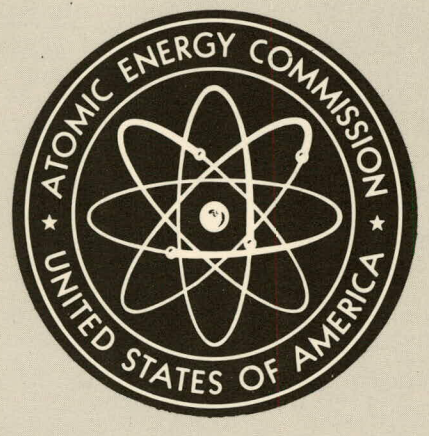
DWR-1582



COO-1183-32

GROWTH CHARACTERISTICS OF TYPE E CLOSTRIDIUM BOTULINUM IN THE TEMPERATURE RANGE 34 TO 50° F

Final Report, January 15, 1969—January 14, 1970

February 24, 1970

Metal Division Research and Development
Continental Can Company, Incorporated
Chicago, Illinois

UNITED STATES ATOMIC ENERGY COMMISSION · DIVISION OF TECHNICAL INFORMATION

DISCLAIMER

LEGAL NOTICE

Printed in USA. Price $3.00. Available from the Clearinghouse for Federal Scientific and Technical Information, National Bureau of Standards, U. S. Department of Commerce, Springfield, Virginia 22151.

USAEC Division of Technical Information Extension, Oak Ridge, Tennessee

COO-1183-32
BIOLOGY AND MEDICINE (TID-4500)

ANNUAL REPORT TO THE UNITED STATES

ATOMIC ENERGY COMMISSION

Growth Characteristics of Type E Clostridium botulinum in in the temperature Range 34 to 50°F.

FINAL REPORT

January 15, 1969 - January 14, 1970

February 24, 1970

Contractor Administrator: D. T. Maunder

Technical Collaborators: W. P. Segner
C. F. Schmidt
J. K. Boltz

Contract No: AT (11-1) 1183

TABLE OF CONTENTS

SUMMARIES OF PHASES XVII AND XVIII

Phase XVII

1. The results of a second inoculated pack study on fresh cod are presented. With incubation at 46°F and an inoculum level of 10^6 spores/g, unirradiated control samples showed Type E toxin development as early as 3/4 X (6 days). Comparable samples given 100 Krads radiation became toxic at 1/2 X (7 days) and samples receiving 200 Krads radiation were toxic at X (15 days), but not at earlier times. With a 10^4 inoculum level, toxin development in control samples was delayed until 1 1/2 X (12 days). In contrast, similar irradiated samples showed detectable toxin much earlier. Samples given 100 Krads radiation showed toxin in one out of three replicates at 3/4 X (10 days), while samples given 200 Krads showed toxin at X (15 days). None of the samples given 200 Krads radiation showed toxin development before X.

2. With incubation at 50°F, control samples inoculated with 10^4 spores showed Type E toxin at 3/4 X (6 days) in one out of three replicates. With 100 and 200 Krads radiation, toxin was detected at 1/2 X (7 days) and at 3/4 X (11 days), respectively. With a 10^2 inoculum level, control samples showed no toxin at X (8 days) but toxin was present in one out of three replicates at 1 1/4 X (10 days). In contrast, samples given 100 and 200 Krads radiation became toxic at 1/2 X (7 days) and 3/4 X (11 days).

Phase XVIII

1. Marine isolates of Cl. botulinum Type C designated as 6812, 6813, and 6814 showed excellent growth and good sporulation in Egg Meat Medium supplemented with $(NH_4)_2\ SO_4$, yeast extract, and glucose. Preheat treatments in the range 140 to 170°F for 15 minutes had no effect on the viable spore count for each strain. Preheating at 180°F produced a two-threefold reduction in count.

2. A comparison of the cultural characteristics of two non-marine Type C strains to those of three marine isolates showed some differences in their sugar fermentation ability. All of the strains fermented glucose. The non-marine strains fermented fructose and ribose weakly. None of the other sugars tested was fermented by the non-marine strains. In contrast, the marine strains actively fermented galactose and ribose; dextrin, inositol, maltose, melibiose, and starch were weakly fermented.

3. Some lots of sodium thioglycollate at a concentration of 0.1% and lower were inhibitory for the growth of Type C. L-cysteine hydrochloride at a concentration of 0.1% failed to show inhibition and was adopted as the reducing system for the Type C work.

4. As reflected by increased colony counts, the incorporation of $NaHCO_3$ had a beneficial effect in the recovery of Type C spores with Beef Infusion agar. In some cases, the beneficial effect of $NaHCO_3$ was offset by longer incubation.

5. The presence of Na_2HPO_4 at a concentration of 0.5% in Beef Infusion Agar for recovery of spores of Type C sometimes caused reduced colony counts. There was no advantage in the addition of phosphate to the recovery medium for making colony counts.

6. A comparison was made of the amount of Type C toxin produced by the marine and the non-marine strains in Jensen's Medium (Modified), Cardella's Medium, Beef Infusion Broth (no phosphate), and Egg Meat Medium. Egg Meat Medium containing $(NH_4)_2SO_4$, yeast extract, and glucose was the best medium for toxin production. The marine Type C strains produced appreciably higher toxin titers than the non-marine strains in all of the media tested.

7. There was no evidence of Type C toxin activation with trypsin like that occurring with Type E toxin.

8. The possible low temperature growth ability of the marine and the non-marine strains was determined. Ground haddock and Egg Meat Medium were used with incubation at 60, 55, 50, and 46°F. The marine strains showed growth at 60°F in both substrates, but no growth at lower temperatures. On the other hand, the non-marine strains showed growth at 60 and 55°F, but not below.

9. The marine and the non-marine Type C strains were very sensitive to low levels of sodium chloride. The non-marine strain 6810 and the marine strain 6814 showed growth with 2.5% salt, but not with 3.0%. The non-marine strain 6811 and the marine strains 6812 and 6813 were even more sensitive to salt with growth being inhibited by 2.5%.

10. The pH sensitivity of the marine and the non-marine Type C strains was studied in Beef Infusion broth with incubation at 85°F. The marine strains were much less pH sensitive than the non-marine strains. At an inoculum level of 2×10^6 spores per tube, the marine strain 6813 showed growth at pH 5.00 in three out of five replicate tubes, but no growth was observed at pH 4.91 during 6 months of incubation. The non-marine strains showed growth at pH 5.62, but not at pH 5.45.

11. The heat resistance of spores of the marine and the non-marine strains was determined in 0.067 M phosphate buffer at pH 7.0 by the partial spoilage technique. The recovery medium was Beef Infusion broth containing glucose, sodium bicarbonate, and L-cysteine hydrochloride. The spores of the non-marine strain 6810 showed the highest heat resistance, giving a D_{220} equal to 0.82 minutes. The slope of the thermal resistance curve for spores of 6810 was z equal 11.5°F. Spores of 6811 showed a D_{220} equal to 0.40 minutes and a z equal to 10.0°F. Spores of the marine strains 6812, 6813, and 6814 gave D_{220} values of 0.094, 0.028, and 0.020 minutes, respectively. The z-values ranged from 9.0 to 10.5°F.

INTRODUCTION

Public health safety is unquestionably paramount to all other considerations for the proposed application of low doses of ionizing radiation to extend the refrigerated storage life of certain seafoods. In this regard, Clostridium botulinum Type E has particular importance. Spores of Type E are known to be widely distributed in nature in a marine environment. In addition, Type E spores are quite resistant to ionizing radiation; therefore, doses of the order of 100 to 200 Krads, which currently are being considered for some marine products, would have no major destructive effect on Type E spores. These aspects, coupled with the fact that Type E can grow and produce toxin within the refrigeration temperature zone, accounts for our concern over this organism with low dose irradiated seafoods.

