

Biochemical Basis for Nitrite-Inhibition of *Clostridium botulinum* in Cured Meat 4533

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ABSTRACT

The biochemistry of the anaerobic spore-forming clostridia and the chemistry of the nitrite reactivity during meat curing are reviewed. Direct and indirect mechanisms for nitrite inhibition of *Clostridium botulinum* growth and toxin formation are presented. The "Perigo effect" produced by heating nitrite in laboratory media may be produced by artifactual oxidants, and is not identical to that produced in cured meats. Inhibition of *C. botulinum* by nitrite in cured meats is most likely due to several interacting mechanisms: (a) reaction and oxidation of cellular biochemicals within the spores and vegetative cells; (b) restriction of use of iron (or other essential metal ions) through inhibition of solubilization, transport, or assimilation, thus interfering with metabolism and repair mechanisms and (c) cell surface membrane activity limiting substrate transport by the outgrowing cell.

Although nitrite salts have been permitted for meat curing for over 50 years (1,131) and were present as reduction products of nitrate salts used in curing since antiquity, the mode of nitrite's inhibitory action on growth and toxin formation by *Clostridium botulinum* is still unknown. These spore-forming organisms, commonly present in Northern Hemisphere temperate zone soils and occasionally in the intestinal contents of birds and mammals (57,118,126), can often occur in foods as contaminants and present the potential hazard of botulism in humans. Canned or vacuum-packaged meat products that have been heated only sufficiently to inactivate bacterial vegetative cells and that lack added inhibitors, such as nitrite, provide excellent anaerobic and nutrient conditions for spore germination and subsequent production of toxin. These conditions have led to incidences of botulism following consumption of home-processed meat products (17,56,118).

Because of concern over safety of nitrite as a food additive, particularly the possibility of nitrosamine formation, the U.S. Department of Agriculture recently proposed (and subsequently withdrew) a reduction in allowable added nitrite in bacon from the former 156 ppm to 40 ppm (with addition of 550 ppm of ascorbate or erythorbate and 0.2% sorbic acid or sorbate

equivalent), a level of nitrite considered to be still effective with the added sorbate in preventing botulinum toxin formation (80,147). Since nitrite may be totally banned in the future as a food additive, research has been directed toward development of alternative anti-clostridial agents. Such research has been hampered by lack of understanding of the mechanism(s) by which nitrite inhibits toxin formation in cured meats. Experiments with model systems and with actual cured meat systems have indicated that the nature of inhibition is complex. No single mechanism appears to be responsible for all the various inhibitory responses of *C. botulinum* reported under differing conditions.

In this paper, the biochemistry of the clostridia, the chemistry of the meat curing process and the various chemical and biochemical mechanisms by which nitrite inhibition of clostridial toxin formation may occur in both experimental systems and actual cured meats are presented. The most probable mechanisms for these separate inhibitory actions are also presented.

BIOCHEMISTRY OF *C. BOTULINUM*

Morphology, physiology, and biochemistry

The clostridia are gram-positive, catalase-negative mesophilic rods, usually motile by peritrichous flagella and with large oval terminal or subterminal spores; they are classified in the family *Bacillaceae* along with the genus *Bacillus* and three other genera (17). Clostridia, however, are strict anaerobes or facultative anaerobes, with the distinction based partially on the lack of superoxide dismutase in obligate anaerobes that protects against the toxic superoxide form of oxygen (44,91,121). Because of their anaerobiosis, clostridia have specific biochemical pathways to furnish energy in the absence of atmospheric oxygen. Unfortunately, knowledge about this metabolism is limited because of the difficulties inherent in maintaining anaerobiosis during all analyses.

