

STUDIES ON THE ASPARTIC ACID DECARBOXYLASE OF RHIZOBIUM TRIFOLII

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Virtanen and Laine (1937) were the first to suggest the existence of an aspartic acid decarboxylase in *Rhizobium trifolii*, by the isolation of *beta*-alanine from a 47-day fermentation mash containing aspartic acid. Shive and Macow (1946), working with hydroxyaspartic acid as a competitive analogue for aspartic acid in the metabolism of *Escherichia coli*, found that the addition of this compound to the growth medium inhibited the proliferation of the organism and that this inhibition was completely prevented by the addition of aspartic acid, pantothenic acid, or *beta*-alanine. They concluded from these results that aspartic acid was the precursor of *beta*-alanine, which in turn becomes part of the pantothenic acid molecule.

It has been reported recently (Mardeshev *et al.*, 1948, 1949) by several Russian workers that they have found an organism, *Pseudomycobacterium*, that is capable of decarboxylating aspartic acid, as measured by CO₂ production in a Warburg vessel. They further state that the enzyme has been prepared in a cell-free state (Mardeshev *et al.*, 1948) and that the coenzyme of the decarboxylase has been found to be pyridoxine phosphate (Mardeshev *et al.*, 1949).

It has more recently been reported from this laboratory (Billen and Lichstein, 1949) that washed cell suspensions of *Rhizobium trifolii* contain an aspartic acid decarboxylase as determined by microbiological analysis for the end product of the decarboxylation, *beta*-alanine. Because of the importance of such an enzyme, further studies have been made and the data are herewith presented.

EXPERIMENTAL METHODS

Preparation of active cell suspensions. The organism employed in most of the work was a strain of *R. trifolii* carried as a departmental stock culture. This organism, as well as others to be reported later, was grown in a medium that consisted of the following: 10 grams each of yeast extract, tryptone, and glucose; 1 gram of DL-aspartic acid; and 1,000 ml of distilled water.

A 1-ml inoculum of a 24-hour culture of *R. trifolii* was introduced into 200 ml of the growth medium. The flask was then incubated on a mechanical shaker at room temperature, which fluctuated between 24 and 30 C. The mechanical shaker provided a convenient means of aeration and made possible the harvest of large quantities of cells. After 16 to 18 hours' incubation (final pH 4.5 to 5.5), the cells were harvested by centrifugation, suspended in distilled water equal in volume to the growth medium, and recentrifuged. This washing was repeated twice to remove as much endogenous material as possible, and the washed cells were re-

suspended in distilled water. The concentration was determined in terms of bacterial nitrogen per ml of suspension by measuring turbidity in a Klett-Summerson photoelectric colorimeter and converting into terms of nitrogen content by the use of a previously standardized table.

Experimental procedure. In general an experiment was run in the following manner: A series of pyrex tubes (13 by 100 mm) was placed in a metal rack. To each tube in the series was added 1 ml of 0.5 M phosphate buffer of the desired pH, 0.1 ml of 0.1 M L-aspartic acid, and finally the cell suspension. This was then brought to a constant volume of 2 ml by the addition of distilled water and the tubes were placed in a water bath at the desired temperature. The reaction was allowed to proceed for a specific time and then stopped by immersing the tubes in boiling water for 5 minutes. Controls were run in the same manner without added

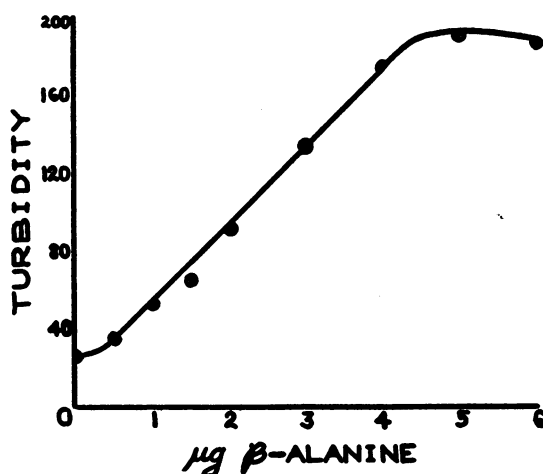


Figure 1. Response of *Saccharomyces fragilis* to beta-alanine (modified Snell medium; 24 hours; 30 C).

aspartic acid. The tubes were then centrifuged and aliquots of the supernatant assayed for beta-alanine.

