INTERMEDIATE METABOLISM OF AEROBIC SPORES

IV. Alanine Deamination during the Germination of Spores of *Bacillus cereus*

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Rapid germination of bacterial spores may be initiated by exposing them to simple chemical agents. Of these, L-alanine has been most frequently reported (Stedman, 1956). Although the interaction of L-alanine with spores during the early stages of germination has been a subject of increasing interest in recent years, little is known of the nature of this interaction or of the "trigger" mechanisms involved in germination. For example, both catalytic (Harrell and Halvorson, 1955) and substrate (Falcone, 1955; Halvorson and Church, 1957) roles have been proposed to explain the action of alanine in germination. The major findings concerning alanine-stimulated germination can be summarized as follows: (a) alanine interacts initially with a stereospecific spore site (Woese *et al.*, 1958); (b) germination is independent of the action of alanine racemase activity (Church *et al.*, 1954); (c) alanine is utilized during activation and germination, presumably to pyruvate and NH_3 (Murrell, 1952; Murty and Halvorson, 1957; Falcone, 1955; Halvorson and Church, 1957); (d) pyruvate oxidation is a prerequisite for germination (Halvorson and Church, 1957).

The purpose of the present investigation was to study the initial interaction of L-alanine with spores during germination in an attempt to determine whether L-alanine serves as a substrate or as a catalyst. The observations reported here indicate that activated spores can deaminate exogenous alanine to pyruvate and NH_3. However, most of the products resulting from the interaction of alanine with spores arise from endogenous sources. In spite of the larger contribution from endogenous reserves, exogenous alanine is preferentially utilized during the early stages of germination.

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MATERIALS AND METHODS

Preparation of spores. Spores of *Bacillus cereus* strain T. (previously called *B. cereus* var. *terminalis*) were used in all experiments. The preparation of washed spores has been described (Church *et al.*, 1954). Lyophilized spores (1 g) were heat-activated in 100 ml 0.066 M phosphate buffer, pH 6.8, at 65 C for 2 hr. The suspension was then centrifuged and the spores washed three times with distilled water before use.

Analytical methods. Ammonia was recovered by microdiffusion (Conway, 1947) and determined by the procedure of Varner *et al.* (1953), using alkaline Nessler's reagent. Pyruvate was measured by the method of Friedemann and Haugen (1943), free amino nitrogen by the procedure of Moore and Stein (1948) using alanine as a standard. Alanine was identified by paper chromatography using Whatman no. 1 paper buffered at pH 12.0 according to the method of McFarren (1951); phenol saturated with buffer at pH 12.0 was the developing solvent and spots were detected by spraying with 0.5 per cent acidic ninhydrin in acetone.

Carbon dioxide was recovered by lowering the pH of the reaction mixture to 3.0 flushing for 30 min with N_2, and finally collecting in barium hydroxide; the washed barium carbonate was then acidified and the evolved CO_2 measured manometrically.

All radioactivity measurements were made with a Nuclear-Chicago scaler and a thin-window Geiger tube suitable for detecting C^{14}. Fluid samples were spread on stainless steel planchets, dried, and counted directly. Correction was made for self-absorption, where necessary.

Kjeldahl digestion for N^{15} determination of DL-alanine-N^{15} was done by the procedure described by Wilson and Knight (1952) using a

mercury catalyst. The ammonia was converted to nitrogen through oxidation by sodium hypobromite in the nitrogen conversion apparatus of Burris *et al.* (1943). A Consolidated-Nier isotope ratio mass spectrometer was employed for mass analysis.

DL-Alanine-1-C^{14} was recovered from the reaction mixture in some experiments by ion exchange chromatography (Stein and Moore, 1949) using Dowex 50 in the H^+ form. The radioactive components from the column were chromatographed as previously described and the alanine spot identified from radioautograms.

Chemicals. DL-Alanine-1-C^{14} (0.5 mc per mmole) was kindly furnished by Dr. W. Meinke, Department of Chemistry, University of Michigan, and DL-alanine-N^{15} (16.4 atom per cent excess N^{15}) by the New England Nuclear Corporation. Dowex 50 was obtained from Microchemical Specialties Company.

