HEAT RESISTANCE OF *BACILLUS SUBTILIS* SPORES IN ATMOSPHERES OF DIFFERENT WATER CONTENTS

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY

Richard Allen Jacobs

1963


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By

Richard Allen Jacobs

AN ABSTRACT

Submitted to the College of Agriculture
Michigan State University of Agriculture and Applied Science
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Food Science Department

1963

Approved by 

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The purpose of this study was to determine what effect an increase of water vapor content in a heated atmosphere would have on the heat resistance of Bacillus subtilis spores. The heat resistance of the spores was measured by D, decimal reduction time, and z, the negative reciprocal of the slope of the thermal resistance curve. D values were determined from survivor curves.

The spores were placed in small cups and vacuum dried; the cups were then sealed in small (208 x 006) cans containing specified quantities of alum, $AlK(SO_4)_2 \cdot 12H_2O$, the water of hydration of which was used to obtain atmospheres of different water contents. The cans were heated in saturated steam in miniature retorts. The dried spores were heated in a total of four different atmospheres (0, 25, 50, and 100% water vapor) at each of three different temperatures (235, 250, and 265°F).

The heat resistance of the spores was found to depend on the atmosphere in which they were heated. The following parameters of thermal resistance, in terms of D at 250°F and z, were found: at 0% moisture (dry air), D was 270 min., z was 41°F; at 25% water vapor content, D was 160 min., z was 25°F; at 50%, D was 73 min., z was 44°F, and at 100% water vapor content (saturated steam), D was 0.48 min., z was 17°F. The heat resistances found at 0 and 100% are comparable to those determined by other workers.

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INTRODUCTION

Bacterial spores have been of interest to the bacteriologist since 1838, when they were first observed by Ehrenberg. This curious and hardy biological adaptation has afforded grounds for much research and, perhaps, more speculation as to why they are formed, their biological role, their changes during formation and germination, and, especially, for more practical reasons, their resistance to adverse environmental conditions.

Even today, despite the enormous amount of study of spores, little is known about the intimate chemical, physical, and metabolic details presumably responsible for this resistance. Examining the work on the heat resistance of spores, one finds many contradictions and discrepancies throughout the published material. In early research, some discrepancies arise because of the lack of a clear definition of heat resistance, and because of variations within the same species of organism. More recently, a lack of precise machinery and refined analytical methods has occasioned some disagreement among researchers.

One known aspect of heat resistance is the striking contrast of the resistance of bacterial spores to wet and dry heat. This study is an investigation of the uncharted area of heat resistance which lies between saturated steam and dry gases.

REVIEW OF LITERATURE

The practical importance of achieving complete destruction of all, or some particular, microbial species which may be in or on material - a definition of sterilization - is sufficiently obvious to need no further discussion here. Historically, heat, used by Spallanzani and others before bacteria were known to be responsible for spoilage and disease, was probably the first sterilizing agent. Two features of sterilization by heat were discovered very early by microbiologists; first, that bacterial spores are among the most resistant forms of life; and, second that whether the heat is supplied as hot air or as hot water makes quite a difference. This difference could be described by saying that, at a given temperature, sterilization requires a much longer time in hot air than in hot water. For various reasons, these two ways of supplying heat have led to somewhat different lines of investigation of bacterial destruction.

When bacteria are heated in an environment in which liquid water and water vapor are present in equilibrium, then the process is known as wet (or moist) heat sterilization.

These conditions prevail in the steam autoclave and in retort canning of food. When bacteria are heated in an environment free of liquid water, such as hot air sterilization of pharmaceuticals and other medical supplies, then the process is known as dry heat sterilization. Sterilization by superheated steam is, by these working definitions, sterilization by dry heat.

The concept of heat resistance[1]

From a practical point of view, sterilization is a side issue: the surgeon knows he needs sterile instruments, but he only asks how long to heat them to be sure they are sterile; the food manufacturer knows he must sterilize his product, but he only asks how long it must be cooked. It is not surprising, therefore, to find that recommendations for sterilization are given in the form of recipes, such as, No. 2 cans of pumpkin should be heated for 70 minutes at 250°F.

Early studies in heat resistance followed this pattern. Among the first notions of resistance is the thermal death point. A series of samples of a bacterial suspension are heated for a fixed time at a series of temperatures and

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[1] The author has drawn heavily from Schmidt's (1957) excellent review of heat resistance studies.

observed for growth or survival; the lowest temperature at which sterility is achieved is known as the thermal death point. According to Bigelow and Esty (1920), when the above procedure was used for vegetative cells, the time chosen was usually 10 minutes; when spores were heated, the temperature chosen was 100°C., and the time was varied. In both instances a thermal death point is recorded; a temperature for vegetative cells, and a time for spores. This form of reporting resistance is misleading, in that, one gets the impression that there is a critical temperature above which death occurs. Bigelow and Esty (1920) showed in their very important study that the resistance measured this way was a function of the number of bacterial cells treated and the pH of the medium. They defined a new quantity, the thermal death time, as: ". . . the time at different temperatures necessary to completely destroy a definite concentration of spores in a medium of known hydrogen-ion concentration." It should be noted that their attention was focused on complete destruction. Their skill as researchers is attested to by the fact that they corrected for lag in heating and cooling of the containers.

Bigelow (1921) showed that the thermal death time data (all on spore formers) obtained by him and Esty, lay on

a straight line if plotted as the heating temperature against the common logarithm of the thermal death time. His thermal death time data for some species of non-spore formers also plotted as straight line. Bigelow called these curves thermal death time curves and his nomenclature has been retained to this day, although now, the temperature is usually plotted in degrees Fahrenheit and as the independent variable.

The slope of this curve has proved to be an important measure of thermal resistance. As these curves were originally plotted by Bigelow, the slope, z, would be the number of degrees Celsius that it takes the curve to cross one log-cycle, and it would be negative since the thermal death time decreases as the temperature increases. As the curves are now conventionally plotted, the slope is -1/z, where z is a positive number and is customarily given in degrees Fahrenheit. Bigelow's curves show a z of about 17°F. for spores and about 8°F. for vegetative cells.

Ball's (1923) formula method of process calculation is based, in part, on the thermal death time curve. He describes the curves by defining, in addition to z, F, the thermal death time at 250°F. According to research thus far (1923), thermal resistance could be defined by F and z and

would mean that such a curve gives the times necessary for complete destruction of the number of organisms upon which the curve was based, and provided the pH was the same.

The work of Townsend, Esty, and Baselt (1938) added to present knowledge the fact that medium as well as pH of the medium is an important factor in fixing F. The weakness of F and z as a description of heat resistance lies, of course, in the dependence of F upon initial numbers of organisms.

Meanwhile, beginning with the work of Madsen and Nyman (1907) and Chick (1908), cited by Schmidt (1957), a different aspect of heat destruction was being studied. These people studied partial destruction instead of sterility. They discovered the form of bacterial death known as the logarithmic order of death (also called exponential survival); that is, for bacteria heated at a constant temperature,

$$N = N_o 10^{-t/D} \qquad (1)$$

where N is the number of survivors at time, t; N_o is the initial number; and the decimal reduction time, D, is a parameter which is a function (among other things) of the temperature.

