THE OXIDATION OF L-ARABINOSE BY PSEUDOMONAS SACCHAROPHILA*

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The importance of the pentoses in the physiology of plants and animals has been known since ribose and deoxyribose were identified as constituents of nucleic acids. Interest in pentose metabolism has been stimulated in the past few years by the discovery that ribose-5-phosphate and ribulose-5-phosphate were integral parts of the oxidative breakdown of glucose (1). The subject of pentose and pentose phosphate metabolism has been reviewed by Lampen (2) and Horecker (3). It has recently been found that the pentose, ribulose-1,5-diphosphate, may play a fundamental rôle in the process of photosynthesis (4).

A study of pentose metabolism in Pseudomonas saccharophila was undertaken as a result of the discovery that glucose is metabolized in this organism by a mechanism involving neither the Emden-Meyerhof scheme nor the pentose phosphates (5). Preliminary studies revealed that the oxidation of L-arabinose by this organism is strikingly different from that of glucose. In the experiments to be reported, it has been shown that this pentose can be converted to α-ketoglutarate by a series of reactions which appear to involve neither phosphorylated intermediates nor the tricarboxylic acid cycle.

Methods*

Cells of P. saccharophila were grown on a liquid mineral medium described previously (6) with L-arabinose as the sole carbon source. Resting cell suspensions were prepared by centrifuging liquid cultures inoculated 18 to 24 hours previously, washing the cells twice, and resuspending them in 0.033 M phosphate buffer at pH 6.8. Oxygen consumption and CO₂ evolution were determined with the Warburg respirometer (7).

Enzyme preparations were obtained either by grinding centrifuged cells

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1 The following abbreviations are used: DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; Tris, tris(hydroxymethyl)aminomethane-HCl buffer; SAS, saturated solution of ammonium sulfate.
Oxidation of L-arabinose

with levigated alumina (8) or by sonic oscillation (9). The cells for enzyme preparations were grown in 15 liter volumes, harvested in a Sharples supercentrifuge, and stored at \(-20^\circ\) until used. For grinding, the cells were thawed and placed in a mortar previously chilled to approximately \(0^\circ\). The alumina was then added in an amount that was 3 times the weight of the cells. After grinding for 10 minutes, the protein was eluted by gradually adding 3 parts of 0.01 \(\text{M} \) Tris, pH 8.0. For sonic oscillation, the centrifuged cells were suspended in 3 parts of the above buffer, placed in the cup of a Raytheon magnstriction oscillator, 50 watts, 9 kc., model S-102A, and vibrated for 10 minutes while iced water was run through the cooling system. Insoluble materials were removed from the enzyme solution prepared by either method by centrifuging in the International refrigerated centrifuge, model PR-1, at 23,000 \(\times g\) for 20 minutes at \(0^\circ\). Both methods produced enzyme preparations of approximately equal activity. The L-arabinose dehydrogenase was stable and could be stored at least 2 weeks at \(-20^\circ\). The enzyme system oxidizing L-arabonate was unstable and was, therefore, used the same day it was prepared.

Pentose was determined with Bial’s color reagent (10). Pyruvic acid and \(\alpha\)-ketoglutaric acid were determined colorimetrically (11) or by ceric sulfate decarboxylation (12). Succinic acid was determined enzymatically with succinic oxidase (7). DPN and TPN reduction was measured at 340 nm in the Beckman quartz spectrophotometer, model DU, equipped with a constant temperature chamber. Silica glass cells with a 1 cm. light path, holding a volume of over 3 ml., were used in all assays. Lactone was measured by the method of Hestrin (13) with L-arabono-\(\gamma\)-lactone as the standard. Optical rotations were measured in a polariscope at room temperature with a sodium lamp as a light source. The polariscope tube had a 10 cm. light path and held a volume of 1.6 ml.

Radioactivity was determined by drying the samples on copper planchets and counting in a thin window Geiger-Müller counting tube. Decarboxylation of radioactive keto acids with ceric sulfate and the preparation of the resulting CO\(_2\) for counting were performed as described previously (14).

The 2,4-dinitrophenylhydrazones of the keto acids were prepared for chromatography as described by Cavallini et al. (15). The free keto acids were prepared for chromatography by treating a reaction mixture with Dowex 50 in the acid form to remove cations. The Dowex 50 and denatured protein were removed by centrifugation and aliquots of the supernatant solution were applied to the paper.

Radial or “pie plate” chromatography was used for the identification of compounds (16). The solvent used for chromatographing the phenylhydrazones was \(n\)-butanol equilibrated with 3 per cent ammonium hydroxide.
(17). The spots were yellow, but greater differentiation of 2,4-dinitrophenylhydrazones could be obtained by spraying the sheet with an alcoholic solution of 0.5 N NaOH. The 2,4-dinitrophenylhydrazone of pyruvate appeared dark brown, that of glyoxylate had a brick-red color, and the α-ketoglutarate derivative appeared olive-drab. The solvent for the chromatographic separation of the free keto acids was a mixture of 70 per cent ether and 30 per cent benzene, made 3 M with formic acid and then saturated with water (18). The acids were detected by spraying the sheet with brom thymol blue indicator (19).

Ascending chromatography was used for the isolation of radioactive α-ketoglutarate. The reaction mixture, containing from 5 to 10 μmoles of the acid and treated with Dowex 50 to remove cations, was chromatographed in one dimension with the ether-benzene-formic acid-water mixture described above. The acid was located with brom thymol blue indicator and eluted with water. The amount of keto acid in the eluate was determined, and a known volume was applied to a planchet for radioactivity assay.

