5-1981

The Effects of Myoglobin, Nitrosylmyoglobin, and Free Iron on the Growth of *Clostridium botulinum* in Cured Meat

Susan K. Fortier Collinge
Utah State University

THE EFFECTS OF MYOGLOBIN, NITROSYLMYOGLOBIN, AND FREE IRON ON THE GROWTH OF *CLOSTRIDIUM BOTULINUM* IN CURED MEAT

by

Susan K. Fortier Collinge

A thesis submitted in partial fulfillment

of the requirements for the degree

of

MASTER OF SCIENCE

in

Nutrition and Food Sciences

UTAH STATE UNIVERSITY
Logan, Utah
1981

ACKNOWLEDGEMENTS

Funding from the National Science Foundation made this work possible. I am grateful for this financial support.

I wish to thank Dr. Art Mahoney for his constant encouragement and advice. Dr. Daren Cornforth was quite helpful with his expertise in the area of meat science. The microbiological aspects of this project were made clearer with the help of Dr. Fred Post.

I appreciate the help of my fellow students and would especially like to thank Farzaneh Vahabzedeh and Randy James for helping with the laboratory analyses.

Finally, I wish to thank my husband Mark for his loving support and my daughter Amy, for waiting until this work was completed before she was born. The entire experience was made more enjoyable by the constant emotional support and friendship of Nicole Bernhard.

Susan K. Fortier Collinge

TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES

ABSTRACT

The Effects of Myoglobin, Nitrosylmyoglobin, and Free Iron on the Growth of *Clostridium botulinum* in Cured Meat

by

Susan K. Fortier Collinge, Master of Science

Utah State University, 1981

Major Professor: Arthur W. Mahoney, Ph.D.
Department: Nutrition and Food Sciences

Although nitrite is a known inhibitor of *Clostridium botulinum* in cured meats, the mechanism of inhibition is not understood. The observation has been made that iron is required for growth of *C. botulinum* and that the role of nitrite may be to alter the pathway of iron uptake by these organisms. Since the color change in cured meats is due to the binding of nitrite to the heme group of meat pigments, it was hypothesized that nitrite may also be tying up an essential iron source, heme. This experiment was an investigation of the possibility that myoglobin added to a meat system would stimulate growth and toxin production by *C. botulinum* much more than myoglobin that had been nitrosylated before inclusion in the product. Treatments were included to compare the effects of a heme iron source, myoglobin, with that of an ionic source, ferric chloride. To help understand the role of free iron in botulinal growth, several treatments contained a metal ion chelator, ethylenediaminetetraacetic acid (EDTA). Nitrite caused a definite delay

of growth, as evidenced by gas bubbles, when compared with a non-nitrite system. Addition of ferric chloride resulted in an increase in the rate of appearance of swollen samples, although growth was enhanced even more when myoglobin was added. When nitrosylated myoglobin was included, growth was inhibited more than in the treatment with nitrite alone. EDTA inhibited growth of C. botulinum but a conclusion should not be made with respect to the chelation of iron since EDTA chelates many other metals. Residual nitrite levels had declined to below 10 ppm by the time swelling occurred. Although swelling did not occur until nitrite had declined in the products, the absence of nitrite alone did not allow growth and toxin production. Since nitrosylated myoglobin and EDTA inhibited botulinal growth even after residual nitrite had declined, it is possible that the inhibitory action of nitrite is creating a nutritional deficiency for C. botulinum.

(83 pages)

INTRODUCTION

Function of Nitrite

Nitrite has long been used in the curing of meats. Its functions include (a.) producing the characteristic pink color of cured meats; (b.) preventing oxidative rancidity; (c.) affecting flavor and texture; and (d.) inhibiting growth and toxin production by *Clostridium botulinum* (Sofos et al., 1979). The pink color is formed when nitrite is converted to nitric oxide by reductants in the meat, which is complexed with oxidized pigments, mainly metmyoglobin, to form nitrosylmetmyoglobin, which is ultimately converted to nitrosyl-hemochrome when heated (Fox and Thomson, 1963). Igene et al. (1979) reported that addition of nitrite and removal of heme pigments from meat significantly inhibited lipid oxidation in cooked meat. Prevention of lipid oxidation would certainly affect the taste of meat. Hustad et al. (1973) reported that the flavor quality of weiners made with nitrite was significantly higher than weiners made without nitrite. A number of researchers have shown that nitrite inhibits growth and toxin formation by *C. botulinum* (Christiansen et al., 1973; 1974; Hustad et al., 1973). When considering the inclusion of a substance which inhibits *C. botulinum* one might wonder how great the risk is of acquiring botulism from sausage. In a survey of 2,358 samples of raw meat from the U.S. and Canada, only one *C. botulinum* spore was confirmed (Greenberg et al., 1966b). If this study were representative, the logical conclusion is that the chance of getting botulism from cured meat is slight.

Safety of Nitrite

Recently, the safety of nitrites has been questioned. Several researchers have reported the presence of nitrosamines in bacon. Nitrosamines can be formed by a reaction of nitrite or nitrous acid with secondary or tertiary amines (Sebranek and Cassens, 1973). Cured meats contain these reactants and the potential for nitrosamine formation. Fazio et al. (1973) isolated N-Nitrosopyrrolidine from eight commercial brands of cooked bacon at levels ranging from 10 to 108 ppb (ug/kg). Nitrosamines are considered one of the most potent groups of carcinogens known. Schmahl and Osswald (1967) reported the presence of liver tumors in eight different species fed diethyl-nitrosamine. The frequency of the liver tumors was practically one hundred percent. Newberne (1979) concluded that nitrite itself was carcinogenic. Lymphoma was increased in all groups fed nitrite. The incidence of lymphoma was 5.4 percent in 573 control rats and 10.2 percent in 1383 treated rats. Holland (1979) reviewed some of the findings from a number of investigations of the Newberne study. Criticisms included unethical data recording procedures, poor quality control, and the presence of urethane, a powerful carcinogen in one of the animal rooms. Pearson et al. (1980) confirmed the carcinogenicity of N-nitrosylpyrrolidine but found no evidence that nitrite alone or in a combination with pyrrolidine was carcinogenic.

While questioning the safety of nitrite, it is important to consider the relative contribution of nitrite to our diets from cured meats. It has been estimated that of the average daily per capita ingestion of nitrite in the U.S., 21.1% is from cured meats and 76.8%

from saliva (White, 1976). Since saliva is the main source of nitrite in our diets, it would be illogical to try to reduce nitrite consumption by excluding nitrite from meat.

Current concern over the possibility that nitrite or reaction products of nitrite may be carcinogenic has led to an investigation of the benefits of this meat additive. Most of this research has been focused on the antibotulinal properties of sodium nitrite, since a critical issue exists with regards to the risk of contracting this often fatal neuroparalytic disease. Even one incident of botulism could destroy an entire meat company as well as undermine public confidence in the meat industry as a whole. At present, there is evidence that nitrite is beneficial as an antibotulinal agent (Christiansen et al., 1973; 1974; Hustad et al., 1973) but may be reacting to form carcinogenic nitrosamines (Fazio et al., 1973). More research is probably needed if a clear decision is to be made concerning this risk-benefit dilemma.

Mechanism of Botulinal Inhibition

Although the antibotulinal property of nitrite has been demonstrated, only speculations have been made as to the mechanism of inhibition. If results of further studies show nitrite or degradative products of nitrite to be a significant risk, it may be necessary to find alternative methods of curing. The ideal substitute for nitrite would impart the same characteristics to the product, including a high degree of botulinal safety, without posing a health risk. If the mechanism by which nitrite inhibits *C. botulinum* growth were

determined, it may facilitate the development of other non-nitrite curing systems.

Johnston et al. (1969) described the following as possible mechanisms of inhibition by sodium nitrite: 1.) enhancement of the destruction of spores in the presence of heat; 2.) causing an increased rate of germination of spores during the heat process followed by death of the germinated spores from the heat process; 3.) prevention of germination of spores that survived the heat process; and 4.) production of more inhibitory substances from nitrite. Of the above possibilities, the fourth has received the most discussion and study. Perigo et al. (1967) speculated that nitrite might, when heated in the presence of the supporting medium, be converted to a much more powerful inhibitor (Perigo factor). Experimentally, they were able to show with C. sporogenes that smaller concentrations of nitrite were inhibitory in a heated culture medium when compared with an unheated system. Perigo and Roberts (1968) confirmed the enhancement of the inhibitory effect of nitrite after heating, using C. botulinum types A, B, E, and F and C. welchii (perfringens). In another study, growth of C. perfringens occurred at nitrite concentrations as high as 600-1000 ppm in a filter sterilized medium, whereas in autoclaved media, growth was observed at nitrite levels of 10-50 ppm (Riha and Solberg, 1975). Attempts have been made to characterize this inhibitor that has been referred to as the Perigo factor. Moran et al. (1975) found that only amino acids and mineral salts were involved in the production of this inhibitor. A complex of cysteine, iron, and nitric oxide was detected in an autoclaved solution containing cysteine, ferrous sulfate, and sodium nitrite. This compound did not appear to be inhibitory at levels that

would be found in the culture medium. Johnston et al. (1969) investigated the possibility that the Perigo factor was formed in meat systems. The conclusion was made that meat may prevent the formation of this factor and that meat and culture work are best considered separately, if the inhibitory mechanism is to be determined.

Residual vs. Initial Nitrite

More recently, researchers have investigated the role of residual nitrite in botulinal inhibition, along with variations in mineral and vitamin composition of the meat. Christiansen et al. (1974) showed that botulinal toxin production depended upon initial nitrite formulation and spore level. At low inoculum levels (210 spores/g before processing), samples became toxic if formulated with 120 μg/g nitrite or less. At the same inoculum level, toxin was not detected in products formulated with 170 or 340 μg/g nitrite. Inoculum levels of 19,000 spores/g resulted in detectable toxin at all nitrite levels.

The question of the relative importance of residual versus initial nitrite level has been addressed by Christiansen (1980). A suggestion was made that considering initial nitrite level as the important factor implies one of three things: 1.) nitrite reacts with C. botulinum spores to cause inhibition; 2.) nitrite reacts with a component of the meat to form a Perigo-type factor; or 3.) the input level of nitrite must cure the meat and provide sufficient residual nitrite to provide inhibition. Christiansen (1980) stated that: "The literature contains no compelling evidence that either of the first two events occurs in meat products." In early studies by Christiansen et al. (1973; 1974) a

conclusion was made that the input level of nitrite predetermines the degree of nitrite inhibition. Later discussion has indicated that the above conclusion was erroneous and Christiansen (1980) has since emphasized the importance of residual nitrite in botulinal inhibition. The variation in response with differing initial nitrite levels was explained as the result of a race between nitrite depletion and death of germinated botulinal spores (Christiansen et al., 1978). This theory described by Christiansen (1980) is based upon the idea that botulinal spores germinate but do not grow and produce toxin in the presence of nitrite. If this were the case, growth would occur if nitrite was depleted because the input level was not adequate. Since the amount of residual nitrite depends to an extent on the input level, it does not seem possible to completely dissociate initial and residual nitrite.

If nitrite is injurious to germinated botulinal spores (Christiansen, 1980), one would expect nitrite to limit growth in a culture medium other than meat. Some strains of C. botulinum (types A, B, E, or F) were not inhibited by nitrite concentrations as high as 100 ppm in culture media (Perigo and Roberts, 1968). Roberts and Ingram (1973) reported growth of C. botulinum in culture media at nitrite concentrations as high as 300 ppm. Growth of C. perfringens occurred at nitrite concentrations as high as 600-1000 ppm at pH 7.2 (Riha and Solberg, 1975). Growth of Clostridia in culture media containing nitrite does not indicate that nitrite itself is injurious to the bacterial cells, nor does it help to validate the theory that residual nitrite is responsible for inhibition of C. botulinum in meats.

Among the more convincing evidence that residual nitrite is important for C. botulinum inhibition, is the acceleration of growth

following nitrite depletion by extended refrigeration (Tompkin et al., 1978e). It is possible that botulinal spores do not germinate until removed from refrigeration and held at 27C. By the time germination occurs, nitrite is depleted, and growth occurs.

In addition to these suggestions of possible implications for the importance of initial nitrite level, is one that is being investigated here. It is possible that nitrite reacts with heme iron so that it becomes unavailable for microbial utilization, thus inhibiting growth and toxin production by limiting uptake of an essential nutrient, iron. This could depend upon the input level of nitrite.

Tompkin et al. (1977) attempted to determine a base line for inhibition of C. botulinum by nitrite in a perishable canned meat product. Growth of C. botulinum was considered positive when cans became swollen with gas. Predicted average times to first swell were 6.7, 29.8, 82.6, and 94.3 days when 0, 50, 100, and 156 μg/g of sodium nitrite was added to the meat. The length of the lag phase was extended with increasing amounts of nitrite, but once swelling began, rate of appearance of swollen cans was not significantly different at 50, 100, and 156 μg/g of sodium nitrite. Within a particular nitrite level, a substantial amount of variability was seen between the six replications.

Iron and C. botulinum

Further studies have been focused on determining the cause of variation within replications of the same treatment. Tompkin et al. (1978b) tested several different kinds of meat to see if botulinal growth varied with meat source. No inhibition was seen with pork or

beef heart, but with turkey breast, turkey thigh, pork ham, beef round, and veal, varying amounts of inhibition were observed. The authors felt that a possible reason for the lack of inhibition with heart meat was the increased amount of pigment. To test this hypothesis, 1 percent hemoglobin (1 percent hemoglobin + pork is approximately equal to heart meat in iron content) was added to a basic pork product, before curing. With equivalent amounts of sodium nitrite, the hemoglobin treatment was much more inhibitory than the heart formulations, but less inhibitory than the pork control. A conclusion was made that heart meat has a higher level of readily available iron than the pork plus hemoglobin system. Residual nitrite had an inhibitory effect in the hemoglobin test but this effect was overcome by an essential factor in the heart meats. Tompkin et al. (1978b) suggested that nitric oxide formed from nitrite reacts with iron in the vegetative cells, blocking an essential step for growth.

To test the hypothesis that loss of inhibition with heart meat was due to the greater availability of iron, products were formulated with ethylenediaminetetraacetic acid (EDTA) and isoascorbate, known metal ion chelators (Tompkin et al., 1979). Neither EDTA (Tompkin et al., 1979) nor isoascorbate (Tompkin et. al, 1978a) were inhibitory alone. Both enhanced the inhibitory effect of nitrite in a pork ham formulation. Only EDTA enhanced inhibition in canned cured pork hearts. The conclusion was made that EDTA more effectively sequesters iron, making iron less available for preventing inhibition by nitrite.

In addition to differences in pigment between heart and most skeletal muscles, other nutritional differences exist. Tompkin et al. (1978c) examined the effects of calcium, iron, manganese, zinc, and

riboflavin content on botulinal growth in perishable canned cured meat. Of the variables tested, only the iron (in addition to residual nitrite level) influenced the degree of botulinal inhibition. A hypothesis derived from this study for the inhibitory effect of nitrite was that nitric oxide reacts with the iron of a compound such as ferredoxin within the germinated cell. This reaction could interfere with energy metabolism to prevent outgrowth.

Tompkin et al. (1979) showed a dose response relationship of available iron to the antibotulinal property of sodium nitrite. This involved the addition of ferric chloride at the level of 0, 10, 20, 30, and 40 μg/g meat. The antibotulinal effect of sodium nitrite decreased as the level of added iron increased. This response coupled with that of EDTA presents a strong case for free iron causing a loss of inhibition by sodium nitrite.

Tompkin et al. (1978c; 1979) showed substantial evidence of the requirement of C. botulinum for iron. Although a complete loss of inhibition was seen with heavily pigmented meats like pork and beef heart, addition of hemoglobin to pork ham did not produce the same effect. Tompkin et al. (1978b) concluded that the iron in heart meat causes a loss of inhibition and that it is more available than the iron of the heme group for this purpose. More heavily pigmented turkey thigh meat also showed a decreased level of inhibition when compared with turkey breast. Although heart meat may contain something in addition to pigment that would stimulate growth of C. botulinum, pigment may be an important factor. Addition of hemoglobin to a pork ham product resulted in a less inhibitory system than the control. Stimulation of C. botulinum growth in the presence of added hemoglobin, with turkey

thigh meat, and heart meat suggests that these organisms can use the iron from the heme group. The same reaction that causes the pink color of cured meat could cause the iron of the heme group to become unavailable for microbial utilization. Mahoney et al. (1979) showed that the bioavailability of meat iron is decreased in a cured meat product, as measured by hematinic responses of rats. It is possible that curing with nitrite reduces the bioavailability of heme iron for utilization by C. botulinum.

C. botulinum certainly has a requirement for iron, but how is the iron utilized? A number of iron containing or iron activated enzymes and proteins have been reported in other Clostridia. Among these are ferredoxins, which play a central role in the transfer of electrons in many anaerobic redox reactions (Lovenberg, 1974). Since free iron may be utilized in the synthesis of a number of essential metabolic proteins, it would be reasonable to expect that a deficiency of available iron would limit Clostridia growth and toxin formation.

Objective

The objective of this study was to investigate the effects of free iron, heme iron, nitrosoheme iron, and EDTA on C. botulinum growth and toxin production in perishable cured meat.

