5-1982

The Relative Roles of Initial and Residual Sodium Nitrite on Germination of *Clostridium botulinum* Spores in Meat

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THE RELATIVE ROLES OF INITIAL AND RESIDUAL SODIUM NITRITE ON GERMINATION OF CLOSTRIDIUM BOTULINUM SPORES IN MEAT

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Nutrition and Food Sciences

Approved:

UTAH STATE UNIVERSITY
Logan, Utah

1982

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation and indebtedness to Dr. Daren P. Cornforth, my Major Professor, for his understanding, advise, help and guidance in writing this thesis.

A very special thanks to Dr. Arthur W. Mahoney, Dr. Darrell T. Bartholomew, and Dr. Donald V. Sisson for their valuable advise and direction, and also their encouragement and patience throughout this study.

I would also like to thank Dr. David L. Turner and Mrs. Maria Norton for their help in computer and statistical analyses.

I wish to thank all my friends for their suggestions, help and friendship.

Finally, I would like to express my greatest thanks to my family for their tremendous support and encouragement in pursuing my education, and to my friend, Bulsakdi Dhimasombat, for his moral support, understanding and patience in fulfilling this study.

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by

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Utah State University, 1982

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Four levels of $NaNO_2$ (0 ppm, 50 ppm, 100 ppm, and 156 ppm) were tested for antibotulinal activity in ground pork inoculated with spores of Clostridium botulinum either at the time of formulation or after cooking. Samples formulated with less than 156 ppm sodium nitrite received additional nitrite to adjust the residual nitrite equal to that found after cooking in samples formulated with 156 ppm nitrite. All samples were subjected to abusive storage at 27 C. Inoculating the spores at the time of formulation resulted in a faster rate of swelling. Heating C. botulinum spores for 30 min. at 77 C before addition to cooked meat also resulted in rapid germination. Total botulinal counts were significantly higher($p=0.05$) in these samples, compared with similarly treated samples formulated with unheated spores.

Samples formulated with 50 ppm initial sodium nitrite and with 78 ppm additional $NaNO_2$ after cooking were the most inhibitory. Initial nitrite concentration was shown to be important for inhibition of C. botulinum growth, probably because of the inhibitory carryover effects of protein-bound nitrite formed during cooking, as well as influencing the concentration of residual nitrite.

(116 pages)

INTRODUCTION

For thousands of years, people have been eating meat cured with salts containing nitrate and nitrite. Nitrite was present originally as a natural impurity in the salts used in curing meat and was a key ingredient in determining the effectiveness of the process (CAST, 1978). Scientists have shown that nitrate is changed to nitrite in meat, and that nitrite reacts with the meat to produce the desired preservative and safety effects as well as the characteristic "cured" flavor and color (CAST, 1978). Today almost all cured meats are produced by addition of sodium nitrite, rather than sodium nitrate.

Nitrite, in the form of its sodium salt, is a unique ingredient of meat curing mixtures because it serves a variety of functions related to chemical and microbial properties that influence the botulinal safety, quality, and identity of cured meat products (Sofos and Busta, 1980). The roles of nitrite in the curing process as described by Kramlich et al. (1973) are: a) to form the characteristic cured meat color and flavor, b) to possess antioxidant properties that delay the onset of rancidity, c) to serve as an antimicrobial agent for preventing growth of yeasts and molds, and d) to prevent Clostridium botulinum growth and toxin production, which involves the main topic of this research.

Since the discovery by Lijinsky and Epstein (1970) that nitrosamines are formed by interaction of secondary amines with sodium nitrite in cooked cured meat, especially in crisp-fried bacon, and that these nitrosamines cause cancers in laboratory animals, extensive efforts were undertaken to reevaluate the functions of nitrite, especially its antibotulinal activity, and to identify possible alternatives. Several hundred compounds have been tested but no single alternative, possessing all the useful functions of nitrite, has been identified (Brown, 1973; CAST, 1978). A combination of five substances was identified (Sweet, 1975) as a potential alternative, but problems were associated with its application. The fact that the ten-year old nitrite controversy remained active, and that only minor changes have been introduced in the Federal regulations, verifies the importances of nitrite in the production of cured meat products (Sofos and Busta, 1980).

Any substances to be considered as an alternative to nitrite in cured meat products should also control other microorganisms of public health significance or delay product spoilage. Those substances should not interfere with beneficial microorganisms such as lactic acid producing cultures, necessary for the manufacture of fermented meat products (Sofos and Busta, 1980). The most attractive way to find a suitable substitute for nitrite would be to

determine the mechanism through which nitrite exerts its activity and then examine compounds which act similarly in meat curing.

Until now, no single compound has been successfully used as a nitrite substitute. Effort has been directed to determination of the lowest level of nitrite that could be used in cured meat products and still produce all the essential functions. Of all the functions of nitrite as mentioned above, the antibotulinal effect of nitrite is the most important. Many scientists have tried to decrease the level of nitrite to a minimum at which the antibotulinal effect was still obtained (Tompkin et al.,1977), but final conclusions have not been reached. The fact is that the exact mechanism of botulinal inhibition by nitrite is not fully understood (Sofos et al.,1979). Thus, further studies on this subject are needed.

REVIEW OF LITERATURE

Factors Affecting Botulinal Safety

The microbiological safety of cured meats has been reported to be the result of a variety of factors (Table 1). The interaction of these factors result in the prevention of Clostridium botulinum growth and toxin production (Roberts, 1975; Lechowich et al.,1978; Sofos et al.,1979).

Table 1: Factors affecting botulinal safety of cured meat products (Sofos and Busta, 1980).

C. botulinum spore incidence	Heat processing (injury)
Nitrite, nitrate	Dehydration (Aw)
Salt (NaCl)	Storage temperature
pH (acid)	Time of consumption-abuse
Product composition	Microbial contamination
Other ingredients	Sanitation-packaging

C. botulinum spore incidence

The relatively low incidence of C. botulinum spores in raw meat and other cured meat product constituents is one major factor contributing to botulinal safety. Only one spore is estimated to be in 1-7 pounds of meat (Lechowich et al.,1978). In spite of the low spore incidence, the potential for botulism outbreaks exist. As indicated by Lechowich et al.(1978) and Sofos et al. (1979), there are

several important factors to be considered when evaluating spore incidence. These factors are: the limitations of the methodology used to estimate spore incidence, the non-uniformity of meat, the potential for abuse before, during and after processing, the tonnage of products manufactured and consumed, the limited number of existing surveys to determine raw ingredient contamination, and the possibility that the spores occupy microenvironments in the form of colonies or higher concentrations (Lechowich et al., 1978; Sofos et al.,1979).

Nitrite, nitrate

Nitrite (as interpreted in the meat industry refers to sodium nitrite, and this term will be used through out this thesis) is one of the most important ingredients that assure the botulinal safety of meat products. Nitrite concentrations higher than those necessary for color and flavor development are needed for reasonable botulinal inhibition (Sofos and Busta, 1980). Nitrate is considered as generally non-essential to the botulinal stability of the products and probably only serves as a source of nitrite (Christiansen et al.,1973; 1974; Hustad et al.,1973). The USDA Expert Panel on Nitrites, Nitrates and Nitrosamines has recommended elimination of nitrate from all cured meat products except fermented sausage and dry-cured products (Sofos et al.,1979).

The relative significance of initial and residual

nitrite concentrations in controlling botulinal growth and toxin production is not completely understood. Initial nitrite has been reported to be an important factor in determining botulinal inhibition (Greenberg , 1972; Christiansen et al., 1973; Bowen and Deibel, 1974; and Chang and Akhtar ,1974). On the other hand, residual nitrite is related to long-term control of C. botulinum. According to van Roon and Olsman (1977), the amount of added nitrite is important but probably during storage the reacted (protein-bound) and residual nitrite will contribute to the stability of the product. However, in the complex food system, other factors such as salt concentration, pH, spore concentration, water availability, and heat treatment might interact to influence the safety of the finished product (Pivnick et al. 1969; Christiansen et al.,1974).

Salt (NaCl)

Salt (NaCl) has been shown to be another important ingredient in cured meat. The combination effect of salt with other factors such as nitrite, pH, heat treatment, meat type and spore level contributes to the increasingly inhibitory effect on C. botulinum (Sofos et al.,1979). Duncan and Foster (1968a) found that sodium chloride at concentrations above 6% completely prevented germination. In the presence of 3 to 6% sodium chloride, most of the spores germinated and produced vegetative cells, but cell division was often blocked (Duncan and Foster, 1968a). When

sodium nitrite was present at a concentration of 75 ppm, the inhibitory concentration of sodium chloride was 5.8%, and with 150 ppm sodium nitrite the inhibitory concentration was 4.9% (Pivnick, et al.,1969). Sodium chloride is commonly added to cured meat at the level of 2 to 3% of the weight of the product (CAST, 1978).

pH (acid)

The average pH of most cured meat products falls within the range of 5.6 to 6.6 (Lechowich et al.,1978). This range of pH is well above the lower pH limit of 4.0 to 5.0 for growth of spores of C. botulinum types A and B (Ingram and Robinson, 1951). Roberts and Ingram (1966) reported that the antimicrobial activity of nitrite increase roughly ten-fold when the pH is changed from 7.0 to 6.0. The low pH in the fermented meat products or in certain products where acid may be added, is an influential factor in controlling botulinal growth (Christiansen et al.,1975). Nordin et al.(1975) also reported that botulinal spore outgrowth increased with pH and decreased with salt and nitrite concentration.

Product composition

The composition of the basic product is another function contributing to the safety of cured meats. It has been shown that addition of hemoglobin to the formulation reduces residual nitrite after processing and consequently

decreases botulinal inhibition (Tompkin et al.,1978a). Different meats yielded different rates of botulinal outgrowth when substituted for fresh pork ham. Tompkin et al.(1978a) also found that pork or beef heart meat had no inhibition of the C. botulinum inoculum (100 spores/g meat) even with a 156 ug/g amount of sodium nitrite added to the product.

Other ingredients

Other product ingredients such as ascorbate or iso-ascorbate, smoke components, polyphosphates, sugars and syrups, antioxidants, chelators and sequestering agents, seasonings and spices, and extenders or binders are found in one or more types of meat products, and may also be influential on the safety of the products (Sofos and Busta, 1980). Ascorbate or its stereoisomer isoascorbate (erythorbate) are used in cured meat products, especially in bacon, mainly as accelerators of the curing reaction (Sofos and Busta, 1980). They also contribute to the development of a more stable cured color and act as synergists to antioxidants. Ascorbate is also reported to decrease the rate of nitrosamine formation, either by increasing the rate of residual nitrite depletion (Bowen and Deibel, 1974; Woolford and Cassens, 1977) or by directly blocking the nitrosation of amines by nitrite (Mirvish et al.,1972, cited in Sofos and Busta, 1980).

Heat processing

The amount of heat to which a spore is subjected is very important. Thermal processing of the product was reported by Silliker et al.(1958), Duncan and Foster (1968a; 1968b; 1968c) and Pivnick et al.(1969) to influence the botulinal spore outgrowth since it may either inactivate some C. botulinum spores or cells, or it may damage the spores and consequently increase their sensitivity to curing ingredients. At relatively low temperatures (158 to 176 F) activation occurs. At somewhat higher temperatures the spore is injured in some unknown manner but still viable. The spore is rendered nonviable if more heat is still applied (CAST, 1978). This low level temperature (158 to 176 F) is essentially a pasteurizing process to injure the spores and kill the vegetative cells of C. botulinum and cause them to be more susceptible to inhibition by the curing agents.

Dehydration (Aw)

Dehydration during processing of various types of meat products can also contribute to inhibition of growth of C. botulinum. A water activity (Aw) of 0.975 (5% NaCl) inhibits growth of type E strains, while types A and B can grow in up to an Aw value of 0.935 (10% NaCl or 50% sugar concentrations)(Murrell, 1974; Tompkin and Christiansen, 1976). The destructive effect of heat on spores was also reported to be related to water activity of the medium by

Murrell and Scott (1966) and Tompkin and Christiansen (1976).

Storage temperature

Storage of products at refrigeration temperatures is a good control against botulinal growth and greatly contributes to the safety of the products. Growth of C. botulinum type A and B spores cannot occur at temperatures below 50 F (Ohye and Scott ,1953, cited in CAST, 1978). Thus continuous refrigeration at 50 F or lower provides adequate protection from this organism.

Time of consumption - abuse

The time of consumption of the products, which is frequently soon after production is another important factor. Depletion of nitrite with time has been reported by many scientists (Fox and Nicholas, 1974; Nordin, 1969). Other factors such as pH, product formulation, and temperature during processing and during storage were also reported by the same scientists to have an effect on the rate of nitrite depletion. Tompkin et al. (1978b) demonstrated that products abused after refrigeration were less inhibitory to botulinal growth when compared to product abused immediately after production, presumably due to nitrite depletion during storage. Thus, the longer the time until consumption, the probability for abuse and resultant microbial growth is higher. Furthermore, other oxidation-

reduction reactions which are essential for anaerobic bacteria (Yoch and Valentine, 1972) might occur during storage and thus favor botulinal growth.

Microbial contamination

Other microorganisms that may be present in the product may directly or indirectly affect C. botulinum growth and toxin production (Riemann et al.,1972; Christiansen et al., 1975). The inhibitory effects of Enterococci, which may be independent of pH, on botulinal growth have been reported by Riemann (1973) and Tabatabai and Walker (1974). Botulinal toxin may be inactivated by proteolytic bacterial species and boticins, substances produced by some type E strains, which may be inhibitory to toxin-producing strains (Kautter et al., 1966).

Sanitation-packaging

High sanitation levels and effective packaging can minimize product contamination and further aid in the safety of meat products (Sofos and Busta, 1980). However, there is no way to guarantee that the product does not undergo temperature abuse sometime prior to consumption.

Regulatory Changes Affecting Nitrite/Nitrate Usage

In the United States, the Department of Agriculture, on a recommendation by an Expert Panel on Nitrites, Nitrates and N-Nitrosamines, published proposed regulatory changes for

nitrite and nitrate usage to reduce residual nitrite in cured meat products, thereby reducing potential for formation of nitrosamines during high temperature cooking (40 Fed. Reg. 5264, Nov. 1975, cited in Gray and Randall, 1979). These included: "a) discontinuing use of nitrate in processed meat and poultry with the exception of dry-cured and fermented sausage products and country ham; b) limiting the amount of ingoing nitrite in bacon to 125 mg/kg in the presence of 550 mg of sodium erythorbate or ascorbate/kg; c) restricting the ingoing level of nitrite to 156 mg/kg in cooked cured sausages; d) limiting the ingoing level of nitrite in processed products; and e) reducing the residual levels of nitrite in the finished product from 200 mg/kg to (i) 50 mg/kg in canned, cured, sterile products such as corn beef hash and deviled ham, (ii) 100 mg/kg in cooked sausage products such as frankfurters, bologna and luncheon meats, and (iii) 125 mg/kg in pickled cured products such as hams, picnics, shoulders, canned cured shelf-stable products such as canned, chopped meat and ham salad spreads, and canned perishable products such as canned ham." The industry began voluntarily to adopt these 1975 recommendations even though the latter have not yet been adopted as regulations (CAST, 1972). In 1978, the USDA issued a regulation to reduce ingoing sodium nitrite levels in bacon to 120 ppm in the presence of 550 ppm of sodium ascorbate or sodium

isoascorbate and also set a maximum of nitrosamine formation as 10 ppb (Butler, 1980).

Mechanism of Botulinal Inhibition by Nitrite

Germination and outgrowth of anaerobic spores include five sequential steps:(i) germination of the spore (becoming nonrefractile, stainable, and heat sensitive); (ii) swelling of the germinated spore; (iii) emergence of the new vegetative cell; (iv) elongation; and (v) cell division (Duncan and Foster, 1968a). The neurotoxins of C. botulinum are synthesized within bacteria cell during growth (Bonventre and Kempe, 1960) and are released during lysis.

Gould (1964) described germination as follows: "The first stage in the growth of spores into vegetative cells (germination) is characterized by an increase in heat sensitivity; loss of dry weight including calcium, dipicolinic acid and mucopeptide (Powell and Strange, 1953); fall in optical density of suspensions; increase in stainability (Powell, 1950); swelling (Hitchins et al. 1963); and darkening of individual cells under phase contrast illumination (Pulvertaft and Haynes, 1951). Subsequent development, often termed outgrowth (Campbell, 1957), involves further swelling of the now metabolically active forms followed by rupture or lysis of the spore wall. The manner in which the spore wall opens is usually characteristic of the strain (Lamanna, 1940) and is not

radically affected by changes in the environment (Gould, 1962). The emergent cells elongate, discarding what remains of the spore walls, and finally become new vegetative organisms which will divide and multiply." Several possible mechanisms have been proposed through the years for the inhibitory effect of nitrite on C. botulinum , but final conclusions have not been reached.

