

# Kinetics of Germination of *Bacillus* Spores<sup>1</sup>

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## ABSTRACT

VARY, J. C. (University of Wisconsin, Madison), AND H. O. HALVORSON. Kinetics of germination of *Bacillus* spores. *J. Bacteriol.* **89**:1340-1347. 1965.—The kinetics of germination of *Bacillus cereus* strain T spores was accurately described by McCormick. To study the mechanism of germination, it is necessary to correlate the characteristic changes in a population of germinating spores with the behavior of the individual spores in the same population. Two microscopic events are apparent during germination: microlag, the time interval between the addition of L-alanine to heat-activated spores and the beginning of loss in refractility, and microgermination time, the time for the actual change in refractility to occur. The frequency distributions of both events are skewed, and appear to be independent. The effects of L-alanine concentration, heat activation, and temperature of germination on three parameters, microlag, microgermination, and per cent germination, were microscopically studied. The data are discussed in relation to the mechanism of germination, and a correlation between microlag and microgermination times with the constants of McCormick's equation has been suggested.

The dormant bacterial spore is characterized by a rigid structure. During germination, this structure is broken down by a number of degradative steps culminating in the emergence of a metabolically active cell and a return to vegetative growth. Characterization of the stages involved in germination has been difficult because of the lack of a complete kinetic description of the germination process. In earlier attempts, germination curves have been mathematically expressed as the fraction of germination within some interval of time (Hachisuka, Asano, and Kato, 1954; Hachisuka et al., 1955) or as a first-order rate equation describing the decrease in the fraction of ungerminated spores (Woese, Morowitz, and Hutchison, 1958). Such expressions describe only a portion of the observed sigmoid-type germination curve. Recently, McCormick (1964, 1965) empirically derived an equation which accurately describes the entire germination curve.

A further complication in understanding the stages in germination is the methodology used to measure germination. Such parameters as loss in optical density, loss of cell components or heat resistance, or changes in metabolic activity are all properties of a large population of cells in which the response of the individuals may be heterogeneous. Ideally, the kinetic description of

germination in a single spore would provide the most direct information on the stages involved. The analysis of individual spore germinations should provide answers to the following questions. (i) Is the sigmoid nature of the germination curve a property of the heterogeneity in the lag periods of the individual spores in a population? (ii) What proportion of the germination time is represented by the lag period? (iii) Do the stages in germination differ in their sensitivity to environmental factors? In this paper, an analysis of the events which occur microscopically during germination has shown that there are two obvious events: a lag time which lasts from the addition of germinating agent to the heat-activated spore until the beginning of loss in refractility, and a measurable time for the actual change in refractility to occur. These two events were statistically analyzed, and a double exponential function (Weibull, 1951; McCormick, 1964) was shown to apply. The effects of several environmental changes on these two events were also studied.

## MATERIALS AND METHODS

*Organism.* *Bacillus cereus* strain T was grown in G medium at 30 C for 36 hr, as described by Church, Halvorson, and Halvorson (1954) except that CaCl<sub>2</sub> was increased from 10 to 100 mg per liter. The free spores were separated from the vegetative cells and debris by centrifugation at 5,000 × *g* for 10 min, and were washed 15 to 20 times with distilled water. If the clean spores were

<sup>1</sup> Preliminary reports of this work have been presented by Halvorson, Vary, and McCormick (*in press*) and Vary and Steinberg (1964).

not immediately used, they were lyophilized and stored at  $-20^{\circ}\text{C}$ . Lyophilized stocks were suspended in distilled water and washed twice before use.

**Measurement of germination.** To measure germination by a spectrophotometric method, clean spores were suspended in distilled water to an optical density of 1.0 to 1.5 at  $625\text{ m}\mu$  and were heat-activated at  $65^{\circ}\text{C}$  for varying periods of time ( $\Delta S$ ) as indicated. Heat-activated spores were washed twice with distilled water and resuspended in  $0.05\text{ M}$  tris(hydroxymethyl)aminomethane (Tris) buffer ( $\text{pH } 8.5$ ). Germination was initiated by the addition of L-alanine to a final concentration of  $0.1\text{ M}$ . Germination was followed by the decrease in optical density at  $625\text{ m}\mu$  on a Beckman DK-2 recording spectrophotometer equipped with a temperature-controlled cuvette compartment maintained at  $30^{\circ}\text{C}$ .

