Determination of the Thermal Death Time of Clostridium Botulinum Type E in Crawfish (Procambarus Clarkii) Tailmeat.

Carmen Alicia oquendo De pantoja
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DETERMINATION OF THE THERMAL DEATH TIME OF CLOSTRIDIUM BOTULINUM TYPE E IN CRAWFISH (PROCAMBARUS CLARKII) TAILMEAT

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DETERMINATION OF THE THERMAL DEATH TIME
OF CLOSTRIDIUM BOTULINUM TYPE E
IN CRAWFISH (PROCAMBARUS CLARKII) TAILMEAT

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in
The Department of Food Science

by
Carmen Alicia Oquendo de Pantoja
B.S. University of Puerto Rico, 1979
M.S. Louisiana State University, 1983

August 1986
DEDICATION

This work is dedicated to my husband, Alberto, for his love, support, and understanding during all our time together, and to my parents, Carmen and Manuel, for their faith and encouragement.
ACKNOWLEDGEMENTS

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The help and moral support of many friends, without whom this struggle would have been impossible, is also

Finally, the author wishes to thank the guidance offered to her by Professors M. James and A. El Koury, University of Puerto Rico; and Dr. R.J. Siebeling, for providing the motivation and challenges that so much helped her in the pursuit of this degree.
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ABSTRACT

The thermal death time of *Clostridium botulinum* type E spores in crawfish tailmeat was determined at temperatures ranging from 80 to 95°C. Crawfish paste containing $10^6$ spores per gram was dispensed into 16 x 125 mm test tubes, which were subsequently placed in water baths preset at the test temperatures. Sample tubes were removed from the baths at predetermined time intervals and incubated anaerobically for up to two months. The D value for the spores in crawfish tailmeat was determined from the corrected time of exposure to the heat and survival data using three methods of calculation. D values were plotted versus temperature and a thermal death time curve was constructed. The values were compared and ranged from 4.9 to 6.9 min at 80°C, from 6.7 to 8.8 at 85°C, from 2.5 to 3.1 at 90°C, and 8.7 to 17.1 at 95°C. The z-values ranged from 8 - 15°C depending on the method of calculation.
INTRODUCTION

The crawfish industry in Louisiana produces 99 percent of all the crawfish consumed in the United States. While most of the product is consumed locally, there has been increased interest in expanding the market for the crustaceans, for both economical and cultural reasons. Fresh crawfish tailmeat is available to the consumer from December to June, and demand for the product during off-season months is usually met by the frozen product. Problems inherent to frozen products include keeping the product frozen during transport and storage, as well as quality losses due to flavor and textural changes.

Recently, Louisiana seafood producers have expressed an interest in extending the availability of crawfish tails by means of pasteurization, a process which has been successfully applied to crabmeat by the same producers. Pasteurization of seafood usually entails heating the food which is packed in hermetically sealed containers at relatively mild temperatures (below the boiling point of water). After processing, the product is refrigerated during storage, thus increasing the shelf life.

Whenever a new thermal process is considered, it is of utmost importance to assess its safety, particularly in regards to the survival of Clostridium botulinum. Because
crawfish are found in freshwater environments, the final product might be contaminated with *C. botulinum* type E spores. Because these spores are capable of growing and producing toxin at refrigeration temperatures, it is extremely important that none survive the heating process.

In order to determine an adequate heat process for the crawfish tailmeat, the thermal resistance of *C. botulinum* type E spores in the product must be determined. The research presented was undertaken in an attempt to obtain such information.
LITERATURE REVIEW

Historical Aspects

*Clostridium botulinum* is the etiological agent of botulism, a neuroparalytic disease which affects man and animals. As defined by Dolman (1964), the disease is relatively rare and always tragic. Three distinct clinical forms of botulism are known presently: foodborne, wound, and infant botulism. Foodborne botulism occurs as a result of the ingestion of foods containing preformed botulism toxin. Wound and infant botulism result from the *in vivo* production of toxin by the organism in the injured tissue or the intestines of the individual, respectively (CDC, 1979; Eklund, 1982).

History of the disease. Botulism has been known for centuries as "sausage poisoning," which in 1822 was made a reportable disease in Germany. In 1870 Muller coined the term "botulism" from *botulus*, Latin for sausage, to describe the neuroparalytic syndrome associated with the disease. In 1896, Professor E. van Ermengen of the University of Ghent, isolated an anaerobic, spore-forming bacillus from ham samples which had been incriminated in a "sausage poisoning-like" outbreak. Injection of culture filtrates into laboratory animals produced characteristic paralysis and death resulted. As a result of his experiments, Professor van Ermengen proposed the name *Bacillus botulinus* for the organism.
As the scientific community became aware of the prevalence of botulism, reports of outbreaks became more common. An outbreak in California prompted an investigation of canned fruits and vegetables, for up to that time these food items were not regarded as vehicles of the disease. A Botulism Commission was established by the United States Public Health Service to study the causes and modes by which to prevent botulism (Dolman, 1964). The Commission stressed the importance of heat treatment in processing canned foods, and examined the geographical distribution of *C. botulinum* spores (Meyer and Dubovsky, 1922). These surveys suggested that the natural habitat for spores was the soil, while more recent studies have reported the isolation of this organism from freshwater and marine sediments (Eklund and Poysky, 1965; Ward et al., 1967a,b) as well as the intestinal tract of man and animals (Smith and Hobbs, 1974).

By 1919, Leuchs and Burke, as reported by Huss (1981a), described two distinct biotypes of *C. botulinum* which they differentiated into types A and B. In 1922, type C was described, followed by type D in 1927, type E in 1935, type F in 1960, and type G in 1970 (Huss, 1981a). Taxonomy of the organism. The genus *Clostridium* (from the Greek *kluster*, or small spindle) was described in 1880 by Prażmowski (Smith and Hobbs, 1974). Organisms in this genus are Gram-positive rods, 2-10 um in length and 0.5 to
2 um in width, which are usually motile by means of peritrichous flagella. All species form ovoid to spherical spores which usually "swell" the sporangium. Clostridia are chemoorganotrophs, able to ferment sugars and other carbohydrates. Many are proteolytic, yet some species are saccharolytic only. Some species fix nitrogen, while none have been shown to reduce sulfate. Most strains do not produce catalase, and thus they are defined as strict anaerobes. However, microaerophilic strains are found in the genus.

Over 300 species of *Clostridium* have been described, which are classified into five groups, I-V, depending upon a) the position of the spore in the sporangium and b) the gelatin metabolism of the vegetative cells. Species in group II, which includes *C. botulinum*, exhibit subterminal spores and will hydrolyze gelatin. As a result, *C. botulinum* is not regarded as a single species, but rather as a conglomerate of several relatively distinct groups (Smith, 1977). Although some effort has been directed towards dividing the group into several species, it has been recommended by the *Clostridium* subcommittee of the VI International Congress of Microbiology to keep all strains in the species *botulinum*, mainly on the basis of their capacity to synthesize neurotoxin (Smith, 1977).

A further classification scheme within the species is based on the serological differentiation of the neurotoxin
into eight types: A, B, C₁, C₂, D, E, F, and G. These types have been classified by metabolic and physiological characteristics, into 4 groups. The cultural characteristics of groups I through IV are presented in Table I.

