Reaction between Nitrite and Low Salt-soluble, Diffusible Fraction of Meat. Partial Purification and Some Properties of Unknown Reaction Products[†]

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The reaction products between ¹⁵N-nitrite and 0.05 M NaCl-soluble fraction of meat were chromatographed on Sephadex G-15 column. The unidentified ¹⁵N compound(s) which correspond to about 25% of added nitrite-¹⁵N eluted as one peak, and separated from peptides, nitrite and nitrate. These compounds had a tendency to be adsorbed to Sephadex gel, and eluted after nitrite and nitrate. They had no UV absorption from 240 to 320 nm, and were not nucleotides, amino acids or peptides. They were subjected to thin-layer chromatography and paper electrophoresis. The findings of paper electrophoresis showed that they were strongly acidic compounds.

Nitrite has been used in meat curing for a long time for the purpose of giving a characteristic heat-stable red color¹⁾ and desirable cured flavor²⁾ to meat products and preventing the growth of *Clostridium botulinum*.³⁾ However, nitrite has recently been found to produce strong carcinogenic nitrosamines⁴⁾ and nitrosopyrrolidine.⁵⁾ Thus it is very important to reveal the fate of added nitrite in cured meat in order to reassess the wholesomeness of the curing agent.

The studies conducted so far have manifested that nitrite added to meat products is rapidly depleted⁶⁾ and converted into other forms such as nitrosomyoglobin,⁷⁾ nitrate,⁸⁾ nitrosothiol⁹⁾ and gaseous nitrogen compounds.¹⁰⁾ We have demonstrated that, in a curing model system composed of myoglobin, nitrite and ascorbate, nitrogen of the added nitrite was almost completely recovered in the forms of nitrite and the four above-mentioned compounds.¹¹⁾ On the other hand, in a curing system comprising whole meat, 34% of the added nitrite-N was unidentified.¹²⁾ When pork was separated into four fractions, 0.05 M NaCl-soluble diffusible fraction (Fr. 1), 0.05 M NaCl-soluble

non-diffusible fraction (Fr. 2), Weber-Edsall solution-soluble fraction (Fr. 3) and Weber-Edsall solution-insoluble fraction (Fr. 4) and each fraction was cured, the unidentified-N compounds were produced in Fr. 1, 2 and 3, and the amount of unidentified nitrite-N was the greatest in Fr. 1 (27% of added nitrite-N).¹²⁾

Sebranek¹³⁾ studied the fate of nitrite in comminuted luncheon meat by applying ¹⁵N as a tracer. In his studies, about 25~30% of the ¹⁵N of the added nitrite was detected as non-nitrite substance in water-soluble fraction. This fraction was subjected to Sephadex G-10 column chromatography and a prominent peak of non-nitrite ¹⁵N was separated. The ¹⁵N compounds in this peak are not yet identified. The true chemical nature of these unidentified-N compounds should be completely elucidated to assess the safety of nitrite.

In the present work, we separated the unidentified-N compounds produced by the reaction between Fr. 1 and nitrite as one peak on Sephadex G-15 column and clarified some of their properties. Attempts were also made to further purify them.

MATERIALS AND METHODS

Diffusible fraction. The diffusible fraction was

[†] Fate of Nitrite Added to Cured Meat. Part III. See References 11) and 12).

prepared in essentially the same manner as previously described.¹²⁾ One kg of commercial pork, trimmed free of external fat and connective tissue, was minced twice. The minced meat (800 g) was homogenized very mildly with an equal weight of 0.05 M NaCl for 15 min, and the slurry was centrifuged at $11,000 \times g$ for 15 min. The supernatant obtained was dialysed against 10 liters of 0.05 M NaCl for 20 hr at 4°C. The diffusate was freeze-dried and then dissolved in 2.5 liters of water. This solution is designated as Fr. 1.

Reaction of nitrite and the meat fraction. A mixture containing 30 ml of Fr. 1, 2 ml of 0.02 M ^{15}N -sodium nitrite (99.0 atom %, from Hikari Kogyo Co., Ltd.), 4 ml of 0.2 M sodium ascorbate, 0.8 g of NaCl, 2 ml of water and 2 ml of 1.6 M acetate buffer (pH 5.5) was placed in a tightly-sealed flask and heated at 70°C for 60 min.

Sephadex G-15 gel filtration. After rapidly cooled to room temperature, the reacted mixture was applied to a 5.5×56 cm column of Sephadex G-15 and eluted with 0.05 M NaCl. The 10.4-ml portions were collected and examined for the following characteristics: total nitrogen, ^{15}N , nitrite, nitrate, UV absorption from 240 to 320 nm and reaction with ninhydrin reagent.