It is not suggested here that all bacteria obey this simple law, but, as Schmidt (1957) says, "The evidence in favor of a logarithmic order of death is considerable and impressive and warrents the full exploitation of the consequences of such order in application to experimental data." Ball (1943) stated that z values for thermal death time curves could be obtained by plotting $\log_{10}D$, determined at various temperatures, against T, the temperature at which each survivor curve was determined. Rahn (1945) emphasizes the significance of death rates (reciprocal of D) this way, "Death rates make it possible to compare the heat resistance of different species at the same temperature, or the heat resistance of one species at different temperatures." He seems to prefer Q_{10} (the ratio of death rates measured at temperatures 10°C. apart) to Ball's z as a measure of the temperature dependence of D.

B. Hypotheses of heat resistance

Williams (1929) discussed and summarized earlier explanations of heat resistance and concluded, "Evidence gleaned from the literature and accumulated during the progress of this work supports the idea that the cause of death in cells exposed to a high temperature is the coagulation of bacterial protein." Low water content and low ash

content are mentioned as contributing to resistance.

Virtanen and Pulkki (1933) associated the high heat resistance of spores with essential enzymes. Rahn (1945) rejected enzyme inactivation as a possible cause of death.

Henry and Friedman (1937) and Friedman and Henry (1938) demonstrated that, even though the water contents of endospores and vegetative cells are almost identical, spores of the Bacillus species have a bound water content, depending on the species, of 70 to 60% (wet basis), whereas only 0 to 28% is bound in the corresponding vegetative cell. Their thermal death time for B. subtilis spores was 6 minutes at 100°C. The bound water, according to them, would make the spores more resistant to heat. Powell and Strange (1953) suggested because of the high bound water content, spore protein is stabilized by bonding to various particles. Waldham and Halverson (1954) discredited Friedman and Henry's work and theorized that heat resistance is a result of "bound protein" rather than "bound water." If the polar groups of spore protein are attached to some particle, rather than hydrogen bonds, the protein would be less susceptible to heat and denaturation.

Curran et al. (1943) found that endospores had a higher calcium content than vegetative cells. High calcium

contents of the endospores were associated with increased heat resistance. Powell and Strange (1956), studying biochemical changes occurring during sporulation in Bacillus species reported that dipicolinic acid, could cause resistance to heat by stabilizing the spore protein either by further chelate linkages between dipicolinic acid, spore protein, and calcium or other divalent heavy metals.

Many other theories for heat resistance of endospores have been presented. Sugiyama (1951) suggested a stabilizing effect due to lipid-protein combinations. Another theory, Rode and Foster (1960, 1961), predicts the existence of a central core kept relatively dry in the dormant spore, possibly by contraction of an internal sheath, Lewis, Burr and Snell (1960). Black and Gerhardt (1962) suggest that the occurrence of an insolubly gelled core with cross-linkage between macromolecules through stable, but reversible bonds, forming a high polymer matrix with entrapped free water, is responsible for the heat resistance of endospores. Bach and Sadoff (1962) reported that heat resistant enzymes in B. cereus, and model systems involving these enzymes are being studied to determine why the enzymes are heat resistant and possibly why endospores are heat resistant. Obviously much research is needed before the

phenomenon of heat resistance can be explained. However, protein stability seems to be the common factor.

C. Destruction kinetics

The similarity of experimental survival curves and the form of the observed dependence of D on temperature has naturally led to a description of bacterial destruction in terms of Eyring's theory of absolute reaction rates. See, for example, Johnson, Eyring, and Polissar (1954). The Eyring equation is:

$$k' = (kT/h) \exp. (\Delta S^*/R) \exp. (-\Delta H^*/RT) \qquad (2)$$

where: $k' = 1/D$ = reaction rate, sec.$^{-1}$

k = Boltzman's constant, 3.28×10^{-24} cal/^{o}K

h = Planck's constant, 1.56×10^{-34} cal sec

R = gas constant, 1.986 cal/^{o}K mol.

T = absolute temperature, ^{o}K

ΔS^* = activation entropy, E. U./mol.

ΔH^* = heat of activation, cal/mol.

Activation used here means the (theoretical) activation complex.

With the Eyring equation, one has a form of the Arrhenius equation giving the variation of k' with temperature.

$$\frac{d \ln k'}{dT} = \frac{E_a}{RT} \qquad (3)$$

where: E_a is the Arrhenius activation energy, and is related to the activation energy, E_o, of the activated complex above by:

$$E_o = E_a - mRT \qquad (4)$$

where: m shows the dependence of k' on T in the equation:

$$k' \sim T^m \exp. \; - E_o/RT \qquad (5)$$

and if log k' is linear in 1/T then m is zero (E_o is not a function of temperature) and $E_o = E_a$, and in liquid systems

$$E_o = \Delta H^* + RT \qquad (6)$$

See, for example, Frost and Pearson (1961).

The free energy of activation, ΔF^*, is given by:

$$\Delta F^* = \Delta H^* - T\Delta S^* \qquad (7)$$

If k' follows the Arrhenius equation and if the energy of activation is constant, then bacterial destruction defined as D and z could equally well be defined by two parameters, say ΔS^* and ΔH^*, at some reference temperature.

D. Previous results

The heat resistance of the B. subtilis spores used in the present study has been determined in wet heat, dry

heat, and superheated steam, (See Table 1.) Anderson (1959), Pflug (1960), Sisler (1961), Pflug, Nicholas, Pheil (1963).

Table 1.--Heat resistance of dried spores of Bacillus subtilis 5230

Source	Wet heat (250°F.)		Dry heat (350°F.)		Superheated steam (350°F.)	
	D, min.	z, °F	D, min.	z, °F	D, min.	z, °F
Anderson 1959	0.35	14.4	-	-	-	-
Pflug 1960	0.35	14.8	-	-	0.57	42
Sisler 1961	0.24	12.8	-	-	0.56	37.1
Pflug et. al. 1963	-	-	0.16	33	0.45	35

Much information describing the effects of moist or dry heat on the heat resistance of bacterial spores is available. However, the area between those two limits hasn't been thoroughly investigated. Apparently only Scott and Murrell (1957) have investigated this area. In their studies several strains of spores were dried, then

equilibrated *in vacuo* over saturated solutions of different water activities. The heat resistance, $D_{110^{\circ}C}$, was found to be a function of equilibrium moisture content of the spore. Murrell (1963), with further refinements of the technique, found that maximum heat resistance was obtained at 0.2 activity (20% relative humidity), which corresponds to a moisture content in the spores of 5 - 10%. The composition of the atmosphere in which the spores were heated is not given directly.

EXPERIMENTAL PROCEDURES

The basic data from which heat resistance was determined came from survivor curves. The general procedure was to fill small cups with aliquots of a spore suspension, dry the spores, and store in desiccator jars until just before heat treatment, seal the cups in small thermal death time cans, and process them in miniature retorts. After processing, the cups were removed from the cans, placed in sterile distilled water, shaken, and appropriate aliquots plated, incubated, and the number of survivors counted.

A. Preliminary Experiments and Results

1. Arbitrarily fixed procedures

The organism selected for this study was a spore forming mesophilic aerobe known as strain 5230. This organism is apparently identical to Bacillus subtilis in biochemical and morphological characteristics, except that it will grow anaerobically in the presence of fermentable carbohydrates, whereas Bacillus subtilis will not (Sisler, 1961). Dr. C. F. Schmidt, Continental Can Company, Chicago, Illinois, provided the original culture.