L-Arabinose-1-C\textsuperscript{14} and L-arabonate-1-C\textsuperscript{14} were obtained as a gift from Dr. A. C. Neish. Totally labeled L-arabinose was generously supplied by Dr. W. Z. Hassid. Potassium L-arabonate, potassium d-arabonate, potassium d-galactonate, and totally labeled potassium L-arabonate were prepared by the oxidation of the corresponding sugars with hypoiodite (20). L-Arabono-γ-lactone was prepared as described by Isbell and Frush (21). Sodium glyoxylate was prepared by the method of Weissbach and Springs (22). Glutaconic acid and L-α-hydroxyglutaric acid were gifts from Dr. M. Rothstein. D-Xylose-1-phosphate was obtained from Dr. W. Z. Hassid. DL-Glyceraldehyde-3-phosphate was obtained from Dr. H. O. L. Fischer. All other chemicals were commercial preparations.

The crude enzyme was fractionated in the following manner to separate the L-arabinose dehydrogenase from the system oxidizing L-arabonate. The extract, in 0.01 M Tris, pH 8.0, was treated with 0.33 volume of SAS. The precipitate was removed by centrifugation and discarded. Then 3.67 volumes of SAS were added to the supernatant solution. The precipitate was collected and dissolved in enough 0.01 M phosphate buffer, pH 6.0, to give a 1 per cent protein solution. To this was added 0.1 volume of 2 per cent solution of protamine sulfate (Lilly) adjusted to pH 5.0 with acetic acid. The precipitate was discarded and the protein in the supernatant solution was precipitated by adding 4 volumes of SAS. The precipitate was dissolved in 0.01 M Tris, pH 8.0, and made up to the original volume of the crude enzyme. This solution was treated with 0.5 volume of SAS. The precipitate was removed by centrifugation, and 0.5 volume of SAS was added to the supernatant solution. The resulting precipitate was col-
lected. The two precipitates were each dissolved in one-third the original volume in 0.01 M Tris, pH 8.0. The lower fraction contained 10 per cent of the original protein, approximately 80 per cent of the L-arabinose dehydrogenase, and no activity toward L-arabonate as measured by DPN reduction at 340 μm. The fraction precipitating between 0.5 and 1.0 volume of SAS contained the remainder of the activity toward L-arabinose and all of the activity toward L-arabonate.

Acetone-dried cells of *Clostridium kluyveri* (kindly supplied by Dr. H. A. Barker) were used as a DPNH oxidase in the first manometric studies on the oxidation of L-arabinose. Later, a supply of a partially purified DPNH oxidase present in the particulate fraction of a cell-free preparation from *Azotobacter vinelandii* was obtained through the generosity of Dr. A. J. Marr. This particulate fraction (which also contains catalase) was used in all subsequent manometric experiments.

**EXPERIMENTAL**

**Experiments with Resting Cell Suspensions**

Resting cell suspensions of *P. saccharophila* grown with L-arabinose as substrate oxidized L-arabinose, sodium pyruvate, and sodium acetate rapidly. Also oxidized, but at a considerably lower rate, were D-galactose and the salts of citric, succinic, malic, fumaric, and α-ketoglutaric acids. There was no increased rate of oxygen uptake above endogenous with D-glucose, D-arabinose, D-xylose, L-xylose, D-ribose, sucrose, and the salts of gluconic, 2-ketogluconic, L-arabonic, and D-arabonic acids. Approximately 60 per cent of the L-arabinose was oxidized having a respiratory quotient of 1.07, the rest being assimilated by the cells. When the cells oxidized L-arabinose-1-Cl4, practically all of the radioactivity (97 per cent) was found in the CO2. Resting cell suspensions, poisoned with 3 × 10^{-4} M iodoacetate, consumed 2.5 moles of oxygen per mole of substrate and produced 2.2 moles of CO2 and 0.9 mole of pyruvic acid. With L-arabinose-1-Cl4 as substrate, 95 per cent of the radioactivity appeared in the CO2.

*Pyruvic acid and α-ketoglutaric acid were identified chromatographically as the 2,4-dinitrophenylhydrazones and by the melting points of these same derivatives. The melting point of the derivative prepared from the reaction mixture poisoned with iodoacetate was 221–223°. When the substance was mixed with the 2,4-dinitrophenylhydrazone of pyruvic acid, the melting point was 222–224°. The melting point of the derivative of pyruvic acid was 222–224°. The melting point of the 2,4-dinitrophenylhydrazone prepared from the reaction mixture poisoned by the arsenite was 227–229°. The melting point of the 2,4-dinitrophenylhydrazone of α-ketoglutaric acid was 228–229°. A mixture of the two melted at 227–229°. All melting points are uncorrected. The keto acid formed in crude enzyme preparations from L-arabonate formed a derivative with 2,4-dinitrophenylhydrazine which was identical in behavior with the derivative of the compound formed by arsenite-poisoned cells.
When the cells were poisoned with 0.01 M arsenite, the respiratory quotient was 0.12, and a keto acid accumulated which was later identified as \(\alpha\)-keto-glutaric acid.\(^2\) As much as 90 per cent of the pentose disappearing could be accounted for as this compound.

