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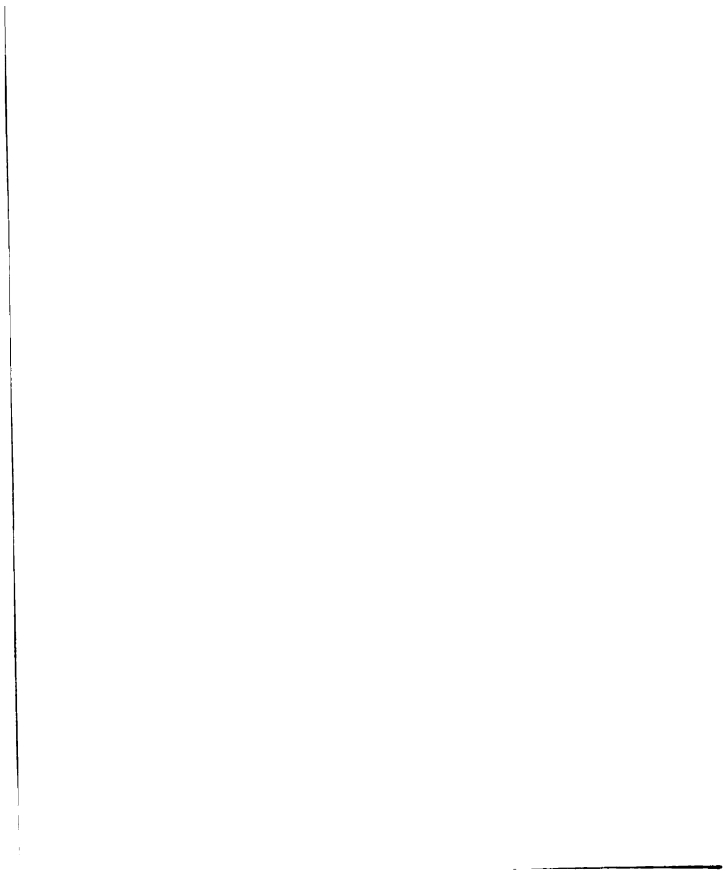

ABSTRACT

RECOVERY PATTERNS OF SPORES OF PUTREFACTIVE ANAEROBE NO. 3679 IN VARIOUS SUBCULTURE MEDIA FOLLOWING MOIST AND DRY HEAT TREATMENT

by Jorg A. L. Augustin

The purpose of this investigation was to study the pattern of recovery of spores of Putrefactive Anaerobe No. 3679 (P.A. 3679) in various recovery media following moist and dry heat treatment.

Two spore crops were produced in beef heart infusion and trypticase-yeast extract broth respectively. The spores produced from these media after having been freed from their vegetative sporangia and any foreign material were dispensed into small metal cups in amounts of 0.01 ml and dried under vacuum at room temperature. Heating these spores in moist and dry heat was accomplished by subjecting them to saturated steam in the thermoresistometer or in miniature retorts, and to hot air enclosed in thermal death time (TDT) cans in miniature retorts using saturated steam as a heating medium for the hot air entrapped in the TDT cans. After the heat treatments, the spores were transferred into the various subculture media which included infusion type media, formulated media and dehydrated media. The effectiveness of the subculture media in recovering surviving spores was established by determining D. values on the basis of endpoint determinations over a temperature range of 230°F to 280°F in moist heat and 255°F to

to 320°F in dry heat. D. Values were calculated by the modified Schmidt method. The z values were obtained graphically.

The results revealed significant differences in decimal reduction times (D values) as affected by the heating medium, the sporulation medium and the subculture medium. The fact that increased D values were obtained when the synthetic medium was supplemented with DNA, RNA and/or vitamin B_{12} indicated that at least in part nutritional deficiencies of some of the media might be responsible for the differences in the D values obtained with the various subculture media. D values were consistently and considerably higher for beef-heart infusion spores and for those spores which were subjected to dry heat treatment.

The z values did not vary greatly with the sporulation medium in which the spores were produced. The largest differences in z values resulted from the use of the two different heating media. Dry heat treatment of the spores resulted in z values of 36°F and 33°F respectively. Moist heat treated spores which were produced in beef-heart infusion showed z values of 19.5°F with the exception of those spores which were subcultured in trypticase or eugonbroth. In those cases the z values were 18.2°F and 17.5°F respectively. Moist heat treated spores which were produced in trypticase-yeast extract broth gave z values ranging from 15.5°F to 17.0°F depending on the type of subculture medium.

In general highest D values were obtained when the heated spores were subcultured in beef infusion. Subculturing of the heated spores in liver infusion, pea infusion or yeast extract resulted in slightly lower D

values. Even lower D values were obtained when eugonbroth or trypticase were used as subculture media. The lowest D values resulted from the use of a synthetic medium. In spite of these similarities found in the recovery of the heated spores regardless of the type of heating medium used, the relative magnitudes of the D values as well as of the z values with reference to the subculture media used were different with moist heat than with dry heat. This led to the conclusion that the requirements for germination and outgrowth are different for spores which were subjected to moist heat than for those that were subjected to dry heat.

The above results and the conclusions drawn therefore lend support to the theory that the logarithmic order of death of bacterial spores is the result of the inactivation of an essential gene and that the differences in D values obtained are the result of random injury of the genetic material of the spores which forms the basis for the nutritional requirements of these spores for germination and outgrowth.

RECOVERY PATTERNS OF SPORES OF PUTREFACTIVE ANAEROBE NO. 3679 IN VARIOUS SUBCULTURE MEDIA FOLLOWING MOIST AND DRY HEAT TREATMENT

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INTRODUCTION

Different food products require different amounts of heat for sterilization. Possible explanations for this are differences in nutritional composition of and/or presence or absence of inhibitory substances.

Little is known about the nutritional requirements for germination and outgrowth of spores of food spoilage organisms following moist or dry heat treatment. What little information is available refers to moist heat only, and in most cases the experimental arrangements do not allow definite conclusions as to which of the subculture media fulfill best the nutritional requirements of these spores.

It is the purpose of this investigation to obtain information regarding the resistance of spores of Putrefactive Anaerobe No. 3679 (P.A. 3679) to moist and dry heat. Of specific interest are answers to questions as to the nutritional requirements of these spores for germination and outgrowth following heat treatments, and as to the nature and mechanism that leads to their destruction.

P.A. 3679 is used in the food canning industry as one of several test organisms for the evaluation of thermal processes for low acid type foods. The goal in designing thermal processes is to destroy all microorganisms which are either of public health significance and/or might cause spoilage during normal procedures of handling, storage or distribution. In low acid type foods, chief attention is paid to bacterial

spores which exhibit considerably higher heat resistance than their vegetative cells.

Canning sterilization procedures are designed according to the moist heat resistance of bacterial spores. However, where the separately sterilized food product is filled aseptically into pre-sterilized containers, sterilization of the containers by moist heat has proved to be impractical. Instead, dry heat in the form of dry air, inert gases, or superheated steam, or in combination with gaseous agents which are known to be sporicidal have been used in the past.

Infusion type media for both sporulation of P. A. 3679 as well as for its recovery following the heat treatment, have been unsurpassed so far by any formulated, dehydrated and synthetic media, from the criterion of sporulation media producing spores of greater heat resistance and as subculture media with reference to efficiency in recovery of the heated spores are concerned. The exact composition of these infusion type media is not known, and are thus difficult to simulate in a synthetic medium. Therefore, the experimental design will be primarily geared to answer questions as to whether the nutritional requirements are the same for the germination and outgrowth of spores which have been produced in different sporulation media, as well as for spores which were subjected to treatment in moist heat compared to those which survived dry heat treatment. Information of this nature will be sought by subjecting dried spores of known concentrations to dry and moist heat for various times and at various temperatures followed by

subculturing in several media. Determination of 90 percent destruction times (D values) at various temperatures, and rates of changes of these D values with temperature with various media will serve as comparative criteria in evaluating this problem.

It is believed that some of the differences in the recovery of the spores in various subculture media following the heat treatments are due to nutritional deficiencies in the subculture media. An effort will be made to prove this theory by supplementing the subculture medium giving lowest recovery with certain compounds which are believed to be contained by the subculture medium giving highest recovery.

REVIEW OF LITERATURE

A detailed review of all aspects of spore bacteriology is obviously beyond the scope of this investigation. Therefore, this discussion will be limited to those studies which are believed to be essential and helpful for the understanding of this investigation. The topics reviewed are: bacterial endospores with particular emphasis on the spores of the test organism used in this study, thermal destruction of bacterial spores, methods of studying heat resistance of bacterial spores and factors affecting the thermal resistance of bacterial spores.

The main sporeforming bacteria are found in the genera Bacillus and Clostridium. In a few instances, the ability to form bacterial endospores has also been observed with certain cocci and spirilla.

Bacterial spores are formed inside the vegetative cells. Normally one spore is formed per mother cell. The endospores become free when the mother cells lyse. Some of the main characteristics which distinguish bacterial spores from their corresponding vegetative cells are: high refractility, greatly reduced metabolic activity, high density, presence of dipicolinic acid (DPA) and increased amounts of calcium, resistance to usual staining techniques, resistance to heat, and to ultraviolet and gamma ray irradiation.

P.A. 3679 was first isolated by E. J. CAMERON in 1927 (TOWNSEND, ESTY AND BASELT, 1938) from a spoiled can of corn. Some controversy exists as to whether this organism is to be considered as

a strain of Clostridium sporogenes, or as a separate species. According to GROSS, VINTON AND STUMBO (1946) the former is serologically different from the latter. However, since the cultural differences reported by these workers, are few, and based upon only one strain of each organism, it is doubtful whether these differences are significant enough to warrant P.A. 3679 to be considered as a separate species. CAMPBELL, JR. AND FRANK (1956) reported P.A. 3679 to have a nutritional difference from C. sporogenes since P.A. 3679 requires serine for growth whereas the latter does not. These organisms are alike, however, in that they both form spores under proper conditions, their vegetative cells are mesophilic, anaerobic, use the Stickland reaction to obtain energy for growth, and produce gas and a typical putrefactive odor in meat products and in most laboratory media.

1. Death of Bacterial Spores

The only practical criterion for death of bacterial spores is their failure to germinate and reproduce under conditions satisfactory for germination and outgrowth of the normal live spore. The study of the rate of destruction of bacterial spores as well as the changes in rates of destruction with changing temperatures have not only opened the path for mathematical treatment of this phenomenon which, in turn, has proven to be extremely valuable in the design of industrial sterilization processes, but also has provided valuable information as to the possible biochemical nature of death and the mechanisms causing it. A brief discussion of some of the literature that has been published in this field of

study, appears to be an integral part of this investigation.

a. The Order of Death of Bacterial Spores:

The mathematical expression for the logarithmic order of death of unicellular organisms is

$$\frac{dN}{N} = -kdt \qquad (1)$$

where N = number of organisms
k = rate of destruction
t = time

Integrating this equation gives

$$\log N = -kt + \text{constant, or} \qquad (2)$$

$$\log N = -kt + \log N_o \qquad (3)$$

where N_o = initial numbers of spores
N = number of spores surviving

Solving equation (3) for k gives

$$k = 1/t(\log N_o - \log N) \qquad (4)$$

KATZIN, SANHOLZER AND STRONG (1943) assuming a 90 percent destruction of the original population arrived at the following equation:

$$k = 1/t \times \log N_o/0.1N_o = 1/t \qquad (5) \quad \text{or}$$

$$k = 1/t$$

The time "t" in this case was defined as the decimal reduction time, that is the time required to reduce the original population by 90 percent, or the time required for the survivor curve to traverse one logarithmic cycle if the logarithm of the number of survivors is plotted against time. Originally the term Z was used to designate decimal reduction time. Later SCHMIDT (1950) suggested the term D because of

possible confusion with z the reciprocal of the slope of the thermal death time curve. The term D is now generally accepted as the designation of decimal reduction time.

Replacing 1/k by D the following equation is obtained:

$$D = t/(\log N_o - \log N) \qquad (6)$$

The assumption of the above mathematical relationship of spore destruction has been the subject of controversy for a number of years. Reports describing deviations from the logarithmic order of death in the forms of curves ranging from concave up to concave down and to sigmoidal have been as numerous as those describing straight-line relationships. The subject has been extensively reviewed by RAHN (1929, 1930, 1943) and later by SCHMIDT (1957). Since then, FRANK AND CAMPBELL (1957), and WALKER, MATCHES AND AYRES (1961) reported survival curves which were concave up. Curves with a shape of concave down were reported by ORDAL AND LECHOWICH (1958), WALKER, MATCHES AND AYRES (1961), EL-BISI ET AL. (1962), LECHOWICH AND ORDAL (1962), and LICCIARDELLO AND NICKERSON (1963). Some of these workers reported the occurrence of a logarithmic survival pattern with various deviations from it in the same publication, and in some cases even with the same organisms, all depending on the experimental circumstances. The various shapes of survival curves reported and the possible reasons for deviations from the straight-line relationship have been discussed by EL-BISI AND ORDAL (1956b), AMAHA AND ORDAL (1957), and BALL AND OLSON (1957). SCHMIDT (1957) while giving

full recognition to the existence of non-logarithmic survival patterns in some instances, accepts the logarithmic order of death as a convenient working hypothesis. For, it is only by making this assumption that survival data can be subjected to any kind of mathematical treatment.

b. Thermal Destruction Curves

A similar controversy appears to exist with reference to the semi-logarithmic relationship between rate of destruction and temperature. BIGELOW (1921) was the first to demonstrate this relationship. He named this curve the thermal death time (TDT) curve. Similar findings were reported later by STUMBO (1948), STUMBO, MURPHY AND COCHRANE (1952), PFLUG AND ESSELEN (1953, 1954), and ANDERSON (1959), PHEIL, NICHOLAS AND PFLUG (1963). Actually BIGELOW (1921) obtained a sigmoidal curve, but he explained the deviations from the straight-line relationship at both ends of the curve as being due to experimental errors at the higher temperatures, and accepted the initial lag at the lower temperatures as an experimental fact. HALVERSEN AND HAYS (1936) reported also a lag in the thermal resistance curve at the lower end of the temperature range studied. GILLESPY (1954) showed that if a logarithmic order of thermal reduction is assumed, then by applying the Arrhenius equation, the thermal resistance curve, although straight over limited temperature ranges, would run asymptotically to both the ordinate as well as to the abscissa. ESSELEN AND PFLUG (1956) reported a less steep slope of the thermal resistance curve at the higher temperatures, and explained this as

being due to possible changes occuring in the spore supporting media during heating. LICCIARDELLO AND NICKERSON (1963) reported a concave down curve with Clostridium sporogenes but a more or less straight line with spores of Bacillus subtilis and Salmonelia senftenberg 775W over temperature ranges of 40°C and 20°C respectively. PHEIL AND PFLUG (1964) reported a break in the thermal resistance curves: at the lower temperatures, 172-230°F they obtained a z value of 25°F and at the higher temperatures of 235°F to 250°F, one of 15°F.

c. Nature and Mechanism of Death

LEWITH (1890) postulated that coagulation of protein was the cause of death of bacterial spores for both moist and dry heat. He attributed the extremely higher resistance of bacterial spores to dry heat as compared to moist heat to a change in sensitivity of proteins toward coagulation in dependence to its water content, and was able to demonstrate this experimentally by heat treating egg albumen containing various amounts of moisture. The protein coagulation theory was substantiated by WILLIAMS (1929) by the facts that conditions inhibiting coagulation result in increased heat resistance.

Furthermore, it was shown by RAHN (1945a) that the temperature coefficients for thermal death of spores and protein coagulation are of the same magnitude. VIRTANEN (1934) agreed with this theory at least as far as the moist heat sensitivity was concerned; however, he extended it to the inactivation of some vitally important enzymes as being the major event leading to the destruction of a cell. RAHN AND SCHROEDER

(1941) demonstrated that bacterial cells can be inactivated and still retain enzyme activity. On the basis of logarithmic order of death, they postulated that death was due to heat inactivation or heat coagulation or a critical and rare molecule in the cell. Further RAHN (1945b) upon analyzing the logarithmic order of death on the basis of probabilities of destruction of bacterial cells, demonstrated that death must be the result of thermal inactivation of one or only a few critical molecules of the cell. Since the probability that two or more molecules within a cell are inactivated is the product of the probabilities of the destruction of each molecule, he showed that, if the destruction of four or five or more molecules were directly associated with thermal death of unicellular organisms, their order of death would cease to be logarithmic and would eventually follow that of multicellular organisms. Since the bacterial cell contains more than several thousand molecules of each enzyme, he rules out enzyme inactivation as the critical event leading to the death of bacterial or sporal cells. On the basis of the bacteriologists' definition of death as the loss of the ability of bacterial cells to reproduce, as well as on the basis that cell division is under genetic control, he suggests that the denaturation of a critical gene molecule be considered as the critical event causing death.

With reference to dry heat destruction, RAHN (1945a) postulates the cause to be oxidation, because dry cells display no life function, that enzymes of spores are not active in the absence of moisture, that all endogenous catabolism was absent in dried spores and finally that

dried proteins would not coagulate even when heated to 100°C. Increased death rate, according to RAHN, is attributable to increased rate of oxidation.

INGRAHAM (1962) without furnishing any experimental evidence tries to explain the heat killing of bacterial cells as being the result of either heat inactivation of an essential gene or the destruction of a certain structure such as the cell membrane. Experimental evidence as to the former possibility has been furnished at least in part by ZAMENHOF (1960) who demonstrated that dried spores of Bacillus subtilis, and dried cells of Escherichia coli when subjected to heating in vacuo, showed an increase in rate of mutation, that the carrier of the mutational injury was deoxyribonucleic acid (DNA), and that with some exceptions the occurrence of a larger percentage of mutants was not the result of higher heat resistance among the mutant cells. On the basis of these results, it is quite conceivable that certain lethal mutations might be induced by the application of heat. Some experimental evidence as to the possibility of structural disruption of the cells as the lethal result of heat treatment has been furnished by HUNNELL AND ORDAL (1961) who followed the structural changes occuring in spores of Bacillus coagulans during heating at 121°C in M/15 phosphate buffer. Microscopic examination of the spores revealed the disappearance of the cortex as the first phenomenon which was followed by a filling of the spore coat by the core. At this stage the spores had lost their viability. During further heating, disintegration of the core occurred, and eventually it was only the spore coat that retained some structural integrity.

Heat has been shown to be an agent lethal to bacterial spores, if imposed upon the latter in sufficient quantity. Its possible mode of action on the sporal cell may aid in explaining the mechanism and possible also the nature of death of bacterial spores. A brief outline of the theories which have been advanced as an explanation for the logarithmic order of death appears, therefore, to be appropriate.

TISCHER AND HURWICZ (1955) reason that in view of the fact of the monomolecular type of mechanism involved in bacterial destruction, heat cannot be visualized as a continuous and homogenous wave front. If it were, the probability of survival would only have two values: zero and one. However, since the probability for survival gradually decreases with increasing time of heat application, the above authors conclude, that the logarithmic order of death of bacterial spores can be explained only by assuming that heat energy consists of discontinuous, homogenous or nonhomogenous quanta of energy.

BALL AND OLSON (1957) visualize the "bullets" causing death as molecules in a medium which greatly exceed the energies, momenta and velocity of the average molecules. On the basis of this theory they are able to explain the effect of pH, the presence of sodium chloride, fats and oils on the rate of destruction of bacterial spores.

CHARM (1958) in his "kinetic effect theory" compares bacterial destruction rates to chemical reaction rates. He visualizes the bacterial cell as being composed of a number of sensitive volumes surrounded by a number of water molecules, all enclosed within the cell wall. When the water molecule next to the sensitive volume exerts sufficient energy

onto this sensitive volume, inactivation of the cell occurs. Due to the distribution of energy among the molecules in the cell, not all cells would be inactivated at the same time.

2. Methods for Studying Heat Resistance of Bacterial Spores

In designing experimental arrangements for thermal destruction studies, it is important to select the type of apparatus most feasible to carry out such studies, the proper experimental procedure and the type of statistical evaluation of the data to be obtained. These three aspects of the experimental design of this thesis will be discussed in the following sections.

a. Apparatus

The early studies of heat resistance of bacterial spores were mostly carried out using test tubes or capillary tubes as containers and a water bath or an oil bath as the heating medium. BIGELOW AND ESTY (1920), and ESTY AND MEYER (1922) placed the spore suspensions into tubes which were subsequently sealed in an oxygen blast flame and then subjected to the desired heat treatment in an oil bath. WILLIAMS, MERRILL AND CAMERON (1937) developed the "tank" method where the spores are heated in some food stuff (mostly pureed food materials) in an agitated tank under pressure. Samples are removed at various intervals and tested for surviving spores. SOGNEFEST AND BENJAMIN (1944) first reported the use of thermal death time (TDT) cans and outlined a method for inserting a thermocouple into the can for the purpose of

obtaining information about the time-temperature relationship during retorting. In this method too, the spores are suspended in some pureed food material, filled into the cans which are then sealed and subjected to the desired heat treatment. Subculturing is not necessary in this case if spores of gas producing organisms are employed in the experiments. The cans are merely placed in an incubator following the heat treatment and examined periodically for gas formation which exhibits itself in the bulging of the cans. MAGOON (1926) heated spores in capillary tubes in an oil bath. This method was later improved by STERN AND PROCTOR (1954) who designed an apparatus with an automatic transfer device for exposing biological suspensions in capillary tubes to high temperatures in an oil bath. FARKAS (1962) developed a modified version of this machine which was made to hold 20 or more capillary tubes and which would be adopted to hold various sizes of tubes and other containers.

While the rate of heat transfer in capillary tubes is greatly improved over that found in test tubes and TDT cans, it is far from instantaneous and therefore correction calculations have to be made for the heating and cooling lag in order to obtain the actual exposure time at the desired temperature. The first apparatus where this problem is eliminated to the point where the times necessary for the heated spores to reach the desired temperature and for cooling are negligible provided the total exposure time exceeds three or four seconds, was designed by STUMBO (1948). This machine which is commonly called a

thermoresistometer, consists of a heating chamber and a sliding plate onto which small metal cups containing the spore suspension are placed, and on which these samples are moved into and out of the heating chamber.

ESSELEN AND PFLUG (1956) describe a similar apparatus using pistons with rings to transport the metal cups instead of the sliding plate. PFLUG (1960), in order to obtain a more positive seal, developed a modified version of this thermoresistometer by constructing a double seal operation. This machine has the further advantage that it can be used for both moist and dry heat.

Studies on thermal resistance of spores in dry heat involve rather long exposure times. The number of replicates that can be treated in the thermoresistometer at any one time is limited to five cups. In studies of this kind ten to thirty or more replicates are run for each time of exposure. PFLUG, AUGUSTIN AND JACOBS (1961), in order to eliminate such time consuming procedures, placed up to ten tin-plated metal cups into TDT cans, sealed the cans, and subjected them to moist heat in miniature retorts. Obviously, inside the cans dry heat conditions exist. Ten or twelve cans can be heat treated at any one time with this method.

KOESTERER AND BRUCH (1962) in their studies of dry heat resistance of bacterial spores, inserted test tubes containing the dried spores into holes of heated cylindrical aluminum blocks. The spores were either dried directly in the tubes, or they were first placed onto

filter strips or mixed into sterile sand, dried and then placed into the tubes.

b. Experimental Methods:

BIGELOW AND ESTY (1921) and ESTY AND MEYER (1922) expressed heat resistance of bacterial spores in terms of thermal death points which they defined as the number of minutes required at a given temperature to destroy a spore suspension of a known concentration. The physical arrangement of their tests was such that they placed a series of tubes, all containing an equal number of spores, into a heated oil bath. At various time intervals one tube was removed and checked for survival. ESTY AND WILLIAMS (1924) reported the occurence of "skips," meaning that spores in single tubes which are exposed to heat for several consecutive time intervals show destruction at one time and survival at a longer time of exposure. As a solution to this problem they suggested the use of replicate tubes for each exposure time. In doing so, they found that at a certain exposure time only a certain percentage of the tubes showed growth, and that plot of the logarithms of the percentages of survivors against time on a linear scale resulted in an approximate straight line relationship. Although their suggested method of plotting "survivor" curves did not find acceptance, their findings introduced the use of replicate samples in most similar studies carried out thereafter.