All spores used in this study came from a master suspension, which was prepared as follows. The sporulation medium was nutrient agar (Difco) plus 0.5% glucose and 1 ppm Mn^{++} from $MnSO_4 \cdot H_2O$. The sporulation medium was poured into sterile plastic petri dishes the day of inoculating. The inoculation culture was transferred in dextrose tryptone starch broth on each of two successive days before inoculating the plates. After the inoculum was spread on the surface with an L-shaped glass rod, the plates were incubated for 96 hours at 37°C, at which time a microscopic examination of several of the plates indicated nearly 100 per cent sporulation. The spores were harvested by first flooding the plate with about 10 ml of cold sterile distilled water, then by scraping the agar surface with an L-shaped glass rod to loosen the spores. The spore suspension was filtered through sterile glass wool to remove small pieces of agar. The filtrate was centrifuged and discarded. The remaining spores were resuspended and centrifuged five times in sterile distilled water. After the fifth washing, the spores were suspended in sterile M/15 phosphate buffer at pH 7.0. The spores were stored at 20°C. in several sterile 16-oz. glass bottles containing glass beads. The spore concentration of each bottle was determined

just before use. The bottle was hand-shaken for five minutes to break up any clumps of spores that might be present. Five ml of the spore suspension were heat shocked for 15 minutes at 100°C. A series of dilutions were made, ten plates were poured for each dilution. Large spreading colonies, which cause difficulty and inaccuracy in plate counting, were avoided by pouring a thin agar overlay on top of the layer containing the spore dilution.

The plates were incubated at 37°C for 48 hours and the colonies were counted. This method indicated that a stock suspension contained 2.5×10^7 spores per ml. A direct count made with a Petroff-Hausser counting chamber indicated 3.0×10^7 spores per ml. in the same suspension.

A sub-stock solution, containing approximately 10^5 spores per ml. was made by diluting a portion of the above stock suspension with phosphate buffer at pH 7.0.

A microscopic examination of each suspension revealed approximately 100 per cent spores, very little foreign matter, and no noticable clumps.

The steel sample cups, Pflug and Esselen (1953), used were punched from 0.008 inch (0.02 cm) thick tinplates (hereafter referred to as cups). These cups have an outside diameter of 1.13 cm and a depth of 0.846 cm. The volume of

the metal is about 0.08 cm^3 per cup. Machine oil was removed from the cups by first washing the cups in methyl ethyl ketone, decanting, and then washing them in ethyl alcohol. The washed cups were placed on paper toweling, covered with cheese cloth and allowed to air dry. Fifty dry cups were placed in each petri dish, and sterilized at 177°C. for 2 hours.

Thermal death time cans (208 x 006), Townsend et al. (1938), (hereafter referred to as cans) were used in these studies. Each can was fitted with a ring made from a 7-in. length of 1/8-inch diameter welding rod. These rings fit next to the can wall and keep the cups close to the can center so when the can is opened, the cups are not damaged by the cutting edge of the can opener. The cans with rings already in place were sterilized at 177°C. for 2 hours. The cans were sealed with an Automatic[1] hand closing machine.

2. Procedures investigated to determine optimum test conditions

a. Syringe

The variance of the number of spores in each cup must be minimal or reproduction of the results will be

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[1]Automatic Can Closure Co., Mannitowoc, Wisc.

difficult. At first, a B-D and Yale, number 1YT, 1cc tuberculin syringe fitted to a micrometer screw was used to dispense the spore solution into the cups. Later a California Laboratories Microsyringe/Burette,[2] hereafter referred to as the Calab syringe, was used for filling the cups.

Each of these syringes was used for filling cups with two different volumes of spore suspension, 0.01 ml and 0.10 ml were dispensed into the cups. Ten plates were made from each cup. An analysis of variance was made on the delivery capability of each syringe. The means and variances computed in these analyses (of the 0.01 ml volume) are summarized in Table 2. The results of these tests of delivery capability are based on filled cups which were not dried to assure that the number of spores recovered would be the same as the number filled into the cups. The Calab syringe was judged to be superior for use in the present study for several reasons: (1) Average delivery was independent of dispensed volume; (2) Variance (cup-to-cup) was smaller for the Calab syringe and was equal to the plate-to-

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[2]California Laboratories Equipment Co., Berkeley 10, Calif.

plate variance; (3) Coefficient of variation was smaller (8.9% for the Calab syringe). The delivery of the Calab syringe was checked by dispensing and weighing water; the delivery was 0.01 $\pm$ 0.001 ml.

Table 2.--Analysis of variance of plate counts to test syringe delivery capability (0.01 ml dispensed).

Syringe	Source	D.F.	Mean Sq.	F	Mean Plate count
Calab	Cups	5	178.6	0.97	153
	Plates	51	184.3		
BD&Y	Cups	2	1459.0	15.6**	84
	Plates	27	93.3		

** significant at 1% level.

b. Filling cups

One-one hundredth of a milliliter was delivered throughout this study to conserve the original suspension and to facilitate drying. Each time the cups were filled, a few additional samples were taken to determine the initial number of spores that each cup probably received. The techniques for determining the initial number are discussed earlier. After filling, the cups were vacuum dried and stored.

c. Drying and storing cups

The filled cups used in this study were vacuum dried at room temperature under approximately 29 inches of vacuum and stored at room temperature in dessicator jars containing a drying agent. Normally, they were used within one week after being placed in the dessicator jar, but in no case were they used after four weeks of storage. The effects of drying method, storage temperature, and length of storage on the viability of the spores were investigated by Augustine (1962). (See Table 3). Cups dried in 29 inches of vacuum and stored at $0^{\circ}F$ retained their original viability after 12 months of storage and therefore this procedure would be the recommended method for prolonged storage. The procedure discussed above, of vacuum drying and storing at room temperature retained only 38% viability after 12 months of storage and isn't recommended for prolonged storage.

Table 3.--Percent viability as a function of drying and storage treatments. (means of 5 cups 2 plates per cup)

Drying treatment	Storage temperature, °F	Storage 6 Months	12 Months
Air in desiccator at room temperature	78	80%	44%
29" vacuum 24 hours	0	100%	100%
	32	92%	78%
	78	98%	38%
Frozen to 0°F; then vacuum dried: 29" vac., 0°F 24 hrs.	78	108%	70%

d. Heat shock and shaking

The results of a heat shock test are shown in Table 4. The spore suspension was dispensed directly into screw-capped test tubes. An analysis of variance of the data shows that heat shocking at 100°C (212°F) for 15 minutes gives significantly higher plate counts than heat shocking at 80°C (176.2°F) for 10 minutes. Therefore all germination and original suspension counts were made after the endospores were heat shocked for 15 minutes at 100°C (212°F).

Table 4.--Plate counts (average 10 plates) of heat shocked spores.

Statistic	Heat Shock Treatment		
	None	10 Minutes 80°C (176.2°F)	15 Minutes 100°C (212°F)
Mean	96.7	185.8	248.5
Standard deviation	6.0	11	14

In the course of obtaining a methodology for the present study, several questions had to be answered: (1) should the screw top test tube, which contained the cup, be shaken before or after heat shocking and (2) what length of time and by what method should these tubes be shaken. The experimental design (1 cup, 2 plates/cup, for each treatment combination) consisted of two methods of shaking, hand shaking and machine shaking by a reciprocating (200 RPM) shaker; shaking times, for each method, of five, ten, or fifteen minutes; and, one lot of tubes heat shocked before shaking, the other after shaking. In this test, the spores were dried for 24 hours under 29 inches of vacuum.

Table 5.--Analysis of variance on method of shaking, time of shaking, and heat shock.

