GLUCONATE OXIDATION BY PSEUDOMONAS FLUORESCENS

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The oxidation of glucose to gluconic acid and then to 2-ketogluconic acid by some species of Pseudomonas has been reported by Lockwood, Tabenkin, and Ward (1). Lockwood and Stodola (2) reported that one species, Pseudomonas fluorescens NRRL B-6, on further oxidation produced α-ketoglutaric acid as a major product. About 0.2 mole of α-ketoglutaric acid was produced per mole of glucose oxidized. Pyruvic and succinic acids were also found in small amounts.1 We are currently interested in establishing this fermentation as an industrial source of α-ketoglutaric acid and have undertaken a detailed study of the chemical mechanism of its formation, with the object of providing information that will be helpful in directing the fermentation toward higher yields with a minimal production of by-products. The great ease with which α-ketoglutaric acid is metabolized has made study of the mechanism highly desirable.

The presence of gluconate and 2-ketogluconate during glucose oxidation by P. fluorescens B-6 suggests that the oxidation proceeds by way of the hypothetical hexose monophosphate shunt reaction sequence (3). The suggestion gains support by the finding of Lockwood and Nelson (4) that this organism will oxidize certain pentoses to corresponding pentonic acids and beyond. In the present work, the oxidation of gluconate by P. fluorescens was studied from the viewpoint of the hexose monophosphate shunt hypothesis. Conditions affecting the accumulation of pyruvate and α-ketoglutarate were investigated, and reaction balances of gluconate oxidation were obtained.

EXPERIMENTAL

Procedure for Conducting and Analyzing Fermentations—Carbon balances, and oxidation-reduction balances if possible, were desired at various stages of oxidation to insure that all major metabolic products would be accounted for. The use of converted suction flasks as fermentation vessels permits the determination of oxygen consumption and carbon dioxide evol...
solution, and also allows frequent aseptic sampling of gases and fermentation liquor during the prolonged incubation period necessary for optimal pyruvate and $\alpha$-ketoglutarate accumulation. The fermentation vessel is shown in Fig. 1.

The medium (195 ml.) is prepared and sterilized in the flask. After inoculating with 5 ml. of 18 to 24 hour inoculum of *P. fluorescens* B-6 propagated in the same medium, the air in the flask is removed by aspiration through the air filter and replaced by oxygen. The oxygen content of the flask is calculated from the flask volume. The flask is placed on a reciprocating shaker and incubated at 27°. Periodically during incubation, a sample of the enclosed gas is removed and analyzed for carbon dioxide and oxygen according to ordinary methods of gas analysis. At
the same time, a sample (usually 10 ml.) of fermentation liquor is withdrawn aseptically. The remaining gas in the flask is replaced by oxygen, and incubation is continued.

The specimen of fermentation liquor is centrifuged. The cells are washed and analyzed for carbon according to Van Slyke and Folch (5). The supernatant liquor is analyzed for bound carbon dioxide by Van Slyke vacuum extraction. 2-Ketogluconate is determined as reducing power with the copper sulfate reagent of Somogyi (6). Pyruvate and α-ketoglutarate are measured simultaneously by a modification of the method of Friedemann and Haugen (7). The initial gluconate concentration is calculated from the weight of potassium gluconate used. Purity of the gluconate is verified by optical rotation and analysis for carbon and hydrogen.

Results

Oxidation of Purified Potassium Gluconate in Synthetic Medium—In order to reduce the amount of extraneous carbon compounds in the fermentations, it was desired to conduct gluconate oxidation in media of as simple composition as possible. Preliminary experiments indicated that oxidation could take place in liquid media containing potassium gluconate as the substrate and the sole source of carbon and ammonium sulfate as the sole source of nitrogen, monobasic potassium phosphate, and magnesium sulfate. No buffer is necessary, since the fermentations usually maintain themselves between pH 6.5 and 7.2. Gluconate oxidation characteristically begins with rapid formation of 2-ketogluconate in essentially stoichiometric yield. As 2-ketogluconate itself is oxidized, carbon dioxide, α-ketoglutarate, and pyruvate appear. However, the rate of fermentation as well as quantities and proportions of products in early experiments was dependent on the specimen of potassium gluconate employed, even though the specimens were essentially pure from an analytical standpoint. It appeared that trace impurities in the gluconate specimens were affecting the course of fermentation.

