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Iron Requirement of Clostridium Botulinum Type A and Characterization of Iron-Sulfur Proteins in Nitrite Treated and Untreated Botulinal Cells

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IRON REQUIREMENT OF <u>CLOSTRIDIUM</u> <u>BOTULINUM</u> TYPE A

AND CHARACTERIZATION OF IRON-SULFUR PROTEINS IN

NITRITE TREATED AND UNTREATED BOTULINAL CELLS

by

Divya Shree A. Reddy

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<div align="right">Divya Shree Reddy</div>

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Iron Requirement of <u>Clostridium</u> <u>botulinum</u> Type A

and Characterization of Iron-Sulfur Proteins in

Nitrite Treated or Untreated Botulinal Cells

by

Divya Shree A. Reddy, Doctor of Philosophy

Utah State University, 1985

Major Professor: Dr. Daren P. Cornforth

Department: Nutrition and Food Science

The effect of added iron on the growth of <u>Clostridium</u> <u>botulinum</u> type A in a chemically defined medium was studied. Growth of <u>C.</u> <u>botulinum</u> was supported by an iron level of 0.05 ug/ml with maximum growth observed at a level of 3 ug iron/ml.

Electron paramagnetic resonance (EPR) studies were conducted to detect the presence of iron-sulfur centers and iron-nitric oxide complexes in untreated and nitrite treated cell-free extracts of <u>C.</u> <u>botulinum</u> type A. Untreated extracts of <u>C.</u> <u>botulinum</u> exhibited EPR signals in the oxidized and reduced states characteristic of a "HiPiP-type" iron-sulfur center (g=2.02) in the oxidized state and a reduced signal at g=1.94, characteristic of a reduced iron-sulfur center. Extracts of <u>C.</u> <u>botulinum</u> treated with nitrite exhibited an EPR signal at g=2.035, characteristic of iron-nitrosyl complexes, with the simultaneous disappearance of the the signal at g=1.94. This indicates that nitrite reacts with the iron-sulfur centers in

botulinal cells to form iron-nitrosyl complexes. Addition of ascorbate with nitrite intensified the EPR signal at g=2.035, probably by enhancing the reduction of nitrite to nitric oxide.

A cytochrome c reduction method was used for the determination of ferredoxin activity in untreated and nitrite treated cells of C. botulinum type A from which ferredoxin had been partially purified. Untreated extracts of C. botulinum reduced cytochrome c which demonstrates ferredoxin activity within the cells. Treatment of the cells with nitrite at a level of 1000 ppm for 45 min was found to inhibit ferredoxin activity by 90%. Boiling the partially purified ferredoxin from the untreated cells for 5 min inactivated the protein.

Pyruvate-ferredoxin oxidoreductase activity in partially purified extracts of nitrite treated and untreated cells of C. botulinum was determined by assaying for FAD reduction and acylhydroxamate formation. Nitrite treated cells exhibited an inhibition of 70% of FAD reducing activity and 80% inhibition of acylhydroxamate formation when compared to the untreated cells. Boiling inhibited the activity of partially purified oxidoreductase activity by more than 90% in both the assays.

(97 Pages)

CHAPTER I

INTRODUCTION

Cured products are frequently subjected to a minimal heat treatment to maintain a satisfactory appearance and texture. A majority of such sub-lethally processed cured meats are completely stable when stored at ambient temperatures (Perigo et al., 1967). This stability results from a number of factors, of which the presence of nitrite in cured meats is one.

Sodium nitrite has long been used as an additive in cured meats, where it has antimicrobial and antibotulinal properties (Pivnick et al., 1970). Nitrite, originally present as an impurity in curing salts has been shown to influence botulinal safety, quality and, identity of cured meat products (Sofos and Busta, 1980).

The addition of nitrite to cured meat products has been source of controversy since it was documented that nitrite may be a precursor to carcinogenic nitrosamines, especially in fried, cured meats such as bacon (Pensabene et al., 1974). Several hundred compounds have been tested but no single alternative posessing all the useful functions of nitrite has been identified. The search for an effective nitrite replacement has been complicated by the fact that the antibotulinal mechanism of nitrite remains unknown.

Several hypotheses have been put forward related to the indirect and direct mechanisms of inhibition of Clostridium botulinum by nitrite. Perigo et al. (1967) showed that nitrite, on heating, reacts with components in the medium, producing a substance which is

extremely inhibitory to the growth of C. botulinum vegetative cells.

Although Ashworth and Spencer (1972) were able to show the Perigo

effect in a model meat system, they stated that from the work

reported, there was no evidence that a Perigo effect was involved,

under practical conditions, with the safety and stability of cured

meats.

Tompkin et al. (1978a) suggested that nitrite reduced the iron

available for botulinal growth by binding with the iron in meat.

Benedict (1980) also postulated that nitrite may inhibit botulinal

growth indirectly by reacting with the vital nutrients like iron,

outside the cell, through chelation or destruction and thus prevent

uptake by the cell.

Riha and Solberg (1973) measured the sodium nitrite level in a

chemically defined microbiological medium for Clostridium perfringens

and found no difference in the amino acid composition between nitrite

containing and nitrite free medium. This work eliminated the

possibility of a reaction between nitrite and amino acids creating

nutritional deficiency of amino acids.

Riha and Solberg (1975) also found the inhibitory effect of

nitrite on C. perfringens to be permanent and concluded that the

inhibition was due to a reaction of some inhibitory agent formed by

nitrite, or the reaction of nitrite with the cell itself to render it

incapable of growth.

A hypothesis by Tompkin et al. (1978a) for the mechanism of

nitrite inhibition which is compatible with existing information is

that nitric oxide reacts with an essential iron containing compound

such as ferredoxin within the botulinal cell, thus preventing growth.

The two iron-sulfur proteins that participate in the phosphoroclastic, oxidative cleavage of pyruvate are ferredoxin and pyruvate-ferredoxin oxidoreductase. Simmons and Costilow (1962) demonstrated the presence of the phosphoroclastic system in C. botulinum. Woods et al. (1981) observed that the phosphoroclastic system in C. sporogenes was inhibited upon treatment with nitrite by the reaction of nitric oxide, produced from nitrite, with the non-heme iron of pyruvate-ferredoxin oxidoreductase. Woods and Wood (1982) also reported that the phosphoroclastic system, as a whole, was inhibited by nitrite treatment in several types of C. botulinum, as measured by an increase in pyruvate accumulation in the medium.

To date, the presence of ferredoxin in C. botulinum has not been reported. It has also not been shown if ferredoxin, or pyruvate-ferredoxin oxidoreductase, or both, are the site of nitrite action in the inhibition of the phosphoroclastic system in C. botulinum.

Consequently, the purpose of this study is (1) to determine the effect of added iron on the growth of C. botulinum type A in a chemically defined medium, (2) to test for the presence of iron-sulfur centers by electron spin resonance spectroscopy in nitrite treated and untreated cells of C. botulinum, and (3) to test for ferredoxin activity in nitrite treated and untreated cells by the cytochrome c reduction method, and to test for pyruvate-ferredoxin oxidoreductase activity in ferredoxin-free protein extracts of nitrite treated and untreated cells of C. botulinum by measuring acylhydroxamate formation and FAD reduction.

CHAPTER II

REVIEW OF LITERATURE

Nitrites in Cured Meats

The first use of nitrites in cured meats is unknown although it is cited that before the Christian era, "nitre" or saltpetre was used in meat in China and India (Binkered and Kolari, 1975). The term nitrite is interpreted in the meat industry to be sodium nitrite, and will be referred to as such.

The sodium salt of nitrite is a unique ingredient in meat curing mixtures that has chemical and microbiological functions in cured meats (Sofos and Busta, 1980). It is associated with the development of the characteristic color (Cassens et al., 1979) and flavor in cured meats and, serves as an antioxidant preventing off-flavors and odors (Wasserman and Tally, 1972). The other major function is the role of nitrite in inhibiting the growth and toxin production of Clostridium botulinum, especially when cured meat products are stored at elevated temperatures (Sofos and Busta, 1980). Nitrate is generally considered as non-essential to the botulinal safety of the products and probably functions as a source of nitrite in cured meats (Christiansen et al., 1973, 1974). The USDA Expert Panel on Nitrites, Nitrates, and Nitrosamines recommended the elimination of nitrates from cured meat products (Sofos et al., 1979).

The level of nitrite needed to produce a satisfactory cured meat color for a limited time is as low as 5 ppm (Ingram, 1974) but upto 20 ppm of nitrite is necessary to provide adequate color stability.

However, nitrite concentrations higher than those necessary for color and flavor development are required for reasonable botulinal inhibition. Grever (1974) concluded from his experiments that to guarantee complete prevention of clostridial growth, 200 mg/Kg nitrite should be added.

The current permitted level of nitrite in most cured meats is 156 ppm (.25 oz per 100 lbs meat). The USDA issued a new regulation in 1978 lowering the level of nitrite in bacon to 120 ppm (.19 oz per 100 lbs meat) with the use of 550 ppm (.88 oz per 100 lbs meat) of sodium ascorbate or isoascorbate (Cerveny, 1980). This change was made because of the doubts regarding the safety of nitrites in meats as a factor in the production of nitrosamines and later as potential carcinogens themselves.

Nitrosamine Formation in Cured Meats

Nitrosamines became suspect in the 1960's. Lijinsky and Epstein (1970) first reported that nitrates and nitrites were precursors in the formation of nitrosamines by interacting with secondary amines, and these nitrosamines caused cancer in laboratory animals. Schreck (1975) also reported the formation of nitroso compounds from the reaction between nitrite, nitrous acid and either secondary amines or tertiary aromatic amines. Many N-nitroso compounds were found to be carcinogenic to a wide range of animal species (Newton et al., 1972) but there was no direct evidence of the carcinogenicity of nitrosamines in man (Crosby and Sawyer, 1976).

Volatile nitrosamines were first reported in bacon in 1971

(Fazio et al., 1971; Crosby et al., 1972). In 1972, the FDA-USDA announced that nitrosopyrrolidine, a nitrosamine, was formed in retail-purchased bacon upon frying while no nitrosamines were detected in raw bacon (Herring, 1973). Fried bacon and some samples of fried country-style ham were the only cured meats in the United States that were found to contain detectable levels of nitrosopyrrolidine (Greenberg, 1977).

Christiansen et al. (1973) on analyzing comminuted ham with varying levels of nitrate and nitrite for 14 different volatile nitrosamines found the samples to be negative at detection levels of 1 ppm nitrite. Nitrosamines, N-nitrosodimethylalanine and N-nitroso-pyrrolidine, were found in all samples of bacon tested by Wasserman et al. (1978) who calculated that frying bacon at low or medium temperatures for less than 10 min could result in less than 10 ug/Kg nitrosamine formation. Gary and Randall (1979) in an update on the nitrosamine problem in cured meats also observed that bacon was still the product of concern where N-nitrosopyrrolidi-ne was commonly detected in cooked samples.

Newberne in 1979 reported, based on a study on rats, that sodium nitrite itself was weakly carcinogenic. The Newberne report on review revealed that certain tissue abnormalities were misinterpreted for cancer and that some cancerous lesions occur spontaneously in rats and have no comparison in human cancer (Anon, 1980). The Nitrite Safety Council report (1980) stated that from a comprehensive study of nitrosamines in cured meat products in the U.S., cooked sausages, semi-dry and dry sausages were free of nitrosamines. Increased

incidence and quantitatively more nitrosamines were detected in fried bacon.

The USDA issued new regulations to reduce the level of sodium nitrite in bacon to 120 ppm and permitted the use of sodium ascorbate or isoascorbate (550 ppm) along with it. They also set the maximum level of nitrosamine formation at 10 ppb (Butler, 1980; Havery and Fazio, 1985).

Reducing Agents in Cured Meats

Sodium nitrite has been observed to be more inhibitory to microorganisms in the presence of reducing agents (Tompkin et al., 1978b) which probably accelerate the reduction of nitrite to nitric oxide, thus enhancing the formation of iron-nitrosyl complexes. Mirvish et al. (1972) reported that the ascorbate-nitrite reaction could be a possible means of blocking the formation of carcinogenic N-nitroso compounds and the extent of blocking depended on the compound nitrosated and on the experimental conditions.

Woolford and Cassens (1977) observed that the use of ascorbate in curing bacon alters the distribution of radioactively labelled nitrogen amoungst the various fractions of protein and lipid and lowered the level of residual nitrite. Tompkin et al. (1978b) found that the addition of sodium isoascorbate to the formulation for perishable canned comminuted cured meat markedly enhanced the efficacy of nitrite against C. botulinum. They suggested that the enhancement of the antibotulinal function of nitrite by isoascorbate may be by the prevention of the repair of damaged material or formation of a new

cation dependent material.

Alternatives to Nitrite in
Cured Meat Products

The search for a suitable replacement for nitrite in cured meats was originally directed to the discovery of a single compound that would have all the beneficial properties of nitrite without being a health hazard (Sebranek, 1979). Extensive research has been conducted in this area and several hundred compounds have been tested without success (Howard et al., 1973; Kemp, 1974). Finding a substitute for nitrite in cured meats which could provide suitable protection against C. botulinum remains the most important problem (Sebranek, 1979).

Widdus and Busta (1982) noted that the most promising alternatives to nitrite, identified by the National Academy of Sciences, National Research Council, were sorbate, sodium hypophosphite, fumerate ester, acidulation by lactic acid producing bacteria, alpha tocopherol, ascorbate and, irradiation.

Although many of these compounds have been found to be effective in a model system, their effectiveness in commercial products is still being studied. The search for a suitable substitute for sodium nitrite in cured meats is complicated by the fact that the mechanism of inhibition of growth of C. botulinum by nitrite remains unknown. Several hypotheses have been put forward regarding the direct and indirect mechanism of nitrite inhibition of clostridial growth in cured meats.

Nitrite Inhibition of C. botulinum

Indirect inhibition of botulinal growth by nitrite: Perigo et al.
(1967) first showed that nitrite upon heating in a laboratory medium
produced an unknown compound that was inhibitory to the growth of
C. sporogenes. This compound differed from unheated sodium nitrite
in that its activity was less pH dependent, its response was less
variable and, it was an extremely potent inhibitor that was formed at
very low nitrite levels.

Perigo and Roberts (1968) confirmed the inhibitory effect of sodium
nitrite heated in a laboratory medium on thirty clostridial strains,
which included fourteen strains of C. botulinum and eight strains of
C. welchii. Sodium nitrite at levels of 5-20 ppm in a laboratory
medium, at pH 6.0 and 7.0, was found to be sufficient to prevent the
growth of test organisms of a wide range of clostridia and not just
C. sporogenes.

The inhibitory factor produced by sodium nitrite in a heated
laboratory medium was termed the "Perigo inhibitor" (Johnston et al.,
1969). They studied the role of nitrite in the formation of the
Perigo inhibitor in a meat system and found that the addition of one
percent meat to the culture medium interferred with the formation of
the inhibitor and twenty percent or more completely prevented it.
Addition of non-fat meat solids to a medium with the inhibitor already
present neutralized its activity. Johnston et al. (1969) concluded
from their results that the Perigo inhibitor produced in the
laboratory medium was of no importance in explaining the role of

nitrite in the safety and stability of commercial canned cured meat products.

Roberts and Smart (1974) also confirmed the formation of an inhibitory compound on heating nitrite in a laboratory medium. Most spores remained inhibited after three months incubation but some of the spores were viable upon resuspension in a fresh nitrite-free medium. The inhibitory effect of nitrite was also found to be temporary because of the growth of cells upon the inoculation of vegetative cells of the test organism into a cell free medium.

Ingram (1974) postulated the mechanism of nitrite inhibition to be the reaction of nitrite via nitrous acid with amino and other groups in proteins with the direct dependence of nitrous acid on pH. The enhanced bacteristatic effect of nitrite under acidic conditions was observed and reported by several researchers (Castellini and Niven, 1955; Tarr, 1941) and was attributed to the presence of nitrous acid at low pH (Shank et al., 1962; Mirna, 1974).

van Roon (1974) noted the presence of iron ions in the Perigo medium and observed that as a transitional metal, iron could react with cysteine and nitric oxide to form coordination complexes. Iron added in small quantities to a beef product caused an increase in the rate of nitrite reduction. Vahabzadeh et al. (1983) found that botulinal inhibition was somewhat decreased when iron (in the form of ferric chloride or myoglobin) was added to samples of ground pork. They concluded that the stimulation of swelling in cured meats associated with iron was more due to the depletion of residual nitrite by the added iron, rather than the direct stimulation of growth by

providing iron as an essential nutrient for growth.