Group I includes all of type A and proteolytic strains of types B and F. This group is further characterized by the production of highly heat resistant spores and minimal growth temperature of about 10°C (Lynt et al., 1982). Some strains are saccharolytic, attacking a variety of sugars, and strongly proteolytic (Smith, 1977). Group I strains represent the majority of the strains implicated in botulism outbreaks for which the source has been identified, as reported by the Center for Disease Control (CDC) in 1979. All types within group I share antigens both in the vegetative cells and the spores, but they do not exhibit antigens in common with strains from other groups or other Clostridium species (Lynt et al., 1982). The G+C ratio for Group I is between 26 and 28%, while DNA homology studies suggest an association among strains which corresponds to their protein metabolism.

Group II includes all type E and the nonproteolytic strains of types B and F (Smith, 1977). The vegetative cells of these strains are characterized by growth at temperatures as low as 3.3°C, and saccharolytic rather
than proteolytic metabolism (Lynt et al., 1982). The spores produced by *C. botulinum* group II are less resistant to heat than the spores of group I. The strains in group II also exhibit group-specific spore and somatic antigens (Lynt et al., 1967, 1982; Smith, 1977; Solomon et al., 1967), and their G+C ratio is similar to that of group I (Smith and Hobbs, 1974).

Group III includes the nonproteolytic types C and D strains (Smith, 1977). These strains are culturally indistinguishable and mostly saccharolytic. They have been involved in outbreaks of animal botulism (Delucca et al., 1973; Dolman, 1964). Group IV includes all type G strains, which are proteolytic but nonsaccharolytic. *C.

### Table 1. Cultural characteristics of *Clostridium botulinum* groups I-IV.

<table>
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<tr>
<td></td>
<td>I</td>
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<tr>
<td>Proteolytic metabolism</td>
<td>+</td>
</tr>
<tr>
<td>Optimum growth temp (°C)</td>
<td>30-40</td>
</tr>
<tr>
<td>Minimum growth temp (°C)</td>
<td>10-12</td>
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<tr>
<td>Inhibition by salt (%)</td>
<td>10</td>
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<tr>
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<td>high</td>
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w = weakly proteolytic
na = not available

botulinum type G was isolated in 1970 and has not been involved in botulism outbreaks (Eklund, 1982).

**Clostridium botulinum type E**

**The organism**

*Clostridium botulinum* type E was first isolated in Russia by Kushner and identified in 1935 by Gunnison and her co-workers from both the Russian samples and smoked salmon from Canada. Type E was later described by Hazen (1937). In the early 1960's, increased interest on *C. botulinum* occurred as a result of several relatively large outbreaks which occurred in 1963. The outbreaks were traced to the consumption of vacuum-packed smoked fish in the U. S. Great Lakes area. Type E strains, which are included in group II, are characterized by nonproteolytic metabolism, although the isolation of proteolytic mutants has been reported (Nakane and Iida, 1977). The cells are mostly saccharolytic, capable of fermenting fructose, glucose, mannose, and sucrose, with the production of acetic and butyric acid as main end products (Smith, 1977). Most type E strains hydrolyze gelatin, hemolyze blood, and reduce sulfites, although not consistently (Huss, 1981a).

**Environmental effects**

*Effect of temperature.* *C. botulinum* type E vegetative cells are capable of growth and toxin
production in various substrates at temperatures as low as 3.3°C (Abrahamsson et al., 1966; Hobbs, 1981; Roberts and Hobbs, 1968). The maximum growth temperature for type E is 45°C (Huss, 1981a), and the optimum is between 30 and 35°C. Maximum toxin production requires slightly lower incubation temperatures, ranging from 25-30°C. Higher temperatures result in the rapid inactivation of the toxin (Huss, 1981a).

**Effect of water activity.** Emodi and Lechowich (1969a) reported that substrates with water activity below 0.975 (when NaCl was used as the solute) were inhibitory to the growth of *C. botulinum* type E vegetative cells. Similarly, the addition of 5% NaCl to growth media inhibits cell growth, and this effect is increased by the addition of nitrite salts. The addition of NaCl and NaN0₂ in various combinations is the basis by which outgrowth of botulinum spores in cured and smoked products is prevented.

**Effect of pH.** Growth and toxin production by *C. botulinum* type E is also affected by fluctuations in pH. Studies by Emodi and Lechowich (1969a) showed that the growth of *C. botulinum* was inhibited at pH 5.3 and below. Recently, Tsang and co-workers (1985) demonstrated growth and toxin production in a system acidified with citric acid to pH 4.2. However, utilization of acetic acid as the acidulant inhibited the growth of the cells at pH
below 5.0. Using acidified food systems as well, Post and her co-workers (1985a) obtained results which confirmed the generally accepted concept of growth inhibition of *C. botulinum* cells at a pH below 4.6.

**Inhibition of *C. botulinum***. The mechanisms which result in the inhibition of *C. botulinum* are not completely understood. Often the synergistic action between inhibiting agents complicate the picture. For example, the concentration of nitrites required to inhibit *C. botulinum* cell growth decreases with the addition of NaCl (Pelroy et al., 1982). Similarly, the use of different acidulants and/or water activity modifying agents results in changes in growth dynamics which are difficult to explain by changes in one factor alone (Gombas, 1983). Lynt et al. (1982) reported that, in general, as the temperature of storage or pH of the substrate are lowered, less salt is required to inhibit outgrowth of the spores. Similarly, as pH decreases, heat resistance of the spores also decreases (Blocher and Busta, 1983; Gombas, 1983).

**Heat resistance of the spores**

The heat resistance of type E spores has been the subject of several studies. Spores of this organism are mildly heat resistant, when compared to the spores of other *C. botulinum* types and other spore-forming bacteria. Spore heat resistance is affected by several factors which
include the inherent resistance of the spores; age, temperature, and constituents of the medium in which the spores are produced, as well as the make-up of the medium in which the spores are suspended during the heating step (Stumbo, 1973). Factors in the medium which can affect the heat resistance include pH, concentration of salts, sugars and fats, and the water activity. Considered collectively, it is not surprising that different strains of the same organism grown and heated in the same medium may exhibit different levels of resistance to heat (Gombas, 1983; Stumbo, 1973).

Using phosphate buffer as the heating medium, Ohye and Scott, as reported by Huss (1981a), determined the D$_{80}$ of *C. botulinum* type E to be somewhere between 0.3-3.3 min. These results are in agreement with those published by Roberts and Ingram (1965), who reported a D$_{80}$ of 0.33-1.25 min. Working with fish paste, Angelotti (as reported by Huss, 1981a) reported a D$_{80}$ of 1.3-4.3 min. Similar values (1.6-4.3 min) were reported by Crisley et al. (1968). It was on the basis of these D values that the FDA Advisory Committee on Botulism Hazard recommended a heat treatment of a minimum of 30 min at a temperature of 82.2°C in order to assure the safety of smoked fish (Anonymous, 1964). This treatment was assumed to be sufficient to destroy up to $10^7$ spores of the more heat resistant strains (Huss, 1981a). Studies by Cockey and
Tatro (1974) revealed that pasteurization at 85\(^\circ\)C for 1 min was adequate to destroy most of the spores which could be present in crabmeat. They cited the excellent record of this product as evidence of the soundness of the process. Lower processing temperatures, however, were not considered safe by Lerke and Farber (1971) unless storage below 36\(^\circ\)F was assured.

