Metabolism and the Triggering of Germination of *Bacillus megaterium*

CONCENTRATIONS OF AMINO ACIDS, ORGANIC ACIDS, ADENINE NUCLEOTIDES AND NICOTINAMIDE NUCLEOTIDES DURING GERMINATION

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A considerable amount of evidence suggests that metabolism of germinants or metabolism stimulated by them is involved in triggering bacterial-spore germination. On the assumption that such a metabolic trigger might lead to relatively small biochemical changes in the first few minutes of germination, sensitive analytical techniques were used to detect any changes in spore components during the L-alanine-triggered germination of *Bacillus megaterium* KM spores. These experiments showed that no changes in spore free amino acids or ATP occurred until 2–3 min after L-alanine addition. Spores contained almost no oxo acids (pyruvate, α -oxoglutarate, oxaloacetate), malate or reduced NAD. These compounds were again not detectable until 2–3 min after addition of germinants. It is suggested, therefore, that metabolism associated with these intermediates is not involved in the triggering of germination of this organism.

Despite intensive study of initial events in bacterial-spore germination, no clear picture has emerged of the mechanism(s) by which common germinants such as L-alanine trigger rapid release from dormancy. The accumulated evidence from several experimental approaches has nevertheless suggested that germinant metabolism or germinant-stimulated metabolism is the initiating signal. This evidence includes studies on the effect of electron-transport inhibitors (Dring & Gould, 1975; Yoshiaki, 1974) and the inhibition of L-alanine-induced germination by inhibitors of pyruvate metabolism (Prasad, 1974; Halvorson & Church, 1957). In addition, studies with *Bacillus subtilis* mutants (Wax *et al.*, 1967; Wax & Freese, 1968; Prasad *et al.*, 1972) strongly suggest a role for glycolysis in germination triggering.

Direct assessment of the role of metabolism during germination by measuring initial changes in metabolite concentrations, is made difficult by the rapid rate of endospore germination. This was attempted by Setlow & Kornberg (1970) and Setlow & Setlow (1977), who followed the concentrations of ATP, 3-phospho-D-glyceric acid and reduced nucleotides in germinating *B. megaterium* QM B1551 spores. Their results showed that both ATP production and reduction of nucleotides was occurring 1 min after addition of germinants. Unfortunately germination is so rapid in this strain of *B. megaterium* that it is not

clear whether these events precede the other changes typically associated with germination. Production of pyruvate and ammonia when *B. cereus* spores were incubated with alanine plus an inhibitor of both pyruvate metabolism and germination has been reported (Halvorson & Church, 1957), but the significance of this observation is not clear, since neither the pyruvate nor the ammonia was derived from the added alanine (O'Connor & Halvorson, 1959).

Strong support for the role of metabolism in triggering germination came from studies on glutamate degradation during germination of *B. megaterium* QMB 1551 (Foerster, 1972), in which it was shown that 50% of the glutamate pool of the spore was degraded via 4-aminobutyrate within 30 s of exposure to germinants. The role of 4-aminobutyrate and the enzyme producing it (glutamate decarboxylase, EC 4.1.1.15) was further emphasized by studies on a mutant requiring 4-aminobutyrate for germination (Foerster, 1971; Foerster & Foerster, 1973).

Since most of the work described above was carried out with a single strain of *B. megaterium* unusual in its lack of specificity for germinants (Foerster & Foster, 1966), it was decided to extend this approach to a different strain of *B. megaterium* more suitable for such investigations. This strain of *B. megaterium* KM germinates only in response to L-alanine or a few related amino acids, but not to nucleosides, glucose or inorganic salts. It also displays a lag phase of 2–3 min after addition of L-alanine

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before many of the changes associated with germination commence, despite the fact that, after 1½ min, 50% of the spores are 'committed' to germination. In this context, 'commitment' is defined as the length of time spores must be exposed to L-alanine to enable them to continue to germinate in the presence of saturating concentrations of the competitive germination inhibitor D-alanine (Scott *et al.*, 1977). If metabolism is involved in the triggering process, there is therefore a period of 1–1½ min in the germination of this strain when metabolites produced by initiation-specific metabolism might be detectable before the general metabolism associated with germination commences.

