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THE EFFECT OF GAMMA RAYS ON HADDOCK AND CLAMS INOCULATED WITH CLOSTRIDIUM BOTULINUM, TYPE E

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SUMMARY

Haddock inoculated with 1 x 10^2, 1 x 10^4 or 1 x 10^6 spores per gram of type E, Cl. botulinum and irradiated at 150, 250 or 350 kilo rad, then held at 33, 35 or 40°F did not become toxic in 1, 2 or 3 times the irradiated storage life at these temperatures (maximum of 65 days at 40°F, 120 days at 33 and 35°F).

Haddock inoculated with 1 x 10^2 spores per gram of type E, Cl. botulinum, irradiated with 150, 250 or 350 kilo rad and stored at 45°F did not become toxic in 1, 2 or 3 times the irradiated storage life at that temperature (maximum of 26, 42 and 54 days respectively).

Haddock inoculated with 1 x 10^4 spores per gram of type E, Cl. botulinum irradiated at 150 kilo rad and stored at 45°F became toxic in 3 times the irradiated storage life (26 days) but was not toxic in 2 times the irradiated storage life (18 days).

Haddock inoculated with 1 x 10^4 spores per gram of type E, Cl. botulinum, irradiated at 250 kilo rad and stored at 45°F became toxic in 3 times the irradiated storage life (42 days) but not in 2 times the irradiated storage life (29 days).

Haddock inoculated with 1 x 10^4 spores per gram of type E, Cl. botulinum irradiated at 350 kilo rad and stored at 45°F became toxic in 3 times the irradiated storage life (54 days) but not in 2 times the storage life (36 days).

Haddock inoculated with 1 x 10^6 spores of type E, Cl. botulinum, irradiated at 150, 250 or 350 kilo rad and stored at 45°F became toxic in the normal, irradiated storage life time (16, 16 and 20 days respectively).

In samples inoculated with 1 x 10^6 spores per gram of type E, Cl. botulinum, some of which were not irradiated, others of which were irradiated at 100 or 200 kilo rad, all of which were stored at 45°F, toxicity occurred in irradiated samples at the same time (8 days of storage).

In samples inoculated with 1 x 10^4 spores per gram of type E, Cl. botulinum some of which were not irradiated, others of which were irradiated at 100 or 200 kilo rad, all of which were stored at 45°F, the unirradiated samples became toxic in 16 days, the irradiated samples in 19 days.

In a preliminary survey in which 18 - 50 gram portions of commercially produced haddock fillets were cultured, none of the cultures were found to contain the toxin of type E, Cl. botulinum.

In a survey to determine the level of contamination of commercially produced haddock fillets with type E, Cl. botulinum 34 - 555 gram samples from 5 different plants have been cultured by the Most Probable Number technique. Among these only 8 contained type E, Cl. botulinum.

The highest concentration of type E, Cl. botulinum cells found in any of the 8 contaminated haddock fillet samples has been found to be 0.17 per gram or 17 per 100 grams.

There is some factor in haddock, and in haddock press juice, which tends to destroy the spores of type E, Cl. botulinum during standing. For instance, if the heat shocked spores of this organism are inoculated into haddock press juice (pH found to be 6.7) to provide a concentration of 1×10^6 per ml. and this suspension is held at 35°F for 24 hours, then counted to determine the number of clostridia, there is a decrease in the number of viable cells to provide a count of only about 1×10^3 per ml.

The thermal destruction time for 10,000 LD_{50} (mouse) units of type E, Cl. botulinum toxin in fish press juice (pH 6.7) has been determined. Approximate times are: 55 minutes at 140°F, 11.5 minutes at 150°F, 2.35 minutes at 160°F, 0.5 minutes at 170°F, 0.1 minute at 180°F and 0.02 minutes at 190°F.

The temperatures attained at the slowest heating point during the deep fat frying of haddock portions and during cooling after removal from the frying medium, have been recorded.

The temperatures attained during deep fat frying, in the sample of haddock reaching the lowest temperatures, have been integrated graphically with the thermal destruction times at various temperatures for the toxin of type E, Cl. botulinum. If it is considered that the heating time required to inactivate a unit amount of type E toxin at a particular temperature would be increased tenfold if 10 unit amounts of toxin were to be inactivated, then during the deep fat frying

and subsequent cooling of haddock (that sample attaining the lowest temperature) approximately 21 - 10,000 LD_{50} mouse units of toxin would be inactivated (approximately 47 man lethal doses calculated on a weight basis).

Maunder et al.[1] have found that in buffer at pH 6.0 approximately 10 times the heating time at a particular temperature is required to inactivate 10 unit amounts of type E, Cl. botulinum toxin as is required to inactivate one unit amount of toxin at this temperature.

In experiments in this laboratory it has been found that when present in haddock press juice (pH 7.05) the relationship between heating destruction time at a particular temperature for 10 times a unit amount of type E toxin as compared to that for a unit amount of toxin is only 5.5 to 1. This provides for an even greater destruction of toxin during the cooking of fish, should toxin be present, than was previously indicated.

In haddock inoculated with 1000 spores per gram of type E, Cl. botulinum, irradiated at 200 kilo rad and held for 42 days at 45°F, the highest titer of toxin which has been found is 200 MLD_{50} units per gram, this is equivalent to 22,680 MLD50 units per 4 oz. serving. The deep fat frying of haddock under standard conditions destroys much more toxin than this amount.

In haddock inoculated with 1000 spores per gram of type E, Cl. botulinum, irradiated at 200 kilo rad and stored at 75°F

for 10 days the highest titer of toxin found (tested daily) was 2000 MLD_{50} units per gram, equivalent to 226,800 MLD_{50} units per 4 oz. serving. Calculated on the basis that the number of minutes required to destroy one unit of toxin; the deep fat frying of haddock under standard conditions would destroy more toxin than 226,800 MLD_{50} units.

The effect of pH on the thermal destruction of type E, Cl. botulinum toxin suspended in fish press juice has been determined. The toxin is much more stable at pH 6.0 than at pH 7.0 when heated at 150°F and much more stable at pH 7.0 than at pH 8.0 when heated at the same temperature.

It has been found that there is a factor in haddock press juice which decreases the potency of type E, Cl. botulinum toxin when this material is produced in a dialysis bag in TPG broth and added to the fish press juice without trypsinization. If the type E, Cl. botulinum toxin material is first trypsinized, then diluted in haddock press juice there is no decrease in the potency of the toxin.

It has been found that when present in such concentrations as to provide a count in the 1:10 dilution, the cells of type E, Cl. botulinum can probably be identified by growth in trypticase-peptone-mannose agar containing brom thymol blue (as an indicator of acid production) and incubating at 45°F. Such cultures should be made by the spin-tube technique[2] and incubated for approximately 26 days. Confirmation could be made, if necessary, by isolating cells from acid-producing

colonies, culturing these in a suitable liquid medium and testing the latter culture for toxin.

The spores of type E, Cl. botulinum have been cultured at 45°F, aerobically and anaerobically in irradiation-sterilized ground haddock tissues without and together with the cells of other bacterial species.

In aerobic culture in which type E, Cl. botulinum alone was present toxin was produced but there was no outgrowth of the organism. In aerobic cultures in which type E, Cl. botulinum and some other organism were present the following results were obtained: with Streptococcus lactis there was outgrowth of the latter, possibly a slight outgrowth of Cl. botulinum but no toxin production; with Streptococcus faecalis there was outgrowth of the latter, no outgrowth of Cl. botulinum and no toxin production; with Lactobacillus viridescens there was outgrowth of the latter, no outgrowth of Cl. botulinum and no toxin production; with Cl. sporogenes there was outgrowth of neither organism and no toxin production. With Pseudomonas fragi as well as with Pseudomonas fluorescens there was outgrowth of the Pseudomonas species in each case and also outgrowth of Cl. botulinum and toxin production in each case.

With Achromobacter there was outgrowth and toxin production, but as with Ps. fluorescens, at least 20 days at 45°F were required for toxin production, whereas with Ps. fragi 10 days at 45°F were required for outgrowth of Cl. botulinum, type E and toxin production.

In anaerobic cultures in which these organisms were inoculated together as previously indicated there was no outgrowth of Cl. botulinum and no toxin production in any case and among the other organisms inoculated separately or together with the latter organisms only S. faecalis, S. lactis and L. viridescens grew out.

It is thus indicated that the type of bacteria which causes spoilage of fresh fish, and which is largely destroyed by irradiation treatment, is among the organisms which facilitate outgrowth and toxin production by type E, Cl. botulinum in haddock. Moreover, this occurs under aerobic conditions. Achromobacter, the spoilage organism of irradiation-pasteurized fish, may do the same thing but requires a longer time at 45°F to set up conditions for growth of type E, Cl. botulinum. This implies that as far as botulism is concerned, irradiation-pasteurized fresh fish products may constitute a lesser public health hazard than fresh fish which are handled in the usual manner.

It is considered that the irradiation-pasteurization of haddock presents no particular public health hazard regarding botulism, for the following reasons:

a) There is no outgrowth or toxin production of type E, Cl. botulinum in haddock when inoculated with this organism at high concentrations, irradiation-pasteurized and held at 33, 35 or 40°F.

b) In haddock inoculated with 1×10^2 spores of type E, Cl. botulinum, outgrowth and toxin production does not occur

when the product is irradiation-pasteurized and held for three times the expected storage life at 45°F.

c) Type E, Cl. botulinum is usually not present in 555 gram portions of commercially produced haddock fillets.

d) The highest concentration of type E, Cl. botulinum which has been found, in those samples of commercial haddock fillets which contained this organism, was 0.17 per gram.

e) It has been shown that the heat applied during the deep fat frying of haddock portions is sufficient to inactivate a number (about 47) of 10,000 LD_{50} mouse units of type E, Cl. botulinum toxin.

f) It has been found that the type of bacteria which causes spoilage of fresh fish enhances growth and toxin production of type E, Cl. botulinum in fish. These bacteria are mostly destroyed by irradiation-pasteurization treatment of haddock.

In a survey to determine the level of contamination of commercially produced shucked soft-shelled clams (from Massachusetts and Maryland) 3 - 11.1 gram samples and 15 - 555 gram samples have been cultured on a Most Probable Number basis and tested for type E, Cl. botulinum toxin. Four of these samples were toxic. In those samples containing toxin, the highest cell concentration of type E, Cl. botulinum indicated was 2 per 100 grams or 0.02 per gram.

METHODS

Production and Harvesting of Spores for the Inoculation of Haddock Fillets

A portion of 10 ml. of Trypticase-Peptone-Glucose broth containing 0.2% of sodium thioglycollate was inoculated with the spores of type E, Cl. botulinum. This suspension was heat shocked at 140°F for 15 minutes, stratified with vaspar then incubated at 30°C for about 18 hours. This culture was transferred to 100 ml. of the same medium which had been preheated, was then stratified and incubated for 24 hours at 30°C. The 100 ml. culture was then transferred to 1 liter of the preheated medium which was stratified and incubated at 30°C for 72 hours.

The spores were recovered by centrifuging 100 ml. portions at 7000 to 8000 RPM for 20 minutes in a refrigerated centrifuge (32°F). The supernatant was poured off in each case and the residue washed with distilled water then centrifuged. This was repeated three times. The final spore crop was finally collected in a sterile 50 ml. bottle to which 20 ml. of sterile, distilled water had been added.

Spores of Beluga, Detroit, 8E and Minneapolis strains of type E, Cl. botulinum were collected separately in this manner and held at 32°C until used.

Inoculation of Haddock Fillets with Spores of Type E, Cl. botulinum.

The spores were heat-shocked at 140°F for 15 minutes after which counts were made on TPG agar containing thioglycollate, 0.05% of ferric citrate and .07% of sodium sulfite. The spin-tube technique was used. The colonies were counted and calculations made as to what portion of a particular dilution of each strain should be inoculated into fillets to provide concentrations in the inoculated product of 1×10^2, 1×10^4 or 1×10^6 spores per gram of the mixed strains. The heat-shocked spores were mixed and inoculated into fillets with a 0.5 ml. tuberculin syringe and $2\frac{1}{2}$ inch, 22 gauge needle. In inoculating, the needle was inserted a distance of $1\frac{1}{2}$ inches through the long side of the fillet, between the front and back, and as the needle was withdrawn through a distance of 3/4 of an inch the suspension was injected.

Quantities of 10 pounds of fillets were inoculated with a particular concentration of spores and after inoculation the 10-pound portion was placed in a polyethylene bag, the bag sealed and placed in a cardboard carton made to hold this quantity of product. The cartons were held at 32°F until the product was irradiated, within a few hours after it had been inoculated with spores.

