PENTOSE FERMENTATION BY LACTOBACILLUS PLANTARUM

IV. L-RIBULOSE-5-PHOSPHATE 4-EPIMERASE

BY D. P. BURMA* AND B. L. HORECKER

(From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service, Bethesda, Maryland)

(Received for publication, September 11, 1967)

Extracts of Lactobacillus plantarum derived from cells grown on L-arabinose contain an active isomerase, which converts L-arabinose to L-ribulose (1, 2), and a ribulokinase, which catalyzes the formation of L-ribulose 5-phosphate (3, 4). Such extracts also catalyze the conversion of L-ribulose 5-phosphate to D-xylulose 5-phosphate, which has been shown to be the key intermediate in pentose fermentation by L. plantarum (5, 6). On the basis of known enzymatic reactions, the accompanying pathway can now be written for the fermentation of L-arabinose in this organism.

The conversion of L-ribulose 5-phosphate to D-xylulose 5-phosphate was first demonstrated by Wolin, Simpson, and Wood (7), with an enzyme isolated from extracts of Aerobacter aerogenes. Since the reaction catalyzed by this enzyme is similar to that catalyzed by UDPG 4-epimerase (8), the enzyme will be named L-ribulose-5-phosphate 4-epimerase. The enzyme has now been obtained in highly purified form from L. plantarum. Its properties and possible cofactor requirements will be considered.

Methods

Materials—L-Ribulose phosphate was prepared from L-ribulose and ATP by the action of ribulokinase (4). D-Xylulose phosphate was obtained by a modification of the method described by Hurwitz and Horecker (9). DPN and TPN were commercial preparations. DPNH was prepared by the method of Ohlmeyer (10). Darco was obtained from the Atlas Powder Com-

* Fellow of the Jane Coffin Childs Memorial Fund for Medical Research, on leave from the Bose Research Institute, Calcutta, India. Present address, Bose Research Institute, Calcutta, India.

1 The following abbreviations are used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; P_i, orthophosphate; UDPG, uridine diphosphoglucose; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; DPNH, dihydrodiphosphopyridine nucleotide; DPNase, diphosphopyridine nucleotidase; Tris, tris(hydroxymethyl)aminomethane; ThPP, thiamine pyrophosphate.

2 The name stereoisomerase (7) has been used for this enzyme; however, in correspondence with Dr. W. A. Wood and his coworkers, it has been agreed that the present nomenclature is more suitable.
pany, Wilmington, Delaware. All other chemicals were commercial preparations.

Potato acid phosphatase was prepared according to Kornberg.\(^3\) Phosphoketolase was purified from extracts of *L. plantarum* as previously described (6). The enzyme purified through ammonium sulfate step I was sufficiently pure for the purposes of this work. Acetokinase was purified from *Escherichia coli* by the method of Rose et al. (11). Spleen and *Neurospora* DPNases were kindly provided by Dr. R. Burton of the National Institute of Neurological Diseases and Blindness and Dr. N. O. Kaplan

\(^3\) Dr. A. Kornberg, unpublished procedure.
of Brandeis University. L-Arabinose isomerase was prepared as already described (2).

\textit{\textbf{D-Xylose Isomerase—}}This enzyme (12), used here for the determination of D-xylose, was partially purified from extracts of xylose-grown \textit{L. plantarum} by the following procedure.

Xylose-grown cells of \textit{L. plantarum} were grown and harvested as previously described (6). The cell paste (2.6 gm.) was placed in the Nossal shaker cartridge (13) with 8 ml. of 0.02 M NaHCO\textsubscript{3} and 8 gm. of Superbrite glass beads and shaken for three 30 second periods, with cooling in ice in between. The mixture was centrifuged, and the residue was washed twice with 6 ml. portions of 0.02 M NaHCO\textsubscript{3}. The combined supernatant solution and washings (18 ml.) were treated with 0.9 ml. of 1 M MnCl\textsubscript{2} and kept at 0° for 30 minutes, and the precipitate was removed by centrifugation and discarded. The supernatant solution (17.5 ml.) was treated with 7.6 gm. of ammonium sulfate and centrifuged. The precipitate which contained about 30 per cent of the total activity was discarded. The supernatant solution (21 ml.) was treated with 3.6 gm. of ammonium sulfate, and the precipitate was collected by centrifugation and dissolved in 2 ml. of 0.1 M Tris buffer, pH 7.0. The final volume was 2.5 ml. The preparation was stable when stored at -16°. D-Ribulose and L-xylulose were inactive with this enzyme, but L-ribulose was slowly converted to L-arabinose, indicating that traces of L-arabinose isomerase were present. With D-xylulose the equilibrium mixture was found to contain 18 per cent of ketopentose at 25°, compared with 13 per cent found by Mitsuhashi and Lampen (12) and 16 per cent found by Hochster and Watson (14).

