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Germination of Single Bacterial Spores

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Changes in refractility and optical density occurring in individual spores of *Bacillus cereus* T and *B. megaterium* QM B1551 during germination were investigated by use of a Zeiss microscope photometer. The curves revealed that the germination process in single spores had two distinct phases; an initial rapid phase was followed by a second slower phase. Under the experimental condition employed, the first phase of germination of *B. cereus* spores lasted for approximately 75 ± 15 sec, whereas the second phase lasted for 3 to 4.5 min. In *B. megaterium* spores, the first phase was observed to last for approximately 2 min and the second phase for more than 7 min. The duration of the second phase was dependent on conditions employed for germination. The kinetics of the first phase were strikingly similar under all conditions of physiological germination. Time-lapse phase-contrast microscopy of germinating spores also revealed the biphasic nature of germination. It was postulated that the first phase represents changes induced by an initial partial hydration of the spore and release into the medium of dipicolinic acid, whereas the second phase reflects degradation of the cortex and hydration of the core.

In recent years, there has been a growing interest in the mechanisms involved in the germination of bacterial spores (5, 10). Germination, triggered irreversibly by physiological germinants such as L-alanine or D-glucose, transforms the activated spores into metabolizing vegetative cells within a short period of time. The evidence indicates that physiological changes accompanying germination involve a series of degradative reactions initially yielding an alteration of the rigid spore structure and leading eventually to the complete hydration of the vital core of the spore. During germination, spores release into the medium dipicolinic acid (DPA), calcium, and peptide containing muramic acid (14, 15). Morphologically, germinated spores lose their characteristic refractility and appear dark when examined by phase-contrast microscopy (12, 16); loss of heat resistance accompanies germination (12). All of these parameters have been used either singly or in combination for measurement of the kinetics of germination in a suspension of bacterial spores (2). Indeed, current knowledge of the stages involved in the germination of bacterial spores is largely based on information obtained from such studies.

It is recognized, however, that these observations reflect only the summation of events occurring in the individual spores of that population (19). Powell (13) called attention to the asynchronous nature of germination of individual spores in a large population and emphasized the importance of the lag period of individual spores in the measurement of the kinetics of germination. The asynchronous nature of the germination process was further analyzed statistically by Vary and Halvorson (19) and Vary and McCormick (21), who concluded that the kinetics of germination of a spore suspension is most critically influenced by the cumulative distribution of the microlag time of individual spores. More recently, a successful recording of the change in optical density of a single spore during germination was made by use of a microscope photometer apparatus (17).

During the course of our work dealing with the germination of single spores of *Bacillus cereus* T and *B. megaterium* QM B1551, we noted that the germination curve of individual spores exhibited a characteristic biphasic nature. A description of our observations and a correlation of these results with those of other workers is the purpose of this paper.

MATERIALS AND METHODS

Preparation of spores. A phage-resistant strain of *B. cereus* strain T, isolated from the original strain at the University of Michigan, was grown and sporulated with vigorous aeration in a modified G medium, as reported earlier (6), by use of a Microferm fermentor (model MF-14, New Brunswick Scientific Co., New Brunswick, N.J.). Complete sporulation was usually attained within 10 to 12 hr after inoculation. The free

spores were collected at 4 C by means of centrifugation in a DeLaval continuous-flow centrifuge; spores were subsequently washed 10 to 15 times with cold distilled water. *B. megaterium* QM B1551, obtained from H. S. Levinson, Pioneering Research Division, U.S. Army Natick Laboratories, was sporulated in the medium of Arret and Kirshbaum (1) without agar. The medium was distributed into 1-liter Erlenmeyer flasks (125 ml each), autoclaved, inoculated with 0.5 ml of inoculum, and incubated on a reciprocating shaker at 30 C for 4 days. The inoculum was prepared by emulsifying a loopful of sporulated cells from an agar slant culture (the same medium plus 1.5% agar) in 10 ml of sterile distilled water, heating the preparation at 70 C for 10 min, and diluting it with approximately 50 ml of sterile distilled water. Subsequent harvesting was as described for *B. cereus*.

Unless the spores were used immediately, 1-ml portions of a dense suspension were dispensed in small glass vials, quickly frozen, and stored at −20 C. Just prior to experiments, spores were gradually thawed, washed twice, and suspended in distilled water [3 to 6 mg (dry weight) of spores/ml]. The spores were then activated by heating at 65 C for various periods of time, washed once, and suspended in distilled water.

Measurement of single-spore germination. A fine Pasteur pipette was used to place a small drop of a spore suspension on a rigorously cleaned cover glass (no. 1 thickness); the suspension was then spread evenly on the surface of the cover glass. The thin film of spores was dried at room temperature (25 C). It was recently observed that most consistent results were obtained if this thin dried film of spores was subsequently immersed in cold absolute methanol for 15 to 20 sec; this treatment did not affect the results and minimized the movement of spores during microscopic observations.

