INCIPIENT GERMINATION IN HEAVY SUSPENSIONS OF SPORES OF *BACILLUS STEAROTHERMOPHILUS* AT SUBMINIMAL GROWTH TEMPERATURES

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ABSTRACT

CURRAN, HAROLD R. (U.S. Department of Agriculture, Washington, D.C.), AND MICHAEL J. PALLANSCH. Incipient germination in heavy suspensions of spores of *Bacillus stearothermophilus* at subminimal growth temperatures. J. Bacteriol. **86**:911–918. 1963.—By use of spore (plate) counts and permeability to stain, labilization was followed periodically in heavy suspensions of washed *Bacillus stearothermophilus* 1518 spores incubated at different temperatures. Although vegetative proliferation did not occur below 38 C, incipient germination was rapid down to 20 C and much slower and incomplete at 14 C. Dilution of the suspension materially reduced the degree and rate of labilization. The degree of washing and use of deionized water had no appreciable influence upon early development of the spores. The results are discussed from the point of view of the possible origin and nature of the germination stimulant.

Wynne and his co-workers (Wynne, Galyen, and Mehl, 1955; Wynne and Galyen, 1956) reported that spores of certain mesophilic clostridia lost their heat stability rapidly when incubated in buffered caramellized glucose at 75 C; vegetative growth of these organisms did not occur at 50 C or above. Although this change in thermostability of the spores was initially regarded as germination, these workers were unable to demonstrate the presence of dipicolinic acid in the external substrate. However, more recently Roberts and Wynne (1962) reported the excretion of dipicolinic acid from spores of *Clostridium sporogenes* and other clostridia heated at 75 C in caramellized glucose, with progressive loss of heat resistance.

This phenomenon of labilization of spores at temperatures far above that supporting vegetative growth may be shown to have its counterpart at subminimal growth temperatures. Evidence on the latter point was obtained with heavy suspensions of the thermophilic test organism *Bacillus stearothermophilus* 1518 (National Canners Association). Details of this study and the observed results are presented in this paper.

MATERIALS AND METHODS

B. stearothermophilus 1518 was cultivated at 53 C on the surface of nutrient agar slopes of the following composition: beef extract, 0.3%; peptone, 1.0%; agar, 2.5%; $MnCl_2$ (5 ppm, soluble manganous ion); starch, 1%; final pH 7.0. As inocula, 17-hr nutrient broth cultures were used. After 5 to 6 days of incubation, the spores were harvested and were washed at least eight times with iced distilled water in a refrigerated centrifuge at 2 C. The washed spores were resuspended in cold, sterile, distilled water and used immediately or held at 3 C. During the wash cycles, the proportion of spores to water was 1:10 by volume.

Quantities (5 ml) of the chilled well-mixed stock spores were dispensed into cold screw-capped 15-ml Pyrex centrifuge tubes. The suspensions were quickly adjusted to the desired incubation temperature by gentle shaking of the tubes in a temperature-controlled water bath. Counts of heat-stable spores were made at the beginning of each experiment (0 hr) and after incubation for the desired time periods. This was accomplished by heating portions of the well-mixed sample at 100 C for 20 min, and then subculturing in glucose (0.5%)-nutrient agar plates. The latter were counted after 48 hr of incubation at 53 C; occasional samples were recounted after an additional 48 hr of incubation. Film preparations of each sample at each plating period were stained with aqueous 0.5% crystal violet solution and examined microscopically; the proportion of deeply staining spores was observed. Deionized water

was prepared by circulating distilled water through a preflushed cartridge of mixed bed resins.

RESULTS

It is well known that among facultative and obligate thermophiles the lowest temperature which supports vegetative growth is, to some extent, nutrient-dependent (Campbell and Williams, 1953; Campbell, 1954; Long and Williams, 1959; Long, 1958). Under the conditions employed in this study, the test organism did not grow vegetatively or produce visible colonies below 38 C, even after prolonged incubation.