EXPERIMENTAL PROCEDURE

Product Formulation and Processing

One large batch (23 kg) of fresh pork picnics (lower half of pork shoulder) was received, ground through a 0.64 cm plate, and portioned into packages the appropriate size for all treatments. The meat was vacuum packaged and kept frozen until time of formulation. All products were formulated to contain 2.5% sodium chloride and 0.5% dextrose and then emulsified in a one gallon stainless steel blender. The finished product contained 68.6% water, 13.3% protein, 15.2% fat, and 2.8% ash. When sodium nitrite was added to the product, the 156 ug/g was based only on the meat fraction, according to standard industry practice. The meat mixture (approximately 80 g) was filled into plastic film pouches as described by Greenberg et al. (1966a). The pouches, laminated of 0.75 mil nylon and 2.25 mil of a co-polymer of 6% ethylene vinyl acetate and 94% polyethylene, are used routinely for vacuum packaging of meats. These pouches are sold under the trade name "Vacu-fresh" by Meat Packers and Butchers Supply Co., 2820 E. Washington Blvd., Los Angeles, Ca., 90023 (Oxygen permeability, 0.78 ml/24 hr/atm). Pouches were vacuum packaged and cooked in a 70C water bath for 30 min. Tompkin et al. (1978b) found that final internal processing temperatures within the range of 63C to 74C did not influence botulinal inhibition. Immediately after cooking, the pouches were placed into ice water until fully cooled.

Inoculum and Spore Counts

A mixture of spores of one type A (ATCC #19397) and one type B (ATCC #17843) strain of *C. botulinum* was used. Each strain was propagated separately in chopped liver media (Appendix A) at 35C for approximately one week. The broth was centrifuged at 3,000 G for 15 min. to sediment the cells. After decanting the broth, the organisms were resuspended in sporulation media (Appendix A). After allowing one week at 35C for spore formation, the suspensions were heat shocked at 85C for 5 min. to inactivate the toxin as well as preserve most of the spores (Woodburn et al., 1979). Spore suspensions were centrifuged at 3,000 G for 15 min, the media decanted, and the spores resuspended in sterile water. Spore counts were determined by making appropriate dilutions in 0.1% peptone water (Appendix A) and plated on anaerobic egg agar (Appendix A). The plates were incubated anaerobically at 35C using a polycarbonate jar with a hydrogen and carbon dioxide generator. A single suspension was made containing equal numbers of both spore types, and mixed into the meat (100 spores/g meat) immediately before bagging. The meat was processed as quickly as possible after spore addition, to minimize the possibility of germination and subsequent death of the vegetative cells during processing.

In some of the treatments (6, 7, 8, and 9), anaerobic and aerobic plate counts were done on two samples from each treatment that were unswollen after 100 days. Aerobic counts were done with plate count agar (Appendix A) and anaerobic counts done as described above. Colonies from both aerobic and anaerobic plates were Gram stained (Appendix A) and examined microscopically.

Holding Conditions

The sealed pouches were abused by holding at 27C and observed for gas production for 100 days. Pouches were removed and analyzed periodically for nitrosoheme iron, heme iron, residual nitrite, and toxin. Each treatment also included an uninoculated control. Samples were checked daily for evidence of gas production.

Experimental Rationale

In this experiment, nine treatments were compared. For each comparison, twenty five pouches of product were formulated, stored, and analyzed as described. The nine treatments were formulated as follows:

1.) This was the basic meat formulation with added dextrose, water, and sodium chloride.

2.) Sodium nitrite (156 μg/g meat) was added to the basic product. This resulted in 128 μg/g in the final product.

3.) Sodium nitrite (156 μg/g meat) and ferric chloride (20 μg/g product) were added.

4.) Sodium nitrite (156 μg/g meat) and myoglobin (2.16 mg/g product) were added. The amount of myoglobin added was equivalent in iron content to 20 μg/g ferric chloride.

5.) Sodium nitrite (156 μg/g meat), myoglobin (2.16 mg/g product), and EDTA (200 μg/g) were included.

6.) Myoglobin (2.16 mg/g product) was first nitrosylated and then added to the meat with 156 μg sodium nitrite/g meat.

7.) EDTA (200 μg/g) was added to the basic nitrite containing product.

8.) Ferric chloride (20 μg/g product) was included in a product with 200 μg/g EDTA and 156 μg/g sodium nitrite.

9.) Ferric chloride (40 μg/g product) was added to a product with 200 μg/g EDTA and 156 μg/g sodium nitrite.

The formulations for the nine treatments are summarized in Table 1.

Preparation of Nitrosylmyoglobin

Nitrosylmyoglobin was prepared by solubilizing 6g lyophilized horse heart myoglobin in approximately 300 ml of a 1000 ppm solution of sodium nitrite, adding 1-2 g ascorbic acid, and heating the mixture in a boiling water bath for approximately 20 min. Ascorbic acid was used to hasten development and stabilize the color of the pigment (Kramlich et al., 1973), although normally the sodium salt is used. Nitrosylmyoglobin was precipitated out of solution by adding a minimum amount of sodium chloride (less than 1 g sodium chloride for 6 g myoglobin). The precipitate was centrifuged for 15 min. at 6,000 G, resuspended with water to wash out excess nitrite, sodium chloride, and ascorbic acid. Washing and centrifuging were repeated four times before the nitrosylmyoglobin was added to the treatment. The above procedure produced a product with 74-87% of the pigment nitrosylated when measured by the Hornsey (1956) method (Appendix D). A distinct color change was noted as the product was converted from the brown color of metmyoglobin to the reddish-pink nitrosylated product.

Table 1. Meat product formulations; including all additives for 2.27 kg meat[1], 65 g sodium chloride, 13 g dextrose, and 410 ml water.

Treatment	NO_2^-	$FeCl_3$	Mb	NOMb	EDTA
	(g)	(mg)	(g)	(g)	(g)
1 No nitrite	-----	-----	-----	-----	-----
2 Nitrite	.3538	-----	-----	-----	-----
3 Nitrite + $FeCl_3$	.3538	55.1	-----	-----	-----
4 Nitrite + Mb	.3538	-----	5.953	-----	-----
5 Nitrite + Mb +EDTA	.3538	-----	5.953	-----	.7020
6 Nitrite + NOMb	.3538	-----	-----	5.953	-----
7 Nitrite + EDTA	.3538	-----	-----	-----	.7020
8 Nitrite + EDTA + 20 ppm $FeCl_3$	.3538	55.1	-----	-----	.7020
9 Nitrite + EDTA + 40 ppm $FeCl_3$	.3538	110.2	-----	-----	.7020

[1]Ground pork picnics (lower portion of pork shoulder).

Chemical Analyses

Nitrite concentration was determined spectrophotometrically (Appendix B). Total iron and soluble iron were determined by atomic absorption spectroscopy (Appendix C). Total heme iron and nitrosohemochrome were determined spectrophotometrically (Appendix D).

Early in the analytical process, it was noted that an unusual amount of variability was seen in the results of the pigment analysis. To verify the validity of the procedure, several experiments were performed. Hematin was nitrosylated by dissolving in dilute NaOH, followed by adding sodium nitrite, and then adding ascorbic acid until the hematin precipitated out of solution. The acidification procedure appeared to be necessary for nitrosylation. Hematin was brought back into solution by careful addition of NaOH. This nitrosylated hematin was added in varying amounts to weiner samples and measured for completeness of nitrosylation over a range of concentrations.

Acetone-water extracts of both nitrosylated myoglobin and purchased weiners were scanned between 350 and 750 nm using a recording spectrophotometer. Another scan was done to show the absorption spectra for total pigments over the same wavelengths.

Toxin Assays

The first five pouches to swell from each test variable were tested for botulinal toxin. Tompkin et al. (1978a) reported that over five years of using this procedure, over 90% of swelled cans were toxic. If swelling did not occur, the first five samples removed from the

treatment were tested for toxin. The procedure for toxin testing was adapted from the Food and Drug Administration Bacteriological Analytical Manual (Kautter and Lynt, 1978). Toxin assays involved blending 5 g of each sample with 10 ml of gel phosphate buffer (Appendix A). The slurry was centrifuged at 3,000 G and the supernatant fluid sterilized by filtration through a sterile 0.2 micron filter. One mouse was injected with 0.5 ml of the supernatant fluid, one with 0.5 ml of the boiled fluid, and one injected with the toxic substance after first being protected with type AB antitoxin. The protected mice received 0.5 ml of each type of antitoxin, diluted to 2 IU/ml with glycerine and 0.9% sterile saline. All mice were observed for botulism symptoms for 72 hrs after injection. Botulinal toxin was confirmed by death of the unprotected mouse and survival of the ones that received antitoxin or boiled toxin.

Statistical Analyses

Data for total and nitroso pigments and total iron were analyzed by Analysis of Variance using a completely randomized block design (Ostle and Mensing, 1975). Least significant difference (LSD) values were determined by using the same source. Mean differences must equal or exceed the LSD values to be statistically significant.

Nitrite Depletion in Uninoculated Samples

Using the same meat and product formulation as the other treatments, an experimental unit (treatment #10) was set up with 156 μg

sodium nitrite/g meat, as in treatment #2. This treatment was not inoculated with C. botulinum. Nitrite was measured daily for 29 days using a randomly selected sample to observe decline of nitrite in uninoculated samples.

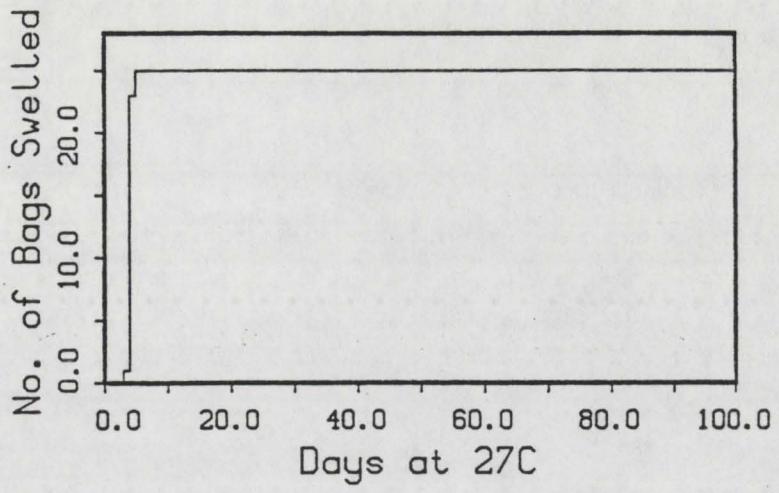
RESULTS

Swelling Rate

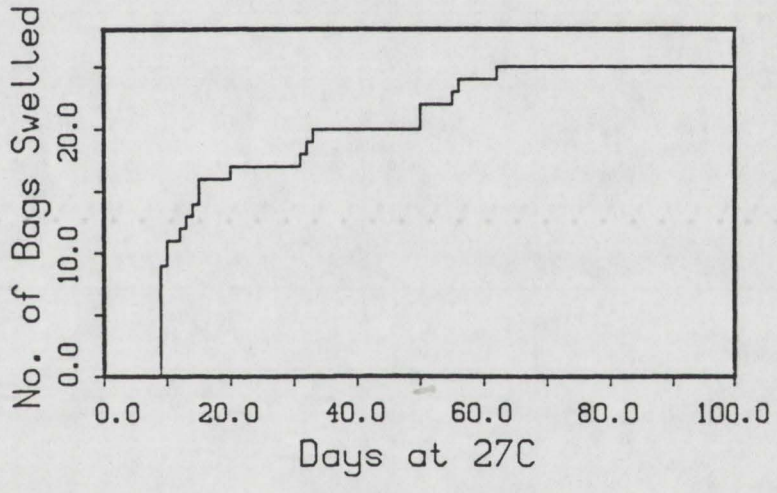
The rate of swelling for all nine treatments is presented graphically in figures 1-3. In the absence of sodium nitrite (tmt. #1, Fig. 1a), all samples had swelled by day 5. The addition of sodium nitrite (tmt. #2, Fig. 1b) had a definite inhibitory effect. When nitrite was added, swelling began on day 9 but all 25 samples were not swollen until day 62. In treatment #3 (Fig. 1c) with added ferric chloride, swelling began at the same time as in treatment #2, but the curve appears to be a little steeper, indicating a slightly faster rate of swelling. When myoglobin was added to the mixture (tmt. #4, Fig. 2a), swelling began in eight days and the rate of swelling was faster than in either treatment 2 or 3, with all bags swelling by day 43. Addition of EDTA to a treatment of similar composition as treatment #4 resulted in a rather inhibitory system (Fig. 2b). In treatment #5 (Fig. 2b), swelling was delayed until day 18 and by day 100 only 14 bags had swelled. Treatment #6 (Fig. 2c), with added nitrosylmyoglobin, proved to be more inhibitory than treatments 2-5. Addition of EDTA to the basic nitrite containing product like treatment #2 proved to be extremely inhibitory (Fig. 3a). Treatment #8 (Fig. 3b) had a composition similar to treatment #5 except that the iron source of #8 was ferric chloride instead of myoglobin. Swelling occurred more slowly in treatment #8 than in treatment #5. Treatment #9 (Fig. 3c) had twice as much ferric chloride as treatment #8 and the response was much like that of treatment #7.

Figure 1. Rate of swelling in a pasteurized meat product formulated (a.) without nitrite (treatment 1), (b.) with 156 μg sodium nitrite/g meat (treatment 2), and (c.) with 156 μg/g sodium nitrite and 20 μg/g ferric chloride (treatment 3).

(a.)


No. of Bags Swelled
0.0
10.0
20.0
0.0
20.0
40.0
60.0
80.0
100.0
Days at 27C

(b.)


No. of Bags Swelled
0.0
10.0
20.0
0.0
20.0
40.0
60.0
80.0
100.0
Days at 27C

(c.)

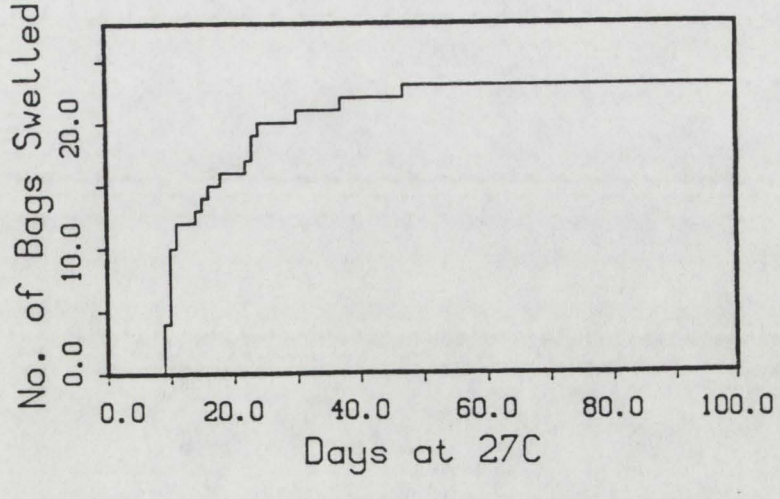
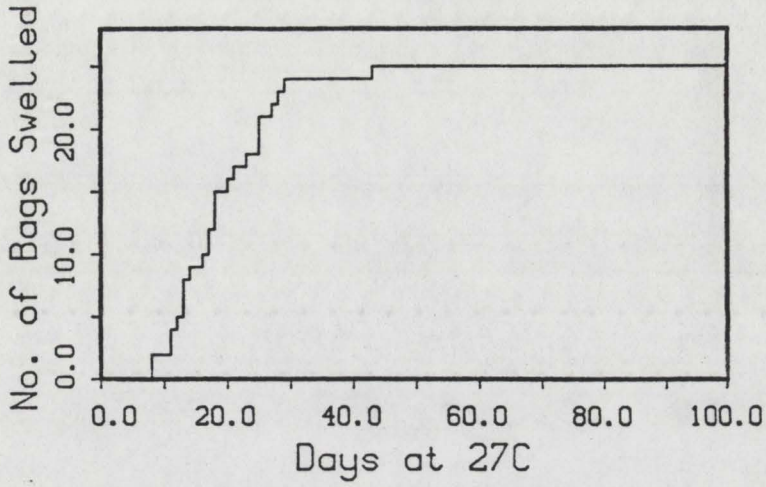
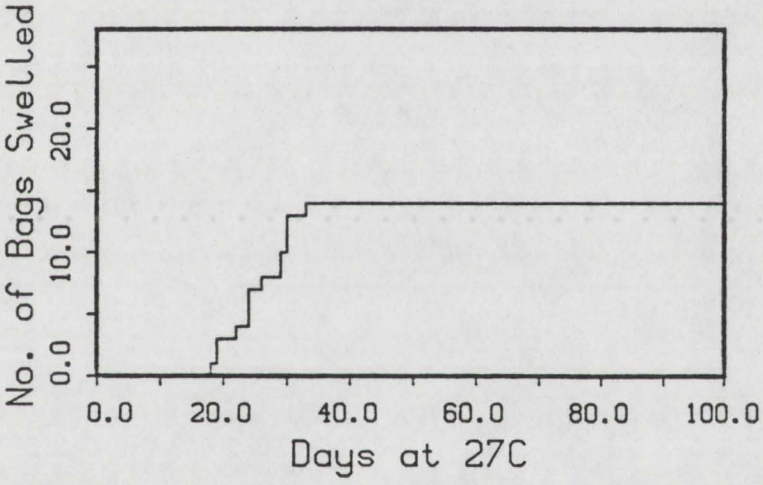

No. of Bags Swelled
0.0
10.0
20.0
0.0
20.0
40.0
60.0
80.0
100.0
Days at 27C

Figure 2. Rate of swelling in a meat product formulated with 156 μg/g sodium nitrite and (a.) 2.16 mg myoglobin/g (treatment 4), (b.) 2.16 mg myoglobin/g, and 200 μg/g EDTA (treatment 5), (c.) 2.16 mg nitrosylated myoglobin/g (treatment 6).

(a.)


No. of Bags Swelled
0.0
10.0
20.0
0.0
20.0
40.0
60.0
80.0
100.0
Days at 27C

(b.)


No. of Bags Swelled
0.0
10.0
20.0
0.0
20.0
40.0
60.0
80.0
100.0
Days at 27C

(c.)

