Influence of Spore Moisture Content on the Dry-Heat Resistance of *Bacillus subtilis* var. *niger*

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The dry-heat resistance of *Bacillus subtilis* var. *niger* spores located in or on various materials was determined as D and z values in the range of 105 through 160 C. The systems tested included spores located on steel and paper strips, spores located between stainless-steel washers mated together under 150 inch-lb and 12 inch-lb of torque, and spores encapsulated in methylmethacrylate and epoxy plastics. D values for a given temperature varied with the test system. High D values were observed for the systems in which spores were encapsulated or under heavy torque, whereas lower D values were observed for the steel and paper strip systems and the lightly torqued system. Similar z values were obtained for the plastic and steel strip systems ($z_D = 21$ C), but an unusually low z for spores on paper ($z_D = 12.9$ C) and an unusually high z for spores on steel washers mated at 150 inch-lb of torque ($z_D = 32$ C) were observed. The effect of spore moisture content on the D value of spores encapsulated in water-impermeable plastic was determined, and maximal resistance was observed for spores with a water activity (a_w) of 0.2 to 0.4. Significantly decreased D values were observed for spores with moisture contents below a_w 0.2 or above a_w 0.4. The data indicate that the important factors to be considered when measuring the dry heat resistance of spores are (i) the initial moisture content of the spore, (ii) the rate of spore desiccation during heating, (iii) the water retention capacity of the material in or on which spores are located, and (iv) the relative humidity of the system at the test temperature.

The dry-heat sterilization cycles presently employed have been empirically derived (14; *U.S. Pharmacopoeia*, 17th revision, p. 811) and are too severe to apply to the sterilization of interplanetary spacecraft. Because of the microbial contamination associated with the interiors of certain electronic parts (15), the National Aeronautics and Space Administration has decided that interplanetary spacecraft shall be sterilized. Dry heat has been assessed as one of the sterilization processes that may be applied to spacecraft, providing that time-temperature combinations can be developed that are compatible with maintaining the functional properties of electronic parts.

In comparison to the accumulated knowledge of the factors that influence microbial resistance to wet heat, little is known about the factors that affect microbial resistance to dry heat, except for the reports of Murrell and Scott (10, 11) on the effects of water activity. In an attempt to identify some of these factors and to generate data that may be useful in developing spacecraft sterilization cycles, this study has been undertaken. This report describes the dry-heat resistance of microbial spores in the range of 105 to 160 C, as influenced by initial spore moisture content and by systems which, because of their physical characteristics, either permit or retard spore desiccation during heating.

MATERIALS AND METHODS

Production of spores. Bacillus subtilis var. *niger* spores were produced by surface culture on agar medium (Seitz-filtered glucose, 0.25%; Casamino Acids (technical grade), 0.25%; yeast extract, 0.5%; $MnSO_4 \cdot H_2O$, 0.001%; $FeSO_4 \cdot 7H_2O$, 0.0014%; agar, 3%) in 6-liter Pavitsky bottles with 7 days of incubation at 35 C. The spores were washed from the surface with double-distilled sterile water, shaken with glass beads, filtered through cotton, and held at 45 C (water bath) overnight. The heated suspension was washed

five times in 400-ml volumes of double-distilled sterile water (4,080 $\times$ g for 20 min at 5 C) and was stored at 5 C. Sufficient spore crops were produced initially so that a single, pooled, stock spore suspension could be used throughout the study. Plate count values of the refrigerated stock spore suspension prepared in tryptone-glucose-beef extract-agar (48 hr of incubation at 35 C), obtained at various intervals over the past 2.5 years, has revealed no change, with time, in the number of spores per ml. In addition, no difference in the total number of spores per ml has been observed over the same period for heat-shocked (80 C for 10 min) versus non-heat shocked samples of the stock spore suspension.

