

STAGES IN GERMINATION OF SPORES OF *BACILLUS LICHENIFORMIS*

M. HALMANN AND A. KEYNAN

The Israel Institute for Biological Research, Ness Ziona, Israel

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ABSTRACT

HALMANN, M. (The Israel Institute for Biological Research, Ness Ziona) AND A. KEYNAN. Stages in germination of spores of *Bacillus licheniformis*. *J. Bacteriol.* **84**:1187-1193. 1962.—This work defines conditions under which the first biochemical step in germination of spores (the so-called trigger reaction) could be studied separately from subsequent steps in germination. Although the initiation of germination of spores of *Bacillus licheniformis* occurred only above 20 C, and at pH 6, spores preincubated for short periods at this temperature and pH will continue to germinate when transferred to a lower temperature or pH. D-Alanine, various salts, and ethyl pyruvate inhibited the trigger reaction, but did not inhibit the continued germination of triggered spores. The results of this experiment are consistent with the notion that the germination reaction is composed of at least two distinct metabolic phases, and that the functioning of the enzyme L-alanine dehydrogenase is necessary for the first phase, the trigger reaction.

The process of germination of the bacterial spore has been defined as "the change from a heat-resistant spore to a heat-labile entity which may not necessarily be a true vegetative cell" (Campbell, 1957). This process, called an example of a "biological trigger reaction" (Halvorson, 1959), is initiated by specific chemicals, such as alanine, and is accompanied by a number of phenomena. Some of these are: decrease in heat resistance and refractivity, increase in permeability, exudation of dipicolinic acid and other substances, as well as imbibition of water (Powell, 1957).

A lag period has been frequently observed between the addition of the germination-inducing agent and the first manifestation of germination (Woese and Morowitz, 1958). It is therefore likely that an initial biochemical step precedes any visual signs of germination. Germination is

probably the outcome of a multiple-step metabolic chain. One of the most interesting steps in this sequence is the reaction which initiates germination, the so-called trigger reaction. The existence of such a triggering step was demonstrated by Harrell and Halvorson (1955), who showed that a very short exposure to L-alanine caused a subsequent germination in spores of *Bacillus cereus* var. *terminalis* (*B. cereus*).

Although many enzyme systems and metabolic steps have been shown to occur in the germinating spore, their sequence, and, therefore, functional relationship to germination, are not known.

It has been suggested (O'Connor and Halvorson, 1961) that L-alanine dehydrogenase is the binding site, and that alanine deamination is the first step in L-alanine-induced germination. It is not evident from these studies whether the L-alanine dehydrogenase itself or other subsequent enzymatic reactions, or a combination, are necessary to induce germination. It would be useful for the elucidation of the biochemical mechanism of germination to achieve a separation of the various steps occurring during germination.

It was therefore the aim of this work to provide conditions in which the initial step, the trigger reaction, could be isolated from subsequent steps in germination.

Several approaches seemed to be possible to achieve this goal. There has been some indication in the literature (Harrell and Halvorson, 1955) that a low temperature, at which germination cannot be induced, will not interrupt germination initiated at a higher temperature. Therefore, the possibility that the trigger reaction has a different temperature dependence than the following steps in germination was investigated. For these experiments, we used a strain of *B. licheniformis*, a species unable to germinate at low temperatures (Wolf and Mahmoud, 1957).

Early experiments indicated the existence of differences in the conditions necessary for induction of germination from those supporting

its continuation. These observations led us to examine the relationship between these two phases of germination. Another approach to this problem was the use of inhibitors. A comparison of the action of inhibitors on the induction of germination, and on preinduced, germinating, spores showed a different response of these two phases.

MATERIALS AND METHODS

A strain of *B. licheniformis* (*B. subtilis* NTCC 9945) was grown on nutrient agar. The spores were suspended in cold distilled water and washed about 30 times by centrifugation at 5 C, until no spontaneous germination could be detected under optimal conditions of pH and temperature (pH 8.0 at 37 C). The length of heat shock necessary to obtain a maximal rate of germination was found to be dependent on the age of the spores after harvest. Freshly harvested spores needed 24 hr at 56 to 60 C, whereas spores 6 months old needed only 4 hr at 56 to 60 C for induction of optimal germination rate.