The ability of the cells to fix CO\(_2\) was determined. 3 ml. of cells, poisoned with either 0.01 M arsenite or \(3 \times 10^{-4}\) M iodoacetate, were placed in a 10 ml. test-tube, and the tube was closed with a serum bottle stopper. The atmosphere within the test-tube was replaced with one composed of 60 per cent oxygen and 40 per cent nitrogen. By means of a syringe and hypodermic needle, 80 \(\mu\)moles of \(L\)-arabinose and 18 \(\mu\)moles of Na\(_2\)CO\(_3\) (3.5 \(\times\) \(10^4\) c.p.m. per \(\mu\)mole) were added to the poisoned cells to give a final liquid volume of 4.0 ml. The reaction mixture was incubated with shaking at 30° for 4 hours. The reaction was stopped by adding sulfuric acid to give a final concentration of approximately 1 N. The keto acids were isolated from the acidified supernatant fluid by continuous ether extraction for 24 hours. It was found that 65.5 \(\mu\)moles of keto acid had been formed in the arsenite-poisoned system and 62.3 \(\mu\)moles in the iodoacetate-poisoned system. The specific activities were 84.4 and 162 c.p.m. per \(\mu\)mole, respectively. In both cases ceric sulfate degradation of the keto acids showed that all the activity was located in the carboxyl carbon adjacent to the carbonyl group.

The above experiments demonstrate that intact cells can fix CO\(_2\) into the carboxyl groups of pyruvic and \(\alpha\)-ketoglutaric acids. Calculations based on the CO\(_2\) production in the presence of iodoacetate showed that the carboxyl group of pyruvic acid was equilibrated to an extent of approximately 6.0 per cent with the labeled CO\(_2\) present at the end of the experiment. However, the carboxyl group of \(\alpha\)-ketoglutarate formed in the presence of arsenite was equilibrated to an extent of about 1.2 per cent. This suggests that CO\(_2\) is not an intermediate in the major pathway of synthesis of either pyruvate or \(\alpha\)-ketoglutarate from arabinose, a conclusion which was supported by further experiments with cell-free preparations.

**Experiments with Enzyme Preparations**

**Oxidation of \(L\)-Arabinose**—Cell-free preparations, made either by grinding with levigated alumina or by sonic oscillation, reduced DPN but not TPN with \(L\)-arabinose as substrate. Addition of TPN, diphosphothiamine, and uridine triphosphate had no effect upon the rate of reduction, while adenosine triphosphate caused a decrease in the rate. The rate of reduction of DPN increased with increasing pH up to pH 12. At pH 13 the reaction was completely inhibited.

When 0.1 ml. of crude cell-free extract in 0.01 M phosphate buffer at pH 7.6 was mixed with 0.1 ml. of 0.1 M MgCl\(_2\), 0.2 ml. of 0.001 M DPN, 0.1 ml.
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of 0.1 M substrate, and 2.5 ml. of 0.1 M phosphate buffer at pH 7.6 or 0.1 M Tris, pH 8.0, only L-arabinose, L-arabonate, D-galactose, D-glucose-6-phosphate, and D-fructose-6-phosphate served as substrates for DPN reduction. The following compounds did not reduce DPN at a demonstrable rate under these conditions: D-arabinose, D-xylose, L-xylose, D-ribose, D-glucose, D-fructose, D-arabonate, D-glucose-1-phosphate, D-fructose-1-phosphate, D-xylose-1-phosphate, D-fructose-1,6-diphosphate, D,L-glycer-aldehyde-3-phosphate, pyruvate, a-ketoglutarate, isocitrate, citrate, cis-aconitate, glyoxylate, D-galacturonate, D-galactonate, glutaconate, and L-α-hydroxyglutarate. D,L-Glyceraldehyde phosphate could not serve as a substrate even under the conditions described for triosephosphate dehydrogenase (23).

Attempts to demonstrate an arabokinase or a pentose isomerase were unsuccessful.

The crude enzyme preparations were erratic in their ability to reduce methylene blue. Therefore, manometric measurements were made by coupling DPN reduction to oxygen by means of acetone-dried cells of C. kluyveri. It was found that approximately 0.5 mole of oxygen was consumed per mole of substrate (Table I). The respiratory quotient in this experiment was 0.1. Acid production accompanied the oxidation, and the values for oxygen uptake and the respiratory quotient suggested arabonic acid as the probable product of the reaction. L-Arabonic acid is also oxidized by crude preparations, but under different conditions. The oxidation of L-arabonic acid will be discussed in a later section. The oxidation was next carried out with larger amounts of substrate by coupling the oxidation of L-arabinose by DPN to the reduction of acetaldehyde with crystalline alcohol dehydrogenase. The reaction was followed by the amount of base required to neutralize the acid formed. When acid production stopped, the reaction mixture was deproteinized by adding Dowex 50, which also removed the cations. The supernatant fluid was neutralized with potassium hydroxide and concentrated 5-fold in vacuo. Upon addition of 4 volumes of 95 per cent ethyl alcohol, crystals formed. The precipitate was collected, recrystallized, and identified as potassium L-arabonate.

A hydroxamic acid accumulated in reaction mixtures in which hydroxylamine was included as a trapping agent. With crude enzyme at pH 8.0 and a concentration of 0.1 M NH₂OH (adjusted to pH 8.0), 100 μmoles of L-arabinose, DPN, and a purified DPNH oxidase from A. vinelandii, it was found that 78 μmoles of pentose were oxidized in 2 hours and 23.7 μmoles

3 The melting point of the benzimidazole derivative of the enzymatically produced compound was 242-243°. The benzimidazole of L-arabonate melted at 241-242°. A mixture melted at 240-242°. All melting points are uncorrected. The rotation ([α]_D^25) was +49.0°. The specific rotation listed by Moore and Link (20) is +49.2°.
of lactone were trapped. Higher concentrations of hydroxylamine could not be used, since it inhibited oxygen consumption. Under the same conditions with L-arabonate as substrate, there were no oxygen consumption and no lactone formation.

