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EFFECTS ON GROWTH AND TOXIN PRODUCTION OF SPORES
OF NON-PROTEOLYTIC CLOSTRIDIUM BOTULINUM EXPOSED
TO SUBLETHAL DOSES OF GAMMA IRRADIATION

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EFFECTS ON GROWTH AND TOXIN PRODUCTION OF SPORES
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TO SUBLETHAL DOSES OF GAMMA IRRADIATION

Approved:

M. A. ...

Chairman

Date approved by Chairman: 3/6/72

謹以本書為我最敬愛的母親
余黃葆秀夫人米秩壽禮。

鐘兒

民六十年三月
於美國喬州

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SUMMARY

The purpose of this investigation was to determine the effects of sublethal doses of gamma irradiation, 0.1 and 0.2 megarad, on growth and toxin production of spores of Clostridium botulinum type B, strain B-17 and type E, strain Beluga, as compared to unirradiated controls.

Spores of Clostridium botulinum type B, strain B-17 and type E, strain Beluga were exposed to 0.1 and 0.2 megarad of gamma radiation from a cesium-137 source. The effects upon post-irradiation growth were determined by microscopic examination and optical density readings. Mouse bioassay determinations were used to ascertain the effects of irradiation on toxin production.

The data obtained from these experiments show that irradiation of spores of Clostridium botulinum type B, strain B-17 did not alter the patterns of growth at 25C, as compared to the unirradiated control, and the titer of toxin produced in the irradiated cultures at 25C was not significantly different from that of the toxin produced in the unirradiated controls. The toxin titers became significantly different after subjection to trypsinization. There was no spore outgrowth and no untrypsinized toxin found at a post-irradiation incubation temperature of 10C but low titers of trypsinized toxin were detected after various periods of incubation at 10C.

The post-irradiation growth response at 25C of spores of Clostridium botulinum type E, strain Beluga, is variable. The titer of toxin produced in the irradiated cultures at 25C was not significantly different from

that produced in the unirradiated control but became significantly different after treatment with trypsin. Spore outgrowth was evident on one occasion in the unirradiated control at a post-irradiation incubation temperature of 10C but there was no outgrowth of spores in the irradiated cultures. At 10C, no toxin was demonstrable without trypsinization and the trypsinized toxin was not found continuously at the intervals tested in either irradiated cultures or unirradiated control cultures.

The finding was that irradiation did not significantly alter the production of untrypsinized toxin by post-irradiation cultures, as compared to controls, but did affect the trypsinized toxin production. The author suggests that, for certain strains, low level irradiation doses affect the metabolism of the cells mutagenically.

CHAPTER I

INTRODUCTION

This thesis is a report on one of a series of studies under the supervision of Dr. N. W. Walls on growth and toxin production by Clostridium botulinum spores which have been subjected to sublethal doses of gamma irradiation. In previous investigations, data have been obtained on the behavior of proteolytic and non-proteolytic strains of Clostridium botulinum type F (Williams-Walls, 1969; LeBlanc, 1970) under the conditions described here, but limited studies have been made with the non-proteolytic type B and type E in similar circumstances. The purpose of the present report is to compare the effects of irradiation on growth and toxin production by spores of these two non-proteolytic types of Clostridium botulinum.

The "threshold dose"* of irradiation for spores of Clostridium botulinum has been determined to be about 0.4 megarad of gamma radiation (Denny and Bohrer, 1959; Morgan and Reed, 1954; Roberts and Ingram, 1965; Wheaton and Pratt, 1962). Therefore, irradiation doses below that level may not have the effect of killing spores but may only inflict some degree of injury to them. Lower doses of 0.1 megarad and 0.2 megarad gamma irradiation doses were chosen for this research because:

1. According to the Food and Drug Administration's stipulations, food irradiated with more than 0.2 megarad of gamma radiation will

*Threshold dose: The minimum level of radiation dosage at which loss of cell viability begins.

not be accepted for commercial distribution. This restriction was imposed to lessen the incidence of color, taste, and odor changes (i.e., organoleptic changes), or chemical changes which might be hazardous to the consumer's health (Ronsivalli and Holston, 1967).

2. Sublethal damage to the spores might be expressed in ways related to subsequent growth and toxin production. These data would be of value to the food industry in explaining the mechanism by which irradiated foods produce toxin sooner and in greater quantity than do unirradiated foods.

Clostridium botulinum is a gram positive, anaerobic, spore-forming bacillus, varying in length from 3 to 8 μ and in width from 0.4 to 1.2 μ . The organism produces a highly potent exotoxin known as botulinum toxin which causes an illness in humans and animals called "botulism". At this time, six (A, B, C, D, E, and F) immunologically distinct types of exotoxins of Clostridium botulinum have been described. The toxins of types A, B, E, and F induce the disease in humans. Type C toxin produces "limber-neck" in fowl and type D toxin causes botulism in cattle.

Botulinum toxin is one of the most powerful neuroparalytic poisons. In purified form, one μ g of this substance contains about 200,000 MLD (minimum lethal dose) for white mice weighing 20 gm each, and it is suspected that not much more than one μ g of the toxin may be fatal for man (Morton, 1961). The disease of botulism usually results from the ingestion of food contaminated with the preformed toxin produced by Clostridium botulinum, although cases resulting from wound contamination have been reported (Foresman et al., 1971).

In the human, symptoms usually appear within 18 to 96 hours after ingestion of the toxin-containing food. Botulinum toxin attacks the human peripheral nervous system, producing symptoms such as difficulty in swallowing and breathing, inability to focus the eyes, and paralysis of the extremities. The central nervous system does not seem to be affected since the victims who succumb to botulism remain conscious to the last. Botulinum toxin enters the body by absorption from the gastrointestinal tract and reaches susceptible neurons by way of the blood stream. Once at the neuron, the botulinum toxin may block acetyl choline release from "demyelinated" ends of cholinergic motor nerves. All six types of botulinum toxin differ greatly in their relative toxicities for different animal species, although they have the same action on the nervous system. For instance, the ratio of the lethal doses for mice and fowl are 1:15 for type A, 1:2000 for type C, 1:100,000 for type D and 1:25 for type E (Davis et al., 1968).

The high mortality rate of botulism disease has left a deep impression on human history, although human outbreaks are relatively rare. From 1899 through 1969, in the United States, 659 outbreaks were reported to the Health Agencies (U.S. Dept. of Health, Education and Welfare, 1971). In these 659 outbreaks, a total of 1969 cases produced 957 deaths, a mortality rate of 56.5 percent (U.S. Dept. of Health, Education and Welfare, 1971). During the period from 1899 to 1949, the death rate for reported cases was even higher, 60 percent. The decline in death-to-case ratio after 1949 is probably due to improvements in intensive care in cases of acute respiratory failure and the therapeutic effects of botulism antitoxin.

Since botulism affects a great variety of animal species in all parts of the world, surveys of distribution have been reported by many investigators. In general, the spores of Clostridium botulinum commonly exist in soils, muds, and the intestinal contents of animals; thus, great opportunities for contamination of foods with this organism are possible.

A summary of outbreaks of botulism may serve to illustrate the widespread distribution of Clostridium botulinum. In Europe, especially in France, Germany and Norway, most of the outbreaks of botulism have proved to be type B, whereas type A is responsible for most of the botulism outbreaks in the Soviet Union (Legroux et al., 1947; Skulberg 1961). In Japan, type E is responsible for the highest incidence rate, probably because of a favorite food of the Japanese, called "izushi", which is made of raw fish, rice and diced vegetables (Nakamura, 1963). In the United States, five western states including California, Washington, Colorado, Oregon, and New Mexico, claim over half of all reported outbreaks for this country. A total of 144 type A outbreaks were recorded from 1899 to 1969, 132 of which (about 92 percent) were in the western states noted above (U.S. Dept. of Health, Education, and Welfare, 1971). Twenty-five out of 37 type B outbreaks (1899-1969) were reported from the eastern states (U.S. Dept. of Health, Education, and Welfare, 1971). Those data confirmed the report made by Meyer (1956), who found in a soil survey that in North America type A spores of Clostridium botulinum predominate west of the Rocky Mountains and type B spores east of the Great Lakes. The high incidence of type A outbreaks also corroborates the surveys by Burke (1919) and Meyer and Dubovsky (1922) in which they found that distribution of type A spores in California soils is

much more common than type B. Unfortunately, there are no reliable data concerning botulism, or the causative organisms, from many parts of the world. This lack of information is probably due to the difficulty in diagnosis of the disease in remote areas, or to the scarcity of scientific reports of incidences.

Incidence of Type B Botulism

The first type B botulism recorded may be traced back to 1880 in the Belgian village of Ellezelles. It was caused by the ingestion of ham consumed by members of a musical society who had just performed at a funeral. Professor Van Ermengem of the University of Ghent isolated an anerobic spore-forming bacillus from the remains of the ham and from the liver of one of the victims. Culture filtrates from this organism were injected into laboratory animals and produced symptoms of paralysis and death - as is observed in humans who have eaten food contaminated with botulism toxin. The isolated organism was called Bacillus botulinum (Van Ermengem, 1897); now we know it as Clostridium botulinum. According to Van Ermengem's observations, the Ellezelles organism would be classified today as a non-proteolytic type B strain.

In 1910, Leuchs showed by means of horse antitoxin serum that two cultures of Bacillus botulinum - the Ellezelles strain of Van Ermengem and a strain isolated by G. Landmann (1904) from preserved bean salad which had caused 11 deaths from botulism at Darmstadt - were morphologically and culturally almost identical but produced immunologically distinct toxins. Leuchs designated these strains simply "E" (Ellezelles) and "D" (Darmstadt). Both cultures were lost soon afterward, but it was the

contention of Meyer and Gunnison (1929) that they should be classified as a non-proteolytic type B (Ellezelles strain) and a type A (Darmstadt strain).

After Van Ermengem's investigation, the non-proteolytic type B was found to be the one most commonly involved in European botulism, but this non-proteolytic form was still unknown in North America until recent years. All the type B strains isolated in North America had been proteolytic until 1958, when two outbreaks, caused by putrescent salmon eggs contaminated with non-proteolytic type B toxin, occurred among Coastal Indians of Northern British Columbia (Dolman et al., 1960). This was 65 years after and 8000 miles away from the tragedy at Ellezelles.

Among 37 type B outbreaks reported in the United States from 1899 to 1969, there were 125 cases of botulism from which 61 deaths ensued, a case fatality ratio of 43.9 percent. Twenty-five of the 37 type B outbreaks were reported from eastern states; New York alone reported ten outbreaks. Vegetables were found to be the vehicles for 22 type B outbreaks, and fruits were responsible for four outbreaks (U.S. Dept. of Health, Education, and Welfare, 1971).

In France, from 1940 through 1944, 202 out of 500 outbreaks of botulism were reported as type B; in the same period of time, there were only 27 type B outbreaks reported in the United States (Dolman, 1964).

These statistics would tend to confirm, along with the reports of Legroux et al., (1947) and Skulberg (1961), that spores of type B Clostridium botulinum predominate over type A spores in some large regions such as Germany, France, Norway, and certain parts of the United States.

Incidence of Type E Botulism

The incidence of type E has been reported in considerable detail in the thesis written by a previous worker in this laboratory (LeBlanc, 1970). At this time, little additional information is available to add to his description of individual outbreaks of type E botulism:

The first outbreak of type E occurred in 1932 at Cooperstown, N. Y., the vehicle being smoked salmon from Labrador (Hazen, 1938). Between 1932 and 1969, a total of 57 cases in 17 outbreaks of type E botulism were recorded in the United States. Twenty-five out of the 57 cases terminated in death, a case fatality ratio of 43.9 percent (U.S. Dept. of Health, Education, and Welfare, 1971). Fish and fish products were incriminated in 16 of the 17 outbreaks; only one outbreak was caused by ingestion of vegetables. Eight of these same 17 outbreaks of type E botulism occurred in Alaska. The main source of the toxin was "muktuk", an Eskimo food prepared by cutting the skin and underlying blubber of beluga whale flippers into chunks or strips and hanging them outdoors on a rack or over a pole until dried.

In Japan, from 1930 to 1964, 46 out of 62 reported outbreaks were caused by type E botulism. "Izushi", a fish preparation which has been described before, was responsible for 90 percent of these type E outbreaks (Dolman, 1964).

In Canada, from 1940 to 1963, there was a total of 14 outbreaks of botulism among the Northwest Pacific Coast Indians, the vehicle being salmon eggs. Nine of these outbreaks proved to be type E botulism (Dolman, 1964) which resulted in 13 deaths out of 26 cases identified.

In addition to these specific examples, high incidences of type E

botulism may be found in the records of the Soviet Union, Sweden, and Denmark.

Since type E botulism occurs so frequently in Japan and Alaska, Dolman and Iida (1963) have developed the hypothesis that spores of type E, transported by trans-Pacific oceanographic currents and reinforced by land drainage into coastal waters, may expose the peoples of Northern Japan, the Eskimos, and the Pacific Coast Indians to type E botulism more frequently because of the greater possibility of contact of their foods with these spores. They also suggest that the capacities of the grey whale and of migratory predatory fish (such as salmon) to serve as intestinal carriers of type E spores make them potential sources of botulism.

Irradiation of Food

Irradiation preservation of foods is one goal of the use of radiation in peace time. Ideally, irradiation would greatly lengthen the storage and shelf life of foods. As examples, irradiation can prevent sprouting of root crops, eliminate insects from grain, or destroy bacteria or other microorganisms contained in various foods. In addition, food scientists could expect that irradiation preservation would have great advantages, such as convenient sterilization or pasteurization of foods, safe storage of foods at room temperature, and no change of odor, taste or color of foods such as is caused by thermal treatment. However, the doses of irradiation required to accomplish each of the above vary greatly. It has been established that doses of 0.02 to 0.05 megarad are required to disinfect grains and cereals from the presence of insects. Furthermore, the pasteurization doses for foods range from 0.2 to 0.5

megarad. Very high doses, ranging from 2 to 4.5 megarads, are needed for "sterilization" of foods destined for long time storage without refrigeration. Unfortunately, after extensive research, scientists found that although pasteurization doses destroyed some microorganisms contained in food, this amount of radiation also changed the odor and taste of food and even affected the vitamin content. In addition, the amount of radiation which inactivates the clostridial organisms may not inactivate other pathogenic organisms such as the Lansing strain of the polio virus.

At present, we know that most of the drawbacks to food irradiation are due to oxidation. Experiments have shown that if oxygen is removed from food, flavor and odor are much less affected by irradiation and most of the changes which do occur will abate during post-irradiation storage. Thus it is certainly possible to improve techniques of food irradiation (Casarett, 1968).

The Atomic Energy Commission (AEC) claims that low doses of ionizing radiation can greatly increase the life of food products without loss of nutrition and flavor, but the Food and Drug Administration (FDA) refuses to permit the use of irradiated food for humans in this country except in the cases of potatoes and wheat flour. The reason given by FDA for its stand is that, in its opinion, the present data do not prove the safety of most irradiated foods to the satisfaction of the law:

- (1) It has not been firmly established that the irradiation of foods produces no carcinogens.
- (2) Proof has not been offered that the approved amount of irradiation does indeed kill potentially pathogenic organisms.
- (3) If these organisms are not destroyed by irradiation, the

possibility exists that this preservation treatment may change them in such a way as to produce worse problems (e.g., subsequent production of more toxin by irradiated spores of Clostridium botulinum). Moreover, large doses of irradiation may increase the radioresistance of organisms and their mutational tendencies.

Because of these objections, irradiation treated foods are not extensively available commercially anywhere in the world. Some of the studies which support or contradict FDA in its conclusion are described below:

Schmidt et al., (1962a) studied the radiation resistances of spores of six strains of Clostridium botulinum type E in canned beef stew substrate. The inoculum was 100 million spores per can. After the cans were irradiated with various doses of gamma radiation, the results were compared with those obtained using spores of six strains of type A and five strains of type B. This comparison indicated that the spores of type E strains possess about 45 to 55 percent of the resistance to ionizing radiation of spores of types A and B. These workers concluded that a radiation dose for food sterilization based on the maximum resistance of types A or B strains would provide ample protection against spores of any type E strains.

Kempe and Graikoski (1962) irradiated vacuum canned pork luncheon meat (PLM) before inoculation with 10,000 to 1,530,000 Clostridium botulinum type A or B spores per can. The radiation doses were 0.186 megarad for type B spores and 2.15 megarad for type A spores. In this experiment, they found the presence of detectable toxin within one month in cans inoculated with type A spores after irradiation and within two months in

those inoculated with type B spores. But, when vacuum canned PLM was irradiated after inoculation, with 0.190 to 4.40 megarad, there was no spoilage for several months at 30C, the same length of shelf life as for the controls. They suggested that the spores of Clostridium botulinum types A and B were relatively easily inactivated when irradiated in PLM, probably because of the presence of curative agents in the meat which increased the effect of irradiation on the spores.

Kazanas et al. (1966) found that, after irradiation with 0.3 or 0.6 megarad gamma radiation from ^{60}Co , the microflora contained in freshwater fish, stored at 1C or 6C, were reduced nearly 100 percent, as shown by plate counts. They concluded that irradiation can significantly extend the shelf life of refrigerated freshwater fish.

Emerson et al. (1966) found that after irradiation with 0.3 and 0.6 megarad gamma radiation from ^{60}Co , the storage life of irradiated samples of freshwater fish held at 33F was four to five times longer than that of unirradiated samples. If, however, the storage temperature was increased to 42F, the shelf life of irradiated samples was decreased to 50 percent of those irradiated and stored at 33F.

CHAPTER II

MATERIALS AND METHODS

Source of Organisms Used

Strain B-17 of Clostridium botulinum type B and strain Beluga of Clostridium botulinum type E were kindly supplied by Dr. M. W. Eklund, Bureau of Commercial Fisheries, Seattle, Washington. Strain B-17 of Clostridium botulinum type B was originally isolated by Dr. Eklund from ocean sediments taken off the Pacific coast. Stock cultures of the organisms in cooked meat medium were quick frozen at -80C in 2.5 ml amounts in a bath of 2-ethoxyethanol with dry ice and stored at 10C in the laboratories of Dr. Nancy Walls, Engineering Experiment Station, Georgia Institute of Technology, Atlanta, Georgia.

Spore Production

A tube of frozen stock culture of non-proteolytic B-17 strain of the Clostridium botulinum type B or the Beluga strain of Clostridium botulinum type E was thawed in tap water, inoculated into 30 ml of Eklund's GPBI medium (see Appendix A) and the culture incubated at 25C. After four days, smears from the culture were stained with malachite green spore stain (Manual of Microbiological Methods, 1957) and examined microscopically to confirm the presence of spores. A ten percent inoculum was then transferred into each of the following media:

For Clostridium botulinum type B, strain B-17:

- (1) 200 ml trypticase-peptone-glucose (TPG) medium plus one

percent yeast extract and 0.1 percent sodium thioglycollate (see Appendix A).

(2) 200 ml phytone medium plus 0.1 percent sodium thioglycollate (see Appendix A).

For Clostridium botulinum type E, strain Beluga:

(1) 200 ml trypticase-peptone-glucose (TPG) medium plus one percent yeast extract and 0.1 percent sodium thioglycollate (see Appendix A).

Both media were adjusted to pH 7.2 with 10 N NaOH before autoclaving. After inoculation, the cultures were incubated at 25C for 36 hours and ten percent inocula were made from this growth into 1500 ml of TPG plus one percent yeast extract medium and, for type B, also into 1500 ml of phytone medium. These cultures were incubated at 25C for 36 hours, after which smears taken at frequent intervals from each culture were stained and examined microscopically to determine the optimum time for harvesting the spores.

Harvesting of Spores

For each medium being harvested for spores, six sterile 250 ml centrifuge bottles were each filled with approximately 200 ml of medium containing cells and spores and centrifuged at 4C for 30 minutes at 2000 rpm (1170 x g) in an International refrigerated centrifuge*. The supernatant portions were decanted, autoclaved, and discarded. The sediments were resuspended in a small amount of cold sterile deionized water and transferred to a single sterile screw cap 250 ml Erlenmeyer flask which was held in an ice bath. The spinning and collecting procedure was repeated until all the mother medium was removed. The pooled spores and

*Model PR-2, International Equip. Corp., Needham Heights, Mass.

cells in the flask were then distributed among four centrifuge bottles and suspended in cold sterile deionized water. The washing and spinning were repeated three times, reducing the number of bottles used each time. When all the sediments were in one bottle, the spinning time was increased to one hour and the spun material left in the centrifuge at 4C overnight. The following morning, the spores were washed and spun once more, then the pellet of spores was transferred to a sterile 50 ml screw cap Erlenmeyer flask containing a few glass beads. The centrifuge bottle was repeatedly washed with small amounts of cold sterile deionized water and the washings added to the collected spores. The spores were then refrigerated at 4C.

Heat Shocking Procedure

Before counting, each spore stock was heat-shocked to kill any vegetative organisms which may have survived the collection procedure. To accomplish this, the spore stock was heated to 60C in a water bath for 13 minutes in a screw cap 50 ml Erlenmeyer flask, then rapidly cooled in ice water.

Spore Cleaning

The spores of non-proteolytic Clostridium botulinum type B, strain B-17 and Clostridium botulinum type E, strain Beluga, were found to be far more sensitive to heat and to lysozyme-and-sonic treatment than the proteolytic strains investigated by previous workers in this laboratory. This sensitivity was confirmed by microscopic examination of enzyme-sonication treated spores stained with methylene blue. Uptake of methylene blue immediately following cleaning of the spores indicated

incipient germination and therefore greater susceptibility to irradiation than refractive spores which do not take up methylene blue stain. Therefore, the spore cleaning procedures for these strains were modified as follows:

For Clostridium botulinum type B, strain B-17, the spore crop was cleaned by treatment with 95 percent ethanol. The spore stock was diluted 1:1 with 95 percent ethanol and the mixture placed in a 50 ml sterile screw cap centrifuge tube. The spore-ethanol mixture was incubated at room temperature for one hour, with frequent agitation. The tube and its contents were then centrifuged for 30 minutes at 3000 rpm (1760 x g) in an IEC refrigerated centrifuge at 4C. The supernatant was decanted and the sediment washed with cold sterile deionized water. The spinning and washing procedure was repeated three times; then the spores were collected in a 50 ml sterile screw cap Erlenmeyer flask, brought up to their original volume with cold sterile deionized water, and stored in a refrigerator at 4C.

For Clostridium botulinum type E, strain Beluga, the spore crop was cleaned by enzyme-sonication treatment. The procedure was:

Stock solution: Trypsin 1:250 (Difco)---to contain 5 mg/ml of solution.

Lysozyme (6000 to 10,000 units/mg*---to contain 10 mg/ml of solution.

These solutions were Millipore-filtered**, tested for sterility, and kept refrigerated until used.

*Nutritional Biochemical Corp., Cleveland, Ohio.

**Millipore Corp., Bedford, Mass.

Into a sterile 150 ml beaker were placed aseptically:

Spore stock	2.0 ml
Cold sterile deionized water	46.0 ml
Trypsin solution	1.0 ml
Lysozyme solution	1.0 ml

The mixture was subjected alternately to ultrasonic oscillation with a Bronwill Biosonik III* probe at a setting of 50, and incubation in a water bath at 37C as noted:

1. Incubation of spore-enzyme mixture one hour
2. Sonic treatment one minute
3. Incubation one hour
4. Sonic treatment one minute
5. Incubation one hour
6. Sonic treatment one minute
7. Incubation one hour
8. Sonic treatment one minute
9. Incubation one hour
10. Sonic treatment one minute

During sonic disruption, sterile foil covered the beaker and the probe was inserted through an opening in the foil. After the last sonic oscillation was completed, the spore material was transferred to two sterile 50 ml screw cap centrifuge tubes. The beaker was washed with small amounts of cold sterile deionized water and the washings added to the spores. The mixture was stored in the refrigerator overnight, then was centrifuged at 3000 rpm (1760 x g) for 30 minutes at 4C. The

*Bronwill Scientific, Rochester, New York.

supernatant was carefully suctioned off and the spores washed with cold sterile deionized water. Washing and spinning were repeated at least three times. Finally the spores were diluted to the original volume (two ml) in sterile deionized water and refrigerated until needed.

Irradiation Procedure

The cleaned spore stock was diluted 1:10 with Borenson's phosphate buffer, pH 7.0. Ten ml of the diluted spore suspension were transferred to each of six sterile 15 x 125 mm screw cap culture tubes. The tubes were immersed in iced water before, during, and after irradiation until inoculated into the growth medium chosen for the experiments. Duplicate tubes containing the spore suspension were subjected to either 0.0, 0.1 or 0.2 megarad irradiation in the center sample hole of a cesium-137 source* having a uniform radiation field. The samples were equidistant from the surrounding cesium-137 rods and therefore were assured equivalent radiation dosage. The average dose rate was 1.1 megarad per hour as measured by both ferrous ion oxidation and Dupont HSC-300 light blue cellophane dosimeters.

Growth Medium

The medium used for growth and toxin production after spore irradiation was freshly prepared trypticase soy broth (BBL) plus 0.1 percent sodium thioglycollate (see Appendix A) contained in 300 ml Nephelo culture flasks**. The pH of the medium was 7.2, requiring no adjustment. After the medium had been autoclaved for 15 minutes at 15 psig and cooled to room temperature, six ml of Millipore-filtered 20 percent ribose were added to each Nephelo culture flask containing 244 ml of growth medium

*The radiation source is a 12,000 curie cesium-137 source located in the Emerson Building at the Georgia Institute of Technology.

**Bellco Glass Inc., Vineland, New Jersey.

(approximately 0.48 percent ribose). The medium was allowed to equilibrate in the 10C or 25C incubator for three hours (for the type B experiments) or five hours (for the type E experiments). Then one percent inocula of spore suspension were added to duplicate flasks for each radiation level (0.0, 0.1 or 0.2 megarad). The effects of irradiation on spore outgrowth were determined for two incubation temperatures: 10C and 25C.

Growth Measurements

Bacterial growth was determined by measuring the turbidity of the cultures with a Bausch and Lomb "Spectronic 20" colorimeter* set at a wavelength of 600 m μ . The cultures were read against a blank of sterile growth medium, which gave a relative value rather than an absolute quantitation of cells per unit volume. The cultures incubated at 25C were read hourly from zero hour through sixty hours after inoculation. The cultures incubated at 10C were measured daily from zero hour through thirty days after inoculation. From the cultures at 25C, smears were made at least once every four hours to fix the time at which the inoculated spores began to germinate.

Trypsinization Procedure

Both trypsinized and untrypsinized culture supernatants were tested for toxicity at each sampling time. To trypsin-activate a sample, 1.2 ml of centrifuged culture supernatant was adjusted to pH 6.0 with dilute hydrochloric acid. To this was added 0.3 ml of five percent Millipore-filtered trypsin solution (Difco 1:250)*. The liquids were thoroughly mixed and incubated in a 37C water bath for 45 minutes.

*Bausch & Lomb, Inc., Rochester, New York.

**One part Trypsin, Difco, 1:250 will digest 250 parts of casein under the conditions of the U.S.P. test for trypsin in pancreatin.

Injections into mice were begun as soon as possible after trypsinization was completed.

