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THE SIMULTANEOUS LETHAL EFFECT OF TEMPERATURE AND GAMMA RADIATION ON BACTERIAL SPORES

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INTRODUCTION

The lethal action of ionizing radiations on bacterial spores has been assumed to be independent of the ambient temperature during irradiation. This conclusion was made by Lea, Haines and Coulson (1936, 1937) after studying the lethal effects of alpha and beta particles on Bacillus mesentericus spores which were exposed at various temperatures. This apparent lack of a temperature coefficient for the lethal effects of ionizing radiations on bacterial spores was used as strong evidence against a chemical or an indirect mechanism by which radiations exert their lethal effects (Lea, 1947). Until the present decade, these experiments represented the only detailed investigation in which bacterial spores were used as a test system in studies with high energy radiations.

Ionizing radiations have been suggested for sterilization purposes. Since the destruction of bacterial spores is usually the limiting factor in sterilization processes, the relative resistance of these spores to the various types of radiations and the conditions prevalent during irradiation become important in the determination of the sterilization dose.

However, when food sterilization is considered a further limiting factor is imposed upon the use of radiation, namely the production of off-flavors and odors in a variety of food stuffs. The extent of off-flavor and odor production is dependent upon the total dosage. Therefore, any method which would help to lower the sterility dose would be of importance in the use of radiation sterilization for food processing.

In this respect the simultaneous action of heat and radiation for killing bacterial spores may become important. Aside from the combined but independent effect of these two forms of energy, the synergistic action of radiation and heat may be of significance. The observation that the coagulation temperature of certain proteins could be lowered significantly by prior exposure to ultra-violet radiation was early recognized in radiation studies (Bovie, 1913; Clark, 1922; Stedman and Mendel, 1926). However, the mechanism of the phenomenon, referred to as photosensitization, has not been elucidated. That cellular protoplasm could also be sensitized by ultra-violet radiation was first demonstrated in Paramecium (Bovie and Klein, 1919; Forbes and Daland, 1923; Bovie and Daland, 1923). Following the development of better techniques in the use of ultra-violet radiation, photosensitization of cellular protoplasm to heat was established in several species of bacterial spores by Curran and Evans (1938), in yeast cells by Duggar and Anderson (1939, 1941) and reconfirmed by Giese and Crossman (1945) in Paramecium.

Giese and Heat (1948) were the first to demonstrate that X-rays were capable of inducing sensitization in Paramecium. Although not well established, these investigations suggested that the reverse procedure of a prior heat treatment did not increase the sensitivity of cellular protoplasm to subsequent radiation treatment.

Morgan and Reed (1954) made a preliminary study of the photosensitization phenomenon by cobalt-60 gamma radiation using spores from certain food spoilage organisms. They found that spores from a thermophilic anaerobe were more sensitive to heat treatment after exposure to gamma radiation. The reverse treatment did not alter the

radiosensitivity of spores from Bacillus coagulans var. thermoacidurans or Bacillus stearothermophilus.

In more extensive experiments, and with spores from strains of Clostridum botulinum, it was demonstrated the F_0 value (heating time in minutes at 250°F) could be lowered as much as four fold if the spores were irradiated with a total dose of 900,000 rep from cobalt-60 (Kempe 1955). The practical significance of this method of sterilization was indicated when it was demonstrated that spores from strains of Clostridium botulinum and a putrefactive anaerobe could be adequately inactivated by lower heat treatments in canned peas, as well as in raw and cooked meat after pre-irradiation treatment (Kempe et al., 1957, 1958, 1959).

Kan et al. (1957) also demonstrated that putrefactive anaerobe spores, as well as aerobic spores of Bacillus cereus could be rendered more heat sensitive by cobalt-60 radiations.

At present, little information is available concerning the effect of irradiation temperature upon bacterial spores, especially those of importance in food spoilage. Those temperature studies which have been undertaken have been mostly concerned with the relative resistance of bacterial spores in frozen and non-forzen states. The results of these investigations are conflicting and no adequate explanation for the diverse results has been advanced.

In other radiobiological studies, where the role of temperature during irradiation has been studied, the significance of photosensitization of proteins has not been adequately considered.

Therefore this study was initiated to explore the lethal effects of gamma radiations on bacterial spores using a wide range of sub-lethal

temperatures during irradiation. Spores produced by food spoilage organisms were selected because of the practical considerations involved and also because of their greater resistance to heat as compared to those spores of the mesophilic, aerobic, spore-forming bacteria.

Aside from any theoretical implications involved, the information obtained should be useful in evaluating the simultaneous effects of heat and radiation as a method of sterilization.

HISTORICAL

Introduction

The interaction of radiation with living cells results in a complex series of reactions before death ensues. For clarity, it is convenient to separate into various stages the sequence of events which occur following radiation exposure. Generally, three stages are recognized. The first, or primary action, is the absorption of the incident radiation in, or near the cell; the second, an intermediate stage, consists of a sequence of biochemical and biological reactions which lead to the third or final stage, death of the cell.

The interaction of radiant energy with molecules of matter is well understood. Also, the lethal effects following radiation exposure has been well documented for many types of cells. But, the mode of energy transfer to the site of the primary lesion within the cell, and the events originating from this lesion which result in lethality are still incompletely understood in radiobiology.

As working models, two theories have been advanced to explain the effects of radiation on living cells. These theories are based upon the method by which the incident energy is absorbed within the cell. The target theory postulates that the primary absorption of energy occurs within or very near the key molecules in the cell. The indirect action theory assumes that the primary absorption of energy occurs in other molecular species, in or near the cell, with resultant production of lethal chemicals that then react at the sensitive sites within the cell.

The effect of temperature during irradiation assumes an important role in evaluating the two models. Since one model assumes a physical reaction for radiobiological effects and the other assumes a chemical reaction, a temperature coefficient could help to determine which model is operative.

Temperature dependency during irradiation was one of the first parameters tested in radiobiological experiments. No satisfactory explanation was advanced for the diverse temperature effects observed with a wide variety of test systems (Muller, 1954). To date, no satisfactory explanation exists for the various ways by which temperature can modify radiobiological reactions (Pollard et al, 1955).

The early experimental basis and arguments for the target theory have been well documented by Lea (1947) and extended recently by Pollard (1953, 1955). The absorption of radiant energy in matter, which results in excitations and ionization, is not considered to be influenced by temperature. According to this model the temperature coefficient for radiobiological reactions should be one or less. Any observed temperature dependency during irradiation would need to be attributed to other operative factors, for example, the effect of temperature on the reactions which lead to the recovery of the cell from the original lesion produced by the radiation.

However, recent investigations have questioned the importance of the direct action model as the modus operandi by which radiations exert their lethal effects. The evidence for this negation is that the alteration of the environmental conditions during exposure of the cells is capable of significantly altering the radiosensitivity of the cells. A notable

factor influencing the radiation sensitivity of the cell is the quantity of oxygen present during irradiation. Subsequent investigations have shown that other chemicals are also capable of altering the sensitivity of the cell. Therefore, a number of radiobiological studies have been directed towards determination of the influence of oxygen on the lethality of ionizing radiations.

Since viable cells contain approximately 80 per cent water and are intimately associated with an aqueous environment, a suggested approach for the mechanism by which oxygen modifies the radio-resistance of cells is based upon the proposed mechanism for the radiolysis of pure water. Early studies with X-rays demonstrated that hydrogen peroxide is formed in oxygenated solutions (Fricke, 1934 and Risse, 1929). A reaction mechanism, which incorporates the transient existence of intermediate free radicals with strong oxidative and reductive powers, has been postulated for production of this compound. These entities may be responsible for the observed radiobiological lesions in the cell (Weiss 1944, 1947). Since the amount of peroxide formation is dependent upon the amount of oxygen present in the system, the oxygen effect has been attributed to the lack or non availability of this compound in the cell. Historically, the activated solvent hypothesis or the indirect action model was proposed by Risse (1930) and Fricke (1934) for the biological effects of radiations.

The quantitation and formulation of the indirect action model has been outlined by Zerkle and Tobias (1953). This model, referred to as the diffusion model, was designed to supplement the direct and indirect hypothesis.

In order to explain the results in which a temperature dependency was noted during irradiation, several attempts have been made to ascertain the role of temperature during irradiation on the rate of free radical and peroxide formation by ionizing radiations in pure water.

Bonet-Maury and LeFort (1948) have demonstrated that the amount of hydrogen peroxide formed in pure water during irradiation is a function of temperature, twice as much peroxide being formed at 20° as at -4°C. A definite discontinuity in peroxide formation occurs in the transition from the liquid to the solid phase. These investigators were not able to detect peroxide formation below -116°C following a dose of 2 x 10^6 r.

Gromley and Steward (1956) were able to detect peroxide formation in ice at a temperature of -196°C. However, the amount detected was independent of the amount of oxygen present in the system. Their results showed more peroxide was formed at -5° than at -78° or -196°C; the amount produced at the former two temperatures depended on the oxygen concentration in the system.

Hochandel (1952) studied peroxide formation in pure water irradiated in the temperature range of 25° to 65°C. He found that less peroxide was formed in pure water as the temperature increased in this range when cobalt-60 was used as the source of gamma radiations. This could indicate that the rates for the back reactions increased as the temperature of the system was elevated, thus resulting in lower effective peroxide concentrations.

Another means by which temperature could exert its influence during irradiation involves a change in the rates of diffusion of free radicals

to sensitive sites within the cell. The diffusion processes would be of considerable importance when irradiation is carried out in the frozen as contrasted with non-frozen states. This concept for temperature dependence during irradiation can be adequately explained with the model proposed by Zirkle and Tobias (1953).

Because of their high thermal resistance, bacterial spores offer an excellent test system for studying the effect of temperature during irradiation. Such spores can be subjected to heat treatments over a wide temperature range without significant loss of viability. Furthermore, because of their low metabolic activity, the influence of temperature, oxygen and other chemical substances on enzyme reactions which might affect the viability of the spores is also minimized. However spores can be considered abnormal cells in that they possess a greater resistance to deleterious influences than the vegetative cells from which they originate. This objection may be outweighed, in part, by a corollary study of spore resistance to two physical agents. The mechanism of spore resistance has not been elucidated, and the phenomenon presents an intriguing problem in microbiology. As a matter of practical significance, factors affecting spore resistance are of importance in the evaluation of sterilization techniques and processes.

Effect of Temperature During Irradiation on Bacterial Spores

The early work by Lea et al. (1936) on the independence of temperature during irradiation on the survival of B. mesentericus spores was used to support the target theory (Lea, 1947). The results of this investigation indicated no difference in the surviving fraction of B. mesentericus

spores contained in dry gelatin and exposed to alpha-particles from polonium at temperatures of 50, 20, 2 and -20°C. Similar effects were observed with spores of the same organism when they were exposed to beta-particles from radium at temperatures of 41, 20 and -20°C.

Edwards et al. (1954) using three mev electrons produced by a Van der Graaf accelerator, observed no significant differences when Bacillus subtilis spores were irradiated at temperatures of 0, 44 and -60°C with 34,000 rep. However, when spores were exposed to 120,000 rep statistically significant differences were noted at these temperatures, for example, survivors to this dosage were 5.46 per cent at 0°C, 3.92 at -60°C, and 15.71 at 44°C. They also observed that the increase in number of survivors at the higher dosage level depended on the concentration of organisms. These investigators did not advance an explanation for the results they observed.

Also using high energy cathode rays produced by a three mev Van der Graaf accelerator and B. subtilis spores, Proctor et al. (1955) observed that the spores were more sensitive when irradiated at -78°C than at 4.4°C. These results showed an opposite trend, which might be expected from a consideration of the indirect versus direct action theories. No explanation for this anomaly was presented except that freezing might alter the sensitivity of the spores to irradiation. These results are in opposition to those obtained by Edwards et al. (1954) using both the same strain of organism and the same type of radiation.

Houtermans (1956) studied the lethal effect of alpha particles, as well as soft (50Kv) and hard X-rays on B. subtilis spores in the temperature range of -184° to 20°C. The spores were exposed to each type of

radiation in the presence and absence of moisture. The degree of hydration did not alter the sensitivity of the spores to hard and soft X-rays at -184°C. However, at room temperature a marked difference in the sensitivity of the spores was noted to X-rays. The dry spores were more sensitive to both wavelengths of X-radiation than the moist spores. However, the slope of the dose inactiviation curve using soft X-rays indicated that this radiation was more efficient for inducing lethality than the shorter wavelengths.

In a series of experiments in which the spores were exposed to a constant dose (60 and 90 second exposures) of hard X-rays and two conditions of hydration, a gradual decrease in sensitivity occurred from -184 to -40°C. At the latter temperature a marked change in sensitivity occurred as evidenced by a break in the survivor curve. No further change in sensitivity of the spores, due to the radiation temperature, was noted from -40° to +20°C. Although, the break in the sensitivity curve at -40°C occurred under both conditions of hydration and dosage levels, it was most pronounced with dry spores which had been exposed for 90 seconds.

With alpha particles from thoruim-B, the least number of survivors occurred with dry spores irradiated at -184°C. The same degree of inactiviation occurred at 20°C with wet and dry spores as with moist spores at -184°C. With hydrated spores, irradiated at two constant dosage levels, no difference in the number of survivors was noted in the temperature range of -184 to 20°C. However, with dehydrated spores a marked change in the sensitivity of the spores to alpha particles occurred between -60 and -40°C.

Proctor et al. (1958) utilized spores of B. thermoacidurans, an organism of significance in food spoilage, to study the effect of various environmental conditions during irradiation on lethal effects of beta and

gamma radiation. Very little difference was noted in the number of surviving spores when they were suspended in saline and irradiated in frozen (-72°) and non-frozen conditions. Comparative results were presented for both gamma-rays from cobalt-60 and 3 mev. electrons, when the spores were irradiated under various physical states and gaseous conditions while suspended in 0.85 per cent sodium chloride. The following conditions were employed; dry ice and room temperature, and in the presence of pure oxygen, nitrogen or air. No appreciable differences in the number of spores surviving irradiation treatment were observed under the various conditions present at the time of irradiation when the spores were suspended in saline; however, when the suspending medium was nutrient broth rather than saline, slight differences were observed. The spores were found to be more resistant to irradiation in the frozen condition in this medium. Also, oxygen increased the radiosensitivity of the spores to irradiation. The over all differences in resistance of spores suspended in broth and saline solutions during irradiation was not appreciable.

Webb et al. (1958) studied the sensitivity of dry spores of Bacillus megatherium to 50Kv X-rays at temperatures between 36° and -268°C. The technique used by these investigators was to dry the spores on Millipore filters and expose them to 50Kv X-rays at various temperatures in an atmosphere of helium. No difference in sensitivity of the spores to radiation was noted in the temperature range of -268° to -121°C. At the latter temperature, a sharp break in radio-sensitivity occurred. In the temperature range of -121° to 36°C it was found that the log of the slope of the inactiviation curve was proportional to the reciporical of the absolute temperature. No conclusions based on the various methods by which temperatures

can alter the radio-sensitivity of spores was advanced by these investigators for their results, other than a discussion of the discontinunity in hydrogen peroxide formation at -168°C as was previously shown by Ghromley and Stewart (1956).

Comparison of these results obtained with B. megatherium spores with those of B. subtilis (Houterman, 1956) showed a striking similarity in that a sharp change in sensitivity occurred at freezing temperatures. The temperature at which this break occurred varied slightly in the two investigations. Some difference was noted in that B. subtilis became more radio-resistant as the temperature decreased to -176°C and no change in sensitivity occurred in the 0° to 30°C temperature range.

Photosensitization of Proteins to Heat

With the development of ultra-violet, X-ray and radium sources at the turn of the century, one of the first effects observed was that visible coagulation of protein solutions occurred when such solutions were exposed to these emanations. Coagulation of proteins was assumed to be the mechanism by which radiations affect cells. At first, it was the contention of the earlier workers that coagulations produced by irradiation and heat were identical. Further studies showed that differences do exist, although the data is meager (McLaren, 1949). The early work has been reviewed by Arnow (1936). It is of interest to note that Effont, in 1913, suggested that the effects of radiations on protein solutions might be due to formation of hydrogen peroxide in water (Arnow, 1936).

An interesting observation with ultra-violet light showed that prior exposure of albumen to ultra-violet radiation resulted in lowering

the coagulation temperature of the protein (Bovie, 1913). Stedman and Mendel (1926) irradiated 12 purified proteins from a wide variety of sources and found that the coagulation temperature was markedly lowered by pre-irradiation with ultra-violet light. Clark (1935) made a systematic study of the sensitization of egg albumin by ultra-violet in order to elucidate the mechanism involved. She separated the reaction into three steps. 1) A slight denaturation of protein wherein a large amount of energy is absorbed. This reaction is independent of temperature, and can occur in the absence of water and over a wide pH range in presence of water. 2) The second step involves the reaction of altered molecules with water (hydrolysis). This step was considered to be chemical in nature and occurs with a high temperature coefficient (temperature coefficient of 10). 3) The third step, a physical reaction, manifests itself by the appearance of the visible coagulum. Although this investigator offered the above mechanism to explain photo-sensitization, the manner in which radiation alters the protein molecule is not apparent. Actually this proposed scheme is a modification of the classical theory of protein coagulation as caused by heat and suggested by Chick and Martin (1910, 1911).

Photosensitization of Protoplasm to Heat

The first observation that electromagnetic radiations rendered protoplasm of living organisms sensitive to heat was observed in Paramecium by Bovie (1919) with ultra-violet rays transmitted through fluorite (the radiations transmitted through fluorite are considered to be in the Schumann region of the electromagnetic spectrum between 1250 - 2000A°). In a series

of controlled and detailed experiments (Bovie and Daland, 1923; Forbes and Daland, 1923) with Paramecium caudatum it was observed, for example, that 88 per cent of the cells formed vesicles when heated at 34.6°C for five minutes after exposure to ultra-violet light, whereas, when treated independently, 30 per cent formed vesicles following heating and 25 per cent subsequent to irradiation. Sensitization of cells by ultra-violet light was also observed at temperatures of 27.5° and 30.5°C, temperatures which were not considered to be lethal for the animals. Preheating the animals did not alter their radiation sensitivity.

Although the experiments of the above mentioned investigators were detailed there was some question as to whether the sensitization was produced directly by the radiation or indirectly as a result of ozone produced by the ultra-violet light sources. With the advent of improved radiation sources and techniques for energy measurements, Curran and Evans (1938) restudied the problem of photo-sensitization using bacterial spores as a test system. In their studies three strains of aerobic organisms were used, Bacillus albolactis and Bacillus cohaerens and an unknown strain belonging to the B. mesentericus group which they designated as CC. Two sources of ultra-violet radiation were used, these were a mercury lamp which emitted 95 per cent of the radiation at a wavelength of 2,537A and a hydrogen discharge tube with a fluorite filter. The emission of the latter lamp was in the 350 - 1600A region. Using consecutive light exposures, heat treatments at 98°C, and time intervals up to nine minutes in length, they observed that an initial exposure to ultra-violet light made the spores of the three organisms more susceptible to subsequent heat treatment. Also,

in order for the heat sensitization phenomenon to manifest itself, it was necessary for the exposure to ultra-violet radiation to be of sufficient duration to be sporicidal for a large number of the organisms. Shorter wavelengths of ultra-violet radiation, in the range 350 - 1600A, were found to be more efficient for inducing heat sensitization. The most heat resistant organism tested, B. cohaerens, was the most susceptible to sensitization.

Duggar and Anderson (1939) observed that prior exposure of cells of Saccharomyces cerevisiae to ultra-violet radiation, (2650A) followed by heat treatment of 50°C, was two to five times more lethal than the reverse procedure. They also observed that pre-irradiation increased the ability of the cells to take up methylene blue after heating, but that the respiration rate was not affected. Respiration of the yeast cells was not affected by ultra-violet light even at exposures which prevented the cells from forming colonies. A conclusion reached by these investigators was that respiration and the ability to form colonies are governed by separate mechanisms. It was suggested that nucleoprotein may be involved in sensitization because nucleoprotein strongly absorbs radiation of this wavelength.

A more extensive investigation by these investigators confirmed their original observation (Anderson and Duggar, 1941). Using the same criteria, namely, inhibition of colony formation, uptake of methylene blue and alteration of the respiration rate, they compared the effectiveness of irradiation at 2200A with that at 2650A in conjunction with heat treatments at 50°C. They observed that irradiation at 2200A was as effective as at 2650A with the added observation that the respiration rate was depressed

when irradiation was carried out at 2200A. They also observed that heat was able to sensitize the cells to radiation at 2650A, but that the reversed treatment, pre-irradiation then heat treatment, was more effective.

Using better radiation sources and more refined intensity measurements, Giese and Crossman (1945) re-investigated the photo-sensitization of Parmacium to heat by ultra-violet radiation. The spectrum at various wavelengths, between 2383A and 3660A, was studied. Maximum sensitization of the animals to heat treatment at 41.5°C occurred at 2383A°, this was the shortest wavelength tried. There was a rapid decline in the sensitizing effect towards the longer wavelengths with no observable effect apparent at 3669A. The spectrum for photo-sensitization of pseudoglobulin was observed to be similar to that for sensitization of the animals.

They observed that recovery was possible after exposure at all wavelengths of radiation, as well as following heat treatment, but the recovery was slow. The slowest recovery of all occurred after the cells were exposed at a wavelength of 2650A. Since nucleoproteins have the maximum absorption at this wavelength and recovery was slowest, indicating greater damage, it was concluded that nucleoprotein was involved in the photo-sensitization phenomenon. Although only one temperature (41.5°C) was used in their studies, these investigators stated that the sensitization phenomenon manifested itself only if lethal temperatures were used after irradiation.

The first observation that ionizing radiations are capable of inducing sensitization in Paramecium was observed by Giese and Heath (1948) with X-rays. An X-ray dose of 140,000 rep and heat treatment for 1.5 minutes

at 42°C killed the animals. Either treatment alone had no effect. The opposite treatment, that is heating the cells at 42°C for 1.5 minutes followed by exposure to X-rays, also had no apparent effect. A direct correlation of heat lethality with radiation dosage was observed.

Although the phenomenon of sensitization to heat by ultra-violet radiation has long been recognized, this study by Giese and Heath with ionizing radiation appears to stand alone. Recently, because of implications of radiation sterilization, the combined effect of heat and irradiation has been studied on spores of importance in food processing. Morgan and Reed (1954) demonstrated that spores of thermophilic anaerobe NCA strain 3814 were more susceptible to heat treatment at 240°F after receiving a total dose of 250,000, 600,000, and 1,000,000 rep from cobalt-60. They also observed that Bacillus coagulans var. thermacidurans and Bacillus stearothermophilis were not rendered more sensitive to gamma radiation by prior heat treatment.

In a much more extensive investigation with Cl. botulinum spores from strains 62-A and 213-B it was observed that the F_0 value in phosphate buffer could be reduced as much as four-fold following a pre-irradiation treatment of 900,00 rep (Kempe, 1956). Preheating the spores for periods as long as 24 minutes at 99°C did not alter their sensitivity to subsequent irradiation. The extent of the sensitization phenomena produced in the spores was dependent on the total radiation dosage received. The practical significance of this method of sterilization was demonstrated with canned beef and peas. Depending upon the amount of gamma radiation used both the spores of Cl. botulinum and putrefactive anaerobe were rendered

significantly more sensitive to subsequent heat treatment in these products. (Kempe et al., 1957, 1958, 1959).

Kan et al. (1957) also observed that pre-irradiation treatment with gamma radiation from cobalt-60 rendered the spores more sensitive to subsequent heat treatment. Further experiments with spores from Bacillus cereus showed the phenomenon to be operative in this species.

The sensitization phenomenon has been observed adventitiously in studies directed towards other objectives. For example Wood (1955), in his study on the influence of temperature and phase state prevalent during radiation on the survival of yeast cells exposed to X-radiation, observed that the sum of radiation and heat treatment was greater than if they had been applied independently. In a series of additive experiments in which the cells were exposed to various combinations of heat at 52.5°C and radiation treatments, his results indicated that the greatest number of cell survivors occurred when the heat treatment was applied prior to irradiation. It is interesting to note that this is exactly opposite to that observed in other investigations.

With bacteriophage strain T-1, the simultaneous application of heat and radiation resulted in greater destruction of the viruses than if either treatment was applied independently (Adams and Pollard, 1952).

The phenomenon of sensitization of protoplasm to heat by electromagnetic radiations seems to be universal in that it occurs in a wide variety of microorganisms. However, the relative importance of this phenomenon in terms of lethality of radiations must yet be ascertained. Also the mechanism by which sensitization develops in the cells needs to be elucidated. With bacterial spores, an explanation of the sensitization phenomenon

assumes added importance; here such knowledge may help to produce an understanding of the basis upon which some bacterial spores exhibit unusually high resistance to damage by heat.

MATERIALS AND METHODS

Organisms

The organisms used in the present study were originally obtained from the following sources:

Clostridium parabotulinum 62-A *Clostridium botulinum* 213-B	Dr. F. K. Meyer Hooper Foundation for Medical Research San Francisco, California
Clostridium parabotulinum 457-A	Dr. L. S. McClung University of Indiana
putrefactive anaerobe NCA strain 3679	Dr. E. J. Cameron National Canners Assoc. Washington, D. C.

Maintenance of Stock Cultures

Stock cultures of *Clostridium* strains were maintained in cooked meat phytone medium (a product of the Baltimore Biological Laboratories). This medium, consisting of dehydrated meat particles and enzymatically digested soy beans, was rehydrated and sterilized for 30 minutes at 121°C. It has been reported that cultures could be stored in this medium for extended periods of time without losing their desired cultural characteristics (Vera, 1944).

These cultures were transferred approximately every six months. For this purpose 0.1 ml of the stock cultures was transferred into cooked meat phytone media which was prepared in 16 x 120 mm screw cap tubes and from which the dissolved oxygen had been removed by heating in boiling water. The cultures were then incubated at 37°C until the meat was blackened; this usually required three or four days. The cultures were then

examined for purity and evidence of sporulation. If spores were present, the tubes were heated in a boiling water bath for 15 minutes in order to kill vegetative cells and to heat-shock the spores. After transferring into fresh media, and incubating at 37°C, the cultures were stored at 4°C.

The purity of the strains was verified by combining filtrates from cultures grown in 10% casitone broth with type specific anti-toxin obtained from the New York State Health Department. Stock cultures, produced as outlined above, have maintained sporogensis and toxicity for several years.

Production of Spore Suspensions

The following medium was used for production of spore crops of Clostridium botulinum 213-B.

