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ENVIRONMENTAL MICROBIOLOGY
AS RELATED TO PLANETARY QUARANTINE
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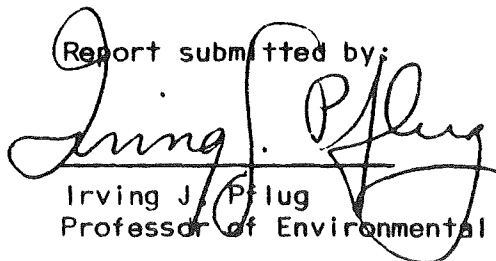
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TABLE OF CONTENTS

Introduction	i
Survival of Microbial Spores Under Several Temperature and Humidity Conditions	1
Detection of Low Levels of Microbial Contamination on Surfaces by Chemical Approaches	9
Dry Heat Destruction Rates of <u>Bacillus subtilis</u> var. <u>niger</u> in a Closed System:	
A. Thermal Destruction Studies	11
B. Measurement of Water	17
Dry Heat Destruction Rates of Microorganisms on Surfaces:	21
A. D-value Studies at 90 and 100°C	23
B. Changes in the D_{125} -value as a Function of Incremental Changes in the Environmental Temperature and Vapor Pressure	39
C. Change in D-value for Small Changes in Temperature	49
Publications and Presentations	55

INTRODUCTION

This report covers research activities during the period June 1, 1970, through November 30, 1970, for the project entitled "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--Lawrence B. Hall, Planetary Quarantine Officer.

This is the fifth semiannual report of progress on NASA project NGL 24-005-160. A limited number of earlier reports on this project are available for those who do not have a complete set but who wish to obtain all of our procedures and results in a given area.

In the studies of the survival of Bacillus subtilis var. niger spores, we found that when these spores were suspended in either distilled water, buffered distilled water or 95% ethanol at 45°C the time for a one-log reduction in the number of spores was in the range of nine weeks. Extrapolation of these data to 30°C suggests a storage time of the order of 900 weeks for a one-log reduction. We do not feel justified in extrapolating these data to 3-5°C, (the normal refrigeration storage temperature for spores); however, it is obvious that the time for a one-log reduction at a temperature of 3-5°C will be 10-100 times longer than at 30°C giving values of the order of hundreds of years. For spores in acetone at 45°C, the time for a one-log reduction in number of spores was half as long as that for the water or 95% ethanol. The effect of spore survival in ethanol and isopropanol at 70 and 35% is also reported.

In the study of water in a closed system, we evaluated the effect of spore atmospheric volume on the D-value: we found that in the presence of a constant initial number of spores the D-value appears to decrease as the atmospheric volume and, necessarily, the surface area increase. As has been postulated previously by other researchers, we believe that this D-value relationship is a function of the water content relationships within the system where water concentration decreases as volume increases.

In studies to determine the dry heat destruction rates of microorganisms on surfaces in the temperature range of 90-125°C we found that the survivor curve (logarithm of the numbers of the survivors plotted as a function of time on the hot plate at a given temperature) could well be represented by a straight line. The D-value, a measure of the slope of the regression line, was smaller in all low humidity experiments (RH of .001% at 125°C) than it was in experiments at higher humidities (RH of 0.4% at 125°C). For the two spores evaluated, the change in D-value with change in relative humidity was greater for the Bacillus

subtilis var. niger spores than for the Bacillus firmus spores. A very interesting result, for which we have not been able to determine full significance, is that the intercept ratio decreased with the decrease in the clean room relative humidity from 1.1% to 0.4% at 125°C for both the Bacillus subtilis var. niger and the Bacillus firmus spores. In evaluating the data at the several temperatures, we concluded that, for spores deposited on stainless steel planchets and heated on a hot plate the residual water content of the spores will be a function of the heating temperature; therefore, the resulting D-value data cannot be used to calculate meaningful z-values.

We carried out a group of experiments designed to observe the change in estimated D-value with changes in vapor pressure in the ambient temperature and vapor pressure range. D-value increased with vapor pressure, however, it appeared that for the narrow range studied the rate of increase of D-value was greater at high vapor pressures than at lower vapor pressures.

An experiment consisting of eight tests, two temperature conditions repeated four times in a single day, was conducted to study experimental variation of D-value of spores heated on hot plates in a clean room. All known parameters were controlled or observed. The results in many regards indicated consistency and reproducibility; however, there apparently are still unrecognized and thus, uncontrolled, environmental parameters that varied during a single day which must be identified in the future and controlled.

Several years ago it was recognized that spores under different physical conditions were killed at different rates when subject to heat. The open, mated and closed systems concept was invented to identify major different physical conditions. Today we are finding that open, mated and closed are simply points on a continuum. Open and closed systems are the extremes with mated being an intermediate condition. Now that we are beginning to understand the range of physical conditions possible, and the causes of variation in the dry heat destruction rate which are associated with specific physical conditions, we can begin to relate conditions more precisely to each other and to experimental conditions in the laboratory. All together this means that we are continuously improving our accuracy and ability to predict spore destruction rates and consequently the heat sterilization cycle which will be necessary to produce a given level of sterility on space hardware.

I. J. Pflug

SURVIVAL OF MICROBIAL SPORES UNDER SEVERAL TEMPERATURE AND HUMIDITY CONDITIONS

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Division of Environmental Health

Project Contributors: J. Bearman and R. Jacobson
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INTRODUCTION

In previous progress reports we have described and given the results of experiments to measure survival rates of Bacillus subtilis var. niger (AAAA) on surfaces at 22, 45 and 60°C as a function of the relative humidity of the surrounding air. In these experiments, silica gel and water were used to achieve the desired relative humidity. In order to continue these studies at 75 and 90°C it was necessary to develop a new experimental system. Work has now been completed on a chamber to control and measure relative humidities at temperatures of 75 and 90°C and experiments have been initiated.

Procedures for evaluating the effect of water activity on spore survival were also described in previous reports. The current report describes experiments performed to determine the D-values for spores suspended in several liquids of varying water activities (A_w) at temperatures of 45, 60, 75 and 90°C.

OBJECTIVE

The objectives of these experiments are: 1) to determine the D-value of spores at temperatures below 90°C suspended in liquids commonly used for the storage of spores, and 2) to determine the effect of available water on the survivor curves of spores suspended in a liquid solution below 90°C.

EXPERIMENTAL PROCEDURES

Water, ethanol, isopropanol and acetone

(Tests GS0096A through D, 098A and B, 180A through E, 225A and B and 279A)

Procedures for determining the D-values of spores in water and buffered distilled water were described in Progress Report 4. The same protocol was used in performing the experiments using ethanol, isopropanol and acetone.

D-values have been determined for spores of Bacillus subtilis var. niger (AAAA) suspended in the following liquids and at the following temperatures:

1. Distilled water and buffered distilled water (Butterfield's Buffer)
at 45, 60, 75 and 90°C.

2. Buffered distilled water (Sorenson's Buffer, pH 7.0) at 90°C.
3. 35, 70 and 95% ethanol at 45 and 60°C.
4. 35, 70 and 99% isopropanol at 45°C and 99% isopropanol at 60°C
5. Acetone at 45°C.

RESULTS AND DISCUSSION

Tests GS0096A through D, GS0098A and B, GS0180A through E, GS0225A and B and GS0279A

We evaluated the survival of spores of Bacillus subtilis var. niger (AAAA) in several liquids including distilled water, buffered distilled water, acetone and 95% ethanol, all of which are commonly used as liquids for holding spores. The results in all of the experiments performed in this series are summarized in Table 1.1. Evaluations of 35 and 70% ethanol and isopropanol, and 99% isopropanol were also made. All of the aforementioned liquids were maintained at 45°C.

TABLE 1.1
Summary of Results

Test No.	Temp. °C	Liquid	D-value (hrs.)	95% C.I.	IR ^c
GS0132A	45	DW ^a	1618	1320-2080	0.967
GS0132B	45	BDW ^a	1526	1310-1820	1.043
GS0132C	45	95% eth.	1525	1340-1770	1.003
GS0132D	45	99% iso.	902	770-1080	1.049
GS0180E	45	acetone	1139	1080-1210	0.965
GS0180A	45	70% eth.	167	160-180	0.822
GS0180C	45	70% iso.	102	87-120	0.870
GS0180B	45	35% eth.	105	97-110	0.980
GS0180D	45	35% iso.	85.9	75-100	0.954
GS0096A	60	DW ^a	183	160-210	0.953
GS0096B	60	BDW ^a	166	140-210	0.985
GS0134A	60	95% eth.	133	86-290	1.040
GS0134B	60	99% iso.	110	75-210	1.037
GS0225A	60	70% eth.	6.4	6-7	0.806
GS0225B	60	35% eth.	10.1	9-11	0.953
GS0096C	75	DW ^a	6.2	5-8	0.897
GS0096D	75	BDW ^a	7.8	6-10	0.931
GS0098A	90	DW ^a	0.43	0.40-0.45	0.859
GS0098B	90	BDW ^a	0.51	0.49-0.54	0.867
GS0279A	90	BDW ^b	0.73	0.69-0.77	0.915

^aButterfield's Buffer

^bSorenson's Buffer, pH 7.0

$$^c IR = \frac{\log Y_0}{\log N_0}$$

Figure 1.1 illustrates the near identity of results with distilled and buffered distilled water. In Table 1.2 the results from these water experiments are compared with the results from previous water experiments using spores AADA. (The survivor curves for the AADA data were shown in Figures 1.8, 1.9 and 1.10 of Progress Report #4.)

TABLE 1.2
Comparison of Results for Spores AAAA and AADA

Test No.	Spores	Temp. °C	Liquid	D-value (hrs)	95% C.I.	IR
GS0096A	AAAA	60	DW	183	160-210	0.953
GS0075A	AADA	60	DW	138	81-490	0.974
GS0096B	AAAA	60	BDW ^a	166	140-210	0.985
GS0075B	AADA	60	BDW	140	120-170	0.873
GS0096C	AAAA	75	DW	6.2	5-8	0.897
GS0075C	AADA	75	DW	7.2	6-9	0.927
GS0096D	AAAA	75	BDW	7.8	6-10	0.931
GS0075D	AADA	75	BDW	7.3	6-9	0.937
GS0098A	AAAA	90	DW	0.43	0.40-0.45	0.859
GS0077A	AADA	90	DW	0.43	0.39-0.48	0.869
GS0098B	AAAA	90	BDW	0.51	0.49-0.54	0.867
GS0077B	AADA	90	BDW	0.50	0.46-0.55	0.873

^aButterfield's Buffer

The data in Table 1.3 suggest that at 45°C there is little, if any, difference in survival of the organisms between the suspensions in distilled water, buffered distilled water, and 95% ethanol. While it appears that the difference in survival between these three suspensions and the acetone suspensions is more than can be attributed to chance (Table 1.1), it may be questioned whether such a reduction in D-value is sufficiently large to be of biological significance (viz., clearly less than a quarter of a log). At the same time, the 99% isopropanol showed almost a quarter of a log reduction in D-value, and this is more than a chance reduction. Figure 1.2 illustrates the survivor curves for the acetone and the two high-concentration alcohol solutions.

The D-values obtained with both the 35% ethanol and the 35% isopropanol solutions as well as the 70% ethanol and the 70% isopropanol solutions were not only statistically, but also biologically, significant reductions of the order of a full log. Figure 1.3 shows these results for ethanol, and the isopropanol results are shown in Figure 1.4.

A similar set of results is found at 60°C. Distilled water, buffered distilled water, and 95% ethanol were compared with 99% isopropanol and with 35 and

70% ethanol. While the best estimate of D-value for 95% ethanol appears to be lower than the values for distilled water and buffered distilled water, this reduction is probably a chance phenomenon since the confidence intervals for the other two are totally contained within the corresponding interval for the 95% ethanol solution. Similarly, the reduction in D-value for the 99% isopropanol is likely to be more apparent than real, since the confidence interval of the 99% isopropanol D-value contains all the other "point-estimates" of D-value for the other solutions. Figure 1.5 shows the survivor curves for the high concentrations of alcohol. When we examine the results for the 35 and 70% ethanol, the D-values are both statistically and biologically significant with the D-value for the 35 and 70% ethanol being approximately 1/10 of the D-value for the distilled and buffered distilled water. Figure 1.6 illustrates the results for these solutions.

The previously described results are summarized in Table 1.3. This makes it clear that the estimated D-values decrease to 1/10 or less in 70% and 35% alcohol over the D-values found in the same materials at higher concentrations.

TABLE 1.3

Summary of the D-values for Bacillus subtilis var. niger in Water Ethanol and Isopropanol as Functions of Heating Temperatures

Liquid	D-value in hours at			
	45°C	60°C	75°C	90°C
DW	1618	183	6.2	0.43
BDW	1526	166	7.8	0.51
95% ethanol	1525	133	---	--
99% isoprop.	902	110	---	--
70% ethanol	167	6.4	---	--
70% isoprop.	102	---	---	--
35% ethanol	105	10.1	---	--
35% isoprop.	85.9	---	---	--

At 75°C, the D-value in buffered distilled water was larger than that in the distilled water, but the difference was not statistically significant. At 90°C, the apparent increase of D-value in the buffered distilled water over distilled water (as at 75°C) is more than can be attributed to chance. Sorensen's Buffer (pH 7.0) exhibited more than a chance protective effect on the spores. The phosphate content of Sorensen's Buffer is M/15 and that of Butterfield's Buffer is .03125M. Since the concentration of the phosphate radical is the only difference between the buffers, it would appear that the increased concentration of the phosphate radical had a protective effect on the spores.

