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Sequence of Events During *Bacillus megaterium* Spore Germination[1]

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ABSTRACT

LEVINSON, HILLEL S. (U.S. Army Natick Laboratories, Natick, Mass.), AND MILDRED T. HYATT. Sequence of events during *Bacillus megaterium* spore germination. J. Bacteriol. **91**:1811–1818. 1966.—An integrated investigation of the sequence of events during the germination of *Bacillus megaterium* spores produced on three different media—Liver "B" (LB), synthetic, and Arret and Kirshbaum (A-K)—is reported. Heat-activated spores were germinated in a mixture of glucose and L-alanine. For studies of dipicolinic acid (DPA) release and increase in stainability and phase-darkening, germination levels were stabilized by the addition of 2 mM $HgCl_2$. Heat resistance was measured by conventional plating techniques and by a new microscopic method. The sequence (50% completion time) of LB spore germination events was: loss of resistance to heat and to toxic chemicals (3.0 min); DPA loss (4.7 min); stainability and Klett-measured loss of turbidity (5.5 min); phase-darkening (7.0 min); and Beckman DU-measured loss of turbidity (7.2 min). The time difference between 50% completion of stainability and complete phase darkening was 1.5 min, in excellent agreement with the microgermination time of 1.49 min as determined by observation of spores darkening under phase optics. Alteration of the sporulation medium modified the 50% completion times of these germination events, and, in some cases, their sequence. In the A-K spores, the rates of loss of heat resistance and DPA were substantially higher than those of the other germination events, whereas in spores produced in the LB and synthetic media all germination events followed an approximately parallel time course. This is discussed from the point of view of spore population heterogeneity and germination mechanisms.

The germination of bacterial spores can be measured in various ways, including: loss of heat resistance; increase in spore stainability, loss of refractility as evidenced by darkening under phase optics and by loss in turbidity of suspensions (2); and by the release of dipicolinic acid (DPA; 15, 18). An equation derived from turbidimetric data (14) describes spore germination kinetics which appear to be identical to those reported by Vary and Halvorson on the basis of phase microscopic observations (17). These authors arbitrarily selected two microscopic end points in germination of individual spores: microlag, the time from addition of germination agent to the first changes in refractility; and microgermination, the time from the end of microlag to completion of the loss in refractility.

The present report on *Bacillus megaterium* constitutes an integrated investigation of the time sequence of these spore germination parameters, and of their possible kinetic equivalence. We have also extended our previous observation (12) on the influence of the sporulation medium on spore germination characteristics to the influence of the sporulation medium on the time sequence of germination events.

The kinetics of turbidity loss during germination are easily followed, but when the kinetics of DPA release or increase in stainability and phase darkening are being estimated, some method must be used to stabilize germination levels at desired intervals, as otherwise spores will continue to germinate while measurements are being made. Stabilization methods involving chilling (4, 18, 19), immediate filtration (9), or acid treatment (18) have previously been reported. We present here a less cumbersome and more

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effective stabilization method based on the $HgCl_2$ (2 mM) inhibition of glucose and L-alanine-induced germination of *B. megaterium* spores (7).

MATERIALS AND METHODS

B. megaterium QM B1551 spores were harvested after 4 days of growth at 30 C on a reciprocal shaker on: (i) Wilson Laboratories Liver Fraction "B" (11), (ii) a synthetic medium (3), and (iii) the complex medium (omitting agar) of Arret and Kirshbaum (1). We refer to these spores as LB, synthetic, and A-K spores, respectively. The germination parameters of the LB spores were studied in detail.

Spores, heat-shocked at 60 C for 10 min in aqueous suspensions, were usually germinated in 10 or 50 mM phosphate buffer (*p*H 7.0) containing 25 mM glucose and 1 mM L-alanine (final concentrations). Occasionally, for DPA determinations, phosphate was omitted from the germination medium. Germination temperature was carefully controlled at 30 C. Spore concentration did not affect the germination rate.