Bacto-Casitone	100. grams/ℓ
Bacto-Beef Extract	0.5 grams/ℓ
Di-Sodium Phosphate	0.5 grams/ℓ
pH 6.8 - 7.0	

This is essentially the same medium employed by Reed et al. (1951) in their studies on the heat resistance of anaerobic bacterial spores except that Bacto-casitone was substituted for the freshly prepared pancreatin digest of casein recommended in their procedure. The inoculum used for seeding the sporulating media was prepared by transferring 0.1 ml of a stock culture into 10 ml of this casitone medium after it had previously been exhausted of dissolved oxygen. The cultures were incubated at 30°C until growth occurred. Further transfers were made at daily intervals until the organisms became adapted to the new medium which was evident by early and vigorous growth in the culture tubes. A 24 hour culture, for

use as an inoculum, was then prepared and used to seed 800 ml. of media contained in 1000 ml Erlenmeyer flasks. Usually a five per cent inoculum was used. The flasks were sealed with paraffin paper and allowed to incubate at 30°C for three weeks. After this, the flasks were removed from the incubator and then were placed in a refrigerator for seven to ten days. This permitted the spores to settle, and the lysis of any remaining vegetative cells.

For harvesting, the supernatant was aseptically aspirated from the settled spores in the culture flask and the spores were washed five times in 200 ml centrifuge tubes with cold, sterile, distilled water. The initial washings were done by sedimentation in an International centrifuge located in the refrigerator. Each centrifugation was carried out at a rotational speed of 1500 rpm for 30 minutes. Additional washings were conducted at room temperature until a spore suspension was obtained that was practically free from vegetative cells and extraneous material as evidenced by microscopic examination. This usually required eleven additional washings. As a final step, the spores were concentrated in a few ml of sterile distilled water and stored over glass beads at 4°C. Yields of approximately 10^6 spores per ml, based on the original culture broth, were obtained by the above procedure.

A fresh liver broth medium was used for the growth of spores of Cl. botulinum 62-A. The preparation of the liver broth medium was as follows: Chopped beef liver (fat free) was mixed with water in the proportion of 500 grams to 1000 ml. This mixture was boiled slowly for one hour, adjusted to approximately pH 7.2 with 10% sodium hydroxide and boiled for an an additional 10 minutes. This mixture was filtered through cheese cloth

and then made up to the original volume. To this extract were added 10 grams of Bacto-peptone and one gram di-potassium phosphate per liter. The medium was dispensed into tubes and five liter Erlenmeyer flasks, a few iron wires were added, and sterilization carried out for 45 minutes at 15 psi. The pH after sterilization was approximately 7.0.

A 24 hour culture, grown at 37°C, was used to inoculate the five liter flasks. The large flasks were allowed to incubate for seven days at 37°C. Sporulation was observed to be complete after this time. The cultures were cooled in a refrigerator after which the cells were harvested. Preparation of the spore suspensions of this organism was essentially the same as described with strain 213-B.

Spore suspensions of putrefactive anaerobe NCA strain 3679 were prepared by the method, outlined by the National Canners Association (79) which is described in the next section.

Fresh pork hams were trimmed of fat and the lean pork was ground and mixed with distilled water using one pound of pork to one liter of water. This was boiled slowly for one hour. The pH was adjusted to 7.4 with 10 per cent sodium hyroxide; approximately 110 ml of alkali were needed for 20 liters of extract. The meat was separated from the hot mixture by filtration through several layers of cheese cloth. A portion of the resulting meat-cake was reserved and dried at 100°C. The pork extract was placed in the refrigerator overnight in order to facilitate removal of the solidified fat. After removal of the fat, the extract was made up to its original volume and the following ingredients were added per liter of extract:

Bacto-peptone	5.00 grams
Bacto-tryptone	1.50 grams
Dextrose	1.00 grams
K_2HPO_4	1.25 grams

After dissolving the above ingredients, the pH was readjusted to 7.4.

The above medium, with the addition of 1.5 per cent agar, was used as a recovery medium. However, during sterilization a troublesome precipitate always developed. This was removed by the following procedure: five liters of medium contained in a Erlenmeyer flask were atuoclaved for 40 minutes at 15 pounds pressure. After autoclaving, the flask was placed in a tilted position in an incubator at 37°C until the precipitate settled. This required about one hour. The liquid was then carefully decanted after which the medium was dispersed into tubes and sterilized at 121.7°C for 15 minutes. It was stored in the refrigerator at 4°C.

The procedure for preparation of spore suspensions was as follows:

First step: Two ml of a stock culture of PA 3679 spores were transferred into each of three tubes each of which contained 10 ml of medium along with a small amount of dried pork in the bottom of the tube. This was stratified with pork agar and incubated at 37°C.

Second step: When good growth was evident, as indicated by gas production, two ml were transferred into each of six tubes containing 10 ml of medium along with dried pork particles and a clean iron wire. These tubes were incubated for one day at 37°C.

Third step: The contents of three of the above tubes were transferred into two 50 ml flasks containing the same medium. These were then incubated at 37°C for two days.

Fourth step: The contents of the two cultures from the third step were transferred to a flask which contained five liters of medium,

dried pork and 10 pieces of iron wire. This was incubated at 37°C for a period of one week and then at 30°C for two weeks. After incubation the flask was chilled in the refrigerator and the pork particles were strained off through a thin cotton pad. The liquid was centrifuged in order to collect the spores in a small volume. Otherwise harvesting and subsequent treatment of the putrefactive anaerobe spores was essentially the same as that previously described for Clostridium botulinum spores. A difficulty was encountered here that was not present with Clostridium botulinum spores, namely, removal of adhering meat particles. Consequently, more washing and filtrations were necessary in order to obtain a clean spore suspension.

Preparation of Spore Suspensions for Irradiation

In order to minimize any variation inherent within the spores, the same spore suspension was used in all experiments where comparative results were to be made. All experiments were performed with spores suspended in sterile M/15 phosphate buffer (Sorensons) at a pH of 7.0. This menstruum was selected because spores have been demonstrated to exhibit maximum heat resistance when suspended in this medium (Esty and Meyer, 1922) and therefore it is widely used in heat resistance studies.

Solutions of M/15 K_2HPO_4 and Na_2HPO_4 were mixed in the desired proportions to give a final pH value of 7.05. They were then sterilized for 15 minutes at 121°C. During sterilization the pH of the solution dropped 0.05 of a unit. After preparation, solutions were stored at 4°C in pyrex flasks. Fresh buffer solutions were prepared every two weeks.

Prior to preparation of spore suspensions for the experiments, appropriate amounts of stock suspensions were heat-shocked to break their

dormancy. Spores of Clostridium botulinum were heated for 15 minutes at 85°C and those of the putrefactive anaerobe, for five minutes at 100°C. These treatments have been shown to be optimum for subsequent germination of these organisms (Reynolds et al., 1945; Desrosier and Heiligman, 1952).

After shaking with glass beads in a rotary type shaker to dispense clumps, stock suspensions containing approximately 10^8 spores per ml were diluted with cold buffer to a final concentration of 10^6 spores per ml. The latter concentration was selected because it represented a convenient dilution for subsequent counting. Previous experiments demonstrated that the sporocidal effect of irradiation is independent of the concentration of spores in the range of 10^4 to 10^7 spores per ml. Four ml of the diluted spore suspension were next aseptically transferred to sterile five ml Neutraglass vials (Kimble) and cooled in a refrigerator at 4°C. The vials were then quickly sealed in an oxygen flame. No appreciable heating of the spore suspensions was noted during this sealing. All vials were stored in a refrigerator at 4°C until used. In order to minimize any storage effect, spore suspensions were always prepared a few hours before each series of experiments and plated immediately after irradiation.

The frozen spore suspensions were prepared by plunging the sealed vials individually into an ethyl alcohol, dry ice bath maintained at -72°C. The spore suspensions were maintained at this temperature until irradiation was completed. After irradiation the vials were stored in a deep-freeze chest until they were removed and thawed for plating. During storage, the temperature rose to -10°C at times. This method of freezing had no effect

on the viability of the spore suspensions as determined by viable counts. Actually alternate freezing and thawing of the spore suspensions as much as five times did not decrease the viability of the spores as determined by plating procedures.

Counting Techniques and Recovery Medium

Several anaerobic techniques for enumeration of anaerobic organisms have been described. Among these are: Brewer Anaerobic Plate (Brewer, 1942), Spray dish (Spray, 1931), modified deep agar shake tube (Miller et al., 1939), roller tube technique (Saleh and Ordal, 1955), incubation of petri dishes in inert atmospheres and Andersons layer plate method (Anderson, 1951). For these studies, the modified deep agar shake tube technique as described by Miller et al. (1939) was selected. Not only were maximum spore counts obtained by this technique but it was adaptable to handling of a large number of samples.

Several different recovery media have been described for counting heated anaerobic spores but so far no agreement exists concerning the best medium for irradiated spores. The pork-extract medium previously described, with the addition of 1.5 per cent agar, was used as the counting medium. This medium was slightly modified by incorporation of 0.1 per cent soluble starch and 0.1 per cent sodium thioglycolate. The former has been shown to increase the recovery of severely heated spores (Olsen and Scott, 1946) while the later assures a sufficiently low redox potential for anaerobic growth. The pork-extract medium, as modified, has been shown in one study to have a definite superiority over media that have been used for recovery of severely heated spores (Frank and Campbell, 1955). A disadvantage of

of this medium is the possible variability in the preparation of different lots of pork-extract. In order to reduce possible effects of such variability, the same batch of recovery medium was used throughout any one set of experiments.

The procedure for counting the spores was as follows: Prior to use, tubes of media were melted, cooled at 50°C and held at this temperature. Vials containing irradiated spores were then aseptically opened and appropriate dilutions of the spore suspensions were made in 99 ml of sterile distilled water. Tubes of media were then inoculated, rotated to disperse the spores uniformly, and immersed in ice water. After solidification two per cent agar containing 0.1 per cent sodium thioglycolate was added to seal the tubes. Duplicate tubes of triplicate dilutions were usually prepared and incubated at 30°C for three to six days. Prolonged incubation after this time did not result in an increase in survivors. Tubes containing between 10 to 100 visible colonies were counted with the aid of a Quebec Colony counter.

Irradiation Facility

For irradiation purposes, a cobalt-60 radiation source was employed. It consisted of 100 cobalt rods encased in aluminum jackets. The rods were six millimeters in diameter, 25 centimeters long, and were supported in two concentric circles by an aluminum rack with an inner diameter of 15 centimeters. The source had 51 rods in the outer and 49 in the inner circle; it was housed in a irradiation room, shielded with concrete, and could be raised into the room or lowered into a 12 foot well filled with water by means of a hand operated elevator. A detailed description of the source has been published (Brownell et al., 1953; Nehemias et al., 1954).

Irradiation Techniques

The center well of the cobalt-60 source was used since this position offered both the zone of maximum flux and uniformity of radiation. Because of the design of the radiation faculty, it was necessary to consider several factors in designing the equipment for irradiating samples at controlled temperatures. These were: (1) The geometry needed to be such that each individual sample received the same dosage. (2) A maximum number of samples should be irradiated at one time. (3) The device needed to be sufficiently simple so that it could be removed when not in use but still be repositioned accurately for each series of individual experiments. (4) A minimum amount of radiation should be absorbed by the system.

The above criteria were quite satisfactorily satisfied by the apparatus diagramatically illustrated in Figure 1 and described below.

The irradiation chamber, which also served as a temperature bath, was fabricated from a 4-1/2 x 6 inch stainless steel beaker. It was insulated with alternate layers of glass wool and asbestos sheets. The chamber was positioned in the center well of the radiation source by means of a wooden support which consisted of circular bases separated by a riser. When the rack containing the cobalt-60 rods was raised into the irradiation room it was enclosed in a wire cage which protected it against accidental spilling of the rods. This cage also acted as a support for the irradiation chamber in the center well. The lower disc of the chamber fitted snugly into the bottom of the cage, the other supported the chamber. A double row brass rack, each row containing spaces for fourteen vials, held the spore suspensions in proper position in the chamber. By proper adjustment of the height of the wooden riser, the irradiation chamber was positioned

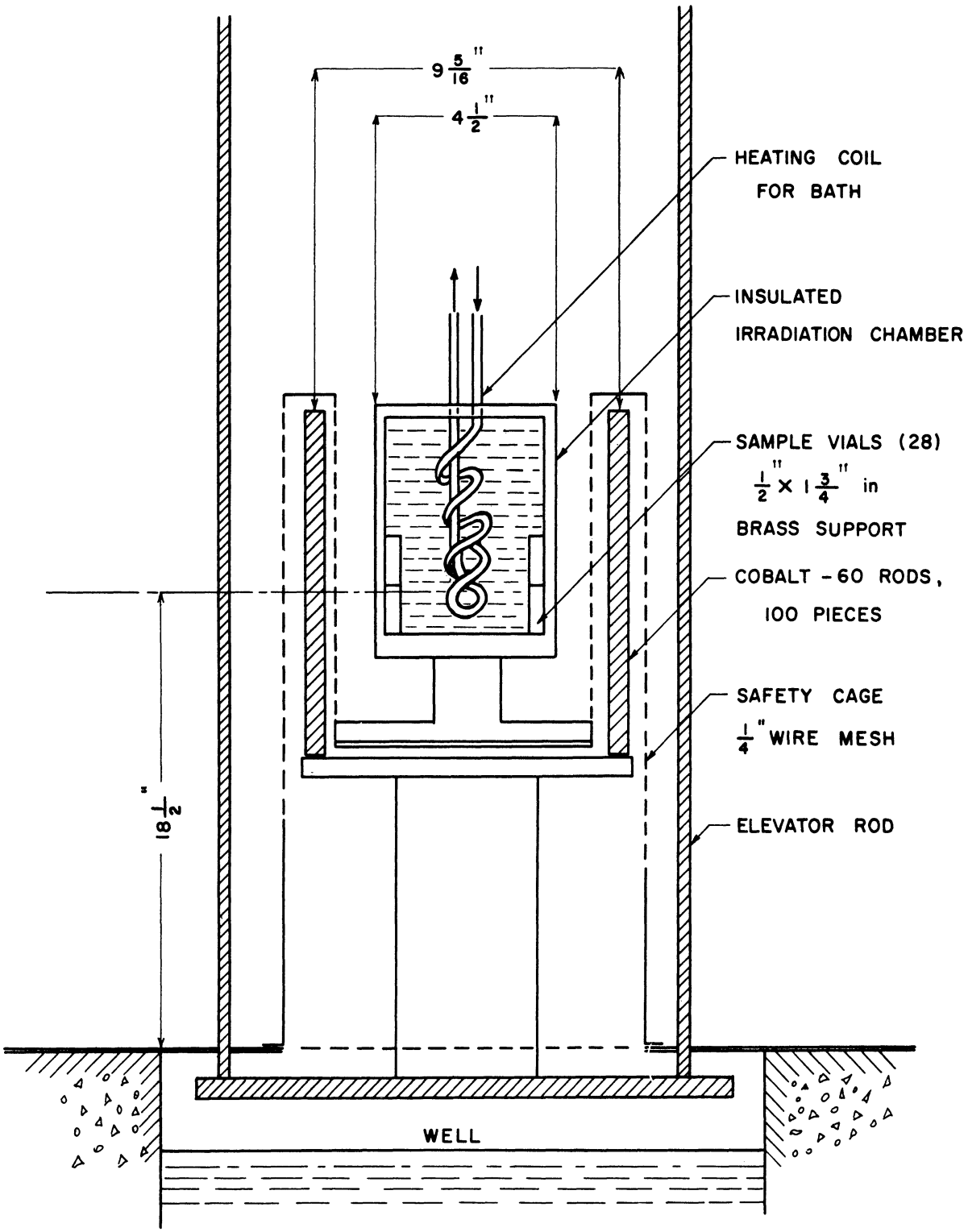

Figure 1. Diagram of Facility for Irradiation of Spore Suspensions with Cobalt-60.

in the center well in such a way that one rack of vials was above and one below the mid-plane of the cobalt rods.

A spiral coil of aluminum tubing (5/16 in. O.D.) was used to heat or cool the bath. This coil fitted into the center of the racks along the vertical axis of the irradiation chamber and was connected by means of insulated pipes to a reservoir of a temperature controlling liquid located outside the irradiation room. During operation, heating or cooling liquids were pumped through the coil in order to maintain the irradiation chamber at a constant temperature. The vials within the irradiation chamber were completely immersed in the bath. After allowing time for the entire system to come to equilibrium, the temperature could be maintained within ±2 °C with this arrangement.

For the experiments in which the spores were subjected to heat alone, an insulated temperature bath was constructed. For the heating menstruum, twelve quarts of dibutyl phthalate was employed. Temperature control was maintained by means of a bimetallic thermoregulator and a time-delay-relay system. The temperature of the bath could be controlled within 0.2 °C with this system. The thermometer used for temperature measurement was calibrated against a Bureau of Standards thermometer. To minimize errors due to extra manipulation, the spore suspensions were heated in the same vials in which they had been irradiated. Under these conditions the time required for the spore suspensions in the vials to come up to the bath temperature after immersion was determined to be two minutes.

Measurement of Dose Rates

In order to carry out a quantitative study of the lethal effects of radiation on bacterial spores it was necessary to calibrate the radiation

field in which the sample vials were placed. Furthermore, since the strength of the radiation field was not the same in all positions within the center-well, calibration at each spot occupied by a vial was necessary. Extensive calibration measurements were carried out initially, and whenever required, usually at monthly intervals.

The ferrous sulphate method as outlined by Weiss (1952) was used for radiation intensity measurements. As he pointed out dilute ferrous sulphate is oxidized quantitatively by ionizing radiation in the dose range of 0 to 50,000 rep. The concentration of ferric ion thus produced can then be determined as a function of ultra-violet light absorption of the solution at a wavelength of 305 mμ.

The procedure and solutions used for dosimetry studies were as follows:

Stock Solution (made from reagent grade stock)

Ferrous Ammonium Sulfate	3.90 g
Sodium Chloride	0.12 g
Sulfuric Acid, concentrated	2.20 ml
Triple Distilled aerated water	100.00 ml

This stock solution could be stored for about a month provided it was protected from light.

Dilute Solution (the actual dosimetric solution)

Stock Solution	1.0 ml
Sulfuric Acid	4.4 ml
Triple distilled water	200.0 ml

The dilute solution was aerated by passing filtered air through it for several hours prior to use. All solutions were prepared from glass-ware which had previously been soaked in acid dichromate cleaning solution thoroughly rinsed and then dried in an oven. The same type of vials used

for dosimetric studies were later used to contain spore suspensions used in the experimental runs. These vials were soaked at least 24 hours in acid dichromate cleaning solution and then thoroughly rinsed. The final rinsing was carried out with triple distilled water. The vials were thoroughly dried in an oven before use. For calibration runs, four ml of the dosimetry solution were carefully pipetted into each vial; then the vials were sealed. These dosimeters were positioned in the irradiation vessel in the exact position in which the spore suspensions were later to be exposed; the vessel was placed in the center-well and the source brought into position. The time of exposure was adjusted to give a total dose between 10 and 45 kilorep since the procedure is most accurate in this range.

The concentration of ferric ion, produced by the radiations, was determined by comparison of the optical densities of the irradiated solution to that of known concentrations of ferric ion. The optical density of the solutions was determined in a Beckman Spectrophotometer model DU at wavelength of 305 mμ and with a slit width of 0.5 mm. The quartz cuvettes used in the procedure were rinsed four times with triple distilled water and dried with acetone between each reading.

For the conversion of ferric ion concentration to radiation dose a value based upon oxidation of 15.4 micromoles of ferric ion per liter per kilorep was employed. This value is based on the absorption of 93 ergs per gram of water exposed to one roentgen of radiation (Weiss, 1952). It defines the unit, rep, used in this thesis.

The standard ferric ion solution used for calibrating the spectrophotometer was prepared as follows: a 0.1N solution of ferric sulphate

was dissolved in 0.8N sulfuric acid. The ferric sulphate solution was then standardized by reduction of adequate portions with high purity aluminum followed by immediate titration using Ferroin (o-Phenanthroline) solution as an indicator. The standardized ferric solution was diluted to concentrations between 50 and 500 micromoles of ferric ion per liter and the optical density of these samples then determined. From this a standard curve was prepared which related the total radiation dose to the ferric ion concentration observed.

Dosimetry Results

The results of the dosimetry study are presented in Table I: Figure 2 shows the relative dose rate in the vials during August 1954, in the various positions that they occupied during irradiation.

TABLE I

DOSE RATE AT VARIOUS POSITIONS
IN THE IRRADIATION CHAMBER, AUGUST, 1954

Bottom Row of Rack

Dosimeter	rep per hour
1	221,000
2	232,000
3	227,000
4	228,000
5	238,000
6	235,000
7	225,000
8	224,000
9	221,000
10	225,000
11	220,000
12	225,000

TABLE I (CONT'D)

Top Row of Rack

Dosimeter	rep per hour
1	225,000
2	214,000
3	213,000
4	216,000
5	204,000
6	202,000
7	219,000
8	200,000

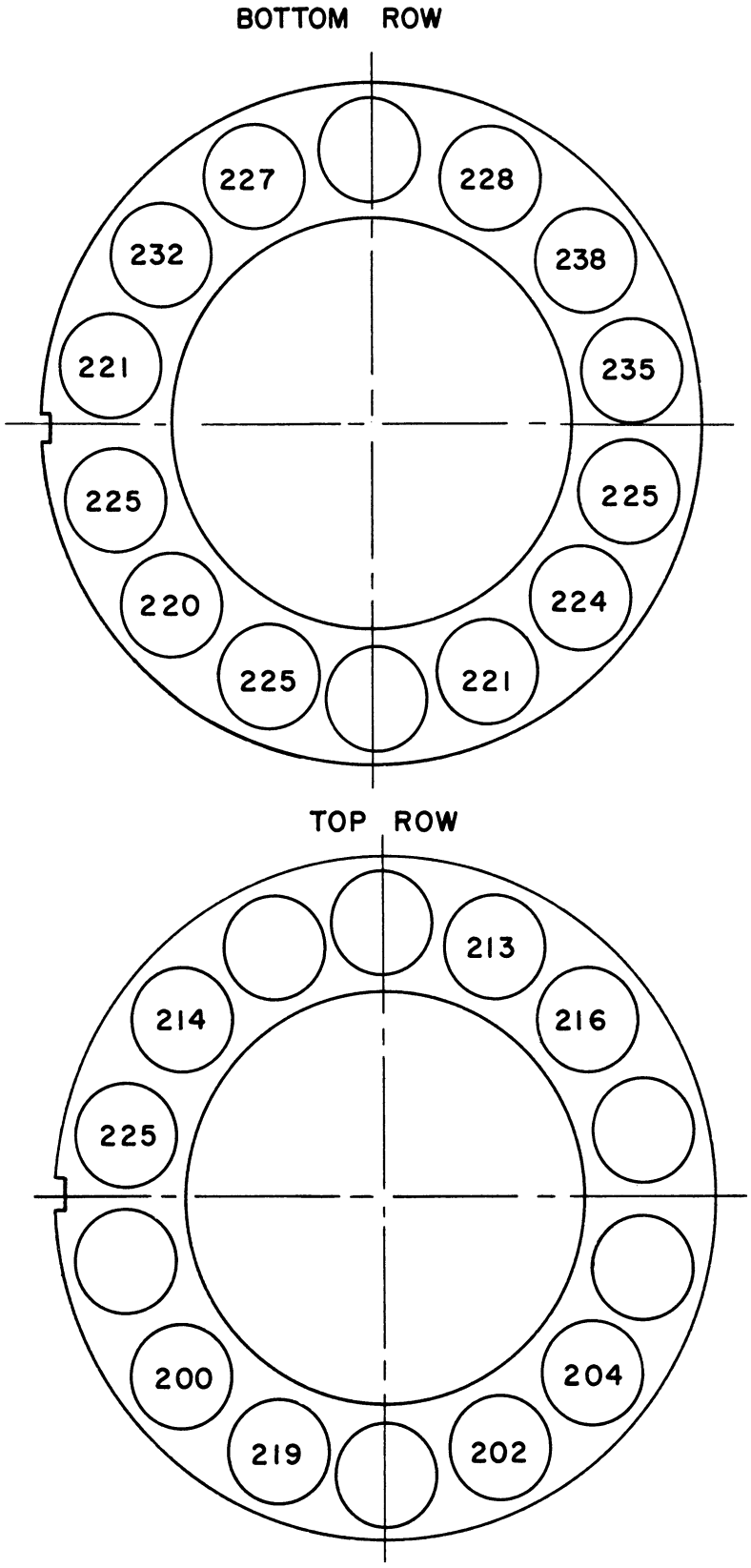

Figure 2. Dose Rate in Rep Per Hour x 10^3 at Various Positions in the Irradiation Chamber.

EXPERIMENTAL RESULTS

The Effect of Temperature During Irradiation on the Survival of Cl. Botulinum and putrefactive anaerobe Spores

The object of the first series of experiments was to determine whether the exposure of bacterial spores to gamma radiation at a wide range of temperature would affect their subsequent survival as determined by colony formation. As outlined in the section on methods, the experimental conditions were so designed that the only variable was the ambient temperature during irradiation. The general procedure followed was to bring the irradiation bath to a desired temperature, add the vials containing the spore suspensions and then allow them to equilibrate to the bath temperature. The time for the spore suspensions, within the vials, to reach the bath temperature was determined to be two minutes. The irradiation chamber was then placed in the radiation field and samples were removed at time intervals which gave the desired dosage levels. In the experiments in which the spores were irradiated at temperatures greater than 30°C the vials were removed as rapidly as possible and plunged into a container containing ice water. This was necessary to minimize any after effect due to post radiation heating of the spores. The time for removal of samples was never more than 30 seconds. The total time for manipulation of the radiation source varied between one to two minutes. After irradiation the samples were stored in a refrigerator until the spores were cultured into the growth medium. In cases where the spores were exposed to temperatures greater than 30°C, appropriate temperature controls were used. In this case the spore suspensions, contained in the

vials, were exposed at the temperature at which the irradiation was performed for a period of time equivalent to that used for the experimental suspensions.

In the experiments in which the spores were frozen during irradiation, the sample used to determine the initial number of spores was also frozen and maintained in that condition during irradiation of the other samples. The frozen spore suspensions were thawed just before subculturing into the recovery medium. As can be seen from the temperature controls, freezing and thawing did not have a deleterious influence on the subsequent survival of the spores as determined by colony formation. The previously mentioned procedures were the protocol for this first series of experiments.