Figure 1.1 - Thermal resistance curves for Bacillus subtilis var. niger spores (AAAA) in distilled and buffered distilled water

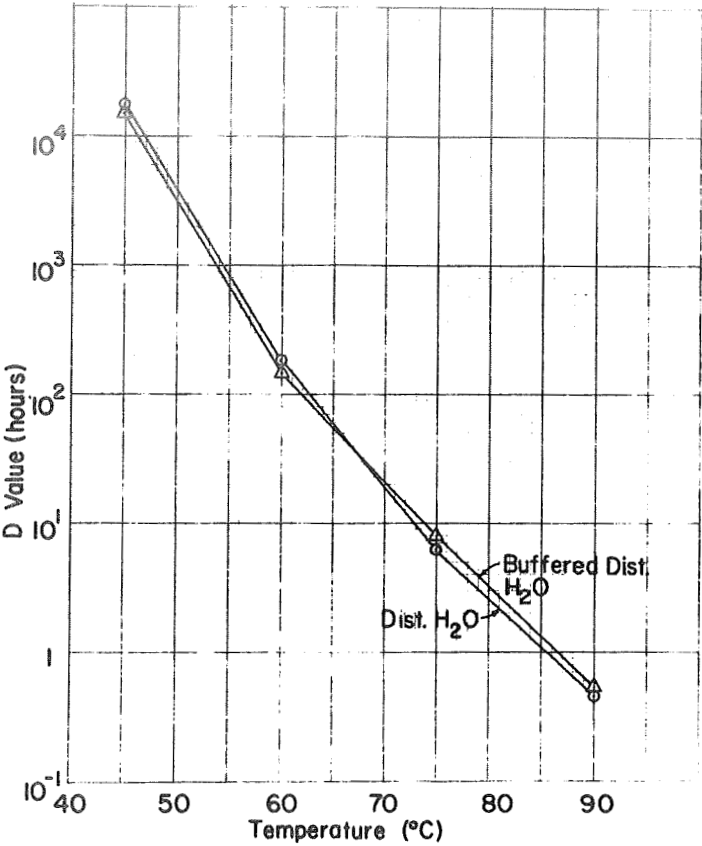
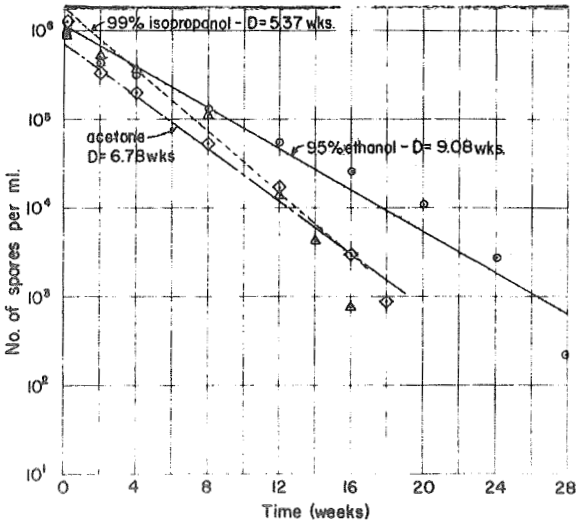


Figure 1.2 - Survivor curves for Bacillus subtilis var. niger spores (AAAA) in 95% ethanol, 99% isopropanol, and acetone at 45°C



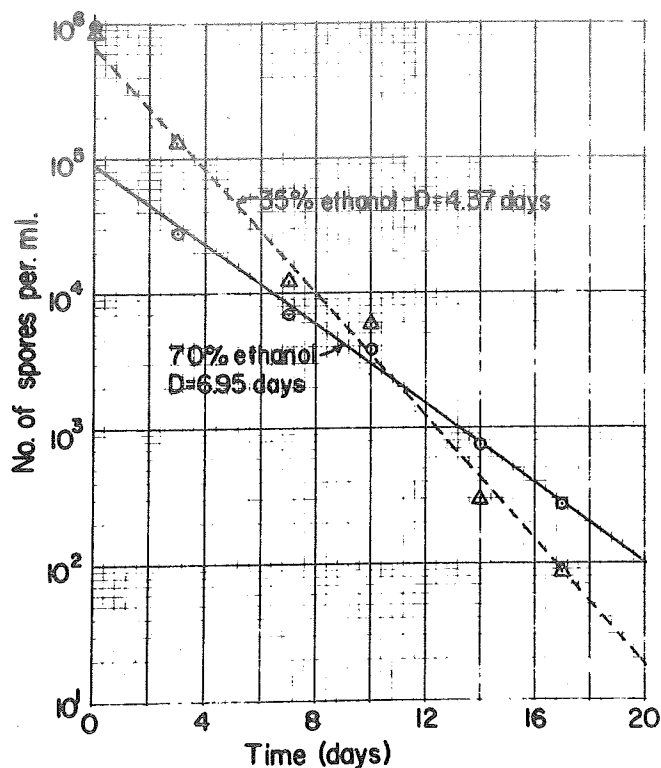


Figure 1.3-Survivor curves for *Bacillus subtilis* var. *niger* spores (AAAA) in 35% and 70% ethanol at 45°C

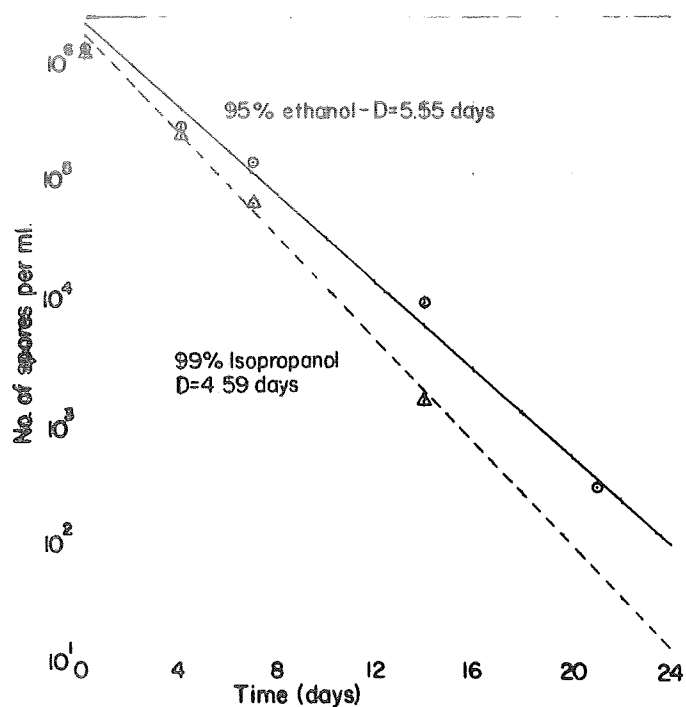


Figure 1.5-Survivor curves for *Bacillus subtilis* var. *niger* spores (AAAA) in 95% ethanol and 99% isopropanol at 60°C

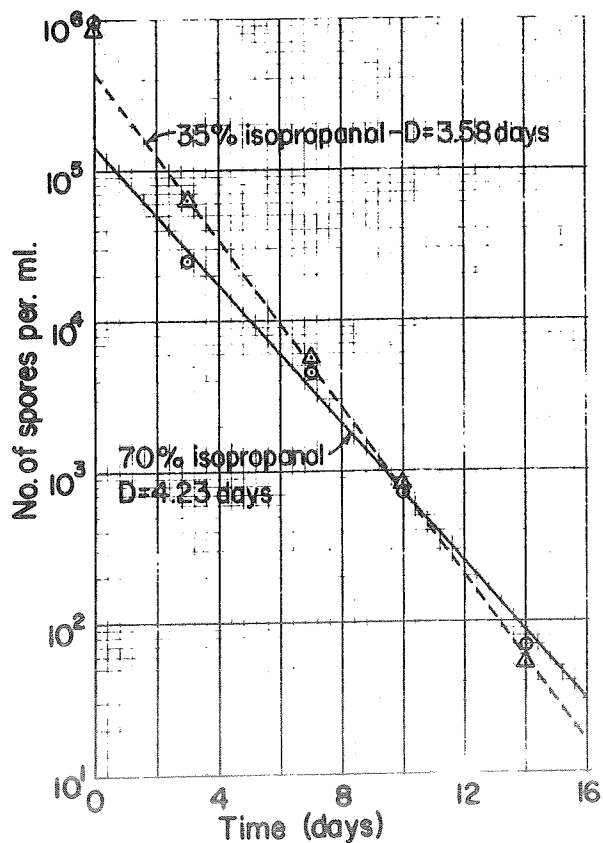


Figure 1.4-Survivor curves for *Bacillus subtilis* var. *niger* (AAAA) in 35% and 70% isopropanol at 45°C

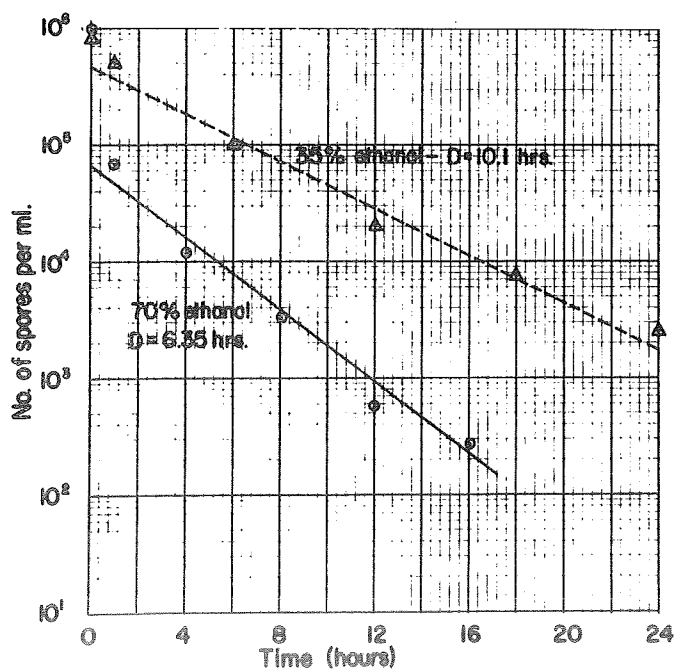


Figure 1.6-Survivor curves for *Bacillus subtilis* var. *niger* spores (AAAA) in 35% and 70% ethanol at 60°C

All of these tests were carried out in blood culture bottles made of soda lime glass; successive samples were removed from each bottle. The variability of the plate counts increased with time particularly at the low temperatures of 45 and 60°C where the length of heating time was in weeks and days respectively. The pH was determined in several bottles of both buffered distilled water (Butterfield's Buffer) and distilled water, all having been stored at 4°C for at least six weeks. The bottles and their contents were then heated for three hours at 90°C and the pH was recorded a second time. The results are given in Table 1.4.

TABLE 1.4
pH of Fluids in Blood Culture Bottles after Storage

Fluid	Bottle No.	pH after 6 wks. at 4°C	pH after 3 hrs. at 90°C
Buffered distilled water	1	6.85	7.3
	2	7.0	7.95
	3	7.0	8.0
	4	7.0	7.25
	5	6.9	7.60
Distilled water	1	7.25	7.85
	2	6.95	8.00
	3	6.95	8.15
	4	6.85	7.85
	5	6.85	8.20

Storage in these bottles at 4°C does not seem to have an appreciable effect. However, after three hours at 90°C, alkali metal ions are leached out of the glass and the pH increases. Heating at 45 or 60°C for daily or weekly periods may raise the pH even more, and in a more variable manner. This factor may account for the increase in the variability of results over long periods of time at temperatures of 45 and 60°C.

Alcohols have been reported to be inhibitors of spore germination. Removal of the alcohol by dilution reverses the inhibition.¹ This phenomenon is probably not responsible for the sporicidal effect of the 70 and 35% alcohols as they would have been effectively removed by the dilution procedures used during plating.

¹Trujillo, R. and N. Laible. "Reversible Inhibition of Spore Germination by Alcohols." Applied Microbiology, 20, 620-623, October, 1970.

CONCLUSIONS

1. There was little difference in the survival of Bacillus subtilis spores in either distilled or buffered distilled water or 95% ethanol at 45°C. The time for a one-log reduction was in the range of 1500-1600 hours (about nine weeks). If we extrapolate these data to 30°C the storage time for a one-log reduction will be about 900 weeks. In other words, our spores are quite stable in both water and 95% ethanol.
2. The spores were relatively stable in acetone but not as stable as in water or 95% ethanol.
3. A reduction in alcohol concentration from 95 or 99% to 70% increased the sporicidal effect by a factor of 10; the same effect is achieved by increasing the temperature 15°C. A further reduction in alcohol concentration from 70 to 35% further increased the sporicidal effect.
4. Similar results generally hold for Bacillus subtilis spores stored at 60°C. However, the reduction of the ethanol concentration from 70 to 35% does not increase the sporicidal effect; on the contrary, the sporicidal action is diminished.

FUTURE WORK

In the future we plan to: 1) complete the destruction rate of spores studies which are now underway at 45, 60, 75 and 90°C and 15, 35, 55 and 75% relative humidity in the experimental chamber using Bacillus subtilis var. niger spores, 2) complete the destruction rate of Bacillus subtilis var. niger spores studies now underway in glycerol and sucrose at .85, .90 and .99 water activity and 3) evaluate additional species of microorganisms in regard to their survival at different temperature and relative humidity conditions.

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

Project Personnel: V. Goppers and I. J. Pflug
Division of Environmental Health

INTRODUCTION

Studies during the past several years have been directed toward developing a chemical method that can be used for quickly enumerating surface microbial contamination. Our program has been based on an analysis of ATP (adenosine triphosphate). We chose to follow an ATP analysis utilizing thin-layer chromatography plus a sensitive photometer system for measuring the fluorescence of the compound on the thin-layer plate.

SUMMARY OF ACTIVITIES

The quantitative reproducibility of the analysis system requires a reproducible chromatogram. We have in general been unable to reproduce quantitatively the fluorescence level of the chromatogram on the chromatographic plate. One of the interfering conditions has been the humidity in the environment during the development of the chromatogram and the measurement of its fluorescence.

Our activity during this reporting period has been directed toward either eliminating or ameliorating the effect of varying humidity on the development of the chromatogram. To date we have not totally solved this problem, however, the development of the chromatographic plate in a controlled humidity glove box appears to have promise for a solution to this problem.

During the next six months, which will be the last six months of this project, we plan to continue our efforts to obtain a reproducible chromatogram and will be preparing the final report.

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DRY HEAT DESTRUCTION RATES OF BACILLUS SUBTILIS VAR. NIGER
IN A CLOSED SYSTEM: A. THERMAL DESTRUCTION STUDIES

Project Personnel: B. Moore, I. Pflug, R. Gove and Y. Thun
Division of Environmental Health

INTRODUCTION

This is a report of the continuation of efforts toward attaining the project objectives outlined in Progress Report #4, June, 1970. Experiments were carried out using the same basic cylindrical heat block system described in Progress Report #3, December 1969.

