STUDIES ON THE SPORES OF AEROBIC BACTERIA

I. The Occurrence of Alanine Racemase[1]

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The unique resistance of bacterial spores to destruction by high temperatures coupled with the lack of convincing evidence of a measurable metabolic rate in free spores has led gradually to the supposition that free spores may not contain active enzymes. Yet spores do germinate, with amazing rapidity, when placed in a suitable medium. Assuming germination to be linked necessarily with enzymatic activity we have focused our attention on the very earliest stages of the germination process in order to study how a spore interacts with the substances initiating its change to a vegetative cell.

Hills and his group have in the past few years demonstrated that in a great many cases the requirements for rapid germination of aerobic bacterial spores are simple. With some strains of bacteria, single amino acids with or without adenosine, glucose alone, or adenosine alone can lead to 90 per cent germination of a 10^9 per ml suspension of spores in a period of 10 to 30 minutes (Hills, 1949*a*, *b*, 1950; Powell, 1950, 1951).

The spores used in this investigation require L-alanine and adenosine for complete germination, adenosine being replaceable by guanosine, but not by adenylic acid or adenine plus ribose. D-Alanine is a powerful inhibitor of germination under these conditions, a fact initially ascertained by Hills (1950). In studying the disappearance of L-alanine in the germination process it has been found that a rapid conversion of L-alanine to D-alanine occurs, in the presence *or absence* of adenosine. Since no germination (defined as loss of heat resistance accompanied by changes in optical properties, following Hills) occurs in the absence of adenosine, this rapid conversion of L-alanine to its isomer constitutes an example of a highly active enzymatic system associated with "resting" spores. It is with this enzymatic system, hereafter referred to as alanine racemase (Wood and Gunsalus, 1951), that this paper is concerned.

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MATERIALS AND METHODS

Organism. The culture used in the main part of this investigation bears the identification *Bacillus terminalis* in the culture collection of the Bacteriology Department of the University of Illinois. It was selected because of its rapid growth and production of spores without pellicle formation or clumping in nutrient broth, the large size of its cells and spores, and the ability of its spores to germinate in simple well defined media. On the basis of cell size (about 40 μ by 1 μ, to 2 μ by 1 μ, depending on the growth phase), spore size (1.5 μ by 1 μ), and mode of germination (absorption of coat and swelling), this organism evidently is related closely to *Bacillus cereus*, but no serious attempt at rigid classification has been made.

Culture media. The spores used were grown in an effectively aerated medium (G) of the following composition: K_2HPO_4, 1 g; $(NH_4)_2SO_4$, 4 g; (Difco) yeast extract, 2 g; $MnSO_4 \cdot H_2O$, 0.1 g; $MgSO_4$, 0.8 g; $ZnSO_4$, 10 mg; $CuSO_4 \cdot 5H_2O$, 10 mg; $CaCl_2$, 10 mg; $FeSO_4 \cdot 7H_2O$, 1 mg; and glucose, 4 g per liter of distilled water. Sterile "DC-Antifoam-A" was added as needed to control foaming.

The nutrient broth employed contained: yeast extract, 100 ml; peptone autolysate, 12 ml; and NaCl, 5 g; made up to 1 liter with distilled water and brought to pH 7 with NaOH. Nitrate broth contained KNO_3, 1 g per liter of nutrient broth. When spores were grown on agar surfaces, large size petri dishes were used, each containing 100 ml of 2 per cent of agar medium. Cultures were incubated at room temperature or in a 30 C incubator.

Preparation of spores. Most of the experiments were performed on a crop of vacuum dried spores, harvested from 16 liters of G medium (above) after 18 hours of growth with heavy

aeration. The spores were separated from the medium in a Sharples air driven centrifuge, resuspended in distilled water, shaken with glass beads to facilitate washing, and centrifuged. This procedure was repeated six times. Finally the suspension in distilled water was brought to pH 8 and allowed to shake at room temperature for 3 hours to assist the autolysis of the few remaining vegetative cells. After the suspension had been frozen and thawed, the spores were washed four more times with distilled water. The resulting suspension was observed microscopically to be clean of vegetative cells and cellular debris, and subsequently was dried from a frozen state *in vacuo* over $CaCl_2$. From this procedure resulted 2.2 g of a fluffy white powder that was stored in an amber bottle at room temperature. The dried spores were 100 per cent viable and required heat shock (65 C for 15 min) for rapid germination, as do freshly harvested "wet" spores of this strain. A suspension of 10^8 of the heat shocked spores per ml will germinate completely in 5 min in the presence of L-alanine (6 mM per ml) and adenosine (12 μM per ml) at 30 C, pH 7.

