the reaction between *S*-nitrosoglutathione (GSNO) and ascorbate [40]. There was a general decrease in absorption between ca. 300 and 450 nm with the maximum changes occurring at 340 nm, indicating the possibility of a simple one-step reduction (see Fig. 2a). It should be noted that the residual absorption (approx. 0.1) at the end of the kinetic trace, in the inset, is due to the presence of excess ascorbate and the newly formed dehydroascorbic acid.

Good spectral changes in the visible region of the electronic spectrum were obtained upon mixing 3 mM of SNOCap with 60 mM L-ascorbic acid solution. Absorbance changes occurred between 450 and 650 nm, with the maximum changes occurring at 547 nm (Fig. 2b). Unlike SNAP, the absorbance changes at ~332 nm were poor, in fact the repetitive scan taken in the UV region (not shown) was very different than that previously obtained in the case of SNAP and GSNO, this was due to an overlap between SNOCap and ascorbate in this region of the electronic absorption spectrum.

3.2. Stoichiometric determination

Spectrophotometric titrations at 547 and 590 nm were used in determining the reaction's stoichiometry for SNOCap and SNAP, respectively, so as to avoid interference from the increasing absorbances in the UV region which is probably due to the decomposition of the expected product dehydroascorbic acid. The results for these systems indicate a 1:1 molar ratio of ascorbic acid to RSNO, as shown in Fig. 3 for the SNAP-ascorbate system. Due to the large concentrations used, distinct colour changes of the reaction mixtures were evident. The green coloured SNAP solution and the red SNOCap solution went to colourless (owing to the decomposition of both RSNOs) as the reaction progressed. The overall redox stoichiometry is shown in the following equation (Eq. (1)).