Bacteria of the genus *Clostridium* are important from a medical standpoint as several of the common species produce toxins that are pathogenic to humans. These include: *Clostridium tetani* (tetanus), *Clostridium welchii* (gangrene), *Clostridium perfringens* (food intoxications), and *C. botulinum* (botulism). The toxin of *C. botulinum* is an extremely potent high molecular weight protein that acts by inhibiting acetylcholine release at myoneural

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Ferredoxins, rubredoxins and metalloproteins

A portion of the iron and sulfur required by *C. botulinum* is used for synthesis of ferredoxin and rubredoxin, electron-transport compounds present in the cell cytoplasm. The ferredoxins are small proteins (6,000-8,000 daltons) with a highly negative oxidation-reduction potential (ca -400 mv at pH 7), and generally lack the thermodynamically less stable amino acids tryptophan and histidine (16). A clostridial ferredoxin molecule contains two iron-sulfur clusters that participate in the oxidation-reduction reactions. Each cluster consists of four atoms of iron and four inorganic sulfur atoms joined in a distorted cube that is connected to the protein by an iron-cysteinyl linkage (16,107,162). Addition of acid to ferredoxin causes release of the labile inorganic sulfur as hydrogen sulfide with a concomitant inactivation of the ferredoxin. The oxidized form of the clostridial ferredoxin can be detected by specific absorption peaks at 390-395 nm; these peaks disappear upon reduction. The spectrophotometric absorption of ferredoxin with other ligands such as nitrite appears not to have been studied. Mossbauer spectroscopy of bacterial ferredoxins indicates an electronic state for iron intermediate between ferrous and ferric in oxidized molecules, and a combination of ferrous and the intermediate state for the reduced molecule (92). In vegetative cells of *C. botulinum*, ferredoxin reduced by hydrogen acts as an electron donor in the reduction of NAD(P) to NAD(P)H, nitrite and hydroxylamine to ammonia, sulfite to sulfide and bisulfite to thiosulfite. Carbon dioxide is assimilated in the presence of ferredoxin and is used for reductive carboxylation of acetyl CoA to pyruvate, succinyl CoA to α -ketoglutarate, propionyl CoA to α -ketobutyrate and isobutyryl CoA to α -ketoisovalerate (16). In other *Clostridium* species, such as *C. perfringens*, that possess nitrate reductase, ferredoxin assists in the reduction of nitrate to nitrite (125).

Rubredoxins have been isolated from many *Clostridium* species and are believed to be present in *C. botulinum*. Anaerobic rubredoxins contain four cysteine residues linked in a tetrahedral arrangement to the single iron atom, and contain no labile or inorganic sulfur. Rubredoxins also participate in electron transport and often have absorption peaks in the visible range when oxidized (114). Flavodoxins, low molecular weight proteins with one flavin mononucleotide prosthetic group, are present in some clostridia, and substitute for ferredoxins in certain reactions. Their presence in *C. botulinum* is unknown.

Ferredoxin and rubredoxin both contain non-heme iron. The presence of heme iron-containing b- or c-type cytochromes or the mono-oxygenase P-450, which may have regulated pre-biotic oxygen toxicity in anaerobes has not been ruled out definitely (90,124,157); if present, however, such molecules are probably not functional. A heme iron "siroheme" has been reported in certain strains of clostridia with sulfite reductases, but its

presence in *C. botulinum* is unknown (100). Other iron-sulfur enzymes (non-heme) that are present in bacteria and that may be present in *C. botulinum* include: hydrogenase, ψ -hydroxylase, pyruvate dehydrogenase, succinic dehydrogenase, formate dehydrogenase, xanthine dehydrogenase and glutamate synthase (162).

Of the other inorganic elements required in synthetic media, magnesium and phosphorous are required for ATP synthesis and for kinase activity. Calcium is necessary for sporulation, and manganese for enzyme reactions. Micromolar quantities of selenium (probably present as sulfur contaminants) are needed for synthesis of glycine reductase (Stickland reaction) and of formate dehydrogenase. Specific requirements for copper, zinc or cobalt have not been reported (45,134,135).

