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Distribution and Correlation of Events During Thermal Inactivation of *Bacillus megaterium* Spores[1]

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Aqueous suspensions of *Bacillus megaterium* QM B1551 spores were heated at temperatures from 75 to 85 C. The rapid initial viability loss, followed by a more gradual, almost exponential decline, was not due to mixed populations with discrete heat resistances. The slight "tailing" below 0.01% survival was not the result of heat adaptation. Loss of viability was more rapid than loss of dipicolinic acid (DPA) and germinability and, although these events could not be correlated by use of simple kinetic plots, they had similar activation energies (80 to 90 kcal/mole). Probability (probit) plots of per cent survival as a function of logarithmic time yielded not the single line expected, if the heat resistances of individuals in the population were log-normally distributed, but two straight lines intersecting at a survival level of 1 to 6%. Probit-intersects occurred at times ranging from 8 min for spores heated at 85 C, to 310 min at 75 C. Probit-intersects for DPA release and loss in germinability occurred at the same time as for survival, but at much higher levels of retention. There appeared to be two subpopulations, both log-normally distributed but with different mechanisms of kill. Ninety-four to 99% of the spores died via injury to the cell-division process but retained germinability; the remaining smaller subpopulation (1 to 6%) was nonviable because of loss of the ability to germinate.

Various types of survival curves of heat-challenged bacteria have been described. These include the strictly exponential (logarithmic death); those with a shoulder or lag, followed by an exponential decline; those with rapid initial loss in viability, followed by a decreasing death rate; and sigmoid curves, showing both a shoulder and a "tail." "Tailing," or the appearance of high resistance at low survival levels, is often overlooked in published reports, either because the heating time was too short, because the original population was too small, or by unwarranted assumption of a logarithmic model with consequent adjustment of the points with a "best fit" straight line. Deviations from strictly exponential death have been variously attributed to the presence of two or more discrete, discontinuous populations of differing resistances; to the acquisition of heat resistance during the initial portion of the heating period; to a high degree of cell clumping; to the requirement for heat activation of spores; to multitarget kinetics; or, in the case of a tail, to the presence of a few highly resistant cells. Many exceptions to the exponential rule, which is based (8, 31) on the assumption that cells are homogeneous in regard to heat resistance, have been reported.

One of the most common aspects of biological populations is their variability (21). It is unlikely that all spores in a population are equally susceptible to heat inactivation. It is more likely that the shape of bacterial survival curves reflects a continuous distribution of the heat resistances of the individuals in a population. However, cases where the normal distribution curve gives the closest approximation to the observed facts are the exception rather than the rule (14). It is usually possible to transform the distribution by means of a function of the actual observation, and this function is, in turn, normally distributed. Among the most useful of such transformations is the log-normal distribution, and a number of workers (12, 35, 37) have shown that the shapes of a variety of survival curves are more consistent with a log-normal than with a normal distribution of resistances.

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[2] Deceased 19 April 1971.

Many workers, without experimental substantiation, have suggested loss of ability of spores to germinate or changes in germination requirements as bases for apparent loss of viability during heat inactivation. J. M. Scharer (Ph.D. thesis, University of Pennsylvania, Philadelphia, 1965) found that heat-injured *bacillus stearothermophilus* spores eventually germinated to the same extent as control (optimally activated) spores, although their germination rate was reduced. However, the germinability at survival levels <3% was not examined. Edwards et al. (10) suggested that a *B. subtilis* germination system was injured by heating, but that an alternative germination system (in $CaCl_2$ and sodium dipicolinate) remained uninjured, accounting for higher survival when heat-damaged spores were plated on media supplemented with these compounds. However, under certain conditions, spores may be inactivated by radiation (24) or by heat (15) via injury to the postgerminative process of cell division, without injury to the germination system.

Rode and Foster (33) concluded that thermal release of dipicolinic acid (DPA) occurred only upon death of the spores. Data of others indicate that, during heating, loss of DPA lagged behind loss of spore viability (36; J. M. Scharer, Ph.D. thesis, University of Pennsylvania, Philadelphia, 1965; T. Tang, Ph.D. thesis, Illinois Institute of Technology, Chicago, 1967; R. G. Wax, Ph.D. thesis, Pennsylvania State University, State College, 1963).

We have examined the distribution of heat resistances of *B. megaterium* QM B1551 spores and have attempted to correlate heat-induced loss of DPA and germinability with loss in viability. These were apparently unrelated on the basis of simple kinetic plots, but, on the basis of probit plots, we have postulated the presence of two log-normally distributed subpopulations of spores, differing in mechanism of death. Most (94 to 99%) of the spores died (i.e., failed to form colonies) because their capacity for cell division was injured, but retained germinability; the smaller subpopulation (1 to 6%) did not form colonies because of loss of ability to germinate.

MATERIALS AND METHODS

Spore preparation. Spores of *B. megaterium* QM B1551, grown at 30 C on the complex medium (omitting agar) of Arret and Kirshbaum (5), were harvested by centrifugation at 4 C, washed twice with 10 mM potassium phosphate (*p*H 4.65) and six times with distilled water, and lyophilized. A pool of lyophilized spores, sufficient for all the experiments described here, was stored under vacuum at 4 C over silica gel. Contrary to the report of Murrell and Scott (28), we found no evidence of damage or change in heat sensitivity when spores were lyophilized. These spores contained (dry weight basis) 12.5% DPA and ca. 5% water (removable in 24 hr at 100 C). No vegetative cells were microscopically detectable in the spore preparations.