A Perigo-type inhibition of C. perfringens growth by sodium
nitrite in a chemically defined medium, at pH 6.3 and heated to 121 C
for 15 min, was demonstrated by Riha and Solberg (1973). The
possibility of nitrite reacting with amino acids to create a
nutritional deficiency was eliminated by these workers. They measured
the sodium nitrite in a chemically defined media for the growth of
C. perfringens and found no difference in the amino acid composition
of the nitrite containing and nitrite-free media.

Tompkin et al. (1978b) suggested that nitrite reduced
iron available for botulinal growth by binding with the iron in meat.
Benedict (1980) observed that nitrite may react indirectly with vital
nutrients such as iron, outside the cell, through chelation or
destruction and thereby inhibit uptake by the cell.

Direct inhibition of botulinal growth by nitrite: Roberts and
Smart (1974) examined the effect of prolonged incubation on the
survival of clostridial spores in a Perigo type medium. Inhibited
cells were reinoculated in fresh basal medium. Heated and unheated
spores of C. botulinum type A and E were found to grow, indicating
that inhibition for three months had occured without complete loss of
viability.

In contrast, Riha and Solberg (1975) found the inhibitory effect
of sodium nitrite on C. perfringens to be permanent in a chemically
defined medium. Cultures that failed to reproduce during the growth
study demonstrated no growth during prolonged incubation and failed to
reproduce after resuspension in a nitrite free medium. They

concluded that the inhibition of C. perfringens was due to the reaction of some inhibitory agent formed by nitrite, or by the reaction of nitrite with the cell itself to render it incapable of growth.

Tompkin et al. (1978a) concluded from their experiments that nitric oxide, formed from nitrite via nitrous acid, reacted with an iron-containing compound such as ferredoxin within the germinated cell and prevented growth of the organism. Benedict (1980) also suggested that nitrite may react directly with the spore or cell to cause destruction or denaturation of cellular enzymes, nucleic acids or membranes that could be essential to the organism.

Ingram (1939) observed that nitrite inhibited respiration in Bacillus cereus by reacting with the amino groups of dehydrogenases. Castellini and Niven (1955) suggested that nitrite inactivated certain bacterial enzyme systems which had active sulfhydryl groups. Morris et al. (1984) postulated that similar to nisin action, nitrite reacts reversably with membrane sulfhydryl groups of B. cereus to inhibit transport of nutrients across the membrane and thereby inhibit growth.

In a study by O'Leary and Solberg (1976), nitrite was found to reduce the concentration of free sulfhydryl groups of soluble cellular components of C. perfringens by about 91%, decrease aldolase activity by 67% and abolish the activity of glyceraldehyde-3-phosphate dehydrogenase (EC1.2.1.12). Bard and Gunsalus (1950) had shown that the glycolytic pathway in C. perfringens required metallic ions, ferrous ions being the most effective, and linked it to the inactivation of aldolase enzyme.

Rowe et al. (1979) reported that nitrite at levels greater than 25 mM (about 1670 ppm) almost completely inhibited the active transport, oxygen uptake and oxidative phosphorylation in Pseudomonas aeruginosa (a strict aerobe). Under denitrifying conditions nitrite can serve as the terminal electron acceptor to support oxidative phosphorylation and active transport in P. aeruginosa. Yarbrough et al. (1980) showed that nitrite inhibited proton dependent, active transport of proline in Escherichia coli (a facultative anaerobe), probably via the inhibition of the cytochrome chain, preventing the formation of a proton gradient.

Yarbrough et al. (1980) noted that nitrite inhibited growth of aerobic bacteria in several ways. Nitrite interferred with energy conservation by preventing oxygen uptake, oxidative phosphorylation and proton-dependent active transport. Nitrite acting as an uncoupler results in the destruction of the proton gradient and thirdly, nitrite inactivates certain metabolic enzymes. They found that nitrite had more than one site of attack in the bacterial cell.

Meyer (1981), comparing carbon monoxide, nitric oxide and nitrite as inhibitors of nitrogenase from C. pasteurianum, found that both nitric oxide and nitrite inhibit acetylene reduction, hydrogen evolution and ATP hydrolysis with the preferential disruption of the iron-sulfur cluster in the protein. The effects of NO and nitrite were observed to be irreversable.

Woods et al. (1981) suggested that, in C. sporogenes, nitrite inhibits the phosphoroclastic system by the reaction of nitric oxide, formed from nitrite, with the non-heme iron of pyruvate-ferredoxin oxidoreductase. Woods and Wood (1982) demonstrated the presence of the

phosphoroclastic system in C. botulinum and concluded that this
system was inhibited by sodium nitrite, although C. botulinum has
not been shown to contain ferredoxin.

Reddy et al. (1983) found that nitrite treatment of actively
growing vegetative cells of C. botulinum type A caused the
disappearance of the EPR signals for reduced iron-sulfur centers and
the appearance of a signal for an iron-nitric oxide complex. They
concluded that inactivation of iron-sulfur proteins in the presence of
nitrite would inhibit growth and could be the most important site for
nitrite reaction and the basis of the antibotulinal activity of
nitrite.

Tu et al. (1984) reported a possible mechanism of nitrite
inhibition of C. botulinum growth, related to the formation of a
thionitrite derivative of coenzyme A (CoA). At 25 C and at pH 6 or
below, nitrite reacted with CoA in aqueous media to form a S-nitroso-
thiol (thionitrite) derivative which was incapable of accepting acyl
groups, thus causing inhibition.

Iron Requirement of Clostridia

Bard and Gunsalus (1950) noted that the importance of iron in the
growth and toxin production of the genus Clostridium was well
documented. Theories dealing with the mechanism of inhibition of C.
botulinum growth and toxin production involve iron, either within the
cell or outside the cell, in the surrounding environment (Riha and
Solberg, 1975; Tompkin, 1978; Johnston et al., 1969).

Feeney et al. (1943) observed that iron in the form of ferrous

sulfate was one of the components of the synthetic growth media for C. tetani. They also noted that iron was the single most impotant limiting growth factor in a casein hydrolysate medium for optimal toxin production of C. tetani. Iron appeared to be the first nutrient in the medium to disappear under conditions of maximal toxin production.

Bonventre and Kempe (1960) noted a considerable decrease in toxicity of the filterate of C. botulinum in the presence of ethylenediaminetetraacetic acid (EDTA) at a final concentration of 0.001 M. The addition of ferrous or magnesium salts reversed the effect of EDTA on cell multiplication. Winarno et al.(1971) also reported a similar inhibitory effect of EDTA in concentrations above 2.5 mM on the germination and outgrowth of spores of C. botulinum type A and to toxin production in a fish homogenate.

Smith (1977) reported that the heat resistance of C. botulinum spores was decreased at lower concentrations of iron and calcium in the growth medium. A similar result was observed by Farkas and Gercz (1980) when EDTA was present in the medium and they suggested that the effect was due to the chelation of divalent cations in the medium which may be important to the stability of the cell membrane.

Pappenheimer and Shaskan (1944) were able to determine that 0.06 ug iron per ml was required for maximum growth of C. welchii. Shankar and Bard (1951) observed that while 0.2 ug/ml of iron was sufficient to support growth, maximum growth of C. perfringens occured at a level of 2 ug iron per ml with some growth inhibition as the level of iron increased.

Curran et al. (1981) observed that absence of added iron always resulted in a significantly longer generation time for C. perfringens than that observed when any level of iron, from 1 ug per ml to 18 ug per ml were added. Vahabzadeh et al. (1983) observed no differences in nitrosoheme or soluble iron content between swollen and unswollen samples of pork inoculated with botulinal spores, and postulated that the iron requirement for the growth of C. botulinum cells must be very small.

Although the necessity of iron for the growth and toxin production of C. botulinum has been well established (Smith, 1977), the iron requrement for growth has yet to be determined.

The Phosphoroclastic System in Clostridia

Nitrogen fixation in C. pasteurianum was demonstrated by Mortenson et al. (1963) to be dependent on the transitory intermediates of pyruvate metabolism. Pyruvate utilization via the phosphoroclastic reaction involves the oxodative decarboxylation of pyruvate to acetate with the production of carbon dioxide.

The reactions involved are as follows (Sagers et al., 1961; Uyeda and Rabinowitz, 1971a):

$$Pyruvate + CoA + Fd_{ox} \xrightarrow{enzymes} Acetyl\text{-}CoA + CO_2 + Fd_{red}$$

$$Acetyl\text{-}CoA + HPO_4^= \longrightarrow Acetyl\text{-}PO \xrightarrow[ADP \quad ATP]{} Acetate$$
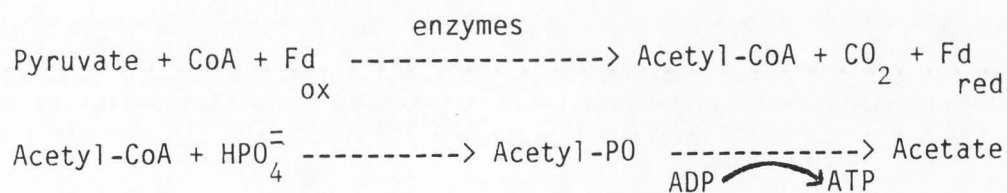
The phosphoroclastic reaction was reported in C. cylindosporum (Rabinowitz and Barker, 1955), C. butyricum (Mortlok et al., 1959), and C. acidiurici (Sagers et al., 1961).

The phosphoroclastic system was also readily demonstrated in glucose adapted and non-adapted cells of C. botulinum by Simmons and Costilow (1962). Mortenson et al. (1963) demonstrated the phosphoroclastic reaction in C. pasteurianum. C. sporogenes was shown to contain the phosphoroclastic system by Woods et al. (1981).

The two iron-sulfur proteins that function in the phosphoroclastic reaction are ferredoxin and pyruvate-ferredoxin oxidoreductase which catalyses the oxidative decarboxylation of pyruvate (Uyeda and Rabinowitz, 1971a).

Ferredoxin: An electron-transferring protein that linked hydrogenase with a variety of electron donors or acceptors was obtained from C. pasteurianum. The new factor which contained iron but no detectable heme or flavin was labelled ferredoxin (Mortenson et al., 1962). Ferredoxin is an iron-sulfur protein which has been found in the cytoplasm of many species of bacteria, with a molecular weight ranging between 6000 and 14000 daltons.

Ferredoxins are widely distributed among bacteria and have been isolated from all the three major physiological groups of bacteria found in nature, that is fermentative, aerobic and photosynthetic (Yoch and Valentine, 1972). Several clostridial species such as C. pasteurianum, C. acidiurici, C. butyricum, C. sporogenes, C. cylindrosporum, C. tetanomorphum, and C. thermosacharolyticum were shown to contain ferredoxin (Lovenberg et al., 1963). All ferredoxins isolated to date have been acidic proteins which have a high affinity for DEAE-cellulose. Tagawa and Arnon (1962) were able to first isolate a crystalline preparation of ferredoxin from C.

pasteurianum using DEAE-cellulose chromatography and ammonium sulfate
fractionization. Mortenson (1964) developed a more rapid acetone
extraction procedure using 50 percent acetone. Polyethyleneimine
precipitation of ferredoxin, after acetone precipitation, with the
use of DEAE-cellulose chromatography was proposed by Schonheit et al.
(1978).

The oxidized form of clostridial ferredoxin can be detected by
specific absorption peaks at 390-395 nm which disappear upon reduction
(Benedict, 1980). In vegetative cells of C. botulinum, ferredoxin
reduced by hydrogen probably acts as an electron doner in the
reduction of NADP to NADPH. Thus, purified ferredoxin can be measured
by the cytochrome c reduction method in the presence of NADPH and
ferredoxin-NADP reductase (FNR) (Shin, 1971). The one to two electron
transfer between ferredoxin (1-electron acceptor) and NADP $+$ (2-
electron doner) is catalyzed by FNR (Zanetti et al., 1984). The
transfer of electrons is carried out via a ternary complex of Fd.FNR.
NADP $\pm$ (Batie and Kamin, 1984).

The characteristic dark brown color of ferredoxin was found to be
due to the iron-sulfur prosthetic group of the protein. A clostridial
ferredoxin molecule contains two iron-sulfur clusters, each cluster
having four atoms of iron and four atoms of inorganic sulfur joined in
a distorted cube that is connected to the protein by an iron-cysteinyl
linkage (Packer et al., 1977).

The iron content of clostridial ferredoxin was reported to be 0.5
umole Fe per mg of protein and of a nonheme nature (Mortenson et al.,
1962). The sulfide which was in equimolar concentration with the iron

was found to be liberated as hydrogen sulfide upon the acidification of clostridial ferredoxin (Lovenberg et al., 1963).

Electron paramagnetic resonance (EPR) spectroscopy of the iron-sulfur prosthetic group of ferredoxins provided direct physical evidence of an iron-sulfide bond in a number of ferredoxins (Yoch and Valentine, 1972). Reduced spinach ferredoxin was observed to have an EPR spectrum that was quantitatively similar to many other non-heme iron proteins with a feature at g= 1.94 (Palmer and Sands, 1966). Palmer et al. (1966) also reported a major band of resonance in an EPR spectra at g=1.94 which was a result of the molecule gaining an unpaired electron upon reduction. This signal was observed in bacterial ferredoxins only at liquid helium temperatures.

The iron-sulfur prosthetic group of ferredoxin functions as a center for electron transfer. The oxidation-reduction potentials of ferredoxin vary, depending on their source (Yoch and Valentine, 1972). Tagawa and Arnon (1968) compared the electron-carrier properties of plant and bacterial ferredoxins. The oxidation-reduction potentials, measured at different partial pressures of hydrogen and at varying pH values, were found to be E = -420 mV for spinach ferredoxin and E = -390 mv for clostridial ferredoxin.

Eisenstein and Wang (1969) observed that each mole of clostridial ferredoxin (from C. pasteurianum) reduced two moles of ferricyanide. They showed that clostridial ferredoxin could hold two electrons, at non-interacting sites, each capable of a one-electron oxidation-reduction step.

Anaerobic bacteria that grow using sugars and organic nitrogen

compounds as nutrient sources often depend on ferredoxin for the essential oxidation-reduction reactions and electron transfer. Yoch and Valentine (1972) reported that reduced ferredoxin produced by the oxidation of pyruvate and other substrates functioned as an electron source for hydrogen formation which seemed to be essential for the growth of many fermentative bacteria.

Carbon dioxide fixation into pyruvate in cell extracts of C. butyricum was shown to require, in addition to carbon dioxide and acetyl phosphate, a strong reductant. Bachofen et al. (1964, cited from Yoch and Valentine, 1972) demonstrated that the physiological reductant for pyruvate synthesis by this reaction, in extracts of C. pasteurianum, was ferredoxin. Mortenson et al. (1963) observed that ferredoxin served in the transport of electrons from pyruvate dehydrogenase to hydrogenase in the formation of hydrogen in the phosphoroclastic reaction.

There are about 18 ferredoxin dependent reactions in fermentative bacteria. Some of the ferredoxin-linked dehydrogenases and reductases that handle the substrates to be oxidized or reduced are pyruvate dehydrogenase, pyruvate synthase, NAD reductase, NADP reductase, sulfite reductase, thiosulfate forming system, and nitrogenase (Yoch and Valentine, 1972).

Pyruvate-ferredoxin oxidoreductase: Pyruvate-ferredoxin oxidoreductase is an enzyme that catalyzes the oxidative decarboxylation of pyruvate in the phosphoroclastic reaction (Uyeda and Rabinowitz, 1971a). It is one of the pyruvate dehydrogenases which are iron-sulfur enzymes that contain thiamine pyrophosphate (TPP). In

saccharolytic clostridia such as C. pasteurianum and C. butyricum,
pyruvate dehydrogenase serves in providing glucose-fermenting cells
with reduced ferredoxin for carbon dioxide and NADP reduction, and
acetyl-COA for ATP synthesis (Yoch and Carithers, 1979).

Pyruvate-ferredoxin oxidoreductase has been purified from C.
acidiurici by passing the cell-free extracts through a DEAE-cellulose
column, the flow-through extract being treated with ammonium sulfate
before being passed through a sephadex column (Uyeda and Rabinowitz,
1971b; Raeburn and Rabinowitz, 1971b).