Johnston et al.(1969) suggested four possible roles of nitrite which were later repeated and reviewed by Ingram (1974; 1976). According to Johnston et al.(1969) the roles of nitrite might be: (a) to enhance destruction of spores by heat; (b) to increase spore germination during thermal processing with subsequent destruction of the germinated spores by heat; (c) to prevent germination or outgrowth of the spores and (d) to react with some type of meat component(s) to form a more inhibitory compound(s).

The possibility of enhancing the destruction of spores by heat was first reported by Jensen and Hess in 1941. Murrell (1964, cited in Duncan, 1970) stated that heat treatments which did not result in complete inactivation of bacterial spores may damage them enough to prevent growth and multiplication under the storage condition that prevail after heating. Roberts and Ingram (1966) indicated that aerobic and anaerobic spores given increasingly severe heat treatments became progressively more sensitive to the effect of curing agents (sodium chloride, sodium nitrite and sodium

nitrate) in the outgrowth medium. The effect of nitrate and salt were roughly equal. Since the nitrate concentration presented in cured meat was much lower than salt concentration, its separate effect would be negligible in practice. The inhibitory effect on the outgrowth of heated spores was pronounced even at a nitrite concentration of 50 ppm. Studies by Duncan and Foster (1968b) confirmed the results of Roberts and Ingram (1966) by showing that heated spores of putrefactive anaerobe 3679h were less tolerant to nitrate, nitrite, or salt than were unheated spores in the outgrowth medium when these curing agents were added only to the heating medium (buffer system), but not to the outgrowth medium. Sodium chloride and sodium nitrate tended to protect the spores against heat injury, but sodium nitrite did not. When the spores were both heated and cultured in the presence of these curing ingredients, at low concentration of salt (0.5 to 1%) the protective effect predominated and the sensitizing effect of salt predominated at higher concentrations (2-4%). However, nitrite was markedly inhibitory, especially at pH 6.0 in both conditions. Experiments by Pivnick et al.(1970) eliminated the possibility that nitrite enhanced destruction of heated spores. Pivnick et al.(1970) heated spores of C. bot[illegible], PA 3679 and PA 3679h individually in meat [illegible] phosphate buffer, pH 6.2, with increasing levels of sodium nitrite, followed by incubation at 30 C. There was no

difference in the destruction of the spores heated at 100 C to F_o =0.1 in the buffer system between nitrite levels of 0 ppm vs 200-1600 ppm (F_o is the time in min. required to destroy a given number of organisms of a reference strain at a temperature of 250 F when Z=18 F. Z is the accepted temperature increase or decrease needed to lower or raise the D value by one log. D is the time in min. required to reduce the viable count by one log (or by 90%). For example, an F_o value of 2.5 is equivalent to a 12 D process or the time required to reduce the spore count by 12 logs at temperature of 250 F and Z=18 F). Moreover, heating spores of C. botulinum type A and PA 3679h at 80 C for 23 minutes in the meat system did not cause destruction of more spores with 1600 ppm of nitrite than with 0 ppm of nitrite.

Increasing the rate of germination of spores during the heat process followed by the death of the germinated spores from the heat process, as described by Johnston et al.(1969), was supported by the results of Duncan and Foster (1968a). Duncan and Foster (1968a) studied the effect of meat-curing agents on germination and outgrowth of PA 3679h spores in microcultures, and suggested that nitrite actually stimulated spore germination, especially in acid conditions. Thus, during normal heat processing, nitrite may induce spores to germinate and make them susceptible to thermal inactivation. Spores that survive the heat treatment may germinate, but outgrowth may be blocked by residual nitrite

in the product. Some spores may survive after the heat process (Silliker et al.,1958; Riemann, 1963). Silliker et al.(1958) stated that 20% of the inoculated spores were not killed after heat treatment and they concluded that the failure of those spores to germinate was probably the result of an inhibitory effect of the curing salts dependent on heat damage. The results of the studies of Pivnick et al. (1970) fail to confirm the findings of Duncan and Foster (1968a). They (Pivnick et al.,1970) found that addition of nitrite to the meat system, up to 1600 ppm, did not cause an increased germination of the organisms tested (C. botulinum type A, B, and PA 3679h) during 1 week of incubation at 30 C. Thus, Sofos et al. (1979) and Ingram (1974) both concluded that nitrite did not affect spore germination.

The third possible role of nitrite as suggested by Johnston et al.(1969) was to prevent the germination of spores that survived the heat process. This possibility was plainly demonstrated by Roberts and Ingram (1966) who found that the safety of cured meat products which received a mild heat treatment arose from the ability of curing ingredients to interfere with some stage in the germination and outgrowth of surviving heated spores. These levels of the curing salts would not be inhibitory to germination and growth of unheated spores. Pivnick et al.(1970) also suggested that the main effect of nitrite in the safety of

canned cured meats was its ability to aid in the inhibition of growth of spores that survived the heat process and germinated during the storage period.

Another possible inhibitory effect of nitrite was the formation of a more inhibitory substance(s) from the reaction of nitrite with other components of the system (Johnston et al.,1969; Ingram, 1974). Perigo et al.(1967) found that sufficient heating of nitrite in a laboratory medium produced some unknown inhibitory substance with differed from inorganic nitrite in three important respects. First, it's activity was less pH-dependent than inorganic nitrite. Second, it's response was less variable. Third, it was a very potent inhibitor, formed even with only 3.5 ppm of nitrite heated at 109 C for 20 min. Not long after Perigo's first report on this subject (Perigo et al.,1967), a study was undertaken by Johnston et al.(1969) to examine whether the same results would be obtained in the meat system. The inhibitory substance in meat was found to be dialysable, while the Perigo Inhibitory substance in the laboratory medium was not. Addition of as little as 1% meat to the culture medium interfered with the development of the inhibitor, and 20% or more meat prevented it. Addition of non-fat meat solids to the culture medium with the inhibitor already present neutralized it's activity. Johnston et al. (1969) concluded that the inhibitor produced in the laboratory media was of little or no consequence in

explaining the role of nitrite in the safety of commercially meat products. Ingram (1976) stated that since the observations made in culture media and in meat media have little relevancy, the term Perigo Factor should only be used in culture media, not in meat media.

It has been demonstrated that nitrite can combine with sulfhydryl groups in meat to form nitrosothiols (Mirna and Holfmann, 1969). This reaction may explain the disappearance of nitrite via bonding to proteins in the form of nitrosothiol. Furthermore, nitrosothiols once formed may undergo a redox reaction resulting in a disulfide and the liberation of nitric oxide which might easily take part in other reactions (Mirna & Holfmann, 1969). It should be mentioned that Incze et al.(1974) found S-nitrosocysteine more inhibitory than nitrite in a beef bouillon-based medium. van Roon and Olsman (1977) also found S-nitrosylcysteine and nitrite inhibitory to clostridia in canned cured pasteurized beef and ham products, while a cysteyl-nitric oxide ferrate complex was ineffective. On the other hand, Lee et al.(1978) studied the effect of SH-groups on the inhibitory effect of nitrite and the results indicated that formation of inhibitor in cured meats did not require the presence of reactive SH groups. Furthermore, the inhibitory activity of the cured meats was probably not because of deficiency in SH compounds that the organism needed for growth.

Ingram (1974) postulated a mechanism of nitrite which is microbiologically important: Nitrite reacts via nitrous acid with amino and other groups in proteins, and this nitrous acid was depending directly on the pH. The concentration of HNO_2 thus increased roughly ten-fold for every unit fall of pH (Ingram, 1974). It was agreed by many scientists that nitrite was more inhibitory under acid conditions, and this might be due to the presence of nitrous acid (Ashworth et al.,1973; Roberts and Ingram, 1966; Shank et al.,1962). The pH range for the anti-microbial activity of nitrite was suggested to be from pH 4.5 to pH 5.5 (Shank et al., 1962; Ingram, 1974) because nitrite existed principally as nitrous acid. Below pH 4.5, nitrous acid was rapidly converted to nitrate and nitric oxide, and neither of these possessed antibacterial characteristics. At higher pH values (pH 6 to pH 7), the equilibrium shifted toward $NaNO_2$, the cycle was prevented from functioning, and no bacterial effects were noted (Shank et al.,1962).

Recent studies by Tompkin et al.(1978c) suggested that nitrite reacted via nitric oxide with an iron-containing compound, such as ferredoxin, within the germinated botulinal cell and consequently could interfere with the energy metabolism of the germinated cell to prevent outgrowth. Ferredoxin is an nonheme iron-sulfur protein which is essential in electron transport, enzyme activity, and energy production (Mortenson et al.,1963). Oxidation-

reduction reactions which are essential for anaerobic bacteria, also depend on the presence of ferredoxin (Yoch and Valentine, 1972). However, in the same paper Tompkin et al.(1978a) also suggested that nitrite somehow bound meat iron, reducing the amount of iron available for botulinal growth.

Benedict (1980) also suggested that nitrite may react either directly on the cell or spore to cause destruction or denaturation of cellular enzymes, nucleic acids or membranes that are essential to the organism or indirectly outside the cell through destruction or chelation of external vital nutrients such as iron, hence inhibiting uptake by the cell.

Botulinal Inhibition as Affected by Initial Nitrite Level

It has been found that a major issue in explaining the inhibitory activity of nitrite is the relative importance of the initial nitrite input versus that of the residual nitrite found in the product after processing and during storage (Sofos et al.,1979). The importance of initial nitrite was supported by Christiansen et al.(1973). They found that increasing the level of nitrite added at the time of heat processing (initial nitrite) resulted in an increase in time necessary for *C. botulinum* toxin production. Similar results have been reported by Greenberg (1972), and Bowen and Deibel (1974). Greenberg (1972) studied the

inhibitory effect at different levels of nitrite and nitrate in canned ham which was inoculated with C. botulinum spores (type A and B). He concluded that the initial nitrite level was the key factor to prevent botulinal toxin formation in the product rather than the residual nitrite level. Chang and Akhtar (1974) specifically stated that initial nitrite rather than residual nitrite level was the importance factor in determining botulinal inhibition. Tompkin et al. (1978c) indicated that residual nitrite could be indirectly related to botulinal inhibition by serving as a reservoir for a reactive intermediate such as nitrous acid. The possibility also existed that some inhibitory substance could be formed during processing by reacting with nitrite and could be dissipated during storage simultaneously with residual nitrite. Christiansen (1980) stated that those researchers who supported the importance of the initial nitrite added at the time of processing may imply that a) nitrite reacted initially with botulinal spores to cause inhibition; b) nitrite reacted with some compound in the meat to form an inhibitor during formulation or during the heat process; and c) higher levels of initial nitrite would result in higher residual nitrite levels, which could produce sufficient botulinal inhibition.

Botulinal Inhibition as Affected by Residual Nitrite Level

Residual nitrite was shown to be another important factor contributing to the safety and stability of canned cured meats. Christiansen et al.(1978) followed spore germination, cell survival and residual nitrite depletion during 27 C storage of a perishable canned cured pork product. They found no difference in the rate of spore germination between the initial nitrite levels of 50 ppm and 156 ppm. However, the germinated cells decreased, simultaneously with residual nitrite. Tompkin et al.(1978c) examined the effect of adding hemoglobin on the inhibitory activity of nitrite against C. botulinum outgrowth. Addition of 1% hemoglobin to product formulated with 50 or 156 ppm of nitrite eliminated the inhibitory effect of nitrite. Tompkin et al.(1978a) also observed that products made from meat containing high levels of myoglobin, such as pork heart, swelled faster than products such as ham with lower myoglobin levels. Tompkin et al.(1978c) postulated that the responses obtained were due to two different phenomena: 1) Hemoglobin addition decreased residual nitrite which in turn decreased botulinal inhibition; and 2) A high level of available iron in heart meats also brought about a loss in botulinal inhibition. Tompkin et al.(1978b) studied the effects of prior refrigeration on growth of C. botulinum spores in a perishable canned cured meat product at abuse

temperature (27 C). They observed a relation between the decrease in botulinal inhibition and reduction of residual nitrite in the product.

Botulinal Inhibition as Affected by Heat Treatment and Spore Load

Pasteurized canned cured meats occupy a unique position amongst canned foods since the thermal processes applied to such products would be grossly inadequate to ensure the safety and stability of other low-acid canned foods (Jarvis et al.,1976). A process of F_o =0.05-0.4 min is usually employed in cured meats by most manufacturers. Such a process is 10-100 times lower than that necessary for low acid foods. It is believed that stability results from the low incidence of clostridial spores and the interactions between the various curing agents and the heat process. Increases in the spore inoculum level can negate the inhibitory effect of nitrite and allow toxin production (Christiansen et al.,1973; Pivnick et al.,1970). For example, meat inoculated at a level of 90 spores/g was toxic when cured with 150 ppm sodium nitrite, but not with 200 ppm sodium nitrite. With 5,000 spores/g of meat, toxin was detected with 400 ppm but not with 500 ppm of sodium nitrite at 27 C (Christiansen et al.,1973). Investigations by many workers have shown that salt (NaCl), nitrite, pH value and thermal process all interact to produce a system which is

more inhibitory to spores than is any one factor alone (Roberts & Ingram, 1973).

The production of a thermally induced inhibitor, derived from nitrite, was first demonstrated in a laboratory medium by Perigo et al.(1967), and was subsequently confirmed by other workers. Using modified techniques, Ashworth and Spencer (1972) demonstrated the production of a thermally induced inhibitor in minced pork, containing sodium nitrite, when given a sterilization heat treatment. Pivnick et al.(1969) reported on the interaction of heat treatment and spore inoculum size. In a ground pork system inoculated with C. botulinum type A and B spores, a thermal process of F_o =0.15 min did not prevent toxin production with 10^4 spores/g. An F_o =0.30 min prevented toxinogenesis with 10^4 spores/g but it did not with 10^6 spores/g inoculum size, which was inhibited by a thermal process of F_o =0.60 min. Viable spores were recovered after 18 months even though the meat was unspoiled and non-toxic.

It has been reported that the sensitivity of heat-damaged spores to inhibition by curing salts is higher than is the sensitivity of unheated spores (Roberts et al., 1966; Roberts and Ingram, 1966). Ingram and Roberts (1971) demonstrated that C. botulinum spores which were subjected to a sublethal heat process at 95 C were inhibited more by nitrite heated in the recovery medium for 15 min at 115 C than by unheated nitrite. Ashworth et al.(1973), working

with a pasteurized meat system, reported that spores heated in the meat for up to 4 hr at 80 C were inhibited by a nitrite level similar to that found to inhibit unheated spores inoculated into meat after heating. The above observations were the reasons Jarvis et al.(1976) suggested that, although spores heated to 90 C were sensitized to the inhibitory action of salt and nitrite, heated spores at pasteurization temperatures of 70-80 C may not be sensitized to the inhibitory action of the curing salts. The same researchers (Jarvis et al.,1976) found lower sensitivity of spores to sodium nitrite heated in meat and unheated sodium nitrite added to the meat after cooking, compared with higher sensitivity in culture media. Thus, data obtained in laboratory media may not be applicable in meat systems.

In summary, previous scientists have shown that nitrite has antibotulinal properties, especially with other curing agents in heated meat systems. Nitrite apparently inhibits outgrowth (development and growth of vegetative cells from spores), but does not inhibit germination. Nitrite may inhibit outgrowth by directly inhibiting various bacterial enzymes, such as ferredoxin, indirectly limiting growth by reducing the amount of available iron, or both. The inhibitory effects of both initial and residual nitrite have been demonstrated, but the relative roles of initial versus residual nitrite in botulinal inhibition have not been shown.

OBJECTIVE

The purpose of this research was to determine the relative importance of initial and residual nitrite levels upon swelling and botulinal spore germination rates in heated meat systems to which botulinal spores were either heated (added before cooking) or unheated (added after cooking).

MATERIALS AND METHODS

Experimental Design

Four levels of sodium nitrite (0 ppm, 50 ppm, 100 ppm, and 156 ppm) were tested. One level of botulinal spores plus uninoculated controls were used. At each level of nitrite, the spores of C. botulinum were added to the meat at the time of formulation or after cooking. This resulted in 8 treatments with 46 bags of samples per treatment for confirmation of spore levels and for chemical analysis.

