The Mechanism of the Nitrosation of α-Amino Acids: Evidence for an Intramolecular Pathway

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The results of a kinetic study of the nitrosation of sarcosine, proline, cysteine, and the ethyl ester of sarcosine are reported. Under the conditions in which sarcosine and proline were studied, both first- and second-order terms were found for nitrite dependence. The experimental results are interpreted by a mechanism which involves NO^+ and N_2O_3 as direct nitrosating agents, while the effect of acidity changes on the first-order term for nitrite shows that the *N*-nitroso compound is also formed in a parallel reaction in which a slow intramolecular rearrangement follows the attack of NO^+ on the carboxylate group of the amino acid. This pathway is confirmed by the absence of such a parallel reaction in the nitrosation of the ethyl ester of sarcosine. The influence of acidity on the nitrosation of cysteine shows that *S*-nitrosation is brought about by the reaction of NO^+ with both the *N*-protonated, $HSCH_2$–$CH(\overset{+}{N}H_3)$–CO_2H, and zwitterion, $HSCH_2$–$CH(\overset{+}{N}H_3)$–CO_2^-, forms of the amino acid. The values obtained for the bimolecular rate constants are compatible with diffusion control, the difference between the values for both forms of the amino acid being explicable in terms of charges of the reagents.

In two recent articles[1,2] we have explained the influence of acetate ion on the rate of nitrosation of amines by invoking the formation of nitrosyl acetate as an effective nitrosating agent, although there has been some disagreement in the literature about the involvement of such a species.[3,4] These findings led us to suspect that similar effects might be found whenever the CO_2^- group is present in the reactants. The nitrosation of amino acids appeared to be a particularly interesting case in point, since the presence of the CO_2^- group in the amino acid itself meant that the nitrosatable substrate and the putative nitrosating agent would both form part of a single molecule.

In a thorough study of the nitrosation of amino acids in which the kinetics of the reaction were investigated by two independent methods, Mirvish *et al.*[5] obtained the rate equation (1),

$$r = k[HNO_2]^2[\text{Amino acid}] \quad (1)$$

according to which the sole nitrosating agent would be N_2O_3. The values of k were of the same order of magnitude as for other aliphatic amines.[6]

The present article reports the results obtained in our own study of the nitrosation of the amino acids sarcosine (Sar), proline (Pro), and cysteine (Cys), and of the ethyl ester of sarcosine (See).

Experimental

Sar, Pro, and See were Fluka puriss. products, Cys was supplied by Sigma, and other reagents were obtained from Merck (pro analysi grade). As an indication of their purity, the m.p.s of Sar, Pro, and Cys were measured. The values obtained were respectively 208—210, 218—220, and 177—180, which may be compared with published values of 210—212, 220—222, and 178—180 °C.[7] The results of a number of experiments using amino acids purified by repeated recrystallization from methanol–water were compared with these obtained using the commercial products as supplied. Since no differences were observed between the results, the remainder of experiments were carried out using the commercial products after drying with no further purification, as were the other reagents.

Kinetic measurements were carried out in a Spectronic 2000 spectrophotometer (Bausch and Lomb) equipped with a thermostatted cell holder, a recorder, and a printer. Acidity was measured using a Radiometer model PHM-82 pH-meter with a GK2401C combined electrode.

The rates of nitrosation of Sar, Pro, and See were measured spectrophotometrically by following the rise in absorbance at 249 nm due to the appearance of the nitroso compounds. The reagents were mixed in the spectrophotometer cell itself to give a final volume of 3.5 cm^3, nitrite being added last with a previously calibrated automatic pipette.

Given the slowness of these reactions, the initial-rate method of kinetic analysis was employed so as to avoid the competitive decomposition of nitrous acid. The rate of formation of the nitroso compounds, r, was thus calculated from expression (2) obtained by differentiating equation (3), where A_t is the

$$r = \frac{d[C]}{dt} = \frac{1}{\Delta\varepsilon}\frac{d(A_t)}{dt} \quad (2)$$

$$A_t = A_0 + \Delta\varepsilon[C] \quad (3)$$

absorbance at time t, A_0 the initial absorbance, $\Delta\varepsilon$ the differential molar absorptivity, and C the nitroso compound. The linearity of the absorption–time data was ensured by never following >2% of the reaction. That the rate measured was indeed the initial rate was shown by the identity of the initial measurement with the calculated value of A_0.

