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S-Nitrosocysteine and Cystine from Reaction of Cysteine with Nitrous Acid. A Kinetic Investigation[1]

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The formation of the *S*-nitrosocysteine (CySNO) in aqueous solution starting from cysteine (CySH) and sodium nitrite is shown to strongly depend on the pH. Experiments conducted within the pH range 0.5−7.0 show that at pH below 3.5 the NO^+ (or $H_2NO_2^+$) is the main nitrosating species, while at higher pH (>3.5) the nitrosating species is most likely the N_2O_3. A kinetic study provided a general kinetic equation, $V_{CySNO} = k_1[HNO_2][CySH]_{eq}[H^+] + k_2[HNO_2]^2$. The first term of this equation is predominant at pH lower than 3.5, in agreement with the literature for the direct nitrosation of thiols with nitrous acid; the value for the third-order rate constant, $k_1 = 7.9 \times 10^2$ L^2 mol^{-2} min^{-1}, was calculated. For experiments at pH higher than 3.5, the second term becomes prevalent and the second-order rate constant $k_2 = (3.3 \pm 0.1) \times 10^3$ L mol^{-1} min^{-1} was calculated. A competitive oxidation process leading to the direct formation of cystine (CySSCy) has been also found. Most likely also for this process two different mechanisms are involved, depending on the pH, and a general kinetic equation, $V_{CySSCy} = k_3[CySH]_{eq}[HNO_2][H^+] + k_3'[CySH]_{eq}[HNO_2]$, is proposed.

Introduction

Thiols are by far one of the most abundant classes of compounds present in vivo, and NO-related species have been shown to react readily with both sulfidryl-containing proteins and low-molecular-weight thiols to form *S*-nitrosothiols.[2] Actually, an increasing number of proteins as well as low-molecular-weight thiols, such as cysteine and glutathione, have been found to be *S*-nitrosylated in vivo, and the resulting *S*-nitrosothiols are strongly believed to transport and store nitric oxide, whose role as neurotransmitter has been recognized since early 1990. All this accounts for the great interest in the chemistry of *S*-nitrosothiols shown by biochemists and chemists in the past decade.

The synthesis of this class of compounds can be readily achieved in aqueous acidic solutions by sodium nitrite. The rate law for the nitrosation at very acidic pH, reported by Williams,[3] is $V_{R-SNO} = k[HNO_2][RSH][H^+]$. Actually, under strong acidic conditions, NO^+ (or H_2O-NO^+) is likely the nitrosating agent, whereas at higher pH N_2O_3 is probably involved.[3,4]

Nitrosation of thiols can be performed in organic solvents as well by treatment with NO and O_2, which rapidly react to give N_2O_3;[5−9] however, nitric oxide alone seems to be nonreactive. Simple low-molecular-weight thiols can also be nitrosated in good yield by using peroxynitrite in acetonitrile/water under acidic conditions, as we recently reported.[10]

Even though biological thiols can be readily nitrosated in aqueous solution under the above conditions, mechanistic details are generally lacking. In particular, as for cysteine (CySH) as regarded, the only available kinetic data seem to be confined to those reported by Stedman[11] and, most recently, by Williams,[12] who suggested the rate law equation $v = k[H^+][HNO_2][CySH]$. Moreover, the reaction leading to cystine (CySSCy) has not been generally taken into account, notwithstanding that its formation always competes with the formation of *S*-nitrosocysteine (CySNO), and it becomes very important by increasing the pH.