Germination was measured by the decrease in the optical density of the spore suspensions in a Coleman Junior spectrophotometer at 500 μ . The percentage decrease in optical density was calculated as follows:

$$\frac{\text{initial optical density} - \text{final optical density}}{\text{initial optical density}} \times 100$$

Experiments were carried out in test tubes of 6-mm diameter in a solution of 7×10^{-3} M phosphate buffer (pH 8.0), 7×10^{-3} M, L-alanine, and spores to give an optical density between 0.25 to 0.40. The total volume of 1.5 ml was equilibrated at 37 C. The temperature of incubation was changed when necessary by transferring the test tubes from the water bath at 37 C with the initial temperature to another one with the second desired temperature, and shaking. Under these conditions, the temperature equilibrated in 30 to 45 sec (as measured by thermocouples).

Changes in pH were obtained by the addition of suitable amounts of HCl and NaOH.

Salts and reagents were all analytical grade. L-Alanine (tested chromatographically for contamination with other amino acids) was obtained from Hoffmann-LaRoche, Inc. (Nutley, N.J.).

RESULTS

Separation of the trigger reaction from following steps by its different temperature requirement. Heat-shocked spores of *B. licheniformis* germinated readily in the presence of L-alanine at temperatures between 20 and 43 C, with an optimum at 37 C. No germination occurred below 20 C during a 2-hr period. Spores exposed to temperatures above 20 C for some minutes would continue germination when transferred to temperatures below 20 C. These results indicated that the first step in germination, the "trigger reaction," has a higher temperature requirement than subsequent germination.

Effect of length of time of exposure to 37 C. To verify that no "triggering" occurs at the lower temperature, experiments were designed to show the dependence of the changes occurring at the lower temperature on conditions of preincubation at higher temperatures. Spore suspensions were kept for different lengths of time, at optimal germination conditions, in the presence of L-alanine at 37 C, then cooled to 0 C. Spores cooled at the time of addition of L-alanine or 2 min later did not germinate at 0 C (Table 1). Spores preincubated for 4 min did not germinate at the time of cooling but showed a drop of 16% of the optical density after 300 min at 0 C. Spores preincubated for 6 min showed a decrease of 10% in the optical density at the time of cooling,

TABLE 1. Influence of time of preincubation at 37 C on extent of germination of *Bacillus licheniformis* spores at 0 C*

Preincubation at 37 C	Decrease in optical density at time of transfer to 0 C	Decrease in optical density after 300 min at 0 C
min	%	%
0	0	0
2	0	0
4	0	16
6	11	25
10	28	41
20	45	50
30	49	53

* Heat-shocked spore suspensions were prepared as described in the text. Germination was induced by the addition of L-alanine to a final concentration of 7×10^{-3} M. Optical density was measured at 500 μ . The end of the decrease in optical density was confirmed by measuring the optical density of the suspension after 24 hr.

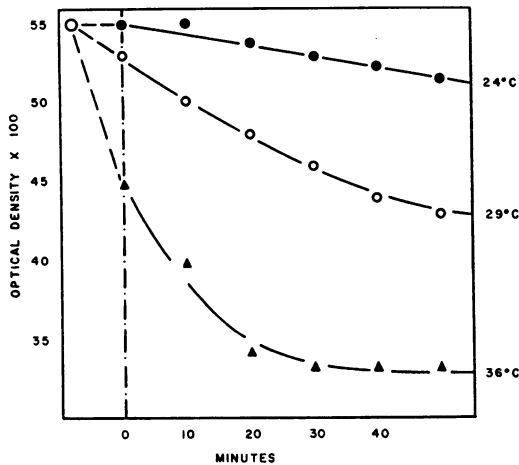


FIG. 1. *Effect of temperature of preincubation on spore germination. Preparation of spore suspension and conditions of germination were as described in Materials and Methods. After addition of L-alanine, the spore suspensions were exposed for 6 min to the temperatures indicated at the right side of the figure, then transferred to 18 C. Germination is given as decrease in optical density vs. time; 0 time is time of transfer to 18 C. No decrease in optical density was observed in spore suspensions with L-alanine maintained at 18 C.*

indicating some germination; the optical density of this suspension continued to drop, to 30%, after a further stay at 0 C. It can be seen that the extent of germination of these spores at 0 C is dependent on the length of time they had been exposed to 37 C.

Effect of the temperature of preincubation. Figure 1 summarizes the results in which three samples of spore suspension were preincubated for 6 min at 24, 29, and 36 C, respectively, and then transferred to 18 C. Controls in which samples of the same suspension were held at 18 C showed that germination did not start at this temperature. It can be seen that the extent of germination at 18 C increases with the temperature of preincubation. The percentage of spores in the suspension which will germinate at the lower temperature depends on the time and temperature of preincubation above 20 C.