The nitrosation of Cys was followed at 330 nm and analysed by the integration method.

N-Nitrososarcosine (Nsar) and *N*-nitrosoproline (Npro), which are not available commercially, were synthesized as per ref. 8. The nitrosation reaction was carried out under optimal rate conditions and with nitrite greatly in excess. After evaporating water off with a high-vacuum line, the nitroso compounds were isolated and recrystallized various times before their molar absorptivities were determined.

The molar absorptivities of nitrosoproline and nitrososarcosine vary with the acidity of the medium due to the resulting changes in the ratio CO_2^-:CO_2H, and accordingly had to be obtained for various values of pH (Table 1). Various determinations of the molar absorptivities of these compounds were also carried out at 260 nm, the wavelength used by Mirvish *et al.*, whose values were found to coincide with our own. The molar absorptivity of *N*-nitrososarcosine ethyl ester (Nsee) is independent of pH and the value determined (3 200 ± 30 dm^3

Table 1. Influence of acidity on the molar absorptivities of Nsar and Npro

pH	ε_{Nsar}/ dm^3 mol^{-1} cm^{-1}	pH	ε_{Npro}/ dm^3 mol^{-1} cm^{-1}
1.00	1 879	1.84	3 073
1.58	1 932	1.90	3 061
1.85	1 964	2.01	3 085
2.04	1 999	2.06	3 112
2.23	2 096	2.13	3 112
2.54	2 190	2.20	3 116
2.81	2 349	2.32	3 117
2.85	2 367	2.41	3 199
2.90	2 376	2.62	3 261
2.97	2 442	2.71	3 289
3.15	2 491	2.81	3 299
3.22	2 482	2.97	3 335
3.34	2 553	3.10	3 348
3.45	2 562	3.23	3 466
3.73	2 602		
12.10	2 686		

mol^{-1} cm^{-1} at 249 nm) is consistent with those obtained by Druckrey *et al.* at other wavelengths.[9]

Finally, the spectra of mixtures left until reaction was complete were found to coincide qualitatively and quantitatively with those of the nitroso compounds synthesized as above. The spectral changes observed during the 2% of the reaction followed in the kinetic experiments were such as produced by the formation of these nitroso compounds.

All the kinetic experiments were duplicated and the values obtained never differed by >3%. All experiments were carried out in water at 25 °C and at constant ionic strength (0.5 mol dm^{-3} for Sar, Pro, and See, and 0.25 mol dm^{-3} for Cys) controlled with $NaClO_4$.

Results and Discussion

(I) *The Nitrosation of Sar and Pro.*—Experiments to investigate the influence of the concentration of Sar upon the initial rate of its nitrosation showed the reaction to be of order one with respect to the amino acid (Figure 1). When the influence of the concentration of nitrite on the reaction rate was studied, both first- and second-order terms were detected. The latter experiments were repeated at various acidities, the results for some of which are shown in Figure 2.

The two sets of experiments together imply an experimental rate equation of the form of equation (4), where [nit] is the stoicheiometric concentration of nitrite and [AA] the total concentration of amino acid. Table 2 shows the values of a and b obtained at the various acidities used.

$$r_0 = (a[\text{nit}]_0 + b[\text{nit}]_0^2)[\text{AA}]_0 \qquad (4)$$

Equation (4) is analogous to the rate equation found for other secondary amines.[10] Hitherto, the second-order term with respect to nitrite has been attributed to a rate-controlling step involving attack by N_2O_3 on the free amino acid, and the other term to attack by NO^+ on the same substrate. However, Table 2 shows that the variation with acidity of the observed values of the ratio $a:b$ does not agree with that predicted by the general mechanism for secondary amines involving diffusion-controlled attack by N_2O_3 and NO^+. Sar must therefore be nitrosated by a different mechanism from that of secondary amines in general, and the results (Table 2) and conclusions for Pro are entirely analogous. Since the CO_2^- group is the only difference between