Source of variation[a]	df	Mean square	F ratio
Total	69	-	
M	1	1,741	1.64
T	2	919	.86
S	1	80	.07
MT	2	1 954	1.84
MS	1	1 531	1.44
TS	2	264	.25
MTS	2	1,802	1.70
Cup	23	1.060	
Error	35	165.6	

[a]M = method of shaking: hand or reciprocating shaker
T = time of shaking: five, ten, or fifteen minutes
S = heat shock: before or after shaking

An analysis of variance (Table 5) of these various treatments demonstrated that there was no significant difference between the methods of shaking, between heat shocking before or after shaking, between the times of shaking, or in any combination of these treatments; the overall mean was 1.66×10^5/cup.

For convenience, and to standardize the procedure, all tubes were shaken by the reciprocating shaker for fifteen minutes. Whenever a heat shock was required, the tubes were heat shocked before shaking. However, the cup-to-cup variance (coefficient of variation 20%) was considerably larger than the plate-to-plate variance (coefficient of variation of 7.8%). Therefore, because of this large cup-to-cup variation, the error control is not governed by the number of plates (at least for tests of spores which were dried in the cups). Increased precision is achieved by treating more cups rather than by increasing the number of plates per cup. In experiments to be described later, the cups used per process time were increased to fifty; with one plate being used per cup.

e. Water vapor contents

Four different salts, $AlK(SO_4)_2 \cdot 12H_2O$, $Na_2S_2O_3 \cdot 5H_2O$, $CuSO_4 \cdot 5H_2O$, and $Na_2SO_4 \cdot 10H_2O$, and water were used to establish different percentages of water vapor in the atmospheres inside the cans. The required amount of salt or water was added to each can, and was weighed on a Mettler automatic balance. Some physical properties of

the compounds and the amounts added to the cans are shown in Table 6. The required amount of water was calculated on the basis of Dalton's law and the perfect gas law. The expression, "25% water vapor content", means that 25% of the total internal can pressure is contributed by water vapor and the remaining 75% by air.

Table 6.--Characteristics[a] and amounts of substances required to produce a water vapor content of 25% and 50% moisture in 208 x 006 cans.

Substance	Characteristics	Desired water vapor content	Weight required (grams)
H_2O	MP 0°C	25%	.00313
MW = 18.02	BP 100°C	50%	.00989
$AlK(SO_4)_2 \cdot 12H_2O$	$-9H_2O$ 64.5°C	25%	.00687
MW = 474.39	MP 92 (84.5)°C	50%	.02060
$Na_2S_2O_3 \cdot 5H_2O$	45-50°C decomposes	25%	.00862
MW = 248.21		50%	.02587
$CuSO_4 \cdot 5H_2O$	$-4H_2O$ 110°C M.P.	25%	.0108
MW = 249.69	$-5H_2O$ 150°C B.P.	50%	.0325
$Na_2SO_4 \cdot 10H_2O$	32.4°C decomposes	25%	.0056
MW = 322.22		50%	.0168

[a]Hodgman, C. D., 1956, Handbook of Chemistry and Physics, Ed. 37, Chemical Rubber Publishing Company, Cleveland, Ohio.

During preliminary tests to find the approximate times required for sterilization, several end-point destruction tests were made. At that time $Na_2SO_4 \cdot 10H_2O$ was selected as the source of water for the water vapor content of the atmosphere inside the cans. The $Na_2SO_4 \cdot 10H_2O$ lost some of its water of hydration to the atmosphere, before it could be weighed. Because of this, the end-point destruction data were not reproducible (evidence not presented). Therefore, the use of this salt was discontinued and distilled water alone was tried. Once again reproduceability seemed too low (evidence not presented). It seemed as if the spores could have absorbed some water from the atmosphere. If they did, perhaps this moisture had an effect on their heat resistance.

Three other salts, $CuSO_4 \cdot 5H_2O$, $Na_2S_2O_3 \cdot 5H_2O$ and $AlK(SO_4)_2 \cdot 12H_2O$, were compared in the same manner. See Table 7. Alum, $AlK(SO_4)_2 \cdot 12H_2O$, compared most favorably with water. The thiosulfate ion may have had some lethal effects on the spores since it gave low results in comparison with the other salts and water. Because of the possible lethality of the thiosulfate ion, the difficulty in handling $Na_2(SO_4)_2 \cdot 10H_2O$, and because of the

desirability of alum, the $CuSO_4 \cdot 5H_2O$ was abandoned. As shown in Table 8, the end-point destruction tests were more uniformly and more easily reproduced when alum was used rather than water. Therefore, throughout the survivor studies alum was used as the source of water.

Table 7.--Water vapor source test (No. of positive tubes/ No. treated). 1,000 spores/cup; 250°F; 50% water vapor. B. subtilis spores

Water vapor source	Process time, minutes						
	60	90	102	120	150	180	210
$CuSO_4 \cdot 5H_2O$	-	3/10	4/32	-	0/10	0/20	-
$Na_2SO_4 \cdot 10H_2O$	-	-	-	10/10	5/10	6/10; 2/18	1/10
$Na_2S_2O_3 \cdot 5H_2O$	1/10	2/10	1/30	0/10	0/10	0/20	-
$AlK(SC_4)_2 \cdot 12H_2O$	10/10	3/10	7/23; 14/30	0/10	0/10	0/20	-
H_2O	-	-	13/30		-		-

Table 8.--Effect of elapsed time between sealing the can and processing on survivors of B. subtilis spores. (No. of positive tubes/No. treated). (1,000 spores/cup; 250°F; 103 min.; 50% water vapor; held at 78°F.)

Time between sealing and processing, min.	Water vapor source		
	Water		$AlK(SO_4)_2 \cdot 12H_2O$
7.5	0/10		-
15	0/10		-
30	1/10		3/10
60	4/10	8/10	3/10
90		4/10	-
120	1/10		5/10
150	1/10		-
240	5/10	2/10	2/10
480	5/10		-
885	-		3/10
1320	-		3/10
1440		4/10	-
2160	-		3/10
Total	35/120 = 0.292		22/70 = 0.315

The amount of salt to be added was calculated as follows. At the test temperature, (subscript 2) after the water has vaporized:

$$P_2=P_w+P_a=(n_w+n_a)RT_2/V_2 \qquad (8)$$

Where: P is pressure, V, can volume, n, moles of gas, R is the gas constant, the subscripts a and w refer to air and water, respectively, and T is temperature.

Before heating (subscript 1)

$$P_1 = n_a R T_1 / V_1 \tag{9}$$

So for vapor fraction of water, x

$$n_w / (n_w + n_a) = x \tag{10}$$

$$n_w = n_a \left(\frac{x}{1-x}\right) = P_1 V_1 / RT_1 \left(\frac{x}{1-x}\right) \tag{11}$$

Where: V_1 is the volume in sealed cans minus the gas volume displaced by the cups and ring (calculated from ring and cup dimensions). The amount of salt is easily calculated.

For the calculation of H_2O added (as the water of hydration of alum) to obtain water vapor contents of 25% and 50%, it is assumed that 100% of the water of hydration is released and in the vapor state; 9 waters of hydration are lost at 64.5°C (146.5°F); the test temperatures are all above the melting point of the salt, 92°C (197°F), but the equilibrium between $AlK(SO_4)_2 \cdot 3H_2O \rightleftharpoons AlK(SO_4)_2 + 3H_2O$ was not calculated.