$$2RSNO + 2HA^- \rightarrow 2RS^- + 2NO^{\bullet} + A + H_2A \quad (1)$$

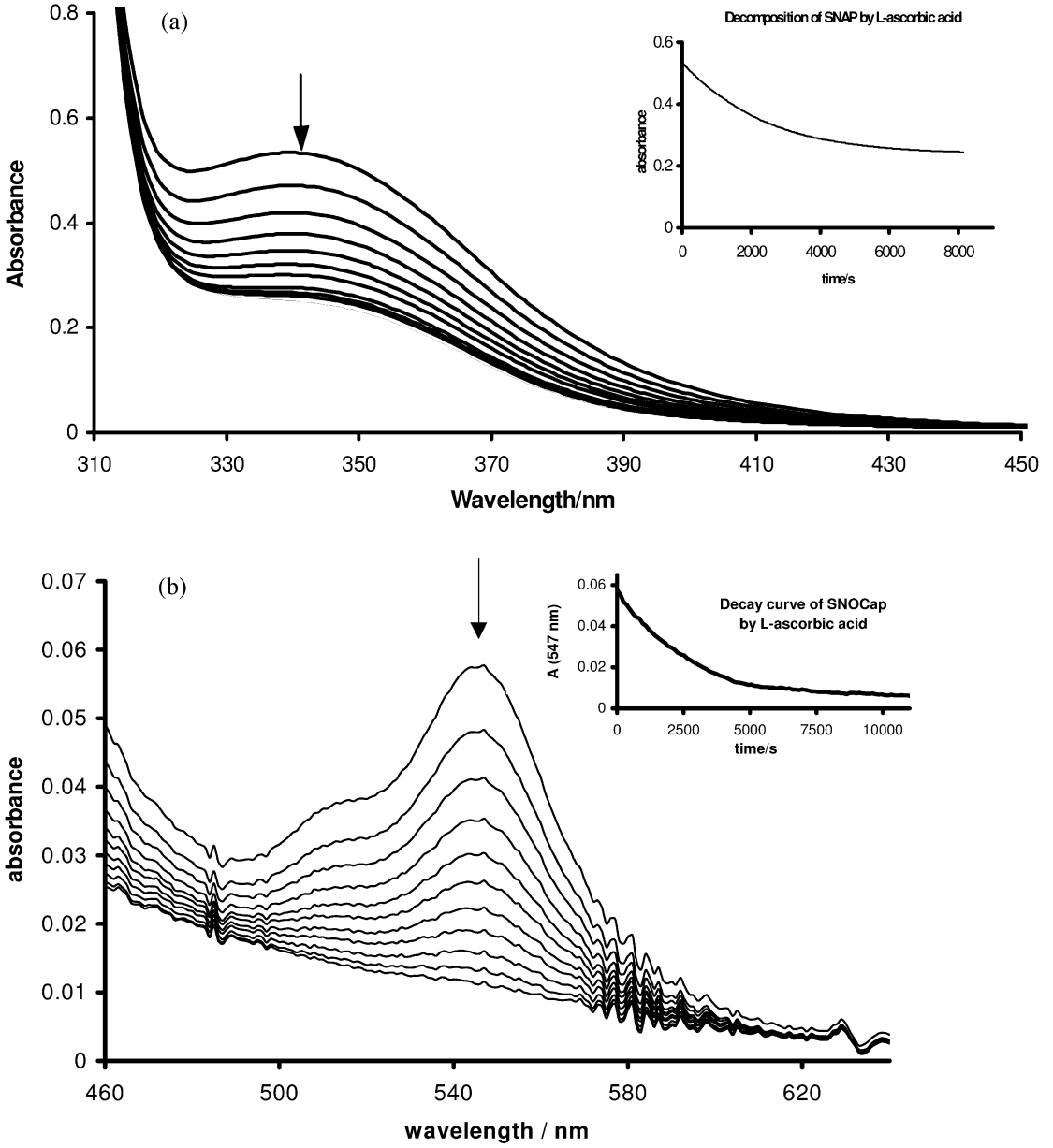


Fig. 2. (a) Spectral changes for the reaction of *S*-nitroso-*N*-acetyl-D,L-penicillamine with ascorbic acid. Conditions: 0.4 mM [SNAP], 30 mM $[H_2A]_t$, pH 7.34 (0.1 M phosphate buffer), 50 μM EDTA, and at 25 °C. Spectra were recorded at 10 min intervals. *inset*: Decay curve of SNAP by ascorbic acid at 340 nm. (b) Spectral changes for the reaction of *S*-nitrosocaptopril with ascorbic acid. Conditions: 3 mM [SNOCap], 60 mM $[H_2A]_t$, pH 7.38 (0.1 M phosphate buffer), 200 μM EDTA, and at 37 °C. Spectra were recorded at 8 min intervals. *inset*: Decay curve of SNOCap by ascorbic acid at 547 nm.

3.3. L-ascorbic acid variation

Linear dependence on the concentration of ascorbic acid was observed (see Table 1) within the range $0.020 \leq [\text{ascorbate}]_T \leq 0.080$ M, when k_{obs} values were plotted against [ascorbate]. Both plots showed zero intercept, re-emphasizing that thermal, photochemical and/or catalytic decomposition do not occur under the conditions employed. The reaction rates, as expected, are slower for SNOCap than for SNAP, owing to the much greater stability exhibited by SNOCap.

Table 1
Pseudo-first-order rate constants for the reaction between SNAP and SNOCap with L-ascorbic acid at various L-ascorbic acid concentrations

[L-ascorbic acid]/mol dm^{-3}	$k_{obs}/10^{-4}$ s^{-1}
SNAP (25°C, pH 7.34, 340 nm)	
0.02	3.15
0.03	4.17
0.04	5.79
0.05	7.43
0.06	9.77
0.04 (37 °C)	12.2
SNOCap (37 °C, pH 7.38, 547 nm)	
0.03	2.10
0.04	2.77
0.06	3.93
0.08	5.71

[SNAP] = 0.4 mM, [SNOCap] = 3 mM, [EDTA] = 50 or 200 μM.

3.4. pH dependence

The reactions of SNAP and SNOCap with ascorbic acid are greatly affected by the pH of the solution. As pH increases within the range pH 5–8 for the reaction of SNAP with L-ascorbic acid, k_{obs} values increase (see Table 3 and Fig. 4a), but more-so above pH 7, where the A^{2-} species becomes more dominant. In the case of SNOCap, the reactions were much slower. Rates also increase as pH increases from pH 7.3 to 10.2. However, below pH 8.3, there is little or no reaction, but after which the rate increases drasti-

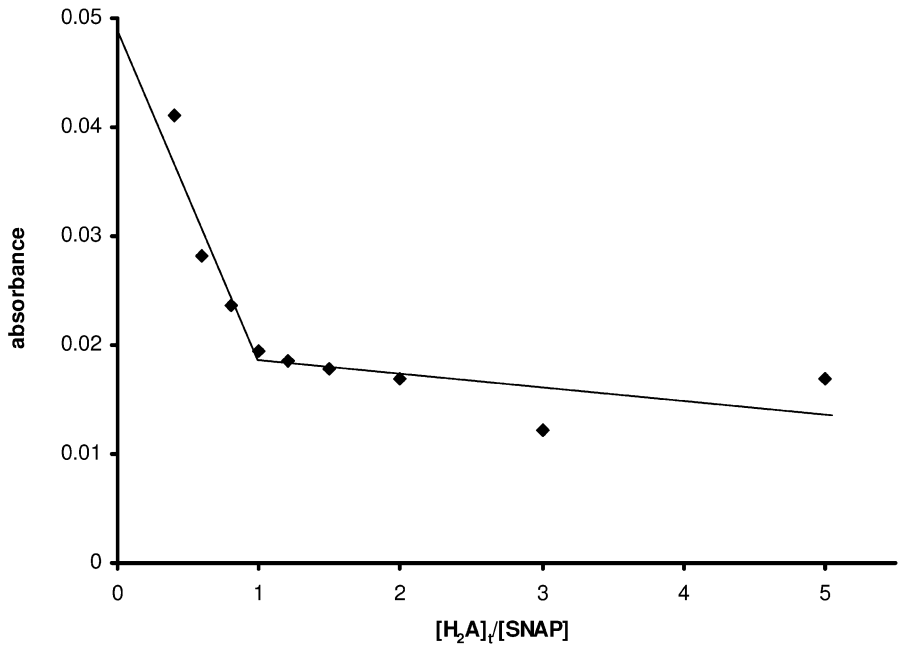


Fig. 3. Stoichiometric plot for the reaction of SNAP with L-ascorbic acid. Conditions: 10 mM [SNAP], 50 μM [EDTA], temp = 25 °C, pH 7.0 (0.1 M phosphate buffer), $\lambda = 590$ nm.