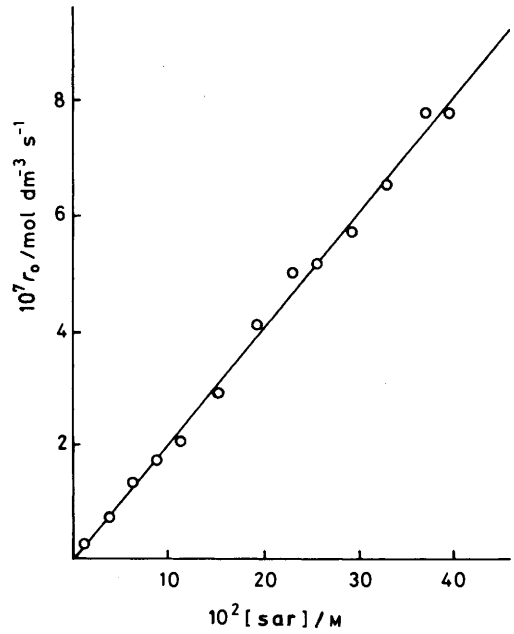


Figure 1. Influence of the concentration of Sar on its initial rate of nitrosation: $[\text{nit}]_0$ 2.57 × 10^{-3}M, pH 2.99 ± 0.02, t 25 °C, μ 0.5M

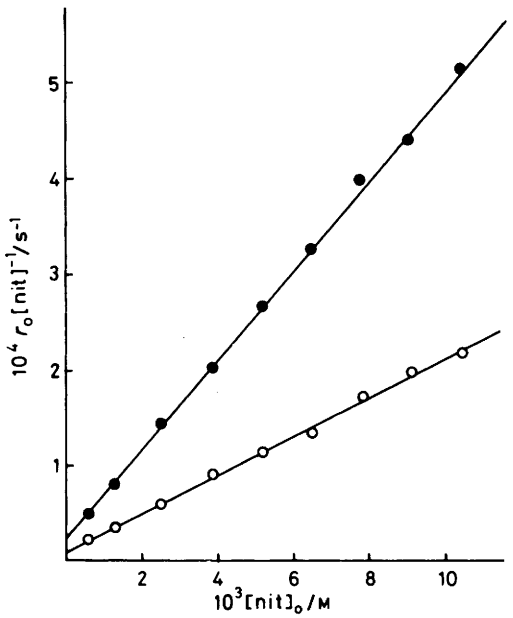


Figure 2. Influence of the concentration of nitrite on the initial rate of nitrosation of Sar: $[\text{Sar}]_0$ 0.115M, t 25 °C, μ 0.5M; ● pH 2.40 ± 0.02, ○ pH 1.56 ± 0.02

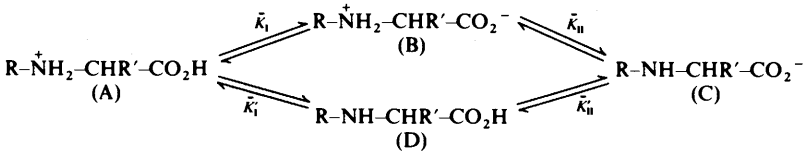
these amino acids and other secondary amines, our initial view concerning its effect on their nitrosation has been justified.

To explain the effects described above, we put forward a mechanism (Scheme 1), which includes new steps in which a nitrosyl carboxylate is formed and attacks the unprotonated amino group, in an intramolecular process.