Uyeda and Rabinowitz (1971b) observed through the use of
acrylamide gel electrophoresis and specific staining methods that the
purified enzyme had multiple forms. The enzyme was estimated to have
a molecular weight of 240,000 and contain six non-heme iron, three
acid-labile sulfurs, 0.5-0.8 moles thiamine/mole protein, and
negligible amounts of riboflavin. Raeburn and Rabinowitz (1971a)
reported that the enzyme contained a non-heme chromophore with
equimolar amounts of iron, sulfide, thiamine and trace amounts of
flavin, with a molecular weight of about 215,000.

Uyeda and Rabinowitz (1971a) observed that the iron- sulfur center
of the enzyme was reduced by pyruvate only in the presence of the of
reduced coenzyme A (CoA) which suggested the formation of an interme-
diate. Mortenson (1963) proposed the mechanism of the reaction as:

Pyruvate + TPP-E -------------> (hydroxyethyl-TPP)-E + CO_2

(Hydroxyethyl-TPP)-E +Fd + CoA.SH --------> acetyl-CoA + Fd + TPP-E
ox red

Acetyl-CoA+ $H_2PO_4^-$ ------> acetyl-PO + CoA.SH

Acetyl-PO_4 + ADP ------> acetate + ATP

$$Fd_{red} + 2H^+ ------> Fd_{ox} + H_2$$
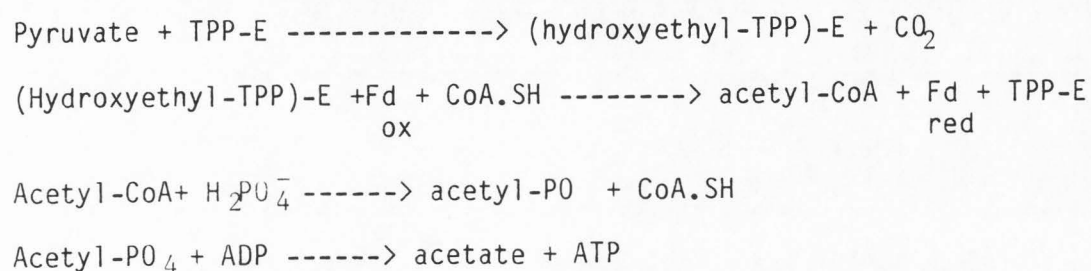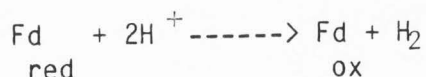
The purified enzyme from C. acidiurici was observed to form one mole of acylhydroxamate (an active intermediate) for each mole of pyruvate degraded (Raeburn and Rabinowitz, 1971a). In the reverse reaction, that is the synthesis of pyruvate from bicarbonate and acetyl phosphate or acetyl-COA in the presence of ferredoxin, the amount of pyruvate synthesized was found to be proportional to the amount of enzyme present.

Woods et al. (1981) determined the effect of nitrite upon the phosphoroclastic system of C. sporogenes. They observed that nitrite treatment of whole cells at a level of 1.5 mM (about 100 ppm) inhibited the production of carbon dioxide and hydrogen by 56% and 48% respectively. In cell-free extracts, 8 mM (about 500 ppm) nitrite caused a 50% inhibition of NAD reduction. In the reconstituted system it was observed that the nitrite treated ferredoxin fraction still showed NAD-reducing activity in the reconstituted system, while the nitrite treated fraction containing the oxidoreductase enzyme showed no NAD-reducing activity in the reconstituted system. They concluded that nitrite inhibition of the phosphoroclastic system in C. sporogenes was by the reaction of nitric oxide, produced from nitrite, with the non-heme iron of pyruvate-ferredoxin oxidoreductase and not by the inhibition of ferredoxin.

Woods and Wood (1982) also observed the inhibition of the phosphoroclastic system as a whole in C. botulinum, as measured by the accumulation of pyruvate, upon nitrite treatment at a level of 4.4mM (about 300 ppm).

Electron Paramagnetic Resonance Spectroscopy

Since the first use of electron paramagnetic resonance (EPR) spectroscopy in the early 1950's EPR, also known as electron spin resonance spectroscopy or ESR, has been finding rapid application in biological and biochemical studies. Carrington and McLachlan (1979) noted that the development of magnetic resonance spectroscopy was one of the most important advances in chemical physics in the last two decades.

Application of magnetic resonance spectroscopy to biological molecules gives information on the structural changes involved in biochemical functions. EPR is sensitive to unpaired electrons and can thus be applied to paramagnetic metalloenzymes, other paramagnetic metalloproteins, and to true radical intermediates in biochemical reactions. As it is unlikely that any one system would have many unpaired electrons, EPR is highly specific (Knowles et al., 1979).

Berliner (1976) also noted that EPR was a sensitive method that could be applied in biochemical and biomedical problems for the identification of free radical intermediates in metabolic reactions, for the observation of stable, naturally occuring paramagnetic species such as transitional metal ions, for the detection of radicals produced by external radiation, and for the analysis of paramagnetic probes introduced into specific biological systems as in spin labeling.

EPR spectroscopy is ideally suited for the study of paramagnetic compounds such as ferredoxin which gain an unpaired electron upon reduction. The EPR spectrum depends upon the spin orientation of the

unpaired electron in a changing external magnetic field. The position of the EPR signal on the spectra, at liquid helium temperatures, gives information as to the type and structure of the compound associated with the paramagnetic electron.

Normally, the unpaired electron exists at a lower energy state where its spin momentum is aligned in the direction of the external magnetic field. Absorption of microwave energy in a molecular environment characteristic for an unpaired electron causes a transition of the unpaired electron to a higher energy state. In this state the electron magnetic moment is aligned opposite to the external magnetic field.

The magnetic moment of the electron (u) is given as

$$u = -g \, B_n \, M_s$$

where, g is the spectroscopic splitting factor which has a value that is dependent on the environment of the electron. B_n is the Bohr magneton, a factor for converting angular momentum to magnetic momentum, and M_s is the angular momentum quantum number with values of plus or minus one half.

In practice, g is calculated as,

$$g = (714.48 \times v) \, / \, H$$

where, v is the microwave frequency in giga hertz (GHz), and H is the magnetic field in gauss.

Each unpaired electron has a characteristic g value by which it can be identified on the EPR spectra. Some characteristic g values for identifying heme and non-heme iron complexes are (Fee,1978):

g = 2.0023 for a free electron

g = 2.02 for a HiPiP (High Potential iron-sulfur Protein) complex

g = 1.94 for a reduced iron-sulfur complex

g = 2.035 for an iron-nitric oxide complex

g = 4.3 for the iron-sulfur complex of rubredoxin

g = 6 for a high spin heme

g = 3 for a low spin heme

Some of the measures taken to check the EPR signals of iron-sulfur complexes on the spectra are to increase the microwave power and the temperature. If the signal is not saturated or, does not disappear on increasing microwave power, and increasing the temperature causes the disappearance of the signal, it is indicative of a fast relaxing $Fe_3S_3^*$ or $Fe_4S_4^*$ center. Fast relaxing centers are those in which the microwave energy that is absorbed is readily dissipated, allowing the complex to relax and be prepared to absorb more energy. This property indicates that the unpaired electron is associated with a large molecule.

Some of the most significant applications of EPR in biological systems have been reported to be in the areas of structural studies of hemoproteins, flavins and flavoenzymes, spin-labelled proteins and interactions with spin-labelled ligands, spin-labelled membranes, the primary photochemical events of photosythesis, studies on irradiated nucleic acids and whole tissues and for the characterization of iron-sulfur proteins and copper proteins (Jones, 1980).

EPR spectroscopy has been extensively used for the identification and study of the mechanism of iron-sulfur centers of enzymes such as

spinach ferredoxin-nitrite reductase (Lancaster et al., 1979), succinate dehydrogenase (Salerno et al., 1976), and for iron-nitric oxide complexes with amino acids, peptides and proteins (Woolum et al., 1968).

Low-temperature EPR spectroscopy is a very sensitive method of detecting iron-sulfur proteins. The demonstration of resonance in appropriately oxidised or reduced samples is taken as presumptive evidence for the presence of iron-sulfur clusters (Orme-Johnson and Orme-Johnson, 1978).

CHAPTER III

EFFECT OF ADDED IRON ON THE GROWTH OF <u>CLOSTRIDIUM</u>

<u>BOTULINUM</u> IN A CHEMICALLY DEFINED MEDIUM

Introduction

Iron has played an important role in elucidating the mechanism of nitrite inhibition of botulinal growth. Theories dealing with the direct or indirect mechanism of inhibition involve iron; either within the cell or outside the cell in the surrounding environment (van Roon, 1974; Riha and Solberg, 1975, and Tompkin, 1978).

Benedict (1980) stated that <u>C. botulinum</u> had a definite growth requirement for iron and in addition, sporulation required a high concentration of iron along with a few other nutrients. He observed that iron could be a factor in the indirect inhibition of growth by nitrite although the action and form of iron in the germinating cell and during outgrowth has not been defined.

The influence of iron on microbial growth and toxin production has been well documented. The products from glucose breakdown by <u>C. welchii</u> were found to depend on the iron content of the cells, with more acid being produced when the concentration of iron was suboptimal for growth. Iron was also the first nutrient in the medium to disappear under conditions of maximal toxin production (Pappenheimer and Shaskan, 1944).

The indispensible requirement of iron for clostridial growth was demonstrated by the essentiality of iron for the aldolase activity and for homolactic fermentation by Bard and Gunsalus (1950) in <u>C.</u>

perfringens. Huhtanen and Wasserman (1975) studied the effect of added iron on the formation of clostridial inhibitors. They observed that a potent anticlostridial inhibitor can be formed in an autoclaved medium with nitrite.

Benedict (1980) postulated that the prevention of uptake of an essential nutrient required for outgrowth, such as iron, may be the principal action of nitrite. Chelation of iron in the medium by nitrite or its reaction products may prevent the solubilization and transport of iron to germinating cells. Cell repair mechanisms, electron transport, metabolic reductions and various cellular enzymes in the cell may be inhibited due to the lack of iron.

The iron requirement of several clostridia have been determined (Feeney et al., 1943; Pappenheimer and Shaskan, 1944; Shankar and Bard, 1951). The growth requirement of iron for C. botulinum has yet to be defined. The purpose of this experiment was to study the effect of added iron on the growth of C. botulinum in a chemically defined medium and to determine the actual requirement of iron for growth.

Materials and Methods

A freeze dried culture of C. botulinum type A vegetative cells (ATCC 19397) was obtained from the American Type Culture Collection, Rockville, Maryland. The cells were propagated in chopped liver broth (Lehninger, 1976; Appendix A) at 35 C for approximately one week.

The cell suspension was centrifuged at 3000xG (International Equipment Co., Boston, MA.) for 15 min. The supernatant was discarded and the cells were resuspended in sporulation medium (Schmidt and

Nank, 1960; Appendix B). After incubation for one week at 35 C the suspensions were heat shocked at 85 C for 5 min to destroy the vegetative cells and toxin, and preserve most of the spores (Woodburn et al., 1979). Spore counts were made by appropriate dilutions in 0.1% peptone water, followed by plating on anaerobic egg agar (Lehninger, 1976, Appendix C).

One hundred spores were added per ml of chemically defined bacteriological medium (Riha and Solberg, 1971, Appendix D) containing 19 amino acids, uracil, adenine, glucose, vitamins and salts. Glucose was passed through a chelex column (Dowex chelating resin, Sigma Chemical Co., Appendix E) before being added to the autoclaved medium. The iron content of the basal medium was determined to be 0.05 ug/ml by atomic absorption spectroscopy (Instrumentation Laboratory Inc., MA., U.S.A.). Filter sterilized ferrous sulfate was added to ten ml of the medium to give levels of 1, 2, 3, 4, and 5 ug of total iron/ml.

Growth was measured after incubation at 35 C for 24, 36, and 48 hr by an increase in turbidity measured at 500 nm (Bausch and Lomb, Spectronic 21) and by plate counts on anaerobic egg agar (Lehninger, 1976, Appendix C). Appropriate dilutions of each sample were made up in 0.1% peptone water and 0.1 ml of each dilution was plated for total cell count.

Double distilled water was used for the preparation of all the solutions. All glassware used was soaked overnight in 30% HCl solution and rinsed in double distilled water. All estimations were carried out in duplicate.

Results and Discussion

Boyd et al. (1948) developed a chemically defined medium to study the factors affecting the growth and toxin production of C. perfringens. Later a chemically defined medium that supports the growth of C. perfringens was developed by Riha and Solberg (1971) containing all the nutrients essential for the growth of the organisms. Several studies have been conducted with C. perfringens using this medium (Riha and Solberg, 1973; Page and Solberg, 1980; Curran et al., 1981).

Preliminary studies showed the Riha and Solberg medium to be suitable for the growth of C. botulinum type A and was used for this study. The experiment was repeated several times with turbidity measurements and cell count taken at 24, 36, and 48 hours of incubation.

The turbidity measurements are presented in Fig. 1. After 24 hr incubation, a slight increase in turbidity was observed in the control sample of basal medium containing 0.05 ug iron/ml. A steady increase in absorbance was recorded after 36 and 48 hr at this level. A corresponding increase in growth was measured by total cell count on anaerobic egg agar (Fig. 2). The initial inoculum level of 100 spores/ml increased to 3.5×10^4 at 24 hr, 1.24×10^6 at 36 hr, and 1.14×10^7 at 48 hr in the basal medium containing 0.05 micrograms iron/ml.

For optimal toxin production by C. tetani in a casein hydrolysate medium Feeney et al. (1943) reported that 0.05 ug iron/ml was required. Iron below this level was found to decrease the growth of

Fig 1. Growth of <u>C. botulinum</u> type A in a chemically

defined medium as measured by an increase in turbidity

at an absorbance of 500 nm.

The iron levels are 0, 1, 2, 3, 4, and 5 ug per ml.

The turbidity was measured at 24, 36, and 48 hours.

The standard deviation of the means is 0.0012, 0.0011,

0.0010, 0.0016, 0.0028, 0.0011 absorbance units at

500nm for total iron levels of 0.05-5 ppm respectively.