The data from the experiments for Cl. botulinum 213-B spores suspended in M/15 neutral phosphate buffer and irradiated in the temperature range from -70° to 95°C are presented in Table II and Figure 3. As can be observed, the number of organisms surviving radiation exposure can be influenced by the ambient temperature during irradiation. The spores are slightly more resistant in the frozen condition when compared with the non-frozen state. There is a trend towards radio-resistance as the temperature is raised above room temperature. With this suspension of 213-B spores the maximum number of survivors occurred at approximately 30°C. In order to more clearly demonstrate this effect the number of survivors at a constant dosage of 740,000 rep are shown in Figure 4 and Table III.

The results of a similar experiment, but with spores of putrefactive anaerobe NCA strain 3679, are presented in Table IV and Figure 5.

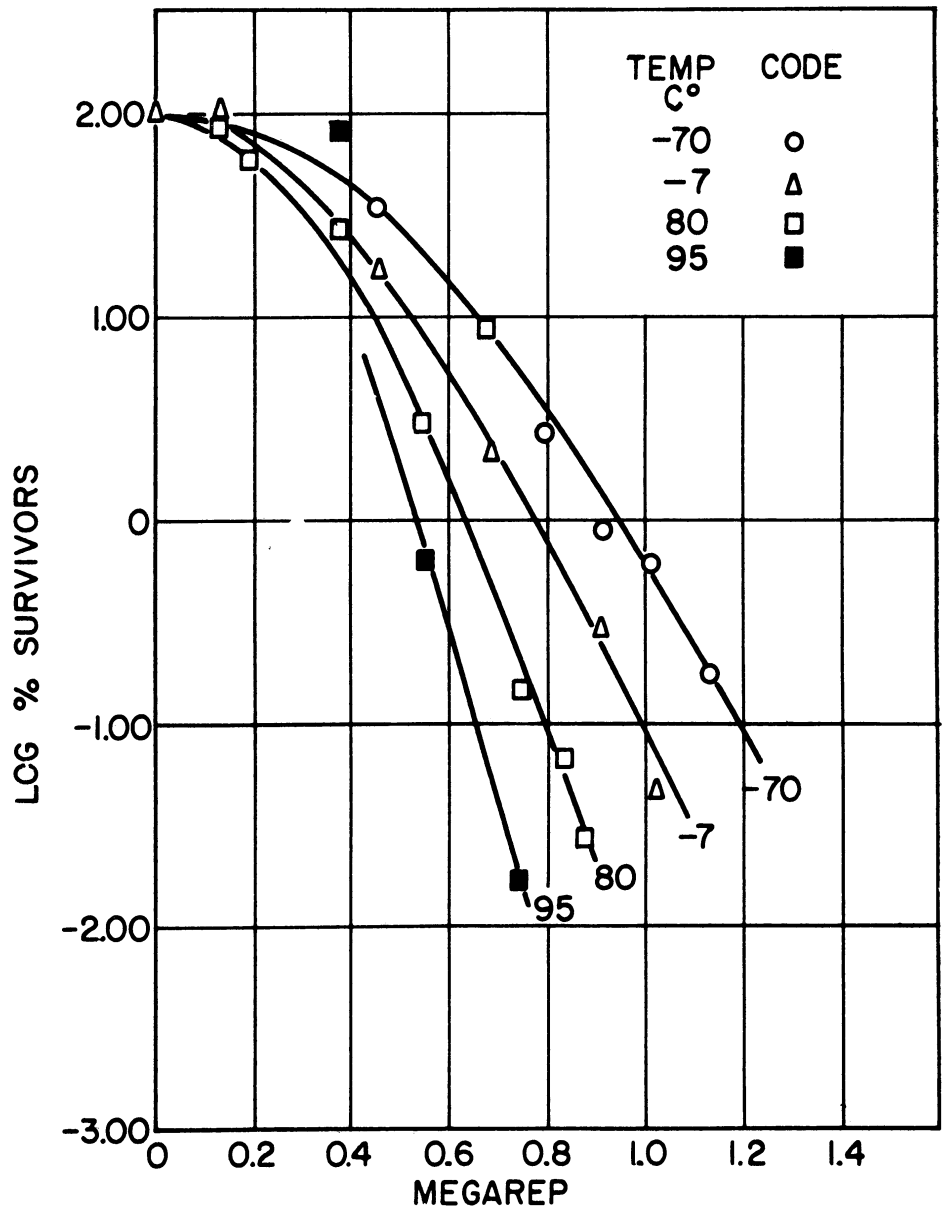

Figure 3. Effect of Temperature During Irradiation with Gamma, Rays from Cobalt-60 on the Survival of Spores of Cl. botulinum 213-B Suspended in M/15 Phosphate Buffer at pH 7.0.

TABLE II

EFFECT OF TEMPERATURE DURING IRRADIATION WITH GAMMA RAYS FROM COBALT-60 ON THE SURVIVAL OF SPORES OF C. BOTULINUM 213B WHEN SUSPENDED IN M/15 PHOSPHATE BUFFER AT pH 7.0

	5° C		30° C		58° C	
Dose* Megarep	Number of Spores	Log % Survivors	Number of Spores	Log % Survivors	Number of Spores	Log % Survivors
0	6,200,000	2.000	2,500,000	2.000	3,500,000	2.000
0.185	2,000,000	1.508	---------	-----	1,300,000	1.570
0.370	5,700,000	0.964	900,000	1.560	5,200,000	1.170
0.550	140,000	0.354	350,000	1.146	100,000	0.456
0.647	---------	-----	110,000	0.644	---------	-----
0.740	41,000	2.820	22,000	-0.055	15,000	-0.369
0.832	---------	-----	1,000	-1.398	---------	-----
0.925	3,900	-2.201	150	-2.222	1,500	-1.369
1.017	150	-3.617	85	-3.469	---------	-----
1.110	3	-4.315	---------	-----	---------	-----

	80° C		95° C		Heat Control		
Dose* Megarep	Number of Spores	Log % Survivors	Number of Spores	Log % Survivors	Hrs	80° C	85° C
0	5,500,000	2.000	2,700,000	2.000	0	5,500,000	2,700,000
0.185	3,200,000	1.765	---------	-----	2	---------	3,000,000
0.370	1,400,000	1.407	2,300,000	1.930	3	---------	2,300,000
0.550	160,000	0.465	14,000	-0.215	4	---------	1,500,000
0.740	7,800	-0.848	45	-1.777	5	3,000,000	1,400,000
0.832	3,500	-1.197	0	-----	-	---------	---------
0.877	1,400	-1.595	0	-----	-	---------	---------

	-70° C		-7° C		27° C	
Dose** Megarep	Number of Spores	Log % Survivors	Number of Spores	Log % Survivors	Number of Spores	Log % Survivors
0	950,000	2.000	520,000	2.000	670,000	2.000
0.227	720,000	1.903	520,000	2.000	530,000	1.898
0.454	350,000	1.522	79,000	1.225	260,000	1.589
0.680	75,000	0.940	9,700	0.314	78,000	1.065
0.794	25,000	0.440	-------	-----	-------	-----
0.907	12,000	-0.060	1,800	-0.523	71,000	1.025
1.020	5,000	-0.246	200	-1.366	-------	-----
1.134	1,500	-0.773	-------	-----	790	-0.928

Frozen Control 9.0×10^5

*Dosage Rate = 0.185 megarep per hour.

**Dosage Rate = 0.227 megarep per hour (vials not immersed in liquid).

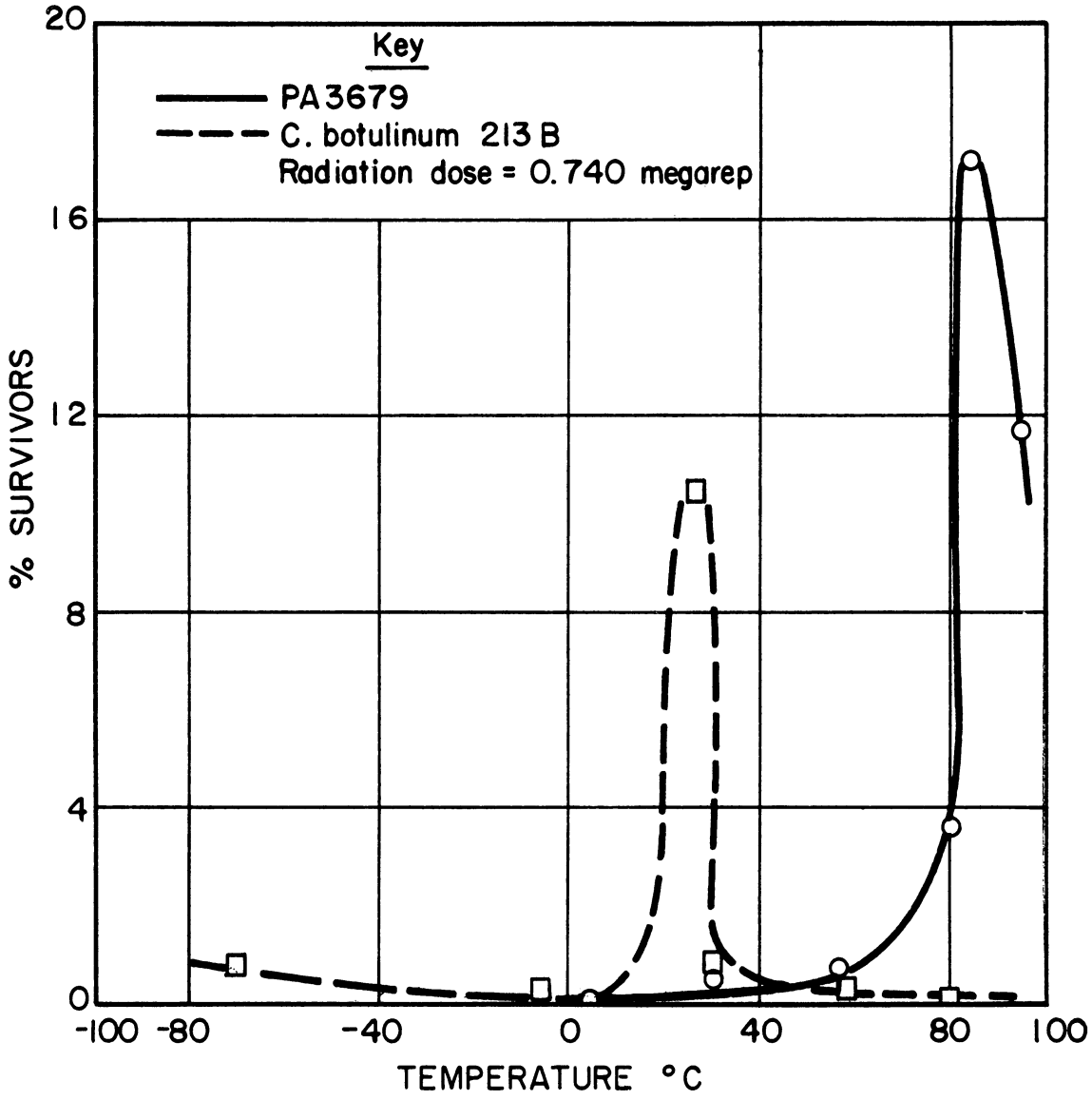

Figure 4. Effect of Temperature During Irradiation with Gamma Rays from Cobalt 60 on the Survival of Anaerobic Bacterial Spores Suspended in M/15 Phosphate Buffer at pH 7.0.

TABLE III

EFFECT OF TEMPERATURE DURING IRRADIATION WITH GAMMA RAYS FROM COBALT-60 ON THE SURVIVAL OF ANAEROBIC BACTERIAL SPORE SUSPENDED IN M/15 PHOSPHATE BUFFER AT pH 7.0

Temp, °C	Percent Survivors		
	Dosage Megarep		
	0.550	0.647	0.740
	PA 3679		
5	1.75		1.05
30	4.65	0.697	0.500
56	8.75		0.675
58	12.3		0.229
80	12.7		3.62
85	58.5	20.0	17.2
95	47.5		11.7
	C. botulinum 213B		
-70	8.7	2.8	0.8
- 7	2.0		0.3
5	2.3		0.06
27	11.6		10.5
30	14.0	4.4	0.88
56	10.0	0.3	
58	2.9		0.4
80	2.9		0.14
95	0.16		

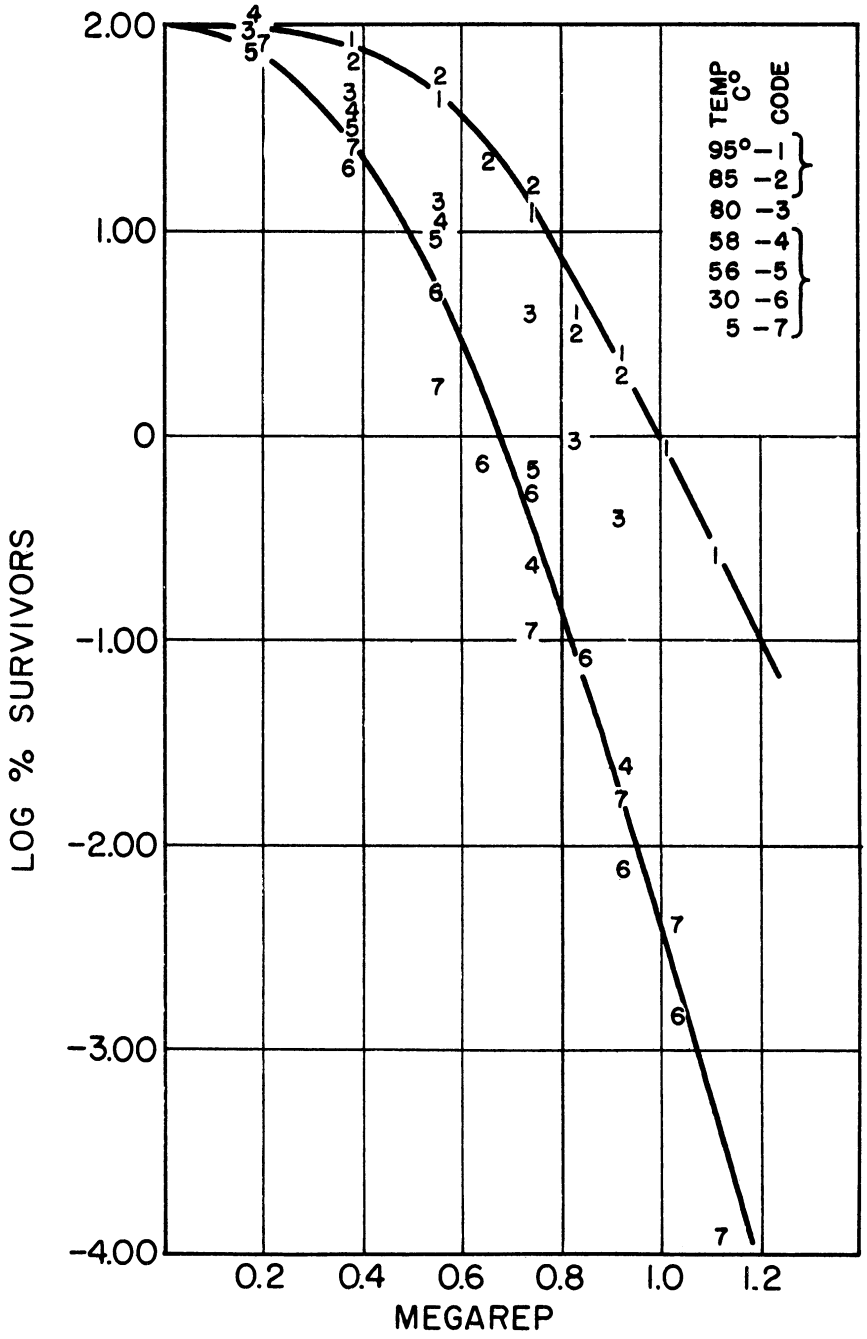

Figure 5. Effect of Temperature During Irradiation with Gamma Radiation from Cobalt-60 on the Survival of Spores of PA 3679 Suspended in M/15 Phosphate Buffer at pH 7.0.

TABLE IV

EFFECT OF TEMPERATURE DURING IRRADIATION WITH GAMMA RAYS FROM COBALT-60 ON THE SURVIVAL OF SPORES OF PA 3679 WHEN SUSPENDED IN M/15 PHOSPHATE BUFFER AT pH 7.0

	5°C		30°C		56°C	
Dose,* megarep	Number of Spores	Log % Survivors	Number of Spores	Log % Survivors	Number of Spores	Log % Survivors
0	850,000	2.000	280,000	2.000	400,000	2.000
0.185	700,000	1.911	-------	-----	300,000	1.875
0.370	220,000	1.409	54,000	1.286	180,000	1.653
0.550	15,000	0.243	13,000	0.668	35,000	0.942
0.647	-------	-----	1,900	-0.157	-------	-----
0.740	900	-0.979	1,400	-0.301	2,700	-0.171
0.832	-------	-----	220	-1.103	-------	-----
0.925	160	-1.731	20	-2.146	-------	-----
1.017	35	-2.392	4	-2.845	-------	-----
1.110	1	-3.935	-------	-----	-------	-----

*Dosage rate = 0.185 megarep per hour

	58°C		80°C		85°C	
Dose,* megarep	Number of Spores	Log % Survivors	Number of Spores	Log % Survivors	Number of Spores	Log % Survivors
0	480,000	2.000	1,100,000	2.000	700,000	2.000
0.185	480,000	2.000	950,000	1.937	-------	-----
0.370	210,000	1.640	530,000	1.684	470,000	1.826
0.550	59,000	1.089	140,000	1.104	410,000	1.767
0.647	-------	-----	---------	-----	140,000	1.301
0.740	1,100	-0.641	40,000	0.558	120,000	1.235
0.832	-------	-----	10,000	-0.041	55,000	0.895
0.925	120	-1.602	4,000	-0.439	13,400	0.267
1.017	-------	-----	---------	-----	-------	-----
1.110	-------	-----	---------	-----	-------	-----

	95°C		Heat Control PA 3679		
Dose, megarep	Number of Spores	Log % Survivors	Hr	85°C	95°C
0	1,200,000	2.000	-	-------	---------
0.185	---------	-----	0	700,000	1,200,000
0.370	900,000	1.875	2	950,000	1,400,000
0.550	570,000	1.676	3	-------	1,600,000
0.647	---------	-----	4	810,000	1,400,000
0.740	140,000	1.068	5	-------	1,200,000
0.832	51,000	0.628	5.5	790,000	---------
0.925	30,000	0.398	-	-------	---------
1.017	10,000	-0.061	-	-------	---------
1.110	3,100	-0.558	-	-------	---------

The number of spores surviving at a constant dose but irradiated at various temperatures is given in Figure 4 and Table III. In this series of experiments the organisms were irradiated in the temperature range of 5° to 90°C. From the data presented it is observed that the spores of this organism are most sensitive to radiation at a temperature of 5°C. The greater number of survivors were obtained at a irradiation temperature of 90°C. As can be observed from the temperature control, holding the spores of this organism at 90°C for five and one half hours did not have an adverse effect on their viability.

Similarily the experimental results obtained when spores of Cl. botulinum 62-A were irradiated in the temperature range of -72° to 87°C are presented in Table V and Figure 6. A curve indicating the number of survivors at a constant dose of 860,000 rep at various irradiation temperatures is given in Figure 7. The results show that the spores of this organism exhibit a slight increase in resistance to radiation when irradiation is carried out at -72°C as compared to 4°C. As was observed with the spores of the other bacteria, there is an increase in resistance to gamma radiation as the irradiation temperature is increased. With spores of this bacterial strain the maximum resistance is obtained at about 70°C. A further increase in the irradiation temperature results in a rapid kill of the organisms. This is demonstrated when irradiation is carried out at 87°C. The temperature control at 87°C shows that some heat inactivation of the spores occurs at this temperature. However, the independent effect of this temperature was not pronounced for the length of time these spores were held at this temperature.

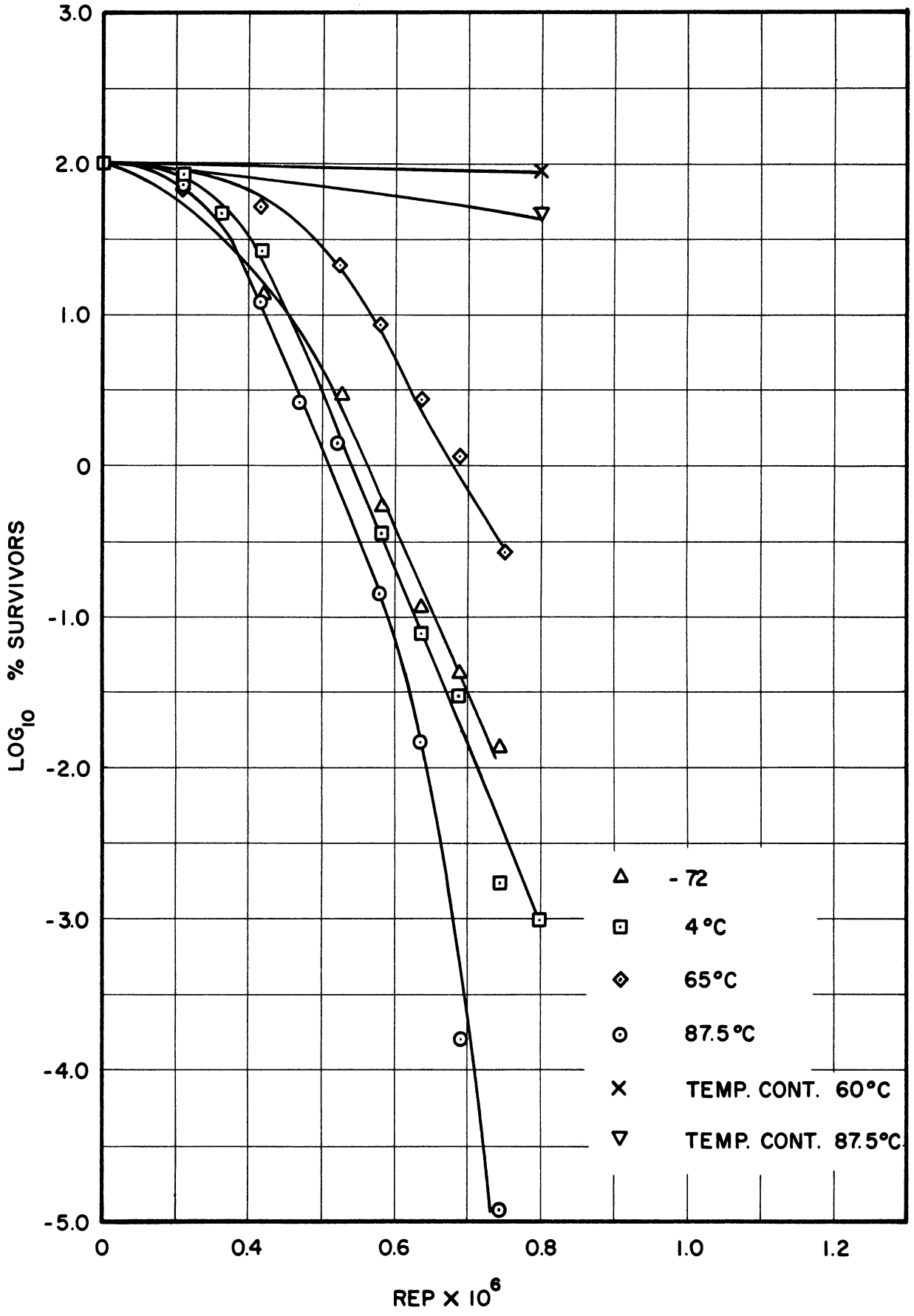

Figure 6. Effect of Temperature During Irradiation on the Survival of Cl. botulinum 62-A Spores Suspended in M/15 Phosphate Buffer at pH 7.0.