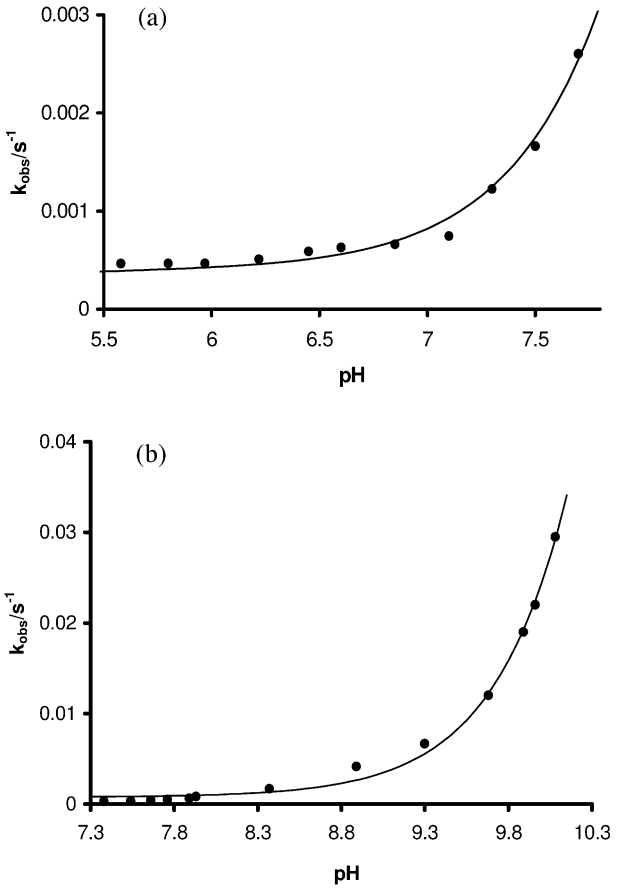


Fig. 4. Observed first-order rates (k_{obs}) vs. pH for the reaction between SNAP and L-ascorbic acid. Conditions: 0.4 mM [SNAP], 40 mM $[H_2A]_t$, 50 μM [EDTA], $\lambda = 340$ nm, and at 37 °C. (●) Experimental points and (—) calculated line from Eq. (6). (b) Observed first-order rates (k_{obs}) vs. pH for the reaction between SNOCap and L-ascorbic acid. Conditions: 3 mM [SNOCap], 30 mM $[H_2A]_t$, 200 μM [EDTA], $\lambda = 547$ nm and at 37 °C. (●) Experimental points and (—) calculated line from Eq. (6).

cally (see Fig. 4b). The pH ranges chosen for the kinetic studies are of physiological interest.

3.5. Effect of Cu^{2+} on the decomposition of SNOCap

The effect of Cu^{2+} on the rates of decomposition of SNOCap was investigated at (i) high ascorbate concentration and (ii) in the absence of ascorbic acid to determine the efficacy of RSNO decomposition by both ascorbate and copper ions. At high ascorbate concentrations, there was no observable catalysis by Cu^{2+} in the range $2.5 \leq [Cu^{2+}] \leq 125$ μM (see Table 2). In the absence of ascorbic acid, there was also no catalysis by Cu^{2+} at micromolar concentrations, however, on adding millimolar quantities of Cu^{2+} in a manner to effect pseudo-first-order conditions with respect to Cu^{2+} concentration, a 100-fold increase in the rate was observed. The results clearly indicate that ascorbic acid will preferentially reduce SNOCap because of the greater reactivity.

3.6. Ionic strength and metal-ion variation

The experimental results (Table 4) imply that the reacting ions possess the same sign, as increasing the ionic strength by the addition of inert ions increases the rate. The formation of a single, highly charged ionic complex from two less highly charged ions is favoured by a high ionic strength because the new ion has a denser ionic atmosphere and interacts with that atmosphere more strongly. No catalytic effect by the various metal ions on the reductive decomposition was observed (see Table 4).