To investigate this possibility, a quantity of potassium gluconate was purified as thoroughly as possible by repeated recrystallization by using infusorial earth and activated charcoal. When oxidized in a medium containing 50 μM of gluconate, 15 μM of nitrogen (1 gm. atom per mole), 4 μM of phosphate, and 1 μM of magnesium sulfate per ml., this purified gluconate was oxidized rapidly to 2-ketogluconate. Further oxidation was very slow, requiring about 12 days for complete utilization of 2-ketogluconate. Pyruvate accumulated in a yield of 0.7 mole per mole of 2-ketogluconate oxidized. No α-ketoglutarate appeared. About 90 per cent of the initial carbon was accounted for as pyruvate, carbon dioxide, and cells.
Effect of Adding Iron—Molecular oxygen acts as an ultimate hydrogen acceptor in these gluconate oxidations. It was felt that the slow rate of oxidation of purified gluconate beyond 2-ketogluconate might be due to a lack of iron for the synthesis of iron-containing respiration catalysts. The addition of 0.02 μM of iron as ferrous ammonium sulfate per ml. to the above medium resulted in very marked stimulation of gluconate oxidation. The initial 50 μM of gluconate were now oxidized so rapidly that no 2-ketogluconate remained at 24 hours. No pyruvate was produced. Carbon dioxide and cells were the only major products.

Additional potassium gluconate (to give 100 μM per ml.) was supplied to the organisms at 48 hours and was oxidized rapidly to 2-ketogluconate. At first, the latter was oxidized only to carbon dioxide, but at 120 hours α-ketoglutarate and at 160 hours pyruvate began to accumulate. Both substances disappeared when the 2-ketogluconate was exhausted.

The extent of stimulation by iron was unchanged between 0.002 and 0.02 μM of iron per ml., which is the range tested. This range is approximately equivalent to between 0.1 and 1 part per million. The marked stimulation brought about by trace quantities of iron demonstrates the marked dependence of overall metabolism on an adequate hydrogen transport system. It has been noted that in the absence of iron the cells, when centrifuged from the medium, have a very pale yellow color and that the medium contains sufficient pigment of a fluorescent nature to give a marked greenish yellow color. The pigment is almost absent in fermentations containing iron, while the cell crop has a pink flesh-like color. This suggests that in the absence of iron the organisms are able to synthesize an alternative hydrogen transport system having limited capacity.

Two-Stage Gluconate Oxidations—In subsequent experiments under these fermentation conditions, it was found that net cell production, as measured by analyses for cellular carbon, was confined to the very early part of the oxidation processes, the phase in which gluconate was being oxidized completely to carbon dioxide. Accumulation of α-ketoglutarate and pyruvate did not begin until net cell production had ceased. This finding makes it possible to separate the phase of cell proliferation from the phase during which pyruvate and α-ketoglutarate are accumulating, so that carbon and oxidation-reduction balances can be obtained in which cell production is eliminated. This is done by conducting the fermentations in two stages.

Cell proliferation is restricted to Stage I by balancing the amounts of potassium gluconate and nitrogen in the medium so that, except for cell production, carbon dioxide will be the only major product and no significant quantities of metabolic intermediates will accumulate. A gluconate level of 40 μM per ml. is preferred for this stage since the amount
of oxygen supplied in one filling of the flask is adequate, with slight excess, for complete oxidation. Although this small amount of gluconate is usually oxidized to carbon dioxide within 21 hours, Stage I is continued to 48 hours to provide excess time.

Stage II is begun at 50 hours by adding fresh potassium gluconate. This is oxidized rapidly (usually within 16 to 24 hours) to 2-ketogluconate. If the proper level of nitrogen has been supplied in Stage I, \(\alpha\)-ketoglutarate accumulation now begins immediately, and pyruvate accumulation begins thereafter. If 2-ketogluconate disappears from the medium during active Stage II metabolism, the amounts of accumulated pyruvate and \(\alpha\)-ketoglutarate decrease and may disappear also. The cellular carbon level remains unchanged, decreasing slightly in late phases of Stage II, apparently because of autolytic changes. Thus reaction balances of Stage II oxidation need not include cell proliferation.

Effect of Nitrogen Level—In two-stage experiments, the amounts of \(\alpha\)-ketoglutarate and pyruvate accumulating, and to some degree the time at which the accumulations begin, are dependent on the amount of nitrogen furnished for cell proliferation. The optimal nitrogen level is about 7.5 \(\mu\)M per ml. At low nitrogen levels (about 4 \(\mu\)M per ml.) too few cells are produced to promote Stage II oxidation beyond 2-ketogluconate before fermentation ceases. At high levels (about 15 \(\mu\)M per ml.), cell proliferation continues well into Stage II, and the accumulation of pyruvate and \(\alpha\)-ketoglutarate is delayed and decreased.