We want to report here kinetic results obtained from a study of the reaction of CySH with sodium nitrite,

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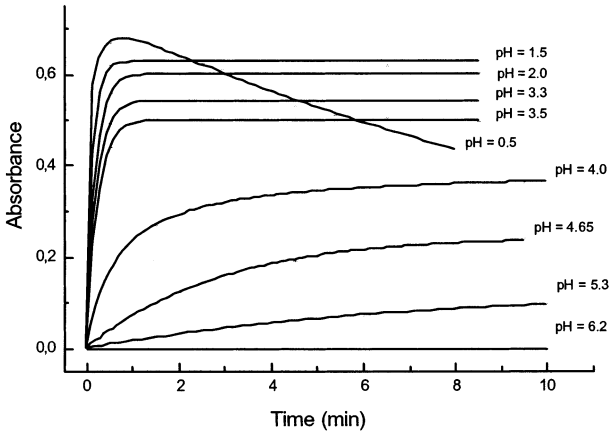

FIGURE 1. Plots of *A* vs time (min) for reactions of 0.04 M CySH with 0.04 M $NaNO_2$ in buffered aqueous solution at pH 0.5−6.2.

leading to both CySNO and CySSCy in a ratio strongly depending on the pH. Our present results can actually throw further light on the mechanism of formation of *S*-nitrosocysteine in vitro and then help elucidate the formation of this compound in vivo.

Results and Discussion

Reactions between CySH and $NaNO_2$ were conducted at 37 °C in buffered aqueous solutions at pH ranging between 0.5 and 7.0. The CySNO formation was continuously monitored measuring the absorbance at 543 nm (ϵ = 16.8 M^{-1} cm^{-1}).[13] The influence of pH on the rate of formation of CySNO for solutions containing 0.04 M CySH and 0.04 M $NaNO_2$ was at first examined. Plots of absorbance versus time are reported (Figure 1).

In a very strong acidic medium (pH = 0.5), the formation of CySNO is fast and quantitative. The absorbance value (A_{max} = 0.670) corresponded to the complete conversion of CySH into CySNO, which was reached in less than half a minute. However, at this pH CySNO is quite unstable; the absorbance quickly drops, and the initially deep red color solution fades completely within 1 h. Capillary electrophoresis analysis of the reaction mixture showed the presence of CySSCy as the only decomposition product. On the contrary, when experiments were conducted at pH of greater than 1, CySNO was stable and no significant decrease of absorbance (A_{max}) was detected after several hours. At pH between 1 and 4.65 the conversion of CySH was still quantitative within 30 min, but the absorbance values strongly decreased with increasing pH (see Figure 1). From A_{max} values (Table 1, column 4), we calculated the formation yield of CySNO. On the contrary, at pH 5.3 and 6.2 only a partial conversion, 50 and 30%, respectively, was obtained after 120 min, while no conversion at all occurred at pH 7 after 60 min.

The Nitrosation Reaction. The rate of formation of CySNO (V_{CySNO}) as a function of pH was obtained from analysis of the initial slope of curves reported in Figure 1. Actually, at the initial stage the concentration of reagents can be considered as the starting concentration, and the use of the equation $V_{CySNO} \propto k[\text{reagents}]_0$ is allowed: values of the rate were thus determined (Table, column 6).

As expected, the V_{CySNO} values are dependent on the pH, since the concentration of the possible reagents, i.e., HNO_2 and CySH, is dependent on the H^+ concentration (Scheme 1).

The concentrations of HNO_2 and CySH at equilibrium were also calculated (Table, columns 2 and 3), using $K_a = 5.6 \times 10^{-4}$ and $K_a = 1.95 \times 10^{-2}$, respectively.[14] In principle, two rate laws, eqs 1 and 2, are possible for the nitrosation reaction.

$$V_{CySNO} = k_1[HNO_2][CySH]_{total} \quad (1)$$

$$V_{CySNO} = k_1[HNO_2][CySH]_{eq} \quad (2)$$

The term $[CySH]_{total}$, eq 1, refers to the analytical concentration of CySH, including both the protonated ($CySH_2^+$) and the unprotonated form, while the term $[CySH]_{eq}$, eq 2, refers to reaction of HNO_2 with the unprotonated cysteine. However, none of the two equations fit the experimental results; thus it was compulsory to hypothesize a nitrosating species different from HNO_2 itself, for example a HNO_2/acid-catalyzed species. Toward this aim eqs 3 and 4 were considered.

$$V_{CySNO} = k_1[HNO_2][CySH]_{total}[H^+] \quad (3)$$

$$V_{CySNO} = k_1[HNO_2][CySH]_{eq}[H^+] \quad (4)$$

Actually, eq 3 did not fit the experimental values obtained for V_{CySNO}, whereas a good linear correlation between V_{CySNO} and the $[HNO_2][CySH]_{eq}[H^+]$ term was obtained, at least at pH lower than 3.5 (Figure 2).