In 1977, Lynt and co-workers reported a D\(_{82}\) of 0.49-0.74 min for spores of \textit{C. botulinum} type E in the meat of the blue crab. These authors attempted to evaluate the safety of the pasteurization process for crabmeat, which prescribes holding the internal temperature of the cans at 185\(^\circ\)F for 1 min. Lynt et al. (1977) considered the treatment to be insufficient to assure the safety of the pasteurized product, suggesting treatments of at least 10 min at the same internal temperature. The same authors recognized that proper storage of the canned product at temperatures below 46\(^\circ\)F will prevent growth and toxigenesis in the crabmeat.

The toxin and the disease

Properties of the toxin. The eight immunologically distinct toxins produced by \textit{C. botulinum} have similar molecular weights and pharmacological action (Simpson, 1981). The toxin is synthesized within the cell during the logarithmic phase of growth, and released as a
protoxin (also called progenitor toxin) upon lysis of the cell. Toxin production appears to be related to lysogeny (Dolman, 1964; Iida et al., 1981; Simpson, 1981), and is not necessary for cell growth.

Botulism toxin is proteinaceous in nature and heat labile. Woodburn et al. (1979), found that a treatment of 5 min at 85°C is required to inactivate $10^5 \text{LD}_{50}$ of the toxin. The toxin has a molecular weight of 200,000 - 900,000. The molecule is made up of two fractions: a very potent neurotoxin and a hemagglutinin, which seems to stabilize and protect the former (Schantz and Sugiyama, 1974). The neurotoxin is made up of a light (MW ~ 50,000) and a heavy (MW ~ 100,000) chain (Middlebrook and Dorland, 1984).

Gordon et al. (1957) and Kitamura et al. (1968) purified botulism type E toxin. Analysis of the two subunits by the same authors revealed a molecular weight of 350,000 for the toxigenic E component and 150,000 for the nontoxigenic E. The toxicity of type E toxin (in MLD50) is smaller than the toxicity for types A, B, and D, but greater than for types F and G (Schantz and Sugiyama, 1974).

As with other types, type E progenitor toxin is released as a partially toxic agent which is rendered fully toxic upon activation by protease action Sakaguchi and Sakaguchi (1959). Duff et al. (1956) reported a 12-
to 72-fold activation rate upon treatment of type E toxin with trypsin. The activated neurotoxin is absorbed through the small intestine and reaches the circulatory system via the thoracic lymph duct. Botulism toxins block the release of acetylcholine at the neuromuscular junction and at other nerve endings that use acetylcholine as the transmitter (Simpson, 1981). The binding of the toxin to its receptor (which has not been identified) is irreversible, thus recovery from the disease is slow.

Although nonspecific gastrointestinal symptoms such as nausea, vomiting and diarrhea may develop after the ingestion of botulism toxin, particularly type E, the mechanism of action of all toxins results in the incidence of neurological impairment. Early symptoms such as weakness, lassitude, dizziness, and vertigo are often followed by blurred vision, diplopia, fixed and dilated pupils, and impaired reaction to light. As the condition progresses, ptosis of eyelids and facial muscle and pharyngolaryngeal paralysis ensue. Impaired salivation and difficulty in speech are also observed. In type E intoxications, abdominal fullness and pain, as well as retention of urine, have been reported (Sakaguchi and Sakaguchi, 1959). Muscle weakness in tongue, neck, diaphragm, and extremities cause prostration. In severe cases, paralysis of the diaphragm results in death due to respiratory failure and airway obstruction. It is
important to note that, while marked somnolence suggests involvement of the central nervous system, mental status is normal (CDC, 1979). Incubation periods for botulism range from 48 hours to 2 weeks and, in general, the shorter the incubation period, the more severe the disease.

**Treatment.** Treatment for botulism is usually limited to symptomatic relief, removal of toxin from the gastrointestinal tract, respiratory assistance (tracheostomy), and neutralization of the toxin in the circulation with antitoxin (Morris, 1981). The latter step is only taken in cases where diagnosis is certain.

**Epidemiology**

**Distribution of cases.** Statistics on the incidence of botulism have been kept in the U.S. since 1899, in Canada since 1924 (CDC, 1979; Dolman, 1964). Botulism outbreaks have occurred in 45 states (CDC, 1979) and Puerto Rico (Rigau-Perez et al., 1982). Most outbreaks have been reported in California and other Western states, mostly due to type A toxin, a finding that reflects the geographical distribution of the spores. Type B botulism has been reported in Eastern states, mostly in New York. Similarly, most type E botulism outbreaks have occurred in states around the Great Lakes and in Alaska. Outbreaks due to type E toxin are also dominant in Japan, Canada and Scandinavian countries (Huss, 1981a).
In a report published by the Center for Disease Control in 1979, a total of 766 botulism outbreaks, which occurred between 1899 and 1977, were reported. Of these, type A accounted for 26%, type B for 7.8%, type E for 4.2%, and type F for 0.1%. In contrast, type E was involved in 47-99% of the cases reported in Japan, Canada, Denmark, Norway, and Sweden (Huss, 1981a). In 61.6% of the cases reported in the U.S., the toxin type was not identified. However, the identification rate for the last three decades has improved, mainly as a result of increased diagnostic capabilities (Feldman et al., 1981).

Up to the early 1960's, most botulism outbreaks in the U.S. involved toxins A and B. Several large outbreaks of botulism caused by type E toxin occurred since 1963. Type F toxin has been involved in at least three cases (CDC, 1979).

Sources of outbreaks. Up to 1977, commercially processed foods have accounted for 8-9% of all cases of foodborne botulism. In many instances, the foods were improperly handled, while a handful of well-documented cases have been due to underprocessing or inadequate sealing of containers (NFPA/CMI Container Integrity Task Force, Microbiological Assessment Group Report, 1984). However, most of the cases up to date have been attributed to home-canned foods, usually improperly preserved vegetables, fruits, meats, and condiments, containing type
A or B toxin. Fish and fish products containing type E toxin have been involved in several outbreaks.

Most outbreaks involving type E spores have been traced to contaminated fish or fish or fish products. In few instances, inadequate processing of canned products was the cause of the outbreak. However, many outbreaks involving type E toxin have been reported, in the U. S. and other countries, following the ingestion of food that was "semi-processed," that is, treated with a process which does not result in a commercially sterile product (e.g. cured, smoked, salted, fermented, etc.). Generally, those products were uncooked or only slightly heated, stored at ambient temperature, and consumed without further heating (Eklund, 1982; Huss, 1981a; Sakaguchi and Sakaguchi, 1959).

Distribution of C. botulinum type E

Type E spores seem to be prevalent in marine sediments from temperate zones and have been found in Northern Japan, Canada, Alaska, the Great Lakes area of the U. S., Slavic countries and the Soviet Union (Dolman, 1964; Dolman and Murakami, 1961; Huss, 1981a, 1981b). Although Dolman and Murakami (1961) reported that type E spores had not been found south of latitude 40°N, Ward and his co-workers (1967a, 1967b, 1967c), as well as Huss (1981) have disproved the theory by reporting the isolation of such spores from the waters of the U. S. Gulf
Coast and Indonesia. While marine and freshwater sediments seem to contain great numbers of *C. botulinum* type E spores, they are rarely found in soils, particularly those with no contact with aquatic environments.

**Assessment of botulism risk from seafoods**

Botulism is a rare and serious disease which usually results from the ingestion of toxin-contaminated foodstuffs. However, the production of toxin in the food system does not always result in the occurrence of the disease, even though many foods are capable of sustaining growth of *C. botulinum*. The occurrence of a botulism outbreak follows a breakdown in the safety net provided by sound food handling, processing, and storage practices. Eklund (1982) cited four conditions necessary for botulism outbreaks to occur.

