# HEAT ACTIVATION KINETICS OF BACILLUS MEGATERIUM SPORES

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SUMMARY

Bacillus megaterium QM B1551 spores were activated by heating in aqueous suspension. Activation was measured by increase in germination rate (on glucose at 30 C) over that of dormant spores. At any one temperature, activation increased linearly with time of exposure. Spores were activated throughout the range of test temperatures (50-75 C). The exposure time required to achieve a given germination rate increased with decreasing temperature. The logarithm of the activation velocity (increase in germination rate/sec of heating) was a linear function of the temperature of heating. The apparent average thermodynamic constants for the system were: $\Delta H^{\ddagger}$ = 72.4 kcal/mol; $\Delta F^{\ddagger}$ = 22.8 kcal/mol; $\Delta S^{\ddagger}$ = 147.4 cal/mol/deg.

Dormant bacterial spores germinate poorly, or not at all, under conditions permitting rapid germination of activated spores (9). Dormancy may be broken by the application of heat (3), and the presence of water is required for this activation (7, 14). The degree of activation can be measured in two ways: (i) by increase in colony count, a method frequently used with highly dormant spores which do not form colonies unless activated; or, (ii) by increase in germination rate in the presence of a defined germination agent. The latter is the more direct and more sensitive method (10). A number of workers (2, 4, 6, 12) have evaluated the time-temperature relationship of heat activation as measured by increase in colony count. Keynan et al. (8) have studied the effects of heat activation on the germination rate of B. cereus T spores, but used only a few exposure times. Powell and Hunter (14) investigated the time-temperature relationship for the spontaneous germination of B. megaterium spores. The results reported here describe the kinetics and thermodynamic properties of heat activation of B. megaterium QM B1551 spores, using increase in germination rate on glucose

(over that of dormant spores) as a measure of activation.

## MATERIALS AND METHODS

Spores of B. megaterium QM B1551, grown on the complex medium, omitting agar, of Arret and Kirshbaum (1), were harvested, washed, lyophilized, and stored under vacuum at 4 C over silica gel.

For heat activation, 0.1 ml samples of a chilled aqueous suspension of spores (50 mg spores/ml) were added to 9.9 ml of water (in 18 X 150 mm Pyrex test tubes) previously equilibrated in circulating water baths to the desired temperature. The resulting suspensions (0.5 mg spores/ml) were stirred magnetically to achieve rapid temperature equilibration and maintenance. The addition of the 0.1 ml of heavy spore suspension lowered the temperature of the heating water less than 0.1 C. After heating for various times at 10 temperatures between 50 and 75 C, further activation was stopped by vigorously shaking the tubes in an ice bath. Only at the higher temperatures (72.5 and 75 C), where heating times were 0.25 min or less, did the time for cooling to non-activating temperatures cause any slight error in estimation of the activation response. For example, spores heated at 70 C were chilled to $< 55$ C in $< 0.1$ min.

After heat activation, spores were germinated at 30 C in 25 mM glucose, buffered with 50 mM potassium phosphate, pH 7.0. Germination was followed kinetically, for 120 min, as the decrease in optical density ($OD_{560}$ nm, Klett-Summerson colorimeter) of spore suspensions (0.33 mg spores/ml, initial Klett reading = ca. 280). The criterion of heat activation was the increase in germination rate (% OD loss/min during the period of most rapid linear decrease in OD after the addition of glucose) over that of the dormant spores. However, the very low germination rate of dormant (unheated) spores permitted us to neglect this value in calculation of the increase in germination rate attributable to heating.

