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I am submitting herewith a thesis written by Shi-Chien Catherine Sung entitled "Effect of Chemical Inhibitors on Toxin Production by Clostridium botulinum in Canned Comminuted Cured Meat." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Technology and Science.

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EFFECT OF CHEMICAL INHIBITORS ON TOXIN PRODUCTION BY *CLOSTRIDIUM BOTULINUM* IN CANNED COMMINUTED CURED MEAT

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Shi-Chien Catherine Sung

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DEDICATION

This thesis is dedicated to my parents, Mr. and Mrs. Yane-Keng Sung, whose love, support, encouragement and prayer in my life will always be treasured.

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ABSTRACT

The purpose of this experiment was to determine the effect of eight antimicrobials on growth and toxin production by Clostridium botulinum in canned, comminuted cured meat. The antimicrobials evaluated were methyl, ethyl, propyl and butyl parabens, sorbic acid, potassium sorbate, sodium dehydroacetate and sodium benzoate.

Chemicals were first evaluated in thioglycollate broth for their ability to inhibit growth of Clostridium sporogenes and C. botulinum 12885A and ATCC 7949. Results showed that methyl, ethyl, propyl and butyl parabens and sorbic acid were very effective in inhibiting growth of C. sporogenes and C. botulinum 12885A and ATCC 7949. Ethyl, propyl and butyl parabens and sorbic acid were also every effective in inhibiting toxin production by C. botulinum 12885A and ATCC 7949.

Since ethyl, propyl and butyl parabens and sorbic acid completely inhibited toxin production, they were individually added to a pork slurry containing salt and sugar, with or without 40 ppm sodium nitrite. Treatments were dispensed into 206x006 thermal death time cans, inoculated with C. botulinum, and processed at 77°C in a water bath to an internal temperature of 68.5°C. The bulk of the canned product (18 cans/treatment) was abused by holding at 27°C and observed over a three month period. However, in canned, comminuted cured pork, ethyl propyl and butyl parabens and sorbic acid inhibited toxin production, by C. botulinum 12885A and ATCC 7949 at 27°C for a maximum of 28 days. Thus, they delayed outgrowth of C. botulinum but did not inhibit it completely.

These experiments have provided an initial evaluation of the effectiveness of eight antimicrobials against growth and toxin production by C. botulinum 12885A and ATCC 7949. Expanded studies using additional strains of C. botulinum and utilizing other food systems are necessary to fully evaluate the potential of the selected chemicals as preservative agents against C. botulinum in our food supply.

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CHAPTER I

INTRODUCTION

The use of preservatives in meats to inhibit growth and toxin production by Clostridium botulinum has been limited to sodium nitrite based on historical curing processes (Christiansen et al., 1973). Recently, a major concern has been the occurrence of carcinogenic nitrosamines in cured meat, arising partly from the presence of nitrite (Roberts et al., 1976; Ivey et al., 1978; Sebranek, 1979; Sofos et al., 1979a). One approach to the solution of this problem has been the consideration of potential substitutes for nitrite to control C. botulinum (Ivey and Robach, 1978; Sebranek, 1979; Sofos and Busta, 1980).

Some common preservatives widely used as antimicrobial agents in the food industry have recently been suggested as potential alternatives to nitrite as antibotulinal agents in meat products. These preservatives include methyl and propyl esters of parahydroxybenzoic acid (parabens) and potassium sorbate or sorbic acid (Tompkin et al., 1974; Ivey and Robach, 1978; Robach and Pierson, 1978; Sofos et al., 1979b,c). Sodium benzoate and sodium dehydroacetate have not been tested for their ability to inhibit growth and toxin production by C. botulinum.

This study was designed to determine the value of these chemicals as inhibitors of the growth and toxin production of C. botulinum in canned comminuted cured meat. The levels of sodium chloride, dextrose, and processing conditions were held constant, all in concert with typical industry practice (Christiansen et al., 1973).

Canned, comminuted cured meat has been suggested as a good system for testing botulinal inhibitors because it offers the best control in terms of formulation and processing (Tompkin et al., 1977). Utilizing canning is necessary for practical evaluation of inhibitors as preservative agents against C. botulinum under normal storage conditions.

CHAPTER II

REVIEW OF LITERATURE

I. *CLOSTRIDIUM BOTULINUM* TOXIGENESIS

Clostridium botulinum food intoxication (botulism) has been recognized as a public health problem since 1793 (Odlaug and Pflug, 1978). Botulism is a severe type of food poisoning caused by the ingestion of foods containing heat-labile protein neurotoxin formed during growth of *C. botulinum* (Riemann, 1973; Johnson and Peterson, 1974; Smith, 1977; CAST, 1978; Losikoff, 1978; Sofos et al., 1979a). Botulinal toxin, the most potent poison known to man, is produced by the growth of the organism in the food material before it is consumed and thus, the disease is an intoxication and not a food-borne infection (Johnson and Peterson, 1974).

C. botulinum is a gram positive, anaerobic, gas-forming motile, rod-shaped soil bacterium which forms spores subterminally. The organism occurs in the soil throughout the world (Johnson and Peterson, 1974; Schantz and Sugiyama, 1974; Smith, 1977).

Seven antigenic types of toxin are produced by different members of the species and these form the basis for classification of the organism into type A through G. Types A, B, E and F produce human botulism (Riemann, 1973; Johnson and Peterson, 1974; Schantz and Sugiyama, 1974; Smith, 1977; Odlaug and Pflug, 1978; Sofos et al., 1979a). Botulism caused by type A or B toxins is associated with canned vegetables, fruits and meats. Botulism caused by type E toxin is

associated with fish or various types of marine products (Schantz and Sugiyama, 1974; Odlaug and Pflug, 1978).

The Toxins of Clostridium botulinum

The toxins are synthesized within the bacterial cell during growth and are released from the cells of C. botulinum during autolysis or cell degeneration (Smith, 1977; Sofos et al., 1979a). Highly purified toxins in acidic solutions behave as simple proteins with molecular weights 200,000 to 900,000. These proteins are the most toxic substances known and cause botulism in man and animals (Schantz, 1968; Schantz and Sugiyama, 1974).

The toxin is a complex of at least two simple proteins: the smaller protein, constituting about 20% of the crystalline toxin, is a neurotoxin and is designated the α fraction, also called the acid-labile antoxigenic (Ea) component. The nontoxic protein has hemagglutinating properties. It constitutes about 80% of the crystalline toxin and is designated the β fraction, also called the nontoxingenic (Eb) component (Schantz and Sugiyama, 1974; Smith, 1977; Sofos et al., 1979a).

The hemagglutinin and the toxic component are distinctly different proteins (Smith, 1977). The nontoxic component of the toxin complex appears to make the neurotoxin more stable. The neurotoxin is particularly labile to some enzymes of the digestive tract. The hemagglutinin portion of the type A molecule forms a shield around the neurotoxin that protects it against adverse effects (Schantz and Sugiyama, 1974). The hemagglutinin of type A is antigenic and its ability to bind with red blood cells is inhibited by antibody (Smith, 1977). The means by which the neurotoxin is bound to the nontoxic

portion of the toxin complex is not understood. Whatever the nature of the bonding, it apparently provides protection to allow some of the neurotoxin to survive the digestive processes and get into the bloodstream. The acidic condition of the stomach would tend to keep the complex bound together, and the alkaline conditions encountered later in the intestine would favor dissociation into units that could be absorbed into the bloodstream. A small toxin molecule would help to explain how the toxin passed through the various membranes of the body to the target nerves by the bloodstream (Schantz and Sugiyama, 1974; Smith, 1977; Sofos et al., 1979a).

After entering the circulatory system, the toxin molecules are bound specifically to the neuromuscular (myoneural) junction, where they act by blocking the presynaptic release of acetylcholine from cholinergic junctions, preventing the transmission of impulses from nerves to muscles, thus blocking normal voluntary muscle movement (Johnson and Peterson, 1974; Schantz and Sugiyama, 1974; Forrest et al., 1975; Smith, 1977; Sofos et al., 1979a).

It appears that the organism does not produce the fully toxic molecule, but rather a progenitor toxin that is activated to its full toxicity by certain enzymes produced in the culture. Type E and non-proteolytic strains of other types do not produce such progenitor activation enzymes. This toxic form (progenitor) could be converted to its full toxicity by treatment with trypsin (Schantz, 1968). Conversion of the type E progenitor toxin to its full potency can increase the toxicity as high as 100-fold or more. The proteolytic organisms that produce types A and B toxins also produce progenitor toxins, but these organisms produce proteinases that activate the toxins to their potential

toxicity. One of these enzymes has been purified and shown to have trypsin-like properties (Schantz and Sugiyama, 1974; Smith, 1977; Sofos et al., 1979a).

The toxins of C. botulinum are heat labile and are therefore readily destroyed by normal cooking. Type A toxin is inactivated at 60°C for 40 minutes. Boiling for 10 minutes provides a reasonable margin of safety against all types (Johnson and Peterson, 1974; Sofos et al., 1979a). The inactivation rate depends to a great extent on the pH of the medium containing the toxin; the more alkaline, the faster the detoxification (Schantz and Sugiyama, 1974).

A unique characteristic of the botulinal neurotoxins is their extreme toxicity. The estimated human lethal dose is about 5×10^{-8} gram or 1 gram of toxin could kill more than 500 million people if properly diluted (Sofos et al., 1979a).

The toxins are good antigens, but natural immunity in people rarely occurs because the dose needed to cause death is less than that required to elicit antibody production. Toxoids of the toxins can be made with formaldehyde, resulting in the complete destruction of toxicity and retention of antigenicity to effectively immunize man and animals. Besides the production of antisera for medical treatment of botulism in man, the toxoids are valuable for the immunization of people that work with the toxin, livestock, and some fur-bearing animals against botulism (Schantz and Sugiyama, 1974; Smith, 1977).

Symptoms

The earlier the onset of symptoms, the higher the toxin concentration and the higher the probability of fatality (Sofos et al.,

1979a). The typical symptoms of botulism usually appear within 12 to 36 hours although a longer or shorter time may be required. Botulinal toxin causes paralysis by blocking motor nerve terminals at the myoneural junction. Paralysis progresses downward, usually starting with the eye and face, progressing to the throat, chest and extremities. When the diaphragm and chest muscles become fully involved, respiration is no longer possible and death from asphyxia results (Johnson and Peterson, 1974; Sofos et al., 1979a).

Early signs are marked: lassitude, weakness, vertigo, diplopia, progressive difficulty in speaking, swallowing and breathing, weakness of other muscles, abdominal distention and constipation. Death usually results from respiratory failure (Johnson and Peterson, 1974; Schantz and Sugiyama, 1974).

II. THE USE OF NITRATE AND NITRITE

Historically

It is established that for many centuries nitrite or its precursor, nitrate, have been accidental or intentional ingredients of meat products (Rubin, 1977; CAST, 1978; Sofos et al., 1979a).

The first form of nitrate to be used was saltpeter or nitre (potassium nitrate, KNO_3). As time passed, use of nitrate became customary; curing techniques were developed; the importance of nitrite over that of nitrate was realized; nitrite's effects and functions were determined; and curing a meat became a science (Sofos et al., 1979a).

The transformation of meat curing from an inexact art to a precise science started toward the end of the 19th century (Sebranek,

1979; Sofos et al., 1979a). One of the achievements of this period was the recognition that nitrite was formed through microbial action on nitrate, and that nitrite instead of nitrate was responsible for cured meat color development (Sofos et al., 1979a).

The Bureau of Animal Industry of the U.S. Department of Agriculture (USDA) subsequently authorized the use of sodium nitrite in quantities not to exceed 1/4 oz per 100 lbs of chopped or comminuted meat (156 parts per million or ppm), with a residual level of sodium nitrite not to exceed 200 ppm in the cured product. This authorization was incorporated into the Meat Inspection Act of 1906 and subsequently became part of the Code of Federal Regulation (CAST, 1978). In 1923, the USDA gave permission for experimentation on the direct use of nitrite in meat products. In 1925, the USDA authorized use of sodium nitrite in meat curing in federally inspected plants (Sofos et al., 1979a). During the 1930s, progress advanced as processors adopted the use of nitrite to accelerate their processes (Sebranek, 1979).