Separation of the two phases of germination by their pH dependence. Since the preceding experiments indicated that conditions for initiation of germination might be different from those sup-

porting its continuation, the possibility of separating these two stages of germination by differences in pH was investigated. It was found that there was no initiation of germination in these spores by L-alanine below pH 6, but spores exposed to L-alanine at a higher pH continued to germinate at pH 5. No germination could be noted at pH 4. This experiment indicated that the trigger reaction occurred at a higher pH than the subsequent stages in germination.

When spore suspensions were preincubated at 36 C with L-alanine at pH 8 for 2 min and then the pH was adjusted to pH 6, only a small percentage of spores germinated. Preincubation for 4 min at pH 8 induced germination of about 60% of the spores when they were subsequently transferred to pH 6. Preincubation for 10 min resulted in activation of all spores present in the suspension.

These findings made it possible to investigate separately the pH dependencies of the "trigger reaction" and those of the following steps.

The influence of pH on the first step of germination was measured in the following way. Spore suspensions were preincubated in the presence of L-alanine for 3 min at 37 C at pH 6.0, 6.6, 7.0, 7.4, 8.0, and 8.5. Samples from these suspensions were transferred to test tubes held at 15 C, containing different amounts of acid or alkali to obtain a final pH of 8.0 in all the tubes. No germination was initiated in control spore suspensions held at 15 C for 2 hr; therefore, the extent of germination measured at 15 C indicated the amount of spores activated during the 3 min of preincubation as a function of the different pH levels. The results (Table 2) demonstrate an increase in the rate of activation with an increase in pH in the range of 6.0 to 7.4. The pH dependence of the reactions following initiation (triggering) was measured as follows: a spore suspension was activated as previously described at pH 8, (in the presence of L-alanine at 37 C) and then transferred to test tubes at 15 C containing varying amounts of HCl to obtain pH levels of 5.0, 6.0, 7.0, and 8.0. The results showed a decrease in the rate of germination with a decrease in pH, but germination was still evident at pH 5.0. From these results, it can be concluded that the pH range supporting the induction of germination is narrower than the pH range which will support its continuation.

TABLE 2. *Extent of induction of germination in spores of Bacillus licheniformis as a function of pH**

Preincubation pH of spores for 3 min at 37 C	Germination†
	%
6.0	14
6.6	22
7.0	27
7.4	36
8.0	36
8.5	36

* Spore suspension and germination conditions were as described in Materials and Methods. Spore suspensions were incubated for 3 min at 37 C and the indicated pH. The suspensions were then transferred to tubes containing enough NaOH or HCl to give pH 8, and incubated at 15 C. No germination with L-alanine occurred at 15 C.

† Germination expressed as per cent decrease in optical density of spore suspension. Spores were preincubated at different pH values for 3 min at 37 C. Germination was at 15 C and pH 8.

Difference in response of the "trigger reaction" and the subsequent metabolic steps to inhibitors. It has been shown in the above experiments that, although the "trigger reaction" occurs only at a temperature above 20 C, the subsequent steps occur at lower temperatures.

Experiments were therefore performed to investigate whether these two steps occurring during germination are also separable by their different responses to inhibitors. Substances tested for their ability to inhibit the trigger reaction were added together with L-alanine to the spore suspension. The suspensions were incubated at 37 C and, during 30 min, the decrease in optical density was measured and compared with that of a suspension without inhibitor under the same conditions.

The ability of a substance to inhibit germination of previously activated spores was measured as follows: spores were preincubated with L-alanine for a few min at 37 C, then transferred to 15 C, and the inhibitor was added. The decrease in optical density of these suspensions was measured and compared with suspensions preincubated for the same time, and then transferred to 15 C without addition of an inhibitor. In this experiment, 0.1 M concentrations of D-alanine and various salts were found to be specific inhibitors of the trigger reaction. They inhibited

germination completely when added together with alanine at 37 C but had no effect when added to spore suspensions germinating at 15 C (preincubated for 6 min at 37 C).

D-Alanine is a well-known inhibitor of L-alanine germination. Salt inhibition has been noted (Woese and Morowitz, 1958), but not much information on this phenomenon exists in the literature. Table 3 gives the concentration of salts which reduced the percentage decrease of optical density of spore suspensions to 50% when added during preincubation at 37 C. Divalent cations were more active than the monovalent ones, CaCl₂ being especially active (Table 3).

