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

Annual Report, January 15, 1968

October 15, 1968

Metal Division Research and Development
Continental Can Company
Chicago, Illinois

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ANNUAL REPORT TO THE
UNITED STATES ATOMIC
ENERGY COMMISSION

January 15, 1968

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Title: Growth Characteristics of Type E
Clostridium botulinum in the
Temperature Range 34 to 50 F.

October 15, 1968

TABLE OF CONTENTS

	<u>Page</u>
Summary Reports:	
Period I - January 15, 1966 to January 14, 1967	1
Period II - January 15, 1967 to January 14, 1968.	6
Period III - January 15, 1968 to January 14, 1969.	11
List of Publications:	14
Introduction:	15
Phase XVII - Toxin Development in Fresh Marine Products	16
Phase XVIII - Outgrowth Properties of <u>Clostridium botulinum</u> type C in Marine Products	21
General Discussion:	25
Appendix:	
Dosimetry procedures and results	27

SUMMARY REPORTS

Period I - January 15, 1966 to January 14, 1967.

During this period, aerobic inoculated pack studies were initiated to determine the potential health hazard of Clostridium botulinum type E in low dose irradiated fresh haddock. These studies were undertaken with the cooperation of the Gloucester Bureau of Commercial Fisheries Laboratory. Estimates of maximal expected product storage life were determined by an untrained consumer type panel at the Gloucester laboratory. Values of storage life are designated as "X" and represent the time for unanimous product rejection by this panel as judged largely by odor. Fresh haddock was shipped by Air Freight to Chicago and the inoculated pack was started. Triplicate samples were prepared for each variable. Samples were inoculated with either 10^6 , 10^4 , or 10^2 spores of the Beluga strain per gram. The samples were given 0.1 and 0.2 Mrad irradiation doses and incubated at either 50, 46, 42, 40, or 38 F along with inoculated unirradiated control samples. Toxin assays were conducted at fractions and multiples of X by the acetate toxin assay procedure. All negative assays were nontoxic without and with trypsin digestion.

The inoculated pack on haddock is summarized as follows:

1. At 50 F, unirradiated control samples inoculated with 10^4 vs. 10^2 spores showed no type E toxin up to 2X (16

days). Samples inoculated with 10^4 spores and given 0.1 Mrad irradiation showed no toxin development up to 2X (24 days); however, similar samples inoculated with 10^2 spores showed toxin in one out of three replicates at 2X, but not at earlier time periods. With 0.2 Mrad irradiation, samples inoculated with 10^4 spores showed toxin at 1/2X (10 days) and at each subsequent sampling time; samples inoculated with 10^2 spores showed toxin at X (20 days), but not at 1/2X.

2. At 46 F, the controls inoculated with 10^6 and 10^4 spores showed no toxin up to 2X (24 days). Samples given 0.1 Mrad irradiation also showed no toxin development up to 2X (26 days). However, samples inoculated with 10^6 spores and given 0.2 Mrad irradiation exhibited toxin in one out of three replicates at X (24 days). Corresponding samples inoculated with 10^4 spores and subjected to 0.2 Mrad irradiation were nontoxic through 2X (50 days).
3. At 42 and 40 F, all of the unirradiated and the irradiated samples inoculated with 10^6 and 10^4 spores failed to show toxin at times corresponding to 1/2X, X, and 2X.

The results summarized above were based on spores produced in TPG medium with incubation at 85 F. In work in ground fish substrates, there was a suggestion that Beluga spores produced at low temperatures showed somewhat faster outgrowth at 38 and 40 F than spores produced at 85 F. To obtain a satisfactory spore yield at 50 F, it was necessary to supplement TPG medium with 1% yeast extract. The highest spore yield at 50 F (40×10^6 /ml) was about half that usually obtained at 85 F.

The inoculated pack on fresh haddock, with incubation at 42 and 40 F, was repeated using spores of the Beluga strain produced at 50 F. Unirradiated and irradiated samples were toxin assayed at 3/4X, X, 1 1/2X and 2X. None of the samples showed detectable toxin at any time period. From these results, it was concluded that there is no reason to expect faster outgrowth or growth at any lower temperature from spores produced at suboptimal temperatures than from spores produced at 85 F.

Prior to the inoculated pack study on fresh haddock, considerable work was done on unsteamed ground haddock and cod substrates which were prepared from frozen product. Triplicate samples were run for each experimental variable. The samples were inoculated with

10^6 or 10^4 spores of the Beluga strain per gram. Samples were given 0.1 and 0.2 Mrad irradiation, vaspax sealed, and incubated at 46, 42, 40 F. With the 10^6 inoculum at each of these temperatures, the irradiated samples showed only a slight or no extension of time for toxin development over similar unirradiated controls. With the 10^4 inoculum, the irradiated samples showed no toxin formation after long incubation times. For example, no toxin was detected in any of the irradiated haddock samples incubated at 42 F up to 70 days or at 40 F up to 60 days, the longest time periods tested. With cod, the irradiated samples incubated at 42 F showed no toxin up to 47 days. Similar irradiated cod samples incubated at 40 F remained nontoxic up to 40 days. With 46 F incubation, samples inoculated with 10^4 spores and given 0.1 and 0.2 Mrad irradiation showed some delay for toxin development over comparable unirradiated controls.

There were notable differences between the times for toxin development in the samples incubated anaerobically and the samples incubated aerobically. Such differences might be explained by the fact that a gram-positive microflora predominated with anaerobic incubation, whereas a gram-negative flora predominated in the aerobic or semi-aerobic environment of these inoculated packs.

