Nitrite in Meat. Effect of Various Compounds on Loss of Nitrite

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Various compounds endogenous to meat or added to cured meats, and known to react with nitrite, were tested in meat slurries for their effects on nitrite loss. The reductants, ascorbate and cysteine, and the amino acid, histidine, caused the only important losses when added to endogenous or normally used concentrations. Reduced nicotinamide adenine dinucleotide caused a short term reduction of nitrite, but the loss was not permanent. Kinetic data show that loss of nitrite is related to the reduction reaction which produces nitric oxide. Production of the latter accounts for a large part of total nitrite loss and evidence indicates that most or all of the remaining nitrite not accounted for is involved in the formation of nitroso-reductant intermediates or products.

In the United States, sodium nitrite is added to cured meat products at levels up to 2.25 m*M* (dry mix) or 3.62 m*M* (10% pumped pickle). During curing, cooking, and aging, nitrite content is greatly reduced and sometimes completely eliminated (AMIF, 1971). Mirna and Hofmann (1969) made a significant discovery with regard to nitrite loss when they found that the loss of added nitrite in meat was equaled by a reduction in free sulfhydryl groups. The sulfhydryl content of meat is about 20 m*M* and is therefore sufficient to completely eliminate nitrite. Other observations however, raise questions about this loss. In practice, even with molar excesses of reductants over nitrite, the latter is not usually completely eliminated. Ascorbate, which is commonly added to cured meats, is even more reactive toward nitrite on a molar basis in forming of nitric oxide than is cysteine (Fox and Ackerman, 1968) suggesting that the roles of ascorbate and cysteine in causing nitrite loss should be compared. Walters and Taylor (1964) reported that not all nitrite loss in meat could be accounted for as nitric oxide, which is as yet the only identified product of the reaction of either cysteine or ascorbate with nitrite (Kelley and Watts, 1957). The relation between nitrite loss and nitric oxide formation with either ascorbate or cysteine has not been quantitated. Mirna and Hofmann (1969) observed the formation of spectrally absorbing nitrosothiol compounds, which they suggested might be involved in nitrite disappearance, possibly from the oxidation of sulfhydryl by nitrite. The oxidation of ascorbate by oxidants other than nitrite has been shown to proceed through the formation of spectrally identifiable intermediates (Herbert *et al.*, 1933; Schauenstein *et al.*, 1948; Fujimura and Ikeda, 1957; Bielski *et al.*, 1971), which suggests that spectral identification of ascorbate or cysteine intermediates might be used to determine the mechanism of nitrite loss.

This investigation was undertaken to determine: first, the relative importance of the cysteine (sulfhydryl) and ascorbate reactions in the loss of nitrite; second, if spectrally distinct compounds are formed during the ascor-

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Table I. Effect of Nitrite Loss of Compounds Added to Meat Slurries Containing 4 m*M* Nitrite, Incubated for 300 Minutes at 60°

Compound	m*M*	Loss, %	Compound	m*M*	Loss, %
Control	...	0–35[a]	Glyceraldehyde	20	0
Ascorbate	3	25–50[b]	Dimethylamine	5	0
Cysteine	30	30–50	Diphenylamine	50	0
Lysine	100	0	Sulfanilic acid	50	75–100
Histidine	50	20–35	Nitrate	200	0
Tyrosine	50	0	Lecithin	80 mg/10 ml	0
Tryptophan	50	0	Fat (pork)	5 g/10 ml	0
NADH	4	15–25	Cytochrome	0.001[c]	0
Creatine	50	0	Thiocyanate	0.025[c]	0
Creatinine	50	0	Spermine	50	0
Adenine	20	0	Spermidine	5	0
Guanine	20	0			

[a] Standard deviation of duplicate controls was ±3%. The range represents batch to batch variation. [b] % Loss values in slurries with added compounds are calculated as excess loss over that of control. [c] Added to test possible catalytic effect on endogenous nitrite reactants.

bate–nitrite reaction which might be related to nitrite loss; third, the kinetics of nitrite disappearance to see if this reaction is related to the formation of nitric oxide or is due to some other mechanism; and fourth, the reactivity of other meat components toward nitrite to determine if minor reactions might occur that would not be detected in the presence of faster reactions. The compounds tested were selected because they occur endogenously in meat and meat products (α-amino acids, purines, coenzymes, etc.), are added to cured meats (ascorbate, nitrate, fat), have been shown to react to some extent with nitrite (primary, secondary, or aromatic amines, sulfanilic acid, etc.), or interfere in nitrous acid reactions (substituted phenols, tyrosine). They were added at levels either endogenous to meat or normally added to cured meats (AMIF, 1960; FAO, 1970; Long, 1961; CFR, Title 9, 1971).