The Role of Nitrite

Experience and scientific knowledge have indicated that when nitrite is added to meats, it performs the following functions: (a) it produces the characteristic cured meat color and affects flavor (Cho and Bratzler, 1970; MacDougall et al., 1975); (b) it has antioxidant activities which prevent "warmed-over" flavor (Rubin, 1977); (c) it retards *C. botulinum* growth and toxin production, which can occur if the product is mishandled and temperature-abused (Christiansen et al., 1973; Gray and Dugan, 1975; Gray, 1976; Rubin, 1977).

Color and Flavor

It has been shown that the main portion of the total nitrite (156 μg/g) added to cured meat products is used for control of C. botulinum, and only a small fraction (about 25 μg/g) is needed for development of the characteristic color and flavor of the products (Sebranek, 1979; Sofos et al., 1979a). Recent studies have shown that as little as 25 to 50 ppm sodium nitrate ($NaNO_2$) is required to give typical color and flavor (Rubin, 1977; Ivey et al., 1978; Sofos et al., 1979a).

Nitrite is responsible for development of cured meat color through its reduction to nitric oxide and reaction with the meat pigment myoglobin (Mb). The compound formed through the reaction of nitric oxide and myoglobin (Mb) is called nitrosomyoglobin or nitrite oxide myoglobin (NOMb) and is relatively unstable (Cho and Bratzler, 1970; MacDougall et al., 1975; Rubin, 1977). The protein moiety of the compound is denatured by heat during thermal processing and a much more stable compound, the cured meat pigment nitrosohemochrome, is formed. However, it is unstable in the presence of light (Sofos et al., 1979a; Sofos and Busta, 1980).

Few studies have reported on the interaction of nitrite and meat constituents that influence flavor. Very little is known of the identity of the volatile substances or the mechanism of the reactions leading to the flavor formation of cured flavors in cooked products (MacDougall et al., 1975).

The relationship of nitrite and nitrate to cured flavor was first described in 1940. The first rigorous test of the essential part played

by nitrite in cured flavor formation was made by Cho and Bratzler in 1970. Nitrite-containing products showed a more intense cured flavor (Cho and Bratzler, 1970; MacDougall et al., 1975). In common with most foods, cured meat products have complex aroma volatile profiles and it is reasonable to assume that cured flavor is a composite sensation derived from the contributions of many individual odoriferous compounds (MacDougall et al., 1975).

Antioxidant

Sodium nitrite has a potent antioxidant effect, eliminating the problem of warmed-over flavor in cured meats. The mechanism of action is probably through the binding of iron in myoglobin and hemoglobin by reaction with nitric oxide. Iron itself is a known potent pro-oxidant (Rubin, 1977).

Antibotulinal

The most important nitrite effect in cured meat products is its inhibitory action against C. botulinum growth and toxin production (Perigo and Roberts, 1968; Christiansen et al., 1973; Roberts, 1975; Miller, 1980). To retard bacterial growth, nitrite concentrations higher than those needed for color and flavor development are necessary (Roberts, 1975; Sofos et al., 1979a). At least 100 to 150 ppm $NaNO_2$ is required to inhibit outgrowth and toxin production by C. botulinum (Christiansen et al., 1973; Ivey et al., 1978).

Although the antibotulinal action of nitrite is well documented (Christiansen et al., 1973), the exact mechanism of action is still unknown (Duncan, 1970; Roberts, 1975; Sofos et al., 1979a). The only

point of agreement is that it acts on the spores of C. botulinum after germination and before release of the toxin (Christiansen, 1980; Sofos and Busta, 1980). Nitrite inhibits by preventing outgrowth of germinated botulinal spores (CAST, 1978; Christiansen, 1980). Recent information, based on indirect but conclusive evidence, indicates that nitrite may be reacting with an iron-containing compound, such as ferredoxin, within the germinated botulinal cell and consequently could interfere with the energy metabolism of the germinating spore to prevent outgrowth (Sofos and Busta, 1980).

The Carcinogenic Hazard Related to Nitrite

Since the mid-1960s, the concern over nitrosamines in cured meat products has greatly increased, and nitrite usage in meat curing has come under attack (Ivey et al., 1978; Sofos et al., 1979a; Miller, 1980). It is due to reports showing that nitrite added to certain meats serves as a precursor of carcinogenic nitrosamines (Rubin, 1977; Ivey et al., 1978; Gray and Randail, 1979; Sebranek, 1979; Sofos et al., 1979a). Although there is no direct evidence that n-nitrosocompounds are carcinogenic to man, indirect proof from animal studies on 12 species would suggest that there is a potential danger to man (Swann, 1975; Gray and Randail, 1979). Most of these compounds are carcinogenic and in addition some exhibit mutagenic, embryopathic and teratogenic effects (Gray, 1976; Gray and Randail, 1979).

Nitrosamines can be produced by a combination reaction of nitrite or nitrous acid and secondary amines (normal protein breakdown products) or tertiary amines. Since cured meats contain both nitrite and amines which are naturally present in raw meats, there is a potential for

nitrosamine formation under the appropriate reaction conditions (Gray and Dugan, 1975; Swann, 1975; Gray, 1976; Roberts et al., 1976; CAST, 1978; Gray and Randail, 1979; Sofos et al., 1979a). The rate of n-nitrosation of secondary amines is directly proportional to the square of the nitrite concentration. N-nitrosamine formation is directly related to the initial concentration of nitrite (Gray, 1976).

When the news about nitrosamines in meats was published in 1970, the cured-meat industry reacted promptly to establish research programs to reevaluate the use of salt, nitrite, and nitrate in the curing process (CAST, 1978).

A recent report (Newberne, 1979) implicated nitrite itself as an agent directly causing cancer to experimental animals. The results of this study, which indicate that nitrite produces a statistically significant increase in cancer of the lymphatic system, was made available to the public in summer, 1978. If the Newberne study is confirmed, USDA and FDA will, under current law, have the clear responsibility to propose a ban on the addition of nitrite to the food supply (Butler, 1980).

In Time magazine on September 1, 1980, it was stated that

> . . . the government last week (around late August) announced that pathologists examined Newberne's experiment and could not confirm the study conclusion. FDA and USDA announced that there is no action to remove nitrite from food at this time. The government in the meantime is asking National Academy of Science to reevaluate nitrite.

III. CONSIDERATION OF ALTERNATIVES

The fact that the ten-year-old nitrite controversy remains active and that only minor changes have been introduced in the federal regulations, verifies the importance of nitrite in the production of

cured meat products. This fact is also an indication that no viable alternative capable of covering the entire range of cured meat products has been identified. The lack of progress in identifying alternatives also indicates the complexity of the problem (Sofos and Busta, 1980).

Since nitrosamine has been implicated as a possible cancer-causing agent and reduction in, or elimination of, nitrite's use in meat curing has been proposed (Gray, 1976; Wasserman et al., 1977; Ivey et al., 1978; Sebranek, 1979; Sofos et al., 1979b) and testing of alternative preservatives has become more important (Ivey and Robach, 1978; Sebranek, 1979; Butler, 1980; Sofos and Busta, 1980).

The major alternative to process or handling modifications would be to develop a replacement or substitute for nitrite. This alternative has received intense attention for several years. Several hundred compounds have been examined, but none has been a suitable food substitute (Sebranek, 1979; Sofos and Busta, 1980). The most recent approach to providing an alternative is to use reduced levels of nitrite, along with either nitrosamine-blocking agents or a C. botulinum inhibitor (Sebranek, 1979).

The first compound to be recognized as a nitrosamine inhibitor was ascorbate or erythorbate (Gray and Dugan, 1975). Although ascorbate and erythorbate are quite effective, they are not completely effective as nitrosamine inhibitors, probably because of their limited solubility in adipose tissue (Gray, 1976). Several lipid-soluble, potential nitrosamine-blocking agents have been studied: propyl gallate, ascorbyl palmitate, alpha-tocopherol, and tertiary butylhydroquinone (TBHQ) (Gray and Dugan, 1975; Pensabene et al., 1978; Sebranek, 1979).

An update on the present status of the efforts to identify antibotulinal alternatives to nitrite is also timely and important (Sebranek, 1979; Sofos and Busta, 1980). Any substance or any other means of botulism protection will be considered as an alternative to nitrite. The lack of information on the nitrite mechanism makes it necessary to explore empirical solutions and to consider alternative ways to inhibit botulinal toxin production based on available information (Sofos and Busta, 1980).

Based on available information it appears that there are at least two ways of approaching the task of identifying alternatives to nitrite as antibotulinal agents. One is the selection based on effectiveness and interaction of optimal combinations of factors presently used in meat products, and the second is to examine substances with potential when feasible. This approach could be examined both in the complete absence as well as presence of nitrite solely for chemical reactions. It would constitute a compromise and could be based on minimizing potential health risks to possible "acceptable limits" (Sebranek, 1979; Sofos and Busta, 1980). The nitrite issue is a real issue. Finding alternatives for nitrite is thus sound business policy, as well as sound health policy (Butler, 1980).

IV. PRESERVATIVES

Salt (NaCL)

A concentration of 10% sodium chloride is well established as sufficient to control all types of C. botulinum (Riemann et al., 1972; Riemann, 1973). This 10% level of salt binds the free water of the

system resulting in reduced water activity. The average salt concentration of most of the cured meat products is in the range of 2.0 to 3.0% (CAST, 1978). Increasing the concentration to a level adequate for complete botulism control (10%) would interfere with the palatability of the products and would minimize their acceptance by the consumer (Greenberg et al., 1959). However, salt concentrations considerably lower than 10% can interact with factors such as pH, nitrite, and thermal processing to exhibit sufficient inhibitory activity against botulinal outgrowth.

Sugar

Sugars are used in cured meat products primarily as flavoring ingredients or to decrease the harshness of salt or both. A sugar level of 50% decreases water activity, which is inhibitory to C. botulinum growth. However, such levels of sugars in meat are not in use. Fermentable carbohydrates such as dextrose at low levels have an indirect effect against C. botulinum growth by serving as substrates for pH reduction by microorganisms (Riemann et al., 1972).

V. OTHER PRESERVATIVES (POTENTIAL ALTERNATIVES)

Some common preservatives widely used as antimicrobial agents in the food industry have been suggested as potential alternatives to nitrite as antibotulinal agents in meat products. These preservatives include methyl and propyl esters of parahydroxybenzoic acid (parabens) and potassium sorbate or sorbic acid.

Acetates

Empirical knowledge of the preservative action of acetic acid in the form of vinegar extends far back into history. Today, besides vinegar and purified acetic acid, the following compounds which yield acetic acid are available for food use: sodium acetate, calcium acetate, potassium acetate and sodium diacetate. The acetic compounds not only have preservative action, but also function as sequestrants, acidulants and flavoring agents (Chichester and Tanner, 1972; Frazier and Westhoff, 1978).

Acetic acid (CH_3COOH) is more effective against yeasts and bacteria than against molds. Acetic acid and its salts' effectiveness increases with a decrease in pH, which would favor the presence of the undissociated acid (Chichester and Tanner, 1972; Frazier and Westhoff, 1978). It may be added for flavor, but it also exerts concurrent antimicrobial action (Chichester and Tanner, 1972).

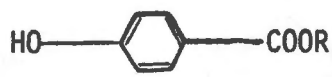
Sodium diacetate ($CH_3COONaCH_3COOH \cdot 1/2H_2O$) has been used in cheese spreads and malt syrups as an antimicrobial agent, and used as treatment to wrappers used on butter (Chichester and Tanner, 1972; Frazier and Westhoff, 1978). Sodium diacetate has an advantage over the simple acetates, because it controls both rope and molds in bread and other baked goods (Chichester and Tanner, 1972).

Under federal regulations in the United States, vinegar, acetic acid, sodium acetate, calcium acetate, sodium diacetate and calcium diacetate are generally recognized as safe (GRAS) for use in foods (Chichester and Tanner, 1972).

Dehydroacetic acid has been used to impregnate wrappers for cheese to inhibit growth of molds and as a temporary preservative for squash (Frazier and Westhoff, 1978).