To permit comparison of changes in various germination parameters, data were normalized and plotted relative to completion of the germination event (15 min for LB and synthetic spores; 30 min for A-K spores). For example, the per cent turbidity loss of LB spore suspensions at any time, t equals $(OD_i - OD_t)/(OD_i - OD_{15}) \times 100$, where OD_i is the initial optical density and OD_{15} is the optical density after 15 min. The 50% completion times were noted as reference points. The 100% completion values for each germination parameter are indicated in the appropriate figure legends.

For microscopic observations, germinating spore suspensions were stabilized at various time intervals by the addition of samples to equal volumes of 4 mM $HgCl_2$. Duplicate smears were prepared from this mixture and allowed to dry. A drop of 0.5% methylene blue (in 2 mM $HgCl_2$) was placed on one dried smear, covered with a glass cover slip, and examined for stainability while wet. A drop of 2 mM $HgCl_2$ was added to the duplicate slide, and it was examined for loss of refractility (darkening) under dark-contrast phase optics.

Microgermination time, the time between beginning of loss of refractility and completion of the process, was determined by microscopic observation of spores darkening under phase optics (17).

Turbidimetric estimations of germination were made by following decrease in optical density at 560 mμ on a Beckman DU spectrophotometer; or on a Klett-Summerson colorimeter, with a no. 56 (530 to 590 mμ) filter. Temperature was maintained at 30 C in the spectrophotometer by use of a thermospacer, and in the colorimeter by alternating readings of numerous tubes removed from a thermostatically controlled water bath. The spores were kept well suspended by frequent shaking.

DPA loss during germination was estimated colorimetrically (8) by measuring DPA remaining in spore pellets or excreted into the supernatant fluid. Germination was arrested by addition of equal volumes of 4 mM $HgCl_2$ to samples removed from spore suspensions during incubation in Erlenmeyer flasks shaking in a water bath at 30 C, or by chilling untreated samples in an ice bath. Phosphate (50 mM), which interferes with the determination of DPA, was removed from spore pellets by washing once in 2 mM $HgCl_2$, or, when germination supernatant fluids were being tested, phosphate was either omitted or was used at 10 mM. Supernatant fluids were freed from residual spores, after centrifugation, by passage through a 0.22-μ (pore size) Millipore filter. $HgCl_2$ precipitated the ascorbic acid component used in the colorimetric assay (8), but this precipitate was removable by centrifugation without affecting DPA determinations.

Loss of heat resistance during germination was tested by conventional plating techniques and by a new microscopic method. In the first method, spores (1 mg/ml) were incubated at 30 C in the germination medium and, at intervals, duplicate 0.5-ml samples were removed, and either heat-challenged at 70 C for 10 min or chilled in an ice bath after addition to 9.5 ml of 50 mM phosphate equilibrated to these temperatures. Samples (1 ml) of appropriate dilutions were plated on Brain Heart Infusion Agar (Difco). Plates were counted after 24 hr of incubation at 30 C, and those spores capable of producing colonies were scored as heat-resistant. In the more rapid and less cumbersome microscopic method, 0.5-ml samples of similarly heat-challenged germinating spore suspensions (and controls that were not heat-challenged) were incubated at 30 C for 2 hr in 1 ml of Brain Heart Infusion (Difco), a medium capable of supporting rapid and synchronous postgerminative development; slides were prepared, stained with methylene blue, and examined microscopically. Heat-resistant spores (those surviving the heat challenge) had germinated and divided once in the Brain Heart Infusion medium (except for a very few which did not germinate and remained unstainable); heat-sensitive spores were stainable and had not developed beyond a slight swelling.

The loss of spore resistance to toxic chemicals ($HgCl_2$) during germination was similarly tested. Duplicate 0.5-ml samples of germinating spore suspensions were added either to 0.5 ml of 4 mM $HgCl_2$ or to water. Appropriate dilutions were made in chilled phosphate buffer and 1-ml samples were plated on Thioglycollate Medium (BBL), which counteracts the toxicity of any residual $HgCl_2$. Resistant spores from the germinating suspension produced colonies, but those which had become sensitive to $HgCl_2$ did not form colonies.

