Comparison of the Fate of Nitrite Added to Whole Meat, Meat Fractions and Model Systems[†]

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The fate of nitrite in cured meat was investigated. Of added nitrite-N, 66~90% was found as nitrite, nitrate, nitrosothiol, denatured nitrosomyoglobin and gaseous nitrogen compounds, and the remainder of N was unidentified. The amount of unidentified N depended on both the curing period and the amount of added sodium ascorbate (AS).

Pork was separated into four fractions: dil. sodium chloride-soluble small-molecular fraction (Fr. 1), dil. sodium chloride-soluble large-molecular fraction (Fr. 2), Weber-Edsall solution-soluble fraction (Fr. 3) and Weber-Edsall solution-insoluble fraction (Fr. 4). Each fraction was cured and the fate of nitrite was examined. In the absence of AS, almost all nitrite-N was detectable, while in its presence, 73~82% of nitrite-N was detectable in both Fr. 1 and Fr. 2, 78~100% in Fr. 3 and 94~100% in Fr. 4. These results suggest that the unidentified nitrite-N in cured meat originated mostly from the reaction between the three meat fractions (Frs. 1, 2 and 3), nitrite and AS.

Since nitrite has been confirmed to be a precursor of strong carcinogenic nitrosamines, the wholesomeness of nitrite as a curing agent has aroused great concern. In spite of many studies, our knowledge of the fate of nitrite in cooked cured meat is still not enough to solve this problem. Quantitative investigations of the fate of nitrite are now necessary.

Our recent work¹⁾ on the fate of nitrite in meat-curing model systems composed of myoglobin, nitrite and ascorbate showed that the added nitrite was almost completely recovered in the form of the following four compounds: nitrite, nitrate, DNOMB* and gaseous nitrogen compounds. The present paper describes the fate of the nitrite in whole meat and its four fractions *i.e.*, dil. NaCl-soluble small-molecular fraction (Fr. 1), dil. NaCl-soluble large-molecular fraction (Fr. 2), Weber-Edsall solution²⁾-soluble fraction (Fr. 3) and Weber-Edsall solution-insoluble fraction (Fr. 4).

Mirna *et al.*³⁾ have reported that meat con-

tains 20 mM sulfhydryl group which can react with nitrite to give nitrosothiol. Saville⁴⁾ has shown that heavy metals split nitrosothiol into nitrite and sulfhydryl. Mirna *et al.*^{3,5)} and Olsman *et al.*⁶⁾ have used this reaction to determine nitrosothiols, revealing that the sulfhydryl group can play a very important role in influencing the fate of nitrite. In a study on the specific interaction between nitrite and sulfhydryl groups of myosin, Kubberød *et al.*⁷⁾ have shown that twenty percent of the total nitrite lost was changed to nitrosothiol-N under the following conditions: molar ratio of SH/nitrite, 1/1; pH, 5.0; temperature, 100°C; and reaction time, 1 hr.

In the present work, determination was conducted on nitrosothiol as well as on nitrite, nitrate, DNOMB and gaseous nitrogen compounds originating from nitrite in cooked cured meat.

MATERIALS AND METHODS

Fractionation of meat. Minced meat (commercial pork) was homogenized very mildly by hand with an equal weight of 0.05 M NaCl for 15 min and centrifuged at $11,000 \times g$ for 15 min. The supernatant obtained was dialyzed against 0.05 M NaCl and the diffusate was concentrated with a rotary evaporator at

[†] Fate of Nitrite Added to Cured Meat. Part II. See Reference 1).

* Abbreviations used in this paper: DNOMB, denatured nitrosomyoglobin; AS, sodium ascorbate; AM-buffer, ammonia-ammonium acetate buffer; Mb, myoglobin.

30°C. The concentrate was used as Fr. 1, and the dialysate as Fr. 2. The residue was homogenized very mildly with a 6-fold volume of Weber-Edsall solution (0.6 M KCl, 0.04 M NaHCO₃ and 0.01 M Na₂CO₃) for 10 min. After standing for 24 hr at 4°C, the mixture was centrifuged at 7300 × *g* for 15 min. The supernatant was used as Fr. 3, and residue as Fr. 4.