The enzyme catalyzing the oxidation of L-arabinose, which will be referred to as "L-arabinose dehydrogenase" was partially purified by the procedure described under "Methods." This enzyme was specific for DPN. When the oxidation of L-arabinose with L-arabinose dehydrogenase was coupled with oxygen reduction through DPNH oxidase, in the presence of hydroxylamine and at pH 8.0, 63.5 μmoles of hydroxamic acid were formed during the oxidation of 82 μmoles of substrate. At pH 7.0, a very considerable accumulation of lactone was observed in the absence of hydroxylamine. This made it possible to determine the structure of the lactone by computing its molecular rotation, which could be calculated either from the total rotation coupled with a chemical analysis of the reaction mixture, or from the change in optical rotation brought about by the partial alkaline hydrolysis of the lactone. An outline of the procedure and the calculations are reported in Table II. The computed rotation of the lactone was found to be very close to -10,600, which is the reported value for L-arabonoy-lactone (24). The δ-lactone, which is believed to be produced on bromine oxidation, is dextrorotatory (25).

### Table I

**Oxidation of L-Arabirwse and L-Arabonate by Crude Enzyme Preparations**

Oxygen consumption (in micromoles) was measured in a Warburg respirometer at 30°.

<table>
<thead>
<tr>
<th></th>
<th>Experiment A, L-arabinose, 20 μmoles, added</th>
<th>Experiment B, L-arabonate, 10 μmoles, added</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>10.7</td>
<td>5.2</td>
</tr>
<tr>
<td>&quot; minus substrate</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>&quot; DPNH oxidase</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>&quot; enzyme</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>&quot; DPN</td>
<td>0.0</td>
<td>0.2</td>
</tr>
</tbody>
</table>

In Experiment A, the main chamber of the Warburg vessel contained 0.5 ml. of enzyme (aged 3 days at 0°) in 0.01 M Tris, pH 8.0; 0.2 ml. of a 20 per cent suspension of acetone-dried cells of _C. kluyveri_ in 0.01 M Tris, pH 8.0; 0.1 ml. of 0.001 M DPN; 1.0 ml. of 0.1 M phosphate buffer, pH 8.0; in the side arm, 0.2 ml. of 0.1 M L-arabinose; center well, 0.2 ml. of 10 per cent KOH.

In Experiment B, the main chamber contained 0.5 ml. of freshly prepared enzyme in 0.01 M Tris, pH 8.0; 0.1 ml. of 0.1 M potassium L-arabonate; 1.5 ml. of 0.1 M Tris, pH 8.0; in the side arm, 0.1 ml. of 0.005 M DPN; 0.1 ml. of 10 per cent suspension of DPNH oxidase from _A. vinelandii_; center well, 0.2 ml. of 10 per cent KOH.

Oxidation of L-arabinose

Table II

Molecular Rotation of Lactone Produced Enzymatically in Oxidation of L-Arabinose

<table>
<thead>
<tr>
<th>Before incubation</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) L-Arabinose added, μmoles per ml.</td>
<td>67</td>
<td>100</td>
</tr>
<tr>
<td>(b) L-Arabinose remaining, μmoles per ml.</td>
<td>0</td>
<td>21.6</td>
</tr>
<tr>
<td>(c) L-Arabinolactone, μmoles per ml.</td>
<td>34.8</td>
<td>51.6</td>
</tr>
<tr>
<td>(d) L-Arabinonic acid, μmoles per ml.*</td>
<td>32.2</td>
<td>26.8</td>
</tr>
<tr>
<td>(e) Optical rotation of reaction mixture, degrees †</td>
<td>-0.40</td>
<td>-0.26</td>
</tr>
</tbody>
</table>

| After alkaline treatment ‡ | (f) L-Arabinolactone, μmoles per ml. | 5.9 | 22.6 |
| | (g) L-Arabinonic acid, μmoles per ml.* | 61.1 | 55.8 |
| | (h) Optical rotation, degrees † | -0.11 | +0.02 |
| Calculations | (i) Optical rotation due to L-arabinose and L-arabinonic acid after incubation \[(b)(+15,700) + (d)(-1630)\] \(10^{-8}\), degrees | -0.05 | +0.29 |
| | (j) Optical rotation due to L-arabinolactone \((e - i)\) | -0.35 | -0.55 |
| | (k) Change in rotation due to alkaline treatment \((h - e)\) | +0.29 | +0.28 |
| | (l) Change in optical rotation due to increase in arabinonic acid \((g - d)\) \((-1630)10^{-8}\) | -0.05 | -0.05 |
| | (m) Change in rotation due to decrease in L-arabinolactone \((k - l)\) | +0.34 | +0.33 |
| | (n) Molecular rotation of L-arabinolactone computed from data before alkaline treatment \((j/c)10^6\) | -10,100 | -10,700 |
| | (o) Molecular rotation of L-arabinolactone computed from data obtained after alkaline treatment \((m/f - e)10^6\) | -11,700 | -11,400 |

1.0 ml. of a partially purified preparation of L-arabinose dehydrogenase in 0.01 M Tris, pH 8.0, was mixed with 0.6 ml. of 0.01 M DPN, 0.6 ml. of DPNH oxidase (10 per cent suspension), 0.2 ml. of L-arabinose solution of the desired concentration, and 3.6 ml. of 0.5 M phosphate buffer, pH 7.0. In Experiment 1, the reaction mixture was incubated in a respirometer vessel at 30° for 3.5 hours, when oxygen consumption ceased. The particulate oxidase was removed by centrifuging for 20 minutes at 23,000 X g at 0°. In Experiment 2, the reaction was stopped after 4.5 hours of incubation (before oxygen consumption had ceased) by removing the oxidase by centrifugation. The optical rotation of the supernatant fluids was measured and, at the same time, the concentration of pentose and lactone was determined. Then, a small amount of NaOH was added to the reaction mixtures to raise the pH to approximately 13.0. After 10 minutes at room temperature, the solutions were carefully neutralized with HCl. Determinations of lactone and optical rotation were again made simultaneously. In a control experiment, in which the substrate had
been omitted, the optical rotation of the reaction mixture was the same before and after alkali treatment.