OBJECTIVE

In review, the objective of this project is to determine the 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 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 adsorption and water vapor transfer characteristics of plastic materials on the D-values of spores encapsulated in plastic and spores on metal strips pressed against a plastic surface.

Results obtained from studies on the effect of atmospheric volume on the D-value of spores with different water contents are presented in this report.

EXPERIMENTAL PROCEDURE

The general test procedure outlined in Progress Report #4 with the improved cleaning procedure described before was used in all experiments. Certain other modifications in the test procedure were made; they are described along with the description of specific experiments.

Block cleaning procedure

Thorough cleaning of the blocks, gaskets and discs was found to be helpful in reducing variability. A rigorous cleaning treatment was developed for the block units.

After several experiments it was noted that the finish on the block units was becoming dull and tarnished even after thorough cleaning with a non-ionic detergent, and rinsing in tap water, distilled water, alcohol, and ether. It was thought that residues on the block units might adsorb varying quantities of

water and contribute to both block-to-block and day-to-day variation. An experiment was designed to explore this idea. One half of the block units were cleaned as usual and the others were cleaned by a more rigorous method using an acid detergent designed for stainless steel. The spores heated in the blocks cleaned by the new method showed lower D-values with less variability. Incidentally, these blocks also appeared visibly cleaner than the blocks cleaned by the old method. From this evidence it was decided to employ the more severe cleaning procedure for the blocks as a standard procedure, yet one which is consistent with the procedures in Appendix C of NASA Standard Procedures for the Microbiological Examination of Space Hardware (August, 1967).

The cleaning procedure now used for the stainless steel blocks and any other reusable stainless steel in the system is outlined below.

Steps in preparing stainless steel blocks

1. cleaned in 1,1,1 - trichloroethane
2. washed in a non-ionic detergent in hot water
3. rinsed with tap water
4. immersed in an acid detergent¹ for stainless steel for at least 2 hours
5. washed with the non-ionic detergent in hot water
6. rinsed with tap water
7. rinsed with distilled water
8. rinsed in 99% isopropyl alcohol
9. rinsed in anhydrous ether
10. heated in dry heat oven for 2 hours at 125°C

Choice of gasket

The most important element in establishing system reproducibility was the use of Teflon gaskets for sealing the block units. The search for a satisfactory seal (i.e., vapor tight and relatively inert to water and gas) has been proceeding for some time. Sealing systems that have been tried to date are: Teflon and Viton o-rings and Teflon, lead and aluminum flat gaskets. Though all of these sealing systems have not been investigated exhaustively, the flat Teflon gasket (O.D.-2.75", I.D.-1.28", thickness-0.032") showed the least block-to-block variation and therefore the narrowest D-values confidence limits.

The Teflon gaskets now used are cleaned with a non-ionic detergent in hot water, rinsed with tap water, rinsed with distilled water and immediately heated

¹ Lime-a-way, a product of the Economics Laboratory, Inc., St. Paul, Minn.

in a dry heat oven for two hours at 125°C in the same way as the other parts of the block system.

RESULTS

Comparative D-values for spores at 3 atmospheric volumes conditioned at 35%RH at 22°C

D-values were determined for spores at three different atmospheric volumes (0.693 cm³, 5.211 cm³ and 9.729 cm³) conditioned for 18 hours in a controlled temperature (22°C ±1°C) and humidity (35% RH ±1%) environment using plastic isolators. The atmospheres in the plastic isolators were adjusted to a constant relative humidity with silica gel and water by means of the method described in Appendix A of Progress Report #3.

Only one block volume was evaluated on a single day. Data for the block volumes are listed in Table 3A.1.

TABLE 3A.1
Block Characteristics

Block Top Description	Inside Surface Area (cm ²)	Inside Atmospheric Volume (cm ³)	Volume/area(cm)
flat	50.103	0.693	0.014
.25" recess	54.936	5.211	0.095
.50" recess	60.936	9.729	0.160

In Table 3A.2 the mean D-values, 95% confidence intervals and the intercept ratio are given along with the conditioning relative humidity for the individual tests.

The results suggest that the D-value is inversely related to some function of atmospheric volume. They also seem to show an inverse relation to some function of inside surface area. So, too, they seem to be inversely related to some function of the volume-to-area ratio. However, it is interesting that the volume-to-area ratio for the flat, 1/4" and 1/2" units are approximately .01, .09 and 0.16 and that the square root of these values are 0.1, 0.3 and 0.4. These values correspond to average D-values of 39, 32 and 28 as shown in the summary table, Table 3A.3. It is interesting to conjecture that the approximate relationship between ΔD and $\Delta\sqrt{\frac{V}{A}}$ is: $\Delta D = -40\Delta\sqrt{\frac{V}{A}}$.

TABLE 3A.2
Individual Test Data

Test No.	Block Top Descrip.	Conditioning RH % ^a	D ₁₂₅ ^b (mins.)	95% C.I.	IR ^c
BM0259A	flat	34	42.8	37-51	1.01
BM0267A	"	34	37.2	34-41	1.05
BM0286A	"	34	35.4	32-40	1.05
BM0295A	"	36	39.9	35-46	1.03
BM0274A	.25" rec.	35	33.8	28-43	1.04
BM0279A	"	35	31.8	27-40	1.10
BM0288A	"	34	26.2	33-43	1.05
BM0300A	"	34	37.0	33-43	1.04
BM0281A	.50" rec.	35	30.0	27-34	1.02
BM0293A	"	35	24.5	22-28	1.06
BM0302A	"	36	29.3	25-35	1.05

^aHoneywell W611A RH indicator

^bInitial populations (N₀): 10⁶ spores; D determined from least-squares fitted straight line

^cIR = $\frac{\log y_0}{\log N_0}$, y₀ = the intercept of the least-squares line

TABLE 3A.3
Summary of D-value Data

Flat (12 blocks/test)	.25" recess (8 blocks/test)	.50" recess (8 blocks/test)
35	26	24
37	32	30
40	34	29
Mean <u>43</u>	<u>37</u>	—
D ₁₂₅ (min.) 39	32	28

The total quantity of water in the closed system is additively made up of four components:

1. The quantity of water in the gas, this quantity being directly proportional to the atmospheric volume;
2. The quantity of water adsorbed on the surface, for constant environmental conditions is directly proportional to the surface area;
3. The quantity of water in any foreign material or dirt particles, this quantity being directly proportional either to the atmospheric volume or the surface area or both, depending on whether the foreign material is in the air or on the surface or both; and

4. The quantity of water in the spores.

The relative magnitude of each of these water components is directly related to the vapor pressure, or, equivalently, to the equilibrium relative humidity of the environment in which the parts of the system are equilibrated.

Since the test procedure was carried out in the clean room and since all elements of the system were carefully cleaned, it is not unreasonable to assume that the third component has been reduced to a point where it can be neglected.

The quantity of water in the atmosphere can be calculated leaving as the missing link the quantity of water adsorbed by the stainless steel and absorbed and/or adsorbed by the spores. These missing links are being pursued under part B of this project. Resolution of this problem must await the discovery of these missing links.

Effect of plastics on spore D-values

Preliminary work has been done comparing D-values of spores with methylmethacrylate² and with Teflon in this closed system. A methylmethacrylate disc (O.D.-2.75", thickness-0.063") was placed either directly on top of the inoculated spore disc or on top of a sterile stainless steel disc covering the inoculated disc. The flat-top block unit and bottom unit were bolted together and sealed with a Viton o-ring. The methylmethacrylate discs were conditioned 18 hours in atmospheres with different relative humidities just prior to bolting the blocks together. The o-rings, blocks and spores were generally conditioned at around 48% relative humidity at 22°C in a clean room atmosphere for 18 hours. Teflon discs (O.D.-2.75", thickness-0.063") were used in the same way the methylmethacrylate discs were used. Results so far show the following relationships:

1. Longer D-values were found for spores separated from the methylmethacrylate sterile cover disc than for those spores in direct contact with the methylmethacrylate discs.

2. Spores generally showed slightly longer D-values when the methylmethacrylate was conditioned at <1% and 15% RH at 22°C for 18 hours than the methylmethacrylate was conditioned at 30%, 48% and 100% RH at 22°C for 18 hours.

3. Spores in the Teflon system always had a D-value several times larger than the spores in the methylmethacrylate system.

4. The D-value for spores in the simple stainless steel system with the Viton o-rings was lower than the D-value for the spores in the Teflon system but much higher than the D-value for the spores in the methylmethacrylate system.

²In these studies, an acrylic resin, Plexiglass, manufactured by the Rohm and Haas Company, was used.

It should be noted that high variability occurred in the methylnmethacrylate data even between duplicates within the same block. On the other hand, the Teflon data showed very low variability within and among blocks. More work is planned to examine the effects of plastics on thermal resistance of spores, including an investigation to determine the origin of the variability of D-values using discs of methylnmethacrylate in the light of the much lower variability using Teflon discs of the same dimensions.

CONCLUSIONS

1. Residual foreign material or oxide coating on blocks affects results. Careful attention to insure that the stainless steel blocks are thoroughly cleaned reduces variability in D-value determinations.
2. A flat Teflon gasket is the most satisfactory seal found.
3. In the experiments concerning spore survival at different atmospheric volumes, it appears that D-value decreases as the atmospheric volume and necessarily the surface area increase. We believe that this relationship is a function of the water content relationships within the system.
4. It appears, however, that methylnmethacrylate directly or indirectly has some yet unexplained lethal effect on heated spores. However, not enough data have been acquired to make any firm conclusions concerning the effect of plastic on the D-value of spores.

FUTURE WORK

The next phase of this program will be to explore the effect of several levels of relative humidity in the environment in which the spores are conditioned. We plan to do this study at several environmental temperatures.

We also plan to continue our efforts to evaluate the quantity of water in the spores and on the stainless steel surface.

DRY HEAT DESTRUCTION RATES OF BACILLUS SUBTILIS VAR. NIGER
IN A CLOSED SYSTEM: B. MEASUREMENT OF WATER

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INTRODUCTION

We believe that the dry heat resistance of microbial spores is a function of the quantity of water in the spores during heating. In order to control the quantity of water, studies of the dry heat resistance of spores are usually carried out under controlled vapor pressure or relative humidity conditions. We believe that we must relate the quantity of water in the spores to the vapor pressure or relative humidity of the atmosphere surrounding them.

OBJECTIVE

The objective of this study is to determine the quantity of water in spores and adsorbed by surfaces in systems where dry heat resistance is being measured.

The study described below was initiated on the premise that very small quantities of water in an organic solvent can be determined by using the infrared spectrophotometer. We speculated that if it is possible to extract the water from our samples with an organic solvent and if small enough quantities of water can be measured quantitatively, then we will have a practical method for measuring the water in our system.

EXPERIMENTAL PROCEDURE

This project was carried out in the following steps:

1. investigation of solvents
2. adjustment of the instrument for the specific purpose
3. developing chemical procedures for preparation of the testing material for the infrared spectrophotometer
4. scanning the prepared material in the infrared spectrophotometer
5. evaluation of the recorded spectrum
6. evaluation of interfering substances and conditions

The instrument used in this study was a Beckman Infrared Spectrophotometer (model-IR 12) with an operating range of $250-4000\text{ cm}^{-1}$. An infrared spectrophotometer requires cells which are not attacked by water. For that reason,

cells with barium fluoride windows were used in this study because they are both transparent to infrared radiation and are not attacked by water. The cells have a capacity of 0.1 ml and are sealed micro-liquid cells assembled with a 0.1 mm spacer.

Impurities which are harmful must be separated out by special treatments according to needs. For example, presence of sulfate ions in the water or solvent will damage the barium fluoride (BaF_2) cells since barium sulfate is much less soluble in water than BaF_2 . Barium sulfate forms an opaque layer inside the BaF_2 cell window and therefore reduces the light transmission of the cells. In these experiments the acetone was treated with barium chloride to precipitate sulfate and other interfering ions. To protect the glass surface from sulfate ions during cleaning, hydrochloric acid was used as the cleaning agent.

To find the most suitable solvent, a group of organic nonpolar solvents was investigated for the following properties:

1. solubility of the water into the solvent
2. the infrared spectrum of the pure solvent
3. ability to extract water from biological material
4. influence of the solvent on the instrument

Considering organic solvents with regard to the requirements listed above, acetone (CH_3COCH_3) was chosen as the most suitable compound. Regarding solubility, water dissolves in acetone in all proportions. Acetone also is an inert, nonpolar solvent. Absorption bands of acetone in the infrared spectrum do not interfere with the specific water absorption band at 3650 cm^{-1} . Acetone itself does not damage the barium fluoride infrared cells. These attributes make acetone potentially a good solvent for extracting water from biological materials.

The following procedure was used for the measurement of water: Acetone Spectrograde (ACS-Eastman Kodak Co.) was treated with anhydrous crystalline barium chloride to precipitate the sulfate ions as barium sulfate. After 15 hours the clear supernatant was poured off from the possible sediments and used for the preparation of the test solutions. Water-acetone solutions were made up by adding different quantities of demineralized glass distilled water to acetone. These standard solutions were prepared in a glove box where the relative humidity was maintained below 2%. This step was necessary to protect the solutions from absorbing water from the surrounding atmosphere. The quantities of water added were measured with a Hamilton syringe, calibrated in $0.1\text{ }\mu\text{l}$. Solutions with water concentrations ($\% \text{H}_2\text{O}$) of 0.1, 0.075, 0.05, 0.04, 0.03, 0.02, and 0.01 were prepared and used to evaluate this method. The prepared standard solutions were filled into the cell with the aid of a Tuberculin syringe. This operation was also

carried out in the low relative humidity glove box. The solutions were used to determine the capability of the instrument to measure water.

In order to increase the sensitivity the Variable Scale expansion attachment was used. This unit permits the adjustment of the scale to read 100% transmittance when two BaF_2 cells, both filled with pure acetone, were placed one in the reference beam and the other in the sample beam. To measure the quantity of water in acetone the cell in the sample beam was emptied and refilled with one of the water-acetone standard solutions.

RESULTS

We were able to determine quantities of water as small as 0.01% in acetone with an error of measurement of $\pm 1\%$. This level of measurement of one part water in 10,000 would be useful if we had a large size sample; however, there is still some distance to go to measure water level changes of 0.1% in 10^5 to 10^6 spores having a dry weight of 10^{-6} or 10^{-7} g. (To weigh to one part in 100 in a sample of 10^{-6} g. requires measurements of the order of one part in 10^8).