Composition of reaction mixture. Reaction mixtures contained minced whole meat or one of its fractions, 1 mM ¹⁵N-sodium nitrite (99.0 atom %, from Hikari Kogyo Co., Ltd.), 0 or 20 mM AS, 2% NaCl and 0.08 M acetate buffer (pH 5.5). The formulation was made according to Tables I and III. Twenty g of the reaction mixture contained 17.6 g of whole meat. The protein concentrations of Fr. 2 and Fr. 3 in the reaction mixtures were 5 and 10 mg/ml, respectively. Fr. 1 was concentrated to the same volume as that of Fr. 2, whereas Fr. 4 was diluted with a three-fold volume of water.

Curing and cooking. Twenty ml (20 g in the case of whole meat) of the reaction mixture was placed in a tightly sealed flask and allowed to cure at 4°C for 0, 3 or 7 days. The mixture was then cooked at 70°C for 60 min and the amounts of DNOMb, nitrite, nitrate, nitrosothiol and gaseous nitrogen compounds were determined.

DNOMb determination. The method was the same as described in the previous paper.¹⁾

Nitrite, nitrate and nitrosothiol determination. Two ml (2 g in the case of whole meat) of the reaction mixture and about 80 ml of 0.1 M AM-buffer solution (pH 9.0) were placed in a 100 ml flask and heated at 80°C. After 10 min, 2 ml of a zinc uranyl acetate solution, prepared by mixing 100 ml of 0.1 M zinc acetate with 10 g of uranyl acetate in 100 ml ammonium acetate, was added and heating was contained for

another 5 min. After cooling the contents rapidly to room temperature, the volume was made up to 100 ml with AM-buffer solution and filtered through a Toyo No. 5C filter paper. The filtrate was used as a test solution.

a) Nitrite and nitrate determination. The same method as described in the previous paper.¹⁾

b) Nitrosothiol determination. Nitrosothiol was determined according to Saville's method,⁴⁾ using 2 ml of saturated mercuric chloride solution instead of zinc uranyl acetate. The amount of nitrosothiol-N was obtained by subtracting the amount of nitrite detected in the absence of mercuric chloride from that in the presence of mercuric chloride.

Gaseous nitrogen compounds determination. The method was the same as described in the previous paper.¹⁾

RESULTS

1. Fate of nitrite in whole meat

Table I shows the fate of nitrite added to whole meat cured with and without AS. In the absence of AS, 77% of added nitrite was recovered from the whole meat on 0-day curing and 46.5% on 7-day curing, while almost all of the nitrite was recovered in Mb-nitrite model system as shown in Table II. Therefore it is clear that nitrite added to whole meat decomposed both during the curing and at the cooking stage. These results suggest that compounds having the same effect as AS are present in meat.

In the presence of AS, the amount of nitrite detected on both 0- and 7-day curing was

TABLE I. FATE OF NITRITE IN WHOLE MEAT

Minced meat (17.6 g) homogenized with 1 ml of 0.02 M sodium nitrite, 1 ml of 0, 0.04 or 0.4 M AS and 0.4 g of NaCl was cooked at 70°C for 60 min after curing for 0 and 7 days.

Conditions		Fate of nitrite ^{a)}					
Curing period (days)	AS ^{b)} (mM)	DNOMb-N	Nitrosothiol-N	Nitrite -N	Nitrate -N	Gaseous -N	Total-N
0	0	1.8	0.9	77.0	10.0	0	89.7
	2	4.9	2.5	56.0	19.7	2.3	85.4
	20	6.2	7.1	30.0	22.7	4.5	70.5
7	0	3.3	1.4	46.5	13.0	4.3	68.5
	2	3.9	0.6	27.3	30.5	2.9	66.2
	20	4.7	4.5	4.8	67.6	3.4	85.0

^{a)} Expressed as N atom % added nitrite-N

^{b)} Final concentration

TABLE II. FATE OF NITRITE IN Mb-NITRITE MODEL SYSTEMS

The model systems composed of 5 ml of 1 mM sperm whale Mb, 5 ml of 5 mM sodium nitrite, 10 ml of 0.2 M acetate buffer (pH 5.5) and 5 ml of water were cooked at 70°C for 60 min after curing for 0 and 7 days.

