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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 been allowed to grow. With one exception, all known cases of Type E botulism have resulted from eating fish, fish products, and sea mammals. C. botulinum Type E bacteria have reportedly been recovered from the intestinal contents of fish and from mud samples taken in Japan, British Columbia, Greenland, Denmark, and France. When Type A or B botulism has resulted from eating fish, it is believed to have been caused by incidental contamination during processing, although actual transmission by fish cannot be overlooked.

In the present study, six representative strains of C. botulinum Type E were obtained and their sporulation characteristics studied. These bacteria were found to sporulate quite readily in a trypticase medium. This medium also was used to produce large numbers of such spores in 10-liter flasks. The Type E spores were found to be considerably less resistant to gamma radiations from cobalt-60 than Type A spores. When suspended in an extract of haddock tissue, Type E spores were more resistant to gamma radiations than they were when suspended in phosphate buffer. Growth, sporulation, and toxin synthesis were found to occur quite readily in clam juice and in haddock and shrimp extracts. Toxin synthesis is being studied in radiation-sterilized foods. The aforementioned studies are being extended, and studies of the growth and toxin synthesis in fish and seafood substrates by C. botulinum Type E bacteria in the temperature range of 0° to 30°C are being initiated.

INTRODUCTION

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.

Generally speaking, botulism intoxication can be transmitted by proteinaceous foods which have a pH of 4.5 or above, whenever such foods are kept under anaerobic conditions at temperatures suitable for the growth of C. botulinum. The foods commonly associated with human botulism are canned vegetables, fruits, fish and fish products, and meat, and animal products.

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 these strains have been identified as the causative agents of human botulism. While only Strains A, B, and E are of major concern at present, the possibility of involvement of the others cannot be overlooked.

Human botulism, resulting from eating of contaminated fish, has so far only been ascribed to Types A, B, and E. However, botulism from fish and fish products is usually caused by Type E and is commonly designated in the literature as "fish poisoning."

Human botulism can be controlled in two general ways: prevention of growth of C. botulinum cultures in foods, and immunization of people eating the foods. The latter method is impractical except in unusual situations, such as protection of laboratory personnel studying this bacterium. Growth of C. botulinum in foods can be prevented in many ways, all of which are known to food technologists. Among the more practical preventive measures are sanitary practices in handling and preparing the food, adequate refrigeration, acidification to pH values below which C. botulinum spores cannot germinate, and processing to kill the spores and vegetative cells of C. botulinum accompanied by suitable packaging to prevent recontamination. It is rather obvious that these preventive measures can and should be used together for food preservation wherever feasible.

Good sanitation, refrigeration, and irradiation pasteurization to inactivate most of the common spoilage bacteria are useful in preserving fish in a "raw" condition. Such a pasteurization treatment will reduce the number of bacterial spores, but generally will not eliminate them. When C. botulinum spores survive the radiation treatment, they may exist in unusually suitable conditions for growth and toxin development since their usual bacterial com-

petitors will have been destroyed. It therefore becomes particularly important to evaluate the physiological conditions under which spores of C. botulinum Types A, B, and E can grow and develop toxin in fish after irradiation.

FISH-BORNE BOTULISM

The first proven incident of Type E botulism occurred 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 Laborador (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 Germany. The organisms involved in the outbreaks were isolated and characterized by Hazen (1937, 1938, 1942) who identified them 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 Fransico, California. It was attributed to mushroom sauce canned in California. The dehydrated mushrooms used were imported from Jugoslovia (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 Nanaimo, British Columbia, in 1944 (Dolman, 1947), due to 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 in Japan was reported from eating "izushi," a fermented relish made from fish, rice, and vegetables (Nakamura et al., 1956). Other reported outbreaks followed: one in France in 1952 (Prévot and Huet, 1951), in Denmark in 1951 (Pederson, 1955), and in Laborador in 1956 (Dolman, 1960).

The most recent outbreak of Type E botulism in the United States apparently occurred in Minneapolis, Minnesota, in September, 1960. Two deaths were reported there from the consumption of smoked ciscos (Anon., 1960). No detailed account appears to be available yet in the literature, specifically designating the strain as Type E.

During the years from 1899 to 1954, fish and seafoods have been incriminated in 32 outbreaks of botulism in the United States. 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, and pickled mackeral and herring. Only three of these outbreaks were caused by Type E. In Alaska, on the other hand, six 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).

Of the 24 recorded cases of botulism before 1960 in Canada, 66.7% were found to have been transmitted by fish, fish products, and sea mammals (Dolman, 1961). The products responsible 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 (Dolman, 1960a). All were due to fish: Type E was 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. This is 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 ten outbreaks that occurred in Norway, one was due to home-canned fish and one to "rakefish" made from lake trout. Type B toxin was isolated in both cases. In Denmark, of the ten outbreaks of botulism, five were due to salted or pickled mackerel and herring (Dolman, 1961). No organism was isolated in four of the cases, but Type E was obtained from one case due to herring (Pederson, 1955). In France, one case involving Type B was traced to tuna soup (Dolman, 1961), and three outbreaks of fish-borne botulism were attributed to Type E (Prévot and Huet, 1951). In the United Kingdom, an outbreak due to pickled herring from which a Type A culture was isolated, has been reported (Mackay-Scollay, 1958). Meyer (1956) lists 19 episodes of botulism in Germany in 1948 and 1949. Nine of these 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.