Fermentation Balances in Gluconate Oxidation during Pyruvate and \(\alpha\)-Ketoglutarate Accumulation—A typical two-stage oxidation experiment in which the nitrogen and Stage I gluconate levels were chosen to bring about accumulation of high pyruvate and \(\alpha\)-ketoglutarate levels, in relation to the 2-ketogluconate consumed, will now be described in greater detail. For Stage I, the medium contained 40 \(\mu\)M of potassium gluconate, 7.5 \(\mu\)M of nitrogen, 0.005 \(\mu\)M of iron as ferrous ammonium sulfate, 4 \(\mu\)M of monobasic potassium phosphate, and 1 \(\mu\)M of magnesium sulfate per ml. For Stage II, sufficient potassium gluconate was added at 50 hours to give a level of 100 \(\mu\)M per ml. The oxidations proceeded as shown in Fig. 2. \(\alpha\)-Ketoglutarate accumulation began within 24 hours and pyruvate accumulation at about 48 hours after Stage II was begun. At 216 hours after inoculation, the pyruvate level decreased and was accompanied by an increase in the \(\alpha\)-ketoglutarate level. This phenomenon has been observed in about half of the oxidations studied; in other cases, the levels of both substances remain approximately constant after reaching their highest point, providing that some 2-ketogluconate is still present.

Balance data for this fermentation are given in Table I. No 2-ketogluconate, pyruvate, or \(\alpha\)-ketoglutarate was present at the end of Stage
I. 27 μm of carbon per ml. remained unaccounted for. This carbon represents soluble carbon such as peptides, products of cell autolysis, pigments, and other unidentified substances. At 216 hours, 23 μm of α-ketoglutarate and 54 μm of pyruvate had accumulated during utilization of 83 μm of 2-ketogluconate, and 18 μm of Stage II carbon remained unaccounted for. At 288 hours, 36 μm of α-ketoglutarate and 42 μm of pyruvate were present from the utilization of 90 μm of 2-ketogluconate, and 12 μm of Stage II carbon were unaccounted for. The relation of these results to contemporary hypotheses of carbohydrate oxidation will be discussed presently.

Search for Reducing Carbohydrates Other Than 2-Ketogluconate—According to the hexose monophosphate shunt oxidation hypothesis, a series of reducing carbohydrates, in phosphorylated form, should occur as intermediates in glucose or gluconate oxidation. If it is assumed, in keeping with this hypothesis, that the extracellular appearance of stoichiometric quantities of 2-ketogluconate in these fermentations results from its excretion by the cell after dephosphorylation of 6-phospho-2-ketogluconate, then it would appear probable that other reducing substances of the shunt sequence may be excreted in the same manner. These substances might be pentose, 2-ketopentonate, tetrose, 2-ketotetronate, and triose.
Several series of experiments were conducted in which reducing substances other than 2-ketogluconate were sought. Two-stage fermentations of the above type, and single stage fermentations of 150 μM of gluconate per ml. at a nitrogen level of 15 μM per ml., with and without iron, were analyzed at the peak of reducing power and at intervals thereafter. In all cases, the optical rotation of the fermentation liquors coincided with that which would be obtained if 2-ketogluconate were the only reducing substance present. The liquors were passed through cation and anion exchange columns. Less than 0.1 per cent of the reducing power appeared in the effluent, indicating that no neutral reducing compounds were present.

**TABLE I**

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Products</th>
<th>After inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Stage I</td>
</tr>
<tr>
<td>Gluconate, μM per ml.</td>
<td></td>
<td>48 hrs.</td>
</tr>
<tr>
<td>Oxygen, μM per ml.</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Cell carbon, μM per ml.</td>
<td></td>
<td>162</td>
</tr>
<tr>
<td>2-Ketogluconate, μM per ml.</td>
<td></td>
<td>40</td>
</tr>
<tr>
<td>α-Ketoglutarate, “” “”</td>
<td>楚</td>
<td>17</td>
</tr>
<tr>
<td>Pyruvate, μM per ml.</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Carbon dioxide, μM per ml.</td>
<td></td>
<td>23</td>
</tr>
<tr>
<td>“” unaccounted for, μM per ml.*</td>
<td></td>
<td>54</td>
</tr>
<tr>
<td>“” accounted for, per cent*</td>
<td></td>
<td>173</td>
</tr>
<tr>
<td>Oxidation-reduction balance, ×100*</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>246</td>
</tr>
<tr>
<td></td>
<td></td>
<td>208</td>
</tr>
<tr>
<td>Stage II</td>
<td></td>
<td>203</td>
</tr>
<tr>
<td>173</td>
<td></td>
<td>264</td>
</tr>
<tr>
<td>208</td>
<td></td>
<td>222</td>
</tr>
<tr>
<td>90</td>
<td></td>
<td>90</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td>98</td>
</tr>
</tbody>
</table>

* These values refer only to substances of the corresponding stage. Carbon unaccounted for in Stage I is disregarded in Stage II values.