Curing period (days)	Fate of nitrite ^{a)}				
	DNOMb-N	Nitrite-N	Nitrate-N	Gaseous-N	Total-N
0	0	100.0	0	trace	100.0
7	0	96.2	3.4	1.0	100.6

^{a)} Expressed as N atom % of added nitrite-N.

less than that in the absence of AS (Table I). In our previous work¹⁾ on Mb-nitrite-AS model system, the amount of nitrite detected was 63% on 0-day curing with 2 mM AS, 41% on 0-day curing with 20 mM AS, and 19% and 2.4% on 7-day curing with respectively 2 mM and 20 mM AS. This model system did not contain NaCl, whereas the system used in the present study contains 2% NaCl. Even if the

effect of the NaCl addition is taken into account, the present results (Table I) are comparable to those obtained from the model system. These results suggest that the compound in meat decomposes nitrite as well as AS does. However, the nitrite decomposition reaction by AS and that by the compound were not synergistic.

It was noteworthy that in the case of whole

TABLE III. FATE OF NITRITE IN EACH OF FOUR MEAT FRACTIONS

The reaction mixtures composed of 17 ml of each meat fraction, 1 ml of 0.02 M sodium nitrite, 1 ml of 0 or 0.4 M AS, 0.4 g of NaCl and 1 ml of 1.6 M acetate buffer (pH 5.5) were cooked at 70°C for 60 min after curing for 0, 3 and 7 days respectively.

Conditions			Fate of nitrite ^{a)}					
AS ^{b)} (mM)	Curing period (days)	Fraction	DNOMb-N	Nitroso- thiol-N	Nitrite -N	Nitrate -N	Gaseous -N	Total-N
0	0	1	0	0.9	90.0	3.8	0	94.7
		2	0	0	92.5	8.1	1.0	101.6
		3	0	1.3	101.9	2.9	trace	106.1
		4	0	0	97.4	3.0	0.9	100.4
	7	1	0	0	93.5	7.1	1.7	102.3
		2	0	1.2	87.6	11.2	1.0	101.0
		3	0	1.0	96.9	6.5	0	104.4
		4	0	0	99.3	4.9	trace	104.2
20	0	1	0	0	54.5	8.3	10.8	73.6
		2	0.5	1.8	51.4	19.1	8.0	80.8
		3	0	4.1	79.2	11.3	5.5	100.1
		4	0	0.4	82.7	16.4	0	99.5
	3	1	0	0.6	38.0	20.8	14.1	73.5
		2	0.6	5.2	43.9	19.0	4.2	73.2
		3	0	4.1	55.1	14.6	6.8	80.6
		4	0	5.5	86.3	12.2	trace	104.0
	7	1	0	1.8	26.3	32.0	13.1	73.2
		2	0.5	3.0	42.7	31.4	4.0	81.6
		3	0	0.8	46.4	23.3	7.8	78.3
		4	0	2.6	78.7	10.2	2.9	94.4

^{a)} Expressed as N atom % of added nitrite-N.

^{b)} Final concentration.

meat the recovery of nitrite-N was 66~90% and a remarkable amount of added nitrite remained unidentified, though the recovery was 100% in the model system of our previous work. On 0-day curing, the unidentified nitrite-N increased as AS increased, but decreased in the case of 7-day curing. Unidentified nitrite-N was detected even if AS had not been added, and increased with the curing period; this finding is different from that of meat fraction (Table III). These results show that some components other than Mb and/or AS were involved in the formation of unidentified-N compound.

2. Fate of nitrite in various meat fractions

In order to determine the components of meat which may contribute to the differences between the fate of nitrite in whole meat and the fate in Mb-AS model system, four fractions extracted from meat were cured with nitrite, and nitrite-N containing substances, nitrite, nitrate, DNOMb, nitrosothiol and gaseous compounds, were determined as shown in Table III.

