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

Annual Report, May 1, 1967—January 31, 1968

April 15, 1968

Metal Division Research and Development
Continental Can Company
Chicago, Illinois

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SUMMARY

Phase VII.

1. Work on the immunodiffusion technique to detect the presence of toxin of C1. botulinum Type E in incubated fish samples was undertaken. Preliminary studies with antiserum obtained from the Pasteur Institute failed to give precipitin bands when tested against toxin of the Beluga and Minneapolis strains. With Porton antiserum, visible bands were seen. Additional work with antiserum which was produced in rabbits is planned.
2. Nine cultures associated with nonspecific toxicities were injected intraperitoneally into mice to determine their possible infectivity. The cultures represent 4 Proteus, one Providence Subgroup B, 2 Aerobacter, and 2 Erysipelothrix insidiosa. All of the cultures produced infections as shown by the isolation of organisms from blood cultures. The cultural characteristics and physiological behavior of the isolates were indistinguishable from those of the cultures injected. In all cases, the blood cultures appeared to be pure cultures of the organism tested for infectivity.

Phase XVII.

1. Inoculated pack studies were conducted on fresh cod. Samples were inoculated with 10^6 , 10^4 , and 10^2 spores/g of the Beluga strain that had been produced at 50 F. The samples were radiated with 0.1 and 0.2 Mrad doses and incubated at 50, 46, 42, and 38 F along with inoculated unirradiated control samples. Triplicate samples per variable were examined for odor, total count, pH, and the presence of Type E toxin at time periods corresponding to fractions and multiples of the maximum expected product storage life. Estimates of the maximum storage life are based on the judgement of an untrained consumer-type panel at the Gloucester BCF Laboratory. Their estimates of maximum storage life are designated as "X".

2. Unirradiated control samples with an inoculum of 10^4 or 10^2 at 50 F and with an inoculum of 10^6 or 10^4 at 46, 42, and 38 F failed to show detectable Type E toxin up to a time period corresponding to $1\frac{1}{2}$ X. Also, with 38 F incubation, irradiated samples inoculated with 10^6 or 10^4 spores showed no toxin. At 42 F with a 10^6 inoculum, 0.2 Mrad irradiated samples showed toxin in one out of 3 replicate samples at $\frac{3}{4}$ X and at X. Detectable toxin was absent at $1\frac{1}{2}$ X under similar conditions. No toxin was shown at any time period

Phase XVII. (Continued)

with a 10^4 inoculum. With 0.1 Mrad irradiation, toxin was detected at 1 1/2 X in one out of 3 replicates with a 10^4 inoculum, but was absent at earlier time periods. Comparable 0.1 Mrad samples with a 10^6 inoculum showed no toxin at each sampling period tested.

3. With incubation at 46 F, 0.1 Mrad samples inoculated with 10^6 spores showed toxin at X and 1 1/2 X in one out of three replicate samples. No toxin was detected with a 10^4 inoculum. Samples given 0.2 Mrad showed toxin at 1 1/2 X with a 10^6 inoculum and at X with a 10^4 inoculum. Again the situation with a 10^4 inoculum was toxin in one out of 3 samples tested.
4. With incubation at 50 F, 0.1 and 0.2 Mrad samples showed the presence of Type E toxin at X and 1 1/2 X with both 10^4 and 10^2 inoculum levels.

Phase XVIII.

1. The effect of various medium formulations on the sporulation of C1. botulinum Type C was studied. Difco Cooked Meat and Egg Meat media were the only formulations producing sporulation of strain 468-C to any degree. The addition of glucose, yeast extract and ammonium sulphate at 1% levels to Egg Meat Medium increased sporulation from an estimated 2-5% to about 80%. The refractile spore count reached $65 \times 10^6/\text{ml}$ after 3 days of incubation at 85 F. The viability of the recovered spores was 88% of the refractile count with Beef Infusion Agar and 85 F incubation. Preheating at temperatures in the range 140 to 170 F for 15 minutes produced only a slight reduction in count as compared to an unheated count. Higher temperatures reduced the viable count appreciably.
2. Botulinum toxin from two Type C strains failed to show activation with trypsin under conditions which activate Type E toxin. Trypsin digestion of toxin of strain 468-C produced an appreciable reduction in toxicity; however, trypsinization of toxin of 571-C caused no significant loss in toxicity.

Phase XIX

Strains 17B and 202F have been obtained from Dr. Eklund. Preliminary experiments have been conducted on sporulation and viable spore counting. The best spore yield was obtained in the cooked meat medium of Eklund. Spore yield in this medium was significantly higher at 70 F than at 85 F. A comparison of various media for viable spore counts showed that Beef Infusion agar incubated at 70 F gave the highest viable spore count as did 5% Peptone agar plus 1% Yeast Extract. Preheating 15 minutes at 140 F neither activated spores or caused any reduction in viable count.

INTRODUCTION

The last annual report for the contract year January 15, 1966 to January 14, 1967 was extended to April 30, 1967 to permit the complete presentation of the data upon the inoculated pack experiments in haddock. This report for the contract period January 15, 1967 to January 14, 1968 will include work conducted since May 1, 1967 to the end of the contract period. No irradiation work has been conducted during this period of time. It was intended that further inoculated pack experiments would be started in October 1967. However, in September 1967, it was learned that the Co⁶⁰ source at IITRI had deteriorated so far as to be unusable for our purposes. Some time was required to arrange for replacement of the source and to locate a supplier of a Co⁶⁰ capsule meeting the dimension requirements of the IITRI facility. Delivery has been delayed on legitimate grounds. It is expected that the capsule will be delivered and installed and dosimetry conducted this month (April) and that the intended irradiation experiments can be started in May.