Sodium dehydroacetate ($C_8H_7NaO_4 \cdot H_2O$) is the sodium salt of dehydroacetic acid. It is a white or nearly white, odorless powder having a slight characteristic taste. It is used in foods as a preservative (Food Chemical Codex, 1972; The Food Chemical News Guide).

Parabens



The alkyl esters of para-hydroxybenzoic acid (parabens) comprise a group of antimicrobial agents which have been used widely in cosmetic and pharmaceutical products (Aalto et al., 1953; Chichester and Tanner, 1972; Robach and Pierson, 1978), since they offer a means for extending upward the limiting pH for effective use of benzoic acid (Chichester and Tanner, 1972).

The antimicrobial action of the parabens was first described in 1924. Regulations permitting the use of these preservatives in foods were adopted in many European countries between 1932 and 1938 (Aalto et al., 1953; Chichester and Tanner, 1972). Approval of these esters as food preservatives in the U.S. was first indicated by the FDA in 1956. The methyl and propyl esters of p-hydroxybenzoic acid are most commonly used in the U.S.A., but the ethyl and butyl esters find some application in other countries (Chichester and Tanner, 1972). Under U.S. Food and Drug Administration regulations, methyl and propyl parabens are "generally recognized as safe" (GRAS) when used as

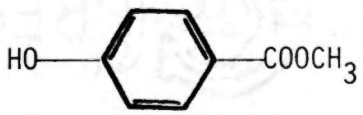
chemical preservatives of foods, with a total addition limit of 0.1% (Chichester and Tanner, 1972; Jay, 1978). Among the seventeen countries discussed in the section on international uses of antimicrobial food additives, about two-thirds of them list the parabens as officially approved for use. In Italy the ethyl, as well as the methyl and propyl, esters may be used as a direct additive. The butyl ester is used in Japan (Chichester and Tanner, 1972).

The antimicrobial activity of the p-hydroxybenzoic esters is directly proportional to the chain length, but water solubility also decreases with increasing chain length (Aalto et al., 1953; Chichester and Tanner, 1972), hence, in practice, the lower esters are commonly used. These compounds, being closely related to benzoic acid, have many properties in common with it, yet differ in ways which enhance their utility (Chichester and Tanner, 1972). The parabens are most active against molds and yeasts. The esters are more fungistatic than fungicidal. They are less effective against bacteria, especially gram-negative bacteria (Aalto et al., 1953; Chichester and Tanner, 1972).

All of the p-hydroxybenzoic acid esters commonly used in foods are white, free-flowing powders (Chichester and Tanner, 1972). The esters are stable in air and are resistant to hydrolysis in cold or hot water and show no hydrolysis under the usual conditions of sterilization. In general, resistance to hydrolysis increased with the size of the alkyl side chain and with branching of the chain (Aalto et al., 1953). The methyl ester of p-hydroxybenzoic acid has a slight, characteristic odor, but the other esters are practically odorless in the usual, purified form (Aalto et al., 1953; Chichester and Tanner, 1972). No particular taste is noticeable in solutions of the esters, but in

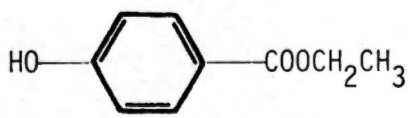
saturated aqueous solutions may exhibit a slight tang or weak burning sensation in the mouth. In terms of equal concentrations, it is most noticeable with the butyl esters. According to taste panel tests, this tang is not noticeable with lower concentration or in the presence of flavorings or fatty materials (Aalto et al., 1953). According to Ayres et al. (1980), alkyl esters of p-hydroxybenzoic acid impart milk but distinct flavors and they are used only in foods having flavors with which the ester flavors blend.

1. Methyl p-hydroxybenzoate (Methylparaben) $C_8H_8O_3$

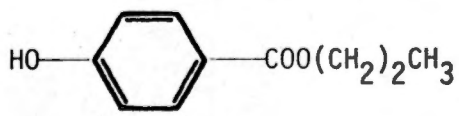


It is small, colorless crystal or a white, crystalline powder. It has a faint, characteristic odor and a slight, burning taste. It is used in foods as a preservative and antimicrobial agent (Food Chemical Codes, 1972).

2. Ethyl p-hydroxybenzoate (Ethylparaben) $C_9H_{10}O_3$

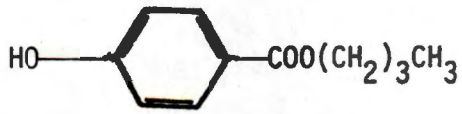


3. Propyl p-hydroxybenzoate (Propylparaben) $C_{10}H_{12}O_3$



Propylparaben is a small, colorless crystal or a white powder. It is used in foods as a preservative and antimicrobial agent (Food Chemical Codex, 1972). It is approved for use in the casings of dry sausages to retard mold growth (Sofos and Busta, 1980). Lee (1973) found propylparaben was the most effective food preservative among 14 food preservatives against V. parahaemolyticus.

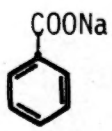
4. Butyl p-hydroxybenzoate (Butylparaben) $C_{11}H_{14}O_3$



Butylparaben is cleared for use as a synthetic flavoring substance and adjuvant (The Food Chemical News Guide).

Robach and Pierson (1978) found methyl- and propyl-parabens very effective against growth and toxin production by C. botulinum strain 10755A in a microbiological medium. Tanaka et al. (1978) working with wieners found effects of parabens against C. botulinum type A and B spores to be only minor. Deibel (1979) reported that they were ineffective in a commerical wiener system. Consequently the outlook on parabens as potential alternatives to nitrite in meat products has not been very promising (Sofos and Busta, 1980).

Sodium Benzoate ($C_7H_5NaO_2$)



Sodium benzoate was the first chemical preservative permitted in foods by the U.S. FDA (Jay, 1978). This compound is still in wide use today in a large number of foods as a preservative and antimicrobial (Chichester and Tanner, 1972; Food Chemicals Codex, 1972; Jay, 1978), and has an especially wide applicability as an antimicrobial for foods (Chichester and Tanner, 1972). Use levels range from 0.05% to 0.10% (Chichester and Tanner, 1972; The Food Chemical News Guide).

This compound, as an article of commerce, is in the form of a white powder or flakes, and is odorless or nearly odorless (Chichester and Tanner, 1972; Food Chemicals Codex, 1972). Sodium benzoate is most

suitable for foods and beverages which are in the pH range below 4.0 or 4.5 (Chichester and Tanner, 1972). Sodium benzoate is generally considered to be most active against yeasts and molds. It acts against microorganisms by inhibiting the cellular uptake of substrate molecules (Jay, 1978).

Benzoates are on the GRAS list in the U.S. (Chichester and Tanner, 1972; The Food Chemical News Guide). There is no danger of accumulation of benzoate in the body. The apparent reason for the high tolerance of the body to benzoic acid is a detoxifying mechanism, whereby the benzoate is conjugated with glycine to produce hippuric acid and is excreted in that form. Among the antimicrobial additives, sodium benzoate has the additional advantage of low cost (Chichester and Tanner, 1972).

Sorbates ($CH_3CH=CHCH=CHCOOR$)

Sorbic acid ($C_6H_8O_2$) and potassium sorbate ($C_6H_7KO_2$), collectively known as the sorbates, have been used as antimicrobial agents in the food industry for over 30 years. Sorbates have been mainly used as antifungal agents in many foods (Deuel et al., 1954a) and have been GRAS food additives for several years (Ivey et al., 1978; Robach and Hickey, 1978; Sebranek, 1979). Sorbic acid has been shown to be harmless for mammals as a component of foods (Deuel et al., 1954a; Smith and Rollin, 1954). Sorbic acid is a 6 carbon, unsaturated fatty acid and is metabolized, like most fatty acids, to CO_2 and H_2O (Deuel et al., 1954b; Sebranek, 1979; Sofos et al., 1979a).

Sorbic acid has been used for years as an effective antifungal agent, but only recently has its antibacterial properties been reported

(Ando, 1973; Tompkin et al., 1974; Robach, 1978; Robach and Hickey, 1978; Robach et al., 1978; Pierson et al., 1979).

Sorbate has been reported to have either no effect or a stimulatory growth effect on C. botulinum in culture media (Hansen and Appleman, 1955). Sorbic acid had been used to selectively culture clostridia rather than inhibit these organisms (Emard and Vaughn, 1952; York and Vaughn, 1954).

However, in a recent report, Tompkin et al. (1974) evaluated potassium sorbate in uncured, cooked sausage and found that sorbate apparently delayed or retarded C. botulinum growth. Subsequent studies have indicated that sorbic acid at 0.2% or potassium sorbate at 0.26% (equivalent to 0.2% sorbic acid) inhibits C. botulinum in canned, comminuted pork (Ivey and Robach, 1978) and in bacon (Ivey et al., 1978). Sofos et al (1979b,c) demonstrated the effectiveness of a combination of sorbic acid and nitrite in preventing toxin production by C. botulinum in chicken frankfurter emulsions. Huhtanen and Feinberg (1980) also reported that sorbic acid can be used to inhibit C. botulinum spore outgrowth in poultry frankfurters.

Research results with potassium sorbate or sorbic acid appear promising. These chemicals have received undoubtedly the most attention as an alternative to current nitrite control of botulism in meat products (Sebranek, 1979; Sofos and Busta, 1980).

In spite of much experimentation with preservation of canned, cured food, no study has yet been published which would permit prediction of required changes in salt and processing levels for a given reduction of the nitrite level.

To date no single alternative has been identified that could substitute for nitrite completely (Sofos and Busta, 1980). Of all the research on the replacement of nitrite, only the alternative of decreasing its level and supplementing it with sorbate has been extensively tested and appears promising. This alternative is the least destructive to current practices, reduces potential nitrosamine formation in bacon, and gives adequate protection against botulism (Sofos and Busta, 1980).

CHAPTER III

MATERIALS AND METHODS

I. TEST ORGANISMS

A pure culture of Clostridium sporogenes obtained from Dr. J. O. Mundt (The University of Tennessee, Knoxville, Tennessee) was grown at 35°C for 24 hours in fluid thioglycollate medium (BBL). Pure cultures of Clostridium botulinum 12885A and ATCC 7949 were obtained from Dr. L. N. Christiansen (Swift & Company, Research and Development Center, Oak Brook, Illinois) and grown at 35°C for 24 hours in thioglycollate medium. The cultures of C. sporogenes and C. botulinum were kept in the refrigerator (5°C) until needed.

II. GROWTH MEDIUM

The chemical inhibitors were kindly supplied by Tri-K Industries. The inhibitors were dissolved in fluid thioglycollate medium. It was dispensed into 16 x 150 mm tubes and autoclaved at 121°C for 15 minutes. The medium was stored at room temperature until needed.

III. GROWTH STUDIES IN PURE CULTURE WITH INHIBITORS

The growth medium was inoculated with one loop pure culture of C. sporogenes and incubated at 35°C. After 24 hours, the number of bacteria in the medium was determined by Direct Microscopic Count. The growth medium was incoulated with one loop pure culture of C. botulinum and incubated at 35°C. After 24 hours, the number of bacteria in the

medium was determined by Direct Microscopic Count. Each of the following chemicals were tested at their maximum allowable concentration (permitted in food) for their ability to inhibit growth of C. botulinum.

Methyl paraben	0.1%	Sorbic acid	0.2%
Ethyl paraben	0.1%	Potassium sorbate	0.26%
Propyl paraben	0.1%	Sodium benzoate	0.1%
Butyl paraben	0.1%	Sodium dehydroacetate	0.1%

IV. TOXIN ASSAY

Bacterial cells of growth medium were centrifuged at 3000 xg for 20 minutes. The toxin was assayed according to the method of Robach and Pierson (1978).

The supernatant was divided into two portions: (1) 0.1 ml of supernatant was injected intraperitonally into four 5-week-old male gray mice obtained from The University of Tennessee Hospital, Knoxville, Tennessee; (2) four mice were injected with 0.1 ml boiled supernatant.

Botulinal toxin was confirmed by death of the four unprotected mice, and by survival of the four mice injected 0.1 ml of boiled supernatant fluid. The mice were observed for typical symptoms of botulism over a period of four days (Smith, 1977).