Inoculated packs were undertaken in our laboratory to assess the health hazard risk of Type E relevant to a marked extension of the refrigerated storage life of certain kinds of low dose irradiated fish. The procedures used in these inoculated packs followed those recommended by the Ad Hoc Committee on Botulism to the United States Atomic Energy Commission. This report finalizes the data for the inoculated pack on cod.

Besides Type E, there has been concern about the possible significance of Cl. botulinum Type C for low dose irradiated seafoods. This report also describes the growth characteristics of three Type C strains that were isolated in our laboratory from marine sediment samples.

Phase XVII

Toxin Development in Fresh Marine Product (Cod)

An experiment on cod was summarized in our annual report dated April 15, 1968. A second inoculated pack experiment on cod was run. The rationale for the inoculated pack to determine the margin of public health safety of radurized marine food products has been previously discussed. Briefly, the concept for such packs is an attempt to show that the low dose irradiated seafood poses no greater health hazard than does similar unirradiated product. Estimates of maximal product storage life ("X" values) for cod were determined by an untrained consumer-type panel at the Bureau of Commercial Fisheries Laboratory at Gloucester. Each "X" value represents the time for unanimous rejection of a sample by this panel. As has been previously stated, the occurrence and unquestionable recognition of spoilage by the consumer appreciably ahead of the earliest time of possible Type E toxin production would provide an adequate margin of public health safety. Because of the doubtful safety of cod given 100 and 200 Krads radiation with incubation at 50 and 46°F, the experiment was repeated.

The protocol followed in the second cod experiment was the same as in the preceding pack with the exception of a minor change in the toxin assay procedure. In the first experiment, samples were blended with an equal quantity of sterile distilled water and then further diluted 1:2 with sterile 0.5 M sodium acetate buffer (pH 5.0) for trypsin digestion. In this experiment, the samples were blended directly with the acetate buffer, thereby reducing the dilution factor twofold. The acetate blended samples were in the range of pH 5.5 - 5.8. Because this is somewhat higher than previously obtained (pH 5.2 - 5.5), the trypsin digestion

time at 98°F was reduced from 3 hours to 2 hours. All samples reported as being nontoxic failed to show toxin either with or without trypsin digestion by the acetate method.

In prior inoculated packs on haddock and cod, samples given 200 Krads radiation never showed nonspecific mouse deaths in assays for Type E toxin. This was not the case in the second cod experiment. Samples given 200 Krads radiation produced nonspecific deaths like those previously encountered with unirradiated and 100 Krads irradiated product (Segner and Schmidt, 1966). To avoid these nonspecific reactions, the antibiotics chloramphenicol and oxytetracycline were routinely injected.

Tables 17-1 through 17-4 present the inoculated pack results on cod. The discrepancies between our acceptance-rejection determinations and the BCF laboratory estimates of maximal storage life are undoubtedly related to the difference in our rejection criterion. While the BCF "X" values were based mainly on odor, the product appearance was also considered. In our laboratory, acceptance or rejection of a sample was based solely on odor and appearance was ignored. In some cases, our samples showed slime and mold growth without showing strong disagreeable odors.

From the toxicity data, there appears to be little advantage in toxin assaying without trypsin disgestion. Considerably more samples showed toxin with digestion than without. In addition, only one of the nontoxic

trypsinized samples showed toxin when reassayed without trypsin digestion. Similar results were obtained in prior inoculated packs.

Table 17-5 summarizes the toxicity data in terms of the earliest time for toxin production. In examining the 50°F results for a 10^4 inoculum level, the irradiated product appears to present no greater health hazard risk than the unirradiated product. However, for the 10^2 inoculum level, the irradiated samples were clearly toxic well ahead of the controls on the basis of equivalent X-values. With incubation at 46°F, the results are somewhat analogous to those obtained with incubation at 50°F. At the lower temperature with a 10^6 inoculum, both control and irradiated samples showed toxin at or before the maximal storage life estimates. However, with a 10^4 inoculum level, the control samples showed no toxin through 1 1/4 X (10 days), although toxin was present at 1 1/2 X (12 days). With a corresponding inoculum, the 100 and 200 Krads irradiated samples showed detectable toxin at 3/4 X (10 days) and at X (15 days), respectively.

Phase XVIII

Outgrowth Properties of Clostridium botulinum Type C in Marine Products

Three strains of Cl. botulinum Type C were isolated in our laboratory from marine bottom-sediment samples, as previously discussed (annual report dated April 15, 1968). The new isolates are designated 6812, 6813, and 6814. The objective of this work was to determine the possible low temperature growth ability of the marine strains and two non-marine strains, designated 6810 and 6811. The non-marine strains were originally isolated by Prevot from animals dead from Type C poisoning.

Little information is available concerning the low temperature growth ability of Type C. Michener and Elliott (1964) reviewed the minimal growth temperature for various organisms, but failed to find evidence in the literature of Type C growth below 50°F. Tanner et al. (1940) reported growth and toxin production from a spore inoculum at 50°F, but not at 41°F, in peas and asparagus. It is believed that all strains tested for low temperature growth ability have been from non-marine sources, although definite proof is unavailable.

There are valid reasons to suspect that marine Type C strains might possess the ability to grow in the refrigeration temperature zone (32-50°F). Surveys for Type E conducted along the Atlantic and Gulf of Mexico coasts by Dr. B. Q. Ward and along the Pacific coast by Dr. M. W. Eklund have shown that Type C is present in a marine habitat with Type E and the nonproteolytic Types B and F. It is known that Type E and the nonproteolytic Types B and F possess low temperature growth ability (Schmidt et al. 1961, Eklund et al. 1967a, and Eklund et al.

1967b). Since all known Type C strains are reportedly nonproteolytic and are similar in other respects to Type E and the nonproteolytic Types B and F, low temperature growth of marine Type C strains was suspected. Evidence to substantiate the low temperature growth ability of marine Type C strains would have considerable relevance to the public health aspects of refrigerated radurized seafoods.

Following the isolation of 6812, 6813, and 6814, spore suspensions were produced in Egg Meat Medium (Difco) supplemented with 1% concentrations each of $(NH_4)_2\ SO_4$, yeast extract (Difco), and glucose. Each strain showed good growth and sporulation. Peak sporulation was considered to occur after about 40 hours at 85°F, whereas, the nonmarine strains appeared to show peak sporulation after about 72 hours of incubation.

The viability of spores of the three suspensions expressed as a percentage of the refractile count with preheating (160°F, 15 min.) and without preheating is shown in Table 18-1. Beef Infusion Agar (See attached Appendix) with additions of filter sterilized $NaHCO_3$ (10%) and heat sterilized L-cysteine (20%) to give final concentrations of 0.14 and 0.10%, respectively, was used to standardize the suspensions. Spores of 6813 and 6814 showed good viability with and without preheating; however, spores of 6812 showed somewhat less viability, the highest percentage being obtained with a preheat treatment.

The effect of preheat temperatures in the range 140 to 190°F at 10°F intervals on the viability of spores of the marine strains was determined.

The heating time at each temperature was 15 minutes. The results are shown in Table 18-2. Spores of each strain showed no reduction in count through 170°F; however, at 180°F a considerable diminution in count was observed.

