THERMAL DESTRUCTION OF BACTERAL SPORES UNDER OPEN SYSTEM DRY-HEAT CONDITIONS

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ABSTRACT

THERMAL DESTRUCTION OF BACTERIAL SPORES UNDER OPEN SYSTEM DRY HEAT CONDITIONS

by Kenneth I. Fox

The sterilization of surfaces by dry heat presents many unknown variables. The thermal resistance of the most heat resistant organisms which could be potential contaminants on various surfaces must be known. The effect of the test surface composition on the resistance of the spores must be determined, and the effectiveness of the heating procedure must be evaluated.

The purposes of this investigation were to study the effect of open system dry heating of Bacillus subtilis spores, and to determine the effects of gas flow rate on the thermal resistance of the bacterial spores. The tests were conducted under conditions of flowing air and nitrogen.

Survivor curve tests were run in a specifically designed dry heat oven. This oven provided accurate temperature control and also allowed air or nitrogen to pass over the spores during the lethal treatment. Experiments were carried out at various flow rates of the two gases (air and nitrogen) and the data were expressed as survivor curves from which the decimal reduction time (D value) was

obtained. Linear regression analysis methods were used to compute the slope of the survivor curves.

The results of these experiments indicated that at temperatures below 285° F., increasing the gas flow rate decreased the D value. However at 285° F., the effect of increasing the gas flow rate was to increase the D value. The resistances observed in the nitrogen tests were about the same as those obtained in the air studies. This seemed to mitigate the possibility of oxidation by atmospheric oxygen as being a major factor in dry heat thermal destruction of bacterial spores.

These results seemed to indicate that as the gas flow rate is increased the effect of temperature on the destruction rate of the spores is lessened, the z value being very large. It is believed that the higher gas flow rates cause a greater dehydration of the spores and that spore moisture loss is the determining factor in dry heat thermal resistance.

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By

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to my parents

TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

I. INTRODUCTION

The wet heat destruction of bacteria has always been important to the medical profession, the food industry and the drug industry. Recently with the advent of the space age, dry heat sterilization has become important for sterilizing interplanetary probes. The sterilization of spacecraft has provided a considerable impetus to dry heat thermal resistance research.

Very little is known about factors affecting dry heat destruction of bacteria. The obvious parameters such as time and temperature necessary for the destruction of bacterial spores are still not completely understood because another variable, moisture level, is implied. The surfaces on which the bacterial spores are present have considerable effects on their resistance to dry heat. The atmosphere in which the spores are heated presents many problems to the scientist who tries to predict bacterial resistance. The moisture content of the atmosphere, its chemical composition and its pressure are all variables which are still not completely understood in their relationship to dry heat resistance of bacterial spores.

It is the purpose of this study to try to understand and gain insight into two of these variables: (1) the effect of nitrogen vs. air and (2) the gas flow rate.

II. LITERATURE REVIEW

A. Characteristics of Spores

1. Composition Related to Heat Resistance

The formation of spores is perhaps one of the most outstanding characteristics of the bacterial genera Bacillus and Clostridium. The spores of these genera are characterized as being highly refractile, and having high density. These spores, by virtue of their dormant state maintain greatly reduced metabolic activity. It has been postulated that in the genus Bacillus the spore consists of a central cortex surrounded by a spore coat of protein, (Warth, Ohye, and Murrell, 1962). The central cortex has been shown by electron microscopy to be a layered matrix (Mayall and Robinow, 1957).

One compound which has been consistently found in the genus Bacillus is dipicolinic acid (DPA) (Church and Halvorson, 1959); the DPA content of Bacillus spores usually ranges from 5 to 14 percent dry weight (Murrell and Warth, 1965). This DPA is always bound by calcium ions so that no matter what the DPA content, the calcium-dipicolinic acid ratio is approximately one (Murrell and Warth, 1965).

These authors (Murrell and Warth, 1965) correlated various factors to heat resistance of the spores. They

developed multiple regression correlation equations for seven variates, and correlated these variates to heat resistance. These variates were: dipicolinic acid, diaminopimelic acid, hexosamine, spore weight, Mg-Ca, Ca, Mg. These equations enable heat resistance to be predicted with a fair degree of precision.

The calcium content appears to have an effect on the heat resistance of bacterial spores; increasing calcium content appears to increase the spore resistance to wet heat (Curran, Brunstetter and Meyers, 1943). Sugiama (1951), Amaha and Ordal (1957) and others have observed the fact that calcium is needed in the development of heat resistant spores. Furthermore, α,ε-diaminopimelic acid (DAP) has also been shown to be a constituent of heat resistant spores (Warth et al., 1962).

Characteristics Under Wet Heat

Amaha and Ordal (1957) showed that the heat resistance of bacterial spores increases with increasing calcium ion content of the spores. The effect of holding the spores in calcium buffers demonstrated by Alderton, Thompson and Snell (1964) may be to increase their calcium ion content and subsequently their heat resistance. It is questionable whether the resistance would increase when held in solutions other than calcium buffers. Alderton, Thompson and Snell (1964) stated that holding of spores in sodium buffer at

pH 5.7 gave only minor enhancement of heat resistance, but at higher pH the sodium ion was effective in conferring heat resistance.

Because of the relationships between DPA content and heat resistance, and observations that spore development temperature affects heat resistance, the central cortex appears to be functional in heat resistance. The moisture content of the spores is believed to have an effect on their heat resistance. Early investigators (Cohn, 1877; Burke, 1923) thought that the spore contained a water impermeable membrane. This has been challenged by Murrell and Scott (1958) and Black and Gerhardt (1962).

Henry and Friedman (1937) postulated a theory for the heat resistance of bacterial spores, which claimed that most of the water in the spore is bound water, and that this bound water may be inactive in so far as its influence on the coagulation of protein material by heat is concerned. Friedman and Henry (1938) determined the bound water content for spores and vegetative cells of Bacillus subtilis, B. megaterium, and B. mycoides. They found that in all cases, the spores had a greater water binding capacity than did the corresponding vegetative cells. Waldham and Halvorson (1954) determined the relationship between equilibrium vapor pressure and moisture content of bacterial spores. They proposed that the spore is heat resistant because the proteins in the spore are immobilized through linkages involving the polar groups and some solid material.

Sadoff et al. (1965) investigated the effect of various solvents on the properties of glucose dehydrogenase from spores of Bacillus cereus in an effort to understand the mechanism of its heat resistance in spores. These authors found a million fold decrease in the heat resistance of the glucose dehydrogenase in vivo when spores germinate. They found that this same range of heat resistances can be produced with isolated enzymes when subjected to various ionic environments. These experiments suggest that the heat resistance of bacterial endospores may be a function of the heat resistance of the enzymes.

It is known that several enzymes which are present in spores show greater heat resistance than the enzymes present in the vegetative cells (Stewart and Halvorson, 1954). There is also evidence that the more heat resistant bacterial spores are possibly more strongly disulfide bonded in the protein layers of the cell (Murrell, 1964).

3. Characteristics of Dry Cells

Black and Gerhardt (1962) found evidence that the spore consists of a central core which is kept relatively low in moisture in the dormant spore. Since dry proteins are more thermostable than moist proteins, this may have some bearing on heat resistance. Black and Gerhardt (1962) in their studies of the permeability of spores found that the water is distributed unevenly, with the core having little water and a high density. Finally these investigators

proposed that the core of the dormant spore exists as an insoluble and thermostable gel, in which cross linkages between macromolecules occur through stable but reversible bonds so as to form a high polymer matrix with entrapped free water.

The theory of a contractile cortex system in the bacterial spore would provide a mechanism to dehydrate the protoplast and a mechanism for maintaining this state. The contractile cortex theory is compatible with the current data available on water permeability of bacterial spores (Lewis, Snell and Burr, 1960). Since it seems probable that there is free passage of water into and out of the bacterial spore (Black and Gerhardt, 1962), this water need not be entirely excluded by mechanical pressure from the cortex.

B. Kinetics of Bacterial Destruction

To discuss the death of bacteria, we must have some suitable criterion of death. Schmidt (1954) has stated that the only single practical criterion of death of microorganisms is their failure to reproduce, when as far as is known, suitable conditions for reproduction are provided. If we assume that this definition of death is satisfactory then we must inquire into what causes a cell to lose its ability to reproduce.

Amaha and Sakaguchi (1957) have stated that the death of bacterial spores by heat is a gradual chemical process

which proceeds step by step. Rahn (1945) proposed a logarithmic order of death for bacteria. This theory stated that the death of a cell is due to the inactivation of a single critical molecule. He therefore asserts that the death rate should follow the same kinetics as does a monomolecular decay reaction.

The expression for this rate of death is then

$$\frac{dN}{N} = -kdt$$

Where,

N = number of organisms remaining alive

k = rate of destruction (constant)

t = time

By integrating this equation

$$\int \frac{dN}{N} = -k \int dt$$

we obtain,

$$\ln N = -kt + C$$

where C = constant of integration

using the initial conditions where $t = 0$ and $N = N_o$,

$$\log N_o = C$$

$$\log N - \log N_o = -kt$$

Katzin, Sanholzer, and Strong (1943) obtained the following equation for a 90 percent reduction in the original population:

$$k = \frac{1}{t} \times \log N_o/0.1N_o$$

Here they defined t as the decimal reduction time. Schmidt (1954) changed the equation slightly by using D for decimal reduction time and substituting it for 1/k obtained

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

This equation then suggests a straight line relationship if we plot log N vs. t. This is the equation of the logarithmic order of death as it was originally proposed by Rahn.

Much evidence appears to support the logarithmic theory, (Madsen and Nyman, 1907; Watkins and Winslow, 1932; Rahn, 1945), however, many investigators have obtained survivor curves which are not linear. A number of workers have obtained wet heat survivor curves which were concave downward (Amaha and Ordal, 1957; El-Bisi and Ordal, 1956; Licciardello and Nickerson, 1963, Lechowich and Ordal, 1962 and Fox, Eder and Pflug, 1967). Other investigators have found wet heat survivor curves which were concave upward (Frank and Campbell, 1957; Walker, Matches and Ayres, 1961; Fox, Eder and Pflug, 1967).

Various explanations have been offered for these nonlogarithmic curves. Rahn (1945) suggests that curves which are concave downward are caused by clumping of the individual bacterial cells. Fox, Eder and Pflug (1967) gave as a possible cause of their concave upward dry heat survivor curves, the fact that a mixed culture might be present. Rahn (1945) suggests that curves which are concave downward are caused by clumping of the individual bacterial cells. El-Bisi and Ordal (1956) explained their concave downward wet heat survivor curves as follows: during relatively short heating times some of the viable spores were not heat activated and as a result germination did not occur when these samples were plated. At longer times, the number not activated diminished. Because the number of spores surviving at short times was underestimated, the survivor curves did not drop logarithmically until all viable spores were heat activated.

C. Destruction of Bacteria by Wet Heat

1. General

Wet heat is defined as heating in an environment of 100 percent relative humidity or an environment in which the water activity is very close to 1.00. The water activity is the moisture content of the spore which is in equilibrium with the moisture in the surrounding atmosphere.

Wet heat refers to the heating medium being saturated steam above 212° F. or water at temperatures below 212° F.

One of the first theories for the destruction of bacteria was proposed by Lewith (1890). He proposed that the coagulation of protein was the cause of death of bacterial spores for both wet and dry heat. His theory regarded the moisture content of the bacterial cell of extreme importance in determining its heat resistance. He therefore believed that bacteria were much more labile to wet heat than to dry heat because of the greater thermolability of moist proteins.

Rahn (1945) in explaining his logarithmic order theory postulated that a single critical molecule was coagulated or denatured and this caused the cell to die. Ingraham (1962) proposed that a hereditary factor in the cell was somehow inactivated by the heat.

To understand the destruction of bacterial spores in wet heat it is necessary to discuss some of the processes involved in producing spores from vegetative cells. The genus Bacillus forms spores under a variety of conditions. These conditions of spore formation affect the heat resistance of the final spores. The transformation of a vegetative cell into a spore is accompanied by a number of changes in the physiology and biochemistry of the bacterial cell. Although the complete mechanism of spore formation has not been elaborated, a number of factors have been shown to promote sporulation in the Bacillus species.

Srinivasan (1965) found that extracts of spore formers possess the ability to transform vegetative cells of B. cereus to granulated cells having the property of sporulating endotrophically i.e., sporulating in distilled water. Amino acids appear to play an important role in the sporulation process; Bernlohr (1965) found that during sporulation there is an increase in the intracellular pool of many amino acids. Isoleucine and valine are oxidized to carbon dioxide during sporulation in B. licheniformis. Many workers (Amaha and Ordal, 1957; Murrell and Warth, 1965) have found that Mn^{++} ions will induce sporulation in the genus Bacillus.

Before spores of bacteria will germinate, they must be either heat activated or somehow chemically treated. Rieman and Ordal (1961) showed that certain B. subtilis spores can germinate without heat activation on alanine initiation, merely by the addition of calcium and DPA. Since the activity of alanine dehydrogenase has been found to be very high in germinating spores, O'Connor and Halvorson (1960) proposed that alanine and some of its analogues initiate germination since they are deaminated by alanine dehydrogenase. The method of heat shocking spores to cause them to germinate was first used by Curran and Evans (1945). Heat activation causes various changes in the spores, stimulates germination, activates enzymes which are dormant in the resting spores, and changes some of the requirements for germination. The exact temperatures and

times required for heat activation vary widely with the particular type of bacterial spore (Busta and Ordal, 1964). Keynan et al. (1964) suggested that the activation changes the tertiary structure of a protein responsible for the maintenance of the dormant state of spores, and that this is a type of reversible denaturation. However, if germination is not induced by placing the spores on a suitable nutrient medium immediately after heat activation, the spores will revert to the dormant state (Curran and Evans, 1945).

After activation has occurred, the spore loses its refractility and assumes the characteristics of a vegetative cell. Levinson and Hyatt (1956) observed that special nutritional requirements are necessary to convert the spore into a vegetative cell and these requirements are different than those for germination. At the initiation of outgrowth new proteins are formed which were not present in the resting spore (Kobayashi et al., 1965). Finally, during outgrowth, a new vegetative cell is produced with all the characteristic properties.

2. Factors Affecting Wet Heat Resistance

a. Medium.--There are a number of factors which can affect the observed wet heat resistance of bacteria. Frank and Campbell (1955) have found that the survival time of spores was longer using certain subculture media which might indicate that a severe reaction is necessary to make

a spore incapable of reproduction in a nutritionally more complete subculture medium.

b. pH.--The pH of the wet heat environment is very important to the wet heat resistance. Most proteins and other biological compounds have their maximum molecular stability at or near pH 6.8. For this reason the observed resistance of bacteria in an acid medium might be much lower than at neutral pH.

c. Salts.--Salts can play an important role in the destruction of bacteria by wet heat. Since proteins, DNA, and other macromolecules present in the bacterial cell are largely stabilized by hydrogen bonds, any change in the ionic strength of the environment may tend to rupture some of these hydrogen bonds, and therefore lower the heat resistance. Certain compounds such as quarternary ammonium salts are believed to interfere with bacterial fermentation or respiration by inhibiting the enzymes of carbohydrate metabolism.

d. Age of Cells.--The age of the cells may have some effect on their exhibited heat resistance. Esty and Meyer (1922) found that young moist spores are more resistant than old spores under wet heat conditions, however, Williams (1929) found no correlation between the age of the spores and their resistance when several species of organisms were examined.

e. Growth and Sporulation Temperature.--The temperature at which the spores are grown appears to have some

effect on the resistance of the spores. Early studies showed that the resistance of spores of Bacillus anthracis increases with increasing growth temperature (Weil, 1899). El-Bisi and Ordal (1956) found the resistance of spores of a strain of Bacillus coagulans to be greater with increased sporulation temperature.

It can be safely said that many compounds will have an effect on the heat resistance of bacterial spores. For example, Sugiama (1951) found an increase in the heat resistance of bacterial spores when heated in a menstruum containing some soluble carbohydrate. Jensen (1945) found an increase in the resistance of Streptocci sp. when heated in a medium high in fat instead of a purely aqueous medium.

D. Destruction of Bacteria by Dry Heat

1. General

The destruction of microorganisms by dry heat is certainly different in magnitude and may also be physiologically different than destruction by wet heat. Unlike wet heat which is precisely defined as heating in a medium consisting of 100 percent relative humidity, dry heat is much less readily defined and is usually taken to refer to the destruction of microorganisms in any environment where the relative humidity is less than 100 percent or where the water activity is less than 1. It is often assumed that dry heat is an equally specific condition as is wet heat;

this is not true: dry heat includes an endless number of combinations of very different conditions. Some of these conditions are: (1) initial water content of organisms before dry heating, (2) the nature of the liquid medium or menstruum from which the organisms are dried, (3) the relative composition of the gas atmosphere in contact with the organisms during dry heating, and (4) physical nature of the material in contact or supporting the dry spores.