Sporulation

Under certain conditions, e.g., stress, *C. botulinum* cells produce metabolically inert spores that are more resistant than the vegetative cells to heat, radiation or germicides (alcohols, phenolics or mercurials) (56,126). Vegetative cells are killed by heat at 60 C for several seconds, whereas a higher temperature (80 C) and longer time (60 min) often enhance spore germination (57,126,131). Sporulation may be a mechanism of population control to reduce microbial numbers to those resistant to various stress conditions (126). The most effective laboratory medium for inducing sporulation is a cooked meat medium that is high in meat content (126). In semi-synthetic media, the yield of spores is proportional to the content of peptone, with the meat-based peptones being more satisfactory for this purpose than soy-based peptones (126). The biochemical signal within the cell for sporulation is unknown, but cellular autolysis products (5) and highly phosphorylated adenine nucleotides or HPN (138) have been proposed as possible agents. Production of adenosine 5'triphosphate-3'diphosphate (pppApp) during sporulation may provide a system for sequestering essential metals in the spore, as HPNs are effective chelators for divalent metal ions (99). The increased quantity of ATP necessary for HPN formation appears to be furnished through increased activity in the citrulline-ornithine pathway mentioned previously and increased Stickland reaction activity (126). The latter pathway could account for the increased metabolic activity, higher intracellular pH and increased production of volatile fatty acids noted during sporulation.

The microbial spore consists of a core of DNA surrounded, in order, by a plasma membrane, cortical membrane, cortex, spore coat and exosporium (126). The outer protective exosporium has a high content of disulfide bonds and resembles keratin in inertness and in resistance to oxidation or reactivity. The exosporium and the outer spore coat may be removed chemically by treatment of the spores with mercaptoacetic acid, urea and EDTA, exposing the cortex. Further treatment with lysozyme, EDTA and sulfhydryl reagents dissolves the cortex which contains calcium dipicolinate and a loosely cross-linked, highly electronegative peptidoglycan (126).

the added cures, other variations may arise from the type of meat and processing used. Meat may be tissues representing specific groups of muscles, as ham or bacon, or a combination of several tissues from different animals, including skeletal muscle, organ (mainly liver), and adipose tissues, as in sausages or wursts. Processing variables related to curing may include chopping, emulsification, salting, brining, heating and drying, usually in specific regimens (2,75). Skeletal muscles and adipose tissues consist of myofibrils and fat cells in a matrix of collagenous connective tissue, lipids and phospholipids. Muscles are constructed of bundles of fibers, and each fiber is composed of numerous myofibrils. These myofibrils differ in appearance (red and white), contraction rate (fast and slow) and metabolism (glycolytic and oxidative), as well as in the content and type of lipids, depending on the muscle tissue selected (85,112). Such tissue factors influence the content of residual nitrite after curing (81) as well as the final pH, color and texture (2,75).

Muscle biochemicals contain various carbonyl-, sulfur-, and nitrogen-containing substances which on being heated in the absence of nitrite produce various complex chemical components including pyrazines, furans, aldehydes, thiazoles, thiophenes and quinoxalines (153). Representative carbonyl precursors include the α -keto and α -hydroxy metabolic acids as lactic and citric, and quinones and phenols as tocopherols and tyrosine. Sulfur components present in the tissues include: sulfhydryls (cysteine and glutathione), disulfides (cystine and thioctic acid), thioether (methionine), thio-imidazole (ergothionine), amino sulfur acids (taurine, hypotaurine and cysteic acids) and thiazole (thiamine). Nitrogenous substances include: imidazole (histidine), indole (tryptophan), guanidino (arginine), purines (inosine, adenine), pyrimidines (thymine, cytosine), pyridines (niacin, pyridoxine), N-methyl imidazole (anserine), N-methyl amine (sarcosine, creatine, creatinine), quaternary amine (carnitine) and various amines and polyamines from decarboxylations or other reactions of amino acids (2). Practically all the above substances react with nitrite (or nitrous acid) in the cured meat products, increasing the complexity of identification of inhibitory substances. Nitrite reacts with certain of the other cure additives, such as spice components, as well as with the meat constituents. Reviews on these reactions of nitrite by Cassens et al. (21) and Sofos et al. (131) may be consulted for specific reactions.