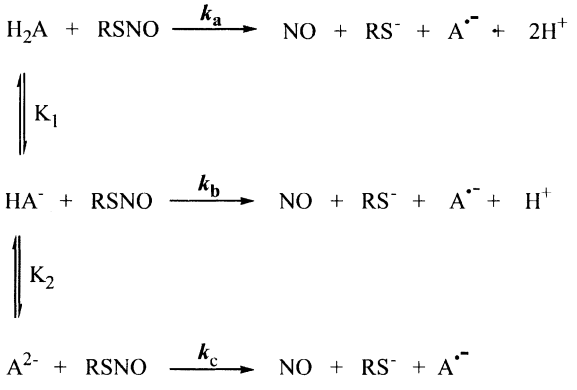
3.7. Products detection

An average of three determinations gave at least 60% of NO in both cases. This low detection of NO may have been as a result of (i) NO loss from the reaction vessel and (ii) aerial oxidation of NO by trace amounts of molecular oxygen due to the

Table 2
Decomposition of SNOCap in the presence of (a) L-ascorbic acid and Cu^{2+}, (b) Cu^{2+} only

$[Cu^{2+}]/\mu M$	$^{a}k_{obs}/10^{-4}s^{-1}$	$^{b}k_{obs}/10^{-6}s^{-1}$
no added	4.9 ± 0.6	–
5	4.9 ± 0.6	–
10	5.6 ± 0.5	2.7 ± 0.2
25	–	2.9 ± 0.2
50	5.4 ± 0.7	3.6 ± 0.3
100	–	3.7 ± 0.3
125	5.1 ± 0.7	–

(a) [SNOCap] = 5 mM, $[H_2A]_t = 50$ mM, $\lambda = 547$ nm, temp = 37 °C, pH 7.5; (b) [SNOCap] = 0.4 mM, $\lambda = 332$ nm, temp = 37 °C, pH 7.5.



Scheme 1.

slow nature of the reactions. According to the Griess test, 4% NO_2^- was detected at the end of the reactions. Approximately, 90% of the expected DHA was obtained upon analysis of the final reaction solution. The missing percentage could be due to (i) an incomplete reaction between RSNO and ascorbic acid; (ii) errors associated with the analysis; and (iii) subsequent reactions involving $NO^{\bullet}$ and ascorbic acid radical species.

4. Discussion

A general mechanism by which NO is lost from two very stable *S*-nitrosothiols in the presence of L-ascorbic acid is proposed from the rate data and the stepwise acid dissociation of ascorbic acid (Scheme 1) [41]. It is apparent from the rate data (Table 3) that although all forms of L-ascorbic acid are capable of reducing both SNAP and SNOCap the fully deprotonated form, A^{2-} species is by far the most reactive. The reaction mechanism can be viewed as involving an initial attack at the nitroso nitrogen atom by the monoanion (HA^-) and/or the dianion (A^{2-}) of ascorbic acid (Scheme 1). The presence of the free radical form of ascorbic acid ($A^{\bullet-}$) at the end of the reaction is confirmed by the chemical identification of DHA. This radical is relatively non-reactive [42], and remains unchanged over the entire pH range of -0.3 to 11.

$$H_2A \overset{pK_1=4.25}{\rightleftharpoons} H^+ + HA^- \overset{pK_2=11.8}{\rightleftharpoons} H^+ + A^{2-} \qquad (2)$$

The reduction of both SNAP and SNOCap by ascorbate under pseudo-first-order conditions occurs as one slow step with no spectral evidence of any intermediate formation, and involves the loss of NO from the parent RSNO due to the reduction of the S–NO bond. The loss of NO is indicated by a decrease in absorption at 340 and 547 nm as for SNAP and SNOCap, respectively.

The stabilities of these *S*-nitrosothiols have established the expected reactivity with ascorbate and hence the differences in the rates. Generally, it is expected that SNAP being a tertiary RSNO would exhibit greater stability compared with the primary SNOCap, and as a result undergo reductive decomposition by ascorbate much slower. Clearly, our observations dispute this. SNAP, although a tertiary RSNO appears to be less stable than the primary SNOCap. Arguments [43–45]

Table 3
pH dependence for the reaction of SNAP and SNOCap with L-ascorbic acid at 37 °C, in the presence of an electrolyte (0.5 M NaCl), and 50 μM EDTA

pH	$k_{obs}/10^{-4}$ s^{-1}	
	SNAP	SNOCap
5.35	2.21	–
5.58	4.65	–
5.80	4.68	–
5.97	4.67	–
6.22	5.09	–
6.45	5.87	–
6.60	6.30	–
6.85	6.60	–
7.10	7.46	–
7.30	12.20	–
7.38	–	3.02
7.50	16.66	3.56
7.70	26.00	4.23
7.80	–	5.09
7.89	–	6.42
7.93	–	8.21
8.37	–	16.8
8.89	–	41.5
9.30	–	66.6
9.68	–	120
9.89	–	190
9.96	–	220
10.08	–	295

Reactions involving SNAP: [SNAP] = 0.4 mM, λ = 340 nm, [ascorbate] = 40 mM; SNOCap: [SNOCap] = 3 mM, λ = 540 nm, [ascorbate] = 30 mM.

have been put forward and are still underway to elucidate the variations observed in the reported stabilities of *S*-nitrosothiols. Some of the reported factors affecting stability are based upon the S–NO bond dissociation energies, the *syn* or *anti* position taken by the S–NO moiety in the RSNO molecule, and the general bulky framework of the RSNO compound, which will ultimately affect the other mentioned factors. These parameters will vary from one RSNO to another.