Labilization of spores at different temperatures. In this experiment, spores washed eight times were resuspended in distilled water to make a heavy suspension (10^{10}/ml), and 5-ml samples were incubated in water baths at each of four temperatures. Changes in the thermostability of the spores with time are shown in Fig. 1. The rate and extent of labilization during a 5-day period was a function of the temperature. At the higher temperatures, most of the spores became thermolabile in a few hours. At 14 C, incipient germination was slow and affected only a part of the population; the rapidity and extent of germination at this temperature varied considerably with different crops of spores. In general, the decrease in the proportion of heat-stable spores closely paralleled the uptake of stain. (Some results with stained preparations are shown in Fig. 2, 3, and 4.) Concurrent with the loss of heat stability and acquired permeability to stain, increasing amounts of dipicolinic acid [method of Janssen, Lund, and Anderson (1958)] appeared in the suspension substrate, reaching a level of 329 μg/ml under the conditions existing in Fig. 4.

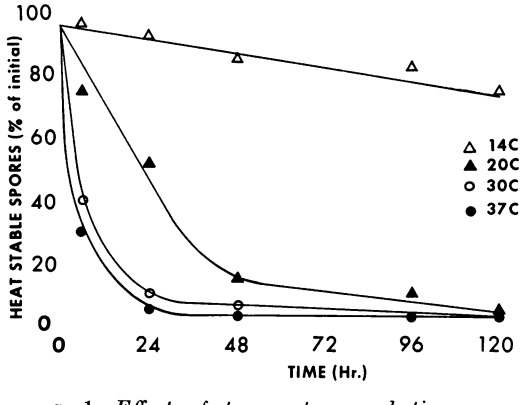

FIG. 1. *Effect of temperature and time upon the heat stability of washed unheated spores of Bacillus stearothermophilus 1518. Concentration, 10^{10}/ml.*

Effect of degree of washing upon labilization. If the observed germination was induced by traces of nutrients derived from the growth medium which were incompletely removed in the washing, preliminary exhaustive washing should progressively reduce the effect. Spores washed the usual 8 times were washed an additional 9 and 22 times, respectively, and tested at the same concentration as before. Under these conditions, the rate and extent of incipient germination was not appreciably different from that of normally (eight times) washed spores.

Effect of reduced spore concentration upon labilization. The effect upon labilization of diluting the spore suspension was investigated, in the belief that this might reveal the source of the germination stimulant. Spores (washed eight times) from the stock batch used in Fig. 1 were diluted with distilled water to yield a concentration of 5.0×10^7/ml heat-stable spores. Figure 5 shows the development of thermolability with time when the spores were incubated at different temperatures. A 200-fold decrease in the concentration of spores (over that used in Fig. 1) materially reduced the rate and extent of labilization.

Effect of deionized water upon labilization. The importance of ions in spore germination has received increasing emphasis in recent years (Levinson and Sevag, 1953; Rode and Foster, 1962*a*, *b*, *c*). Rode and Foster, on the basis of observations of *B. megaterium* and *B. cereus*, proposed that ions play a primary role in spore germination, with organic substances assuming a merely augmentative action. Although results in the preceding experiment made it improbable that the wash water was concerned with the observed labilization, the possible effect of ions in the wash water upon germination was considered. To test this point, spores that had been washed eight times were washed an additional nine times with deionized water, and their germination at different temperatures was compared with control spores from the same crop washed a comparable number of times in plain distilled water. Under these conditions, washing the spores in deionized water had no measurable effect upon labilization.