RESULTS

LB spores. $HgCl_2$ (2 mM) was an effective stabilizer of germination. Slides prepared 30 min after addition of $HgCl_2$ to suspensions of germinating spores showed no more stainability or phase-darkening than slides prepared immediately after stabilization. (Germination of spores of *B. cereus* strain T, or of *B. subtilis*, was not stabilized by 2 mM $HgCl_2$, but other more specific inhibitors of the germination of

these species might prove effective.) Suspensions which were chilled in an ice bath in an attempt to achieve stabilization did increase in percentage of stainability.

Varying the spore concentration (0.2, 0.5, 1.0, 4.0, or 8.0 mg/ml) or varying the phosphate concentration (0, 10, or 50 mM) had no effect on the final degree or relative rate of stainability or phase-darkening of these spores. Stainability with 0.5% methylene blue started after about 2 min of incubation (Fig. 1). By 15 min, 82% of the spores had become stainable, and the time for 50% completion of this event was 5.5 min. These LB spores never became fully phase-dark, a small central area remaining somewhat refractile. However, spores which had attained their maximal phase-darkening began to appear at about 4 min, and the time for 50% completion of this event (82% of the spores had become dark in 15 min) was 7.0 min, 1.5 min after stainability (Fig. 1). If spores were scored for all stages of phase-darkening, from those just beginning to lose refractility, to those which had completed darkening, the plot coincided with that for stainability; LB spores in all stages of phase-darkening were fully stainable.

Microgermination time (17) for these spores averaged 1.49 min. This was an average of observations of the phase-darkening period of several hundred germinating spores, there being a wide range of microgermination times from 0.79 to 2.22 min. There was no correlation between microlag (time between addition of substrate and first change in refractility) and microgermination time. Spores with a short microlag did not necessarily have a short microgermination time—these were ordered but random events (17).

Optical-density decreases accompanying germination were followed on both the Klett and on the Beckman DU instruments. Spore concentration and wavelength (440 to 625 mμ) affected the initial optical-density reading and the percentage of turbidity loss, but plots relating turbidity loss to the final value were identical. These LB spores appeared, under phase contrast, to be less refractile than other spores, and, as noted above, did not appear to lose their refractility completely. Only about 30% of the original turbidity (560 mμ) was lost in 15 min when spores were used at 0.5 mg/ml. The relative optical-density loss in the Klett colorimeter (Fig. 1) coincided kinetically with stainability increase (50% completed in 5.5 min for both parameters). Optical density loss, measured with the DU spectrophotometer (50% completed in 7.2 min), trailed behind Klett-measured optical density loss and approximated phase-darkening (50% completed in 7.0 min). The divergence in

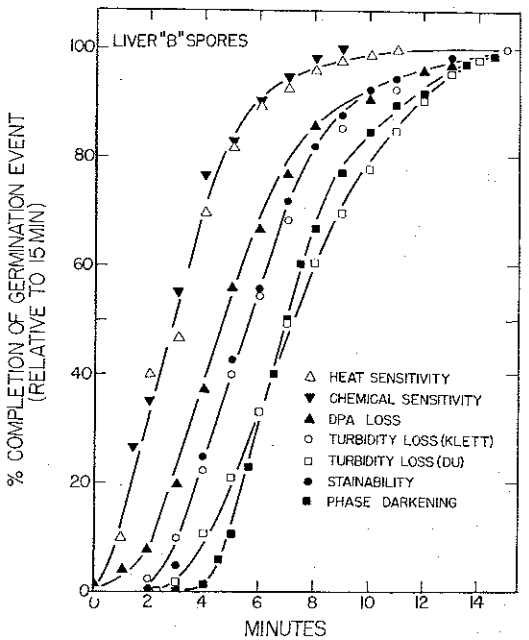

FIG. 1. *Summary of the kinetics of germination events in Bacillus megaterium spores, produced on Liver "B" (LB) medium. Heated (60 C, 10 min) spores were incubated at 30 C in a mixture of glucose (25mM) and L-alanine (1 mM). In 15 min, 88% of the spores were sensitive to heat and $HgCl_2$; 82% were stainable and phase-dark; spore suspensions had lost 34% (Klett) and 26% (DU) of their original turbidity; and 68 μg of their original (85 μg per mg of spores) DPA.*

Klett and DU measurements of optical density was an artifact resulting from use of round rather than plane-sided cuvettes in the Klett instrument. If rectangular cuvettes were used in the Klett colorimeter, results coincided with those of the DU spectrophotometer.