Steel and paper test surfaces. By means of a microburette, 0.01-ml samples of an appropriate aqueous dilution of the stock spore suspension were placed on one surface of strips [1 $\times$ 0.25 inch (2.54 $\times$ 0.64 cm)] of stainless steel (202, #4 finish) and filter paper (Whatman #2). The strips were placed in borosilicate thermal death time (TDT) tubes (13 $\times$ 100 mm), which were then constricted to form a well in the upper third of the tube. A loose plug of glass wool was placed in the constriction, and the well was filled with silica gel and loosely plugged with cotton (Fig. 1). The tubes were placed in a forced air oven at 50 C for 1 hr; then they were placed in a desiccator over silica gel and held overnight at 20 C. After drying, the tubes were sealed at the constriction with an oxy-gas torch. The silica gel in the well above the constriction prevented the reintroduction of moisture from the ambient air during sealing.
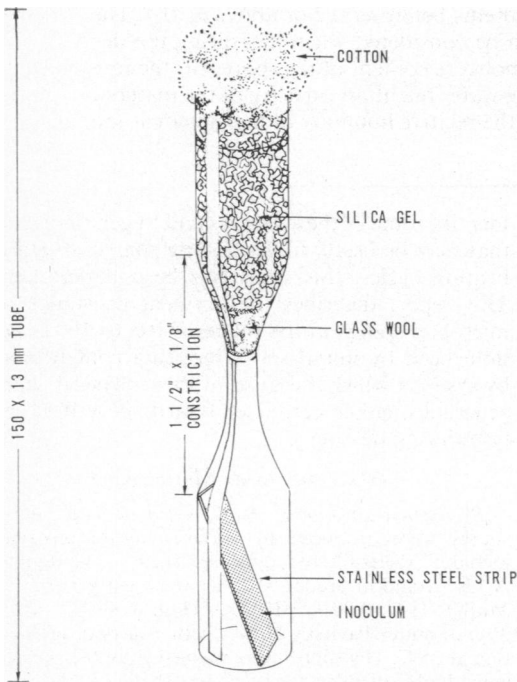
Stainless-steel washers [202, #4 finish, 0.5 inch (1.27 cm) outer diameter and 0.25 inch (0.64 cm) inner diameter] were also inoculated on one surface with 0.01 ml of the aqueous spore suspension and dried at 50 C for 1 hr as described above. The dry, inoculated washers were mounted on the male lug (*see* Fig. 2), with the inoculated surface adjacent to the uninoculated surface of a second washer. Male and female lugs were loosely threaded together and placed in borosilicate TDT tubes (15 $\times$ 100 mm), and the tubes and torque wrench were sealed in a polyethylene bag containing silica gel. The flexible desiccator was stored overnight at 20 C. Working from outside of the plastic bag, the units were torqued the desired amount, the bag was opened, and a constriction was made in the TDT tubes. Silica gel was placed in the upper well of the tubes, and the tubes were stored in a desiccator over silica gel overnight at 20 C; then the tubes were sealed at the constriction. Again, these precautions were taken to assure that moisture from the ambient air did not contact the dried spores located on the mated surfaces.

Fabrication of Lucite (methylmethacrylate) rods. To remove the polymerization inhibitor, methylmethacrylate monomer was washed twice with equal volumes of 2% NaOH, followed by two additional washings with equal volumes of distilled water. The washed monomer was then mixed with an excess of anhydrous sodium sulfate (Na_2SO_4) and allowed to stand overnight to remove water. The sodium sulfate was removed by filtration, and the monomer was stored in the cold (5 C) until ready for use.

A 1-ml amount of an aqueous dilution of the stock spore suspension was distributed over 50 g of methylmethacrylate powder in a sterile drying pan. The pan was placed in a forced-air drying oven for 30 min at 50 C; the pan was then removed and the powder was placed in a sterile mortar and ground by hand until the dried spore inoculum appeared to be evenly
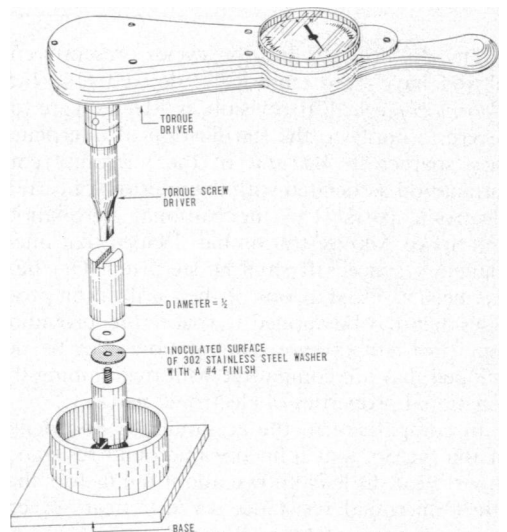

FIG. 1. *Drying tube for inoculated stainless-steel strips.*

FIG. 2. *Mated surface assembly (150 inch-lb).*

distributed throughout the powder. The powder was returned to the drying pan, heated for an additional 30 min in the oven, and once more ground. The desired quantity of inoculated, dried powder was weighed into a shallow pan, placed in a desiccator over silica gel, and held overnight at 20 C. Following overnight storage, the powder was transferred to a beaker, and to each 50 g of powder, 50 ml of methylmethacrylate monomer was added and mixed with a spatula. Though some moisture may have contacted the inoculated powder during the transfer to the beaker, it was assumed that, after the addition of the monomer, absorption of moisture would be negligible because of the relative water impermeability of the finished plastic. The liquid mixture was placed in a vacuum flask and evacuated with a water pump until bubbles no longer formed. The viscous, partially polymerized plastic was poured into TDT tubes, and the tubes were cotton-stoppered and placed in a 50 C water bath for 2 hr to complete the polymerization. After polymerization, the TDT tubes were sealed in the oxy-gas flame. This method consistently yielded Lucite rods that were hard, clear, free of bubbles, and from which approximately 10^8 spores per gram were consistently recovered (see Recovery methods). An analysis of the distribution of spores in the rods revealed that no significant differences ($\alpha = 0.05$) in spore concentrations were detected within a rod or among rods prepared from separate batches of plastic, and that the viable spore population within the rods remained stable when the rods were stored for several days at room temperature.