Experimental

Materials

The principal organism used was a sporogenic strain of *Bacillus megaterium* KM that remains lysozyme-sensitive throughout sporulation (Ellar & Posgate, 1974). *Bacillus cereus* T was obtained from Professor G. W. Gould, Unilever Research, Colworth House, Sharnbrook, Bedford, U.K.

Glutamate dehydrogenase (EC 1.4.1.3, type I), lactate dehydrogenase (EC 1.1.1.27, type II), creatine phosphokinase (EC 2.7.3.2, type I), myokinase (EC 2.7.4.3, type III), desiccated firefly lanterns, NAD⁺ (grade III), NADH (grade III), NADPH (grade III), disodium ATP, L-alanine, inosine and pyruvic acid (type I) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Malate dehydrogenase (EC 1.1.1.37) and oxaloacetic acid were obtained from Boehringer Corp. (London) Ltd., Ealing, London W.5, U.K. Creatine phosphate (A grade) was obtained from Calbiochem (London, W.1, U.K.). L(–)-Malic acid and α-oxoglutaric acid were obtained from Koch-Light, Colnbrook, Bucks., U.K. O-Carbamoyl-D-serine was a gift from Dr. P. E. Reynolds of this department. Swinnex filters were purchased from Millipore (U.K.) Ltd. (London NW10 7SP, U.K.).

Methods

Preparation of spores. Both organisms were grown in modified CCY medium (Wilkinson *et al.*, 1977) as described by Ellar & Posgate (1974). Spores were harvested by centrifugation (10000g, 3 min, 4°C) when more than 95% had been released from the mother cell, and were washed at least six times in deionized water (4°C) until the surface of the pellet was free from contamination by cells or debris as judged by phase-contrast microscopy.

All spore weights referred to are dry weights and were measured by heating spore suspensions at 105°C to constant weight.

Germination of spores. Immediately before germ-

ination, spores were heat-shocked in deionized water at 70°C for 30 min and cooled in ice. Unless otherwise stated, germination was in 50 mM-potassium phosphate, pH 7.5, containing 1 mM-L-alanine at 37°C. In this system, germination as measured by ATP production was identical at spore densities of 0.2, 2 and 10 mg/ml.

Extraction and assay of free amino acids. For the determination of free amino acid concentrations during germination, 10 mM-potassium phosphate, pH 7.5, was used, and the L-alanine concentration decreased to 0.5 mM as higher concentrations caused overloading of the amino acid analyser. These changes did not affect the germination rate.

Samples (0.5 ml) containing 2.5 mg of germinating spores were rapidly mixed with 4 vol. of deionized water at 100°C and maintained at 100°C for 30 min. This procedure has been shown to extract all free amino acids from spores (Nelson & Kornberg, 1970). The samples were cooled, and a measured amount of norleucine was added, together with sufficient 1 M-HCl to decrease the pH to 2. They were then centrifuged (2000g, 5 min, 4°C) and a portion of the supernatant freeze-dried, redissolved in the running buffer of the analyser and analysed with a Rank-Hilger Chromaspek (model J 180 mark 1) amino acid analyser. Quantities of the amino acids were calculated with reference to the internal norleucine standard.

Extraction and assay of malate, pyruvate and α-oxoglutarate. Spores were germinated at 10 mg/ml, and samples (8 ml) mixed with 4 vol. of boiling redistilled propan-1-ol. These were boiled for 5 min, then chilled in acetone/solid CO₂ and dried *in vacuo*. The residue was suspended in 8 ml of deionized water, incubated at room temperature for 30 min with frequent shaking, and clarified by filtration through a 2.5 cm Swinnex syringe filter, fitted with a 0.45 μm-pore-size Millipore filter. The filtrate was assayed for malate, pyruvate and α-oxoglutarate as described by Goldberg & Passonneau (1974), Passonneau & Lowry (1974) and Narins & Passonneau (1974) respectively, with an Aminco-Bowman spectrophotofluorimeter attached to an Aminco ratio photometer. Standard samples of these three compounds were recovered quantitatively when added to the propanol before the dormant spores, but oxaloacetate was completely destroyed, producing an almost equimolar amount of pyruvate. Values for pyruvate from this procedure therefore include oxaloacetate. This method of extraction completely removes 3-phosphoglyceric acid and nucleotides (Setlow & Kornberg, 1970), amino acids (K. Johnstone: personal communication) and dipicolinic acid (I. R. Scott, unpublished work) from spores and was therefore assumed to extract all small water-soluble compounds completely from the spore.