TOXIN ASSAY PROCEDURES

Phase VII.

Detection of botulinum toxins by the immunodiffusion technique.

Dr. H. Sugiyama's work on the immunodiffusion technique to detect botulinum toxin appears to offer possible advantages over the conventional mouse assay test. The applicability of his plate double diffusion test to detect the development of Type E toxin is being examined with inoculated fish samples incubated at low temperatures. Our preliminary studies with Type E antisera of equine origin have not been encouraging; however, the fact that Sugiyama is employing antiserum of rabbit origin may account for his reported success. It is known that antibodies produced in horses against certain proteins often form weak and reversible combinations with their antigens. In contrast, antibodies produced in rabbits against these same proteins tend to form less reversible combinations (Crowle, 1961).

The medium used in the double diffusion experiments consisted of 1% Ionagar (Colab) in 0.2M Tris buffer at pH 7.2, plus 0.85% NaCl and 1:50,000 merthiolate. Ten ml of the medium were poured into a plastic, disposable petri dish (100 x 15 mm) and permitted to solidify. A central well 8 mm in diameter was cut with a No. 4 cork borer. Five satellite wells were cut around the central well so that the distance from center to center of the central well and each of the satellites was 14 mm. The agar plugs were removed with a glass rod attached to a suction system. The central well was loaded with 0.05 ml of antitoxin and the peripheral wells with

Phase VII. (Continued)

0.05 ml of samples being tested for Type E toxin. Each plate was placed in a screw-cap jar and stored at 85 F. After 2-7 days, the plates were examined for lines of precipitation.

In initial experiments the reaction of toxins of the Beluga and the Minneapolis strains were tested against antiserum obtained from the Pasteur Institute. Visible precipitin bands were not distinguishable when various dilutions of antitoxin and toxin were tried. However, use of Porton antiserum at a 1:10 dilution gave discernible bands against Type E toxin.

Type E antitoxin of rabbit origin has been obtained through the generosity of Dr. Sugiyama. Comparative tests are planned to determine the sensitivity of the double diffusion technique and the conventional mouse assay test to detect botulinum toxin. The results of these tests will be presented in our next annual report.

Nonspecific toxicities in the mouse assay test for botulinum toxin.

The problem of nonspecific mouse deaths in Type E toxin assays in inoculated pack experiments was previously discussed (annual report dated April 30, 1967). The principal cause of these deaths is a septicemia. This was shown by obtaining blood cultures from mice injected with samples giving nonspecific deaths.

Phase VII. (Continued)

Out of 23 purified isolates, 8 were originally identified as Proteus vulgaris, 3 Proteus morgannii, one Proteus rettgerii, 2 Aerobacter aerogenes, one Actinobacillus, 3 Enterococci, one Alcaligenes and 4 Erysipelothrix insidiosa. Subsequent work on isolate 9-16 identified as P. morgannii shows it belongs to the Providence Subgroup B. Of the organisms isolated, only the Proteus, Providence, Aerobacter, and Erysipelothrix cultures produced illness and deaths when injected intraperitoneally into mice.

Proof of the infectivity of the organisms producing nonspecific mouse deaths was sought. Nine isolates with at least one representative culture from each group were selected (Table 7-1) and injected intraperitoneally into healthy mice. At the first indication of illness, blood cultures were taken by means of a cardiac puncture. All of the mice injected became ill and gave positive blood cultures. Each blood culture obtained appeared to be pure. In each case, the organisms from these blood cultures were indistinguishable culturally and physiologically from the organisms injected. These results leave no doubt that the Proteus, Providence, A. aerogenes and E. insidiosa cultures isolated are infectious for mice. Moreover, blood cultures on 10 healthy mice which had not been injected showed no growth indicating the aseptic techniques used here were satisfactory.

Use of the antibiotics Chloromycetin and Terramycin in the control of organisms causing nonspecific deaths were previously discussed. The antibiotics are administered simultaneously by intraperitoneal injection.

Phase VII. (Continued)

Two injections are given, one about 20 hours before a sample giving a nonspecific toxicity is injected and the second at the time the mouse is injected with the sample. Each mouse is given 2.0 mg of Chloromycetin and 0.25 mg of Terramycin. These levels can be increased fivefold without any apparent adverse effects. Antibiotic protected mice generally survive up to 4 days or the end of the test when samples with nonspecific toxicities are tested. However, some exceptions might be encountered. Eklund (1967) reported that some nonspecific toxicities were still obtained by following the procedure outlined above. He has found that the injection of the two antibiotics 4 hours before and 24 hours after injecting samples causing nonspecific deaths to be more effective. Possible adoption of Eklund's technique might be considered if nonspecific deaths are particular troublesome in the future.

Not all nonspecific mouse deaths in assays for botulinum toxin are caused by bacterial infections. Occurrence of a septicemia usually is characterized by mouse deaths about 24 hours or later after injection. Cultures of various marine sediments have often produced mouse deaths within a few minutes after injection. Such rapid deaths suggests the presence of some toxic by-product of bacterial growth, for example, ammonia or some potent amine.

TOXIN DEVELOPMENT IN FRESH MARINE PRODUCTS

Phase XVII.