\textbf{Analytical Procedures—}Unless otherwise indicated the analytical methods used were those described in Paper III. DPNase assays were carried out by the method of Kaplan \textit{et al.} (15). Acetyl phosphate was measured by the method of Lipmann and Tuttle (16). Since this procedure was less sensitive than other methods to be described, it was used only for quantitative measurements and not for routine assay.

In addition to the colorimetric procedures for pentose and ketopentose (2), the following enzymatic methods were employed. For L-ribulose, a specific method was available based on the conversion of this sugar to L-arabinose by L-arabinose isomerase (2). The decrease in cysteine-carbazole-reactive pentose upon treatment of the mixture with L-arabinose isomerase was equal to 90 per cent of the original content of L-ribulose. The presence of other keto or aldopentoses did not interfere in this assay. A similar procedure was employed for the assay of D-xylulose, with the D-xylene isomerase preparation described above. However, in this case, since the enzyme preparation showed some activity with L-ribulose, it was necessary to treat complex mixtures first with L-arabinose isomerase and
then with D-xylose isomerase. The first density change was divided by the factor 0.90 and the second density change by the factor 0.82. In this way, by the successive addition of L-arabinose isomerase and D-xylose isomerase both ketopentoses could be determined in the same reaction mixture (Fig. 1).

![Graph showing isomerase assay for mixtures of L-ribulose and D-xylose](image)

**Fig. 1.** Isomerase assay for mixtures of L-ribulose and D-xylose. The incubation was carried out at 23°C in a total volume of 0.35 ml. containing 2.0 μmoles of L-ribulose, 1.9 μmoles of D-xylose, and 15 μmoles of Tris buffer, pH 7.5. The reaction was begun by the addition of 50 μl of L-arabinose isomerase. Aliquots were taken at the intervals indicated and assayed for ketopentose by the cysteine-carbazole color test. At 60 minutes, when the first equilibrium was reached, 700 μl of D-xylose isomerase were added.

**Enzyme Assay**—For the assay of 4-epimerase the following reactions were used.

(1) \[ \text{L-Ribulose 5-phosphate} \rightleftharpoons \text{D-xylose 5-phosphate} \]

(2) \[ \text{D-Xylulose 5-phosphate} + P_{i} \rightarrow \text{acetyl phosphate-P}\text{P}_{2} + \text{triose phosphate} \]

(3) \[ \text{Acetyl phosphate-P}_{3}\text{P}_{2} + \text{ADP} \rightarrow \text{acetate} + \text{ATP}\text{P}_{3}\]

ATP\text{P}_{3} formed in this system was adsorbed on charcoal (17) and counted as such. In the presence of an excess of phosphoketolase (Reaction 2) and acetokinase (Reaction 3) the rate of formation of ATP\text{P}_{3} was proportional to the quantity of 4-epimerase (Reaction 1) (Fig. 2). The formation of 0.005 μmole of D-xylose 5-phosphate can readily and accurately be measured by this method. P_{32} was counted in an end window counter.

4 This assay procedure was suggested by Dr. J. Hurwitz.
Purification Procedure—L. plantarum, strain 124-2 (ATCC 8041), was grown on L-arabinose as previously described (6). The cell paste was stored at $-16^\circ$ and used as needed for the preparation of the enzyme. Such frozen cells showed no loss of activity in several months. The subsequent operations were carried out at 0–2$^\circ$ unless otherwise stated.

![Graph](http://www.jbc.org/)  
**Fig. 2.** Proportionality of reaction rate to quantity of L-ribulose 5-phosphate 4-epimerase. The incubation mixture (0.13 ml.) contained 12 μmoles of succinate buffer, pH 6.0, 1.0 μmole of MgCl₂, 1.5 μmoles of glutathione, 0.025 μmole of ThPP, 0.9 μmole of ADP, 0.16 μmole of L-ribulose phosphate, 27 units of phosphoketolase, 1.2 units of acetokinase, and 0.8 μmole of orthophosphate-$^{32}$P (10,000 c.p.m.). The reaction was begun by the addition of 4-epimerase and, after 15 minutes at 37$^\circ$, was stopped by the addition of 1 ml. of 2.5 per cent HClO₄. To this solution was added 0.2 ml. of a suspension of charcoal in water (Darco, 10 per cent (w/v)) to adsorb the adenine nucleotides. The charcoal was collected by centrifugation, washed three times with 10 ml. portions of 2.5 per cent HClO₄, filtered onto a sintered disk, washed with water, and transferred to the counter. The quantity of ATP$^{32}$ found was calculated from the known specific activity of the inorganic phosphate added. The heated fraction (specific activity = 179 units per mg., see Table I) was used in this experiment.

