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Nitrosylation of Myoglobin and Nitrosation of Cysteine by Nitrite in a Model System Simulating Meat Curing

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ABSTRACT: Demand is growing for meat products cured without the addition of sodium nitrite. Instead of the direct addition of nitrite to meat in formulation, nitrite is supplied by bacterial reduction of natural nitrate often added as vegetable juice/powder. However, the rate of nitrite formation in this process is relatively slow, and the total ingoing nitrite is typically less than in conventional curing processes. The objective of this study was to determine the impact of the rate of addition of nitrite and the amount of nitrite added on nitrosylation/nitrosation reactions in a model meat curing system. Myoglobin was preferentially nitrosylated as no decrease in sulfhydryl groups was found until maximum nitrosylmyoglobin color was achieved. The cysteine−myoglobin model retained more sulfhydryl groups than the cysteine-only model ($p < 0.05$). The rate of nitrite addition did not alter nitrosylation/nitrosation reactions ($p > 0.05$). These data suggest that the amount of nitrite but not the rate of addition impacts the nitrosylation/nitrosation reactions this system.

KEYWORDS: *sodium nitrite, cysteine, myoglobin, nitrosylation, cured meat model*

INTRODUCTION

Curing meat incorporates a complex set of chemical reactions, some of which are not fully understood. Meat preservation by meat curing has been documented for over 5000 years and likely began by using salt contaminated with saltpeter (calcium or potassium nitrate) to preserve meat.[1] In the 1890s, it was determined that nitrite, not nitrate, was necessary for cured meat production.[2] During the 1950−1970s, concerns about nitrate, nitrite, and *n*-nitrosamine formation surfaced following illnesses in animals fed fishmeal produced with sodium nitrite.[3,4] The National Academy of Science has supported the safety and continued use of sodium nitrite and nitrate in food products.[5,6] Growing evidence now supports the importance of nitrite and nitrate in many biological functions.[7] Still, a significant number of consumers have shunned the use of these and other common food ingredients as indicated by the rapid growth observed in the natural and organic food market.[8,9] Although research does not show health benefits in consuming organic versus conventionally produced foods,[10] the perception of improved healthfulness is one of the commonly cited reasons for purchasing these categories of foods.[11,12]

Because of U.S. Department of Agriculture (USDA) regulations governing natural and organic foods, sodium nitrite and nitrate are among the many commonly used ingredients for conventional products that are not allowed in natural or organic foods.[13−16] However, by utilizing natural nitrate sources, primarily celery juice/powder, and a nitrate-reducing starter culture, nitrite can be formed in natural and organic processed meats and will produce characteristics similar to conventionally cured products that are produced with direct addition of sodium nitrite.[17,18] While these naturally cured products look and taste like traditionally cured meats, Jackson et al.[19] and Schrader[20] found increased risk for the growth of *Clostridium perfringens* and *Listeria monocytogenes*, respectively. Many factors can impact pathogen growth in cured meats, but it is likely related at least in part to the curing process. Lower ingoing nitrite concentrations have been reported for naturally cured meats,[17] but this observation could also be affected by the rate that nitrite is formed or added during the curing process. For example, when using bacterial reduction of nitrate in naturally cured meats, nitrite is slowly formed in the system, which could shift the reactions in favor of those with greater substrate reactivity. The addition of all of the nitrite at once, which occurs in conventional curing, might result in a different proportional distribution of nitrite among the various reaction substrates in a meat mixture. This has implications for differential effects of nitrite for creating the typical cured meat properties of antimicrobial protection, color development, and flavor protection.

Meat is a complex system that makes measurement of chemical or biological reactions difficult. Early work used ^{15}N isotopes to determine the fate of nitrite in cured meats[21−23] and identified the partition of nitrite in a meat mixture but could not identify specific reactions and did not clarify the complexity. During curing, myoglobin and cysteine are known to undergo nitrosation/nitrosylation.[24] Myoglobin−nitrite chemistry is among the most well understood of many cured meat reactions.[25,26] Cysteine has been shown to act as a nitrite-reducing compound and nitrosating/nitrosylating agent in cured meats.[27,28] Creating a model system with these compounds could provide a simplified method to determine nitrite reactions as a result of the rate of addition of nitrite to the system. These compounds can be measured relatively easily and could provide insight into the alteration of nitrosated/nitrosylated compounds in natural and traditional meat-curing systems. The objective of this study was to use a simplified model system of cysteine and myoglobin to test the hypothesis

