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NASA CR 110431

ENVIRONMENTAL MICROBIOLOGY AS RELATED TO PLANETARY QUARANTINE

Semiannual Progress Report 3

December 1969

UNIVERSITY OF MINNESOTA

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AS RELATED TO PLANETARY QUARANTINE

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Supported by

NASA Grant NGL 24-005-160

Submitted to the

National Aeronautics and Space Administration

Washington, D.C.

by the

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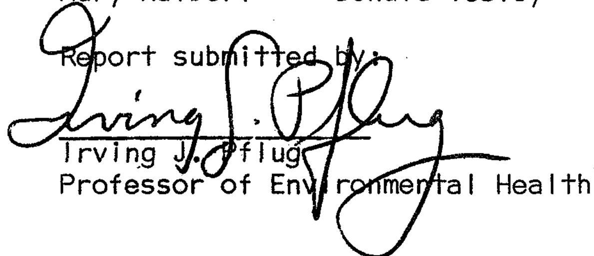

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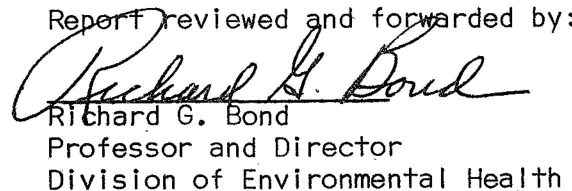

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INTRODUCTION

This report covers research activities during the period June 1, 1969 through November 30, 1969 on the project Environmental Microbiology as Related to Planetary Quarantine. These studies were conducted by the Division of Environmental Health, School of Public Health, at the University of Minnesota, under the auspices of the National Aeronautics and Space Administration.

This report pertains to research conducted in a continuous project,therefore, details regarding background and procedures for some of the tasks will be found in previous project reports. The research we have been conducting is reported under five task headings. These five areas are a combination of the tasks (letter to L.B. Hall, October 28, 1969) that were outlined in our revised work plans for this project year.

In the introduction of our progress report No. 2, (June, 1969) we listed three questions that seemed pertinent in the dry heat area of Planetary Quarantine. The first question was "What true effect does the quantity of water in the spore at heating medium temperature have on the D-value?" During the past few years a rather descriptive expression has grown up amongst Planetary Quarantine microbiologists studying the dry heat destruction of microorganisms. This expression is "water is the big problem in the dry heat destruction of microorganisms". It has been almost fifteen years since the first report was made of the significant effect of water on dry heat destruction rates. In the last five years a great many people have been actively engaged in trying to elucidate these relationships. We have had success in developing generalities, however, we have not been successful in specifically explaining all that is involved.

In my opinion, part of the water problem stems from a confusion in terminology and a failure to understand the physical behavior of water in mixtures of water vapor and air. Relative humidity is a property of air that can be measured indirectly using one of several psychometric devices. Water activity is the condition of water in a solution or inside a cell. By definition, when an equilibrium condition exists

between a spore and its surroundings, the internal water conditions (described by the term "water activity") and the external water conditions (described by the term "relative humidity") are equal. Although it is impossible to measure the water activity of a microbial spore directly, many experimenters report their data in terms of water activity, when the measured variable is relative humidity. I believe that if the measured variable in an experiment is relative humidity then the data should be reported as a function of relative humidity.

When we talk about water conditions external to the microbial spore, we approach another semantic hazard area. Relative humidity is defined as the ratio of the partial pressure of the water vapor in the air to the water vapor pressure in the air if it were saturated at the same temperature (the saturated water vapor pressure at the described temperature). In environmental microbiology we are interested in both the relative humidity as a ratio value and as a measure of the water vapor pressure in the air. To obtain the water vapor pressure value from relative humidity measurements we must know the temperature at which the relative humidity was measured. Throughout this report and in future work we at the University of Minnesota plan to include a temperature with all relative humidity values. We plan to use the general form--37% RH at 22°C.

I propose that we consider the use of either vapor pressure in millimeters of mercury, or dew point in degrees C as a replacement for our present terms of relative humidity and temperature in order to indicate the water vapor condition in the atmosphere surrounding a dry heat experiment. If we use either vapor pressure or dew point temperature we will only have to specify the test temperature, (e.g. 125°C) and either vapor pressure or dew point to give a complete picture of the water vapor conditions of the experiment.

SURVIVAL OF MICROBIAL SPORES UNDER SEVERAL TEMPERATURE AND HUMIDITY CONDITIONS

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INTRODUCTION

Experimental procedures for measuring the survival rates of bacterial spores as a function of relative humidity in sealed plastic containers at 22° and 45°C were described in the previous progress reports (December, 1968 and June, 1969). During the current reporting period a number of experiments previously initiated have been completed and additional experiments have been performed.

OBJECTIVE

The objective of this project is to determine the effect of relative humidity and relatively low temperatures (<90°C) on the long-term survival of bacterial spores on surfaces. It is possible that there may be temperature and humidity conditions acceptable to spacecraft engineers which will produce a relatively rapid rate of reduction in the microbial spore population. The effect of the conditions during either space hardware assembly or post-assembly storage could be used to reduce the terminal sterilization bio-load.

EXPERIMENTAL PROCEDURES

The procedures for obtaining the desired relative humidity conditions in sealed plastic containers were previously described in Progress Report #1, December, 1968. Details of the silica gel preparation are included in Appendix A of this report.

Experiments which have been concluded during the report period can be summarized as follows:

1. Final data analyses have been completed for a 28-week experiment to determine survival rates of *Bacillus subtilis*

var. niger (spore code AAAA) at 22°C and 45°C at <10%, ∿50%, and >90% RH in the closed system and 22°C and 30% RH with the spores exposed to the airstream of a laminar downflow room. Data through twenty weeks were presented in the previous report.

2. A 12-week experiment using the same species of microorganisms at 45°C and <10%, ∿25%, ∿35%, and ∿50% has also been completed and analyzed. The main purpose of this experiment was to determine the effect of relative humidities intermediate between <10% and ∿50% because these humidities will be more acceptable to spacecraft engineers for storage of components than humidity of 50% or higher.
3. An 8-week experiment has been completed and analyzed, again using the same suspension of Bacillus subtilis var. niger at 60°C and <10%, ∿50%, and >90% RH.
4. A 12-week experiment has been completed and analyzed using a suspension of spores isolated from garden soil. The soil suspension was stored at 50°C for 48 hours and then suspended in ethanol to destroy the vegetative cells. Survival rates were determined at 45°C and <10%, ∿50%, and >90% RH.
5. Based on data from the above experiments, a definitive series of experiments has been initiated at 45°C. This series can be summarized as follows:

   The objective of these experiments is to evaluate the following elements in the procedure used to determine D-values of Bacillus subtilis var. niger spores stored at <1%, <2%, ∿30%, ∿50% and >90% RH at 45°C.

   a) Strip material; glass vs. stainless steel
   b) Suspending fluid of the spores; 95% ethanol vs. water
   c) Different spore crops; spores produced on Tam agar vs. spores produced in SSM-10 liquid medium
   d) Box-to-box variation

   Three experiments will be performed. The relative humidities will be <1%, <2%, ∿30%, ∿50%, and >90%. A summary of the experimental design is shown in Table 1.1.

Table 1.1 - Summary of Experiments on the Survival of Bacillus subtilis var. Niger Spores Stored at 45°C - <1%, <2%, ~30%, ~50%, and >90% Relative Humidity

Relative Humidity	# of Strips	Spore Susp.*	Strip Mat.	# of Strips	Spore Susp.*	Strip Mat.	# of Strips	Spore Susp.	Strip Mat.
<1% Dry Air	16	AAAA	SS**	16	AAAA	SS	16	AAAA	SS
<2% Silica Gel	8 8	AAAA AAAA	SS g***	8 8	AAAA AAAB	SS SS	8 8	AAAA AAND	SS SS
~30%	16	AAAA	SS	16	AAAA	SS	16	AAAA	SS
~50%	8 8	AAAA AAAA	SS g	8 8	AAAA AAAB	SS SS	8 8	AAAA AAND	SS SS
>90%	8 8	AAAA AAAA	SS g	8 8	AAAA AAAB	SS SS	8 8	AAAA AAND	SS SS

* See Appendix B for details of the spore code

** 1 x 2 inch stainless steel strips

*** 1 x 2 inch glass strips

To achieve the <1% relative humidity, pint jars that can be hermetically sealed will be used to contain the strips. Strips will be inoculated, equilibrated for 20-24 hours, placed in jars and sealed inside a glove box with an atmosphere of flowing dry air (relative humidity ≤1%). Jars, covers, strips, etc. will be dried over silica gel before being placed in the glove box. The moisture in the atmosphere of the glove box will be analyzed with a Bell and Howell (C.E.C.) moisture analyzer. From these data the relative humidity in the jar at 45°C will be calculated. There are no plans to check the humidity in the jars after they have been sealed. The other relative humidity conditions will be achieved as previously reported using silica gel and water.

Two containers at <1%, <2%, and ~30% relative humidity will be analyzed every two weeks for a period of ten weeks. Strips held at ~50% and >90% RH will be analyzed weekly for a period of five weeks. Where two strip materials or spore suspensions are being evaluated, four strips of each material will be placed in a container. Sterile teflon strips will be used to separate strips placed back to back.

Eight strips from the glove box and eight from the downflow room will be processed as zero-time controls.

To date only Experiment I has been carried out but the data analysis for this experiment has not yet been completed.

6. A supplementary experiment was also carried out to ascertain the effect of utilizing higher agar concentrations when plating greater quantities of diluent. This practice has been followed in all previous experiments in an effort to equalize the available nutrient per given volume of diluent. The experiment was carried out as follows:

Approximately 10^5 spores were deposited on each of five 1" x 2" stainless steel strips and were allowed to dry. Each strip was placed in a 125 ml flask, 50 ml of buffer were added, and the flask was then insonated for two minutes.

Dilutions of the eluate were then made with sterile buffer so that by plating 0.1, 1.0, 10, and 30 ml aliquots, the same number of organisms would be present per dilution. (See Figure 1.1, page 5)

Twenty ml of single strength TSA were used for plating the .1 and 1.0 ml dilutions, fifteen ml of 1 1/2 strength TSA for the 10 ml dilutions, and thirty ml of double strength TSA for the 30 ml dilutions.

7. A second supplementary experiment was carried out to determine whether two different pipettes would deliver equal quantities (.02 ml) of spore suspension. We also wanted to find out whether there was a trend toward either increasing or decreasing deposit concentrations with time between mixing sequences for the spore suspension. The experiment was carried out as follows:

Thirty-two 1" x 2" stainless steel strips were contaminated with .02 ml of an alcohol suspension of <u>Bacillus subtilis</u> var. <u>niger</u> spores, sixteen using pipette A (downflow room) and sixteen using pipette B (crossflow room). A vortex mixer was used on the spore suspension after inoculating each of the eight strips with each pipette. Immediately after drying, the strips were transferred to sterile 125 ml flasks and processed alternately.

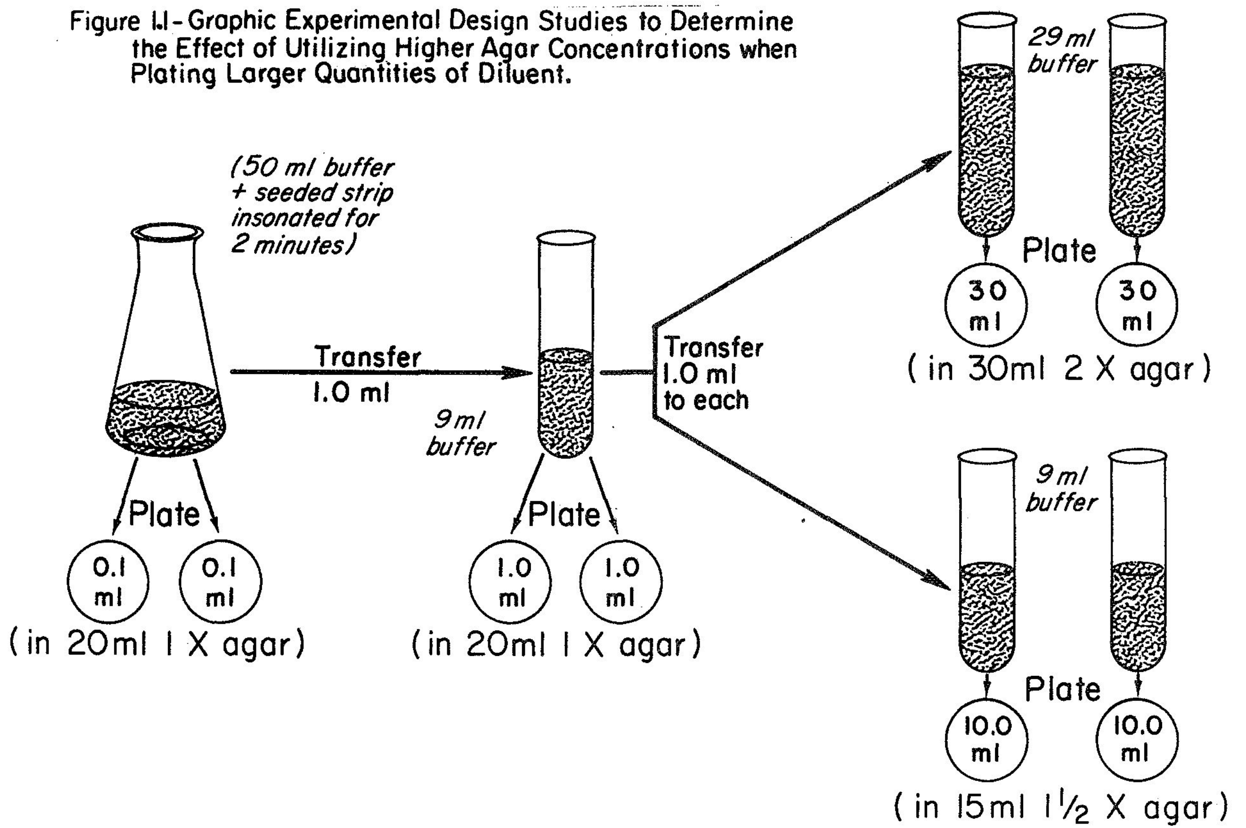

Figure 1.1 - Graphical experimental design scheme for studies to determine the effect of utilizing higher agar concentrations when plating larger quantities of diluent

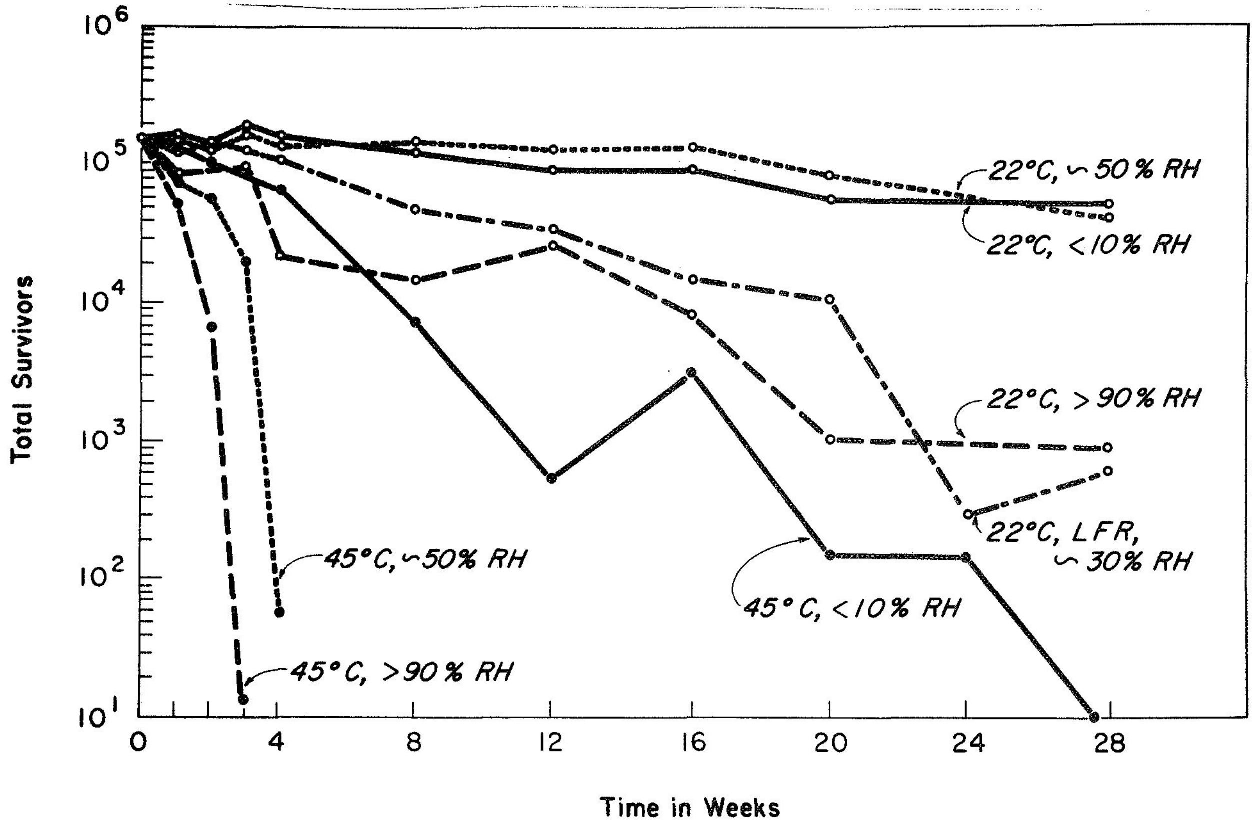

Figure 1.2 - Survival of Bacillus subtilis var. niger spores at 22 and 45°C as a function of relative humidity during a 28-week experiment period

RESULTS

Figure 1.2 represents the survival of the *Bacillus subtilis* var. *niger* spores at 22°C and 45°C during the 28-week experiment. Figure 1.3 (see page 8) illustrates the survival at 45°C at the intermediate relative humidities during the 12-week experiment, while Figure 1.4 (see page 8) depicts the same information at 60°C during the 8-week experiment at that temperature. The data from all of these experiments are summarized in Table 1.2 and included are the range of relative humidities recorded and the D-values calculated from the slope of the least squares regression lines of log N vs. time.

Table 1.2 - D-values of *Bacillus subtilis* var. *niger* Spores as a Function of Relative Humidity and Temperatures of 22°, 45°, and 60°C

Temperature °C	% RH Range	Length of Experiment (weeks)	N_o (x 10^5)	D-value (days)
22	<2	28	1.5	391.5
	30-40 (LFR)	28	1.5	74.3
	50-59	28	1.5	540.1
	88-96	28	1.5	89.1
45	<2-9	28	1.5	47.1
	<2-8	12	2.6	26.5
	24-27	12	2.6	18.9
	35-38	12	2.6	15.2
	55-57	28	1.5	7.1
	53-55	12	2.6	7.6
	96-100	28	1.5	4.4
60	<2-8	8	1.7	11.4
	53-55	8	1.7	2.7
	94-95	8	2.1	2.2

The relationship between D-value and relative humidity at each temperature tested is illustrated in Figure 1.5 (see page 8). It is apparent that in the ranges tested both the temperature and humidity significantly affected spore survival. The most rapid die-away occurred at the higher temperatures and at the higher humidities. Relatively little die-away was observed at 32°C, particularly in the closed systems and where the humidity was below 60%. In tests where the spores on strips were exposed to the laminar flow air stream the die-away was more rapid than it was in the very high humidity condition in the closed containers. However, even these conditions at 22°C resulted in considerably longer D-values than those in the lowest humidity in experiments at 45°C. The D-values of less than twenty days observed at 45°C, even at relative humidity as low as ∿25%, indicate that important reductions in surface spore contamination may be achieved during storage of the components prior to terminal sterilization. If circumstances permit storage at 60°C, even greater advantages may accrue.

In all of the foregoing experiments, it should be pointed out that D-value calculations have been used primarily for convenience in reporting the results. Observation of actual survival curves (see Figures 1.2-1.4), indicates that logarithmic count reduction plotted against time does not necessarily follow a straight line. Die-off appears to accelerate after a certain time period, particularly at high humidities, thus making the D-value estimates conservatively higher than they might otherwise be. Further investigation of the shape of the survival curves will be carried out.

The results of experiment 4, which measured the survival of natural spores from a soil sample, are illustrated in Figure 1.6 (see page 8) and are summarized in Table 1.3.