The acetate procedure to detect low levels of type E toxin showed greater sensitivity than the conventional phosphate buffer method with trypsin digestion. The acetate procedure, like the phosphate buffer method, employs trypsin digestion, but at a lower pH and for a longer time. Out of 78 samples of ground haddock and cod inoculated with 10^6 spores/ tube and incubated at 40 F, only 10 samples showed detectable toxin by the phosphate buffer procedure versus 30 toxic samples by the acetate procedure. Without trypsin digestion, 24 samples showed toxin.

Nonspecific toxicities were frequently encountered in toxin assays of unirradiated and 0.1 Mrad irradiated samples. Samples given 0.2 Mrad irradiation failed to produce nonspecific reactions. The symptoms and the pattern of these deaths suggested a septicemia. Subcultures of heart blood from mice showing nonspecific symptoms indicated an infectious process. Among 23 pure culture isolates from the blood, eight were identified as Proteus vulgaris, two Proteus morganii, one Proteus rettgeri, one Providence Subgroup B, two Aerobacter aerogenes, one Actinobacillus, three Enterococci, one Alcaligenes marshalli and four Erysipelothrix insidiosa. The E. insidiosa, aerobacter, Providence and most of the Proteus isolates produced deaths when injected intraperitoneally into white mice. The other isolates failed to cause mouse deaths and probably represent secondary invaders. The four E. insidiosa

showed growth in the temperature range 50 to 40 F; this is the first known report of their low temperature growth ability. The nonspecific toxicities were avoided by protecting the mice with the antibiotics Chloramphenicol and Oxytetracycline.

Finally, during this contract period work was completed on the resistance to cobalt-60 irradiation of spores of the Beluga strain in TPG medium and blended haddock homogenate. The irradiation D-values of spores in TPG medium closely paralleled those in haddock. With both substrates, D-values from the exponential portion of the survivor curves equalled 0.21 Mrad. Calculated from 10^6 inactivation, the values of spores in haddock and TPG medium equalled 0.23 and 0.25 Mrad, respectively. The log values (dose for the survivor curve to cross the first log cycle of reduction) equalled 0.39 and 0.45 Mrad for spores in haddock and TPG medium, respectively. The results suggested that TPG medium may be a suitable substrate to simulate marine or other food products for preliminary determination of the irradiation resistance of spores of other type E strains.

Period II - January 15, 1967 to January 14, 1968.

The only method currently available to detect botulinum toxins is the mouse bioassay test. Improvements in this test have been sought which might be faster and more sensitive than the

conventional assay procedure. Use of the acetate toxin assay procedure has shown somewhat greater sensitivity than the common phosphate buffer procedure. During this period, some preliminary work started to determine the sensitivity of the immunodiffusion technique to detect botulinum toxins. The reaction of type E toxin of the Beluga and Minneapolis strains was tested against antiserum from the Pasteur Institute by employing the plate double diffusion method. Visible precipitin bands failed to develop with this antiserum. However, when Porton type E antiserum was used, weak bands were recognizable. Both the Pasteur and Porton antisera are of equine origin. Further work is planned using antiserum of rabbit origin.

During this contract period, an inoculated pack experiment on cod was completed. The procedures used were identical to those described for the haddock experiment (Period I). Triplicate samples for each variable were inoculated with spores of the Beluga strain and given 0.1 and 0.2 Mrad irradiation. The irradiated and inoculated unirradiated controls were incubated at 50, 46, 42, and 38 F. The toxin assay results are summarized as follows:

1. At 38 F, the controls and all of the irradiated samples inoculated with 10^6 and 10^4 spores showed no detectable toxin up to 1 1/2X, the longest incubation times tested.
2. At 42 F, the controls inoculated with 10^6 and 10^4 spores showed no toxin development up to 1 1/2X (29 days). Samples inoculated with 10^6 spores and given 0.1 Mrad irradiation failed to show toxin up to 1 1/2X (39 days); however, with a 10^4 inoculum, similar samples showed toxin in one out of three samples at 1 1/2X, but not at earlier time periods. Samples inoculated with 10^6 spores and given 0.2 Mrad irradiation showed toxin at 3/4X and X, 24 days and 31 days, in one out of three replicates. At 1 1/2X (46 days), none of the samples showed detectable toxin. With a 10^4 inoculum, all of the 0.2 Mrad irradiated samples remained nontoxic through 1 1/2X.
3. At 46 F, the control samples inoculated with 10^6 and 10^4 spores showed no toxin development up to 1 1/2X (13 days). Samples inoculated with 10^6 spores and given 0.1 Mrad irradiation were toxic at 3/4X (14 days), whereas

comparable samples inoculated with 10^4 spores failed to show toxin up to 1 1/2X (26 days). Samples inoculated with 10^6 spores and given 0.2 Mrad irradiation showed toxin at 1 1/2X (35 days); corresponding samples inoculated with 10^4 spores showed toxin in only one out of three replicates at X (24 days).

4. At 50 F, samples inoculated with 10^4 and 10^2 and given 0.1 and 0.2 Mrad irradiation doses showed toxin at X (13 days and 18 days, respectively). Toxin assays were not conducted before X. In contrast, the unirradiated samples inoculated with 10^4 and 10^2 levels failed to show toxin at X (6 days) and 1 1/2 X (9 days).

Towards the end of this contract period some introductory work on C1. botulinum type C was started. Various marine sediment samples reported to contain type C were obtained from Dr. B. Q. Ward, of the University of Florida. Since little is known concerning the low temperature outgrowth ability of spores of type C, attempts were made to isolate pure type C cultures. Our initial efforts were directed toward the development of a suitable sporulation medium. It was reasoned that the possibility of isolating pure type C cultures might be greatly improved if sporulation could be induced. Various media formulations were screened against two stock type C cultures (468-C and 571-C) that are very difficult to sporulate.