Fabrication of epoxy rods. Solid blocks of epoxy plastic were formed by mixing Scotchcast Electrical Resin No. 5 (Minnesota Mining and Manufacturing, St. Paul, Minn.) Part A (syrup) with Part B (hardener) in a 2:1 ratio in a beaker that had been previously coated with Mold Release 225 (Ram Chemicals, Inc., Gardena, Calif.). The release agent was applied to the beaker with a cotton swab, and the coated beaker was baked at 107.2 C (225 F) for 1 hr. The beaker of casting syrup was placed in a 50 C water bath for 3 hr, during which time the plastic polymerized. The epoxy block was disintegrated by means of an electrical sanding belt, and the shavings were collected, autoclaved for 15 min at 121 C, and dried overnight in the 50 C forced-air oven. Sixteen grams of shavings, in a drying pan, were inoculated with 6.4 ml of the undiluted, aqueous stock spore suspension. The inoculum was dried and ground twice, as described for methylmethacrylate, and was stored over silica gel overnight at 20 C. To each 16 g of inoculated and desiccated shavings, 64 g of Scotchcast Resin Part A was added, mixed thoroughly, and followed by the addition of 48 g of Part B. Mixing with a spatula was continued until the whole appeared homogenous. The syrup was evacuated similarly to methylmethacrylate, poured into cotton-stoppered TDT tubes that had been previously coated with release agent (see above), and placed in the 50 C water bath for 3 hr to polymerize. After polymerization, the tubes were sealed in the oxy-gas flame. This method consistently yielded rods that were rigid, clear, free of bubbles, and from which approximately 10^8 spores per gram

were consistently recovered (see Recovery methods).

Equilibration of spore moisture content. Water activity (a_w) is a property of aqueous solutions and is defined as $a_w = p/p_0$, where p and p_0 are the vapor pressure of the solution and solvent, respectively. The connection between relative humidity (RH) and a_w is that a_w is numerically equal to the RH expressed as the fraction RH/100. Under conditions of water vapor equilibrium or equilibrium relative humidity (ERH), the terms a_w, RH, and ERH are interchangeable and define each other. [For a detailed discussion of the a_w concept, see Scott (18, 19).]

To determine the effect of spore moisture content on resistance, methylmethacrylate powder was inoculated and dried as described above. The desired quantity (50 g) of dried and inoculated powder was placed in a pan and spread out to achieve a shallow layer of powder. The pan was placed in a desiccator containing 500 ml of a saturated salt solution. The desired saturated salt solution required to yield a vapor pressure of known a_w or ERH at 25 C in a sealed container was selected from Robinson and Stokes (16). The desiccator was sealed and stored for 14 days at 25 $\pm$ 0.5 C; immediately upon removal of the desiccator lid, 50 ml of methylmethacrylate monomer was added to the equilibrated powder. Lucite rods were formed from this material and sealed in TDT tubes as previously described.

Determination of dry-heat resistance. Replicate TDT tubes containing the various inoculated test materials were heated by complete immersion in a silicone bath operating at the desired test temperatures ($\pm$0.1 C). Following the heat exposure, the TDT tubes were plunged immediately into ice water to cool for 15 min, followed by washing in detergent solution to remove the silicone. After washing and rinsing, the tubes were immersed in saturated alcoholic iodine solution for 10 min, dried with sterile towels, scored, and snapped open. The contents were removed aseptically and examined bacteriologically, as described under Recovery methods.

Determination of rate of heat penetration. Five replicate heat penetration determinations were done for each test material at each temperature studied. This was accomplished by attaching individual copper-constantan thermocouples (B & S, 30 gauge) to the test materials and placing them in TDT tubes. The thermocouples were soldered to the center of one surface of the stainless-steel strip, soldered to one surface of the stainless-steel washers, which, in turn, were placed between the threaded lugs and torqued, inserted into the paper strip, and inserted into the liquid plastics already in TDT tubes prior to polymerization. The TDT tubes were sealed with a rubber cap bound with wire, and the leads extended through the cap to a Bristol 20-point Dyna Master Recorder. Each of these TDT tubes with attached thermocouple was submerged singly into the test bath, and the temperature of the material to which the thermocouple was attached was recorded once per second. The time interval required to raise the temperature of the test material within the TDT tube to the bath temperature, as registered by a control thermocouple, was recorded. After attaining bath temperature, the TDT tube was

plunged into ice water, and the time required for the test material to cool to 50 C (nonlethal temperature) was recorded. With the recorded values, heat penetration curves were drawn for each of the test systems at each temperature studied: semilogarithmic paper was rotated 180°, and time (seconds) was plotted on the abscissa against the number of degrees below bath temperature on the logarithmic coordinate, with 0.1 C below bath temperature as the end point. Corrections for the amount of lethality that occurred during "thermal lag" (time required for the test material to attain bath temperature after immersion, and the time required for the test material to cool to a nonlethal temperature when placed in ice water) were calculated by the graphical method (2). The values obtained by these calculations were subtracted from the total exposure time in the bath to give end points of survival corrected for lethality due to "thermal lag." These corrections are insignificant for experiments in which long exposure times at temperature are involved; consequently, they were applied only when the combined "heat-up" and "cool-down" time was equivalent to or exceeded 3% of the total experimental time exposure.

Calculation of D and z values. Identical experiments (I and II) were duplicated for each test temperature. Paired samples (a and b) were tested at each exposure time within an experiment. Plate counts, in duplicate (a_1 and a_2; b_1 and b_2), were obtained for each of the paired samples. This resulted in the use of four observations per time interval for each of the paired samples in a given experiment. Linear regressions were calculated for the data from each experiment. The regression so obtained was tested for homogeneity of regression to the data obtained for the second experiment at the same temperature. At times, a nonhomogeneity of regression was observed for the data from duplicated experiments. In these instances, the experiments were repeated, taking special care to observe good laboratory technique, and nonhomogeneity of regression was eliminated. The proportion of the sum of squares of deviations (R^2) due to linear regression were calculated, as were the D value (the time interval at the test temperature required to obtain a 90% reduction in the number of viable spores) and the 95% confidence interval for the D value. To obtain z_D values (slope of the thermal destruction curve or the number of degrees required for the thermal destruction curve to traverse one log cycle), linear regressions were calculated of the D values so derived. The general method and assumptions for fitting linear regression, testing of homogeneity, and calculating confidence intervals were those of Ostle (13).

