THE RELATIONSHIP BETWEEN THE CLEAVAGE OF PURINE RIBOSIDES BY BACTERIAL SPORES AND THE GERMINATION OF THE SPORES[1]

N. L. LAWRENCE

New York State Agricultural Experiment Station, Cornell University, Geneva, New York

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Since adenosine is required for the initiation of rapid germination of spores of several species of the genus *Bacillus* (Hills, 1949; Stewart and Halvorson, 1953; Church *et al.*, 1954; Lawrence, 1955) including a strain of *Bacillus cereus* var. *terminalis*, it appeared worth while to investigate the role of adenosine in the germination process. It has been found (Lawrence, 1955) that spores of *B. cereus* are capable of cleaving adenosine to yield adenine and ribose.

It is the purpose of this report to compare the conditions optimum for cleavage of adenosine with conditions optimum for germination, to evaluate the effects of related nucleic acid derivatives and to describe the correlation between the germination requirements of certain spores and their ability to cleave adenosine.

MATERIALS AND METHODS

Washed and dried spores of *B. cereus* var. *terminalis* were obtained from the batch described by Stewart and Halvorson (1953). Clean dry spores of *Bacillus globigii* and of *Bacillus polymyxa* were obtained through the courtesy of Dr. Harlyn Halvorson. The spores were stored at −20 C until they were used.

Germination of the spores in liquid media was followed by noting the decrease in plate count at 37 C after heating at 80 C for 5 minutes and by measuring the increase in per cent transmission at 630 mμ in the Lumetron colorimeter model 402 (Powell, 1951).

The nucleic acid derivatives were commercial products and were essentially pure as tested chromatographically. The analytical procedures used are described by Lawrence (1955).

EXPERIMENTAL RESULTS

The effectiveness for germination of compounds related to L-*alanine.* It has been found (Stewart and Halvorson, 1953; Church *et al.*, 1954, and others) that the germination of spores of *B. terminalis* and other species is markedly stimulated by the presence of L-alanine and that D-alanine inhibits the activity of L-alanine. In order to define more closely the requirements of spores of *B. cereus* var. *terminalis* for germination, attempts were made to substitute for L-alanine the related compounds, D-alanine, DL-alanine, sodium pyruvate or lactic acid. Spores were incubated in water solution containing 3.7 μM per ml adenosine and approximately 10 μM per ml of each compound to be tested. In one set of tubes, the spores were heated in the presence of substrate for 15 minutes at 60 C. In another set of tubes, the heating was carried out in water, and the substrates added after cooling to room temperature. The rate of germination was followed by measuring the per cent light transmission during incubation at room temperature. It was observed that no germination occurred even with L-alanine and adenosine at temperatures of incubation of 53, 48 or 41 C, while at 37 and 27 C rapid germination ensued.

The results (figure 1) indicate that DL- or D-alanine, sodium pyruvate or lactic acid will replace L-alanine for the germination of spores in the presence of adenosine, if the spore suspension is heated in the presence of the substrates. The rate of germination in the presence of L-alanine is not affected by such heat treatment. It was noted that there was no measurable decrease in the concentration of lactate or pyruvate during the incubation of spores with these compounds.

The effectiveness for germination of compounds related to adenosine. Stewart and Halvorson (1953) reported that guanosine would replace adenosine for the rapid germination of *B. terminalis* spores. Therefore, a number of purine ribosides and related compounds, each at a concentration of approximately 2 μM per ml, were tested for this ability in the presence of 5.6 μM per ml of L-alanine. The rate of increase in light transmission of the suspension was used as the criterion of rate of

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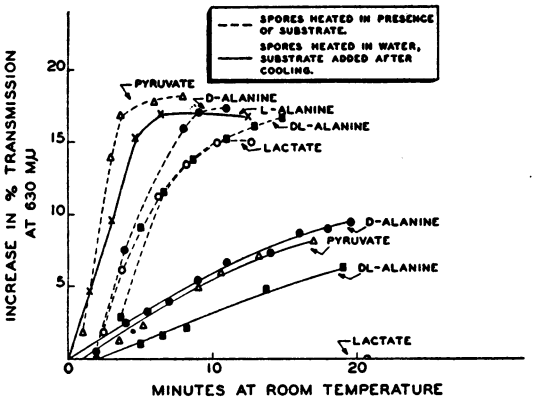

