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Microgermination of *Bacillus cereus* Spores

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The biphasic nature of germination curves of individual *Bacillus cereus* T spores was further characterized by assessing the effects of temperature, concentration of germinants, and some inorganic cations on microgermination. Temperature was shown to affect both phases of microgermination as well as the microlag period, whereas the concentration of L-alanine and supplementation with adenosine exerted a significant effect only on the microlag period. The germination curves of individual spores induced by inosine were also biphasic and resembled those of spores induced by L-alanine. High concentrations (0.1 M or higher) of calcium and other inorganic cations prolonged both phases of microgermination, particularly the second phase, and had a less pronounced effect on the microlag period. The second phase of microgermination was completely inhibited when spores were germinated either in the presence of 0.3 M $CaCl_2$ or at a temperature of 43 C; this inhibition was reversible. Observations on the germination of spore suspensions (kinetics of the release of dipicolinic acid and mucopeptides, loss of heat resistance, increase in stainability, decrease in turbidity and refractility) were interpreted on the basis of the biphasic nature of microgermination. Dye uptake by individual spores during germination appeared also to be a biphasic process.

Ideally, the kinetics of germination of bacterial spores should be investigated in individual spores, because germination curves obtained by use of spore suspensions represent the summation of events occurring in the individual members of that population. Although loss of refractility under a phase-contrast microscope is frequently used for following germination, the lack of reliable methods to quantitate phase-darkening has limited its use for following the germination of individual spores (17; H. Riemann, Ph.D. Thesis, Univ. of Copenhagen, Copenhagen, Denmark, 1963).

The successful application of microscope photometry to quantitate phase-darkening has provided a new tool for exploring the kinetics of germination of individual spores (15). Hashimoto et al. (2) recently demonstrated that microgermination of *Bacillus cereus* T and *B. megaterium* QM B1551 spores is a biphasic process, and it was suggested that each phase of microgermination is affected differently by certain environmental factors.

Although the effects of environmental factors on microlag and microgermination have been previously studied by direct phase-contrast microscopy and by employing statistical analysis (18, 19), the development of a more precise technique for analyzing microgermination has led us to reinvestigate the kinetics of microgermination.

This communication deals with the effects of temperature, type and concentration of germinants, and some divalent cations, particularly calcium, on the kinetics of microgermination of *B. cereus* T spores.

Events occurring during each phase of microgermination are correlated further with data on germination obtained by use of spore suspensions.

MATERIALS AND METHODS

Spores. Spores of *B. cereus* strain T were produced, harvested, and stored as previously described (2).

Heat activation and temperature effects. Spores were activated by heating at 65 C for 4 hr and were germinated in 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 8.3) under the conditions specified.

The effect of temperature on germination of single spores was studied in an adjustable constant-temperature room (range of 16 to 33 C); similar studies on spore suspensions were carried out in the range of 10 to 45 C with the desired temperature maintained by use of a variable-temperature water bath.

Heat sensitivity. Loss of heat resistance in spore suspensions during germination was followed by removing 1-ml samples at intervals and transferring them to 9.0-ml distilled water blanks equilibrated to

80 C. Samples were heat-challenged at 80 C for 10 min and chilled in an ice bath; then 0.1-ml amounts of appropriate dilutions were plated on Trypticase Soy Agar. After 24 hr of incubation at 30 C, the colonies were counted with a New Brunswick colony counter.

Optical density. The decrease in optical density of spore suspensions was measured at 420 nm in a Bauch & Lomb Spectronic-20 colorimeter.

Refractility and stainability. Germinating spore suspensions were stabilized by removing samples at various time intervals and transferring them to a predetermined amount of sulfuric acid to lower the *p*H to 2.0 (1). Duplicate smears were prepared from this mixture and quickly dried over gentle heat. To one smear, a drop of 1% crystal violet was added and mounted with a cover slip; spores were examined for stainability while wet. To a duplicate slide, a drop of sulfuric acid (0.05 M) was added, and spores were examined for loss of refractility (phase-darkening) by use of dark phase-contrast optics. Preparations examined 2 hr after the addition of sulfuric acid showed no more stainability or phase darkening than specimens prepared immediately after stabilization with acid.

