# NO-Group transfer (transnitrosation) between *S*-nitrosothiols and thiols. Part 2[1]

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The kinetics of NO-group transfer have been measured for the reaction between a nitrosothiol ($HOCH_2CH_2SNO$) and nine thiols, mostly based on the cysteine structure. The reaction is second-order and there is evidence for a steric effect for thiols containing 1,1-dimethyl substituents (penicillamine derivatives). Reaction occurs *via* the thiolate anion as shown by the pH–rate constant profile, and a full kinetic analysis for the reactions of two thiols (*N*-acetylcysteine and glutathione) is quantitatively in agreement with this mechanism. Variation of the nitrosothiol structure for reaction with *N*-acetylcysteine shows that electron-withdrawing substituents in the nitrosothiol promote reaction; there is a similarity with the corresponding reactions of alkyl nitrites.

Direct transfer of the NO group from a nitroso compound to a nucleophilic centre (nucleophilic attack at the nitroso-nitrogen atom) is well known for many different types of nitroso compounds, including alkyl nitrites,[2] nitrosoamines,[3] nitrosoamides and a nitrososulfonamide.[4] Usually reactions have been studied under basic conditions involving reactions with amines, alkoxides, thiolates and carbanions, but in some cases, notably for nitrosoamines reaction can occur in acid solution[5] with non-basic nucleophiles such as halide and thiocyanate ions and a range of sulfur nucleophiles. Recently there has been much interest in the chemistry of *S*-nitrosothiols[6] (or thionitrites) RSNO, henceforth referred to as nitrosothiols, because of their possible therapeutic use as vasodilatory drugs (by release of nitric oxide) and also they could be involved *in vivo* in some of the remarkable physiological processes brought about and controlled by nitric oxide,[7–9] which have been recently discovered. One area of interest is the possibility of NO-group transfer to another thiol (and also possibly to other sites such as the iron atom in a haem) which might account for the question of nitric oxide storage *in vivo*,[9] since decomposition rates of RSNO species to give nitric oxide are very dependent on the structure of the nitrosothiol.[10] These reactions are strongly catalysed by $Cu^{2+}$. In the complete absence of $Cu^{2+}$, *e.g.* when EDTA is added the reaction rate is reduced to negligible levels. Often there is enough $Cu^{2+}$ in the distilled water/buffer components to bring about reaction. The mechanism is not fully understood, but it is believed that reduction to $Cu^{+}$ occurs and that $Cu^{+}$ is the true catalyst. Generally however these reactions are much slower than any involving direct NO group transfer.

Transnitrosation from RSNO to thiols has been reported on a number of occasions,[11,12] where the final isolated products are the products of decomposition of nitrosothiols, the disulfides. All three possible disulfides have been identified, for example, as shown in eqn. (1) for the reaction of nitrosoglutathione

$$RSNO + R'SH \longrightarrow RSSR + RSSR' + R'SSR' \quad (1)$$

(GSNO) with cysteine. More recently a direct spectrophotometric observation of the primary products of transnitrosation has been noted,[1,13] before subsequent decomposition to the disulfides has occurred. One of these studies showed that for two nitrosothiols, reaction occurs with the thiolate anion derived from the thiol [eqn. (2)], as expected by analogy with the

$$RSNO + R'S^- \rightleftharpoons RS^- + R'SNO \quad (2)$$

corresponding reactions of alkyl nitrites with thiolate anions.[14] Also as expected, the reaction is reversible and some equilibrium constants have been measured[13] at one pH value.

In view of the potential importance of transnitrosation from nitrosothiols *in vivo*, we have significantly extended our earlier studies[1] to include a large range of thiol and nitrosothiol species in order to establish (*a*) the generality of the reaction and (*b*) the quantitative aspects of reactivity.

## Results and discussion

We chose initially to work with the nitrosothiol derived from 2-hydroxyethanethiol, *i.e.* $HOCH_2CH_2SNO$, in its reaction with a range of thiols. Using this system it was possible to get excellent reproducible kinetic data, noting the increase or decrease in absorbance in the 300–400 nm range, depending on the relative magnitudes of the extinction coefficients of RSNO and R'SNO. Throughout we worked with $[R'SH]_0 \gg [RSNO]_0$ and at 0.1 mol $dm^{-3}$ $[OH^-]$ to ensure that the thiol was effectively fully converted to the thiolate anion. For all nine thiols used we always obtained excellent first-order kinetic behaviour. Plots of the first-order rate constant ($k_0$) *vs.* $[R'S^-]$ were excellent straight lines and values of the second-order rate constant $k_2$ [defined by eqn. (3)] obtained. In some cases there was evidence