Among 13 media examined, Egg Meat Medium (Difco) supplemented with Yeast Extract (Difco), ammonium sulphate, and glucose, each at 1% concentration, gave good sporulation and satisfactory spore yields. For example, strain 468-C gave a refractile spore count equal to $65-80 \times 10^6/\text{ml}$ after three days of incubation at 85 F. The viability of this spore suspension, as determined with Beef Infusion Agar and 85 F incubation, was 88% of the refractile count. Preheat temperatures in the range 140 to 170 F for 15 min produced only a slight reduction in the viable count; however, temperatures at 170 and 180 F reduced the count appreciably.

The effect of trypsin on type C toxin of strains 468-C and 571-C was determined with conditions commonly used to activate type E toxin. No suggestion of type C toxin activation was observed. On the contrary, trypsin digestion of toxin of 468-C produced a substantial reduction in toxicity. No significant change in toxicity occurred with digestion of toxin of 571-C.

Finally, during this period various sporulation media were tested against 17B and 202F, nonproteolytic strains of C. botulinum. The B and F strains were obtained from Dr. M. W. Eklund of the Seattle Bureau of Commercial Fisheries Laboratory. Sporulation was best in Eklund's cooked meat medium. Substantially higher spore yields were obtained at 70 F than at 85 F. A comparison of

several media for viable spore counts showed that Beef Infusion Agar or 5% Peptone Agar plus 1% Yeast Extract with incubation at 70 F gave the highest count. Preheating at 140 F for 15 min neither activated nor caused any reduction in viable count.

Period III - January 15, 1968 to January 14, 1969.

The inoculated pack results on haddock summarized in Period I suggested that 0.2 Mrad irradiated samples were more susceptible to type E toxin development than either unirradiated or 0.1 Mrad samples. To determine the validity of the data at 46 and 50 F, a second experiment was run. The procedures used were the same as previously described with the exception that each inoculum level was preheated at 140 F for 15 min. In the first haddock experiment, only the 10^6 level was preheated. The toxicity data is summarized below:

1. At 46 F, unirradiated controls inoculated with 10^6 spores showed no toxin at each sampling period up to 4X (48 days). Similar samples given 0.1 Mrad irradiation were toxic at X (12 days), but not at 3/4X (9 days). Samples given 0.2 Mrad irradiation showed toxin at 1/2X (12 days), the earliest time tested. With 10^4 spores, the controls showed no toxin up to 3 1/3X (40 days). Samples subjected to 0.1 Mrad irradiation showed no toxin at times up to 2X (26 days), the longest time tested. Samples given

0.2 Mrad irradiation showed toxin in one out of three replicates at 1/2X and 3/4X (12 and 18 days, respectively).

2. At 50 F, the controls inoculated with 10^4 spores showed toxin at 1 1/2X (12 days), but not at X (8 days). Toxin was absent at 24, 30 and 40 days, suggesting the possibility of toxin destruction. With 0.1 Mrad irradiation, none of the samples showed toxin up to 2X (24 days). In contrast, with 0.2 Mrad irradiation, toxin was present at 1/2X (10 days). With 10^2 spores, the control samples failed to show toxin up to 4X (30 days) the longest time tested. Corresponding samples given 0.1 Mrad irradiation showed toxin in one out of three replicates at 1 1/2X (18 days); all of the other samples were nontoxic. Samples given 0.2 Mrad irradiation showed toxin at 3/4X (15 days), but not at 1/2X (10 days).

The X-values for control and irradiated product in most cases are not based on equivalent incubation times. Nevertheless, 0.1 and 0.2 Mrad samples incubated at 50 and 46 F seemed to show type E toxin production somewhat earlier than unirradiated samples. When compared on the basis of equivalent X-values, the 0.2 Mrad irradiated samples definitely showed toxin formation ahead of either the unirradiated or the 0.1 Mrad irradiated samples. The

results with 0.1 Mrad irradiation appear quite encouraging for haddock, but the data with 0.2 Mrad irradiation are considerably less favorable.

An inoculated pack experiment on haddock also was run at 60 F with 10^2 spores and 10^1 spores of the Beluga strain/g. For each experimental variable, type E toxin was detected at 1/2X without and with irradiation.

Work started on C1. botulinum type C during the preceding period was continued. Strain 571-C showed good growth and sporulation in the Egg Meat Medium used to sporulate strain 468-C. Spore suspensions of the two type C strains showed good viability or high counts in Beef Infusion agar as compared to the refractile spore counts. Experiments on the resistance to heat of spores of 571-C gave results similar to that for spores of 468-C; both showed considerable heat resistance up to 170 to 180 F.

The Egg Meat formulation used to sporulate 468-C and 571-C was used to recover type C subcultures from marine sediment samples supplied by Dr. B. Q. Ward. More of the subcultures showed type C toxin in connection with a preheat of 160 F for 15 min than without any preheat. Two pure type C cultures were isolated from the sediment samples, one from New Dorp Lane, Staten Island, New York and one from Yacht Club, Edgewood, Maryland.

Following is a list of papers published during the last three contract periods:

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.