Toxin Titration

Toxin was assayed by the standard mouse bioassay procedure using SAF/ICR strain white mice, each 13-18 g in weight. Each mouse was injected with 0.5 ml centrifuged culture supernatant fluid, trypsinized or untrypsinized, diluted when necessary with sterile gelatin diluent (see Appendix A) to the desired titration level. Two mice were used for each toxin titration level and the results expressed as LD₅₀.

For the cultures incubated at 25C, toxin was measured at four hour intervals from initiation of the experiment (zero hour) to 60 hours; thereafter, at 24 hour intervals to 240 hours. Toxin was measured at zero hour and at 5, 8, 11, 16, 20, 23, 28, and 30 days for the cultures incubated at 10C.

Spore Titration

The parameter for viability of spores after irradiation was their ability to form colonies after inoculation into the proper solid growth medium. The spore suspension was diluted in 0.1 percent peptone water (see Appendix A) and inoculated in triplicate samples of appropriate dilutions into 25 ml volumes of freshly prepared Schmidt's counting medium (see Appendix A) contained in Prickett tubes (Fisher modified agar slant tubes). The filled tubes were held at 45C in a water bath until inoculated, after which each tube was rotated between the palms of the investigator to insure uniform mixing and then rapidly cooled in an ice water bath until solidified. When firm, each tube of medium was

sealed with a plug containing 2 percent agar and 0.1 percent sodium thioglycollate; then the original plug was replaced. The tubes were incubated at 25C for two to three days before counting the colonies with the aid of a "Bactronic"* colony counter.

*Model CC-110, New Brunswick Scientific Co., New Jersey.

CHAPTER III

RESULTS

I. Non-proteolytic *Clostridium botulinum* Type B, Strain B-17: Growth and Toxin Production After Irradiation at 0.0, 0.1, or 0.2 Megarad.A. Growth and Toxin Production at 25C.

(1) Experiment II-B, Spores Produced from Phytone Medium.

Figures 1, 2, 3, and 4 show the growth response and toxin production pattern of vegetative cells of *Clostridium botulinum* type B, strain B-17 grown from irradiated (or control) spores inoculated into trypticase soy broth (TSB) medium and incubated at 25C post-irradiation. The spores, which had been produced in phytone medium, were inoculated into TSB medium after treatment with 0.0, 0.1, or 0.2 megarad gamma irradiation in a cesium-137 source. To establish the germination time of these spores, slides were made from the cultures at hourly intervals and at least 25 fields per slide were carefully examined under the oil immersion lens of a bright-field microscope, noting both stainability of the spores with malachite green or methylene blue dye and beginning emergence of vegetative cells. Approximately 11 hours after inoculation (zero hour), the unirradiated spores showed evidence of germination (see Table 1) as determined by their appearance on microscopic examination of stained smears from the culture. At this point, spores were beginning to lose refractivity (as evidenced by the inability to absorb malachite green stain) and were starting to stain with methylene blue at the perimeters of the spores. The same method was used to determine that germination

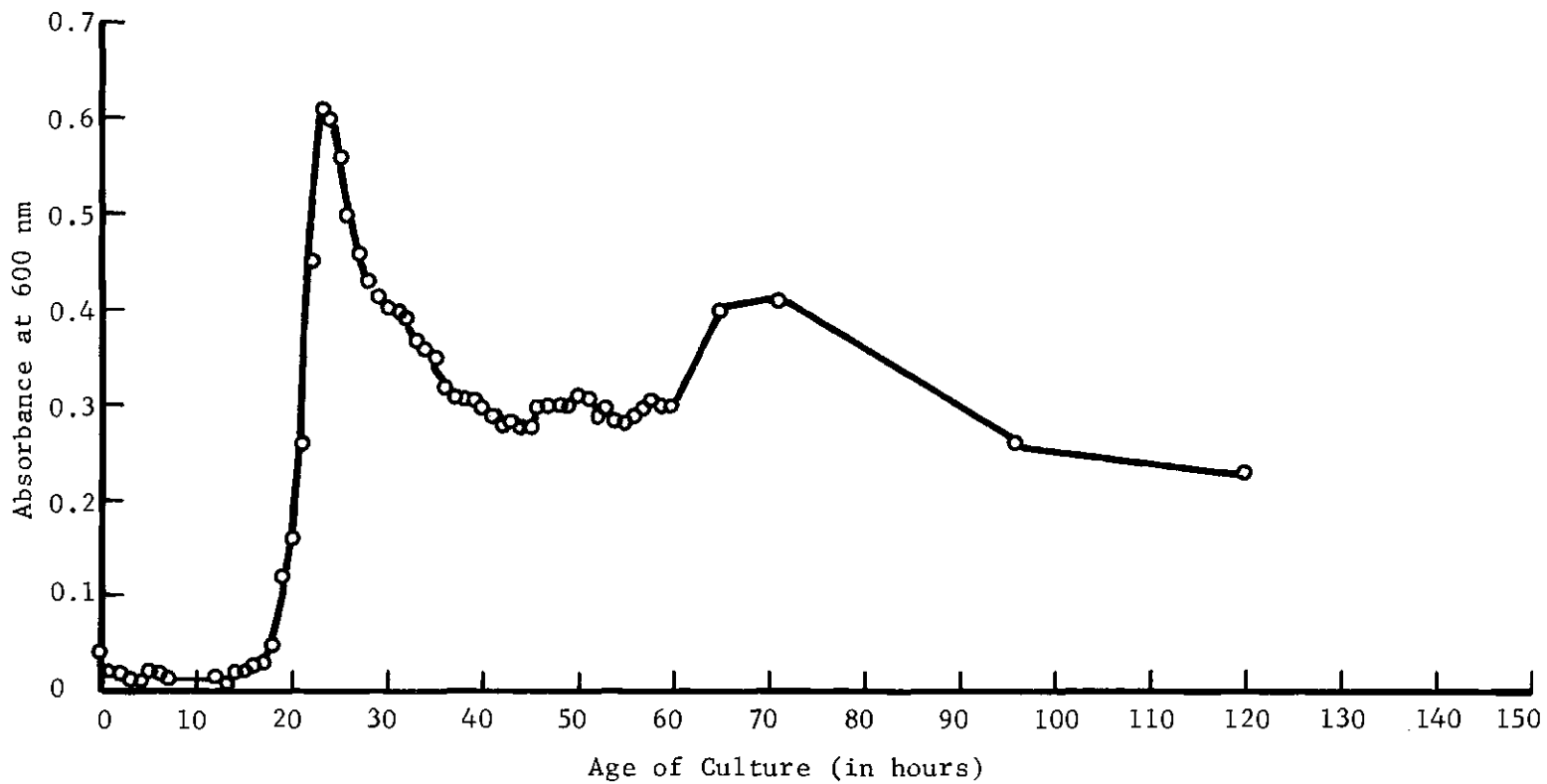


Figure 1. Growth Response of Unirradiated Spores of Clostridium botulinum Type B, Strain B-17 in Trypticase Soy Broth at 25C (Experiment II-B). Spores Produced in Phytone Medium.

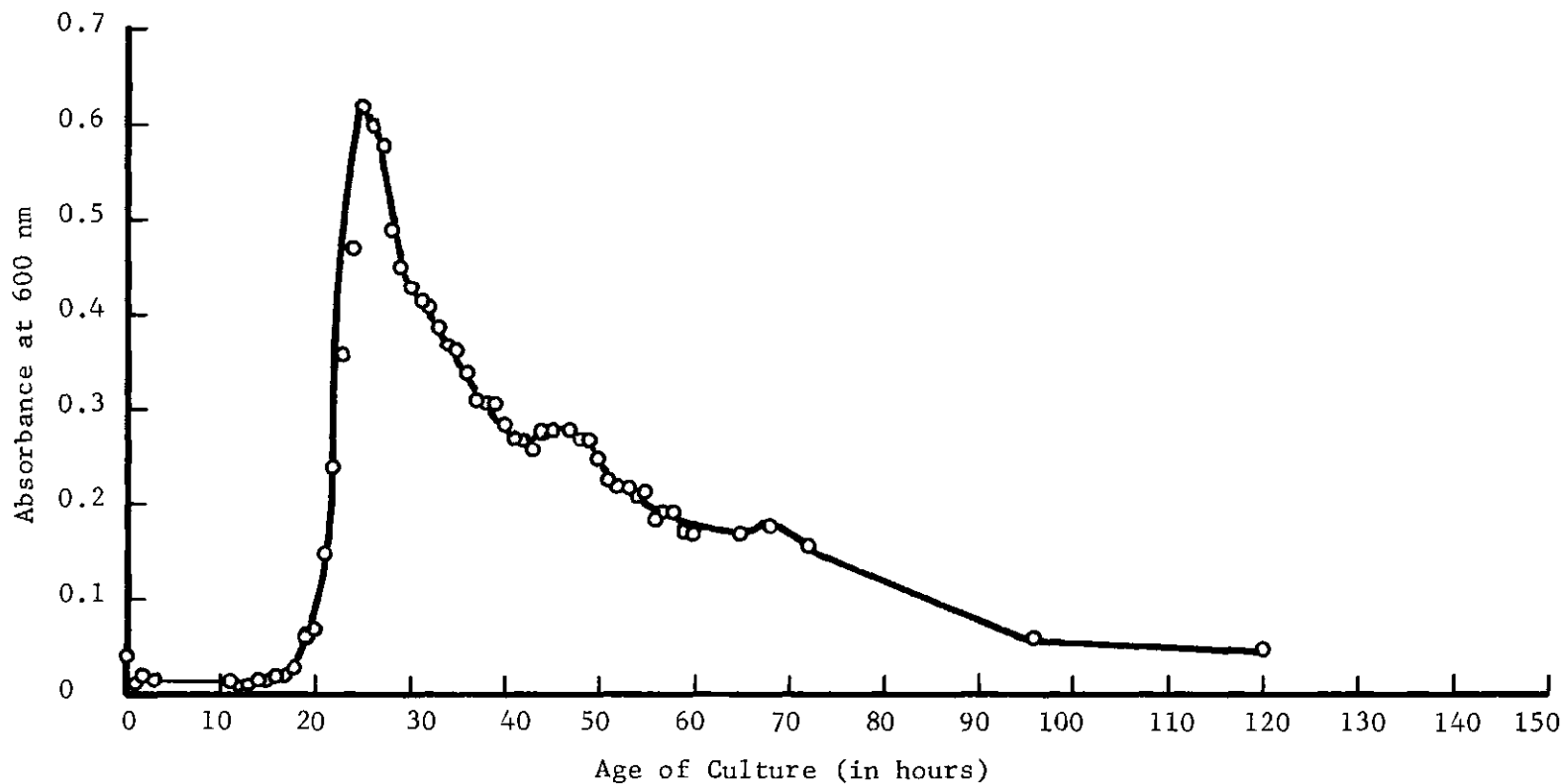


Figure 2. Effect of 0.1 Megarad of Cesium-137 Gamma Irradiation on Growth Response of Spores of *Clostridium botulinum* type B, Strain B-17 in Trypticase Soy Broth at 25C (Experiment II-B). Spores Produced in Phytone Medium.

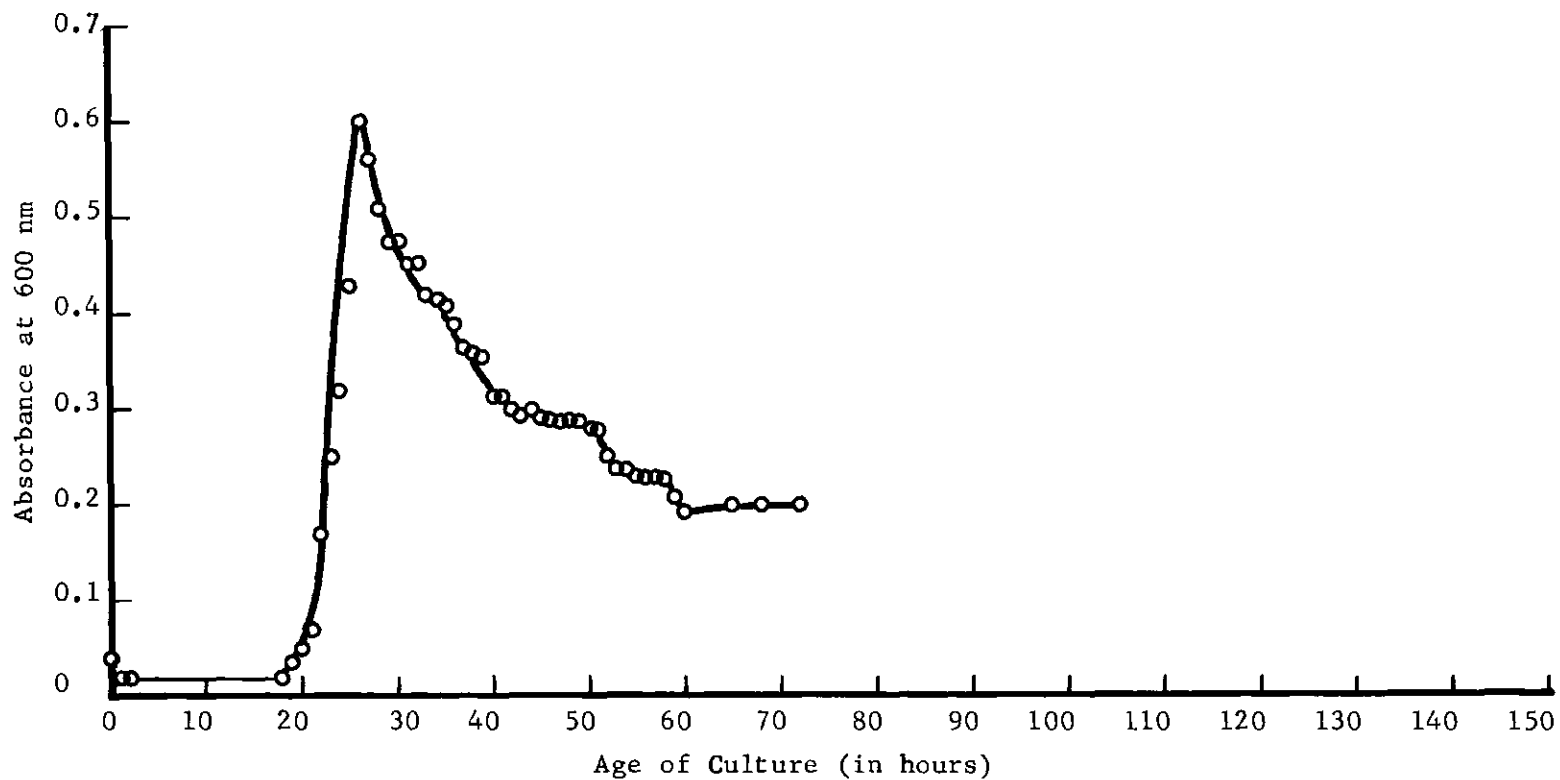


Figure 3. Effect of 0.2 Megarad of Cesium-137 Gamma Irradiation on Growth Response of Spores of Clostridium botulinum Type B, Strain B-17 in Trypticase Soy Broth at 25C (Experiment II-B). Spores Produced in Phytone Medium.

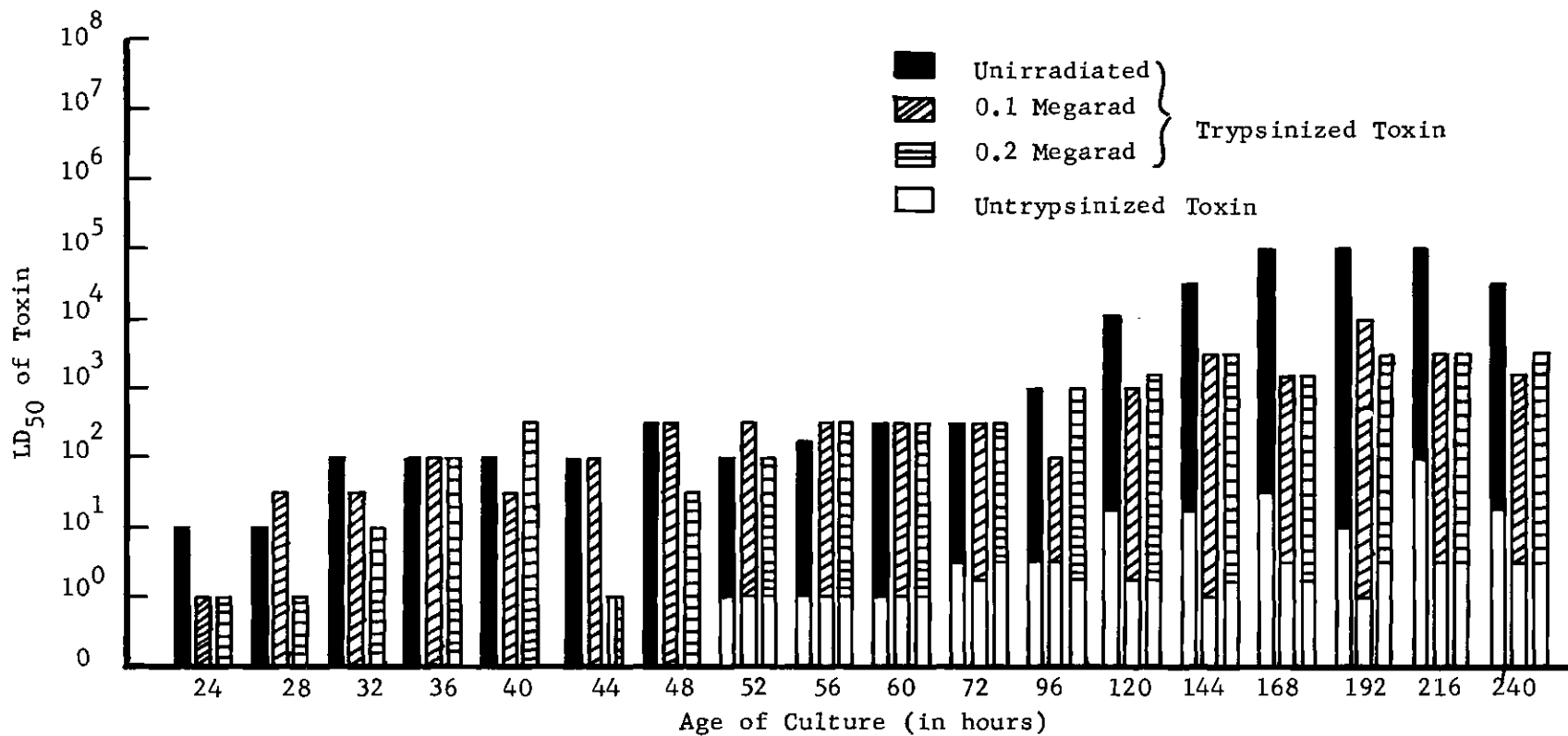


Figure 4. Toxin Production of Unirradiated and Irradiated Spores (0.1 Megarad and 0.2 Megarad of Cesium-137 Gamma Irradiation) of *Clostridium botulinum* Type B, Strain B-17 in Trypticase Soy Broth at 25C (Experiment II-B).

began in the 0.1 and 0.2 megarad irradiated cultures at 12 hours (see Table 1).

Outgrowth was evidenced by an increase in optical density readings at 14 hours after inoculation for the unirradiated culture, at 14 hours for the 0.1 megarad irradiated culture, and at 19 hours for the 0.2 megarad irradiated culture.

The logarithmic growth phase continued for ten hours in the unirradiated culture, reaching a maximum optical density reading of 0.61 at 23 hours after inoculation (see Table 2), a peak which was followed by rapid autolysis. In the 0.1 megarad culture, the logarithmic growth phase continued for 12 hours, reaching a maximum optical density reading of 0.63 at 25 hours after inoculation, again followed by rapid autolysis. In the 0.2 megarad irradiated culture, the logarithmic growth phase also continued for eight hours, reached a maximum optical density reading of 0.60 at 26 hours after inoculation, and then the culture rapidly autolyzed.

After trypsin activation, toxin was detected at 24 hours in all three culture supernatants. Without trypsin activation toxin was first found at 52 hours for all three cultures. Toxin titers for both the trypsinized and untrypsinized culture supernatants were higher for the unirradiated culture than for the irradiated cultures, a condition which persisted throughout the 240 hours of toxicity testing. Consistently, throughout 240 hours of sampling, trypsinization increased the toxin titers from a minimum of 50 fold to a maximum of 10,000 fold for the unirradiated culture, 20 to 10,000 fold for the 0.1 megarad irradiated culture, and one to 2000 fold for the 0.2 megarad irradiated

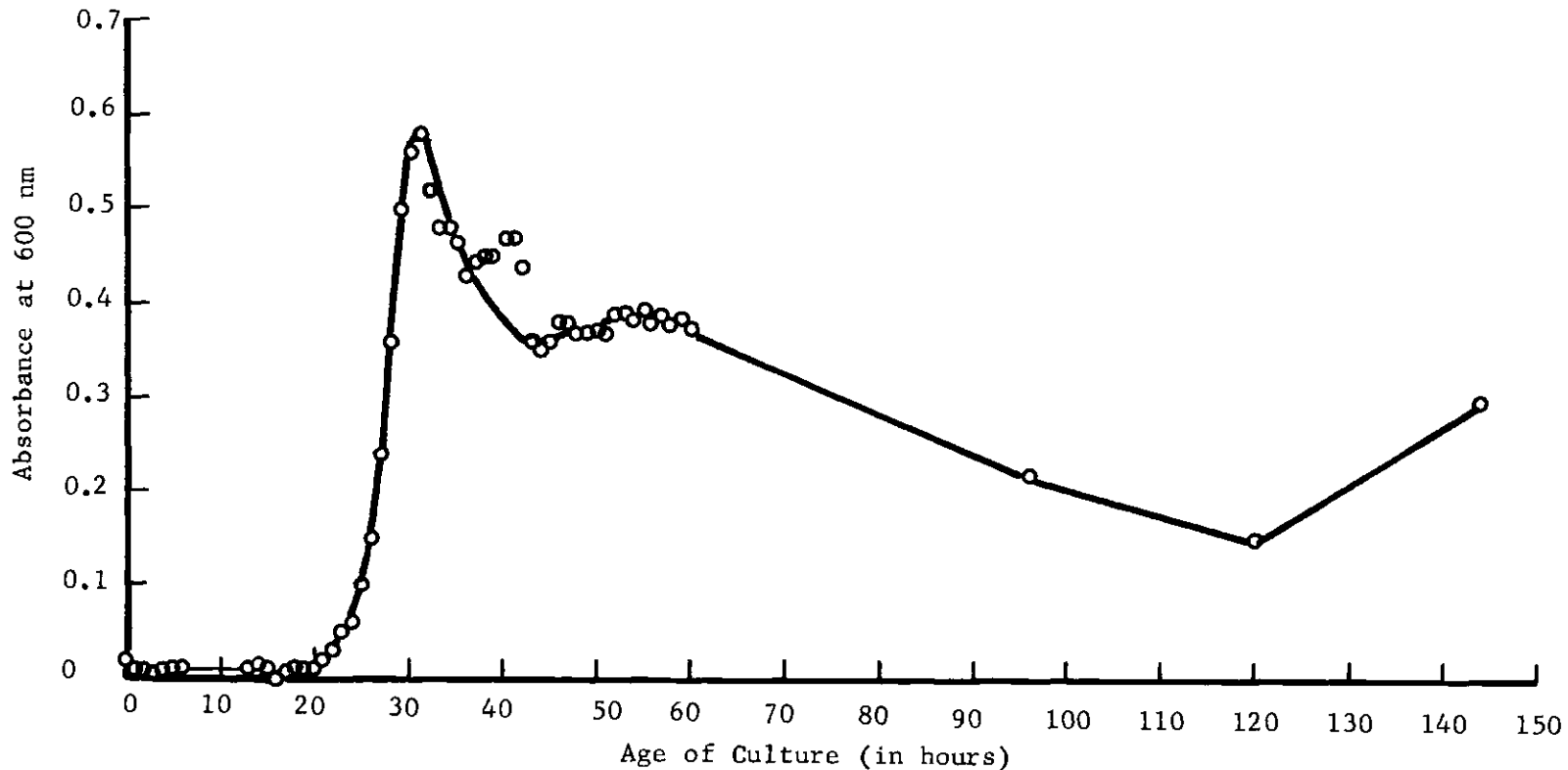


Figure 5. Growth Response of Unirradiated Spores of Clostridium botulinum Type B, Strain B-17 in Trypticase Soy Broth at 25C (Experiment III-B). Spores Produced in TPG Medium.

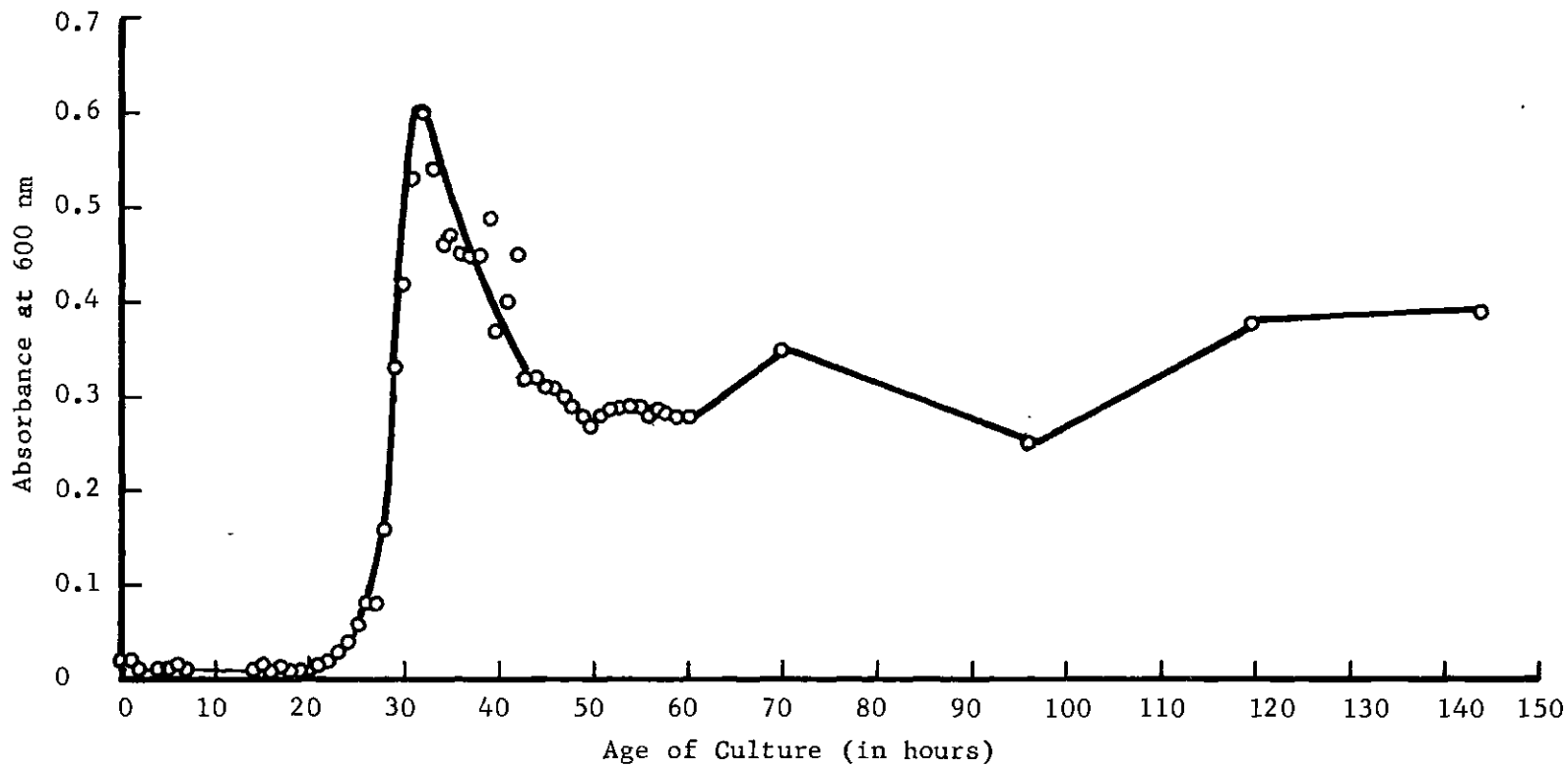


Figure 6. Effect of 0.1 Megarad of Cesium-137 Gamma Irradiation on Growth Response of Spores of Clostridium botulinum Type B, Strain B-17 in Trypticase Soy Broth at 25C (Experiment III-B). Spores Produced in TPG Medium.