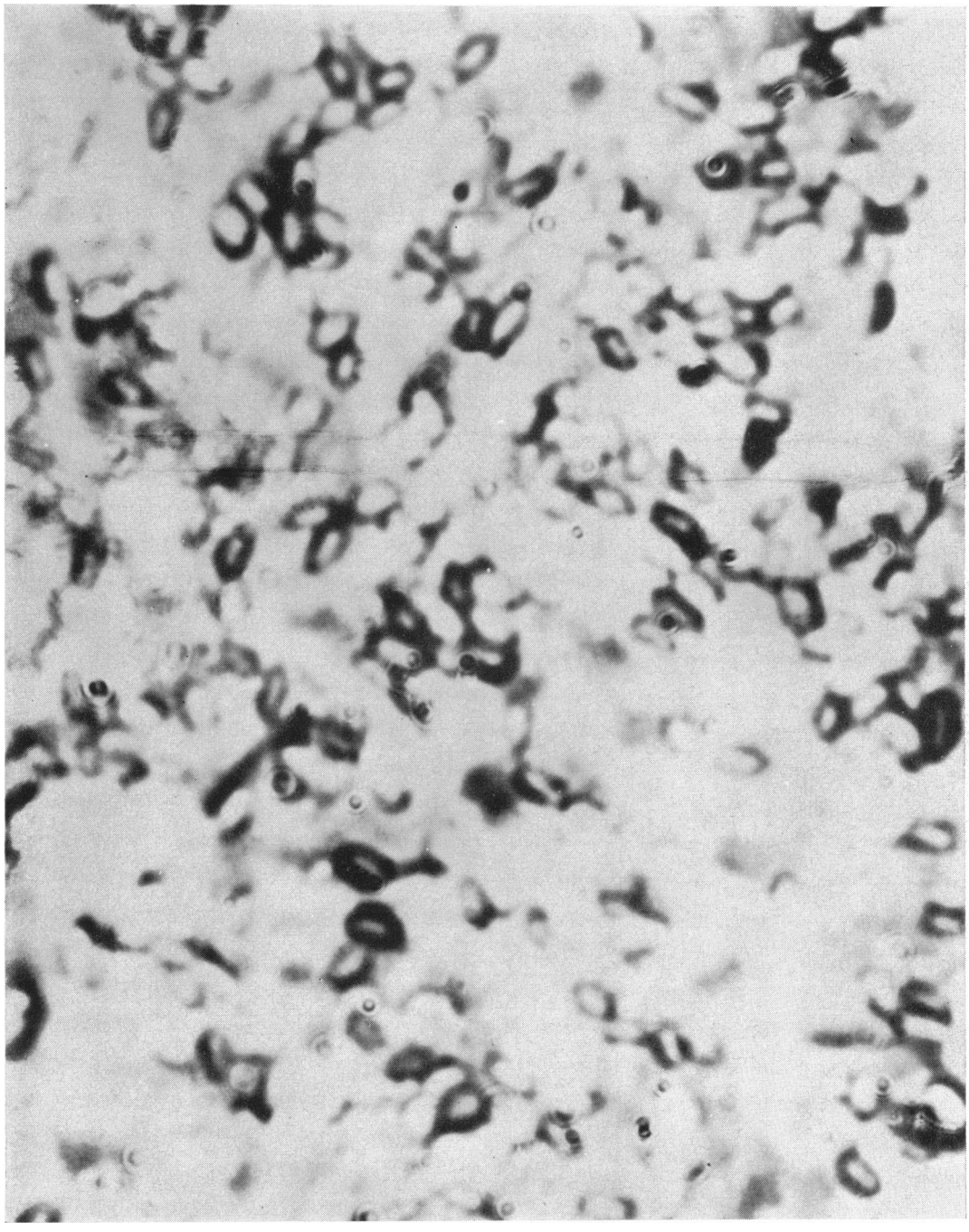

FIG. 2. *Spores of Bacillus stearothermophilus 1518. Concentration, 10^{10}/ml. Stained with crystal violet, ×1350, 0 hr.*