While these early studies did not involve any calculations and were only concerned with the establishment of the thermal destruction time,

more emphasis has been placed recently on establishing rates of destruction of bacterial spores expressed as D values. Such values can be calculated from survivor data or determined graphically from survivor curves. Such procedures however involve much time consuming processes as dilution of the heated spores, plating and counting. Furthermore, it has the disadvantage that the accuracy of the counts becomes questionable if the number of survivors fall below a certain minimum. For these reasons, several workers, among them STUMBO (1948), STUMBO, MURPHY AND COCHRANE (1950) and SCHMIDT (1957) prefer to establish D values on the basis of end-point determinations. In this method, multiple samples are run at successive time intervals with appropriate spacing so that with the shortest time most or all replicate samples show growth and with the longest time of exposure either a very small fraction of the tubes or none exhibit growth. Details on the various methods of calculation will be outlined in the following paragraph.

c. Methods of Calculating D Values:

STUMBO (1948) used equation (6) replacing t by U, N_o by A and N by B, where A designated the total number of samples heated multiplied by the number of spores per sample, and B was calculated by assuming one surviving spore per container when less than the total number of containers showed survival. STUMBO, MURPHY AND COCHRANE (1950) modified this method by calculating B by means of the "most probable number" technique which was developed by HALVORSON AND ZIEGLER (1933). In this case B equals the most probable number of

spores per replicate times the number of replicates. This is calculated from the following equation:

$$\bar{x} = 2.303 \log n/q \qquad (7)$$

where $\bar{x}$ = the most probable number of survivors per replicate
n = the total number of replicates
q = the total number of sterile replicates

A number of exposure times are chosen at a certain temperature. For each exposure time the corresponding D value is calculated. The final D value is the average of all these D values. This method is often called the unweighted average method.

The probability method developed by SCHMIDT (1957) is a modification of the method which was first developed by REED (1936), and has been worked out on the basis of the following two assumptions: First, any sample that does not show survivors at a given exposure time, does not show survivors at a longer time of exposure at the same temperature, and second, any sample showing survivors at a given exposure time will also show survivors at a shorter exposure time at the same temperature. These assumptions permit the data of all process times of a given temperature to be combined for the computation of the D value. The probability of sterility is first calculated on the basis of the cumulative number of samples showing growth and the cumulative number of sterile samples, according to the following equation:

$$P = (n + 1)/(m + n + 2) \qquad (8)$$

where P = the probability of sterility at a given time
n = the cumulative samples not surviving each exposure time obtained by adding the

negative samples downward from the shortest exposure time to the longest

m = the cumulative samples surviving each exposure time obtained by adding the positive samples upward from the longest exposure time to the shortest

The P values corresponding to the various exposure times are plotted on probability paper. A line is drawn between these points. From this curve the time corresponding to P = 0.50 is determined. This time point is called the L. D. 50 value which is the time at which 50 percent sterility occurs. This time corresponds to 0.69 organisms per tube according to HALVORSON AND ZIEGLER (1933). D is then calculated according to the following equation:

$$D = L.D.50/(\log A - \log 0.69) = L.D.50/(\log A + 0.16) \quad (9)$$

where A = number of spores per sample prior to the heat treatment.

LEWIS (1956) in reviewing the various methods for calculating D sharply criticized the STUMBO, MURPHY AND COCHRANE method for its bias. With reference to the SCHMIDT method, his only criticism is the fact that no detailed analysis is available for it. He suggests the use of the Spearman-Karber method or the maximum likelihood method, often called the loglog method, both described by FINNEY (1952). The Spearman-Karber method uses a different procedure to calculate the mean sterility time V_t and the variance of this time. Equation (9) is used to calculate D with the modification the L. D. 50 is replaced by V_t. The loglog method is suggested by LEWIS (1956) as an alternative of the Spearman-Karber method mainly for reasons of saving in time

consuming experimentation. Mainly as a result of the criticism by LEWIS, SCHMIDT (1957) added to his original procedure a method for estimating the precision of the D values. He suggested the following method to calculate the 95 percent confidence limits for D:

$$95\%\ C.L._D = \frac{L.D.50 \pm 95\%\ C.L.}{\log A + 0.16} \qquad (10)$$

where $95\%\ C.L. = 1.96 \times 2s/2N$

where $2s$ = the time difference in heat dosage between $P_{0.84}$ and $P_{0.16}$

N = the total number of tubes in the time groups showing partial survival.

Equation (10) was later modified by PFLUG (1962) to the point where N was taken as the total number of replicates between $P_{0.16}$ and $P_{0.84}$. Several workers compared the D values obtained by various methods of calculations. ESSELEN AND PFLUG (1956) compared the results obtained by the STUMBO, MURPHY AND COCHRANE method and the SCHMIDT method. The values obtained by these two methods were generally in good agreement. LEWIS (1956) compared D values obtained by the methods of STUMBO, MURPHY AND COCHRANE, Spearman-Karber and maximum likelihood did not find very substantial differences. Also ANDERSON (1959) compared D values calculated by the STUMBO method, the STUMBO, MURPHY AND COCHRANE method and the SCHMIDT method and the probit method.

3. Some Factors Affecting the Heat Resistance of Bacterial Spores

SCHMIDT (1957) classifies these factors into three general groups: inherent resistance, environmental influences active during the growth

and formation of the spores, and environmental influences active during the time of heating of the spores. The latter two factors being closely associated with this investigation will be discussed in the following.

a. Effect of the Sporulation Medium

ESTY AND MEYER (1922) reported that the type of sporulation medium used has a significant effect on the heat resistance of bacterial spores. Furthermore they observed some variation in the heat resistance of spores which were grown in different batches of identical media. It was indicated by the authors that this effect might be attributed to variations in the pH of the media at the end of the sporulation process. Recently, WHEATON AND PRATT (1961) found differences in heat resistance of spores of one strain of Putrefactive Anaerobe 3679 produced in four different media. The highest degree of heat resistance was found in those spores which were produced in beef heart infusion, the lowest in those grown in trypticase medium. The spores with an intermediate degree of heat resistance were those grown either in liver infusion or pork infusion.

Numerous efforts have been made in the past to determine the identity of those components in the sporulation medium that affect the heat resistance of the spores. SUGYIAMA (1951) found increased heat resistance of spores of Clostridium botulinum grown in casitone medium with increasing amounts of various fatty acids, increased degree of unsaturation of these fatty acids, and up to a certain concentration level, increasing amounts of iron, calcium and magnesium. The effect of the

calcium ions was especially apparent when the sporulation medium was deprived of its calcium ions. EL-BISI AND ORDAL (1956a) found a decrease in heat resistance as well as in the calcium content of spores of Bacillus coagulans var. thermoacidurans with increasing phosphate concentration in the sporulation medium. It was concluded from these experiments that the phosphate in the sporulation medium interfered with the availability of bivalent cations to the sporulating cells.

AMAHA AND ORDAL (1957) found that heat resistance of spores of Bacillus coagulans, var. thermoacidurans could be increased by the addition of calcium and manganese to the sporulation medium. The addition of magnesium was without any effect. SLEPECKY AND FOSTER (1958) demonstrated an increasing heat resistance of spores of Bacillus megaterium, when the sporulation medium was supplemented with calcium ions, but a decrease in heat resistance when increasing amounts of manganese and zinc ions were added to the sporulation medium. LEVINSON AND HYATT (1964) working with spores of Bacillus megaterium reported an increase in heat resistance of these spores when a liver-type sporulation medium was supplemented with calcium, and a decrease in heat resistance when the phosphate concentration of this medium was increased, or supplemented with either L-proline or L-glutamic acid. An increased heat resistance was the result of the addition of L-glutamic acid to a synthetic medium. The addition of manganese, L-alanine, L-leucine, L-valine or D-glucose did not appear to affect the heat resistance of the spores.

As a result of the above findings, efforts have been made to determine any possible relationship between the concentration of certain components of the spores that are a characteristic part of the spores, such as calcium and dipicolinic acid (DPA), and their heat resistance.

LECHOWICH AND ORDAL (1962) found an increase in heat resistance with increasing amounts of calcium, magnesium, manganese and DPA in the spores of Bacillus subtilis. This relationship however did not hold for spores of Bacillus coagulans. In this case the DPA content was lower in spores which exhibited a higher degree of heat resistance. CHURCH AND HALVORSON (1959) reported a decrease in heat resistance of spores of Bacillus cereus with decreasing amounts of DPA, whereas BYRNE, BURTON AND KOCH (1960) found the opposite to be true for spores of Clostridium roseum.

The heat resistance of bacterial spores appears not only to be dependent on the composition of the sporulation medium, but also on the temperature of sporulation. SUGIYAMA (1951) found highest heat resistance when spores of Clostridium botulinum were produced at 37°C. Both higher and lower sporulation temperatures resulted in decreased heat resistance.

WILLIAMS AND ROBERTSON (1954), EL-BISI AND ORDAL (1956b), ORDAL AND LECHOWICH (1958) and LECHOWICH AND ORDAL (1962) all reported an increased heat resistance with increasing temperatures of sporulation up to 37°C.

b. The Effect of the Initial Spore Concentration

Reports about this effect on the heat resistance of bacterial spores are rather conflicting. ESTY AND MEYER (1922) and WILLIAMS (1929) indicated that the increase in survival time with increasing spore concentration is somewhat different from that expected from the logarithmic order of death. AMAHA (1961) reported an increase in survival time with increasing initial spore concentration with spores of C. sporogenes, B. natto, and B. megaterium. He and his co-workers established the following relationship between the heat resistance of these spores and change in their initial spore concentration:

$$\log t = a + b \log N$$

where t is the survival time, N is the initial number of spores per sample, and a and b are constants depending on bacterial genus, species and strain, the temperature and the nature of the heating medium.

SISLER (1961) reported very little change in heat resistance of spores of B. subtilis CCC 5230 with changing spore concentration in moist heat. In superheated steam however, there was an increase in D values at the lowest spore concentration used.

c. Effect of the Medium Supporting the Spores During Heating

ESSELEN AND PFLUG (1956) reported that with spores of Putrefactive Anaerobe 3679 a lower heat resistance was found when the spores were heated in previously canned vegetable purees than when heated in purees which were prepared either from fresh or previously frozen vegetables. The highest resistance was found in those cases where

the spores were suspended in M/15 phosphate buffer during heating. REYNOLDS ET AL. (1952) also reported that the heat resistance of the same spores varies according to the medium in which they were suspended during heating. STUMBO, MURPHY AND COCHRANE (1950) reported highest heat resistance of spores of Putrefactive Anaerobe 3679 and Clostridium botulinum which were heated in a suspension of M/15 phosphate buffer, and lowest heat resistance when heated in distilled water. Heating of these spores in various vegetable and meat purees resulted in an intermediate heat resistance. ORDAL AND LECHOWICH (1958) showed that spores of Bacillus coagulans, var. thermoacidurans exhibited highest heat resistance when heated in M/40 phosphate buffer as compared to heating in phosphate buffers of higher or lower concentrations, M/100 glycylglycine, M/100 trihydroxymethylaminemethane buffer, M/100 ethylenediaminetetraacetic acid solution or distilled water. ANDERSON (1959) observed no significant difference in heat resistance of spores of Bacillus subtilis 5230 which were heated either in M/15 phosphate buffer as a free suspension or on filter paper strips, or dried from a M/15 phosphate buffer solution.

As already pointed out, the effect of the presence of other components in the spores suspension during heating on the heat resistance of the spores has been investigated. SUGYIAMA (1951) noticed that the heat resistance of spores of Clostridium botulinum Type A and Type B suspended in M/15 phosphate buffer could be considerably increased by the addition of increasing amounts of sucrose, up to a concentration of

50 percent. Supplementation of the suspension containing 0.15M sodium chloride with 0.001M calcium chloride or 0.001 M magnesium chloride greatly decreased the heat resistance of the spores. AMAHA (1961) reported that with Clostridium sporogenes the presence of sucrose or glycerol in concentrations ranging from 10 to 50 percent in the spore suspension did not affect the survivor pattern of the spores. The addition of starch also showed no effect. However, when serum albumen, ovalbumen or either two different types of laboratory peptones were added in concentrations of up to 0.5 percent, the heat resistance of the spores was significantly increased. This held true when the spore suspension was supplemented with yeast nucleic acid in concentrations of up to 1 percent, or heat coagulated serum albumen or ovalbumen. With reference to the dry heat resistance of spores, the only work reported in reference to the effect of the supporting medium during heating comes from BRUCH, KOESTERER AND BRUCH (1963) who observed that several species of bacilli and clostridia showed a higher heat resistance when heated in sand than when heated on a filter strip or on a glass wall.

d. The Effect of the Heating Medium

Most of the studies on heat resistance of bacterial spores were carried out using moist heat in the form of saturated steam or aqueous solutions as the heating medium. One of the first reports regarding the relatively high heat resistance of bacterial spores in dry heat stems from LEWITH (1890). In 1922, TANNER AND DACK mentioned the extremely high resistance of five strains of Clostridium botulinum

spores to dry heat. OAG (1940) studied the dry heat resistance of spores of Bacillus anthracis, Bacillus welchii, and Bacillus anthracoides. These spores were heated on glass slides in an electric oven. The temperatures applied ranged from 120°C to 400°C. He reported a sharp change in heat resistance of these spores at 160°C. Above this temperature, the spores were killed significantly more rapidly than at temperatures below 160°C.

The study of dry heat resistance of bacterial spores gained momentum with the advent of aseptic canning and space craft sterilization. COLLIER AND TOWNSEND (1956) studied the resistance of spores of Bacillus stearothermophilus, Bacillus polymyxa, and of Putrefactive Anaerobe 3679 to saturated and superheated steam. All spores showed considerably more resistance to superheated steam than to saturated steam. However, no correlation was found between the relative heat resistance of these spores in superheated steam and in saturated steam. PFLUG (1960) repeated these experiments with spores of Bacillus subtilis strain 5230 with essentially the same results. SISLER (1961) reported a threefold increase in the z value of spores of Bacillus subtilis strain 5230 in superheated steam as compared to the z value of saturated steam.

Similar results were reported by PFLUG AND AUGUSTIN (1961), and KOESTERER AND BRUCH (1962) who were subjecting spores to hot air instead of superheated steam. The latter further reported that no correlation existed between the relative heat resistance of various

genera of spores in dry and in moist heat. MURRELL AND SCOTT (1957) studied the heat resistance of spores of Bacillus stearothermophious, Bacillus megaterium, and Clostridium botulinum in heating media of various water activities (a_w). Contrary to all expectations, the heat resistance of all these spores was not found to be highest at an a_w of or near zero (absence of water, complete dryness), but at an a_w of approximately 0.8. By far the lowest degree of heat resistance was found to exist with spores heated in a suspension of M/15 phosphate buffer (a_w = 1.0). The authors concluded that dry heat resistance of bacterial spores under conditions of low water activities was largely dependent on the maintenance of some part of the spore contents in a relatively dry state.

JACOBS (1963) and JACOBS, NICHOLAS AND PFLUG (1964) studied the heat resistance of spores of Bacillus subtilis strain 5230-10 by subjecting them to steam of various water contents. They found that the decimal reduction time was a function of the water vapor content of the heating atmosphere within the temperature range studied. The slope of the thermal reducation time curve (1/z) was found to be lowest at 50 percent water vapor content, and nearly as high in a completely dry atmosphere. The respective values of z were 44 and 41°F. At 25 percent water vapor content of the atmosphere a z of 25°F was reported, and at a complete water vapor saturation of the atmosphere, z was found to be 17°F.

PHEIL, NICHOLAS AND PFLUG (1963) studied the effect of various gases used as heating media on the thermal resistance of Bacillus

subtilis strain 5230. All the gases used appeared to exert a similar effect on the spores. The slopes of all thermal resistance curves were identical. However, the decimal reduction times obtained increased in the order of helim, nitrogen, oxygen, air and carbon dioxide. The values obtained with the first three gases were almost identical, but clearly distinct from the last two gases.

e. The Effect of the Composition of the Subculture Medium

SUPFLE AND DENGLER (1916) reported that spores of Bacillus anthracis after being subjected to heating showed survival times of 4 minutes when subcultured in nutrient broth, and 30 minutes when subcultured in the same broth which had been supplemented with 3 percent glucose and 5 percent horse serum. MORRISON AND RETTGER (1930a, 1930b) reported similar findings. CURRAN AND EVANS (1937) found only minor differences in survival with different enrichments added to the nutrient agar with spores of Bacillus cohaerens, Bacillus subtilis and Bacillus albolactis that were not heated prior to counting. However, differences were found after the same spores had been subjected either to ultraviolet irradiation or moist heat. The conclusion was made from these studies, that spores become more exacting in their nutrient requirements following heating or irradiation. REYNOLDS ET AL. (1952) were able to increase the extent of recovery of spores of Putrefactive Anaerobe 3679 which had been subjected to moist heat in various low acid type foods such as meat and vegetable purees, if following the heat treatment, the mixtures were supplemented with certain enrichments.

Several media have been reported to give maximum counts for heated spore of clostridia. WHEATON AND PRATT (1961) made an extensive comparative study of several recovery media, infusion type, dehydrated and formulated media. The differences reported in counts of spores of Putrefactive Anaerobe 3679 which were heat treated were quite drastic. Fresh meat infusions generally yielded the highest survival counts. With the exception of yeast extract-starch-bicarbonate agar, as described by WYNNE, SCHMIEDING AND DAYE (1955), the formulated media generally resulted in somewhat lower counts. By far the lowest number of survivors were obtained with the so-called dehydrated media such as eugonagar.

Infusion-type media are time consuming to prepare. Therefore, FRANK AND CAMPBELL (1955), in an effort to find a formulated medium which would give comparable results of heated bacterial spores to infusion-type media screen tested a number of media. Spores of Putrefactive Anaerobe 3679 were used in these tests. The results revealed the highest number of survivors with Yesair's pork infusion which had been fortified with 0.05 percent sodium thioglycollate. Recovery was, however, almost as high with the yeast-extract-starch-sodium thioglycollate-sodium bicarbonate agar as described by WYNNE, SCHMIEDING AND DAYE (1955), if the last two components were omitted from the medium. Eugonagar with or without supplements, such as starch, malt extract or liver infusion resulted in significantly lower results. These results were more or less in accordance with those

reported by WHEATON AND PRATT (1961) with the exception that the latter reported an even higher count with beef infusion and pork-pea infusion agar.

AMAHA (1961) reports an increase in survival time with heat treated spores of Bacillus natto when glucose was added to nutrient broth. When other carbohydrates were added, he found only those sugars to be effective in extending the survival time that were also able to support good growth of the organism in a synthetic medium when added as the sole source of carbon. It was further demonstrated that the survival time of the spores in moist heat varied greatly with the addition or omission of certain vitamins and amino acids in the recovery medium.

ESTY AND MEYER (1922) reported that spores often remain dormant for considerable periods of time--up to 378 days--following a heat treatment. Since very few workers exposed their heat treated spores to such prolonged incubation times, it is often not known whether the failure of the spores to germinate and outgrow in a certain medium is actually the result of the absence of essential nutrients in the recovery medium or the presence of some compound which retards or inhibits germination and outgrowth. The study made by AMAHA (1961) indicates that the addition of glucose, certain amino acids and vitamins serve the purpose of satisfying the nutritional requirements of the spores. On the other hand, FOSTER AND WYNNE (1948) clearly demonstrated that the germination of spores of several species of clostridia was inhibited by the presence of unsaturated fatty acids such as oleic acid, linoleic and linolenic acids.

This inhibitory effect could be overcome by the addition of 0.1 percent starch to the recovery medium. The growth of the vegetative cells of these organisms was not inhibited by these acids. Similar studies were carried out with a number of strains of Clostridium botulinum by OLSON AND SCOTT (1950) and with a number of bacillus species by MURRELL, OLSON AND SCOTT (1950). They also demonstrated that the addition of starch, but also that of charcoal, or serum albumen, was efficient in overcoming inhibitory factors present in the recovery media. AMAHA AND ORDAL (1957), however, reported that the addition of starch to nutrient agar recovery medium failed to alter the survivor counts of heat treated spores of Bacillus coagulans, var. thermoacidurans.

MATERIALS AND METHODS

The experimental procedure employed in this investigation is designed to evaluate the differences in recovery in various subculture media of moist and dry heat treated spores of P. A. 3679 and to investigate the mechanisms involved in the thermal destruction of these spores.

1. Preparation of Spore Suspension

Spores of P. A. 3679 were prepared in two different sporulation media which in the past have been shown to produce spores of different moist heat resistance. The organism of known history was obtained from Dr. C. F. Schmidt of Continental Can Company in Chicago, Illinois.

a. Spores Grown in a Beef Heart Infusion Medium:

A large number of spores were grown in beef heart infusion according to a procedure described by WHEATON AND PRATT (1961) and WHEATON (1963). The medium was prepared as follows:

- Boil 15 lbs of cleaned, defatted, chopped beef heart from freshly slaughtered animals for one hour in 15 liters of distilled water.
- adjust pH to 7.4 with sodium hydroxide,
- filter material through cheese cloth and cotton,
- air dry solid meat particles, and make fluid up to 15 liters with distilled water,
- add 150 grams gelatin and 150 grams Bacto-tryptone,
- adjust pH to 8.5 with sodium hydroxide,
- add 75 grams isoelectric casein (DIFCO) and dissolve completely in broth, then add 4.5 grams glucose, 4.0 grams dipotassium phosphate and 45 grams sodium citrate,

- readjust pH to 7.2 with hydrochloric acid,

- dispense and autoclave for 30 minutes at 121°C,

- just prior to inoculation, exhaust medium for 20 to 30 minutes in free flowing steam and cool to 32°C.

Using this medium, spores of P.A. 3679 were prepared according to the following schedule:

- Transfer 1 ml of a heat shocked stock spore suspension into each of five 16 x 150 mm screw cap test tubes containing approximately a 1 cm deep layer of dried beef heart particles, a pinch of electrolytically reduced iron powder, and 15 ml of the beef heart extract. Stratify this mixture with a 1:1 mixture of mineral oil and paraffin, and incubate at 32°C until the tubes show good growth as indicated by gas formation.

- Transfer the entire content of each of these tubes into each of five 1" x 8" test tubes containing approximately a 1 cm deep layer of beef heart particles, a pinch of iron and 25 ml of beef heart extract, stratify and incubate as outlined above.

- Transfer the entire content of each of these tubes into each of five 500 ml Erlenmeyer flasks containing a small layer of dried beef heart particles, a pinch of iron and 400 ml of bee heart extract, and incubate at 32°C for a period of 24 hours.

- Transfer the entire contents of each of the above flasks into each of five 2000 ml Erlenmeyer flasks each containing a small layer of dried beef heart particles, 3 pinches of iron and 1500 ml of the beef heart extract. Incubate for approximately three weeks at 32°C.

b. Spores grown in Trypticase Medium:

This sporulation medium which was recommended by BROWN (1963) was prepared as follows:

- Dissolve 30.0 g trypticase (BBL), 1.0 g yeast extract (BBL) and 1.0 gram ammonium sulphate in 1000 ml distilled water,

- adjust pH to 7.3 with sodium hydroxide,

- dispense in 16 x 150 mm screw cap test tubes, 250 ml Erlenmeyer flasks and 2000 ml Erlenmeyer flasks and autoclave for 15, 20 and 30 minutes respectively at 121°C.