$$\text{Rate} = k_2[RSNO][R'S^-] \quad (3)$$

of a small positive intercept, which would be consistent with an element of reversibility of the reaction, but this was too small to quantify. With R'SH in large excess over RSNO the reaction is effectively driven in one direction. A typical plot of $k_0$ *vs.* $[R'S^-]$ is shown in Fig. 1 for the reaction with the ethyl ester of cysteine. The collected $k_2$ values for the nine thiols are given in Table 1. We did not set out specifically to examine a wide range of thiolate ion reactivity but rather to concentrate mainly on nucleophiles which might have some biological significance. Consequently the variation in thiolate ion structure is not large and the resulting reactivity range is quite small as borne out by the $k_2$ values in Table 1. One feature of interest does stand out; the reactivity of penicillamine and its *N*-acetyl derivative is significantly smaller than that of the corresponding cysteine derivatives. Consideration of electronic effects alone would suggest that the 1,1-dimethyl derivatives should be the more reactive. Experimentally this is not the case, so the electronic effect must be outweighed by a steric effect resulting from a structure that is close to a *tert*-butyl group.

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**Table 1** Values of $k_2$ [eqn. (3)] for the reaction of $HOCH_2CH_2SNO$ ($5 \times 10^{-4}$ mol $dm^{-3}$) with nine thiolate anions ($1–8 \times 10^{-2}$ mol $dm^{-3}$)

| $R'SH \longrightarrow R'S^- + H^+$ | | $k_2$/$dm^3$ $mol^{-1}$ $s^{-1}$ |
|---|---|---|
| Cysteine | $^-O_2CCH(\overset{+}{N}H_3)CH_2SH$ | 445 ± 7 |
| Cysteine ethyl ester | $EtO_2CCH(NH_2)CH_2SH$ | 416 ± 10 |
| *N*-Acetylcysteine | $HO_2CCH(NHAc)CH_2SH$ | 432 ± 6 |
| Penicillamine | $^-O_2CCH(\overset{+}{N}H_3)CMe_2SH$ | 23 ± 0.5 |
| *N*-Acetylpenicillamine | $HO_2CCH(NHAc)CMe_2SH$ | 56 ± 2 |
| Cysteamine | $H_2NCH_2CH_2SH$ | 300 ± 7 |
| Thiomalic acid | $HO_2CCH_2CH(CO_2H)SH$ | 251 ± 5 |
| Methyl thioglycolate | $MeO_2CCH_2SH$ | 712 ± 37 |
| Glutathione | $^-O_2CCH(\overset{+}{N}H_3)[CH_2]_2CONHCH(CH_2SH)CONHCH_2CO_2H$ | 257 ± 4 |

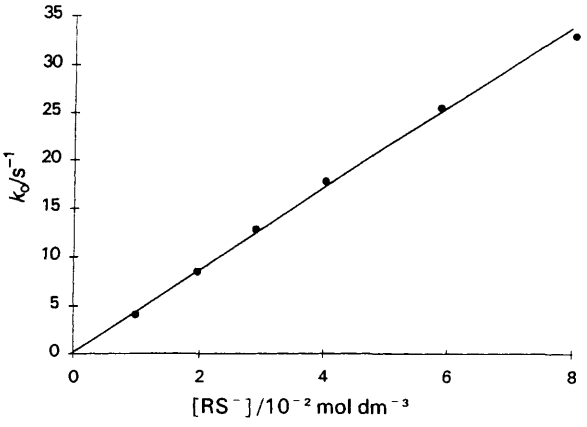


**Fig. 1** Plot of $k_0$ *vs.* [$RS^-$] for the reaction of $HOCH_2CH_2SNO$ with the thiolate of cysteine ethyl ester

**Table 2** Values of $k_0$ as a function of pH for the reaction of $HOCH_2CH_2SNO$ ($5 \times 10^{-4}$ mol $dm^{-3}$) with *N*-acetylcysteine (0.10 mol $dm^{-3}$) and with glutathione (0.010 mol $dm^{-3}$)

