Enzymatic Basis for \(d\)-Arabitol Production by Saccharomyces rouxii

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ABSTRACT

INGRAM, JORDAN M. (Michigan State University, East Lansing), AND W. A. WOOD. Enzymatic basis for \(d\)-arabitol production by Saccharomyces rouxii. J. Bacteriol. 89: 1186-1194. 1965.—The enzymatic steps in \(d\)-arabitol synthesis by Saccharomyces rouxii were studied. The fermentation of \(d\)-glucose-\(\delta\)-\(C^{14}\) gave rise to \(d\)-arabitol labeled at C-5; \(d\)-ribose of ribonucleic acid had the same isotope pattern. Crude extracts were able to reduce \(d\)-ribulose with reduced nicotinamide adenine dinucleotide phosphate (NADPH\(_2\)) and \(d\)-xylulose with reduced nicotinamide adenine dinucleotide (NADH\(_2\)). These extracts also oxidized \(d\)-arabitol with nicotinamide adenine dinucleotide phosphate and xylitol with nicotinamide adenine dinucleotide. No reduction of \(d\)-ribulose-\(5\)-phosphate or \(d\)-xylulose-\(5\)-phosphate was observed. An enzyme which reduced \(d\)-ribulose with NADH\(_2\) was purified 33-fold and characterized as a xylitol (→ \(d\)-xylulose) dehydrogenase. Similarly, an enzyme reducing \(d\)-ribulose with NADPH\(_2\) was purified 12-fold and characterized as a \(d\)-arabitol (→ \(d\)-ribulose) dehydrogenase. Alkaline and acid phosphatases were purified 50- and 40-fold, respectively, and their specificities were determined. Only the acid phosphatase had detectable activity on \(d\)-ribulose-\(5\)-phosphate. The data support the postulate that \(d\)-arabitol arises by dephosphorylation of \(d\)-ribulose-\(5\)-phosphate and reduction of \(d\)-ribulose by a NADPH\(_2\)-linked \(d\)-arabitol (→ \(d\)-ribulose) dehydrogenase.

Certain osmophilic yeasts grown in the presence of high glucose concentrations produce, in addition to ethanol and \(CO_2\), a variety of polyhydric alcohols; glycerol, erythritol, \(d\)-arabitol, and mannitol have been identified (Spencer and Sallans, 1956). The effects of environmental conditions, salt concentrations, nitrogen sources, and aeration upon production of polyols have been studied in detail (Spencer and Shu, 1957; Onishi, Saito, and Koshiyama, 1961). Saccharomyces rouxii P\(_8\)A, grown on specifically labeled glucose, produced \(d\)-arabitol with labeling patterns consistent with the postulate that the pentitol arose from \(d\)-ribulose-\(5\)-phosphate (Spencer et al., 1956). With the demonstration of a pyridine nucleotide-linked \(d\)-arabitol (→ \(d\)-xylulose) dehydrogenase in bacteria (Wood, McDonough, and Jacobs, 1962), attention has been directed to the mechanism of \(d\)-arabitol formation by these yeasts. Recently, two conflicting reports have appeared stating that \(d\)-xylulose (Blakely and Spencer, 1962) and \(d\)-ribulose (Weinberg, 1962) were immediate precursors of \(d\)-arabitol. It is currently unclear whether \(d\)-arabitol arises by reduction of a ketopentose phosphate followed by a subsequent dephosphorylation, or by reduction of a ketopentose produced by dephosphorylation or other processes. The present report deals with the enzymatic steps leading to the biosynthesis of \(d\)-arabitol in S. rouxii P\(_8\)A.

MATERIALS AND METHODS

Bacteriological. The P\(_8\)A strain of S. rouxii was obtained from the National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan, Canada. The yeast was maintained on a solid medium composed of 60% honey, 1% yeast extract, 0.28% urea, and 2% agar (Spencer and Sallans, 1956). The fermentation medium was identical, except that 20% glucose was substituted for the honey. The yeast was grown aerobically for 96 hr prior to harvesting.

