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A STUDY OF THE EFFECT OF IONIZING RADIATION ON RESISTANCE, GERMINATION, AND TOXIN SYNTHESIS OF *CLOSTRIDIUM BOTULINUM* SPORES, TYPES A, B, AND E

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SUMMARY

Human botulism has been reported to result from eating fish and seafood products in which Clostridium botulinum Types A, B, and E have grown. With one exception, all known cases of Type E botulism have resulted from eating fish, fish products and sea mammals.

To date, Type E botulism as transmitted by fishery products has resulted in 81 outbreaks involving 432 cases and 125 deaths.

The latest outbreaks which occurred in Michigan in September 1963 and in Tennessee, Alabama, and Kentucky in October of the same year had resulted in a great interest in Clostridium botulinum Type E.

C. botulinum Type E has been reportedly recovered from the intestinal contents of fish and mud samples taken in Japan, British Columbia, Greenland, Denmark, France, Mediterranean coast, Gulf of Mexico, and The Great Lakes region of the United States.

In the present study six representative strains of C. botulinum Type E were used initially. During the course of the study isolates from the smoked fish outbreaks were also obtained and their heat resistance compared.

Growth and toxin production in haddock homogenates and broth were studied with incubation at temperatures between 0-37°C. At 37.5°C, 33°C, and 18.0°C growth occurred within three days. At 8.3°C, and with some strains at 5.4°C and 3.3°C growth as measured by gas production was variable. However, low levels of toxin were detected in samples at these temperatures within 38 days of incubation. At 0°C no growth occurred in any of the samples after 290 days of incubation. Toxin assays of the samples incubated at 3.3°C and 0°C for 250 days did not demonstrate any toxin, although a low level was detected after 38 days at 3.3°C. Transfer of cultures incubated at 3.3°C and 0°C for 250 days to a 30°C incubator resulted in growth within 48 hours and the production of toxin indicating spore viability. Activation of the cultures with trypsin was necessary in order to detect low levels of toxin in the haddock homogenates.

A pre-irradiation treatment of spores used as an inoculum for the haddock homogenate cultures delayed the onset of growth. The size of the inoculum, did not effect the outgrowth time of the spores appreciably in haddock homogenates.

Toxin production in various seafood products was also studied. Growth, sporulation, and toxin synthesis was found to occur readily in clam juice,

haddock, and shrimp homogenates previously sterilized by gamma radiation. No toxin was detected in oyster juice previously sterilized by gamma radiation, inoculated with Type E spores and incubated at several temperatures. However, toxin was detected in similar experiments using oyster juice that had been both irradiated and heated. With haddock homogenates, toxin was produced both in the radiation sterilized media and in the irradiated and heated media. Activation of the preformed toxin by trypsin in the tissue homogenates was variable. Variation of pH of broth cultures in the range of 6.5-7.5 had little effect on the production of toxin. Although growth was slightly greater at the higher pH, resulting in a slight increase in the amount of toxin, recovered. As long as a fermentable carbohydrate was present growth and toxin synthesis occurred.

The thermal resistance of spores of 11 strains of Type E were studied in phosphate buffer at pH 7.0 in the temperature range of 60°-90°C. All strains had a similar resistance to heat. Very little inactivation occurred at 60°C and 65°C. At temperatures of 70°-90°C there is a rapid initial kill of the majority of the spores in the population within 10 minutes of heating with a small fraction surviving longer heating times giving a tailing effect. With two strains slight activation of dormant spores was apparent at 70°C. Toxin production was detected by the spores surviving heating at higher temperatures and for longer periods of time. However, in some cases non-toxic isolates were obtained. Spores produced by the heat surviving spores had a resistance similar to the stock spore suspensions.

Resistance of the spores of six strains of Type E to gamma radiation in phosphate buffer was found to be similar, but less than Type A spores. Irradiation of the spores in haddock homogenates and oyster juice resulted in an increase in survivors which was 100-1000 fold greater than that resulting from irradiation in phosphate buffer.

The outbreak of Type E botulism in Michigan and by fishery products processed in Michigan prompted a study in the possible existence of Type E in fish in The Great Lakes. A brief study of Lake Michigan fish resulted in the isolation of 3 toxic cultures neutralized by Type E antitoxin and one culture neutralized by Type A antitoxin from chubs and perch.

Dose rate measurements about the two cobalt-60 sources which were available for this investigation were performed. The two cobalt-60 sources orginally used in the studies were replaced by ones of higher intensity. Dose rate measurements around these sources was also performed. Some preliminary work on the possible uses of oxalic acid for high dose rate measurements was evaluated.

INTRODUCTION

SEAFOODS AND BOTULISM

Although human botulism is generally considered to be comparatively rare, the universal distribution of C. botulinum spores poses a continuing, potential hazard in food processing. This fact must be recognized whenever new food-processing techniques are considered.

Based on the nature of the toxin produced, six serologically distinct types of C. botulinum are known to exist. These are identified as Types A, B, C, D, E, and F. All of these strains have been identified as the causative agents of human botulism. While only Types A, B, and E are of major concern at present, the possibility of involvement of the other types cannot be overlooked. In the past, fish and related fishery products have not been extensively involved in the transmission of botulinum poisoning in the United States. During the years from 1899 to 1954, fish and seafoods have been incriminated in 32 outbreaks of botulism in the United States, excluding Alaska. These constituted only 6.2% of the total number of cases of botulism recorded (Meyer, 1956). Most of the outbreaks were attributed to commercially canned fish products. Among the products involved were salmon, sardines, crab, trout, sprats, tuna, clams, clam juice, and pickled mackerel and herring.

Types A and B were isolated from the foods involved in some of the outbreaks. In two outbreaks due to processed fish, Type E was isolated. In Alaska, seven recorded outbreaks have been traced to the consumption of products of sea mammals and salmon eggs, and all of these were due to Type E toxin (Dolman, 1960a).

In 1963 the situation as related to transmission of botulism by fish changed drastically. Of the total of 12 recorded outbreaks, 4 were related to smoked fish and one to commercially canned tuna. These four outbreaks involved 22 cases out of a total 46 cases and accounted for 9 out of 14 deaths. The outbreaks were all attributed to Type E. (MMWR 1964)

Of the 24 cases of botulism recorded in Canada before 1960, 66.7% were found to have been transmitted by fish, fish products, and sea mammals (Dolman, 1961). The products responsisble for the outbreaks were home-processed canned salmon, salmon eggs, pickled herring and trout, seal liver, seal flippers, and dried seal meat. Seven Type E, one Type B, and one Type A strains were isolated from the incriminated foods.

In Japan there have been 33 outbreaks of botulism between 1951 and 1960; all due to fish (Dolman, 1960a). Type E organisms were isolated in each case, all but two of which were transmitted through the consumption of "izushi," a relish consisting of fermented fish, rice, and vegetables, a popular food of the people of Hokkaido and North Honshu. The other two cases were due to canned mackerel and trout eggs.

In Europe, of the 13 outbreaks of botulism that have occurred in Norway, six involved fish. Four Type B and one Type E organisms were isolated. One of the outbreaks was due to home canned trout, the others to "rake fish." (Skulberg, 1961)

In Denmark there has been a total of 12 outbreaks of botulism, seven of which were caused by the consumption of fish (salted or pickled mackerel or herring). Type E was isolated in three of the outbreaks (Johannsen, 1964).

In Sweden fish have been involved in seven of the eight recorded outbreaks of botulism in that country. One case was transmitted by pickled herring, another by a commercially cooked fish product. Type E was isolated from the foods involved in three of the outbreaks.

In France, one case of botulism involving Type B was traced to tuna soup (Dolman, 1961). Three other outbreaks attributed to fish from which Type E was isolated have been reported (Prevot and Huet, 1951).

In the United Kingdom, an outbreak due to pickled herring (imported) from which a Type A culture was isolated has been reported (Mackay-Scallay, 1958).

Meyer (1956) lists 19 episodes of botulism in Germany in 1948 and 1949. Of these, nine were attributed to fish.

In Russia, botulism was considered to be a serious problem with fisheries products, although details are lacking. Meyer (1956) lists 1,117 cases as having occurred in Russia and the USSR between 1878 and 1937. Of these, 609 were designated as fish poisoning, due mostly to Types A and B botulism. An episode due to Type E botulism in Leningrad in 1938 is mentioned (Dolman, 1961). However, more outbreaks due to Type E may have occurred, since the prototype E, identified by Gunnison et al. (1936), was obtained from Russia.

An examination of the literature points out that Types A, B, and E have been involved in the transmission of botulism by fish and fishery products and sea mammals. Most significant however, is that with one exception, Type E intoxications have always been associated with marine products. Also, the incidence of botulism is highest in those regions in which there are workers in the field of anaerobic bacteria.

A fairly accurate compilation of the proven outbreaks of Type E botulism is as follows: a total of 80 outbreaks, 400 cases, and 124 deaths, distributed as indicated by Table 1.

TABLE 1

DISTRIBUTION OF C. BOTULINUM TYPE E OUTBREAKS

Place	Outbreaks	Cases	Deaths
Japan	44	293	76
U.S.A.	15	58	23
Canada	12	32	20
Sweden	3	5	1
Denmark	3	8	2
Norway	1	3	1
U.S.S.R.	2	2	2
France	2	?	?
Totals	81	432	125

HISTORY OF TYPE E BOTULISM

The first proven incident of Type E botulism occurred in the United States in Cooperstown, New York, on July 31, 1932. In this instance a girl died and her parents became ill after consuming smoked salmon imported from Labrador (Mackenzie, 1934). A second case involved three people, with one death. It occurred in Westchester County, New York, in 1934, and was due to canned sprats (small herring) imported from Kiel, Germany. The organism involved in the outbreak was isolated and characterized by Hazen (1937, 1938, 1942) who identified it as Type E. Previously, Gunnison, Cummings, and Meyer (1936) had described a new strain which they could not characterize by available botulinum antitoxins and which they then designated as Type E, in keeping with Burke's classification. Their two cultures had been sent to them by Dr. L. Bier of the Bacteriological Institute at Dniepropetrowsk, Ukrania, USSR, in March, 1936.

Another episode occurred in San Francisco, California. It was attributed to mushroom sauce canned in California. The dehydrated mushrooms used in the manufacture of the sauce were imported from Yugoslavia (Geiger, 1941). This is the only recorded case of Type E botulism in which fish, fish products, or marine mammals were not the food responsible for the outbreak.

The next reported outbreak of Type E botulism occurred in 1944 in Nanaimo, British Columbia (Dolman, 1947). It was caused by canned salmon. This was followed by an outbreak in Alaska in 1950 due to consumption of meat from flippers of the beluga whale (Dolman, 1953).

The first recorded case of Type E botulism occurred in 1951 in Japan from the eating of "izushi," a fermented relish made from fish, rice, and vegetables (Nakamura et al., 1956). Other reported outbreaks followed: one in France in 1951 (Prevot and Huet, 1951), in Denmark in 1951 (Pederson, 1955), in Labrador in 1956 (Dolman, 1960), Sweden in 1960 (Johannsen, 1964), Norway in 1963 (Skulberg, 1964).

In the past four years several outbreaks of Type E botulism have occurred and all are related to fish.

In Minneapolis, Minnesota, two fatal cases are reported from the consumption of smoked ciscos. (MMWR Vol. 12) This was subsequently proven to have been caused by Type E. This episode was followed by an outbreak in Detroit, Michigan in March, 1963. In this outbreak commercial canned tuna was involved which resulted in three cases and two deaths. The organism was isolated from one the patients and the can lid which contained the tuna. The tuna was packed in California; the tuna was imported.

In September of the same year two people died of botulism food poisoning in Kalamazoo, Michigan after consuming smoked whitefish. A Type E organism was isolated from remnants of the fish. Shortly after this outbreak in October another outbreak occurred in Tennessee, Alabama, and Kentucky, also due to smoked fish. In this incident vacuum packed whitefish chubs which were processed in Michigan were involved. In this outbreak there were seventeen cases and five deaths (MMWR 13, No. 1).

It is interesting to speculate on the reservoir for Type E spores. Most of the epidemological evidence points to an aquatic rather than a terrestrial habitat for these spores. If this assumption is valid, it should be possible to isolate these spores from fish and from the sea. Spores of C. botulinum Type E have been isolated from the terraqueous deposits and benthic sludges along the coast of British Columbia (Dolman, 1960 a). Some of these spores were capable of surviving several months of storage at 5°C. Also, a toxic culture was obtained from the intestine of a salmon caught in the Straits of Georgia, Vancouver (Dolman, 1957 a).

Surveys of samples collected from littoral mud of Lake Abashiri and sands from the coast of the Sea of Okhotsk have yielded 31 toxic cultures, from which nine pure strains of Type E were isolated. No positive cultures were isolated from 247 soil samples collected from the various districts of Hokkaido. Toxic cultures were isolated from the intestinal tracks of fish caught in Lake Abashiri (Nakamura et al., 1956). Pederson (1955) was able

to isolate Type E cultures in 16 out of 19 samples of mud taken from Copenhagen harbor. Also, a mud sample from the east coast of Greenland yielded a toxigenic Type E culture. In France, Prevot and Huet (1951) were able to obtain one culture of Type E from a perch after the examination of the intentinal contents of 163 fresh- and 13 salt-water fish. In surveys of samples obtained from the Baltic Sea and various districts of Sweden, Johannsen (1964) has found that C. botulinum Type E organisms to be widely distributed.

PHYSIOLOGICAL CHARACTERISTICS OF C. BOTULINUM TYPE E

Some of physiological characteristics of Type E organisms no doubt account for the peculiarities and lack of understanding concerning Type E botulism. Compared with Types A and B, Type E strains demonstrate lower growth temperatures, greater sensitivity to heat, marked potentiation of their toxin by proteolytic enzymes, and the ability of toxic cultures to revert easily to non-toxic states.

Of major concern in the pathogenicity of Type E botulism is the ability of the spores to germinate and grow with toxin synthesis at low temperatures. Cases of Type E botulism due to "izushi" prepared in winter months have been noted (Nakamura et al., 1956). Dolman (1950) demonstrated slight but definite toxin production in herring that had been artifically inoculated with a Type E culture and then incubated at 6°C.

Ohye and Scott (1956) studied the growth characteristics of nine Type E strains in the temperature range of 2.5 to 40°C. Six of the nine strains grew from spore inocula in a peptone-yeast extract medium at 5°C. However, all of the strains grew at 5°C when germination of the spores was first carried out at 10°C and then the cultures were transferred to 5°C. In cooked meat media, all nine strains grew at 5°C after three to four weeks of incubation. This shows the importance of the type of culture medium when assessing the growth characteristics of this organism. Four of these nine Type E cultures contained approximately 10 MLD of toxin for mice per ml when grown in cooked meat media at 5°C. It would therefore appear that a slightly higher temperature is necessary for toxin synthesis than that for cell growth. In these studies, however, toxin activation by trypsin was not attempted, so the culture media may have had a higher titre for toxin than was demonstrated. Cultures which had been incubated for five months at 2.5°C were removed from the incubator, heated to 60°C for 30 minutes, and then reincubated at 25°C. Growths developed readily within 24 hours, indicating that the spores were still viable.

However, it should be noted that Wagenaar (1956) found that one strain of Type E did not grow after 26 weeks of incubation at 10°C. Growth with toxin production occurred at 12.8°C after four weeks, and in two weeks at 15.5°C. Cultures of Types A and B grew and were toxic after one week at

15.5°C and after 6 weeks at 12.8°C. No growth occurred at 10°C.

Schmidt et al. (1962a), showed that spores of three strains of C. botulinum Type E, inoculated into beef stew and into pea-peptone media, were able to grow and synthesize toxin at temperatures of 43°F and 49°F within 16 to 22 days. Furthermore, in studies involving the radiation resistance of Type E spores in beef stew, the spores that survived an irradiation exposure of 0.8 megarad were able to germinate and produce toxin after incubation at 43°F for 75 days.

Schmidt et al. (1962), reported further observations on the minimum growth temperatures for Type E cultures. They report that mildly heat-shocked spores of four strains of Type E cultures that were inoculated into heat-sterilized beef stew did not show gas or toxin production after 104 days of incubation at 34 or 36°F. At 38°F, however, visible growth and toxin production occurred in 31 to 45 days.

These conflicting statements in the literature regarding the temperatures at which Type E cultures will germinate, grow, and develop toxin are very important for the proposed refrigerated storage of fish pasteurized with ionizing radiations. It would seem likely that any error in assigning temperatures to these physiological characteristics of C. botulinum Type E cultures would tend to indicate lower temperatures than the ones actually occurring. Therefore these temperatures for growth, toxin production, etc., by C. botulinum Type E need to be very carefully confirmed in fish substrates using irradiated systems. The greater heat sensitivity of the spores of various strains of Type E cultures, as compared with Types A and B, could account for the fact that Type E are not generally found when surveys are made for the distribution of C. botulinum in soils and food. An usual procedure for the isolation of bacterial spores involves heating the sample for about 30 minutes at 85°C. This will not inactivate Types A and B spores, but will usually kill Type E. For example, Gunnison et al. (1936), found that 5×10^6 Type E spores per ml in phosphate buffer were inactivated in six minutes at 80°C; and Nakamura et al. (1956), observed that heating for 20 minutes at 80°C destroyed spores of eight Type E strains. In more detailed studies it was observed at a 3 sec exposure at 105°C reduced the numbers of Type E spores by 90%, whereas 10 to 12 minutes were required for an equivalent reduction of Type A spores (Dolman, 1957a). Ohye and Scott observed that Types A and B spores were 1000 times more resistant than Type E spores (1957).

A disconcerting fact, recognized early in the study of Type E botulism in man, was the apparent high toxicity of the ingested toxin as compared with the low toxicity of the toxin in the food before ingestion (Dolman and Chang, 1953; Sakaguchi et al., 1954). Indeed, the examination of the stomach contents of a patient who died from Type E botulism revealed a much higher titre (20-200 fold) of Type E toxin than was present in the ingested food (Dolman, 1960b). It has been demonstrated that proteolytic enzymes of

bacteria (Dolman, 1957b and Sakaguchi et al., 1954) and pure trypsin can activate preformed E toxin resulting in a substantial increase in the titre. This increase may be as much as 1000 fold (Duff et al., 1956, Dolman, 1957b, Iida et al., 1958).

In this connection it should be noted that Sakaguchi and Tohyama (1954, 1955a and b) isolated an anaerobic spore forming, non-toxic, and proteolytic bacterium which when grown simultaneously with a Type E strain, resulted in a much higher titre of Type E toxin than was obtained from the same strain when it was grown in pure culture. This is important when considering the possibilities in radiation-pasteurized fish and seafoods. Radiation pasteurization does not inactivate all the bacteria, rather it is selective; hence resistant species and spores will survive. Therefore the symbiotic relation described above might result in the enhancement of any Type E toxin that might be present. It is also possible that the reverse situation might occur; that is, the symbiotic growth of other microorganisms in the presence of Type E spores might inhibit the germination, growth, and toxin synthesis by this organism. A few of the strains isolated from "izushi" by Sakaguchi and Tohyama (1954) adversely affected toxin synthesis of the Type E strain. Preformed toxin in the presence of C. sporogenes has been shown to be destroyed (Jordan and Dack, 1924 and Dack, 1926). A third possibility in this connection should not be overlooked: it would seem possible that native trypsin or other proteolytic enzymes present in foods might affect Type E toxin formed in the food. The question remains, would this effect be to enhance or to destroy the toxin?

Type E strains are reported to be quite unstable, i.e., they readily transform from toxigenic to non-toxigenic types (Dolman, 1957b). The isolation of atoxic strains of Type E from natural habitats has been reported (Nakamura et al., 1956 and Dolman, 1957a). Such instability is very disconcerting when studying these bacteria in the laboratory. The mechanism and conditions producing the instability have not been clearly defined. It is even possible that radiation, acting as a mutagenic agent, could increase the interconversions between toxic and atoxic phases. Altogether, it is evident that there is a great deal of importance that needs to be learned about botulism, and about Type E botulism in particular, when irradiation pasteurization of fish and fish products is contemplated.

EXPERIMENTAL METHODS

It is apparent from the discussion of the physiological characteristics of C. botulinum Type E that much more information is needed to evaluate confidently food preservation processes where these organisms may be involved. Our broad objectives therefore were to gather information on the germination and outgrowth of Type E spores under optimal conditions and in fish and fish products in the temperature range of 34° to 85°F. Concomitantly, the rate and extent of toxin synthesis should be considered under the same conditions. The heat and radiation resistance and factors affecting the resistance of the spores need study to ascertain the effect of irradiation and heat on the subsequent germination and outgrowth of the spores. Particular emphasis was placed on C. botulinum Type E spores, but Types A and B spores were used where applicable.

STOCK CULTURES

Orginally six strains of C. botulinum Type E were used in this study. The strains represent certain differences in physiological characteristics and geographical distribution. The following strains were obtained from Dr. L. E. Dolman, University of British Columbia; Nanaimo (NA), Vancouver herring (VH), Beluga (Bl), Iwanai (IW), E-74, and Forks (F). The cultures were received in a lyophilized condition in small vials. The vials were asceptically opened and a small quantity of sterile broth added. Transfers were made into fresh beef-extract media. The cultures were incubated at 33°C until sporulation was evident, at which time aliquots were transferred to two ml ampoules and frozen to -72°C. These stock cultures were maintained at -30°C.

The Types A and B cultures used were those that have been maintained in our laboratory for the past several years. Strains 62-A and 213-B were originally obtained from Dr. K. F. Meyer at the Hooper Foundation for Medical Research. The Types A and B cultures have been carried in cooked-meat media and stored at 2°C; they have maintained their sporogenic and toxigenic properties for years.

During the course of this study three outbreaks of botulism occurred in which fish were involved and Type E was the organism isolated. Isolates of these organisms were obtained and some of their characteristics studied. The Type E isolates obtained were the "Eloise" (El) isolated by Dr. D. M. Rosebaum (Wayne County Hospital, Eloise, Michigan) from the can which contained the tuna responsible for the outbreak in Detroit, Michigan, in March, 1963. Two isolates designated as Smoked Chub Detroit (SCD) and Smoked Chub Tennessee (SCT) were isolated from the smoked chubs involved in the Tennessee, Kentucky, and

Alabama outbreak. The SCD was obtained from Ralph Johnston of Food and Drug Laboratories, Detroit, Michigan. The SCT and the Minneapolis strain were obtained from K. A. Kautter of Food and Drug Laboratories in Washington, D.C. The Kalamazoo isolate was obtained from remnants of the smoked whitefish involved in the outbreak which caused two deaths in Kalamazoo, Michigan, in September, 1963.

The procedure used for handling Type E cultures was to transfer an inoculum from the stock culture into a fresh tube of the medium under consideration. To minimize the possibility that the culture might revert to a non-toxic phase no more than three transfers were made from the original stock culture. The Types A and B cultures had previously been tested with homologous antitoxin and were shown to be type specific. The newly received cultures were checked with Proton, Type E antitoxin, obtained from M. A. Cardella at Fort Detrick. For this purpose, an aliquot from the supernatant of a beef-extract culture was diluted with gelatin-phosphate buffer and part of this was combined with antitoxin; 0.5 ml amounts were injected to 15-20 gram mice. At least 200 MLD of toxin per ml for mice were developed by the various strains.

The ability of the various strains to ferment carbohydrates was also determined. Acid and gas was formed by all strains from glucose, maltose, sucrose, fructose, sorbitiol, and trehalose. Fermentation of adonitol was variable.

GROWTH AND SPORULATION

Before we can study such subjects as the resistance, germination conditions, culture development, etc., of C. botulinum, it is necessary to have large stock supplies of Types A, B, and E spores. It is desirable that these spores have maximum resistance to irradiation and heat and that they be stable during storage and use.

Although Type E strains sporulate in various media, their exact sporulation requirements have not been determined. Media consisting of meat extracts and meat particles have been used for sporulation. However, the presence of meat particles makes it difficult to separate the spores and obtain a clean spore suspension. Schmidt et al. (1962) used a 5% trypticase, 0.5% peptone, and 0.2-0.4% glucose medium in their studies on the radiation resistance of Type E spores. They reported adequate yields of spores in this medium. However, they found that the spores germinated and lysed after reaching a peak refractile state. This can also occur in the manipulation and harvesting procedures. It would be advantageous to develop a medium or procedure in which this lysis does not occur.

A brief survey was made of the growth and sporulation characteristics of the five Type E strains in media commonly used for growing anaerobes. The following media were reviewed: brain-heart infusion, papain digest of fresh beef, beef-heart digest, pea-peptone infusion, liver-extract broth, and thioglycollate broth. All these media contained added glucose. Sporulation was followed in the cultures incubated at 30°C by microscopic observation of the growths, using aqueous methylene blue or crystal violet stain. The ratio of the number of spores to vegetive cells was noted; in some cases, part of the culture was centrifuged to estimate the total yield of spores. Good growth and sporulation occurred in all media except that two strains did not grow in thioglycollate broth, and one strain, Forks, grew much better in the brain-heart infusion broth.

Growth of Type E strains is characterized by vigorous gas production within 12 hours, which continues for two or three days with increasing turbidity, followed by decreasing gas production. The spores then settle and the medium becomes clear again in four to seven days. Examination of the sediment shows a few scattered, gram-variable, pleomorphic cells together with free, refractile spores, germinated spores, and much cellular debris.

The ability of the cultures to develop spores was determined in the medium used by Schmidt (1962a), which consisted of 5% trypticase, 0.5% Bacto-peptone, 0.1% sodium-thioglycollate, and 0.5% glucose. The last two ingredients were added asceptically. The medium was used in 150 ml prescription bottles. To initiate growth, one ml of a spore inoculum containing 10^6 spores per ml was added. These spores were heat-shocked at 60-65°C for 10 minutes before being used as an inoculum. Good growth usually developed within 24 hours of incubation at 30°C; sporulation occurred by 48 hours. At this time, 10^7 to 10^8 viable spores were present in the culture media. This was determined by counting the number of colonies developing in pork-extract media, using Prickett tubes and suitable dilutions.

When the 0.5% Bacto-peptone was omitted from the medium, only a slight growth occurred with no gas production using strains E-74 and VH; after an initial lag the other four strains reached maximal growth in 48 hours. Substitution of 0.5% of the following ingredients for the Bacto-peptone was tested: beef extract, yeast extract, phytone, neo-peptone, casamino acids, and thiotone. Yeast extract stimulated the Iwanai, E-74, Nanaimo, and Forks strains; beef extract stimulated the E-74 and VH strains. The animal peptones were more stimulatory than the plant peptones. Glucose was found to be necessary for growth of all strains, although concentrations in excess of 0.8% inhibited sporulation. No combination of ingredients was found that would prevent the lysis of the spores that was previously mentioned. The stimulatory effect of the above ingredients was not apparent when large inocula were used. It was found that the trypticase-peptone medium was suitable to produce adequate spore crops for the experiments contemplated. Furthermore, since Schmidt et al. (1962b) used this medium previously in radia-

tion resistance studies of Type E spores, we decided to use the same medium so that the results of the two laboratories could be comparable.

