

Water Vapor, Aqueous Ethyl Alcohol, and Heat Activation of *Bacillus megaterium* Spore Germination¹

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Dormant spores of *Bacillus megaterium* were activated for germination on glucose by heating them in aqueous suspension (but not if heated dry), by treating them with aqueous ethyl alcohol at 30 C, or by exposing them to water vapor at room temperature. The degree of water vapor activation depended upon the relative humidity, the time, and the temperature of exposure. Activation increased the extent and rate of glucose-induced germination and decreased the average microlag. Extended water vapor treatment also activated spores for germination induced by KI and by L-alanine. Spores activated by any of the three treatments were deactivated by treatment at 66 C, either for 18 hr in 100% ethyl alcohol or for 40 hr over P₂O₅. Deactivated spores were reactivated by heat, by 5 M ethyl alcohol, or by water vapor. It is postulated that heating and ethyl alcohol may change the structure of liquid water, so that it is more like water vapor and can more readily penetrate to and hydrate a critical (enzymatic?) spore site, leading to activation.

Activation, a reversible process, results in a spore which retains its typical heat-resistance, refractivity, nonstainability, and dipicolinic acid (DPA) content, but which is no longer dormant—that is, it is conditioned for germination. The term dormancy, as used here, describes not the apparent metabolic inactivity of the spore but rather the necessity for some form of activation for rapid and complete germination (15). Activation requirements depend upon the sporulation medium, the chemical composition of the spore, its storage history, and the nature of the germination agent. Activation increases the total number of spores germinating and the initial germination rate under a particular condition, decreases the lag before germination becomes perceptible (31), decreases the concentration of germinants required and increases the variety of effective germinants (11, 17, 24), and activates certain enzymes of the resting spore (4).

Curran and Evans (5) first clearly recognized that the bacterial spore could be activated by the application of heat. Later workers found that spores were also activated by reducing agents and by low pH (14), by high or low pH (8), by exposure to Ca-DPA solutions (16), by dimethyl-

formamide and dimethylsulfoxide (33), by aging (23), by aqueous solvents (9), and by water vapor without heating (10).

The present report extends our observations on water vapor activation to include variables of time, temperature, and relative humidity. Activation, deactivation, and reactivation characteristics of spores activated by exposure to water vapor are compared with those activated by heat and by aqueous alcohol. We propose that activation of bacterial spore germination by these three treatments involves the hydration of a critical spore site and that these treatments enable water to reach this site.

MATERIALS AND METHODS

Spore preparation. Spores of *Bacillus megaterium* QM B1551, grown on the complex medium (omitting agar) of Arret and Kirshbaum (1), were harvested, washed, and lyophilized, and were stored under vacuum at 4 C in a desiccator over either CaSO₄ or silica gel. These A-K spores contained (dry-weight basis) 2.6% calcium, 10% DPA, and ca. 5% water (removable in 24 hr either by drying at 100 C or by evacuating). In a few cases, *B. megaterium* QM B1551 spores were grown on 0.5% Liver Fraction "B" (Wilson and Co., Chicago, Ill.); these are referred to as LB spores.

Water uptake. The water uptake characteristics of spores were determined at 20 and 30 C, by using a McBain-Bakr (21) sorption apparatus modified so

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that relative humidity was controlled through the control of the temperature of a water reservoir (condenser), with special Ni-Span-C springs in place of quartz springs. Duplicate samples (ca. 70 mg) of lyophilized spores, evacuated at 0.1 mm of Hg for 24 hr to constant weight (5% weight loss as water), were exposed to water vapor at the indicated relative humidities. Weight changes due to sorption of water were followed for 18 hr.

Activation. For heat activation, 10 ml of spores in aqueous suspension (2 mg/ml) were heated for 10 min in water baths at the indicated temperatures. Activation was stopped by chilling in an ice bath.

Similar spore suspensions (10 ml) were activated by exposure to aqueous ethyl alcohol at 30 C for 5 min (9). Routinely, when spores were activated in 5 M ethyl alcohol, activation was stopped by diluting the ethyl alcohol to ineffective levels with 30 ml of water. The diluted suspension was then centrifuged and the spore pellet resuspended in 10 ml of water. In experiments where higher concentrations of ethyl alcohol were used (as in examining a range of ethyl alcohol concentrations), and where dilution with 30 ml of water would not reduce the ethyl alcohol to ineffective concentrations, activation was arrested by discarding most of the ethyl alcohol after centrifugation at 4 C, washing the spore pellet with 40 ml of water, and resuspending it in 10 ml of water.

For activation by water vapor, certain preparative procedures were necessary to ensure uniform exposure of the spores and to minimize activation by ambient humidity. Experiments designed to examine the effects of various relative humidities at short exposure times were not done on days of high relative humidity, since some activation occurred during weighing of the lyophilized spore samples. Spore-coated no. 13 Ballotini beads (10) were evenly distributed in open 6-cm petri dishes and exposed in 160-mm desiccators (volume, ca. 2,750 ml), previously equilibrated to the desired relative humidity for 24 hr at various temperatures with 100 ml of various H₂SO₄-water controlling solutions (26). (We describe here, only the atmospheric relative humidity to which the spores were exposed, and not necessarily the water activity reached by the spores.) H₂SO₄, per se, made no contribution to the observed water vapor activation, as similar activation levels were attained with NaOH-water controlling solutions (data not shown). In some cases, spores (in 15 ml flasks, similar to Warburg vessels) were water vapor-activated by continuous flushing with air (50 ml/min) previously passed through two towers containing the various H₂SO₄-water mixtures. Activation was stopped by the addition of 10 ml of liquid water. Control spores similarly coated on glass beads, but exposed in a desiccator over P₂O₅ or mixed with liquid water without prior exposure to water vapor, were not activated for germination, ruling out the possibility that mechanical abrasion during the coating of the beads with spores contributed to the observed activation.