Vegetative cell preparation. Brain Heart Infusion (BHI), at 125 ml/1-liter Erlenmeyer flask, was inoculated with 0.5 ml of an actively growing (6 hr, 30 C) BHI culture of *B. megaterium* QM B1551 and incubated, with shaking, at 30 C for 16 hr, at which time the resulting vegetative cells were in stationary phase. The cells were harvested by centrifugation at 4 C and, after washing twice with *p*H 7.1 Na-K phosphate buffer (Na_2HPO_4, 6 g; KH_2PO_4, 3 g; per liter), were resuspended in the same buffer to a concentration of ca. 8.3×10^9 cells/ml. Vegetative cells were not lyophilized and were tested for heat resistance immediately after harvest. No spores were microscopically detectable in the vegetative cell preparations.

Heating methods. Pyrex (borosilicate) glassware, leached twice (1) with water (122 C, 1 hr), was used to avoid the possibility of alteration of heat resistance by contaminating ions such as Ca^{2+} (3). Glass-distilled water (resistivity $>10^6$ ohm·cm) was used throughout.

Spores were routinely heated at a concentration (0.5 mg of spores/ml, ca. 2.5×10^8 spores/ml) which was high enough to permit detection of a resistant tail, which had an initial optical density (OD) high enough to measure germination turbidimetrically, and which provided detectable levels of DPA. A fresh stock spore suspension (50 mg of lyophilized spores per ml of water) was prepared for each experiment. This suspension, freed of large clumps of spores by filtration through a coarse sintered-glass filter, was kept chilled for ca. 1 hr before use. Similar patterns of heat inactivation were obtained when clumps were disintegrated in a Waring Blendor. The stock suspension was diluted 1:100, usually by adding 1.5 ml of suspension to 148.5 ml of water (in 250-ml Erlenmeyer flasks), which had been previously equilibrated in circulating water baths to the desired temperatures (2.5 C increments between 75 and 85 C). The resulting suspension (*p*H 7.16), containing 0.5 mg of spores/ml, was stirred magnetically during heating to achieve rapid temperature equilibration and temperature maintenance within ±0.1 C. No measurable temperature decrease occurred after the addition of spore suspension to the heated water. By using a single large heating flask, permitting pipetting of the spores directly into the water, and containing enough spore suspension for sampling at all times of exposure, reproducible results were attained. Spore samples were removed at appropriate intervals, rapidly cooled in an ice bath, and kept chilled until tested for survival, DPA excretion, and ability to germinate (germinability).

Vegetative cell suspensions were prepared by adding 4.5 ml of freshly harvested cells to 145.5 ml of Na-K phosphate buffer, previously equilibrated in a water bath to 46 C. The resulting suspension (ca. 2.5×10^8 cells/ml), at 46 C, was magnetically stirred and samples were withdrawn at appropriate time intervals.

Survival. Viability is defined here as the ability to form a visible colony on agar medium. One-milliliter samples of heated spore suspensions were added to 9 ml of chilled water, and appropriate further decimal

dilutions in chilled water were made immediately. Vegetative cells were diluted in Na-K phosphate buffer to avoid the rapid loss in viability of even unheated, water-suspended vegetative cells. Dilution tubes were kept chilled until plated (within 1 hr of dilution) on Thioglycollate Medium (BBL), Formula 135 C, supplemented with 1.5% Agar (Difco). This Thioglycollate Medium Agar (TMA) gave the best recovery of heat-damaged spores of any of the other media examined, including: BHI Agar; Tryptone Glucose Extract Agar; Nutrient Agar; Wynne Agar (all Difco formulations); or these media supplemented, singly or in combination, with 0.2% glucose, sodium thioglycolate, or soluble starch. The more exacting requirements for germination and growth of heat-damaged spores and their greater sensitivity to inhibitory substances have recently been reviewed (32). Supplementation of various media with either 2 or 40 mM $CaCl_2$ and sodium dipicolinate (7, 10) appeared to inhibit vegetative growth of *B. megaterium* QM B1551. TMA (18 ml per 25 by 200 mm Pyrex test tube) was freshly prepared and sterilized only once, on the day it was to be used. Portions of appropriately diluted suspensions were added to 18 ml of molten TMA (maintained at 50 C for spores, or at 43 C for vegetative cells), poured into 10-cm petri dishes, allowed to harden, and overlaid with an additional 5 ml of TMA to minimize spreading of surface colonies, which might obscure late-developing colonies.

The standard recovery temperature was 30 C. Although colonies grew more rapidly at 37 C, higher survival levels were attained at 30 C. A few more colonies eventually appeared at 25 C than at 30 C, but slower growth hindered counting. Although incubation time was routinely set at 48 hr, at survival levels >1 to 6%, full counts were reached in 24 hr; at lower survival levels the colonies were smaller and slower to appear, but by 48 hr they had increased in size and number and did not increase in number with further incubation. The percentage of survival of spores heated for various times between 75 and 85 C was based on comparison with colony counts of spores heat-activated at 65 C for 10 min, a treatment which permitted enumeration of the entire spore population.

Germinability. Samples (4 ml) of heated spore suspensions, added at appropriate time intervals to 2 ml of three times concentrated Thioglycollate Medium (TM) to give a spore concentration of 0.33 mg/ml, were incubated at 30 C. The extent of germination (120 min) on TM, which contains 0.6% glucose, a potent germination agent for *B. megaterium* QM B1551 spores, was determined as percentage of decrease in $OD_{560\ nm}$ (initial OD = ca. 280 Klett units) and as percentage of stainability with 0.5% methylene blue. The percentage of OD loss was normalized relative to the 64% OD loss accompanying near-complete (95%) germination. Germination on 25 mM glucose, buffered with 50 mM potassium phosphate (*p*H 7.0), was essentially identical to germination on TM. The germination rate was calculated as the percentage of OD loss per minute during the period of most rapid linear decrease in OD after the addition of phosphate-buffered glucose.