**Presence of spores in the food**

In order for botulism to occur, the food must be contaminated with spores or vegetative cells of *C. botulinum*. The presence of *C. botulinum* spores in fish is clearly documented. Several surveys have resulted in the isolation of the organism from marine and freshwater sediments as well as animals. Wide distribution of *C. botulinum* type E spores in sediments from the Great Lakes was reported by Bott et al. (1966); in Alaska by Miller.
Spores have been found in marine fish and shellfish from many areas, including the U. S. Gulf coast (Kautter et al., 1974; Ward et al., 1967b; Ward et al., 1977). In these surveys, *Clostridium botulinum* type E was isolated from the gills and viscera of fish and shellfish, including croaker, black drum, flounder, mullet, red snapper, speckled trout, spot, crab, and shrimp. Although the incidence of *C. botulinum* spores in most samples is relatively low, their presence is significant, because of the potential for introduction of spores in food processing operations (Licciardello, 1983). Furthermore, the presence of spores in the viscera of fish may result in the production of toxin, which was detected in the edible portion of fish after brief incubation at temperatures as low as 15°C by Huss (1981). The presence of toxin in fish has possible health implications and could be a source of contamination for workers (Zaleski, 1981).

**Adequacy of the processing treatment**

The processing treatment must be adequate to destroy the spores, or the product will be contaminated following processing. Several instances of botulism outbreaks have
been due to underprocessing (NFPA/CMI Container Integrity Task Force, Microbiological Assessment Group Report, 1984). Furthermore, it has been clearly established that certain processes and techniques used for the preservation of seafood are not adequate to destroy the spores of *C. botulinum* type E even though those spores are relatively heat sensitive. Processes which result in the selective destruction or inhibition of spoilage bacteria, but do not affect *C. botulinum* spores, were shown to be inadequate (Eklund, 1982).

**Hot-process smoking**. The process of hot smoking, which calls for holding the fish at 82.2°C for at least 30 min (Anonymous, 1964), has been found to be inadequate in preventing the outgrowth of and toxin production by *C. botulinum* type E (Cann et al., 1965; Christiansen et al., 1968; Kautter, 1964; Zaleski, 1981).

**Irradiation**. Irradiation of fish resulted in a faster rate of toxin production by surviving spores (Cann et al., 1965). Radurization processes (with doses above 100 Krad) were found to be potentially hazardous, especially when followed by storage above 3.3°C (Eklund, 1982; Jimes, 1967). Moreover, botulism toxin is resistant to irradiation. Thus, the use of practical doses of radiation cannot be relied upon to remove the hazard (Skulberg, 1965).
**Modified atmosphere storage.** The use of modified atmosphere storage to preserve seafoods also results in the inhibition of spoilage bacteria (Stier et al., 1981; Wang and Brown, 1983; Wolfe, 1980), thus enhancing the shelf life of the fresh product (Parkin and Brown, 1982). However, Silliker and Wolfe (1980) questioned the safety of the process and suggested that an evaluation of the effect of modified atmosphere storage on the growth and toxin production by *C. botulinum* type E was badly needed. Stier and his co-workers (1981) observed toxin production only after 2-3 days incubation at 22°C, but not when the product was refrigerated. Such an evaluation was also done by Llobrera (1983), who observed toxin production in inoculated fish samples which had been stored at 10°C for 6 days. Similar results were reported by Post and her co-workers (1985b). Using both inoculated and noninoculated fish fillets, the authors reported the formation of toxin in both samples and controls, either before or simultaneously with sensory rejection. All authors stressed the importance of adequate refrigerated storage as means of preventing health hazards.

**Pasteurization.** Pasteurization processes are widely used as means of preserving fish and shellfish (Lerke and Farber, 1971; Rodriguez-Solano, 1985; Ward et al., 1982). The treatment is relatively mild and it usually involves heat processing the food in hermetically sealed
containers at waterbath temperatures, below 100°C (Thomas and Thomas, 1983). By definition, a pasteurized product is not sterile, but may contain some bacteria, which can grow under favorable conditions, thus the product usually requires refrigerated storage (Ward et al., 1982). Although type E spores are sensitive to mild heat, several researchers have found that conventional pasteurization processes do not destroy them completely (Lerke and Farber, 1971; Lynt et al., 1977). The use of refrigerated storage is imperative in order to insure a safe product. Another key to the safety of fish and shellfish is that they are usually, but not necessarily, cooked before consumption.

**Other conditions necessary for botulism outbreaks**

As reported by Eklund (1982), any process which destroys or inhibits normal spoilage flora from fish samples is removing a safety index from the product, thereby producing a health hazard. Since *C. botulinum* type E is characterized by nonproteolytic metabolism, the formation of off-odors cannot be relied upon as an index of spoilage.

Botulism outbreaks only result from the consumption of food which supports growth and toxin production by *C. botulinum* type E when it is stored above 3.3°C. This is particularly important, because seafoods might be
contaminated with the organism and some processes are not effective in destroying its spores.

One last condition necessary for botulism outbreaks to occur is that the food is not heated further before consumption and is acceptable as such. The safety record of fresh and frozen fish and shellfish is excellent (Eklund, 1982). These products require heating, which inactivates botulism toxin. In addition, it is possible that objectionable aromas decrease their acceptability to the consumer before toxin has been released. In the case of nonproteolytic strains, objectionable odors might not be produced prior to toxin production. It is in these cases where the destruction of natural spoilage flora is of paramount importance. Without this spoilage flora, no index of decomposition is present.

**Determination of a Thermal Process**

Before a thermal process for a new product is established, the heat resistance of the spores of significance in the product must be determined (Lopez, 1981). The most commonly used method for determining the heat resistance of bacteria involves the determination of their thermal death time (TDT). There are several ways of determining this value for a specific strain. All methods are based on the general assumption that bacteria, as well as spores, are killed in a logarithmic fashion, that is, the number of viable cells present in a medium is reduced
exponentially with the time of exposure to the lethal temperature.

Methods. The methods for measuring the thermal resistance of bacteria have been reviewed by Stumbo (1973), and include the following.

**TDT tube method.** In this method, inoculated medium (water, buffer, food material) is distributed in small diameter tubes which are subsequently heat-sealed. The tubes are immersed in a bath heated at the test temperature. At predetermined intervals, replicate tubes are removed from the bath and cooled. After cooling, the tubes are opened aseptically, and their contents transferred to culture medium or, if the menstruum itself is favorable for growth, the tubes are incubated directly. In the latter case, growth is indicated by the production of gas (National Canners Association Research Laboratories, 1968).

The use of unsealed ("plugged") tubes spares the worker the task of heat sealing and subsequent opening of the tubes, which can be time consuming. The use of capillary tubes allows for rapid heating and cooling of the media, but filling the tubes can be tedious, especially in the case of thick products. The TDT tube method is, in general, simple but time consuming. Furthermore, the method is accurate only at temperatures below 240°F.
TDT can method. This method involves the use of small cans as containers for the product. The cans are heated in small retorts, but otherwise treated as TDT tubes described above. The method has the disadvantages of adding the possibility of post-heating contamination through leakage, and that special equipment (can sealers, retorts) is needed. On the other hand, it makes possible the study of a system which closely resembles commercial canning operations.