EXPERIMENTAL SECTION

A meat slurry was prepared from fat-free flank steak, blended with water (1:2 g/ml) for 30 seconds in a blender (for best results the blades must be sharp). Ten-milliliter volumes of the resulting slurry were dispensed from a 50-ml irrigating syringe into the appropriate reaction vessel, and the slurries made 4.0 m*M* in nitrite.

Nitrite Loss in Slurries. The compounds tested for reactivity with nitrite are listed in Table I. The reactions were run in 30-ml centrifuge tubes. The various reagents were added, the tubes stoppered, and the reaction started by placing the tubes in a water bath controlled to within 0.1° of the desired temperature. At desired times, the tubes were removed, cooled in an ice bath, and centrifuged at 480 × g for a few seconds to pack the solids lightly. Twenty-five microliters of supernatant was withdrawn for nitrite analysis; the residue was resuspended and incubated further. Volume changes in the supernatant of 10 ml of slurry were negligible; removal of 10 samples decreased the total volume only 5.0%. Control slurries containing the nitrite only were used for each group of samples. Nitrite loss for any given sample was calculated as the excess loss in that sample over the loss in its group's control.

The pH of meat slurries ranged from 5.45–5.65. When following nitrite loss, we adjusted all reagent solutions to this range and buffered the aqueous systems at pH 5.5, except in two of the experiments where, to get more rapid changes, we followed the reaction at pH 4.5.

Nitrite Analysis. Twenty-five microliters of slurry supernatants or buffered solutions were pipetted into 10-ml volumetric flasks to which 1 ml of Griess reagent (AOAC Official Methods of Analysis, 1970) had been added. Volume was brought to 10 ml with water and after 0.5 hr, the absorption was read at 525 nm. Standard nitrite solutions analyzed in this way gave linear readings up to 20 μ*M* nitrite with a Beer–Lambert relation expressed by: $A_{525\text{nm}}{}^{1\text{ cm}} = (0.0350 \pm 0.0009) \times \mu M$ nitrite.

Residual nitrite in the slurry supernatants also was determined by reduction with dithionite, and measurement of the amount of nitric oxide thus formed by complexing with myoglobin (Mb) to form MbNO. A solution containing 1 m*M* nitrite and 1 m*M* ascorbate, pH 4.5, was incubated at 85°; 0.1-ml samples were transferred at appropriate times to 3 ml of 0.05 m*M* metmyoglobin (MetMb) at pH 4.5. Powdered $Na_2S_2O_4$ was added to reduce MetMb and nitrite. Optical absorbance of the resulting mixture of Mb and nitrosylmyoglobin (NOMb) was read in a Cary 14 spectrophotometer at 549 nm (isosbestic point) and at 480 nm, the wavelength of greatest spectral difference in the visible region. The absorbance values used were 13.51 mM^{-1} cm^{-1} at 549 nm for both pigments, and 8.46 and 5.20 mM^{-1} cm^{-1} for NOMb and Mb, respectively, at 480 nm. The formula for the concentration of NOMb is

$$[\text{NOMb}] = 0.307A_{480} - 0.119A_{549} \quad (1)$$

Nitrite recovery was 97% by this technique.

To measure gaseous nitric oxide production, a cell was made by sealing the open ends of two syringe barrels together (Figure 1). A side arm, sealed with a serum bottle cap, allowed addition of slurries, reagents, and gases from a syringe and needle. Nitrogen gas, after passing through alkaline pyrogallol to remove residual oxygen, was swept over the surface of the solution in the cell. The gas stream was passed through a water trap into a sealed 1-cm optical cuvette containing alkaline sulfite (10 g Na_2SO_3 + 1 g NaOH in 50 ml H_2O) (Treadwell and Hall, 1935), and finally vented to the air. The nitric oxide in the gas stream was trapped in the sulfite as sodium *N*-nitrosohydroxylamine-*N*-sulfonate ($Na_2(NO)_2SO_3$) which absorbs in the ultraviolet at 258 nm. The sulfite itself has an absorption band at ~260 nm, which gave a basal absorption greater than 4 Absorption Units. We therefore read the increase in absorption at 265 nm where the basal absorption was low enough to permit recording for $Na_2(NO)_2SO_3$. $\Delta\epsilon_{265\text{ nm}}$ was 0.417 mM^{-1} cm^{-1}, and we were able to detect quantities of NO as low as 0.1 μmol.