TABLE V

EFFECT OF TEMPERATURE DURING IRRADIATION ON THE SURVIVAL OF *Cl. botulinum* 62-A SPORES SUSPENDED IN PHOSPHATE BUFFER AT PH 7.0

	4° C			20° C		
Dose Megarep	Number of Spores	% Survivors	Log % Survivors	Number of Spores	% Survivors	Log % Survivors
0	1,400,000	100.00	2.000	785,000	100.00	2.000
0.218	1,200,000	85.7	1.933	-	-	-
0.327	660,000	47.1	1.673	-	-	-
0.436	360,000	25.7	1.41	465,000	55.4	1.744
0.545	-	-	-	615,000	21.0	1.322
0.654	-	-	-	61,500	7.82	0.893
0.763	4,900	.35	-0.456	14,350	1.83	-0.265
0.872	1,100	.0785	-1.105	4,350	0.554	-0.257
0.981	410	.0293	-1.533	1,500	0.191	-0.719
1.090	24	.00171	-2.766	455	0.058	-1.237
1.199	14	.00100	-3.000	-	-	-

	32° C			43° C		
Dose Megarep	Number of Spores	% Survivors	Log % Survivors	Number of Spores	% Survivors	Log % Survivors
0	7,250,000	100.00	2.000	5,350,000	100.00	2.000
0.218	-	-	-	6,650,000	124.00	
0.327	-	-	-	-	-	-
0.436	2,400,000	33.1	1.54	3,450,000	64.5	1.82
0.545	-	-	-	1,800,000	33.6	1.528
0.654	230,000	3.17	0.50	850,000	15.8	1.2
0.763	98,000	1.35	0.013	420,000	7.85	0.904
0.872	14,900	.205	-0.688	245,000	4.57	0.66
0.981	610	0.0084	-2.075	55,000	1.02	0.0086
1.090	145	.002	-2.699	2,850	.0533	-1.274
1.199	-	-	-	-	-	-

	54° C			60° C		
Dose Megarep	Number of Spores	% Survivors	Log % Survivors	Number of Spores	% Survivors	Log % Survivors
0.	7,600,000	100.0	2.000	3,550,000	100.00	2.000
0.218	4,500,000	59.3	1.77	2,550,000	71.80	1.856
0.327	-	-	-	-	-	-
0.436	4,150,000	54.5	1.73	1,900,000	53.50	1.728
0.545	2,500,000	32.9	1.52	-	-	-
0.654	860,000	11.3	1.05	485,000	13.65	1.350
0.763	360,000	4.73	0.665	300,000	8.45	0.927
0.872	99,000	1.30	0.114	94,000	3.78	0.578
0.981	58,500	0.77	0.115	37,000	1.04	0.017
1.090	8,950	0.118	0.928	9,600	0.27	-0.569
1.199	-	-	-	-	-	-

TABLE V (CONT'D)

	73° C			84° C		
Dose Megarep	Number of Spores	% Survivors	Log % Survivors	Number of Spores	% Survivors	Log % Survivors
0.	5,750,000	100.00	2.000	4,900,000	100.00	2.000
0.218	2,000,000	34.800	1.541	4,600,000	93.9	1.8690
0.327	-	-	-	-	-	-
0.436	1,900,000	33.0	1.519	2,300,000	46.8	1.294
0.545	-	-	-	-	-	-
0.654	870,000	15.1	1.179	220,000	4.48	0.281
0.763	700,000	12.2	1.086	255,000	5.20	0.180
0.872	108,000	1.88	0.2742	12,700	0.259	-0.818
0.981	43,000	0.748	-0.126	2,150	0.0438	-1.726
1.090	6,700	0.116	-0.935	-	-	-
1.199	-	-	-	-	-	-

	87.5° C			-65° C		
Dose Megarep	Number of Spores	% Survivors	Log % Survivors	Number of Spores	% Survivors	Log % Survivors
0	4,350,000	100.00	2.000	15,250,000	100.00	2.000
0.218	3,700,000	86.00	1.935	-	-	-
0.327	-	-	-	-	-	-
0.436	520,000	11.9	1.076	4,450,000	29.2	1.465
0.545	110,000	2.53	0.401	-	-	-
0.654	7,300	0.168	-0.775	540,000	3.52	0.547
0.763	6,300	0.161	-0.791	285,000	1.87	0.272
0.872	455	0.01445	-1.839	109,000	0.718	-0.145
0.981	7	0.00016	-3.793	29,000	0.190	-0.722
1.090	.5	0.0000115	-4.939	20,000	0.131	-0.883
1.199						

	-72° C			4° C		
Dose Megarep	Number of Spores	% Survivors	Log % Survivors	Number of Spores	% Survivors	Log % Survivors
0	1,800,000	100.00	2.000	7,150,000	100.0	2.000
0.218	-	-	-	-	-	-
0.327	-	-	-	-	-	-
0.436	200,000	11.1	1.045	1,650,000	21.1	1.361
0.545	-	-	-	620,000	8.68	0.938
0.654	55,000	3.05	0.484	245,000	3.43	0.535
0.763	8,500	0.472	-0.326	46,500	0.642	-0.192
0.872	2,400	0.133	-0.976	15,600	0.218	-0.661
0.981	760	0.0422	-1.375	4,250	0.0594	-1.276
1.090	240	0.0133	-1.877	315	0.0044	-2.355
1.199	-	-	-	126	0.00177	-2.752

TABLE V (CONT'D)

Temperature Controls

60° C		73° C	
Hours	Number of Spores	Hours	Number of Spores
0	3,500,000	0	4,750,000
1	3,450,000	1	5,100,000
2	4,100,000	2	5,000,000
4	3,450,000	4.2	5,850,000
4.5	3,450,000	5	4,900,000

84° C		87.5° C	
Hours	Number of Spores	Hours	Number of Spores
0	4,900,000	0	4,350,000
3	4,500,000	1	3,450,000
16.2	1,520,000	2	3,400,000
1	2,700,000	4	1,950,000
3	3,450,000	6	1,950,000
4.5	3,750,000		

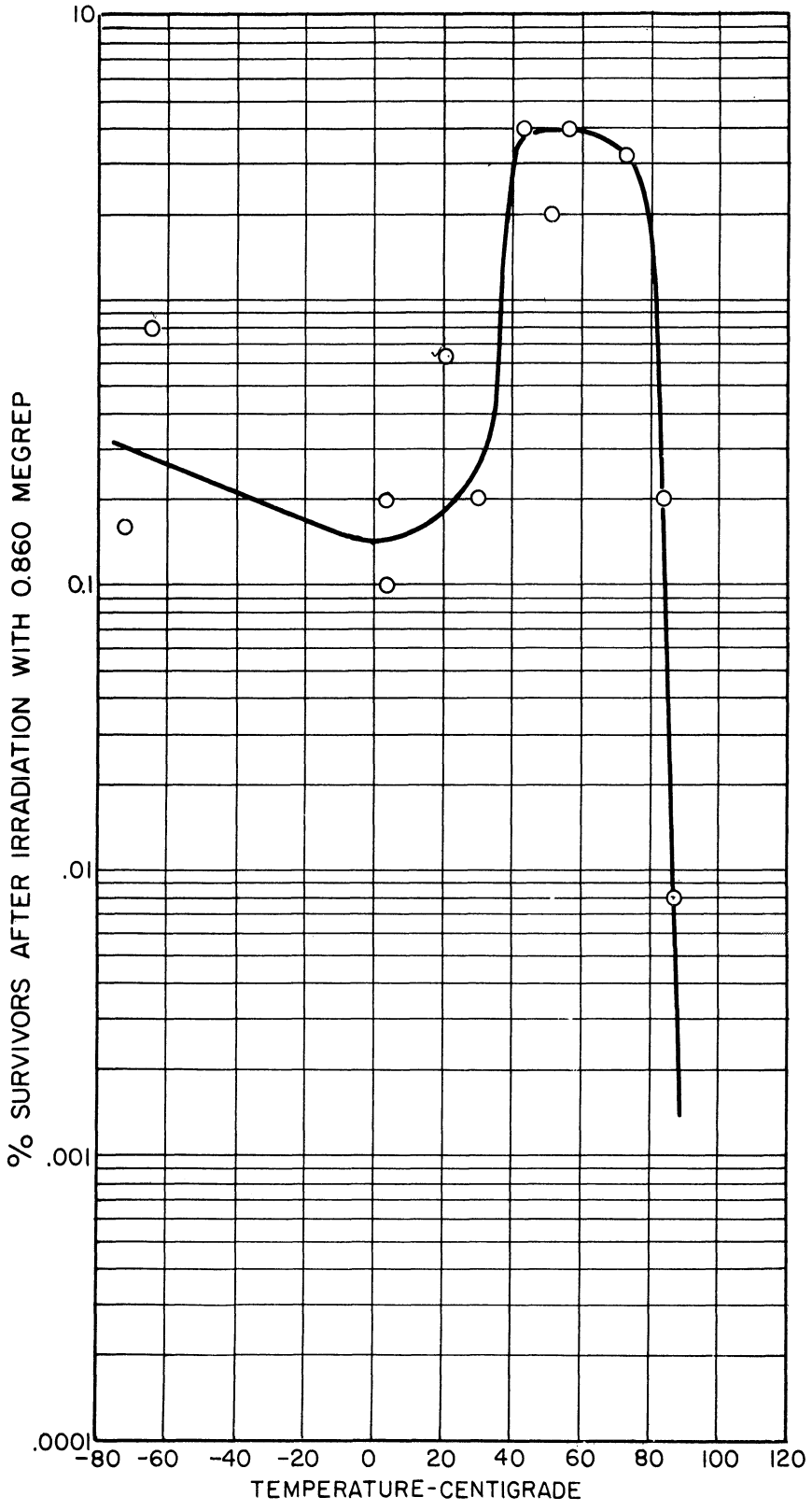

Figure 7. Effect of Temperature During Irradiation on the Lethality of Gamma Radiation from Cobalt 60 for the Spores of Cl. Botulinum 62A When They are Suspended in M/15 Phosphate Buffer at pH 7.0.

In order to substantiate the above results, a series of experiments was performed using different spore suspensions of the same organism. In this case only a few temperatures were selected in order to strengthen the observation that bacterial spores are more resistant at a high temperature and also when irradiated in the frozen condition at a temperature of -72°C. In this series, spores of putrefactive anaerobes and Cl. botulinum 213-B were irradiated at -72°C, 10°C, and 90°C. Another botulinum strain designated as 457-A was also included. The spores of this strain were irradiated at -72°C, 5°C, 25°C, and 90°C. The results from this series of experiments are presented in Tables VI and VIII and Figures 8 through 10. The trend observed in previous experiments is again evident in that irradiation at high temperatures and in the frozen state increases the resistance of the spores.

The Effect of Temperature During Irradiation on the Survival of Bacillus subtilis var. niger Spores

In order to determine if the temperature of irradiation has an effect on spores of another genus, spores of the aerobic bacterium, Bacillus subtilis var. niger, were irradiated. In this case the number of spores surviving at irradiation temperatures of 5° and 65°C was determined. A temperature of 65°C was selected since this represents a sub-lethal temperature for this organism. This can be observed from the temperature control. The results presented in Table IX and Figure 11 show a slight but definite increase in number of surviving spores at all dosage levels at 65°C. The other aspect for which this experiment was performed will be presented subsequently.

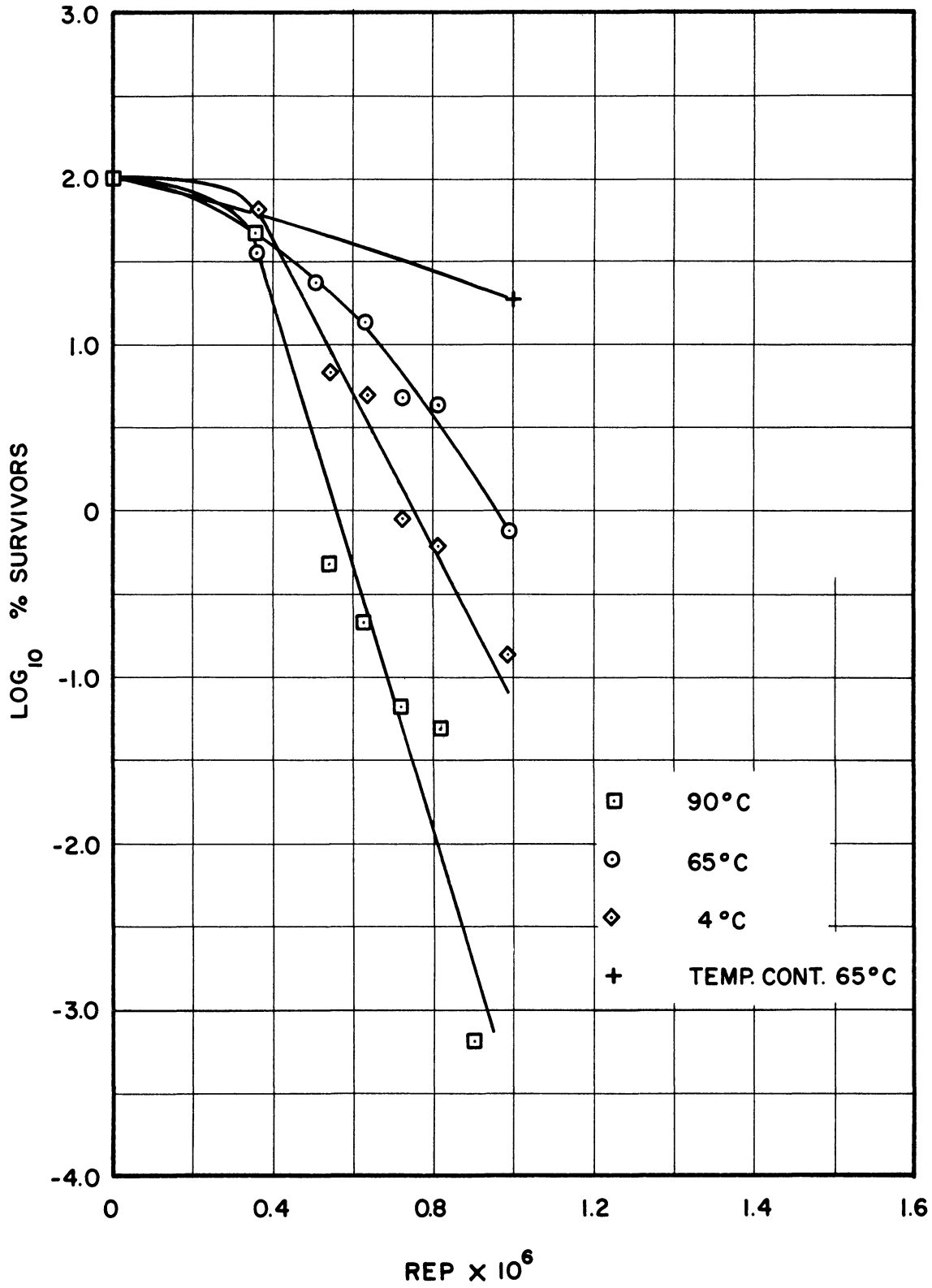

Figure 8. Effect of Temperature During Irradiation on Survival of Cl. botulinum 213-B Spores Suspended in M/15 Phosphate Buffer at pH 7.0.

TABLE VI

EFFECT OF TEMPERATURE DURING IRRADIATION ON SURVIVAL OF Cl. botulinum 213-B SPORES

Dose Rep.	Number Of Spores	% Survivors	Log % Survivors
90°C			
0	7,000,000	100.0	2.00
360,000	290,000	4.14	0.670
540,000	33,000	0.472	-0.326
630,000	16,000	0.229	-0.649
720,000	4,700	0.0672	-1.173
810,000	3,400	0.0485	- .314
900,000	48	0.000675	-3.171
	Temperature Control 90° C		
Time Hrs.			
0	7,000,000	100.0	2.000
2	910,000	13.0	1.114
4	140,000	2.0	0.301
6	140,000	2.0	0.301
8	200,000	2.89	0.456
65°C			
0	10,000,000	100.0	2.000
360,000	3,420,000	34.2	1.534
540,000	2,358,000	23.6	1.369
630,000	1,340,000	13.4	1.127
720,000	482,000	4.82	0.683
810,000	432,000	4.32	0.635
990,000	7,450	0.075	-0.125
25°C			
0	6,300,000	100.0	2.000
360,000	3,700,000	58.7	1.386
540,000	1,210,000	19.2	1.156
765,000	117,000	1.81	0.152
900,000	10,200	.162	-0.693
1,080,000	6,300	.100	-0.874

cont'd.

TABLE VI (CONT'D)

Dose Rep.	Number Of Spores	% Survivors	Log % Survivors
4°			
0	3,100,000	100.0	2.000
360,000	955,000	30.50	1.484
540,000	215,000	6.93	0.841
630,000	157,000	5.06	0.704
720,000	32,000	1.03	0.013
810,000	19,000	0.61	-0.212
990,000	4,700	0.15	-0.821
	Temperature Control 65° C		
Time Hrs.			
0	10,000,000	100.00	2.000
3	8,000,000	80.00	1.9031
6	1,900,000	19.00	1.2788

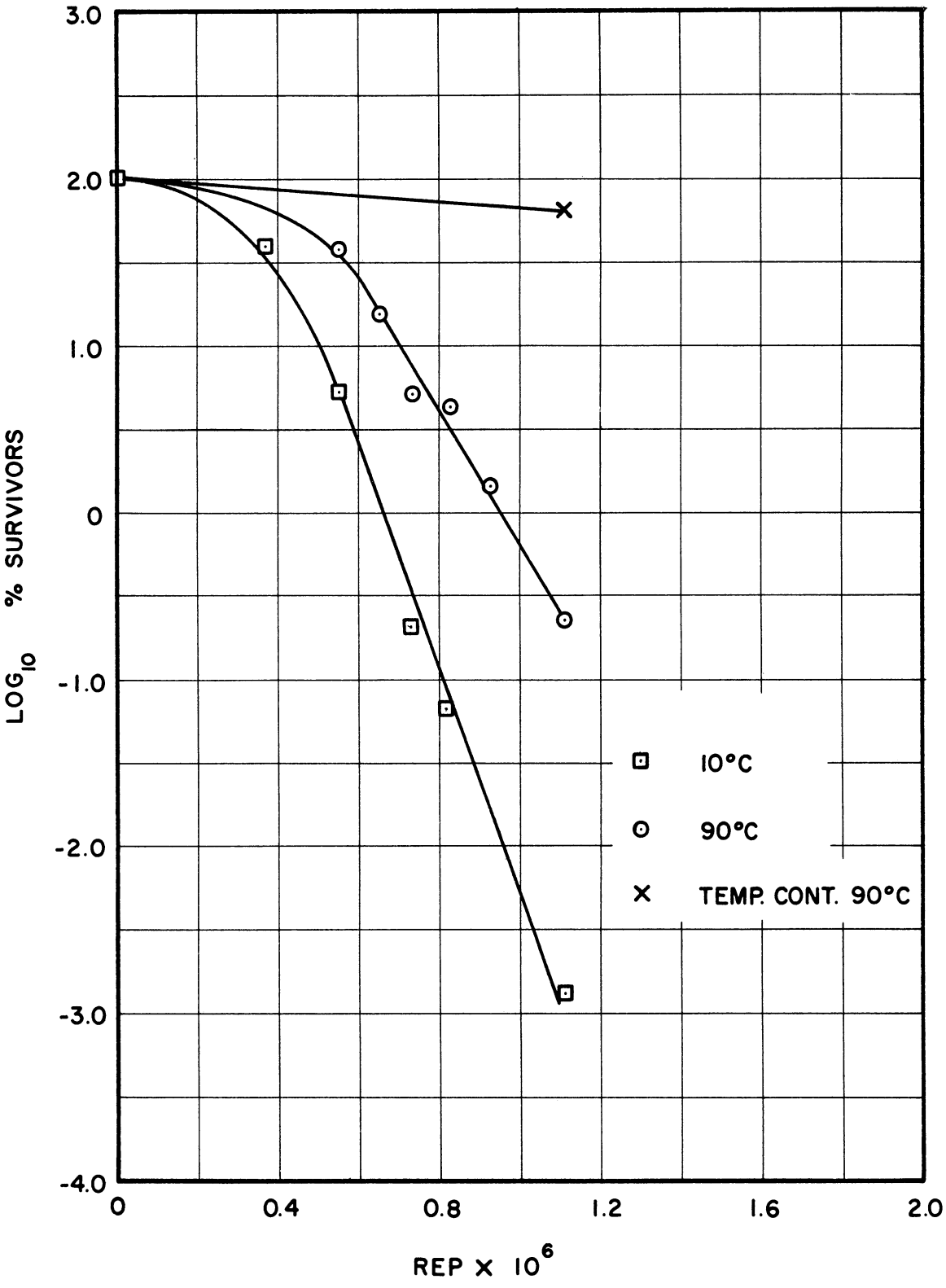

Figure 9. Effect of Temperature During Irradiation on The Survival of putrefactive anaerobe Spores NCA 3679 Suspended in M/15 Phosphate Buffer at pH 7.0.

TABLE VII//

EFFECT OF IRRADIATION AT 10°C AND 90°C
ON SURVIVAL OF PUTREFACTIVE ANAEROBE NCA 3679

	Irradiated at 10° C			Irradiated at 90° C		
Dose Rep	Spores per ml	% Survivors	Log % Survivors	Spores per ml	% Survivors	Log % Survivors
0	380,000	100.0	2.000	590,000	100.0	2.000
370,000	150,000	39.5	1.593	340,000	37.6	1.575
555,000	18,000	5.15	0.712	200,000	33.9	1.530
647,500	-	-	-	90,000	15.3	1.185
736,000	800	0.21	-0.678	30,000	5.1	0.708
826,500	260	0.0684	-1.165	25,000	4.23	0.626
925,000	-	-	-	8,400	1.42	0.152
1,175,000	5	0.00131	-2.8827	1,300	0.222	-0.6517

Temperature Control

0 Hrs	590,000	100.00	2.000
2 Hrs	510,000	86.5	1.937
3 Hrs	410,000	69.5	1.842
5 Hrs	380,000	64.5	1.809
6 Hrs	410,000	69.5	1.842

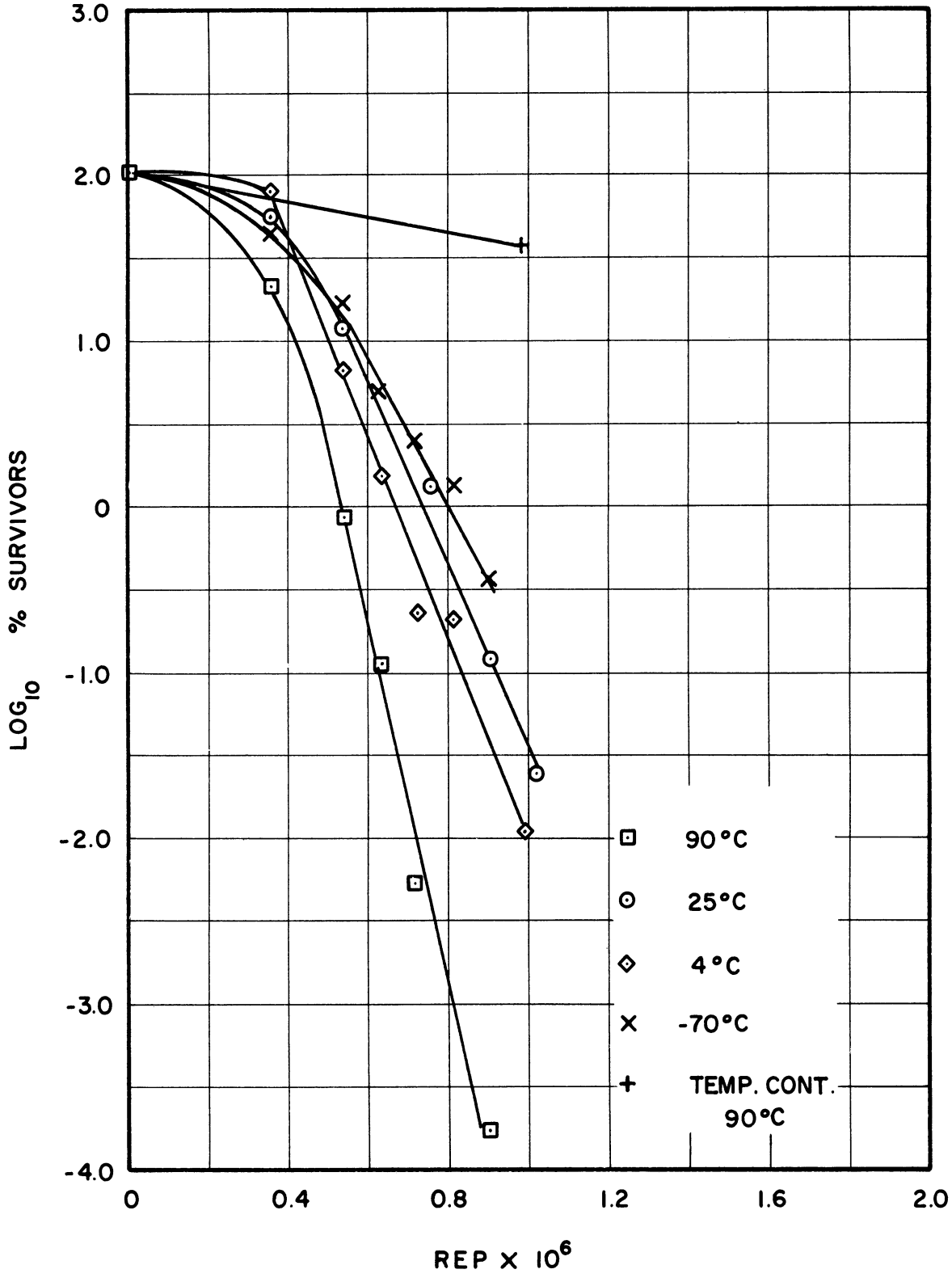

Figure 10. Effect of Temperature During Irradiation on the Survival of Cl. botulinum 457-A Spores Suspended in M/15 Phosphate Buffer at pH 7.0.

TABLE VIII

EFFECT OF TEMPERATURE DURING IRRADIATION
ON SURVIVAL OF Cl. parabotulinum 457-A SPORES
DARA

Dose Rep.	Number Of Spores	% Survivors	Log % Survivors
90°C			
0	3,200,000	100.0	2.000
360,000	660,000	20.6	1.314
540,000	27,000	0.843	-0.074
630,000	3,600	0.112	-0.9508
720,000	17	0.0053	-2.276
810,000	-	-	-
900,000	7	0.000218	-3.773
	Temperature Control 90° C		
Hours			
0	3,200,000	100.0	2.000
2	3,400,000	106.0	2.025
4	1,600,000	50.0	1.699
6	1,030,000	32.5	1.511
8	30,000	9.36	0.971
25° C			
0	3,600,000	100.0	2.000
360,000	2,100,000	58.4	1.7664
540,000	430,000	12.2	1.0864
765,000	52,000	1.44	.1584
900,000	3,900	.107	- .9706 -.9706
1,108,000	670	.0181	-1.6258 -1.6258
4° C			
0	900,000	100.0	2.000
360,000	745,000	82.7	1.9143
540,000	59,000	6.55	.8162
630,000	14,000	1.56	.1931
720,000	2,100	.232	-0.6345 -0.6315
810,000	1,450	.161	-0.6932 -0.6932
990,000	940	.0104	-1.9820 -1.9820

TABLE VIII (CONT'D)

Irradiated at -70°C

Dose Rep	Spores per ml	% Survivors	Log % Survivors
0	1,900,000	100.0	2.000
Frozen	2,300,000	121.0	2.083
360,000	1,030,000	44.8	1.651
540,000	385,000	16.7	1.223
630,000	123,000	5.4	0.732
720,000	58,000	2.52	0.401
810,000	17,100	0.774	-0.111
900,000	7,700	0.334	-0.476

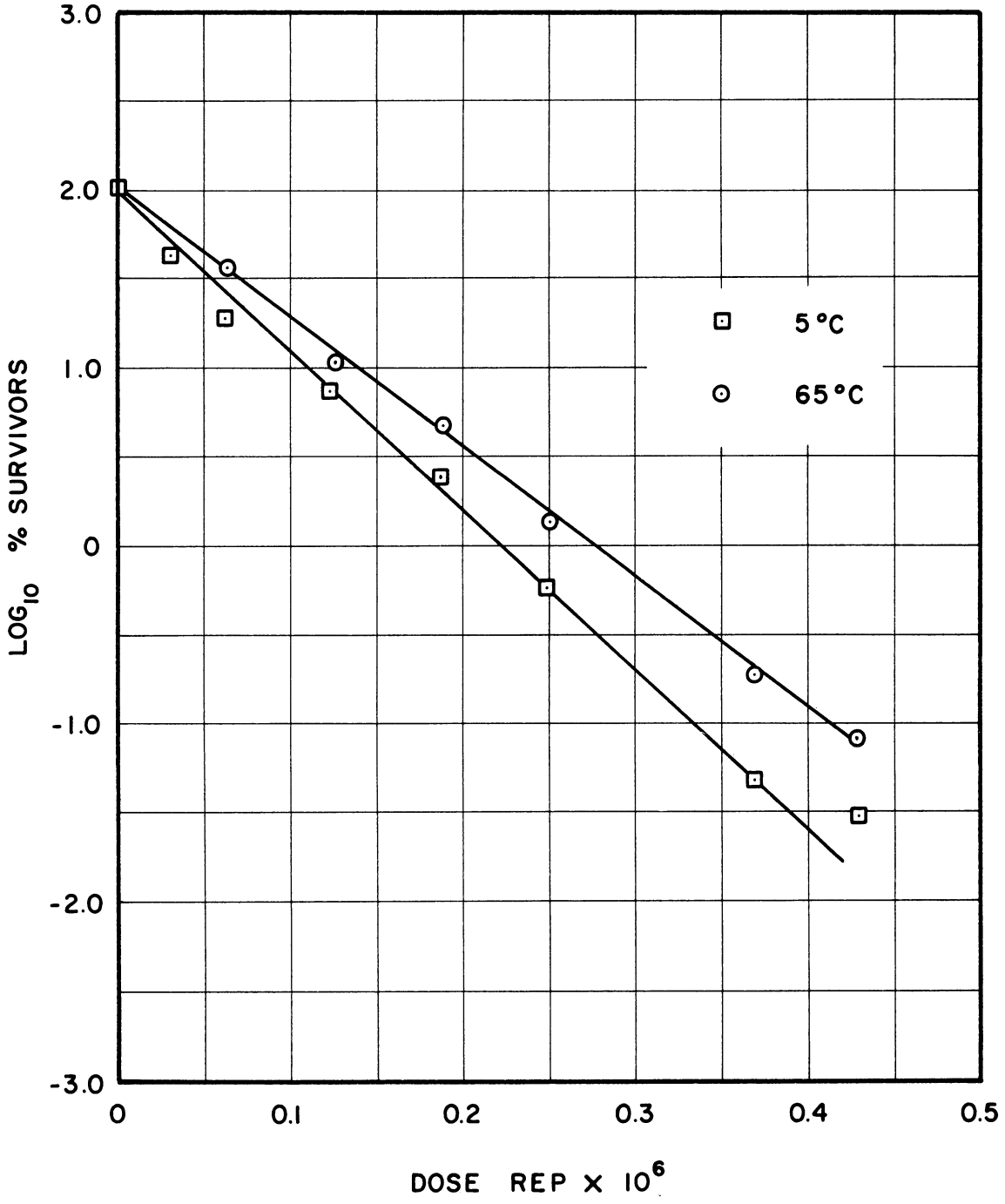

Figure 11. Effect of Temperature During Irradiation on Survival of Bacillus subtilis var. niger Spores.