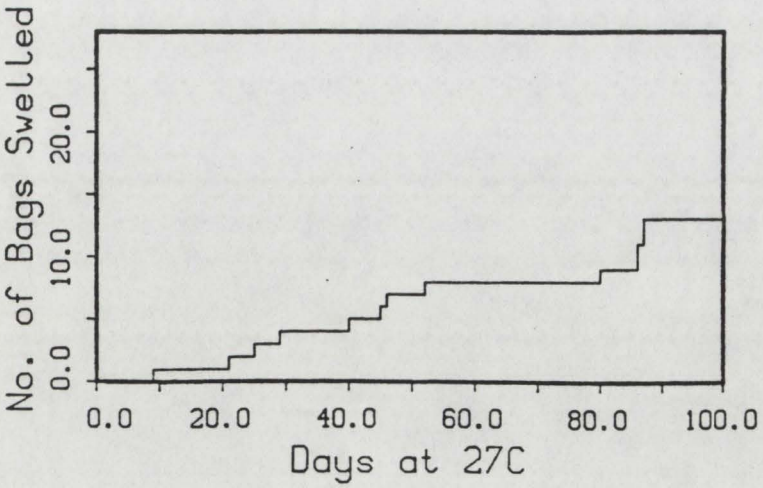
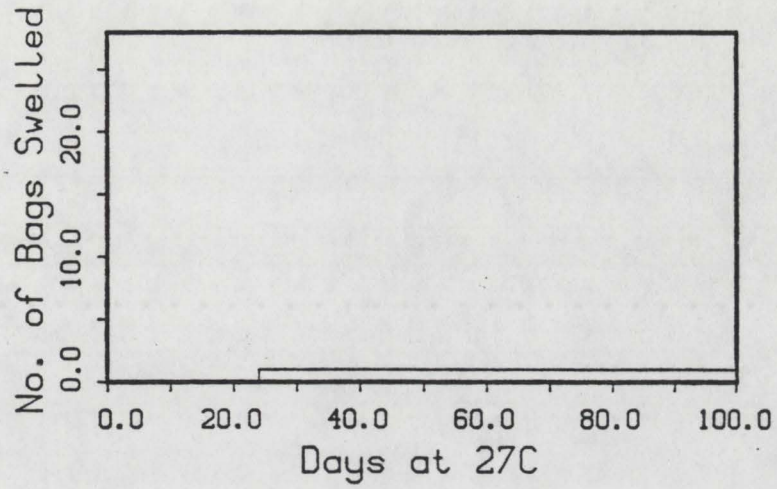
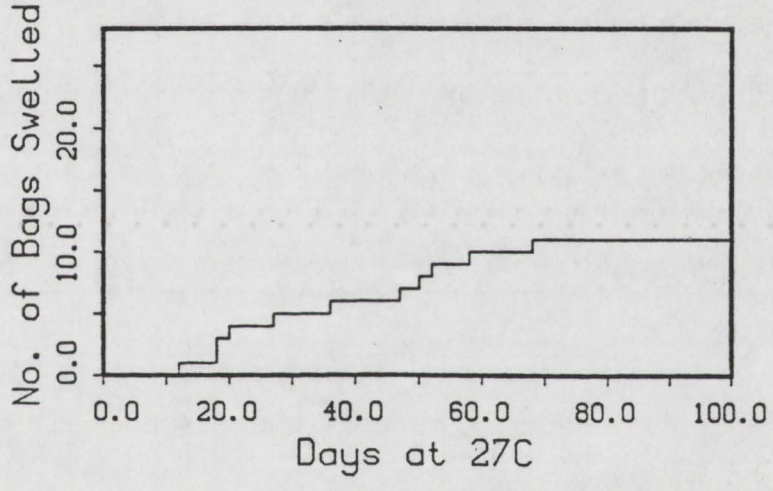

No. of Bags Swelled
0.0
10.0
20.0
0.0
20.0
40.0
60.0
80.0
100.0
Days at 27C

Figure 3. Rate of swelling in a meat product formulated with 156 µg/g sodium nitrite and (a.) 200 µg/g EDTA (treatment 7), (b.) 200 µg/g EDTA and 20 µg/g ferric chloride (treatment 8), (c.) 200 µg/g EDTA, and 40 µg/g ferric chloride (treatment 9).

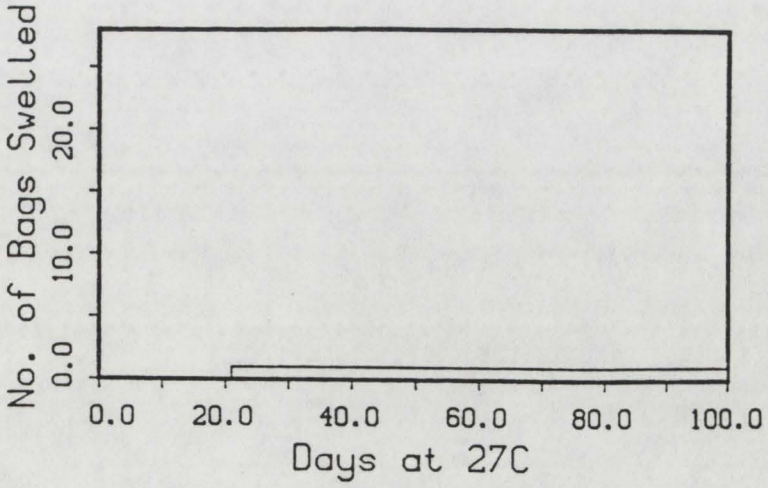
(a.)


No. of Bags Swelled
0.0
10.0
20.0
0.0
20.0
40.0
60.0
80.0
100.0
Days at 27C

(b.)


No. of Bags Swelled
0.0
10.0
20.0
0.0
20.0
40.0
60.0
80.0
100.0
Days at 27C

(c.)


No. of Bags Swelled
0.0
10.0
20.0
0.0
20.0
40.0
60.0
80.0
100.0
Days at 27C

Toxin Assays

Results of the toxin assay are presented in Table 2. In treatments 1-3, all samples tested were confirmed to contain toxin, however in the remainder of the treatments some discrepency was observed between swollen and toxic bags. Of the 36 swollen samples tested, only 23 (64%) were confirmed to contain toxin. None of the boiled samples caused the death of a mouse. Of the protected mice, only one death occurred, confirming that Botulism types A and B were the etiological agents of death.

Nitrite Level

The decline of nitrite over time is presented for treatments 2-9, in Figures 4-7. At the time of swelling, nitrite had decreased to less than 10 ppm in all treatments. Initial nitrite in treatment 1 (no nitrite) was 3.36 μg/g, which is probably near the limit of sensitivity of the procedure. Although swelling was accompanied by low nitrite levels, the absence of residual nitrite was not always associated with swelling as is evidenced by treatments 6, 7, 8, and 9.

Heme Pigments

Total and nitroso pigments for the nine treatments are presented graphically in figures 8 through 10. Theoretically, the amount of heme added to treatments 4, 5, and 6 was 80 μg hematin/g meat, doubling the amount in the product. From the data (Fig 8-10, Table 3), one can see

Table 2. *C. botulinum* toxin in meat samples.[a]

Treatment	Swollen	Unswollen	Protected	Boiled	Toxin
	(No. of Samples Tested)		(Number of Deaths)		
1 No nitrite	5	0	0	0	5
2 Nitrite	5	0	0	0	5
3 Nitrite + 20 ppm $FeCl_3$	5	0	0	0	5
4 Nitrite + Mb	5	0	1	0	4
5 Nitrite + Mb + EDTA	5	0	0	0	3
6 Nitrite + NOMb	4	1	0	0	0
7 Nitrite + EDTA	1	4	0	0	0
8 Nitrite + EDTA + 20 ppm $FeCl_3$	5	0	0	0	1
9 Nitrite + EDTA + 40 ppm $FeCl_3$	1	4	0	0	0

[a]Five bags were tested in each treatment by mouse assay.

Table 3. Mean values of total and nitroso pigments, calculated amount of heme iron (μg/g).

Treatment	Total[1] Pigment	Nitroso[2] Pigment	Heme Iron	Total Iron	Percent of Iron as Heme
1 No nitrite	75.5^{a} n=11	4.49^{d} n=11	6.66	11.3 n=6	58.9
2 Nitrite	92.8^{b} n=15	58.8^{b} n=14	8.18	11.3 n=6	72.4
3 Nitrite + 20 ppm $FeCl_3$	84.9^{ab} n=18	45.6^{a} n=18	7.49	17.6 n=5	42.6
4 Nitrite + Mb	163.8^{cd} n=18	126.9^{c} n=17	14.4	15.6 n=4	92.3
5 Nitrite + Mb + EDTA	168.4^{d} n=17	79.9^{e} n=16	14.8	14.9 n=4	99.3
6 Nitrite + NOMb	154.5^{c} n=20	121.7^{c} n=19	13.6	17.7 n=5	76.8
7 Nitrite + EDTA	93.2^{b} n=8	48.2^{ab} n=7	8.22	11.7 n=4	70.3
8 Nitrite + EDTA + 20 ppm $FeCl_3$	87.9^{b} n=17	44.5^{a} n=16	7.75	18.3 n=5	42.4
9 Nitrite + EDTA + 40 ppm $FeCl_3$	93.2^{b} n=9	49.5^{ab} n=8	8.22	20.3 n=6	40.5

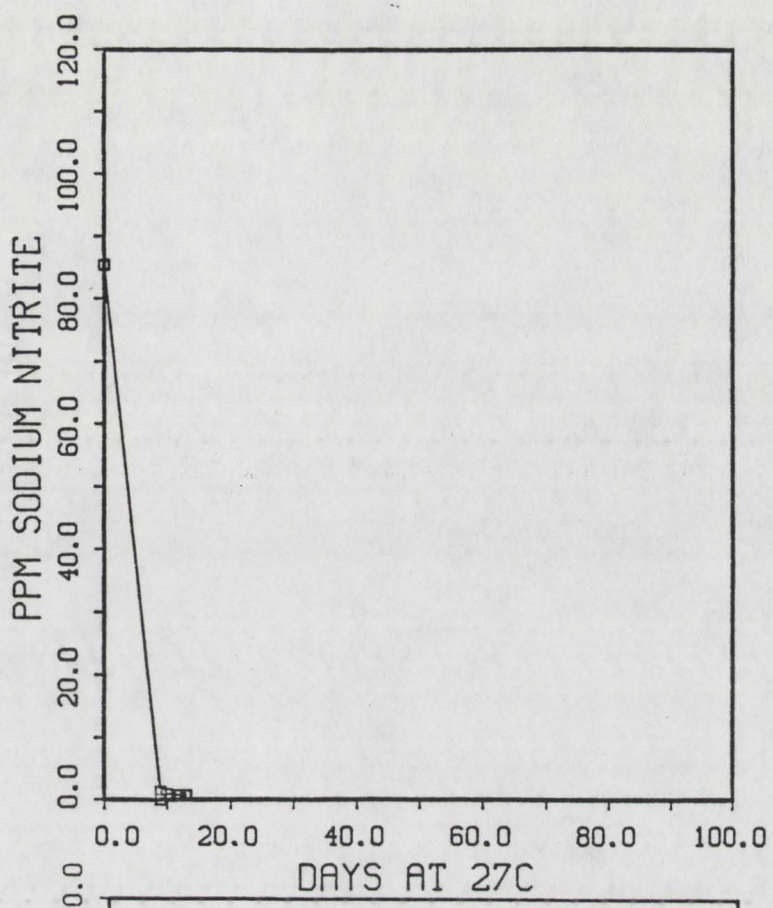
[1]LSD .01/.05=9.78/7.39

[2]LSD .01/.05=11.73/8.86

[abcde]Mean values with the same letter are not significantly different at the 1% level of probability.

Figure 4. Nitrite depletion in a meat product formulated with (a.) 156 µg sodium nitrite/g meat (treatment 2), (b.) 156 µg/g sodium nitrite and 20 µg/g ferric chloride (treatment 3).

(a.)


PPM SODIUM NITRITE
0.0
20.0
40.0
60.0
80.0
100.0
120.0
0.0
20.0
40.0
60.0
80.0
100.0
DAYS AT 27C

(b.)

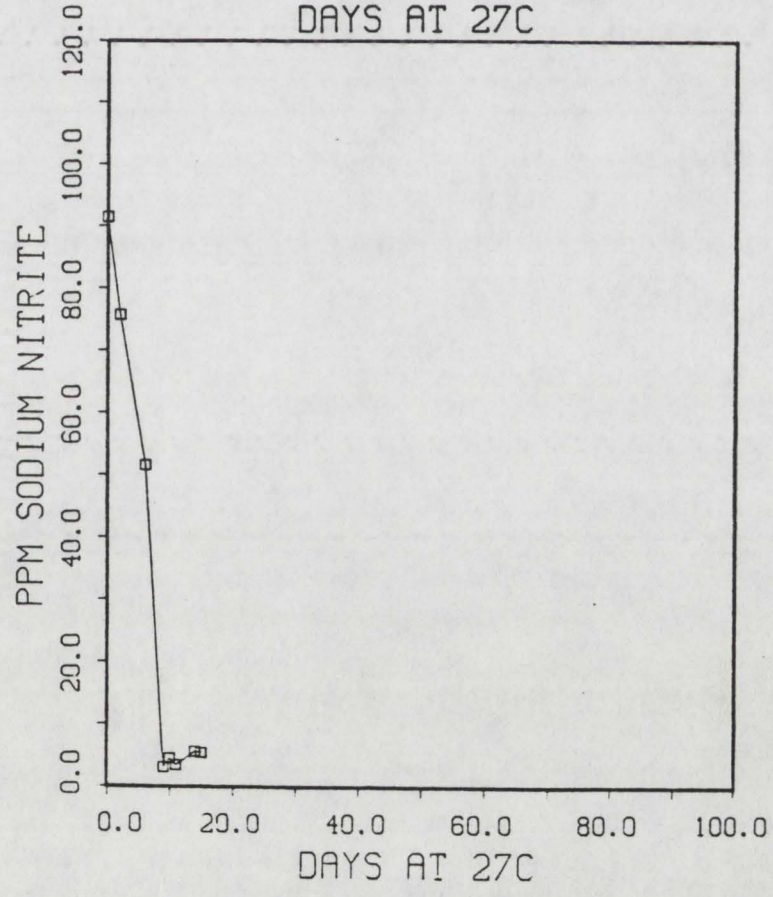
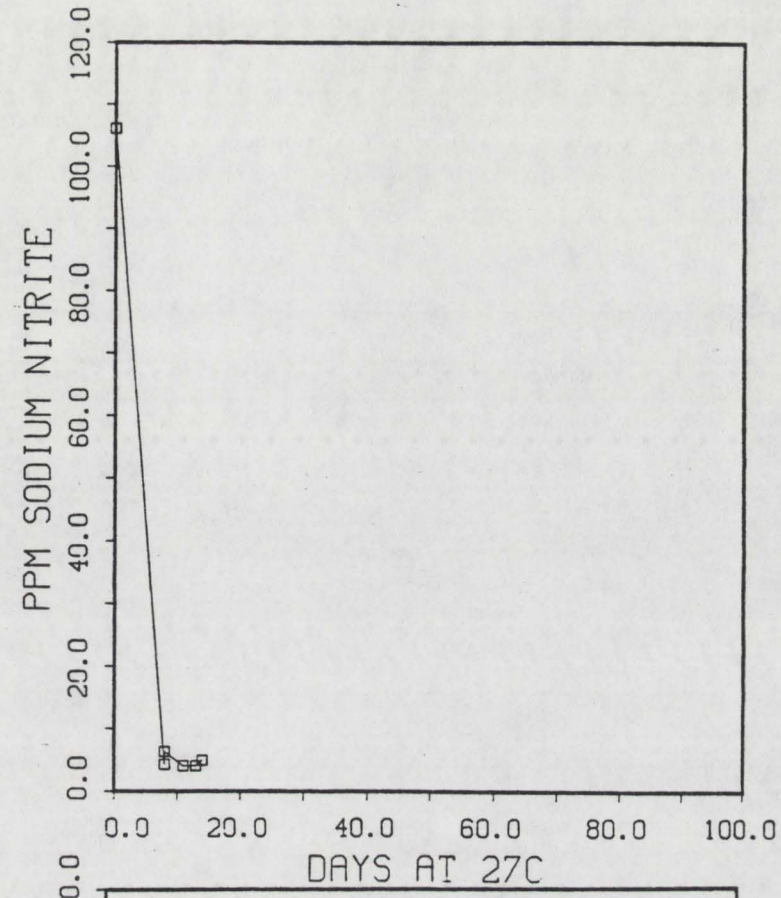

PPM SODIUM NITRITE
0.0
20.0
40.0
60.0
80.0
100.0
120.0
0.0
20.0
40.0
60.0
80.0
100.0
DAYS AT 27C

Figure 5. Nitrite depletion in a meat product formulated with 156 μg/g sodium nitrite and (a.) 2.16 mg myoglobin/g (treatment 4), (b.) 2.16 mg/g myoglobin and 200 μg/g EDTA (treatment 5).

(a.)


PPM SODIUM NITRITE
0.0
20.0
40.0
60.0
80.0
100.0
120.0
0.0
20.0
40.0
60.0
80.0
100.0
DAYS AT 27C

(b.)