The ability of heat-injured (80 C) spores to germinate on CaDPA [40 mM each of $CaCl_2$ and sodium dipicolinate in 50 mM tris(hydroxymethyl)aminomethane buffer, *p*H 8.0, with 0.4% gelatin] was also examined turbidimetrically and by staining.

DPA excretion. Samples (7 ml) of heated spore suspensions were centrifuged (17,000 × *g*), and DPA in the supernatant fluids was determined colorimetrically (18) and calculated as percentage of the total spore DPA (DPA in supernatant fluids of autoclaved spore suspensions—125 μg/mg of spores).

RESULTS

Survival. Heating produced a rapid initial drop in viability to the 20 to 35% survival level (Fig. 1A), followed by a less abrupt, almost exponential decline, and finally by a slight "tailing" at survival levels below 0.01 to 0.001% (Fig. 1B). The duration of the first phase of rapid decline in viability depended upon the temperature of heating and ranged from less than 2 min at 85 C to 60 min at 75 C. At 75 or 77.5 C, survival did not go below 0.01% in the 8-hr heating time, and no "tailing" was apparent. The time required to reduce survival to 10% ranged from 5 min at 85 C to 204 min at 75 C (Table 1, Fig. 1).

Survival curves of this type (sometimes described as concave-upward) have been attributed to the presence of populations with two discrete levels of resistance (29). Indeed, Y-intercepts of the apparent second component of survival curves at various temperatures were all at approximately the same value (ca. 40% survival), an indication that 40% of the spores might represent a discrete resistant population. If the spore stock had consisted of 60% sensitive and 40% resistant spores, one would expect survival curves of spores from randomly isolated colonies to have either the steep slope characteristic of the sensitive population or the less abrupt slope characteristic of the more resistant spores. However, survival curves of spores grown from six different isolates (three from unheated spores and three

TABLE 1. *Retention of germinability and DPA during heating, at the time when 10% of Bacillus megaterium spores remain viable*

Temp (C)	Time (min)[a]	Germinability[b]		DPA retained (%)
		OD loss[c] (%)	Stainability (%)	
75	204	78	83	70
77.5	74	81	87	76
80	20	89	87	80
82.5	10	86	87	86
85	5	85	85	90

[a] With 10% viability.

[b] Spores were germinated 120 min in Thioglycollate Medium at 30 C.

[c] Data were normalized relative to the 64% OD loss, accompanying near-complete (90 to 95%) germination.

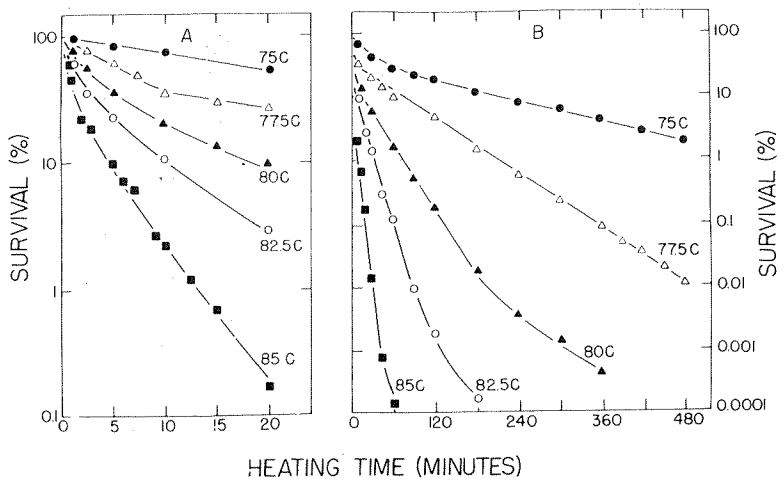

FIG. 1. *Survival of Bacillus megaterium spores heated at various temperatures. A, Survival over the first 20 min of heating, showing rapid initial loss in viability; B, survival over the entire heating period.*

from survivors of spores heated at 80 C for 300 min) were similar to those of the original spore stock (*data not shown*), indicating that the initial rapid decline and subsequent less steep decline were not due to spore populations with two discrete resistance levels. Vas and Proszt (35) and Vas (34) also reported that spores derived from apparently resistant fractions were no more resistant than the original population.

Similarly shaped spore-survival curves have been postulated as being due to transformation of sensitive spores to resistant spores during heating (3, 23), perhaps as a result of conversion of spores to a more resistant "ionic" form (25) or through a protective effect by spore exudates. The use of specially cleaned glassware (1) and high resistivity water negated the possibility of ionic conversion. A mechanism of heat adaptation involving protection by spore exudates (which contain Ca^{2+}, DPA, and amino acids) was also rendered untenable since, in our experiments, survival curves of spores heated at a concentration of 2.5×10^8 spores/ml were unaltered by heating in the presence of supernatant fluids of heated (80 C, 360 min) spores (*data not shown*). Furthermore, neither heat-killed spores nor their exudates affected the thermal death rate of *B. pumilus* spores (23). The slight change in *p*H during heating in water (*p*H dropped from 7.16 to 6.66 after 300 min at 80 C) was not a factor, as similar survival curves were obtained with spores heated at 80 C in 50 mM potassium phosphate, *p*H 7.2, or in *p*H 7.2 Na-K phosphate buffer (*data not shown*).

Vegetative cells, heated at a much lower temperature (46 C), also suffered a rapid initial loss in viability (to 1% survival), followed by a less rapid, approximately exponential decline (Fig. 2). The final decline in death rate ("tailing") did not occur until fewer than 0.0001% survivors remained.