Determination of alanine racemase. 1. Static assay. In this assay racemization was carried out in 15 ml plastic centrifuge tubes with a total reaction volume of 2 ml usually containing 0.3 ml 0.3 M L-alanine at the required pH (normally pH 8.5) and a conveniently diluted suspension of spores or cells. After 10 min incubation at 30 C the tubes were chilled rapidly in an ice bath, the cells centrifuged, and the supernatant assayed for D-alanine. D-Alanine was determined manometrically as described by Wood and Gunsalus (1951), using D-amino acid oxidase prepared from acetone-dried pig kidney cortex.

2. Dynamic assay. An assay preferred for some experiments involved carrying out the racemization in Warburg cups in the presence of the D-amino acid oxidase. This enzyme does not attack L-alanine but oxidizes D-alanine with the uptake of ½ mol of oxygen per mol of D-alanine. When the oxidase is present in excess, the rate limiting step becomes the formation of D-alanine from the added L-alanine by the enzyme in the spore (*see* Wood and Gunsalus, 1951). Under these conditions, the oxygen uptake is proportional to the amount of racemizing enzyme present. Details of this assay, which is a modification of the one given by Wood and Gunsalus, are as follows: The main cup of the Warburg vessel contained 1 ml of D-amino acid oxidase, 0.4 ml M/1 phosphate buffer pH 8.3, and 0.3 ml 0.3 M L-alanine. The side arm contained 0.2 ml of a suspension of spores suitably diluted in water. Two-tenths ml 20 per cent KOH with filter paper was added to the center well. The cups were incubated at 30 C for ten minutes, in which time the small amount of D-alanine contained in the substrate was oxidized, the side arms tipped, the reaction allowed to proceed for ten minutes, and then rate readings taken over the next thirty min. The rate was proportional to the racemization up to about 10 μM alanine racemized per hour.

L-Alanine. The L-alanine used was acquired from Nutritional Biochemicals Corporation. It contained 0.6 per cent D-alanine when tested with D-amino acid oxidase.

Pyruvate determination. In order to be sure that it was D-alanine and not some other D-amino acid that was being oxidized in the assay, the contents of a Warburg run after an assay had been completed were tested for the presence of pyruvic acid. 2,4-Dinitro-phenylhydrazine derivatives were made of cup contents and a purified sample of pyruvic acid according to the procedure given by Friedemann and Haugen (1943). After extraction with toluene and Na_2CO_3 the red colors developed on addition of NaOH to the carbonate solution were compared in a Beckman spectrophotometer over the range of 420 to 560 mμ. The two curves of cup contents and pyruvic acid were exactly similar as would be the result if D-alanine were being oxidized to pyruvic acid by D-amino acid oxidase in the assay system.

RESULTS

Occurrence of alanine racemase in spores. Using the dynamic assay, the dried crop of spores of *B. terminalis* racemized 84 μmoles of L-alanine per hour per mg dry wt. This rate is about 20 times greater than the value reported by Wood and Gunsalus (1951) for dried cells of *Streptococcus faecalis*. In order to ascertain whether or not this enzyme is of general occurrence in bacterial spores, a survey was carried out with some available *Bacillus* strains under identical conditions. For this purpose log phase cultures were spread thickly on large petri plates each containing 100 ml nutrient agar. The plates were incubated at room temperature for 2 to 8

days, harvested when microscopic observation indicated at least 90 per cent sporulation, washed several times with distilled water, and assayed for alanine racemase in the dynamic assay system described above. A two milliliter aliquot of each suspension was pipetted into each of two small aluminum weighing cups and placed in an oven at 105 C for 24 hours to determine dry weight. The data in table 1 show the comparative values obtained, expressed as Q = μM D-alanine formed/hour/mg dry wt.

TABLE 1

Occurrence of alanine racemase in spores and vegetative cells

ORGANISM	Q IN VEGETATIVE CELLS	Q IN SPORES
Bacillus terminalis	5	84
Bacillus fulminans		18
Bacillus megaterium		22
Bacillus subtilis sp		47
Bacillus subtilis, strain C-4†	4	10
Bacillus cereus		37
Bacillus stearothermophilus		10
Bacillus polymyxa	1*	10
Streptococcus faecalis	3*	
Staphylococcus aureus, strain 2	2*	
Escherichia coli, strain B	1*	
Pseudomonas fluorescens, strain A.3.12	4*	
Clostridium welchii, strain BP6K	1*	

Q = μmoles D-alanine formed/mg dry wt/hour.