TABLE IX

EFFECT OF TEMPERATURE DURING IRRADIATION
ON SURVIVAL OF *B. subtilis var.* niger SPORES

DOSE REPX10^6	TEMPERATURE 5° C COUNT	5° C % SURVIVORS	5° C LOG % SURVIVORS	65° C COUNT	65° C % SURVIVORS	65° C LOG % SURVIVORS	TEMP CONTROL AT 65° C
0	$1.0x10^6$	100.0	2.000	$1.1x10^6$	100.0	2.000	$1.1x10^6$
0.031	$4.0x10^5$	40.0	1.602	$4.6x10^5$	41.8	1.621	$1.2x10^6$
0.062	$1.9x10^5$	19.0	1.279	$3.0x10^5$	37.3	1.573	$1.1x10^6$
0.124	$7.9x10^4$	7.9	0.898	$1.2x10^5$	10.9	1.037	$1.3x10^6$
0.186	$2.4x10^4$	2.4	0.380	$5.5x10^4$	5.0	0.699	-
0.248	$5.7x10^3$	0.57	-.244	$1.5x10^4$	1.37	0.137	$1.1x10^6$
0.372	$4.9x10^2$	0.049	-1.310	$2.0x10^3$	0.182	-.740	-
0.434	$3.0x10^2$	0.030	-1.523	$9.0x10^2$	0.082	-1.087	$1.2x10^6$

The Effect of Post-Irradiation Heating on the Survival of Bacterial Spores

At the beginning of this study it was anticipated that an increase in the temperature during irradiation would result in a significant reduction in the number of viable spores after such a treatment. Such a result would be expected if the coagulation temperature of proteins is lowered by a pre-irradiation treatment as Stedman and Mendel (1926) have shown occurred with ultra-violet radiation. However, the results of the experiments of the present study, in which the spores were irradiated at various temperatures, indicated that the reverse was occurring; the spores were more resistant to radiation as the temperature was raised above room temperature.

In the investigations in which photo-sensitization was demonstrated in various living cells the experimentors generally used temperatures which were lethal for the cells. Therefore, it was not clear if a lowering of the inactivation temperature occurred as a result of the pre-irradiation treatment.

A series of experiments was performed to determine whether the temperature for spore inactivation was lowered by a pre-irradiation treatment. The second objective for this experiment was to determine whether the sequence of pre-irradiation plus heat would lead to an increase in the number of surviving spores. In this series of experiments the spores were exposed to various dosage levels of radiation at 4 °C and then subjected to heat treatment for one hour at various temperatures.

The results for the experiment in which Cl. botulinum 213-B spores were irradiated at various dosage levels at 4 °C and then subjected to heat treatment at various temperatures between 50° and 100 °C are given in Table X.

The results for an identical but separate experiment with the same organism are given in Table XI. In order to better illustrate the effect, the results of Table X are plotted in Figure 12. The results of a similar experiment but using putrefactive anaerobes are presented in Table XII and Figure 13.

The most significant conclusions which can be derived from these experiments is that the thermal lethal threshold of the spores is not lowered by a pre-irradiation treatment, even at radiation dosages which cause considerable inactivation of the spores. However, a pre-irradiation treatment accelerates the rate of thermal inactivation at lethal temperatures. With Cl. botulinum spores no significant inactivation occurs until 90°C is attained. At this temperature non-irradiated spores are inactivated. With putrefactive anaerobe spores, a temperature over 100°C must be attained before inactivation occurs. However, it is noticed that some inactivation of irradiated spores can occur even at the sublethal holding temperatures.

The Effect of Heating Cl. Botulinum and Putrefactive Anaerobe Spores Prior to Irradiation

An important factor to be considered in any study of the effect of heat on bacterial spores is the phenomenon of "dormancy". Dormancy in relation to bacterial spores refers to a delayed germination of the spores. It was early recognized that a mild heat treatment, commonly referred to as "heat shock", could increase the number of Cl. botulinum spores germinating in a suitable medium (Burke, 1923). Subsequent investigations have demonstrated that a wide variety of bacterial spores require a mild heat treatment to attain maximum germination (Curran and Evans, 1937). In many cases, suitable cultural factors can replace the initial heat treatment

TABLE X

SURVIVAL OF Cl. botulinum 213B SPORES SUSPENDED IN M/15 PHOSPHATE BUFFER AT pH 7.0, WHICH HAVE BEEN IRRADIATED AT 5°C WITH GAMMA RAYS FROM COBALT-60 AND THEN HELD FOR ONE HOUR AT THE INDICATED TEMPERATURE

	Control		100,000 rep		200,000 rep		400,000 rep	
Temp °C	Spores per ml	Log % Survivors	Spores per ml	Log % Survivors	Spores per ml	Log % Survivors	Spores per ml	Log % Survivors
5	1,050,000	2.000	820,000	2.000	730,000	2.000	370,000	2.000
50	1,050,000	2.000	1,000,000	2.083	630,000	1.940	320,000	1.937
60	930,000	1.947	670,000	1.912	970,000	2.124	240,000	1.813
70	960,000	1.961	620,000	1.879	550,000	1.877	450,000	2.083
80	590,000	1.749	620,000	1.879	720,000	1.984	330,000	1.950
90	630,000	1.778	520,000	1.803	240,000	1.513	106,000	1.457
95	310,000	1.450	11,000	1.335	50,000	0.914	2,800	0.013
100	1,700	-0.791	170	-1.894	40	-2.261	0	

TABLE 11

EFFECT OF HOLDING IRRADIATED SPORES OF Cl. BOTULINUM 213 B
FOR ONE HOUR AFTER IRRADIATION

Temp. C	Control Spores per ml	Control % Survivors	Control Log % Survivors	400000 Rep Spores per ml	400000 Rep % Survivors	400000 Rep Log % Survivors
0	13,000,000	100.0	2.000	5,600,000	100.0	2.000
60	15,000,000	115.0	2.061	5,400,000	96.5	1.984
80	15,000,000	115.0	2.061	4,610,000	73.3	1.865
90	12,000,000	92.5	1.966	2,300,000	41.0	1.613
100	660,000	5.07	0.705	1,400	0.025	- 1.602
110	0	0	0	0	0	-

Temp. C	800,000 Rep Spores per ml	800,000 Rep % Survivors	800,000 Rep Log % Survivors	1,000.000 Rep Spores per ml	1,000.000 Rep % Survivors	1,000.000 Rep Log % Survivors
0	730,000	100.0	2.000	7,500	100.0	2,000
60	280,000	38.3	1.583	14,000	187.0	2.272
80	360,000	49.3	1.692	7,400	99.0	1.996
90	82,000	11.4	1.057	100	1.46	0.164
100	0	0	-	0	0	-
110	0	0	-	0	0	-

Exposed for one hour at each temperature

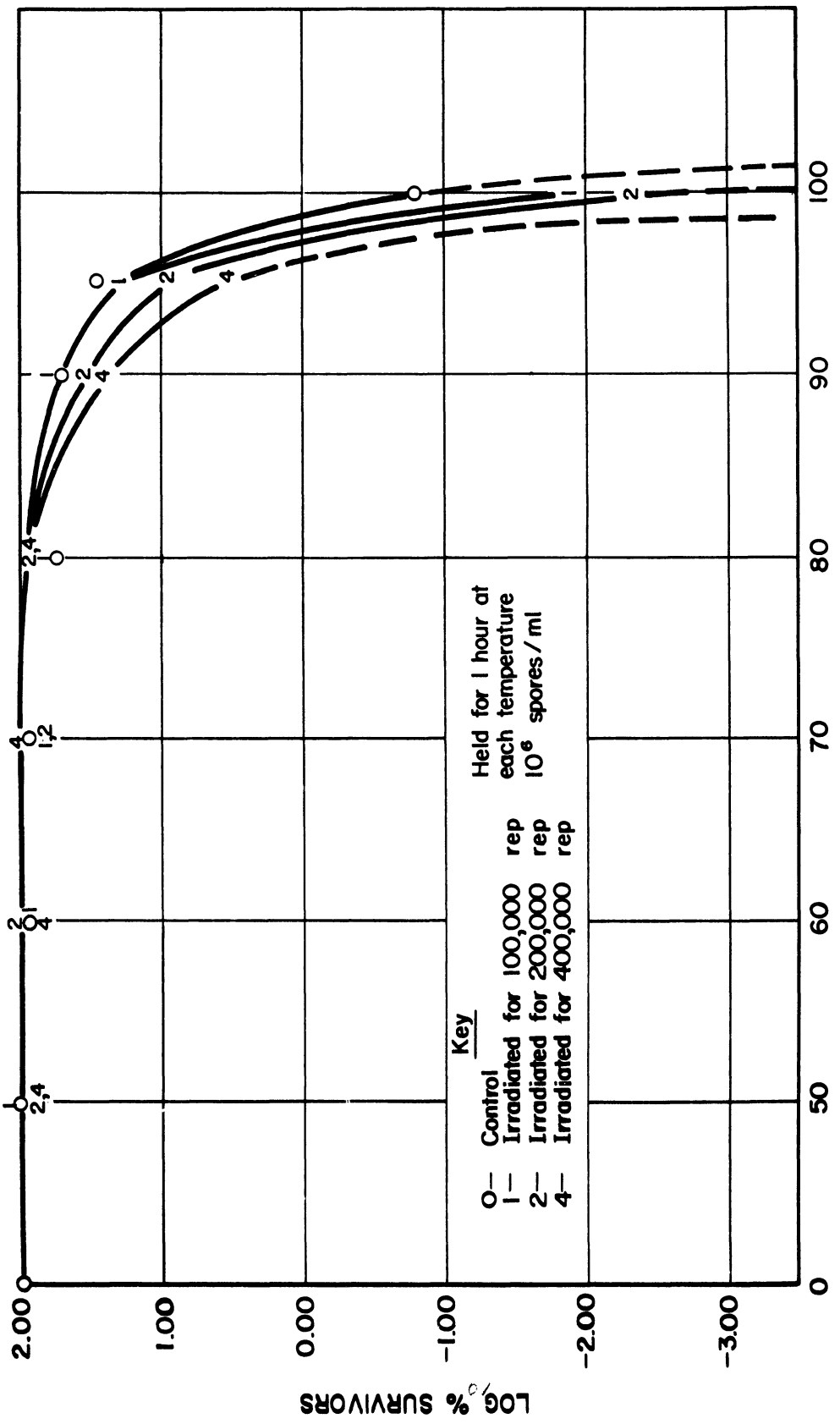

Figure 12. Survival Curves for Cl. botulinum 213-B Spores Suspended in M/15 Phosphate Buffer at pH 7.0, Which have been First Irradiated with Gamma Rays from Cobalt-60 and Then Held for One Hour at the Indicated Temperatures.

TABLE XII

EFFECT OF POSTIRRADIATION HEATING FOR ONE HOUR AT VARIOUS TEMPERATURES ON PREVIOUSLY IRRADIATED PA 3679 SPORES

Temperature, °C	Spores per ml	Percent Survivors	Log Percent Survivors
a) Nonirradiated			
Control	860,000	100	2.000
70	650,000	75.5	1.878
80	660,000	76.7	1.885
90	640,000	74.5	1.872
95	500,000	58.2	1.765
100	580,000	67.5	1.829
105	310,000	34.9	1.543
110	0	0	-
b) Irradiated with 400,000 rep			
Control	150,000	100	2.000
70	170,000	113	2.053
80	160,000	106	2.025
90	162,000	108	2.033
95	175,000	116	2.065
100	134,000	89.5	1.952
105	3,600	2.40	0.380
110	0	0	-
c) Irradiated with 800,000 rep			
Control	1,340	100	2.000
70	200	14.9	1.1732
90	305	22.8	1.358
95	560	41.7	1.620
100	65	4.85	0.686
105	0	0	-
110	0	0	-

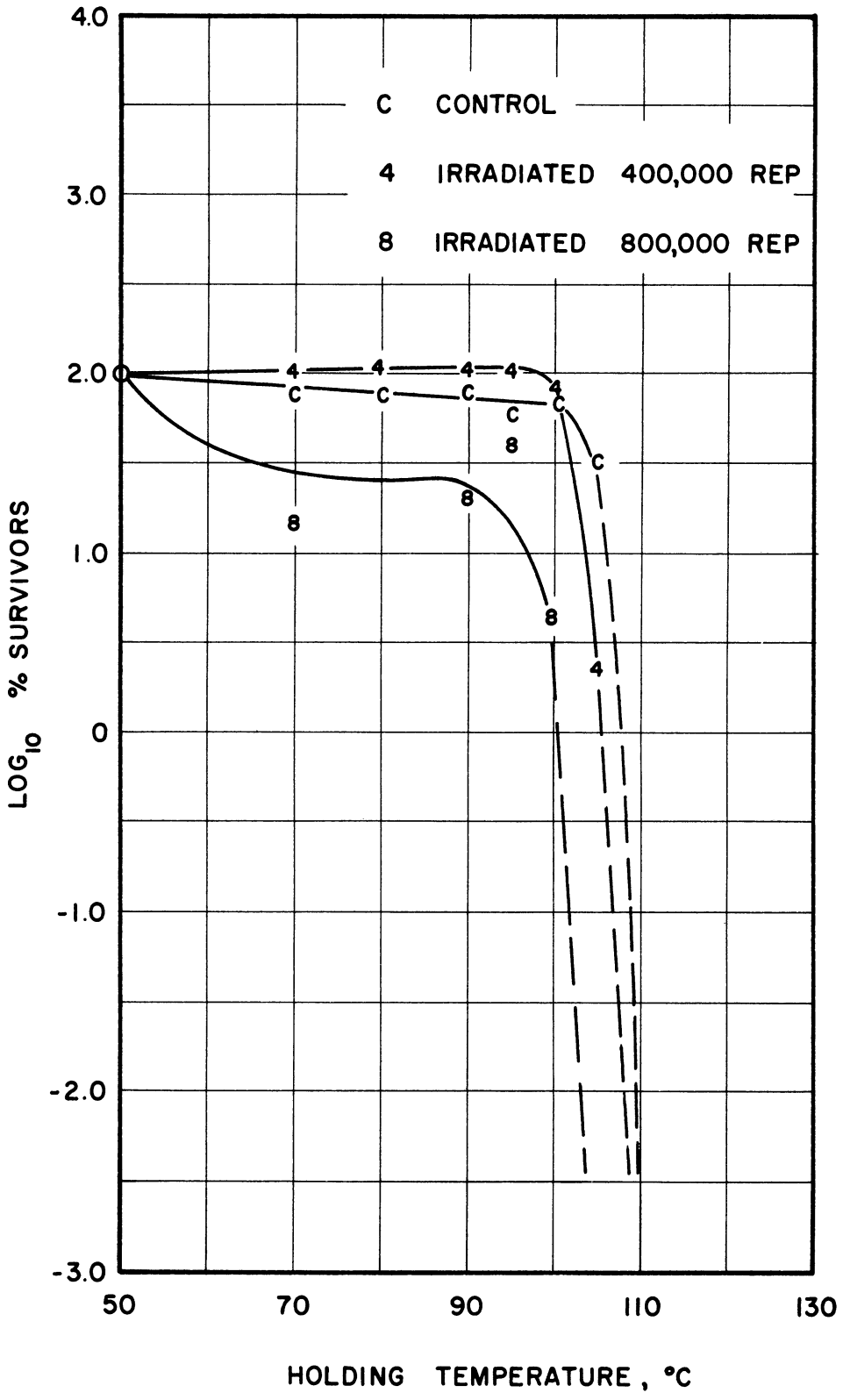

Figure 13. Effect of Post-Irradiation Heating for One Hour at Various Temperatures on Previously Irradiated putrefactive anaerobe NCA 3679 Spores.

(Curran and Evans, 1937). Dormancy in bacterial spores has been the subject of extensive investigation in the past decade and has been adequately reviewed (Knaysi, 1938; Curran, 1950; Schmidt, 1955; Stedman, 1956; Halvorson and Church, 1957). However, it can be generally concluded that those factors which are capable of supplying energy to the bacterial spores are responsible for the induction of the germination process (Halvorson and Church, 1957).

Since the present study concerns the simultaneous lethal effect of heat and radiation, the possible activation of bacterial spores by heat must be considered. The question arises whether the apparent increase in resistance by the spores to gamma radiation at the elevated temperatures is due to activation by heat of any dormant spores which may be present in the suspensions. However, the rationale of the experimental procedure rules out this possibility. All spores used in these experiments were heat shocked prior to each experiment. The time and temperature selected for this treatment was considered maximal for germination of the bacterial strains employed. Not only was this evident from the results of this study but the investigation of Desrosier and Heiligman (1952) is significant in this respect. They demonstrated that a time temperature relationship existed for breaking dormancy of several bacterial species including the putrefactive anaerobe strain 3679. Their results showed no increase in germination, as measured by a viable count, if the spores were heated for a prescribed time at a given temperature. The maximum number of spores germinated after a heat treatment of five minutes at 95°C. With Cl. botulinum spores a heat treatment for 20 minutes at temperatures between 70° and 90°C was sufficient

to obtain maximum viability of the spores as determined by colony forming ability (Reynolds and Lichtenstein, 1949).

However it would seem advisable to determine what effect a prolonged heat exposure at a high but sub-lethal temperature before irradiation would have on the resistance of the spores to irradiation. Previous studies with several strains of food spoilage organisms have shown that a preheat treatment at lethal temperatures and for time intervals which were sporicidal for a large number of the spores did not affect the resistance of the survivors to gamma radiation (Kan et al., 1957; Kempe, 1955; Morgan and Reed, 1954).

For this experiment spores of Cl. botulinum 213-B contained in vials were held at 65°C for 14 hours. After this heat treatment the spore suspensions were irradiated for various lengths of time at 4°C. The results indicated that a prolonged heat treatment at 65°C did not render the spores more sensitive to radiation. Table XIII and Figure 14.

In a similar experiment, spores of P.A. 3679 were preheated at 90°C for seven hours and then subjected to gamma radiation. The number of spores surviving this treatment are shown in Table XIV and Figure 15. Here the results would seem to indicate that preheating made the spores slightly more resistant, rather than making them more sensitive to subsequent radiation treatment.

The Effect of Irradiation on the Subsequent Germination of Bacterial Spores

A question which arises relative to heat activation is whether radiant energy can be substituted for thermal energy in breaking dormancy of bacterial spores. If this were so then the apparent increase in

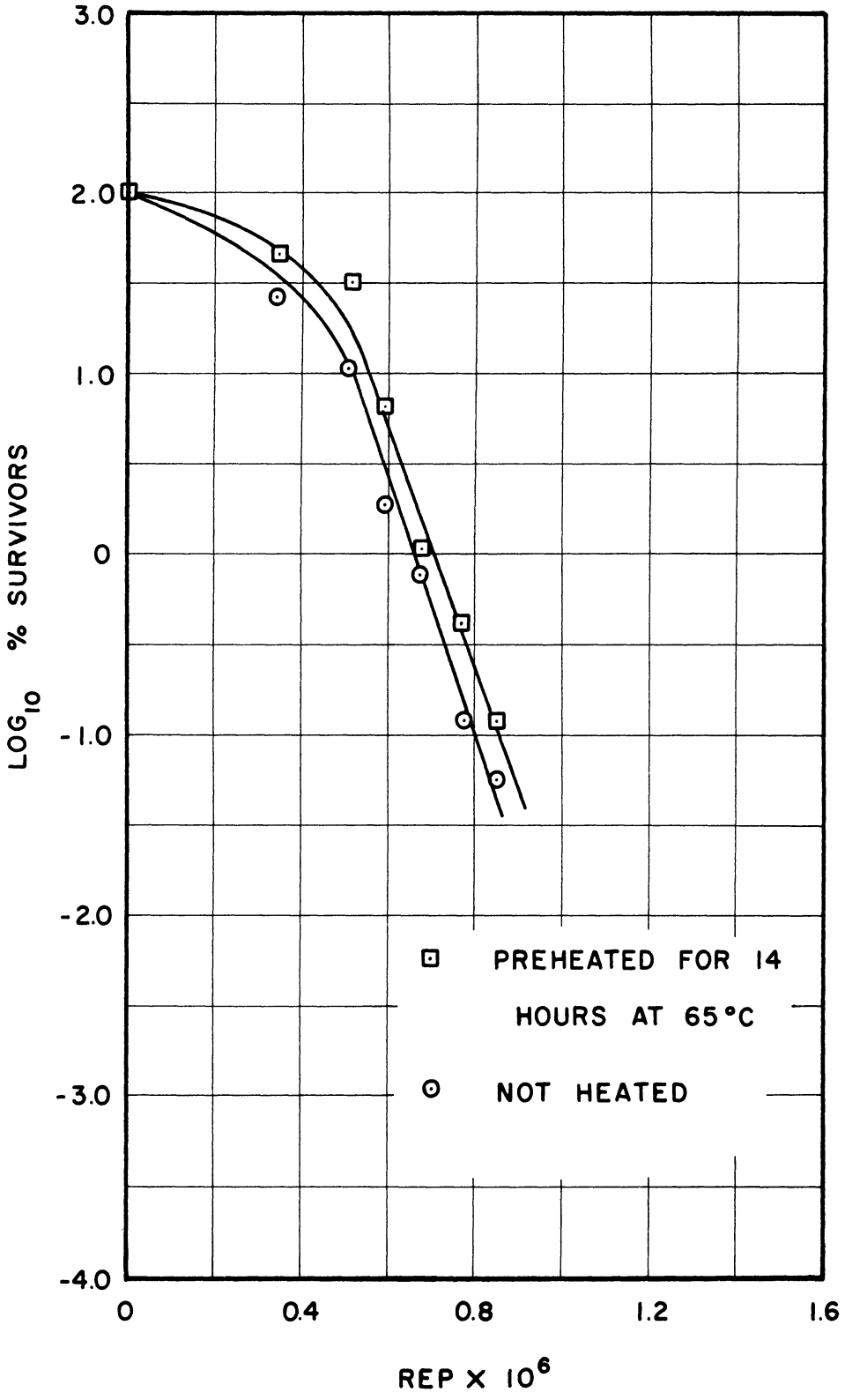

Figure 14. Effect of Pre-Heating Cl. botulinum 213-B Spores at 65°C for 14 Hours Prior to Irradiation.