Early studies on the dry heat resistance of bacterial spores of Clostridium botulinum brought a new era into the study of heat resistance of microorganisms (Tanner and Dack, 1922). The study of dry heat resistance was then relatively slow due to the difficulty in defining dry heat and the apparent greater variability of resistance to dry heat than to wet heat. Later Collier and Townsend (1956) studied the resistance of Bacillus stearothermophilus, B. polymyxa and Putrefactive anaerobe 3679 under superheated steam. Pflug (1960) performed dry heat resistance experiments on Bacillus subtilis spores in superheated steam. It was found that the resistance to superheated steam was much greater than the resistance to saturated steam.

Pflug and Augustin (1961) found larger resistances when spores of B. subtilis were exposed to dry hot air than to steam. Jacobs, Nicholas and Pflug (1965) studied the heat resistance of Bacillus subtilis spores in atmospheres of different water content. They held the test spores in

thermal death time cans (Pflug and Augustin, 1961) maintained at water vapor contents ranging from 0 to 100 percent controlled by various hydrated salts. Their results showed that the resistance of the spores was very much dependent upon the moisture content of the atmosphere in which the spores were heated.

2. Factors Affecting Dry Heat Resistance

Murrell (1964) classified factors affecting dry heat resistance as occurring before heating, during heating, and after heating. The factors present during heating are those which are pertinent to a study of dry heat resistance.

a. Initial Number of Organisms.--Sisler (1961) determined survivor curves for different initial numbers of spores of Bacillus subtilis heated in wet steam at 250° F. and in superheated steam at 320° F. The D value for the 10^6 spores/ml was greater than the D value for the 10^7 spores/ml when tested in superheated steam. These differences were not observed with wet steam.

b. Water Activity.--The effect of water activity has been found in many cases to affect the exhibited resistance of spores to dry heat (Murrell and Scott, 1957; Marshall, Murrell and Scott, 1963). Recently Murrell and Scott (1966) have reported a method in which they controlled the water activity (a_w) of the spores during the actual heat treatment. These workers found a maximum resistance when the controlling water activity was around 0.3. In all cases

they found a decrease in resistance as the water activity approached either 0 or 1, the resistance being much lower at water activity of 1.

c. Menstruum.--Fry and Greaves (1951) found that the survival of spores during and after drying was very dependent upon the liquid or menstruum from which they were dried. They found that when bacterial cells were dried from plain distilled water, their survival time in the dried state was shorter than if the cells were dried out of some other solutions. When they dried cells of Staphlococcus aureus from glucose solutions, they found a much greater survival time. These workers proposed that the glucose acts as some sort of protective colloid. They claimed, however, that a much more probable explanation of the sugar effect is that the presence of glucose insures the retention of a certain amount of moisture in the dry spores which is necessary for survival. Annear (1956) found that freeze dried bacteria could be preserved for long periods of time by suspending them in peptone solutions prior to freeze drying. He attributed this increased survival to the fact that the peptone protects the cells from becoming too dry during the freeze drying process.

Heller (1941) found that significant decreases occurred in the death rates of freeze dried Streptococcus pyogenes and Escherichia coli when the organisms were dried in a menstruum containing crystalline compounds of high

solubility and which the organism was capable of utilizing in normal fluid media. When Streptococci were dried in the presence of colloidal substances, the death rates decreased with increases in the hydrophylic property and in the protective colloid effect of these compounds.

d. Support Medium.--Another factor which appears to influence the dry heat resistance of bacterial spores is the support medium. Augustin (1964) found that the dry heat resistance of Putrefactive anaerobe 3679 was higher on tin than on aluminum. Pflug and Fox (1966) determined the dry heat resistance of Bacillus subtilis spores on various support media. They found that the highest resistance occurred when the spores were heated on tin. Aluminum, glass and filter paper showed respectively decreasing spore resistances. The reason for the very low resistance of the spores on the filter paper might be attributed to the fact that on this medium the spore suspension diffused through the paper and thus the spores were relatively scattered apart as compared to clumping of the spores on the tin or aluminum. It is possible that when the spores are very close together as on the tin and glass, there might be some protective effect of the cells themselves. Another possibility is that when the spores are clumped they might retain just enough water to permit them to survive longer (an effect similar to that found by Fry and Greaves (1951)).

Many workers have experienced differences in resistance of spores to dry heat when supported on different surfaces (Angelotti, 1967; Fox and Pflug, 1967; Bruch, 1963). These workers in trying to determine sterilization parameters for the National Aeronautics and Space Administration (NASA) have found that one of the main problems in this area is the recovery of the organisms from the surfaces. At the present time there are many different methods available and in use for recovering the spores from the test surfaces. Some of these include soaking the surface in water solutions, shaking these solutions, scraping the surface to loosen the spores, using ultrasonicators for removing the spores, and breaking up the surface. Puleo et al. (1966) have reported a system in which they used two methods for removing spores from surfaces and within various solids. In one method they pulverized the surface material (usually a plaster-like material) in a Waring blender; in the other method they used a mortar and pestle. They found that the best recovery was obtained using the mortar and pestle. Other workers (Favero, 1966) have obtained satisfactory results using an ultrasonicator to remove the heated spores from the test surface material.

The fact that the surface spores are very difficult to remove may be the reason for some of the variation reported in dry heat data. Bruch, Koesterer, and Bruch (1963) determined the dry heat resistance of several

species of spores using various support media. These investigators used paper, glass and sand for supporting the dry spores for thermal destruction testing. They found that in most cases the highest D value occurred when the support medium was sand and the lowest D value occurred using the paper strip. These investigators also determined the z value for B. subtilis v. niger in dry heat. They found a z value of 49° F. for this organism. This is quite high when compared with a z of 17° to 25° F. usually obtained with this organism in wet heat.

e. Effect of Test Atmosphere.--Pheil et al. (1967) studied the effect of various gas atmospheres on the dry heat resistance of bacterial spores. They found that the resistances of B. subtilis in air and carbon dioxide were essentially the same, but higher in helium and nitrogen. Oxygen gave values close to those obtained with air and carbon dioxide. These results seem to lessen the possibility of some type of oxidation mechanism being involved in dry heat bacterial destruction.

Silverman (1966) found that the dry heat resistance of various species of spores was greatly reduced when the spores were heated in an atmosphere with dry air flowing over the spores at the rate of 3 liters per minute. He also found that the resistance increased as the moisture content of the air increased.

3. Variability of Dry Heat Data

At the present time the data resulting from dry heat experiments are rather inconsistent and very difficult to reproduce. Many workers have found difficulty in replicating plate counts during various dry heat resistance experiments (McDade, 1967; Angelotti, 1967; Fox, 1967). These workers have found variability among replicate samples during dry heat testing to be as much as ten fold and often greater. The causes of this variability have not yet been explained. One of the most promising explanations is the poor recovery of spores from surfaces experienced in most cases as previously discussed. Other factors which might cause some variation have been listed by Bruch (1963) as factors which appear to affect the dry heat resistance of bacterial spores: (1) effect of spore carrier on dry heat resistance of bacterial spores; (2) effect of spore nutrition on dry heat resistance of bacterial spores; (3) effect of sporulation environment on the dry heat resistance of bacterial spores; (4) effect of recovery environment on the survival of spores exposed to dry heat; (5) effect of exposure environment on microbial dry heat resistance; (6) effect of combination of sterilizing agents on the rate of microbial destruction.

The variability of dry heat resistance data is very great as exemplified by the findings of the Spacecraft Sterilization Advisory Committee. This committee found

decimal reduction times for B. subtilis v. niger heated on stainless steel surfaces at 125° C. to vary from 8 minutes to 40 minutes when determined in various different dry heat testing apparatus (Spacecraft Sterilization Advisory Committee, 1967).

III. EXPERIMENTAL PROCEDURE

A. Design of Apparatus and Description of Equipment

One of the primary objects of this research was to provide an accurate method for heating the spores. Existing devices were not satisfactory, therefore a new heating device was needed that would satisfy the following conditions:

1. The temperature of the spores should remain constant throughout the test period. An accuracy of plus or minus one degree F was desired.
2. The device would hold six sets of samples, each set consisting of six replicates, each set of replicates removable from the oven without disturbing the other samples.
3. Reproducible heating and cooling times are needed; the come-up time of the samples should be as short as practicable and the cool down time of the samples after removal from the oven as rapid as practicable.
4. The method of removing the samples from the oven after heating must be aseptic.

5. Provision should be made for preheating air or other gases used in gas flow tests.

A simple device was designed and constructed to meet the above criteria; it was constructed and performed satisfactorily. The apparatus or oven that was constructed can be thought of as a large metal block 5 x 8.5 x 9 inches with six slots for the six sets of samples. The large mass of metal with its considerable thermal capacity simplifies temperature control problems. Five sides of the block were insulated leaving only the face of the block exposed.

The oven was constructed from two large aluminum blocks each 2.5 x 8.5 x 9.0 inches. Figure 1-a is a photograph of the bottom block illustrating the heating chambers; each chamber is fitted with a sample bar containing six positions for samples. Each sample bar has a push rod made of heat resistant material. The push rods are adhered to the sample bars with epoxy resin.

Figure 1-b shows the assembled top and bottom blocks; each block contains two electrical cartridge heaters. A Honeywell L7038 thermistor is located in the bottom block. This thermistor, when used with a Honeywell R7081C temperature controller (described later) gives $\pm$ 0.3° F. temperature control over the range 220° F. to 320° F.

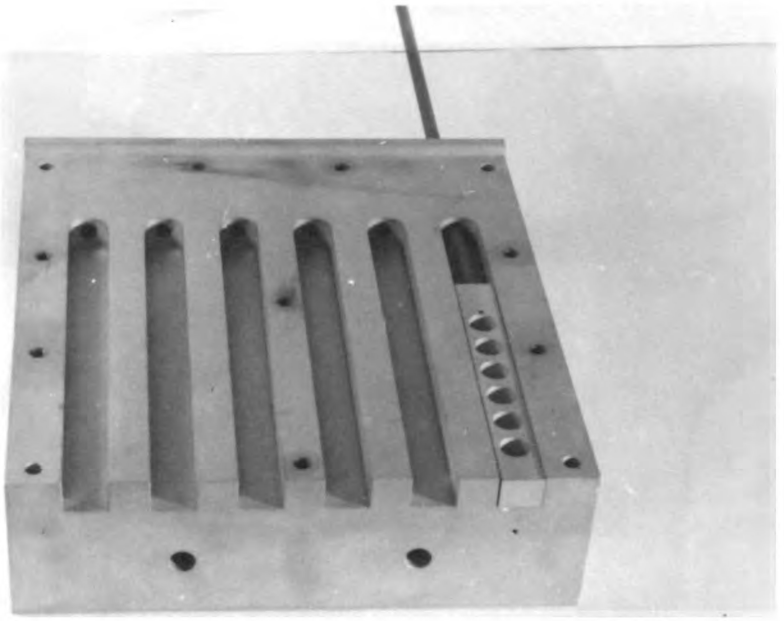
Slits 1/16-inch in width, 5-inches in length and 1-inch deep to heat the incoming gas are located in the top block. The gas enters through an opening on the side of the unit and goes into a manifold that feeds the six

sets of slits. The gas flows to the rear of the oven through the heating slits. Each set of slits has a hole at the rear which carries the gas to the rear of the sample heating chambers. The gas then flows over the spore samples and out the front of the oven. Figure 2 shows a diagramatic view of the gas flow path through the oven. An aluminum plate 1/8-inch thick placed on top of the upper block seals the gas heating area.

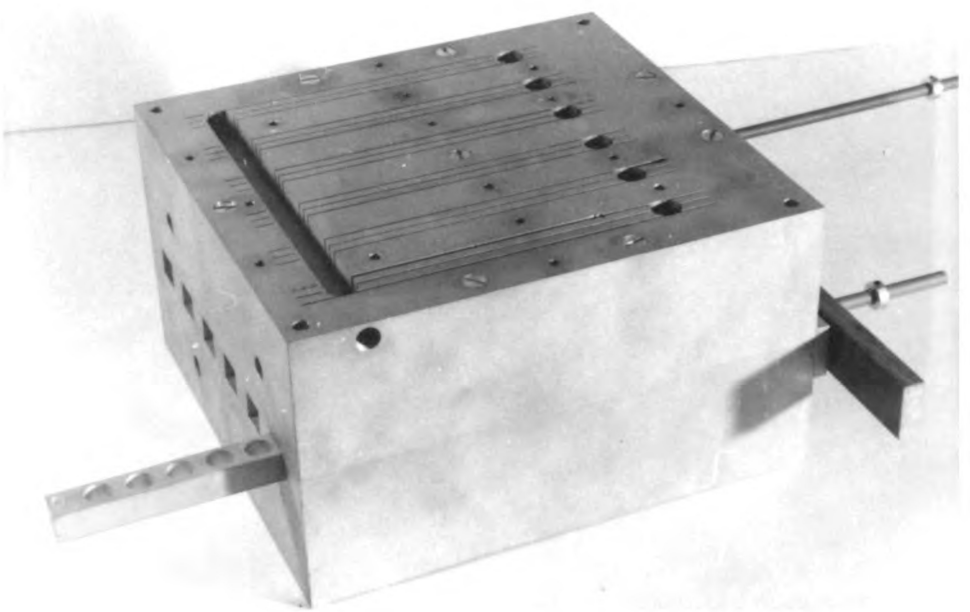
To minimize the heat loss from the unit the entire device is located inside a 3/4-inch thick plywood box. Spacers keep the plywood 1/2-inch from the surface of the aluminum.

Attached to the front of the oven is a movable shelf that can be located in two positions. The first position is directly under the sample bars as shown in Figure 1-c. With the shelf in this position, the sample cups are placed in the bars. The shelf enables all 36 sample cups to be loaded at the same time. The six bars are then pulled into the oven simultaneously by means of the cross bar located at the rear of the oven.

After loading, the shelf is moved to its second position, about 1-inch below the previous level. In this position the shelf can hold a sterile petri dish for receiving the sample cups. As shown in Figure 1-d a bar has been pushed out and the sample cups have fallen into the sterile petri dish. The detailed operation of the oven will be described in a later section.

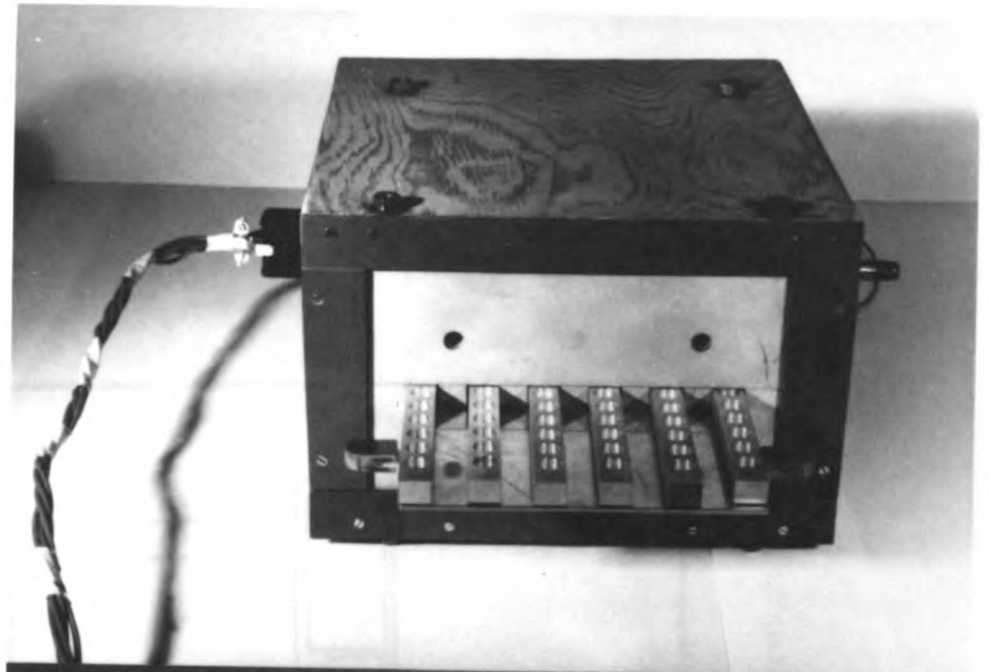


1-a



1-b

Figure 1-a (Top).--Bottom block of dry heat oven.
1-b (Bottom).--Assembled top and bottom blocks of dry heat oven.



