Pathway of L-Sorbose Metabolism in Aerobacter aerogenes*

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SUMMARY

The pathway of L-sorbose metabolism in Aerobacter aerogenes was determined to be: L-sorbose → L-sorbose 1-phosphate → D-glucitol 6-phosphate → D-fructose 6-phosphate. Phosphorylation of L-sorbose at C-1 is mediated by a phosphoenolpyruvate-dependent phosphotransferase system, whereas L-sorbose 1-phosphate reduction and D-glucitol 6-phosphate oxidation are mediated by pyridine-nucleotide-linked dehydrogenases. Mutants deficient in L-sorbose 1-phosphate reductase, D-glucitol 6-phosphate dehydrogenase, or a component (Enzyme I) of the phosphotransferase system did not grow on L-sorbose, whereas revertants which had regained these activities grew normally. Extracts of the Enzyme I-deficient mutant failed to catalyze the phosphorylation of L-sorbose with phosphoenolpyruvate, and adenosine 5′-triphosphate could not replace the phosphoenolpyruvate requirement for L-sorbose phosphorylation in extracts of the wild type strain.

Previously reported metabolic reactions of L-sorbose involve oxidation to 2-keto-L-gulonate by various bacteria (1) and oxidation to 5-keto-L-sorbose (5-keto-D-fructose) by Gluconobacter suboxydans (2) and by a basidiomycete, Trametes sanguinea (3). Rat liver fructokinase can catalyze the phosphorylation of L-sorbose with adenosine 5′-triphosphate, but the reaction is considered not to be of physiological significance (4).

In contrast to the organisms in which L-sorbose metabolism has been previously investigated, Aerobacter aerogenes PRL-R3 can utilize L-sorbose as a sole source of carbon and energy, both aerobically and anaerobically. In this paper we present evidence that the pathway by which L-sorbose is metabolized in A. aerogenes PRL-R3 is as follows: L-sorbose → L-sorbose 1-phosphate → D-glucitol 6-phosphate → D-fructose 6-phosphate. Thus, this pathway is distinct from those previously described.

MATERIALS AND METHODS

Bacterial Strains and Bacteriological Procedures—The "wild type" organism used was A. aerogenes PRL-R3 or a uracil auxotroph derived from it. The methods for cell growth and the preparation of cell extracts have been described previously (5, 6), with one exception: cells to be assayed for L-sorbose 1-phosphate reductase were suspended in 0.04 M MES buffer, pH 6.15, prior to sonic treatment.

Enzyme Assays—D-Glucitol 6-phosphate dehydrogenase activity was measured spectrophotometrically with the phosphoglucoisomerase-glucose 6-phosphate dehydrogenase-linked assay described previously (7).

The reaction mixture (0.15 ml) for L-sorbose 1-phosphate reductase contained: 20 μmoles of MES buffer, pH 6.15; 1.0 μmole of L-sorbose 1-phosphate, and 0.05 μmole of NADPH. The reaction was stopped at 340 nm with a Gilford model 2400 absorbance-recording spectrophotometer thermostated at 25°.

The assay for phosphoenolpyruvate-L-sorbose 1-phosphotransferase activity was a modification of the phosphoenolpyruvate:L-fructose 1-phosphotransferase assay of Hanson and Anderson (8). The reaction mixture contained: 8 μmoles of Tris-HCl buffer (pH 7.5); 3 μmoles of NaF; 0.01 μmole of MgCl₂; 1.2 μmoles of phosphoenolpyruvate; 40 μmoles of L-sorbose; and cell extract (usually 2 to 3 mg of protein) in a total volume of 0.2 ml. The tubes were incubated at 30° for 10 min, immersed in a boiling water bath for 6 min to stop the reaction, and clarified by centrifugation. Accumulated L-sorbose 1-phosphate in the supernatant solution was quantitated in an end point assay identical with the assay for L-sorbose 1-phosphate reductase except that the supernatant solution was used as limiting substrate and L-sorbose 1-phosphate reductase was added in excess.

In all three of the above assays, the rates of conversion of substrate to product were constant with time and proportional to the amount of enzyme assayed in the ranges used. Units of enzyme are defined as the amount (nanomoles) of substrate converted per min, and specific activities are reported as the units per mg of protein. Protein was determined by the method of Lowry et al. (9).

