Metabolism and the Triggering of Germination of *Bacillus megaterium*

USE OF L-[³H]ALANINE AND TRITIATED WATER TO DETECT METABOLISM

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(Received 19 December 1977)

L-[2,3-³H]Alanine was used to probe for metabolism of alanine during triggering of germination of spores of *Bacillus megaterium* KM. No detectable incorporation of label into any compound, including water, was found, indicating that any metabolism involving the alanine germinant must be at a very low rate and also that alanine racemase is absent from spores of this strain. Spores were germinated in ³H₂O to find if any of the many metabolic reactions causing irreversible incorporation of ³H into reaction products took place during triggering of germination. No incorporation was detected until 2–3 min after addition of germinants. It is therefore concluded that a wide variety of metabolic routes, including glycolysis, the tricarboxylic acid cycle, the pentose phosphate pathway and amino acid metabolism are either not involved in the reactions causing the triggering of germination or operate at an extremely low rate during this process.

L-Alanine is capable of triggering the germination of a wide range of bacterial endospores (Gould, 1970), and the mechanism of this effect has been the subject of several investigations. These studies have generally concluded that metabolism of the alanine is an important part of the germination mechanism (Halvorson & Church, 1957; O'Connor & Halvorson, 1959; Hermier *et al.*, 1970; Prasad, 1974; Freese *et al.*, 1964; Wax & Freese, 1968). One of the most obvious lines of investigation is to study the catabolism of alanine during germination induced by radiolabelled alanine. Surprisingly little work of this type has been published, although it has been stated in reviews (Gould, 1970) that metabolism of [¹⁴C]alanine could not be detected early during germination.

Harrell & Halvorson (1955) also reported that metabolism of [¹⁴C]alanine by *Bacillus cereus* spores could not be detected during the first 45 s after alanine addition and that less than 200 molecules of alanine were bound to each spore during the period. These experiments were, however, carried out with a 1 μ M racemic mixture of DL-alanine instead of the 6 mM-L-alanine used in the rest of their experiments. The fact that such a low concentration of L-alanine in the presence of inhibitory D-alanine had the same effect as 6 mM-L-alanine makes the role of the alanine uncertain.

Watabe *et al.* (1974) showed an initial uptake of [¹⁴C]alanine by spores of *Bacillus thiaminolyticus* immediately on exposure to alanine. However, this cannot serve as evidence for metabolism, since the alanine was shown to be subsequently released

unchanged into the medium. The actual quantity of alanine absorbed in this way was not calculated in their paper, and the extent of alanine metabolism that could be detected is therefore not known.

The significance of the [¹⁴C]alanine uptake described by Martin & Harper (1965) is again uncertain, since their methodology did not distinguish between radioactivity in the spore cytoplasm and that trapped in the interstitial water of the spore pellet on centrifugation of the germinating-spore suspension. Thus reliable quantitative data on the possible extent of alanine metabolism are not available.

Wilson (1964) used a novel isotopic method to study metabolism. He pointed out that a wide variety of enzyme reactions resulted in the incorporation or exchange of protons from water into the substrate and that these protons were often then non-exchangeable with water. Thus the non-exchangeable incorporation of ³H when cells are suspended in ³H₂O will reflect any enzyme reactions occurring in the cell. This technique has been successfully used to study the early metabolism of germinating seeds (Missen & Wilson, 1970; Spedding & Wilson, 1967) and fern spores (Lever, 1971), and offers several important advantages for studies of bacterial-spore germination. The method is extremely sensitive, since ³H₂O of very high activity is available. Problems of permeability are avoided, since spores are readily permeable to water (Lewis *et al.*, 1960) and the technique is ideally suited to detect metabolism of endogenous substrates. Finally, incorporation of the isotope is likely to be catalysed by a wide range of different enzymes. This latter advantage is particularly valuable in these studies, where the precise nature of the metabolism is unknown.

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The present paper describes the use of isotopically labelled L-alanine and $^3\text{H}_2\text{O}$ to detect metabolism either of the alanine germinant or of endogenous substrates during the period after 'commitment' to germination has taken place but before the onset of the general activation of spore metabolism associated with germination [the preceding paper, Scott & Ellar (1978)]. It is during this period that the changes in the spore responsible for the triggering of germination take place.