1-c

1-d

Figure 1-c (Top).--Dry heat oven showing upper position of sample shelf.
1-d (Bottom).--Dry heat oven showing petri dish in position for receiving sample cups.

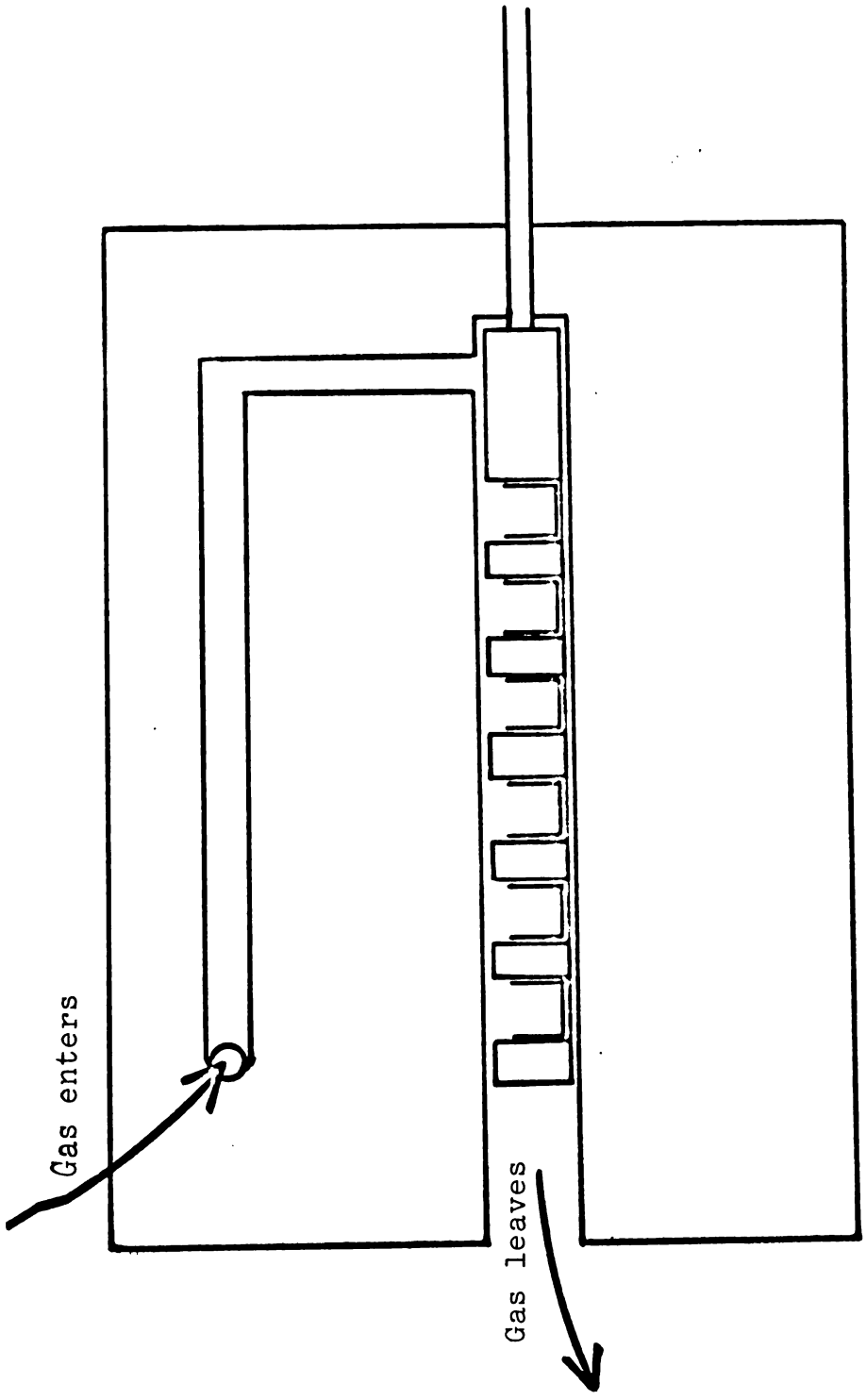


Figure 2.--Diagramatic pattern of gas flow through dry heat oven.

The temperature controller is a Honeywell Versa-Tran Transistorized amplifier relay #R7081C, designed to control a heating load. Using the L7038 thermistor sensor it was possible to control the oven heaters so the oven temperature variation was within a temperature differential of approximately 0.3° F. This temperature controller actuated a normally open 10 amp relay which in turn controled the input current to the four heating elements; the heating elements are 3/8-inch diameter x 6-inches long and are rated at 250 watts at 120 VAC. Figure 3 shows a schematic block diagram of the oven electrical control circuit. Eighty minutes were required for the oven to heat from room temperature to 260° F.; a timing device was included in the electrical circuit so the oven could be automatically turned on 2 hours before testing was to be started.

To study the effects of gas flow rates on the rate of destruction of the bacterial spores a system was designed to carry and meter the gas from the compressed gas cylinders to the oven. The gas flow rate was controlled by a diaphragm pressure reducing valve attached to the gas cylinder. The gas was metered using a previously calibrated rotameter.

It was important that the gas flowing over the spores be at the same temperature as the spores. By inserting thermocouples in the chambers within the oven just above the positions where the spores are held in the cups, it was found that the gas temperature was always within 1° F.

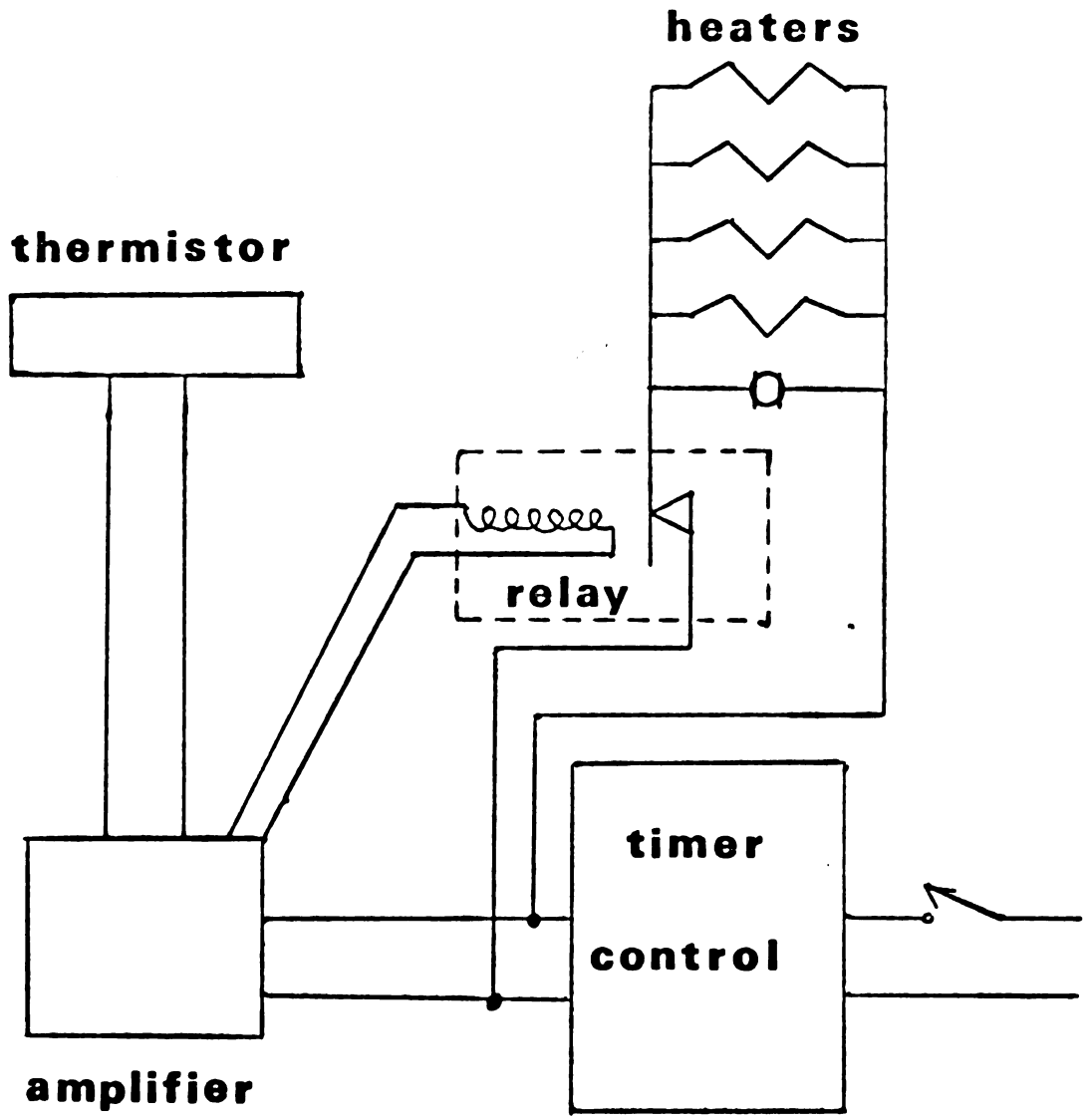


Figure 3.--Schematic block diagram of electrical circuit controlling dry heat oven.

of the oven temperature. This was verified at both the high flow rate (4 cfm) and the low flow rate (1.4 cfm).

B. Calibration of Dry Heat Oven

To calibrate the oven, it was necessary to determine a number of parameters: temperature control cycle and fluctuation, heating up time, heating and cooling times of the sample cups, temperature variation among cups within the same bar, temperature variation between bars, and operating range of the oven.

1. Temperature

The temperature of the oven was found to follow a cyclical pattern; the magnitude of the fluctuation at 260° F. was about 0.5° F., and the cycle time was 5.5 minutes. Since the oven was provided with several thermocouple holes, it was possible to measure the temperature at various points across the block. It was found that the greatest temperature differential in the block was about 1° F.

2. Heating Time

The oven required about 80 minutes to heat from an initial temperature of 75° F. to an operating temperature of 260° F. The heating curve (temperature vs. time) is linear.

3. Heating of Cups

To determine the heating and cooling times of the sample cups a small (30 gauge) thermocouple was soldered in the bottom of a sample cup. This cup was then loaded into the special bar and pulled into the oven. Using a Honeywell Recording Potentiometer, the temperature was recorded every 5 seconds during the heating of the cups. These time-temperature data were then plotted by the method described by Ball and Olson (1957). This method involves plotting the temperature difference, heating medium temperature-sample cup temperature, on inverted semi-logarithmic coordinates versus heating time. The temperature response parameter f is defined as the time required for the resulting linear heating curve to traverse one log cycle (Kopelman, 1966). The heating lag is expressed by

$$j = \frac{T_1 - T_a}{T_1 - T_o}$$

where T_a is the ordinate of the origin of the asymptote

T_1 is the temperature of the heating medium

T_o is the initial temperature of the sample cups

4. Cooling of Cups

To determine how rapidly the cups cool after removal from the oven into the sterile petri dish, cups with the

small thermocouples were pushed from the oven and allowed to cool while the temperature was recorded on the potentiometer. These data were plotted as cooling curves (Ball and Olson, 1957) and the f_c (temperature response parameter for cooling) and j_c (cooling lag) were calculated.

Figure 4 shows a sample heating curve for the cups. Table 1 shows the f and j values of the sample cups for various oven temperatures determined from the various heating curves and also the lag correction factors. The lag

TABLE 1.--Lag correction factors, and f and j values for cups heated in dry heat oven in still air.

Temp. °F.	f_h (sec)	f_c (sec)	j_h	j_c	Lag Correction Factor (min)					
					z=10	20	30	40	60	80
255	117	80	0.71	1.0	1.8	1.3	0.9	0.67	0.28	0.02
265	120	91	0.75	1.0	2.5	1.9	1.5	1.4	0.87	0.61
275	110	78	0.70	1.1	1.9	1.4	1.0	0.80	0.45	0.20
285	135	84	0.33	1.0	1.8	1.1	0.67	0.40	--	--

correction factor is the time which should be subtracted from the actual heating time in a thermal resistance study since it represents the initial heating time where no significant lethality is accrued and the lethality of the cool that occurs after the end of the heating time. The lag correction factors were calculated by the method of Pflug (1966). Since these values were relatively small,

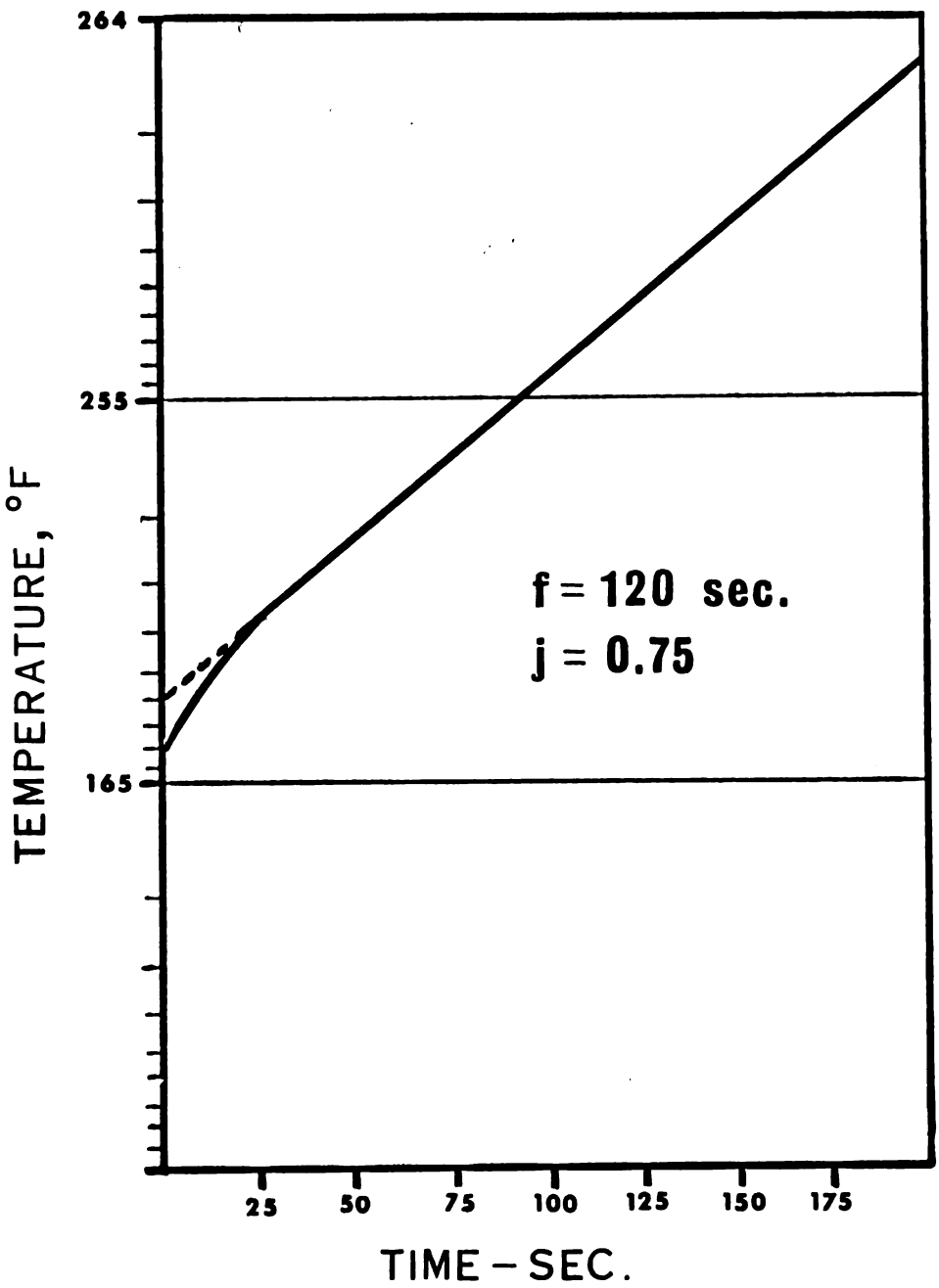


Figure 4.--Heating curve for sample cup at 265° F. in still air.

it was decided to discount them as being insignificant in plotting the survivor curves.

5. Temperature Variation of Cups Within the Same Bar

To determine the temperature variation of the six cups within one bar a special bar was prepared with a hole drilled as shown in Figure 5. A sample cup with a 30 gauge thermocouple soldered to the bottom was placed in each hole and the six pairs of lead wires passed through the hole in the bar through the push rod out the rear of the oven and then to the potentiometer. The bar was moved into the oven, and after the cups had reached oven temperature the temperatures were recorded on the 12 point recording potentiometer. The temperatures of the six cups within the same bar agreed to within 0.5° F. It is felt that part of this temperature difference may have been due to the thermocouples themselves.