TABLE XIII

EFFECT OF PRE-HEATING Cl. botulinum 213-B SPORES
AT 65°C FOR 14 HOURS PRIOR TO IRRADIATION

Dose Megarep	Heated Samples*			Non-Heated Samples		
	Number of Spores	% Survivors	Log % Survivors	Number of Spores	% Survivors	Log % Survivors
Control	630,000	100.0	2.000	630,000	100.0	2.000
Heated 4 hrs.	535,000	85.1	-	-	-	-
Heated 14 hrs.	525,000	83.4	-	-	-	-
340,000 rep	250,000	46.7	1.669	172,000	27.3	1.438
510,000 rep	75,000	14.2	1.52	72,000	11.4	1.057
595,000 rep	35,000	6.67	0.824	11,500	1.83	0.263
680,000 rep	7,100	1.32	0.121	6,100	.968	-0.141
765,000 rep	2,100	0.400	-0.398	760	.121	-0.917
850,000 rep	625	0.119	-0.925	370	.0587	-1.231

* heated at 65°C for 14 hours

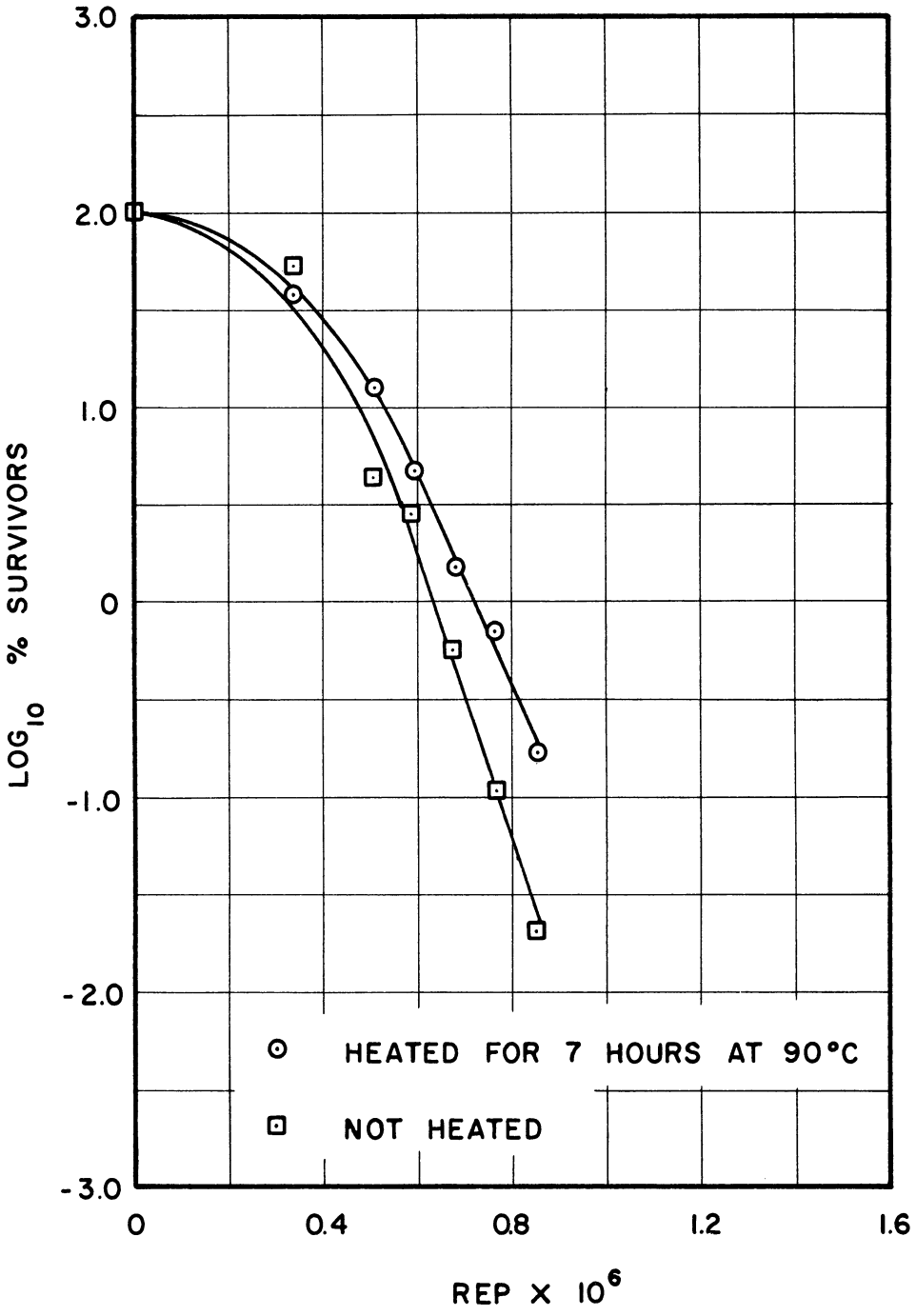

Figure 15. Effect of Pre-Heating putrefactive anaerobe Spores at 90°C for Seven Hours Prior to Irradiation.

TABLE XIV

EFFECT OF PRE-HEATING P.A. 3679 SPORES
AT 90°C FOR 7 HOURS PRIOR TO IRRADIATION

	Heated Samples *			Non-Heated Samples		
Dose Megarep	Number of Spores	% Survivors	Log % Survivors	Number of Spores	% Survivors	Log % Survivors
Control	860,000	100.0	2.000	860,000	100.00	2.000
Heated 3.0 hrs.	500,000	58.2	-	-	-	-
Heated 4.8 hrs.	540,000	62.8	-	-	-	-
Heated 7.0 hrs.	600,000	69.8	-	-	-	-
340,000 rep	235,000	39.2	1.593	365,000	42.4	1.627
510,000 rep	76,500	12.7	1.104	35,100	4.07	0.610
595,000 rep	29,000	4.84	.685	24,500	2.85	0.455
680,000 rep	8,900	1.48	.170	5,000	0.582	-0.235
765,000 rep	4,350	0.725	-0.140	950	0.110	-0.959
850,000 rep	1,040	0.173	-0.762	240	0.0245	-1.694

* heated at 90°C for 7 hours

survivors at the elevated temperatures might be due to increased numbers of germinating spores. To test this possibility with anaerobic spores is difficult since the requirements for their complete and rapid germination without subsequent outgrowth has yet to be determined. Therefore a strain of an aerobic spore former, whose exact germination requirements has been accurately determined, was selected. For this experiment spores of B. cereus var. terminalis were employed. This organism germinates rapidly without outgrowth in the presence of adenosine and l-alanine after a heat treatment of 15 minutes at 65°C (Church, 1955). In this experiment nonheated spores were irradiated for one and three hours which correspond to 180,000 to 540,000 rep respectively. After irradiation, aliquots of irradiated spores along with nonirradiated controls were added to the germination solution and heat shocked for 15 minutes at 65°C. The rate of germination at 30°C was followed by changes in optical density of the spore suspensions. Also uptake of methylene by the various spore samples was determined. The results of this experiment are presented in Table XV. As can be observed, radiation does not induce the spores to germinate as measured by the above criteria.

A difference was noted in the straining characteristics of heat and radiation inactivated spores. Botulinum spores, subjected to steam sterilization (10 minutes at 121°C), if subsequently stained by the Gram method will stain entirely gram positive. However, no differences were noted in the staining characteristics of the spores which had been subjected to high dosages of radiation; although, if the irradiated spores were placed in a complete growth medium, incubated and then stained by the

TABLE XV

EFFECT OF PRE-IRRADIATION TREATMENT ON
GERMINATION OF B. cereus var. terminalis SPORES

Germination Time	Optical Density of Spore Suspensions				
Minutes	Control	Heated *	Irradiated 1 hr. Plus Heat *	Irradiated 3 hr. Plus Heat	Irradiated No Heat
0	.564	.564	.536	.536	.536
10	.536	.360	.346	.324	.492
20	.522	.320	.320	.300	.480
30	.516	.320	.302	.292	.478
40	.516	.306	.300	.282	.474
120	.500	.296	.286	.276	.460

* Heat Treatment 15 min. 65° C

Germination Solution: 3.2 mgm adenosine, + 2.67 mgm l-alamine in .067 M pyrophosphate buffer pH 7.3.

Number of Spores as Determined by Methylene Blue Uptake

Sample	Spores Staining With Methylene Blue Spores	Germinated Spores*
After Irradiated 1 hour	100.0 %	0
After Irradiated 3 hours	100.0 %	0

After Germination 40 minutes

	Spores	Germinated Spores
Not Heated	266	31
Heated	7	239
Irradiated 1 hour + Heat	16	225
Irradiated 3 hour + Heat**	11	173
Irradiated + No Heat	291	53

** Germination Time 20 Minutes
* Those Staining (with MB)

Gram method, a discrete but irregular gram positive area was observed within the spore. It would seem that drastic heating causes complete polymerization of the cellular components responsible for the Gram stain, whereas radiation is more selective in its action on the spores. The results of Henry and Stacey (1943, 1954) on the mechanism of the Gram stain is pertinent in this respect. They observed the gram positive staining character of Clostridium welchii is due to a complex formed by protein and nucleic acids in the presence of magnesium and formaldehyde.

The Effect of Temperature During Irradiation on the Subsequent Heat Resistance of Bacterial Spores

Since a pre-irradiation treatment of bacterial spores makes them more susceptible to heat, one could conclude that a common mechanism within the spore, responsible for its' viability, is acted upon by both forms of energy. On the other hand the fact that a preheat treatment of the spores does not alter their subsequent sensitivity to radiation would seem to indicate that the same mechanism is not involved. However, this conclusion may not be valid since both radiation and heat could affect the same mechanism but the manner in which these two forms of energy are operative might be different. It would seem that if less damage occurs to the spores when irradiation is carried out at an elevated temperature, and if the spores are subsequently heated, any difference in the number of surviving organisms would indicate whether a joint mechanism is responsible for the lethal action.

An experiment was devised to test whether irradiation at different temperatures would be reflected in differences in the sensitivity of the spores to subsequent heat treatments.

In the first experiment spores of putrefactive anaerobe NCA 3679 were irradiated at 95° and 5°C at various dosage levels. After irradiation they were heated at 99.5°C for a period of one hour. A control spore suspension, which received no irradiation treatment, was also heated for 5 hours, the length of time needed to deliver the maximum radiation dosage used. The results of this experiment are presented in Table XVI and Figures 16 and 17. The results indicate that an irradiation temperature which produces a maximum number of survivors is also a temperature at which greater numbers of survivors are found when the organisms are heated after irradiation. The differences in the number of spores surviving heat treatment after being irradiated at the two temperatures is not great, but the holding temperature of 99.5°C is a sub-lethal temperature for this organism. This can be observed from the temperature control which indicates no inactivation of nonirradiated spores. Previous experiments have demonstrated that in order for the maximum expression of the sensitization phenomenon to occur, heating must be carried out at or above the thermal, lethal threshold for that particular organism.

The results of this experiment indicate that there is a slight reduction in the number of spores at a high but sublethal temperature at all radiation dosage levels. Furthermore, there is a difference in heat sensitivity when irradiation is carried out at two extremes of temperature. That is, those spores which exhibit greater resistance during irradiation at the higher temperatures are also the more resistant to heat after irradiation.

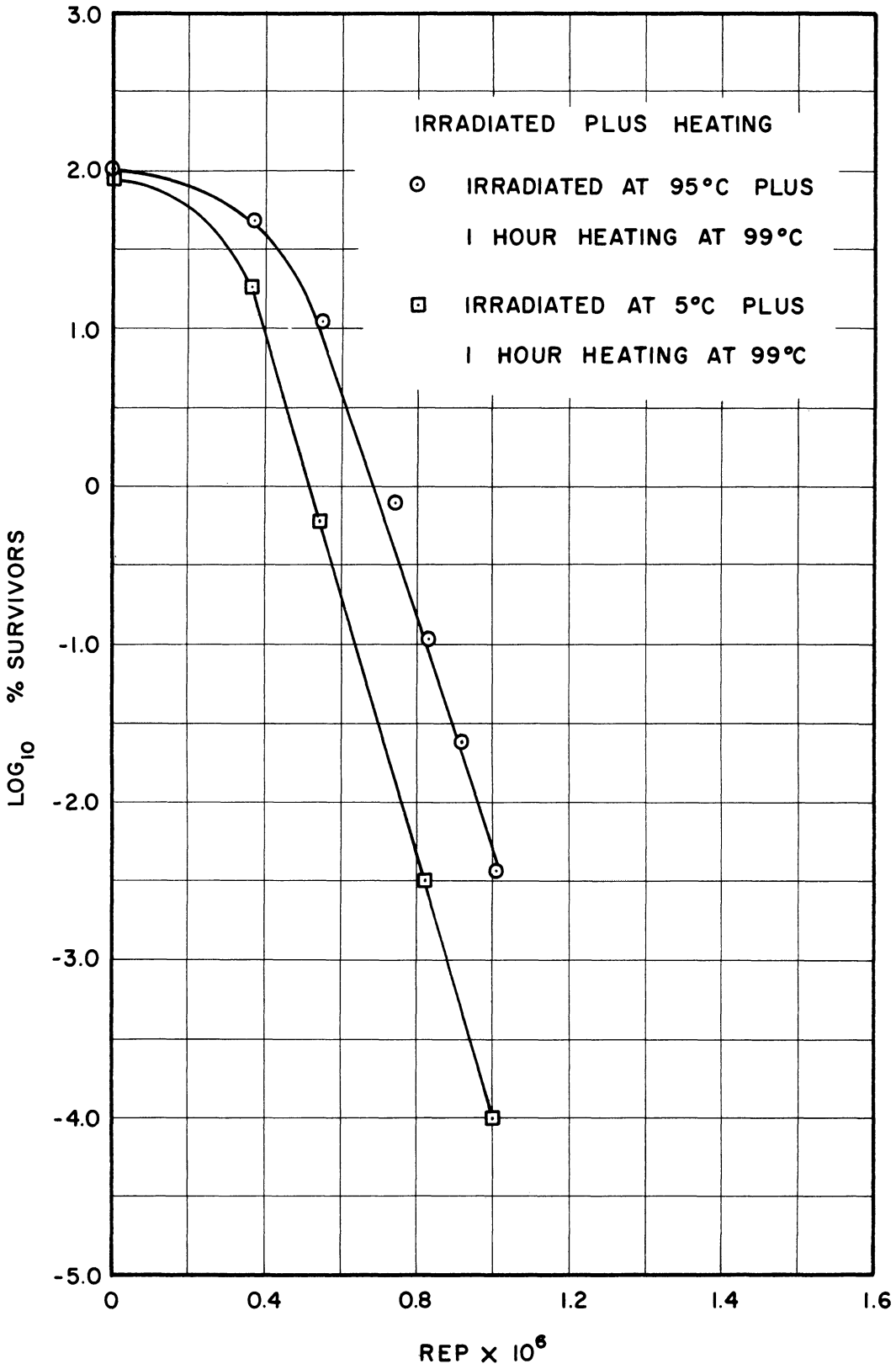

Figure 16. Effect of a Combined Treatment Consisting of Irradiation with Gamma Radiation from Cobalt-60 Followed by Heating for One Hour at 99°C on Survival of putrefactive anaerobe Spores Suspended in M/15 Phosphate Buffer at pH 7.0.

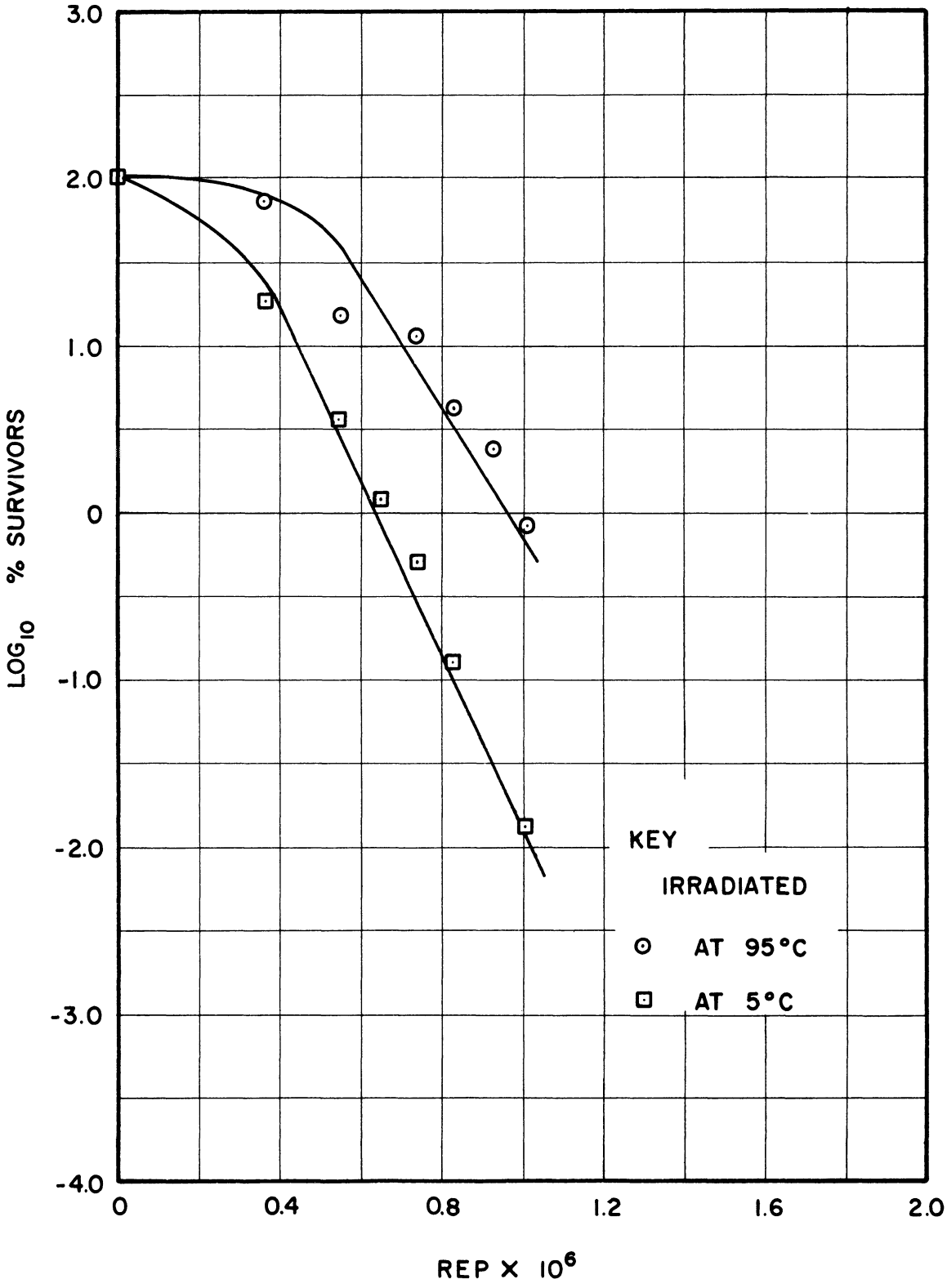

Figure 17. Effect of Irradiation at 5° and 95°C on the Survival of putrefactive anaerobe NCA 3679 Spores.

TABLE XVI

EFFECT OF A COMBINED TREATMENT CONSISTING OF IRRADIATION WITH GAMMA RAYS FROM COBALT-60 FOLLOWED BY HEATING FOR 1 HOUR AT 99°C ON THE SURVIVAL OF PA 3679 SPORES SUSPENDED IN M/15 PHOSPHATE BUFFER AT pH 7.0

Dosage, rep	Spores per ml	% Survivors	Log % Survivors
1a Irradiated at 5°C			
0	2,700,000	100.	2.00
370,000	460,000	17.0	1.230
550,000	95,000	3.51	0.545
650,000	32,000	1.18	0.0719
740,000	14,000	0.519	-0.2848
833,000	3,200	0.118	-0.9281
1,015,000	340	0.0126	-1.8996
1,100,000	54	0.002	-2.699
1b Irradiated at 5°C and Heated for 1 Hour at 99°C			
0	2,300,000	92.0	1.964
370,000	450,000	19.5	1.29
550,000	14,000	0.61	-0.2147
832,000	700	0.0304	-2.5171
1,000,000	2	0.00008	-4.0605
2a Irradiated at 95°C*			
0	1,200,000	100.0	2.00
370,000	900,000	75.0	1.875
550,000	570,000	47.5	1.676
740,000	140,000	11.7	1.068
832,000	51,000	4.25	0.628
925,000	30,000	2.5	0.398
1,017,000	10,400	0.866	-0.063
1,100,000	3,100	0.258	-0.588
2b Irradiated at 95°C and heated 1 hour at 99°C			
0	1,100,000	100.0	2.00
370,000	600,000	54.5	1.7364
550,000	120,000	10.9	1.0374
740,000	8,700	0.791	-0.1018
832,000	1,200	0.109	-0.9626
925,000	260	0.0236	-1.6271
1,000,000	36	0.00328	-2.4840
1,100,000	8	0.000726	-3.1391

* Control

Held at 95°C for 5 hours (the time required for 925,000 rep); 1,200,000 spores per ml remain, which is the same number as was originally present.

In a similar experiment B. subtilis var. niger spores were irradiated at 5° and 65°C for various dosage levels and then heated, after irradiation at 99.5°C, for a total of four minutes. The results of this experiment are given in Table XVII and illustrated in Figure 18. As was observed previously, irradiation of this organism at 65°C resulted in more spores surviving at this temperature than at 50°C. This temperature is not lethal for this organism. The spores which exhibit the greatest resistance during irradiation also show a greater resistance to subsequent heat treatment after irradiation.

In order to more clearly demonstrate that spores irradiated at different temperatures also exhibit differences during post-irradiation heating another approach was used. In this series of experiments vials of spores were irradiated for a constant dose at two extremes of temperatures and then a thermal inactivation curve was determined for the irradiated organisms.

In the first experiment spores of Cl. botulinum 213-B were irradiated at 5°C and -70°C for a total dose of 250,000 and 500,000 rep. The vials of spore suspensions were then heated in an oil bath maintained at 100°C. The results of this experiment appear in Tables XVIII and XIX Figures 19 and 20. As can be observed, sensitization of the spores occurred when spores were irradiated at 5° and -70°C. However, post-irradiation heating of the spores indicated differences in thermal sensitivity; the spores irradiated at -70°C being the more resistant. As previously shown in this study, irradiation of these spores at -70°C results in greater numbers of survivors. The degree of sensitization at two different

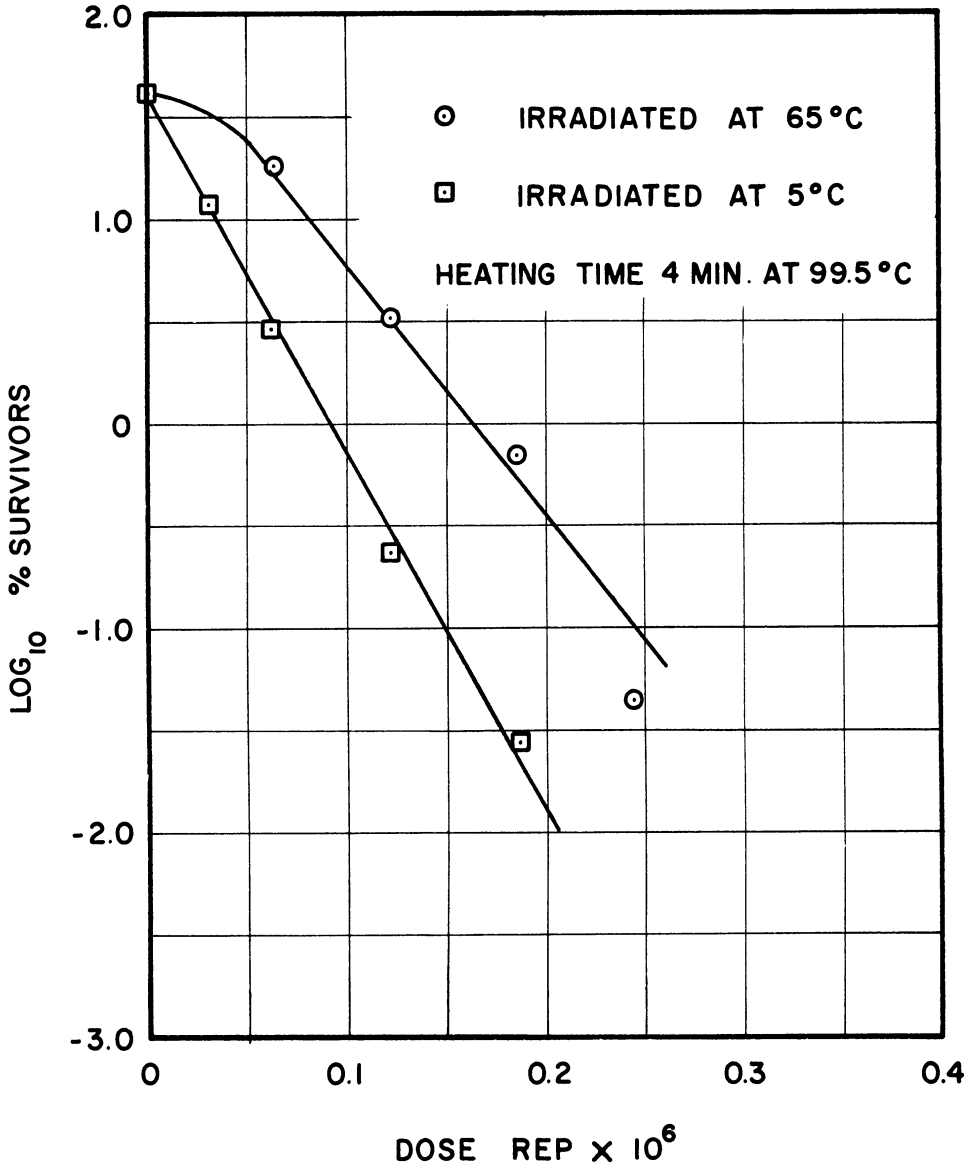

Figure 18. Effect of Post-Irradiation Heating on the Survival of Bacillus sublilis var. niger Spores Irradiated at 5°C and 65°C.

TABLE XVII

EFFECT OF POST-IRRADIATION HEATING AT 99°C FOR FOUR MINUTES ON SURVIVAL OF *B. subtilis var.* niger SPORES IRRADIATED AT 5° AND 65°C

	TEMPERATURE					
	5° C			65° C		
DOSE	COUNT	% SURVIVORS	LOG % SURVIVORS	COUNT	% SURVIVORS	LOG % SURVIVORS
0	4.2×10^5	42.0	1.623	4.2×10^5	42.0	1.623
0.031	1.2×10^5	12.0	1.079	-	-	-
0.062	2.9×10^3	2.9	0.462	1.9×10^4	19.0	1.279
0.124	2.4×10^2	0.24	$\bar{1}.380$ $-.620$	3.2×10^3	3.2	0.505
0.186	2.8×10^1	0.028	$\bar{2}.447$ -1.552	7.0×10^1	0.7	$\bar{1}.845$ $-.155$
0.246	-	-	-	5.0×10^1	0.05	$\bar{2}.699$ -1.301

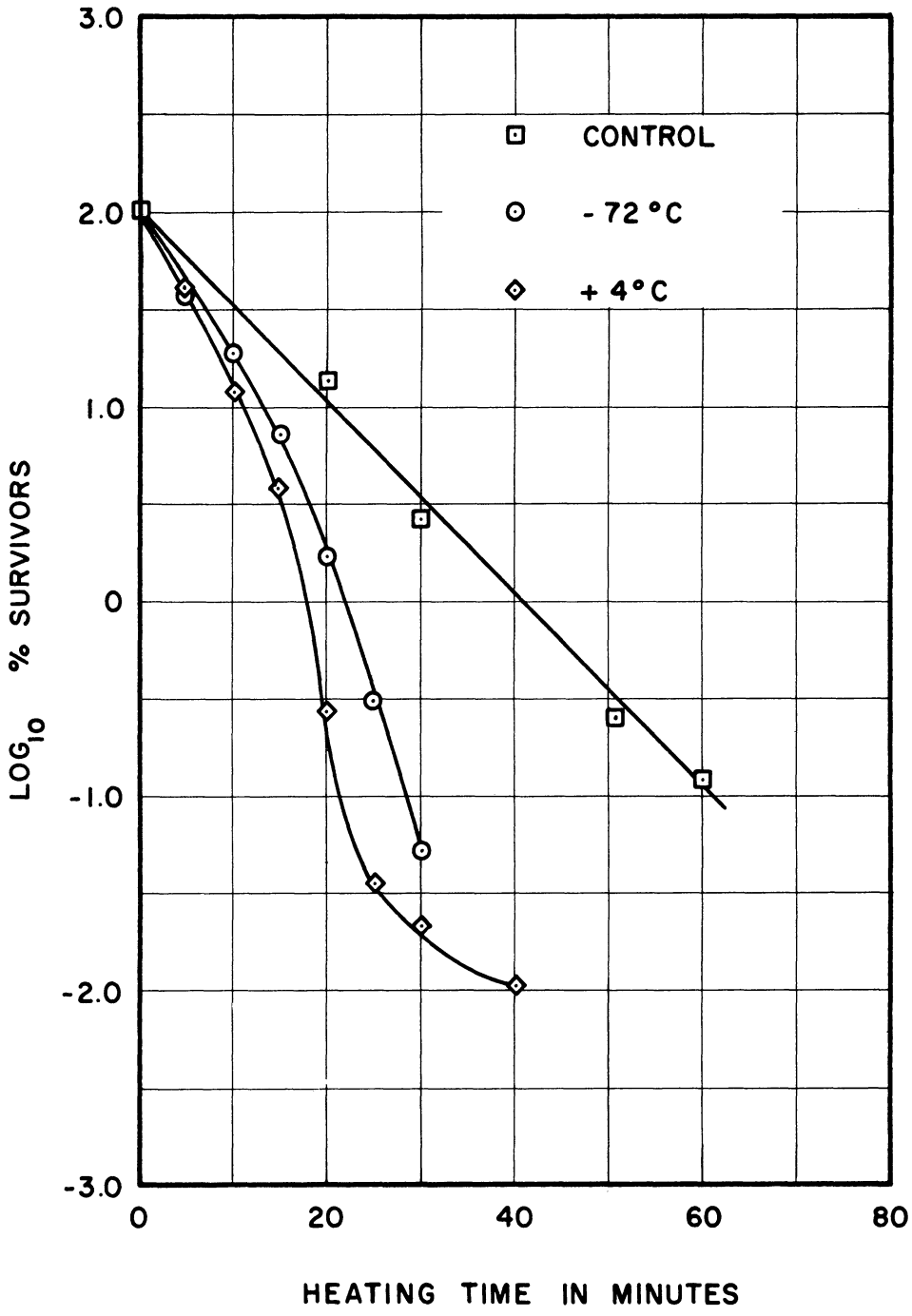

Figure 19. Effect of Pre-Irradiation at 4 °C and -70 °C with Subsequent Heating at 100 °C on Survival of Cl. botulinum 213-B Spores.

