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: $H^+ + NO_2^- + CysSH \rightleftarrows CysSNO + H_2O$. 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 transitrosate 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 instability 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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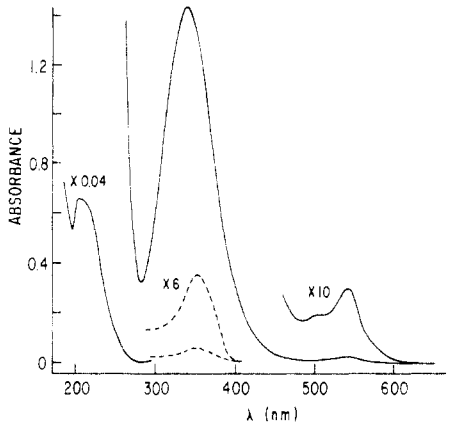

Figure 1. Absorption spectra of 2.64×10^{-3} M *S*-nitrosocysteine (solid line) at pH 2.0 and of 2.64×10^{-3} M sodium nitrite (dashed line) at pH 4.0.

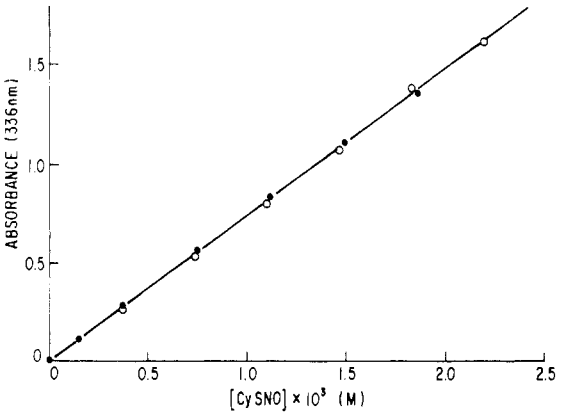

Figure 2. Absorbance at 336 nm vs. concentration of *S*-nitrosocysteine at pH 2.0, assuming completion of reaction 1. Open circles: dilutions of a buffered solution containing initially 4.752×10^{-3} M cysteine and 1.867×10^{-3} M nitrite. Solid circles: buffered solutions with initial cysteine/nitrite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

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 ~1 °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 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

$$H^+ + NO_2^- + CysSH \rightleftarrows CysSNO + H_2O \quad (1)$$

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 ~220 nm (ϵ = 9700 M^{-1} cm^{-1}), 336 nm (ϵ = 738 M^{-1} cm^{-1}), 503 nm (sh) (ϵ = 5 M^{-1} cm^{-1}), and 543 nm (ϵ = 14 M^{-1} cm^{-1}). The first band, although most intense, overlaps a moderately strong band at 198 nm (ϵ = 1370 M^{-1} 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 NO_2^- and HONO at 336 nm is merely 19 M^{-1} cm^{-1}. Neither free cysteine nor the buffers absorb in this spectral region.

To determine ϵ_{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 (0.15×10^{-3} to 1.9×10^{-3} M CysSNO) deviations from linearity are below experimental error. The value found for ϵ_{CysSNO} at 336 nm is 738 M^{-1} cm^{-1}. [Mirna and Hofmann (1969) report a value 800 M^{-1} cm^{-1}; Dennis et al. (1979) state that ϵ_{CysSNO} = 870 M^{-1} 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:

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

Here $[nit]_0$ is the initial stoichiometric concentration of all nitrite species present, i.e., $[nit]_0 = [NO_2^-] + [HONO]$. ϵ_{CysSNO} is obtained from Figure 2 as described above; ϵ_{nit} = 19 M^{-1} cm^{-1}.

Rearrangement of eq 2 yields

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

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 ± 0.5 °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 °C. The initial cysteine concentration

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

run	$\frac{[CysSH]_0}{[nit]_0}$ [a]	$[CysSH]_0$ [b]	$[nit]_0$ [b]	pH	A_{336} [c]	$[CysSNO]_{eq}$ [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 ± 0.26 [f]	6.26 ± 0.06 [f]

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

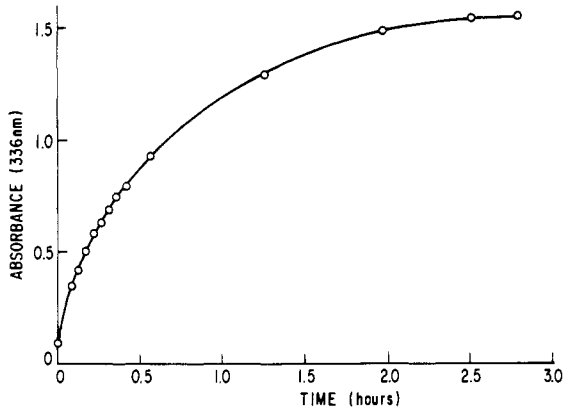

Figure 3. Extent of reaction of cysteine with nitrite as a function of time as monitored by CysSNO absorbance at 336 nm. pH 3.62. $[CysSH]_0 = 4.128 \times 10^{-3}$ M. $[NO_2^-]_0 = 4.129 \times 10^{-3}$ M. $[CysSNO]_{eq} = 2.029 \times 10^{-3}$ M. HOAc/NaOAc buffer (0.05 M).