Using this medium, 8000 ml of spores of P.A. 3679 were prepared according to the following:

- Transfer 1 ml of a heat shocked stock spore suspension into each of four 16 x 150 mm screw cap test tubes containing 20 ml of the above medium, stratify with a 1:1 paraffin mineral oil mixture, and incubate at 30°C until good growth has developed as evidenced by gas formation.

- Transfer the contents of each of these tubes into each of four 250 ml Erlenmeyer flasks containing 200 ml of the above medium and incubate at 30°C for 12 hours.

- Transfer the entire contents of each of these flasks into each of four 2000 ml Erlenmeyer flasks containing 1700 ml of the above medium and incubate at 30°C until maximum sporulation is attained.

The time for the cultures to reach maximum sporulation was 96 hours.

c. Harvesting and Cleaning of Spores

The beef heart grown spores were harvested and cleaned according to the method described by TOWNSEND ET AL. (1956) as follows:

- Filter the entire sporulation mixture through a sterile layer of fiber glass with cheese cloth on both sides in order to remove the meat particles.

- Place the separated broth containing the spores into sterile 250 ml screw cap centrifuge bottles, and subject it to centrifugation in an International Centrifuge for 30 minutes at 1500 rpm.

- After the entire crude spore suspension has been subjected to this treatment, add some sterile water and some sterile glass beads to the precipitated spores, shake this mixture vigorously for several minutes and recentrifuge. Repeat this step several times until the supernatant appears relatively clear.

- Resuspend the spores in neutral phosphate buffer (equal amounts of M/15 KH_2PO_4 and M/15 K_2HPO_4), shake for several minutes and

centrifuge. Repeat this step three times, except do not centrifuge last time.

- Store the concentrated spore suspension in a screw cap bottle in a refrigerator at 40°F.

Microscopic examination of this spore suspension revealed the presence of debris as well as sporangia. In order to free the spores from their sporangia and to achieve further removal of the debris material the spore suspension was subjected to an enzymatic treatment that was described by GRECZ, ANELLIS AND SCHNEIDER (1963) and to further cleaning as follows:

- Prepare an aqueous M/15 phosphate buffer solution containing 80 mg lysozyme* and 40 mg trypsin*.
- Sterilize this solution by filtration,
- Add this solution to 200 ml of the spore suspension and incubate it for six hours at 45°C under constant stirring,
- Pass through a sterile glass filter, centrifuge and wash with M/15 phosphate buffer ten times,
- Store the cleaned spore suspension in a screw cap bottle in a refrigerator at 40°F.

Microscopic examination revealed complete removal of sporangia from the spore suspension, and also of most of the debris material.

The spore crop obtained from the trypticase-yeast extract medium was cleaned in the same manner with the omission of the initial glass fiber-cheese cloth filtration. Also in this case Na_2HPO_4 was used instead of K_2HPO_4 in the preparation of the phosphate buffer. This

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*California Corporation for Biochemical Research, Los Angeles 63, California.

appeared to facilitate drying of the diluted spore suspension. The cleaned spore suspensions were finally diluted to the desired concentration, and stored in screw cap bottles under refrigeration.

2. Media for Standardization and Recovery

a. Infusion type media:

- Beef Infusion: Preparation of infusion: Mix 500 g lean ground beef with 1000 ml distilled water in a Waring Blender, boil for 1 hour, cool and filter through 2 layers of cheese cloth.

Formulation:		
	beef infusion	1000 ml
	Bacto-peptone	5.0 g
	Bacto-tryptone	1.6 g
	soluble starch	1.0 g
	K_2HPO_4	1.25 g
	Final pH	7.4

- Pea Infusion: Preparation of infusion: Mix 100 g of frozen peas with 1000 ml distilled water in a Waring Blender, boil for 30 minutes, cool and filter through 2 layers of cheese cloth.

Formulation:		
	pea infusion	1000 ml
	Bacto-tryptone	10.0 g
	K_2HPO_4	1.0 g
	Final pH	7.4

- Liver Infusion: Preparation of infusion: Mix 500 g of fresh chopped beef liver with 1000 ml of distilled water in a Waring Blender, boil for 1 hour, cool and filter through 2 layers of cheese cloth.

Formulation:		
	liver infusion	1000 ml
	Bacto-peptone	10.0 g
	K_2HPO_4	1.0 g
	Final pH	7.4

b. Formulated Media:

- Yeast Extract:

Yeast Extract (BBL)	10.0 g
starch (BBL)	1.0 g
K_2HPO_4	2.0 g
distilled water	1000 ml
Final pH	7.2

- Trypticase:

Trypticase	50.0 g
K_2HPO_4	2.0 g
distilled water	1000 ml
Final pH	7.2

- Synthetic:

1. amino acides:

L-arginine	3.00 g
L-aspartic	0.45 g
L-cysteine	0.25 g
glycine	0.20 g
L-glutamic	0.50 g
L-histidine	0.20 g
DL-isoleucine	0.50 g
L-leucine	1.50 g
L-lysine, HCL	0.85 g
DL-methionine	0.60 g
DL-phenylalanine	2.00 g
L-proline	0.45 g
DL- threonine	1.00 g
L-tryptophane	0.10 g
L-tyrosine	0.40 g
DL -valine	2.00 g
DL-serine	0.25 g
DL-alanine	0.42 g

2. vitamins:

biotin	0.05 mg	riboflavin	1.00 mg
folic acid	0.20 mg	pyridoxal, HCl	0.50 mg
niacin	4.00 mg	thiamine, HCl	1.00 mg
DL-Ca-pan-thothenate	1.00 mg	p-aminobenzoic acid, K-salt	0.24 mg

3. mineral salts:

K_2HPO_4	1.00 g
KH_2PO_4	1.00 g
$MgSO_4x7H_2O$	40.0 mg
NaCl	2.0 mg
$FeSo_4x2H_2O$	2.0 mg
$MnSO_4xH_2O$	1.5 mg

4. distilled water 1000 ml

Final pH 7.2

c. Dehydrated Media:

- eugonbroth: eugonbroth (BBL) 30.0 g
  distilled water 1000 ml

  Final pH 7.2

  The 30 g eugonbroth contain:

Trypticase	15.0 g
Phytone	5.0 g
NaCl	4.0 g
cystine	0.2 g
Na_2SO_3	0.2 g
Na-citrate	1.0 g
dextrose	5.0 g

Media which were to be used to study the recovery of heat treated spores were filled into 16 x 125 mm disposable culture tubes in amounts of 8 ml per tube. After the tubes were filled and stoppered, they were sterilized according to the following procedure:

- infusion type media 25 minutes at 121°C
- formulated media 15 minutes at 121°C
- synthetic medium 12 minutes at 116°C

The eugonbroth medium, prior to being filled into the culture tubes, was boiled for 2 minutes, then filled into the tubes. The tubes were then stoppered, and the medium was sterilized for 15 minutes at 118°C. Immediately following sterilization, all media were cooled by immersion in cold water. Innoculation of the media with spores normally occured within less than 12 hours following the preparation of the media.

Immediately prior to innoculation, the following additions were aseptically made to each tube:

0.1 ml of a 10 percent sodium thioglycollate solution
0.1 ml of a 40 percent dextrose solution
0.2 ml of a 4 percent sodium bicarbonate solution

Dextrose was not added to eugonbroth media. While dextrose was sterilized by filtration, the other two compounds were sterilized by heat.

Following innoculation with the heat treated spores, the tubes were stratified with a 1:1 mixture of pre-sterilized paraffin-mineral oil. (See Figure 1)

Media which were used for the determination of initial spore counts were supplied with 15 g agar per 1000 ml of medium. The sterilization procedures for these media were the same as outlined above with the exception that the time of sterilization was prolonged for 5 minutes in this case. All media were sterilized in amounts of 300 ml in 500 ml Erlenmeyer flasks. Prior to being poured into Prickett tubes containing the spore suspension, each flask was supplied with 4 ml of a 10 percent sodium thioglycollate solution and 8 ml of a 4 percent sodium bicarbonate solution. Following solidification of the agar, a small overlayer of the basal medium was added to each tube.

The criteria for growth were colony formation in the case of agar counts, and gas formation and/or turbidity development with the broth type media used for end-point determinations.

All inoculated tubes were stored at 37°C. The storage times ranged from 24 to 48 hours for the agar samples, and two weeks after no more positive tubes developed, which amounted to approximately six weeks, for the samples containing the broth media.

FIGURE 1. Stratification with paraffin - mineral oil mixture

3. Enumeration of Initial Spore Count

Comparative spore counts were made to determine the initial spore concentration with each medium used as a subculturing medium. Also periodic determinations of the initial spore count were carried out throughout the period of experimentation using the yeast extract medium only. These periodic checks served to note any possible changes in initial spore concentration during storage.

Prior to innoculation, the spore samples were heated shocked in a boiling water bath for 8 minutes and cooled in ice water. Following this treatment, the samples were diluted and then pipetted into the Prickett tubes in such amounts as to yield a spore count of approximately 20 to 80 per tube. A 0.85 percent aqueous saline solution was used as the dilution menstruum.

The spores grown in trypticase-yeast extract germinated spontaneously in the various culture media. Therefore no heat shock treatment was applied to them prior to innoculation.

4. Preparation of the Spore Suspensions for Thermal Treatment

Sample cups (11 mm outside diameter by 8 mm deep and formed from tinplate 0.008 in. thick or from aluminum plate 0.02 in. thick) were first washed in butanone, then in absolute alcohol, dried, placed into petri dishes and sterilized at 320°F for 2 hours. The flask containing the standardized spore suspension was placed on a magnetic stirrer which kept the suspension constantly agitated. The sample cups were loaded with spores using a micro syringe burette which held 0.25 ml

calibrated in 0.0001 ml. The micro syringe burette was filled from the agitated flask containing the spore suspension. A volume of 0.01 ml of the spore suspension was measured into each cup. Twenty-five cups could be loaded each time the syringe was filled. The petri dishes containing the loaded cups were placed in a vacuum drying chamber and held there under reduced pressure for at least 24 hours at room temperature. The dried samples were stored in a desiccator at room temperature until used for the heat treatment. This storage time amounted to no longer than 48 hours.

5. Heat Treatment

The thermal resistance of the spores was determined by exposing the spore samples in the cups to different heat treatments either in the thermoresistometer, or open or enclosed in TDT cans placed in miniature retorts, depending on the type of heating medium the spores were to be exposed to as well as the time of exposure, and then subcultured in various media. The results, in numbers of positive and negative tubes at each time-temperature combination were used to calculate D values which in turn were used to plot the thermal resistance curves. D values were established on the basis of the recovery of the heat treated beef-heart-grown spores in all subculture media listed in paragraph 2 of this chapter. Identical studies were performed with the trypticase spores limiting however the number of subculture media to three, namely beef infusion broth, yeast extract, and eugonbroth.

a. Moist Heat:

Heat treatments lasting less than three minutes were carried out in the thermoresistometer described by PFLUG (1960). This apparatus is shown in Figure 2. It can be used for dry heat up to 380°F and for moist heat to 310°F, and insures immediate heating and cooling of the spore samples. The exposure chamber is connected to a steam reservoir where the temperature is accurately controlled by a thermocouple temperature sensing controller and an appropriate air operated pressure control valve. The temperature of the steam reservoir was checked by an industrial thermometer. In order to insure agreement in temperature between the thermoresistometer heating chamber and the steam reservoir periodic checks were made through thermocouples located at both locations.

The desired exposure time was set on two automatic timers,[1] and a Precision timer recorder[2] was used to measure the actual exposure time of the spore samples. Upon activation of the time controlling device, the cups were introduced into the steam chamber, at the end of the preset treatment period, the cups were released automatically from the chamber, and dropped into the subculture medium. Five cups were treated at one time.

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[1]Manufactured by Eagle Signal Corp., Moline, Ill. and Dimco-Gray Co., Dayton, Ohio.

[2]Manufactured by The Standard Electric Time Co., Springfield, Mass.

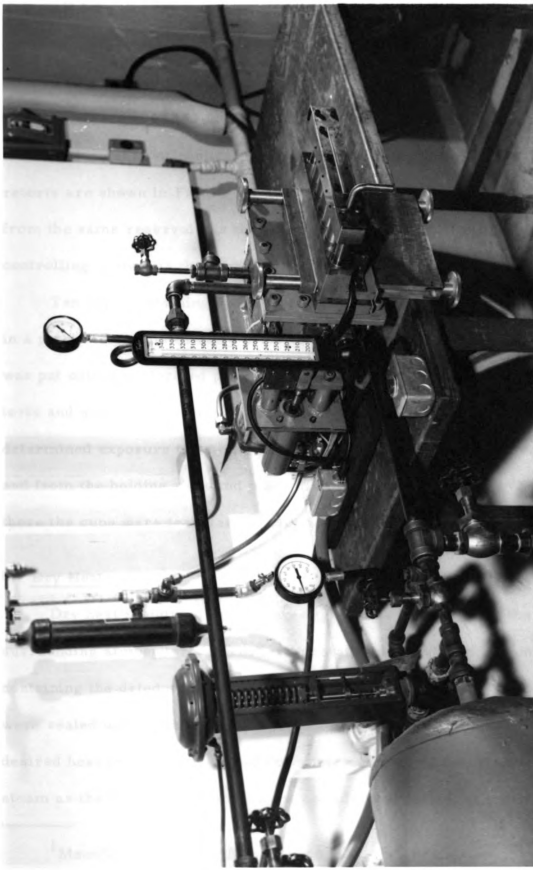

FIGURE 2. The thermoresistometer

All long time heat treatments (longer than 3 minutes) were carried out in miniature retorts. These heating devices which have been demonstrated to produce identical results to those obtained with the thermoresistometer by PFLUG AND AUGUSTIN (1961) were used because of the larger number of replicates that can be treated at one time. These retorts are shown in Figure 3. They are supplied with saturated steam from the same reservoir as the thermoresistometer. The temperature controlling system is the same as described for the thermoresistometer.

Ten cups containing spores were placed into a sterile open TDT can in a petri dish. Immediately prior to the heat treatment, the TDT can was put onto a perforated plate of a holding rack, put into one of the retorts and subjected to the desired heat treatment. At the end of the predetermined exposure time, the TDT can was removed from the retorts and from the holding rack and placed into a sterile petri dish. From there the cups were transferred aseptically into the subculture medium.

b. Dry Heat:

Dry heat is defined in this investigation as heated air of the normal surrounding atmosphere. In order to obtain this condition 10 metal cups containing the dried spores were placed into each TDT can. The cans were sealed with a Dixie Automatic Can Sealer, [1] and subjected to the desired heat treatment in one of the miniature retorts using saturated steam as the heating medium. Upon completion of the heat treatment,

[1] Manufactured by the Dixie Canner Co.

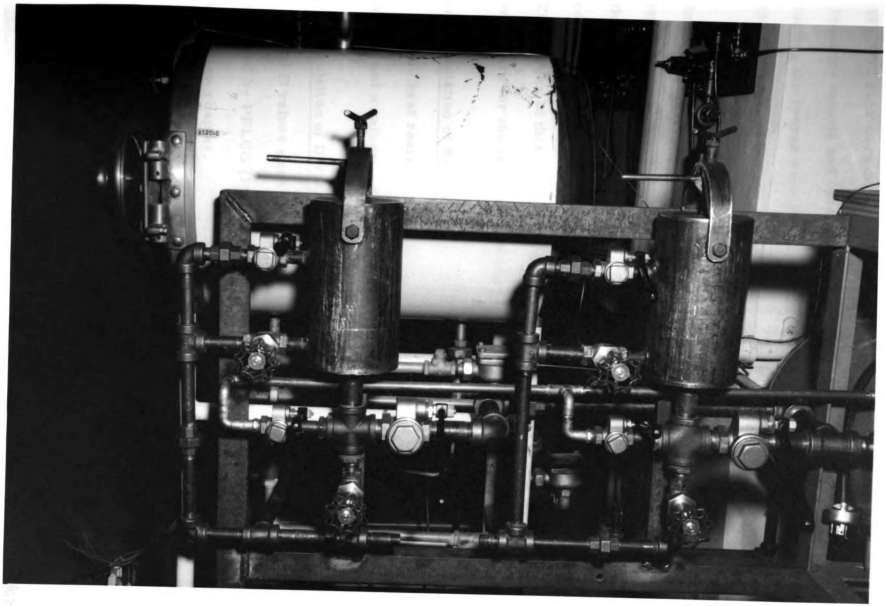

FIGURE 3. The miniature retorts

the TDT cans were cooled in running cold water in the retort, removed from the retorts and opened aseptically. Their content, the cups containing the spores were placed aseptically into the test tubes containing the recovery medium.

In this type of heat treatment, heating and cooling was not immediate. In order to evaluate the extent of the heating and cooling lag, heat penetration studies were made in which the time-temperature pattern inside the TDT cans was followed by a means of a copper constantant thermocouple inside the cans, and a Honeywell-Brown temperature recorder. The results of this study are shown in the Appendix and the calculations necessary for the lag corrections are outlined in paragraph 6c.

6. Calculation of Results

The heat resistance of the spores was evaluated by determining their D and z values, which were defined previously.

a. Calculation of D Values:

All D values were calculated using the modified Schmidt method described by PFLUG (1962). This method has been outlined in the literature review chapter.

Four different methods of calculations were used to determine D values from heat resistance data obtained with beef heart grown spores which were heated in moist and dry heat, and recovered in yeast extract medium. They were: the modified Schmidt method, the Stumbo, Murphy and Cochrane method, often called the unweighted average method, the

Spearman-Karber method and the loglog or maximum likelihood method. The first two methods have been outlined in the literature review chapter.

The Spearman-Karber method which has been described by LEWIS (1956) is outlined in the following: D is calculated from equation (6). Just as this was the case with the modified Schmidt method, N represents the number of surviving spores per tube corresponding to the mean sterility time, namely 0.69 organisms. The mean sterility time t is calculated as follows:

$$t = t_u + d/2 - d\, r_i/n_i$$

where t_u = the highest exposure time included in the calculation
d = the time interval between two exposure times
r_i = the number of sterile samples
n_i = the number of replicates.

The 95 percent confidence limits for D were computed as follows:

$$95\%\ C.L._D = (t \pm 1.96\ s)/(\log N_o - \log 0.69)$$

where $s = v\ 1/2$, and

$$V = d^2\, r_i(n_i - r_i)/(n_i^2 - 1)$$

The loglog method has been outlined in detail by FINNEY (1952) and LEWIS (1956). In this method the rate constant is calculated as k according to the following equation:

$$k = \frac{(\log_e No) \sum w_i - \sum w_i y_i}{\sum w_i t_i}$$

where t_i = the time of exposure
$y_i = Y_i - \eta_i$

w_i = the weighing coefficient obtained from Y_i

η_i = the working deviate obtained from Y_i

Y_i is obtained from the following equation:

$$Y_i = \log_e N_o - kt_i$$

w_i is obtained by interpolation from FINNEY"S appendix Table XVII.

η_i is obtained from either one of the following equations:

$$\eta_i = \eta_o + pA$$
$$\eta_i = \eta_1 - qA$$
$$\eta_i = q\eta_o - p\eta_1$$

η_o, η_1 and A are all obtained from the above Table. p and q refer to the fractions of negative and positive samples respectively.

This method consists of several successive cycles of computations which give estimates of k that near the theoretical maximum likelihood estimate asymptotically. In order to eliminate possibly several cycles of computations, k is first calculated by any of the other methods of calculating D. Then k is calculated from the equation D = 2.303/k. From this k, Y_i is calculated as described above. The entire cycle of computation is then carried out resulting in a new k value. This new k is used in the next cycle. The series of calculations is repeated until the k values are in agreement. D is then calculated according to the above equation.

b. Determination of z Values:

The z values were determined graphically by plotting the logarithms of the D values against the linear values of temperature. The z value is defined as the negative reciprocal slope of this line commonly expressed as the number of degrees Fahrenheit necessary for a tenfold change in D values.

c. Lag Correction:

The corrected heating time of the samples was obtained from the following equation:

$$t_c = t - (u) = t - t_m + t_e$$

where t_c = the corrected time of exposure of the samples
t = the time of exposure of the samples in the retort
t_m = the time required for the can interior to reach maximum temperature
t_e = the time equivalent of t_m at maximum temperature.
u = lag correction

The term t_e was determined according to the general method as outlined by BALL AND OLSON (1957). The characteristics of the heat penetration curves which were established on the basis of three determinations in each of two TDT cans with temperature measurements taken at five second intervals and are compiled for each temperature at which lag correction might be necessary in Table 1. The heat penetration curve for 320°F is shown in Figure 1A.

The destruction rates L for any value of T associated with a particular thermal reduction curve were calculated from the following equation:

$$L = \log^{-1}(T - T_1)/z$$

where T = selected temperatures corresponding to certain times on the heat penetration curve

T_1 = the retort temperature
z = the time required for the thermal resistance curve to traverse one logarithmic cycle.

L values were computed for T values which were taken from the heating curve at two seconds' intervals. The sum of these L represent t_e. U values are for each temperature and z values are compiled in Table 2. A sample calculation is shown in Table 1A.

Lag corrections were only included in the calculation of the D values if the former amounted to less than 1 percent of the L. D. 50 values. It was found that such corrections were only necessary in a few cases at temperatures of 315°F and higher.

TABLE 1. Heat penetration data for cups in TDT cans heated in steam in miniature retort.*

T_1 °F	T_o °F	j	f_1 sec.	t_p sec.	f_2 sec.
285	67	1.0	6.04(3.3-13.5)[a]	7.9	22.2
300	65	1.0	4.3 (3.5-5.3)	6.0	21.7
315	65	1.0	4.25(3.6-4.6)	5.7	23.6
320	66	1.0	4.2 (3.7-4.8)	6.4	25.2

T_1: retort temperature, °F
T_o: initial temperature in TDT can
j : Lag factor = $(T_1 - T_A)/(T_1 - T_o)$
f_1 : slope of the first straight line of the heating curve, expressed in seconds required for this line to traverse one logarithmic cycle
f_2 : slope of the second straight line of the heating curve expressed as for f_1
t_p : Time point of intersection of the two straight lines of the heating curve in seconds
* data are average of 6 runs
[a] range is listed after each average ratio

TABLE 2. Lag correction factors

z°F	U, min.			
	285°F	300°F	315°F	320°F
33	--	--	0.18	0.17
36	0.19	0.15	0.17	0.17
39	--	--	0.15	0.16

RESULTS

1. Preliminary Studies

Two types of cup materials were available as containers for spores during heat treatment: aluminum and tinplate. In anticipation of possible effects of corrosion products derived from these materials during drying of the spore suspension and during the heat treatment and as well, and probably above all during incubation in the subculture medium, a preliminary comparative study was undertaken with both kinds of cups.

The results, as shown in Table 3, reveal considerably higher D values with tinplate cups as compared to aluminum cups regardless of the type of heating medium. The reason for this difference is not known. However, since both yeast extract medium and pea infusion contain sufficient iron to satisfy the nutritional requirements of the germinating and outgrowing spores, the supply of iron-type corrosion products from the tinplate cups appear hardly to be the reason for this difference in D values. Rather, it seems that some inhibitory factor associated with the aluminum cups might account for the low D values obtained with these cups. Tinplate cups were used throughout the remaining experiments.