INTRODUCTION

Low doses of ionizing radiation or so called radurization doses in the range 0.1 to 0.2 Mrad when combined with adequate refrigeration cause a considerable extension of storage life of many seafoods. However, a marked extension of storage life might create a health hazard problem due to Clostridium botulinum type E. Ecological surveys for Cl. botulinum type E have definitely established the presence of the organism in a marine environment. It is also known as a result of work in our laboratory that radiation doses of the magnitude being considered have little significant destructive effect on spores of this organism. Moreover, the ability of type E to grow and to produce toxin at temperatures well within the refrigeration zone has been sufficiently documented in our laboratory and elsewhere. The preceding statements indicated the necessity of definitive experiments to properly assess the risk of type E toxin development in various seafoods of interest in the United States Atomic Energy Commission marine products program. The major portion of our work during the last three years has concerned inoculated pack studies to determine the importance of this public health problem.

Phase XVII

Toxin Development in Fresh Marine Products (Haddock).

The inoculated pack experiment on haddock discussed in our annual report for January 15, 1966 to April 30, 1967 was repeated. The results of the first experiment suggested that 0.2 Mrad irradiated samples were somewhat more susceptible to type E toxin production than either 0.1 Mrad irradiated or unirradiated samples. To determine the validity of these results, a second inoculated pack was run. The protocol followed in the second experiment was the same as in the first with the exception that each inoculum level was preheated at 140 F for 15 min.

A new spore suspension of the Beluga strain was produced in TPG medium with incubation at 85 F. Toxin assays were made at 1/2X, 3/4X, X, 1 1/2X and 2X. Each sample was assayed with and without trypsin digestion by the acetate procedure. The antibiotics chloramphenicol and oxytetracycline were used routinely to avoid nonspecific mouse deaths. Each mouse was given two intraperitoneal injections of the antibiotics, one about 20 hr before injection of the fish sample and the second with injection of the sample. Each mouse was given 0.1 ml volume containing 5.0 mg of Chloromycetin and 0.625 mg of Terramycin. The antibiotics were not used with samples given 0.2 Mrad because nonspecific reactions failed to occur

A total bacterial count was made on the incoming haddock fillets and on the samples immediately after 0.1 and 0.2 Mrad irradiation (Table 17-1). No attempt was made to follow the changes in the total count with incubation time, as was done in the first inoculated pack experiment on haddock. Irradiation with 0.1 and 0.2 Mrad doses resulted in a reduction in count equal to about 94% and 98%, respectively.

Spoilage of the unirradiated and the irradiated haddock samples was characterized by the development of strong putrid odors. In addition, many of the samples showed obvious slime growth. The 0.2 Mrad samples also exhibited considerable mold growth as spoilage developed, whereas no mold growth was seen in either unirradiated or 0.1 Mrad irradiated samples (Table 17-2).

Tables 17-3 through 17-6 show the inoculated pack data. The tables are arranged to show the irradiation dose, the incubation time and the corresponding value of X, the pH range of triplicate samples, our spoilage rejection determinations, and the toxin assay results without and with trypsin digestion. All of the samples showed a rapidly increasing pH, reaching more than pH 8.0 in many cases. Usually, the samples were rated as rejected by our staff at the

maximum storage life estimates determined by the BCF consumer type panel. Our rejection determinations were based solely on odor. The rejection determinations of the BCF Laboratory were based largely on odor, but undoubtedly the panel's judgements were influenced by the product appearance as well.

The toxin assay results with 0.1 Mrad irradiation and 50 and 46 F incubation appear favorable, since no toxin was detected at times up to twice the maximum storage life with a 10^4 inoculum level.

At 46 F, toxin was detected at X with a 10^6 inoculum level, but not at earlier sampling periods. As previously discussed, we believe that negative toxin assay results with 10^4 spores/g for twice the maximum product storage life would be ample evidence of the absence of a type E hazard, even though occasional toxin production might be detected earlier with a challenge dose of 10^6 spores/g.

The results with 0.2 Mrad irradiation are less favorable than those without irradiation or with 0.1 Mrad irradiation. Samples inoculated with 10^6 and 10^4 spores, irradiated at 0.2 Mrad and incubated at 46 F showed toxin at 1/2X, the earliest time period tested. With 50 F incubation, samples inoculated with 10^4 and 10^2 spores and given 0.2 Mrad irradiation were toxic at 1/2X.

and 3/4X, respectively.

Examination of the toxicity data shows some evidence of destruction of type E toxin. For example, unirradiated samples inoculated with 10^4 spores and incubated at 50 F (Table 17-3) showed toxin development at 1 1/2X (12 days). Similar samples examined at 2X (18 days) and at 2 1/4X (18 days) also showed the unquestionable presence of toxin. However, samples assayed at 3X (24 days) and at even longer incubation times were nontoxic. Samples given 0.2 Mrad and incubated at 46 F seemed to show a trend that might be interpreted as an indication of toxin destruction (Tables 17-5 and 17-6). The cause of this apparent loss in toxicity is not known. It might be caused either by the proteolytic enzymes of the fish or by microbial degradation by the normal flora.

From the results shown in Tables 17-3 to 17-6, there appears to be little advantage in toxin assaying simultaneously with and without trypsin digestion. Out of 180 samples assayed both ways, 32 showed detectable toxin with trypsin digestion versus 13 without digestion. In only two cases, toxin was detected without digestion from samples that failed to show toxin with digestion. These results suggest the desirability of first assaying with digestion and then retesting all apparently nontoxic samples without digestion.

Table 17-7 summarizes the toxicity data on the second haddock experiment. In most cases, the results are not based on equivalent incubation times. However, 0.1 and 0.2 Mrad irradiated samples seemed to show toxin development somewhat earlier at 50 and 46 F than comparable unirradiated samples. When compared on the basis of nearly equivalent values of X, 0.2 Mrad irradiated samples definitely showed toxin formation earlier than either 0.1 Mrad irradiated or unirradiated samples.