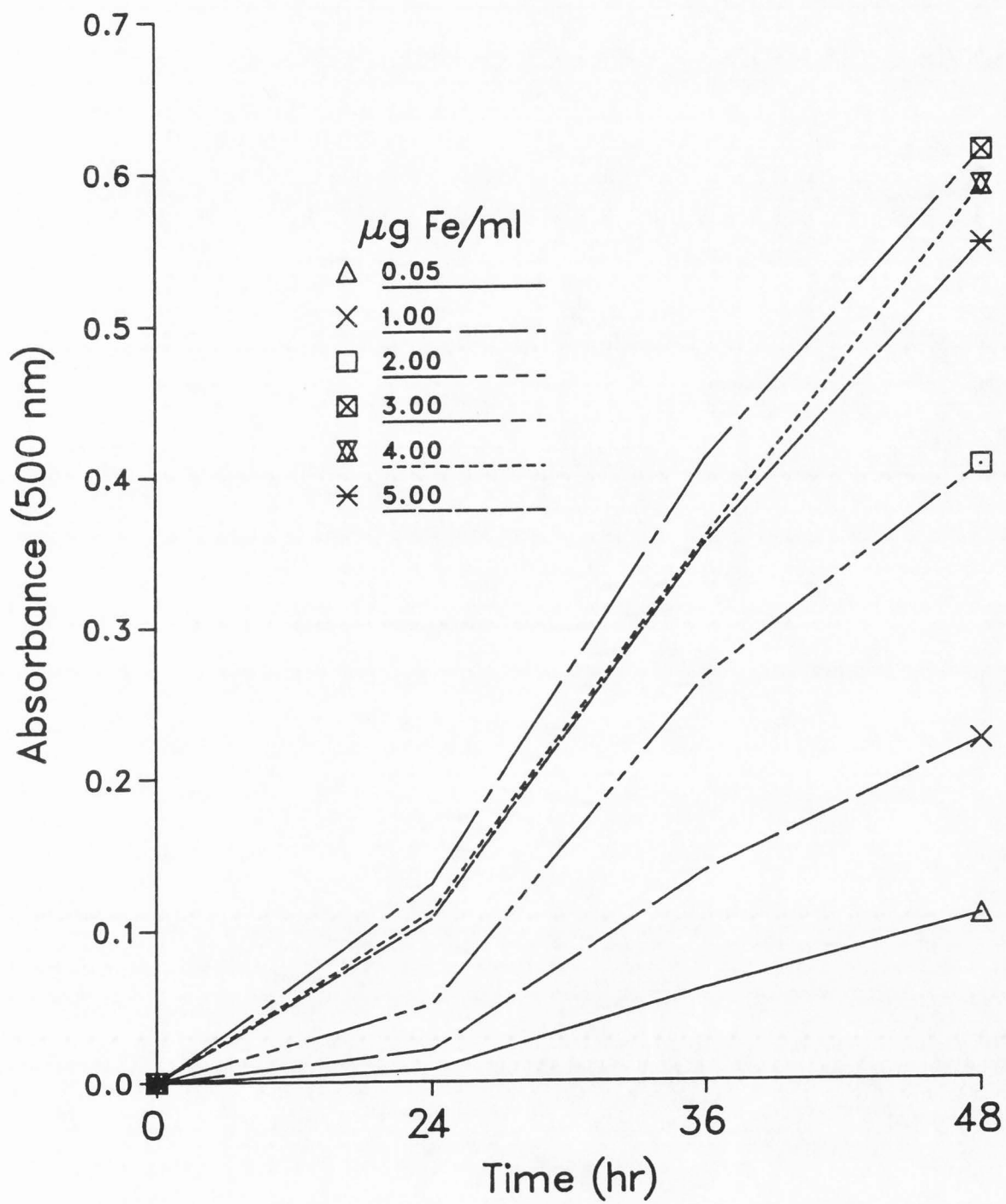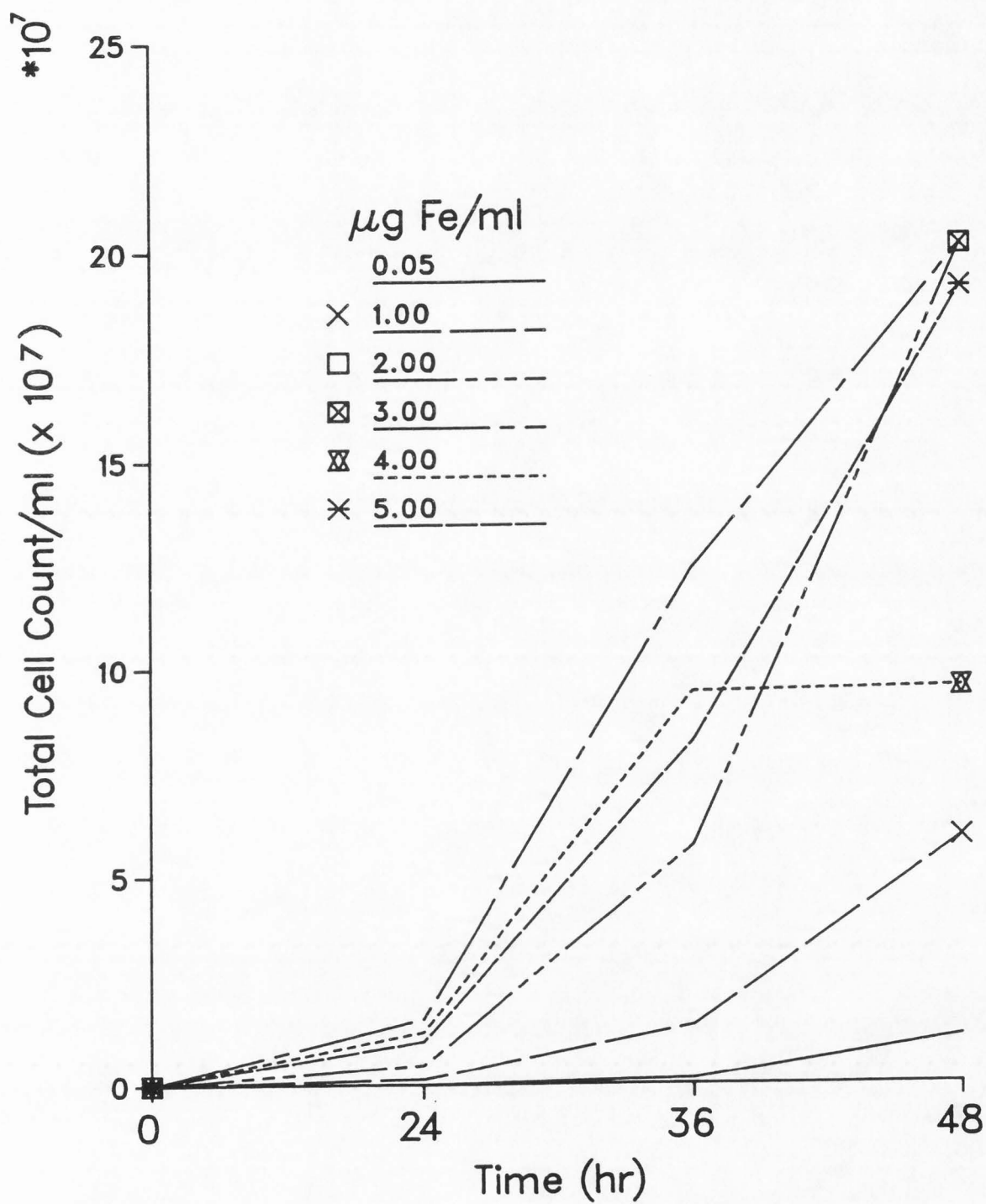Fig 2.   Growth of <u>C. botulinum</u> type A in a chemically defined

medium as measured by total cell count on anaerobic egg

agar.

The iron levels are 0, 1, 2, 3, 4, and 5 ug per ml.

The plating was done using appropriate dilutions at

24, 36, and 48 hours.

The standard deviation of the means is 2.2, 13.7, 23.7,

28.3, 39.2, 49.6 total cell count/ml for iron levels

from 0.05-5.0 ppm respectively.

the organism while larger quantities improved growth but diminished
toxin production. Pappenheimer and Shaskan (1944) determined that
0.06 ug iron/ml was required for optimum growth of C. welchii in a
gelatin hydrolysate medium. The results also support the postulation
by Vahabzadeh et al. (1983) that C. botulinum had a low iron
requirement for growth.

Turbidity was observed to increase significantly with higher
levels of iron. An absorbance of 0.44 was recorded at 36 hr and 0.78
at 48 hr at a level of 3 ug iron/ml, compared to a value of 0.00 at
time zero. Similarly the initial cell count of a 100 spores/ml also
increased to maximum values of $1.24X10^8$ at 36 hr and $2.01X10^8$ at 48 hr
for samples with 3 ug/ml added iron. Maximum growth was measured at
three ug iron/ml. Shankar and Bard (1951) observed that while 0.2
ug/ml iron was sufficient to support the growth of C. perfringens,
maximum growth occured at a level as low as 2 ug iron/ml. Curren et
al. (1981) noted that absence of added iron always resulted in a
significantly lower generation time for C. perfringens than that
observed when any level of iron, from one to 18 ug/ml was added to the
chemically defined medium of Riha and Solberg (1971).

At iron levels above 3 ug/ml, a slight decrease in growth was
observed after 24, 36, and 48 hr of incubation. The difference in
growth between the levels of iron was found to be significantly
different (Appendix F, Table 1). A similar growth inhibition was
reported by Shankar and Bard (1951) at iron levels over two ug/ ml.
Suggi and Sakaguchi (1977, cited from Benedict, 1980) observed that
high levels of iron in the growth medium depressed the toxigenic

effect of C. botulinum in vegetables with the production of lower
molecular weight forms of toxin which had lowered toxicity.

These results show that, similar to other clostridia, C. botulinum
type A has a low requirement for iron with growth being supported at
levels as low as 0.05 micrograms iron/ml in a chemically defined
medium. Three ug iron/ml is required for maximum growth with a slight
decrease in growth observed at levels of iron above three ug/ml.

CHAPTER IV

ELECTRON PARAMAGNETIC RESONANCE DETECTION OF IRON-SULFUR

CENTERS AND FORMATION OF IRON-NITRIC OXIDE COMPLEXES

IN C. BOTULINUM ON NITRITE TREATMENT

Introduction

The addition of sodium nitrite to cured meat products has been a source of controversy as nitrite may be a precursor to the carcinogenic nitrosamines, especially in fried, cured meats such as bacon (Pensabene et al., 1974; Wasserman at al., 1978). A number of compounds have been tested as nitrite substitutes, but no single compound having all the properties of nitrite has been found (Sofos and Busta, 1980). The search for an effective nitrite replacement has been complicated by the fact that the antibotulinal mechanism of nitrite remains unknown, although several mechanisms have been proposed (Tompkin et al., 1978a; Riha and Solberg, 1975).

Mirna (1974) and van Roon and Olsman (1977) demonstrated that nitrite via nitrous acid may react with sulfur-containing amino acids. Tompkin et al. (1978b) suggested that nitrite may react directly with an iron-containing protein or enzyme within the vegetative cell, such as ferredoxin, necessary for energy production in some clostridial vegetative cells (Yoch and Valentine, 1972), thus inhibiting growth.

Woods and Wood (1982) showed that nitrite does inhibit the phosphoroclastic reaction, presumably catalyzed by ferredoxin in C. sporogenes and C. botulinum, although C. botulinum has not

been shown to contain ferredoxin. Electron paramagnetic resonance
(EPR) spectroscopy is ideally suited for the study of iron-sulfur
proteins such as ferredoxin. It has been used extensively for the
identification and study of the mechanism of iron-sulfur centers of
enzymes such as succinate dehydrogenase (Salerno et al., 1976), and
spinach ferredoxin-nitrite reductase (Lancaster et al., 1979). Woolum
et al. (1968) studied the complexes formed in the reaction of ferrous
ions and nitric oxide with amino acids, peptides and a variety of
protiens by EPR spectroscopy.

The purpose of this experiment was to determine if C. botulinum
contains ferredoxin or other iron-sulfur proteins by electron spin
resonance spectroscopy, and to determine the effects if any of
treatment of the cells with nitrite or nitrite and ascorbate.

Materials and Methods

Spores of C. botulinum type A prepared as previously described
were grown in chopped liver broth (Lehninger, 1976; Appendix A) at
35 C for one week. After centrifugation at 3000xG for 15 min the
cells were resuspended in anaerobic growth medium (Schmidt and Nank,
1960, Appendix B) for one week at 37 C.

Actively growing cells were centrifuged at 7000xG for 20 min,
then resuspended in 0.1 percent peptone water. The cell suspension
was divided into three equal volumes. One volume of cells was used
as the untreated sample. The second volume of cells was treated with
200 ppm of sodium nitrite for 45 min at 35 C. The third volume of
cells was treated with 200 ppm sodium nitrite and 500 ppm of ascorbate

for 45 min at 35 C.

All the cell suspensions were then centrifuged at 7000xG for 20 min and resuspended in 0.1 percent peptone water three times to obtain washed, packed cells of C. botulinum.

The packed cells were then sonicated with a microtip sonicator (Heat Systems-Ultrasonics, Inc., Plainsview, New York) and centrifuged until the lysed cells were packed down and a clear supernatant was obtained. The supernatant of each sample was filled in EPR tubes, frozen in liquid nitrogen and stored at 77 K until analyzed in the EPR spectrophotometer.

The EPR spectrophotometer components included a source of liqid helium, a microwave source of constant frequency and variable amplitude, a sample chamber, a microwave bridge to supply the sample with microwave power, a detector of microwave power absorption, a variable AC magnetic field superimposed on the steady field to sweep continuously through the resonance absorption of the sample, and a chart recorder.

The instrument settings were made according to the sample and are listed with the figures.

Results and Discussion

The EPR spectra for untreated, air-oxidized samples of C. botulinum type A is presented in Fig. 3A. The oxidized, untreated sample exhibited an EPR signal with a g value of 2.02. This is the characteristic g value reported for a "HiPiP-type" iron-sulfur center (Palmer, 1973).

Fig 3. EPR spectra for untreated preparations of C. botulinum
cells in the oxidized and reduced states.

Instrument settings: microwave frequeny, 9.136 GHz;
microwave power, 10 mW; modulation frequency, 100 KHz;
and modulation amplitude, 12.5 G (6.5 K).
Instrument gain is 500 in (A) and 800 in (B).
The X axis represents the magnetic field strength in
gauss.
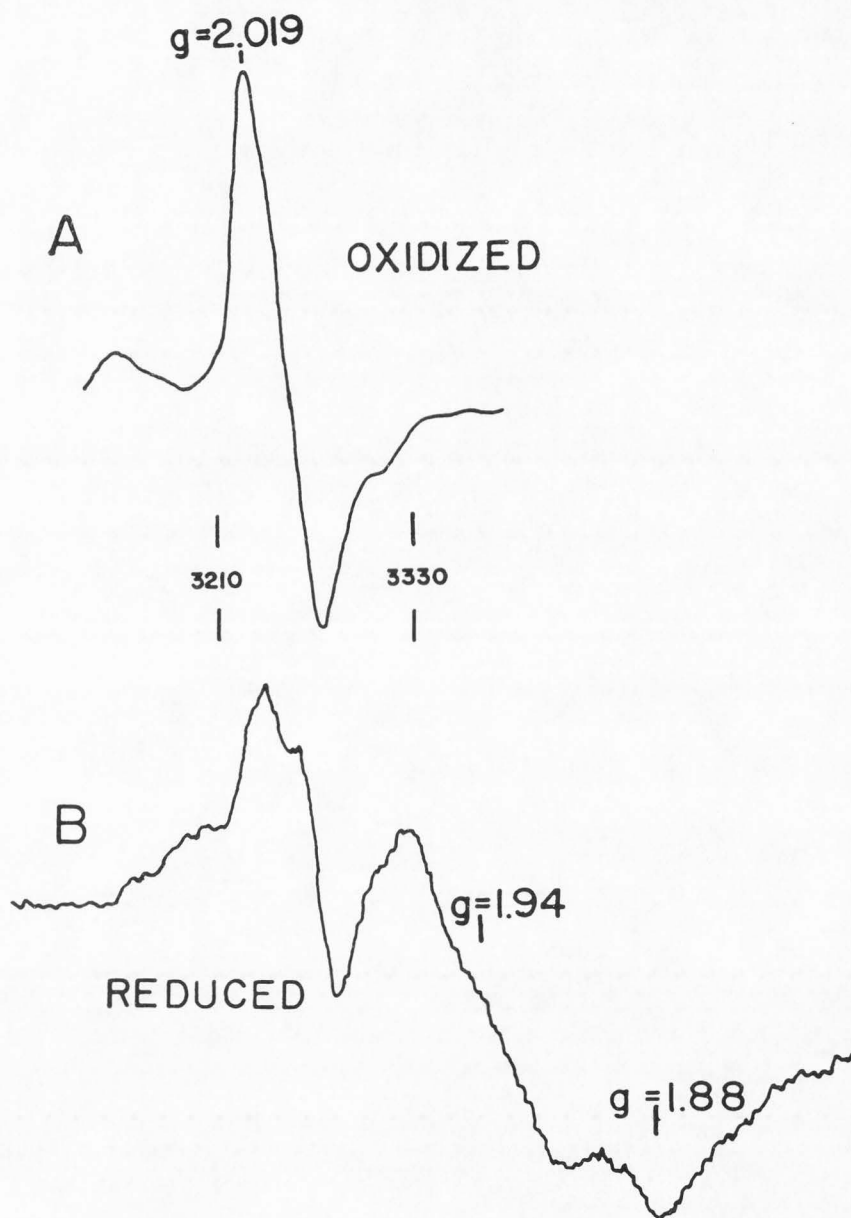
With an increase in microwave power to 40 mW at 7 K, the signal at 2.02 was not appreciably saturated, and dissapeared when the temperature was increased to 30 K (Fig. 4). These properties of an axial signal with g= 2.02 are indicative of a fast-relaxing Fe_3-S_3^* or Fe_4-S_4^* center. A crude sonicated cell suspension of C. botulinum could probably have other metal-protein complexes, but these would not exhibit a fast-relaxing axial signal at g= 2.02 at liquid helium temperatures, which is characteristic for iron-sulfur centers.

The untreated sample of C. botulinum was reduced with excess sodium dithionite. This resulted in the disappearance of the axial signal at g= 2.02 and the appearance of features characteristic of reduced "g= 1.94 type" iron-sulfur centers (Fig. 3B). The feature at g= 1.94 also relaxed rapidly at 7 K and was broadened significantly, although still present, at 30 K (Fig. 5). This furthur confirms that the signal was due to the presence of reduced iron-sulfur centers.