After cooking, additional sodium nitrite was added to some samples formulated with 0 ppm, 50 ppm and 100 ppm of nitrite, so that the residual nitrite levels for all samples were equal to that found in samples formulated with 156 ppm nitrite. The meat was then remixed, put into the bags, sealed and incubated as indicated in the materials and methods.

A sublethal "heat shock" was studied as follows: Spores of C. botulinum were heated at 77 C for 30 min. (Christiansen et al.,1973) and then added to the meat formulated with 50 ppm of nitrite after cooking. For part of the samples, residual nitrite was adjusted to the level found in samples formulated with 156 ppm of nitrite. Part of the samples did not have the residual nitrite adjusted. Samples were inoculated with spores either before or after cooking. This provided controls for studying the

effects of residual nitrite on germination of the spores. All the samples were incubated at 27 C. The treatments were as followed:

1) Sodium nitrite (156 ug/g meat) was added to the basic meat formulation with added dextrose, water, and sodium chloride. Spores of C. botulinum were inoculated before cooking.

2) Sodium nitrite (156 ug/g meat) was added. The spores were inoculated after cooking.

3) Sodium nitrite (100 ug/g meat), inoculum before cooking. Residual nitrite was adjusted to 104 ppm.

4) Sodium nitrite (100 ug/g meat), inoculum after cooking. Residual nitrite was adjusted to 104 ppm.

5) Sodium nitrite (50 ug/g meat), inoculum before cooking. Residual nitrite was adjusted to 104 ppm.

6) Sodium nitrite (50 ug/g meat), inoculum after cooking. Residual nitrite was adjusted to 104 ppm.

7) No initial sodium nitrite added, inoculum before cooking. Residual nitrite was adjusted to 104 ppm.

8) No initial sodium nitrite added, inoculum after cooking. Residual nitrite was adjusted to 104 ppm.

9) Sodium nitrite (156 ug/g meat), no inoculum (a control).

10) Sodium nitrite (50 ug/g meat), inoculum before cooking. Residual nitrite was not adjusted.

11) Sodium nitrite (50 ug/g meat), inoculum after cooking. Residual nitrite was not adjusted.

12) Sodium nitrite (50 ug/g meat), spores were heated before inoculating into the meat. Residual nitrite was not adjusted.

13) Sodium nitrite (50 ug/g meat), spores were heated before inoculating into the meat. Residual nitrite was adjusted to 104 ppm.

The formulations were summarized in Figure 1 and 2.

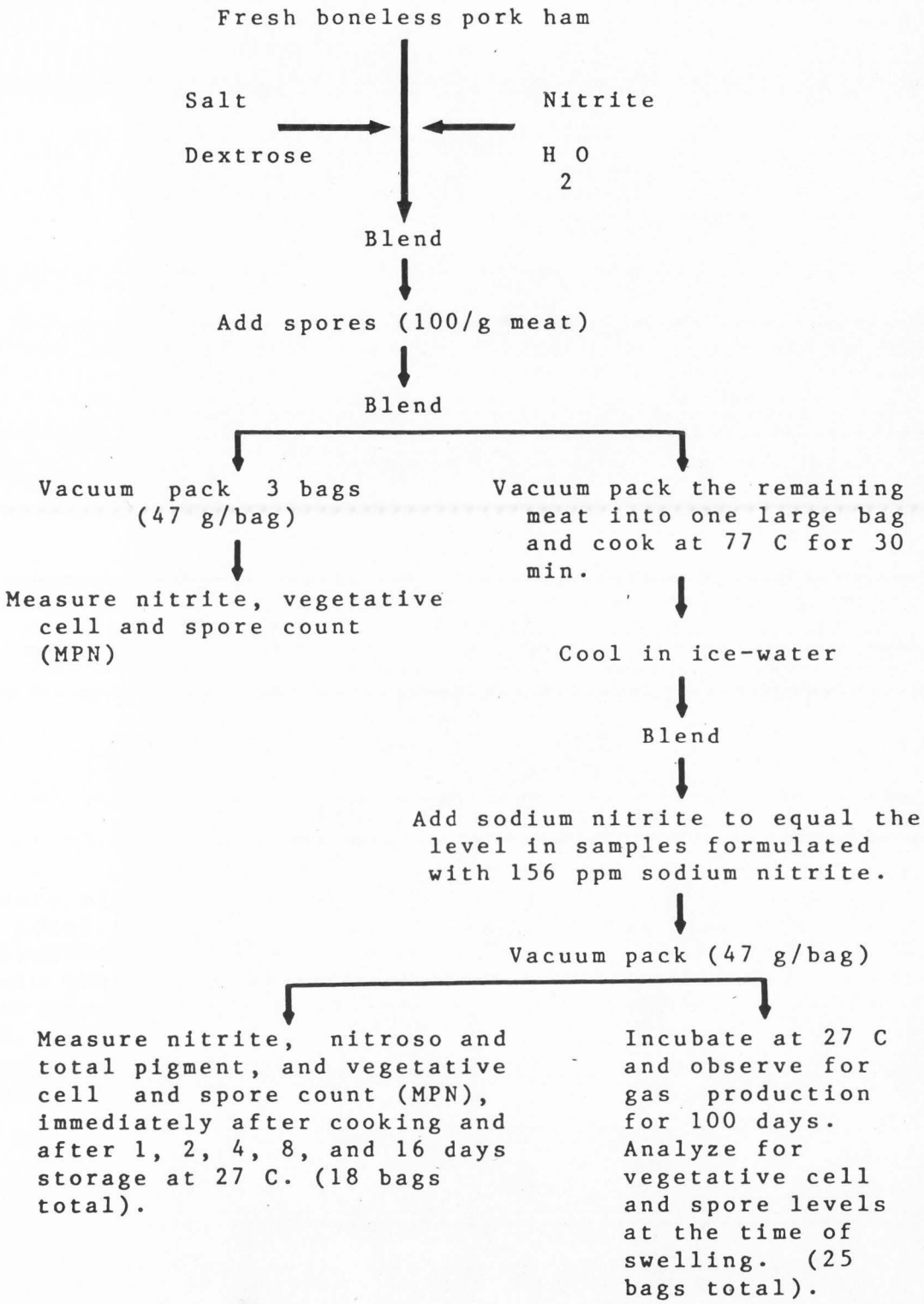

Figure 1: Preparation of treatments inoculated with *C. botulinum* spores before cooking.

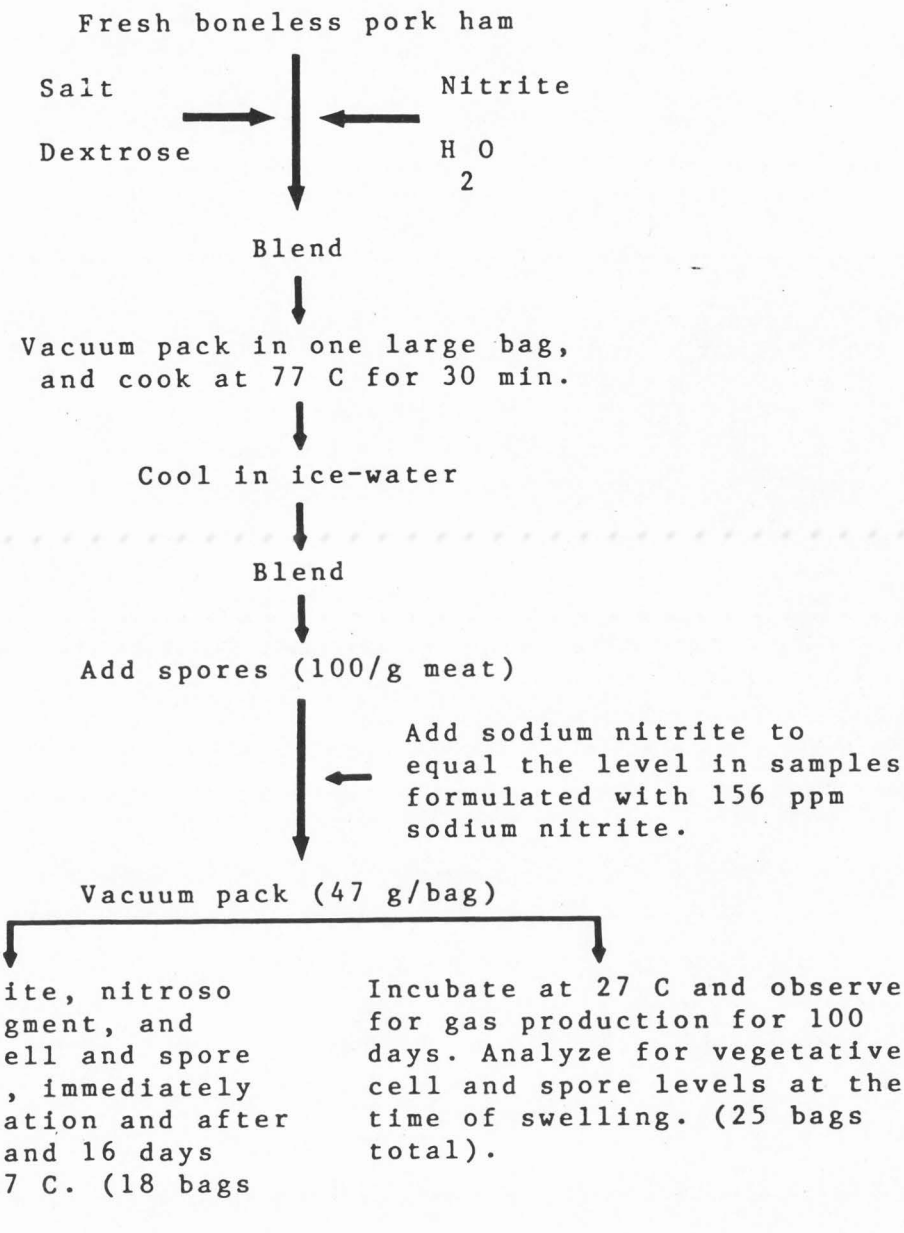

Figure 2: Preparation of treatments inoculated with *C. botulinum* spores after cooking.

Product Preparation

Fresh pork legs were boned, trimmed of fat, and then ground through a 0.64 cm plate, using a Hobart grinder (Hobart Corporation, Troy, Ohio, 45374, Fig.1). The meat, in approximately 4 pound portions, was vacuum sealed in polyethylene bags and kept frozen until the time of formulation. All products were formulated to contain 2.5% sodium chloride, 0.5% dextrose, 0 ppm, 50 ppm, 100 ppm, or 156 ppm sodium nitrite, based on the weight of the meat, and about 10% (w/w) distilled water. The mixture was then emulsified in a one gallon stainless steel blender. The finished product contained 15% added water, based on the final batch weight. About 50 ml of water was set aside for dilution and later addition with the spores. The spore suspension (C. botulinum type A and B in equal numbers) was added to the meat mixture at a concentration sufficient to give 100 spores per gram final product either at the time of formulation or after cooking. If the spores were added after cooking (Fig.2), the meat was cooled in an ice water bath for 15 min and then blended for 2-3 min. Approximately 45 g of the meat mixture were packed into air impermeable plastic film pouches. The laminated pouches, consisting of 0.75 mil nylon and 2.25 mil of a co-polymer of 6% ethylene vinyl acetate and 94% polyethylene, with oxygen permeability of 0.78 ml/24 hr/atm. (Meat Packers and Butchers Supply Co., Los Angles, CA),

are used routinely for vacuum packaging of meats. Pouches were vacuum packaged, double bagged, and cooked in a 77 C water bath for 30 min. Immediately after cooking, the pouches were cooled in an ice water bath for 15 min.

For each treatment, three bags of meat were sampled for heme pigment, nitrite, and MPN (Most Probable Number) tests after inoculum and before or after cooking. Three bags were also taken from the incubator and sampled for heme pigment, residual nitrite and botulinal spore levels (MPN test) at 0, 1, 2, 4, 8 and 16 days of incubation and also at the time of swelling.

Inoculum and Spore Counts

A mixture of spores of one type A (ATCC#19397) and type B (ATCC#17843) strain of *Clostridium botulinum* was used. Each strain was propagated separately in chopped liver media (Lehninger,1976, Appendix A) at 35 C 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 medium (Schmidt and Nank, 1960, Appendix A). After allowing one week at 35 C for spore formation, the suspensions were heat shocked at 85 C for 5 min to inactivate the toxin as well as preserve most of the spores (Woodburn et al.,1979). Spore counts were determined by making appropriate dilutions in 0.1% peptone water and plated on anaerobic egg agar

(Lehninger, 1976, Appendix A). The plates were incubated anaerobically at 35 C using a polycarbonate jar with a hydrogen and carbon dioxide generator (Gas Pak, Becton, Dickinson and Co., Cockeysville, Md.). Spore suspensions of C. botulinum type A and type B were made by diluting the stock suspension with 50 ml distilled water and mixed into the meat (100 spores/g meat) immediately before bagging. The meat was processed as quickly as possible (less than 30 min.) after spore addition, to minimize the possibility of germination and subsequent death of the vegetative cells during processing.

Holding Conditions

The sealed pouches were abused by holding at 27 C and observed for gas production for 100 days. Pouches were removed and analyzed periodically as well as at the time of swelling for nitrosoheme iron, total heme iron, residual nitrite and tested for the spore levels.

Vegetative Cell and Spore Count

Vegetative cells and spore levels were measured by using the MPN method (Appendix A). Three unswollen bags were removed at each sampling time and tested for botulinal spore and vegetative cell levels. Botulinal spore levels were obtained by heating a 1:10 dilution of the product at 80 C for 15 min before doing MPN determinations,

to kill vegetative cells. An unheated portion was analyzed to obtain total viable botulinal counts (spores plus vegetative cells). Modified peptone colloid (Greenberg et al.,1966, Appendix A) was used as the recovery medium. The MPN procedure consisted of three tubes per dilution. All tubes were incubated at 37 C for 1 week. Blackening of the recovery medium accompanied with a putrid aroma was considered adequate evidence of botulinal growth in the medium.

Chemical Analyses

Nitrite concentration was determined spectrophotometrically using a modification of method 24:014 of AOAC (1980, Appendix B). Total heme iron and nitrosohemochrome were determined spectrophotometrically by the procedure of Hornsey (1956), as modified by Kramlich et al.(1973, Appendix C).

Statistical Analyses

Data for rate of swelling, nitrite, nitroso and total pigments were analyzed with a factorial split plot in time, and by the analysis of variance (Ostle and Mensing, 1975). The difference is significant at $p=0.05$. Least significant difference (LSD) values were also computed (Ostle and Mensing, 1975). Mean differences must equal or exceed the LSD values to be statistically significant.

RESULTS

Effect of Heat Treatment and Nitrite Level on Swelling Rate

The rate of swelling for all 9 treatments including the control (Trt.9) is presented graphically in figures 3-5.

For samples formulated with higher initial nitrite levels the rate of swelling was significantly greater ($p=0.05$) when the spores were heated, i.e.,added before cooking, than for samples formulated with unheated spores (Fig.3). For example, when 156 ppm nitrite was added along with the spores before cooking, 18 bags were swollen after 48 days of storage (Fig.3a). However, when the spores were not heated, no swelling was observed (Fig.3b). Similar results were obtained when 100 ppm nitrite was added initially (Fig.3c-d).