Germination by L-alanine was initiated as follows. The cover glass (smeared side down) was mounted over a glass slide on which a droplet of a germinant solution of desired concentration had been placed. The edges of the cover slip were sealed immediately with melted Vaspar, and the preparation was quickly placed under a Zeiss Universal Microscope equipped with an MPM microscope photometer. To determine the microlag period (19), a stopwatch was used to measure elapsed time; the initial contact of germinant solution with the dried spore film was taken as time zero.

Physiological germinants used in the present study included L-alanine (5 mg/ml) and adenosine (2.5 mg/ml) for *B. cereus* spores and L-alanine (5 mg/ml) and D-glucose (1.8 mg/ml) for *B. megaterium* spores.

For studies of the kinetics of influx of water into spores, an 80% aqueous solution of formic acid was used as a nonphysiological germinant. When spores were treated with this agent, they rapidly became phase-dark, apparently by a one-step process after a lag period of 30 to 90 sec. The *p*H of the germinant solutions was adjusted by means of appropriate buffers as specified in Tables 1 and 2.

Microscopic observations of the germination process of individual spores were carried out in a controlled-temperature room (25 C) as follows. A healthy spore which was well isolated in the field was randomly selected and focused in a restricted (2 μm diameter) field. [The criteria for "healthy" *B. cereus* T spores is based on earlier observations (6) that normal mature spores resist a heat treatment of 85 C for 30 min, appear highly refractile by phase-contrast microscopy, and are uniform in size.] The total magnification of the system was adjusted by means of the Optovar system or by controlling the projection lens system of the photometer so that the refractile portion of the individual spore sufficiently covered the restricted area (which is open to the photosensitive detector system) to give accurate readings. A total magnification of 2,000 times was found to be most appropriate for observations of the germination process. All observations were carried out with monochromatic (540 nm) light. Monochromatic illumination was obtained by the combined use of a filter monochromator and a pin-hole diaphragm (0.1 mm diameter) inserted into the filter holder.

Changes in refractility. A dark phase-contrast microscope equipped with a 100 × objective lens (Neofluar phase 100/1.30, Zeiss) was used for observing changes in refractility. As soon as a selected spore was focused in the designated restricted area, the total amount of light refracted from the ungerminated spore was set at 100% light transmittance. A recorder (Laboratory Recorder V.O.M.-7, Bausch & Lomb, Inc., Rochester, N.Y.) was used to record the changes in refractility occurring during germination as a function of time; unless otherwise specified the recorder was operated at a speed of 2.54 cm per min.

Optical density changes. Since most studies on the kinetics of spore germination have used the change in optical density of the suspension as a parameter, a comparable experiment with a single spore was attempted by use of a bright-field optical system. A spore was initially focused under a 100 × objective (Planapo 100/1.30, Zeiss) in an area adjacent to the photosensitive spot, and the optical density of the clear background of the microscopic field was adjusted to zero (100% light transmission). The focused spore was then carefully brought into the photosensitive area by adjustment of the stage control knobs. The optical density reading caused by interpositioning of the spore was taken as the initial optical density of this spore. During germination of the spore, there was a decrease in optical density (increase in light transmission) which was recorded by use of the previously described apparatus.

Phase-contrast photomicroscopy. To correlate the germination curve of a single spore as recorded above with the actual microscopic appearance of the germinating spore, a number of the spores undergoing germination were followed by use of time-lapse photomicrography. Exposures were made on panchromatic film (Kodak plus X) every 15 sec with a Nikon camera and an automatic exposure system attached to the phase-contrast microscope.

The experimental conditions used for the photomicrographic studies were identical to those employed in following the changes in refractility which accompany germination.

Determination of DPA. The DPA content of dormant spores was determined colorimetrically by the method of Janssen et al. (7), with purified DPA (Matheson, Coleman, and Bell, Norwood, Ohio) as a standard. The percentage of DPA released into the medium after 30 min of germination was calculated by the method of Levinson and Hyatt (9).