Irradiation and Storage of Inoculated Fillets.

For doses of 150 and 250 kilo rad the boxes of fillets were merely placed in the center irradiation chamber of the Mark I Co^{60} source, the chamber lowered into the well and allowed to remain there for the time required to provide the required dose. Due to the longer time required, when a dose of 350 kilo rad was applied, the cartons in the center irradiation chamber were surrounded with crushed ice during irradiation treatment. After irradiating, the cartons were placed in storage at 33, 35, 40 or 45°F until tested for toxin.

The concentrations of spores of mixed strains of type E, Cl. botulinum per gram of fish flesh used for inoculation, the irradiation doses applied and the storage temperatures and times used after irradiation and before testing for toxin are listed in the following:

Inoculum Level (spores per gram)	Irradiation Dose (rad.)	Storage Temp. (°F)	Storage Time (normal storage life in days)	Additional Storage Time (days)	
10^2	150,000	33	15	90	120
10^4	150,000	33	15	90	120
10^6	150,000	33	15	90	120
10^2	250,000	33	25	90	120
10^4	250,000	33	25	90	120
10^6	250,000	33	25	90	120
10^2	350,000	33	35	90	120
10^4	350,000	33	35	90	120
10^6	350,000	33	35	90	120

continued

Inoculum Level (spores per gram)	Irradiation Dose (rad.)	Storage Temp. (°F)	Storage Time (normal storage life in days)	Additional Storage Time (days)	
10^2	150,000	35	13	90	120
10^4	150,000	35	13	90	120
10^6	150,000	35	13	90	120
10^2	250,000	35	21	90	120
10^4	250,000	35	21	90	120
10^6	250,000	35	21	90	120
10^2	350,000	35	30	90	120
10^4	350,000	35	30	90	120
10^6	350,000	35	30	90	120
10^2	150,000	40	10	20	30
10^4	150,000	40	10	20	30
10^6	150,000	40	10	20	30
10^2	250,000	40	17	34	51
10^4	250,000	40	17	34	51
10^6	250,000	40	17	34	51
10^2	350,000	40	22	44	66
10^4	350,000	40	22	44	66
10^6	350,000	40	22	44	66
10^2	150,000	45	8	16	24
10^4	150,000	45	8	16	24
10^6	150,000	45	8	16	24
10^2	250,000	45	14	28	42
10^4	250,000	45	14	28	42
10^6	250,000	45	14	28	42
10^2	350,000	45	18	36	54
10^4	350,000	45	18	36	54
10^6	350,000	45	18	36	54

Testing of Fillets for Toxicity After Inoculation, Irradiation and Storage.

After a particular storage period had ended the liquid which had accumulated in the package was trypsinized (0.1 ml of 1% trypsin to 1.0 ml. of drip) at 98.6°F for 45 minutes

then centrifuged (32°F) at 8000 RPM for 10 minutes, the centrifuged supernatant was diluted 1:10 with gel-phosphate buffer (0.4% Na_2HPO_4 and 0.2% gelatin in 1000 ml. distilled H_2O; adjusted to pH 6.2). This was injected intraperitoneally into unprotected mice and into mice protected with type E, Cl. botulinum antitoxin. Untrypsinized drip liquid was also injected and in this case the drip was centrifuged (as above) and diluted 1:10 in gel-phosphate buffer prior to injection.

For each test one protected and two non-protected mice were used for both trypsinized and non-trypsinized extracts.

Mice were observed for symptoms after 24 and 48 hours following injection.

Examination of Commercial Haddock Fillets and Shucked Soft-Shelled Clams for the Presence of Type E, Cl. botulinum.

The haddock fillets or clams were ground with a sterile hand grinder, mixed and placed in preheated and cooled TPG broth containing 0.2% of sodium thioglycollate. The medium was stratified with vaspar and incubated at 86°F for 3 days prior to testing for toxin. This was done by MPN technique, so that for each sample, five 100-gram portions of fish or clams were cultured in five 1000-ml. portions of medium, five 10-gram portions of fish or clams in five 100-ml. portions of medium and five 1-gram portions of fish or clams in five 10-ml. portions of medium. The total sample, therefore, consisted of 555 grams of ground haddock fillets or clams. This was the

case with all but 3 samples of clams in which case 10, 1 and 0.1-gram samples were cultured, the total sample consisting of 11.1 grams. Each culture was tested for toxins by mouse injection. In this case the cultures were first centrifuged, the supernatant trypsinized, diluted 1:10 in gel-phosphate buffer and injected. Non-trypsinized, 1:10 dilutions in gel-phosphate buffer were also injected. Otherwise the test was made in the manner previously indicated.

Anaerobic and Aerobic Counts on Inoculated, Irradiated and Unirradiated and Stored Fish Fillets.

Dilutions of the fish press juice were cultured in TPG agar containing 0.2% sodium thioglycollate, 0.05% ferric citrate and 0.07% sodium bisulfite and 0.05% of sodium bicarbonate (sterile solution). The Tsuji spin-tube technique was used, the inoculated liquid agar being spun in a cold water bath to provide a layer of medium around the inside of the tube. The cotton stopper was pushed in, 1 gram of pyrogallic acid powder and 1 ml. of 20% sodium carbonate added on top of the stopper, a rubber stopper inserted and the tube inverted. Cultures were incubated at 86°F and counts were made after 18, 24 and 36 hours. Black colonies were considered to be indicative of clostridia.

In making aerobic counts the diluted drip liquid was surface plated on Eugon agar. Plate cultures were incubated for 5 days at 68°F before colony counts were made.

Test to Determine the Effect of Fish Press Juice on the Spores of Type E, Cl. botulinum.

Fish press juice was obtained by freezing and thawing ground haddock fillets. This was heated to 158°F and filtered through cheesecloth. About 25% of the solids were resuspended in the broth and blended with it. This was placed in tubes and autoclaved for purposes of sterilization.

The spores of 8E and Minneapolis strains of type E, Cl. botulinum were heat-shocked (140°F for 15 minutes) and counts were made on TPG agar containing 0.2% sodium thioglycollate which was stratified with 2% agar. Cultures were incubated at 86°F. When the counts had been made a tube of sterile fish-press juice was inoculated with such dilutions of spores as to contain approximately 1 x 10^6 per ml. Minneapolis and 8E strains of type E, Cl. botulinum were inoculated into separate tubes of fish press juice and both were placed at 35°F. After 24 hours at this temperature, counts were again made on the fish-press juice as indicated above.

Determination of the Thermal Destruction Time of Type E, Cl. botulinum Toxin.

One ml. of a mixture of the heat-shocked spores of 8E, Minneapolis, Detroit and Beluga strains of type E, Cl. botulinum was inoculated into 100 ml. of preheated TPG medium with thioglycollate. This was stratified with vaspar and incubated at 86°F for 3 days. This culture was centrifuged for 20 minutes at 8000 RPM in a refrigerated centrifuge and the supernatant

collected in a sterile flask and held at 35°F until used. Portions of the supernatant were trypsinized, diluted in fish press juice and allowed to stand at 35°F for several hours. This was diluted in gel-phosphate buffer and injected intraperitoneally into protected and non-protected mice. Six non-protected mice were used in each case and calculations were made from the dilution at which 50% of the unprotected mice died to obtain the LD_{50}. In fish press juice the concentration was 10,000 LD_{50} units of toxin. In this form it was heated for various times and temperatures after which 0.5 ml. of the heated then trypsinized material was injected into 3 non-protected mice and one protected mouse and deaths recorded. The time of heating at a particular temperature at which no deaths occurred was considered to be the inactivation time. The spread in heating times at which deaths occurred and that at which no deaths occurred was 0.5 to 10 minutes. A curve was drawn on semi-log paper in which temperature was plotted on the abscissa and time on the ordinate (log scale).

Determination of the Temperatures Attained in Haddock Portions During Deep Fat Frying and Subsequent Cooling.

Portions of haddock fillets were cut to such a size that 9 oz. of fish would be cooked in one gallon of oil. A thermocouple, attached to a continuous temperature recorder, was inserted into the center of the thickest portion of the piece of fish and the portion was placed in oil at 375°F.

The temperature of the interior of the fish was recorded continuously during cooking and cooling. The maximum fish thickness varied between 1/2 and 1 inch and cooking times varied between 3.5 and 6.5 minutes. Since the temperature continued to rise for some time after removal of the fish from the cooking oil and to decrease slowly after the maximum temperature had been attained, the temperature was recorded during cooling down to a temperature of 140°F.

Integration of the Time-Temperature Effect on the Destruction of Type E, Cl. botulinum Toxin.

A table was first prepared in which certain data from the cooking and cooling curve (that portion of fish attaining the lowest temperature) and the thermal destruction curve for 10,000 LD_{50} mouse units of toxin were recorded. This included (1) the time of cooking or cooling, (2) the temperature in the fish at that time, (3) the destruction time in minutes at that temperature for 10,000 LD_{50} mouse units of toxin, and (4) the destruction rate for the toxin at this temperature (reciprocal of the destruction time at the particular temperature).

A curve was then drawn on Cartesian coordinates in which the destruction rate (for toxin) per minute was plotted on the ordinate and the time of heating on the abscissa. Since one unit on the ordinate was equivalent to the destruction of two-tenths of 10,000 LD_{50} units of toxin and one unit on the

abscissa equal to one minute, one square under the curve was equivalent to the destruction of 0.2 x 1 or (0.2 x 10,000) = 2000 LD_{50} units of toxin. That being the case 1/0.2 or 5 squares under the curve would be equivalent to the destruction of 10,000 LD_{50} mouse units of toxin.

Toxin Concentration in Haddock Fillets Inoculated with Type E Cl. botulinum, Irradiated at 200 Kilo Rad and Held at 45°F or at 75°F.

The fillets were cut into 50 gram portions and inoculated with type E, Cl. botulinum to contain a concentration of 1000 spores per gram. Inoculation was carried out by injection within the flesh with hypodermic needles. Inoculated portions were placed in polyethylene bags, the bags sealed, placed in cartons and irradiated in the Mark I Co_{60} source to a dosage of 200 kilo rads.

After irradiation some samples were held at 75°F, others at 45°F. Samples were tested for toxin titer, aerobic and clostridial counts. Toxin titer determinations were made on the ground fish diluted with buffer as previously described. Aerobic counts were made by surface plating on Eugon agar with 0.5% yeast extract, after incubating for 5 days at 68°F. Clostridial counts were made by culturing in TPG agar with ferric citrate, 0.07% sodium sulfite, 0.05% of sterile (filtered solution) sodium bicarbonate and 0.2% of sodium thioglycollate. The medium was melted and poured into Miller-Prickett tubes

after the inoculum had been added and was stratified with 2% agar containing 0.2% of sodium thioglycollate. Cultures were incubated at 86°F and counts were made after 18, 24 and 36 hours. Black colonies were considered to be indicative of clostridia.

Samples held at 75°F were tested for toxin and examined for aerobic bacteria and clostridia daily. The same tests were made every 7 days on samples held at 45°F.

Production of High Titer Type E, Cl. botulinum Toxin.

The following liquid media were inoculated with spores of the 8E strain of Cl. botulinum: TPG with thioglycollate and 0.5% glucose, TPG with thioglycollate and 0.1% glucose, brain heart infusion broth with thioglycollate and 0.2% glucose, and liver broth (pH 7.2). After incubation at 75°F for 6 days the various media were tested for toxin titer in the regular manner.

Since TPG medium with thioglycollate and 0.1% glucose gave the highest titer an attempt was made to obtain a higher titer by growing a mixture of the four strains of type E, Cl. botulinum in this medium in a cellulose bag containing 70 ml. of medium immersed in a flask containing 1.5 liters of medium. The liquid medium could dialyze through the bag but cells and toxin could not. The culture was centrifuged, diluted, trypsinized and then tested for toxin concentration in the usual manner. When the culture material was diluted

in fish press juice, then trypsinized to test for toxin, it was found that the toxin had a titer of 1000 LD_{50} mouse units. However, when trypsinized and then diluted in fish press juice to test for toxin, the titer was 500,000 LD_{50} mouse units.

