

# Spectroscopic Estimation of the Extent of *S*-Nitrosothiol Formation by Nitrite Action on Sulfhydryl Groups

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A study of the interaction between cysteine (CysSH) and nitrite ion in weakly acidic media to give *S*-nitrosocysteine (cysteine thionitrite, CysSNO) shows that this is an equilibrium reaction:  $H^+ + NO_2^- + CysSH \rightleftharpoons CysSNO + H_2O$ . Absorbance measurements of the 336-nm band of *S*-nitrosocysteine over a range of reactant concentrations and in buffers of varying pH (3.6–4.2) yield an apparent equilibrium constant (in terms of concentration),  $K_c = 1.84 (\pm 0.26) \times 10^6$ . Calculations based on this value for  $K_c$  imply that, at nitrite concentrations typically used in cured meat products, as much as 3–12% of the nitrite added is converted to thionitrite groups. The formation of CysSNO was directly observed spectroscopically even at pH values as high as 5.5. These results suggest that, under conditions found in nitrite-treated foods, nitrite may also be able to react with free sulfhydryl groups of bacterial enzymes, disrupting their function. Thus, this may represent one mechanism by which nitrites can inhibit bacterial growth.

The reaction of nitrite with the sulfhydryl group of cysteine (CysSH) and with cysteine residues of proteins to produce *S*-nitrosocysteine (cysteine thionitrite, CysSNO) is important in a variety of contexts. In the first place, in addition to being a preservative, nitrite could be a precursor of carcinogenic nitrosamines. A number of workers have studied the formation of *S*-nitrosocysteine groupings in meat products and model systems to elucidate their possible role in nitrosamine formation. Mirna and Hofmann (1969) prepared CysSNO as a model compound and studied its stability. They also presented indirect evidence for the formation of *S*-nitrosothiol (thionitrite, -SNO) groups in nitrite-cured meats and hypothesized that the -SNO moiety could act as a reactive intermediate in the formation of nitrosylmyoglobin and *N*-nitrosamines. Kubberod et al. (1974) investigated the specific interaction between nitrite and sulfhydryl groups of myosin. Emi-Miwa et al. (1976) studied thionitrite formation in meat and model systems as part of the more general problem of tracing nitrite depletion in cured meats; related studies were carried out by Sebranek et al. (1976). Massey et al. (1978) compared *S*-nitrosation to *C*- and *N*-nitrosation in a protein-based model system. These workers (Dennis et al., 1979, 1980) also studied the reaction of *S*-nitrosocysteine with secondary amines in relation to *N*-nitrosamine formation in cured meats. In addition, Oae et al. (1977, 1978) have demonstrated the ability of *S*-nitrosothiols to transnitrosate secondary amines in nonaqueous environments to yield *N*-nitrosamines. A more recent report (Natake et al., 1979) indicates that reaction systems containing nitrite and cysteine exhibit both DNA-damaging and mutagenic activity toward *Salmonella typhimurium* TA1535 and TA98. These researchers infer that the active product of this system is CysSNO.

Some evidence has been presented indicating that thionitrites are more potent bacterial growth inhibitors than inorganic nitrites and that they might be related to the active antibacterial agent remaining in cured meats after the original nitrite has largely disappeared (Incze et al., 1974; Hansen and Levin, 1975). Nonetheless, the in-

stability of these substances is such that at pH conditions, temperatures, and nitrite concentrations usually employed their most important role may be as reactive intermediates (Mirna and Hofmann, 1969; Hansen and Levin, 1975; Moran et al., 1975). Some later work suggests that CysSNO is probably not the substance responsible for the bacteriostatic response observed in heat-treated, nitrite-containing media known as the Perigo effect (Asan and Solberg, 1976; Perigo et al., 1967). Although *S*-nitrosocysteine can act as an antioxidant in cured meat (Kanner, 1979), later studies with comminuted turkey meat suggest that its anticlostridial activity is limited (Kanner and Juven, 1980).