Fate of nitrite in Fr. 1

When Fr. 1 was cured without AS, the added nitrite was almost completely recovered as nitrite-N on 7-day as well as on 0-day curing, recoveries being 102 and 95%, respectively.

In the presence of AS, nitrite decreased remarkably with the formation of a notable amount of gas. With increase on the curing period, nitrite-N decreased and both nitro-

sothiol and nitrate-N increased, while gaseous-N remained constant at a level of 11~14%. It was notable that the production of gaseous-N was larger in this fraction than the other meat fractions. The decomposition rate of nitrite in this fraction was larger than that of AS-nitrite model system in both 0- and 7-day cured samples (Table IV). This suggests that Fr. 1 causes nitrite breakdown in the presence of AS, both during the curing period and at the cooking stage.

The recovery of the added nitrite was about 73% after any of the three curing periods. In other words, 27% of nitrite changed into unidentified compounds. This reaction seems to be unaffected by curing period.

Fate of nitrite in Fr. 2

The effect of Fr. 2 on the fate of nitrite in the absence of AS was very similar to that of Fr. 1. About 90% of added nitrite-N was detected as nitrite and its recovery was 100%.

In the presence of AS, almost all Mb in this fraction was covered to DNOMb with generation of gas in each case. On 0-day curing nitrite decreased remarkably at the same rate as in Fr. 1. However, the decrease during 7-days curing was smaller in Fr. 2 than in Fr. 1, and was comparable to that observed in AS-nitrite model system (Table IV). This suggests that Fr. 2 provokes nitrite breakdown at the cooking stage, but not during the curing.

The recovery of the added nitrite lay between 73 and 82% in Fr. 2. The uniden-

TABLE IV. FATE OF NITRITE IN NITRITE-AS MODEL SYSTEM

The model systems composed of 5 ml of 5 mM sodium nitrite, 5 ml of 10 or 100 mM AS, 10 ml of 0.2 M acetate buffer (pH 5.5) and 5 ml of water were cooked at 70°C for 60 min after curing for 0 and 7 days.

Conditions		Fate of nitrite ^{a)}			
AS ^{b)} (mM)	Curing period (days)	Nitrite-N	Nitrate-N	Gaseous-N	Total-N
2	0	89.0	8.0	2.2	99.2
	7	78.4	24.0	0	102.4
20	0	76.0	15.2	4.8	96.0
	7	64.4	22.4	5.4	92.2

^{a)} Expressed as N atom % of added nitrite-N.

^{b)} Final concentration.

tified nitrite-N comprised 20% of the added nitrite-N in the case of 0-day curing (Table III). In the 3-day cured sample the unidentified nitrite-N level increased to 27%, whereas in the 7-day cured sample it was almost equal to the original level (20%).

Recently Knowles *et al.*⁸⁾ investigated the interaction of nitrite with bovine serum albumin at gastric pH and obtained 3-nitrotyrosine, dopa and 6-hydroxynorleucine. In the present work the interaction of nitrite with proteins in Fr. 2 was also investigated. However, acid analysis of such nitrite-treated proteins showed no significant changes.

Fate of nitrite in Fr. 3

In the absence of AS, nitrite hardly changed and the amount of nitrosothiol was very small, although this fraction contained a large amount of sulfhydryl groups and the molar ratio of nitrite to SH was estimated to be approximately 1:2,⁷⁾ on the assumption that only myofibrillar proteins constituted this fraction.

In the presence of AS, only about 4% of the added nitrite was determined as nitrosothiol-N on 0- as well as 3-day curing. The percentage of nitrosothiol-N in the total loss of nitrite-N was calculated as 20, 9 and 1.5% on 0-, 3- and 7-day curing, respectively. These results were similar to the observation of Kubberød *et al.*⁷⁾

In contrast to the case of Fr. 2, the amount of nitrite was large on 0-day curing, and decreased remarkably in the 3- or 7-day cured samples. This finding indicates that Fr. 3 produces the breakdown of nitrite at the cooking stage and also during the curing.