Cell extracts were prepared with the Nossal shaker in the same manner as for xylose isomerase. Four batches, corresponding to 12 gm. of packed cells, were combined and centrifuged, and the residue was washed twice with 24 ml. of 0.02 m NaHCO₃. The extracts and washings were combined ("Crude extract," 78 ml.).

The crude extract was treated with 3.9 ml. (0.05 volume) of 1 m MnCl₂, kept for 30 minutes, and centrifuged. The precipitate was discarded. The supernatant solution (75 ml.) was diluted with 44 ml. of cold water,
and 11 ml. of 0.5 m sodium acetate were added. Subsequent fractionation with acetone was carried out at $-10^\circ$. Acetone was added slowly, and the solution was cooled in an alcohol-dry ice bath ($-15^\circ$) so that the final temperature of the solution was about $-8^\circ$. After the first addition of acetone (17.8 ml.), the mixture was kept for 2 minutes and centrifuged for 1 minute in a Servall SS-1 centrifuge at $-10^\circ$. The precipitate was discarded. The supernatant solution was treated in the same way with 18 ml. of acetone, and the precipitate was collected and dissolved in 8 ml. of 0.05 m Tris buffer, pH 7.5 ("Acetone fraction," 8.5 ml.). This fraction was found to be stable when kept frozen overnight, and the following steps were conveniently carried out on the next day.

The acetone fraction was treated with 2.45 gm. of ammonium sulfate and centrifuged, and the precipitate was discarded. The supernatant solution was treated with 0.54 gm. of ammonium sulfate, and the precipitate was collected and dissolved in 5 ml. of 0.05 m Tris buffer, pH 7.5 ("Ammonium sulfate fraction," 5 ml.).

The ammonium sulfate fraction was placed in a bath at 70$^\circ$ and heated to 65$^\circ$. This required about 2 minutes, after which the vessel was transferred to a bath at 65$^\circ$ where it was kept for 5 minutes. The solution was rapidly cooled, and the voluminous precipitate was removed by centrifugation. The precipitate was washed with 1 ml. of cold water. The combined supernatant solution and washing (4.7 ml.) were treated with 1.7 gm. of ammonium sulfate, and the precipitate was collected and dissolved in 1 ml. of 0.05 m Tris buffer, pH 7.5 ("Heated fraction," 1.1 ml.).

The heated fraction was further purified by charcoal treatment. 1 ml. of 10 per cent (w/v) charcoal suspension in water was centrifuged, and the water phase was removed by aspiration. To the charcoal residue was added 1.0 ml. of the heated fraction. After 15 minutes at 0$^\circ$ the solution was centrifuged, and the supernatant solution was decanted ("Charcoal fraction," 1.0 ml.).

A summary of the purification procedure is given in Table I. The final product was about 175-fold purified compared with the crude extract; about 25 per cent of the original activity was recovered. For most purposes the heated fraction was employed.

Properties of Enzyme

Dependence upon pH and Cofactors—The enzyme showed maximal activity in a broad range from pH 7 to 9 and was about 50 per cent of maximal at pH 6.0. However, the routine assay was carried out at pH 6.0 to provide optimal conditions for the action of phosphohexokinase.

In the case of UDPG 4-epimerase, Maxwell (18) showed DPN to be an essential cofactor. With L-ribulose-5-phosphate 4-epimerase no cofactor
requirement has been established. The enzyme preparations showed significant absorption at 260 m\(\mu\), but were not inactivated by treatment with charcoal or anion exchange resins. However, these procedures did not completely remove the material responsible for the absorption at 260 m\(\mu\), indicating the presence of nucleic acid or tightly bound nucleotides.

**TABLE I**

*Purification of L-Ribulosephosphate 4-Epimerase*

<table>
<thead>
<tr>
<th>Step</th>
<th>Total units*</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>795</td>
<td>1.9</td>
</tr>
<tr>
<td>Acetone fraction</td>
<td>564</td>
<td>11.7</td>
</tr>
<tr>
<td>Ammonium sulfate fraction</td>
<td>410</td>
<td>16.7</td>
</tr>
<tr>
<td>Heated fraction</td>
<td>410</td>
<td>179.0</td>
</tr>
<tr>
<td>Charcoal fraction</td>
<td>220</td>
<td>330.0</td>
</tr>
</tbody>
</table>

* A unit was defined as the amount of 4-epimerase which yields 1 \(\mu\)mole of ATP\(^{32}\) under the assay conditions.