6. Temperature Variation Among Bars

To determine if there was any temperature variation among the six bars, each bar was provided with a thermocouple inserted through the push rod into the thermocouple hole at the rear of the bar. A set screw at the rear of the bar held the thermocouple in place. In this manner each bar temperature could be measured each 5 sec. using the recording potentiometer. It was found, after observing the recorder output for this test for several hours, that

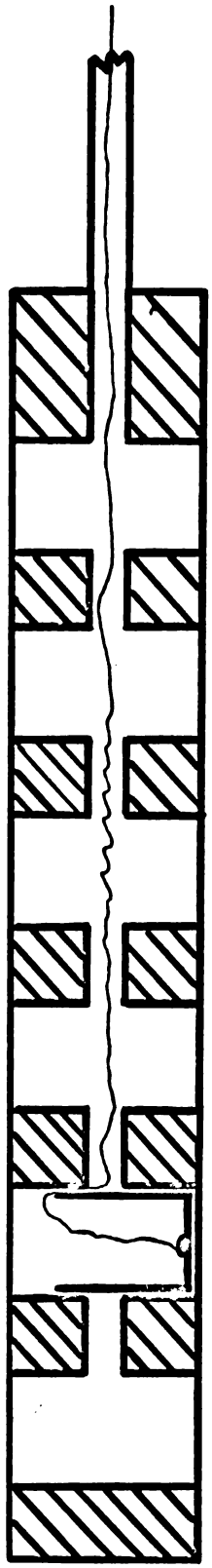


Figure 5.--Special sample bar allowing passage of up to six thermocouple wires.

there were essentially no differences in bar temperatures. Due to the method of printing of the temperatures and the inherent error in the potentiometer only differences greater than about 0.4° F. can be discerned.

7. Operating Range of the Oven

As a final test it was necessary to determine the limits of temperature within which the oven could be operated. To do this the oven was set at different temperatures and the temperature fluctuation observed. It was found that at all the temperatures tested from 230° F. to 315° F. the temperature control was within 0.4° to 1° F. This fluctuation is due in part to the temperature profile set up in the block. Temperatures above or below this range were not tested since it was not desired to utilize such temperatures.

8. Calibration of Thermistor Amplifier

It was necessary to calibrate the thermistor amplifier so the temperature could be preset to within 3° F. The remaining fine control was accomplished using the potentiometer. A dial plate on the thermistor amplifier was simply rotated and reset to assure calibration. In this manner it was possible to set the temperature to within 3° F.

Since all the bar temperatures were the same the monitoring of the system during dry heat testing was accomplished by measuring the temperature of the third bar.

C. Description of Operation of Oven

The following discussion will describe an actual detailed operation of the oven under zero air flow conditions. (For convenience this discussion will assume that the oven is operated at 270° F. for a total time of 3 hours, and that the samples will be withdrawn at 30-minute time intervals.) The procedure is as follows:

1. Adjust temperature control (approximately ± 3° F.).
2. Set preheating timer.
3. When oven stabilizes readjust control so that oven cycles ± 0.5° F. of the control temperature.
4. Load 36 samples.
5. At t = 0 all samples are drawn into oven and the timer is started.
6. Shelf is lowered to unloading position.
7. Just before t = 30 a sterile petri dish is placed on the shelf.
8. At t = 30 the first push rod is operated to remove samples which fall into petri dish.
9. Using sterile tongs the sample cups are transferred to sterile 10 ml water blanks.

Steps 8 and 9 are repeated for each time interval for the duration of the test.

D. Preparation of Spores

1. Growth and Harvesting

The organism used in this study was Bacillus subtilis 5230 (15 μ). This strain of B. subtilis will grow anaerobically in the presence of fermentable carbohydrate (Sisler, 1961). The original culture was provided by Dr. C. F. Schmidt of Continental Can Company. He originally obtained the culture from H. Curran as a 15 μ strain.

A few ml of the stock suspension were heat shocked and placed in five tubes of dextrose, tryptone, starch (DTS) broth with bromcresol purple indicator. These tubes were incubated for 24 hours at 98° F. after which a loop of suspension was transferred to another 5 tubes of DTS broth. After 24 hours incubation at 98° F., one loop of suspension from each of these five tubes was inoculated onto five plates of sporulation media which were then incubated for 108 hours at 98° F.

The sporulation media consisted of Difco nutrient agar containing 0.5 percent glucose and 1 ppm Mn^{++} from $MnSO_4 \cdot H_2O$. To prepare the media, 0.5 percent glucose was added to the nutrient agar and then autoclaved for 15 minutes at 250° F. Then a solution of $MnSO_4 \cdot H_2O$ (100 ppm) was prepared, sterilized and added to the agar just prior to pouring the plates. The plates were allowed to harden before streaking.

After the plates had been incubated for the appropriate time, 50 ml of sterile distilled water were poured over each plate and an L-shaped glass stirring rod was used to wipe the spores from the surface of the agar. The water which now contained the spores was poured through a sterile glass wool filter through a sterile funnel into centrifuge bottles (250 ml). The surface of the agar was washed once more with 20 ml of sterile water and this solution was also poured into the centrifuge bottles. The suspension was distributed throughout four centrifuge bottles. These spores were then centrifuged for about 30 minutes at 2800 rpm, and the supernatant discarded. The spores were then resuspended in sterile water, shaken to break up any clumps of spores and centrifuged again. This procedure was repeated four times. Finally the spores were resuspended in M/15 phosphate buffer, pH 7.0 and washed in the buffer three more times. They were then stored in this buffer at 35° F.

2. Initial Count

The spore suspension was plated (pour plate) to determine the number of spores. A small sample was removed from the stock bottle and heat shocked for 10 minutes at 212° F. Appropriate dilutions were then made and the spores were plated in duplicate. The media used for this determination was dextrose, tryptone, starch agar (DTS). The plates were incubated for 48 hours at 98° F. and the colonies were

counted. The counts showed that this stock solution contained 8.5×10^8 spores/ml. Microscopic examination of this culture showed that it consisted essentially of 100 percent spores as determined by Malachite Green-Safranin spore stain.

A 1/100 dilution of this suspension was made using the M/15 phosphate buffer and poured into a small jar containing glass beads and a magnetic stirrer. This jar was used as the stock suspension for the entire set of experiments. The magnetic stirrer and the glass beads were used so that each time it was necessary to withdraw samples from the jar, the contents could be mixed to break up any clumps and distribute the spores evenly. This suspension was plated to determine the number of spores and the count showed that it contained 8.5×10^6 spores/ml.

3. Preparation for Heating Tests

To prepare the spores for heat treatment, 0.01 ml of an approximately 10^7 spores/ml suspension was dispensed using a micrometer syringe (Roger Gilmont Instruments, Inc.) into small 11 mm O.D. x 8 mm deep tin plate cups (Pflug and Esselen, 1953). Prior to innoculation the cups had been washed, placed in petri dishes, sterilized in the autoclave at 250° F. for 30 minutes, and dried in an air oven at 250° F. The inoculated cups were dried at 70° F. for 24 hours at 29 in. mercury vacuum and stored in a dessicator over $CaCl_2$ until used.

To determine the initial number of organisms for each test, five inoculated cups were dropped into each of five screw cap dilution tubes containing 10 ml sterile distilled water. These tubes were placed in a boiling water bath for 10 minutes to heat shock the spores. After the heat shock, the caps were tightened and the tubes were shaken vigorously for 10 minutes for removal of the spores from the surface of the cups, and to distribute the spores throughout the 10 ml suspension. A 1/100 dilution was made of each tube and one ml of the dilution was plated (pour plate) with dextrose, tryptone, starch agar. When the agar had solidified, a thin layer of DTS agar was poured over the surface to prevent any spreading of surface colonies. The plates were inverted and incubated for 48 hours at 98° F. The plates were counted, and the average count of the five plates taken as the initial number of organisms. The initial number varied slightly from test to test over the period during which the experiments were conducted. The initial number for various dates throughout the testing program are given in Table 2.

For the heating studies, the spores were prepared as outlined above, placed in the dry heat oven for the appropriate heat treatment time, dropped into a sterile petri dish and transferred into screw cap dilution tubes containing 10 ml of sterile distilled water. The tubes were shaken vigorously for 10 minutes for removal of the spores from

TABLE 2.--Initial spore counts throughout testing period.

Date	Initial Count Spores/Cup
10-17	7.0×10^4
10-26	6.8
11-7	7.3
12-5	6.4
12-13	6.5
12-20	7.0
1-9	6.5

the surface of the cups. Appropriate dilutions were made according to the particular heat treatment which the samples had received, and one ml of the dilution was plated (pour plate) onto DTS agar as outlined above.

E. Methods of Analysis

There are two general methods available for the analysis of heat resistance data: Fraction Negative (FN) and Survivor curve.

The Fraction Negative is a procedure in which the bacterial suspensions to be tested are heated in replicate tubes until the viable population in each tube has reached the point where some of the tubes contain at least one viable cell and some of the tubes are sterile. The method of analysis consists of a most probable number technique

for determining the number remaining and from this value the D value can be determined. This method has been adequately described by Eder (1966).

The other common method and the one used in this study consists of reporting the data as survivor curves. In this method the number of viable organisms is counted after various heating treatments and the number of viable cells is plotted against the time. The plate count-survivor curve method gives more information about the order of death of the bacteria than does the FN analysis. Furthermore the survivor curve does not depend upon any assumptions as does the fraction negative method.

The principle advantage of the fraction negative method of analysis is the fact that it allows one to calculate the D value in the very low survival ranges. By using survivor curve techinque we are limited to about 30 colonies as a lower limit of survival.

In many sterilization experiments it is indeed the low levels of survival that we are interested in. If this is the case it would be a good idea to use the Fraction Negative method in the low survival range and a standard most probable number technique in the upper range. This would generate a survivor curve which would range from the initial number to a very low survival level.

The method used in this thesis involves plotting the number of viable spores at various time intervals against the time on semi-logarithmic coordinates.

The data were analyzed by means of linear regression using the least square approach. The plate counts at the various time intervals were typed onto punched computer cards and the least square coefficients, i.e., the D value (-1/slope) and the apparent initial number N_a (ordinate intercept) were calculated. Eighty percent and ninety-five percent confidence limits were calculated on the D value. To perform this analysis a program was written for the Michigan State University CDC 3600 digital computer. This program calculated for each experiment the D value, the apparent initial number N_a and the confidence limits on the D value. It was found that the confidence limits of some of the D values were large as shown by the analysis which was a modification of the t test. These large confidance limits were caused by the great variability in the replicate plate counts at a given time.

F. Method of Linear Regression

The linear regression D value was calculated by the least squares method of Dixon and Massey (1957). The method will be described here in detail. Each survivor curve test resulted in a series of six replicate plate counts for each time interval. For the analysis, the plate counts (corrected for dilution) represent the P values and the times represent the x values. Each corrected plate count was first converted to the corresponding logarithm so that for this analysis:

$$Y = \log P$$

The x values were left as natural numbers.

The following quantities are then calculated:

(1) $$\bar{X} = \sum_{i=1}^{N} X_i / N$$ N = total number of data points per test

(2) $$\bar{Y} = \sum_{i=1}^{N} Y_i / N$$

(3) $$S_x^2 = \frac{\sum_{i=1}^{N} X_i^2 - \frac{\left[\sum_{i=1}^{N} X_i\right]^2}{N}}{N - 1}$$ variance of x

(4) $$S_x = \sqrt{S_x^2}$$ standard deviation of x

(5) $$S_y^2 = \frac{\sum_{i=1}^{N} Y_i - \frac{\left[\sum_{i=1}^{N} Y_i\right]^2}{N}}{N - 1}$$ variance of Y

(6) $$S_y = \sqrt{S_y^2}$$ standard deviation of Y

(7) $$b = \frac{\sum_{i=1}^{N} X_i Y_i - \frac{\sum_{i=1}^{N} X_i \Sigma Y_i}{N}}{\sum_{i=1}^{N} X_i^2 - \frac{\left[\sum_{i=1}^{N} X_i\right]^2}{N}}$$ slope of regression line

(8) $$D = \frac{-1}{b}$$ decimal reduction time

since by definition

$$D = \frac{t}{\log N_a/N}$$

$$- \log N_a/N = -t/D$$

$$\log N = \frac{-t}{D} + \log N_a$$

This is the equation of a straight line in the form of

$$Y = m\ x + b$$

where

$\log N$ = ordinate value

$- \frac{1}{D}$ = slope

$\text{Log } N_a$ = ordinate intercept

(9) $$\log N_a = \frac{\sum_{i=1}^{N} Y_i}{N} - b\bar{x}$$ apparent initial number

(10) $$Sy \cdot x^2 = \frac{N - 1}{N - 2} (Sy^2 - b^2 - Sx^2)$$ mean square deviation of sample points from regression line

(11) $$Sy \cdot x = \sqrt{Sy \cdot x^2}$$ standard error of estimate

(12) $$b \pm t \frac{Sy \cdot x}{S_x \sqrt{N - 1}}$$

confidence limits of slope

where t is the .975 percentile of the t distribution for N-1 degrees of freedom

(13) $$\frac{-1}{b \pm t \frac{Sy \cdot x}{S_x \sqrt{N - 1}}}$$

confidence limits of D value

IV. RESULTS AND DISCUSSION

A. Decimal Reduction Times

The data from all experiments were used to calculate linear regression survivor curves, as previously described under analysis. Table 3 summarizes the D values obtained for the individual tests showing 80 percent confidence limits for each D value. Table 4 shows the same D values with their 95 percent confidence limits.

Table 5 shows the average linear regression D values after discarding some tests which were felt to be in error. In preparing Table 5, those D values which showed opposition to the theory that as the temperature increases the D value decreases, were discarded. The following D values were discarded:

Temperature (°F.)	Flow Condition	D Value (min)
255	0 flow air	110
275	1.4 cfm air	62
285	1.4 cfm air	109

In those cases where more than one D value was obtained at the particular condition an average was taken of the values.

On examining Table 5 we can see that the resistance at 255° F. (D value) decreased as the flow rate was increased. The D value obtained for the low flow rate (1.4 cfm) of nitrogen was the same as that of the high flow rate of air. However, the high flow rate of nitrogen gave the lowest D value of all.

We see a slight decrease in resistance between the zero flow condition and the low air flow condition at 265° F. However, there is no difference between the D value obtained at the low air flow rate and the high air flow rate. The D values for the nitrogen tests were lower than those for air, but no difference was observed between the two flow rates.

There appeared to be no difference in resistance between the still air condition and the flow conditions at 275° F. No difference was observed between the nitrogen flow rates; however, the nitrogen D values were about 1/2 the value of the D values obtained in the air tests.

At 285° F. the effect of increasing the flow rate of air has been to increase the D value slightly. For the nitrogen tests there is a slight increase in D value with higher nitrogen flow.

Table 6 summarizes the z values (temperature change required to produce a ten fold change in D value) for the various experimental conditions. The data for the closed system dry heat and for the wet heat are from Fox, Eder and Pflug (1967). These data are listed to show the

TABLE 3.--D values (min) for individual thermal destruction tests showing 80 percent confidence limits.

Temperature (°F.)	Air 0 cfm.	Air 1.4 cfm.	Air 4.0 cfm.	Nitrogen 1.4 cfm.	Nitrogen 3.0 cfm.
255	255 (164-575) 110 (83-167) 511 (307-1509)	216 (135-551)	169 (116-315)	151 (115-221)	117 (93-159)
265	144 (104-235) 149 (87-531) 203 (111-1224)	107 (82-155)	108 (71-215) 116 (88-173)	93 (72-131)	97 (75-137)
275	106 (68-241) 94 (71-142)	62 (47-91) 95 95 (70-147)	141 (98-250) 71 71 (56-97	48 (40-60)	57 (48-70)
285	43 (37-51) 50 (41-65)	109 (67-284) 74 (54-120)	128 (76-420) 61 (45-98	64 (45-112)	107 (78-173) 77 (60-107)

TABLE 4.--D values (min) for individual thermal destruction tests showing 95 percent confidence limits.

