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Effect of Sodium Nitrite and Sodium Nitrate on Botulinal Toxin Production and Nitrosamine Formation in Wieners

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Wieners were formulated and processed approximating commercial conditions as closely as possible. Twenty-four batches of product were made with the addition of six levels of sodium nitrite (0, 50, 100, 150, 200, and 300 μg/g), four levels of sodium nitrate (0, 50, 150, and 450 μg/g), and two levels of *Clostridium botulinum* (0 and 620 spores/g). After formulation, processing, and vacuum packaging, portions of each batch were incubated at 27 C or held for 21 days at 7 C followed by incubation at 27 C for 56 days. The latter storage condition approximated distribution of product through commercial channels and potential temperature abuse at the consumer level. Samples were analyzed for botulinal toxin, nitrite, and nitrate levels after 3, 7, 14, 21, 28, and 56 days of incubation. When nitrite was not added, toxic samples were detected after 14 days of incubation at 27 C. At the lowest level of nitrite added (50 μg/g), no toxic samples were observed until 56 days of incubation. Higher levels of nitrite completely inhibited toxin production throughout the incubation period. Nine uninoculated samples, representing various levels and combinations of nitrite and nitrate, were evaluated organoleptically. The flavor quality of wieners made with nitrite was judged significantly higher ($P = 0.05$) than of wieners made without nitrite. The nine samples were negative for 14 volatile nitrosamines at a sensitivity level of 10 ng/g. The results indicated that nitrite effectively inhibited botulinal toxin formation at commercially employed levels in wieners and that detectable quantities of nitrosamines were not produced during preparation and processing of the product for consumption.

The use of nitrite and nitrate in cured meat products is receiving considerable attention throughout the world. The advisability of totally eliminating nitrite is questionable. The potential for growth of *Clostridium botulinum* in the absence of nitrite has not been completely assessed, and no substitute has been found for nitrite which provides an organoleptically acceptable product. This study is one of a series undertaken cooperatively by The American Meat Institute, the Food and Drug Administration, and the United States Department of Agriculture in an effort to determine the role of nitrite and nitrate on botulinal toxin production, nitrosamine formation, and manufacturing needs in cured meat products. Toxin assays in this study were performed by the Food Research Institute of the University of Wisconsin. This study is focused on toxin and nitrosamine formation in finely comminuted cured meat products.

MATERIALS AND METHODS

Experimental design. Six levels of nitrite (0, 50, 100, 150, 200, and 300 μg/g), four levels of nitrate (0, 50, 150, and 450 μg/g), two inoculum levels (0 and 620 spores/g, arithmetic mean), seven incubation periods (0, 3, 7, 14, 21, 28, and 56 days) and three storage conditions (7 C, controls only; 27 C; and 27 C preceded by 21 days at 7 C) constituted the variables. The nitrite, nitrate, and spore levels were initial levels

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added at formulation. One inoculated replicate of a factorial design for toxin assay and one uninoculated replicate of a factorial design for nitrite, nitrate, and proximate analyses were included. Uninoculated wieners were evaluated organoleptically and analyzed for nitrosamines. A total of 24 batches of wieners consisting of 2,131 units was prepared for this study, and 1,320 inoculated units were analyzed for toxin. In addition, 36 units of uninoculated product were examined for toxin as a control. A unit consisted of one inoculated or eight uninoculated wieners per package. Toxin assays were performed on uninoculated wieners when gas production was evident during the incubation period or at the end of the period, whichever occurred first.

Inoculum. Ten strains of *C. botulinum*, five of type A (33A, 52A, 77A, 36A, and ATCC 12885) and five of type B (53B, 41B, 7949B, 40B, and Lamanna), were used to inoculate the meat mixture. Washed spore suspensions of the cultures were provided by L. N. Christiansen (Swift and Co., Oak Brook, Ill.). The spore suspensions were heat shocked (80 C for 15 min), and the numbers were estimated by using a five-tube most-probable-number procedure. A stock spore suspension was prepared in which equal numbers of each of the 10 strains were employed. Spores from this suspension were heat shocked prior to inoculating the meat mixture.