Germinability. Irradiation (24) or heating (15), sufficient to destroy the capacity of spores for cell division and subsequent colony formation, does not necessarily affect their ability to germinate. Loss of germinability on glucose-containing TM, whether measured turbidimetrically (Fig. 3) or by stainability (Fig. 4), was slower than, and could not be directly correlated with, loss of via-

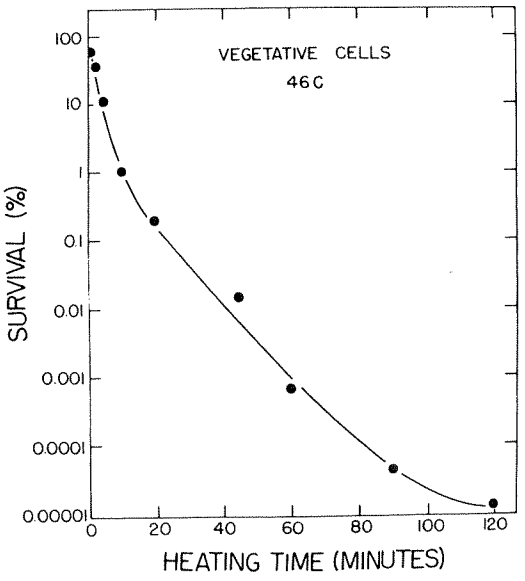

FIG. 2. *Survival of vegetative cells of Bacillus megaterium, heated at 46 C.*

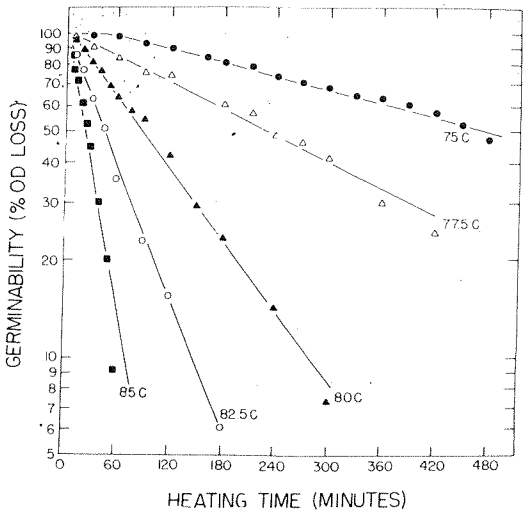

FIG. 3. *Retention of germinability (estimated by OD loss) by Bacillus megaterium spores heated at various temperatures. Germination, estimated as percentage of OD loss (120 min) in Thioglycollate Medium at 30 C, was normalized relative to the 64% OD loss accompanying near-complete (ca. 95%) germination.*

bility. When only 10% of the spores were viable, 78 to 89% of them were still capable of germination (Table 1). After prolonged heating at 80 to 85 C, when only ca. 5% of the spores could still germinate, <0.001% of the spores were still viable; 50 to 55% of the spores heated at 75 C for 480 min could still germinate (Fig. 3, 4), but only 2% were still viable.

Spores heated for longer times at the higher temperatures might have lost germinability on TM (probably glucose-induced germination) but might still be able to germinate on 40 mM $CaCl_2$ and sodium dipicolinate, as has been demonstrated for heat-damaged *B. subtilis* spores (10). However, spores heated for varying times at 80 C showed the same pattern of germinability loss on CaDPA as on TM (*data not shown*).

Sublethal heat activates *B. megaterium* spores for germination, increasing both the rate and extent of germination (26). The effects, on glucose-induced germination, of heating at higher temperatures are shown in Table 2. At temperatures between 75 and 85 C, heating for only 0.1 min increased the germination rate over that of unheated spores (<0.05% OD loss per min). Spores heated for 1 min germinated near maximally (86 to 98%), but not necessarily at a maximum rate. The germination rate of heated spores increased or remained essentially unchanged between 1 min and a heating time which decreased with increasing heating temperature. The increase in germination rate with heating time was most evident at the lower temperatures, a rate of 6.45% OD loss per min being attained with spores heated at 75 C for 180 min. Further heating resulted in decreased germination rates. Viability decreased after all exposures beyond 1 min, although the germination rate may have increased, remained stable, or decreased. The fact that viability may be lost while germination rate increased suggests that highly activated (rapidly germinating) spores may be less capable of outgrowth than more slowly germinating spores (16). The subsequent depression of germination rate with continued heating may argue for inactivation of the germination system as a mechanism, under certain circumstances, for spore injury and consequent inability to form colonies (F. F. Busta, Bacteriol. Proc., 1967).

DPA excretion. DPA was released from the spores during heating at various temperatures (Fig. 5). A short period of rapid initial loss (10 to 20%) was followed by a less steep exponential loss. The zero-order kinetics for DPA release described by El-Bisi et al. (11) were not observed. The colorimetric assay (18) was too insensitive for accurate estimation of levels of DPA retention below 5%. DPA was lost from the spores at a much lower rate than was viability and could not be correlated directly with it. Thus, at the time when 10% of the spores retained viability, 70 to 90% of their DPA was retained (Table 1); more DPA was lost from

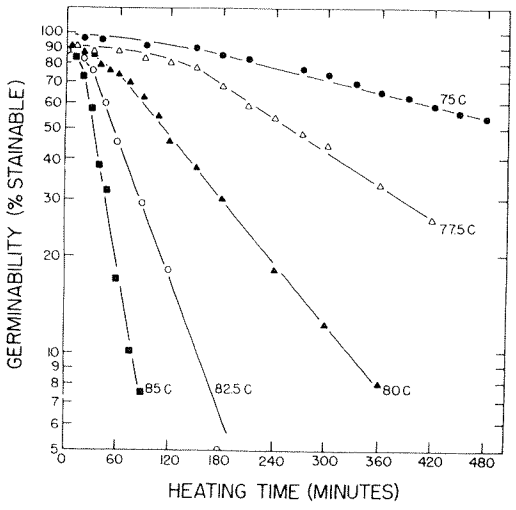

FIG. 4. *Retention of germinability (estimated by percentage of stainability) by Bacillus megaterium spores heated at various temperatures. Spores were germinated for 120 min at 30 C in Thioglycollate Medium. Some germinability was lost after exposure at the shortest time at temperatures above 75 C.*