In agreement with the kinetic equation reported for the nitrosation of simple thiols at pH < 1,[3] these results proved that, at very acidic pH, a nitrosating species such as NO^+ (or H_2O-NO^+) is involved. However, it needs to be emphasized that the nitrosation cannot take place on the protonated form, $CySH_2^+$, which at pH < 1.7 predominates on the zwitterionic form CySH (Scheme 1). From the slope of the curve of Figure 2, the value of the third-order rate constant k_1, eq 4, can be determined as 7.9×10^2 L^2 mol^{-2} min^{-1}.

However, none of the above equations, 1−4, seem to fit the experimental values at pH > 3.5. Most probably, at these pH's, neither NO^+ (or H_2O-NO^+) nor HNO_2 can be the nitrosating agent. To obtain evidence on the actual species at less acidic conditions, we carried out experiments in a buffered solution at pH 4.65 and we calculated V_{CySNO} for reactions of 0.02 and 0.04 M CySH with 0.02 and 0.04 M $NaNO_2$. Plots of V_{CySNO} versus $[HNO_2]^2$ are reported in Figure 3.

Two important aspects were evidenced: (i) the rate of formation of CySNO is independent of the initial cysteine concentration; (ii) V_{CySNO} is not linearly correlated to $[NaNO_2]$, but rather to the square term $[NaNO_2]^2$.

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TABLE 1. Yields (%) of CySNO and CySSCy and Corresponding Rates of Formation, V_{CySNO} and V_{CySSCy} (mol L^{-1} min^{-1}), for Reactions of 0.04 M CySH with 0.04 M $NaNO_2$ in Buffered Aqueous Solution at 37 °C[a]

pH	$[HNO_2] \times 10^{-2}$	$[CySH]_{eq} \times 10^{-2}$	CySNO, %	CySSCy, %	$V_{CySNO} \times 10^{-2}$	$V_{CySSCy} \times 10^{-2}$
0.5	3.99	0.19	100	0	7.75	
1.5	3.93	1.52	92.1	7. 9	7.35	0.63
2.0	3.79	2.64	90.2	9.8	6.68	0.73
2.5			85.8	14.2		
3.3	1.89	3.88	79.3	20.7	5.89	0.98
3.5	1.44	3.94	75.1	24.9	5.25	1.38
4.0	0.61	3.98			3.0	1.32
4.65	0.15	4.0	35.1	64.9	0.47	0.87
5.3	0.043	4.0			0.12	

[a] Values in columns 2 and 3 are referred to $[HNO_2]$ and [CySH], at equilibrium, respectively.

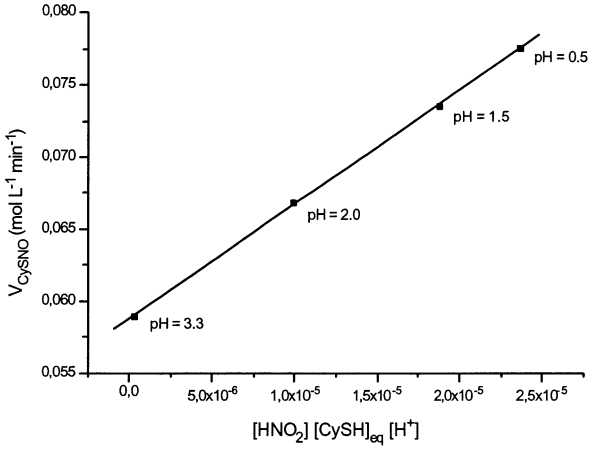


FIGURE 2. Plot of V_{CySNO} vs $[HNO_2][CySH]_{eq}[H^+]$ for reactions of 0.04 M CySH with 0.04 M $NaNO_2$.