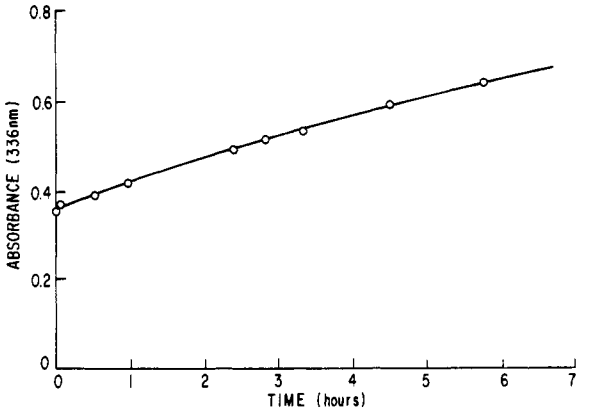

Figure 4. Extent of reaction of cysteine with nitrite as a function of time as monitored by CysSNO absorbance at 336 nm. pH 5.52. $[CysSH]_0 = 2.064 \times 10^{-2}$ M; $[NO_2^-]_0 = 2.065 \times 10^{-2}$ M. HOAc/NaOAc buffer (0.05 M).

was varied between the limits of ca. 4×10^{-3} M and 20×10^{-3} M; the nitrite concentration ranged between ca. 3×10^{-3} and 5.7×10^{-3} M. The ratio of the initial cysteine concentration to the initial nitrite concentration, $[CysSH]_0/[NO_2^-]_0$, 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)

$$2CysSNO \rightarrow CysSSCys + 2NO\uparrow \quad (4)$$

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

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

where $[nit] = [NO_2^-] + [HONO]$. That is, [nit] is the total stoichiometric concentration of nitrite species at equilibrium. All solutions were sufficiently dilute for molarities 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 H^+ concentrations involved in this study, reaction 1 is strictly speaking better represented as the sum of two chemical equations:

$$H^+ + NO_2^- \rightleftarrows HONO \qquad K_1 = 1/K_a \quad (6a)$$

$$HONO + CysSH \rightleftarrows CysSNO + H_2O \qquad K_2 \quad (6b)$$

$$H^+ + NO_2^- + CysSH \rightleftarrows CysSNO + H_2O \qquad K_c \sim Q_c \quad (1)$$

Here K_a is the dissociation constant for nitrous acid and has the value 4.37×10^{-4} (Cassens et al., 1979). Other species such as N_2O_3, $H_2NO_2^+$, and 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 1.84×10^6. The standard deviation of the calculated values of Q_c from the mean value of Q_c is 0.26×10^6. 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 8.03×10^2.

Figure 5 shows the value of log Q_c as a function of the initial cysteine/nitrite ratio, $[CysSH]_0/[NO_2^-]_0$. This demonstrates the constancy of Q_c with respect to variations

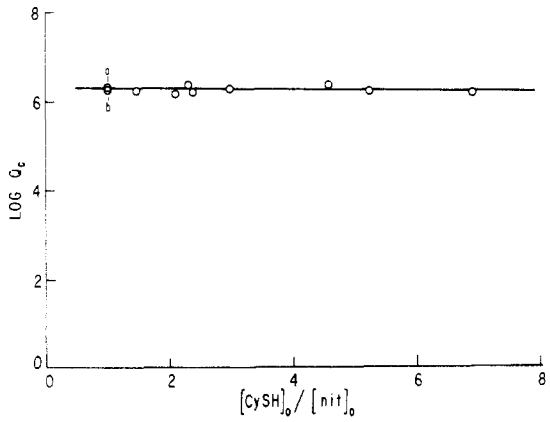

Figure 5. log Q_c at increasing ratios of initial cysteine to nitrite concentration. pH 3.89 ± 0.01, except for first two points. Point a: pH 3.62. Point b: pH 4.24.