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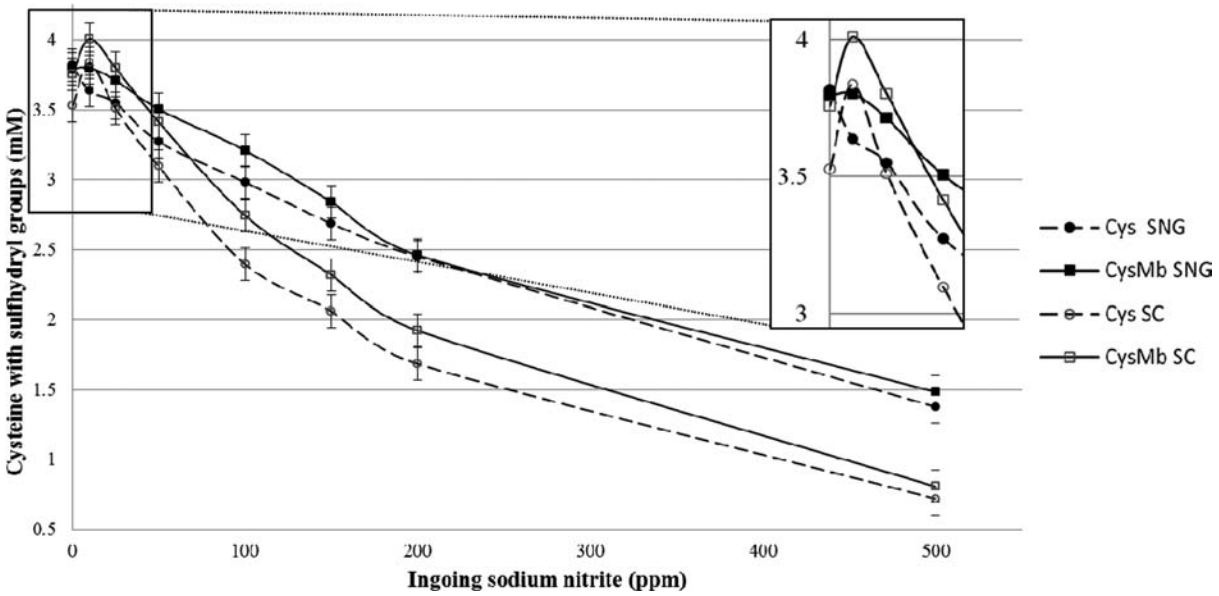

Figure 1. Concentration of cysteine with intact sulfhydryl groups . Cys SNG = cysteine-only model evaluated following simulated bacterial nitrite generation; CysMb SNG = cysteine plus myoglobin model evaluated following simulated bacterial nitrite generation; Cys SC = cysteine-only model evaluated following cooking simulation; and CysMb SC = cysteine plus myoglobin model evaluated following cooking simulation.

that the amount or rate of addition of sodium nitrite will affect some of the reactions commonly occurring during meat curing.

MATERIALS AND METHODS

Solution Preparation and Model System. A cysteine and myoglobin model was prepared to evaluate nitrosation/nitrosylation reactions. The final concentrations in the cysteine plus myoglobin model solution were cysteine (5.06 mM), myoglobin (0.029 mM), and nitrite (0, 0.072, 0.181, 0.362, 0.725, 1.087, 1.450, and 3.623 mM). The final concentrations in the cysteine-only model solutions were cysteine (5.06 mM) and nitrite (0, 0.072, 0.181, 0.362, 0.725, 1.087, 1.450, and 3.623 mM) equivalent to 0, 10, 25, 50, 100, 150, 200, and 500 ppm of ingoing nitrite. U.S. regulations allow the addition of up to 200 ppm of sodium nitrite to meat when nitrite is added as in solution.[29] Prior to the addition of nitrite in this study, the concentrations of myoglobin and cysteine used in the cysteine plus myoglobin and cysteine-only model system were approximately half of those found in fresh ham.[30,31] Cysteine and cysteine plus myoglobin solutions were prepared, and all nitrosylation and nitrosation reactions occurred in a pH 5.6 buffered phosphate solution. This is a typical pH of meat following rigor mortis.