RESULTS

Typical germination curves of single spores of *B. cereus* are shown in Fig. 1 and 2. In Fig. 1 germination is recorded as the refractility change as a function of time, whereas in Fig. 2 the change in optical density with time is recorded. The germination of both spores was induced by L-alanine and adenosine at *p*H 8.3. These curves were selected because both spores had similar microlag periods: 3 min 35 sec (Fig. 1) and 3 min 30 sec (Fig. 2). It is apparent from these figures that the germination curve of a single spore is not sigmoid; this is in contrast to the results obtained with spore suspensions, for which the germination curve has generally been described as being sigmoid in nature (21). Two distinct phases can be differentiated in the germination curves of individual spores. The first or initial rapid phase is characterized by a relatively rapid 50 to 70% decrease in refractility and optical density; this first phase, under our experimental conditions, had a duration of 75 ± 15 sec. In contrast to this, the second phase of the germination curve exhibited a slower rate of decrease in refractility and optical density; this second phase usually had a duration of more than 3 min. The tapering portion of the second phase of the curve was taken as the end point of germination. The germination curves of the more than 50 individual spores of *B. cereus* invariably exhibited biphasic characteristics, regardless of the previous treatment of the spores (time and temperature of heat activation, age of spores, etc.) or of the germination conditions (*p*H or "triggering" agent used).

The duration of the microlag and microgermination time (19) of 10 fresh randomly selected spores (five non-heat-activated and five optimally heat-activated) is presented in Table 1. It is immediately apparent that fresh spores, if not heat-activated, are characterized by a significantly longer microlag time than those of heat-activated spores, thus confirming the observations of Vary and Halvorson (19). However, once germination commences, the first phase of the process is completed by the individual spores within a surprisingly narrow time range. Observations made on a limited number of fresh spores, heat-activated at 55 C for 30 min or at 65 C for 30 min or 1, 2, or 3 hr, and on spores stored in the sporulated medium for 15 months at room temperature revealed that the duration of the first phase of germination is very similar to those observed for non-heat-activated spores (Table 1). Under the conditions specified in Table 1, the duration of the second phase was approximately

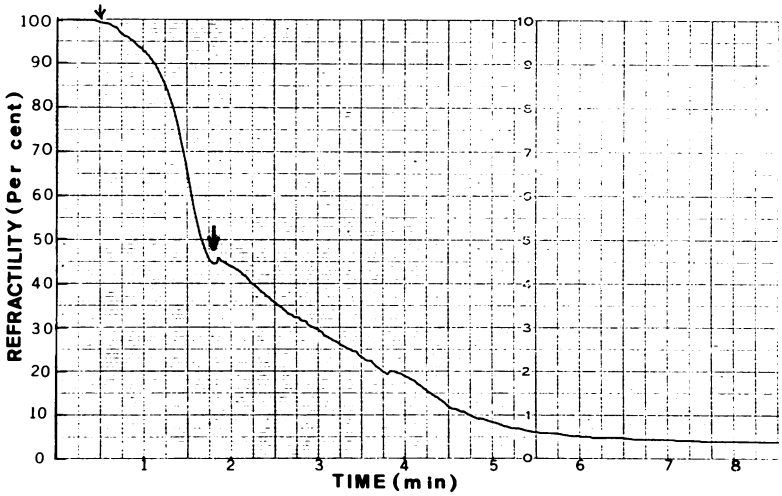

FIG. 1. *Germination curve of a single spore of B. cereus T. Germination was followed by monitoring the loss of refractility. There is an approximately 55% loss of refractility during the first phase of germination. The small arrow indicates the initiation of the first phase; the large arrow indicates the transition of the first phase to the second phase. The second phase tapers off after a 95% loss of refractility. This particular spore initiated germination at room temperature (25 C) after a microlag of 3 min and 35 sec in the presence of* L-*alanine (5 mg/ml) and adenosine (2.5 mg/ml) at pH 8.3 [0.05* M *tris(hydroxymethyl)aminomethane buffer].*

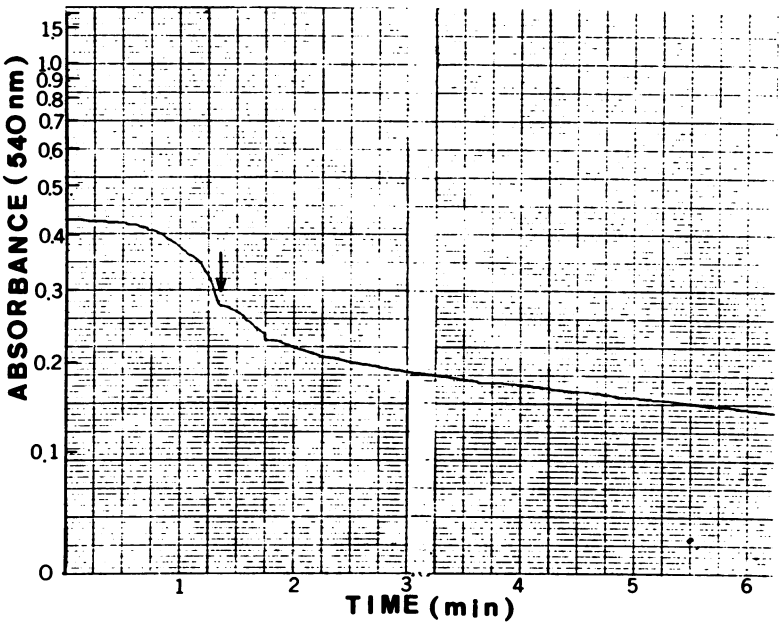

FIG. 2. *Germination curve of a single spore of B. cereus T. The germination process is expressed as the change of optical density with time. The transition region between the first and second phase of germination is indicated by the arrow. The microlag time of this spore was 3 min and 30 sec. Conditions for germination as described in Fig. 1.*