EXPERIMENTAL RESULTS

Effect of heat-activation. When intact heat-activated spores are incubated with L-alanine, there is an immediate and constant evolution of NH_3 for at least 1 hr (figure 1). Heat activation alone leads to a similar endogenous release of ammonia. NH_3 can not be detected from unheated spores incubated in the presence or absence of L-alanine. The dependency of the rate of NH_3 release on heat activation at 65 C is shown in figure 2. Four hour treatment at 65 C was required to obtain maximal NH_3 evolution from freshly harvested spores. Longer periods of activation lead to diminishing rates of NH_3 release. In general, spores which had been harvested, dried and stored at −20 C for 6 months were used for the following experiments. These spores are maximally activated by heating for 2 hr at 65 C.

Localization of the L-alanine-deaminating system. Berger and Marr (1957) have provided a methodology whereby the localization of spore components can be detected by determining the kinetics of their solubilization during sonic oscillation. For example, the immediate first order loss of exosporium and adenosine deaminase during sonic oscillation suggested that the enzyme was associated with that structure. Since their findings further raised the possibility that the exosporium might serve as the locus of the enzymes initially active on germinating agents,

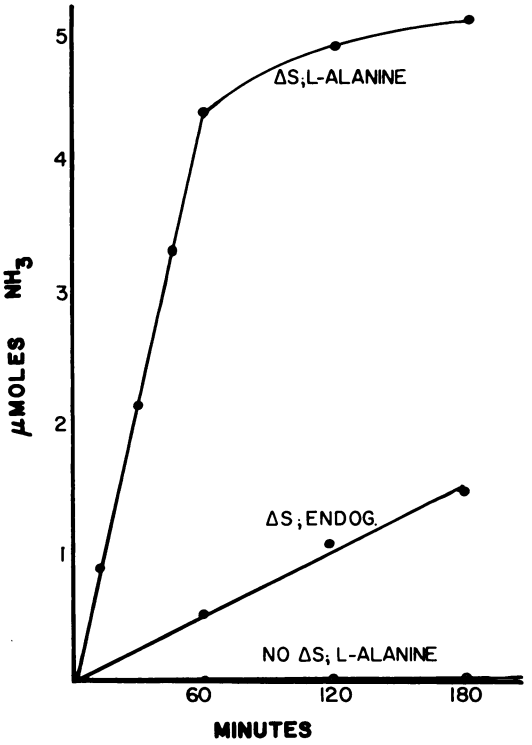

Figure 1. Ammonia release from spores exposed to L-alanine. Each Conway unit contained 25 mg spores and 133 μmoles phosphate buffer, pH 6.8, plus 5 μmoles L-alanine where indicated in a total volume of 2 ml. The culture was incubated at 30 C. Heat shock (ΔS) prior to incubation is described in Materials and Methods. Endog. = above reaction mixture without L-alanine.

the following experiment was carried out. Spores were treated in the sonic oscillator under the conditions described in figure 3. The turbidity and viability of the suspension decreased by single and multihit curves respectively, confirming the observations of Berger and Marr (1957). The rate of decrease in the activity of L-alanine deaminase during sonic oscillation shows that the enzyme is not associated with the exosporium, but is an integral part of the spore itself.

Stoichiometry of L-alanine deamination. In previous studies we found that when heat-activated spores were incubated with 2.5 μmoles L-alanine per ml and an inhibitor of pyruvate oxidation nearly stoichiometric yields of NH_3 and pyruvate were recovered. However, because later experiments suggested a wide variation in the recoveries of NH_3 and pyruvate, it seemed desirable to re-