Assay of adenine nucleotides. ATP was determined by the luciferin/luciferase chemiluminescence method of Strehler & Totter (1952). Firefly lanterns (0.5 mg/ml) were extracted in 50 mM-sodium arsenate/HCl, pH 7.5, containing 20 mM-MgCl₂. The extract was clarified by filtration and used after overnight storage at 0°C. The ATP sample, either in aqueous solution (0.1 ml) or dried down, in a 2 cm × ½ cm glass vial was rapidly mixed with 1 ml of firefly-lantern extract. Both vial and extract were at room temperature. Exactly 15 s after mixing, the light-production was measured for 0.4 min in a Unilux liquid-scintillation counter. ADP was measured after conversion into ATP by incubation of samples with 20 µg of creatine phosphokinase/ml, 0.66 mg of creatine phosphate/ml, 1 mg of bovine serum albumin/ml and 5 mM-MgCl₂. The final volume was 0.2 ml, and, after 30 min incubation at 37°C, 1 ml of luciferase extract was added and ATP measured as above. AMP was assayed in the same way by including 10 µg of myokinase/ml in the incubation mixture. Standard curves were prepared for each experiment by adding standard solutions of ATP to extracts of dormant spores.

Extraction of adenine nucleotides during germination. Samples of suspensions of germinating spores (0.2 ml) were injected into 1 ml of boiling propan-1-ol, boiled for 5 min, cooled and dried *in vacuo*. When spore suspensions of ≥1 mg/ml were used, the dry residue was taken up in a suitable volume of water, centrifuged, and portions of the supernatant assayed. When spore suspensions of 0.2 mg/ml were used, the dried residue was assayed directly. The presence of the small amount of insoluble matter affected neither the luciferase reaction nor the creatine phosphokinase/myokinase reaction. This low concentration of spores was necessary when germinating spores of *B. cereus* to prevent inhibition of germination by D-alanine produced by the active alanine racemase of this organism. O-Carbamoyl-D-serine was included in the germination mixture for this organism to inhibit the alanine racemase. The low concentration of spores also allowed absorbance measurements to be made directly on the germinating suspension.

Extraction and assay of NADH. Spores were germinated at 10 mg/ml anaerobically, and samples (5 ml) were taken and injected into 25 ml of boiling propan-1-ol containing 0.5 mM-cysteine and 10 mM-NaOH. After 5 min the samples were cooled, neutralized with 3 ml of 0.2 M-KH₂PO₄ and dried in the dark *in vacuo*. The residue was resuspended in 1.35 ml of 50 mM-Tris/HCl, pH 7.5, filtered through a 2.5 cm Swinnex filter with a 0.45 µm-pore-size Millipore filter and assayed fluorimetrically for NADH and NADPH as described by Klingenberg (1974). Anaerobiosis was ensured by bubbling both spores and germinants (at 0°C) with O₂-free N₂ for 2 h before warming to 37°C and mixing. The germina-

tion vessel was sealed with a rubber Suba-Seal and all additions and samplings were made with a syringe. A flow and positive pressure of N₂ was maintained over the spore suspension throughout the experiment.

Results and Discussion

Concentrations of free amino acids during germination

The concentrations of free amino acids during the first 7 min of *B. megaterium* KM spore germination are shown in Table 1. No significant net change in any amino acid was observed until about 3 min after L-alanine addition. The changes which occur after 3 min undoubtedly reflect the spore proteolysis described by Setlow (1975). These data also show that the glutamate pool of this spore is stable, and no 4-aminobutyric acid is produced even 7 min after L-alanine addition. Thus metabolism of amino acids in general, and of glutamate via 4-aminobutyric acid in particular, cannot be detected during the lag phase of germination in this organism.

Concentrations of malate, α-oxoglutarate and pyruvate during germination

These three acids (plus oxaloacetate, which was measured as pyruvate) were selected for analysis because they are convenient intermediates of the two halves of the tricarboxylic acid cycle and of the lower half of glycolysis. Pyruvate is also the product of alanine dehydrogenase (EC 1.4.1.1), which has often been suggested to be the site of action of L-alanine as a germinant, and α-oxoglutarate is necessary for the action of the transaminases, which have been implicated in spore germination (Prasad, 1974).