Inoculated pack experiments on fresh cod were conducted according to the procedures described for haddock in our annual report dated April 30, 1967. The purpose of these inoculated packs was to determine the relationship between the earliest time for toxin production by C1. botulinum Type E and the maximum expected product storage life. The occurrence of unquestionable spoilage as recognized by a potential consumer markedly ahead of the earliest time of possible toxin production would give a considerable margin of public health safety for low dose irradiated marine food products.

The protocol for the inoculated pack experiments was that recommended by the Ad Hoc Committee on botulism to the AEC. A spore suspension of the Beluga strain produced at 50 F in TPG medium containing 1% yeast extract was used as the inoculum. Triplicate samples of fresh cod were prepared per variable and inoculated with 10^6 , 10^4 , or 10^2 spores/gram. The 10^6 inoculum was preheated at 140 F for 15 minutes to reduce the possibility of toxin carryover. The lower inoculum levels were not preheated. Samples were radiated with 0.1 and 0.2 Mrad and incubated at 50, 46, 42, and 38 F along with inoculated unirradiated control samples. At sampling periods based on the Gloucester BCF estimates, triplicate samples for each variable were removed from incubation. Acceptance or rejection of the samples based solely on odor was determined by a six member panel of our microbiology staff. At certain examinations fewer than six people were available to evaluate the samples. Total plate counts and pH determinations were made after blending the samples with an equal amount of sterile distilled water. Toxin assays were conducted

Phase XVII. (Continued)

without and with trypsin digestion according to acetate procedure previously described.

The data for cod are summarized in Tables 17-1 through 17-8. The tables are arranged to show incubation temperature and inoculum level. It can be seen that the total counts increased rapidly at each incubation temperature tested. In general, the unirradiated samples showed increasing counts with each successive time period. However, the irradiated samples usually showed the highest count at $3/4$ X or at X (maximum expected storage life) and then a reduction in count after longer incubation. Not shown are the initial counts. Unirradiated control samples had a count of 200,000/g, 0.1 Mrad samples 12,000/g, and 0.2 Mrad samples 4,100/g.

The pH results on cod, as with haddock, show an appreciable increase with aerobic incubation, often reaching 8.0-8.3. The pH determinations have little value in assessing the health hazard of C1. botulinum Type E and will be greatly curtailed in future inoculated pack work. Also, the aerobic plate counts have limited practical value. The counts on a sample with a comparatively large surface area as used here may or may not be comparable to counts on whole fillets. It appears that the incoming fish quality should be monitored, but that further attempt to follow counts during incubation should be reduced or possible eliminated.

The toxin assay results may be easier to comprehend by referring to Tables 17-9 and 17-10. The tables are arranged to show incubation

Phase XVII. (Continued)

temperature, irradiation dose, the fraction or multiple of X, and the toxin assay result. The toxin assay data at 46 F and below appear quite favorable. However, the appearance of toxin at X in one out of three samples inoculated with 10^4 spores and irradiated with 0.2 Mrad is disturbing because no toxin was encountered at the same time period in samples inoculated with 10^6 spores. A similar situation is shown at 42 F with 0.1 Mrad irradiation at 1 1/2 X. While such results were not frequent, running additional samples might have shown toxin at the next higher inoculum level used.

The toxin assay results at 50 F for cod, as for haddock, are less favorable than those at lower temperatures. Type E toxin was detected at X with both 0.1 and 0.2 Mrad radiation, but none was detected with unirradiated samples at X or at 1 1/2 X. The failure to demonstrate toxin production in unirradiated product may merely reflect the shorter incubation times tested. It is also recognized that without radiation, growth and toxin development by Type E may be delayed or inhibited by a competitive microflora which would be eliminated by radiation.

GROWTH CHARACTERISTICS OF CLOSTRIDIUM BOTULINUM

TYPE C AT LOW TEMPERATURES

Phase XVIII.

INTRODUCTION:

Mixed cultures containing Cl. botulinum Type C, as determined by mouse protection tests, were obtained from Dr. B. Q. Ward, University of Florida. The cultures were acquired by Dr. Ward during a survey of the Gulf and Atlantic Coasts for Cl. botulinum Type E. The appearance of Type C in a marine environment has stimulated interest in the possible ability of the organism to grow at low temperatures. Little is known concerning the low temperature growth characteristics of Type C. An attempt is being made to isolate pure Type C cultures from the grossly contaminated cultures supplied and, if successful, to determine their ability to grow at temperatures below 50 F.

In an effort to isolate pure Type C cultures from marine sediments, preliminary work was done with known Type C cultures to learn more about the sporulation requirements of the organism. The possibility of isolation of pure Type C cultures would be greatly improved if sporulation could be induced. Our initial efforts have been to develop a suitable sporulation medium for two Type C cultures which do not sporulate under most conditions. The cultures are designated 468 C and 571 C. The cultures were originally in the K. F. Meyer collection.

Phase XVIII. (Continued)

Sporulation media. Various broth formulations were tested in an attempt to sporulate 468 C and 571 C at 85°F. Table 18-1 shows a summary of the 13 different media tested with respect to growth initiation and sporulation of 468-C. Sodium thioglycollate (20%) was added to all of the broth media not containing meat particles to give a final concentration of 0.1%. The composition of the non-commercial media is given in the appendix to this report. Difco Cooked Meat and Difco Egg Meat were the only media giving excellent growth and any degree of sporulation. Sporulation in these particulate media was estimated microscopically at 2-5% at the end of 3 days of incubation. Longer incubation showed a reduction in spore yield. Since Egg-Meat Medium has no glucose in its composition, it was selected for further sporulation experiments.