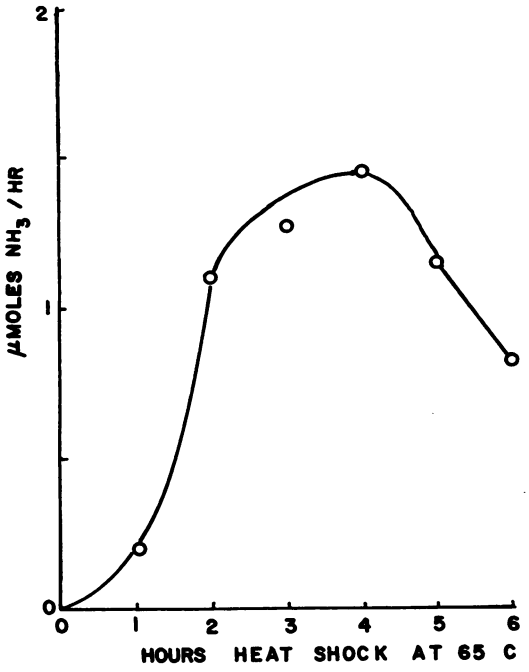

Figure 2. Effect of heat activation on ammonia release during alanine activation. One gram of spores was suspended in 100 ml 0.066 M phosphate buffer, pH 6.8, and immersed in a water bath at 65 C. At the indicated time intervals, 10 ml of the suspension were removed, chilled, centrifuged and resuspended in 0.066 M phosphate buffer, pH 6.8. The rate of ammonia release per 0.1 g of spores was determined as described in figure 1 with a correction for the endogenous release of NH_3.

investigate the stoichiometry at various concentrations of L-alanine. The experiment is described in figure 4. It is clear that when the concentration of L-alanine is less than 2.5 μmoles per ml, the yield of ammonia exceeds that of the substrate alanine, thereby suggesting a catalysis of endogenous NH_3 release at low levels of L-alanine.

These results raise the question whether the amino group of L-alanine contributes to the recovered NH_3. An analysis of alanine disappearance during NH_3 evolution from alanine-exposed spores (figure 5) indicated that L-alanine was utilized during the NH_3 release. However, the rate of NH_3 release was consistently higher than that of the alanine utilization. During the initial 30 min incubation, NH_3 was released in the absence of detectable alanine utilization.

A more direct test of the relationship between substrate and product was provided by measuring the flow of N and C from alanine to NH_3 and pyruvate. Heat-activated spores were incubated with DL-alanine-N^{15} and the ammonia recovered by microdiffusion. The specific activity of NH_3 was determined as described in table 1. Analysis of the NH_3 after incubation for 15 min revealed the presence of significant quantities of N^{15}; however, only 10 per cent of the NH_3 was derived from exogenous alanine. Since 91 per cent germination occurs during this period, L-alanine-induced germination involves the rapid release of NH_3 from endogenous sources. Little change in the specific activity occurred throughout the remainder of the incubation period.

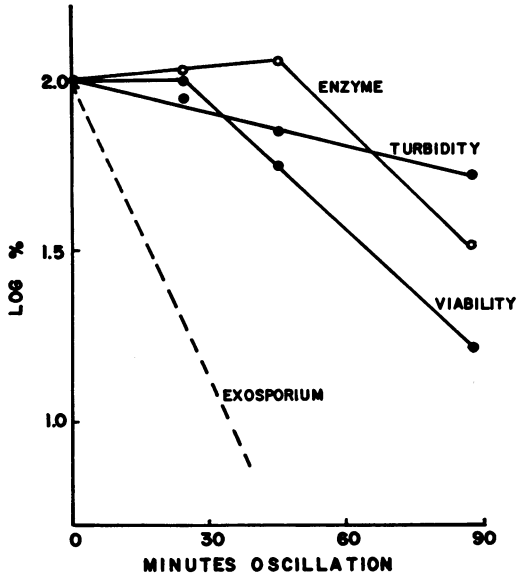

Figure 3. Localization of alanine deaminase within the spore. Fifty milliliters of 0.066 M phosphate buffer, pH 6.8, containing 1 g of heat-activated spores were treated in a Raytheon 10 kc sonic oscillator at 4 C. At the indicated times 7 ml samples were removed and replaced by an equal volume of buffer. Viability was determined by plate count using nutrient agar (Difco). Turbidity was measured with a Klett colorimeter (red filter). Enzyme release was determined by measuring the remaining alanine deaminase activity of the sediment after centrifuging at 10,000 × G for 15 min. The sediment was resuspended in 4 ml 0.066 M phosphate buffer, pH 6.8. The rate of ammonia release was determined by the method described in figure 1 with 0.3 μmole diphosphopyrydine nucleotide added. Correction was made for endogenous release of NH_3.