TABLE 2. *Effect of heating on rate and extent of germination of Bacillus megaterium spores in glucose*

Temp (C)	Time of heating[a] (min)	Germination[b]		Survival (%)
		Rate (% OD loss/min)	Extent (%)	
75	0.1	1.90	79	100
	1	2.55	98	100
	180	6.45	85	10.3
	300	4.15	71	6.5
77.5	0.1	2.30	81	100
	1	2.60	92	92
	90	3.50	85	8
	300	1.88	44	0.22
80	0.1	2.60	78	100
	1	2.52	92	80
	30	2.90	84	6.5
	300	0.43	14	0.0015
82.5	0.1	1.95	82	100
	1	1.45	87	77
	30	1.40	76	1.51
	120	0.25	18	0.0016
85	0.1	1.24	80	100
	1	1.00	86	47
	20	1.00	73	0.17
	60	0.25	18	0.00015

[a] Shortest heating time was 0.1 min. The germination rate either increased or remained essentially unchanged after heating at times between 1 min and the third time indicated for each temperature. When heating was extended beyond the third time, the germination rate decreased rapidly. The fourth indicated heating time for each temperature was either the longest exposure used in study of germination rate (300 min) or the time, later than which, the germination rate was <0.1% OD loss/min.

[b] Heated spores were germinated at 30 C in 25 mM glucose, buffered with 50 mM potassium phosphate, *p*H 7.0. Rate was estimated as the highest percentage of OD loss per minute attained during germination; extent was estimated as the percentage of spores staining after 120 min. The germination rate of unheated spores was <0.05% OD loss/min.

spores heated at the lower temperatures, where longer exposure was required to achieve 90% loss in viability. Spores heated at higher temperatures eventually lost most of their DPA, but only after survival levels had dropped below 0.001%. Our results tend to confirm the conclusion of El-Bisi et al. (11) regarding the existence of more than one DPA pool in the spore. A portion of the DPA, apparently not associated with spore resistance, was gradually released during heating at the lower temperatures. On the other hand, some DPA, requiring severe or prolonged heating for its release, may be associated with thermal resistance.

Distribution of resistances (a reexamination of the data). The inability to explain the survival curves on the basis of either heat adaptation or the presence of discrete spore populations, and the lack of any apparent correlation (on simple kinetic plots) between loss of DPA or germinability and loss of viability, prompted a reexamination of the data on the basis of a continuous distribution of resistances in the spore population.

Defining resistance as the length of time a spore survives at a given temperature, a frequency distribution (including ca. 95% of the population) of resistances of spores heated at 80 C is shown in terms of arithmetic (Fig. 6A) and logarithmic (Fig. 6B) time. The sharp positive skewness of the arithmetic plot is transformed to a distribution approaching the normal in logarithmic time, indicating an approximately log-normal distribution of resistances of our spore stock. The heat resistances (46 C) of vegetative cells were also approximately log-normally distributed.

In a log-normal distribution, the mean survival time (M) can be calculated as the logarithm of the time when 50% of the cells survive; the standard deviation (λ) from the mean is the difference between M and the logarithm of the time when either 84 or 16% of the cells survive. Values for M and λ of spores and vegetative cells, expressed in log seconds and calculated from our data on the basis of a log-normal distribution, are shown in Table 3. Although M of

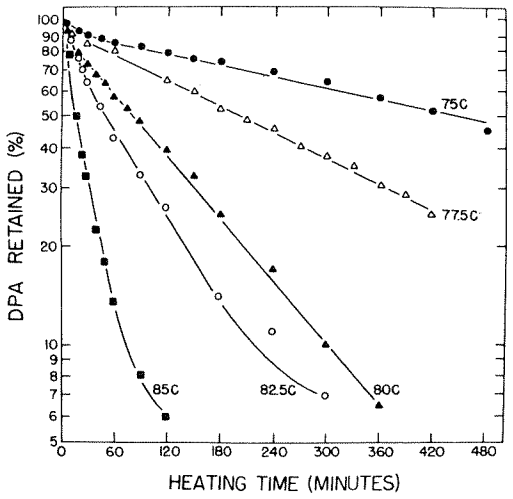

FIG. 5. *Retention of dipicolinic acid (DPA) by Bacillus megaterium spores heated at various temperatures. Based on DPA released by autoclaving (125 μg of DPA/mg of spores).*

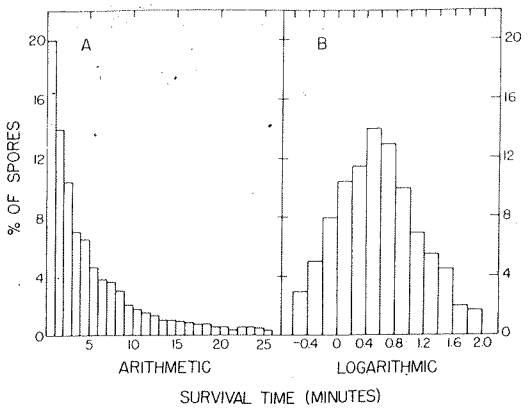

FIG. 6. *Frequency distribution of survival times of Bacillus megaterium spores heated at 80 C. The ordinates represent the percentage of the original spore population killed per 1.0-min increment of arithmetic time (A) and per 0.2-increment of logarithmic time (B). Each curve represents ca. 95% of the total spore population.*

spores ranged from 1.7 to 3.1, λ varied little with temperature and was quite high (0.58 to 0.69). Such values (M and λ) can be used to describe the survival curves of a culture (35).