Figure 1. The ability of D-alanine, DL-alanine, sodium pyruvate or lactic acid to replace L-alanine for the rapid germination of spores. The upper group of curves represents the germination obtained at room temperature after incubation of spores with substrate at 60 C for 15 minutes. The lower group of curves represents the germination at room temperature following heating in the absence of substrate. The rate of germination obtained with L-alanine is not dependent on the heat treatment. The substrates were used in a concentration of approximately 10 μM per ml, with 3.75 μM per ml of adenosine.

germination of the spores. These rates were compared to the rates obtained in the presence of adenosine and L-alanine. It was found that the purine ribosides, inosine, guanosine and xanthosine were equally as effective as adenosine in stimulating germination. In contrast to this, it was found that adenine plus ribose, the pyrimidine nucleosides cytidine and uridine, and the purine nucleotides adenosine-5-phosphate and guanosine-5-phosphate were completely inactive in stimulating germination of the spores. The cleavage of each of these compounds into the corresponding free base is described below.

Studies of germination and cleavage in the presence of adenosine alone. The results reported above appear to implicate adenosine and other purine ribosides as critical in germination of spores of this organism. For this reason it appeared advisable to investigate the role of adenosine and compounds related to adenosine in the absence of alanine or other additives, and under conditions both favorable and unfavorable for the germination of the spores.

A suspension of spores in water containing 3.7 μM per ml adenosine was heated at 60 C for various periods of time, then cooled rapidly to

TABLE 1

The effect of time of pre-incubation at 60 C on the subsequent rate of germination at room temperature in a water solution containing 3.7 μM of adenosine per ml

Time Held at 60 C	Increase in Per Cent Transmission after Incubation at Room Temperature for:			
	10 min	30 min	60 min	120 min
min				
0	0	0	0	0
30	0.3	2.8	5.0	6.6
60	1.2	2.1	5.7	6.8
90	0.6	1.1	6.6	9.0
120	1.5	7.0	16.7	17.5
0*	17.0	17.2	17.6	17.6

* With alanine added.

room temperature. After cooling, the rate of germination during incubation was followed by the optical method described. The results (table 1) indicate that prolonged heating at an elevated temperature and prolonged incubation are necessary for germination of spores, if adenosine is the only added material. Even then, the rate of germination is low and not comparable to the rate obtained in the presence of both adenosine and alanine.

The supernatant fluid from cells germinated in this manner did not stimulate the germination of another suspension of spores. Even after 11.2 μM per ml of L-alanine was added the spores would not germinate. However, after further addition of adenosine, rapid germination occurred.

These results are consistent with the finding (Lawrence, 1955) that adenosine is rapidly cleaved by these spores and that adenine and ribose together will not induce germination. It was observed (Lawrence, 1955, table 2) that 5 mg of spores would convert 1.85 μM of adenosine into adenine and ribose in 15 minutes at 37 C. It was also found that the cleavage would proceed rapidly at elevated temperatures, although at these temperatures germination did not occur. As described elsewhere (Lawrence, 1955) spores were able to cleave adenosine after heat treatment sufficient to prevent their germination.