Two 0.1 M phosphate (potassium phosphate, monohydrate) buffer solutions were prepared at pH 5.6 and 7.4. A 0.117 mM stock myoglobin solution was prepared using 0.3 g of myoglobin from equine skeletal muscle (Sigma Aldrich Co., St. Louis, MO) in 150 mL of pH 5.6 phosphate buffer solution. A 20.25 mM stock cysteine solution was prepared with 0.7980 g of L-cysteine hydrochloride hydrate (Acros Organic, Geel, Belgium) in 250 mL of pH 5.6 phosphate buffer solution. Cysteine solutions were utilized immediately following preparation to limit the reduction of sulfhydryl groups due to oxidation and disulfide bond formation.

Sodium nitrite stock solution was prepared by mixing 1 g of sodium nitrite in 1 L of distilled water (14.49 mM) and diluting with distilled water to 0.144, 0.362, 0.724, 1.449, 2.173, 2.899, and 7.246 mM solutions. Distilled water was used for 0 nitrite concentration. Ellman reagent to measure sulfhydryl concentration was prepared with 0.1586 g of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) mixed with 20 mL of phosphate buffer, pH 7.4 (20 mM). Reagents to measure residual nitrite concentration, sulfanilamide and *N*-(1-naphtyhyl) ethylenediamine dihydrochloride (NED), were prepared as described by AOAC method 973.31.[32]

The stock cysteine solution was mixed 1:1 with stock myoglobin solution for the cysteine plus myoglobin model. The stock cysteine solution was also mixed 1:1 with pH 5.6 phosphate buffer for the cysteine-only model. Duplicate test tubes containing 5 mL of cysteine plus myoglobin or cysteine-only solutions were prepared for each nitrite concentration. Samples used to simulate traditional curing had all of the sodium nitrite added at the beginning, while for the simulated natural cure, nitrite was added over time to simulate bacterial generation that occurs in natural curing. For tubes simulating traditional curing, 5 mL of diluted nitrite solution was added at time 0 min of the experiment. To simulate natural curing, 1 mL of diluted nitrite solution was added at time 0 min of the experiment. All nitrite concentrations were evaluated. All tubes were capped and placed in a 35 °C water bath for 60 min to simulate a bacterial generation of nitrite found in natural curing. In the natural curing model, 1 mL of nitrite solution was added every 10 min until a total of 5 mL was added. After 60 min, samples were placed in a 75 °C water bath for 30 min to simulate a cooking process. Three independent replicates were conducted, and duplicate samples were prepared for each treatment combination in each replicate.

Sulfhydryl Concentration. Sulfhydryl concentrations in the model system mixtures were determined using a modified Ellman's reaction.[33] Decreases in sulfhydryl groups were used as a measure of cysteine nitrosylation. In test tubes, 2.97 mL of phosphate buffer, pH 7.4, and 0.03 mL of the model solutions were combined with 0.015 mL of DTNB. Samples were vortexed immediately following DTNB addition. After color development, sample absorbance was measured using a spectrophotometer at 412 nm using a 1 cm cuvette with phosphate buffer as a blank. The absorbance of 0.015 DNTB in 3.0 mL of phosphate buffer (7.4 pH) and 0.015 DTNB in 0.0725 mL of myoglobin stock solution with 2.9925 mL of phosphate buffer (pH 7.4) was recorded to adjust for background absorbance. A conversion factor of 1.415 M^{-1} cm^{-1} was used to determine sulfhydryl group concentration in millimolar. Samples were read and recorded following the simulated bacterial reduction and cooking steps. Each sample was prepared and analyzed in duplicate.

Cured Color. Cured color (nitrosylhemochromogen) was measured directly on the sample solution using absorbance at 535 nm in a 1 cm cuvette. Only samples in the cysteine plus myoglobin model were measured for cured color following the cooking step.