At 50 ppm initial nitrite, the swelling rate was very low for both treatments (i.e.,spores added before or after cooking, Fig.4a-b). When the spores were added before cooking, only 1 bag swelled after 100 days storage, compared with only 4 swollen bags when the spores were added after cooking. The amount of sodium nitrite added after cooking was also higher in these treatments. Seventy-eight ppm sodium nitrite was added after cooking to adjust the residual nitrite level equal to that measured after cooking in the treatments formulated with 156 ppm nitrite, while

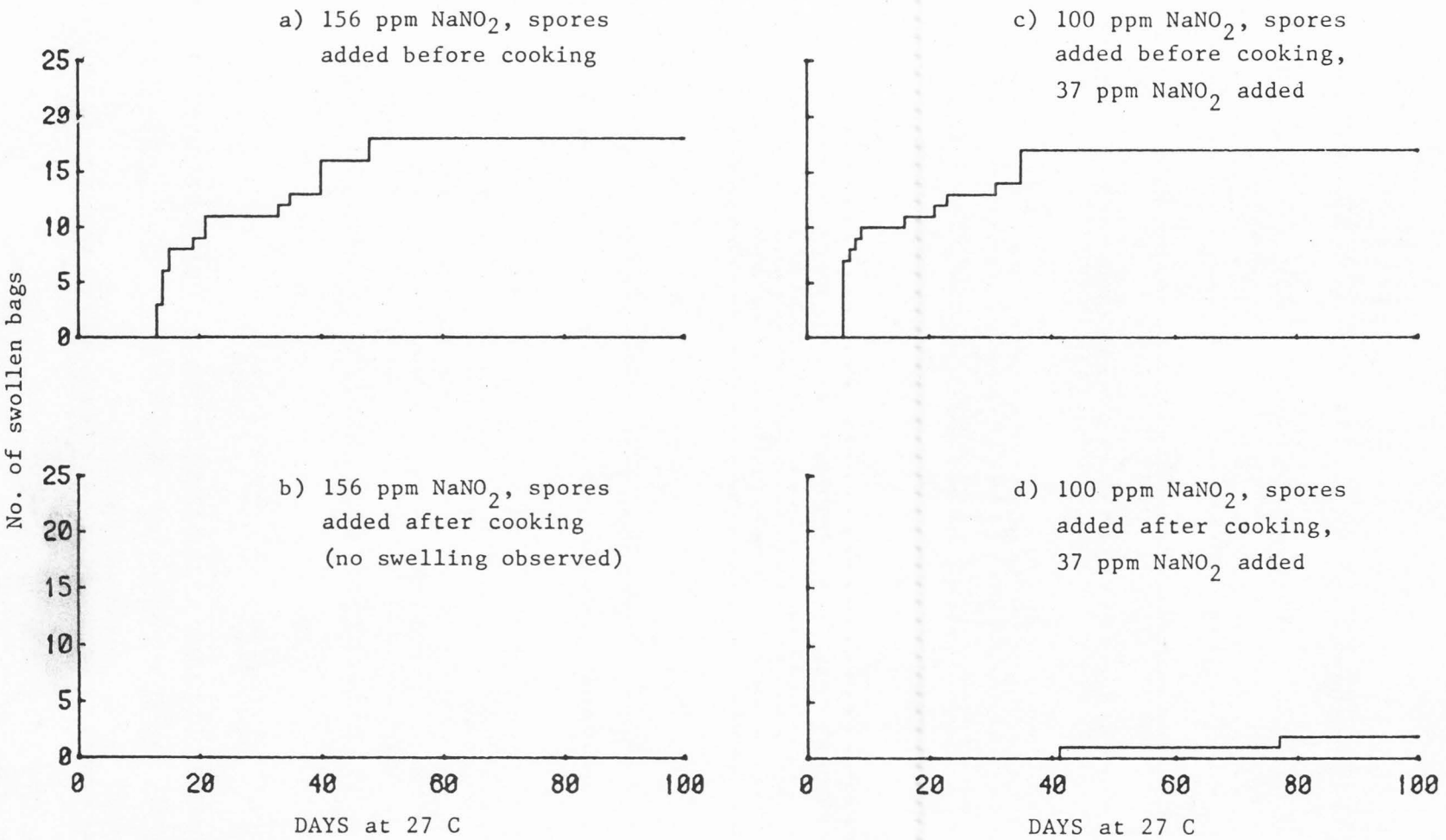

Figure 3. Rate of swelling in bags of ground pork formulated with 156 ppm nitrite (a,b) or 100 ppm nitrite (c,d), inoculated with 100 C. botulinum spores/g meat, either before (a,c) or after cooking (b,d), 37 ppm nitrite added after cooking then incubated at 27 C for 100 days.

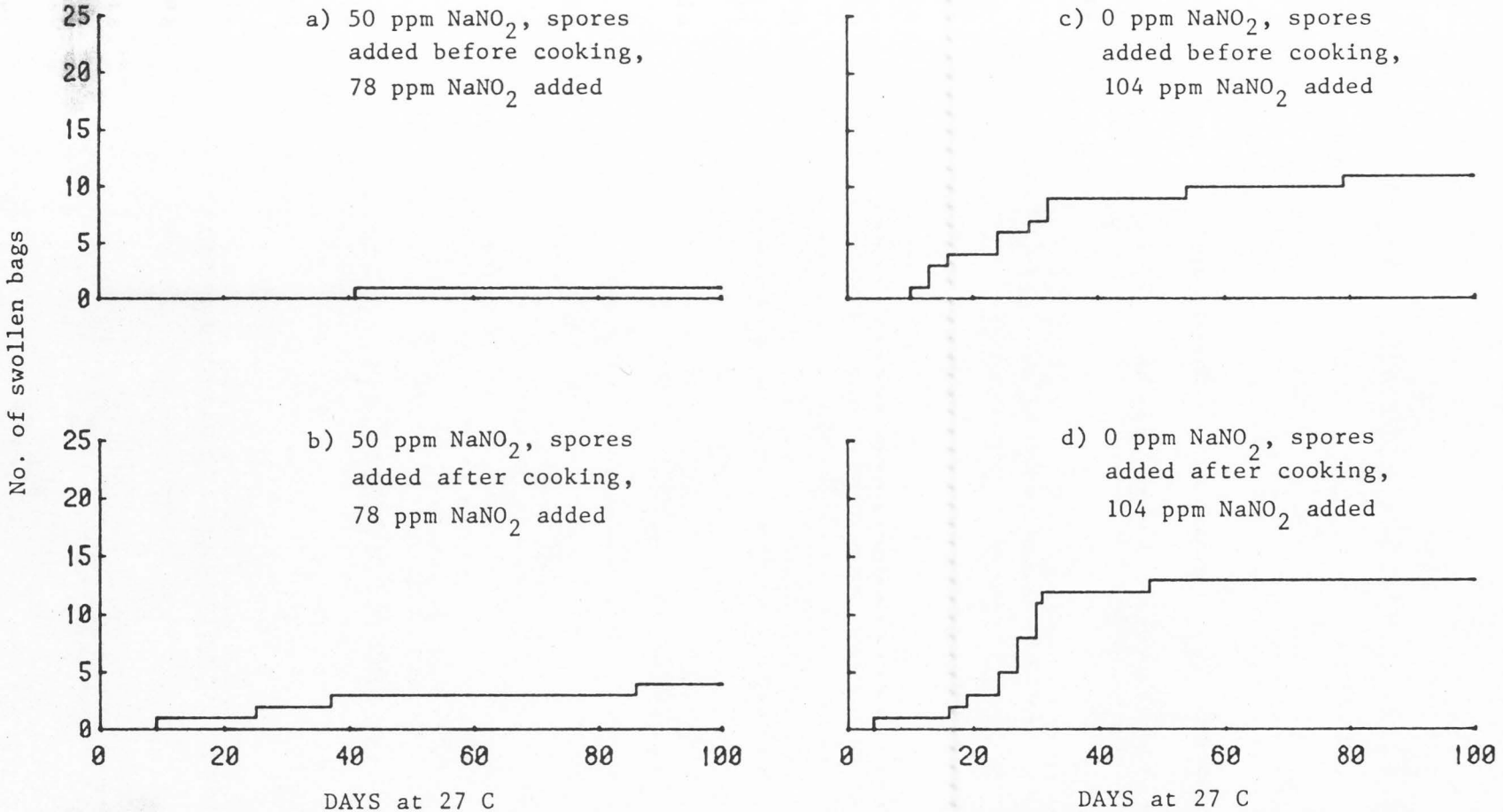

Figure 4. Rate of swelling in bags of ground pork formulated with 50 ppm nitrite (a,b) or 0 ppm nitrite (c,d), inoculated with 100 C. botulinum spores/g meat, either before (a,c) or after cooking (b,d), 78 ppm nitrite (a,b) or 104 ppm nitrite (c,d) added after cooking, then incubated at 27 C for 100 days.

only 37 ppm nitrite was needed to bring the samples formulated with 100 ppm nitrite to the same level (Fig.4a-b, 3c-d).

When samples were prepared with 0 ppm initial nitrite, the swelling rate was significantly higher ($p=0.05$) than for samples prepared with 50 ppm nitrite, even though 104 ppm nitrite was added after cooking. Eleven and 13 bags were swollen when spores were added before and after cooking, respectively (Fig.4c-d), while only 1 and 4 bags were swollen, respectively, when 50 ppm nitrite was added initially (Fig.4a-b).

As expected, the swelling rate was very low when no botulinal spores were added. Only 4 bags were swollen after 100 days storage (Fig.5), compared with 18 swollen bags in the treatment formulated with the same level of initial nitrite (156 ppm), but with the addition of 100 spores/g meat (Fig.3a).

Residual Nitrite Depletion

Residual nitrite levels decreased rapidly in all unswollen meat samples stored at 27 C (Fig.6-8). Most samples contained less than 5 ppm nitrite after 8 days of storage. However, those samples formulated with 156 ppm initial nitrite (Fig.6a-b,8c) had significantly higher ($p=0.05$) nitrite levels (Appendix D, table 21) than any other treatments, even though the other treatments received additional nitrite to adjust all samples to the same level

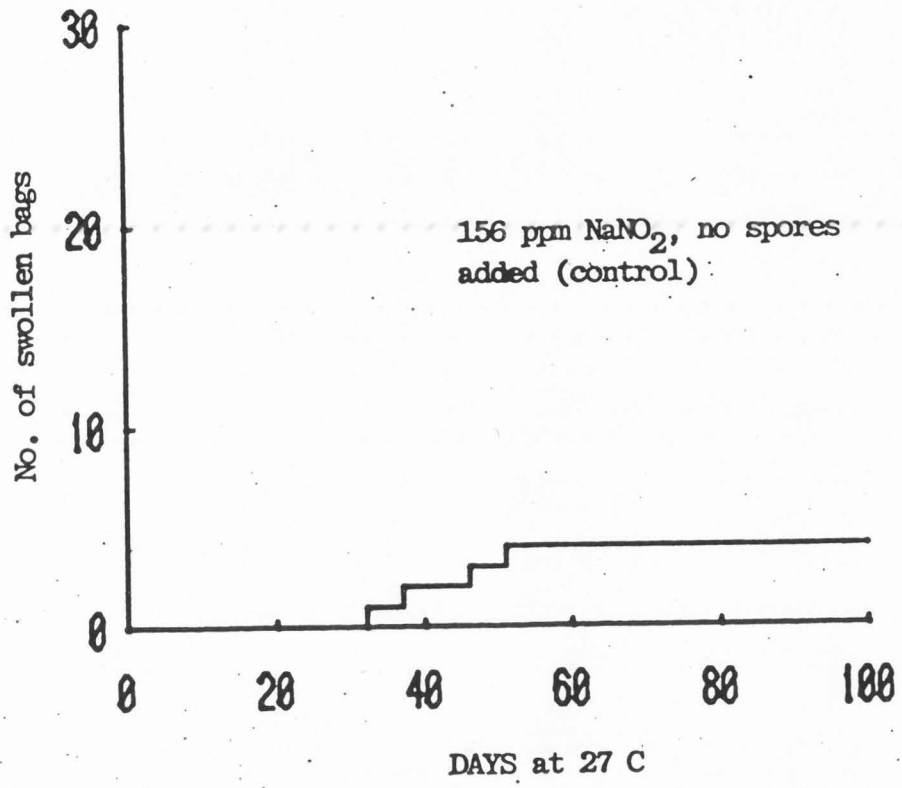

Figure 5. Rate of swelling in uninoculated ground pork formulated with 156 ppm nitrite.

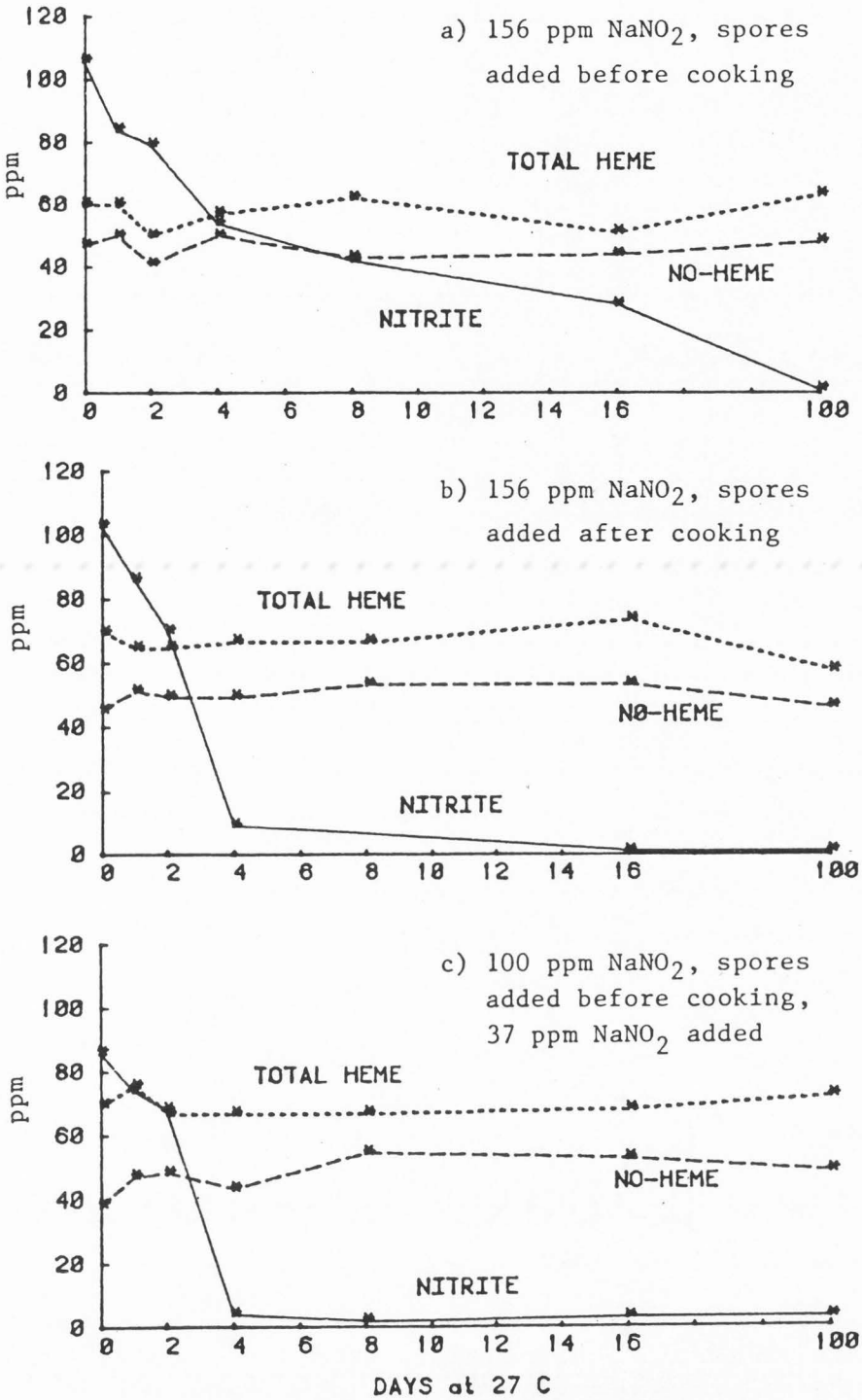

Figure 6. Nitrite, total and NO-heme pigments in unswollen bags of ground pork formulated with 156 ppm nitrite(a,b) or 100 ppm nitrite(c), inoculated with 100 C. botulinum spores/g meat, either before(a,c) or after cooking(b), 37 ppm nitrite added after cooking(c), then incubated at 27 C for 100 days. The initial sample(0 days) was taken immediately after cooking(a,b) or after blending with supplemental sodium nitrite.