Recovery methods. Paper strips were placed in 100 ml of sterile phosphate-buffered dilution water (1) contained in a micro-Waring Blendor cup and were blended for 2 min at slow speed. Additional 10-fold serial dilutions were prepared in the same buffer and were plated in tryptone-glucose-beef extract-agar. The plates were counted after incubation for 48 hr at 35 C.

Steel strips and washers were examined by placing the individual strips or washers in separate test tubes (15 × 150 mm) containing either 5 ml (for washers)

or 6 ml (for strips) of phosphate-buffered dilution water. The tubes were placed in an ultrasonic bath (power output of 300 w at a frequency of 25 kc per sec) and treated for 12 min. A dilution plate count of the liquid contents of the tubes was made as described for paper.

Lucite rods were removed aseptically from the TDT tubes, placed in a sterile screw-capped tube, and weighed. After weighing, the rod was aseptically introduced through an entry port to a modified Waring Blendor cup containing 200 ml of sterile acetone (Fig. 3). The blendor modification consisted of a stainless-steel disc that had been cut from a vegetable grater and was mounted in place of the rotary knives. The closure for the jar consisted of a threaded lid fitted with a piece of 0.5 inch (1.27 cm) diameter stainless-steel tubing (the entry port) that projected through the lid. The exposed end of the steel tubing was fitted with a Morton closure, and the open end, inside the blendor cup, was positioned approximately $\frac{3}{16}$ of an inch (0.47 cm) above the grinding disc (Fig. 4). The plastic rod was dropped down the steel tubing, and a sterile steel rod, which acted as a piston to drive the rod against the grinding disc, was inserted (Fig. 5). Approximately 0.5 inch (1.27 cm) of plastic was ground from the rod, and the remainder of the rod was withdrawn from the tubing by impaling it with an elongated sterile bodkin. The entry port was again sealed with the Morton closure, and the blendor was placed upon a reciprocating shaker (144 strokes per min) for 0.5 hr at room

Fig. 3. *Modified Waring Blendor jar. The closure for the jar consisted of a threaded lid fitted with a piece of stainless-steel tubing (the entry port) that projected through the lid. A Lucite rod is being aseptically introduced through the entry port to the jar containing 200 ml of sterile acetone.*
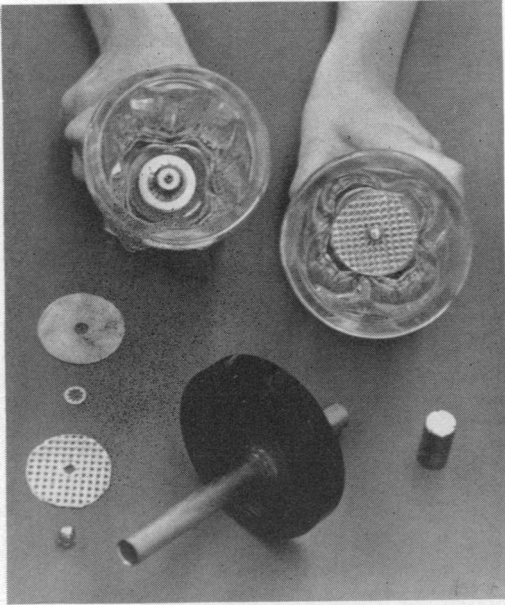
FIG. 4. *Modified Waring Blendor jar. A stainless-steel disc that had been cut from a vegetable grater was mounted in place of the rotary knives.*

temperature, during which time the plastic shavings were completely dissolved. Serial 10-fold dilutions of the blendor contents were prepared in sterile acetone, and 1 ml or more, as appropriate, of the dilution was passed through a Gelman alpha 6 metricel membrane filter, followed by two 10-ml rinses with sterile acetone. The membrane was placed in a sterile petri dish to dry. Drying was usually completed within 30 sec; then the membrane was overlaid with approximately 20 ml of sterile tryptone-glucose-beef extract-agar. The membranes were incubated for 18 to 24 hr at 35 C, and colonies were counted (*see* Tables 1 and 2 for the effect of acetone on the outgrowth and colonial development of heat-treated spores). The weight of the plastic rod was again obtained after grinding, and the number of surviving spores per gram of plastic was calculated.

Epoxy rods also were placed in tubes and weighed. After weighing, the rod was inserted into the entry port of the modified Waring Blendor described above. In this case, however, the stainless-steel grinding disc used to disintegrate methylmethacrylate rods was replaced with a disc of silicon carbide grinding paper (220 A grit) that had been previously leached to remove soluble toxic residues (100 discs autoclaved in 5 liters of distilled water, followed by distilled water rinsing and air drying). The blendor cup contained 200 ml of sterile tryptone-glucose-beef extract-broth with 0.004% Dow Corning antifoam AF added. A 0.5 inch (1.27 cm) section of the rod was ground by applying a 2,000-g weight to the piston. Ten-fold serial dilutions were prepared of the ground suspension in phosphate-buffered water; the dilutions were plated in

tryptone-glucose-beef extract-agar made up to contain 5 ml of Tween 80 and 0.7 g of Asolectin per liter (phenol neutralizer). The plates were incubated for 72 hr at 35 C and counted.