The atmospheres inside the cans, during processing, have been calculated according to certain assumptions and an experimental curve that describes the internal can volume change as a function of the pressure difference

between the inside and outside of the can. This curve, fitted to a parabola by the method of averages, is

$$\Delta V = 0.0588 + 0.05239\ \Delta p + 0.002826\ (\Delta p)^2$$

where Δp is the pressure difference in psi and ΔV is the volume change in cm^3.

The assumptions are

1. both air and water vapor obey the perfect gas law;
2. initial conditions at the time of sealing, $T = 25^{\circ}C$ ($77^{\circ}F$), $P = 1$ atm;
3. total pressure in the can is the sum of the partial pressures of water vapor and air (Dalton's law);
4. all the water of hydration (of alum) is released as vapor;
5. if internal pressure is less than the external, there is no volume decrease.

Table 9 gives the calculated internal pressure and relative humidity as a function of temperature and water vapor content. The values were computed by iteration. The net volume of a sealed can, with 10 cups and a ring, is 12.7 $cm.^3$

Table 9.--Calculated internal pressure and relative humidities in 208 x 006 cans. Closing conditions T = 25°C (77°F); P = 1 atm.

Temp. °F		Water vapor content, %			
		0	25	50	100
235	P, atm.	1.29	1.70	2.59	1.55
	RH,%	0	27.5	76.9	100
250	P, atm.	1.32	1.76	2.42	2.03
	RH,%	0	21.8	62.5	100
265	P, atm.	1.35	1.80	2.68	2.64
	RH,%	0	17.1	50.9	100

f. Retorting procedures

All of the cans were placed in the miniature retorts inverted and stacked one above the other. This method was chosen after experimental evidence (not reported) indicated that there was no difference between cans stacked one on top of the other and cans which were randomly (any position) placed in the retort. Stacking in an inverted position helped to standardize experimental procedures.

A special rack was made to separate the cans to ascertain if stacking would have an effect on the heat penetration into the cans (Table 10).

Table 10.--Analysis of variance ($\log_{10}$ No. of counts) of stacked and separated cans (heated 0.5 hours at 250°F; 50% water vapor content).

Source of variation	d f	Log mean square	F ratio
Total	49	-	
Stacking method	1	0.0144	0.72
Location	4	0.0327	1.64
Interaction	4	0.0557	2.79*
Error	40	0.0200	-

*Significant at 5% level

The analysis of variance of the results of these various treatments demonstrated that there was no significant difference between stacking and non-stacking nor between specific locations of cans. However the interaction of stacking and location was significant at the 95% confidence level. When the number of survivors were plotted with respect to the position of the can, for both stacked and non-stacked cans, there was no orderly relationship for the number of survivors, thus accounting for the significance of the interaction term. The log mean average for stacked and separated cans was 2.23 and 2.20 respectively. If the two data for the stacked cans which were

significant at the 95% confidence level were rejected, then their log mean average was 2.20 also. The over-all mean count was 169; the coefficient of variation is about 40%. This marked increase in variation is characteristic of all cans heated with salts and dried spores.

g. Effect of splitting cups

Splitting cups down two sides should allow more uniform circulation of the heating atmosphere around the spores. This should give a more uniform amount of destruction of the spores among the cups in the can, and hence a more uniform number of surviving spores. Experimental results (not reported) indicated that the same number of spores survived whether or not the cups were split. Therefore, none of the cups were split. An analysis of variance showed no difference between means or variances.

B. Major Experiment

1. Scope

The thermal resistance of Bacillis subtilis spores was measured by decimal reduction times, D, at several temperatures, and by z, the negative reciprocal of the slope of the thermal resistance curve. All D values in this

study were determined from survivor curves (generally, 5 cans a time and 10 cups a can) for each of four vapor contents studied. In addition, another set was studied at 100% R.H, but less than 100% water vapor content. The process times, temperatures, initial spore concentration, and water vapor contents are given in Table 11. In order to get a reasonable number of survivors at reasonable processing times it was necessary to use more than one initial spore concentration. It is assumed without proof that D is independent of concentration (See Sisler, 1961).

2. Summary of procedures

The procedure used throughout the study of thermal resistance was as follows.

1. Filled the cups, 0.01 ml/cup, with a Calab syringe.
2. Dried the cups, 29 inches vacuum - 24 hours at room temperature and stored them in dessicator jars.
3. Salt added to presterilized cans fitted with rings.
4. Cups were put in cans, and the cans were then sealed.
5. Cans were heated in miniature retorts.
6. Cans were stored in the refrigerator until plated (experimental results (not reported) showed no loss of total count with up to 10 days of storage in refrigerator).
7. Cans were opened aseptically and cups placed in screw capped test tubes containing 10 ml sterile distilled water.
8. Tubes were shaken 15 minutes on a reciprocal-action shaker (about 50 tubes at a time).

Table 11.--Treatment combinations, B. subtilis, 5230 spores.

Temp., °F	Water Vapor Conc., %	Initial Number per cup	Process times, minute
235	0	8.3×10^2	180, 300, 420
	25	"	30, 60, 120, 180, 240, 300
	50	6.6×10^4	30, 60, 120, 180[a]
	57.6	"	0.5, 1.0, 3.0, 4.0, 5.0
	100	"	0.5, 2.0, 3.0, (4.0, 5.0)[b]
250	0	8.3×10^2	15, 30, 60, (120, 240, 360)[b]
	25	"	30, 60, 90, 120, 150, 180
	50	6.6×10^4	60, 90[a], 120, 150
	63.6	"	0.5, 1.0, 2.0, 3.0, 4.0
	100	"	0.5, 1.0, 1.5, (2.0, 2.5)[b]
265	0	8.3×10^2	(60) 180, 300)[b]
	25	"	30, 60, 90, (120)[b]
	50	6.6×10^4	30, 60, 90
	69.1	"	0.5, 1.0, 2.0[c], 3.0
	100	"	(0.5, 1.0, 1.5, 2.0, 2.5)[b]

[a] variation considered to be too large
[b] ( ) too few survivors to analyze
[c] too many survivors, wrong dilution

9. Appropriate aliquots were plated on Dextrose Tryptone Soluble Starch Agar (one plate for each cup).
10. Plates were incubated at 37°C, and a final count was made at 48 hours.

3. Analysis of data

A survivor curve was determined by linear regression of log N vs. t at each water vapor-temperature combination, where N is the number of survivors and t is the adjusted process time (process time minus lag factor). The linear regression analysis requires, among other things, that the variance of log N be the same at each process time, so, first, an analysis of variance was made of can-to-can variance at each time, and the cup-to-cup variance among times.

A preliminary plot of log N (using all data) against time was drawn; in one instance (30 min., 235°F, 25% water vapor) the mean did not fit a straight line survivor curve and therefore it was not used to compute D.

The data (for a survivor curve) were adjusted by discarding any can means and any cup data that were outside 90% confidence limits for the mean and using the adjusted variance in each case. Table 12 gives the observed and adjusted means. This adjustment of data was continued until the cup-to-cup variance of all of the cans within any given temperature-water vapor content combination were equal so that a regression analysis could be made.