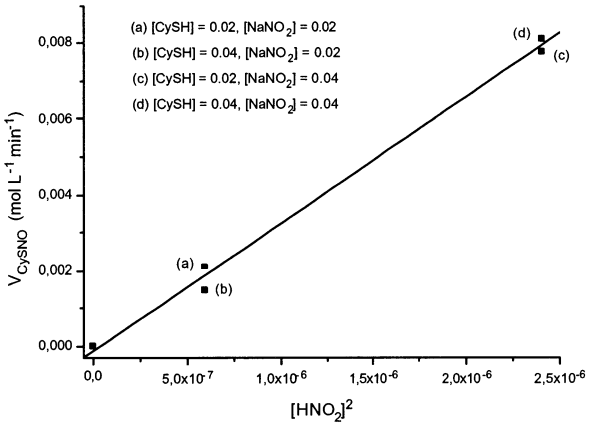
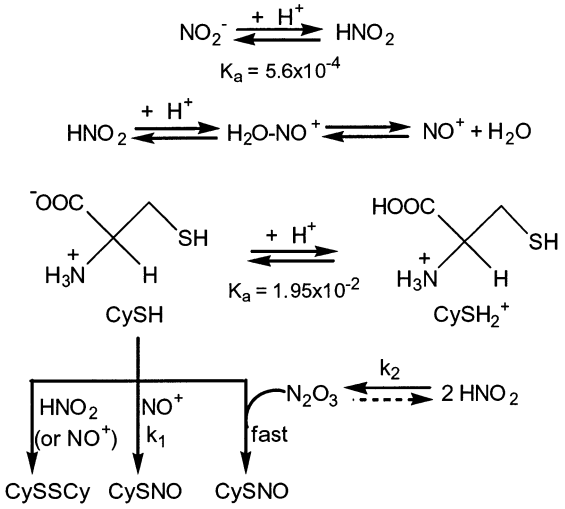


FIGURE 3. Plots of V_{CySNO} vs $[HNO_2]^2$ for reactions of 0.02 and 0.04 M CySH with 0.02 and 0.04 M $NaNO_2$ at pH = 4.65.

SCHEME 1



These results seem to imply N_2O_3 as the nitrosating species, which should come from HNO_2 through a bimolecular reaction. The same claim was already suggested for the nitrosation of simple thiols at pH greater than 2.[3] On this basis, the general rate law for the nitrosation of the cysteine becomes

$$V_{CySNO} = k_1[HNO_2][CySH]_{eq}[H^+] + k_2[HNO_2]^2 \quad (5)$$

From the slope of the curve, Figure 3, the value $k_2 = (3.3 \pm 0.1) \times 10^3$ L mol^{-1} min^{-1} was calculated. The first term of eq 5 is predominant at acidic pH, i.e., where the acid catalysis can be efficient, while the second term becomes predominant by increasing the pH. The finding that the second term, eq 5, is independent of the cysteine concentration suggests the formation of N_2O_3 as the rate-determining step (Scheme 1). At pH around neutrality no nitrosation was observed, most likely because the HNO_2 concentration, and then N_2O_3, tends to zero.