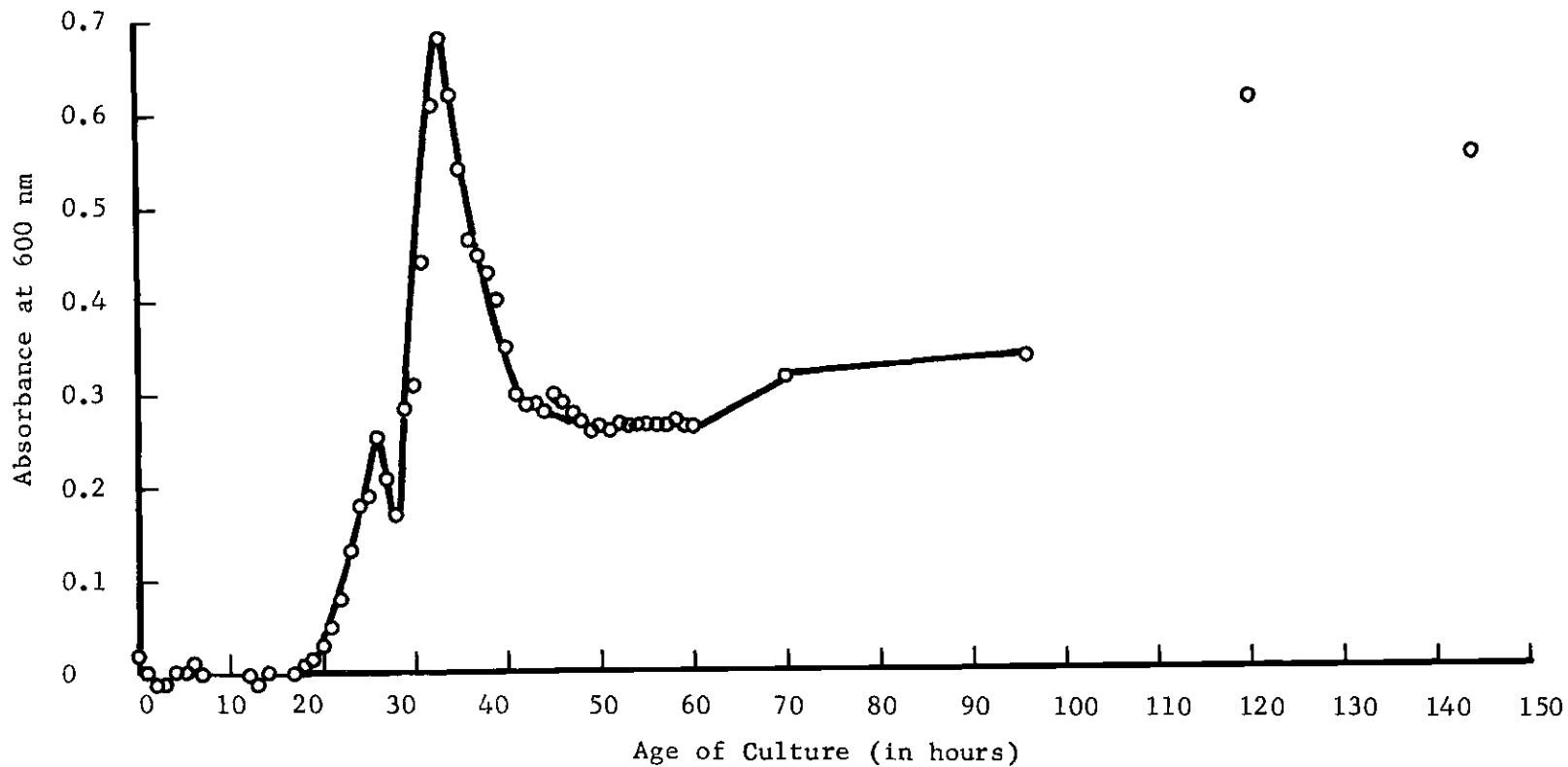


Figure 7. Effect of 0.2 Megarad of Cesium-137 Gamma Irradiation on Growth Response of Spores of Clostridium botulinum Type B, Strain B-17 in Trypticase Soy Broth at 25 C (Experiment III-B). Spores Produced in TPG Medium.

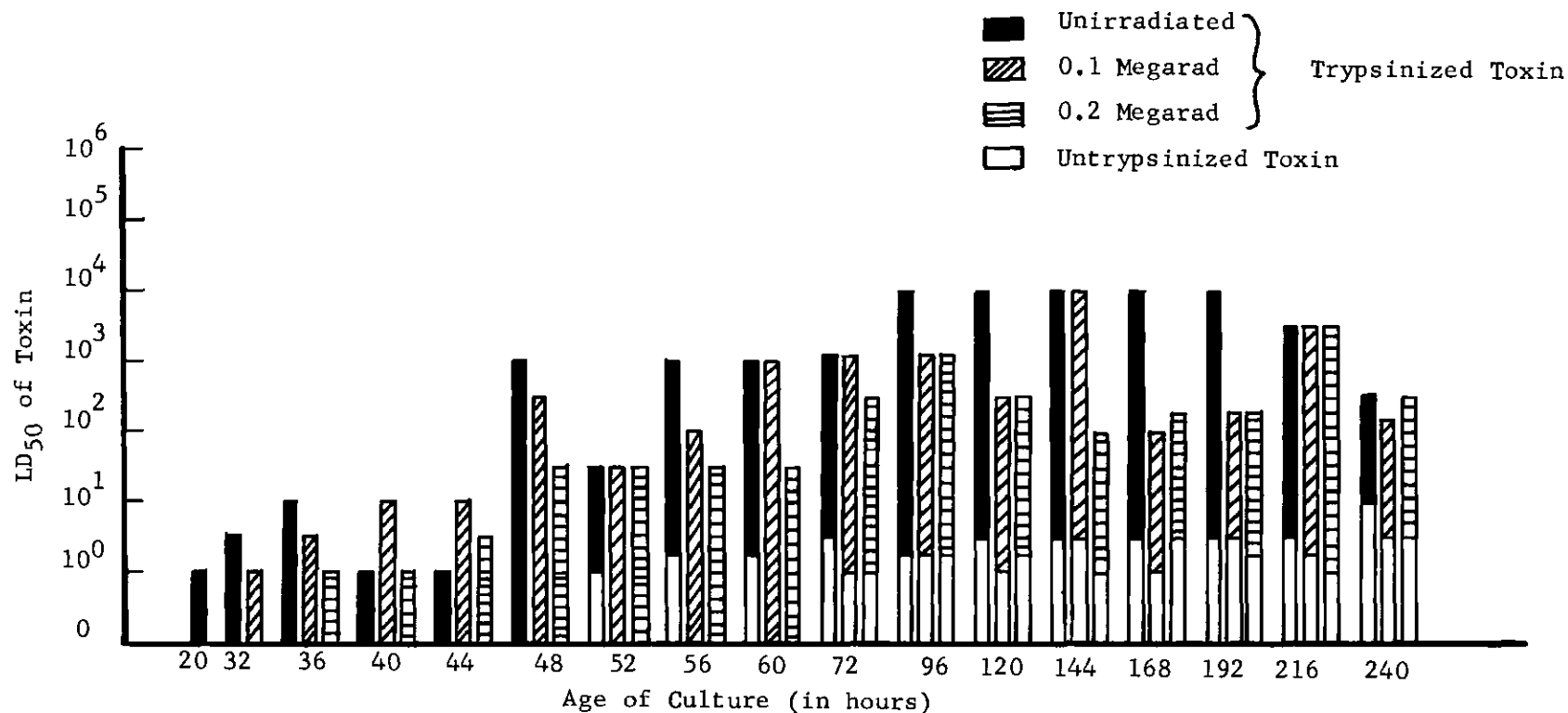


Figure 8. Toxin Production of Unirradiated and Irradiated Spores (0.1 Megarad and 0.2 Megarad of Cesium-137 Gamma Irradiation) of Clostridium botulinum Type B, Strain B-17 in Trypticase Soy Broth at 25C (Experiment III-B).

culture.

(2) Experiment III-B, Spores Produced from TPG Medium.

Figures 5, 6, 7, and 8 show the results of a second experiment using Clostridium botulinum type B, strain B-17. The spores, this time produced in trypticase-peptone-glucose (TPG) medium, were inoculated into TSB medium after they were treated with 0.0, 0.1, or 0.2 megarad gamma radiation in a cesium-137 source. Spores of all three cultures showed signs of germination at 11 hours after inoculation (see Table 1). The cultures grown from the unirradiated and 0.1 megarad irradiated spores both entered the logarithmic growth phase 21 hours after inoculation. The logarithmic growth phase continued for 10 hours in the unirradiated culture and for 11 hours in the 0.1 megarad irradiated culture. The cultures reached maximum optical density readings of 0.58 at 31 hours and 0.60 at 32 hours, respectively. Rapid autolysis then followed in both cultures. The 0.2 megarad irradiated culture entered the logarithmic growth phase at 18 hours after inoculation, about three hours earlier than the unirradiated and 0.1 megarad irradiated cultures. In contrast to the response seen in the other experiments with spores of this organism, the 0.2 megarad irradiated culture underwent partial lysis at eight hours after entering the logarithmic growth phase. An additional eight hours were required for this culture to reach the maximum optical density reading of 0.68 at 33 hours after inoculation, followed by rapid autolysis.

Toxin (after trypsin activation) was first detected at 20 hours for the unirradiated control and at 32 hours for the 0.1 megarad irradiated culture, but at 36 hours for the 0.2 megarad irradiated culture. Without trypsin activation, toxin was first detected at 52, 72, and 72 hours,

respectively, after inoculation. Toxin titers were again higher for the unirradiated and 0.1 megarad irradiated cultures than for the 0.2 megarad irradiated culture. Trypsinization increased the toxin titers ranging from a minimum of 50 to 4000 fold for the unirradiated culture, 40 to 2000 fold for the 0.1 megarad irradiated culture, and 40 to 5000 fold for the 0.2 megarad irradiated culture.

(3) Experiment IV-B, Spores Produced from TPG Medium.

Figures 9, 10, 11, and 12 show the results of the third repetition of this investigation on growth response and toxin production at 25C of cultures grown from irradiated or control spores of Clostridium botulinum type B, strain B-17. As in the previous experiment, the spores were produced in trypticase-peptone-glucose (TPG) medium. Again the spore germination times are shown in Table 1. Both the unirradiated culture and the 0.1 megarad irradiated culture entered the logarithmic growth phase at 21 hours after inoculation. The logarithmic growth phase of the unirradiated culture continued for nine hours, reached a maximum optical density reading of 0.48. Then rapid autolysis began at 30 hours after inoculation into the growth medium. The logarithmic growth phase of the 0.1 megarad irradiated culture continued for 12 hours and reached a maximum optical density reading of 0.48 at 33 hours after inoculation before beginning rapid autolysis. The 0.2 megarad irradiated culture entered the logarithmic growth phase at 22 hours after inoculation, which continued for 11 hours, reached a maximum optical density reading of 0.54 at 33 hours, and was followed by rapid autolysis. Table 2 shows that the maximum optical density reading reached was somewhat higher in the 0.2 megarad irradiated culture than in the unirradiated culture and 0.1 megarad irradiated culture.

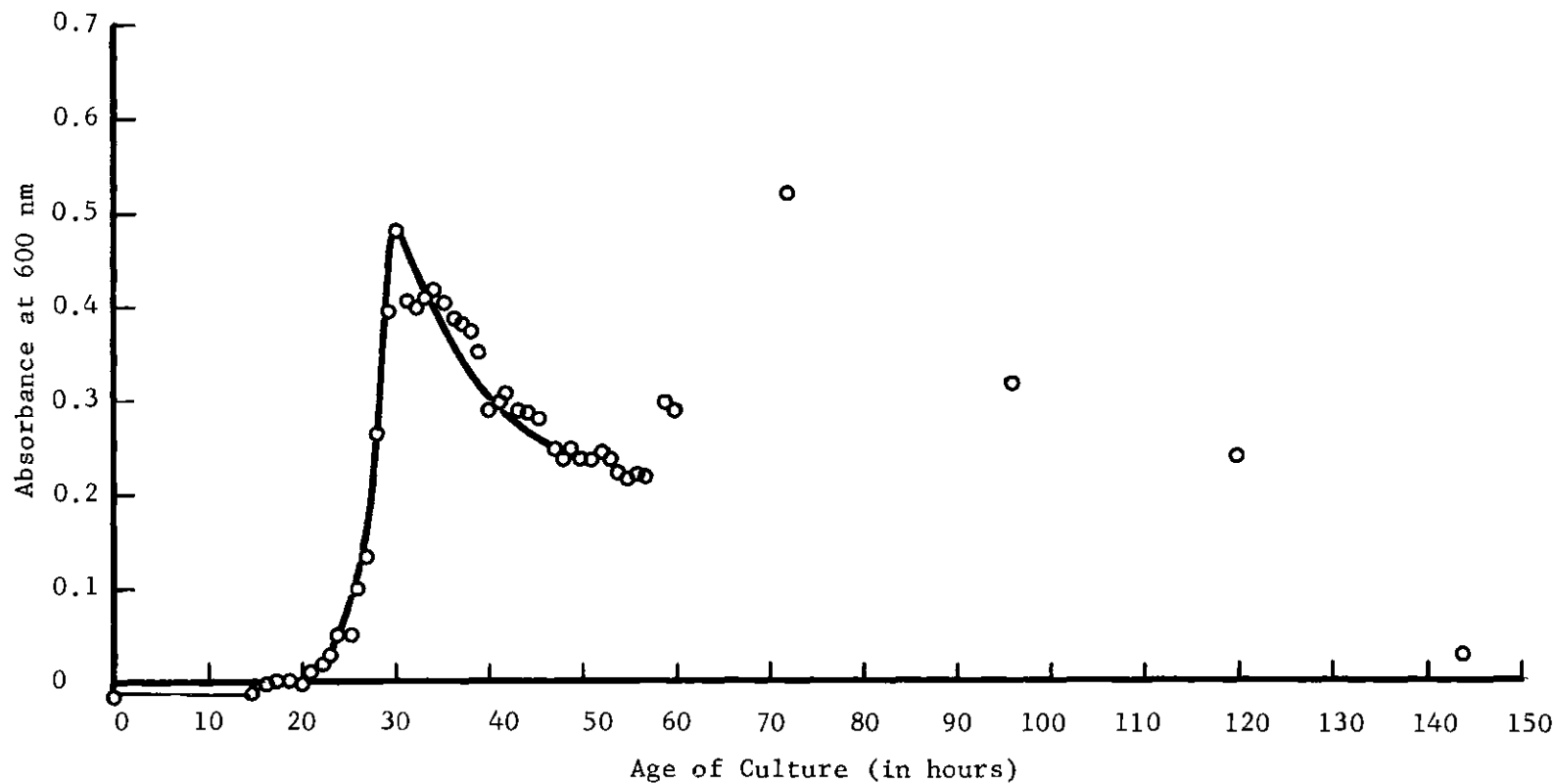


Figure 9. Growth Response of Unirradiated Spores of Clostridium botulinum Type B, Strain B-17 in Trypticase Soy Broth at 25C. (Experiment IV-B). Spores Produced in TPG Medium.

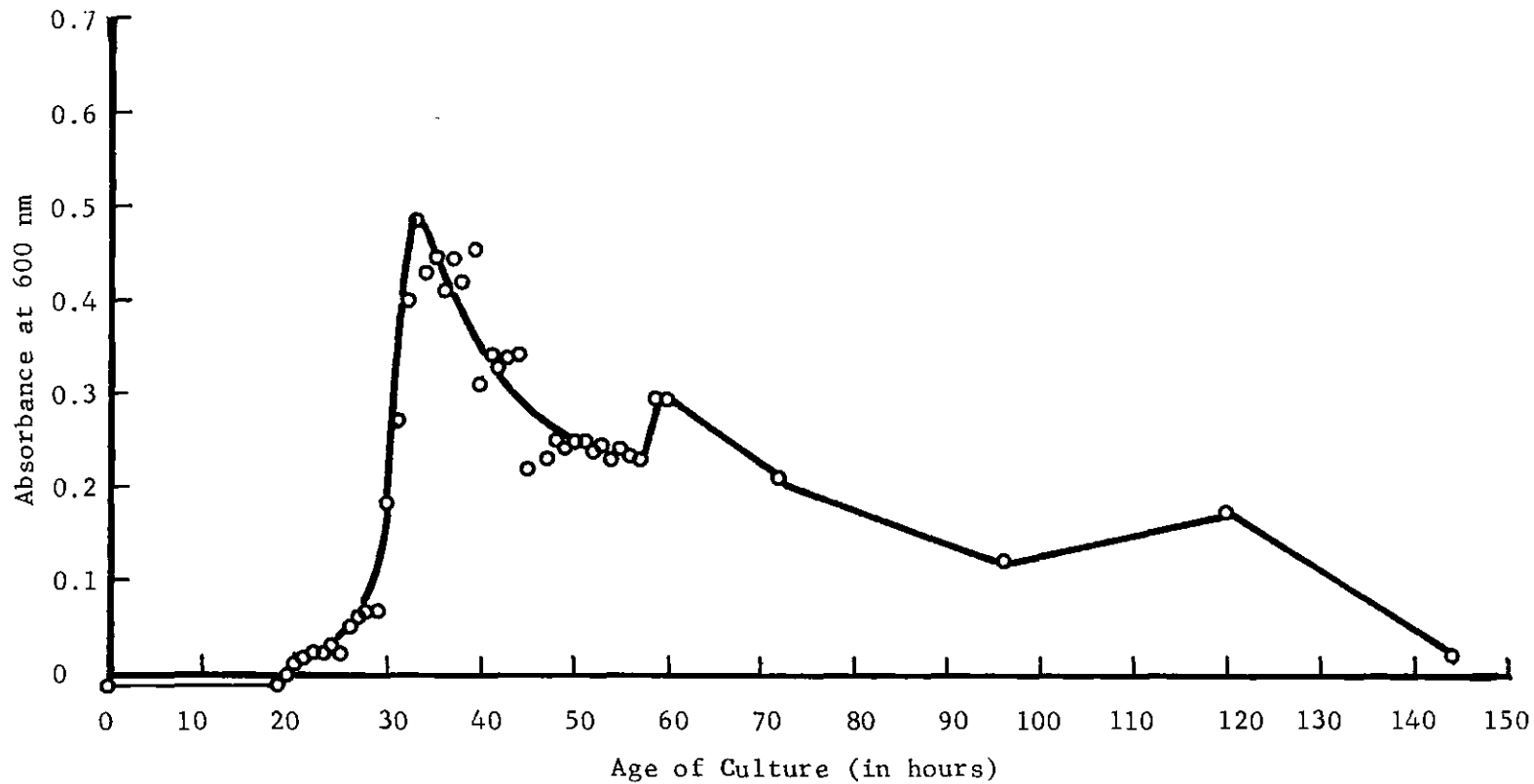


Figure 10. Effect of 0.1 Megarad of Cesium-137 Gamma Irradiation on Growth Response of Spores of Clostridium botulinum Type B, Strain B-17 in Trypticase Soy Broth at 25C (Experiment IV-B).

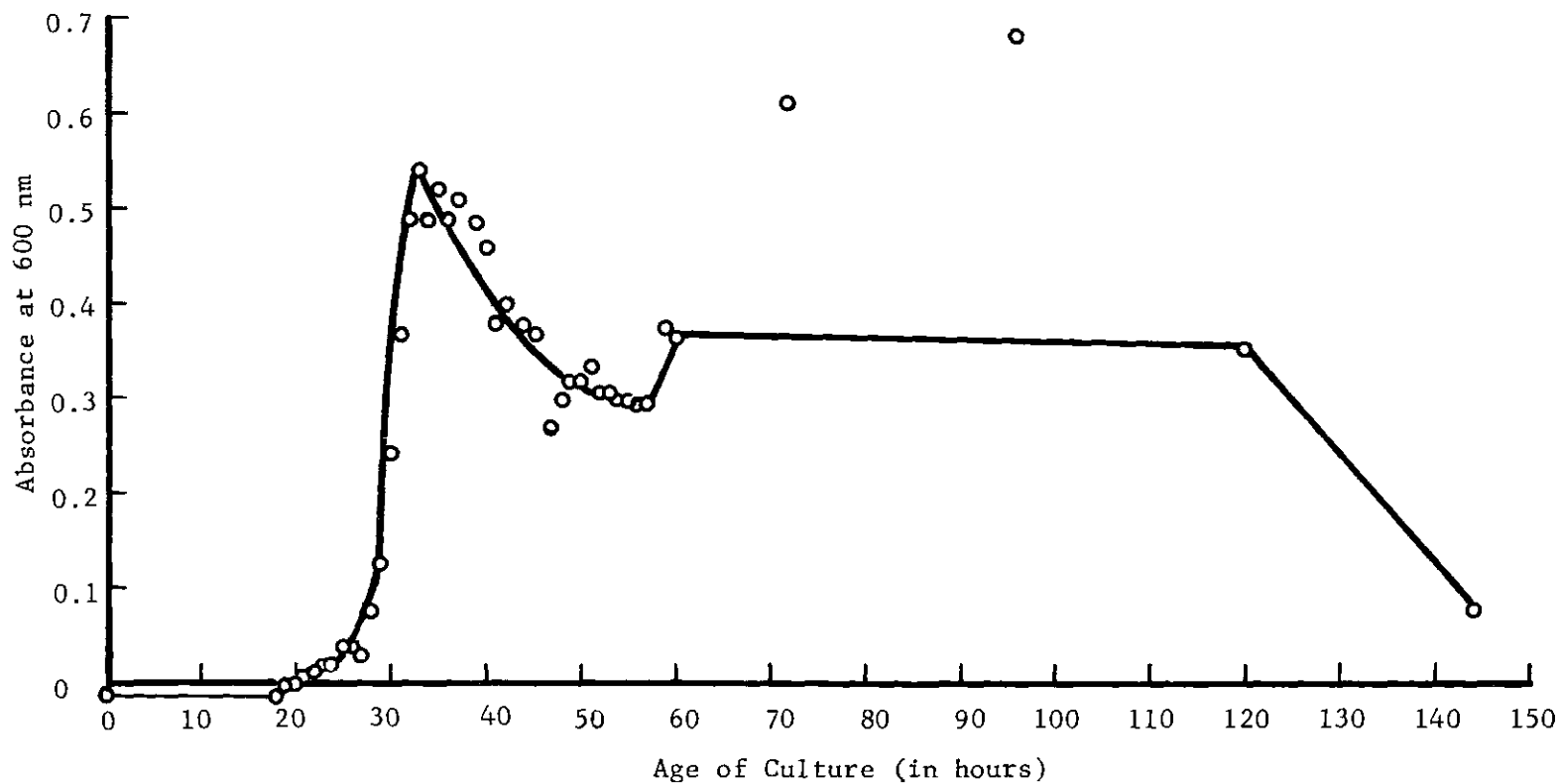


Figure 11. Effect of 0.2 Megarad of Cesium-137 Gamma Irradiation on Growth Response of Spores of Clostridium botulinum Type B, Strain B-17 in Trypticase Soy Broth at 25C (Experiment IV-B).

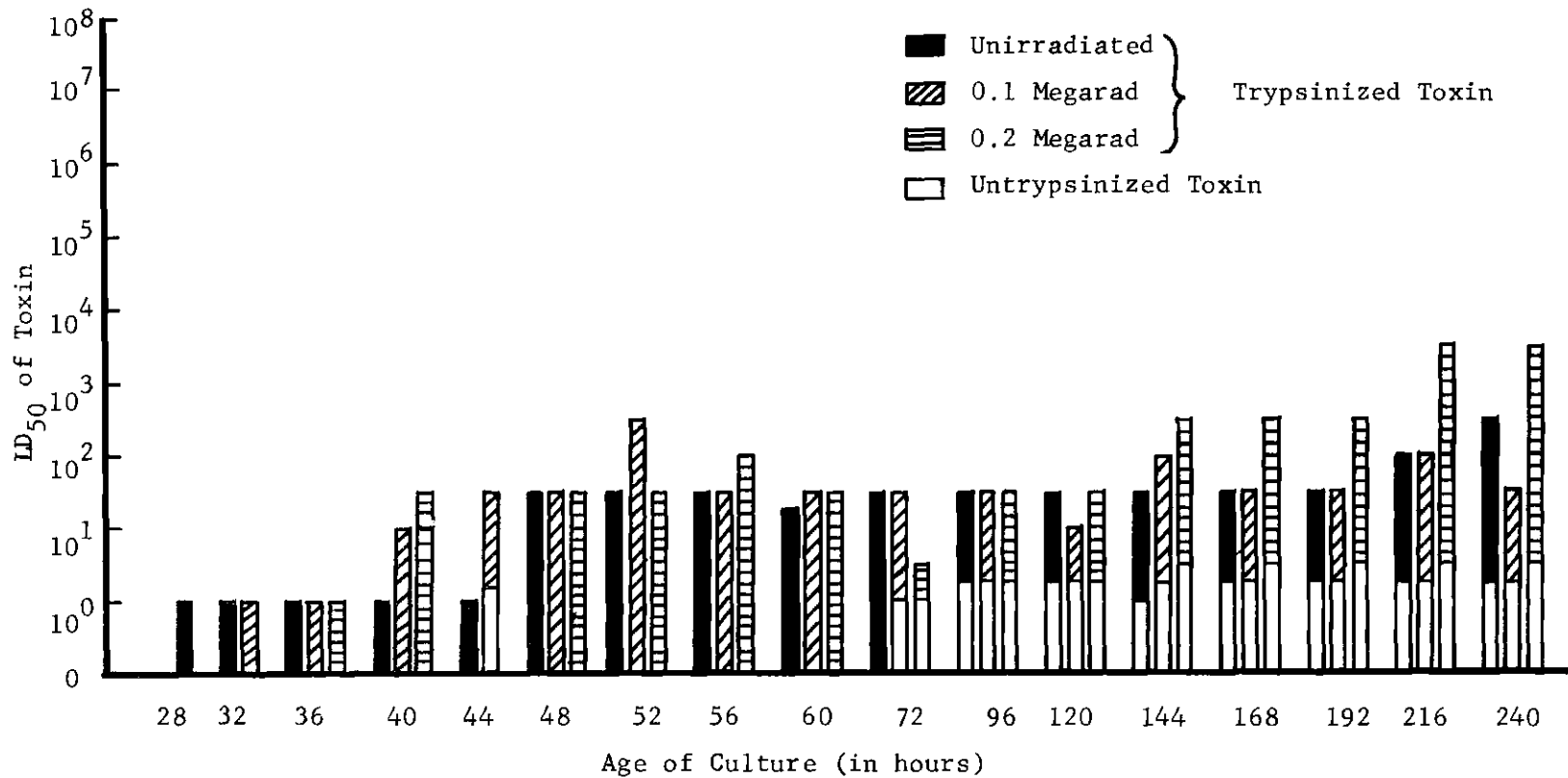


Figure 12. Toxin Production of Unirradiated and Irradiated Spores (0.1 Megarad and 0.2 Megarad of Cesium-137 Gamma Irradiation) of *Clostridium botulinum* Type B, Strain B-17 in Trypticase Soy Broth at 25C (Experiment IV-B).