* Calculated by assuming that all the initially added substrate unaccounted for as unchanged pentose and lactone after incubation was the free acid \( a - (b + c) \) = free acid.

† Corrected for control without substrate (−0.06°).

‡ All values corrected for dilution during alkali treatment to correspond to original volumes.

Crude extracts of cells were found to be capable of enzymatically catalyzing the delactonization of L-arabono-\( \gamma \)-lactone. When 1.0 ml. of a 0.02 M solution of L-arabono-\( \delta \)-lactone, produced either chemically or enzymatically, was incubated at 30° with 0.1 ml. of a freshly prepared crude enzyme preparation at pH 7.0, 35 to 38 per cent of the lactone disappeared in 2 hours. However, when the lactone was mixed with buffer at pH 7.0 or with boiled enzyme, only 8 per cent disappeared in 2 hours. Under the same conditions at pH 8.0, almost 100 per cent of the lactone was hydrolyzed enzymatically in 1 hour, while 40 per cent was hydrolyzed in buffer in the same time. The product of the reaction appears to be L-arabonic acid, since boiling the incubated mixtures for 5 minutes in 1 N HCl gave an almost quantitative recovery of lactone. The lactone present before enzymatic treatment and that formed by boiling in HCl had the same \( R_f \) when chromatographed in a solvent composed of 95 per cent butanol, saturated with water, and 5 per cent formic acid.

Oxidation of L-Arabonic Acid—Since L-arabonate had been shown previously to be oxidized by cell-free preparations as measured by DPN reduction, the reason for its accumulation when large amounts of L-arabinose were used as substrate was investigated. It was found that the crude enzyme, warmed to 30° in the absence of substrate, became inactive toward L-arabonate within 5 minutes. However, when L-arabonate was added to the enzyme at 0° and the mixture was allowed to come to 30°, the rate of loss of enzymatic activity was greatly diminished. Glutathione and MgCl\(_2\) did not protect the enzyme at 30° either with or without substrate.

The optimal pH for the oxidation of L-arabonate as determined from the rate of DPN reduction was about 8.0. The reaction was inhibited by 0.003 M hydroxylamine or 0.001 M ethylenediaminetetraacetic acid. The effect of the latter could be counteracted with magnesium ion. The rate of reduction was slightly decreased in the presence of 0.01 M arsenite, but was completely unaffected by 0.002 M iodoacetate. The use of 0.1 M pyrophosphate or 0.1 M borate as buffer at pH 9.0 completely inhibited the reduction.
While the enzyme system oxidizing L-arabinose could use only DPN as the hydrogen acceptor, the system oxidizing L-arabonate could reduce either DPN or TPN. TPN was reduced at a greater rate than DPN. Adenosine triphosphate and diphosphothiamine had no effect upon the rate of reduction. Crude preparations were inactivated by dialysis for 24 hours against 0.01 M Tris at pH 8.0 and could not be reactivated with glutathione, MgCl₂, the supernatant fluid from a boiled enzyme preparation, or any combination of the three.

No evidence for the phosphorylation of L-arabonate could be found when disappearance of 7 minute-hydrolyzable phosphate was used as a criterion for the transphosphorylation from ATP.

Manometric studies were carried out by coupling the oxidation of L-arabonate to oxygen through the DPNH oxidase of A. vinelandii. If the substrate and enzyme were mixed in the main compartment of a Warburg flask while the enzyme was cold, activity could be maintained long enough to attach the flask to a manometer, permit temperature equilibration for 5 minutes, and then add DPN and DPNH oxidase from the side arm. The reaction must be over in 60 minutes or less because the system becomes inactivated in that length of time. L-Arabonate was found to be oxidized, with the uptake of 0.5 mole of oxygen per mole of substrate (Table I). The keto acid produced by this reaction was identified as α-ketoglu taric acid by chromatographing the free acid and by chromatography and melting point determinations of the 2,4-dinitrophenylhydrazone. As further proof, the keto acid was decarboxylated with ceric sulfate and the resulting succinic acid quantitatively identified by using succinic oxidase (Table III). By following the same precautions for the oxidation of L-arabinose as just described for L-arabonate, L-arabinose was also shown to be quantitatively converted to α-ketoglutarate (Table III).

From the curves obtained by measuring DPN reduction with L-arabonate as the substrate, it appeared that L-arabonate was not the immediate substrate for the oxidative step. When DPN and substrate were added at the same time to a cuvette containing buffer and enzyme, there was a lag before any reduction could be detected (Fig. 1, Curve A). By increasing the amount of substrate added initially, the lag could be somewhat shortened. Under conditions in which enough substrate was added to reduce all the DPN in 15 minutes, it was observed that the rate of reduction increased continuously until all the DPN had been reduced. With L-arabonolactone as substrate, the lag period was considerably lengthened, indicating that the lactone had to be hydrolyzed to the acid before being further metabolized (Fig. 1, Curve E).