The recovery according to percent transmission was $\pm 0.2\%$ to $\pm 1\%$ depending upon quantities of water measured. The transmission values measured on the infragram and plotted on the ordinate (log axis) of semilog paper versus the concentration on the abscissa produced nearly a straight line, following Beer's law. The slight deviation observed was expected since the infrared absorption intensities cannot be expressed in terms of a universal constant; further, the band width is of the same order of magnitude as the slit width, which causes the measured optical density to be influenced strongly by the slit width. This implies that the percentage of transmittance does not present the true intensity of a band. For this reason, the area of the band should be integrated and used in calculations representing band intensity.

Inaccuracy in measurement is due to the inability to determine precisely the I_0 (initial transmittance) and errors in the measurement of transmission imposed by the "noise level". Using a pair of calibrated cells, the first problem was reduced by the "differential spectrum" technique.

Instrument adjustments used to improve the precision of the measurements and thus make it possible to determine smaller quantities of water were the adjustment of the light window width, sample cell holders and other minor things.

CONCLUSIONS

The method developed will detect 1 part water in 10,000. This level of measurement is not adequate for our requirement; however, the method may be useful for rapidly determining the quantity of water in materials where the sample size is larger.

FUTURE WORK

The objective of this study, which was to measure the quantity of water in spores and adsorbed on surfaces, has not been reached; therefore another approach to the solution must be found. Next we plan to evaluate the du Pont "water analyzer" used by Dr. Campbell of Cincinnati in his spore-water studies and see if we can measure the low levels of water in our system.

DRY HEAT DESTRUCTION RATES OF MICROORGANISMS ON SURFACES

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INTRODUCTION

This is a report on the continuation of experiments previously described in Progress Reports 3 and 4 (December, 1969, and June, 1970). The equipment and methods for obtaining data on dry heat destruction rates on surfaces as a function of relative humidity (RH) were described in Progress Report 3. D-value data at 110° and 125°C for two spore suspensions, Bacillus subtilis var. niger (AAOE) and Bacillus firmus (VDLB), in a dry nitrogen or dry air environment (or both) compared with a clean room environment of ~35% RH at 22°C were presented in Progress Report 4.

During the current reporting period, three separate studies were carried out in conjunction with the general objectives of this project: A) D-value data were obtained for spore suspensions AAOE and VDLB at 90°C for the clean room environment (35% RH at 22°C) and at 100°C in dry air, dry nitrogen and the clean room environment; B) Studies which were carried out in the clean room to determine changes in $D_{125^{\circ}\text{C}}$ -values as a function of incremental changes in the environmental temperature and vapor pressure have been completed; and C) A one-day study to evaluate the change in D-value for small changes in temperature has also been completed.

OBJECTIVES

The specific objectives of these studies were as follows:

A. To develop surface D-values for two spore suspensions (AAOE and VDLB) at 100°C as a function of relative humidity (<1% vs. ~35% at 22°C) and surrounding gas (air or nitrogen) and at 90°C for the ambient clean room environment of ~35% RH at 22°C.

B. To determine the effect that a range of temperatures and vapor pressures in the clean room environment has on $D_{125^{\circ}\text{C}}$ -values, using AAOE as the test spore suspension.

C. To determine the D-value of AAOE spores at 123° and 127°C simultaneously by using two hot plates.

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A. D-VALUE STUDIES AT 90 AND 100°C

INTRODJCTION

The experiments at 90 and 100°C complete the four-temperature study. We have also included the summary data for 110 and 125°C so that these data are available to support our analysis covering all four temperatures.

EXPERIMENTAL PROCEDURE

In the experiments carried out at 100°C we used the same general procedure as we used in experiments at 110°C and 125°C. This procedure was described in the previous report.

Four sets of experiments (MH0166, 0173, 0187 and 0194) were carried out using a heating temperature of 100°C. Each experiment consisted of four tests (A, B, C, D).that were carried out simultaneously. The AAOE spores were used in tests A and B and the VDLB spores were used in tests C and D. Tests A and C were carried out in the clean room environment with tests B and D in a dry gas environment(MH0166 and 0173, dry air; MH0187 and 0194, dry nitrogen). All spores were conditioned in the clean room.

Two experiments(MH0201 and 0208) were carried out at 90°C in the clean room environment. Each experiment consisted of two tests(A and B). The AAOE spores were used in tests A and VDLB spores were used in tests B. All spores were conditioned in the clean room.

In all experiments, deposits of 10^6 spores were made on stainless steel planchets which were then conditioned in the clean room for about 24 hours. Copper boats (each containing three planchets) were randomly allocated either to the dry gas or the ambient atmosphere. Two boats of each of the two spore suspensions were randomly selected for removal from each atmosphere at each heating time. Heating times of 1.25, 2.50, 3.75 and 5.0 hours were used for the AAOE spores in the clean room. Heating times were 2.5, 5.0, 7.5 and 10.0 hours for VDLB spores in the dry gas glove box, and 6.0, 10.0, 23.0 and 30.0 hours for those spores in the clean room. The heating times were based on expected $D_{100^\circ\text{C}}$ -values calculated using the 110° and 125°C data.

Heating times of 24, 48, and 72 hours for both suspensions were utilized for the 90°C clean room experiments.

Data for the 90 and 100°C experiments using Bacillus subtilis var. niger together with previously reported 110 and 125°C data are reported in Table 4A.1. and in Table 4A.2 for Bacillus firmus (VDLB).

TABLE 4A.1

Summary of D-value Experiments - *Bacillus subtilis* var. *niger* (AAOE)

Temp. °C	Ambient gas during heating	Test No.	D-value, min.		N _o	y _o	IR**
			Mean	95% CI			
90	air ~35% RH ~22°C (1)	MH0201A	1219	1000-1560	1.25E6	5.09E6	1.10
		MH0208A	1190	920-1680	1.68E6	5.86E6	1.09
100	air ~35% RH ~22°C (1)	MH0166A	429	380-480	1.48E6	2.65E6	1.04
		MH0183A	365	320-440	1.62E6	3.96E6	1.06
		MH0187A	432	410-460	1.60E6	2.47E6	1.03
		MH0194A	411	340-520	1.67E6	3.13E6	1.04
	air <1% RH ~22°C (1)	MH0166B	98.6	58-340	1.62E6	4.63E5	0.91
		MH0173B	109	100-130	1.44E6	5.38E5	0.93
110	air ~35% RH ~22°C (1)	MH0187B	133	100-190	1.60E6	1.90E5	0.85
		MH0194B	162	140-190	1.70E6	3.55E5	0.89
		MH9308D	163	150-170*	2.16E6	1.52E6	0.98
		MH9322D	167	160-180*	2.52E6	1.31E6	0.96
		MH9328E	140	120-160*	1.96E6	1.27E6	0.97
		MH0069B	161	140-200*	2.03E6	7.63E5	0.93
	air <1% RH ~22°C (1)	MH0069B	158	123-230	2.47E6	1.21E6	0.95
		MH0076B	136	76-620	2.71E6	1.20E6	0.95
	air <1% RH ~22°C (1)	MH9308C	40.4	38-43*	2.03E6	6.25E5	0.92
		MH9322C	42.5	39-47*	2.23D6	6.31E5	0.91
	N ₂ <1% RH ~22°C (1)	MH9328C	43.3	39-48*	1.92E6	4.78E5	0.90
		MH9336C	37.7	35-42*	2.24E6	4.99E5	0.90
		MH0069A	68.5	58-84	2.10E6	4.02E5	0.89
		MH0076A	69.0	61-80	2.37E6	7.49E5	0.92
	air ~35% RH ~22°C (2)	MH9308B	141	130-160*	1.77E6	1.60E6	0.99
		MH9322B	142	130-150*	1.90E6	1.81E6	1.00
	air <1% RH ~22°C (2)	MH9308A	43.1	39-48*	1.72E6	2.82E5	0.87
		MH9322A	42.3	38-47*	1.92E6	6.87E5	0.93
	air ~35% RH ~22°C (3)	MH9328B	178	160-190*	1.99E6	5.59E5	0.91
		MH9336B	148	140-160*	1.85E6	1.45E6	0.98
	N ₂ <1% RH ~22°C (3)	MH9328A	38.6	37-41*	1.79E6	1.13E6	0.97
		MH9336A	49.1	46-53*	1.72E6	2.94E5	0.88
125	air ~35% RH ~22°C (1)	MH9344D	32.3	19-110	2.00E6	4.35E4	0.90
		MH0048D	18.8	15-26	2.03E6	6.23E5	0.92
		MH0055D	17.9	15-22	1.77E6	9.21E5	0.95
		MH0062D	16.6	14-20	2.01E6	9.55E5	0.95
	air <1% RH ~22°C (1)	MH0048C	6.9	5-10	1.88E6	8.50E5	0.95
		MH0062C	6.2	5-9	1.94E6	1.08E6	0.96
	N ₂ <1% RH ~22°C (1)	MH9344C	7.4	5-13	1.74E6	1.27E6	0.98
		MH0055C	8.5	7-13	1.97E6	7.53E5	0.93
	air ~35% RH ~22°C (2)	MH0048B	15.9	11-33	2.02E6	6.65E5	0.92
		MH0062B	20.7	15-32	1.64E6	2.76E5	0.88
	air <1% RH ~22°C (2)	MH0048A	9.2	4-∞	2.08E6	1.99E5	0.84
		MH0062A	6.7	5-10	2.14E6	6.16E5	0.91
	air ~35% RH ~22°C (3)	MH9344B	29.4	23-41	1.91E6	3.73E5	0.89
		MH0055B	16.4	14-21	1.93E6	1.02E6	0.96
	N ₂ <1% RH ~22°C (3)	MH9344A	6.5	5-11	2.19E6	2.20E6	1.00
		MH0055A	7.6	6-11	1.94E6	9.04E5	0.95

Conditioning environment:

- (1) Clean room
- (2) Dry air
- (3) Dry nitrogen

*Based on arithmetic mean

(others based on geometric)

$$** \frac{\log y_o}{\log N_o}$$

TABLE 4A.2

Summary of D-value Experiments - *Bacillus firmus* (VDLB)
(conditioned in the clean room)

Temp. °C	Ambient gas during heating	Test No.	D-value, min.		N ₀	y ₀	IR**
			Mean	95% CI*			
90	air ~35% RH ~22°C	MH0201B	1028	981-1080	7.33E5	3.88E6	1.12
		MH0208B	1375	1130-1760	6.91E5	3.57E6	1.12
100	air ~35% RH ~22°C	MH0166C	631	479-925	5.67E5	7.83E5	1.02
		MH0173C	461	406-534	6.39E5	2.01E6	1.09
		MH0187C	699	587-866	7.39E5	8.55E5	1.01
		MH0194C	447	406-497	6.45E5	1.83E6	1.08
	air <1% RH ~22°C	MH0166D	226	208-247	6.06E5	2.05E5	0.92
		MH0173D	248	199-327	6.23E5	1.95E5	0.91
110	air ~35% RH ~22°C	MH0187D	347	243-607	7.40E5	8.86E4	0.84
		MH0194D	214	171-289	5.71E5	1.26E5	0.89
	N ₂ <1% RH ~22°C	MH0069C	196	127-437	1.03E6	2.58E6	1.07
		MH0076C	198	141-332	8.38E5	1.22E6	1.03
125	air ~35% RH ~22°C	MH0069D	127	104-163	9.55E5	1.59E5	0.87
		MH0076D	103	89-122	9.71E5	9.61E4	0.83
	air ~35% RH ~22°C	MH0055E	43.6	36-54	1.09E6	5.10E5	0.95
		MH0062E	37.4	24-86	1.03E6	6.76E5	0.97
	air <1% RH, 22°C	MH0062F	22.0	21-24	8.59E5	2.38E5	0.91
		MH0055F	23.8	19-32	9.94E5	3.52E5	0.92

*Based on geometric mean

$$**IR = \frac{\log y_0}{\log N_0}$$

D-values and their means for all temperatures studied are presented in Tables 4A.3 and 4A.4.

TABLE 4A.3

Summary of AAOE Spore D-value Data
Used in z-value Analysis

Temp.	Heating Environment			
	Dry gas		Clean room	
125°C	6.9	7.4	18.8	15.9
	6.2	8.5	16.6	20.7
	9.2	6.5	32.3	29.4
	6.7	7.6	17.9	16.4
	mean =	7.5	mean =	21.0
110°C	40.4	43.1	163	136
	42.5	37.7	167	141
	43.3	68.5	140	142
	42.3	69.0	161	178
	38.6	49.1	158	148
100°C	mean =	47.5	mean =	153
	98.6	133	429	365
	109	162	432	411
90°C	mean =	126	mean =	409
			1219	1190
			mean =	1205

TABLE 4A.4
Summary of VDLB Spore D-value Data
Used in z-value Analysis

Temp.	Heating Environment	
	Dry gas	Clean room
125°C	23.8 22.0 mean = 22.9	43.6 37.4 mean = 40.5
110°C	127 103 mean = 115	196 198 mean = 197
100°C	226 248 347 214 mean = 259	631 699 461 447 mean = 560
90°C		1028 1375 mean = 1202

Typical survivor curves for the 90 and 100°C experiments are illustrated in Figures 4A.1, 4A.2, 4A.3, and 4A.4.

In this project a series of experiments has been carried out in which microbial spores deposited on plachets have been heated on hot plates located in a low water content environment (glove box) and a moderate water content environment (clean room). In these tests the temperature and water concentration (relative humidity) of these environments were maintained constant within certain limits. In Table 4A.5 are listed estimates of a single relative humidity value to be associated with the experiments carried out at the specific heating temperatures. Relative humidity values were calculated using the saturated water vapor pressure at the respective heating temperatures.

The estimated values for the clean room conditions are the means of all measured values. In the case of the dry gases the estimate in each case is the low level in our measurement range. The water content of the dry gas system was controlled by the water content of the gas used in the experiment. We monitored the water content of the gas used in the glove box chamber. However, the water content of the compressed nitrogen and air was a function of the manufacturing process and therefore was beyond our control. It was usual for the water content of the gas to be less than our minimum level of measurement. However, periodically there would be high water contents as indicated by the upper levels in the range of values given in Table 4A.5.