Table 18-3 summarizes the cultural characteristics of the marine and the non-marine strains. In general, the procedures used were those recommended by Dowell and Hawkins (1969). All of the reactions shown were obtained with incubation at 85°F. For the fermentation studies, Brewer's Agar Medium without thioglycollate, resazurin, or glucose was used. Andrade's indicator (Harleco) was employed. Each of 22 sugars tested were filter sterilized and added aseptically to the melted base medium to give a final concentration 0.5%. A deep-tube technique was selected. Based on their fermentative ability, the marine strains can be separated from the non-marine strains. Culturally, the marine strains appeared to be very similar to each other. The non-marine strains also showed no individual characteristic which would distinguish strain 6810 from 6811.

Some lots of sodium thioglycollate were inhibitory for Type C growth. In our initial work on Type C, the lot of thioglycollate being used showed no detectable inhibitory effect. However, subsequent lots from various suppliers all showed some degree of inhibition. The reducing compound L-cysteine hydrochloride produced no detectable inhibition as determined by comparative colony counts and refractile spore counts

made with a Petroff-Hausser chamber. The optimal concentration of L-cysteine (Table 18-4) was considered as 0.1%. In using L-cysteine, the precaution must be taken to compensate for the pH reduction caused by the addition of this compound to the growth medium. This was accomplished by the addition of sterile I N NaOH after the addition of the L-cysteine. Filter sterilized or heat sterilized (250°F for 10 min.) L-cysteine gave about the same colony counts. Heat sterilized L-cysteine was routinely used throughout the remainder of the work.

In making colony counts, the addition of $NaHCO_3$ to Beef Infusion Agar gave somewhat higher counts than when $NaHCO_3$ was omitted from the medium. This was particularly evident when comparative counts were made after 48 hours of incubation (Table 18-5). In some cases, longer incubation appeared to partially offset the beneficial effect from the addition of $NaHCO_3$. The counts suggested that the addition of $NaHCO_3$ was desirable for the maximal recovery of spores of Type C.

The composition of the Beef Infusion Agar medium used in making counts of Type C is given in the Appendix. For our usual Beef Infusion broth formulation, 0.5% $Na_2H\ PO_4$ is added. The presence of phosphate at this concentration was shown to greatly reduce the colony counts for spores of 6811, 6812, and 6813 (Table 18-6). For this reason, phosphate was deleted from the Beef Infusion Agar and from the broth formulation for the Type C work.

Cardella et al. (1958) and Jensen (personal communication) have used different media for the production of Type C toxin. The compositions

of their media are given in the Appendix. A comparison of the levels of toxin produced in Cardella's and Jensen's (modified) media, Beef Infusion broth, and Egg Meta medium is shown in Table 18-7. Egg Meat medium with 1% concentrations of $(NH_4)_2\ SO_4$, yeast extract, and glucose was the best medium for toxin production. The marine strains produced appreciably higher levels of toxin than the non marine strains. The toxin titers for the marine strains were as high as those produced by some Types A and B strains.

Savin (1966) reported that certain animal enzymes, including trypsin, were capable of activating Type C toxin to a greater potency. However, toxin produced by our non-marine and marine stains showed no evidence of toxin activation (Table 18-8). If Type C toxin from the marine or the non-marine strains is trypsin activatable, then the conditions for activation must be different from those used for Type E toxin activation.

Tables 18-9 and 18-10 show the results of two experiments to determine the low temperature growth ability of the marine and non-marine strains. The tests were conducted with ground haddock and with Egg Meat Medium containing $(NH_4)_2\ SO_4$, yeast extract and glucose at 1% concentrations. The substrates were dispensed in 20 X 150 m m screw cap tubes, with five-tube replicate sets being prepared for each experimental variable. The incubation temperatures studied were 60, 55, 50, and 46°F. For incubation at 60 and 55°F, the haddock substrate was autoclaved at 250°F for 15 minutes. For incubation at 50 and 46°F, the substrate was heated in flowing steam for 30 minutes. The Egg Meat Medium was autoclaved at 250°F for 15 minutes for each incubation temperature used. The

inoculum consisted of 2 x 10^6 spores/tube, based on a 160°F, 15 minute preheat prior to inoculation. After inoculation the tubes were vaspar sealed and incubated. The tubes were examined for the presence of gas periodically during incubation. The marine strians showed growth at 60°F, but not at lower temperatures in both substrates. On the other hand, the non-marine strains grew at 60 and 55°F, but not below. At the completion of incubation, the tubes showing no growth at the temperature just below that permitting growth were examined microscopically for growth, and each was assayed for the presence of Type C toxin. None showed detectable growth or toxin production.

Table 18-11 summarizes the NaCl sensitivity of the marine and the non-marine strains with Beef Infusion broth. Strains 6811, 6812, and 6813 showed growth in 2.0%, but not in 2.5% salt. Strains 6810 and 6814 tolerated only a slightly higher salt level with growth in 2.5% salt, but not in 3.0%.

The limiting pH for the growth of Type C at 85°F in Beef Infusion broth with and without glucose is shown in Tables 18-12 and 18-13. The inoculum level consisted of 2 x 10^6 spores per tube, with five-tube replicates per variable. Beef Infusion was adjusted to varying pH levels by the addition of diluted hydrochloric acid then sterilized at 250°F for 15 min. Immediately before pouring the medium, sterile L-cysteine (10%) was added to give a final concentration of 0.05%. The pH levels shown were those obtained after the addition of L-cysteine. After incubation for 180 days, uninoculated control tubes at each pH tested showed no perceptible change in the adjusted pH levels. While the pH levels between the two

experiments were not directly comparable, there was no suggestion that the presence or the absence of glucose in the medium had any effect on the minimal pH permitting growth. The marine strains grew at considerably lower pH levels than the non-marine strains, with 6813 and 6814 showing growth at a somewhat lower pH than 6812.

The heat resistance of spores of the marine and the non-marine strains in neutral phosphate buffer was determined. Sets of 10 replicate tubes (16 x 150 mm screw cap) per variable were inoculated with 0.1 ml per tube, equivalent to 1 x 10^6 spores. The inoculum level was based on a viable preheated count (160°F for 15 minutes). After inoculation, 0.9 ml of 0.067 M phosphate buffer at pH 7.0 was pipetted into each tube. Ten-tube replicate sets were heated to give partial spoilage endpoints. D-values were determined for each strain at three or four different temperatures. For heating temperatures of 215°F and above, thermal death time retorts were used. For temperature of 205°F and lower, a constant temperature water bath was used. In using the water bath, the tubes were completely submerged during heating. After heating, each tube was poured with Beef Infusion broth containing 1% glucose, 0.1% L-cysteine, and 0.14% $NaHCO_3$. Sterile I N NaOH was added to the medium prior to pouring to neutralize the addition of the L-cysteine. The poured tubes were vaspar sealed and incubated at 85°F. The tubes were examined for growth of survivors over an incubation period of not less than two months. D-values were calculated from the partial spoilage data (Stumbo, 1948). Table 18-14 through 18-18 summarize the partial spoilage data and the D-values for each time-temperature combination studied.

Thermal resistance curves were prepared by plotting the D-values versus temperature on three cycle semi-logarithmic paper. The curves are characterized by D_{220} values and z-values (slope of the curve in °F) tabulated in Table 18-19. As shown, spores of the marine strains were appreciably more heat sensitive than those of the non-marine strains. Among the marine strains, spores of 6812 showed the greatest heat resistance.

