FACTORS AFFECTING THE FORMATION OF ACETYLMETHYLCARBINOL BY LACTOBACILLUS ARABINOSUS

ALBERT G. MOAT AND HERMAN C. LICHSTEIN

Department of Bacteriology and Immunology, University of Minnesota, Minneapolis, Minnesota

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During the past few years there has been considerable renewed interest in the production of acetylmethylcarbinol by microorganisms. Rowatt (1951), Nossal (1951, 1952), Dolin and Gunsalus (1951), Juni (1950, 1952), and Watt and Werkman (1951) have investigated acetylmethylcarbinol formation in representative species of the genera Lactobacillus, Streptococcus, Aerobacter, Bacillus, Serratia, and Micrococcus. These workers found that two moles of carbon dioxide and one mole of acetylmethylcarbinol were produced for every two moles of pyruvate utilized although the conditions necessary for optimal acetylmethylcarbinol production varied considerably from one organism to another.

In regard to the mechanism of acetylmethylcarbinol formation, Krapfiz (1948) demonstrated the decarboxylation of α-acetolactate by bacterial preparations capable of acetylmethylcarbinol formation from pyruvate. Juni (1950, 1952) has extended this work by the resolution of the enzymatic components of the acetylmethylcarbinol forming system of Aerobacter into two fractions. One fraction acted upon pyruvate to form α-acetolactate and carbon dioxide, whereas the other decarboxylated α-acetolactate to form acetylmethylcarbinol. Cocarboxylase was found to be required for the first but not the second reaction. No other cofactor requirements were reported for this system.

This paper deals with investigations of the conditions necessary for the formation and activity of the enzyme system in Lactobacillus arabinosus, strain 17-3, responsible for acetylmethylcarbinol production from pyruvate.

MATERIALS AND METHODS

The medium of Skeggs and Wright (1944), modified by the addition of 0.5 μg of folic acid per 100 ml, was used to obtain cells for enzyme studies. Glucose was autoclaved separately as a 20 per cent solution and added aseptically prior to inoculation. Resting cellular suspensions were obtained by growing L. arabinosus, strain 17-5, in this medium at 32°C for 24 hours or longer using a 1 per cent inoculum from a yeast extract-casitone broth culture. Harvesting was accomplished by centrifugation, the cells washed twice with distilled water, and resuspended to the desired turbidity. Cell nitrogen was determined from a standard curve relating turbidity to mg of cell nitrogen.

Dried cells were prepared by growing the organism as described and drying under vacuum in thin layers over “drierite” at room temperature. Such dried cell preparations were found to have a high degree of acetylmethylcarbinol producing activity from pyruvate and remained active for considerable periods of time (over 4 months) if stored under vacuum at room temperature.

Carbon dioxide production was measured manometrically by conventional Warburg techniques. Acetylmethylcarbinol was determined by the method of Westerfeld (1945), pyruvic acid by the method of Friedemann and Haugen (1943), and lactic acid by the method of Barker and Summerson (1941). After manometric measurement of the carbon dioxide produced, the reaction was stopped by the addition of 1 ml of 10 N sulfuric acid. The contents of the cup were transferred to graduated test tubes and brought to a total volume of 10 ml by washing the cups with small volumes of distilled water. Cells were removed by centrifugation and aliquots of the supernatant fluid used for chemical analyses.

The sample of α-acetolactic acid used in these experiments was kindly supplied by Dr. D. J. O’Kane of the University of Pennsylvania as N-methyl-acetoxyethyl acetoadetate. This compound was hydrolyzed in excess alkali for 30 minutes at room temperature and neutralized before use. To ascertain the amount of acetylmethylcarbinol produced from α-acetolactate acid, the determination was carried out immediately.
without acidification because of the lability of α-acetolactic acid in strong acid solution. Boiled cell controls were included in order to correct for the spontaneous decarboxylation of α-acetolactic acid.

EXPERIMENTAL RESULTS AND DISCUSSION

Products of pyruvate dissimilation. Fermentation balances on resting cells at pH 3.8 are presented in table 1. It is manifest that the usual 2:1 ratio of CO₂:acetylmethylcarbinol was obtained when pyruvate was used as substrate. In addition, CO₂ and acetylmethylcarbinol accounted for essentially all of the pyruvate dissimilated under these conditions.

pH optimum for the formation and activity of the acetylmethylcarbinol system. Alteration of the pH of the reaction mixture through a range of pH values from 2.7 to 4.6 using 0.03 M phosphate buffer indicated that the optimum pH for CO₂ and acetylmethylcarbinol production by L. arabinosus was quite critical. It is apparent (figure 1) that there was a sharp peak of maximum activity between pH 3.0 and 4.0, with activity falling off sharply below or above this range. Identical results were obtained with dried cells of this organism.

In the course of conducting the initial experiments on this enzyme system it was noted that greater activity was obtained if harvesting of the cells was prolonged until the final growth pH had reached that of maximum enzyme activity. Consequently, studies were conducted to determine the effect of pH on the production of this enzyme system. Incubation of cultures at 32 °C and harvesting of the cells prior to reaching a growth pH of 4.0 resulted in cells having little or no pyruvate dissimilating activity at pH 3.8. Such cells, after incubation at 32 °C in the presence of glucose and small quantities of the growth medium, exhibited a marked ability to produce CO₂ and acetylmethylcarbinol from pyruvate if the initial pH of incubation was the same as that required for optimum enzyme activity. Little or no activity was found when the initial pH of incubation was near neutrality (figure 2). The activity of the cells incubated under the two conditions was determined at pH 3.8. Although turbidity measurements indicated that some growth occurred during the incubation of these cells, it is significant to note that the greatest increase in cell numbers occurred at the initial pH of 6.8 where essentially no increase in enzymatic activity was observed.