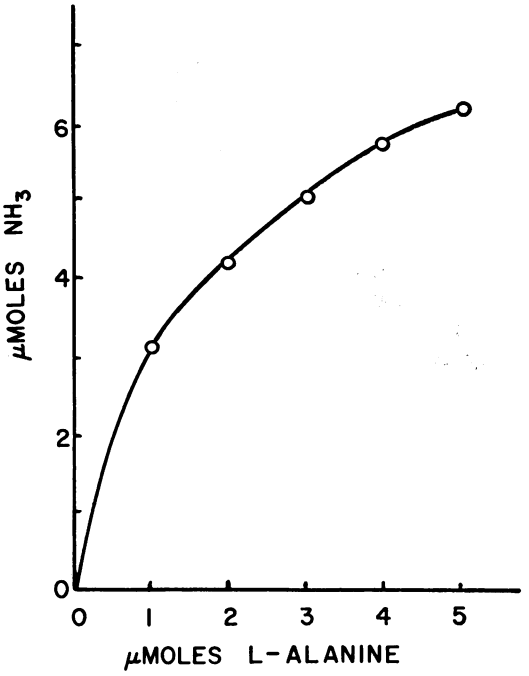

Figure 4. Stoichiometry of ammonia release from L-alanine by heat-activated spores. Ammonia release from the intact heat-activated spore was determined by the method described in figure 1, after an incubation period of 3 hr.

The demonstration of pyruvate as a product of L-alanine metabolism requires the accumulation of a sufficient amount to permit its recovery. Of the various inhibitors of pyruvate oxidation tested (10^{-2} M arsenite, cyanide, hydroxylamine, hydrazine, semicarbazide, bisulfite, and 10^{-4} M *bis* - 1,3 - β - ethylhexyl - 5 - methyl - 5 - amino-hexahydropyrimidine), only arsenite consistently provided an accumulation of pyruvate. The effectiveness of this inhibition is indicated by the fact that spores exposed to 52 μmoles of DL-alanine-1-C^{14} in the presence of arsenite yielded no CO_2 that could be detected manometrically; spores exposed to 20 μmoles DL-alanine-C^{14} in the absence of arsenite yielded 20.8 μmoles of CO_2.

When spores were exposed to DL-alanine-1-C^{14} for 3 hr in the presence of arsenite (table 2), significant quantities of radioactive pyruvate were recovered. Decarboxylation of this pyruvate by the method of Meister (1952) resulted in complete recovery of the radioactivity as $C^{14}O_2$, indicating that the pyruvate was labeled only in the COOH position. Nearly equimolar quantities of NH_3 (21.8 μmoles) and pyruvate (17.1 μmoles) were recovered, confirming the previous reports (Halvorson and Church, 1957). However, the specific activity of the recovered pyruvate was only 11 per cent of the added alanine, indicating that almost 90 per cent of the pyruvate was produced from endogenous sources. This equimolar relationship between recovered ammonia and pyruvate suggests that alanine, or amino-bearing compounds with a carbon skeleton similar to alanine, was providing the endogenous ammonia and pyruvate. The specific activity of the alanine recovered from the medium after the incubation period (table 2) was essentially the same as that added initially. If this endogenous