Table 1.3 - Survival of Spores from Natural Soil at 45°C
(12-week Experiment, $N_o = 1.1 \times 10^3$)

Relative Humidity*	D-value (Days)
< 2%	127.0
50-56%	42.4
88-92%	19.5

* RH at 45°C

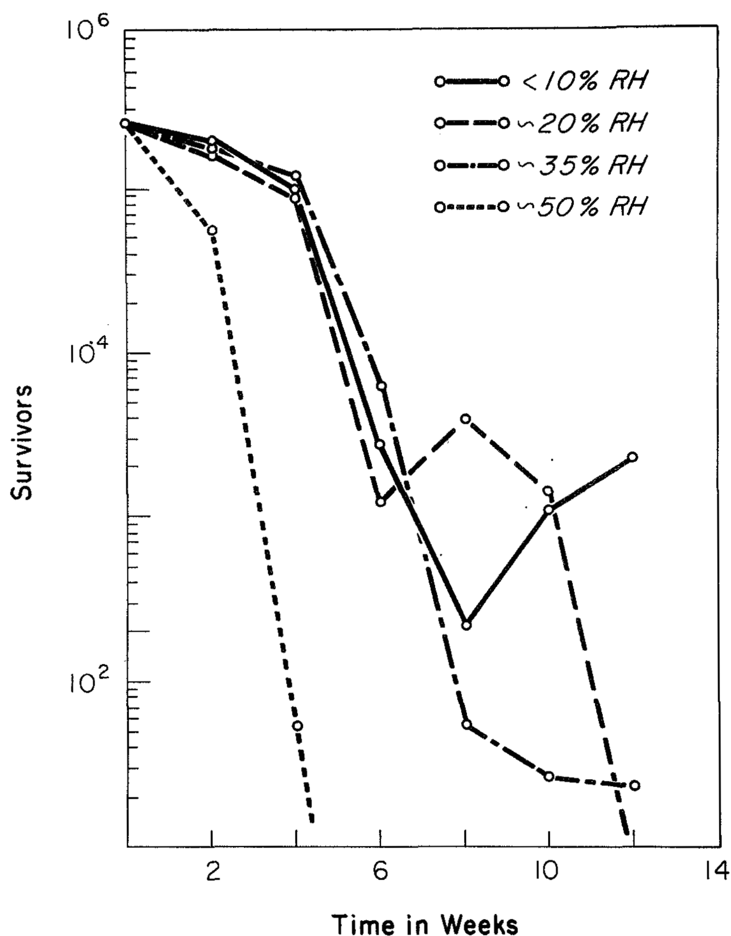

Figure 1.3 - Survival of Bacillus subtilis var. niger spores at 45°C at intermediate relative humidities.

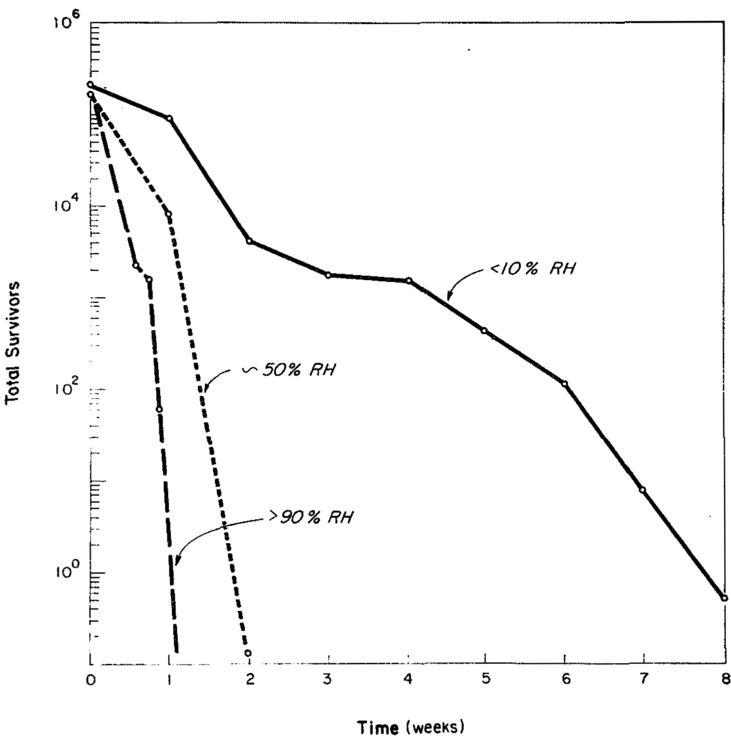

Figure 1.4 - Survival of Bacillus subtilis var. niger spores at 60°C as a function of relative humidity.

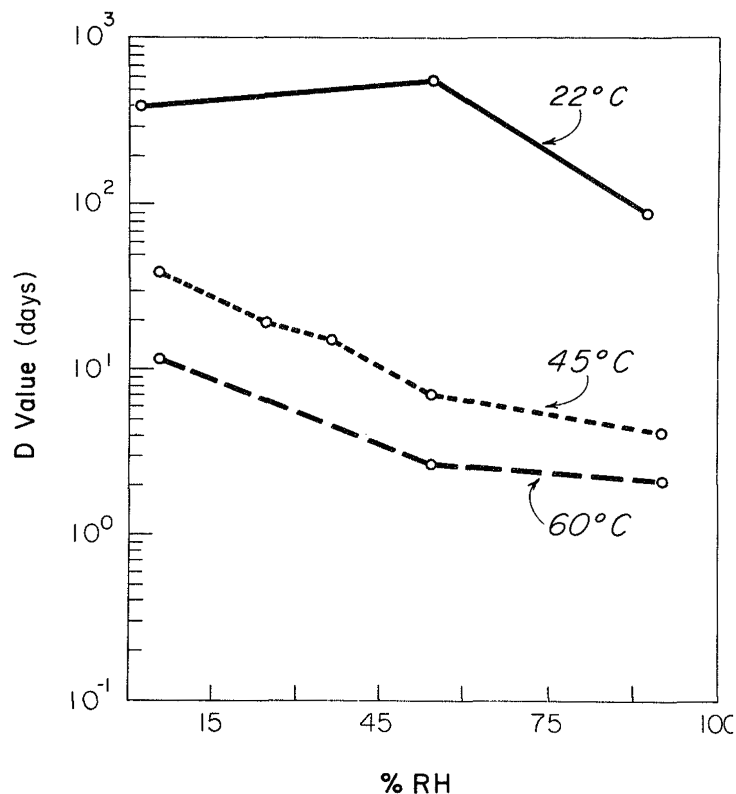

Figure 1.5 - The relationship of the D-value in day with relative humidity in percent for the data at 22, 45 and 60°C.

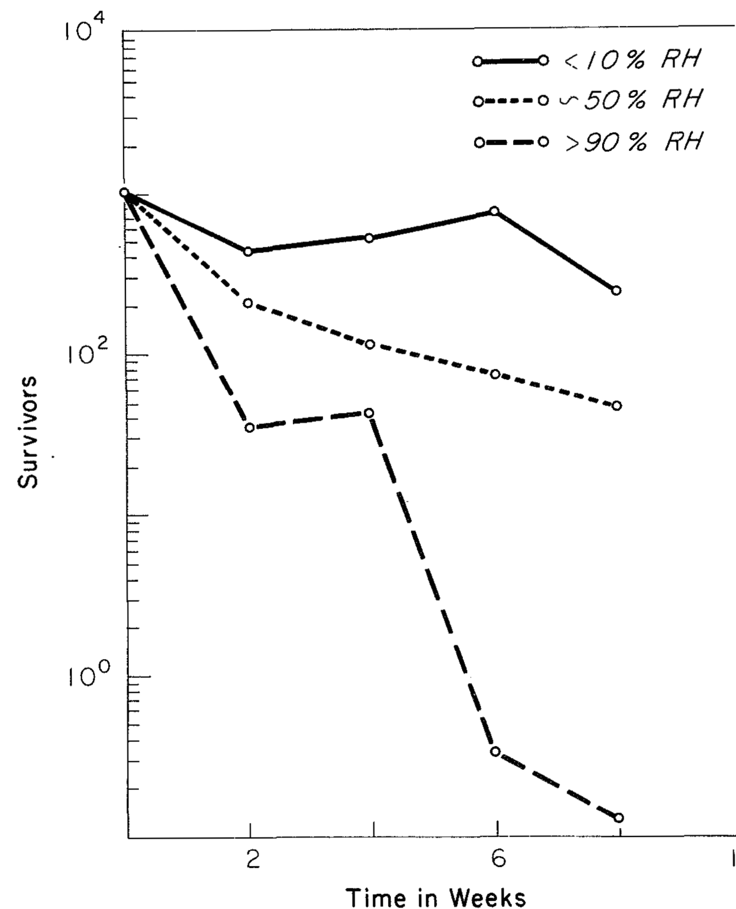

Figure 1.6 - Survival studies of spores obtained fro soil held for different periods of time at 45°C at several relative humidity levels.

It can be seen that the humidity effect at 45°C was similar to that observed for Bacillus subtilis var. niger spores. However, D-values for these spores are considerably longer. The results are not directly comparable, however, as N_o in this case was several logs lower than in other experiments.

Table 1.4 summarizes the results to date of the series of experiments at 45°C.

Table 1.4 - Survival of Bacillus subtilis var. niger Spores at 45°C

% RH	Strip Material	Colonies Surviving After: (weeks)						
		0	1	2	3	4	5	6
0.26-0.40*	SS	2.20E5	-	9.84E4	-	9.42E4	-	6.48E4
<2%	SS	3.09E5	-	1.45E5	-	1.41E5	-	6.22E4
	Glass	2.74E5	-	1.72E5	-	1.10E5	-	7.59E4
27-32	SS	3.09E5	-	2.23E5	-	1.10E5	-	1.74E4
49-55	SS	3.09E5	2.31E5	8.68E4	3.49E3	7.17E2	1.71E2	**
	Glass	2.74E5	8.38E2	**	-	-	-	-
90-95	SS	3.09E5	1.33E5	2.84E4	2.25E2	2.5E-1	7.5E-1	**
	Glass	2.74E5	8.90E1	**	-	-	-	-

* Humidity calculated at 45°C but measured during preparation only (at 23°C) -- not during storage.

** No colonies observed.

The spores used in this series are the same as those previously used. Different spore suspensions will be evaluated later. The results through six weeks are reported. There is no indication as yet, of a difference in survival rate between those spores in the less than 2% RH silica gel boxes and those prepared in the special dry gas environment at <1% RH. The very rapid die-away of the spores which were deposited on glass strips and stored at ∿50% or >90% RH was surprising. These spores died away so quickly that additional experiments will have to be performed in order to obtain sufficient data points to calculate the

D-values.

A secondary objective of this experiment has been to compare the data from replicate boxes, which each contained four strips, under identical conditions for each relative humidity. Data analyses from this objective will be presented in the next report.

Data from the experiment to determine the effect of agar concentration changes on the number of microorganisms recovered are summarized in Table 1.5.

Table 1.5 - Effect of Agar Concentration on Colony Counts

Quantity of Diluent Plated (a)	Agar Strength (b)	Quantity of Agar Added to Plate(ml) (c)	Relative Agar Strength in Pour Plate (d)*	Colonies per Strip times E-5	Mean Colonies per Strip times E-5
0.1	1 x	20	20/20.1	1.74 1.78 2.27 2.16 0.97	1.78
1.0	1 x	20	20/21	1.42 1.51 2.18 1.94 0.73	1.57
10.0	1 1/2 x	15	22.5/25	1.31 1.38 1.81 1.65 0.74	1.38
30.0	2 x	30	60/60	1.44 1.46 1.83 1.64 0.78	1.43

$$* \ (d) = \frac{(c) \times (b)}{(c) + (a)}$$

It appears that the original 0.1 ml from the 50 ml of diluent yielded somewhat higher counts than the other dilutions, however, no significant

differences were observed among the other three dilutions. This indicates that the variation in agar concentration generally achieved the goal of equalizing relative agar strength in the pour plates.

The results of experiment 7 are summarized in Table 1.6.

Table 1.6 - Comparison of Two Eppendorf Pipettes in Delivering 0.02 ml of Spore Suspension

	Pipette A		Pipette B	
	Colonies per Spore Deposit	Coefficient of Variation	Colonies per Spore Deposit	Coefficient of Variation
Trial 1 (8 deposits)	2.10E5 2.10E5	.11	2.37E5	.12
Trial 2 (8 deposits)	2.12E5	.17	2.15E5	.13
Mean of Two Trials	2.11E5	.14	2.26E5	.12

An analysis indicated that there was no significant difference which could be attributed to the pipettes even at $\alpha = 0.10$. Results also disclosed that there was no trend toward increased or decreased concentration of the spore deposit over the eight deposits made between the mixing sequences of the suspension.

CONCLUSIONS

At this time it is apparent that significant die-away of *Bacillus subtilis* var. *niger* spores does occur at relatively low temperatures (45°-60°C). This die-away is most pronounced at very high relative humidity (> 90%) but is also significant at intermediate humidities (∿ 50% or even lower). The same effect was noted for natural spores but with considerably longer D-values. Glass strips, when compared to stainless steel, appear to greatly accelerate the die-away at >50% RH, but the significance of this finding must await further exploration.

FUTURE WORK

In the immediate future experiments will continue to evaluate differences between glass and stainless steel, and the effect of different spore suspension histories. Later, priority will be given to experiments at higher temperatures (up to 90°C), using different surface materials and other spore species.

THE EFFECT OF HUMIDITY, LOCATION, SURFACE FINISH AND SEPARATOR THICKNESS ON THE DRY HEAT DESTRUCTION OF BACILLUS SUBTILIS VAR. NIGER SPORES LOCATED BETWEEN MATED SURFACES

Project Personnel: D. W. Drummond, I. J. Pflug, and J. Haugen
Division of Environmental Health

INTRODUCTION

This is a continuation of studies reported in Progress Reports #1 and #2. Report #1 described the mated surface experimental system and summarized the preliminary experiments which were performed. These indicated that relative humidity before and during treatment and position within the mated surface were the most important parameters affecting microbial destruction. Other variables of interest were the surface finish of the mated surface and the presence or absence of separators between the surfaces.

A series of experiments was conducted to explore the effects of the parameters listed above. In addition an open system was prepared and tested to provide experimental data comparable to that for the mated surface system. Preliminary data were reported in Progress Report #2. The present report presents the analyzed results of experiments performed after November, 1968.

OBJECTIVE

The objective of this project is to measure the thermal death parameters of *Bacillus subtilis* var. *niger* spores in a mated surface system and to determine the effect of certain variations of the environment and surfaces on thermal destruction.

EXPERIMENTAL PROCEDURE

Details of the experimental procedure are contained in Reports 1 and 2 (December, 1968 and June, 1969). A sketch of the treatment apparatus with mated surface package in place is shown in Figure 2.1. The effort in this project during the reporting period was used to carry out the

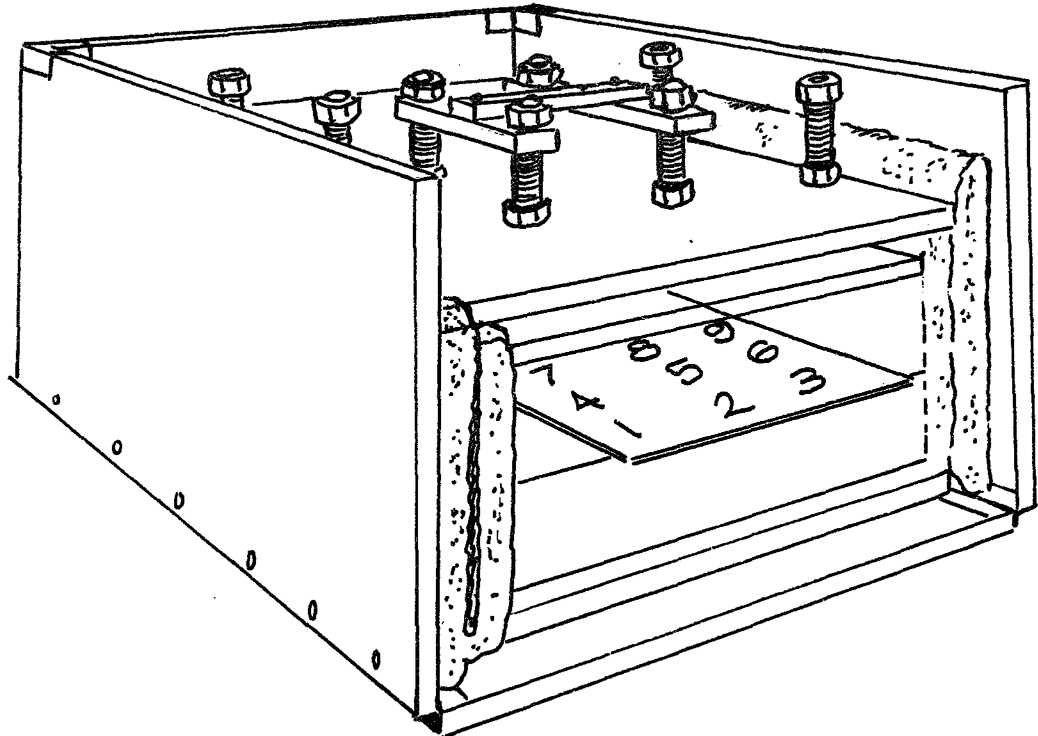

Figure 2.1 - The heat block used for treating mated surface packages is shown with a package in place. The numbers represent the location of the spore deposits on the 6" x 8" mated surface.

following new experiments:

1. Mated System
   a. Measurement of microbial destruction parameters of spores after conditioning in a clean room .
   b. The first of a series of tests to compare the thermal resistance of *Bacillus subtilis* var. *niger* with the thermal resistance of spores isolated by Jet Propulsion Laboratory from the Mariner Spacecraft.
   c. A test of the effect of the concentration of spores in a mated surface area on the D-value.
2. Open System
   a. Experiments at high and low conditioning and treatment humidities were repeated with the time for movement from the conditioning to the treatment environment reduced from four minutes to approximately twenty seconds.
   b. An experiment with spore conditioning and treatment in a clean room provides data at intermediate relative humidity conditions.
3. Development of detailed data analysis computer programs.
   a. Analysis of variance. The analysis of variance was carried out by the statistical services group of the Biometry Department of the School of Public Health, University of Minnesota. The model used to analyze all data was a combination of factorial and split-plot approaches. In both the mated surface and the oven systems each group of samples which received a given time-temperature treatment combination was considered to be a whole plot. The individual samples within the time-treatment combination (i.e.the positions within the mated surface package) were the sub-plots. A factorial analysis of the whole plot allowed separation of the effects of day of treatment, conditioning humidity, treatment humidity, surface finish, etc. as appropriate to a given experiment. The split-plot analysis determined whether there were variations among positions; such variations would be expected in the mated

system but not in the open system. Since analysis of variance assumes that the variance of individual samples is a constant, it was necessary to modify the data so that the assumption would hold. The analysis therefore, was carried out on the logarithms of the data values. Since all data values were included, even those which were zero, the actual modifying function used was the logarithm of datum value +1.0. The modification introduces a small error in the data, but it is not significant above an estimated value of twenty spores where it produces a 5% error. The modification allows the direct computer analysis of the data.

b. Linear regression. The linear regression was carried out by a computer program developed specifically for the analysis of microbiological data. The program performs the following tasks:

1. Lists the raw data
2. Computes and lists the logarithm of datum value +1.0
3. Computes and lists the geometric mean, standard error and the 95% confidence interval for replicate values for each exposure time
4. Performs a least-squares linear regression analysis on the data
5. Calculates and prints the D-values, the y-intercept and their 95% confidence intervals
6. Calculates separate regression lines and D-values for each column (e.g. position)

RESULTS AND DISCUSSION

The results are summarized experiment-by-experiment in this section. Where applicable, an analysis of variance was used to determine statistical significance. The D-values and y-intercepts of the regression lines, the 95% confidence interval for each, and the intercept ratio (IR) where IR equals $\log y_o/\log N_o$ are recorded in the data table. The IR is a gross measure of the shape of the survivor curve during the initial

portion of the heat treatment (i.e. the portion between t=o and the shortest treatment time.) If the IR is greater than 1.0 there may be a trend for the D-value to decrease with increasing heating times; that is for the curve to be concave downwards, the curve may be thought to have a shoulder. If the IR is less than 1.0 the initial portion of the curve is concave upwards. The logarithms were used to calculate the IR since the intercept of the regression line and the value of N_o are both calculated using the logarithms of the data. If further analysis of the IR is desired at a later time it will be less complex than the analysis of a statistic which was composed of functions of other statistics.

The analysis of variance was performed using all recovered data. In all cases where the analysis was performed, six positions from the mated surface plate were included. To make the present report manageable, however, only the results for positions 1 and 5 have been reported in the tables.

Experiment DD8320

The experiment evaluated the effect of four treatment humidities on the D-value of spores in the mated surface system. All samples were conditioned at 40% relative humidity and 23°C in a clean room; the treatment humidities were .36%, .66%, .79%, and 1.1% at 125°C.

The treatment humidities were obtained by placing the treatment apparatus in a walk-in refrigerator, an air-conditioned laboratory, a humidified clean-room, and a humidified incubator respectively. Each time-treatment combination was replicated four times. The replicates were prepared, treated, and processed together. The data are summarized in Table 2.1.

The spores used in the experiment have the same genetic composition as the AAAC spores used in subsequent experiments but they are from an earlier crop whose production was less well-controlled. Therefore, the results are not directly comparable with the results of later experiments. The spores used in the present experiment were stored in alcohol for about a year before they were re-suspended in water in preparation for this experiment. The present results along with those of experiment 13 reported in Progress Report #1, suggest that spores produced under less than ideal conditions, and/or spores

which have been stored for a long time show a greater sensitivity to treatment humidity than do fresh, more-resistant spores such as those used in experiment DD9066; this possibility should be explored with additional species and varieties of spores.