3 to 4.5 min, and loss of approximately 95% of the original refractility was observed at the end of this phase. Thus, the total microgermination time ranges between 4 and 6 min, which is a considerably longer period than that reported by Vary and Halvorson (19) and Rodenberg et al. (17). Therefore, an attempt was made to determine the appearance of the spore, under the phase microscope, at various stages of germination. Photomicrographs were taken under conditions which were virtually identical to those employed for obtaining germination curves. Figure 3 reveals that the most drastic drop in refractility occurs in the first 75 sec (Fig. 3, 0- to 75-sec period), which is in close conformity with the results obtained by means of the microscope photometer analysis. The second phase of germination is characterized in the photomicrographs by a gradual disappearance of residual refractility from the spore (Fig. 3, 75- to 195-sec period). The visual evidence thus supports the photometric evidence, and it appears that germination of *B. cereus* T spores takes more time than generally has been assumed. Although not illustrated, a similar study with *B. megaterium* spores also revealed a convincing correlation between the germination curve and the actual phase-contrast appearance of individual germinating spores.

The observation that the pattern and the duration of the first phase of germination in single

TABLE 1. *Effect of optimal heat activation on the duration of the first and second phases of germination of B. cereus spores*[a]

Heat activation	Expt	Microlag time (min:sec)	Microgermination time (min:sec)	
			First phase[b]	Second phase[c]
None	1	5:38	1:15	4:00
	2	11:25	1:00	4:00
	3	15:40	1:15	3:30
	4	18:18	1:15	4:00
	5	25:05	1:30	4:30
65 C, 4 hr[d]	1	2:15	1:15	3:00
	2	2:40	1:25	3:15
	3	2:55	1:15	3:00
	4	3:10	1:05	3:00
	5	3:20	1:30	3:25

[a] The spores were used immediately after harvesting. Germination was induced by L-alanine and adenosine at *p*H 8.3 (0.05 M tris(hydroxymethyl)aminomethane buffer).

[b] These spores lost 50 to 70% of their initial refractility after completion of this phase.

[c] These spores lost more than 95% of their initial refractility after completion of this phase.

[d] The maximal rate of germination by L-alanine and adenosine was obtained when spores were heat-activated under these conditions.