V. FORMULATION AND PROCESSING

Preparation of the Spores

A pure culture of C. botulinum 12885A and ATCC 7949 was grown at 30°C for 7 days in 15 ml fluid thioglycollate medium to produce spores. After 7 days, the number of spores in the medium was determined by

Direct Microscopic Count. The spore suspension was heat shocked for 10 minutes at 80°C, then diluted in differential reinforced clostridial medium (DRCM) to the level required for inoculation before using (Speck, 1976).

Preparation of the Meat Slurry

Canned comminuted cured pork was formulated as described by Rhodes and Jarvis (1976) and processed according to Christiansen et al. (1973). Pork shoulder was trimmed of skin and fat and ground. The ground pork was formulated to contain 2.5% sodium chloride, 0.5% dextrose and maximum tolerance selected chemical inhibotors with or without 40 ppm sodium nitrite. After dissolving the additives in autoclaved distilled water, the fresh ground pork was added to it, in a bottom-drive homogenizer and blended for 60 seconds. The meat slurry consisted of a blend of equal parts by weight of ground pork and brine containing appropriate quantities of additives.

Processing Conditions

The meat slurry (18g/can) was poured into 206 x 006 thermal death time (TDT) cans. This meat slurry was inoculated with 100 spores per gram of meat with 12885A and ATCC 7949 strain of C. botulinum using a sterile 1 ml pipette. Cans were closed and then cooked in 77°C water bath to an internal temperature of 68.5°C in 25 minutes by temperature monitor. The product was then chilled in ice water to less than 27°C within 10 minutes.

The four chemicals demonstrating the greatest inhibition of toxin production and growth in the pure cultures were tested in the meat slurry individually and in combination with 40 ppm sodium nitrite (Table 1).

Table 1. Ingredients Added to Basic Comminuted, Cured Pork Formulated for *Clostridium botulinum* Inhibition

Test Lot No.	Sodium Nitrite	Chemical
1	0 ppm	0 (Control)
2	0 ppm	Ethyl paraben
3	40 ppm	Ethyl paraben
4	0 ppm	Sorbic acid
5	40 ppm	Sorbic acid
6	0 ppm	Butyl paraben
7	40 ppm	Butyl paraben
8	0 ppm	Propyl paraben
9	40 ppm	Propyl paraben

Holding Conditions

The bulk of the canned product (18 cans) was abused by holding at 27°C and observed over a three-month period. Cans were removed according to the leakage and swell. Two cans for each treatment were randomly selected and tested for toxicity immediately after canning. Therefore, a total treatment consisted of 20 cans.

Toxin Assay of Can

Toxin assays were made by blending each can of product with equal weight of gelatin phosphate buffer (GPB). The slurry was centrifuged and the supernatant was collected. The supernatant was then divided into two portions and handled as indicated under Toxin Assay.

VI. STATISTICAL ANALYSIS

1. The two response variables being tested were: (a) swell or no swell; (b) toxic or not toxic to mice.

2. Each chemical treatment was compared to the control treatment (no chemical) and to other treatments to evaluate its ability to inhibit C. botulinum toxin production.

3. Significant differences among treatments was determined by Duncan's Multiple Range Test to test differences among means.

CHAPTER IV

RESULTS AND DISCUSSION

I. EFFECT OF ANTIMICROBIALS ON GROWTH OF *CLOSTRIDIUM SPOROGENES*

The results obtained for the effect of antimicrobials on growth of *C. sporogenes* in microbiological medium are presented in Table 2. The number of bacteria per ml in each medium was determined by the Direct Microscopic Count. Differences in the effect of the chemicals on growth of *C. sporogenes* was expressed as percentage inhibition. Ethyl, propyl and butyl parabens and sorbic acid inhibited the growth of *C. sporogenes* by greater than 99%. Methyl paraben inhibited growth of *C. sporogenes* by greater than 97%. Potassium sorbate, sodium dehydroacetate and sodium benzoate inhibited the growth of *C. sporogenes* by 65.8%, 46.0%, and 24.1%, respectively. Methyl, ethyl, propyl and butyl parabens and sorbic acid were very effective inhibitors of growth of *C. sporogenes*.

Statistical analysis showed that methyl, ethyl, propyl and butyl parabens and sorbic acid were significantly different from the control treatment and from the other antimicrobials ($p < 0.05$). Methyl, ethyl, propyl and butyl parabens and sorbic acid were not significantly different from each other ($p < 0.05$). These five antimicrobials are markedly more effective than sodium dehydroacetate, sodium benzoate and potassium sorbate in inhibiting growth of *C. sporogenes* in fluid thioglycollate medium. Raw data for these experiments showing variations among test tubes and fields are shown in Tables A-1, A-2, A-3 and A-4 (Appendix).

Table 2. Effect of Selected Chemicals on Growth of Clostridium sporogenes in the Test Tubes Containing Thioglycollate Broth.

Chemical	No. of Bacteria per ml [1] ± Standard Deviation	Range[2]	% Inhibition[1]
Control I	$1.0 \times 10^8 \pm 0.5 \times 10^8$	1070	0.0[a3]
Methyl paraben (0.1%)	$2.5 \times 10^6 \pm 2.7 \times 10^6$	29	97.7[e]
Ethyl paraben (0.1%)	$3.6 \times 10^4 \pm 9.8 \times 10^4$	1	99.9[e]
Propyl paraben (0.1%)	$0.9 \times 10^5 \pm 1.8 \times 10^5$	3	99.9[e]
Butyl paraben (0.1%)	$2.8 \times 10^5 \pm 8.2 \times 10^5$	14	99.6[e]
Control II	$2.8 \times 10^8 \pm 0.9 \times 10^8$	1350	0.0[a]
Sodium dehydroacetate (0.1%)	$1.4 \times 10^8 \pm 0.6 \times 10^8$	860	46.0[c]
Sodium benzoate (0.1%)	$2.3 \times 10^8 \pm 1.6 \times 10^8$	1850	24.1[b]
Sorbic acid (0.2%)	$0.7 \times 10^5 \pm 1.8 \times 10^5$	3	99.9[e]
Potassium sorbate (0.26%)	$1.0 \times 10^8 \pm 0.5 \times 10^8$	660	65.8[d]

[1]The data are the mean of two replicates with five observations per replicate.

[2]Range = (maximum-minimun) number of bacteria per observation area.

[3]Values followed by the same letter are not significantly different ($p < 0.05$).

The Direct Microscopic Count was used rather than the Plate Count Method for counting cells of C. sporogenes. Difficulties were encountered with the Plate Count Method since we could not get stable results. When the growth medium was poured into the plate it became oxidized and exposed C. sporogenes to oxygen. Since C. sporogenes is an anaerobe, oxygen is toxic to it and it could not grow under the oxidized condition. Even when plates were placed in the BBL Gas Pak anaerobe jar, oxygen was was reduced sufficiently to allow C. sporogenes to grow.

II. EFFECT OF ANTIMICROBIALS ON GROWTH OF CLOSTRIDIUM BOTULINUM 12885A

The results obtained for the effect of selected chemicals on growth of C. botulinum 12885A (Type A) in fluid thioglycollate medium are shown in Table 3. Ethyl, propyl and butyl parabens and sorbic acid inhibited the growth of C. botulinum 12885A by greater than 99%. Methyl paraben and potassium sorbate inhibited growth of C. botulinum 12885A by 98.8% and 93.4%, respectively. Statistical analysis showed that methyl, ethyl, propyl and butyl parabens and sorbic acid were significantly different from the control treatment ($p < 0.05$). These five chemicals were not significantly different from each other ($p < 0.05$). These five antimicrobials are obviously more effective than sodium dehydroacetate (48.4% inhibition) and sodium benzoate (78.4% inhibition) in inhibiting growth of C. botulinum 12885A in microbiological medium.

The range of counts of C. botulinum 12885A (max-min) in media containing methyl, ethyl, propyl and butyl parabens and sorbic acid per observation area were 8, 6, 3, 3 and 2, respectively. The variation of observation area for these five antimicrobials was very small (Tables A-5, A-6, A-7 and A-8, Appendix).

Table 3. Effect of Selected Chemicals on Growth of *Clostridium botulinum* 12885A (Type A) in the Test Tubes Containing Thioglycollate Broth

Chemical	No. of Bacteria per ml [1] ± Standard Deviation	Range[2]	% Inhibition[1]
Control I	$4.4 \times 10^7 \pm 2.3 \times 10^7$	270	0.0[a3]
Methyl paraben (0.1%)	$4.6 \times 10^5 \pm 6.4 \times 10^5$	8	98.8[d]
Ethyl paraben (0.1%)	$1.9 \times 10^5 \pm 3.7 \times 10^5$	6	99.5[d]
Propyl paraben (0.1%)	$1.2 \times 10^5 \pm 2.3 \times 10^5$	3	99.7[d]
Butyl paraben (0.1%)	$1.2 \times 10^5 \pm 2.3 \times 10^5$	3	99.7[d]
Control II	$4.1 \times 10^7 \pm 3.0 \times 10^7$	340	0.0[a]
Sodium dehydroacetate (0.1%)	$2.4 \times 10^7 \pm 2.9 \times 10^7$	370	48.4[b]
Sodium benzoate (0.1%)	$1.1 \times 10^7 \pm 1.5 \times 10^7$	170	78.4[c]
Sorbic acid (0.2%)	$0.9 \times 10^5 \pm 1.6 \times 10^5$	2	99.7[d]
Potassium sorbate (0.26%)	$2.8 \times 10^6 \pm 5.0 \times 10^6$	80	93.4[c,d]

[1]The data are the mean of two replicates with five observations per replicate.

[2]Range = (maximum-minimum) number of bacteria per observation area.

[3]Values followed by the same letter are not significantly different ($p < 0.05$).

III. EFFECT OF ANTIMICROBIALS ON GROWTH OF *CLOSTRIDIUM BOTULINUM* ATCC 7949

The effect of selected chemicals on growth of *C. botulinum* ATCC 7949 (Type B) in thioglycollate medium is shown in Table 4. Methyl, ethyl, propyl and butyl parabens and sorbic acid again inhibited the growth of *C. botulinum* ATCC 7949 by greater than 99%. Potassium sorbate and sodium benzoate inhibited growth of *C. botulinum* ATCC 7949 by 68.6% and 41.7%, respectively. Sodium dehydroacetate inhibited the growth of *C. botulinum* ATCC 7949 by only 3.3%. Statistical analysis reported that methyl, ethyl, propyl and butyl parabens and sorbic acid were significantly different from the control and the other antimicrobials, and they were not significantly different from each other ($p < 0.05$). Methyl, ethyl, propyl and butyl parabens and sorbic acid were very effective inhibitors of growth of *C. botulinum* ATCC 7949 in microbiological medium.

The range of counts in thioglycollate medium containing methyl, ethyl, propyl and butyl parabens and sorbic acid per observation area was small. The range of methyl, ethyl, propyl and butyl parabens and sorbic acid are 1, 3, 3, 1 and 2, respectively (Tables A-9, A-10, A-11 and A-12, Appendix).

The results obtained from Tables 2, 3 and 4 indicated that methyl, ethyl, propyl and butyl parabens and sorbic acid were very effective inhibitors of growth of *C. sporogenes*, *C. botulinum* 12885A and ATCC 7949 in fluid thioglycollate medium.

Why are methyl, ethyl, propyl and butyl parabens and sorbic acid much more effective inhibitors than sodium dehydroacetate, sodium

Table 4. Effect of Selected Chemicals on Growth of Clostridium botulinum ATCC 7949 (Type B) in the Test Tubes Containing Thioglycollate Broth

Chemical	No. of Bacteria per ml [1] ± Standard Deviation	Range[2]	% Inhibition[1]
Control I	$3.1 \times 10^7 \pm 2.1 \times 10^7$	280	0.0[a3]
Methyl paraben (0.1%)	$0.5 \times 10^5 \pm 1.1 \times 10^5$	1	99.8[d]
Ethyl paraben (0.1%)	$0.7 \times 10^5 \pm 2.1 \times 10^5$	3	99.7[d]
Ethyl paraben (0.1%)	$0.9 \times 10^5 \pm 2.1 \times 10^5$	3	99.7[d]
Butyl paraben (0.1%)	$0.7 \times 10^5 \pm 2.1 \times 10^5$	1	99.7[d]
Control II	$6.4 \times 10^7 \pm 3.0 \times 10^7$	350	0.0[a]
Sodium dehydroacetate (0.1%)	$5.9 \times 10^7 \pm 4.5 \times 10^7$	630	3.3[a]
Sodium benzoate (0.1%)	$3.4 \times 10^7 \pm 1.8 \times 10^7$	230	41.7[b]
Sorbic acid (0.2%)	$1.0 \times 10^5 \pm 1.8 \times 10^5$	2	99.8[d]
Potassium sorbate (0.26%)	$2.0 \times 10^7 \pm 1.9 \times 10^7$	190	68.6[c]

[1]The data are the mean of two replicates with five observations per replicate.