## RESULTS AND DISCUSSION

Unheated spores of B. megaterium QM B1551 were essentially dormant for glucose-induced germination, losing only ca. 5% of their initial OD in 120 min, with a germination rate of $< 0.05\%$ OD loss/min. Heating increased both the extent and rate of germination. A typical plot of OD loss during germi-

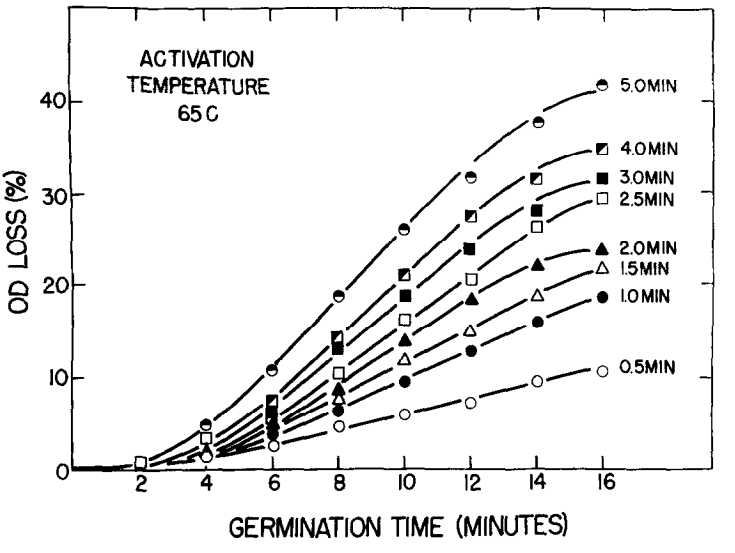


Figure 1. Germination of Bacillus megaterium spores, heat activated at 65 C for various times. Germination rates (% OD loss/min in glucose at 30 C) were calculated from the rectilinear portions of these plots, and from similar plots of the germination of spores, heat activated at other temperatures between 50 and 75 C.

nation of spores heated for various times at 65 C is shown in Fig. 1. Activation, as measured by increase in germination rate, occurred over the whole range of 10 exposure temperatures. However, the time of heating required to achieve a given level of activation increased with decrease in temperature (Fig. 2). For example, the time of heating necessary to achieve a germination rate of 1.5% OD loss/min ranged from 0.08 min at 75 C to 225 min at 50 C. With B. megaterium spores, activation at any one temperature appeared to increase linearly with exposure time (Fig. 2), and not logarithmically as reported for other organisms, where increased colony count was the index of activation (2, 4). The germination rate of B. cereus T spores has also been found to increase linearly with time of heating at 65 C (13). However, at any one time of exposure to heat, activation, as measured by total extent of germination, increased exponentially with temperature of heating (11).

We have previously postulated (11) that the depression in the maximum germination rate and increase in the lag before attainment of this rate by spores heated at higher temperatures, may indicate that the germination