Germination was also measured by a microscopic method. A suspension of heat-activated spores ( $20\ \mu\text{liters}$ ) was placed on a slide, and germination was initiated by the addition of  $20\ \mu\text{liters}$  of  $0.2\text{ M}$  L-alanine in  $0.05\text{ M}$  Tris buffer ( $\text{pH } 8.5$ ) containing  $0.4\%$  (w/v) hydroxyethyl cellulose (Hercules Powder Co., Wilmington Del.). The latter reagent was added to the germination medium to prevent evaporation and had no effect on germination. A cover slip was placed over the suspension, and the slide was placed under an oil immersion lens of a phase-contrast microscope at  $1,600\times$  magnification.

Statistical analyses of microgerminations required repeated observations of germination on small populations of activated spores. To avoid reversal of activation during this period, activated spores were rapidly cooled to  $4^{\circ}\text{C}$ , and the suspension was used no longer than 1 hr. At the moment that L-alanine (on an inverted cover slip) was added to spores (on a slide), a stopwatch was started. The slide was immediately placed under the microscope, and a field was randomly chosen which contained 8 to 15 spores. A suitable field was usually found within 20 to 40 sec after the addition of L-alanine, but, if one was not found

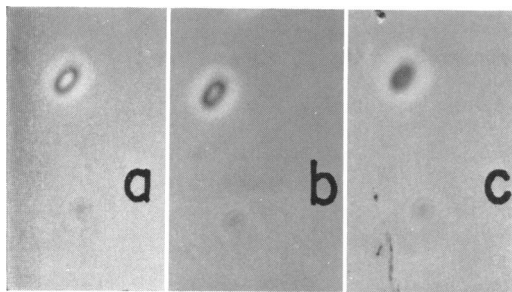


FIG. 1. Stages during germination of spores. (a) Refractile ungerminated spore during microlag. (b) Spore at the end of microlag and beginning microgermination. (c) Spore at the end of microgermination.

within 100 sec, the preparation was discarded. In the event that a preparation dried out during an observation, those results were discarded. The field was observed for 600 sec, and the time of germination was recorded for each spore. Two reproducible end points were arbitrarily chosen: microlag and microgermination times. The first of these was defined as that period of time after the addition of L-alanine until a spore showed the first change in refractility. The second was defined as that length of time from the end of microlag to the complete loss in refractility accompanied by the first hint of swelling. Both time intervals were recorded for each spore, and an example of these end points is shown in Fig. 1. To avoid heating of the slide by the microscope lamp, microscopic observations were carried out in a constant temperature room, and a  $1\%$  (w/v) aqueous solution of  $\text{CuSO}_4$  placed between the light source and microscope condenser was used as a heat filter. By these means, the temperature of the preparation on the slide remained approximately constant at  $28^{\circ}\text{C}$ .

**Germination rates.** Germination rate constants were calculated from the equation  $Y = Y_0 e^{-ct}$  (McCormick, 1964), where  $Y$ , the fraction germinated at any time  $t$ , is  $(\text{OD}_i - \text{OD})/(\text{OD}_i - \text{OD}_f)$ ;  $\text{OD}$  is the optical density at time  $t$ , and  $\text{OD}_i$  and  $\text{OD}_f$  are the initial and final optical densities, respectively.  $Y_0$  is the fraction germinated at  $t = 1$ , and, from a plot of  $\ln \ln 1/Y$  versus  $\ln t$ , the slope constant ( $c$ ) is calculated from  $\ln \ln 1/Y = \ln \ln 1/Y_0 - c \ln t$ . For further discussion on definitions, see McCormick (1964).

## RESULTS

The kinetics of germination of a suspension of spores represent the summation of events occurring in the individual spores of that popula-

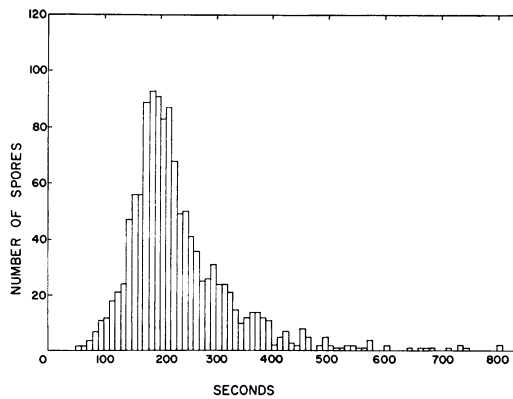


FIG. 2. Frequency distribution of germination times. Suspensions of spores, heat-activated for 1.0 hr, were observed microscopically as described in Materials and Methods. The ordinate is the number of spores that have completely lost their refractility within the indicated times.