Strain 8E, type E, Cl. botulinum was next cultured in dialysis bags containing 70 ml. of media immersed in a flask containing 1½ liters of medium. The medium in this case consisted of TPG broth (1% glucose) with thioglycollate, the trypticase, peptone and glucose and thioglycollate being dissolved in a haddock extract (1 liter of distilled water to 1 pound of haddock, simmered for 30 minutes and filtered through cheesecloth). Incubation was carried out at 75°F and tests were made for toxin by diluting in fish press juice then trypsinizing, and injecting into mice. This material diluted in fish press juice had a titer of 500,000 LD_{50} mouse units after 8 days of incubation and the fish press juice used for dilution did not lower the titer. This material was used to determine the effect of concentration of toxin on heat inactivation time.

Effect of Toxin Concentration on Heat-Inactivation Time.

Starting with a 500,000 LD_{50} mouse unit concentration of toxin regulated to pH 7.05 the concentration was diluted with sterile TPG-haddock press juice broth to obtain concentrations of 50,000, 5,000 and 500 LD_{50} mouse units. All of these concentration of toxin were placed in capillary tubes, heated for various times at 150°F, then trypsinized and tested

for toxin in the usual manner without further dilution. An inactivation curve was then drawn in which the inactivation time was plotted on the log scale against toxin titer on the Carteisan coordinate.

Effect of pH on the Heat-Inactivation Time of Type E, Cl. botulinum Toxin.

The original pH of the toxin concentrate produced in TPG-thioglycollate-fish extract broth was 6.15. This was used as such to determine heat-inactivation time at that pH and was adjusted to pH 7.05 and 8.10 with 20% sodium hydroxide solution. The thermal inactivation time at 150°F was determined at each of the adjusted pH's in the usual manner. A curve was then drawn in which inactivation time was plotted on the log scale and pH on the Cartesian scale.

Development of Methods to Determine Type E, Cl. botulinum in Foods When Present in Sufficient Concentrations.

Pure cultures of 8E, Detroit, Minneapolis and Beluga strains of type E, Cl. botulinum were cultured on trypticase-peptone-mannose (1%) agar with 0.2% of sodium thioglycollate. This medium contained as acid indicators, 2.4 PPM of neutral red, or 8 PPM of brom thymol blue, brom cresol purple or methyl red, to detect acid formation from mannose.

Cultures were made by the spin tube technique and anaerobiosis was obtained by adding solid pyrogallic acid on top of the pushed in cotton stopper, adding 20% sodium

carbonate solution to this, stoppering the tube with a rubber stopper and inverting the tube. Cultures were incubated at 45°F and observed for colonies producing acid as indicated by a change in the color of the dye.

Effect of the Growth of Other Bacteria on Growth and Toxin Production by Type E, Cl. botulinum.

Haddock fillets were ground, placed in impervious containers and sterilized with an irradiation dose of 4.0 megarad in the Co^{60} source. This material was held for 24 to 48 hours at 36 to 40°F to obviate any effect of the irradiation treatment on the ground fish which might influence the growth of bacteria in this material.[3]

Portions of 50 grams were now placed in plastic bags and inoculated and mixed with the Detroit strain of type E, Clostridium botulinum, Streptococcus lactis, Streptococcus faecalis, Lactobacillus viridescens, Clostridium sporogenes, Pseudomonas fragi, Pseudomonas fluorescens, or three mixed strains of Achromobacter. (These Achromobacter cultures were obtained from Dr. J. S. Lee, Oregon State University, who isolated them from spoiled, irradiated dover sole.) Cultures of this type were also prepared in which one of each of the other organisms was inoculated (approximately the same cell concentration of each) together with the Detroit strain of type E, Cl. botulinum.

These cultures were incubated aerobically at 45°F and tests were made for toxin together with aerobic and clostridial counts after 0, 1, 5, 10, 20 and 30 days. Aerobic counts for P. fragi and P. fluorescens or Achromobacter were made by surface plating on Eugon agar with 0.5% yeast extract incubated 5 days at 68°F, and for S. lactis, S. faecalis and L. viridescens by surface plating on APT agar incubated at 68°F for 5 days. Clostridial counts were made by the spin tube method on TPG agar containing 0.2% of sodium thioglycollate, 0.05% of ferric citrate and 0.07% of sodium sulfite. The cultures were made anaerobic with pyrogallic acid and sodium carbonate solution and were incubated at 30°C. Counts for black colonies were made after 24, 48 and 72 hours. For anaerobic counts in samples in which Achromobacter and type E, Cl. botulinum were inoculated together, the same medium was used in Miller-Prickett tubes. The medium was stratified with 2% agar containing thioglycollate.

Anaerobic cultures for each individual organism and for type E, Cl. botulinum together with each of the other organisms in ground, pre-sterilized haddock, were prepared as follows: portions of 10 grams of ground fish in a sterile glass tube, were inoculated with one organism alone or with type E, Cl. botulinum together with one of the other bacteria and then mixed. A sterile plastic "dispo" plug was then shoved into the tube, solid pyrogallic acid and 20% sodium carbonate added and the tube plugged with a rubber stopper. It was

unnecessary to invert the tube. Tubes were incubated at 45°F. Tests for toxin and aerobic and anaerobic bacterial counts were made after 0, 1, 5, 10, 20 and 30 days of incubation.

In testing for toxin the liquid from the ground fish culture was diluted 1:10 in gel-phosphate buffer, trypsinized and injected intraperitoneally into 3 unprotected and one protected mouse. The 1:10 dilution of the culture liquid was also injected into 2 unprotected mice without trypsinization.

RESULTS AND DISCUSSION

The results of toxicity tests and of aerobic and clostridia bacterial counts on haddock, uninoculated and irradiated or inoculated with type E, Cl. botulinum and irradiated, both of which were stored at 33, 35, 40 or 45°F, are listed in Tables 1, 2 and 3. It is evident from the results that no uninoculated sample became toxic during holding periods equivalent to three times the storage life of the irradiated (150, 250 or 350 kilo rad) product at any of the above temperatures.

In product inoculated with mixed strains of type E, Cl. botulinum (levels of 1×10^2, 1×10^4 or 1×10^6 spores per gram) none of the irradiated products became toxic in three times the irradiated storage life at 33, 35 or 40°F.

In products inoculated with spores of type E, Cl. botulinum, irradiated and held at 45°F, fillets inoculated with 1×10^2 spores per gram did not become toxic in three times the irradiated storage life. Product inoculated with 1×10^4 or 1×10^6 spores per gram and irradiated became toxic in either three times the irradiated storage life (1×10^4 spores per gram) or in the normal irradiated storage life time (1×10^6 spores per gram). However, two things should be noted: a) that excepting in one case, when samples became toxic the spore count never reached the level which the fillets were originally inoculated to contain, and b) the highest

level of contamination with type E, Cl. botulinum that has been found in commercial haddock fillets has been 0.17 per gram. The level, therefore, of 1×10^2 per gram (the lowest level used for inoculation) is more than 100 times the contamination level which may be expected in commercial haddock fillets and none of the product inoculated at this level irradiated and stored at 33, 35, 40 or 45°F has become toxic in three times the irradiated storage life at any of the temperatures indicated.

The results of toxicity tests on haddock inoculated with 1×10^4 or 1×10^6 spores per gram of type E, Cl. botulinum and held at 45°F without irradiation treatment or after irradiating at 100 or 200 kilo rad are listed in Table 4. It is evident that while irradiated and unirradiated samples inoculated at a level of 1×10^6 spores per gram became toxic in the same period of time, in samples inoculated at a level of 1×10^4 spores per gram the unirradiated samples became toxic in a shorter storage time than did the irradiated samples.

Since, in the past, samples have become toxic without outgrowth of spores to the level at which they were originally inoculated, it may be possible that at high levels of inoculation proteolytic enzymes, in the fillets themselves or produced by the growth of other psychrophilic bacteria, activate residual toxin precursor in the spores and cause toxicity.

The results of tests to determine the presence of type E, Cl. botulinum in commercially produced haddock fillets are tabulated in Tables 5 and 6. It is evident that among 34 555-gram samples only 8 have been found to contain this organism. It is also evident that when contaminated with type E, Cl. botulinum the concentration of this organism in haddock fillets is very low.

Counts on haddock press juice held 24 hours at 35°F after inoculation with type E, Cl. botulinum, are listed in Table 7. It is evident that compared to the numbers inoculated there has been a decrease to approximately one thousandth of the numbers inoculated.

The curve for thermal destruction time at various temperatures for 10,000 LD_{50} units of type E, Cl. botulinum toxin in fish press juice is shown in Figure 1. In Table 8 the temperatures attained in four different samples during the deep fat frying and subsequent cooling of haddock portions are listed. Tabulation of the temperatures reached at various times in the portion of haddock attaining the lowest temperature, the destruction times for 10,000 LD_{50} units of toxin at these particular temperatures and the destruction rates (1/destruction time in minutes) for these temperatures has been made in Table 9.

In Figure 2 the curve integrating destruction rates with time of heating or cooling during cooking, has been drawn. According to this curve one unit on the ordinate is

equivalent to the destruction of 0.2 x 10,000 LD_{50} mouse units of toxin and one unit on the abscissa is equivalent to 1 minute. One square under the curve is therefore equivalent to the destruction of 0.2 x 1 x 10,000 LD_{50} units of toxin and 1/0.2 or 5 squares under the curve are equivalent to the destruction of 10,000 LD_{50} units of toxin. This area, equivalent to the destruction of 10,000 LD_{50} mouse units of toxin, has been cross-hatched under the curve. On a weight basis 5000 LD_{50} mouse units of toxin is about one man lethal dose. The total area under the curve is about equivalent to the destruction of 21 - 10,000 LD_{50} mouse units or 42 man lethal doses. Ordinary deep fat frying of haddock would, therefore, destroy considerable amounts of type E, Cl. botulinum toxin, were this material present.

It should be pointed out, however, that the above calculations have been based on the findings of Maunder et al.[1] which indicate that at pH 6.0, in buffer, it requires 10 times a unit amount of heating at a particular temperature to destroy 10 times a unit amount of toxin. In our work in this laboratory in fish press juice at pH 6.7, it has been found that only about 5.5 times a unit amount of heating is required to destroy 10 times a unit amount of toxin (Figure 3). This means that during the deep fat frying of haddock, as calculated previously, about 38 x 10,000 LD_{50} mouse units of type E, Cl. botulinum toxin would be destroyed

The amounts of toxin produced in haddock fillets inoculated with 1000 spores per gram of type E, Cl. botulinum irradiated at 200 kilo rad and stored for 42 days at 45°F are listed in Table 12. Since at 45°F 0.5 ml. was toxic in the 1:100 dilution, 1 gram of tissue would contain 200 MLD_{50} units of toxin.

The amounts of toxin produced in haddock fillets inoculated with 1000 spores per gram of type E, Cl. botulinum irradiated at 200 kilo rad and stored at 75°F for 10 days are listed in Table 13. Since at 75°F 0.5 ml. was toxic in the 1:1000 dilution, 1 gram of tissue would contain 2000 MLD_{50} units of toxin.

It should be noted that irradiation with 200 kilo rad would probably reduce the spore concentration in the fish to about 100 per gram at the start since the D value is about 140,000 and there is a shoulder on the curve which causes the first log cycle reduction to be greater than 140 kilo rad.

The effect of pH on the destruction time of type E, Cl. botulinum toxin in fish press juice is indicated in Figure 4. According to this curve, 50,000 LD_{50} mouse units of toxin at pH 6.0 are destroyed in about 340 minutes, at pH 7.0 in about 130 minutes and at pH 8.0 in 29 minutes, when heating is carried out at 150°F. The toxin is much less stable to heat, therefore, at the higher pH levels. Moreover, there appears to be a change in the slope of the curve at about pH 7.0, the slope of the curve decreasing above this pH.

It has been found that, when present in numbers sufficient to be cultured in the 1:10 dilutions, the spores of type E, Cl. botulinum can be selected from some other types of clostridia by growth under anaerobic conditions (spin tube method) at 45°F in Trypticase Peptone Mannose agar containing sodium thioglycollate and a suitable dye to indicate acid. Absolute identification would require isolation from typical colonies, growth in liquid medium and testing for toxin by mouse injection.

The results of tests to indicate the presence of type E, Cl. botulinum when grown in bacteriological media are listed in Table 12.

Bacterial counts and toxicity tests on ground, irradiation-presterilized haddock inoculated with various bacteria alone or with type E, Cl. botulinum together with another organism then held at 45°F for periods up to 30 days are listed in Table 13. The other organisms used were: S. faecalis, S. lactis, L. viridescens, Cl. sporogenes, Ps. fragi and Ps. fluorescens and mixed strains of Achromobacter. Cultures were incubated both aerobically and anaerobically.

