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Excretion of Glyoxylate, Pyruvate and α-Ketoglutarate during Oxidation of Acetate

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Oxidations of several organic acids were investigated with the whole cells of coli-aerogenes grown aerobically on either citrate or acetate-medium. A considerable amount of α-ketoglutarate was obtained from acetate and lactate, when the washed cells were aerobically incubated in the absence of semicarbazide. This C₅-ketonic acid accumulated only in a very small amount during the oxidation of citrate by a citrate-grown cell. When the whole cells oxidized acetate in the presence of semicarbazide, a remarkable excretion of either pyruvate or glyoxylate occurred, under which conditions the accumulation of α-ketoglutarate was extremely decreased. The yields of pyruvate and glyoxylate from acetate were found to vary with the kinds of the bacterial strains. The oxidations of both acetate and glycollate were also investigated with the dried cells. Mechanisms for microbial oxidation of acetate were propounded and discussed.

INTRODUCTION

In the previous papers¹⁻⁵), the authors have studied the chemical pathway of α-ketoglutaric acid-fermentation in coli-aerogenes bacteria. The mechanism for the bacterial respiration has also been studied, and it has been indicated that an enzyme, isocitritase catalyzing the degradation of isocitrate to glyoxylate and succinate may play an important role in the terminal oxidation of substrate-carbon⁶⁻⁷). Further investigations have revealed that a glyoxylic acid reductase-glycollic acid oxidase system may function in the respiration of microorganisms⁸⁻¹⁰). The present paper deals with the excretion of ketonic acids including pyruvate, glyoxylate and α-ketoglutarate occurring during the oxidation of substrates, especially acetate, by the whole cells of coli-aerogenes.

EXPERIMENTAL AND RESULTS

Methods. The organisms used : Escherichia coli (strain G-2); Aerobacter aerogenes (strain B-2); Aerobacter cloacae (strain A-c). These organisms were grown on a shaker at 30°C for 20-26 hours in the media containing 2.0% either Na-citrate or Na-acetate, 1.0% peptone, 0.5% yeast extract, 0.1% KH₂PO₄, 0.3% (NH₄)₂SO₄, 0.05% MgSO₄·7H₂O and 0.2% NaCl. Washed cells : Cells were harvested by a centrifuge, washed with and suspended in distilled water. Dried cells : Washed cells were dried by an electric fan at room temperature for 5-7 hours.

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Analytical procedures were performed by methods mentioned in the previous paper⁹.

Oxidation of several organic acids by washed cells. The washed cells of coli-aerogenes grown on either citrate or acetate were incubated with several substrates. The typical results of experiments are shown in Tables 1 and 2. α-Ketoglutarate was obtained in a remarkable amount as the oxidation-products of substrates such as; acetate, lactate, pyruvate and C₄-dicarboxylic acids.

Table 1. Oxidation of acetate and citrate by washed cells of strain B-2 of A. aerogenes grown on citrate.

<table>
<thead>
<tr>
<th>Vessel No.</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrates added</td>
<td>Acetate</td>
<td>Acetate</td>
<td>Citrate</td>
<td>Citrate plus acetate</td>
<td>DL-Lactate plus acetate</td>
</tr>
<tr>
<td>Inhibitors (M)</td>
<td>0</td>
<td>Semicarbazide</td>
<td>0</td>
<td>0</td>
<td>α,α'-Dipyridyl</td>
</tr>
<tr>
<td>Products (μmoles):</td>
<td>Glyoxylate</td>
<td>0</td>
<td>8.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Pyruvate</td>
<td>0</td>
<td>1.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>α-Ketoglutarate</td>
<td>17.8</td>
<td>3.2</td>
<td>3.5</td>
<td>21.0</td>
</tr>
</tbody>
</table>

Each flask contained 1.5 m moles phosphate buffer, pH 7.4, 40 μmoles MgSO₄, 2.0 m moles substrates (Na-salts), the indicated amounts of inhibitors and 110 mg (as dry weight) washed cells of A. aerogenes; total volume, 20 ml; 6 hours' incubation at 30°C on a shaker.

Table 2. Oxidation of acetate and citrate by washed cells of strain A-c of A. cloacae grown on citrate.