* = values taken from paper of Wood and Gunsalus (1951) calculated in terms of Q above.

† Strain obtained through the courtesy of Dr. I. C. Gunsalus.

In some cases vegetative cells also were harvested, long before the onset of sporulation. These cells were washed and resuspended in M/15 phosphate buffer pH 7, instead of water but otherwise assayed in the same manner. As indicated in table 1, the values for vegetative cells are invariably lower than the rates in the corresponding spore suspensions. For ease of comparison, some of the figures of Wood and Gunsalus also are included in the table, converted to the same Q value. From these data it is evident that relative to vegetative cells of bacilli and the cells of other bacterial strains the spores of aerobic bacteria generally contain a high order of activity of alanine racemase.

Relation of racemase content to growth of culture. The discrepancy between the content of alanine racemase in spores and vegetative cells of the same culture indicated in table 1 made it of some interest to follow more closely the association of enzyme with the growth curve of the culture. The results of such an experiment are shown in figure 1. In this experiment, a Fernbach flask containing 1.5 L of nitrate broth was inoculated with 15 ml of a log phase culture of *B. terminalis* which had been grown in the same medium. The flask was placed on a rotary shaker in a 30 C incubator and samples of appropriate volume were taken at intervals. The samples were spun down, washed three times with M/40 phosphate buffer pH 7, resuspended to the desired density in buffer, and assayed for alanine racemase. As the cells change drastically in size and association pattern during growth, dry weight determinations were made on the final resuspension of each sample, from which, knowing the original volume taken, were computed the points on the growth curve. In most cases the samples were placed in the deep freeze for a 12 hour period before the static assay of the enzyme as this treatment was shown not to alter the results.

It is evident that the activity of alanine racemase stays rather uniformly low, with one exception, until the cells have formed fully matured spores. The exceptional point on the curve, occurring after 4 hours of incubation, was checked in a repeat experiment and evidently reflects a real peak in activity with cells at a morphological age corresponding to four hours' growth under these conditions. Such cells are in the log phase of growth and are extremely long, averaging 1 by 40 μ at this stage. The magnitude of the morphological changes which occur regularly during the growth of a culture of *B. terminalis* facilitates accurate comparison of developmental culture age. The sample taken at 40 hours, which is the last point shown on the curve in figure 1, shows virtually 100 per cent sporulation, but the spores are still enclosed in their parent sporangia. At 80 hours this culture contained all free spores, and a sample taken at that time also gave a $Q_{alanine}$ of 60.

Properties of alanine racemase of spores. The response of the racemase in spores to changes in pH is shown in figure 2. It was necessary to use a static assay to obtain these data, as D-amino acid oxidase activity is also a function of pH.

