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Effect of Nitrite and Nitrate on Toxin Production by *Clostridium botulinum* and on Nitrosamine Formation in Perishable Canned Comminuted Cured Meat

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Comminuted ham was formulated with different levels of sodium nitrite and nitrate, inoculated with *Clostridium botulinum*, and pasteurized to an internal temperature of 68.5 C. When added to the meat, nitrite concentrations decreased, and cooking had little effect on them. Nitrite concentrations decreased more rapidly during storage at 27 than at 7 C; however they remained rather constant at formulated levels throughout the experiment at both incubation temperatures. The level of nitrite added to the meat greatly influenced growth and toxin production of *C. botulinum*. The concentration of nitrite necessary to effect complete inhibition was dependent on the inoculum level. With 90 *C. botulinum* spores/g of meat, botulinum toxin developed in samples formulated with 150 but not with 200 μg of nitrite per g of meat. At a spore level of 5,000/g, toxin was detected in samples with 400 but not with 500 μg of nitrite per g of the product incubated at 27 C. At lower concentrations of nitrite, growth was retarded at both spore levels. No toxin developed in samples incubated at 7 C. Nitrate showed a statistically significant inhibitory effect at a given nitrite level; however, the effect was insufficient to be of practical value. Analyses for 14 volatile nitrosamines from samples made with varying levels of nitrite and nitrate were negative at a detection level of 0.01 μg of nitrite or nitrate per g of meat.

There is much conflicting information in the earlier literature concerning the effect of curing salts on botulinal toxin production in cured meats. More recent information has indicated that nitrite in particular plays a significant role in inhibiting growth of *C. botulinum* and other organisms in cured meat products (6, 10, 13-15, 17, 18).

Ham, bacon, dry sausage, frankfurters, and other cured meats differ from each other in many respects (e.g., formulation, processing techniques, packaging, and manner of marketing). For this reason, the determination of nitrite and nitrate levels needed to control the botulinal hazard must be made separately for each class of product.

Perishable canned ham does not receive sufficient thermal processing to prevent the possibility of botulinal toxin production in the product and thus is labeled "keep under refrigeration." It is also recognized that this class of product is often temperature abused at the retail and consumer level. In addition to the role played by curing salts in reducing the botulinal hazard, there is current interest in the role of these compounds in the formation of nitrosamines in cured meat products (5). This paper is the first in a series reporting collaborative studies by the meat industry, United States Department of Agriculture, and Food and Drug Administration on the minimal levels of nitrite or nitrate, or both, necessary for control of botulinal toxin production in several types of cured meat products.

MATERIALS AND METHODS

Experimental design. Eight levels of nitrite (0 to 500 μg/g of meat) and four levels of nitrate (0 to 2,000 μg/g of meat) were tested. This includes levels above and below current levels permitted by federal regulations (nitrite, 200 μg/g of meat; nitrate, 500 μg/g of meat). Two levels of botulinal spores plus uninoculated controls were included as outlined in Table 1. Analysis of both cooked and raw inoculated product yielded virtually the same levels of 90 and 5,000 spores per g. The experiment consisted of one-half replicate of a factorial design for toxin assay plus one replicate of a factorial design for chemical analysis of nitrite and nitrate plus necessary controls. This resulted in 1,285 cans of product for toxin assay. Additional cans were used for chemical analyses and confirmation of spore levels.

Inoculum. A mixture of spores of five type A (77A, 62A, 33A, 12885A, and 36A) and five type B (9B, 40B, 41B, 51B, and 53B) strains of *C. botulinum* were used. Spores of the individual strains were produced in the medium of Schmidt and Nank (16) consisting of 5% trypticase (BBL), 0.5% peptone (Difco), and 0.05% sodium thioglycolate using the conventional procedure of Anellis and Rowley (2). The composite spore suspension was heat shocked (15 min at 80 C) and mixed into the meat immediately after the nitrite and nitrate were added.

Product formulation. Fresh, whole, boneless pork hams were ground and formulated to contain 2.5% sodium chloride, 0.5% dextrose, and 0.02% sodium isoascorbate. Analysis of the product showed 2.2% salt, 55.9% moisture (3.75% brine), 27.4% fat (pH 6.24), and a water activity of 0.96 to 0.97.