RESULTS

Survivor curves of *B. subtilis* var. *niger* spores were plotted from the data obtained in each heating experiment. With the exception of the Lucite system, a straight line, logarithmic order of death was observed for all the systems. A typical curve is presented in Fig. 6 (epoxy 115 C). The Lucite curves generally were diphasic, and a sharp decrease in numbers (approximately 99%) was noted during the initial heating period, followed

FIG. 5. *Plastic rod was dropped down the steel tubing (entry port) of the blendor jar, and a sterile steel rod, which acted as a piston to drive the rod against the grinding disc, was inserted.*

TABLE 1. *Effect of pretreatment with acetone on the dry heat inactivation of Bacillus subtilis var. niger spores at 125 C*

Exposure time	No. of survivors recovered per strip	
	Acetone suspension of spores dried on paper	Aqueous suspension of spores dried on paper
hr		
0	295×10^6	290×10^6
3	4.25×10^6	5.05×10^6
6	750	5.7×10^4

TABLE 2. *Number of Bacillus subtilis var. niger spores on paper strips surviving 1-hr exposure to 125 C dry heat in sealed TDT tubes*

Suspension	Blended and diluted in acetone	Blended and diluted in water
Aqueous suspension of spores dried on paper[a]	13.5×10^7	17×10^7
Acetone suspension of spores dried on paper[a]	8×10^7	9×10^7

[a] Number of spores per strip of paper: paper inoculated from water suspension = 20×10^7 per strip; paper inoculated from acetone suspension = 15×10^7 per strip.

by a slower rate of death for the remainder of the exposure. The R^2 value for the Lucite experiments often was 0.90 or more despite the initial die-off, and D values were calculated similarly to those for the other systems.

The D values for the test spores obtained with the various sytems are presented in Table 3. Spore resistance to a given dry-heat temperature varied with the carrier in or on which the spores were located. Spores encapsulated in plastics or trapped between stainless-steel surfaces under heavy torque (150 inch-lb) displayed greater resistance than those located on paper and on stainless-steel strips or located between steel surfaces under light torque (12 inch-lb). For example, at 125 C,
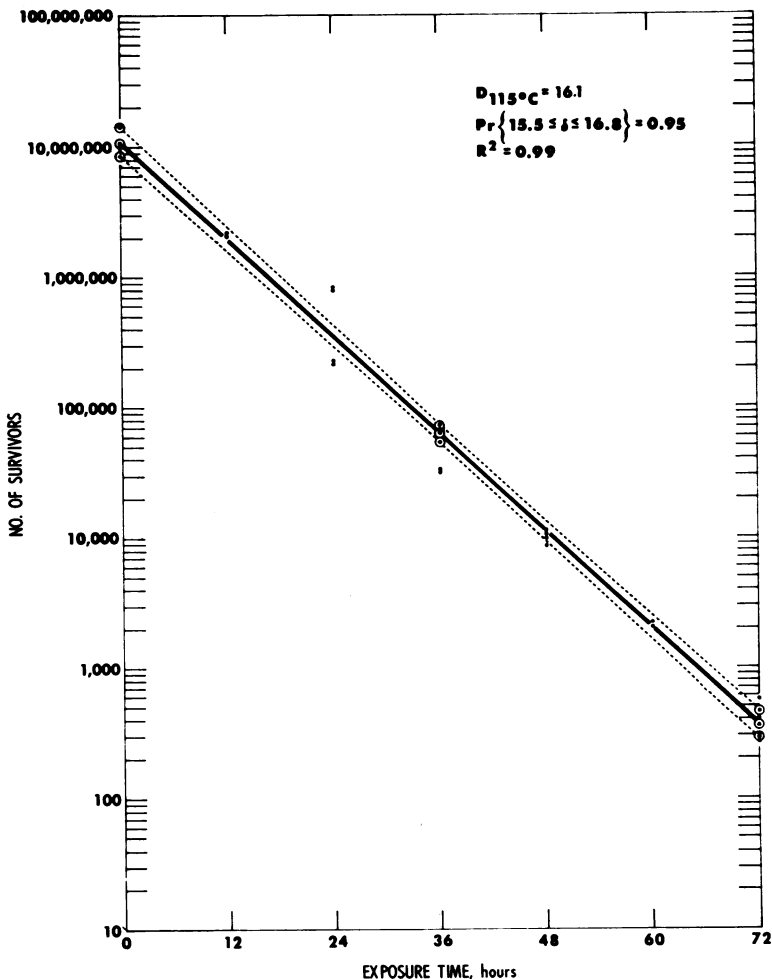

FIG. 6. *Dry-heat inactivation at 115 C of Bacillus subtilis var. niger spores encapsulated in epoxy resin. The solid line represents the linear regression of the survival points; the dashed lines represent the 95% confidence interval for the linear regression.*

TABLE 3. *D value at various temperatures for Bacillus subtilis var. niger[a] spores located in or on different materials*