When the synthetic medium was first used for the subculturing of heated spores, zero survival was obtained. As a result of this a comparative study was undertaken using two compounds which were known to stimulate the germination and possibly the outgrowth of spores: Ethylene-diaminotetraacetic acid (EDTA) and sodium bicarbonate. The

TABLE 3. The effect of the type of cup used as a container for spores during heat treatment

Heating Medium	Temperature °F	D values	
		Tinplate cups	Aluminum cups
Dry Heat	255	37.5 minutes	30.1 minutes
Moist Heat	250	88.0 seconds	50.0 seconds

Recovery media: Dry Heat : Yeast Extract
Moist Heat : Pea Infusion

Spore suspension: Dry Heat : Beef heart spores, crude suspension
Moist Heat : Beef heart spores, clean suspension

results of these experiments in which the addition of minerals was included as another variable are compiled in Table 4. It is obvious from these data, that only sodium bicarbonate enhanced the recovery of the spores following the heat treatment. EDTA had no effect whatsoever at the concentration in which it was used. However, RIEMANN (1963) and BROWN (1956) noted that the germinating effect of EDTA is lost, if it is added to the medium in too high concentrations. In such a case, EDTA not only removes metals inhibitory to germination but possibly also some of the essential minerals. This possibility might be without any effect on germination, but could seriously limit outgrowth. That EDTA is acting as an inhibitor is indicated by the fact that recovery is somewhat reduced in the case where EDTA is added to the medium containing sodium bicarbonate with or without minerals.

A somewhat more extensive experiment was carried out comparing the effect of sodium bicarbonate on the recovery of spores in the synthetic medium following both moist and dry heat treatments. The results which are shown in Table 5 confirm the above findings, and those reported by WYNNE, SCHMIEDING AND DAYE (1955) and RIEMANN (1963) with reference to P.A. 3679. WYNNE AND FOSTER (1948) reported carbon dioxide only to have a favorable germinating effect on spores of Clostridium botulinum but not on P.A. 3679. From these findings, it appears that the germination-stimulatory effect of sodium bicarbonate varies not only with the organism, but also with the type of culturing medium used. Since sodium bicarbonate has never been reported to have

TABLE 4. Preliminary studies of the effect of several additions to the synthetic medium on the recovery of spores of P.A. 3679 following dry heat treatment at 300°F.

Recovery Media	No. of Replicates	No. of Tubes Showing Growth	
		5 minutes	15 minutes
Synthetic w/o minerals	5	0	0
Synthetic with minerals	5	0	0
Synthetic w/o minerals, with $NaHCO_3$	5	5+	2+
Synthetic with minerals and $NaHCO_3$	5	5+	5+
Synthetic w/o minerals, with EDTA	5	0	0
Synthetic with minerals and EDTA	5	0	0
Synthetic w/o minerals, with $NaHCO_3$ and EDTA	5	5+	1+
Synthetic with minerals, $NaHCO_3$ and EDTA	5	5+	4+

-minerals refer to all salts added to the medium as outlined in the experimental part of this thesis with the exception of phosphates which were added in all cases
-concentration of $NaHCO_3$: 0.5 percent
-concentration of EDTA : 10 M

TABLE 5. The effect of the addition of $NaHCO_3$ to the synthetic medium on the recovery of beef heart spores following their heat treatment

Heating Medium	Temperature °F	D values	
		Control	+ 0.5% $NaHCO_3$
Moist Heat	260	7.5 sec.	9.50 sec.
	275	1.4 sec.	1.60 sec.
Dry Heat	315	1.39 min.	2.12 min.

any inhibitory effect on either germination or outgrowth of spores, it was added to all the subculturing media used in this work.

2. Resistance of Spores to Saturated Steam

A summary of the D_{250} values and the z values as well as their corresponding energies of inactivation of beef heart grown spores is given in Table 6. All D values obtained with these spores at various temperatures are compiled in Table 7. The thermal resistance curves are shown in Figures 4 through 10.

The z values, and therefore also the energies of inactivation are the same for all media, namely 19.5°F and 64,700 calories with the exception of eugonbroth with a z of 17.5°F and an E of 70,500 calories, and trypticase with a z of 18.2°F and an E of 68,200 calories. Due to the differences in z values, the relative magnitude of the D values obtained with the last three subculturing media changes with increasing temperatures. At 280°F the D values decrease in the following order: synthetic medium, trypticase, eugonbroth. This points out clearly that comparative heat resistance studies are of little value unless D values are established at least at two different preferably extreme temperatures.

At 230°F the D values obtained with the various subculture media can be grouped into four classes of decreasing distinctly different magnitudes: The highest D values are obtained with either beef infusion or pea infusion. The second class includes liver infusion yeast extract and eugonbroth. To the third and fourth class belong trypticase and the synthetic medium respectively. At 275°F again beef infusion gives highest

TABLE 6. D_{250} and z values and corresponding heats of inactivation for moist heat resistance as shown in figures

Recovery Medium	Beef Heart Spores				Trypticase Spores			
	D_{250}, min	z, °F	E*	S*	D_{250}, sec	z°F	E*	5*
Beef Infusion	1.4	19.5	64,700	99	0.35	16.0	82,100	144
Liver Infusion	0.9	19.5	64,700	96	--	--	--	--
Pea Infusion	1..35	19.5	64,700	76	--	--	--	--
Yeast Extract	1.2	19.5	64,700	96	0.35	17.0	76,200	130
Trypticase	0.52	18.2	68,200	109	--	--	--	--
Eugonbroth	0.71	17.5	70,500	114.5	0.18	15.5	83,200	146
Synthetic	0.48	19.5	64,700	98	--	--	--	--

*see TABLE 8
Value will be changed to number

TABLE 7. D values and their corresponding 95 percent confidence limits in minutes for moist heat resistance of beef-heart infusion spores and various recovery media

Temp. °F	Beef Infusion	Liver Infusion	Pea Infusion	Yeast Extract	Trypticase	Eugonbroth	Synthetic
230	13.13 12.21-14.04	10.1 10.23 9.71-10.76	13.78 13.27-14.29 13.3 12.92-14.00	11.8 10.5-13.1	6.60 6.20-7.02	9.05 -- 9.78 9.48-10.09	4.11 3.04-5.17 4.92 4.63-5.20
250	1.38 1.31-1.43 1.32 1.29-1.36 1.47 1.43-1.51	.935 -- .747 .60-.898 -- --	1.60 1.47-1.73 -- -- -- --	.86 -- 1.09 1.00-1.18 1.24 1.21-1.27	.497 -- .437 -- .550 .517-.583	.797 -- .693 .641-.775 -- --	.432 .415-.448 .446 .405-.487 .512 --
260	.468 .416-.522	.273 .260-.286	--	.334 .313-.355	.132 .120-.144	.164 .134-.194	.158 .148-.168
270	--	--	.114 -- .117 .107-.127	.108 .105-.117 --	-- --	.0533 .0515-.057 	-- --
275		.0492 .0465-.0517	--	--	.0253 .0227-.0280	--	.0267 .0238-.0295
280	.0403 .0373-.0432	--	.0383 .0357-.0400 .0322 .0290-.0353	.0333 .0313-.355 .0335 .0322-.0350	-- --	-- --	-- --

TABLE 8. D values and their corresponding 95 percent confidence limits in minutes for moist heat resistance of trypticase-yeast extract spores and various recovery media

Temp. °F	Beef Infusion	Yeast Extract	Eugonbroth
230	6.74 6.26-7.21 --	5.27 -- 5.12 4.75-5.37	3.77 3.50-4.05 --
240	--	--	0.841 0.809-0.878
250	0.329 0.298-0.361	0.337 0.313-0.360	0.166 0.157-0.175
260	0.0805 0.0748-0.0863	0.0792 0.0690-0.0893	0.0378 0.0347-0.041
270	0.0273 0.0259-0.0290	0.0225 0.0707-0.0245	--

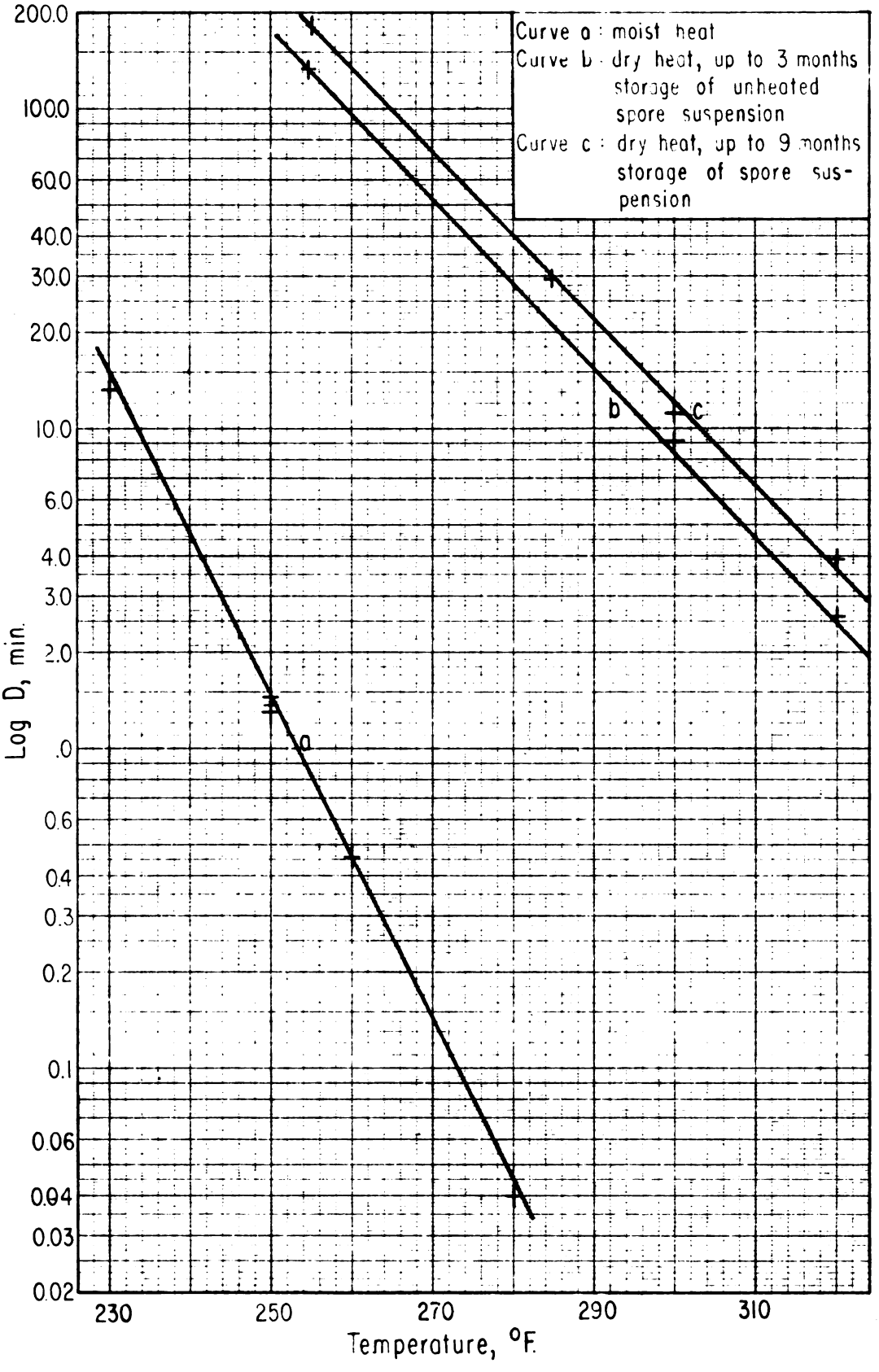

FIGURE 4. Thermal resistance curves of beef-heart infusion spores subcultured in beef infusion

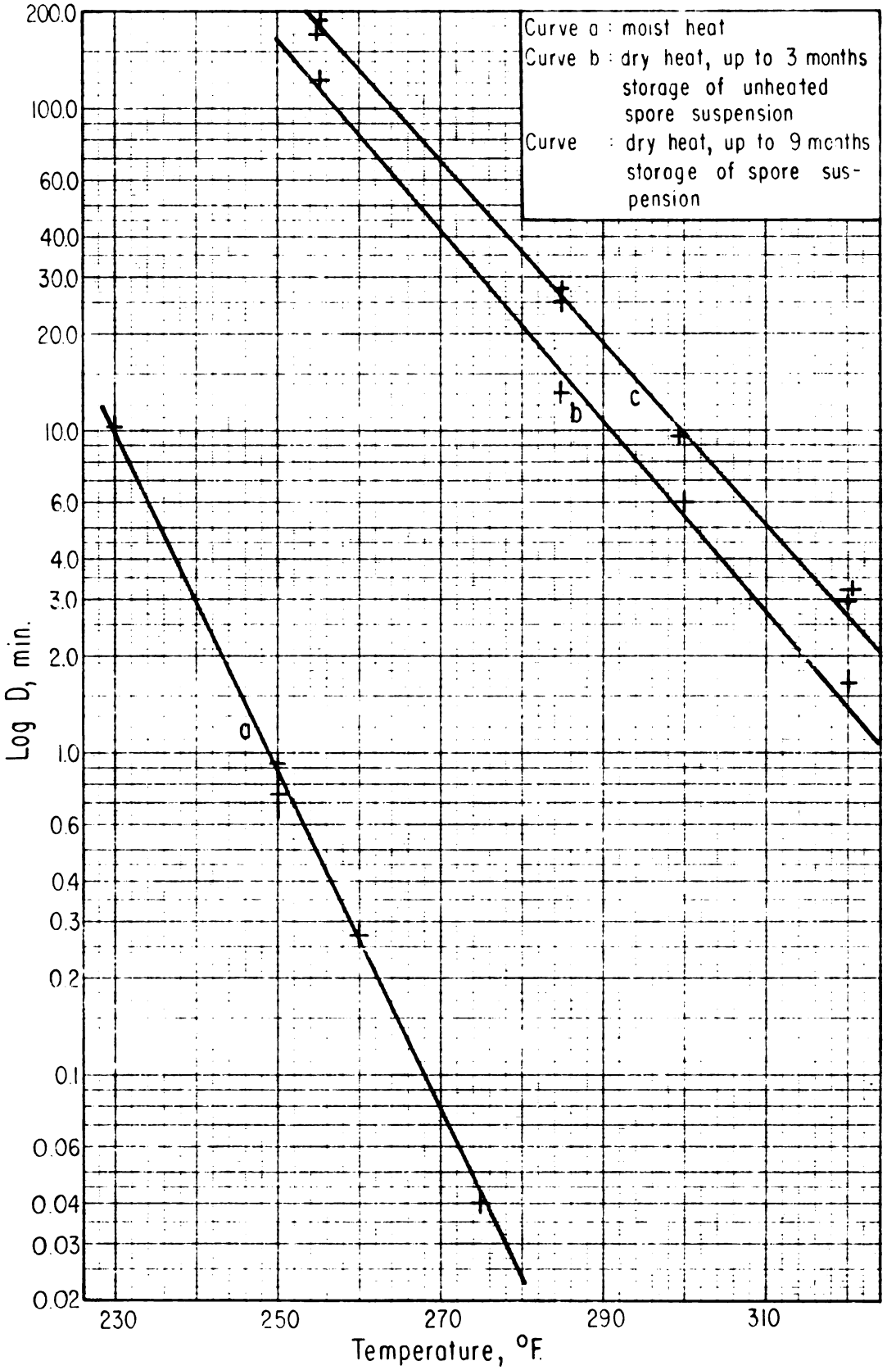

FIGURE 5. Thermal resistance curves of beef-heart infusion spores subcultured in liver infusion

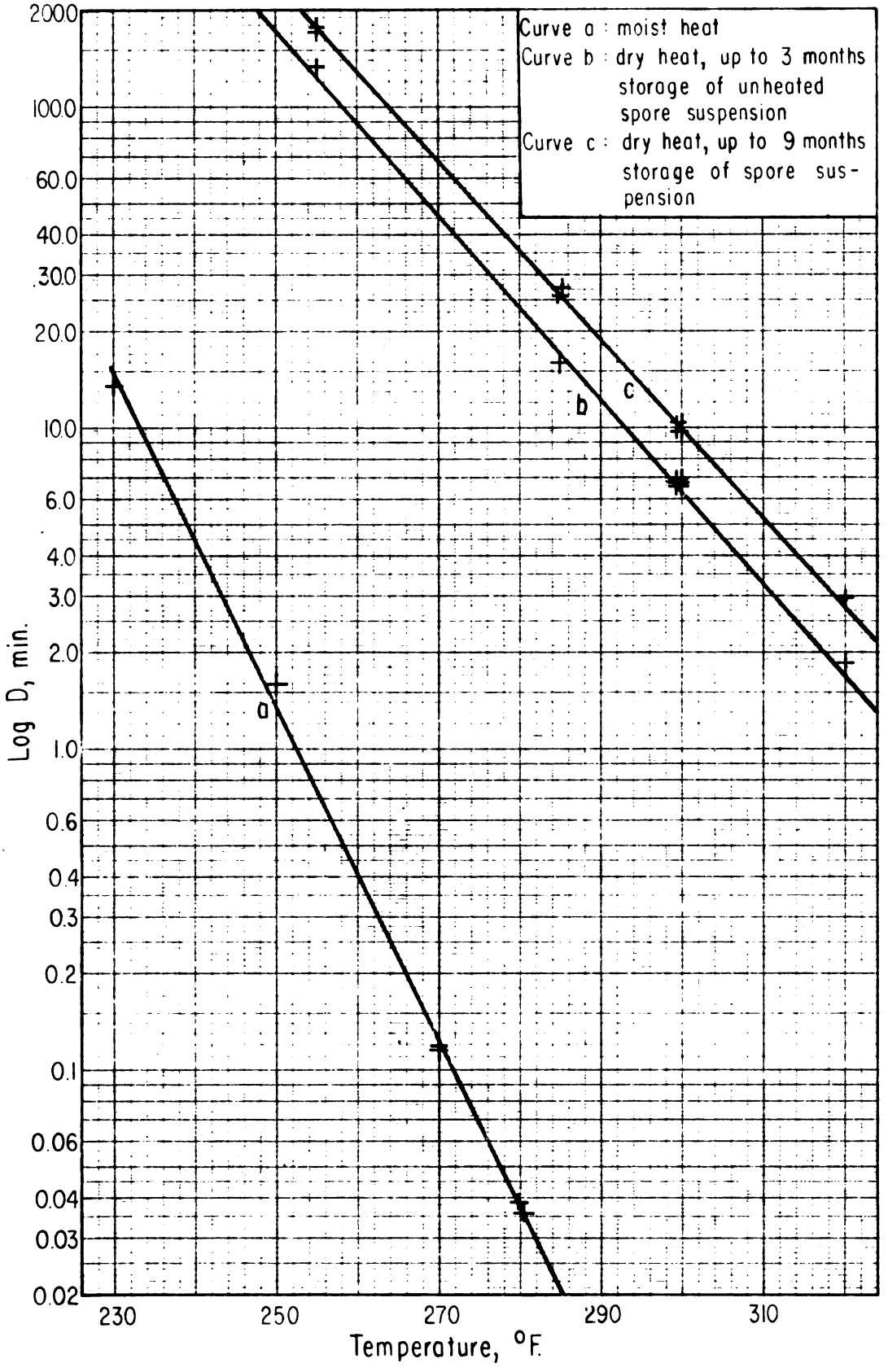

FIGURE 6. Thermal resistance curves of beef-heart infusion spores subcultured in pea infusion

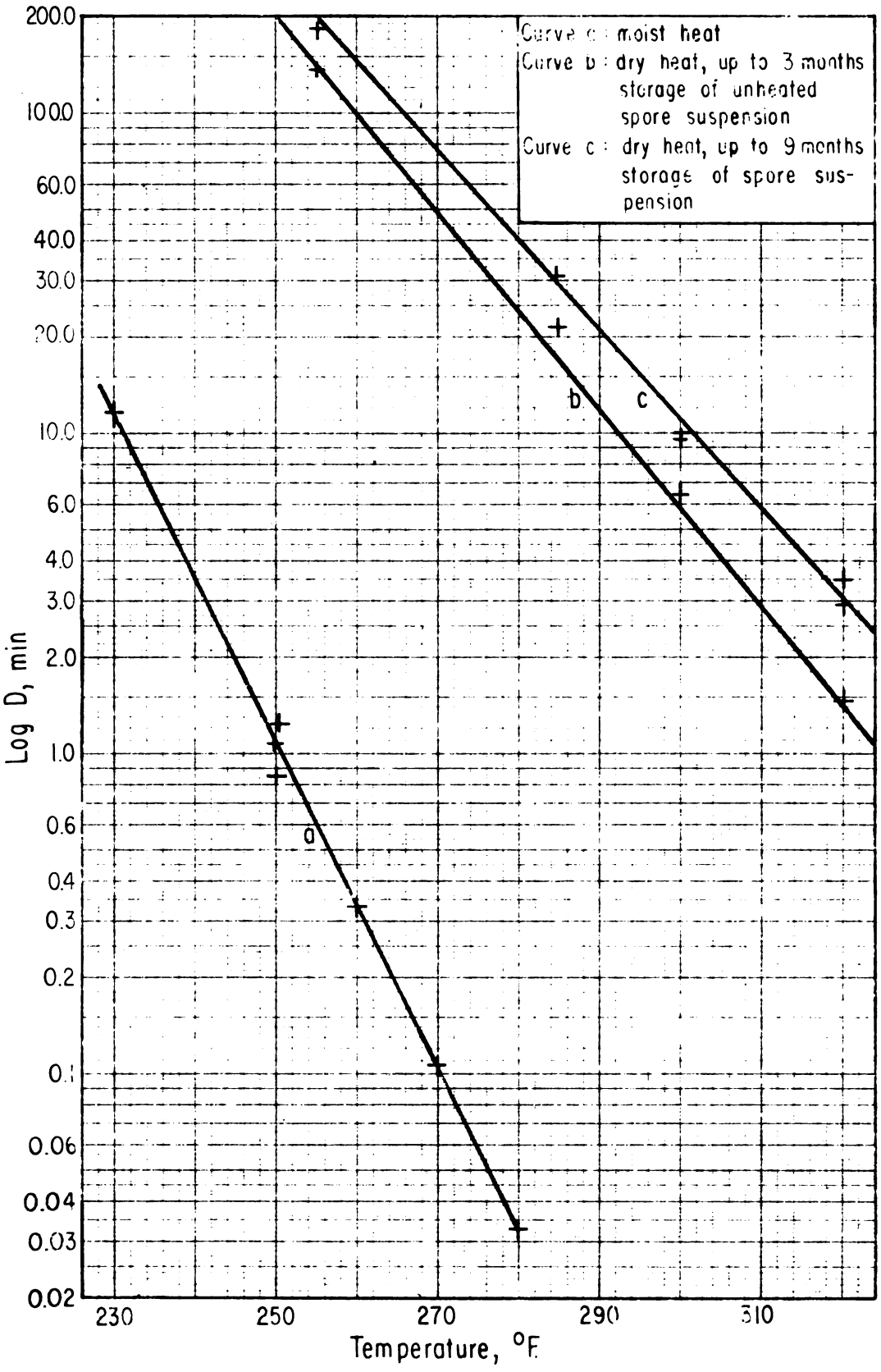

FIGURE 7. Thermal resistance curves of beef-heart infusion spores subcultured in yeast extract