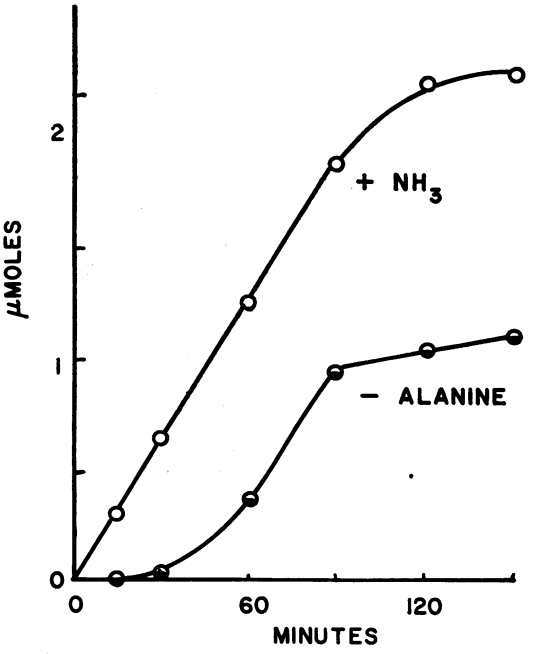

Figure 5. Rate of ammonia release and alanine utilization during alanine activation. The reaction mixture (250-ml Erlenmeyer flask) contained 100 μmoles L-alanine, 500 mg heat activated spores, and 2.67 mmoles phosphate buffer in a total volume of 40 ml. The reaction was incubated on a reciprocal shaker at 30 C. At intervals, 5 ml aliquots were removed and centrifuged. NH_3 corrected for endogenous evolution was determined on the supernatant as previously described. Alanine was measured by the ninhydrin assay, corrected for NH_3, and identified by paper chromatography. The results are expressed in terms of μmole per ml of the original reaction mixture.

TABLE 1

NH release from DL-alanine-N^{15} during germination*

The 80 ml reaction mixture contained 200 μmoles DL-alanine-N^{15}, 1.0 g spores, and 5.32 mmoles phosphate buffer, pH 6.8 in a 1-L Erlenmeyer flask. The reaction mixture was incubated on a reciprocal shaker at 30 C. The NH_3 was recovered, converted to N_2, and its specific activity determined. Germination was determined by stainability with 1 per cent (w/v) methylene blue.

Incubation Time	Germination	NH_3 Released	Specific Activity $N^{15}H_3$	NH_3 from DL-Alanine-N^{15}
min	%	*μmoles*	*atom % excess N^{15}*	%
10	91.3	68.7	0.125	10.0
150	99.2	273.6	0.104	8.3

* Specific activity of DL-alanine-N^{15} added initially; 1.248 atom per cent excess N^{15}.

precursor of pyruvate is alanine, then it must not be in equilibrium with the exogenous alanine. Two unidentified radioactive components were also obtained, but only when pyruvate oxidation was blocked with arsenite, suggesting that these components are intermediates of pyruvate metabolism. Analyses of these products for lactate, keto acids, and free amino groups were negative. There was only negligible incorporation of the DL-alanine-C^{14} into spore protein (table 2). In the presence of arsenite, germination was completely suppressed, confirming the observation that the inhibition of pyruvate oxidation precludes germination (Halvorson and Church, 1957).

When arsenite was omitted from the reaction mixture, $C^{14}O_2$ was recovered (table 3), probably as a result of the decarboxylation of pyruvate. The specific activity of this CO_2 was 14 per cent of that of the substrate alanine. If the CO_2 were derived solely from the carboxyl group of pyruvate, then 86 per cent of it must have been derived from endogenous sources. Since spores contain an active acetate-oxidizing system (Halvorson and Church, 1957), as much as 42 per cent of the CO_2 may have been derived from substrate alanine. The distribution of the radioactivity is shown in table 3. Only a negligible amount of radioactivity was incorporated into the cold trichloroacetic acid-insoluble fraction.

The alanine recovered after 3 hr incubation with DL-alanine-1-C^{14} had a specific activity only 55 per cent of that of the substrate alanine, indicating that spores release a large amount of alanine during germination. This dilution was not unexpected since germination exudate is known to contain large quantities of alanine (Powell and Strange, 1953).