The extent of activation was determined by measuring the increase in the rate and extent of germination (over that of the untreated spores) at 30 C in 25 mM glucose, buffered with 50 mM potassium phosphate

(pH 7.0). Germination was followed kinetically by decrease in optical density (OD) of spore suspensions (0.4 mg spores/ml) at 560 m μ (Klett-Summerson colorimeter); total spore germination was also estimated microscopically by stainability with 0.5% methylene blue. A 25% loss in OD approximated 50% germination (stainability); loss of 55 to 60% of the original OD corresponded to 98 to 100% germination. The germination rate was calculated as the per cent of decrease in OD per minute during incubation in glucose over the first 10 or 20 min (whichever was higher). Average microlag (31) was estimated as the time for 50% completion of the total OD loss (18).

Deactivation. Spores treated under conditions giving approximately equivalent degrees of activation (water vapor at 90% relative humidity, 30 C, 30 min; 5 M ethyl alcohol, 30 C, 5 min; aqueous suspension, heated at 58 C, 10 min) were centrifuged and washed with 40 ml of water before deactivation. For absolute ethyl alcohol deactivation, spore pellets were suspended in 50 ml of absolute ethyl alcohol, incubated at 66 C for 18 hr, centrifuged, and washed with 40 ml of water. Spore pellets to be deactivated over P₂O₅ were lyophilized for 4 hr; the dried pellets were exposed in 6-cm petri dishes in evacuated desiccators over P₂O₅ for 2 hr at 30 C and for an additional 40 hr at 66 C.

Reactivation. After deactivation, spore pellets were either suspended in liquid water for reactivation by heat (65 C, 10 min), in 5 M ethyl alcohol for ethyl alcohol reactivation (30 C, 5 min), or were lyophilized for 4 hr before reactivation by water vapor (90% relative humidity, 30 C, 30 min).

Viability and heat resistance. Viability and heat resistance (75 C, 10 min) were determined by conventional plating techniques (colony counts after 24 hr of growth on nutrient agar, supplemented with 0.2% yeast extract).

DPA. DPA in untreated and activated spores was determined colorimetrically (13).

RESULTS

The emphasis in the present study was on the activation of *B. megaterium* spores by exposure to water vapor. Water vapor activation was also compared with activation by heating or by treatment with aqueous alcohol.

Heat activation. Untreated *B. megaterium* A-K spores were essentially dormant for glucose-induced germination. After 120 min of incubation with 25 mM glucose, only 8% of untreated spores were stainable, with a concomitant loss of ca. 4% of the OD (Fig. 1). The rate of germination of the untreated spores was <0.1% loss/min (Fig. 1, Table 1), and the average microlag time was 36 min (Table 1). Heating of spores in aqueous suspension at 60 to 70 C for 10 min resulted in maximal activation for subsequent germination; after 30 min in glucose, 98% of such heated spores were stainable and 55% of the original OD was lost. The rate of germination of spores heated at 65 C for 10 min was increased to 3.3% OD loss

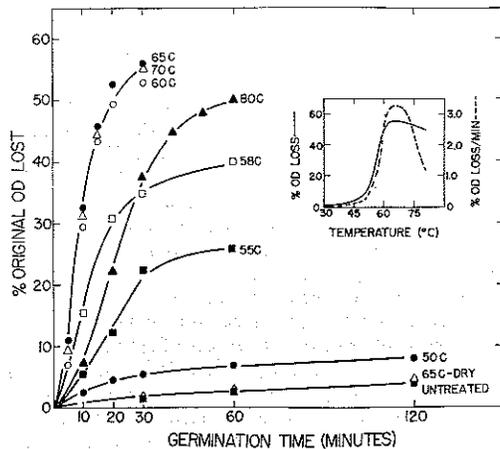


FIG. 1. Heat activation of the germination of *Bacillus megaterium* spores. Spores were heated in aqueous suspension for 10 min at the indicated temperatures and then germinated at 30 C in 25 mM glucose buffered with 50 mM potassium phosphate, pH 7.0. Untreated spores were those suspended in water for 10 min at room temperature (25–30 C). Insert compares total germination (% OD loss) with germination rate (% OD loss/min).

per min, and the average microlag was decreased to 8.5 min (Table 1). Increasing the heating time to 60 min did not further increase the germination rate. There appeared to be a critical temperature of activation, with little activation of spores heated for 10 min at temperatures of 50 C or lower. Heating at 80 C for 10 min reduced activation from maximal levels, with a greater effect on the germination rate than on the number of spores germinating (Fig. 1, insert). Spores were not activated when heated dry (over P_2O_5 for 2, 24, or 40 hr at 65 C), but such dry-heated spores were fully activable when subsequently suspended in water and heated at 65 C for 10 min. Spores activated by heating at 65 C for 10 min were nonstainable, refractile, heat resistant, and had lost only 5% of their total DPA.