It was concluded that the reducing substance detected during gluconate oxidation consists solely of 2-ketogluconate.

**DISCUSSION**

From these experiments, it appears that the accumulation of α-ketoglutarate and pyruvate may represent an abnormal or impaired mode of metabolism in gluconate oxidation by *P. fluorescens* B-6. Normal oxidation apparently constitutes complete oxidation of gluconate to carbon dioxide. Under our experimental conditions, complete oxidation was found early in the fermentation, and especially during the period when cell proliferation was most active. In time, the complete pathway appears to suffer an impairment or series of impairments, so that incompletely oxidized carbon residues, α-ketoglutarate and later pyruvate, accumulate...
successively. It thus seems probable that these substances are intermedi-
ates in the normal pathway, whose further oxidation has been throttled
or blocked entirely, but it is nevertheless possible that their accumula-
tion represents a diversion from the normal metabolic mechanism.

Superficial similarity between glucose oxidation by \textit{P. fluorescens} B-6
and the hypothetical hexose monophosphate shunt reaction sequence,
reviewed by Potter (3), has been mentioned earlier. This similarity is
based on the transient successive appearance of gluconate and 2-keto-
gluconate during glucose oxidation by the bacteria, and the ability of these
organisms to oxidize pentoses to pentonic acids. Evidence for further
conformity to the hypothesis was sought by searching for copper-reducing
carbohydrates other than 2-ketogluconate during fermentation. None
could be detected. This does not, of course, exclude the possibility that
such reducing compounds are formed during gluconate oxidation, since
metabolic intermediates are, in general, retained within the cell. Thus,
direct evidence on the immediate fate of 2-ketogluconate was not ob-
tained.

Information on the fate of 2-ketogluconate in relation to the contempo-
rary shunt hypothesis may be deduced indirectly, however, from the
yields of pyruvate and \( \alpha \)-ketoglutarate obtained. According to the con-
temporary hypothesis, pyruvate would arise from the postulated 3-carbon
residue, and its production would be accompanied by evolution of 3
molecules of carbon dioxide per molecule of 2-ketogluconate oxidized.
\( \alpha \)-Ketoglutarate would be considered to arise during further oxidation of
pyruvate. The latter assumption is supported by the apparent transfor-
mation of pyruvate into \( \alpha \)-ketoglutarate during late phases of our experi-
ments.

However, if only the carbon atoms of pyruvate are involved in \( \alpha \)-ket-
glutarate formation, then pyruvate plus \( \alpha \)-ketoglutarate should account
for less than 3 of the carbon atoms of dissimilated 2-ketogluconate. In-
stead, from data given in Table I at 216 and 288 hours, it is calculated
that 3.34 and 3.40 carbon atoms, respectively, of dissimilated 2-keto-
gluconate are accounted for. We consistently obtain values between 3.2
and 3.6 in calculations of this type. It should be noted that both pyru-
vate and \( \alpha \)-ketoglutarate are transient substances and that their accumula-
tion is dependent on the balance between formation and destruction. It
is possible that \( \alpha \)-ketoglutarate destruction was not blocked completely
in our experiments and that greater amounts than those which accumu-
lated may have been formed.

Several explanations for these findings are possible. If 2-ketogluconate
is dissimilated to form pyruvate and 3 molecules of carbon dioxide as
postulated in the contemporary shunt hypothesis, then some of the carbon
dioxide produced must be taken up in further oxidation of pyruvate. Alternatively, instead of proceeding according to the contemporary hypothesis, 2-ketogluconate dissimilation may yield a 3-carbon or a 2-carbon fragment in addition to pyruvate, and α-ketoglutarate may arise by condensation of pyruvate with these fragments.

Additional experiments now under way should lead to further information on this fermentation mechanism.

**SUMMARY**

1. Conditions for the oxidation of gluconate by *Pseudomonas fluorescens* in synthetic medium, leading to the accumulation of high levels of pyruvate and α-ketoglutarate in relation to the gluconate oxidized, are described. Trace quantities of iron cause marked stimulation of gluconate oxidation. The accumulation of α-ketoglutarate and pyruvate is dependent on the level of nitrogen provided for cell proliferation.

2. The yields of α-ketoglutarate and pyruvate obtained are discussed in relation to contemporary hypotheses of gluconate oxidation suggested by the presence of 2-ketogluconate, α-ketoglutarate, and pyruvate among the oxidation products.

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**BIBLIOGRAPHY**

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