

Oxidative Activation of *Bacillus cereus* Spores

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A study was made of the activation of *Bacillus cereus* strain T spores by using the oxidizing agent sodium perborate. The degree of activation was measured with constant germination conditions by using L-alanine, inosine, adenosine, and L-alanine plus adenosine as germination stimulants. The germinal response following the various treatments was compared with the responses obtained with heat activation. It was concluded that the optimal time for activation with 30 mM sodium perborate at room temperature was about 4 hr. If the exposure time was greatly extended, the spores would germinate spontaneously. When the perborate treatment followed heat activation, the germinal response to L-alanine was stimulated, to inosine retarded and without apparent effect for adenosine or L-alanine plus adenosine. Results of experiments designed to demonstrate deactivation by slow oxidation showed that spores activated with sodium perborate were not deactivated by slow oxidation, whereas those activated by heat were. A deactivation study using mercaptoethanol as the deactivation agent showed that both methods of activation could be deactivated after a 24-hr exposure, but this deactivation was reversible by extending the exposure to mercaptoethanol. The results of heat-sensitivity studies revealed that about 70% of the sodium perborate-activated spores were heat sensitive after 60 min in a germination medium of L-alanine plus adenosine, whereas similarly treated heat-activated and nonactivated spores were about 99.99% heat sensitive, respectively.

Activation is the process of conditioning the spore to germination. Activation is not strictly definable, nor is there a readily apparent visual, chemical, or physical difference between an activated and a dormant spore. When activated spores are exposed to a suitable environment, germination takes place. During germination, the spore loses its typical dormant properties. To evaluate activation it is necessary to test the germinal response of the spore. Germination has been measured several ways (1). A common method of measuring germination is to determine the change in refractility of a spore suspension by measuring its change in optical density.

A common method of activation is that of heat activation (2). A detailed study of the heat-activation conditions for optimal germinal response has been made for the test spores used in the present study (8). A standardization of germination conditions was necessary before the activation treatments studied could be compared by the secondary response of phase darkening due to germination. The germinal conditions for the overall optimal kinetic

response of the test spores to the germination stimulants used has been determined, and these conditions were used throughout the study (8).

It has been suggested (6, 8) that there are different pathways or various recognition sites on the spore which respond to germination stimulants. One purpose for studying oxidative activation with different types of germination stimulants was to obtain further evidence of multiple pathways for germination. A second reason for studying the germinal response of the activation treatments with different types of germination stimulants was to obtain evidence that oxidative activation differed from heat activation. This report describes the altered kinetics of the germinal response of test spores to the germination stimulants when the activation treatment was varied.

MATERIALS AND METHODS

Spores. Spores of *Bacillus cereus* strain T were produced and harvested by procedures described previously (7). The cleaned spores were stored at -20°C either as a frozen, concentrated (9×10^{11} spores per

ml), distilled water suspension or as a lyophilized preparation.

Heat activation. The spores were adjusted to the desired concentration in 40 mM phosphate buffer (pH 8) and heated at 70 C for 30 min. After activation, the spores were centrifuged and resuspended in 80 mM phosphate buffer (pH 8) for germination.

Oxidative activation. The spores were adjusted to the desired concentration in 40 mM phosphate buffer (pH 8) containing 30 mM sodium perborate as an oxidizing agent, and allowed to activate at room temperature for the specified time. After activation, the spores were centrifuged and resuspended in 80 mM phosphate buffer (pH 8) for germination.

Deactivation by oxidation. The activated spores were suspended to the desired concentration in twice-distilled water. The suspension was held at room temperature and stirred slowly while water-saturated air was slowly bubbled into it. After the desired time of deactivation, the suspension was centrifuged and resuspended in 80 mM phosphate buffer (pH 8) for germination.

Deactivation by mercaptoethanol. The activated spores were suspended to the desired concentration in 40 mM phosphate buffer (pH 8) containing 50 mM mercaptoethanol. The suspension was sealed in a container and held at room temperature. After the desired time of deactivation, the suspension was centrifuged and resuspended in 80 mM phosphate buffer (pH 8) for germination.