To determine the spore inoculum level in the sausage mixture and finished product, 10-g samples were blended in 90 ml of diluent containing 0.1% peptone. The five-tube most-probable-number procedure was used, and the growth medium consisted of Trypticase soy broth (BBL). The toxicity of each tube evidencing growth was tested as described under "Toxicity testing."

After inoculation of the meat mixture, an average of 620 spores/g was detected (average of 15 trials). In the processed product, this level decreased to approximately 325 spores/g (average of 9 trials).

Incubation of cultures. All cultures were incubated anaerobically by using the Gas-Pac system (BBL) at 37 C.

Toxicity testing. To detect toxin in the incubated product (i.e., held as described in the "Holding conditions" section), 20 g of sample was blended with 40 ml of gel-phosphate buffer (gelatin, 2.0 g; Na_2HPO_4, 4.0 g; water, 1 liter; pH 6.2) in a Waring blender. The sample was centrifuged, and 0.5-ml amounts of the supernatant fluid were injected into two mice by the intraperitoneal route. If a mouse died within 4 days, the toxin was identified by the usual protection tests by using pooled antitoxins A and B. Type A antitoxin was provided by H. Sugiyama (Food Research Institute of the University of Wisconsin) and type B antitoxin was obtained from Burroughs-Welcome and Co. (Research Triangle Park, N.C.).

Product formulation. The following formula was used to prepare the meat mixture (values in percent): pork (50% lean), 42.50; beef (95% lean), 31.41; ice, 17.13; water, 3.20; salt, 2.51; dried corn syrup, 1.80; dextrose, 1.02; spice, 0.39; and sodium ascorbate, 0.04.

The meat trimmings were preground through a 0.32-cm plate and mixed with the other ingredients in a Rietz ribbon mixer for 10 to 15 min at approximately −5 C. Sufficient ingredients were mixed to prepare four batches per day (125.6 kg). Part of the mixture was used to prepare the inoculated wieners (3.63 kg/batch) and part was used to prepare the uninoculated wieners (24.95 kg/batch). Water was withheld from the above formula to allow for the addition of nitrite and nitrate solutions.

Preparation procedure. A model 84142 Hobart silent chopper was used to prepared 3.63 kg of the mixture for the inoculated product. The product was chopped for 30 s, the spore suspension was added, and the mixture was chopped for an additional minute. The appropriate nitrite and nitrate solutions were added, and the mixture was chopped for an additional 8 to 9 min. The final temperature of the mixture was approximately 20 C. A comparable procedure was employed for the uninoculated product.

The sausage mixture was stuffed into cellulose casings (size 25 Nojax castings, Union Carbide Corp., Chicago, Ill.) and linked to form links weighing approximately 45 g each. The wieners were immersed in liquid smoke (C-6 Charsol, Red Arrow Products Co., Manitowoc, Wis.) for 30 s at about 21 C and then rinsed with tap water.

Processing and packaging procedures. The links were processed as follows: 30 min at 49 C, 20 min at 71 C, and about 15 min at 87.7 C (or until an internal temperature of 71 C was reached). After heat processing, the wieners were chilled with tap water to 32 C and immersed in a 5.0% sodium chloride brine solution at about 0 C until an internal temperature of 4 C was reached.

The casings were removed from the inoculated wieners, and each link was individually vacuum packaged in Saran-coated Mylar by using a Giglio Luigi (model Brevattatto) closing machine. A portable bar sealer was used to apply a second seal to each package to guard against leakage. The uninoculated wieners were vacuum packaged (8 links per unit) in Saran film.

Holding conditions. After packaging, one-half of each batch of inoculated product and one-fourth of the uninoculated product were incubated at 27 C for 56 days. During this time samples were examined for toxin and levels of nitrite and nitrate.