Dry heat exposure temp	Stainless-steel strips		Paper strips		Stainless-steel washers (150 inch-lb torque)		Lucite rods		Epoxy rods	
	D value (min)	95% CI[b] (min)	D value (min)	95% CI (min)	D value (min)	95% CI (min)	D value (hr)	95% CI (hr)	D value (hr)	95% CI (hr)
C 105							32.0	26.4–33.6		
							28.8	26.4–33.6		
115	24.0	21.7–27.0			102.6	95.4–111.0	15.6	13.4–17.8	16.1	15.2–17.2
	24.4	22.2–27.0			97.8	90.6–106.2	15.1	13.7–16.2	16.1	15.2–16.8
120							6.2	5.6–6.7		
							5.9	5.0–6.8		
125	8.3	7.9–8.9	102.1	96.1–108.2	47.5	43.9–51.8	3.1	2.5–3.6	5.3	5.1–5.7
	8.9	8.5–9.4	102.1	96.1–108.2	44.5	41.8–48.0	3.4	2.9–3.9	5.3	5.0–5.6
135	2.6	2.6–3.0	16.1	15.3–16.9	25.5	20.7–33.3	1.4	1.3–1.5	1.9	1.7–2.1
	2.9	2.3–3.8	17.2	16.0–18.3	22.0	18.2–27.7	1.3	1.2–1.4	1.9	1.7–2.1
140			7.2	6.8–7.6						
			7.1	6.6–7.5						
160							4.6[c]	4.4–4.8[c]		
							4.1[c]	3.6–4.5[c]		

[a] In all tests, the initial number of spores equaled 10^8 per surface or per gram.
[b] Confidence interval.
[c] These values are in minutes, not hours.

an approximate 38-fold increase in resistance was noted for spores encapsulated in epoxy versus that observed for spores on stainless-steel strips. The relative resistance of the spores at 135 C for the various test systems is presented graphically in Fig. 7.

Thermal destruction (TD) curves and their corresponding z_D values were calculated from linear regressions of the semilogarithmic plot of D values and their corresponding temperatures. The values so obtained, as well as their 95% confidence intervals, are presented in Table 4, whereas the curves derived from these plots are shown in Fig. 8. On conversion to the Fahrenheit scale and with the exception of the paper data, these values are large (38 to 58 F) in comparison to the z_D value of 16 to 20 F in the temperature range of 220 to 270 F associated with spores subjected to wet-heat systems (17). By way of contrast, the value for the paper system (z_D = 12.9 C or 23.2 F) approximates that of a wet-heat system.

The influence of water activity upon the dry-heat resistance of spores encapsulated in Lucite is shown in Fig. 9. These data indicate that spores of intermediate moisture content are more resistant

than spores of lesser or greater moisture content. Maximal resistance occurred in the range of 0.2 to 0.4 a_w, and an approximate eightfold increase in resistance was noted between minimally resistant (0.9 a_w, D_{135} = 10.9) and maximally resistant (0.4 a_w, D_{135} = 88.7) spores.

DISCUSSION

Before proceeding with a discussion of the results outlined above, it may be profitable to review momentarily the definition of dry heat and some of the recent findings pertaining to spore permeability and water activity.

The term "dry heat" obviously implies the application of heat in the absence of water. On closer examination, however, one becomes aware that some finite value must be established to define the term "dry" or rather its antithesis "wet". A wet-or moist-heat sterilization cycle may be defined as one in which the organism is in contact with an environment having an a_w of 1.0 or a water-saturated atmosphere. These conditions are met only when the organism is heated in contact with pure water or saturated steam. This definition of wet heat implies that dry heat is not an equally specific
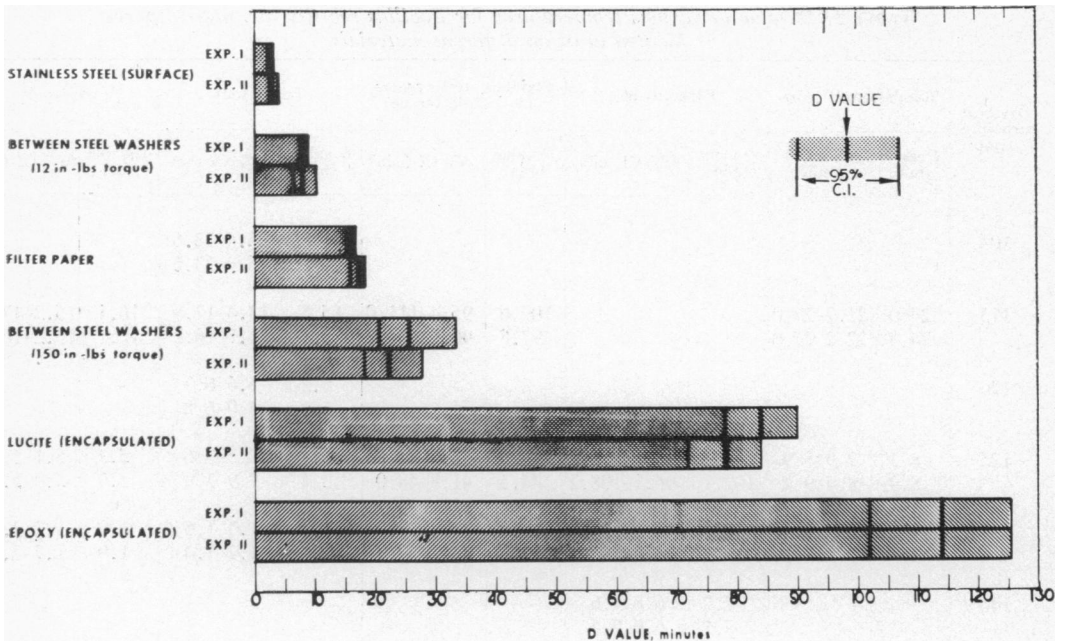
FIG. 7. *Inactivation of Bacillus subtilis var. niger spores in or on various materials exposed to a dry-heat temperature of 135 C.*