Activation measurement. The process of activation was measured by observing the secondary response of germination to a standardized set of germination conditions. The germination conditions used had been previously described (8) and were followed except that the temperature of germination was 30 C. The germination stimulants and the concentrations at which they were used were: 10 mM L-alanine, 1 mM inosine, 1 mM adenosine, and 10 mM L-alanine plus 1 mM adenosine.

Heat sensitivity. Loss of heat resistance during germination was followed by removing samples at intervals and transferring them to sterile distilled water blanks pre-equilibrated to 80 C. After holding for 10 min at 80 C, 0.1-ml samples of appropriate dilutions were surface plated on Trypticase soy agar plates. The plates were incubated at 28 C for 18 to 20 hr and then counted with the aid of a Quebec colony counter.

RESULTS

Prior investigations have indicated that oxidizing agents can be used to activate spores (3). The results of a preliminary investigation (A. J. Cutaia, Ph.D. thesis, Univ. of Illinois, Urbana-Champaign, 1968) which examined several chemical oxidizers as activators resulted in our choice of 30 mM sodium perborate. The extent of activation resulting from the various treatments was assessed by an evaluation of the kinetics of the germinal response to the four germination stimulants.

Our initial efforts demonstrated that if the

spores were exposed to 30 mM sodium perborate for 24 hr, the majority of spores became phase dark. A time sequence of these changes is presented in Fig. 1. No phase darkening was apparent after a 4-hr exposure period, but slight but detectable darkening was apparent in an 8-hr exposure. Spores which were removed from the perborate solution after 4 hr and resuspended in distilled water were stable and did not spontaneously phase darken.

The nature of the response to the specific germinant and the effect of oxidative activation times in the sodium perborate are shown in Fig. 2. In each case, the response is compared with the response to heat activation and the response to no activation treatment. Although four germination stimulants were used, the response to adenosine stimulation in all experiments was so slight that it was used as a negative control and as a check for amino acid contamination of the germinating suspensions. Hence these data are not presented.

The shapes of the response curves to L-alanine plus adenosine stimulation (Fig. 2A) indicate that activation has taken place in the sodium perborate since the overall response, although slightly less than that for heat activation, is greater than that for no activation. The maximum response to L-alanine plus adenosine resulted after a time of oxidative activation of about 4 hr. The overall kinetics for this stimulant improved with time up to 4 hr. The lag times shortened, the maximum rate increased, and the percentage of the population which became phase dark increased. At times greater than 4 hr, although the lag times and maximum rates are similar to those of 4 hr, there is an apparent decrease in the percentage of the population which germinates.

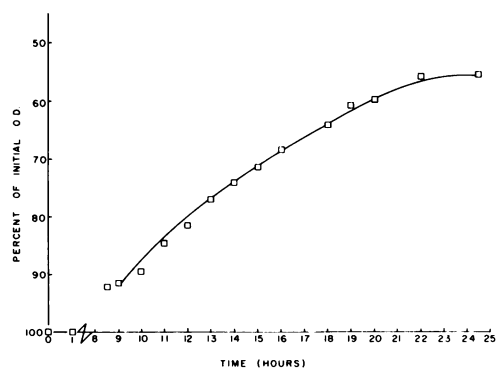


FIG. 1. Phase darkening of *Bacillus cereus* spores following various periods of time in 40 mM sodium phosphate (pH 8) containing 30 mM sodium perborate, as measured by change in optical density.