In a strictly log-normal distribution, a plot on probability (probit) paper of the percentage of survival as a function of logarithmic time is linear. Such plots have been used to linearize data (12, 37). A logarithmic probability plot (probit values from reference 6) of survival of vegetative cells at 46 C was linear (Fig. 7), confirming the log-normal distribution of the heat resistances of vegetative cells. Probit survival plots for spores, however, yielded not the single line expected from a log-normal distribution, but two straight lines of differing slopes intersecting at about 1 to 6% survival (Fig. 7). The probit-intersects occurred at times ranging from ca. 8 min for spores heated at 85 C to ca. 310 min at 75 C (Table 4, Fig. 7). Each of the two straight-line segments of the probit plot may represent a separate log-normally distributed fraction of the spore population; the frequency distribution of Fig. 6 represents only the larger fraction (ca. 95%). Probit plots representing the larger fraction at each temperature were approximately parallel, reflecting approximately equal λ (in terms of logarithmic time). Probit plots of the smaller fraction also approached parallelism.

Neither the point of change in the survival plot (Fig. 1) from the rapid initial decline nor the "tail" corresponded with the probit-intersect at 1 to 6% survival (Fig. 7). The rapid initial decline to ca. 20 to 35% survival may reflect the high standard deviation of the larger log-normally distributed population. The "tail" was apparent only after <0.01% of the spores survived. The differences in slope of the probit plots of the two subpopulations may reflect the differences in standard deviations (λ), the larger population (average λ = ca. 0.63) being more heterogeneous than the smaller subpopulation, where average λ = ca. 0.35.

Probit-log time plots of germinability (Fig. 8) and of DPA retentions (Fig. 9) each yielded two series of intersecting lines which were parallel for the various temperatures. Each segment (early or late) of the bilinear germinability and DPA probit plots (Fig. 8, 9) was approximately parallel to the corresponding segment of the survival probit plot (Fig. 7). Probit-intersects for germinability and DPA retention occurred at about the same time as probit-intersects for viability, but at higher levels of germinability or DPA retention (Table 4). For example, after 24 min (time of probit-intersect at 82.5 C), only 2.5% of the spores were viable, but 76% of the germinability and 73% of the DPA were retained.

It might be argued that a single, gradually curving line could be drawn through our data points on probit paper. A strictly exponential order of death yields such a gradually curving line (17), with no possibility of a straight line through any but very short segments. However, our data appear to show really abrupt changes in slope, and, for each temperature, the probit-intersects occurred at the same time though at different levels for retention of viability, germinability, and DPA.

Activation energy. Velocity constants for survival and for retention of germinability and of

TABLE 3. *Mean (M) and standard deviation (λ) from mean, assuming a log-normal distribution of Bacillus megaterium heat resistances at various temperatures*

	Temp (C)	Mean[a] (log sec)	λ[a] (log sec)
Spores	75	3.10	0.69
	77.5	2.68	0.67
	80[b]	2.26	0.61
	82.5	1.98	0.63
	85	1.71	0.58
Vegetative cells	46	2.08	0.34

[a] $M = \log t_{50}$; $\lambda = M - \log t_{84} = \log t_{16} - M$, where subscript indicates percentage of survival at t sec. Time, t, was converted from minutes to seconds to avoid use of negative numbers.

[b] At 80 C, for example, 50% of the spores survived antilog 2.26, or 182 sec; 84% survived antilog (2.26 − 0.61), or 45 sec; and 16% survived antilog (2.26 + 0.61), or 741 sec.

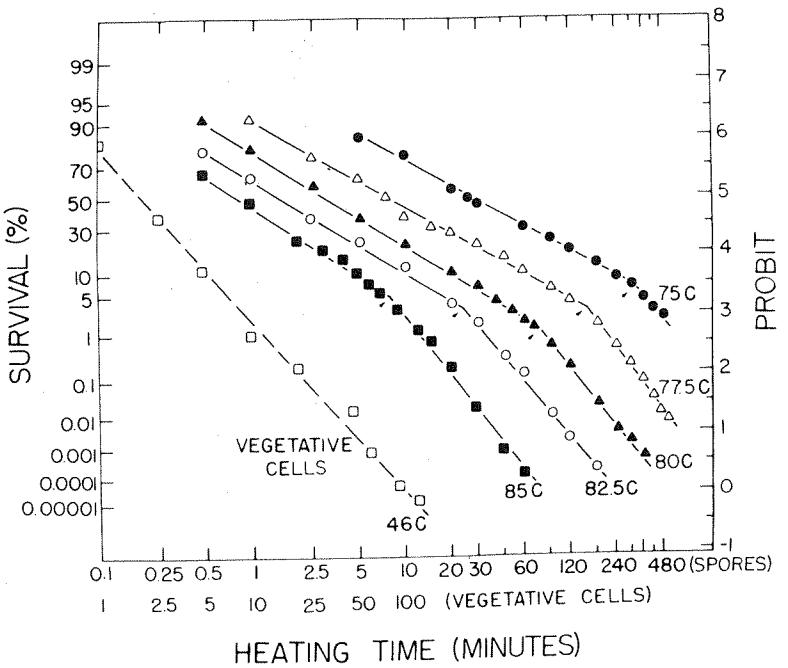

FIG. 7. *Probits of survival of Bacillus megaterium spores (heated at 75 to 85 C) and of vegetative cells (heated at 46 C) as functions of logarithmic time. Data from Fig. 1 and 2. Arrows indicate probit-intersects. Note different logarithmic time scales for spores and vegetative cells.*