The cleavage of other nucleic acid derivatives by spores of B. cereus. In order to ascertain whether spores of this organism would cleave nucleic acid derivatives other than adenosine, 5 mg per ml of spores were incubated for 40 minutes at room temperature with approximately 4 μM per ml of

TABLE 2

The cleavage of various nucleic acid derivatives by spores of Bacillus cereus var. terminalis

Compound Tested	Rf Value	Stability of Spot to HNO_2 Method of Reguera and Asimov (1950)	Deduced Cleavage Product
Inosine-spore supernatant	0.26	+	Hypoxanthine
Inosine control	0.14	−	
Hypoxanthine control	0.23	+	
Guanosine-spore supernatant	0.45	not tested	Guanine
Guanosine control	0.68		
Guanine control	0.42		
Xanthosine-spore supernatant	0.16	+	Xanthine
Xanthosine control	0.04	−	
Xanthine control	0.17	+	
Adenosine-5-phosphate-spore supernatant	0.38	+	Adenine
Adenosine-5-phosphate control	0	−	
Adenine control	0.41	+	
Guanosine-5-phosphate-spore supernatant	0.44	not tested	Guanine
Guanosine-5-phosphate control	0.79		
Cytidine-spore supernatant	0.31	+	Cytosine
Cytidine control	0.17	−	
Cytosine control	0.30	+	
Uridine-spore supernatant	Conversion of uridine to uracil verified by shift in peak absorption in 0.01N NaOH from 261 to 284 mμ.		

The solvent used for chromatography was n-butanol saturated with a 10 per cent aqueous solution of urea, for all of the compounds except the guanine derivatives. For guanine derivatives a two phase system consisting of isoamyl alcohol and a 5 per cent aqueous solution of disodium phosphate was used. The methods are described by Carter (1950).

each of a number of such derivatives. After removing the cells by treatment with perchloric acid and centrifugation, paper chromatograms were prepared in the manner described (Lawrence, 1955). All of the nucleic acid derivatives used were cleaved (table 2) to yield the free base. This cleavage occurred even though some of the compounds were not effective germinating agents. No attempt was made to determine whether more than one enzyme was involved in the cleavage of the various substrates.

Effects of pH on germination of spores and on the cleavage of adenosine. In order to determine the effect of pH on germination of spores, the rate of germination at room temperature was measured at various pH values. Phosphate and acetate buffers (0.017 M) were used in the range from pH 3.8 to 11.2. Germination was followed in the presence of 5.6 μM per ml adenosine and 11.2 μM per ml L-alanine. The optimum pH for germination (figure 2) was found to be near pH 8. The pH-activity curve for the cleavage of adenosine by spores (Lawrence, 1955) included in figure 2 shows that this optimum is near pH 5.

It is evident from figure 2 that at pH of 4.5, reducing sugar is produced at nearly maximum rate, while germination proceeds at only 20 per cent of the maximum rate. It follows that it is possible to obtain partial separation of rate of germination and rate of adenosine cleavage by choosing the proper pH. Complete separation of cleavage from germination can be obtained by preheating the spores (Lawrence, 1955) or by incubation at elevated temperatures. In all these

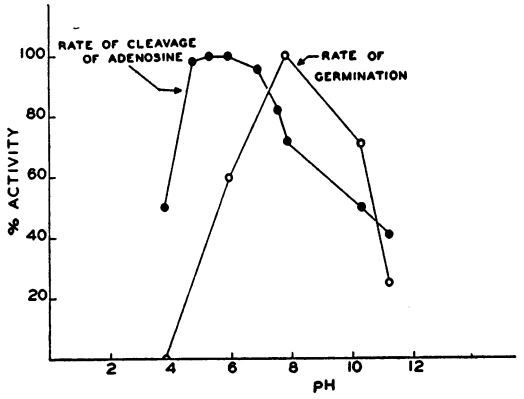

Figure 2. The effect of pH on the rate of germination of spores and on the rate of production of reducing sugar from adenosine. The curve of rate of production of reducing sugar was taken from another paper (Lawrence, 1955). The substrate was adenosine in a concentration of 5.62 μM per ml. The rate of germination plotted was the maximum obtained at each pH value, using as substrate 5.62 μM adenosine and 11.2 μM L-alanine per ml. The buffers in each case were 0.017 M phosphate and acetate.

cases, the germination process is the more sensitive of the two.

