STUDIES ON THE ROLE OF L-ALANINE IN THE GERMINATION OF SPORES OF BACILLUS TERMINALIS

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In the past few years several investigators have demonstrated the importance of the role of L-alanine for the germination of spores from aerobic bacilli. Hills (1949) found that the germination of *Bacillus anthracis* spores was greatly stimulated by L-alanine, L-tyrosine, and adenosine. The requirement for L-alanine could not be replaced by related compounds, and in addition its activity was strongly inhibited by D-alanine. The germination of spores of *B. subtilis* suspended in a buffered medium containing only L-alanine has also been described (Hills, 1950; Powell, 1950). Stewart and Halvorson (1953) reported the germination of *B. terminalis* in the presence of L-alanine and adenosine. Recently Church, Halvorson, and Halvorson (1954) have shown that L-alanine is stimulatory for the germination of *B. terminalis*, *B. cereus*, *B. polymyxa*, *B. subtilis*, and *B. globigii*.

Although an active alanine racemase has been demonstrated in spores of *B. terminalis* (Stewart and Halvorson, 1953), Church *et al.* (1954) have shown that its activity is not necessary for the germination of these spores. Thus it would appear that, although L-alanine plays an important role in the germination of spores of aerobic bacilli, its role is as yet obscure. The present report represents an attempt to control the germination process in order to analyze the stages involved and to quantitate the levels of L-alanine utilized.

MATERIALS AND METHODS

The production and purification of spores, the determination of viability, and the measurement of germination have been previously described by Church *et al.* (1954).

Germination studies. All germination studies were carried out on clean heat-shocked spores suspended in 0.067 M pyrophosphate buffer at pH 8.2. The spores were heat-shocked by exposing them in buffer to 65 C for 15 min. Unless otherwise stated, the spores were distributed so that each ml of buffer contained 0.2 mg of spores (2×10^7 spores). Following equilibration of the spore suspension at 30 C, L-alanine and adenosine were added and the subsequent germination followed by changes in optical density, uptake of dye, and loss of heat resistance.

Analytical procedures. Pyruvate was determined by the method of Friedemann and Haugen (1943) using a Beckmen spectrophotometer, and lactate by the colorimetric method of Barker and Summerson (1941) employing a Klett-Summerson photoelectric colorimeter with a no. 56 filter. Amino nitrogen determinations were carried out by the method of Moore and Stein (1948) and paper chromatography by the method of McFarren (1951).

Chemicals. Purified, crystalline samples of L-alanine, D-alanine, adenosine, and pyruvate were obtained from Nutritional Biochemical Corporation. These were used without further purification. The C^{14} carboxyl labeled DL-alanine (0.5 mc per mM) was obtained from Dr. W. Meinke, Department of Chemistry, University of Michigan.

RESULTS

Optimal germination. Nearly 100 per cent of the spores of *B. terminalis* germinated in 20 min in the presence of 6 mM of L-alanine and 12 μM of adenosine (Stewart and Halvorson, 1953; Church *et al.*, 1954). In figure 1 it will be observed that in the presence of L-alanine alone, approximately 50 per cent of the spores germinate, while with adenosine alone less than 5 per cent germinate in the 60 min test period. When the concentration of L-alanine is dropped to 3 mM, only 30 per cent of the spores germinated in 60 min in the presence of 12 μM of adenosine (figure 2). Further dilutions of L-alanine markedly reduce the extent of germination.

H. Orin Halvorson has recently observed (*personal communication*) that adenosine will partially spare the requirement for L-alanine.

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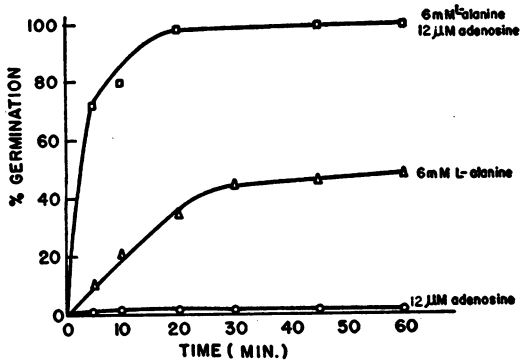

Figure 1. The germination of spores of *Bacillus terminalis* in various substrates. One mg of heat-shocked, clean spores was suspended in 5 ml of 0.067 M pyrophosphate, pH 8.3, at 30 C with various supplements. Suspension was incubated until maximal germination occurred.