Reagent Enzymes—L-Sorbose 1-phosphate reductase was partially purified to remove enzymes that might interfere with its use in end point assays for L-sorbose 1-phosphate. Twenty milliliters of a crude extract (40 mg of protein per ml) from L-sorbose-grown PRL-R3 were chromatographed on a column (38 × 2.6 cm) of Sephadex G-200. Five-milliliter fractions were collected, and those containing the most activity (Fractions 27 and 28) were pooled and concentrated 8-fold with a Diaflo model 50 ultrafiltration cell (Amicon Corp.). This preparation of L-sorbose 1-phosphate reductase was about 4-fold purified and 1 The abbreviation used is: MES, 2-(N-morpholino)ethane sulfonate.
possessed only a noninterfering amount of NADPH oxidase activity. However, it had 6-phosphogluconate dehydrogenase activity when had to be taken into account when it was included in end point assays involving glucose 6-phosphate formation, such as in the experiment presented in Fig. 2.

\( ^{-}\)Glucitol 6-phosphate dehydrogenase was partially purified as described previously (7); this preparation was free from detectable 6-phosphogluconate dehydrogenase when used in end point assays (as in Fig. 2). Other reagent enzymes were obtained from commercial sources.

**Analytical Methods**—Ketohexoses were quantitatively determined by a modification of Roe's method as discussed by Putnam (10). Sorbose was distinguished from other 2-ketohexoses by the colorimetric method of Dische and Devi (11). Total phosphate and inorganic orthophosphate were determined by the modified method of Fiske and SubbaRow (12). Trimethylsilyl derivatives of sugar phosphates were prepared and subjected to gas-liquid chromatography as described by Sweeley, Wells, and Bentley (13). Gas chromatography was performed on a Hewlett and Packard gas chromatograph, model 402, employing a 1.2-meter column of 3% OV-1 at a temperature of 190°. Trimethylsilyl O-methyloxime derivatives of sugar phosphates were prepared and subjected to combined gas-liquid chromatography and mass spectrometry as described by Laine and Sweeley (14).

**Chemicals**—L-Sorbose 1-phosphate was prepared as described by Mann and Lardy (19); the starting compound, diacetone-\( ^{-}\)sorbose, was a gift from Hoffman-LaRoche, Inc., Nutley, N. J. \( ^{-}\)Glucitol 6-phosphate was prepared as described by Wolff and Kaplan (16). Other chemicals were obtained from commercial sources or as described previously (5, 6).

**RESULTS**

**Growth Response on L-Sorbose of Hexose- and Hexitol-negative Mutants**—An analysis of the growth response on L-sorbose of mutants of *A. aerogenes* PRL-R3 blocked at various points of hexose and hexitol metabolism (Table I) permitted construction of a hypothetical pathway of L-sorbose metabolism. The pathway had to be unrelated to the metabolism of \( ^{-}\)-fructose, its C-5 epimer, since mutants missing \( ^{-}\)-fructokinase, \( ^{-}\)-fructose 1-phosphate kinase, both of these enzymes, or the "\( ^{-}\)-fructose specifier protein" of the phosphoenolpyruvate:hexose phosphotransferase system all grew normally on L-sorbose. On the other hand, a mutant (PL-122) missing Enzyme I of the phosphoenolpyruvate:hexose phosphotransferase system did not grow on L-sorbose, whereas a revertant (PL-122R) did, suggesting that L-sorbose metabolism is initiated by this system. Furthermore, mutants missing \( ^{-}\)glucitol 6-phosphate dehydrogenase (strain 4) and \( ^{-}\)fructose 6-phosphate kinase (strain A9-1) failed to grow on L-sorbose, indicating that \( ^{-}\)glucitol 6-phosphate and \( ^{-}\)fructose 6-phosphate were likely intermediates in L-sorbose metabolism. This suggested the probable participation of another previously unreported enzyme, an L-sorbose 1-phosphate reductase, the product of which would be \( ^{-}\)glucitol 6-phosphate. Evidence for the occurrence of the postulated enzymes (phosphoenolpyruvate:L-sorbose 1-phosphotransferase and L-sorbose 1-phosphate reductase) and evidence for their participation, along with that of \( ^{-}\)glucitol 6-phosphate dehydrogenase, in the metabolism of L-sorbose is presented below.