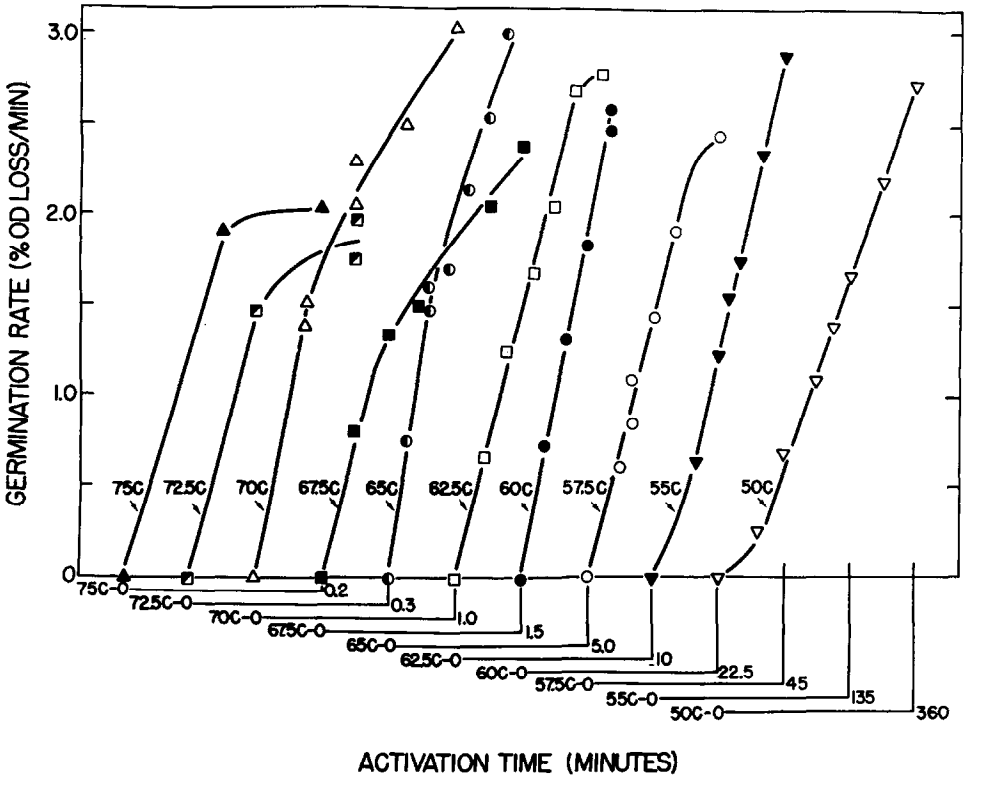


Figure 2. Heat activation of Bacillus megaterium spores at ten temperatures. Germination rate (% OD loss/min) is plotted against a time scale which has been manipulated to accommodate the longer heating times necessary for activation at the lower temperatures. For example, the time axis for 75 C represents 0.2 min; the same length of time axis at 50 C represents 360 min. The rectilinear portions of curves at each temperature were used to calculate activation velocities (increase in germination rate/sec of heating).

system is the site of the initial damage in heat inactivation of bacterial spores. In the present experiments, as the temperature of activation was increased above 65 C, germination rates tended to level off with increased time of exposure (Fig. 2), although the spores eventually germinated to the full extent. For example, the germination rate leveled off after exposure at 75 C for as short a time as 0.2 min, probably reflecting initial stages of heat inactivation. Our data for activation at 75 C may give a minimum value for activation velocity at this temperature. Activation velocity, the increase in germination rate/sec of heating, was calculated from the slopes of the linear portions of the curves in Fig. 2. Some inactivation,

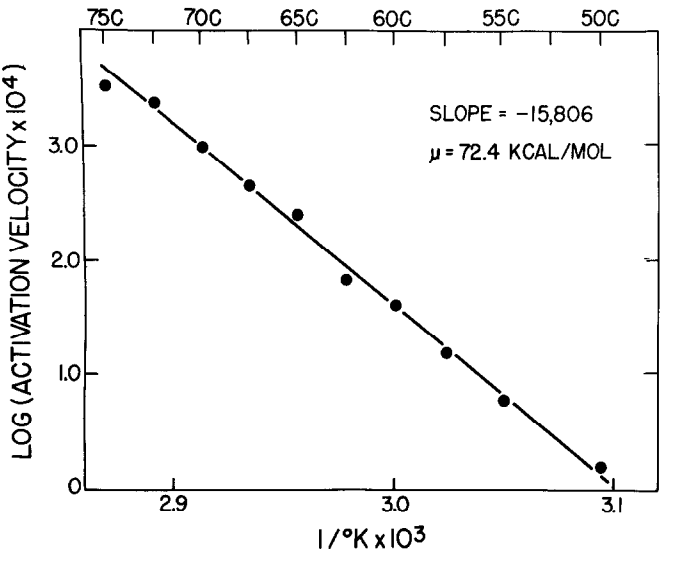


Figure 3. Effect of temperature on activation velocity (increase in germination rate/sec of heating) of Bacillus megaterium spores (an Arrhenius plot).

with consequent plateauing of the germination rate, may already have occurred during exposure at this temperature for 0.1 min - the shortest heating time feasible with the heating methods employed.

The temperature coefficient ($Q_{10}$ = ca. 27.5) for the activation process (based on activation velocities calculated from Fig. 2) was considerably higher than that of ca. 5.5 found by Murrell (12) for B. coagulans spores, heated at temperatures between 95 and 110 C, using the colony count method. An Arrhenius plot of the activation velocities is shown in Fig. 3. The method of the sum of least squares (by computer analysis) was used for the best fit of a straight line curve, which had a slope of - 15,806. Accordingly, an activation energy ($\mu$) of 72,392 cal/mol was determined for the system (5). Comparable values for $\mu$ (70,000 - 80,000 cal/mol) were also obtained using parameters other than initial germination rates [i.e., extent of germination in 120 min (using only exposure times where response to heat was still linear with time); % OD loss in 6, 10, or 20 min; or the germination rate of the fraction of spores remaining available to enter into the heat activation response].