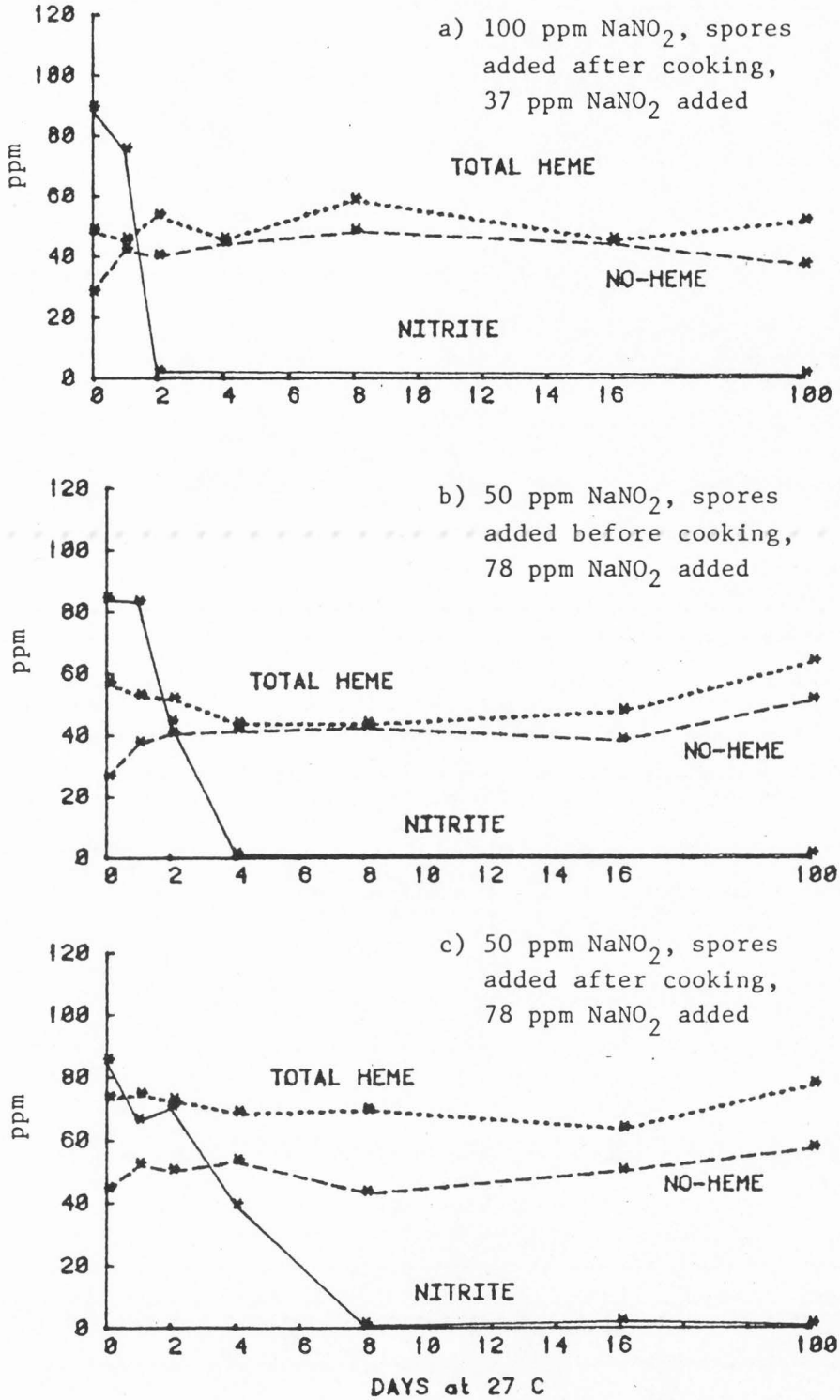

Figure 7. Nitrite, total and NO-heme pigments in unswollen bags of ground pork formulated with 100 ppm nitrite(a) or 50 ppm nitrite(b,c), inoculated with 100 C. botulinum spores/g meat, either before(b) or after cooking(a,c), 37 ppm nitrite(a) or 78 ppm nitrite(b,c) added after cooking, then incubated at 27 C for 100 days.

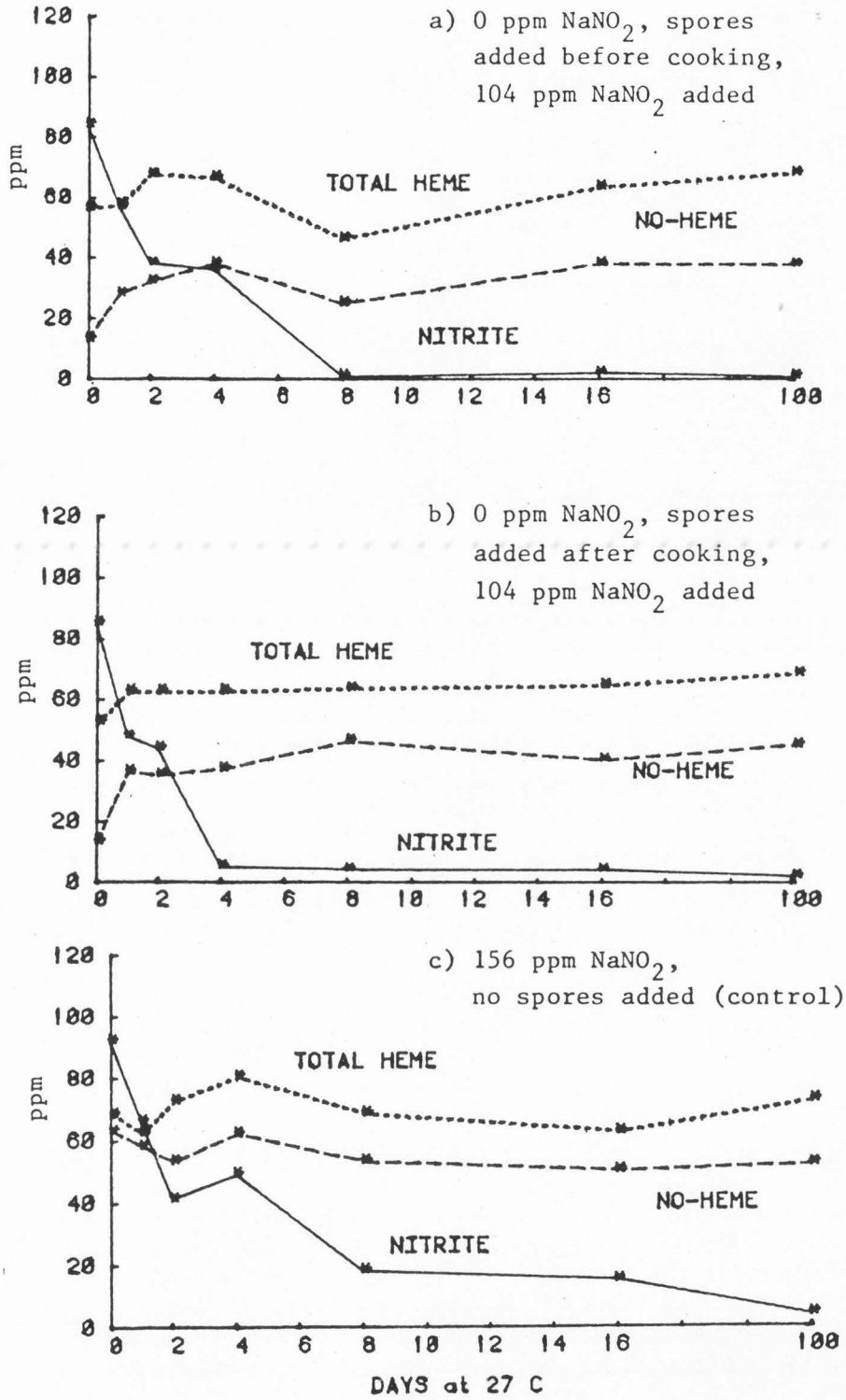

Figure 8. Nitrite, total and NO-heme pigments in unswollen bags of ground pork formulated with 0 ppm nitrite(a,b) or 156 ppm nitrite(c), inoculated with 100 C. botulinum spores/g meat, either before(a) or after cooking(b), or not inoculated(c), 104 ppm nitrite added after cooking(a,b), then incubated at 27 C for 100 days.

of nitrite (104 ppm) after cooking.

Four additional treatments were formulated with 50 ppm initial nitrite with or without additional nitrite, and with unheated or heated spores, to determine the effect of these variables on residual nitrite level (Fig.9-10). As expected, samples with more nitrite (78 ppm) added after cooking (Fig.7b-c,10b) did have significantly higher residual nitrite values ($p = 0.05$, Appendix D, table 23) than did similar samples without additional nitrite (Fig.9a-b, 10a, respectively). Nevertheless, residual nitrite levels usually declined faster in samples with nitrite added after cooking (Fig.7b-c), compared with similar samples with no added nitrite (Fig.9a-b, respectively).

When spores were heated (77 C for 30 min) before addition to the meat formulated with 50 ppm nitrite (Fig.10a) no significant difference was observed in the rate of nitrite depletion, compared with similar meat samples with unheated spores (Fig.9b). In contrast, comparisons were observed in which heat treatment of spores (Fig.10b) resulted in significantly ($p=0.05$, Appendix D, table 23) lower residual nitrite levels in the sample, compared with similar samples containing unheated spores (Fig.7c).

There was a reduction of nitrite as a result of contact with raw meat (Table 2). Approximately 1/4 of the added nitrite was lost within 30 minutes after formulation. There was only a slight decrease of nitrite due to cooking (Table 2).

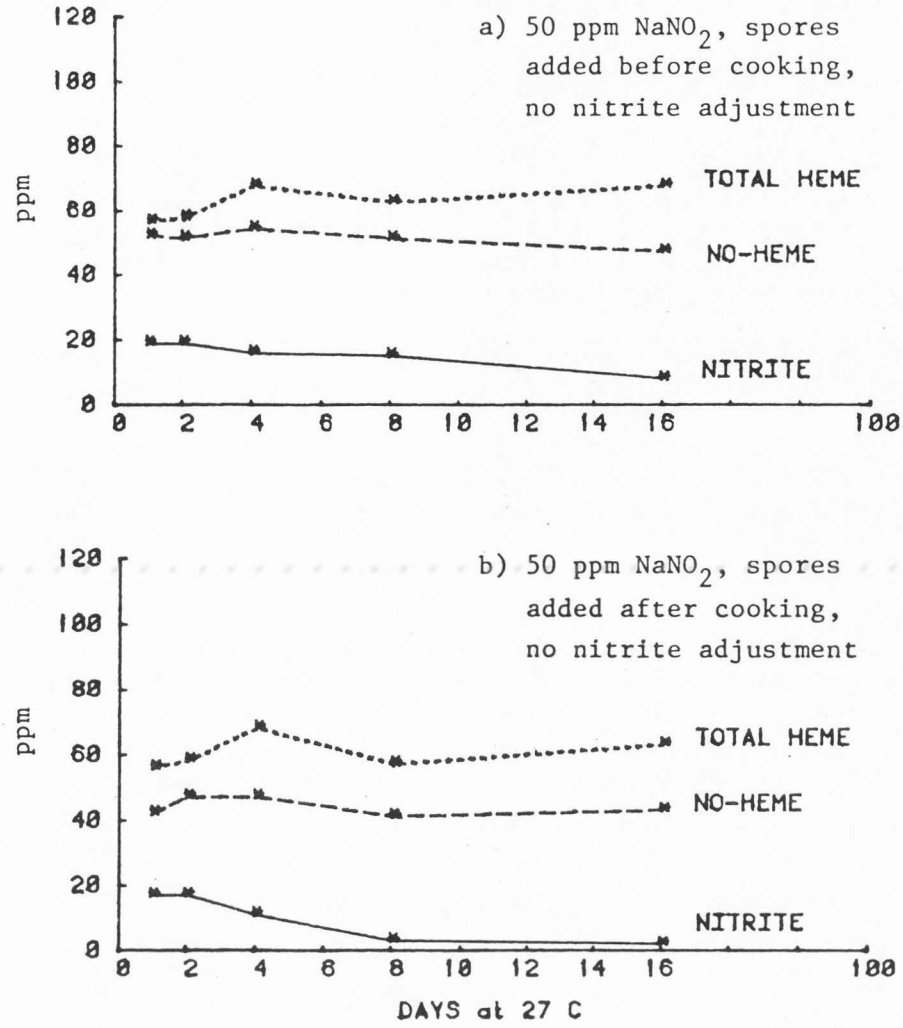

Figure 9. Nitrite, total and NO-heme pigments in unswollen bags of ground pork formulated with 50 ppm nitrite, inoculated with 100 C. botulinum spores/g meat, either before(a) or after cooking(b), then incubated at 27 C for 16 days.

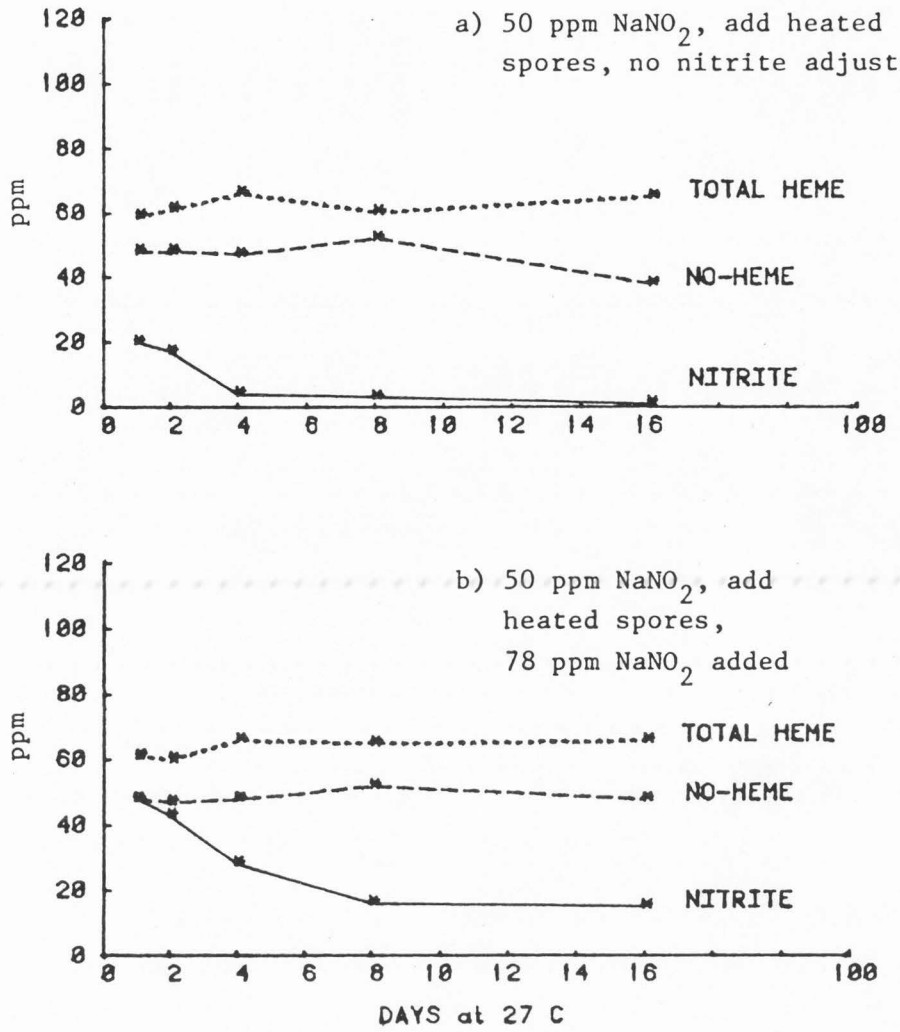

Figure 10. Nitrite, total and NO-heme pigments in unswollen bags of ground pork formulated with 50 ppm nitrite, inoculated with heated spores (100 C. botulinum spores/g meat) after cooking, and either with 0 ppm nitrite(a) or 78 ppm nitrite (b) added after cooking, then incubated at 27 C for 16 days.

Table 2: Sodium nitrite levels (ppm) as formulated and as analyzed.

Time of analysis	Nitrite level as formulated			
	0 ppm	50 ppm	100 ppm	156 ppm
Raw meat, after formulation	0	25	66	115
After cooking	0	22	60	104

In swollen bags, nitrite had decreased to less than 10 ppm in almost all treatments (Tables 3a, 4-6). Treatment 9, the control, formulated with no added spores, did have swollen bags containing nitrite levels ranging from 10-17 ppm (Table 3b).

Heme Pigments

The data for nitroso and total heme pigment content of swollen samples are presented in Tables 3-6. Swollen bags did contain significantly different levels of nitroso and total heme pigment, compared to unswollen bags ($p=0.05$, Appendix D, table 20). However, in the inoculated samples formulated with the higher initial nitrite levels of 100 and 156 ppm nitrite (Trts.1-4), there were no significant differences in either nitroso or total heme pigment content among swollen and unswollen bags (Appendix D, table 21). At the lower initial nitrite levels of 0 and 50 ppm (Trts.5-8),

Table 3: Residual nitrite, nitrosoheme and total heme pigment in swollen bags of ground pork processed with 156 ppm nitrite, and either inoculated with C. botulinum spores (100/g) before cooking (a) or not inoculated (b), and incubated at 27 C (Trt.1 and 9).

	Days at 27 C	Nitrite (ug/g)	NO-heme pigment (ug hematin/g)	Total heme pigment (ug hematin/g)
(a)	13	1	39	55
	14	1	41	56
	15	4	50	53
	19	5	44	57
	21	1	47	59
	33	0	53	62
	35	0	33	48
	40	2	44	65
	48	4	37	64
(b)	32	10	47	66
	37	17	46	61
	46	12	47	63
	51	13	41	67

All values were an average of duplicate samples from each swollen bag.