DPA excretion associated with germination was effectively stabilized by 2 mM $HgCl_2$. Spore suspensions to which $HgCl_2$ had been added at various time intervals during germination did not show subsequent excretion of DPA over a 60-min period. Suspensions which were chilled in an ice bath in an attempt to achieve stabilization did continue to release DPA. Varying the spore concentration (1, 2, 4, or 8 mg per ml), the phosphate concentration (0, 10, or 50 mM), or the method of measurement (DPA remaining in the pellet or excreted into the supernatant fluid) did not change the relative rate plot of DPA loss associated with germination. In a typical experiment (Fig. 1), DPA release ($HgCl_2$-stabilized) was 50% complete in 4.7 min, preceding stainability by 0.8 min.

Resistance to heat and toxic chemicals was also determined. Dormant *B. megaterium* spores were resistant to heating at 70 C for 10 min. As incubation in the germination medium progressed, the number of cells capable of growing after exposure to this temperature declined. Germinated spores, not heat-challenged, were all recovered by plating, indicating that they survived the diluting and plating manipulations. Loss of heat resistance (plotted as increase in heat sensitivity in Fig. 1) was measured either by conventional plating techniques or by microscopic observation of cells capable of division. The microscopic method involved a slightly different definition of a heat-resistant spore, not as a heat-challenged spore that produced a visible colony, but as one which was capable of at least one cell division. However, the 50% completion time by both methods was approximately 3.0 min (Fig. 1). Loss of heat resistance paralleled, but preceded, the other germination events. The conversion of the heat-resistant spore population to heat sensitivity was 50% complete at a time when spores had just started to become stainable (Fig. 1). Sensitivity to toxic chemicals ($HgCl_2$) occurred at the same time as heat sensitivity, 50% completion of this event being at 3.0 min (Fig. 1).

In summary (Fig. 1, Table 1), as germination progressed, LB spores first lost resistance to heat and toxic chemicals (50% complete in 3.0 min). By 4.7 min, DPA loss was 50% complete. This was followed by acquisition of stainability and loss of Klett-measured turbidity (5.5 min), by phase-darkening (7.0 min), and by DU-measured loss of turbidity (7.2 min). The slopes of the curves for all of these germination parameters were approximately parallel, however.

Synthetic spores. Germination occurred rapidly with spores produced on the synthetic medium, and germination events were plotted relative to their completion at 15 min (Fig. 2, Table 1).

In 15 min, 96% of the synthetic spores became stainable with 0.5% methylene blue; the average time for completion of this event was 4.3 min. These spores became more fully phase-dark than LB spores. The average time for phase-darkening was 5.4 min, following stainability by 1.1 min. Microgermination time as determined by direct observation of spores darkening under phase microscopy was 1.0 min.

Compared with the LB spores, the synthetic spores lost a higher percentage of their original turbidity (69% Klett-measured loss and 58% DU-measured loss, when spores were used at a concentration of 0.25 mg/ml). Turbidity loss as measured on the DU spectrophotometer lagged only a very short time behind the Klett measurements, 50% completion of turbidity loss occurring at 4.3 min (Klett) and at 4.7 min (DU). Increase in stainability coincided with turbidity losses measured on the Klett colorimeter, but phase-darkening trailed behind DU-measured loss of turbidity.

In these spores, DPA loss was concomitant with increase in stainability and Klett-measured turbidity loss, the 50% completion time being 4.3 min. Acquisition of heat sensitivity paralleled but preceded the other germination events, and was 50% complete by 3.0 min (microscopic method).

A-K spores. Turbidity loss and increase in stainability of spores produced on the Arret-Kirshbaum (1) medium were not complete until 30 min, and germination events were plotted relative to this time (Fig. 3, Table 1).