From the available information presented above, it is evident that Type E intoxications have always, with one exception, been associated with marine products. The single exception was an outbreak in California due to canned mushroom sauce. Dolman (1961) has compiled the data on the proven outbreaks of Type E botulism in the world as of 1960. A total of 50 outbreaks involving 242 cases and 85 deaths (35.1% fatalities) are recorded. The cases are

distributed as follows: Japan, 32 (Hokkaido, 19 and North Honshu, 13); Canada, 7 (British Columbia, 6 and Laborador, 1); United States, 9 (Alaska, 6; New York, 2; and California, 1); and one each for the USSR and Denmark. The outbreak in 1960 in Minneapolis should be added to the United States total.

The epidemological evidence points to a marine rather than a terrestrial habitat for the spores. If this assumption is valid, it should be possible to isolate Type E 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, 1960a). 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, 1957a).

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 tracts 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, Prévot and Huet (1951) were able to obtain one culture of Type E from a perch after the examination of the intestinal contents of 163 fresh- and 13 salt-water fish.

It is possible that the existence of C. botulinum Type E spores can be overlooked in soil samples because of the isolation techniques used. Usually soil samples are heated before sub-culturing; the heat treatment involved could be expected to inactivate Type E spores because of their lower heat resistance as compared to Types A or B. A toxigenic Type E culture was isolated from a soil sample taken from a chicken run in the yard of a home in Nanaimo, British Columbia, where a case of Type E botulism occurred. This same organism, which had caused the outbreak, was isolated from canned chicken and canned salmon found in the house. Thus, this finding of Type E spores in the soil may have been one of contamination rather than endogenous existence in the soil, since it is common practice in rural areas to bury the viscera of fish in the yard. In this instance the chickens could have dug up the viscera, which is even more likely because the man of the house indicated that he spaded up the spot because it "stank," indicating that the viscera were not buried deeply. The organism was isolated from a one-gram sample, thus indicating a high concentration of organisms in the soil.

The presence of C. botulinum Types A and B in marine muds has been reported only on infrequent occasions. Lang (1935) reported the isolation of a Type A strain from the slime on a tuna and also from fish just prior to the pre-cooking operation in canning. He concluded that his finding represented

incidental contamination during handling of the fish. A Type A strain was isolated from the bottom mud obtained in Bute Inlet, British Columbia (Dolman, 1960a). Ohye and Scott (1957) reported obtaining two toxigenic cultures from 22 mud samples collected off the coast of Tasmania and New South Wales. The toxic samples were protected by Type E but not by Types A or B antitoxin. Attempts to isolate the organism were unsuccessful. There is some possibility that these may have been Type B cultures since Type E toxin has been reported to cross-react with some Type B antitoxins. In another situation, Meyer (1956) reported that the intestines of sturgeon-type fish in Russia yielded 5% Type A and 11% Type B cultures.

It is generally conceded that the *C. botulinum* Type A and Type B intoxications transmitted by fish are due to contamination of the products during processing, whereas Type E intoxications are presumed to be due to Type E spores present with the fish when they are caught. This is borne out by the observation that of the 49 out of 50 proven outbreaks of Type E botulism listed above, all were of marine origin.

Therefore, it is evident that the conditions which lead to Type E botulism in man are important considerations in the radiation pasteurization of fish and fish products.

PHYSIOLOGICAL CHARACTERISTICS OF C. BOTULINUM TYPE E

Some of the 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 kind 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 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 alive.

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 of 43°F for 75 days.

In a paper presented at the Twenty First Annual Meeting of the Institute of Food Technologists, on May 8, 1961, Schmidt et al. (1961), reported further observations on the minimum growth temperatures for Type E cultures. In this paper 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 soil 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 $5x10^6$ 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 that 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; 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, proteolytic bacterium from "izushi," which was involved in an outbreak of botulism. When this bacterium was grown simultaneously with a Type E strain, a much higher

titre of Type E toxin resulted 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 might even possibly be destroyed, as has been shown to occur for Type A toxin in the presence of C. sporogenes (Jordan and Dack, 1924; 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; 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 RESULTS AND DISCUSSION

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 are to gather information on the germination, growth, and sporulation of representative strains of Type E, the factors which govern the synthesis of their toxin, and the resistance of these spores to radiation and heat. More specifically, the germination and outgrowth of Type E spores must be studied 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 radiation resistance and factors affecting the resistance of the spores to radiation need study to ascertain the effect of irradiation on the subsequent germination and outgrowth of the spores. Particular emphasis will be placed on C. botulinum Type E spores, but Types A and B spores will be also used where applicable.

STOCK CULTURES

Six strains of C. botulinum Type E are being 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, Vancouver herring (VH), Beluga, Iwani, E-74, and Seattle Forks Emesis. The cultures were received in a lyophilized condition in small vials. The vials were aseptically 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 are 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, and Strains 457-A, 169-A and 109-B were obtained from Dr. McClung of Indiana University. 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.

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 cultures 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, sorbitol, and trehalose. Fermentation of adonitol was variable.

GROWTH AND SPORULATION

Before we can study such subjects as the radiation 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 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 vegetative 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 of the five 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, refractible 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 aseptically. 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 at about 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 was tested: beef extract, yeast extract, phytone, neo-peptone, casamino acids, or thiotone for the Bacto-peptone. Yeast extract stimulated the Iwani, E-74, Nanaimo, and Seattle 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 irradiation experiments contemplated. Furthermore, since Schmidt _et al._ (1962b) used this medium previously in radiation resistance studies of Type E spores, we decied 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 an inoculum of 10^4 spores per ml of media, good growth and vigorous gas production occurred with three strains within 12 hours of 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 Sorvall 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.