<table>
<thead>
<tr>
<th>PYRUVATE UTILIZED</th>
<th>CO₂ PRODUCED</th>
<th>ACETYL-METHYL CARBINOL PRODUCED</th>
<th>CARBON RECOVERY</th>
<th>O/R INDEX</th>
<th>OBSERVED CALCULATED CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>µM</td>
<td>µM</td>
<td>µM</td>
<td>%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.40</td>
<td>2.60</td>
<td>1.27</td>
<td>107</td>
<td>1.05</td>
<td>1.02</td>
</tr>
<tr>
<td>3.60</td>
<td>3.62</td>
<td>1.84</td>
<td>95</td>
<td>0.96</td>
<td>0.98</td>
</tr>
<tr>
<td>9.09</td>
<td>7.36</td>
<td>4.13</td>
<td>86</td>
<td>0.85</td>
<td>0.89</td>
</tr>
<tr>
<td>3.86</td>
<td>3.48</td>
<td>1.48</td>
<td>101</td>
<td>1.02</td>
<td>1.18</td>
</tr>
</tbody>
</table>

Cells grown in experimental medium for 20 hr at 32 °C. Experiments conducted in Warburg flasks using 0.03 M phosphate buffer, pH 3.8, 0.0033 M sodium pyruvate as substrate, 0.00005 M MnCl₂, 1 mg cell N per cup. Gas phase, N₂. Temp = 37 C.

The effect of glucose on carbon dioxide and acetylmethylcarbinol production. The stimulation of CO₂ and acetylmethylcarbinol production by catalytic amounts of glucose reported by Rowatt
(1951) and Nossal (1952) for lactobacilli was confirmed in this laboratory. It was of interest

TABLE 2
Effect of glucose on pyruvate dissimilation by Lactobacillus arabinosus, strain 17-5

<table>
<thead>
<tr>
<th>ADDITIONS</th>
<th>QCO₂ (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Washed cellular</td>
</tr>
<tr>
<td>None</td>
<td>110</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>360</td>
</tr>
<tr>
<td>Pyruvate + 1 μM glucose</td>
<td>1,160</td>
</tr>
<tr>
<td>Pyruvate + 1 mg adenosine triphosphate</td>
<td>—</td>
</tr>
</tbody>
</table>

0.2 mg cell N per cup for washed cellular suspension, 10 mg dried cells per cup. 0.0033 M sodium pyruvate as substrate. Other conditions as for table 1.

Figure 2. Effect of pH on the formation of the acetylactylcarbinol producing system in Lactobacillus arabinosus, strain 17-5.

Cells grown in experimental medium for 16 hr at 32 C. After harvesting they were incubated at pH 3.8 or 6.8 for the time periods indicated and tested for activity at pH 3.8. Other conditions as for table 1.

to determine the nature of this effect. The possibility of a permeability barrier to pyruvate was considered. The data obtained (table 2) indicate that a permeability barrier to pyruvate in intact cells of this organism is the major factor in glucose stimulation at this pH. This permeability barrier was not removed completely by desiccation until dried cell preparations had been stored for a period of time. After storage under vacuum, the dried cells retained their ability to produce CO₂ and acetylactylcarbinol from pyruvate, but the rate of this reaction was no longer stimu-

![Graph showing effect of pH on CO₂ production](https://example.com/graph.png)

Figure 3. Decarboxylation of α-acetolactic acid (AAL) by vacuum dried cells of Lactobacillus arabinosus, strain 17-5.

Cells harvested from experimental medium after 48 hr growth at 32 C, dried by vacuum desiccation, and stored under vacuum at room temperature for 2 months. 20 mg dried cells per cup, 0.0133 M DL-α-acetolactic acid as substrate. Control for spontaneous decarboxylation = cells boiled 10 minutes to inactivate enzymes. Other conditions as for table 1.

lated by glucose. The replacement of glucose by adenosine triphosphate in freshly prepared vacuum dried cells suggests the need of energy for membrane penetration.

Mechanism of acetylactylcarbinol formation. Since Juni (1952) had demonstrated that other bacteria produce acetylactylcarbinol from pyruvate via the decarboxylation of α-acetolactic acid, it was of interest to determine if L. arabinosus could utilize this compound. Vacuum dried
cells of this organism were shown to decarboxylate \( \alpha \)-acetolactic acid to one mole of \( \text{CO}_2 \) and one mole of acetylmethylcarbinol (figure 3). The enzyme acted only upon the natural isomer of \( \alpha \)-acetolactic acid. No attempt was made, however, to demonstrate this compound as a normal intermediate in the production of acetylmethylcarbinol by this organism.

**SUMMARY**

*Lactobacillus arabinosus*, strain 17-5, has been found to convert pyruvate quantitatively to \( \text{CO}_2 \) and acetylmethylcarbinol. The optimum pH for this enzyme system is between pH 3 and 4, and the pH of the culture medium during growth of the organism must reach that required for optimum activity before maximum enzyme formation occurs.

Vacuum dried cells retain their ability to convert pyruvate to \( \text{CO}_2 \) and acetylmethylcarbinol under the same conditions found for resting cells. Such preparations decarboxylate \( \alpha \)-acetolactic acid to \( \text{CO}_2 \) and acetylmethylcarbinol.

The reported stimulation of this system by glucose apparently is due to a permeability barrier to pyruvate in intact cells of *L. arabinosus*.

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