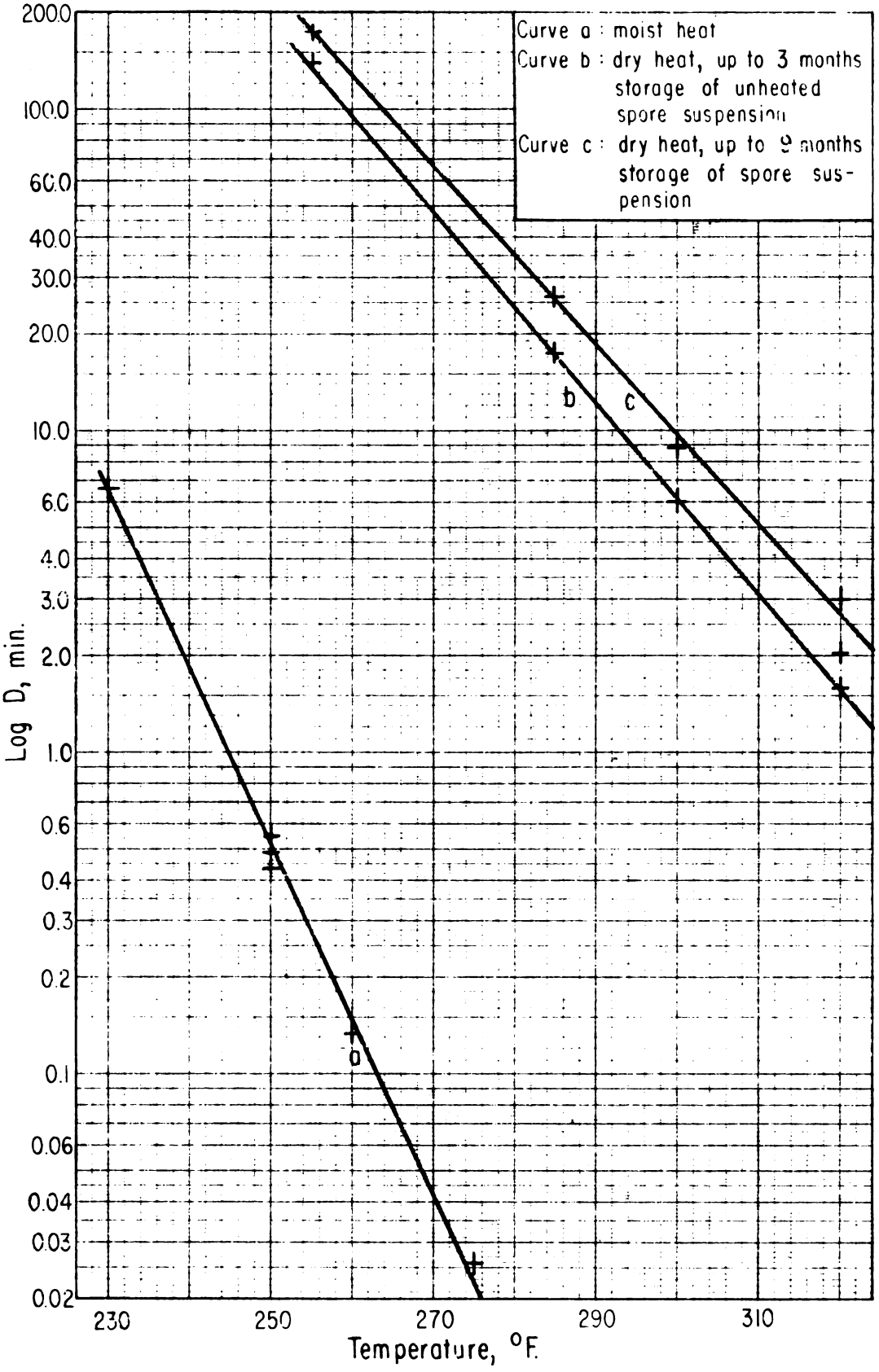

FIGURE 8. Thermal resistance curves of beef-heart infusion spores subcultured in trypticase

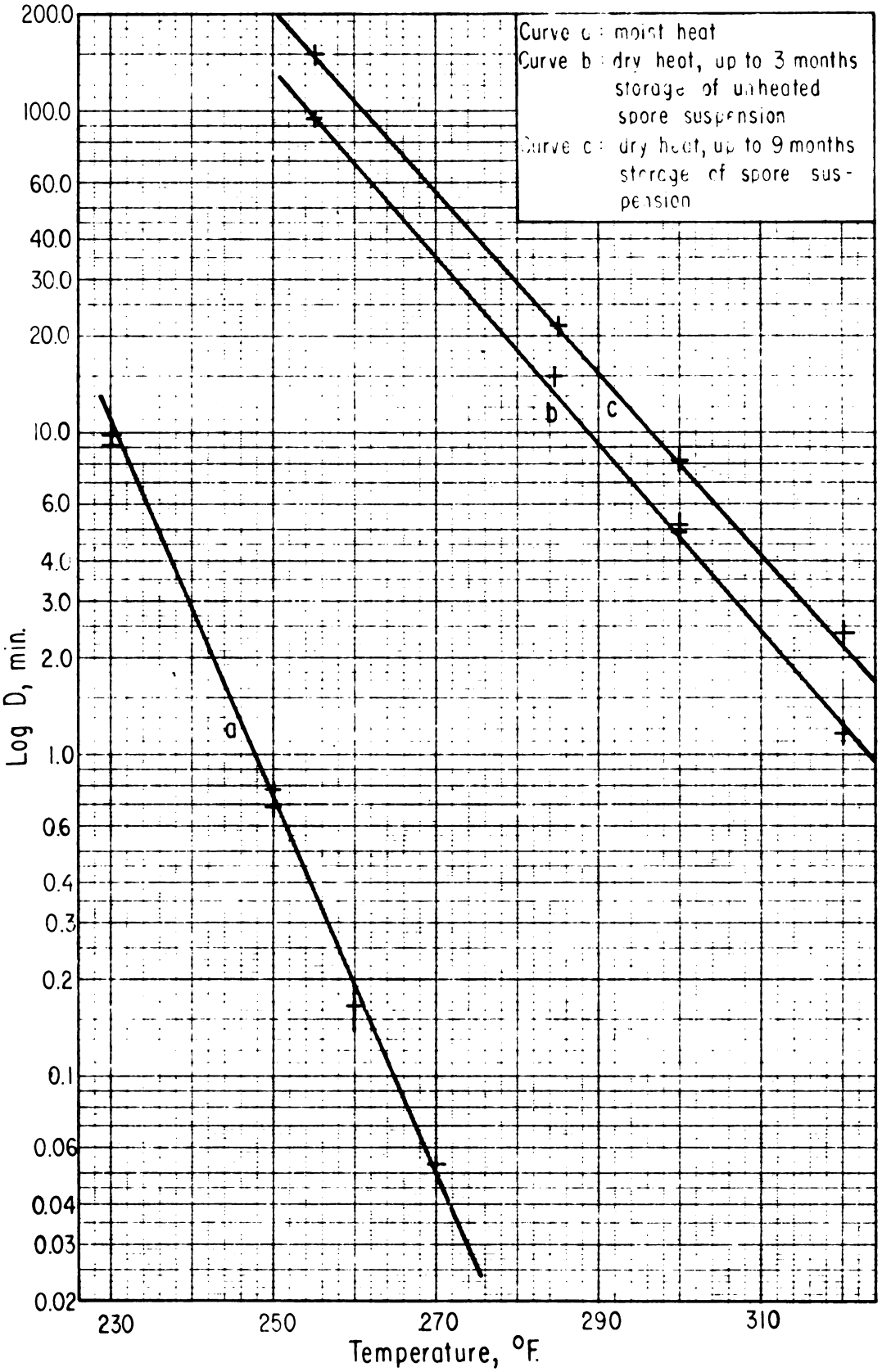

FIGURE 9. Thermal resistance curves of beef-heart infusion spores subcultured in eugonbroth

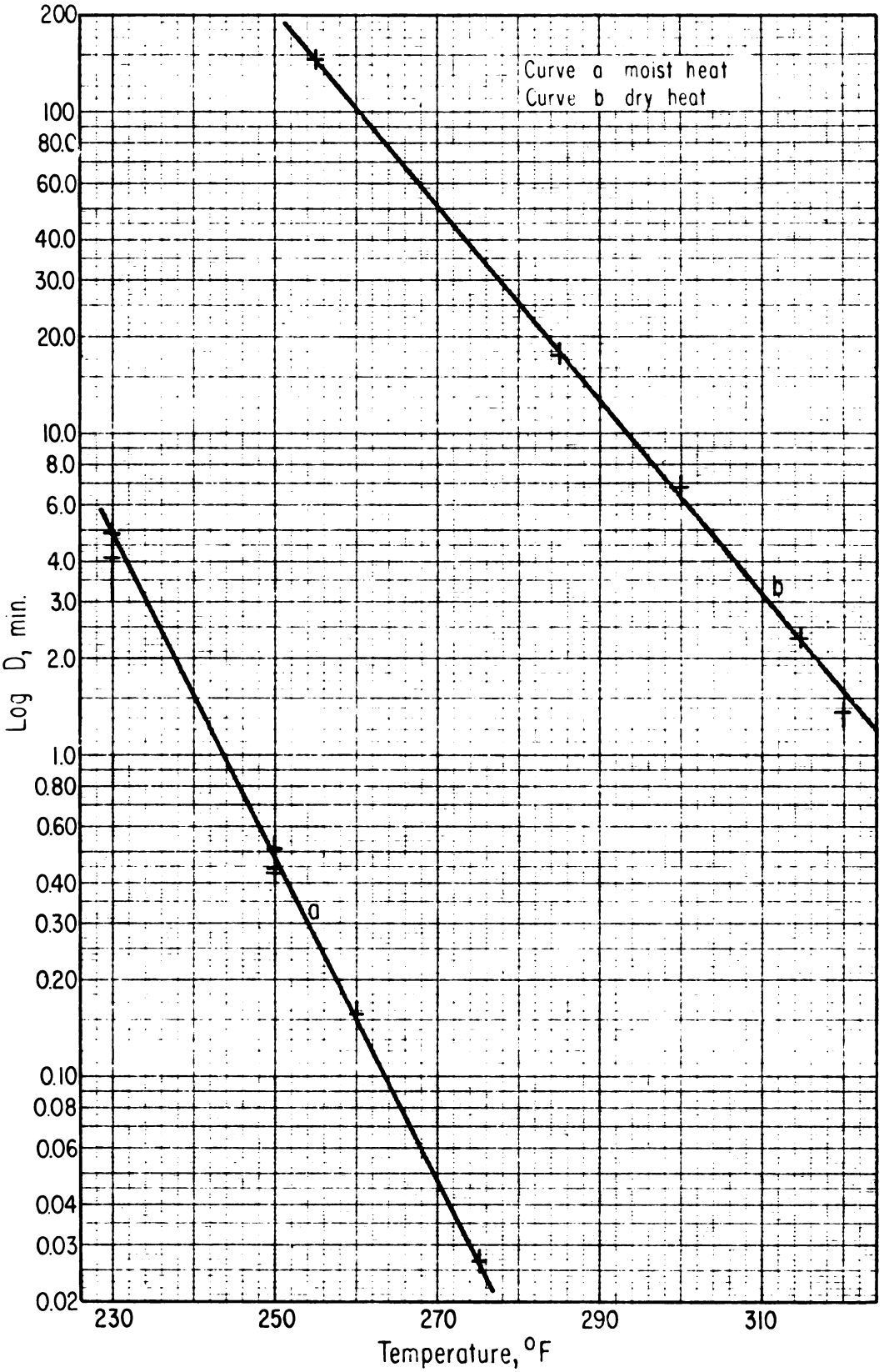

FIGURE 10. Thermal resistance curves of beef-heart infusion spores subcultured in synthetic medium

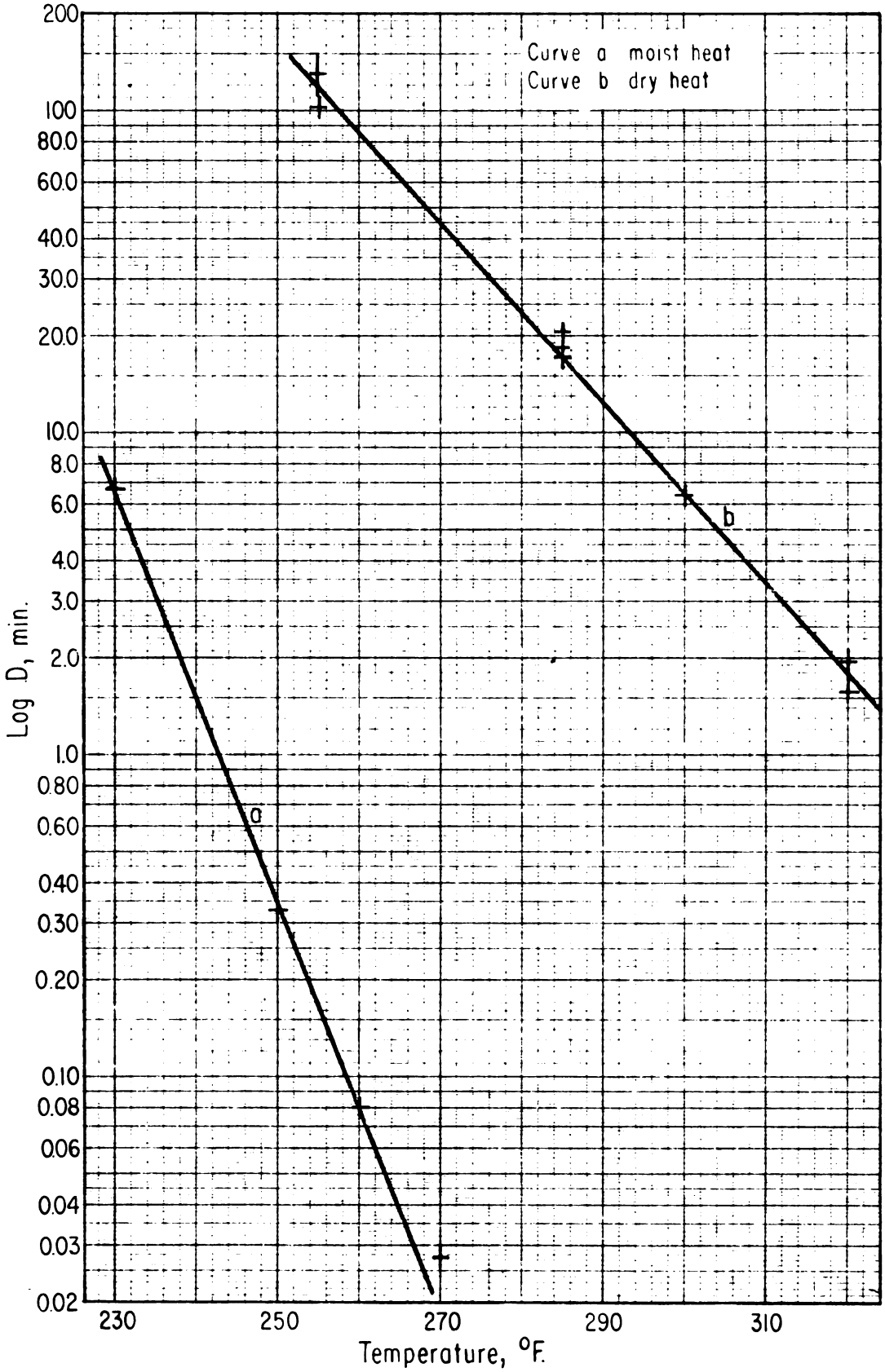

FIGURE 11. Thermal resistance curves of trypticase-yeast extract spores subcultured in beef infusion

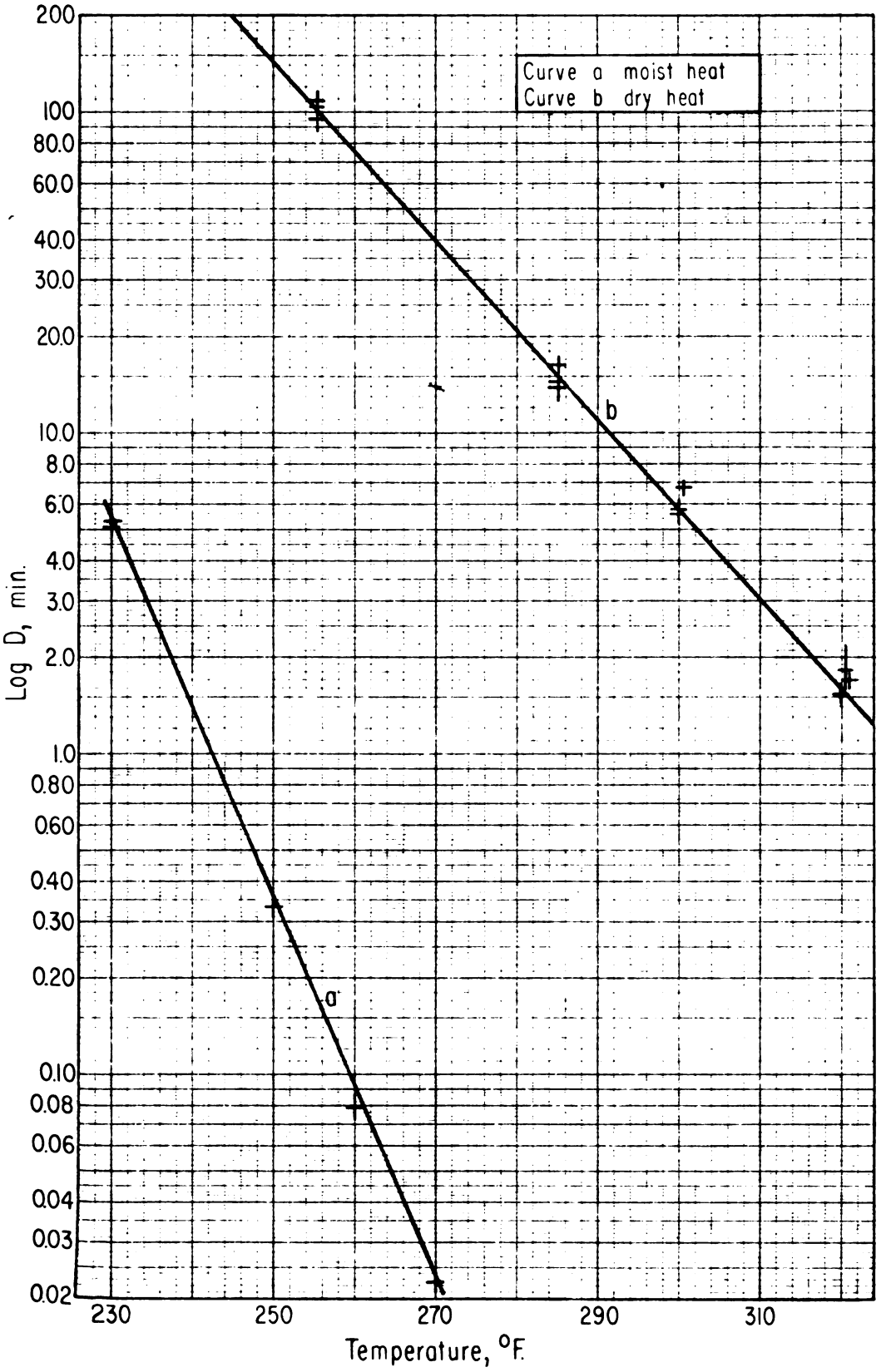

FIGURE 12. Thermal resistance curves of trypticase-yeast extract spores subcultured in yeast extract

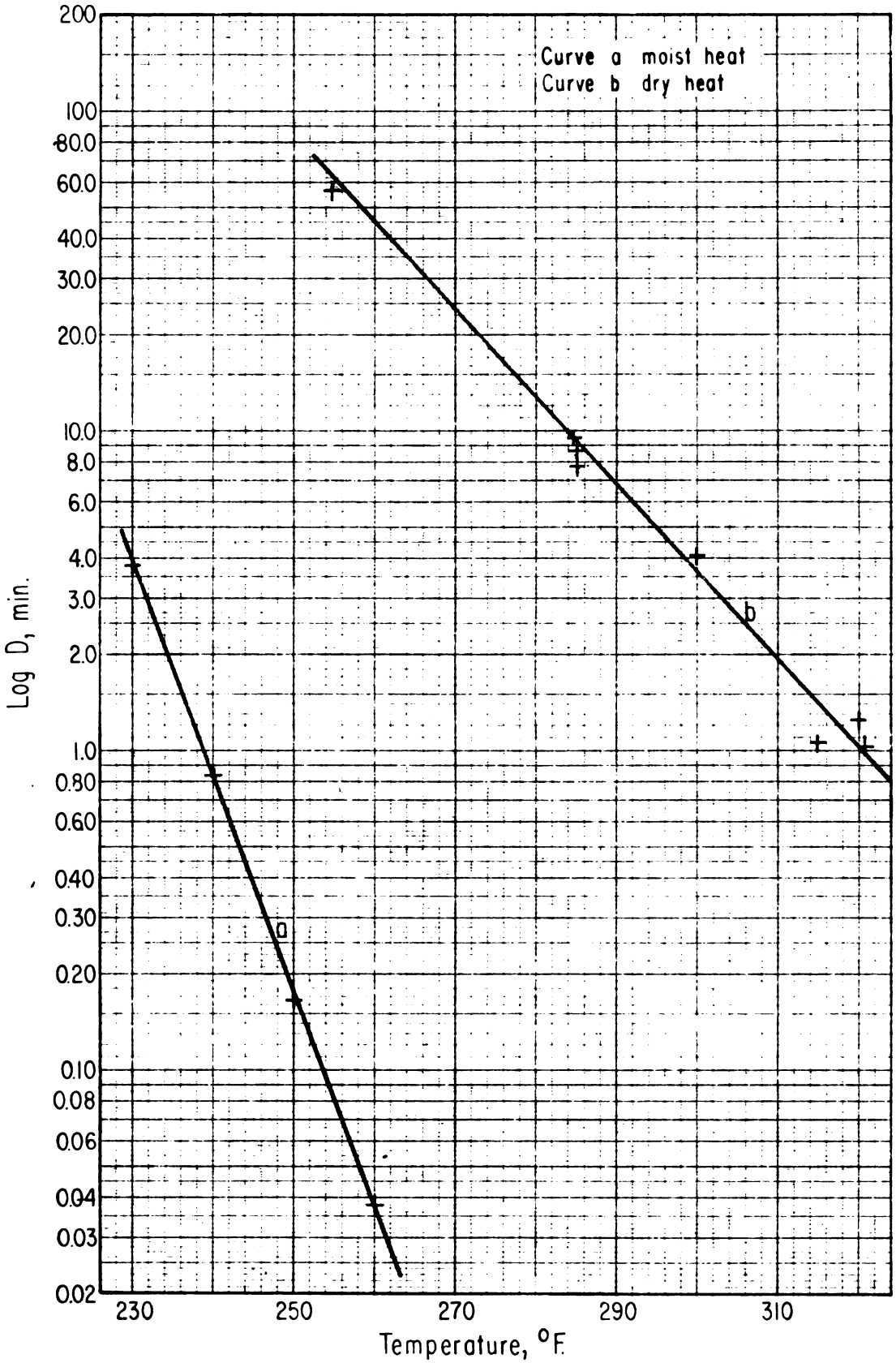

FIGURE 13. Thermal resistance curves of trypticase-yeast extract spores subcultured in eugonbroth

D values. Distinctly different and decreasing D values in the following order were obtained with pea infusion, yeast extract and liver infusion. The lowest D values that were all of the same magnitude were found when either trypticase, eugonbroth or the synthetic medium were used for subculturing. The fact that the D values obtained with the infusion type media and yeast extract are changed in their relative magnitude at the two temperatures is explained as being due to very slight differences in their z values.

The spores grown in trypticase yeast extract are of considerably lower heat resistance. As shown in Tables 6 and 8 and Figures 11 to 13, their D_{250} values decrease with the recovery medium in the following order: beef infusion, yeast extract, eugonbroth. The z values vary from 17.0°F for yeast extract to 16.0°F for beef infusion and to 15.5°F for eugonbroth.