The first step of this mechanism represents the protonation equilibria of the amino acid. K_I and K_{II} are the macroscopic constants for the loss of respectively the first and second protons. The values of the microscopic constants can be estimated from K_I and K_{II} by assuming the microscopic constant

Table 2. Influence of acidity on parameters a and b of equation (4) for the nitrosation of Sar and Pro. Comparison of the ratio $a:b$ observed with the values predicted by mechanism exclusively N_2O_3 and NO^+

pH	$10^5\ a$/dm^3 mol^{-1} s^{-1}	$10^2\ b$/dm^6 mol^{-2} s^{-1}	$10^3\ (a:b)$/mol dm^{-3} observed	predicted
Sarcosine				
1.22	6.52	8.43	0.77	0.30
1.39	7.45	13.74	0.54	0.21
1.56	7.48	17.82	0.42	0.14
1.67	8.61	22.60	0.38	0.11
1.87	9.48	32.60	0.29	0.072
2.20	10.00	41.47	0.24	0.036
2.40	10.78	42.43	0.25	0.026
2.60	14.96	44.78	0.33	0.018
2.80	16.10	36.35	0.44	0.013
2.96	19.82	29.22	0.68	0.011
Proline				
0.98	0.71	1.20	0.58	0.58
1.08	0.66	2.01	0.33	0.42
1.22	0.69	2.32	0.30	0.30
1.39	0.67	3.20	0.21	0.21
1.61	0.70	4.41	0.16	0.13
1.81	0.65	6.30	0.10	0.082
1.90	0.55	7.23	0.076	0.068
2.16	0.60	8.58	0.070	0.040
2.18	0.56	8.80	0.063	0.038
2.51	0.52	8.20	0.063	0.020
2.80	0.48	6.12	0.079	0.013



(1)

K_I K_{II}

$HNO_2 \rightleftharpoons NO_2^- + H^+$ K_a (2)

$HNO_2 + H^+ \rightleftharpoons NO^+ + H_2O$ K_2 (3)

$2\ HNO_2 \rightleftharpoons N_2O_3 + H_2O$ K_3 (4)

$NO^+ + D \longrightarrow$ Products k_a (slow) (5)

$N_2O_3 + D \longrightarrow$ Products k_b (slow) (6)

$NO^+ + B \rightleftharpoons R\overset{+}{N}H_2\text{-}CHR'\text{-}COONO$ K_4 (7)

$R\overset{+}{N}H_2\text{-}CHR'\text{-}COONO \rightleftharpoons RNH\text{-}CHR'\text{-}COONO + H^+$ K_5 (8)

$RNH\text{-}CHR'\text{-}COONO \longrightarrow$ Products k_c (slow) (9)

Scheme 1.

K'_I to be equal to the acidity constant of an ester of the amino acid (K_e).[11] The concentrations of (A)—(D) in step (1) can then be expressed as functions of the acidity and the total concentration of the amino acid in the working conditions used [equation (5)].

$$[A] = \frac{[AA][H^+]}{K_I + [H^+]} \quad [B] = \frac{K_I[AA]}{K_I + [H^+]} \quad [C] = \frac{K_IK_{II}[AA]}{(K_I + [H^+])[H^+]} \quad [D] = \frac{K_e[AA]}{K_I + [H^+]} \tag{5}$$

As (A) cannot undergo nitrosation because it is completely protonated, and the concentration of (C) at the pH values used is negligible compared with that of (D), only (B) and (D) have been taken into account in the remainder of the analysis. Steps (2)—(6) are common to the nitrosation of all secondary amines. The novelty of the present mechanism lies in steps (7)—(9), which represent the formation of a nitrosyl carboxylate analogous to nitrosyl acetate, the loss of a proton, and subsequent internal rearrangement involving the migration of

Table 3. Optimized values[a] of the kinetic parameters α, β, γ, and K_1 [equation (6)] and values of k_a[b] and k_b[c], calculated from α and β, for Sar and Pro