One-half of each batch of both inoculated and uninoculated product was held at 7 C for 21 days followed by incubation at 27 C for 56 days. Again, samples were periodically examined for toxin and levels of nitrite and nitrate. This phase of the study was intended to approximate the time interval between manufacture and purchase by the consumer who might abuse the product by storing it at improper temperatures.

The remaining one-fourth of the uninoculated product was held at 7 C for 56 days and intermittently analyzed for nitrite and nitrate.

Analytical methods. Analysis for nitrite was a modified colorimetric method (1) of the Association of Official Analytical Chemists (AOAC) wherein sulfanilic acid and α-napthylamine were added separately to the meat sample. The analysis for nitrate

was a modified AOAC method (1) involving the use of urea at an acid pH to destroy nitrite. Nitrate was then reacted with *meta*-xylenol followed by distillation and colorimetric estimation. Proximate analyses were performed by using AOAC methods for meat samples (1).

Organoleptic evaluation of nine wiener samples containing different levels of nitrite and nitrate was performed by an 18-member expert panel. The nine samples contained the following initial levels of added nitrite and nitrate (μg/g), respectively: 0, 0; 50, 0; 50, 50; 100, 0; 100, 150; 150, 0; 150, 450; 200, 0; and 300, 0. Wieners were cooked for 7 min in water brought to boiling and then maintained in a warming unit until sampled. Triplicate evaluations of each sample for tenderness, juiciness, and flavor quality were performed by using 0 to 9 hedonic scales. No attempt was made to disguise the appearance of the samples. Each of these three characteristics was statistically analyzed by using two-way analysis of variance.

After packaging, three eight-link packages of each of the above nine samples were frozen at −54 C before being sent to the Food and Drug Administration Laboratories, Washington, D. C., for nitrosamine analyses. Samples were analyzed at the 10 ng/g detection level by using the 14 volatile nitrosamine multidetection method, which includes gas-liquid chromatography and mass spectroscopy as the confirmatory method (T. Fazio, J. W. Howard, and R. H. White, 1971, Proceedings on Analysis and Formation of Nitrosamines, Heidelberg, Germany, Oct. 13–15, in press). Wieners were analyzed as received (no further heating), after water heating (as described for taste panel evaluations), or after pan frying.

TABLE 1. *Effect of processing and storage conditions on sodium nitrite (μg/g) in vacuum-packaged wieners*

Time of analysis	Levels of nitrite (μg/g)					
	0[a]	50[a]	100[a]	150[a]	200[a]	300[a]
Before process	2	41	77	131	175	256
After process	2	13	29	51	74	115
Days storage at 7 C						
3	2	8	18	33	25	56
7	1	7	17	25	25	49
14	2	6	10	17	15	32
21	1	5	7	11	11	15
28	2	4	6	5	10	12
56	5	9	4	5	5	8
Days storage at 27 C						
3	2	5	11	16	9	29
7	2	3	5	6	5	7
14	1	3	3	4	3	3
21	1	2	3	3	4	4
28	2	3	3	4	4	5
56	2	3	3	3	2	2
Days storage at 27 C after 3 weeks at 7 C[b]						
3	2	4	5	9	7	5
7	3	4	5	5	6	5
14	3	3	3	4	6	5
21	3	4	3	3	4	4
28	3	4	3	2	4	3
56	2	3	3	3	2	2

[a] Amount of nitrite added initially (micrograms per gram).

[b] Represents an average of four determinations at various nitrate levels. All other nitrite values represent an average of two determinations at various nitrate levels.

TABLE 2. *Effect of processing and storage conditions on sodium nitrate (μg/g) in vacuum-packaged wieners*

Time of analysis	Levels of nitrate (μg/g)			
	0[a]	50[a]	150[a]	450[a]
Before process[b]	37	83	185	483
After process[b]	47	85	168	415
Days storage at 7 C				
3	50	91	156	436
7	46	90	169	434
14	49	61	165	382
21	38	58	166	409
28	38	52	150	390
56	33	43	137	339
Days storage at 27 C				
3	29	46	112	280
7	0	26	60	275
14	10	31	56	258
21	6	22	51	213
28	1	24	15	178
56	3	9	8	215
Days storage at 27 C after 3 weeks at 7 C[b]				
3	22	40	115	320
7	18	21	67	260
14	9	23	63	212
21	8	19	77	222
28	5	22	60	245
56	5	22	69	243

[a] Amount of nitrate added initially (micrograms per gram).