Residual Nitrite. Residual nitrite was measured using AOAC method 973.31[28] with modifications. For each sample, 3.6 mL of water

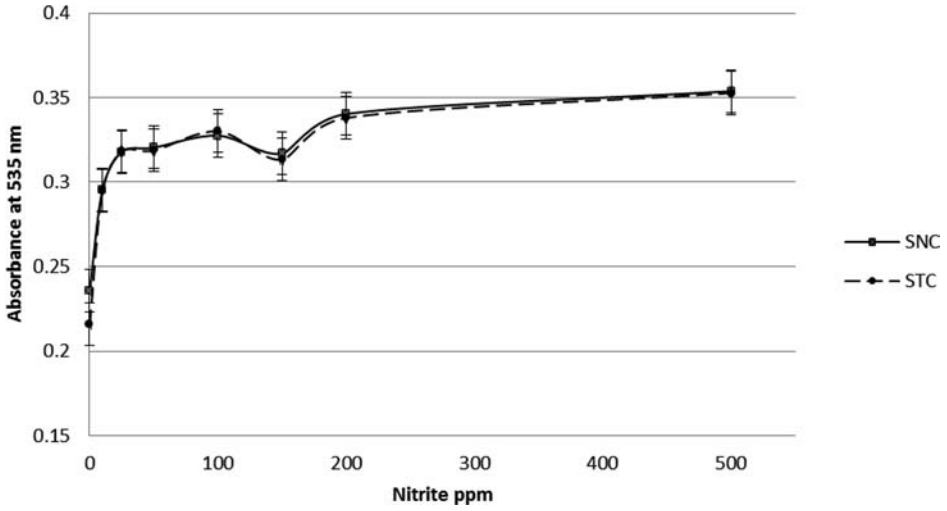

Figure 2. Absorbance at 535 nm of the cysteine plus myoglobin model system as an indicator of nitrosylhemochromogen formation. SNC = sodium nitrite solution added in 1 mL increments for simulated bacterial generation of nitrite to simulate natural curing; and STC = entire sodium nitrite solution added at beginning to simulate traditional curing.

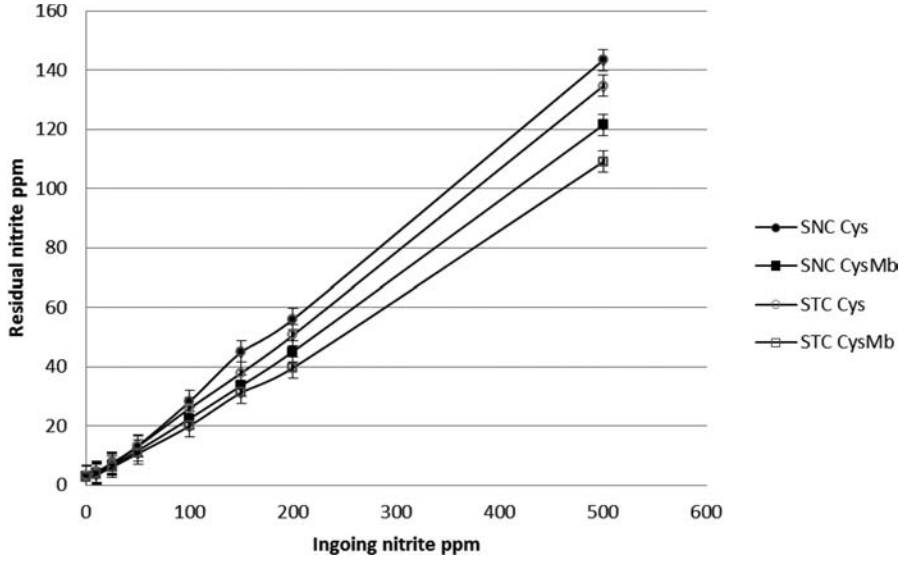

Figure 3. Residual nitrite content as a result of ingoing nitrite, rate of addition, and model system following simulated bacterial generation of nitrite and before simulated cooking. SNC Cys = cysteine-only model, sodium nitrite solution added in 1 mL increments for simulated bacterial generation of nitrite; SNC CysMb = cysteine plus myoglobin model, sodium nitrite solution added in 1 mL increments for simulated bacterial generation of nitrite; STC Cys = cysteine-only model, entire sodium nitrite solution added at beginning to simulate traditional curing; and STC CysMb = cysteine plus myoglobin model, entire sodium nitrite solution added at the beginning to simulate traditional curing.

and 0.4 mL of sample solution were placed in a test tube. Sulfanilamide reagent, 0.22 mL, was added to each test tube and vortexed. After 5 min, 0.22 mL of NED reagent was added, vortexed, and allowed to stand for 15 min. Samples were read at 540 nm in a 1 cm cuvette on a spectrophotometer. A solution of 4.5 mL of water and 0.25 mL of each sulfanilamide and NED reagents was used for the blank. A standard curve to calculate residual nitrite concentration was created as described in the original method. Residual nitrite was measured in the samples following the simulated bacterial reduction and cooking steps.