"Tank" method. A steel cylinder or heating chamber is filled to capacity with the inoculated substrate and heated. Tubes fitted with sampling valves are at the bottom of the chamber. Through these tubes, samples of the treated substrate are obtained periodically. The samples are diluted and colony counts provide the data for survivor curves.

Flask method. In this method, a three-neck flask contains the substrate. The flask is heated in a bath containing mineral oil. When the substrate approaches the bath temperature, the inoculum is introduced. Samples are withdrawn at predetermined intervals and subcultured for counting of survivors. This method is only suitable for the determination of the resistance of "low resistance" organisms, because it is limited to the use of sub-boiling water temperatures.
Thermoresistometer method. Samples of inoculated medium are subjected to steam under pressure in three adjacent chambers in a continuous transfer process. At the end of the process, the samples, contained in small cups, are dropped into tubes of culture medium. Because of automatic subculture, the resistometer method reduces the chances of contamination. Because of its continuous operation, it is labor- and time-saving. This method is particularly suited for studying the resistance of highly resistant spores.

Treatment of thermal resistance data

The determination of thermal resistance is based on the logarithmic rate of death of bacterial spores. The thermal death time is defined as the time in minutes required to destroy completely a certain spore population at a given temperature (Russell, 1982). Because it depends on the number of spores present in the suspension, a thermal death time is not very valuable.

The D-value, or decimal reduction time value, refers to the time in minutes required to destroy 90% (or one log cycle) of the spore population at a given temperature. The D value is usually expressed as a function of the temperature at which it was determined, e.g. $D_{90}$. The D value can be obtained in several ways, which are each suitable for the type of data available.
**Direct plotting.** The usual method is to plot the number or percent survivors on the logarithmic scale versus heating time on the arithmetic scale, as shown in Fig. 1. When the curve is a straight line, the D value can be read directly from the graph. As an alternative, the following formula may be applied to obtain the D value (Russell, 1982; Schmidt, 1954; Stumbo, 1973).

\[
D = \frac{t}{\log a - \log b}
\]

where \( t = \) exposure time at a given temperature

\( a = \) initial number of spores

\( b = \) final number of spores

**Most probable number (MPN) technique.** When the surviving spores are incubated in the same medium in which they were heated, the number of replicate containers showing survival at a given time is assumed to be equal to the number of surviving spores. Following this approach, Stumbo (1948) used the following formula:

\[
Z \text{ or } D = \frac{U \text{ or } t}{\log A - \log B}
\]
Figure 1. Time versus percent survival curve used for the direct determination of D-values.
where \( A \) = total number of samples heated multiplied by the number of spores per sample;
\[
    B = \text{number of survivors, calculated assuming one surviving spore per container when less than the total number of containers showed survival; and,}
\]

\( U \) or \( t \) = exposure time.

Stumbo et al. (1950) used the same formula described above, but used the following formula to calculate the "most probable number" of survivors.

\[
\bar{X} = 2.303 \log \frac{n}{q}
\]

where \( \bar{X} \) = most probable number of spores surviving per replicate
\( n \) = total number of replicates
\( q \) = number of sterile replicates

Then, \( B = \bar{X} \) times the number of replicates.

**Probability method.** A third method for calculating \( D \) values was described by Schmidt (1954). It is based on two assumptions: first, that any sample not showing survivors at a given exposure time, would not show survivors at a longer exposure time. Second, any sample showing survivors at a given exposure time would show survivors at a shorter exposure time. These two assumptions permit the data to be subjected to a cumulative test similar to any \( LD_{50} \) bioassay.
The probability of sterility, $P$, at any given time is calculated from the following formula:

$$P = \frac{n + 1}{m + n + 2}$$

where $n = \text{cumulated samples not surviving each time exposure, obtained by adding the negative samples downward from the shortest time to the longest}$; and,

$m = \text{cumulated samples surviving each exposure time, obtained by adding the positive samples upward from the longest exposure to the shortest.}$

The probability of sterility is plotted versus time on probability paper, and the $LD_{50}$ point is read directly. Then, $D$ is calculated from:

$$D = \frac{LD_{50}}{\log A + 0.16}$$

where $A = \text{initial number of organisms per tube.}$

Obtaining the $D$-value

Generally, the $D$ values calculated by any of these three methods show acceptable agreement (Schmidt, 1954). However, the probability method was considered best by Lynt et al. (1977), mainly because it allowed handling of the skips and tailing present in the data. $D$ values calculated as described are reproducible, and basically
the same, regardless of spore concentration (Schmidt, 1954).

Once all D values at different temperatures are obtained, a thermal resistance curve is drawn on semilogarithmic paper, by plotting the D values on the logarithmic scale versus temperature on the linear scale.
MATERIALS AND METHODS

Culture media

All media used in the preparation and enumeration of spore crops were obtained from Difco Laboratories (Detroit, MI), unless otherwise indicated. Media were used the same day they were prepared, or otherwise steamed for 15 min in order to remove dissolved oxygen. The following culture media were used during the course of these experiments.

1. Cooked meat medium (CMM). Rehydrated by the method outlined by Scott and Bernard (1982). Approximately 1.25 g dehydrated meat particles were placed in 20 x 150 mm screw-capped tubes. Fifteen ml of a solution containing 0.3% glucose and 0.2% starch were added to each tube. The meat particles were allowed to rehydrate for 30 to 60 min, after which the tubes were sterilized.

2. Tryptone-peptone-glucose-starch (TPGS) broth. Prepared according to the formula published by Scott and Bernard (1982). One liter of TPGS contained 50 g tryptone, 5 g peptone, 2 g starch, and 0.5 g cysteine (Sigma Chemical Company, St. Louis, MO).

3. Biphasic sporulation medium (BSM). Prepared as described by Scott and Bernard (1982). The agar phase contained 500 g ground defatted beef heart, 10 g
neopeptone, 10 g tryptone, 10 g gelatin, and 20 g agar per liter of distilled water. The medium was dispensed into Roux flasks and sterilized for 60 min at 121°C. The agar phase was then allowed to solidify as a flat surface, after which it was used.

4. **Tryptone peptone agar (TPA).** Prepared as described by Polvino and Bernard (1982). It contained 50 g tryptone, 5 g peptone, 1 g thioglycollate, and 15 g agar per liter of distilled water.

**Spore stocks**

Three strains of *Clostridium botulinum* type E (Beluga, Minnesota and Whitefish) were obtained from the National Food Processors Association (NFPA), Washington, D.C. The spores were kept refrigerated and used as the stock from which each spore crop was originated.

**Preparation of spores.** A biphasic technique, similar to the one described by Bruch and her co-workers (1968), was used to obtain spores. As suggested by Scott and Bernard (1982), a loopful of the original spore stock was transferred to several tubes containing CMM. Those tubes were incubated at 30°C for 48 h. Subsequently, one ml aliquots from the CMM supernatant fluid were transferred to several 20 x 150 mm screw-capped tubes containing 15 ml TPGS broth. These latter tubes were incubated at 30°C for 48 h, after which the contents from each tube were
transferred aseptically to Roux flasks containing 200 ml BSM. The solid phase of the BSM was overlaid with 250 ml TPGS. The flasks were incubated at 30°C for at least 7 days, during which sporulation was monitored using phase contrast microscopy (Anellis et al., 1972).