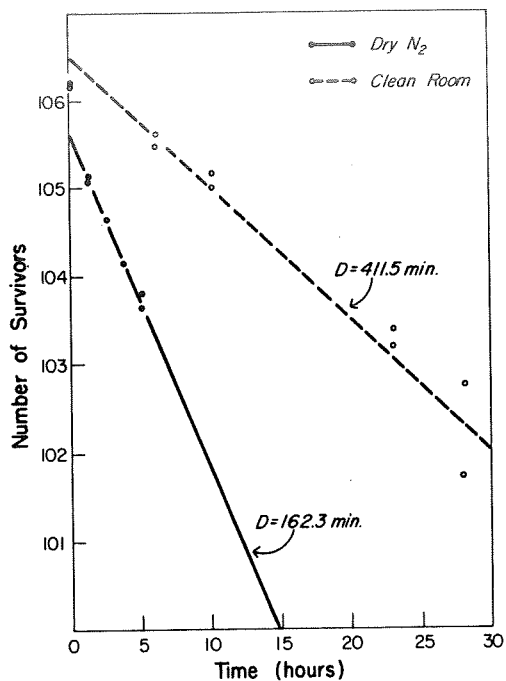
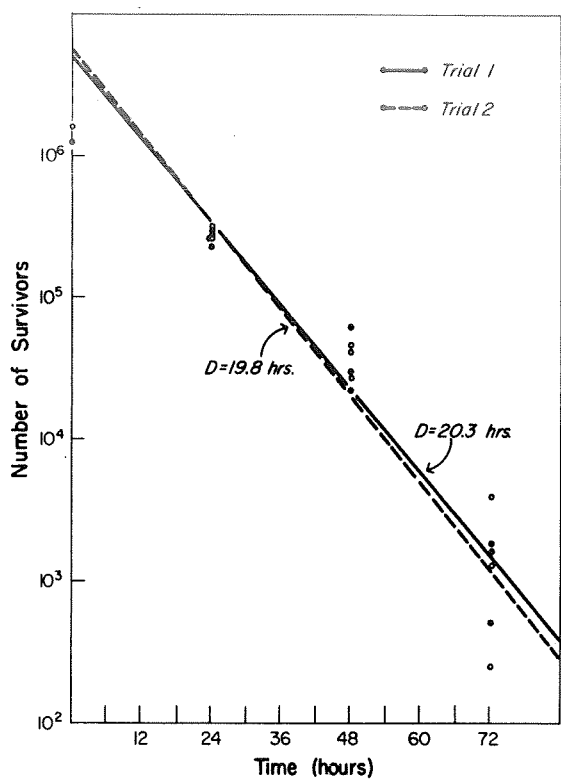


Figure 4A.1-Survivor curves for *Bacillus subtilis* var. *niger*(AAOE) at 90°C

Figure 4A.2-Survivor curves for *Bacillus subtilis* var. *niger*(AAOE) at 100°C

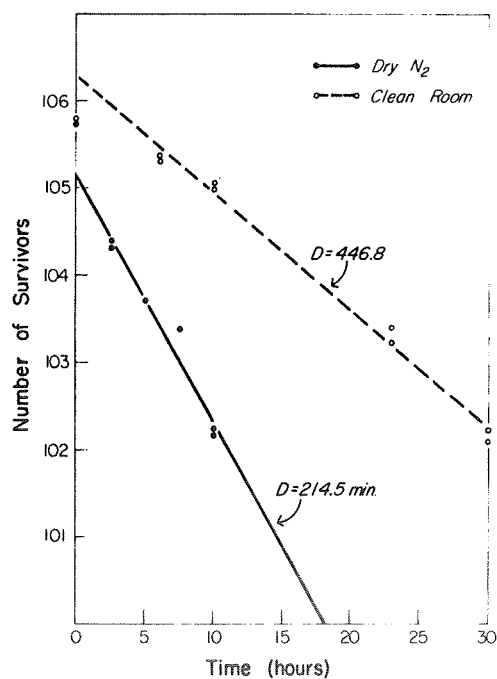
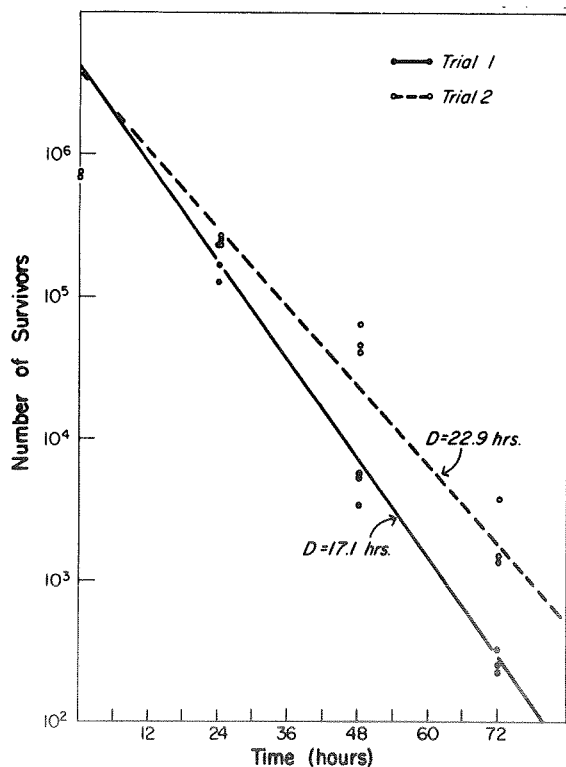


Figure 4A.3-Survivor curves for *Bacillus firmus* (VDLB) at 90°C

Figure 4A.4-Survivor curves for *Bacillus firmus* (VDLB) at 100°C

TABLE 4A.5

Estimate of a Single Relative Humidity Value and the
Range of Values Measured in Experiments at each Temperature

Heating temp. °C	Dry gas		Clean room	
	Estimate(%)	Range of measurement(%)	Estimate(%)	Range of measurement(%)
90	--	--	1.5	1.30 to 1.71
100	0.003	0.003 to 0.039	1.1	0.93 to 1.41
110	0.002	0.002 to 0.022	0.7	0.57 to 0.75
125	0.001	0.001 to 0.006	0.4	0.33 to 0.42

Since for the great majority of test times the water content was below the minimum level that our equipment could measure we believe that we are justified in using this value as our estimate.

RESULTS

The D-value data are summarized in Table 4A.6 and the intercept data in Table 4A.7. The salient results regarding the effect of relative humidity on D-value are as follows:

1. For tests at a specific temperature, the D-value is lower at the lower relative humidity.
2. As the relative humidity decreased from a range of 0.4 to 1.5% in the clean room to .001 to .003 in the dry gas atmosphere, the D-value decreased to 30 to 50% of the clean room value.
3. The effect of humidity on D-value was consistent at all temperatures studied.
4. The D-values of the Bacillus subtilis spores (AAOE) were affected to a greater extent than the D-values of the Bacillus firmus (VDLB).

We have introduced the intercept-ratio concept to clarify reporting and discussion of changes in the y-intercept of the survivor curve. When analyzing the effect of test conditions we believe that we should consider both the intercept and the slope(D-value) of the survivor curve rather than just the slope.

The mean intercept-ratios (IR) for all test conditions are shown in Table 4A.7.

TABLE 4A.6

Mean D-values (in minutes) for All Test Conditions

Spores	Environment	Heating Temperature °C			
		90	100	110	125
<u>B. subtilis</u> (AAOE)	Clean room	1205	409	153	21.0
	Dry gas	--	126	47.5	7.5
<u>B. firmus</u> (VDLB)	Clean room	1202	560	197	40.5
	Dry gas	--	259	115	22.9

TABLE 4A.7

Mean Intercept Ratios (IR=log y_0/N_0) for All Test Conditions

Spores	Environment	Heating Temperature °C			
		90	100	110	125
<u>Bacillus subtilis</u> (AAOE)	Clean room	1.10	1.04	0.96	0.92
	Dry gas	--	0.90	0.91	0.94
<u>Bacillus firmus</u> (VDLB)	Clean room	1.12	1.05	1.05	0.96
	Dry gas	--	0.89	0.85	0.92

Interestingly, there seems to be a consistent pattern of a decrease in the clean room environment intercept-ratios as we go from 90 to 125°C for both spores, and a hint of an increase in the IR for the dry gas environment. The clean room relative humidities and the intercept-ratios for the two spores evaluated at the four temperatures are listed in Table 4A.8.

TABLE 4A.8

Relative Humidity and Intercept Ratios for AAOE and VDLB for the Clean Room Tests at the Four Temperatures

Temperature of test °C	Clean room * relative humidity (%)	Intercept ratios (IR)	
		AAOE	VDLB
90	1.5	1.10	1.12
100	1.1	1.04	1.05
110	0.7	.96	1.05
125	0.4	.92	.96

*calculated at test temperatures

Here we see that the decrease in the intercept-ratio appears to parallel the decrease in the clean room relative humidity, but this is confounded completely with temperature. From time to time general observations have been reported in the literature to the effect that as the water content of the spores decreases the intercept-ratio also decreases. Another way of stating this is, that at high initial water contents, the initial phase of the survivor curve is concave downward, whereas with low water content the initial phase of the survivor curve is concave upward. The results of these experiments appear to substantiate these general observations.

At 125°C there is essentially no difference in the intercept-ratio between the clean room and dry gas conditions. There is also no clear-cut effect of humidity on the intercept-ratio for tests carried out in the dry gas atmosphere. The results suggest that changes in the intercept-ratio accompany changes in relative humidity down to 0.4%. We shall look forward to IR data for humidities above 1.5% to observe whether the intercept-ratio increases at the higher relative humidities.

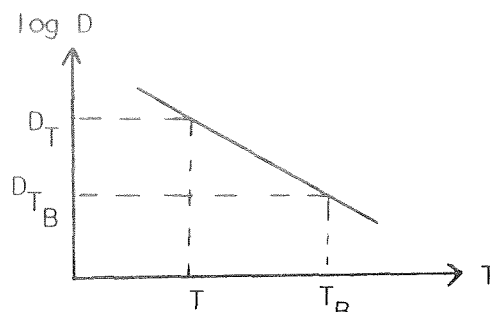
The dry heat destruction rate data that we have developed at several temperatures were evaluated with regard to temperature coefficients. The temperature coefficient is an important consideration in the design of a sterilization process.

The dry heat destruction rate of microorganisms is a function of temperature and time. This destruction rate is temperature-dependent; increasing the temperature decreases the D-value. In the sterilization of a spacecraft it will not be possible to heat the spacecraft instantly to an elevated temperature, hold the spacecraft at that temperature until sterilization is accomplished, and then instantly cool the spacecraft to ambient temperature. Both heating and cooling will take place slowly. To optimize the thermal process, the heat lethality will be integrated over the whole cycle--heating, holding at temperature, and cooling. This integration requires a model that includes the effect of both temperature and time.

The temperature coefficient model of Bigelow^a that has been widely used in the food and pharmaceutical industries is, in our opinion, the preferred method for treating the effect of temperature on D-value. If we let T_B equal the base temperature, the D-value at T_B is specified and is represented by D_{T_B} . Limiting ourselves to a sufficiently short interval on the temperature scale so that the change in log D-value is essentially linear with temperature, z measures the slope of the thermal resistance curve, now assumed to be linear. The temperature increment needed to change the D-value by a factor of 10 is represented by z . The accompanying diagram

^a Bigelow, W. D., 1921. The logarithmic nature of thermal death time curves. J. Infectious Diseases., 29, pp. 528-536.

illustrates the relationships.



If T is some new temperature for which we wish to determine the corresponding D -value, call it D_T , from the diagram and the definition of z (the slope of the line) it follows that

$$z = \frac{T_B - T}{\log D_T - \log D_{T_B}}, \text{ or } z(\log D_T - \log D_{T_B}) = T_B - T, \text{ and dividing}$$

by z : $\log D_T - \log D_{T_B} = z^{-1}(T_B - T)$, from which $\log D_T = z^{-1}(T_B - T) + \log D_{T_B}$.

This is the equation for Bigelow's model for determining D_T corresponding to any new temperature, T , as long as the thermal resistance curve remains linear.

In the actual dry heat sterilization process, the relative effectiveness of a time unit of the heat process at any temperature T in terms of a base temperature T_B can be calculated if the z -value is known. The z -value is related to $Q_{10^\circ\text{C}}$ in the equation $z^\circ\text{C} = 10/(\log Q_{10^\circ\text{C}})$ and Q_{10} is related to the reaction rate K_T in the equation $Q_{10^\circ\text{C}} = (K_T + 10^\circ\text{C})/K_T$.

The z -value of the Bigelow model can be related to the energy of activation in the equation $E^* = 2.303RT^2 z^{-1}$ where E^* is the activation energy, R is the gas constant and T is the absolute temperature.

An initial plot of all D -values versus temperature indicated that the z -value for our experiments over the temperature range 90 – 125°C did not appear to be constant. We next proceeded to calculate z -values using D -values for two adjacent temperatures. We calculated the z -values for both the mean D -values and for the extreme range of D -values shown in Tables 4A.3 and 4A.4. The resulting z -values are shown in Tables 4A.9, 4A.10, 4A.11 and 4A.12.

We have the most data for the AAOE spores heated in the clean room. In Table 4A.9 we have shown the data at four different temperatures plus data from a special experiment at 123°C and 127°C (described later in this report) which allowed us to accurately determine z over this narrow range centered at 125°C . In Table 4A.9 we observe that D -values calculated using data at 90 , 100 and 110°C give z -values of 21.3°C for 90°C and 100°C ; and a z -value of 23.4°C for 100°C and 110°C . For all spore-environment combinations (Tables 4A.9, 4A.10, 4A.11, 4A.12) the calculated z -value for 110 to 125°C is less than the z -value for 100 to 110°C .