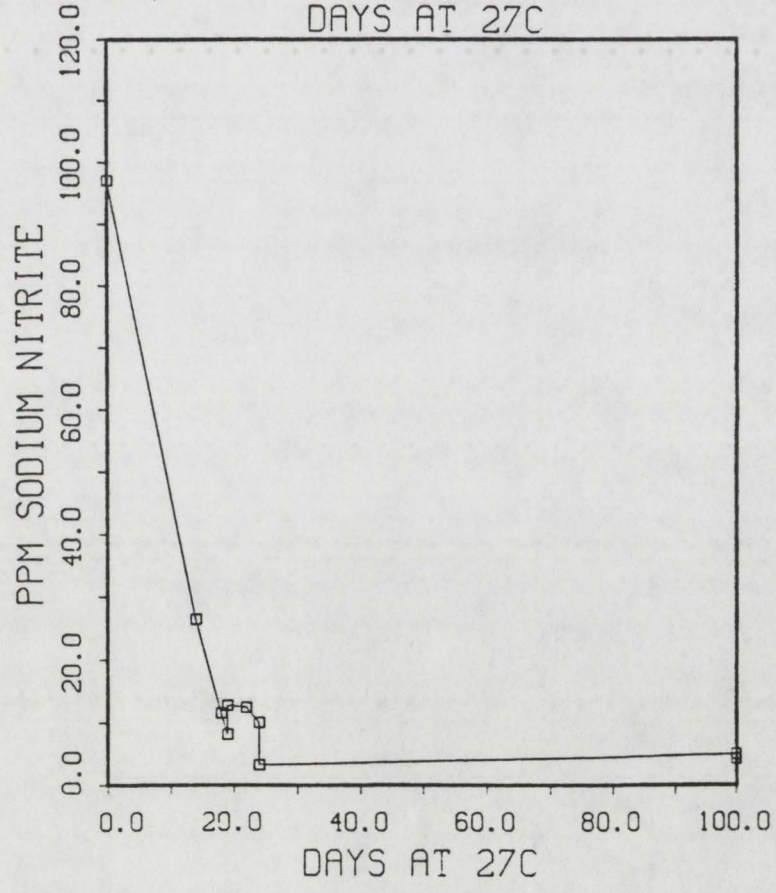
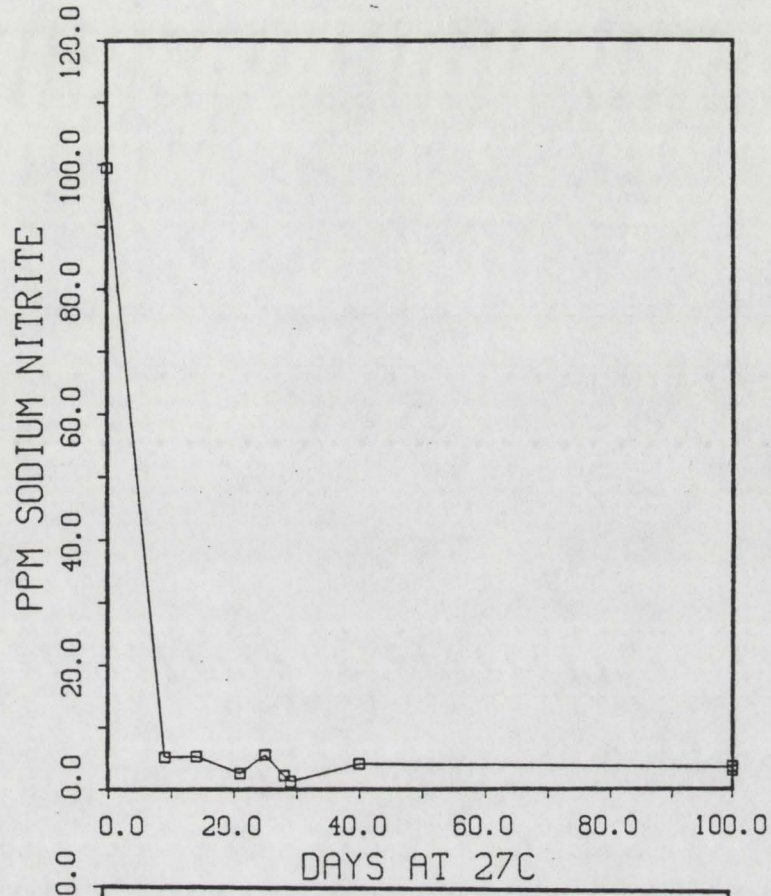

PPM SODIUM NITRITE
0.0
20.0
40.0
60.0
80.0
100.0
120.0
0.0
20.0
40.0
60.0
80.0
100.0
DAYS AT 27C

Figure 6. Nitrite depletion in a meat product formulated with 156 μg/g sodium nitrite and (a.) 2.16 mg/g nitrosylmyoglobin (treatment 6), (b.) 200 μg/g EDTA (treatment 7).

(a.)


PPM SODIUM NITRITE
0.0
20.0
40.0
60.0
80.0
100.0
120.0
0.0
20.0
40.0
60.0
80.0
100.0
DAYS AT 27C

(b.)

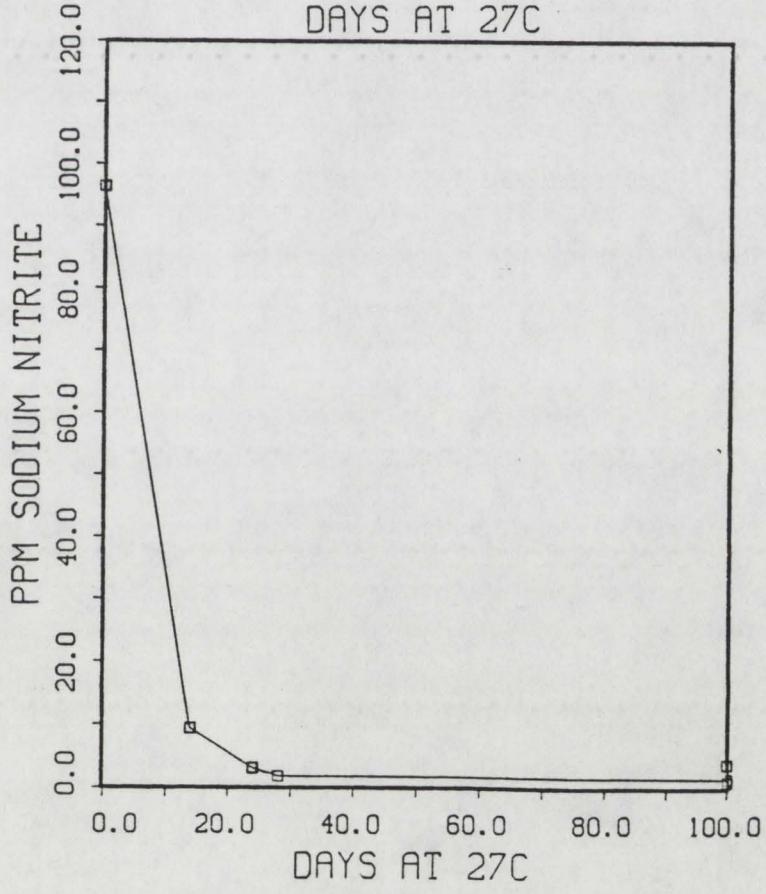
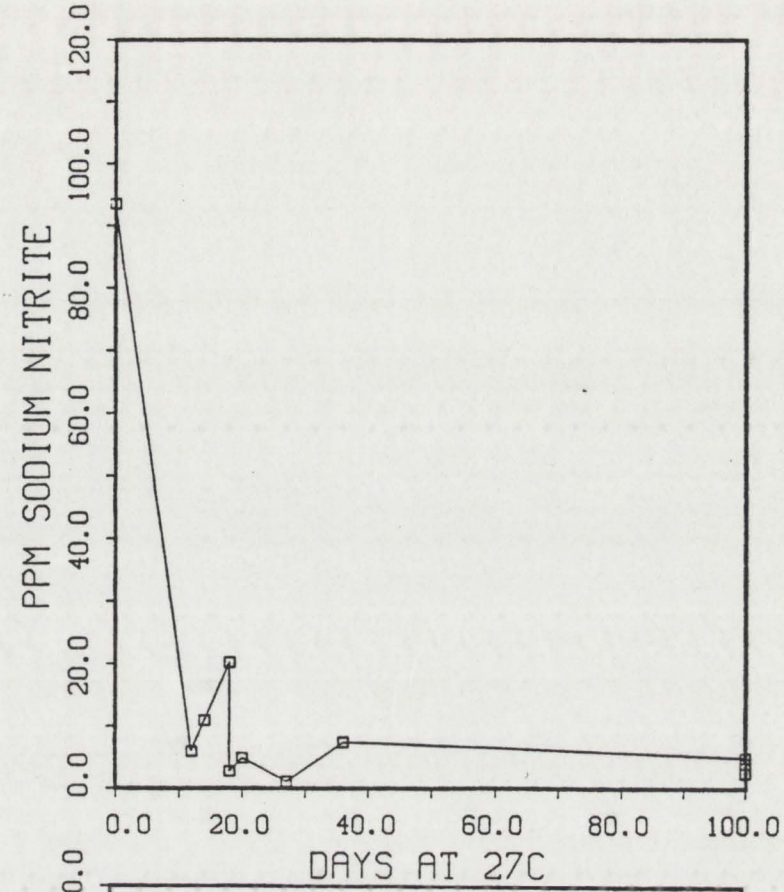

PPM SODIUM NITRITE
0.0
20.0
40.0
60.0
80.0
100.0
120.0
0.0
20.0
40.0
60.0
80.0
100.0
DAYS AT 27C

Figure 7. Nitrite depletion in a meat product formulated with 156 µg/g sodium nitrite, 200 µg/g EDTA, and (a.) 20 µg/g ferric chloride (treatment 8), (b.) 40 µg/g ferric chloride (treatment 9).

(a.)


PPM SODIUM NITRITE
0.0
20.0
40.0
60.0
80.0
100.0
120.0
0.0
20.0
40.0
60.0
80.0
100.0
DAYS AT 27C

(b.)

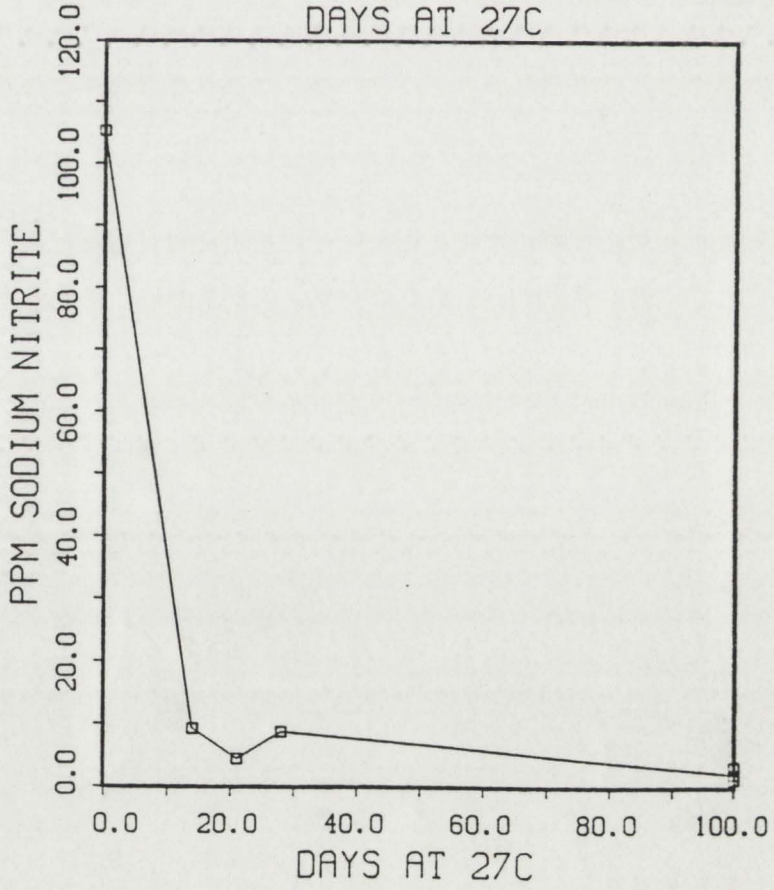
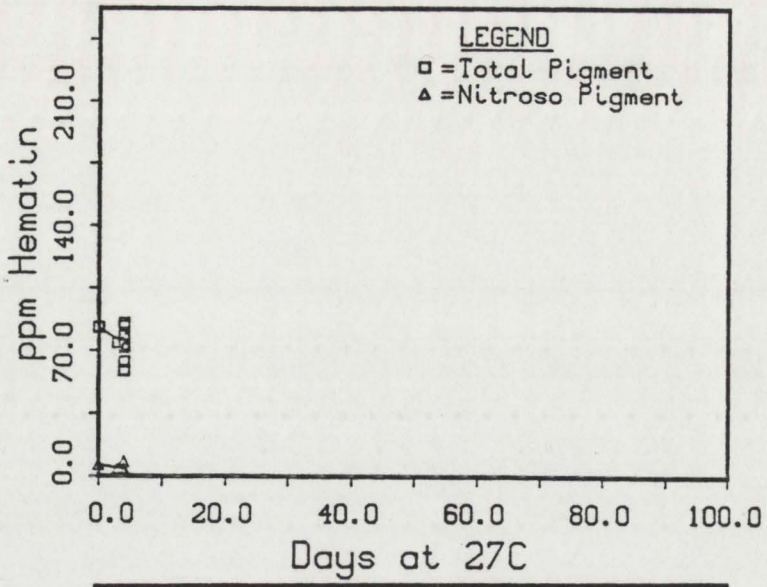
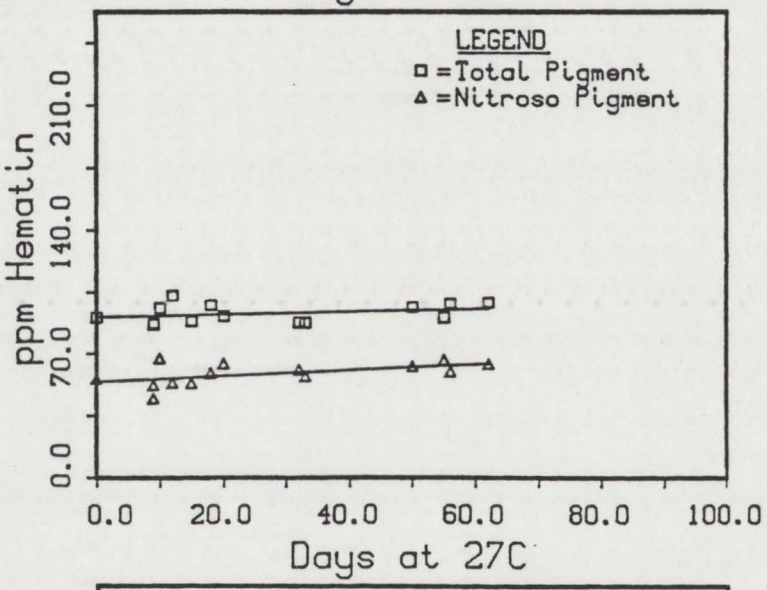

PPM SODIUM NITRITE
0.0
20.0
40.0
60.0
80.0
100.0
120.0
0.0
20.0
40.0
60.0
80.0
100.0
DAYS AT 27C

Figure 8. Total and nitroso pigments in a meat product (a.) without sodium nitrite (treatment 1), (b.) with 156 μg/g sodium nitrite (treatment 2), (c) with 156 μg/g sodium nitrite and 20 μg/g ferric chloride (treatment 3).

(a.)


LEGEND
□ =Total Pigment
△ =Nitroso Pigment
ppm Hematin
0.0
70.0
140.0
210.0
0.0
20.0
40.0
60.0
80.0
100.0
Days at 27C

(b.)


LEGEND
□ =Total Pigment
△ =Nitroso Pigment
ppm Hematin
0.0
70.0
140.0
210.0
0.0
20.0
40.0
60.0
80.0
100.0
Days at 27C

(c.)