TABLE 1. *Similar distributions*

Definition	Weibull (1951)	McCormick (1964)
Cumulative frequency of events.....	$1 - P_m$	$Y$
Intercept constant.....	$x_0$	$k$
Class intervals.....	$x - x_u$	$y$
Slope constant.....	$m$	$c$

\* Distribution functions of Weibull (1951) and of McCormick (1964), respectively:

$$P_m = 1 - e^{-\frac{(x-x_u)^m}{x_0}} \quad \text{and} \quad Y = e^{-kt^c}.$$

tion. To study these events, heat-activated spores were microscopically observed under phase contrast during L-alanine-induced germination. As shown in Fig. 2, the number of spores germinating within different time intervals is a skewed distribution. A statistical treatment of this distribution was carried out by testing several types of functions for "goodness of fit." For a normal distribution, the coefficient of skewness was +5.628, which indicated that 50% of the deviations occur at values less than the median. Also, for the same distribution, the  $X^2$  for the median value was 36.6 with, for 15 degrees of freedom,  $P \ll 0.01$ , indicating that the normal distribution may be discarded. The calculated  $X^2$  for a Poisson distribution was 49.99 for 5 degrees of freedom, which greatly exceeds the 1% significance level and therefore may be rejected.

A distribution function derived by Weibull (1951) was found to describe the distribution of germinating spores. This expression is almost identical to that derived by McCormick (1964), as shown in Table 1. There are only two minor differences: first, the  $P$  of the Weibull function represents the total fraction of events *not* completed within some time interval  $x$ , whereas the  $Y$  of the McCormick equation represents the total fraction of events that *do* occur within a given interval  $t$ ; second, the sign of the slope constant is different. If McCormick's equation describes the frequency distribution of germinating spores, then a graph of  $\ln \ln 1/Y$  versus  $\ln t$  should show a linear relationship with a slope  $c$ . If  $Y$  is known for any three times such that  $t_1/t_2 = t_2/t_3$ , then the curve which this equation predicts may be calculated and compared with the actual frequencies measured both microscopically and spectrophotometrically.

Using the values of  $Y$  at 2.5, 5, and 10 min, the expected frequencies of germination were calculated from the equation  $Y = e^{-kt^c}$  as shown in Fig. 3. If the expected frequencies of germina-

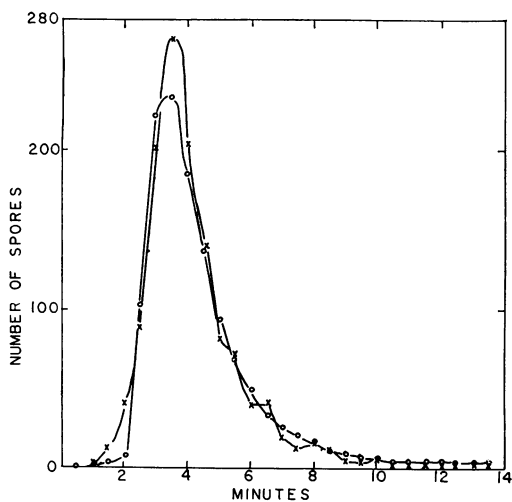


FIG. 3. Comparison of observed and calculated frequency distribution of germinating spores. Symbols: X, observed distribution frequency from Fig. 2; O, calculated distribution frequency from the equation  $Y = Y_0 t^{-c}$  (McCormick, 1964).

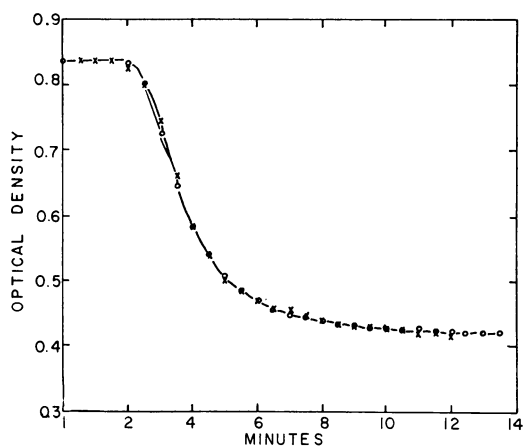


FIG. 4. Optical-density profile of a suspension of germinating spores. Spores were heat-activated for 1.0 hr and observed spectrophotometrically during germination as described in Materials and Methods. Symbols: X, observed optical-density values, O, optical-density values calculated from the equation  $Y = Y_0 t^{-c}$  (McCormick, 1964).