Toxin was produced under aerobic conditions in cultures containing Cl. botulinum alone but this required 35 days at 45°F and there was no outgrowth of the organism. The only other aerobic cultures containing toxin were those in which both Cl. botulinum, type E and either Ps. fragi, Ps. fluorescens

or Achromobacter (mixture of three isolates of Achromobacter) were inoculated into the fish tissues as a mixed culture. Together with Ps. fragi, type E, Cl. botulinum produced toxin in 10 days at 45°F; with Ps. fluorescens, type E, Cl. botulinum produced toxin in 20 days; with Achromobacter, type E, Cl. botulinum produced toxin in one instance in 20 days and in another instance in 30 days. In all instances of toxin in mixed culture samples (type E, with Pseudomonads or Achromobacter) there was outgrowth of the aerobic organism and some outgrowth of type E, Cl. botulinum. It can be seen from Table 11 that when outgrowth and toxin production occurred in mixed culture samples the aerobic count was approximately 100,000,000 per gram. The reason why outgrowth and toxin production by type E, Cl. botulinum eventually occurs in such mixed cultures, however, appears not to depend solely on the cell concentration of aerobes (Pseudomonas or Achromobacter) since all of these organisms appear to grow in haddock tissues at approximately the same rate.

Pseudomonas fragi is the type of organism which spoils fresh fish[4] and Achromobacter is probably the type of organism that spoils irradiation-pasteurized fish.[5] Also it is a fact that irradiation-pasteurized fish cannot be held at 45°F for two times the storage life of fresh fish at this temperature, without becoming spoiled prior to this time. Considering these things it would appear that fresh refrigerated haddock is as much or more of a public health hazard

from the standpoint of type E botulism as is irradiation-pasteurized haddock.

There was one instance of possible outgrowth of Cl. botulinum without toxin production. This occurred when the organism was cultured together with S. lactis, the latter of which grew out to high concentrations.

There is the possibility that both Achromobacter species (the organisms usually causing spoilage of irradiation-pasteurized haddock)[5] and Pseudomonas species (the organisms causing spoilage of fresh haddock)[4], when grown in haddock to large concentrations may set up conditions which facilitate the growth of type E, Cl. botulinum. However, cell concentrations far greater than that present at spoilage appear to be necessary in both cases.

The concentration of type E, Cl. botulinum found in 3 - 11.5 gram samples and in 15 - 555 gram samples of commercial shucked soft-shelled clams is listed in Table 14. It can be seen that this organism was found in 4 samples. According to estimations made from the number of positive tubes and Most Probable Number tables, the highest level of contamination was 2 cells per 100 grams or 0.02 per gram. It should be noted that product harvested in both Massachusetts and Maryland (Chesapeake Bay) have been examined.

REFERENCES

(1) Maunder, D. T., W. P. Segner, C. F. Schmidt and J. K. Balty. 1964. Growth characteristics of type E, Clostridium botulinum in the temperature range of 34 to 50°F. Annual Report to the United States Atomic Energy Commission. Contract No. AT(11-1)-1183.

(2) Tsuji, K. 1959. Thermal destruction characteristics of spore forming bacteria within the intermediate pH range. Ph.D. Thesis. University of Massachusetts.

(3) Solberg, M. and J. T R. Nickerson. 1963. Growth support potential of irradiated chicken for Escherichia coli and Staphylococcus aureus. Jour. Food Technol. 17: 915.

(4) Castel, C. H. and M. F. Greenough. 1957. The action of Pseudomonas on fish muscle. Part 1. Organisms responsible for odours produced during insipient spoilage of chilled fish muscle. Jour. Fisheries Bd. Can. 14: 617.

(5) Lee, J. S. 1965. Effect of irradiation on the microbial flora surviving irradiation pasteurization of seafoods. Monthly Progress Report-25. May 1 to May 31. Atomic Energy Commission Contract Number AT(04-3)-502.

LIST OF DOCUMENTS

Monthly Progress Reports

Monthly Progress Letter
Contract: AT(30-1)-3325. The Effect of Gamma Rays on Haddock and Clams Inoculated with *Clostridium botulinum*, Type E.
Period Covered: January 1 to January 31, 1965

Monthly Progress Letter
Contract: AT(30-1)-3325. The Effect of Gamma Rays on Haddock and Clams Inoculated with *Clostridium botulinum*, Type E.
Period Covered: February 1 to February 28, 1965

Monthly Progress Letter
Contract: AT(30-1)-3325. The Effect of Gamma Rays on Haddock and Clams Inoculated with *Clostridium botulinum*, Type E.
Period Covered: March 1 to March 31, 1965.

Monthly Progress Letter
Contract: AT(30-1)-3325. The Effect of Gamma Rays on Haddock and Clams Inoculated with *Clostridium botulinum*, Type E.
Period Covered: April 1 to April 30, 1965.

Monthly Progress Letter
Contract: AT(30-1)-3325. The Effect of Gamma Rays on Haddock and Clams Inoculated with *Clostridium botulinum*, Type E.
Period Covered: May 1 to May 31, 1965.

Monthly Progress Letter
Contract: AT(30-1)-3325. The Effect of Gamma Rays on Haddock and Clams Inoculated with *Clostridium botulinum*, Type E.
Period Covered: June 1 to June 30, 1965.

Monthly Progress Letter
Contract: AT(30-1)-3325. The Effect of Gamma Rays on Haddock and Clams Inoculated with *Clostridium botulinum*, Type E.
Period Covered: July 1 to July 31, 1965.

Monthly Progress Letter
Contract: AT(30-1)-3325. The Effect of Gamma Rays on Haddock and Clams Inoculated with *Clostridium botulinum*, Type E.
Period Covered: August 1 to August 31, 1965.

Monthly Progress Letter MIT Report No. B21
Contract: AT(30-1)-3325. The Effect of Gamma Rays on Haddock and Clams Inoculated with Clostridium botulinum, Type E.
Period Covered: September 1 to October 31, 1965.

Annual Report

S. A. Goldblith and J. T. R. Nickerson, "The Effect of Gamma Rays on Haddock and Clams Inoculated with Clostridium botulinum, Type E." Contract: AT(30-1)-3325. 1965 (published in September) MIT Report No. B21

Final Report

S. A. Goldblith and J. T. R. Nickerson, "The Effect of Gamma Rays on Haddock and Clams Inoculated with Clostridium botulinum, Type E." Contract: AT(30-1)-3325. 1965 MIT Report No. B22

Publications

J. T. R. Nickerson and J. J. Licciardello. July 1965. Storage of Eviscerated Haddock in Refrigerated Sea Water Treated with Gamma Radiation. Journal of Milk and Food Technology, Vol. 28, No. 7 (P. 223-226).

Table 1

Toxicity Data and Aerobic and Anaerobic Counts for Skinless Haddock Fillets, Non-Inoculated and Inoculated with Spores of Type E, Cl. botulinum Irradiated at 150,000 rad and Stored at 33, 35, 40 and 45°F.

Storage Temperature (°F)	Inoculum Level (spores/g)	Storage Time (Days)	Aerobic Counts (per gram) Sample 1	Aerobic Counts (per gram) Sample 2	Anaerobic Counts (Clostridia per gram) Sample 1	Anaerobic Counts (Clostridia per gram) Sample 2	Increases in Toxicity Sample 1	Increases in Toxicity Sample 2
45	0	8	3.8×10^7	1.1×10^7	$<10^1$	$<10^1$	-	-
	0	16	6.9×10^7	4.9×10^7	$<10^1$	$<10^1$	-	-
	0	23	1.4×10^8	1.8×10^8	$<10^1$	$<10^1$	-	-
	10^2	8	6.7×10^5	5.0×10^5	$<10^1$	$<10^1$	-	-
	10^2	18	7.6×10^6	6.3×10^8	$<10^1$	$<10^1$	-	-
	10^2	26	6.6×10^7	1.8×10^6	$<10^1$	$<10^1$	-	-
	10^4	8	7.6×10^6	8.4×10^6	1.0×10^2	1.0×10^2	-	-
	10^4	18	1.4×10^8	1.3×10^8	3.0×10^1	7.0×10^1	-	-
	10^4	26	1.8×10^8	1.4×10^8	5.0×10^2	3.0×10^2	+	+
	10^6	8	7.5×10^6	1.5×10^8	4.0×10^1	8.0×10^1	-	-
	10^6	15	1.2×10^8	1.3×10^8	6.2×10^4	2.3×10^4	+	+
40	0	9	2.5×10^5	2.9×10^5	$<10^1$	$<10^1$	-	-
	0	20	1.3×10^7	4.6×10^7	$<10^1$	$<10^1$	-	-
	0	30	8.1×10^7	6.7×10^7	$<10^1$	$<10^1$	-	-
	10^2	11	1.8×10^6	1.1×10^6	$<10^1$	$<10^1$	-	-
	10^2	20	2.0×10^7	1.8×10^7	$<10^1$	$<10^1$	-	-
	10^2	32	3.2×10^7	8.4×10^7	$<10^1$	$<10^1$	-	-
	10^4	11	3.2×10^6	3.9×10^6	$<10^1$	$<10^1$	-	-
	10^4	20	6.0×10^7	1.2×10^8	$<10^1$	$<10^1$	-	-
	10^4	32	1.2×10^8	6.7×10^7	$<10^1$	$<10^1$	-	-
	10^6	9	6.4×10^7	4.1×10^7	1.4×10^3	1.0×10^3	-	-
	10^6	21	6.4×10^7	1.7×10^7	3.0×10^2	2.0×10^2	-	-
	10^6	31	4.5×10^7	6.1×10^7	$<10^1$	$<10^1$	-	-

Table 1 - continued

Storage Temperature (°F)	Inoculum Level (spores/g)	Storage Time (Days)	Aerobic Counts (per gram)		Anaerobic Counts (Clostridia per gram)		Increases in Toxicity	
			Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2
35	0	13	4.0×10^5	5.9×10^5	$< 10^1$	$< 10^1$	-	-
	0	89	---	---	$< 10^1$	$< 10^1$	-	-
	0	120	2.6×10^7	1.0×10^8	$< 10^1$	$< 10^1$	-	-
	10^2	13	3.4×10^6	2.3×10^6	$< 10^1$	$< 10^1$	-	-
	10^2	90	7.1×10^7	---	$< 10^1$	$< 10^1$	-	-
	10^2	120	8.0×10^7	4.1×10^8	$< 10^1$	$< 10^1$	-	-
	10^4	13	3.9×10^6	1.7×10^6	0.5×10^1	0.5×10^1	-	-
	10^4	90	5.7×10^7	4.2×10^7	$< 10^1$	$< 10^1$	-	-
	10^4	120	7.7×10^7	5.7×10^7	$< 10^1$	$< 10^1$	-	-
	10^6	22	1.1×10^8	1.0×10^8	2.0×10^1	$< 10^1$	-	-
	10^6	90	2.8×10^8	5.8×10^8	1.0×10^1	$< 10^1$	-	-
	10^6	120	6.4×10^8	8.6×10^7	10^1	$< 10^1$	-	-
33	0	15	1.5×10^7	3.8×10^7	$< 10^1$	$< 10^1$	-	-
	0	89	7.0×10^7	1.5×10^8	$< 10^1$	$< 10^1$	-	-
	0	120	9.6×10^7	1.1×10^8	$< 10^1$	$< 10^1$	-	-
	10^2	16	4.8×10^6	5.8×10^6	$< 10^1$	$< 10^1$	-	-
	10^2	90	1.2×10^8	1.9×10^8	$< 10^1$	$< 10^1$	-	-
	10^2	120	1.8×10^8	2.2×10^8	$< 10^1$	$< 10^1$	-	-
	10^4	16	1.5×10^7	2.8×10^8	$< 10^1$	$< 10^1$	-	-
	10^4	90	3.6×10^8	5.0×10^8	$< 10^1$	$< 10^1$	-	-
	10^4	120	2.3×10^8	2.7×10^8	$< 10^1$	$< 10^1$	-	-
	10^6	15	4.9×10^5	6.6×10^5	$< 10^1$	1.1×10^2	-	-
	10^6	90	1.0×10^9	8.0×10^8	4.0×10^2	$< 10^2$	-	-
	10^6	120	1.4×10^9	1.3×10^9	1.2×10^4	1.3×10^2	-	-

\+ indicates that the sample is toxic in 1:10 dilution

\- indicates that the sample is not toxic in 1:10 dilution

Table 2

Toxicity Data and Aerobic and Anaerobic Counts for Skinless Haddock Fillets, Non-Inoculated and Inoculated with Spores of Type E, Cl. botulinum Irradiated at 250,000 rad and Stored at 33, 35, 40 and 45°F.