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 portions 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 resistant spores. Spores of Strains 457-A, 169-A, 109-B, and 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.

The ability of Type E strains to grow and sporulate in various seafood media is presently being investigated. Using spore inocula, growth of each of five strains was observed in clams suspended in their juice, as well as in shrimp extract. Both of these media were first pasteurized with radiation. All five strains produced cultures and spores in these media. Toxin was found in one set of cultures tested. This effort is being extended.

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 proteolytic enzymes coming from other sources such as bacteria. Irradiation offers a method for separating these two proteolytic effects, since irradiation doses used for sterilization are not sufficient for the inactivation of enzymes. This concept was used in the experiment described next.

To compare toxin synthesis in raw and cooked seafoods, 300 ml of shucked clams were sterilized in their juice by irradiation; another set was heat sterilized. The samples were then inoculated with heat-shocked spores of the Iwani and Seattle Forks strains. The inoculated clams were incubated at 33°C for two days and then at 20°C for five days. Growth and vigorous gas production occurred in all of the bottles in less than 24 hours. Toxin was present

in the clam juice inoculated with both strains; it titered between 200 and 2000 MLD for mice. The supernatant liquids in both the irradiation and heat-sterilized samples, which had been inoculated with the Iwani strain, were further tested for toxin after activation with trypsin. Aliquots of the supernatant were incubated in the presence of 0.1% trypsin for one hour at 35°C. Suitable portions were then injected into mice. The heat sterilized clams produced a titre greater than 20,000 MLD, while the radiation sterilized clams developed between 2000 and 20,000 MLD. This result was unexpected, a higher titre being anticipated in the irradiated clams since they could contain active proteolytic enzymes. The lower titre actually obtained in the irradiated clams could be due to their being a poorer growth- or toxin-producing medium for the Type E strain. Whatever the reason, the experiment will be repeated.

IRRADIATION STUDIES

The resistance of Type E spores to ionizing radiations has not been extensively reported. 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.

Our studies are being designed to determine the irradiation resistance of six strains of *C. botulinum* Type E spores in fish and seafood substrates as well as in phosphate buffer. Survivor curves are being used for this purpose. Survivors remaining after different levels of irradiation are counted by a dilution technique using pork-extract agar in Prickett tubes and incubation at 30°C. The experiments are being designed to allow comparisons of irradiation resistance between various strains of Type E and between Types A, B, and E. The effects of the environment during irradiation are being evaluated. This includes the effects of temperature, pH, added salts such as $NaNO_2$ etc.

The cobalt-60 radiation source used principally in this study is located in the Phoenix Radiation Facility, although another radiation source is available to us on this campus. The Phoenix Radiation Facility previously was named the Fission Products Laboratory. The location of this cobalt-60 source in the same building as our bacteriological laboratory allows adequate control throughout the processing of samples.

The radiation source itself consists of approximately 1800 curies of cobalt-60. The cobalt is present in rods jacketed with aluminum, held in a cylindrical rack, and enclosed in a stainless steel can. When not in use, this source is stored in a well of water 14 feet deep. For irradiation purposes, the rack is raised by remote control into the irradiation room. The center of the cylindrical source is large enough to hold a large Dewar flask which permits refrigeration during irradiation. Spore samples are usually placed in 5 ml ampoules and irradiated in an ice-water mixture. The ampoules are accurately positioned in the Dewar flask by a rack that was especially designed for this purpose. The rack has spaces for 28 vials.

The amount of gamma radiation absorbed is determined by Fricke dosimetry using detailed procedures given in the ASTM Bulletin, D-1671-579, and which was discussed at the Contractor's Dosimetry Conference at Brookhaven National Laboratories during January, 1962.

A new calibration curve relating Fe^{+3} to the radiation dose has been constructed for our Model DU Beckman Spectrophotometer. For this purpose, a stock solution of ferric sulfate was passed through a Jones Reductor, an aliquot was standardized by potassium permanganate titration, diluted with 0.8 N H_2SO_4 to desired concentrations, and then tested in the spectrophotometer. A wavelength of 305 mμ and a slit width of 0.5 mm were used for the readings against an 0.8 N H_2SO_4 blank. The spectrophotometer and all solutions were kept in a constant temperature room at 22°C. The concentrations of ferric ion solution, whose optical densities were measured, were converted into equivalent radiation dosages using a G value of 15.6. By using the method of least squares the molecular extinction coefficient for ferric ion in 0.8 N sulfuric acid was found to be 2131.4 at 22°C. By correcting this value with a temperature coefficient of +0.7% per degree centigrade, a value of 2176.2 was obtained for 25°C.

A molecular extinction coefficient was also determined at a wavelength of 275 mμ since this constant is reported to be independent of temperature at this wavelength. We obtained a value of 1780 for the molecular extinction coefficient of ferric ion in 0.8 N H_2SO_4 at 22°C. Preliminary data show that this value is invariant with temperature at this wavelength. The optical density values of known ferric ion concentration is shown in Fig. 1, the conversion of ferric ion concentration to absorbed dose in rads in Fig. 2. The optical densities of ferric ion obtained at a wavelength of 275 mμ is given in Fig. 3.