Thus, employing 1 mg per ml of adenosine, nearly 100 per cent of the spores of *B. terminalis* germinate in the presence of only 1.12×10^{-3} μM per ml of L-alanine. Adenosine alone was ineffective.

Experimental control of the germination process. In order to further analyze the kinetics and the amount of L-alanine required for germination, a series of experiments was carried out to stop the germination process at various stages without irreversibly inactivating the spores. Attempts to control germination by chilling at various intervals after the addition of L-alanine were unsuccessful due to the rapidity of the germination process. Arsenate and azide, at levels which completely inhibit the vegetative cells (10^{-2} M), have no effect on the germination of the spores. Similar results were previously observed by others (Powell, 1951) and might merely reflect the inability of these agents to penetrate the spore.

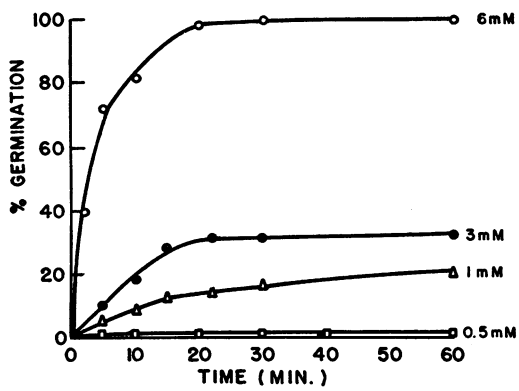

Figure 2. The effect of various concentrations of L-alanine on the germination of spores of *Bacillus terminalis* in the presence of 12 μM adenosine. (See text and figure 1 for conditions.)

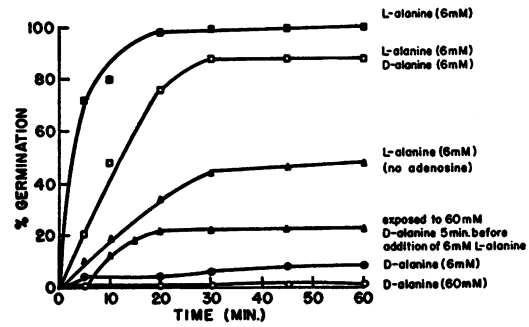

Figure 3. The inhibition of germination of spores of *Bacillus terminalis* by D-alanine in the presence of 12 μM adenosine. (See figure 1 for conditions.)

Hills (1949) reported that D-alanine inhibited the germination of aerobic spores. Therefore, experiments were designed to examine the feasibility of controlling germination with D-alanine. Although the germination of the spores is considerably retarded by the preincubation of the spores with 60 mM of D-alanine or by adding it 1 to 2 min after the addition of L-alanine, complete inhibition was not obtained (figure 3).

A possible solution to the control of the germination process was suggested by the work of Church *et al.* (1954). They observed that germination of *B. terminalis* spores would not occur at either low or high pH. Preliminary experiments demonstrated that dropping the pH to 3.5 or below would completely inhibit germination. Upon returning the pH to 8.5, germination continued normally. When the pH was maintained at 2, no additional germination was observed over a 60 min period. It was thus possible to study the effect of L-alanine at any desired time interval by the addition of a predetermined amount of acid to the suspension.