Storage Temperature (°F)	Inoculum Level (spores/g)	Storage Time (Days)	Aerobic Counts (per gram)		Anaerobic Counts (Clostridia per gram)		Increases in Toxicity	
			Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2
45	0	14	5.1×10^7	4.3×10^7	$<10^1$	$<10^1$	-	-
	0	28	1.2×10^8	1.3×10^8	$<10^1$	$<10^1$	-	-
	0	42	1.9×10^8	1.3×10^8	$<10^1$	$<10^1$	-	-
	10^2	14	4.0×10^6	7.0×10^6	$<10^1$	$<10^1$	-	-
	10^2	29	1.5×10^8	2.8×10^8	$<10^1$	$<10^1$	-	-
	10^2	42	1.6×10^8	2.2×10^8	1.4×10^4	2.3×10^4	-	-
	10^4	14	2.0×10^7	4.9×10^7	$<10^1$	4.0×10^1	-	-
	10^4	29	9.7×10^7	6.5×10^7	3.0×10^2	4.0×10^1	-	-
	10^4	42	1.2×10^8	1.2×10^8	2.3×10^4	8.6×10^2	+	+
	10^6	14	1.1×10^8	1.0×10^8	2.9×10^3	1.5×10^3	+	+
40	0	16	8.5×10^6	6.2×10^6	$<10^1$	$<10^1$	-	-
	0	34	1.7×10^8	4.4×10^8	$<10^1$	$<10^1$	-	-
	0	52	9.6×10^7	1.6×10^8	$<10^1$	$<10^1$	-	-
	10^2	16	1.2×10^7	1.6×10^7	$<10^1$	$<10^1$	-	-
	10^2	34	2.2×10^8	8.4×10^7	$<10^1$	$<10^1$	-	-
	10^2	52	1.7×10^8	5.7×10^7	$<10^1$	$<10^1$	-	-
	10^4	16	1.8×10^6	6.1×10^6	$<10^1$	$<10^1$	-	-
	10^4	34	7.6×10^7	1.3×10^8	$<10^1$	$<10^1$	-	-
	10^4	52	1.1×10^8	3.3×10^8	$<10^1$	$<10^1$	-	-
	10^6	16	3.1×10^6	2.4×10^6	1.0×10^2	1.9×10^2	-	-
	10^6	35	2.5×10^6	1.0×10^6	$<10^1$	$<10^1$	-	-
	10^6	51	8.4×10^7	8.6×10^7	$<10^1$	$<10^1$	-	-

Table 2 - continued

Storage Temperature (°F)	Inoculum Level (spores/g)	Storage Time (Days)	Aerobic Counts (per gram) Sample 1	Aerobic Counts (per gram) Sample 2	Anaerobic Counts (Clostridia per gram) Sample 1	Anaerobic Counts (Clostridia per gram) Sample 2	Increases in Toxicity Sample 1	Increases in Toxicity Sample 2
35	0	22	3.1×10^7	9.6×10^7	$<10^1$	$<10^1$	-	-
	0	90	3.1×10^8	6.0×10^8	$<10^1$	$<10^1$	-	-
	0	120	4.4×10^8	1.3×10^8	$<10^1$	$<10^1$	-	-
	10^2	21	4.4×10^6	9.3×10^6	$<10^1$	$<10^1$	-	-
	10^2	90	3.2×10^8	2.5×10^8	$<10^1$	$<10^1$	-	-
	10^2	120	1.1×10^9	8.1×10^8	$<10^1$	$<10^1$	-	-
	10^4	21	8.8×10^6	1.8×10^7	$<10^1$	$<10^1$	-	-
	10^4	90	---	---	$<10^1$	$<10^1$	-	-
	10^4	120	3.4×10^8	5.0×10^8	$<10^1$	$<10^1$	-	-
	10^6	22	7.6×10^6	1.0×10^7	$<10^1$	1.0×10^1	-	-
	10^6	90	5.0×10^8	7.7×10^8	$<10^1$	1.0×10^1	-	-
	10^6	120	2.9×10^8	8.6×10^8	$<10^1$	$<10^1$	-	-
33	0	24	6.4×10^7	1.4×10^8	$<10^1$	$<10^1$	-	-
	0	90	4.1×10^8	2.5×10^8	$<10^1$	$<10^1$	-	-
	0	120	1.6×10^8	1.7×10^8	$<10^1$	$<10^1$	-	-
	10^2	27	1.4×10^7	3.1×10^6	$<10^1$	$<10^1$	-	-
	10^2	90	3.5×10^8	3.5×10^8	$<10^1$	$<10^1$	-	-
	10^2	120	1.3×10^8	1.3×10^8	$<10^1$	$<10^1$	-	-
	10^4	27	3.2×10^7	8.5×10^7	$<10^1$	$<10^1$	-	-
	10^4	90	2.1×10^8	---	$<10^1$	$<10^1$	-	-
	10^4	120	2.4×10^8	2.1×10^8	$<10^1$	$<10^1$	-	-
	10^6	24	4.0×10^8	4.0×10^8	$<10^1$	1.0×10^1	-	-
	10^6	90	2.8×10^8	1.7×10^8	$<10^1$	$<10^1$	-	-
	10^6	120	7.7×10^8	1.0×10^9	2.3×10^2	5.0×10^2	-	-

+ indicates that the sample is toxic in 1:10 dilution

- indicates that the sample is not toxic in 1:10 dilution

Table 3

Toxicity Data and Aerobic and Anaerobic Counts for Skinless Haddock Fillets, Non-Inoculated and Incculated with Spores of Type E, Cl. botulinum Irradiated at 350,000 rad and Stored at 33, 35, 40 and 45°F.

Storage Temperature (°F)	Inoculum Level (spores/g)	Storage Time (Days)	Aerobic Counts (per gram)		Anaerobic Counts (Clostridia per gram)		Increases in Toxicity	
			Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2
45	0	20	4.0×10^7	5.4×10^7	$<10^1$	$<10^1$	-	-
	0	36	8.0×10^6	7.8×10^6	$<10^1$	$<10^1$	-	-
	0	54	3.8×10^8	8.5×10^8	$<10^1$	$<10^1$	-	-
	10^2	18	9.2×10^7	8.4×10^7	$<10^1$	$<10^1$	-	-
	10^2	36	2.8×10^8	3.9×10^8	$<10^1$	1.0×10^1	-	-
	10^2	54	2.1×10^8	1.2×10^8	1.3×10^4	1.1×10^4	-	-
	10^4	18	8.4×10^7	1.3×10^8	3.7×10^2	6.6×10^2	-	-
	10^4	36	2.2×10^8	2.2×10^8	6.5×10^2	3.7×10^4	-	-
	10^4	54	1.9×10^8	2.1×10^8	3.9×10^3	4.4×10^4	+	+
	10^6	20	5.4×10^7	8.5×10^7	8.3×10^4	2.9×10^4	+	+
40	0	24	2.6×10^7	3.2×10^7	$<10^1$	$<10^1$	-	-
	0	42	1.8×10^7	2.2×10^7	$<10^1$	$<10^1$	-	-
	0	68	1.1×10^8	1.5×10^8	$<10^1$	$<10^1$	-	-
	10^2	24	1.9×10^6	2.0×10^7	$<10^1$	$<10^1$	-	-
	10^2	46	4.9×10^8	4.0×10^8	$<10^1$	$<10^1$	-	-
	10^2	66	7.0×10^8	6.4×10^8	$<10^1$	$<10^1$	-	-
	10^4	24	3.5×10^7	5.7×10^7	$<10^1$	$<10^1$	-	-
	10^4	46	1.7×10^8	1.6×10^8	$<10^1$	$<10^1$	-	-
	10^4	66	1.4×10^8	3.5×10^8	$<10^1$	$<10^1$	-	-
	10^6	23	1.1×10^7	1.2×10^7	1.0×10^1	8.0×10^1	-	-
	10^6	44	3.0×10^8	3.0×10^8	$<10^1$	$<10^1$	-	-
	10^6	65	7.2×10^7	1.4×10^8	$<10^1$	$<10^1$	-	-

Table 3 - continued

Storage Temperature (°F)	Inoculum Level (spores/g)	Storage Time (Days)	Aerobic Counts (per gram) Sample 1	Aerobic Counts (per gram) Sample 2	Anaerobic Counts (Clostridia per gram) Sample 1	Anaerobic Counts (Clostridia per gram) Sample 2	Increases in Toxicity Sample 1	Increases in Toxicity Sample 2
35	0	30	2.1×10^5	3.2×10^5	$<10^1$	$<10^1$	-	-
	0	91	2.3×10^8	1.9×10^8	$<10^1$	$<10^1$	-	-
	0	120	8.7×10^7	1.1×10^8	$<10^1$	$<10^1$	-	-
	10^2	31	2.2×10^7	2.6×10^7	$<10^1$	$<10^1$	-	-
	10^2	90	3.8×10^8	1.7×10^8	$<10^1$	$<10^1$	-	-
	10^2	120	5.7×10^8	3.1×10^8	$<10^1$	$<10^1$	-	-
	10^4	31	8.5×10^6	1.1×10^7	$<10^1$	$<10^1$	-	-
	10^4	90	1.0×10^5	3.0×10^5	$<10^1$	$<10^1$	-	-
	10^4	120	2.8×10^8	4.9×10^8	$<10^1$	$<10^1$	-	-
	10^6	31	7.0×10^6	7.8×10^6	$<10^1$	$<10^1$	-	-
	10^6	90	1.0×10^8	5.2×10^7	$<10^1$	$<10^1$	-	-
	10^6	120	6.4×10^7	8.6×10^7	$<10^1$	$<10^1$	-	-
33	0	35	3.6×10^6	8.0×10^6	$<10^1$	$<10^1$	-	-
	0	91	8.6×10^7	1.4×10^8	$<10^1$	$<10^1$	-	-
	0	120	4.5×10^8	4.6×10^8	$<10^1$	$<10^1$	-	-
	10^2	35	6.5×10^7	7.7×10^7	$<10^1$	$<10^1$	-	-
	10^2	90	1.2×10^8	1.6×10^8	$<10^1$	$<10^1$	-	-
	10^2	120	7.0×10^8	5.0×10^8	$<10^1$	$<10^1$	-	-
	10^4	35	1.9×10^7	4.6×10^7	$<10^1$	$<10^1$	-	-
	10^4	90	4.8×10^8	4.1×10^8	$<10^1$	$<10^1$	-	-
	10^4	120	6.8×10^8	3.1×10^8	$<10^1$	$<10^1$	-	-
	10^6	35	2.8×10^7	4.9×10^7	$<10^1$	5.0×10^1	-	-
	10^6	90	4.7×10^7	9.4×10^7	$<10^1$	$<10^1$	-	-
	10^6	120	3.5×10^8	1.1×10^8	5.8×10^2	6.6×10^2	-	-

+ indicates that the sample is toxic in 1:10 dilution

- indicates that the sample is not toxic in 1:10 dilution

Table 4

Toxicity Data for Skinless Haddcck Fillets Inoculated with Spores of Type E Cl. botulinum (mixture of four strains), Non-Irradiated and Irradiated, and Stored at 45°F

Inoculum Level (spores/gm)	Storage Time (days)	Toxicity								
		Non-Irradiated			Irradiated (100,000 rad)			Irradiated (200,000 rad)		
		Sample Number			Sample Number			Sample Number		
		1	2	3	1	2	3	1	2	3
10^6	8	+	-	+	+	+	+	not done	not done	not done
10^6	10	++	lost	++	+	-	++	+	+	+
10^4	9	-	-	-	-	-	-	not done	not done	not done
10^4	10	not done	not done	not done	not done	not done	not done	-	-	-
10^4	14	-	-	-	-	-	-	-	-	-
10^4	16	+	+	+	-	-	-	-	-	-
10^4*	16	+	+	+	-	-	-	-	-	-
10^4	19				-	+	-	-	-	-
10^4*	19				-	+	-	+**	-	+**
10^4	21				+	+	-	+	-	+
10^4	21				+	+	-	-	-	+

\+ indicates toxic in 1:10 dilution
++ indicates toxic in 1:100 dilution
* indicates samples not trypsinized prior to testing for toxin. All other samples were trypsinized
+** only one of the three mice injected died

Table 5

Toxicity of 50 Portions of Haddock Fillets Cultured in TPG Broth Containing Thioglycollate