An inoculated pack on haddock also was conducted at 60 F with 10^2 and 10^1 spores per gram. The results are shown in Tables 17-8 and 17-9. For each variable, toxin was detected at incubation times corresponding to $1/2X$. The results suggest that at 60 F and at higher temperatures of abuse, irradiated product presents no greater health hazard than unirradiated product.

Phase XVIII

Outgrowth Properties of Clostridium botulinum

type C in Marine Products

Little is known concerning the possible low temperature outgrowth ability of spores of Clostridium botulinum type C. Work is in progress to isolate pure type C cultures from the marine sediment samples collected by Dr. B. Q. Ward. These samples were taken at various locations along the Atlantic seaboard and the Gulf coastal area in a survey for Cl. botulinum type E. As previously discussed in our annual report for the period May 1, 1967 to January 31, 1968, our initial efforts were aimed toward developing a suitable medium to permit satisfactory sporulation of two type C strains that are difficult to sporulate. These strains are designated 468-C and 571-C. Among various media screened, good growth and adequate sporulation were obtained in Egg Meat Medium (Difco) supplemented with yeast extract, ammonium sulphate, and glucose each at 1% levels. Spore yields were highest after three days of incubation at 85 F.

Spore suspensions of 468-C and 571-C were standardized with Beef Infusion (BI) agar. Sodium bicarbonate and sodium thioglycollate were used at respective concentrations of 0.14%

and 0.1%. Based on the refractile spore counts, the viability of spores of both strains was high in BI agar (Table 18-1).

The effect of preheat treatments in the range 140 to 190 F for 15 min was determined for spores of the two type C strains (Table 18-2). Spores of 468-C and 571-C showed considerable heat resistance up to 170 and 180F.

The fortified Egg Meat Medium was used to obtain cultures containing type C from Ward's sediment samples. The medium was prepared in 20 x 150 mm screw cap tubes. A duplicate set of tubes with three to five tubes per set was inoculated for each sediment sample. Each tube was inoculated with 1 to 1.5 g of the sample. One set of tubes was preheated at 160 F for 15 min to eliminate the nonsporeforming microflora, while the other set of tubes was not preheated. Both sets of tubes were incubated at 85 F for 3 days and assayed for type C toxin. More cultures showing type C toxin were obtained with preheating than without (Table 18-3). Moreover, higher toxin titers were obtained with preheating.

Sediment sample 613 was collected at New Dorp Lane, Staten Island, New York. One of the preheated tubes contained 10,000 MLD of type C toxin/ml. Sample 616 was taken at Yacht Club, Edgewood, Maryland. From this sample, one of the preheated tubes showed 50,000 MLD of toxin. Sample 345 was taken at Lagund Madre, Corpus Christi, Texas. With a preheat, seven of the eight tubes of sample 345 contained type C toxin, but only one tube showed 10,000 MLD of toxin; the other type C cultures showed less than 2,000 MLD of toxin.

Two type C isolations were made, one from sediment sample 613 and one from sample 616. The preheated culture of sample 613 showed an appreciable increase in toxicity after two successive transfers to Egg Meat Medium. With the preheated culture of sample 616, a marked increase in toxicity occurred after the first transfer (Table 18-4). These high toxin levels suggested a substantial increase in the number of type C cells and possibly in the number of spores of these cultures. (Table 18-4). The culture of sediment sample 345 showed a moderate increase in toxicity after the first transfer, but no further increase after additional transfers. Recently, the last transfer was inoculated into fresh Egg Meat Medium and preheated at 190 F for 30 min. After 3 days of incubation, the culture showed 500,000 MLD of type C toxin/ml. Work is now in progress to obtain a pure type C isolate from high toxin titer culture.

A deep tube technique was used to obtain the purified type C cultures. Tubes measuring 6 mm (O.D.) and about 4 inches in length were prepared from glass tubing. Rubber stoppers were inserted into both ends. These tubes were sterilized at 250 F for 15 min. A preliminary deep tube viable count was made on the culture transfer with the highest toxin titer. Using appropriate dilutions to give one or two colonies per tube, 30 to 40 rubber stoppered tubes were inoculated. The inoculated tubes were poured with about 6 ml of BI Agar medium containing 0.14% sodium bicarbonate and 0.1% sodium thioglycollate. After solidification of the medium, the tubes were incubated at 85 F for about 48 hours. Well isolated colonies were picked into Egg Meat Medium. In some cases, a colony was picked directly from a tube; however, colonies usually were picked after removal of each cylinder of agar to a sterile petri dish. The inoculated tubes of Egg Meat Medium were incubated at 85 F for 3 days. Tubes showing blackening were discarded. Tubes not showing blackening were tested for the presence of type C toxin. Each type C isolate was subjected to a second purification step using the deep tube technique.

GENERAL DISCUSSION

A continuation of the cooperative study between the Bureau of Commercial Fisheries Laboratory at Gloucester and our laboratory is planned. The inoculated pack experiment on cod described in our preceding annual report will be repeated with more frequent toxin assay determinations. In addition, the inoculated pack concept will be extended to determine the public health safety of other marine food products with respect to C1. botulinum type E. Among such seafoods having possible importance in the AEC marine products program are picked blue crab meat, clams, and scallops. The order in which these products are examined will depend upon the preference of the AEC. The protocol followed in the inoculated pack experiments on haddock and cod appears to be the best approach to assess the health hazard risk of C1. botulinum type E growth and toxin production.