Processing conditions. The meat mixture (approximately 80 g) was filled into 208 × 107 aluminum tear-top cans, closed under vacuum, and cooked in 77 C water to an internal temperature of 68.5 C per standard industry practice. The product was then chilled in ice water to less than 27 C within 15 min. Temperature was monitored by thermocouples placed in the center of several cans.

Holding conditions. The bulk of the canned product was abused by holding at 27 C and observed over 6 months. A lesser number of the canned product was held at 7 C. Cans were removed according to a predetermined sampling schedule or at time of swelling, whichever occurred first. Uninoculated product was tested to determine the residual levels of nitrite and nitrate.

Assays for spore levels and toxin. Modified peptone colloid (7) was used for viable spore counts. All samples were heat shocked (15 min at 80 C) before analysis. Toxin assays were made by blending half of each can of product with an equal weight of gelatin phosphate buffer. The slurry was centrifuged, and the supernatant fluid (0.5 ml) was injected into Swiss strain white mice of weights varying from 18 to 20 g. Botulinal toxin was confirmed with one unprotected mouse, one mouse protected with AB botulinal antitoxin, and one mouse injected with 0.5 ml of boiled supernatant fluid. Death of the unprotected and survival of the latter two mice indicated botulinal toxin in the sample. Samples yielding questionable results were reconfirmed by additional testing on the remaining portion of the sample which had been frozen.

Chemical analyses. Nitrite and nitrate concentrations were determined spectrophotometrically by using modifications of methods 24.014 and 24.011 of the Association of Official Analytical Chemists (4). The nitrite procedure involved the separate additions of sulfanilic acid and alpha-naphthylamine to the sample. For nitrate analysis the sample was treated with urea in acid solution to destroy the nitrite. The nitrate then was reacted with meta-xylenol, the reaction product was distilled off, and the distillate was diluted for analysis. Samples with various combinations of nitrite and nitrate levels were analyzed for nitrosamine content by using the Food and Drug Administration's multidetection system (Fazio, et al., 1971. Proceedings on Analysis and Formation of Nitrosamines, Heidelberg, Germany, 13-15 October, in press).

TABLE 1. *Experimental design*

Factor	Variables
Nitrite level	0; 50; 100; 150; 200; 300; 400; 500 μg/g of meat
Nitrate level	0; 500; 1,000; 2,000 μg/g of meat
Inoculum level	100; 10,000 spores/g of meat and uninoculated
Storage time	0; 3; 7; 14; 21; 28; 42; 84; 168 days
Holding temp	7 C; 27 C

RESULTS

The effect of cooking and storage on nitrite depletion is shown in Table 2. An approximate one-fourth reduction in nitrite occurred after its addition to the meat. Cooking had little, or no, effect on nitrite levels. The rate of nitrite

TABLE 2. *Effect of processing, storage time (days) and temperature on depletion of added nitrite*

Added nitrite (μg/g of meat)	Nitrate values (μg/g of meat)								
	Before[a] cooking	After[a] cooking	No. days stored at 7 C[b]				No. days stored at 27 C[b]			
			3	7	28	168	3	7	28	168
0	0	0	0	4	1	8	19	19	—[c]	—[c]
50	29	37	28	29	10	7	30	28	13	6
100	70	65	50	56	27	7	39	35	5	5
150	110	96	84	81	35	8	66	49	6	6
200	144	139	105	108	167	62	68	57	9	4
300	158	155	117	127	60	18	78	75	13	4
400	280	268	191	230	117	58	154	135	40	6
500	378	352	273	303	184	103	240	197	84	9

[a] Each value before and after cooking is an average of four samples (different NO_3 values).

[b] Each value at 7 C and 27 C is an average of two samples.

[c] All cans swollen prior to 28 days and not analyzed chemically.

depletion was dependent upon storage temperature. A best-fitting (least squares) line through the nitrite data showed a geometric decline of nitrite with time (Fig. 1). Nitrite reduction was more rapid during storage at 27 C than at 7 C. Predicted residual nitrite dropped from 500 μg of added nitrite per g of meat to below 100 μg per g of meat within 2 weeks of storage at 27 C.