Statistical Analysis. Data were analyzed using the proc GLIMMIX procedure of SAS (v 9.2, SAS Corp, Cary, NC) in a factorial design including solution (cysteine-only or cysteine plus myoglobin), sodium nitrite concentration, and rate of sodium nitrite addition. When significant treatment effects ($p < 0.05$) were identified, means separation was conducted using LSMEASN procedure. Results following the bacterial reduction and cooking simulations were analyzed separately.

RESULTS

Nitrite reacts with myoglobin and cysteine during meat curing.[24] Changes in cysteine sulfhydryl groups are shown in Figure 1. A decrease in sulfhydryl groups was used as an indication of cysteine nitrosylation. Following both simulated bacterial generation of nitrite and cooking, significant treatment effects were found for the cysteine-only and cysteine plus myoglobin models and for ingoing nitrite concentration ($p < 0.05$). Sulfhydryl groups decreased as ingoing nitrite increased. Following the simulated bacterial generation of nitrite, 75.4% of the cysteine sulfhydryl groups were recovered when no nitrite was added. No statistically significant differences for sulfhydryl groups were found for the rate of addition of nitrite within a given nitrite concentration following both the simulated bacterial nitrite generation or the cooking steps ($p > 0.05$). Following the simulated bacterial generation of nitrite step, the

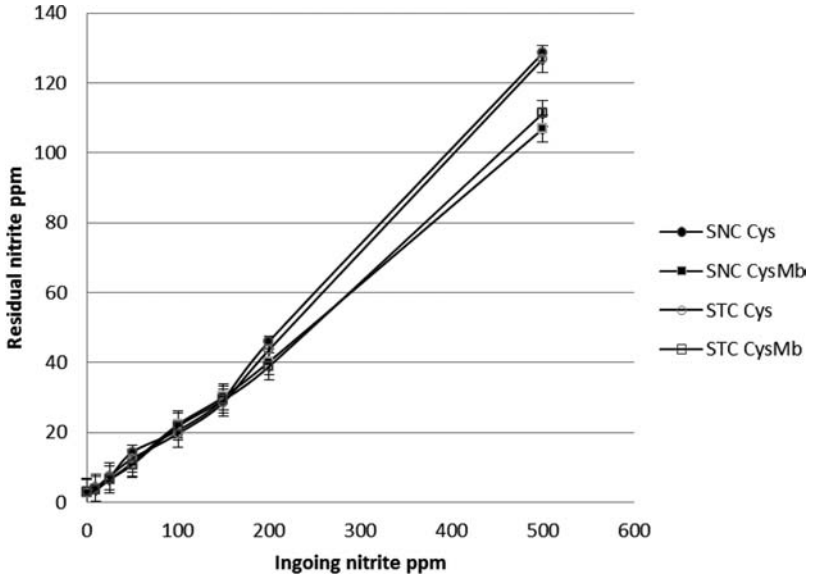

Figure 4. Residual nitrite content as a result of ingoing nitrite, rate of addition, and model system following simulated bacterial nitrite generation and simulated cooking. SNC Cys = cysteine-only model, sodium nitrite solution added in 1 mL increments for simulated bacterial gerneration; SNC CysMb = cysteine plus myoglobin model, sodium nitrite solution added in 1 mL increments for simulated bacterial generation of nitrite; STC Cys = cysteine-only model, entire sodium nitrite solution added at beginning to simulate traditional curing; and STC CysMb = cysteine plus myoglobin model, entire sodium nitrite solution added at the beginning to simulate traditional curing.

cysteine plus myoglobin model had greater sulfhydryl groups remaining than the cysteine-only model. Equine myoglobin does not contain cysteine,[34] and preliminary work showed that myoglobin alone did not react with Ellman's reagent. Ingoing nitrite concentrations of 0, 10, and 25 ppm resulted in similar sulfhydryl group concentrations, while all other concentrations of nitrite were significantly different from each other ($p < 0.05$). Following simulated cooking, similar results were found. The cysteine-only model had fewer sulfhydryl groups than the cysteine plus myoglobin model. Samples with 10 ppm ingoing nitrite resulted in the greatest number of sulfhydryl groups, followed by 0 and 25, which were similar. Within model and simulated processing steps, all other ingoing nitrite concentrations were statistically different from each other and declined with increased ingoing nitrite.