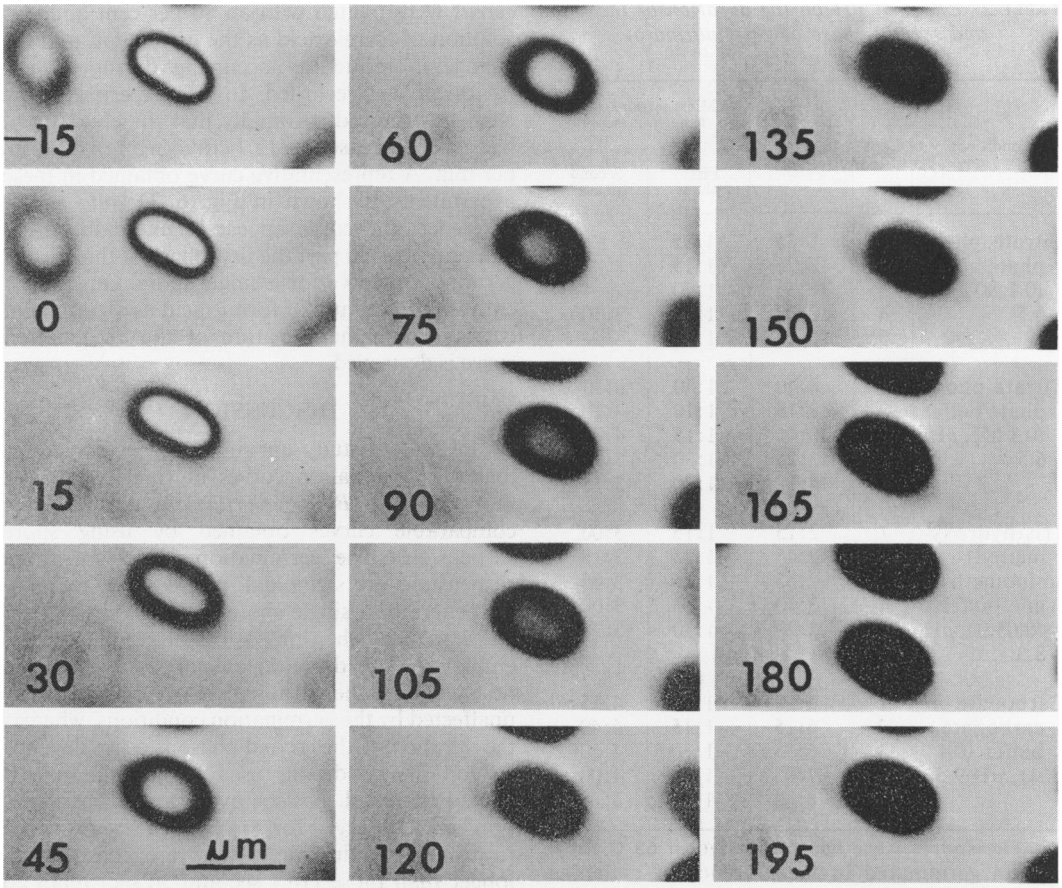

FIG. 3. *Time-lapse phase-contrast micrographs of a germinating spore of B. cereus T. Each frame represents the change in refractility occurring in the spore during a 15-sec interval. The figures on the micrographs indicate time (seconds) elapsed after the initiation of germination. The most rapid loss of refractility occurs in the first 75 sec. Note the persistence and gradual loss of residual refractility in the core region of the spore up to the last frame (195 sec after initiation). The bar in the micrograph represents 1 μm. Conditions for germination as described in Fig. 1.*

spores remains relatively constant despite extreme differences in the physiological condition of the spore (age of spores, conditions of heat activation) prompted us to examine the effects of some environmental factors on each phase of germination (Table 2). It is evident that *p*H of the medium considerably influences both the microlag time and duration of the second phase, and affects the first phase to an insignificant degree or not at all. At *p*H 5.5 (0.1 M citrate-phosphate buffer), only 80% of the spore population underwent incomplete germination (incomplete germination refers to the situation where spores that do germinate only complete the first phase of the process). Even under these suboptimal conditions, the first phase of germination was completed within 75 ± 15 sec, which is comparable to the times observed under better conditions for germination. At *p*H 5.5, the second phase of the curve tended to taper off at the end of the first phase or proceeded very sluggishly, and often lasted for more than 30 min (Fig. 4). Phase-contrast microscopy revealed that these spores retained some detectable refractility and remained in this state almost indefinitely. This type of partial or incomplete germination was frequently observed when the spores were induced to germinate under suboptimal conditions. Although the spores underwent only partial germination at *p*H 5.5, they released most of their DPA into the medium (Table 3).

The type of buffer used in studies on the effect of *p*H on germination may be quite critical, since we noted that germination of *B. cereus* spores occurs, although incompletely, at *p*H 5.5 in 0.1

TABLE 2. *Effect of pH on the duration of the first and second phase of germination of B. cereus spores*[a]

Conditions	Expt	Microlag time (min:sec)	Microgermination time (min:sec)	
			First phase	Second phase[b]
Citrate, phosphate buffer (0.1 M), *p*H 5.5[c]	1	8:35	1:05	—[d]
	2	13:25	1:15	—
	3	18:35	1:20	—
	4	19:05	1:15	30:00
	5	20:05	1:30	—
Citrate phosphate buffer (0.1 M), *p*H 6.5	1	4:40	1:30	3:30
	2	8:15	1:10	4:10
	3	10:45	1:15	4:00
	4	13:25	1:30	3:30
	5	14:10	1:20	3:15
Tris(hydroxymethyl)-aminomethane buffer (0.05 M), *p*H 8.3	1	2:15	1:15	3:00
	2	2:45	1:25	3:15
	3	2:55	1:15	3:00
	4	3:10	1:05	3:00
	5	3:20	1:30	3:25
Carbonate bicarbonate buffer (0.1 M), *p*H 9.5	1	5:20	1:20	4:15
	2	6:15	1:15	4:40
	3	6:35	1:30	4:50
	4	7:05	1:45	4:00
	5	8:40	1:05	5:15

[a] The spores were heat-activated at 65 C for 4 hr and germinated in the presence of L-alanine and adenosine at 25 C.

[b] Loss in refractility at the end of this phase at *p*H 5.5, <80%; *p*H 6.5, 8.3, and 9.5, >95%.

[c] No germination occurred if citrate phosphate buffer was replaced by acetate buffer of the same *p*H.

[d] The second phase was absent and the spores remained semirefractile.