Composition of the uncured and cured meats with respect to the molar concentration of selected reactants for stoichiometric comparisons are shown in Table 1. As literature values for the most part were not available for the processed products, these values were calculated from cured meat recipes and standard handbook compositional data for uncured meats adjusted for cure additives; they reflect levels before reaction and dehydration (3,4,6,55,58,70,87,109,155,146). The 166 μ moles/100 g of product for the nitrite concentration

(equivalent to 115 ppm) is from 1 to 100 times lower than most of the listed reactants, and exceeds only the listed molar concentrations of iron. The level of 115 ppm was selected as representative of those present in meat (1), and is lower than the present permitted maximum level.

Action of nitrite in curing

After addition of the cure ingredients to the meat products, heat, physical treatments and smoke constituents, where employed, increase the variety of chemical reactions and the kind of products that can form. High salt levels solubilize some of the myofibrillar proteins and, with added sugar, increase the osmolarity of the water phase, leading to membrane transfer reactions and partial cell lysis (2,75). The nitrite-nitrous acid combination is considered as amphiphilic with both hydrophilic properties (nitrite) and lipophilic properties (nitrous acid) (43). Adipose tissues in many cured meat products have interfaces between lipid globules and cytoplasmic tissue proteins (22) which should permit reaction of nitrite under certain catalysts in pseudo-enzymic and phase-transfer type reactions, leading to products distinct from those occurring in a single phase. Heating is required for pork products that have not been frozen or treated under prescribed regimens to destroy cysts of the nematode worm, *Trichinella spiralis* (trichinae). Some cured meats made from certified trichina-free pork such as specialty sausages need not be so heated (75). Frankfurters, fully cooked hams, and cured hams are treated to 160-165 F, uncooked cured hams to 142 F, and bacon to 127-128 F. Although an internal temperature of 137 F is required to destroy trichina, bacon will always be cooked to higher temperatures before being consumed (75). These processing temperatures alone might be insufficient to inactivate vegetative cells of *C. botulinum* (depending upon time-temperature regimen), but have been adequate for anti-botulinal protection because of the supplementary effect of the added nitrite and salt and the very low content of anaerobic bacteria present (80). Botulinal toxin preformed in the food may be inactivated by heat (80 C for 10 min) and by shorter times at higher temperatures.

Reaction of nitrite (as nitrous acid) with various components of meat and the products formed from these reactions are shown in Table 2 [revised from (21) and (117)]. Numerous studies on the fate of added nitrite (38,40-42,47,82,103,156,161) indicate that most of these reactions do occur, particularly the color-producing reactions with heme pigments. The nitrite reaction products (Table 2) are in addition to the products formed through heating of the uncured meats mentioned above and as such constitute only a portion of the total formed substances of cured meat. Cassens' group (21,161) have determined the relative reactivity of nitrite labeled with ¹⁵N with the meat fractions of bacon as percentage ranges of the total nitrite. These values have been listed in Table 3, along with calculated values for the actual molar concentration in the various fractions for an initial

TABLE 3. Estimation of fate of added ^{15}N nitrite in bacon^a.