The data presented thus far do not clearly establish whether the role of L-alanine in germination is that of substrate or catalyst. While quantities of exogenous alanine are metabolized to pyruvate and ammonia, contact of the spores with L-alanine also results in endogenous metabolism yielding even higher levels of pyruvate and ammonia. If subsequent pyruvate oxidation is blocked, no germination occurs. Therefore,

TABLE 2

Distribution of C^{14} derived from DL-alanine-1-C^{14} (arsenite added)*

The 20 ml reaction mixture contained 52 μmoles DL-alanine-1-C^{14}, 130 mg spores, 0.96 μmole arsenite, and 1.33 mmoles phosphate buffer, pH 6.8, in a 250-ml Erlenmeyer flask. The reaction mixture was incubated on a reciprocal shaker for 3 hr at 30 C. After recovery of the CO_2, samples of the reaction mixture were removed and analyzed for pyruvate and ammonia. The spores were then centrifuged from the remaining medium and fractionated by cold trichloroacetic acid extraction. Pyruvate was recovered as the 2,4-dinitrophenylhydrazone (Shriner and Fuson, 1948), alanine by column chromatography using Dowex 50-(H^+) (Stein and Moore, 1949).

Fraction	Total Radioactivity	Specific Activity	C Derived from DL-Alanine-1-C^{14}
	cpm	*cpm/μmole*	%
1. Pyruvate	7,380	433	11.8
2. CO_2	862		
3. Spores:			
a. Cold trichloroacetic acid-soluble	1,120		
b. Cold trichloroacetic acid-insoluble	None		
4. Reaction Mixture			
a. Alanine	163,300	3,653	94.7
b. Unknown substances	30,700		

* Specific activity of DL-alanine-1-C^{14} added initially: 3853 cpm per μmole.

TABLE 3

*Distribution of C^{14} derived from DL-alanine-1-C^{14}**

The 8.0 ml reaction mixture contained 20 μmoles DL-alanine-1-C^{14}, 100 mg spores, and 532 μmoles phosphate buffer, pH 6.8 in a 50-ml Erlenmeyer flask. The reaction mixture was incubated on a reciprocal shaker for 3 hr at 30 C. The radioactivity was recovered as described in table 2.

Fraction	Total Radioactivity	Specific Activity	C Derived from DL-Alanine-1-C
	cpm	*cpm/μmole*	%
1. CO_2	17,389	833	14–41
2. Spores			
a. Cold trichloracetic acid soluble	1,188		
b. Cold trichloroacetic acid insoluble	836		
3. Medium:			
a. Alanine	81,400	3,300	54

* Specific activity of the DL-alanine-1-C^{14} added initially: 6036 cpm per μmole.

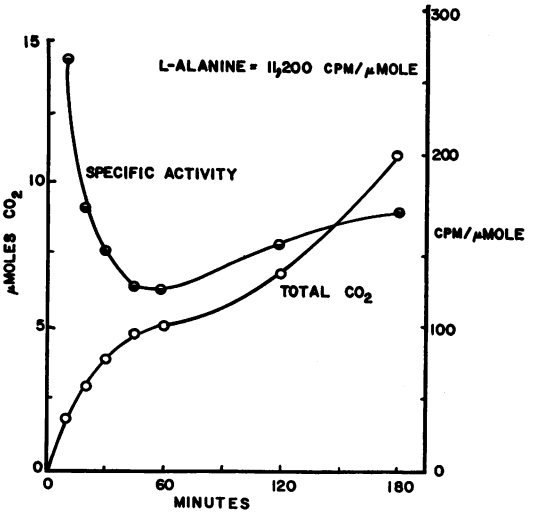

Figure 6. Evolution of CO_2 during the utilization of DL-alanine-1-C^{14}. Each 50-ml Erlenmeyer flask contained 25 μmoles DL-alanine-1-C^{14}, 125 mg spores, and 6.65 μmoles phosphate buffer in a total fluid volume of 10 ml. At the indicated time intervals, CO_2 was recovered as indicated in the text.

accelerated catabolic activity is necessary for germination. If alanine plays the role of substrate in germination, then the catabolic activity accompanying germination should reflect the preferential oxidation of exogenous alanine.

The contribution of substrate alanine-1-C^{14} to the respiratory activity during germination can be determined by measuring the specific activity of the evolved CO_2. If the oxidation of alanine-derived pyruvate is the initial step in alanine-induced germination, then the radioactive CO_2 evolved during the initial stages of germination should have a relatively high specific activity.