Table 4: Residual nitrite, nitrosoheme and total heme pigment in swollen bags of ground pork processed with 100 ppm nitrite, inoculated with *C. botulinum* spores (100/g) either before (a) or after cooking (b), 37 ppm nitrite added after cooking, and then incubated at 27 C (Trt.3,4).

	Days at 27 C	Nitrite (ug/g)	NO-heme pigment (ug hematin/g)	Total heme pigment (ug hematin/g)
(a)	6	2	45	71
	7	1	42	68
	8	1	46	63
	9	2	43	61
	16	2	54	64
	21	3	54	68
	23	1	60	76
	31	0	55	69
	35	2	55	63
(b)	41	1	39	51
	77	0	38	49

All values were an average of duplicate samples from each swollen bag.

Table 5: Residual nitrite, nitrosoheme and total heme pigment in swollen bags of ground pork processed with 50 ppm nitrite, inoculated with C. botulinum spores (100/g) either before (a) or after cooking(b), 78 ppm nitrite added after cooking, and then incubated at 27 C (Trt.5,6).

	Days at 27 C	Nitrite (ug/g)	NO-heme pigment (ug hematin/g)	Total heme pigment (ug hematin/g)
(a)	41	1	60	75
(b)	9	1	34	60
	25	0	65	92
	37	1	50	62
	86	3	51	74

All values were an average of duplicate samples from each swollen bag.

Table 6: Residual nitrite, nitrosoheme and total heme pigment in swollen bags of ground pork processed with 0 ppm nitrite, inoculated with C. botulinum spores (100/g) either before (a) or after cooking (b), 104 ppm nitrite added after cooking, and then incubated at 27 C (Trt.7,8).

	Days at 27 C	Nitrite (ug/g)	NO-heme pigment (ug hematin/g)	Total heme pigment (ug hematin/g)
(a)	10	1	23	62
	13	1	29	61
	16	1	42	66
	24	1	51	81
	29	1	47	63
	32	2	52	68
	54	0	34	54
	79	1	34	56
(b)	4	8	30	60
	16	4	40	64
	19	3	44	63
	24	2	52	71
	27	1	49	61
	30	2	54	63
	31	4	45	62
	48	5	46	57

All values were an average of duplicate samples from each swollen bag.

the values for nitroso pigment actually were higher in the swollen samples (Appendix D, table 21), although the total pigment content was about the same, in most cases.

The nitroso pigment levels were significantly lower in those samples formulated with 0 ppm initial nitrite, as expected ($p=0.05$, Appendix D, table 21). Both treatments formulated with 0 ppm initial nitrite had 38 ppm or lower of nitroso pigment in unswollen bags, compared to values ranging from 40-51 ppm for other treatments (Appendix D, table 21).

Comparison of nitroso and total heme pigment content of all treatments formulated with 50 ppm initial nitrite is presented in Appendix D, table 23. Whenever additional nitrite was added (Trt.6, 13), the nitroso pigment values of unswollen bags increased, compared with similar treatments (Trt.11, 12, respectively) without additional nitrite, with one observed exception. Unswollen bags from treatment 10 (50 ppm nitrite, spores added before cooking, no additional nitrite) had the highest nitroso pigment content of all samples formulated with 50 ppm initial nitrite, i.e., 52.1 ppm nitroso pigment, compared to 39.6 ppm for bags from treatment 5, which received 78 ppm additional nitrite. However, treatment 10 was the only treatment in this comparison to be blended only once. Meat for all other treatments was reblended to mix in either spores or additional nitrite. This second blending may have caused

some decrease in the nitroso pigment content of these samples.

The nitroso and total heme pigment content of unswollen bags is presented graphically in figures 6-10. The values for both nitroso and total heme pigment were quite constant during storage, with the exception of the samples formulated with 0 ppm initial nitrite (Fig.8a-b), which increased in nitroso pigment content during the first few days of storage, as previously noted.

C. botulinum Spore and Total Count

The number of C. botulinum spores and total botulinal organisms (spores plus vegetative cells) per gram meat were determined at various time intervals during storage for unswollen bags, and also at the time of swelling (Tables 7-19). Nitrite level, heat treatment of spores, and time of storage were all significant sources of variation for total botulinal count but not spore count (Appendix D, table 24).

Spore and Total Count in Unswollen Bags

For unswollen bags, the samples formulated with 156 ppm nitrite, and with spores added before cooking (Trt.1) had a lower spore and total botulinal count during the first 16 days of storage than any other treatment (Appendix d, table

25). Samples formulated with 100 ppm sodium nitrite, and with spores added before cooking (Trt.3) had a significantly higher ($p=0.05$) total botulinal count than any other treatment (Appendix D, table 25). When spores were heated for 30 min at 77 C before addition to cooked meat, they germinated rapidly. Total botulinal counts were significantly higher ($p=0.05$, Appendix D, table 27) in unswollen bags when heated (Trt.13, 12) rather than unheated (Trt.6, 11, respectively) spores were added to cooked meat. Examination of the actual data reveals that mean total botulinal count usually exceeded 1000/g after 1 or 2 days storage in samples with heated spores added (Tables 18-19). Similar samples receiving unheated spores had total botulinal counts that never exceeded 150/g during the first 4 days of storage (Tables 17, 12, respectively).

The addition of unheated spores to raw meat also appeared to increase spore germination rate. However, this may not be the case. For example, addition of 100 spores/g meat, as determined by a direct count on anaerobic egg agar, gave a mean total botulinal count of 460 (Table 7) when samples were taken 30 min. later (after blending, vacuum packaging, and freezing for later MPN analysis). Since the tabulated MPN 95% confidence interval for 460 is 71-2,400, the estimate of 460 total botulinal count may not reflect a true increase in botulinal count from the 100 spores added. Similarly, the spore count of 28 (Table 7) may not reflect a

Table 7: Number of spores and total botulinal organisms in meat product formulated with 156 ppm sodium nitrite, inoculated with 100 botulinal spores/g meat before cooking, and then incubated at 27 C (Trt.1)

Days at 27 C	No.of bags per sample	Status of bag	Residual nitrite (ug/g)*	Total count/g	Spore/g
0 (pre-cook)	3	unswollen	115	460 (71-2400)**	28 (10-150)
0 (post-cook)	3	"	104	23 (4-120)	16 (3-44)
1	3	"	84	25 (4-120)	17 (3-44)
2	3	"	79	23 (4-120)	10 (3-36)
4	3	"	54	102 (15-380)	9 (1-36)
8	3	"	42	36 (7-130)	8 (1-36)
16	3	"	28	7 (1-23)	5 (0.5-20)
13	3	swollen	1	>2,400	>2,400
14	3	"	1	>2,400	>2,400
15	2	"	4	>2,400	>2,400
19	1	"	5	>2,400	>2,400
21	2	"	1	>2,400	>2,400
33	1	"	0	>2,400	>2,400
35	1	"	0	>2,400	4 (<0.5-20)
40	3	"	2	>2,400	>2,400
48	2	"	4	>2,400	460 (71-2400)
100	3	unswollen	0	4 (<0.5-20)	<3
100	1	"	0	9 (1-36)	<3
100	1	"	1	>2,400	>2,400

* Values were an average of duplicate samples from 1 bag.
** 95% Confidence Limits.

Table 8: Number of spores and total botulinal organisms in meat product formulated with 156 ppm sodium nitrite, inoculated with 100 botulinal spores/g meat after cooking, and then incubated at 27 C (Trt.2).

Days at 27 C	No.of bags per sample	Status of bag	Residual nitrite (ug/g)*	Total count/g	Spore/g
0 (post-cook)	3	unswollen	103	53 (7-210)**	18 (7-89)
1	3	"	86	142 (30-440)	11 (3-36)
2	3	"	70	144 (30-440)	79 (14-230)
4	3	"	9	142 (30-440)	20 (7-89)
8	3	"	0	46 (7-210)	48 (7-210)
16	3	"	1	47 (7-210)	30 (10-150)
100	1	"	2	43 (7-210)	43 (7-210)
100	1	"	1	9 (1-36)	4 (<0.5-20)
100	1	"	1	75 (14-230)	43 (7-210)
100	1	"	0	93 (15-380)	43 (7-210)
100	1	"	0	23 (4-120)	43 (7-210)

* Values were an average of duplicate samples from 1 bag.
** 95% Confidence Limits.

Table 9: Number of spores and total botulinal organisms in meat product formulated with 100 ppm sodium nitrite, inoculated with 100 botulinal spores/g meat before cooking, 37 ppm nitrite added after cooking, and then incubated at 27 C (Trt.3).

Days at 27 C	No.of bags per sample	Status of bag	Residual nitrite (ug/g)*	Total count/g	Spore/g
0 (pre-cook)	3	unswollen	66	174 (30-440)**	23 (4-120)
0 (post-cook)	3	"	86	174 (30-440)	23 (4-120)
1	3	"	74	461 (71-2400)	67 (15-380)
2	3	"	68	1,753	22 (4-120)
4	3	"	4	>2,400	16 (3-44)
8	3	"	2	>2,400	26 (10-150)
16	3	"	3	44 (7-210)	23 (4-120)
6	3	swollen	2	>2,400	16 (3-44)
7	1	"	1	43 (7-210)	7 (1-23)
8	1	"	1	>2,400	4 <(0.5-20)
9	1	"	2	>2,400	43 (7-210)
16	1	"	2	>2,400	9 (1-36)
21	1	"	3	>2,400	9 (1-36)
23	1	"	1	>2,400	>2,400
31	1	"	0	>2,400	>2,400
35	3	"	2	1,967	955 (150-2400)
100	1	unswollen	2	23 (4-120)	7 (1-23)
100	1	"	3	9 (1-36)	4 (<0.5-20)
100	1	"	2	4 (<0.5-20)	4 (<0.5-20)

* Values were an average of duplicate samples from 1 bag.
** 95% Confidence Limits.

Table 10: Number of spores and total botulinal organisms in meat product formulated with 100 ppm sodium nitrite, inoculated with 100 botulinal spores/g meat, and with 37 ppm nitrite added after cooking, and then incubated at 27 C (Trt.4).

Days at 27 C	No.of bags per sample	Status of bag	Residual nitrite (ug/g)*	Total count/g	Spore/g
0 (post-cook)	3	unswollen	89	161 (30-440)**	199 (35-470)
1	3	"	75	429 (71-2400)	36 (7-130)
2	3	"	2	36 (7-130)	60 (3-37)
4	3	"	0	36 (7-130)	13 (3-37)
8	3	"	0	36 (7-130)	17 (3-44)
16	3	"	0	106 (15-380)	30 (10-150)
41	1	swollen	1	43 (7-210)	43 (7-210)
77	1	"	0	23 (4-120)	93 (15-380)
100	1	unswollen	1	39 (7-130)	21 (4-47)
100	1	"	1	150 (30-440)	93 (15-380)
100	1	"	1	23 (4-120)	23 (4-120)
100	1	"	0	93 (15-380)	93 (15-380)

* Values were an average of duplicate samples from 1 bag.
** 95% Confidence Limits.

Table 11: Number of spores and total botulinal organisms in meat product formulated with 50 ppm sodium nitrite, inoculated with 100 botulinal spores/g meat before cooking, 78 ppm nitrite added after cooking, and then incubated at 27 C (Trt.5).

Days at 27 C	No.of bags per sample	Status of bag	Residual nitrite (ug/g)*	Total count/g	Spore/g
0 (pre-cook)	3	unswollen	25	125 (30-380)**	54 (15-380)
0 (post-cook)	3	"	84	76 (14-230)	76 (14-230)
1	3	"	83	76 (14-230)	12 (3-36)
2	3	"	44	182 (35-470)	46 (7-210)
4	3	"	1	79 (14-230)	12 (3-36)
8	3	"	0	125 (30-380)	72 (14-230)
16	3	"	0	134 (3-380)	71 (14-230)
41	1	swollen	1	43 (7-210)	4 (<0.5-20)
100	1	unswollen	0	43 (7-210)	23 (4-120)
100	1	"	0	23 (4-120)	23 (4-120)
100	1	"	0	43 (7-210)	43 (7-210)
100	1	"	0	39 (7-130)	75 (14-230)

* Values were an average of duplicate samples from 1 bag.
** 95% Confidence Limits.

Table 12: Number of spores and total botulinal organisms in meat product formulated with 50 ppm sodium nitrite, inoculated with 100 botulinal spores/g meat, and with 78 ppm nitrite added after cooking, and then incubated at 27 C (Trt.6).

Days at 27C	No.of bags per sample	Status of bag	Residual nitrite (ug/g)*	Total count/g	Spore/g
0 (post-cook)	3	unswollen	85	60 (15-380)**	60 (15-380)
1	3	"	66	136 (30-380)	119 (30-380)
2	3	"	70	72 (14-230)	34 (70-130)
4	3	"	39	191 (35-470)	36 (7-130)
8	3	"	1	52 (15-380)	36 (7-130)
16	3	"	2	36 (7-130)	53 (15-380)
9	1	swollen	1	43 (7-210)	93 (15-380)
25	1	"	0	23 (4-120)	43 (7-210)
37	1	"	1	43 (7-210)	23 (4-120)
86	1	"	3	39 (7-130)	9 (1-36)
100	1	unswollen	0	93 (15-380)	21 (4-47)
100	1	"	0	43 (7-210)	15 (3-44)
100	1	"	0	23 (4-120)	43 (7-210)
100	1	"	0	210 (35-470)	93 (15-380)

* Values were an average of duplicate samples from 1 bag.
** 95% Confidence Limits.

Table 13: Number of spores and total botulinal organisms in meat product formulated with 0 ppm sodium nitrite, inoculated with 100 botulinal spores/g meat before cooking, 104 ppm nitrite added after cooking, and then incubated at 27 C (Trt.7).

Days at 27 C	No.of bags per sample	Status of bag	Residual nitrite (ug/g)*	Total count/g	Spore/g
0 (pre-cook)	3	unswollen	0	193 (35-470)**	12 (3-36)
0 (post-cook)	3	"	84	248 (36-1300)	36 (7-130)
1	3	"	57	36 (7-130)	119 (30-380)
2	3	"	38	36 (7-130)	36 (7-130)
4	3	"	36	36 (7-130)	34 (7-130)
8	3	"	1	23 (4-120)	16 (3-44)
16	3	"	2	8 (1-23)	40 (7-210)
10	1	swollen	1	>2,400	4 (<0.5-20)
13	2	"	1	235 (36-1300)	122 (30-380)
16	1	"	1	>2,400	4
24	2	"	1	1,202 (150-4800)	1,202 (150-4800)
29	1	"	1	93 (15-380)	9 (1-36)
32	2	"	2	43 (7-210)	<3
54	1	"	0	>2,400	>2,400
79	1	"	1	>2,400	>2,400
100	1	unswollen	1	4 (<0.5-20)	<3
100	1	"	0	39 (7-130)	<3
100	1	"	1	9 (1-36)	4 (<0.5-20)

* Values were an average of duplicate samples from 1 bag.
** 95% Confidence Limits.

Table 14: Number of spores and total botulinal organisms in meat product formulated with 0 ppm sodium nitrite, inoculated with 100 botulinal spores/g meat, and with 104 ppm nitrite added after cooking, and then incubated at 27 C (Trt.8).

Days at 27 C	No.of bags per sample	Status of bag	Residual nitrite (ug/g)*	Total count/g	Spore/g
0 (post-cook)	3	unswollen	85	89 (15-380)**	87 (15-380)
1	3	"	48	175 (30-440)	36 (7-130)
2	3	"	44	54 (15-380)	64 (15-380)
4	3	"	5	53 (15-380)	42 (7-210)
8	3	"	4	60 (15-380)	36 (7-130)
16	3	"	4	29 (10-150)	36 (7-130)
4	1	swollen	8	>2,400	>2,400
16	1	"	4	93 (15-380)	43 (7-210)
19	1	"	3	150 (30-440)	23 (4-120)
24	2	"	2	92 (15-380)	14 (3-37)
27	3	"	1	70 (14-230)	37 (7-130)
30	3	"	2	53 (15-380)	27 (10-150)
31	1	"	4	93 (15-380)	43 (7-210)
48	1	"	5	93 (15-380)	23 (4-120)
100	1	unswollen	0	23 (4-120)	15 (3-44)
100	1	"	4	43 (7-210)	7 (1-23)
100	1	"	3	23 (4-120)	9 (1-36)
100	1	"	2	23 (4-120)	15 (3-44)

* Values were an average of duplicate samples from 1 bag.
** 95% Confidence Limits.