A tentative procedure was developed for growing the spore crops in 10-liter bottles. For this purpose, the media consisted of 4.5% trypticase and 0.5% Bacto-peptone. To this was added 0.5% glucose, which had been sterilized separately. Using a large inoculum of good growth and vigorous gas production occurred with three strains within 12 hours, incubation at 30°C. Sporulation was evident in 24 hours; however, at this time the spores were still contained within the sporangia. Further incubation resulted in recycling, but free spores developed nevertheless. Spores were harvested after incubation for three days using a Szent-Gyorgyi and Blum continuous-flow attachment to an RC2 Serval refrigerated centrifuge. The concentrated spores were resuspended in large volumes of distilled water and then recentrifuged. These spores then became the stock suspensions that were stored at 4°C in a refrigerator. Use of the continuous-flow attachment was discontinued after several tries because of leakage into the rotor making decontamination difficult. The batch method of pooling spores in several bottles grown in 200 ml of TPG broth contained in screw-cap prescription bottles was adequate.

Before use, aliquots of the spore suspensions were further washed from six to eight times. This produced suspensions that were relatively free from vegetative cells and debris.

Several difficulties were encountered in harvesting and maintaining spore suspensions. For example, during the first centrifugation from the mother liquor, the spore fraction became "sticky" and viscous. This "stickiness" disappeared upon further washing. The substance causing this "stickiness" was not determined. Another problem involved lysis of the spores in some of the suspensions stored for long periods in the refrigerator. When this occurred, swollen spores and spore "coats" were observed on stained slides viewed under the microscope. Whether actual lysis of the spores occurred, or whether the spores germinated and then lysed is not known.

Spores of C. botulinum Type 62-A were grown in five liters of liver-extract media, a method used in this laboratory for several years which yields large numbers of stable and resistent spores. Spores of Strain 213-B were grown in 10% casitone broth. Cultures of these organisms, grown at 30°C for one week, have yielded more than 10^8 viable spores per ml of media.

COUNTING MEDIA

Since part of the study was to involve enumeration of viable spores in the spore suspensions after exposure to various doses of gamma radiation and to heat, a survey of the counting media in use for enumeration of C. botulinum Type E spores was made. The difficulty with C. botulinum Type E culture is

that excessive gassing occurs making it difficult to count the colonies after 48 hours of incubation. Schmidt et al. (1962) used liver infusion agar for enumeration but they also reported excessive gassing which was partially overcome by lower incubation temperatures, which resulted in longer incubation times. Routinely we have used Yesair's pork infusion agar and Anderson's pork-pea infusion agar for the enumeration of anaerobic spores. A comparative study of the various media for the enumeration of anaerobic spores has shown that fresh meat extract media to be superior (Wheaton 1958). Excessive gassing occurred in these media. This could be minimized by the omission of the glucose.

The following media were compared:

Beef-Infusion Broth

Beef infusion*	1 liter
Proteose-peptone	1%
Di-sodium phosphate	0.2%
Sodium chloride	0.5%
Sodium thioglycollate	0.1%
Soluble starch	0.1%
Agar	15.0%

pH 7.2-7.4

*one pound of beef/liter distilled water, simmer 1/2 hour—chilled, filter.

Pork-Infusion Agar

Pork infusion	1 liter
Bacto-peptone	0.5%
Bacto-tryptone	0.16%
di-potassium phosphate	0.1%
soluble starch	0.1%
Sodium thioglycollate	0.1%

pH 7.2-7.4

Pork-Pea Extract

Pork infusion	800 ml
Fresh pea infusion	200 ml
Other ingredients as above	

Mossel's Media

Bacto-tryptone	1.5%
Bacto-yeast extract	1.0%
Bacto-agar	1.5%
Distilled water	1 liter
Iron citrate*	0.05%
Sodium sulfite*	5 ml of 10% solution

pH 7.0

*omitted

Peptone Media

Bacto-peptone	5%
Distilled water	1 liter
pH 7.2	

In the comparative experiment, spores of the Nanaimo and Forks strain were heat-shocked for 15 minutes at 65°C, diluted, and added to the tubes. The various media were added and overlayed with agar and sealed with vaspar. One set of tubes was incubated at 33°C and the other at 19°C for 30 days. Counts were made at periodic intervals. The counts obtained from duplicate tubes of duplicate dilutions are presented in Tables 2, 3, and 4.

The results of the survey of counting media showed that the beef infusion and pork infusion media to be comparable and superior to the other media tested. The beef infusion agar however, gave less gas production than the pork infusion agar. The beef infusion agar was selected for all our counting procedures for Type E spores because it was easier and cheaper to prepare than the pork infusion media. For one strain, Forks, the simple peptone media was very satisfactory. This should be studied further with more strains.

TABLE 2

COMPARISON OF COUNTS IN VARIOUS COUNTING MEDIA AT TWO INCUBATION TEMPERATURES FOR THE NANAIMO STRAIN

Time Counted	Media									
	Beef Infusion		Pork Infusion		Mossels		Pork-Pea Infusion		Pork-Pea + $NaHCO_3$	
	Number of Viable Spores x 10^6									
	10^{-5}	10^{-6}	10^{-5}	10^{-6}	10^{-5}	10^{-6}	10^{-5}	10^{-6}	10^{-5}	10^{-6}
					33°C Incubation					
24 hr	19	21	14	19	1.7	2.0	17	18 G*	14	20
48 hr	19	23	15	18	2.7	4.7	12	20 G	gas	20
5 days	17	20	14	22	3.5	4.3	gas	17	gas	gas
12 days	16	19	14	20	4.0	6.3	gas	15	gas	gas
31 days	14	21	14	20	3.9	4.7	gas	17	gas	gas
					19°C Incubation					
24 hr	TSTC**	TSTC	TSTC	TSTC	TSTC	TSTC	TSTC	TSTC	13	13
48 hr	18	17	16	15	2.5	0.7	16	24 G	16	15
5 days	18	15	17	15	1.4	6.7	gas	24 G	gas	19 G
12 days	13	13	17	16	2.9	14.0	gas	21 G	gas	13 G
31 days	F***	11	17	15	3.3	13.0	gas	18 G	gas	9 G

*G = gas

**TSTC = Too small to count

***F = fading

TABLE 3

COMPARISON OF COUNTS IN VARIOUS COUNTING MEDIA AT TWO INCUBATION TEMPERATURES FOR THE FORKS STRAIN

Time Counted	Media									
	Beef Infusion		Pork Infusion		Mossels		Pork-Pea Infusion		Pork-Pea + $NaHCO_3$	
	Number of Viable Spores x 10^6									
	10^{-5}	10^{-6}	10^{-5}	10^{-6}	10^{-5}	10^{-6}	10^{-5}	10^{-6}	10^{-5}	10^{-6}
					33°C Incubation					
24 hr	13	14	12	15	4.5	6.7	13	23	12	13
48 hr	13	14	12	14	4.7	10.0	15	20	12	15
5 days	13	13	12	13	5.0	7.3	15	20	18	20
12 days	10	13	11	13	5.0	9.0	14	21	13	19
31 days	11	12	12	14	4.0	5.0	15	17	12	18
					19°C Incubation					
24 hr	13	9	0	0	0.2	0	12	13	13	10
48 hr	14	21	13	15	4.8	3	13	12	13	14
5 days	14	19	13	14	10.0	6	13	13	13	14
12 days	14	20	13	14	9.5	6	14	13	12	16
31 days	14	18	12	15	10.0	7	14	13	12	15

TABLE 4

COMPARISON OF COUNTS IN VARIOUS COUNTING MEDIA
FOR FORKS AND NANAIMO STRAINS

Time Counted	Number of Viable Spores x 10^6			
	Forks		Nanaimo	
	Beef Infusion	Peptone Agar	Beef Infusion	Peptone Agar
		33°C Incubation		
64 hr	38	111	80	68
5 days	37	104	81	72
9 days	41	100	81	69
14 days	37	102	77	72
23 days	36	100	76	68
28 days	35	95	83	64

EFFECTS OF MEDIA VARIATION ON TOXIN PRODUCTION

Because of the reported variation in toxin production by C. botulinum Type E strains, a comparison was made of the toxin producing ability of the strains used in our studies. Several laboratory media as well as represented seafoods were included in the comparison. The effect of carbohydrate and pH was also checked. The three media selected were beef infusion broth, trypticase peptone media and the media used by Duff (1956) in his comparison of toxin production by various Type E strains. The extent of toxin production in 200 ml of media was checked after incubation at 33°C for seven days. As can be seen from Table 5, the amount of toxin produced by the six strains in the three different media was not consistent. Wide variation in toxin titres can be expected, depending upon the strain of organism and medium used.

TABLE 5

TOXIN PRODUCTION IN THREE MEDIA BY C. BOTULINUM TYPE E INCUBATED AT 33°C FOR 7 DAYS

Strain	MLD/ml		
Media:	D	TP	BI
Iwanai	$2\text{-}20 \times 10^2$	$2\text{-}20 \times 10^2$	200×10^2
Forks	$2\text{-}20 \times 10^2$	$20\text{-}200 \times 10^2$	$.2\text{-}2 \times 10^2$
E-74	$20\text{-}200 \times 10^2$	$20\text{-}200 \times 10^2$	20×10^2
Beluga	2×10^2	$2\text{-}20 \times 10^2$	2×10^2
Nanaimo	$20\text{-}200 \times 10^2$	$200\text{-}2000 \times 10^2$	---
Vancouver Herrring	$2\text{-}20 \times 10^2$	200×10^2	20×10^2

D = Proteose Peptone - YE - 1% Dextrin
TP = Trypticase-Peptone - 1% Dextrose
BI = Beef infusion - 1% Dextrose

EFFECT OF pH ON GROWTH AND TOXIN PRODUCTION

The extent of toxin production by strain Iwanai in trypticase peptone media containing glucose was tested at pH 6.58, 6.9, 7.0, and 7.51. Growth after 72 hours of incubation did not occur at pH 4.98. As determined turbidimetrically, the greatest growth occurred at pH 7.8. In this narrow pH range no differences in toxin titres were found except at pH 7.51, where greater growth and gas production were observed (see Table 6).

TABLE 6

TOXIN PRODUCTION BY C. BOTULINUM TYPE E (IWANAI)
INCUBATED IN TRYPTICASE PEPTONE BROTH AT 33°C
FOR 72 HOURS AT VARIOUS pH LEVELS

pH	MLD/Toxin
6.88	200-2000
6.90	200-2000
7.00	2000
7.51	2000-20,000

SEAFOOD SUBSTRATES

No difficulty was experienced in initiating growth from spore inoculum of the various strains in clam juice, shrimp,and haddock homogenates, whether heated or sterilized by irradiation. The studies were extended to test what effect the various seafoods would have on toxin production by the several Type E strains used in our studies.

A question arises whether the potency of Type E toxin produced in seafood can be affected by the endogenous, proteolytic enzymes present in the food as has been reported to occur with trypsin and proteolytic enzymes coming from other sources such as bacteria. Irradiation offers a method for separating these proteolytic effects, since irradiation doses used for sterilization are not sufficient for the inactivation of tissue enzymes, but will prevent the bacteria from proliferating. This concept was used in the experiment described next.

To compare toxin production in raw and cooked seafoods, 300 ml of shucked clams were sterilized in their juice by radiation; another set was heat sterilized. The bottles were inoculated with approximately 10^6 heat-shock spores of the Iwanai and Forks strain and then incubated at 33°C for two days and 20°C for five days. For comparison of toxin titres a bottle of TPG media was similarly treated. Growth and vigorous gas production occurred in all bottles. Toxin was produced in the clams by both strains whether treated by heat or radiation with a titre between 200-2000 MLD for mice. The supernatant liquids in both the irradiated and heat-sterilized samples which were inoculated with the Iwanai strain were further tested after activation by trypsin. The results are summarized in Table 7. For comparison, toxin production in TPG is included.

TABLE 7

PRODUCTION OF TOXIN IN SHUCKED CLAMS
BY C. BOTULINUM TYPE E (IWANAI)

Sample	MLD Toxin
Irradiated plus Trypsin*	2000-20,000
Heated plus Trypsin	720,000
TPG	200-2000
TPG plus Enz	200-2000

*.1% Trypsin, 60 min, 35°C, pH 6.0.

Under conditions of this experiment toxin titres in the radiation sterilized clams was lower than the heated sample. There was no activation of the toxin by the tryptic treatment either in the clam supernatant or the TPG media.

Since the results of this experiment indicated that variable results can occur between heat and radiation sterilized substrates, a similar comparison was made between toxin production in haddock homogenates, which were not sterilized, and in homogenates which were sterilized by radiation and by heat. After sterilization the tubes of haddock homogenates were inoculated with approximately 10^6 spores per ml, incubated at 33°C, and then tested for toxin. Table 8 summarizes the results of this experiment. Significantly, no toxin was detected in the untreated sample, which was contaminated with other organisms.

TABLE 8

PRODUCTION OF TOXIN IN HADDOCK HOMOGENATE
INOCULATED WITH SPORES OF C. BOTULINUM TYPE E (IWANAI)
AND INCUBATED AT 33°C FOR 48 HOURS

Sample Treatment	MLD Toxin
None	0
Heated	20-200
Irradiated	20-200
Irradiated and Heated	2000

In another experiment the toxin producing ability of the organism was tested in haddock homogenates at incubation temperature of 33°C and in oysters after they had been heat or radiation sterilized. Tables 9 and 10 summarize the results of these experiments. No toxin was detected in the irradiation sterilized oyster juice in tubes which had been inoculated with spores of three different strains of Type E. However, toxin was produced in the oyster juice when heated after irradiation. A heat sensitive factor which is present in oysters but not in haddock homogenates has a deleterious effect on toxin production.

TABLE 9

TOXIN PRODUCTION BY C. BOTULINUM TYPE E IN HADDOCK HOMOGENATE AND OYSTER JUICE STERILIZED WITH GAMMA RADIATION AND INCUBATED AT 33°C FOR 5 DAYS

Strain	MLD Toxin	
	Haddock	Oysters
Vancouver Herring	>20	0
Forks	>20	0

TABLE 10

TOXIN PRODUCTION BY C. BOTULINUM TYPE E (IWANAI) IN HADDOCK HOMOGENATE AND OYSTER JUICE INOCULATED WITH C. BOTULINUM TYPE E SPORES AND INCUBATED AT 20°C FOR 7 DAYS

Sample	MLD Toxin
Haddock Homogenate	
Irradiated and Heated	2000
Irradiated	2000-5000
Oysters	
Irradiated	0
Irradiated and Heated	20,000

Because of the peculiar results on toxin production obtained with the various strains in radiation sterilization, further studies with another strain of Type E and in more detail were performed to further confirm the phenomena.

In this experiment, a fresh shipment of oysters in the shell were obtained and shucked in the laboratory. They were blended slightly to break them up in small pieces and then dispensed in large screw cap tubes along with the juice. The blend had a pH of 6.0. One batch of tubes received approximately 2.5 megarads. One part of the irradiated tubes received a heat treatment of five minutes at 121°C. Several tubes did not receive any treatment, i.e., to act as control or raw, all tubes which received the various treatments were inoculated with 10,000 heat shocked spores of the Nanaimo strain. For comparative purposes, a set of tubes containing TPG medium was also inoculated. The tubes were incubated at 33°C, 19°C, and 8°C. Table 11 summarizes the growth observed as evidence by gas production at the various temperatures.

TABLE 11

GROWTH OF C. BOTULINUM TYPE E IN OYSTERS AT VARIOUS TEMPERATURES

Temperature, °C	Sample	Growth, Gas Production
33	Raw oysters	+4 48 hr
	Irradiated and Heated	+4 48 hr
	Irradiated	+4 48 hr
	TPG	+4 48 hr
19	Raw oysters	+4 48 hr
	Irradiated and Heated	+4 48 hr
	Irradiated	+2 72 hr
8	Raw oysters	within 2 wk
	Irradiated and Heated	within 2 wk
	Irradiated	+3 48 hr

A preliminary check for toxin in the cultures was made by centrifuging the cultures and injecting 0.5 ml into mice. Table 12.

TABLE 12

TOXIN PRODUCTION IN OYSTERS INOCULATED WITH SPORES OF C. BOTULINUM TYPE E

Sample	Deaths
33°C 48 hr	
Raw oysters	0/2
Irradiated	0/2
Irradiated and Heated	2/2
TPG broth	2/2
20°C 48 hr	
Irradiated	0/2

The same samples which had been previously checked for presence of toxin were tested further for titre of toxin and possible activation of the pro-toxin by trypsin. The supernatant fluid was treated with 0.1% trypsin at 37°C for one hour at pH 5.8 - 6.0. Also, the TPG broth culture was similarly treated. Table 13.

The samples which had been incubated at 20°C for eight days were similarly checked for toxin. The results of the preliminary titration are presented in Table 14 and the determination of toxin titres with and without activation by trypsin of the same samples are presented in Table 15.

The cultures incubated at 8°C for 19 days were also checked for presence of toxin. However, toxin titres were not determined in this sample (Table 16).

The results of this series of experiments support our previous results which showed that toxin production did not occur in radiation sterilized oysters, whereas in the irradiated oysters which were subsequently heated, toxin production occurred very readily. Two exceptions were noted. At the incubation temperature of 33°C for 48 hours, toxin was detected in the irradiated sterilized oysters after treatment with trypsin; however, the titre was low as indicated by the length of time for the animals to die and none was present in the non-trypsinized sample. In no other sample at the other incubation temperatures was toxin demonstrated with or without treatment

TABLE 13

TOXIN PRODUCTION IN OYSTERS BY C. BOTULINUM TYPE E
INCUBATED AT 33°C FOR 48 HOURS

Sample	Dilution (0.5 ml injected)	Deaths	Time, hr
Raw and trypsin	0	0/2	
Irradiated and trypsin	0	2/2	within 18
Irradiated, heat, and trypsin	0	2/2	2
	1/10	2/2	2
	1/100	2/2	2
	1/1000	2/2	18
Irradiated and heat, no trypsin	1/10	2/2	18
	1/100	0/2	
TPG media, no trypsin	1/10	2/2	2
	1/100	2/2	2
	1/1000	2/2	18
TGP media and trypsin	1/10	2/2	18
	1/100	2/2	18
	1/1000	0/2	

TABLE 14

TOXIN PRODUCTION IN OYSTERS BY C. BOTULINUM TYPE E INCUBATED AT 20°C FOR 8 DAYS

Sample	Dilution	Deaths	Time, hr
Raw	0	2/2	4
Irradiated	0	0/2	
Irradiated and Heat	0	2/2	4
	1/10	3/3	4
	1/100	2/3	4
Raw and trypsin	0	0/2	
Irradiated and trypsin	0	0/2	
Irradiated, heat, and trypsin	0	2/2	4

TABLE 15

TOXIN PRODUCTION IN OYSTERS BY C. BOTULINUM TYPE E INCUBATED AT 20°C FOR 8 DAYS

Sample	Dilution	Deaths
Irradiated and heat	0	2/2
	1/10	3/3
	1/100	3/3
	1/1000	0/3
	1/10,000	0/3
Irradiated, heat, and trypsin	0	1/1
	1/10	3/3
	1/100	3/3
	1/1000	3/3
Raw, no trypsin	0	0/2
	1/100	0/2
	1/1000	0/2

TABLE 16

TOXIN PRODUCTION IN OYSTERS INOCULATED WITH C. BOTULINUM TYPE E SPORES AND INCUBATED AT 8°C FOR 19 DAYS

Sample	Dilution	Deaths
Raw	0	0/2
Raw and trypsin	0	0/2
Irradiated	0	0/2
Irradiated and trypsin	0	0/2
Irradiated and heat	0	2/2
Irradiated, heat, and trypsin	0	2/2

with trypsin, in oysters sterilized by radiation. The other exception, toxin was detected in the raw sample incubated at 20°C for eight days. However, further mouse inoculations with the same sample treated and not treated with trypsin did not result in deaths.

A hundred-fold activation of the toxin occurred by trypsin treatment in the irradiated sterilized and heated oysters. Significantly in the TPG cultures incubated at 33°C for 48 hours which contained a slightly greater amount of toxin before activation in comparison to the oyster cultures, treatment with trypsin resulted in a lower titre, indicating toxin destruction.

HEAT RESISTANCE STUDIES OF C. BOTULINUM TYPE E SPORES

In order to more fully characterize the spores of the various strains used in our investigation the heat resistance of the several strains were compared in phosphate buffer. The possible heat activation of any dormant spores which may be present in the spore suspensions was also studied.

Gunnison, Cummings, and Meyer (1936) checked the heat resistance of the Russian strains they isolated. They observed that 5 x 10^6/cc and 50 x 10^6/cc spores in phosphate buffer were inactivated in 2 minutes at 100°C, 6 minutes at 80°C and 5 minutes at 100°C and 40 minutes at 80°C respectively. Hazen (1937) observed that the thermal resistance of the salmon and sprat strains was low, the spores being inactivated at 80°C in 10 minutes. Nakamura et al. (1956) observed that 10^4 spores/cc of 7 strains were destroyed within 10 minutes at 80°C, one strain survived 3 minutes of heating at 90°C. Ohye and Scott (1957) studied the heat resistance of two type E strains at temperatures of 70, 75 and, 80°C. Decimal reduction times for one strain was 36, 10, 3 minutes, respectively, at these temperatures. For the other strain the equivalent times were 7.8, 3.1, and 0.4 minutes.

In a tunafish substrate it was observed that less than a 3-second exposure at 105°C reduced the number of spores by 90% (work of Stumto quoted by Dolman (1957). Dolman also mentions that some spores of type E could withstand a temperature of 100°C for 1/2 hour in beef infusion broth, others were killed in 5 minutes.

EXPERIMENTAL

The procedures for production of spores and counting are described in the section on experimental methods.

Two methods of heating the spore suspensions were employed in these studies. In one case 5 milliliters of the spore suspensions were heated in individual 16 x 150 mm test tubes. These tubes were immersed to approximately to 1/3 of the top of the tube. In the other case, 4 ml of the spore suspension was dispensed in 5 ml neutral glass vials (Kimble), sealed with oxygen flame and were heated while completely emersed in the bath. In each case individual tubes were removed at the desired time intervals for plating. The individual tubes were heated in a bath maintained at $\pm$ 0.3°C.

The open tube method is frequently used in the determination of the thermal destruction of bacteria mostly because of convenience. It is useful in obtaining comparative heat resistance data on the thermal resistance of

several organisms. However, the technique is open to question when studying the absolute thermal resistance of the organisms. It is possible that some spores may escape the heat treatment while adhering to the sides of the tube which is not immersed in the bath. Therefore the few surviving spores which one recovers may not have received the full heat treatment.

In our first experiments the open tube technique of heating was employed, since our interest was to obtain a comparison of the heat resistance of the spores used in our studies. During the course of the study the question of the absolute resistance of Type E spores arose and some of the results with the open tube technique were questionable especially at higher temperatures of heating so the sealed ampoules were employed.

For the heat studies 1 ml of the concentrated stock spore suspension was diluted 1:100 with M/15 PO_4 buffer of pH 7.0 to give a final concentration of approximately 10^6- 10^7 spores/ml. The spores were heated in the temperature range of 65-90°C for various periods of time.

The results of the various experiments are presented as log % of survivors versus heating time in minutes. The time to come up to temperature in the sealed vials were determined. The effective heating time is used for these experiments. The results of the open tube experiments are presented first. In this case the results are expressed as the actual heating time for comparative purposes.

In all cases non-heat shocked spores were used in order to determine if the heat treatment at the various temperatures would increase the count, indicating any heat activation of the spores.

RESULTS

In the first experiment a comparison was made between the open tube and sealed ampoule technique of heating. In this case spores of the Forks strain were heated at 75°C for various periods of time up to 60 minutes. The data as expressed in Table 17 and plotted on Fig. 1 show that in this case the method of heating gave comparable results.

The data for the heat resistance of the spores of Eloise, Nanaimo, and Forks strains heated at temperatures between 60°-90°C for periods up to 90 minutes in open tubes is presented in Tables 18 and 19 and plotted in Figs. 2, 3, and 4. There is slight killing at 60° and 65°C. The spores used in these experiments were not previously heated so some of the inactivation observed at these temperatures may be due to the inactivation of vegetative cells and germinated spores present in the suspensions. However, the counts obtained at these temperatures indicate very few heat sensitive cells in the spore suspension used in these studies. At temperatures of 70°, 75°, and 85°C

TABLE 17

COMPARISON OF TWO METHODS OF HEATING ON
THE NUMBER OF SURVIVING SPORES

Actual Time Minutes	Strain—Forks			
	Sealed Ampoules		Open Tubes	
	c/ml	% Survivors	c/ml	% Survivors
0	5.8×10^{8}	100.0	3.1×10^{7}	100.0
5	3.7×10^{5}	.064	3.2×10^{4}	.103
10	2.4×10^{4}	.0041	7.3×10^{2}	.00235
15	7.3×10^{3}	.0013	---	---
20	3.6×10^{3}	.00062	9.7×10^{1}	.000313
25	2.4×10^{3}	.00041	---	---
30	2.1×10^{3}	.00036	1.7×10^{1}	.000548
40	7.2×10^{2}	.00012	4.3×10^{1}	.000139
60	4.5×10^{2}	.000078	2.4×10^{1}	.0000774

there is a rapid initial kill of the majority of the spores in the population within 15 minutes, with a small fraction of spores surviving heat treatment for a period up to 2 hours at 85°C. Spores of the Nanaimo strain heated at 70°C for 15 minutes showed no inactivation, indicating that some activation of the more heat resistant spores in the suspension may be occurring. This is more apparant in the heat studies performed in the sealed ampoules which will be presented later. Also, in the case of the spores of the Forks strain, heating at 70°C for 20 minutes gave a slight increase in count indicating some activation may be occurring, although the data are not sufficient to be dogmatic. Apparently, a small number of the spores in the suspension case resist heating at a temperature of 80°-85°C for long period of time. These spores are sensitive when heated at a temperature of 90°C. With the spores of the Eloise strain the results are similar in respect to resistance as those obtained with the Forks and Nanaimo strain. However, the inactivation curves are more uniform indicating a spore suspension with a more homogenous heat resistance. The approximate decimal reduction times at 70°, 75°, 85°, and 90°C is 10, 3, 2, and 1 minute for the heat sensitive portion of the spore population of the Eloise strain. These values are intermediate to those obtained by Ohye and Scott (1958).