4.1. Rate expressions

The second-order rate constants for the decomposition of SNAP and SNOCap by ascorbic acid were elucidated from Eqs. (3)–(6) and are assigned as k_a, k_b and k_c denoting reduction of the RSNO via the H_2A, HA^- and A^{2-} pathways, respectively. The expressions/equations are written in a generalized manner where RSNO denotes *S*-nitrosothiol, and are arrived at from the mechanism shown in Scheme 1. The calculated and experimental k_{obs} values are plotted against pH as shown in Fig. 4a,b and indicate good agreement between these sets of data, which further speaks to the accuracy of the mechanism put forward for these reactions. The second-order rate constants and the calculated k_{obs} values were obtained by incorporating Eq. (6) along with the experimental pH data and Sigma Plot non-linear regressional calculations. k_c is magnitudes greater than k_b, while k_a remains negligible in all cases. This further confirms the much greater reactivity of the A^{2-} over the HA^- species.

$$\text{Rate} = (k_a[H_2A]_e + k_b[HA^-] + k_c[A^{2-}])[\text{RSNO}] \quad (3)$$

At equilibrium, in aqueous solution, the total ascorbic acid concentration:

$$[H_2A]_t = [H_2A]_e + [HA^-] + [A^{2-}] \quad (4)$$

$$\text{Rate} = \frac{-\text{d}[\text{RSNO}]}{\text{d}t} = \frac{k_a[H^+]^2 + k_bK_1[H^+] + k_cK_1K_2}{[H^+]^2 + K_1[H^+] + K_1K_2}[H_2A]_t[\text{RSNO}] \quad (5)$$

and the pseudo-first-order rate constant (k_{obs}) will have the following expression:

$$k_{obs} = \frac{k_a[H^+]^2 + k_bK_1[H^+] + k_cK_1K_2}{[H^+]^2 + K_1[H^+] + K_1K_2}[H_2A]_t \quad (6)$$

As expected, the rate constant via the HA^- pathway, k_b, is much smaller than the rate constant via A^{2-}, and is characterized by a relatively high activation enthalpy, $\Delta H^{\ddagger}_{b\text{SNAP}} = 93 \pm 7$ kJ mol^{-1}, $\Delta H^{\ddagger}_{b\text{SNOCap}} = 63 \pm 11$ kJ mol^{-1}. The higher of the two $\Delta H^{\ddagger}_b$ has the lower k_b (see Table 5). The activation entropy ($\Delta S^{\ddagger}_b$) is small and positive in the case of SNAP ($\Delta S^{\ddagger}_b = 15 \pm 2$ J K^{-1} mol^{-1}) and could relate to an unstable, disordered transition state intermediate. In the case of SNOCap, $\Delta S^{\ddagger}_b$ is negative and denotes the formation of a possibly more ordered transition state intermediate prior to the complete reduction of the S–N bond in SNOCap. However, the activation enthalpies for the A^{2-} pathway are found to be relatively large and positive although the rate constants (k_c) are large. The enthalpy of activation for SNOCap is approximately twice that obtained for SNAP, and may be a direct result of the higher stability of SNOCap, compared to SNAP, resulting in its greater resistance to reductive decomposition by the A^{2-} species. There is, however, a compensation of this large $\Delta H^{\ddagger}_c$ by a large and positive $\Delta S^{\ddagger}_c$ in the case of SNOCap. With respect to SNAP, negative activation entropy was obtained which implies the formation of a relatively ordered transition state intermediate in the reaction pathway via the A^{2-} species. The converse would apply to SNOCap. In both cases, the free energy of activation for the HA^- pathway (i.e. $\Delta G^{\ddagger}_b$) is greater than that via the A^{2-} pathway ($\Delta G^{\ddagger}_c$). Specifically, $\Delta G^{\ddagger}_{b\text{SNAP}} = 88 \pm 7$ kJ mol^{-1}, $\Delta G^{\ddagger}_{b\text{SNOCap}} = 85 \pm 11$ kJ mol^{-1}; $\Delta G^{\ddagger}_{c\text{SNAP}} = 60 \pm 5$ kJ mol^{-1}, $\Delta G^{\ddagger}_{c\text{SNOCap}} = 66 \pm 7$ kJ mol^{-1}, and hence as expected $k_c \gg k_b$. The free energy of activation for the HA^- pathway for both SNAP and SNOCap are approximately equivalent. The same applies to the values of $\Delta G^{\ddagger}_c$ for both systems. This leads us to conclude that the mechanism at work with these reaction systems is similar.