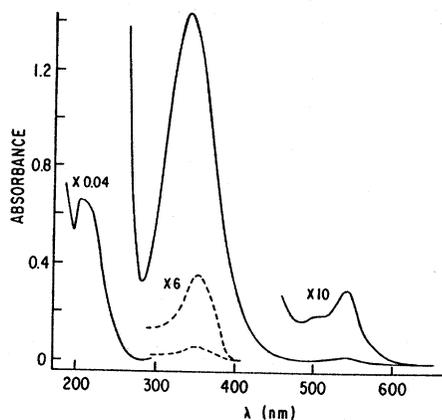
Of much greater importance with respect to determining plausible mechanisms by which nitrite functions to inhibit bacterial growth is the possibility that nitrites may react with the sulfhydryl groups of specific bacterial enzymes, such as glyceraldehyde-3-phosphate dehydrogenase, thereby disrupting their function (O'Leary and Solberg, 1976). Earlier work with this dehydrogenase and with rabbit muscle aldolase indicating that enzyme activity decreased when the free sulfhydryl groups were chemically modified further supports this hypothesis (Boyer and Schulz, 1959). Interactions of nitrites with enzymes to form -SNO groups may occur at lower acidities and nitrite levels than are necessary to yield simple thionitrites (O'Leary, 1974). Mechanistic studies of such interactions should provide some of the basic information required to develop safe antibacterial additives.

*S*-Nitrosothiols serve other functions of biochemical interest besides the ones observed in nitrite-treated food products. In a continuing series of papers, a group at Tulane University School of Medicine has shown that CysSNO is a potent activator of the enzyme guanylate cyclase (Ignarro et al., 1981, and references cited therein). This ubiquitous enzyme catalyzes the transformation of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). The data imply that nitrosothiols are likely intermediates in the pharmacological action of alkyl nitrites, nitroglycerin, and related species as coronary and pulmonary vasodilators.

In all these ramifications concerning the role of nitrosothiols in nitrite-related problems, the extent of formation and the stability of *S*-nitrosocysteine under different conditions are of paramount importance. Yet these aspects of nitrite activity have heretofore received little systematic attention. We are engaged in a study of the equilibria and

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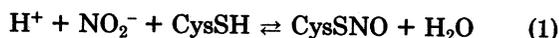
**Figure 1.** Absorption spectra of  $2.64 \times 10^{-3}$  M *S*-nitrosocysteine (solid line) at pH 2.0 and of  $2.64 \times 10^{-3}$  M sodium nitrite (dashed line) at pH 4.0.

the kinetics of the formation of the *S*-nitrosothiol grouping. The present communication deals with the spectroscopic determination of an empirical equilibrium constant for *S*-nitrosocysteine formation under various experimental conditions.

#### EXPERIMENTAL SECTION

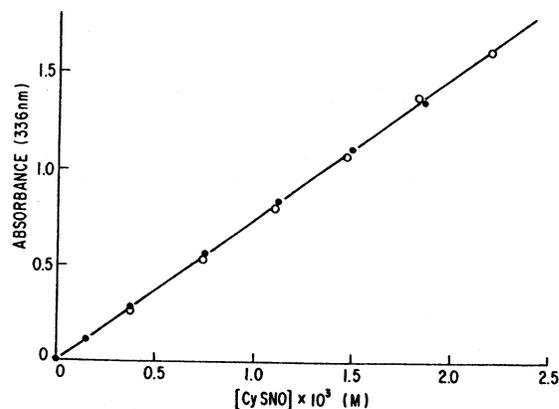
**Chemicals and Instrumentation.** All chemicals used for this study were commercially available, reagent-grade materials. The pH 2.0 buffer was a commercially prepared solution (Fisher certified) of HCl/KCl, 0.05 M in concentration. The 0.05 M acetic acid/sodium acetate buffers (pH 3.62 to pH 4.24) were prepared by us. Buffered solutions of sodium nitrite and cysteine were freshly prepared prior to each set of runs and kept at  $\sim 1^\circ\text{C}$ . The purity of the crystalline materials was verified by infrared spectroscopy. UV-visible spectra were run on either a Cary 14 spectrophotometer or a Zeiss Model PMQ LL by using matched, 1.0-cm, fused quartz cells. The cell compartments were purged with dry  $\text{N}_2$ . All pH measurements were made with a Radiometer pH meter, type PHM4c, and a glass electrode standardized with commercially prepared buffer solutions.