Fraction	Reaction	Range of % of of ^{15}N found	Calculated maximum con- tent ($\mu\text{moles}/$ 100 g bacon)
Non-heme proteins	various	20-30	50
Free nitrite	none	5-20	33
Nitrosothiols	sulphydryls	5-15	25
Myoglobin	heme iron	5-15	25
Free nitrate	oxidation	1-10	16
Lipids	unknown	1-5	8
Gases (NO, N_2)	Van Slyke, reduction	1-5	8

^aRanges of label in the various meat fractions after addition of added ^{15}N nitrite were reported by Cassens et al. (21). With an assumed nitrite concentration of 115 ppm or 166 $\mu\text{moles}/100$ g product, the maximum content of nitrite in each of the fractions was calculated.

added level of 115 ppm nitrite. Under these conditions, therefore, formation of the postulated di-nitrosyl iron heme complex (Table 2) may occur. In bacon, 100 g contain approximately 12.7 μmoles of heme iron (Table 1), with the myoglobin heme iron fraction containing approximately 25 μmoles of nitrite (Table 3), allowing di-nitrosyl iron formation. Reactivity of iron in this form may be altered from that of the uncured meat or the mono-nitrosyl complex. Reportedly, bioavailability of iron in cured meat products is lower than that in uncured meats when fed to rats (88); differences, however, were not great. Formation of nitrosothiols (Table 3) with a concentration of 25 $\mu\text{moles}/100$ g bacon obviously accounts for only a small part of the cysteine thiol (0.44 m moles from Table 1) and less of the total thiols (58). Nitrosothiols, however, have been postulated as intermediates in the reaction with heme iron to produce nitrosated heme iron and disulfide (93), and in the transnitrosation of secondary amines for form N-nitrosamines (30). Additional nitrosation reactions can occur through a possible reaction with chloride ions which are in excess in cured meats to produce the nitrosating species nitrosyl chloride (24).

Although most of the studies on nitrite action in meat have concentrated on the protein constituents, the contribution of the non-protein components such as lipid peroxides, unsaturated fatty acids, carbonyls, choline, phospholipids, tocopherols, carotene and selenium from fatty tissue, aromatic and sulfur compounds from spices and hydroxyproline from connective tissue should not be overlooked for other reaction mechanisms. Reaction of nitrite with unsaturated fatty acids in model systems has been reported recently, but highly acidic conditions were required and reaction products were unidentified (77).

Since the concentration of nitrite added is in the micromolar range in cured meat products, the problem of identifying the various compounds formed and their inhibitory effect on *C. botulinum* is formidable, as their formation and concentration are functions of both chemical and physical factors.

MECHANISM FOR INHIBITION BY NITRITE

Basic considerations

Nitrite (or nitrous acid) has been assumed by many investigators to be the "active" inhibitor, but such inhibitions may also occur indirectly with reaction products of nitrite or with a combination of both nitrite and its reaction products. These inhibitors may act positively or directly on the cell or spore to produce destruction or denaturation of essential cellular enzymes, nucleic acids or membranes. Alternatively, inhibitors may act negatively or indirectly outside the cell through destruction or chelation of external essential nutrients, thus preventing uptake by the cell (131). The mechanisms of inhibition, both direct and indirect, are examined separately.

Direct inhibition by nitrite

Nitrite is able to react as either an oxidant or a reductant. It can form nitrous acid, which is highly reactive chemically or which can be dehydrated to form gaseous nitrogen oxides. In addition, nitrous acid can react with certain transition metal ions to form complexes. Specific chemical reactions relative to meat constituents have been listed (Table 2).

Nitrite and nitrous acid are effective inhibitors of bacterial growth. When nitrite was allowed to react with citric acid and moisture within a permeable packet, the generated nitrous acid effectively reduced bacterial surface growth of aerobes to <100 colony forming units/g on meat that was physically separated from the packet but maintained within the same package container (14). The mechanism for such inhibitions were not studied. With the aerobic *Staphylococcus aureus*, added nitrite interfered with glucose catabolism, possibly by an initial interference with the phosphoenolpyruvate-dependent phosphotransferase system for transport of glucose across the cell membrane. Inhibition of other enzymic reactions occurs following diffusion of nitrite into the cell (18). With *C. perfringens*, unheated nitrite produced a loss of glyceraldehyde-3-phosphate dehydrogenase activity and a 90% decrease in free sulphydryls (105). Nitrite in unheated laboratory media inhibited growth of vegetative cells of *C. botulinum*. This inhibition was strongly pH-dependent. Perigo and Roberts (111) found a 10-fold increase in inhibition by nitrite for a decrease in 1 pH unit, indicating the probable involvement of free nitrous acid ($\text{pK}_a = 3.29$) rather than nitrite anion. The pH-dependent inhibition was not noted when the medium containing nitrite plus iron and sulfur compounds was heated before inoculation; however, there was an increased inhibition of microbial growth (111).