Cured color development was measured by absorption in the red visible region where cured meat pigment absorption maximum occurs (Figure 2). The amount of ingoing nitrite ($p < 0.0001$) but not rate of addition ($p = 0.643$) had significant main effects. Regardless of the rate of addition, 0 ppm ingoing nitrite had a significantly lower absorbance than all other nitrite concentrations ($p < 0.05$). The ingoing nitrite concentration of 200 and 500 ppm resulted in greater absorbance from nitrosylhemochromogen than 10 ppm ($p < 0.05$), while all other ingoing nitrite concentrations (25−500 ppm) had similar absorbance. This plateauing effect of color formation was expected. General consensus suggests that 40−50 ppm of ingoing nitrite is required for stable cured color in cured meat, but higher concentrations are needed for bacteria suppression.[29]

Residual nitrite concentrations following simulated bacterial generation of nitrite and cooking simulations are found in Figures 3 and 4, respectively. Significant treatment effects were found for the rate of addition ($p = 0.020$) and ingoing nitrite × model interaction ($p = 0.002$). When nitrite was added slowly to simulate a natural curing process, higher residual nitrite concentrations were found than in the traditionally cured simulation, 34.3 and 31.3 ppm, respectively, across all concentrations, as shown in Figure 3. A significant interaction of ingoing nitrite and model system was found following the simulated bacterial generation of nitrite ($p < 0.05$). Residual nitrite was similar between models with lower concentrations of ingoing nitrite, but the cysteine-only model had significantly greater residual nitrite than the cysteine plus myoglobin model at 150, 200, and 500 ppm of ingoing nitrite. For results averaged across substrate and curing model treatments following the simulated bacterial generation of nitrite step, the percentage of ingoing nitrite recovered as residual nitrite at ingoing nitrite concentrations above 25 ppm varied very little (47.9−50.8%), but a greater portion of ingoing nitrite was recovered as residual nitrite (80.8 and 55.4%) for 10 and 25 ppm ingoing nitrite, respectively. Following the simulated cooking step, no significant treatment effects were found for the rate of addition for simulated cooking ($p = 0.780$). Similar to the simulated bacterial generation of nitrite, a significant ingoing nitrite × model interaction ($p = 0.008$) was found following simulated cooking. Residual nitrite in the cysteine-only model was greater than the cysteine plus myoglobin model for 500 ppm of ingoing the nitrite, while all other concentrations-by-model combinations were similar. This is likely due to the ratio of substrate: nitrite in the models. For results averaged across substrate and curing system models following the simulated cooking step, the proportion of ingoing nitrite recovered ranged from 39.2 to 47.4% for the 100−500 ppm treatments. Residual nitrite changed less than 1 ppm for 10, 25, and 50 ingoing nitrite treatments between the simulated bacterial generation of nitrite and cooking steps.

The rate of addition of sodium nitrite was only significant for residual nitrite following the simulated bacterial generation of nitrite. The rate of addition had no impact on sulfhydryl concentration at either time point or on cured color formation and residual nitrite concentration following the cooking simulation step.

DISCUSSION

S-Nitrosothiol groups are formed through the reaction of a sulfhydryl group and a nitrosylating agent such as dinitrogen trioxide, N_2O_3, but not nitric oxide directly.[35] Peterson et al. produced *S*-nitrosocysteine in mildly acidic conditions with equal molar concentrations of nitrite and cysteine and reported the formation of over 90% nitrosocysteine[36] in conditions similar to this experiment. Nitrosation of cysteine and other thiol groups has been shown to have many important biological functions as cellular signaling molecules that can[37] release nitric oxide to regulate blood flow[38] and modify metabolic rates and oxygen consumption[39] among many others. When aqueous solutions were exposed to oxygen, Rehder and Borges[40] found that disulfide bonds formed nonenzymatically via a sulfenic acid, RSOH, intermediate. The presence of trace metals such as iron or copper increased the rate of disulfide bond formation. The nonenzymatic disulfide bond formation observed by Rehder and Borges[40] may explain why only 75% of the ingoing cysteine sulfhydryl groups remained intact in our study even when no nitrite was added. Interestingly, in this experiment, with the addition of 10 ppm of sodium nitrite, greater remaining sulfhydryl concentrations were observed than with 0 ppm of sodium nitrite. Nitric oxide is able to stabilize heme groups, bind free iron,[41] and rapidly consume oxygen,[42] which may limit nonenzymatic disulfide bond formation. When greater than 10 ppm of nitrite was added, the decrease in sulfhydryl groups was likely due to nitrosation of cysteine. It is unlikely that nitrosocysteine remained in the mixture following the cooking simulation due to the thermal instability of nitrosocysteine and likely disulfide bond formation and nitric oxide release.[35] In biological systems and cured meats, it is likely that *S*-nitrosothiols serve as a reaction intermediate and nitric oxide donor or reducing agent.