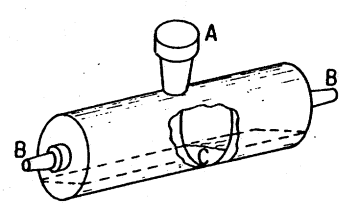

Figure 1. Reaction vessel for degassing meat slurries. A, serum bottle cap; B, male luer fittings; C, solution.

Table II. Nitrite Loss and Nitric Oxide Formation, 70°

System with 4 m*M* nitrite	Nitrite loss, m*M*/hr	NO formed, m*M*/hr	NO/NO_2^-,[a] %
Slurry, pH 5.6	0.212	0.0268	12.6
Slurry, pH 5.6, 4 m*M* ascorbate	1.02	0.207	20.3
Slurry, pH 5.6, 4 m*M* ascorbate	1.33	0.241	18.1
Slurry, pH 5.6, 20 m*M* cysteine	1.17	0.0740	6.3
Slurry, pH 5.6, 20 m*M* cysteine	1.49	0.0860	5.8
Slurry, pH 5.6, 40 m*M* histidine	1.20	0	...
Slurry, pH 5.45, before ascorbate		0.0552	
Slurry, pH 5.45, with 4 m*M* ascorbate		0.512	
Buffer, pH 5.5	0.256	0.003	1
Buffer, pH 5.5, 4 m*M* ascorbate	0.779	0.416	53.4
Buffer pH 5.5, 20 m*M* cysteine	1.32	0.057	4.3

[a] NO formed/NO_2^- lost.

RESULTS

Nitrite Reactivity. The results of adding various compounds to meat slurries containing nitrite are shown in Table I. In meat slurries to which only nitrite was added, some nitrite was always lost, but the loss was variable. Meat from which the slurries were made was kept frozen at −21° and in an impermeable wrap, yet the rate of nitrite depletion in the slurries decreased as the meat aged. Ascorbate, cysteine, and histidine caused a loss of nitrite. The reaction was first-order with respect to nitrite concentration. Reduced nicotinamide adenine dinucleotide (NADH) caused a 70–80% decrease in the nitrite content immediately on addition, followed by an increase in the concentration back to 75–85% of the original value. Sulfanilic acid did not affect the initial reading, but after 15-minute incubation, the nitrite concentration decreased to zero or almost so.

Nitric Oxide Production. We determined the loss of nitrite by Griess analysis and formation of nitric oxide in slurries at 70° (Table II). Nitrite alone in pH 5.5 buffer produced a detectable amount of NO by the termolecular reaction of nitrous acid (Table II):

$$3HNO_2 \longrightarrow 2NO + HNO_3 + H_2O \qquad (2)$$

Although histidine caused a loss of nitrite in slurries, we could not demonstrate nitric oxide production. Since nitric oxide readily couples with myoglobin, we tried to detect NOMetMb or NOMb formation from histidine and nitrite, both in slurries and model systems, but at histidine concentrations up to 300 m*M*, we observed no NO–heme complex formation. The effect of histidine on nitrite loss was observed only in the meat system; no loss occurred in buffered systems.

Order of the Reaction. Previously, Fox and Ackerman (1968) demonstrated that the formation of nitric oxide is dependent on the square root of the reductant concentration. Experiments were run with ascorbate, cysteine, and histidine to determine if the kinetics were the same with respect to total nitrite loss. Figure 2 shows that the first-order rate constants for nitrite loss were dependent on the 0.5 power of the concentration of each of the three compounds. In confirmation of these findings, when the data of Adriaanse and Robbers (1969) are calculated in terms of nitrite reacted with ascorbate (initial nitrite minus nitrite as analyzed by Griess reagents), we find the same fractional order dependence.