The decomposition of SNAP was accomplished in the pH range 5–8, where HA^- is the predomi-

Table 4
Effect of metal-ion and ionic strength variation on the reaction between SNAP and L-ascorbic acid

Parameter	$k_{obs}/10^{-4}$ s^{-1}
M^+	
Li^+	1.12
Na^+	1.16
K^+	1.13
Cs^+	1.25
I_{TOTAL}/mol dm^{-3}	
0.2	1.50
0.3	2.02
0.4	2.37
0.5	2.75

[SNAP] = 0.4 mM, [ascorbate] = 20 mM, [EDTA] = 50 μM, temp = 25 °C, pH 7.2 (Tris–HCl).

nant species (i.e. >99%) compared to A^{2-}, which exists in trace amounts, and since HA^- is of much lower reactivity, the reaction is still slow. However, the decomposition of SNOCap by ascorbate was investigated in the pH range pH 7–10 and clearly re-emphasizes that A^{2-} is the predominantly reactive species, as HA^- concentration over this pH range, although in greater amounts remains basically unchanged. Generally, the reactions involving SNOCap were much slower than SNAP even at 25 °C, and as such the temperature variation for SNOCap excluded experiments at 25 °C and at pHs less than pH 7. Reactions involving $A^{\bullet-}$ species are notably very fast, as such it can be assumed that this radical does not take part in the decomposition of SNAP and SNOCap by ascorbic acid, as these reactions are established as relatively slow.

The mechanism of decomposition of SNAP and SNOCap by ascorbic acid occurs either by an outer sphere electron transfer process [40,46] or by nucleophilic attack [27,47] by the ascorbate anion/dianion on the sulfur of the S–NO bond. However, the data obtained from the metal ion variation experiment (Table 4), strongly indicates the absence of any mediator that would facilitate electron transfer from ascorbate to RSNO. This information contradicts what is expected for an outer sphere electron transfer process between reacting species with similar charges. Owing to this, a reaction involving nucleophilic attack is more favourable. This is further supported when the rates of decomposition are correlated with the molecular structure of the RSNO. Williams et al. [27] has established that at high ascorbate concentration, the reaction pathway is interpreted in terms of nucleophilic attack by ascorbate at the nitroso-nitrogen atom, leading to thiol and *O*-nitrosoascorbate which breaks up by a free radical pathway, to give dehydroascorbic acid and NO. Nucleophilic attack by ascorbate on the nitrosyl moiety would be significantly more affected by bulkiness in the RSNO molecular structure than would be for an outer-sphere mechanism. Bulky RSNOs, such as GSNO, SNAP and SNOCap are more stable and decompose much slower than their lighter molecular weight counterparts irrespective of whether they are primary, secondary or tertiary RSNOs [48]. There is also the concept of the effect of specific functionalities within the RSNO molecule that could affect decomposition rates. Electron-releasing groups such as acetyl and alkyl that are bonded near to the S–NO group can strongly deactivate and thus stabilize the S–N bond (i.e. making it less polar), rendering it less susceptible to nucleophilc attack by the ascorbate ion [48]. This will definitely affect the rate at which NO is released from the specific RSNO. This has been established in a recent report [27], where the second-order rate constants for the decomposition of a variety of RSNOs by ascorbate are listed. Some of these are 2540×10^{-4} M^{-1} s^{-1}, 27.3×10^{-4} M^{-1} s^{-1}, for *S*-nitroscysteine and *S*-nitroso-*N*-acetylcysteine, respectively; also, 1400×10^{-4} M^{-1} s^{-1} and 32.2×10^{-4} M^{-1} s^{-1} for *S*-nitrosopenicillamine and *S*-nitroso-*N*-acetylpenicillamine, respectively. It can also be concluded that the presence of an acetyl group as in the case of SNAP (compared to *S*-nitrosopenicillamine) has a dual effect on the rates of decomposition, as it not only stabilizes the S–NO bond as previously mentioned, but also increases steric hindrance to nucleophiles. It is important to note that most of the second-order rate constants obtained by Williams et al. [27] were evaluated as the slope of plots of k_{obs} vs. $[H_2A]$ at fixed pH. These rate constants do not, therefore, reflect the true reactivity/mechanism that exists between these RSNOs and ascorbate, as the behaviour of the reductant is

best established when pH is varied, as its various species which may be responsible for the reactions observed, are all taken into consideration.

4.2. Copper ion catalysis

At high ascorbate concentration, there was no observable catalysis by Cu^{2+} within the range studied (Table 2), however, on adding millimolar quantities of Cu^{2+} in a manner to effect pseudo-first-order conditions with respect to Cu^{2+} concentration, a remarkable difference in the rates was observed. Under conditions of: [SNOCap] = 0.4 mmol dm^{-3}, [Cu^{2+}] = 4 mmol dm^{-3}, I = 0.5 mol dm^{-3} (NaCl), pH 3.0 (HCl) and at a temperature of 37 °C, a value of 3.04×10^{-4} s^{-1} was obtained as the pseudo-first-order rate constant. Data in Table 2 demonstrate that ascorbic acid will preferentially reduce SNOCap because of the greater reactivity. The better chelating property of ascorbic acid/dehydroascorbic acid with Cu^{2+} (K = 37 at 25 °C and ionic strength of 0.1 mol dm^{-3}) than SNOCap with Cu^{+}/Cu^{2+}, may also help to explain why there is approximately no catalysis at micromolar copper ion concentrations. Since at low copper concentrations, Cu^{2+} would be preferentially chelated by AA/DHA rendering it unavailable to catalyze the decomposition of SNOCap.