	Sarcosine	Proline
α/dm³ mol⁻¹ s⁻¹	(6.07 ± 0.23) × 10⁻³	(1.62 ± 0.08) × 10⁻³
β/dm³ mol⁻¹ s⁻¹	(5.49 ± 0.70) × 10⁻⁵	(6.83 ± 0.26) × 10⁻⁶
γ/s⁻¹	(8.39 ± 0.79) × 10⁻⁷	(5.69 ± 0.31) × 10⁻⁸
K_1/mol dm⁻³	(5.08 ± 0.43) × 10⁻³	(8.76 ± 0.97) × 10⁻³
k_a/dm³ mol⁻¹ s⁻¹	1.8 × 10¹⁰	6.0 × 10⁹
k_b/dm³ mol⁻¹ s⁻¹	2.0 × 10⁸	1.4 × 10⁸

[a] A value of $K_a = 8 \times 10^{-4}$ mol dm⁻³ was used. [b] Calculated using $K_2 = 3 \times 10^{-7}$ dm³ mol⁻¹ (ref. 14) and $K_e = 1 \times 10^{-8}$ mol dm⁻³ for Sar and $K_e = 5 \times 10^{-9}$ mol dm⁻³ for Pro (ref. 5). [c] Calculated using $K_3 = 3 \times 10^{-3}$ dm³ mol⁻¹ (ref. 13) and $K_e = 1 \times 10^{-8}$ mol dm⁻³ for Sar and $K_e = 5 \times 10^{-9}$ mol dm⁻³ for Pro (ref. 5).

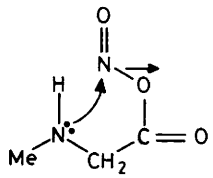


Figure 3. Steric facility for an internal rearrangement mechanism in the nitrosation of Sar

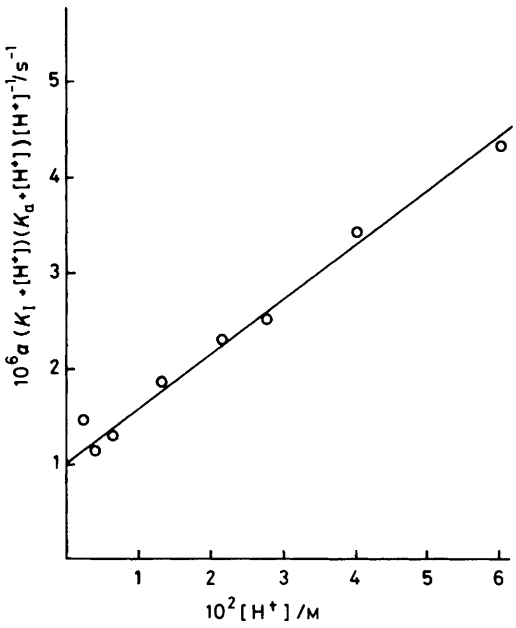


Figure 4. Acidity dependence of the first-order nitrite term of equation (4) according to equation (6) for the nitrosation of Sar

the –N=O group, as in other reactions described in the literature.[12] This would involve a sterically favourable five-membered ring mechanism as outlined in Figure 3. No steps in which the nitrosyl carboxylate attacks another molecule of amino acid are included because any such process would have shown up kinetically as a term of order two with respect to the amino acid.

Bearing in mind that in the working conditions used [nit] = $[HNO_2] + [NO_2^-]$, then the proposed mechanism leads to the theoretical rate equation (6), where $\alpha = k_b K_3 K_e$,

$$r = \alpha \frac{[AA][nit]^2[H^+]^2}{(K_1 + [H^+])(K_a + [H^+])^2} + \beta \frac{[AA][nit][H^+]^2}{(K_1 + [H^+])(K_a + [H^+])} + \gamma \frac{[AA][nit][H^+]}{(K_1 + [H^+])(K_a + [H^+])} \quad (6)$$

$\beta = k_a K_2 K_e$, and $\gamma = k_c K_4 K_5 K_1 K_2$. The first term represents the reaction between N_2O_3 and the amino acid, the second attack on the amino acid by NO^+, and the third internal rearrangement *via* the nitrosyl carboxylate.