Oxidation of Cysteine. As we have already noted above, in all experiments carried out at pH 0.5−4.65 complete conversion of CySH was achieved, but the yield of CySNO strongly decreased with increasing pH (see Figure 1 and Table 1, column 4). To determine the formation of other reaction products, the reaction mixtures were decomposed to CySSCy at pH < 1 (see Experimental Section) and then analyzed by capillary electrophoresis. In all cases CySSCy was found to be present in almost quantitative yield. So, it can be argued that both CySNO and CySSCy were produced as the exclusive reaction products. Since CySNO was found to be stable at pH ≥ 1, both in the absence and in the presence of added CySH, CySSCy was not a byproduct deriving from the decomposition of CySNO or from a possible reaction between CySNO and CySH, but it was directly formed from CySH in competition with the nitrosation reaction. The yield of CySSCy can be easily achieved from the yield of CySNO through the equation CySSCy% = (100 − CySNO%) (Table 1, column 5). The rate of formation of CySSCy (V_{CySSCy}) as a function of pH can be obtained from the equation $V_{CySSCy} = [V_{CySNO} \times (CySSCy\%/CySNO\%)]$.

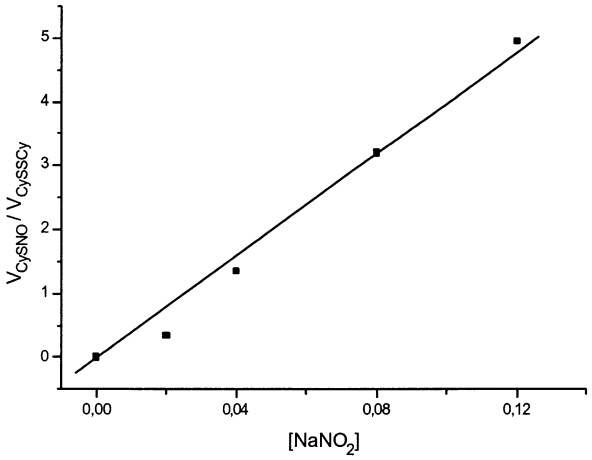


FIGURE 4. Plot of (V_{CySNO}/V_{CySSCy}) vs [$NaNO_2$] for reactions of 0.02 M CySH with $NaNO_2$ at pH = 4.65.

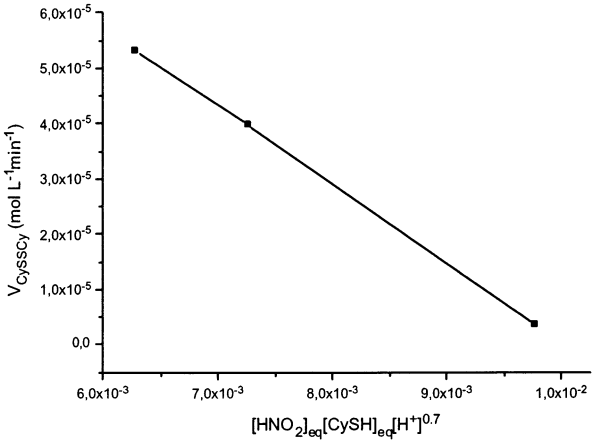


FIGURE 5. Plot of V_{CySSCy} vs $[HNO_2]_{eq}[CySH]_{eq}[H^+]^{0.7}$ for reactions of 0.04 M CySH with 0.04 M $NaNO_2$ in the pH range 1.5−3.3.

Different experiments were performed to establish the dependence of V_{CySSCy} on both HNO_2 and CySH concentration. Experiments conducted at pH 2.5, at different $NaNO_2$ concentrations (0.02 and 0.04 M), showed that V_{CySNO}/V_{CySSCy} (calculated from the yield of CySNO and CySSCy) is independent of $[HNO_2]$; because at this pH V_{CySNO} = cst $[HNO_2]$, we can claim that also V_{CySSCy} is dependent on $[HNO_2]$. Experiments carried out at pH = 4.65 with 0.02 M CySH in the presence of variable amounts of $NaNO_2$ (0.02−0.12 M) showed the rate ratio V_{CySNO}/V_{CySSCy} (similarly calculated from the yield of CySNO and CySSCy) to be linearly dependent on the HNO_2 concentration, at least in the presence of an excess of $NaNO_2$, i.e., conditions in which we can assume the process to have a pseudo-first-order kinetic behavior (Figure 4).