Table 15: Number of spores and total botulinal organisms in an uninoculated meat product formulated with 156 ppm sodium nitrite, and then incubated at 27 C (Trt.9).

Days at 27 C	No.of bags per sample	Status of bag	Residual nitrite (ug/g)*	Total count/g	Spore/g
0 (pre-cook)	3	unswollen	96	<3	<3
0 (post-cook)	3	"	92	<3	<3
1	3	"	66	<3	<3
2	3	"	41	<3	<3
4	3	"	49	<3	<3
8	3	"	18	<3	<3
16	3	"	15	<3	<3
32	1	swollen	10	<3	<3
37	1	"	17	<3	<3
46	1	"	12	<3	<3
51	1	"	13	<3	<3
100	1	unswollen	1	<3	<3
100	1	"	7	<3	<3
100	1	"	7	<3	<3
100	1	"	8	>2,400	4 (<0.5-20)**

* Values were an average of duplicate samples from 1 bag.
** 95% Confidence Limits.

Table 16: Number of spores and total botulinal organisms in meat product formulated with 50 ppm sodium nitrite, inoculated with 100 botulinal spores/g meat before cooking, and then incubated at 27 C (Trt.10).

Days at 27 C	No.of bags per sample	Status of bag	Residual nitrite (ug/g)*	Total count/g	Spore/g
1	3	unswollen	19	43 (7-210)**	53 (15-380)
2	3	"	19	23 (4-120)	18 (7-89)
4	3	"	16	53 (15-380)	30 (10-150)
8	3	"	15	30 (10-150)	9 (1-36)
16	3	"	8	46 (7-210)	7 (1-23)

* Values were an average of duplicate samples from 1 bag.
** 95% Confidence Limits.

Table 17: Number of spores and total botulinal organisms in meat product formulated with 50 ppm sodium nitrite, inoculated with 100 botulinal spores/g meat after cooking, and then incubated at 27 C (Trt.11).

Days at 27 C	No.of bags per sample	Status of bag	Residual nitrite (ug/g)*	Total count/g	Spore/g
1	3	unswollen	17	125 (30-380)**	30 (10-150)
2	3	"	17	95 (15-380)	27 (10-150)
4	3	"	11	30 (10-150)	193 (35-470)
8	3	"	3	888 (150-4800)	23 (4-120)
16	3	"	2	43 (7-210)	53 (15-380)

* Values were an average of duplicate samples from 1 bag.
** 95% Confidence Limits.

Table 18: Number of spores and total botulinal organisms in meat product formulated with 50 ppm sodium nitrite, inoculated with 100 heated botulinal spores/g meat after cooking, and then incubated at 27 C (Trt.12).

Days at 27 C	No.of bag per sample	Status of bag	Residual nitrite (ug/g)*	Total count/g	Spore/g
1	3	unswollen	20	600 (71-2400)**	527 (71-2400)
2	3	"	17	1,100 (150-4800)	142 (30-440)
4	3	"	4	600 (71-2400)	199 (35-470)
8	3	"	3	460 (71-2400)	36 (7-130)
16	3	"	0	70 (14-230)	43 (7-210)

* Values were an average of duplicate samples from 1 bag.
** 95% Confidence Limits.

Table 19: Number of spores and total botulinal organisms in meat product formulated with 50 ppm sodium nitrite, inoculated with 100 heated botulinal spores/g meat and with 78 ppm nitrite added after cooking, and then incubated at 27 C (Trt.13).

Days at 27 C	No.of bags per sample	Status of bag	Residual nitrite (ug/g)*	Total count/g	Spore/g
1	3	unswollen	48	1,533 (150-4800)**	313 (36-1300)
2	3	"	43	1,247 (150-4800)	125 (30-380)
4	3	"	28	813 (150-4800)	107 (15-380)
8	3	"	16	764 (71-2400)	64 (15-380)
16	3	"	15	34 (7-130)	46 (7-210)

* Values were an average of duplicate samples from 1 bag.
** 95% Confidence Limits.

Similarly, the spore count of 28 (Table 7) may not reflect a true decrease from the 100 spores added, since the 95% confidence interval ranges from 10-150.

Among treatments formulated with 50 ppm initial nitrite, the addition of more nitrite after cooking had no significant effect on the total botulinal or spore count, when similarly prepared samples were compared (Trt.5 vs Trt.10, Trt.6 vs Trt.11, Trt.12 vs Trt.13, respectively, Appendix D, table 27).

Effect of Heat Treatment on Spore and Total Count in Swollen Bags

In most instances, spore and total botulinal counts were higher for swollen than for unswollen bags. However, some unswollen bags sampled after 100 days storage did contain total botulinal counts exceeding 2400/g (Tables 7, 15). Swollen samples formulated with 156 or 100 ppm sodium nitrite and with spores added before cooking (Tables 7, 9) contained spore and total botulinal count often exceeding 2400/g meat, while samples formulated similarly, but with spores added after cooking never had spore or total counts of botulinal organisms exceeding 95/g (Tables 8,10). No differences were observed in spore and total botulinal count due to heat treatment of spores (addition of spores before or after cooking) in samples formulated with 50 ppm nitrite (Tables 11, 12). At 0 ppm initial nitrite, the

spore and total botulinal count was again higher when the spores were added before cooking (Tables 13, 14).

Effect of Nitrite Level on Spore and Total Count in Swollen Bags

Somewhat surprisingly, the samples formulated with higher initial nitrite (156 or 100 ppm) also had highest spore and total botulinal count in swollen bags, if spores were also heated (i.e.,added before cooking, Tables 7, 9). Samples formulated with 50 ppm initial nitrite had much lower spore and total botulinal counts, but received 78 ppm nitrite after cooking compared with 37 and 0 ppm nitrite added after cooking for samples formulated with 100 and 156 ppm initial nitrite, respectively (Tables 7-12).

Samples formulated with 0 ppm initial nitrite (Tables 13, 14) had the most nitrite added after cooking (104 ppm) to adjust the residual nitrite level equal to that found after cooking in samples formulated with 156 ppm nitrite. However, the spore and total botulinal counts were again exceeding 2400/g in some swollen bags. Apparently, a combination of a small initial nitrite (50 ppm) with a relatively high nitrite addition (78 ppm) after cooking was the most effective in reducing botulinal numbers in swollen bags. In addition, the degree of swelling was not as

extensive as in those treatments where the total botulinal counts were high.

DISCUSSION

Effect of Heat on Spore Germination

Rate of swelling in treatments where spores were added before heat treatment were usually higher than in treatments where spores were added after cooking. Thus, heating the spores (as would normally occur in cooking) apparently stimulates spore germination and thereby increased the swelling rate, especially in treatments with little or no (37 or 0 ppm) nitrite added after cooking. A number of previous studies have concluded that heat activation (heat shock at about 70 C for 15 min) increased the germination rate, activating enzymes which are dormant in resting spores, and broadening the variety of agents that will induce initiation (Keynan, Issahary-Brand and Evenchik, 1965). For instance, Hyatt and Levinson (1961) concluded that sublethal heat shock might "reduce the spore requirement for germination". They suggested that heating released certain substances within the spore, and these substances interacted with exogenously added compounds to stimulate germination. Levinson (1957) postulated the internal release of germination stimulating substances through the mediation of proteases. Goddard (1939) postulated that the spore germination rate of Neurospora tetrasperma may be partly determined by the internal concentration of stimulatory compound whose supply is limited by a heat-activated reaction. Heated spores release

dipicolinic acid into the medium (Levinson and Hyatt, 1960; Rode and Foster, 1960). Young (1959) suggested that in the resting spore, dipicolinic acid is complexed with amino acids such as alanine. This led Hyatt and Levinson (1961) to suggest that heating renders alanine, or a related compound, available as an internal germination stimulant. Heating may activate an enzyme system similar to lysozyme, which may in turn cause release of alanine or related compounds from some constituent of the spore coat (Strange and Dark, 1956). Walker and Matches (1965) also reported that heating bacterial spores causes a progressive loss of dipicolinic acid, peptides and other constituents from the spores. Fleming (1963) and Duncan (1967) suggested that prevention of dipicolinic acid release by sodium chloride in the heating medium (Duncan, 1970) may in part account for the protective effect of salt against thermal destruction of the spores at higher temperatures.

Under more severe heat treatment, spores may be damaged or sensitized to the presence of antimicrobial agents, rather than simply stimulated to germinate. For example, Jarvis et al. (1976) found that spores heated at 95 C were sensitized to the inhibitory action of 3.5% salt, but spores heated at pasteurization temperatures (e.g. 70-80 C) were not sensitized. In contrast, heat or radiation damaged spores (to a 1% survival level) of C. botulinum type A and E and of C. sporogenes were shown to be no more sensitive to heated nitrite than undamaged spores (Roberts and Smart,

1974).

Contrary to the results obtained here, Ashworth et al.(1973) found that the inhibitory effect of nitrite on spores of C. sporogenes was similar whether spores were heated with the meat or added post processing. However, they were not using C. botulinum spores, and probably more importantly their incubation temperature was 37 C, rather than 27 C used in the present study. This may in part explain the different results of the two studies.

In the present study, the rate of germination as measured by MPN determination of total botulinal count was also higher when spores were heated separately, then added to cooked meat (Trt.13), rather than addition of unheated spores to cooked meat (Trt.6). Thus, the enhancing effect of heat shock (77 C for 30 min) on spore germination must be due to the direct effect of heat on the spore structure or chemical composition, possibly by the swelling of the spore wall with release of dipicolinic acid, resulting in an increased permeability of the spore to stimulatory agents (amino acids and sugars) in the medium. Duncan (1970) also concluded that mild heat treatment, as used for cooking cured meats, may be sufficient to activate the spores and "prepare" them for germination. However, the presence of curing salts would be inhibitory to germination of at least some of the spores with subsequent deactivation occurring on storage. On the other hand, a large proportion of the spores in cured meats are not inactivated by the heat process, and

are able to germinate and grow if transferred to a suitable nutritive environment (Ingram and Roberts, 1971).

Effect of Nitrite on Swelling Rate and Spore Germination

Adding nitrite at the time of formulation had a distinct inhibitory effect compared with samples formulated with 0 ppm initial nitrite. This is in similar to previous studies in which higher initial nitrite levels decreased the toxicity of cured meat samples inoculated with botulinal spores and stored at abuse temperatures (Greenberg, 1972; Bowen and Deibel, 1974; Christiansen et al.,1973). However, residual nitrite levels were also found to be important for botulinal inhibition. Those samples receiving the least nitrite addition after cooking had the fastest swelling rate and the highest botulinal counts by MPN, even though these samples (i.e.,156 ppm and 100 ppm nitrite with spores added before cooking) had high levels of initial nitrite. For samples in which spores were added after cooking, there was no difference in the botulinal counts between samples in which additional nitrite was added, compared to samples with no added nitrite. The botulinal counts were low even in the swollen samples, compared with the botulinal counts in swollen samples in which spores were added before cooking.

In treatments 5 and 6, (50 ppm initial nitrite, 78 ppm nitrite added after cooking) 1 and 4 bags swelled during

storage, respectively. Even these low values were probably over estimates of the swelling rate for these treatments, because these bags had very low botulinal counts when examined by MPN. The reason that some swollen bags had low total counts and spore counts may have been due to the 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 clostridial growth. All 4 swollen bags from uninoculated samples (Trt.9) also had counts lower than 3/g for both total counts and the spore counts. Nitric oxide gas from added nitrite may be formed but the amount would be very small. In general, only 1-5% of nitrite originally added was recovered in gas (Cassens et al,,1977).

The importance of residual nitrite for botulinal inhibition has been shown in studies by several investigators. Tompkin et al. (1978c) showed that addition of hemoglobin reduced residual nitrite levels and increased swelling rate. He suggested that residual nitrite was inhibitory. Tompkin et al. (1978b) also showed that cured pork refrigerated until residual nitrite levels were low swelled faster on subsequent incubation with botulinal spores and storage at 27 C. Christiansen et al. (1978) measured botulinal spore and total counts, as well as residual nitrite in canned cured pork inoculated with botulinal spores and stored at 27 C. They concluded that the time at which botulinal outgrowth occurs depends upon

the relative levels of residual nitrite and surviving botulinal cells.

Thus, strong evidence exists for the inhibitory effects of both initial and residual nitrite levels. In this study, swelling rates were lowest when a low level of initial nitrite (50 ppm) was supplemented with a relatively high amount (78 ppm) of nitrite after cooking. When 0 ppm initial nitrite was added, the swelling rate was much faster, even though the highest level of supplemented nitrite (104 ppm) was added. Johnston et al. (1969) also found that nitrite added after cooking was more inhibitory if some initial nitrite was also included. When there was no initial nitrite at least 150 ppm nitrite was necessary after cooking to obtain an inhibitory effect in canned meat product. Ashworth and Spencer (1972) similarly observed that both initial and residual nitrite was inhibitory to botulinal organisms. They stated that adding nitrite before heating enhanced inhibition, since the minimum level of nitrite necessary for inhibition of 50% of the spores of C. sporogenes (ED_{50}) was 66 ppm for initial nitrite, compared to 99 ppm for nitrite added after cooking.

In most treatments, approximately 1/4 of added sodium nitrite was lost within 30 min. after formulation. Residual nitrite decreased slightly due to cooking. However, nitrite levels declined rapidly upon storage at 27 C. The variations in residual nitrite level in unswollen bags within treatments were quite small, since most samples

contained less than 5 ppm nitrite after day 8 of storage. The reasons why some bags within a treatment swelled while the others did not may be due to the spores themselves. Spores may differ in many characteristics, other than size. Hyatt and Levinson (1961) stated that the thickness of the spore coat, permeability to substrates, concentration and degree of polymerization of dipicolinic acid, internal concentration of amino acids and other compounds capable of stimulating germination, may vary among individual spores, and these variations may then determine the extrinsic requirements of individual spores for germination.

Residual nitrite in most of the swollen bags was below 10 ppm (Table 3-6). However, all the unswollen bags remaining after 100 days also contained low residual nitrite (less than 10 ppm). The difference in nitrite level between swollen and unswollen bags was significant at $p=0.05$ (Appendix D, table 20).

Residual nitrite in all treatments (Trts.1-9) was adjusted to the same level as found in samples formulated with 156 ppm initial sodium nitrite, but nitrite levels measured after additional nitrite was added after cooking was usually between 84-89 ppm (Table 9-14). This loss of additional nitrite may be due to the effect of blending.

Residual nitrite adjustment was found to have an effect on the level of NO-heme pigment but not total heme pigment (Appendix D, table 22). NO-heme pigment values in samples with nitrite added after cooking were higher than samples

with no added nitrite (Appendix D, table 23), only one exception was observed as mentioned earlier. Mirna (1974) also found that a higher NO-heme pigment values could be observed in the experiments with subsequent addition of nitrite.

NO-Heme and Total Heme Pigment in the Swollen and Unswollen Bags

Nitroso and total heme pigments were measured on unswollen bags periodically and also on day 100 , as well as at the time of swelling. Significant variations were observed for both nitroso and total heme pigments among treatments (Appendix D, table 20). These variations may be due to the fact that treatments were prepared from different batches of meat. Sufficient meat was originally prepared to complete the entire experiment. However, the shut down of the electricity in the cold room that was used to store the meat made it necessary to use a new batch of meat to replace the old rotten one. Treatments 1-5 were prepared from the same batch of meat, and all other treatments were formulated from a second batch of meat.

Mechanism of Nitrite Inhibition

While both initial and residual nitrite have inhibitory properties, it is difficult to determine their relative importance. The effects of initial nitrite levels are especially hard to evaluate, since higher initial nitrite

levels always produce higher residual nitrite levels. Three possible effects of initial nitrite are:

1) initial nitrite levels bind iron, reducing the amount of this nutrient available for botulinal growth (Tompkin et al.,1978a).

2) initial nitrite kills or inactivates spores either before or during cooking.

3) initial nitrite levels simply produce proportional levels of residual nitrite, plus a "protein bound" nitrite fraction (Olsman, 1977; van Roon and Olsman, 1977).

van Roon (1974) and Tompkin et al. (1978a) observed that added ferrous ions (or hemoglobin, Tompkin et al.,1978c) reduced botulinal inhibition by nitrite. Tompkin et al. (1978a) concluded that botulinal organisms have a requirement for iron, and nitrite reduces the amount of iron available for growth. The results of the present study make this possibility seem unlikely. First, not all meat iron is nitrosylated. Secondly, the amount of nitroso pigment does not decrease even in swollen bags. Third, the iron requirement of botulinal organisms, if any, is probably quite small. Shankar and Bard (1951) showed that C. perfringens require only 2 ug Fe^{++} per ml for optimum growth. The observed increase of botulinal growth upon addition of iron is more likely due to a decrease in residual nitrite level. In fact, a patent (Danner et al., 1973) has been issued wherein ferrous salts are added to meat cured with NO gas to promote better cure color

formation and stability, indicating that ferrous ions can indeed in some way react with nitrite or related compounds.