| *N*-Acetylcysteine | | Glutathione | | |
|---|---|---|---|---|
| pH | $k_0$/s$^{-1}$ | pH | $k_0$/s$^{-1}$ | $k_0^a$/s$^{-1}$ |
| 8.49 | 2.27 ± 0.03 | 7.38 | 0.103 ± 0.004 | 0.117 |
| 9.05 | 6.04 ± 0.07 | 7.72 | 0.19 ± 0.01 | 0.25 |
| 9.33 | 9.9 ± 0.5 | 8.01 | 0.50 ± 0.01 | 0.40 |
| 9.56 | 15.0 ± 0.4 | 8.26 | 0.63 ± 0.02 | 0.60 |
| 9.80 | 19.4 ± 0.4 | 8.53 | 0.82 ± 0.02 | 0.86 |
| 9.94 | 23.7 ± 0.3 | 8.79 | 1.05 ± 0.03 | 1.11 |
| 10.14 | 27.8 ± 1.0 | 9.45 | 1.85 ± 0.05 | 1.80 |
| 10.33 | 31.2 ± 0.5 | 9.94 | 2.50 ± 0.09 | 2.46 |
| 10.55 | 34.7 ± 0.7 | 10.19 | 2.7 ± 0.1 | 2.82 |
| 10.68 | 37.2 ± 0.8 | 10.32 | 3.0 ± 0.1 | 3.01 |
| | | 11.28 | 3.8 ± 0.3 | 3.75 |

[a] Calculated from eqn. (6).

The effect of changing pH was examined for two reactions and a kinetic analysis undertaken. Earlier we have shown that reaction occurs *via* the thiolate anion but the data were not sufficiently accurate to allow a kinetic analysis. We have obtained rate constants for the reactions of $HOCH_2CH_2SNO$ with *N*-acetylcysteine and with glutathione. Both reactions gave excellent first-order behaviour (again with $[R'SH]_0 \gg [RSNO]_0$) over the pH ranges and $k_0$ values are given in Table 2. In both cases the $k_0$ *vs.* pH plot is the characteristic S-shaped curve indicative of reaction *via* the thiolate anion. For *N*-acetylcysteine there is only one form of $R'S^-$ and if reaction occurs exclusively *via* $R'S^-$ then the expected expression for the first-order rate constant is given by eqn. (4) where $k_2$ is as before

$$k_0 = \frac{k_2 K_a [R'SH]_{total}}{K_a + [H^+]} \quad (4)$$

the bimolecular rate constant for reaction of $R'S^-$ with RSNO, $K_a$ the acid dissociation constant for R'SH and $[R'SH]_{total}$ the total stoichiometric concentration of the thiol. A reciprocal plot of $(k_0)^{-1}$ *vs.* $[H^+]$ is thus predicted to be linear with a positive slope and intercept. Such a plot is shown in Fig. 2. The data fit the equation very well. From the slope and intercept we readily obtain values of 408 $dm^3$ $mol^{-1}$ $s^{-1}$ for $k_2$ and 9.80 for the p$K_a$ of *N*-acetylcysteine. The former agrees reasonably well with the value of 432 $dm^3$ $mol^{-1}$ $s^{-1}$ obtained earlier for reaction in 0.1 mol $dm^{-3}$ sodium hydroxide, and the latter is in reasonably good agreement with a literature value [15] of 9.76.

For glutathione the reaction is more complicated. In the pH range studied the four forms **A**, **B**, **C** and **D** will be in equilibrium (see Fig. 3). We have assumed that the two reactive forms are **B** and **D**. There is no evidence to suggest from earlier experiments including those with alkyl nitrites, that the thiol form is sufficiently nucleophilic to bring about reaction. The reaction rate is then given by eqn. (5). The resulting expression for $k_0$ in

$$\text{Rate} = k'[\mathbf{B}][RSNO] + k''[\mathbf{D}][RSNO] \quad (5)$$

terms of $[R'SH]_{total}$ is given by eqn. (6). Using an equation-

$$k_0 = \frac{k'[R'SH]_{total} + k''[R'SH]_{total}K_3/[H^+]}{\left(1 + \frac{K_3}{[H^+]} + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}\right)} \quad (6)$$

fitting computer program we obtained the calculated values of $k_0$ given also in Table 2, using values for $k'$ and $k''$ of 190 and 390 $dm^3$ $mol^{-1}$ $s^{-1}$, respectively, and p$K_1$, p$K_2$ and p$K_3$ values of 8.55, 9.12, 9.97 (literature values [16] are 8.72, 9.47 and 9.47). The calculated and observed values of $k_0$ agree reasonably well. So both sets of results for reaction of *N*-acetylcysteine and glutathione are quantitatively consistent with the proposed scheme.