TABLE XVIII

EFFECT OF PRE-IRRADIATION AT 5° AND -70°C WITH SUBSEQUENT HEATING AT 100°C ON SURVIVAL OF *Cl. botulinum* 213B

Minutes at 100°C	Spores Per ml	% Survivors	Log % Survivors
Control			
0	3,600,000	100.00	2.000
20	500,000	13.9	1.143
30	99,000	2.74	0.443
50.2	9,300	0.258	-0.588
60.1	4,500	0.125	-0.903
Irradiated -70° C*			
0 (Cont Irrad.)	3,300,000	100.00	2.000
5.3	1,270,000	38.5	1.586
10.0	680,000	20.6	1.314
15.0	250,000	7.58	0.880
20.0	57,000	1.78	0.241
25.2	10,400	0.316	-0.500
30.0	1,300	.0394	-1.405
Irradiated 5°C*			
0	2,500,000	100.00	2.000
5.3	950,000	38.0	1.580
10.0	390,000	15.6	1.1931
15.0	114,000	4.56	0.659
20.0	7,100	0.284	-0.5467
25.2	870	.0348	-1.458
30.0	550	.022	-1.658
40.0	270	.0108	-1.967

* Irradiated for 250,000 Rep.

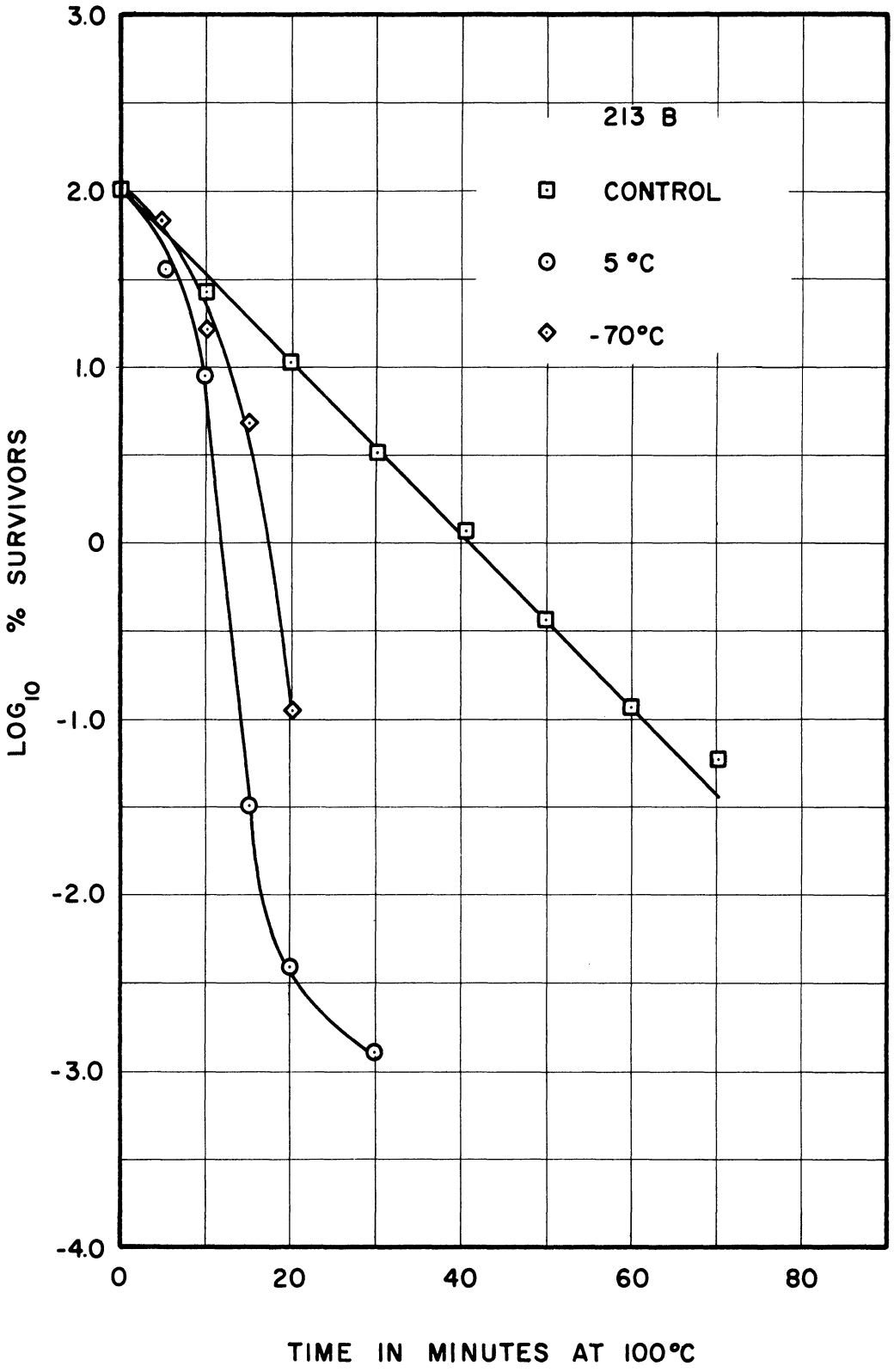

Figure 20. Effect of Pre-Irradiation at 5 °C and -70 °C with Subsequent Heating on Survival of Cl. botulinum 213-B Spores.

TABLE XIX

EFFECT OF PRE-IRRADIATION AT 5° AND -72° C
WITH SUBSEQUENT HEATING ON SURVIVAL OF
Cl. botulinum 213-B SPORES

Control Not Irradiated

Heating Time in Min.	Spores Per ml	% Survivors	Log % Survivors
0	2,800,000	100.0	2.000
10	840,000	30.0	1.447
20	310,000	11.1	1.045
31	89,000	3.17	0.5011
41.5	35,000	1.25	0.0969
50.0	10,000	0.358	-0.4461
60.0	3,500	0.125	-0.9131
70.0	1,700	0.0607	-1.2168
	Irradiated at -70° C*		
0 (Irrad.)	1,100,000	100.00	
5.	830,000	75.2	1.896
10	190,000	17.3	1.238
15	19,000	1.73	0.738
20	1,200	0.109	-0.963
	Irradiated at + 5° C*		
0 (Irrad.)	460,000	100.00	2.000
5.2	66,000	14.3	1.553
10.0	4,200	9.15	0.961
15	140	0.030	-1.517
20	18	0.00391	-2.408
30	6	0.0013	-2.886

*500,000 Rep.

temperatures when spores are irradiated with 250,000 rep is not great. However, it has been previously reported that the sensitization phenomenon is not significantly manifested until a total dosage of 300,000 rep is delivered to the spores (Kempe, 1955). As can be observed in the radiation survival curve for Cl. botulinum spores, significant radiation inactivation does not occur until the spores have received approximately 300,000 rep.

The results of another experiment with the same organism are presented in Table XX and Figure 21. In this case the irradiation was carried out at 10° and 90°C. As is apparent from the results, irradiation at 90°C results in greater thermal inactivation than if the spores are irradiated at 10°C. Spores of this organism are the most sensitive when irradiation is carried out at 90°C.

In still another experiment, spores of Cl. botulinum 62-A were irradiated at -70° and 5°C for a total dosage of 500,000 rep before being heated at 100°C. The results shown in Table XXI and Figure 22 indicate that there is a 10-fold difference in survivors to subsequent heating at 100°C when irradiation is carried out at these two different temperatures. The spores irradiated at -70°C were the more resistant to heat.

The results of an experiment in which putrefactive anaerobe spores were treated as described above for Cl. botulinum spores are presented in Table XXII and Figure 23. As noted previously, the spores of this organism are many times more resistant to heat than Cl. botulinum strains. This is adequately demonstrated in the temperature control for this experiment. Heating for three hours had no apparent effect on the viability of these spores. A difference in their heat sensitivity is again demonstrated when

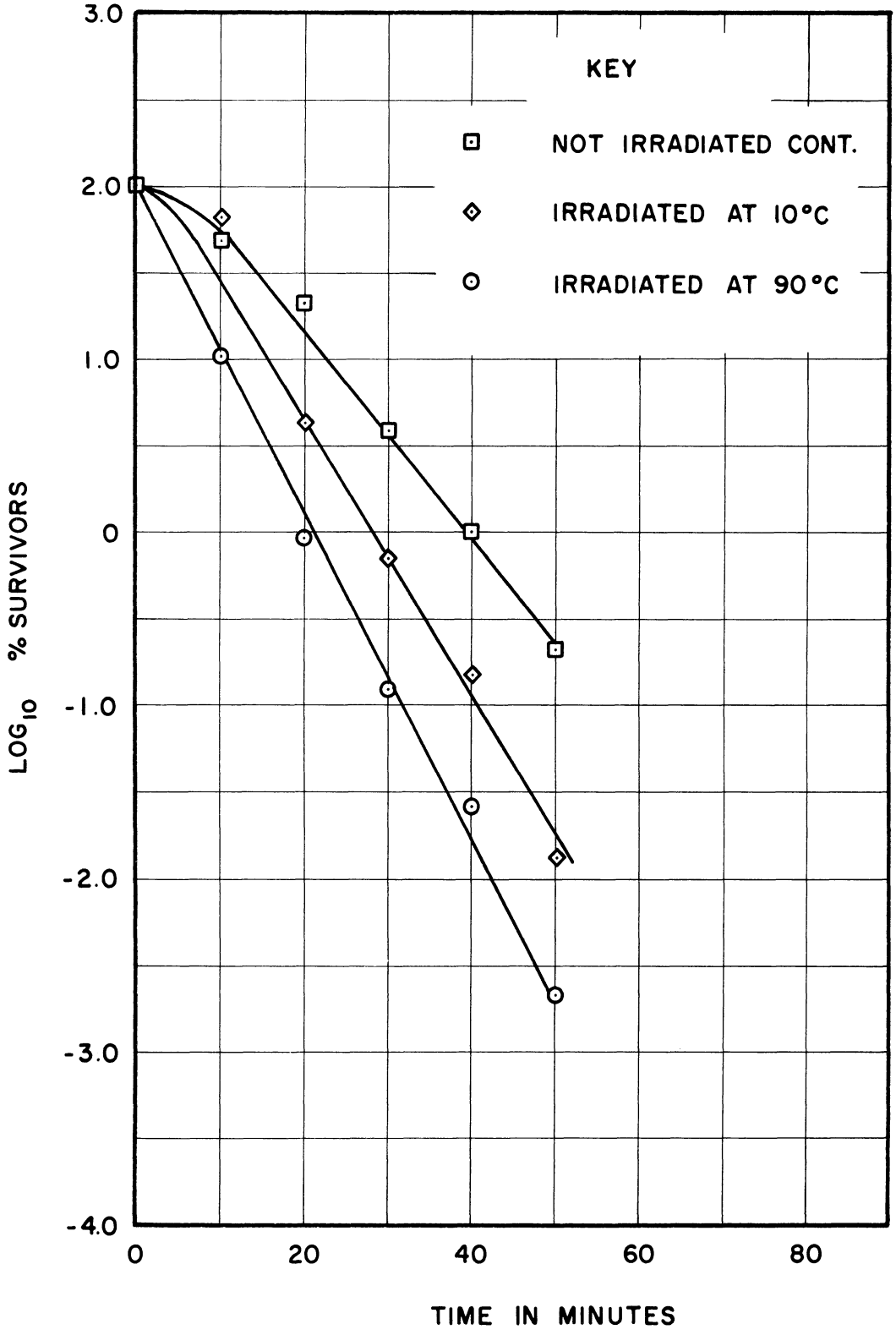

Figure 21. Effect of Irradiation at 10°C and 90°C with Post-Irradiation Heating on 100°C on Survival of Cl. botulinum 213-B Spores.

TABLE XX

EFFECT OF IRRADIATION AT 10°C. AND 90°C. WITH POST-IRRADIATION HEATING AT 100.0°C ON SURVIVAL OF *CL. BOTULINUM* 213-B SPORES

Time (Minutes)	Spores per ml	% Survivors	% Survivors
Control			
0	2,200,000	100.0	2.000
10	1,100,000	50.0	1.699
20	460,000	20.9	1.320
30	85,000	3.86	0.5866
40	22,000	1.00	0.000
50	4,400	0.200	-0.699
Irradiated at 10°C			
0	230,000	100.0	2.000
10	160,000	69.5	1.842
20	9,700	4.21	0.624
30	1,600	0.695	-0.158
40	360	0.156	-0.807
50	30	0.0130	-1.886
Irradiated at 90°C			
0	600,000	100.0	2.000
10	61,000	10.2	1,0086
20	3,200	0.533	-0.273
30	750	0.125	-0.903
40	160	0.0267	-1.574
50	13	0.00216	-2.666

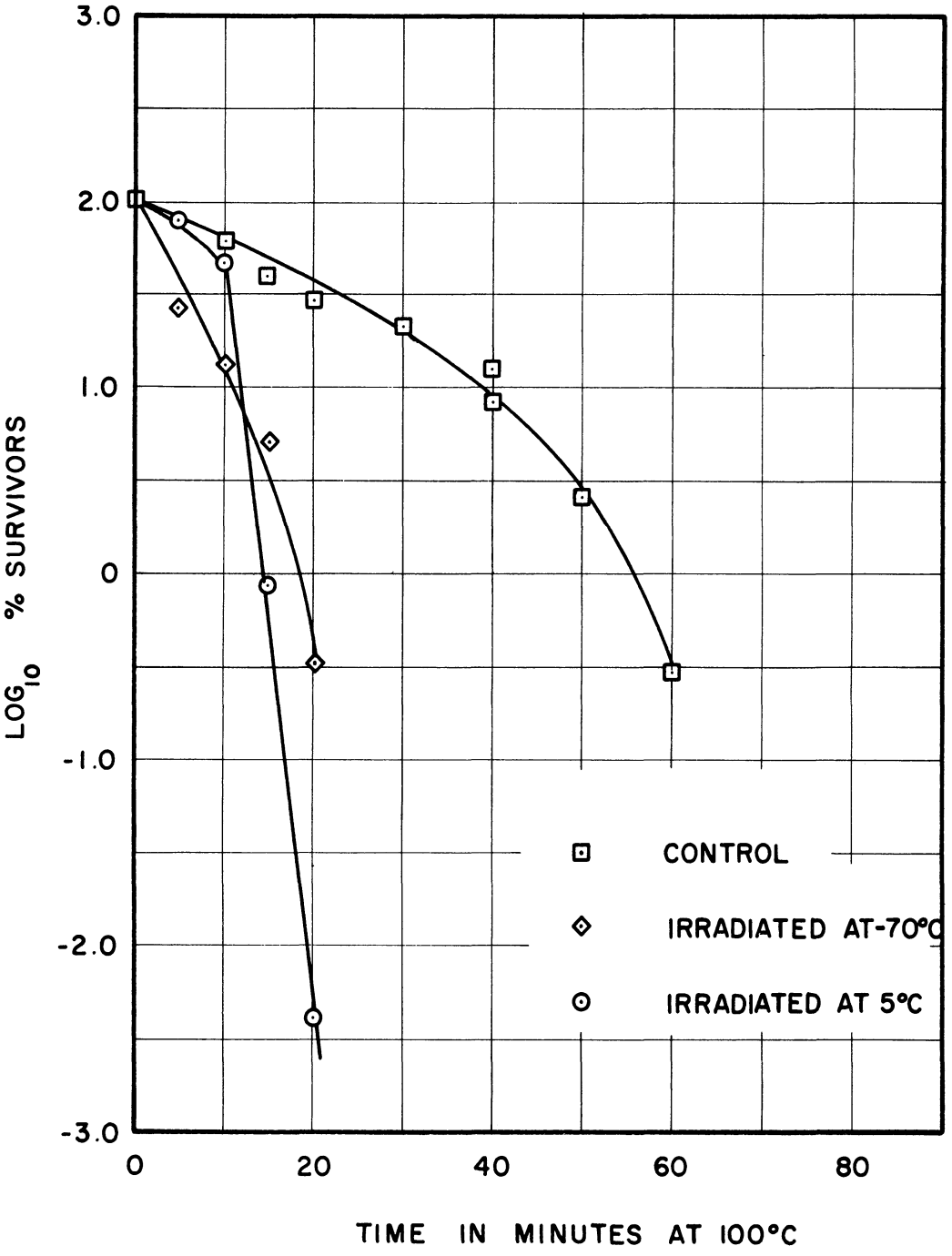

Figure 22. Effect of Temperature During Irradiation on the Subsequent Heat Resistance of Cl. botulinum 62-A Spores to Heating at 100°C.

TABLE XXI

EFFECT OF TEMPERATURE DURING IRRADIATION ON THE SUBSEQUENT RESISTANCE OF C. BOTULINUM 62A SPORES TO HEATING AT 100°C

Heating Time, min	Control			Irradiated		
	Spore Count per ml	% Survivors	Log % Survivors	Spore Count per ml	% Survivors	Log % Survivors
A) (500,000 rep at -70°C)						
0	370,000	100.0	2.00	90,000	100.0	2.00
5				58,000	64.5	1.4416
10	220,000	59.5	1.774	16,000	17.7	1.150
15	150,000	40.5	1.608	7,700	8.55	0.7161
20	110,000	29.8	1.474	290	0.322	-0.4939
30	26,000	7.03	0.847			
40	9,900	2.68	0.428			
50	3,000	0.811	-0.091			
60	1,100	0.298	-0.526			
B) (500,000 rep at +5°C)						
0				86,000	100.0	2.00
5				71,000	82.5	1.9165
10	Same control as (A)			4,300	5.0	0.699
15				1,000	1.16	0.065
20				3.5	0.04	-2.

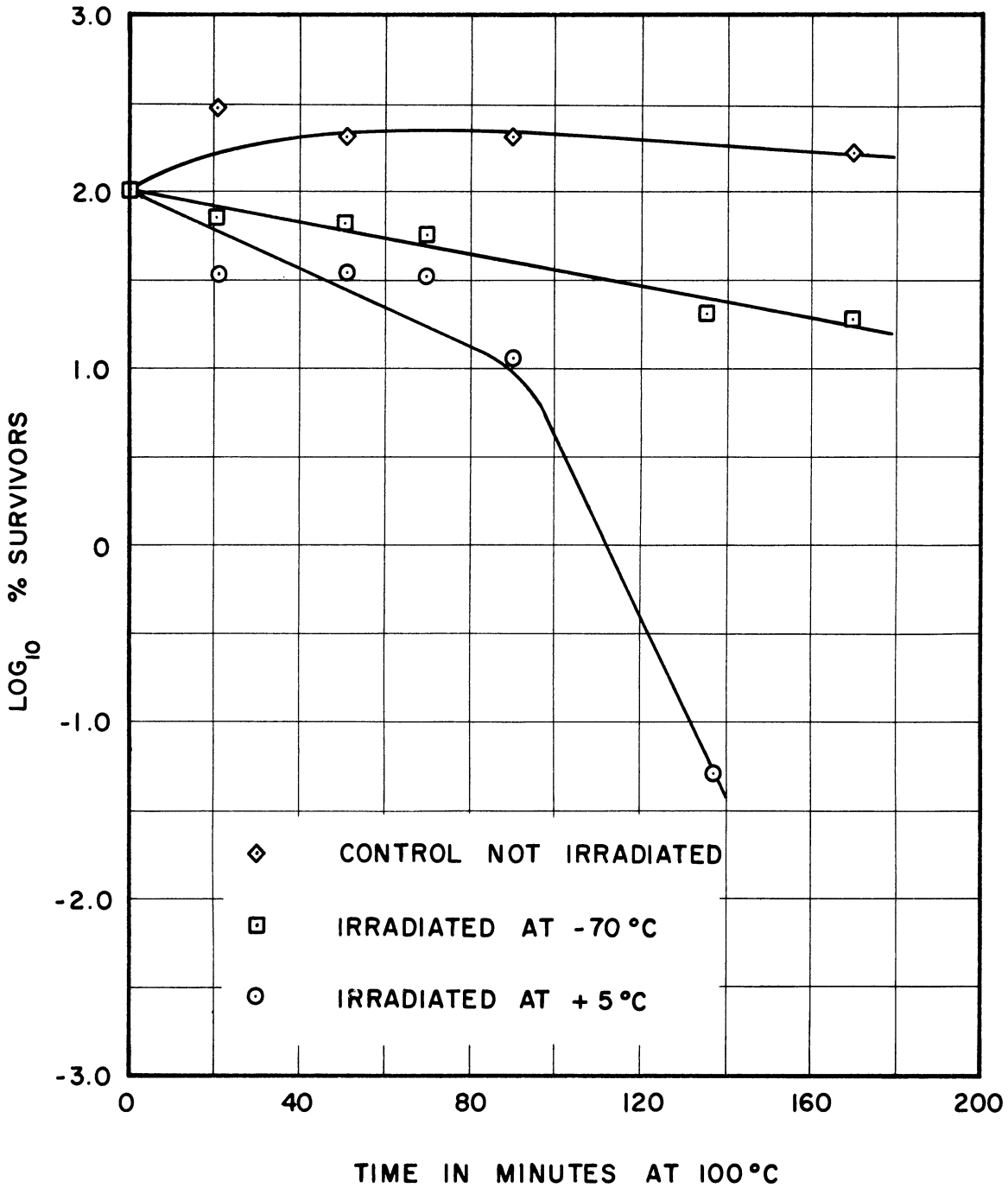

Figure 23. Effect of Temperature During Irradiation on the Subsequent Heat Resistance of putrefactive anaerobe Spores to Heating at 100°C.

TABLE XXII

EFFECT OF TEMPERATURE ON THE SUBSEQUENT HEAT RESISTANCE OF P.A. 3679 SPORES TO HEATING AT 100° C

Heating Time in Min.	Spores Per ml	Control Spores Per ml	Log % Survivors
Control	34,000	2.00	2.000
51	110,000	303	2.481
90	77,000	226	2.354
136	77,000	226	2.354
170	65,000	190	2.788
	Irradiated at -70° C		
Control	34,000		
Control Irrad.	10,000	100.0	2.000
21	7,300	73.0	1.863
51	6,700	67.0	1.826
70	6,100	61.0	1.785
136	2,200	22.0	1.342
170	2,100	21.0	1.322
	Irradiated at 5° C		
Control	34,000		
Control Irrad.	5,300	100.0	2.000
21	1,800	33.9	1.530
51	1,900	35.8	1.553
70	1,800	33.9	1.530
90	750	1.41	0.149
137	110	0.208	-0.06819

Irradiated for 500,000 rep.

the spores are irradiated at two different temperatures. With these spores slight thermal inactivation occurs at 100°C using spores irradiated at -70°C. Furthermore these results also demonstrate that the spores are slightly heat sensitized by irradiation. Spores irradiated at 5°C and heated for short periods of time show an equivalent degree of resistance at 100°C as do those irradiated at -70°C. However, after heating the spores for two hours, those irradiated at 5°C become more sensitive to heat. It would seem that an irradiation treatment, followed by heating of sufficient duration, results in loss of thermal resistance.

The results of the previously outlined experiments all indicate that irradiation at different temperatures results in differences in the subsequent heat resistance of the spores. This is supporting evidence for the observation, previously reported in this thesis, that spores vary in their resistance to radiation when irradiation is carried out at different temperatures since variation in thermal resistance of these spores also occurs after such treatment.

DISCUSSION

Several observations can be made from the results of this investigation. Depending on the ambient temperature during irradiation, the bacterial spores used in this study exhibit a varying degree of resistance to gamma radiation. The degree of resistance is slightly greater when irradiation is carried out at -70 °C as compared to the non-frozen condition at 4 °C. Furthermore, there is a trend towards radio-resistance as the ambient irradiation temperature is increased above room temperature. The maximum degree of resistance to radiation occurs at higher temperatures and is greater just before thermal inactivation of the spores becomes significant. A second observation indicates that a pre-irradiation treatment of the spores, at a dose which is sporocidal for large numbers of the spores, does not significantly lower the temperature threshold necessary for thermal inactivation. These results indicate that radio-sensitization of the bacterial spores is induced by gamma radiation but in order for the phenomenon to be significantly expressed the spores must be heated at their thermal, lethal threshold. A third observation is that spores irradiated at different temperatures also exhibit differences in thermal resistance. Those spores which exhibit the greater resistance to radiation at various temperatures are also the more resistant to heat. These results suggest that a common mechanism is responsible for spore survival and radio-sensitization and that this mechanism is acted upon by both forms of energy.

An interpretation of the results of the simultaneous effect of temperature and radiation on bacterial spores observed in this study is

difficult under the proposed mechanisms which have been advanced for the biological effects of radiation.