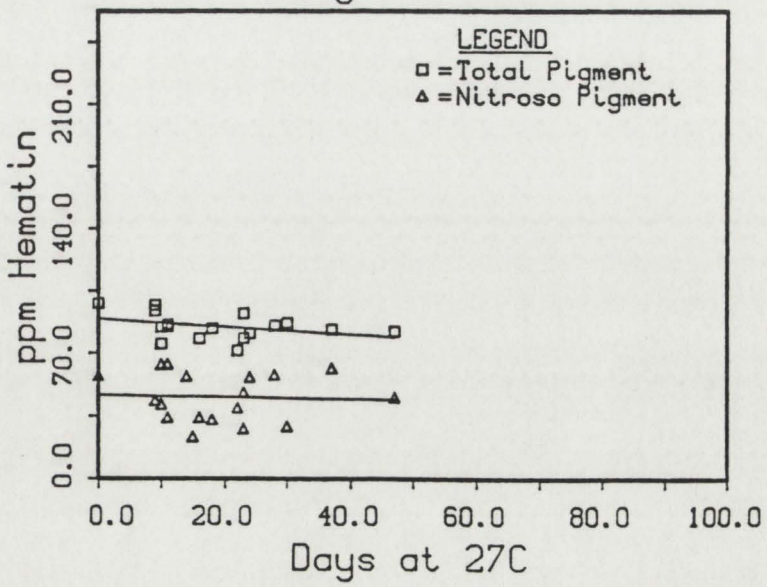
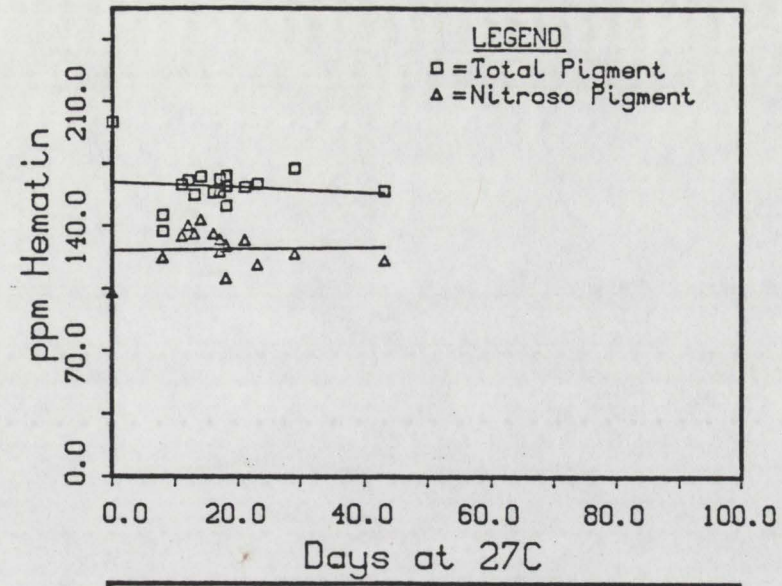
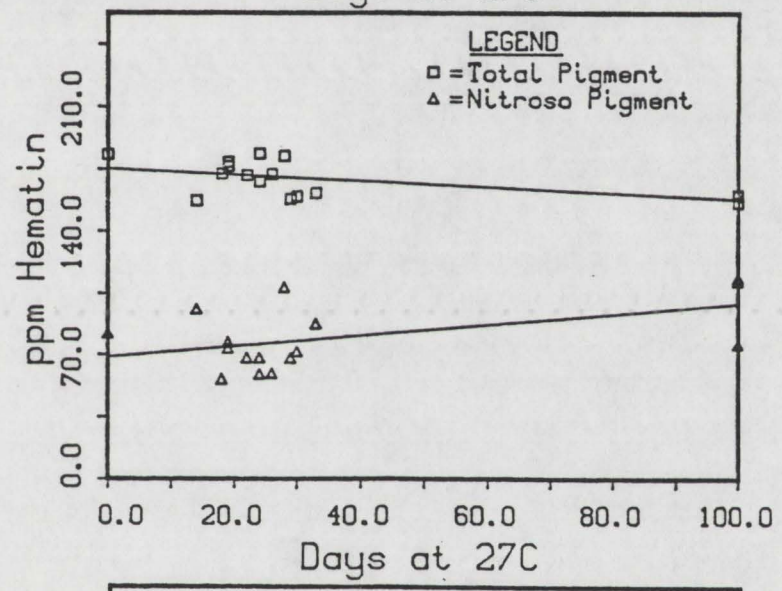

LEGEND
□ =Total Pigment
△ =Nitroso Pigment
ppm Hematin
0.0
70.0
140.0
210.0
0.0
20.0
40.0
60.0
80.0
100.0
Days at 27C

Figure 9. Total and nitroso pigments in a meat product formulated with 156 μg/g sodium nitrite and (a.) 2.16 mg/g myoglobin (treatment 4), (b.) 2.16 mg/g myoglobin and 200 μg/g EDTA (treatment 5), (c.) 2.16 mg/g nitrosylmyoglobin (treatment 6).

(a.)


LEGEND
□ =Total Pigment
△ =Nitroso Pigment
ppm Hematin
0.0
70.0
140.0
210.0
0.0
20.0
40.0
60.0
80.0
100.0
Days at 27C

(b.)


LEGEND
□ =Total Pigment
△ =Nitroso Pigment
ppm Hematin
0.0
70.0
140.0
210.0
0.0
20.0
40.0
60.0
80.0
100.0
Days at 27C

(c.)

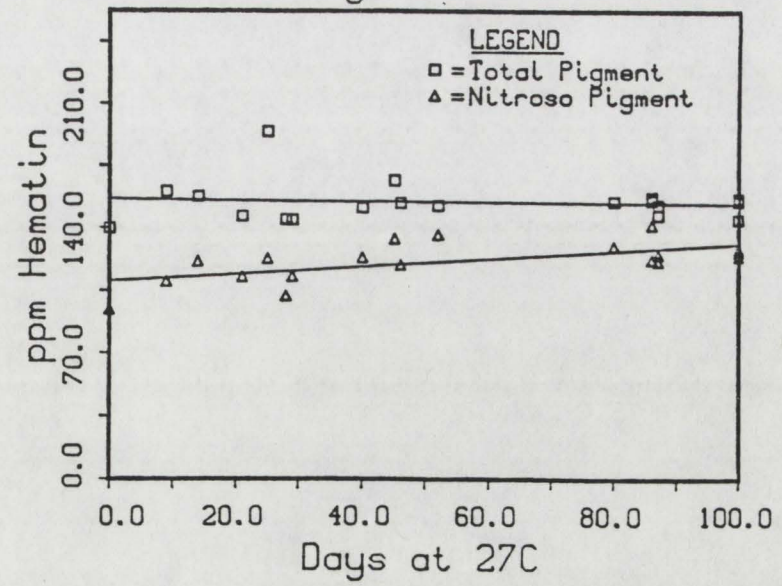
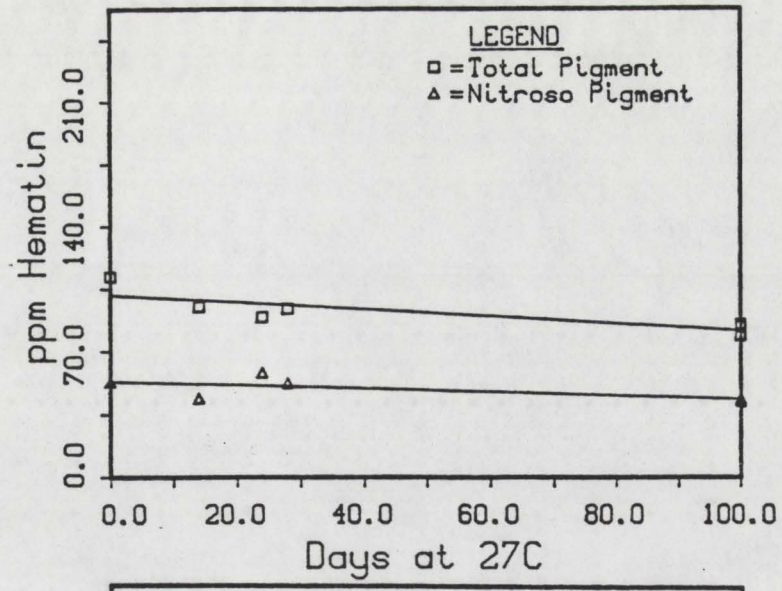
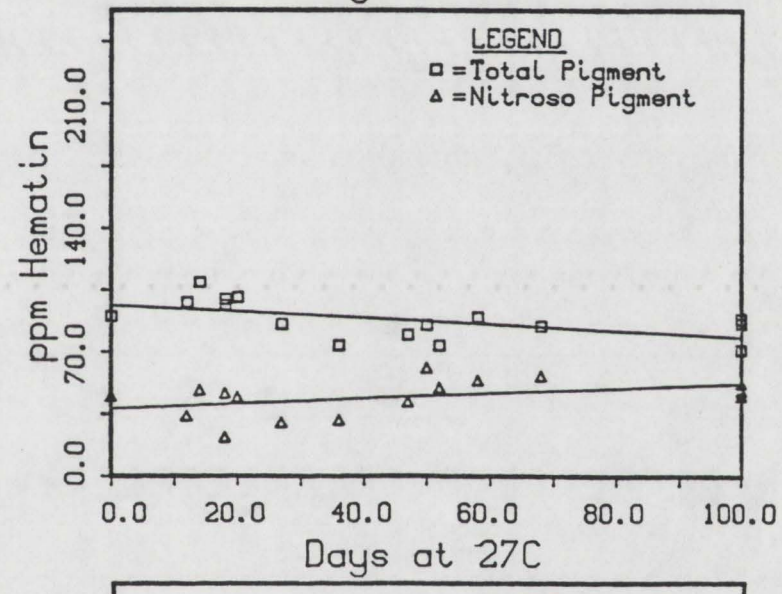

LEGEND
□ =Total Pigment
△ =Nitroso Pigment
ppm Hematin
0.0
70.0
140.0
210.0
0.0
20.0
40.0
60.0
80.0
100.0
Days at 27C

Figure 10. Total and nitroso pigments in a meat product formulated with 156 μg/g sodium nitrite and (a.) 200 μg/g EDTA (treatment 7), (b.) 200 μg/g EDTA and 20 μg/g ferric chloride (treatment 8), (c.) 200 μg/g EDTA and 40 μg/g ferric chloride (treatment 9).

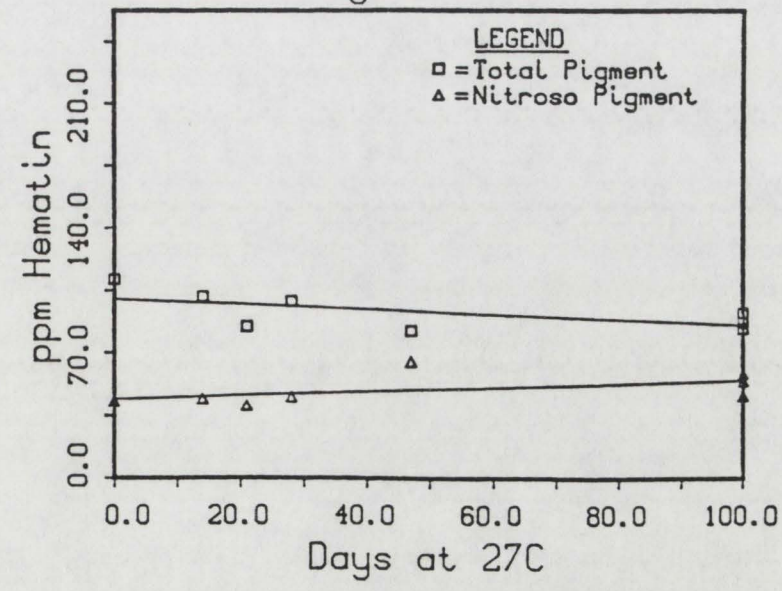
(a.)


LEGEND
□ =Total Pigment
△ =Nitroso Pigment
ppm Hematin
0.0
70.0
140.0
210.0
0.0
20.0
40.0
60.0
80.0
100.0
Days at 27C

(b.)


LEGEND
□ =Total Pigment
△ =Nitroso Pigment
ppm Hematin
0.0
70.0
140.0
210.0
0.0
20.0
40.0
60.0
80.0
100.0
Days at 27C

(c.)


LEGEND
□ =Total Pigment
△ =Nitroso Pigment
ppm Hematin
0.0
70.0
140.0
210.0
0.0
20.0
40.0
60.0
80.0
100.0
Days at 27C

that this added heme was measured by the analytical procedure for total heme pigment. In the scan of the extracted pigment from one of the samples, there was a definite peak at 640 nm, the anticipated wavelength for measuring acid hematin (Fig. 12a). Table 3 includes the mean and LSD values for total and nitroso pigments, along with the calculated amount of heme iron. The analysis for nitroso pigments in treatment #6 (Table 3.), did not show the anticipated increase over treatment #4. In an experiment with nitrosylated hematin, 83.1% to 90.1% of the pigment was measured as nitrosylated over a concentration of 225 to 290 ppm total hematin. With a range of 7%, measured percent nitrosylation appears to be consistent over the concentrations investigated. In addition to these data supporting the validity of the Hornsey (1956) procedure, in a scan of an acetone and water extract of nitrosylated myoglobin (Fig. 12b) or purchased weiners, no definite peak was seen at 540 nm, where nitroso pigments were to be measured.

Nitrite Depletion in Uninoculated Samples

In treatment number 10, the observed decline of nitrite over 29 days of sampling was not as expected (Figure 11a). These data were much more variable than data from mainly swollen samples (Figures 4-7).

Total Iron

The expected and measured values for all nine treatments are presented in Table 4. Total iron content was increased in all treatments with added iron, as measured by atomic absorption

Table 4. Theoretical and measured total iron and soluble iron (μg/g). The basic product without added iron (tmts. 1 and 2), contained 11.3 μg/g Fe.

Treatment	Theoretical Added Fe	Theoretical Total Fe	Measured Total Fe[1]	Soluble Fe[2]
1 No nitrite	0	11.3	11.3^{a} n=6	1.42^{ab} n=4
2 Nitrite	0	11.3	11.3^{a} n=6	$.67^{a}$ n=9
3 Nitrite + 20 ppm $FeCl_3$	6.89	18.2	17.6^{c} n=5	$.78^{ab}$ n=9
4 Nitrite + Mb	6.89	18.2	15.6^{d} n=4	1.07^{ab} n=9
5 Nitrite + Mb + EDTA	6.89	18.2	14.9^{b} n=4	2.52^{c} n=9
6 Nitrite + NOMb	6.89	18.2	17.7^{c} n=5	1.59^{b} n=8
7 Nitrite + EDTA	0	11.3	11.7^{a} n=4	3.65^{d} n=7
8 Nitrite + EDTA +20 ppm $FeCl_3$	6.89	18.2	18.3^{c} n=5	4.64^{e} n=7
9 Nitrite + EDTA +40 ppm $FeCl_3$	13.78	25.1	20.3^{d} n=6	8.06^{f} n=4

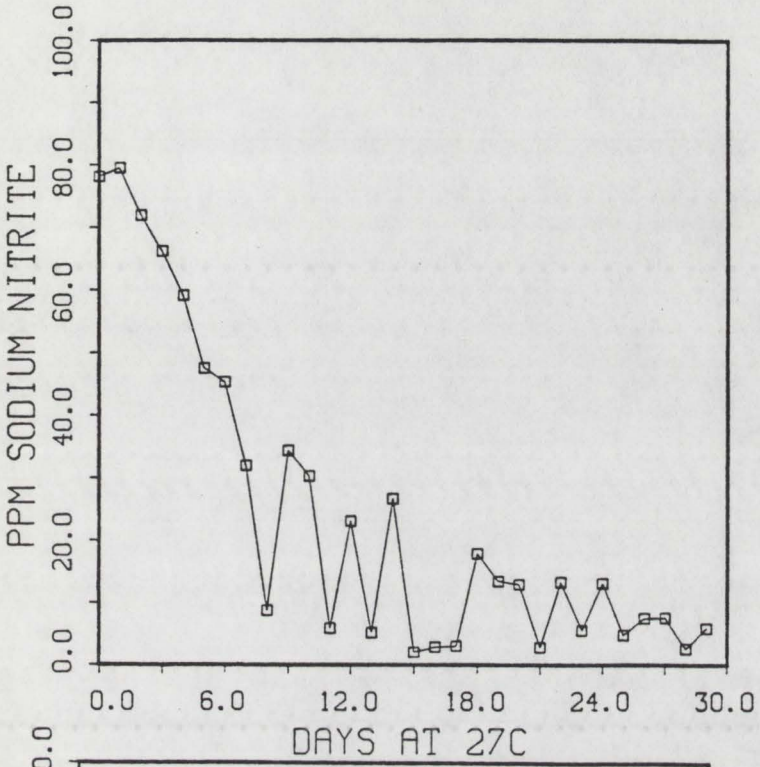
[1]LSD .01/.05 = 1.47/1.11

[2]LSD .01/.05 = .88/.66

^{abcdef}Mean values with the same letter are not significantly different at the 1% level of probability.

Figure 11. Nitrite depletion over time in an uninoculated meat product formulated with 156 μg/g sodium nitrite: (a.) connecting all points and (b.) showing possible dichotomy of values.

(a.)


PPM SODIUM NITRITE
0.0
20.0
40.0
60.0
80.0
100.0
0.0
6.0
12.0
18.0
24.0
30.0
DAYS AT 27C

(b.)