After incubation, the culture fluid from each flask was filtered through several layers of sterile cheesecloth to remove large particulate matter (mainly agar and beef particles). The filtrate was dispensed in sterile 250 ml plastic centrifuge tubes and centrifuged at 10,000 rpm for 30 min in a Sorvall model RC5C automatic refrigerated centrifuge (Sorvall Instruments, Dupont, Newtown, CT). The supernatant was discarded, and the spore pellet was rinsed by resuspending in sterile distilled water. The centrifuge bottles were then refrigerated overnight to allow for the lysis of any vegetative cells present in the preparation.

The following day, the spores were centrifuged and rinsed by resuspending twice more. After the final centrifugation step, the spores in each bottle were resuspended in a small amount of sterile distilled water (usually 3-5% of the original TPGS volume). The spore suspension was later tested for the presence of aerobic contaminants by streaking a loopful of the suspension onto conventional aerobic plate count media. Plates showing no aerobic growth were considered to come from
"noncontaminated" bottles. The contents of such bottles were pooled, supplemented with glycerol at the 5% level, and divided into 5 ml aliquots, which were then stored in small vials and frozen at -80°C in a Revco Ultra Low Temperature Freezer (Rheem Manufacturing Co., Asheville, NC).

Spore crops were repeated until a large quantity of spores was available for experiments.

**Enumeration of spores.** Spores were enumerated using a modification of the method outlined by Polvino and Bernard (1982). Three 0.5 ml samples of the spore suspension were diluted 1:10 with 4.5 ml phosphate buffer in a screw-capped 16 x 125 mm tube. The diluted spore suspension was heated in a 60°C waterbath, in order to activate the spores (Scott and Bernard, 1982). Subsequently, the suspension was diluted appropriately and enumerated in TPA, which was overlaid with sterile 2% agar. The plates were incubated anaerobically in GasPakR anaerobic jars (BBL Microbiological Systems, Cockeysville, MD) at 30°C for 96 h. Colonies were counted using a Quebec darkfield colony counter, and reported as an average of two plates per dilution. An overall average of the three samples was obtained to determine the spore count in each spore crop, and to calculate the volume of suspension needed to seed the crawfish paste. Spore counts for each spore crop are presented in Table 2.
Table 2. Counts from spore suspensions obtained from each spore crop.

<table>
<thead>
<tr>
<th>Spore crop (date)</th>
<th>Count (spores/ml)</th>
<th>ml suspension/lb crawfish paste</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (06-27-84)</td>
<td>$6.9 \times 10^6$</td>
<td>65.8</td>
</tr>
<tr>
<td>2 (07-23-84)</td>
<td>$3.7 \times 10^7$</td>
<td>12.3</td>
</tr>
<tr>
<td>3 (11-13-84)</td>
<td>$1.2 \times 10^9$</td>
<td>0.4</td>
</tr>
<tr>
<td>4 (03-25-85)</td>
<td>$2.8 \times 10^7$</td>
<td>16.2</td>
</tr>
<tr>
<td>5 (09-30-85)</td>
<td>$7.3 \times 10^6$</td>
<td>62.2</td>
</tr>
<tr>
<td>6 (10-28-85)</td>
<td>$1.4 \times 10^7$</td>
<td>32.4</td>
</tr>
<tr>
<td>7 (11-29-85)</td>
<td>$1.9 \times 10^7$</td>
<td>23.9</td>
</tr>
<tr>
<td>8 (11-30-85)</td>
<td>$2.3 \times 10^7$</td>
<td>19.7</td>
</tr>
<tr>
<td>9 (03-07-86)</td>
<td>$1.0 \times 10^7$</td>
<td>45.4</td>
</tr>
<tr>
<td>10 (04-06-86)</td>
<td>$9.6 \times 10^6$</td>
<td>47.3</td>
</tr>
</tbody>
</table>
Thermal Resistance Determinations

Preliminary heat resistance determination. A preliminary heat resistance test was conducted with the objective of determining the relative resistance of the three samples available for the study. A shrimp:water (1:2) suspension was inoculated with approx. 10,000 spores per g of suspension and two 16 x 125 mm test tubes per strain were filled with the suspension. The tubes were heated in an 80°C waterbath. The tubes were removed from the waterbath 5 and 10 min after the tubes reached temperature. The spore counts of both heated samples as well as that of a nonheated reference were obtained as described above. C. botulinum type E strain Minnesota E was found to be more heat resistant than strains Beluga and Whitefish. Therefore, the Minnesota strain was used in the remainder of this study.

Preparation and inoculation of the crawfish paste. Peeled crawfish tails packed in 1 lb bags were obtained from a local supplier and frozen until needed. Three pounds of crawfish tails were placed on a shallow tray and sterilized for 60 min. The tray was stored in the refrigerator overnight. The following morning, the crawfish tailmeat was inoculated with enough spore suspension to achieve a concentration of $10^6$ spores per g of tailmeat. The meat was blended to distribute the spores evenly, while adding water to facilitate the
process. The quantity of water added varied from batch to batch, and depended on the amount of water present in the crawfish as well as the water added along with the spores. Due to differences in spore crop counts, some batches were more diluted than others (Table 2). Efforts to standardize the amount of water added to the meat-spore suspension resulted sometimes in differences in the thickness (consistency) of the paste, particularly in cases where spores from different crops were used. To compensate for these differences, the consistency of the paste was judged visually.

At later stages of the experiments, standardization of the paste was achieved by adding 500 ml distilled water to 1362 g (3 lb.) sterile crawfish tails. Changes in the consistency of the paste were minimized by using a blender jar which was large enough to allow the blending of a large amount of paste, enough for several replicates of the experiment.

**Heat resistance determinations.** The unsealed TDT tube method, as described by Stumbo (1973), was used to determine the heat resistance of the spores of *C. botulinum* type E strain Minnesota E. The TDT experiments were conducted following a procedure similar to the ones outlined by Lynt et al. (1977, 1979, 1981) and Stumbo et al. (1945).
The inoculated crawfish paste was dispensed into 16 x 125 mm screw-capped tubes by means of a stainless-steel calf forced-feeder syringe. The filled tubes were stored in an ice-bath until all tubes were filled.

Temperature reference tubes were fitted with stainless-steel needle type (copper constantan) thermocouples (O.F. Ecklund, Cape Coral, FL). A similar thermocouple and a mercury-in-glass thermometer were used to measure the temperature of the water in the bath. All thermocouples were attached to a multi-point data-logger (Model 205, Omega Engineering, Stamford, CT). Both tubes and waterbath temperatures were recorded every minute.

All tubes (except 5 nonheated reference tubes) were dropped simultaneously into the waterbath set at the test temperature. Sufficient time was allowed for the waterbath to return to its original temperature. As soon as the slowest heating reference tube reached one degree below test temperature, five duplicate tubes were withdrawn from the waterbath (time 0), and timing of the experiment was started. Subsequent five tube samples were withdrawn from the bath at predetermined time intervals, which were adjusted in successive experiments, as the results warranted. As the samples were removed from the bath, they were immediately plunged in an ice bath and kept there until enough tubes were gathered to fill a
GasPak$^R$ jar, where the tubes were placed for anaerobic incubation.

The tubes were incubated anaerobically at 30°C for 2 weeks, after which they were observed for gas production. Tubes which showed gas production were considered positive, while tubes showing no signs of gas production were considered negative. Negative tubes were overlaid with sterile melted wax and stored at 30°C for two months. After this incubation period, the tubes were observed for gas production, and the number of positive tubes was recorded. Tubes which showed no signs of growth after the prolonged incubation period were considered sterile.