At pH = 4.65 V_{CySNO} is dependent on $[HNO_2]^2$, pointing out that V_{CySSCy} is dependent on $[HNO_2]$. So, it appears that V_{CySSCy} is dependent on $[HNO_2]$ at both strong and mild acidic conditions.

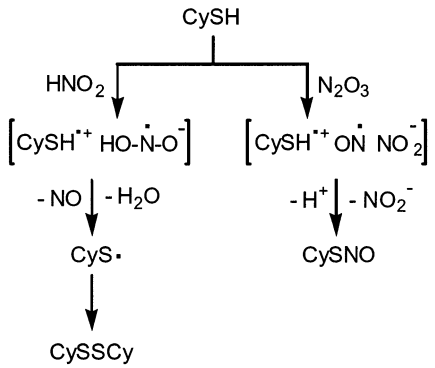
Moreover, experiments conducted at pH 2.5 and 4.65 with CySH, 0.02 and 0.04 M, in the presence of 0.04 M $NaNO_2$ showed that V_{CySNO}/V_{CySSCy} is independent of the CySH concentration at pH = 2.5, conditions in which V_{CySNO} = cst $[CySH]_{eq}$, whereas it is proportional to the CySH concentration at pH = 4.65, conditions in which V_{CySNO} is independent of [CySH]. As for the effect of $[H^+]$ on V_{CySSCy}, the rate values reported (Table, column 7) indicate a nonlinear dependence. Initially V_{CySSCy} increases with decreasing $[H^+]$ up to pH = 3.5, and afterward a decrease is observed.

Apparently, experimental results were fitted by eq 6 with $1 > n \geq 0$. However, the coefficient n is in turn dependent on the pH.

$$V_{CySSCy} = \text{cst}\ [CySH]_{eq}[H\ NO_2][H^+]^n \qquad (6)$$

In particular, we found $n \approx 0$ at the higher pH values (3.5 and 4.65). That is, at these milder acidic conditions the oxidation of CySH is largely independent of H^+ concentration. In contrast, at higher acidic conditions a good linear correlation was obtained for $n \approx 0.7$ (Figure 5).

SCHEME 2



From the mechanistic point of view, eq 6 points out that the formation of CySSCy can occur through two different routes: one dependent on and the other independent of $[H^+]$, but both depending on [CySH] and $[HNO_2]$. On this basis we suggest the following rate law:

$$V_{CySSCy} = k_3[CySH]_{eq}[HNO_2][H^+] + k_3'[CySH]_{eq}[HNO_2] \qquad (7)$$

However, eq 7 deserves some comments. The first term ($V_{CySSCy} = k_3[CySH]_{eq}[HNO_2][H^+]$) indicates that the same species (NO^+ and/or X−NO^+, X = H_2O, Cl^-) responsible for the nitrosation of CySH to CySNO, eq 4, is also capable of oxidizing CySH to CySSCy, possibly through an initial SET mechanism (Scheme 2).

Since the formation of the oxidizing/nitrosating species (NO^+ and/or X−NO^+, X = H_2O, Cl^-) is largely inhibited by decreasing $[H^+]$, this mechanism occurs only at strong acidic conditions. The second term ($V_{CySSCy} = k'_3[CySH]_{eq}[HNO_2]$) suggests that the oxidation of CySH can be promoted by HNO_2 itself. This mechanism becomes largely predominating (or exclusive) at the higher pH. It is worth noting that nitrous acid can behave as an oxidizing agent for CySH, while it is incapable of behaving as a nitrosating agent.

The Role of NO and NO_2. The role of NO and NO_2 as possible oxidizing and/or nitrosating species has also

been taken into account. As reported above, NO is a good nitrosating agent in the presence of air, but almost unreactive in anaerobic conditions: actually, no evidence of CySNO formation was evidenced. It has been also reported that NO slowly reacts with thiols through a reaction catalyzed by traces of iron ions, invoked to be present in the reaction medium;[13] however, results of our preliminary tests on the role of iron ions seem not to confirm this behavior.