Regarding possibility 2, it is indeed possible that nitrite could inactivate botulinal spores before or during cooking. However, in this study the total botulinal count usually increased before cooking but the total count decreased after cooking. This probably due to the death of vegetative cells during cooking, rather than to any inhibitory effect of nitrite.

Thus, possibility 3 seems to be the most likely role for initial nitrite. More initial nitrite in a product certainly produces more residual nitrite. Residual nitrite is likely inhibitory due to its reactivity, via nitrous acid, with sulfur amino acids (Mirna and Holfmann, 1969; Olsman, 1977; van Roon, 1980), leading to inactivation of an iron sulfur enzyme (Tompkin et al.,1978a, O'Leary and Solberg, 1976), especially ferredoxin. Also, initial nitrite levels rapidly decrease in cooked cured meats, with formation of a substantial fraction of protein bound nitrite (Mirna, 1974; van Roon and Olsman, 1977). One such product, S-NO-cysteine, has been shown to a) have antimicrobial properties (Incze et al.,1974; Moran et al.,1975; Hansen and Levin, 1975; Huhtanen, 1975), and b) reversibly form NO (Olsman, 1977). Thus, the increased antibotulinal effects of low initial nitrite levels supplemented with nitrite after cooking are probably due to the formation and

antimicrobial properties of the bound nitrite fraction formed during (and possibly after) cooking.

CONCLUSION

Inoculating the spores at the time of formulation resulted in higher rate of spore germination. Heat, somehow, increased the rate of germination possibly by releasing substances within the spores which interacted with exogenously added compounds to stimulate germination as described by Hyatt and Levinson (1961). Spores heated before inoculum also resulted in a higher rate of germination.

Addition of nitrite at the time of formulation was shown to be important to inhibit C. botulinum, but during storage some of the protein-bound and residual nitrite probably contributed to the stability of the product.

Subsequent addition of nitrite seemed to have an effect on the inhibition of C. botulinum in treatments in which spores were added before cooking. The higher the amount of nitrite added after heat treatment, the greater the inhibitory effects on spore germination. Since the rate of nitrite depletion was very high in samples where spores were added after cooking and when additional nitrite was added after cooking, but the swelling rate of these samples was very low, nitrite might in some way, react with other components in meat to form products that can inhibit growth. S-nitrosothiols have been reported to be a group of reaction product of nitrite (Mirna and Holfmann, 1969; Olsman, 1974; 1977) that have proved inhibitory in culture media (Incze et

al.,1974; Moran et al.,1975). Olsman (1977) suggested that decomposition of this compound to nitric oxide which easily reacted with other endogenous substances to enhance inhibition by nitrite was of cardinal importance in relation to the significance of residual nitrite.

Rate of germination of spores between samples in which residual nitrite was adjusted and samples in which residual nitrite was not adjusted, showed no significant difference during 16 days of incubation.

The interaction between nitrite and heat treatment was shown to be very important for the inhibition of C. botulinum. This suggests that the interplay between the curing ingredients, the heat processing, and the number of spores presence all contribute to the stability and safety of cured meats.

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APPENDICES

Appendix A

Bacteriological Media and Procedures

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Chopped Liver Media

Ground beef liver	500.0 g
Soluble starch	1.0 g
Peptone	10.0 g
Dipotassium phosphate	1.0 g
Distilled water	1.0 l

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 121 C. Before use, exhaust for 20 min in flowing steam.

Peptone Water Diluent

Dissolve 1 g peptone in 1 liter distilled water. Adjust to pH 6.8.

Sporulation Media

Tryptone	50.0 g
Peptone	5.0 g
Distilled water	1.0 l

Sodium thioglycollate 1 g in 10 ml water.

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

Reference: Schmidt and Nank, 1960.

Anaerobic Egg Agar

Fresh eggs	3
Yeast extract	5.0 g
Tryptone	5.0 g
Proteose peptone	20.0 g
Sodium chloride	5.0 g
Agar	20.0 g
Distilled water	1.0 l

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 121 C for 15 min. Let the agar mixture cool to 45 to 50 C, add 80 ml of the egg yolk emulsion, mix thoroughly, and pour plates immediately. Dry plates at room temperature 2-3 days, or 24 hr at 35 C. Discard contaminated plates. Store sterile plates in the refrigerator until use.

Gram Stain

Reagents:

1. Crystal violet: Mix 0.8 g of ammonium oxalate with 80 ml water. Mix together with 2.0 g crystal violet dissolved in 20 ml of 95% ethyl alcohol.

2. Iodine: Mix together 1.0 g iodine, 2.0 g KI, and 300 ml water.

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

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

Procedure:

1. Prepare the smear. Air dry and heat fix.

2. Apply Gram's crystal violet for 1-2 min.

3. Wash off the excess stain by holding the slide under a steam of water.

4. Flood the slide with Gram's iodine and allow it to react for 1 min or longer.

5. Rinse as in step No.3. Shake the excess water off or blot lightly, but not to dryness.

6. Holding the slide at an angle, carefully add the decolorizing solution one drop at a time. As soon as color stops coming off the slide, after about 8-10 seconds, rinse with water to stop the decolorizing action.

7. Flood the slide with Gram's safranin and allow it to react for 30-60 seconds.

8. Drain the excess stain from the slide and thoroughly wash it.

9. Carefully blot the stained slide, do not rub.

Modified Peptone Colloid Broth

Tryptose	20.0 g
Salt (NaCl)	5.0 g
Agar	1.0 g
Distilled water	1.0 l
Dextrose	1.0 g
Ferrous sulfate ($FeSO_4$)	0.2 g
Sodium thiosulfate	0.3 g

Add tryptose, NaCl and agar to the distilled water and boil until the clear solution is obtained. Adjust the broth to pH 7.3+.2. Add dextrose, $FeSO_4$, and sodium thiosulfate to the broth and boil again for 15-20 min. Transfer the 10 ml of broth into the test tubes, cover with friction-fit cap. Sterilize for 15 min at 121 C.

Ten grams of the sample were homogenized in a sterile blender with 90 ml of 0.1% peptone water, for a 10^{-1} dilution. The sample was further diluted to 10^{-2} and 10^{-3}.

From each dilution bottle, 1.0 ml was transferred to each of 3 tubes of modified peptone colloid broth, for a total of 9 tubes. This was repeated for another set of 9 tubes. One set of tubes was heat shocked at 80 C for 15 min to kill vegetative cells. All the tubes were incubated at 37 C for 1 week, then observed for a yellow to black color change of the media, along with a putrid odor, indicative of growth. Growth in the heat shocked tubes was due to spores. MPN tables (FDA, 1978) at the appropriate dilutions was used to estimate the number of organisms present.

Appendix B

Nitrite Analysis

Nitrite Analysis

Reagents and Apparatus:

1. NED reagent: Dissolve 0.2 g N-(1-naphthyl)-ethylenediamine 2 HCl 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; 1,000 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 80 C 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 80 C water bath and let stand for 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 ug 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

Meat Pigment

Meat Pigment

Reagent:

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

2. 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. Weight 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 paper into a test tube.

6. Transfer filtrate into 1 cm cuvette and read absorbance within 1 hr at 540 nm. 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 nm. and calculate as total pigment.

Calculations:

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

$$\text{ppm nitroso pigment} = A_{540} \times 290$$

$$\text{ppm total pigment} = A_{640} \times 680$$

If volume or sample sizes were varied:

$$\text{ppm nitroso pigment} = 57.7 \times A_{540} \times \text{total vol. (ml)/sample(g)}$$

$$\text{ppm total pigment} = 135.8 \times A_{640} \times \text{total vol. (ml)/sample(g)}$$

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

Appendix D

Statistical Analyses

Table 20: Analysis of co-variance of treatment (nitrite level and heat treatment) and bag status (swollen or unswollen) on nitrite, nitroso and total heme pigment level in samples stored for 100 days (Trts.1-9).

		Nitrite		NO-heme pigment		Total heme pigment	
Source of variation	D.F.	MS	F-ratio	MS	F-ratio	MS	F-ratio
Treatment	16	3987.55	8.56*	600.84	11.08*	629.48	11.76*
Bag status	1	73404.27	157.59*	994.84	18.34*	679.53	12.69*
Error	260	465.78		54.23		53.54	

* Significant at p=0.05.

Table 21: Comparison of estimated means during a 100 day storage period for nitrite, nitroso and total heme pigment in treatments 1-9 for both swollen and unswollen bags.

Treatment	Nitrite (ug/g)	NO-heme pigment (ug hematin/g)	Total heme pigment (ug hematin/g)
Trt.1 (unswollen)	42.39^{a}	46.35^{beg}	58.56^{bd}
Trt.1 (swollen)	-2.91^{bg}	43.21^{bde}	58.06^{bd}
Trt.2 (unswollen)	30.05^{ac}	47.57^{bc}	61.72^{bef}
Trt.3 (unswollen)	26.82^{cf}	47.56^{bc}	69.06^{ae}
Trt.3 (swollen)	-6.44^{b}	51.18^{cf}	68.35^{ae}
Trt.4 (unswollen)	18.86^{cde}	40.92^{de}	51.20^{c}
Trt.4 (swollen)	10.13^{bc}	37.35^{deh}	49.30^{c}
Trt.5 (unswollen)	24.14^{cf}	42.55^{eij}	54.11^{cd}
Trt.5 (swollen)	3.08^{bcf}	59.76^{af}	74.80^{ae}
Trt.6 (unswollen)	29.29^{ac}	51.45^{ac}	71.76^{a}
Trt.6 (swollen)	2.82^{bdg}	49.72^{cfgj}	70.37^{ae}
Trt.7 (unswollen)	24.53^{cf}	31.97^{h}	61.98^{bef}
Trt.7 (swollen)	-2.10^{bg}	39.49^{dik}	65.38^{efg}
Trt.8 (unswollen)	23.37^{cef}	38.85^{di}	63.68^{f}
Trt.8 (swollen)	-1.62^{bg}	46.52^{bj}	64.25^{ef}
Trt.9 (unswollen)	33.69^{af}	54.80^{af}	69.09^{ag}
Trt.9 (swollen)	14.17^{cg}	44.98^{bejk}	63.78^{befg}

Values in the same column with the same letter superscript are not significantly different at p=0.05 (LSD test).

Table 22: Analysis of variance of spore treatment (spore addition before or after cooking, or addition of heated spores after cooking) on nitrite, nitroso and total pigment during 16 days of storage in samples formulated with 50 ppm sodium nitrite (Trts.5, 6, 10-13).

		Nitrite		NO-heme pigment		Total heme pigment	
Source of variation	D.F.	MS	F-ratio	MS	F-ratio	MS	F-ratio
Spore treatment	2	62.55	29.50*	12.02	3.41	617.87	32.32*
$NaNO_2$ adjustment	1	5320.42	2509.63*	38.40	10.88*	33.75	1.77
Spore trt. x $NaNO_2$ adjustment	2	309.52	146.00*	466.55	132.17*	695.00	36.35*
Error A	6	2.12		3.53		19.12	
Days	4	3457.98	683.40*	28.32	3.42	31.81	10.23*
Error B	4	5.06		8.27		3.11	
Spore trt. x Days	8	207.93	102.43*	33.27	3.76*	14.35	1.57
$NaNO_2$ adjustment x Days	4	1322.96	651.70*	16.48	1.86	87.46	9.59*
Spore trt. x Days x $NaNO_2$ adjustment	8	296.06	145.84*	15.76	1.78	28.77	3.15*
Error C	20	2.03		8.84		9.12	

* Significant at p=0.05.

Table 23: Comparison of estimated means during a 16 day storage period of nitrite, nitrosoheme and total heme pigment in treatments formulated with 50 ppm sodium nitrite (Trts. 5, 6, 10-13).

Treatment	Nitrite (ug/g)	NO-heme pigment (ug hematin/g)	Total heme pigment (ug hematin/g)
Trt.5 (78 ppm nitrite added)	25.50^{c}	39.60^{e}	47.10^{c}
Trt.10 (residual nitrite not adjusted)	15.30^{d}	52.10^{a}	61.60^{b}
Trt.6 (78 ppm nitrite added)	35.60^{a}	49.60^{b}	69.00^{a}
Trt.11 (residual nitrite not adjusted)	10.00^{e}	43.70^{d}	60.50^{b}
Trt.13 (78 ppm nitrite added)	29.70^{b}	48.30^{bc}	63.70^{b}
Trt.12 (residual nitrite not adjusted)	9.00^{e}	46.50^{c}	62.20^{b}

Values in the same column with the same letter superscript are not significantly different at p=0.05 (LSD test).

Table 24: Analysis of variance of initial nitrite level, heat treatment of the spores (spore addition before or after cooking), and days on total botulinal and spore count in unswollen bags from treatments 1-8 for a 16 day storage period.

		Total count		Spore count	
Source of variation	D.F.	MS	F-ratio	MS	F-ratio
$NaNO_2$ level	3	3145546.60	55.72*	6307.30	2.23
Heat treatment	1	2343961.00	41.52*	9328.34	3.30
$NaNO_2$ level x Heat treatment	3	2674030.20	47.36*	1365.14	0.48
Error A	16	56457.54		2829.25	
Days	5	421214.58	8.76*	5320.49	1.53
Error B	10	48084.80		3476.52	
$NaNO_2$ level x Days	15	393381.56	9.22*	2282.60	1.14
Heat treatment x Days	5	542827.18	12.72*	2741.59	1.37
$NaNO_2$ level x Days x Heat treatment	15	552981.71	12.96*	4698.48	2.34*
Error C	70	42681.14		2003.81	

* Significant at p=0.05.

Table 25: Comparison of estimated means for total botulinal and spore count during a 16 day storage period in unswollen bags from treatments 1-8.

Treatment	Total count	Spore count
Trt.1	36.11^{b}	10.94^{b}
Trt.2	95.78^{b}	34.39^{ab}
Trt.3	1205.39^{a}	29.61^{ab}
Trt.4	134.11^{b}	59.00^{a}
Trt.5	112.28^{b}	48.11^{ab}
Trt.6	91.22^{b}	56.28^{a}
Trt.7	64.56^{b}	46.83^{ab}
Trt.8	76.56^{b}	50.22^{ab}

Values in the same column with the same letter superscript are not significantly different at p=0.05 (LSD test).

Table 26: Analysis of variance of spore treatment (spore addition before or after cooking, or addition of heated spores after cooking) on total botulinal and spore count during 16 days storage in samples formulated with 50 ppm nitrite (Trts.5, 6, 10-13).

		Total count		Spore count	
Source of variation	D.F.	MS	F-ratio	MS	F-ratio
Spore treatment	2	3645806.70	20.64*	134477.34	9.58*
$NaNO_2$ adjustment	1	161544.10	0.91	5921.11	0.42
Spore trt. x $NaNO_2$ adjustment	2	381592.03	2.16	11431.41	0.81
Error A	12	176603.78		14041.32	
Days	4	449491.08	6.06*	55269.21	5.89*
Error B	8	74199.70		9389.34	
Spore trt. x Days	8	453928.15	2.57*	46709.21	3.00*
$NaNO_2$ adjustment x Days	4	132985.68	0.75	12703.53	0.82
Spore trt. x Days x $NaNO_2$ adjustment	8	155555.95	0.88	8475.33	0.54
Error C	40	176794.39		15553.40	

* Significant at p=0.05.

Table 27: Comparison of estimated means for total botulinal and spore count during a 16 day storage period in samples formulated with 50 ppm nitrite (Trts.5, 6, 10-13).

Treatment	Total count	Spore count
Trt.5 (residual nitrite adjusted)	119.47^{c}	42.47^{bc}
Trt.10 (residual nitrite not adjusted)	39.00^{c}	23.47^{c}
Trt.6 (residual nitrite adjusted)	97.53^{c}	55.60^{bc}
Trt.11 (residual nitrite not adjusted	236.20^{bc}	65.07^{bc}
Trt.13 (residual nitrite adjusted)	878.27^{a}	131.07^{ab}
Trt.12 (residual nitrite not adjusted)	565.87^{ab}	189.27^{a}

Values in the same column with the same letter superscript are not significantly different at p=0.05 (LSD test).