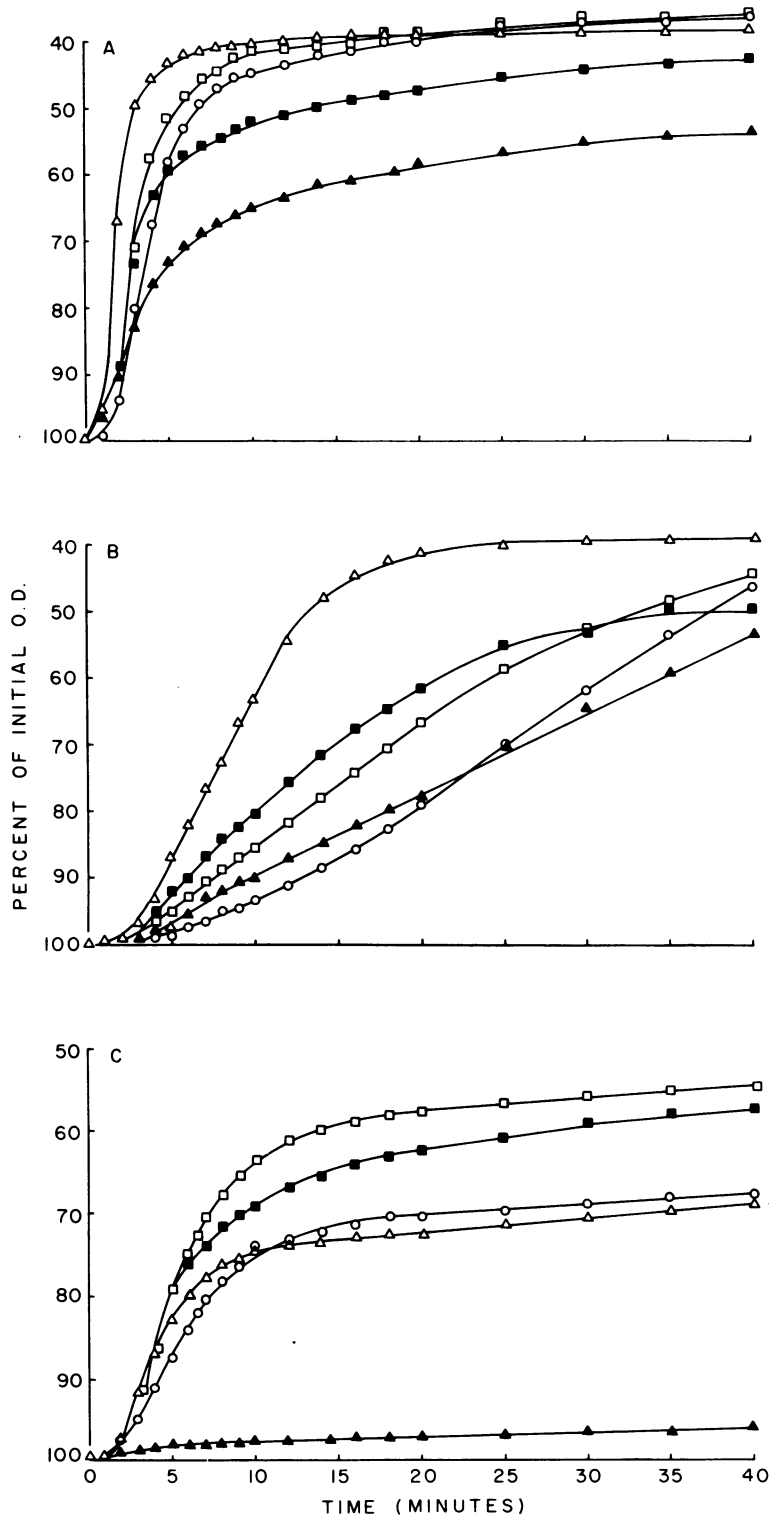


FIG. 2. Germinal response of *Bacillus cereus* spores as affected by selected activation conditions. Optical density changes were followed in response to different germination stimuli. A, Response to 10 mM L-alanine plus 1 mM adenosine; B, Response to 1 mM inosine; C, Response to 10 mM L-alanine. Symbols: O, 2-hr activation in 30 mM sodium perborate; □, 4-hr activation in 30 mM sodium perborate; ■, 8-hr activation in 30 mM sodium perborate; Δ, heat activated; ▲, no activation.

The kinetics of the germinal response to inosine (Fig. 2B) for the various activation treatments are different from those to L-alanine plus adenosine stimulation. The overall response for sodium perborate activation never approaches the response for heat activation. The germinal responses for sodium perborate activation do indicate that activation has taken place since, as the length of time in the sodium perborate increases, the lag time decreases and the maximum rate increases. The percentage of the total population which becomes phase dark increases with increasing time, with the exception of time greater than 4 hr, when the response levels off after a 30-min exposure to the stimulant.

The overall responses for the various activation treatments to L-alanine (Fig. 2C) follow the pattern of decreasing lag times, increasing maximum rates with increasing lengths of time, and increasing percentages of the total population which germinate with lengths of time up to 4 hr. When compared with the response expected for heat activation, it was found that times greater than 2 hr gave increased germinal responses.