Loss of spore components. Loss of dipicolinic acid (DPA) and mucopeptides during germination was followed by measuring the DPA or mucopeptides remaining in spore pellets. Appropriate volumes of germinating spore suspension were removed at various intervals and immediately transferred to sufficient sulfuric acid to lower the *p*H to 2.0. Sulfuric acid effectively stabilized germination with respect to DPA and loss of mucopeptides, for spore suspensions to which sulfuric acid had been added at various intervals showed no subsequent excretion of DPA or mucopeptides over a 60-min period. Spore pellets were obtained by centrifuging at 6,000 × *g* for 15 min at 4 C. DPA was estimated colorimetrically by the method of Janssen et al. (4) with purified DPA (Matheson, Coleman and Bell, Norwood, Ohio) as a standard, and hexosamine was estimated after hydrolysis by the method of Rondle and Morgan (16) with glucosamine as a standard. Hydrolysis for 5 hr at 100 C in 6 N HCl was carried out in sealed glass ampoules.

Normalization of data. To compare the time sequence of the various germination parameters, each event was plotted relative to completion of that event in 30 min. Such normalization of the data permits comparison of the rate of change among all of the parameters (7).

Measurement of single spore germination. Except for the few minor modifications described below, the techniques employed for measuring germination of single bacterial spores were essentially the same as reported earlier (2). To keep the number of spores per slide relatively constant, the following procedure was regularly employed: approximately 2.5 μliters of aqueous spore suspension (optical density of 0.8 at 420 nm) was spread evenly over the entire surface of a cover glass (#1 thickness) by use of a 5-μliter capillary pipette. After drying, without prior fixation, the cover glass was mounted over a microscope slide on which approximately 2.5 μliters of the desired concentration of germinant had been placed. The edges of the cover glass were sealed immediately with melted vaspar, and the preparation was placed under a Zeiss Universal microscope equipped with an MPM microscope photometer. The instrument was modified in this study by using a 3.2-mm rather than a 2.0-mm photometer stop. The 3.2-mm photostop was used because we could include the entire spore in the photosensitive area, thus enabling us to measure absorbancy or refractility changes more accurately during germination. The initial contact of germinant solution with the dried spore film was taken as time zero in calculating the microlag period. Germination of individual spores was followed for up to 30 min with the use of monochromatic illumination (540 nm).

Measurement of dry uptake by single bacterial spores during germination. A cover glass smeared with a thin film of spores was mounted over a microscope slide on which a germinant solution containing 0.3 to 0.7% crystal violet had been placed; most experiments were run at a dye concentration of 0.5%. For measuring dye uptake, the phase-contrast optical system of the microscope was replaced with a bright-field objective (Planapo 100/1.30, Zeiss) and condenser for optical density measurements. An area adjacent to a selected ungerminated spore was brought into the photosensitive area, and the instrument was adjusted to give an optical density reading of zero. The spore was carefully brought into the photosensitive area, and the optical density reading caused by this interpositioning was recorded as the initial optical density. Uptake of crystal violet by individual spores during germination was recorded as a gradual increase of optical density of the spore at 590 nm.

Phase-contrast microscopy. A Zeiss Universal Microscope equipped with a 100 × objective lens (Neofluar phase 100/1.30, Zeiss) was used for routine dark phase-contrast microscopy. Photomicrographs were taken on panchromatic film (Kodak plus X).

RESULTS

Effect of temperature. Temperature appears to be by far one of the most significant factors affecting bacterial spore germination. As shown in Table 1, both the microlag period and duration of the first and second phases of microgermination were significantly affected by temperature. It is evident that there was a wide fluctuation in microlag and microgermination times when germination occurred at low temperatures (16 C) as compared with higher temperatures (25 C or higher). Representative microgermination curves of single *B. cereus* T spores obtained at 16 and 30 C are shown in Fig. 1.