tion are compared with the microscopically observed frequencies 2 min after the initiation of germination,  $X^2 = 30.542$  for 21 degrees of freedom which corresponds to  $P = 0.083$ . Similarly, from the calculated values of  $Y$ , optical densities may be predicted and compared with those values observed spectrophotometrically (Fig. 4). From this comparison,  $X^2 = 2.198$  for 17

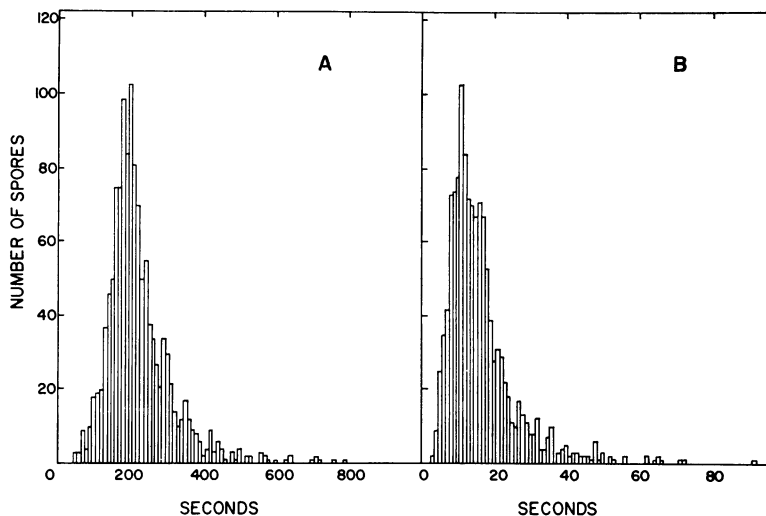


FIG. 5. Frequency distributions of microlag and microgermination times. The ordinate is the number of spores with given microlag (A) and microgermination (B) times as described in Materials and Methods.

degrees of freedom which corresponds to  $P \gg 0.99$ .  $X^2$  does not exceed the 5% significance level, indicating that the distribution of germinating spores measured both spectrophotometrically and microscopically is described by McCormick's equation.

Many investigators have observed that activated spores remain refractile for various periods of time after the addition of L-alanine and that then they change their refractility within a relatively short period of time. To quantitate these observations, two end points were arbitrarily chosen, microlag and microgermination times, as defined in Materials and Methods. Frequency distributions for microlag and microgermination times were constructed after recording the times for each event in a large number of individual spore germinations (Fig. 5). The frequencies of the microlags and the microgermination times appear to be skewed distributions, and the length of microlag relative to microgermination time seems to be the important factor in the time scale of events. From a suspension of spores heat-activated for 1 hr, the average microlag time was found to be 218 sec with a standard deviation of the mean of 2.6 sec; the average microgermination time, 16.7 sec with a standard deviation of the mean of 0.13 sec.

It is of interest to know whether a relation exists between microlag and microgermination time, in addition to the fact that they are ordered events. For example, does a spore with a long microlag have a long microgermination time? To answer this question, a correlation diagram of

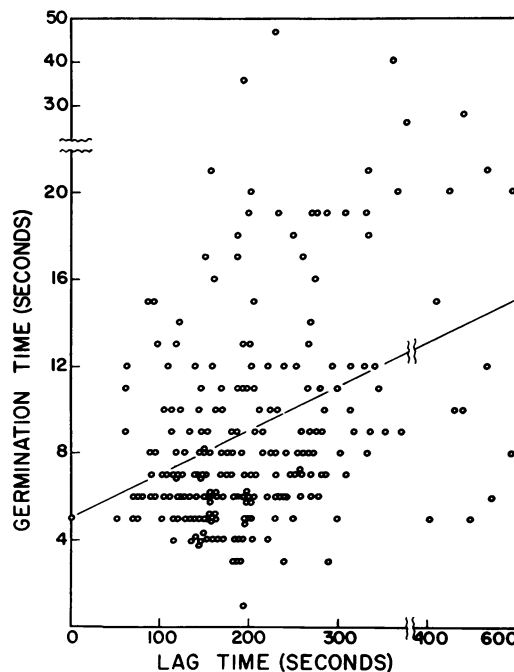


FIG. 6. Correlation diagram of microlag and microgermination times. The microgermination time was plotted versus the corresponding microlag time for each spore from the data in Fig. 5. The straight line was obtained by a least-square analysis.