M citrate-phosphate buffer but not at all at *p*H 5.5 in 0.1 M acetate buffer.

Figure 5 illustrates the germination curve of a single spore of *B. megaterium* QM B1551 germinated under the specified conditions. The biphasic nature of the germination curve is clearly shown. It was calculated that the first phase lasted for 105 sec and the second phase for more than 7 min. The linear relationship between the second phase and time is also evident in Fig. 5.

We have assumed that the first phase of the germination curve of single spores reflects a rapid influx of water into the spore. To study this aspect of germination further, the loss in refractility of spores was investigated by use of the lethal chemical germinant formic acid. Spores of *B. cereus* were treated with an 80 per cent aqueous solution of formic acid as the germinant, and the change in refractility occurring during such a treatment was recorded. In this experiment, the changes occurred so rapidly that the chart speed had to be increased to 12.7 cm instead of 2.54 cm per min. A representative curve obtained in these experiments is shown in Fig. 6. Despite a rate difference, the curve appears sigmoid in nature and resembles in part the first phase of the normal germination curve for a single spore. One apparent result of the use of formic acid as a germinant is that the biphasic nature of the germination curve is destroyed.

DISCUSSION

The present study unequivocally demonstrates that the germination curves of single spores of *B. cereus* and *B. megaterium* do not resemble comparable curves obtained by using spore suspensions. The germination curves for spore suspensions are sigmoidal, whereas the germination curve of a single spore is invariably of a biphasic nature when physiological germinants are employed. The duration and appearance of the first phase of the germination curve is generally unaffected by the germination conditions, whereas the duration of the second phase is quite dependent on the conditions used for germination. It should be noted that the average microgermination times observed for spores of *B. cereus* range from 4 to 6 min; these times are considerably longer than those (16.7 sec and 36 sec) reported by Vary and Halvorson (19) and Rodenberg et al. (17). This large discrepancy is probably due to the difference in temperatures used for germination (all of our experiments were conducted at 25 C instead of 30 C) and the highly improved method we developed for measuring microgermination times. It is pertinent that our selection of spores in the field was nonrandom, since we followed germination only in healthy spores.

The presence of the distinct second phase of the germination curve, the persistence of residual refractility in the core region during this period, and its gradual disappearance were more readily observed in spores of *B. megaterium* than in those of *B. cereus*.

One plausible explanation for this situation is that the thickness of the spore cortex may influence the time required for release of spore components and subsequent hydration of the core. We made measurements of the width of the spore cortex of *B. megaterium* QM B1551 [measurements made on previously published electron micrographs of spores of *B. megaterium* QM B1551 (3), and unpublished micrographs of spores

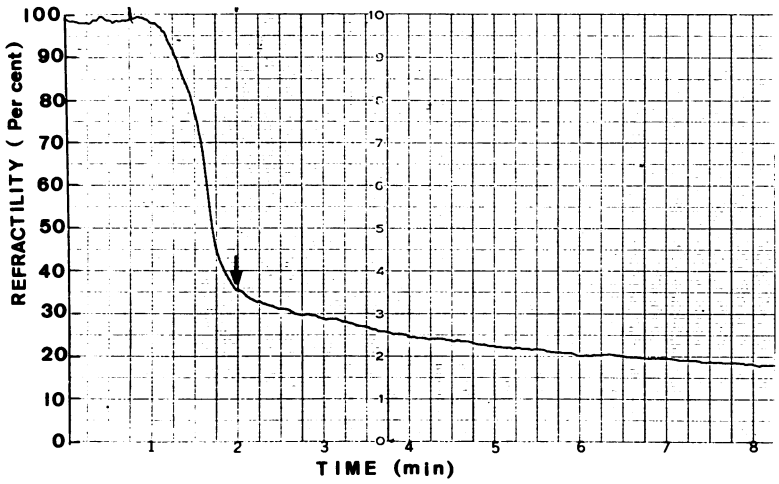

FIG. 4. *Germination curve of a single spore of B. cereus T. Germination was induced by* L-*alanine and adenosine at pH 5.5 with the use of citrate-phosphate buffer (0.1* M*). Germination occurred after a microlag period of 19 min and 5 sec. During the first phase of germination (1 min and 15 sec), an approximately 65% loss of refractility occurs. The slope of the second phase is significantly shallow (compare with Fig. 1); the spore failed to complete full germination under these conditions. The arrow indicates the transitional region between the first and second phases of germination.*

of *B. cereus* T]. These measurements reveal that the spore cortex of *B. megaterium* QM B1551 (100 nm wide) is twice as thick as that of *B. cereus* T (45 nm).

The striking similarity in the basic pattern of the germination curve of spores of *B. cereus* and *B. megaterium*, however, strongly suggests that germination involves a sequence of events common to all bacterial spores.