Experiment IV-B, toxin (after trypsin activation) was first demonstrated at 28 hours after inoculation for the unirradiated culture, at 32 hours for the 0.1 megarad irradiated culture, and at 36 hours for the 0.2 megarad irradiated culture. Plain toxin (without trypsin activation) was first detected at 72 hours after inoculation for the irradiated cultures and at 96 hours for the unirradiated control culture. Both the trypsinized toxin and untrypsinized toxin of the unirradiated and 0.1 megarad irradiated cultures persisted from their first detection through 240 hours of testing for toxin production. In the 0.1 megarad irradiated culture, trypsinized toxin was first found at 32 hours after inoculation and untrypsinized toxin at 44 hours. Trypsinized toxin was detected from the 32nd through the 240th hour of observation, but untrypsinized toxin could not be demonstrated again after its first appearance at 32 hours until the 72 hour sample was tested. It then was present throughout the remainder of the 240 hours of sampling. In contrast to the results obtained in the first two experiments, the toxin titers were higher for the 0.2 megarad irradiated culture supernatants than for the unirradiated and 0.1 megarad irradiated samples in this replicate experiment. Trypsinization increased the toxin titers ranging from a minimum of 20 fold to a maximum of 200 fold for the unirradiated culture, 4 to 50 fold for the 0.1 megarad irradiated culture, and 10 to 1000 fold for the 0.2 megarad irradiated culture.

B. Growth and Toxin Production at 10C.

In addition to the studies at a post-irradiation growth temperature of 25C, spores of Clostridium botulinum type B, strain B-17, were also

incubated at 10C in trypticase soy broth (TSB) medium after irradiation and observed for growth response and toxin production. For all experiments, no growth response for any radiation level (0.0, 0.1 or 0.2 megarad) could be detected, by the colorimetric method used, over a period of 30 days observation. Although some spores germinated, as shown by microscopic examination, only a few showed evidence of division or further outgrowth.

Likewise, without trypsin activation, no toxin was demonstrated in any culture of four different experiments. In Experiment I-B (Figure 13), trypsinized toxin was found in the unirradiated culture supernatants at 15 days (50 LD₅₀), 23 days (100 LD₅₀) and 27 days (500 LD₅₀). In the 0.1 megarad irradiated culture, one LD₅₀ was found at 19 days, and in the 0.2 megarad, one LD₅₀ at 19 days and at 30 days. In Experiment II-B (Figure 14), toxin after trypsin activation was detected in the unirradiated culture at 11 days (one LD₅₀) and in the 0.2 megarad irradiated culture at 23 days (one LD₅₀). In this experiment, no toxin was found in the 0.1 megarad irradiated culture through 30 days of investigation. In Experiment III-B (Figure 15), trypsinized toxin was found at 11 days (one LD₅₀) in the 0.2 megarad irradiated culture; no toxin was detected in the unirradiated and 0.1 megarad irradiated culture during 30 days of investigation. In Experiment IV-B (Figure 16), no toxin (with or without trypsin treatment) was found in the 0.1 megarad irradiated culture for the length of the experiment. One LD₅₀ of trypsinized toxin was found at five days in the unirradiated control culture. In the 0.2 megarad irradiated culture trypsinized toxin was found at 23 days (five LD₅₀), 28 days (one LD₅₀), 30 days (25 LD₅₀), 33 days (7.5 LD₅₀) and 36 days (ten LD₅₀).

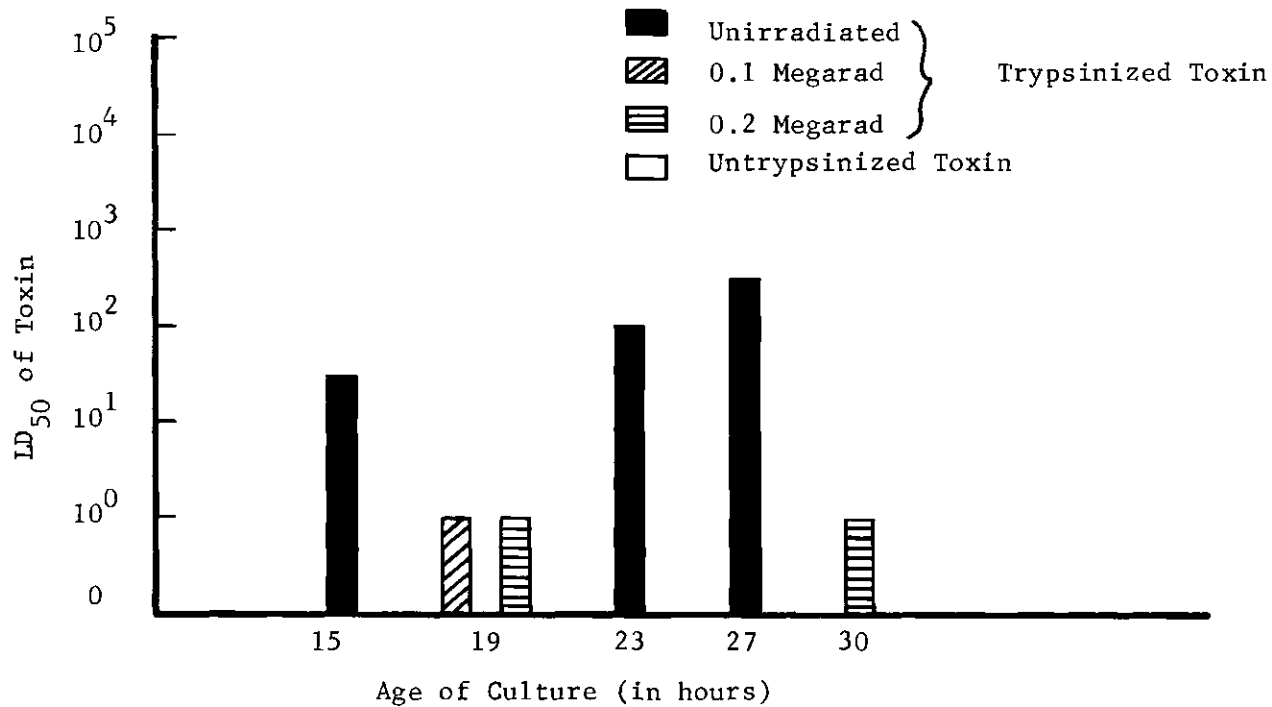


Figure 13. Toxin Production of Unirradiated and Irradiated Spores (0.1 Megarad and 0.2 Megarad of Cesium-137 Gamma Irradiation) of Clostridium botulinum Type B, Strain B-17 in Trypticase Soy Broth at 10C (Experiment I-B).

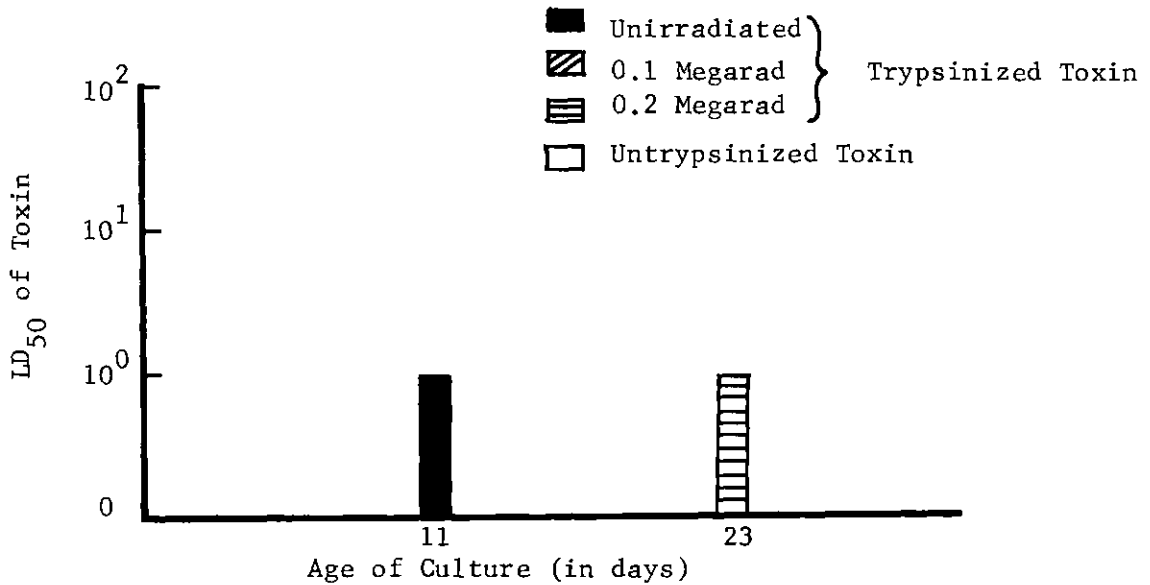


Figure 14. Toxin Production of Unirradiated and Irradiated Spores (0.1 Megarad and 0.2 Megarad of Cesium-137 Gamma Irradiation) of Clostridium botulinum Type B, Strain B-17 in Trypticase Soy Broth at 10C (Experiment II-B).

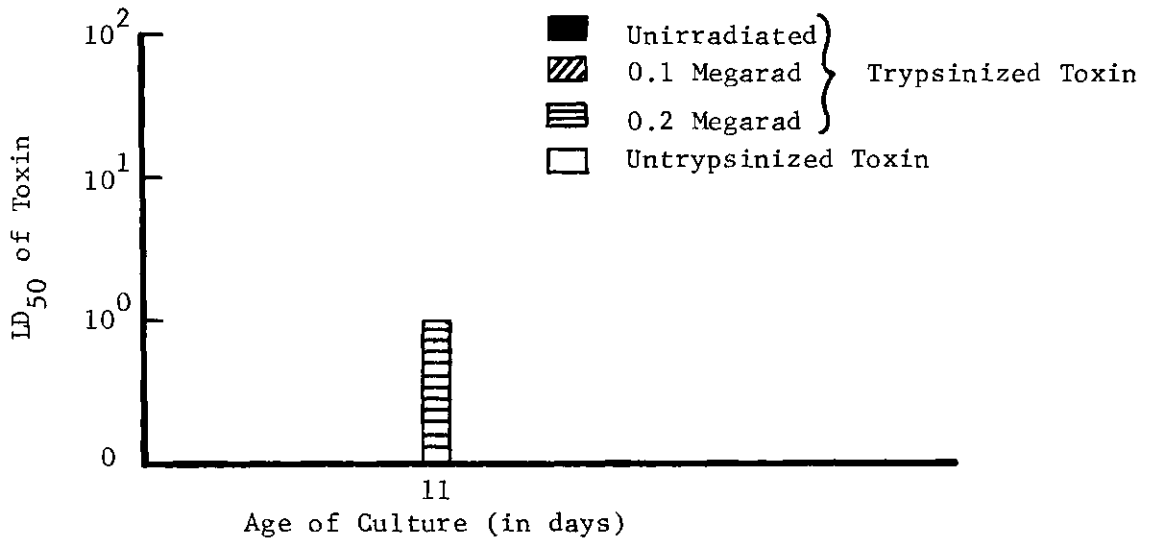


Figure 15. Toxin Production of Unirradiated and Irradiated Spores (0.1 Megarad and 0.2 Megarad of Cesium-137 Gamma Irradiation) of Clostridium botulinum Type B, Strain B-17 in Trypticase Soy Broth at 10C (Experiment III-B).

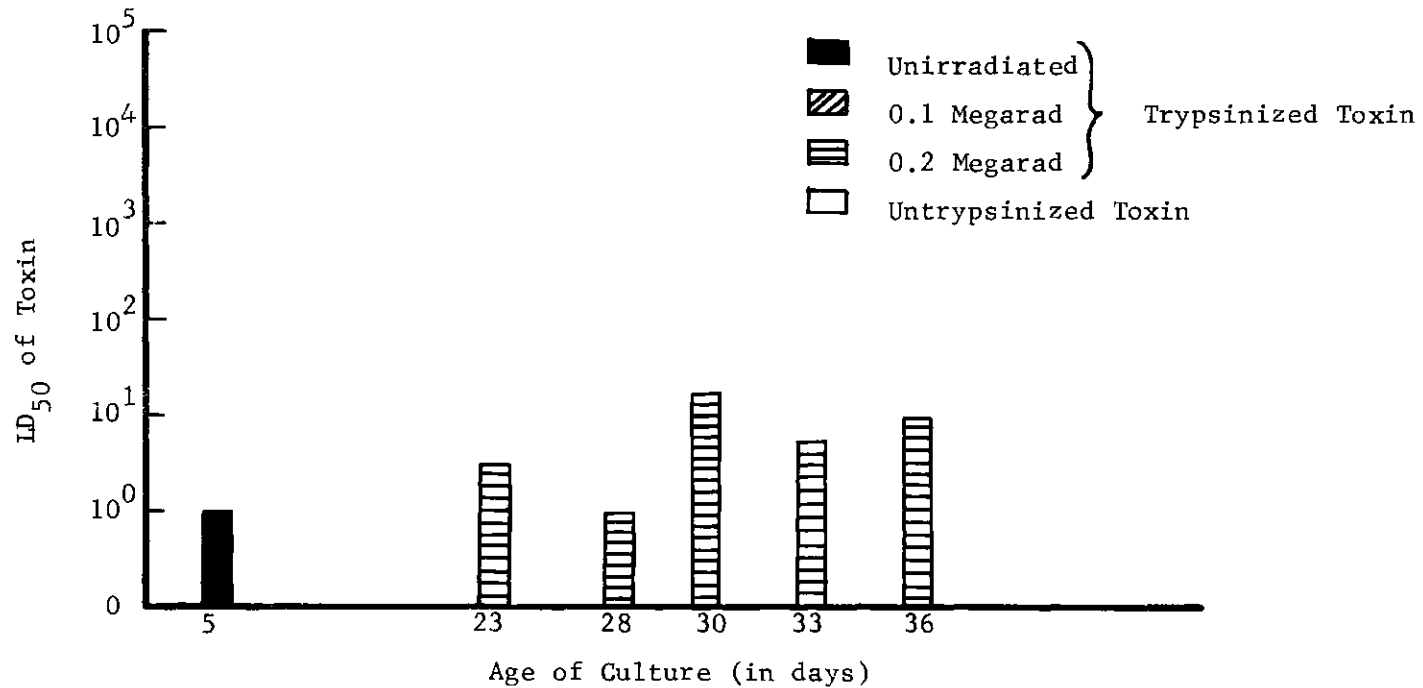


Figure 16. Toxin Production of Unirradiated and Irradiated Spores (0.1 Megarad and 0.2 Megarad of Cesium-137 Gamma Irradiation) of Clostridium botulinum Type B, Strain B-17 in Trypticase Soy Broth at 10C (Experiment IV-B).

II. Non-proteolytic *Clostridium botulinum* Type E, Strain Beluga: Growth and Toxin Production After Gamma Irradiation of Spores at 0.0, 0.1, or 0.2

Megarad.

A. Growth and Toxin Production at 25C.

The spores of *Clostridium botulinum* type E, strain Beluga used in these experiments were all produced in TPG medium.

(1) Experiment I-E.

Figure 17, 18, 19, and 20 show the results obtained in an experiment on the growth response and toxin production in trypticase soy broth medium of vegetative cells grown from irradiated (0.1 or 0.2 megarad) or unirradiated (0.0 megarad control) spores of the non-proteolytic Beluga strain of *Clostridium botulinum* type E, incubated at 25C. Spores of all three cultures showed signs of germination about ten hours after inoculation into TSB medium (see Table 3). The unirradiated control culture entered the logarithmic phase at 21 hours after inoculation (judging by the first detectable optical density reading) and continued logarithmic growth for the next 16 hours, reaching a maximum optical density reading of 1.30 at 36 hours after inoculation. Then the culture underwent partial lysis. Eight hours later, this culture reached a second peak of growth with an optical density reading of 1.23 at 44 hours after inoculation. Rapid and complete autolysis followed. The 0.1 megarad irradiated culture entered the logarithmic growth phase 23 hours after inoculation, remained in the logarithmic growth phase for the next 15 hours, and reached a maximum optical density reading of 1.50 at 37 hours after inoculation. Thereafter, this culture also underwent partial lysis, and again an additional eight hours were required for the culture to reach a second

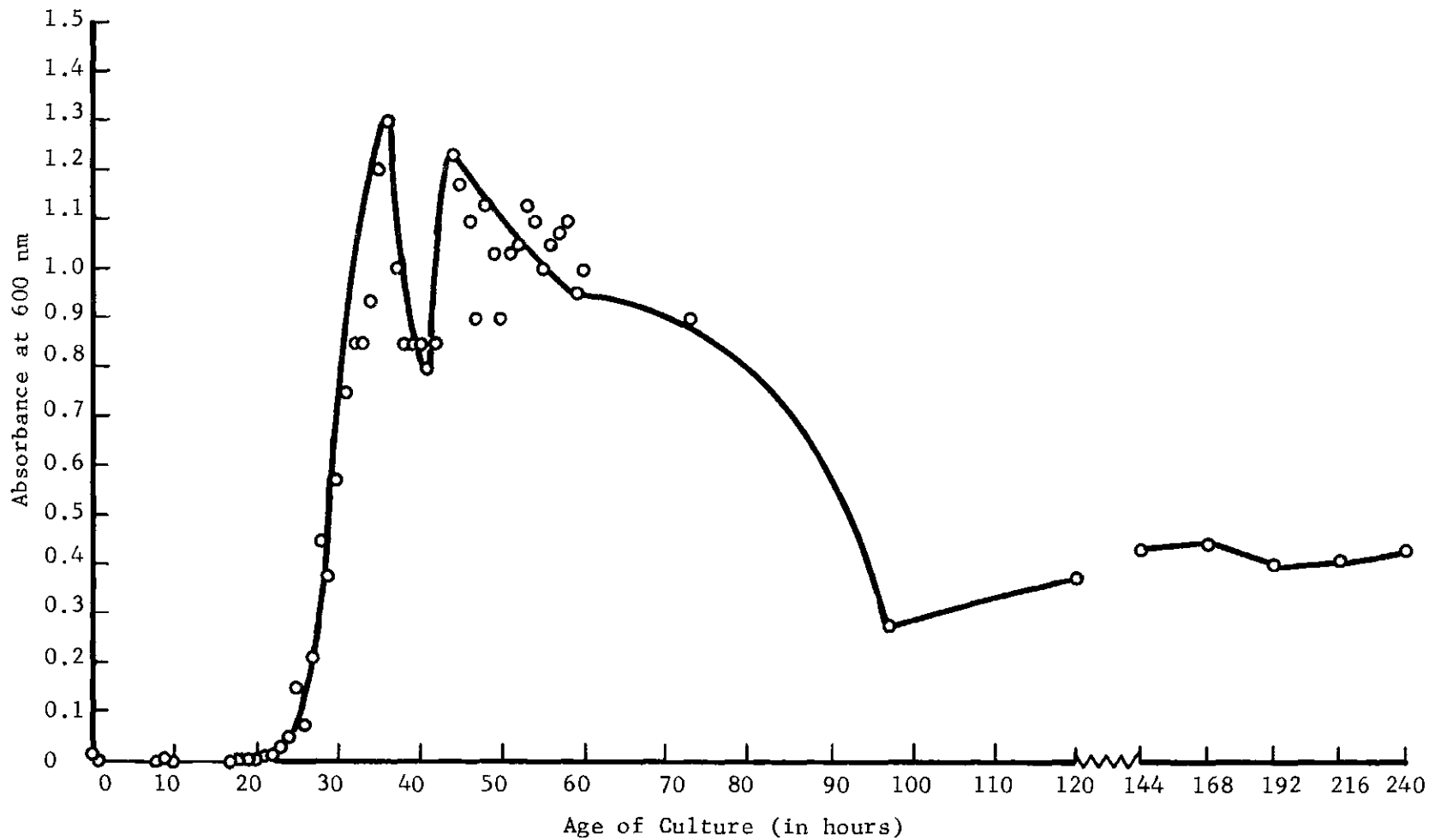


Figure 17. Growth Response of Unirradiated Spores of Clostridium botulinum Type E, Strain Beluga in Trypticase Soy Broth at 25C (Experiment I-E).

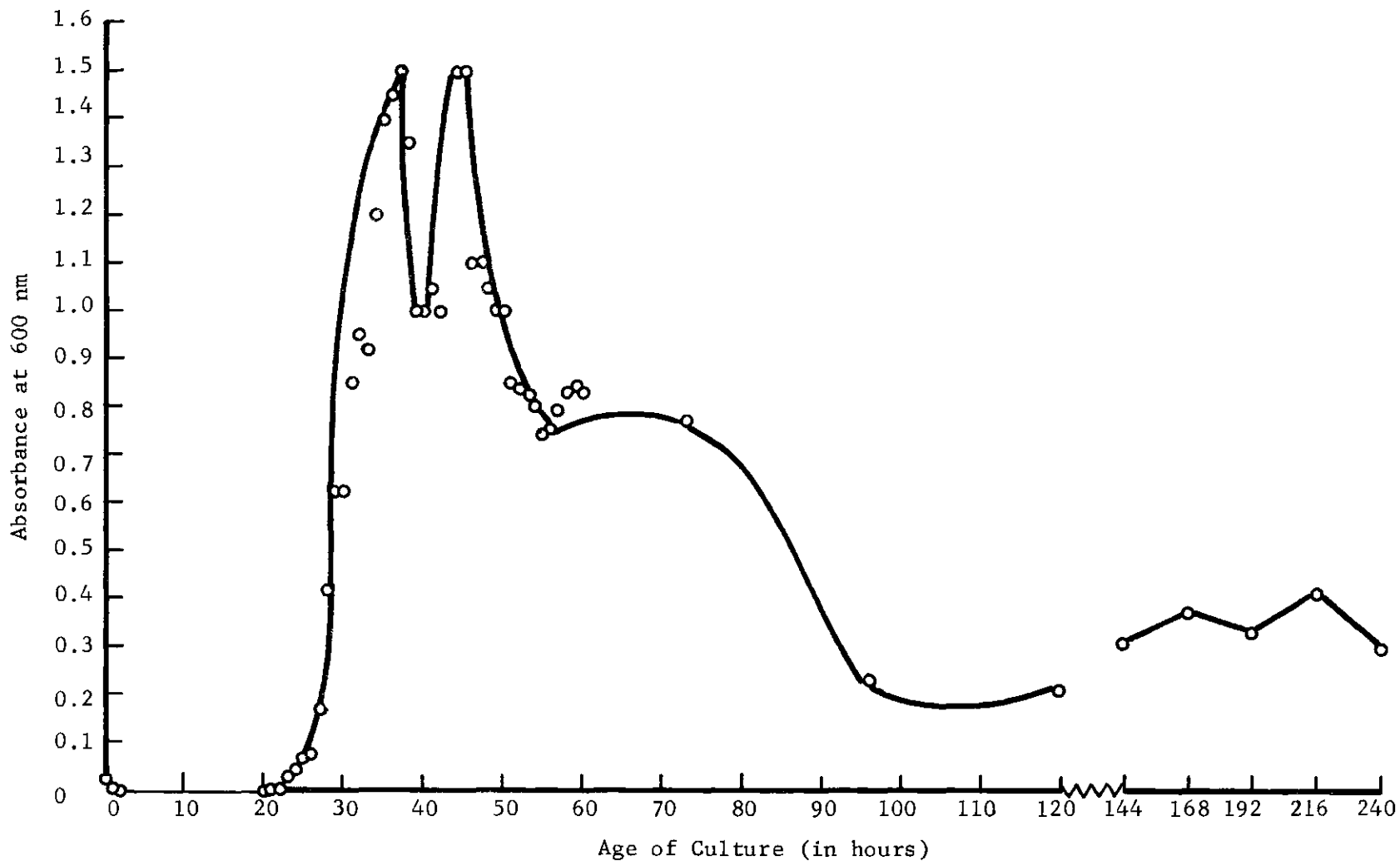


Figure 18. Effect of 0.1 Megarad of Cesium-137 Gamma Irradiation on Growth Response of Spores of Clostridium botulinum Type E, Strain Beluga in Trypticase Soy Broth at 25C (Experiment I-E).

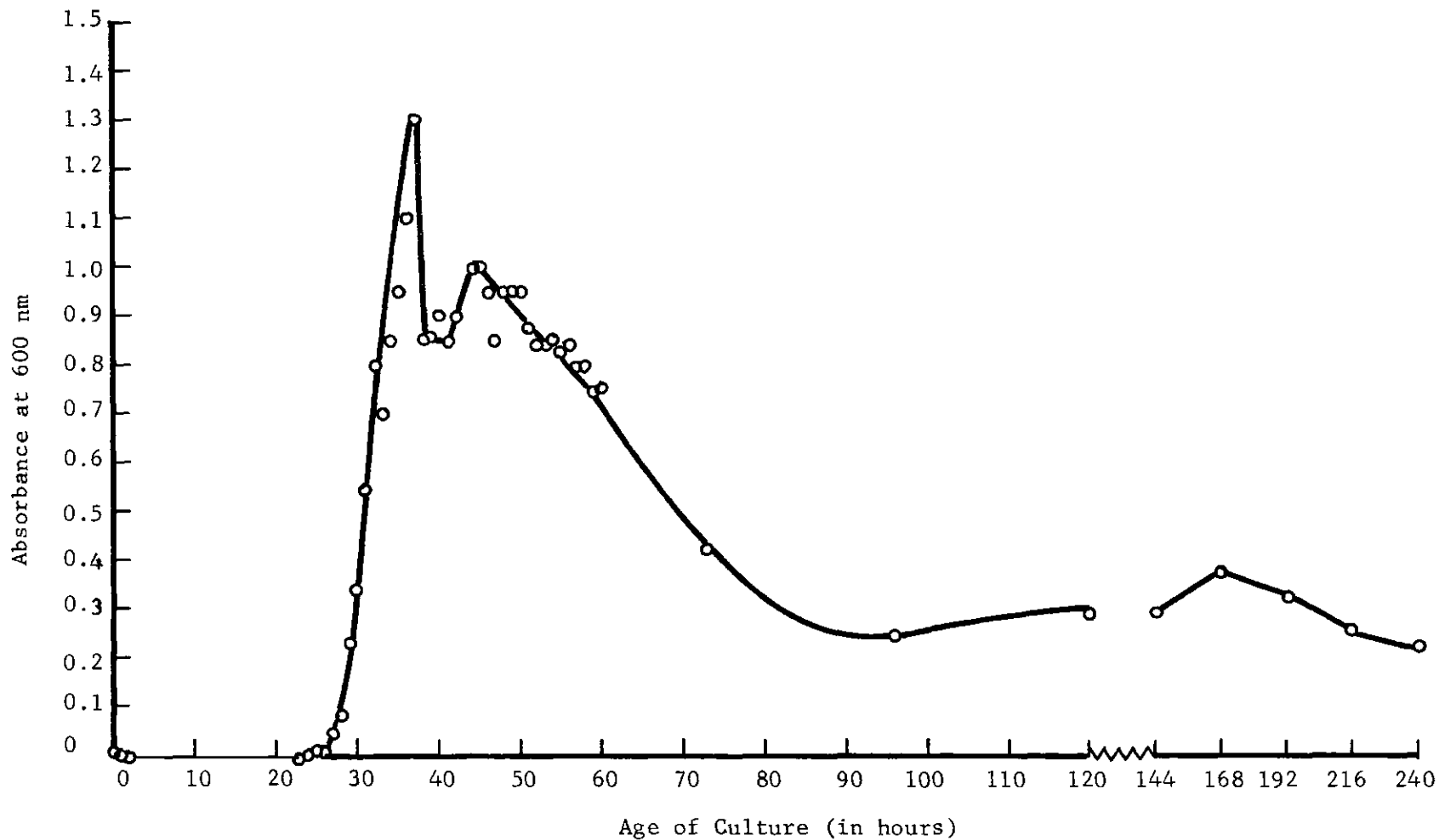


Figure 19. Effect of 0.2 Megarad of Cesium-137 Gamma Irradiation on Growth Response of Spores of Clostridium botulinum Type E, Strain Beluga in Trypticase Soy Broth at 25C (Experiment I-E).