At optimal temperatures (30 C), microgermination occurred rapidly and in a relatively narrow range of time, i.e., 40 to 75 sec for the first phase and 1 min 30 sec to 2 min 30 sec for the second phase. As a result of this considerable shortening of the duration of both the first and second phases

of microgermination, the characteristic biphasic nature of the microgermination curve tended to be obscured. Indeed, we obtained several microgermination curves (Fig. 1C) at optimal temperatures in which the transitional portion was virtually absent, thereby resulting in sigmoid curves similar to those reported by Rodenberg et al. (15). However, it should be emphasized that we observed such sigmoidal curves only rarely.

One interesting feature of germination at high temperatures is that the second phase of microgermination of *B. cereus* spores was almost completely blocked at 43 C. The core of spores germinated at this temperature remained semirefractile for an indefinite period of time (Fig. 2a) but became phase-dark when the temperature was lowered below 38 C. It therefore was essential to treat such spores with weak acid or aqueous $HgCl_2$ prior to microscopic observation to prevent initiation of the second phase of microgermination. Examination of the heat resistance and DPA content of spores germinated at 43 C (Table 2) revealed that they were viable, they were completely heat-sensitive, and they had released practically all of their DPA. However, such spores retained a considerable portion of their glucosamine and were partially resistant to staining with basic dyes.

Effect of L-alanine concentration. Microgermination curves of *B. cereus* T spores were strikingly similar regardless of the concentration of L-alanine employed to induce germination. It is evident (Table 3) that the concentration of L-alanine within the range of 0.05 to 50 mg/ml primarily affected the microlag period and exerted little effect on microgermination of spores which were initiated within 30 min.

Effect of adenosine supplementation. Adenosine is known to have a synergistic effect on L-alanine-induced germination of bacterial spores. Its effect, however, appears to be limited to the microlag period (Table 4), since the kinetics of microgermination of single spores induced in the presence of L-alanine and adenosine or L-alanine alone were essentially identical.

Kinetics of inosine-induced germination. Certain nucleosides, particularly inosine, have been shown to be effective germinants for *B. cereus* T spores (11). Although statistical data are not presented, microgermination induced by inosine alone is similar to that induced by L-alanine or L-alanine and adenosine. Representative microgermination curves obtained at 25 and 30 C are shown in Fig. 3A and 3B. The biphasic nature of the curves is apparent at both temperatures. It thus appears that, as long as certain environmental conditions (temperature, *p*H, etc.) are constant, the kinetics of physiological germina-

TABLE 1. *Effect of temperature on L-alanine- and adenosine-induced germination of single B. cereus T spores*[a]

Temp	Extent of germination[b]	Microlag time[c]	Microgermination time[c]	
			First phase	Second phase
C	%	*sec*	*sec*	*sec*
16	80	545 ± 172 (260–775)	99 ± 17[c] (75–125)	216 ± 24[c] (185–225)
25	>99	296 ± 151 (135–615)	77 ± 8 (65–110)	189 ± 14 (170–235)
30	>99	245 ± 83 (140–455)	54 ± 9 (40–75)	104 ± 11 (85–155)
33	>99	235 ± 74 (123–320)	54 ± 8 (40–70)	106 ± 12 (90–135)

[a] The spores were heat-activated at 65 C for 4 hr and germinated at the specified temperature in the presence of L-alanine (5 mg/ml) and adenosine (2.5 mg/ml) in Tris (*p*H 8.3) buffer (0.05 M).

[b] Percentage of spore population germinating under these conditions in 30 min as determined by phase-contrast microscopy.

[c] The means and standard deviation were calculated by averaging data from 20 curves obtained for each set of conditions. The figures within the parentheses are the minimum and maximum times recorded for each specified condition.