Table 12.--Raw and adjusted survivor data

Temp. °F	Water vapor content, per cent	Process times, minutes	Orig. no. cups	Crude ave.	Adj. no. cups	Adj. ave.	vari-ance of log n
235	0	180	46	120	28	48.5	.042
		300	45	26	22	30.4	.030
		420	49	7.5	12	20.0	.015
	25	60	50	221	44	280	.011
		120	49	248	49	248	.023
		180	48	231	48	231	.041
		240	50	180	50	180	.025
		300	46	152	46	152	.025
	50	30	48	14,700	23	13,500	.016
		60	49	4,560	24	7,300	.010
		120	49	1,120	16	1,190	.0085
		0.5	48	2,910	35	3,770	.0290
		1.0	48	4,600	31	4,230	.0198
	57.6	3.0	48	2,790	28	3 400	.0227
		4.0	48	1,110	17	2,100	.0216
		5.0	49	650	32	615	.0209
	100	0.5	25	34,200	22	35,500	.0022
		2.0	25	17,900	25	17,900	.0065
		3.0	23	15,100	17	14,900	.0037
250	0	15	46	208	9	88.43	.0250
		30	46	106	29	78.05	.0245
		60	34	25.3	8	60.60	.0062
	25	30	49	277	49	277	.0861
		60	48	161	48	161	.0876
		90	46	87	46	87	.0727
		120	48	20.6	26	29.3	.0508
		150	48	75.6	17	65.4	.0247
		180	50	37.8	30	33.0	.0459
	50	60	48	1,130	48	1,130	.1111
		120	49	193	49	193	.2843
		150	49	64.6	49	64.6	.1155
		0.5	47	6,140	42	6,990	.0464
	63.6	1.0	48	7,342	48	7,342	.0458
		2.0	48	3,457	48	3,457	.0820
		3.0	47	795	37	813	.0883
		4.0	35	25.6	31	19.2	.0895

Table 12.--Con't.

Temp. °F	Water vapor content, per cent	Process time, minutes	Orig. no. cups	Crude ave.	Adj. no. cups	Adj. ave.	Vari-ance of log n
	100	0.5	48	2,325	48	2,325	.0397
		1.0	25	254.4	25	254.4	.0321
		1.5	30	17.81	30	17.81	.0440
265	25	30	48	173.5	48	173.5	.0489
		60	49	59.2	49	59.2	.0192
		90	49	16.9	46	18.8	.0428
	50	30	50	6,660	18	6 380	.0125
		60	44	1,620	38	1 777	.0363
		90	49	110	33	169	.01384
	69.6	0.5	50	3,470	34	7,809	.1302
		1.0	47	5,164	47	5,164	.0704
		3.0	49	134	31	230	.1035

Table 13.--Typical analysis of variance (265°F; 50%; 1 hour); Original counts (expressed as number survivors/cup).

Can Number									
1		2		3		4		5	
n	log n	n	log n	n	log n	n	log n	n	log n
2840	3.95	1680	3.23	900	2.95	1420	3.15	3260	3.51
1160	3.06	1480	3.17	1880	3.27	520*		2260	3.35
300*		4680*		380*		1800	3.26	2720	3.43
480*		2240	3.35	1280	3.11	2780	3.44	1020	3.01
1140	3.06	920	2.96	1220	3.09	2560	3.41	3160	3.50
1840	3.26	2600	3.42	2640	3.42	2880	3.46	1340	3.13
2860	3.46	2940	3.47	880	2.94	1580	3.20	4200*	
760	2.88	2600	3.42			2740	3.44	3260	3.51
2240	3.35	1240	3.09			1180	3.07	1300	3.11
		1120	3.05						
$\overline{\log n}$	3.22		3.24		3.13		3.31		3.31

$\overline{\log n} = 3.2484$ N = 1777

*discarded because values were outside 95% confidence limits

Table 14.--Analysis of Variance of counts in Table 13.

Source of variation	df.	Mean Square	F- ratio
Total	37	-	
Between	4	.0389	1.07
Within	33	.0363	

Table 15.--Typical test for linearity of regression (265°F, 25%)

Source	df	Mean square	F - ratio
Within	140	.0364	
Regression	1		3.18
Among	1	.1156	
Total	2		

$s^2_{\log n} = .24268$ $s^2_{\log nt} = .03698$

$s^2_t = .1654$ $\log n = 2.9273 - 1.11589^t$

$b = 1/D = .8961$ $n_o' = 846$

$D = 53.8$ min.

Table 16.--Typical test for confidence limits and independence (265°F, 25%).

Confidence limits on slope

(b, 95%)

$\pm 1.96\ (.03698/.1654x142)^{1/2} = \pm (0.07756)$

57.79 min. $\geq D \geq$ 50.27 min.

Test for independence

$t = (1.11589/.03957) = 28.20^{**}$

** Significant at 1% level

RESULTS AND DISCUSSION

Table 17 gives the heat resistance (D and z) of Bacillus subtilis spores as a function of the amount of water vapor in the surrounding atmosphere. These D-values are plotted as a function of water vapor content in Figure 1. Except for 0% and 25% water vapor (both at 235°F), the 95% confidence limits are about as large as the radius of the plotted circles. At 0%-235°F the limits are 477 to 884 min., and 555 to 2,330 min. at 25%-235°F. The thermal resistance curves for each per cent water vapor are shown in Figure 2. The table and the two figures present the same data, but in different aspects.

One of the central issues upon which hypotheses of heat resistance are based, is the survivor curve. Is it linear with respect to log N vs. t? Experimental evidence obtained in this study indicated that in 8 of 13 cases, one accepts the hypothesis with five per cent risk. These linearity tests were based on adjusted data. Thus, most of the data obtained appear to demonstrate logrithmic order of death. Five of the thirteen curves failed the linearity test,