Temperature (°F.)	Air 0 cfm.	Air 1.4 cfm.	Air 4.0 cfm.	Nitrogen 1.4 cfm.	Nitrogen 3.0 cfm.
255	255 (137-1713) 110 (72-234) 511 (254- --)	216 (112-3079)	169 (99-582)	151 (102-293)	117 (83-195)
265	144 (90-351) 149 (70- --) 203 (80- --)	107 (73-203)	108 (60-487) 116 (78-233)	93 (64-168)	97 (67-175)
275	106 (56-830) 94 (62-194)	62 (42-123) 95 (42-206)	141 (83-446) 71 (50-121)	48 (36-71)	57 (44-80)
285	43 (34-57) 50 (36-78)	109 (56-2781) 74 (47-177)	128 (62- --) 61 (39-144)	64 (39-190)	107 (68-255) 77 (54-135)

TABLE 5.--Average linear regression D values in minutes after discarding data points believed to be in error.

Temperature (°F.)	Air			Nitrogen	
	Still	1.4 cfm	4.0 cfm	1.4 cfm	3.0 cfm
255	383	216	169	151	117
265	165	107	112	93	97
275	100	95	106	48	57
285	47	74	95	64	77

TABLE 6.--z values for various gas flow conditions.

Flow Condition	z value (°F.)	D_{250} (min)
dry heat closed system-TDT can	31	600
wet heat closed system-TDT can	25	0.50
still air-dry heat oven	32	800
1.4 cfm air flow-dry heat oven	50	340
4.0 cfm air flow-dry heat oven	100	120
1.4 cfm nitrogen flow-dry heat oven	42	270
3.0 cfm nitrogen flow-dry heat oven	73	150

relative values of the open system resistances when compared to the closed system and wet heat system.

Figures 6 through 12 show the thermal resistance curves (plot of log D value versus temperature) for the various heating conditions. Figures 6 and 7 are from Fox, Eder and Pflug (1967).

It is important to realize that there is a major difference between the open heating systems and the closed systems. The open dry heat system provides a relatively low humidity and it is probable that as the spores are heated, they also become drier. For this reason the survivor curve has a slightly different meaning in the open system than in the closed system. In the open system the moisture content of the spores may not be constant throughout the heating period; the spores may become drier as they are heated due to the loss of moisture from the open system. In the wet heat system, the spores are maintained at a water activity near 1.0 throughout the heating time, and in closed system dry heat methods there is an equilibrium established between the water in the spores and the water vapor in the atmosphere inside the container. If the water activity of the spores were held constant during the heating period we could be more certain that the D value obtained was a true D value. However, it is not possible at the present time to hold the water activity of the spores constant and at the same time maintain an open

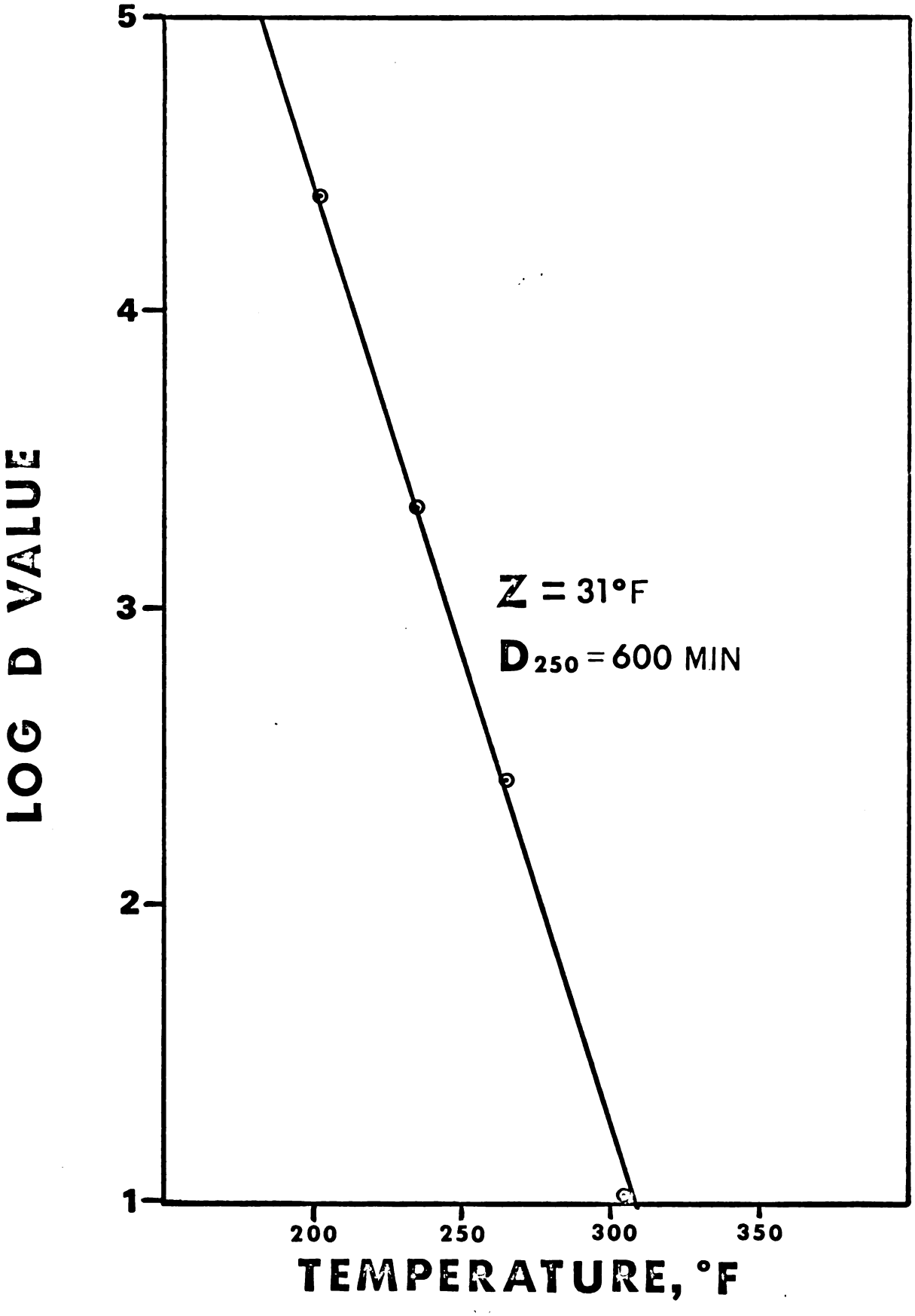


Figure 6.--Thermal resistance curve for dry heating in thermal death time cans.

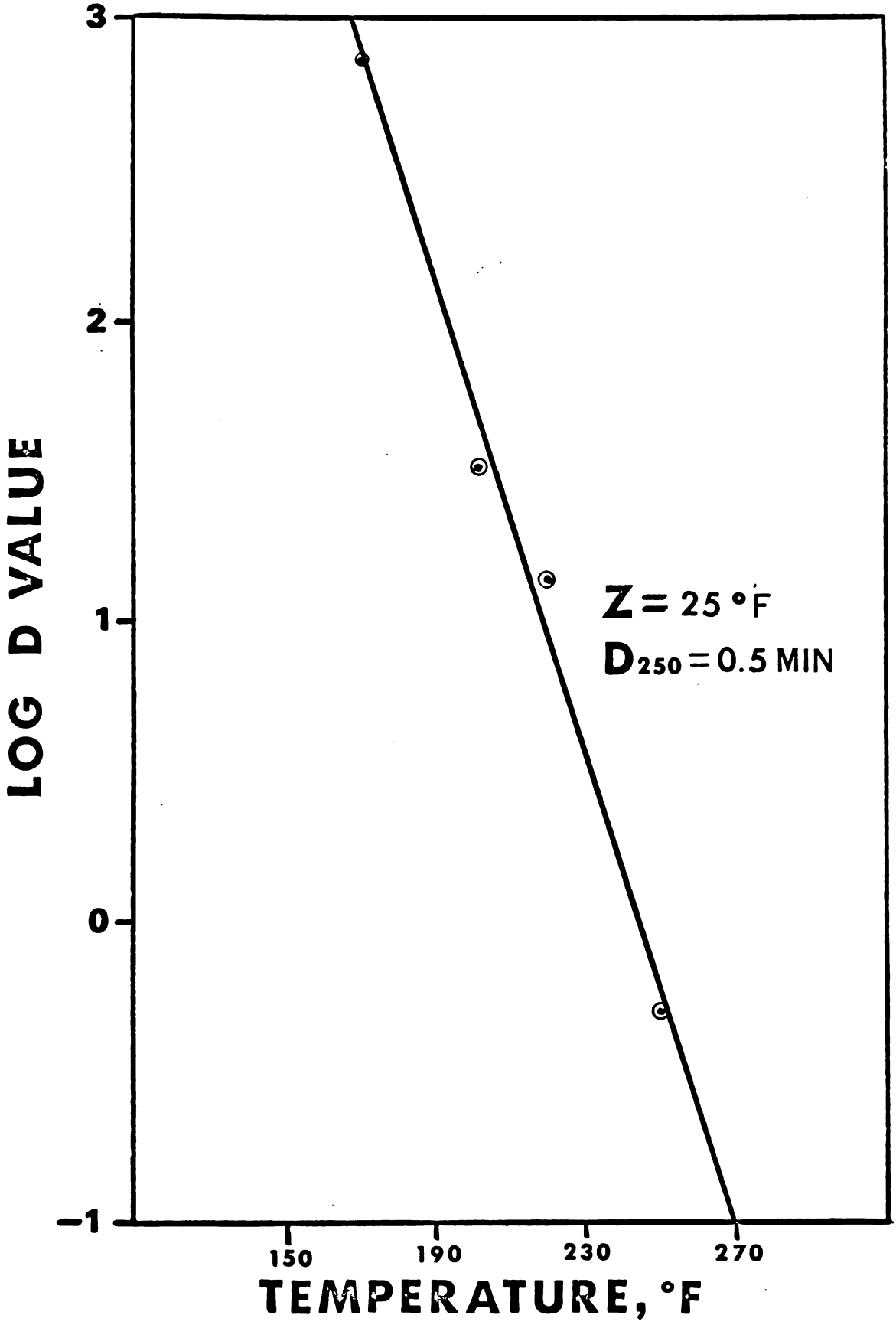


Figure 7.--Thermal resistance curve for wet heating in thermal death time cans.

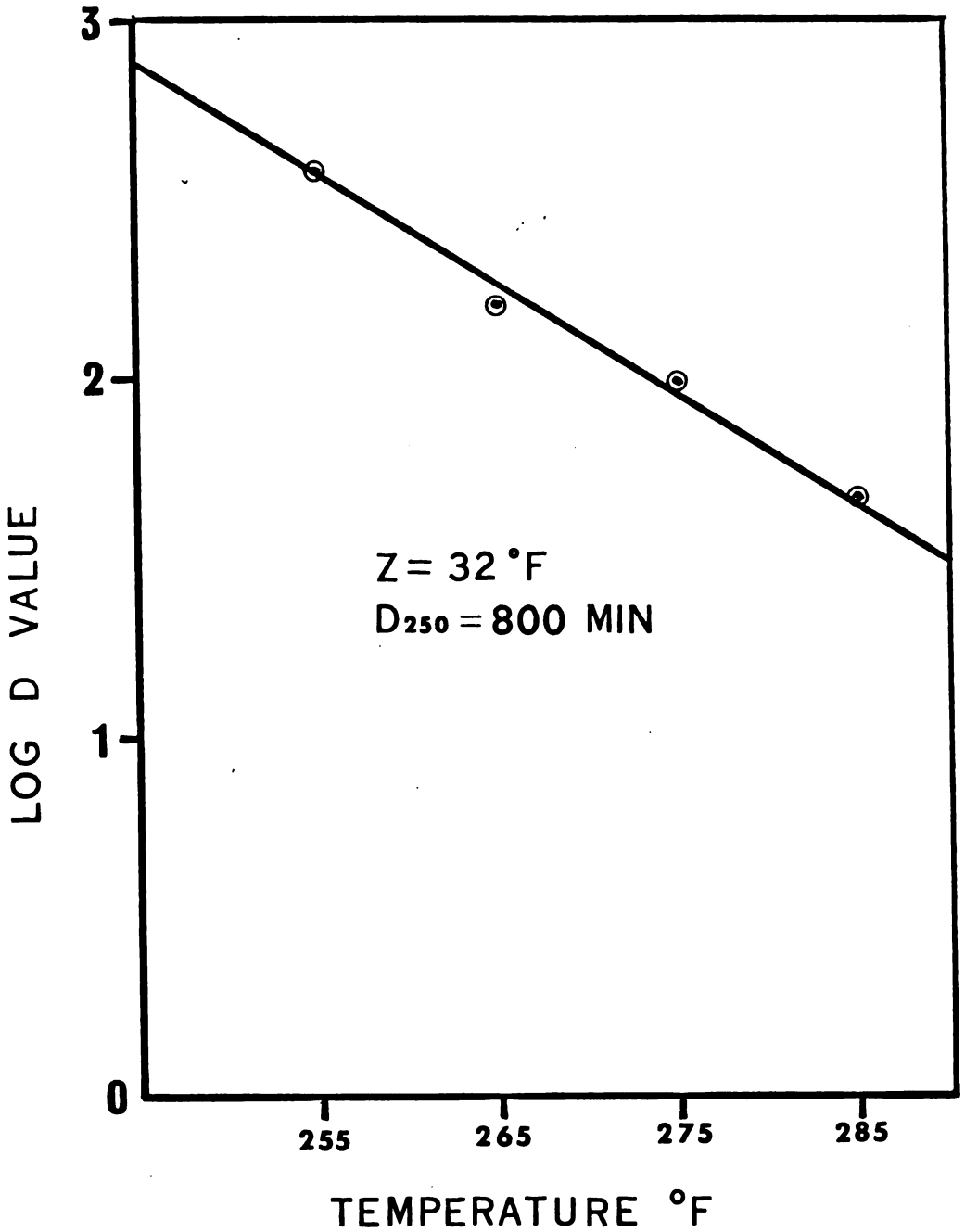


Figure 8.--Thermal resistance curve for still air in dry heat oven.

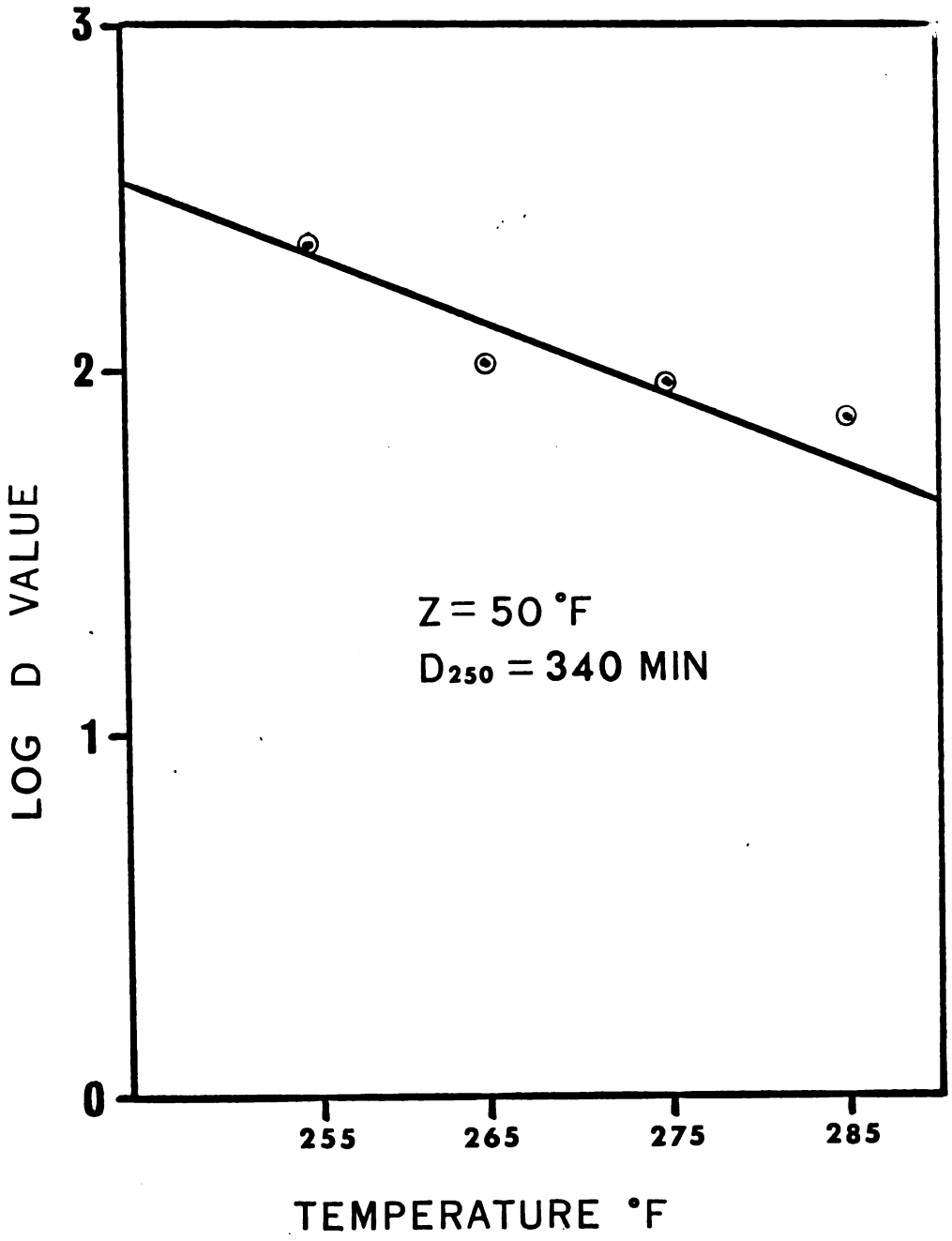


Figure 9.--Thermal resistance curve for 1.4 cfm air flow in dry heat oven.