If nitrite or nitrous acid acts directly on the vegetative cell or spore, the most likely sites of action are enzymes, enzyme co-factors, nucleic acids and cellular membranes. Inhibition of particular enzyme systems in other organisms by nitrite has been mentioned above (18,105). Their involvement in inhibition of *C. botulinum* is

Slyke reaction (45). At pH values above 3 rearrangements also occur resulting in altered alkyl products, but the importance of these reactions in cured meat systems is unknown. Under certain conditions with secondary, tertiary or quaternary amines, nitrous acid produces N-nitrosamines that have been implicated as potential health hazards through formation of mutagenic alkylation products and carcinogens (160). When tested on *C. botulinum*, selected N-nitrosamines were found to be non-inhibitory (154). Attempts to produce mutants of *C. botulinum* with nitrosamines resulted in asporogenous mutants that were nontoxic (57).

In addition to the possible reaction of thiols with nitrite to form nitrosating nitrosothiols mentioned above, the thiols, heme iron and unsaturated lipids may be involved with the added nitrite in the formation of oxidants. *C. botulinum* is extremely sensitive to molecular oxygen and to oxygen radicals such as peroxide, superoxide anion, hydroxyl radical and singlet oxygen since it lacks catalase and superoxide dismutase (76). From this aspect, the production and effects of such oxidants in meat or media should be considered both in the absence and the presence of nitrite. The oxygenated heme pigment in fresh meats, oxymyoglobin, reacts with unsaturated lipids to produce ferric metmyoglobin and lipid hydroperoxide which with further reaction forms various carbonyls (13,84,95). The rates of these oxidations are increased by the cure additives sodium chloride and ascorbic acid (37,69), and may be of importance in development of oxidized fats in meats cured without added nitrite. Transition metal- or sulfur-containing compounds are particularly involved in formation of oxygen radicals (159). Autoxidation of sulfhydryls, heme proteins, ferredoxins, iron-ascorbate complexes or pteridine compounds (44,94), as well as the heating of reducing sugars or cysteine with transition metals (20), leads to formation of hydrogen peroxide, superoxide anion and other bactericidal products. Reducing sugars and the iron and sulfur compounds are present in meat and in bacteriological media. Heating of anaerobic broth media or their exposure to atmospheric oxygen produce bactericidal substances (19). The toxicity of these media (to anaerobic *Peptostreptococcus*) could be abolished through addition of catalase, horseradish peroxidase or metal ion chelating agents (20). Scavengers of superoxide anion, singlet oxygen or hydroxyl radical were ineffective, indicating direct involvement of peroxide (104). With spores of the aerobic *Bacillus subtilis*, direct application of hydrogen peroxide increased the lag phase and incubation time for colony growth, but incorporation of both ferrous and manganous ions in the subsequent recovery medium produced optimum recovery from such treatments (149). Manganese (II) can replace iron in certain heme-type reactions (106), and both ions may be required for specific membrane enzyme activity, such as cytidylate cyclase (23), or for reversal or repair of oxidative degradations. These degradative effects can result from combinations of hydrogen peroxide with

ferrous iron salts to produce Fenton's reagent-type oxidants (150), with pseudoperoxidases such as iron-ascorbate or iron-histidinyl complexes to produce peroxidation of cellular substrates (54) or with peroxidase and halogens (or pseudohalogens, e.g., thiocyanate) to produce bactericidal substances (72).