DPA were calculated from the straight-line portions of the plots in Fig. 1, 3, and 5, respectively; rate functions describing time of probit-intersect for survival were derived from Fig. 7 as the reciprocal of probit-intersect time. Arrhenius plots (*not shown*), over the temperature range 75 to 85 C, were linear for all of these functions, and activation energies (μ), calculated from the slopes of the Arrhenius plots, were (kcal/mole): 91.3 for survival, 80.7 for germinability retention, 78.8 for DPA retention, and 96.1 for time of probit-intersect. These activation energies, in the range associated with denaturation of proteins and other macromolecules, were of similar magnitudes to those reported by R. G. Wax (Ph.D. thesis, Pennsylvania State University, State College, 1963) for release of DPA and loss of viability during heating of spores of a thymine-requiring strain of *B. subtilis*.

DISCUSSION

Many mathematical models have been postulated to explain the shape of survival curves, but these, in general, were applicable only to the particular data being analyzed. Kinetic modeling can be useful for elucidating mechanisms of death, but models must be substantiated by pertinent biological experiments (30). In contrast, in this report we have treated heat resistance as a continuously distributed function and have attempted, in this way, to correlate loss of viability with other events (loss of germinability and DPA) occurring during thermal inactivation of spores.

Probit plots of survival of *B. anthracis* and *B. subtilis* spores (12) indicated a single log-normal distribution of resistances. Other reported data replotted on probit paper also indicated single, log-normal distributions of heat resistances of

TABLE 4. *Probit-intersects of events during heat inactivation of Bacillus megaterium spores*

Temp (C)	Probit-intersect for					
	Viability		Germinability[a]		DPA	
	Time (min)	Retention (%)	Time (min)	Retention (%)	Time (min)	Retention (%)
75	310	6	310	68.5	314	65
77.5	155	2.5	156	68	152	60
80	75	1.1	73	60	75	54
82.5	24	2.5	22	76	24	73
85	8	4.3	13	72.5	8	83

[a] Germinability, measured as percentage of OD loss in 120 min in Thioglycollate Medium at 30 C, was normalized relative to the 64% OD loss accompanying near-complete (90 to 95%) germination.

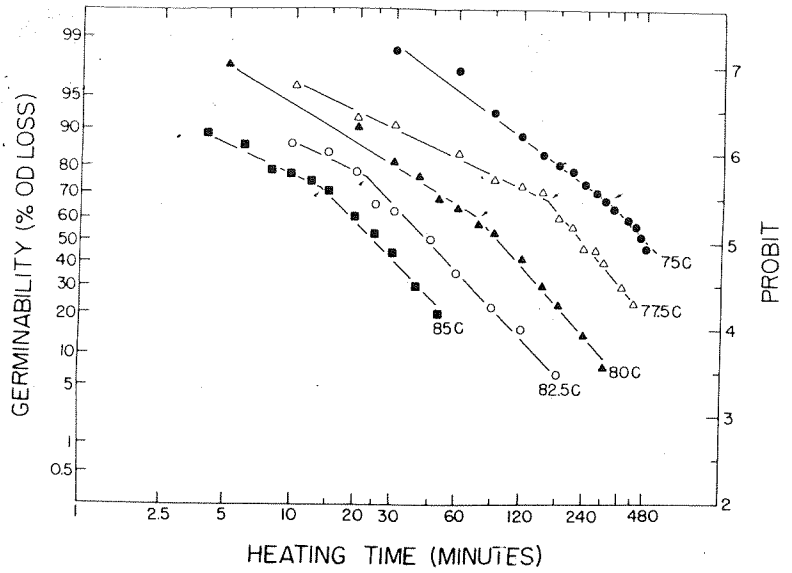

FIG. 8. *Probits of retention of germinability (estimated by OD loss) by heated Bacillus megaterium spores as functions of logarithmic time. Data from Fig. 3. Arrows indicate probit-intersects.*

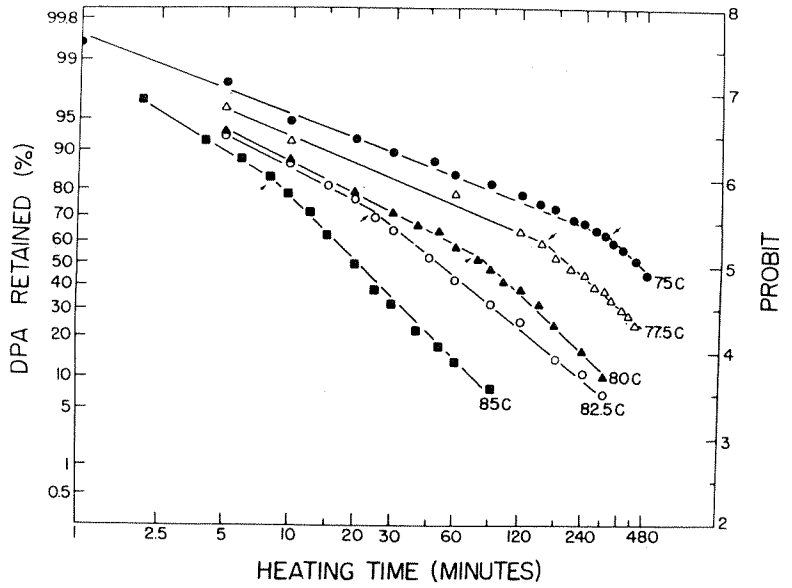

FIG. 9. *Probits of retention of dipicolinic acid (DPA) by heated Bacillus megaterium spores as functions of logarithmic time. Data from Fig. 5. Arrows indicate probit-intersects.*

spores (2, 4, 9; T. Tang, Ph.D. thesis, Illinois Institute of Technology, Chicago, 1967).