The results obtained in general are in agreement with those reported for P.A. 3679 in the literature: infusion type media generally yield a higher recovery than formulated media with the exception of yeast extract which in turn gives a better recovery than the dehydrated media. FRANK AND CAMPBELL (1955) reported contrary to the findings of WYNNE, SCHMIEDING AND DAYE (1955) a higher recovery of spores of P.A. 3679 with yeast extract starch agar than with yeast extract starch bicarbonate agar. This discrepancy is believed to stem from the fact that the former authors used the same media for both initial and survivor counts, while FRANK AND CAMPBELL derived their initial counts

only from one medium namely eugonagar, regardless of the type of medium used to count the survivors. As shown in Table 9, initial counts vary considerably with the type of medium used. It is therefore quite conceivable, that if FRANK AND CAMPBELL had used the same media for both the initial and survivor counts, their results would have been in agreement with those reported by WYNNE, SCHMIEDING AND DAYE. As far as the use of eugonagar is concerned, FRANK AND CAMPBELL reported lowest D values when compared to any of the other media tested. Even supplementation of eugonagar with soluble starch, malt extract, yeast extract or liver infusion resulted only in a very slight increase in the D value.

WHEATON AND PRATT (1961) obtained similar results. By far the best recovery was obtained with pork pea infusion as the recovery medium. Beef infusion gave somewhat lower results, as did yeast extract. Eugonagar again gave the lowest recovery. Yeast extract however resulted in a higher number of survivors than liver infusion.

With reference to the relative heat resistance of beef heart grown spores and trypticase yeast extract grown spores the results obtained are also in agreement with those of WHEATON AND PRATT (1961). As pointed out earlier, D values in some cases have been reported to be affected by the initial spore concentration. However, according to the reports cited earlier, ten or one hundred fold differences in initial spore concentration result in relatively small changes in D values. It is therefore concluded that the small differences in the initial spore

TABLE 9. Comparative initial counts in various recovery media

Recovery Medium	Beef Heart Spores	Trypticase Spores
Beef Infusion	4.35×10^4/cup	11.0×10^3/cup
Liver infusion	3.93×10^4/cup	---
Pea Infusion	3.94×10^4/cup	---
Yeast Extract	4.29×10^4/cup	11.3×10^3/cup
Trypticase	3.10×10^4/cup	---
Eugonbroth	2.98×10^4/cup	9.46×10^3/cup
Synthetic	2.54×10^4/cup	

concentrations between the two types of spores as shown in Table 7, are not large enough to account for the extreme difference in their relative heat resistance, and that therefore these results are to be considered factual rather than coincidental.

The thermodynamical data reported should be viewed with caution. These values are meant to give information regarding the events that take place during heat inactivation of the spores. Therefore they are only applicable in the case where the subculture medium completely fulfills the nutritional requirements of the surviving spores. In the case of this investigation, beef infusion is believed to represent such a medium. Therefore, the energies of activation and the entropy values calculated from the data obtained with this medium are to be considered the true values. Nevertheless, the thermodynamical data calculated from any of the other recovery media, provide useful indications in comparing moist and dry heat resistance of bacterial spores.

Table 10 shows the results of an experiment in which the effect of the addition of vitamin B_{12}, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) to the synthetic medium was studied with the purpose of finding out, whether the addition of certain compounds would result in a higher recovery. The results indicate that supplementing this medium with either vitamin B_{12} or RNA or a combination of any of two of these compounds or all three of these compounds resulted in an increased D value. However, experimentation was not extensive enough to allow firm

TABLE 10. The effect of the addition of RNA, DNA, and Vitamin B_{12} to the synthetic medium on the recovery of moist heat treated beef heart spores

Compounds added	No. of replicates	No. of positive	D_{260}, sec.
Control	20	0	8.65
DNA (400mg/lt)	20	0	8.65
RNA (800mg/lt)	20	2	8.60
Vitamin B_{12}	20	2	8.60
DNA, RNA, B_{12}	20	4	9.40
DNA, RNA	20	4	9.40
RNA, B_{12}	20	6	9.70
DNA, B_{12}	20	4	9.40

conclusions. Rather, the results are to be taken as indicative to the direction in which further work might be fruitful.

3. Resistance of Spores to Dry Heat

In accordance with other reportings, the spores of P.A. 3679 exhibit considerably higher resistance to dry heat than to saturated steam. Just as this was the case with moist heat treatments, both D and z values vary as shown in Tables 11 and 12 and Figures 4 to 10 are dependent on the type of subculture medium used. With the exception of beef infusion with a z value of 39°F and the synthetic medium with a z value of 33°F, z values obtained with all other subculture media are of the magnitude of 36°F. At 255°F the D values obtained with the various subculture media are lowest when the heated spores were recovered in the synthetic medium. A slightly but distinctly higher D value was found when eugonbroth was used as the recovery medium. With all other subculture media substantially higher D values but of the same magnitude were obtained. At 320°F the use of beef infusion as the subculture medium gave by far the highest D values. D values obtained with yeast extract were slightly lower. Third highest D values were obtained with liver infusion, followed by the D values obtained with pea infusion and trypticase, which were of the same magnitude. Again, the use of eugonbroth resulted in second lowest, and that of the synthetic medium in the lowest D values.

Results in Table 12 and Figures 4 to 9 show an increase in the D values during storage of the spores. The first D values were obtained no longer than 90 days following sporulation, whereas the last values,

TABLE 11. D_{300} and z values and corresponding heats of inactivation for dry heat resistance as shown in figures

Recovery Medium	Beef Heart Spores				Trypticase Spores			
	D_{300}, min	z, °F	E*	S*	D_{300}, min	z, °F	E*	S*
Beef Infusion	12.0	39.0	37,000	17.0	6.4	36.0	40,600	27
Liver Infusion	10.0	36.0	40,600	25.6	---	---	---	
Pea Infusion	10.0	36.0	40,600	25.6	---	---	---	
Yeast Extract	11.2	36.0	40,600	25.6	5.6	36.0	40,600	27
Trypticase	9.4	36.0	40,600	25.6	---	---	---	
Eugonbroth	8.2	36.0	40,600	26.0	3.2	36.0	40,600	28
Synthetic	6.4	33.0	44,000	34.5	---	---	---	

*E = Energy of activation = $2.303R_x \frac{T_2 x T_1}{T_2 - T_1} x \log k_2/k_1$

S = Entropy of activation = $R(2.303 \log K_1 - 2.303 \frac{K T_1}{h} + \frac{\Delta H}{RT_1})$

TABLE 12. D values and their corresponding 95 percent confidence limits in minutes for dry heat and various subculture media of beef heart grown spores.

Temp., °F	Beef Infusion	Liver Infusion	Pea Infusion	Yeast Extract	Trypticase	Eugonbroth	Synthetic
255	133	125	134	138	140	96	--
	(129-137)	(119-131)	(127-141)	(132-144)	(135-145)	(91.5-100)	
	181	189	179	183	174	150	143
	(169-193)	(174-204)	(163-197)	(173-193)	(165-183)	(145-155)	(136-151)
	--	174	175	--	--	--	--
		(165-183)	(163-187)				
285	--	13.3	16.1	--	17.7	15.0	--
		(12.5-14.1)	(14.8-17.3)		(17.1-18.3)	(14.3-15.7)	
	29.6	28.0	26.0	21.8	--	--	18.2
	(28.6-30.6)	(27.0-29.0)	(25.1-26.9)	(21.0-22.6)			
	29.4	25.7	27.5	31.5	26.7	21.2	17.7
	(27.9-30.8)	(24.2-27.2)	(26.1-28.9)	(30.8-33.2)	(26.4-27.0)	(20.6-21.7)	(17.5-17.9)
300	--	6.1	6.8	6.6	6.1	4.9	--
		(5.4-6.6)	(6.1-7.4)	(6.2-7.0)	(5.6-6.5)	--	
	9.1	9.7	6.9	--	--	5.2	--
	(8.6-9.5)	(9.3-10.0)	(6.5-7.4)			(4.8-5.6)	
	11.1	--	9.8	9.7	8.9	8.3	--
	(10.5-11.7)		(9.4-10.2)	(9.2-10.2)	(8.6-9.2)	(8.0-8.6)	
	--	9.6	10.3	10.2	8.8	--	6.9
		--	(9.8-10.7)	(9.4-11.1)	(8.4-9.2)		(6.6-7.2)
320	--	1.67	1.84	1.47	1.67	1.15	2.30 (315°F)
		(1.59-1.75)	(1.74-1.95)	(1.38-1.57)	(1.57-1.76)	(1.06-1.21)	(2.21-2.38)
	2.59	2.98	2.94	2.94	2.01	--	--
	(2.45-2.69)	(2.88-3.08)	(2.79-3.09)	(2.87-3.01)	(1.89-2.12)		
	3.94	3.20	2.89	3.50	2.93	2.36	1.34
	(3.80-4.10)	(3.02-3.38)	(2.78-3.01)	(3.41-3.58)	(2.72-3.14)	(2.20-2.52)	(1.23-1.46

that is those which are substantially higher, were obtained up to 9 months following sporulation. This is contrary to the findings reported in the literature which cite either no change or a decrease in heat resistance.

The trypticase yeast extract grown spores again exhibit considerably lower heat resistance than the beef heart grown spores, as shown in Tables 11 and 13 and Figures 11 to 13. The results reveal a decrease in D values with the recovery medium in the following order: beef infusion, yeast extract, eugonbroth. It is interesting to note that the E values which are all of the same magnitude, namely 40,600 calories are approximately half of those obtained with the same type of spores with moist heat. This is not the case with the beef heart grown spores.

The difference in recovery of the nonheated spores in the various subculturing media as shown in Table 9 follows more or less the same pattern as those reported for their corresponding D values, although to considerably smaller degree. The same holds true for the trypticase grown spores.

4. Statistical Methods of Analysis

The results of a comparative study of four different methods of statistical analysis of the thermal resistance data are compiled in Table 14 and shown in Figures 14 and 15. With the moist heat resistance data, the D values obtained with the loglog or maximum likelihood method are consistently lower than those obtained with the Schmidt or the Spearman-Karber method. With the dry heat resistance data, an opposite trend appears to hold. However the z values resulting from any of

TABLE 13. D values and their corresponding 95 percent confidence limits in minutes for dry heat resistance of trypticase yeast extract spores and various recovery media.

Temp., °F	Beef Infusion	Yeast Extract	Eugonbroth
255	101.9 (96.8 - 107.8) 130 (108 - 151) -- -- --	100 (72.4) 96.0 (84.2-107.7) 105 (90.5-111) 109 (103-115)	56.5 (48.3-65.0) 57.0 (52.4-61.7) -- -- --
285	17.3 (16.7-17.9) 20.7 (19.2-22.2) 18.5 (17.4-19.7) --	16.6 (15.5-17.7) 14.7 (13.8-15.5) 14.0 (12.3-15.7) 16.3 (15.8-16.9)	9.66 (9.1-10.5) 8.74 -- 7.85 (6.55-9.14) --
300	6.33 (5.94-6.72) --	6.82 (6.42-7.20) 5.82 (5.44-6.19)	4.01 (3.51-4.41) 3.22 --
315	--	--	1.05 (0.84-1.26)
320	1.55 (1.42-1.69) 1.91 (1.76-2.06) 1.89 (1.78-1.99)	1.56 (1.39-1.72) 1.53 (1.37-1.68 1.80 (1.62-1.98) 1.68 (1.56-1.80)	1.03 (0.94-1.11) 1.23 (1.04-1.41) --

TABLE 14. D values and their corresponding 95 percent confidence limits utilizing different methods of calculation

Heating Medium	Temp., °F	D values and 95% CL*			
		Modified Schmidt	Maximum likelihood (LEWIS)	STUMBO ET AL.	Spearman-Karber
Moist heat	230	11.83 (10.50-13.08)	11.5 --	12.8 --	11.9 (10.50-13.33)
	250	1.09 (1.0-1.18)	1.00 --	1.08 --	1.12 (1.07-1.16)
	260	0.33 (0.31-0.37)	0.32 --	0.33 --	0.33 (0.31-0.35)
	280	0.033 (0.031-0.037)	0.031 --	0.033 --	0.033 (0.032-0.035)
Dry heat	255	181 (169-193)	192 --	192 --	191 (187-195)
	285	29.4 (27.9-30.8)	31.1 --	31.6 --	31.5 (29.2-33.7)
	300	11.1 (10.4-11.6)	10.9 --	11.0 --	11.0 (10.7-11.4)
	320	3.94 (3.8-4.10)	3.87 --	3.84 --	3.85 (3.73-3.98)

*Moist heat: Yeast extract recovery medium, D in seconds
Dry heat: Beef infusion recovery medium, D in minutes

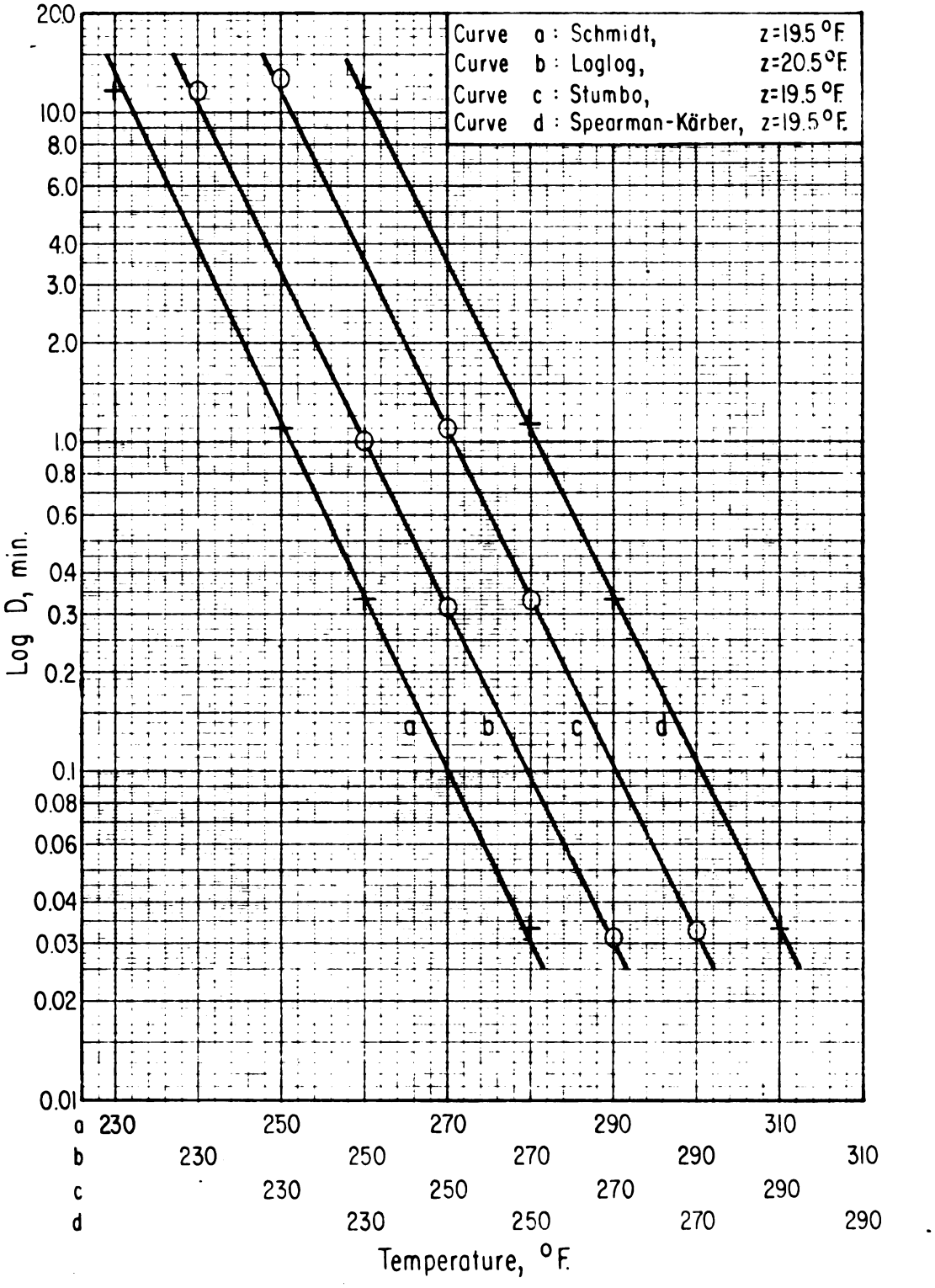

FIGURE 14. Thermal resistance curves obtained from D values calculated by four different methods from moist heat resistance data of beef-heart infusion spores subcultured in yeast extract

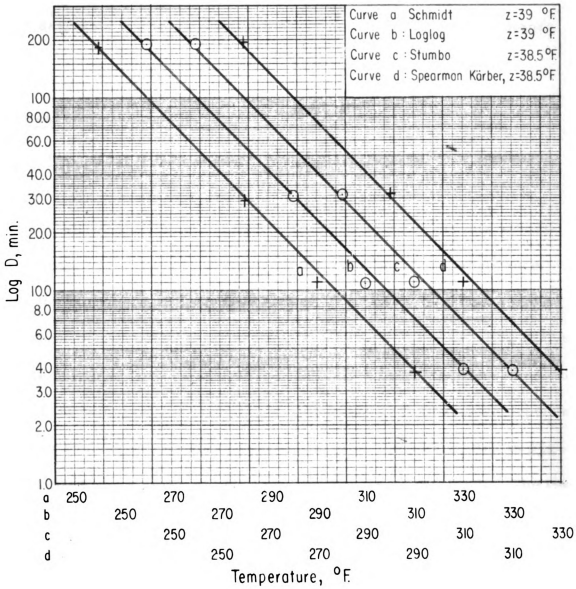

FIGURE 15. Thermal resistance curves obtained from D values calculated by four different methods from dry heat resistance data of beef-heart infusion spores subcultured in beef infusion

the four methods used were identical. Therefore, and because the differences in the D values obtained by the four methods were only small and for ease of calculation the Schmidt method was considered to be the most desirable one for the statistical treatment of all heat resistance data.

5. Rearrangement of Heat Resistance Data

As shown earlier, the z values not only vary with the heating medium, but also in some cases with the type of recovery medium used. This means that the D values obtained with the various subculture media are somewhat different in their temperature dependence. Thus the statement made by CURRAN AND EVANS (1937) that severe heat treatment causes bacterial spores to become more exacting in their nutritional requirements might show not only a time dependence regardless of the temperature used, but it also might be temperature dependent. In order to demonstrate this more drastically, the data plotted in Figures 4 to 10 were rearranged. Equation (6) was solved for N. The time t was selected to give an N of 1000 with the beef infusion medium and held constant with each temperature for the calculation of the N values calculated for the other subculture media. The results were compiled in Tables 15 and 16 and shown in Figures 16 through 18.

The results demonstrate very clearly the differences in surviving spores with reference to the subculture media. Above all they point out that the reaction of the spores surviving dry heat is quite different from that of spores surviving moist heat with reference to the subculture

TABLE 15. Number of surviving beef heart spores of P. A. 3679 recovered in various media following heat exposure at constant times

Subculture Medium	Initial Count %	Moist Heat				Dry Heat			
		230°F	250°F	270°F	275°F	255°F	285°F	300°F	320°F
Beef Infusion	100	1000	1000	1000	1000	1000	1000	1000	1000
Liver Infusion	90.3	159	148	166	159	871	562	407	257
Pea Infusion	90.4	661	646	646	661	939	537	417	219
Yeast Extract	98.4	501	458	501	479	1150	742	603	398
Trypticase	71.0	7.8	1.5	0.4	0.03	646	347	240	138
Eugonbroth	68.5	120	19.5	3.6	1.2	295	162	115	66.5
Synthetic	58.4	6.0	5.6	6.0	6.0	276	38	26	15.5

TABLE 16. Number of surviving trypticase spores of P.A. 3679 recovered in various subculture media following heat exposure at constant times.

Subculture Medium	Initial Count %	Moist Heat				Dry Heat			
		230°F	240°F	250°F	260°F	255°F	285°F	300°F	320°F
Beef Infusion	100	1000	1000	1000	1000	1000	1000	1000	1000
Yeast Extract	102.7	457	646	851	1073	759	725	759	725
Eugonbroth	86.0	112	91.2	74.1	52.5	83.2	75.9	79.5	91.3

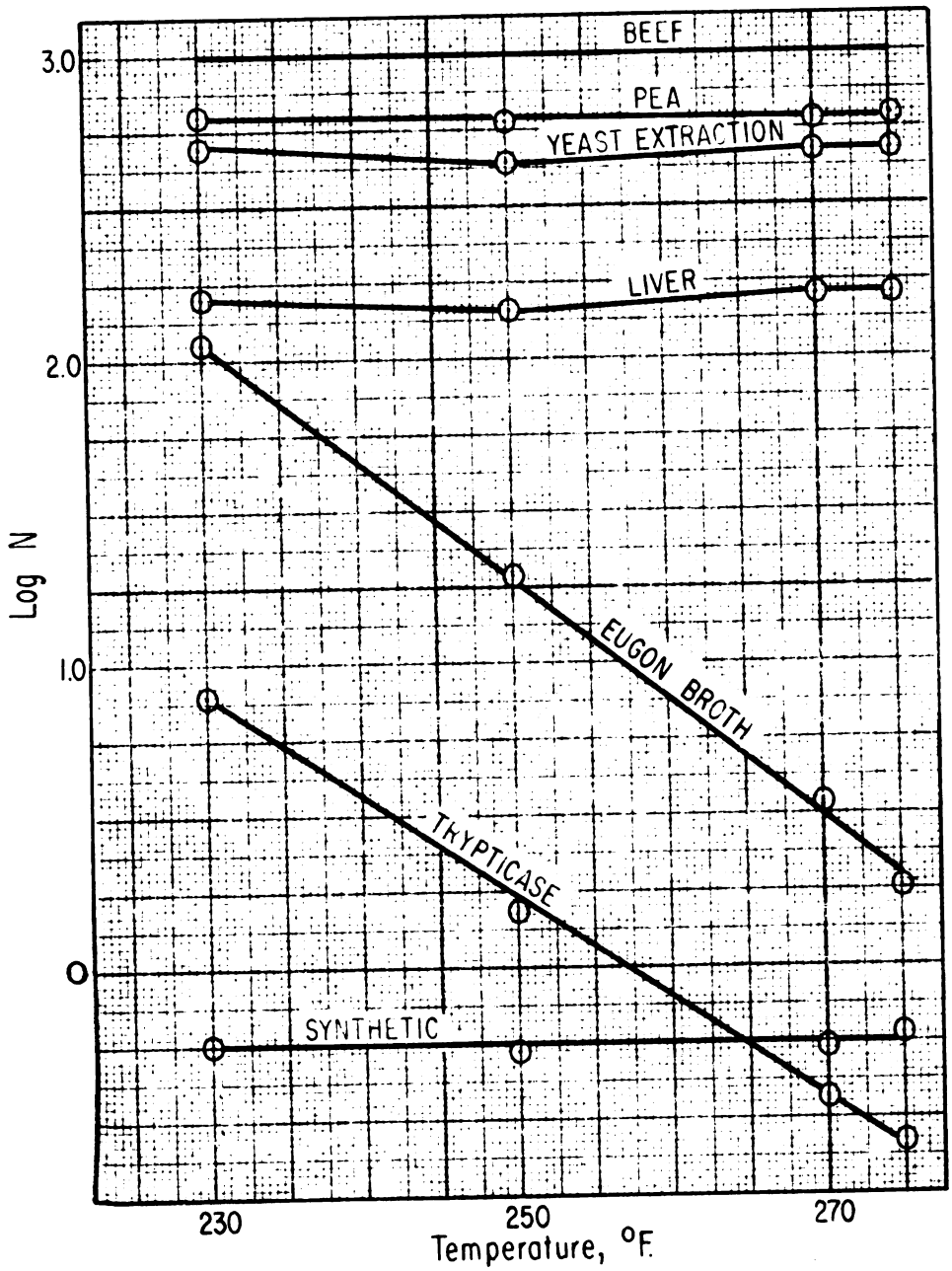

FIGURE 16. Logarithms of the number of surviving beef-heart spores in various subculture media following moist heat treatment with heating times chosen to yield 10^3 survivors with beef infusion as the subculture medium.