Table 2.1 - D_{125}-values, Y-intercepts (y_o) and Their .95 Confidence Intervals and Intercept Ratios for Experiment DD8320. Comparison of the Effects of Four Treatment Humidities at One Conditioning Humidity

Pooled Data (4 replicates)

Conditioning RH at 23°C	Treatment RH at 125°C		Position 1	Position 5
40%	.36%	D y_o IR*	17.0 (12.4,26.7) 8.78E3 (8.00E2,9.65E4) .78	24.1 (17.8,37.2) 3.04E4 (5.92E3,1.56E5) .88
40%	.66%	D y_o IR	22.4 (16.3,36.1) 2.68E4 (4.05E3,1.77E5) .87	84.5 (24.7,∞) 1.26E4 (5.14E2,3.11E5) .81
40%	.79%	D y_o IR	20.7 (14.1,38.6) 1.73E4 (1.40E3,2.13E5) .83	31.5 (23.9,45.9) 2.75E4 (9.01E3,8.42E4) .87
40%	1.1%	D y_o IR	17.8 (12.9,28.7) 5.07E4 (4.69E3,5.48E5) .93	32.5 (25.5,44.9) 5.87E4 (2.27E4,1.52E5) .94

* $IR = \frac{\log y_o}{\log N_o}$ N_o = 1.20E5 (9.45E4,1.52E5)

The analysis of variance (Table 2.2) shows that treatment humidity has a significant effect on the thermal destruction of these spores in contrast to the results of experiment DD9066. As in all of the experiments, position has a significant effect with survival being lower near the edge of the mated surface plate than near the center. There is also evidence of interaction between humidity and position and also between treatment time and position; this means that the effect of treatment humidity and the effect of treatment time are probably dependent upon the location of the spores within the mated surface.

Table 2.2 - Analysis of Variance for Experiment DD8320: Comparison of the Effects of Four Treatment Humidities (.36,.66,.79,1.1% RH at 125°C) at One Conditioning Humidity (40% RH at 23°C)

Source	SS	df	MS	F
Mainplot				
Between 6" x 8" Sheets	214.8963	47	4.5723	
Humidity	40.7343	3	13.5781	14.97 (P<.001)
Treatment Time	132.3241	2	66.1621	72.94 (P<.001)
H x T	9.1819	6	1.5303	1.69 (not sig.)*
Mainplot Residual	32.6561	36	.9071	
Subplot				
Within 6" x 8" Sheets	104.6253	240	.4359	
Position	66.6957	5	13.3391	132.04 (P<.001)
Humidity x Position	3.3064	15	.2204	2.18 (P<.01)
Time x Position	12.6251	10	1.2625	12.50 (P<.001)
H x T x P	3.8142	30	.1271	1.26 (not sig.)*
Subplot Residual	18.1839	180	.1010	
Total	319.5217	287		

* P>.1

Data Analysis of Experiment DD9066

The experiment evaluated the effect of two levels of conditioning humidity (<2% and 90% RH at 23°C) and two levels of treatment humidity (.34% and 1.1% RH at 125°C) on thermal destruction. The experiment was carried out five times, each on different days. Each experiment used four treatment times (15,40,65, and 90 minutes) in addition to the various humidity treatments for a total of sixteen 6" x 8" plates or 96 1" x 2" strips per experiment, exclusive of controls. The preliminary results of the experiment were reported in Progress Report #2; the data are summarized in Table 2.3 and are shown in graphical form in Figure 2.2.

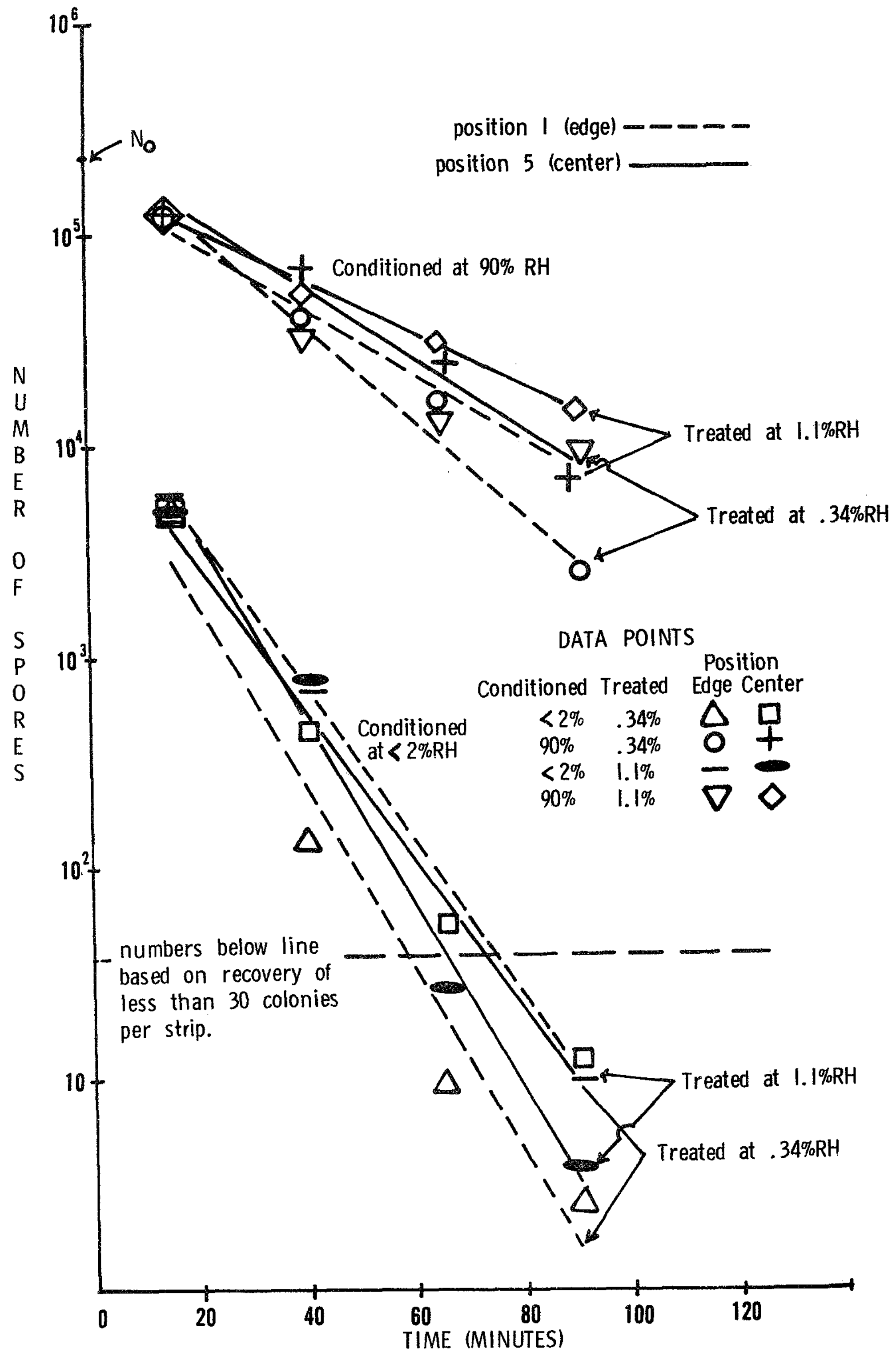

Figure 2.2 - Data points and least squares regression lines for Experiment DD9066: Comparison of the effects of two conditioning humidities (<2% and 90% RH at 23°C) and two treatment humidities (.34% and 1.1% RH at 125°C) in a mated surface system. Each datum point is the geometric mean recovery from five strips.

Table 2.3 - D_{125}-values, Y-intercepts (y_o) and Their .95 Confidence Intervals and Intercept Ratios for Experiment DD9066. Comparison of the Effects of Two Conditioning Humidities and Two Treatment Humidities in a Mated Surface System

Pooled Data(5 replicates)

Conditioning RH at 23°C	Treatment RH at 125°C		Position 1	Position 5
low <2%	low .34%	D y_o IR	22.8 (16.2,38.7) 1.28E4 (1.09E3,1.52E5) .77	28.5 (18.0,69.3) 1.33E4 (7.91E2,2.25E5) .77
low <2%	high 1.1%	D y_o IR	27.2 (21.2,37.9) 2.02E4 (4.87E3,8.37E4) .81	23.0 (16.3,38.8) 2.68E4 (2.37E3,3.04E5) .81
high 90%	low .34%	D y_o IR	45.4 (37.4,57.9) 2.81E5 (1.47E5,5.37E5) 1.02	60.6 (44.5,94.9) 2.49E5 (1.10E5,5.64E5) 1.01
high 90%	high 1.1%	D y_o IR	65.6 (44.5,125.3) 1.72E5 (6.38E4,4.65E5) .98	82.5 (59.4,135.0) 1.81E5 (9.47E4,3.44E5) .99

N_o = 2.13E5 (1.27E5,3.55E5)

If the results reported here are compared with those reported in Progress Report #2, a considerable difference in D-values will be noted; in the earlier report the arithmetic means were plotted for each time-treatment combination and a regression line was estimated visually. In the present experiment, the regression line was calculated using the logarithms of the data. The logarithm transformation makes the variance of the points independent of the mean, a necessary pre-condition for linear regression and analysis of variance. An equivalent visual procedure would be to plot the geometric means for each time-treatment combination and then estimate the regression line visually.

When spores are conditioned at <2% RH at 23°C and treated at .34% RH at 125°C, the D-value at position 1 is clearly less than the D-value at position 5. The results suggest that there is less water at position 1 than at position 5. When spores are conditioned at the lower humidity and treated at the higher humidity, however, position 1 at first appears to have a higher D-value than position 5; however, a look at the confidence intervals will show that the intervals overlap almost completely. We may say, and it is confirmed by the

analysis of variance, that the position effect is smaller at higher humidities; in other words, the difference in behavior between position 1 and position 5 is smaller at high treatment humidities than at low treatment humdities; probably because water vapor is lost from the exposed edge of the mated surface more slowly at higher treatment humidities.

When the conditioning humidity is high the results show a considerably larger D-value at the center position than at the edge position for both the low and high treatment conditions. This is compatible with the reasoning above since the surfaces conditioned at a higher humidity start the treatment cycle with a larger water content. Methods to measure the water content of a spore sample may soon be available; a direct measurement of water content combined with an analysis of the diffusion characteristics of the mated surface package should permit a relationship to be developed between water loss and thermal destruction. A revised mated system is proposed under "Future Work" which will make such an analysis more feasible.

The analysis of variance (Table 2.4) shows that the day on which an experiment is performed has a significant effect on the results. This result appears frequently in experiments with this system as well as with other systems; it requires further exploration. Conditioning humidity has a very significant effect, although treatment humidity does not. In the sub-plot analysis, however, a significant interaction between treatment humidity and position is detected. This is the variation in the effect of position with humidity which was discussed above. Position within the mated surface has a significant effect on the death parameters.

D-value data for the same spores (AAAC) at the same conditioning and treatment humidities but in an open system are reported as experiment DD9304.

Table 2.4 - Analysis of Variance for Experiment DD9066. Comparison of the Effects of Two Conditioning Humidities (<2% and 90% RH at 23°C) and Two Treatment Humidities (.34% and 1.1% RH at 125°C) in a Mated Surface System

Source	SS	df	MS	F
Mainplot				
Between 6" x 8" Sheets	1166.7464	79	14.7689	
Days	44.5468	4	11.1367	3.96 (P≐.09)
Conditioning Humidity	623.9927	1	623.9927	221.72 (P<.001)
Treatment Humidity	2.9505	1	2.9505	1.05 (not sig.)*
C x T	.3639	1	.3639	.13 (not sig.)*
Time	276.8597	3	92.2866	32.79 (P<.001)
Higher Interactions	49.1762	9	5.4640	1.94 (P≐.08)
Subjects within Groups	168.8566	60	2.8143	
Subplot				
Within 6" x 8" Sheets	44.6408	400	.1116	
Positions	2.0103	5	.4021	4.1 (P<.001)
Conditioning Humidity x Position	.3716	5	.0743	.76 (not sig.)*
Treatment Humidity x Position	2.6060	5	.5212	5.34 (P<.001)
Time x Position	1.3504	15	.0900	.92 (not sig.)*
Other Interactions with Position	7.0626	50	.1413	1.45 (P<.025)
Positions x Subjects within Groups	31.2399	320	.0976	
Total	1211.3873	479		

* P>.1

Experiment DD9141

The experiment repeated one of the large D-value conditions of experiment DD9066 using a treatment time sufficient to reduce the initial spore population by a factor of 1000; this helps to assure that no unusual behavior of the curve occurs after long treatment times. Another objective of the experiment was to investigate the day-to-day variability which occurs in this and other experimental systems. The question was: does

the variability appear among replicates handled on the same day or does it appear from day to day? In the former case, unsuspected variations in the treatment package or the experimental system would be suspected; in the latter case a variation in the preparation or processing or some other extraneous factor would be more likely.

The experiment was repeated on two different days. On each day two sets of mated surface plates were prepared, treated, and processed together. Three treatment times (1, 3.5, and 6 hours) were used. All test samples were conditioned at <2% RH at 23°C and were treated at .34% RH at 125°C. The results are summarized in Table 2.5.

Table 2.5 - D_{125}-values, Y-intercepts (y_o) and Their .95 Confidence Intervals and Intercept Ratios for Experiment DD9141. Up to Six-hour Treatment of Samples Conditioned at 90% RH at 23°C and Heated at .34% RH at 125°C

		Position 1	Position 5
Day 1	D	72.8 (56.6,101.9)	105.7 (65.4,275.5)
	y_o	3.29E5 (3.67E4,2.95E6)	3.02E5 (1.15E4,7.92E6)
	IR	1.05	1.04
Day 2	D	64.1 (55.8,75.3)	72.3 (66.6,79.1)
	y_o	3.37E5 (9.15E4,1.24E6)	5.28E5 (2.71E5,1.03E6)
	IR	1.05	1.08
Pooled Data(2 replicates)	D	68.2 (59.7,79.4)	85.9 (67.0,119.4)
	y_o	3.33E5 (1.04E5,1.07E6)	3.99E5 (6.40E4,2.49E6)
	IR	1.05	1.06

N_o = 1.90E5 (1.42E5,2.55E5)

The survivor curves behaved in the usual manner; however, the D-values obtained are somewhat longer than those for the same

conditions in experiment DD9066. The analysis of variance (Table 2.6) shows that the day-to-day variation is significant at the .05 level. The results confirm that it is better to carry out replicate experimental runs on different days rather than all on the same day. By carrying out the runs on different days, the mean D-values and intercepts will approach the real value more closely. The cause of the day-to-day variation is still unexplained; however, within days the inter-relationships of treatments are very well preserved, therefore the cause of the variation is probably external to the treatment system. Variations in barometric pressure or in some other atmospheric factor seem most likely, although variations in the recovery buffer or nutrient medium may be responsible. Further studies are most desirable to discover the source of the day-to-day variation.

Table 2.6 - Analysis of Variance for Experiment DD9141. Up to Six-hour Treatment of Samples Conditioned at 90% RH at 23°C and Heated at .34% RH at 125°C

Source	SS	df	MS	F
Mainplot				
Between 6" x 8" Sheets	217.6707	11	19.7882	
Days	7.9672	1	7.9672	6.42 (P<.05)
Treatment Time	199.5931	2	99.7965	78.97 (P<.001)
Mainplot Residual	10.1104	8	1.2638	
Subplot				
Within 6" x 8" Sheets	9.2740	60	.1546	
Position	3.9868	5	.7974	9.32 (P<.001)
Position x Process	1.4384	10	.1438	1.68 (not sig.)*
Subplot Residual	3.8488	45	.0855	
Total	226.9444	71	3.1964	

* P>.1

Experiment DD9155

The experiment compared the thermal destruction of spores deposited on type 301 stainless steel (rolled finish) with the destruction of spores

on type 302 stainless steel (brushed finish). Spores were conditioned at <2% RH at 23°C and were treated at .34% RH at 125°C. The experiment was completely replicated on two different days. The results are summarized in Table 2.7.

Table 2.7 - D_{125}-values, Y-intercepts (y_o) and Their .95 Confidence Intervals and Intercept Ratios for Experiment DD9155. Comparison of the Effects of Rolled Finish on Type 301 with Brushed Finish on Type 302 Stainless Steel Conditioned at <2% RH at 23°C and Heated at .34% RH at 125°C

		Position 1	Position 5
Type 301	D y_o IR	52.4 (33.3,122.4) 6.19E4 (1.39E4,2.76E5) .91	77.6 (52.1,151.8) 8.56E4 (3.61E4,2.03E5) .93
Type 302	D y_o IR	106.6 (62.5,361.4) 4.61E4 (1.86E4,1.14E5) .88	82.9 (62.6,122.7) 1.32E5 (7.68E4,2.25E5) .97

N_o = 1.94E5 (1.15E5,3.29E5)

Experiment 13 (reported in Progress Report #1) showed the major difference between the two surfaces with type 302 having a smaller D-value. This was attributed to the slightly rougher finish on the type 302 steel. However, the present experiment shows a reversal of the results of experiment 13 with the type 301 showing a smaller D-value in the present experiment. Although the difference between D-values was much smaller in the present experiment, the analysis of variance (Table 2.8) shows that the differences were significant at the .05 level.

The spores in experiment 13 were later found to be unreliable and were abandoned for experiment use; the large effect of surface finish in that experiment was probably due to the physico-chemical characteristics of the spores. The sensitivity of spores to humidity conditions seems to be increased by long storage and by poor spore preparation conditions. As mentioned above, a visual comparison of the results of experiments DD8320 and DD9066 suggests that, especially during the short period when the spores are in the treatment environment, newer and more resistant spores are less likely to show differences in death parameters, due to differences in the treatment system. Further investigation of the effects

of surface finish on various types of spores may be desirable.

Table 2.8 - Analysis of Variance for Experiment DD9155. Comparison of the Effects of Rolled Finish on Type 301 with Brushed Finish on Type 302 Stainless Steel Conditioned at <2% RH at 23°C and Heated at .34% RH at 125°C

Source	SS	df	MS	F
Mainplot				
Between 6" x 8" Sheets	19.5467	15	1.3031	
Days	.0248	1	.0248	.10 (not sig.)*
Surface Finish	1.6050	1	1.6050	6.43 (P<.05)
Treatment Time	15.3837	3	5.1279	20.55 (P<.001)
S x T	.7865	3	.2622	1.05 (not sig.)*
Mainplot Residual	1.7467	7	.2495	
Subplot				
Within 6" x 8" Sheets	4.7085	80	.0589	
Positions	3.1653	5	.6331	35.11 (P<.001)
Position x Process	.8222	35	.0235	1.30 (not sig.)*
Subplot Residual	.7211	40	.0180	
Total	24.2552	95	.2553	

* P>.1

Experiment DD9161

The experiment compared the thermal destruction of spores in the usual mated surface package, with the destruction in a package in which a longitudinal .010 inch separator was placed between the plates. The shape of the separator used is shown in Figure 4.2 of Progress Report #1. The spores were conditioned at <2% RH at 23°C and were treated at .34% RH at 125°C. The experiment was replicated on two different days. The results are shown in Table 2.9.

Table 2.9 - D_{125}-values, Y-intercepts (y_o) and Their .95 Confidence Intervals and Intercept Ratios for Experiment DD9161. Comparison of the Effects of Packages With and Without a .010 inch Separator Between the Mated Surfaces

Pooled Data(2 replicates)

		Position 1	Position 2
Without Separator	D	36.4 (24.1,73.5)	43.3 (28.2,93.0)
	y_o	1.61E5 (2.31E4,1.13E6)	1.77E5 (3.15E5,9.96E5)
	IR	.99	1.00
With .010 inch Separator	D	40.0 (36.0,45.0)	48.6 (38.5,65.7)
	y_o	1.01E5 (6.87E4,1.49E5)	1.08E5 (5.08E4,2.29E5)
	IR	.95	.96

N_o = 1.76E5 (1.28E5,2.42E5)

The analysis of variance (Table 2.10) shows that the presence or absence of a separator has no significant effect on spore destruction.

Table 2.10 - Analysis of Variance for Experiment DD9161. Comparison of the Effects of Packages With and Without .010 Inch Separator Between the Mated Surfaces. Conditioned at <2% RH at 23°C and Heated at .34% RH at 125°C

Source	SS	df	MS	F
Mainplot				
Between 6" x 8" Sheets	39.4088	11	3.5826	
Days	.0975	1	.0975	.22 (not sig.)*
Separator Thickness	.1944	1	.1944	.43 (not sig.)*
Treatment Time	36.8231	2	18.4115	41.16 (P<.001)
S x T	.0575	2	.0288	.06 (not sig.)*
Mainplot Residual	2.2363	5	.4473	
Subplot				
Within 6" x 8" Sheets	2.1648	60	.0361	
Positions	.8632	5	.1726	7.51 (P<.001)
Position x Process	.6117	25	.0245	1.06 (not sig.)*
Subplot Residual	.6899	30	.0230	
Total	41.5736	71		

* P>.1

Experiment DD9169

The experiment was carried out using spores suspended in water and having no history of alcohol storage. It had been suspected that water-stored spores behaved differently from spores with a history of alcohol storage and it was hoped that the results of this survey experiment might provide additional information with which to interpret the results of previous experiments. Three types of treatment packages were used: the ordinary package prepared with type 301 (rolled surface) stainless steel, the package with .010 inch separators as used in experiment DD9161 and the ordinary package prepared with type 302 (brushed surface) stainless steel. The test samples were conditioned at 40% RH and treated at .34% RH at 125°C. The results are shown in Table 2.11. Only four samples per plate were recovered and the experiment was carried out on only one day since only relative values were desired and the experiment was performed as a survey.