Treatment of Sample	Sample Number	Number of Mice Dying				Presence of Type E, Cl. botulinum
		Unprotected		Protected		
		Material Trypsinized	Material Not Trypsinized	Material Trypsinized	Material Not Trypsinized	
Not Heat Shocked	1	0 in 3	0 in 2	0 in 3	0 in 2	Not present
"	2	3 in 3	1 in 2	0 in 3	0 in 2	Present
"	3	3 in 3	2 in 2	0 in 3	0 in 2	Present
"	4	0 in 3	0 in 2	0 in 3	0 in 2	Not present
Heat Shocked	5	0 in 3	0 in 2	0 in 3	0 in 2	Not present
"	6	3 in 3	0 in 2	0 in 3	0 in 2	Present
"	7	0 in 3	0 in 2	0 in 3	0 in 2	Not present
"	8	0 in 3	0 in 2	0 in 3	0 in 2	Not present
Not Heat Shocked	9	0 in 3	--	0 in 3	--	Not present
"	10	0 in 3	--	0 in 3	--	Not present
"	11	0 in 3	--	0 in 3	--	Not present
Heat Shocked	12	0 in 3	--	0 in 3	--	Not present
"	13	0 in 3	--	0 in 3	--	Not present
"	14	0 in 3	--	0 in 3	--	Not present

Table 6

Presence of Type E, Cl. botulinum in Commercial Haddock Fillets (tests completed to date)

Source of Sample	Sample Number	Number of Culture Tubes (among 5) Containing Toxin			MPN Type E per gram
		100g fish per tube	10g fish per tube	1g fish per tube	
Plant A	1	5	4	1	0.17
	2	5	1	0	0.03
	3	0	0	0	none
	4	0	0	0	none
	5	1	1	0	0.004
	6	3	0	0	0.008
	7	0	0	0	none
	8	0	0	0	none
	9	1	0	0	0.002
	10	0	0	0	none
	11	0	0	0	none
	12	0	0	0	none
	13	0	0	0	none
	14	0	0	0	none
	15	0	0	0	none
	16	0	0	0	none
	17	0	0	0	none
	18	0	0	0	none
Plant B	1	2	0	0	0.005
	2	2	0	0	0.005
	3	0	0	1	0.002
	4	0	0	0	none
Plant C	1	0	0	0	none
	2	0	0	0	none
	3	0	0	0	none
	4	0	0	0	none
Plant D	1	0	0	0	none
	2	0	0	0	none
	3	0	0	0	none
	4	0	0	0	none
Plant E	1	0	0	0	none
	2	0	0	0	none
	3	0	0	0	none
	4	0	0	0	none

Table 7

The Effect of Haddock Press Juice on the Viability of Type E, Cl. botulinum Spores

Botulinum Type	Count Which the Fish Press Juice Should Have Contained (per ml)	Count (per ml) Indicated by Culturing in TPG Agar, After Holding in Fish Press Juice for 24 Hrs. at 35°F
Minneapolis	5×10^6	5×10^4
8E	5×10^6	8×10^3

Table 8

Temperatures of Deep Fat Fried Haddock Fillets During and After Cooking at 375°F

Time (Minutes)	1/2" Thick Sample 1	5/8" Thick Sample 2*	3/4" Thick Sample 3	1" Thick Sample 4**
0.00	51.7°F	53.0°F	42.0°F	48.8°F
0.25	51.7	56.0	42.4	50.5
0.50	53.5	60.0	43.0	52.2
0.75	61.0	70.0	43.7	53.0
1.00	69.0	80.0	47.0	55.7
1.25	80.0	88.0	50.0	57.5
1.50	90.0	95.0	54.0	60.5
1.75	100.0	102.0	60.0	65.1
2.00	110.0	108.0	64.0	68.7
2.25	120.0	114.0	70.0	73.1
2.50	128.3	120.0	75.0	78.1
2.75	137.0	125.0	80.2	83.2
3.00	142.5	130.3	85.2	88.1
3.25	151.0	137.0	92.0	94.2
3.40	158.0 (end of cooking)	140.2	98.0	98.3
3.75	166.0	145.0	105.0	103.2
4.00	171.0	149.2	109.3	108.3
4.25	175.8	153.7	115.0	112.3
4.50	178.3	158.0 (end of cooking)	121.9	117.0
4.75	180.5	163.6	127.0	121.7
5.00	181.7	166.5	135.2 (end of cooking)	126.9
5.25	182.4	168.7	147.0	130.8
5.50	182.5	170.0	158.2	135.0
5.75	182.7 (max)	171.0	164.2	140.0
6.00	182.6	171.3	168.5	143.5
6.25	182.5	172.0	170.0	147.2
6.50	182.5	172.2	172.2	151.0 (end of cooking)
6.75	182.4	172.2	174.0	154.4
7.00	182.3	172.4 (max)	175.0	157.1
7.25	182.2	172.4	176.0	160.1
7.50	182.1	172.4	177.0	162.5
7.75	181.6	172.4	177.3	165.7
8.00	181.0	172.3	177.8	168.0
8.25	180.2	172.3	178.0 (max)	168.6
8.50	179.9	172.3	178.0	171.2
8.75	179.2	172.3	178.0	172.7
9.00	178.4	172.2	178.0	173.7
9.25	178.2	172.2	178.0	174.9
9.50	177.9	172.1	178.0	175.3
9.75	177.3	171.9	178.0	175.9

Table 8 - continued

Time (Minutes)	1/2" Thick Sample 1	5/8" Thick Sample 2*	3/4" Thick Sample 3	1" Thick Sample 4**
10.00	176.4	171.6	178.0	176.1
10.25	176.2	171.0	177.8	176.1
10.50	175.8	170.3	177.3	176.2
10.75	175.2	170.0	176.3	176.2
11.00	174.3	169.3	175.7	176.2
11.25	174.1	168.4	174.3	176.3 (max)
11.50	173.7	168.1	174.3	176.3
11.75	173.3	167.7	173.8	176.3
12.00	172.5	167.0	173.0	176.2
12.25	172.0	166.2	172.0	176.1
12.50	171.2	165.8	171.3	175.9
12.75	170.7	165.3	170.3	175.6
13.00	170.2	164.4	170.0	174.6
13.25	169.7	164.0	169.2	174.0
13.50	169.7	163.3	168.2	173.4
13.75	169.0	162.8	167.7	172.9
14.00	168.2	162.0	166.9	172.2
14.25	168.1	161.6	166.0	171.3
14.50	167.5	160.6	153.3	170.4
14.75	167.1	160.2	164.4	169.8
15.00	166.1	159.7	164.0	169.5
15.25	166.0	159.0	163.0	168.6
15.50	165.8	158.3	162.1	168.0
15.75	165.0	158.0	161.8	167.1
16.00	164.3	157.3	161.0	166.2
16.25	163.9	156.4	160.1	165.7
16.50	163.3	155.9	159.7	165.0
16.75	162.7	155.0	158.8	164.1
17.00	162.0	154.3	158.0	163.3
17.25	161.2	154.0	157.5	162.6
17.50	160.4	153.3	156.8	162.0
17.75	160.0	152.6	156.0	161.3
18.00	159.1	152.0	155.7	160.3
18.25	158.2	151.6	155.0	159.8
18.50	157.8	150.5	154.0	159.0
18.75	157.0	150.0	153.4	158.3
19.00	156.3	149.8	152.6	157.8
19.25	155.8		152.0	157.0
19.50	155.0		151.5	156.2
19.75	154.3		150.5	155.6
20.00	153.9		150.0	154.8
20.25	153.2		149.5	154.2
20.50	152.3		149.0	153.2
20.75	151.9			152.5
21.00	151.3			152.1
21.25	150.7			151.3
21.50	150.2			150.5
21.75	150.0			150.0
22.00				149.6

* 245 grams
** 250 grams

Table 9

Calculation of Toxin Destruction Rates

Time (Minutes)	Temperature (°F) at Point of Slowest Heating (See Table 8 Column 2)	Destruction Time (From Fig. 2) (Minutes)	Destruction Rate (See Fig. 1)
3.25	137.0	84.0	1/84 or 0.012
4.0	149.2	12.2	1/12.2 or 0.082
5.0	166.5	0.8	1/0.8 or 1.25
6.0	171.3	0.36	1/0.36 or 2.78
7.0	172.4	0.32	1/0.32 or 3.11
8.0	172.3	0.33	1/0.33 or 3.0
9.0	172.2	0.33	1/0.33 or 3.0
10.0	171.6	0.36	1/0.36 or 2.78
11.0	169.3	0.53	1/0.53 or 1.88
12.0	167.0	0.74	1/0.74 or 1.38
13.0	164.4	1.15	1/1.15 or 0.87
14.0	162.0	1.75	1/1.75 or 0.57
15.0	159.7	2.4	1/2.4 or 0.42
16.0	157.3	3.5	1/3.5 or 0.28
17.0	154.3	5.6	1/5.6 or 0.18
18.0	152.0	8.0	1/8.0 or 0.12

Table 10

Fillets Inoculated With Three Strains (Detroit, Minneapolis and 8E) Cl. botulinum, Type E Irradiated 200 Kilo Rad and Stored at 45°F

Storage Time (weeks)	Bacteria per Gram		Toxicity	Remarks
	Clostridial	Aerobic		
3	10^2	187×10^5	-	
4	10^1	81×10^7	-	Slightly putrid odor
5	15×10^4	58×10^7	-	Trace of NH_3
6	2×10^6	32×10^7	T 0/3* N 3/3* -** -**	NH_3 odor

T = trypsinized extract
N = nontrypsinized extract
* = 1:10 dilution
** = 1:100 dilution

Table 11

Haddock Fillets Inoculated with Cl. botulinum, Type E (mixture of strains of Detroit, Minneapolis, 8E) Irradiated for 200 Kilo Rad and Stored at 75°F

Storage Time (days)	Bacteria per Gram		Toxicity Dilution Level			
	Clostridial	Aerobic	10^{-1}	10^{-2}	10^{-3}	10^{-4}
0	10^1	10^1	-	-	-	-
3	13×10^6	30×10^7	T - N +	-	-	-
4	27×10^6	69×10^7	T + N -	T + N -	-	-
5	7×10^7	46×10^7	T + N +	T + N +	T - N +	-
6	5×10^7	40×10^7	T + N +	T + N +	-	-
7	4×10^7	50×10^7	T + N +	T - N +	T - N +	-
10	2×10^7	58×10^7	T + N +	T - N +	T - N +	-

Remarks: The 3 day sample had a slight NH_3 odor; as the storage time increased, the odor became more intensified. The samples were very gaseous at 3 days.

Table 12

Growth at 45°F and Production of Acid by Four Strains of Type E, Cl. botulinum on Trypticase-Peptone-Mannose Medium Containing Thioglycollate and Acid Indicator Dyes

Strains of Type E, Cl. botulinum	Dye Used as Indicator of Acid	Growth Time (days)	Time at Which Acid Production Was Indicated
Beluga	None	12	--
	Brom thymol blue	12	26
	Brom cresol purple	12	No acid indicated
	Methyl red	12	No acid indicated
Detroit	None	11	--
	Brom thymol blue	11	22
	Brom cresol purple	11	26
	Methyl red	11	No acid indicated
8E	None	12	--
	Brom thymol blue	12	26
	Brom cresol purple	12	No acid indicated
	Methyl red	12	No acid indicated
Minneapolis	None	11	--
	Brom thymol blue	11	26
	Brom cresol purple	11	No acid indicated
	Methyl red	11	No acid indicated

Table 13

Results of Bacterial Counts and Toxicity Tests on Ground Irradiation-Sterilized Haddock After Inoculation With Type E, Cl. botulinum or with Type E, Cl. botulinum plus Another Bacterium and Incubating at 45°F Aerobically or Anaerobically.