It therefore seemed of interest to determine whether or not spores so treated would continue to germinate upon removal of L-alanine from the medium. To test this, the spores were suspended in pyrophosphate buffer at pH 8.3 and exposed to 6 mM of L-alanine and 12 μM of adenosine for 45 sec. At the end of this period a predetermined amount of concentrated sulfuric acid was added

to lower the pH to 2.0. The spores were then washed six times in an alanine-free acetate buffer at pH 2.0. Following the last washing the spores were resuspended in pyrophosphate buffer, pH 8.3, 12 μM of adenosine added, and the subsequent germination followed by the usual means. The results recorded in figure 4 demonstrate that the 45 sec exposure to L-alanine at pH 8.3 was sufficient to enable about 40 per cent germination of the alanine treated, washed spores. Control experiments in which the acid was added either prior to or at the same time as the L-alanine and adenosine failed to germinate. Attempts to reduce the 40 per cent germination by repeated washings were unsuccessful and indicate that either the L-alanine was firmly bound or that some energy requiring reaction had previously taken place during the exposure period.

Several experiments seem to indicate that this stimulation process is not a simple matter of alanine adsorption. The spores were suspended in pyrophosphate buffer, pH 8.3, and preincubated at 0 C for 5, 10, and 20 min with 6 mM of L-alanine and 12 μM of adenosine. After washing and resuspending in cold buffer and adenosine, the spores failed to germinate when incubation was continued at 30 C. Furthermore, when the 45 sec exposure to L-alanine (described in figure 4) was conducted under anaerobic conditions, subsequent germination did not occur. Should L-alanine be involved in some metabolic reaction, the failure to activate germination during the short exposure period under anaerobic conditions is easily understood in an organism with a predominantly aerobic metabolism.

Use of C^{14} carboxyl labeled alanine to determine the amount of L-alanine utilized or bound during the 45 sec exposure period. A typical experiment may be detailed as follows: 10 mg of clean, heat-shocked spores were added to a 250 ml flask containing 25 ml of pyrophosphate buffer, pH 8.3, and 25 mg of adenosine. The flask was then placed in a 30 C water bath and connected to a CO_2 trap containing 2.5 M CO_2-free NaOH. After flushing the system with CO_2-free air, 2.5×10^{-3} mg of carboxyl labeled DL-alanine (0.5 mc per mM) were added. After a 45 sec exposure period, a predetermined amount of concentrated sulfuric acid was added to lower the pH to 2.0. The mixture was continually aerated for 1 hour and the CO_2 evolved trapped. The spores were subsequently washed 8 times with acetate buffer

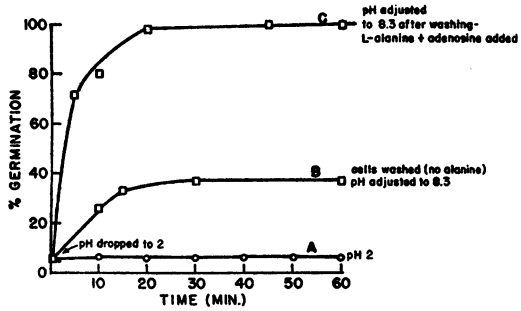

Figure 4. The effect of pH on the germination of spores of *Bacillus terminalis*. One mg of heat-shocked, clean spores was suspended in three tubes containing 5 ml of 0.067 M pyrophosphate buffer, pH 8.3, and L-alanine and adenosine at final concentrations of 6 mM and 12 μM. After 45 sec at 30 C, concentrated HCl was added to lower the pH to 2.0. The tubes were then treated as follows: (A) continued incubation at 30 C; (B) cells washed 6 times in pH 2.0 buffer, resuspended in 5 ml of pyrophosphate buffer, pH 8.3, containing 12 M adenosine and incubated at 30 C; and (C) treated the same as (B) except 6 M L-alanine was included after washing the spores.

at pH 2.0 and converted completely to CO_2. The supernatant fluids were combined, diluted to 50 ml, and a total combustion carried out on a 1.0 ml aliquot. A control run was conducted in a similar manner with the exception that the labeled alanine and sulfuric acid were added simultaneously. All the samples were degraded and their radioactivity determined by the gas phase counting method described by Bernstein (1953). The results obtained are shown in table 1. As indicated in table 1, it was not possible to obtain a significant decrease of radioactivity in the supernatant fluids following a 45 sec germination period. The CO_2 evolved during this period can be attributed either to bound CO_2 or acid decarboxylation of the labeled alanine. The 34 cpm in the spores recovered from the experimental run appear to be significantly higher than the 20 cpm of the control spores. This difference has been obtained in three separate experiments. In all cases there is no subsequent germination of the control spores and approximately 40 per cent germination of the spores from the experimental run. Whether the increased cpm in the spores from the experimental run is due to alanine or to some product formed from alanine is not known.