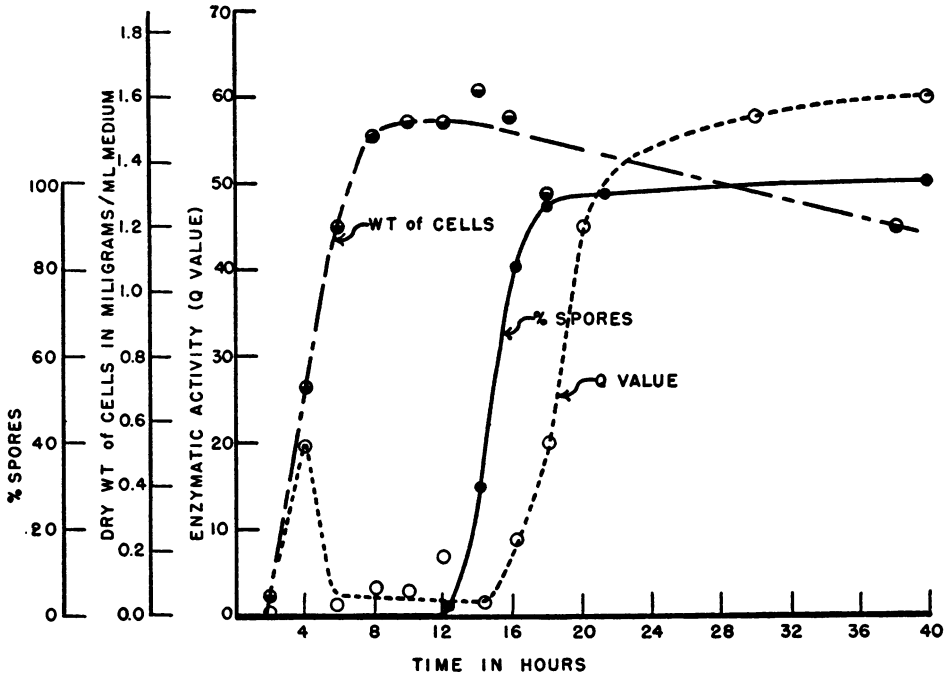

Figure 1. Static assay. Each tube contained 1 ml cell suspension, 0.3 ml 0.3 M L-alanine, and 0.3 ml M/1 phosphate buffer pH 8.3, plus 0.4 ml water. Reaction time 10 min at 30 C. Supernatant analyzed for D-alanine manometrically using D-amino acid oxidase.

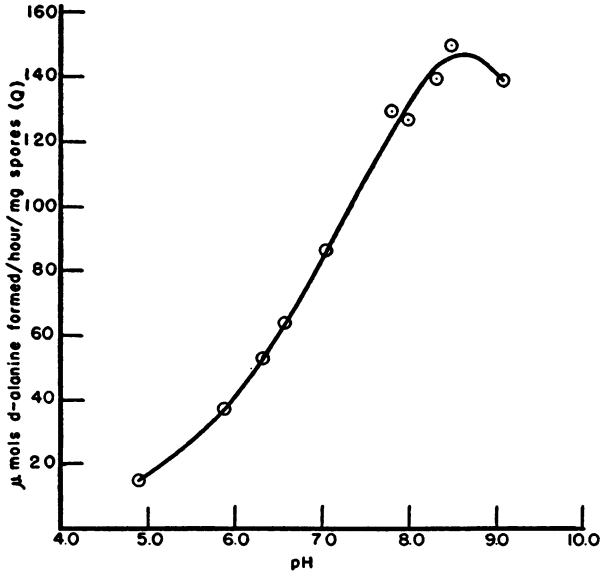

Figure 2. Static assay. Each tube contained 0.5 ml 0.3 M L-alanine, 0.5 ml spore suspension (1 mg dry wt), and 1 ml veronal-acetate buffer plus NaOH or HCl to required pH. Tubes incubated 15 min at 30 C; supernatant analyzed manometrically for D-alanine. pH of final supernatant recorded in graph.

The distribution of activities between pH 5 and 9 indicates a pH optimum of around 8.6. This checks roughly with the data of Wood and Gunsalus which show for the alanine racemase of *S. faecalis* a pH optimum of above 8.

Measurement of enzyme activity at different concentrations of substrate indicates that it takes quite high concentrations of L-alanine to saturate the enzyme. Lineweaver-Burk (1934) plots of such data give straight lines from which a calculation of the Michaelis-Menton constant yields a value of $K_s = 5.8 \times 10^{-3}$ M. This low affinity of enzyme and substrate is in good agreement with the findings of Wood and Gunsalus who report a value of 8.5×10^{-3} M for the enzyme from *S. faecalis*.

Although pyridoxal phosphate is a cofactor requirement of purified alanine racemase from *S. faecalis* (Wood and Gunsalus, 1951), the addition of this substance was not stimulatory in our system. Using static assays in order to rule out any possible contribution of pyridoxal phosphate from the D-amino acid oxidase test system, a $Q_{alanine}$ of 84 was obtained with spores alone, and a value of 85 on addition of 20 μg pyridoxal phosphate. Either the enzyme has no such cofactor requirement, or the cofactor is already present in excess in the spores. To test the possibility that the low activity evidenced by vegetative cells represents merely a lack of cofactor, pyridoxal phosphate was added to an assay of the vegetative cells of *B. terminalis*. Whereas vegetative cells alone gave a Q value of 0.6, the assay with 20 μg added pyridoxal phosphate was 0.8, an insignificant difference. Since in no case was the need for the cofactor pyridoxal phosphate indicated, the addition of this substance was omitted from the assay procedure.

Of the amino acids tested, no other racemization activity has been detected in spores. L-Leucine, L-hydroxyproline, L-histidine, L-lysine, L-proline, L-cysteine, and D-glutamic acid[2] are evidently not suitable substrates for the enzyme system of spores.

In connection with studies on the activation of spore germination by "heat shock" procedures, to be reported in a subsequent paper, some data on the thermal stability of alanine racemase in spores were obtained. An inactivation curve run at 65 C shows that in 15 min, which is the period normally used for heat shock at that temperature and which results in 100 per cent activation, no appreciable enzyme activity is lost. After 120 min heating at 80 C the activity drops to 97 per cent of its original value.