**Phosphoenolpyruvate:L-Sorbose 1-Phosphotransferase**—L-Sorbose 1-phosphate synthesis in extracts of wild type cells (strain PRL-R3) was dependent on phosphoenolpyruvate (Table II). The specific activity was comparable to those reported for other substrates of the phosphoenolpyruvate-dependent phosphotransferase system in this and in other organisms (see Reference 7). ATP could not replace phosphoenolpyruvate (Table II), indicating the absence of L-sorbose kinase. Furthermore, an intact phosphoenolpyruvate:sugar phosphotransferase system was re-

**TABLE I**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Missing enzyme and reference</th>
<th>Growth on L-sorbose*</th>
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</thead>
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<tr>
<td>PL-122</td>
<td>Enzyme I of P-enolpyruvate-dependent phosphotransferase system (7)</td>
<td>~</td>
</tr>
<tr>
<td>PL-122R (Revertant of PL-122)</td>
<td>None (7)</td>
<td>+</td>
</tr>
<tr>
<td>QQ17</td>
<td>&quot;( ^{-})-Fructose specifier protein&quot; of P-enolpyruvate-dependent phosphotransferase system (8)</td>
<td>+</td>
</tr>
<tr>
<td>A9-1</td>
<td>( ^{-})-Fructose 6-phosphate kinase (5)</td>
<td>~</td>
</tr>
<tr>
<td>DD31</td>
<td>( ^{-})-Fructose 1-phosphate kinase (6)</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>( ^{-})-Fructose 1-phosphate kinase and ( ^{-})-fructokinase (6)</td>
<td>+</td>
</tr>
<tr>
<td>HRAF 4</td>
<td>( ^{-})-Fructokinase (6)</td>
<td>+</td>
</tr>
<tr>
<td>4R (Revertant of 4)</td>
<td>None (7)</td>
<td>+</td>
</tr>
</tbody>
</table>

* Minus (~) indicates no growth; + indicates a rate and extent of growth similar to that of the wild type (strain PRL-R3), i.e. a doubling time of about 50 min at 30-32°.

**TABLE II**

<table>
<thead>
<tr>
<th>Strain</th>
<th>&quot;Inducer&quot;</th>
<th>Reaction mixture</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRL-R3 (wild type)</td>
<td>L-Sorbose</td>
<td>Complete</td>
<td>8.5</td>
</tr>
<tr>
<td>PRL-R3 (wild type)</td>
<td>L-Sorbose</td>
<td>P-enolpyruvate</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>PRL-R3 (wild type)</td>
<td>L-Sorbose</td>
<td>P-enolpyruvate, plus ATP</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>PRL-R3 (wild type)</td>
<td>L-Sorbose</td>
<td>L-sorbose and P-enolpyruvate</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>PRL-R3 (wild type)</td>
<td>L-Sorbose</td>
<td>Complete</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>PL-122 (Enzyme I)*</td>
<td>L-Sorbose</td>
<td>Complete</td>
<td>7.5</td>
</tr>
<tr>
<td>PL-122R (Enzyme I)*</td>
<td>L-Sorbose</td>
<td>Complete</td>
<td>9.4</td>
</tr>
<tr>
<td>PRL-R3 (wild type)</td>
<td>L-Glucitol</td>
<td>Complete</td>
<td>13.0</td>
</tr>
<tr>
<td>PRL-R3 (wild type)</td>
<td>L-Glucose</td>
<td>Complete</td>
<td>9.4</td>
</tr>
</tbody>
</table>

* Specific activity: nanomoles of L-sorbose-1-P formed per min per mg of protein.

* Isolated as described in Reference 7.
Phosphoenolpyruvate: L-sorbose 1-phosphotransferase specific activity was similar in extracts of cells grown on L-sorbose, D-glucose, and D-glucitol (Table II). Other experiments showed that half-maximal rates for L-sorbose 1-phosphate formation were in the range of 1 to 4 mM L-sorbose in extracts of cells grown on either L-sorbose, D-glucitol, or D-glucose.