Nitrate concentrations were not reduced by contact with the meat, cooking, or storage at 7 C or 27 C (Table 3). Small positive nitrate values were found in samples to which no nitrate had been added. These samples were negative for nitrate before cooking; however, all had detectable nitrate levels after cooking or during the storage period, indicating some generation of nitrate during cooking, or storage, or both. Low levels of nitrite were detected in some samples to which nitrite had not been added (Table 2).

Both rate of toxin production and number of toxic cans were dependent upon the level of nitrite added to the meat. Increasing the level of added nitrite reduced the number of cans in which toxin was produced and increased the length of time for toxin development (Tables 4 and 5).

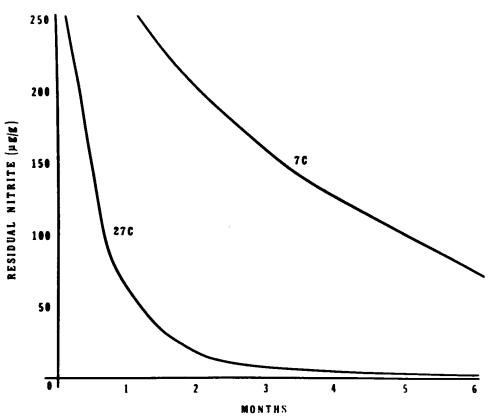

FIG. 1. *Predicted rate of nitrite depletion in perishable canned comminuted cured meat at 7 C and 27 C in product formulated initially to 500 μg of sodium nitrite per g of meat.*

The level of nitrite necessary to inhibit toxin production was dependent upon the inoculum level. At the low spore inoculum level (Table 4), toxin was confirmed in the product with up to 150 μg of nitrite per g of meat. At the high inoculum level (Table 5), toxin was confirmed up to 400 μg of nitrite per g of meat. However, only 8 of 280 samples with nitrite levels of 200 μg/g of meat or greater were botulinogenic with the high spore inoculum.

As expected, toxin production was generally more rapid at a given nitrite level in the product with the high inoculum as compared with the low inoculum. Also, there were more toxic cans at the high inoculum level (135 cans) than at the low inoculum level (95 cans).

The data were analyzed by multiple linear regression analysis to determine the type and degree of relationship between toxin development and the test conditions. Confirmed toxic samples were assigned a probability of 1.0, and nontoxic samples were assigned a probability of 0.0. These response data were analyzed to develop prediction equations on the probability of toxicity for specified conditions. All of the 27 C inoculated data were pooled for an analysis of toxicity as a function of time, initial nitrite and nitrate levels, and spore levels. The resulting curves (Fig. 2 and 3) confirm the observations: (i) as the level of nitrite increased, the probability of botulinal toxin production decreased; and (ii) at a given nitrite concentration the probability of toxin production was greater at the high spore inoculum level than at the low spore inoculum level.

The same type of statistical analysis was performed by using the predicted residual nitrite level associated with each sample. Comparison of the equations indicated that the risk

TABLE 3. *Effect of processing, storage time (days) and temperature on depletion of added nitrate*

Added nitrate (μg/g of meat)	Nitrate values (μg/g of meat)								
	Before[a] cooking	After[a] cooking	No. days stored at 7 C[b]				No. days stored at 27 C[b]			
			3	7	28	168	3	7	28	168
0	0	55	81	34	78	38	46	24	57	2
500	536	548	543	540	431	519	501	529	498	509
1,000	1,004	1,019	893	996	1,062	1,014	902	970	985	923
2,000	2,069	2,025	2,079	1,995	1,979	2,089	1,933	1,897	2,026	2,108

[a] Each value before and after cooking is an average of eight samples (pooled NO_2 values).
[b] Each value at 7 C and 27 C is an average of three or four samples.

