ROLE OF AMINO ACIDS IN GERMINATION OF BACILLUS LICHENIFORMIS SPORES. I. UPTAKE OF RADIOACTIVE AMINO ACIDS BY GERMINATING AND DORMANT SPORES

J. H. MARTIN AND W. J. HARPER
Department of Dairy Technology, The Ohio State University, Columbus

ABSTRACT

The uptake of amino acids by Bacillus licheniformis spores was investigated using radioactive L-alanine, L-valine, DL-glutamic acid, and glycine.

The uptake of L-alanine and L-valine was rapid in germinating spores, with the major portion of the radioactivity being incorporated into the cytoplasm during and following germination, rather than in the spore coat. Approximately 80% of the radioactivity in the cytoplasm of spores germinated with radioactive L-alanine was soluble in cold trichloroacetic acid, and was not associated with the ribosomal fraction of the spore cytoplasm.

Radioactive glycine and DL-glutamic acid entered the dormant spore readily, even though germination did not occur, and became almost entirely associated with the cytoplasmic portion. The uptake of glycine in the dormant spore increased continuously during 2 hr of incubation; whereas, the uptake of DL-glutamic acid was independent of incubation time. When L-alanine was added as a spore germinant, along with radioactive glycine, the incorporation of radioactive glycine occurred immediately, with essentially no increase during germination and post-germinative development. When radioactive DL-glutamic acid was present with L-alanine, the uptake of DL-glutamic acid increased dramatically following germination.

Since a previous paper presented results which showed that L-alanine, L-cysteine, and L-valine were capable of inducing germination of spores of Bacillus licheniformis (2), this study was undertaken to explore the role of these amino acids in the germination phenomenon.

EXPERIMENTAL PROCEDURE

Methods for preparation of the spore suspension and for determining amino acid-induced germination have been described previously (2).

Preparation of radioactive amino acids. Radioactive L-alanine and L-valine, uniformly labeled with carbon 14, and radioactive glycine-1-C14 and DL-glutamic acid-1-C14 were obtained from the Volk Radiochemical Corporation. Stock solutions of the radioactive amino acids were prepared in 2 N HCl in concentrations sufficient to yield 0.1 μc/ml in the germination medium (M/100 phosphate buffer, pH 7.2).

Received for publication September 13, 1964.

Preparation of cellular fractions. Thirty milliliters of the spore suspension were removed at intervals during incubation and centrifuged (3-5 C) at 15,000 × g for 15 min to remove all the spores. The spores were resuspended in 30 ml of sterile distilled water and ruptured by combining with an equal volume of glass beads (Minnesota Mining Co., No. 130-5005) and mixing in a Gifford-Wood Mini-Mill at a speed of 22,000 rpm for 1 hr at 2-5 C. Over 95% of the spores were ruptured by this treatment, as determined by phase-contrast microscopy, and by the number of viable spores which survived.

The glass beads were removed from the spore fractions by suction filtration, using Whatman No. 42 filter paper. The filtrate was centrifuged at 15,000 × g for 15 min to separate the cytoplasm and the spore coats. The supernatant contained the cytoplasmic fraction. The sedimented spore coats were resuspended in 10 ml of sterile distilled water.

Determination of radioactivity. The radioactivity (cpm./0.5 ml) of these fractions was determined with a gas-flow counter (Nuclear-Chicago, Model D47) in conjunction with a decade sealer (Nuclear-Chicago, Model 181A). The sealer was equipped with an automatic sample changer and recorder, and 1,000 counts
were recorded for each sample to minimize the counting error. The counts were corrected for background activity.

Separation of TCA-soluble components of the cytoplasm. Ten-milliliter volumes of the cytoplasmic fraction were treated with 2 ml of 50% trichloroacetic acid (TCA). This mixture was centrifuged at 15,000 × g and the TCA removed from the supernatant by five washings with ethyl ether. Nitrogen was bubbled through the samples for 15 min to remove the ether, and the radioactivity remaining in the TCA-soluble supernatant was determined.

Determination of the radioactivity of the soluble and particulate fractions of the cytoplasm. Distribution of the radioactivity in the spore cytoplasm between the soluble and particulate material was determined following ultracentrifugation. A 13.5-ml aliquot of the cytoplasmic fraction was centrifuged at 104,500 × g for 1 hr in a Spinco Preparative Ultracentrifuge. The sediment, containing the particulate components, was resuspended in 1.0 ml of distilled water. The supernatant was assumed to contain the soluble material. The radioactivity of the supernatant and sediment was determined as previously described.