A direct involvement of NO_2 until now had not been explored. Toward this aim, we ran experiments, directly preparing NO_2 by addition of NO to a sealed tube containing dioxygen. The brown-colored nitrogen dioxide, instantaneously formed, was added with a 0.04 M CySH aqueous solution, buffered at pH = 2.5. Two experiments with 1 and 2 molar equiv of NO_2 showed that the conversion of CySH was 45% and 90%, respectively, showing a stoichiometry of 1:2 CySH/NO_2.

In both cases CySNO and CySSCy were formed in a 78:22 and 80:20 ratio, respectively. These values are those forecasted for the reaction of 0.04 M CySH with 0.04 M $NaNO_2$ at pH 2.5, suggesting that HNO_2, and then NO^+ (or $H_2O{-}NO^+$), is once again the reactive species. In fact, HNO_2 was expected to be derived from the disproportionation reaction of NO_2 with water ($2\ NO_2 + H_2O \rightarrow HNO_2 + HNO_3$).

Conclusions

The reaction of CySH with $NaNO_2$ in aqueous solution leads to CySNO and CySSCy with a rate of formation, and relative yield, strongly dependent on the pH. The rate of formation of CySNO (V_{CySNO}) as well as CySSCy (V_{CySSCy}) decreases by increasing the pH, while the ratio CySSCy,%/CySNO,% largely increases with the pH. Different species seem to be involved as nitrosating and oxidizing species. Under strong acidic conditions the main reactive species is NO^+ (or $H_2O{-}NO^+$), deriving from nitrous acid through an acid-promoted reaction. This species mainly leads to the nitrosation product, CySNO, and, to a lesser extent, to the oxidation product, CySSCy. It is important to underline that the formation of both CySNO and CySSCy takes place on the unprotonated CySH instead of the protonated form, $CySH_2^+$, which prevails at pH < 1.7.

By increasing the pH the acid-catalyzed reaction leading to the nitrosating/oxidizing species NO^+ (and/or $H_2O{-}NO^+$) is progressively inhibited, and HNO_2 and N_2O_3 are mainly involved as oxidizing and nitrosating agent, respectively. The kinetic equation obtained from experiments carried out at pH 4.65 ($V_{CySNO} = k_2[HNO_2]^2$, $k_2 = (3.3 \pm 0.1) \times 10^3$ L mol^{-1} min^{-1}) suggests that the formation of N_2O_3 from HNO_2 is the rate-determing step in the nitrosation of CySH.

The reason HNO_2 behaves as oxidant, while N_2O_3 as nitrosating agent, is not clear. We can hypothesise that both species are involved in an initial SET process leading to the formation of a radical ion pair, which *in cage* behaves differently depending on the nature of the radical anion. In the former case proton transfer from $CySH^{\bullet+}$ might lead to the free $CyS^\bullet$ radical and then to CySSCy by dimerization. In the latter case radical ion coupling, followed by loss of HNO_2, would lead to the nitrosated product, CySNO (Scheme 2).

However, no evidence of formation of the cysteine radical, $CyS^\bullet$, could be achieved. At the present time we cannot exclude that the oxidation of CySH could give some intermediates (for instance CySOH and/or $CySNO_2$), which by decomposition can lead to CySSCy.

Experimental Section

The reaction were conducted in a thermostated bath at 37 °C. The formation of CySNO was constantly monitored by measuring the absorbance at $\lambda = 543$ nm ($\epsilon = 16.8$ M^{-1} cm^{-1}), flowing the reacting solution, by a peristaltic pump, in a UV/vis spectrophotometer equipped with a flow-cell. The CySNO percent yield was calculated on the basis of reacted CySH.

