CALCIUM DIPICOLINIC ACID-INDUCED GERMINATION OF *BACILLUS CEREUS* SPORES

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ABSTRACT

KEYNAN, A. (University of Wisconsin, Madison) AND H. O. HALVORSON. Calcium dipicolinic acid-induced germination of *Bacillus cereus* spores. J. Bacteriol. **83**:100–105. 1962.—The germination of spores of *Bacillus cereus* strain T can be initiated by calcium dipicolinic acid. The kinetics of germination are characterized by a long lag period followed by a rapid loss of refractility. The lag period displays the temperature dependence of a metabolic reaction, whereas the rate of germination is relatively independent of temperature. Germination induced by calcium dipicolinic acid is insensitive to L-alanine analogues, is sensitive to metabolic poisons, and proceeds without a detectable activation stage. It was concluded that calcium dipicolinic acid-induced germination has a metabolic basis and differs, at least in its initial phases, from L-alanine-induced germination.

In recent years a number of different types of germination of aerobic spores have been described. These include "physiological germination" in response to various nutrients, "mechanical germination" in response to various mechanical treatments (Rode and Foster, 1960*a*), and "chemical germination" in response to surface-active (Rode and Foster, 1960*b*) or chelating agents (Brown, 1957).

Riemann and Ordal (1961) observed that calcium and dipicolinic acid (DPA) in equimolar concentrations will induce germination. This phenomenon could be viewed as another example of "chemical germination," which was shown by Rode and Foster (1961) not to require endogenous metabolism. Since CaDPA is released during "physiological germination," however, the ability of these substances added exogenously to stimulate germination poses the question whether externally added and endogenously released CaDPA have a similar role in germination.

This paper is a further characterization of the CaDPA-induced germination. In particular, an attempt was made to determine if this phenomenon is a metabolism-mediated process.

MATERIALS AND METHODS

Preparation of spore suspensions. Spores of *Bacillus cereus* strain T were obtained as previously described (Church, Halvorson, and Halvorson, 1954). The preparations used in the present experiments were lyophilized and stored at −20 C for 17 to 18 months. Unless indicated otherwise, a spore suspension (1 mg/ml) in water was heat shocked at 65 C for 1 hr, centrifuged, and resuspended in 0.02 M tris(hydroxymethyl) aminomethane (tris) buffer (pH 8) containing 0.4% gelatin (Difco Laboratories, Detroit, Mich.). The gelatin was found necessary to prevent precipitation during CaDPA-induced germination. Gelatin is not a germinating agent itself, nor does it have any influence on the rate of CaDPA-induced or L-alanine-induced germination.

Germination conditions and measurement. To 2 ml of spore suspension, 0.6 ml of 0.4 M $CaCl_2$ solution was added. Germination was initiated by the addition of 1 ml of 0.2 M DPA at 20 C. The final volume was 5 ml. Germination was followed either by microscopic examination of stained spores in a phase contrast microscope (Keynan, Murrell, and Halvorson, *in press*) or by measuring the decrease in optical density at 625 mμ. In most cases, the kinetics were followed continuously with a DK-2 Beckman recording spectrophotometer. Germination rates were calculated according to the method of Woese, Morowitz, and Hutchison (1958).

Measurement of calcium excretion. Radioactive spores were prepared by sporulation in G medium (Church et al., 1954) containing $Ca^{45}Cl_2$. At

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intervals during germination, samples were rapidly filtered through 0.45-μ Millipore filters, and the radioactivity of the filtrates measured with a gas flow windowless counter. Thin samples, in which self-absorption was negligible, were counted. Ca^{45} was not adsorbed on the filter.

Materials. DPA was obtained from the Aldrich Chemical Co., Milwaukee, Wis. Ethyl pyruvate was a gift from E. Kosower. $Ca^{45}Cl_2$ (0.55 mc/mmole) was obtained from the Oak Ridge National Laboratory, Oak Ridge, Tenn.

RESULTS

Inhibition of CaDPA-induced germination. If CaDPA-induced germination is mediated by metabolic reactions, then one might expect that it would be sensitive to metabolic inhibitors of physiological germination. Table 1 summarizes the effect of various inhibitors on germination induced by L-alanine, L-cysteine, and CaDPA. Both L-alanine-induced and L-cysteine-induced germination were subject to stereospecific inhibitors, whereas CaDPA-induced germination was unaffected by these same agents, and thus presumably operates initially by a different pathway. On the other hand, all three may have reactions in common since they are all sensitive to $HgCl_2$, low pH, and to inhibitors of pyruvate metabolism (ethyl pyruvate) and electron transport (atabrine).