Non-linear optimization of the parameters of equation (6) by applying an algorithm based on Marquardt's method to all the data produced the values shown in Table 3. The agreement between the values so calculated for the acidity constants of the two amino acids and those obtained by other authors by non-kinetic procedures[7] supports the mechanism proposed here. The optimized values of α and β allow the constants k_a and k_b to be calculated[13,14] (Table 3). The values of k_a, which represents attack by NO^+, are both of the order of 10^{10} dm³ mol⁻¹ s⁻¹ and are in agreement with those of reactions of other amines, whether aliphatic[15,16] or aromatic[16] and the same is true of the value of *ca.* 10^8 dm³ mol⁻¹ s⁻¹ found for k_b,[6] k_e cannot be calculated because the equilibrium constants K_4 and K_5 are unknown.

Figure 4 shows the variation with acidity of the first-order dependence on nitrite of the rate of nitrosation of Sar when calculated from the second and third terms of equation (6). It should be noted that the existence of a positive intercept can only be explained if this third term is considered.

Although this discussion has been carried out with reference to Sar, the results and conclusions for Pro are entirely analogous.

The failure of a first-order nitrite term to show up in the work of Mirvish *et al.*[5] is quite comprehensible in view of their experimental methods (discontinuous monitoring, analysis by the integration method even when very low percentages of reaction were followed, and graphical methods to obtain tangents in the initial rate method). With these techniques, which are much less precise than those employed in the present study, it is easy for a relatively small first-order term to be hidden by a dominant second-order term. It is worth pointing out that the second-order nitrite term obtained in the present study agrees satisfactorily with that deduced by Mirvish *et al.*[5]

(II) *The Nitrosation of See.*—In order to confirm that the observed effects in Sar and Pro are mediated by the formation of a COONO group, experiments were carried out with the ethyl ester of Sar, in which the formation of this group is impossible. The nitrosation of See was found to be of first order with respect to the ester and of mixed first and second orders with respect to nitrite at low pH (Figure 5), but was different from the nitrosation of Sar and Pro in that at pH 2 the first-order nitrite contribution is negligible, as for other secondary amines of similar pK_a values when such high concentrations of nitrite are used. There is therefore no evidence of any term other than simple attack by NO^+ in the first-order dependence on nitrite of the nitrosation of See, which must take place *via* the same mechanism as other secondary amines. This implies the theoretical rate equation (7), where $\delta = k_b K_2 K_e$ and $\theta = k_a K_3 K_e$.

Equation (7) is of the same form as the experimental rate equation, and comparison of the two yields the values $\delta = 3.9 \times 10^{-5}$ dm⁶ mol⁻² s⁻¹, $\theta = 6.0 \times 10^{-3}$ dm⁶ mol⁻² s⁻¹, and

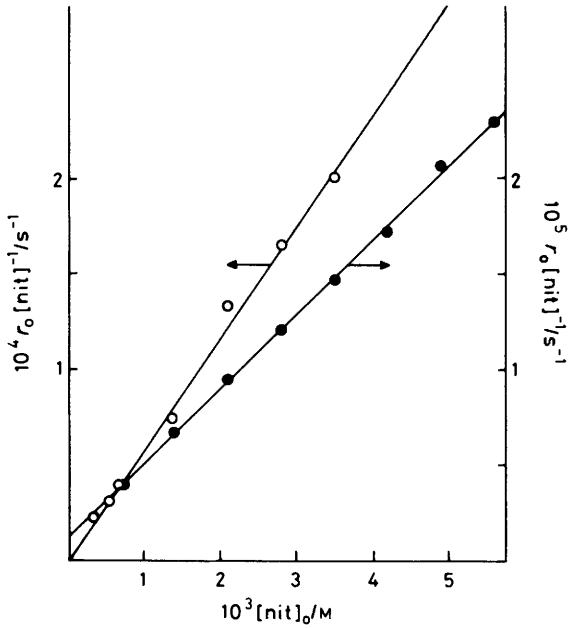


Figure 5. Influence of the concentration of nitrite on the initial rate of nitrosation of See: $[See]_0$ 0.037M, t 25 °C, μ 0.5M; ● pH 1.18 ± 0.02, ○ pH 2.70 ± 0.02