RESULTS

Radioactive L-alanine in spore germination. When radioactive L-alanine was used to germinate *B. licheniformis* spores, the germination percentages were 0, 85.9, 98.7, 99.4, and 99.6% after 0, 5, 15, 30, and 120 min of incubation, respectively. Microscopic examination revealed swelling of the germinated spores after 30 min of incubation.

The uptake and distribution of radioactive L-alanine in the spore coats, cytoplasm, and TCA-soluble cytoplasm are illustrated in Figure 1. The data reveal that the uptake of radioactivity increased continuously during the entire incubation time. Initially, the uptake was slow in the cytoplasm and TCA-soluble fraction, and practically nil in the spore coats. There was a marked increase in the rate of uptake after 15 min of incubation, when about 99% of the spores had germinated. The radioactive content of the cytoplasm and TCA-soluble cytoplasm was greater than that of the spore coats at all times, and the rate of uptake in these fractions was also much greater than in the spore coats. The spore coats did not display any significant uptake of radioactivity until after germination was complete and the swelling stage of post-germinative development was observed. The TCA-soluble cytoplasm

<table>
<thead>
<tr>
<th>Incubation time at 35°C (min)</th>
<th>Percentage germination</th>
<th>Cytoplasm (cpm/0.5 ml)</th>
<th>Spore coats (cpm/0.5 ml)</th>
<th>Percentage germination</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>117</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>128</td>
<td>2</td>
<td>82.7</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>286</td>
<td>4</td>
<td>98.9</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
<td>288</td>
<td>4</td>
<td>99.1</td>
</tr>
<tr>
<td>120</td>
<td>0</td>
<td>304</td>
<td>5</td>
<td>99.3</td>
</tr>
</tbody>
</table>

**TABLE 1**

Uptake and distribution of radioactive glycine during germination induced by L-alanine.

<table>
<thead>
<tr>
<th>Incubation time at 35°C (min)</th>
<th>Percentage germination</th>
<th>Cytoplasm (cpm/0.5 ml)</th>
<th>Spore coats (cpm/0.5 ml)</th>
<th>Percentage germination</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>117</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>128</td>
<td>2</td>
<td>82.7</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>286</td>
<td>4</td>
<td>98.9</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
<td>288</td>
<td>4</td>
<td>99.1</td>
</tr>
<tr>
<td>120</td>
<td>0</td>
<td>304</td>
<td>5</td>
<td>99.3</td>
</tr>
</tbody>
</table>
curve reveals that at all periods this fraction contained the major portion of the radioactivity of the cytoplasm.

The radioactivity of the ultracentrifugal supernatants (Figure 2) was essentially the same as for the original supernatants in Figure 1. When the ultracentrifugal supernatants were treated with cold TCA, a small portion of the radioactivity was removed, but again the major portion of the radioactivity remained in TCA-soluble components. The radioactivity associated with the ultracentrifugal sediments, which would include the ribosomal fraction of the cytoplasm, was practically negligible during the entire incubation period.

Radioactive L-valine in spore germination. Attention was focused on the uptake and distribution of C\textsuperscript{14}-L-valine by B. licheniformis spores during germination induced by this amino acid. The germination percentages were 0, 11.9, 70.4, 84.9, and 96.7\% after 0, 5, 15, 30, and 120 min of incubation, respectively. Swelling of some of these spores was observed after 30 min of incubation, but was not extensive until after 60 min.

Data for L-valine (Figure 3) reveal that, as observed with L-alanine, there was a much higher level of radioactivity in the cytoplasm as contrasted to the spore coats, and the rate of uptake was also much greater at all times. L-valine entered the cytoplasm of the spore immediately after it was added to the germination medium. This was followed by a decrease in radioactivity at the 5-min incubation interval and a subsequent rapid increase in uptake for the remainder of the incubation period. A significant increase in the rate of uptake of radioactivity was observed after the 30-min period, at which time 84.9\% of the spores had germinated.

As observed with L-alanine, the level of radioactivity in the spore coat fraction was low in comparison to that of the cytoplasm. Essentially no uptake was observed in the coats during the first 30-min period, and the uptake increased slowly thereafter.