Spores were incubated with DL-alanine-1-C^{14} for 3 hr in seven Erlenmeyer flasks, 50-ml. At intervals, flask contents were acidified by adding H_2SO_4 to pH 3.0. Carbon dioxide was displaced in the reaction mixture with CO_2-free nitrogen gas, and trapped in $Ba(OH)_2$. After 3 hr incubation with 25 μmoles of DL-alanine-1-C^{14}, 11 μmoles of radioactive CO_2 were recovered (figure 6). Carbon dioxide evolution was rapid throughout the first 60 min; the rate of liberation then decreased and remained nearly constant for 2 hr. The radioactive CO_2 recovered after a short incubation period had a higher specific activity than that recovered after a longer period. This initial high specific activity indicates that there is a preferential utilization of the exogenous alanine during the early stages of germination, accompanied by a rapid dilution with unlabeled CO_2 from endogenous sources.

DISCUSSION

The present isotope experiments, demonstrating that L-alanine is converted by spores to pyruvate and NH_3, confirm previous observations that L-alanine is utilized during the activation (Murrell, 1952; Murty and Halvorson, 1957) and germination (Falcone, 1955; Halvorson and Church, 1957) of aerobic spores. One of the interesting findings is that most of the pyruvate and NH_3 recovered during alanine-stimulated germination originates from endogenous sources. The labilization of these reserve materials is consistent with the current view that germination is essentially a degradative process. For example, germination is accompanied by a 30 per cent loss in dry weight, partly derived from loss of spore coats (Powell and Strange, 1953) and spore cortex (Mayall and Robinow, 1957), decrease in protein-bound P (Fitz-James, 1955), activation of hydrolytic enzymes (Powell, 1958; Levinson, 1958), and an increase in the intra-

sporal amino acid pool (Foster, 1958). The endogenous release of NH_3 may also be mediated through the heat-activated transaminases of spores described by Falcone and Caraco (1958).

Although most of the catabolic activity accompanying germination involves the metabolism of endogenous material, exogenous alanine is preferentially utilized during the early stages. The tracer studies reported here indicate that NH_3, pyruvate, and eventually CO_2 are products of its metabolism. That pyruvate metabolism is essential for germination, originally observed by Halvorson and Church (1957), has been confirmed. Yet the inhibition of pyruvate metabolism did not prevent the release of endogenous pyruvate and NH_3 within the spore. This observation indicates that alanine stimulates endogenous degradation prior to the oxidation of alanine-derived pyruvate. Alanine could serve to induce the degradation of spore macromolecules into low molecular weight components that serve as precursors for subsequent cell synthesis. The energy required for this synthesis could then be provided by the oxidation of pyruvate derived from substrate alanine and endogenous sources. Since germination does not occur when pyruvate metabolism is blocked, the gross changes (such as stainability and increase in respiration) by which germination is measured actually reflect the energy-requiring stage.

An understanding of the trigger role of L-alanine in germination requires a further analysis of the site of the initial interaction. One might imagine, for example, that the alanine deaminating system described here is the primary site of L-alanine action. On the other hand, the intrasporal distribution of the enzyme and the need for heat activation to initiate its activity, suggest that the deamination may be a secondary rather than a primary step in germination. We are currently investigating this possibility by comparing the specificity of the purified spore enzyme with the specificity of germination initiated by L-alanine and its analogues.

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SUMMARY

Intact spores of *Bacillus cereus* strain T. contain an intrasporal heat-activation-dependent system that converts exogenous alanine as well as endogenous reserves to pyruvate and NH_3. The deamination of alanine can occur in the absence of germination. Pyruvate, produced from either endogenous or exogenous sources, is rapidly metabolized. Inhibition of pyruvate metabolism leads to a suppression of germination. During the early stages of germination, the exogenous alanine is preferentially deaminated.

REFERENCES

BERGER, J. A. AND MARR, A. G. 1957 Structure of endospores of *Bacillus cereus*. Bacteriol. Proc., **1957**, 41.