condition, but rather a range of conditions that includes such factors as the moisture content of the microorganisms prior to and during heating, the water vapor pressure and flow rate of the gaseous atmosphere in contact with the microorganisms, the chemical and physical composition of the material on or in which the spores are located, and the total pressure of the system.

The works of Gerhardt and Black and their associates (3, 4, 5; P. Gerhardt, S. H. Black, and R. E. MacDonald, Intern. Congr. Microbiol., 7th, Stockholm, p. 22, 1958), of Murrell and Scott (Intern. Congr. Microbiol., 7th, Stockholm, p. 26, 1958), Murrell (9), Murrell and Warth (12), and of Lewis and co-workers (6) have demonstrated that spores are highly permeable and that a free exchange of water occurs between the spore and its environment. The water activity of spores may be expected, therefore, to change in relation to the water activity of the suspending fluid or with the relative humidity of the atmospheric environment. The ability of spores to come to water vapor equilibrium with their environment is an important consideration in establishing dry-heat sterilization cycles. This has been demonstrated by Murrell and Scott (10, 11) in experiments which revealed that: (i) the amount of moisture associated with spores as a result of equilibration

to various water activities prior to heating affects dry-heat resistance; (ii) spores of intermediate moisture content (0.2 to 0.4 a_w) are more resistant than spores of greater or lesser moisture content; and (iii) increased D values may be expected when spores of intermediate a_w are subjected to dry heating conditions that prevent a change in the moisture content of the spores during heating.

The above information is useful in interpreting the results presented earlier. In all of the experiments, except those in which the a_w of spores in Lucite was purposely varied, an attempt was made to achieve an initially uniform, but unfortunately unknown, moisture content for spores in all the

TABLE 4. Z_D *values of Bacillus subtilis var. niger spores obtained in various test systems*

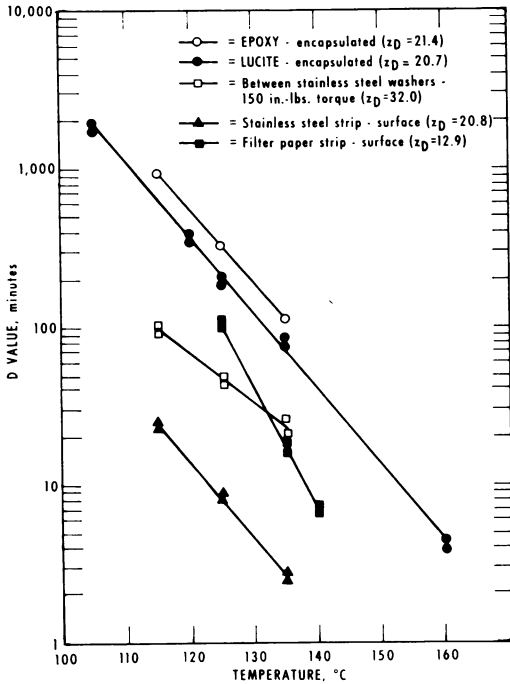| Test system	Z_D value (C)	95% Confidence interval (C)
Filter paper................	12.9	12.5–13.4
Stainless steel (surface)...	20.8	19.3–22.6
Between stainless-steel washers (150 inch-lb torque).................	32.0	28.2–36.8
Lucite (encapsulated).....	20.7	19.3–22.1
Epoxy (encapsulated).....	21.4	20.8–22.1

FIG. 8. *Thermal destruction curves for Bacillus subtilis var. niger spores.*

systems tested. Precautions were taken to prevent or at least minimize any gross change in moisture content of the spores or the carrier systems during manipulations. Additionally, previous tests had demonstrated to our satisfaction that the spore carriers were nontoxic and that quantitative recovery of survivors was being achieved. The data of Murrell and Scott (10, 11) and those of this study (Fig. 9) reveal that one of the factors which affects dry-heat resistance of spores is a_w or the moisture content of the spore prior to and during heating. If the moisture content of the spores and the systems was equivalent at the beginning of each of the heating tests reported here and the systems were nontoxic, then an explanation must be sought for the differences in resistance noted for the various systems (Fig. 7).