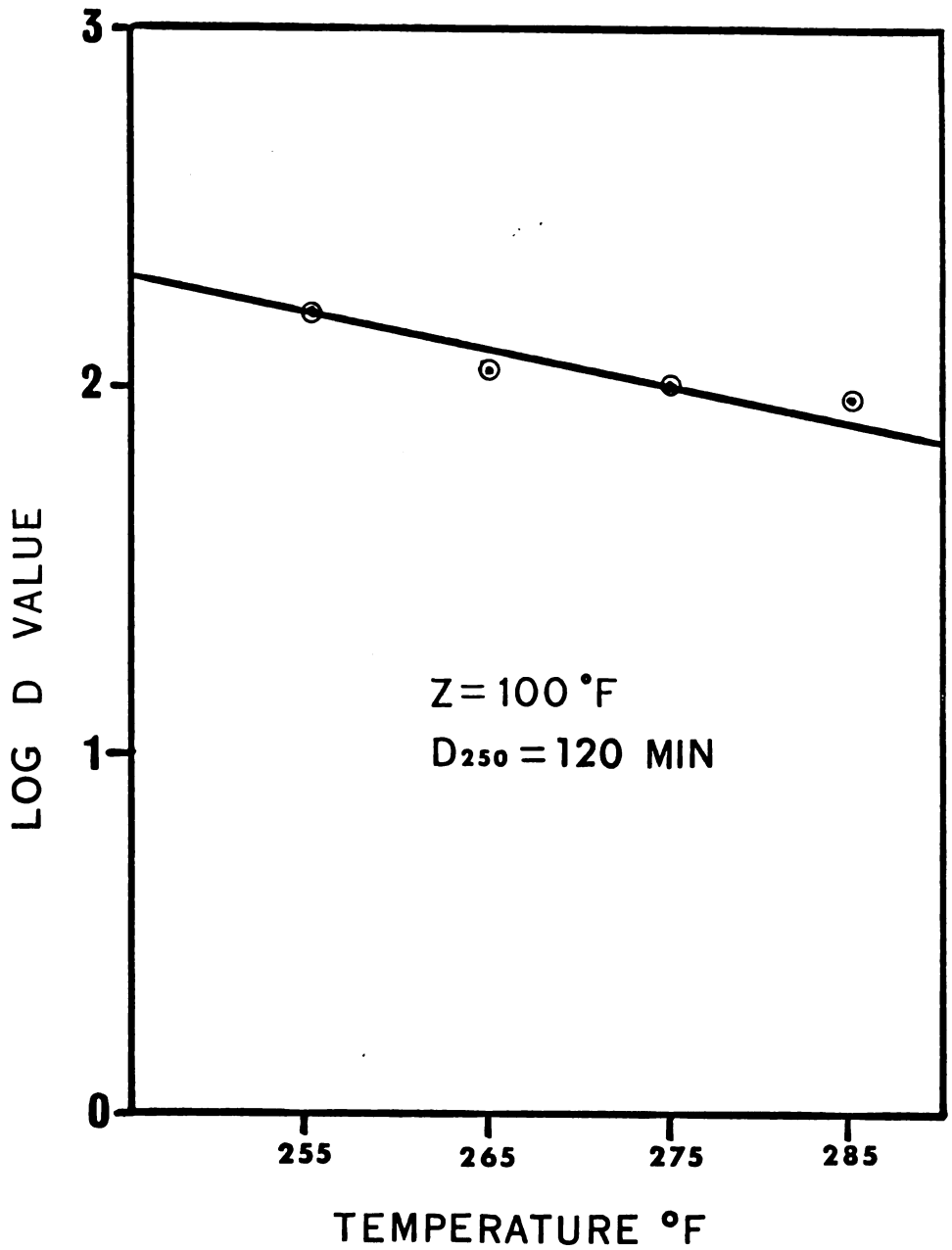


Figure 10.--Thermal resistance curve for 4.0 cfm air flow in dry heat oven.

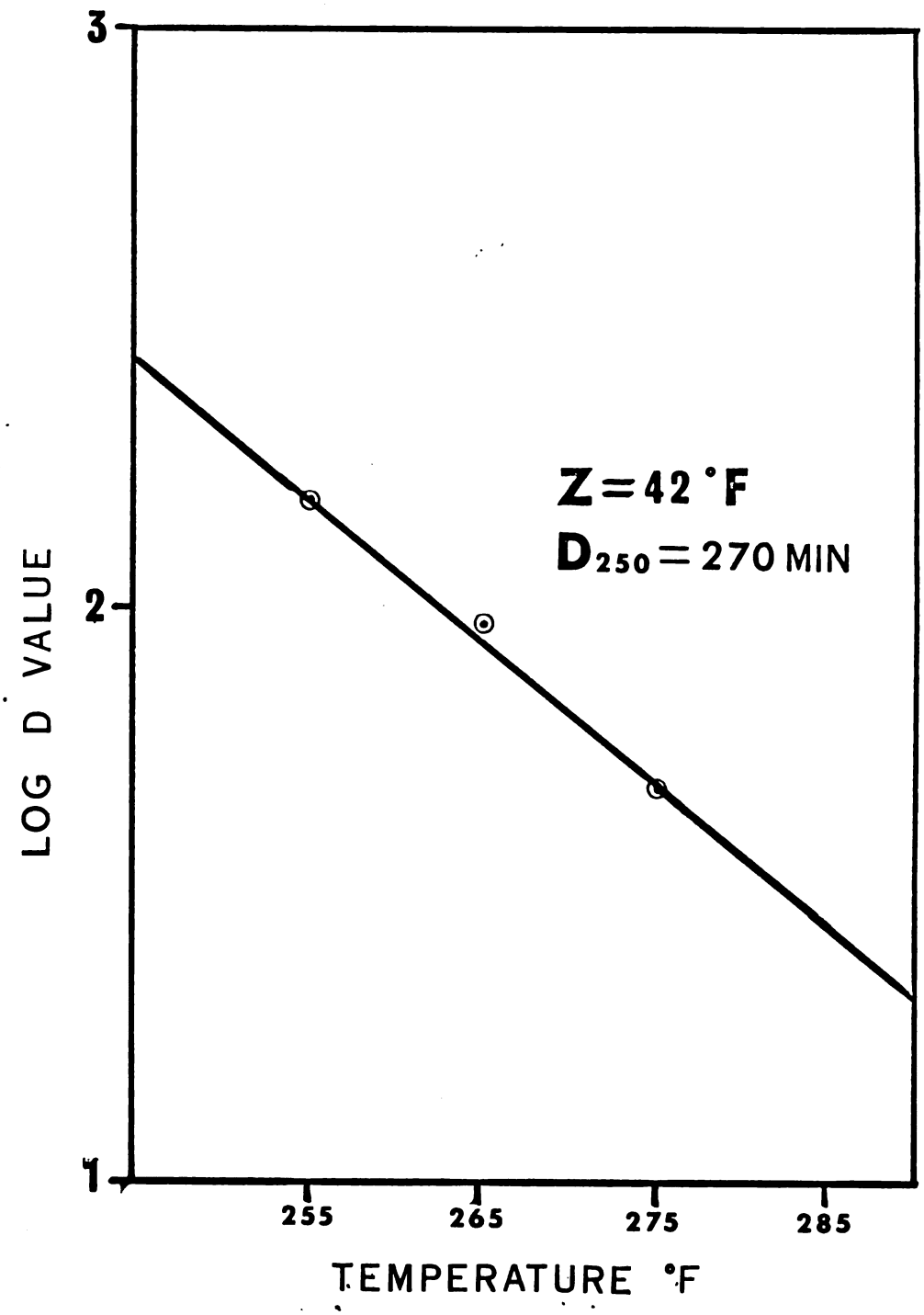


Figure 11.--Thermal resistance curve for 1.4 cfm nitrogen flow in dry heat oven.

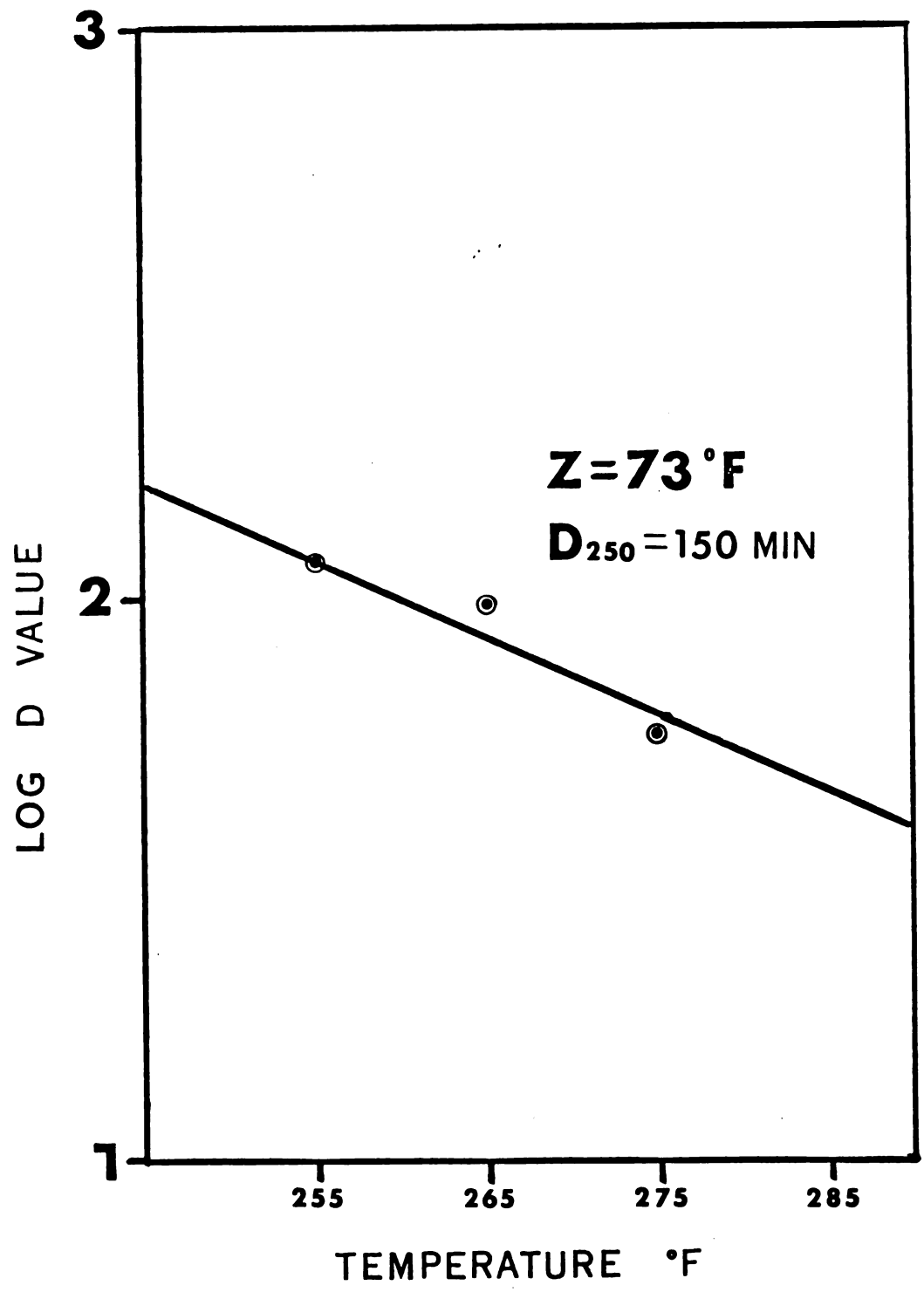


Figure 12.--Thermal resistance curve for 3.0 cfm nitrogen flow in dry heat oven.

system. Murrell and Scott (1966) reported that they held the water activity fairly constant throughout the heating schedule, but to do this it was necessary to use sealed test tubes containing certain salts to keep the relative humidity constant.

If we assume (1) that as the flow rate of gas over the spores is increased, the rate of dehydration of the spores is accelerated and (2) inactivation of the spores is increased by extreme dessication then it would seem that spores are much more sensitive to thermal destruction in the more dessicated state. Davis, Silverman and Keller (1963), however, reported that up to 40 percent of the initial number of organisms of B. subtilis var. niger could survive for 5 days at 60° C. under ultra high vacuum (approximately 10^{-10} Torr). At the same temperature these workers found that under atmospheric pressure about 70 percent of the initial number of spores remained viable for 5 days. It is apparent then from these studies that the effect of extreme vacuum (dessication) does cause some death of bacterial cells by itself. When these workers held the same spores under the ultrahigh vacuum for 5 days at higher temperatures, they found that at 88° C. less than 0.01 percent survived and at 100° C. and 107° C. there were no survivors remaining.

The effect of nitrogen has been to give resistances slightly lower than those obtained in air. It was originally thought that if oxidation of dry bacterial

spores was contributing some lethal effect, the nitrogen should increase the resistance since there would be little if any oxidation during the heating in the nitrogen. The results show, however, that the resistance in nitrogen as compared to air was higher only at 285° F. Since at this temperature the D value of the spores heated in air generally increased as the air flow rate increased we cannot infer anything concerning oxidation as a cause of death. If oxidation were occurring during dry heating in air we might expect the D value to decrease as we increase the air flow (as it did at temperatures below 285° F.), but we would expect the D values under nitrogen to be very high. Pheil, Pflug, Nicholas and Augustin (1967) suggested that if the oxygen content of the gas is responsible for the major destructive effect in dry heat sterilization, then the dry heat resistance should be less in oxygen. However, in their studies these workers found that the resistance was not measurably different between oxygen and air. These authors also tested the resistance of Clostridium sporogenes and Bacillus subtilis under inert gas atmospheres. They found that in general these inert gases (helium, nitrogen) gave the largest D values.

B. Thermal Resistance--z Values

From Table 6 we can see that for both the nitrogen and the air tests, the largest z value was obtained at the

highest flow rate which means that as the flow rate of the gas increases, the thermal resistance becomes less temperature dependent.

In the dry heat closed system using thermal death time cans, the open system with zero air flow, and the open system having low flow rate we have observed essentially the same z values. The low flow rate of nitrogen has given a slightly higher z value. Therefore in systems where the rate of dehydration (rate of flow) of the spores is relatively low (closed systems and low flow rate systems) the main influencing factor of the thermal resistance is the temperature.

Eder (1966) studied the resistance of Bacillus subtilis spores in simulated flexible package seals. This study was an effort to determine how the resistance of the spores is affected when the spores are confined to a very small closed system, i.e., the seal interface. He proposed that if moisture loss from the spore during dry heating decreased the resistance, then conditions which restrict the water loss might increase dry heat resistance. He found that at high temperatures (305° F.) the resistance was higher in simulated seals than on thermoresistometer cups, but at lower temperatures the two resistances were about the same.

C. Shape of Survivor Curves

Figure 13 shows a sample survivor curve. This curve is representative of those obtained for all the heating tests. In all cases there is an apparent initial rapid drop in number of survivors. This shape is generally known as concave upward, or broken survivor curve. Alderton, Thompson and Snell (1964) stated that their data supports the hypothesis that concave upward survivor curves result from a gradual increase in heat resistance developed during heating. In their experiments Bacillus megaterium spores were first stripped of metallic ions by holding them in nitric acid, then stored in calcium buffer solutions and finally tested for heat resistance and Ca^{++} ion uptake at preselected time intervals.