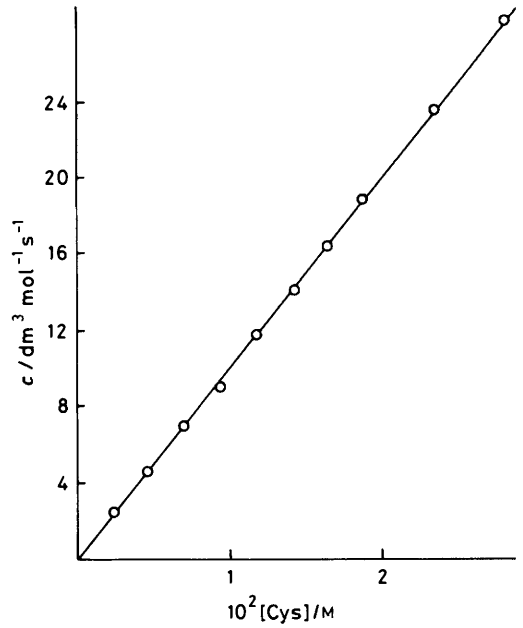


Figure 6. Influence of the concentration of Cys on the second-order rate constant [equation (9)]: [nit] 2.06 × 10^{-4}M, pH 2.71 ± 0.02, t 25 °C, μ 0.25M

$$r_0 = \delta \frac{[AA][nit][H^+]^2}{(K_a + [H^+])} + \theta \frac{[AA][nit]^2[H^+]^2}{(K_a + [H^+])^2} \quad (7)$$

K_a = 7.0 × 10^{-4} l mol. Since K_e, K_3, and K_2 are known (see Table 3), the rate constants for N_2O_3 and NO^+ attack can be calculated as respectively 2 × 10^8 dm^3 mol^{-1} s^{-1} and 1.3 × 10^{10} dm^3 mol^{-1} s^{-1}, which are quite similar to those calculated for Pro and Sar and to the published values for other amines.[6,10] This supports the absence of a second first-order nitrite term in the nitrosation of See, which in turn confirms that this term in the nitrosation of Pro and Sar is due to the presence of the CO_2^- group.

(III) *The Nitrosation of Cys.*—According to Stedman and co-workers,[17] the nitrosation of Cys occurs at the HS group: RSH + NO^+ → RSNO + H^+. They measured the rate of nitrosation of Cys and put forward a mechanism including as its slow step the reaction between the nitrosating agent NO^+ and the Cys SH group. From this mechanism they deduced the rate

$$r = k[HNO_2][H^+][Cys] \quad (8)$$

equation (8), where k = 456 dm^6 mol^{-2} s^{-1}. Dix and Williams[18] later confirmed this rate equation and further investigated the variation of the rate constant with acidity and added nucleophiles, but on plotting k_0 against $[H^+]$ (k_0 being the observed first-order rate constant defined by $-d[HNO_2]/dt = k_0[HNO_2]$), they obtained a non-zero ordinate at the origin which cannot be explained by the mechanism assumed.

The results of the present study also showed the nitrosation of Cys to be of first order with respect to nitrite and amino acid (Figure 6) [see equation (9)].

$$r = c[nit][Cys] \quad (9)$$

However, Figure 7 shows that the results of experiments carried out to study the influence of acidity on the second-order rate constant fail to support the linear dependence of $[H^+]^2/c$ on $[H^+]$ predicted by the mechanism hitherto accepted. The