After finding that oxidative activation using 30 mM sodium perborate could replace heat activation in stimulating the germinal response to L-alanine plus adenosine and that it resulted in an increased response to L-alanine germination, it was decided to see if the sodium perborate treatment had an effect on heat-activated spores. In these experiments, the spores were first heat activated and then resuspended in the 30 mM sodium perborate solution for the desired time. The results of this examination are graphically presented in Fig. 3.

The response curve to L-alanine plus adenosine stimulation (Fig. 3A) is only slightly different from the response curve for heat activation alone. The lag time is slightly longer, but the maximum rate and the percentage of the spore population remaining phase bright are about the same.

The inosine germinal response for the sodium perborate treatment following heat activation (Fig. 3B) was markedly different from the response for heat activation alone. Overall, the response to inosine was inhibited by the treatment. The lag times were lengthened and the germination rates were retarded.

In contrast to inosine stimulation, when L-alanine was used as a germination stimulant (Fig. 3C), the spores responded in what could be considered an additive manner. After 1 hr of the treatment, the total germinal response was much greater than that of heat activation alone and, in fact, was greater than the response to

4-hr sodium perborate activation. The germinal response after 4 hr of treatment was even greater.

Activation has been termed a reversible process. Other investigators have demonstrated that activation by a heat treatment or by a reducing agent is reversible by slowly oxidizing the activated spores (3, 5). Table 1 compares the germination rates of heat-activated spores with those of sodium perborate-activated spores for two opposing types of deactivation, deactivation by slow oxidation and deactivation with a reducing agent (50 mM mercaptoethanol). Each type of deactivation elicited different germinal responses.

Deactivation by slow oxidation resulted in a substantial lowering of the response of heat-activated spores to L-alanine and inosine stimulation; the germinal response to L-alanine plus adenosine showed a lengthened lag period but then accelerated so the response was essentially equal to the control at 20 min. Slow oxidation did not function as a deactivation method to any of the germinal stimulants used for spores which had been activated with sodium perborate. The reducing agent, 50 mM mercaptoethanol, functioned both as a deactivator and as an activator, the degree being dependent on the type of activation and the stimulant. The mercaptoethanol acted first as a deactivator; then, as the treatment continued, the spores were reactivated. The response to inosine stimulation at 20 min of germination does not show reactivation, but reactivation became apparent after an extended period of time.

A heat-sensitivity study was done to determine if sodium perborate activated some of the "superdormant" spores found in similar studies with heat-activated spores (4). Figure 4 compares the heat sensitivity of germinating spores that were heat activated, sodium perborate activated or nonactivated. Each activation treatment resulted in substantial difference in heat sensitivity. After 60 min in the germination menstruum, about 99.99% of the heat-activated spores were heat sensitive to the test conditions compared with about 95% of the nonactivated spores and only about 70% of the sodium perborate-activated spores. The data indicated that not only did sodium perborate not activate the "superdormant" spores, but that it somehow had made the phase-dark spores more heat resistant. Further work is being undertaken to explain this phenomenon.