Production of toxic oxidants may occur through treatment with electromagnetic radiation. Irradiation of foods has been claimed to produce free hydroxyl radicals ($\bullet\text{OH}$) from water that prevent or slow growth of organisms within the food (122). Such radicals may cause single strand breaks (SSB) within the organism's nuclear DNA, which must be repaired before growth can occur (71). Resistance to irradiation by spores of certain strains of *C. botulinum* is related to their tolerance to high salt levels in the recovery medium. Kiss et al. (71) report the NaCl blocks some unknown essential metabolic event occurring before the first cell division and suggest that it could be the SSB repair mechanisms (ligases or excisions) or DNA replication, but not RNA transcription, protein synthesis or cell membrane permeability.

As nitrous acid can be both an oxidant and reductant, its addition to meat products or to laboratory media might be expected to alter their oxidizing capacities. Nitrite initially oxidizes the heme iron in meat to the brown metmyoglobin form which, when subsequently heated and reacted with additional nitrite, is transformed to the familiar pink nitrosyl heme compounds (38). If the meat with nitrite is not heated, lipid oxidation occurs; if heated no detectable lipid oxidation takes place (136,163). Formation of hydroperoxide in lipids occurs in cured meats only after the nitrite concentration has been reduced to < 30 ppm by long term storage; it is followed by increases in the thiobarbituric acid reactive material (presumably malonaldehyde) (164). Nitrate complexes of transition metals which might arise in the unheated cured meat are strong oxidants (48). In the heated cured meat, the antioxidant effect may occur because of inactivation by nitrite complexation of potential metal ion catalysts or from removal of reactive oxygen radicals through trapping by nitrogen oxides. Alternatively, N-nitrosocysteine formation in meats has been postulated as the effective preventative of lipid oxidations (68). Nitrosothiols have been investigated in model systems as possible antibotulinal substances, as the heating of thiols, such as cysteine or thioglycols, in the presence of nitrite and iron salts leads to formation of inhibitory substances (8,148). The iron (II)-amino acid complex may react with nitric oxide from nitrite to produce an iron (III) species, various isolable iron-nitrosyls and a disulfide coordinated to the iron nitrosyl (when cysteine is used) (78). This combination—thiol and transition metal—as noted above, produces the oxidants of peroxide and superoxide anion potentially lethal to anaerobes (19,104). The action of nitrite in modifying such oxidants is unknown, but such oxidants might be responsible for the inhibitions noted in "Perigo effect" studies. In some of these studies, the laboratory

contribute more to the cells' resistance (108). Among species of clostridia, *C. perfringens*, which possesses both nitrate and nitrite reductases, tolerates much higher levels of nitrite than does *C. botulinum* (8,105). During curing of meat products, vegetative cells of nitrite-susceptible organisms are exposed to finite levels of the added nitrite and salt. Definite changes probably occur within these cells as a result of such exposure. The increased salt concentration would alter intracellular toxicity, lower water activity and possibly inactivate certain enzyme systems. Nitrite, in addition, could act directly on the vegetative cell membrane to limit substrate uptake (43,105) or on essential sulfhydryl-containing enzymes (105). Reaction with intracellular iron to produce an inactive form may occur with that present in iron-sulfur enzymes (162) or in ferredoxin or rubredoxin (16,114). Direct reaction with the nucleic acids and nucleotides is another possibility (89). If oxidants within the cell were formed similar to those produced during irradiation (122), the possibility of single strand breaks in the DNA and interference with repair mechanisms by high salt levels is also present (71). Through these mechanisms and with the subsequent heating, it is probable that all vegetative cells of *C. botulinum* (and others) have been destroyed or inactivated.