TABLE 4. *Effect of sodium nitrite and nitrate on toxin production by Clostridium botulinum types A and B in cured, canned, perishable meat incubated at 27 C with an inoculum of 90 spores/g of meat*

Added concn (μg/g of meat)		No. of toxic samples at weekly intervals[a]																						Total no. of toxic samples
Nitrite	Nitrate	0.5[b]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
0	500		4	28																							32
0	2,000		1	1	8	3																					13
50	0		2	18	1																						21
50	1,000					1	1	9																			11
100	500							3	1	1			1	1							1						8
100	2,000					2				1	1	4															8
150	0																										0
150	1,000											1		1													2
200	500																										0
200	2,000																										0
300	0																										0
300	1,000																										0
400	500																										0
400	2,000																										0
500	0																										0
500	1,000																										0

[a] Each nitrite, nitrate level initially contained 40 test samples.
[b] Numbers across represent weekly intervals.

TABLE 5. *Effect of sodium nitrite and nitrate on toxin production by Clostridium botulinum types A and B in cured, canned perishable meat incubated at 27 C with an inoculum of 5,000 spores/g of meat*

Added concn (μg/g of meat)		No. of toxic samples at weekly intervals[a]																						Total no. of toxic samples
Nitrite	Nitrate	0.5[b]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
0	0		30	4																							34
0	1,000		3	26																							29
50	500			3	4			1																			8
50	2,000			3	4		2	1																			10
100	0			9	4	1			1	1	1																17
100	1,000				1	1			11	2		1															16
150	500							1	1	2																	4
150	2,000										1	7		1													9
200	0											1		1	1	1											4
200	1,000																									1	1
300	500																										0
300	2,000																									1	1
400	0				1																						1
400	1,000											1															1
500	500																										0
500	2,000																										0

[a] Each nitrite, nitrate level initially contained 40 test samples.
[b] Numbers across represent weekly intervals.

of toxicity can most accurately be predicted from initial rather than residual nitrite levels.

The effect of nitrate on toxin production was slight but statistically significant ($P = 0.05$). Increased nitrate levels at a given nitrite level generally resulted in a delay in toxin development and fewer samples with botulinal toxin. This effect was most apparent at the nitrite levels (0 and 50 μg/g of meat) with the low spore inoculum (Table 4).

It is possible that the positive nitrate effect may have been caused by nitrite. During storage at 27 C, the concentration of residual nitrite at a given added nitrite level generally was higher in samples containing the higher level of nitrate (Table 6). This suggests that

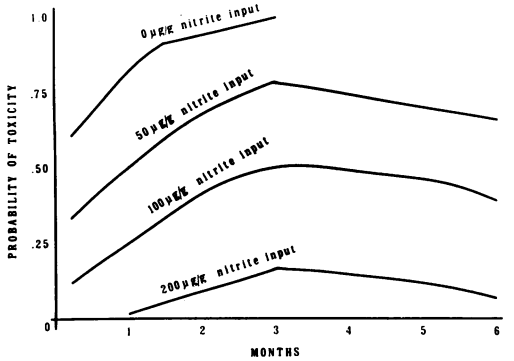

FIG. 2. *Influence of nitrite on probability of toxicity in ham held at 27 C with 90 Clostridium botulinum spores/g of meat and formulated without nitrate.*

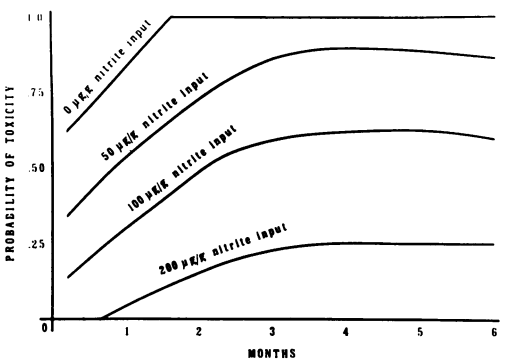

FIG. 3. *Influence of nitrite on probability of toxicity in ham held at 27 C with 5,000 Clostridium botulinum spores/g of meat and formulated without nitrate.*

there may have been some conversion of nitrate to nitrite.