DISCUSSION

The demonstration of a highly active alanine racemase affords positive evidence for the existence of active enzymes in spores. This has been a matter of some controversy since Virtanen and Pulkki (1933) failed to find enzymatic activity (i.e., catalase, polypeptidase, and glucose fermentation) in spores and intimated that this might be the key to their thermostability. Earlier reports ascribing catalytic activity to spores by Effront (1917), Cook (1931), and especially Ruehle (1923) were due almost certainly to the use of insufficiently washed spore suspensions and inadequate techniques for studying enzymatic activity. Ruehle's spore suspensions were washed only twice and gave positive tests for catalase and gelatinase. Holst and Sturtevant (1940) found that suspensions of *B. larvae* spores could be washed and centrifuged three times without losing all associated proteolytic activity but that further washings yielded spore suspensions showing no ability to hydrolyze casein or liquefy gelatin even after prolonged incubation. Spores also could be "cleaned" of extraneous enzymes by heating to 93.5 C for 15 min. Such spores were viable but showed no proteolytic activity. Similarly we have found that suspensions of *B. terminalis* spores contain catalase sometimes even after 7 or 8 washings with distilled water. But with persistence a spore preparation can be washed free of all measurable catalase and remain 100 per cent viable. It seems safe to assume that such elutable enzymes are not intrinsically associated with spores but are contaminating protein from the autolysed mother cells.

The situation with respect to respiratory catalysts in resting spores is equally uncertain. Tarr (1933) was evidently following the respiration of germinating spores. Keilin and Hartree (1947) report endogenous respiration in spores which had been heat treated to destroy contaminating enzymes, but this activity could have been due to germinating spores. Recently Crook (1952) has used a microrespirometer to settle the question of the existence of endogenous respiration in resting spores. He used a well cleaned

[2] We wish to thank Mr. S. A. Narrod and Dr. W. A. Wood for performing the assay for glutamic acid racemase.

spore suspension, but the respiration he found either with or without glucose was barely above the limit of detection of his sensitive apparatus.

A recent paper by Spencer and Powell (1952) has appeared which attributes diaphorase activity to spores of *B. subtilis* and *B. megaterium*. Suspensions of spores broken by shaking in a Mickle disintegrator were capable of oxidizing reduced coenzyme I with the reduction of methylene blue. These authors also analyzed spores and vegetative cells for flavin-adenine dinucleotide and found that spores contain about a third as much of this substance as vegetative cells with no increase immediately evident on germination. It is interesting that no D-amino acid oxidase, L-amino acid oxidase, nor xanthine oxidase could be detected in the spores. Up to 30 mg dry wt of *B. subtilis* spores in a micro-Warburg respirometer showed no endogenous respiration, but after heating at 60 C for 15 min the spores did oxidize glucose with a Q_{O_2} of 0.42. They suggested that this low rate conceivably might be due to a low percentage of germinating spores, escaping detection by staining.

Alanine racemase thus represents the most active enzyme so far detected in resting spores. The much higher concentration of racemase in spores than in the vegetative cells from which the spores are derived suggests that this enzyme may have some unique function in spore physiology. The fact, that in forming D-alanine from L-alanine a powerful and specific inhibitor of germination is generated, may be significant. It is interesting to speculate on the possibility that this negative feed-back type of regulation of spore germination is an effective survival mechanism, ensuring that the supply of viable spores will not all be used up by a single set of favorable circumstances.

Finally, the discovery of an active enzyme in spores provides us with a much sought-after tool to facilitate the comparison of the effect of heat and other protein denaturants on the proteins of spores vs. vegetative cells. Until we found a protein whose denaturation could be measured, i.e., an enzyme whose inactivation could be followed in association with spores and vegetative cells, such a comparison was impossible. Work on this aspect of the problem is in progress.

ACKNOWLEDGMENTS

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SUMMARY

Free spores of aerobic sporeforming bacilli contain a highly active enzyme which catalyzes the conversion of L-alanine to D-alanine. The properties of this enzyme are similar to the alanine racemase found in *Streptococcus faecalis* by Wood and Gunsalus.

The content of alanine racemase in spores is 3 to 16 times greater than in vegetative cells. The increase in enzyme of a culture follows closely the percentage sporulation.

The pH optimum of alanine racemase in spores is close to 8.6. The enzyme is specific for alanine, of the substances tried, and has a low affinity for the substrate.

The racemization of alanine by spores does not require pyridoxal phosphate and is remarkably heat stable.

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