Experimental

Materials

L-[2,3- ^3H]Alanine of specific activity 30–50 Ci/mmol and $^3\text{H}_2\text{O}$ of specific activity 5 Ci/ml were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Zerolit 225 (>200 mesh) ion-exchange resin was obtained from Hopkin and Williams, Chadwell Heath, Essex, U.K. L-Alanine was obtained from Sigma, St. Louis, MO, U.S.A.

Methods

Preparation of spores. *Bacillus megaterium* KM was grown and spores prepared and cleaned as described in the preceding paper (Scott & Ellar, 1978). For experiments with $^3\text{H}_2\text{O}$ the spores were further cleaned by centrifuging six times from ice-cold deionized water (10000g, 3 min, 4°C), each time removing and discarding the uppermost layer of spores. The recovery of spores from this procedure was approx. 50%. The spores were stored at 0°C and used within 1 h.

Purification of L-[2,3- ^3H]alanine. The isotope as supplied contained appreciable quantities of non-alanine radioactivity, presumably as a result of autoradiolysis. It was purified on the day of an experiment as follows. A sample (0.6 ml) of 0.2 mM-L-alanine containing 100 μCi of L-[2,3- ^3H]alanine was mixed with 0.2 ml of 2 M- NH_3 and freeze-dried. The residue was redissolved in 2 $\times$ 0.5 ml of deionized water and applied to a 3 cm $\times$ 1 cm column of Zerolit 225 cation-exchange resin (>200 mesh) in the H^+ form. The column was washed with 8 ml of deionized water and the alanine eluted with 2 ml of 2 M- NH_3 , freeze-dried and redissolved in 0.6 ml of 100 mM-potassium phosphate, pH 7.5.

Purification of $^3\text{H}_2\text{O}$. $^3\text{H}_2\text{O}$ as supplied by the Radiochemical Centre was diluted in 50 mM-potassium phosphate, pH 7.5, to produce twice the specific activity required during germination and distilled *in vacuo* in a sealed system.

Extraction and fractionation of spores germinating in [^3H]alanine. Spores (200 mg/ml) were heat-activated at 70°C for 30 min, cooled to 37°C and mixed with an equal volume of purified [^3H]alanine in phosphate buffer. Samples (0.2 ml) were taken at intervals,

injected into 1 ml of boiling redistilled propan-1-ol and boiled under reflux for 5 min. The samples were then cooled and fractionated as shown in Scheme 1. For the zero-time sample, 0.1 ml samples of the heat-activated spores and the alanine/phosphate germinant were added successively to 1 ml of boiling propanol and treated as above.

Germination in this system was slightly slower than that in 1 mM-L-alanine, with 30% instead of 50% of the spores phase-dark after 10 min.

Germination of spores in $^3\text{H}_2\text{O}$. Spores additionally cleaned as described above were suspended at 200 mg/ml in deionized water and mixed with an equal volume of purified $^3\text{H}_2\text{O}$ at 0°C to give a final specific activity of 0.6 Ci/ml. The spores were held at 0°C for 10 min to allow equilibrium of the ^3H label, then warmed to 37°C. Samples (0.1 ml) were taken at intervals for 5 min, then a mixture of 1.25 M-potassium phosphate and 25 mM-L-alanine was added to one-half of the spore suspension to give a final concentration of 50 mM-potassium phosphate, pH 7.5, and 1 mM-L-alanine. Samples (0.1 ml) were taken at intervals from both the spores alone and the spores plus germinants.

The samples were rapidly mixed with 5 ml of ice-cold 80% (v/v) propan-1-ol containing 0.1 ml of 50 mM-potassium phosphate, pH 7.5, for samples of spores not containing phosphate buffer. The samples were then transferred to a boiling-water bath for 12 min, chilled in an acetone/solid CO_2 bath and dried down *in vacuo*. The residue was suspended in 3 ml of deionized water, heated to 90°C for 10 min, cooled and centrifuged (2000g, 10 min, 4°C). A portion (2.5 ml) of the supernatant was freeze-dried, redissolved in 1 ml of water and repeatedly freeze-dried until the radioactivity measured in the portions remained constant.