Dose-rate measurements were determined in the same vials in which the spores are irradiated. The dosimeters were placed in the same position occupied by spore suspensions during irradiation. At the present time, the mean dose rate was found to be 83,000 rads per hour at the position occupied by the vials. This source is rather weak for our purposes. A larger source would materially accelerate our studies.

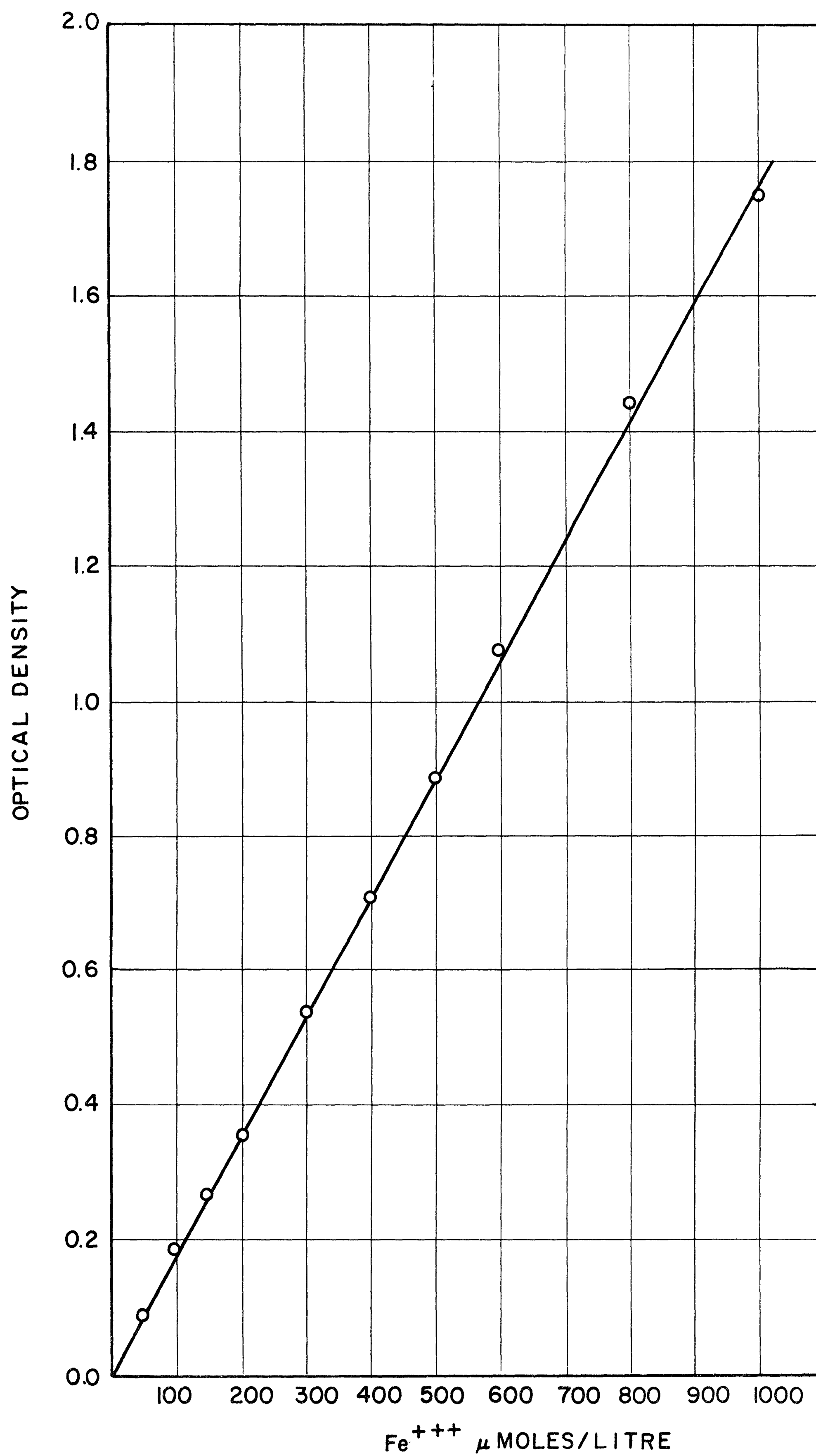

Fig. 1. Optical density vs. concentration of Fe^{+3} in 0.8 N H_2SO_4; wavelength 305 mμ, temperature 22°C.