The spores of the Smoked Chub Detroit strain were heated at 65°, 75°, 85°C, and 90°C for periods up to 90 minutes. Table 20 and Fig. 5. The pattern of inactivation is similar to that of the previous strains. A rapid initial inactivation of the greater number of the spore population within 10 minutes of heating at the various temperatures, with a tailing

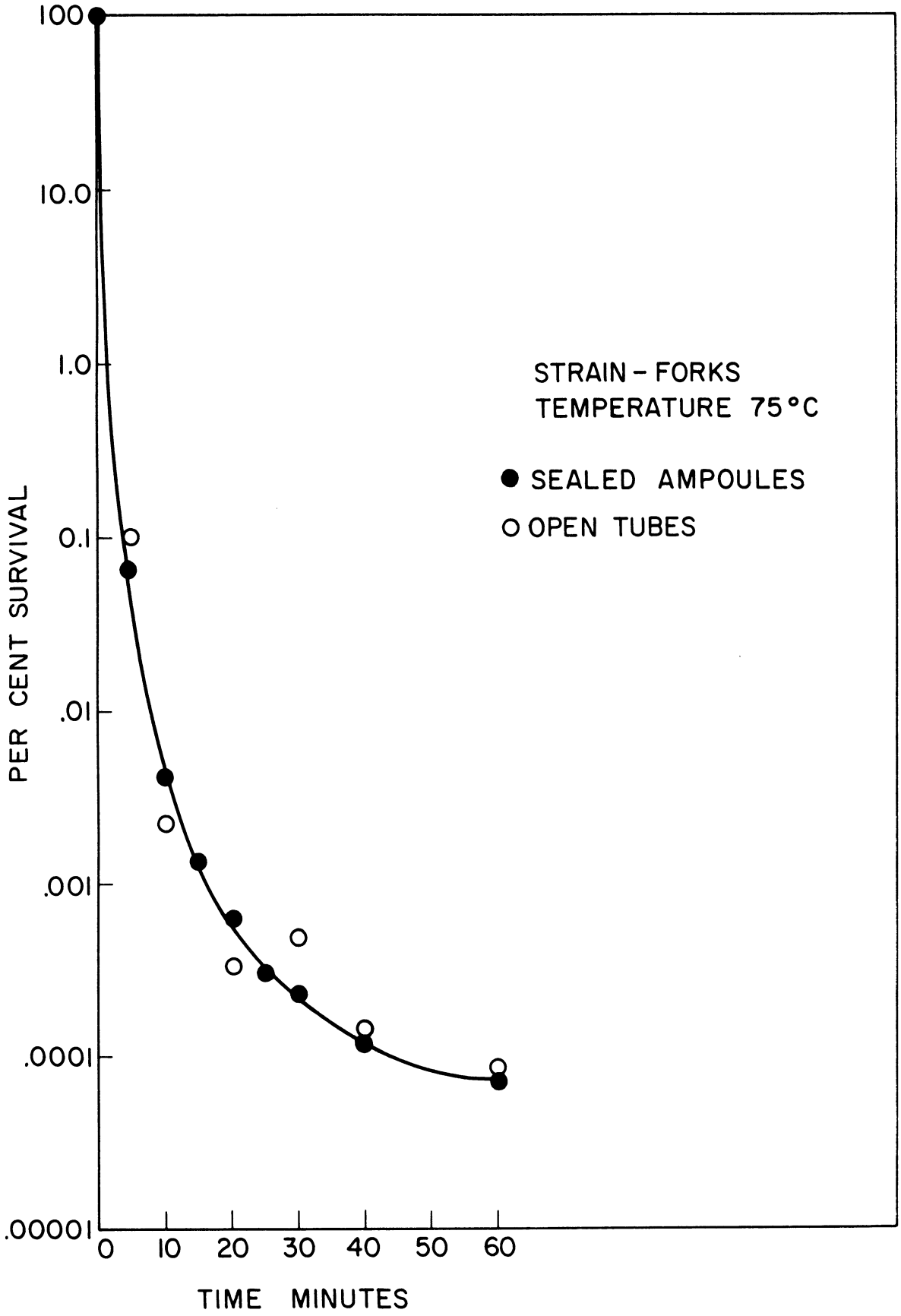

Fig. 1. Comparison of two methods of heating on the number of surviving spores.

TABLE 18

HEAT RESISTANCE OF C. BOTULINUM TYPE E SPORES
IN M/15 PHOSPHATE BUFFER pH 7.0

Temp, °C	Actual Time Minutes	Strain			
		Eloise		Forks	
		c/ml	% Survivors	c/ml	% Survivors
60	0	3.6×10^6	100.0	3.1×10^6	100.0
	15	1.9×10^6	53.0	3.3×10^6	106.0
	30	1.3×10^6	36.0	1.9×10^6	61.0
	60	1.0×10^6	28.0	2.0×10^6	64.0
65	0	3.4×10^6	100.0	3.4×10^5	100.0
	15	1.2×10^6	34.0	2.4×10^5	71.0
	30	1.3×10^6	20.0	1.3×10^5	38.0
	60	2.0×10^5	10.0	2.0×10^5	5.9
70	0	3.4×10^6	100.0	2.8×10^5	100.0
	20	6.1×10^4	1.8	1.1×10^2	.039
	30	4.6×10^3	.14	1.6×10^2	.057
	40	2.3×10^3	.068	1.8×10^2	.064
	60	4.4×10^2	.013	1.0×10^2	.036
	90	39	.0011	87	.0031
75	0	3.4×10^5	100.0	3.4×10^6	100.0
	15	1.0×10^2	.029	1.3×10^3	.0391
	30	4.4×10^2	.0129	1.1×10^2	.0032
	60	1.0×10^2	.0029	48	.0014
85	0	2.4×10^6	100.0	4.0×10^6	100.0
	15	250	.0104	363	.0091
	30	19	.0008	36	.009
	60	4	.00017	49	.0012
90	0	2.3×10^6	100.0	2.7×10^6	
	15	54	.002	130	.005
	30	24	.001	15	.0005
	60	.3	.00001	61.3	.00005

TABLE 19

HEAT RESISTANCE OF C. BOTULINUM TYPE E SPORES
IN M/15 PHOSPHATE BUFFER pH 7.0

Temp, °C	Actual Time Minutes	Strain—Nanaimo	
		c/ml	% Survivors
60	0	4.4×10^6	100.0
	15	2.4×10^6	54.0
	30	1.7×10^6	39.0
	60	1.3×10^6	30.0
65	0	4.0×10^6	100.0
	15	1.6×10^6	40.0
	30	7.0×10^5	17.5
	60	2.2×10^5	5.5
70	0	2.1×10^6	100.0
	20	2.3×10^4	110.0
	30	4.2×10^2	.02
	40	3.6×10^2	.017
	60	5.0×10^2	.024
	90	69	.003
75	0	4.0×10^5	100.0
	10	9.0×10^2	.023
	30	1.2×10^2	.03
	60	4.1×10^2	.01
85	0	4.3×10^6	100.0
	15	146	.011
	30	453	.0034
	60	84	.0019
90	0	2.0×10^6	100.0
	15	49	.0024
	30	15	.0007
	60	6	.00029

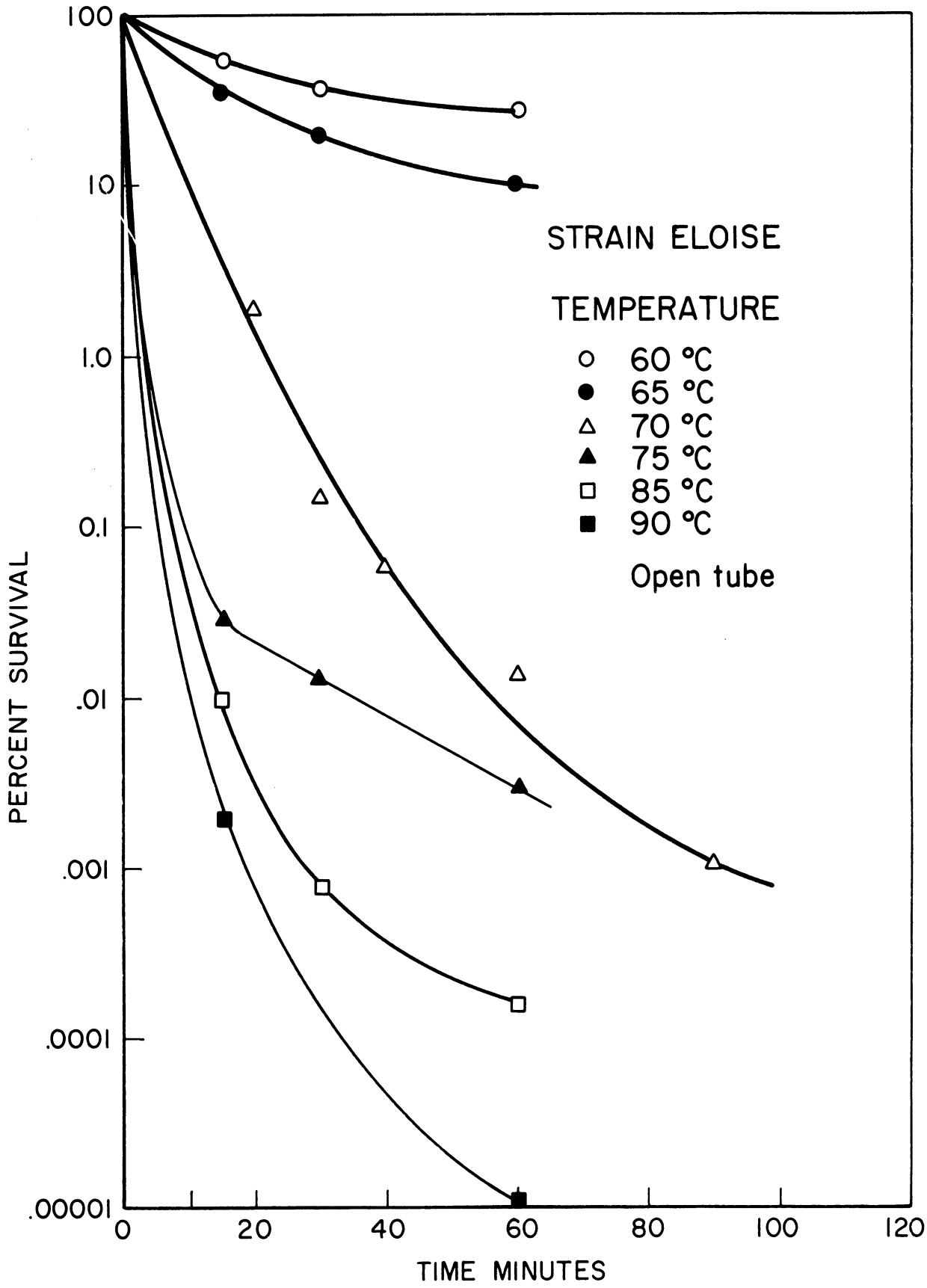

Fig. 2. Heat resistance of C. botulinum Type E spores in M/15 phosphate buffer pH 7.0.

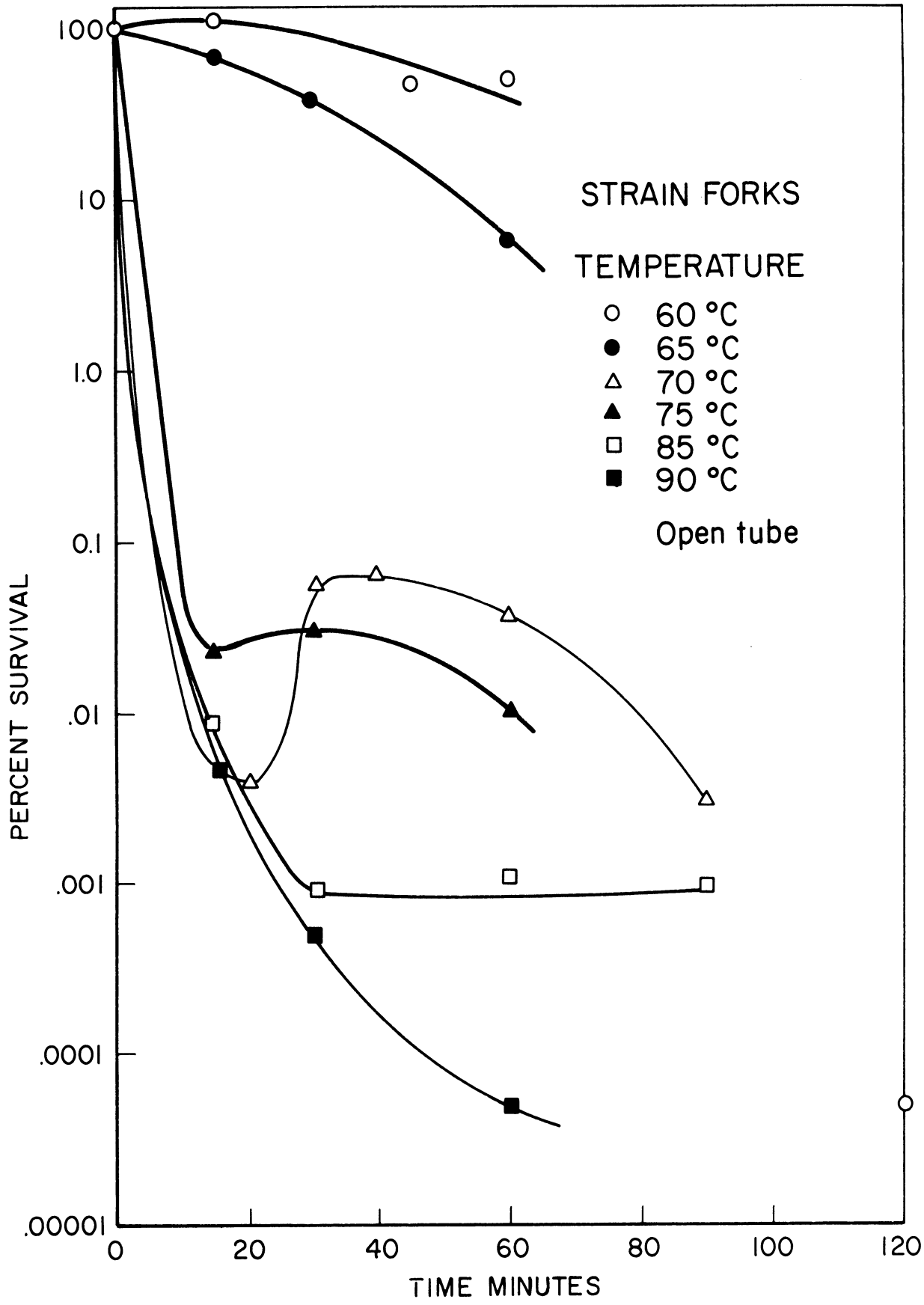

Fig. 3. Heat resistance of C. botulinum Type E spores in M/15 phosphate buffer pH 7.0.

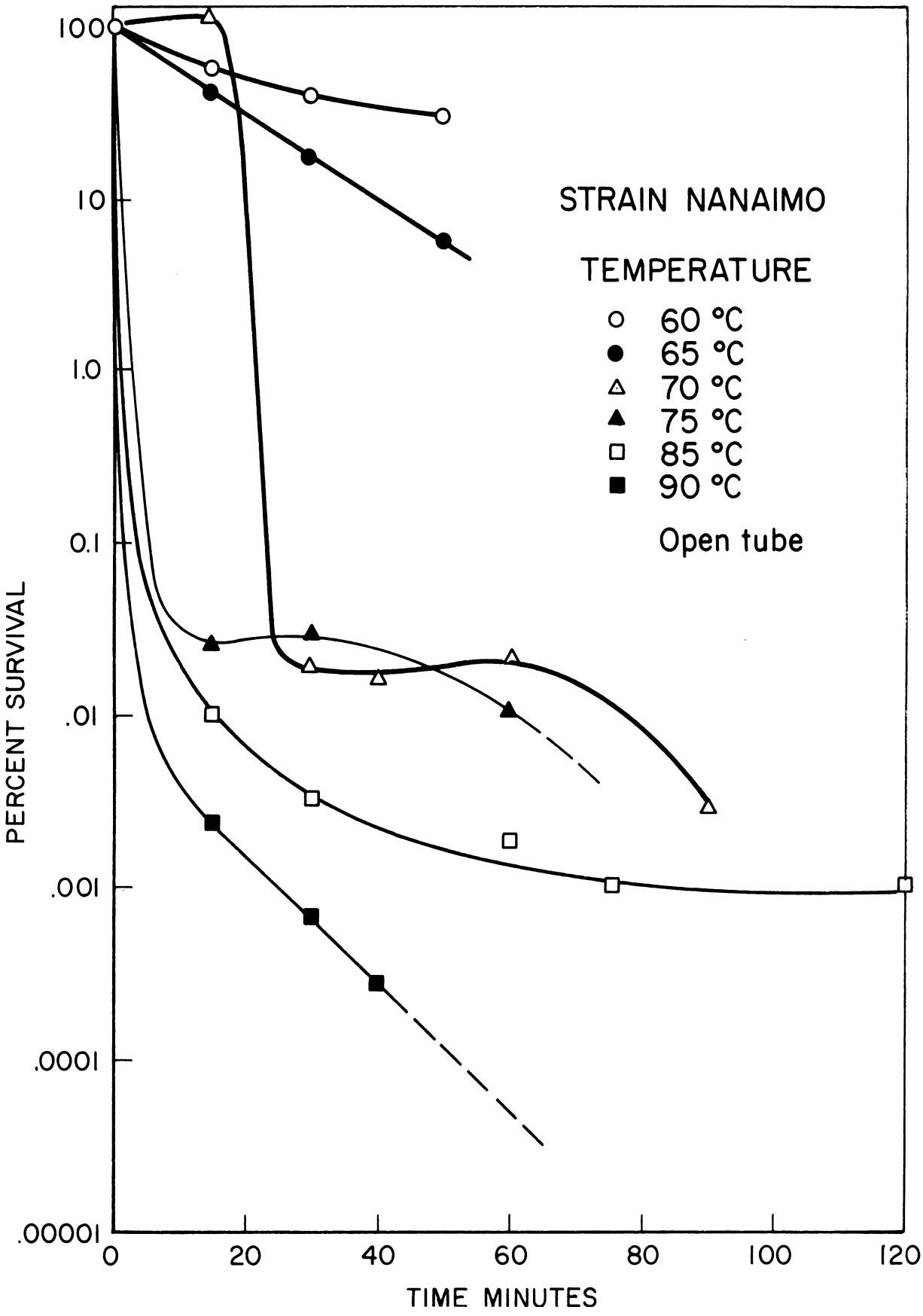

Fig. 4. Heat resistance of C. botulinum Type E spores in M/15 phosphate buffer pH 7.0.

TABLE 20

HEAT RESISTANCE OF C. BOTULINUM TYPE E SPORES
IN M/15 PHOSPHATE BUFFER pH 7.0

Temp. °C	Actual Time Minutes	Strain—Smoked Chub Detroit c/ml	% Survivors
65	0	1.0×10^6	100.0
	15	6.1×10^5	61.3
	30	8.2×10^5	81.6
	60	4.0×10^5	39.6
	120	4.8×10^5	47.6
75	0	6.7×10^5	100.0
	5	3.7×10^3	.055
	10	1.3×10^2	.019
	30	27	.0041
	60	27	.0040
	90	23	.0034
85	0	7.3×10^5	100.0
	5	1.1×10^2	.01
	10	23	.003
	30	2.0×10^2	.02
	60	7	.00089
	90	10	.0014
90	0	1.2×10^6	100.0
	10	8	.00067
	36	2	.00017
	60	1	.000083

occurring with prolonged heating at 75° and 85°C. A partial explanation for the inhomogeniety of the spore suspensions in respect to heat resistance of the Nanaimo and Forks strain is these suspensions were stored for several weeks after preparation at 0°C whereas the Eloise and Smoked Chub spore suspensions were used very soon after preparation. It has been known that aging by storage for prolonged periods of time can introduce dormancy in bacterial spores.

With the spores produced by the various other strains used in our studies, extensive heat studies at various temperatures were not performed. Spores from strain Iwanai was checked at temperatures of 65° and 85°C, Table 21 and

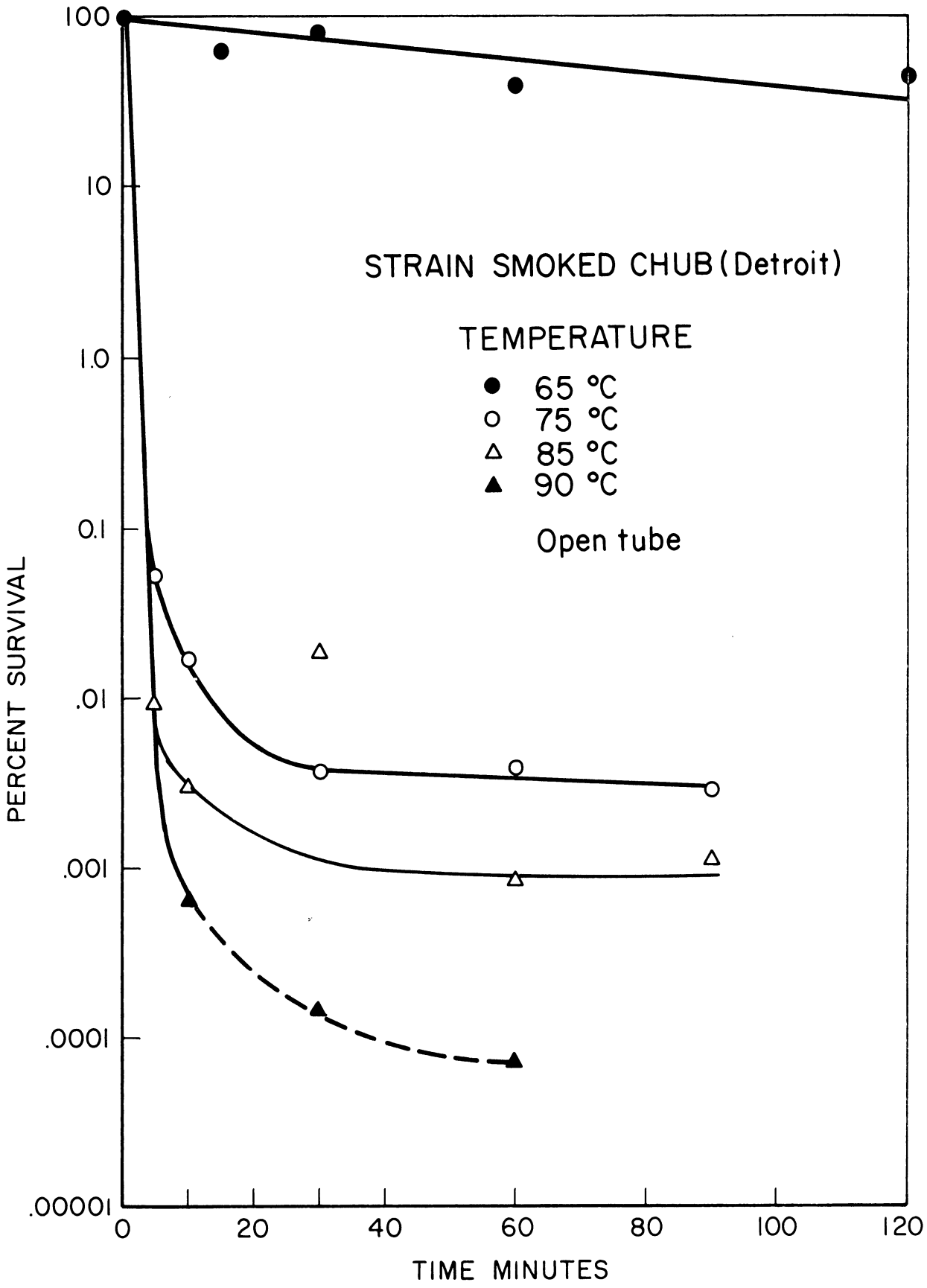

Fig. 5. Heat resistance of C. botulinum Type E spores in M/15 phosphate buffer pH 7.0.

TABLE 21

HEAT RESISTANCE OF C. BOTULINUM TYPE E SPORES
IN M/15 PHOSPHATE BUFFER pH 7.0

Temp, °C	Actual Time Minutes	Strain—Iwanai	
		c/ml	% Survivors
65	0	2.5×10^6	100.0
	15	6.8×10^5	27.0
	30	4.7×10^5	19.0
	60	2.6×10^5	10.0
85	0	2.5×10^5	100.0
	15	173	.0069
	30	37	.0015
	60	12	.00048

Fig. 6. Results are similar to those obtained with the other strains. Spores produced by strains Beluga, E-74, and Vancouver Herring strains were tested for their resistance at 85°C only. The number of spores surviving each heat treatment by the three strains is presented in Table 22. The results are not plotted. From the counts one observes that with the spores of these strains there is a small number of spores surviving even with heating up to 2 hours at 85°C.

The question arises whether the spores surviving the prolonged heat treatment at these various temperatures are able when subcultured into appropriate media to produce toxin. In order to check this possibility one ml of the spore suspensions of the various strains which survived heat treatments were subcultured into TPG broth and incubated at 33°C for 48 hours. The cells were centrifuged down and 0.5 ml of the supernatant was injected into mice to detect the presence of toxin. Table 23 gives the results of toxin production by the spores of the various strains which survived the various heat treatments. Of the eight strains tested, with one exception all the heat surviving spores when subcultured gave rise to toxin cultures. The exception observed was heating the spores of the Forks strain at 90°C for 10 and 30 minutes. It must noted that the spores of this strain heated at 75°C for one and two hours gave rise to toxic cultures. In this series of experiments all the spores were heated in open tubes. The surviving spores used for inoculum were those that gave rise to the tailing effect observed on the curves.