TABLE 4A.9

Mean and Range of D-values and Two-point z-values
for Spores AAOE Heated in the Clean Room

Temp. (°C)	D-value(min.)		z-value °C	
	Mean	Range	Mean D-value	Extreme D-value
90	1205	1190-1219		
100	409	365-432	21.3	19.5-22.7
110	153	136-178	23.4	19.9-32.1
125	21.0	15.6-32.3	17.4	14.3-24.0
123*	24	22-27		
127*	12.8	12.2-13.1	13.1	11.5-17.8

*Single-day tests described in section 4C

TABLE 4A.10

Mean and Range of D-values and Two-point z-values
for Spores VDLB Heated in the Clean Room

Temp. (°C)	D-value(min.)		z-value °C	
	Mean	Range	Mean D-value	Extreme D-value
90	1202	1028-1375		
100	560	447-699	30.1	20.5-59.7
110	197	196-198	22.0	18.7-28.3
125	40.5	37.4-43.6	21.8	20.7-23.0

TABLE 4A.11

Mean and Range of D-values and Two-point z-values
for Spores AAOE Heated in the Dry Gas

Temp. (°C)	D-value(min.)		z-value °C	
	Mean	Range	Mean D-value	Extreme D-value
90	126	98.6-162		
110	47.5	37.7-69.0	23.6	15.8-64.5
125	7.4	6.2-9.2	18.6	14.3-24.7

TABLE 4A.12

Mean and Range of D-values and Two-point z-values
for Spores VDLB Heated in the Dry Gas

Temp. (°C)	D-value (min.)		z-value °C	
	Mean	Range	Mean D-value	Extreme D-value
100	259	214-347	28.4	19.0-44.1
110	115	103-128	21.4	19.7-23.6
125	22.9	22.0-23.8		

The z-value calculated from the mean D-value at 123 and 127°C is 13.1°C (Table 4A.9). This is probably the most accurate measure of z that we have. Using the extreme values at 123 and 127°C the z-value is only 17.8°C.

Considering the fact that the z for 110-125°C is less than the z for 100-110°C and the fact that the 123-127°C z-value is less than the z-value for 110-125°C, we conclude that in our test situation the z-value decreases with increasing temperature--at least those temperatures above 110°C.

The rate of change of D-value with temperature as measured by z indicates that when the z-value is calculated for a specific spore at a specific atmospheric water condition using D-value data at two adjacent temperatures, the z-value is not a constant over the temperature range from 90 to 125°C but appears to decrease as the temperature increases. Having made these calculations, we now question whether it is legitimate for us even to calculate z-values considering the varying water conditions in our particular experimental system.

The primary result of these experiments has been to show that when heating was carried out in a low water-content environment, the D-value was smaller than when heating was carried out in a higher water-content environment at all temperatures and for both spores. If we consider spores on planchets which were heated on a hot plate in two environments (low and high water content) at the same temperature, the water content of the surrounding atmosphere has a significant effect only on the water content of the spores, not on their temperature or other variables. The water content of the atmosphere surrounding the spores will either increase the water content of the spores (in which case water will diffuse from the atmosphere into the spores), or decrease the water content of the spores (in which case water diffuses from the spores into the surrounding atmosphere).

In both our dry gas and clean room environments, the water content in terms of relative humidity at the hot plate temperature is relatively low. We believe that we are working in an area in which the equilibrium water content of the spores

at the start of heating is considerably higher than the equilibrium spore water content for this heating temperature and relative humidity condition. Therefore the spores will lose water during the heating period.

The rate of water vapor diffusion from the spores into the surrounding gaseous environment will be a function of the water vapor pressure in the gaseous environment and the water vapor pressure in the voids inside the spore. The water vapor pressure inside the spore will be a function of the relative water(molecule) holding capacity of the macromolecules that make up the spore. The water(molecule) holding capacity of the spore is given by the water content-equilibrium relative humidity graph for the spore at the temperature under study. To analyze what we think is going on inside the spore, let us review the relationship of the quantity of water in the spore to the equilibrium relative humidity. In general, increasing the spore water content will increase the equilibrium relative humidity and vice versa.

If we assume that the equilibrium relative humidity versus water content curve is the same at 90, 100, 110 and 125°C, we can theorize regarding what happens to the spore water content when we heat spores at higher temperatures. Relative humidity is the ratio of the actual to the saturated water vapor pressure at the same temperature; the saturated water vapor pressure is a function of the spore temperature. The saturated vapor pressures for temperatures of 90, 100, 110 and 125°C are: 525.8, 760.0, 1074.5 and 1740.5 mm Hg. respectively. Therefore, as the temperature increases, the vapor pressure inside the spore must increase in proportion to the saturated vapor pressure if the equilibrium relative humidity and the bound water content of the spores are to remain constant. This seem impossible. If we increase the spore temperature, then the tendency of the spore to lose water will increase, other conditions remaining constant including the temperature and relative humidity of the surrounding environment; and for the same initial water condition, at the higher temperature the final water content will be reduced. It follows then, that for the same initial water conditions in the spore, the water content of the spores during heating will decrease as temperature increases, which will increase the rate of water loss from the spore. We are dealing with two in-balance type water conditions: water absorbed or adsorbed by macromolecules is in equilibrium with the water vapor in the voids of the spore and the water vapor pressure in the voids of the spore must be kept in balance with the external vapor pressure through the outward flow of water molecules from the spore to the surrounding environment as temperature increases. It appears to us that the only way we can have the same amount of water in the spores during heating at several temperatures is for the macromolecules to hold water molecules more tenaciously at the higher

temperature. This does not seem likely.

The thermal death of microbial spores is the result of lethal bio-chemical reactions. Chemical reaction rates increase with temperature; therefore, when we heat spores at 125°C we expect a smaller D-value than when we heat spores at 110°C. The D-value is the same magnitude as the reciprocal of the chemical reaction rate K. The mean D-values obtained for AAOE spores heated at 110 and 125°C in the clean room were 153 min. and 21.0 min. respectively. From Table 4A.9 we observe that the calculated z-value for these data bits is 17.4. According to the analysis above, the spores heated at 125°C will contain less water than the spores heated at 110°C, so the decrease in the D-value from 153 min. at 110°C to 21.0 min. at 125°C is the combination of both the change in the D-value due to increasing the temperature from 110 to 125°C and the change in the D-value due to a change in the water content. The z-value is related to the activation energy which is an indication of the type of chemical reaction. In order to be meaningful, the z-value should be measured under comparable conditions (including water content) at the different temperature levels. Therefore, it is basically wrong to conclude from the results reported here, that the z-value and, consequently, the activation energy change with temperature; it is basically wrong because we have not been able to separate the temperature coefficient effect from the change in water content effect. It is our opinion therefore, that while we may calculate a temperature coefficient z, and show that it does change with temperature, it is wrong to discuss it in any meaningful way in terms of the temperature coefficient of the thermal destruction reaction rate or the activation energy at this time. When we have data where the temperature coefficient effect has been separated from the water content effect then we may be able to begin to draw conclusions regarding mechanisms of death of the spores.

The change in D-value for a one degree change in temperature

The sensitivity of the D-value of a straight-line survivor curve with a change in temperature is important in adjusting D-values from one temperature to another temperature and in estimating the errors in D-values that will result from errors in temperature.

We have determined the change in D-value (D) for a 1.0°C change in temperature (T) assuming that the survivor curve is a straight line and that when log D is plotted vs. temperature the result is a straight-line thermal resistance curve.

The thermoresistance curve can be written

$$\log D_T^z = \alpha - \frac{T}{z} \quad (1)$$

where D_T^z = negative inverse of the slope of the survivor curve for a particular organism exposed to a particular environment with a temperature T ($^{\circ}\text{C}$);

z = negative inverse of the slope of the thermoresistance curve for a particular organism exposed to a particular environment;

α = intercept of the thermoresistance curve.

For an increase of 1°C

$$\log D_{T+1}^z = \alpha - \frac{T+1}{z}; \quad (2)$$

thus

$$\log D_T^z - \log D_{T+1}^z = -\frac{T}{z} + \frac{T+1}{z}; \quad (3)$$

and

$$\log \frac{D_T^z}{D_{T+1}^z} = \frac{1}{z}; \quad (4)$$

or

$$\frac{D_T^z}{D_{T+1}^z} = 10^{1/z}; \quad (5)$$

and

$$D_{T+1}^z = (10^{-1/z}) D_T^z. \quad (6)$$

For a decrease of 1°C

$$D_{T-1}^z = (10^{1/z}) D_T^z. \quad (7)$$

Using equations (6) and (7) we can examine how $\pm 1^{\circ}\text{C}$ change in temperature affects the D_T^z -value.

Consider the following example:

$$z = 21,$$

$$1/z = 0.0476.$$

From equation (6),

$$D_{T+1}^z = (0.896) D_T^z.$$

From equation (7),

$$D_{T-1}^z = (1.116) D_T^z.$$

A relevant question is: What is the expected change in the number of survivors if the temperature T_1 changes to $T_1 + 1^{\circ}\text{C}$ or $T_1 - 1^{\circ}\text{C}$? The answer to this question is illustrated in the following example.

Let N_u = number of survivors at time u (minutes). Assume $N_0 = 10^6$ and $D_{T_1}^{21} = 20$ minutes. Then we observe the following:

<u>T($^{\circ}\text{C}$)</u>	<u>D-value(min)</u>	<u>N₆₀</u>
T_1	20.00	10^3
$T_1 + 1^{\circ}\text{C}$	17.92	4.5×10^2
$T_1 - 1^{\circ}\text{C}$	22.32	2.1×10^3

CONCLUSIONS

The survivor curve, where the logarithm of the numbers of survivors is plotted as a function of time on the hot plate at a given temperature, could well be represented by straight lines.

The D-value, a measure of the slope of the regression line, was smaller in all experiments carried out at a low humidity than in the corresponding experiments carried out at a higher humidity.

The change in the D-value with change in relative humidity was greater for the AAOE spores than for the VDLB spores.

The intercept ratio decreased with a decrease in the clean room relative humidity, at least down to a relative humidity of 0.4% at 125°C. These results hold for both spores AAOE and VDLB.

D-value data for spores deposited on stainless steel planchets heated on a hot plate under conditions that permit the water content in the spores to be a function of heating temperature cannot be used to calculate meaningful z-values.

The change in D-value for $|\Delta T| = 1^\circ\text{C}$ for a z-value of 21°C is: $D_{T+1} = (0.896)D_T$, and $D_{T-1} = (1.116)D_T$. An example illustrates the affect of a change in the heating temperature (T) on the number of spores surviving after 60 minutes of heating.

FUTURE WORK

A more accurately controlled glove box system has been constructed for studying dry heat resistance of spores on surfaces under low water vapor pressure conditions. In this new system a range of vapor pressure conditions can be obtained and the respective levels controlled. We plan to use this system to further elucidate the relationship between vapor pressure and spore survival at low water vapor pressure levels.

Naturally occurring soil organisms have been found to exhibit very high thermal resistance. We plan to examine these spores in order to determine whether the heating environment has an effect on the resistance of these spores.

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B. CHANGES IN THE D_{125} -VALUE AS A FUNCTION OF INCREMENTAL
CHANGES IN THE ENVIRONMENTAL TEMPERATURE AND VAPOR PRESSURE

INTRODUCTION

As previously reported, we observed that the survival pattern of Bacillus subtilus var. niger(AAOE) when subjected to a heat treatment at 125°C was affected by changes in relative humidity at a given temperature and by changes in temperature for a given relative humidity. Further investigation has suggested that vapor pressure is the critical factor when the spore survival pattern is related to these variables.

The following series of experiments was designed and conducted for the purpose of providing information regarding the role of vapor pressure in the survival pattern of spores.

Three classes of experiments were planned. The conditions for each class were as follows: Class I - vapor pressure constant, saturated vapor pressure varied(clean room temperature varied); Class II - vapor pressure varied, saturated vapor pressure constant(temperature constant); Class III - vapor pressure constant, saturated vapor pressure constant(temperature constant).

In the Class I experiments the vapor pressure was held constant while the saturated vapor pressure was varied to predetermined levels(by varying the room temperature) to give the desired 25 and 45% relative humidity levels. (The saturation vapor pressure used to calculate relative humidity is a tabulated value that is a function of temperature.) The results would be spore destruction rates at the 25 and 45% relative humidity levels for a constant vapor pressure in the clean room.

In the Class II experiments the saturated vapor pressure was held constant (clean room temperature constant) and the vapor pressure was varied. Hopefully, the results of the Class II experiments would be spore destruction rates at the 25 and 45% relative humidity levels with two different vapor pressure levels in the clean room.

In the Class III experiments the spore destruction data were obtained with both vapor pressure and saturated vapor pressure constant. The results of these experiments were to reflect reproducibility of results within days.

EXPERIMENTAL PROCEDURE

Previous experimental results suggest that some uncontrolled factor in the experimental system caused a detectable change in the day-to-day survival

pattern of the spores. The variations in the environmental physical conditions are assumed to increase as the experimental time period increases. Hence, to increase the precision of the experimental results each pair of experiments was conducted on the same day.

The experiments were conducted in a crossflow clean room equipped with an environmental control system which allowed for regulation of both the temperature and relative humidity. The spores were heated using the hot plate-boat-planchet method. Each boat was heated on a hot plate at 125°C for one of four possible times: 15, 30, 45, and 60 minutes. Heating times for experiments CH0126A and CH0126B were 20, 40, 60, and 80 minutes. There were three planchets per boat and two boats for each heating time. Experiments conducted in the morning are designated by the letter A, those conducted in the afternoon by the letter B. The D-value and the intercept ratio (IR) were used to characterize the survival pattern of the spores. The estimated number of spores at time zero (N_0) was not included in the calculation of the D-value and y-intercept.

The experimental conditions actually achieved for Class I, II, and III are shown in Table 4B.1, 4B.2 and 4B.3. These conditions differ from the experimental objectives because there were some difficulties in controlling the humidifier system for the experimental environment. This appeared to be due to a faulty control valve that did not function consistently at high relative humidities resulting in some fluctuation of environmental conditions during a test.