DISCUSSION

The inoculated pack data on cod incubated at 50 and 46°F appears to be quite unfavorable. At 50°F with a 10^2 inoculum level, Type E toxin was produced in samples given 100 and 200 Krads radiation at incubation times appreciably ahead of the BCF storage life estimates. Comparable unirradiated control samples showed toxin at 1 1/4 X (10 days), but not at earlier times. With incubation at 46°F and a 10^4 inoculum level, the irradiated samples became toxic at or before the maximal storage life estimates; the controls failed to show toxin development until 1 1/2 X (12 days). Below 46°F, the inoculated pack data obtained from a preceding experiment were much more favorable. At 42°F, samples inoculated with 10^6 spores and given 200 Krads radiation showed toxin development in one out of three replicates at 3/4 X (24 days). Samples given 100 Krads radiation and controls failed to produce toxin at 1 1/2 X values (39 and 13 days, respectively). With a 10^4 inoculum, only one out of three samples given 100 Krads radiation became toxic, but not until 1 1/2 X (39 days); the controls and the samples subjected to 200 Krads radiation remained nontoxic through 1 1/2 X. With incubation at 38°F, samples inoculated with 10^4 or 10^6 spore levels showed no detectable toxin through 1 1/2 X. We believe that negative toxin assay data with an inoculum of 10^4 spores/g for 1 1/2 X to twice the maximal storage life estimate would provide a sufficient margin of public health safety, even though a few occassional samples may show toxin development earlier with an inoculum level of 10^6 spores/g.

Our inoculated pack results on cod at 50°F are in compartively good agreement with those reported by Graikoski (1968). If the BCF estimates of maximal product storage life on cod are accepted, then Graikoski's data show toxin development at 50°F with 100 and 200 Krads radiation (10^6, 10^4, and 10^2 inoculum levels) at times preceding the storage life estimates. In contrast, unirradiated control samples did not show toxin development prior to the maximal storage life values. With incubation at 44.6°F, the inoculated pack results appeared more encouraging. None of the unirradiated or irradiated samples became toxic before the approximated storage life estimates. An approximation of the 44.6°F storage life was necessary, because actual BCF data were not secured at this specific temperature. Our results show that Type E toxin production in low dose irradiated cod might be possible before the maximal storage life estimates unless the product is handled and distributed at temperatures no higher than 42°F.

There is some evidence that low doses of radiation may promote Type E toxin development with incubation at low temperatures. Ajmal (1968) reported that aerobically packed herring and cod given 650 Krads radiation and incubated at 50°F were appreciably more susceptible to Type E toxin production than comparable unirradiated product. Our inoculated pack on haddock at 46 and 50°F showed that samples given 200 Krads radiation usually became toxic earlier than samples given 100 Krads radiation or not irradiated. (Annual report dated October 15, 1968.) However, the second inoculated pack on cod at 46° and 50°F showed little evidence that toxin was produced more rapidly with 200 Krads radiation than with

100 Krads radiation or without radiation. It is apparent that further work will be necessary before a definite conclusion can be drawn concerning the possible stimulatory effect of low doses of radiation on Type E toxin production.

Work on Type C has failed to show that marine strains are capable of growth at any lower temperature than non-marine strains. The results suggest that inoculated pack studies on Type C like those conducted on Type E will not be necessary.

Experiments involving the sodium chloride tolerance and the pH sensitivity of marine and non-marine strains of Cl. botulinum Type C were completed. It appears that Type C possesses less salt tolerance than the Types A, B, or E for which data are available. In Beef Infusion broth, the inhibitory salt level ranged from 2.5-3.0% for the five stains tested. Spores of the marine Type C strains tolerated lower pH levels then spores of the non-marine strains. Marine strain 6813 showed growth at pH 5.00, but not at pH 4.91. There is no indication that Type C will grow at any lower pH than that recognized as completely inhibitory for strains of the other toxin Types of Cl. botulinum.

Heat resistance studies on spores of the marine and non-marine strains showed they occupy a position intermediate with respect to the heat resistance of spores of Type A or B and those of Type E. In comparison to spores of the non-marine strains, spores of the marine strains were appreciably more heat sensitive.

SUMMARY OF ACCOMPLISHMENTS

1. Studies conducted during the first contract year (June 1962 to June 1963) were aimed at defining the conditions under which spores of Cl. botulinum Type E would germinate, leading to growth and toxin production at temperatures within the refrigeration temperature zone. During this period, much was learned about the preparation and the standardization of Type E spore suspensions. The Beluga, 8E, Alaska, and the Minneapolis strains were selected from among several possible test strains. Trypticase, Peptone, glucose broth (TPG) proved to be an excellent medium for sporulation of these strains. For standardization, 5% Peptone agar gave good recovery without the problem of gas production that had been previously experienced with TP agar and liver infusion agar media. Twenty-two commercially available protein hydrolysates and four complex formulated media were individually screened for their ability to support Type E growth at 46°F. The results showed that the composition of the growth medium had a significant effect on the spore outgrowth time (earliest time for visible gas production under a vaspar seal). Among the various media tested, Peptone and Trypticase - Peptone broths gave the shortest outgrowth times. Experiments using steamed ground haddock and sole substrates showed both supported rapid and good growth at temperature of 50, 46, and 42°F. Addition of 1% sodium chloride to a haddock substrate permitted Type E growth as low as 38°F, while tubes of haddock without salt failed to show growth.

2. During the contract year June 1963 to June 1964, studies were completed to determine the concentration of sodium chloride required for complete inhibition of Type E growth in the temperature range 46 to 85°F. In studies using TPG medium with incubation at 85, 70, and 60°F, 5% salt was necessary to completely inhibit growth. At 46° and 50°F, the concentration of salt required for inhibition was only slightly less (4.5%) than that shown at higher temperatures. With 4.0% salt, some extension of outgrowth time was observed.

The limiting pH permitting growth of Type E from a spore inoculum was determined with TPG medium. With an inoculum of 2×10^6 spores and incubation at 85°F, spores of one Type E strain showed growth at pH 5.21, but not at pH 5.10. With a tenfold higher inoculum level, the same strain showed growth at pH 5.03 in one out of five replicate tubes. With incubation at 46°F, the Beluga, 8E, Alaska, and Minneapolis strains grew at pH 5.9 but not at pH 5.7 with an inoculum of 2×10^6 spores per replicate tube.

In heat resistance studies, spores of the Minneapolis strain suspended in TPG medium showed a D_{176} equal to 1.9 min and a z-value of 16°F. In phosphate buffer, the thermal resistance curve was characterized by a D_{176} equal to 2.3 min and a z-value equal to 15°F. The respective D_{176} values for spores of the Beluga, 8E, and Minneapolis strains in phosphate buffer were 1.06, 1.38 and 0.93 min.

In addition to heat resistance studies on spores of Type E, the heat resistance of Type E toxin was also investigated. With a toxin titer

of 10,000 MLD/ml in 0.067 M phosphate buffer at pH 6.0, heating at 150°F yielded detectable toxin at 60 min., but none at 65 min. With a tenfold lower toxin level, the inactivation time was decreased about tenfold. Toxin inactivation proceded much more slowly in the pH range 5.2 to 5.6 than at pH 6.0 or 7.0.

3. During June 1964 to January 1966, several possible chemical food additives were screened for their inhibitory effects against Type E. Among the compounds showing, some inhibition in blended fish homogenate at 46°F were sodium benzoate, sodium nitrite, sodium parahydroxybenzoate (PHB), and the calcium-disodium and the disodium salts of ethylenediamine - tetraacetic acid (EDTA). Calcium propionate and potassium sorbate, each at 1,000 ppm concentrations, sodium citrate at 2,000 ppm, or sodium tripolyphosphate at 5,000 ppm failed to delay Type E growth. With radurizing doses of gamma radiation, benzoate, nitrite, PHB, and Na_2 EDTA showed some synergistic effects which delayed the growth of Type E.