The question also arises on whether the spores surviving the extreme heat treatments are able when subcultured into sporulating media to produce

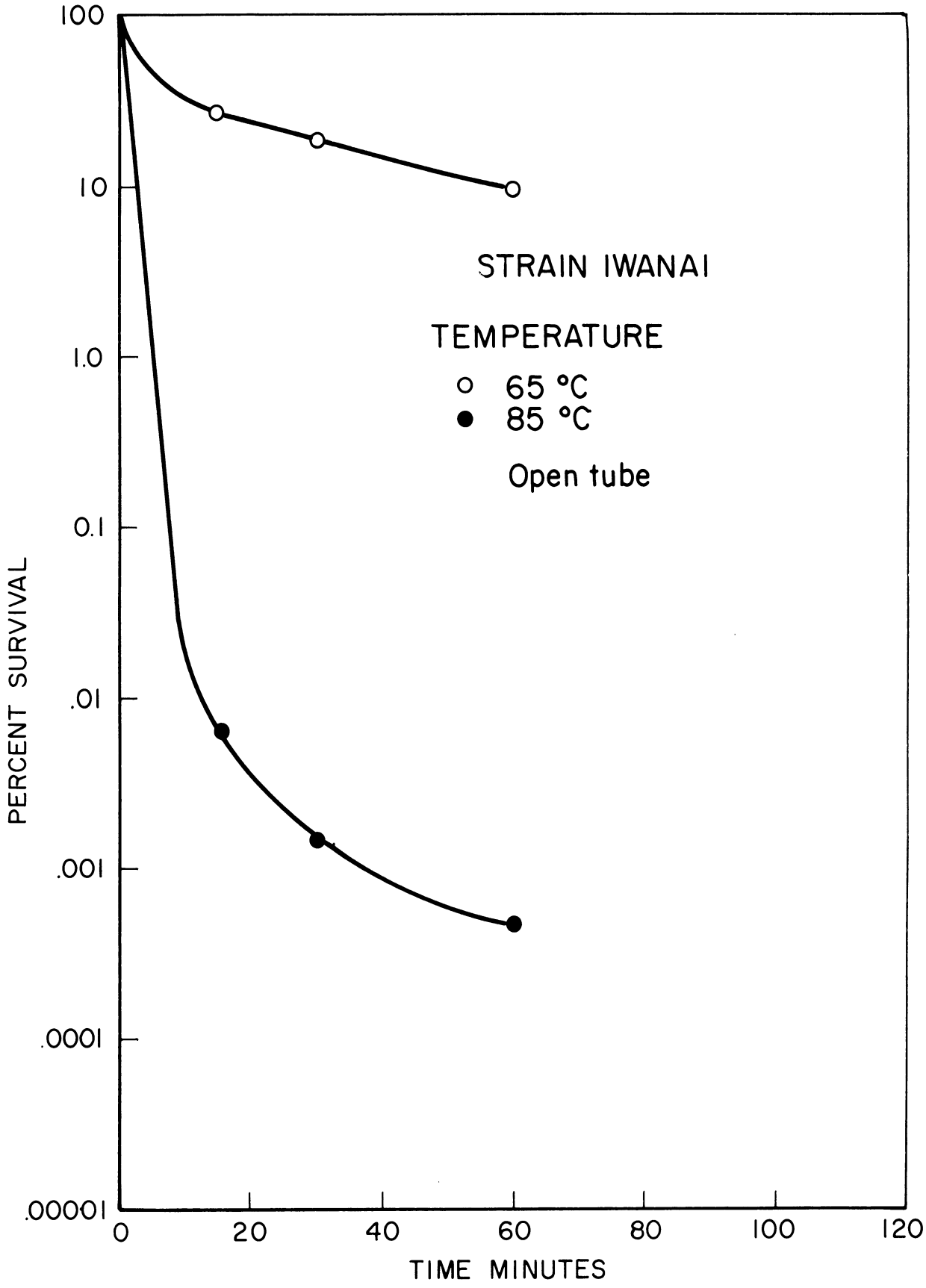

Fig. 6. Heat resistance of C. botulinum Type E spores in M/15 phosphate buffer pH 7.0.

TABLE 22

HEAT RESISTANCE OF C. BOTULINUM TYPE E SPORES
IN M/15 PHOSPHATE BUFFER pH 7.0

Strain	Effective Time Minutes	c/ml	% Survivors
		Temperature 85°C	
Beluga			
	0	3.4×10^6	100.0
	15	1.76×0^2	.0052
	30	74	.0022
	60	7	.00021
	90	19	.00056
	120	2	.000059
E-74	0	3.4×10^6	100.0
	15	1.3×10^2	.0037
	30	1.9×10^2	.0057
	60	---	---
	90	6	.00019
	120	35	.0010
Vancouver Herring	0	3.7×10^6	100.0
	15	1.6×10^2	.0042
	30	46	.0012
	60	17	.00046
	90	1	.000035
	120	12	.00032

spores of increased heat resistance. Spores of the Nanaimo, Forks, and Iwanai strains which had survived heating at 85°C for at least 30 minutes were used as inocula to produce a new spore suspension. These spores were washed, etc., as described previously. The resistance of these new spores was determined at 85°C using the open tube method. The resistance of the spores produced by the heat survivors was compared with the heat resistance obtained with the stock spore suspensions. Tables 24, 25, 26, and Figs. 7, 8, and 9 show the results of this comparison. It is obvious that the spores produced by the heat surviving spores gave rise to a spore suspension which was similar in heat resistance to the stock spore suspension.

The next series of studies on heat resistance was using sealed ampoules in order to determine whether the tailing effect observed in the open tube

TABLE 23

TOXIN PRODUCTION BY C. BOTULINUM SPORES HEATED AT VARIOUS TEMPERATURES

Strain	Heat Treatment		Deaths
	Temp., °C	Time	
Forks	none		2/2
	75	1 hr	2/2
	75	2 hr	2/2
	none		4/4
	90	10 min	0/4
	90	30 min	0/4
Nanaimo	none		2/2
	75	1 hr	2/2
	75	2 hr	2/2
	none	2/2	
	90	10 min	2/2
	90	30 min	2/2
Iwanai	75	2 hr	2/2
Smoked Chub (Detroit)	none		4/4
	85	10 min	4/4
	85	90 min	4/4
Beluga	90	0	2/2
	90	30 min	2/2
E-74	90	0	2/2
	90	30 min	2/2
Kalamazoo	85	10 min	4/4
Smoked Chub (Tenn.)	85	10 min	4/4

method of heating was due to the technique of heating. The preparation of the spore suspensions in these experiments were the same. The only difference was that the spore suspensions were contained in sealed ampoules and completely immersed in the bath during heating.

All strains were not tested for their heat resistance by this method. The heat resistance of spores of the Beluga and Nanaimo strains was studied most extensively at several temperatures, Tables 27 and 28, Figs. 10 and 11.

TABLE 24

HEAT RESISTANCE OF C. BOTULINUM TYPE E SPORES PRODUCED BY HEAT SURVIVING SPORES IN M/15 PHOSPHATE BUFFER pH 7.0

Strain	Temp, °C	Actual Time Minutes	c/ml	% Survivors
Forks				
	65	0	4.4×10^6	---
	85	0	71.0×10^4	100.0
	85	10	150	.0211
	85	30	42.5	.006
	85	60	4.5	.0064
	85	120	.5	.000071

TABLE 25

HEAT RESISTANCE OF C. BOTULINUM TYPE E SPORES PRODUCED BY HEAT SURVIVING SPORES IN M/15 PHOSPHATE BUFFER pH 7.0

Strain	Temp, °C	Actual Time Minutes	c/ml	% Survivors
Nanaimo				
	65	0	2.6×10^6	---
	85	0	97.0×10^4	100.0
	85	10	225	.024
	85	30	35.5	.0037
	85	60	16.5	.0020
	85	120	2	.0002

TABLE 26

HEAT RESISTANCE OF C. BOTULINUM TYPE E SPORES PRODUCED BY HEAT SURVIVING SPORES IN M/15 PHOSPHATE BUFFER pH 7.0

Strain	Temp, °C	Actual Time Minutes	c/ml	% Survivors
Iwanai				
	65	0	1.2×10^6	---
	85	0	49.5×10^4	100.0
	85	10	10^2	.02
	85	30	16.5	.003
	85	60	5.5	.001
	85	120	1	.0002

The heating times were shortened in order to more clearly define the first part of the curve. In these studies the time of heating was corrected for the time that spore suspension came up to the desired temperatures. With the Beluga and Nanaimo strains, there is a slight but definite increase in count at 70°C, indicating some heat activation of the spores. This confirms the similar observation in the study with the open tube. The heat inactivation curve obtained with this technique was similar, an initial kill of the majority of the spores with a small fraction surviving the longer heating times giving a tailing effect. At 78°C the spores of the Beluga strain were heated up to 60 minutes and survivors were still obtained. Spores of the Minneapolis, Kalamazoo, and Smoked Chub strains were checked at several temperatures. The results were similar to those obtained with the other strains, Tables 29, 30, and 31, and Figs. 12, 13, and 14.

Spores of the Beluga strain which survived heating at 78°C for 60 minutes and the Nanaimo strain which survived heating at 90°C for 3 minutes were used as inocula to produce another spore suspension. The heat resistance of these two suspension was checked at 80°C, Tables 32 and 33, and Figs. 15 and 16. As can be observed, the heat surviving spores produced a spore suspension which was as heat sensitive as the stock spore suspensions.

The ability of the heat surviving spores at various temperatures and heating times in the sealed ampoules to produce toxin in broth cultures was also tested. Tables 34, 35, and 36 summarize the data obtained in this study. In summary the ability of the surviving spores to produce toxic cultures was variable. For example with spores of the Beluga strain heated at 78°C for

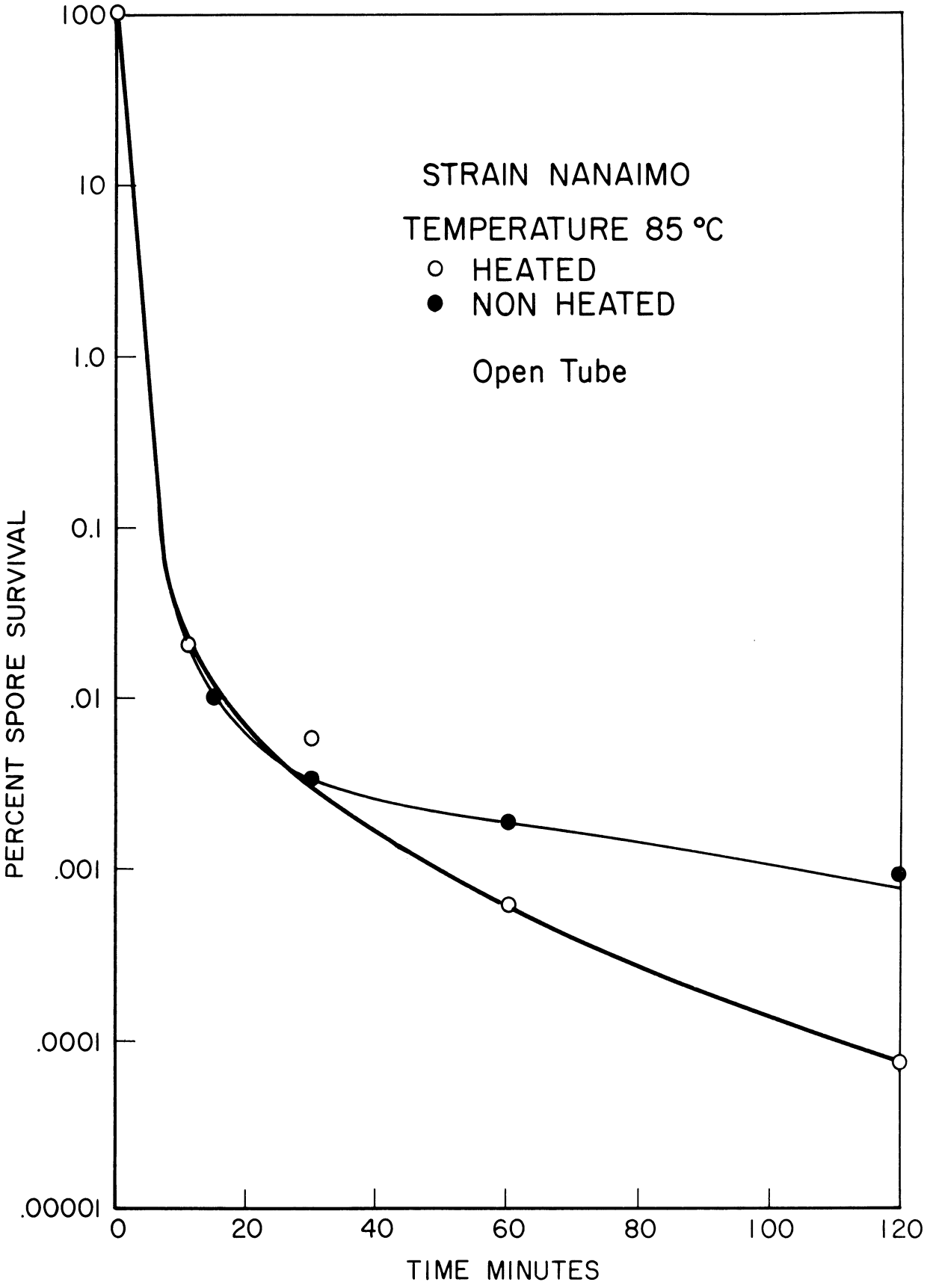

Fig. 7. Heat resistance of C. botulinum Type E spores produced by heat surviving spores in M/15 phosphate buffer pH 7.0.

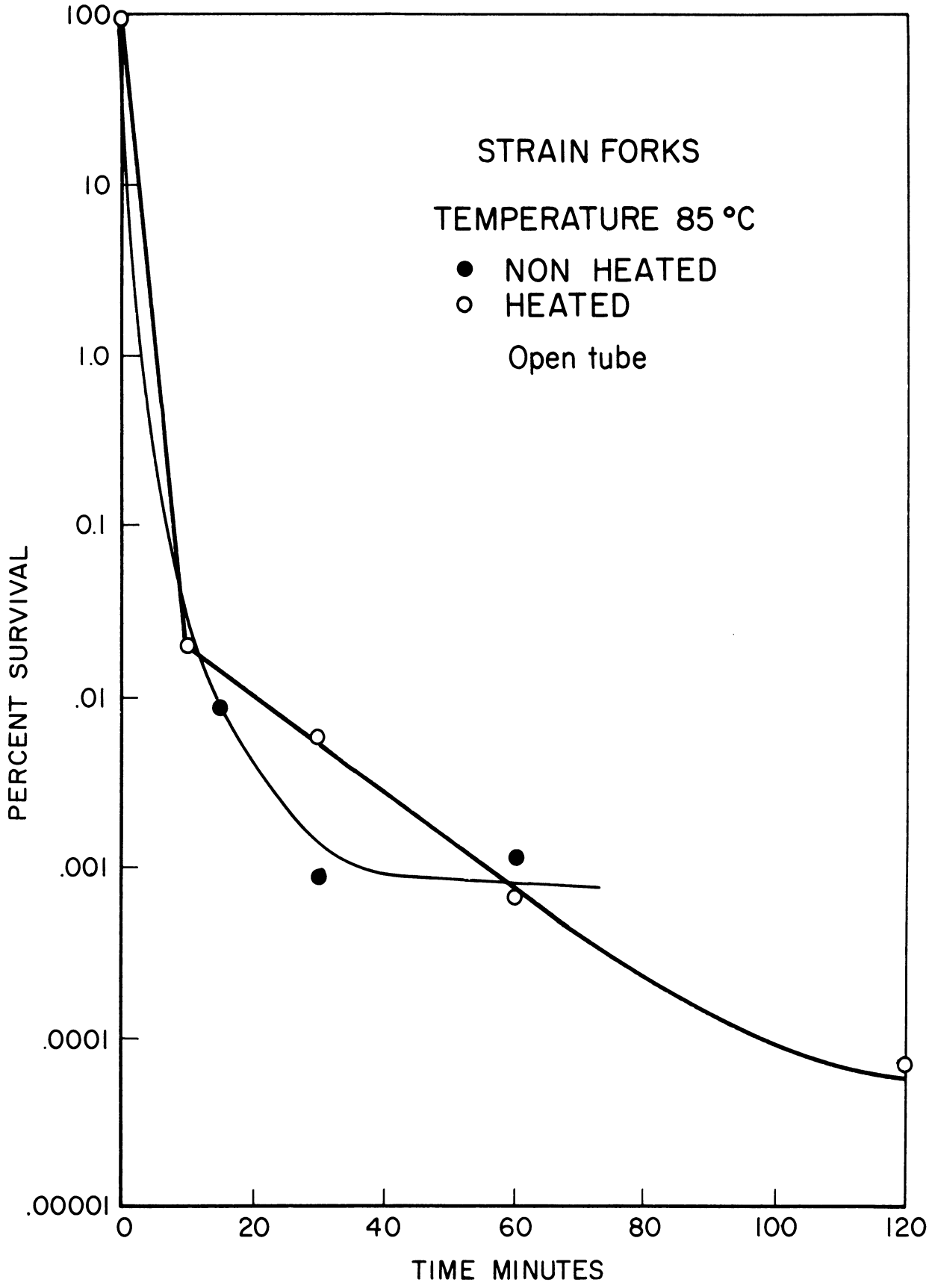

Fig. 8. Heat resistance of C. botulinum Type E spores produced by heat surviving spores in M/15 phosphate buffer pH 7.0.

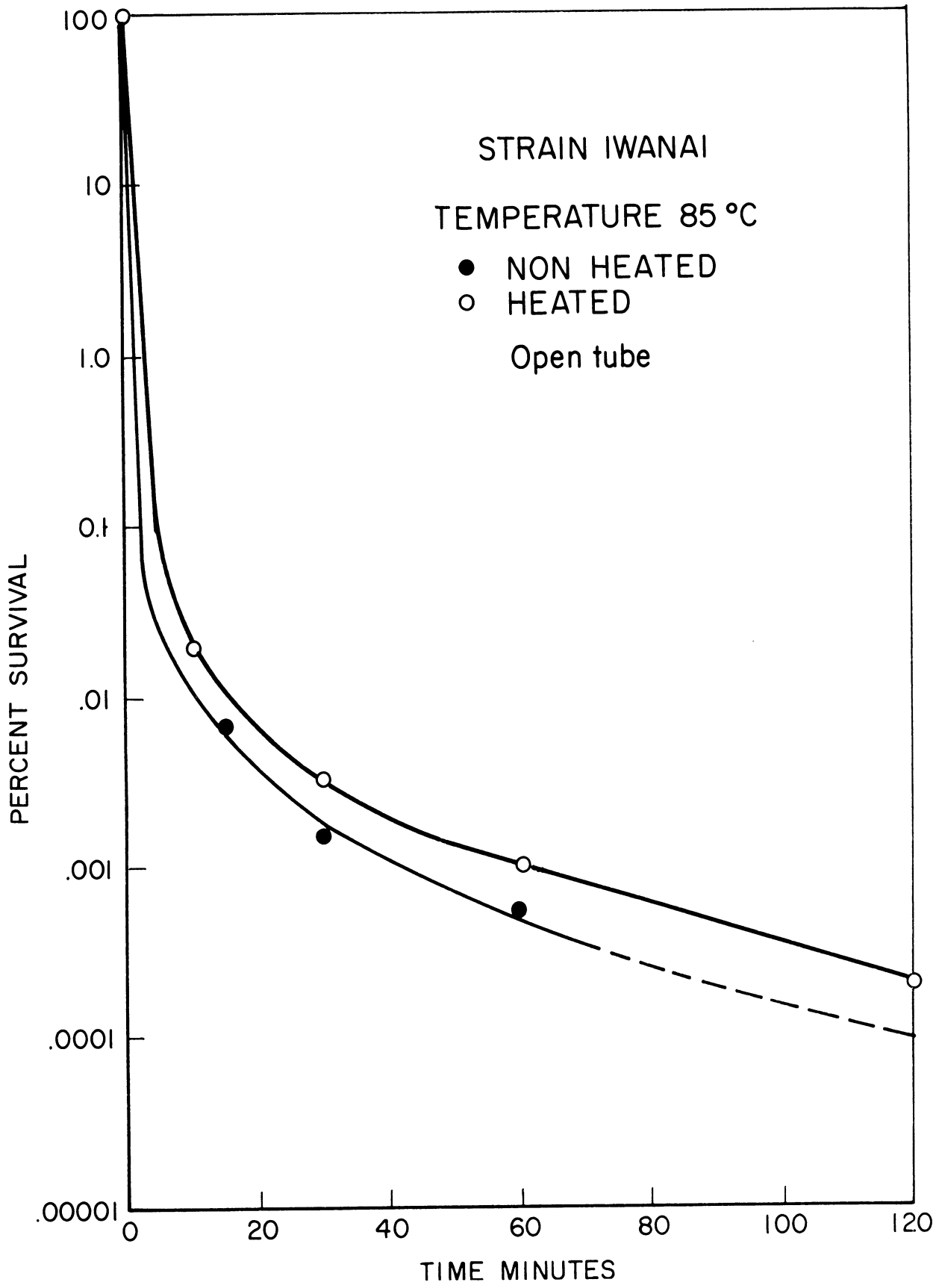

Fig. 9. Heat resistance of C. botulinum Type E spores produced by heat surviving spores in M/15 phosphate buffer pH 7.0.

TABLE 27

HEAT RESISTANCE OF C. BOTULINUM TYPE E SPORES
IN M/15 PHOSPHATE BUFFER pH 7.0

Temp, °C	Effective Time Minutes	Strain—Beluga c/ml	% Survivors
65	0	6.1×10^7	100.0
	10	1.3×10^8	210.0
	20	3.9×10^7	63.0
	30	3.6×10^7	58.0
70	0	6.1×10^7	100.0
	5.5	2.3×10^7	38.0
	8.5	1.2×10^7	18.0
	13.5	2.0×10^7	3.3
	20.5	2.7×10^5	.43
	28.5	1.8×10^4	.029
	58.5	7.9×10^2	.00013
72	0	6.1×10^7	100.0
	1.5	4.2×10^7	69.0
	3.5	2.2×10^7	36.0
	5.5	6.5×10^6	10.6
	8.5	1.1×10^6	1.7
	13.5	5.0×10^4	.082
	20.5	6.7×10^3	.011
	28.5	1.2×10^3	.00197
	58.5	9.0×10^1	.000147
75	0	6.1×10^7	100.0
	5.5	1.9×10^3	.00315
	8.5	2.8×10^2	.000466
	13.5	2.1×10^2	.00035
	20.5	65	.000107
	28.5	25	.00004
	58.5	66	.000108
78	0	6.1×10^7	100.0
	1.25	1.7×10^4	.028
	3.25	1.8×10^2	.00029
	5.25	4.4×10^2	.00072
	9.25	2.9×10^2	.000495
	13.25	3.6×10^2	.0059
	20.25	4.0×10^2	.000655

TABLE 27 (Concluded)

Temp, °C	Effective Time Minutes	Strain—Beluga c/ml	Strain—Beluga % Survivors
78	28.25	2.4×10^2	.00039
	58.25	1.5×10^2	.000243
80	0	7.0×10^7	100.0
	1.3	3.7×10^2	.00053
	3.3	1.1×10^2	.000157
	5.3	2.8×10^2	.00039
	8.3	1.7×10^2	.00024
	13.3	1.3×10^2	.00018
90	0	8.2×10^7	100.0
	1.15	1.1×10^2	.00014
	3.15	28	.000034
	5.15	28	.000034
	8.15	19	.000023

TABLE 28

HEAT RESISTANCE OF C. BOTULINUM TYPE E SPORES
IN M/15 PHOSPHATE BUFFER pH 7.0

Temp, °C	Effective Time Minutes	Strain—Nanaimo	
		c/ml	% Survivors
70	0	5.0×10^7	100.0
	1.5	7.2×10^6	144.0
	3.5	7.2×10^6	144.0
	5.5	2.2×10^6	43.0
75	1.4	5.4×10^6	10.8
	3.4	2.5×10^3	.005
	5.4	1.1×10^2	.00021
80	1.3	115	.00023
	3.3	43	.000086
	5.3	32	.000064
85	1.2	30	.00006
	3.2	62	.000124
	5.2	53	.00011
90	0	1.8×10^7	100.0
	1.1	10	.000052
	3.1	3	.000014
	5.1	2	.000011
	8.1	3	.000014

60 minutes produced toxic culture. A further check on specificity of toxin was made and it was Type E toxin. At 80°C heating for 15 minutes the survivors did not produce a toxic culture. The same situation was observed with the other strains. At the lower temperatures and heating times the surviving cultures consistently produced toxic cultures. However at the higher heat treatments variable results occurred in the production of toxic cultures. For example with the Beluga strain the spores surviving heating at 90°C for 3 minutes produced a toxic culture whereas those surviving heating at 5 and 7 minutes did not. In the case of the Nanaimo strain heat survivors at 90°C for 3, 5, and 10 minutes did not produce toxic cultures. As mentioned previously the spores of this strain surviving heating at 90°C for 3 minutes were used to produce a new spore suspension to test whether these produced spores with an unusual resistance to heat. These spores when

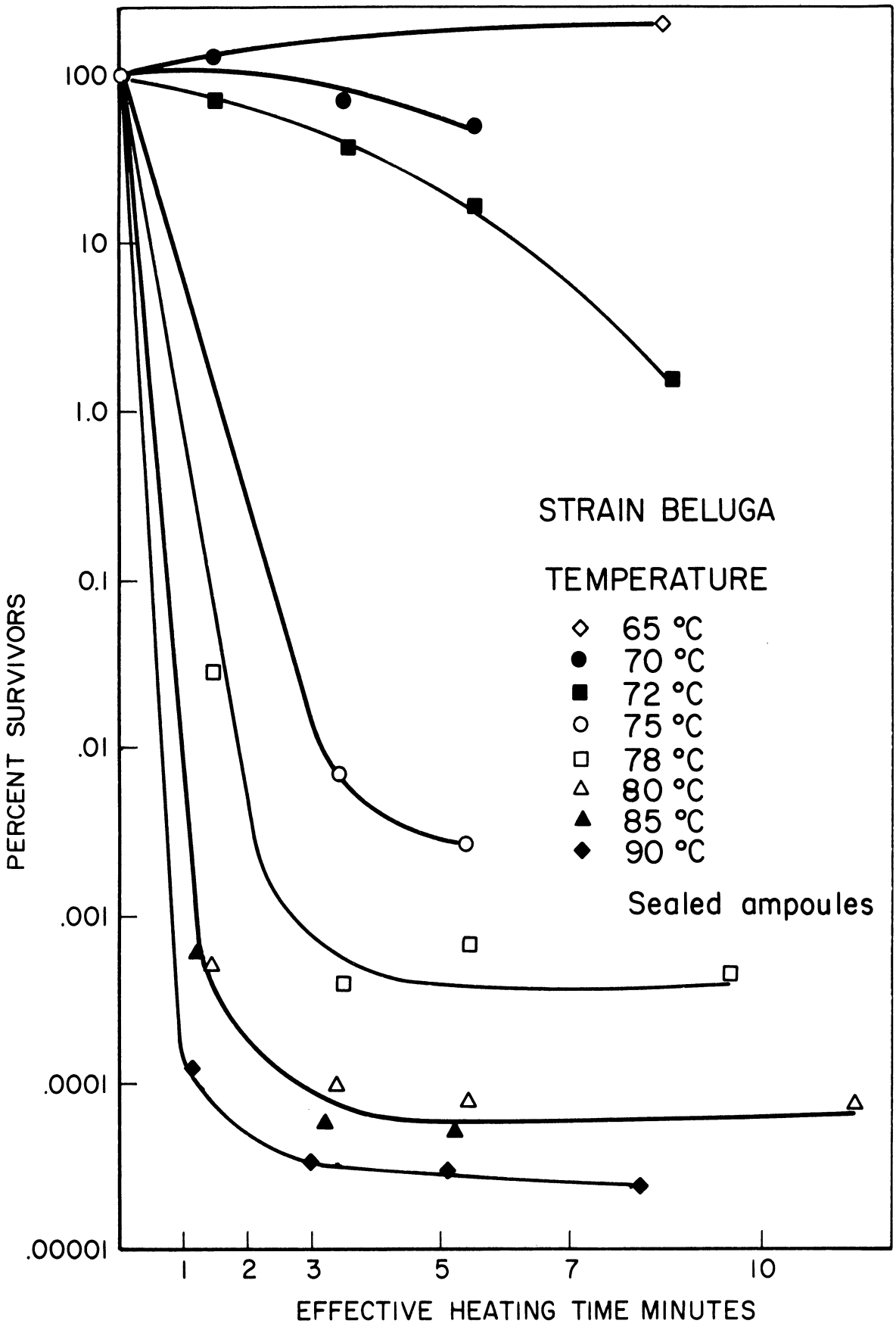

Fig. 10. Heat resistance of C. botulinum Type E spores in M/15 phosphate buffer pH 7.0.