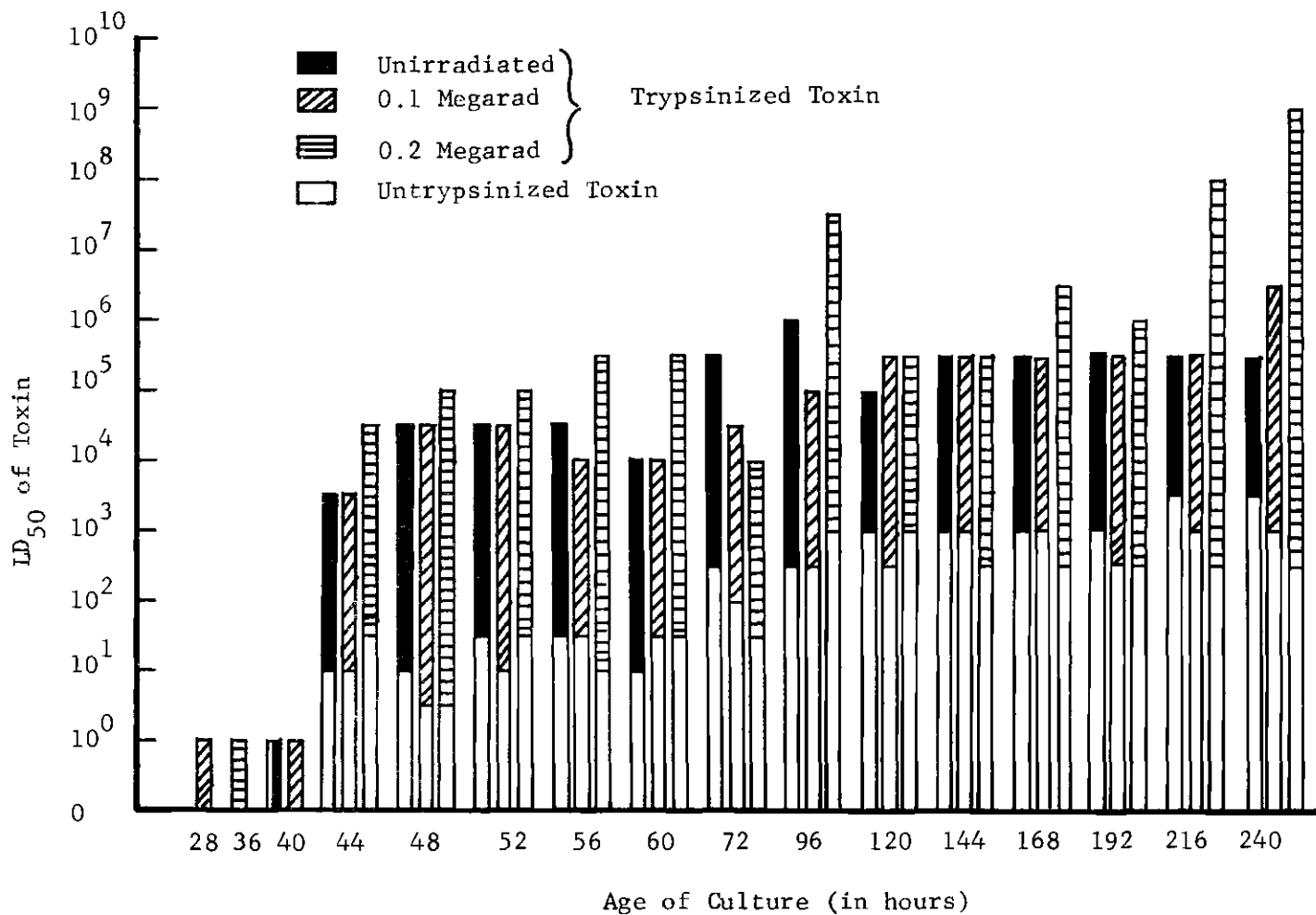


Figure 20. Toxin Production of Unirradiated and Irradiated Spores (0.1 Megarad and 0.2 Megarad of Cesium-137 Gamma Irradiation) of *Clostridium botulinum* Type E, Strain Beluga in Trypticase Soy Broth at 25C (Experiment I-E).

peak optical density reading of 1.50 at 44 and 45 hours after inoculation. This was again followed by rapid autolysis. The 0.2 megarad irradiated culture entered the logarithmic growth phase at 25 hours after inoculation. This phase continued for the next 12 hours, reaching a maximum optical density reading of 1.30 at 37 hours after inoculation. In this culture also, partial lysis occurred and seven hours were required for the culture to reach its second peak optical density reading of 1.00 at 44 hours, followed by rapid and complete autolysis.

After trypsinization treatment, the presence of toxin was detected at 28 hours after inoculation in the 0.1 megarad irradiated culture, at 36 hours after inoculation in the 0.2 megarad irradiated culture, and not until 40 hours after inoculation in the unirradiated control culture. Untrypsinized toxin was also first detected at 40 hours in the unirradiated control culture but at 44 hours in both the 0.1 megarad and 0.2 megarad irradiated cultures. The maximum titer of trypsinized toxin was 1×10^9 LD₅₀ per ml, found in the 0.2 megarad irradiated culture at 240 hours. This figure has not been corroborated thus far. Trypsinization increased the toxin titers to as much as 5000 fold for the unirradiated control culture, and 500 to 2,000,000 fold for the 0.2 megarad irradiated culture.

(2) Experiment II-E.

Figures 21, 22, 23, and 24 show the results of the second replicate of this investigation on growth and toxin production at 25C of both irradiated cultures and an unirradiated control of Clostridium botulinum type E, strain Beluga. The spore germination times are shown in Table 3. The unirradiated control culture, 0.1 megarad, and 0.2

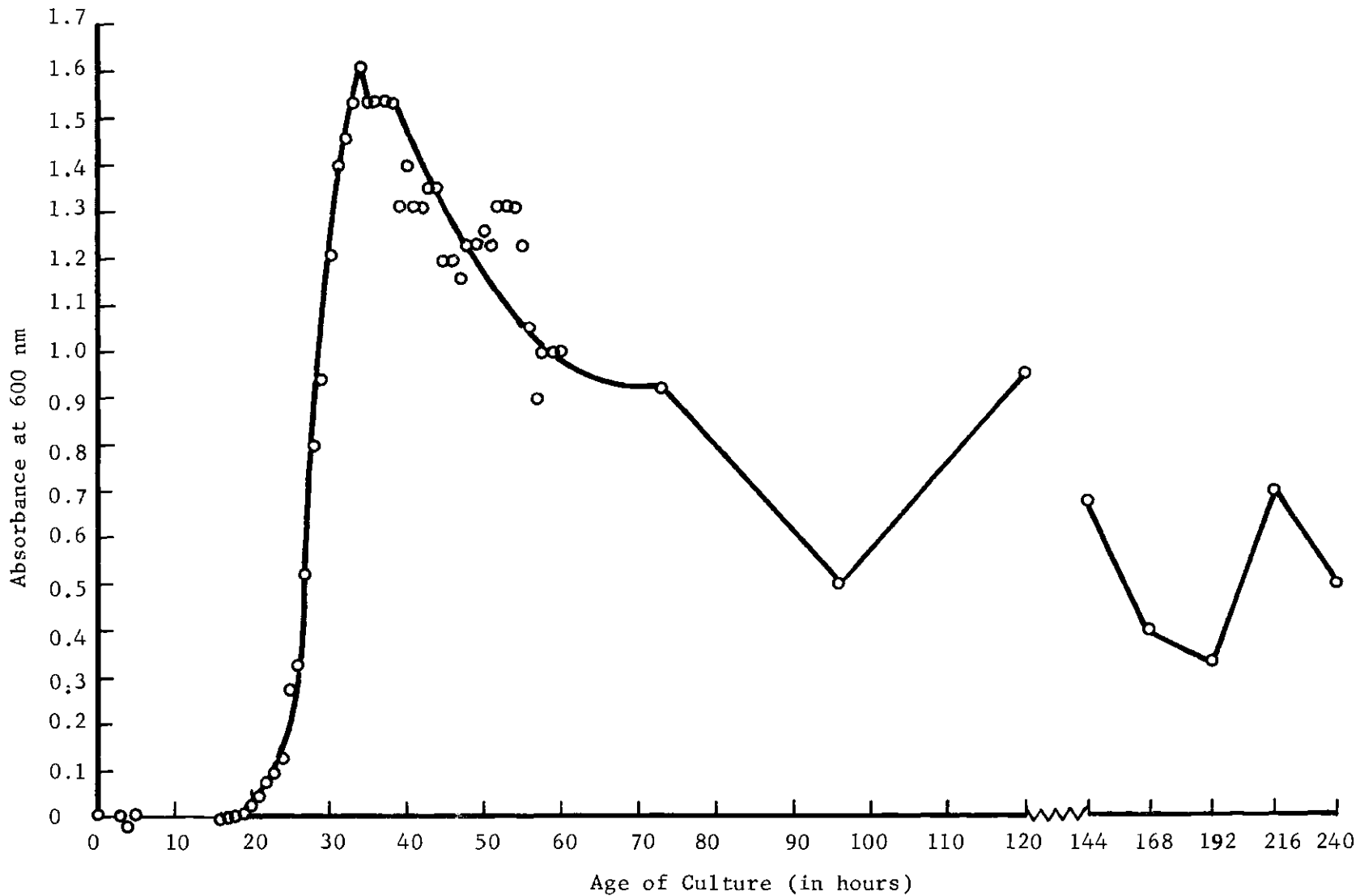


Figure 21. Growth Response of Unirradiated Spores of *Clostridium botulinum* Type E, Strain Beluga in Trypticase Soy Broth at 25C (Experiment II-E).

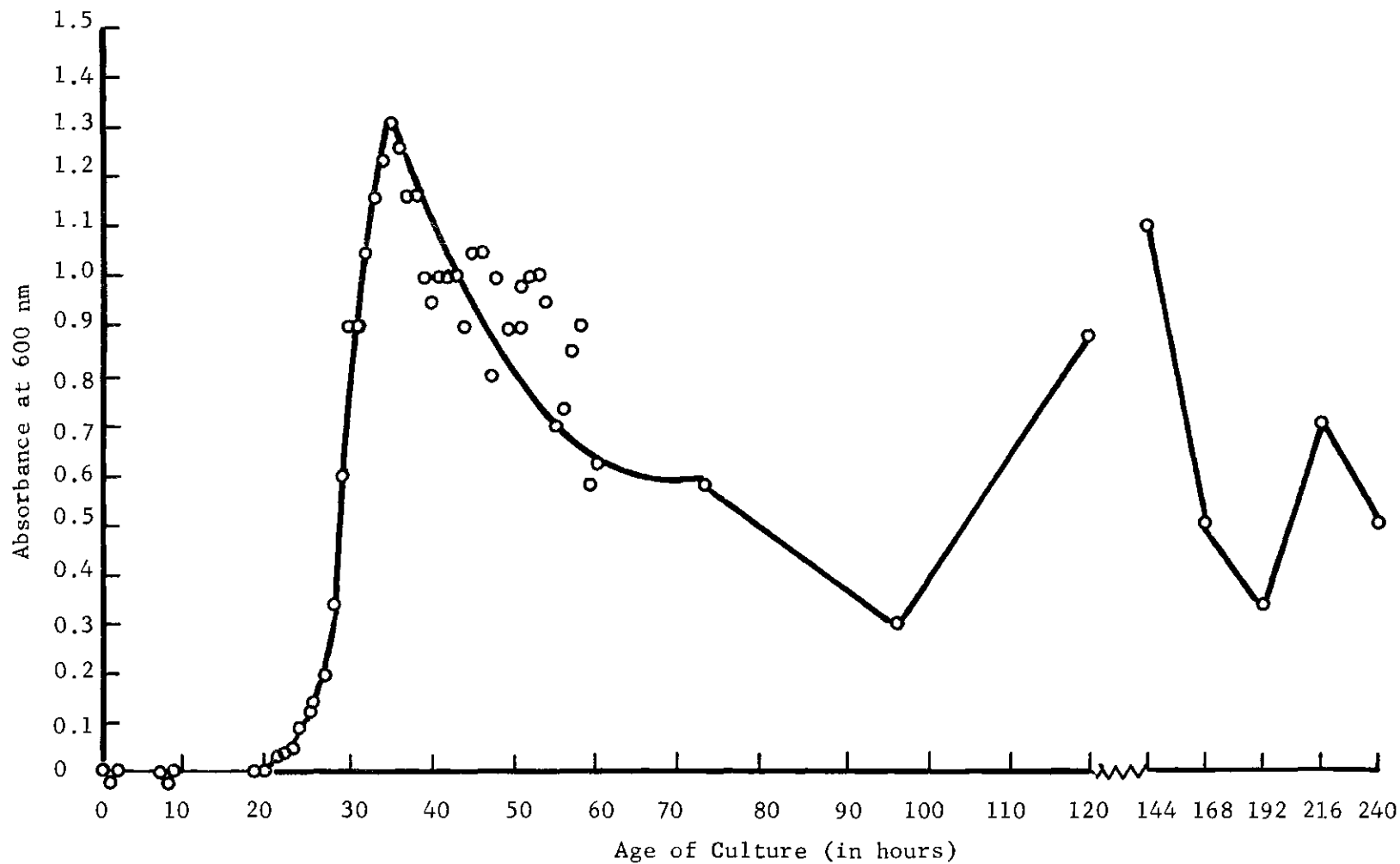


Figure 22. Effect of 0.1 Megarad of Cesium-137 Gamma Irradiation on Growth Response of Spores of Clostridium botulinum Type E, Strain Beluga in Trypticase Soy Broth at 25C (Experiment II-E).

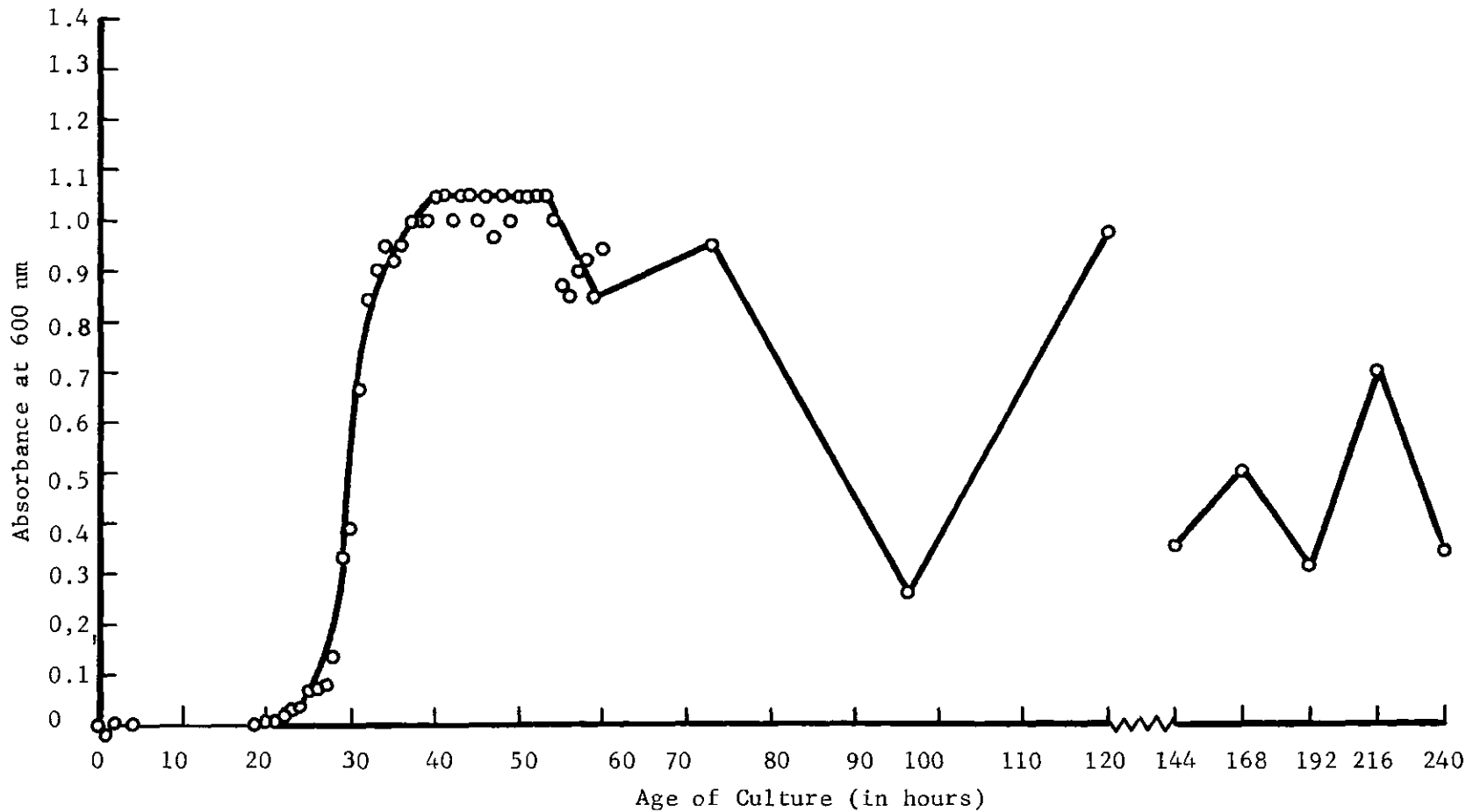


Figure 23. Effect of 0.2 Megarad of Cesium-137 Gamma Irradiation on Growth Response of Spores of Clostridium botulinum Type E, Strain Beluga in Trypticase Soy Broth at 25C (Experiment II-E).

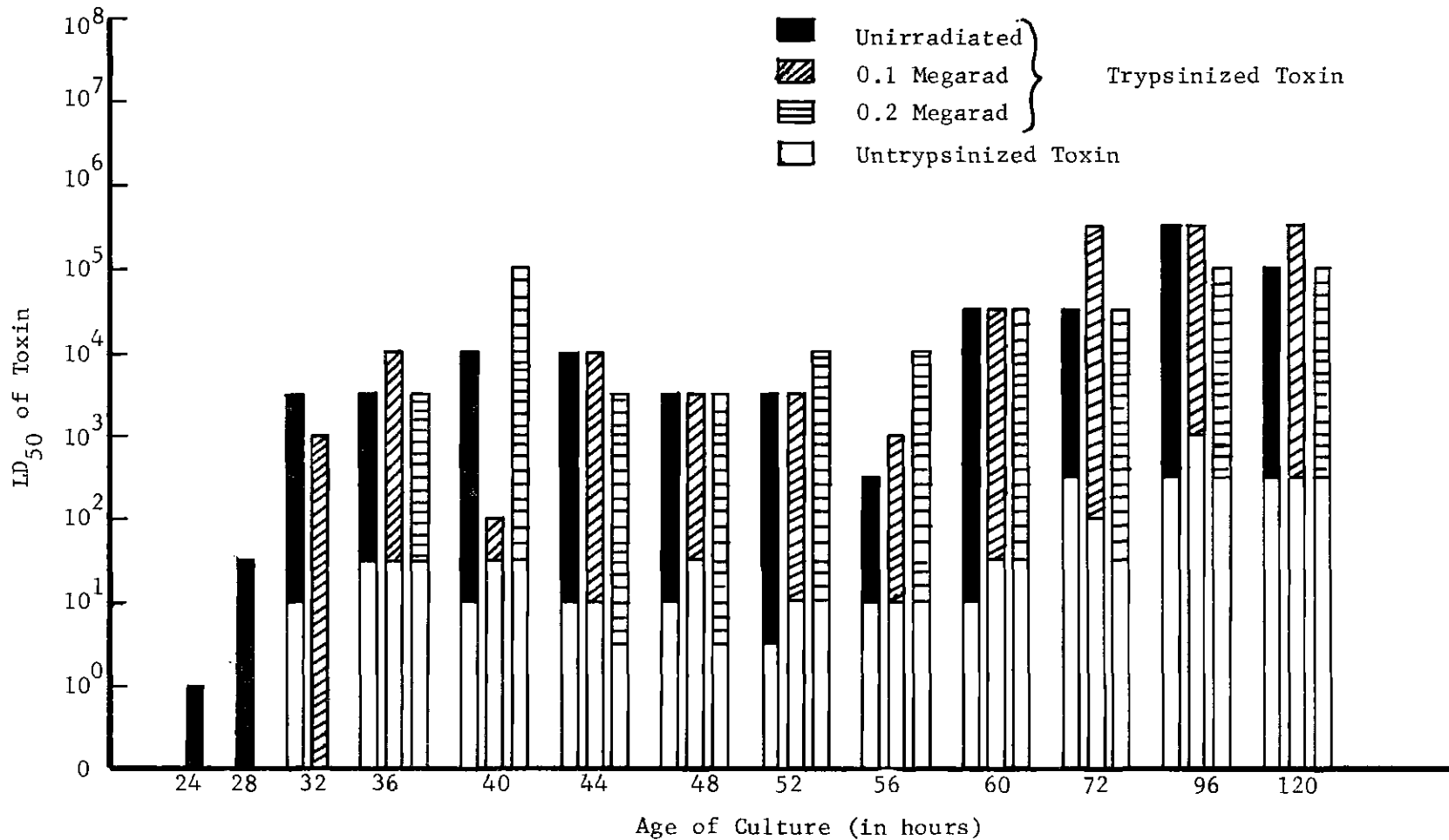


Figure 24. Toxin Production of Unirradiated and Irradiated Spores (0.1 Megarad and 0.2 Megarad of Cesium-137 Gamma Irradiation) of Clostridium botulinum Type E, Strain Beluga in Trypticase Soy Broth at 25C (Experiment II-E).

megarad irradiated cultures entered the logarithmic growth phase at 17, 20, and 20 hours, respectively, after inoculation. The unirradiated control culture continued logarithmic growth for 16 hours, reaching a maximum optical density reading of 1.61 at 34 hours. A stationary growth phase ensued, followed by autolysis beginning 39 hours after inoculation. The 0.1 megarad irradiated culture remained in the logarithmic growth phase for 15 hours, reached a maximum optical density reading of 1.31 at 35 hours after inoculation, then autolyzed rapidly. The 0.2 megarad irradiated culture continued logarithmic growth for 18 hours and reached a maximum optical density reading of 1.05 at 40 hours. A stationary growth phase ensued and then rapid autolysis occurred, starting 53 hours after inoculation.

Toxin after trypsinization was first detected at 24, 32, and 36 hours in the 0.0, 0.1, and 0.2 megarad irradiated cultures, respectively. Untrypsinized toxin was first found in the unirradiated control at 32 hours and in both the 0.1 megarad and 0.2 megarad irradiated cultures at 36 hours. Trypsinization increased toxin titers ranging from a minimum of 50 fold to a maximum of 5000 fold for the unirradiated culture, two to 5000 fold for the 0.1 megarad irradiated culture and 100 to 2000 fold for the 0.2 megarad irradiated culture.

B. Growth and Toxin Production at 10C.

No outgrowth was observed in any of the cultures of Experiment I-E through 30 days of investigation at 10C, although some spores showed signs of germination as determined by microscopic examination. Outgrowth was observed in the unirradiated control of Experiment II-E but not in either of the irradiated cultures. The highest optical density reading obtained in the unirradiated control of Experiment II-E was 0.32 at 25

days after inoculation (see Figure 25).

In Experiment I-E, trypsinized toxin was first found at a level of one LD₅₀ at 28 days for all the 10C cultures and at 23 days (one LD₅₀) for 0.1 megarad irradiated culture (see Figure 26); one LD₅₀ was found only in the unirradiated control of Experiment II-E after five days incubation (see Figure 27). Untrypsinized toxin could not be detected in any 10C culture of Experiments I-E and II-E throughout the investigation.

III. Survival of Spores of *Clostridium botulinum* Type B, Strain B-17 and Type E, Strain Beluga, Subjected to 0.0, 0.1, or 0.2 Megarad Doses of Gamma Irradiation.

Counts were made repeatedly from unirradiated control and irradiated spores immediately after irradiation to assay the number of organisms capable of outgrowth and the subsequent formation of macroscopically visible colonies. This was done to eliminate the possibility that the greater cell mass of some of the irradiated cultures (as indicated by optical density readings) might be due to more spores germinating after gamma irradiation because of an activation effect.

Figure 28 shows the results for Irradiation Experiments I-B and II-B. The values were taken from the number of viable spores of *Clostridium botulinum* type B, strain B-17, surviving irradiation at the designated levels. Spores for these two experiments were harvested from phytone medium. As shown in the graph, the 0.1 megarad dose apparently caused no significant change while the 0.2 megarad dose resulted in a reduction of viable spores as compared to the unirradiated control.