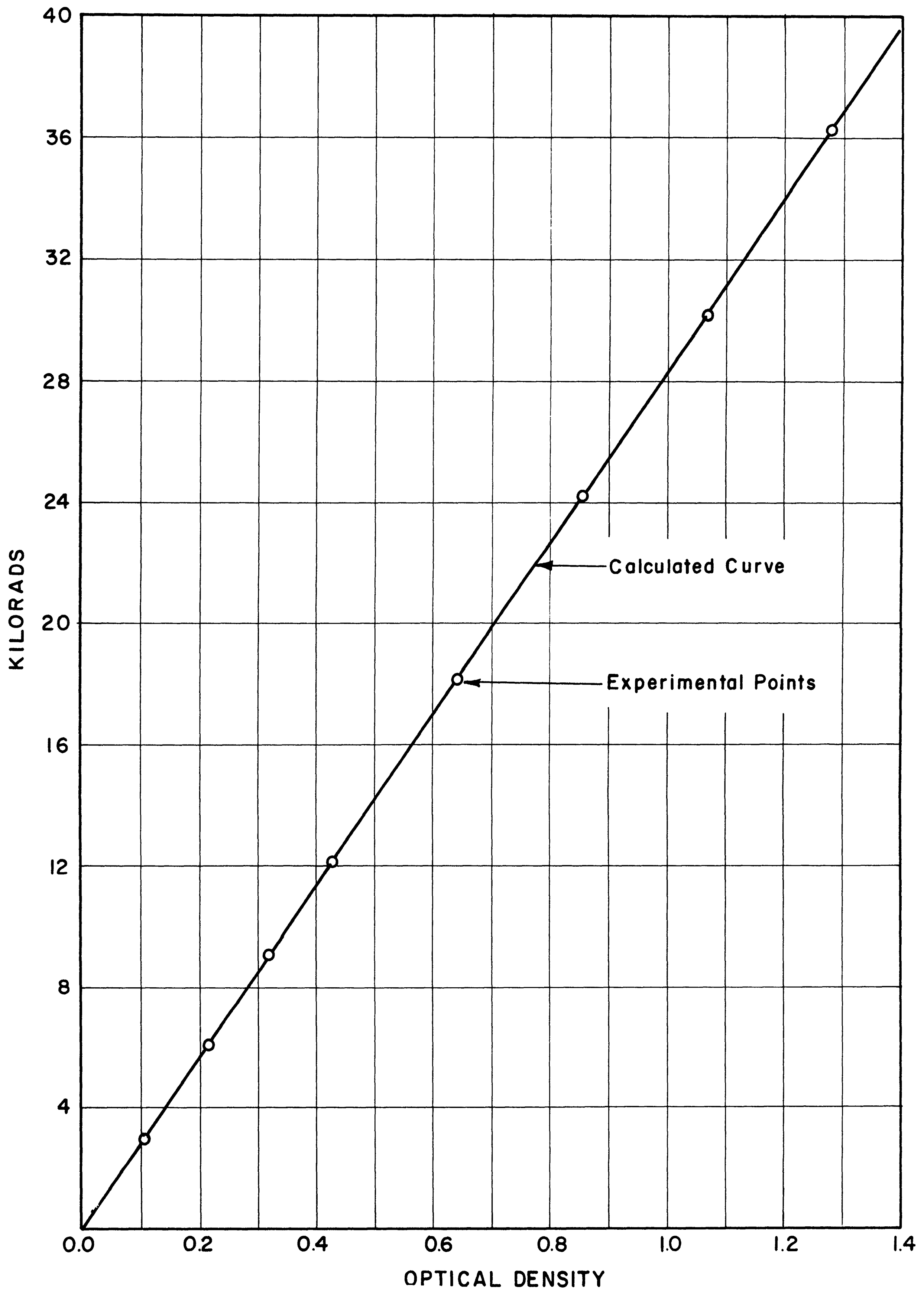

Fig. 2. Calculated gamma irradiation dose vs. optical density of Fe^{+3} solutions in 0.8 N H_2SO_4; wavelength 305 mμ, temperature 22°C.