Fig. 1 shows the concentrations of these intermediates during the first 7 min of germination. The dormant spore lacks all four intermediates and they do not appear until 2–3 min after the addition of germinants. It appears therefore that the reactions of the lag phase of germination do not involve these intermediates.

Adenine nucleotide metabolism during germination

Fig. 2 shows that ATP synthesis only commences 1½–2 min after the addition of germinants, and therefore ATP synthesis is also unlikely to be associated with the triggering of germination. This conclusion is strengthened by the observation that ATP synthesis can be almost completely inhibited without affecting germination, as is shown in Fig. 3. Fluoride at the concentration used in the experiments shown in Fig. 3 completely inhibits the action of enolase (EC 4.2.1.11) *in vitro* and *in vivo* (Setlow & Kornberg, 1970), thus preventing utilization of the large 3-phosphoglycerate pool of the spore. The fact that fluoride further inhibited the residual ATP synthesis that occurred under anaerobic conditions suggests

Table 1. Concentrations of amino acid pools during germination of *Bacillus megaterium*

Free amino acids were extracted from spores germinating in 10 mM-potassium phosphate, pH 7.5, and 0.5 mM-L-alanine as described under 'Methods'. The alanine was added after the zero-time sample had been taken. Abbreviation used: γ Abu = 4-aminobutyric acid.

		Concentration (nmol/mg of spores)							
Time (min) after adding alanine ...	0	$\frac{1}{2}$	1	2	3	4	5	6	7
Asp	1.5	1.5	1.2	1.4	1.4	2.0	3.0	4.7	4.4
Thr	1.9	1.9	1.9	2.5	2.3	4.1	6.4	12.5	13.5
Ser	2.1	2.1	2.9	2.1	3.2	4.7	7.1	12.4	17.3
Glu	78	81	76	83	80	90	75	88	100
Gly	2.4	2.4	3.3	2.8	3.7	5.1	7.9	15.1	21.0
Ala*	1.9	126	123	135	130	146	116	135	148
Val	0.6	0.6	2.2	1.2	3.6	4.3	5.8	10.1	12.5
Met	0.4	0.4	0.6	0.4	0.5	0.8	1.3	2.7	3.2
Ile	0.5	0.5	0.9	0.6	0.8	1.3	2.1	4.1	5.2
Leu	0.7	0.7	0.9	0.8	1.2	2.0	3.4	6.5	8.1
Tyr	0.5	0.5	0.6	0.4	0.5	1.0	1.5	3.0	3.6
Phe	1.3	1.3	1.3	1.0	1.1	1.9	2.5	4.1	5.2
γ Abu	2.5	2.5	2.5	2.8	3.0	3.0	3.4	2.7	2.9
His	1.7	1.7	1.7	1.4	1.7	2.1	2.2	2.9	3.3
Trp/Orn	2.4	2.4	2.1	1.9	1.7	1.6	1.6	2.1	2.0
Lys	12	12	10	12	12	14	15	23	26
Arg	26	28	27	26	27	27	28	31	31

* The increase in alanine at 0.5 min is entirely accounted for by its addition as the germinant.

Fig. 1. Production of organic acids during germination. Spores were germinated at 10 mg/ml in 50 mM-potassium phosphate plus 1 mM-L-alanine and samples were extracted and assayed as described under 'Methods'. $\circ$, Malate; $\square$, α -oxoglutarate; $\triangle$, pyruvate plus oxaloacetate.

Fig. 2. Timing of synthesis of ATP during germination of *Bacillus megaterium* by 1 mM-L-alanine. Spores were germinated at 1 mg/ml in 50 mM-potassium phosphate, pH 7.5, and 1 mM-L-alanine. Samples were extracted and assayed as described under 'Methods'.

that this residual synthesis may be partly due to degradation of phosphoglycerate. However, under aerobic conditions, fluoride had no detectable effect on ATP synthesis (data not shown) so that phosphoglycerate utilization does not seem to be the main

source of ATP during normal germination, by contrast with the findings of Setlow & Kornberg (1970)

Fig. 3. Effect of inhibitors on ATP synthesis and germination of *Bacillus megaterium*