Further work on C1. botulinum type C is planned for the proposed contract period January 15, 1969 through December 31, 1970.

Further attempts will be made to isolate pure type C cultures from marine sediment samples representing different geographical areas of the United States. Each isolate will be screened for its ability to grow and to produce toxin in a fish substrate in the refrigeration temperature zone.

During the contract period January 15, 1967 to January 14, 1968 work was done on the nonproteolytic types B and F strains of C1. botulinum and on the immunodiffusion technique to detect botulinum toxins. While no work was done in these two areas during the present contract year, additional research is planned. Experiments will be conducted to compare the low temperature outgrowth ability of spores of nonproteolytic types B and F to that of spores of type E. Work on the immunodiffusion technique will involve the determination of the sensitivity of the method as compared to the conventional mouse assay test.

IIT Research Institute
10 West 35 Street, Chicago, Illinois 60616
312/225-9630

June 24, 1968

Dr. Wayne Segner
Continental Can Company
12000 West 76th Street
Chicago, Illinois 60620

Dear Dr. Segner:

With the enplacement of the three U.S. Nuclear cobalt 60 sources dosimetry profiles have been determined within the geometry of the refrigerated sample holding apparatus designed by Continental Can Company. The dosimetry method was the Ferric Sulfate gamma irradiation technique as used in previous determinations. Irradiation counters and the chemical dosimetry substantiated that the three sources are evenly matched at 1,730 curies each.

Irradiation profiles were determined for the outer ring cannisters holding three-tier stacked 3 oz. jars which contained 50 grams (cc) of the indicating solution. Samples were exposed to the three stacked sources for 15 minutes. However the samples were not rotated 180° as done in the past, since solution dispersion is non critical. The solutions were located at the holes indicated in the diagram at a temperature of 25.5°C.

The following table presents the absorption data at 304 λ compared with unexposed sample in a Beckman Spectrophotometer with the sensitivity setting at 5, and the split opening 0.4:

Jars and Aluminum cannister

50 cc sample jar

Source Holder

Cannister-Source Orientation

Cannister hole no. Top Middle Bottom	Optical Density for ferric sulfate at 304 λ	Optical Density times 2.69×10^4 yields Rads $\times 10^3$
I	0.64 0.57 0.485	1.72 1.53 1.30
III	0.65 0.60 0.53	1.75 1.61 1.43
IV	0.65 0.585 0.51	1.75 1.57 1.37
IX	0.64 0.585 0.468	1.72 1.57 1.26
XI	0.72 0.55 0.55	1.94 1.48 1.48

Blank jars to calibrate the Beckman Spectrophotometer read (1) +0.002; (2) +0.002 and (3) +0.003. Temperature during reading 25.5°C
Extinction coefficient (temperature corrected) 2246. Yield constant for Cobalt 60 gamma ray 15.6

$$\begin{aligned}
 \text{ev/gm/hr} &= \frac{\text{O.D.} \times 50 \text{ cc} \times 6.025 \times 10^{23} \times 100}{2246} \\
 &= \frac{15.6 \times .25 \text{ hrs} \times 50 \text{ gms} \times 1024 \times 10^3}{15.6 \times .25 \text{ hrs} \times 50 \text{ gms} \times 1024 \times 10^3} \\
 &= \text{O.D.} \times 6.72 \times 10^{18} \times 1.602 \times 10^{-12} \\
 &= \text{O.D.} \times 1.08 \times 10^7
 \end{aligned}$$

$$\text{Factor (Rad/hr): } 1.08 \times 10^7 \times 10^{-2} = 1.08 \times 10^5$$

June 24, 1968

This factor, 1.08×10^5 multiplied by the Optical Density-mean yields Rads/hr for the dosimetric analysis for these new sources. Continued dosimetric runs will be made and submitted in a supplemental report with that of the dosimetry of the exposure ring for the biological tubes.

Respectfully submitted

IIT RESEARCH INSTITUTE

William P. McDonald
Assistant Chemist
Physical Chemistry Research

Approved by:

Eli S. Freeman
Assistant Director
Chemistry Research

WPMC:emh

TABLE 17-1

Total aerobic plate counts on haddock
samples before and after irradiation¹

Day ²	Replicate count/g			Avg. Count	% Reduction ³
	1	2	3		
Wednesday	49 x 10 ⁴	36 x 10 ⁴	62 x 10 ⁴	49 x 10 ⁴	
Thursday	31 x 10 ⁴	78 x 10 ⁴	---	54 x 10 ⁴	
Friday	83 x 10 ⁴	290 x 10 ⁴	---	136 x 10 ⁴	
0.1 Mrad	62 x 10 ³	69 x 10 ³	124 x 10 ³	85 x 10 ³	93.7
0.2 Mrad	20 x 10 ³	58 x 10 ²	70 x 10 ²	11 x 10 ³	98.0

¹ Counts determined in TPY agar medium at 70 F.

² The fish was received on Tuesday and the experiment started on Wednesday.

³ % reduction is based on avg. count on Friday.

TABLE 17-2

Incubation time for obvious slime and mold
growth at 50 and 46 F.¹

Dose (Mrad)	X-value	50 F		46 F	
		Slime	Mold	Slime	Mold
0	1/2	5/6	0/6	3/6	0/6
	3/4	4/6	0/6	4/6	0/6
	1	6/6	0/6	6/6	0/6
	1 1/2	5/6	0/6	6/6	0/6
	2	6/6	0/6	5/6	0/6
0.1	1/2	0/6	0/6	1/6	0/6
	3/4	4/6	0/6	2/6	0/6
	1	5/6	0/6	3/6	0/6
	1 1/2	6/6	0/6	6/6	0/6
	2	6/6	0/6	6/6	0/6
0.2	1/2	2/6	0/6	6/6	0/6
	3/4	1/6	5/6	5/6	2/6
	1	6/6	5/6	6/6	4/6
	1 1/2	6/6	4/6	6/6	1/6
	2	6/6	1/6	6/6	0/6

¹ Results show fraction of replicate samples with slime and mold growth
at comparable values of X.