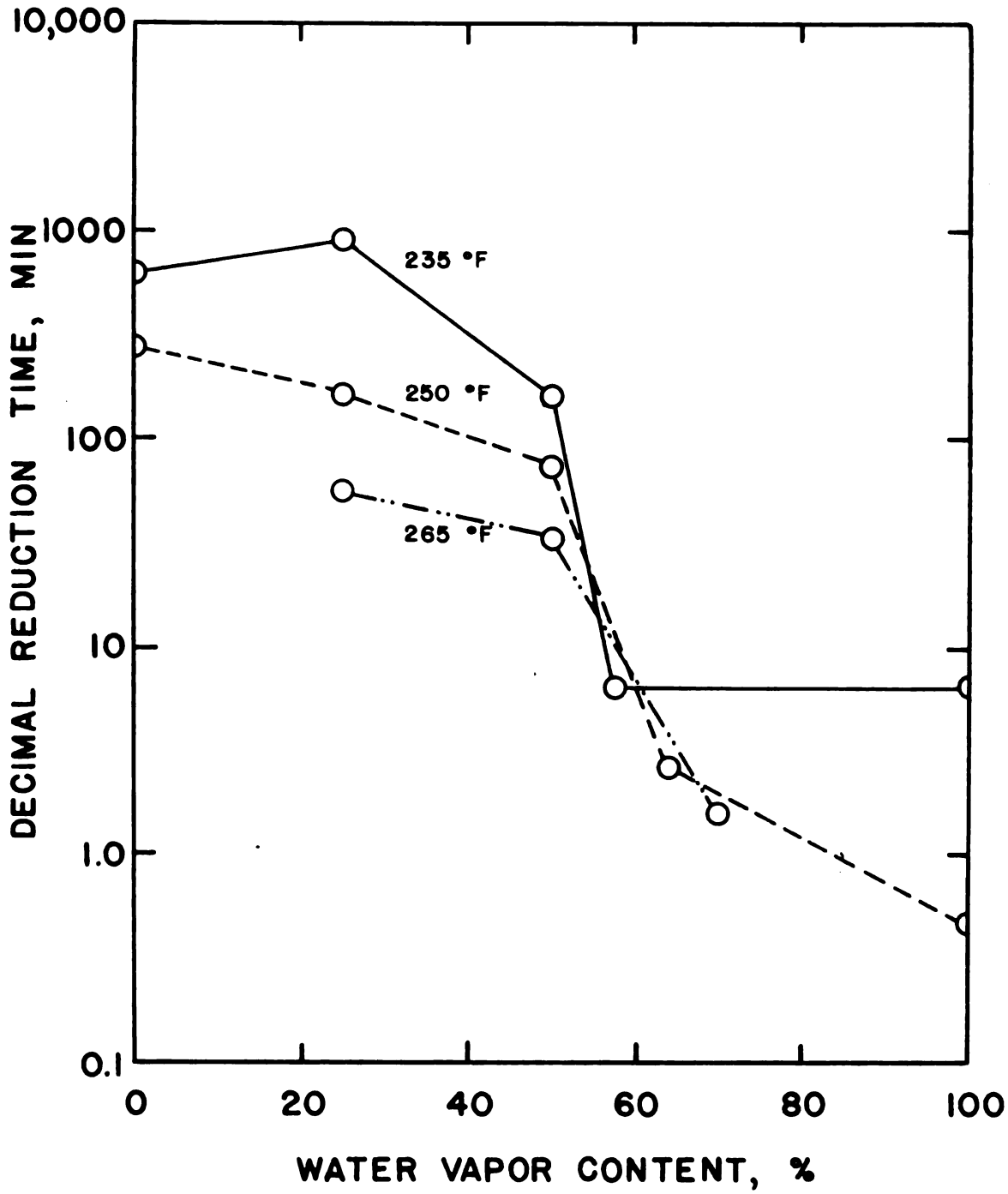

Figure 1.--Decimal reduction time as a function of water vapor content (mean and 95% confidence limits) for B. subtilis spores.

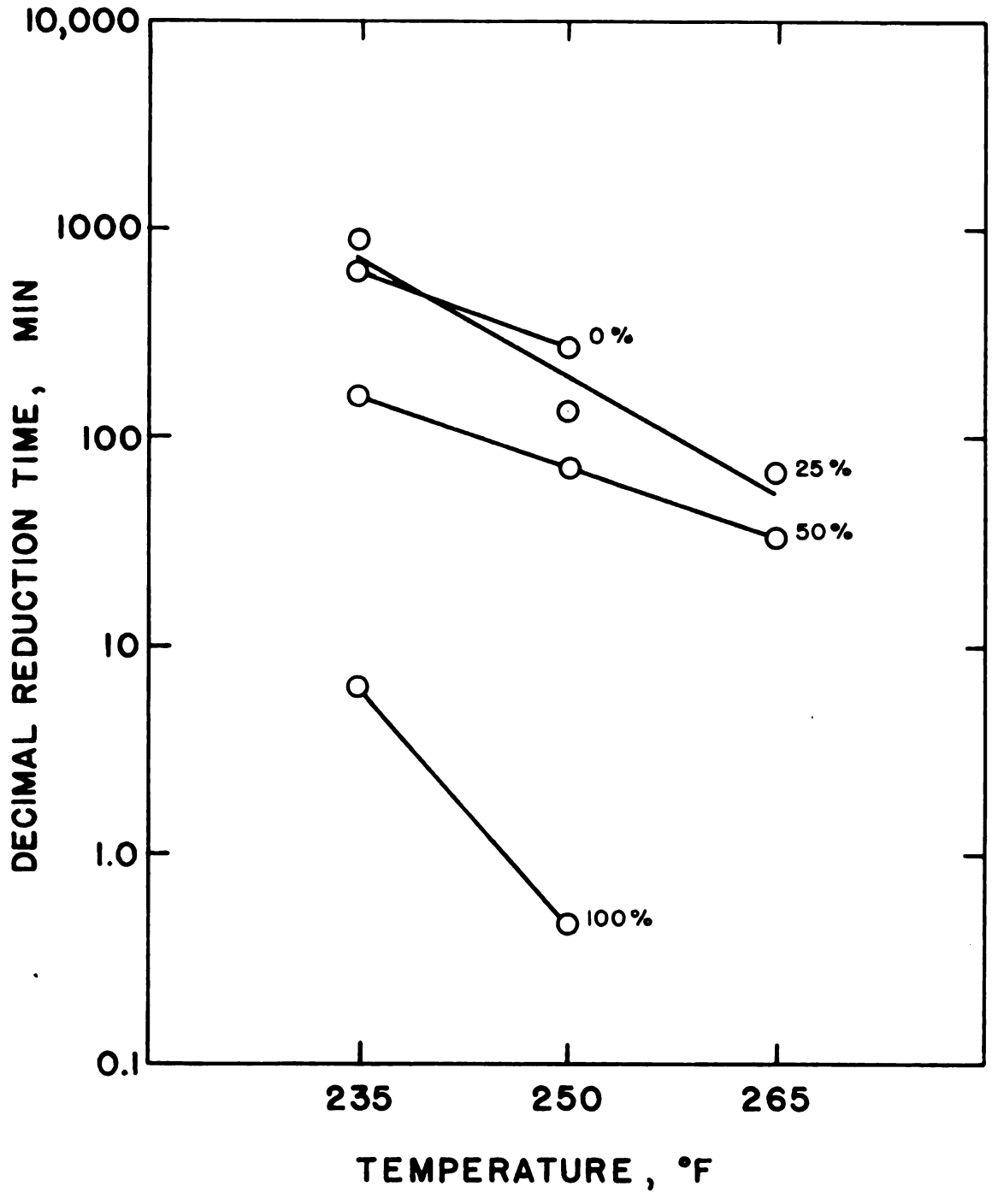

Figure 2.--Thermal resistance curves of B. subtilis spores heated in atmospheres of different water vapor content.

Table 17.--Heat resistance of B. subtilis spores in atmospheres of different water vapor contents.

Water vapor content, %	Decimal reduction time, min. at 235°F	at 250°F	at 265°F	Z, °F
0	620	270	-	41
25	900	160	54	25
50	160	73	33	44
57.6	6.3	-	-	-
63.6	-	2.6	-	-
69.1	-	-	1.6	-
100	6.3	0.48	-	17

four of them at 1% risk; of the five, four were convex (that is, no evidence of tailing).

An important feature of these survivor curves is that they did not, in general, pass through the known initial number. The intercept of the survivor curve on the t = 0 axis was either equal to or less than the known initial number. The intercepts of the survivor curves on the t = 0 axis are given in Table 18, and are expressed as the ratio of the apparent initial number (the intercept determined from the regression analysis) to the known initial number.

Adjusting the means appreciably affected the observed means of only three temperature-water vapor combinations, 235°F-0%, 250°F-0%, and 265°F-69.1%. Of these, the adjusting

Table 18.--Apparent initial number of B. subtilis spores (as % of known initial number) determined from the regression analysis.

Water vapor content, %	Temperature, °F		
	235	250	265
0	11	13	-
25	41	44	102
50	26	12	111
51.6	9	-	-
63.6	-	23	-
69.1	-	-	29
100	62	40	-

changed the heat resistant fraction from 100% to 10% and 56% to 13% at 235°F-0% and 250°F-0% respectively. By adjusting the means, there was no significant change between the observed and calculated heat resistant fractions of any of the other temperature-water vapor combinations. There seems to be no systematic trend to the predicted heat resistant fractions, either with temperature or with the percentage of water vapor.