Aqueous ethyl alcohol activation. Permeation of a spore site by water has been postulated (9) as the basis for both heat and aqueous ethyl alcohol activation of *B. megaterium* LB spores. *B. megaterium* A-K spores were also activated by incubation in a broad concentration range of ethyl alcohol in water (Fig. 2, Table 1). There was little or no activation at concentrations of ethyl alcohol below 2 M, or with absolute alcohol. Spores which had been exposed to 8 to 15 M ethyl alcohol germinated completely in glucose (Fig. 2), but their rate of germination was lower and their average microlag was longer than those of maximally heat-activated spores (Table 1). Increasing the time in aqueous ethyl alcohol from

TABLE 1. Effect of activation on the rate and average microlag of *Bacillus megaterium* spore germination^a

Activation treatment	Germination rate ^b	Avg microlag ^c
	% OD loss/min	min
None	0.05	36
Heated in aqueous suspension (10 min) at:		
55 C	0.6	20
58 C	1.55	15
65 C	3.3	8.5
Aqueous ethyl alcohol (5 min, 30 C):		
5 M	1.7	16
10 M	2.25	14
Water vapor (30 C) at relative humidity:		
90%, 30 min	1.7	17
120 min	1.75	14
75%, 120 min	1.7	14
18 hr	2.0	12
90 hr ^d	3.15	8.5
60%, 168 hr	3.05	10

^a After activation, spores were germinated at 30 C in 25 mM glucose, buffered with 50 mM potassium phosphate, pH 7.0.

^b Germination rate was calculated as percentage of OD loss/min during the first 10 or 20 min after addition of glucose.

^c Average microlag was estimated as the time for 50% completion of the total OD loss.

^d Under these conditions, 17% of the spores germinated spontaneously (without added glucose).

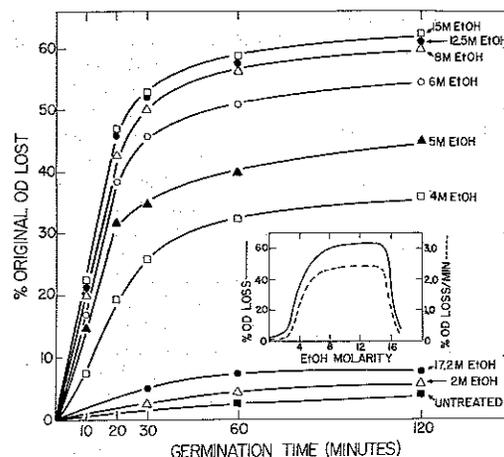


FIG. 2. Aqueous ethyl alcohol activation of the germination of *Bacillus megaterium* spores. Spores were treated with aqueous ethyl alcohol (EtOH) at the indicated concentrations for 5 min at 30 C. Germination conditions and insert as in Fig. 1. Absolute EtOH = 17.2 M.

5 to 30 min did not result in higher germination rates.

Water vapor activation. Spores were exposed to water vapor at 30 C for periods of 15, 30, 60, and 120 min and at 60, 75, and 90% relative humidities, either by continuous flushing or in desiccators. The flushing system, in which humidity equilibrium was not disturbed by introduction of the spore sample, gave slightly higher activation, but only at the shortest exposure time. Under all other conditions of exposure time and relative humidity, activation levels were similar in both systems. Ease of handling and potential for examining many variables simultaneously (in separate desiccators) made desiccator-exposure the method of choice. To avoid the problem of condensation of water vapor with slight variations in temperature, 100% relative humidity was not used in detailed studies of water vapor activation.

Exposure of dry lyophilized spores to water vapor, at temperatures well below those required for heat activation, activated them for subsequent germination on glucose, thus increasing the extent and rate of germination and decreasing the average microlag. Water vapor-activated (90% relative humidity, 30 C, 30 min) spores suspended in water retained the typical spore characteristics of nonstainability, refractility, and heat resistance, had lost only 2% of their total DPA, and had no detectable endogenous respiration (75 mg of activated spores per Warburg vessel, 4 hr). The degree of water vapor activation depended upon the relative humidity, time, and temperature of exposure.

With time (120 min) and temperature (30 C) of water vapor exposure held constant, spores exposed to water vapor at 75 or 90% relative humidity germinated on glucose to the same (maximal) extent as those which had been heated in aqueous suspension at 65 C for 10 min (Fig. 1, 3). Spores exposed to water vapor at 50 to 60% relative humidity were activated for glucose-induced germination to approximately the same extent as those heated in aqueous suspension at 55 C for 10 min.

Maximal levels of activation for glucose-induced germination were also attainable at 60% relative humidity by increasing the time of exposure to water vapor to 90 hr (Fig. 4). Spores exposed at 25 and 35% relative humidities for 120 min were only slightly activated, but extended exposure at these relative humidities resulted in appreciable activation. For example, after 168 hr at 35% relative humidity, 45% of the spores germinated (were stainable) on glucose, with a 24% OD loss.

The germination rate of water vapor-activated spores (75 or 90% relative humidity, 120 min,

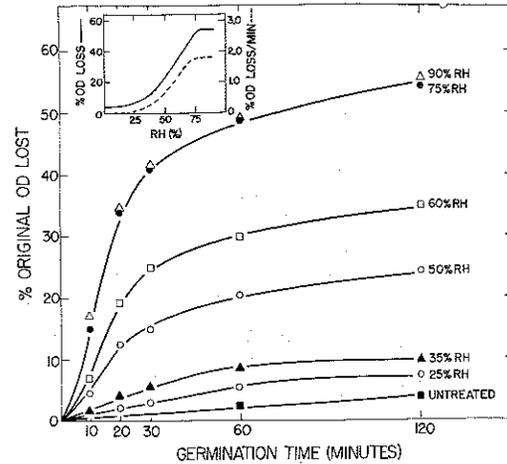


FIG. 3. Effect of relative humidity during water vapor activation on the germination of *Bacillus megaterium* spores. Lyophilized spores were exposed to water vapor at the indicated relative humidities (RH) for 120 min at 30 C, then suspended in liquid water. Untreated spores were those suspended in liquid water for 120 min at 30 C, without prior exposure to water vapor. Germination conditions and insert as in Fig. 1.