If L-arabonate and enzyme were incubated together in a cuvette before the addition of DPN, there was no lag in reduction when coenzyme was added. Instead, there was an initial high rate of reduction which gradu-
ally decreased (Fig. 1, Curves B and C). The amount of the material which caused the rapid reduction of DPN in a reaction mixture was roughly

**Table III**

*Quantitative Estimation of α-Ketoglutaric Acid Produced in Oxidation of L-Arabinose and Potassium L-Arabonate with Crude Enzyme*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Substrate oxidized</th>
<th>Acid produced</th>
<th>α-Ketoglutaric acid produced</th>
<th>Colorimetric determination</th>
<th>Ceric sulfate decarboxylation</th>
<th>CO₂ produced</th>
<th>Succinic acid produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Arabinose</td>
<td>500 µmoles</td>
<td>926 µeq.</td>
<td>420 µmoles</td>
<td>470 µmoles</td>
<td>392 µmoles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Arabonate</td>
<td>500 µmoles</td>
<td>495 µeq.</td>
<td>502 µmoles</td>
<td>500 µmoles</td>
<td>445 µmoles</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1.0 ml. of enzyme in 0.01 M Tris, pH 8.0, was mixed with 1.0 ml. of 0.005 M DPN, 0.5 ml. of 10 per cent suspension of DPNH oxidase from *A. vinelandii*, 1.0 ml. of 0.5 M substrate, and 6.5 ml. of 0.2 M Tris, pH 8.0. The reaction mixture was incubated aerobically with shaking at 30° until keto acid production had stopped.

**Fig. 1.** Enzymatic DPN reduction with potassium L-arabonate and L-arabono-γ-lactone. 0.5 ml. of enzyme in 0.01 M Tris, pH 8.0, was mixed with 13.0 ml. of 0.1 M Tris, pH 8.0, and 1.0 ml. of 0.01 M potassium L-arabonate and incubated at 30°. After various time intervals, 2.9 ml. aliquots were placed in a cuvette, and 0.1 ml. of 0.01 M DPN was added and the reduction of DPN measured. The preincubation periods were as follows: Curve A, 0 time; Curve B, 15 minutes; Curve C, 45 minutes; Curve D, 120 minutes; Curve E, same as Curve A except that the substrate was L-arabono-γ-lactone.

proportional to the length of the preincubation period provided this was not too long. With prolonged preincubation, it appeared that this compound (or compounds) was destroyed (Fig. 1, Curve D).
All attempts to accumulate or identify the rapidly oxidized compound or compounds have been unsuccessful to date. The substrate for the dehydrogenation is apparently extremely labile, since it disappears on prolonged incubation of L-arabonate and enzyme and is destroyed by such deproteinizing agents as boiling, the addition of methanol or of perchloric, trichloroacetic, or mineral acid, or the treatment of the reaction mixture with Dowex 50 in the cold.

In order to gain an insight into the mechanism of the oxidation of L-arabonate to α-ketoglutarate, experiments with radioactive substrates were undertaken. The crude enzyme, with DPNH oxidase, was allowed to oxidize L-arabonate-1-Cl4 for 2 hours. The reaction was stopped by adding sulfuric acid to give a final concentration of about 1 N, and the proteins were removed by centrifugation. The supernatant fluid was continuously extracted with ether for 24 hours. The α-ketoglutaric acid in the ether extract was then isolated by ascending chromatography. A portion of the isolated keto acid was decarboxylated with ceric sulfate, and the Cl4 content of the carbon dioxide derived from the carboxyl carbon adjacent to the carbonyl group was determined. Another portion was dried on a planchet for a direct assay of the specific activity of the acid. The specific activities of the α-ketoglutarate and of the CO2 produced by ceric sulfate decarboxylation were found to be the same as that of the substrate (Table IV). A similar experiment was conducted with L-arabinose-1-C14 as the substrate, except that the α-ketoglutarate was not isolated and the decarboxylation was carried out with the entire reaction mixture. Again, the specific activity of the CO2 was equal to that of the original substrate (Table IV).

Since α-ketoglutarate is a well known member of the tricarboxylic acid cycle, it appeared possible that other members of this cycle could be intermediates in the oxidation of L-arabonate to α-ketoglutarate. The compounds tested were citrate, cis-aconitate, isocitrate, and pyruvate. Since both DPN and TPN were reduced with L-arabonate in the presence of a crude enzyme preparation, the ability of the above compounds to serve as substrates for both DPN and TPN reduction was determined. In no case did any of them cause a reduction of DPN. However, TPN was rapidly reduced with isocitrate and relatively slowly with cis-aconitate. Pyruvate and citrate were inactive. The product of the oxidation of isocitrate was shown to be α-ketoglutarate by chromatography. A keto acid also accumulated in reaction mixtures containing isocitrate when TPN was not present. This keto acid was tentatively identified as glyoxylic acid by chromatography.

The possibility that the above compounds or products of their metabolism may be intermediates in the oxidation of L-arabonate to α-keto-
glutarate was further checked by determining their ability to dilute the specific activity of the α-ketoglutarate formed in the oxidation of totally labeled L-arabonate. The enzyme was incubated with L-arabonate-C¹⁴, DPN, and DPNH oxidase under aerobic conditions in the presence of an excess of each of the unlabeled compounds, separately as well as in their absence. As controls, the same reaction mixtures were prepared except that the L-arabonate was excluded. There was no α-ketoglutarate formed in any control experiment. The α-ketoglutarate formed in the reaction mixtures containing L-arabonate was isolated by ascending chromatography. As shown in Table V, the specific activity of the α-ketoglutarate was essentially unchanged by the added compounds.