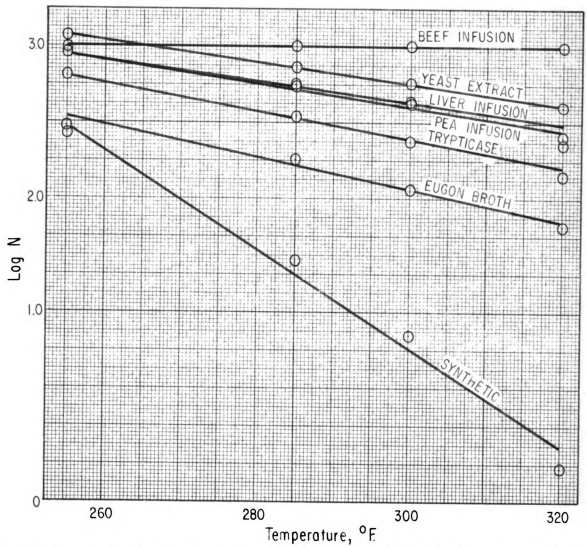

FIGURE 17. Logarithms of the number of surviving beef-heart spores in various subculture media following dry heat treatment with heating times chosen to yield 10^3 survivors with beef infusion as the subculture medium.

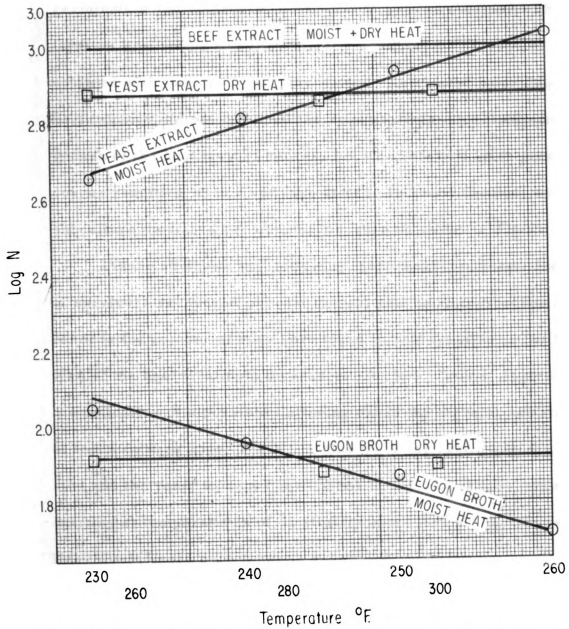

FIGURE 18. Logarithms of the number of surviving trypticase spores in various subculture media following heat treatment with times chosen to yield 10^3 survivors with beef infusion as the subculture medium.

medium. The same dependence holds with regard to the sporulation medium.

The question that presents itself when taking a closer look at Figure 17 is: What course do the survivor lines obtained with the various subculture media take when they approach the survivor line obtained with beef infusion? If beef infusion medium fulfills all the requirements for germination and outgrowth, a break in the curve obtained with the beef infusion line. This line theoretically could not be crossed by the curves obtained with the other recovery media. As to what would happen if the beef infusion did not fulfill all the requirements for germination and outgrowth of the heat treated spores, becomes obvious when looking at the curves resulting when trypticase and the synthetic medium were used as subculture media: a crossing of the lines as shown in Figure 16.

Figure 18 shows that following moist heat treatment, the number of survivors recovered in yeast extract gradually approaches and finally exceeds the number of survivors found in beef infusion as the temperature of the heat treatment increases. A closer look at Figure 11 reveals the indication of a possible flattening out of the thermal resistance curve at temperatures above 265°F. If this were the case, then the beef infusion and the yeast extract curve obtained with moist heat would run parallel starting at temperatures of or slightly above 265°F. If, however, the two lines would cross each other in reality, the only reasonable explanation that can be given is the same one which was put forward above for trypticase and the synthetic medium under the assumption that beef infusion

does not fulfill all the requirements for germination and outgrowth of trypticase spores which have been subjected to moist heat.

GENERAL DISCUSSION

The only criteria for survival of bacterial spores following exposure to any kind of lethal agent are the abilities of the spores to germinate and reproduce. When outgrowth of the heat exposed surviving spores, as evidenced in the case of P. A. 3679 by the formation of gas and the development of turbidity in the subculture medium is chosen as the criterion for survival, germination is a logical prerequisite for outgrowth. In order to properly evaluate the heat resistance of spores, it is imperative that all conditions for germination and outgrowth of these spores be fulfilled. The only study in which the nutritional requirements for heat treated spores for germination and outgrowth were determined was done with spores of B. natto as reported by AMAHA (1961). However, ideal conditions for germination and outgrowth of the heat treated spores of P. A. 3679 in terms of their nutritional requirements have never been defined. Therefore the existence of such ideal conditions can only be judged on a comparative basis. Media yielding highest recovery are considered to fulfill these conditions unless some other medium is found which gives an even higher survival count. Once these optimal conditions are established in the form of a medium giving highest recovery, the factors which are contributory to these optimal conditions have to be isolated and their identity has to be established. Questions as to whether these factors act as inhibitors or stimulants to

germination and/or outgrowth, or whether they are of a biochemical or physical nature have to be determined.

These experiments provide in part an answer to the first problem, namely that of creating the conditions which are ideal for recovery of the spores following treatment in moist and dry heat. However, the results also provide certain evidence regarding the nature of some of the factors which might be responsible in contributing to the prevalence of these optimal conditions for germination and outgrowth.

The results of this study show clearly that relative to the subculture media employed, beef infusion best fulfills these ideal conditions for germination and outgrowth of the heated spores, since the use of this medium results in the highest D values, the exceptions being trypticase grown spores which were subjected to moist heat at temperatures higher than 257°F, in which case higher D values were obtained when yeast extract was used as the subculture medium. In other words, the use of beef infusion results in the recovery of the largest number of spores surviving a given heat treatment. Subculturing of the heated spores in pea infusion, liver infusion or yeast extract results in slightly lower D values than when these same spores are placed into beef infusion, yet higher D values as compared to subculturing in trypticase, eugonbroth or the synthetic medium. However, in comparing the D and z values obtained with heated spores in the different subculture media, it becomes quite obvious that these values are quite different relative to each other, depending on the type of heating medium used. These differences in the

reaction of the spores to their subculture surroundings suggest that different types of heating media induce different changes in the spores regarding their requirements for germination and outgrowth. It is quite conceivable that such changes include changes in the nutritional requirements of the spores. If this were the case, the addition of one component to a subculture medium in which the spores recover poorly regardless to what type of heating medium they were exposed, would increase the degree of recovery and thus the D value if the spores were subjected to one type of heating medium, such as moist heat, but fail to improve the degree of recovery of spores which were subjected to a different type of heating medium such as dry heat, or, of course, vice versa.

The fact of the small differences in the initial spore counts obtained with various subculture media as compared to the much larger differences in survivor counts found with the same media following exposure to heat, are in agreement with the findings made by CURRAN AND EVANS (1937). However the conclusions drawn by these authors, that spores become more exacting in their nutritional requirements following heat treatment cannot be confirmed on the basis of the results obtained, because the factors contributing to these differences in recovery are not known. The differences obtained could be the result of nutritional deficiencies, or of inhibitory factors in the form of either direct inhibitors or improper ratios of some of the components of the media such as amino acids or vitamins. For this reason, the results obtained only allow the conclusion that heat treatment of P. A. 3679 spores resulted in their increased

sensitivity to their nutritional environment. But no matter what these factors are which contribute toward the differences in recovery of the surviving heat treated spores, the requirements of the heated spores for germination and outgrowth vary at least to some degree depending on the type of heating medium. This becomes especially obvious when comparing Figures 16 through 18.

That nutritional deficiencies might be responsible at least in part for the differences reported in recovery with various media is indicated by the fact that the recovery of the spores in the synthetic medium can be increased if the latter is supplemented with certain components notably RNA, DNA and vitamin B_{12}. Tables on the composition of the various subculture media might provide further material to substantiate this conclusion. However such data are often fragmentary and of insufficient accuracy to be of any value. Furthermore exact data on the composition of infusion type media do not exist.

This change in sensitivity of the spores to their nutritional environment manifests itself not only in relation to the non-heated spores versus the heat treated spores depending on the type of recovery media, but also within the heat treated spores there is in most cases a gradual increase in sensitivity with increasing temperature.

An explanation of this phenomenon can be derived on the basis of the findings reported by ZAMENHOF (1960) who demonstrated that dry heat treatment of bacterial spores in vacuo resulted in mutational injury in these spores, and that the site of this injury was the genetic material

of the spores i. e. the deoxyribonucleic acid (DNA) molecules. This thermal injury revealed itself in the form of alterations in the nutritional requirements of the spores. The differences in D values obtained with different recovery media as well as the gradual departure of the number of survivors recovered in some media relative to those recovered in beef infusion, suggests that increased severeness of the heat treatment creates an increased rate of random denaturation of the genetic material i. e. the DNA molecules of the spores. The denaturation of some of the DNA molecules merely results in an alteration in the nutritional requirements of these spores. Depending on the nutrient composition of the subculture medium spores can be expected to exhibit different recovery patterns in these media. Supplementation of these media with the proper nutrients would then most likely overcome the additional nutritional requirements which were developed in the spores during heating. If, however, a so called critical gene were denatured by the heat effect, any improvement of the nutritional environment would fail to receive any responses from the spores.

The fact that considerably higher energies of activation and entropy values are obtained with moist than with dry heat destruction does not rule out this theory. POLLARD (1953) who reported similar differences with reference to the thermal inactivation of viruses concluded that in the case of moist heat, higher energy barriers have to be overcome to permit biological inactivation. He hypothesized that this is due to a tighter binding of the entire structure, i. e. the molecules in the wet

state. The considerably higher entropy values associated with moist heat inactivation he suggests are due to a large extent to hydration.

On the basis of this theory, the mechanism of destruction of the spore is expected to be the same regardless of the type of heating medium to which the spores are subjected. Both moist and dry heat cause denaturation of the genetic material. However, certain parts of the genetic material are more susceptible to dry heat denaturation whereas other parts of the genetic material are more susceptible to moist heat denaturation.

If different degrees of hydration were positively correlated with changes in the size of the spores, this hypothesis could be extended as a possible explanation for the differences in heat resistance found among the spores which were produced in two different sporulation media. Substantial support for this type of reasoning could possibly be gained by determining the density of the spores grown in the two different sporulation media. It would be expected that the spores grown in trypticase would be of a lower density than those grown in beef heart infusion. It is therefore not unthinkable that a positive correlation could be established between the calcium content, the heat resistance of the spores and their densities. If such correlations could be verified experimentally, it could be possible to predict heat resistance of spores on the basis of their density.

According to this theory a gradual decrease from maximum heat of activation and entropy values required for the destruction of completely

hydrated spores to minimum heat of activation and entropy values needed for the destruction of dry spores is to be expected. That this is the case at least for the extreme degrees of hydration and dryness has been demonstrated in the results of this study which clearly reveal the values of the heats of activation and entropy obtained from moist heat destruction of the spores to be close to double those obtained with dry heat destruction.

INGRAHAM (1962) mentions, as another possible explanation of the logarithmic order of death, the theory of destruction of a certain structure such as the cell membrane. The work by HUNNELL AND ORDAL (1961) offers some support to this theory. However, the results obtained in this investigation appear to disprove it. If their theory would hold, then the differences in survivors recovered in the various subculture media following the heat treatments would be identical to the differences found in the non-heated spore samples in the same media.

The theories that have been advanced as explanation of the mode of action of heat on the sporal cell and which have been outlined in the literature review chapter all fall well in line with the findings of this study. The mechanism of death suggested by BALL AND OLSON (1957) however deserves some comment. While no evidence can be presented against their postulation that the agents of death are molecules having at a given time energies, momenta, or velocities greatly in excess of the average, their theory that death is due to the action of such molecules on the cell wall cannot be accepted in view of the rather strong

evidence presented above which rules out cell rupture as the critical event associated with death of bacterial spores and favors instead the theory of denaturation of DNA. These molecules are located inside the cell. Therefore the moelcules of the heating medium causing lethal effects have to penetrate the spore wall, or have to transfer their energies on some carrier molecule or a series of carrier molecules which in turn act on the DNA. This however anticipates permeability of the spore wall to molecules of such size. That spores are permeable to inorganic and organic molecules has been demonstrated by BLACK AND GERHARDT (1961) who estimated the pore size of moist spores of B. cereus var. terminalis to range from 10 to 200 angstroms. This pore size is sufficient enough to allow penetration of such molecules as water, oxygen, nitrogen, all of which are parts of the heating media employed in this study. Chances that these "lethal" molecules act on DNA by direct hit from outside the cell wall are however small. Rather it has to be assumed that they either gradually work their way through to the DNA molecule or that they activate some other molecules inside the cell which in turn confer their lethal dose on to the DNA molecule. It is quite conceivable that the former theory holds with dry heat destruction, and the latter with moist heat inactivation, in which case water molecules inside the cells act as transfer molecules. It is quite conceivable that, if a number of transfer molecules in the form of water molecules were involved in conveying lethal energies from the molecules of the heating medium to the DNA molecules, some energy inherent to the molecules

of the heating medium would be lost as a result of such transfers. This could serve as another explanation for the increased heat of activation required for the destruction of bacterial spores in moist heat as compared to dry heat.

SUMMARY AND CONCLUSIONS

A comparative study has been made of the heat resistance of spores of P. A. 3679 grown in two different sporulation media and the recovery pattern of these spores in several subculturing media following moist and dry heat treatment.

The heat resistance of the spores was characterized in the form of D and z values. The z values were obtained by the graphical method by plotting the logarithm of the D values against temperature on a linear basis. The D values were determined by using the modified Schmidt method after a preliminary comparative study of four different methods of calculating D revealed no significant difference at a 95 percent confidence level of the D values obtained by these methods.

The results revealed significant differences in D values depending on the heating medium, the sporulation medium and the subculturing medium. D values were consistently and considerably higher when spores were subjected to dry heat.

Spores were grown in two different sporulation media. Among them beef heart infusion yielded spores with a substantially higher heat resistance than the trypticase yeast extract medium.

Seven subculture media were tested and evaluated; among them beef infusion gave the best recovery of the heat treated spores; pea infusion, liver infusion and yeast extract approached beef infusion in spore recovery. Lower D values are obtained with trypticase and eugonbroth

with the synthetic medium showing the lowest recovery. However, the relative magnitudes of the D values and z values with reference to the subculture media used were different with moist heat than with dry heat.

Initial spore counts depend also on the type of culturing medium used. However the differences are incomparably smaller than those obtained with the heat treated spores.

The largest differences in z values were obtained as a function of the heating medium used. Dry heat treatment of the spores resulted in z values of $36^{\circ}F$ except with beef heart grown spores which were subcultured in beef infusion and the synthetic medium in which case z amounted to $37.5^{\circ}F$ and $33^{\circ}F$ respectively. Moist heat treated spores which were grown in beef infusion showed z values of $19.5^{\circ}F$ with the exception of those spores which were subcultured in trypticase and eugonbroth in which case z was $18.2^{\circ}F$ and $17.5^{\circ}F$ respectively. Slightly lower z values were obtained with the trypticase yeast extract grown spores ranging from $15.5^{\circ}F$ to $17.0^{\circ}F$.

Two theories which have been advanced as possible reasonable explanations of the logarithmic order of death of spores have been discussed.

On the basis of the results obtained the following conclusions are made:

1. The differences in recovery obtained with the various subculture media are at least in part due to nutritional deficiencies of these media.

2. Different types of heating media induce different changes in the spores regarding their requirements for germination and outgrowth.
3. The results obtained in these experiments together with the findings of other workers favor the theory that heat treatment of spores results in an increased rate of random denaturation of the genetic material of the spores including that of a critical gene.
4. The sequence of events leading to death of the spores is the same for moist and dry heat.
5. The differences between moist and dry heat resistance of the spores can be explained by differences in the stability of the genetic material depending on its degree of hydration.
6. The differences in the recovery of the spores in the various subculture media following moist and dry heat treatments are believed to be due to the fact that certain parts of the genetic material are more susceptible to denaturation in the dry state whereas other parts are more susceptible to denaturation when in the hydrated state.

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APPENDIX

TABLE 1A. Calculation of data for lethal rates

Time Sec.	Sec.	Temp T. °F	$\frac{315-T}{33}$ log		$L = 1/T$	t x l
0	2	65				
2	2	230	2.576	378	0.003	0.006
4	2	286	0.879	7.58	0.132	0.264
6	2	304.5	0.318	2.08	0.481	0.962
8	2	306.5	0.258	1.81	0.552	1.104
10	2	308.	0.212	1.63	0.613	1.226
12	2	309.2	0.176	1.50	0.667	1.334
14	2	310.3	0.142	1.39	0.719	1.438
16	2	311.15	0.117	1.31	0.763	1.526
18	2	311.85	0.095	1.24	0.806	1.612
20	2	312.4	0.079	1.20	0.833	1.666
22	2	312.9	0.064	1.16	0.862	1.724
24	2	313.25	0.053	1.13	0.885	1.770
26	2	313.58	0.043	1.10	0.909	1.818
28	2	313.82	0.036	1.08	0.926	1.852
30	2	314.0	0.030	1.07	0.935	1.870

$U = t_m - t_e = 31 - 20.17 = 10.83$ sec.

$U = 10.83/60 = 0.18$ min

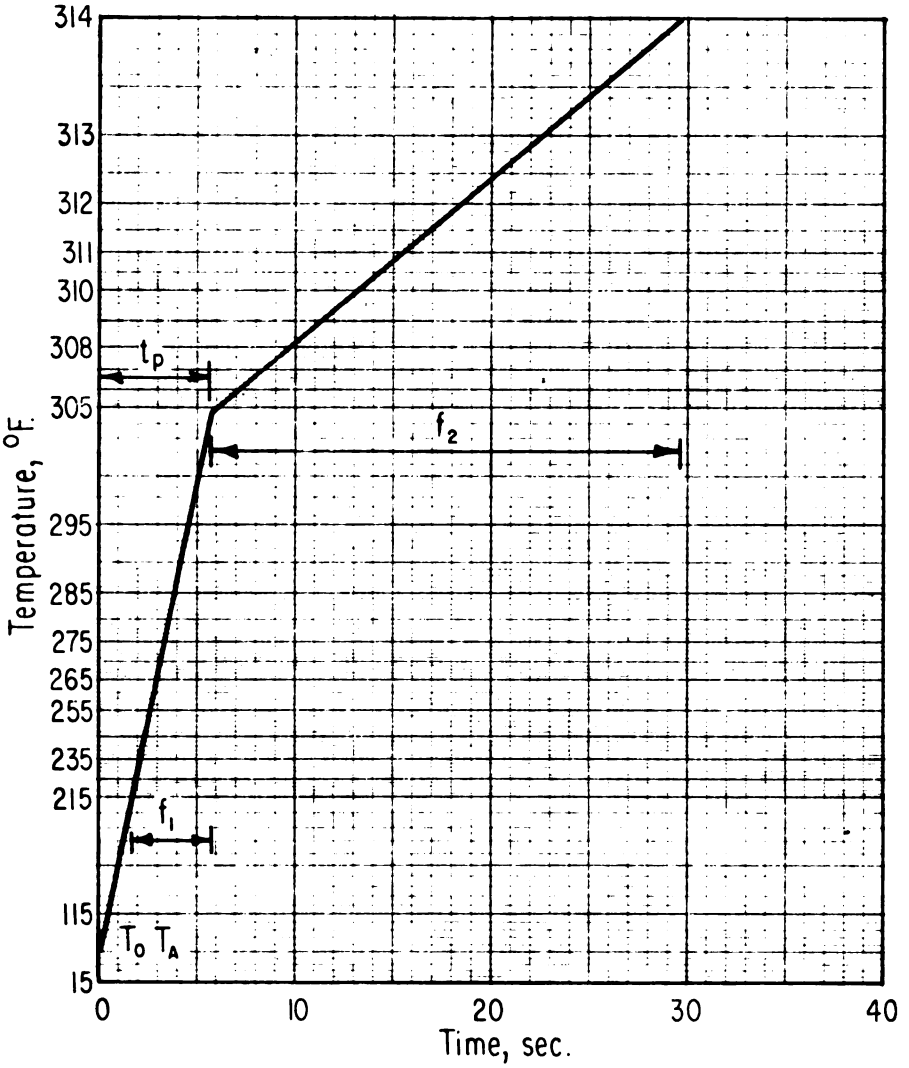

FIGURE 1A. Heat penetrations curve for cups in TDT cans heated in saturated steam

TABLE 2A. Results of tests of beef-heart infusion spores of P.A. 3679 subcultured in beef infusion following moist heat treatment at various temperatures.

230°F		250°F				260°F		280°F	
Time, min.	No. pos. tubes	Time, min., sec.	No. of positive tubes			Time, sec.	No. pos. tubes	Time, sec.	No. pos. tubes
56	18	5	20			96	18	9	20
64	9	6	17	18	9	108	17	10	19
72	0	6:30	-	4	-	120	10	11	9
80	0	6:40	-	-	7	132	-	12	9
88	0	7	2	2	-	144	9	13	8
96	0	7:20	-	-	6	156	5	14	0
		7:30	-	0	-	168	4	15	0
		8	0	0	0				
		8:30	-	1	0				
		9	1						
		10	0						
Date of tests	11/15		11/15	12/28	3/7		12/22		12/22
No. of repl.	20		20	20	10		20		20
D, min.	13.13		1.38	1.32	1.47		0.468		0.0403
95% C. L. D, min.	12.21-14.04		1.31-1.43	1.29-1.51	1.43-1.51		0.416-0.522		0.0373-0.0432

TABLE 4A. Results of tests of beef-heart infusion spores of P. A. 3679 subcultured in pea infusion following moist heat treatment at various temperatures.

230°F			250°F		260°F		270°F			280°F		
Time min.	No. Pos. tubes		Time min.	No. pos tubes	Time sec.	No. pos tubes	Time sec.	No. Pos, tubes		Time sec.	No. pos. tubes	
50		20	4	10	30	20	24		20	6	20	20
56		10	5	10	36	20	30		14	7	20	--
58	8	--	6	8	42	20	36	4	6	8	20	15
62	-	8	7	8	48	20	42	0	1	9	20	--
64	8	-	8	4	54	20	48	0	1	10	9	6
68	-	7	9	1	60	20	54	0	0	11	7	--
72	1	5			66	20	60	0	0	12	2	1
80	0				72	20	66	0	0	13	4	--
88	0						72	0	0	14		0
96	0									15		0
										16		0
										17		0
										18		0
										19		0
										20		0
Date of tests	11/10	12/4		11/10		11/10		12/17	1/2		12/17	1/2
No. repl.	10	20		10		20		20	20		20	20
D, min.	13.78	13.3		1.60		-		0.114	0.117		0.0383	0.0322
95% C. L. D, min.	13.27-14.29	12.92-14.00		1.47-1.73				--	0.107-0.127		0.0357-0.0400	0.0290-0.0353

TABLE 3A. Results of tests of beef-heart infusion spores of P. A. 3679 subcultured in liber infusion following moist heat treatment at various temperatures.