To prepare the product for identification, the standard assay mixture was scaled up 50-fold and was incubated with crude extract (12 mg of protein) from D-glucose-grown cells at 28° for 3 hours. The reaction was terminated by heating at 100° for 7 min, and the precipitated protein was removed by centrifugation. The product in the supernatant was purified and separated from unreacted substrates by chromatography on a column of Dowex I-bicarbonate (1 × 7 cm, 200 to 400 mesh), with a stepwise gradient (100 ml each) of water and 0.15, 0.30, and 0.45 M KHCO₃. Fractions containing ketose phosphate, which eluted with 0.15 M KHCO₃, were combined and treated with Dowex 50(H⁺) to lower the pH to 2. The compound was then lyophilized to dryness, dissolved in 3 ml of water, and the pH of the solution adjusted to 7.0 with NaOH. The yield was 24.4 μmoles of ketose phosphate.

The product was identified as L-sorbose 1-phosphate on the basis of the following data. The ketohexose to phosphate ratio was 0.99. The 50% hydrolysis time in 1 N HCl at 100° was 10 min for both the product and for chemically synthesized L-sorbose 1-phosphate; this is similar to a reported value (15) and distinct from that of L-sorbose 6-phosphate (15). Reaction with cysteine-H₂SO₄ (11) for 15 hours at room temperature gave an A₄₁₂/A₆₀₆ ratio of 0.4 for the product, authentic L-sorbose 1-phosphate, and L-sorbose; all other 2-ketohexoses give values greater than 3.0 (11, 17). Gas chromatography (13) of trimethylsilyl derivatives of the product and of authentic L-sorbose 1-phosphate gave identical patterns for each (three peaks with retention times of 9.5, 11.0, and 11.5 min) and n-fructose 6-phosphate (major peak at 10.7 min) standards. Mass spectrometry of trimethylsilyl O-methyloxime derivatives (14) of the product (Fig. 1) and of authentic L-sorbose 1-phosphate supported the expected molecular weight of 721 and yielded identical fragmentation patterns; important ions for identification occurred at m/e 103, 205, 217, 307, 414, and 516 (14), and at m/e 299, 314, 315, and 387 (18). And finally, in a separate reaction mixture (Fig. 2), the product reacted quantitatively through the sequence: L-sorbose 1-phosphate → D-fructose 6-phosphate → D-glucose 6-phosphate → 6-phosphoglucono-6-lactone through the participation of L-sorbose 1-phosphate reductase, D-glucitol 6-phosphate dehydrogenase, D-glucose 6-phosphate isomerase, and D-glucose 6-phosphate dehydrogenase.

L-Sorbose 1-Phosphate Reductase—The specific activity of this enzyme in L-sorbose-grown cells was about 80 nmoles of L-sorbose 1-phosphate reduced per min per mg of protein. L-sorbose 1-phosphate reductase activity could not be detected in extracts of cells grown on D-glucose, n-glucitol, or glycerol, indicating that the enzyme is specifically induced by growth on L-sorbose. NADPH and NADH served equally well as cofactors with both the crude and partially purified enzyme. Half-maximal velocity was achieved at an L-sorbose 1-phosphate concentration of about 0.4 mM. The equilibrium of the reaction under the conditions of the standard assay, but with the L-sorbose 1-phosphate concentration limiting, was greatly in favor of D-glucitol 6-phosphate formation (> 95%), thus permitting the use of this enzyme in end point assays for L-sorbose 1-phosphate.

Intermediate of L-Sorbose Metabolism—The time course of formation of intermediates of L-sorbose metabolism in a phosphoenolpyruvate-supplemented cell extract is shown in Fig. 2. After 20 min of incubation, 91% of the L-sorbose added was accounted for as L-sorbose 1-phosphate and D-glucitol 6-phosphate. The time of appearance of the two intermediates was consistent with a precursor-product relationship in which L-sorbose 1-phosphate was formed first and then converted to D-glucitol 6-phosphate.

Isolation and Analysis of Mutants Lacking L-Sorbose 1-Phosphate Reductase and D-Glucitol 6-Phosphate Dehydrogenase—A mutant (strain 1a) lacking L-sorbose 1-phosphate reductase was obtained by minor modifications of methods detailed previously for the selection of other mutants (5, 6). It was selected as a pale colony on L-sorbose-MacConkey agar after mutagenesis of the uracil auxotroph of strain PRL-R3 with ethyl methane sulfonate and allowance of 10 generations of growth on n-glucose-mineral salts medium. A spontaneous revertant (strain 1aR3) which had regained L-sorbose 1-phosphate reductase was selected as a normal-appearing colony on L-sorbose-mineral salts agar.