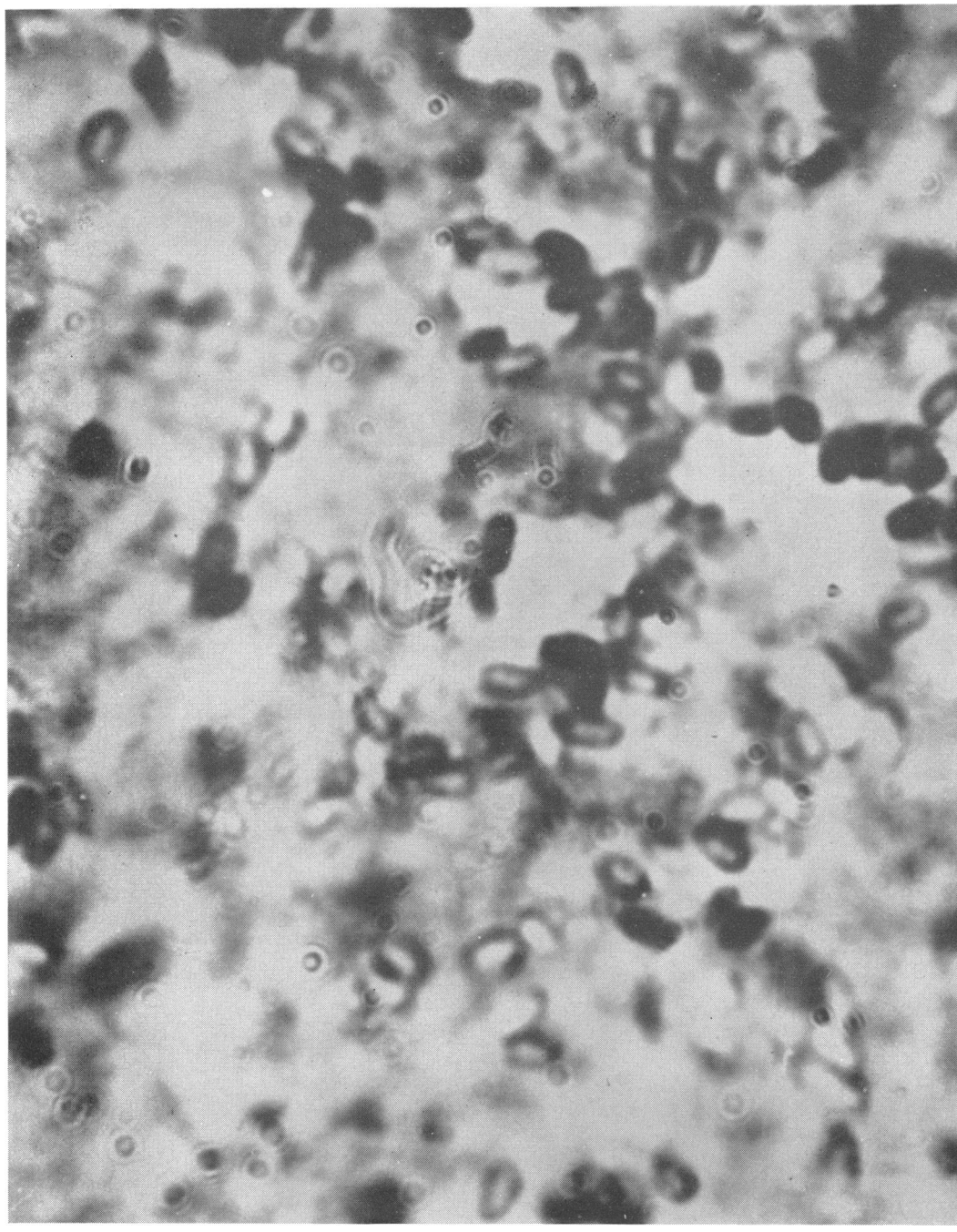

FIG. 3. *Spores of Bacillus stearothermophilus 1518. Concentration, 10^{10}/ml. Stained with crystal violet, ×1350, after 6 hr at 30 C.*

