ANAEROBIC DISSIMILATION OF C¹⁴-LABELED GLUCOSE AND FRUCTOSE BY PSEUDOMONAS LINDNERI*

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Ethanol formation during the heterolactic fermentation of glucose by *Leuconostoc mesenteroides* has been shown to proceed via a pathway differing from the Embden-Meyerhof-Parnas glycolysis scheme (1–3). Ethanol arising during yeast fermentation via the Embden-Meyerhof-Parnas scheme is derived from carbon atoms 1 (aldehyde carbon), 2, 5, and 6 of glucose (4), the methyl group representing carbons 1 and 6, while in the heterolactic fermentation only carbon atoms 2 and 3 give rise to ethanol, the methyl group representing carbon atom 2. It was, therefore, of interest to determine whether ethanol formation in other bacterial fermentations involved the Embden-Meyerhof-Parnas or other pathways.

*Pseudomonas lindneri* (*Zymomonas lindneri, Termobacterium mobile*) was investigated because of its high yield of ethanol during glucose or fructose fermentation. This fermentation has been studied by Kluyver and Hoppenbrouwers (5) using growing cultures, and their results may be expressed in the following equation:

\[
\text{Glucose} \rightarrow 0.2 \text{lactic acid} + 1.8 \text{ethanol} + 1.8 \text{CO}_2
\]

In the present work the products of glucose fermentation by resting cell suspensions of *P. lindneri* were determined and found to be in essential agreement with those previously observed with growing cultures.

Preliminary data for labeling in the ethanol and CO₂ arising from the fermentation of C¹⁴-labeled glucose by *P. lindneri* (6) indicated that the anaerobic dissimilation might proceed via a mechanism similar to that in *Leuconostoc*. However, the present experiments, which represent a more detailed investigation, indicate that the anaerobic dissimilation of glucose and fructose by *P. lindneri* involves a route of ethanol formation differing from either the usual Embden-Meyerhof-Parnas pathway or the hexose monophosphate pathway in the heterolactic bacteria. The results suggest a close resemblance to the aerobic dissimilatory mechanism proposed for *Pseudomonas saccharophila* by Entner and Doudoroff (7).

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Methods

Bacteriological—A culture of P. lindneri, obtained from Professor A. J. Kluyver, was used throughout these experiments. The culture was maintained by monthly transfer on a stock agar containing, per 100 ml., 1 gm. each of glucose, tryptone, and yeast extract, 0.5 gm. of KH$_2$PO$_4$, and 2 ml. of salt mixture (per 100 ml., 0.8 gm. of MgSO$_4$.7H$_2$O, 0.16 gm. of MnSO$_4$.4H$_2$O, and 0.04 gm. each of NaCl and FeSO$_4$.7H$_2$O), and 2 gm. of agar. To obtain cells for carbon balance and tracer studies, the culture was grown in the same medium less agar. This medium required no pH adjustment and was sterilized at 120° for 15 minutes. For the experiments involving fructose, the glucose in the medium was replaced by fructose.

For inoculum a transfer was made from stock agar to a test-tube containing approximately 10 ml. of growth medium; the tube was incubated 20 to 24 hours at 30°, and 0.05 ml. was transferred to a second tube of medium, which after 12 to 14 hours at 30° was used to inoculate 100 ml. of growth medium (1 per cent inoculum). After 12 hours, the cells were collected by centrifugation and washed once with 10 ml. of water, suspended in water, and the concentration was adjusted to 20 mg. of dry weight per ml.

Chemical—In the balance studies, carbon dioxide formation in a nitrogen atmosphere was measured at 30° in the Warburg respirometer. After gas formation had ceased, the contents of the Warburg vessel were acidified with 0.1 ml. of 5 N H$_2$SO$_4$. After removal of cells by centrifugation, the supernatant solution and washings were made to volume and aliquots removed for the determination of ethanol (8), glucose (9), and lactic acid (10). The analyses indicated that in all the experiments the total added hexose was fermented.