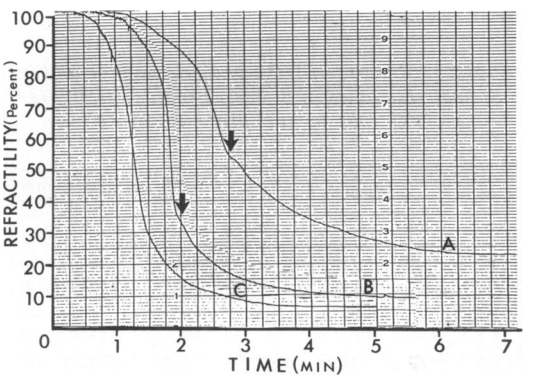

FIG. 1. *Microgermination curves of B. cereus T spores at 16 C (A) and 30 C (B and C), illustrating the effects of temperature on the kinetics of germination of single spores. The biphasic nature of the microgermination curves (A and B) is occasionally obscured at 30 C (see text for discussion). Arrows indicate the transitional portion of the biphasic curve; germination with L-alanine and adenosine at pH 8.3. The duration (seconds) of the microlag (ML), first phase (MG-1), and second phase (MG-2) for each spore was as follows: curve A, ML = 305, MG-1 = 105, MG-2 = 225; curve B, ML = 195, MG-1 = 60, MG-2 = 150; curve C, ML = 190, MG-1 = 60, MG-2 = 120. Figures 1, 3, 4, 5, and 6 are composites of actual curves recorded for individual spores.*

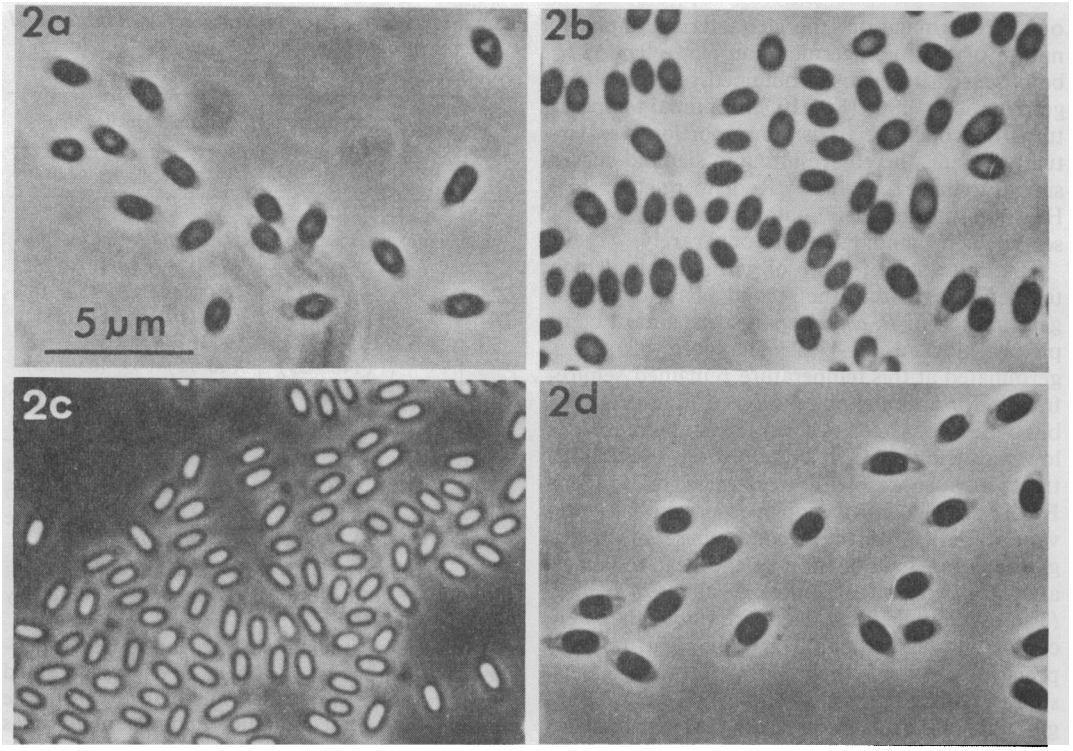

FIG. 2. *Phase-contrast micrographs of B. cereus T spores germinated with* L-*alanine and adenosine at 43 C (2a) and in the presence of 0.3* M *$CaCl_2$ (2b). Ungerminated (2c) and completely germinated spores (2d) are controls.* ×*4,000.*