Total nitrogen (^{14}N plus ^{15}N) and ^{15}N determination. Seven ml of each portion eluted from Sephadex G-15 column was freeze-dried and then dissolved in 0.2 ml of water. This condensed solution was used for total nitrogen and ^{15}N determination. The ^{15}N content was measured by optical emission spectrometry.¹⁴⁾ The discharge tubes for emission were prepared as shown in Fig. 1.

FIG. 1. Method of Discharge Tube Preparation.

A few microtubes (length, 1 cm; volume, about 5 μl) which had absorbed sample solution (about 5 μg N) were mildly dried and put into the discharge tube. After CuO powder (about 5 mg) and CaO (about 10 mg) were placed in it, the tube was sealed in vacuum.

For sample solutions containing nitrite or nitrate, copper (about 20 mg) was also placed in the tube. The sample was decomposed by heating at 560°C for 7 hr. After cooling to room temperature, the tube was analysed for ^{15}N abundance with a ^{15}N -analyser (Nihonbunko, JASCO NIAI) (A).

The total nitrogen content of each fraction was determined by the dilution method¹⁵⁾ as follows: 0.15 ml of $(^{15}\text{NH}_4)_2\text{SO}_4$ (^{15}N : 30 atom %, 500 μg $^{15}\text{N}/\text{ml}$) was added to the sample solution of each tube, and the ^{15}N atom % (B) was analysed as stated above. Total nitrogen (x $\mu\text{g}/\text{ml}$) and ^{15}N content (y $\mu\text{g}/\text{ml}$) of each fraction were obtained by the following equations.

$$x = [(150 - 500A)/(B - A)] \times 0.2/7$$

$$y = Ax/100$$

Nitrite and nitrate determination. Nitrite and nitrate were determined as described in the previous paper.¹²⁾

Thin-layer chromatography. The unidentified ^{15}N compounds were subjected to cellulose thin-layer chromatography. *n*-Butanol-acetic acid-water (4:1:1) was used as a solvent system. Materials were located with iodine vapor and ninhydrin. After development, the thin-layer was divided into several parts. Each part was scraped off and extracted with ethanol-water (1:1). The extracts were analysed for ^{15}N abundance.

Paper electrophoresis. The unidentified ^{15}N compounds were subjected to paper electrophoresis. The following operating conditions were used: paper, Toyo filter paper No. 51 (12.5×26 cm); solvent systems, pyridine-acetic acid-water (10:0.4:90, pH 6.4; 1:8:90, pH 4.0) and 80% formic acid-acetic acid-water (5:15:80, pH 1.8); current, 0.8 mA/cm; duration, 30 min. After running, the paper was divided into several pieces, extracted with ethanol-water (1:1). The extracts were analysed for ^{15}N abundance.

RESULTS

Sephadex G-15 gel chromatography

Table I shows the fate of nitrite added to 0.05 M NaCl-soluble diffusible meat fraction (Fr. 1). These data were picked out from Table III in the previous paper¹²⁾ to facilitate discussion of further results in the present paper. The added nitrite-N was recovered at most 73.6% as DNOMb (denatured nitrosomyoglobin)-N, nitrosothiol-N, nitrite-N, nitrate-N and gaseous-N. The remainder, 26.4%, was not identified.

The reaction products of nitrite and diffusible fraction of meat were chromatographed on

TABLE I. FATE OF NITRITE IN DIFFUSIBLE MEAT FRACTION (Fr. 1)

The reaction mixture composed of 30 ml of Fr. 1, 2 ml of 0.02 M ^{15}N -sodium nitrite, 4 ml of 0.2 M sodium ascorbate, 0.8 g of NaCl, 2 ml of water and 2 ml of 1.6 M acetate buffer (pH 5.5) was heated at 70°C for 60 min.

Fate of nitrite ^{a)}					
DNOMb-N	Nitroso-thiol-N	Nitrite-N	Nitrate-N	Gaseous-N	Total-N
0	0	54.5	8.3	10.8	73.6

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

a Sephadex G-15 column to separate these unidentified-N compounds. Figure 2 shows the total nitrogen and ^{15}N contents of every portion eluted off the column. Total nitrogen eluted mostly from tubes 65 to 85. ^{15}N elution gave three major peaks. The first eluting peak from tubes 68 to 84, the second from tubes 89 to 101 and the third from tubes 102 to 114 were designated as P1, P2 and P3, respectively.