TABLE 17-3

Toxin production in unirradiated and irradiated haddock
inoculated with 10^4 spores/g and incubated at 50 F.

Dose (Mrad)	Inc Time (days)	X-Value ¹	pH	Rejection ²	Toxin	
					U ³	T ⁴
0	4	1/2	7.6-7.8	15/15	0/3	0/3
	6	3/4	7.2-7.7	15/15	0/3	0/3
	8	1	7.7-7.9	15/15	0/3	0/3
	12	1 1/2	7.6-8.0	15/15	0/3	1/3
	16	2	7.5-8.1	18/18	2/3	2/3
	18	2 1/4	---	---	1/3	2/3
	24	3	---	---	0/3	0/3
	30	3 3/4	---	---	0/3	0/3
	40	5	---	---	0/3	0/3
0.1	6	1/2	7.0-7.3	7/15	0/3	0/3
	9	3/4	---	6/12	0/3	0/3
	12	1	7.3-7.6	18/18	0/3	0/3
	18	1 1/2	7.8-7.9	18/18	0/3	0/3
	24	2	7.8-8.0	18/18	0/3	0/3
0.2	10	1/2	6.9-7.7	10/18	0/3	2/3
	15	3/4	7.2-7.5	9/18	0/3	3/3
	20	1	8.0-8.2	18/18	0/3	0/3
	30	1 1/2	7.8-8.1	18/18	2/3	2/3
	40	2	7.9-8.1	18/18	2/3	2/3

¹ BCF tentative storage life estimates.

² Number samples rejected/number samples examined.

³ Fraction of samples toxic without trypsin digestion.

⁴ Fraction of samples toxic with trypsin digestion.

TABLE 17-4

Toxin production in unirradiated and irradiated haddock
inoculated with 10^2 spores/g and incubated at 50 F.¹

Dose (Mrad)	Inc Time (days)	X-Value	pH	Rejection	Toxin	
					U	T
0	4	1/2	7.6-8.4	15/15	0/3	0/3
	6	3/4	7.4-7.6	13/15	0/3	0/3
	8	1	7.2-7.9	15/15	0/3	0/3
	12	1 1/2	8.0-8.2	18/18	0/3	0/3
	16	2	7.5-7.8	18/18	0/3	0/3
	18	2 1/4	8.2-8.4	---	0/3	0/3
	24	3	8.2-8.3	---	0/3	0/3
	30	4	---	---	0/3	0/3
0.1	6	1/2	7.1-7.3	8/15	0/3	0/3
	9	3/4	7.5-7.8	9/15	0/3	0/3
	12	1	7.2-7.6	16/18	0/3	0/3
	18	1 1/2	7.5-7.8	18/18	0/3	1/3
	24	2	7.9-8.0	18/18	0/3	0/3
0.2	10	1/2	7.0-7.5	7/18	0/3	0/3
	15	3/4	7.3-7.7	9/18	0/3	1/3
	20	1	7.5-8.1	18/18	2/3	2/3
	30	1 1/2	7.9-8.1	18/18	1/3	0/3
	40	2	7.9	18/18	1/3	1/3

¹ See footnotes Table 17-3

TABLE 17-5

Toxin production in unirradiated and irradiated haddock
inoculated with 10^6 spores/g and incubated at 46 F.¹

Dose (Mrad)	Inc Time (days)	X-Value	pH	Rejection	Toxin	
					U	T
0	6	1/2	7.2-7.7	4/15	0/3	0/3
	9	3/4	7.8-8.0	11/12	0/3	0/3
	12	1	7.3-8.0	18/18	0/3	0/3
	18	1 1/2	7.4-8.1	18/18	0/3	0/3
	24	2	8.1-8.3	18/18	0/3	0/3
	36	3	---	---	0/3	0/3
	48	4	---	---	0/3	0/3
0.1	6	1/2	7.0-7.4	6/15	0/3	0/3
	9	3/4	---	8/12	0/3	0/3
	12	1	7.5-7.7	10/18	0/3	3/3
	18	1 1/2	7.8-8.0	18/18	0/3	0/3
	24	2	7.8-7.9	18/18	2/3	2/3
0.2	12	1/2	7.4-7.6	10/18	0/3	3/3
	18	3/4	7.9-8.0	14/15	0/3	2/3
	24	1	7.9-8.0	18/18	0/3	1/3
	36	1 1/2	7.5-7.8	18/18	0/3	1/3
	48	2	---	18/18	1/3	1/3

¹

See footnotes Table 17-3.