Another interesting point to note is the necessity for the coordination of RSNOs to copper ions to effect their decay to produce NO [40,49]. Complexation of Cu^{+} to RSNO occurs via the sulfur, nitrogen and/or oxygen atoms in the RSNO molecule [50]. In the case of SNOCap, the only nitrogen atom present is in a 5-membered ring, which may pose a low probability of establishing complexation with Cu^{+} owing to the rigidity of the molecule posed by the presence of the ring. Complexation by way of –N–Cu^{+}–S– may also not be possible based on the distance between the N and S atoms. Complexation via the oxygen and sulfur atoms may also be limited due to the latter reason and also because Cu^{+} has a higher affinity for bonding to S and N atoms.

The complexity of the role played by the Cu^{2+}/Cu^{+} system is again emphasized by their interaction with disulfides. It has been reported [5,51,52] that these ions form stable complexes with disulfides, thus limiting their catalytic behaviour. At large Cu^{2+} concentrations (i.e. 2 moles [Cu^{2+}] to 1 mole complex), the disulfide-Cu^{2+} complexes have been isolated and their crystal and spectral properties described [53]. The UV/vis spectrum is characterized by a well-defined band at 530–600 nm. The unreactivity of *S*-nitrosocaptopril at millimolar concentrations may also possibly arise from the Cu^{2+}-complexing ability of the product disulfide [5]. This hypothesis is yet to be tested. Since the human body contains 0.1 g of copper per 75 kg of body weight and it is widely distrib-

Table 5
Second-order rate constants and activation parameters for the reductive decomposition of both SNAP and SNOCap by L-ascorbic acid

T/°C	SNAP		SNOCap	
	$k_b/10^{-3}$ dm^3 mol^{-1} s^{-1}	k_c/dm^3 mol^{-1} s^{-1}	$k_b/10^{-2}$ dm^3 mol^{-1} s^{-1}	k_c/dm^3 mol^{-1} s^{-1}
19.7	1.07 ± 0.15	195 ± 4		
25.0	2.02 ± 0.28	324 ± 7		
30.1	3.63 ± 0.28	467 ± 8	1.64 ± 0.88	17.7 ± 0.8
37.0	9.81 ± 1.39	662 ± 38	2.57 ± 1.29	49.7 ± 1.3
42.2			4.60 ± 3.54	88.6 ± 4.7
	$\Delta H_b^{\ddagger}$/kJ mol^{-1}	$\Delta H_c^{\ddagger}$/kJ mol^{-1}	$\Delta H_b^{\ddagger}$/kJ mol^{-1}	$\Delta H_c^{\ddagger}$/kJ mol^{-1}
	93 ± 7	51 ± 5	63 ± 11	103 ± 7
	$\Delta S_b^{\ddagger}$/J K^{-1} mol^{-1}	$\Delta S_c^{\ddagger}$/J K^{-1} mol^{-1}	$\Delta S_b^{\ddagger}$/J K^{-1} mol^{-1}	$\Delta S_c^{\ddagger}$/J K^{-1} mol^{-1}
	15 ± 2	−28 ± 3	−71 ± 20	118 ± 8

k_b and k_c are derived from Eq. (6).
Note: k_a values are ca. zero.