DISCUSSION

The data indicate that the time for optimal

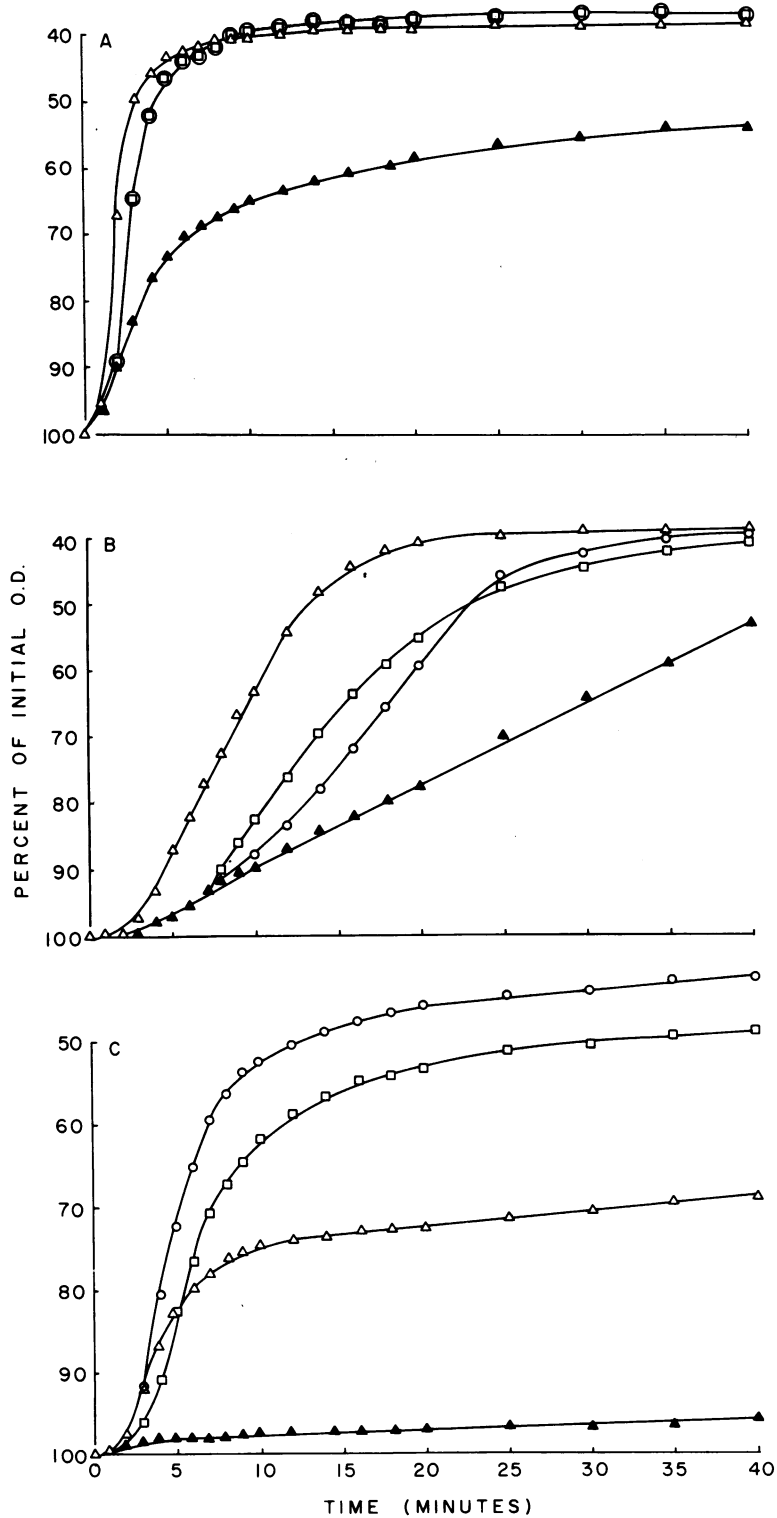


FIG. 3. Germinal response of *Bacillus cereus* spores as affected by selected activation conditions. Optical density changes were followed in response to different germination stimuli. A, Response to 10 mM L-alanine plus 1 mM adenosine; B, Response to 1 mM inosine; C, Response to 10 mM L-alanine. Symbols: □, heat activated plus 1 hr in 30 mM sodium perborate; ○, heat activated plus 4 hr in 30 mM sodium perborate; △, heat activated; ▲, no activation.

TABLE 1. Effect of deactivation treatments on heat-activated and sodium perborate-activated spores of *Bacillus cereus*

Germination stimulant ^a	Activation treatment	Deactivation resulting from deactivation treatment (%) ^b		
		Slow oxidation	24-hr mercapto-ethanol	48-hr mercapto-ethanol
L-Alanine	Heat	82	50	33
Inosine	Heat	73	100	0
L-Alanine plus adenosine	Heat	0	20	0
L-Alanine	NaBO ₃	0	30	15
Inosine	NaBO ₃	0	99	99
L-Alanine plus adenosine	NaBO ₃	0	30	0

^a The representative time of 20 min was chosen for the estimation of the germination rate.