[2]Range = (maximum-minimum) number of bacteria per observation area.

[3]Values followed by the same letter are not significantly different ($p < 0.05$).

benzoate and potassium sorbate? One reason that the parabens and sorbic acid are better inhibitors is because of the pH of the medium. The pH of fluid thioglycollate medium is 6.5 to 7.2. The optimum pH range for microbial inhibition of parabens is 3.0-9.0. The optimum pH range for microbial inhibition of sorbic acid is 3.0-6.5. The optimum pH range for microbial inhibition of acetic acid is 3.0-5.0. The optimum pH range for microbial inhibition of benzoic acid is 2.5-4.0, thus benzoates are well adapted to the preservation of foods which are acid, or readily acidified (Chichester and Tanner, 1972). Parabens are effective through a wider pH range than benzoic acid and acetic acid. Therefore, parabens and sorbic acid are better inhibitors than other antimicrobials in fluid thioglycollate medium.

The pH of the thioglycollate broth was not adjusted down to pH 4.0 to 5.0 because the chemicals were also to be tested in a meat system. Data in the 4.0 to 5.0 pH range would be interesting but could not be used in this study since the pH range of canned comminuted pork is 5.8-6.5 (Ivey and Robach, 1978). Therefore we thought that the pH of the thioglycollate medium should approximate that of the meat system in testing effectiveness of antimicrobials in inhibiting C. sporogenes and C. botulinum.

IV. EFFECT OF ANTIMICROBIALS ON TOXIN PRODUCTION BY CLOSTRIDIUM BOTULINUM

The results obtained for the effect of chemicals inhibiting toxin production by C. botulinum 12885A and ATCC 7949 are shown in Table 5. The death of the mice which were injected with supernatant and survival of the mice which were injected with boiled supernatant indicated that

Table 5. Effect of Antimicrobials on Toxin Production by *Clostridium botulinum*

Chemical	Strain[1]	Mice Injected Supernatant	Mice Injected Boiled Supernatant
Control	12885A	4/4[2]	0/4[3]
	7949[4]	4/4	0/4
Methyl paraben (0.1%)	12885A	4/4	0/4
	7949	4/4	0/4
Ethyl paraben (0.1%)	12885A	0/4	0/4
	7949	0/4	0/4
Propyl paraben (0.1%)	12885A	0/4	0/4
	7949	0/4	0/4
Butyl paraben (0.1%)	12885A	0/4	0/4
	7949	0/4	0/4
Sodium dehydroacetate (0.1%)	12885A	4/4	0/4
	7949	4/4	0/4
Sodium benzoate (0.1%)	12885A	4/4	0/4
	7949	4/4	0/4
Sorbic acid (0.2%)	12885A	0/4	0/4
	7949	0/4	0/4
Potassium sorbate (0.26%)	12885A	4/4	0/4
	7949	4/4	0/4

[1]Strain: *Clostridium botulinum* strain.

[2]4/4: Number of death mice/number of mice injected.

[3]0/4: Number of death mice/number of mice injected.

[4]7949: ATCC7949.

the death of the mice was due to the toxin, and not other components present in the supernatant fluid.

Ethyl, propyl and butyl parabens and sorbic acid completely inhibited toxin production by C. botulinum 12885A, ATCC 7949. The other antimicrobials did not completely inhibit toxin production by C. botulinum 12885A and ATCC 7949. If any toxin was produced, mice died. There was no variation.

Methyl paraben was an excellent antimicrobial for inhibiting growth of C. botulinum 12885A, ATCC 7949 (98% and 99% inhibition, respectively), but it did not effectively inhibit toxin produced by C. botulinum 12885A, ATCC 7949. Therefore, this indicates that an antimicrobial could be an excellent inhibitor of growth of C. botulinum, but might not be an effective inhibitor of toxin production by C. botulinum. It also showed that any chemical being considered as an inhibitor of toxin production by C. botulinum should inhibit growth of C. botulinum by at least 99% and preferably 100%.

As the chain length of the esterified acid is increased, so is the inhibitory capacity of the parabens (Aalto et al., 1953). This could explain why the longer chained ethyl, propyl and butyl esters were more effective than the methyl ester in inhibiting the toxin production by C. botulinum 12885A and ATCC 7949.

Because ethyl, propyl and butyl parabens and sorbic acid completely inhibited toxin production by C. botulinum 12885A and ATCC 7949, they were chosen as antimicrobial agents to be tested in canned comminuted cured meat with or without sodium nitrite (Table 1, p. 27).

V. EFFECT OF ANTIMICROBIALS ON CANNED, COMMINUTED CURED MEAT

Two cans of each treatment were randomly selected and tested for toxicity immediately after canning to assure that reaction between antimicrobial and spores was minimal. One mouse was injected for each can to test the toxicity of the meat and antimicrobial mixture. After 96 hours of observation, all mice survived. This demonstrated that spores in the cans did not immediately germinate to produce toxin after canning. It also indicated that no toxin was added to the can during inoculation. Heat shock at 80°C for 10 minutes assured the destruction of toxin and vegetative cells.

The rate and number of swollen and leaking cans in each test lot are depicted in Figures 1 to 5. Toxin assays of the first three swollen and leaking cans in each test confirmed the presence of botulinal toxin. Thus, it is reasonable to assume that all swollen cans were a result of botulinal growth.

Botulinal toxin was detected after 12 days at 27°C in the control comminuted meat and in the butyl paraben treatment. Toxicity and swollen cans were detected after 19 days at 27°C with samples treated with sorbic acid, butyl paraben + $NaNO_2$, propyl paraben and propyl paraben + $NaNO_2$ treatment. Botulinal toxin was not detected until 28 days in the comminuted meat treated with ethyl paraben, ethyl paraben + $NaNO_2$ and sorbic acid + $NaNO_2$ (Table A-13, Appendix). After the 3-month treatment period, all treatments had various numbers of swollen cans. The number of cans containing toxin of C. botulinum 12885A and ATCC 7949 was highest for the product with propyl paraben and lowest in the product containing sorbic acid + $NaNO_2$.

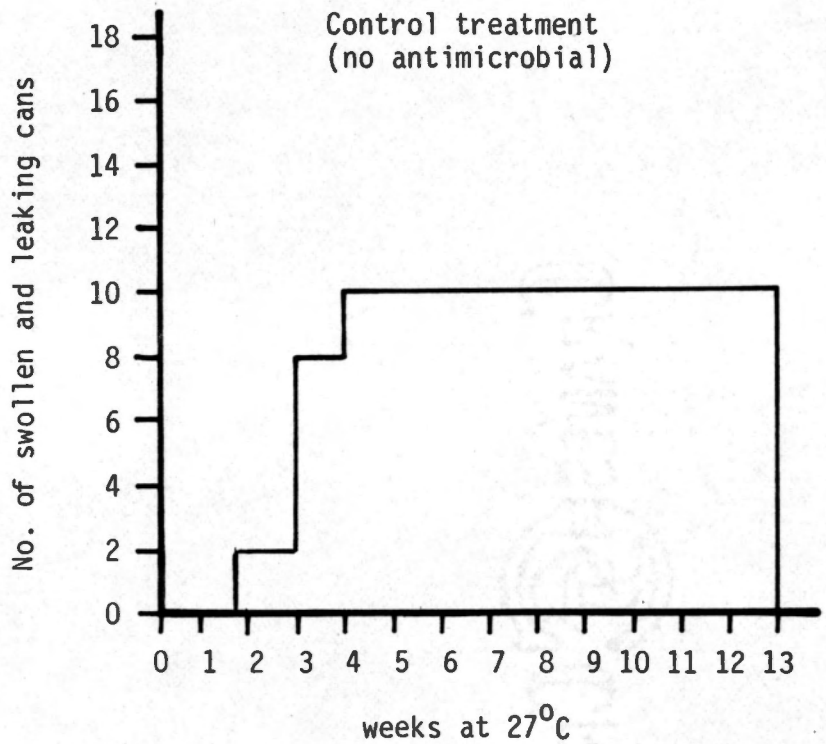


Figure 1. Number of cans in control treatment with time swelled during incubation of cured, comminuted pork inoculated with 100 spores/g of Clostridium botulinum 12885A and ATCC 7949(B) at 27°C. This figure shows the time at which each of 18 cans swelled.

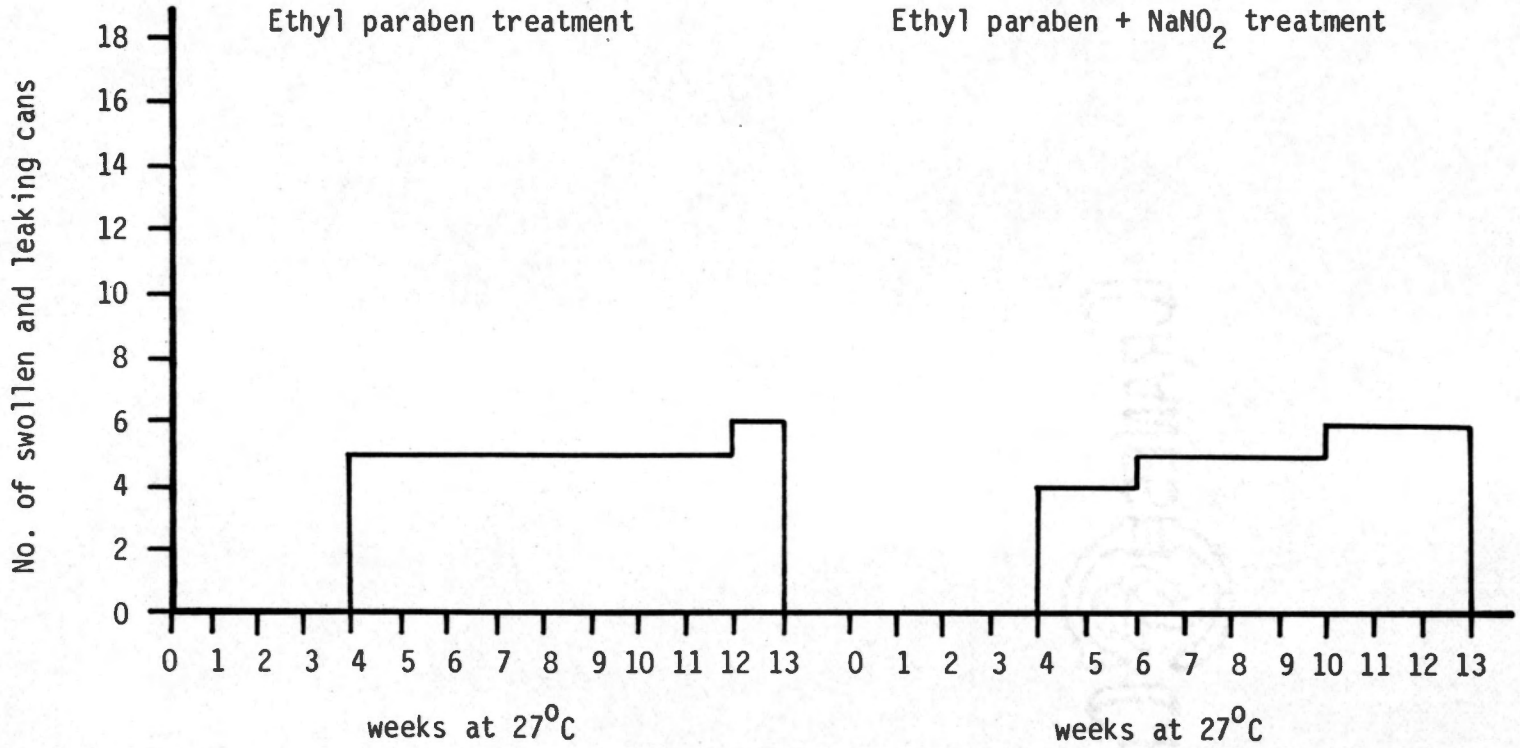


Figure 2. Number of cans in ethyl paraben and ethyl paraben + $NaNO_2$ treatment with time swelled during incubation of cured, comminuted pork inoculated with 100 spores/g of Clostridium botulinum 12885A and ATCC 7949(B) at 27°C. This figure shows the time at which each of 18 cans swelled.