The radiation resistance of spores of Type E in neutral phosphate buffer and in blended haddock homogenate was determined. The radiation survivors at varying doses were determined with Peptone-Gelatin medium and with incubation at 68 and 46°F. Survivor curves showed a pronounced "shoulder" during the first log cycle of reduction followed by exponential destruction. D-values for spores in haddock, based on colony counts at 68°F, ranged from 200 to 220 Krads. With incubation at 46°F, the D-values were about half those obtained at 68°F. D-values for spores in buffer, based on counts at 68°F, ranged from 80

to 110 Krads. Similar D-values for spores in buffer based on counts at 46°F were obtained. D-values determined from partial spoilage data and calculated for 10^6 inactivation were in close agreement to those shown by the exponential portion of the survivor curves.

4. Inoculated pack studies were initiated during the contract period January 1966 through April 1967. The purpose of these studies was to determine the public health safety of a low dose irradiated marine product in comparison to that existing in the unirradiated product. The work was undertaken with the cooperation of the Gloucester Bureau of Commercial Fisheries Laboratory. Estimates of maximal product storage life were determined by an untrained consumer-type panel at the BCF laboratory. The storage life estimates were designated as "X" and represent the time in days for unanimous rejection of a product by this panel. Similar product was shipped by BCF to our laboratory for the inoculated pack. Triplicate samples were prepared for each experimental variable. The samples were inoculated with 10^6, 10^4 or 10^2 Beluga spores/g. The samples were given 100 and 200 Krads radiation and incubated at 50, 46, 42, 40, or 38°F along with comparable inoculated unirradiated control samples. Toxin assays for Type E toxin were made at fractions and multiples of the BCF "X" values.

The first inoculated pack study was on haddock. At 40 and 42°F incubation, unirradiated controls and irradiated samples inoculated with 10^6 or 10^4 spores showed no toxin up to 2 X (twice the maximal expected storage life). At 46°F, samples inoculated with 10^6 spores and given 100 Krads radiation showed toxin at X, but not at earlier times; controls were non-toxic through 2X. With a 10^4 inoculum, control and 100 Krads irradiated samples with both inoculum levels (10^6 and 10^4) showed detectable toxin at 1/2X, the earliest time tested. At 50°F, control and 100 Krads irradiated samples with 10^4 and 10^2 inoculum levels were non-toxic up to X. However, 200 Krads irradiated samples showed detectable toxin at 1/2X and 3/4 X with 10^4 and 10^2 inoculum levels. While these data for haddock with 100 Krads radiation appeared encouraging, the results with 200 Krads were much less favorable.

In conducting the inoculated pack experiment on haddock, nonspecific toxicities were often encountered by the mouse assay test for Type E toxin. These nonspecific toxicities occurred only with unirradiated and 100 Krads irradiated samples. Nonspecific reactions were not observed with 200 Krads irradiated samples (although subsequent work on cod showed nonspecific toxicities with this radiation dose). The symptoms and the pattern of the nonspecific toxicities suggested that a septicemia was involved. Heart-blood cultured from mice showing nonspecific illness confirmed an infectious process. Among 23 isolates obtained, twelve were identified as species of the genus Proteus. The other isolates included two strains of Aerobacter aerogenes,

one Actinobacillus, three enterococci, one Alcaligenes marshalli, and four strains of Erysipelothrix insidiosa. The E. insidiosa isolates produced symptoms and deaths in mice resembling those seen in mice with typical nonspecific toxicities. It was shown that the E. insidiosa cultures were capable of growth as low as 40°F. This is the first known report of their low temperature growth ability. The nonspecific deaths in assays for toxin were avoided by the intraperitoneal injection of mice with the antibiotics chloramphericol and oxytetracycline.

5. An inoculated pack experiment on cod was completed during the next period (May 1967 through January 1968). Unirradiated controls and irradiated samples (100 and 200 Krads) inoculated with 10^6 and 10^4 spores and incubated at 38°F showed no detectable toxin up to 1 1/2 X, the longest times tested. With incubation at 42°F, the controls inoculated with 10^6 and 10^4 spores showed no toxin at 1 1/2 X. Samples with 10^6 spores and given 100 Krads radiation showed no toxin up to 1 1/2X, however, with a 10^4 inoculum, toxin was detected at 1 1/2 X in one of three replicate samples, but not at earlier times. Samples with 10^6 spores and given 200 Krads radiation showed toxin at 3/4 X and at X in one out of three replicates, yet failed to show toxin at longer times. With a 10^4 inoculum, none of the 200 Krads irradiated samples became toxic up to 1 1/2 X. With incubation at 46°F, control samples inoculated with 10^6 or 10^4 spores failed to become toxic through 1 1/2X. Samples inoculated with 10^6 spores and receiving 100 Krads radiation became toxic at

3/4X; with 10^4 spores, samples remained nontoxic through 1 1/2 X. Samples inoculated with 10^6 and 10^4 spores and given 200 Krads radiation showed toxin at 1 1/2 X and at X, respectively. In the latter case, only one out of three samples were toxic with a 10^4 inoculum. With incubation at 50°F, control samples inoculated with 10^4 and 10^2 spores failed to show toxin at X or at 1 1/2X. In contrast, the irradiated samples showed toxin at X, the earliest sampling time tested.

6. The inoculated pack experiment on haddock was repeated during the contract period January 1968 to January 1969. The results of the second experiment were quite similar to those obtained earlier. Samples given 100 or 200 Krads radiation showed toxin development at 50 and 46°F somewhat earlier than unirradiated control samples at about equivalent incubation times. When compared on the basis of equivalent X-values, the 200 Krads irradiated samples were clearly toxic before either the 100 Krads irradiated samples or the unirradiated controls.

In addition to the inoculated pack study on haddock, work was started on Cl. botulinum Type C. The purpose of this work was to determine the possible low temperature growth ability and growth characteristics of Type C strains originating from a marine environment. The results of the early work on Type C are briefly discussed in Phase XVIII of this report.

7. Accomplishments from January 1969 through January 14, 1970 which terminates this contract, are presented in the summary to this report.

Following is a list of published papers.

1. Segner, W. P., C. F. Schmidt, and J. K. Boltz. 1966. Effect of sodium chloride and pH on the outgrowth of spores of Type E Clostridium botulinum at optimal and suboptimal temperatures. Appl. Microbiol. 14:49-54.

2. Segner, W. P., and C. F. Schmidt. 1966. Radiation resistance of spores of Clostridium botulinum Type E. Proc. Intern. Symposium on Food Irradiation. Karlsruhe, Federal Republic of Germany. Intern. Atomic Energy, Vienna, Pages 287-298.

3. Segner, W. P., and C. F. Schmidt, 1968. Nonspecific toxicities in the mouse assay test for botulinum toxin. Appl. Microbiol., 16: 1105-1109.

4 Segner, W. P., and C. F. Schmidt. Inoculated pack studies on Clostridium botulinum Type E in unirradiated and irradiated haddock. Proc. UJNR Conference on Toxic Microorganisms. Honolulu, Hawaii. October 7-10, 1968. In Press.

5. Schmidt, C. F., and W. P. Segner, 1964. The bacteriology of Type E Clostridium botulinum. Proc. Sixteenth Research Conference, Research Council of the American Meat Institute Foundation. p. 13-19.

Papers in Preparation for Publication

6. Segner, W. P., C. F. Schmidt, and J. K. Boltz. Interaction of selected chemical additives and low-dose ionizing radiation against spores of Clostridium botulinum Type E.

7. Segner, W. P., and C. F. Schmidt. Isolation and Characterization of marine strains of Clostridium botulinum Type C.

8. Segner, W. P., and C. F. Schmidt. Low temperature growth and heat resistance studies on spores of Clostridium botulinum Type C.

APPENDIX

Media composition used for Type C; all figures are in grams/liter.