BURRIS, R. H., EPPLING, F. J., WAHLEN, H. B., AND WILSON, P. W. 1943 Detection of nitrogen fixation with isotopic nitrogen. J. Biol. Chem., **148**, 349–357.

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

CONWAY, E. J. 1947 *Microdiffusion analysis and volumetric error*. Crosby Lockwood and Son Ltd., London.

FALCONE, G. 1955 Metabolismo della L-alanina germinazione della spore. Giorn. microbiol., **1**, 185–192.

FALCONE, G. AND CARACO, A. 1958 Transaminase activities in germinating and heat-activated spores of *Bacillus subtilis*. Intern. Congr. Microbiol., 7th Congr., Stockholm. Rept. Proc., p. 34.

FITZ-JAMES, P. C. 1955 The phosphorus fraction of *Bacillus cereus* and *Bacillus megaterium*. II. A correlation of the chemical with the cytological changes occurring during spore germination. Can. J. Microbiol., **1**, 525–548.

FOSTER, J. W. 1958 Discussion of chemical changes occurring during spore germination. In *Spores*, pp. 76–82. Edited by H. O. Halvorson. Am. Inst. Biol. Sci., Washington, D. C.

FRIEDEMANN, T. E. AND HAUGEN, G. E. 1943 Pyruvic acid. II. The determination of keto acids in blood and urine. J. Biol. Chem., **147**, 415–422.

HALVORSON, H. AND CHURCH, B. D. 1957 Intermediate metabolism of aerobic spores. II. The relationship between oxidative metabolism and germination. J. Appl. Bacteriol., **20**, 359–372.

HARRELL, W. K. AND HALVORSON, H. 1955 Studies on the role of L-alanine in the germination of spores of *Bacillus terminalis*. J. Bacteriol., **69**, 275–279.

LEVINSON, H. S. 1958 Non-oxidative enzymes of spore extracts. In *Spores*, pp. 120–143. Edited by H. O. Halvorson. Am. Inst. Biol. Sci., Washington, D. C.

MAYALL, B. H. AND ROBINOW, C. F. 1957 Observations with the electron microscope on the organization of the cortex of resting and germinating spores of *B. megaterium*. J. Appl. Bacteriol., **20**, 333–341.

MCFARREN, E. F. 1951 Buffered filter paper chromatography of the amino acids. Anal. Chem., **23**, 168–174.

MEISTER, A. 1952 Enzymatic preparation of α-keto acids. J. Biol. Chem., **197**, 309–317.

MOORE, S. AND STEIN, W. R. 1948 Photometric ninhydrin method for use in the chromatography of amino acids. J. Biol. Chem., **176**, 376–384.

MURRELL, W. G. 1952 Metabolism of bacterial spores and thermophilic bacteria. Ph.D. Thesis, Oxford, England.

MURTY, G. G. K. AND HALVORSON, H. O. 1957 Effect of duration of heating, L-alanine, and spore concentration on the oxidation of glucose by spores of *Bacillus cereus* var. *terminalis*. J. Bacteriol., **73**, 233–240.

POWELL, J. F. 1958 Chemical changes occurring during spore germination. In *Spores*, pp. 72–82. Edited by H. O. Halvorson. Am. Inst. Biol. Sci., Washington, D. C.

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

SHRINER, R. L. AND FUSON, R. C. 1948 *The systematic identification of organic compounds*. John Wiley and Sons, Inc., New York.

STEDMAN, R. L. 1956 Biochemical aspects of bacterial endospore formation and germinations. Am. J. Pharm., **128**, 84–97, 114–130.

STEIN, W. H. AND MOORE, S. 1949 Chromatographic determination of the amino acid composition of proteins. Cold Spring Harbor Symposia Quant. Biol., **14**, 179–190.

VARNER, J. E., BULEN, W. A., VANECKO, S., AND BURRELL, R. C. 1953 Determination of ammonia, amide, nitrite, and nitrate nitrogen in plant extracts. Anal. Chem., **25**, 1528–1529.

WILSON, P. W. AND KNIGHT, S. G. 1952 *Experiments in bacterial physiology*. Burgess Publishing Co., Minneapolis.

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