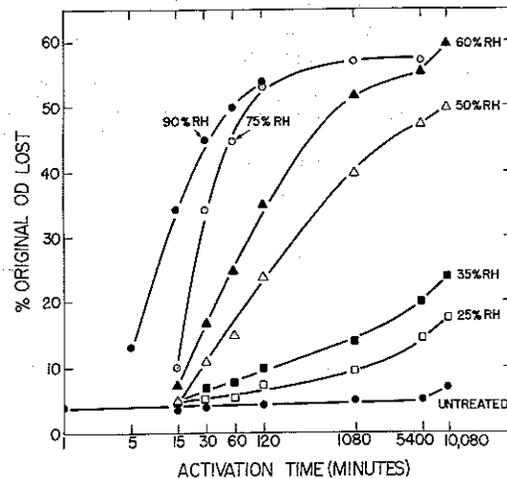


FIG. 4. Effect of time of water vapor activation on the germination of *Bacillus megaterium* spores. Lyophilized spores, exposed to water vapor at the indicated relative humidities (RH) for various times at 30 C, were suspended in liquid water and germinated as in Fig. 1. Untreated spores were those suspended in liquid water for the indicated time at 30 C, without prior exposure to water vapor. Note: 1,080 min = 18 hr; 5,400 min = 90 hr; 10,080 min = 168 hr.

30 C) was lower and the average microlag was longer (Table 1) than those of heat-activated spores (65 C, 10 min), although the extent of germination was equal (maximal) after the differ-

ent activation treatments. Extending the time of exposure to water vapor at 75% relative humidity to 90 hr resulted in a germination rate and in an average microlag more closely approximating that of heat-activated spores, but the changes in these parameters were obscured by spontaneous germination of the water vapor-activated spores, i.e., 17% of them germinated in aqueous suspension without added glucose (see below for further results on spontaneous germination). However, maximal germination, with germination rate and average microlag close to those obtained by heat activation, was attained without spontaneous germination by exposing spores at 60% relative humidity at 30 C for 168 hr (Table 1).

The degree of activation attained after 120 min of exposure to water vapor reached a maximal plateau level at 75% relative humidity, and the magnitude of the plateau depended on the temperature of exposure (Fig. 5A). Spores activated at 75% relative humidity at 35 C had a faster germination rate (2.1% OD loss/min) than those activated at 30 C (1.7% OD loss/min). With activation replotted (Fig. 5B) as a function of the calculated vapor pressure (12), it was apparent that the effects of temperature and relative humidity were not solely attributable to the resultant vapor pressure. At 15 mm of Hg vapor pressure, for example, there was more activation at 25 C than at 30 or 35 C. Since the relative humidity necessary to attain this vapor pressure was higher at the lower temperature, relative humidity, per se, may influence activation aside from its effect on vapor pressure.

Water uptake isotherms of *B. megaterium* QM B1551 spores at 20 C and at 30 C were essentially identical (Fig. 6, insert) and closely approximated that obtained at 18 C by Lewis (19). Since the rates of water uptake at 20 C and at 30 C were also essentially equal (data not shown), the greater activation attained in 120 min at 30 C than at 20 C (Fig. 5) could not be explained on the basis of greater water uptake at the higher temperature. It may be that reactions following water sorption and resulting in activation were temperature-dependent. Activation levels at the various relative humidities (Fig. 4) should not be compared kinetically with water uptake (Fig. 6), as the latter was measured under vacuum and activation was done in the presence of air. However, in the few experiments in which activation was done under vacuum (data not shown), activation occurred slightly more rapidly than in air, but water uptake was measurable before activation was detected and leveled off before maximal activation was achieved. This suggests that gross water uptake was not the prime factor in determining the extent of water vapor activation and that the reactions leading to activation occurred subsequent to water uptake.

Exogenous oxygen was not required for activation by water vapor. Spores were activated for 30 min at 30 C at 100% relative humidity by being continuously flushed with either water-saturated air or water-saturated prepurified nitrogen, the oxygen dissolved in the water having been replaced by nitrogen during a 1-hr

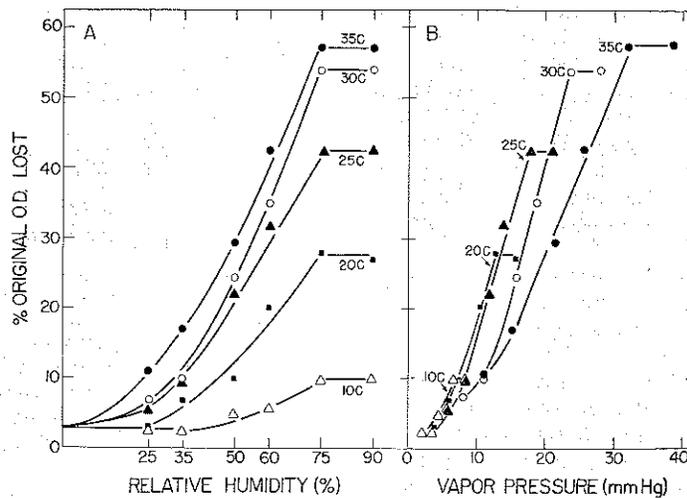


FIG. 5. Effect of temperature during water vapor activation on the germination of *Bacillus megaterium* spores. Lyophilized spores, exposed to water vapor at the indicated relative humidities and temperatures for 120 min, were suspended in liquid water and germinated as in Fig. 1. (A) data plotted against relative humidity; (B) same data plotted against calculated vapor pressure.