In a few cases, replotting of published spore survival data on logarithmic probit paper revealed distributions similar to ours, i.e., two straight lines intersecting at a definite survival level. Nonexponential *B. coagulans* survival curves (13) showed a single log-normal distribution of resistances when survival levels reached 2%, but with survival data given to 0.2%, a probit-intersect at ca. 1.5% survival was evident. Data replotted from Silverman (National Aeronautics and Space Administration Report NASA-CR 70029, Massachusetts Institute of Technology, Cambridge, Mass., 1966), for *B. subtilis* var. *niger* spores, and from J. M. Scharer

(Ph.D. thesis, University of Pennsylvania, Philadelphia, 1965) for *B. stearothermophilus* spores, indicated probit-intersects at ca. 5% survival. A number of probit plots for *B. subtilis* var. *niger* spore survival presented by Schalkowsky and Wiederkehr (National Aeronautics and Space Administration Report NAS-w 1340, Exotech. Inc., Washington, D.C., 1966) showed evidence of two straight lines intersecting at various survival levels. Since their main interest was heat sterilization, they considered only the later stages of inactivation and reported a model based on a single log-normal distribution of resistances, not considering the possibility that the initial loss in viability might represent another log-normally distributed population. Detection of more than a single distribution of resistances in a bacterial spore population probably requires heating temperatures sufficiently low to permit estimation of high survival levels, and exposure time sufficiently extended to permit accurate measurement of survival at low levels.

Among nonsporulating organisms, Withell (37) found that probit plots of survival times indicated an approximately log-normal distribution of resistances to both heat and chemicals. Moats (27), using survival data for three nonsporulating organisms, proposed a model involving the inactivation of multiple critical sites, occurring at random in a homogeneous population, and postulated that the frequency of sites inactivated per bacterium was normally distributed. Koch (22), however, pointed out that such multihit kinetics simulate the log-normal distribution, and replots of Moats' data do indeed yield straight-line plots on logarithmic probability paper. Jordan and Jacobs (20) concluded that probit-log time plots of *Escherichia coli* survival at 51 C were very closely approximated by two lines intersecting at ca. 30% survival. Although these authors suggested that a continuous curve might provide a better fit, they did propose, in an analysis of phenol resistance (19), that a bilinear probit plot might indicate two log-normally distributed populations of different resistances.

A log-normal distribution of resistances is not, however, a valid basis for postulating the mechanism of death without collateral evidence (22). The similarity of our logarithmic probability curves for survival to those for DPA release and for germinability is, however, suggestive of a relationship between these events. It may be that spores which lose DPA were either more susceptible to loss in germinability or that these two effects resulted from a common mechanism such as disruption of the cell membrane. At the time of probit-intersect for survival (1 to 6% survival), there was also a probit-intersect for retention of germinability and of DPA. Up to the time of the survival probit-intersect, only a small portion of the germinability and DPA had been lost. We postulate that the probit-intersect in survival plots marks the separation of two subpopulations, each log-normally distributed in heat resistance. Approximately 95% of the spores are included in the first subpopulation, ca. 5% of the spores are in the second. We feel that a likely postulate is that, in the larger population (95%), the capacity to germinate was only slightly damaged by heating and that death of individuals in this subpopulation occurred through injury to the cell division process. Individuals in the smaller subpopulation, we believe, were unable to form colonies owing to injury to their capacity to germinate, whether on TM, glucose, or CaDPA. Macrocolony formation from the smaller fraction of spores was much slower (plates countable only after 48 hr of incubation) than that from spores of the larger subpopulation (plates countable after 24 hr of incubation), suggesting delayed germination. Vegetative cells, which have no need to germinate in order to form colonies, exhibited a single log-normal distribution, with no probit-intersect.

The shoulder or lag in survival curves has been attributed to clumping, to the necessity for heat activation, or to multihit kinetics. However, a log-normally distributed population implies a sigmoidal survival curve with both a lag and a tail, the exact shape of the curve depending on the mean survival time and on the standard deviation (35). In two populations, each with the same mean survival time, the smaller the standard deviation, the more pronounced the shoulder. Since log-normal distributions with a large standard deviation from the mean tend to result in survival curves with a shortened lag, it is not surprising that our curves do not generally exhibit an appreciable shoulder. Another spore crop with a smaller standard deviation might show the shoulder typical of many published survival curves. Furthermore, treatment of heat resistance as a log-normally distributed function avoids the frequently encountered necessity for disregarding the "tail" in postulating models for thermal inactivation (27). With some spore populations, unlike ours, a more substantial fraction of the spores may die via loss of ability to germinate. The sporulation and harvesting procedures (including washing at *p*H 4.65) which we used may have resulted in a high percentage of spores sensitive to injury via the cell division process. Even with our spores, if higher temperatures of heating had been used so that 1 to 6% survival levels were reached after very short heat exposures, the fraction of spores dying via injury to

cell division might have been overlooked, and we would have seen a single logarithmic probability plot of the same slope as that of the smaller fraction (dying via injury to the germination system).

Spore resistance to heat can, in most cases, be interpreted as more closely reflecting a log-normal than a normal distribution. The shape of the survival curve is dependent on the mean and on the standard deviation from the mean. Assumption of a continuous distribution of resistances does not allow postulation of the molecular mechanism of death, but it does permit graphic analysis of time-temperature effects, extrapolation to low survival levels, and correlation of various events occurring during thermal inactivation.

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