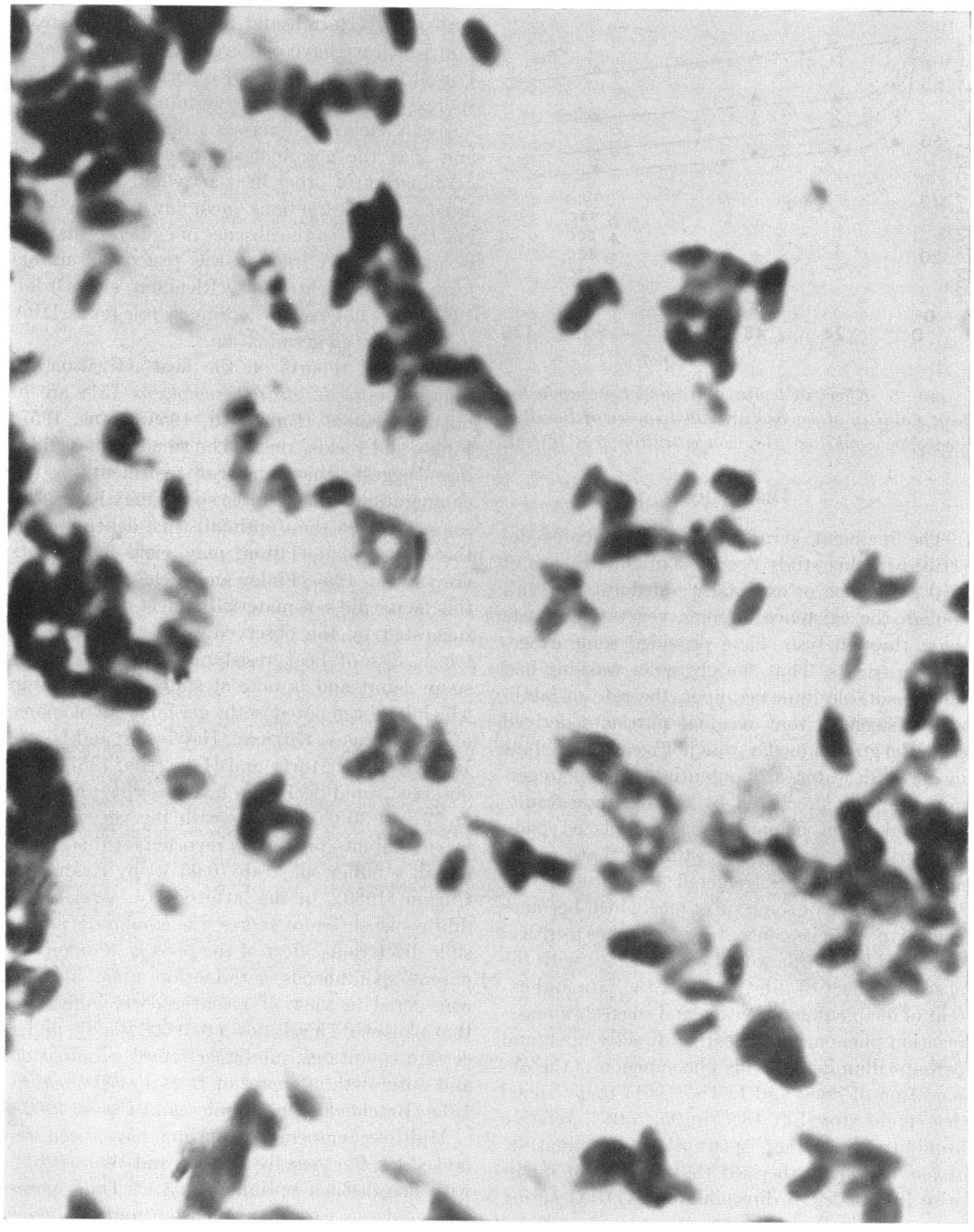

FIG. 4. *Spores of Bacillus stearothermophilus 1518. Concentration 10^{10}/ml. Stained with crystal violet, ×1350, after 30 hr at 30 C.*

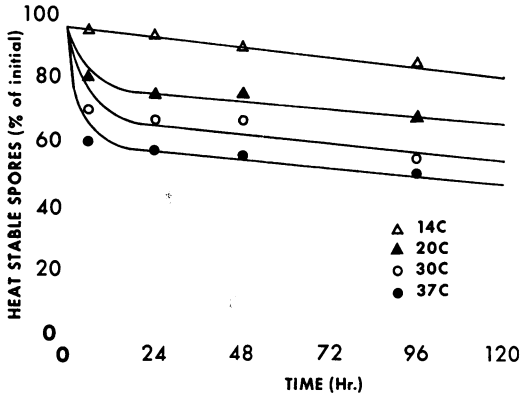

FIG. 5. *Effect of temperature and time upon the heat stability of washed unheated spores of Bacillus stearothermophilus 1518. Concentration, 5 × 10⁷/ml.*