**Spectroscopic Measurements.** The reaction under investigation may be stoichiometrically formulated as



This reaction is a strongly pH-dependent equilibrium. As will be discussed in the next section, when the pH is approximately 2 or less, use of a moderate excess of CysSH forces the reaction essentially to completion, converting nearly all of the nitrite initially present into CysSNO.

The spectrum of buffered CysSNO (pH 2.0) from 190 to 650 nm is presented in Figure 1. CysSNO exhibits four maxima at  $\sim 220$  nm ( $\epsilon = 9700 \text{ M}^{-1} \text{ cm}^{-1}$ ), 336 nm ( $\epsilon = 738 \text{ M}^{-1} \text{ cm}^{-1}$ ), 503 nm (sh) ( $\epsilon = 5 \text{ M}^{-1} \text{ cm}^{-1}$ ), and 543 nm ( $\epsilon = 14 \text{ M}^{-1} \text{ cm}^{-1}$ ). The first band, although most intense, overlaps a moderately strong band at 198 nm ( $\epsilon = 1370 \text{ M}^{-1} \text{ cm}^{-1}$ ) due to both the carboxylate and the sulfhydryl groups of unreacted, free cysteine. The two, weak bands in the visible region (at 503 and 543 nm) are essentially free from overlap but lack sufficient intensity for quantitative measurement of CysSNO at concentrations below  $\sim 1.0 \times 10^{-2}$  M. We therefore used the moderately strong band at 336 nm for determining CysSNO concentration. A weak absorption band of nitrite ion in equilibrium with nitrous acid, with a maximum at 350 nm (dashed line, Figure 1), overlaps this CysSNO absorption. Fortunately, at pH 4.0, the total absorptivity of  $\text{NO}_2^-$  and HONO at 336 nm is merely  $19 \text{ M}^{-1} \text{ cm}^{-1}$ . Neither free cysteine nor the



**Figure 2.** Absorbance at 336 nm vs. concentration of *S*-nitrosocysteine at pH 2.0, assuming completion of reaction 1. Open circles: dilutions of a buffered solution containing initially  $4.752 \times 10^{-3}$  M cysteine and  $1.867 \times 10^{-3}$  M nitrite. Solid circles: buffered solutions with initial cysteine/nitrite ratios of 89/1 to 15/1, based on an initial cysteine concentration of  $3.270 \times 10^{-2}$  M. ( $R = 0.99973$ .)

buffers absorb in this spectral region.

To determine  $\epsilon_{\text{CysSNO}}$ , the molar absorptivity of CysSNO at 336 nm, we ran reaction 1 in a pH 2.0 buffer using an excess of CysSH relative to nitrite (see the legend of Figure 2 for details). These conditions ensure that the reaction runs essentially to completion. Figure 2 gives the observed absorbance at 336 nm as a function of CysSNO concentration. Over the concentration range studied ( $0.15 \times 10^{-3}$  to  $1.9 \times 10^{-3}$  M CysSNO) deviations from linearity are below experimental error. The value found for  $\epsilon_{\text{CysSNO}}$  at 336 nm is  $738 \text{ M}^{-1} \text{ cm}^{-1}$ . [Mirna and Hofmann (1969) report a value  $800 \text{ M}^{-1} \text{ cm}^{-1}$ ; Dennis et al. (1979) state that  $\epsilon_{\text{CysSNO}} = 870 \text{ M}^{-1} \text{ cm}^{-1}$ . Neither group, however, describes how their values are determined.]