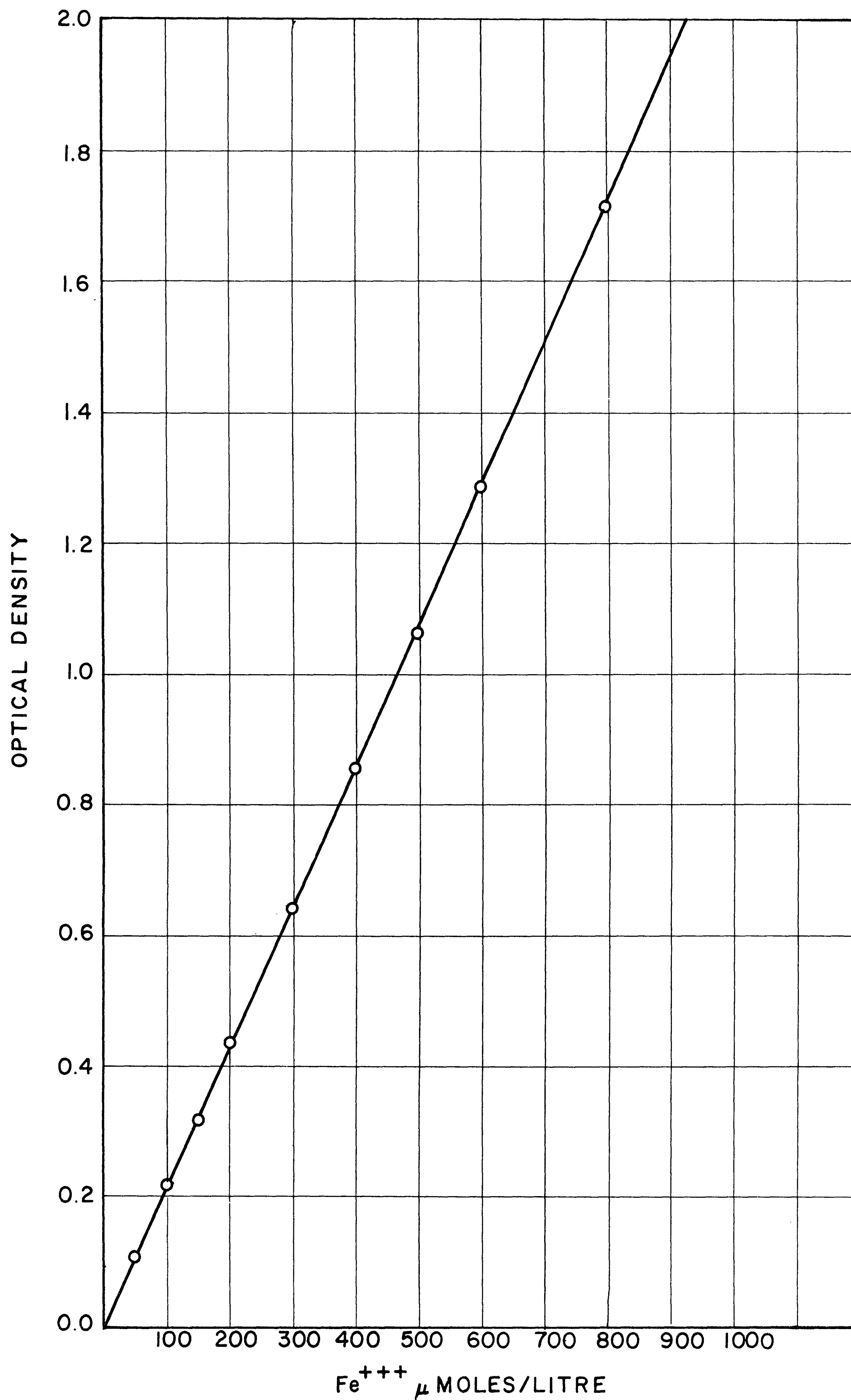

Fig. 3. Optical density vs. concentration of Fe^{+3} in 0.8 N H_2SO_4; wavelength 275 mμ, temperature 22°C.

RADIATION-RESISTANCE STUDIES

The radiation resistances of various strains of *C. botulinum* spores in phosphate buffer and other desired substrates require thoroughly washed spores. For a series of radiation tests, a suitable quantity of spores are pipetted from the stock spore suspension. This is washed by repeated centrifugation until the suspension is free of vegetative cells and debris. The spores are then heat-shocked; Type E spores being heated at 60 to 65°C for 15 minutes and Type A or Type B spores at 85°C for 15 minutes. Following heat-shocking, the spores are diluted into cold 15/M phosphate buffer or other suspending media being teated. Normally the finally prepared suspensions contain between 10^6 and 10^7 spores per ml.

The suspensions are kept cold before, during, and after irradiation. Samples are subcultured into the counting media within two hours after irradiation. The counting medium employed is Yesair's pork-extract agar without added glucose and contained in Prickett tubes. The inoculated tubes are incubated for 36 to 48 hours at 30°C, after which the number of colonies developed are enumerated with the aid of a Quebec colony counter.

A typical survivor curve for Type E spores irradiated in M/15 phosphate buffer at pH 7.0 is given in Fig. 4. These data indicate a D value for the Seattle Forks strain of 0.134 megarad when the irradiation is carried out in phosphate buffer.

Survivor curves for spores of the Seattle Forks strain of Type E and for Type A spores are given in Fig. 5. 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. 6 and Table I. It can be seen that the haddock extract protects these spores against the lethal effect of gamma radiations.