When the salts (KCl, CaCl₂) were removed by washing in the centrifuge 10 to 15 min after their addition to these spores, the washed spores could readily be germinated with alanine. Spores exposed to salts for 2 hr did not respond readily to alanine after the salts were removed by centrifugation.

Other inhibitors, such as HgCl₂, inhibited at whatever stage they were added. The demonstration of inhibitors which affect only the triggering reaction but have no effect on spores that have been previously triggered is additional evidence of the existence of the two distinct steps during germination.

Analysis of the biochemical nature of the trigger reaction by means of inhibitors. It has been sug-

TABLE 3. *Inhibitory concentration of various salts on germination of Bacillus licheniformis**

Salt	Concn needed for 50% inhibition
KCl.....	4 × 10 ⁻²
NaCl.....	7 × 10 ⁻²
LiCl.....	3 × 10 ⁻²
RbCl.....	3 × 10 ⁻²
KNO ₃	7 × 10 ⁻²
NaNO ₃	8 × 10 ⁻²
KI.....	7 × 10 ⁻²
KBr.....	5 × 10 ⁻²
Na ₂ SO ₄	4 × 10 ⁻²
K ₂ SO ₄	3 × 10 ⁻²
NH ₄ Cl.....	7 × 10 ⁻³
MgCl ₂	5 × 10 ⁻³
CaCl ₂	6 × 10 ⁻⁴

* These concentrations of salts reduced by 50% the decrease in optical density of the spore suspension when added before or together with alanine, after 10 min of incubation at 36 C.

gested that the enzyme L-alanine dehydrogenase might be the first binding site, and its reaction the first step "triggering" germination in spores of *B. cereus* var. *terminalis*. This assumption is in agreement with the inhibition by D-alanine, which is also a strong inhibitor of this enzyme. An attempt was made to see whether the inhibition pattern of the trigger reaction agrees with the concept described by O'Connor and Halvorson (1961).

The experimental design described above did not differentiate between inhibition of the trigger reaction itself or of a subsequent reaction whose activity is essential for germination. Experiments were therefore designed to investigate whether substances inhibit the activation of spores by preventing the "alanine triggering" or whether they prevented "alanine-triggered" spores from germinating. This was done by exposing spore suspensions with L-alanine alone and with L-alanine and inhibitor to 37 C for 6 to 10 min and then removing the alanine and the inhibitor by centrifugation (four times in the cold, 5 to 7 C). The washed spores were resuspended in the original volume, and the decrease in optical density was measured after transfer to higher temperatures. The substances which prevented induction of germination in this experiment were assumed to be true inhibitors of the trigger reaction. When D-alanine, a strong inhibitor of L-alanine dehydrogenase, was tested in this system, it could be shown to inhibit L-alanine triggering completely.

Another substance tested in this system was octyl alcohol, an inhibitor of germination (Hal-

vorson, 1959) which has also been shown to prevent the oxidative deamination of L-amino acids (Krebs, 1935), as well as to kill germinated spores. In our experiments, octyl alcohol inhibited germination when added together with L-alanine, and stopped the decrease in optical density when added to preincubated spores. To investigate whether octyl alcohol prevents the triggering of the spores in addition to its killing effect on already germinated ones, some spores were exposed to alanine for 10 min at 37 C with and without octyl alcohol, washed five times in the cold, resuspended in the original volume, incubated, and their decrease in optical density measured. Data (Table 4) show that suspensions exposed to alanine alone germinated readily after resuspension, while those which were exposed to alanine and octyl alcohol did not germinate.

Viable counts showed that octyl alcohol, when added together with alanine, did not kill the spores. Spores exposed to octyl alcohol and alanine and then washed could be readily germinated by addition of alanine, showing that the spores were not "injured." This is in favor of the assumption that octyl alcohol inhibits the trigger reaction, besides its action on other metabolic steps.