A final part of this study was concerned with the investigation of structural variation within the nitrosothiol in reaction with a common nucleophile. Again we have in the main worked with RSNO species which might be of biological interest. We chose to work with *N*-acetylcysteine and six RSNO species listed in Table 3. As in the first part of this paper we have worked throughout using 0.1 mol $dm^{-3}$ sodium hydroxide which ensures that the thiol is fully deprotonated and the $k_2$ values are easily obtained. Again for all nitrosothiols good straight line plots of $k_0$ *vs.* $[RS^-]$ were obtained, which passed through

or very near the origin. The $k_2$ values are given in Table 3. In three cases small quantities of dioxan were used to ensure full solubility in the reaction solutions. As a check that there was no significant solvent effect the reaction of $(CH_3)_2CHSNO$ with *N*-acetylcysteine was examined over a range of solvent composition. The data in Table 4 show that such a solvent effect is indeed small and can be neglected within the context of the results in Table 3.

**Table 3** Values of $k_2$ for the reaction of the thiolate anion derived from *N*-acetylcysteine (1–8 × $10^{-2}$ mol $dm^{-3}$) with a range of RSNO species (5 × $10^{-4}$ mol $dm^{-3}$)

| RSNO | $k_2$/$dm^3$ $mol^{-1}$ $s^{-1}$ | Dioxan in solvent (%) |
|---|---|---|
| $CH_3CH_2SNO$ | 84 ± 3 | 0 |
| $(CH_3)_2CHSNO$ | 110 ± 2 | 2 |
| $(CH_3)_3CSNO$ | 95 ± 1 | 4 |
| $HOCH_2CH_2SNO$ | 432 ± 6 | 0 |
| $CH_3OCOCH_2CH_2SNO$ | 646 ± 10 | 0 |
| $Cl\text{-}C_6H_4CH_2SNO$ | 1016 ± 17 | 15 |

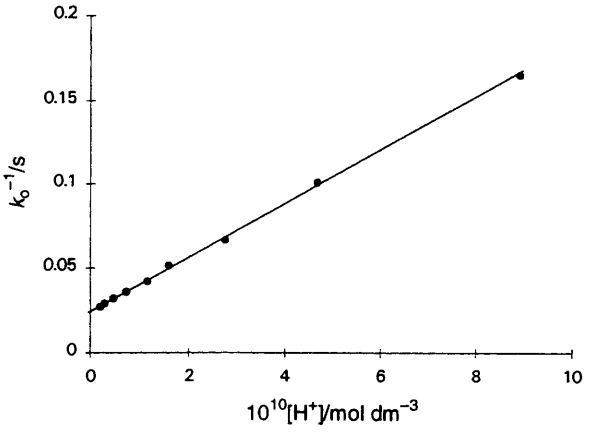


**Fig. 2** Reciprocal plot of $k_0^{-1}$ *vs.* [$H^+$] for the reaction of $HOCH_2CH_2SNO$ with *N*-acetylcysteine

The main feature which stands out from these results is that the introduction of electron-withdrawing groups in the nitrosothiol results in a rate-enhancement. This is clear for the substituent groups $HOCH_2$–, $CH_3OCOCH_2$– and $ClC_6H_4$-$CH_2$–, as expected for reaction involving nucleophilic attack by $R'S^-$ at the nitrogen atom of RSNO and shows that the same trend as has been established[14] for alkyl nitrite reaction with $R'S^-$ (and also for the reaction of other nucleophiles[17] with alkyl nitrites) applies also to the corresponding reactions of nitrosothiols. There is very little change of rate constant along the series ethyl-, isopropyl- and *tert*-butyl- nitrosothiols which is in contrast with the behaviour of the corresponding alkyl nitrites[14] with thiolate ions, where a steric effect is believed to operate. Maybe the larger sulfur atom (compared with the oxygen atom in alkyl nitrites) reduces the impact of the steric effect of the *tert*-butyl group at the nitroso nitrogen atom.

We believe that we have now covered a sufficiently wide range of nitrosothiol and thiolate ion structure to establish that NO-group exchange between them is completely general. For a

**Table 4** Effect of added dioxan on the value of $k_0$ for the reaction of $(CH_3)_2CHSNO$ with *N*-acetylcysteine in water containing sodium hydroxide (0.1 mol $dm^{-3}$)

| Dioxan (%) | $k_0$/$s^{-1}$ |
|---|---|
| 2 | 2.90 ± 0.2 |
| 4 | 2.89 ± 0.05 |
| 10 | 2.62 ± 0.04 |
| 15 | 2.42 ± 0.06 |
| 20 | 2.19 ± 0.06 |