Since no known iron-sulfur center exhibits an EPR signal in both the oxidized and reduced states, the findings of a signal at g= 2.02 in the oxidized state and g= 1.94 in the reduced state indicates that C. botulinum type A has at least two types of iron-sulfur centers. Woods et al., 1981) have shown that C. sporogenes also contains two iron-sulfur proteins (ferredoxin, and the iron-sulfur enzyme, pyruvate-ferredoxin oxidoreductase).

Packer et al. (1977) noted that a clostridial ferredoxin molecule contained two iron-sulfur clusters with each cluster consisting of four atoms of iron and four atoms of inorganic sulfur that is attached to the protein by an iron-cysteinyl linkage. Palmer and Sands (1966)

Fig 4. EPR spectra of untreated, oxidized preparations of

C. botulinum cells.

At (A) 30 K; microwave power, 10 mW, (B) 8 K; microwave

power, 10mW, and (C) 40 mW microwave power at 7 K.

Instrument settings: microwave frequency, 9.138 GHz;

modulation frequency, 100 KHz; modulation amplitude,

12.5 G.

Instrument gain is 2000 in (A) and (B), and 100 in (C).

The x axis represents the magnetic field strength in

gauss.

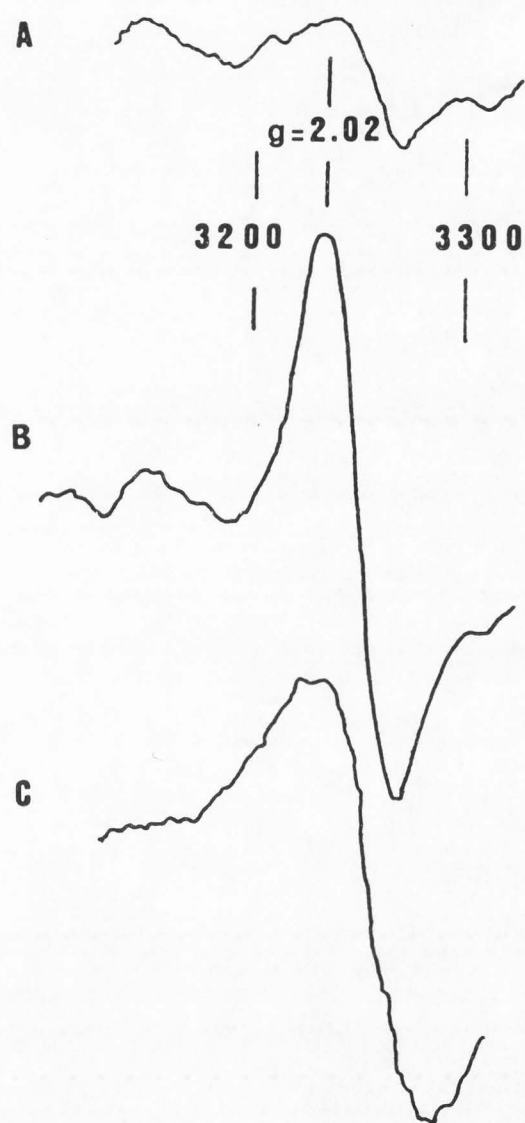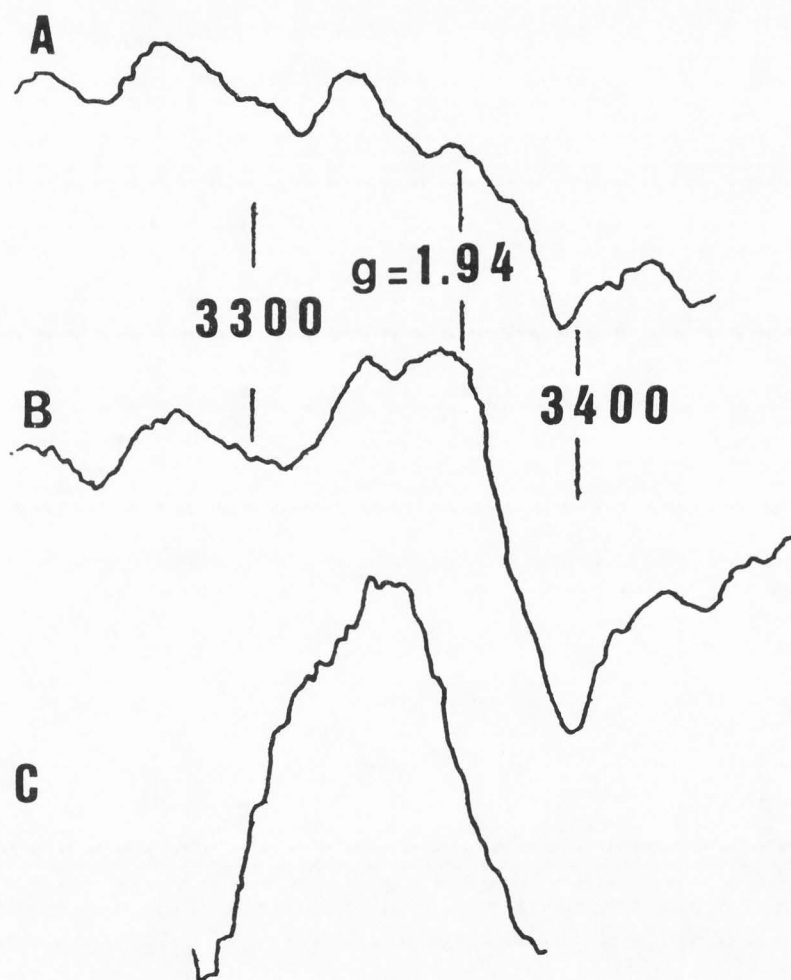Fig 5. EPR spectra of untreated, reduced preparations of

C. botulinum cells.

At (A) 30 K; microwave power, 10 mW, (B) 8 K; microwave

power, 10 mW, and (C) 100 mW at 7 K.

Instrument settings: microwave frequency, 9.136 GHz;

modulation frequency, 100 KHz; modulation amplitude,

12.5 G.

Instrument gain is 800.

The x axis represents the magnetic field strength in

gauss.

A

g=1.94

3300

B

3400

C

observed that the EPR spectra of reduced spinach ferredoxin had a g

value of 1.94. This g= 1.94 signal could not be saturated with

increased microwave power and was highly temperature sensitive.

Palmer et al. (1966) also reported that like spinach ferredoxin,

C. pasteurianum ferredoxin also exhibited an EPR signal at g= 1.94

which was the result of the molecule gaining an unpaired electron upon

reduction. This signal was observed in bacterial ferredoxins only at

liquid helium temperatures.

Samples of C. botulinum type A treated with 200 ppm sodium nitrite

were studied at 77 K and the results are presented in Fig. 6A. The

axial signal has a principal upward feature at g= 2.035. This g value

is characteristic for nitrosyl complexes of a variety of iron-

containing proteins, peptides and chelate complexes as shown by Woolum

et al. (1968) and by Salerno et al. (1976) in succinate dehydrogenase.

Therefore it can be concluded that the addition of nitrite to C.

botulinum vegetative cells results in the formation of iron-nitrosyl

complexes.

Addition of 500 ppm of ascorbic acid along with sodium nitrite

increased the intensity of the axial signal at g= 2.035 although it

was still identical in shape (Fig. 6B, note the difference in

instrument gain). This signal did not decrease in intensity on

dialysis with 0.075 M EDTA in 0.1 M phosphate buffer (pH 7.0), furthur

evidence that the signal was due to the presence of iron-nitrosyl

complexes. The increase in intensity of the signal in the presence

of ascorbate is in agreement with the observation by Tompkin et al.

(1978b) that nitrite is more inhibitory to microorganisms in the

Fig 6. EPR spectra of preparations of C. botulinum cells

treated with nitrite and nitrite plus ascorbate, and

reduced untreated vs nitrite plus ascorbate treated.

(A) and (B): EPR spectra of preparations of C.

botulinum cells at 35 C for 45 min with 200 ppm sodium

nitrite (A), and with 200 ppm sodium nitrite plus 500

ppm ascorbic acid (B).

Instrument settings: microwave frequency, 9.03 GHz;

microwave power, 10 mW; modulation frequency, 100 KHz;

modulation amplitude, 12.5 G (77 K).

Instrument gain is 10000 in (A), and 2000 in (B).

(C): EPR spectra of preparations of C. botulinum

untreated and reduced (upper trace) or treated with 200

ppm sodium nitrite plus 500 ppm ascorbic acid and

reduced (lower trace).

Instrument settings: microwave frequency, 9.113 GHz;

modulation frequency, 100 KHz; microwave power 10 mW;

and modulation amplitude, 12.5 G (10 K).

Instrument gain is 10000.

The x axis represents the magnetic field strength in

gauss.
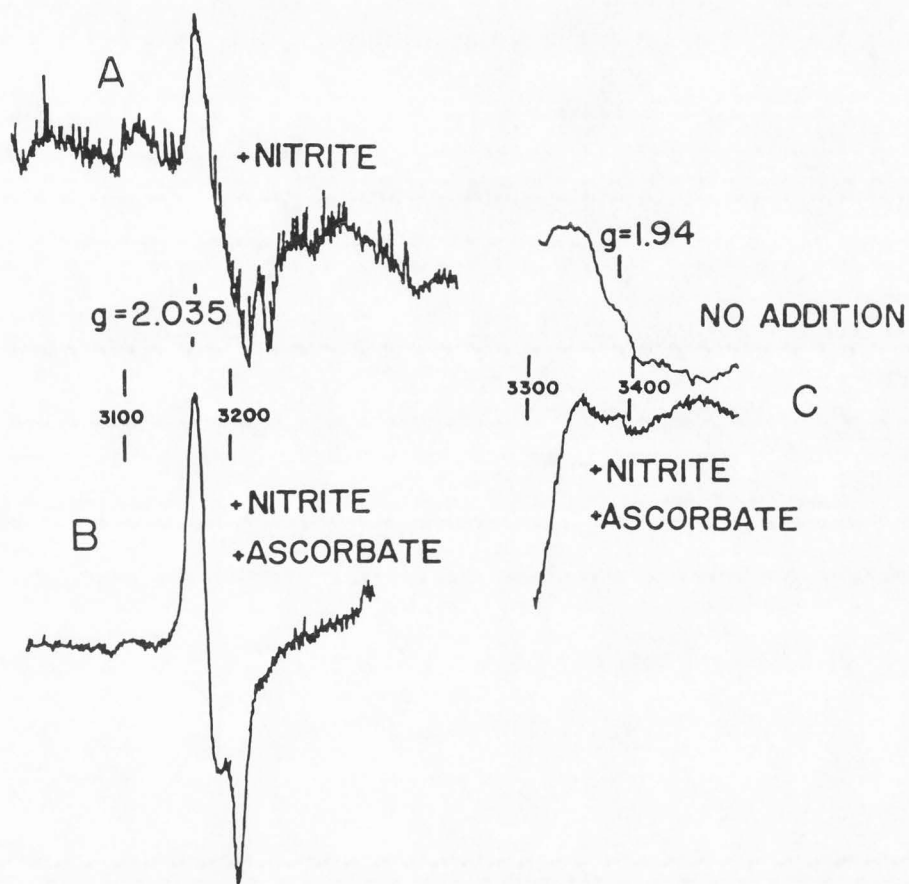
presence of reducing agents. Ascorbate enhances the formation of iron-nitrosyl complexes, probably by accelerating the reduction of nitrite to nitric oxide.

Neither untreated nor nitrite-treated samples of C. botulinum exhibited an EPR signal characteristic of heme centers of high or low spin. This is consistant with the reported absence of cytochromes in clostridia (Pappenheimer and Shaskan, 1944; Stanier et al., 1976), typical of strict anaerobuc organisms.

Fig. 6C compares the reduced iron-sulfur signal (upper trace) in untreated samples with the nitrite plus ascorbate treated signal (lower trace). The reduced signal at g= 1.94 is decreased significantly in intensity on treatment with nitrite and ascorbate. The loss of the iron-sulfur signal at g= 1.94 (Fig. 6C) and the appearance of the iron-nitrosyl signal at g=2.035 (Fig. 6A,B) on addition of nitrite and ascorbate is evidence that iron-sulfur complexes in botulinal cells react with nitrite to form iron-nitrosyl complexes. This effect of nitrite is definitely inhibitory if not lethal to the organism.

Destruction of iron-sulfur centers of purified succinate dehydrogenase under similar conditions was also reported by Salerno et al. (1976). Meyer (1981) found nitric oxide to rapidly and irreversibly inactivate the ferredoxin protein of the nitrogenase from C. pasteurianum, by disrupting the iron-sulfur cluster of the protein.

Similarly, Hosein and Palmer (1983) found that in oxidized spinach ferredoxin, addition of nitric oxide results in the destruction of the

iron-sulfur chromophore as monitored by the loss in optical absorption. They concluded that the goemetry of the active center of ferredoxin cannot tolerate the addition of a nitrosyl ligand.

Woods et al. (1981) reported that nitric oxide, produced from nitrite, reacted with the non-heme iron of pyruvate-ferredoxin oxidoreductase in C. sporogenes thus resulting in the inhibition of its growth in bacteriological media.

The EPR results demonstrate that vegetative cells of C. botulinum type A contain at least two types of iron-sulfur proteins. These react with added nitrite to form iron-nitric oxide complexes, with the resultant destruction of the iron-sulfur cluster. Although iron-sulfur destruction would certainly result in antibotulinal effects, nitrite addition may have other inhibitory actions as demonstrated by Yarbrough et al. (1980) and O'Leary and Solberg (1976).

Because of the function of iron-sulfur proteins in electron transport and adenosine triphosphate generation in anaerobic bacteria, inactivation by binding with nitric oxide would almost certainly inhibit growth. This could be the most important site of reaction and the basis of the antibotulinal activity of nitrite in foods.

CHAPTER V

DETERMINATION OF FERREDOXIN AND PYRUVATE-FERREDOXIN

OXIDOREDUCTASE ACTIVITY IN NITRITE TREATED AND

UNTREATED CELLS OF C. BOTULINUM

Introduction

Electron paramagnetic resonance spectroscopy of cell-free extracts

of C. botulinum type A showed the presence of iron-sulfur centers in

untreated cells. The EPR signal for the reduced iron-sulfur centers

disappeared upon nitrite treatment, with the simultaneous appearance

of iron-nitric oxide complexes (Reddy et al., 1983). These findings

led to the conclusion that in C. botulinum the two iron-sulfur

proteins involved in the phosphoroclastic reaction, namely ferredoxin

and pyruvate-ferredoxin oxidoreductase, may react with nitrite

resulting in their inhibition. Thus, this could very probably be the

site of nitrite action within the botulinal cell to inhibit growth.

The phosphoroclastic reaction is the oxidation of pyruvate to

acetate and the series of reactions involved are as follows

(Mortenson, 1963):

Pyruvate + TPP-E ------------> (hydroxyethyl-TPP)-E + CO_2

(Hydroxyethyl-TPP)-E + Fd_{ox} +CoA.SH -------> acetyl-CoA + Fd_{red} + TPP-E

Acetyl-CoA + $H_2PO_4^-$ ------> acetyl-PO_4 + CoA.SH

Acetyl-PO_4 + ADP ------> acetate + ATP

Fd_{red} + $2H^+$ -------> Fd_{ox} + H_2
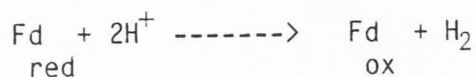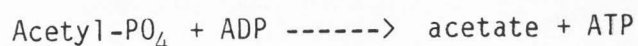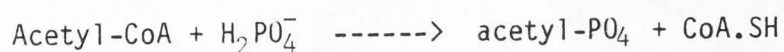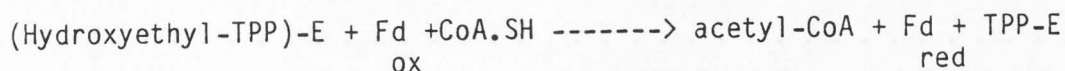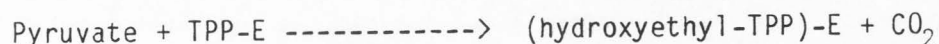
Pyruvate ferredoxin oxidoreductase is a thiamine pyrophosphate

(TPP) containing iron-sulfur enzyme (Yoch and Carithers, 1979) which catalyzes the oxidative decarboxylation of pyruvate in the phosphoro-clastic reaction (Uyeda and Rabinowitz, 1971a). Ferredoxin, an electron-transferring iron-sulfur protein first isolated from C. pasteurianum (Mortenson et al., 1962) was observed to serve in the transfer of electrons from pyruvate dehydrogenase to hydrogenase in the formation of hydrogen in the phosphoroclastic reaction (Mortenson et al., 1963).