Another explanation of the characteristic shape of these dry heat survivor curves may be explained as possibly being due to the fact that the culture under observation is non-homogeneous in resistance, and that there are mostly very liable or low heat resistant organisms which are killed very rapidly as indicated by the first portion of the curve. The second portion of the curve could be due to the higher heat resistance of the remaining organisms. Stumbo (1965) gives two possible explanations for these concave upward survivor curves. First he discusses the mixed flora theory which has been mentioned. As a second explanation he gives the flocculation theory. Flocculation

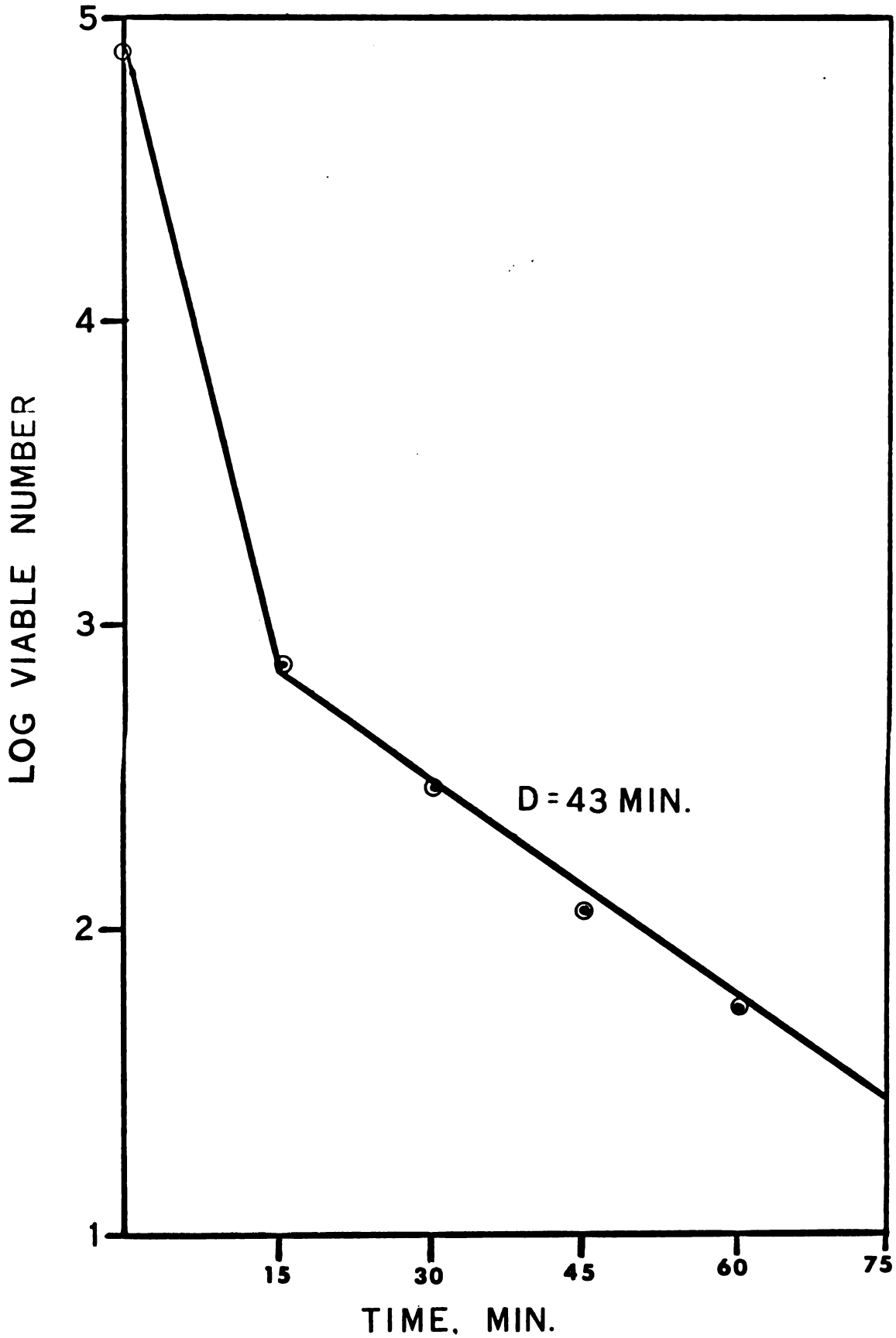


Figure 13.--Sample survivor curve for Bacillus subtilis in dry heat oven (still air) at 285° F.

of the spores occurs early in the heating procedure and

> . . . as clumping nears completion, the colony count will tend to level off during the time the number of surviving cells per clump is being reduced to one cell per clump. Only when virtually all clumps in which viable cells remain contain only one viable cell per clump will the rate of reduction in colony count assume that of a logarithmic order.

In testing the heat resistance of six species of spores at various water activities (a_w) ranging from 0 to 1, Murrell and Scott (1966) obtained survivor curves for two species which were similar in shape to the dry heat survivor curves found in this study. They attributed the sudden initial decrease in viable number as a result of damage during freeze drying of the spores.

It is possible that the culture used in the present studies contained some spores which were very susceptible to dehydration. Therefore these susceptible cells were not destroyed during the initial drying procedure and appeared in the initial count of the organisms. However, when the sample was placed in the dry heat oven, these susceptible cells were dehydrated further and killed immediately, thus accounting for the sudden drop in the survivor curve.

It is also possible that the spore suspension is essentially uniform but during the first few minutes of dry heating, the heat energy induces changes in the spores that cause the spores still viable to move into a more resistant state. This explanation of developing resistance

seems to parallel Alderton, Thompson, and Snell's (1964) observations of an increase in resistance during heating when stripped spores were heated in a system containing cations.

The method of heat shocking the spores used in this research was that of Curran and Evans (1945). The spores were suspended in distilled water and heated at 212° F. for 10 minutes before being plated. It is possible that since this heat shock was a wet heat treatment in reality the count obtained by this method would not fall on the straight line formed by the dry heated spores.

D. Possible Causes for Variability Among Replicate Plate Counts

The large variability among replicate plate counts has caused the confidence limits on the D values to be very large in some instances. These large variations may be caused by:

1. Variations in the original numbers of spores per sample. This is a very improbable reason since the spores were pipetted into each cup using an accurate microsyringe and the initial counts which were obtained before heating showed very consistent results. Table 2 summarizes the initial numbers obtained for the various heating tests over a period of 3 months.

2. Variations in flow rates over each sample cup during actual heating treatments. Due to small convection currents which are probably set up in the sample chambers there may be some differences in the air flow rate within each cup.
3. Recovery of spores from cups. This is the most promising explanation. The fact that the method of plate counting depends upon the removal of the spores from the test surface makes it necessary that the same proportion of spores are removed from all cups. It is quite possible that during the heating process these spores become "baked" onto the surface and that when the cups are soaked for 10 minutes to remove the spores, some spores are retained on the surface of the cup. If the percentage retained on the surface is not constant in each cup, this could explain some of the variation.

An effort was made to correlate the variation in D value with the date of the experiment. It was thought possible that as the spores were stored in the stock suspension throughout the testing period their resistance might have changed. However, when the D values were examined in relation to when they were obtained, no trend could be seen. There were slightly more instances when the D value dropped as storage time of the stock suspension

increased, however, there were not enough data available to reach any definite conclusions.

V. CONCLUSIONS

From the results of this study it is concluded that:

1. There is a difference in the response of bacterial spores when heated in dry heat open systems and dry closed systems.
2. As the rate of gas flow is increased, the z value of thermal destruction of the spores becomes larger.
3. There appears to be no difference in the thermal destruction in air versus nitrogen and therefore oxidation of cellular components is probably not a major factor in the dry heat thermal destruction of bacterial spores.
4. The decimal reduction time (D value) obtained in an open dry heat system may have a slightly different significance than the corresponding D value obtained for wet heat and a closed dry heat system. In the open system the composition of the spores may not be constant throughout the heating period.

APPENDIX

Run #2. Temperature = 285° F. Still Air. Date: 10-12

Time (min)	Viable Counts					
15	530	1670	1470	1560	190	780
30	220	340	330	320	300	
45	260	330	40	90	70	
60	70	40	140	50	30	60
75	30	10	70	20	130	

D value of regression line = 43.0 min.
Apparent initial number = 1576.3.

Run #3 Temperature = 275° F. Still Air. Date: 10-12

Time (min)	Viable Counts					
15	1800	1600	2000	800	1500	800
30	690	3600	4150	4520	2490	1120
45	680	340	620	230	870	3900
60	200	210	590	220	150	270
75	1010	900	3310			

D value of regression line = 106.3 min.
Apparent initial number = 2211.0.

Run #4 Temperature 265° F. Still Air. Date: 10-17

Time (min)	Viable Counts					
15	5000	6000	5000	5000	3000	5000
30	4800	400	200	900	200	400
45	1330	2500	230	3800	1090	120
60	400	630	1100	3200	900	180
75	280	680	900	1790	1030	2210
90	390	1049	180	1740	5150	2450

D value of regression line = 203.9 min.
Apparent initial number = 1963.2.

Run #5. Temperature: 295° F. Still Air. Date: 10-17

Time (min)	Viable Counts					
10	3000	4000	3000			
20	200	800	1700	300	500	
30	470	310	20	930	320	320
40	20	140	120	250	70	400
50	160	0	10	110	10	290
60	140	240				

D value of regression line = 27.8 min.
Apparent initial number = 3445.8.

Run #6. Temperature: 275° F. Still Air. Date: 10-19

Time (min)	Viable Counts					
15	7900	4700	6600	4400	1500	2600
30	700	4000	700	8600	100	1900
45	1540	980	1790	200	2750	
60	680	1010	2450	4700	2750	
75	430	300	1250	1200	570	
93	900	710	300	120	370	

D value of regression line = 94.5 min.
Apparent initial number = 4189.4.

Run #7. Temperature: 265° F. Still Air. Date: 10-19

Time (min)	Viable Counts					
15	1000	2600	5800	13400	1700	
30	340	6000	1470	4250	2560	1710
45	470	1020	4950	420	5200	
60	120	60	410	100	2250	
75	6950	6900	2700	1920	1400	920

D value of regression line = 149.1 min.
Apparent initial number = 3337.1.

Run #9. Temperature: 255° F. Still Air. Date: 10-24

Time (min)	Viable Counts					
30	3000	18000	5000	4000	5000	12000
60	3700	400	1300	800	1100	3300
90	8600	2000	2200	1600	100	1200
120	370	490	320	1900	8600	7650
150	4950	6150	130	1120	400	4900
180	1670	1370	3850	280	260	900

D value of regression line = 255.2 min.
Apparent initial number = 4295.0.

Run #10. Temperature: 255° F. Still Air. Date: 10-26

Time (min)	Viable Counts					
20	5600	2500	7300	1900	4600	17500
40	210	1150	2010	1660	4100	1290
60	500	570	1720	6050	4000	5450
80	1060	450	620	750	640	
100	720	340	410	1040	5850	
120	370	440				

D value of regression line = 110.6 min.
Apparent initial number = 5243.7.

Run #11. Temperature: 265° F. Still Air. Date: 11-1

Time (min)	Viable Counts					
20	7500	14600	17000	21000	14500	2700
40	5400	810	8200	7200	10100	790
60	660	1720	600	5400	5600	1900
80	1340	810	560	1980	6750	7750
100	970	5100	1070	1680	1000	1540
120	960	390	750	6600	4600	5000

D value of regression line = 144.3 min.
Apparent initial number = 8316.3.

Run #12. Temperature: 285° F. Still Air. Date: 11-7

Time (min)	Viable Counts					
15	3100	710	2300	3300	2430	
30	2450	1500	1490	510	1950	3750
45	1710	370	2750	1180	470	170
60	430	780	250	210	1120	1600
75	20	110	310	40	220	510

D value of regression line = 50.1 min.
Apparent initial number = 5626.2.

Run # 15. Temperature: 275° F. 1.4 CFM Air Flow. Date: 11-30

Time (min)	Viable Counts					
15	490	420	1910	5850	3950	5250
30	1340	1540	3450	270	3050	
45	1810	2420	140	2080	1020	640
60	370	1500	200	1670	2350	120
75	330	590	90	210	70	130

D value of regression line = 62.1 min.
Apparent initial number = 4129.7.

Run #17. Temperature: 285° F. 1.4 CFM Air Flow Date: 12-5

Time (min)	Viable Counts					
15	3700	3500	700	2300	3100	500
30	1250	480	100	50	150	340
45	1230	190	190	40	340	1690
60	350	1020	630	130	80	90
75	740	260	770	380	180	200

D value of regression line = 108.8 min.
Apparent initial number = 1057.8.

Run #18 Temperature: 255° F. 1.4 CFM Air Flow Date: 12-6

Time (min)	Viable Counts					
20	12400	7000	12700	4300	12200	12100
40	1400	460	4000	2750	1030	1440
60	1000	990	960	2250	5150	6600
80	1700	8000	6200	1260	7500	6950
100	7050	6700	6750	830	1900	6300
120	530	1280	160	600	6500	2950

D value of regression line = 216.2 min.
Apparent initial number = 5889.1.

Run #19 Temperature: 255° F. 4.0 CFM Air Flow Date: 12-6

Time (min)	Viable Counts					
15	9100	5300	13000	9100	7300	6400
30	2350	790	3750	6750	3800	2700
45	1600	7750	3500	3150	5550	7750
60	5150	930	6650	2050	7650	890
75	1000	490	5250	900	4900	
90	1900	4400	1600	3150	5900	1900

D value of regression line = 169.2 min.
Apparent initial number = 6728.9.

Run #20 Temperature: 265° F. 4.0 CFM Air Flow Date: 12-6

Time (min)	Viable Counts					
15	1300	5600	1400	12200	3600	1500
30	4400	1010	6650	850	3850	5500
45	2750	2000	2850	4000	650	4550
60	260	420	3400	500	1250	3300
75	3150	3200	1100	290	150	2300

D value of regresslin line = 107.1 min.
Apparent initial number = 4768.2.

Run #21 Temperature: 275° F. 4.0 CFM Air Flow Date: 12-7

Time (min)	Viable Counts					
15	3200	2900	7400	4400	5600	5500
30	4350	1900	760	830	1300	1120
45	4150	3900	700	4150	3750	
60	710	1510	2200	3700	2350	790
75	1710	1550	930	1550	770	1390

D value of regression line = 140.9 min.
Apparent initial number = 4233.6.

Run #22 Temperature: 285° F. 4.0 CFM Air Flow Date: 12-8

Time (min)	Viable Counts					
15	1300	1000	1500	6900	1400	800
30	850	680	2400	150	480	2000
45	70	50	720	1140	2650	1080
60	270	1190	3910	1010	2290	150
75	1080	540	160	200	740	230

D value of regression line = 128.3 min.
Apparent initial number = 1591.6.

Run #23 Temperature: 285° F. 4.0 CFM Air Flow Date: 12-8

Time (min)	Viable Counts					
15	1600	6000	900	1000	5000	2600
30	490	70	290	980	780	520
45	340	420	1430	2800	250	1600
60	690	120	190	80	220	900
75	660	630	50	60	10	1850

D value of regression line = 61.7 min.
Apparent initial number = 2636.1.

Run #24 Temperature: 275° F. 4.0 CFM Air Flow Date: 12-9

Time (min)	Viable Counts					
15	17500	11500	11000	3300	16000	13000
30	4250	8320	6500	780	1830	7600
45	7400	5250	5850	8300	5400	8850
60	2270	740	960	2200	1550	
75	2000	1400	500	2600	2100	

D value of regression line = 71.1 min.
Apparent initial number = 15368.6.

Run #25 Temperature: 265° F. 4.0 CFM Air Flow Date: 12-12

Time (min)	Viable Counts					
15	8900	5100	2900	13800		
30	1900	4100	5900	4200	4450	8350
45	450	3700	4900	5150	810	4300
60	4000	1520	5000	1700	3750	6700
75	1250	1600	380	4300	3650	4300

D value of regression line = 116.5 min.
Apparent initial number = 7918.6.

Run #26 Temperature: 255° F. Still Air Date: 12-13

Time (min)	Viable Counts					
30	10700	6000	3500	17500	4100	6400
60	1500	15500	5100	2400	3200	4600
90	1240	490	5600	5250	4150	1550
120	6000	7050	1700	1500	2750	1900
150	3300	2150	3200	4200	5100	3500
180	2800	950	3600	5500	3400	

D value of regression line = 510.6 min.
Apparent initial number = 5524.5.

Run #27 Temperature: 265° F. 1.4 CFM Air Flow Date: 12-14

Time (min)	Viable Counts					
15	3500	9800	12100	11800	6000	4100
30	900	4600	1250	5300	4600	540
45	3000	2200	4650	5700	1200	1600
60	1450	920	1060	1030	4400	490
75	1850	1150	3750	1100	1650	
90	390	3900	520	900	3300	410

D value of regression line = 107.7 min.
Apparent initial number = 6277.4.