<table>
<thead>
<tr>
<th>Vessel No.</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrates added</td>
<td>Acetate</td>
<td>Acetate</td>
<td>Citrate</td>
<td>Citrate</td>
<td>Citrate</td>
</tr>
<tr>
<td>Conditions</td>
<td>Anaerobically</td>
<td>Aerobically</td>
<td>Aerobically</td>
<td>Aerobically</td>
<td></td>
</tr>
<tr>
<td>Semicarbazide added (M)</td>
<td>1×10⁻¹</td>
<td>1×10⁻¹</td>
<td>1×10⁻¹</td>
<td>1×10⁻¹</td>
<td></td>
</tr>
<tr>
<td>Products (μmoles):</td>
<td>Glyoxylate</td>
<td>0</td>
<td>0</td>
<td>3.0</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>Pyruvate</td>
<td>0</td>
<td>0</td>
<td>36.8</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>α-Ketoglutarate</td>
<td>0</td>
<td>35.7</td>
<td>5.8</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Each flask contained 1.5 m moles phosphate buffer, pH 7.4 40 μmoles MgSO₄, 2.0 m moles substrates (Na-salts), the indicated amounts of semicarbazide and 185 mg (as dry weight) washed cells of A. cloacae; total volume, 20 ml; aerobically, on a shaker; anaerobically, Thunberg tube in vacuo; 4 hours' incubation at 30°C.

However, the formation of α-ketoglutarate was strongly inhibited in the presence of semicarbazide or α,α'-dipridyridyl. On the other hand, the cells formed a large quantity of pyruvate from C₄-dicarboxylic acids such as succinate when incubated aerobically in the presence of semicarbazide.

Oxidation of acetate. In the previous papers of this series, the effect of semicarbazide has been investigated on both the anaerobic and aerobic degradation of C₆-tricarboxylic acids by the bacteria. In the present paper, the bacterial oxidation of acetate was carried out under the presence or absence of semicarbazide. From the results of experiment shown in Tables 1-3, it has been found
Table 3. Oxidation of acetate by washed cells of *coli-aerogenes* grown on acetate.

<table>
<thead>
<tr>
<th>Bacteria used</th>
<th>A-c strain of <em>A. cloacae</em></th>
<th>G-2 strain of <em>E. coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Semicarbazide added (M)</td>
<td>0</td>
<td>$1 \times 10^{-1}$</td>
</tr>
<tr>
<td>Products (µmoles):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\alpha$-Ketoglutarate</td>
<td>50.2</td>
<td>Trace</td>
</tr>
<tr>
<td>Glyoxylate</td>
<td>0</td>
<td>2.9</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0</td>
<td>12.5</td>
</tr>
</tbody>
</table>

Each flask contained 1.5 m moles phosphate buffer, pH 7.4, 40 µmoles MgSO$_4$, 1.5 m moles Na-acetate, indicated amounts of semicarbazide and 100 mg (as dry weight) washed cells of the bacteria; total volume 15 ml; 4 hours’ incubation at 30°C on shaker.

Table 4. Oxidation of acetate and glycollate by dried cells of *coli-aerogenes*.

<table>
<thead>
<tr>
<th>Bacterial strains and growth-media</th>
<th>Dried cells of B-2 strain of <em>A. aerogenes</em> grown on citrate</th>
<th>Dried cells of G-2 strain of <em>E. coli</em> grown on acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrates added</td>
<td>0 (Endogenous)</td>
<td>Acetate</td>
</tr>
<tr>
<td>Glyoxylate</td>
<td>Trace</td>
<td>11.5</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>Trace</td>
<td>7.5</td>
</tr>
<tr>
<td>$\alpha$-Ketoglutarate</td>
<td>Trace</td>
<td>Trace</td>
</tr>
</tbody>
</table>

Each flask contained 2 m moles phosphate buffer, pH 7.4, 50 µmoles MgSO$_4$, 2 m moles semicarbazide, 200 mg dried cells of indicated bacteria and 500 µmoles substrates (Na-salts); total volume, 20 ml; 4 hours’ incubation at 30°C on shaker.

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that the washed cells are able to excrete three kinds of ketonic acids including glyoxylate, pyruvate and $\alpha$-ketoglutarate when they oxidize acetate in the presence of semicarbazide. Under anaerobic conditions, little or no formation of ketonic acids took place, as shown in Table 2. No ketonic acids were also formed in a control without acetate.