The phosphoroclastic system has been demonstrated in several clostridial species (Rabinowitz and Barker, 1955; Mortlok et al., 1959; Sagers et al., 1961). Simmons and Costilow (1962) readily demonstrated the presence of the phosphoroclastic system in C. botulinum. Woods et al. (1981) observed the phosphoroclastic system in C. sporogenes and reported that nitrite inhibited the system by the reaction of nitric oxide, produced from nitrite, with pyruvate-ferredoxin oxidoreductase and not ferredoxin. Woods and Wood (1982) also observed the inhibition of the phosphoroclastic system in C. botulinum upon the addition of nitrite by measuring the accumulation of pyruvate in the medium.

The presence of ferredoxin in C. botulinum has yet to be reported. It has also not been shown if ferredoxin or pyruvate-ferredoxin oxidoreductase or both are the site of nitrite action in the inhibition of botulinal growth.

The purpose of this experiment was to determine the presence of ferredoxin in C. botulinum, and if present, to seperately study the effect of nitrite on ferredoxin and pyruvate-ferredoxin oxidoreduc-

tase, the two iron-sulfur proteins of the phosphoroclastic system.

Materials and Methods

C. botulinum type A (ATCC 19397) spores obtained from the American Type Culture Collection, Rockville, Maryland, were propagated in chopped liver broth (Lehninger, 1976; Appendix A) at 35 C for one week.

The cell suspension was transferred to reinforced clostridial medium (Hirsh and Grinsted, 1954; Appendix G) and incubated at 35 C for one week. The actively growing cells were divided into two equal volumes. One volume of cells was treated with 1000 ppm sodium nitrite for 45 min at 35 C. Although only 156 ppm sodium nitrite is used in cured meats, because of the large volume of cells being treated, a higher concentration of nitrite was used to ensure that there was sufficient nitrite in the media to react with the cells. The sodium content of trhe media upon addition of sodium nitrite increased from 0.06 M (0.13%) to 0.075 M (0.16%), which could not affect the growth of botulinal cells in the media.

The nitrite treated and untreated cells were collected by centrifugation at 3000 RPM for 15 min. The cells were resuspended in distilled water and centrifuged three more times to remove traces of growth medium and excess nitrite. The washed packed nitrite treated and untreated cells were assayed for ferredoxin and pyruvate-ferredoxin oxidoreductase activity.

The assays were repeated with two batches of cells grown separately and all assays were done in triplicate.

Ferredoxin assay: The packed cells of nitrite treated and untreated C. botulinum were lysed by suspending the cells in 1.5 ml of water containing 1 mg of lysozyme and 0.1 mg of deoxyribonuclease per gm of cells and incubated at 37 C for 45 min (Schonheit et al., 1978). The lysed cell suspension was centrifuged at 14,000 rpm for 15 min. The ferredoxin in the supernatant was separated by the Mortenson (1964) procedure using DEAE-cellulose chromatography (Appendix H). The ferredoxin fraction collected by elution with 0.5 M Tris-HCl buffer, pH 8.0 was dialyzed overnight versus 0.05 M Tris-HCl buffer, pH 7.3.

The partially purified ferredoxin extract from nitrite treated and untreated cells was assayed for protein by the Bio-Rad protein assay (Appendix I) and samples of equal protein content were used for the determination of ferredoxin activity by the cytochrome c reduction method (Shin, 1971; Appendix J).

The reduction of cytochrome c follows the path:

$$\text{NADPH} + \text{H}^+ \quad\quad \text{FNR ox} \quad\quad \text{Fd red} \quad\quad \text{Cyt c ox}$$

$$\text{NADP} \quad\quad \text{FNR red} \quad\quad \text{Fd ox} \quad\quad \text{Cyt c red}$$
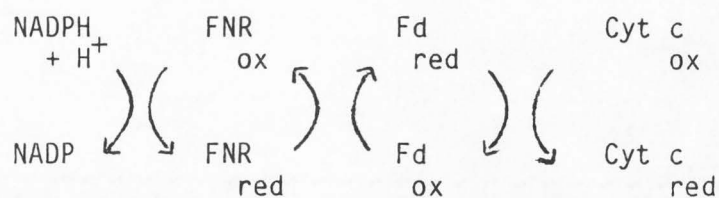
Addition of ferredoxin in the presence of NADPH and FNR results in the reduction of cytochrome c which can be measured by an increase in absorbance at 550 nm. An aliquot of the control was treated with 1000 ppm nitrite for 45 min at 35 C. Another sample of the control was boiled for 5 min and assayed for ferredoxin activity.

Dilutions of the untreated sample were assayed for activity of cytochrome c reduction by ferredoxin. The micromoles of cytochrome c

reduced was calculated as the change in absorbance at 550 nm per min for each sample.

Pyruvate-ferredoxin oxidoreductase assay: The packed cell samples of nitrite treated and untreated C. botulinum cells were suspended in distilled water and sonicated with a microtip sonicator (Heat Systems-Ultrasonics, Inc., Plainview, New York). The lysed cells were then centrifuged at 14,000 RPM for 20 min to obtain a clear supernatant cell-free extract.

The cell-free extracts were passed through a DEAE-cellulose column (Appendix H) to remove the ferredoxin. The flow-through fraction containing the oxidoreductase (partially purified enzyme) was collected and assayed for protein by the Lowry method (Lowry et al., 1951; Appendix K).

Equal protein samples of nitrite treated and untreated extracts were assayed for FAD-reducing activity (Uyeda and Rabinowitz, 1971a; Appendix L) and for acylhydroxamate formation (Raeburn and Rabinowitz, 1971a; Appendix M) as a measure of pyruvate-ferredoxin oxidoreductase activity. The oxidation of pyruvate involves the reduction of electron acceptors such as ferredoxin or FAD and the formation of acylhydroxamate which is an active acyl group intermediate.

An aliquot of the untreated enzyme extract was treated with 1000 ppm sodium nitrite for 45 min at 35 C. An aliquot of the untreated sample was boiled for 5 min and both the samples were assayed for FAD-reduction and acylhydroxamate formation activity. Dilutions of the untreated sample were assayed for enzyme activity.

Results and Discussion

The results of the ferredoxin assay are presented in Fig 7. The partially purified ferredoxin from untreated cells was observed to reduce about 3 umoles of cytochrome c/min/ug of protein. Commercialy prepared ferredoxin from C. pasteurianum (Sigma Chemical Co., St. Louis, MO) reduced 39 micromoles of cytochrone c/min per ug of protein. The ability of untreated ferredoxin extracts to reduce cytochrome c clearly demonstrates the presence of ferredoxin activity in C. botulinum. Ferredoxin was first isolated and purified from C. pasteurianum (Mortenson et al., 1962) and has since been demonstrated in several clostridial species (Lovenberg et al., 1963).

Treatment of the cells with nitrite before ferredoxin isolation decreased cytochrome c reduction rate by the extracts to about 12% of the control (Fig 7). Since the cells had been washed several times before lysis, the nitrite must actually have penetrated the cells to react with ferredoxin. Also, since the cells had been washed prior to lysis, any inhibition of cytochrome c observed could not be due to the presence of excess, unreacted nitrite in the sample.

Hosein and Palmer (1983) reported that spinach ferredoxin was unstable to the bubbling of nitric oxide gas through the solution. Nitric oxide reacted with the oxidized ferredoxin resulting in the destruction of the iron-sulfur chromophore, as monitored by a loss in optical absorption. They concluded that the geometry of the active site could not tolerate the addition of a nitrosyl ligand.

Zanetti et al. (1984) reported that spinach ferredoxin formed a complex with FNR and NADP . The iron-sulfur center of the cross-

Fig 7: Effect of dilution, nitrite treatment or boiling on

cytochrome c reduction activity of partially purified

ferredoxin from C. botulinum.

X axis is ug protein in untreated, nitrite treated and

boiled samples.

Y axis is umoles of cytochrome c reduced calculated as

the change in absorbance at 550 nm per min for the first

minute of the reaction.

Each value is an average of two trials done in

triplicate.
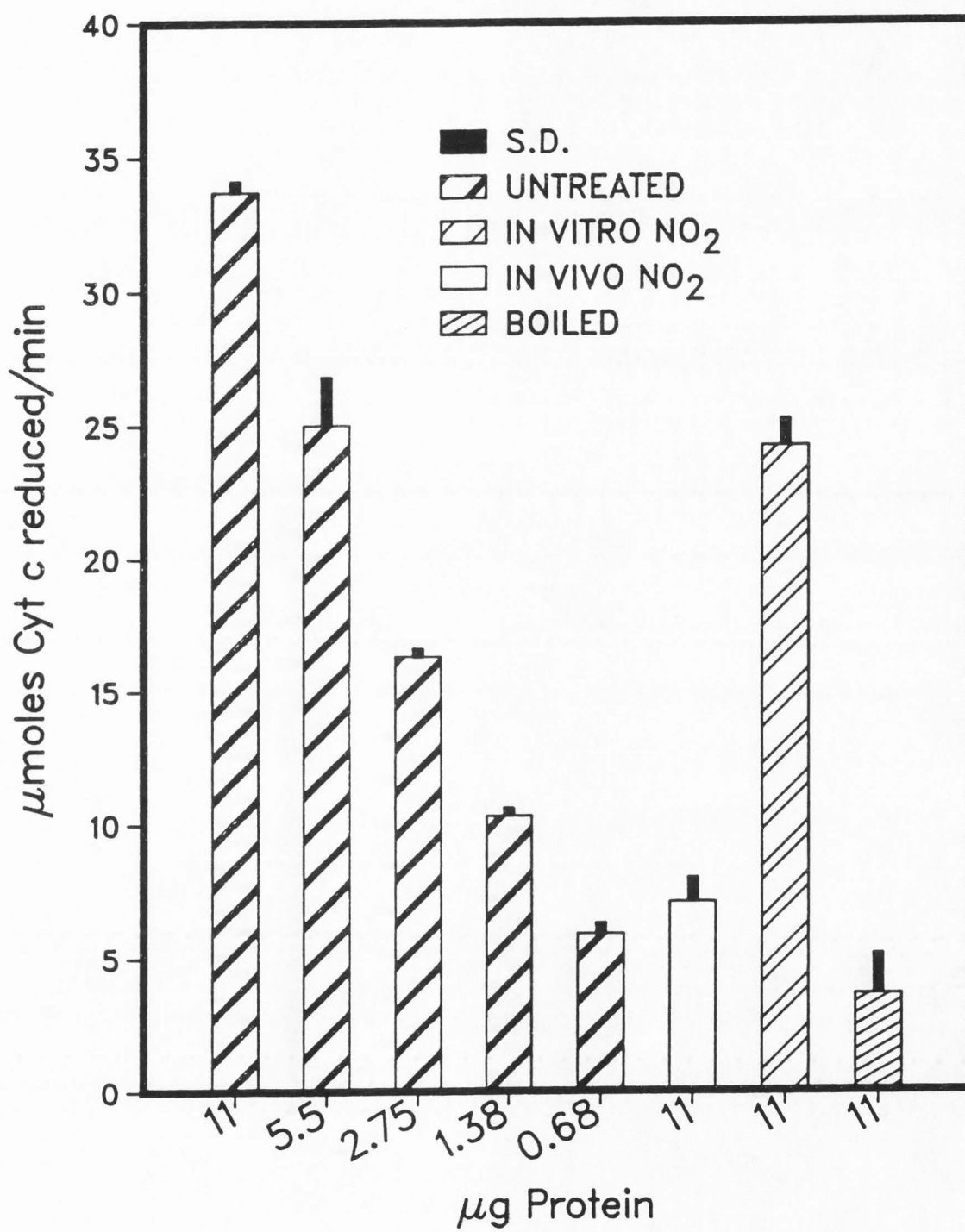
linked complex was found to be necessary for the catalysis of the NADPH-cytochrome c reductase reaction. They observed that the destruction of the iron-sulfur center brought about a dramatic decrease in the cytochrome c reductase activity.

In studies conducted by Carpenter and Cornforth (unpublished data), treatment of C. pasteurianum cells with 1500 ppm nitrite for 30 min at 35 C was observed to inhibit ferredoxin activity by 88%.

Treatment of the ferredoxin extract with nitrite also showed a similar decrease in cytochrome c reduction activity, lowering activity to less than 50% of the untreated control. The in vitro treated ferredoxin extracts were dialyzed to remove any residual nitrite present. The inhibition observed was therefore not due to the inhibition of assay components by any carry-over nitrite. The difference in the extent of inhibition between in vivo treated cells and in vitro treated extracts can be explained by the absence of reducing agents in the partially purified extract of ferredoxin when compared to the whole cells leading to the incomplete conversion of nitrite to nitric oxide.

Woods et al. (1981) had previously reported that the in vitro treatment of purified ferredoxin from C. sporogenes with nitrite did not inhibit ferredoxin activity. Preincubation with 6 mM (about 400 ppm) nitrite caused less than 20% inhibition of the phosphoroclastic reaction in a reconstituted system in which the ferredoxin and oxidoreductase fractions were isolated, separately treated and mixed together. Their results were however complicated by the presence of residual nitrite in the assay system carried over in the ferredoxin

fraction after in vitro nitrite treatment. This could have been avoided by the dialysis of the extract prior to reconstitution.

Boiling of the untreated sample was found to inhibit ferredoxin activity by more than 90% indicating almost complete denaturation of the protein upon heat treatment (Fig 7).

The spectrophotometric measurement of FAD-reduction was used as a measure of pyruvate-ferredoxin oxidoreductase activity (Uyeda and Rabinowitz, 1971a). The micromoles of FAD reduced was calculated based on the change in absorbance at 450 nm for the first min of the reaction. The umoles of FAD reduced per min by the control, nitrite treated and boiled samples is presented in Fig 8.

The enzyme extract of untreated cells of C. botulinum had relatively high FAD reducing capability, indicating the presence of pyruvate-ferredoxin oxidoreductase activity.

The FAD-reducing activity of the enzyme was observed to decrease upon nitrite treatment. Partially purified samples of oxidoreductase from C. botulinum cells treated with 1000 ppm nitrite for 45 min at 35 C exhibited an activity that was less than 30% of the untreated cells (Fig 8). The partially purified pyruvate-ferredoxin oxidoreductase that was treated with 1000 ppm nitrite in vitro also showed a significantly decreased enzyme activity, i.e. less than 30% of the control (Fig 8). Boiling the untreated sample also decreased the FAD-reducing activity to less than 5% of the control indicating heat denaturation of the enzyme (Fig 8).

Woods et al. (1981) reported that the pyruvate dependent reduction of NAD in cell-free extracts of C. sporogenes was inhibited upto 50%

Fig 8: Effect of dilution, nitrite treatment or boiling on FAD

reduction activity of partially pyruvate-ferredoxin

oxidoreductase from C. botulinum.

X axis is ug protein in untreated, nitrite treated and

boiled samples.

Y axis is micromoles of FAD reduced calculated as the

change in absorbance at 450 nm per min for the first

minute of the reaction.