The effect on sporulation of 468-C by adding glucose, yeast extract, and ammonium sulphate to the the Egg Meat Medium was determined. Each additive was tested at a 1% concentration. Individually, the additives had little effect on increasing sporulation. However, in combination, there was a marked increase in spore yield, with an estimated 80% of the cells present showing refractile spores after 3 days of incubation.

The optimal concentrations of glucose, yeast extract, and ammonium sulphate for sporulation of 468-C are shown in Table 18-2. While glucose and yeast extract at concentrations of 0.5% were as effective as higher levels, the concentrations of these two additives was maintained at 1% in subsequent experiments. Levels of ammonium sulphate lower or higher than 1% somewhat reduced the yield of refractile spores.

Phase XVIII. (Continued)

Sporulation of 468 C at 85 F commenced about 30-48 hours after inoculation with an unheated recently sporulated culture and reached a peak after 72 hours of incubation.

Preparation of Type C spore suspensions. The following procedures were adopted to prepare Type C spore suspensions. Two, one liter flasks each containing 75g of Egg Meat Medium, 500 ml distilled water, and 1% concentrations of Yeast Extract and ammonium sulphate were prepared and sterilized (250 F for 15 minutes). Sterilized glucose (20%) was then added aseptically to give a final concentration of 1%. The flasks were inoculated with an active sporulated culture and incubated at 85 F. An unheated inoculum was used, since preheating tended to delay sporulation. As a safety precaution against possible explosions due to vigorous gas evolution, the flasks were attached to a water trap and the entire assembly was placed in a pressure cooker. Growth and sporulation were followed by Petroff-Hausser chamber counts.

The spore crop was harvested when peak sporulation was attained (80-85%). In harvesting, the liquid portion was decanted and filtered through sterile cheese cloth in a Buchner funnel into a sterile screw-cap flask. About 100 ml of sterile distilled water was added to the meat particles and decanted to recover some of the remaining spores. The suspension was chilled and sonicated with a Biosonik III ultrasonic unit to disintegrate the vegetative cells and accompanying meat particles. The suspension flowed by gravity from a separatory funnel into a continuous flow chamber and out into a sterile suction flask. During sonication, the suspension was maintained cold by circulating water from an ice-water bath through the jacket of the continuous flow chamber. After concentrating

Phase XVIII. (Continued)

the spores by centrifugation, the liquid was decanted and the sediment resuspended in prechilled sterile distilled water to about 1/20 of its original volume. Finally, the aqueous suspension was stored at 36 F in a screw-cap bottle containing glass beads.

Standardization of Type C spore suspensions. A deep tube counting technique was used with 5 replicate tubes per variable. The recovery medium consisted of Beef Infusion Agar. Filter sterilized NaHCO_3 (20%) and sodium thioglycollate (20%) was added to the melted medium just before use to give respective concentrations of 0.14% and 0.1%. After pouring and solidification, the medium was overlaid with additional Beef Infusion Agar to act as an oxygen barrier. The tubes were incubated at 85 F. Colony counts were made after 48 hours and one week of incubation.

Table 18-3 shows the refractile yield and percent viability for spores of 468-C produced in the above manner.

Effect of preheating on the viability of spores of Cl. botulinum Type C.

The effect of preheating at temperatures from 140 to 190 F for 15 minutes was determined for spores of 468-C. Viable counts were determined for each preheat treatment with Beef Infusion Agar and 85 F incubation. As shown in Table 18-4, preheat temperatures in the range 140 to 170 F caused a slight reduction in count as compared to the unheated count. Preheating at 180 F produced an appreciable reduction in count and an even greater reduction at 190 F as compared to the lower preheat treatments.

Phase XVIII. (Continued)

Effect of trypsin digestion of Type C toxin. The effect of trypsin on the activation of Type E toxin is well known (Duff, Wright and Yarinsky, 1956). The potency of Type E toxin produced with a pure culture can be increased 50 to 200 fold with trypsin digestion. Since both Cl. botulinum Type E and Type C are nonproteolytic, it was considered that Type C toxin might also show activation. However, Type C toxin showed no increase in potency after trypsin digestion (Table 18-5). Toxin from 468-C actually showed an appreciable reduction in potency after trypsin digestion, whereas toxin from 571-C showed no significant change in potency. If Type C toxin can be activated with trypsin, it is apparent that the conditions necessary for activation are definitely different than required for activation of Type E toxin.

Isolation of Type C cultures from marine sediments - Efforts to purify Type C cultures from crude cultures and various marine sediments so far have not been successful. However, work in this area will be continued. Studies with 468-C and 571-C indicate the unlikely possibility of isolating Type C from streaked plates incubated anaerobically. Neither strain has given growth when streaked on Beef Infusion Agar and incubated anaerobically at 85 F. Anaerobiosis was obtained with a Brewer Anaerobic Jar and by using a BBL Gaspak envelope. While this system permits growth of Cl. botulinum Type E and Type A and PA3679, it does not permit growth of the two Type C strains tested. For this reason, a deep tube technique is being employed in an attempt to isolate pure Type C cultures from Ward's samples.

Phase XIX - OUTGROWTH PROPERTIES AT LOW TEMPERATURE OF NONPROTEOLYTIC
CLOSTRIDIUM BOTULINUM TYPES B AND F IN MARINE PRODUCTS

INTRODUCTION

Since non-proteolytic Type B and Type F cultures have been isolated from surveys conducted on the West Coast by Dr. Eklund there is a reasonable presumption that they might be present in marine products subjected to low dose irradiation. Therefore, it is intended to conduct studies on the outgrowth properties at low temperature in marine products of spores of these two types. Pure cultures of the non-proteolytic strains 17B and 202 F have been obtained from Dr. Eklund for this work.