Assay for beta-alanine. The aspartic acid decarboxylase activity of the washed cells was determined by microbiological assay for beta-alanine in the supernatant. A synthetic medium described by Snell *et al.* (1940) was employed first. The response by the assay organism was found to be limited to a narrow range of approximately 0.1 to 0.2 µg of beta-alanine in this medium. The addition of nicotinic acid (100 µg), para-aminobenzoic acid (20 µg), and 50 ml of a 10 per cent vitamin-free acid-hydrolyzed casein to 950 ml of the assay medium, followed by adjustment to pH 5.5, increased the range of assayable beta-alanine 30- to 40-fold (figure 1).

The organism employed for the assay was *Saccharomyces fragilis* (ATCC 2360). This organism was grown for 24 hours in a medium composed of 1 per cent each of yeast extract, tryptone, and glucose; harvested by centrifugation; washed twice with distilled water; and resuspended in distilled water. The assay medium was inoculated with washed cells until a faint turbidity was noted. Five ml of

inoculated assay medium were added to aliquots of the supernatant made to a volume of 2 ml with distilled water that had been steamed in an autoclave at 2 to 5 pounds steam pressure for 10 minutes and cooled. The assays were made in pyrex glass tubes (24 by 200 mm) with cotton plugs inserted before steaming to avoid condensation of fluid into their contents. The tubes were then incubated at 30 C and turbidity measurements made after 20 to 24 hours in a Klett-Summerson photoelectric colorimeter. The concentration of *beta*-alanine in the supernatants was calculated from standard curves included in each experiment.

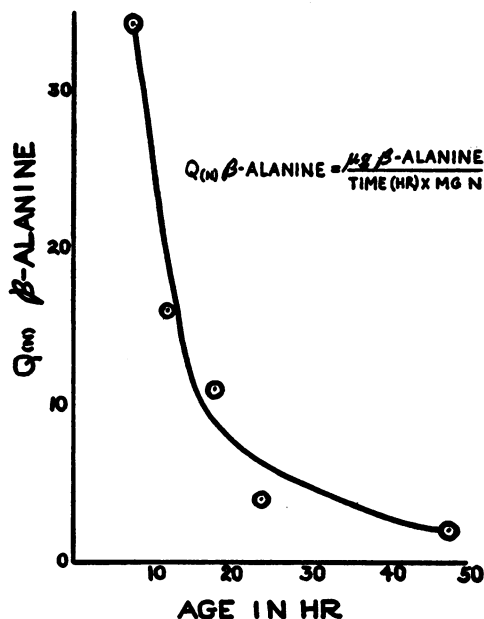


Figure 2. Effect of age of culture on enzyme activity (30 minutes; 30 C; 0.5 M phosphate buffer, pH 6).

EXPERIMENTAL RESULTS

The results of several experiments indicated little effect of temperature on enzyme production when the organisms were grown between 22 to 38 C. The $Q_{(N) \text{ beta-alanine}}$ ¹ for several temperatures was 5.5 at 22 C, 6.5 at 30 C, and 6.4 at 38 C.

The age of the culture had marked effect on enzyme activity. It was found that the activity was greatest during the logarithmic phase (8 to 12 hours) and decreased rapidly with time (figure 2). In this experiment 1 ml of a 12-hour culture, aerated on the mechanical shaker, was used as the inoculum in order to obtain significant growth from the 8- and 12-hour cultures.

An experiment was made using 0.5 M phosphate buffer of varying pH values from 3 to 9 to determine the effect of hydrogen ion concentration on enzymatic activity. The final pH of each tube was determined after the cell suspension had been added. The reaction was stopped by boiling, and the pH of the supernatant

¹ $Q_{(N) \text{ beta-alanine}} = \mu\text{g beta-alanine produced per mg bacteria nitrogen per hour.}$

was adjusted by the addition of 1 ml of 1 M phthalate buffer (pH 5) in order to equalize the pH of all the supernatants, thus preventing possible adverse effects of widely varying pH's on the results of the assay. The enzyme shows optimal activity between pH 5.2 and 6.2, with activity falling off sharply on either side of this range (figure 3). This is in agreement with the general range of optimal pH for the other known amino acid decarboxylases, which vary between 2.5 and 6.0 (Gale, 1946).