A mutant (strain 4) lacking D-glucitol 6-phosphate dehydrogenase and a revertant (strain 4R6) derived from it were those previously isolated in an investigation of D-glucitol metabolism (7).

Table III gives data on the growth rates and enzyme levels for these two mutants and two revertants grown on or induced by L-sorbose, D-glucitol, and D-glucose. The mutant lacking
L-Sorbose metabolism in Aerobacter aerogenes

Enzyme activities in and growth rates of wild type (PRL-R3) A. aerogenes and L-sorbose-negative mutants

For the enzyme analyses, cells were induced as described in Table II.

| Strain    | L-Sorbose | D-Glucose | D-Glucitol | Growth rate
<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>PRL-R3</td>
<td>83.0</td>
<td>&gt;0.5</td>
<td>&gt;0.5</td>
<td>1.2</td>
</tr>
<tr>
<td>1a</td>
<td>&lt;0.5</td>
<td>&gt;0.5</td>
<td>&gt;0.5</td>
<td>No growth</td>
</tr>
<tr>
<td>1aR3</td>
<td>75.0</td>
<td>&lt;0.5</td>
<td>194</td>
<td>1.3</td>
</tr>
<tr>
<td>4</td>
<td>3.0</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>No growth</td>
</tr>
<tr>
<td>4R6</td>
<td>50.0</td>
<td>&lt;0.5</td>
<td>73</td>
<td>1.2</td>
</tr>
</tbody>
</table>

*Specific activity: nanomoles of substrate converted per min per mg of protein.

Growth rate: divisions per hour at 32°C.

Table III

Examination of Possible Alternate Pathways of L-Sorbose Metabolism—We have been unable to detect reactions of L-sorbose in extracts of L-sorbose-grown cells other than phosphorylation with phosphoenolpyruvate. The data in Table II indicated the absence of L-sorbose kinase (ATP:L-sorbose 1-phosphotransferase). Similarly, with a pyruvate kinase-lactate dehydrogenase-linked assay (19), which would detect ATP-dependent phosphorylation at C-6 as well as C-1, we were unable to detect L-sorbose kinase activity (< 10 nmoles of substrate phosphorylated per min per mg of protein, with 27 mm L-sorbose as the substrate). Also, we could not detect NADH- or NADPH-dependent oxidation of D-glucitol (< 0.05 nmoles per min per mg of protein) in extracts of L-sorbose-grown wild type cells.

DISCUSSION

These results establish that the degradation of L-sorbose in A. aerogenes PRL-R3 involves phosphoenolpyruvate-dependent phosphorylation of L-sorbose at C-1, pyridine-nucleotide-dependent reduction of L-sorbose 1-phosphate, and oxidation of the resulting D-glucitol 6-phosphate (Fig. 3). These conclusions are based on the demonstration of phosphoenolpyruvate:L-sorbose 1-phosphotransferase, L-sorbose 1-phosphate reductase, and D-glucitol 6-phosphate dehydro-
genase activities in extracts of L-sorbose-grown cells, the inability of mutants deficient in these activities and D-fructose 6-phosphate kinase to grow on L-sorbose, a quantitative conversion of L-sorbose to intermediates (L-sorbose 1-phosphate and L-glucitol 6-phosphate) of the pathway in extracts, and the inability to detect other enzyme activities (i.e. L-sorbose kinase, L-sorbose reductase, and L-sorbose and D-glucitol dehydrogenases) that might participate in alternate sequences or reactions. This represents a new pathway of L-sorbose metabolism which is distinct from any previously reported (see the introduction).

Phosphoenolpyruvate:L-sorbose 1-phosphotransferase and L-sorbose 1-phosphate reductase are new enzymes which have not been previously reported for any organism, whereas D-glucitol 6-phosphate dehydrogenase has previously been shown also to participate in n-glucitol metabolism (7).

The previous demonstration that D-glucitol failed to induce D-glucitol 6-phosphate dehydrogenase in strain PL-122 (Enzyme I-negative) led to the suggestion that D-glucitol 6-phosphate rather than D-glucitol was the actual inducer of D-glucitol 6-phosphate dehydrogenase (7). The finding that D-glucitol 6-phosphate dehydrogenase is induced in strain 1a (L-sorbose 1-phosphate reductase-negative) by D-glucitol but not by L-sorbose (Table III) lends support to this supposition.

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