microlag versus microgermination time for each spore was drawn. As shown in Fig. 6, there is apparently no relation between the microgermina-

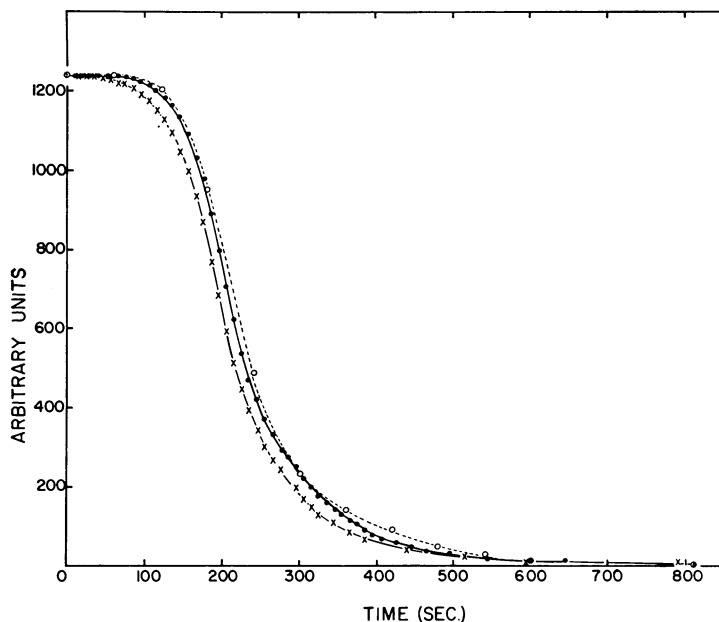


FIG. 7. Influence of microlag on germination. A cumulative plot of the total number of ungerminated spores (●) versus time was made from the data in Fig. 2. Similarly, a cumulative plot of the total number of spores that had not begun to germinate, *i.e.*, microlag times (×), versus time was made from the data in Fig. 5. The corresponding optical-density curve (○) was normalized for comparison by equating the change in optical density to the total number of spores counted microscopically.

tion time and microlag for individual spores. Application of a least-squares fit gives the indicated straight line with a positive slope of 0.02, and the correlation coefficient ( $r$ ) is +0.553; both of these statistics indicate an interdependency. A positive correlation coefficient does not define a dependence but only indicates a given probability of correlation which may or may not be real. The probability of a correlation is real in this case, because  $r$  is +0.553 for 229 samples, which is significantly different from zero and much greater than +0.254, the value that  $r$  must approach for a significance level of 1% or greater with 100 samples (Fisher, 1930). But two points presented by Eisenhart and Wilson (1943) must be considered. First, if complete independence in a bivariate function is to be shown, the above correlation coefficient is a serious contradiction, but, on the other hand, if complete correlation is to be proven,  $r$  must be greater than 0.8 since  $100(1 - r^2) = 69\%$  of the variation in each event is independent of that in each of the other events. It can only be concluded that the present data do not indicate a real dependence between microlag and microgermination time, nor do they prove a complete independence. The two events are ordered, because microlag precedes germination, and, once germination begins, it must

terminate within a short period of time relative to microlag (approximately one-tenth).

From these observations, one would predict that the influential event occurring during germination is the length of the microlag periods for individual spores. As shown in Fig. 7, if the cumulative number of spores germinated at any given time is plotted on the same ordinate as the optical-density curve (where optical-density values are in arbitrary units), the two curves coincide. Also, a cumulative plot of the number of spores that have just begun to germinate, that is, microlag time, tracks behind and parallel to the optical-density curve. The time difference between the two curves is 15 to 20 sec, which is the range for the average microgermination time of 16 sec.