SPORULATION AND SPORE SUSPENSIONS

Eklund has used a cooked meat medium for growth and sporulation of the non-proteolytic strains. This is composed of Beef infusion plus the following ingredients per liter: NaCl 5 gm, $K_2 HPO_4$ 0.8 gm, Peptone 5 gm, Yeast Extract 10 gm, Trypticase 10 gm, glucose 2 gm. This is combined with the ground infused beef in either tubes or bottles. Since this medium was not available when the cultures were received, a modified cooked meat medium was used temporarily. This was prepared by dissolving the constituents added to the beef infusion and adding Difco Egg Meat in the proportion of 1 gm to 20 ml in tubes or 5 gm to 200 ml in bottles. Subsequently, a lot of Beef Infusion was prepared and with the cooked meat used to prepare the Eklund cooked meat medium.

Phase XIX (Continued)

Transfers from the original cultures grew well in the modified cooked meat medium and showed sporulation at 85 F. A small lot of spore suspension was prepared with each of these cultures in a bottle of medium at 85 F. Other small spore crops of each culture were prepared in the Eklund cooked meat medium at 85 F and at 70 F. The results in terms of refractile spore yield per ml of medium are shown in Table 19-1. The yield of refractile spores of these strains at 85 F in the modified cooked meat medium was disappointly low as compared to the yields obtained in TPG medium with Type E strains. Also at 85 F the refractile spore yields in the Eklund cooked meat medium were no better. However, in the Eklund cooked meat medium at 70 F the refractile spore yield of 17 B increased about 5.9 fold and the 202 F about 11 fold. In each case the refractile spores appeared to form about 95 % of the total population. It is intended for the present at least to prepare spore suspensions of these strains in Eklund cooked meat medium at 70 F.

Using the first suspensions prepared in the modified cooked meat medium experiments were conducted to determine the medium and conditions which would yield the maximum viable count. The results of these experiments are shown in Table 19-2. The viable counts were conducted in 16 x 125 mm screw-cap tubes with all media containing 1.5% agar and 0.1% Na thioglycolate. In general, maximum colony count was reached in 3-4 days at 85 F and in 6-7 days at 70 F.

From Table 19-2, it may be observed that maximum viable spore counts were obtained at 70 F in Beef Infusion agar, 5% Peptone agar plus 1% Yeast Extract and TP agar plus 1% Yeast Extract. At 85 F the viable count in each of these three media appears lower. In the only test conducted

Phase XIX (Continued)

on preheating using 5% Peptone-Yeast Extract at 85 F for viable count a preheat of 15 minutes at 140 F does not appreciably affect the viable count while 15 minutes at 176 F eliminates the small number of spores present. In the dilution used for counting there were theoretically 150 refractile spores per ml of 17 B and 80 refractile spores per ml of 202 F. Therefore, the viable count as percent of the refractile count for 17 B is 23% and for 202 F is 18%. These percentages are considerably lower than that found with Type E suspensions.

It is intended that the viable spore counts on these two strains will be conducted in Beef Infusion agar at 70 F until further comparative studies may completely justify replacement with either of the media.

GENERAL DISCUSSION

Inoculated pack studies on other marine food products showing promise under the AEC program are planned. Such products include crab meat, clams and scallops. The order in which these products will be tested will depend upon the preference of the AEC. The protocol followed in haddock and cod experiments will be used to determine the public health safety of these low dose irradiated products with respect to growth and toxin development by Cl. botulinum Type E.

Work is in progress at the Gloucester BCF Laboratory on crab meat and scallops. Acceptability and storage life data on these products are encouraging (Monthly Progress Report, Contract AT [49-11] 1889, August 1-30, 1967).

Similar work on shucked soft-shelled clams at the Massachusetts Institute of Technology appears equally encouraging. The clam results are summarized in TID - 19861 entitled, "A Study of the Effects of Sub-Sterilization Doses of Radiation on the Storage Life Extension of Soft-Shelled Clams and Haddock Fillets" (Annual Report, May 1, 1962 - April 30, 1963). Inoculated pack experiments will be started once storage life estimates for crab meat, scallops, and clams are firmly established.

Further inoculated pack work on haddock and cod is planned. The failure to detect Type E toxin in inoculated unirradiated samples may merely reflect the shorter incubation times used. It seems desirable that toxin assays on unirradiated samples should be made at time periods corresponding to the earliest time of toxin development in irradiated product. Such studies are particularly revelant to samples incubated at 50 F and possibly at 46 F.

There is concern over the development of toxin ahead of the maximum expected storage life estimates with irradiated samples incubated at 50 F. Irradiated samples often show slime and mold growth before they are judged unacceptable by odor. It seems reasonable that a potential consumer would consider appearance as well as odor in judging the quality of irradiated marine food products. Therefore, consideration of both appearance and odor would seem desirable. From the practical standpoint this might cause a reduction in the maximum storage life estimates at 50 F. A reduction of X-values might give a more favorable picture with respect to toxin development in haddock and cod at 50 F. It is proposed that further inoculated pack experiments should be conducted at 50 F taking into consideration X-values established on the basis of product odor and appearance.