TABLE 2. *Release of DPA and hexosamine and loss of heat resistance in B. cereus spores germinated*[a] *at 43 C and in the presence of calcium*

Condition	DPA released	Hexosamine released	Loss of heat resistance
	%	%	%
Heat activation only	10	13	0
30 C	99	81	99
43 C[b]	88	42	99
$CaCl_2$ (0.3 M, 30 C)	98.6	30	99

[a] Germination of heat-shocked spores (65 C for 4 hr) was induced by L-alanine (5 mg/ml) and adenosine (2.0 mg/ml) in Tris buffer (0.05 M, *p*H 8.3); incubated for 30 min.

[b] At this temperature of incubation, only 90% of the spores germinated, and about 10 to 15% were fully germinated.

tion of single spores is quite similar regardless of the germinants employed.

Effect of cations. Calcium ions, at concentrations ranging from 0.2 to 0.4 M were found to block the second phase of microgermination reversibly. Two representative microgermination curves obtained in the presence of 0.3 and 0.2 M Ca^{++} in addition to L-alanine and adenosine are shown in Fig. 4A and 4B. Phase-contrast microscopy of these spores revealed that the core was semirefractile (Fig. 2b) and partially resistant to staining. It is apparent from these observations that 0.3 M Ca^{++} blocked the induction or progress of the second phase and possibly a part of the first phase of microgermination. This inhibition, however, was reversed either by the physical removal of Ca^{++} (centrifugation and resuspension in Ca^{++}-free buffer) or by the addition of equimolar ethylenediaminetetraacetic acid to the spore suspension. Once the inhibition was reversed by either procedure, the spores completed the second phase of microgermination when placed in 0.05 M, *p*H 8.3 Tris buffer. These spores developed into vegetative cells provided suitable nutrients and growth conditions were supplied. At relatively high concentrations of Ca^{++} (0.2 M), a short lag often preceded the secondary decrease of refractility (Fig. 4B).

Ca^{++} appeared also to affect the kinetics of the first phase of microgermination, as evidenced by a considerable prolongation of the duration of this phase (Fig. 4A and 4B). Indeed, the duration of

TABLE 3. *Effect of L-alanine concentration on germination of single B. cereus T spores*[a]

L-Alanine concn	Extent of germination[b]	Microlag time[b]	Microgermination time[b]	
			First phase	Second phase
mg/ml	%	*sec*	*sec*	*sec*
50	99	585 ± 166 (215–950)	52 ± 10 (40–70)	108 ± 13 (85–130)
15	99	535 ± 242 (240–1,210)	54 ± 12 (35–75)	103 ± 15 (85–140)
8	99	516 ± 295 (210–1,415)	53 ± 11 (40–75)	102 ± 14 (85–145)
4	95	599 ± 291 (285–1,143)	55 ± 13 (33–75)	109 ± 14 (90–130)
1	60	1,075 ± 274 (615–1,765)	54 ± 10 (40–75)	103 ± 13 (85–125)
0.1	40	1,288 ± 291 (1,105–1,465)	54 ± 10 (40–75)	103 ± 12 (85–125)
0.05	25	1,456 ± 233 (1,000–1,605)	53 ± 12 (40–65)	103 ± 13 (80–125)

[a] The spores were heat-activated at 65 C for 4 hr and germinated in the presence of various concentrations of L-alanine at *p*H 8.3 (Tris buffer, 0.05 M) at 30 C.
[b] See Table 1 for explanation of figures.