Several other compounds which, for various reasons, appeared to be possible intermediates were tested in the same manner. These were glucose-6-phosphate, glyoxylate, L-α-hydroxyglutarate, and glutaconate. Of these four compounds, only glucose-6-phosphate served as a substrate for the reduction of DPN and TPN. The others were inactive toward both coenzymes. Pyruvic acid and glyceraldehyde-3-phosphate had previously

### Table IV

**Determination of Radioactivity in α-Ketoglutaric Acid Produced Enzymatically from L-Arabinose-1-C¹⁴ and Potassium L-Arabinonate-1-C¹⁴**

<table>
<thead>
<tr>
<th>(a) Substrate, μmoles</th>
<th>L-Arabinose</th>
<th>r-Arabinose</th>
</tr>
</thead>
<tbody>
<tr>
<td>(b) Specific activity*</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>(c) α-Ketoglutarate in reaction mixture, μmoles</td>
<td>1032</td>
<td>95</td>
</tr>
<tr>
<td>(d) CO₂ produced on ceric sulfate decarboxylation of reaction mixture, μmoles</td>
<td>26.0</td>
<td>45.5</td>
</tr>
<tr>
<td>(e) Specific activity</td>
<td>†</td>
<td>43.2</td>
</tr>
<tr>
<td>(f) Specific activity of α-ketoglutarate isolated chromatographically</td>
<td>†</td>
<td>95.6</td>
</tr>
<tr>
<td>(g) Specific activity of CO₂ produced by ceric sulfate decarboxylation of isolated α-ketoglutarate</td>
<td>1028</td>
<td>†</td>
</tr>
<tr>
<td>(h) Specific activity of CO₂ vs. specific activity of substrate (e/b)</td>
<td>966</td>
<td>†</td>
</tr>
<tr>
<td>(i) Specific activity of α-ketoglutarate vs. specific activity of substrate (f/b)</td>
<td>0.94</td>
<td>1.01</td>
</tr>
</tbody>
</table>

0.5 ml. of enzyme in 0.01 M Tris, pH 8.0, 0.25 ml. of 10 per cent suspension of DPNH oxidase from *A. vinelandii*, 0.5 ml. of 0.005 M DPN, 0.50 ml. of 0.1 M substrate, and 3.25 ml. of Tris, pH 8.0, were incubated aerobically with shaking at 30° for 2 hours.

* Counts per minute per micromole.
† Not determined.
been shown to be the products of the oxidation of glucose-6-phosphate by preparations from cells grown with glucose (5). In the present experiments, pyruvate was also identified chromatographically as the main keto acid produced. No α-ketoglutarate was formed from any of the above compounds, nor did any of them show any dilution of the specific activity of the α-ketoglutarate produced from L-arabonate (Table V).

The possibility that acetate, formate, or CO₂ may be involved in the formation of α-ketoglutarate was also tested. 10 µmoles of inactive L-arabonate were incubated with the enzyme system and each of the follow-

<table>
<thead>
<tr>
<th>TABLE V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ability of Various Compounds to Dilute Specific Activity of α-Ketoglutaric Acid Produced Enzymatically from Totally Labeled Potassium L-Arabonate</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Unlabeled compound</th>
<th>Specific activity of α-ketoglutarate c.p.m. per µmole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>113</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>113</td>
</tr>
<tr>
<td>Isocitrate</td>
<td>115</td>
</tr>
<tr>
<td>Citrate</td>
<td>109</td>
</tr>
<tr>
<td>cis-Aconitate</td>
<td>105</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>117</td>
</tr>
<tr>
<td>Glyoxylate</td>
<td>112</td>
</tr>
<tr>
<td>Glutaconate</td>
<td>113</td>
</tr>
<tr>
<td>L-α-Hydroxyglutarate</td>
<td>114</td>
</tr>
</tbody>
</table>

10 µmoles of L-arabonate-C₁⁴ (111 c.p.m. per µmole), 30 µmoles of unlabeled compound, 0.5 µmole of DPN, 900 µmoles of Tris, pH 8.0, were mixed with 0.1 ml. of enzyme (33 per cent extract in 0.01 M Tris, pH 8.0) and 0.05 ml. of 10 per cent suspension of DPNH oxidase from *A. vinelandii* to give a final volume of 1.0 ml. The reaction mixtures were incubated aerobically at 30° for 2 hours. The α-ketoglutarate was isolated chromatographically.

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<td>114</td>
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</table>

Experiments with both intact cells and cell-free preparations of *P. saccharophila* have demonstrated that the metabolism of L-arabinose in this organism proceeds by a mechanism entirely different from that described for the breakdown of glucose (5). It also differs from the metab-
The overall oxidation does not appear to involve any phosphorylated intermediates or the classical tricarboxylic acid cycle. It may be summarized by the accompanying scheme.

In the first step, L-arabinose is oxidized with a DPN-specific "L-arabinose dehydrogenase" to the L-arabono-γ-lactone, suggesting that the furanose form of the sugar is attacked. It is interesting to note that the enzymatic oxidation of both glucose and glucose-6-phosphate yields the corresponding δ-lactones (34–38) and that the δ-lactones are produced in the bromine oxidation of glucose, L-arabinose, and a variety of other sugars (25).