It was also noteworthy that in the 0-day cured sample all the added nitrite was recovered in Fr. 3 and in the 3- and 7-day cured samples the recovery was reduced to 81 and 78%, respectively. The values of nitrosothiol, nitrite and gaseous-N were almost similar for 0- and 3-day curing, and the decrease *i.e.*, 24.1% of the added nitrite-N from 0-day to 3-day curing was equal to the increase in unidentified nitrite-N during these 3 days.

These results suggest that changes in contractile proteins during the curing accelerated the nitrite breakdown and produced unidentified compounds.

Fate of nitrite in Fr. 4

In the absence of AS, the added nitrite hardly changed. In the presence of AS, nitrite decreased at a rate similar to that in the nitrite-AS model system (Table IV). The recovery of the added nitrite was almost 100% under the conditions shown in Table IV, even when AS was present. These results led to the conclusion that Fr. 4 hardly affects the decomposition of added nitrite.

DISCUSSION

The present work indicates that the fate of nitrite in whole meat is different from that in the Mb-nitrite (Table II) and the Mb-nitrite-AS model system (our previous paper¹⁾) in the following points.

a) In whole meat there are components that can decompose nitrite without AS, both during the curing and at the cooking stage.

b) Unidentified nitrite-N is produced by curing as well as by cooking, and its production can be observed even without AS. However, AS can increase its amount.

c) A significant amount of nitrite-N changed into gaseous products even on 7-day curing.

These findings on the fate of nitrite in the meat fraction-nitrite-AS system contribute to the understanding of the components in meat which are responsible for the above-mentioned differences.

Concerning the first point, it is estimated that it was observed that when cured without AS none of the meat fractions decomposes nitrite as does whole meat. This difference in nitrite decomposition between the individual meat fractions and whole meat can be attributed either to a loss in the activity of the effective component during the preparation of the meat fractions or to the decomposition of nitrite resulting from the cooperative reaction

of some of the fractions.

In the presence of AS, nitrite detected decreased greatly in both Fr. 1 and Fr. 2 on 0-day curing. The amount of nitrite detected was more than expected when nitrite was decomposed only by AS (see Table IV). This finding shows that there is a cooperative reaction between AS and some components of meat fractions. By comparing the decomposition rate of nitrite in the nitrite-AS system with that in the nitrite-Mb-AS system, it becomes clear that Mb in Fr. 2 is one of these components. However, possible components other than Mb are still unknown.

On the second point, it is probable that in the presence of AS a notable amount of unidentified nitrite-N is produced on 0-day curing in both Fr. 1 and Fr. 2, but not in Fr. 3 or in Fr. 4. On 7-day curing, the amount of unidentified nitrite-N in Fr. 1 and in Fr. 2 was unchanged, but increased greatly in Fr. 3. These results suggest that in the presence of AS, Fr. 1 and Fr. 2 are involved in the production of unidentified nitrite-N on 0- and 7-day curing, whereas Fr. 3 is concerned with that on 7-day curing.

In the absence of AS, the amount of unidentified nitrite-N in whole meat reached 10% on 0-day and 31.5% on 7-day curing. However, the results with meat fraction-nitrite system showed that significant amounts of unidentified nitrite-N were not produced by the individual fractions. The reason for this difference appears to be the same as that responsible for the difference between the rate of nitrite decomposition in the whole meat and that in any of the meat fractions without AS.

As far as the third point is concerned,

Frs. 2, 3 and in particular Fr. 1 may be concerned with the gas production.

Besides these three points it is interesting that nitrosothiol can be produced by the reaction of added nitrite with every meat fraction, especially with Fr. 4. It should also be noted that Fr. 4 has only a little, if any, effect on the decomposition of the nitrite.

Since unidentified nitrite-N detected in whole meat is very important to assess the safety of nitrite, the true chemical nature of unidentified nitrite-N should be completely elucidated. The results shown in Table III suggest that a detailed investigation on the products of the reaction between the three meat fractions (Frs. 1, 2 and 3) and nitrite to a certain extent may resolve the controversial aspects of the safety of nitrite curing.

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