TABLE 4. *Effect of adenosine on L-alanine-induced germination of single B. cereus T spores*[a]

Germinant		Extent of germination[b]	Microlag time[b]	Microgermination time[b]	
L-Alanine	Adenosine			First phase	Second phase
mg/ml	*mg/ml*	%	*sec*	*sec*	*sec*
1	0	60	1,075 ± 274 (615–1,765)	54 ± 10 (40–75)	103 ± 13 (85–125)
5	0	99	505 ± 245 (210–1,050)	55 ± 9 (44–75)	109 ± 16 (85–145)
1	2.5	99	484 ± 140 (320–750)	55 ± 10 (40–75)	105 ± 15 (90–135)
5	2.5	99	245 ± 83 (140–455)	54 ± 9 (40–75)	104 ± 11 (85–155)

[a] The spores were heat-activated to 65 C for 4 hr and germinated in the presence of specified germinants at *p*H 8.3 (Tris buffer, 0.05 M) at 30 C.
[b] See Table 1 for explanation of figures.

the first phase was almost doubled in the presence of 0.3 M Ca^{++} when compared with controls. Some inhibitory effects of Ca^{++} on physiological germination of *B. cereus* T spores were observed at concentrations as low as 10^{-2} M when studied by microscope photometry. The kinetics of inhibition of microgermination by Ca^{++} appeared to be quite similar in the spores initiated with L-alanine alone, inosine alone, or L-alanine and adenosine combined.

Spores germinated in the presence of 0.3 M $CaCl_2$ appeared to have released all of their DPA and to have lost heat resistance completely, whereas only a fraction of their glucosamine content was released; loss of DPA and heat resistance thus appear to be events associated with the first phase of microgermination (Table 2).

Strontium and barium exhibited a similar inhibitory effect on microgermination.

Events occurring during germination of spores. Figure 5 illustrates the events occurring during germination of *B. cereus* T spores. The curves are very similar to those reported by Levinson and Hyatt (6, 7) for *B. megaterium* QM B1551 spores.

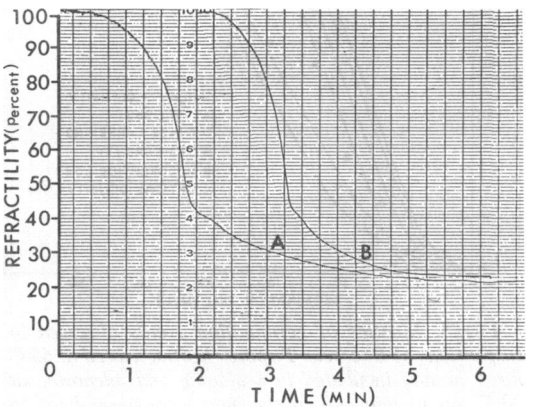

FIG. 3. *Microgermination curves of B. cereus T spores germinated with inosine (5 mg/ml, pH 8.3) at 25 C (A) and 30 C (B). Curve A, ML = 215, MG-1 = 90, MG-2 = 195; curve B, ML = 335, MG-1 = 65, MG-2 = 110.*

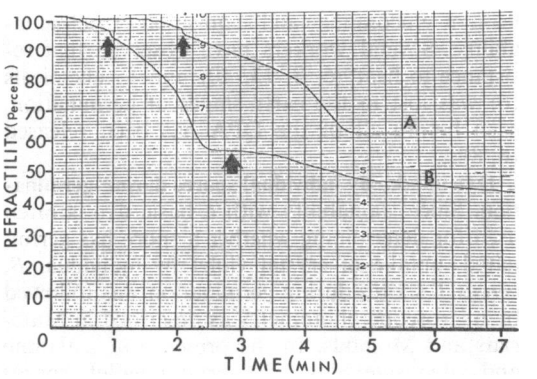

FIG. 4. *Microgermination curves of B. cereus T spores induced by L-alanine and adenosine in the presence of 0.3 M (A) and 0.2 M (B) $CaCl_2$. Calcium markedly influences the kinetics of the first and second phases of microgermination; the extent of refractility loss in both curves is significantly reduced. A secondary lag (B, large arrow) occurred between the first and second phase of microgermination when 0.2 M $CaCl_2$ was employed. Short spikes (small arrows) were usually observed in the early part of the first phase. Curve A, ML = 435, MG-1 = 180, MG-2 = absent; curve B, ML = 635, MG-1 = 120, MG-2 = ∞.*