Each value is the average of two trials done in

triplicate.
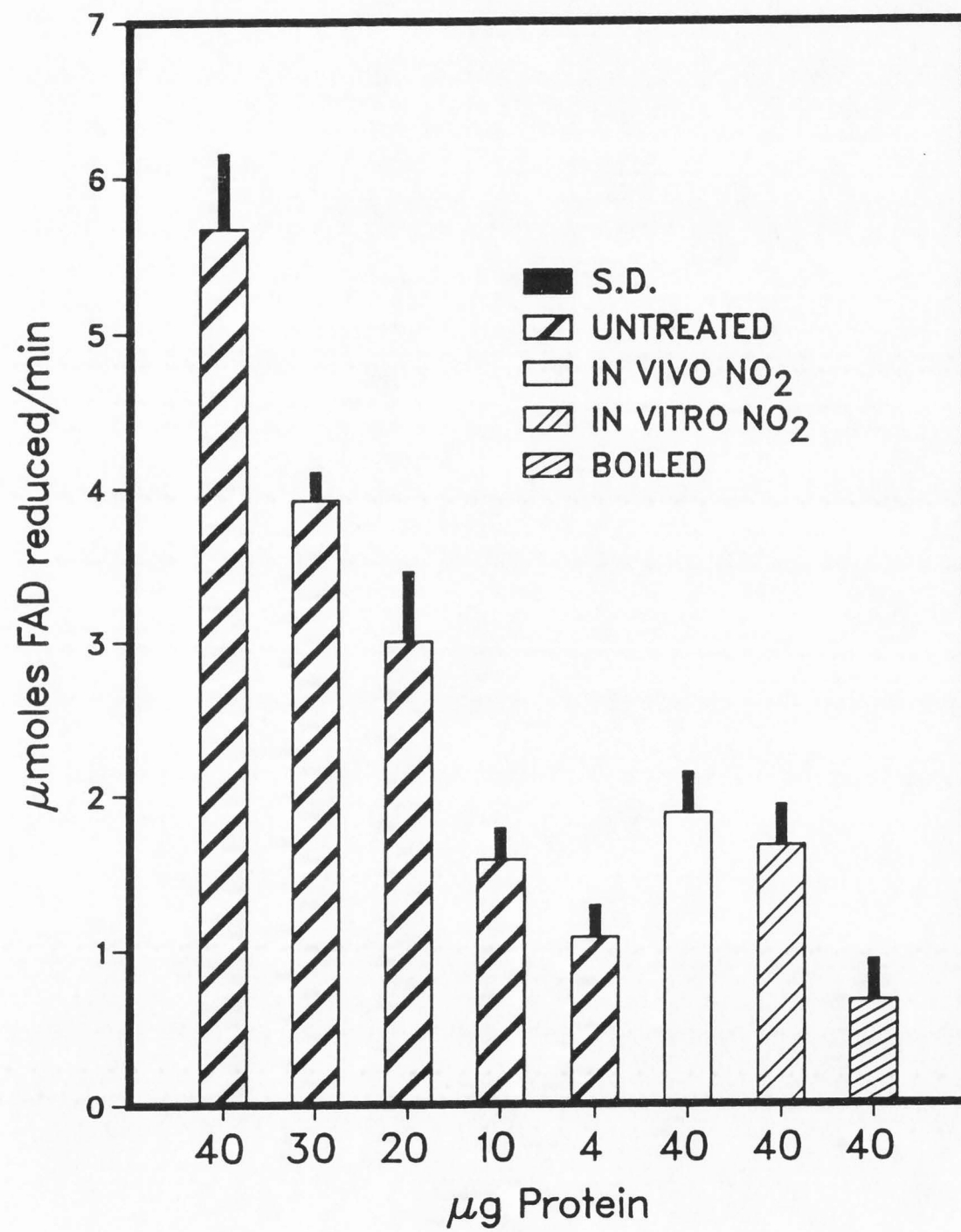
by 8mM (about 530 ppm) nitrite. They also reported that nitric oxide gas bubbled through the protein fraction containing the oxidoreductase caused 40% decrease in NAD reduction activity.

The formation of acylhydroxamate, an active acyl group generated in the conversion of pyruvate to acetate was also determined spectrophotometrically as another measure of oxidoreductase activity (Raeburn and Rabinowitz, 1971a). The acylhydroxamate formed by different dilutions of the untreated, nitrite treated and boiled samples is presented in Fig 9.

Raeburn and Rabinowitz (1971a) demonstrated that one mole of acylhydroxamate was formed for every mole of pyruvate consumed with FAD as the electron acceptor. Simmons and Costilow (1962) also observed that cell-free extracts of C. botulinum cleaved and oxidized pyruvate, with one mole each of acetyl phosphate, carbon dioxide and hydrogen being produced per mole of pyruvate.

The formation of acylhydroxamate by nitrite treated and boiled samples was found to be decreased when compared to the control. The treatment of C. botulinum cells with 1000 ppm nitrite decreased the acylhydroxamate formation to less than 20% of the control (Fig 9). Nitrite treatment of the partially purified enzyme in vitro also inhibited acylhydroxamate formation that was less than 14% of the control.

The boiled, untreated extract had less than 10% of the activity of the control, also indicating the heat inactivation of the enzyme (Fig 9). The decrease in the rate of FAD reduction and acylhydroxa-mate formation after treatment of cells or partially purified enzyme

Fig 9: Effect of dilution, nitrite treatment or boiling on

acylhydroxamate formation by partially purified

pyruvate-ferredoxin oxidoreductase from C. botulinum.

X axis is ug protein in untreated, nitrite treated and

boiled samples.

Y axis is umoles of acylhydroxamate formed calculated

based on absorbance at 540 nm per min.

Each value is the average of two trials done in

triplicate.
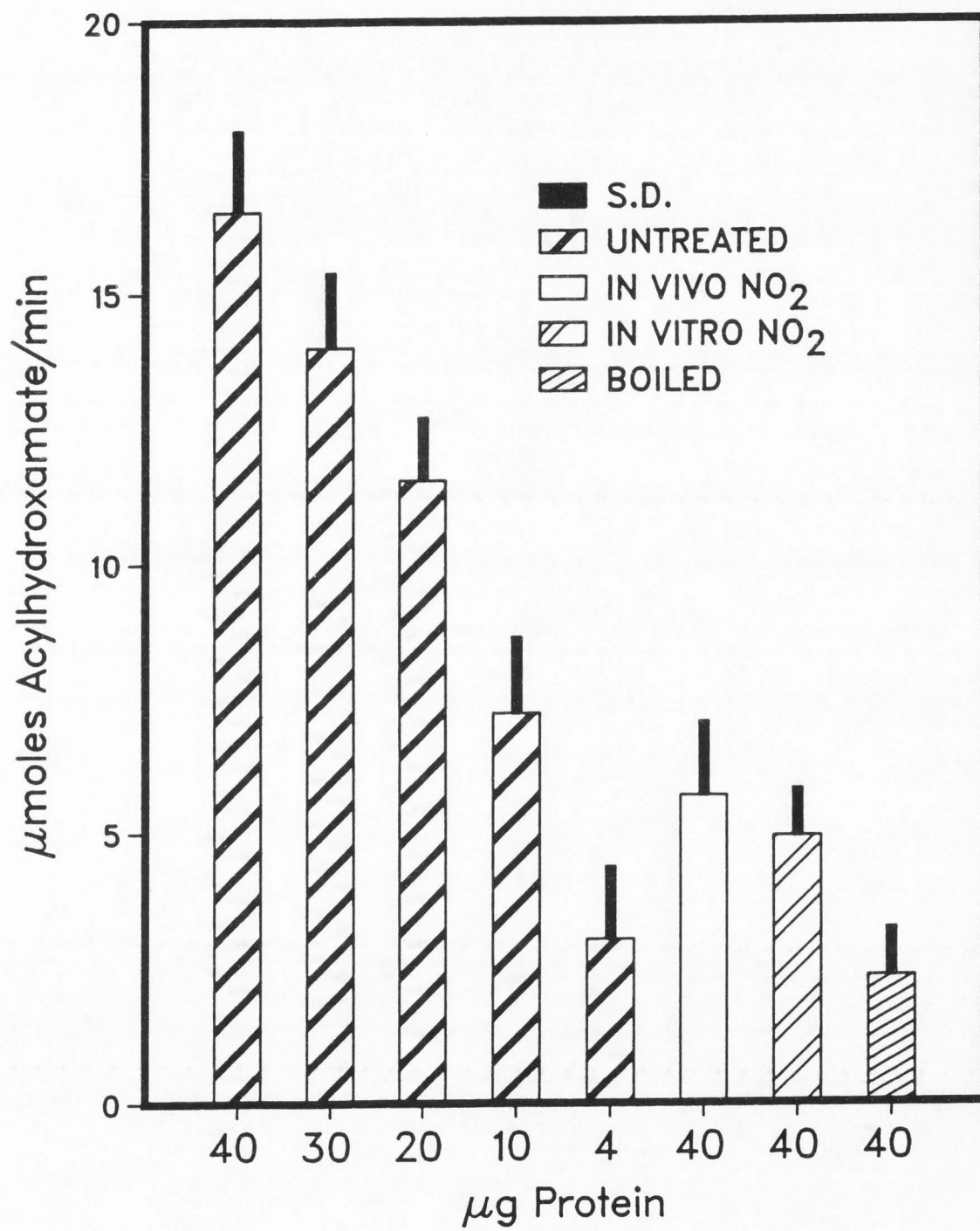
with nitrite demonstrates that nitrite inhibits the activity of pyruvate-ferredoxin oxidoreductase in C. botulinum.

This is in agreement with the work of Woods et al. (1981), who observed that the reaction of nitric oxide with the non-heme iron chromophore of the enzyme caused the inhibition of the phosphoro-clastic reaction in C. sporogenes. Uyeda and Rabinowitz (1971a) observed that the non-heme iron chromophore of pyruvate-ferredoxin oxidoreductase isolated from C. acidiurici was active in the phosphoroclastic reaction. The reaction of nitrite with this iron chromophore of the enzyme would certainly lead to the inhibition of enzyme activity.

Tu et al. (1984) observed that in an aqueous solution with a pH of 2.0, acetyl CoA reacted with nitrite to form an S-nitrosothiol derivative which could contribute in part to the inhibition of botulinal growth. However, the rate and extent of the reaction was found to decrease, although still present, by raising the pH to 6.0.

The results of the cytochrome c reduction assay, FAD reduction assay, and acylhydroxamate formation assay clearly demonstrate the presence of ferredoxin and pyruvate-ferredoxin oxidoreductase in C. botulinum. Nitrite at a level of 1000 ppm inhibits both the iron-sulfur proteins probably by the reaction of nitrite as nitric oxide with the non-heme iron centers.

These findings correlate with the results of previous EPR studies (Reddy et al., 1983) which showed the disappearance of iron-sulfur centers and the simultaneous appearance of iron-nitric oxide complexes upon nitrite treatment of C. botulinum cells.

Contrary to the observation by Woods et al. (1981) that the site of nitrite action in C. sporogenes was pyruvate-ferredoxin oxidoreductase and not ferredoxin, the results of this experiment demonstrate that the site of action of nitrite in the inhibition of the phosphoroclastic system in C. botulinum is both ferredoxin and pyruvate-ferredoxin oxidoreductase. Ferredoxin activity was inhibited by 90% and FAD reduction and acylhydroxamate formation, a measure of oxidoreductase activity, was inhibited by 70% and 80% respectively.

CHAPTER VI

CONCLUSIONS

The possible mechanism for nitrite inhibition of botulinal growth suggested by Tompkin et al. (1978 b) was that nitrite acts on an essential bacterial nutrient in meat, such as iron , thus making it unavailable for bacterial growth. It has been postulated that nitrite may also directly inhibit botulinal growth by inactivating an iron containing enzyme such as ferredoxin within the cell (Tompkin et al., 1978b; Benedict, 1980).

Thus, in the present study, the iron requirement for the growth of C. botulinum type A vegetative cells was determined in an attempt to test the hypothesis of indirect inhibition of botulinal growth by nitrite. It was observed that iron at a level of 0.05 ug per ml media supported the growth of C. botulinum cells with maximum growth at 3 ug iron/ml media. The reported values for the iron content of meat is about 28 ug iron/gram of beef round of which 8-10 ug/gm is non-heme iron (Jansuittivechukal, 1983). In addition, not all of the heme-iron in cured meat is nitrosylated (Vahabzadeh et al., 1983). The above factors lead to the conclusion that cured meats contain sufficient available iron to support botulinal growth and the hypothesis of indirect inhibition is not applicable.

The direct inhibition of botulinal growth by nitrite inactivation of an iron-containing compound within the cell was tested. First, EPR studies conducted to detect the presence of iron-sulfur proteins in untreated C. botulinum type A vegetative cells showed the presence of

a high potential iron-sulfur protein (HiPiP, g= 2.02) in the oxidized state and an EPR signal at g= 1.94, also reported for spinach and clostridial ferredoxin, in the reduced state. Treatment of the cells with nitrite or nitrite and ascorbate resulted in the disappearance of the reduced EPR signal at g=1.94 and the appearance of a signal at g= 2.035 characteristic of an iron-nitric oxide complex. This leads to the conclusion that vegetative cells of C. botulinum do contain iron-sulfur proteins which are destroyed by the addition of nitrite.

The cytochrome c reduction method for the determination of ferredoxin demonstrated the presence of ferredoxin activity in C. botulinum cells. Treatment of botulinal cells with 1000 ppm nitrite for 45 min at 35 C was found to inhibit ferredoxin activity by about 90%. The presence of pyruvate-ferredoxin oxidoreductase in C. botulinum was also determined by FAD reduction assay and by an acylhydroxamate formation assay. FAD reduction and acylhydroxamate formation were inhibited by about 70% and 80% respectively upon treatment of botulinal cells with 1000 ppm nitrite. Therefore both ferredoxin and pyruvate-ferredoxin oxidoreductase may react with nitrite, inhibiting the phosphoroclastic system of C. botulinum.

The phosphoroclastic system is the major pathway for ATP sythesis in clostridia and depends on the activity of two iron-sulfur proteins, ferredoxin and pyruvat-ferredoxin oxidoreductase. Because of the importance of these iron-sulfur proteins in electron transport and ATP generation in anaerobic bacteria inactivation by nitrite would inhibit growth and is certainly one of the most important sites of nitrite reaction.

Cured meat products are extensively consumed in the U.S. and nitrite has been a controversial additive in cured meats, since it may under certain conditions be a precursor to the carcinogenic nitrosamines. The controversy has subsided, since recent research has shown that nitrosamines are only detected in severely heated products such as fried bacon. Even in bacon, nitrosamine formation can be avoided by decreasing the nitrite level at formulation, and by using ascorbate to furthur reduce nitrite levels after processing.

The importance of nitrite as an antibotulinal agent in cured meats has been long recognized. Nonetheless, the exact mechanism of botulinal inhibition by nitrite remains to be established. Nitrite inactivation of iron-sulfur proteins such as ferredoxin and pyruvate-ferredoxin oxidoreductase appears to be the most likely mechanism of nitrite inhibition in botulinal cells.

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APPENDICES

Appendix A. Chopped Liver Broth

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

Add finely chopped ground beef liver to the distilled water and boil for one hour. Adjust the broth to ph 7.0 and boil another 10 min. Press through cheese cloth and make broth up to one lit. 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 one cm deep), cover with 8-10 ml broth. Sterilize for 20 min. at 121 C. Before use, exhaust for 20 min in flowing steam.

Reference: Lehninger, 1976.

Appendix B. Anaerobic Growth Medium

Tryptone	50.0 g
Peptone	5.0 g
Distilled water	1.0 L
Sodium thiglycollate	10 g %

Mix tryptone and peptone in water. Heat to dissolve and cool the solution. Adjust to pH 7.0. Pour into dilution bottles and sterilize for 15 min at 121 C. Immediately prior to inoculation, add one ml of 10 % sodium thiglycollate solution to each bottle of medium (100ml). Sterilize again for 5 min at 121 C.

Reference: Schmidt and Nank, 1960.

Appendix C.   Anaerobic Egg Agar

Fresh eggs	3
Yeast extract	5.0 g
Tryptone	5.0 g
Protease 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 for 10 to 15 min; remove and allow eggs to dry. Crack eggs aseptically; separate and discard the whites. Add the yolks to an equal volume of sterile saline solution (0.9%) and mix thoroughly.

Combine the remainder of the ingredients, dissolve in water, adjust to pH 7.0, 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 for 2-3 days or for 24 hours at 35 c. Discard contaminated plates. Store sterile plates in the refrigerator until use.

Reference: Lehninger, 1976.

Appendix D. Chemically Defined Medium

Comp.	Final conc. (mg/ml)	Comp.	Final conc. (mg/ml)
DL-Alanine	0.50	Uracil	0.01
L-Arginine	5.00	Adenine sulfate	0.174
L-Aspartic acid	0.50		
		Ascorbic acid	0.25
L-Cystine	0.40		
		Riboflavine	0.0005
Glycine	0.50		
		Ca-d-pantoth- nate	0.001
L-Glutamic acid	1.00		
L-Histidine	0.25	Pyridoxamine	0.0005
L-Hydroxy proline	0.20	Biotin	0.000005
DL-Isoleucine	0.50	Nicotinic acid	0.001
L-Lycine	0.50	Thiamine	0.001
L-Leucine	0.50	Sodium phosphate	2.85
DL-Methionine	0.35	Potassium phosphate	0.70
L-Proline	0.25		
DL-Phenylalanine	0.50	Magnesium sulfate	0.10
DL-Serine	0.75	Manganese chloride	0.05
DL-Threonine	0.50		
		Zinc sulfate	0.05
L-Tryptophan	0.25		
		Ferrous sulfate	0.05
L-Tyrosine	0.25		
		Glucose	12.50
DL-Valine	0.375		

Procedure: 1. Amino acids (except cystine) and ascorbic acid were weighed out into a flask. Distilled water was added and the amino acids were dissolved by bringing the solution to boil.