Table 2.11 - D_{125}-values, Y-intercepts (y_o) and Their .95 Confidence Intervals and Intercept Ratios for Experiment DD9169. Effects of Three Types of Mated Surfaces on Spores (AABF) Conditioned at 40% RH at 23°C and Heated at .34% RH at 125°C

		Position 1	Position 5
301 Surface Without Separator (standard package)	D y_o IR	29.8 (8.7,∞) 2.43E5 (1.75E2,3.38E8) .98	36.5 (4.5,∞) 2.95 (9.35E-3,9.32E12) 1.00
301 Surface .010-inch Separator	D y_o IR	25.3 (3.0,∞) 2.26E5 (1.28E-6,3.99E16) .98	30.3 (10.1,∞) 3.46E5 (9.65E2,1.24E8) 1.01
302 Surface Without Separator	D y_o IR	42.5 (22.4,414.0) 1.96E5 (3.00E4,1.28E6) .97	62.4 (14.8,∞) 2.65E5 (2.72E3,2.55E7) .99

N_o = 2.94E5 (2.46E5,3.51E5)

The presence or absence of a separator seems to have no effect. The type 302 surface seems to produce an increase in D-value which might be found to be significant if replicate experiments were carried out. The results are similar to the results of experiments DD9155 and DD9161 which

measured the behavior of spores with a history of alcohol storage.

Experiment DD9176

The spores in this experiment were conditioned at <2% RH at 23°C and treated at .46% RH at 115°C. The results are shown in Table 2.12.

Table 2.12 - D_{115}-values, Y-intercepts (y_o) and Their .95 Confidence Intervals and Intercept Ratios for Experiment DD9176. Conditioned at <2% RH at 23°C and Heated at .46% RH at 115°C

	Position 1	Position 5
D	211.8 (205.7,218.3)	234.6 (63.2,∞)
y_o	1.05E5 (9.75E4,1.14E5)	1.73E5 (2.66E2,1.12E8)
IR	.95	.99

N_o = 1.97E5 (1.62E5,2.39E5)

The results represent only one mated surface package per time-treatment combination. If the average D-value for the six recovered positions on the plate (292 minutes) is compared with the weighted average of the portions of experiments DD9155,DD9161 and DD9066 which were similarly treated, a Z-value of 11°C is obtained. This value is smaller than most of the dry heat Z-values reported in the literature; it is important that we determine whether this is an artifact or is characteristic of the mated surface system. The D-value confidence interval for the pooled data of experiment DD9176 is 218 minutes to 440 minutes. Using these extremes gives a range for the Z-value of 9-13 minutes which is roughly analogous to a .95 confidence interval. It should be noted that the treatment humidity for experiment DD9176 is slightly higher than that of the experiments treated at 125°C due to limited control on the treatment environment used.

Further study at several humidity conditions and treatment temperatures using the same treatment humidity at each temperature will be needed.

Experiment DD9273

The experiment evaluated the effect of three conditioning humidities. Samples were conditioned at <2%, 36% and 90% relative humidities at 23°C and were treated at .34% RH at 125°C. The experiment was replicated on two different days. The data are summarized in Table 2.13.

Table 2.13 - D_{125}-values, Y-intercepts(y_o) and Their .95 Confidence Intervals and Intercept Ratios for Experiment DD9273. Comparison of the Effects of Three Conditioning Humidities at One Treatment Humidity (.34% RH at 125°C)

Pooled Data (2 replicates)

Conditioning RH at 23°C		Position 1	Position 5
low <2%	D y_o IR	20.8 (12.7,57.2) 3.44E4 (9.33E2,1.27E6) .88	33.1 (20.7,84.0) 4.92E4 (5.71E3,4.25E5) .91
medium 36%	D y_o IR	38.1 (27.5,61.8) 4.32E4 (1.32E4,1.42E5) .90	38.9 (30.0,55.6) 1.26E5 (5.09E4,3.12E5) .99
high 90%	D y_o IR	68.4 (54.7,91.3) 2.22E5 (1.44E5,3.43E5) 1.04	145.6 (53.0,) 1.26E5 (3.06E4,5.20E5) .99

N_o = 1.41E5 (6.64,3.00E5)

As has been shown in previous mated surface experiments, conditioning humidity has a large effect on D-value. This is confirmed in the analysis of variance in Table 2.14. The results show again that the y-intercept of the survivor curve as well as its slope is affected by the conditioning humidity.

Table 2.14 - Analysis of Variance for Experiment DD9273. Comparison of the Effects of Three Conditioning Humidities (<2%, 36% and 90% RH at 23°C) at One Treatment Humidity (.34% RH at 125°C)

Source	SS	df	MS	F
<u>Mainplot</u>				
Between 6" x 8" Sheets	95.4911	17	5.6171	
Days	.5797	1	.5797	2.37 (not sig.)*
Humidity	56.3923	2	28.1962	115.51 (P<.001)
Treatment Time	30.9255	2	15.4628	63.35 (P<.001)
H x T	5.6412	4	1.4103	5.78 (P<.005)
Mainplot Residual	1.9524	8	.2441	
<u>Subplot</u>				
Within 6" x 8" Sheets	18.2419	90	.2027	
Positions	9.5284	5	1.9057	28.07 (P≤.001)
Humidity x Position	2.5324	10	.2532	3.73 (P<.005)
Time x Position	1.5505	10	.1551	2.28 (P<.05)
H x T x P	1.5745	20	.0787	1.16 (not sig.)*
Subplot Residual	3.0561	45		
Total	113.7331	107	.0679	

* P>.1

The extreme conditioning humidities of this experiment and the conditioning humidities of experiment DD9066 are the same so the results may be compared if the confounding factor of day-to-day variation is kept in mind. The data are compared in Table 2.15.

Table 2.15 - D-values, Y-intercepts(y_o) and Intercept Ratios for Comparable Conditions from Experiments DD9066 and DD9273 (treated at .34% RH at 125°C)

		Position 1		Position 5	
		DD9066	DD9273	DD9066	DD9273
Conditioned at <2% RH at 23°C	D	22.8 (16.2,38.7)	20.8 (12.7,57.2)	28.5 (18.0,69.3)	33.1 (20.7,84.0)
	y_o	1.28E4 (1.09E3,1.52E5)	3.44E4 (9.33E2,1.27E6)	1.33E4 (7.91E2,2.25E5)	4.92E4 (5.71E3,4.25E5)
	IR	.77	.88	.77	.91
Conditioned at 90% RH at 23°C	D	45.4 (37.4,57.9)	68.4 (54.7,91.3)	60.6 (44.5,94.9)	145.6 (53.0,∞)
	y_o	2.81E5 (1.47E5,5.37E5)	2.22E5 (1.44E5,3.43E5)	2.49E5 (1.10E5,5.64E5)	1.26E5 (3.06E4,5.20E5)
	IR	1.02	1.04	1.01	.99

The D-values for the low humidity tests were of the same magnitude in both experiments and in general the D-value at position 1 did not differ greatly from the D-value at position 5 although in both cases the D-values at position 5 were larger. When the spores were conditioned at 90% RH at 23°C, considerably higher D-values were obtained; the high humidity D-values in experiment DD9273 were from 50% to more than 150% larger than the D-values of experiment DD9066. In both cases the D-values at position 5 were also 50-150% greater than the D-values at position 1. The significance of these results is not understood at this time.

Experiment DD9277-open system

The experiment was carried out to provide data on the dry heat destruction of spores in an open system at an intermediate conditioning and treatment humidity. Spores were conditioned at 36% RH at 23°C and were treated at .44% RH at 125°C. Two sets of five samples were prepared, treated, and processed for each time-treatment combination. The experiment was carried out on only one day. The treatment system is described in Progress Report #2. The results are shown in Table 2.16.

Table 2.16 - D_{125}-values, Y-intercepts(y_o) and Their .95 Confidence Intervals and Intercept Ratios for Experiment DD9277. Conditioned at 36% RH at 23°C and Heated at .44% RH at 125°C in an Open System

Replicate 1	D y_o IR	16.2 (13.2,21.0) 1.56E4 (3.49E3,6.96E4) .81
Replicate 2	D y_o IR	18.8 (13.8,29.4) 9.26E3 (1.22E3,7.03E4) .77
Pooled	D y_o IRF	17.4 (14.5,21.6) 1.20E4 (3.64E3,3.97E4) .78

N_o = 1.47E5 (1.26E5,1.71E5)

Experiment DD9304-open system

The experiment is similar to the open-system experiment reported in Progress Report #2. Samples were conditioned at less than 2% RH or at 89% RH at 23°C and were treated at .34% or 1.1% RH at 125°C. The conditions are comparable to those of experiment DD9066 for a mated system. The experiment was completely replicated on two different days. In each experiment, three 1" x 2" strips, each carrying one spore deposit were assigned to each time-treatment combination. This gives a total of six replicate samples for each time-treatment combination.

In the earlier experiment there was an unavoidable delay between removal of the strips from the conditioning environment and their insertion into the treatment environment. In the present experiment, the time was

reduced from four minutes to 20 seconds. The temperature of each strip was monitored throughout treatment and the final counts were corrected to a treatment temperature of 125°C using a Z-value of 21°C. The results are shown numerically in Table 2.17 and graphically in Figure 2.3.

Table 2.17 - D_{125}-values, Y-intercepts(y_o) and Their .95 Confidence Intervals and Intercept Ratios for Experiment DD9304. Comparison of the Effects of Two Conditioning Humidities and Two Treatment Humidities in an Open System

Conditioning RH at 23°C	Treatment RH at 125°C		Day 1(3 strips)	Day 2(3 strips)	Pooled(6 strips)
low 2%	low .34%	D	13.7 (11.1,18.1)	19.3 (16.0,24.3)	16.1 (13.1,20.1)
		y_o	6.13E4 (9.79E3,3.85E5)	7.85E4 (2.56E4,2.41E5)	6.94E4) (1.62E4,2.97E5)
		IR	.89	.91	.90
low 2%	high 1.1%	D	17.5 (14.2,22.6)	23.9 (21.9,26.4)	20.6 (18.5,23.1)
		y_o	4.83E5 (1.23E5,1.90E6)	3.06E5 (2.03E5,4.60E5)	3.78E5 (2.13E5,6.69E5)
		IR	1.06	1.02	1.04
high 89%	low .34%	D	15.8 (13.2,19.8)	22.0 (19.0,26.2)	18.4 (14.7,24.5)
		y_o	1.22E5 (3.20E4,4.63E5)	3.29E5 (1.53,7.07E5)	2.00E5 (4.79E4,8.36E5)
		IR	.95	1.03	.99
high 89%	high 1.1%	D	32.5 (24.2,49.8)	44.5 (34.7,62.1)	37.6 (29.5,51.9)
		y_o	1.62E5 (5.28E4,5.00E5)	2.16E5 (1.10E5,4.22E5)	1.87E5 (8.65E4,4.05E5)
		IR	.97	.99	.98

N_o = 2.34E5 (1.74E5,3.15E5)

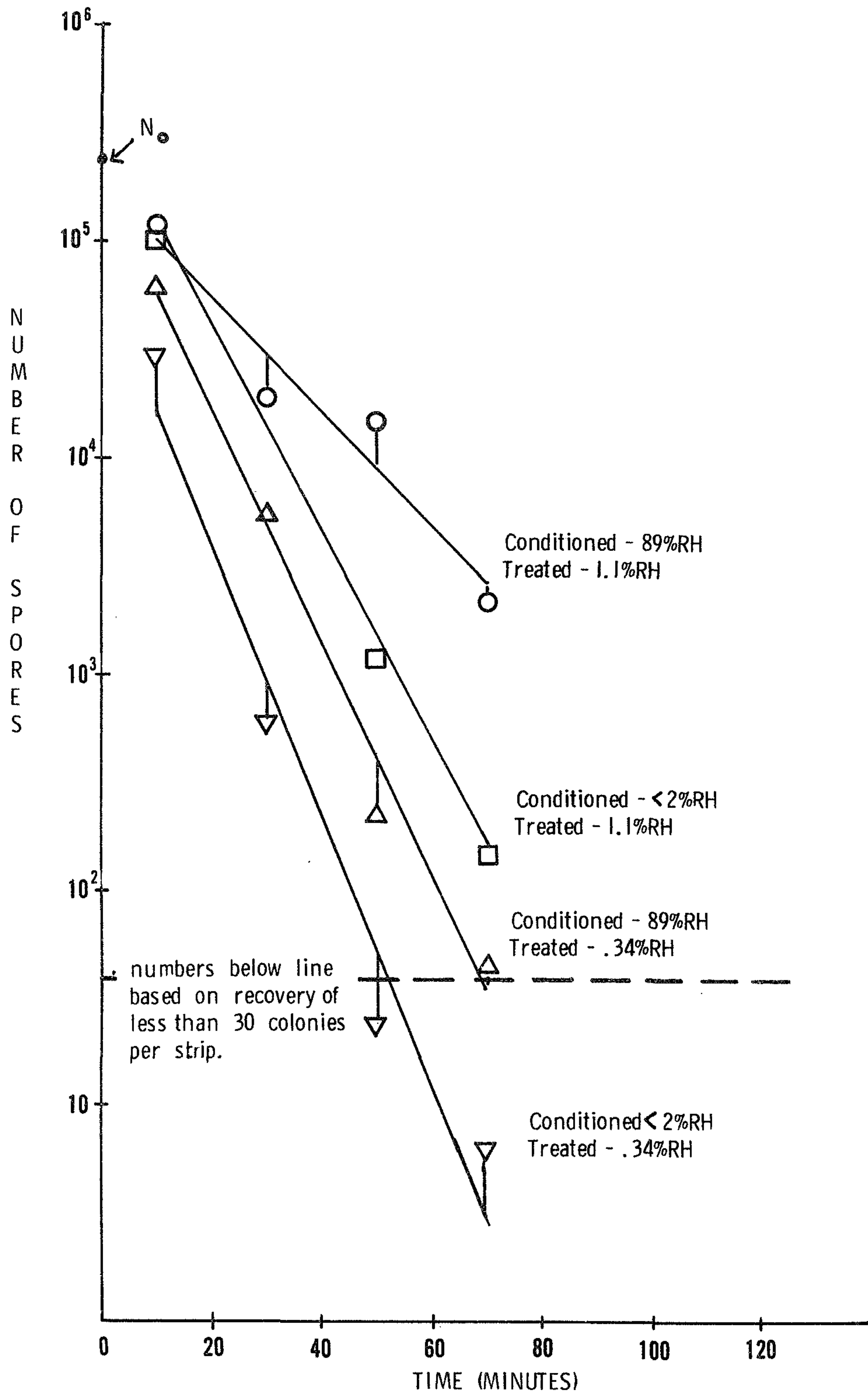

Figure 2.3 - Data points and least squares regression lines for Experiment DD9304: Comparison of the effects of two conditioning humidities (<2% and 90% RH at 23°C) and two treatment humidities (.34% and 1.1% RH at 125°C) in an open system. Each datum point is the geometric mean recovery from six strips.

The analysis of variance (Table 2.18) shows that in the open system, both conditioning and treatment humidity have an effect on the thermal destruction of spores. In the mated system, it will be remembered, conditioning humidity is clearly the most important factor. The analysis of variance also shows that position did not have a significant effect.

A comparison of Figure 2.2 (mated system, page 20) and Figure 2.3 (open system) shows the relatively larger effect of treatment RH in the open system.

Table 2.18 - Analysis of Variance for Experiment DD9304. Comparison of the Effects of Two Conditioning Humidities(<2% and 90% RH at 23°C) and Two Treatment Humidities(.34% and 1.1% RH at 125°C) in an Open System

Source	SS	df	MS	F
Mainplot				
Between Racks of 3 Strips	185.9220	31	5.9975	
Days	12.6575	1	12.6575	30.48 (P<.001)
Conditioning RH	11.0480	1	11.0480	26.60 (P<.001)
Treatment RH	33.4900	1	33.4900	80.64 (P<.001)
C x T	.2465	1	.2465	.59 (not sig.)*
Treatment Time	111.1031	3	37.0344	89.18 (P<.001)
Other Interactions	11.1478	9	1.2386	2.98 (P<.05)
Mainplot Residual	6.2291	15	.4153	
Subplot				
Within Racks of 3 Strips	5.8389	64	.0898	
Position	.1632	2	.0816	.82 (not sig.)*
Position x Process	2.4000	30	.0800	.81 (not sig.)*
Subplot Residual	3.2757	32	.0993	
Total	191.7607	95	2.0185	2.0185

* P>.1

Experiment DD9329

The experiment was a comparison between the AAAC spores which have been used in nearly all the mated surface experiments, the same spores diluted tenfold, and VDLA spores (Mariner Isolate #4-47A grown on synthetic sporulation medium) supplied by Jet Propulsion Laboratory. The two different dilutions of the AAAC spores were used to see whether deposition from a more dilute suspension, which theoretically would decrease clumping, would have an effect on thermal destruction. The spores were conditioned at 36% RH at 23°C and were treated at .34% RH at 125°C. The results are shown in Table 2.19.

Table 2.19 - D_{125}-values, Y-intercepts(y_o) and Their .95 Confidence Intervals and Intercept Ratios for Experiment DD9329. Comparison of AAAC Spores with VDLA Spores Conditionedaat 36% RH at 23°C and Heated at .34% RH at 125°C

		Position 1	Position 5
AAAC N_o=1.92E5	D y_o IR	29.3 (23.2,39.5) 2.15E5 (5.30E4,8.76E5) 1.01	42.9 (36.5,51.9) 1.81E5 (9.55E4,3.45E5) .99
AAAC N_o=1.92E4	D y_o IR	31.7 (10.0,∞) 1.59E4 (5.34E0,4.76E7) .98	72.3 (66.9,78.7) 9.82E3 (8.60E3,1.12E4) .93
VDLA N_o=1.92E5	D y_o IR	159.8 (48.5,∞) 1.45E5 (1.49E4,1.41E6) .98	131.4 (70.5,966.0) 2.70E5 (9.53E4,7.63E5) 1.03

Up to the 105-minute maximum treatment time, the VDLA spores showed much greater heat resistance than did the AAAC spores. A final judgment on the resistance of the VDLA isolate will require repeated experiments using longer treatment times. The treatment time should be long enough for the survivor curve to traverse at least three log cycles. This is especially true in this case since some workers have reported a decreasing D-value with increasing treatment times for this and related spores. The results of the comparison between the usual concentration of AAAC in these tests (2×10^5 per .02 ml) and the

diluted concentration (2×10^4 per .02 ml) of the AAAC spores is also inconclusive at this time.

CONCLUSIONS

1. Treatment humidity has a much larger effect on D-values in an open system than it does in a mated-surface system. The treatment humidity has an effect in mated systems, however, it may be neglible as far as the design of spacecraft sterilization cycles are concerned.
2. In the open system used here, both conditioning and treatment humidity affect D-value, y-intercept and IR.
3. Both the D-value and y-intercept must be considered in a discussion of microbial survival. The intercept ratio (IR) appears to be useful for discussion.
4. The mated-surface system shows a greater variability than does the open system. This is not unexpected and is probably due to variabilities in the mated-surface package and in the off-the-shelf stainless steel used for the mated surfaces. It emphasizes, however, the need for replicating data where high precision is desired.
5. The location of spores in a mated surface has an important effect on their thermal destruction rate. The spores near the edge tend to have smaller D-values than spores at the center of the 6" x 8" plate.

FUTURE WORK

The measurement of Z-values and of D, y_o, and IR in dry nitrogen will complete phase I of the mated-surface study. These are the last experiments contemplated for the present heat block.

For phase 2 of the study, a new disc-shaped treatment apparatus will be constructed. A disc-shaped mated surface without packaging material would lend itself to engineering analysis more readily than would the rectangular mated surfaces used in the phase I study. The apparatus will be constructed in several sizes to simulate different

sizes of mated surfaces. In addition, a number of units of each size will be constructed so that multiple samples can be treated simultaneously; this will be particularly important as we attempt to measure the survival of very small numbers of organisms.

Several surface materials, both metallic and plastic, will be used to simulate materials present on the spacecraft. The effect of storage time in dry nitrogen after assembly and conditioning under clean room conditions and before treatment in dry nitrogen should be explored, since mated surfaces re-equilibrate to new humidity conditions more slowly than do open surfaces.

DETECTION OF LOW LEVELS OF MICROBIAL CONTAMINATION ON SURFACES BY CHEMICAL APPROACHES

Project Personnel: Velta Goppers
Division of Environmental Health

INTRODUCTION

Studies are in progress to develop chemical methods which will quickly enumerate surface microbial contamination. At the present stage of development a microscope photometer is being used in order to measure and evaluate the quantities of the separated nucleotides from the microbial cells found on thin-layer plates.

OBJECTIVE

The objective of these studies is to make it possible to use the developed chemical method for the determination of the number of microbial cells on surfaces.

EXPERIMENTAL PROCEDURE

As was previously reported, nucleotides were extracted from microbial cells and separated by thin-layer chromatography. The developed chromatoplates were examined visually under ultraviolet light at 3600 and 2500 angstroms. In order to increase the sensitivity of the chemical methods, that is,to detect a smaller number of microbial cells on surfaces, a microscope photometer is being used. This unit measures the intensity of the fluorescent light which is reflected from the separated nucleotides--adenosine triphosphate.

At the present time, the chemical procedure and the microscope photometer are under a strict standardization process. This process includes:

1. Analyzing the prepared and chromatographically purified blank chromatoplates for interfering substances and the homogeneity of the layer.

2. Conducting studies which check the background chemical contamination from the surrounding environment.
3. Taking readings from the separated fluorescent nucleotides on thin-layer plates. These plates are prepared from bacteria E. coli in growth phase, Bacillus subtilis var. niger and standard compounds.