(a) Heat-shocked spores were germinated at 2 mg/ml in 50 mM-potassium phosphate, pH 7.5, and 1 mM-L-alanine with inhibitors as shown. Anaerobiosis was achieved by bubbling both spore suspension and germinants with O_2 -free N_2 for 2 h at $0^\circ C$. Samples (0.2 ml) were extracted and assayed as described under 'Methods'. (b) Heat-shocked spores were germinated at 0.1 mg/ml in 50 mM-potassium phosphate, pH 7.5, and 1 mM-L-alanine containing inhibitors as shown. Anaerobiosis was achieved by bubbling the buffer solution with O_2 -free N_2 for 1 h then adding 2 mg/ml of sodium dithionite to the spore suspension before adding the alanine. The A_{600} was monitored continuously with a Unicam SP. 1750 spectrophotometer. $\diamond$, No inhibitor; $\square$, anaerobic; $\triangle$, 10 mM-KCN plus 0.1 mM-2-heptyl-4-hydroxyquinoline N-oxide; $\circ$, anaerobic plus 10 mM-KF.

Fig. 4. Effect of inosine on adenine nucleotide concentrations during germination of *Bacillus megaterium*

Spores were germinated at 2 mg/ml in 50 mM-potassium phosphate, pH 7.5, containing (a) 1 mM-L-alanine only or (b) 1 mM-L-alanine plus 1 mM-inosine. Samples were extracted and assayed as described under 'Methods'. $\circ$, ATP; $\square$, ATP+ADP; $\triangle$, ATP+ADP+AMP.

for *B. megaterium* QMB1551. Some endogenous substrate must, however, be used for this purpose, since the L-alanine germinant is not detectably

metabolized during germination [the following paper, Scott & Ellar (1978)].

The observation that anaerobiosis or even reducing conditions did not affect germination (Fig. 3) confirms the studies of Roth & Lively (1956) on three species of *Bacillus*. If, therefore, electron-transport-linked reactions are involved in germination (Dring & Gould, 1975), endogenous acceptors other than oxygen must be reduced.

Inosine, although having little or no effect on germination of this strain of *B. megaterium*, greatly

stimulates L-alanine-induced germination in many strains of *Bacillus* (Foerster & Foster, 1966). Fig. 4 shows the effect of inosine on adenine nucleotide concentrations during germination of *B. megaterium*. The possibility that the dramatic rise in both ATP and total adenine nucleotide concentrations in the presence of inosine might be responsible for the stimulation of germination by inosine could not be investigated in this strain of *B. megaterium*, since its germination is not stimulated by inosine. These experiments were therefore repeated with spores of *Bacillus cereus* T, which show a considerable stimulation by inosine. Fig. 5 shows that inosine had a similar effect on adenine nucleotide concentrations to that seen with *B. megaterium*. However, comparable ATP synthesis was obtained by germination in alanine plus glucose, or alanine plus inosine under anaerobic conditions, without altering the difference in rate of germination due to the presence of the inosine (Fig. 6); some other effect of inosine must therefore exist.

Fig. 5. Effect of inosine on adenine nucleotide concentrations and rate of germination of *Bacillus cereus* T

Spores were germinated at 0.2mg/ml in 50mM- $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pH7.5, containing (a) 10mM-L-alanine and 10mM-O-carbamoyl-D-serine and (b) 1mM-L-alanine, 1mM-O-carbamoyl-D-serine and 1mM-inosine. Samples were extracted and assayed as described under 'Methods'. Absorbance measurements were made directly on the germinating suspension. $\circ$, ATP; Δ , ATP+ADP+AMP; $\diamond$, % of initial A_{600} .

Fig. 6. Stimulation of germination of *Bacillus cereus* by inosine without stimulation of ATP synthesis

Spores were germinated at 0.2mg/ml in 50mM- $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pH7.5, containing (a) 10mM-L-alanine, 10mM-O-carbamoyl-D-serine and 10mM-glucose, and (b) 1mM-L-alanine, 1mM-inosine and 1mM-O-carbamoyl-D-serine. In (b) germination was under anaerobic conditions. The symbols are the same as for Fig. 5.

A final important point from these experiments is that whereas ATP synthesis and fall in absorbance occurred in parallel when *B. cereus* spores germinated, as was found for *B. megaterium* QMB1551 (Setlow & Kornberg, 1970), in the *B. megaterium* KM used in the present study the rise in ATP concentration was virtually complete after 10 min, when only half of the final decrease in absorbance had taken place (Fig. 3). At this time approximately half of the spores were still phase-bright as viewed in the phase-contrast microscope, which implies that a spore may be metabolically active while still at least partly refractile.