Figure 29 shows the results of viable counts of the spores of *Clostridium botulinum* type B, strain B-17, used in Experiments III-B and

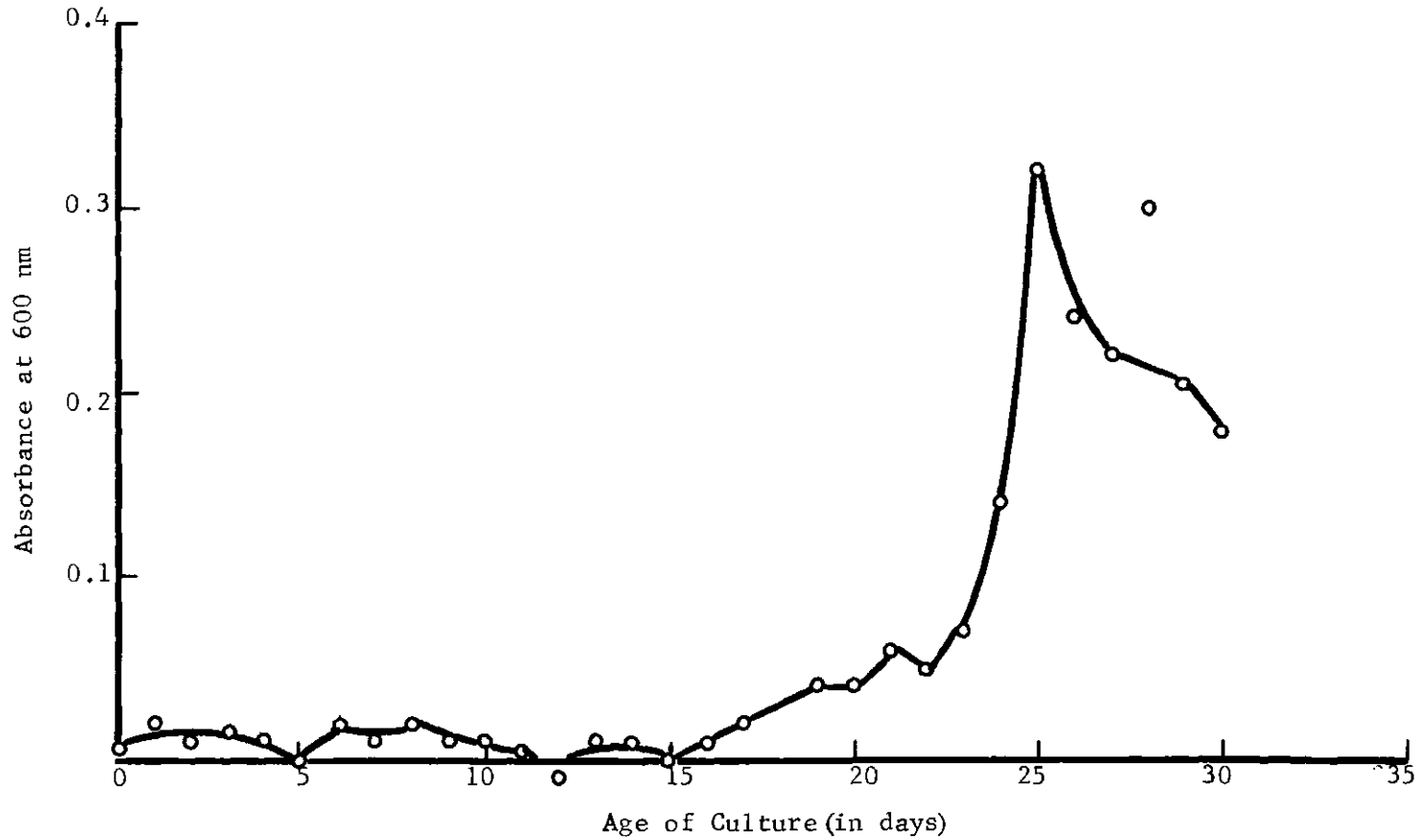


Figure 25. Growth Response of Unirradiated Spores of Clostridium botulinum Type E, Strain Beluga in Trypticase Soy Broth at 10C (Experiment II-E).

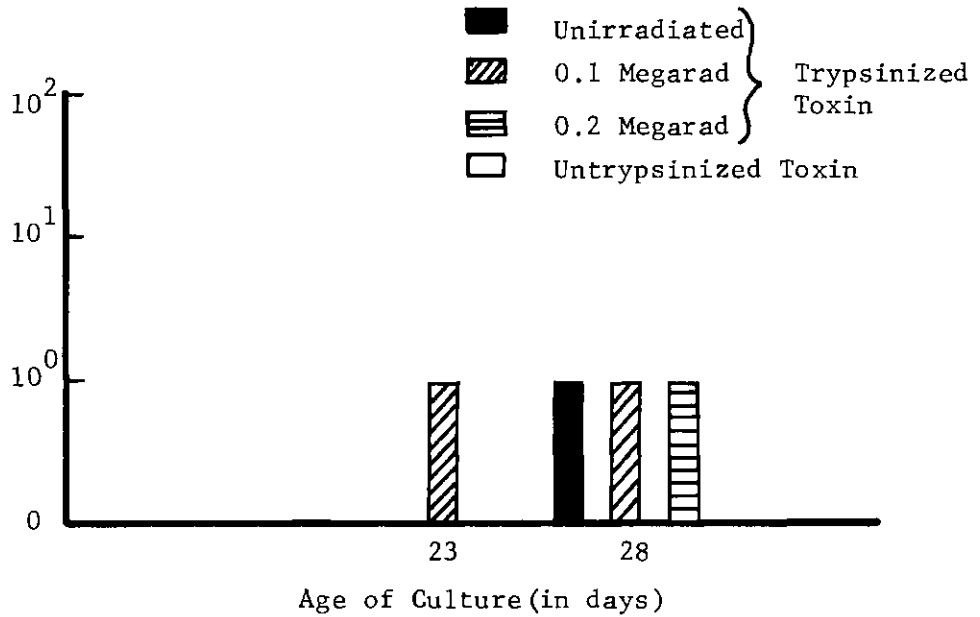


Figure 26. Toxin Production of Unirradiated and Irradiated Spores (0.1 Megarad and 0.2 Megarad of Cesium-137 Gamma Irradiation) of *Clostridium botulinum* Type E, Strain Beluga in Trypticase Soy Broth at 10C (Experiment I-E).

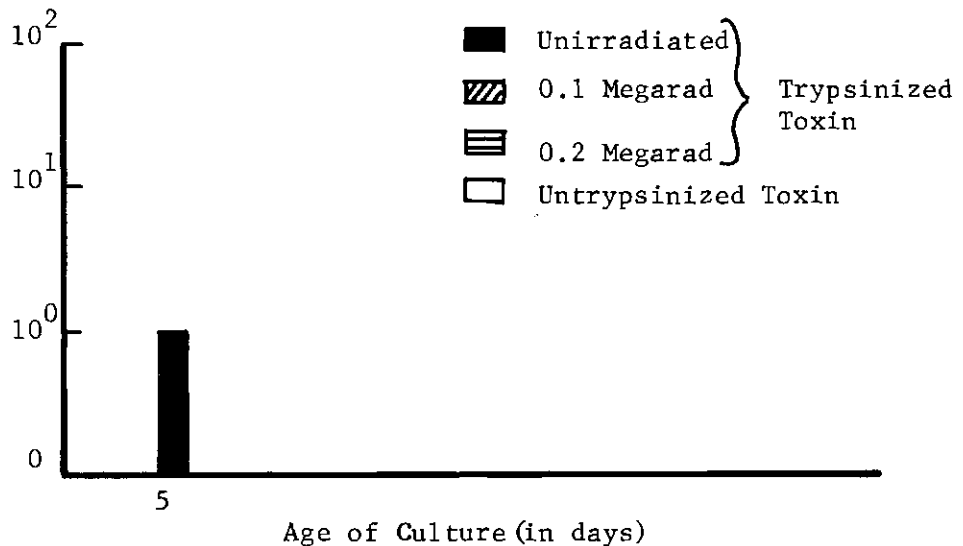


Figure 27. Toxin Production of Unirradiated and Irradiated Spores (0.1 Megarad and 0.2 Megarad of Cesium-137 Gamma Irradiation) of *Clostridium botulinum* Type E, Strain Beluga in Trypticase Soy Broth at 10C (Experiment II-E).

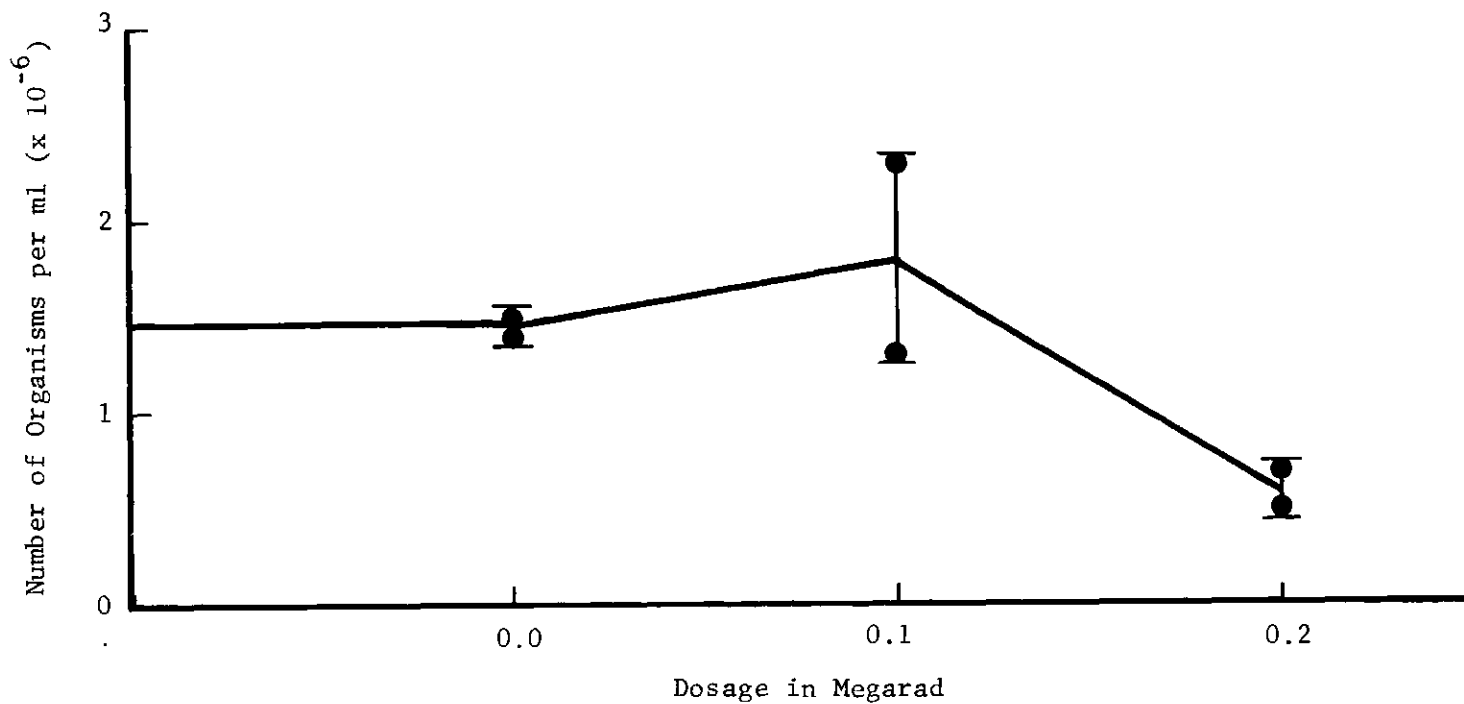


Figure 28. Effect of Cesium-137 Gamma Irradiation on Survival of Spores of Clostridium botulinum Type B, Strain B-17 Harvested from Phytone Medium and Suspended in Sterile Sorenson's Phosphate Buffer (pH 7.0) During Irradiation (Experiment I-B and II-B).

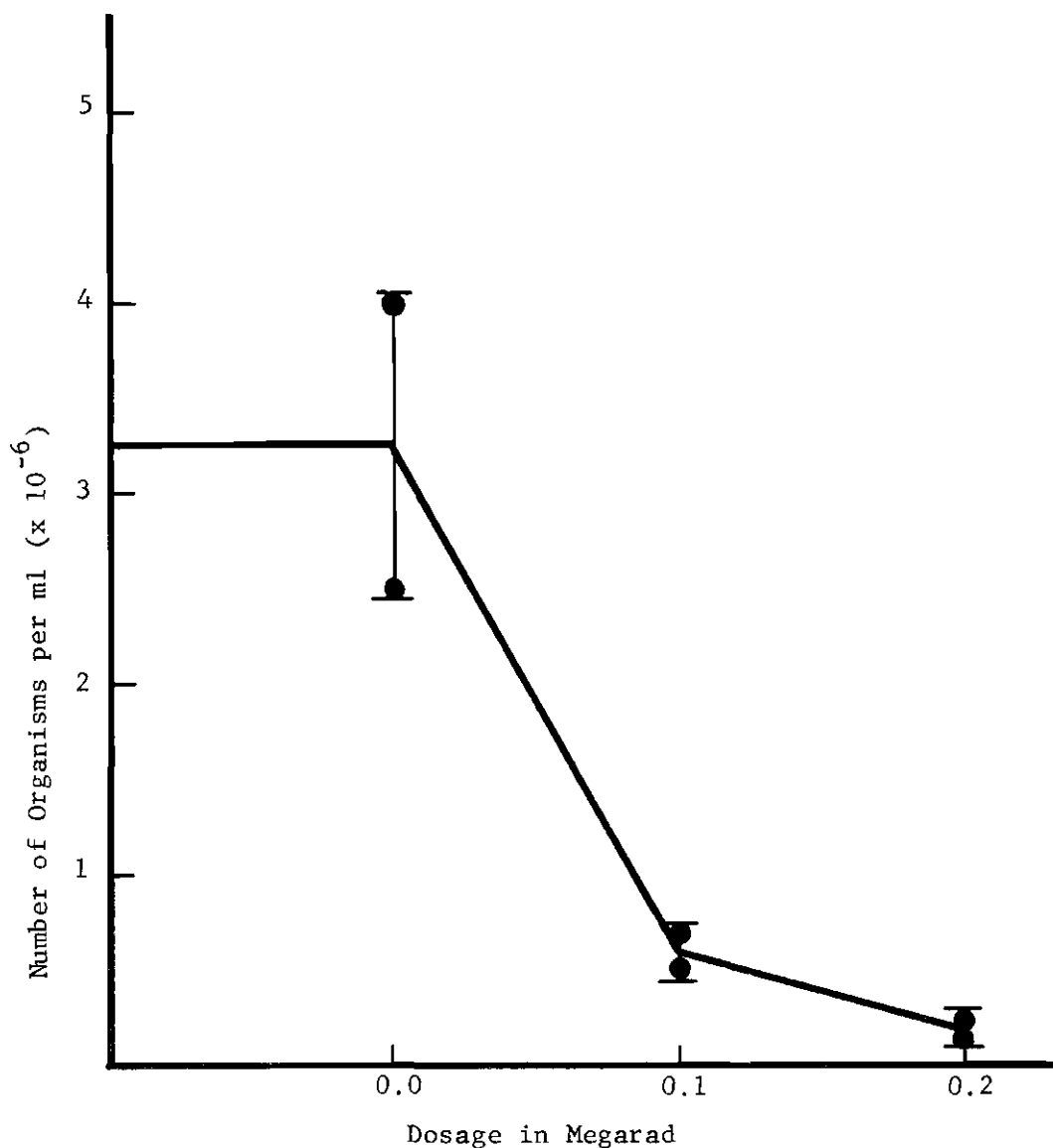


Figure 29. Effect of Cesium-137 Gamma Irradiation on Survival of Spores of Clostridium botulinum Type B, Strain B-17 Harvested from Trypticase-Peptone-Glucose Medium and Suspended in Sterile Sorenson's Phosphate Buffer (pH 7.0) During Irradiation (Experiments III-B and IVB).

IV-B. In these two experiments, the spores used were produced in TPG medium. In contrast to the results shown in Figure 28, the 0.1 megarad and 0.2 megarad doses resulted in appreciable reductions in viable spores. The degree of reduction was slightly higher for the 0.2 megarad dose than for the 0.1 megarad dose but the loss of viability was not logarithmic between 0.1 and 0.2 megarad at the same rate as between 0.0 and 0.1 megarad.

Figure 30 shows the results of viable counts of spores of Clostridium botulinum type E, strain Beluga, subjected to 0.0, 0.1 or 0.2 megarad doses of gamma irradiation. In this case, the spores were harvested from TPG medium. The 0.1 megarad and 0.2 megarad doses resulted in appreciable reductions in viable spores. The degree of reduction was higher in the 0.2 megarad dose than in the 0.1 megarad dose and the loss of viability was a logarithmic function.

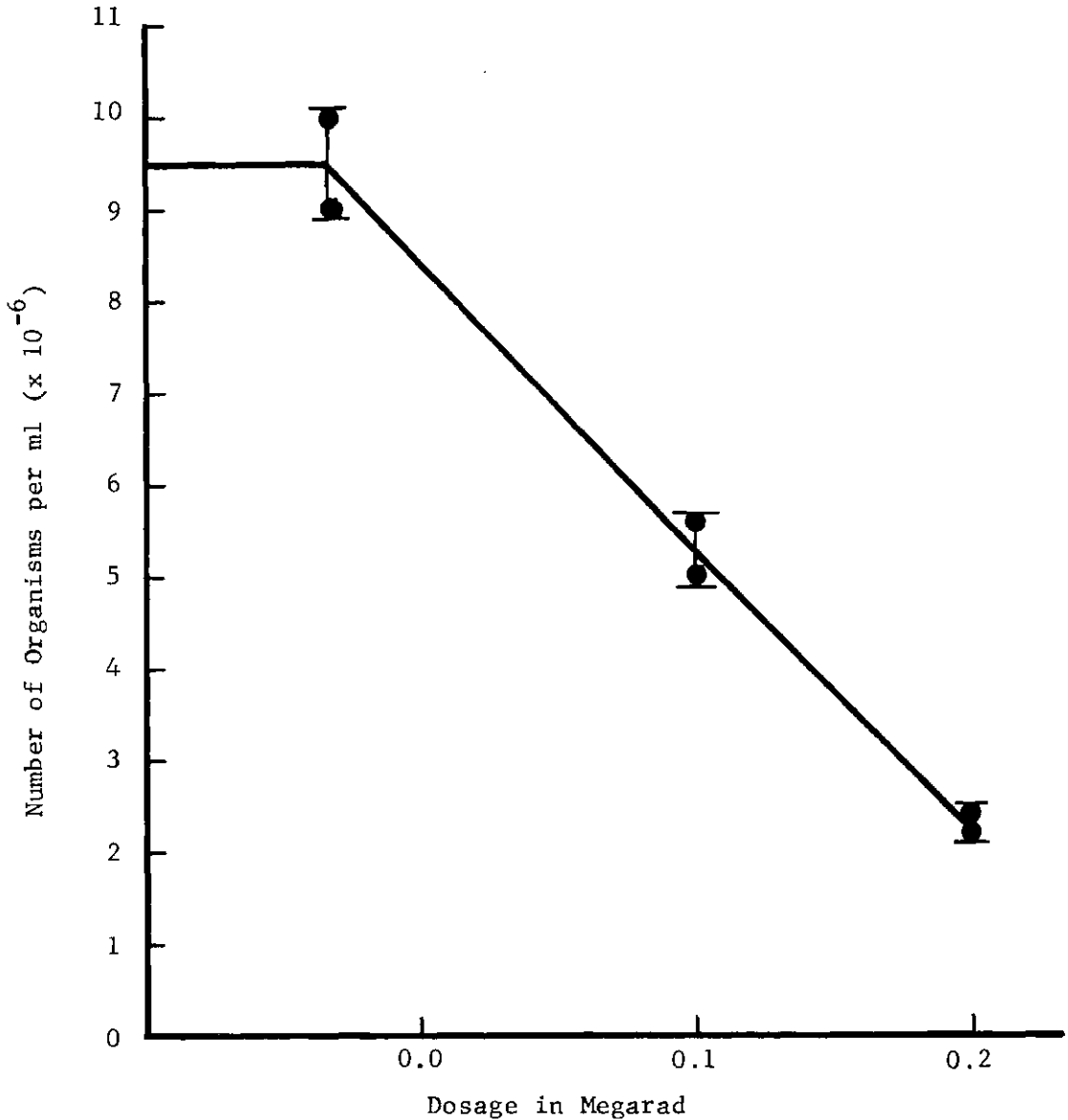


Figure 30. Effect of Cesium-137 Gamma Irradiation on Survival of Spores of Clostridium botulinum Type E, Strain Beluga Harvested from Trypticase-Peptide-Glucose Medium and Suspended in Sterile Sorenson's Phosphate Buffer (pH 7.0) During Irradiation (Experiments I-E and II-E).

Table 1. Summary of Germination Times for 0.0, 0.1, and 0.2 Megarad Doses of Gamma Irradiation in Experiments on Clostridium botulinum Type B, Strain B-17, Incubated at 25C in Trypti- case Soy Broth Medium.

Experiment Number	Germination Time In Hours		
	0.0 Megarad	0.1 Megarad	0.2 Megarad
II-B	11	12	13
III-B	10	12	11
IV-B	13	17	18

Table 2. Maximum Absorbance and LD₅₀'s Obtained in Experiments on Clostridium botulinum type B, Strain B-17 Incubated at 25°C in Trypticase Soy Broth Medium.

Radiation Dosage	Experiment Number	Maximum OD Obtained	Age of Culture (in Hours)	Initial Appearance of Toxin (in Hours)		Maximum LD ₅₀ Toxin (x 10 ³)		Age of Culture, Time of Maximum Titer	
				T*	U**	T	U	T	U
0.0 Mrad	II-B	0.61	23	24	52	100.00	0.10	168 192 216	216
	III-B	0.58	31	20	52	10.0	0.01	96 120 144 168 192	240
	IV-B	0.48	30	28	96	0.50	0.0025	240	96 120 168 192 216 240
0.1 Mrad	II-B	0.63	25	24	52	10.00	0.005	192	168 192 216 240
	III-B	0.60	32	32	72	10.00	0.005	144	144

Table 2. (Continued)

Radiation Dosage	Experiment Number	Maximum OD Obtained	Age of Culture (in Hours)	Initial Appearance of Toxin (in Hours)		Maximum LD ₅₀ Toxin ($\times 10^3$)		Age of Culture, Time of Maximum Titer	
				T*	U**	T	U	T	U
									192
									240
	IV-B	0.48	33	32	44	0.10	0.005	144	96
								216	120
									144
									168
									192
									216
									240
0.2 Mrad	II-B	0.60	26	24	52	5.00	0.005	144	192
								192	216
								216	240
								240	
	III-B	0.68	33	36	72	5.00	0.005	216	168
									240
	IV-B	0.54	33	36	72	5.00	0.005	216	144
								240	216
									240

*Trypsinized Toxin

**Untrypsinized Toxin

Table 3. Summary of Germination Times for 0.0, 0.1 and 0.2 Megarad Doses of Gamma Irradiation in Experiments on Clostridium botulinum Type E, Strain Beluga, Incubated at 25C in Trypticase Soy Broth Medium.

Experiment Number	Germination Time in Hours		
	0.0 Megarad	0.1 Megarad	0.2 Megarad
I-E	10	10	12
II-E	8-12	8-12	12-16

Table 4. Maximum Absorbance and LD₅₀'s Obtained in Experiments on Clostridium botulinum Type E, Strain Beluga Incubated at 25C in Trypticase Soy Broth Medium.

Radiation Dosage	Experiment Number	Maximum OD Obtained	Age of Culture (in Hours)	Initial Appearance of Toxin (in Hours)		Maximum LD ₅₀ Toxin (x 10 ³)		Age of Culture, Time of Maximum Titer	
				T*	U**	T	U	T	U
0.0 Mrad	I-E	1.30	36	40	40	10.00	0.05	96	216 240
	II-E	1.61	34	24	32	5.00	0.005	96	72 96 120
0.1 Mrad	I-E	1.50	37 44 45	28	44	50.00	0.01	240	144 166 240
	II-E	1.31	35	32	36	5.00	0.05	72 96 120	96
0.2 Mrad	I-E	1.30	37	36	44	10000.00	0.01	240	96 120
	II-E	1.05	45	36	36	1.00	0.005	40	96

Table 4. (Continued)

Radiation Dosage	Experiment Number	Maximum OD Obtained	Age of Culture (in Hours)	Initial Appearance of Toxin (in Hours)		Maximum LD ₅₀ Toxin ₃ (x 10 ³)		Age of Culture, Time of Maximum Titer	
				T*	U**	T	U	T	U
			46					96	120
			48					120	
			50						
			51						
			52						
			53						

*Trypsinized Toxin

**Untrypsinized Toxin

CHAPTER IV

DISCUSSION

I. Survival of Spores of *Clostridium botulinum* Type B, Strain B-17, Subjected to 0.0, 0.1, or 0.2 Megarad Doses of Gamma Irradiation.

Spores of *Clostridium botulinum* type B, strain B-17, collected from phytone medium were tested for viability after irradiation. The results indicated that for these spores there was a "threshold dose" between 0.1 megarad and 0.2 megarad of cesium-137 gamma irradiation. Irradiation with 0.1 megarad caused no loss of viability while 0.2 megarad killed about 70 percent of the spores. Thus the spores harvested from phytone were radiation resistant at a dose of 0.1 megarad. In contrast, spores of the same organism collected from trypticase-peptone-glucose (TPG) and irradiated were killed at the 0.1 megarad dose level (86 percent loss in viability). The experimental data also seems to demonstrate at least two levels of radiation resistance in this spore population since the loss of viability was not linear with increasing radiation dosage (0.2 megarad). This indicated that the spores collected from TPG medium were not homogenous and did not possess a uniform sensitivity to the irradiation dosages employed. Some of the spores were more radiation-resistant than others. In addition, the presence of a threshold dose level in spores collected from phytone medium and the absence of a comparable threshold dose in those collected from TGP medium may be due to differences in chemical composition of the spore production media,

differences which may contribute to radiation resistance of the resulting spores.

II. Post-Irradiation Growth at 25C of Spores of Clostridium
botulinum Type B, Strain B-17.

Compared to the unirradiated control cultures in the type B experiments, the numbers of viable spores initially inoculated into the growth medium for the irradiated cultures were significantly fewer, except for the 0.1 megarad irradiated culture of Experiment II-B. However, it should be noted that, in all cases but one, the first detectable optical density readings of irradiated cultures in Experiments II-B, III-B, and IV-B were seen almost at the same hour, or a little earlier, than the control cultures, even though the germination times for the irradiated spores were slightly more delayed than the controls. These observations indicate that the irradiated cultures of the type B experiments (with the exception noted above) must have entered the logarithmic phase earlier than the control cultures. Irradiation may be responsible for this difference but this phase of the research was not pursued since no fruitful experimental approach appeared feasible. The exception of the 0.1 megarad irradiated culture of Experiment II-B probably occurred because this radiation dose was below the "threshold dose" for that particular group of spores (see Section I).

The post-irradiation growth curves of Clostridium botulinum type B, strain B-17 were subjected to a t-Test (Goldstein, 1964) to determine the significance of the difference between the slopes of the cultures grown from spores exposed to different levels of gamma irradiation. The

results of this statistical treatment indicated that there were no significant differences between the slopes of the regression lines of the unirradiated and irradiated cultures within each experiment, or between experiments, at a 99 percent confidence level (see Appendix B). This is evidence that, for spores of Clostridium botulinum type B, strain B-17, there is no difference in the rate of growth of irradiated and unirradiated cultures. In the experiments performed, there also were no significant differences in the maximum growth attained by irradiated or unirradiated control cultures. Therefore, irradiation of spores of type B, strain B-17 did not cause any detectable alteration of post-irradiation growth of their vegetative cell progeny.