Under conditions where reaction 1 does not proceed to completion, both CysSNO and nitrite are simultaneously present in solution. The observed absorbance at 336 nm is now the sum of the absorbances of both species:

$$A_{336} = \epsilon_{\text{CysSNO}}[\text{CysSNO}] + \epsilon_{\text{nit}}([\text{nit}]_0 - [\text{CysSNO}]) \quad (2)$$

Here  $[\text{nit}]_0$  is the initial stoichiometric concentration of all nitrite species present, i.e.,  $[\text{nit}]_0 = [\text{NO}_2^-] + [\text{HONO}]$ .  $\epsilon_{\text{CysSNO}}$  is obtained from Figure 2 as described above;  $\epsilon_{\text{nit}} = 19 \text{ M}^{-1} \text{ cm}^{-1}$ .

Rearrangement of eq 2 yields

$$[\text{CysSNO}] = (A_{336} - \epsilon_{\text{nit}}[\text{nit}]_0) / (\epsilon_{\text{CysSNO}} - \epsilon_{\text{nit}}) \quad (3)$$

Because thionitrites are unstable both thermally (Cantoni et al., 1975) and photochemically (Barrett et al., 1966), all reactions were run by using an ice bath (the temperature of the reaction mixture itself was  $1.0 \pm 0.5^\circ\text{C}$ ) under minimal incandescent lighting. To prevent decomposition of the product in the UV light of the absorption spectrophotometer, fresh aliquots were taken just before each measurement. Ionic strength was kept approximately constant by the 0.05 M acetic acid/acetate buffers.

Figure 3 shows a typical plot of the observed absorbance at 336 nm vs. time as the reaction proceeds toward equilibrium. Under the given conditions the reaction reaches equilibrium in about 3 h.

#### RESULTS AND DISCUSSION

Table I gives the initial composition data and the results of 10 experimental runs under various conditions designed to establish the numerical value of an apparent equilibrium constant for the formation of *S*-nitrosocysteine from cysteine and nitrite at  $0^\circ\text{C}$ . The initial cysteine concentration

Table I. Empirical Equilibrium Constant  $Q_c$  for *S*-Nitrosocysteine Formation from Nitrite and Cysteine at 0 °C

run	$[\text{CysSH}]_0^a$ [nit] <sub>0</sub>	$[\text{CysSH}]_0^b$	$[\text{nit}]_0^b$	pH	$A_{336}^c$	$[\text{CysSNO}]_{\text{eq}}^{b,d}$	$Q_c \times 10^{-6}^e$	$\log Q_c^e$
1	0.99	4.128	4.131	4.24	0.816	1.005	1.79	6.25
2	1.00	4.128	4.128	3.62	1.550	2.029	1.91	6.28
3	1.46	5.416	3.694	3.90	1.266	1.671	1.75	6.24
4	2.09	6.201	2.955	3.90	1.056	1.397	1.48	6.17
5	2.30	6.779	2.947	3.89	1.330	1.780	2.37	6.37
6	2.37	7.018	2.955	3.90	1.175	1.563	1.64	6.21
7	2.98	16.832	5.653	3.98	3.10	4.075	1.94	6.29
8	4.57	17.207	3.768	3.98	2.20	2.898	2.23	6.35
9	5.23	15.423	2.947	3.89	1.625	2.191	1.70	6.23
10	6.91	20.039	2.947	3.89	1.700	2.296	1.54	6.18
							mean: $1.84 \pm 0.26^f$	$6.26 \pm 0.06^f$

<sup>a</sup>  $[\text{CysSH}]_0$  = initial cysteine concentration;  $[\text{nit}]_0$  = initial nitrite concentration. <sup>b</sup> Concentration in units of  $10^{-3}$  M. <sup>c</sup> Absorbance at 336 nm. <sup>d</sup> *S*-Nitrosocysteine concentration at equilibrium, calculated by using  $A_{336}$  and eq 4. <sup>e</sup>  $Q_c$ , in terms of molar concentrations (M), as defined by eq 5. <sup>f</sup> Standard deviation from the mean.