Beef Infusion Agar

1000 ml filtrate of beef infusion (1 lb. fresh beef/1)

NaCl 5g

Peptone (Difco) 10g

pH 7.4

$Na_2 HPO_4$ (5g), normally added, was not added for the Type C work.

Jensen's Medium (Modified)

Lactalysate (BBL) 30g

Yeast Extract (Difco) 20g (Substituted for Albimi Yeast Autolysate)

Glucose 10g (Jensen recommended 5g)

Na - citrate 3.5g

pH 7.3 - 7.4

Cardella's Medium

Proteose - Peptone (Difco) 40g

Trypticase (BBL) 20g

Yeast extract (Difco) 20g

Glucose 10g

pH 7.0

Egg Meat Medium (Difco)

Egg Meat 150g

$(NH_4)_2\ SO_4$ 10g

Yeast Extract (Difco) 10g

Glucose 10g

pH 7.4

References

Ajmal, M. 1968. Clostridium botulinum Type E: Growth and toxins production in food. J. Appl. Bacteriol. 31: 124-132.

Cardella, M. A., J. J. Duff, C. Gottfried, and J. S. Begel. 1958. Studies on immunity to toxins of Clostridium botulinum IV. Production and purification of Type C toxin for conversion to toxoid. J. Bacteriol. 75: 360-365.

Eklund, M. W., F. T. Poysky, and D. I. Wieler. 1967a Characteristics of Clostridium botulinum Type F isolated from the Pacific Coast of the United States. Appl. Microbiol. 15: 1316-1323.

Eklund, M. W., D. I. Wieler, and F. T. Poysky. 1967b Outgrowth and toxin production of nonproteolytic Type B Clostridium botulinum at 3.3 to 5.6C. J. Bacteriol. 93: 1461-1462.

Graikoski, J. T. 1970. Outgrowth of Clostridium botulinum Type E in radiation pasteurized fish. In M. Herzberg [ed.] Proc. First U.S. - Japan Conf. on Toxic Microorganisms. Unnumb. Pub. [In Press] U.S. Dept. of Interior and UJNR Panels on Toxic Microorganisms, Washington, D.C.

Michener, H.D., and R. P. Elliott, 1964. Minimum growth temperatures for food poisoning, fecal-indicator, and psychrophilic microorganisms, p. 349-396. In C. O. Chichester, E. M. Mrak, and G. F. Stewart [ed.], Advances in Foods Research 13, Academic Press, New York.

Savin, V. R. 1967. The influence of certain animal and fungal enzymes on the toxins of Clostridium botulinum Types A, B, C, and E., p. 258-263. In M. Ingram and T. A. Roberts, [ed.], Botulism 1966. Chapman and Hall, Ltd., London.

Schmidt, C. F., R. V. Lechowich, and J. F. Folinazzo. 1961. Growth and toxin production by Type E Clostridium botulinum below 40°F. J. Food Sci. 26: 626-630.

Segner, W. P., and C. F. Schmidt. 1968 Nonspecific toxicities in the mouse assay test for botulinum toxin. Appl. Microbiol. 16: 1105-1109.

Stumbo, C. R. 1948. A Technique for studying resistance of bacterial spores to temperatures in the higher temperature range. Food Technol. 2: 228-240.

Tanner, F. W., P. R. Beamer, and C. J. Rickher. 1940. Further studies on development of Clostridium botulinum in refrigerated foods. Food Res. 5: 323-333.

TABLE 17-1

Toxin development in unirradiated and irradiated cod inoculated with 10^4 spores of the Beluga type E strain and incubated at 50°F.

Dose Krads	X-Value[1]	Time (Days)	Rejection[2]	Toxin[3] Undigested	Toxin[3] Trypsinized
0	1/2	4	3/9	0/3	0/3
	3/4	6	9/9	0/3	1/3
	1	8	9/9	0/3	0/3
	1 1/4	10	9/9	0/3	2/3
	1 1/2	12	9/9	0/3	2/3
	2	16	9/9	0/3	2/3
100	1/2	7	4/9	0/3	2/3
	3/4	10	4/9	1/3	3/3
	1	14	0/9	3/3	---
	1 1/4	17	13/15	---	---
	1 1/2	21	13/15	---	---
	2	28	12/12	---	---
200	1/2	7	2/9	0/3	0/3
	3/4	11	0/9	1/3	3/3
	1	15	---*	3/3	---
	1 1/4	19	8/12	---	---
	1 1/2	24	7/9	---	---
	2	30	10/12	---	---

[1] Storage life values based on the Gloucester untrained, consumer-type panel estimates.

[2] No. samples rejected/No. possible rejections.

[3] Fraction of triplicate samples toxic.

* Result not determined.

2/24/70

TABLE 17-2

Toxin development in unirradiated and irradiated cod inoculated with 10^2 spores and incubated at 50°F.

Dose Krads	X-Value	Time (Days)	Rejection	Toxin Undigested	Toxin Trypsinized
0	1/2	4	4/9	0/3	0/3
	3/4	6	6/9	0/3	0/3
	1	8	6/9	0/3	0/3
	1 1/4	10	9/9	0/3	1/3
	1 1/2	12	9/9	0/3	0/3
	2	16	9/9	0/3	0/3
100	1/2	7	4/9	0/3	1/3
	3/4	10	6/9	0/3	2/3
	1	14	5/9	1/3	1/3
	1 1/4	17	14/15	1/3	3/3
	1 1/2	21	6/15	1/3	3/3
	2	28	12/12	---	---
200	1/2	7	4/9	0/3	0/3
	3/4	11	1/9	0/3	1/3
	1	15	---	2/3	2/3
	1 1/4	19	7/12	0/3	1/3
	1 1/2	24	8/9	2/3	3/3
	2	30	11/12	3/3	3/3

2/24/70

TABLE 17-3

Toxin development in unirradiated and irradiated cod inoculated with 10^6 spores and incubated at 46°F.

Dose Krads	X-Value	Time (Days)	Rejection	Toxin Undigested	Trypsinized
0	1/2	4	6/12	0/3	0/3
	3/4	6	4/12	0/3	3/3
	1	8	8/9	0/3	3/3
	1 1/4	10	7/9	0/3	2/3
	1 1/2	12	8/9	1/3	2/3
	2	16	9/9	0/3	3/3
100	1/2	7	1/9	0/3	3/3
	3/4	10	0/9	0/3	2/3
	1	14	5/9	1/3	2/3
	1 1/4	17	4/15	1/3	3/3
	1 1/2	21	12/15	---	---
	2	28	8/12	---	---
200	1/2	7	0/9	0/3	0/3
	3/4	11	0/9	0/3	0/3
	1	15	---	0/3	3/3
	1 1/4	19	5/12	---	---
	1 1/2	24	4/9	---	---
	2	30	9/12	---	---

TABLE 17-4

Toxin development in unirradiated and irradiated cod inoculated with 10^4 spores and incubated at 46°F.

Dose Krads	X-Value	Time (Days)	Rejection	Toxin Undigested	Toxin Trypsinized
0	1/2	4	2/12	0/3	0/3
	3/4	6	11/12	0/3	0/3
	1	8	8/9	0/3	0/3
	1 1/4	10	9/9	0/3	0/3
	1 1/2	12	9/9	0/3	1/3
	2	16	9/9	0/3	1/3
100	1/2	7	0/9	0/3	0/3
	3/4	10	0/9	0/3	1/3
	1	14	4/9	0/3	1/3
	1 1/4	17	4/9	0/3	3/3
	1 1/2	21	13/15	---	---
	2	28	9/12	---	---
200	1/2	7	0/9	0/3	0/3
	3/4	11	0/9	0/3	0/3
	1	15	---	0/3	2/3
	1 1/4	19	6/12	3/3	---
	1 1/2	24	5/9	---	---
	2	30	10/12	---	---

TABLE 17-5

Summary of toxin assay results on cod with incubation at 50 and 46°F.