**Confirmation of positive tubes.** Sample tubes from the longest exposure time which showed growth were tested for the presence of botulism toxin using the mice bioassay (Kautter and Lynt, 1980; CDC, 1979; Hatheway, 1986). The fluid accumulated at the bottom of each positive tube to be tested was transferred aseptically to CMM. The CMM tube was incubated for at least 48 h at 30°C. The CMM fluid was centrifuged and the crude supernatant separated from the particulate matter. The supernatant was divided in two portions. One portion was boiled at 100°C for 10 min, in order to inactivate any toxin present, thus serving as a control. The second portion was mixed with diluted trypsin (Difco Laboratories, Detroit, MI) as indicated in the Bacteriological Analytical Manual.
(Kautter and Lynt, 1980). One gram Difco 1:250 trypsin was dissolved in 10 ml distilled water to make a saturated aqueous solution of trypsin, two-tenths ml of which was added to each 1.8 ml toxic fluid. The mixture was incubated at 37°C for 1 h, agitating occasionally.

The trypsinized toxic fluid was divided in two portions, one of which was diluted 1:5 with sterile physiological saline. One milliliter of each diluted, trypsinized sample was neutralized with 0.25 ml type E monovalent antitoxin (obtained from Dr. C.L. Hatheway, CDC, Atlanta, GA), and incubated at 37°C for 30 min.

Portions of 0.5 ml of either heated, trypsinized, or neutralized samples were injected i.p. into white mice (20-25 g each). The mice were observed for botulinogenic signs for up to 72 h.

**Analysis of the results.** The results were analyzed and the D values calculated using the methods of Stumbo (1948), Stumbo et al. (1950) and Schmidt (1954).
RESULTS AND DISCUSSION

A total of 23 heating experiments, in which inoculated meat samples were treated, were done in the last phase of this project. Six replicate experiments were run at 85 and 90°C, and four replicates were done at 80 and 95°C. One experiment was performed at 70°C and two were done at 75°C. The observed and corrected endpoints for the experiments are presented in Table 3.

The endpoint for each experiment was defined as the time after which none of the five tubes in the sample showed discernible bacterial growth. Variation in the endpoints was frequently observed for each temperature. For example, no endpoint was obtained in experiments 85E, 90E, and 95B. The variation may be attributed to the differences in the come up times for each experiment.

Two replicates at 80, 85, 90, and 95°C yielded endpoints which were satisfactory for further analysis.

Determination of Corrected Exposure Time

Prior to the calculation of the D values from each experiment, the time of exposure to heat must be corrected to account for the thermal lag time, which is the time it takes for the substrate to reach the temperature in the waterbath. In addition, another term which needs to be considered is that one which accounts for the lethality afforded to the spores by the exposure to temperatures
Table 3. Observed and corrected endpoints for the replicate experiments done at six temperatures.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Replicate code (date)</th>
<th>Observed endpoint (min)</th>
<th>Corrected endpoint (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>70A (10-11-85)</td>
<td>ner b</td>
<td>ner</td>
</tr>
<tr>
<td>75</td>
<td>75A (08-13-85)</td>
<td>ner</td>
<td>ner</td>
</tr>
<tr>
<td></td>
<td>75B (01-21-86)</td>
<td>ner</td>
<td>ner</td>
</tr>
<tr>
<td>80</td>
<td>80A (08-20-85)</td>
<td>ner</td>
<td>ner</td>
</tr>
<tr>
<td></td>
<td>80B (01-20-86)</td>
<td>ner</td>
<td>ner</td>
</tr>
<tr>
<td></td>
<td>80C (04-08-86)</td>
<td>60</td>
<td>62.3</td>
</tr>
<tr>
<td></td>
<td>80D (04-09-86)</td>
<td>180</td>
<td>182.6</td>
</tr>
<tr>
<td>85</td>
<td>85A (08-22-85)</td>
<td>ner</td>
<td>ner</td>
</tr>
<tr>
<td></td>
<td>85B (01-16-86)</td>
<td>ner</td>
<td>ner</td>
</tr>
<tr>
<td></td>
<td>85C (02-25-86)</td>
<td>120</td>
<td>122.3</td>
</tr>
<tr>
<td></td>
<td>85D (03-20-86)</td>
<td>ner</td>
<td>ner</td>
</tr>
<tr>
<td></td>
<td>85E (04-08-86)</td>
<td>neo c</td>
<td>neo</td>
</tr>
<tr>
<td></td>
<td>85F (04-09-86)</td>
<td>60</td>
<td>62.7</td>
</tr>
<tr>
<td>90</td>
<td>90A (10-01-85)</td>
<td>ner</td>
<td>ner</td>
</tr>
<tr>
<td></td>
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<td>ner</td>
</tr>
<tr>
<td></td>
<td>90C (02-24-86)</td>
<td>120</td>
<td>122.4</td>
</tr>
<tr>
<td></td>
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<td>ner</td>
<td>ner</td>
</tr>
<tr>
<td></td>
<td>90E (04-08-86)</td>
<td>neo</td>
<td>neo</td>
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<tr>
<td></td>
<td>90F (04-09-86)</td>
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<tr>
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<td>240</td>
<td>243.4</td>
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<tr>
<td></td>
<td>95B (02-18-86)</td>
<td>neo</td>
<td>neo</td>
</tr>
<tr>
<td></td>
<td>95C (02-21-86)</td>
<td>210</td>
<td>212.4</td>
</tr>
<tr>
<td></td>
<td>95D (03-17-86)</td>
<td>ner</td>
<td>ner</td>
</tr>
</tbody>
</table>

a. See text for explanation of the correction.

b. ner = no endpoint reached. Tubes heated for the longest time were positive for C. botulinum growth, as determined by the mouse bioassay.

c. neo = no endpoint observed. No tubes gave evidence of C. botulinum growth, even at the shortest heating time.
below the test temperature. The correction for the exposure time in these experiments was done using a modification of the formula of Anellis et al. (1954). They corrected the exposure time as outlined below.

Corrected exposure time = total exposure time in bath - thermal lag time + lethality due to come-up and come-down time.

In the experiments reported here, timing began after the substrate reached a temperature of one degree below waterbath temperature; therefore, no correction was made for the thermal lag time. At the conclusion of each time interval, the tubes were immediately cooled in ice and, as a result, the lethality due to come-down time was negligible. Consequently, the recorded exposure time was corrected for the lethality due to the come-up time only.

The lethal rate due to the come-up time was calculated with the following formula, as described by Stumbo (1973).

\[ L = \log^{-1} \frac{T_s - T_r}{z} \]

where \( T_s \) = temperature of the substrate

\( T_r \) = reference temperature (waterbath)

\( z \) = the number of degrees C required for the thermal death curve to traverse one log cycle. The \( z \)-value also identifies the slope of the thermal death curve.
In the calculations, a z-value of 8°C was used (Russell, 1982). The lethal rates for each time exposure at each temperature were added to obtain the accumulated lethal rate inherent to the heating rate of the substrate. The lethality obtained represents the accumulated lethal effect of the thermal lag time which is expressed in minutes (Scott, 1986). Accumulated lethal rates for each of the eight replicate experiments are listed in Table 4. The accumulated lethality value for each experiment was rounded to 0.1 min and then added to the exposure time to give the corrected exposure time.