Reaction Products. Both ascorbate and cysteine formed products with nitrite that absorbed in the ultraviolet region. Figure 3 shows a set of curves for the formation of the ascorbate–nitrite reaction at pH 4.5. Similar products were formed at pH 5.5, but much more slowly and less distinctly. Incubation of ascorbate without added nitrite did not produce any of the observed bands in the reported time. Absorption reached a maximum after 90 minutes, then began to decrease and disappeared after 4–5 hr, when no free nitrite could be detected. At pH 4.5, cysteine produced an absorption band at 318 nm, but at neither pH 4.5 nor pH 5.5 did we observe the 335- and 540-nm bands reported by Mirna and Hofmann (1969).

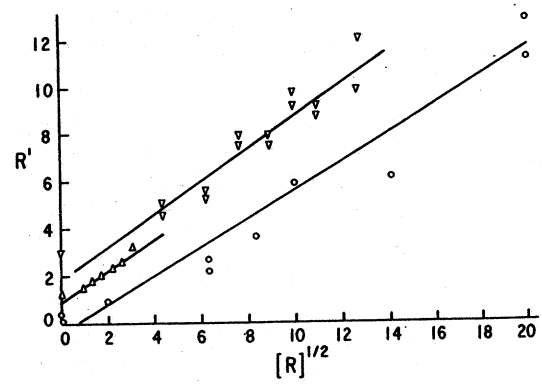

Figure 2. Dependence of the rate constant of the nitrosation reaction on the concentration of histidine, ascorbate, and cysteine. O, [R] = histidine in m*M*, $R' = k_{1st} \times 10^2$ in min^{-1}. Δ, [R] = ascorbate in m*M*, $R' = k_{1st} \times 10^3$ in min^{-1}. ∇, [R] = cysteine in m*M*, $R' = k_{1st} \times 10$ in hr^{-1}.

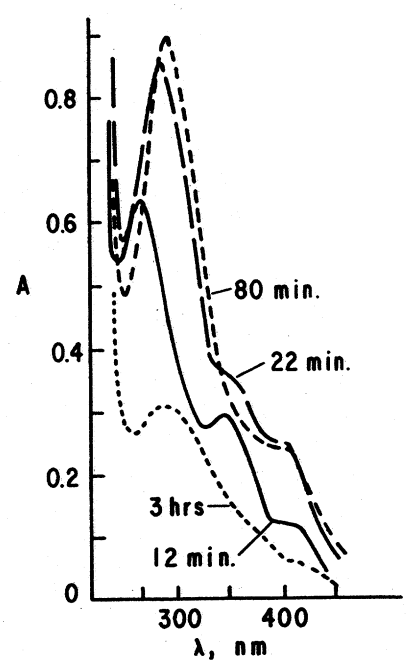

Figure 3. Uv absorption spectra of the reaction products of 1 m*M* ascorbate and 1 m*M* nitrite incubated at 85°, pH 4.5.

As part of a previous attempt to develop an alternative method to the standard colorimetric methods of nitrite analysis, we had devised the procedure of forming NOMb

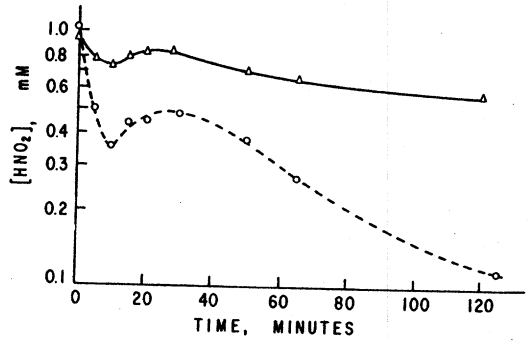

Figure 4. Free nitrite as analyzed by two different methods in a mixture of 1 m*M* ascorbate and 1 m*M* nitrite, 70°, pH 4.5. O, NOMb method; Δ, Griess method.

from nitrite, dithionite, and MetMb, using a molar excess of MetMb over nitrite. With nitrite standards, the NOMb and the Griess method gave equivalent results within 3% of theoretical. However, when nitrite was analyzed in meat or model systems, the Griess reagent gave higher values than the NOMb method (Figure 4). Since both methods were quantitative for free nitrite, the difference between the two curves may represent nitrosoascorbate intermediates not cleaved under the less acid conditions of the NOMb method. Such results are not unusual for reactions in which unstable intermediates accumulate.