The CO$_2$ released during the fermentation of the C$^{14}$-hexose was collected in alkaline and assayed as previously described (3). The cells were removed by centrifugation and washed with two 2 ml. portions of water. The supernatant liquid plus washings was adjusted to pH 8 and the ethyl alcohol collected by distillation. The alcohol was either converted to CO$_2$ by persulfate oxidation (11) or oxidized to acetic acid with 0.5 gm. of K$_2$Cr$_2$O$_7$ in 4 N H$_2$SO$_4$. The acetic acid was degraded by the method of Phares (12). Since the amount of lactic acid formed was too small for assay of radioactivity, it was discarded.

All C$^{14}$ samples were assayed as barium carbonate with a methane flow β proportional counter (13).

The glucose-1-C$^{14}$, glucose-2-C$^{14}$, and fructose-1,6-C$^{14}$ were kindly furnished by Dr. H. Isbell of the National Bureau of Standards. The glucose-6-C$^{14}$ was kindly supplied by Dr. J. C. Sowden, Washington University, St. Louis. The glucose-3,4-C$^{14}$ was prepared from rat liver glycogen (14).
Results

The products of glucose fermentation by resting cell suspensions of \textit{P. lindneri} are shown in Table I. The yields of products in these experiments are essentially the same as those obtained with growing cultures (5).

\begin{table}[h]
\centering
\begin{tabular}{lcc}
\hline
\textbf{Products of Glucose Fermentation by Resting Cell Suspensions of \textit{P. lindneri}} \\
\text{Per Warburg vessel, 0.3 ml. of 0.6 M phosphate buffer, pH 6.0, 0.1 ml. of 0.1 M glucose, 0.5 ml. of cell suspension (10 mg. of dry weight), water to 3 ml.; atmosphere N\textsubscript{2}; 30\textdegree C.} \\
\hline
\textbf{Glucose used, \textmu M} & 10 & 10 \\
\textbf{Products formed} & & \\
\text{CO\textsubscript{2}, \textmu M} & 18.0 & 18.0 \\
\text{Ethanol, \textmu M} & 19.6 & 19.6 \\
\text{Lactate, \textmu M} & 0.51 & 0.51 \\
\text{Carbon recovery, \%} & 97.9 & 97.9 \\
\text{Oxidation-reduction balance} & 0.92 & 0.92 \\
\hline
\end{tabular}
\caption{Products of Glucose Fermentation by Resting Cell Suspensions of \textit{P. lindneri}}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{lcccc}
\hline
\textbf{Experiment No.} & \textbf{Carbohydrate} & \textbf{CO\textsubscript{2}} & \textbf{Ethanol} \\
 & \textbf{Label} & \textbf{Specific activity} & \textbf{Total activity} & \textbf{Specific activity} & \textbf{Total activity} & \textbf{Specific activity} & \textbf{Total activity} \\
\hline
1 & Glucose-1-\textsuperscript{14}C & 20.8 & 15.0 & 76.5 & 15.8 & 0.15 & 0.05 \\
2 & \textsuperscript{14}C & 20.8 & 15.0 & 74.5 & 15.6 & 0.20 & 0.09 \\
3 & Glucose-6-\textsuperscript{14}C & 88.0 & 18.0 & 0.0 & 0.0 & 119 & 14.1 \\
4 & Glucose-3,4-\textsuperscript{14}C & 20.5 & 14.8 & 34.0 & 7.5 & 14.8 & 6.5 \\
5 & \textsuperscript{14}C & 22.0 & 15.3 & 46.7 & 9.1 & \ & \ \\
6 & \textsuperscript{14}C & 22.0 & 15.3 & 42.6 & 8.8 & \ & \ \\
7 & Fructose-1,6-\textsuperscript{14}C & 21.0 & 15.0 & 30.5 & 7.4 & 11.2 & 6.8 \\
8 & \textsuperscript{14}C & 21.0 & 15.0 & 28.6 & 7.2 & 12.6 & 6.9 \\
\hline
\end{tabular}
\caption{\textsuperscript{14}C-Glucose and \textsuperscript{14}C-Fructose Fermentation with \textit{P. lindneri}; Total Oxidation of Ethanol}
\end{table}