Some changes have been introduced in order to transfer the E. coli cells onto the chromatographic plate:

1. The bacteria E. coli cells were removed from the surface of the agar plate with a tarred glass capillary loop and were then weighed on an analytical balance. The weighed material was immediately transferred to distilled water, mixed thoroughly and spotted on thin-layer plates for chemical separation.
2. The cell-cleaning procedure (washing with distilled water) was omitted for the time being until certain questions can be clarified.

Besides using E. coli cells which were cultivated on the three different kinds of media mentioned above, limited work was also done with Bacillus subtilis var. niger.

The quantity of E. coli used in this investigative phase was large enough to make it possible to visually locate the significant substances on thin-layer plates in ultraviolet light. In this case the concentration was: one lambda equal to 1×10^{-6} grams. The concentration of the next group of dilutions prepared was: one lambda equal to 1×10^{-9} grams. These dilutions were applied to chromatographic plates in quantities of three, two and one lambda.

RESULTS AND DISCUSSION

The results which were obtained by using the microscope photometer are very encouraging. At present, we are not supplying groups of numbers and charts because the purpose of this phase was to standardize the method and the unit itself. Results were observed only on a readout unit. In the near future they will be recorded with a connected recorder.

The baseline for the blank, prepared chromatoplate, according to the microscope photometer readout unit, is within ±2%. This deviation from a straight line is caused by the reflection of the particles on the thin-layer and will be lowered by adding some additional material in order to

make the surface more even. This baseline is quite good and can be tolerated if it is not contaminated by the environment.

The chemical background contamination was definitely visible after the above scanned plates were exposed to a natural laboratory environment for the length of time which is required in regular procedure. On the readout unit the peaks produced from the contaminated particles were as high as 20% and covered longer or shorter distances according to the size of the particles which were deposited from the air. This proves that after the plates are chromatographically cleaned they must be protected from chemical contamination. For the same reason certain phases of the separation procedure must be conducted in a controlled environment.

The readout unit registered definitely every area where the nucleotides were located and the intensity of the peaks reached as much as 40%. The results were not linear because of the large quantities used. This is an indication that the unit will be able to detect small quantities of the extracted and separated nucleotides from a small number of microbial cells.

It is apparent that the E. coli cells were not washed with distilled water due to the obvious changes in chemical pattern which varied with the length of time the material was handled. The negative effect of distilled water on microbial cells is also reported in the literature.

CONCLUSION

The results indicate that the chemical method using thin-layer chromatography in connection with the microscope photometer produces very promising results in detecting low numbers of microbial cells on surfaces.

FUTURE WORK

Future work will involve completion of the research phase to determine how many bacteria there are on the surface. We will continue to standardize the microscope photometer and adjust the chemical procedure according to our needs. The quantity range of the unknowns for the given condition will be established. More work will be done with standard compounds, E. coli, Bacillus subtilis and other species.

The last phase of research will be devoted to analyzing different surfaces for the number of microbial cells present.

DRY HEAT DESTRUCTION RATES OF *BACILLUS SUBTILIS* VAR. *NIGER* IN A CLOSED SYSTEM

Project Personnel: B. Moore, I. J. Pflug and J. Haugen
Division of Environmental Health

INTRODUCTION

During the past six months we have begun a study of dry heat destruction rates of spores in a closed system. To date we have developed one type of apparatus for use in this project and have obtained some preliminary data.

OBJECTIVE

The objective of this project is to determine dry heat D-values of microorganisms in closed systems: specifically it is to determine 1) the relationship of spore D-value to spore water content, 2) the effect of the atmospheric volume per spore on the D-value of spores with different initial water contents 3) the effect of pressure on D-value, 4) the effect of water absorption and water vapor transfer characteristics of plastic materials on the D-values of microorganisms adjacent to or imbedded in these materials, and 5) if there are differences between the D-values of spores encapsulated in metals vs. spores encapsulated in plastics.

LITERATURE REVIEW AND BACKGROUND

The most fundamental need of both NASA and the scientific community is to know the precise relationship of water and spore D-value. The problem is severely complicated by two factors: 1) it is nearly impossible to measure the quantity of water in the spore and 2) it is extremely difficult to keep the water in the spores constant.

It is apparent at this time that the spore D-value is very sensitive to minute changes in water content. Starting with spores at ambient conditions of 35% RH at 22°C and heating to 100 or 125°C in this atmosphere (35% RH at 22°C) will result in a loss of water from

spores on surfaces and a decrease in D-value. Encapsulated microorganisms may also lose water; however, their water loss will depend on the surrounding physical system. If a device or system is assembled under normal ambient water vapor conditions (35% RH at 22°C) the encapsulated microorganisms will have a larger D-value than they would have under any lower water condition. Therefore any drying of the system will result in a smaller D-value.

One of the critical locations of encapsulated microorganisms is in and under potting compounds or conformal coatings. At the present time the $D_{125°C}$ for encapsulated microbial spores is five hours. This value was obtained from laboratory experiments where Bacillus subtilis spores were encapsulated in Lucite and epoxy rods and the rods were placed in sealed thermal-death-time tubes to be heated in an oil bath.[1] The conditions to which those spores were subjected will certainly be similar to the conditions to which spores encapsulated in large pieces of Lucite or epoxy will be subject to on the spacecraft. However, were the conditions used by Angelotti the same as the conditions of spores located under thin layers (less than 0.5 inches) of plastic? Since the sealed tube retained the water vapor from the plastic rod it is possible that the D-value of the spores in the rod under these conditions was higher than if the rod were heated outside the glass tube. We believe that the potential gain from a reduction in D-value is sufficient to warrant experiments in this area.

In the sterilization cycles for spacecraft hardware the come-up time for the hardware before there is a significant lethal effect may be five to ten hours. During this time water may be lost from both the plastic rod and the encapsulated spores; this will result in a reduced spore D-value.

Encapsulated spores were studied extensively in the early part of the NASA Planetary Quarantine Program. Most of the studies of encapsulated spores were carried out when there was little understanding of the critical role played by water in the dry heat system. The present research has been designed to start where earlier studies ended, using the new

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[1] Angelotti, et. al. "Influence of Spore Moisture Content on the Dry-Heat Resistance of Bacillus subtilis var. niger," 16 Appl. Microbiol. 735-745 (1968)

knowledge of dry heat spore destruction to design experiments that will hopefully give a more complete picture of the dry heat destruction characteristics of encapsulated microorganisms.

Development and Use of Cylindrical Heat Block Apparatus

The cylindrical heat block apparatus has been developed to make it possible to study dry heat destruction under a wide variety of closed system conditions. The heat block apparatus consists of a top and bottom unit held together with Allen cap ccrews. The units are made out of type 303 stainless steel. The bottom unit (Figure 4.1) contains the DASH No. 222 O-ring groove and a 1.275" diameter by 0.020" recess to hold the cylindrical sample disc. To date three types of top units (Figure 4.2) have been constructed: a flat unit, a unit with a 1.185" x 0.25" recess and a unit with a 1.185" x 0.50" recess.

Auxiliary devices required when using the heat-block apparatus are: a torque wrench (Craftsman No. 12-9G44441) for uniformly tightening the cap screw (Figure 4.3), a magnetic wand for removing the sample carrying discs from the recess in the bottom unit (Figure 4.4) and a rack with a handle for holding the block when it is placed in the oil bath (Figure 4.5).

Heating has been carried out using a model MW-1155C-1 "Magni-Whirl" (Blue M Electric Co.) oil bath filled with "U con" HTF-100 heat transfer fluid. A large stainless steel container filled with ice cubes and tap water is used to cool the heat block after heating.

Temperatures in the oil bath are monitored during testing using a model 15305836 Honeywell temperature-recording potentiometer. The temperature is sensed by bare 24-gauge copper constantan thermocouples.

Evaluation of Heating and Cooling Time Lags

The considerable mass of the heat blocks plus the fact that they are made out of stainless steel means that they will not instantly heat and cool. The heating and cooling characteristics of a heat block with a flat top have been evaluated. Heating studies were made both with a thermocouple soldered to the spore-sample disc and with the thermocouple in the air in recess. A lag correction factor of 12.5 minutes was

calculated. Details of the heating and cooling time lag study will be included in the next progress report.

Test Procedure

The Bacillus subtilis var. niger spore crop No. AAAB (see spore code details, Appendix B) was used in all experiments. The stock spore suspension was resuspended in distilled water so that an 0.02 ml droplet would contain 5×10^5 spores. The spores were deposited on the discs using an 0.02 ml push-button (Eppendorf) pipette.

Stainless steel discs were punched out of type 302 full-hard stainless steel shim stock. The discs were thoroughly cleaned. As many as ten discs were placed on a sterile stainless steel sheet in a clean room for inoculation. Four 0.02 ml spore suspension deposits were placed on each disc. Before starting the pipetting operation the spore suspension was mixed for a few seconds using a vibrating automatic mixer (Vortex) and was remixed after each ten pipettings. The inoculating scheme order is shown in Figure 4.6. The discs were randomly assigned to heating times. Both the strips and the blocks with O-rings were allowed to equilibrate in the clean room at 35% RH at 22°C for 18 to 24 hours before assembly.

The blocks were assembled by placing an inoculated disc, with a sterile disc on top of it, into the recess of the bottom block. The screws were then inserted and tightened and the sealed block was immersed into the constant-temperature (125°C ±1°C) oil bath for the specified length of time. At the end of heating, the blocks were cooled for two minutes in the 0°C ice bath and were then wiped dry. The dry block was inserted into the block holder, the screws were removed and the top unit was aseptically removed. The magnetic wand was chemically sterilized using peracetic acid and was then used to remove the two discs. Using a sterile forceps to hold the inoculated disc, it was cut into four pieces (one spore inoculum per piece) using a metal-cutting bench shears (Whitney-Jensen No. 39) that was also sterilized in peracetic acid.

Each stainless steel piece was assayed individually according to NASA Standard Procedures for the Microbiological Examination of Space Hardware, October, 1968.

The data were analyzed to determine the D-value, y-intercept and the respective confidence intervals.

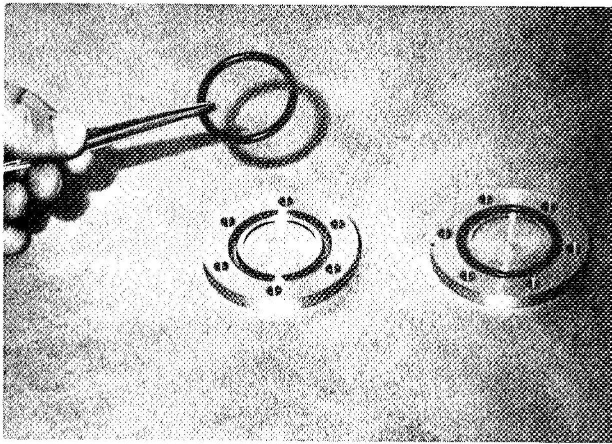

Figure 4.1 - Bottom unit of heat block system and O-ring

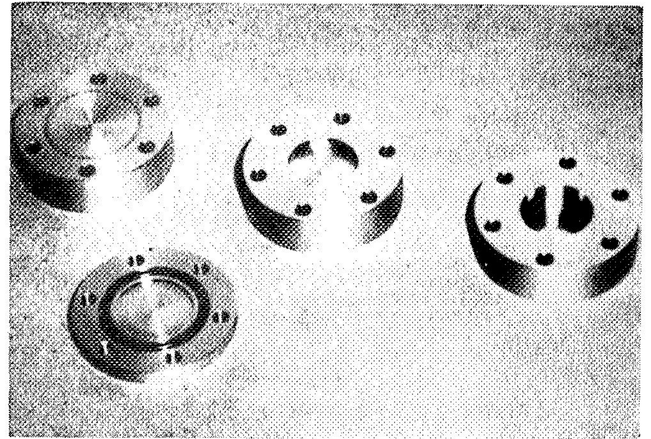

Figure 4.2 - Three types of top units with one bottom unit

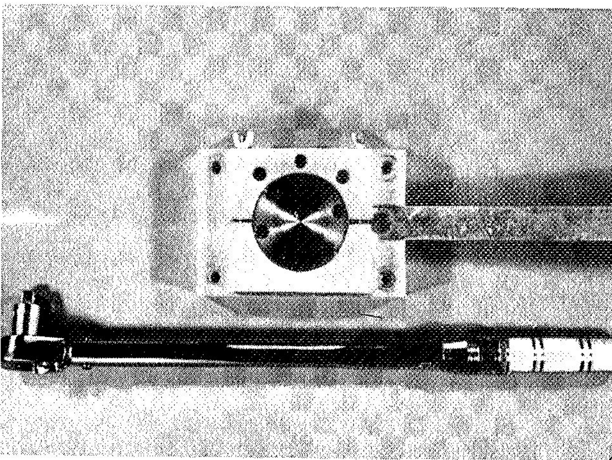

Figure 4.3 - Torque wrench and block-holding apparatus

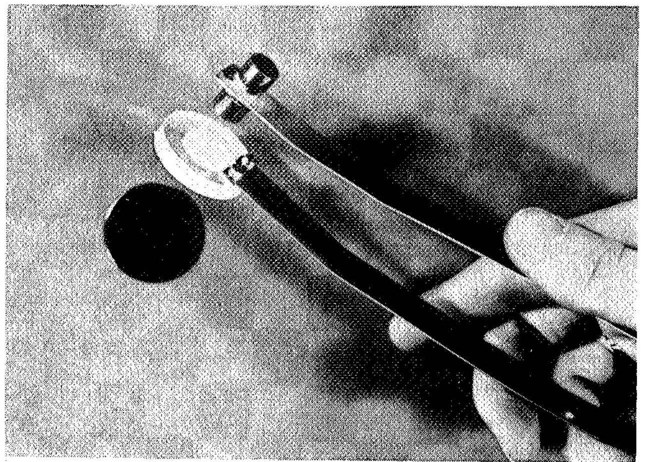

Figure 4.4 - Magnetic wand for removing the sample discs from the recess in the bottom block

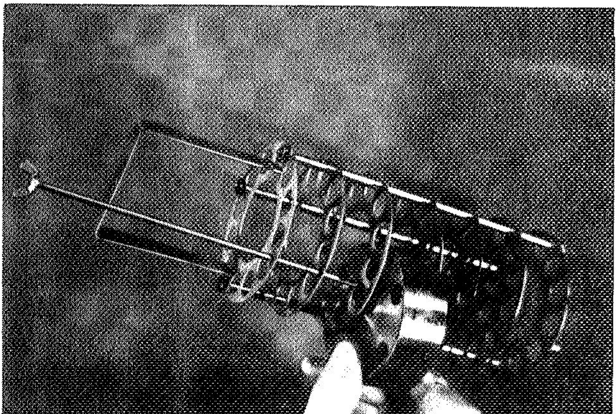

Figure 4.5 - Rack for supporting block during handling in and out of the oil bath

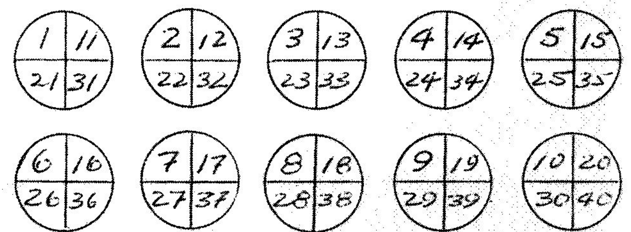

Figure 4.6 - The inoculating-scheme order for the discs

RESULTS AND DISCUSSION

The results of the two sets of replicated tests completed to date are summarized in Table 4.1. Five tests were carried out at 35% RH at 22°C using the Teflon O-rings and for three tests the Viton[2] O-rings were used.

Table 4.1 - Preliminary D-value Results Showing the Effect of Two Kinds of O-rings Using the Cylindrical Heat Block System with a Flat Top Unit with Two .010-inch-thick Discs in Bottom Unit Recess

O-ring Material	Equilibration Condition	D_{125} min.
Teflon	Clean Room 40% RH at 23°C	27-42
Viton	Clean Room 35% RH at 23°C	80-91

Teflon O-rings were initially chosen as the block seal because it was decided that the 0-water absorbing characteristics and heat resistance of Teflon were necessary requirements for this study at 125°C. Unfortunately, Teflon becomes amorphous at 121°C and loses its resiliency. This property of Teflon probably permitted periodic leakage. After deciding that it was possible that the Teflon allowed water vapor to escape, Viton O-rings were chosen because of their good sealing characteristics at high temperatures (up to 204°C) and their low water absorbing properties.

The increase in the D-value of the tests where Viton O-rings were used as compared to the tests using Teflon is difficult to explain on any other than a pressure and water vapor-loss basis.

The heat block system that has been developed appears to perform very well and will allow us to proceed to carry out experiments to answer the several questions regarding the dry heat destruction rates of microorganisms in closed systems.

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[2]Viton - a synthetic rubber able to withstand a temperature of 200°C in contact with most oils, chemicals, solvents, and fuels. Has good mechanical properties, also resistance to ozone, oxygen, and weathering. Commercial development: DuPont. The Merck Index. 7th edition. (Rathway, New Jersey: Merck and Co. Inc., 1960).

FUTURE WORK

In the immediate future, we will attempt to relate the quantity of water in and surrounding the spore to dry heat destruction characteristics. Initially we will use Bacillus subtilis var. niger spores. However, we will ultimately want these data for several species of microorganism.

In the first series of studies, those spores on stainless steel discs will be equilibrated at several relative humidities (≈0, 20, 35, 50, and 75%) and will be carried out at 125°C with subsequent tests at other temperatures so that Z-value data can be obtained. This series will be completed by evaluating at least one other species of microbial spore.

In the second series of tests we plan to relate variation in initial water to variation in the volume of atmosphere per spore. Initial studies will be carried out at 125°C using Bacillus subtilis var. niger and at least one other species will also be studied.

A series of experiments will be carried out to evaluate the effect of total gas pressure during heating on the D-value as a function of spore water-content, temperature, and microbial species.

The effect of plastic materials in contact with spores during heating will be studied in a series of experiments. The variables will be: type of plastic, initial water content of plastic, initial spore water-content, temperature, and species of microorganism.

DRY HEAT DESTRUCTION RATES OF MICROORGANISMS ON SURFACES AS A FUNCTION OF RELATIVE HUMIDITY

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Division of Biometry

INTRODUCTION

The plan to use a dry nitrogen environment for terminal sterilization in the Viking '75 mission has created an immediate need for information concerning surface spore-destruction rates under dry-nitrogen conditions. Considerable data are available concerning dry heat destruction rates of spores (particularly *Bacillus subtilis* var. *niger*) which have been equilibrated and heated in ambient clean-room environments. Since the Viking '75 lander will be sterilized in a dry-nitrogen atmosphere, it is imperative to obtain comparable data for that environment. In this regard, a test system has been advised and a series of experiments has been initiated to obtain D-values for spores in a dry gas (either air or nitrogen) as well as in clean room environments.

OBJECTIVE

The specific objective of this project was to determine dry heat destruction rates (D-values) for several species of microbial spores deposited on surfaces and subjected to temperatures in the range of 90°-125°C as a function of relative humidity and types of atmosphere in contact with the spores.

EXPERIMENTAL PROCEDURE

Microbial Spores

In the experiments carried out thus far, *Bacillus subtilis* var. *niger* spores obtained from suspensions with several different

histories have been utilized. These were as follows:

Suspension Spore Code AAAB: Spores obtained from the Phoenix NCDC Laboratory approximately one year earlier, regrown in our laboratory (growth procedure #1), stored in ethanol and then re-suspended in deionized distilled water.

Suspension Spore Code AANE: Spores obtained from the Phoenix NCDC Laboratory (procedure #2) which had been stored in buffered distilled water and were then re-suspended in deionized distilled water.(A new crop of this spore was received later in a frozen condition and was designated as AAOE.

Suspension Spore Code AADB: Spores recently harvested from the Phoenix Laboratory crop,(procedure #1) stored in ethanol, and then re-suspended in deionized distilled water.

(See Appendix B for details of spore preparation.)

Heating System

Spore deposits were made on 1/2" x 1/2" stainless steel planchets previously placed on rectangular copper boats allowing five strips/boat (See Appendices C and D). In all experiments carried out thus far, spores have been heated at 110°C on a specially designed hot plate. The temperature was determined using thermocouples attached to the copper boats; two boats with thermocouples were placed on each of the hot plates. The thermocouples were connected to a temperature recording potentiometer so that we had a record of the temperature of the hot plate during the experiment. For each condition tested, five boats were heated; one additional boat was always processed after equilibration (but before heating) for determination of N_o and a second additional boat was carried along as a spare. One randomly allocated boat for each condition was removed at each of five time-intervals for processing. In all instances, four planchets from each boat were processed according to the standard procedure. The fifth planchet (the middle one) was not processed unless one of the other four was inadvertently mishandled. This procedure was initiated to minimize the possibility of missing data. Additional control procedures related to plating media consistency are outlined in Appendix F.

Gas Control System

In all experiments, heating was carried out in the special dry gas environment and in a laminar crossflow clean room used as a control. The clean room ran consistently at 22-24°C and 34-44% RH during the experimental period. During heating the spore deposits were protected from the direct air stream by a perforated plastic box. (See Figure 5.1)

The dry gas environment was set up in a double glove-box arrangement (Figures 5.2 and 5.3). One box was used for equilibration and the other for heating; each had its own access antechamber. In experiments thus far we have utilized either predried dry air or dry nitrogen from compressed gas tanks to pressurize the two glove-box chambers independently. A bellows arrangement maintained a consistent positive pressure in the two chambers and their access chambers. A Bell and Howell (C.E.C.) moisture analyzer was connected to the apparatus so that a direct readout of moisture content could be obtained from either chamber. During experiments thus far, relative humidity has ranged from 0.1 to 2.0% at 25°C in the chamber air. Figure 5.4 outlines the basic glove-box set-up.