Another known inhibitor of germination is ethyl pyruvate. This substance, which is a pyruvate-metabolism antagonist, inhibited germination at 37 C, and had only a very weak effect when added to "triggered" spores germinating at 15 C. The action of ethyl pyruvate was investigated in the same way as described for octyl alcohol. From this experiment (Table 5),

TABLE 4. Influence of octyl alcohol on germination of *Bacillus licheniformis* spores under various conditions*

Conditions	Extent of germination†	
	After pre-incubation	End of expt
	%	%
Spore suspension incubated with alanine for 10 min at 37 C, washed five times in cold water, resuspended in initial vol, and incubated for 3 hr at 15 C.....	13	33
Spore suspension incubated with octyl alcohol and alanine for 10 min at 37 C, washed five times in cold water, resuspended in initial vol, and incubated for 3 hr at 15 C.....	0	0
Spore suspension incubated with alanine for 10 min at 37 C, washed five times in cold water, resuspended in initial vol with octyl alcohol, and incubated for 3 hr at 15 C.....	12	18

* Spore suspensions were prepared as indicated in Materials and Methods. The amount of octyl alcohol added was calculated as 0.01 M (final concentration).

† Measured as per cent decrease in optical density.

TABLE 5. Influence of ethyl pyruvate on germination of *Bacillus licheniformis* spores under various conditions*

Conditions	Extent of germination†	
	After pre-incubation	End of expt
	%	%
Spore suspension preincubated with L-alanine at 37 C for 10 min, then transferred to 15 C for 150 min.....	22	54
Spore suspension preincubated with alanine at 37 C for 10 min, ethyl pyruvate added, then transferred to 15 C for 150 min.....	20	42
Spore suspension incubated with alanine at 37 C for 10 min, washed four times at 7 C, resuspended in original vol, and incubated at 37 C for 180 min.....	25	60
Spore suspension incubated with alanine and ethyl pyruvate at 37 C for 10 min, washed four times at 7 C, resuspended in original vol, and incubated for 180 min at 37 C.....	0	0

* Spore suspensions were prepared as indicated in Materials and Methods. Amount of ethyl pyruvate added was calculated as 0.01 M (final concentration).

† Measured as per cent decrease in optical density.

it is evident that a 6-min exposure to L-alanine at 37 C is sufficient to activate the trigger system. This activation process is inhibited by ethyl pyruvate.

DISCUSSION

The process of spore germination is usually considered a biological "trigger reaction" (Halvorson, 1959). This has been shown to be correct for *B. cereus* var *terminalis* by Harrell and Halvorson (1955). The experiments described here support this concept for spores of *B. licheniformis*. It has been shown that temperatures and pH conditions for induction of germination are different from those necessary for its continuation. These findings support the view that a distinctive metabolic trigger reaction exists. The reaction is apparently necessary to initiate spore germination and can be inhibited by D-alanine, various salts, ethyl pyruvate, and octyl alcohol. This "trigger reaction" is apparently followed by different metabolic steps which cannot be inhibited by salts and D-alanine, and only slightly by ethyl pyruvate.

The studies with inhibitors support the concept that L-alanine dehydrogenase is the first binding site of L-alanine in spores, and represents an essential step in the trigger reaction (O'Connor and Halvorson, 1961), as D-alanine is a strong inhibitor of L-alanine dehydrogenase and prevents triggering of germination. The trigger reaction itself seems to be composed of more than one enzymatic step, since the inhibition by ethyl

pyruvate indicates that pyruvate metabolism is necessary for this reaction.

Some work on the influence of ions on germination has been reported in the literature. Ions of many metals have been shown to inhibit germination, and Mn⁺⁺ has been shown to stimulate it in *B. megaterium* (Levinson and Hyatt, 1956). K⁺ has been shown to inhibit germination (Krask, 1961); that Ca, Na, Li, and the other salts inhibit L-alanine-induced germination has not been reported. No explanation of this effect can be given at this time, but the fact that, after some time of exposure, the inhibitory effect cannot be reversed by washing indicates a binding of the salts to the spore or an exchange with internal ions. An example of the latter was shown by Halvorson and Howett (1960).

From this study, the properties of the trigger mechanism in *B. licheniformis* can be summarized as follows: (i) the mechanism can be activated only above a temperature of 20 C, and a pH above 6; (ii) the trigger mechanism is inhibited by octyl alcohol, ethyl pyruvate, and D-alanine; and (iii) the trigger mechanism is competitively inhibited by various salts, Ca⁺⁺ and Mg⁺⁺ being the most active.

There have been suggestions that more than one kind of trigger mechanism exist in the same individual spore (Keynan, Murrell, and Halvorson, 1961; Levinson and Hyatt, 1962). It would therefore be interesting to see whether the two steps described here can be shown to exist in germination induced by substances other than L-alanine.

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