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 ± 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):

$$2CysSNO + 2CysSH \rightarrow 2CysSSCys + N_2O + H_2O \quad (7)$$

Large excesses of nitrite, on the other hand, can lead to the formation of thiirancarboxylic acids (Maycock and Stoodley, 1976). Limiting the $[CysSH]_0/[NO_2^-]_0$ 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 °C, the absorption spectra of buffered aqueous solutions of $NaNO_2$ (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 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, $[nit]_{eq}$. Because nitrite is the limiting reagent, we set $x = [nit]_{eq}$, $[CysSNO]_{eq} = [nit]_0 - x$, and $[CysSH]_{eq} = [CysSH]_0 - [nit]_0 + x$. Solving for x we find $[nit]_{eq} = x = 3.41 \times 10^{-5}$ M. This shows that under these conditions ~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 21×10^{-3} to 25×10^{-3} M (Hamm and Hofmann, 1966). When a typical amount of sodium nitrite (100 ppm) is added and if an average density of 1 g cm^{-3} is assumed, $[nit]_0 = 1.5 \times 10^{-3}$ M. Again using eq 5, but now with $x = [CysSNO]_{eq}$, we calculate that $[CysSNO]_{eq}$ ranges from 5.57×10^{-5} to 1.89×10^{-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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An Investigation into the Potential Formation of N-Substituted Amides and Their Nitrosated Derivatives during the Frying of Bacon

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The formation of N-substituted amides was investigated by using both model systems and bacon. Fatty acids were shown to react readily with selected α-amino acids in model systems at 200 °C to give N-substituted amides. However, formation of primary amines via the decarboxylation of α-amino acids appears to be unlikely at temperatures normally encountered in pan frying of bacon due to insufficient energy for the decarboxylation step. Under these conditions, only amines would react readily with fatty acids to yield secondary amides. N-substituted amides were shown to be nitrosated readily under acid conditions in a model system. It was also demonstrated that *N*-nitrosamides are very unstable under conditions commonly encountered in cooking bacon and thus are unlikely to be present in heat-processed foods.

N-Nitrosamides which arise from the reaction of secondary amides with nitrite (Mirvish, 1977) usually have one alkyl residue and an acyl residue. They are chemically reactive compounds and are relatively easily hydrolyzed to alkylating diazoalkanes (Preussmann, 1974). They are considered to exert both local and systemic activity in the carcinogenesis of experimental animals (Preussmann, 1974). Although their powerful carcinogenic responses are well-known, there has been only a limited number of studies on the occurrence of nonvolatile *N*-nitroso compounds in food systems, due in part to their instability under neutral and alkaline conditions (Mirvish, 1971). However, the precursors of *N*-nitrosamides have been reported in certain foods. Such compounds include uridine and ureas which have been isolated from fish by Mirvish (1975). High concentrations of agmatine, a decarboxylation product of arginine, have been reported in fresh abalone (Kawabata et al., 1978), and citrulline has been reported by Wada (1930) in watermelon.

Recently, Sims and Fioriti (1975) reported that heating fatty acids (or esters) and triglycerides with α-amino acids at temperatures above 150 °C resulted in the formation of N-substituted amides. These results were confirmed by Kakuda and Gray (1980a) using a model system containing amino acids or free amines and fatty acids. They reported that the presence of a secondary amino group in these compounds makes them susceptible to nitrosation. These compounds may thus represent another source of nitrosatable species available for reaction with nitrite.

The present study was undertaken to establish whether N-substituted amides can be formed under conditions encountered in the processing and cooking of bacon and thus be potential precursors of *N*-nitrosamides. Specific objectives of this study were (1) to investigate the formation of N-substituted amides from reactions between fatty acids and/or triglycerides with α-amino acids and amines in both model and bacon systems, (2) to investigate the nitrosation of N-substituted amides in both model and bacon systems, and (3) to study the thermal stability of *N*-nitrosamides during the frying process.

MATERIALS AND METHODS

Note: Because of the extremely hazardous nature of N-nitroso compounds, all work was carried out in efficient fume cupboards whenever possible, and extreme caution was exercised in handling these components.

Reagents. All chemicals and solvents employed were of analytical grade and were used without further purification. Fatty acids and their methyl esters were purchased from Fisher Scientific Co., Fair Lawn, NJ. Pentylamine, norleucine, valine, and methionine were purchased from Eastman Kodak Co., Rochester, NY. Column packing materials were obtained from Supelco, Inc., Bellefonte, PA. Pork bellies were purchased from a local supplier soon after slaughter and stored in a cooler at 2 °C until used.

Preparation of N-Substituted Amides in Model Systems. A series of N-substituted amides were prepared by reacting norleucine with lauric, myristic, palmitic, stearic, oleic, and linoleic acids at 200 °C for 1 h. The unreacted amino acid was removed by suction filtration after the addition of warm (30 °C) diethyl ether to the reaction flask. After removal of the solvent by evaporation, the residue was redissolved in a minimum of warm (40 °C) petroleum ether and then left at room temperature to crystallize. Further purification of the prepared amides was carried out as previously described by Kakuda and Gray (1980a).

Analysis and Identification of N-Substituted Amides. The purity of the synthesized *N*-pentyllauramide, *N*-pentylmyristamide, *N*-pentylpalmitamide, *N*-pentyl-

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