^b Deactivation (%) = $(G_t - G_n / G_a - G_n) \times 100$, where G is the estimation of the germination rate (per cent optical density loss per min), G_t is the estimated germination rate of the deactivation treatment, G_a is the estimated germination rate of the activated spores, and G_n is the estimated germination rate of nonactivated spores for the individual stimulant.

germinal response to oxidative activation using sodium perborate varies with the specific stimulant used. After consideration of the shapes of the curves obtained for the various activation treatments and the germination stimulants used, it was concluded that the optimal exposure time to the perborate which resulted in good germinal response was 4 hr. This activation time gave germinal responses which, when compared with the response for heat activation under the same set of standardized conditions, was comparable for L-alanine plus adenosine stimulation, increased for L-alanine stimulation, and retarded for inosine stimulation.

It was discovered during the course of this study that sodium perborate activation was, in reality, a continuous system in that it had the ability to ultimately cause the spores to germinate or become phase dark (Fig. 1.). This is similar to the finding of Gould and Hitchins (3) that hydrogen peroxide caused phase darkening in spores exposed to agents known to cause rupture of disulfide bonds. However, they did not report phase darkening as a result of their use of the oxidizing agent performic acid in activating the spores. Some of the spores activated in sodium perborate started to appear phase dark at 6 hr.

The data clearly support the suggestion by others (6, 8) that there are different pathways or

at least different recognition sites on the spore for the various germination stimulants. The investigation for the optimal time for oxidative activation with sodium perborate resulted in germination curves with shapes different from those for heat activation. The response to L-alanine stimulation was greater for sodium perborate activation than for heat activation, whereas the response to inosine was less for sodium perborate activation than for heat activation. The effects of the sodium perborate treatment following heat activation resulted in opposite effects to L-alanine and to inosine stimulation. The L-alanine germinal response reacted in an additive manner to the treatment, whereas the inosine germinal response was inhibited. The two deactivation methods acted to a different degree, depending on the stimulant used.

The data also indicate that heat activation and oxidative activation activate the spore by different mechanisms. Several of the experiments support this suggestion. The effect of the sodium perborate treatment following heat

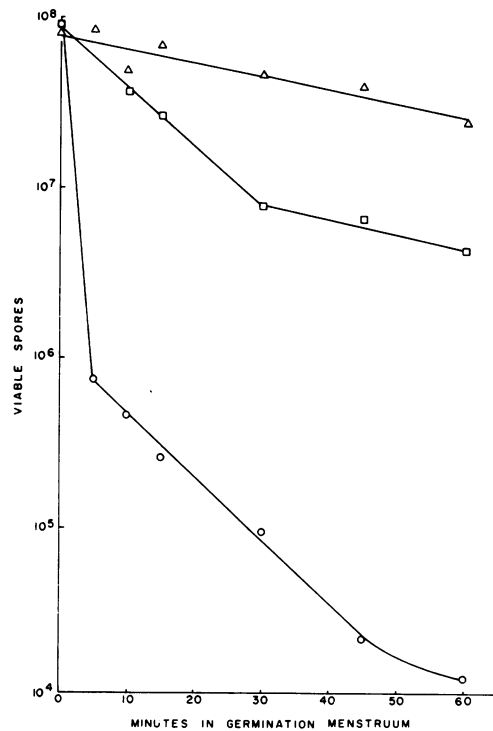


FIG. 4. Comparison of activation treatments on the number of heat-resistant *Bacillus cereus* T spores at various times in a germination medium of L-alanine plus adenosine. Samples were heat challenged at 80 C for 10 min. Symbols: □, nonactivated; ○, heat activated; △, NaHBO₃ activated.

activation resulted in an additive germinal response to inosine stimulation. The suggestion of differing mechanisms of activation is more strongly supported by the data from the deactivation and heat-sensitivity studies. Spores activated with the sodium perborate treatment were not deactivated by slow oxidation, whereas those activated by heat were deactivated. The heat-sensitivity study revealed that although the germinal response to L-alanine plus adenosine stimulation as measured by phase darkening was similar for both heat-activated and sodium perborate-activated spores, the percentage of the test population which was heat sensitive after like times in the germination menstruum was substantially different. In fact, the activation treatment with sodium perborate resulted in the germinating spores having a much greater heat resistance than even the germinating spores which received no activation treatment.

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