DISCUSSION

Amine Reactions. Except for histidine, none of the amine compounds tested reacted appreciably to cause nitrite loss. These results are in agreement with Mirna and Hoffman's (1969) results with respect to sulfhydryl groups, where all the loss was due to sulfhydryl oxidation, and Möhler's (1965) negative results with respect to Van Slyke reactions. The reaction with histidine was not anticipated since imidazoles have been reported not to react with nitrite (Hofmann, 1953). The kinetic evidence (see below) indicates that the rate-limiting steps with histidine were the same as those of the reducing compounds. Since the histidine effect on nitrite loss was observed only in meat-containing systems, it would appear that histidine is acting only as an intermediary or catalyst in some other reaction. It may also be noted that Riha and Solberg (1973) observed no nitrite loss in incubation media containing histidine. Recently, Olsman and Krol (1972) found that masking sulfhydryl groups in meat with either $HgCl_2$ or *N*-ethylmaleimide reduced protein-bound nitrite by only 60%; histidine-reacted nitrite may account for the other 40%.

Sulfanilic acid originally was added to meat slurries to test the effect of a very reactive amine on nitrite loss. The sulfanilic acid was nitrosated since all the nitrite disappeared, but the nitrososulfanilic acid was no longer available for coupling to 1-naphthylamine when the Griess reagent was added. Apparently nitrososulfanilic acid coupled with tyrosine and histidine, which is the Pauly reaction (Hofmann, 1953).

Reductants. Titratable reductants in meat have been estimated to range as high as 100 m*M* (Fox and Ackerman, 1968). Free sulfhydryl groups have been found in the range of 21–25 m*M* (Hamm and Hofmann, 1966; Hofmann, 1971), which is about the concentration of cysteine in meat (AMIF, 1971; FAO, 1970) and is more than adequate to account for total nitrite depletion. Since ascorbate is usually added to levels equivalent to nitrite (2.75 m*M* *vs.* 2.25 in mixed cures and 3.45 m*M* *vs.* 3.62 m*M* in pumped products), the amount of ascorbate is also adequate to account for total loss of nitrite. From the data of Table I, it is seen that the addition of reductants or histidine with nitrite to meat slurries caused an extra nitrite loss of the same magnitude as the slurries alone with nitrite. Since the reaction in meat slurries is due primarily to sulfhydryl reaction, we conclude from the similarity in rates that the bulk of the cysteine sulfhydryls in meat are available for reaction with nitrite. Any nitrite reacted with cysteine or other reactive groups in the insoluble proteins could not have been analyzed as nitrite since such bound nitrite would have been precipitated by the centrifugation step. Oxidation of protein sulfhydryl groups and other reductants during storage may be the cause of the high variability in residual nitrite commonly observed in cured meats (AMIF, 1971). Oxidation by nitrite of sulfhydryl groups in proteins could result in protein cross-linking through formation of disulfide bonds; cross-linking has been shown to be related to toughness (Dubé *et al.*, 1972). Confirming this view, Ackerman and Swift (1972) found that nitrite increases the shear values of frankfurters.

The mechanism of the NADH–nitrite reaction is obscure. Fox and Ackerman (1968) found that high levels of NADH produced nitric oxide from nitrite to form NOMb; Koizumi and Brown (1971) found it necessary at low levels to add flavins. Both nitric oxide reactions followed normal decay-type kinetics, in contrast to the nitrite loss kinetics which did not (immediate high loss followed by recovery). The concentration of NADH we used was quite arbitrary. Total NAD in tissue ranges from 0.5–1.0 m*M*, but it is not known what proportion is in the reduced state under the specified conditions. In view of these considerations, we would say the role of NADH in causing nitrite loss appears small at this time.

Reaction Intermediates. Dahn *et al.* (1960) found that the end product of the reduction of nitrite by ascorbic acid is nitric oxide, and they postulated that the reaction proceeded through the formation of a nitrosoascorbic acid intermediate. This conclusion was supported by the studies of Fox and Thomson (1963) and of Fox and Ackerman (1968) on the basis of the kinetics of the formation of nitrosylmyoglobin. The latter also demonstrated that nitrite formed similar reaction intermediates with cysteine, NADH, and hydroquinone. The present study and Walters and Taylor's (1964) study of nitric oxide formation suggest that these reaction intermediates either bind nitrite or react further to produce a form of nitrite that does not react with sulfanilic acid (Griess) or dithionite (nitrosylmyoglobin formation). The formation of these unreactive products is greater, in slurries and in buffered systems, with cysteine than with ascorbate because cysteine produced lower percentages of nitric oxide in relation to nitrite loss (Table II).