TABLE 1

Analysis of fixed L-alanine by spores after a 45 second exposure to C^{14} labeled alanine

Sample	Experimental Total	Control Total
	*cpm**	*cpm**
Original supernate (alanine)	1,126,000	—
Experimental supernate (spores)	1,106,000	—
CO_2	99	104
Spores	34	20

* Counted to an accuracy of 2 per cent.
See text for details.

Attempts to detect the disappearance to alanine from the supernatant or the appearance of some products that might be formed from alanine were unsuccessful. Following the germination of the spores, the resulting cells were removed by centrifugation. The cell-free supernatants were then tested for alanine disappearance chromatographically and by the amino nitrogen method of Moore and Stein (1948). Although the amino nitrogen test is quite sensitive, no detectable difference between the supernatants of the experimental and the control could be found. Likewise, paper chromatography revealed no detectable decrease in the spot corresponding to L-alanine nor the appearance of other amino nitrogen compounds. Determinations for pyruvic acid and lactic acid in the supernatants were negative. Thus it would appear that if L-alanine is metabolized, the levels involved are such as to preclude their detections by the methods employed.

DISCUSSION

It seems obvious that information on the biochemical reactions involved in the initial stimulation of the germination of spores of aerobic bacilli is requisite to an understanding of the process itself. In view of the rather general requirement of a variety of strains for L-alanine, it is therefore of interest to examine its role in the initial stages of germination. The rapidity of the germination process has, however, largely precluded investigations of the stages involved. In the studies presented here an experimental control over the germination of spores of *B. terminalis* has been described enabling such an analysis. Briefly this procedure is based upon the observation that germination can be arrested at any period desired by lowering the pH to 3.5 or below. Normal germination continues when the pH is raised to 8.3.

Employing such a system it was demonstrated that there is sufficient activation by L-alanine during a 45 sec exposure period at pH 8.3 to subsequently permit 40 per cent of the spores to germinate in the absence of L-alanine. One can thus clearly recognize an activation stage preceding germination. Although the reactions in which L-alanine are involved are not understood, a few of the properties of the activation are evident. During the 45 sec incubation period there is either a fixation or chemical binding to the spore of a small amount of labeled alanine or products formed from alanine. Based on the amino acid added, this represents between 2.8 to 8.5 $\times$ 10^{-21} moles per spore. Several experiments indicate that this is not a matter of simple adsorption. If the spores are exposed to alanine either under anaerobic conditions at low temperatures or at a low pH, the normal germination following the removal of alanine is prevented. Furthermore, the spores appear incapable of decarboxylating L-alanine.

Although L-alanine is an important requirement in the germination of a wide variety of spores, it is important to point out that the germination process can be activated in a variety of apparently unrelated ways. Thus, in some cases glucose can replace alanine (Church *et al.*, 1954). Powell (1951) has also observed a slight stimulatory effect of pyruvate, lactate, glycero-pyruvate, and ribose on the germination of *B. subtilis*. Although pyruvate, lactate, and glycerol are ineffective in germinating spores of *B. terminalis*, their presence partially spares the alanine requirement (Church and Halvorson, *unpublished results*). Adenosine itself in high concentrations can also partially spare the requirement for L-alanine (H. Orin Halvorson, *personal communication*). It would appear that an unraveling of these interrelationships must first await a better understanding of the role that L-alanine plays in the activation of germination.

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SUMMARY

The conditions for controlling the rapid germination of spores of *Bacillus terminalis* by adjusting the pH have been described. Employing such a system an activation of the spores prior to germination was detected following a 45 second exposure to L-alanine. Activation does not occur either at low temperatures, low pH, or under anaerobic conditions.

In studies involving carboxyl labeled alanine, only trace amounts of radioactivity were fixed in the spores during the activation period. There was no detectable metabolism of L-alanine during spore germination.

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