The cleavage of adenosine by spores of other species of the genus Bacillus. In order to determine whether the adenosine-splitting activity of spores would correlate with the requirement of the spores for adenosine for germination, strains of two other species were tested. Spores of *B. polymyxa* require adenosine for germination (Church *et al.*, 1954). These spores were found to cleave adenosine at the same rate as spores of *B. cereus*. Spores of *B. globigii*, which do not require adenosine for germination, did not cleave this compound. It was also found that vegetative cells from a 24 hour culture of *B. cereus* were without adenosine-cleaving activity.

DISCUSSION

A number of workers have established that spores of several species of the genus *Bacillus* will germinate rapidly under defined conditions. In general, the requirements are various combinations of adenosine, alanine, phosphate and glucose. In particular, the limiting requirements for rapid germination of spores of this strain of *B. cereus* (called *B. terminalis* in several papers) have been reported to be 67 mM pyrophosphate, 6 mM L-alanine and 12 μM adenosine (Church *et al.*, 1954).

Although germination will proceed at a low rate in either L-alanine or adenosine solution alone, the two compounds together will induce rapid germination of spores of this organism in the absence of added phosphate or glucose. It has been shown here that the other purine ribosides, inosine, guanosine or xanthosine will replace adenosine, and together with L-alanine, will initiate rapid germination of spores of *B. cereus*. It has also been shown that after applying heat to the spore suspension in the presence of the substrates, D- or DL-alanine, sodium pyruvate or lactic acid will replace L-alanine for rapid germination.

The data reported here and in another paper (Lawrence, 1955) indicate that adenosine and other ribosides are rapidly broken down to the free base and the free sugar upon incubation with spores of this organism. No evidence could be obtained for reversal of the cleavage of adenosine nor of synthesis of inosine from adenosine and hypoxanthine, although both phosphorolytic and hydrolytic cleavage of purine and pyrimidine ribosides and desoxyribosides have been shown to be reversible in other systems (Kalcker, 1945; Kalcker *et al.*, 1952; Greenberg, 1951 and others).

Germination of the spores of *B. cereus* var. *terminalis* in the presence of adenosine or other purine ribosides was always accompanied by cleavage of the ribosides; in no case was it possible to obtain rapid germination in the absence of cleavage. However, if the pH of the medium containing adenosine and L-alanine was adjusted below pH 5, or if the incubation temperature was above 40 C, cleavage of the adenosine did occur (Lawrence, 1955) in the absence of germination. Adenosine-5-phosphate, guanosine-5-phosphate and pyrimidine ribosides were also cleaved in the presence of the spores, but these compounds were not active in promoting rapid germination.

The possibility that the cleavage of purine ribosides represents a first and necessary step in the germination of spores of certain species was strengthened by the finding that of three species tested, the two (*B. cereus* and *B. polymyxa*) which require adenosine for germination cleaved this compound at equal rates, while the spores of *B. globigii*, which do not require adenosine for germination had no effect on adenosine.

SUMMARY

The germination of spores of *Bacillus cereus* in solutions containing adenosine was always found

to be accompanied by cleavage of the riboside with the liberation of free adenine and free ribose. The cleavage was also shown to occur under conditions where germination of the spores did not occur. These conditions involved the adjustment of the suspension to pH below 5–6, a temperature of incubation above 40 C, or prior heating of the spore suspension at 100 C for 1 to 4 hours.

Of the compounds tested only other purine ribosides would replace adenosine for rapid germination of these spores. However, it was observed upon incubation with spores that pyrimidine ribosides and purine nucleotides were also cleaved to yield the free bases.

Rapid germination of these spores could be obtained by substituting for L-alanine either D-alanine, DL-alanine, sodium pyruvate or lactic acid if the spores were first heated in the presence of these substrates with adenosine at 60 C for 15 minutes. Such heating was not necessary in the case of L-alanine.

Spores of a strain of each of the species *B. cereus* and *Bacillus polymyxa*, both of which require adenosine for germination were found to cleave this compound at equal rates. Spores of a strain of *Bacillus globigii*, which does not require adenosine for germination had no effect on this compound, nor did the vegetative cells of *B. cereus*.

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