Further efforts will be made to isolate pure Type C cultures from the grossly contaminated cultures and marine sediments supplied by Dr. Ward. If successful, the low temperature outgrowth characteristics of spores of C1. botulinum Type C of marine and non-marine origin will be compared.

A nonproteolytic Type F strain and a nonproteolytic Type B strain capable of growth as low as 38 F were obtained from Dr. M. W. Eklund of the Bureau of Commercial Fisheries Laboratory at Seattle, Washington. Now that spore suspensions of these two strains have been prepared, their outgrowth properties in selected marine products will be studied. Possible inoculated pack studies will be conducted if the outgrowth times of spores of the two strains are of the order observed for spores of C1. botulinum Type E.

APPENDIX

Media composition for Clostridium botulinum Type C.

Beef Infusion Broth (University of Michigan formulation)

Filtrate of Beef infusion (1 lb. fresh beef/liter)
NaCl 5g
Na₂HPO₄ 5g
Peptone (Difco) 10
pH 7.4 - 7.6

Liver Infusion Broth (NCA formulation)

Filtrate of liver infusion (1 lb. fresh liver/liter)
Tryptone (Difco) 10g
Soluble Starch 1g
K₂HPO₄ 1g
pH 7.2

Jensen's Medium (Dr. W. I. Jensen)

Lactalysate (BBL) 30g
Yeast Extract (Difco) 20g (Substituted for Albimi
(Yeast Autolysate)

Glucose 5g
Na - citrate 3.5g
pH 7.3-7.4

PE-2 Medium (See Folinazzo & Troy, Food Technology 8: 1954. pp. 280-281)

Eklund Cooked Meat Medium

Filtrate of beef infusion 1000 ml (1 lb. fresh beef/l)
NaCl 5g
Na₂HPO₄ 0.8g
Peptone (Difco) 5.0g
Yeast Extract 10.0g
Trypticase (BBL) 10.0g
Meat particles 1g/10 ml

TPG (See Schmidt, Nank, and Lechowich, Food Science 27: 1962.
pp. 77-84).

TPGYE

TPG above plus 1% Difco Yeast Extract

All media formulation on a liter basis.

REFERENCES

1. Crowle, A. J. 1961. Immunodiffusion. Academic Press, New York. p. 14.
2. Duff, J. T., G. G. Wright, and A. Yarinsky 1956. Activation of Clostridium botulinum Type E by trypsin. J. Bacteriol. 72: 455-460.

TABLE 7-1

Isolates associated with nonspecific mouse deaths.

Culture No.

480-23	<u>Proteus vulgaris</u>
317-20	<u>P. vulgaris</u>
61-5	<u>P. vulgaris</u>
9-16	<u>P. morganii</u> (Providence subgroup B)
480-25-2	<u>P. rettgeri</u>
31-2	<u>Aerobacter aerogenes</u>
61-2	<u>Aerobacter aerogenes</u>
314-17	<u>Erysipelothrix insidiosa</u>
315-18	<u>Erysipelothrix insidiosa</u>

TABLE 17-1

Toxin production in unirradiated and irradiated cod inoculated with 10^4 spores/g and incubated at 50 F. ¹

Dose (Mrad)	Inc time (days)	X-value ²	Count ($\times 10^8$)	pH	Rejection ³	Toxin	
						U ⁴	T ⁵
0	6	1	14	7.5-7.9	17/18	0/3	0/3
	9	1 1/2	33	7.9-8.0	18/18	0/3	0/3
0.1	13	1	21	7.6-7.9	12/18	3/3	3/3
	18	1 1/2	16	8.0-8.1	13/15	3/3	3/3
0.2	18	1	15	7.6-7.9	12/15	3/3	3/3
	27	1 1/2	10	7.9-8.2	11/12	3/3	3/3

¹ Spores of the Beluga strain produced at 50 F.

² Maximum expected storage life based on the Gloucester untrained panel.

³ No. samples rejected/no. sample examined by CCC panel.

⁴ No. samples toxic without trypsinization/no. examined.

⁵ No. samples toxic with trypsinization/no. examined.

TABLE 17-2

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

Dose (Mrad)	Inc time (days)	X-value	Count ($\times 10^8$)	pH	Rejection	Toxin	
						U	T
0	6	1	15	7.5-8.0	17/18	0/3	0/3
	9	1 1/2	26	7.4-7.9	18/18	0/3	0/3
0.1	13	1	19	7.4-7.6	11/18	2/3	3/3
	18	1 1/2	18	7.3-7.9	12/15	3/3	3/3
0.2	18	1	15	7.5-7.5	15/15	3/3	3/3
	27	1 1/2	8.0	8.0-8.2	12/12	3/3	3/3

TABLE 17-3

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

Dose (Mrad)	Inc time (days)	X-value	Count ($\times 10^8$)	pH	Rejection	Toxin	
						U	T
0	6	3/4	9.0	7.6-7.7	14/18	0/3	0/3
	8	1	19	7.8-7.9	18/18	0/3	0/3
	13	1 1/2	30	7.7-7.9	18/18	0/3	0/3
0.1	14	3/4	27	7.4-7.7	10/15	0/3	2/3
	19	1	13	7.6-7.7	10/15	1/3	1/3
	26	1 1/2	8.0	7.6-8.0	14/15	1/3	1/3
0.2	17	3/4	17	7.4-7.8	7/15	0/3	0/3
	24	1	14	7.7-7.8	8/12	0/3	0/3
	35	1 1/2	7.0	7.9-8.2	13/15	2/3	1/3