In 30 min, 96% of the spores had become stainable with 0.5% methylene blue, the time for 50% completion of this event being 11.9 min (compared with 5.5 min for the LB spores). Also, 93% of the spores became fully phase-dark, and the average time for darkening was 13.1 min, 1.2 min later than stainability. The microgermination time, determined by observation of

TABLE 1. *Effect of sporulation medium on time for 50% completion and on the rate of various events in Bacillus megaterium spore germination*

Germination event	Time for 50% completion			Germination rate*		
	LB	Synthetic	A-K	LB	Synthetic	A-K
	min	*min*	*min*	*%/min*	*%/min*	*%/min*
Heat sensitivity	3.0	3.0	3.3	21	19	18
Dipicolinic acid loss	4.7	4.3	5.8	17	19	14
Stainability	5.5	4.3	11.9	16	19	7
Turbidity loss (Klett)	5.5	4.3	10.8	16	19	7
Phase darkening	7.0	5.4	13.1	19	17	7
Turbidity loss (DU)	7.2	4.7	10.8	14	16	7

* Germination rate calculated from rectilinear portion of curves (Fig. 1–3) as per cent completion of germination event per minute.

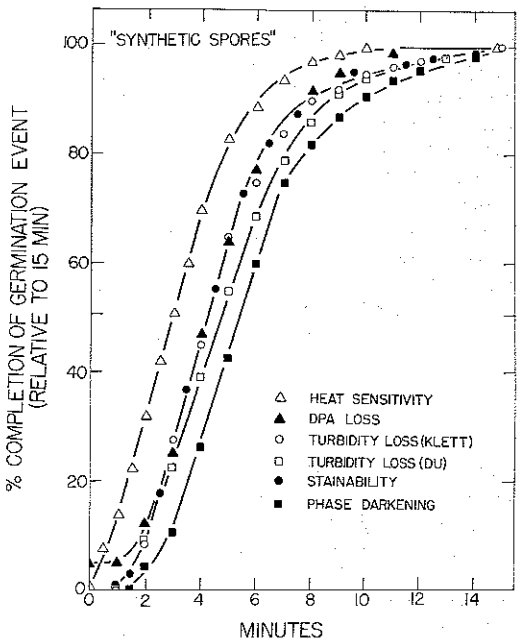

FIG. 2. *Summary of the kinetics of germination events in Bacillus megaterium spores, produced on a synthetic medium. Heated (60 C, 10 min) spores were incubated at 30 C in a mixture of glucose (25 mM) and* L*-alanine (1 mM). In 15 min, 99% of the spores were heat-sensitive; 96% were stainable and 92% were phase-dark; spore suspensions had lost 69% (Klett) and 58% (DU) of their original turbidity; and 96 μg of their original (141 μg per mg of spores) DPA.*

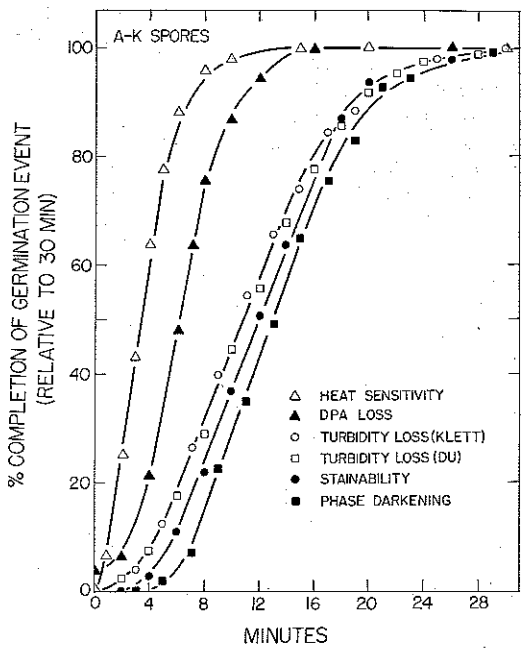

FIG. 3. *Summary of the kinetics of germination events in Bacillus megaterium spores, produced on Arret-Kirshbaum (A-K) medium. Heated (60 C, 10 min) spores were incubated at 30 C in a mixture of glucose (25 mM) and* L*-alanine (1 mM). In 30 min, 98% of the spores were heat-sensitive; 96% were stainable and 93% were phase-dark; spore suspensions had lost 54% of their original turbidity (both on the Klett and DU); and 117 μg of their original (128.5 μg per mg of spores) DPA.*

spores darkening under phase microscopy, was 1.18 min.