The initial experiments in this project were designed to determine the basic workability of the apparatus and to select suitable specific equilibration times, spore suspensions, and initial concentrations of spores. In addition they were used to finalize specific processing techniques and equipment operation procedures, to aid in the selection of suitable time intervals and plating dilutions and to perfect computer analysis programs. While much valuable information has resulted from the initial experiments, the specific procedures have necessarily been somewhat variable.

All experiments carried out thus far at 110°C can be summarized as follows:

1. Comparison of the dry heat destruction rates of the three spore suspensions (AAAB, AANE, AADB) as a function of relative humidity of the gas surrounding the spore deposit.
2. Comparison of the dry heat destruction rates as a function of equilibration time and relative humidity.

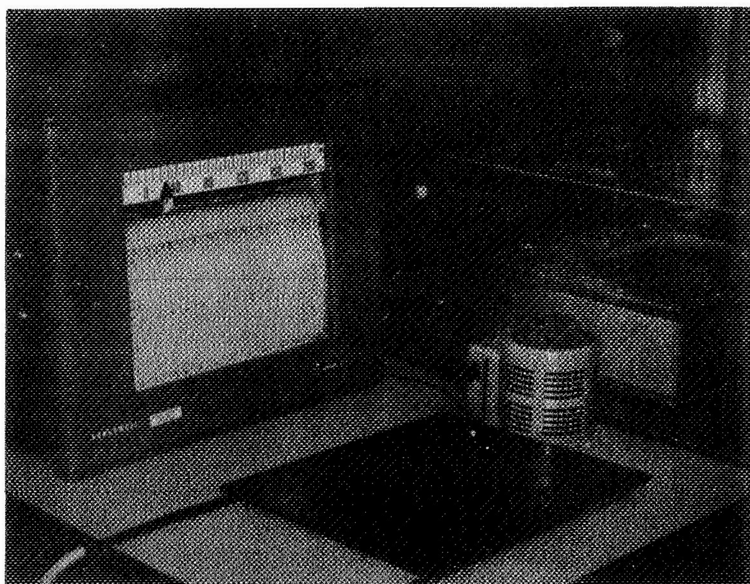

Figure 5.1 - Hot plate, cold plate and temperature recorder in the clean room

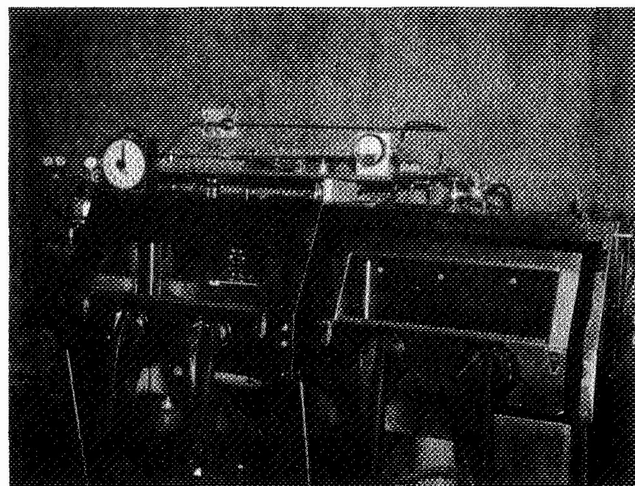

Figure 5.2 - Two-chamber glove box and auxiliary equipment

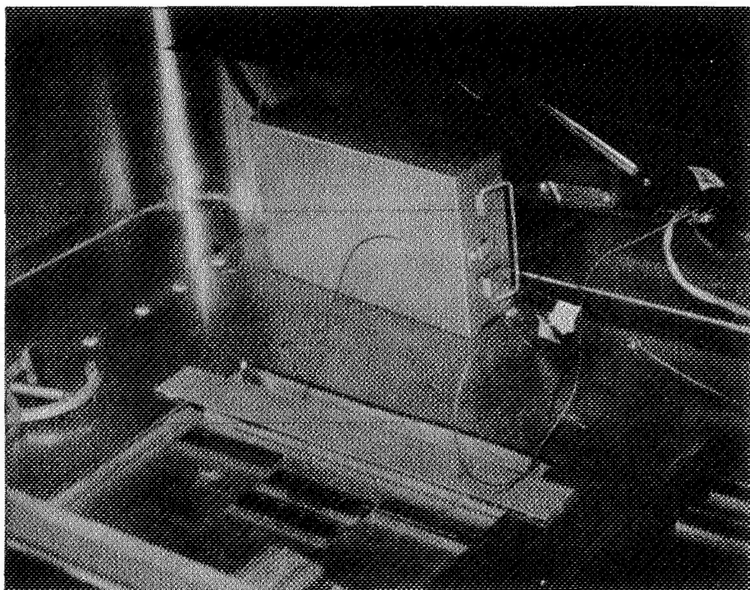

Figure 5.3 - Hot plate built into the bottom of the glove box with boats in place on the hot plate

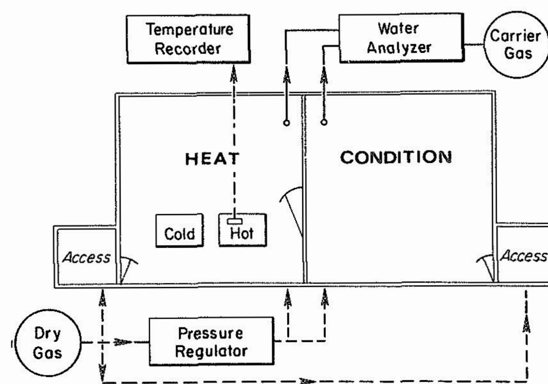

Figure 5.4 - Schematic diagram of the glove box system

3. Comparison of the dry heat destruction rates of a frozen and a non-frozen spore suspension.
4. Comparison of dry heat destruction rates as a function of initial spore concentration per volume of water per deposit.
5. On the basis of information from the above experiments a series of experiments at 110°C was initiated to complete the objectives of the project for that temperature and was designed as follows:*

   Experiment A: Using dry air for the <1% heating and equilibration

   a. Spores were equilibrated at <1% RH and heated at <1% RH
   b. Spores were equilibrated at ∿35% RH and heated at <1% RH
   c. Spores were equilibrated at <1% RH and heated at ∿35% RH
   d. Spores were equilibrated at ∿35% RH and heated at ∿35% RH

   Experiment B: Using dry nitrogen for the <1% heating and equilibration

   a. Spores were equilibrated at <1% RH and heated at <1% RH
   b. Spores were equilibrated at ∿35% RH and heated at <1% RH
   c. Spores were equilibrated at <1% RH and heated at ∿35% RH
   d. Spores were equilibrated at ∿35% RH and heated at ∿35% RH

   *In both experiments the RH was measured at 22-25°C.

Spores AAOE with an equilibration time of 24 hours, were used in all tests. Each experiment was performed at least two times. Details of the experimental procedure follow.

General

All operations (prior to incubation of the plates) except those performed in the dry gas chamber, were carried out in a clean room. The dry gas chamber was located immediately adjacent to the clean room. Aseptic technique was used at all times. Freshly laundered clean-room coats and hats were worn in the clean room when performing microbiological tasks or upon entering the clean room when experimental work was being carried out.

Twenty (5 ml) aliquots of the spore suspension coded AAOE (See Appendix B) were placed into sterile glass vials, covered tightly and stored at 4°C for use in these experiments. A different vial was

used for each trial. After spore deposits were made, the vial was covered, dated, and stored at 4°C. If problems were encountered, it would be possible to go back to the exact spore suspension for further investigation.

Specific Method

1. Copper boats were arranged in order on a stainless steel sheet on the table in the clean room and spore deposits were made.*
2. When all spore deposits were made, a metal air deflector, which was closed on the top and three sides, was placed over the stainless steel sheet on which the copper boats were arranged. The closed end of the deflector faced the HEPA filter wall. The deposits were allowed to dry until no visible water remained. Conditioning time in the clean room (35% RH and 25°C) began when the spore deposits appeared visibly dry.
3. The copper boats to be conditioned at <1% RH, were put into two separate metal transfer boxes, one for each of two treatment groups (<1% RH conditioning and heating and <1% RH conditioning and 35% heating). The boats to be conditioned at 35% RH remained on the stainless steel sheet, under the air deflector.
4. The transfer device (See Figure 5.5, page 58), which was used to transfer the copper boats conditioned at <1% RH and those to be heated at <1% RH, was placed on its side in the conditioning chamber (right side) of the double-chambered, environmentally controlled glove box outside of the clean room. The hood was flushed until the RH was less than 1% (overnight). The two metal transfer boxes to be conditioned at <1% RH were placed in the pass-thru of the conditioning chamber. The transfer box covers were then opened and the pass-thru door was closed. The pass-thru was then flushed for thirty minutes with the same type of gas being used in the test system, (either dry air

* See Method of Randomization of Treatment Combinations and Spore Deposit Method (Appendices C and D)

or dry nitrogen); this involved about ten gas exchanges. Immediately before and after the transfer boxes were placed in the conditioning chamber, the water content of the gaseous environment of the conditioning chamber was determined. Results were then compared to check the efficiency of the flushing method. Conditioning time (24 hours) began when the metal boxes were transferred from the pass-thru into the conditioning chamber.

5. After 24 hours conditioning, the pass-thru of the conditioning chamber was flushed again. By means of the special transfer device (Figure 5.5) the boats conditioned at <1% RH and those to be heated at 35% RH were transferred thru the pass-thru to the hot plate in the clean room. All boats were simultaneously transferred from the stainless steel sheet to the hot plate. The boats conditioned at 35% RH and those to be heated at 35% RH were placed on the hot plate by means of the holding tool (See Figure 5.6) The water content of the gas in the chamber was determined before and after opening the pass-thru door to effect the transfer.
6. Using the special transfer device, the copper boats conditioned at 35% RH and those to be heated at <1% RH were transferred into the pass-thru of the conditioning chamber. The pass-thru was flushed and the transfer device was placed in the conditioning chamber. Again the water content of the gas in the chamber was determined before and after opening the pass-thru.
7. The heating chamber (left side) of the double-chambered, environmentally controlled hood was flushed until the RH was less than 1%. The temperature of the chamber was approximately 25°C, and was maintained by cooling coils located inside of the chamber. A thermocouple located six inches above the surface of the hot plate gave a continuous read-out of the chamber temperature.

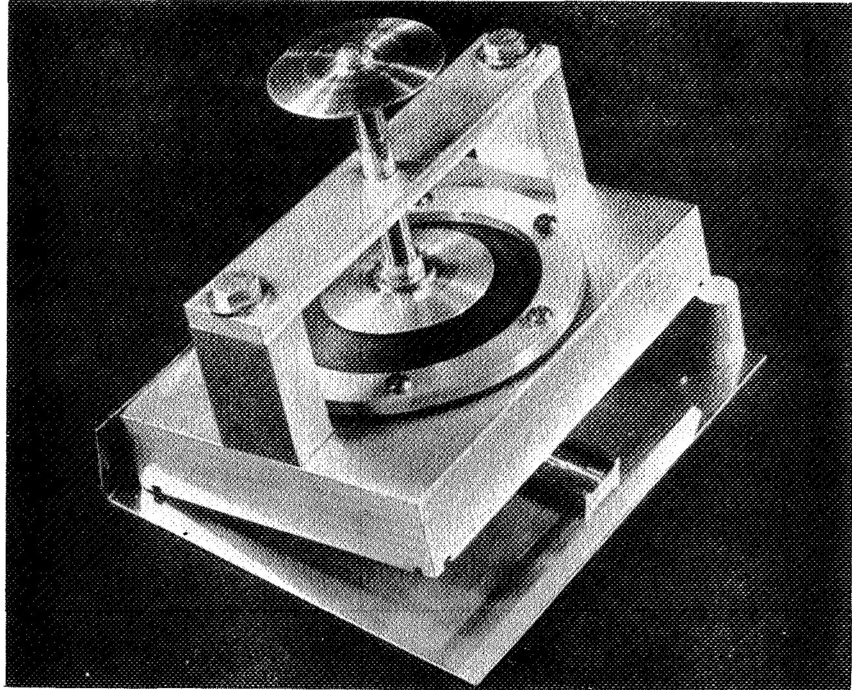

Figure 5.5 - Transfer device

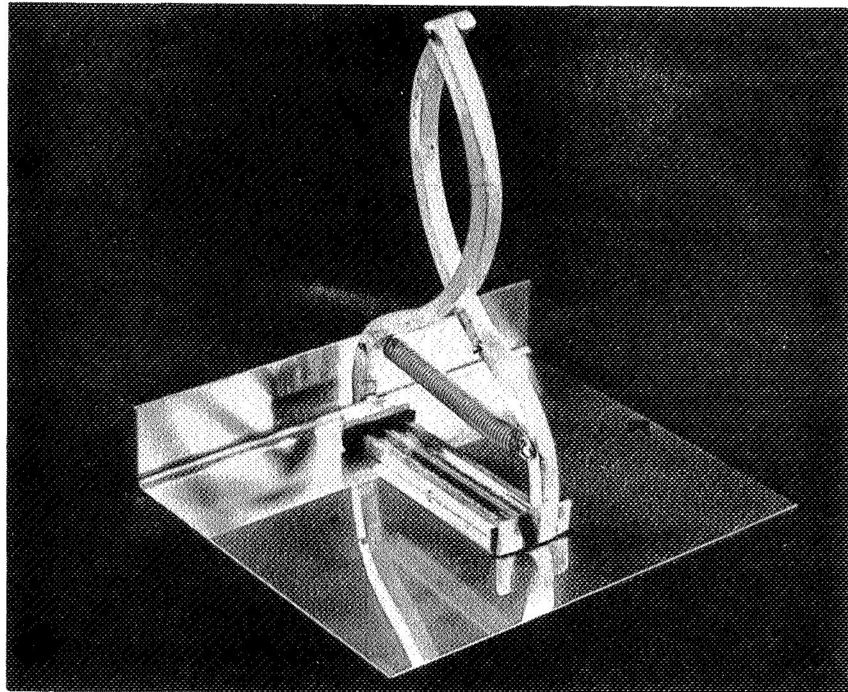

Figure 5.6 - Boat-holding tool

8. The metal transfer box (with strips conditioned at <1% RH and the transfer device (with strips conditioned at 35% RH) were passed from the conditioning chamber into the heating chamber via the inter-chamber pass-thru. The water content of the gas was determined before and after opening the pass-thru door.
9. The copper boats were moved to the hot plate simultaneously from the stainless steel sheet of the transfer device. The boats from the transfer box were placed on the hot plate using the special holding tool (Figure 5.6).
10. Two copper boats which were not involved in the experiment were located on the hot plates in the clean room and heating chamber, one on the upper-left and one on the upper-right corner of each hot plate. They were attached by means of thermocouples to a continuous multichannel recording potentiometer. The pass-thru doors of the heating chamber were never opened until all of the specified heating times had been completed. Heating intervals in the dry gas environment were 25, 50, 75, 100, and 125 minutes. In the clean room they were 85, 170, 255, 340, and 425 minutes for the 110° experiments.
11. Immediately upon completion of each heating interval the designated boat was placed on the cooling plate for a minimum of three minutes. The special holding tool was used to transfer boats. A stainless steel sensing disk was laid on the surface of the cooling plate which was connected by means of a thermocouple to the continuous multi-channel recording potentiometer.
12. All five heating times were completed before processing the strips. The boats were placed in their respective boxes and transferred to the clean room for processing.
13. The copper boat was picked up by means of the special holder and transferred to a sterile-gloved hand. Each of four replicates (planchets) was transferred with a sterile forceps (same one for four replicates from a single boat), spore-side down into a dry, sterile, pre-labeled 125 ml Erlenmeyer Flask. The fifth (center) planchet was not analyzed but was retained in its respective boat in case another strip was lost. If time

did not permit immediate processing the strips were stored dry in their respective labeled flasks until processed. Strips were processed the morning following the heating treatment, but no later than that.

14. To begin processing, 50 ml of sterile phosphate buffer was added to each flask. (Actual observation revealed that the bottles contained 50 ±1 ml of buffer.
15. A single flask, containing the strip and buffer solution was suspended in the ultrasonic tank using a rubber-coated, Stoddard-type test tube holder attached to the wall of the bath as the support. The bottom of the flask was parallel to the bottom of the tank and the tank solution (0.3% V/V Tween 80, aqueous) was level with the buffer solution in the flask.
16. Each flask was insonated at 25 Khz per second for two minutes.
17. Three different aliquots were plated in duplicate, using 15 x 100 mm disposable petri plates and disposable pipettes. Very small volumes (0.01 and 0.05 ml) were measured with a 10 µl or 50 µl Eppendorf Microliter Pipette. Five and ten aliquots were plated using 15 ml of one-and-one-half strength Trypticase Soy Agar (TSA); all other volumes were plated with 20 ml single-strength TSA. The expected D-value was used as the basis for selecting the heating time and the volumes to be plated so the results would yield countable plates at each time interval, with the last heating time calculated to yield approximately 1×10^2 spores per strip.
18. Plates were inverted and incubated for 48 hours at 32°C and counted with the aid of a Bactronic Counter. A single duplicate plate from each aliquot of each treatment combination was counted by each of two counters. If more than two counters were used, then the plates to be counted by each counter had to be randomly allocated.
19. Preliminary D-values and y-intercepts were determined by plotting (on semilogoarithmic paper), the number of survivors (on the logarithmic scale) versus the heating time in minutes (on the linear scale) and drawing a "best fit" line using the number of survivors at the five heating times but not N_o.

20. The estimated number of surviving spores at each heating time was recorded and forwarded to the biomedical computer center for computer analysis. The computer program utilized a least squares regression analysis (again ignoring N_o) to estimate a D-value and y-intercept for each time-temperature treatment combination.

RESULTS

Experiment 1

Results of the comparison of the $D_{110°C}$-values of the three spore suspensions are summarized in Table 5.1, while typical survivor curves for each suspension are shown in Figures 5.7, 5.8, and 5.9. It is obvious that regardless of which suspension was used, the dry gas environment resulted in significantly lower (about four-fold) $D_{110°C}$-values than did the ambient clean room environment. Of the three suspensions the water-stored spores and the new preparation of ethanol spores were more resistant than the older ethanol-stored spores.

Table 5.1 - $D_{110°C}$-values of Three Bacillus subtilis var. Niger Suspensions as a Function of Relative Humidity

Heating Environment	% RH Range		$D_{110°C}$-Value in Min. (No. of Trials)		
	At 25°C	At 110°C	Spores AAAB	Spores AANE	Spores AADB
Dry Gas Box (using dry air)	0.10-1.70	0.018-0.025	21.1 (2)	32.5 (4)	32.7 (8)
Laminar Crossflow Clean Room	40-42	0.89 0.93	99.3 (4)	145.4 (8)	121.3 (2)

Experiment 2

The effects of equilibrium time on the $D_{110°C}$-values of the three spore suspensions in both the dry-gas and clean-room environments are shown in Table 5.2. Survivor curves for the several conditions are shown in Figure 5.10. In all cases the spores were equilibrated in the same environment in which they were heated. Extension of the equilibration time did

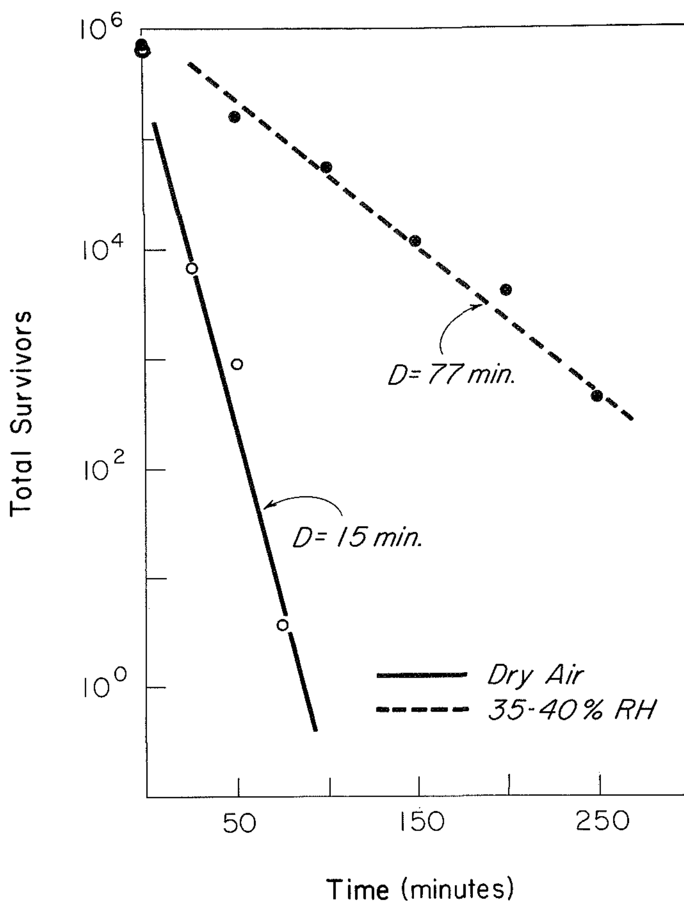

Figure 5.7 - Survivor curves for spores AAAB in dry and 35-40% RH air.