TABLE 1. *Inhibition of germination of Bacillus cereus spores**

Inhibitor	L-Alanine-induced germination	CaDPA-induced germination	L-Cysteine-induced germination
D-Alanine	0.2	No inhibition†	0.03
Glycine	0.04	No inhibition†	0.04
D-Cysteine	Stimulation	No inhibition‡	0.03
Ethyl pyruvate	0.01	0.01	0.01
Atabrine	0.001	0.001	0.001
$HgCl_2$	0.001	0.01	—

* Germination induced by L-alanine (0.1 M) and L-cysteine (0.03 M) was carried out at 30 C. CaDPA-induced germination is described in Materials and Methods. Germination was considered to be inhibited completely when no change in optical density occurred during 45 min; the figures in the table refer to the molar concentration of inhibitor required to produce this effect.

† Normal germination rates as compared to controls in the presence of 3 × more inhibitor than necessary to give total inhibition of L-alanine-induced germination.

‡ Normal germination rates as compared to controls in the presence of 5 × more inhibitor than necessary to give total inhibition of L-cysteine-induced germination.

Kinetics of germination. The kinetics of L-alanine-induced and CaDPA-induced germination differ significantly (Fig. 1). An 8 to 10 min lag and a slower germination rate are typical for CaDPA-induced germination. In L-alanine-induced germination, the lag period is only 1 to 2 min, followed by a germination rate about five times faster than obtained with CaDPA.

The effect of prior heat-shock treatment on the rate of germination also depends on the germination agent employed. L-Alanine-induced (O'Connor and Halvorson, 1961) and L-cysteine-induced (Krask, 1961) germination are heat-shock dependent. On the other hand, the rate of germination induced by CaDPA is unaffected by heat shock (Keynan, Murrell, and Halvorson, 1962).

The temperature dependence of CaDPA-induced germination was measured both on the lag period and on the rate of germination. The latter was found to be relatively temperature independent, and was, as shown by Riemann and Ordal (1961), inhibited by temperatures at which L-alanine-induced germination is optimal. When the temperature was lowered, the germination lag in response to CaDPA was greatly extended (Fig. 2). The insert is an Arrhenius plot of this same data. Between 8 to 20 C a linear relationship, characteristic of an enzymatic reaction, is observed. The ΔH calculated from this data, 14,500 cal/mole, is very similar to the ΔH of the dependence of the *rate* of L-alanine-induced germination on temperature, 17,000 cal/mole (O'Connor and Halvorson, 1961). These results suggest that the metabolic reaction(s) responsible for induction of germination in response to CaDPA occurs during the lag period.

Analysis of the metabolic properties of the lag period. One explanation for the lag period during CaDPA-induced germination is that this period is required for enzymatic reactions involved in the breaking of dormancy. Such reactions may be part of an activation stage common to many dormant systems, and are characterized by increased metabolic activity and decreased dependence on exogenous germinating stimulants.

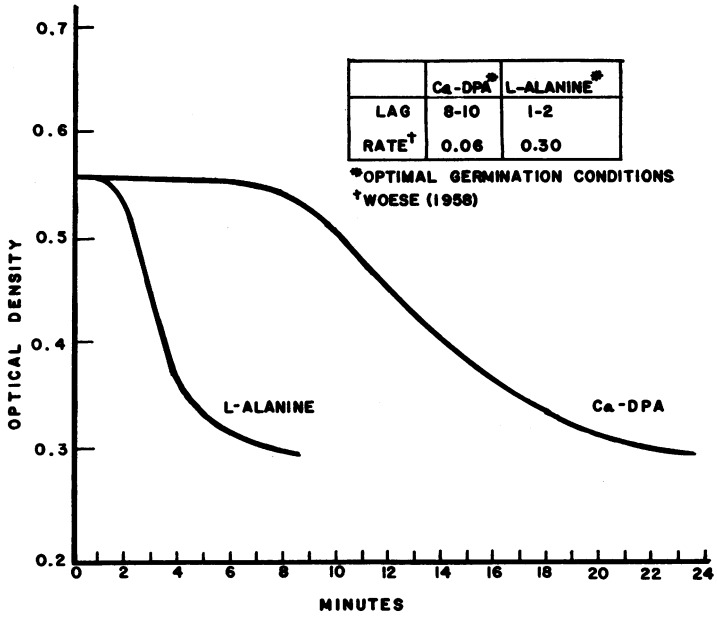

FIG. 1. *Optical density decrease during* L-*alanine and CaDPA-induced germination. Alanine germination was induced at 30 C by 10 mg/ml* L-*alanine in 0.02* M *tris buffer (pH 8). CaDPA germination was induced at 20 C, as described in Materials and Methods. The optical density was recorded at 625 mμ.*