Although we used a narrower range of test temperatures than did Busta

and Ordal (2), we agree with their conclusion that heat activation is a continuous response throughout the range of test temperatures and is a function of time of heating. However, their lower activation energy requirement ($\mu$ = 27,900 cal/mol) may reflect an actual difference between B. subtilis spores and B. megaterium spores, or may be due to their method (colony formation) which is a more complex and less sensitive measure of loss of spore dormancy than is increase in germination rate. The activation energy for Clostridium botulinum spores (6), as determined by colony count, was somewhat higher (42 kcal/mol). Keynan et al. (8) reported that activation (increased germination rate) of B. cereus T spores required a critical temperature which decreased with increased heating times. However, our calculations from their data (heating times limited to 45 min and 48 hr) show activation to be a continuous exponential response (between 20 and 60 C) with $\mu$ = ca. 32,000 cal/mol.

The kinetic data of Busta and Ordal (2), over the range 5 - 94 C, indicated to them little difference between high temperature heat activation, and what was termed "aging at low temperature". If the phenomenon of aging does occur with B. megaterium spores, and if it represents an extension of the same heat response obtained here, we calculate that it would take ca. 20,000 years before spores stored at 5 C would attain a germination rate of 1.0% OD loss/min. It is obvious that, at least with spores having a high $\mu$ for activation, any observed "aging" at low temperatures must be due to changes in the spore not related to heat.

Other thermodynamic constants were calculated from the data, as outlined by Stearn (15). For an activated complex $\Delta H^{\ddagger}$, $\Delta F^{\ddagger}$, and $\Delta S^{\ddagger}$ represent respectively, the standard changes in heat content or enthalpy, free energy, and entropy. The standard enthalpy, $\Delta H^{\ddagger}$, was approximately equal to $\mu$ (72.4 kcal/mol) since the maximum difference (RT) between $\mu$ and $\Delta H^{\ddagger}$ was only 0.69 kcal. Standard free energy for the ten temperatures ranged from 21.3 to 24.6 kcal/mol (average $\Delta F^{\ddagger}$ = 22.8 kcal/mol). Standard entropy, determined by difference, ranged from 146.8 to 147.9 cal/mol/deg (average $\Delta F^{\ddagger}$ = 147.4 cal/mol/deg). While the interpretation and significance of these constants may be open to question, they have been reported here for comparison with re-

sults of other investigators.

Keynan et al. (10) have postulated that heat activation represents a reversible denaturation of one or more proteins which control the dormant state of the spore. The thermodynamic properties of the activation process reported here are characteristic of protein denaturation (high activation energy, large increase in entropy, and high temperature coefficient), and are consistent with the Keynan postulate, but do not constitute proof of its validity. Assuming that this high energy is used in the breaking of bonds, one can calculate, using the approaches suggested by Stearn (15), that, in our activation system, either a number of weak bonds (ca. 14.6), or one strong bond plus a number of weak bonds (ca. 13.2) are broken. These values would yield entropy increases of ca. 167 or 159 cal/mol/deg, which are in good agreement with the experimental value obtained ($\Delta S^{\ddagger}$ = 147.4 cal/mol/deg). The breaking of 1 to 2 strong bonds, as suggested by Busta and Ordal (2) for B. subtilis spore activation, having a much lower $\mu$ than our system, would not yield the large increase in entropy which we found. However, we do not mean to imply that bond-breakage is the only process involved. Other high energy-requiring processes, such as water transport, must also be considered. We have previously postulated that heating alters the structure of liquid water, enabling it, like water vapor, to reach and hydrate a specific spore site (possible a protein) resulting in activation (7) While temperature-induced alterations in water structure may be involved in heat activation, no reliable physical measurements of such changes are presently available. If heat activation is effected by denaturation of a protein, then the effects of heat on the whole protein-water complex must be considered.

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