When we attempted to correlate the spectra of the nitrite reaction intermediates and/or products with similar data from the literature, Table III, we found some disagreement as to what these spectra represent. In particular, the major peak at 290 nm has been identified both as a product (Herbert *et al.*, 1933; Jackson *et al.*, 1960) and as a radical intermediate (Bielski *et al.*, 1971). We are continuing studies of the nitrite–ascorbate reaction in the hope we may resolve or reconcile these viewpoints.

Kinetics of the Loss of Nitrite. Both nitrite loss (this study) and nitric oxide formation (Fox and Thomson, 1963) follow the same kinetics, that is, first order in nitrite and 0.5 order in reductant. A mechanism for the formation of free NO has been derived by mathematical analysis of the kinetic data (Fox and Thomson, 1963) (eq 3–6).

$$2HNO_2 \longrightarrow N_2O_3 + H_2O \quad (3)$$

$$RH + N_2O_3 \longrightarrow RNO + HNO_2 \quad (RH = \text{reductant}) \quad (4)$$

$$2RNO + H_2O \longrightarrow 2RH + N_2O_3 \ (\text{or } HNO_2) \quad (5)$$

$$RNO \longrightarrow R\cdot + NO \quad (6)$$

It is the backward reaction of eq 5 that introduces the square root dependence on the reductant and the first-order dependence on nitrite. Since the kinetics for nitrite

Table III. Absorption Maxima and Coefficients for the Reaction of Nitrite and Other Oxidants with Ascorbate and Cysteine

Reductant/oxidant	λ_{max}	ϵ, mM	Identified as	Authors
Ascorbic acid/I_2–air	290	...	Diketogulonic acid	Herbert *et al.* (1933)
Ascorbic acid/H_2O_2	297	0.38	Conjugated >C=O	Schauenstein *et al.* (1948)
Ascorbic acid/Br_2	300	...	Diketogulonic acid	Fujimura and Ikeda (1957)
Ascorbic acid/O_2	290	...	Diketogulonic acid	Jackson *et al.* (1960)
Ascorbic acid/I_2	343	...	Unidentified	Herbert *et al.* (1933)
Ascorbic acid/pulse radiolysis	290	8.8	Ascorbyl radical	Bielski *et al.* (1971)
Ascorbic acid/pulse radiolysis	360	4.9	Ascorbyl radical	Bielski *et al.* (1971)
Nothing/nitrite	343	~0.01	...	This work
Ascorbic acid/nitrite	290	>1.16	Nitrite oxidation products of ascorbate	This work
	347	>0.12		This work
	408	>0.05		This work
Cysteine/nitrite	335	...	Nitrosothiol compounds	Mirna and Hofmann (1969)
	540	...	Nitrosothiol compounds	Mirna and Hofmann (1969)
Cysteine/nitrite	318	...	Nitrosothiol compounds	This work

loss are the same as for production of nitric oxide, we propose that the above reactions (3 to 5) are also the first steps in nitrite loss. In the case of histidine, reactions 3 to 5 would occur as shown, but the electron transfer of reaction 6 would not. Dahn *et al.* (1960) established that the ascorbate–nitrite reaction in N_2 ultimately produces 97% NO. However, as shown in Table II, at any given time during the reaction with cysteine or ascorbate under nitrogen, the loss of nitrite was greater than nitric oxide production. This means that either there are semistable nitroso intermediates that will not release nitrite on Griess analysis, or the intermediates undergo some reaction other than the release of free NO or nitrite.

Nitric Oxide. Free nitric oxide from reaction 6 may react in either of two ways in air: oxidation to nitrogen dioxide which dismutates in water to form nitrate and nitrite, or direct oxidation back to nitrite (Hardy *et al.*, 1957). Möhler (1970) has observed the first reaction in cured meats but the second reaction has not been demonstrated as far as we know. The route the oxidation reaction takes probably depends on the reaction conditions, which in a system as complex as meat can be highly variable. The effect of oxidation of nitric oxide was observed during color formation in frankfurter emulsions (Fox *et al.*, 1967), in the form of a lag period before color formation commenced. The length of the lag period was determined by the amount of exposure of the emulsion to oxygen.

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