Measurement of radioactivity in experiments with L-[^3H]alanine and $^3\text{H}_2\text{O}$. Aqueous samples were mixed with 15 vol. of scintillation fluid containing 7 g of 2,5-diphenyloxazole and 0.35 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 1 litre of toluene/Triton X-100 (2:1, v/v). Samples were counted for radioactivity after overnight refrigeration in a Packard Tri-Carb liquid-scintillation spectrometer and corrected for background, measured with a sample of deionized water in 15 vol. of scintillant. s.d. values for the radioactivity counts ranged from 2.5 to 1.0%. Counting efficiencies were determined with an internal standard of L-[2,3- ^3H]alanine.

Results and Discussion

Germination in L-[^3H]alanine

The experiments described in the introduction designed to detect incorporation or metabolism of radiolabelled alanine all used the ^{14}C isotope. The

Scheme 1. Fractionation of spores germinated in L-[³H]alanine

reasons for this choice are firstly that [^{14}C]alanine is available in a considerably more pure form than [^3H]alanine and secondly, that many spores have an active alanine racemase (EC 5.1.1.1) that would catalyse release of ^3H from the 2-position of [^3H]alanine. The observation that concentrated and dilute suspensions of *B. megaterium* KM spores germinated at similar rates, suggested that this strain lacks alanine racemase activity. A major reason for using [^3H]alanine in the present study was to investigate one possible route of alanine metabolism that would be undetectable if [^{14}C]alanine were used. Conceivably alanine could act as a catalyst in a reaction, emerging unchanged at the end. For example, the coupled action of alanine dehydrogenase (EC 1.4.1.1) and glutamate pyruvate transaminase (EC 2.6.1.2) would form a functional glutamate dehydrogenase (EC 1.4.1.2) that would be dependent on the presence of alanine. If [2,3- ^3H]alanine were used, however, the action of this couple would result in liberation of the 2- ^3H of alanine into water.

Table 1 shows the results of such an experiment. There was no increase in radioactivity in any of the fractions during the course of the experiment, showing that alanine was not metabolized either during the period of triggering or during the later stages of germination. This latter finding probably reflects the rapid excretion of amino acids from the spore at these times (Setlow, 1975). The absence of radioactivity from fraction A, which contains ^3H released into water, further shows that alanine is not functioning catalytically as outlined above and also confirms that alanine racemase is absent from spores of this organism. Table 2 shows which of the fractions in Table 1 contain the various possible products of alanine metabolism and the limits of production of these products during triggering of germination.

Germination in $^3\text{H}_2\text{O}$

For experiments using $^3\text{H}_2\text{O}$, non-heat-shocked spores were used, since it was found that when heat-

Table 2. *Maximum production of alanine derivatives during initiation of germination*

Fraction	Compounds	Limit of possible production (pmol/mg of spores)
A, A'	Ethylamine, water, acetaldehyde	1.0
B	Covalently bound alanine	0.1
C	Sugars, phosphorylated compounds, di- and tri-carboxylic acids	1.0
D	Monocarboxylic acids	0.25

Fig. 1. *Incorporation of ^3H from water during germination* Non-heat-activated spores were germinated at 100mg/ml in 50mM-potassium phosphate, pH7.5, as described under 'Methods'. Samples of dormant- and germinating-spore suspensions were taken and extracted as described under 'Methods'. $\circ$, Dormant spores; $\triangle$, germinating spores.

Table 1. [^3H]Alanine metabolism during germination Spores were germinated at 100mg/ml in 50mM-potassium phosphate, pH7.5, and 0.1mM-L-[^3H]alanine at 37°C. Samples were extracted and fractionated as described under 'Methods' and in Fig. 1.