III. Toxin Production at 25C by Clostridium botulinum Type B, Strain B-17.

Irradiation of spores of Clostridium botulinum type B, strain B-17 did not produce definite evidence of earlier release of untrypsinized toxin as judged from toxin production data collected from three type B experiments. The toxin production data were also subjected to analysis of variance (Croxtton, 1953) to ascertain (1) the effect of trypsinization on toxin titers, (2) the effect of the amount of irradiation on toxin production, and (3) the effect of the age of the culture on toxin production. The determinations were judged at a 99 percent critical confidence level for all these statistical treatments (see Appendix C). The results of the statistical analysis show that there were no significant differences in the amount of untrypsinized toxin produced at any age for any of the cultures in the three type B experiments, and that there were no significant effects of irradiation on toxin production except for untrypsinized toxin production by the control culture in Experiment II-B.

The higher LD₅₀ of untrypsinized toxin found in this control culture may be attributed to a late secondary growth of this culture which produced more cells capable of releasing additional toxin molecules into the medium after autolysis of the culture. The results of the statistical analysis showing that the production of untrypsinized toxin was not a function of the culture confirm the experimental data, which show that the titers of untrypsinized toxin increased very little after first detection. This information implies that the release of untrypsinized toxin from the bacterial cells may occur at certain stages during incubation. The most likely time for the release of untrypsinized toxin into the culture fluid is after cell lysis; in support of this statement was the fact that untrypsinized toxin was detected in these cultures only after cell lysis had occurred. Statistical treatment of the data for toxin production also shows that there were significant differences in the titers of trypsinized toxin produced between irradiated cultures and unirradiated control cultures, although, as noted above, there were no significant differences in the amounts of untrypsinized toxin produced by cultures of three type B experiments except for the control culture of Experiment II-B.

The trypsinization treatment of culture supernatants collected from irradiated cultures or unirradiated controls was performed under the same controlled conditions of pH, amount of trypsin, temperature of incubation, and length of incubation. If the reactants were the same in each culture, then the products should be the same and have the same characteristics. However, the experimental data and the statistical analysis indicated that the titers of trypsinized toxins from the irradiated cultures were significantly different from those of the unirradiated

controls. Thus, one might theorize that the untrypsinized toxin produced by the irradiated cultures was different from that produced by the unirradiated controls. This difference could result from changes in the structure of the toxin molecules produced by irradiated cultures from that produced by the unirradiated control culture. If this were true, the irradiation of spores could be responsible for the alteration in structure of the untrypsinized toxin molecules produced by irradiated cultures.

On the basis of the data collected from three type B experiments, it may be stated that:

- (a) Very low titers of trypsinized toxin were first detected in the later stages of, or at the end of the logarithmic growth phases in all of the cultures tested.
- (b) The titers of trypsinized toxin gradually increased after the cultures began to autolyze but the maximum titers were found only after the cultures had completely lysed.
- (c) Untrypsinized toxin was detected only after cultures began to lyse.

Gerwing et al. (1968) have stated that toxin is synthesized during a late stage of the logarithmic growth phase and is released into the culture supernatant fluid during lysis of the cells. The small quantity of trypsinized toxin detected in the culture supernatants before autolysis has occurred may be attributed to the process of simple diffusion of the toxin from within the organisms (Bonventre and Kempe, 1960).

The lower toxin production in all of the cultures of Experiment IV-B, as compared to those of Experiments II-B and III-B, may be

attributed to less growth of those cultures (as shown by optical density readings). This assumes that there was a direct relation between cell mass and the amount of toxin produced by a given culture.

IV. Post-Irradiation Growth of *Clostridium botulinum* Type B,
Strain B-17 at 10C.

At an incubation temperature of 10C, most of the cells in both the unirradiated control and the irradiated cultures initiated from spores of *Clostridium botulinum* type B, strain B-17, failed to multiply after germination. This was at variance with the report of Eklund et al., (1967) in which these investigators found outgrowth of this organism after 17 to 18 days at an incubation temperature of 5.6C, and 24 to 28 days at an incubation temperature of 4.4C. This disagreement could be due to the following:

- (a) A difference in the growth medium. Eklund employed cooked meat medium, a highly complex particulate medium, whereas the trypticase soy broth used in this research contains no particles. Particulate media are known to support growth and toxin production of this group of organisms better than non-particulate media (Holdeman, 1964).
- (b) Differences in the experimental conditions imposed. In Eklund's experiments cultures were left undisturbed until production of gas (evidence of growth) appeared, whereas frequent sampling, with resulting aeration, was necessary in the investigation reported here. Even though the amount of oxygen introduced by the sampling technique was small, it was apparently significant at this critical stage in the development of spores.

V. Toxin Production by Clostridium botulinum type B, strain B-17 at 10C.

At an incubation temperature of 10C, toxin production by the cultures inoculated with 0.0, 0.1 or 0.2 megarad irradiated spores of Clostridium botulinum type B, strain B-17 varied between experiments. For the four type B experiments, the highest titer of trypsinized toxin was found to be 500 LD₅₀ in the unirradiated control of Experiment I-B at 27 days. All trypsinized toxin demonstrated in the four type B experiments was found at intermittent intervals, except in the 0.2 megarad irradiated culture of Experiment IV-B. The titers of trypsinized toxin found in the type B cultures varied, but were rarely more than 10 LD₅₀. Judging from the data collected from four type B experiments, trypsinized toxin was found more frequently in the 0.2 megarad irradiated cultures than in the other cultures. Trypsinized toxin was found only once, at a level of one LD₅₀, in the 0.1 megarad irradiated cultures in all four type B experiments. No. untrypsinized toxin was found in the four type B experiments throughout 30 days investigation. Although, at present, we cannot explain why toxin was detected at intermittent intervals in the 0.2 megarad cultures, the frequency with which it was found certainly implies that foods irradiated at this dose might also be more likely to produce toxin than unirradiated foods or those irradiated at a lower dose than 0.2 megarad.

VI. Survival of Spores of *Clostridium botulinum* Type E, Strain Beluga, Subjected to 0.0, 0.1, or 0.2 Megarad of Gamma Irradiation.

An experiment on the effect of gamma irradiation on the viability of type E, strain Beluga spores shows that 0.1 megarad and 0.2 megarad irradiation reduced the number of spores capable of forming macroscopic colonies by 44 percent and 76 percent, respectively. There was no threshold dose present. The survival curve of the irradiated spores of type E, strain Beluga was a linear function of dosage. Therefore, the data indicated that the spores of type E, strain Beluga, collected from trypticase-peptone-glucose medium, were homogenous and have uniform radio-sensitivity at these doses.

VII. Post-Irradiation Growth of *Clostridium botulinum* Type E, Strain Beluga, at 25C.

The data collected from two experiments using a post-irradiation growth temperature of 25C were subjected to a t-test (Goldstein, 1954) to ascertain the statistical significance of differences between slopes. Statistical treatments of these data verified that there were no significant differences in the slopes of the regression lines of the unirradiated culture and the irradiated cultures in each type E experiment. These results indicated that there were no differences in the growth rate between irradiated and unirradiated cultures.

There was no definite evidence which indicated that there has been some change in the irradiated spores (collected from trypticase-peptone-glucose medium) prior to their germination and post-irradiation growth in trypticase soy broth medium. Although the lag phase in irradiated cultures

in both experiments was quite prolonged the investigator felt that this was accounted for by the initial inoculation of fewer viable spores into the irradiated cultures than into the control cultures.

In Experiment I-E, all three cultures underwent partial lysis after they had attained their respective maximum optical density readings. Seven to eight hours were required for each of these cultures to reach a second peak of optical density, after which rapid autolysis ensued in all cultures. This partial lysis phenomenon, seen earlier in the logarithmic phase, was observed previously in this investigation in the 0.2 megarad irradiated culture of Experiment III-B. It has also been found for several strains of Clostridium botulinum type F investigated by others (Williams-Walls, 1969; Dolman and Murkami, 1961; LeBlanc, 1970). However, others (Eklund and Poysky, 1971) who have studied the growth of the same organisms used in this investigation (Clostridium botulinum type B, strain B-17 and type E, strain Beluga), have not reported this lytic phenomenon for either. Those who observed it in the growth of type F organisms surmise that either the presence of bacteriophage (Williams-Walls, 1969) or the dissociation or mutational tendencies of the organism (Dolman and Murakami, 1961) might be responsible for the partial lysis found in broth or solid cultures of Clostridium botulinum type F. These suggestions may also explain the lytic phenomena observed in this investigation.

VIII. Toxin Production at 25C of Clostridium botulinum Type E,
Strain Beluga.

Irradiation of spores of Clostridium botulinum type E, strain Beluga did not affect the initial release of toxin into culture supernatant

fluid as compared to the unirradiated control. As in the type B experiments, the data collected from toxin production of type E were subjected to analysis of variance (Croxtton, 1953) to ascertain: (1) the effect of trypsinization on toxin, (2) the effect of amount of irradiation of spores on toxin production, and (3) the effect of the age of the culture on toxin production. All the significance determinations were made at a 99 percent critical confidence level.

The statistical treatment showed that there were significant differences in titers between trypsinized toxin produced and untrypsinized toxin produced (at the same time in the same culture) in both type E experiments. This indicated that trypsinization significantly increased titers of the untrypsinized toxin. The statistical results also showed, however, that there were no significant differences in untrypsinized toxin produced at the various irradiation levels. This indicated that the irradiation of spores of Clostridium botulinum type E, strain Beluga, did not alter the titers of untrypsinized toxin produced. In addition, the results also showed that there were significant differences in titers of both the trypsinized and untrypsinized toxin produced according to the age of the culture.

The statistical results confirm the experimental finding that the titers of toxin (trypsinized and untrypsinized) increased as the incubation time lengthened, an observation which also implies that the toxin was gradually released into culture fluids. This behavior was different from that found in the type B experiments. The statistical treatment also showed that there were significant differences in titers of trypsinized toxin produced at the various irradiation levels for Experiment I-E, but

not for Experiment II-E. The contradictory results from the two type E experiments may be due to the termination of toxin testing at 120 hours for Experiment II-E, resulting in insufficient data on toxin production to subject to statistical analysis. The statistical findings for untrypsinized toxin production in Experiment I-E showed no significant difference between cultures for the amount of irradiation received, but revealed significant differences in toxin titers after trypsinization of these same culture supernatants. Trypsinization was accomplished under the same conditions for all of the cultures, both control and irradiated. Therefore, if the trypsinized toxins are different, by inference untrypsinized toxins are also different. This means that the untrypsinized toxin produced in the irradiated cultures of Experiment I-E was somehow different from that produced in the unirradiated control. The most likely explanation is a structural change in the toxin molecules produced by the irradiated cultures that becomes evident only after the molecule is unfolded by enzymatic disruption of certain peptide bonds in the protein. Irradiation of the spores from which these cultures were grown must be responsible for this alteration in the toxin molecule.

Although the statistical results apparently showed radiation received by the spores caused no significant differences in untrypsinized toxin production in both of the type E experiments, growth of irradiated cultures differs from that of controls the former in some cases showing a greater optical density and in other cases showing a less optical density than the control. This may indicate that irradiated cultures released more, or less, toxin per cell than the control culture. To illustrate, the maximum growth of the 0.2 megarad irradiated culture of

the 0.2 megarad irradiated culture of Experiment I-E was the same as that of the unirradiated culture, but the titer of trypsinized toxin in the 0.2 megarad irradiated culture was much higher than that for the unirradiated control. In Experiment II-E, the maximum growth (as indicated by optical density) of the 0.2 megarad irradiated culture was much less than the unirradiated control but the titers of trypsinized toxin were about equal for both cultures. These data implied that there were differences in the irradiated culture as compared to the unirradiated culture which caused the production of more toxin per cell in the former -- indirect evidence of disturbances in protein metabolism of the cells, probably caused by irradiation of the parent spores. These data support the theories of N. W. Walls (1969), who, in working with type F, concluded that (1) irradiation disturbance of protein metabolism could have been responsible for increased toxicity of toxin produced by cells of type F, (2) a stimulatory effect of the radiation could have caused more toxin production per cell.

IX. Post-Irradiation Growth of *Clostridium botulinum* Type E,
Strain Beluga at 10C.

At an incubation temperature of 10C, outgrowth of *Clostridium botulinum* type E, strain Beluga was observed in the unirradiated control of Experiment II-E 19 days after inoculation, but was not observed in the irradiated cultures of either type E experiment. These data confirm the reports made by Schmidt et al., (1962), Ohye and Scott (1957), and Dolman et al., (1950), all of whom found growth and toxin production by several unirradiated strains of *Clostridium botulinum* type E at low

temperatures of incubation. The failure of the irradiated cultures to achieve outgrowth in these experiments may be attributed to the double insult of irradiation damage to the spore coupled with a suboptimal incubation temperature of 10C.

X. Toxin Production of *Clostridium botulinum* Type E,
Strain Beluga at 10C.

The toxin production of cultures of Experiment II-E incubated at 10C was measured only at zero hours and five days. Those cultures at 10C in Experiment I-E were measured for toxin content at zero hours and every five days for a total of 28 days. In Experiment I-E, toxin initially appeared in the 0.1 megarad irradiated culture at 23 days and was detected in all three cultures after 28 days incubation, although the titer did not exceed one LD₅₀ for any culture. In contrast to Experiment I-E, trypsinized toxin was first found in Experiment II-E in the unirradiated control, at a level of one LD₅₀ on the fifth day of incubation. The trypsinized toxin found so early in the unirradiated control of Experiment II-E could be toxin from within the spores themselves, released after the spores lost their refractivity and became permeable to dyes (Boroff and Dasgupta, 1971).

CHAPTER V

CONCLUSIONS

Based on the research reported in the preceding text, the following conclusions are made:

I. Irradiation of spores of Clostridium botulinum type B, strain B-17, 0.1 megarad and 0.2 megarad of cesium-137 gamma radiation shows the following:

A. (1) Spores of type B, strain B-17, collected from phytone medium, exhibited a threshold dose between 0.1 megarad and 0.2 megarad of gamma irradiation.

A. (2) Spores of type B, strain B-17, collected from trypticase peptone glucose medium, did not have a threshold dose and the spore survival curve was not a linear function of dosage.

Therefore, the conclusion was that radiation sensitivity is influenced by the chemical composition of the medium in which the cells are produced.

B. Irradiation of spores of type B, strain B-17 did not alter the patterns of growth of the resulting vegetative cells in trypticase soy broth growth medium at an incubation temperature of 25C.

C. The titers of toxin (with or without trypsinization) produced by cultures grown from the irradiated spores of type B, strain B-17, collected from phytone medium, were less than those un-irradiated control cultures at an incubation temperature of 25C.

Irradiation of spores of type B, strain B-17, collected from trypticase-peptone-glucose medium did not alter the titers of untrypsinized toxin produced by cultures of their vegetative cell progeny, but did change the titers of trypsinized toxin produced by these same cultures at an incubation temperature of 25C.

D. No outgrowth was detectable in irradiated or unirradiated control cultures at an incubation temperature of 10C. No untrypsinized toxin was demonstrated in the irradiated or unirradiated cultures inoculated with spores of type B, strain B-17, at 10C. The time of appearance and the amount of trypsinized toxin found at any one age of sampling varied from experiment to experiment at an incubation temperature of 10C. All of these reflect the double insult to the spores of irradiation and suboptimal incubation temperature for this organism.

II. Irradiation of spores of Clostridium botulinum type E, strain Beluga, with 0.1 megarad and 0.2 megarad cesium-137 gamma radiation indicated the following:

A. Spores of type E, strain Beluga did not exhibit a threshold irradiation dose; the spore survival curve was a linear function of dosage.

B. Irradiation of spores did not alter the patterns of growth of the resulting vegetative cell cultures at an incubation temperature of 25C.

C. Irradiation of spores did not alter the titers of untrypsinized toxin produced in the cultures but did change the titers of trypsinized toxin produced, as compared to the unirradiated control

cultures, at an incubation temperature of 25C. In the case of the 0.2 megarad irradiated culture, this change was an increase (in spite of less cell mass), an indication that more toxin or toxin of greater potency was produced per cell by this culture.

D. Irradiation of spores of type E, strain Beluga, prevents the outgrowth of cultures at 10C in trypticase soy broth medium.

E. No untrypsinized toxin was demonstrated at 10C in any of the cultures, irradiated or unirradiated, of type E, strain Beluga. The initial appearance of trypsinized toxin varied from experiment to experiment, and its presence in the culture supernatant was erratic after initial appearance, at an incubation temperature of 10C.

CHAPTER VI

RECOMMENDATIONS

Based on the results of this study, the following recommendations are made for future research:

1. A study of the bacterial genetics of spores of Clostridium botulinum type B, strain and type E, strain, Beluga, after subjection to 0.1 megarad or 0.2 megarad gamma radiation. This study would be very useful in determining the genetic effect of irradiation on spores and in explaining the mechanisms responsible for partial lysis.

2. A chemical analysis of untrypsinized toxin produced in irradiated cultures inoculated with spores of Clostridium botulinum type B, strain B-17 and type E, strain Beluga in trypticase soy broth medium at 25C after treatment with 0.1 megarad or 0.2 megarad gamma irradiation. This study would determine if there was really a change in protein metabolism in the bacterial cells resulting from irradiated spores.

3. A chemical analysis of cultures inoculated with spores of Clostridium botulinum type B, strain B-17 or type E, strain Beluga and incubated at 10C. This type of study should attempt to explain why the toxin was present in the culture supernatants only intermittently, once it had appeared. The knowledge gained might well benefit the food industry.

APPENDICES

APPENDIX A

MEDIA AND STAINING METHODS

Eklund's GPBI Medium

Eklund's GPBI medium is prepared in the following manner:

First Day

1. Trim away fat. Cut meat into small pieces before putting through the fine attachment of a meat grinder.
2. Place the fine ground beef in a clean round stainless steel pot with lid and add one liter of distilled water per pound of beef. Mix well and bring to a boil, stirring frequently. Allow meat mixture to cool to room temperature. Cover and refrigerate at 10C overnight.

Second Day

3. Skim off solidified fat and discard. Allow meat and broth mixture to warm to room temperature.
4. Adjust to pH 8.0 with 10 N NaOH.
5. Heat to boiling. Cool to room temperature, then adjust to pH 8.0 with 10 N NaOH again.
6. Heat to boiling once more. Cool to room temperature, and make a final adjustment to pH 7.2.
7. Strain off meat particles through eight layers of cheesecloth and set aside in a clean container. Catch broth in a clean large glass container. One pound of top round beef produces

approximately 800 ml of liquid medium.

8. Measure the broth. Add:

NaCl (analytical reagent)	0.5%
Na ₂ HPO ₄ (analytical reagent)	0.08%
Bacto-Peptone (Difco)	0.5%
Trypticase (BBL)	0.5%
Dextrose (Bacto)	0.2%
Soluble starch (Merck)	0.1%

9. Heat to dissolve, then dispense 20 ml of enriched broth and 5 gm of meat into each of the required number of 15 x 125 mm screw cap culture tubes.

10. Autoclave tubes containing the medium for 30 minutes at 15 psi. Cool to room temperature, tighten caps, and store in 10C refrigerator.

11. If medium is not used the same day it is made, exhaust each tube in a boiling water bath for 10 minutes and cool quickly before inoculating.

Sorenson's Phosphate Buffer pH 7.0

M/15 Na ₂ HPO ₄	61.1 ml
M/15 KH ₂ PO ₄	38.9 ml

Confirm pH 7.0 with a pH meter or narrow range pH paper. Dispense into a 125 ml screw cap Erlenmeyer flask, autoclave for 15 minutes at 15 psi, then cool rapidly in ice water before using to dilute spores for irradiation.

Peptone Water

Bacto-Peptide	1.0 gram
Deionized water	1000.0 ml

Dissolve peptone in the water. Dispense 99 ml aliquants into 150 ml milk dilution bottles. Autoclave the filled bottles, and Escher stoppers immersed in water in a separate container, for 20 minutes at 120 C and 15 psi, then insert stoppers into bottles aseptically. After bottles have cooled seat stoppers firmly and store dilution bottles at room temperature.

Gelatin Diluent (Duff et al., 1957)

Bacto-Gelatin (Difco)	2.0 grams
$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$	4.0 grams
Deionized water	1000.0 ml

Add gelatin and Na_2HPO_4 to cold water. Warm to dissolve and cool to room temperature. Adjust the mixture to pH 6.8 with 10 percent HCl, dispense into screw cap prescription bottles, and autoclave at 121 C, 15 psi, for 15 minutes. Gelatin diluent may be stored at room temperature until used.

Trypticase Soy Broth Medium

The medium was formulated in these proportions:

Trypticase Soy Broth (BBL)	30.0 grams
Sodium Thioglycollate (Difco)	1.0 grams (0.1%)
Deionized Water	976.0 ml

The medium was mixed thoroughly and warmed gently until solution was complete. Then 244 ml of medium were dispensed into each 300 ml

Nephelo flask to be used in the experiment and the flasks autoclaved for 15 minutes at 15 psi. After autoclaving, the medium was allowed to cool to room temperature. To each Nephelo flask containing 244 ml of medium, 6 ml of filter sterilized 20 percent ribose were added aseptically, making a total of 250 ml of medium in each flask.

Schmidt's Counting Medium

Bacto-Peptone (Difco)	5.0%
Yeast Extract (BBL)	1.0%
Sodium Thioglycollate (Difco)	0.1%
Bacto-Agar	1.0%
Deionized Water	1000.0 ml

All dry components except the agar were added to the deionized water. The mixture was heated with constant stirring until the components were dissolved. The liquid was cooled to room temperature and adjusted to pH 7.2 with 10 N NaOH. The agar was then added to the medium and heated until it had dissolved. The medium was dispensed into Prickett tubes (approximately 25 ml per tube) which were cotton plugged and placed in a rack. The tops of the tubes were covered with a wood plank cut to size which was weighted down with a brick to prevent the cotton plugs from blowing out of the tubes in the autoclave. The tubes were autoclaved for 15 minutes at 15 psi, then removed to a 45 C water bath until used.

Schmidt's counting medium must be freshly prepared on the day it is used for optimum recovery of the inoculated spores.

Spore Stain

An adaptation of Conklin's modification of Wirtz's method (Manual

of Microbiological Methods; Society of American Bacteriologists, 1957) was employed to stain spore and cells. The staining procedure follows:

1. Prepare and heat-fix smears on cleaned glass slides.
2. Flood smears with 5 percent aqueous malachite green dye. Apply just enough heat to produce slight steaming for 5 minutes. Add malachite green as necessary to keep slides from drying.
3. Wash slides with tap water until no more dye comes off.
4. Counterstain for 1 minute with 1 percent safranin.
5. Wash with tap water. Allow to air dry.

Under microscopic examination, the highly refractive spores appear bright green in color; the vegetative cells appear red.

As spores approach germination, they start to lose refractivity (as evidenced by a failure to acquire green color) in the procedure and begin to take up methylene blue stain. This stage of development can be detected by microscopic examination of smears stained with methylene blue as follows:

1. Prepare and heat-fix smears.
2. Flood slides with methylene blue. Stain 2 minutes.
3. Wash slides thoroughly in running tap water and allow to air dry.

A spore undergoing germination will be deep blue in color, staining beginning at the circumference and finally spreading throughout the spore prior to emergence.

Trypticase-Peptone-Glucose (TPG)

Trypticase (BBL)

5.0%

Bacto-Peptone (Difco)	0.5%
Glucose (Difco)	0.4%
Sodium Thioglycollate (Difco)	0.1%
Deionized Water as required	

The medium was mixed thoroughly and warmed gently until solution was complete, then cooled to room temperature and adjusted to pH 7.2 with 10 N NaOH. The medium was dispensed into screw cap Erlenmeyer flasks, autoclaved for 15 minutes at 15 psi, and rapidly cooled in an iced water bath before inoculation.

Phytone Medium

Trypticase (BBL)	5.0%
Phytone (BBL)	2.0%
Sodium Thioglycollate (Difco)	0.1%
Deionized Water as required	

The ingredients were added to deionized water and gently warmed to dissolve, then cooled to room temperature. The pH of the medium was adjusted to 7.2 with 10 N NaOH and dispensed into screw cap Erlenmeyer flasks which were then autoclaved for 15 minutes at 15 psi. If the medium was to be inoculated immediately, the flasks were cooled rapidly in an iced water bath.

APPENDIX B

STATISTICAL ANALYSIS I: SLOPES OF GROWTH CURVES

Symbols Used in Statistical Analysis I (t-Tests)

- X: Hours of incubation following inoculation
- Y: Optical density reading obtained at the corresponding value for hours of incubation
- N: The number of items in a sample
- Σ : Upper case Greek sigma, meaning "take the sum of"
- b: Slope of the regression line

$$= \frac{\Sigma XY - \frac{(\Sigma X)(\Sigma Y)}{N}}{\Sigma X^2 - \frac{(\Sigma X)^2}{N}}$$

- SS_x : Sum of the squares of the x values = $\Sigma X^2 - \frac{(\Sigma X)^2}{N}$

- $S_{y \cdot x}$: A standard deviation of individual y values about the regression

$$\text{line} = \sqrt{\frac{1}{N-2} \left[\Sigma Y^2 - \frac{(\Sigma Y)^2}{N} - b \left(\Sigma XY - \frac{(\Sigma X)(\Sigma Y)}{N} \right) \right]}$$

- $\bar{S}_{y \cdot x}$: Pooled standard deviation = $\sqrt{(S_{y \cdot x})_1^2 + (S_{y \cdot x})_2^2}$

- t: Students t-Test computed = $\frac{b_1 - b_2}{\bar{S}_{y \cdot x} \sqrt{\frac{1}{SS_{x_1}} + \frac{1}{SS_{x_2}}}}$

Critical value of t taken at P = 0.01

Computations shown in detail only for Experiment II-B.

Computation of significance of difference between slopes obtained during the experiments on Clostridium botulinum type B, strain B-17 and type E, Beluga strain was determined in the following manner:

Computation of Significance of Difference Between Two Slopes

Experiment II-B on Clostridium botulinum Type B, Strain B-17

Unirradiated Spores

$$b = \frac{65.3800 - \frac{(220)(2.8850)}{11}}{4510 - \frac{(220)^2}{11}} = 0.0698.$$

$$SS_x = 4510 - \frac{(220)^2}{11} = 110$$

$$s_{y \cdot x} = \sqrt{\frac{1}{9} \left(1.3602 - \frac{(2.8850)^2}{11} - 0.0698 \left[65.3800 - \frac{(220)(2.8850)}{11} \right] \right)}$$

$$= 0.0866.$$

Spores Irradiated with 0.1 Megarad

$$b = \frac{78.6630 - \frac{(273)(3.232)}{13}}{5915 - \frac{(273)^2}{13}} = 0.0592$$

$$SS_x = 5915 - \frac{(273)^2}{13} = 182$$

$$s_{y \cdot x} = \sqrt{\frac{1}{11} \left(1.5215 - \frac{(3.232)^2}{13} - 0.0592 \left[78.6630 - \frac{(273)(3.232)}{13} \right] \right)}$$

$$= 0.0848$$

Spores Irradiated with 0.2 Megarad

$$b = \frac{76.0550 - \frac{(235)(2.9950)}{10}}{5605 - \frac{(235)^2}{10}} = 0.0687$$

$$SS_x = 5605 - \frac{(235)^2}{10} = 82.5000$$

$$s_{y \cdot x} = \sqrt{\frac{1}{8} \left(1.3210 - \frac{(2.9950)^2}{10} - 0.0687 \left[76.0550 - \frac{(235)(2.9950)}{10} \right] \right)}$$

$$= 0.0648$$

Significance of Difference Between Slopes for Unirradiated Spores of *Clostridium botulinum* Type B, Strain B-17 Versus Spores Subjected to 0.1 Megarad in the Experiment II-B.