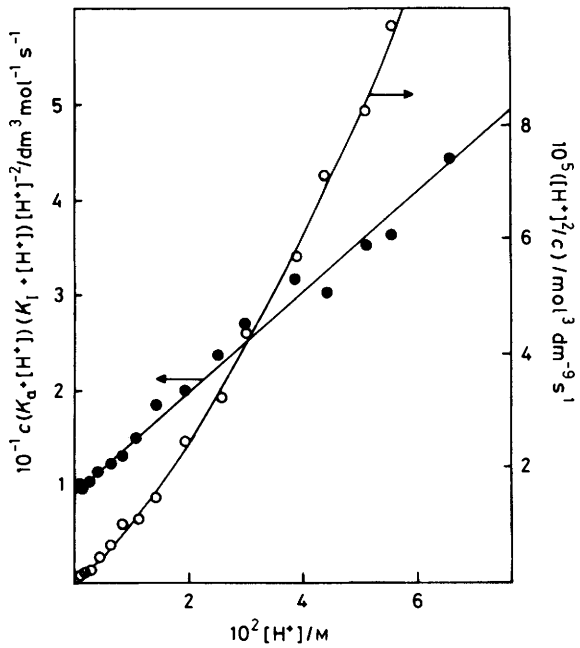


Figure 7. Dependence of the second-order rate constant of the nitrosation of Cys on acidity. ● According to equation (10); ○ without distinguishing between the protonated and the zwitterion forms of Cys

reason would seem to be that since the net charges of the two predominant forms of Cys, (E) and (F), are different, they react with the charged species NO^+ at different rates and must be

$$r = \sigma \frac{[\text{Cys}][\text{nit}][\text{H}^+]^3}{(K_a + [\text{H}^+])(K_1 + [\text{H}^+])} + \pi \frac{[\text{Cys}][\text{nit}][\text{H}^+]^2}{(K_a + [\text{H}^+])(K_1 + [\text{H}^+])} \quad (10)$$

considered separately in the reaction mechanism. The mechanism proposed is shown in Scheme 2 [it is not possible to detect kinetically whether in the k_f step NO^+ attacks the SH group directly, or whether it is the CO_2^- group which is attacked and there is subsequent internal rearrangement to give *S*-nitrosocysteine (Ncys)].

$$\begin{aligned} HNO_2 + H^+ &\rightleftharpoons NO^+ + H_2O & K_2 \\ NO^+ + E &\longrightarrow NCys & k_e \\ NO^+ + F &\longrightarrow NCys & k_f \end{aligned}$$

Scheme 2.

The above reaction mechanism leads to the overall rate equation (10), where $\sigma = k_e K_2$ and $\pi = k_f K_2 K_1$. This equation fits the experimental data well with the optimized values $\sigma = 514 \pm 34$ dm^6 mol^{-2} s^{-1} and $\pi = 9.87 \pm 1.84$ dm^3 mol^{-1} s^{-1}. The value of K_1 implies pK_1 2.29, which is not unreasonable when compared with those of other amino acids.

Equation (10) implies the linear dependence of $r(K_a + [H^+]) \cdot (K_1 + [H^+])/[H^+]^2$ on $[H^+]$. In the rate equation derived from the mechanism proposed hitherto, in which no distinction is made between the protonated and negatively charged forms of the Cys acid group, only the first term of equation (10) is accounted for, and a linear dependence of $[H^+]^2/r$ on $[H^+]$ is accordingly predicted. Figure 7 shows that the experimental data favour equation (10) and the need to distinguish between the CO_2H and CO_2^- forms of Cys.

The values obtained for σ and π allow k_e and k_f to be calculated as 1.7×10^9 dm^3 mol^{-1} s^{-1} and 6.4×10^9 dm^3 mol^{-1} s^{-1}, respectively. These values confirm that the positively charged species reacts more slowly than the neutral zwitterion, as is to be expected. Both values may be considered to indicate diffusion-controlled processes; although the value of k_e appears at first sight to be still too low for a diffusion-controlled reaction, this might be due simply to both the reagents involved having net positive charges. As has already been mentioned, it is impossible to discover by kinetic means whether the value of k_f is due only to attack by NO^+ on the sulphur atom, or also to its reaction with CO_2^- preceeding internal rearrangement.

Finally, another important consequence of our results is that the presence of the CO_2^- group in compounds of great biological interest, such as amino acids, can be the source of carcinogenic *N*-nitroso compounds through a hitherto unreported pathway.

Acknowledgements

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