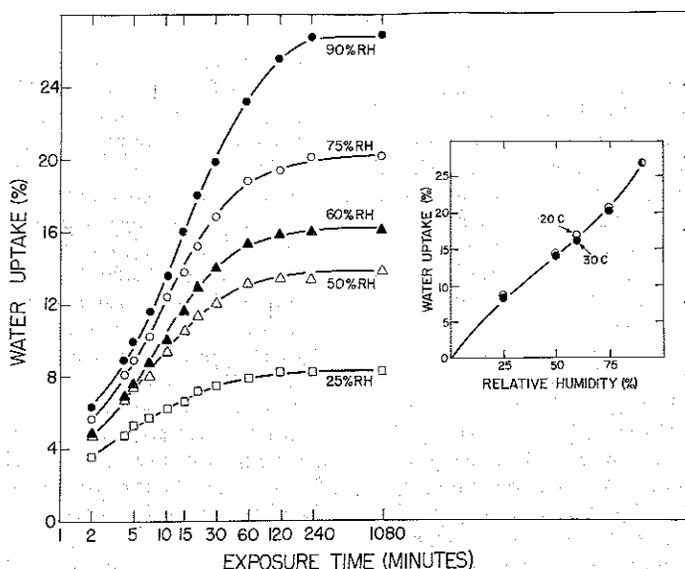


FIG. 6. Water uptake by *Bacillus megaterium* spores exposed to water vapor at various relative humidities. Spores were desorbed to constant weight (5% water loss) before being exposed at the indicated relative humidities (RH) at 30 C. Water uptake is expressed as per cent of the total solids. Insert compares water uptake isotherms (18 hr) at 20 C (○) and at 30 C (●). Note: 1,080 min = 18 hr.

equilibration. Total germination (50% OD loss) and germination rate (1.7% OD loss/min) were the same whether the spores were activated under air or nitrogen.

Supernatant fluids from suspensions of water vapor-activated spores (100% relative humidity, 30 min, 30 C) did not stimulate the glucose-induced germination of untreated spores. Centrifuged and washed activated spores, resuspended in supernatant fluids from untreated spores, lost none of their activity, suggesting that activation was intrasporal and was not due to stimulatory substances secreted into the medium.

Activation for germination on other substrates. In addition to increasing total germination and germination rate on a specific germination agent, activation broadens the variety of agents that induce germination (11, 17, 24). *B. megaterium* A-K spores exposed to water vapor (100% relative humidity, 60 min, 25 C) were not activated for germination on 5 mM KI, or for germination on 100 mM L-alanine (10). Heating in water or treatment at 30 C with aqueous ethyl alcohol (9) did activate *B. megaterium* LB spores for germination on these compounds. We now find that the A-K spores required higher temperatures of heating for maximal activation of germination on L-alanine (75 C, 10 min) or on KI (70 C, 10 min) than for germination on glucose (65 C, 10 min). Analogously longer periods

of water vapor activation (75% relative humidity, 30 C, 18 hr) did promote L-alanine-induced germination (untreated spores, 21% stainable; activated spores, 40% stainable, after 5 hr of incubation) and KI-induced germination (untreated spores, 4% stainable; activated spores, 64% stainable, after 2 hr of incubation). However, no water vapor exposure tested activated for 100% germination on these substrates.

B. megaterium A-K spores required a lower level of heat or water vapor activation for germination on a combination of 25 mM glucose and 1 mM L-alanine than for germination on glucose alone. Indeed, 50% of them germinated in 120 min (25% OD loss; germination rate 0.6% OD loss/min) without activation. At 30 C, maximal water vapor activation (60% OD loss; germination rate, 2.5% OD loss/min) was attained in 15 min at 90% relative humidity, in 30 min at 75% relative humidity, in 120 min at 60% relative humidity, or in 18 hr at 50% relative humidity. Spores heated in aqueous suspension at 55 C for 10 min germinated near maximally (50% OD loss) on this combination of substrates; on glucose, there was only 26% OD loss.

Deactivation and reactivation. If activation by water vapor were due to hydration of a spore site, then removal of water might result in the reversal of this activation. However, relatively mild dehydration treatments (24 hr in vacuo,

40 hr at 66 C, or 40 hr over P_2O_5 at 30 C) did not reverse activation by water vapor. Spores exposed to 90% relative humidity (30 min, 30 C) in a closed system took up ca. 20% of their dry weight as water (Fig. 6), but 99.08% of this water could be desorbed under vacuum (24 hr) without loss of activation for glucose-induced germination. The water involved in activation may be too firmly bound in the spore to be easily removed by vacuum, it may be too small a fraction of the total water uptake to be detected with the spring balance, or the reaction(s) by which water vapor effected activation may have already taken place, and activation was therefore not easily reversible by dehydration.

More rigorous dehydration methods, 100% ethyl alcohol at 66 C for 18 hr (9) or over P_2O_5 at 66 C for 40 hr, did, however, partially reverse water vapor activation, reducing the initial germination rate (Table 2) and increasing the lag (Fig. 7). With extended incubation in glucose, the extent of germination of the deactivated spores approached that of activated spores. Deactivated spores were completely reactivated by heating at 65 C for 10 min (Fig. 7). Spores deactivated by 100% ethyl alcohol were only partially reactivated by water vapor and by 5 M ethyl alcohol, but spores deactivated over P_2O_5 were almost completely reactivated by these treatments. Of the ethyl alcohol-deactivated

spores, 75% were viable and heat resistant, those deactivated over P_2O_5 were essentially nonviable (less than 0.01%), but, nevertheless, could be reactivated for germination. The lethal effect of heating over P_2O_5 was undoubtedly on post-germinative development rather than on germination.

Spores activated by other methods (sublethal heat or aqueous ethyl alcohol) were also deactivated by treatment over P_2O_5 (40 hr, 66 C) and were reactivable (Table 2).