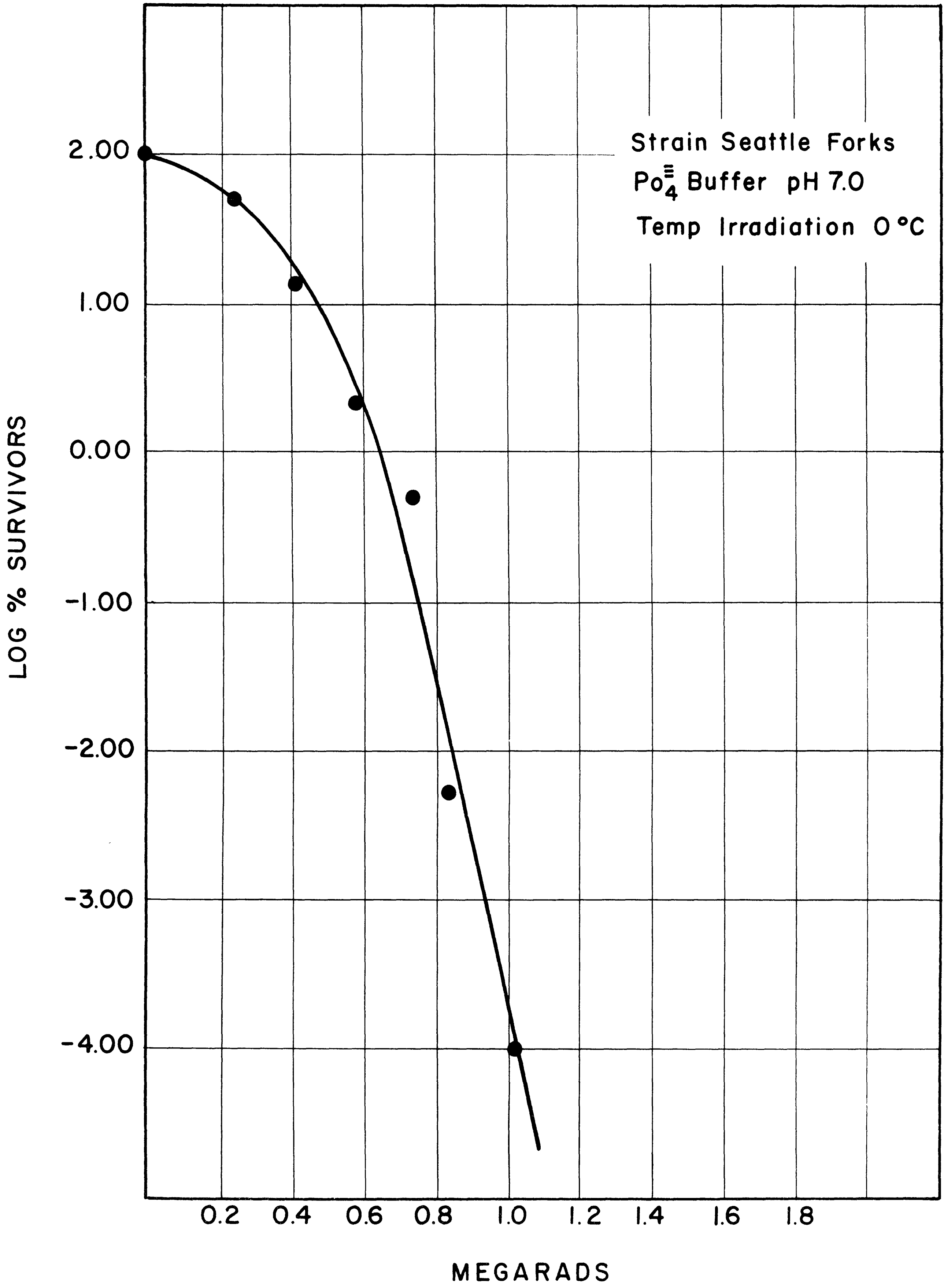

Fig. 4. Survivor curve for C. botulinum Type E spores to gamma radiation.

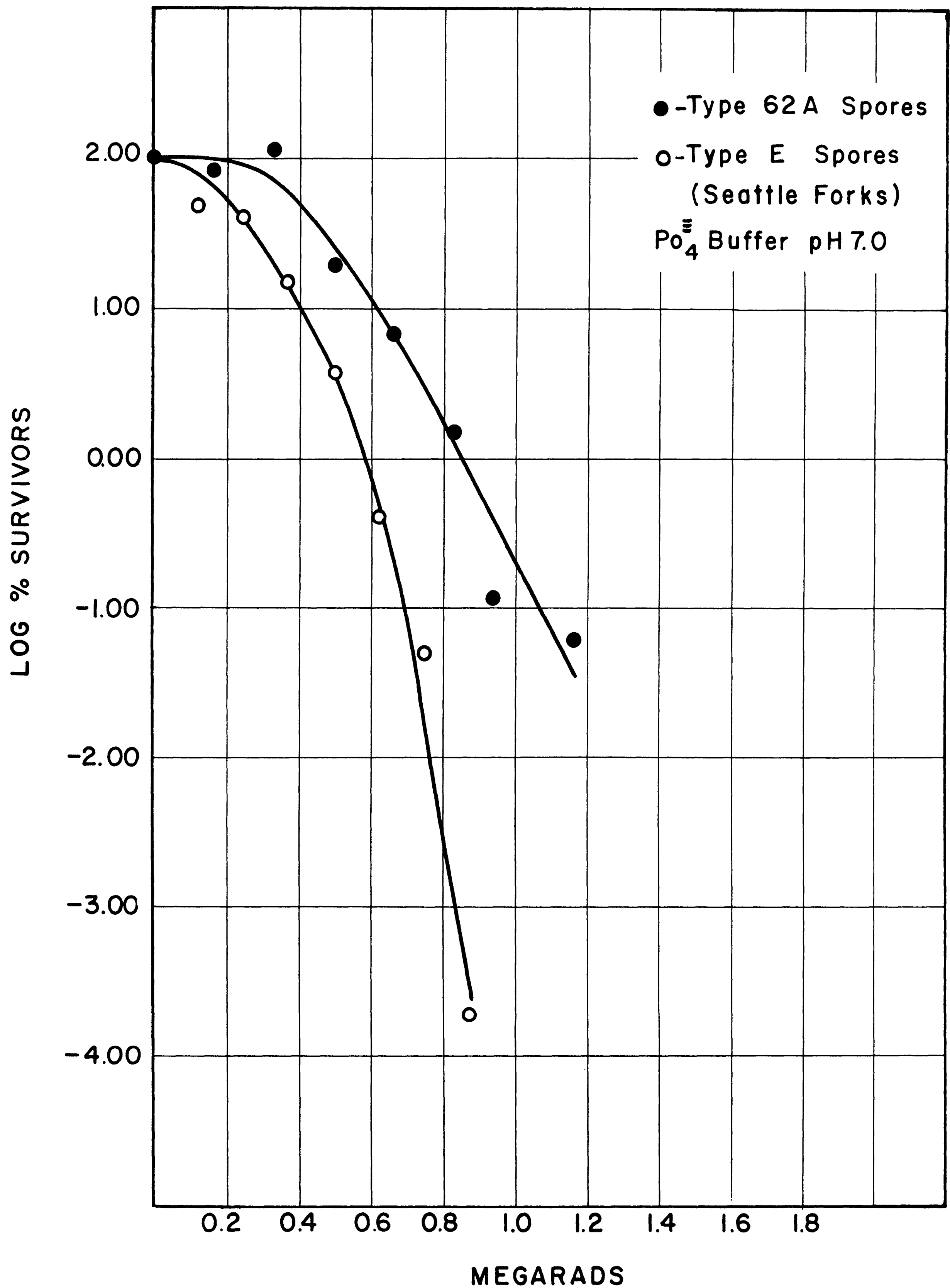

Fig. 5. Comparative resistance to gamma radiation of C. botulinum Type E and Type A spores.