**Determination of the D-values**

The D-values for *C. botulinum* strain Minnesota E were calculated by three methods reported previously (Stumbo, 1948; Stumbo et al., 1950; Schmidt, 1954), and the findings are presented in Table 5. The thermal death time curves for the D-values obtained by each of the three methods are shown on Figures 2, 3, and 4, respectively.

**Comparison of D-values**

According to Schmidt (1954), D-values, calculated by any method, should be reproducible, yet, in this investigation, this was not the case. Moreover, in some cases, the heat resistance of the spores seemed to increase at higher temperatures.
Table 4. Accumulated lethal rate for each thermal death time experiment.

<table>
<thead>
<tr>
<th>Experiment replicate</th>
<th>Lag time (min)</th>
<th>Accumulated lethality^a (min)</th>
<th>Correction factor (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80C</td>
<td>37</td>
<td>2.2849</td>
<td>2.3</td>
</tr>
<tr>
<td>80D</td>
<td>42</td>
<td>2.6220</td>
<td>2.6</td>
</tr>
<tr>
<td>85C</td>
<td>11</td>
<td>2.2859</td>
<td>2.3</td>
</tr>
<tr>
<td>85F</td>
<td>42</td>
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</tr>
<tr>
<td>90C</td>
<td>21</td>
<td>2.4385</td>
<td>2.4</td>
</tr>
<tr>
<td>90F</td>
<td>14</td>
<td>2.4444</td>
<td>2.4</td>
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<tr>
<td>95A</td>
<td>60</td>
<td>3.3663</td>
<td>3.4</td>
</tr>
<tr>
<td>95C</td>
<td>22</td>
<td>2.3914</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Table 5. Decimal reduction time values obtained by three different methods of calculation.\(^a\)

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Stumbo (1948)</th>
<th>Stumbo et al. (1950)</th>
<th>Schmidt (1954)</th>
</tr>
</thead>
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<tr>
<td>80</td>
<td>7.000</td>
<td>6.067</td>
<td>4.928</td>
</tr>
<tr>
<td>85</td>
<td>8.807</td>
<td>6.701</td>
<td>7.911</td>
</tr>
<tr>
<td>90</td>
<td>2.460</td>
<td>2.986</td>
<td>3.112</td>
</tr>
<tr>
<td>95</td>
<td>17.146</td>
<td>10.755</td>
<td>8.689</td>
</tr>
</tbody>
</table>

\(^a\) See text for formula.
Figure 2. Thermal death time curve, as calculated by the method of Stumbo (1948).
Figure 3. Thermal death time curve, as calculated by the method of Stumbo et al. (1950).
Figure 4. Thermal death time curve, as calculated by the method of Schmidt (1954).
D_{80} values ranged from 4.928 to 6.995. These values are higher than the value reported previously for type E spores in fish by Crisley (1968) and Huss (1981a). Similarly, D_{82} values, which were obtained by interpolation in the thermal death time graphs, and ranged from 10.1 to 10.9 min, were higher than those reported for type E in crabmeat by Lynt et al. (1977).

D_{85} values ranged from 6.7005 to 7.911 min, while D_{90} values ranged from 2.460 to 3.112 min. Unexpectedly, D_{95} values were higher than those obtained at lower temperatures.

For each calculation method used, the heat resistance of the spores (as reflected by the D value) increased slightly at 85°C over the D value which was observed at 80°C. This may be attributed to late activation of the more heat resistant spores that could be present in the population. Although the spores of nonproteolytic strains of *C. botulinum* are usually activated at 60°C (Scott and Bernard, 1982), Roberts and Ingram (1965) reported that the degree of activation among clostridia varies largely and unpredictably. Furthermore, spores of high heat resistance usually require high activation temperatures. The spores of *C. botulinum* type E used in the present study appear to be more heat resistant than those used in other studies.
Similarly, the resistance of the spores included in the present study was unexpectedly high at 95°C, while resistance was observed to be the lowest at 90°C. This unusual D value for the highest temperature tested may be attributed to several factors. First, although care was taken to prevent "splashing" the crawfish paste along the sides of the test tubes, there is a possibility that small amounts of inoculated paste present on the sides of the tube provided a "dry" environment in which the spores were protected. It is known that spores are more resistant to "dry" heat than to "moist" heat. In addition water at 95°C is very close to boiling, and considerable losses due to evaporation take place. Although an effort was made to maintain the level of the water it is possible that portions of the crawfish paste inside the tube were not exposed to the heat for the entire duration of the experiment, again causing "dry heat" conditions.

Because data points at 80 and 95°C did not seem to conform to similar data available in the literature, the points were excluded from the thermal death time curve. Furthermore, their exclusion would circumvent the possibility of underprocessing because of a decrease in the z value (slope of the curve) if those points were included in the line.

When the D-values were plotted in the thermal death time curves, a downward trend was observed for at least
two points on each graph. Since the other two points were disregarded, a z value (the slope of the line) can be obtained. The z values vary depending upon the method of calculation used to obtain the D values, and were approximately 8, 12, and 14.5°C, respectively, when calculated by the methods of Stumbo (1948), Schmidt (1954) and Stumbo et al. (1950). The z-value obtained when the D-value was calculated by the method of Stumbo (1948) is in agreement with the z-value reported for C. botulinum type E by Russell (1982). Variability in z-values should not be considered unusual, because it is possible that the sporulation and recovery media utilized may affect the value (Russell, 1982).

Although high heat resistance for type E spores has not been reported, it is important to consider that thermal death time data for crawfish meat is not available. Unpublished data obtained by Scott (1986) indicate that shrimp and other seafood might lend a protective effect to type E spores.

**Determination of the thermal process**

A pasteurization process usually requires a heat treatment equal to four to seven times the D-value. This entails heating the product in the container until the cold point reaches the reference temperature, and holding that temperature for as much time as needed to complete a four to seven D-value.
Usual pasteurization processes applied to crabmeat by local processors involve the use of water at 85°C. Since the same equipment will probably be used for crawfish tailmeat processing, an adequate process at such temperature should be calculated. The $D_{80}$ obtained in this research ranged from 6.7 to 8.8 min. Thus, the suggested treatment for the crawfish tailmeat would range from 26.8 to 35.2 min for a 4D treatment and from 46.9 to 61.6 min for a 7D treatment.

Data observed in the present series of experiments indicate that an appropriate treatment would unfortunately result in darkening of the crawfish tailmeat to a point beyond what would be considered esthetically acceptable.

The results of this research suggest the need for additional research on the thermal resistance of type E spores in crawfish. The use of additional strains would be the primary recommendation. The use of smaller diameter test tubes would decrease the required amount of spores, as well as shorten the come up and thermal lag times. In addition, timing and sampling should be started as the tubes are dropped into the water bath. This might prevent those instances in which no endpoint was observed, that is, all spores were inactivated during the thermal lag period.
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She entered the University of Puerto Rico in August, 1974; obtained an Associate Degree in Chemical Engineering Technology in May, 1976; and obtained a Bachelor of Science degree in Medical Technology in August, 1979. She married Alberto Pantoja in July, 1980.

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Major Field: Food Science

Title of Dissertation: Determination of the Thermal Death Time of *Clostridium botulinum* Type E in Crawfish (*Procambarus clarkii*) Tailmeat

Approved:

[Signature]
Major Professor and Chairman

[Signature]
Dean of the Graduate School

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Date of Examination:

July 10, 1986