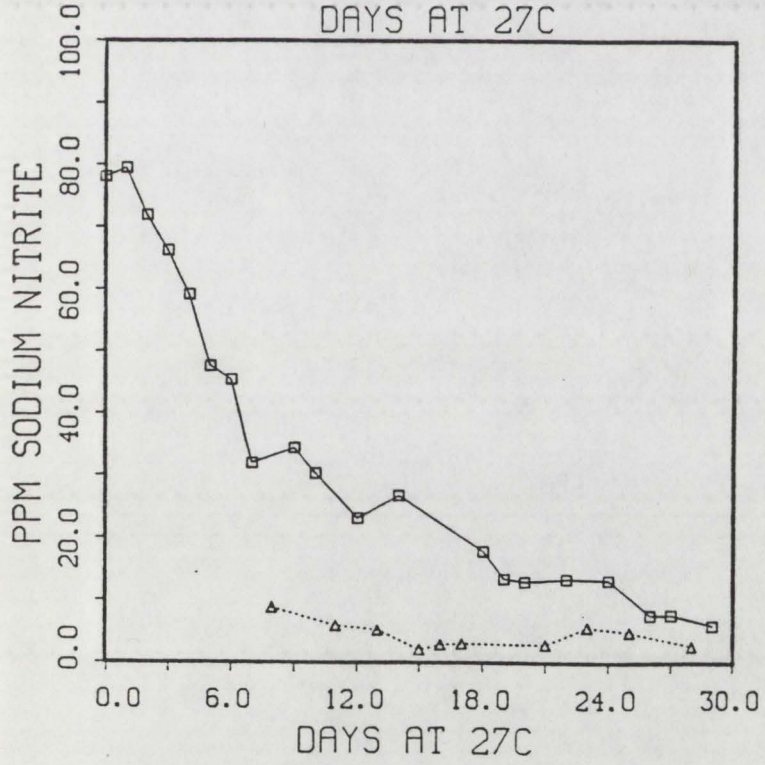
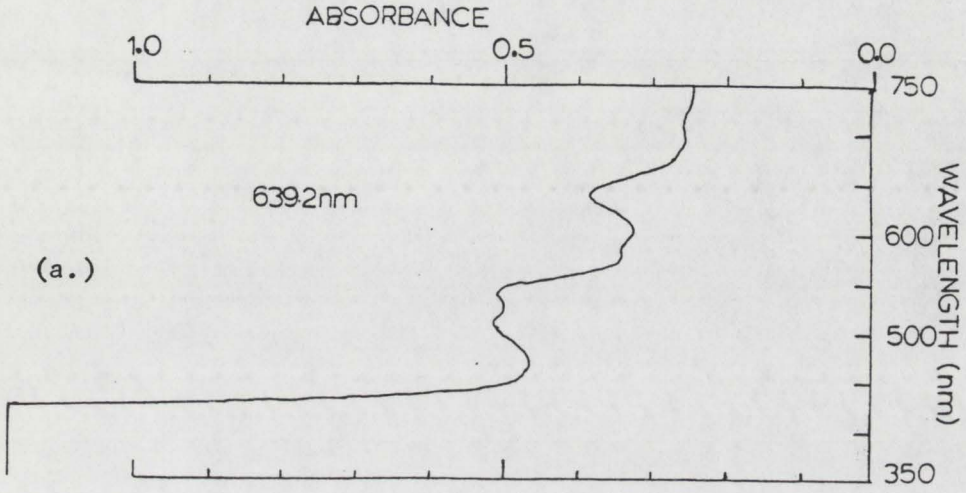
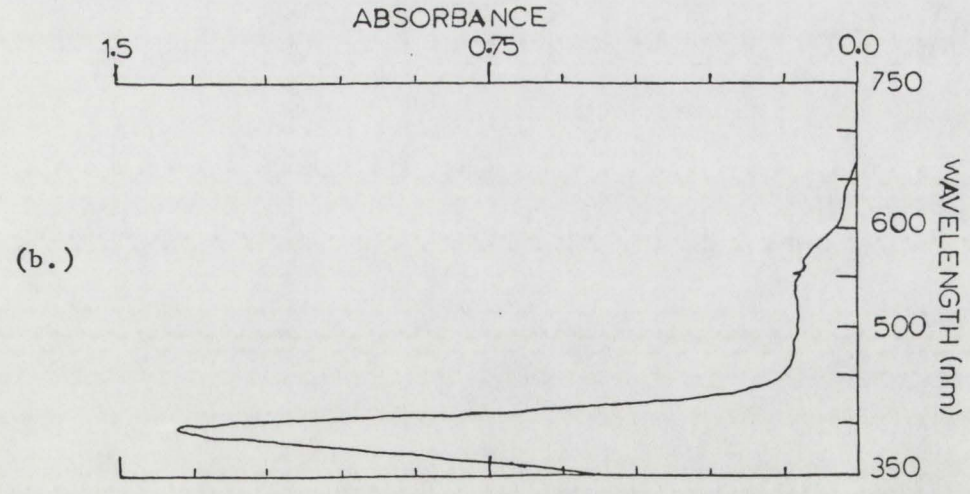

PPM SODIUM NITRITE
0.0
20.0
40.0
60.0
80.0
100.0
0.0
6.0
12.0
18.0
24.0
30.0
DAYS AT 27C

Figure 12. Scan of (a.) extracted acid hematin for measurement of total pigment at 640 nm and (b.) extracted nitroso hematin for measurement of nitroso pigment at 540 nm.


ABSORBANCE
1.0
0.5
0.0
750
639.2nm
600
500
350
WAVELENGTH (nm)
(a.)


ABSORBANCE
1.5
0.75
0.0
750
600
500
350
WAVELENGTH (nm)
(b.)

spectroscopy. Means of the three treatments without added iron (1, 2, and 7) were not significantly different. Treatments 3, 4, 5, 6, and 8 should have had equivalent mean total iron levels, but treatments 4 and 5 were considered significantly different from 3, 6, and 8.

Soluble Iron

Figures 13 through 15 include values of soluble iron for the nine treatments. Mean and LSD values for the nine treatments are included in Table 4. Very little difference was seen in the soluble iron content over time. Several of the treatments showed a slight decline in soluble iron of the swollen samples, when compared to an unswollen sample (day 0). There were some striking differences between treatments, however. All treatments containing EDTA (tmts. 5, 7, 8, 9; Figs. 14b,15a-c) had more water soluble iron than the other treatments.

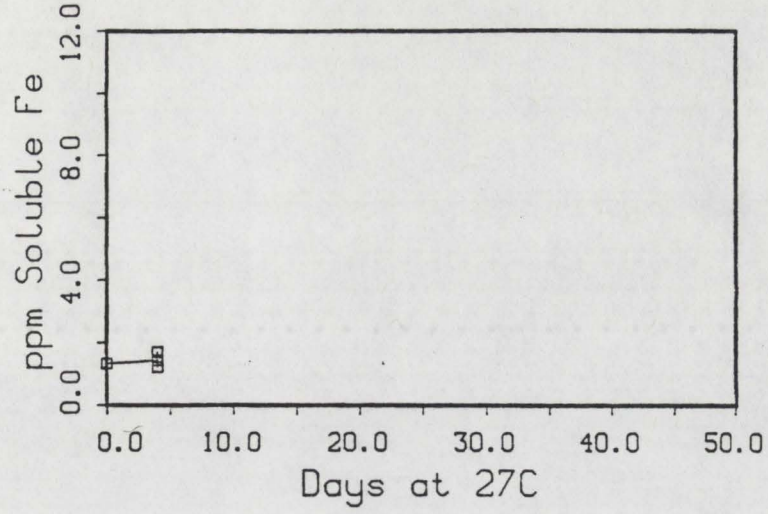
Spore Counts

The stock suspension of Type A spores contained 7.6×10^5 spores/ ml. To a pooled inoculum, 1.72 ml of a 1:10 dilution of this stock suspension was added. The type B spore suspension contained 4.7×10^6 spores/ml. From a 1:100 dilution of this suspension, 2.7 ml was added to the pooled inoculum for a concentration of 5.8×10^4 spores/ml. Of the pooled inoculum, 4.42 ml was mixed with 2700 g of treatment mix to give approximately 100 spores/g meat.

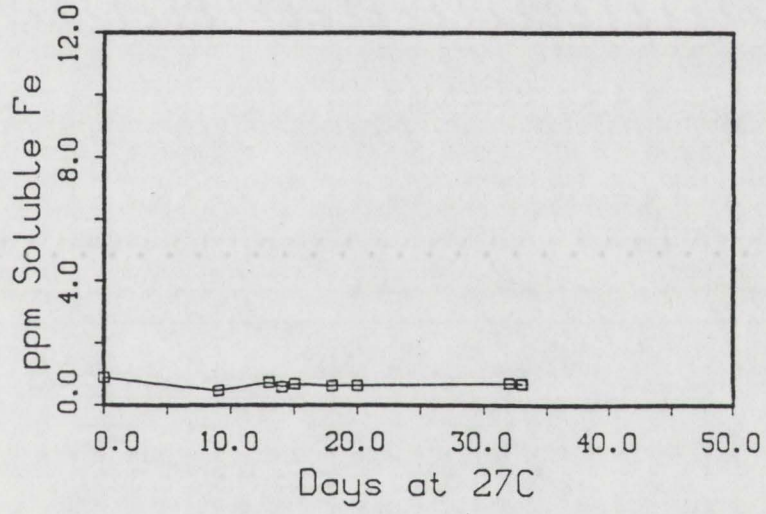
Organism counts from unswollen samples of treatments 6, 7, 8, and 9 are presented in Table 5. No Clostridia were identified in any of the

Figure 13. Soluble iron in a pasteurized meat product (a.) without nitrite (treatment 1). Uncooked sample had 4.62 µg/g; (b.) with 156 µg/g sodium nitrite (treatment 2). Uncooked sample contained 2.08 µg/g; (c.) with 156 µg/g sodium nitrite and 20 µg/g ferric chloride (treatment 3). Uncooked sample contained 4.17 µg/g.

(a.)


ppm Soluble Fe
0.0
4.0
8.0
12.0
0.0
10.0
20.0
30.0
40.0
50.0
Days at 27C

(b.)


ppm Soluble Fe
0.0
4.0
8.0
12.0
0.0
10.0
20.0
30.0
40.0
50.0
Days at 27C

(c.)

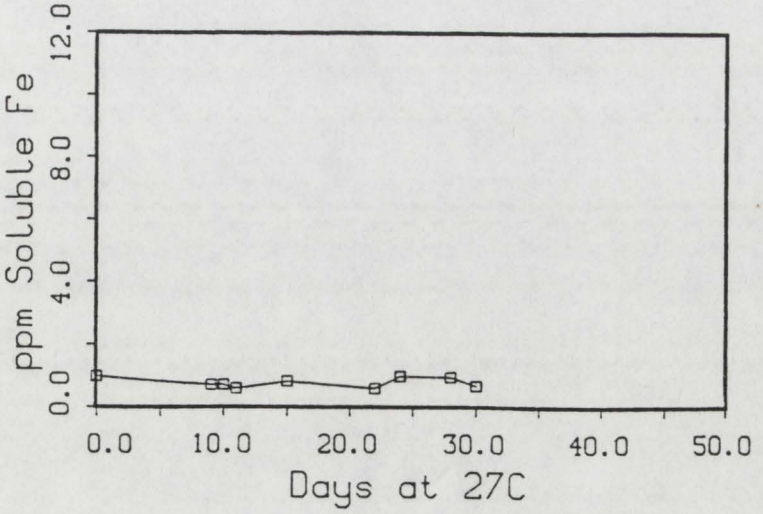
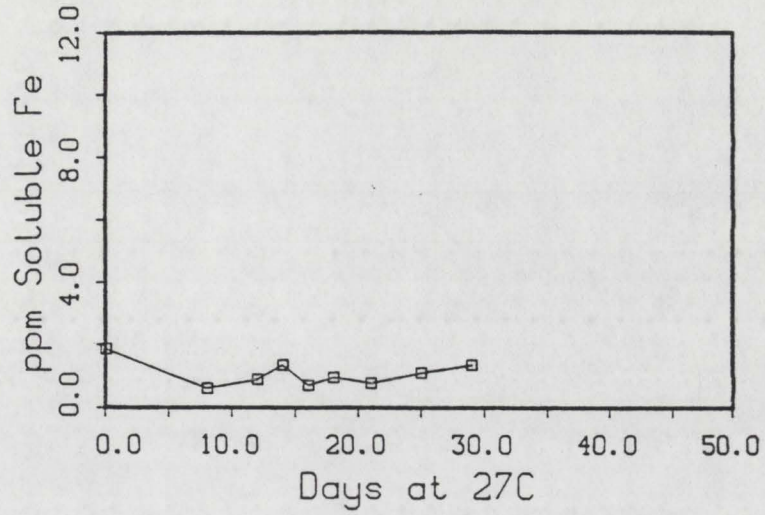
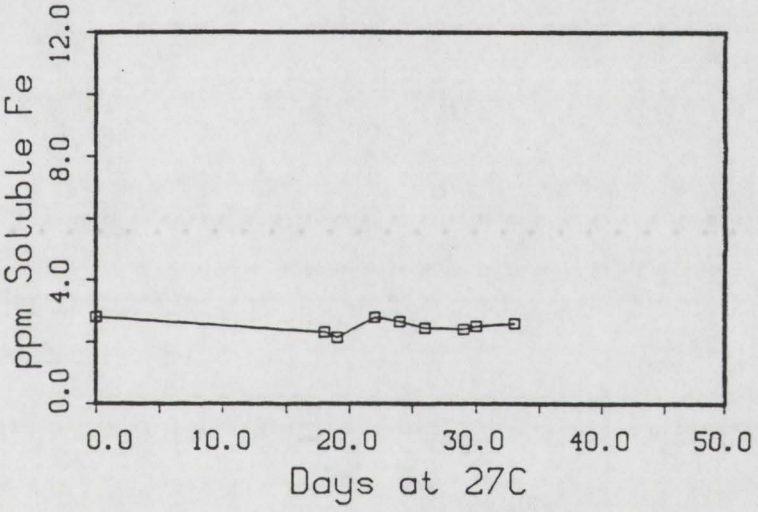

ppm Soluble Fe
0.0
4.0
8.0
12.0
0.0
10.0
20.0
30.0
40.0
50.0
Days at 27C

Figure 14. Soluble iron in a meat product formulated with 156 μg/g sodium nitrite and (a.) 2.16 mg/g myoglobin (treatment 4). Uncooked sample had 7.51 μg/g; (b.) 2.16 mg myoglobin/g and 200 μg/g EDTA (treatment 5). Uncooked sample had 7.22 μg/g; (c.) 2.16 mg/g nitrosylated myoglobin (treatment 6). Uncooked sample had 3.74 μg/g.

(a.)


ppm Soluble Fe
0.0
4.0
8.0
12.0
0.0
10.0
20.0
30.0
40.0
50.0
Days at 27C

(b.)


ppm Soluble Fe
0.0
4.0
8.0
12.0
0.0
10.0
20.0
30.0
40.0
50.0
Days at 27C

(c.)

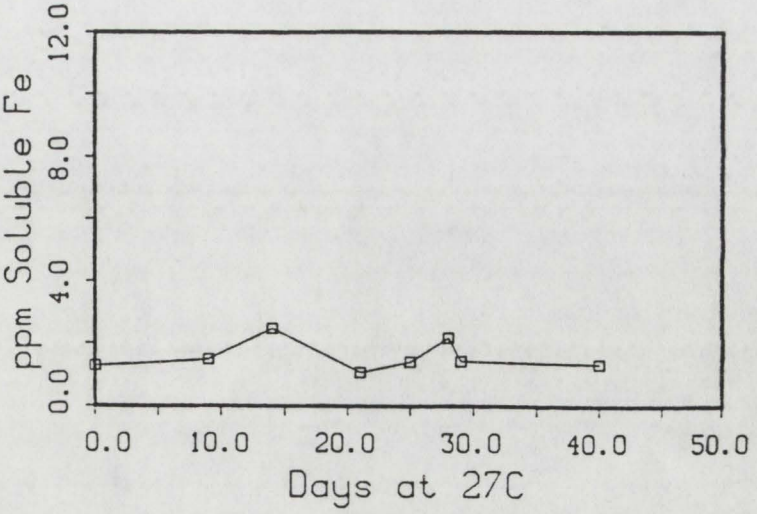
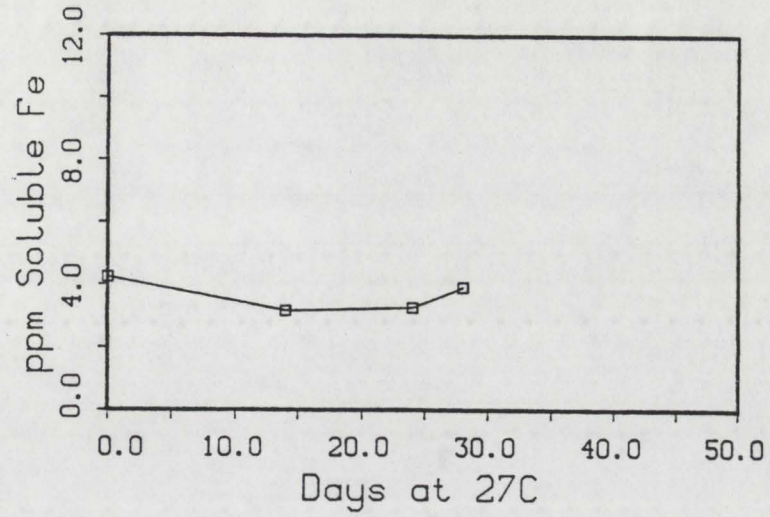
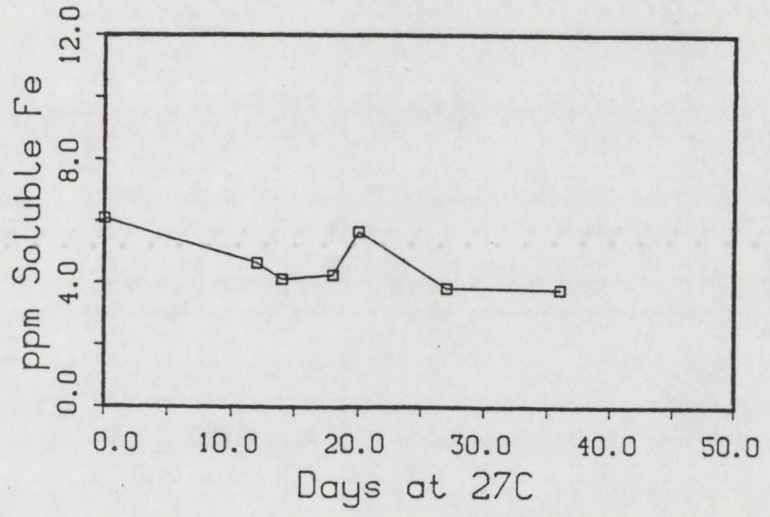

ppm Soluble Fe
0.0
4.0
8.0
12.0
0.0
10.0
20.0
30.0
40.0
50.0
Days at 27C

Figure 15. Soluble iron in a meat product formulated with 156 μg/g sodium nitrite and (a.) 200 μg/g EDTA (treatment 7). Uncooked sample had 6.45 μg/g; (b.) 200 μg/g EDTA and 20 μg/g ferric chloride (treatment 8). Uncooked sample had 8.46 μg/g; (c.) 200 μg/g EDTA and 40 μg/g ferric chloride (treatment 9). Uncooked sample had 10.6 μg/g.