Paper chromatography of the 2:4-dinitrophenylhydrazones showed that $\alpha$-ketoglutarate was the sole ketonic acid produced during acetate oxidation in the absence of added semicarbazide, while a considerable amount of either pyruvate or glyoxylate was obtainable in the presence of the trapping agent, under which conditions $\alpha$-ketoglutarate was strikingly decreased. The amounts of pyruvate and glyoxylate excreted were moreover observed to vary with the kinds of the bacterial strains: using B-2 strain of *A. aerogenes*, glyoxylate was detected in higher yield, whereas using A-c strain of *A. cloacae*, pyruvate detected in much higher yield. Table 4 shows the oxidation of acetate and glycollate by the dried cells. It will be seen that the dried cell preparations possess the ability of producing glyoxylate and pyruvate from acetate.

The preparations also oxidized glycollate to yield glyoxylate under same conditions, indicating that glycollic oxidase was contained in the preparation. Properties of the bacterial glycollic oxidase have already been reported in the previous papers.$^{8,10}$
Subsequently, $^{14}$CO$_2$ was added to the reaction mixture and the incorporation of radioactivity into glyoxylate and pyruvate measured. Reaction mixture contained 2.5 mmole phosphate buffer, pH 7.8, 40 μmoles MgSO$_4$, 500 μmoles Na-acetate, 210 mg (as dry weight) washed cells of B-2 strain of *A. aerogenes* grown on acetate, 2 mmoles semicarbazide and 40 μmoles Na$^{14}$CO$_3$-Na$_2^{14}$CO$_3$ (400,000 c.p.m.); total volume, 20 ml in 500 ml-shaking flask with a rubber plug; incubation for 3 hours at 30°C on a shaker.

After incubation, ketonic acids were obtained in the following yields: 3.0 μmoles of pyruvate and 3.5 μmoles of glyoxylate. However, little or no incorporation of $^{14}$CO$_2$ into these ketonic acids occurred. Thus, it appears that CO$_2$-fixation reaction is not concerned in the formation of both glyoxylate and pyruvate from acetate. Tables 5–7 show the effect of reagents on the oxidation of acetate. The yield of glyoxylate appeared to decrease in the presence of arsenite. Antibiotics including chloramphenicol and tetracycline pro-

Table 5. Effect of inhibitors on the formation of pyruvate and glyoxylate by washed cells of *A. aerogenes* grown on citrate.

<table>
<thead>
<tr>
<th>Inhibitors added (5×10$^{-3} M$)</th>
<th>0</th>
<th>NaF</th>
<th>NaAsO$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate found (μmoles)</td>
<td>1.4</td>
<td>1.9</td>
<td>3.2</td>
</tr>
<tr>
<td>Glyoxylate found (μmoles)</td>
<td>3.4</td>
<td>3.8</td>
<td>Trace</td>
</tr>
</tbody>
</table>

Each flask contained 1.5 m moles phosphate buffer, pH 7.4, 40 μmoles MgSO$_4$, 1.0 m mole Na-acetate, 1.2 m moles semicarbazide, 100 mg (as dry weight) washed cells of strain B-2 of *A. aerogenes* and inhibitors as indicated; total volume 20ml; 2 hours' incubation at 30°C on shaker.

Table 6. Effect of inhibitors on the oxidation of acetate by washed cells of strain G-2 of *E. coli* grown on acetate.

<table>
<thead>
<tr>
<th>Inhibitors added (500 μl/ml)</th>
<th>0</th>
<th>Chloramphenicol</th>
<th>Tetracycline</th>
<th>Lederkyn*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate found (μmoles)</td>
<td>1.56</td>
<td>1.39</td>
<td>2.23</td>
<td>1.70</td>
</tr>
<tr>
<td>Glyoxylate found (μmoles)</td>
<td>2.11</td>
<td>1.56</td>
<td>1.85</td>
<td>1.58</td>
</tr>
</tbody>
</table>

* Lederkyn=3-sulfanil amido-6-methoxypyridazine

Each flask contained 1.5 m moles phosphate buffer, pH 7.4, 40 μmoles MgSO$_4$, 2.0 m moles Na-acetate, 2 m moles semicarbazide, 95mg (as dry weight) washed cells of *E. coli* and inhibitors as indicated; total volume 17ml; 5 hours’ incubation at 30°C on shaker.

Table 7. Inhibiting effect of antibiotics on the formation of α-ketoglutarate from acetate by washed cells of *E. coli* grown on acetate.