The effects of various environmental stimuli on three parameters, microlag time, microgermination time, and per cent germination, are summarized in Fig. 8 to 10 and Table 2. Microlag times decrease with either increasing L-alanine concentrations or increasing times of heat activation; they increase with temperatures of germination below 28 C. Microgermination times are unaffected by either changes in the concentration of L-alanine or the extent of heat activation; however, temperatures of germination below 28

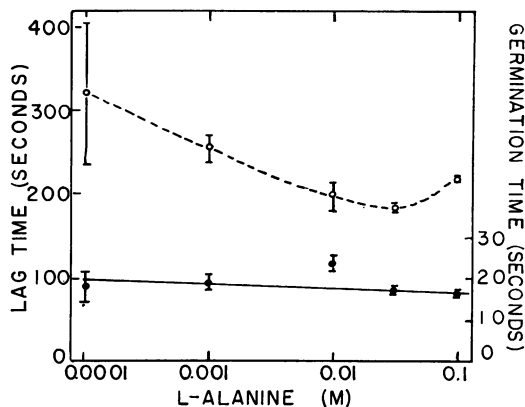


FIG. 8. Effect of L-alanine concentration on micro-lag and microgermination times. Spores were heat-activated for 1.0 hr, and germination was triggered by L-alanine of the indicated final concentrations on the slide. Both micro-lag and microgermination times were recorded as described in Materials and Methods. The average micro-lag (○) and average microgermination (●) times with their respective standard deviations of the mean were calculated for each L-alanine concentration. The wide variation at low L-alanine concentrations is caused by the low per cent germination.

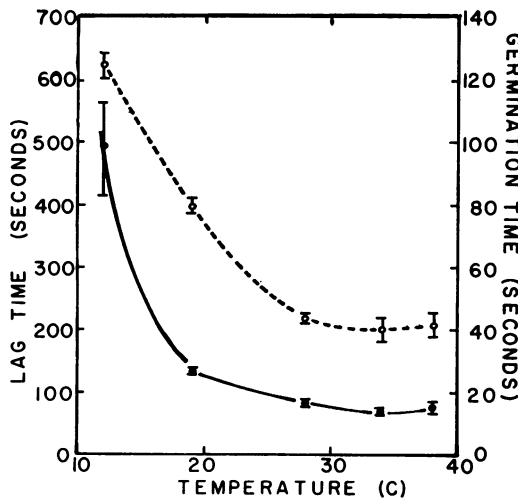


FIG. 10. Effect of temperature of germination on micro-lag and microgermination times. Spores were heat-activated for 1.0 hr and observed microscopically during germination at the indicated temperatures. Both micro-lag (○) and microgermination (●) times were recorded as described in Materials and Methods and in Fig. 8.

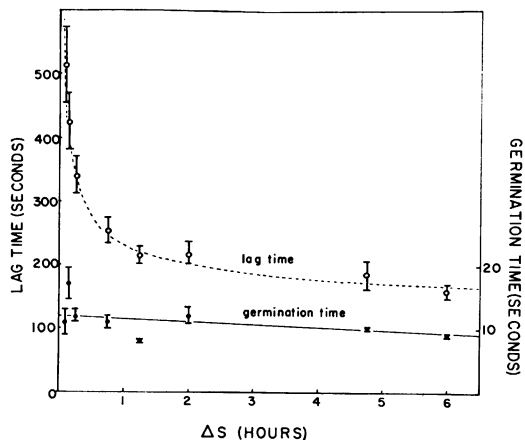


FIG. 9. Effect of heat activation on micro-lag and microgermination times. Spores were heat-activated ( $\Delta S$ ) for the indicated times, and both micro-lag (○) and microgermination (●) times were recorded as described in Materials and Methods and in Fig. 8. Germination was initiated by L-alanine at a final concentration of 0.1.

C increase the microgermination times. The per cent germination increases with either increasing L-alanine concentration or heat activation times, whereas an apparent optimal temperature of germination is evident at 28 C. Differences be-

TABLE 2. Effect of various environmental stimuli on microgermination

L-Alanine concn <sup>a</sup>	Per cent germination <sup>b</sup>	$\Delta S$ time <sup>c</sup>	Per cent germination <sup>b</sup>	Temp <sup>d</sup>	Per cent germination <sup>b</sup>
		<i>M</i>			
0.1	98.2	0	0	38	47.1
0.05	97.0	0.083	24.7	34	89.2
0.01	85.7	0.167	54.6	28	98.2
				19	58.8
0.001	52.3	0.25	81.3	12	3.0
0.0001	5.8	0.75	87.5		
		1.25	86.4		
		2.00	100.0		
		4.75	93.3		
		6.00	100.0		
		<i>C</i>			

<sup>a</sup> See Fig. 8.  
<sup>b</sup> Per cent germination observed microscopically within 600 sec after the initiation of germination.  
<sup>c</sup> See Fig. 9.  
<sup>d</sup> See Fig. 10.

tween micro-lag and microgermination times in Fig. 8 and 9 resulted from the use of different batches of spores for the two experiments.