TABLE 4B.1
Environmental Conditions for the Class I Experiments
(constant observed vapor pressure)

Test No.	Date	Temp. °C	Average Relative Humidity(%)	Average Vapor Pressure (mm Hg)
CH0096A	4-7	27.3	26	7.15
CH0096B	4-7	18.6	45	7.16
CH0098A	4-9	26.8	25	6.57
CH0098B	4-9	18.2	45	6.98
CH0117A	4-28	17.8	49	7.50
CH0117B	4-28	27.1	36	9.68

TABLE 4B.2

Environmental Conditions for the Class II Experiments
(constant saturated vapor pressure and temperature)

Test No.	Date	Temp. °C	Average Relative Humidity(%)	Average Vapor pressure (mm Hg)
CH0103A	4-14	21.9	30	5.96
CH0103B	4-14	21.9	46	9.03
CH0105A	4-16	22.1	30	6.03
CH0105B	4-16	22.2	43	8.43
CH0119A	4-30	22.2	54*	10.52*
CH0119B	4-30	22.8	38	7.84
CH0124A	5-5	22.5	31	5.91
CH0124B	5-5	22.0	60	11.88
CH0126A	5-7	21.1	33	6.19
CH0126B	5-7	22.2	60	11.83

*The relative humidity was at a high of 60% at the beginning of the one-hour experiment and reached a low of 48% in about 30 minutes.

TABLE 4B.3

Environmental Conditions of the Class III Experiments
(constant temperature and observed vapor pressure)

Test No.	Date	Temp. °C	Average Relative Humidity(%)	Average Vapor Pressure (mm Hg)
CH0110A	4-21	21.8	36	6.94
CH0110B	4-21	21.8	37	6.95

RESULTS

Results of the Class I experiments are given in Table 4B.4. Observed D-values range from 18.0 to 28.3. The variation between observed D-values appears to be related, in part, to the measured variation in vapor pressure, in this set of experiments designed to hold vapor pressure constant.

TABLE 4B.4

Results of the Experiments in Class I
(constant observed vapor pressure)

Test No.	Average Vapor Pressure (mm Hg)	D-value(min.)		IR*
		Estimate	95% CI	
CH0096A	7.15	19.2	16.3,23.3	0.36
CH0096B	7.16	18.7	16.0,22.6	0.30
CH0098A	6.57	18.0	16.6,19.7	0.50
CH0098B	6.98	20.3	18.8,22.0	0.69
CH0117A	7.50	24.0	20.8,28.5	1.05
CH0117B	9.68	28.3	24.7,33.1	1.03

$$*IR = \frac{\log y_0}{\log N_0}, \quad y_0 = y\text{-intercept of estimated survival curve}$$

N_0 = estimated number of viable spores per
planchet at time zero

The results of Class II experiments are given in Table 4B.5. In general it appears that vapor pressure and D-value are positively correlated.

TABLE 4B.5

Results of the Experiments in Class II
(constant saturated vapor pressure
and clean room temperature)

Test No.	Average Vapor Pressure (mm Hg)	D-value(min.)		IR*
		Estimate	95% CI	
CH0103A	5.96	20.4	18.3,23.2	1.24
CH0103B	9.03	21.3	19.2,24.0	1.61
CH0105A	6.03	17.8	16.5,19.3	1.72
CH0105B	8.43	19.0	16.6,22.3	2.28
CH0119A	10.52*	22.9	21.4,24.7	2.95
CH0119B	7.84	18.6	16.1,21.1	4.93
CH0124A	5.91	19.3	17.0,22.4	0.79
CH0124B	11.88	30.8	26.7,36.5	0.79
CH0126A	6.19	22.7	18.9,28.5	0.51
CH0126B	11.83	29.5	26.4,33.5	1.17

*See Table 4B.2

The results of the Class III experiments are given in Table 4B.6. Little difference was noted between the D-values.

TABLE 4B.6
Results of the Experiments in Class III
(constant temperature and RH)

Test No.	Average Vapor Pressure (mm Hg)	D-value(min.)		IR
		Estimate	95% CI	
CH0110A	6.94	18.6	17.2,20.2	0.66
CH0110B	6.95	18.6	15.9,22.5	1.13

Discussion

These experiments were conducted during the spring of 1970 when there were wide fluctuations in the outdoor temperature and vapor pressure. During the first few weeks of the project it was possible to obtain predetermined low relative humidities for the experimental environment. However, as the ambient moisture content increased (outdoor temperature increased) the system capacity was inadequate to lower the room vapor pressure condition to the predetermined level. Minimum moisture levels then were determined by the capacity of the system. Efforts to increase the capability of the system by lowering the temperature of the cooling coils were not always successful. On some occasions the moisture on the cooling coils would freeze.

To obtain a measure of the change in D-value with change in vapor pressure we calculated the change in D-value with change in the observed vapor pressure for each pair of Class II experiments performed on the same day. These data are tabulated in Table 4B.7.

TABLE 4B.7
Estimated Rates of Change of D-value with Respect to
Change in Vapor Pressure within Days Between Tests

Test No.	Average observed vapor pressure (mm Hg)	Difference between vapor pressures (mm Hg)	Difference in D-values (min.)	Estimated rate (min./mm Hg)
CH0103	7.50	3.07	0.9	0.3
CH0105	7.23	2.40	1.2	0.5
CH0119	9.18*	2.68	4.3	1.6
CH0124	8.90	5.97	11.5	1.9
CH0126	9.01	5.64	6.8	1.2

*See Table 4B.2

In this table the average observed vapor pressure is the arithmetic mean of the average morning test and afternoon test vapor pressure from Table 4B.5. The difference in vapor pressure is the difference between the average morning test and afternoon test vapor pressure from Table 4B.5. The results of the analysis suggest that the rate of change of D with observed vapor pressure increases with increasing vapor pressure.

The D-value data from Tables 4B.4, 4B.5 and 4B.6 are shown graphically in Figure 4B.1.

Due to the apparent day-to-day variability in D-values in all of these experiments, it is reasonable to conjecture that the true D-value for a particular day is different from the true D-value on the next day even though the experimental conditions are identical. The variation of the D-value between days suggests an interpretation of the confidence intervals of Tables 4B.4, 4B.5 and 4B.6 in a particular way. If certain statistical assumptions are valid, the confidence interval can be interpreted as follows: given that the true D-value for a particular day is fixed, then the observed D-value is randomly distributed around the true D-value, which is the mean. The probability is 0.95 that the confidence interval constructed on the observed D-value will bracket the true D-value. Nothing is implied about bracketing tomorrow's true D-value with today's confidence interval even though the experimental conditions are identical. Therefore, comparison of results obtained within the same day can be made with greater precision than comparison of results obtained on different days.

We believe it is desirable to investigate the unexplained day effects. The following methods are suggested for investigating this source of variation:

- 1) Monitoring the uncontrolled variables that affect D-value, and
- 2) Carrying out a standard experiment each day to use as a covariate with the results of other experiments conducted on the same day.

For method 1 to be effective the uncontrolled variables that affect D-value must be identified. Evaluating intuitively reasonable sources of uncontrolled variability does not appear to be a straightforward task. One method that might be employed is regression analysis as described by Draper and Smith in Applied Regression Wiley, 1966. Analysis of the data might result in the identification of likely candidates that affect survival patterns of spores. Acceptance or rejection of these candidates would depend upon their future performance observed in additional experiments.

The covariate of method 2 would presumably reflect the effects of the uncontrolled variables. Assuming that these effects would be similar to those in the experiments of interest, comparability of results between days could possibly be enhanced by use of the covariate.

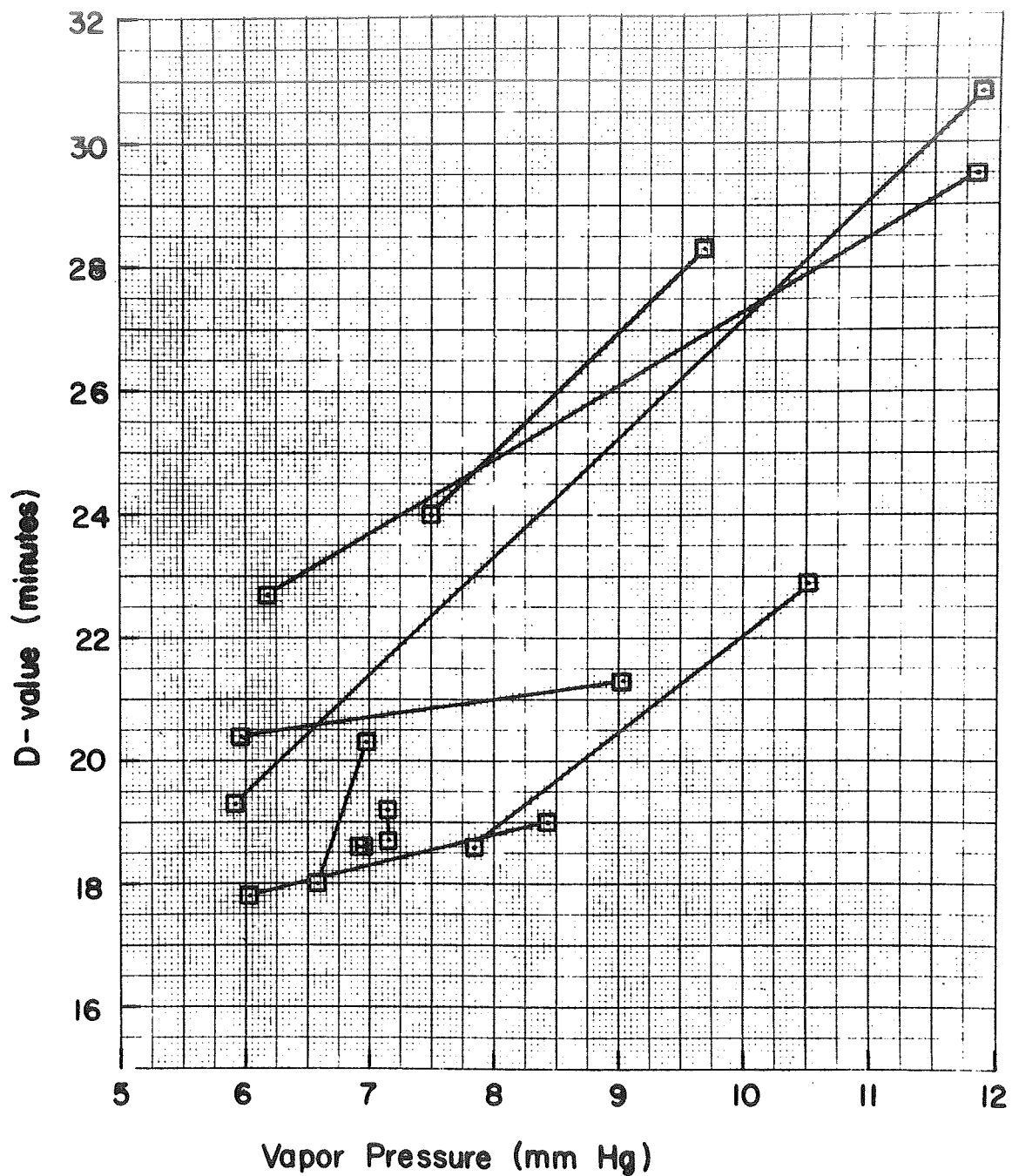


Figure 4B.1 - D-value as a Function of Vapor Pressure for all Tests. Data points for tests carried out on the same day are connected by straight lines.

Four uncontrolled variables were observed during this series of experiments. Those variables suspected as possibly affecting spore survival patterns were barometric pressure, cold plate temperature, initial number of spores deposited and outdoor vapor pressure. Observed values for these variables are presented in Tables 4B.8, 4B.9 and 4B.10. Estimates of their correlations with observed D-values and vapor pressures are presented in Table 4B.11.

TABLE 4B.8

Observed Values of Variables that Possibly Affect Spore Survival Patterns
(Class I Experiments)

Test No.	D-value estimate (min.)	Vapor pressure (mm Hg)	Barometric pressure (mm Hg)	Cold plate temp.(°C)	N _o estimate	Outdoor V.P.(mm Hg)
CH0096A	19.2	7.15	736.0	7.0	2.10E6	5.16
CH0096B	18.7	7.16	732.5	6.0	1.92E6	7.87
CH0098A	18.0	6.57	739.5	7.0	1.80E6	3.88
CH0098B	20.3	6.98	739.7	7.0	1.73E6	3.77
CH0117A	24.0	7.50	728.5	11.0	2.06E6	13.23
CH0117B	28.3	9.68	728.0	12.0	1.96E6	14.36

TABLE 4B.9

Observed Values of Variables that Possibly Affect Spore Survival Patterns
(Class II Experiments)

Test No.	D-value estimate (min.)	Vapor pressure (mm Hg)	Barometric pressure (mm Hg)	Cold plate temp.(°C)	N _o estimate	Outdoor V.P.(mm Hg)
CH0103A	20.4	5.96	741.8	8.0	1.98E6	4.20
CH0103B	21.3	9.03	741.3	8.5	1.93E6	4.35
CH0105A	17.8	6.03	738.6	7.0	2.27E6	4.60
CH0105B	19.0	8.43	738.5	7.5	2.15E6	4.81
CH0119A	22.9	10.52*	736.7	13.5	1.72E6	4.95
CH0119B	18.6	7.84	736.0	13.0	1.69E6	5.83
CH0124A	19.3	5.91	748.0	16.5	1.74E6	4.44
CH0124B	30.8	11.88	748.2	17.5	1.87E6	4.00
CH0126A	22.7	6.19	735.0	18.0	1.90E6	9.28
CH0126B	29.5	11.83	732.0	16.0	1.70E6	11.45

*See Table 4B.2

TABLE 4B.10

Observed Values of Variables that Possibly Affect Spore Survival Patterns
(Class III experiments)

Test No.	D-value estimate (min.)	Vapor pressure (mm Hg)	Barometric pressure (mm Hg)	Cold plate temp.(°C)	N _o estimate	Outdoor V.P.(mm Hg)
CH0110A	18.6	6.94	737.0	8.5	1.74E6	4.36
CH0110B	18.6	6.95	737.5	9.5	1.59E6	4.73

TABLE 4B.11

Calculated Correlation Coefficients Among Observed Variables
Using All Data from Tables 4B.8, 4B.9 and 4B.10

Observed Variables	D-value estimate	Vapor pressure	Barometric pressure	Cold plate temp.(°C)	N _o estimate	Outdoor V.P.
D-value estimate	1.00	0.81	-0.15	0.65	-0.08	0.56
Vapor pressure		1.00	-0.09	0.44	-0.18	0.28
Barometric pressure			1.00	0.14	-0.13	-0.80
Cold plate temp.				1.00	-0.37	0.31
N _o estimate					1.00	0.13

Another factor possibly affecting the survival of spores was the observed moisture on the cold plate. When test conditions included a high relative humidity and a low cold plate temperature, water could condense on the planchet during the cooling period. The cold plate temperature ranged from a low of 6°C on the first day of the series of experiments to a high of 18°C on the last day.