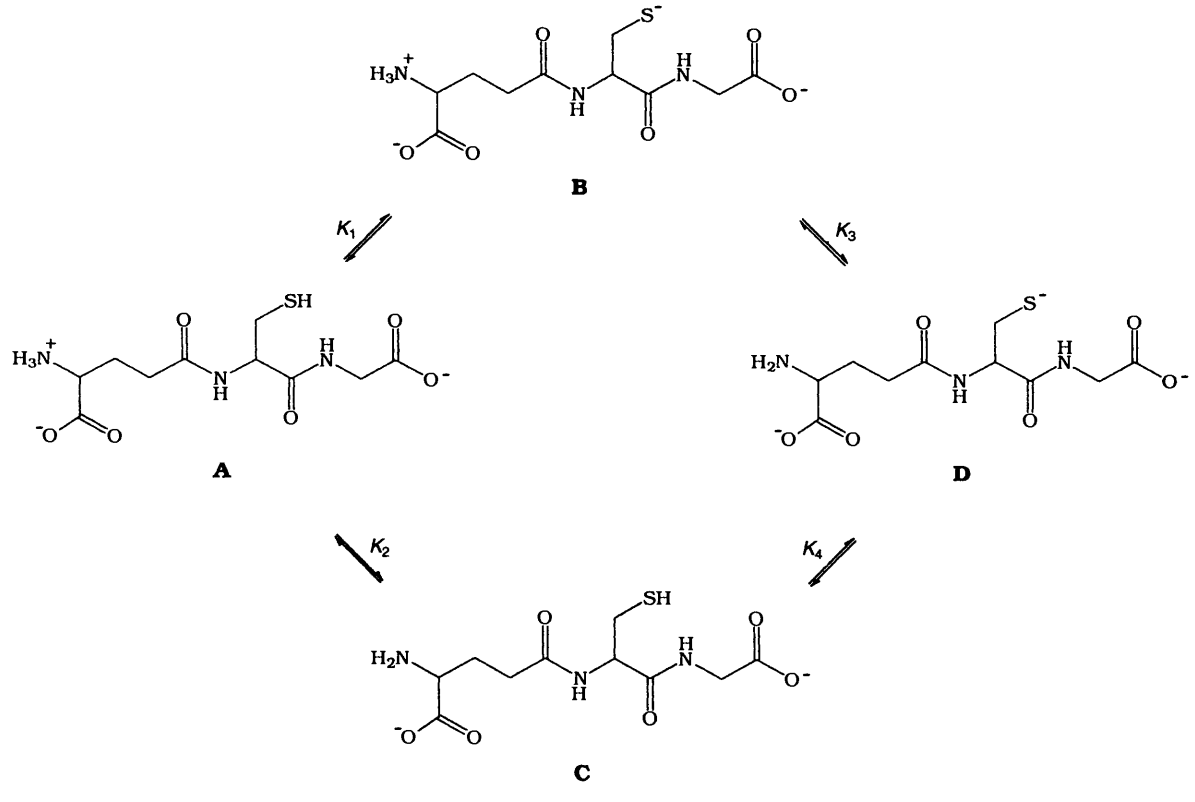


**Fig. 3** Protonation equilibria in glutathione

given pH of the medium, reaction will be more rapid when there are electron-withdrawing substituents within the nitrosothiol structure and reaction will also be more favoured for the thiols with low $pK_a$ value. Thus it is quite conceivable for these processes to occur *in vivo* which could account for the way in which nitric oxide might be stored and transported, given that NO release from a nitrosothiol is very dependent upon the nitrosothiol structure.

## Experimental

All of the materials used were of the highest purity grade available. Nitrosothiols were generated *in situ* from the corresponding thiol and aqueous nitrous acid, diluted and neutralised to the required pH for the kinetic experiments. The solutions of all the nitrosothiols studied were stable at pH 7.4 over the timescale involved in the experiments. The kinetics were all carried out at 25 °C in aqueous buffer or aqueous sodium hydroxide, and in all cases with at least a 20-fold excess of the thiol over the nitrosothiol. A low concentration of EDTA was present in most of the experiments to avoid decomposition by adventitious $Cu^{2+}$ (see ref. 10), but this had no effect on the measurements. Absorbance changes were measured in the 300–340 nm range noting the change of absorbance with time in a stopped-flow spectrophotometer (Applied Photophysics Model SX-17MV). The total absorbance change was in the range 0.02–0.08 which was well within the capability of the instrument. The extinction coefficients of all of the reactant and product nitrosothiols were determined independently. The measured absorbance changes (sometimes an increase and at other times a decrease) were all consistent with these extinction coefficient values. Excellent first-order behaviour was found throughout this study and the quoted values of $k_0$ (the first-order rate constant) are mean values of about ten separate measurements. The standard error was typically ± 3–5%.

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