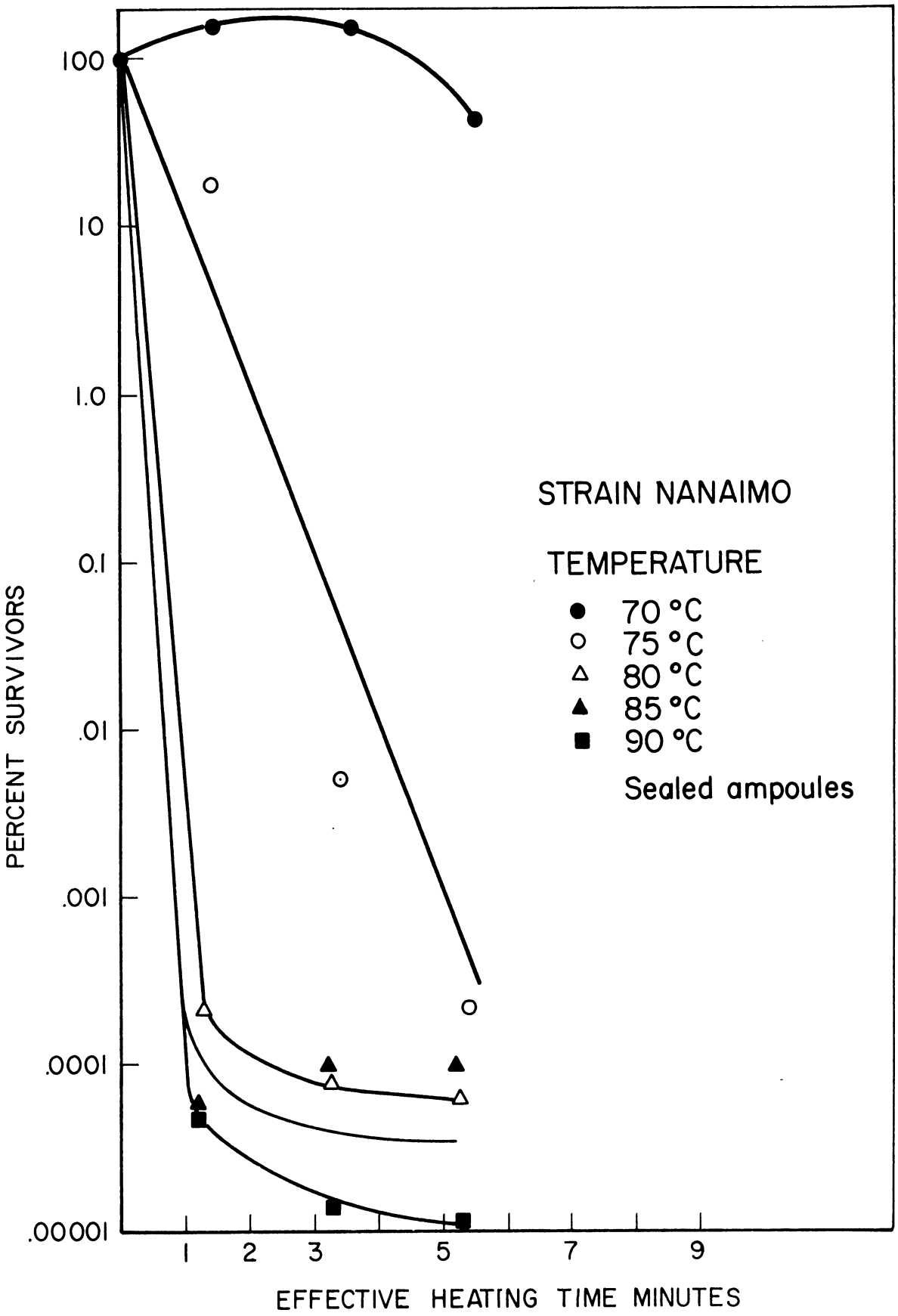

Fig. 11. Heat resistance of C. botulinum Type E spores in M/15 phosphate buffer pH 7.0.

TABLE 29

HEAT RESISTANCE OF C. BOTULINUM TYPE E SPORES
IN M/15 PHOSPHATE BUFFER pH 7.0

Actual Time Minutes	Strain—Minneapolis			
	70°C		85°C	
	c/ml	% Survivors	c/ml	% Survivors
0	4.0×10^6	100.0	4.0×10^6	100.0
5	1.3×10^6	31.7	93	.0023
10	1.3×10^5	32.5	5	.0001
30	5.0×10^4	1.3	20	.0005
60	1.7×10^2	.004	12	.00029
120	---	---	6	.00015

TABLE 30

HEAT RESISTANCE OF C. BOTULINUM TYPE E SPORES
IN M/15 PHOSPHATE BUFFER pH 7.0

Temp, °C	Effective Heating Time	Strain—Minneapolis	
		c/ml	% Survivors
70	0	1.2×10^6	100.0
	1.5	1.2×10^6	100.0
	3.5	4.6×10^5	42.0
	5.5	3.8×10^5	35.0
75	1.4	4.6×10^5	.42
	3.4	8.5×10^3	.77
	5.4	9.3×10^3	.85
80	1.3	70	.0064
	3.3	120	.01
	5.3	8	.00073
85	1.2	2	.00018
	3.2	7	.00058
	5.2	2	.00013

TABLE 31

HEAT RESISTANCE OF C. BOTULINUM TYPE E SPORES
IN M/15 PHOSPHATE BUFFER pH 7.0

Temp, °C	Effective Time Minutes	Strain—Smoked Chub Tennesse	
		c/ml	% Survivors
70	0	9.3×10^6	100.0
	1.5	2.5×10^6	26.0
	3.5	2.0×10^6	22.0
	5.5	2.0×10^6	22.0
75	1.4	6.4×10^5	6.9
	3.4	1.3×10^4	.14
	5.4	6.9×10^3	.076
80	1.3	34	.00037
	3.3	32	.00035
	5.3	7	.000072
85	1.2	7	.000078
	3.2	7	.000078
	5.2	5	.000056

TABLE 32

HEAT RESISTANCE OF C. BOTULINUM TYPE E SPORES, STRAIN BELUGA
PRODUCED BY SPORES SURVIVING HEATING AT 78°C FOR 60 MINUTES

Effective Time Minutes	c/ml		% Survivors	
0	1.3×10^7	5.0×10^6	100.0	100.0
1.3	1.8×10^2	1.8×10^2	.035	.0035
3.3	75	63	.0057	.0012
5.3	51	51	.0039	.0010
8.3	50	34	.0038	.00067
13.3	8	8	.00061	.00015

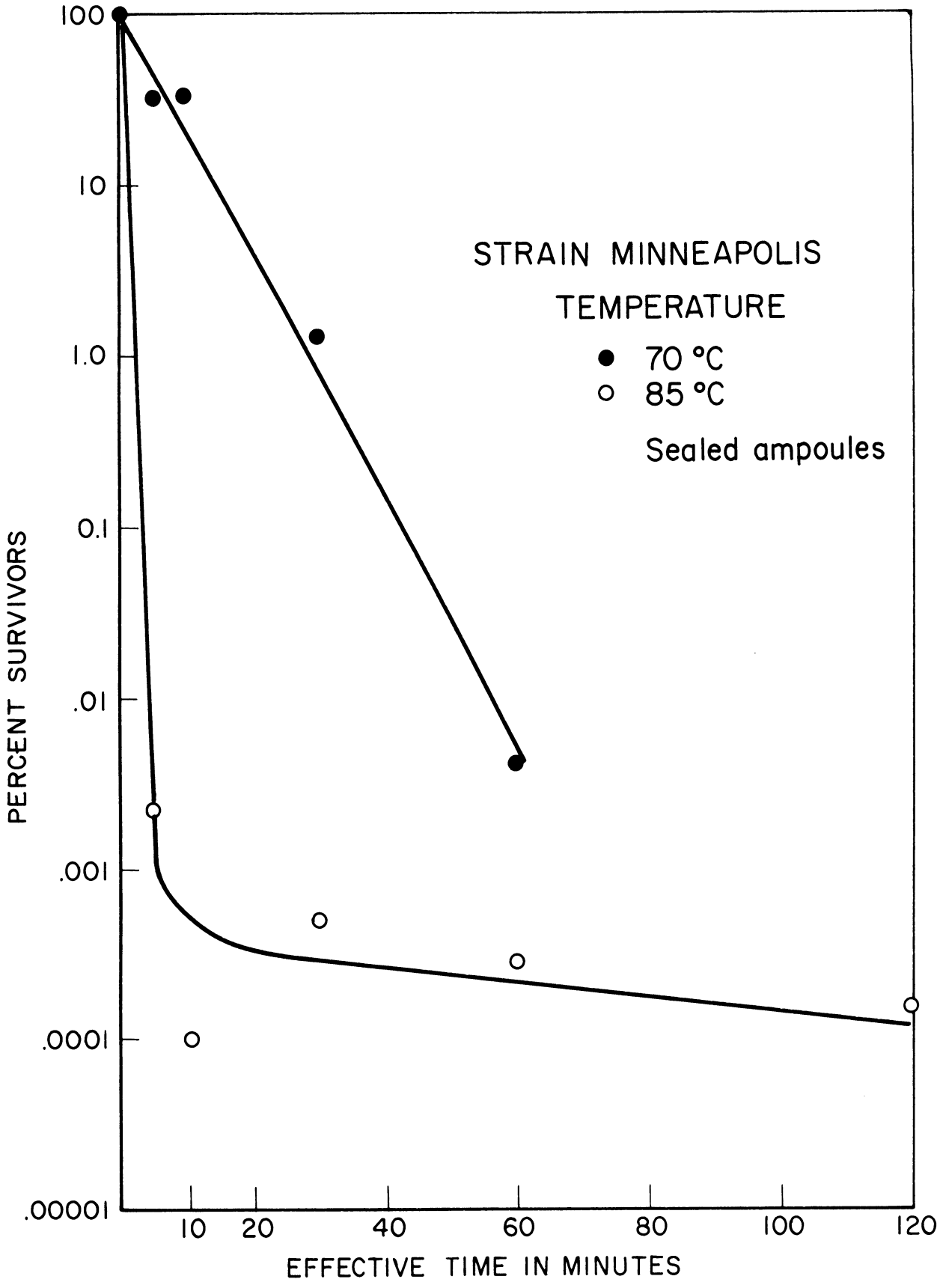

Fig. 12. Heat resistance of C. botulinum Type E spores in M/15 phosphate buffer pH 7.0.

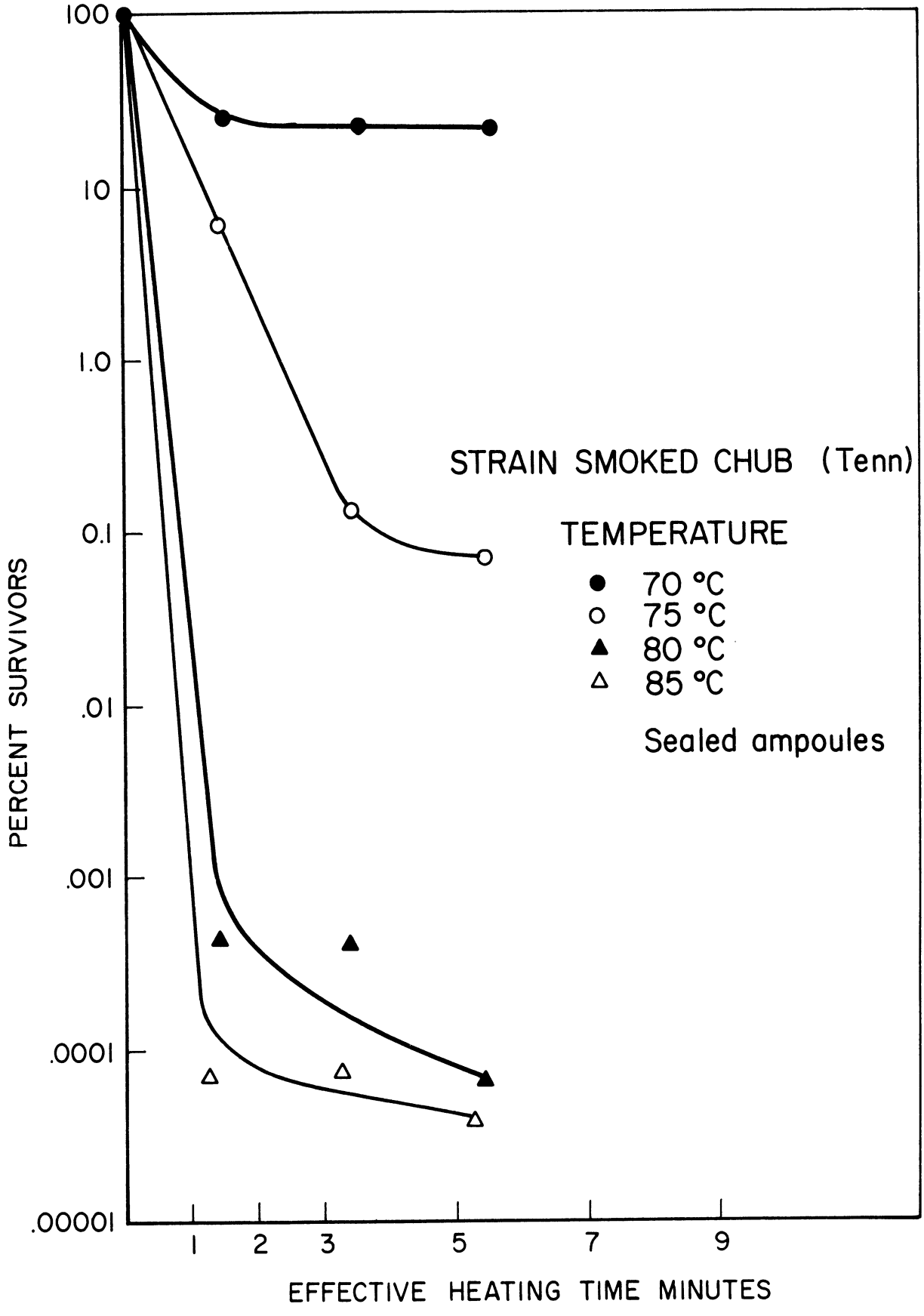

Fig. 13. Heat resistance of C. botulinum Type E spores in M/15 phosphate buffer pH 7.0.

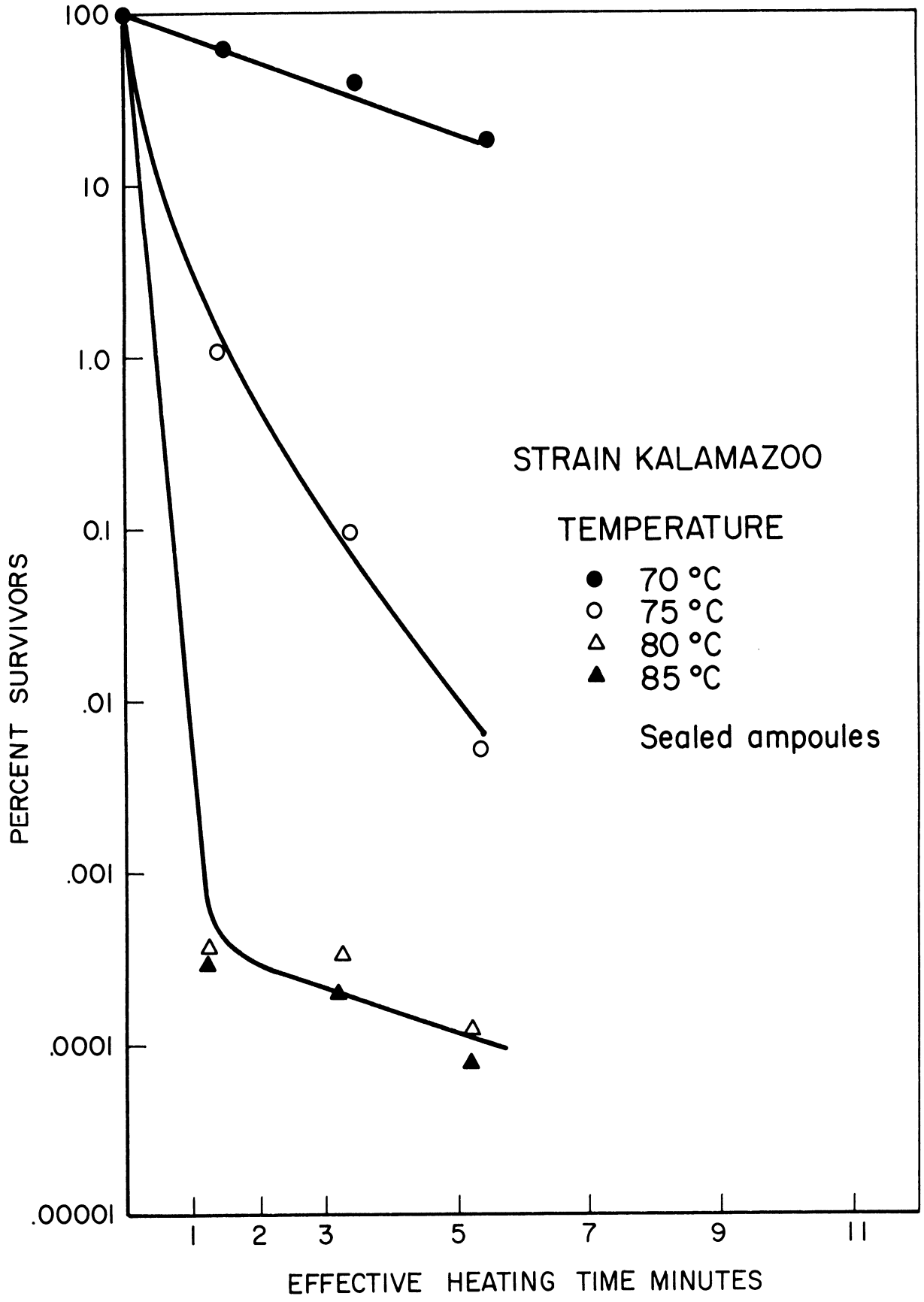

Fig. 14. Heat resistance of C. botulinum Type E spores in M/15 phosphate buffer pH 7.0.

TABLE 33

HEAT RESISTANCE OF C. BOTULINUM TYPE E SPORES, STRAIN NANAIMO PRODUCED BY SPORES SURVIVING HEATING AT 90°C FOR THREE MINUTES

Effective Time Minutes	c/ml	% Survivors
0	6.3×10^5	100.0
1.3	65	.01
3.3	18	.003
5.3	9	.0014
8.3	6	.00095
13.3	11	.0017

TABLE 34

TOXIN PRODUCTION BY HEAT SURVIVING SPORES OF C. BOTULINUM TYPE E SPORES

Strain	Heat Treatment		Deaths
	Temp., °C	Time, min	
Minneapolis	70	7	3/3
	75	5	3/3
Eloise	75	5	3/3
Nanaimo	75	7	3/3
	85	7	3/3
Smoked Chub (Detroit)	75	7	3/3
Beluga	85	7	3/3
Kalamazoo	75	5	3/3

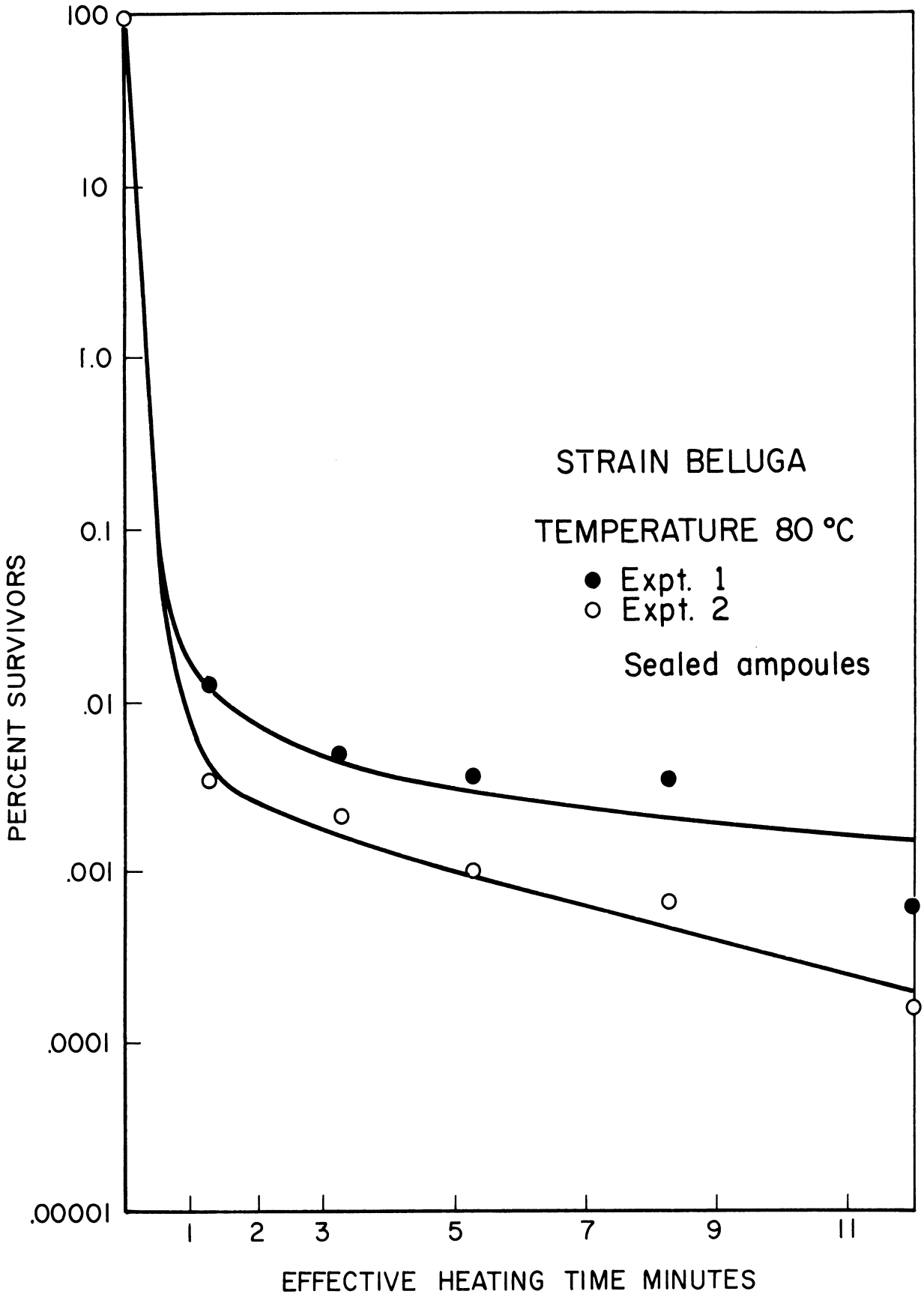

Fig. 15. Heat resistance of C. botulinum Type E, strain Beluga spores produced by spores surviving heating at 78°C for 60 minutes.

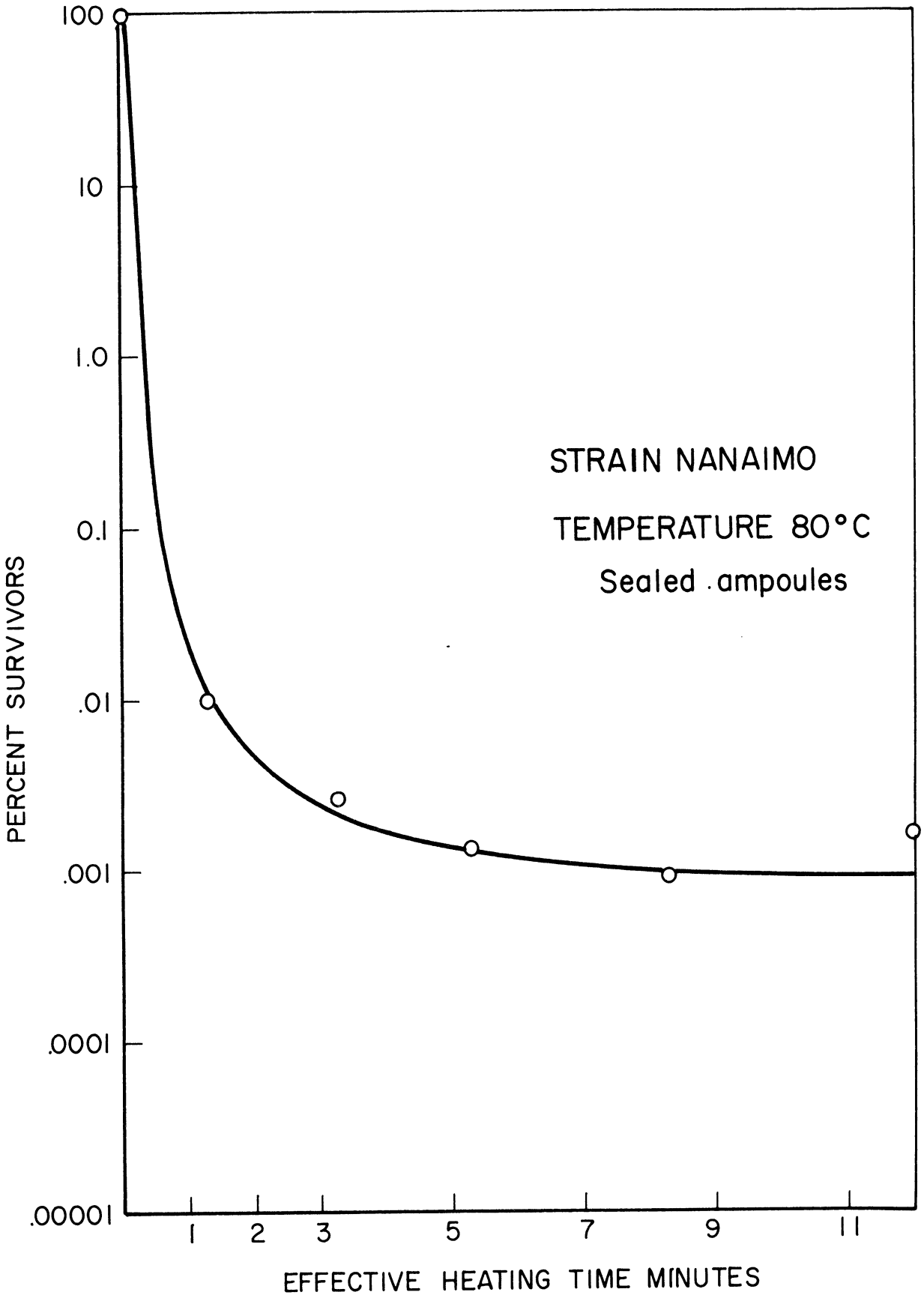

Fig. 16. Heat resistance of C. botulinum Type E, strain Nanaimo spores produced by spores surviving heating at 90°C for 3 minutes.