	230°F		250°F			260°F		275°F	
Time, min.	No. positive tubes		Time, min.	No. positive tubes		Time, sec.	No. pos. tubes	Time, sec.	No. pos. tubes
40		20	4		4	42	20	8	20
44		13	5	3	2	48	20	9	20
48		6	6	0	1	54	20	10	20
52		6	7	0	1	60	20	11	20
56	2	2	8	0	0	66	19	12	16
60	0	1				72	16	13	15
64	0	-				78	11	14	12
72	0					84	3	15	3
80	0								
88-96	0								
Date of tests	11/20	12/2		11/20	12/2		12/22		12/22
No. of repl.	20	20		20	20		20		20
D. min.	10.1	10.23		0.935	0.747		0.273		0.0492
95%C. L. $D_{min.}$	--	9.71-10.76		--	0.600-0.898		0.260-0.286		0.0465-0.0517

TABLE 5A. Results of tests of beef-heart infusion spores of P. A. 3679 subcultured in yeast extract following moist heat treatment at various temperatures.

230°F			250°F				260°F		270°F		280°F		
Time min.	No. pos. tubes		Time sec.	No. pos. tubes			Time sec.	No. pos tubes	Time sec.	No. pos tubes	Time sec.	No. pos. tubes	
26	20		150	19			84	17	27	9	5	20	
32	20		195	18			96	9	30	6	6	20	
38	20		240	18	19	10	108	4	33	2	7	20	9
44	20		270	-	16	10	120	1	36	2	8	18	9
50	19	18	285	19	-	-	132	0	39	0	9	15	6
56		12	300		12	9	144	0	42	1	10	6	5
62		7	330		-	9	156	0			11	4	1
68		2	360		6	8	168	0			12	1	0
			390		1	4							
Date of tests	11/23	12/5		11/23	12/5	2/8		12/19		12/19		12/19	2/8
No. repl.	20	20		20	20	10		20		10		20	10
D, min.	-	11.8		0.86	1.09	1.24		0.334		0.108		0.0333	0.0335
95% C. L. D min.		10.5-13.1		-	1.00-1.18	1.21-1.27		0.313-0.355		0.105-0.117		0.0313-0.0355	0.0322-0.0350

TABLE 6A. Results of tests of beef-heart infusion spores of P. A. 3679 subcultured in trypticase following moist heat treatment at various temperatures.

230°F		250°F				260°F		275°F	
Time, min.	No. pos. tubes	Time, min., sec.	No. of positive tubes			Time, sec.	No. pos. tubes	Time, sec.	No. pos. tubes
26	18	135	-	2	16	36	8	5	18
32	5	150	5	1	9	42	7	6	15
38	1	165	-	1	7	48	3	7	13
44	1	180	-	0	2	54	2	8	4
50	1	195	0	0	3	60	1	9	1
		210	-	0	1	66	1	10	1
		240	0						
		285	0						
Date of tests	11/23		11/23	12/26	1/29		12/17		12/17
No. of repl.	20		20	20	20		20		20
D. min.	6.60		0.497	0.437	0.550		0.132		0.0253
95% C. L. $_D$, min.	6.20-7.02		-	-	0.517-0.583		0.120-0.144		0.0227-0.0280

TABLE 7A. Results of tests of beef-heart infusion spores of P. A. 3679 subcultured in eugonbroth following moist heat treatment at various temperatures.

230°F			250°F			260°F		270°F	
Time min.	No. pos. tubes		Time sec.	No. pos. tubes		Time sec.	No. pos tubes	Time sec.	No. pos. tubes
34	20		180	20		44	8	12	15
40	16	20	195	-	8	52	4	14	15
44	-	11	240	5	1	60	2	16	6
48	1	2	285	-	1	68	4	18	2
52	-	1	300	0	-	76	3	20	1
56	1		330	-	0	84	0	22	1
64	0		360	0		92	0	24	0
			420	0		100	0	26	0
			480	0					
Date of tests	11/20	12/2		11/20	12/2		12/21		12/21
No. repl.	20	20		20	20		20		20
D, min.	9.05	9.78		0.797	0.693		0.164		0.0533
95% C.L. D, min.	-	9.45-10.09		-	0.641-0.775		0.134-0.194		0.0515-0.0570

TABLE 8A. Results of tests of beef-heart infusion spores of P. A. 3679 subcultured in synthetic medium following moist heat treatment at various temperatures.

230°F			250°F				260°F				275°F		
Time min.	No. pos. tubes		Time sec.	No. pos. tubes			Time sec.	No. pos. tubes			Time sec.	No. pos. tubes	
10	20		84		19		16			10	6	5	4
12	20		90		-	10	18			10	7	5	3
14	19		96		16	-	20			10	8	4	2
16	17		105		-	7	22			10	9	3	0
17	-	10	108		13	-	24	10	10	10	10	1	0
18	13	-	120		9	6	26	-	-	9	11	0	0
19	-	9	132		6	-	27	10	9	-	12	1	0
20	5	-	135	3	-	4	30	10	9		13	0	0
21	-	7	144	-	3	-	33	10	4				
22	4	-	150	0	-	0	36	9	2				
23	-	4	156	-	0	-	39	9	1				
24	2	-	165	2		1	42	5	2				
25		2					45	5	1				
27		0					48	2	0				
29		0					51	0	0				
Date of tests	2/8	2/28		1/2	2/8	2/28		1/29	2/28	2/28		1/29	1/29
No. repl.	20	10		10	20	10		10	10*	10		5	5*
D, min.	4.11	4.92		0.512	0.432	0.446		0.158	0.135	-		0.0267	0.0233
95% C.L. D, Min.	3.04-5.17	4.63-5.20		-	0.415-0.448	0.405-0.487		0.148-0.168	-	-		0.0238-0.0295	-

* No $NaHCO_3$ added to subculture medium.

TABLE 9A. Results of tests of beef-heart infusion spores of P.A. 3679 subcultured in beef infusion following dry heat treatment at various temperatures.

255°F			285°F			300°F			320°F				
Time min.	No. pos. tubes		Time min.	No. pos. tubes			Time min.	No. pos. tubes		Time min.	No. pos. tubes			
480	18		78	20			24	20		3	20			
540	17		84	20			28	20		4	20			
600	15		90	20	20		32	19		5	20			
660	13		96	20	-		36	19		6	20			
720	5	10	100		20	20	38	-	20	7	19			
780		10	110		19	20	40	12	-	8	20	20		
840		7	120		20	20	42	-	20	9		20		
900		7	130		16	18	44	9	-	10		20		
960		4	140		10	17	46	-	19	11		20		
			150		7	11	48	5	-	12		17		
			160			6	50		14	13		17		
							54		9	14		16	19	20
							58		6	15		11	20	-
										15.5			-	20
										16			20	-
										17			19	19
										18.5				13
										20				11
										21.5				7
Date of tests	10/21	3/7		1/3	2/19	3/7		10/22	2/19		10/22	1/3	2/19	3/7
No. repl.	20	10		20	20	20		20	20		20	20	20	20
D, min.	133	181.2		-	29.6	29.4		9.1	11.1		-	2.59	-	3.94
95% C. L.D, Min.	129-137	169-193		-	28.6-30.6	27.9-30.8		8.6-9.5	10.5-11.7		-	2.45-2.69		3.80-4.10

TABLE 10A. Results of tests of beef-heart infusion spores of P. A. 3679 subcultured in [illegible] liver infusion following dry heat treatment at various temperatures.

255°F				285°F				300°F				320°F			
Time min.	No. pos. tubes			Time min.	No. pos. tubes			Time min.	No. pos. tubes			Time min.	No. pos. tubes		
420	19			42	20			20	20			7	14		
480	19			50	14			24	12			8	11		
540	13			58	14			28	13			9	7		
600	13	10		64	9			30	-	10		10	2	10	
660	3	10		70	-	10		32	-	-		11		10	
720		9		72	10	10		33		10		12		10	
780		9	4	80		10		36	5	10		13		8	
840		8	6	90		10		39	-	9		13.5		-	9
900			2	100		10		40	1	-	10	14		8	-
960			1	110		10	8	42		8	-	15		2	6
				120		8	9	44		-	10	16.5			1
				130		8	6	45		7	-	18.0			2
				140		2	2	48		4	10	19.5			0
				150			2	52			3	21			1
				160			0	56			0				
								60			0				
Date of tests	10/30	3/11	4/22		10/30	3/11	4/22		10/30	3/11	4/22		10/30	3/11	4/22
No. repl.	20	10	10		20	10	10		20	10	10		20	10	10
D,min.	125	189	174		13.3	28.0	25.7		6.1	9.7	9.6		1.67	2.98	3.20
95% C.L.D, Min.	119-131	163-197	165-183		12.5-14.1	27.0-29.0	24.2-27.2		5.4-6.6	9.3-10.0			1.59-1.75	2.88-3.08	3.02-3.38

TABLE 11. Results of tests of beef-heart infusion spores of P. A. 3679 subcultured in pea infusion following dry heat treatment at various temperatures.

255°F				285°F				300°F					320°F				
Time min.	No. pos. tubes			Time min.	No. pos. tubes			Time min.	No. pos. tubes				Time min.	No. pos. tubes			
480	19			50	10			22	-	17			5	20			
540	15			57	9			24	14	-			6	20			
600	11	10		64	9			26	-	18			7	20	14		
660	12	10		70	-	10		28	10	-			8	20	13		
720	6	9		71	6	-		30	-	15	10		9		7		
780		9	5	78	4	-		32	9	-	-		10		3	10	
840		7	5	80	-	10		33	-	-	10		11		1	10	
900			1	85	2	-		34	-	9	-		12			9	
960			0	90	-	10		36	10	-	10		13			7	
				92	2	-		38	-	7	-		13.5			-	8
				99	1	-		39	-	-	10		14			7	-
				100		10		40	7	-	-	9	15			4	1
				110		8	10	42	-	2	9	-	16.5				0
				120		8	7	44	1	-	-	9	18				0
				130		3	4	45			7	-	19.5				0
				140			2	48			4	7	21				0
				150			2	52				1					
				160			0	56				0					
								60				0					
Date of tests	12/4	3/11	4/22		9/30	3/11	4/22		10/17	12/4	3/11	4/22		10/17	10/31	3/11	4/22
No. repl.	20	10	10		10	10	10		20	20	10	10		20	20	10	10
D, min.	134	179	175		16.1	26.0	27.5		6.85	6.9	9.85	10.3		-	1.84	2.94	2.89
95% C.L.D, Min.	127-141	163-197	163-187		14.8-17.3	25.1-26.9	26.1-28.9		6.1-7.4	6.5-7.4	9.4-10.2	9.8-10.7		-	1.74-1.95	2.79-3.09	2.78-3.01

TABLE 12A. Results of tests of beef-heart infusion spores of P.A. 3679 subcultured in yeast extract following dry heat treatment at various temperatures.

255°F			285°F					300°F				320°F			
Time min.	No. pos. tubes		Time min.	No. pos. tubes				Time min.	No. pos. tubes			Time min.	No. pos. tubes		
420	9		50	8				24	19			3	19		
510	9		57	9				28	11	20		4	17		
500	7		60	-	10			32	7	20		5	20		
690	6		64	10	-			36	7	20		6	20	12	
780	0	10	68	-	10			40	3	20	9	7	17	15	
840	-	6	71	6	-			44	0	10	9	8		9	
870	1	-	76	-	10			48		6	7	9		2	
900	-	2	78	6	-			52		5	1	10		0	
960	1	1	84	-	10			56		2	0	11		0	
			85	1	-			60			0	12		0	
			92	1	10							13		0	
			99	1	-							14			19
			100		9	13	20					15			18
			108		10	-	-					16			16
			110		-	6	20					17			9
			116		9	-	-					18			1
			120			1	20								
			130				18								
			140				17								
			150				11								
			160				6								
Date of tests	10/3	3/10		10/3	1/18	1/29	3/7		10/17	1/9	4/22		10/17	10/31	3/7
No. repl.	10	10		10	10	20	20		20	20	10		20	20	20
D, min.	138	183		-	-	21.8	31.5		6.55	9.7	10.2		1.47	2.94	3.50
95% C.L.$_D$, Min.	132-144	173-193				21.0-22.6	30.8-33.2		6.15-7.0	9.2-10.3	9.4-11.1		1.38-1.57	2.87-3.01	3.41-3.58

TABLE 13A. Results of tests of beef-heart infusion spores of P. A. 3679 subcultured in trypticase following dry heat treatment at various temperatures.

255°F			285°F			300°F				320°F			
Time min.	No. pos. tubes		Time min.	No. pos. tubes		Time min.	No. pos. tubes			Time min.	No. pos. tubes		
420	19		43	20		24	20			3	20		
480	20		50	19		25	-	20		4	20		
540	19		57	20		28	10	20		5	20		
600	17	10	60	20		31	-	19		6	20		
660	9	10	71	12		32	2	-		7	18	16	
720	3	8	78	15		34	-	20		8	10	14	
780	0	7	85	8		37		19		9		10	
840	0	5	90	-	10	38		-	17	10		7	10
			92	2	-	40		13	-	11			10
			100		10	42		-	7	12			10
			110		10	43		7	-	13			8
			120		7	46		-	2	14			8
			130		2					15			2
Date of tests	10/9	3/11		10/9	3/7		10/18	1/29	3/7		11/23	3/11	4/22
No. repl.	20	10		20	10		20	20	20		20	20	10
D, min.	140	174		17.7	26.7		6.1	8.9	8.8		1.68	2.01	2.93
95% C. L. $_D$, Min.	135-145	165-183		17.1-18.3	26.4-27.0		5.6-6.5	8.6-9.2	8.4-9.2		1.57-1.76	1.89-2.12	2.72-3.14

TABLE 14A. Results of tests of beef-heart infusion spores of P.A. 3679 subcultured in eugonbroth following dry heat treatment at various temperatures.

255°F			285°F			300°F				320°F		
Time min.	No. pos. tubes		Time min.	No. pos. tubes		Time min.	No. pos. tubes			Time min.	No. pos. tubes	
420	13		42	20		20	-	13		3	19	
480	3		50	19		24	6	8	10	4	14	
540	2		57	19		27	-	-	10	5	16	
600	0	7	60	14	10	28	0	8	-	6	8	10
660	1	6	70	-	10	30	-	-	10	7	2	9
720		4	71	9	-	32	0	2	-	8	0	10
780		4	78	3	-	33	-	-	9	9	0	9
840		0	80	-	10	36	0		7	10		7
			85	1	-	39	-		6	11		4
			90	-	8	40	0			12		4
			92	1	-	44	0			13		2
			100	-	3							
			110		2							
Date of tests	10/9	3/11		10/8	3/11		10/8	12/2	3/11		10/18	3/11
No. repl.	20	10		20	10		20	20	10		20	10
D, min.	96	150		15.0	21.15		4.9	5.2	8.3		1.15	2.36
95% C. L. D, Min.	91.5-100	145-155		14.3-15.7	20.6-21.7		-	4.8-5.6	8.0-8.6		1.06-1.24	2.20-2.52

TABLE 15A. Results of tests of beef-heart infusion spores of P. A. 3679 subcultured in synthetic medium following dry heat treatment at various temperatures.

255°F			285°F			300°F		315°F			320°F	
Time min.	No. pos. tubes		Time min.	No. pos. tubes		Time min.	No. pos. tubes	Time min.	No. pos. tubes		Time min.	No. pos. tubes
360	20		50	19	20	20	19	8	10	1	3	20
420	14		55	20	-	22	20	9	10	1	4	20
480	9		60	20	20	24	18	10	7	0	5	15
540	8	10	65	20	-	26	16	11	1	0	6	15
600	14	10	70	20	13	28	14	12	2	0	7	2
660	13	8	75	17	-	30	16	13	1	0	8	2
720		6	80	12	0	32	12	14	0	0	9	2
			85	10	0	34	7	15	0	0	10	0
Date of tests	1/11	2/28		1/11	1/6		1/13		1/29	1/29		1/6
No. repl.	20	10		20	20		20		10	10*		20
D, min.	-	143		17.7	18.2		6.9		2.30			1.34
95% C. L. $_D$, Min.	-	136-151		17.5-17.9	-		6.6-7.2		2.21-2.38			1.22-1.46

* No $NaHCO_3$ added to subculture medium.

TABLE 16A. Results of tests of trypticase spores of P. A. 3679 subcultured in beef infusion following moist heat treatment at various temperatures.

230°F		250°F		260°F		270°F	
Time min.	No. pos. tubes	Time sec.	No. pos. tubes	Time sec.	No. pos. tubes	Time sec.	No. pos. tubes
22	10	70	5	10	10	4.5	10
25	7	80	5	12	10	5.0	10
28	4	90	4	14	10	5.5	9
31	2	100	4	16	10	6.0	7
34	3	110	0	18	6	6.5	7
37	0	120	0	20	7	7.0	5
40	0	130	1	22	4	7.5	5
Date of tests	2/19		2/19		2/19		2/19
No. repl.	10		10		10		10
D, min.	6.74		0.329		0.0805		0.0273
95% C. L. $_D$, Min.	6.26-7.21		0.298-0.361		0.0748-0.0963		0.0207 0.0245

TABLE 17A. Results of tests of trypticase spores of P. A. 3679 subcultured in yeast extract following moist heat treatment at various temperatures.

230°F			250°F		260°F			270°F	
Time min.	No. pos. tubes		Time sec.	No. pos. tubes	Time sec.	No. pos. tubes		Time sec.	No. pos. tubes
21		5	80	6	20		3	4.5	6
22	3	-	90	3	23		3	5.0	4
24	-	1	100	1	26		1	5.5	2
25	1	-	110	0	27	0	-	6.0	5
27	-	3	120	1	29	-	0	6.5	4
28	0	-			30	1	-	7.0	4
30	-	1			32	-	1	7.5	3
31	0	-			33	1			
33	-	0			36	0			
34	0	-			39	0			
36	-	1			42	0			
37	1	-			45	0			
40	0								
Date of tests	2/10	2/21		2/21		2/10	2/21		2/21
No. repl.	10	10		10		10	10		10
D, min.	5.27	5.12		0.337		-	0.0792		0.0225
95% C. L. D, Min.	-	4.75-5.37		0.313-0.360			0.0690-0.0893		0.0207-0.0245

TABLE 18A. Results of tests of trypticase spores of P.A. 3679 subcultured in eugonbroth following moist heat treatment at various temperatures

230°F			240°F		250°F		260°F		
Time min.	No. pos. tubes		Time sec.	No. pos. tubes	Time sec.	No. pos. tubes	Time sec.	No. pos. tubes	
8	10		120	10	27	9	4.0	10	
9	10		135	10	30	10	4.5	10	
10	10		150	10	33	8	5.0	10	
11	10		165	10	36	8	5.5	10	
12	10		180	10	39	7	6.0	10	
13	10		195	7	42	4	6.5	10	
14	10	7	210	6	45	5	7.0	10	9
16		3	225	2			8.0		8
18		2					9.0		4
20		1					10.0		6
22		0					11.0		1
Date of tests	2/14	2/21		2/14		2/14		2/14	2/21
No. repl.	10	10		10		10		10	10
D, min.	-	3.77		0.841		0.166		-	0.0378
95% C. L. $_{D}$, Min.	-	3.50-4.05		0.809-0.878		0.157-0.175		-	0.0347-0.0410

TABLE 19A. Results of tests of trypticase spores of P. A. 3679 subcultured in beef infusion following dry heat treatment at various temperatures

255°F			285°F				300°F		320°F			
Time min.	No. of pos. tubes		Time min.	No. pos. tubes			Time min.	No. pos tubes	Time min.	No. pos. tubes		
300	10		56	10	10	-	22	9	5.0		10	
330	9		62	10	10	8	24	8	6.	9	10	10
360	9		68	9	9	9	26	4	7	3	8	9
385	8		74	3	9	6	28	5	8	1	5	1
420	7	9	80	1	6	5	30	2	9	0	2	3
450	5	-	86	0	4	4	32	3	10	0	3	0
480		6	92	0			34	1	11	0		
540		6										
600		5										
Date of tests	2/19	4/15		2/20	4/14	4/15		2/20		2/19	4/14	4/15
No. repl.	10	10		10	10	10		10		10	10	10
D, min.	101.9	130		17.3	20.7	18.5		6.33		1.55	1.91	1.89
95% C. L. D, Min.	96.8-107.8	108-151		16.7-17.9	19.2-22.2	17.4-19.7		5.94-6.72		1.42-1.69	1.76-2.06	1.78-1.99

TABLE 20A. Results of tests of trypticase spores of P. A. 3679 subcultured in yeast extract following dry heat treatment at various temperatures.

255°F					285°F					300°F			320°F				
Time Min.	No. pos. tubes				Time min.	No. pos. tubes				Time min.	No. pos. tubes		Time min.	No. pos. tubes			
270	9				56	8	7		9	22	9		5	10	10		10
300	10				62	8	4	2	8	24	9	5	6	9	6	6	10
330	7				68	5	1	2	5	26	8	-	7	3	4	9	6
360	9	6		10	74	4	3	0	4	27	-	2	8	1	1	0	2
390	10	-		-	80	3	0	0	0	28	7	-	9	0	0	0	0
400	-	-		8	86	0	0	0	0	30	0	0	10	0	0	0	0
420	5	5	4	-	92	1				33		0	11	0			
440	-	-	-	7						36		0					
480		2	2	4						39		1					
540		1	1														
600			0														
Date of tests	2/13	2/21	4/21	4/25		2/12	4/14	4/15	4/25		2/12	4/25		2/12	4/14	4/15	4/25
No. repl.	10	10	10	10		10	10	10	10		10	10		10	10	10	10
D, min.	100	96.0	105	109		16.6	14.7	14.0	16.3		6.82	5.82		1.56	1.53	1.80	1.68
95% C. L. D', Min.	-	84.2-107.7	90.5-111	103-115		15.5-17.7	13.8-15.5	12.3-15.7	15.8-16.9		6.42-7.2	5.44-6.19		1.39-1.72	1.37-1.68	1.62-1.98	1.56-1.80

TABLE 21A. Results of tests of trypticase spores of P.A. 3679 subcultured in eugonbroth following dry heat treatment at various temperatures.

255°F			285°F				300°F			315°F			320°F	
Time min.	No. pos. tubes		Time min.	No. pos. tubes			Time min.	No. pos. tubes		Time min.	No. pos. tubes		Time min.	No. pos. tubes
210		6	30	10			12	10		4	8	8	4	9
240	3	5	34	8		2	14	6		5	5	1	5	1
270	-	3	35	-	4	-	15	-	3	6	3	0	6	0
280	3	-	37	-	-	1	16	5	-	7	2	0	7	0
320	0		38	8	-	-	18	4	0	8	0	1	8	0
360	0		40	-	0	1	20	3	-	9	0		9	0
390	0		42	3	-		21	-	0	10	0			
420	0		45	-	1		22	0	-					
			46	1	-		24	1	0					
			50	0	1		27	-	0					
			55		0		30		0					
Date of tests	2/11	2/21		2/11	2/21	4/14		2/13	2/21		2/13	2/21		4/14
No. repl.	10	10		10	10	10		10	10		10	10		10
D, min	56.5	57.0		9.66	8.74	7.85		4.01	3.22		1.05	1.03		1.23
95% C. L. D, Min.	48.3-65.0	52.4-61.7		9.1-10.15	-	6.45-9.14		3.61-4.41	-		0.84-1.26	0.94-1.11		1.04-1.41