Like the synthetic spores, the A-K spores lost a higher percentage of their original turbidity than the LB spores (55% loss on both the Klett and DU) when spores were used at a concentration of 0.2 mg/ml. Turbidity losses as measured on the Klett and the DU were coincident, 50% completion time being 10.8 min. Stainability did not coincide with Klett-measured loss of turbidity, as it did in the other spores, but trailed 1.1 min behind; phase-darkening followed DU-measured loss of turbidity by 2.3 min.

DPA loss was 50% complete in 5.8 min. Heat sensitivity, measured by the microscopic method, preceded DPA loss and was 50% complete by 3.3 min. In the A-K spores, the slopes of the curves for DPA loss and heat sensitivity were significantly higher than the slopes of the curves for stainability and turbidity loss.

DISCUSSION

It is becoming increasingly evident that it may be possible to separate the events occurring during germination. Two microscopic events (microlag and microgermination) have been described for *B. cereus* strain T spores, and these were variously affected by degree of heat activation, L-alanine concentration, and temperature of incubation (14, 17). We find that alteration of the sporulation medium differentially affects these two events; *B. megaterium* spores grown on the A-K medium had a longer microlag than the LB spores (11.8 versus 5.5 min) but a shorter microgermination time (1.2 versus 1.5 min). Uehara and Frank (Bacteriol. Proc., p. 36, 1965) stated that L-alanine-induced germination of Putrefactive Anaerobe (PA) 3679h spores occurred in at least two stages. The spores first lost turbidity, became only partially phase dark, but did not stain. In the second stage, phase-darkening and stainability were complete. Spores incubated at elevated temperatures or in the presence of D-alanine only went through the first stage—turbidity loss had been separated from stainability.

Conflicting estimates of the sequence of germination events are scattered throughout the literature. Comparison or reconciliation of the differing results is difficult since they reflect such a wide range of experimental variables, including: species differences, sporulation and germination media, methods for stabilization (including differential effectiveness of stabilizing techniques for various germination parameters), definition of end point (e.g., does phase-darkening refer to all stages of phase-darkening or only to fully phase-dark spores?), and techniques of measurement and analysis. For example, the sequence of germination events in PA 3679h was: loss of heat resistance, excretion of DPA and of calcium, loss of turbidity coincident with phase-darkening (all stages), and, finally, stainability (Riemann, Ph.D. Thesis, Univ. of Copenhagen, Copenhagen, Denmark, 1963). With *Clostridium roseum* spores (19), staining and loss of heat resistance occurred concomitantly, and preceded calcium and DPA loss, but stainability may not have been stabilized. On the other hand, working with the same species, Hitzman et al. (6) concluded that loss of heat resistance, susceptibility to staining, and loss of turbidity were simultaneous events, but this observation was based on a single reading at 2 min with the reaction being virtually complete by 5 min. In *B. subtilis* (4), loss of heat resistance was followed in turn by the appearance (in electron microscopy) of translucent areas and by decrease in turbidity. The number of spores with translucent areas increased with time (perhaps comparable to microgermination), and optical-density loss reflected the completion of this event. In *B. anthracis* (5), heat resistance loss and optical-density decrease were concomitant. We have found that alteration of the sporulation medium modified the sequence of germination events (Table 1). In LB and A-K spores, DPA loss preceded increase in stainability and turbidity loss, but in the synthetic spores DPA loss was concomitant with increase in stainability and with Klett-measured loss of turbidity. Furthermore, the degree of coincidence of optical-density loss (Klett and DU) and microscopic change (stainability and phase-darkening) was affected by the sporulation medium. Sporulation media, degree of heat activation, and germination conditions may all have their effects on the sequence of germination events.