More effective deactivation treatments are being sought. Storage at 28 C for 70 hr (14) deactivated water vapor-activated spores only slightly (OD loss after 60 min in glucose reduced from 44 to 37%; germination rate reduced from 1.6 to 1.1% OD loss/min). Deactivation by treatment with 12 mM phosphoric acid (16) was more effective, but, as this treatment may have had effects on the spore other than dehydration, it will be the subject of a separate report.

Spontaneous germination. *B. megaterium* spores exposed at 100% relative humidity at 25 C for 240 min germinated spontaneously (i.e., without added substrate) immediately upon addition of liquid water. All of the spores were stainable, phase-dark, and heat-sensitive, and 50% were still viable (10). We now find that spontaneous germination also occurred when spores were exposed for extended periods at relative humidity

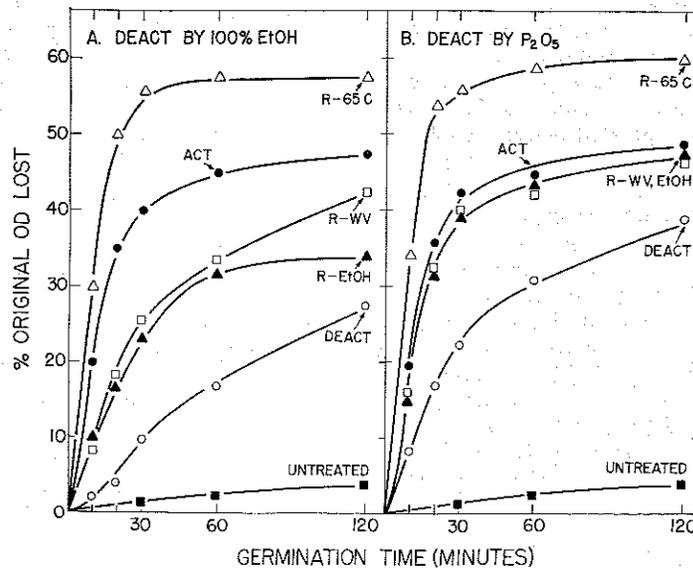


FIG. 7. Deactivation and reactivation of water vapor-activated spores of *Bacillus megaterium*. Lyophilized spores were activated (ACT) by water vapor at 90% relative humidity for 30 min at 30 C. Deactivation (DEACT) at 66 C was (A) by 18-hr treatment with 100% ethyl alcohol, or (B) by 40 hr over P_2O_5 . Reactivation (R) was by heating (65 C in aqueous suspension for 10 min), by water vapor (WV; same conditions as for activation), or by aqueous ethyl alcohol (EtOH; 5M, for 5 min at 30 C). Germination conditions as in Fig. 1.

TABLE 2. Germination rate of activated, deactivated, and reactivated *Bacillus megaterium* spores

Treatment	Germination rate ^a of spores activated by		
	Water vapor ^b	Aqueous ethyl alcohol ^c	Heat ^d
	(% OD loss/min)	(% OD loss/min)	(% OD loss/min)
No activation.....	0.05	—	—
Activated.....	1.8	1.8	1.7
Deactivated ^e	0.85	0.7	0.6
Reactivated-water vapor ^b	1.6	1.2	1.5
Reactivated-aqueous ethyl alcohol ^c	1.55	1.0	0.9
Reactivated-heat ^f	3.4	2.6	2.8

^a Germination rate calculated as percentage of OD loss/min during the first 10 or 20 min after addition of 25 mM glucose in 50 mM potassium phosphate, pH 7.0, at 30 C.

^b Relative humidity, 90%, at 30 C for 30 min.

^c Ethyl alcohol (5 M) at 30 C for 5 min.

^d Heated in aqueous suspension at 58 C for 10 min.

^e Over P₂O₅ at 66 C for 40 hr.

^f Heated in aqueous suspension at 65 C for 10 min.

ties lower than 100%. At 90% relative humidity (30 C), 12% of the spores germinated spontaneously after 4 hr, and all of them germinated spontaneously after 90 hr. After 168 hr at 30 C, 23% of the spores exposed at 75% relative humidity germinated spontaneously; at 60% relative humidity, spores were maximally activated with no spontaneous germination. Perhaps more water uptake was required to induce spontaneous germination than was necessary for activation; this greater water uptake did not occur at lower relative humidities, even with extended exposure (Fig. 6). It has been suggested that the spontaneous germination of spores exposed to high relative humidities may be due to calcium dipicolinate acting catalytically in the minute amount of water of condensation present on the spores (19). In contrast to the reversibility of activation, the spontaneous germinability of spores exposed at 100% relative humidity (4 hr, 30 C) was not reversed by dehydration in 100% ethyl alcohol for 18 hr at 66 C before the addition of liquid water.

Spores exposed at high relative humidity for long periods and then suspended and examined under phase optics in 100% ethyl alcohol, rather than in water, appeared to have reached the end of microlag (31); they had just begun to darken. Immediately after admixture of a trace of liquid

water, the spores became intensely phase-dark, i.e., they appeared to have completed microgermination. Spores germinated in glucose were phase-dark when examined in 100% ethyl alcohol. Perhaps spores initiated germination in water vapor to the end of microlag, but liquid water was necessary for completion of the germination process.

It is important to note that a form of spontaneous germination of heat-activated *B. megaterium* spores was described by Powell and Hunter (24). These investigators found that thick suspensions of heated and centrifuged spores germinated immediately on resuspension (without added substrate).