Run #28 Temperature: 285° F. 1.4 CFM Air Flow Date: 12-15

Time (min)	Viable Counts					
15	2200	6800	4800	5100	1100	
30	1030	620	390	510	400	340
45	470	2200	1650	2450	270	2300
60	1400	1250	180	510	170	630
75	200	90	670	0	1850	100
90	60	310	320	500	1070	470

D value of regression line = 74.4 min.
Apparent initial number = 2902.2.

Run #29 Temperature: 285° F. 3.0 CFM N_2 Flow Date: 12-19

Time (min)	Viable Counts					
15	300	4100	3000	1100	800	3700
30	1130	460	310	110	1450	680
46	220	1160	120	1600	160	470
60	1030	80	420	320	30	480
75	170	160	350	400	1200	420
90	70	330	300	230	130	610

D value of regression line = 107.6 min.
Apparent initial number = 1298.8.

Run #32 Temperature: 275° F. 3.0 CFM N_2 Flow Date: 12-20

Time (min)	Viable Counts					
15	500	1200	400	1400	9200	500
30	1440	420	720	190	470	560
45	860	200	80	130	320	220
60	230	240	60	730	2000	290
75	80	120	140	70	90	220
90	50	120	40	10	100	10

D value of regression line = 57.0 min.
Apparent initial number = 1963.0.

Run #33 Temperature: 265° F. 3.0 CFM N_2 Flow Date: 12-21

Time (min)	Viable Counts					
20	3100	800	1000	10200	4500	10300
40	2200	250	860	470	450	1100
60	1050	140	480	70	250	4350
80	590	790	3350	190	130	3500
100	330	390	210	460	500	130
120	120	30	630	100	600	180

D value of regression line = 96.7 min.
Apparent initial number = 3025.5.

Run #34 Temperature: 255° F. 3.0 CFM N_2 Flow Date: 12-21

Time (min)	Viable Counts					
20	2300	4900	2000	1900	6200	1400
40	500	790	2850	1700	700	360
60	4900	2150	710	150	260	890
80	190	2800	1010	620	150	640
100	340	440	950	470	280	470
120	630	310	290	300	400	60

D value of regression line = 116.9 min.
Apparent initial number = 2833.3.

Run #35 Temperature: 275° F. 1.4 CFM N_2 Flow Date: 12-22

Time (min)	Viable Count					
15	800	300	1100	700	1200	700
30	470	1040	40	460	300	1300
45	700	190	160	140	100	240
60	380	150	170	60	70	70
75	20	20	20	10	100	280

D value of regression line = 47.9 min.
Apparent initial number = 1653.6.

Run #36 Temperature: 265° F. 1.4 CFM N_2 Flow Date: 12-27

Time (min)	Viable Count					
15	1600	2300	1700	3100	2600	1700
30	650	1110	840	420	520	
45	650	450	1050	800	490	3350
60	500	80	80	610	190	740
75	230	240	1050	590	410	650
90	220	60	140	110	420	3550

D value of regression line = 92.8 min.
Apparent initial number = 2141.8.

Run #38 Temperature: 285° F. 1.4 CFM N_2 Flow Date: 12-28

Time (min)	Viable Count					
15	700	1200	600	1600	400	700
30	50	90	420	330	390	510
45	640	1090	30	1400	310	100
60	70	30	150	240	1050	380
75	50	390	0	120	400	50

D value of regression line = 64.3 min.
Apparent initial number = 1124.3.

Run #40 Temperature: 255° F. 1.4 CFM N_2 Flow Date: 1-2

Time (min)	Viable Count					
20	4300	5000	4400	4700	3900	2900
40	1010	820	2600	1000	430	1150
60	400	630	520	1250	530	730
80	470	260	250	690	550	570
100	230	720	750	850	190	1080
120	5400	460	790	320	710	510

D value of regression line = 151.1 min.
Apparent initial number = 2562.3.

Run #42 Temperature: 275° F. 1.4 CFM Air Flow Date: 1-9

Time (min)	Viable Count					
15	3700	500	400	2500	4500	1400
30	320	180	530	510	1200	1090
45	280	1720	200	270	660	160
60	220	240	70	180	1420	210
75	150	500	20	290	350	120
90	110	2250	470	140	40	480

D value of regression line = 95.0 min.
Apparent initial number = 1355.2.

Run #43 Temperature: 285° F. 3.0 CFM N_2 Flow Date: 1-9

Time (min)	Viable Count					
20	1400	2100	2500	1100	200	1200
40	120	300	190	220	1100	490
60	170	180	50	240	10	
80	200	70	10	0	80	50
100	30	110	20	120	80	10
120	70	120	60	210	10	110

D value of regression line = 77.1 min.
Apparent initial number = 953.0.

LITERATURE CITED

Alderton, G., P. A. Thompson, and N. Snell. 1964. Heat adaptation and ion exchange in Bacillus megaterium spores. Science. 143:141.

Amaha, M. and Z. J. Ordal. 1957. Effect of divalent cations in the sporulation medium on the thermal death rate of B. coagulans v. thermoacidurans. J. Bacteriol. 74:596.

Amaha M. and K. Sakaguchi. 1957. The mode and kinetics of death of the bacterial spores by moist heat. J. Gen. Appl. Microbiol. 3:163.

Angelotti, R. 1967. Unpublished data.

Annear, D. I. 1956. The preservation of bacteria by drying in peptone plugs. J. Hyg. 54:487.

Augustin, J. A. L. 1964. Recovery patterns of spores of putrefactive anaerobe 3679 in various subculture media following moist and dry heat treatment. Ph.D. Thesis. Michigan State University.

Ball, C. O. and F. C. W. Olson. 1957. Sterilization in Food Technology. New York: McGraw Hill Book Co.

Bernlohr, R. W. 1965. Role of amino acids in sporulation. In Spores III. American Society for Microbiology, Ann Arbor.

Black, S. H. and P. Gerhardt. 1962. Permeability of Bacterial spores. IV. Water content, uptake and distribution. J. Bacteriol. 83:960.

Bruch, C. W. 1963. Some biological and physical factors in dry heat sterilization: A general review. Life Sciences and Space Res. II. Int. Space Sci. Symp. Warsaw, June 3-12, for COSPAR.

Bruch, C. W., M. G. Koesterer, and M. K. Bruch. 1963. Dry heat sterilization: Its development and application to exobiological space probes. Dev. Indust. Microbiol. 4:334.

Burke, G. S. 1923. Studies on the thermal death time of spores of Clostridium botulinum 2. The differential staining of living and dead spores. J. Inf. Diseases. 32:433.

Busta, F. F. and Z. J. Ordal. 1964. Heat activation kinetics of endospores of Bacillus subtilis. J. Food Sci. 29:1.

Church, B. D. and H. Halvorson. 1959. Dependence of the heat resistance of bacterial endospores on their dipicolinic acid content. Nature. 183:124.

Cohn. F. 1877. Untersuchungen uber Bakterien. IV. Beitrage zur Biologie der Bacillen Beitr. Biol. Pflanz. 2:249.

Collier, C. P. and C. T. Townsend. 1956. The resistance of bacterial spores to superheated steam. Food Technol. 10:477.

Curran, H. R., B. C. Brunstetter, and A. T. Myers. 1943. Spectrochemical analysis of vegetative cells and spores of bacteria. J. Bacteriol. 45:485.

Curran, H. R. and F. R. Evans. 1945. Heat activation inducing germination in spores of thermotolerant and thermophilic aerobic bacteria. J. Bacteriol. 49:335.

Davis, N. S., G. J. Silverman, and W. H. Keller. 1963. Combined effects of ultrahigh vacuum and temperature on the viability of some spores and soil organisms. Appl. Microbiol. 11:202.

Dixon, W. J. and F. J. Massey, Jr. 1957. Introduction to Statistical Analysis. New York: McGraw Hill Book Co.

Eder, B. D. 1966. Temperature and Microbial destruction conditions in flexible package seals. Unpublished M. S. Thesis. Michigan State University

El-Bisi, H. M. and Z. J. Ordal. 1956. The effect of sporulation temperature on the thermal resistance of Bacillus coagulans v. thermoacidurans. J. Bacteriol. 71:10.

Esty, J. R. and K. F. Meyer. 1922. The heat resistance of the spores of Bacillus botulinus and allied anaerobes. J. Inf. Diseases. 31:650.

Favero, M. S. 1966. Personal communication.

Fox, K. I. 1967. Unpublished data.

Fox, K. I. and I. J. Pflug. 1967. Unpublished data.

Fox, K. I., B. D. Eder, and I. J. Pflug. 1967. Comparison of wet and dry heat survivor curves of Bacillus subtilis. Presented at Annual American Society for Microbiology Meeting, April.

Frank, H. A. and L. L. Campbell, Jr. 1955. The influence of recovery media on thermal resistance values of spores of a putrefactive anaerobic bacterium. Appl. Microbiol. 3:300.

Friedman, C. A. and B. S. Henry. 1938. Bound water content of vegetative spore forms of bacteria. J. Bacteriol. 36:99.

Fry, R. M. and R. I. N. Greaves. 1951. The survival of bacteria during and after drying. J. Hyg. 49:220.

Heller, G. 1941. A quantitative study of environmental factors involved in survival and death of bacteria in the dessicated state. J. Bacteriol. 41:109.

Henry, B. S. and C. A. Friedman. 1937. Water content of bacterial spores. J. Bacteriol. 33:323.

Ingraham, J. L. 1962. "Temperature relationships" in I. C. Gunsalus and R. Y. Stainier: The Bacteria, Vol. IV. New York: Academic Press, p. 265.

Jacobs, R. A., R. C. Nicholas, and I. J. Pflug. 1965. Heat resistance of Bacillus subtilis spores in atmospheres of different water contents. Michigan State University Quarterly Bulletin. 48:238.

Jensen, L. B. 1945. Microbiology of Meats. 2nd ed. Champaign, Illinois: Garrard Press.

Katzin, L. I., L. A. Sandholzer, and M. E. Strong. 1943. Application of the decimal reduction time principle to a study of the resistance of coliform bacteria to pasteurization. J. Bacteriol. 45:265.

Keynan, A., Z. Evenchik, H. O. Halvorson, and J. W. Hastings. 1964. Activation of bacterial endospores. J. Bacteriol. 88:313.

Kobayashi, U., W. Steinberg, A. Higa, H. O. Halvorson, and C. Levinthal. 1965. Sequential synthesis of macromolecules during outgrowth of bacterial spores. In Spores III. American Society for Microbiology, Ann Arbor.

Kopelman, I. J. 1966. Transient Heat Transfer and Thermal Properties in Food Systems. Ph.D. Thesis. Michigan State University.

Lechowich, R. V. and Z. J. Ordal. 1962. The influence of the sporulation temperature on the heat resistance and chemical composition of bacterial spores. Canad. J. Microbiol. 8:289.

Levinson, H. W. and M. T. Hyatt. 1956. Correlation of respiratory activity with phases of spore germination and growth in Bacillus megaterium as influenced by manganese and L.-alanine. J. Bacteriol. 72:176.

Lewis, J. C., N. S. Snell, and H. K. Burr. 1960. Water permeability of bacterial spores and the concept of a contractile cortex. Science. 132:544.

Lewith, S. 1890. Uber die Ursache der Widerstandsfaehigkeit der Sporen gegen Hohen Temperatures. Arch. Exp. Path. und Bakt. 26:341.

Licciardello, J. J. and J. T. R. Nickerson. 1963. Some observations on bacterial death time curves. Appl. Microbiol. 11:476.

Madsen, T. and M. Nyman. 1907. Zur Theorie der disinfection. Z. Hyg. Infectionskrahkh. 57:388.

Marshall, B. J., W. G. Murrell, and W. J. Scott. 1963. The effect of water activity, solutes, and temperature on the viability and heat resistance of freeze-dried bacterial spores. J. Gen. Microbiol. 31:451.

Mayall, B. H. and C. F. Robinow. 1957. Observations with the electron microscope on the organization of the cortex of resting and germinating spores of B. megaterium. J. Appl. Bacteriol. 20:333.

McDade, J. 1967. Unpublished data.

Murrell, W. G. 1964. Heat resistance of microorganisms. Aust. J. Pharmacy, May.

Murrell, W. G. and W. J. Scott. 1957. Heat resistance of bacterial spores at various water activities. Nature. 179:481.

Murrell, W. G. and W. J. Scott. 1958. The permeability of bacterial spores to water. Intern. Congr. Microbiol. Stockholm, p. 26.

Murrell, W. G. and W. J. Scott. 1966. The heat resistance of bacterial spores at various water activities. J. Gen. Microbiol. 43:411.

Murrell, W. G. and A. D. Warth. 1965. Composition and heat resistance of bacterial spores. In Spores III. American Society for Microbiology, Ann Arbor.

O'Connor, R. J. and H. O. Halvorson. 1960. Intermediate metabolism of aerobic spores. V. The purification and properties of L-alanine dehydrogenase. Arch. Biochem. Biophys. 91:290.

Pflug, I. J. 1960. Thermal resistance of microorganisms to dry heat: design of apparatus, operational problems and preliminary results. Food Technol. 14:483.

Pflug, I. J. 1966. Unpublished data.

Pflug, I. J. and J. A. L. Augustin. 1961. Unpublished data.

Pflug, I. J. and W. B. Esselen. 1953. Development and application of an apparatus for study of thermal resistance of bacterial spores and thiamine at temperatures above 250° F. Food Technol. 7:237.

Pflug, I. J. and K. I. Fox. 1966. Unpublished data.

Pheil, C. G., I. J. Pflug, R. C. Nicholas, and J. A. L. Augustin. 1967. Effect of various gas atmospheres on destruction of microorganisms in dry heat. Appl. Microbiol. 15:120.

Puleo, J. R., M. S. Favero, N. S. Peterson, and G. S. Oxborrow. 1966. Recovery of viable microorganisms from solids. I. Model systems. NASA contract R-137, Report #13, June 8.

Rahn, O. 1945. Injury and Death of bacteria by chemical agents. Biodynamica, Normandy, Missouri.

Rieman, H. and Z. J. Ordal. 1961. Germination of bacterial endospores with calcium and dipicolinic acid. Science. 133:1703.

Sadoff, H. L., J. A. Bach, and J. W. Kools. 1965. Significance of multiple forms of glucose dehydrogenase in relation to its heat resistance. In Spores III. American Society for Microbiology, Ann Arbor.

Schmidt, C. F. 1954. Antiseptics, disinfectants, fungicides, and chemical and physical sterilization. Ed. C. F. Reddish. Chpt. 32. "Thermal resistance of microorganisms." Philadelphia, Pa.: Lea and Febiger, 1st Ed.

Silverman, G. 1966. Unpublished data.

Sisler, W. A. 1961. A study of the thermal resistance of bacterial spores to moist and dry heat by use of the thermoresistometer. Unpublished M.S. Thesis. Michigan State University.

Spacecraft Sterilization Advisory Committee. 1967. National Aeronautics and Space Administration. Meeting held Jan. 15, 1967, Phoenix, Arizona.

Spinivasan, V. R. 1965. Intracellular regulation of sporulation of bacteria. In Spores III. American Society for Microbiology, Ann Arbor.

Stewart, B. T. and H. O. Halvorson. 1954. Studies on the spores of aerobic bacteria. II. The properties of an extracted heat stable enzyme. Arch. Biochem. Biophys. 49:168.

Stumbo, C. R. 1965. Thermobacteriology in Food Processing. Academic Press, New York.

Sugiama, H. 1951. Studies on factors affecting the heat resistance of spores of Clostridium botulinum. J. Bacteriol. 62:81.

Tanner, F. W. and G. G. Dack. 1922. Clostridium botulinum. J. Inf. Diseases. 31:92.

Waldham, D. G. and H. O. Halvorson. 1954. Studies on the relationship between equilibrium vapor pressure and moisture content of bacterial endospores. Appl. Microbiol. 2:333.

Walker, H. W., J. R. Matches, and J. C. Ayres. 1961. Chemical composition and heat resistance of some aerobic bacterial spores. J. Bacteriol. 83:960.

Warth, A. D., D. F. Ohye, and W. G. Murrell. 1962. Spore cortex formation, composition and heat resistance. (Abstr). Intern. Congr. Microbiol. 8th. Montreal, p. 16.

Watkins, J. H. and C. A. Winslow. 1932. Factors determining the rate of mortality of bacteria exposed to alkalinity and heat. J. Bacteriol. 24:243.

Weil, R. 1899. Zür Biologie der milzbrandbazillen. Arch. Hyg. Bakteriol. 35:355.

Williams, O. B. 1929. The heat resistance of bacterial spores. J. Inf. Diseases. 44:421.