The second step appears to be the hydrolysis of the lactone to L-arabonic acid by a "delactonizing" enzyme. A delactonizing enzyme has been isolated from yeast (38), which catalyzes the hydrolysis of the δ-lactones of both gluconic and 6-phosphogluconic acids but not of L-arabonono-γ-lactone. The specificity of the delactonizing enzyme in P. saccharophila has not been studied.

The further oxidation of L-arabonic acid to α-ketoglutaric acid, indicated as in the third step, is certainly a complex series of reactions which has not as yet yielded to analysis, since at least some of the intermediate compounds and enzymes appear to be extremely unstable. The following conclusions may be drawn from the experiments with crude cell-free extracts: (a) The sum total of the reactions in the third step results in the quantitative conversion of L-arabonic acid to α-ketoglutaric acid, in which the carboxyl carbon adjacent to the carbonyl group is derived from the carboxyl carbon of L-arabonic acid. The tricarboxylic acid cycle is not a major mechanism of α-ketoglutarate synthesis from L-arabonate, since DPN as well as TPN can serve as hydrogen acceptor in this oxidation. DPN cannot be reduced in the oxidation of isocitrate by the same preparations. Also, with DPN as the coenzyme, no part of the following compounds is incorporated into the end-product: CO₂, pyruvic acid, iso-
Oxidation of L-arabinose

citrates, cis-aconitic acid, and citric acid. In addition, formic, acetic, glyoxylic, glutaric, and L-α-hydroxyglutaric acids are not precursors in whole or in part for the α-ketoglutaric acid. Unlabeled glucose-6-phosphate, which, under the conditions of the experiment, can be a source of pyruvic acid and glyceraldehyde-3-phosphate (5), also does not dilute the specific activity of the α-ketoglutarate. (b) The initial reaction involved in the transformation of L-arabonate is not oxidative in nature. Furthermore, it appears that the γ-lactone of this sugar acid precedes the acid in the overall mechanism and that the acid is not a side product. (c) At least one of the substrates for the subsequent steps is unstable in the presence of enzyme and is destroyed by mild chemical treatment. (d) Either DPN or TPN can be used as the hydrogen acceptor in the oxidative step. TPN is used more rapidly than DPN. Since some TPN might have been present in the crude extracts, the possibility existed that this coenzyme might be the primary hydrogen acceptor. However, a TPNH-DPN transhydrogenation reaction appeared to be ruled out, since the same enzyme preparation catalyzed the oxidation of isocitrate with TPN as hydrogen acceptor but did not reduce DPN.

The further metabolism of α-ketoglutaric acid could not be observed with cell-free preparations from P. saccharophila grown with L-arabinose as substrate. However, from evidence obtained in experiments with intact cells, it would seem that pyruvic acid is a metabolite subsequent to α-ketoglutarate. Iodoacetate-poisoned cells oxidize L-arabinose to CO₂ and pyruvate. On the other hand, arsenite-poisoned cells, which oxidize glucose to pyruvic acid (5), produce α-ketoglutaric acid from L-arabinose. It has also been demonstrated by isotope dilution experiments that pyruvic acid is not a precursor of α-ketoglutaric acid in the oxidation of L-arabonate by cell-free preparations.

Preliminary experiments with resting cell suspensions indicate that the mechanism for the oxidation of L-arabinose does not appear to be utilized by P. saccharophila for the oxidation of the other pentoses. The products of the oxidation of d-xylose and d-ribose by poisoned cells are the same as those obtained in glucose oxidation (5), while the oxidation of d-arabinose results in the accumulation of a different group of compounds which have not as yet been identified.

The oxidation of L-arabinose has been studied in other microorganisms (2, 39–45). The results of these studies indicate that different organisms may metabolize this pentose by mechanisms other than that utilized by P. saccharophila. Most of these investigations have not been very extensive and were conducted with obligate or facultative anaerobes. Whether or not the pathway described in these studies is present in other organisms remains to be determined. The occurrence of L-arabonic acid
in the oxidation of L-arabinose by several species of Pseudomonas (39), and Brucella melitensis (2) suggests that these bacteria may use this pathway, at least in part.

The authors wish to express their deep gratitude to the many bacteriologists and biochemists of their acquaintance who have contributed to this work by their generous gifts of chemicals and for their interest and valuable suggestions. A few of the many people to whom we are particularly indebted are Dr. A. C. Neish, H. A. Barker, W. Z. Hassid, E. Putman, M. Rothstein, V. Ginsburg, R. Y. Stanier, N. Palleroni, and J. MacGee.

**SUMMARY**

1. Arsenite-poisoned intact cells and cell-free preparations of Pseudomonas saccharophila oxidize L-arabinose to α-ketoglutaric acid.

2. L-Arabinose is oxidized to L-arabono-γ-lactone with DPN by enzyme preparations. A delactonizing enzyme is present in crude preparations which hydrolyzes the γ-lactone to L-arabonic acid.

3. L-Arabonic acid is further metabolized to α-ketoglutaric acid, in which the carboxyl carbon adjacent to the carbonyl group is derived from the carboxyl carbon of arabonic acid. Either DPN or TPN may act as hydrogen acceptor for this system.

4. Evidence is presented to show that L-arabonic acid is converted to an unstable intermediate which is rapidly oxidized by crude enzyme preparations.

5. There is no evidence for the participation of any phosphorylated intermediates or any of the common metabolites, such as members of the tricarboxylic acid cycle, in the formation of α-ketoglutarate.

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THE OXIDATION OF L-ARABINOSE BY PSEUDOMONAS SACCHAROPHILA
R. Weimberg and Michael Doudoroff


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