TABLE 35

TOXIN PRODUCTION BY HEAT SURVIVING SPORES OF C. BOTULINUM TYPE E SPORES

Strain	Sample	Deaths
Beluga	90°C 3 min	6/6
	90°C 5 min	0/3
	90°C 7 min	0/3
	85°C 7 min	0/3
	80°C 7 min	3/3
Kalamazoo	90°C 3 min	0/3
	80°C 5 min	3/3
Smoked Chub (Tennessee)	90°C 3 min	0/3
	80°C 3 min	3/3
Minneapolis	80°C 3 min	3/3
	80°C 5 min	4/4

Strain	Heat Treatment		Deaths
	Temp., °C	Time, min	
Smoked Chub (Detroit)	90	3	0/2
	90	10	0/2
	78	30	2/2
Kalamazoo	90	3	2/2
	90	10	0/2
Nanaimo	90	3	0/2
	90	5	0/2
	90	10	0/2

TABLE 36

TOXIN PRODUCTION BY HEAT SURVIVING SPORES
OF C. BOTULINUM TYPE E SPORES

Strain--Beluga

Sample	Toxin	
	First Transfer	Second Transfer
78°C 15 min	2/2	2/2
78°C 30 min	2/2	2/2
78°C 60 min	2/2	2/2
80°C 15 min	0/2	2/2
78°C 60 min + AT E	0/2	
78°C 60 min + AT AB	2/2	
78°C 60 min + NO AT	2/2	

subcultured into TPG broth media produced toxin, indicating a reversion to toxin formation. Because of time limitation this phenomena was not pursued further. But the results of these studies demonstrate that some of the survivors from the tail portion of the curve are able to produce toxic cultures. A few of the more heat resistance are of the non-toxic variety. The exact nature of this relationship needs further study.

Because of the interest of the heat resistance of Type E spores in smoked fish products, a preliminary heat study in smoked fish homogenates was performed at 85°C. A homogenate of smoked fish plus 3% sodium chloride (1:1 ratio) was prepared. A mixture of heat smoked spores of the Beluga and Nanaimo strains was blended into the homogenate and dispensed into ampoules and heated. The ampoules were heated at 85°C for various periods of time up to 90 minutes. One set of vials was placed in the incubator at 30°C for 7 days. One set was opened up and subcultured into beef infusion agar to obtain number of surviving spores, the other into trypticase broth to test for toxin production. The results are presented in Table 37. Toxin was not detected in haddock homogenates after incubation of the sample heated at 85°C for 15 minutes, although growth occurred in all ampoules. In the broth subculture toxin was detected with those samples heated for 30 minutes but not 60 minutes. Also growth occurred in all tubes. The homogenates were not pre-sterilized, so it may have contained some heat resistance spores. In case of the outgrowth in the smoked homogenate, the 3% NaCl prevented the few spores surviving the larger heating times to produce toxic cultures.

TABLE 37

HEAT RESISTANCE OF TYPE E SPORES IN SMOKED FISH HOMOGENATE AT 85°C

Strains—Nanaimo and Beluga

Actual Time, min	Beef Infusion Agar	Growth			
		Trypticase Broth		Homogenate	
		Gas	Toxin	Gas	Toxin
0	14×10^6	+	---	+	---
5	23×10^2	+	3/3	+	3/3
10	gas + 4	+	---	+	3/3
15	gas	+	4/4	+	0/3
30	gas	+	4/4	+	---
60	No gas	± putrid	0/4	+	0/4
90	No gas	± putrid	0/4	+	0/4

IRRADIATION RESISTANCE STUDIES

The resistance of Type E spores to ionizing radiations has not been extensively studied. Erdman et al. (1961) found that spores of the VH strain of Type E were less resistant to gamma radiation from cobalt-60 than were Type A spores. They also found that spores from non-toxigenic variants of Type E were more resistant to radiation than the toxigenic variants. Schmidt et al. (1962a and b), studied the resistance of six strains of Type E to gamma radiations from spent fuel elements. The spores were suspended in beef stew. These investigators observed a mean D value of 0.132 megarad for all six strains, the minimum being 0.125 and the maximum 0.138 megarad. Such values compare with mean D values of 0.279 megarad for Type A and 0.238 megarad for Type B spores in the same food product under similar conditions.

IRRADIATION TECHNIQUES

The description of the radiation source and dosimetric procedures used in all of these studies is given in the Appendix.

Thoroughly washed spores were used in all of the work in order to minimize carry-over of medium constituents which may affect the radiation resistance of the organisms. Prior to use, the spores were heated at 60-65°C for 15 minutes. Following heat treatment, the organisms were diluted to the desired concentration in the substrate being tested. Usually a concentration of 10^6-10^7 spores per ml was employed.

The spores for irradiation were contained in 5 ml sealed vials. These vials were positioned in a rack which fits into a Dewar flask and has spaces for 28 vials. The Dewar flask was positioned in the center well of the cobalt-60 source. Dose rate measurements were determined in the same vials in which the spores were irradiated. During dosimetry determinations the vials were placed in the same position occupied by vials containing spores suspension during irradiation. Figure 17 shows the dose rates in positions occupied by the vials during irradiation. All irradiations were carried out at melting ice temperatures (0°C).

After irradiation, the spores were diluted in sterile water and appropriate dilutions were sub-cultured into a counting medium. The medium employed for counting was fresh beef infusion agar.

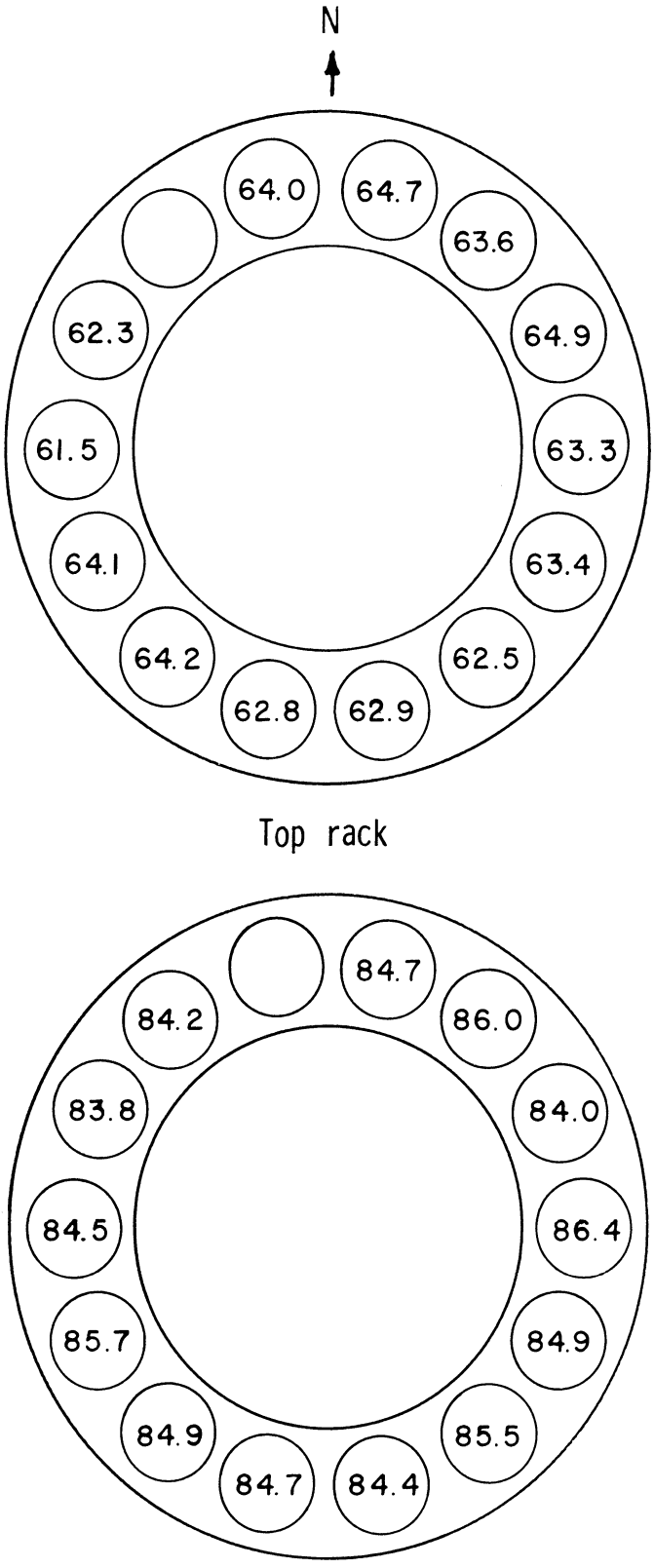

Fig. 17. Gamma radiation dose rates in the position occupied by the spore suspensions during irradiation on the Phoenix Radiation Facility cobalt-60 source.

RESULTS

The results of the irradiation of spores of the six Type E strains in M/15 phosphate buffer are presented in Figs. 18 and 19. The six strains show little difference in resistance to gamma radiation.

Survivor curves for spores of the Forks strain of Type E and for Type A spores are given in Fig. 20. These spores were suspended in M/15 phosphate buffer at pH 7.0 during irradiation. It is evident from this figure that Type A spores are much more resistant to gamma radiations from cobalt-60 than are Type E spores.

The influence of an aqueous extract of haddock tissue as the suspending medium on the radiation resistance of Type E spores is shown in Fig. 21 and Tables 38 and 39. It can be seen that the haddock extract protects these spores against the lethal effect of gamma radiations.

The results of irradiation of spores suspended in haddock homogenate and oyster juice is presented in Table 39. The haddock homogenate and oyster juice offer appreciable protection to the spores during irradiation.

RESISTANCE OF *Clostridium perfringens* TYPE A SPORES TO IONIZING RADIATION*

Since very little work has been done on the radiation resistance of *C. perfringens*, it is of interest to compare the radiation resistance of these spores with that of *C. botulinum* spores.

Food poisoning has been known to result from eating foods in which cultures of *C. perfringens* Type A have inadvertently grown. Within the past few months, radiation sterilized bacon has been approved by the Federal Government as an acceptable food product. Other irradiated foods are being studied for similar clearance. These include foods treated with both irradiation sterilization and irradiation pasteurization processes. No data have been published regarding the radiation resistance of *C. perfringens* spores, which could conceivably be involved in determining the wholesomeness of irradiation processed foods. The present study is intended to apply to this void.

Spores of a Hobbs, "heat-resistant" strain were produced in a trypticase, soy broth medium. Purity of the culture was established by suitable identification criteria, such as: Gram reaction, anaerobiosis, lecithovitellin reaction, absence of motility, biochemical reactions, aerologic testing, and heat-resistance of the spores. In each instance, reactions

*This section was written by T. F. Midura, J. T. Graikoski, L. L. Kempe, and N. A. Milone, of the Departments of Environmental Health and Chemical Engineering, The University of Michigan.

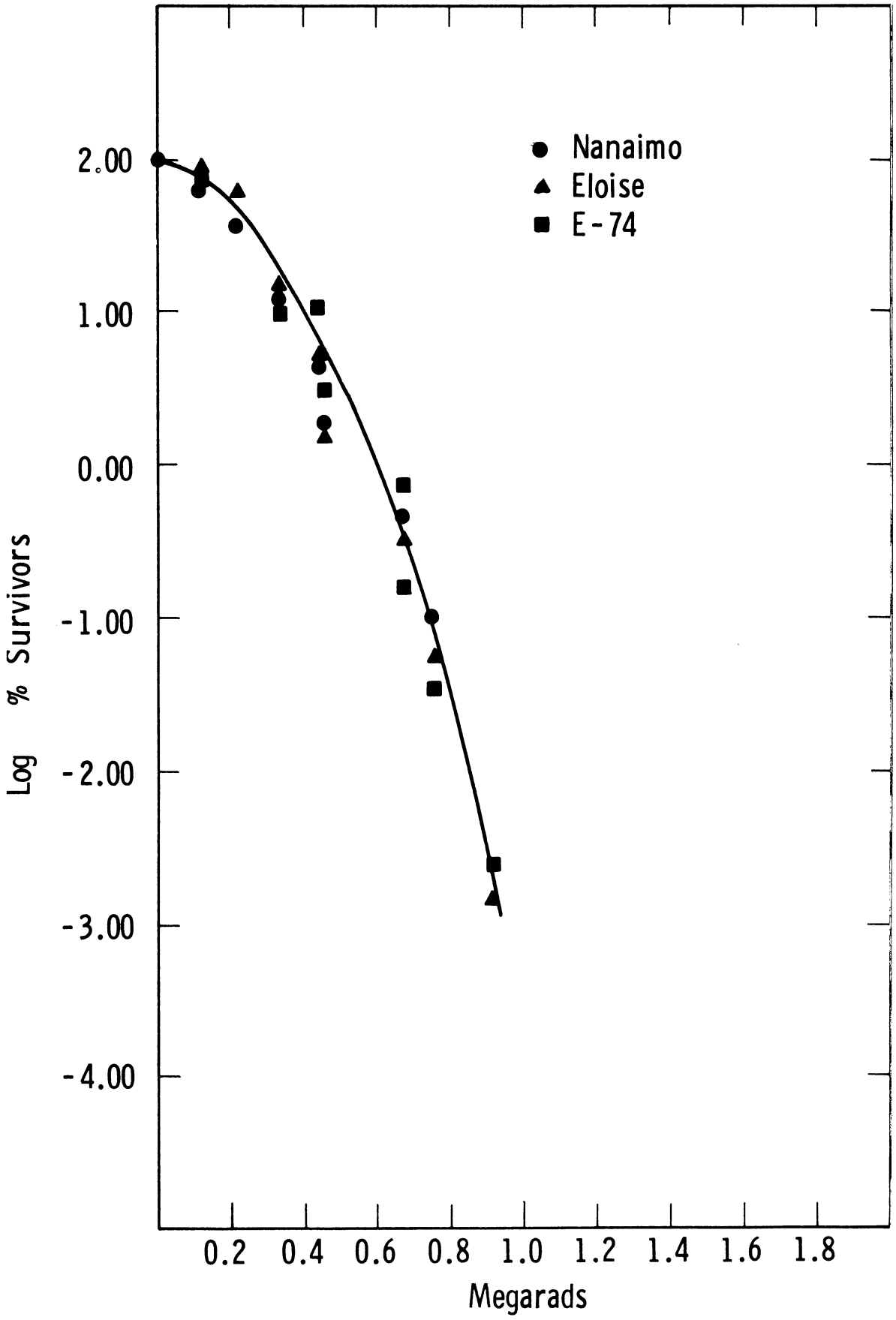

Fig. 18. Comparative resistance to gamma radiation of strains of C. botulinum Type E (Nanaimo, Eloise, E-74).

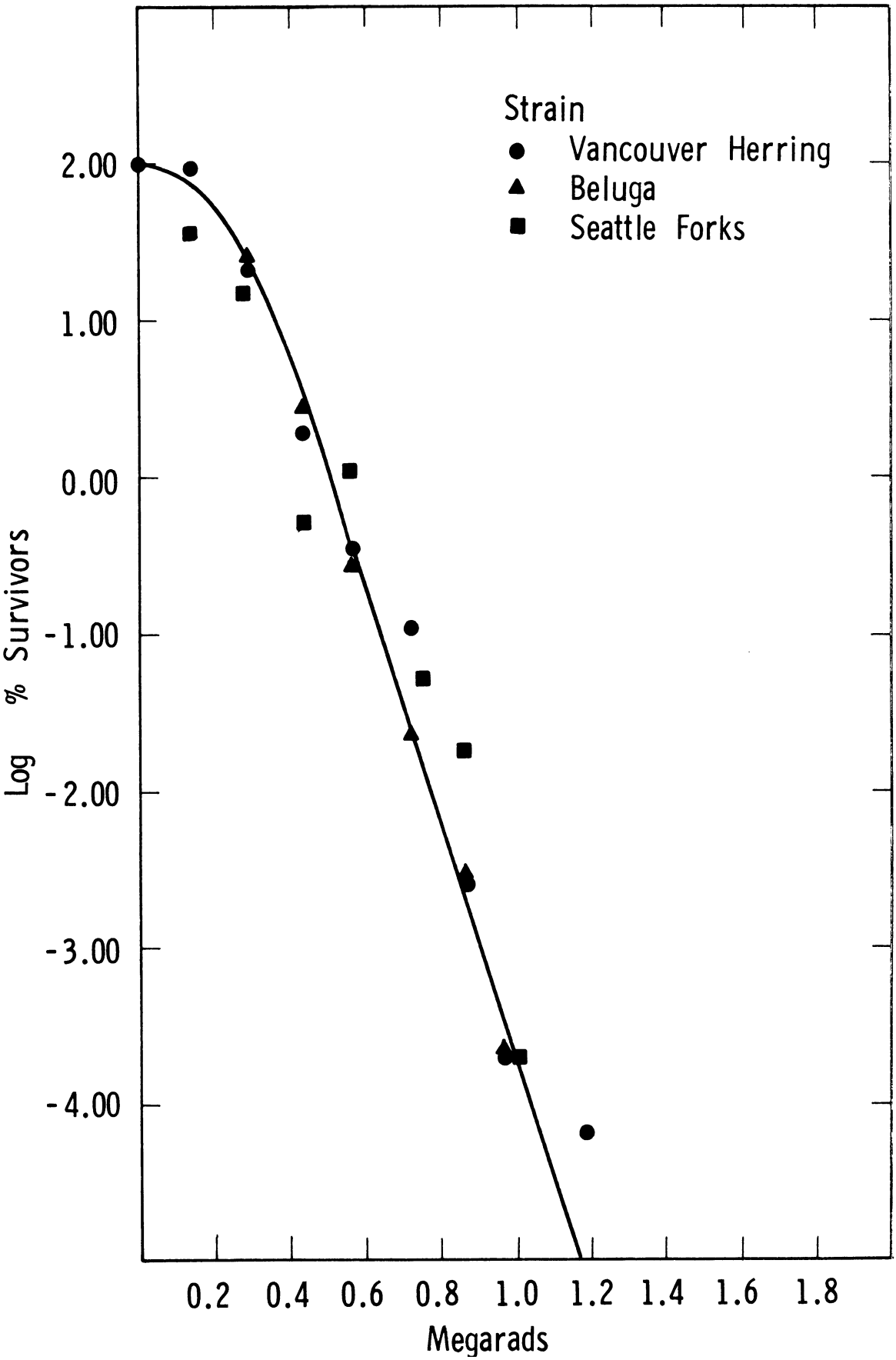

Fig. 19. Comparative resistance to gamma radiation of strains of C. botulinum Type E (Vancouver Herring, Beluga, Forks).

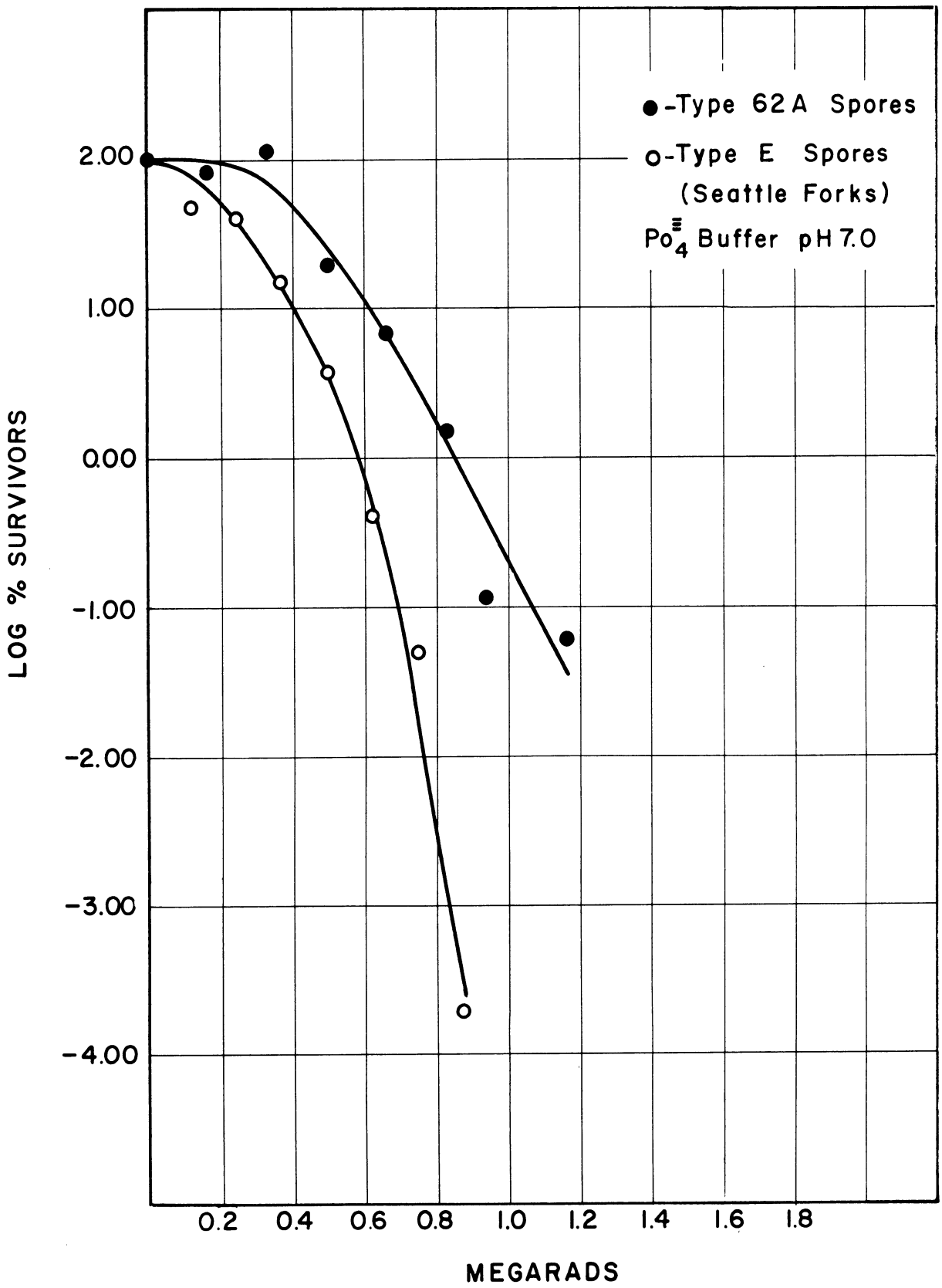

Fig. 20. Comparative resistance to gamma radiation of C. botulinum Type E and Type A spores.

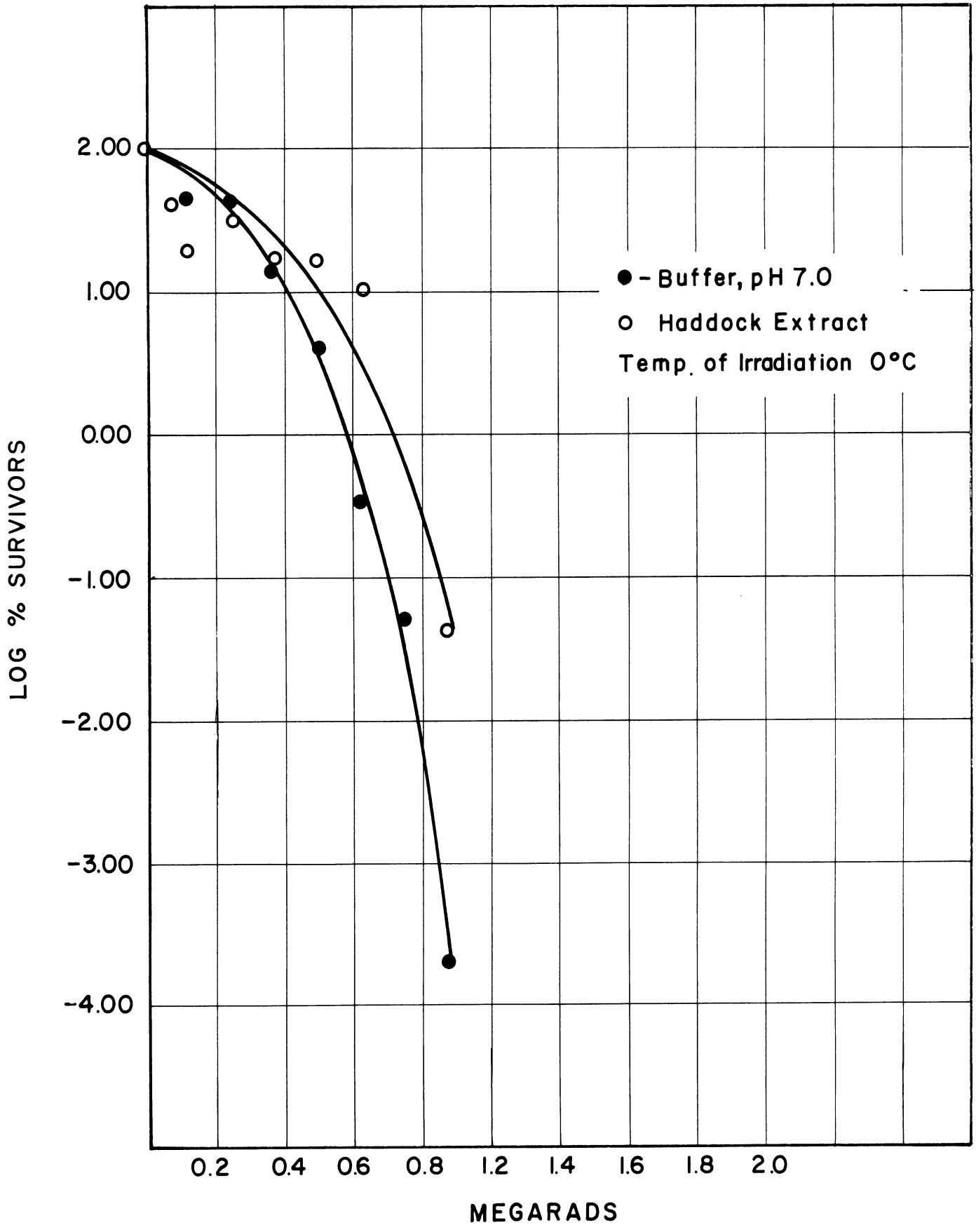

Fig. 21. Comparative sensitivity to gamma radiation of C. botulinum Type E spores suspended in buffer and haddock extract.