During fermentation of glucose-1-\textsuperscript{14}C, resting cell suspensions of \textit{P. lindneri} quantitatively converted the \textsuperscript{14}C to \textsuperscript{14}CO\textsubscript{2}, with approximately the specific activity predicted from carbon balances and preliminary observations. Ethanol contained only traces of the isotope (Table II). On the other hand, glucose-6-\textsuperscript{14}C yielded unlabeled CO\textsubscript{2} and labeled ethanol. The \textsuperscript{14}C
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of either glucose-3,4-C\textsuperscript{14} or fructose-1,6-C\textsuperscript{14} appears in both CO\textsubscript{2} and ethanol, with about equal distribution.

Table III indicates that carbon 2 of glucose is converted to the carbinol carbon of ethanol, while carbon 6 serves as a precursor of the methyl carbon of ethanol. The data also show that the tracer in glucose-3,4-C\textsuperscript{14} appears in both CO\textsubscript{2} and the methyl carbon of ethanol.

**Table III**

*C\textsuperscript{14}-Glucose Fermentation with *P. lindneri*; Degradation of Ethanol

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Glucose Label</th>
<th>Specific activity</th>
<th>Total activity</th>
<th>CO\textsubscript{2} Specific activity</th>
<th>Total activity</th>
<th>Ethanol CH\textsubscript{2}OH Specific activity</th>
<th>Total activity</th>
<th>CH\textsubscript{3} Specific activity</th>
<th>Total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-2-C\textsuperscript{14}</td>
<td>17.3</td>
<td>62.0</td>
<td>0.0</td>
<td>0.0</td>
<td>39.2</td>
<td>48.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>-3,4-C\textsuperscript{14}</td>
<td>14.0</td>
<td>15.0</td>
<td>25.4</td>
<td>7.3</td>
<td>0.68</td>
<td>0.24</td>
<td>15.5</td>
<td>6.2</td>
</tr>
<tr>
<td>3</td>
<td>-6-C\textsuperscript{14}</td>
<td>88.0</td>
<td>12.0</td>
<td>3.2</td>
<td>0.2</td>
<td>183</td>
<td>10.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**DISCUSSION**

Since the results with fructose-1,6-C\textsuperscript{14} were the same as those obtained with glucose-1-C\textsuperscript{14} and glucose-6-C\textsuperscript{14}, the organism apparently metabolizes both sugars via the same pathway.

Carbon dioxide is derived from carbon 1 (aldehyde carbon) and, presumably, carbon 4 of glucose. The alcohol is derived from carbon atoms 2 and 6 and, by elimination, carbons 3 and 5 of glucose. The degradation of the alcohol further demonstrates that carbon 6 and presumably carbon 3 form methyl carbons, while carbon 2 and, by inference, carbon 5 form carbinol carbons.

The data show that *P. lindneri* produces 1.8 moles of CO\textsubscript{2}, 1.9 moles of ethanol, and 0.5 mole of lactic acid per mole of glucose fermented. Even though the stoichiometry of hexose fermentation by *P. lindneri* approaches a yeast type fermentation, it is evident that the pathway is not that of classical glycolysis. If the latter were operative in *P. lindneri*, C\textsuperscript{14} from position 1 of glucose would be found in the methyl carbon of ethanol and not in the CO\textsubscript{2}. Although *P. lindneri* converts carbon 1 of glucose to CO\textsubscript{2} and carbon atoms 2 and 3 to ethanol, as in the *Leuconostoc* fermentation, the two pathways exhibit a marked difference. Since carbon 2 is converted
to the carbinol carbon by *P. lindneri*, and to the methyl carbon by *Leuconostoc*.