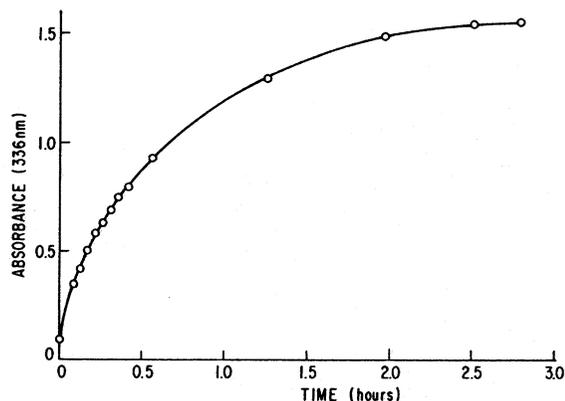


Figure 3. Extent of reaction of cysteine with nitrite as a function of time as monitored by CysSNO absorbance at 336 nm. pH 3.62.  $[\text{CysSH}]_0 = 4.128 \times 10^{-3}$  M.  $[\text{NO}_2^-]_0 = 4.129 \times 10^{-3}$  M.  $[\text{CysSNO}]_{\text{eq}} = 2.029 \times 10^{-3}$  M. HOAc/NaOAc buffer (0.05 M).

was varied between the limits of ca.  $4 \times 10^{-3}$  M and  $20 \times 10^{-3}$  M; the nitrite concentration ranged between ca.  $3 \times 10^{-3}$  and  $5.7 \times 10^{-3}$  M. The ratio of the initial cysteine concentration to the initial nitrite concentration,  $[\text{CysSH}]_0/[\text{NO}_2^-]_0$ , thus spanned values from about 1 to 7. The pH range was from 3.62 to 4.24 units. At low pH values the reaction approaches completion so closely that accurate equilibrium values cannot be obtained. At higher pHs reaction 1 becomes so slow that decomposition via the reaction (Dennis et al., 1979; Oae et al., 1977, 1978)



becomes significant, making quantitative equilibrium determinations difficult, if not impossible. Nevertheless, as shown in Figure 4, we can still observe the formation of appreciable quantities of CysSNO even at pH 5.5. (As discussed below, this falls within the pH limits encountered in cured meat products.)

Equilibrium concentrations of CysSNO were calculated from observed absorbances at 336 nm ( $A_{336}$  in Table I) by means of eq 3. Based on the stoichiometry of reaction 1 these values were in turn used to obtain equilibrium concentrations of unreacted cysteine and nitrite. Hydrogen ion concentration was taken from pH measurements of the buffered reaction mixtures. The reaction quotient  $Q_c$ , which gives the most nearly constant value for these data, is

$$Q_c = [\text{CysSNO}]/([\text{H}^+][\text{nit}][\text{CysSH}]) \quad (5)$$

where  $[\text{nit}] = [\text{NO}_2^-] + [\text{HONO}]$ . That is,  $[\text{nit}]$  is the total stoichiometric concentration of nitrite species at equilibrium. All solutions were sufficiently dilute for molarities

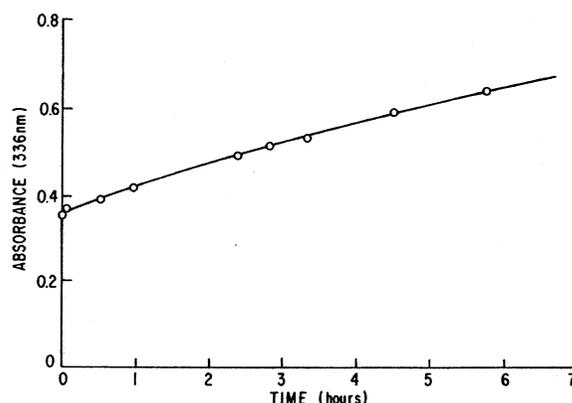
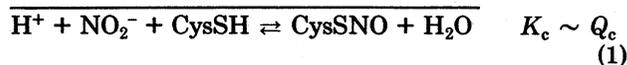