The relationship of D-value and observed vapor pressure could be further investigated by a set of experiments like those of Class II, at a series of temperatures. Each day two experiments could be conducted at vapor pressures that differ by a constant, say 5 mm Hg. The number of experiments in the series would be determined by the range of vapor pressures of interest. Experimental results hopefully would provide estimates of the rate of change of D-value with respect to vapor pressure, thus an estimate of the form of the relationship might be obtained.

CONCLUSIONS

It was observed that for the conditions studied the estimated D-value increased with an increase in vapor pressure. The rate of increase of D-value

did not appear to be constant with respect to vapor pressure; it appeared that the rate was greater at high vapor pressures than at lower vapor pressures.

Similar D-values were observed when environmental conditions had the same vapor pressures even though different combinations of temperature and relative humidity were used (see tests CH0096 and CH0098).

Reproducibility of experimental results within days appeared to be good. The observed variation of results between days can not be explained at this time.

C. CHANGE IN D-VALUE FOR SMALL CHANGES IN TEMPERATURE

INTRODUCTION

In experiment MH0154, the hot plate that had previously been used in the dry gas double glove box was moved into the clean room and placed beside the clean room hot plate. The two hot plates were arranged so that they were side by side and both about 25 inches from the laminar-flow filter wall. The objectives were to determine the D-values of spores treated on the two hot plates at 1) the same temperature and 2) two different temperatures. The experiments were designed so that we could compare the performance of the two hot plates as well as determine the change in the D-value with temperature.

EXPERIMENTAL PROCEDURE

The design of the experiment is diagrammed below where the experiments coded A, C, E, and G refer to plate #1 and B, D, F, and H refer to plate #2:

Code	Time	Temp. °C	
		Hot Plate #1	Hot Plate #2
MH0154 A & B	9:00 a.m.	123°C	127°C
MH0154 C & D	11:00 a.m.	127°C	127°C
MH0154 E & F	1:00 p.m.	127°C	123°C
MH0154 G & H	3:00 p.m.	123°C	123°C

Spore deposits using Bacillus subtilis var. niger(AAOE), were made on the 1/2" x 1/2" stainless steel planchets. Three planchets were located on each of the boats. Boats were arranged in a column of six (parallel to the air flow) on each hot plate and all boats were equidistant from the filter wall. Boats were randomly allocated to hot plate and run. For each run, three boats from each hot plate were randomly selected for removal after a 15-minute heating period while the other three were removed after 45 minutes. Planchets were processed and D-values computed using the previously described techniques.

The experimental unit was assumed to be the copper boat. The response variable associated with the experimental unit was designated to be the average count of spores on six petri dishes (two dishes for each of the three planchets on the boat). The counts obtained at 15 minutes and 45 minutes were analyzed separately. The following model was assumed.

$$y_{ijkl} = \mu + p_i + t_j + (pt)_{ij} + \gamma_k + \epsilon_{ijkl}$$

where y_{ijkl} = average count associated with the i -th hot plate, j -th temperature, k -th run(time of day), and l -th boats, $l=1,2,3$;

μ = mean

p_i = effect of i -th hot plate, $i = 1, 2$;

t_j = effect of j -th temperature, $j = 1, 2$;

$(pt)_{ij}$ = interaction effect of (ij) th hot plate, temperature;

γ_k = effect of k -th run, $k = 1, 2, 3, 4$;

ϵ_{ijkl} = stochastic component;

and

$$\sum_i p_i = \sum_j t_j = \sum_i (pt)_{ij} = \sum_j (pt)_{ij} = 0;$$

$$\gamma_k \sim N(0, \sigma_r^2), \epsilon_{ijkl} \sim N(0, \sigma^2).$$

RESULTS

Table 4C.1 presents the average plate counts obtained for the various treatment combinations. The dilution factor of 1/12750 was constant for all samples at 15 minutes. At 45 minutes the dilution factor for samples at 123°C was 1/500 and at 127°C it was 1/50.

TABLE 4C.1

Average Plate Counts* Related to Number of Surviving
Spores After Heating for 15 and 45 Minutes

Treatment Time	Hot Plate 1			Hot Plate 2		
	Temp.	15 min.	45 min.	Temp.	15 min.	45 min.
9 a.m.	123°C	74.3	101.4	127°C	48.9	42.4
11 a.m.	127°C	38.1	49.8	127°C	43.2	55.9
1 p.m.	127°C	44.1	62.2	123°C	77.2	150.7
3 p.m.	123°C	70.0	84.6	123°C	85.6**	92.4

*Each average value is the average count of spores on 18 plates (3 boats/heating period, 3 planchets/boat, 2 plates/planchet) with one exception(**) which is the average of 16.

Table 4C.2 presents the estimated D-values and the intercept ratios. Figure 4C.1 is a display of the estimated survivor curves for the eight experiments.

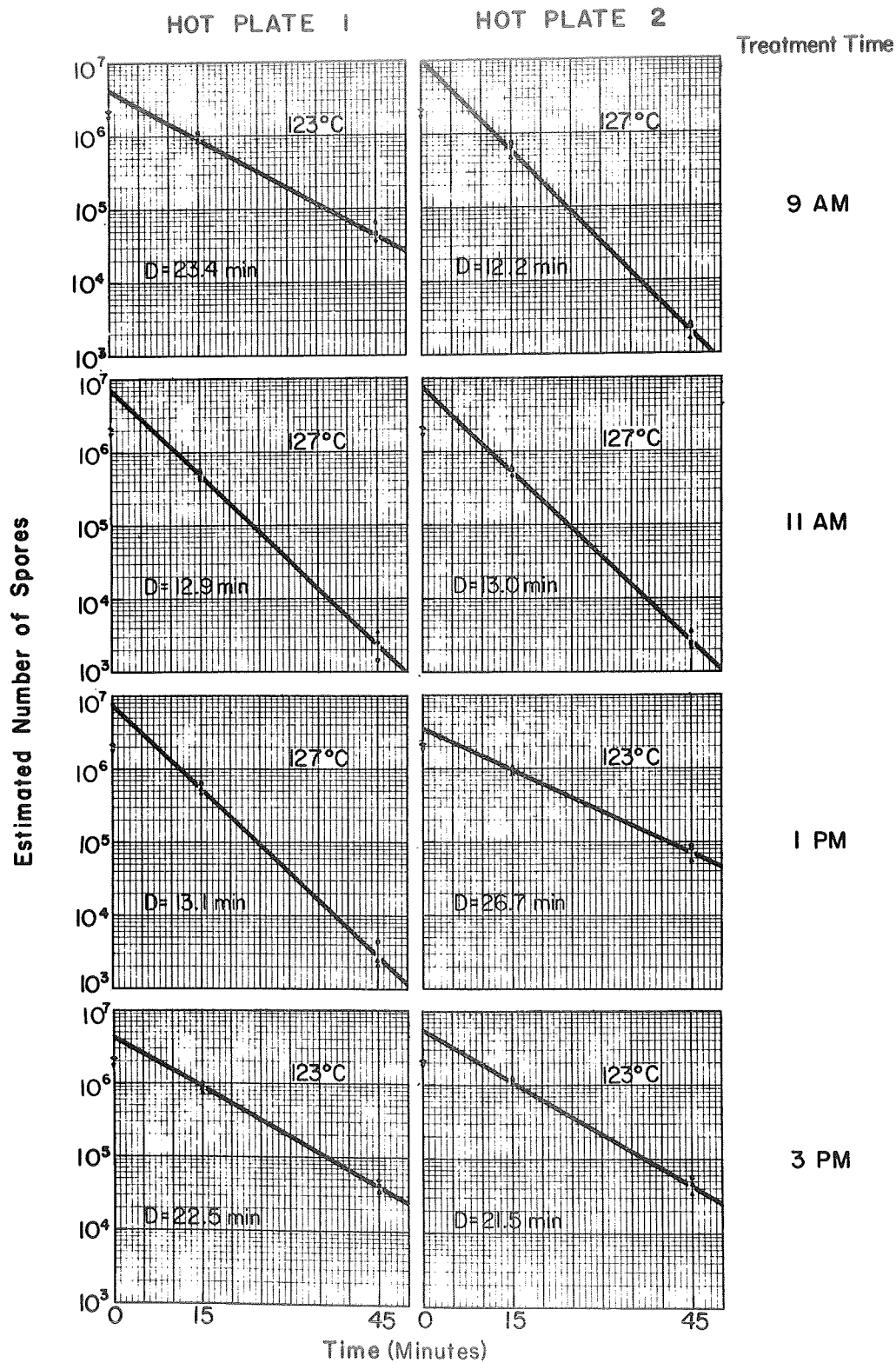


Figure 4C.1-Survivor data and least squares lines through the data points at 15 and 45 minutes for the eight tests carried out in a single day.

TABLE 4C.2
Estimated D-values and Intercept Ratios

Treatment Time	Hot Plate 1			Hot Plate 2		
	Temp.	Estimated D-value (min.)	IR*	Temp.	Estimated D-value (min.)	IR*
9 a.m.	123°C	23.4	1.05	127°C	12.2	1.12
11 a.m.	127°C	12.9	1.09	127°C	13.0	1.10
1 p.m.	127°C	13.1	1.10	123°C	26.7	1.04
3 p.m.	123°C	22.5	1.05	123°C	21.5	1.07

*IR = $\frac{\log y_0}{\log N_0}$ where y_0 = y-intercept of estimated survivor curve

N_0 = estimated number of spores for control at time zero

Results of the analysis of variance are given in Table 4C.3 and 4C.4.^b It should be noted that both the effects of run and hot plate and the effects of run and temperature are non-orthogonal. In the analysis the effect of temperature at 45 minutes is altered because of the use of two different dilution factors. The term "adjusted" in the analysis of variance tables implies that the sum of squares associated with the adjacent source of variation is the difference in the residual sums of squares obtained by fitting the full model and by fitting the full model less the component of the model related to the source of variation of interest.

For both sets of variables (average counts at 15 minutes and 45 minutes) the model appears to be adequate since the deviations from the model (Tables 4C.3 and 4C.4) are minimal; possible interactions between runs and other parameters of interest were not detected. On this basis the error term is the appropriate divisor in the remaining tests.

The effect of runs was judged to be significant at 45 minutes and not significant at 15 minutes. This might imply that some factor in the environment or test system produces a detectable change at 45 minutes but not at 15 minutes. The direction of the change might be related to the run (time of day) but is not explained. Because of the confounding of runs with temperature, it is possible that the observed differences in run can be explained in part by the difference in temperature; since the F-value for "run" in the 45-minute data is just beyond the significance level, these results should be interpreted with caution.

^b In the format as presented, for example, by Kempthorne(1952), Design and Analysis of Experiments, Wiley, Table 6.1., p. 72.

TABLE 4C.3

Analysis of Variance Results
(heating period = 15 minutes)

Source	Degrees of freedom	Sums of squares	Mean squares	F statistic
Mean	1	86860.61		
Hot plate	1	302.22	302.22	6.616*
Temperature(adjusted)	1	2566.69	2566.69	
Hot plate x temp.(adjusted)	1	81.81	81.81	1.791
Run(adjusted)	3	172.90	57.63	1.262
Deviation from model	1	62.83	62.83	1.375
Error	16	730.88	45.68	
Total	24	94774.02		

$$*P(F_{1,16} > 6.616) < 0.02$$

TABLE 4C.4

Analysis of Variance Results
(heating period = 45 minutes)

	Degrees of freedom	Sums of squares	Means squares	F statistic
Mean	1	153306.80		
Hot plate	1	709.59	709.59	1.393
Temperature(adjusted)	1	16317.19	16317.19	
Hot plate x temp.(adjusted)	1	2.08	2.08	0.004
Run(adjusted)	3	5922.74	1974.24	3.876*
Deviation from model	1	90.09	90.09	0.177
Error	16	8150.66	509.41	
Total	24	188013.52		

$$*P(F_{3,16} > 3.876) < 0.05$$

The interaction effects of plate and temperature were judged to be chance differences for both sets of observations.

It was interesting to note that the effect of hot plates was judged to be significant for the set of observations at 15 minutes while not significant at 45 minutes. This inconsistency is unexplainable at present. The significant effect of the run only at 45 minutes might be related to the above inconsistency; the interaction of plate and run might be significant. At 15 minutes the average numbers of spores counted per petri dish were 56.6 on hot plate 1 and 63.7 on hot plate 2. At 45 minutes they were 74.5 and 85.4 respectively. Although

the effect of the hot plates was judged to be not significant at 45 minutes, hot plate 2 appeared to be not as lethal as hot plate 1. Thus it appears that even though the temperature-monitoring devices were recording similar temperatures on the two hot plates, differences unique to the individual hot plates (or systematic differences in the monitoring devices) caused a significantly different response in the bacterial spores, particularly at the 15 minute heating period. The restricted nature of the data collected does not allow the resolution of the above conjectures.

In these four tests which were all carried out in a single-day, there was a significant unexplained test-to-test variation for the 45 minute heating periods. This result suggests that there are still unrecognized and hence, uncontrolled environmental parameters associated with the clean room and hot plate system that vary during a single day.

If we assume that the spore temperature and water condition are the determining factors in the stability of spores to dry heat; it seems probable that the unknown factor relates to water in the spore rather than temperature since equivalent temperature effects would be expected at 15 minutes as well as at 45 minutes, whereas, if this environmental effect relates to the rate of water loss from the spore it would tend to manifest itself more at 45 minutes than at 15 minutes.

CONCLUSIONS

The single conclusion that we can draw from this experiment is that there are still unrecognized, and hence, uncontrolled environmental parameters that vary during a single day.

PUBLICATIONS AND PRESENTATIONS

Publications in the Open Literature

Vesley, D., A. K. Pryor, W. G. Walter, and J. G. Shaffer. "A cooperative microbiological evaluation of floor-cleaning procedures in hospital patient rooms." A committee report. Health Laboratory Sciences, 7, (4), 256-264, October 1970.

Presented at Meetings

Vesley, D. and W. G. Walter. "Microbiological Standards for Surfaces in the Hospital Environment." Presented at the annual meeting of the American Public Health Association, Houston, Texas. October 28, 1970.