**TABLE II**

*Inactivation of 4-Epimerase by DPNase*

<table>
<thead>
<tr>
<th>Condition</th>
<th>With preincubation*</th>
<th>Without preincubation†</th>
</tr>
</thead>
<tbody>
<tr>
<td>No DPNase</td>
<td>0.073</td>
<td>0.070</td>
</tr>
<tr>
<td>With DPNase</td>
<td>0.001</td>
<td>0.076</td>
</tr>
</tbody>
</table>

* 4-Epimerase (0.12 mg. of heated fraction, specific activity = 179 units per mg.) was incubated with or without DPNase, 0.01 ml. (68 units), in a total volume of 0.1 ml. containing 4 \(\mu\)moles of Tris buffer, pH 7.5. After 20 minutes at 37° the solutions were diluted with 4.9 ml. of cold water, and an aliquot (0.01 ml.) was taken for the enzyme assay.

† In this experiment a comparable aliquot of 4-epimerase was assayed directly and with and without 0.01 ml. of 500-fold diluted DPNase added directly to the 4-epimerase assay mixture. A direct effect of DPNase on the assay system is thus excluded.

The enzyme was completely inactivated by incubation with *Neurospora* DPNase (Table II) but was not affected by spleen DPNase. The inactivated enzyme could not be reactivated by the addition of DPN or TPN or boiled crude extract. Furthermore, no DPN or DPNH could be detected in the purified enzyme preparation. With a sensitive catalytic method adequate to detect less than 0.25 mole of DPN or DPNH per 100,000 gm. of protein, none was found.

**Effect of Substrate Concentration**—The effect of L-ribulose 5-phosphate
concentration on the reaction velocity is shown in Fig. 3. The $K_e$ calculated from the Lineweaver-Burk plot (insert in Fig. 3) was $1.1 \times 10^{-2} \text{ M}$. In the absence of a suitable assay method for the reaction in the reverse direction, it was not possible to determine the $K_e$ for $\alpha$-xylulose 5-phosphate. Attempts to measure the disappearance of $\alpha$-xylulose phosphate by assay with phosphoketolase (6) gave inconsistent results. However, it was clear from the equilibrium measurements (see below) that the initial reaction rate was similar with $L$-ribulose phosphate and $\alpha$-xylulose phosphate at comparable concentrations, indicating that the affinity of the enzyme for the two substrates is similar.

**Equilibrium Studies**

Preliminary measurements of the equilibrium carried out with crude enzyme extracts have already been reported (5). However, interfering enzymes present in the crude preparations prevented the attainment of true equilibrium. These measurements have now been carried out with the purified enzyme (Fig. 4). The reaction was followed by measuring the formation or utilization of $\alpha$-xylulose 5-phosphate. With either $L$-ribulose 5-phosphate or $\alpha$-xylulose 5-phosphate as substrate, an equilibrium ratio of $\alpha$-xylulose phosphate to $L$-ribulose phosphate of 1.2 was obtained. To check the final values after equilibrium was reached, the mixtures of phosphate esters were hydrolyzed with potato acid phosphatase and assayed.
for L-ribulose with L-arabinose isomerase and then for d-xylulose with d-xylose isomerase. The values were in good agreement with those obtained by d-xylulose 5-phosphate assay (see Table III).

![Graph showing equilibrium between D-ribulose 5-phosphate and D-xylulose 5-phosphate.](http://www.jbc.org)

**Fig. 4.** The equilibrium between L-ribulose 5-phosphate and d-xylulose 5-phosphate. The reaction was carried out at 37° in a total volume of 3.0 ml. containing 4.0 μmoles of either d-xylulose 5-phosphate or L-ribulose 5-phosphate, 125 μmoles of Tris buffer, pH 7.0, and 12.0 γ of 4-epimerase (heated fraction). 0.5 ml. aliquots were analyzed for d-xylulose 5-phosphate with phosphoketolase as follows: Each aliquot was treated with 0.02 ml. of 0.1 M phosphate buffer, pH 6.0, and 0.05 ml. of 0.5 M succinate buffer, pH 6.0, and heated in a boiling water bath for 1 minute. The turbid suspension was cooled and treated with 3 μmoles of MgCl₂, 9 μmoles of glutathione, 0.1 μmole of ThPP, and 24 units of phosphoketolase; the final volume was made up to 1 ml. After incubation at 37° for 20 minutes, to permit the phosphoketolase reaction to go to completion, the acetyl phosphate formed was measured as described by Lipmann and Tuttle (16).