[b] Represents an average of six determinations at various nitrite levels. All other nitrate values represent an average of three determinations at various nitrite levels.

RESULTS AND DISCUSSION

The average composition of the 24 batches of wieners was: 54.9% moisture, 10.9% protein, 29.5% fat, and 2.6% salt.

The effect of processing and storage conditions on nitrite depletion is given in Table 1. After contact with the meat in the chopper, an average nitrite reduction of 16% occurred. A further nitrite reduction of about 51% occurred during processing. Thus, approximately 33% of the added nitrite remained in the product after preparation. This level is in agreement with previously published data on commercially pre-

pared wieners containing sodium ascorbate which retain 10 to 30% of the added nitrite (2, 4). Low levels of nitrite were detectable in product to which no nitrite was added (Table 1).

The rate of nitrite depletion was dependent on the temperature of storage. Nitrite reduction at 7 C was less rapid than at 27 C. After 21 days at 7 C, only 15 μg of nitrite per g were present at the highest level of addition (300 μg/g). At 27 C, the greatest loss in nitrite occurred during the first 3 days. After 7 days, only 7 μg/g or less was present at any level of nitrite addition. Storing the wieners for 21 days at 7 C before storage at 27 C resulted in only small differences in residual nitrite.

Nitrate concentration was also influenced by storage temperature (Table 2). Decreases in nitrate of 12 to 53% occurred during storage at 7 C and were not dependent on the level of added nitrate. During storage at 27 C, or 21 days at 7 C followed by 27 C, the depletion in average nitrate concentration ranged from 23 to 93% and 24 to 77%, respectively. Small quantities of nitrate were present in samples to which no nitrate was added. Some depletion of this nitrate during storage was evident (Table 2). The observed depletion of nitrate was possibly a result of the growth of microbial contaminants.

The development of toxin in the wiener samples was markedly influenced by the level of nitrite added to the meat (Table 3). Only two samples containing 50 μg of added nitrite per g became toxic after 56 days storage at 27 C. Nitrite concentrations above this level completely suppressed toxin formation in all samples. Toxin was present in 79 of 220 nitrite-free samples. Eighty-one of the 1,320 samples examined were toxic. Toxin was not detected until the product was incubated at 27 C for at least 14 days.

A factor that may govern growth and toxin production is the relatively uniform distribution of nitrite in the product. The probability of spore contact with nitrite, or some reaction product of nitrite, would be enhanced in this finely comminuted, well-mixed product, as compared to a coarsely comminuted or larger particle-type product that is not thoroughly mixed.

Although residual levels of nitrite were generally low during storage (Table 1), toxin was not detected in inoculated product initially formulated to contain 100 μg or more of nitrite per g. The level of nitrite at the time of manufacture rather than the level of residual nitrite is the key

TABLE 3. *Effect of nitrite and nitrate on incidence of toxic samples during incubation*

Days of storage	Levels of nitrite and nitrate (μg/g), respectively					
	0, 0[a]	0, 50[a]	0, 150[a]	0, 450[a]	50, 0[a]	50–300, 0–450[a, b]
Storage at 27 C						
3	0/5[c]	0/5	0/5	0/5	0/5	0/5[d]
7	0/5	0/5	0/5	0/5	0/5	0/5[d]
14	5/5	2/5	0/5	0/5	0/5	0/5[d]
21	4/5	14/14	0/5	2/6	0/7	0/5[d]
28	5/5	0/1	0/5	2/5	0/3	0/5[d]
56	4/5	—	0/5	0/4	1/5	0/5[d]
Total toxic	18	16	0	4	1	0
Storage at 27 C after 21 days at 7 C						
3	—[e]	—[e]	—[e]	—[e]	—[e]	—[e]
7	0/5	0/5	0/5	0/5	0/5	0/5[d]
14	4/5	5/5	0/5	5/5	0/5	0/5[d]
21	2/5	5/5	0/5	1/5	0/5	0/5[d]
28	4/5	2/5	0/5	2/5	0/5	0/5[d]
56	4/5	3/5	0/5	4/5	1/5	0/5[d]
Total toxic	14	15	0	12	1	0