The direct action model assumes no effect of temperature during irradiation. A proposed explanation for a temperature effect during irradiation by this theory has been on the recovery of the cell from the original lesion produced by the radiation treatment. In case of bacterial spores, such an explanation is not plausible since the spores can be considered to be metabolically inert. However, the possibility that temperature may in some way alter the target sites within the cell and thus change their response to radiation can not be ruled out. Also thermal agitation of intracellular molecules could affect the transfer of radiation energy within the cell.

A more adequate interpretation of an observed temperature dependency during irradiation on bacterial spores can be made by the indirect and diffusion models. The relatively greater resistance of bacterial spores irradiated at -70°C can be interpreted as resulting from a decrease in the hydrogen peroxide concentration, and indirectly to radicals which lead to its production, as has been observed in pure water systems. Similarly, the rate of diffusion of toxic substances, produced by radiation, to the sensitive sites within the cell would be retarded by the frozen condition. However, an explanation of the increase in radiation resistance of the spores at elevated temperatures, as observed in this study, is not possible by the above model. As pointed out in a previous section, an increase in temperature results in decreased hydrogen peroxide formation during irradiation. If one can project the observations made in pure water systems to

the one under consideration, the number of spores surviving radiation exposure at elevated temperatures should thus be less than that observed at lower irradiation temperatures. However, an opposite trend has been observed in this investigation with bacterial spores. If the temperature during irradiation were acting on an extrinsic mechanism, then the apparent increase in radiation resistance observed with the various species of spores would be uniform at the same irradiation temperature. Then the degree of resistance of the particular organism under study would solely be dependent on its inherent resistance.

The possibility exists that free radicals and peroxides can be produced in molecules present in the cell other than the water molecules. The detection and importance of these moieties has yet to be ascertained. As the temperature during irradiation is increased one could assume that the decay of these radicals and peroxides would be hastened thus decreasing their effectiveness in causing death of the cell. However, any temperature dependency during irradiation on cellular death would still be at the same temperature and would not vary with the organism being studied.

The results of this investigation do not permit identification of the model by which radiation energy is dissipated to the target sites within the cell. Results of other investigations have indicated that the hydrogen peroxide produced may be the basis upon which gamma radiation exerts its lethal effect. These results show that if catalase, an enzyme capable of decomposing hydrogen peroxide rapidly, is present during irradiation, the lethality of gamma radiations for anaerobic spores is significantly reduced (Williams and Kempe, 1959). Also the presence of other

compounds during irradiation which may affect the concentration of hydrogen peroxide has been shown to alter the sensitivity of bacterial spores to ionizing radiation (Proctor et al., 1955, 1958; Walls, 1959).

A discrepancy is noted in certain published results in that an oxygen effect during irradiation in the absence of moisture has been reported for aerobic spores (Tallentire, 1958). The investigation by Houtermans (1956) showed a greater sensitivity of B. subtilis spores to radiation when irradiated at room temperature in the dehydrated condition as compared to wet spores irradiated at the same temperature. It appears that oxygen must have an additional role in inducing radiation sensitivity in certain bacterial spores other than that of increasing hydrogen peroxide formation in water.

An alternate approach to that of an effect of temperature on extrinsic factors which may modify the lethal effects of radiation is the involvement of an intrinsic mechanism of the cell. A variation in temperature could act in some way on this mechanism which then would be reflected in a varied sensitivity of the spore to the lethal effects of radiation. The question to be resolved is the nature of the factor within the spore and the manner by which it is affected by changes of temperature in order to vary the resistance of the spore to radiation.

In order to arrive at an adequate explanation for the results delineated in this study several aspects of spore physiology in conjunction with radiation induced mechanisms must be considered. These include the phenomena of bacterial spore dormancy, factors responsible for spore resistance and certain aspects of protein denaturation. Each of the previously named fields has been the object of extensive investigation. A great deal

of experimentation will be needed before the involved mechanisms will be elucidated. Nevertheless, certain conjectures can be made at this time in the light of the present knowledge. Therefore, the object of this discussion is to consider certain aspects of each topic which would be of importance in arriving at an interpretation of the results of this study and the pertinent results which have appeared in the literature.

Certain factors concerning the heat activation of dormant spores were considered in the section on experimental results. A ramification of dormancy demonstrated with thermal sterilization was the observation that severly heated spores of Cl. botulinum present in food substrates would only grow out after prolonged periods of incubation (Burke, 1919, 1923). This phenomenon is probably more in the nature of a recovery from injury rather than delayed germination of the spores. The slow recovery of the spore may come about by the retarded replacement of protein molecules within the cell by the undamaged synthetic mechanisms. Evidence for this is that severly heated spores when added to media which normally support growth of untreated spores need enrichment factors in order for growth to occur without prolonged periods of incubation (Curran and Evans, 1937). It would seem that the presence of all the necessary growth factors in the culture medium would simplify the resynthesis of essential cellular components necessary for the outgrowth of the damaged spore. This would then shorten the incubation time for visible growth to occur. Also incubation at lower temperatures, which is more conducive to synthetic processes, will shorten the time of incubation and increase the outgrowth of drastically heated spores (Williams and Reed, 1942). In this connection it has been demonstrated that a nonspecific factor, soluble starch, added to the recovery

medium will also increase the number of survivors following heat treatment (Foster and Wynne, 1948; Olson and Scott, 1946). However, the exact function of this substance is not known. Whether radiation-inactivated spores can recover from the damage has not been adequately demonstrated.

In any study on the effect of deleterious influences on bacterial spores consideration must be given to the resistance mechanism. Although of practical significance in the evaluation of sterilization processes, the basic factors of this mechanism have not been elucidated.

As evident from the early work on thermal inactivation of bacterial spores, the magnitude of the temperature coefficient of death adds support to the theory that organisms subjected to moist heat are killed by the denaturation of protein. Since protein denaturation occurs most readily in the presence of moisture, and since bacterial spores are capable of withstanding long periods of desiccation, attention was drawn to the relative amounts of water in spores as compared to the vegetative cells as an attempt to explain the resistance mechanism. No doubt the thick wall which encompasses the spore and which distinguishes it morphologically from the vegetative cell was also thought to act as a permeability barrier for the water molecules. However, early studies on the relative amount of water present in spores and vegetative cells showed no quantative differences in the water content of the two types of cells (Henry and Friedman, 1937). Therefore, the concept was advanced that if the total amount of water was the same in the vegetative cells and spores of bacteria, it must be present in some inert form and not be available for chemical reactions. This led to the concept of bound water as an explanation for the extreme resistance of bacterial spores to deleterious agents (Friedman and Henry, 1936).

The concept of bound water as a mechanism of resistance is still controversial. One difficulty arises in the accurate determination of the water content of the cell. Recent investigations are contradictory on the absolute amounts of water present in the bacterial spores. The results obtained by Waldhalm and Halvorson (1954), using differences in vapor pressures, indicate that the water content of spores and cells is similar. By another technique, namely differences in the refractive index of spores and cells, the water content of several aerobic spores was observed to be much less than that of the vegetative cells (Ross and Billing, 1957).

Another approach has been followed in order to obtain an understanding of the resistance mechanism of the bacterial spore to deleterious influences. This has been to determine if chemical differences exists which are unique to the spore and not the vegetative cell and which would contribute to its characteristic resistance. However, prior to 1953, it was generally recognized that with a few notable exceptions the chemical constituents of the spores were similar to those of the vegetative cell. The most significant exception recognized was in the differences in the inorganic chemical composition of the cells. Curran et al. (1943) observed, by means of spectral analysis of ashed vegetative cells and spores of 14 species of bacteria, a greater amount of calcium in the spores.

The evidence that calcium and another divalent cation, manganese, are involved in the thermostability of Cl. botulinum, B. megatherium and B. coagulans var. thermoacidurans spores is demonstrated by the fact that spores produced in media with sub optimal concentrations of these ions also demonstrated decreased thermal resistance (Sugiyama, 1951; Grelet, 1952; Amaha and Ordal, 1957).

A significant finding in spore physiology was the discovery of dipicolinic acid (DPA) in spores of B. megatherium by Powell in 1953. This is the first demonstration of a substance unique to the spore. Subsequent investigations have demonstrated this compound to be present in all the spores tested but not in the vegetative cells. The fact that dipicolinic acid is involved in spore resistance also seems to be established. During germination, and at a time when heat resistance is lost, there is a release of DPA (Powell, 1943; Woese, 1958). Also during sporulation of Clostridium roseum an increase in heat resistance is correlated with the synthesis of DPA (Halvorson, 1957). Furthermore, there is evidence that DPA is related in some way to the calcium content of the spore. Powell (1953) originally observed that dipicolinic acid is released as the calcium salt during germination. However, the exact relation of calcium and DPA needs to be elucidated. Perry and Foster (1955) observed that DPA and calcium are not present in equivalent amounts in B. cereus.

The mechanism by which the divalent cations, calcium and manganese, increase the resistance of the spore needs to be ascertained. A suggestion has been made that divalent cations may be capable of combining with proteins in a manner which would impart new strength to their intra-molecular linkage (Sugiyama, 1951).

Similarily the mechanism of DPA involvement in spore resistance is unknown. Suggestions have been advanced that it may act as a chelating agent (Harrel, 1957; Powell, 1957).

The relationship to radiation resistance of the factors which alter the thermalstability of bacterial spores has not been established. There is a suggestion that ultra-violet resistance preceeds heat resistance

which, in turn, preceeds DPA synthesis (Romey and Wyss, 1957). Similarily, it has also been observed that the resistance to gamma radiation by spores of B. cereus var. terminalis precedes heat resistance (Black et al., 1960). Halvorson (1957) observed that heat resistance preceeded DPA synthesis in Cl. roseum. Also, a certain amount of correlation exists between DPA content and radiation resistance of certain aerobic spores (Woese, 1959). Those spores which exhibit greater resistance to X-radiation have a slightly higher DPA content.

An explanation of the exponential order of death of many organisms is difficult without assuming the existence of an essential molecule or, at best, a few molecules which are responsible for cellular death. The hypothesis has been advanced that the thermal death of a cell is due to a lethal gene mutation (Rahn, 1945). This same concept has been advanced for radiation induced cellular death by Lea (1946). The validity of the latter hypothesis is based on the observation that bacteria, when exposed to radiation, are still capable of growing, as evidenced by the long filamentous forms present in the growth medium. This would indicate that the cells have lost their capability of reproduction (Lea, 1936).

Protein denaturation is any irreversible, non-proteolytic modification of the unique structure of a native protein giving rise to definite changes in its chemical and physical properties. The most commonly observed change in proteins, which have undergone denaturation, is the reduction in solubility which is manifested by the appearance of a visible coagulum. However, other changes can occur such as changes in viscosity, unmasking of sulphydryl groups, etc. These changes can be detected only by special means. In bacteria, denaturation of essential cellular

components results in death. Depending on the extent of heat treatment, different degrees of damage can occur because of the differences in the types of cellular protein which have different temperature inactivation coefficients.

A theory of protein denaturation has been advanced by Mirsky and Pauling (1936) which is based on their conception of the structure of the protein molecule. Their conception of a protein molecule is one (or more) polypeptide chain which is held in a unique position by means of hydrogen bonds between two electronegative atoms. As a result of an increase in temperature or attack by denaturing agents such as urea, acids, etc., the side chain bonds are broken leaving the protein molecule to assume any of a large number of configurations. If a large number of bonds are broken then the integrity of the molecule is lost and hence, denaturated. Since the energy of activation of the protein denaturation process is equal to 150,000 cal/mole and the strength of the hydrogen bond is equal to 5, - 8,000 cal/mole, Mirsky and Pauling proposed that approximately 30 hydrogen bonds must be ruptured before the molecule loses its native state. The basis of their theory is the high entropy of activation needed for the denaturation process which indicates hydrogen bond disruption, and the fact that compounds which are specific in attacking hydrogen bonds are also responsible for the denaturation of proteins. However, these authors do not imply that hydrogen bond breakage is the sole mechanism of protein denaturation.

In any discussion of lethal gene mutation, consideration must be given to the mechanism of hereditary transfer by the cell. It has been well established that hereditary transfer in cells occurs through chromosomes which are composed of nucleoproteins. Furthermore, the evidence is

quite conclusive that the hereditary mechanism in bacteria is the deoxyribonucleic acid (DNA) component of the nucleoprotein. The evidence for this is the transformation of certain bacterial cells by purified preparations of deoxyribonucleic acid. However the exact nature of the gene is not known.

Since protein denaturation is based on molecular structure, attention must be directed to the structure of deoxyribonucleic acid for an understanding of the mechanism of denaturation of this compound. On the basis of available information Watson and Crick (1953 a,b) proposed a model for the structure of deoxyribonucleic acid. The basis of the model is a double strand helix consisting of a phosphate ester linked backbone of pentose molecules with the purine and pyrimidine bases guanine, cytosine, thymine and adenine projecting inward. These bases are linked together with hydrogen bonds in the ratio of 1:1. The hydrogen bonds link the $-N_1=C_6(NH_2)-$ groups of adenine or cytosine on one of the chains with the $-N_{(1)}H-C_6O-$ group of thymine or guanine respectively in the other chain. Guanine and cytosine are linked with three hydrogen bonds while thymine and adenine have only two.

Heat denaturation of DNA solutions can occur by the splitting of the hydrogen bonds holding the two helices together (Thomas, 1954) (Cavalieri, Rosenberg, 1957), the guanine and cytosine bond system being more resistant since three hydrogen bonds are involved (Cox and Peacocke, 1956a, 1956b). In this investigation, with DNA from herring sperm, it was observed that the hydrogen bonds were not broken permanently until a temperature of 75°C was reached. Heating the solutions for as long as one hour at temperatures up to 75°C did not cleave the bonds for a long

enough time so that they could not heal. However, above this temperature a critical number were broken irreversibly and denaturation of the substance occurred. A unique characteristic of DNA obtained from a wide variety of sources shows that they must be heated to a specific temperature before any appreciable denaturation occurs. This specific temperature varies with the source of DNA and seems to be a reflection of the structure of this substance (Geiduschek, 1958).

The effect of gamma radiation on sodium deoxyribonucleate can result in cleavage of the inter-nucleotide-phosphate-ester bonds and the cross linking hydrogen bonds (Cox et al., 1955). However, the number of phospho-ester bonds which are broken along the main chain are proportionally small. Preferential breakage of the bonds between the adenine and thymine occurs with gamma radiation from cobalt indicating that the denaturation of DNA by radiation is a non-random process (Cox and Peacocke, 1957). It has been observed that pre-irradiation with 9,000 rep followed by a heat treatment at 100°C markedly changes the sedimentation coefficient of the treated solutions (Shooter et al., 1956). However, it was found necessary to heat irradiated DNA solutions at a high temperature before appreciable inactivation would occur (Butler, 1956).

Another concept which must be considered in any study of the effect of temperature on biological systems is the activation hypothesis. This hypothesis was proposed by Erying and Laidler (1940) to account for the high energies required to initiate a wide variety of chemical reactions. They proposed that the reactants needed to overcome an energy barrier or "hump" prior to going to completion. The individual reactants are considered to be in an activated state when present in this "hump". The

energy needed to initiate the reaction is localized in this activated complex of reactants. The exact fate of the energy within the activated complex is not known. However, the energy can go into the formation of new intermolecular bonds, healing of ruptured bonds, or in strengthening existing bonds of the reacting molecules (Sizer, 1943; Glasstone et al., 1941). Because of the very transient existence of the activated complex, direct experimental evidence for its existence is meager. The detection of new bonds formed during the decomposition of ethylidene diacetate (and esters of the general formula $R\text{-}CH(O\cdot CO\cdot R)_2$) has been demonstrated (Glasstone, Laidler and Eyring, 1941).

Because of the high temperature coefficient of most biological reactions such as heat denaturation, enzyme catalyzed reactions, etc., the activation hypothesis has found wide application in biology. The evidence for this theory to a wide variety biological reactions has been complied into a review by Johnson, Eyring and Polissar (1954). These authors consider heat activation of dormant spores as an example to which the activation hypothesis can be applied.

On the basis of seemingly unrelated observations concerning spore physiology, protein denaturation and the activation hypothesis, an explanation can be advanced for the results in which a temperature dependency has been observed during irradiation of bacterial spores. As the temperature during irradiation is increased, the molecules within the spore are placed in an activated state. The time-temperature relationship for the maximum expression of the excited state will depend on the

individual molecules involved in the spore and will vary with the organisms. Therefore, a different response would be expected at various irradiation temperatures with different organisms. This is reflected in the results of this study in that Cl. botulinum and putrefactive anaerobe spores exhibit maximal resistance to radiation at different temperatures during radiation. The putrefactive anaerobe spores are more heat resistant and more resistant to radiation at higher irradiation temperatures than the Cl. botulinum spores. A similar phenomenon has been observed during irradiation with maize seeds. Dried maize seeds exposed to X-radiation in the temperature range of -187° to 66°C exhibit the greatest radiation resistance at 50° to 60°C (Kempton, 1941). This temperature is just below the lethal thermal threshold for these seeds. A further increase in irradiation temperature results in greater sensitivity of the seeds as measured by subsequent seedling height. These results would indicate a similarity of temperature dependency during irradiation which is not limited to bacterial spores.

The idea that bond breakage is involved in the lethal action of radiation is obtained by inference. The energy of a photon from radioactive cobalt when absorbed within the cell is sufficiently high to rupture any of the various types of chemical bonds present in the cell. The results of Curran and Evans (1938) on the photosensitization of bacterial spores to heat by ultra-violet radiation suggests that bond rupture is involved. These investigators observed that it was necessary to irradiate the spores for a sufficiently long time at 2537A° for the sensitization to be adequately expressed. Irradiation of the spores at 300 - 1600A°

had the greatest effect for heat sensitizing the spores. Also Giese and Crossman (1945) observed that irradiation at 2383A° was most effective in inducing photo-sensitization in Paramecium. The amount of energy corresponding to a wavelength of 2537A° is 112 k cal/mole. This amount of energy, if completely absorbed, is just below the threshold needed for irreversible denaturation to occur. Also, most stable atoms and molecules have ionization potentials in the shorter ultra-violet region (below 1000A°). Therefore, if bond breakage is involved in the process, one would not expect that irradiation at 2537A° or at longer wavelengths to be as effective as higher energy wavelengths in inducing sensitization.

The suggestion has been made that the DNA component of nucleo-protein is involved in spore resistance and hence in survival. Furthermore the hypothesis was advanced that both thermal and radiation energy are operative on the same factor within the spore. The results of this study in which the irradiated spores were heated at various temperatures after irradiation resulted in a curve, Figure 12, which is similar to the published results obtained with DNA solutions treated in a similar manner (Butler, 1956). In both cases it was found necessary for heating to be at a sufficiently high temperature in order for inactivation to occur even after the radiation treatment. With spores of Cl. botulinum and putrefactive anaerobe this temperature varied. Putrefactive anaerobe spores, which are more heat resistant than botulinum spores needed to be heated at higher temperatures before sensitization was demonstrated.

With purified protein solutions, a pre-irradiation treatment with ultra-violet light results in a lowering of the coagulation temperature of the proteins (Stedman and Mendel, 1926). The sensitization of purified proteins to heat by ionizing radiation has not been investigated

thoroughly. It was reported that the coagulation temperature of human serum albumen in lowered by irradiation with high speed electrons (Kan et al., 1957). In the case in which the coagulation temperature of the protein is lowered, it would be anticipated that the kinetics of the thermal inactivation of the irradiated protein would result in a curve more typical of an Arrhenius plot. This type of curve is different from what has been observed here with pre-irradiated spores, heated at various sub-lethal temperatures. This can be interpreted on the basis of a difference in the molecular structure of the component being acted upon.

Several lines of experimentation indicated that the reproductive mechanism, hence the nucleoprotein, of the cell is involved in radiosensitization. Duggar and Anderson (1939) concluded that nucleoprotein must be involved in the photosensitization of yeast cells since the organisms were incapable of producing visible colonies, but still retained their respiratory capacities. Giese and Crossman (1945) observed that Paramecium recovered more slowly from the damage when irradiated at a wavelength of 2650A°. They arrived at this conclusion since absorption by nucleoprotein occurs at this wavelength. It has been recognized for sometime that radiation inhibits the ability of bacterial spores to form colonies but not their ability to germinate (Lea, 1946). The fact that bacterial spores are capable of germinating even after an irradiation treatment which prevents colony formation indicates that the reproductive function and the mechanism of germination are separate. It can be assumed that other protein components of the cell are capable of being heat sensitized by irradiation. However it appears that this does not impair their

subsequent function since the spores are still capable of germinating. However, injury to the germinating mechanism within the spore could be capable of repair or replacement. The irreversible effect of radiaion, which manifests itself in lethality of the spore, is confined within the mechanism which is responsible for the outgrowth and reproduction of the spore. Although photosensitization of other cellular proteins can conceiveably occur, this does not adversely affect certain metabolic functions of the cell.

The phenomenon of dormancy, as separate from that induced by heat injury, is related to spore resistance in that those spores exhibiting greater thermal resistance must be heated for a greater length of time before dormancy is broken (Desrosier and Heiligman, 1952). Furthermore, the spores must be activated before germination can be initiated. As demonstrated in this study, a pre-irradiation or heat treatment of bacterial spores at sub-lethal temperatures does not cause them to germinate as measured by loss in heat resistance and staining. It is conceivable that radiations of lower energy thresholds, which, when absorbed, would not inactivate the spore, might be substituted for heat in breaking dormancy. The observation reported in the present study showed that heating botulinum and putrefactive anaerobe spores for prolonged periods prior to irradiation did not alter their resistance to radiation, demonstrates that heat alone, without the presence of a substrate, cannot break dormancy. It was anticipated that such a prolonged heat treatment would induce the spores to germinate or would alter them in some way that would decrease their resistance to radiation. It can be inferred from the results of these experiments that heat must be applied simultaneously with radiation

in order for the observed temperature effect during irradiation to be demonstrated. If calcium and dipicolinic acid are acting as bonding or chelating agents within the spore, the bonding must be quite tenacious to withstand such drastic heat treatment.

As mentioned previously, DPA and calcium have been implicated in the thermal resistance of bacterial spores. However, the site of action within the spore has not been postulated. The structure of dipicolinic acid would make it easy for it to function as a bonding agent via hydrogen bonds. Conceivably, DPA could act as a bridge between the bases of DNA and in this manner increase the stability of the molecule. This linkage could be formed with or without the participation of calcium. An observation made with another cell system has implicated the importance of calcium in mutagenesis. Stephansen (1956) observed an increase in the number of chromosomal aberrations produced by radiation in Tradescantia microspores grown in calcium deficient media. Although divalent cations seem to be associated with nucleoproteins, the function of these substances is not known (Williamson and Grelek, 1944).

The fact that pre-heating does not alter the sensitivity of the spores to radiation can be interpreted on the basis of the differences in the manner of absorption of the two forms of energy. Application of heat will result in the random absorption of the thermal energy within all the molecular bonds within the spore. Breakage of molecular bonds will occur once the threshold of energy for a particular bond is attained. In the case of ionizing radiations the absorption of the incident photons must be localized within the sensitive area of the spore. After absorption of a photon irreversible damage occurs to the molecules which are

responsible for maintaining the integrity of the cell. However, the absorption of insufficient photons within the critical areas results in only partial bond breakage without inducing lethality. Since the theory of protein denaturation, stated in this discussion, maintains that a critical number of bonds must be ruptured before the integrity of the molecule is lost, a pre-irradiation treatment may occasion a decrease in the number of bonds which must be subsequently inactivated by a heat treatment in order to cause denaturation. This view is in accord with the observation that irradiated spores have a lower susceptibility to heat. The reverse, a preheat treatment, would not cause the spores to be more sensitive to radiation under the above considerations since irreversible breakage of the molecular bonds occur once the thermal threshold energy is attained.

SUMMARY

The object of this investigation has been to study the role of temperature during irradiation on the subsequent survival of bacterial spores in order to arrive at an explanation for some of the conflicting results which have appeared in the literature. The phenomenon of photosensitization of proteins by electromagnetic radiations was also considered.

Previously published work offers no satisfactory explanation for the diverse ways that temperature can affect the lethal effects of ionizing radiation. From the results delineated in this study however, several clarifying observations can be made. Thus, anaerobic bacterial spores, when exposed to gamma radiation in the temperature range of -70° to 95°C, exhibited a varying degree of response to irradiation as determined by their colony forming ability: the spores were slightly more resistant to radiation at -70° than at 4°C, and the maximum number of spores surviving radiation exposure occurred at a temperature just below the thermal lethal threshold for the particular organism under investigation. The thermal lethal threshold for Cl. botulinum spores was found to be approximately 85°C. A further increase above this temperature resulted in rapid inactivation of the spores by radiation. In the case of putrefactive anaerobic spores, the greatest number of survivors were observed in the temperature range of 90° to 100°C, with progressively greater numbers of survivors being obtained as the temperature was increased above room temperature.

Although radiation increases the sensitivity of bacterial spores to heat, the thermal lethal temperature is not lowered by this treatment.

This is contrary to the published results which show that the coagulation temperature of purified proteins is progressively lowered by exposure to ultra-violet radiation. The spores which exhibit greatest resistance at the various temperatures during irradiation, also exhibit greater thermal resistance.

In the protocol of this investigation, all spores were heat treated in order to break dormancy. The heating time and temperatures employed were considered to be maximal for subsequent germination of the organisms used. This would rule out the possibility that the apparent increase in survivors at the elevated temperatures was caused by heat activation of any dormant spores present in the spores suspensions. Furthermore, it was observed that gamma radiation from cobalt-60 was not capable of inducing the spores of an aerobic species to germinate.

On the basis of the observations obtained in this study and pertinent evidence in the literature, it is postulated that the lethal effects of heat and radiation, as well as the radio-sensitization phenomenon are operative on a joint mechanism within the spore. It is suggested that the key mechanism is the reproductive mechanism and that nucleoprotein, or more specifically the deoxyribonucleic acid component of nucleoprotein, is critical in this regard.

Several practical aspects concerning the application of heat and radiation for sterilization purposes can be delineated from the results of this investigation viz.: an accurate determination of the sterility dose must consider the temperature at which irradiation is carried out: if the sensitization of bacterial spores to heat by radiation is to be utilized, the irradiation must be conducted at the thermal, lethal

threshold of the organism under consideration: the most thermally resistant organism must be used in the evaluation of this method of sterilization: for a combined process where radiation and heat is to be utilized, a pre-irradiation treatment, followed by post-irradiation heating at a lethal temperature, must be employed to take full advantage of the sensitization of bacterial spores to heat.

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