In a suspension of germinating spores, individual spores have a wide distribution of microlag times, after which they lose their refractility very rapidly (14, 17). Plots describing kinetics of refractility change, therefore, represent a summation of the changes which occur at different times in individual spores of this heterogeneous population. Stainability changes, corresponding to end of microlag, also occur at varying times in individual spores. Time course plots of all-or-none phenomena like heat resistance also reflect spore heterogeneity. Release of DPA from individual spores may be similarly rapid; or single spores may release DPA over a substantial portion of the entire germination period (18). In the latter case, time course plots would represent, not release of DPA by a heterogeneous spore population, but continued release from individual spores. With the possible exception of DPA release, then, the 50% completion time, which we have used as a reference point, approximated the time when one-half of the spores in a population had completed a particular germination event. The rate of germination (slope of curves in Fig. 1–3; Table 1) for a particular parameter reflected spore population heterogeneity—the steeper the slope, the more homogeneous the population for that event. Parallelism of the curves for the various germination events, then, might indicate approximate equivalence in spore heterogeneity for the various events; divergence from parallelism might indicate that the spores were not as homogeneous in relation to one germination event as to another and might further suggest differing mechanisms for different germination events. Thus, in the LB and synthetic spores, heat resistance and DPA were lost in the spore population at approximately the same rate as stainability increase or turbidity loss (Table 1). In the A-K spores, the heat sensitivity and DPA curves were steeper than the curves for the other parameters, suggesting that these spores were more homogeneous with regard to loss of heat resistance and DPA than with regard to other germination criteria. Since, in *B. subtilis*, DPA release and turbidity changes followed the same slope and time course, it was concluded (18) that these events were measures of the same basic germination process. We suggest that, while one mechanism(s) might be responsible for stainability, phase-darkening, and turbidity loss, another mechanism (less sensitive to modification through alteration of the sporulation medium) might govern loss of heat resistance and perhaps of DPA.

Investigators reporting or interpreting results based on turbidimetry should be aware of the apparently artifactual differences between Klett and DU measurements. The percentage loss of original turbidity does not bear a constant relationship to the percentage of stainability or phase-darkening, but depends, to some degree, on the initial refractility of the spores. For example, LB spores lost approximately 30% of their

original turbidity at completion of germination, when 85% of the spores were stainable. The much more refractile synthetic spores, on the other hand, lost about 63% of their original turbidity, and 98% of the spores were stainable at completion of germination. Ionically germinated spores of *B. megaterium*, grown on a different medium, lost 60% of their original turbidity and were 100% stainable (16). Keynan et al. (10) found that calcium DPA-germinated spores of *B. cereus* strain T with low (2.3%) DPA content lost less turbidity than spores with normal (7%) DPA, although all spores were stainable, and they concluded that a staining method was more suitable than the turbidimetric method for measurement of germination of low DPA spores. However, their data, recalculated relative to the final limiting value for each set of experimental conditions, give kinetic plots which are in good agreement for the two types of *B. cereus* spores.

B. megaterium spores, $HgCl_2$-stabilized at all stages of phase-darkening, from those just beginning to lose refractility to those which were fully phase-dark, were stainable with methylene blue (*see also* 13). Inception of stainability, being coincident with the beginning of phase-darkening, corresponded to the end of microlag (17), the average microlag (50% completion of stainability) for the LB spores being 5.5 min. Microgermination time, calculated as the time between 50% completion of stainability and phase-darkening, corresponded to microgermination time as determined by the method of scoring individual spores (17). Although our method, with the use of stainability and phase-darkening of $HgCl_2$-stabilized spores, is probably not as suitable as the Vary and Halvorson (17) technique for the detection of extremely short microgermination times (i.e., in the order of the 16 sec reported for *B. cereus* strain T), we hope that it will prove an adequate and convenient supplement in studies on spore germination kinetics.

Selection of any one parameter or time of germination may give a distorted picture of germination. With *B. megaterium* LB spores, when a 15-min incubation time is used, germination determined by all of the parameters would be identical, and microscopic techniques (staining or phase-darkening) would afford a reasonably accurate measure of the final percentage of germination. However, after 5 min of incubation, depending on the germination criterion, one might conclude that germination had progressed to 82% (heat or chemical sensitivity), 56% (DPA loss), 43% (stainability), 21% (DU-measured turbidity loss), or 11% (phase darkening) of completion. Perhaps by using various substrates that induce germination by different pathways, and by employing other more specific inhibitors, one could demonstrate independence of, or interdependence among, these parameters of germination.

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