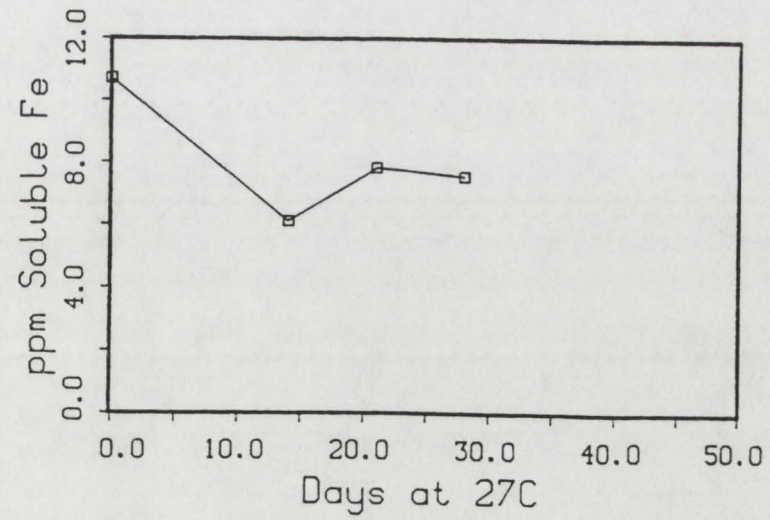
(a.)


ppm Soluble Fe
0.0
4.0
8.0
12.0
0.0
10.0
20.0
30.0
40.0
50.0
Days at 27C

(b.)


ppm Soluble Fe
0.0
4.0
8.0
12.0
0.0
10.0
20.0
30.0
40.0
50.0
Days at 27C

(c.)


ppm Soluble Fe
0.0
4.0
8.0
12.0
0.0
10.0
20.0
30.0
40.0
50.0
Days at 27C

samples. Nearly all the colonies were small, button shaped, opaque, and convex. The same type of colonies was seen on both the anaerobic and aerobic plates. Gram positive cocci in chains and clumps were observed in Gram stains from both aerobic and anaerobic plates.

Table 5. Organisms counted from unswollen samples after 100 days at 27C.

Treatment	Sample Number	Aerobic (Colonies/g)	Anaerobic (Colonies/g)
6 Nitrite + NOMb	6-26	4.1×10^6	5.4×10^7
	6-19	1.9×10^7	2.1×10^7
7 Nitrite + EDTA	7-20	7.5×10^4	6.3×10^4
	7-23	1.4×10^5	1.8×10^5
8 Nitrite + EDTA + 20 ppm $FeCl_3$	8-21	4.7×10^5	5.9×10^5
	8-24	7.3×10^5	9.4×10^5
9 Nitrite + EDTA + 40 ppm $FeCl_3$	9-11	1.5×10^5	2.0×10^5
	9-18	4.0×10^5	4.1×10^5

DISCUSSION

Gas Production

Rate of swelling in a non-nitrite treatment (#1) occurred as expected. Tompkin et al. (1977) reported that the average time to first swell in a non-nitrite treatment was 6.7 days. This compares favorably with the observed average of 4 days until swelling. Once swelling began, all variables were swollen within a few days as has been presented (Tompkin et al., 1977; 1978a) for a non-nitrite product. Pork ham products without nitrite but containing sulfur dioxide (Tompkin et al., 1980), EDTA (Tompkin et al., 1979), or isoascorbate (Tompkin et al., 1978a) all have swelling profiles similar to a non-nitrite treatment without any of these additives.

Addition of 156 μg sodium nitrite/g meat had a distinct inhibitory effect, with swelling beginning in 9 days. A similar swelling pattern was reported (Tompkin et al., 1978a) for a treatment formulated with 156 μg sodium nitrite/g meat. However, Tompkin et al. (1977; 1978b) have also indicated that 156 μg/g sodium nitrite creates a much more inhibitory system, with significant swelling beginning at about day 60 to 80. There is a large difference between these results and those presented by Tompkin et al. (1978a), in which significant swelling began at approximately day 10, as is presented here. The plastic bag assay used in this study may have allowed earlier detection of gas than the aluminum cans used by Tompkin et al. (1977; 1978a; 1978b). All treatments had similar formulations and target spore levels of 100 spores/g. No obvious explanation can be given for the great difference

in swelling time from the data reported here and one study by Tompkin et al. (1978a) beginning in 10 days and other similar treatments resulting in swelling in 60 to 80 days (Tompkin et al., 1977; 1978b).

When iron as ferric chloride (tmt. #3) or myoglobin (tmt. #4) was added to the basic formulation, growth was slightly stimulated. Stimulation of growth by the addition of ferric chloride or myoglobin provides further evidence that C. botulinum requires iron (Tompkin et al., 1978c; 1979). Pre-nitrosylation of myoglobin (tmt. #6) negated the stimulatory effect and caused significant inhibition compared with myoglobin alone. The observed inhibition in treatment #6 could be due to reduced availability of the iron in nitrosylated myoglobin. Since the pre-nitrosylation of myoglobin included a cooking step to denature the protein to facilitate precipitation and centrifugation, this may have created a Perigo-type inhibitor. In further studies, it might be wise to include a non-nitrosylated control in which the myoglobin was heated and denatured, in the absence of sodium nitrite.

Inclusion of EDTA (tmt. #5) also caused a decrease in the stimulatory effect of myoglobin but not as extensively as nitrosylation of the added myoglobin. Since EDTA is a non-specific metal ion chelator, the observed inhibition could be due to chelation of iron as well as other metal ions. Addition of EDTA to the basic nitrite containing formulation proved to be quite inhibitory with a swelling profile almost exactly like that reported by Tompkin et al. (1979). They concluded that EDTA sequesters iron, making iron less available for preventing nitrite inhibition. Addition of 20 ppm ferric chloride to an EDTA and nitrite formulation stimulated growth (tmt. #8) when compared with a similar treatment without added iron (tmt. #7). At the same iron

concentration, myoglobin (tmt. #5) stimulated growth of C. botulinum more than ferric chloride (tmt. #8). Ferric iron probably was not as available as heme iron for microbial utilization in the presence of EDTA. When 40 ppm ferric chloride was added to the basic nitrite and EDTA formulation (tmt. #9), the resulting product had the same growth response as the treatment without added iron (tmt. #7). It was expected that the 40 ppm increment of ferric chloride (tmt. #9) would override the inhibitory effect of EDTA, even more than 20 ppm ferric chloride (tmt. #8). Instead, the 40 ppm ferric chloride treatment was more inhibitory than the 20 ppm treatment.

Toxin Assay

The swelling data may be explained further if the results of the toxin test are discussed. In treatments such as #6 (NOMb) and #8 ($FeCl_3$ +EDTA) there was a substantial discrepancy between the number of swollen samples and those that were toxic. Presence of gas in these treatments apparently was not an adequate assay for growth and toxin production by C. botulinum. In actuality, the growth and toxin production by C. botulinum in treatments 6 and 8 may have been similar to that of tmt. #7, the gas being produced by other organisms. Of 36 swollen samples tested, only 23 (64 percent) were confirmed to contain toxin. Sixty-four percent of swollen samples were confirmed toxic whereas Tompkin et al. (1978a) reported that over 5 years of using this procedure, over 90% of swelled cans were toxic. The reason fewer swelled products were toxic in the work presented here, may have been due to the greater sensitivity of the plastic bag procedure, because it

is easy to observe gas bubbles in the clear plastic bags. Small bubbles of gas from contaminating organisms may have been mistaken for Clostridia growth. Possibly, these bubbles would not have distended aluminum cans.

Nitrite Depletion

Residual nitrite declined in all treatments at a rate similar to that reported by Christiansen (1980). In all treatments containing EDTA (5, 7, 8, and 9), nitrite depletion occurred more slowly than it did in the other treatments. It is possible that EDTA exerts its inhibitory effect by decreasing the rate of nitrite depletion. Although Tompkin et al. (1977) reported that the primary effect of varying the quantity of initial nitrite was to vary the length of the lag phase, this lag phase length is not well correlated with the presence of residual nitrite. Christiansen (1980) has emphasized the importance of residual nitrite in inhibition of C. botulinum. When the input level of sodium nitrite was 156 μg/g, residual nitrite declined to below 10 μg/g (the level considered critical for inhibition) in 20 days at 27C (Christiansen, 1980). Predicted average time to first swell at the same nitrite level was 94.3 days (Tompkin et al., 1977). What is occurring between day 20 and day 90? If residual nitrite is necessary for C. botulinum inhibition, swelling should have occurred before 70 days after nitrite was depleted. Tompkin et al. (1978a) showed significant swelling to occur in as few as 10 days or as many as 60 to 90 days with the same formulation (Tompkin et al., 1977; 1978b). The role of residual nitrite in botulinal inhibition appears to be quite ambiguous.

In treatments 6-9, swelling proceded slowly even after residual nitrite had declined. Residual nitrite may play a role in botulinal inhibition, but it probably is not the most important factor if swelling does not occur when nitrite is depleted.

It was expected that residual nitrite in an uninoculated sample (Fig. 11) would decline as depicted in the classic graphs of Christiansen (1980). Initial decline (Fig. 11a) was constant with time, but after day 8, nitrite values became quite variable. It appears that two completely different trends were seen in this homogeneous treatment (Fig. 11b). Possibly, this dichotomy occurs in many of these assays, but only the values of the lower curve might be measured if swollen samples had lower nitrite values. Many researchers (Christiansen et al., 1973; 1974) only analyzed for nitrite at the time of swelling and would not determine if unswollen samples had higher nitrite values. Some of the treatments in which swelling was delayed (5, 7, 8, and 9) show a curve much like the upper curve in figure 11b (solid line). At this point, no substantial conclusions should be drawn, since more samples should be measured to get a more definite characterization of nitrite decline over time. From the evidence presented here, it appears that nitrite decline in a homogeneous product may be quite erratic.

Heme Pigments

If *C. botulinum* organisms were using the iron from the heme group, one would expect the concentration of total heme to be less in a swollen sample. This was the case in treatment four where the initial total pigment concentration was over 40 ppm greater than the mean value

for the treatment. None of the other treatments show any definite trends and the data appear to be quite variable.

One reason for the variability in the data may have been due to problems in the Hornsey (1956) procedure. The assay for total pigment is probably quite accurate, but the one for nitroso pigment may yield questionable quantitative results. Using the procedure for total pigments, measurement of added hematin was closely associated with the theoretical increase. A distinct peak at 640 nm on the scan of the pigment extract (Fig. 12a) would also support the validity of this procedure. Measurement of nitrosylated hematin was consistent over a concentration range of 225-290 ppm total pigment. However, a scan of nitrosylated myoglobin (Fig. 12b) did not contain a distinct peak at 540 nm, the anticipated wavelength for measuring nitroso pigment. Although this procedure is the standard one used by meat analysts and processors, wavelength scans indicated that it may not be very accurate. Hornsey (1956) included a wavelength scan of a nitroso-heme-acetone complex. Nitroso pigment was quantitated by a rather flat-topped peak between 530 and 570 nm, that may have been the shoulder of a much larger peak at 400 nm. This scan in the paper of Hornsey (1956) showed a more distinct peak than the one obtained from the myoglobin or weiners analyzed in this study (Fig. 12b). In either case, the proximity of the large peak at 400 nm may interfere with quantitative measurement of nitroso heme at 540 nm. This procedure may need a thorough review and revision if it is to be in continued use.

The amount of nitroso pigment was not significantly different between treatments 4 and 6, although treatment #6 should have measured considerably higher (Table 3). It is possible that 156 μg sodium

nitrite/g product was enough to result in an equal proportion of nitrosylated myoglobin (tmt. #4) as in the product with pre-nitrosylated myoglobin (tmt. #6). A more appropriate test of the effect of nitrosylation of myoglobin on C. botulinum growth, might include addition of both myoglobin and pre-nitrosylated myoglibin to the control formulation (156 μg sodium nitrite/g) after curing. One might conclude that nitrosylation was just as complete in treatment #4 as it was in treatment #6 in which nitrosylated myoglobin was added. This could be true but it may also reflect a problem in the analytical procedure. Either way, the pre-nitrosylation of myoglobin appears to have caused a substantial inhibition of botulinal growth and toxin production.

A substantial amount of variation was seen in both total and nitroso pigments within a treatment. This variation may have been due to the storage conditions after swelling. Goutefongea (1980) reported that with a low level of residual nitrite, only a very good vacuum is able to maintain color characteristics. Some of the samples were stored opened in the freezer before analysis. In the absence of a vacuum, pigment may have been oxidized in open packages, resulting in smaller amounts of measurable pigment.

Total Iron

In all treatments with added iron, an increase in iron was measured by atomic absorption analysis, although the measured values differed from the expected values (Table 4). It is possible that there was this much variation in the formulation, but there also might have been some analytical error. Although atomic absorption analysis for iron is done

routinely in this laboratory, in recent studies, problems have been encountered both with the method and the atomic absorption spectrophotometer. The three treatments without added iron (1, 2, and 7) had similar iron levels, indicating a high degree of consistency in formulation.

Soluble Iron

Very little change in soluble iron was observed over time in all of the treatments. In all products, the largest change was seen between the uncooked and cooked sample. Visual observation indicated that much of the pigment was solubilized in the uncooked sample, but not in a cooked product. One might hypothesize that a proteolytic organism like C. botulinum would cause iron to become more soluble as the meat matrix is degraded. If this were the case, the magnitude of change was not measurable by the method employed here. Absolute differences in the amount of soluble iron between treatments were associated with total amount of iron and all treatments formulated with EDTA (5, 7, 8, and 9) contained higher levels of soluble iron. Extractability of iron apparently has little association with C. botulinum growth, since these were among the more inhibitory treatments.

Organism Counts

No Clostridia were isolated from unswollen packages after 100 days of incubation. The Gram-positive cocci found were probably a thermoduric facultative organism. If the environment was not conducive

to germination and growth of C. botulinum spores, any other organisms that survived pasteurization could have grown and altered the media to suppress the growth of Clostridium spores. The contaminating organisms proteolyzed the meat, but it was often sour, not putrefied.

Microbial Iron Metabolism

What might be the physiological consequences of iron deficiency for C. botulinum orgainsms? Although there is a dearth of information regarding iron metabolism in C. botulinum, an iron containing protein, ferredoxin, has been reported in C. pasteurianum (Mortenson et al., 1962; 1963; Mortenson, 1964). Ferredoxin, which contains iron but no detectable heme or flavin, functions as an electron transferring protein between pyruvic dehydrogenase and hydrogenase (Mortenson et al., 1963). Carbon dioxide is produced in the pyruvic dehydrogenase reaction and hydrogen gas is formed in the hydrogenase reaction.

Ferredoxin plays an essential role in pyruvate metabolism, which would be important in utilization of this important energy source. Additionally, ferredoxin is a link in the evolution of hydrogen gas and carbon dioxide gas. Ferredoxin also participates in the reduction of nitrite and hydroxylamine with molecular hydrogen as the reductant (Valentine et al., 1963). A reduction in the availability of iron for C. botulinum growth may result in a deficiency of ferredoxin, which could affect growth of C. botulinum and interpretation of experiments like the one presented here. An interruption in pyruvate metabolism could slow or halt growth since energy for growth and metabolism might be limited. If this occurred, and iron were unavailable for Clostridia

growth, it would definitely inhibit gas and toxin production by C. botulinum Even if growth were occurring in the absence of ferredoxin, hydrogen and carbon dioxide gas might not be produced. This reason alone could account for a reduction in the rate of gas production in iron deficient or iron chelated meat systems. In an iron limited meat system, it may be possible for gas production to be inhibited, while toxin is still being produced. This was not observed here, but could easily be overlooked if only swollen products were tested for toxin. The observation made here that addition of EDTA slowed nitrite depletion (Fig. 22, 24, 25, and 26) may be another manifestation of reduced ferredoxin levels due to the chelated iron being unavailable for microbial utilization, since nitrite can be reduced to ammonia in the presence of ferredoxin (Valentine et al., 1963).

Another iron containing protein, rubredoxin, has been isolated from some Clostridia and is believed to be present in C. botulinum (Probst et al., 1978). It is also involved in electron transport.

CONCLUSION

The addition of pre-nitrosylated myoglobin to a cured meat product resulted in effective inhibition of C. botulinum when compared with a product with the same amount of untreated myoglobin. At the time of swelling, residual nitrite had declined to below 10 ppm, but the decline of nitrite alone was not enough to allow swelling to occur in all treatments. These results contradict those of Tompkin et al. (1977), in which 0, 50, 100, and 156 μg sodium nitrite/g meat was added to four different treatments. In the Tompkin (1977) study, the quantity of nitrite affected the length of the lag phase, but once swelling began, the rate of swelling was not significanly different for the four treatments.

Addition of ferric iron or myoglobin stimulated gas and toxin production by C. botulinum, whereas pre-nitrosylated myoglobin and EDTA inhibited growth. In the treatment with added myoglobin alone, there was an observed decrease in total pigments in the swollen samples when compared with the initial level. This, along with the inhibition observed when heme iron was nitrosylated is evidence that C. botulinum is able to use the iron within a heme group. The effectiveness of sodium nitrite may result from the tying up of heme iron in the nitrosylated hemochrome pigment. Since more than half of the iron in these meat products was in the heme form, this could have a large impact.

The approach taken here was to see if nitrite is responsible for tying up a nutritional requirement of C. botulinum, iron. From the evidence presented above, this is a logical possibility. This approach

is quite contradictory to the explanation by Van Roon (1980), that both heme and non-heme iron are involved in the formation of complexes at the cost of nitrite necessary for growth inhibition. If residual nitrite were necessary for botulinal inhibition, growth should have accelerated greatly in treatment #6 after nitrite was depleted. Inhibition must have been due to something else since this was not the case.

LITERATURE CITED

AOAC. 1980. "Official Methods of Analysis," 13th ed. Association of Official Analytical Chemists, Washington, DC, p. 381.

Christiansen, L.N. 1980. Factors influencing botulinal inhibition by nitrite. Food Tech. 34:237.

Christiansen, L.N., R.W. Johnston, D.A. Kautter, J.W. Howard, and W.J. Aunan. 1973. Effect of nitrite and nitrate on toxin production by Clostridium botulinum and on nitrosamine formation in perishable canned comminuted cured meat. Appl. Microbiol. 25:357.

Christiansen, L.N., R.B. Tompkin and A.B. Shaparis. 1978. Fate of Clostridium botulinum in perishable canned cured meat at abuse temperature. J. Food Protection 41:354.