A consideration of the position of C\(^{14}\) in the CO\(_2\) and ethanol derived from the variously labeled sugars suggests that the *P. lindneri* fermentation may be closely related to the pathway of glucose oxidation of *P. saccharophila* recently demonstrated by Entner and Doudoroff (7). These workers demonstrated that *P. saccharophila* oxidizes glucose to 2 molecules of pyruvic acid, the carboxyl carbon arising from glucose carbon atoms 1 and 4. Pyruvic acid formed in such a manner could give rise to CO\(_2\) and ethanol labeled as found in the *P. lindneri* fermentation.

Based on the tracer data obtained in these experiments and on well-known pathways of carbohydrate dissimilation, at least two possible schemes of hexose degradation can be suggested for *P. lindneri*: (1) an anaerobic hexose monophosphate pathway in which glucose-6-phosphate is converted to CO\(_2\) (C-1) and a pentose phosphate (15), followed by a C\(_5\)-C\(_3\) cleavage of the pentose (16). The C\(_2\)-fragment (C-2 and C-3 of glucose) serves as precursor for one of the ethanol molecules, while the C\(_3\) moiety, presumably triose phosphate, is then converted to CO\(_2\) (C-4) and ethanol (C-5 and C-6) via the known enzyme system, which includes pyruvic carboxylase (6); (2) an anaerobic hexose monophosphate mechanism which converts glucose-6-phosphate to 6-phosphogluconate, followed by dehydration and cleavage of the latter (15) into pyruvic acid (C-1, C-2, and C-3 of glucose) and triose phosphate, both of which may be converted to ethanol and CO\(_2\) by known enzyme systems.

It is evident that lactic acid formed from glucose-1-C\(^{14}\) by the *P. saccharophila* pathway would be radioactive provided the 2 pyruvate molecules produced from each glucose could be equilibrated, in contrast to lactic acid produced by a pathway involving a cleavage of 6-phosphogluconate into C\(^{14}\)O\(_2\) and unlabeled pentose phosphate. However, sufficient labeled sugar was not available to test this point.

Characterization of the enzymes involved is in progress and should permit a definitive choice of the operative mechanism.

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**SUMMARY**

The anaerobic dissimilation of glucose by resting suspensions of *Pseudomonas lindneri* yields 1.8 moles of CO\(_2\), 1.9 moles of ethanol, and a trace of lactic acid per mole of hexose fermented. The distribution of C\(^{14}\) in
the fermentation products has been investigated with glucose-1-C\textsuperscript{14}, glucose-2-C\textsuperscript{14}, glucose-3,4-C\textsuperscript{14}, glucose-6-C\textsuperscript{14}, and fructose-1,6-C\textsuperscript{14}. The results obtained have led to the following conclusions:

1. CO\textsubscript{2} arises from carbon atoms 1 (aldehyde carbon) and 4 of glucose.
2. Carbon atoms 2, 3, 5, and 6 of glucose enter ethanol; carbon atoms 2 and 5 become the carbinol carbons of ethanol.
3. Fructose and glucose were fermented by the same pathway, since fructose-1,6-C\textsuperscript{14} yielded C\textsuperscript{14}O\textsubscript{2} and methyl-labeled ethanol which was similar to that found with glucose-1-C\textsuperscript{14} and glucose-6-C\textsuperscript{14}.
4. The conversion of glucose to CO\textsubscript{2} and ethanol by P. lindneri involves a mechanism differing, at least in part, from both the Embden-Meyerhof-Parnas glycolytic scheme and the Leuconostoc mesenteroides hexose monophosphate pathway. A consideration of the tracer data suggests a mechanism similar to the aerobic pathway of glucose oxidation in Pseudomonas saccharophila.

BIBLIOGRAPHY

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