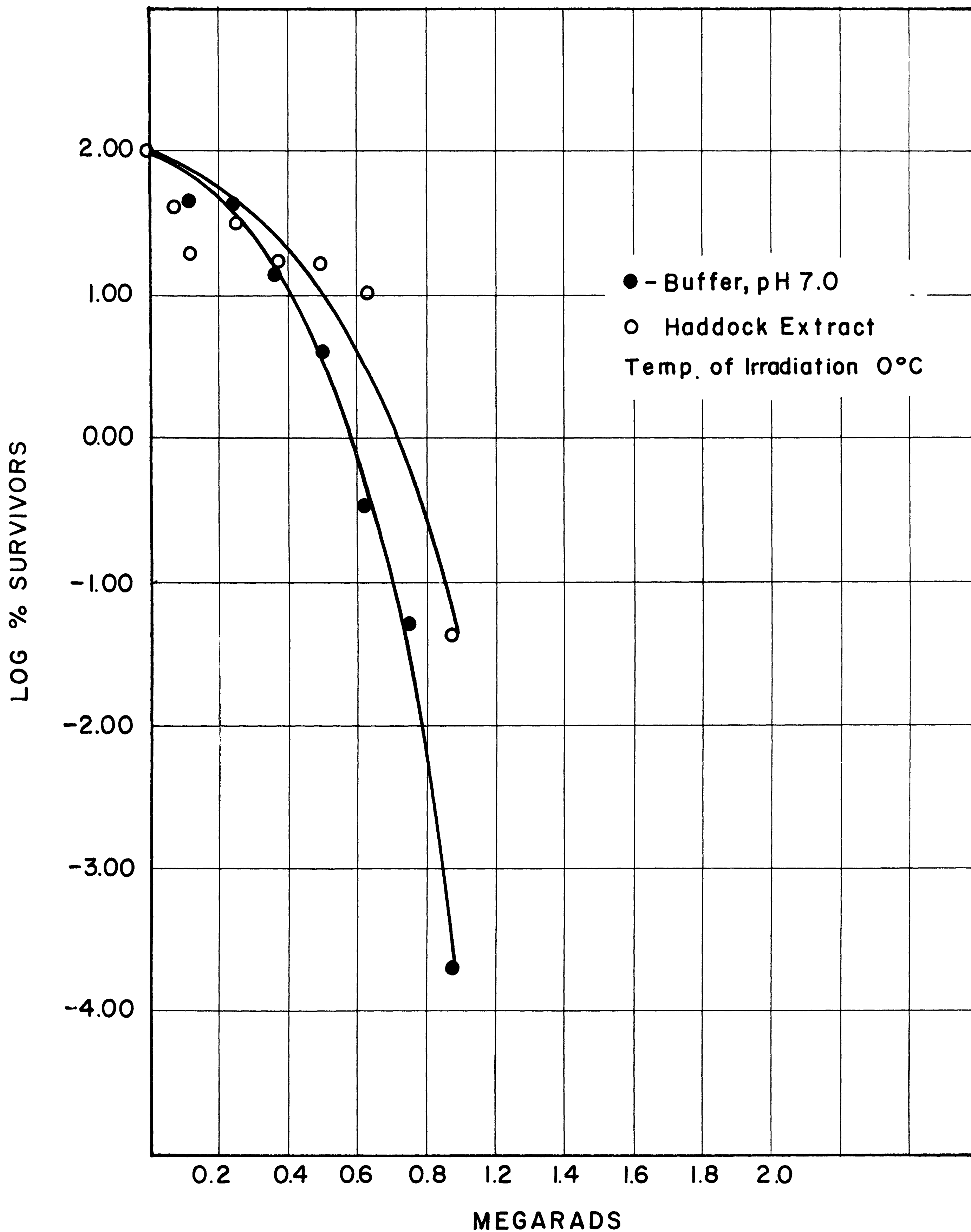

Fig. 6. Comparative sensitivity to gamma radiation of C. botulinum Type E spores suspended in buffer and haddock extract.

TABLE I

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	15.85	19.3
.498	4.12	16.5
.623	0.392	12.1
.747	0.049	---
.842	0.00017	0.037

CURRENT STUDIES

(Scheduled for Completion by Oct. 1, 1962)

1. Survivor curves will be determined for spores of all six Type E strains in phosphate buffer. These curves will be compared with those obtained with Types A and B spores irradiated under similar conditions.

2. The influence of a fish-type suspending medium on the radiation resistance of one strain of Type E spores will be determined. Clam or oyster juices or fish extracts will be used for these tests because it is necessary to have liquids that can be pipetted.

3. The synthesis of toxin by Type E cultures growing in fish extract and clam juices sterilized with irradiation and with heat will be compared.

4. Pilot experiments will be carried out to establish protocols for determination of the minimum temperatures for growth and toxin production by Type E spores in irradiated fish substrates. These experiments will be used as the basis for future studies of this problem.

5. Germination requirements for Type E spores will be studied.

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