Fraction	[Alanine] (pmol/mg of spores)				
	Time (min) ...	1	2	3	4
A	2.44	1.99	2.27	2.09	2.79
A'	0.10	0.10	0.10	0.07	0.09
B	0.24	0.28	0.21	0.20	0.23
C	0.48	1.05	1.00	1.25	1.36
D	0.16	0.30	0.14	0.19	0.35

shocked spores were suspended in water at 37°C, a very small fraction germinated spontaneously. This small amount of germination, which was undetectable by the usual criteria for measuring germination,

showed up as a high and logarithmically increasing ^3H incorporation during the experiment. Even non-heat-shocked spores showed this effect to a lesser extent, but in this case the background incorporation was sufficiently low and the increase sufficiently small to enable any stimulation in the rate of ^3H incorporation on addition of germinants to be detected.

It was further found that spores cleaned in the normal way gave a relatively high background ^3H incorporation that could be much decreased by the very thorough spore-cleaning procedure described under 'Methods'. This presumably reflected a very slight contamination of normal spore preparations with either germinated spores or residual vegetative cells. The specially cleaned preparations were used within 1 h to minimize any possible contamination of the preparations by germination of the spores during storage. The fact that these problems arose was an indication of the sensitivity of this technique.

Fig. 1 shows the results of a typical experiment. ^3H incorporation was not detectable until 2–3 min after addition of L-alanine, indicating that no reactions of the type that lead to non-exchangeable incorporation of ^3H occur during the lag period of germination. However, it must be remembered that these spores were not heat-shocked. For both heat-shocked and non-heat-shocked spores there is always a lag of about 2 min, when germination is monitored by either, fall in $A_{600\text{m}}$ by the uptake of oxygen or by release of calcium dipicolinate (I. R. L. Scott & D. J. Ellar, unpublished work). The difference between the two types of spore is primarily in the synchrony of germination and we are therefore justified in assuming that the timing of changes occurring during the lag period is unaffected by heat shock.

The experiment shown in Fig. 2 demonstrates that less than 20 pmol of ^3H are incorporated during the 2 min after L-alanine addition. This result eliminates the majority of enzyme-catalysed reactions as candidates for the trigger event in germination, since they would be accompanied by a non-exchangeable incorporation of ^3H . For example, all reactions involving pyridoxal phosphate, such as amino acid racemization, decarboxylation and transamination will lead to exchange of the proton on the α -carbon atom with water. For the same reason, reduction of compounds such as fumarate or pyruvate, which might act as endogenous acceptors in electron-transport-linked metabolism, would be detected. Nicotinamide nucleotide-mediated reductions would only be detected if the proton taken up from water becomes non-exchangeably bound, as the hydride ion carried by the reduced nucleotide would not equilibrate with water. Since the method would also detect hexose, pentose and triose phosphate isomerizations, the results rule out the involvement of the upper half of the glycolytic sequence and the linking

reaction of the upper and lower halves of glycolysis. Finally, the knowledge that non-exchangeable ^3H incorporation would also result from the activity of aldolase, transaldolase, enolase, phosphoglycerate mutase and aconitase, eliminates the pentose phosphate pathway, the lower glycolytic sequence and the tricarboxylic acid cycle as sites for germination initiation.

Clearly, since the operation of most of the main metabolic pathways would be detected by this technique, these pathways are either not involved in triggering or operate at an extremely low rate. There is at least one important class of reactions, however, that is not detectable by this technique, namely hydrolytic reactions. Hydrolysis of ester, amide, peptide and glycosidic bonds results in both protons from water being incorporated into exchangeable positions in the molecule. Enzyme-catalysed hydrolyses must therefore remain as possible mechanisms by which germination is initiated.

In view of the considerable accumulated indirect evidence that in certain strains of *Bacillus* the glycolytic pathway is operative during triggering of germination (Prasad *et al.*, 1972; Wax & Freese, 1968; Halvorson & Church, 1957) it may well be worthwhile to apply the versatile $^3\text{H}_2\text{O}$ technique to germination of those strains to attempt to identify directly those reactions that have been predicted to occur. In the strain of *B. megaterium* KM used in the present study, however, it seems probable that a purely degradative or biophysical mechanism exists for the initiation of germination.

These investigations were carried out with the financial assistance of the Medical Research Council and the Science Research Council.

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