Production of reduced nucleotides during germination

It is difficult to extract nicotinamide nucleotides from biological material without altering the NAD^+/NADH ratio (Klingenberg, 1974). These extraction difficulties are likely to be even more pronounced for bacterial spores and consequently no attempt was made to estimate NADH concentrations *in vivo* in dormant spores. Instead an extraction method using boiling 80% propan-1-ol under totally anaerobic reducing conditions was developed (see the Experimental section). Since NADH and NADPH were completely stable for up to 12 min in the boiling propan-1-ol/cysteine/NaOH mixture, we considered that this extraction method would be more likely to reveal the earliest time of reduction of NAD^+ than other procedures (Setlow & Setlow, 1977) that might permit reoxidation of NADH during the extended (15 min) extraction.

Fig. 7 shows the results of these experiments. NADH production could not be detected until 4 min after L-alanine addition, and therefore it is unlikely that NADH-linked metabolism is important in

Fig. 7. Reduction of NAD^+ during germination of *Bacillus megaterium*.

Spores were germinated, extracted and assayed for NADH as described under 'Methods'.

triggering germination. At no stage during germination up to 20 min was NADPH detectable. This may reflect a genuinely very low concentration of NADPH, or it may point to its oxidation by endogenous compounds during the extraction. This latter possibility could also apply to NADH, i.e. if NADH was being used to reduce some substance whose reduction caused triggering of germination, then detectable concentrations of NADH might not accumulate until a late stage in germination. For this to be tenable, however, the equilibrium of the reduction reaction would have to lie well towards NAD^+ and the reduced product.

Conclusions

The action of many proposed mechanisms for the triggering of spore germination would result in the production of metabolic intermediates during the lag phase between spore 'commitment' to germination and the activation of spore metabolism resulting from germination. In the present paper a number of these intermediates were measured and shown not to accumulate to detectable concentrations during this period.

In particular, glutamate metabolism via the 4-aminobutyrate pathway, which has been suggested to be involved in the initiation of germination of *B. megaterium* QMB1551 (Foerster, 1972), could not be detected in this strain of *B. megaterium* KM. Crucial intermediates of the tricarboxylic acid cycle could also not be found, and the absence of α -oxoglutarate from the dormant spore makes a role for glutamate-pyruvate transaminase (EC 2.6.1.2) in alanine-induced germination (Prasad, 1974) unlikely. No pyruvate could be detected (produced from alanine by alanine dehydrogenase), suggesting that the production of pyruvate by spores inhibited from germination by an inhibitor of pyruvate metabolism (Halvorsen & Church, 1957) may be due to the presence of the inhibitor rather than to a germination-related event. If this is the case, the relationship between the substrate specificity of alanine dehydrogenase and the activity of amino acids as germinants (O'Connor & Halvorsen, 1961; Hermier *et al.*, 1970) may be coincidental.

The cofactors ATP and NADH were also shown not to accumulate in detectable concentrations during this period. Although it is conceivable that consumption of these cofactors might be so rapid during triggering of germination that they do not accumulate, the thermodynamics of the situation argues against this. During the first 2 min of germination the ATP/ADP ratio is less than 1:100 and the free energy of hydrolysis of the ATP will therefore be much decreased. Under these conditions, ATP hydrolysis would be quite incapable of driving reactions such as the reversal of glycolysis suggested by Prasad *et al.* (1972). Similar arguments apply to the possibility of

NADH acting as a reducing agent when the NADH/NAD⁺ ratio is less than 1:25. Only redox reactions favouring the reduced product strongly could be driven by NADH under these conditions. One such reaction would be the reduction of oxygen via the electron-transport system. However, the fact that germination is unaffected by total anaerobiosis means that if electron transport were to be involved in germination, acceptors other than oxygen must be used, and in general these would be less powerfully oxidizing.

It should be noted, however, that most of the evidence suggestive of a role for metabolism in germination has been obtained with different species of bacterial spores, particularly *B. cereus* and *B. subtilis*. It is evident from a comparison of the present work with that of Foerster (1972) that great differences can be found in the germination process even within a single species. Although the existence of a single mechanism for germination of all bacterial spores is an attractive concept in view of the similarity of the changes that take place during germination of different species, the extremely wide variety of ways of triggering germination (Gould, 1970) perhaps suggests that diversity rather than uniformity may be the rule.

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