There was no botulinal toxin production or putrefaction of product in the samples stored up to 6 months at 7 C. Seven of 80 cans at 7 C swelled. The seven cans were from variables with no added nitrite. The product in swollen cans was sour and had a greenish discoloration. No swelling occurred at 7 C in the product to which nitrite was added.

Assays for *C. botulinum* on 16 nonswollen samples (nitrite, 150 to 500 μg/g of meat) after 6 months of storage at 27 C did not detect viable *C. botulinum* (<10 per g) in the low-inoculum portion of the study. Assays on 12 nonswollen samples from the high-inoculum product (initial nitrite, 200 to 500 μg/g of meat) showed counts at <10 (8 samples), 10 (2 samples), 100 (1 sample), and 10,000 (1 sample) *C. botulinum* per g. Heated and nonheated counts were similar, indicating that the viable counts were due to spores rather than vegetative cells. No toxin was found in any of these samples, suggesting that substantial botulinal growth had not occurred.

Results on rate and number of swollen cans generally paralleled the toxicity data. Swelling was affected by the level of added nitrite and to a much lesser degree by nitrate.

Not all toxic cans were swollen, but all toxic product was putrid and proteolyzed. Many samples (211) were both swollen and toxic, 91 were swollen but not toxic, and 67 were not swollen but were toxic.

Analyses of selected samples containing various levels of nitrite or nitrate, or both, were negative for 14 volatile *N*-nitrosamines at a sensitivity of 0.01 μg of nitrite or nitrate per g of meat with recoveries ranging from 70 to 95%. The analyses included the following volatile *N*-nitrosamines: dimethylamine, methylethylamine, diethylamine, methylpropylamine, ethylpropylamine, dipropylamine, ethylbutylamine, propylbutylamine, methylamylamine, dibutylamine, piperidine, pyrrolidine, morpholine, and diamylamine.

DISCUSSION

The data clearly show that nitrite significantly inhibits toxin production by *C. botulinum* in perishable canned comminuted cured meat if it is temperature abused. The results show further that it is the nitrite level at the time of formulation which is important

TABLE 6. *Effect of added nitrite and nitrate on residual nitrite concentrations in comminuted cured meat stored at 27 C*

Added nitrite (μg/g of meat)	Added nitrate (μg/g of meat)	Analytical nitrite (μg/g of meat)[a]	
		3 days	7 days
0	0	0	4
0	1,000	37	34
50	500	20	24
50	2,000	40	32
100	0	32	28
100	1,000	46	42
150	500	56	48
150	2,000	76	50
200	0	66	55
200	1,000	70	58

[a] Results of individual determinations.

rather than the residual nitrite level in the product during storage.

This experiment was designed for the express purpose of determining the value of nitrite and nitrate in this class of product. The level of sodium chloride, other ingredients, and processing conditions were held constant, all in concert with typical industry practice.

Botulinal spores have rarely been found in raw meat (8), but they have been found in packaged processed meat products at the retail level (1, 9, 19).

Some postulated roles of nitrite (10) include: (a) enhancement of destruction of spores by heat; (b) increasing the rate of spore germination during thermal processing, with subsequent killing of the germinated spore by heat; (c) prevention of growth of the germinated spores which survive thermal processing; and (d) reaction with some component of the meat to form an antimicrobial compound. The last mechanism has been demonstrated in broth culture (12) and in meat (3).

Our results demonstrate that the viable population of *C. botulinum* declined during storage in samples where actual growth did not occur. It appears that the major role of nitrite might be its ability to prevent growth of germinated spores (14) by either affecting the spore at time of initial contact, or by forming some inhibitory substance that is not analytically nitrite, or by both.

Our results agree with earlier observations that the rate of nitrite depletion is influenced by temperature of storage (11, 15). We found that an initial reduction in nitrite occurred at the time of contact with the meat and that heat processing had no appreciable effect.

It was found that omission of nitrite from the formulation led to nontoxic spoilage (7 of 10 cans) of the product within 1 month's storage at 7 C. This occurred in the uninoculated product and demonstrates that, if this class of product is to be commercially practical under normal refrigeration conditions, nitrite is required to achieve the semiperishable status.

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