Spores that have been dried on surfaces and stored and sealed in TDT tubes in the manner described earlier are in water vapor equilibrium with the gaseous environment within the sealed tube. The ERH within the tube is temperature-dependent and, as the temperature increases inside the tube as a result of immersion in the silicone bath, the moisture-bearing capacity of the gas increases and the RH decreases. This results in the diffusion of moisture from the spore to the hot gaseous environment. This process continues

until the tube contents attain the bath temperature, at which time a new water vapor equilibrium is achieved. At this point, the moisture content of the spores and the relative humidity of the system is greatly reduced. To prevent this change in spore moisture content during heating, Murrell and Scott (11) heated spores in sealed tubes containing salt solutions that maintained the same a_w during heating as that to which the spores were initially adjusted. Another method of preventing spore water loss during heating is by encapsulating spores in nonpermeable plastics. Methylmethacrylate absorbs from 0.3 to 0.4% moisture after immersion in water for 24 hr at room temperature, whereas epoxy absorbs from 0.05 to 0.1% moisture under the same conditions (8). Because of their impermeability, the diffusion of water from the spores to the plastic, or the reverse, would be negligible.

That little, if any, water loss during heating occurred in spores encapsulated in plastic is made evident by a comparison of the data in Fig. 9 to those of Murrell and Scott (11), which were collected in a system employing a controlling solution that maintained the desired vapor pressure at the test temperature. The shape of the survivor
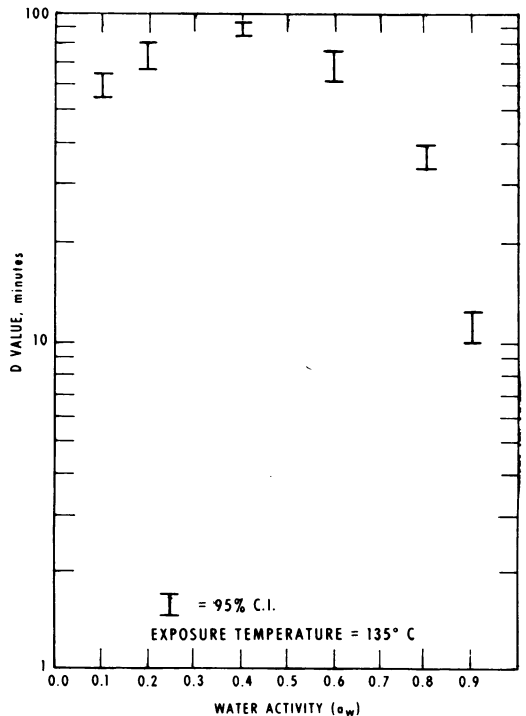

FIG. 9. *Influence of water activity on dry-heat resistance of Bacillus subtilis var. niger spores encapsulated in Lucite.*

curves observed by both sets of experimenters is similar, and both groups observed maximal spore resistance in the range of 0.2 to 0.4 a_w. From these data, one may conclude that there appears to be a critical moisture content of spores which provides them with maximal protection under dry-heating conditions. This moisture content is equivalent to that obtainable by spores when they are equilibrated at RH values of 20 to 40% (a_w 0.2 to 0.4); according to Marshall et al. (7), this moisture content is in the range of 5.5 to 12.4% of the dry weight of the spores, depending on the species.

The differences in resistance displayed by the spores located in or on the various test materials (Fig. 7) may be related to the rate at which the spore moisture content was reduced during heating. Naked systems represented by steel and paper strips would be expected to give up their water to the hot air rapidly, with consequent desiccation of the spores on their surfaces. Water would diffuse from mated surfaces more slowly than from naked surfaces as a function of the mating pressure, and water would diffuse extremely slowly from the plastics. Rapid diffusion of water from the spore to the hot atmosphere within the sealed tube would result in rapid spore desiccation to moisture contents below some critical level (presumably a_w 0.2), at which point rapid destruction rates occur (low D values). Systems which retard or prevent a change in spore moisture content during heating result in slower spore destruction rates (high D values). It appears logical then to attribute the differences in D values shown in Fig. 7 to this mechanism, rather than to some unknown character of the material in or on which the spores were located.

A D value is a measure of the rate of death of an organism at a given temperature (time required to obtain a 90% reduction), whereas a z value is a measure of the change in rate of destruction with temperature and mathematically is equal to the reciprocal of the slope of the thermal destruction curve (TD). The TD curve is constructed by plotting D values logarithmically and temperature arithmetically. Though the D values observed for each of the systems differed considerably, indicating, as previously mentioned, a carrier related effect that may be due to the differences in the water retention capacities of the systems, it is noteworthy that the change in destruction rate with temperature (z) essentially was similar for the plastic and steel-strip systems ($z_D = 21$ C), but quite different for the paper ($z_D = 13$ C) and mated-surface ($z_D = 32$ C) systems. These differences indicate that the "kill mechanism" may vary among systems. For example, the z_D value for paper indicates a wet-heat "kill mechanism,"

whereas the high z_D value for mated surfaces is indicative of a mechanism distinct from that observed in any of the other systems. Insufficient information is presently available to explain the differences in observed z_D values. Studies are in progress, however, to establish the relationship between z_D and spore moisture content and to establish whether significant differences existed in the relative humidities within the sealed TDT tubes of the systems studied.

For the present, then, our results indicate that (i) the initial moisture content of the spores, (ii) the rate of spore desiccation during heating, and (iii) the ERH of the system at temperature influence the dry-heat resistance of *B. subtilis* var. *niger* spores. The rapidity with which the ERH is achieved within a sealed TDT tube appears to be related to the rate at which water vapor is diffused from the carrier system to the hot atmosphere within the TDT tube. Closed systems (mated systems and water-impermeable, plastic encapsulated systems) retard or prevent spore moisture loss during heating, whereas rapid moisture loss during heating occurs in open systems (naked surfaces).

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