[a] Amount of nitrite and nitrate, respectively, added initially (micrograms per gram).
[b] Refers to following levels: nitrite (50, 100, 150, 200, 300 μg/g) and nitrate (0, 50, 150, 450 μg/g).
[c] Number of toxic samples/total samples examined.
[d] Toxin not detected in any of 1,045 samples containing various combinations of nitrite and nitrate in the indicated range of values.
[e] Samples not examined.

protective factor in inhibiting toxin production (3).

Storage at 7 C for 21 days prior to incubation at 27 C had very little influence on the incidence of toxic samples. Toxin was not detected in uninoculated wieners.

The data indicate that nitrate had little effect on toxin production (Table 3). In the absence of added nitrite, 32, 31, 0, and 16 toxic samples were detected with 0, 50, 150, and 450 μg of added nitrate per g, respectively. The samples containing 150 μg/g were further examined and shown to contain viable spores. The reason these spores failed to grow and produce toxin is not known.

Characteristic cured meat color was absent in the nitrite-free samples. No differences were observed in the color of wieners containing the various levels of nitrite. The color of wieners made without nitrite but smoked with hickory sawdust has been described as brown of varying intensity on the surface and grey in the interior (6). Smoke apparently contains sufficient oxides of nitrogen to react with myoglobin to form small amounts of the nitroso meat pigment on the surface of the product. In the study reported herein, liquid smoke imparted a characteristic reddish-brown surface color to the wieners made with added nitrite; nitrite-free wieners had a light brown surface color.

TABLE 4. *Organoleptic evaluation and nitrosamine analyses of wiener samples containing different levels of nitrite and nitrate*

Added nitrite (μg/g)	Added nitrate (μg/g)	Organoleptic evaluation[a]			Nitrosamine analyses of uncooked, water cooked, or pan fried samples[b]
		Tenderness	Juiciness	Flavor quality	
0	0	5.70	5.42	4.09[c]	Negative
50	0	5.80	5.55	5.41	Negative
100	0	5.48	5.44	5.59	Negative
150	0	5.98	5.80	6.18	Negative
200	0	6.14	4.85	5.33	Negative
300	0	5.15	5.01	5.32	Negative
50	50	6.19	5.39	5.21	Negative
100	150	6.01	5.19	5.44	Negative
150	450	5.81	5.15	5.63	Negative

[a] Mean panel scores using a 0 to 9 hedonic scale.

[b] The analyses sensitive to 10 ng/g included the following volatile *N*-nitrosamines: dimethylamine, methylethylamine, diethylamine, methylpropylamine, ethylpropylamine, dipropylamine, ethylbutylamine, propylbutylamine, methylamylamine, dibutylamine, piperidine, pyrrolidine, morpholine, and diamylamine.

[c] Significantly different at the 5% level.

The flavor quality of wieners containing 50 to 300 μg of nitrite per g (Table 4) was judged significantly higher ($P = 0.05$) than for wieners made without nitrite. Flavor quality was not affected by nitrate. Similar data on the effect of nitrite and nitrate on flavor have been reported (5). Taste panel scores for tenderness and juiciness were not affected by either nitrite or nitrate.

Nine samples containing various levels of nitrite and nitrate were analyzed for 14 volatile nitrosamines (Table 4). Each wiener sample was analyzed before heating, after water heating for 7 min, and after pan frying. The 27 results were negative for nitrosamines. This is in agreement with a recent study concerning the effect of sodium nitrite concentration on *N*-nitrosodimethylamine formation in wieners (2).

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