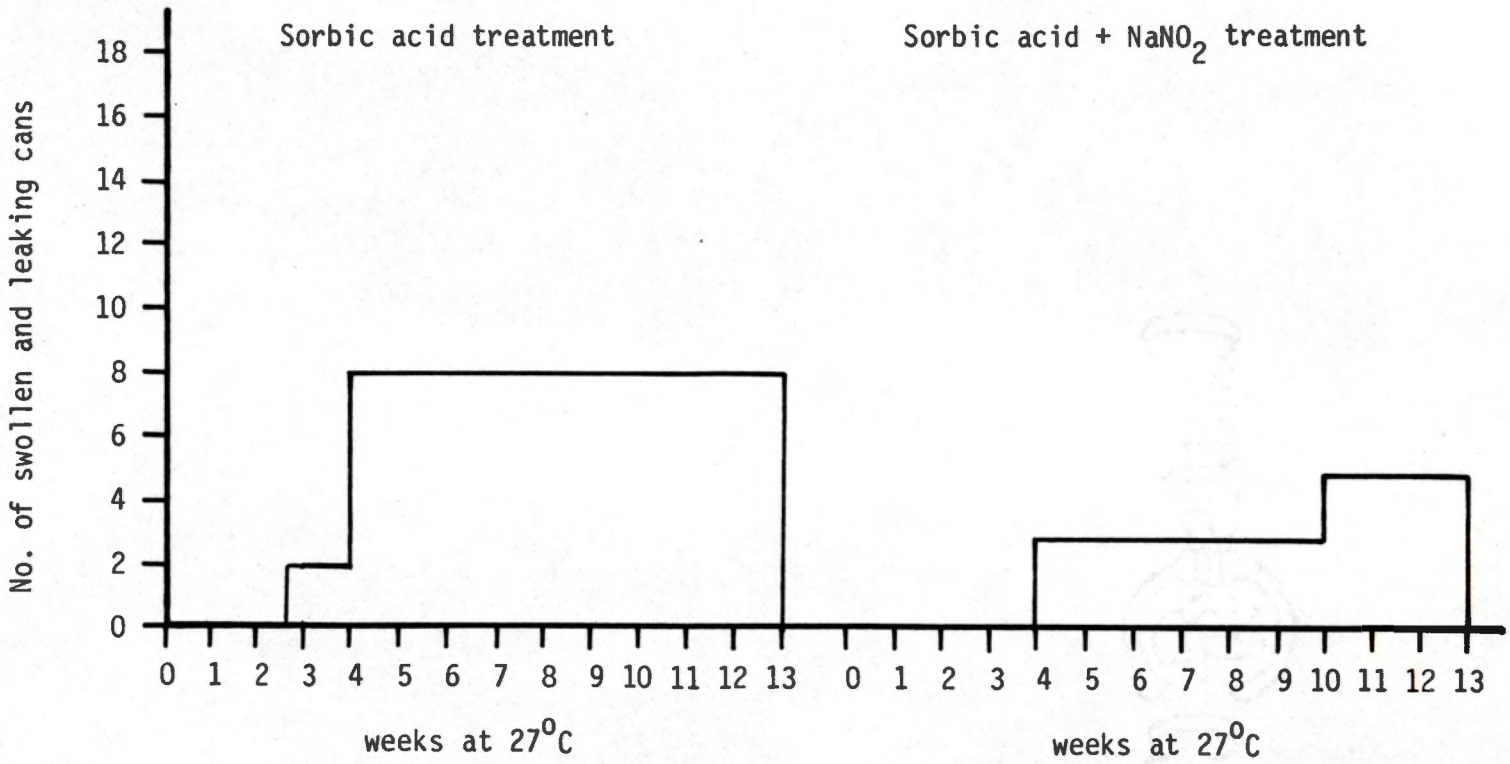


Figure 3. Number of cans in sorbic acid and sorbic acid + $NaNO_2$ treatment with time swelled during incubation of cured, comminuted pork inoculated with 100 spores/g of Clostridium botulinum 12885A and ATCC 7949(B) at 27°C. This figure shows the time at which each of 18 cans swelled.

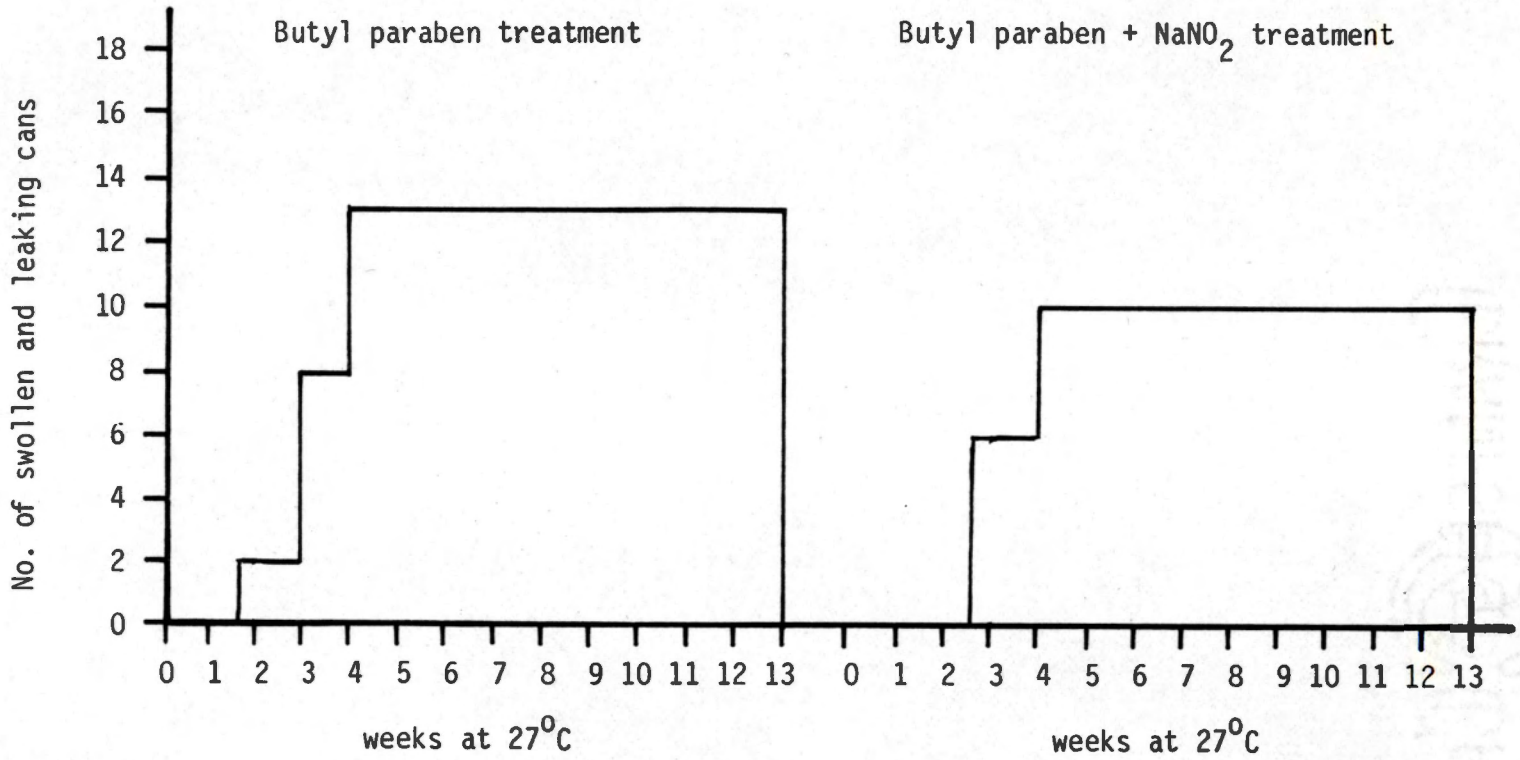


Figure 4. Number of cans in butyl paraben and butyl paraben + $NaNO_2$ treatment with time swelled during incubation of cured, comminuted pork inoculated with 100 spores/g of Clostridium botulinum 12885A and ATCC 7949(B) at 27°C. This figure shows the time at which each of 18 cans swelled.

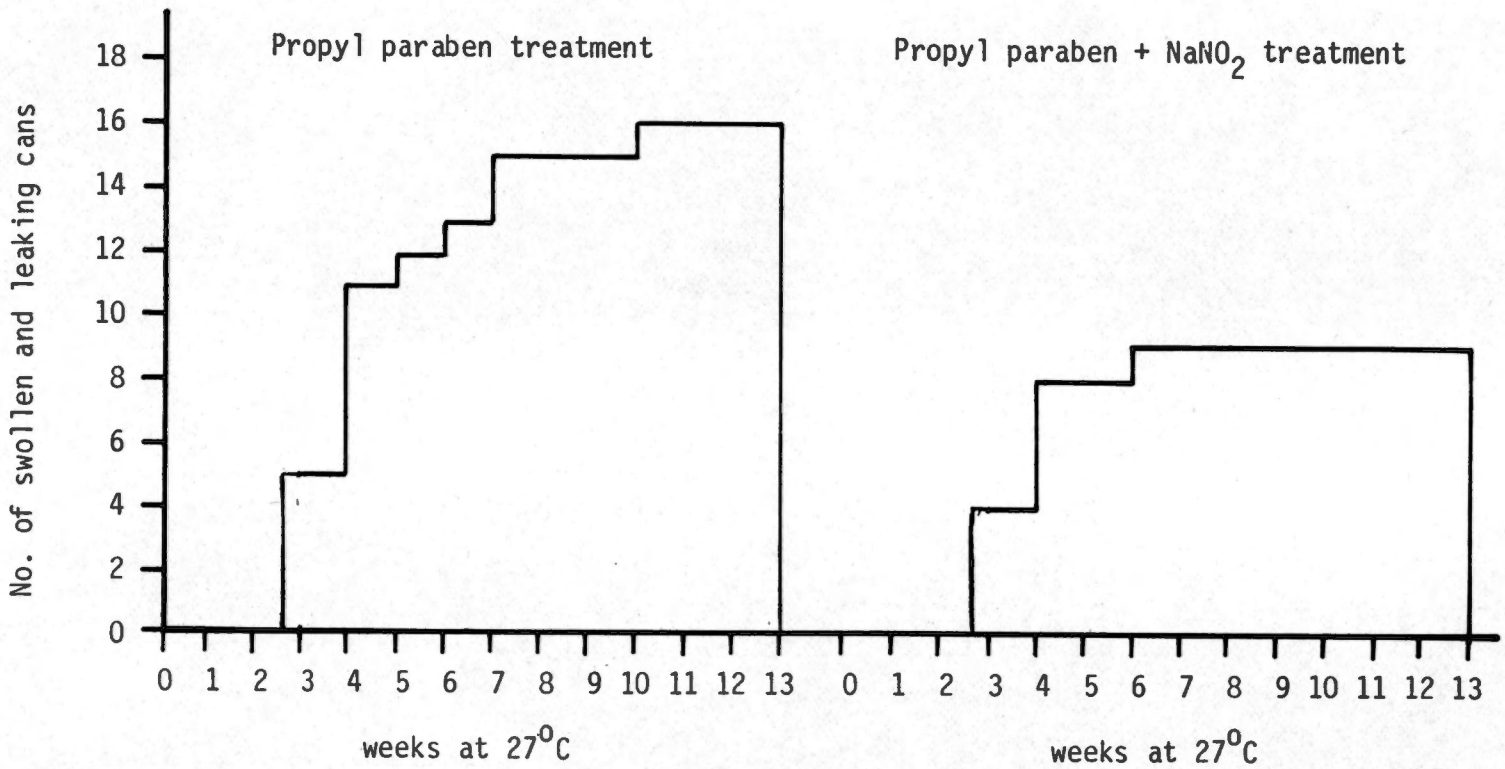


Figure 5. Number of cans in propyl paraben and propyl paraben + $NaNO_2$ treatment with time swelled during incubation of cured, comminuted pork inoculated with 100 spores/g of Clostridium botulinum 12885A and ATCC 7949(B) at 27°C. This figure shows the time at which each of 18 cans swelled.