Christiansen, L.N., R.B. Tompkin, A.B. Shaparis, T.V. Kueper, R.W. Johnston, D.A. Kautter, and O.J. Kolari. 1974. Effect of sodium nitrite on toxin production by Clostridium botulinum in bacon. Appl. Microbiol. 27:733.

Farmer, B.R., A.W. Mahoney, D.G. Hendricks and T.A. Gillett. 1977. Iron bioavailability of hand-deboned and mechanically deboned beef. J. Food Sci. 42:1630.

Fazio, T., R.H. White, L.R. Dusold, and J.W. Howard. 1973. Nitrosopyrrolidine in cooked bacon. J. Assoc. Off. An. Chem. 56:919.

Fox, J.B. and J.S. Thomson. 1963. Formation of bovine nitrosylmyoglobin. I. pH 4.5-6.5. Biochemistry. 2:465.

Goutefongea, R. 1980. Effect of the level of residual nitrite and packaging conditions on color stability in cooked ham. Proceedings of the 26th European meeting of meat research workers. Vol 2. Published by American Meat Science Assoc. p. 219.

Greenberg, R.A., B.O. Bladel and W.J. Zingelmann. 1966a. Use of the anaerobic pouch in isolating C. botulinum spores from fresh meats. Appl. Microbiol. 14:223.

Greenberg, R.A., R.B. Tompkin, B.O. Bladel, R.S. Kittaka, and A. Anellis. 1966b. Incidence of mesophilic Clostridium spores in raw pork, beef, and chicken in processing plants in the U.S. and Canada. Appl. Microbiol. 14:789.

Holland, G.E. 1979. Projection of trends in the food industry. Presented at the Nov. 15, 1979, meeting of the Canadian Institute of Food Science and Technology. In: Q.C.-R.&D. 6:1-18. Research Bulletin of the Meat Packers Council of Canada, Islington, Ont.

Hornsey, H.C. 1956. The colour of cooked cured pork. J. Sci. Food Agric. 7:534.

Hustad, G.O., J.G. Cerveny, H. Trenk, R.H. Deibel, D.A. Kautter, T. Fazio, R.W. Johnston and O.J. Kolari. 1973. Effect of sodium nitrite and sodium nitrate on botulinal toxin production and nitrosamine formation in weiners. Appl. Microbiol. 26:22.

Igene, J.O., J.A. King, A.M. Pearson and J. Ian Gray. 1979. Influence of heme pigments, nitrite, and non-heme iron on development of warmed-over flavor (WOF) in cooked meat. J. Agric. Food chem. 27:838.

Johnston, M.A., H. Pivnick and J.M. Samson. 1969. Inhibition of Clostridium botulinum by sodium nitrite in a bacteriological medium and in meat. Can. Inst. Fd. Technol. J. 2:52.

Kautter, D.A. and R.K. Lynt. 1978. Clostridium botulinum. In "Food and Drug Administration. Bacteriological Analytical Manual." 5th ed. Association of Official Analytical Chemists. Washington, DC.

Kramlich, W.E., A.M. Pearson, and F.W. Tauber. 1973. Meat pigments in cured meat products. In: Processed Meats, AVI Publishing Co., Inc. Westport, Conn. p. 287.

Leininger, H.V. 1976. Equipment, media, reagents, routine tests, and stains. In "Compendium of Methods for the Microbiological Examination of Foods," M.L. Speck (ed.). Am. Public Health Assoc. Washington, DC. p. 10.

Lovenberg, W. 1974. Ferredoxin and rubredoxin. In: Microbial Iron Metabolism. J.B. Neilands (ed.) Academic Press, New York and London. p. 161.

Mahoney, A.W., D.G. Hendricks, T.A. Gillett, D.R. Buck and C.G. Miller 1979. Effect of sodium nitrite on the bioavailability of meat iron for the anemic rat. J. Nutrition. 109:2182.

Moran, D.M., S.R. Tannenbaum and M.C. Archer. 1975. Inhibitor of Clostridium perfringens formed by heating sodium nitrite in a chemically defined medium. Appl. Microbiol. 30:838.

Mortenson, L.E. 1964. Purification and analysis of ferredoxin from Clostridium pasteurianum. Biochim. Biophys. Acta. 81:71.

Mortenson, L.E., R.C. Valentine and J.E. Carnahan. 1962. An electron transport factor from Clostridium pasteurianum. Biochem. Biophys. Res. Commun. 7:448.

Mortenson, L.E., R.C. Valentine and J.E. Carnahan. 1963. Ferredoxin in the phosphoroclastic reaction of pyruvic acid and its relation to nitrogen fixation in Clostridium pasteurianum. J. Biol. Chem. 238:794.

Newberne, P.M. 1979. Nitrite promotes lymphoma incidence in rats. Science. 204:1078.

Ostle, B. and R.W. Mensing. 1975. Statistics in research. 3rd ed. Iowa State University Press. p. 289.

Pearson, A.M., S.D. Sleight, D.P. Cornforth and B.T. Akoso. 1980. Effects of nitrosamines, nitrite and secondary amines on tumor development in mice. In: Proceedings of the 26th European meeting of meat research workers. Vol. 2. Published by American Meat Science Assoc. p. 216.

Perigo, J.A. and T.A. Roberts. 1968. Inhibition of Clostridia by nitrite. J. Fd. Technol. 3:91.

Perigo, J.A., E. Whiting and T.E. Boshford. 1967. Observations on the inhibition of vegetative cells of Clostridium sporogenes by nitrite which has been autoclaved in a laboratory medium, discussed in the context of sub-lethally processed cured meats. J. Food Tech. 2:377.

Probst, I., J.J.G. Moura, I. Moura, M. Brusch, and J. LeGall. 1978. Isolation and characterization of a rubredoxin and an (8 Fe-8 S) ferredoxin from Desulfuromonas acetoxidans. Biochim Biophys Acta 502:38.

Riha, W.E. and M. Solberg. 1975. Clostridium perfringens inhibition by sodium nitrite as a function of pH, inoculum size and heat. J. Food Science. 40:439.

Roberts, T.A. and M. Ingram. 1973. Inhibition of growth of C. botulinum at different pH values by sodium chloride and sodium nitrite. J. Food Technol. 8:467.

Schmahl, D. and H. Osswald. 1967. Carcinogenesis in different animal species by diethylnitrosamine. Experentia 23:497.

Schmidt, C.F. and W.K. Nank. 1960. Radiation sterilization of food. I. Procedures for the evaluation of the radiation resistance of spores of C. botulinum in food products. Food Res. 25:321.

Sebranek, J.G. and R.G. Cassens. 1973. Nitrosamines: A review. J. Milk Food Technol. 36:76.

Sofos, J.N., F.F. Busta and C.E. Allen. 1979. Botulism control by nitrite and sorbate in cured meats: A review. J. Food Protect. 42:739.

Tompkin, R.B., L.N. Christiansen and A.B. Shaparis. 1977. Variation in inhibition of C. Botulinum by nitrite in perishable canned comminuted cured meat. J. Food Sci. 42:1046.

Tompkin, R.B., L.N. Christiansen and A.B. Shaparis. 1978a. Enhancing nitrite inhibition of Clostridium botulinum with isoascorbate in perishable canned cured meat. Appl. Environ. Microbiol. 35:59.

Tompkin, R.B., L.N. Christiansen and A.B. Shaparis. 1978b. Causes of variation in botulinal inhibition in perishable canned cured meat. Appl. Environ. Microbiol. 35:886.

Tompkin, R.B., L.N. Christiansen and A.B. Shaparis. 1978c. The effect of iron on botulinal inhibition in perishable canned cured meat. J. Fd. Technology. 13:521.

Tompkin, R.B., L.N. Christiansen and A.B. Shaparis. 1978d. Antibotulinal role of isoascorbate in cured meat. J. Food Sci. 43:1368.

Tompkin, R.B., L.N. Christiansen, and A.B. Shaparis. 1978e. Effect of prior refrigeration on botulinal outgrowth in perishable canned cured meat when temperature abused. Appl. Environ. Microbiol. 35:863.

Tompkin, R.B., L.N. Christiansen and A.B. Shaparis. 1979. Iron and the antibotulinal efficacy of nitrite. Appl. Environ. Microbiol. 37:351.

Tompkin, R.B., L.N. Christiansen and A.B. Shaparis. 1980. Antibotulinal efficacy of sulfur dioxide in meat. Appl. Environ. Microbiol. 39:1096.

Valentine, R.C., L.E. Mortenson, H.F. Mower, R.L. Jackson, and R.S. Wolfe. 1963. Ferredoxin requirement for reduction of hydroxylamine by Clostridium pasteurianum. J. Biol. Chem. 238: PC856.

VanRoon, P.S. 1980. Clostridial growth inhibitors, derived from nitrite. Proc. of the 26th European meeting of meat research workers. Vol 2. Published by American Meat Science Assoc. p. 227.

White, J.W. 1976. Relative significance of dietary sources of nitrate and nitrite. J. Agric. Food Chem. 24:202.

Woodburn, M.J., E. Somers, J. Rodriguez and E.J. Schantz. 1979. Heat inactivation rates of botulinum toxins A, B, E, and F in some foods and buffers. J. Food Sci. 44:1658.

APPENDICES

Appendix A

Bacteriological Media and Procedures

Chopped Liver Media

Ground beef liver.....500g

Soluble Starch.....1.0g

Peptone.....10.0g

Dipotassium phosphate.....1.0g

Distilled water.....1.0l

Add finely ground beef liver to the distilled water and boil for 1 hr. Adjust broth to pH 7.0 and boil another 10 minutes. Press through cheese cloth and make broth to 1 liter with distilled water. Add peptone and dipotassium phosphate and adjust to pH 7.0. Place liver particles from the pressed cake in the bottom of culture tubes (about 1 cm deep), cover with 8-10 ml broth. Sterilize for 20 min. at 121C. Before use, exhaust for 20 minutes in flowing steam.

Peptone Water Diluent

Mix 1 g peptone in 1 liter distilled water. Adjust to pH 6.8. Prepare dilution blanks with this solution, dispensing a sufficient quantity to allow for loss during autoclaving.

Anaerobic Egg Agar

Fresh eggs.....3

Yeast estract.....5.0g

Tryptone.....5.0g

Proteose peptone.....20.0g

Sodium chloride.....5.0g

Agar.....20.0g

Distilled water.....1l

Wash eggs with a stiff brush and drain. Soak in 70% alcohol 10 to 15 min.; remove and allow eggs to air dry. Crack eggs aseptically; separate and discard the whites. Add the yolks to an equal volume of sterile saline (0.9%) and mix thoroughly.

Combine the remainder of the ingredients, dissolve, adjust to pH 7.0, dispense, and sterilize at 121C for 15 min. Let the agar mixture cool to 45 to 50C, add 80 ml of the egg yolk emulsion, mix thoroughly, and pour plates immediately.

Gram Stain

Reagents:

Crystal Violet: Mix 0.8 g of ammonium oxalate with 80 ml water. Mix together with 2 g crystal violet dissolved in 20 ml of 95% ethyl alcohol.

Iodine: Mix together 1g iodine, 2 g KI, and 300 ml water.

Alcohol: Mix together 80 ml 95% ethyl alcohol and 20 ml acetone.

Safranin: Dissolve 2.5 g of Safranin O in 100 ml of 95% ethyl alcohol. Mix 10 ml of first solution with 100 ml water.

Procedure:

1. Cover the smear with crystal violet for 1/2 to 1 min.
2. Rinse with water and shake any excess water off.
3. Cover with grams iodine for 1 min.
4. Rinse with water.
5. Blot excess water with a blotter but not to dryness.
6. Hold the slide over a sink and let acetone-alcohol flow evenly over the smear for about 10 s. Do not overdo this step.
7. Rinse and blot almost dry.
8. Counterstain with safranin for about 1/2 min.
9. Rinse and blot dry.

Plate Count Agar

Tryptone (Pancreatic digest of casein USP) or Trypticase.....5.0g

Yeast extract.....2.5g

Glucose.....1.0g

Agar.....15.0g

Distilled water.....1.0l

Dissolve ingredients in distilled water by boiling, and adjust to pH 7.1. Dispense into tubes or flasks and autoclave 15 min. at 121C. Final pH should be 7.0.

Gel Phosphate Buffer

Gelatin.....2.0g

Disodium phosphate.....4.0g

Distilled water.....1.0l

Dissolve gelatin and phosphate in distilled water with gentle heat. Sterilize at 121C for 20 minutes. Final pH should be 6.2.

Reference: Leininger, 1976.

Sporulation Media

Tryptone.....50g

Peptone.....5g

Distilled water.....1l

Sodium thioglycollate, 1g in 10 ml water.

Mix first 3 ingredients together. Adjust to pH 7.0. Autoclave 15 min. at 121C. Just prior to inoculation, each bottle of medium (100 ml) receives 1.0 ml of 10% sodium thioglycollate. Sterilize again 5 min. at 121C.

Reference: Schmidt and Nank, 1960.

Appendix B

Nitrite Analysis

Reagents and Apparatus:

1. NED reagent. Dissolve 0.2g N-(1-naphthyl)-ethylenediamine 2HCl in 150 ml 15% (v/v) acetic acid. Filter, if necessary, and store in brown glass bottle.

2. Sulfanilamide reagent. Dissolve 0.5 g sulfanilamide in 150 ml 15% (v/v) acetic acid. Filter, if necessary, and store in brown glass bottle.

3. Nitrite Standard.

a. Stock solution; 1000 ppm sodium nitrite. Dissolve 1.0 g sodium nitrite in water and dilute to 1 liter.

b. Intermediate solution; 100 ppm sodium nitrite. Dilute 100 ml stock solution to 1 liter with water.

c. Working solution; 1 ppm sodium nitrite. Dilute 10 ml intermediate solution to 1 liter with water.

4. Test filter paper for nitrite contamination by analyzing 3-4 sheets from box. Filter about 40 ml water through each sheet. Add 4 ml sulfanilamide reagent, mix, let stand 5 min, add 4 ml NED reagent, mix, and wait 15 min. If any sheets are positive, discard entire box.

Procedure:

1. Weigh 5 g finely comminuted and thoroughly mixed sample into 50 ml beaker.

2. Add about 40 ml of 80C water. Mix thoroughly with glass rod, breaking up all lumps, and transfer to 500 ml volumetric flasks.

3. Wash beaker and rod with successive portions of the hot water, adding all washings to the flask.

4. Add enough hot water to bring volume to about 300 ml, transfer flask to steam bath, and let stand 2 hr., shaking occasionally.

5. Cool to room temperature, dilute to volume with water, and remix.

6. Filter, add 2.5 ml sulfanilamide reagent to aliquot containing 5-50 μg sodium nitrite in 50 ml volumetric flask and mix.

7. After 5 min., add 2.5 ml NED reagent, mix, dilute to volume, mix, and let color develop 15 min.

8. Transfer portion of solution to photometer cell and read A_{540} against blank of 45 ml water, 2.5 ml sulfanilamide reagent, and 2.5 ml NED reagent.

9. Prepare standard curve by adding 10, 20, 30, and 40 ml of working sodium nitrite solution to 50 ml volumetric flasks, add 2.5 ml sulfanilamide reagent, mix, and proceed as above, beginning with step 7.Standard curve is a straight line to 1 ppm sodium nitrite in final solution.

Reference: AOAC, 1980, Method 24.041-24.042.

Appendix C

Iron Analyses

Total Iron

1. Weigh 3-5 g sample into tared crucibles.
2. Ash in muffle furnace at 550C for 48 hrs.
3. Solubilize ash in 5 ml of 6N HCl.
4. Dilute ash solution to 25 ml.
5. Analyze for iron content by atomic absorption spectrophotometry at 2483 Angstroms.

Soluble Iron

1. Weigh 5 g sample into a 50 ml polypropylene centrifuge tube.
2. Macerate with a glass rod and add 20 ml demineralized water. Shake vigorously and let stand 1 hr. at room temperature.
3. Centrifuge at 5C for 15 min. at 3,000G.
4. Remove fat from top of sample.
5. Analyze for iron content by atomic absorption spectrophotometry at 2483 Angstroms.

Reference: Farmer et al., 1977.

Appendix D

Meat Pigment

Reagents:

Acetone a: Place 90 ml distilled water in a 1 liter volumetric flask; add acetone, mix and bring to volume.

Acetone b: Mix water with 20 ml concentrated HCl and bring to 100 ml volume. Transfer the diluted HCl to a 1 liter volumetric flask, add acetone, mix and bring to volume with additional acetone.

Procedure:

Do the following in subdued light to lessen fading of pigment:

1. Weigh out 2.0 g sample in 50 ml polypropylene centrifuge tube.
2. Pipet 9.0 ml acetone a into centrifuge tube.
3. Macerate meat mass for 2-3 min. with a glass rod.
4. Stopper centrifuge tube and mix by gentle swirling.
5. Let stand 10 min., then filter through two Whatman #42 filter papers into a test tube.
6. Transfer filtrate into 1 cm cuvette and read Absorbance within 1 hr at 540 mu and calculate as nitroso pigment.
7. Prepare another 2.0 g sample, using acetone b.
8. Macerate and allow to stand 1 hr before filtering.
9. Filter the extract into another test tube and read Absorbance at 640 mu. Calculate as total pigment.

Calculations: The calculations were made using extinction coefficients from Hornsey (1956).

ppm nitroso pigment = A_{540} X 290

ppm total pigment = A_{640} X 680

If volume or sample sizes were varied:

ppm nitroso pigment = 57.7 X A_{540} X Total vol. (ml)/sample (g)

ppm total pigment = 135.8 X A_{640} X Total vol. (ml)/sample (g)

References: Hornsey, 1956; Kramlich et al., 1973.