Organisms Inoculated	Haddock Incubated	Days Stored	Bacteria per Gram (average of 2 counts) Aerobic Count	Anaerobic Count (Clostridia)	Toxicity of Culture
Cl. botulinum alone	aerobic	0	not done	44×10^2	-
		1	"	$< 10^1$	-
		5	"	$< 10^1$	-
		10	"	$< 10^1$	-
		20	"	$< 10^1$	-
		30	"	$< 10^1$	-
Cl. botulinum alone	anaerobic	0	not done	44×10^2	-
		1	"	$< 10^1$	-
		5	"	$< 10^1$	-
		10	"	1×10^2	-
		20	"	5×10^2	-
		30	"	3×10^2	+
Cl. botulinum alone	aerobic	1	not done	3×10^3	-
		5	95×10^2	2×10^2	-
		10	1000×10^4	$< 10^3$	-
		20	184×10^5	1×10^1	-
		30	53×10^7	17×10^3	-
		35	275×10^6	50×10^1	-(trypsinized) +(non-trypsinized)

Table 13 - continued

Organisms Inoculated	Haddock Incubated	Days Stored	Bacteria per Gram (average of 2 counts) Aerobic Count	Anaerobic Count (Clostridia)	Toxicity of Culture
Cl. botulinum alone	anaerobic	1	not done	8×10^2	-
		5	12×10^2	1×10^2	-
		10	$< 10^1$	$< 10^3$	-
		20	53×10^2	2×10^1	-
		30	39×10^1	$< 10^1$	-
		35	10^1	10×10^1	-
Cl. botulinum alone	aerobic	5	*	3×10^2	-
		10	$< 10^1$	1×10^1	-
		20	$< 10^1$	1×10^1	-
		32	not done	1×10^1	-
Cl. botulinum alone	anaerobic	5	$< 10^1$	8×10^2	-
		10	**	1×10^2	-
		20	$< 10^1$	$< 10^1$	-
		32	not done	18×10^1	-
Cl. botulinum and S. lactis	aerobic	0	58×10^3	61×10^2	-
		1	13×10^5	$< 10^1$	-
		5	46×10^6	$< 10^1$	-
		10	$> 10^8$	4×10^1	-
		20	$> 10^8$	42×10^2	-
		30	$> 10^3$	64×10^3	-

* about 1000×10^1 contaminated, yellow colonies, rod shaped.
** about $10,000 \times 10^1$ contaminated, yellow colonies, rod shaped.

Table 13 - continued

Organisms Inoculated	Haddock Incubated	Days Stored	Bacteria per Gram (average of 2 counts) Aerobic Count	Anaerobic Count (Clostridia)	Toxicity of Culture
Cl. botulinum and S. lactis	anaerobic	0	60×10^2	60×10^2	-
		1	13×10^4	$< 10^1$	-
		5	50×10^6	$< 10^1$	-
		10	$> 10^8$	$< 10^1$	-
		20	$> 10^8$	40×10^1	-
		30	$> 10^8$	45×10^1	-
Cl. botulinum and S. faecalis	aerobic	0	82×10^3	70×10^1	-
		1	13×10^5	$< 10^1$	-
		5	43×10^6	$< 10^1$	-
		10	$> 10^8$	$< 10^1$	-
		20	$> 10^8$	$< 10^1$	-
		30	$> 10^8$	$< 10^1$	-
Cl. botulinum and S. faecalis	anaerobic	0	82×10^3	70×10^1	-
		1	14×10^5	19×10^2	-
		5	87×10^6	5×10^1	-
		10	$> 10^8$	$< 10^1$	-
		20	$> 10^8$	54×10^1	-
		30	$> 10^8$	84×10^1	-
Cl. botulinum and L. viridescens	aerobic	0	39×10^3	40×10^3	-
		1	54×10^5	4×10^1	-
		5	$> 10^8$	$< 10^1$	-
		10	$> 10^8$	31×10^1	-
		20	$> 10^8$	50×10^1	-
		30	$> 10^8$	$< 10^1$	-

Table 13 - continued

Organisms Inoculated	Haddock Incubated	Days Stored	Bacteria per Gram (average of 2 counts) Aerobic Count	Anaerobic Count (Clostridia)	Toxicity of Culture
Cl. botulinum and L. viridescens	anaerobic	0	40×10^3	41×10^3	-
		1	54×10^5	48×10^1	-
		5	32×10^6	$< 10^1$	-
		10	$> 10^8$	28×10^1	-
		20	$> 10^8$	21×10^1	-
		30	$> 10^8$	39×10^1	-
Cl. botulinum and Cl. sporogenes	anaerobic	0	not done	14×10^2	-
		1	"	1×10^1	-
		5	"	$< 10^1$	-
		10	"	$< 10^1$	-
		20	"	$< 10^1$	-
		30	"	$< 10^1$	-
Cl. botulinum and P. fragi	aerobic	0	16×10^2	20×10^1	-
		1	25×10^3	$< 10^1$	-
		5	77×10^6	18×10^1	-
		10	10×10^7	10×10^2	+
		20	20×10^7	15×10^3	+
		30	22×10^7	35×10^4	+
Cl. botulinum and P. fragi	anaerobic	0	not done	91×10^2	-
		1	"	4×10^1	-
		5	"	3×10^1	-
		10	"	15×10^2	-
		20	"	32×10^2	-
		30	"	11×10^1	-

Table 13 - continued

Organisms Inoculated	Haddock Incubated	Days Stored	Bacteria per Gram (average of 2 counts) Aerobic Count	Anaerobic Count (Clostridia)	Toxicity of Culture
Cl. botulinum and P. fluorescens	aerobic	0	16×10^2	14×10^2	-
		1	43×10^3	$< 10^1$	-
		5	59×10^6	$< 10^1$	-
		10	16×10^7	$< 10^1$	-
		20	17×10^7	14×10^3	+
		30	16×10^7	26×10^4	+
Cl. botulinum and P. fluorescens	anaerobic	0	not done	94×10^2	-
		1	"	15×10^1	-
		5	"	9×10^1	-
		10	"	$< 10^1$	-
		20	"	43×10^1	-
		30	"	95×10^1	-
Cl. botulinum and Achromobacter	aerobic	1	20×10^3	11×10^2	-
		5	90×10^5	2×10^2	-
		10	12×10^7	$< 10^3$	-
		20	52×10^7	50×10^3	-
		30	50×10^7	145×10^5	-(trypsinized) +(non-trypsinized)
Cl. botulinum and Achromobacter	anaerobic	1	17×10^4	12×10^2	-
		5	22×10^6	8×10^2	-
		10	$< 10^4$	$< 10^3$	-
		20	958×10^1	$< 10^1$	-
		30	6×10^1	14×10^4	-
		35	$< 10^1$	200×10^4	-(trypsinized) +(non-trypsinized)

Table 13 - continued

Organisms Inoculated	Haddock Incubated	Days Stored	Bacteria per Gram (average of 2 counts) Aerobic Count	Anaerobic Count (Clostridia)	Toxicity of Culture
Cl. botulinum and Achromobacter	aerobic	0	4×10^3	4×10^3	-
		5	about 1064×10^4	10×10^1	-
		10	90×10^7	5×10^1	-
		20	56×10^7	10×10^4	+*
		32	not done	14×10^6	-(trypsinized) +(non-trypsinized)
Cl. botulinum and Achromobacter	anaerobic	5	100×10^5	48×10^1	-
		10	12×10^5	13×10^1	-
		20	$<10^5$	500×10^4	-
		32	not done	84×10^4	-(trypsinized) +(non-trypsinized)

* one of three non-protected mice died in both trypsinized and non-trypsinized.

- indicates that the sample was not toxic to mice in the 1:10 dilution
+ indicates that the sample was toxic to mice in the 1:10 dilution

Table 14

Toxicity of Commercial Shucked Soft-Shelled Clam Cultures

Plant No.	Sample No.	Toxicity in 5 tubes (no. of tubes positive) 100 g	10 g	1 g.	Most Probable Number of Type E Cl. botulinum Ocells (cells per 100 g.)
A	*1	0	0	0	none present
	*2	0	0	0	none present
	*3	0	0	0	none present
	4	0	0	0	none present
	5	1	0	0	2.0
	6	0	0	1	2.0
B	1	0	0	0	none present
	2	0	0	0	none present
	3	0	0	0	none present
	4	0	0	0	none present
	5	0	0	0	none present
	6	0	0	0	none present
C	1	0	1**	0	none present
	2	0	0	0	none present
	3	1	0	0	2.0
	4	0	0	0	none present
	5	0	0	1	2.0
	6	0	0	0	none present

* The first 3 samples were cultured as five tubes each of 10 g, 1 g, and 0.1 g while other samples in the table were cultured as 5 tubes each of 100 g, 10 g, and 1 g.

** This sample contained type B, Cl. botulinum but did not contain type E, Cl. botulinum.

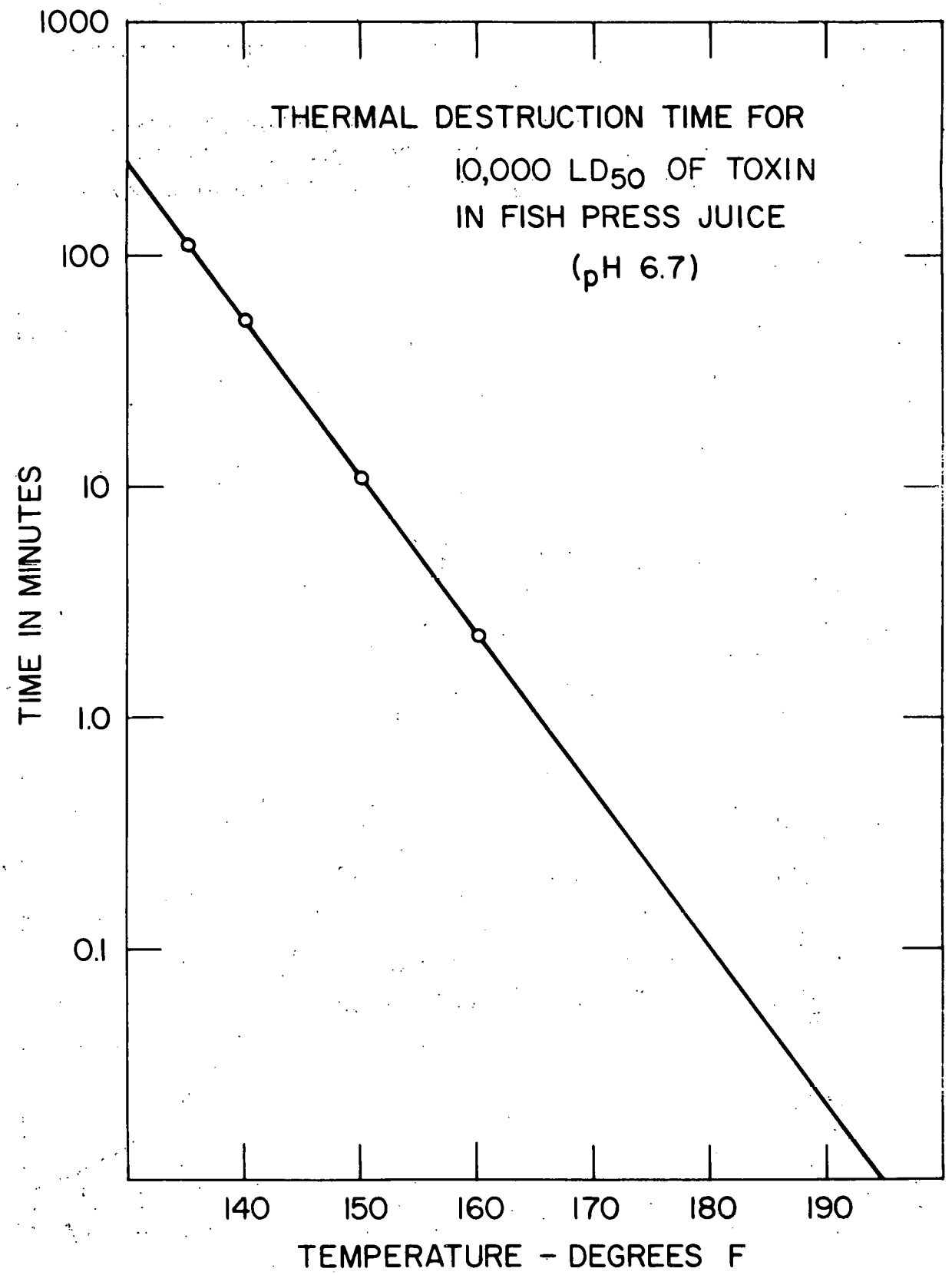
THERMAL DESTRUCTION TIME FOR
10,000 LD50 OF TOXIN
IN FISH PRESS JUICE
(pH 6.7)
1000
100
10
1.0
0.1
TIME IN MINUTES
140
150
160
170
180
190
TEMPERATURE - DEGREES F