When sorbic acid, butyl paraben and propyl paraben treatments were compared to the same treatment containing $NaNO_2$, the number of cans swelling decreased as the level of sodium nitrite increased from 0 to 40 ppm. The rate of swelling of cans in the six treatments are presented in Figures 3, 4 and 5. Sorbic acid, sorbic acid + $NaNO_2$, butyl paraben and butyl paraben + $NaNO_2$ treatment inhibited not only the total number of swollen cans but also delayed the first swell (Figures 3 and 4). In propyl paraben and propyl paraben + $NaNO_2$ the rates of swelling and total number of swollen cans were different, although the days to first swell and leakage were the same (Figure 5). In ethyl paraben and ethyl paraben + $NaNO_2$, the days to first swell and total number of swollen cans were the same, but the rate of swollen cans were different. These experiments agree with Christiansen et al. (1973) who reported that the rate of toxin production and number of toxic cans were dependent on the level of nitrite added to the meat. Increasing the level of added nitrite reduced the number of cans in which toxin was produced and increased the length of time for toxin development. Low nitrite concentrations (less than 50 μg/g) have also been reported to be ineffective against C. botulinum growth and toxin inhibition in various types of cured meat products or meat systems (Christiansen et al., 1973; Rhodes and Jarvis, 1976; Roberts et al., 1976; Ivey and Robach, 1978).

Our data support these reports, since treatments containing 40 ppm sodium nitrite could delay the toxin production by C. botulinum 12885A and ATCC 7949, but could not completely inhibit toxin production. The reason for using only 40 ppm sodium nitrite in this experiment was not only antibotulinal, but also for color and flavor. Generally, nitrite-sorbic acid, nitrite-butyl and nitrite-propyl parabens

combinations have been found more effective in retarding C. botulinum growth and toxin production than sorbic acid, butyl and propyl parabens used singly. These data are summarized in Table A-13, Appendix).

From each treatment two cans which were not swollen or leaking after 90 days were tested for toxicity in mice. Three mice were injected per can and all survived. Therefore, cans not leaking were presumed free of toxin.

The results obtained for the effects of ethyl, butyl and propyl parabens and sorbic acid are depicted in Figures 2, 3, 4 and 5. The products with 0.1% ethyl paraben produced a marked inhibition of botulinal toxin. No toxin was detected through 0 to 28 days of incubation (Figure 2). Toxic samples were found after 28 days at 27°C. Compared to other treatments, the ethyl paraben products extended the time of incubation necessary for toxin formation. Ethyl paraben had the best inhibitory effect on outgrowth and toxin production by C. botulinum in these 4 treatments, followed by sorbic acid and butyl paraben; propyl paraben had the least effect in all treatments.

One possible reason that the antimicrobial effect of sorbic acid was low is its poor solubility in water. In our meat slurry system, the quantity of water was one-half of the total meat system. The water content in the slurry system is higher than would be observed in normal commercial cooked cured meats (Rhodes and Jarvis, 1976). Sorbic acid is only slightly soluble in water. Although the longer chained esterified acids (parabens) had more inhibitory effect than shorter chained esterified acid, the solubility of the paraben in water decreases with increasing chain length. So, the reason for the parabens not completely

inhibiting outgrowth and toxin production by C. botulinum 12885A and ATCC 7949 was probably their poor solubility in water. Ethyl paraben was more effective than butyl or propyl paraben in inhibition outgrowth and toxin formation. Therefore, a combination of two esters (ethyl and butyl) is suggested to take advantage of the higher water solubility of the ethyl paraben and the greater antimicrobial activity of the butyl ester in a 2:1 or 3:1 ethyl to butyl ratio.

CHAPTER V

CONCLUSIONS

Methyl, ethyl, propyl and butyl parabens and sorbic acid were very effective for inhibiting growth of C. sporogenes and C. botulinum 12885A and ATCC 7949 in microbiological medium. Ethyl, propyl and butyl parabens and sorbic acid were also very effective against toxin production by C. botulinum 12885A and ATCC 7949. However, in canned, comminuted cured pork, ethyl, propyl and butyl parabens and sorbic acid inhibited toxin production by C. botulinum 12885A and ATCC 7949 at 27°C for a maximum of 28 days. Thus, they delayed outgrowth of C. botulinum but did not inhibit it completely.

These experiments have provided an initial evaluation of the effectiveness of methyl, ethyl, propyl and butyl parabens, sorbic acid, potassium sorbate, sodium dehydroacetate and sodium benzoate in inhibiting growth and toxin production by C. botulinum 12885A and ATCC 7949 in a meat slurry. Expanded studies using additional strains of C. botulinum and utilizing other food systems are necessary to fully evaluate the potential of the selected chemicals as preservative agents against C. botulinum in our food supply.

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APPENDIX

Table A-1. Parabens Inhibiting Growth of Clostridium sporogenes in the Test Tubes Containing Thioglycollate Broth (Replication 1)

Chemical	No. Tube	No. of Bacteria per Area	Mean	No. of Bacteria per ml	% Inhibition
Control (no chemical)	1	460, 140, 170, 1200, 200	402.00	1.21×10^8	0.0000
	2	540, 380, 390, 380 330			
	3	180, 250, 210, 280, 220			
	4	400, 330, 430, 350, 400			
	5	490, 560, 570, 620, 570			
Methyl paraben (0.1%)	1	20, 9, 17, 11, 29	12.12	0.36×10^7	96.9850
	2	12, 6, 6, 1, 3			
	3	22 22, 26, 28, 26			
	4	8, 7, 12, 8, 8			
	5	6, 5, 4, 3, 4			
Ethyl paraben (0.1%)	1	0, 0, 0, 0, 0	0.04	1.20×10^4	99.9900
	2	0, 0, 0, 0, 0			
	3	0, 0, 0, 0, 0			
	4	0, 0, 1, 0, 0			
	5	0, 0, 0, 0, 0			
Propyl paraben (0.1%)	1	1, 0, 0, 0, 0	0.24	0.72×10^5	99.9403
	2	1, 1, 1, 1, 0			
	3	0, 0, 1, 0, 0			
	4	0, 0, 0, 0, 0			
	5	0, 0, 0, 0, 0			
Butyl paraben (0.1%)	1	1, 0, 0, 0, 0	0.12	0.36×10^5	99.9701
	2	0, 0, 0, 0, 0			
	3	0, 0, 0, 0, 1			
	4	0, 0, 0, 0, 0			
	5	0, 1, 0, 0, 0			

Table A-2. Parabens Inhibiting Growth of Clostridium sporogenes in the Test Tubes Containing Thioglycollate Broth (Replication 2)

Chemical	No. Tube	No. of Bacteria per Area	Mean	No. of Bacteria per ml	% Inhibition
Control (no chemical)	1	450, 350, 290, 290, 350	281.20	0.84×10^8	0.0000
	2	270, 240, 280, 270, 220			
	3	190, 290, 210, 130, 130			
	4	180, 300, 300, 250, 340			
	5	340, 330, 380, 400, 250			
Methyl paraben (0.1%)	1	0, 0, 0, 1, 0	3.92	1.18×10^6	98.6060
	2	2, 1, 1, 4, 0			
	3	19, 19, 13, 18, 20			
	4	0, 3, 0, 0, 0			
	5	4, 0, 0, 2, 1			
Ethyl paraben (0.1%)	1	0, 0, 0, 0, 0	0.20	0.60×10^5	99.9289
	2	0, 0, 1, 0, 0			
	3	0, 0, 0, 0, 0			
	4	1, 1, 1, 0, 1			
	5	0, 0, 0, 0, 0			
Propyl paraben (0.1%)	1	0, 0, 0, 1, 0	0.36	1.08×10^5	99.8720
	2	0, 0, 0, 1, 2			
	3	0, 0, 3, 0, 1			
	4	0, 0, 0, 0, 0			
	5	0, 0, 0, 0, 1			
Butyl paraben (0.1%)	1	0, 0, 0, 0, 0	1.72	5.16×10^5	99.3883
	2	0, 0, 0, 0, 0			
	3	0, 0, 2, 0, 0			
	4	0, 0, 0, 0, 1			
	5	6, 2, 10, 14, 8			

Table A-3. Chemicals Inhibiting Growth of Clostridium sporogenes in the Test Tubes Containing Thioglycollate Broth (Replication 1)

Chemical	No. Tube	No. of Bacteria per Area	Mean	No. of Bacteria per ml	% Inhibition
Control (no chemical)	1	690, 940, 960, 980,1170	1174.40	3.52×10^8	0.0000
	2	1160,1370,1320,1330,1240			
	3	590, 670, 590, 790, 720			
	4	1240,1310,1200,1090,1110			
	5	1030,1250,1140,1470,1720			
Sodium dehydro-acetate (0.1%)	1	210, 260, 180, 140, 80	390.80	1.17×10^8	66.7234
	2	500, 570, 740, 840, 750			
	3	210, 340, 390, 460, 480			
	4	400, 360, 360, 350, 400			
	5	240, 430, 420, 320, 340			
Sodium benzoate (0.1%)	1	730, 800, 950,1110,1340	1029.60	3.09×10^8	12.3297
	2	1270,1930,1840,1800,1700			
	3	820, 910,1370,1280,1140			
	4	410, 500, 380, 520, 490			
	5	670, 840, 920, 970,1070			
Sorbic acid (0.2%)	1	0, 0, 0, 0, 0	0.40	1.20×10^5	99.9659
	2	0, 1, 1, 1, 1			
	3	3, 1, 0, 0, 0			
	4	0, 0, 0, 0, 0			
	5	2, 0, 0, 0, 0			
Potassium sorbate (0.26%)	1	190, 450, 420, 340, 670	359.20	1.08×10^8	60.4142
	2	330, 180, 240, 310, 230			
	3	360, 370, 360, 430, 350			
	4	480, 570, 660, 690, 350			
	5	230, 180, 200, 200, 190			

Table A-4. Chemicals Inhibiting Growth of *Clostridium sporogenes* in the Test Tubes Containing Thioglycollate Broth (Replication 2)

Chemical	No. Tube	No. of Bacteria per Area	Mean	No. of Bacteria per ml	% Inhibition
Control (no chemical)	1	790, 930,1000,1040,1150	758.80	2.28×10^8	0.0000
	2	620, 900, 800,1020, 980			
	3	630, 600, 660, 570, 580			
	4	740,1190, 650, 830, 830			
	5	560, 370, 450, 570, 510			
Sodium dehydro-acetate (0.1%)	1	600, 650, 680, 640, 650	566.80	1.70×10^8	25.3031
	2	630, 840, 660, 840, 940			
	3	240, 260, 300, 200, 200			
	4	420, 430, 540, 520, 440			
	5	740, 580, 640, 820, 710			
Sodium benzoate (0.1%)	1	120, 120, 190, 80, 90	486.40	1.46×10^8	35.8988
	2	630, 770, 990,1150, 930			
	3	190, 130, 230, 120, 160			
	4	160, 160, 210, 140, 270			
	5	810, 840,1120,1190,1360			
Sorbic acid (0.2%)	1	0, 0, 0, 0, 0	0.08	2.40×10^4	99.9895
	2	1, 1, 0, 0, 0			
	3	0, 0, 0, 0, 0			
	4	0, 0, 0, 0, 0			
	5	0, 0, 0, 0, 0			
Potassium sorbate (0.26%)	1	210, 180, 310, 270, 350	285.60	0.86×10^8	62.3616
	2	280, 400, 300, 400, 440			
	3	300, 350, 240, 440, 530			
	4	270, 390, 390, 460, 400			
	5	40, 50, 30, 50, 60			