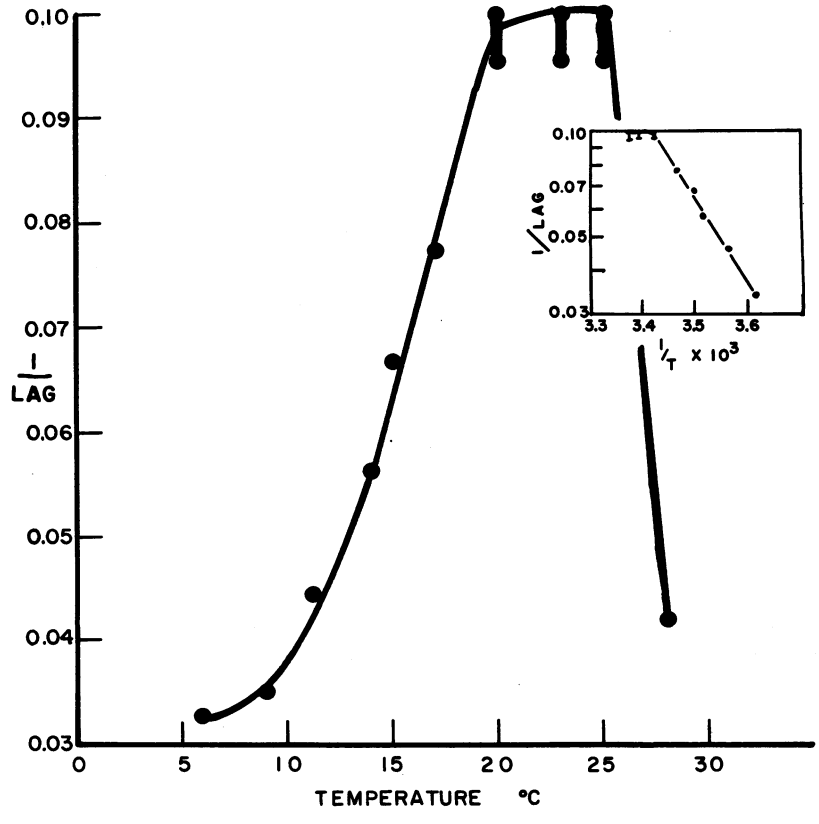

FIG. 2. *Effect of temperature on the lag period of CaDPA-induced germination. The inverse lag time in minutes is plotted as a function of temperature. Lag time is the time from addition of CaDPA to spore suspension until decrease in optical density can be detected photometrically. The insert is an Arrhenius plot of the same data, using time of lag as reaction rate.*

Glucose oxidation is a sensitive measure of metabolic activity in spores. In preliminary experiments it was observed that during germination glucose oxidation could be quantitatively measured by the reduction of tetrazolium salts. As shown in Fig. 2, CaDPA germination can be stopped by raising the temperature to 37 C, thus permitting the removal of samples for examination of their metabolic activity. When samples were removed during the lag period and subsequently exposed to glucose and tetrazolium salts, there was no detectable reduction of tetrazolium.

The kinetics of germination were also used to determine whether an activation stage occurred during the lag period. Spores were removed during the lag period at 20 C, transferred to 37 C, and held at this temperature for 1 hr. When the spores were returned to 20 C, which is optimal for CaDPA-induced germination, a normal germination lag characteristic of the lower temperature was observed. There was no evidence of a shortened lag period as a consequence of the initial CaDPA exposure at 20 C.

To test the possibility that an activation stage exists which is inactivated at higher temperatures, an attempt was made to activate spores by a brief exposure to CaDPA. At intervals during the lag period, samples were removed and the CaDPA removed by filtration on Millipore filters. The spores were washed, rinsed off the filters, and resuspended in buffer at 20 C. Over the entire lag period there was no indication of subsequent germination unless CaDPA was readded to the mixture. Thus, the activation often observed after brief exposure to L-alanine is not observed with CaDPA in this strain. These findings may be specific, since Ordal (*personal communication*) has observed such an activation stage with other spore-forming organisms.