Leuconostoc mesenteroides MAC 246, used for the degradation of \(d\)-xylulose, was supplied by A. C. Blackwood, MacDonald College of McGill University, Montreal, Quebec, Canada. The cultures were maintained and grown on APT medium (Difco).

Analytical. Ketopentoses and pentitols were separated on Dowex-1-borate, as described by Khym and Zill (1952). Ketopentoses were assayed

1 Contribution No. 3520 of the Michigan Agricultural Experiment Station.
by the cysteine-carbazole method (Anderson and Wood, 1962a; Dische and Borenfreund, 1951). Pentitols were determined by the periodate-formaldehyde method of West and Rappoprt (1949). Inorganic phosphate was determined by the method of Fiske and SubbaRow (1925). Protein was determined by the 290:260 ratio method of Warburg and Christian (1942).

Enzymatic. Crude extracts of S. rouxi were prepared from cells washed twice by treatment in a 10-ke, 250-w oscillator for 25 min, followed by centrifugation to remove cytoplasm. Dehydrogenase assays were performed spectrophotometrically with the aid of a Gilford Log Converter (Wood and Gilford, 1961) by observing the rate of reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidation in the presence of ketopentose oxidase. One unit is described as the amount of dehydrogenase producing an absorbance change of 1.0 per minute at 340 nm in a reaction volume of 0.15 ml (1 cm light path). Kinase assays were performed in a manner described by Anderson and Wood (1962a).

The activity of alkaline phosphatases eluted from a diethylaminoethyl (DEAE)-cellulose column was determined from the hydrolysis of p-nitrophenyl phosphate, as described by Torrani (1960). Acid phosphatase was assayed by the release of inorganic phosphate from the various substrates (Fiske and SubbaRow, 1925).

Ribitol (→ d-ribulose) and d-arabitol (→ d-xylulose) dehydrogenases were prepared from Aerobacter aerogenes, as described previously (Wood et al., 1961). Xyitol (→ d-xylulose) dehydrogenase was prepared by gradient elution with KCl of a crude extract of Acetobacter suboxydans from DEAE cellulose (Kersters, Wood, and Deley, J. Biol. Chem., in press). Xyitol (→ l-xylulose) dehydrogenase from guinea pig liver was prepared by the method of Hickman and Ashwell (1959).

Chemical. d-Xylose was isolated after growth of A. suboxydans on d-arabitol (Reichstein, 1934). d-Ribulose was isolated as the D-ribulose monophosphate during isomerization of d-arabinose (Glahar and Reichstein, 1933). d-Xylose-5-phosphate and d-ribulose-5-phosphate were prepared by oxidation of the ketose by phosphatase eluted from the gel of d-xylulokinase and d-ribulokinase from A. aerogenes (Mortlock et al., 1965), according to the method of Simpson and Bhuyan (1962). Glucose-6-C14 was obtained from Volk Radiochemical Co., Skokie, Ill. All other preparations were from commercial sources.

Radiochemical. d-Ribose from ribonucleic acid (RNA) was isolated after growth on glucose-6-C14, as described by Bernstein (1953). The d-ribose was not converted to d-ribose, but was chromatographed on Whatman no. 3 filter paper in ethyl acetate-acetic acid-water (3:1:3, v/v). The d-ribose was detected with silver nitrate, eluted and rechromatographed in butanol-acetic acid-water (4:1:5, v/v). After addition of carrier, the d-ribose-C14 was fermented to acetate and lactate by Lactobacillus arabinosus (Bernstein and Wood, 1957).

d-Arabinol-C14 produced from the fermentation of glucose-6-C14 was converted to d-xylulose-C14 with d-arabitol (→ d-xylulose) dehydrogenase from A. aerogenes. The d-arabitol was incubated in the presence of nicotinamide adenine dinucleotide (NAD), pyruvic acid, lactic acid dehydrogenase, and tris(hydroxymethyl)aminomethane (Tris) buffer, pH 10.0 (Wood et al., 1961). The reaction was followed to completion by determining d-xylulose formation with the cysteine-carbazole test. d-Xylose-C14 was chromatographed in butanol-acetic acid-water, eluted, and diluted with carrier d-xylulose. d-Xylulose-C14 was fermented to acetate and lactate with L. mesenteroides (Spence et al., 1956).