There is a difference in values of decimal reduction time D, for the unadjusted mean data as well as for the adjusted mean data of each temperature-water vapor concentrations as is evident in Table 17 and Figures 1 and 2.

Since a straight line can be drawn within the confidence limits of D at each temperature in Figure 2, z can be determined and used with D to define the heat resistance of Bacillus subtilis spores. Other researchers (Pflug, Nicholas and Pheil 1963), working with the same strain, have found no clearly demonstrable curvature in the thermal resistance curve even over a wider temperature range. Since z for 0% and 100% water vapor contents were determined from only two D-values, the uncertainty in these z-values may be large. However, the z-values obtained from the present study for 0% and 100% water vapor compare favorably with the results given in Table 1.

The thermodynamic properties of the inactivation of spores of Bacillus subtilis in atmospheres of different water vapor contents were calculated and are given in Table 19. Arrhenius plots of destruction rates are shown in Figure 3. The values obtained are similar to the values obtained when protein is denatured. Even though there are similarities, which may be typical of protein denaturation, the evidence is not adequate to point to a specific reaction.

Table 19.--Thermodynamic properties of the inactivation of spores of B. subtilis in atmospheres of different water vapor contents.

	Water vapor concentration, %			
	0	25	50	100
Activation energy, E_a K cal/mole	31	48	30	99
Enthalpy of activation K cal/mole (250°F)	30	47	29	98
Entropy of activation, e.u./mole (250°F)	1.5	-41	1.8	-180
Free energy of activation K cal/mole (250°F)	31	31	30	26

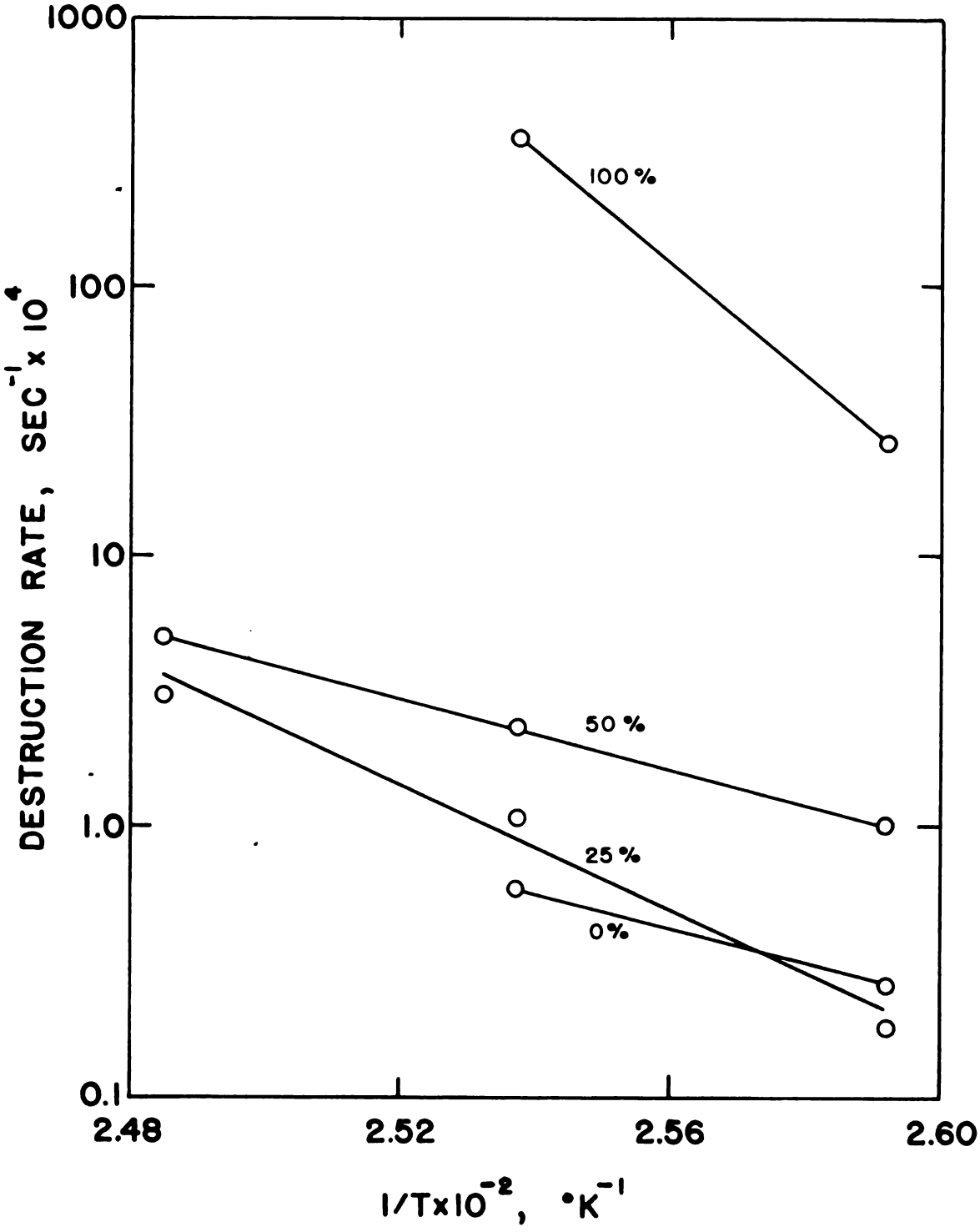

Figure 3.--Arrhenius plots of heat destruction of B. subtilis spores heated in atmospheres of different water vapor content.

CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER STUDY

The heat resistance of Bacillus subtilis spores was determined by decimal reduction time, D, at several temperature-water vapor combinations, and by the negative reciprocal of the slope of the thermal resistance curve.

The decimal reduction time, D, was found to be a function of the water vapor content of the heating atmosphere. This study has described a methodology by which the problem of heat resistance may be studied. Much work remains before one will be able to determine what agent within the spore is responsible for its failure to reproduce.

One important practical application of this study would be shortening the time required to sterilize such items as surgical equipment. For instance, if a tunnel type dryer were used, moist air could be added to produce an atmosphere of, say, 50% water vapor. This water vapor would shorten the sterilizing time considerably. After sterilization is achieved, dry air could be circulated into the heating chamber to remove most of the moisture and thus there would be no condensation on the surgical syringes etc.

Refinement of techniques and areas for further investigation include:

(1). Determine exactly how much water vapor is released from the alum into the atmosphere and how this variable is affected by temperature.

(2). Determine the extent to which the water vapor mixes with the air inside the can. Perhaps poor mixing was the cause of the high variation in counts, from cup to cup, when the cans have some air and some water.

(3). Determine why four curves were convex.

(4). Determine if spores could be dried on filter discs instead of cups, then fifty discs could be used per can, perhaps reducing the variance in the partially moist atmosphere.

(5). Determine why there was so much variance in the heat resistant fraction.

(6). Determine why the variance was higher for 25%, 50% water vapor contents than for 0% and 100%.

(7). Determine survivor curves over a broad range of temperatures, with times long enough to traverse at least two log cycles of destruction.

(8). Determine the effect of the equilibration of the atmospheric moisture with the spore on the heat resistance of the spore.

(9). Examine the very short processing times and determine any differences between the D-values obtained by survivor curves and those determined by end-point destruction studies.

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