**Table III**

<table>
<thead>
<tr>
<th>Phosphate ester added</th>
<th>L-Ribulose* μmoles</th>
<th>D-Xylulose* μmoles</th>
<th>Per cent d-xylulose</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Ribulose 5-phosphate</td>
<td>1.8</td>
<td>2.0</td>
<td>52.6</td>
</tr>
<tr>
<td>D-Xylulose</td>
<td>1.7</td>
<td>2.1</td>
<td>55.3</td>
</tr>
</tbody>
</table>

4.0 μmoles of total pentose in each experiment.

*The final equilibrium reaction mixtures (45 minutes) in Fig. 4 were adjusted to pH 5.5 with 0.04 ml. of 1.0 N acetic acid and heated in a boiling water bath for 1 minute. To the cooled solutions were added 0.025 ml. of 1 M MgCl₂ and 0.05 ml. of potato acid phosphatase. After 2 hours at 37° aliquots were assayed as described in the text for L-ribulose and d-xylulose.
The enzyme has been called L-ribulose-5-phosphate 4-epimerase, because it catalyzes a change in configuration at the 4 position. Phosphoketopentosepimerase (19) which catalyzes the interconversion of \( \beta \)-ribulose phosphate and \( \beta \)-xylulose 5-phosphate carries out a similar inversion at the 3 position and a more appropriate name for this enzyme is \( \beta \)-ribulose-5-phosphate 3-epimerase.

Although the reactions bear a superficial resemblance to each other, it is possible that the mechanisms are entirely different. In the case of interconversion catalyzed by the 3-epimerase, since the carbon atom involved is adjacent to a carbonyl group, an ene-diol intermediate is possible (19). Supporting evidence for this mechanism has been obtained by Topper and Hurwitz\(^6\) who have demonstrated the incorporation of tritium into \( \beta \)-xylulose 5-phosphate incubated with 3-epimerase. Purified 3-epimerase, after about a 700-fold purification, shows no cofactor requirement.

The interconversion of the 4-epimers presents a somewhat different problem, since there is no adjacent carbonyl group. A similar epimerization occurs in the formation of uridine diphosphate galactose from uridine diphosphate glucose (20), but in this case Maxwell has shown the enzyme to require DPN (18), although the function of this coenzyme is not clear. With the L-ribulose-5-phosphate 4-epimerase no DPN or other cofactor has been encountered. No inactivation was obtained with charcoal or Dowex treatment; however, the former procedure left significant quantities of nucleotide while none of the material absorbing at 260 m\(\mu\) was removed by Dowex and no definite conclusions can be drawn from these results.

Since the inactivation by DPNase was not reversed by the addition of DPN or TPN or their reduced forms, it cannot be attributed to a destruction of bound DPN, particularly since no DPN could be detected in the purified enzyme preparations.

\(^{6}\) Y. J. Topper and J. Hurwitz, personal communication.
With the isolation of 4-epimerase, all of the enzymatic steps in the fermentation of L-arabinose by L. plantarum are now defined, and the importance of d-xylulose 5-phosphate in the fermentation pathway becomes clear. All of the pentoses which are fermented by this organism are converted to xylulose 5-phosphate which is then cleaved to yield the 2- and 3-carbon fragments (6) which ultimately give rise to acetic acid and lactic acid, the end products of fermentation.

SUMMARY

Extracts of Lactobacillus plantarum, prepared from cells grown on L-arabinose, catalyze the reversible conversion of L-ribulose 5-phosphate to d-xylulose 5-phosphate. This enzyme, L-ribulose-5-phosphate 4-epimerase, has been purified about 170-fold. The equilibrium constant for the reaction L-ribulose 5-phosphate \( \rightleftharpoons \) d-xylulose 5-phosphate is 1.2.

No cofactor requirement has been established for the enzyme. It is not inactivated by treatment with charcoal or anion exchange resin. The enzyme preparations after such treatment still show considerable absorption at 260 nm. 4-Epimerase is inactivated by Neurospora diphosphopyridine nucleotidase, but the inactive enzyme cannot be reactivated by diphosphopyridine nucleotide or by boiled extracts.

A procedure for the preparation of d-xylose isomerase is described. This enzyme, together with L-arabinose isomerase, can be used for the quantitative assay of mixtures of d-xylulose and L-ribulose.

BIBLIOGRAPHY

PENTOSE FERMENTATION BY LACTOBACILLUS PLANTARUM: IV. 1-RIBULOSE-5-PHOSPHATE 4-EPIMERASE
D. P. Burma and B. L. Horecker


Access the most updated version of this article at http://www.jbc.org/content/231/2/1053.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/231/2/1053.citation.full.html#ref-list-1