Standard Deviation

$$\bar{s}_{y \cdot x} = \sqrt{(0.0866)^2 + (0.0848)^2} = 0.1204$$

$$t = \frac{0.0698 - 0.0592}{0.1204 \sqrt{\frac{1}{110} + \frac{1}{182}}} = 0.7361$$

The t value of 0.7361 is < 2.84; $n_1 = 11$ $n_2 = 13$. Therefore the slopes are not significantly different.

Significance of Difference Between Slopes for Unirradiated Spores of *Clostridium botulinum* Type B, Strain B-17 Versus Spores Subjected to 0.2 Megarad in the Experiment II-B.

Standard Deviation

$$\bar{s}_{y \cdot x} = \sqrt{(0.0866)^2 + (0.0648)^2} = 0.1072$$

$$t = \frac{0.0698 - 0.0687}{0.1072 \sqrt{\frac{1}{110} + \frac{1}{82.5000}}} = 0.0709.$$

The t value of 0.0709 is < 2.90 ; $n_1 = 11$ $n_2 = 10$. Therefore the slopes are not significantly different.

Significance of Difference Between Slopes for Spores of
Clostridium botulinum Type B, Strain B-17 Subjected to
0.2 Megarad Versus Spores Subjected to 0.1 Megarad in the
Experiment II-B.

Standard Deviation

$$\bar{s}_{y \cdot x} = \sqrt{(0.0648)^2 + (0.0848)^2} = 0.112$$

$$t = \frac{0.687 - 0.0592}{0.112 \sqrt{\frac{1}{82.5000} + \frac{1}{182}}} = 0.6834$$

The t value of 0.6834 is < 2.86 ; $n_1 = 13$ $n_2 = 10$. Therefore the slopes are not significantly different.

Table 5. Optical Density Readings Representing the Growth of Spores of Clostridium botulinum Type B, Strain B-17 after Subjection to 0.0, 0.1 or 0.2 Megarad Gamma Irradiation (Experiment II-B).

Case	0.0 Mrad		0.1 Mrad		0.2 Mrad	
	Age of Culture (in Hours)	O.D. Reading	Age of Culture (in Hours)	O.D. Reading	Age of Culture (in Hours)	O.D. Reading
1	15	0.02	15	0.015	19	0.035
2	16	0.025	16	0.019	20	0.050
3	17	0.030	17	0.020	21	0.070
4	18	0.050	18	0.028	22	0.170
5	19	0.120	19	0.060	23	0.250
6	20	0.160	20	0.070	24	0.320
7	21	0.260	21	0.150	25	0.430
8	22	0.450	22	0.240	26	0.600
9	23	0.610	23	0.360	27	0.560
10	24	0.600	24	0.470	28	0.510
11	25	0.560	25	0.620		
12			26	0.600		
13			27	0.580		

Table 6. Summary of t-Test Values, Degrees of Freedom and Critical Values for t-Test at the 0.01 Level for Data from Experiment II-B on Clostridium botulinum Type B, Strain B-17.

Source of Data	t-Test Values	Degrees of Freedom	Critical Value of t
Unirradiated vs 0.1 Megarad	0.7361	20	2.84
Unirradiated vs 0.2 Megarad	0.0709	17	2.90
0.2 Megarad vs 0.1 Megarad	0.6834	19	2.86

Table 7. Optical Density Readings Representing the Growth of Spores of Clostridium botulinum Type B, Strain B-17 after Subjection to 0.0, 0.1, or 0.2 Megarad Gamma Irradiation (Experiment III-B).

Case	0.0 Mrad		0.1 Mrad		0.2 Mrad	
	Age of Culture (in Hours)	O.D. Reading	Age of Culture (in Hours)	O.D. Reading	Age of Culture (in Hours)	O.D. Reading
1	21	0.020	21	0.015	18	0.010
2	22	0.030	22	0.020	19	0.015
3	23	0.050	23	0.030	20	0.030
4	24	0.060	24	0.040	21	0.050
5	25	0.100	25	0.060	22	0.080
6	26	0.150	26	0.078	23	0.130
7	27	0.245	27	0.078	24	0.180
8	28	0.356	28	0.156	25	0.190
9	29	0.500	29	0.330	26	0.255
10	30	0.560	30	0.420	27	0.212
11	31	0.580	31	0.530	28	0.175
12	32	0.520	32	0.600	29	0.285
13			33	0.540	30	0.310
14					31	0.440
15					32	0.610
16					33	0.680
17					34	0.620

Table 8. Optical Density Readings Representing the Growth of Spores of Clostridium botulinum Type B, Strain B-17 after Subjection to 0.0, 0.1, or 0.2 Megarad Gamma Irradiation (Experiment IV-B).

Case	0.0 Mrad		0.1 Mrad		0.2 Mrad	
	Age of Culture (in Hours)	O.D. Reading	Age of Culture (in Hours)	O.D. Reading	Age of Culture (in Hours)	O.D. Reading
1	21	0.010	21	0.015	22	0.010
2	22	0.020	22	0.020	23	0.020
3	23	0.030	23	0.020	24	0.020
4	24	0.050	24	0.030	25	0.040
5	25	0.050	25	0.020	26	0.038
6	26	0.100	26	0.048	27	0.032
7	27	0.135	27	0.062	28	0.075
8	28	0.265	28	0.065	29	0.125
9	29	0.395	29	0.065	30	0.245
10	30	0.485	30	0.180	31	0.370
11	31	0.405	31	0.270	32	0.490
12	32	0.400	32	0.400	33	0.540
13			33	0.485	34	0.490
14			34	0.430	35	0.518
15			35	0.455	36	0.485

Table 9. Summary of t-Test Values, Degrees of Freedom, and Critical Values for t-Test at the 0.01 Level for Data from the Experiments II-B, III-B and IV-B on Clostridium botulinum, Type B, Strain B-17.

Source of Data	t-Test Values	Degrees of Freedom	Critical Value of t
Unirradiated (Exp.II-B) vs 0.1 Megarad (Exp.III-B)	0.4560	21	2.83
Unirradiated (Exp.III-B) vs 0.2 Megarad (Exp.III-B)	1.9019	25	2.79
0.1 Megarad (Exp.III-B) vs 0.2 Megarad (Exp.III-B)	1.3047	26	2.78
Unirradiated (Exp.IV-B) vs 0.1 Megarad (Exp.IV-B)	0.8807	23	2.81
Unirradiated (Exp.IV-B) vs 0.2 Megarad (Exp.IV-B)	0.0636	23	2.81
0.2 Megarad (Exp.IV-B) vs 0.1 Megarad (Exp.IV-B)	0.9175	26	2.78
Unirradiated (Exp.II-B) vs Unirradiated (Exp.III-B)	0.7357	19	2.54
Unirradiated (Exp.II-B) vs 0.1 Megarad (Exp.III-B)	1.0322	20	2.53
Unirradiated (Exp.II-B) vs 0.2 Megarad (Exp.III-B)	2.3760	24	2.49
Unirradiated (Exp.II-B) vs Unirradiated (Exp.IV-B)	1.6690	19	2.54
Unirradiated (Exp.II-B) vs 0.1 Megarad (Exp.IV-B)	2.2013	22	2.51
Unirradiated (Exp.II-B) vs 0.2 Megarad (Exp.IV-B)	1.5933	22	2.51
0.1 Megarad (Exp.II-B) vs Unirradiated (Exp.III-B)	0.4736	21	2.52

Table 9. (Continued)

Source of Data	t-Test Values	Degree of Freedom	Critical Value of t
0.1 Megarad (Exp.II-B) vs 0.1 Megarad (Exp.III-B)	0.4285	22	2.51
0.1 Megarad (Exp.II-B) vs 0.2 Megarad (Exp.III-B)	1.8725	26	2.48
0.1 Megarad (Exp.II-B) vs Unirradiated (Exp.IV-B)	1.0413	21	2.52
0.1 Megarad (Exp.II-B) vs 0.1 Megarad (Exp.IV-B)	2.0181	24	2.49
0.1 Megarad (Exp.II-B) vs 0.2 Megarad (Exp.IV-B)	1.1981	24	2.49
0.2 Megarad (Exp.II-B) vs Unirradiated (Exp.III-B)	0.7022	18	2.55
0.2 Megarad (Exp.II-B) vs 0.1 Megarad (Exp.III-B)	1.0347	19	2.54
0.2 Megarad (Exp.II-B) vs 0.2 Megarad (Exp.III-B)	2.3252	23	2.50
0.2 Megarad (Exp.II-B) vs Unirradiated (Exp.IV-B)	1.7000	18	2.55
0.2 Megarad (Exp.II-B) vs 0.1 Megarad (Exp.IV-B)	2.4573	21	2.52
0.2 Megarad (Exp.II-B) vs 0.2 Megarad (Exp.IV-B)	1.7538	21	2.52
Unirradiated (Exp.III-B) vs Unirradiated (Exp.IV-B)	1.1120	20	2.53
Unirradiated (Exp.III-B) vs 0.1 Megarad (Exp. IV-B)	2.0642	23	2.53
Unirradiated (Exp.III-B) vs 0.2 Megarad (Exp.IV-B)	1.2252	23	2.50
0.1 Megarad (Exp.III-B) vs Unirradiated (Exp.IV-B)	0.5806	21	2.52

Table 9. (Concluded)

Source of Data	t-Test Values	Degree of Freedom	Critical Value of t
0.1 Megarad (Exp.III-B) vs 0.1 Megarad (Exp.IV-B)	1.4867	24	2.49
0.1 Megarad (Exp.III-B) vs 0.2 Megarad (Exp.IV-B)	0.6929	24	2.49
Unirradiated (Exp.IV-B) vs 0.2 Megarad (Exp.III-B)	0.6372	25	2.48
0.2 Megarad (Exp.III-B) vs 0.1 Megarad (Exp.IV-B)	0.3563	28	2.47
0.2 Megarad (Exp.IV-B) vs 0.2 Megarad (Exp.III-B)	0.6590	28	2.47

Table 10. Optical Density Readings Representing the Growth of Spores of Clostridium botulinum Type E, Beluga Strain after Subjection to 0.0, 0.1, or 0.2 Megarad Gamma Irradiation (Experiment I-E).

Case	0.0 Mrad		0.1 Mrad		0.2 Mrad	
	Age of Culture (in Hours)	O.D. Reading	Age of Culture (in Hours)	O.D. Reading	Age of Culture (in Hours)	O.D. Reading
1	21	0.010	22	0.005	26	0.012
2	22	0.010	23	0.025	27	0.048
3	23	0.030	24	0.035	28	0.088
4	24	0.050	25	0.062	29	0.230
5	25	0.150	26	0.070	30	0.340
6	26	0.080	27	0.175	31	0.540
7	27	0.210	28	0.410	32	0.800
8	28	0.450	29	0.620	33	0.700
9	29	0.380	30	0.620	34	0.850
10	30	0.580	31	0.830	35	0.950
11	31	0.750	32	0.950	36	1.100
12	32	0.850	33	0.920	37	1.300
13	33	0.850	34	1.200	38	0.850
14	34	0.930	35	1.400	39	0.860
15	35	1.200	36	1.450	40	0.900
16	36	1.300	37	1.500	41	0.850
17	37	1.000	38	1.350	42	0.900
18	38	0.850	39	1.000	44	1.000
19	39	0.850	40	1.000		

Table 10. (Continued)

Case	0.0 Mrad		0.1 Mrad		0.2 Mrad	
	Age of Culture (in Hours)	O.D. Reading	Age of Culture (in Hours)	O.D. Reading	Age of Culture (in Hours)	O.D. Reading
20	40	0.850	41	1.050		
21	41	0.800	42	1.000		
22	42	0.850	44	1.500		
23	44	1.230				

Table 11. Optical Density Readings Representing the Growth of Spores of Clostridium botulinum Type E, Strain Beluga after Subjection to 0.0, 0.1 or 0.2 Megarad Gamma Irradiation (Experiment II-E).

Case	Age of		Age of		Age of	
	Culture (in Hours)	O.D. Reading	Culture (in Hours)	O.D. Reading	Culture (in Hours)	O.D. Reading
1	17	0.005	20	0.010	23	0.03
2	18	0.005	21	0.030	24	0.03
3	19	0.01	22	0.350	25	0.068
4	20	0.025	23	0.500	26	0.070
5	21	0.040	24	0.800	27	0.075
6	22	0.075	25	0.125	28	0.130
7	23	0.090	26	0.130	29	0.325
8	24	0.130	27	0.185	30	0.390
9	25	0.265	28	0.240	31	0.660
10	26	0.32	29	0.600	32	0.84
11	27	0.52	30	0.900	33	0.900
12	28	0.80	31	0.900	34	0.950
13	29	0.94	32	1.050	35	0.920
14	30	1.23	33	1.160	36	0.950
15	31	1.40	34	1.230	37	1.000
16	32	1.44	35	1.310	38	1.000
17	33	1.53			39	0.99
18	34	1.61			40	1.05

Table 12. Summary of t-Test Values, Degrees of Freedom, and Critical Values for t-Test at the 0.01 Level for Data from Experiment I-E and Experiment II-E on Clostridium botulinum Type E, Beluga Strain.

Source of Data	t-Test Values	Degree of Freedom	Critical Values of t
Unirradiated (Exp.I-E) vs 0.1 Megarad (Exp.I-E)	1.7	41	2.58
Unirradiated (Exp.I-E) vs 0.2 Megarad (Exp.I-E)	0.444	37	2.58
0.1 Megarad (Exp.I-E) vs 0.2 Megarad (Exp.I-E)	0.866	36	2.58
Unirradiated (Exp.II-E) vs 0.1 Megarad (Exp.II-E)	1.400	30	2.75
Unirradiated (Exp.II-E) vs 0.2 Megarad (Exp.II-E)	2.266	32	2.58
0.1 Megarad (Exp.II-E) vs 0.2 Megarad (Exp.II-E)	0.333	30	2.75

APPENDIX C

STATISTICAL ANALYSIS II: TOXIN PRODUCTION

Symbols Used in Statistical Analysis II (Analysis of Variance)

- K_c : The number of columns
- K_r : The number of rows
- n_1 : Degrees of freedom associated with variation between column means or variation between row means
- n_2 : Degrees freedom associated with residual variation
- N_c : Number of items in a column
- N_r : Number of items in a row
- N : Number of items in a sample
- Σ : Upper-case Greek sigma, meaning "take the sum of"
- $\sum_{1}^{K_c}$: A summation over the K_c columns
- $\sum_{1}^{K_r}$: A summation over the K_r rows
- \sum_{1}^N : A summation over the N_c items in a column
- $\sum_{1}^{N_r}$: A summation over the N_r items in a row
- X : Log of LD_{50} of toxin production

Computations for analysis of variance shown in detail only for Experiment II-B.

Formulae used in computation of analysis of variation of data of toxin production of Clostridium botulinum type B, strain B-17 and type E, Beluga Strain.

Total Variation:

$$\Sigma X^2 - \frac{(\Sigma X)^2}{N}$$

Variation Between Column Means:

$$\frac{\sum^k \left(\frac{\sum^N X}{N_c} \right)^2}{N_c} - \frac{(\Sigma X)^2}{N}$$

Variation Between Row Means:

$$\frac{\sum^r \left(\frac{\sum^N X}{N_r} \right)^2}{N_r} - \frac{(\Sigma X)^2}{N}$$

Residual Variation:

Total variation - (variation between column means plus variation between row means).

Estimated Variance:

$$\frac{\text{Variation}}{\text{Degrees of Freedom}}$$

F Test:

$$\frac{\text{Estimated Variance Between Column Means}}{\text{Residual Estimated Variance}}$$

at: n_1 = Degrees of freedom between column means.

n_2 = Degrees of freedom of residual.

or

$$\frac{\text{Estimated Variance Between Row Means}}{\text{Residual Estimated Variance}}$$

at: n_1 = Degrees of freedom between row means.

n_2 = Degrees of freedom of residual.

Table 13. Logs of LD₅₀ of Trypsinized and Untrypsinized Toxin Production of Experiment II-B, Using Spores of Clostridium botulinum Type B, Strain B-17, Harvested from Phytone Medium, at 25C.

Age of Culture (in Hours)	0.0 Mrad		0.1 Mrad		0.2 Mrad	
	Trypsinized Toxin	Untrypsinized Toxin	Trypsinized Toxin	Untrypsinized Toxin	Trypsinized Toxin	Untrypsinized Toxin
52	2.3010	0	2.6989	0	2.0000	0
56	2.0969	0	2.6989	0	2.6989	0
60	2.6989	0	2.6989	0	2.6989	0
72	2.6989	0.6989	2.6989	0.3979	2.6989	0.6989
96	3.0000	0.6989	2.0000	0.6989	3.0000	0.3979
120	4.0000	1.3010	3.0000	0.3979	3.3010	0.3979
144	4.6989	1.3979	3.6989	0	3.6989	0.3979
168	5.0000	1.6989	3.3010	0.6989	3.3010	0.3979
192	5.0000	1.0000	4.0000	0	3.6989	0.6989
216	5.0000	2.0000	3.6989	0.6989	3.6989	0.6989
240	4.6989	1.3979	3.3010	0.6989	3.6989	0.6989

Table 14. Summary of Computations of Analysis of Variance of Data of Trypsinized Toxin Production of Clostridium botulinum Type B, Strain B-17 in Experiment II-B, at 25C.

Source of Variation	Amount of Variation	Degrees of Freedom	Estimated Variance	F-Test	Critical Values of F*
Between Column (Radiation Level) Means	3.0333	2	1.51	$\frac{1.51}{0.21} = 7.18$	5.849
Between Row (Age of Culture) Means	16.4902	10	1.64	$\frac{1.64}{0.21} = 7.80$	3.564- 3.231+
Residual	4.3310	20	0.21		
Total	23.8545	32			

*Significance was determined at a probability value of 0.01.

Table 15. Summary of Computations for Analysis of Variance of Data of Untrypsinized Toxin Production of Clostridium botulinum Type B, Strain B-17 in Experiment II-B.

Source of Variation	Amount of Variation	Degrees of Freedom	Estimated Variance	F-Test	Critical Values of F*
Between Column (Radiation Level) Means	2.2760	2	1.1380	22.76	5.849
Between Row (Age of Culture) Means	4.7603	10	0.476	0.9520	3.564
Residual	2.2733	20	0.05		
Total	9.3096	32			

*Significance was determined at a probability value of 0.01.

Table 16. Summary of Computations for Analysis of Variance of Data of Trypsinized Toxin Production versus Untrypsinized Toxin Production in Unirradiated Control Culture of Experiment II-B, Using Spores of Clostridium botulinum Type B, Strain B-17.

Source of Variation	Amount of Variation	Degrees of Freedom	Estimated Variance	F-Test	Critical Values of F*
Between Column (T vs U)** Means	43.6818	1	43.6818	225.1639	10.044
Between Row (Age of Culture) Means	17.2316	10	1.7231	8.8819	5.057
Residual	1.9408	10	0.1940		
Total	62.8542	21			

*Significance was determined at a probability value of 0.01.

**T = Trypsinized toxin, U = Untrypsinized toxin.

Table 17. Summary of Computation for Analysis of Variance of Data of Trypsinized Toxin Production Versus Untrypsinized Toxin Production in 0.1 Megarad Irradiated Culture of Experiment II-B, Using the Spores of Clostridium botulinum Type B, Strain B-17.

Source of Variation	Amount of Variation	Degrees of Freedom	Estimated Variance	F-Test	Critical Values of F*
Between Column (T vs U)** Means	40.6477	1	40.6477	184.4269	10.044
Between Row (Age of Culture) Means	2.0621	10	0.2062	0.9355	3.564- 3.231†
Residual	2.2044	10	0.2204		
Total	44.9142	21			

*Significance was determined at a probability value of 0.01.

**T = Trypsinized toxin, U = Untrypsinized toxin.

Table 18. Summary of Computation for Analysis of Variance of Data of Trypsinized Toxin Production Versus Untrypsinized Toxin Production in 0.2 Megarad Irradiated Culture of Experiment II-B, Using the Spores of Clostridium botulinum Type B, Strain B-17.

Source of Variation	Amount of Variation	Degrees of Freedom	Estimated Variance	F-Test	Critical Values of F*
Between Column (T vs U)** Means	41.2017	1	41.2017	488.8149	10.044
Between Row (Age of Culture) Means	3.1971	10	0.3197	3.7924	4.706 ⁺ 5.057 ⁻
Residual	0.8436	10	0.0843		
Total	45.2425	21			

*Significance was determined at a probability value of 0.01.

**T = Trypsinized toxin, U = Untrypsinized toxin.

Table 19. Logsof LD₅₀ of Trypsinized and Untrypsinized Toxin Production of Experiment III-B, Using Spores of Clostridium botulinum Type B, Strain B-17, Harvested from TPG Medium.

Age of Culture (in Hours)	0.0 Mrad		0.1 Mrad		0.2 Mrad	
	Trypsinized Toxin	Untrypsinized Toxin	Trypsinized Toxin	Untrypsinized Toxin	Trypsinized Toxin	Untrypsinized Toxin
72	3.0413	0.6989	3.0413	0	2.6989	0
96	4.0000	0.3979	3.0413	0.3979	3.0413	0.3979
120	4.0000	0.6989	2.6989	0	2.6989	0.3979
144	4.0000	0.6989	4.0000	0.6989	2.0000	0
168	4.0000	0.6989	2.0000	0	2.3979	0.6989
192	4.0000	0.6989	2.3979	0.6989	2.3979	0.3979
216	3.6989	0.6989	3.6989	0.3979	3.6989	0
240	2.6989	1.0000	2.3010	0.6989	2.6989	0.6989

Table 20. Logs of LD₅₀ of Trypsinized and Untrypsinized Toxin Production of Experiment IV-B, Using Spores of Clostridium botulinum Type B, Strain B-17, Harvested from TPG Medium.

Age of Culture (in Hours)	0.0 Mrad		0.1 Mrad		0.2 Mrad	
	Trypsinized Toxin	Untrypsinized Toxin	Trypsinized Toxin	Untrypsinized Toxin	Trypsinized Toxin	Untrypsinized Toxin
96	1.6989	0.3979	1.6989	0.3979	1.6989	0.3979
120	1.6989	0.3979	1	0.3979	1.6989	0.3979
144	1.6989	0	2	0.3979	2.6989	0.6989
168	1.6989	0.3979	1.6989	0.3979	2.6989	0.6989
192	1.6989	0.3979	1.6989	0.6989	2.6989	0.3979
216	2	0.3979	2	0.3979	3.6989	0.6989
240	2.6989	0.3979	1.6989	0.3979	3.6989	0.6989

Table 21. Log₁₀ of LD₅₀ of Trypsinized and Untrypsinized Toxin Production of Experiment I-E, Using Spores of Clostridium botulinum Type E, Beluga Strain, Harvested from TPG Medium.

Age of Culture (in Hours)	0.0 Mrad		0.1 Mrad		0.2 Mrad	
	Trypsinized Toxin	Untrypsinized Toxin	Trypsinized Toxin	Untrypsinized Toxin	Trypsinized Toxin	Untrypsinized Toxin
44	3.6989	1	3.6989	1	4.6989	1.6989
48	4.6989	1	4.6989	0.6989	5	0.6989
52	4.6989	1.6989	4.6989	1	5	1.6989
56	4.6989	1.6989	4	1.6989	5.6989	1
60	4	1	4	1.6989	5.6989	1.6989
72	5.6989	2.6989	4.6989	2	4	1.6989
96	6	2.6989	5	2.6989	7.6989	3
120	5	3	5.6989	2.6989	5.6989	3
144	5.6989	3	5.6989	3	5.6989	2.6989
168	5.6989	3	5.6989	3	6.6989	2.6989
192	5.6989	3	5.6989	2.6989	6	2.6989
216	5.6989	3.6989	5.6989	3	8	2.6989
240	5.6989	3.6989	6.6989	3	9	2.6989

Table 22. Log₁₀ of LD₅₀ of Trypsinized and Untrypsinized Toxin Production of Experiment II-E, Using Spores of Clostridium botulinum Type E, Beluga Strain, Harvested from TPG Medium.

Age of Culture (in Hours)	0.0 Mrad		0.1 Mrad		0.2 Mrad	
	Trypsinized Toxin	Untrypsinized Toxin	Trypsinized Toxin	Untrypsinized Toxin	Trypsinized Toxin	Untrypsinized Toxin
36	3.6989	1.6989	4	1.6989	3.6989	1.6989
40	4.0000	1	2	1.6989	5.0000	1.6989
44	4.0000	1.0000	4	1	3.6989	0.6989
48	3.6989	1.00	3.6989	1.6989	3.6989	0.6989
52	3.6989	0.6989	3.6989	1.00	4.00	1.00
56	2.6989	1.0000	3.0000	1.0000	4.0000	1.0000
60	4.6989	1.0000	4.6989	1.6989	4.6989	1.6989
72	4.6989	2.6989	5.6989	2.0000	4.6989	1.6989
96	5.6989	2.6989	5.6989	3.0000	5.6989	2.6989
120	5.6989	2.6989	5.6989	2.6989	5.0000	2.6989

Table 23. Summary of Results of Analysis of Variance of Data of Trypsinized Toxin Production Versus Untrypsinized Toxin Production in Experiments II-B, III-B, IV-B, I-E, and II-E.

Source of Variation	Experiment Number														
	II-B			III-B			IV-B			I-E			II-E		
	0.0 Mrad	0.1 Mrad	0.2 Mrad	0.0 Mrad	0.1 Mrad	0.2 Mrad	0.0 Mrad	0.1 Mrad	0.2 Mrad	0.0 Mrad	0.1 Mrad	0.2 Mrad	0.0 Mrad	0.1 Mrad	0.2 Mrad
Between Radiation Levels	S*	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Between Ages of Cultures	S	N.S**	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	S	S	N.S.	N.S.	N.S.	S

*Significant difference at 0.01 confidence level.

**No significant difference.

Table 24. Summary of the Results of Analysis of Variance of Data of Trypsinized Toxin Production in Experiments II-B, III-B, IV-B, I-E and II-E.

Source of Variation	Experiment Number				
	II-B	III-B	IV-B	I-E	II-E
Between Radiation Levels	S*	S	S	S	N.S.
Between Ages of Cultures	S	N.S.**	N.S.	S	S

*S = Significant difference.

**N.S. = No significant difference.

Table 25. Summary of the Results of Analysis of Variance of Data of Untrypsinized Toxin Production in Experiments II-B, III-B, IV-B, I-E and II-E.

Source of Variation	Experiment Number				
	II-B	III-B	IV-B	I-E	II-E
Between Radiation Levels	S*	N.S.	N.S.	N.S.	N.S.
Between Ages of Cultures	N.S.**	N.S.	N.S.	S	S

*S = Significant difference.

**N.S. = No significant difference.

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