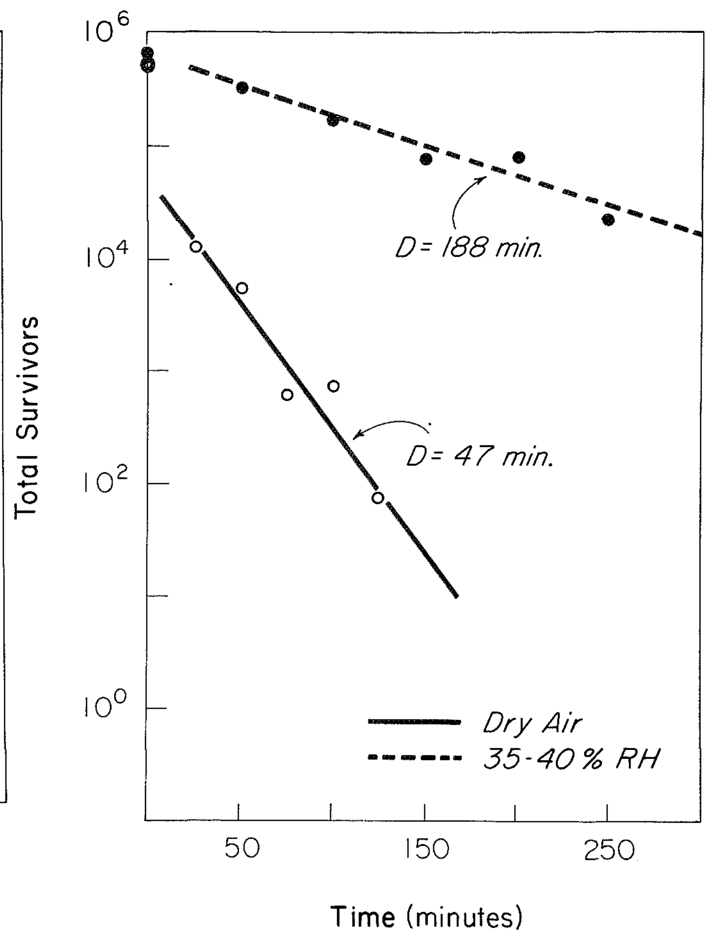

Figure 5.8 - Survivor curves for spores AANE in dry and 35-40% RH air.

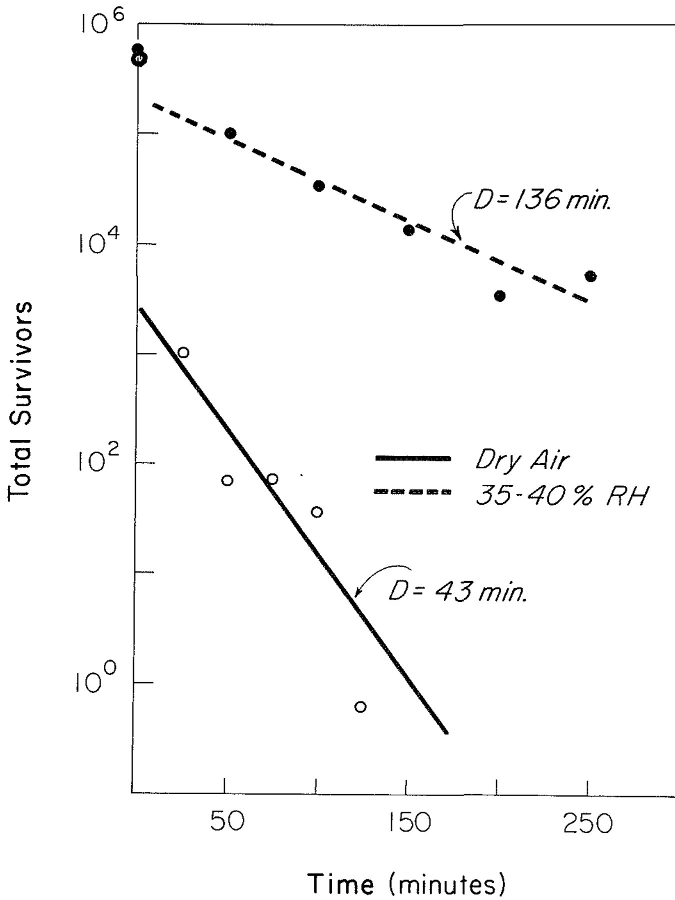

Figure 5.9 - Survivor curves for spores AABD in dry and 35-40% RH air.

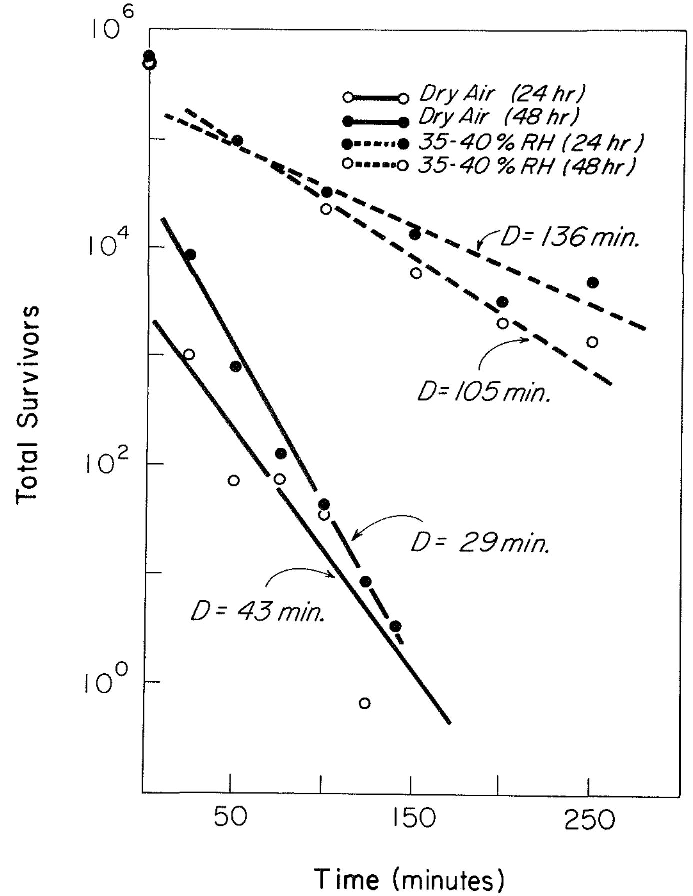

Figure 5.10 - A comparison of 24 and 48 hour equilibration for spores AABD in dry and in 35-40% RH air.

not affect the $D_{110°C}$-value significantly in the dry-gas environment. In the clean room the longer equilibration was associated with a slightly higher $D_{110°C}$-value.

Table 5.2 - Effect of Equilibration Time on $D_{110°C}$-values of Three Spore Suspensions

Equilibration Time	$D_{110°C}$-value in Min. (No. of Trials)					
	Spores AAAB		Spores AANE		Spores AADB	
	Dry Gas	Clean Room	Dry Gas	Clean Room	Dry Gas	Clean Room
24 Hours	22.0 (1)	97.1 (2)	35.8 (1)	148.0 (2)	32.5 (4)	116.9 (1)
48 Hours	20.1 (1)	101.5 (2)	37.2 (1)	165.4 (2)	30.4 (4)	125.6 (1)

Experiment 3

Because the spore coded AAOE generally appeared to be at least as resistant as the other spore suspensions, additional work was done with that suspension. As the original suspension of those spores (AANE) had been stored in water at 4°C and the new suspension had been frozen during shipment, comparative $D_{110°C}$-values were obtained for the old, non-frozen suspension and the new, frozen suspension. The results of these experiments are summarized in Table 5.3. While results were not conclusive from the limited number of trials, there did not appear to be a major effect of freezing on the D-value determination.

Table 5.3 - Effect of Freezing on the $D_{110°C}$-value of a *Bacillus subtilis* var. *niger* suspension Stored in Water

	$D_{110°C}$-value in Min. (No. of Trials)	
Spore Suspension	Dry Gas	Clean Room
Frozen (AAOE)	41.7 (2)	133.7 (2)
Non-frozen (AADB)	30.9 (3)	141.3 (4)

Experiment 4

All experiments described previously utilized spore suspensions which had been pipetted onto stainless steel planchets to yield approximately 1×10^6 spores per deposit before heating. An experiment was carried out in which deposits of approximately 1×10^4 and 1×10^8 spores in equal volumes of water were also evaluated. The results of these tests are summarized in Table 5.4.

It appears that the $D_{110°C}$-values were slightly lower when the lowest concentration (10^4) was used. The very high concentration of spores was very difficult to work with. We hope to make additional comparisons between 10^4 and 10^6 spores per deposit in the future.

Table 5.4 - $D_{110°C}$-values of Bacillus subtilis var. niger Spores (Frozen Suspension B, Spores AAOE) as a Function of Initial Concentration of Spores per Deposit

Initial Concentration per Deposit	$D_{110°C}$-value in Min.	
	Dry Gas Environment	Clean Room Environment
10^4	21.8	136.0
10^6*	41.7	153.0
10^8	42.1**	143.7

* Based on all previous observations

** Based on only two data points

Experiment 5

The results of the series of tests at 110°C in the two conditioning and heating environments with nitrogen as well as air in the low humidity environment are listed in Table 5.5. The data for the several conditions are summarized in Table 5.6. The effect of the relative humidity in the gas surrounding the hot plate during both heating and conditioning is dramatic in that the average for the four "d" conditions (Table 5.5) is 161 minutes compared to an average of 43.3 minutes for the four "a" conditions. The results also suggest

Table 5.5 - Results of Dry Heat Destruction-Rate Studies of *Bacillus subtilis* var. *niger* Spores AAOE on Stainless Steel Surfaces as a Function of Conditioning and Heating Environment

Test Number	Conditioning		Heating		$N_o \times 10^{-6}$	$D_{110°C}$-value in Min.	Y-intercept $\times 10^{-6}$
	% RH at 22°C	Environment	% RH at 110°C	Environment			
1 a	0.26-.98	Air	0.010-0.014	Air	1.72	43.1	.36
1 b	34-36	Clean Room	0.010-0.014	Air	2.03	40.4	.63
1 c	0.26-.98	Air	0.65-0.68	Clean Room	1.72	140.7	1.60
1 d	34-36	Clean Room	0.65-0.68	Clean Room	2.16	163.4	1.52
2 a	0.29-0.72	Air	0.009-0.019	Air	1.92	42.3	.62
2 b	34-36	Clean Room	0.009-0.019	Air	2.23	42.5	.63
2 c	0.29-0.72	Air	0.65-0.68	Clean Room	1.90	142.0	1.81
2 d	34-36	Clean Room	0.65-0.68	Clean Room	2.52	166.6	1.30
3 a	0.17-2.01	Nitrogen	0.005-0.008	Nitrogen	1.79	38.6	1.13
3 b	34-36	Clean Room	0.005-0.008	Nitrogen	1.92	43.3	.48
3 c	0.17-2.01	Nitrogen	0.65-0.68	Clean Room	1.99	177.6	.56
3 d	34-36	Clean Room	0.65-0.68	Clean Room	1.96	139.6	.71
4 a	0.05	Nitrogen	0.0009	Nitrogen	1.72	49.1	.29
4 b	34-36	Clean Room	0.0009	Nitrogen	2.24	37.7	.52
4 c	0.05	Nitrogen	0.65-0.68	Clean Room	1.85	147.7	1.45
4 d	34-36	Clean Room	0.65-0.68	Clean Room	2.03	161.2	.76

that there is no difference in the D-value that can be associated with the type of dry atmosphere. Mean D-values for spores conditioned and heated in the dry gas environment were 42.7 minutes for the dry air and 43.9 minutes for the dry nitrogen. Mean D-values for spores conditioned in the clean room and heated in the dry-gas environment were 41.5 for dry air and 40.5 for dry nitrogen. The moisture-analysis readings indicate that the overall relative humidity range during the heating period was higher for the dry air than it was for dry nitrogen (0.009%-0.022% for air vs. 0.0009-0.008 for nitrogen). Thus it is possible that the D-values are lower for spores in air than for those in nitrogen for an equivalent relative humidity condition. Subsequent experiments will be carried out to explore that possibility.

Table 5.6 - A Summary of the Data in Table 5.5 Showing the $D_{110°C}$-values for the Replicate Tests as a Function of the Specific Environmental Condition

Heated		Conditioned	$D_{110°C}$-values in Min.
Dry Air	a	Dry Air	43.1, 42.3
	b	Clean Room	40.4, 42.5
Dry Nitrogen	a	Dry Nitrogen	38.6, 49.1
	b	Clean Room	43.3, 37.7
Clean Room	c	Dry Air	140.7, 142.0
	c	Dry Nitrogen	177.6, 147.7
	d	Clean Room	163.4, 166.6, 139.6, 161.2

It is apparent that in the two dry nitrogen experiments, the relative humidity effect did not operate in that manner. During the second experiment, the relative humidity was appreciably lower than during the first experiment (0.0009-0.0012 compared to 0.005-0.008), yet the D-value was considerably higher at the lower humidity (49.1 minutes vs. 38.6 minutes). It appears then that either the effect of minute quantities of water was more complex than anticipated or the technique for measuring humidity was sufficiently imprecise that it makes the data comparisons meaningless.

A comparison of the a vs. b data and the c vs. d data will give an indication of the effect of the conditioning treatment (initial level of water in the spores) on D-value. The average D-value for "a" Condition is 43.3 minutes and that for "b" condition is 41.0 minutes suggesting that the initial water content probably has no significant effect. Comparing c and d, the average for c is 152 minutes and that for d is 158 minutes; suggesting that again there is no significant effect of initial spore water content on D-value.

It is also interesting to note that in seven out of eight measurements N_o was lower following dry gas conditioning than it was after conditioning in the clean room. In addition, in three out of four of the experiments (1, 2, and 4) the y-intercept was lower following dry gas heating than it was following ambient environment heating.

CONCLUSIONS

The experiments to date, at 110°C have shown that heating in a dry-gas environment (<0.03% RH at 110°C) results in D-values considerably lower than those obtained by heating in an ambient environment (∿34-36% RH at 22°C or 0.65-0.86% at 110°C). A D-value ratio of approximately 1:4 was noted in almost every trial carried out.

A comparison of dry nitrogen and dry air did not reveal any difference between the two gases; although drier conditions were associated with nitrogen than with air. Thus it is possible that a difference might exist under equivalent moisture conditions.

There was an indication that heating in a dry gas may have reduced the y-intercept (indicating that a sharper than usual reduction took place during the first heating interval), compared to heating in the clean room.

FUTURE WORK

Priorities for additional experiments related to this task include the following:

1. Determination of D-values in the dry gas environment for *Bacillus subtilis* var. *nniger* (AAOE) at temperatures both

higher and lower than 110°C. Data will be obtained at 125°C and 90°C, again utilizing both dry air and dry nitrogen in comparison with ambient clean room environments.

2. Determination of D and Z-values in the range of 90-125°C for highly resistant spores isolated from Mariner '69.
3. Determination of the effect of varying relative humidity in the range of 25-45% on $D_{125°C}$-values as measured on a hot plate in a 22°C clean room.
4. Determination of the effect on $D_{125°C}$-values of varying the room temperature (16-26°C) and relative humidity (25-45%) while holding vapor pressure constant in the clean room.
5. Determination of $D_{125°C}$-values at a relative humidity intermediate between the 0.006% and 0.4% currently being achieved (as calculated at 125°C in the dry gas and clean room environments).
6. Determination of dry gas D-values for spores contained on surfaces other than stainless steel (e.g. glass, plastic, or painted surfaces).
7. Spore species other than _Bacillus subtilis_ var. _niger_ will also be studies to determine the consistency of the dry gas phenomena.

APPENDIX A: THE USE OF SILICA GEL PRE-LOADED WITH WATER TO PRODUCE A CONSTANT RELATIVE HUMIDITY IN A CLOSED CHAMBER

Introduction

Water content appears to be the most important variable in the dry heat destruction rate of microbial spores. One method of varying the water content of spores is to equilibrate the spores in chambers of different relative humidity.

Objective

Throughout the scientific world, saturated salt solutions are used to produce and maintain a fixed equilibrium relative humidity. We were concerned about minute quantities of salt being depositid with the spores on the metal strips. For this reason, we investigated other systems for producing a constant relative humidity. Silica gel pre-loaded with water appeared to have considerable merit for this application.

Our research group's decision to use silica gel to control the relative humidity of environmental cabinets grew out of conversations in March, 1968, with Dr. Elias J. Amdur, Design Supervisor, Apparatus Controls Division, Honeywell Corporation. The following important points resulted from those discussions:

1. Salt solutions often are not satisfactory for relative humidity control for the following reasons: 1) the solutions exhibit creep, i.e., a tendency to climb up the walls of their container, 2) in the usual application, when a container of salt solution is placed in the bottom of a jar, the equilibration time is very long, e.g. 24 hours for a one-gallon jar, and 3) the relative humidity sensing elements become contaminated when stored over salt solutions, even though they are protected from direct contact with the solution.
2. The Honeywell Corporation uses silica gel pre-loaded with water to produce controlled relative humidity levels. A curve of equilibrium relative humidity versus the quantity of water added can be developed for each lot of silica gel. The curve can be

reliably used to pre-load silica gel with water to a desired relative humidity.

3. Silica gel is less stable for moisture control over a long period of time than salt solutions since the relative humidity is a continuous function of the water added to the silica gel.
4. If a sterile humidity-control medium is desired, silica gel and water can be heat sterilized separately and mixed aseptically.
5. Air movement is needed in a humidity control chamber to prevent unduly long equilibration times. A fan is satisfactory but the motor should be kept outside the cabinet in order to avoid heat problems. Alternatively, a rheostat or other control may be used to lower power consumption or the cabinet may be designed to dissipate heat rapidly. To minimize air circulation requirements, the silica gel may be suspended in small packages at many locations in the controlled cabinet.
6. If a humidity cabinet is not temperature controlled, the quality of air conditioning in the surrounding room may cause problems. A 2°F variation in room temperature is likely in an air-conditioned room; in addition to causing fluctuations in the relative humidity, a 2° temperature change may cause condensation in a high relative humidity chamber.

Experimental Procedure - General Recommendations for Loading Silica Gel with Water

The loading procedure consists of adding the water to the silica gel in several portions in a sealed jar, mixing gently but thoroughly after each addition. An overnight (4-15 hours) stabilizing period should be allowed before the equilibrium relative humidity is checked and the gel put into use. Since there is a lot-to-lot variation in the water-loading characteristics of silica gel, the equilibrium relative humidity must be checked and a relative humidity versus water-loading curve developed for each lot of silica gel if accurate relative humidity conditions are to be produced. A typical loading curve from our laboratory is shown in Figure A-1.

The water-loaded silica gel should be stored and used at a constant temperature.

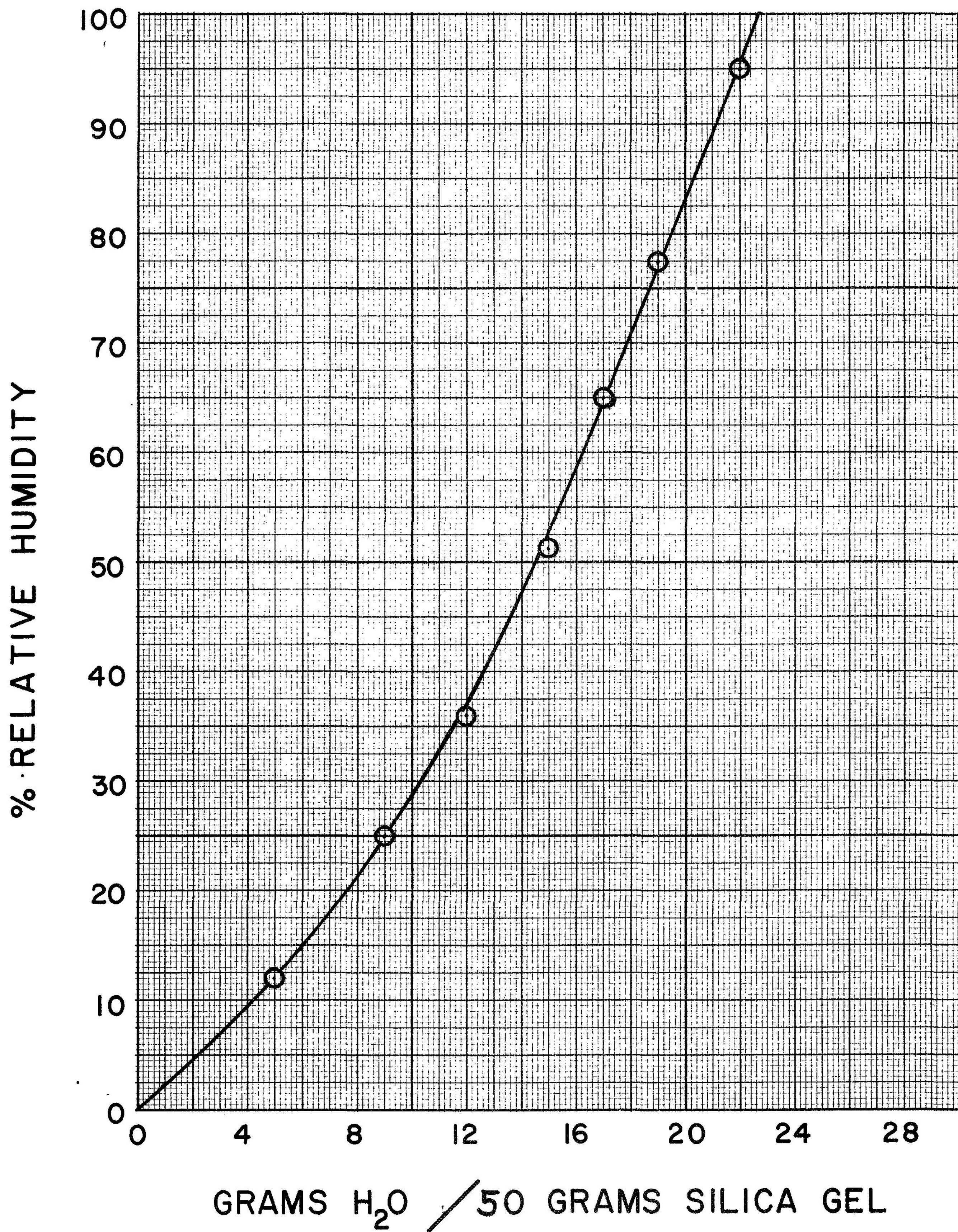

Figure A-1 - Relative humidity as a function of the quantity of water added to one lot of silica gel. Temperature 45°C

In our laboratories, silica gel loaded with water has been used to produce a controlled relative humidity environment in both small plastic (200 ml) and glass (500 ml) containers and in a small (0.2 m^3) plastic glove box.