TABLE 17-6

Toxin production in unirradiated and irradiated haddock
inoculated with 10^4 spores/g and incubated at 46 F.¹

Dose (Mrad)	Inc Time (days)	X-Value	pH	Rejection	Toxin	
					U	T
0	6	1/2	7.6-7.8	5/15	0/3	0/3
	9	3/4	7.5-7.6	12/12	0/3	0/3
	12	1	7.6-7.8	18/18	0/3	0/3
	18	1 1/2	7.7-7.8	18/18	0/3	0/3
	24	2	8.1-8.5	18/18	0/3	0/3
	36	3	---	---	0/3	0/3
	40	3 1/3	---	---	0/3	0/3
0.1	6	1/2	7.0-7.1	3/15	0/3	0/3
	9	3/4	---	5/12	0/3	0/3
	13	1	7.3-7.8	13/18	0/3	0/3
	19	1 1/2	7.8-7.9	18/18	0/3	0/3
	26	2	7.8-7.9	18/18	0/3	0/3
0.2	12	1/2	7.2-7.5	6/18	0/3	1/3
	18	3/4	7.6-7.8	15/15	0/3	1/3
	24	1	7.7-8.2	18/18	0/3	0/3
	36	1 1/2	7.6-7.8	18/18	0/3	0/3
	48	2	7.8-8.0	18/18	0/3	0/3

¹ See footnotes Table 17-3

TABLE 17-7

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

Temp °F	Inoc/g	Mrad dose	Inc time (days)	
			No toxin	Toxin
50	10^4	0	8 (1)*	12 (1 1/2)
		0.1	24 (2)	--
		0.2	--	10 (1/2)
	10^2	0	30 (4)	--
		0.1	12 (1)	18 (1 1/2)
		0.2	10 (1/2)	15 (3/4)
	10^6	0	48 (4)	--
		0.1	9 (3/4)	13 (1)
		0.2	--	12 (1/2)
46	10^4	0	30 (3 1/3)	--
		0.1	24 (2)	--
		0.2	--	12 (1/2)

* Values in parenthesis show fraction or multiple of X.

TABLE 17-8

Toxin production in unirradiated and irradiated haddock
at 60 F with a 10^2 spores/g.¹

Dose (Mrad)	Inc Time (days)	X-Value	pH	Rejection	Toxin	
					U	T
0	3	1/2	7.3-7.4	11/12	0/3	2/3
	5	1	7.1-7.5	12/12	3/3	3/3
	7	1 1/2	7.6-7.8	15/15	--- ^a	3/3
	10	2	7.5-7.7	12/12	---	3/3
0.1	4	1/2	6.9-7.0	3/15	0/3	3/3
	8	1	7.5-7.8	13/15	---	3/3
	12	1 1/2	7.4-8.0	---	---	3/3
	16	2	8.0-8.3	9/9	---	3/3
0.2	5	1/2	6.4-7.1	9/12	3/3	3/3
	10	1	7.7-7.8	10/12	---	3/3
	15	1 1/2	7.5-7.9	8/12	---	3/3
	20	2	8.0-8.3	12/12	---	3/3

¹ See footnotes Table 17-3.

^a Means result not determined.

TABLE 17-9

Toxin production in unirradiated and irradiated haddock
at 60 F with 10 spores/g.¹

Dose (Mrad)	Inc Time (days)	X-Value	pH	Rejection	Toxin	
					U	T
0	3	1/2	7.2-7.6	12/12	0/3	2/3
	5	1	7.4-7.5	12/12	3/3	3/3
	7	1 1/2	7.6-7.7	15/15	--- ^a	3/3
	10	2	7.8-7.9	12/12	---	3/3
0.1	4	1/2	7.2-7.3	4/15	0/3	3/3
	8	1	7.5-7.6	14/15	---	3/3
	12	1 1/2	7.9-8.0	---	---	3/3
	16	2	8.1-8.2	9/9	---	3/3
0.2	5	1/2	6.8-7.2	3/12	3/3	3/3
	10	1	7.3-7.9	10/12	---	3/3
	15	1 1/2	7.5-7.8	8/12	---	3/3
	20	2	8.0-8.2	12/12	---	3/3

¹ See footnotes Table 17-3.

^a Means result not determined

TABLE 18-1

Viability of spores of 468-C and 571-C.*

Strain	Refractile Count X10 ⁶	Unheated Count X10 ⁶	% Viability of Refractile
468-C	120**	105	88
571-C	200**	190	95

* Recovery medium was Beef Infusion Agar with 0.14% NaHCO₃ and 0.1% sodium thioglycollate.

**Refractile count of stock spore suspensions. Spores produced in fortified Egg Meat Medium.

TABLE 18-2

Effect of preheating on the viability of
spores of 468-C and 571-C

Preheat Temp °F ¹	Viable Count X10 ⁶ /ml	
	468-C	571-C
Unheated	105	190
140	88	190
150	86	210
160	85	200
170	90	180
180	56	180
190	32	34

¹ Preheat time equalled 15 min at temperature shown.

TABLE 18-3

Recovery of Clostridium botulinum type C toxin
from marine sediment samples without and with preheating.

Sediment Sample	Number of cultures toxic ¹	
	Unheated	Preheated ²
613	2/5	4/5
616	0/8	1/8
345	4/8	7/8
TOTALS	6/21	12/21

¹ Number of cultures toxic/number of cultures assayed for type C toxin.

² Preheat equalled 160 F for 15 min.

TABLE 18-4

Effect of successive transfers on the production
of type C toxin in mixed culture.

Sample	Transfer ¹	MLD toxin/ml ²	
		Unheated	Preheated ³
613	0		10,000
	1	100,000	20,000
	2	500,000	500,000
	3	500,000	200,000
616	0		50,000
	1	200,000	1,000,000
	2	500,000	1,000,000
345	0		10,000
	1	2,000	50,000
	2	2,000	50,000
	3	50,000	50,000
	4	50,000	50,000

¹ The original culture is designated 0.

² The unheated or the preheated culture showing the highest titer was used as the inoculum for each transfer. Where the unheated and the preheated cultures showed the same toxicity, the preheated culture was selected for transfer.

³ Preheat equalled 160 F for 15 min.