Temp °F	Inoc	Krads	Inc Time (Days) No Toxin	Inc Time (Days) Toxin
50	10^4	0	4 (1/2)*	6 (3/4)
		100		7 (1/2)**
		200	7 (1/2)	11 (3/4)
	10^2	0	8 (1)	10 (1 1/4)
		100		7 (1/2)
		200	7 (1/2)	11 (3/4)
46	10^6	0	4 (1/2)	6 (3/4)
		100		7 (1/2)
		200	11 (3/4)	15 (1)
	10^4	0	10 (1 1/4)	12 (1 1/2)
		100	7 (1/2)	10 (3/4)
		200	11 (3/4)	15 (1)

* Values in parenthesis show fraction or multiple of X.

** Shortest incubation time tested.

TABLE 18-1

Yield and viability of spores of 6812, 6813, and 6814 produced in fortified Egg Meat Medium.

Strain	Refractile Count $X10^6$/ml Culture	Refractile Count $X10^6$/ml Stock	Viability as % of Refractile Count Unheated	Viability as % of Refractile Count Preheated
6812	85	510	26	41
6813	168	780	100	86
6814		840	83	86

Preheat = 160°F, 15 min. Viable counts based on Beef Infusion Agar containing 0.14% $NaHCO_3$ and 0.1% L-cysteine.

2/24/70

TABLE 18-2

Effect of preheating on the viability of spores of 6812, 6813, 6814.

Preheat Temp °F	Colony Count $X10^6$/ml		
	6812	6813	6814
None	130	800	700
140	200	780	780
150	170	760	760
160	210	680	720
170	260	580	700
180	120	160	160
190	50	2	2

Preheat time = 15 min. Counts are the averages of five-tube replicate sets per variable as determined with Beef Infusion agar containing 0.14% $NaHCO_3$ plus 0.1% L-cysteine.

2/24/70

TABLE 18-3

Cultural characteristics of non-marine and marine strains of Clostridium botulinum Type C at 85°F.

TEST	Non-Marine		Marine		
	6810	6811	6812	6813	6814
Dimensions (u)					
Vegetative Cells	1.2 x 6.0	1.7 x 5.0	1.0 x 5.0	1.4 x 5.0	1.2 x 5.0
Spores	1.0 x 1.4	1.0 x 1.2	0.9 x 1.5	1.0 x 1.6	0.6 x 1.2
Protein Hydrolysis					
Gelatin (12%)	+	+	+	+	+
Coag Egg White	-	-	-	-	-
Indole	-	-	-	-	-
Nitrate Reduction	-	-	-	-	-
Motility	-	-	-	-	-
Catalase	-	-	-	-	-
Egg Meat Medium					
Gas	+	+	+	+	+
Digestion	-	-	-	-	-
Sugar Fermentations					
Dextrin	-	-	(AG)	(AG)	(AG)
Fructose	(AG)	(AG)	-	-	-
Galactose	-	-	AG	AG	AG
Glucose	AG	AG	AG	AG	AG
Inositol	-	-	(A)	(A)	(A)
Maltose	-	-	(AG)	(AG)	(AG)
Melibose	-	-	(AG)	(AG)	(AG)
Ribose	(AG)	(AG)	AG	AG	AG
Starch	-	-	(AG)	(AG)	(AG)

* Adonitol, arabinose, dulcitol, glycerol, inulin, lactose, mannitol, raffinose, salcin, sorbitol, sucrose, trehalose, and xylose were not fermented.

A = acid; AG = Acid and gas; reaction in parenthesis shows a weak or delayed reaction (fermentation after 72 hours incubation); - means sugar not fermented during one month of incubation.

2/24/70

TABLE 18-4

Effect of sodium thioglycollate versus L-cysteine hydrochloride on the viable counts with Beef Infusion agar.

Reducing Cpd.	Conc. (%)	Unheated Count $x10^6$/ml				
		6810	6811	6812	6813	6814
Thio	0	28	90	38	66	180
	0.02	75	90	100	720	460
	0.05	<10	<10	120	520	380
	0.10	<10	<10	20	64	160
L-cysteine	0.02	91	42	150	740	620
	0.05	97	100	140	870	660
	0.10	110	180	150	760	880
	0.15	100	150	100	870	870

* Counts of stock suspensions based on BI agar containing 0.14% sodium bicarbonate with incubation at 85°F. Each count shown represents the average of a five-tube replicate set. Counts were made after 4 days and after about one month of incubation.

2/24/70

TABLE 18-5

Effect of the addition of sodium bicarbonate (0.14%) to Beef Infusion agar on the recovery of spores of Clostridium botulinum type C.

Suspension	Colony Count $x10^5$/ml*			
	No $NaHCO_3$		$NaHCO_3$ Added	
	48h	30 Days	48h	30 Days**
6810	60	50	103	90
6811	3	8	97	86
6812	1	34	93	81
6813	44	81	102	89
6814	22	103	115	105

* Counts based on a 160°F, 15 min. preheat. Counts are the averages of five-tube replicate sets. Suspensions used were standardized to contain 100 x 10^5 spores/ml.

** Reduced counts are due to the lysing of colonies after one month of incubation at 85°F.

2/24/70

TABLE 18-6

Effect of the addition of phosphate to BI agar on the viable counts of Type C spore suspensions.

	Colony Count $x10^5$/ml*	
Suspension	No Phosphate	Phosphate Added**
6810	98	71
6811	97	42
6812	93	34
6813	108	68
6814	103	94

* BI agar containing 0.14% $NaHCO_3$ and 0.1% L-cysteine. Counts are based on a 160°F, 15 min. preheat. Each count represents the average of five replicate tubes with incubation at 85°F for 48 hours.

** Sodium phosphate (Na_2HPO_4) concentration = 0.5%.

2/24/70

TABLE 18-7

Toxin production at 85°F by Type C in various media.

Medium[1]	Inc. Time (Days)	MLD Toxin x10^3/ml				
		6810	6811	6812	6813	6814
Jensen's	3	20	20	200	200	200
	6	100	50	500	500	500
	9	50	50	500	200	200
	30	50	50	500	500	500
Cardella's	3	100	100	200	200	500
	6	100	100	500	200	1,000
	9	200	200	1,000	500	500
	30	200	200	2,000	500	1,000
Egg-Meat	3	500	500	1,000	1,000	100
	6	500	500	1,000	1,000	500
	9	500	500	2,000	2,000	500
	30	500	500	5,000	2,000	2,000
BI	3	200	20	500	200	200
	6	<20	20	500	--	100
	9	20	20	100	200	200
	30	10	20	200	100	100

[1] Each medium contained 1% glucose. Sterile L-cysteine was added to Jensen's, Cardella's, and Beef Infusion (BI) medium to give a final concentration of 0.1%. Egg Meat Medium (Difco) was supplemented with 1% concentrations of $(NH_4)_2\ SO_4$ and yeast extract (Difco) in addition to glucose. Each medium was prepared in 150 ml quantity in a swrew-cap bottle and sterilized at 250°F for 15 min. The inoculum used consisted of 10^7 (unheated) spores/bottle.

2/24/70

TABLE 18-8

Effect of Trypsin on Type C toxin produced in Egg-Meat Medium

	MLD $x10^3$/ml	
Strain	Untrypsinized	Trypsinized[1]
6810	200	50
6811	500	500
6812	200	500
6813	100	200
6814	500	500

1. Trypsin digestion with 0.5% trypsin (Difco, 1:250) final concentration and incubation for one hour at 98°F. The cultures tested had been incubated at 85°F for three days.