DISCUSSION

The incipient germination of the spores described in this study occurred in the absence of heat activation or externally added nutrients, although the existence of some vegetative ghosts may, through lysis, have provided some exogenous nutrients. That the degree of washing had no measurable influence upon the rate of labilization suggests that residual nutrients derived from the growth media were not responsible. Ions in the wash water, as a potential stimulus to germination, are precluded by the negative results obtained when deionized water replaced plain distilled water. It may be postulated that the germination process is triggered by an excretion from the spore of some substance which becomes operative when its concentration reaches a critical threshold level; this would be consistent with the observed effect of dilution upon the rate and extent of incipient germination and suggests a mass-reaction phenomenon. Bearing directly upon and perhaps illuminating this phenomenon is the observation of Snell and Lewis (1961) that washed dry spores stored at 18 C in 95 to 98% relative humidity underwent spontaneous germination. It has been hypothesized that this result might arise from calcium dipicolinate (CaDPA) acting autocatalytically in the minute amount of water of condensation present on the spores. This interpretation is quite plausible in view of the known germination-triggering action of this compound (Riemann and Ordal, 1961; Keynan and Halvorson, 1962) and the apparent slow leakage of dipicolinic acid (presumably as CaDPA) that occurs with aging (Harrell and Mantini, 1957), a reaction which we have observed (*unpublished data*). Powell and Hunter (1955) reported that washed spores of *B. megaterium* germinated spontaneously in packed-cell masses after heat activation and that the germination substrate stimulated germination of other heat-activated spores. Isolated exudate fractions including CaDPA were inactive. This, in the absence of evidence that the isolated CaDPA fraction was tested in suitable form and concentration (Riemann and Ordal, 1961), does not exclude a primary role for CaDPA in the observed germination.

Published reports on the heat-activation requirements of *B. stearothermophilus* 1518 are in poor agreement (Brachfield, 1955; Titus, 1957; Finley and Fields, 1962). The trend of these findings suggests that our heat treatment for the enumeration of heat-stable spores may have been somewhat less than optimal; this, depending on the degree of deviation, may yield low counts (Brachfield, 1955; Finley and Fields, 1962). That this factor did not materially affect our results is suggested by the observed close agreement between loss of heat resistance as measured by spore count and uptake of stain, a relationship which has been noted in the germination of spores of other species (Hitzman, Halvorson, and Ukita, 1957; Krishna Murty and Halvorson, 1957). The observed rapid decline in heat stability of spores at 37 and 30 C contrasts with the very gradual loss of viability previously reported for this organism in a buffer substrate (0.06 M) by Evans and Curran (1960). In the latter study, a relatively dilute suspension of spores was employed; a possible deleterious effect of the phosphate upon apparent spontaneous germination must also be considered in view of recent reports indicating that phosphate levels down to 0.008 M may, under certain conditions, inhibit or retard germination and outgrowth of spores of *B. stearothermophilus* 1518 (Brachfield, 1955; Finley and Fields, 1962).

Multiple temperature optima have been reported for *B. cereus* by Thorley and Wolf (1961), with one distinct optimum at 3 C. That spores may undergo partial germination under adverse conditions, including low temperature, which do not permit further development or even survival is well known (Powell, 1951; Mundt, Mayhew, and Stewart, 1954; Wolf and Mahmoud, 1957). That this may occur at temperatures more than 20 degrees below that at which the vegetative

cells can proliferate has not been recognized. Of further interest is the implication that, under suitable conditions, incipient germination may be autocatalytic. The resulting almost complete labilization of the spore population contrasts with the autoinhibition of germination of *B. globigii* described by Stedman et al. (1956). These authors demonstrated that in a glucose-alanine substrate germination leveled off after 60 to 85% of the population became heat-labile and permeable to stain.

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