<table>
<thead>
<tr>
<th>Antibiotics added (500 μl/ml)</th>
<th>0</th>
<th>Chloramphenicol</th>
<th>Tetracycline</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Ketoglutarate found (μmoles)</td>
<td>48.9</td>
<td>15.7</td>
<td>14.3</td>
</tr>
</tbody>
</table>

Each flask contained 1.5 m moles phosphate buffer, pH, 7.4, 40 μmoles MgSO$_4$, 2.0 m moles Na-acetate, antibiotics as indicated and 100 mg (as dry weight) washed cells of strain G-2 of *E. coli* ; total volume, 15 ml; 3 hours' incubation in the absence of semicarbazide at 30°C on shaker.
produced no remarkable inhibition on the formation of both glyoxylate and pyruvate but produced a striking inhibition on the formation of \( \alpha \)-ketoglutarate.

**DISCUSSION**

The authors' interest has been directed to the mechanisms for microbial oxidation of acetate. In the case of the organic acids-grown cells of *coli-aerogenes*, it may be considered that some portion of acetate is oxidized via ketonic acids including pyruvate, glyoxylate and \( \alpha \)-ketoglutarate. Isocitritase has been demonstrated in the bacterial cells of *coli-aerogenes* grown with various carbon sources, as already reported in the previous papers[16,17]. Thus, the excretion of both pyruvate and glyoxylate occurring during the bacterial oxidation of acetate, may be explained according to the following Reaction A, in which condensing enzyme[11], malate synthetase[12] and isocitritase are concerned.

Reaction system A:

**Pyruvate excretion by way of glyoxylate cycle**

\[
\text{Acetate} + \text{oxalacetate} \rightarrow \text{tricarboxylic acids} \rightarrow \text{succinate} \rightarrow \text{glyoxylate} \rightarrow \text{pyruvate}
\]

**Glyoxylate excretion**

\[
\text{Acetate} + \text{oxalacetate} \rightarrow \text{tricarboxylic acids} \rightarrow \text{succinate} \rightarrow \text{glyoxylate}
\]

The authors have recently found that the dialyzed cell-free extracts of *E. coli* were able to decompose glyoxylate under such conditions that triphosphopyridine nucleotid was absent, and succinate was anaerobically formed from glyoxylate[13]. Therefore, some portion of acetate may be oxidized to \( \text{CO}_2 \) and \( \text{H}_2\text{O} \) according to the following cycle.

\[
\text{acetate} \rightarrow \text{oxalacetate} \rightarrow \text{citrate} \rightarrow \text{malate} \rightarrow \text{fumarate} \rightarrow \text{succinate} \rightarrow \text{glyoxylate} \rightarrow \text{CO}_2 + \text{H}_2\text{O}
\]

On the other hand, the authors have presented the hypothesis that some portion of acetate may be metabolized to glycollate by way of an oxygenation reaction; \( \text{CH}_3 \) of acetate being oxidized to \( \text{CH}_3\text{OH} \) by an oxygenase action.

\[
\text{CH}_3\text{COOH} + \text{O} \rightarrow \text{CH}_3\text{OH} \rightarrow \text{COOH}
\]

The formation of either glyoxylate or pyruvate from acetate may also be explained according to the following Reaction B.
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Reaction system B:

Glyoxylate excretion

\[
\text{Acetate} \xrightarrow{+O} \text{glycollate} \xrightarrow{-2H} \text{glyoxylate}
\]

Pyruvate excretion

\[
\text{Acetate} \rightarrow \text{glycollate} \rightarrow \text{glyoxylate} \rightarrow \text{malate} \rightarrow \text{pyruvate}
\]

Glycollic acid oxidase is already demonstrated in the bacterial cells of *coli-aerogenes*. This hypothesis implies that the terminal oxidation of two carbon fragments proceeds by way of the following cycle.

\[
\text{acetate} \xrightarrow{+O} \text{glycollate} \rightarrow \text{glyoxylate}
\]

\[
\text{CO}_2 \xrightarrow{+O} \text{pyruvate} \rightarrow \text{oxalacetate} \rightarrow \text{malate} \rightarrow \text{CO}_2 \pm 2\text{H}
\]

Sum: Acetate + 20₂ → 2CO₂ + 2H₂O

In this cycle, acetate is oxidized to CO₂ and H₂O by the combination of the following enzyme reactions: oxygenation of acetate to form glycollate, glycollic oxidase, malate synthetase, C₄-dicarboxylic acid system and pyruvic oxidase.

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(13) H. Katagiri and T. Tochikura, Unpublished.