Figure 4. Extent of reaction of cysteine with nitrite as a function of time as monitored by CysSNO absorbance at 336 nm. pH 5.52.  $[\text{CysSH}]_0 = 2.064 \times 10^{-2}$  M;  $[\text{NO}_2^-]_0 = 2.065 \times 10^{-2}$  M. HOAc/NaOAc buffer (0.05 M).

to be substituted for mole fractions. If all activity coefficients are nearly unity (unit activity may be assumed for water because the solutions are dilute),  $Q_c$  approximates the true thermodynamic equilibrium constant  $K_c$ . Because nitrite ion is in equilibrium with nitrous acid at the  $\text{H}^+$  concentrations involved in this study, reaction 1 is strictly speaking better represented as the sum of two chemical equations:



Here  $K_a$  is the dissociation constant for nitrous acid and has the value  $4.37 \times 10^{-4}$  (Cassens et al., 1979). Other species such as  $\text{N}_2\text{O}_3$ ,  $\text{H}_2\text{NO}_2^+$ , and  $\text{NO}^+$ , although of potential importance kinetically, exist in extremely low concentration in aqueous solutions except at very acid pHs (Turney and Wright, 1959). The simple form of the empirically determined mathematical expression for  $Q_c$  (eq 5) corroborates this.

The average value in terms of molar concentrations for the apparent equilibrium constant  $Q_c \sim K_c$ , as derived from the data of Table I, is  $1.84 \times 10^6$ . The standard deviation of the calculated values of  $Q_c$  from the mean value of  $Q_c$  is  $0.26 \times 10^6$ . Because  $Q_c \sim K_c = K_1K_2 = K_2/K_a$  (eq 6a, 6b, and 1) we find that  $K_2$ , the equilibrium constant for eq 6b, has a value of  $8.03 \times 10^2$ .

Figure 5 shows the value of  $\log Q_c$  as a function of the initial cysteine/nitrite ratio,  $[\text{CysSH}]_0/[\text{NO}_2^-]_0$ . This demonstrates the constancy of  $Q_c$  with respect to variations

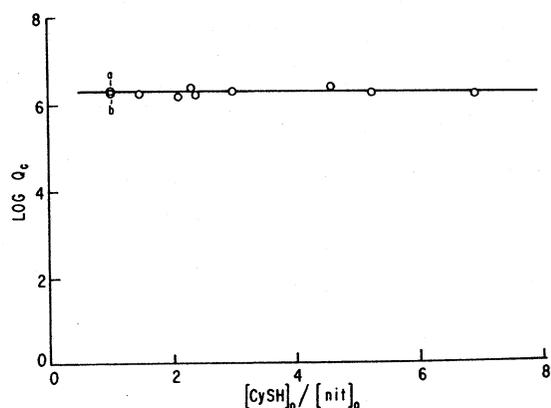


Figure 5.  $\log Q_c$  at increasing ratios of initial cysteine to nitrite concentration. pH  $3.89 \pm 0.01$ , except for first two points. Point a: pH 3.62. Point b: pH 4.24.

in the initial nitrite concentration as well as the initial cysteine concentration.  $\log Q_c$  was used by analogy to the frequently used terms pH and pK, because such a presentation best depicts variations in quantities which in principle can assume values ranging over several orders of magnitude. The calculated mean for  $\log Q_c$  is  $6.26 \pm 0.06$ .

Independence of the observed  $Q_c$  values of pH (within experimental error) is indicated by the closeness of the first two points in Figure 5, which were obtained at pH 3.62 and pH 4.24, respectively. More exhaustive studies of the pH dependence of  $\log Q_c$  are not feasible because of previously cited reasons.