The formation of CySSCy cannot be spectrophotometrically monitored. However, CySNO and CySSCy being the only reaction products (see below), the CySSCy yield was calculated as CySSCy % = (100 − CySNO%).

Capillary electrophoresis analysis was performed with an instrument equipped with a UV-detector at 200 nm. A fused-silica capillary column (75 μm i.d., 67 cm length), a 100 mM boric acid/25 mM Tris background electrolyte (pH 8.2), and a 30 kV voltage were used. Under these conditions the migration time of CySSCy was 3.43 ± 0.01 min.

The following buffer solutions were used: $H_2C_2O_4/NaHC_2O_4$ (pH = 1.5), H_3PO_4/KH_2PO_4 (pH = 2.0 and 2.5), phthalic acid/monosodium phthalate (pH = 3.3 and 3.5), $NaHC_2O_4/Na_2C_2O_4$ (pH = 4.0), acetic acid/sodium acetate (pH = 4.65), monosodium phthalate/disodium phthalate (pH = 5.3), KH_2PO_4/K_2HPO_4 (pH = 7.0).

Reaction of 0.04 MCySH with 0.04 M $NaNO_2$ at pH = 0.5. An aqueous solution (24.0 mL) containing CySH (1.0 mmol, 121 mg) and $NaNO_2$ (1.0 mmol, 69 mg) was added with 12 M HCl (1.0 mL); a deep red color, due to the formation of CySNO, immediately developed. From $A_{max} = 0.670$, reached in less than 30 s, the CySNO yield was calculated to be 100% (100% conversion yield). The absorbance quickly decreased, and in less than 1 h the solution was completely faded. Capillary electrophoresis analysis showed the presence of CySSCy alone.

Reaction of 0.04 MCySH with 0.04 M $NaNO_2$ in Buffered Solution. An aqueous solution buffered at the appropriate pH (25.0 mL) containing CySH (1.0 mmol, 121 mg) was added with $NaNO_2$ (1.0 mmol, 69 mg), and the formation of CySNO monitored spectrophotometrically. For reactions carried out at pH 1.5−4.65 the A_{max} values were reached within 1−30 min and were found not to decrease over 2 h. The reaction mixtures were assayed to assess the conversion of CySH (see below), which in all cases was found to be quantitative. Reactions carried out at pH = 5.2 and 6.3 were stopped after 2 h; after this time conversion of CySH was found to be 50% and 30%, respectively. The reaction carried out at pH = 7.0 showed no conversion at all of CySH after 60 min.

Assessment of CySH Conversion. After the A_{max} value was reached, an extra aliquot of $NaNO_2$ (0.5 molar equiv) was added and then 12 M HCl to set the pH at 0.5. Under these conditions the CySH is quantitatively converted into CySNO, as reported before. The amount of unreacted CySH can be calculated from the enhancement of absorbance. After complete decomposition of CySNO occurred, the reaction mixture was analyzed by capillary electrophoresis. In all experiments CySSCy was the only detected product in 100% yield.

Reactions of CySH 0.04M with NO_2. A 35 mL glass tube was filled with dioxygen, and then 23 mL of gaseous NO was added at atmospheric pressure: the brown color of NO_2 immediately appeared. The reaction tube was placed in a thermostated bath, and a CySH (1 mmol, 121 mg) aqueous solution (25 mL), buffered at pH = 2.5, was then added. The A_{max} value was reached after 15 min. The reaction mixture was tested to determine the conversion of CySH (45%). From

A_{max} = 0.235 the yield of CySNO (78%) and CySSCy (22%) was calculated. In a repeated reaction, 46 mL of NO (2 mmol) was added to the sample tube containing dioxygen and then the CySH (1 mmol, 121 mg) aqueous solution buffered at pH = 2.5. The conversion of CySH, after 15 min, was 90%. From A_{max} = 0.481 the yield of CySNO (80%) and CySSCy (20%) was calculated.

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