Nonstimulatory amino acids. In another phase of the study, attention was directed to determining the uptake by B. licheniformis spores of amino acids found previously not to stimulate germination (2). For this work, glycine and glutamic acid were used. These radioactive amino acids were added to spore suspensions in 0.1\% concentrations. In one series, non-radioactive L-alanine was also added, to induce germination. Data obtained with C\textsuperscript{14}-glycine are presented in Table 1. Results indicate that this amino acid entered the cytoplasmic portion of dormant spores continuously during the incubation, although no germination occurred. The radioactivity in the cytoplasm increased from 117 cpm/0.5 ml to 304 cpm/0.5 ml during
the 2 hr. In contrast, an extremely small amount of radioactivity was incorporated into the spore coats during this period.

When L-alanine was added to germinate the spores, the uptake of radioactive glycine in the cytoplasm was much greater than in the dormant spores, but the uptake did not follow a consistent pattern during incubation, with values varying from 291 cpm to 168 cpm. This occurred even though germination proceeded at a rapid rate, being essentially complete within 15 min. As with the dormant spores, only slight radioactivity was incorporated into the coats of the germinated spores.

Table 2 contains data on the uptake and distribution of radioactive DL-glutamic acid by dormant and germinating *B. licheniformis* spores. Considerable uptake occurred quickly in the cytoplasm of dormant spores, but the amount varied widely during incubation, and did not yield a consistent trend. As observed in the previous studies, the spore coat contained extremely small quantities of radioactivity.

Results obtained with DL-glutamic acid during spore germination induced by L-alanine were strikingly different from those obtained with glycine. From an initial count of 185 cpm/0.5 ml in the cytoplasmic portion of the spore, the incorporation of radioactive DL-glutamic acid increased for the first 5 min of incubation, remained relatively stable through 30 min, and increased markedly thereafter, reaching a total of 1,125 cpm/0.5 ml after 2 hr. Since approximately 99% of the spores had germinated during the first 30 min of incubation, these data reveal that DL-glutamic acid was utilized rapidly during post-germinative development of the spores. Again, the spore coats exhibited only slight radioactivity in contrast to the cytoplasm, but the concentration increased perceptibly near the end of the incubation.

**DISCUSSION**

Results obtained in this study revealed a rapid uptake of radioactive L-alanine and L-valine in the cytoplasm of germinating *B. licheniformis* spores, with a marked increase in the rate of uptake after germination was complete. Since only a small amount of these amino acids was taken up by the spores during the germination process, it would appear that the initiation of germination by these amino acids may be primarily physical-chemical in nature. The marked increase in the rate of uptake of L-alanine and L-valine during post-germinative development suggests that the germinated spore utilizes these amino acids in an entirely different manner than it does during initiation of germination. Also, the major portion of L-alanine was taken up by the cytoplasm and incorporated into components not removed by treatment with TCA nor by ultracentrifugation, indicating that most of the L-alanine was being utilized metabolically as an energy source instead of being incorporated into cellular protein.

Although breaching of a permeability barrier has been suggested as a possible explanation as to why spores germinate in response to certain chemical compounds (4), a rapid uptake of nonstimulatory amino acids (glycine and DL-glutamic acid) was observed in this study. This supports the conclusion of Black and Gerhardt (1), that selective permeability is not a major factor in spore germination.

The uptake of glycine by dormant spores suggests that the nongerminating spore is either capable of storing or metabolically utilizing glycine. Such utilization of glycine under germinating conditions did not occur. Failure

**TABLE 2**

Uptake and distribution of radioactive DL-glutamic acid during germination induced by L-alanine

<table>
<thead>
<tr>
<th>Incubation time at 35 C</th>
<th>Percentage germination</th>
<th>Radioactivity (epm/0.5 ml) in</th>
<th>Spore coats</th>
<th>Radioactivity (epm/0.5 ml) in</th>
<th>Spore coats</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 0</td>
<td>175 3</td>
<td>0 185 4</td>
<td>136 30</td>
<td>120 1.1</td>
<td>136 30</td>
</tr>
</tbody>
</table>
of the spores to take up large quantities of glycine following germination may be significant, since O'Connor and Halvorson (3) reported that glycine was a competitive inhibitor of the L-alanine dehydrogenase obtained from spores of B. cereus strain T. In contrast to glycine, dL-glutamic acid was apparently utilized metabolically during post-germinative development.

REFERENCES