Effect of lyophilization on the dormancy of spores. The germination characteristics of *B. megaterium* A-K spores, harvested and suspended in water (ca. 2 mg of spores/ml) and stored at 4 C without having been lyophilized ("wet" spores), were compared with those of spores stored in the lyophilized state. Both "wet" and lyophilized spores were essentially dormant on glucose, and both were fully activated when heated in aqueous suspension at 65 C for 10 min (Fig. 8). After heating at a lower temperature (55 C, 10 min), or after treatment with 5 M ethyl alcohol, there was less glucose-induced germination of "wet" spores than of lyophilized spores (Fig. 8). Furthermore, lyophilized spores, without any form of activation, germinated (25% OD loss) on a combination of 25 mM glucose and

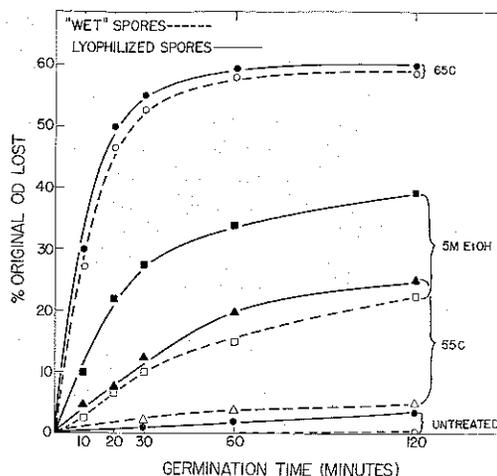


FIG. 8. Germination of "wet" or lyophilized spores of *Bacillus megaterium*. Aqueous suspensions of spores that had been stored either "wet" or in lyophilized state were germinated as in Fig. 1, either untreated, after heating at 55 or 65 C for 10 min, or after treatment with 5 M aqueous ethyl alcohol (EtOH) at 30 C for 5 min.

1 mM L-alanine (see above), but "wet" spores were essentially dormant on this combination of substrates (2.5% OD loss). Suspensions of "wet" spores were not activated by storage (aging) at 4 C for 6 months.

It appeared, then, that there had been some spore activation during the freezing and drying accompanying lyophilization. "Wet" spores, frozen for 30 min in dry ice and then thawed at 30 C, were unchanged in their germination responses, i.e., they had not been activated by freezing. "Wet" spores (10 mg) were collected on 25-mm Millipore filters (0.22 μ porosity; Millipore Corp., Bedford, Mass.), "dried" (equilibrated to various water activity levels) in desiccators at various relative humidities for 20 or 90 hr, and then tested for activation of glucose-induced germination (Table 3). Spores exposed at a relative humidity low enough to allow for some drying, but a high enough water activity level to promote activation (50% relative humidity), were rapidly and maximally activated. The activation during "drying" at 25% relative humidity (Table 3) undoubtedly occurred while the sample was temporarily at a higher water activity level than that afforded by 25% relative humidity. Spores exposed for 20 hr at 75 or 90% relative humidity were not substantially activated (perhaps because of insufficient drying) but, in 90 hr at 75% relative humidity, near maximal activation had occurred, and, at 90% relative humidity, the spores had germinated spontaneously. Similarly, spores lost some dormancy (lower activation requirements) during lyophilization, presumably during the period when they were at a water activity level suitable for activation by water vapor.

Other sporulation media and other organisms. *B. megaterium* LB spores were similar to the A-K spores in their dormancy for glucose-induced germination and in their susceptibility to water vapor activation (56% OD loss after exposure at 90% relative humidity for 30 min at 30 C). These LB spores also germinated spontaneously when exposed at 100% relative humidity at 30 C for 4 hr.

Our strain of *B. megaterium* QM B1551 has been described as unique (7) in that its germination can be induced by certain ions in the absence of such physiological germinants as sugars or amino acids. Several other organisms were therefore tested to determine whether *B. megaterium* QM B1551 was also unique in its water vapor activability. Spores of *B. megaterium* Texas (29) were grown on the A-K medium; they did not germinate on glucose, but were activated for germination on a combination of L-alanine (0.25 mM) and inosine (0.1 mM), either

TABLE 3. Activation during "drying" of "wet" *Bacillus megaterium* spores at various relative humidities^a

Relative humidity during "drying"	Activation ^b of spores dried for	
	20 hr	90 hr
(%)	(% OD loss)	(% OD loss)
Liquid water	4.0	4.0
90	4.0	— ^c
75	7.5	48.5
50	42.0	51.5
25	22.5	29.0

^a Unlyophilized spores were "dried" on Millipore filters by exposure over H₂SO₄-water relative humidity controlling solutions.

^b Activation determined by germination (percentage of OD loss in 120 min) at 30 C on 25 mM glucose in 50 mM potassium phosphate, pH 7.0.

^c All of the spores germinated spontaneously (without added glucose).

by heating (65 C, 10 min) or by exposure to water vapor (90% relative humidity, 30 C, 120 min). These spores all germinated spontaneously after 24 hr, but not after 4 hr, at 100% relative humidity. *B. cereus* strain T spores (received from R. S. Hanson) grown on "G" medium required either extended heating (65 C, 3 hr) or water vapor exposure (75% relative humidity, 30 C, 90 hr) for maximal L-alanine-induced germination. The activated spores lost 50% OD, compared to 10% for the untreated spores. These spores did not germinate spontaneously even after 1 month at 100% relative humidity (30 C). However, *B. cereus* strain T spores grown on 0.5% Liver Fraction "B" germinated spontaneously after 24 hr at 100% relative humidity. Susceptibility to water vapor activation is not, therefore, unique to *B. megaterium* QM B1551 spores, although they are more responsive to this type of activation than the other organisms which we have tested.