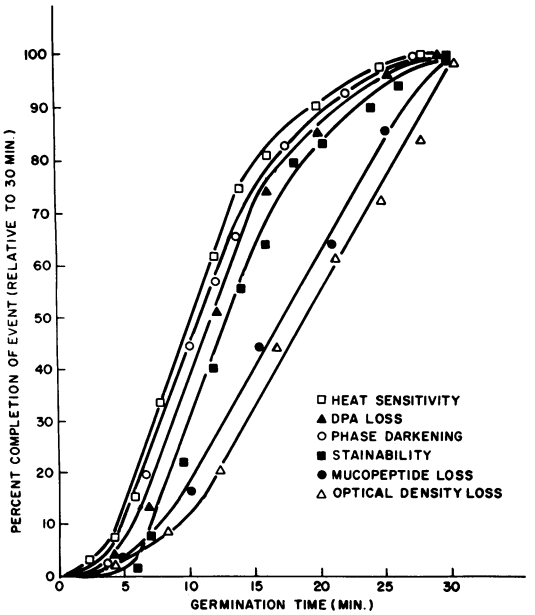

FIG. 5. *Kinetics of germination events observed in suspensions of B. cereus T spores heat-activated at 65 C for 4 hr and incubated in* L-*alanine and adenosine at 30 C for 30 min. All of the data were normalized as described in Materials and Methods. All spores that were still partially resistant to the stain were scored as stainable.*

These data indicate that the release of DPA and loss of heat resistance occurred prior to the release of glucosamine and acquisition of full stainability. Several workers have observed that resistance to heat (6, 8, 13, 20) and to chemicals (6) was lost in the early stages of germination, even before all of the DPA has been released (6, 8).

Dye uptake by individual spores during germination. Since stainability with a basic dye is often used for determining whether a spore has germinated, uptake of crystal violet by individual *B. cereus* T spores during germination was followed by microscope photometry as described in Materials and Methods. In the presence of L-alanine and adenosine plus 0.5% crystal violet, spores initiated germination after a prolonged microlag period (10 to 15 min instead of 3 to 4 min). As shown in Fig. 6A and 6B, dye uptake, reflected by an increase in optical density at 590 nm, takes place also in a biphasic fashion both at 25 and 30 C. In both curves, a slight decrease in optical density (indicated by arrows) occurs prior to the rapid increase. Despite a considerable fluctuation in the rate of dye uptake at a given temperature, the curve of dye uptake by individual spores appears to be biphasic.

DISCUSSION

When a bacterial spore germinates, two events are observed by phase-contrast microscopy: the spore remains fully refractile for some time and then rapidly loses its refractility, thereby becoming phase-dark. These two events were referred to by Vary and Halvorson (18) as microlag and microgermination, respectively. The importance of these separate events in observations on the kinetics of germination of a large population of spores was recognized in the pioneering studies of Powell (10) and Pulvertaft and Haynes (12). However, no detailed investigations of these two events were undertaken until Vary and Halvorson (18) studied the germination of individual spores using direct microscopy and statistical analyses. More refined studies on germination of individual spores are now possible by the introduction of microscope photometry to observations of the microgermination process (2, 15).

As reported by Vary and Halvorson (18), the pronounced effect of temperature on microlag as well as microgermination times of individual spores was quite evident. Although the microgermination times that these authors (18) and Rodenberg et al. (15) reported (16.7 sec and 36 sec, respectively) are considerably shorter than those we obtained (2.5 to 3.5 min), their conclusions were essentially confirmed. The differences in microgermination times may well be due to the sensitivity of the techniques we employed.