Although the apparent equilibrium constant is certainly valid over the concentration range studied, extrapolation beyond these conditions must be done with care. This becomes especially important under conditions where known side reactions may occur. Cystine (CysSSCys) formation via decomposition of the thionitrite (eq 4), particularly at ambient temperatures and above, and at pH approaching neutrality, has already been mentioned. When very large excesses of free thiol are present, a related reaction may take place (Oae et al., 1977, 1978):



Large excesses of nitrite, on the other hand, can lead to the formation of thiirancarboxylic acids (Maycock and Stoodley, 1976). Limiting the  $[\text{CysSH}]_0/[\text{NO}_2^-]_0$  ratio, as in this study, to values from 1 to 7 largely circumvents these difficulties.

Two other potential complications do not arise under the present conditions. One is nitrous acid decomposition at low pH (Cotton and Wilkinson, 1980). At  $0^\circ\text{C}$ , the absorption spectra of buffered aqueous solutions of  $\text{NaNO}_2$  (pH 2.5 to pH 5.5) were observed to be unchanged over a period of several hours. The second is deamination of the amino group on the cysteine. Apparently this reaction, which yields  $\text{N}_2$  gas as a byproduct, is sufficiently slow relative to the formation of the thionitrite (Massey et al., 1978) that we observe no appreciable interference. Indeed, no gas bubbles were seen during our equilibrium measurements.

With the value for  $Q_c$  in hand, we may now verify the validity of our previous assumption that reaction 1 runs nearly to completion at pH 2.0. Taking values for the initial reactant concentrations as described for the first run shown in Figure 2, and using eq 5, we may calculate the equilibrium concentration of all nitrite species,  $[\text{nit}]_{\text{eq}}$ . Because nitrite is the limiting reagent, we set  $x = [\text{nit}]_{\text{eq}}$ ,  $[\text{CysSNO}]_{\text{eq}} = [\text{nit}]_0 - x$ , and  $[\text{CysSH}]_{\text{eq}} = [\text{CysSH}]_0 - [\text{nit}]_0$

+  $x$ . Solving for  $x$  we find  $[\text{nit}]_{\text{eq}} = x = 3.41 \times 10^{-5}$  M. This shows that under these conditions  $\sim 98\%$  of the original nitrite was converted to CysSNO.

In a similar manner the value of  $Q_c$  may be used to estimate the maximum quantity of *S*-nitrosothiol present in a nitrite-cured meat product under ordinary conditions. According to Cassens et al. (1979), the pH of meat (muscle) becomes more acidic after slaughter due to post-mortem glycolysis. Characteristic values range from 6.0 to 5.5. Free sulfhydryl groups in meat vary in concentration from  $21 \times 10^{-3}$  to  $25 \times 10^{-3}$  M (Hamm and Hofmann, 1966). When a typical amount of sodium nitrite (100 ppm) is added and if an average density of  $1 \text{ g cm}^{-3}$  is assumed,  $[\text{nit}]_0 = 1.5 \times 10^{-3}$  M. Again using eq 5, but now with  $x = [\text{CysSNO}]_{\text{eq}}$ , we calculate that  $[\text{CysSNO}]_{\text{eq}}$  ranges from  $5.57 \times 10^{-5}$  to  $1.89 \times 10^{-4}$  M under these conditions. This is equivalent to 3.7–12.6% conversion of added nitrite to *S*-nitrosothiol. These calculations agree well with measurements reported by Cassens et al. (1979), which indicate that reaction of nitrite with free sulfhydryl groups accounts for 5–15% of the observed nitrite loss in cured meat products. Direct observation of CysSNO formation at pH 5.5 (Figure 4) corroborates these calculations. To reiterate, such extrapolations provide but an upper limit on the extent of *S*-nitrosothiol formation in nitrite-processed foods, because competing reactions (for example, eq 4 and 7) may become important. Nonetheless, in spite of the inherent uncertainties, these results suggest to us that under such conditions significant interaction may also occur between nitrite and sulfhydryl groups of bacterial enzymes.

Registry No. L-Cysteine, 52-90-4; *S*-nitrosocysteine, 51209-75-7; nitrite, 14797-65-0.

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