Several properties of the lag period are evident from the experiments of Riemann and Ordal (1961). They observed, during the lag period preceding a loss in refractility, both a decrease in heat resistance and a release of peptides. Since Ca^{++} is generally believed to be involved in heat resistance, one would expect a release of endogenous Ca^{++} during the lag period. To test this possibility, the following experiments were carried out. Spores were grown in the presence of $Ca^{45}Cl_2$, harvested, and washed as previously described for normal spores. The spores were

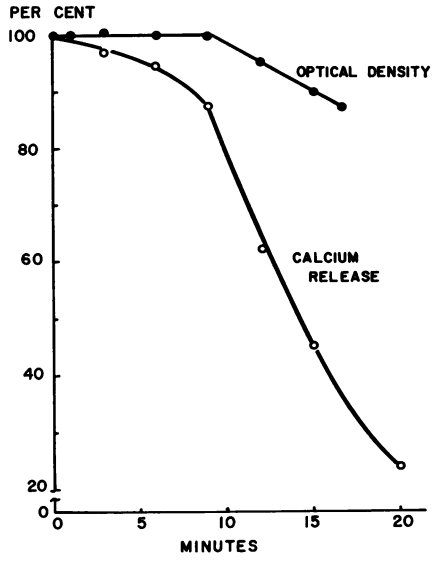

FIG. 3. *Kinetics of Ca^{45} release during CaDPA-induced germination. The optical density of the spore suspension was 0.65 and the radioactivity was 4,800 count/min. Germination was induced by the addition of 0.04 M CaDPA. Samples were withdrawn every 3 min, and the radioactivity of filtrates assayed as described in Materials and Methods. The fall in optical density is included to show the time when germination started.*

exposed to CaDPA and the kinetics of germination followed. At intervals, samples were removed, filtered by Millipore filters, and the release of Ca^{45} measured by the appearance of radioactivity in the filtrate. The results of a typical experiment (Fig. 3) indicate that Ca^{45} excretion starts immediately after the addition of CaDPA, and precedes any other measurable change. In control experiments there was no detectable release or exchange of Ca^{45} when spores were incubated in buffer or exposed to Ca^{++} or DPA alone. Similarly, in L-alanine-induced germination (Fig. 4), Ca^{++} release occurs prior to the loss in refractility.

DISCUSSION

The results presented here support the hypothesis that in CaDPA-induced germination the lag period is associated with a metabolic reaction involved in the loss of heat resistance. Since a metabolic breaking of dormancy has also been shown to exist in L-alanine-induced germination (O'Connor and Halvorson, 1961), these findings raise the question of whether there is

one common, basic mechanism for the physiological germination of aerobic spores.

From the available information it is clear that L-alanine-induced and CaDPA-induced germination differ in several fundamental respects. Exposure to L-alanine involves a stereospecific complexing with a spore site (L-alanine dehydrogenase), activating the spore and leading subsequently to a metabolically controlled rate of germination (O'Connor and Halvorson, 1961). On the other hand, although the initial binding site of the spore with CaDPA is unknown, an activated stage has not been recognized and the metabolic dependency is associated with the lag rather than the rate of germination. In both cases, the over-all process is associated with a release of endogenous Ca^{++}, DPA, peptides, and other components (Powell and Strange, 1953; Riemann and Ordal, 1961). The initial distinction between these two physiological routes of germination is also illustrated with studies on spores containing very little DPA (Keynan et al., *in press*). These spores germinate normally in the presence of CaDPA, but have a very poor response to L-alanine unless exogenous DPA is added. DPA alone will not initiate germination. The DPA stimulation of L-alanine-induced germination is sensitive to D-alanine, indicating that it is acting via the L-alanine dehydrogenase.

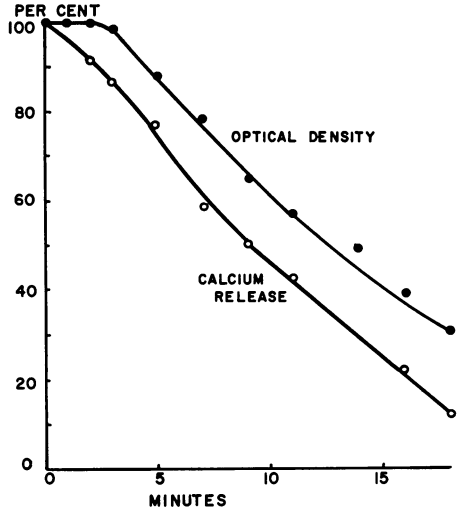

FIG. 4. *Kinetics of Ca^{45} release during L-alanine-induced germination. Procedure as in Fig. 3. Spores were heat activated for 150 min at 65 C in water. Germination was induced by 10 mg/ml L-alanine in the presence of 0.02 M tris buffer (pH 8) at 28 C. Ca^{45} release is plotted as per cent of total. Decrease in optical density plotted as* $\frac{OD - OD_f}{OD_i - OD_f} \times 100$, *where OD is residual density; OD_i initial density; and OD_f, final density (Woese et al., 1958).*