2. Cystine was dissolved in a few ml of .1N HCl and added to cooled solution.

3. Stock solutions of vitamins and nucleic acids were perpared and samples were added from the stock to the cooled amino acid solution.

4. Salts were weighed and added directly to the solution.

5. The medium was adjusted to pH 6.3 before sterilization at 121 C for 15 min.

6. A stock solution of glucose (50% w/w) was made up and autoclaved at 121 C for 15 min, and added aseptically to the medium.

Some of the modifications made to the procedure were (1) cystine was dissolved in 1N HCl instead of 0.1N HCl, (2) iron was omitted from the stock medium, and added aseptically as required, and (3) glucose was passed through a chelex column (Appendix B) before autoclaving.

Reference: Riha and Solberg, 1971.

Appendix E. Chelex Column for Removal

of Iron from Glucose

Step 1: Mix acetone and ethanol in a ratio of 1:1 to make 500 ml.
Soak 50 gm of the dowex chelating resin in the above
solution overnight. Discard the supernatant solution.

Step 2: Wash the chelating resin with 500 ml of acetone.

Step 3: Wash with double distilled water 10-12 times until all
traces of acetone are removed.

Step 4: Pour the chelex column (bed volume about 1.5 cmx25 cm)
and pass two bed volumes of 1N HCl through the column.
Wash the column with 1.5 l of double distilled water.

Step 5: Pass two bed volumes of 1N NaOH through the column.
Wash the column with 1.5 l double distilled water.
The pH is very basic, about pH 11.0.

Step 6: Pass the glucose solution through the column. Discard
the first 100 ml. Autoclave the chelated glucose solution
at 121 C for 15 min.

Step 7: Wash the column with double distilled water and repeat
steps 4 and 5 to re-use the column.

Appendix F. Two-Way Analysis of Variance of

the Effect of Iron level on <u>C. botulinum</u>

Growth in a Chemically Defined Medium

Treatments Iron level (ug/ml)	Turbidity		Plate Count	
	Mean	S.D	Mean	S.D
0.05	0.059*	0.0012	114*	2.2
1.00	0.127*	0.0011	593*	13.7
2.00	0.240*	0.0010	1040*	23.7
3.00	0.385*	0.0016	2010*	28.3
4.00	0.359*	0.0028	1961*	39.2
5.00	0.336*	0.0011	1910*	46.9
LSD value	0.002		34.99	

Least Significant Difference at $p < 0.05$

* Significant at $p < 0.05$

Appendix G.   Reinforced Clostridial Medium

Yeast extract	0.30%
Peptone	1.00%
Beef extract	1.00%
Glucose	0.50%
Sodium acetate	0.50%
Cysteine	0.05%
Soluble starch	0.10%

Dissolve all the ingredients except starch in distilled water. Autoclave the media at 121 C for 15 min. Cool. Dissolve the starch in boiling water and autoclave separately at 121 C for 15 min. Aseptically add starch and 10 ml/l of 10% sterile sodium thioglycollate solution to the medium.

Reference: Hirsh and Grinsted, 1954.

Appendix H.   DEAE-Cellulose Chromatography

Column preparation: DEAE-cellulose was suspended in a large volume of 0.5 N sodium hydroxide in a large vessel for about 12 hours. The liquid was washed by suction and the cellulose was washed with water three times. It was then suspended in 0.5 N HCl for 12 hours, and washed with water three times. The cellulose was once again suspended in 0.5 N sodium hydroxide for 12 hoursg and the material was washed with water by decantation. The washed DEAE-cellulose was suspended in 0.5 M potassium phosphate, pH 6.5 until use. The material was washed with about 10 column volumes of water before use. Pour the column (bed volume 1 cmx 2.5 cm).

Preparation of sample: To the lysed cell homogenate an equal volume of reagent grade acetone at 0 C was added and the mixture was stirred for 15 min. The mixture was centrifuged at 9000 rpm for 10 min at 0 C.

The clear 50% extract was passed directly through a the DEAE-cellulose column at 5 C. A dark brown band was formed at the top of the column.

The contaminating flavins were preferentially eluted with 0.5-1 l of 0.2 M Tris-HCl buffer at pH 8.0. The ferredoxin was removed from the column by elution with 0.5 M Tris-HCl at pH 8.0.

Final purification and crystallization of Fferredoxin is obtained by ammonium sulfate fractionization.

Reference: Rabinowitz, 1971; Mortenson, 1964.

Appendix I. Bio-Rad Protein Assay

The Bio-Rad dye reagent concentrate purchased from the Bio-Rad laboratories, Richmond, CA, was diluted with distilled water in a concentration of 1:4. To 2 ml of the diluted dye reagent. 0.5 ml of the appropriately diluted protein sample was added. The absorbance was measured at 595 nm.

The protein concentration was determined by comparison with a standard curve prepared from crystalline bovine serum albumin (Fig 10).
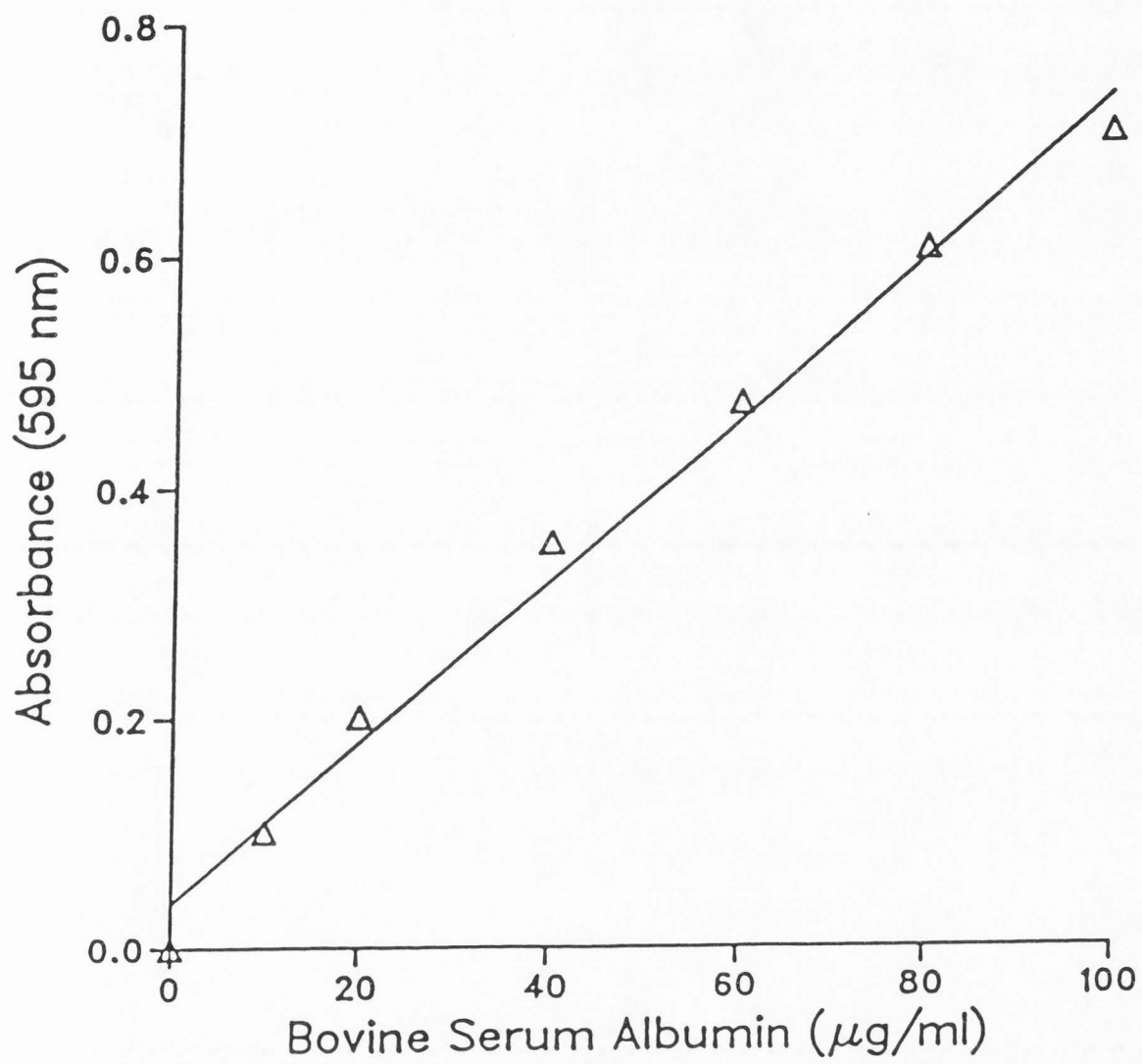
Fig 10. Standard graph for the determination of protein by the
Bio-Rad assay

Appendix J. Determination of Ferredoxin Activity

by a Cytochrome C Reduction Method

Reagents

    Cytochrome c, 0.5 mM

    NADPH, 20 mM

    Tris.HCl buffer, 0.1 M, pH 7.8

    Ferredoxin-NADP reductase (spinach)

Procedure: To a cuvette add 0.1 ml each of Tris-HCl buffer,
cytochrome c, ferredoxin extract, and enzyme (FNR) in a final volume
of 1.0 ml. The reaction is initiated by the addition of 10 ul of
NADPH and the increase in absorbance at 550 nm is measured.

Reference: Shin, M. 1971.

Appendix K. Lowry Protein Determination

Reagents

Solution A: 20 g Sodium carbonate
per liter
    4 g Sodium hydroxide

    0.2 g Potassium sodium tartrate

Solution B: 6 g Cupric sulfate

Solution C: Mix solution A in a 50:1 ratio with

    solution B

Prepare phenol solution immediately prior to use by
diluting Folin and Ciocalteu phenol reagent 1:1 with
water.

To assay for for protein 5 ml of Lowry solution c was added to 1 ml
appropriately diluted protien sample, and the mixture was incubated at
room temperature for 20 min.   Then 0.5 ml of the diluted phenol
solution was rapidly added and mixed.   The mixture was allowed to
stand with occasional shaking for 45 min. at room temperature for
color development.   Absorbance was measured at 660 nm against a
control consisting of water and plus all the other reagents.   The
protein concentration was determined by comparison with a standard
curve prepared from crystalline bovine serum albumin (Fig 11).

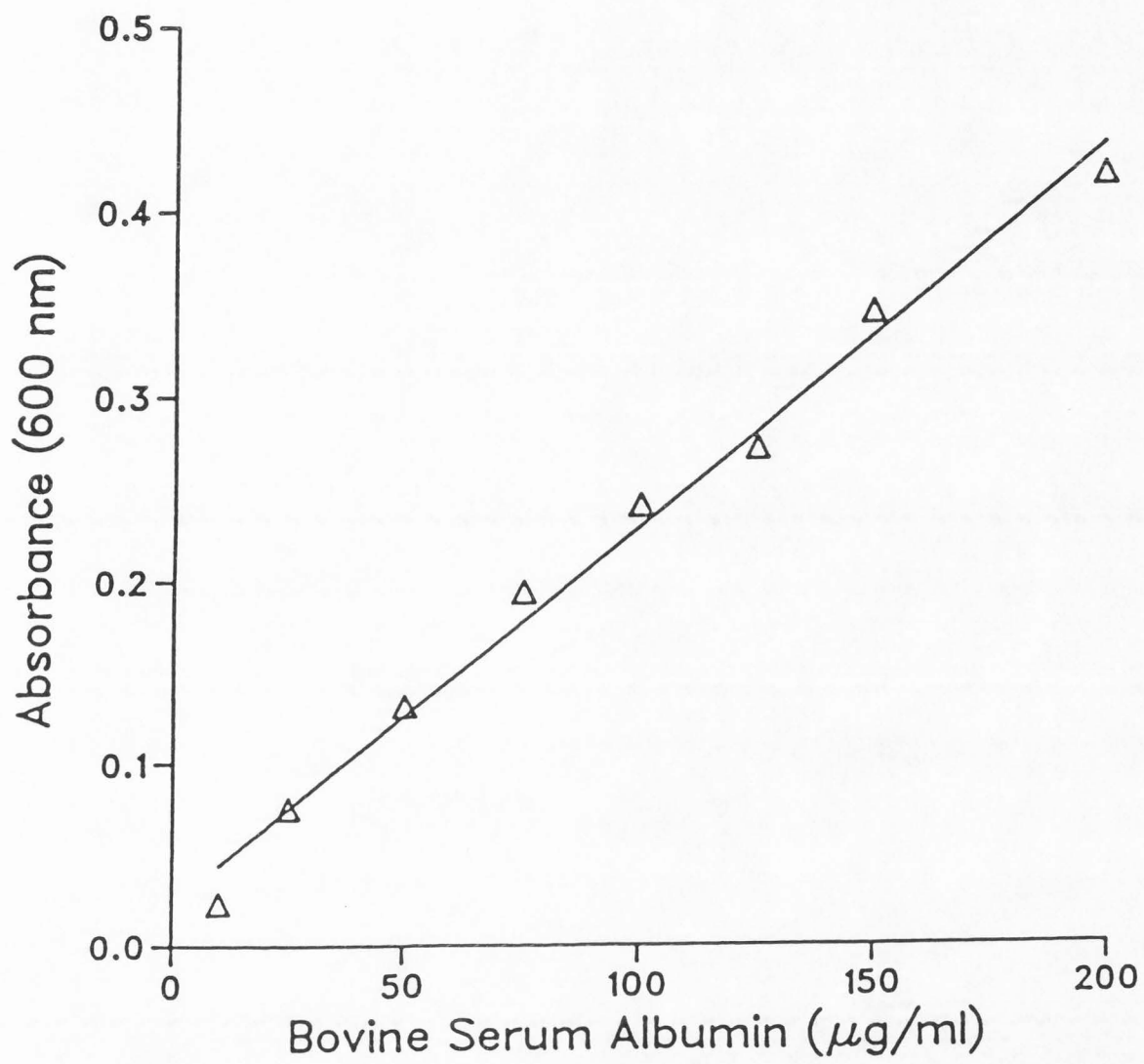Reference: Lowry et al., 1951.

Fig 11. Standard graph for the determination of protein by the Lowry method

Appendix L.   Determination of Pyruvate-Ferredoxin

Oxidoreductase Activity by a FAD Reduction Method

The reaction mixture (0.8 ml) contains:

62.5 mM Potassium phosphate buffer, pH 6.8

25 mM 2-mercaptoethnol

12.5 mM Sodium pyruvate

0.5 mM CoA

0.0625 mM FAD

The reaction mixture was transferred to a 1 ml quartz cuvette, stoppered with a septum rubber gasket.   Two syringe needles were inserted through the gasket to flush the cuvette with nitrogen before the addition of the enzyme.   The reaction was initiated by the addition of 0.1 ml of the enzyme through the septum with a 1 ml syringe.

The change in absorbance at 450 nm at 25 C was determined with a recording spectrophotometer.

Reference: Uyeda and Rabinowitz. 1971b.

Appendix M.   Determination of Pyruvate-Ferredoxin

Oxidoreductase Activity as Measured

by Acylhydroxamate Formation

The reaction mixture (2 ml) contains:

2 mM   Sodium pyruvate

200 mM Potassium phosphate buffer, pH 6.8

0.575 mM CoA

0.5 mM FAD

25 mM 2-mercaptoethnol

Add 0.1 ml enzyme extract containing 40 micrograms protein.
Terminate the reaction by the addition of 1 ml of neutralized 28%
hydroxylamine solution to 2 ml of reaction mixture.   After 10 min,
add 1 ml of 2 M ferric chloride solution.   Remove denatured protein
by centrifugation.

Determine absorbance at 540 nm.

One micromole of acylhydroxamate gives an optical density reading
of 0.17 at 540 nm.

In this study the reaction was allowed to proceed for two min
before termination by the addition of hydroxylamine solution.

Reference: Raeburn and Rabinowitz. 1971a.