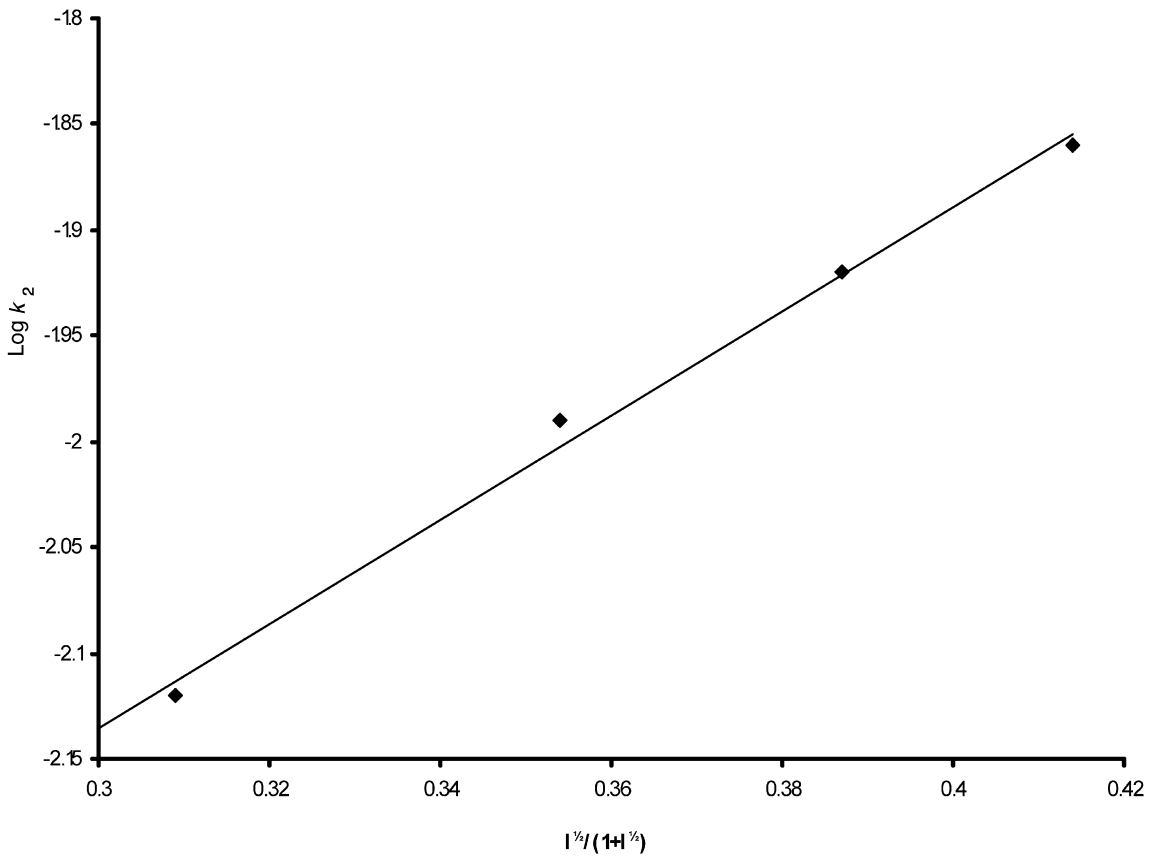


Fig. 5. Plot of log k_2 vs. $I^{1/2}/(1+I^{1/2})$ for the reaction between SNAP and ascorbate—Brønsted–Bjerrum plot. Conditions: 0.4 mM [SNAP], 20 mM $[H_2A]_n$ 50 μM [EDTA], temp = 25 °C, pH 7.0 (0.1 M phosphate buffer), $\lambda = 340$ nm.

uted in the blood, bone and muscle, the decomposition of RSNO catalyzed by copper continues to draw the most attention [54]. There is also a claim [55], that Cu^{2+} bound to proteins and peptides is reduced to Cu^{+} with the latter species being active in generating NO from RSNO compounds. However, ascorbic acid is present up to millimolar quantities in tissue fluid and would probably be more responsible for SNOCap decomposition to give NO in situ.

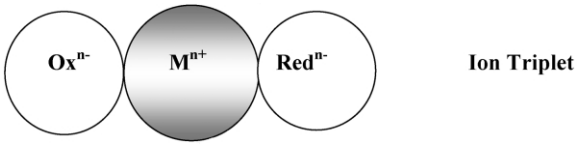
Applying the data obtained in Table 4 to the Brønsted–Bjerrum relationship, shown in Eq. (7), gives the plot of log k_2 vs. $I^{1/2}/(1+I^{1/2})$ as shown in Fig. 5. The resulting slope was 2.4, establishing the product of $z_A z_B$ as ~ 2. The charge on SNAP (z_A) would be $1-$ on the loss of the carboxylic proton in aqueous solution. Therefore, z_B would have to be 2, establishing the dianion (A^{2-}) as the form of ascorbic acid involved in the activated complex of the rate-determining step under the conditions stated.

$$\log k_2 = \log k_o + 1.018\, z_A z_B\, (I^{1/2}/(1+I^{1/2})) \qquad (7)$$

where k_2 is the second-order rate constant at a particular ionic strength, k_o is the rate constant at zero ionic strength, I is the ionic strength and z_A and z_B denote the charge on SNAP and ascorbate, respectively.

Group I metal cations are known to facilitate the electron transfer process between species that possess overall negative charges [56], and in these

reactions RSNO and ascorbate are negatively charged. Under the reaction conditions employed, it can be postulated that the presence of these somewhat large group I metal ions will not affect the electron-transfer process. Therefore, there is no formation of any possible ion-triplet (IT) between metal-ion, oxidant and reductant as shown below, which would facilitate electron transfer from reductant to oxidant. These observations support the proposed mechanism of a nucleophilic attack by ascorbate on the nitrosyl moiety of the RSNO, than an outer-sphere electron transfer mechanism, which would be more duly affected by M^+ catalysis.



The occurrences of IT formation are more typical with the more inorganic-type compounds, such as cyano–metal nitrosyl compounds [56,57], which are generally smaller highly negatively charged complexes, when compared to RSNOs.

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