The observation that spores germinated at high temperature, releasing all of their DPA while the core remains semirefractile [as seen in *B. cereus* T

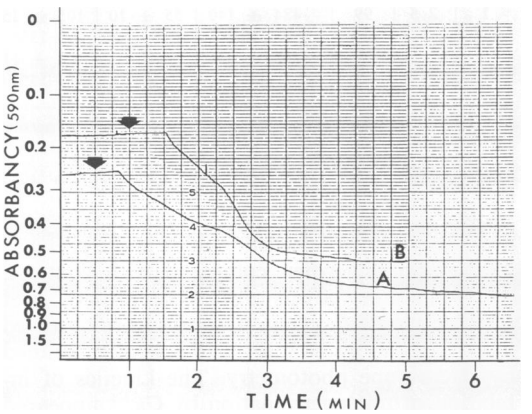

FIG. 6. *Time sequence of dye (crystal violet) uptake by individual B. cereus T spores during germination at 25 C (A) and 30 C (B). Note the biphasic nature of the curves and gradual uptake of the dye throughout the course of microgermination. A slight decrease in absorbance (arrows) is observed prior to dye uptake. Curve A, ML = 875, MG-1 = 75, MG-2 = 180; curve B, ML = 985, MG-1 = 50, MG-2 = 110.*

(Fig. 2a and Table 2) and in a putrefactive anaerobe (17)], strongly supports the view that DPA is released during the first phase of microgermination.

The curves of the kinetics of germination of *B. cereus* T spores (Fig. 5) and other spores (5–8) indicate that the release of DPA and loss of resistance to heat and toxic chemicals occurs prior to the release of cortical materials and development of full stainability. Refractility is lost gradually throughout the course of microgermination. The view that virtually all of the DPA is released during the first phase and the bulk portion of glucosamine during the second phase of microgermination is further supported by our observation that *B. cereus* T spores germinated in the presence of a high concentration of calcium. Although a suboptimal temperature (<30 C) may be used in following the kinetics of germination of spore suspensions because of a more pronounced expression of the biphasic nature of microgermination, the distribution of microlag times at lower temperatures makes the choice of a lower temperature less desirable.

Our data support the conclusion of Vary and Halvorson (18) that microlag times decrease with increasing L-alanine concentration, whereas microgermination times are unaffected. Thus, it appears that the kinetics of microgermination are independent of the concentration of germinant, including supplementation with adenosine, and the extent of heat activation (2, 18). However, whether spores germinating with microlag times longer than 30 min would exhibit kinetics of microgermination similar to those observed in the present study remains to be seen. It should be noted that only 25% of a spore population germinating in the presence of 0.05 mg of L-alanine per ml have undergone germination within 30 min.

The complete inhibition of the second phase of microgermination by Ca^{++} and its reversibility are further indications of the independence of the first and second phases of microgermination. Our assumption is that this inhibition by Ca^{++} of the second phase is due to its interference with lytic enzymes involved in the breaching of the secondary spore barrier or its interference with solubilization of cortical or core components. It is pertinent to recall that the autolysis of germinated spores of *B. megaterium* 9885 was prevented by fairly high concentration of Ca^{++} (3) and that Ca-spores of *B. megaterium* Texas germinated in the presence of L-alanine and inosine were reported to have a dark ring surrounding a somewhat lighter core (14).

The kinetics of crystal violet uptake by germinating individual spores indicate that some dye uptake began during the first phase of microgermination and continued throughout the course of microgermination. It is tempting to interpret this as indicating that imbibition of water also occurs at this time, as an aqueous solution of the dye was used. However, interpretation of the kinetics of dye uptake is complicated by the concomitant loss of optical density during germination as a result of the release of spore components. Therefore, our curves of dye uptake reflect a composite of the increase in absorbance due to dye uptake and the decrease caused by loss of spore components. The only conclusion which can be drawn from our data is that dye uptake by individual germinating spores also occurs in a biphasic fashion.

On the basis of data presented in this paper and elsewhere (2), we can conclude that microgermination curves of bacterial spores are biphasic. Since germination curves of a suspension are invariably sigmoidal (9, 19), we suggest that the sigmoidal curves are due largely to the heterogeneity in the microlag periods of individual spores in a suspension. We further conclude that the stages of the germination process (microlag, first and second phases of microgermination) have a different sensitivity to various factors affecting germination.

Further analysis of the kinetic curves of microgermination combined with ultrastructural studies should provide more useful information as to the nature of spore germination.

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