TABLE 38

COMPARATIVE SENSITIVITY TO RADIATION OF C. BOTULINUM TYPE E SPORES SUSPENDED IN PHOSPHATE BUFFER AND HADDOCK EXTRACT

Dose Megarad	Percent Survival	
	PO_4	Haddock
0	100.0	100.0
.124	49.0	22.5
.249	41.2	32.3
.373	14.85	19.3
.498	4.12	16.5
.623	0.392	12.1
.747	0.049	--
.842	0.00017	0.037

TABLE 39

COMPARATIVE SENSITIVITY TO GAMMA RADIATION OF C. BOTULINUM TYPE E (FORKS) SPORES SUSPENDED IN PO4 BUFFER, OYSTER JUICE, AND HADDOCK HOMOGENATE

Megarads	PO4 %	Log % Survivors in Oysters	Megarads	Log % Survivors in Haddock
0	100.0	100.0	0	100.0
.14	34.2	3.6	.11	14.8
.29	14.9	36.0	.22	14.3
.43	0.51	19.1	.34	17.5
.57	1.08	10.3	.45	12.3
.76	.046	5.91	.56	16.0
.86	.019	1.43	.67	8.1
1.1	.000021	0.236	.78	3.1

were characteristically those of C. perfringens.

Spore suspensions were heat-shocked at 80°C for 10 minutes, which treatment also kills vegetative cells. Numbers of viable spores were determined by dilution counts using the Prickett tube technique and a modified Mossel's medium.

Approximately 10^6 spores per ml were suspended in M/15 phosphate buffer (pH 7.0). Quantities of 4 ml were dispensed into 5-ml ampoules. The ampoules, were heat sealed and immersed in ice water, and the spore suspentions were irradiated with gamma rays from a cobalt-60 source at a rate of 74,000 rads per hour. A non-irradiated control sample was kept at 4°C. Surviving spores were counted as previously described.

As shown in Fig. 22 and Table 40, the D value of C. perfringens Type A spores in M/15 phosphate buffer was 0.23 megarad. Thus, C. perfringens spores appear to be more radiation resistant than C. botulinum Type E spores, which are stated[2] to have an approximate D value of 0.14 megarad.

This will be of significance in evaluating the survival of C. perfringens Type A spores in irradiated foods. It should be recognized that experience with other spores has indicated greater radiation resistance in foods than in phosphate buffer.[3]

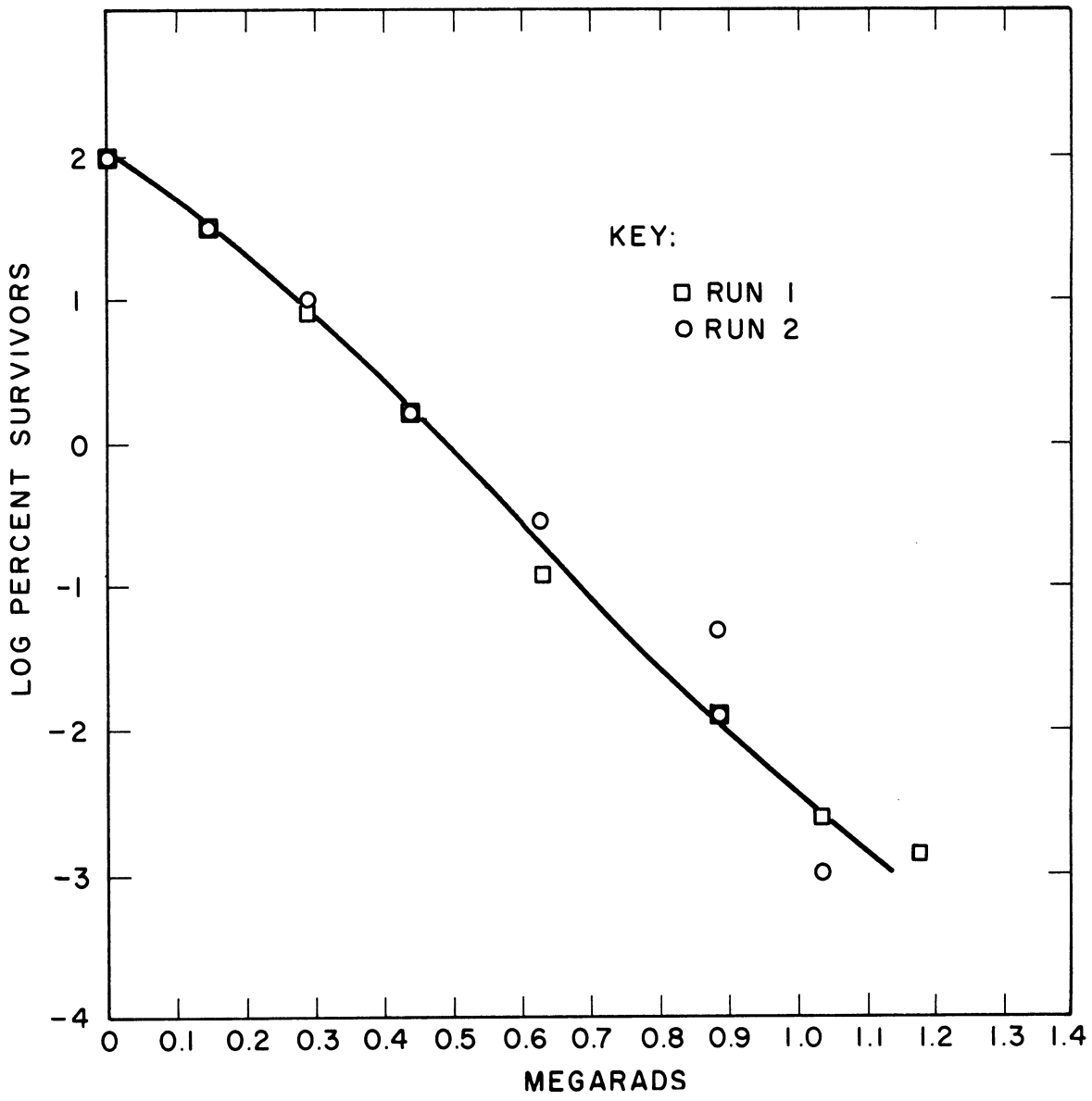

Fig. 22. Radiation resistance of C. perfringens Type A spores suspended in M/15 phosphate buffer to gamma radiation from cobalt-60.

TABLE 40

RADIATION RESISTANCE OF C. PERFRINGENS TYPE A SPORES, SUSPENDED IN M/15 PHOSPHATE BUFFER AT pH 7.0, TO GAMMA RADIATION FROM COBALT-60

Megarad	Spores/ml	% Survivors	Log % Survivors
Run 1			
0.000	800,000	100.0	2.0
0.148	230,000	28.8	1.46
0.296	60,000	7.5	0.88
0.444	12,000	1.5	0.18
0.629	1,000	0.125	-0.90
0.888	100	0.0125	-1.90
1.036	20	0.0025	-2.60
1.184	10	0.00125	-2.90
Run 2			
0.000	1,000,000	100.0	2.0
0.148	300,000	30.0	1.48
0.296	110,000	11.0	1.04
0.444	17,000	1.7	0.23
0.629	3,000	0.3	-0.52
0.888	500	0.05	-1.30
1.036	10	0.001	-3.00

THE EFFECT OF TEMPERATURE ON GROWTH AND TOXIN PRODUCTION

The rates of growth and toxin development was studied in three media and at seven incubation temperatures between 0-37°C (32-98.6°F) using six strains of C. botulinum Type E.

PROCEDURES

Media.—The three media currently employed in the study are haddock homogenate, beef infusion and trypticase peptone broth.

The haddock homogenate was prepared by blending 1 part of frozen haddock fillets (Newfoundland) with two parts of distilled water in a Serval Omni-Mixer. The homogenate was filtered at 2°C. Part of the tissue pulp was dispensed into 20x150 mm (25 ml) screw cap tubes and then filled with filtrate. The tubes were immediately sterilized with 3 megarads of gamma radiation. The pH of the haddock homogenate was 7.2.

The composition of the broth media was as follows:

Beef Infusion Broth (BI)

Beef Infusion*	1 liter
Bacto Peptone	1%
Di-Sodium Phosphate	0.2%
Glucose	0.2%
Sodium Chloride	0.5%
Sodium Thioglycollate	0.1%
Dried Meat Particles	
pH 7.2-7.4	

Trypticase Peptone Broth (TP)

BBL Trypticase	5%
Bacto-Peptone	0.5%
Sodium Thioglycollate	0.1%
Glucose**	0.2%
pH 7.2-7.4	

The broth media was contained in 16x150 mm screw cap tubes.

*1 pound of beef/liter distilled water, boiled 1/2 hour, chilled, filtered.
**Added after sterilization.

Inoculum.—The six Type E strains used in this experiment were Vancouver Herring, Forks, Beluga, E-74, Nanaimo, and Iwanai. Spores of the organisms to be used for inocula were grown in 200 ml trypticase peptone broth at 33°C for 36 hours. The spores were washed six times in cold distilled water and stored in distilled water under refrigeration. Viable counts of the stock suspensions were made in beef infusion agar (see Table 41).

TABLE 41

VIABLE COUNTS OF STOCK SPORE SUSPENSIONS OF C. BOTULINUM, TYPE E

Strain	Count* (Spores/ml)
Nanaimo	8.0×10^8
Vancouver Herring	2.7×10^9
Forks	4.7×10^9
Beluga	4.3×10^9
E-74	1.3×10^9
Iwanai	3.0×10^8

*Pre-heated 10 minutes at 65°C.

For inoculation of the culture tubes an aliquot of the stock suspension was heated and detoxified at 65°C for 10 minutes. The suspension was diluted 1000-fold in sterile distilled water to produce a final concentration of 10^6 spores per ml. One ml of the spore suspension was used to inoculate tubes of haddock homogenate, while 0.1 ml was used for the same purpose in the tubes of broth. Immediately after inoculation, all tubes were layered with 3% agar containing 0.4% sodium thioglycollate. During the procedure of inoculation, all tubes were kept cold in an ice water bath. After inoculation, the tubes were placed in the incubators. Four tubes of haddock homogenate and beef infusion were inoculated per strain of organism for each incubation temperature. One tube of clear TP media was inoculated with each strain in order to observe changes in turbidity during growth.

Incubation.—The culture tubes were incubated at the following temperatures: 37.5°C (99.5°F), 33.0°C (91.5°F), 18.0°C (64.4°F), 8.3°C (46.9°F), 5.4°C (41.8°F), 3.3°C (38.0°F), and 0.0°C (32.0°F), ± 0.5°C (0.9°F).

BOD-type incubators were used for the lower temperatures.

In order to minimize temperature fluctuations in the culture tubes when the incubator doors were opened, the tubes were placed in a water bath. Temperatures were monitored by placing two thermocouples in a blank culture tube, one on the top and one on the bottom of the tube. The thermocouples were connected to both a manual and a recording potentiometer. Under these conditions, the temperatures within the incubators were found to vary between ± 0.5°C (0.9°F). The low temperature incubators for the cultures are shown in Fig. 23.

Growth and Toxin Assay.—Growth was ascertained by the observation of gas bubbles collecting beneath the agar layers, by the development of off odors and by clearance of the opalescence of the homogenates. Confirmation of growth was established by the presence of toxin. Where criteria of growth were absent, toxin assay alone was used.

For toxin determination in the cultures, the contents of the tubes were mixed and centrifuged to remove suspended particles. Mice were injected intraperitoneally with 0.5 ml of the supernatant fluid. Toxin was checked with homologous antitoxin. Titres of toxin were determined by making serial dilutions of the toxin in gelatin-phosphate buffer.

Samples which gave low titres or no deaths were treated with 0.1% trypsin at 37°C for 1-2 hours at pH 5.8-6.0.

RESULTS

At the incubation temperatures of 37.5°C and 33°C, cultures developed gas in all culture tubes within 24 hours, whereas at 18°C, the average time required was 2-3 days. All of the strains but one produced cultures in haddock homogenates within 9-12 days at 8.3°C; in broth, cultures developed 1-2 days earlier at this temperature. Two strains, the Iwanai and Vancouver Herring, have not developed gas in haddock homogenates after 250 days of incubation at 5.4°C; the other four strains developed gas within 15 days. In broth, all the strains developed gas at this temperature within 138 days, but the time of gas production varied with the different strains. At 3.3°C, only one tube out of four inoculated with spores of strain E-74 showed definite gas production. The Beluga strain grew in beef infusion and trypticase broth in 20 days; the Forks strain grew in beef infusion in 12 days. At 0°C no growth has become evident in any strain after 250 days of incubation. Data for the growth experiment are summarized in Fig. 24 and Table 42.

The results of the toxin assays performed on the cultures developed in haddock homogenates are summarized in Fig. 25. Except with strain E-74, no toxin was detected in the haddock homogenates after two days of incubation at 37°C. Maximum titres occurred at 18°C, being of the order of 200-20,000 MLD per ml, without activation. At 5°C low titres, 2 MLD, of toxin were obtained after 38 days of incubation. The titres were increased to 8-16 MLD

Fig. 23. Incubators used for low temperature growth studies of bacterial cultures.

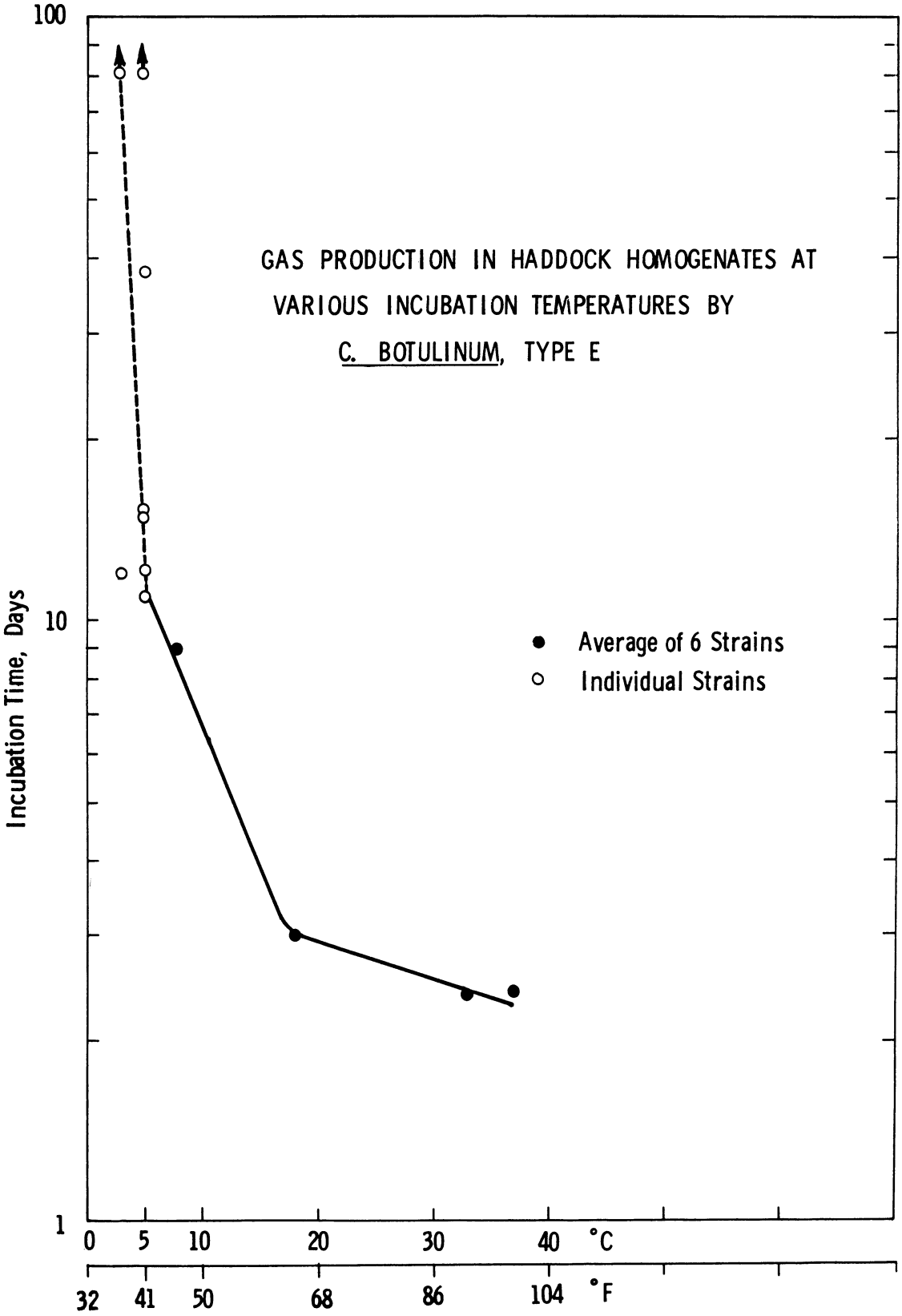

Fig. 24. Gas production in haddock homogenates at various incubation temperatures by C. botulinum Type E.

TABLE 42

TIME FOR GAS FORMATION BY C. BOTULINUM TYPE E IN VARIOUS MEDIA AND AT VARIOUS TEMPERATURES

Type E Strain	Incubation Temperature		Time to Develop Growth					
			Haddock Homogenate		Trypticase Peptone		Beef Infusion	
	°C	°F	hr	days	hr	days	hr	days
Nanaimo	37.5	99.5	48		<24		<24	
	33.0	91.5			<24		<24	
	18.0	64.4	72					
	8.3	46.9		10		12		
	5.4	41.8		12		38		50
	3.3	38.0		<290		<290		<290
	0.0	32.0		<290		<290		<290
Vancouver Herring	37.5	99.5	48		<24		<24	
	33.0	91.5	48		<24		<24	
	18.0	64.4	72		48		48	
	8.3	46.9		12		12		9
	5.4	41.8		38		50		50
	3.3	38.0		<290		<290		<290
	0.0	32.0		<290		<290		<290
Forks	37.5	99.5	48		<24		<24	
	33.0	91.5	48		<24		<24	
	18.0	64.4	96		48		48	
	8.3	46.9		12		8		7
	5.4	41.8		15		38		12
	3.3	38.0		<290		<290		138
	0.0	32.0				<290		<290
Beluga	37.5	99.5	48		<24		<24	
	33.0	91.5	48		<24		<24	
	18.0	64.4	72				48	
	8.3	46.9		10		8		7
	5.4	41.8		15		21		12
	3.3	38.0		<290		20		20
	0.0	32.0		<290		<290		<290
Iwanai	37.5	99.5	48		<24		<24	
	33.0	91.6	48		<24		<24	
	18.0	64.4	96		48			
	8.3	46.9		38		12		12
	5.4	41.8		38		38		80
	3.3	38.0		<290		<290		<290
	0.0	32.0		<290		<290		<290
E-74	37.5	99.5	48		<24		<24	
	33.0	91.5	48		<24		<24	
	18.0	64.4	72		48		48	
	8.3	46.9		9		10		7
	5.4	41.8		11		12		12
	3.3	38.0		12		<290		<290
	0.0	32.0		<290		<290		<290

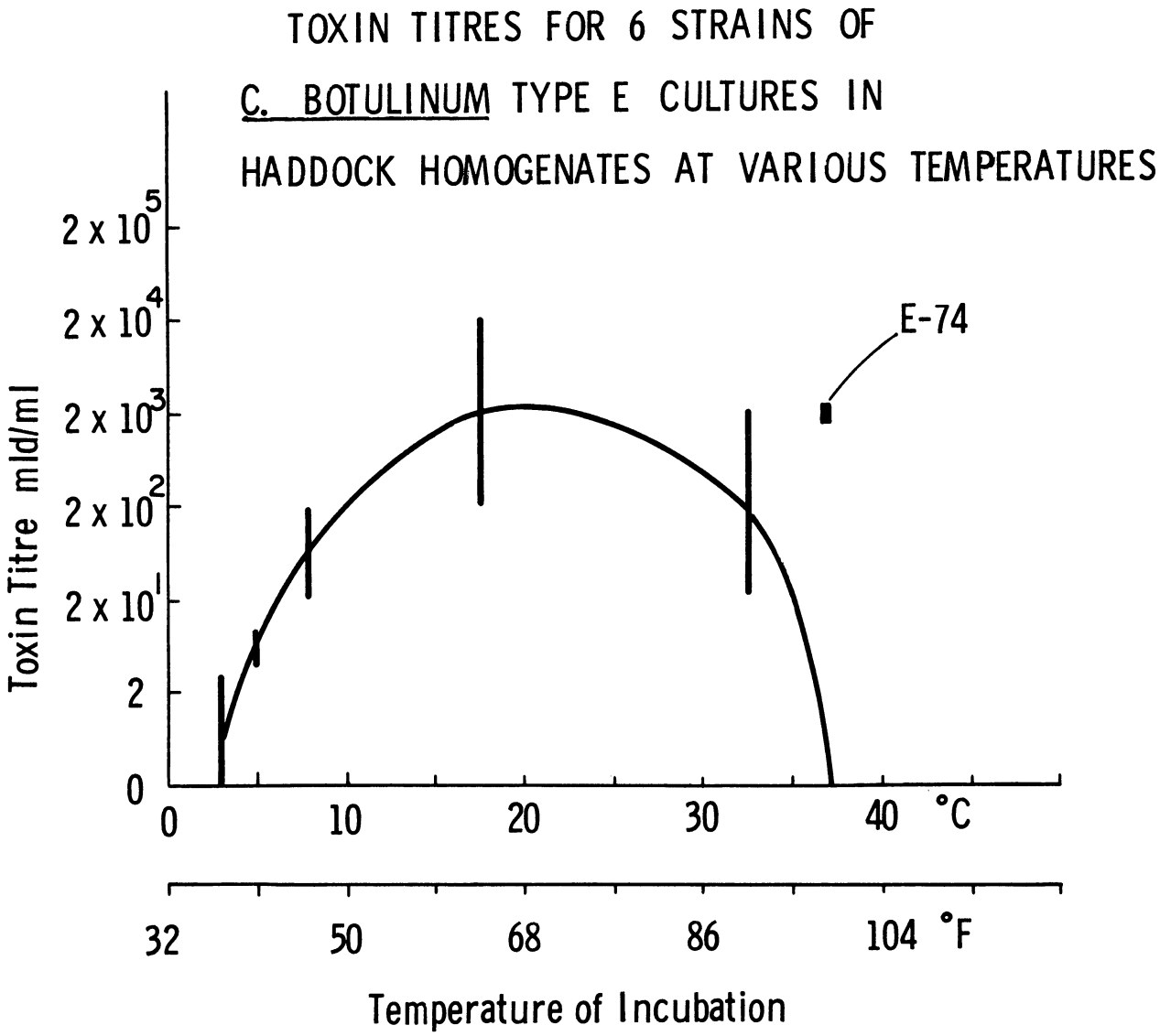

Fig. 25. Toxin titres for 6 strains of C. botulinum Type E cultures developed in haddock homogenates at various temperatures.

by activation with trypsin. At 3.3°C no toxin was detected in any strain until activation with trypsin after 38 days incubation; then low titres, 4 MLD, were yielded. At 0°C no toxin was detected after 80 days incubation.

After approximately 250 days of incubation the samples which were incubated at 3°C and 0°C were checked for the presence of toxin and viable spores in the culture tubes. Periodic checks of the cultures incubated at these low temperatures did not indicate any growth as evidenced by gas formation. Table 43 shows that no toxin was detected in cultures incubated for 250 days at 3.3°C with or without treatment with trypsin. However, when

TABLE 43

TOXICITY CHECK OF HADDOCK HOMOGENATE SAMPLES INCUBATED AT 3°C FOR 250 DAYS

Strain	Deaths	
	No Enzyme	With Enzyme
Beluga	0/4	0/4
Vancouver Herring	0/4	0/4
E-74	0/4	0/4
Iwanai	0/4	0/4
Nanaimo	0/4	0/4
Forks	0/4	0/4

these cultures were transfered to 33°C growth readily occurred within 48 hours and toxin was detected in all cultures, Table 44. Similarly, a set

TABLE 44

TOXICITY OF HADDOCK HOMOGENATE SAMPLES INCUBATED AT 3°C FOR 250 DAYS THEN AT 33°C FOR 48 HOURS

Strain	Growth	Deaths
Beluga	+	4/4
Vancouver Herring	+	4/4
E-74	+	4/4
Iwanai	+	4/4
Nanaimo	+	4/4
Forks	+	4/4

of cultures which had been incubated at 0°C for the same length of time when transfered to 33°C grew out readily, Table 45. In this case one culture that was

TABLE 45

TOXICITY OF HADDOCK HOMOGENATE SAMPLES INCUBATED AT 0°C FOR 280 DAYS THEN AT 33°C FOR 5 DAYS

Sample	Growth	Deaths
Beluga	+	0/4
Vancouver Herring	+	4/4
E-74	+	4/4
Forks	+	4/4
Iwanai	+	4/4
Nanaimo	+	4/4

inoculated with the Beluga strain did not produce toxin. It is evident that the spores are capable of maintaining their viable state after prolonged incubations at low temperatures in haddock homogenates.

DISCUSSION

It was observed that low levels of toxin may be present even though outward manifestations of growth, such as gas production in the cultures, are absent. Furthermore, samples that are suspected of containing toxin must be activated with trypsin before the absence of toxin can be established. In our studies, however, activation or potentiation of the performed toxin in haddock homogenates and other seafood substrates has been variable. In some cases, titres were increased slightly; in a few cases, actual decreases in titres occurred. This procedure needs further investigation. Since tissue enzymes are not inactivated by gamma radiation, such enzymes may conceivably potentiate the toxin precursor or degrade the toxin, or even accomplish both concurrently.