Reuse of the gel is not recommended since the relative humidity reproducibility decreases after the first use.

Procedures for Small Plastic and Glass Containers

Refrigeration-grade silica gel, mesh size 8-20 is used. The gel is dried in a forced air oven at 100°C, for 24 hours, weighed into screw cap bottles, reheated to 100°C, for 24 hours, tightly capped and stored until used.

Twenty-four hours before setting up the relative humidity chamber the correct amount of distilled water is added to the silica gel. Thorough mixing is accomplished by gentle agitation of the bottle. Hard shaking is not recommended since it appears to break up the crystals. The water and gel are then equilibrated for 24 hours before being added to the humidity chamber.

The boxes are fitted with a bulkhead connector and relative humidity-temperature sensor so that humidity can be measured without opening the inner box. After the addition of 50 grams of silica gel plus the appropriate amount of water to the box, the cover is taped in place with masking tape; the box is then heat sealed in double polyethylene bags and placed in a controlled-temperature chamber.

The plastic bag is cut open at intervals to measure the relative humidity (a Honeywell Indicator, #W611A was used for the relative humidity measurement.) The boxes are immediately resealed in plastic bags. The taped box itself is not opened.

The reproducibility of a specific relative humidity in replicate boxes varies with the humidity. The actual relative humidity recorded over a two-month period on boxes intended to be at <2%, 50% and >90% is shown in Table I. On replicate boxes dry silica gel always produced a relative humidity of less than 2% while at higher humidities, reproducibility was ±5%.

Table I - Relative Humidity Levels in Individual 5 1/4", 3 1/2" and 2 1/2" Plastic Boxes at 45°C as a Function of Time During an Experiment

Time	Box #1	Box #2	Box #3
Desired RH	<2%	50%	90%
After 2 days	<2%	55%	97%
After 30 days	<2%	56%	100%
After 60 days	3%	57%	96%

Procedure for Glove Box

Silica gel pre-loaded with water has been used to produce and maintain a controlled humidity condition in plastic glove boxes of about 0.2 cubic meter volume. Stainless steel pans were fastened together and placed on the floor of the glove box so that the entire floor area of the glove box was covered. 900 grams of silica gel pre-loaded with water was spread in a thin layer over the pans. A perforated expanded metal floor was placed over the pans to provide a surface on which the samples could be placed for equilibration.

To insure that the temperature and humidity remained uniform throughout the glove box, a four-inch diameter propeller fan circulating 200 cfm was mounted in each box. Although the fan was operated at full-line voltage the heat output from the fan produced a temperature rise of less than 2°C. In the normal use of the glove box, the controlled relative humidity level is reestablished in about thirty minutes after the glove box is opened for addition or removal of samples.

Results

The system has been used over a five-month period during which period the relative humidity in the room in which the glove boxes were located was very low. As might be expected, the low humidity box maintained a less than 2% relative humidity. The high humidity glove box, however, required weekly additions of water to maintain a constant relative humidity level. The amount of water required varied from week to week and was determined by trial each time. A relative humidity above 92% could not

be reliably attained. When the humidity was above 92%, water condensed on the wall of the plastic glove box because the temperature in the room surrounding the box was a few degrees lower than the temperature inside the glove box.

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APPENDIX B: CODING SPORE CROPS AT THE UNIVERSITY OF MINNESOTA

Introduction

Organization is the foundation of science and understanding. One researcher working with two or three spore crops has a few problems in identifying his spores and keeping track of the results from the different spore crops. When there are people in several laboratories working with more than ten different spore crops, it is necessary to codify these spores so that the identification of all crops is similar. Provision is made in the system for a large number of variations in spore crops with a continuity of identity. Some of the variables that have to be included are the basic genus and species of the culture, the place of isolation or the source, the methods of culturing the organism and perhaps the final suspending medium. We have attempted to put together a spore code that will allow us to fit all of our spore crops into one identification system. The continuity of this system is such that when we look at data from different spore crops the code letters will allow us to have a feel for the spores without having to go back and look up the details on each spore crop.

Explanation of the Spore Crop

The code consists of four letters. The first letter designates the genus, species and subspecies of the organism if they are known. If the spore is an unknown, any identifying number and the place of isolation are given.

The second letter designates the place from which this laboratory originally received the spores.

The third letter identifies the specific spore crop. Information includes date of the crop, culture place and method, cleaning procedures, suspending fluid and any other pertinent information.

The fourth letter designates the storage conditions in this laboratory.

Coded Spore Crops Used in this Progress Report

AAAA

A *Bacillus subtilis* var. *niger*

A Dr. Favero, USPHS, Phoenix, Arizona

A Date cultured: August, 1968

Place cultured: Minnesota

Culture procedure: No. 1

Suspending fluid: 95% ethanol

A 95% ethanol, -20°C

AAAB

A *Bacillus subtilis* var. *niger*

A Dr. Favero, USPHS, Phoenix, Arizona

A Date cultured: August, 1968

Place cultured: Minnesota

Culture procedure: No. 1

Suspending fluid: 95% ethanol

B 95% ethanol, -20°C, resuspended in deionized distilled water at +4°C

AAAC

A *Bacillus subtilis* var. *niger*

A Dr. Favero, USPHS, Phoenix

A Date cultured: August, 1968

Place cultured: Minnesota

Culture procedure: No. 1

Suspending fluid: 95% ethanol

C 95% ethanol, -20°C, resuspended in deionized distilled water at -20°C

AABF

A *Bacillus subtilis* var. *niger*

A Dr. Favero, USPHS, Phoenix

B Date cultured: August, 1968

Place cultured: Minnesota

Culture procedure: No. 1

Suspending fluid: Deionized distilled water

F Deionized distilled water at +4°C

AADB

A Bacillus subtilis var. niger

A Dr. Favero, USPHS, Phoenix

D Date cultured: June, 1969

Place cultured: Minnesota

Culture procedure: No. 1

Suspending fluid: 95% ethanol

B 95% ethanol, -20°C, resuspended in deionized distilled water at +4°C

AAND

A Bacillus subtilis var. niger

A Dr. Favero, USPHS, Phoenix

N Date cultured: March, 1969

Place cultured: USPHS, Phoenix, Arizona

Culture procedure: No. 2

Suspending fluid: Buffered distilled water

Date received: March, 1969

Condition when received: Packed in ice

D Buffered distilled water at +4°C

AANE

A Bacillus subtilis var. niger

A Dr. Favero, USPHS, Phoenix

N Date cultured: March, 1969

Place cultured: USPHS, Phoenix, Arizona

Culture procedure: No. 2

Suspending liquid: Buffered distilled water

Date received: March, 1969

Condition when received: Packed in ice

E Buffered distilled water at +4°C, resuspended in deionized distilled water at +4°C

AAOE

A Bacillus subtilis var. niger

A Dr. Favero, USPHS, Phoenix

O Date cultured: March, 1969

Place cultured: USPHS, Phoenix, Arizona

Culture procedure: No. 2

Suspending fluid: Buffered distilled water

Date received: September, 1969

Condition when received: Packed in dry ice, frozen

E Buffered distilled water at +4°C, resuspended in deionized distilled water at +4°C

VDLA

V Unknown, Isolate #4-47A, Mariner '69

D Jet Propulsion Lab

L Date cultured: October, 1969

Place cultured: JPL, Pasadena, California

Culture procedure: No. 2

Suspending fluid: 95% ethanol

Date received: December, 1969

Condition when received: Packed in dry ice

A 95% ethanol, -20°C

VDLB

V Unknown, Isolate #4-47A, Mariner '69

D Jet Propulsion Lab

L Place cultured: JPL, Pasadena, California

Culture procedure: No. 2

Suspending fluid: 95% ethanol

Date received: December, 1969

Condition when received: Packed in dry ice

B 95% ethanol, - 20°C, resuspended in deionized, distilled water at +4°C

Procedure No. 1 - Spore Production Using Supplemented TAM Sporulation Medium

1. Use Difco TAM Sporulation Agar #0892. The medium is prepared according to the directions on the bottle. Prior to pouring the medium into the plates it is supplemented with two salt solutions:
   a. Calcium chloride: Make a 10% $CaCl_2$ solution, filter sterilize and add 0.8 ml per liter after medium has cooled.
   b. Magnesium sulfate: Make a 10% $MgSO_4$ solution, filter sterilize and add 0.2 ml per liter after cooling.

2. Heat shock an aqueous suspension of spores at 80°C for fifteen minutes. Using the heat shocked suspension inoculate the surface of a supplemented TAM plate with a cotton swab. Incubate at 41°C for 24 hours or until sporulation occurs.
3. Prepare a heavy suspension of spores from the above TAM plate in sterile deionized distilled water and heat shock at 80°C for fifteen minutes. Inoculate a fresh plate of TAM with a cotton swab and incubate at 41°C for three hours.
4. Swab the above plate and inoculate another plate of TAM that has been equilibrated at 41°C. Incubate for three hours and repeat this procedure.
5. You should now have a culture that is in the log phase of growth. Use this plate to inoculate ten plates of TAM that are at 41°C. Incubate at 41°C for at least 24 hours. Check each plate microscopically for sporulation. Any plate that does not have at least 90% sporulation after 48 hours should be discarded.
6. Collect the growth from the plates using a minimal amount of cold sterile deionized distilled water. Using a Branson Sonogen A Series ultrasonic bath, insonate in cold 0.3% Tween 80 for thirty minutes at 25 Khz/sec. to break up vegetative cells. The temperature of the bath is monitored and maintained at 5-10°C by the addition of ice.
7. Centrifuge the suspension at 2400 rpm, 4°C using a refrigerated centrifuge. The spores will separate from the cellular debris. Wash and recentrifuge six times. If the final suspension is to be stored in 95% ethanol, rinse twice in ethanol.
8. The final spore suspension is checked microscopically by making a smear and staining by the Schaeffer and Fulton Modification of the Wirtz Method of Staining Bacterial Spores.

Procedure No. 2 - Spore Production Using Synthetic Sporulation Medium-10
(This medium was described by Lazzarini and Santangelo, J. Bact.94:125-130

1. This procedure using Synthetic Sporulation Medium-10 is a liquid culture system. The following materials with the exception of the metal salts are dissolved in 900 ml of distilled,deionized water

and sterilized for fifteen minutes at 121°C. The metal salt solution is made up in ten-fold concentration. 100 ml are added aseptically to the base medium after it has cooled

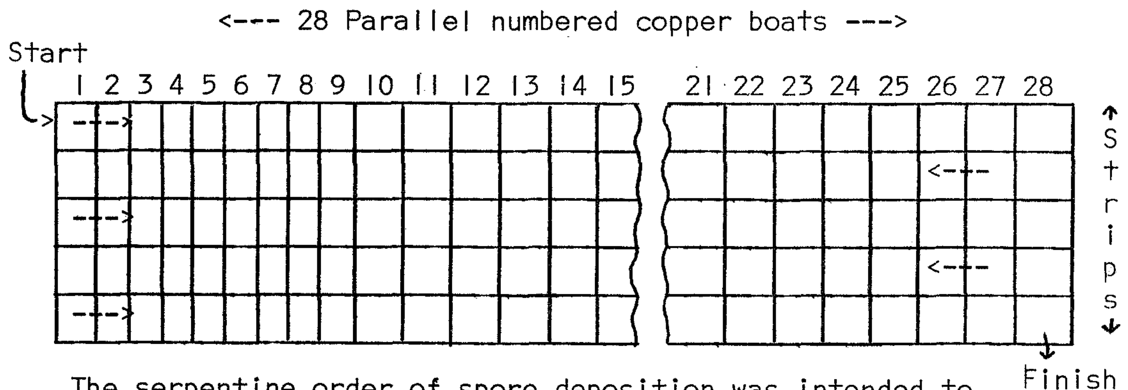
	Per liter
$K_2HPO_4 \cdot 3H_2O$ (K_2HPO_4)	7.3 gm
KH_2PO_4	2.4 gm
Sodium Citrate	1.0 gm
Tris (hydroxymethyl) Aminomethane (free base)	3.6 gm
Glutamic acid	4.4 gm
Glucose	1.5 gm
Tryptophan	25.0 mg
Methionine	25.0 mg
$CaCl_2$ ($CaCl_2 \cdot 2H_2O$)	111.0 mg
$MgSO_4 \cdot 7H_2O$	246.5 mg
$ZnCl_2$	6.81 mg
$MnCl_2 \cdot 4H_2O$	9.89 mg
$FeCl_3$	2.70 mg

2. Three hundred ml portions of the complete medium in Bellco one liter baffled flasks are inoculated with 5 ml of a 24-hour SSM-10 broth culture. Flasks are then incubated at 136 rpm and 32°C for five days (ca. 90% spores) in a Psychro-Therm Incubator Shaker (New Brunswick Scientific Co.).
3. After incubation, growth in 800 ml of medium is harvested with a refrigerated continuous flow centrifuge at 800 x g. Pellets are polled, placed in an ice bath and exposed to ultrasonic energy using a probe operating at 60% maximum intensity for 90 seconds, to disrupt clumps and cell debris.
4. The spore suspension is then recentrifuged and washed four times. If the spores are to be stored in ethanol, rinse them twice with ethanol.
5. The final spore suspension is checked microscopically by making a smear and staining by the Schaeffer and Fulton Modification of the Wirtz Method of Staining Bacterial Spores.

APPENDIX C: METHOD OF RANDOMIZATION OF TREATMENT CONDITIONS

(This example is for Experiment 5. In other experiments the method was the same but treatment combinations differed.)

1. Each trial utilized 28 permanently-numbered rectangular copper boats each containing a row of five 1/2"-square, sterile, stainless steel* strips (planchets) which lay flat in a shallow groove running the length of each boat.
2. The boats were arranged in numerical order, parallel to each other on a clean stainless steel sheet.
3. By means of a twenty µl Eppendorf Microliter Pipette** 0.02 ml of aqueous spore suspension ($\sim 1 \times 10^6$ spores) was deposited onto each strip in a serpentine manner starting at the upper left-hand corner and continuing to the upper right, following the diagram as indicated:



The serpentine order of spore deposition was intended to reduce <u>between-boat</u> variation at the possible expense of increased <u>within-boat</u> variation.

4. There were four treatment combination groups: (RH measured at 22-24°C)
   a. <1% RH conditioning and <1% RH heating
   b. 35% RH conditioning and <1% RH heating
   c. <1% RH conditioning and 35% RH heating
   d. 35% RH conditioning and 35% RH heating

*Type 302 fullhard spring-temper stainless steel shim stock, 0.015-inch thickness

**See Procedure for the Deposition of a Spore Suspension by Use of the Eppendorf Microliter Pipette (Appendix D)

5. For each treatment-combination group, (a thru d) seven copper boats were randomly assigned. Following is a sample random-allocation of boats to treatment.

Treatment	Copper Boat Number			
	a	b	c	d
N_o	11	1	28	3
1st heating time	2	16	19	13
2nd heating time	18	25	4	22
3rd heating time	24	17	26	7
4th heating time	9	15	14	10
5th heating time	27	5	20	23
Spare	12	6	21	8

Four of the five planchets were processed from each copper boat. The center one was not analyzed unless another was lost, up to the point where plates were placed safely on the incubator shelf. Experience to date indicates that this safety margin was adequate.

APPENDIX D: PROCEDURE FOR THE DEPOSITION OF A SPORE SUSPENSION BY USE OF THE EPPENDORF MICROLITER PIPETTE*

1. A sterile, dry tip was placed over the conical lower part of the pipette (yellow for 5 to 100 µl, red for 101 to 1000 µl).
2. The spore suspension was mixed on the Vortex Mixer, maximum power for five to six seconds. The pipette was rinsed once with spore suspension before making the first deposit. It was mixed again after every nine deposits, the cover was replaced before mixing.
3. The stem of the pipette was grasped so that the thumb could operate the colored button on top.
4. The pushbutton was depressed to first stop, then the tip was immersed slightly into the spore suspension.
5. The pushbutton was allowed to return to starting position;solution entered the tip.
6. The tip was withdrawn from the spore suspension and moved slowly along the well of the tube; the edge was touched slightly to remove excess.
7. The tip was placed close to the surface of the stainless steel strip.
8. The pushbutton was depressed to first stop, and after one second it continued all the way down to the second stop.
9. With the pushbutton remaining fully depressed the pipette was slowly withdrawn. The spore deposit was not spread so as to obtain maximum challenge effect.
10. The pushbutton was allowed to return to starting position.

*The filled pipettes were always held upright. A special rack was used for temporary upright storage.

APPENDIX E: GUIDELINES FOR THE ESTIMATION OF THE NUMBER OF SURVIVING SPORES FOLLOWING DRY HEAT TREATMENT

1. Count colonies on all plates and record plates with >600 colonies as TNTC.
2. Do not use any plated aliquot which has a TNTC count or a zero count in any calculation.
3. When making calculations, if possible, use results from that dilution yielding a mean count (mean of 8 colony counts; 2 each from 4 strips) in the range of 30-300 (refer to <u>Standard Methods of Dairy Products</u>, 12th Edition, 1967.) If two such dilutions appear, use the one which yields the <u>highest</u> extrapolated mean count.
4. If no such dilution appears, then use a dilution with a mean of 20 or greater, or less than 500. If there are two such dilutions, again use the highest extrapolated mean.
5. Under no circumstances are data from two or more different aliquots used. If no dilution yields applicable counts, that data time-point is considered lost.

APPENDIX F: GUIDELINES FOR QUALITY CONTROL OF CULTURE MEDIUM (TRYPTICASE SOY AGAR, BALTIMORE BIOLOGICAL LABORATORIES, PRODUCT NUMBER 11043)

1. Record the lot number of the dehydrated medium.
2. When sterilizing a batch of medium, which has been prepared according to the specifications of the manufacturer*, place a maximum-registering thermometer in the chamber of the autoclave during the sterilization cycle. (Capillary contraction allows the mercury to rise when heated but prevents it from receding until it is shaken down.) Remove it from the autoclave at the completion of the sterilization cycle, and allow it to cool to room temperature before reading. (Errors may result if reading is taken before thermometer has cooled.) A vacuum exists above the mercury column and readings must be made with the thermometer in upright position. Record the reading along with an assigned batch number on a separate sheet of paper. Indicate the specific sterilization cycle on the recording chart which indicates temperature and time and save the chart. Periodically check the accuracy of all thermometers used against a standard. Check the recorded time of the sterilization cycle by actual observation of built-in thermometers. If discrepancies occur, adjust the autoclave to correlate with the stated time and temperature of the chart and the stated pre-set temperature of the chamber during the sterilization cycle.
3. Store the prepared medium in the refrigerator at 4°C, in tightly-closed, screw-cap tubes, for a period not exceeding one month.
4. Melt the medium on the same day it is to be used. (Medium melted at the beginning of the day is used at the beginning of plating for that day.)
5. When melting tubes of medium, use a fixed time; in the large autoclave,five minutes; in the small autoclave,three minutes. Upon completion of the melting cycle, transfer the tubes immediately to the media conditioner (50-53°C) or water bath (50-53°C). Any tubes with a decrease in volume should be discarded.
6. Any tubes of medium containing a precipitate should be discarded, as their use may result in reduced capacity for growth promotion.

Precipitation is usually a result of overheating.

7. Discard unused tubes of melted medium at hhe end of the working day and do not remelt prepared medium more than once.
8. Prepare all media used in a single experiment from the same "lot" of dehydrated media.

*<u>Method of Preparation</u>: Suspend 40 grams of the powder in a liter of distilled water and mix thoroughly. Heat with frequent agitation, boil for one minute and dispense and sterilize at 118°C to 121°C (not over fifteen pounds of steam pressure for fifteen minutes. Do not overheat.)

PUBLICATIONS AND PRESENTATIONS

Publications in the Open Literature

Greene, V. W. and D. Vesley. 1969. Microbial Contamination Control in Hospitals, Part 4 - Role of Housekeeping. *Hospitals*. 43(23): 69-80.

Presented at Meetings

Vesley, Donald. "Cooperative Microbial Evaluation of Hospital-Patient-Room Floor Cleaning Procedures," Report of APHA Laboratory Section Committee on Microbial Contamination of Surfaces at the annual meeting of the American Public Health Association, November 11, 1969.