The optimal temperature of reaction is approximately 46 C, the increment of activity with temperature being at a maximum at this point (figure 4). It will

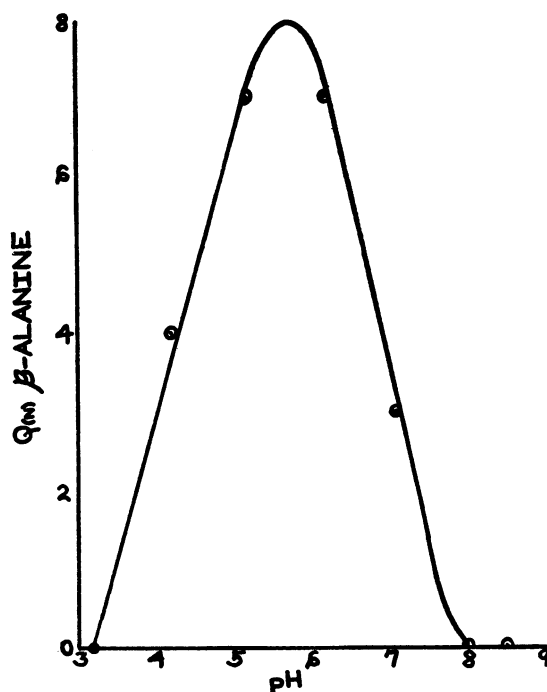


Figure 3. Effect of pH on enzyme activity (120 minutes; 35 C; 0.65 mg bacterial nitrogen per tube; 0.5 M phosphate buffer).

be noted that the optimal temperature is considerably higher than that of growth, namely, 30 C. The probability exists that the enzyme deteriorates rather rapidly at the higher temperatures (54 and 46 C) and that over a longer period of time the total decarboxylation would be greater if incubated at a lower temperature.

An experiment was performed to determine whether aspartic acid decarboxylation goes to completion as determined by complete conversion to *beta*-alanine. One series of tubes contained a relatively large concentration of substrate (1,330 μ g) and another series of tubes contained a limited concentration (66 μ g). It was found that the reaction proceeds normally for 5 hours in the presence of large amounts of substrate, whereas in the tubes containing the smaller concentration, the activity stops after 2 hours (figure 5) and the rate of decarboxylation is con-

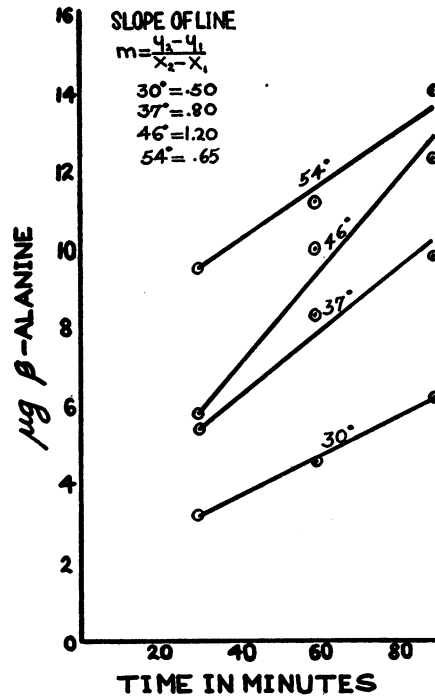


Figure 4. Effect of temperature on enzyme activity (1.4 mg bacterial nitrogen per tube; 0.5 M phosphate buffer, pH 5.5).