Spores of *C. botulinum*, however, survive normal meat processing temperatures (126), and their outgrowth must be controlled by other methods. Nitrite addition has been effective in cured meats. Tompkin (139) recently summarized his group's work (140-143,144,145) on nitrite inhibition and possible nitrite substitutes in cured meats. (a) Nitrite levels decline during storage as a function of storage temperature, initial concentration, and time. "A race occurs between spore germination, cell death, and depletion of residual nitrite," with the relative levels of residual nitrite and viable botulinal cells determining the rate of botulinal outgrowth. (b) The minimum inhibitory concentration of nitrite required decreases as the clostridial cell proceeds from a dormant spore to cellular division of the germinated cell. (c) Subtle differences in thermal processing do not affect the degree of botulinal inhibition. (d) With antioxidants, addition of cysteine, sodium ascorbate, isoascorbate and EDTA to perishable canned cured pork formulated with 50 or 156 ppm of nitrite increased botulinal inhibition, but addition of phenolic antioxidants BHA or TBHQ was not as effective. EDTA alone had no inhibitory effect. (e) An excess of "available" iron negates the inhibitory effect of residual nitrite.

Addition of iron salts (ferrous or ferric), reduced iron powder, heart muscle or hemoglobin increased the content of iron in the test medium, generally reduced the content of residual nitrite and markedly reduced the inhibitory activity of added nitrite. Incorporation of liver in place of skeletal or heart muscle did not cause a loss of inhibition. Although the iron content in liver is equal to or higher than that of heart or skeletal muscle, such iron

may be complexed as ferritin.

Tompkin (139) concluded: "residual nitrite serves as a reservoir for the formation of nitric oxide which can react with iron. A plausible hypothesis for the inhibitory effect of nitrite upon *C. botulinum* is the reaction of nitric oxide with an iron-containing compound, such as ferredoxin, within the germinated cell. Such a reaction could interfere with the energy metabolism of the germinated cell and prevent outgrowth."

Involvement of nitrite with iron appears to be a major factor in the inhibition of outgrowth of the botulinal spore. Sites of action for nitrite other than ferredoxin are probable, since clostridial ferredoxin appears not to be inactivated by nitrite in those species possessing nitrate and nitrite reductases (125). As with vegetative cells, reaction of nitrite with iron-containing enzymes, such as succinic dehydrogenase, lipoygensases or xanthine dehydrogenase, or other metalloproteins could be important (123,139,162). Complexes of ferrous iron may be important for enzyme activations. The ferrous-3-aminopicolinate complex (related to cell wall picolinic acid) can activate phosphoenopyruvate carboxykinase whereas the thiol analog acts to inhibit them (86). Additionally, the metal iron may be involved with toxin formation. High levels of iron appear to depress the toxigenic effect of *C. botulinum* in vegetables by causing production of lower molecular weight forms of the toxin with lowered oral toxicity (137). Most other metal ions tested had no effect.

The action and form of iron within the germinating cell and during outgrowth has not been defined. With non-biochemical reactions, iron as well as certain reducing agents can catalyze formation of hydroxyl radicals from superoxide (53), but a ferrous iron-diimine complex can retard the ferric ion catalyzed decomposition of hydrogen peroxide (28). A nitric oxide complex of iron with cysteine appears to resemble lipoyxygenase in its ESR spectra (123), and may be involved in oxygen transfer reactions. However, addition of nitrite (or EDTA) to cooked meat reduces significantly the oxidation of lipids catalyzed by the iron released from bound heme pigments (63). An examination of the possible involvement of spore iron-nitrite complexes in oxidative reactions should indicate whether inhibition might arise from production of superoxide or hydroxyl radicals. Either could be lethal to the spore (11,19,44,71), but superoxide anion often can be dismutated to less harmful forms by copper complexes. This might be a factor in the experiments mentioned above in which liver meat gave divergent results from other meats, since liver tissue has quite high concentrations of copper (155).

Because the inhibitory action of nitrite decreases with time of storage, organisms, particularly spores, cannot be completely destroyed during inhibition. If the enzymes or the nucleic acids of the cells were denatured or mutated, then reduction or removal of free nitrite might not allow growth of these cells. When repair mechanisms are present for correcting such damage, then these

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