The equation derived by McCormick (11) seems to describe most accurately the process of the germination of a large population of *B. cereus* spores under various environmental conditions. By means of direct measurement of the microlag and microgermination time of many individual spores under various physiological conditions, and by thorough statistical analyses of the data, Vary and Halvorson (19) and Vary and McCormick (21) convincingly showed that both microlag and microgermination times for a number of individual spores had a skewed distribution pattern; they concluded that the frequency distribution for germination of single spores was in fact described by the McCormick equation. They emphasized the microlag time as the most important variable, because the cumulative distribution of the microlag time of individual spores is the determinant in the optical density profile of a suspension of germinating spores. It was also reported that both the duration of heat activation and the concentration of L-alanine affected the length of the microlag but not microgermination time (19).

TABLE 3. *Release of DPA from the spores*[a] *of B. cereus strain T germinated at different pH*

*p*H[b]	DPA released in the medium[c]	Germination after 30 min
	%	%
5.5	66.3	80
6.5	80.2	100
8.3	82.8	100
9.5	92.5	100

[a] The spores contained 11.2% DPA on a dry-weight basis.

[b] Conditions as described in Table 2.

[c] Expressed as percentage of total DPA contained in the spore suspension.

The observation that the germination process occurs in two distinct phases immediately poses an obvious question: can specific events occurring during germination be associated with a particular phase? We observed that when spores of *B. cereus* were allowed to germinate in the presence of L-alanine at a suboptimal *p*H (*p*H 5.5 in citrate-phosphate buffer) the majority of spores (approximately 80%) completed the first phase of germination after a relatively long and variable lag period, but the second phase was either completely blocked or proceeded sluggishly. An analysis of the DPA content of spores 30 min after the initia-

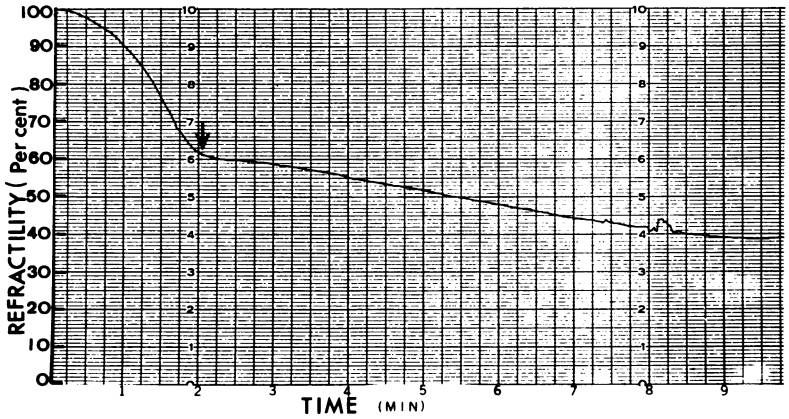

FIG. 5. *Germination curve of a single spore of B. megaterium QM B1551. Germination was induced by* L-*alanine (5 mg/ml) and* D-*glucose (1.8 mg/ml) at pH 8.3 [0.05 M tris (hydroxymethyl)aminomethane buffer] after heat activation at 65 C for 1 hr. During the first phase of germination, the spore loses approximately 40% of the initial refractility. The arrow indicates the transitional region between the first and second phases of germination. This spore initiated germination after a microlag period of 3 min and 35 sec and took approximately 105 sec to complete the first phase of germination. The second phase was longer than 7 min and, at the end of that time, the spore appeared partially refractile when observed under a phase-contrast microscope.*

tion of germination revealed that most of the DPA had been released by that time (Table 3), indicating that the partial residual refractility observed in the core region was due to elements other than DPA. It is pertinent to recall that spores deficient in DPA, produced either by sporulating the cells in the absence of calcium or by autoclaving, similarly appear semirefractile when examined by phase-contrast microscopy. These observations support the view that the first phase of germination involves a breaking of an outer barrier, which subsequently permits the release of DPA into the medium. It is most likely that calcium associated with DPA is also released at this time. It has been shown that DPA and calcium (8, 9, 23) are excreted into the medium prior to the detection of changes in refractility or optical density. Heat resistance is known to be lost prior to DPA release (9); Powell (12) noted that heat resistance was lost prior to observable changes in refractility.