DISCUSSION

Water appeared to be the factor common to the three methods of activation of bacterial spore germination described here: activation by heat, by aqueous ethyl alcohol, or by water vapor. We confirmed previous observations that dry spores were not activated by heat (9, 24). Exposure of spores to aqueous ethyl alcohol or to water vapor resulted in activation at room temperatures, but spores in aqueous suspension were not activated unless heated. We postulate that heating or ethyl alcohol alters the structure of liquid water, permitting it, like water vapor, to reach and hydrate a specific critical spore

site, thus resulting in activation. Although deactivation of activated spores by P_2O_5 suggests the validity of our postulate that water uptake is critical to activation by the three treatments, the evidence is not unequivocal. The other desiccant (absolute ethyl alcohol) or the high temperature (66 C) effective in deactivation could be acting by mechanisms other than dehydration.

A number of structural models have been postulated for liquid water, based on evidence that hydrogen bonds in water form an extensive but transient three-dimensional network. Most of these models can be grouped into two general categories: "mixture" and "continuum" models. The "mixture" models describe liquid water as an equilibrium mixture of discrete molecular species with different numbers of hydrogen bonds per molecule. HDO infrared absorption data (6), showing a broad single-peaked distribution of hydrogen bond strengths, favor the "continuum" models which describe liquid water as an essentially completely hydrogen-bonded network with a normal frequency distribution of hydrogen-bond energies and geometries. Heating of liquid water changes its structure so that it becomes more "vapor-like," either by disruption of hydrogen bonds with consequent increase in the monomer fraction and decrease in cluster size ("mixture" models) or by decrease in strength of hydrogen-bonds ("continuum" models).

The addition of ions to water may have the same effect as increased temperature in changing its structure. The rank of effectiveness of ions in weakening the structure of liquid water was $ClO_4^- > F^- > Br^- > NO_3^- > Cl^-$ (3). These ions have the same rank of effectiveness in supporting ionic germination (28) of *B. megaterium* QM B1551 spores (*unpublished results*). Ions, therefore, may also function in germination by first allowing water to hydrate a spore site. Ethyl alcohol may act by changing water structure or by lowering the surface tension of water, facilitating its entrance into specific spore sites (9). The germination of spores as the result of mechanical abrasion (27) is also interpretable as being due to breaching of a permeability barrier permitting entrance of water into critical spore sites.

The aging phenomenon (decrease in spore dormancy on storage) could be explained on the basis that "vapor-like" water (water monomers or weakly hydrogen-bonded species) present as a small fraction in liquid water could enter into and hydrate a spore site; this penetration would increase with time of storage of spores in liquid water. This interpretation is consistent with the

theory that aging is a form of heat activation occurring at low temperature over a long period of time (2).

Spores stored in the dry state would tend to be activated by atmospheric water (especially on humid days) each time a storage vial was opened to remove a sample. This would account for disparate germination results with apparently identical spores. In nature, with alternate periods of humid and dry atmospheres, activation by water vapor might play a more important role in breaking the dormancy of bacterial spores than would short-term exposure to elevated (and nonphysiological) temperatures.

"Although spores have been found to be freely permeable to water, the evidence fails to indicate whether a small anhydrous region surrounded by an impermeable barrier exists in the spore" (Murrell, 22). Since spores remained activated even after more than 99% of the water sorbed during water-vapor activation had been desorbed under vacuum, the site whose hydration might be necessary for spore activation could occupy an extremely small portion of the spore volume. Spores may be accessible to water, but the spore core may actually contain a reduced amount of water, possibly owing to a compressive cortex (20).

It has been suggested that activation by heat, reducing agents, and low pH may involve a reversible denaturation of proteins (e.g., by reduction of S-S bonds) resulting either in an "unblocking" of an enzyme system, or in a change in the permeability of a structure controlling the dormant state of the spore (14). Polar solvents which change protein conformation also activate spores (33). The high refractive index of spores may be attributable to dehydrated protein (30) which is not easily hydrated (32). We believe that the crucial event in activation by heat, water vapor, and aqueous ethyl alcohol is hydration of a spore site in the relatively anhydrous core of the dormant spore, or in spore membranes. We postulate that these treatments alter the properties of liquid water so as to enable it to reach this site, without necessarily altering spore structure. Whatever the identity of the activation site, we propose that it must be wetted before activation can occur. Hydration would be required regardless of the germinant employed. A heteroporous structure surrounding the postulated anhydrous site might differentially restrict permeation of liquid water but not of water vapor. It is possible that some activation treatments, e.g., reducing agents (14), might "open" spore structure, permitting water to reach and hydrate the site.

We envision the site of activation as an en-

zyme or enzyme system, dehydrated and inactive in the dormant spore, the activity of which is required for germination. It is possible that an enzyme hydrated by one of the activation treatments may be dissociated (at a temperature-dependent rate) from an inactive complex, perhaps the inactive Ca-DPA-enzyme complex suggested by Riemann (25); the activated enzyme would then be able to use added or intrasporal substrates for reactions leading to germination. The hypothesis outlined above would suggest a dual role for heat in the activation of bacterial spore germination: (i) hydration of an enzyme(s) permitting it to be functional, and (ii) increasing the activity of the hydrated enzyme. In contrast, treatment with water vapor would only hydrate and activate the enzyme (i.e., render it functional). Indeed, our data show that heat-activated spores germinated faster than those activated by water vapor, and that water vapor activation was more effective at slightly elevated temperatures.

Our postulated concept of activation through hydration of specific spore sites, as the result of treatments which change the structure of water to facilitate its access to these sites, may provide an integrated explanation for bacterial spore activation by heating, aging, aqueous solvents, and water vapor.

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