Table A-5. Parabens Inhibiting Growth of Clostridium botulinum 12885A (Type A) in the Test Tubes Containing Thioglycollate Broth (Replication 1)

Chemical	No. Tube	No. of Bacteria per Area	Mean	No. of Bacteria per ml	% Inhibition
Control (no chemical)	1	110, 150, 160	120.70	3.62×10^7	0.0000
	2	170, 150, 250			
	3	140, 80, 80			
	4	200, 100, 110			
	5	30, 50, 30			
Methyl paraben (0.1%)	1	0, 0, 0	1.93	5.79×10^5	98.4010
	2	0, 0, 0			
	3	8, 3, 8			
	4	1, 1, 2			
	5	0, 2, 4			
Ethyl paraben (0.1%)	1	2, 0, 2	0.80	2.40×10^5	99.3372
	2	2, 0, 0			
	3	0, 0, 0			
	4	0, 0, 6			
	5	0, 0, 0			
Propyl paraben (0.1%)	1	2, 0, 0	0.33	0.99×10^5	99.7266
	2	1, 1, 0			
	3	0, 0, 0			
	4	0, 0, 0			
	5	0, 0, 1			
Butyl paraben (0.1%)	1	0, 0, 0	0.40	1.20×10^5	99.6686
	2	3, 1, 0			
	3	1, 0, 1			
	4	0, 0, 0			
	5	0, 0, 0			

Table A-6. Parabens Inhibiting Growth of Clostridium botulinum 12385A (Type A) in the Test Tubes Containing Thioglycollate Broth (Replication 2)

Chemical	No. Tube	No. of Bacteria per Area	Mean	No. of Bacteria per ml	% Inhibition
Control (no chemical)	1	250, 300, 240	170.00	5.1×10^7	0.0000
	2	260, 210, 120			
	3	220, 160, 240			
	4	80, 30, 100			
	5	80, 130, 130			
Methyl paraben (0.1%)	1	2, 0, 2	1.13	3.39×10^5	99.3353
	2	0, 0, 0			
	3	3, 1, 1			
	4	3, 2, 3			
	5	0, 0, 0			
Ethyl paraben (0.1%)	1	0, 0, 1	0.46	1.38×10^5	99.7294
	2	1, 0, 0			
	3	0, 0, 1			
	4	0, 1, 0			
	5	1, 1, 1			
Propyl paraben (0.1%)	1	0, 1, 0	0.47	1.41×10^5	99.7235
	2	0, 0, 0			
	3	0, 0, 0			
	4	0, 0, 0			
	5	3, 2, 1			
Butyl paraben (0.1%)	1	0, 0, 2	0.40	1.20×10^5	99.7647
	2	0, 0, 1			
	3	0, 0, 1			
	4	0, 0, 0			
	5	0, 0, 2			

Table A-7. Chemicals Inhibiting Growth of Clostridium botulinum 12885A (Type A) in the Test Tubes Containing Thioglycollate Broth (Replication 1)

Chemical	No. Tube	No. of Bacteria per area	Mean	No. of Bacteria per ml	% Inhibition
Control (no chemical)	1	140, 140, 230	177.40	5.32×10^7	0.0000
	2	120, 350, 180			
	3	100, 110, 110			
	4	60, 80, 90			
	5	310, 340, 300			
Sodium dehydro-acetate (0.1%)	1	30, 10, 20	138.00	4.14×10^7	22.2097
	2	20, 80, 50			
	3	140, 100, 150			
	4	250, 160, 230			
	5	200, 260, 370			
Sodium benzoate (0.1%)	1	10, 10, 10	64.00	1.92×10^7	63.9233
	2	30, 0, 0			
	3	70, 90, 60			
	4	80, 60, 170			
	5	80, 160, 130			
Sorbic acid (0.2%)	1	1, 2, 1	0.40	1.20×10^5	99.7745
	2	0, 0, 0			
	3	0, 0, 0			
	4	1, 0, 1			
	5	0, 0, 0			
Potassium sorbate (0.26%)	1	0, 10, 0	13.30	3.99×10^6	92.5028
	2	0, 0, 10			
	3	10, 10, 10			
	4	40, 80, 30			
	5	0, 0, 0			

Table A-8. Chemicals Inhibiting Growth of Clostridium botulinum 12885A (Type A) in the Test Tubes Containing Thioglycollate Broth (Replication 2)

Chemical	No. Tube	No. of Bacteria per Area	Mean	No. of Bacteria per ml	% Inhibition
Control (no chemical)	1	160, 250, 160	94.70	2.84×10^7	0.0000
	2	160, 210, 180			
	3	40, 20, 10			
	4	60, 70, 40			
	5	20, 30, 10			
Sodium dehydroacetate (0.1%)	1	10, 20, 0	24.00	7.20×10^6	74.6568
	2	30, 20, 0			
	3	60, 30, 10			
	4	50, 20, 10			
	5	50, 30, 20			
Sodium benzoate (0.1%)	1	0, 0, 0	6.70	2.01×10^6	92.9250
	2	0, 0, 0			
	3	10, 10, 10			
	4	10, 10, 20			
	5	10, 10, 10			
Sorbic acid (0.2%)	1	1, 0, 0	0.20	0.60×10^5	99.7888
	2	0, 0, 0			
	3	0, 0, 0			
	4	0, 0, 0			
	5	1, 1, 0			
Potassium sorbate (0.26%)	1	0, 0, 0	5.30	1.59×10^6	94.4034
	2	10, 10, 30			
	3	0, 0, 10			
	4	0, 0, 0			
	5	0, 10, 10			

Table A-9. Parabens Inhibiting Growth of Clostridium botulinum ATCC 7949 (Type B) in the Test Tubes Containing Thioglycollate Broth (Replication 1)

Chemical	No. Tube	No. of Bacteria per Area	Mean	No. of Bacteria per ml	% Inhibition
Control (no chemical)	1	30, 20, 10	120.70	3.62×10^7	0.0000
	2	90, 70, 160			
	3	220, 290, 260			
	4	100, 80, 140			
	5	100, 80, 160			
Methyl paraben (0.1%)	1	0, 0, 0	0.33	0.99×10^5	99.7266
	2	0, 0, 0			
	3	0, 0, 0			
	4	1, 1, 0			
	5	1, 1, 1			
Ethyl paraben (0.1%)	1	1, 0, 0	0.40	1.20×10^5	99.6686
	2	0, 1, 0			
	3	0, 0, 1			
	4	0, 0, 3			
	5	0, 0, 0			
Propyl paraben (0.1%)	1	0, 0, 0	0.47	1.41×10^5	99.6106
	2	0, 0, 1			
	3	0, 1, 0			
	4	0, 0, 0			
	5	3, 2, 0			
Butyl paraben (0.1%)	1	1, 0, 0	0.20	0.60×10^5	99.8343
	2	0, 0, 0			
	3	0, 0, 0			
	4	1, 0, 0			
	5	0, 1, 0			

Table 10. Parabens Inhibiting Growth of Clostridium botulinum ATCC 7949 (Type B) in the Test Tubes Containing Thioglycollate Broth (Replication 2)

Chemical	No. Tube	No. of Bacteria per Area	Mean	No. of Bacteria per ml	% Inhibition
Control (no chemical)	1	80, 170, 140	85.30	2.56×10^7	0.0000
	2	40, 50, 30			
	3	160, 150, 90			
	4	60, 60, 70			
	5	50, 70, 60			
Methyl paraben (0.1%)	1	0, 0, 0	0.00	0.00	100.0000
	2	0, 0, 0			
	3	0, 0, 0			
	4	0, 0, 0			
	5	0, 0, 0			
Ethyl paraben (0.1%)	1	0, 0, 0	0.07	2.01×10^4	99.9215
	2	0, 0, 0			
	3	0, 0, 0			
	4	0, 0, 0			
	5	0, 0, 1			
Propyl paraben (0.1%)	1	0, 0, 1	0.13	3.90×10^4	99.8476
	2	0, 0, 0			
	3	0, 0, 0			
	4	0, 0, 0			
	5	1, 0, 0			
Butyl paraben (0.1%)	1	0, 0, 0	0.13	3.90×10^4	99.8476
	2	0, 0, 0			
	3	1, 0, 0			
	4	0, 0, 0			
	5	1, 0, 0			

Table A-11. Chemicals Inhibiting Growth of Clostridium botulinum ATCC 7949 (Type B) in the Test Tubes Containing Thioglycollate Broth (Replication 1)

Chemical	No. Tube	No. of Bacteria per Area	Mean	No. of Bacteria per ml	% Inhibition
Control (no chemical)	1	110, 40, 50	251.30	7.54×10^7	0.0000
	2	290, 390, 220			
	3	330, 310, 300			
	4	190, 240, 240			
	5	340, 390, 330			
Sodium dehydroacetate (0.1%)	1	170, 160, 120	188.00	5.64×10^7	25.1890
	2	210, 220, 270			
	3	260, 250, 140			
	4	150, 130, 80			
	5	220, 210, 230			
Sodium benzoate (0.1%)	1	10, 10, 10	79.30	2.38×10^7	68.4441
	2	70, 50, 20			
	3	180, 100, 120			
	4	100, 130, 160			
	5	50, 90, 90			
Sorbic acid (0.2%)	1	1, 2, 0	0.47	1.41×10^5	99.3130
	2	1, 0, 0			
	3	0, 1, 1			
	4	0, 0, 0			
	5	0, 1, 0			
Potassium sorbate (0.26%)	1	140, 100, 180	69.30	2.08×10^7	72.4234
	2	30, 0, 10			
	3	10, 10, 10			
	4	130, 140, 150			
	5	40, 50, 40			

Table A-12. Chemicals Inhibiting Growth of Clostridium botulinum ATCC 7949 (Type B) in the Test Tubes Containing Thioglycollate Broth (Replication 2)

Chemical	No. Tube	No. of Bacteria per Area	Mean	No. of Bacteria per ml	% Inhibition
Control (no chemical)	1	210, 120, 100	173.30	5.20×10^7	0.0000
	2	170, 140, 120			
	3	110, 140, 180			
	4	210, 300, 130			
	5	160, 330, 180			
Sodium dehydroacetate (0.1%)	1	170, 120, 110	205.30	6.16×10^7	-18.4650
	2	550, 640, 560			
	3	150, 230, 240			
	4	80, 70, 100			
	5	10, 40, 10			
Sodium benzoate (0.1%)	1	160, 200, 160	147.10	4.41×10^7	15.1183
	2	110, 120, 120			
	3	100, 140, 120			
	4	170, 170, 70			
	5	150, 240, 180			
Sorbic acid (0.2%)	1	0, 0, 0	0.20	0.60×10^5	99.3846
	2	2, 0, 1			
	3	0, 0, 0			
	4	0, 0, 0			
	5	0, 0, 0			
Potassium sorbate (0.26%)	1	0, 30, 30	60.70	1.82×10^7	64.9740
	2	80, 80, 170			
	3	60, 20, 30			
	4	190, 110, 60			
	5	20, 20, 10			

Table A-13. Outgrowth and Toxin Production of Clostridium botulinum in Cured, Comminuted Pork with Chemical Inhibitors with or without Sodium Nitrite

Chemical	No. Swells and Leakage/ Total Cans	Days to First Swell and Leakage	No. Cans on First Swell
Control (no chemical)	10/18	12	2
Ethyl paraben	6/18	28	5
Ethyl paraben & $NaNO_2$	6/18	28	3
Sorbic acid	8/18	19	2
Sorbic acid & $NaNO_2$	5/18	28	3
Butyl paraben	13/18	12	2
Butyl paraben & $NaNO_2$	10/18	19	6
Propyl paraben	16/18	19	5
Propyl paraben & $NaNO_2$	9/18	19	4

VITA

Shi-Chien Catherine Sung was born on August 24, 1954, in Taiwan, Republic of China. She is the youngest of four children of Mr. and Mrs. Yane-Keng Sung. She graduated from Provincial Kaohsiung Girls' High School, Kaohsiung, Taiwan, in 1972. In the fall of 1973 she enrolled at National Chung-Hsing University, where she graduated with a Bachelor of Science degree in Animal Husbandry in June 1977.

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