FIG. 2. Sephadex G-15 Gel Chromatogram of the Reaction Products of Fr. 1 Traced with Total Nitrogen and ^{15}N Content.

Forty ml of the reacted products was applied to a 5.5×56 cm column and eluted with 0.05 M NaCl. Eluate was collected in 10.4 ml portions and, total nitrogen (^{14}N plus ^{15}N) and ^{15}N content were measured for every tube.

The effluences were also scanned with ninhydrin reagent. As shown in Fig. 3, the major

FIG. 3. Sephadex G-15 Gel Chromatogram of the Reaction Products of Fr. 1 Traced with Ninhydrin Positive Substances.

Each tube obtained from the chromatography shown in Fig. 2 was traced with ninhydrin reagent.

portion of ninhydrin-positive compounds was detected in the same position as P1. Since these compounds were supposed to be low molecular peptides, ^{15}N included in P1 may be bound with these peptides in such forms as nitroso compounds. However, nitrosothiol-N was not observed in the reaction products of diffusible meat fraction and nitrite (Table I).

Figure 4 indicates the results of the colorimetric determination of nitrite on the effluents

FIG. 4. Sephadex G-15 Gel Chromatogram of the Reacted Products of Fr. 1 Traced with Colorimetric Nitrite.

Each tube obtained from the chromatography shown in Fig. 2 was traced with colorimetric nitrite.

of the same column. The main peak was observed from tubes 90 to 107, and tube 94

showed the highest value. This position was exactly the same as that of P2. Nitrate also eluted in the same position as nitrite (not shown in the Figure). Thus, ^{15}N of P2 was attributable to nitrite- ^{15}N and nitrate- ^{15}N .

Table II shows the amounts of total N and ^{15}N , ^{15}N atom % and the recovered quantities

TABLE II. AMOUNTS OF TOTAL N, ^{15}N , ^{15}N ATOM % AND YIELD OF ADDED NITRITE- ^{15}N IN EACH PEAK OBTAINED FROM SEPHADEX G-15 COLUMN

	P1 (tubes 68~84)	P2 (tubes 89~101)	P3 (tubes 102~114)
Total N (μg)	78000	1400	830
^{15}N derived from added nitrite (μg)	91	167	158
^{15}N atom % excess	0.12	11.9	19.0
Yield of added nitrite- ^{15}N (%)	15.2	27.8	26.3

of added nitrite- ^{15}N in P1, P2 and P3. Since P1 contained a large amount of ^{14}N , its ^{15}N atom % excess was only 0.12%. Such a low value may inevitably affect the accuracy of the determination of ^{15}N content in P1. The ^{15}N content of P2 amounted to 27.8% of the added nitrite-N. However, this value was considerably lower than 62.8%, the value expected from the nitrite and nitrate contents listed in Table I. This result means that, on determination by optical emission spectrometry, nitrite and nitrate were hardly converted into nitrogen gas even in the presence of reducing agent such as copper. P3 showed the highest value of ^{15}N atom % excess, 19.0%, among the three peaks, and its ^{15}N content amounted to 26.3% of the added nitrite-N. This value was comparable to the value of the unidentified nitrite-N, 26.4%. Since the results described above clearly demonstrated that P3 could not be ascribed to DNOMb, nitrite, nitrate, nitrosothiol or gaseous nitrogen, the ^{15}N -compounds of P3 were concluded to be the unidentified compounds derived from the added nitrite.

P3 contained no or a little nitrite, nitrate, hydroxyl amine¹⁶⁾ and ammonium (checked by distillation method). UV absorption of each

tube obtained from the chromatography in Fig. 2 was scanned from 240 to 300 nm. Tubes 68~74 had maximum absorbance at 250 nm, tubes 75~91 at 266 nm, tubes 103~112 at 289 nm, tubes 116~128 at 251 nm and tubes 133~145 at 270 nm. The absorbance at 266 nm of tubes 75~91 seems to be attributable to ascorbate and its derivatives. P3 (tubes 102~114) had maximum absorbance at 289 nm, and the highest absorbance at 289 nm occurred in tube 108. However, the highest value of ^{15}N did not occur in this tube, but in tube 105. Therefore, this UV absorbance at 289 nm in P3 may derive from contaminants other than ^{15}N -compounds.