TABLE 18-9

Effect of incubation temperature on the outgrowth time of spores of Cl. botulinum Type C in ground haddock.

	Avg. and Range of Outgrowth Time (Days)[1]				
Temp °F	6810	6811	6812	6813	6814
60	36(7-152)	7(5-7)	6(5-7)	6(5-7)	5(5-6)
55	23,25,25,25,>270	19	>270	>270	>270
50	>270	>270	>270	>270	>270
46	>270	>270	>270	>270	>270

Inoculum equaled 2 x 10^6 spores per replicate tube with a 160°F, 15 min preheat. Where fewer than five replicates showed growth, the outgrowth time is shown in parenthesis. All tubes were vaspar sealed.

2/24/70

TABLE 18-10

Effect of incubation temperature on the outgrowth time of spores of Cl. botulinum type C in Egg-Meat medium.

	Avg. and Range of Outgrowth Time (Days)[1]					
Temp °F	6810	6811	6812	6813	6814	6815
60	8(5-12)	6(5-9)	15(7-26)	7(5-9)	9(7-12)	7(5-9)
55	40(19-47)	31(28-33)	>250	>250	>250	>250
50	>250	>250	>250	>250	>250	>250
46	>250	>250	>250	>250	>250	>250

[1] See footnote Table 18-9. The medium contained 1% concentrations of $(NH_4)_2 SO_4$, yeast extract, and glucose.

2/24/70

TABLE 18-11

Effect of sodium chloride concentration on the outgrowth of spores of Cl. botulinum type C in Beef Infusion broth with incubation at 85°F.

	Avg. and Range of Outgrowth Time[1] (Days)				
% Nacl	6810	6811	6812	6813	6814
2.0	3	5(3-6)	29(6-48)	7(6-8)	4(3-6)
2.5	84,84,>200	>200	>200	>200	20,>200
3.0	>200	>200	>200	>200	>200

[1] See footnote Table 18-9.

TABLE 18-12

Effect of pH on the outgrowth time of spores of five Type C strains in Beef Infusion broth without glucose with incubation at 85°F.

	Avg. and Range of Outgrowth Time[1] (Days)				
pH	6810	6811	6812	6813	6814
5.62	4	4	4	4	4
5.45	>180	>180	4	4	4
5.30	>180	>180	4,4,4,7,>180	4	4
5.14	>180	>180	>180	4	4
5.00	>180	>180	>180	4,7,11,>180	>180
4.91	>180	>180	>180	>180	>180

[1] See footnote Table 18-9. Reducing agent was L-cysteine at a concentration of 0.05%. The inocula were not preheated.

TABLE 18-13

Effect of pH on the outgrowth time of spores of five Type C strains in Beef Infusion broth containing 1% glucose and with incubation at 85°F.

	Avg. and Range of Outgrowth Time[1] (Days)				
pH	6810	6811	6812	6813	6814
5.54	>180	>180	4	4	4
5.39	>180	>180	4,4,11,>180	4	4
5.25	>180	>180	4,6,6,6,>180	4	4
5.10	>180	>180	>180	4	4
4.94	>180	>180	>180	>180	>180
4.81	>180	>180	>180	>180	>180

See footnotes Tables 18-9 and 18-12.

TABLE 18-14

Heat Resistance of spores of 6810 in Neutral Phosphate Buffer[1]

Temp °F	Heating Time (Min)[2]	Partial Spoilage Data[3]	D-Value (Min)
215	12.0	10/10	--
	13.0	9/10	2.15
	14.0	9/10	2.31
	15.0	3/10	2.31
	16.0	5/10	2.54
	17.0	1/10	2.43
			Avg 2.35
220	4.0	10/10	---
	4.5	8/10	0.74
	5.0	8/10	0.82
	5.5	8/10	0.90
	6.0	1/10	0.86
	6.5	3/10	1.00
	7.0	0/10	--
			Avg 0.86
225	1.4	10/10	--
	1.6	4/10	0.25
	1.8	3/10	0.29
	2.0	2/10	0.30
	2.5	0/10	--
			Avg 0.28
230	0.4	10/10	--
	0.6	5/10	0.10
	0.8	0/10	--
	1.0	1/10	0.14
			Avg 0.12

[1] Recovery medium was BI broth with 1% glucose, 0.1% L-cysteine, and 0.14% $NaHCO_3$. Inoculum equaled 1 x 10^6 spores per tube with 10 replicates.

[2] Corrected for come up time.

[3] Fraction of tubes showing growth; minimal incubation time at 85°F was two months.

TABLE 18-15

Heat Resistance of Spores of 6811 in neutral phosphate buffer[1]

Temp °F	Heating Time (Min)	Partial Spoilage Data	D-Value (Min)
215	6.5	8/10	1.07
	7.0	3/10	1.07
	7.5	2/10	1.12
	8.0	1/10	1.14
	8.5	2/10	1.27
	9.0	0/10	---
			Avg 1.13
220	2.0	10/10	---
	2.5	5/10	0.04
	3.0	0/10	---
			Avg 0.40
225	0.60	6/10	0.10
	0.80	4/10	0.12
	1.00	0/10	---
			Avg 0.11

[1] See footnotes Table 18-14

2/24/70

TABLE 18-16

Heat resistance of spores of 6812 in neutral phosphate buffer[1]

Temp°F	Heating Time (Min)	Partial Spoilage Data	D-Value (Min)
205	12.0	10/10	---
	14.0	8/10	2.29
	16.0	7/10	2.58
	18.0	3/10	2.77
	20.0	1/10	2.85
			Avg 2.62
215	1.0	10/10	---
	1.2	9/10	0.20
	1.4	3/10	0.22
	1.6	3/10	0.25
			Avg 0.22
220	0.4	10/10	---
	0.6	4/10	0.09
	0.8	5/10	0.13
			Avg 0.11

[1] See footnotes Table 18-14

2/24/70

TABLE 18-17

Heat resistance of spores of 6813 in neutral phosphate buffer[1]

Temp °F	Heating Time (Min)	Partial Spoilage Data	D-Value (Min)
195	30.0	2/10	4.5
	35.0	1/10	5.0
	40.0	0/10	---
			Avg 4.8
200	14.0	7/10	2.26
	15.0	2/10	2.31
	16.0	1/10	2.29
	17.0	0/10	---
			Avg 2.29
205	4.5	1/10	0.64
	5.0	2/10	0.75
			Avg 0.69
215	0.2	5/10	0.03
	0.4	2/10	0.06
	0.6	1/10	0.09
	0.8	1/10	0.11
			Avg 0.07

[1] See footnotes Table 18-14

2/24/70

TABLE 18-18

Heat resistance of spores of 6814 in neutral phosphate buffer[1]

Temp °F	Heating Time (Min)	Partial Spoilage Data	D-Value (Min)
200	18.0	3/10	2.77
	20.0	5/10	3/17
	22.0	1/10	3.14
	24.0	0/10	---
			Avg 3.03
205	5.0	10/10	---
	5.5	7/10	0.89
	6.0	4/10	0.94
			Avg 0.91
215	0.2	10/10	---
	0.4	9/10	0.066
	0.6	0/10	---
			Avg 0.066

1 See footnotes Table 18-14

2/24/70

TABLE 18-19

Summary of thermal resistance characteristics of spores of non-marine and marine strains of Cl. botulinum Type C heated in phosphate buffer.

Strain	D_{220} Value (Min)	Z-Value (°F)
6810	0.182	11.5
6811	0.40	10.0
6812	0.094	10.5
6813	0.028*	10.5
6814	0.020*	9.0

* By extrapolation of the heat resistance curve obtained at lower temperatures.

2/24/70