TABLE 17-4

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

Dose (Mrad)	Inc time (days)	X-value	Count ($\times 10^8$)	pH	Rejection	Toxin	
						U	T
0	6	3/4	10	7.5-7.6	11/18	0/3	0/3
	8	1	14	7.6-7.9	18/18	0/3	0/3
	13	1 1/2	39	7.9	18/18	0/3	0/3
0.1	14	3/4	16	7.6-7.7	3/15	0/3	0/3
	19	1	15	7.6-7.9	7/15	0/3	0/3
	26	1 1/2	10	7.5-7.9	14/15	0/3	0/3
0.2	17	3/4	14	7.5-7.6	4/15	0/3	0/3
	24	1	16	7.5-8.0	8/12	0/3	1/3
	35	1 1/2	8.0	8.0-8.2	12/15	0/3	0/3

TABLE 17-5

Toxin production in unirradiated and irradiated cod inoculated with 10^6 spores/g and incubated at 42 F.

Dose (Mrad)	Inc time (days)	X-value	Count ($\times 10^8$)	pH	Rejection	Toxin	
						U	T
0	7	3/4	8.0	7.5-7.6	10/12	0/3	0/3
	9	1	20	7.6-7.8	18/18	0/3	0/3
	13	1 1/2	29	7.9-8.0	18/18	0/3	0/3
0.1	19	3/4	24	7.5-7.7	6/15	0/3	0/3
	26	1	12	7.6-7.8	5/15	0/3	0/3
	39	1 1/2	9.4	8.1-8.3	12/12	0/3	0/3
0.2	24	3/4	17	7.4-7.6	4/12	0/3	1/3
	31	1	8.0	7.5-7.8	9/12	0/3	1/3
	46	1 1/2	--	8.2-8.3	15/15	0/3	0/3

TABLE 17-6

Toxin production in unirradiated and irradiated cod inoculated with 10^4 spores/g and incubated at 42 F.

Dose (Mrad)	Inc time (days)	X-value	Count ($\times 10^8$)	pH	Rejection	Toxin	
						U	T
0	7	3/4	7.3	7.3-7.5	8/12	0/3	0/3
	9	1	10	7.6-7.7	18/18	0/3	0/3
	13	1 1/2	29	7.9-8.0	18/18	0/3	0/3
0.1	19	3/4	16	7.4-7.8	9/15	0/3	0/3
	26	1	20	7.4-8.0	9/15	0/3	0/3
	39	1 1/2	7.8	8.2-8.3	11/12	0/3	1/3
0.2	24	3/4	21	7.5-7.8	8/12	0/3	0/3
	31	1	15	7.7-7.8	6/12	0/3	0/3
	46	1 1/2	--	7.8-8.2	11/15	0/3	0/3

TABLE 17-7

Toxin production in unirradiated and irradiated cod inoculated with 10^6 spores/g and incubated at 38 F.

Dose (Mrad)	Inc time (days)	X-value	Count ($\times 10^8$)	pH	Rejection	Toxin	
						U	T
0	8	3/4	1.6	7.1-7.5	7/18	0/3	0/3
	10	1	7.8	7.5-7.7	18/18	0/3	0/3
	16	1 1/2	24	7.6-8.0	18/18	0/3	0/3
0.1	25	3/4	16	7.6-7.8	5/12	0/3	0/3
	33	1	19	7.8-7.9	4/12	0/3	0/3
	48	1 1/2	--	8.0-8.3	10/16	0/3	0/3
0.2	27	3/4	14	7.7-8.0	6/12	0/3	0/3
	38	1	12	7.8-8.0	8/12	0/3	0/3
	55	1 1/2	--	8.2-8.3	10/15	0/3	0/3

TABLE 17-8

Toxin production in unirradiated and irradiated cod inoculated with 10^4 spores/g and incubated at 38 F.

Dose (Mrad)	Inc time (days)	X-value	Count ($\times 10^8$)	pH	Rejection	Toxin	
						U	T
0	8	3/4	7.0	7.2-7.4	12/18	0/3	0/3
	10	1	9.1	7.3-7.4	15/18	0/3	0/3
	16	1 1/2	24	7.8-8.0	18/18	0/3	0/3
0.1	25	3/4	18	7.4-8.0	8/12	0/3	0/3
	33	1	12	7.6-7.8	1/12	0/3	0/3
	48	1 1/2	--	7.8-8.3	7/12	0/3	0/3
0.2	27	3/4	13	7.5-7.8	0/12	0/3	0/3
	38	1	10	7.8-7.9	8/12	0/3	0/3
	55	1 1/2	--	8.0-8.2	12/15	0/3	0/3

TABLE 17-9

Summary of toxin assay results on cod with incubation at 50 and 46 F
(Spores produced at 50 F).

Temp°F.	Mrad	Inc (days)	X-value	Presence of Toxin ¹			
				Inoc	10 ⁶	10 ⁴	10 ²
50	0	6	1	-	-	-	-
		9	1 1/2	-	-	-	-
	0.1	13	1	-	-	+	+
		18	1 1/2	-	-	+	+
	0.2	18	1	-	-	+	+
		27	1 1/2	-	-	+	+
46	0	6	3/4	-	-	-	-
		8	1	-	-	-	-
		13	1 1/2	-	-	-	-
	0.1	14	3/4	-	-	-	-
		19	1	++	-	-	-
		26	1 1/2	++	-	-	-
	0.2	17	3/4	-	-	-	-
		24	1	-	-	++	-
		35	1 1/2	+	-	-	-

¹ + Means toxin detected; - Means no toxin detected in three replicate samples.