FIG. 1

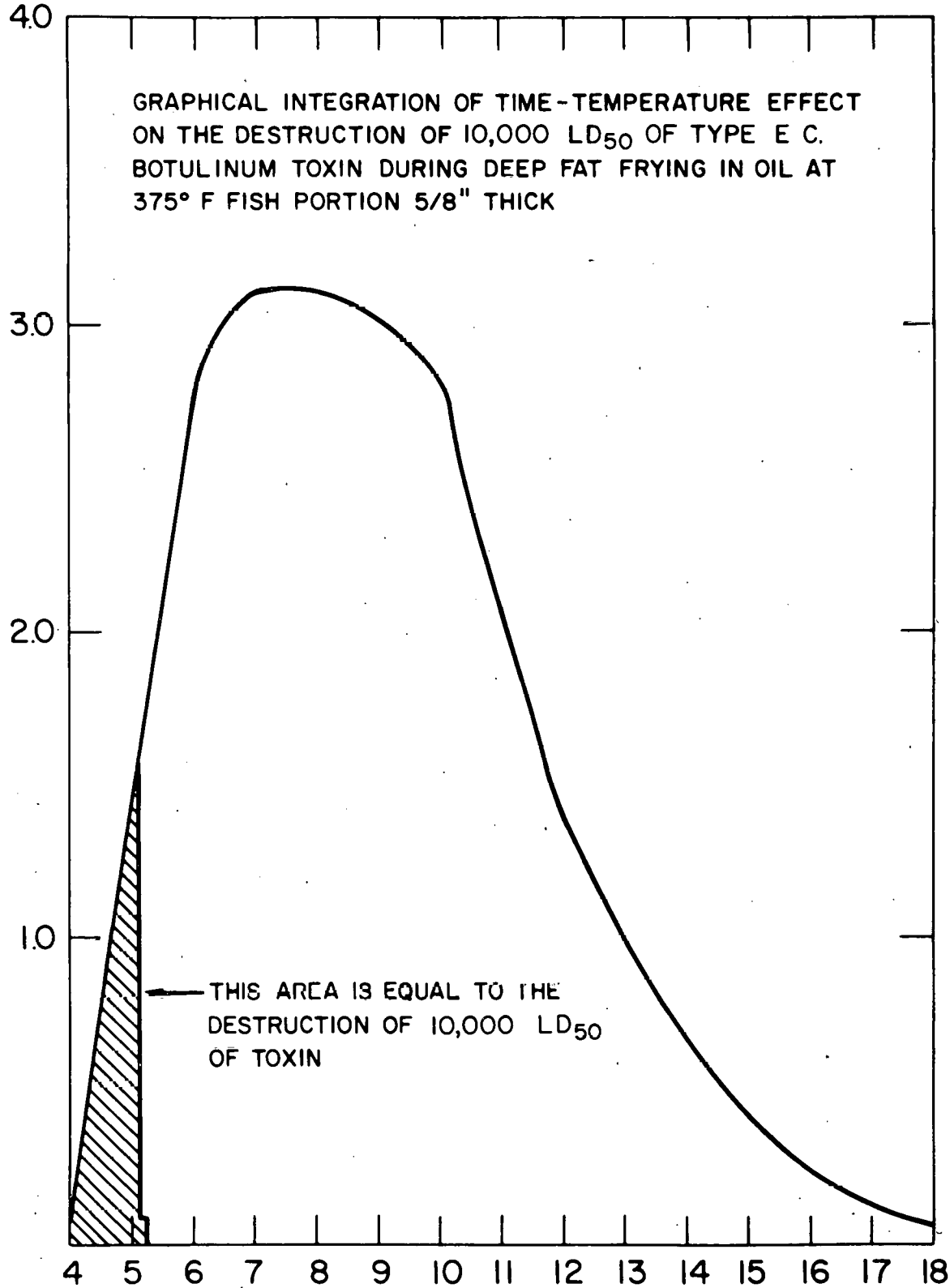
GRAPHICAL INTEGRATION OF TIME-TEMPERATURE EFFECT ON THE DESTRUCTION OF 10,000 LD50 OF TYPE E C. BOTULINUM TOXIN DURING DEEP FAT FRYING IN OIL AT 375° F FISH PORTION 5/8" THICK
4.0
3.0
2.0
1.0
THIS AREA IS EQUAL TO THE DESTRUCTION OF 10,000 LD50 OF TOXIN
4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

FIG. 2

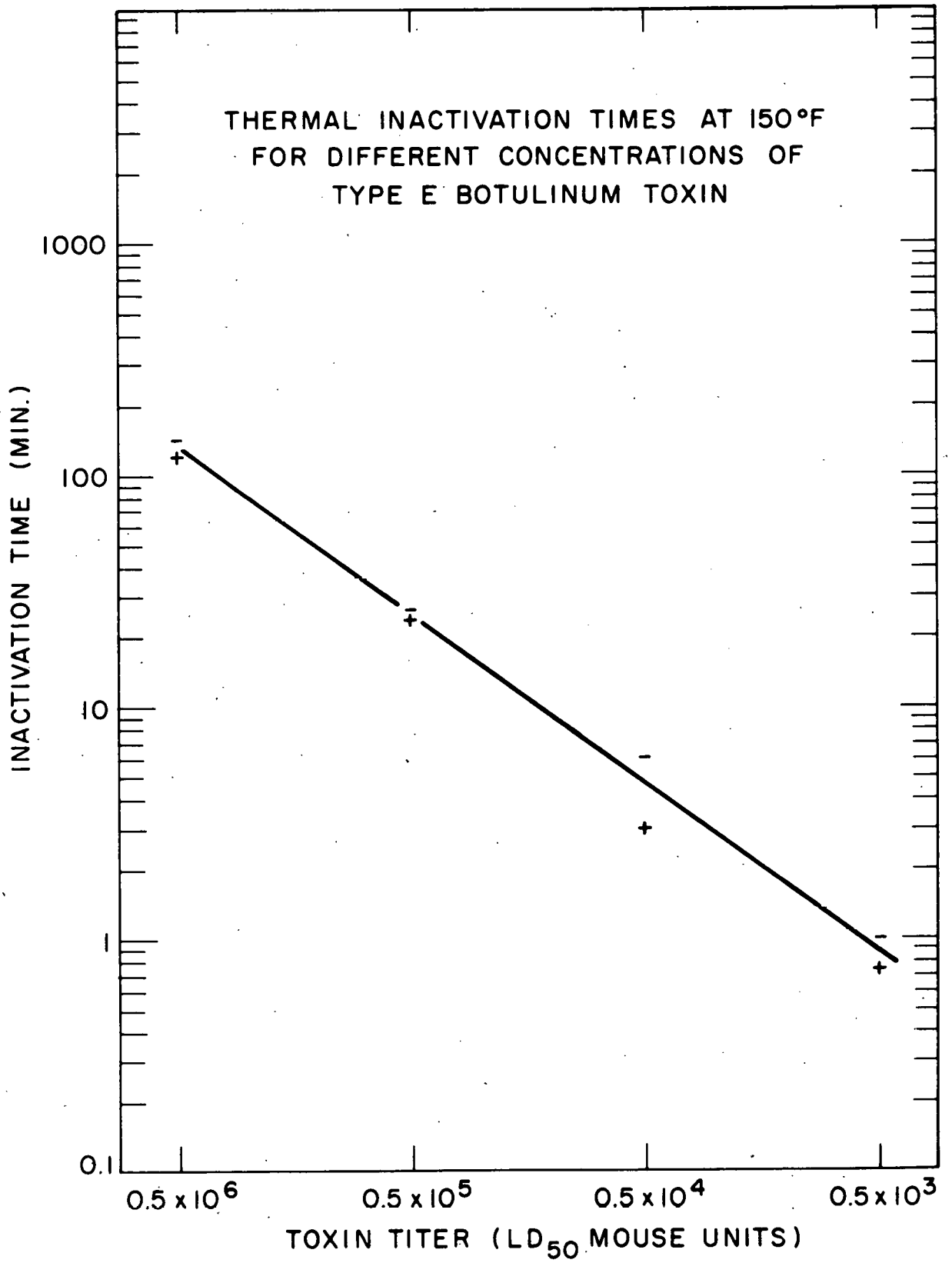
THERMAL INACTIVATION TIMES AT 150°F
FOR DIFFERENT CONCENTRATIONS OF
TYPE E BOTULINUM TOXIN
INACTIVATION TIME (MIN.)
1000
100
10
1
0.1
0.5 x 10^6
0.5 x 10^5
0.5 x 10^4
0.5 x 10^3
TOXIN TITER (LD50 MOUSE UNITS)

FIG. 3

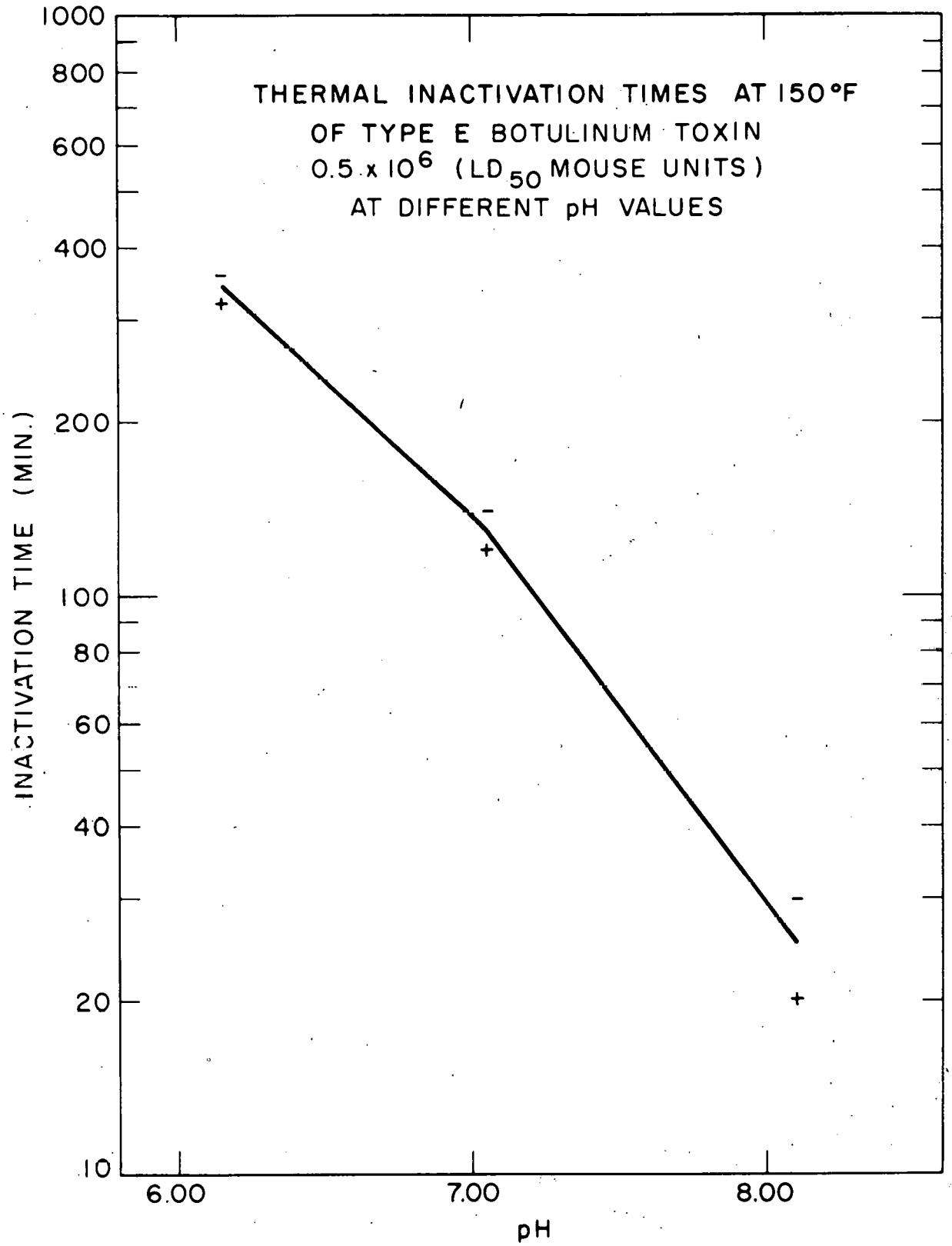
THERMAL INACTIVATION TIMES AT 150°F
OF TYPE E BOTULINUM TOXIN
0.5 x 10^6 (LD50 MOUSE UNITS)
AT DIFFERENT pH VALUES
INACTIVATION TIME (MIN.)
1000
800
600
400
200
100
80
60
40
20
10
6.00
7.00
8.00
pH
+
-

FIG. 4