OTHER GROWTH STUDIES

The turbidity of media containing tissue particles makes it difficult to follow the parameters of growth, such as the length of the lag phase and the extent of growth. Another method being employed in our studies is that

of following turbidity changes in clear broth cultures in order to obtain corollary information.

Results of an experiment in which spores of E-74 were inoculated in trypticase peptone broth and incubated at various temperatures are presented in Fig. 26. In this experiment approximately 10^5 spores were inoculated into 7 ml of trypticase peptone broth (containing 0.2% glucose) contained in screw cap tubes. One tube was withdrawn from incubation at the time intervals indicated, and turbidity was measured.

The rate of growth was greatest at 37°C, but the extent of growth was greatest at 33°C, which is the appproximate optimum temperature for these organisms. There was a progressively greater lag and lessened growth as the temperature was lowered. At 5°C very little turbidity developed.

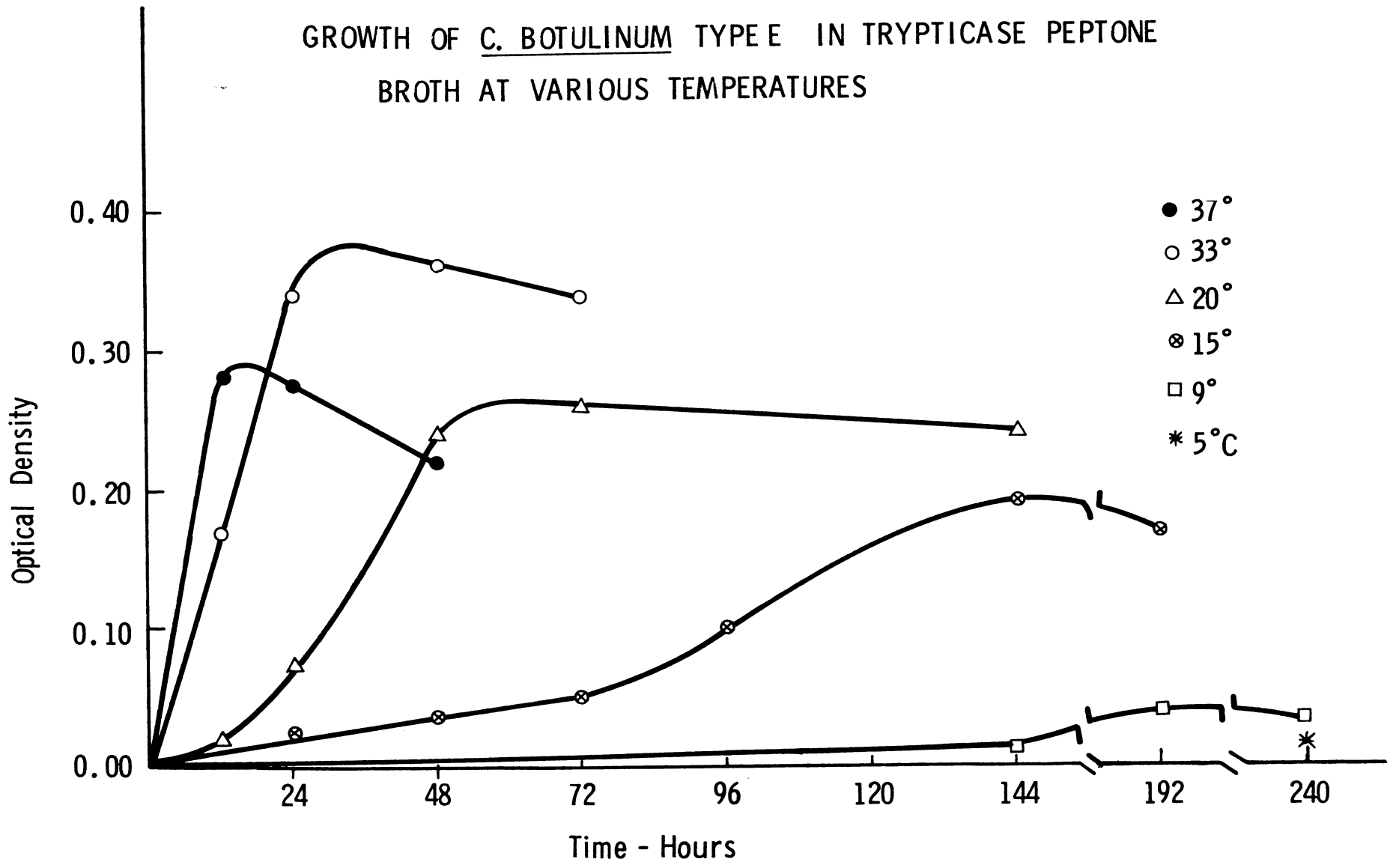

Fig 26. Growth of C. botulinum Type E in trypticase peptone broth at various temperatures.

EFFECT OF PRE-IRRADIATION TREATMENT ON THE OUTGROWTH OF C. BOTULINUM TYPE E SPORES IN HADDOCK HOMOGENATES

The objective of the present experiment was to determine what effect, if any, a pre-irradiation treatment of the spores used as an inoculum would have on the germination and outgrowth in haddock homogenates. In our previous growth experiment the spores used for inoculum were not irradiated; the haddock homogenate was radiation sterilized. It was observed in the previous growth experiment, that non-irradiated spores would germinate and grow and produce toxin when inoculated into radiation sterilized homogenates. The radiation sterilized media did not seem to have an adverse effect on the outgrowth potential of the organisms. The question then arises whether pre-irradiation of the spores would stimulate or possibility delay the germination and outgrowth of the spores in the same media.

EXPERIMENTAL

The preparation of the haddock homogenates, preparation of cultures, and incubation methods were the same as outlined in the growth experiment.

Washed, detoxified spores of 3 strains, Beluga, Nanaimo, and Forks, were used as inocula. An initial viable count of the stock spore suspensions was made and then an aliquot of the stock suspension was irradiated with approximately 350,000 rads, a dose in the radiation pasteurization range. These treatments resulted in a 10-fold decrease in viable cells. Table 46 gives the viable counts of the spores before and after irradiation. For preparation

TABLE 46

NUMBER OF VIABLE SPORES USED AS INOCULUM FOR GROWTH STUDIES

Strain	Not Irradiated	Irradiated
Forks	1.2×10^7	1.9×10^5
Nanaimo	4.2×10^7	1.4×10^5
Beluga	5×10^6	1.4×10^5

of the inoculum, the non-irradiated spores were diluted with sterile water to give an approximate concentration as that obtained after irradiation treatment. This concentration was equal to 10^5 viable spores per ml. One ml was used to inoculate each of 5 tubes of haddock homogenate.

An additional experiment, to test lower levels of inocula size was also run simultaneously. In this experiment the spore suspension of the Beluga strain was diluted further, to given concentrations of 10^6, 10^5, 10^4, and 10^3.

Three temperatures, 20°, 8°, and 4°C, were selected for incubation of the cultures. The 20°C incubation temperature acted as a control to show if growth would occur. At 8°C all tubes should show growth, however the temperature is low enough that a long lag period would occur before growth would be evident. Therefore, any effect of the inoculum on initiation of growth should be apparent at this temperature. A 4°C incubation temperature is borderline for Type E. That is, growth may or may not occur at this temperature depending on the strain and media.

RESULTS AND DISCUSSION

All tubes incubated at 20°C showed gas production within 48 hours indicating that the conditions were satisfactory for growth to occur.

With the Beluga strain growth as shown by gas production occurred at 8°C within 10 days both with the irradiated and non-irradiated spore inocula, Table 47. At 4°C, growth occurred only in the tubes inoculated with

TABLE 47

EFFECT OF PRE-IRRADIATION TREATMENT ON THE OUTGROWTH OF C. BOTULINUM TYPE E SPORES IN HADDOCK HOMOGENATES

Strain—Beluga

Sample	Growth		
	20°C	8°C	4°C
Not Irradiated 10^5 spores/tube	1/1 2 days	4/4 10 days	5/5 19 days
Irradiated 10^5 spores/tube	1/1 2 days	5/5 10 days	0/5 19 days
Not Irradiated 10^4 spores/tube	1/1 2 days	5/5 10 days	0/5 19 days

the non-irradiated spores after 19 days of incubation.

The third column, that is tubes inoculated with 10^4 non-irradiated spores per tube, acted as control to determine growth at a 10-fold less concentration of spores than used in the tubes inoculated with the irradiated spores. That is if irradiation of the spore inocula had an effect, growth in these tubes could be used to compare the magnitude of the effect. In the tubes inoculated with 10^5 and 10^4 non-irradiated spores gas production observed was more vigorous than that observed with tubes inoculated with 10^5 irradiated spores. It is apparent that a pre-irradiation treatment had some effect on the spores since the irradiated spores did not grow out at 4°C during the length of this experiment, that is 20 days, and gas production at 8°C was much less.

With tubes inoculated with spores of the Forks strain, Table 48, growth occurred with the non-irradiated spores within 10 days at 8°C. With the

TABLE 48

EFFECT OF PRE-IRRADIATION TREATMENT ON THE OUTGROWTH OF C. BOTULINUM TYPE E SPORES IN HADDOCK HOMOGENATES

Strain—Forks

Sample	Growth		
	20°C	8°C	4°C
Not Irradiated 10^5 spores/tube	1/1 2 days	5/5 12 days	0/5 19 days
Irradiated 10^5 spores/tube	1/1 2 days	5/5 19 days	0/5 19 days
Not Irradiated 10^4 spores/tube	---	5/5 19 days	0/5 19 days

irradiated spores growth was delayed till 19 days at this temperature. No growth in any of the tubes occurred at 4°C within 20 days.

With spores of the Nanaimo strain, growth at 8°C occurred only in the tubes inoculated with the non-irradiated spores, and this was only after 19 days of incubation, Table 49. Gas production was weak in these tubes. No growth was observed in the tubes inoculated with the irradiated spores at 8°C and with either spore suspensions at 4°C.

The results of this experiment would indicate that a pre-irradiation treatment of the spores has some effect on the germination and subsequent growth.

TABLE 49

EFFECT OF PRE-IRRADIATION TREATMENT ON THE OUTGROWTH OF C. BOTULINUM TYPE E SPORES IN HADDOCK HOMOGENATES

Strain—Nanaimo

Sample	Growth		
	20°C	8°C	4°C
Irradiated 10^5 spores/tube	1/1 2 days	5/5 19 days	0/4 19 days
Not Irradiated 10^5 spores/tube	1/2 2 days	0/5 19 days	0/5 19 days
Not Irradiated 10^4 spores/tube	---	0/5 19 days	0/5 19 days

A more definitive system of measuring growth is needed to more clearly establish the phenomena. In haddock homogenates growth is difficult to ascertain other than by gas production. Gas production can be variable, especially at lower temperatures of growth, with these strains. Even with this gross index of growth there seems to be an effect of pre-irradiation of the spores on the subsequent development at low temperatures.

The results obtained with the Beluga strain are more clear cut because of the ability of this organism to grow at lower temperatures of incubation than the other two strains. The results with the Forks strain at the 8°C temperature of incubation which showed the lag period for outgrowth of the irradiated spores would also indicate that pre-irradiation has an effect on the outgrowth potential.

The effect of spore concentration on initiating growth in haddock homogenates at 8°C does not seem to be important in the range of 10^6-10^3 spores per tube. No delay in growth with this range of inocula was observed, Table 50.

TABLE 50

EFFECT OF SPORE CONCENTRATION ON OUTGROWTH OF *C. BOTULINUM* TYPE E SPORES IN HADDOCK HOMOGENATE AT 8°C

Strain—Beluga

Spore Concentration per Tube	No. Spoiled	Days to Spoil
5×10^6	5/5	11
5×10^5	5/5	11
5×10^4	5/5	11
5×10^3	5/5	11

ISOLATION OF *CLOSTRIDIUM BOTULINUM* FROM FISH FROM LAKE MICHIGAN

During the course of this investigation an outbreak of Type E botulism occurred in Michigan involving smoked fish. The source of the large smoked whitefish which caused the death of a elderly couple in Kalamazoo in October 1963 was not ascertained. The smoked whitefish chubs which were responsible for the second outbreak of Type E botulism outbreaks in Tennessee, Kentucky, and Alabama during October 1963 were processed at Grand Haven, Michigan. The source of the chubs was reportedly from Lake Michigan. The question arose in the many discussions which followed the outbreaks on the possible source of contamination of the fish involved in the outbreaks. As pointed out in the historical part of this report all outbreaks of Type E botulism, with one exception were due to the consumption of fish, fish products, or sea mammals. Therefore, it would be reasonable to expect that the fish were the source of the contamination. At the time of the outbreak there was no information indicating that *Clostridium botulinum* Type E was present in the Great Lakes. Personal communication with Dr. E. M. Foster of the University of Wisconsin after the outbreaks indicated that he had obtained cultures from fish which were toxic for mice and were neutralized by Type E antitoxin.

The object of this survey was to determine if Type E organism was present on the fish in the general area which may have been the source of the chubs which caused the outbreaks.

Arrangements were made through Dr. S. Smith of the U.S. Bureau of Commercial Fisheries early in November to obtain some specially caught and handled fish from the area frequented by commercial fishermen.

A catch of fish was obtained by gill nets due west of Saugatuck, Michigan on December 4, 1964. Two catches were made, one at 20-21 fathoms the other at 10-11 fathoms. The fish were individually removed from the nets by the Bureau personnel and immediately placed into pre-sterilized mason jars, one fish per jar. Special care was taken to prevent cross contamination. The jars were immediately iced thoroughly and transported to Ann Arbor the same day. The catch consisted of a total of 55 fish comprising perch, chubs, 1 smelt, and 6 alewife.

For bacteriological examination Defco brain heart infusion broth +0.1% agar was used. The broth was contained in larger 50 ml screw cap tubes or 200 ml bottles with 120 ml of media. The media was autoclaved at 121°C for 40 minutes, cooled and immediately used for subculturing.

Two techniques were used for sampling. In one case the fish was carefully removed from the jars, the surface slime layer swabbed with sterile cotton swab, and the entire swab subcultured into a tube of BHI broth. The fish then was asceptically opened and the entire digestive tract and other organs were excised and placed into the jars containing media. Some of the jars were placed in anaerobe jars others were incubated without anaerobiosis. The cultures were incubated for 7 days at 30°C. They were then transferred to a refrigerator.

For toxin assay, a sample was centrifugal at 6000 rpm for 10 minutes and 0.5 ml of the supernatant was injected in each of 4 20-gram mice. Mice were observed for botulism symptoms for 48 hours before being discarded.

The following code was used in identifying samples.

L = Large mouth jars = Depth 20-21 fathoms
S = Small mouth jars = Depth 10-11 fathoms
P = Perch
C = Chub
A = Alewife
S = Smelt
S = Swab
G = Gut
1, 2, 3 ... etc. Number designation of fish
H = Head

The first series of samples checked for presence of toxin in the primary cultures is presented in Table 51. These samples represent swabs of individual fish. Two supernatant samples, S-P-2-S (perch) caught at 10-11 fathoms

TABLE 51

TOXIN CHECK OF FISH SWAB SUBCULTURES

Sample	Total Deaths, 48 hr	Time of Deaths		
		1 hr	5 hr	12 hr
L-C-5-S	1/4	sick	sick	1/4
L-P-3-S	1/4	---	---	---
S-C-1-S	1/4	---	3 sick atypical	3 sick
S-P-1-S	1/4	---	---	---
S-P-2-S	4/4	---	2 sick 2/4 bot sym	2/4
L-C-1-S	4/4	1 dead 5 min sick bot sym	3/4	3/4
L-C-4-S	0/4	sick	---	---

bot sym = botulinum symptoms

and L-C-1-S (Chubs) caught at 20-21 fathoms proved to be toxic for all four mice. Several of the other samples caused a reaction in mice but were atypical. The two samples definitely produced botulinum symptoms in all mice. All mice were dead within 12 hours, in one case within 5 hours. This rapidity of death is characteristic with Type E intoxication.

The next day the supernatant of the two toxic cultures was checked for type specific toxin. The supernatant was diluted 1:10 with gelatin phosphate buffer. The dilution was separated into several aliquots. One was heated at 60°C for 10 minutes, one combined with 0.2 ml of antitoxin E and one with bivalent A-B antitoxin. After allowing the neutralization test to incubate for 1 hour at refrigerator temperatures, 0.5 ml of each sample was injected into mice. The results present in Table 52 show that the toxin in both cultures was definitely neutralized by Type E antitoxin.

TABLE 52

CHECK OF TOXIC SUBCULTURES FROM FISH SWABS

Sample	Deaths, Number	Time
S-P-2-S		
Supernatant 0	4/4	3/4 7 hr
Supernatant diluted 1/10	4/4	4/4 17 hr
Supernatant heat 60°C 10 min	0/4	---
Supernatant diluted 1/10 + AT E	0/4	---
Supernatant diluted 1/10 + AT AB	1/4	*
L-C-1-S		
Supernatant 0	4/4	7 hr
Supernatant 1/10	4/4	7 hr
Supernatant + heat 60°C 10 min	0/4	---
Supernatant 1/10 + AT E	0/4	---
Supernatant 1/10 + AT AB	4/4	7 hr

*1/4, 3 sick at 17 hr recovered.

With sample S-P-2-S and A-B antitoxin, the mice showed typical botulinum symptoms, however only 1 died and the other 3 recovered. The time for deaths was delayed in the mice injected with the 1/10 dilution of the supernatant indicating a low level of toxin being present. The addition of 0.2 ml of antitoxin to approximately 2.2 ml of supernatant may have been sufficient to dilute the toxin further. The other possibility that a slight cross reaction between the E toxin and either A or B antitoxin cannot be ruled out.

The precipitates of the primary isolation culture which were obtained in preparing the supernatants for mouse tests were all heated at 50°-55°C for 30 minutes. Approximately one ml was transferred to TPG tubes and incubated at 33°C for 48 hours. After incubation the supernatant was tested for the presence of toxin by injecting 0.5 ml in each of 4 mice. The results of this test, Table 53, show that only cultures S-P-2-S and L-C-1-S which were toxic in primary culture were also toxic on subculture. All the precipitates when examined microscopically showed the presence of spores.

TABLE 53

TOXICITY OF SWAB CULTURES SUBCULTURED INTO TRYPTICASE BROTH

Sample	Deaths	Time
S-C-1-S	0/4	
S-P-2-S	4/4	2/4 3 hr, 4/4 12 hr
S-P-3-S	0/4	
L-C-1-S	4/4	1/4 3 hr, 2/4 3-1/2 hr, 4/4 12 hr
L-C-5-S	0/4	
S-P-1-S	0/4	

The supernatant of the S-P-2-S and L-C-1-S of the trypticase broth culture were checked further for toxin titres and with the S-P-2-S culture for specific toxin neutralization. The results of this test, Table 54, show that a titre of toxin in excess of 200 MLD was produced in the cultures; a titre characteristic of Type E cultures. Furthermore the S-P-2-S isolate definitely was producing Type E toxin and the slight discrepancy in neutralization with A and B antitoxin observed previously in the primary culture was not apparent in the subculture.

A second series of cultures was then tested for toxicity, Table 55. This series included subcultures of the intestines of the chubs along with two cultures which included the head only of the chub. In this series great difficulty was experienced upon the injection of the supernatant material into mice. The mice reacted violently immediately upon injection. Many mice died very shortly after injection with atypical symptoms. Any observation for botulinum symptoms was overshadowed by this reaction. Only one intestine subculture and the 2 fish head subcultures did not produce this reaction.

Two cultures, L-C-1-G and L-C-4-G, were checked further in order to characterize the toxic factor present in the supernatnat. Part of the supernatant material was heated and some of the unheated material diluted with gela-

TABLE 54

TOXICITY OF TRYPTICASE BROTH CULTURES OF S-P-2-S AND I-C-1-S

Sample	Dilution	Deaths, Total	Time
S-P-2-S	1/10	4/4	6 hr
	1/100	4/4	2 sick 6 hr 4/4 16 hr
	1/10 + AT AB	4/4	6 hr
	1/10 + AT B	4/4	6 hr
	1/10 + AT E	0/4	6 hr
L-C-1-S	1/10	4/4	6 hr
	1/100	4/4	6 hr

TABLE 55

TOXICITY OF FISH SUBCULTURES

Sample	Deaths	Time
L-C-1-G*	4/4	2/4 3 hr, 4/4 4-1/2 hr
L-C-2-G	4/4	Sick 30 min, 4/4 12 hr
L-C-3-G	4/4	2/2 45 min, 4/4 4-1/2 hr
L-C-4-G*	4/4	4/4 30 min
L-C-5-G	4/4	2/4 30 min, 4/4 4-1/2 hr
L-C-6-G	1/4	48 hr
L-C-1-H	0/4	48 hr
L-C-1-H	0/4	48 hr

tin phosphate. The results of the animal inoculation, Table 56, indicate that in the case of the L-C-4-G culture the toxic factor was heat stable and could be diluted out a hundred fold without loss in potency. In the case of the L-C-1-G culture heat lability was noted and dilution did not eliminate the toxicity. In this case deaths were delayed for considerable time. Conceivably this culture could have contained botulinum toxin, but it was not tested further. It must be pointed out in this test the mice did not have typical bolutinum symptoms.

TABLE 56

TOXICITY OF FISH SUBCULTURES

Sample	Deaths, Total	Time
L-C-1-G		
0	4/4	16 hr
0 + heat	1/4	16 hr
Diluted 1/10	4/4	16 hr
Diluted 1/100	3/4	16 hr
L-C-4-G		
0	4/4	15 min
0 + heat	3/4	15 min
Diluted 1/10	4/4	16 hr
Diluted 1/100	4/4	16 hr

A third series of primary subcultures were next tested. This series included the rest of the swab subcultures from the fish and some intestinal subcultures from perch, Table 57. The object here being to test if the acute toxicity for mice observed with subcultures of chub intestines would also occur with perch. In case of the swab subcultures only one S-P-9-S showed toxicity which was typical of botulinum intoxication although death was delayed for some time. In the case of the subcultures of the intestinal tract of the perch, the same results were experienced as with the chub cultures. The mice reacted violently and death occurred rapidly. However, by careful observation the mice injected with the supernatants of S-P-7-G and S-P-6-G cultures showed typical botulism symptoms. These were selected for further tests. Three intestinal cultures did not kill mice, although two of the cultures produced a reaction in mice and they recovered.

TABLE 57

TOXICITY OF FISH SUBCULTURES

Sample	Deaths, Total	Time
Smelt 1,2 S	0/4	
S-C-1,2 S	0/4	
S-C-11-S	0/4	
S-P-11-S	0/4	
S-P-8-S	0/4	
S-P-9-S	4/4	3/4 20 hr, 1/4 44 hr
S-C-16-S	0/4	
S-C-8-S	0/4	
S-C-10-S	0/4	
S-C-9-S	0/4	
Alewife-2-G	0/4	Sick recovered
S-C-5-G	0/4	Sick recovered
S-P-7-G	4/4	Sick 30 min
Alewife-1-G	4/4	Sick 30 min
S-P-6-G	4/4	1/4 30 min, 3/4 bot sym 2 hr
S-P-1-G	1/4	48 hr
S-C-3-G	4/4	4/4 20 min
S-C-4-G	4/4	4/4 15 min
Smelt-1-G	0/4	---

Cultures S-P-7-G, S-P-6-G, and S-P-9-S were rechecked in mice for toxicity after diluting 1-2 with gelatin phosphate buffer and after heating at 60° for 10 minutes in order to test for heat lability of the toxic material. The results of Table 58 show that the toxic factor in cultures S-P-7-G and S-P-6-G was heat labile. The slight toxicity observed with S-P-9-S in primary culture was lost by 1-2 dilution of the supernatant material. Toxin neutralization tests with the supernatant liquid of S-P-7-G and S-P-6-G show that Type E toxin was present in the S-P-6-G culture, Table 59. With the S-P-7-G culture either A or B toxin was present but not E. A further neutralization test with the supernatant of the S-P-7-G culture indicated the toxic factor to be neutralized by Type A antitoxin, Table 60. One notices here that there is a slight delay in deaths in culture at the various dilutions. This further demonstrates the slower action of Type A toxin for mice as compared to Type E toxin.

TABLE 58

TOXICITY CHECK OF FISH SUBCULTURES

Sample	Dilution	Deaths, Total	Time
S-P-7-G	1:2	3/4	4 hr
S-P-6-G	1:2	4/4	1-1/2 hr
S-P-9-S	1:2	0/4	
S-P-7-G	1:2 heated	1/4	2 days
S-P-6-G	1:2 heated	0/4	
S-P-9-S	1:2 heated	0/4	

TABLE 59

TOXIN NEUTRALIZATION OF TOXIC FISH SUBCULTURES

Sample	Deaths	Time
S-P-7-G		
Supernatant	4/4	4 hr
Supernatant + AT AB	0/4	
Supernatant + AT E	4/4	4 hr
S-P-6-G		
Supernatant	4/4	4 hr
Supernatant + AT AB	4/4	4 hr
Supernatant + AT E	0/4	

The precipitate of the cultures obtained from the preparation of supernatant for assay was heated at 50-55°C for 30 minutes and then subcultured into TPG broth. After incubation at 33°C for 48 hours the supernatant was tested for toxin. In case of S-P-7-G and S-P-6-G cultures, the supernatant proved toxic for mice, Table 61. However, in the case of the S-P-9-G, culture toxicity was not observed. The slight toxicity observed previously in the primary cultures would indicate that the organism was present in a very low number and did not sporulate sufficiently to produce an inoculum to initiate a subculture as in the case of the other two cultures.

TABLE 60

TOXIN NEUTRALIZATION OF TOXIC SUBCULTURES

Sample	Dilution (Supernatent)	Deaths	Time
S-P-7-G	0	2/2	7 hr
	1/10	4/4	1/4 7 hr, 3/4 18 hr
	1/100	2/4	24
	1/1000	0/4	---
	1/10 + AT AB	0/4	---
	1/10 + AT B	4/4	1/4 7 hr, 3/4 18 hr
	1/10 + AT E	4/4	3/4 18 hr

TABLE 61

TOXICITY OF TRYPTICASE BROTH CULTURES
OF FISH SUBCULTURES

Sample	Deaths
S-P-7-G Supernatant	2/2
S-P-6-G Supernatant	1/2
S-P-9-S Supernatant	0/2

The remaining cultures to be tested consisted of subcultures of the intestines of the various fish. Since great difficulty was experienced with atypical deaths in mice when injected with this material, results would be meaningless in respect to determining the presence of botulinum toxin in the primary subcultures with the present technique. Further tests would require subculturing from the primary cultures into plates and picking typical Type E colonies. Since this would involve considerable effort and time the study was terminated.

However the primary objective was accomplished, the isolation of C. botulinum Type E from fish caught in the lake.

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