DISCUSSION

A statistical analysis of microscopic and spectrophotometric data has provided for the

first time a comparison of the kinetics of germination of individual spores with those of populations of spores. The results show that the frequency distributions of germination are described by the McCormick (1964) equation. This equation accurately describes both the decrease in optical density of a suspension of germinating spores and the corresponding event, loss in refractility of a population of individual spores.

Two obvious events occur microscopically during germination of individual spores. Both microlag and microgermination times for a large number of individual germinations are skewed distributions. Microlag is the more important variable, because the cumulative distribution of microlag is the determinant in the optical-density profile of a suspension of germinating spores.

It was shown previously that the rate of germination of *B. cereus* T spores is affected by the concentration of L-alanine, temperature of germination, and the length of time of heat activation (O'Connor and Halvorson, 1961). It was shown that the rate of germination increased with increasing L-alanine concentrations and increasing times of heat activation, whereas there was a single optimum for the temperature of germination (30 C). Recently, these effects were re-examined by McCormick (1965) to determine the manner in which they changed the two constants,  $k$  and  $c$ , in his equation. His results showed that both increased duration of heat activation and variable L-alanine concentrations changed only  $k$  (the intercept term), but variable temperatures of germination changed both  $k$  and  $c$  (the slope term). In addition, the per cent germination within a given time interval in all of the above experiments was determined, and was found to change in the following manner: the concentration dependence of the per cent germination on L-alanine resembled an adsorption isotherm; the temperature of germination showed a single optimum at 25 C, and the length of heat activation (65 C) proportionally increased the per cent germination.

Both the duration of heat activation and the concentration of L-alanine affect only the length of the microlag times. Apparently, there is a certain probability, dependent on the pretreatment, that a spore will germinate; spores react to these treatments according to saturation-type kinetics. For example, it might be necessary that a given number of binding sites for L-alanine be saturated or a certain number of spore-coat protein bonds be ruptured by heating before germination begins. When these pretreatments are suboptimal, the probability that a particular

spore will begin to germinate is lowered such that not only is the average microlag longer but the per cent germination is lower within a given time interval. However, once the threshold value of events necessary to permit the initiation of germination has occurred, the actual process of loss in refractility proceeds rapidly, and at a rate quite independent of the pretreatment.

If germination rates are measured spectrophotometrically, there is an optimal temperature for germination at 30 C (O'Connor and Halvorson, 1961). Recently, Knaysi (1964) showed, by direct microscopic examination of the per cent germination, that *B. cereus* C<sub>3</sub> spores have an optimal temperature for germination of 30 C. By modification of the germination equation of McCormick (1964), which accurately describes the kinetics of germination, McCormick (1965) included a term  $(1 - \alpha)$ , which compensates for the per cent germination. This form of the equation,  $OD_t = OD_i (1 - (1 - \alpha)e^{-kt^c})$ , includes a rate constant ( $c$ ) and two parameters ( $k$  and  $1 - \alpha$ ) which account for the beginning of the reaction and fraction of the reaction completed, and provides a more accurate method to measure the effects of environmental stimuli on the system. McCormick (1965) determined that the optimal temperature for germination was 25 C, since this was the temperature at which both  $c$  and  $1 - \alpha$  were maximal. These latter results have been confirmed herein by microgerminations. Microlag and microgermination times decrease with increasing temperatures of germination up to 30 C (Fig. 10). In addition, the per cent germination was found to be optimal at 28 C (Table 2). These results do not contradict the above discussion of the L-alanine and heat-activation effects, since one would expect a temperature dependency of microgermination times as in any biological reaction. Although the evidence does not definitely establish the point as yet, we believe that there is a relationship between the constants  $k$  and  $c$  of the McCormick equation and the two end points, microlag and microgermination times. Microlag and  $k$  are decreasing functions of temperature, though they do not decrease in exactly the same fashion; they also decrease with increasing L-alanine concentration. Similarly,  $c$  represents the rapidity with which the initiated event is completed and is only dependent on temperature, as are the microgermination times.

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