* Toxin present in one out of three replicate samples.

TABLE 17-10

Summary of toxin assay results on cod with incubation at 42 and 38 F
(Spores produced at 50 F).

Temp °F.	Mrad	Inc (days)	X-value	Presence of Toxin ¹	
				Inoc	
				10 ⁶	10 ⁴
42	0	7	3/4	-	-
		9	1	-	-
		13	1 1/2	-	-
	0.1	19	3/4	-	-
		26	1	-	-
		39	1 1/2	-	++
	0.2	24	3/4	++	-
		31	1	++	-
		46	1 1/2	-	-
38	0	8	3/4	-	-
		10	1	-	-
		16	1 1/2	-	-
	0.1	25	3/4	-	-
		33	1	-	-
		48	1 1/2	-	-
	0.2	27	3/4	-	-
		38	1	-	-
		55	1 1/2	-	-

¹+ Means toxin detected; - means no toxin detected in three replicate samples.

* Toxin present in one out of three replicate samples.

TABLE 18-1

Growth and sporulation of 468 C in various broth media with incubation at 85 F.

Medium ¹	Growth	Sporulation ²
1. Beef Infusion Broth	Good	None
2. Liver Infusion Broth	Good	None
3. NCA Sporulation Medium (Fisher)	None	
4. Jensen's Medium	Excellent	None
5. PE-2 Medium	Good	None
6. Eklund Cooked Meat	Good	1%
7. Cooked Meat (Oxoid)	Fair	1-2%
8. RCM (Oxoid)	Poor	None
9. Egg Meat (Difco)	Excellent	2-5%
10. Cooked Meat (Difco)	Excellent	2-5%
11. TPG	Fair	None
12. TPGYE	Good	None
13. TPGYE + 1% (NH ₄) ₂ SO ₄	Good	None

¹ Composition of non-commercial media given in appendix.

² Estimated microscopically after 3 days of incubation.

TABLE 18-2

Optimal concentrations of glucose, yeast extract, and ammonium sulphate for sporulation of 468-C in Egg-Meat Medium.

<u>Additive</u> ¹	<u>Conc (%)</u>	<u>Refractile spores x 10⁶/ml</u>
Glucose	0.1	6.5
	0.5	63
	1.0	69
	1.5	40
Yeast Extract	0.5	67
	1.0	65
	2.0	60
	5.0	58
(NH ₄) ₂ SO ₄	0.5	27
	1.0	50
	2.0	30

¹ When the concentration of an additive above was varied, the concentration of the other two additives shown was maintained at the 1% level.

TABLE 18-3

Refractile yield and viability of spores of C1. botulinum Type C strain
468-C.

Refractile count/ml $\times 10^6$		Viable unheated	
<u>Culture</u>	<u>Stock Susp</u>	<u>Count of stock $\times 10^6$</u>	<u>% Viability of refractile ¹</u>
65	120	105	88

¹ Beef Infusion Agar + 0.14% NaHCO_3 + 0.1% sodium thioglycollate and 85 F incubation.

TABLE 18-4

Effect of preheating on the viability of spores of 468-C.

<u>Preheat Temp °F</u> ¹	<u>Viable count x 10⁶</u>
	<u>468-C</u>
Unheated	105
140	88
150	86
160	85
170	90
180	56
190	32

¹ Total preheat time = 15 minutes.

TABLE 18-5

Effect of trypsin digestion on C1. botulinum Type C toxin
produced in Egg-Meat Medium ¹

<u>Strain</u>	<u>Test</u>	<u>MLD toxin/ml</u>	
		<u>Untrypsinized</u>	<u>Trypsinized¹</u>
468-C	1	200,000	50,000
	2	100,000	10,000
571-C	1	500,000	200,000
	2	500,000	500,000

¹ Toxicity after 5 days of incubation at 85 F.

² Digestion with 0.5% trypsin (Difco 1:250)
in 0.067M phosphate buffer at pH 6.2 for 1 hr
at 98 F.

TABLE 19-1

Refractile spore yield per ml of medium of strains 17 B and 202 F.

MEDIUM	TEMP°F.	TIME DAYS	REFRACTILE SPORES/ml X 10 ⁶	
			17B	202F
Modified CM	85	5	19	9
Eklund CM	85	5	18	6
Eklund CM	70	7	107	66

TABLE 19-2

Viable spore count of suspensions of strains 17 B and 202 F.

MEDIUM	TEMP°F.	PREHEAT	VIABLE COUNT/ml OIL SUSPENSION	
			17B	202F
5%	85	0	1.2 (ng)	0
5% P + Y.E.	85	0	25 (ng)	9 (ng)
5% P + Y.E.	85	15'/140 F	22 (ng)	6 (ng)
5% P + Y.E.	85	15'/176 F	0	0
5% P + Y.E.	70	0	34 (g)	10 (ng)
TP + Y.E.	85	0	18 (g)	9 (g)
TP + Y.E.	70	0	34 (g)	10 (ng)
TP + 0.2% G	70	0	4 (ng)	5 (ng)
TPG + Y.E.	70	0	14 (g+)	10 (ng)
BI	85	0	16 (g)	2 (g+)
BI	70	0	34 (ng)	14 (ng)

Y.E. 1% Yeast Extract

P Peptone

TP 5% Trypticase + 0.5% Peptone

G Glucose

BI Beef Infusion

ng = no gas

g = slight gas

g+ = excessive gas