Thin-layer chromatography of ^{15}N -compounds in P3

All effluents of P3 were collected, freeze-dried and kept in vacuum at -20°C until use. This dried material contained a large amount of NaCl. ^{15}N -compounds were effectively extracted with ethanol from this material, unaccompanied by NaCl. The extract was subjected to thin-layer chromatography. Figure 5

FIG. 5. Thin-layer Chromatography of Ethanol Extracts from P3 and Distribution of ^{15}N on Chromatogram.

Layer, cellulose; Solvent, *n*-butanol-acetic acid-water (4:1:1); Development, (a) iodine, (b) ninhydrin.

showed the results. The color development with iodine vapor gave 5 spots, and that with ninhydrin reagent, 2 spots. This cellulose-layer was then divided into 9 parts, and ^{15}N % of each part was measured. The ^{15}N concentration was the highest in part 4, and it

was 16.88 atom % excess. Since there were no ninhydrin spots in part 4, the ^{15}N compounds in this part were neither amino acids nor peptides.

Paper electrophoresis

Ethanol extract of P3 was subjected to paper electrophoresis. On running at pH 6.4, ^{15}N concentration was the highest in part 6 among the 7 parts into which the extract was divided, as shown in Fig. 6, and almost all of the ^{15}N -

FIG. 6. Paper Electrophoretic Pattern of Ethanol Extracts of P3.

Arrows show the starting points of samples. Glu, Tyr, and Lys in the pattern indicate their locations when they were co-run.

* ^{15}N atom % excess.

compounds moved to the anode. On running at either pH 4.0 or 1.8, the ^{15}N -compounds also moved to the anode. To investigate whether the ^{15}N -compounds decomposed under the low pH, IR spectra of ^{15}N -compounds separated under pH 1.8 (part 3,4 and 5 in Fig. 6) were compared with that of the separated one under pH 6.4 (part 6 in Fig. 6) (Fig. 7). Since these spectra were almost identical, ^{15}N -compounds were shown to be stable under the low pH. From these results, the ^{15}N compounds in P3 are presumed to be strongly acidic compounds.

DISCUSSION

In the reaction between nitrite and low salt-soluble diffusible fraction of meat, 74% of added nitrite-N was recovered as DNOMB,

FIG. 7. IR Spectra of ^{15}N -compounds Separated by Paper Electrophoresis under pH 1.8 and 6.4.

These spectra were taken in film made from ethanol solution.

—, pH 1.8 (parts 3,4 and 5 in Fig. 6); ----, pH 6.4 (part 6 in Fig. 6).

nitrosothiol, nitrite, nitrate and gaseous N compounds (Table I). Therefore, 26% of added nitrite was shown to be converted into unidentified nitrite-N compounds.¹²⁾ Separation on Sephadex G-15 column chromatography indicated that these compounds eluted as one peak, had no UV absorption maxima from 240 to 340 nm, and were not nucleotides, peptides or amino acids.

Sebranek¹³⁾ described in his studies on the fate of nitrite in comminuted luncheon meat that a large proportion of added nitrite- ^{15}N was included in water-soluble non-nitrite substance, and he separated non-nitrite ^{15}N as one peak by Sephadex G-10 column chromatography. The peak had 16% of the total ^{15}N added originally, and did not involve nucleotides, peptides or proteins. These properties were very similar to those of P3 obtained in the present study. However, a noticeable difference existed in the eluting position on gel filtration. The peak demonstrated by Sebranek (water-soluble non-nitrite ^{15}N peak) eluted fairly faster than nitrite, whereas P3 eluted more slowly than nitrite possibly because of its tendency to be adsorbed to Sephadex gel. We need further studies to know whether this difference indicated the distinction between the unidentified-N compounds produced by whole meat and those by low salt-soluble diffusible fraction of meat, or was caused by the variation in separating con-

ditions.

The thin-layer chromatogram of P3 indicated that ^{15}N derived from added nitrite was concentrated in a narrow range ($R_f=0.3$), where there were no spots developed with iodine vapor or ninhydrin reagent. The paper electrophoretic pattern showed that there was only one site including higher ^{15}N . The ^{15}N compounds in this site behaved as strongly acidic compounds. We have as yet no effective means to check whether these ^{15}N compounds were completely purified or not. However, the strong acidity of these ^{15}N compounds may prove a key to purification of these compounds. We are now investigating what kind of functional group is related to this acidic property.

Several workers¹⁷⁾ have revealed the role of a low-molecular meat fraction in nitrite decomposition. To clarify the nitrite-decomposable compounds associated with the production of the unidentified ^{15}N compounds is also helpful in identifying this unidentified compound. Our studies from this point of view will be reported in an other paper.

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