Figure 5 shows a diagrammatic description of the interrelstionships between the germination dathways. DPA has been shown to stimulate the reduced diphosphopyridine nucleotide oxidase activity (Halvorson, Doi, and Church, 1958); L-alanine triggering is thus dependent on this reaction. L-Alanine triggering also releases internally bound DPA from spores (Powell and Strange, 1953). Therefore, a type of "feedback" stimulation of germination can be envisaged. One might assume that exogenous CaDPA activates the release of endogenous L-alanine. That this is unlikely was seen by the fact that CaDPA-induced germination is unaffected by stereospecific inhibitors of L-alanine-induced and L-cysteine-induced germination.

The present studies lead to the conclusion that added CaDPA acts in the same way as endogenously released CaDPA. DPA itself appears to have at least two functions: it drives the L-alanine dehydrogenase reaction, and it induces a "metabolically dependent" germination, independent of the L-alanine trigger mechanism.

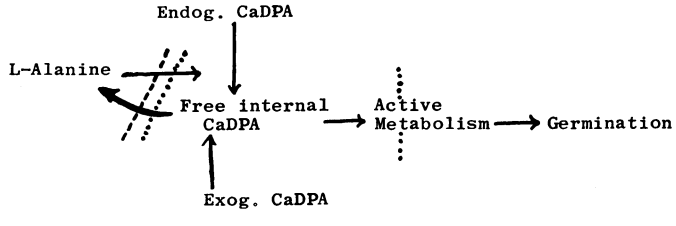

FIG. 5. *Interrelationship between L-alanine and CaDPA-induced germination. The sites of actions of inhibitors of germination are indicated as a dashed line for stereospecific inhibitors and a dotted line for metabolic inhibitors.*

ACKNOWLEDGMENTS

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LITERATURE CITED

BROWN, W. L. 1957. Discussion. *In* H. O. Halvorson [ed.], Spores. American Institute of Biological Sciences, Washington, D. C.

CHURCH, B. D., H. HALVORSON, AND H. O. HALVORSON. 1954. Studies on spore germination: its independence from alanine racemase activity. J. Bacteriol. **68**:393–399.

HALVORSON, H., R. DOI, AND B. D. CHURCH. 1958. Dormancy of bacterial endospores: regulation of electron transport by dipicolinic acid. Proc. Natl. Acad. Sci. U. S. **44**:1171–1180.

KEYNAN, A., W. G. MURRELL, AND H. O. HALVORSON. 1962. Germination properties of spores with low dipicolinic acid contents. J. Bacteriol. **83:** (in press).

KRASK, B. J. 1961. Discussion. *In* H. O. Halvorson [ed.], Spores II. Burgess Publishing Co., Minneapolis.

O'CONNOR, R. J., AND H. O. HALVORSON. 1961. L-Alanine dehydrogenase: a mechanism controlling the specificity of amino acid-induced germination of *Bacillus cereus* spores. J. Bacteriol. **82**:706–713.

POWELL, J. F., AND R. E. STRANGE. 1953. Biochemical changes occurring during the germination of bacterial spores. Biochem. J. **54**:205–209.

RIEMANN, H., AND J. ORDAL. 1961. Germination of bacterial endospores with calcium and dipicolinic acid. Science **113**:1703–1704.

RODE, L. J., AND J. W. FOSTER. 1960*a*. Mechanical germination of bacterial spores. Proc. Natl. Acad. Sci. U. S. **46**:118–128.

RODE, L. J., AND J. W. FOSTER. 1960*b*. Germination of bacterial spores by long-chain alkyl amines. Nature **188**:1132–1134.

RODE, L. J., AND J. W. FOSTER. 1961. Germination of bacterial spores with alkyl primary amines. J. Bacteriol. **81**:768–779.

WOESE, C. R., H. H. MOROWITZ, AND C. A. HUTCHISON, III. 1958. Analysis of action of L-alanine analogues in spore germination. J. Bacteriol. **76**:578–588.