On the basis of these observations, we interpret our data as suggesting that the second phase of germination involves a further gradual degradation, possibly by enzymes, of a second barrier and release of the peptidoglycan components from the cortex, with eventual hydration of the vital core of the spore. It is probable that at this time the spore becomes fully stainable and completely darkened when examined in a phase-contrast microscope. The further drop in residual refractility and optical density occurring during

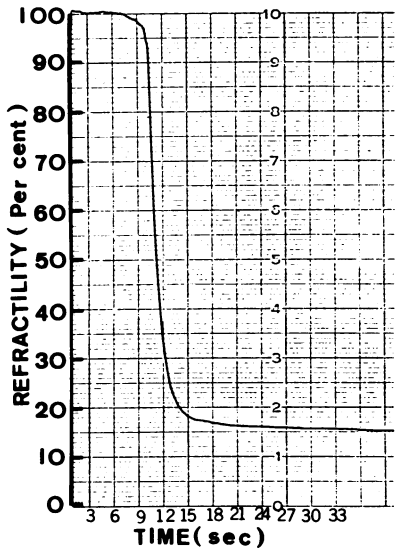

FIG. 6. *Germination curve of a single spore of B. cereus strain T induced by treatment with 80% formic acid. The spore became phase-dark quite rapidly after a short lag period. Though there is no evidence of the presence of residual refractility in a spore germinated under these conditions, darkening normally never extended beyond an 80 to 85% loss of the initial refractility; there is a 95% loss under normal physiological germination conditions. The darkening of the spore appears to occur by a single-phase process as suggested by the sigmoidal appearance of this curve.*

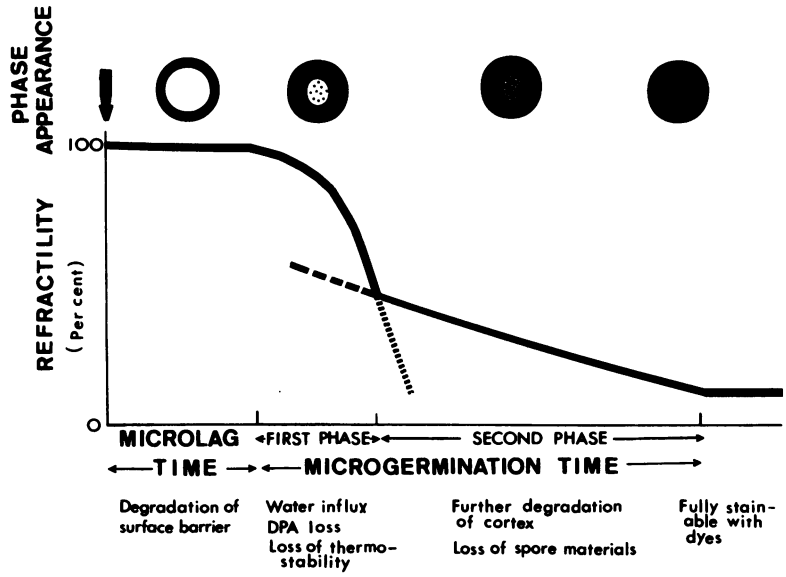

FIG. 7. *Change in refractility, as revealed by phase-contrast microscopy and microscope photometry, and other physiological events presumably occurring during various phases of germination in single spores. Broken lines in the germination curve are the suggested curves occurring independently of each other within a spore. The curve (solid line) reflecting experimental observations may represent a composite curve of these two separate curves.*

the second phase of germination is presumably due to these overall reactions. The kinetic data reported by Levinson and Hyatt (9) for spores of *B. megaterium* QM B1551, and recent phase-contrast micrography of germinating putrefactive anaerobe 3679 (18), are most interesting and informative in this connection, and can be best interpreted in terms of the biphasic nature of the germination process.

The overall process of germination of bacterial spores may then be summarized as follows. Firstly degradation or modification of a surface barrier of the spores occurs by enzymatic reaction(s) (4, 20) triggered by specific germinants. The rapidity of the initial degradative reaction(s), which proceed incipiently, is represented by the microlag time. The termination of the initial reaction culminates in the emergence of the initial rapid drop in refractility (first phase) during which release of DPA and loss of heat resistance occur; this event appears to be completed almost independently of environmental conditions. Secondly, following the release of DPA and partial hydration of the spore, further degradative reactions involving the dissolution of the cortex are initiated. These reactions, dependent on environmental factors, presumably terminate with the release into the medium of components of the cortical peptidoglycan (22). At the end of this series of reactions, the spores are completely hydrated, fully stainable with basic dyes, and nonrefractile when examined by phase-contrast microscopy.

The germination curve of a single spore is most likely a composite of two separate curves which represent the kinetics of two independent phenomena occurring in sequence during germination. Whether the termination of the first phase is essential for the initiation of the second phase or occurs independently still remains to be determined. The events presumed to be occurring in a single spore during physiological germination are diagrammatically illustrated in Fig. 7.

Further studies of the kinetics of germination of single spores with the use of different germination conditions and a statistical analysis of the data should provide significant new information on the mechanism of germination of bacterial spores.

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