The effect of nitrite on heme pigments has been studied for over 140 years. In 1868, Gamgee reported the browning of blood that we now know as methemoglobin formation when nitrite was added.[43] At the turn of the 20th century, Haldane characterized cured meat pigment as nitric oxide hemochromogen.[44] Ingoing nitrite above 25 ppm did not provide increased cured pigment formation in this study. Because the myoglobin concentration in the model was about half that of fresh ham, the ratio of nitrite:myoglobin is similar to that needed to achieve acceptable cured color in cured meat.[29] No change in sulfhydryl concentration was observed until ingoing nitrite reached 25 ppm and after cured pigment formation had plateaued. Additionally, fewer sulfhydryl groups remained in the cysteine-only model than in the cysteine plus myoglobin model following simulated bacterial generation of nitrite. This suggests that myoglobin is nitrosylated more quickly than cysteine is nitrosated and likely explains the formation of cured color prior to cysteine nitrosation. The binding rate constants for nitrosylation of sulfhydryl and heme groups are 4.5×10^5 and 2×10^7 mol^{-1} s^{-1}, respectively, which further supports these findings.[45,46] Previous research has shown that nitrosocysteine added to turkey provided cured color, antioxidant activity, and anticlostridial activity similar to those produced with sodium nitrite,[28] suggesting that nitrosocysteine can donate nitric oxide to myoglobin, which, in turn, suggests preferential binding of nitric oxide to heme iron over sulfhydryl groups. The concentration of ingoing nitrite impacts pathogen growth. O'Leary and Solberg determined that between 100 and 180 ppm of ingoing nitrite inhibited *C. perfringens* and decreased glycolytic enzyme activity of the microorganism by sulfhydryl nitrosation.[47] Xi et al. found that greater ingoing nitrite up to 150 ppm nitrite resulted in lower *L. monocytogenes* growth.[48] The USDA recommends 120 ppm of ingoing nitrite to ensure product safety in all "keep refrigerated" cured meats unless other methods of microbial control are utilized.[29] All of these reports suggest that greater ingoing nitrite concentrations are needed to provide antimicrobial control than for color development in cured meats. The data in this study indicate that myoglobin is preferentially nitrosylated before cysteine is nitrosated, suggesting that meat color is developed before many other nitrite reaction products and that the rate of addition of nitrite does not shift nitrosation/nitrosylation products formed during meat curing.

Differences were found for the rate of addition of nitrite following simulated bacterial generation of nitrite, but no differences were found following simulated cooking. Cassens identified many factors that impact residual nitrite,[49] most of which were controlled in this model. While both cysteine and myoglobin have been shown to have nitrite-reducing capabilities,[27,50] the differences in residual nitrite within treatments and ingoing nitrite concentration were likely due to the total amount of nitrite reactive compounds (myoglobin and cysteine) in the models. In meat products, maintenance of a small amount of residual nitrite is important for cured color stability and for sustained pathogen control during storage.[51,52]

Naturally cured products have cured meat characteristics similar to conventionally cured meats,[18] but the naturally cured products have less pathogen controls.[19,20] While the amount of ingoing nitrite affects the extent of nitrosation/nitrosylation reaction product formation, the natural curing process does not appear to result in a significant shift of nitrite reaction products. This would suggest that it is more important to increase the amount of ingoing nitrite in naturally cured products than increase the rate of nitrite formation from nitrate. This may not hold true for other nitrosation/nitrosylation reaction substrates found in meat or with the use of cure accelerators. Thus, from the results of this study, it appears that the slow release of nitrite in naturally cured products does not affect or shift the amount of nitrite between reaction intermediates. However, the role of other substrates and reactions should be investigated, and the model system used for this study provides a basis for additional research to evaluate reducing agents and substrates.

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Notes

The authors declare no competing financial interest.

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