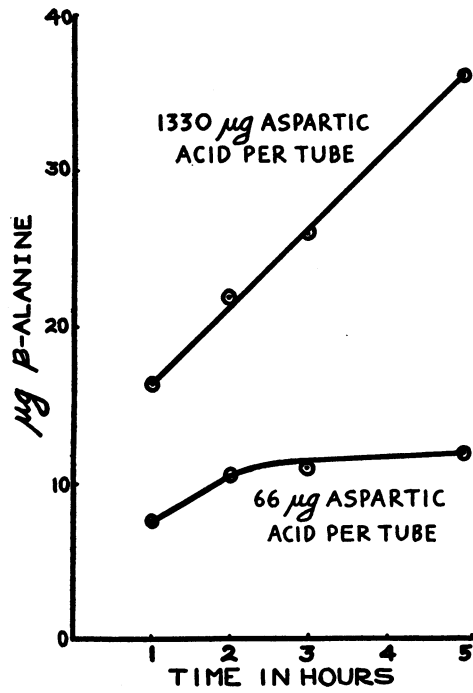


Figure 5. Effect of substrate concentration on enzyme activity (0.5 mg bacterial nitrogen per tube; 36 C; 0.5 M phosphate buffer, pH 5).

siderably lower than in the former case. It is evident from this that the rate of activity is dependent on substrate concentration and is probably influenced by the law of mass action over a certain range of substrate-enzyme concentration. It is also to be noted that only 15 to 20 per cent of the substrate can be accounted for as *beta*-alanine when the reaction is stopped after 2 hours. A possible explanation for this lies in the competition for the substrate by other enzyme systems. These include decarboxylation to *alpha*-alanine by a change in the site of decarboxylation, deamination to fumaric acid, and transamination. This problem is under investigation in our laboratory at the present time. Preliminary data suggest that this organism contains an aspartic acid deaminase that is active at pH 5.5.

Several other organisms were tested for their decarboxylase activity. The only other organism studied that showed activity was *Escherichia coli* (Texas). A $Q_{(N)}^{\text{beta-alanine}}$ of 1 was obtained using cells from a 22-hour culture. This is approximately half of the activity of *R. trifolii* under similar conditions. *Lactobacillus bulgaricus*, *Streptococcus faecalis* R, *Corynebacterium diphtheriae*, *Saccharomyces fragilis*, *Micrococcus pyogenes* var. *albus*, and *Aerobacter aerogenes* gave negative results.

DISCUSSION

The results of these experiments provide evidence for the presence of an enzymatic aspartic acid decarboxylase system in *R. trifolii* and *E. coli* (Texas), although in small quantity. The importance of such an enzyme system can readily be appreciated when it is realized that *beta*-alanine is part of the pantothenic acid molecule. If aspartic acid decarboxylase is concerned with the supply of adequate amounts of *beta*-alanine for the synthesis of pantothenic acid, the low activity found should not be surprising as pantothenic acid is needed in relatively small amounts by those organisms that require it for growth.

The low activity might also present an explanation for the negative results obtained in our laboratory when CO₂ production was employed as a measure of activity. Assuming, from the results of the work reported, that approximately 20 to 30 μg of *beta*-alanine would be produced in a reaction under optimal conditions over a period of 2 hours, by calculation it would mean that 10 to 15 μg or 5 to 8 μl of CO₂ would be produced. This quantity, produced over a period of 2 hours, would not be detectable by the conventional manometric measurements.

SUMMARY

A modified medium for the microbiological assay of *beta*-alanine is described. *Rhizobium trifolii* was found to contain an enzyme system capable of decarboxylating aspartic acid to *beta*-alanine, as determined by microbiological assay for this compound.

A study of the properties of the system was undertaken with the following results: (1) The age of the culture markedly effected enzyme activity, the greatest activity occurring during the logarithmic phase of growth. (2) No marked effect of growth temperature between 22 and 38 C was noted on enzyme activity. (3)

The optimum temperature of activity of washed cells appeared to be 46 C. The calculated slope of *beta*-alanine production versus time was greatest at this temperature. (4) The enzyme shows a pH optimum between pH 5.2 and 6.2 in phosphate buffer, with the activity falling off sharply on either side of this range. (5) The production of *beta*-alanine is markedly influenced by substrate concentration.

The enzyme was found to be present in *Escherichia coli* (Texas) and *Rhizobium trifolii*, but absent under the conditions of the experiments in *Lactobacillus bulgaricus*, *Streptococcus faecalis* R, *Corynebacterium diphtheriae*, *Saccharomyces fragilis*, *Micrococcus pyogenes* var. *albus*, and *Aerobacter aerogenes*.

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