The acetate was separated from lactate by steam distillation, and the lactate was recovered by continuous ether extraction. Each was then purified by Celite chromatography (Bernstein and Wood, 1957). Lactate was oxidized with permanganate to give acetate from C-2 and C-3 of lactate and CO2 from C-1 (Abraham and Hassid, 1957). The acetate was purified by Celite chromatography and degradative procedures as described by Phares (1951) in the apparatus described by Krichevsky and Wood (1961). CO2 from C-1 of acetate was collected and counted in 0.25 mM NaOH. The methylenic from C-2 of C-3 of lactate was distilled and isolated as the hydrochloride salt. Radioactivity was determined in the Packard Tri-Carb liquid scintillation spectrometer in the counting system described by Baldwin, Wood, and Emery (1963).

RESULTS

Labeling patterns of d-ribose from RNA and d-arabitol produced from glucose-6-C14. Theoretically, d-arabitol could arise either by reduction of d-ribose, d-xylulose, or their respective phosphate esters. Production of either d-ribose or d-xylulose from glucose-6-C14 via the hexose monophosphate pathway or from fructose-6-phosphate via transketolase and transaldolase would yield these structures labeled at C-5. However, reduction of d-ribose-5-C14 would yield d-arabitol-5-C14, whereas reduction of d-xylulose-5-C14 would yield d-arabitol-1-C14. d-Ribose-C14 from RNA was examined as an indicator of the labeling pattern of the metabolic pool of pentose phosphates. The d-ribose and d-arabitol were isolated, fermented to lactate and acetate, and the acids degraded carbon by carbon as described in Materials and Methods. Table 1 shows the distribution of radioactivity in the pentose and pentitol. It is evident that the C-6 of glucose becomes C-5 of both d-ribose and d-arabitol, and that virtually no activity is found in the other carbon atoms. Also, the recovery of the activity at the completion of the procedure
TABLE 1. Isotopic patterns in d-ribose and d-arabitol from Saccharomyces rouxii grown on glucose-6-C\textsuperscript{14}

<table>
<thead>
<tr>
<th>Carbon no.</th>
<th>d-Ribose</th>
<th>d-Arabitol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.07</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>3</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>4</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>5</td>
<td>1.11</td>
<td>1.40</td>
</tr>
</tbody>
</table>

* Expressed as disintegrations per minute per millimole. Initial specific activity: d-ribose, 1.29 × 10\textsuperscript{4}; d-arabitol, 1.60 × 10\textsuperscript{4}.

Fig. 1. Reduction of ketopentoses in the presence of NADPH\textsubscript{2} (left) and NADH\textsubscript{2} (right). Each reaction contained: 0.1 μmole of NADPH\textsubscript{2} or NADH\textsubscript{2}; 5.0 μmoles of substrate; 25 μmoles of cacodylate buffer (pH 6.0); and enzyme and water to a total volume of 0.15 ml (1-cm light path).

was 85%, indicating the acceptability of the procedure. The absence of cross-contamination in the degradation procedures for lactate and acetate has been established (Baldwin et al., 1963).

Pentitol dehydrogenases of crude extracts. Crude extracts of S. rouxii catalyzed the reduction of d-ribulose with NADPH\textsubscript{2} and d-xylulose with NADH\textsubscript{2} (Fig. 1). These represent the principal polyol-producing reactions of extracts and, hence, probably of whole cells (Table 2). It is particularly significant that reductions of d-ribulose-5-phosphate and d-xylulose-5-phosphate were not observed. Table 3 illustrates the ability of crude extracts to oxidize the four pentitols. These reactions performed against an unfavorable equilibrium were aided by trapping the ketopentose formed in semicarbazide buffer, thereby favoring the accumulation and measurement of reduced nicotinic acid adenine dinucleotide. In harmony with the results shown in Fig. 1, xylitol was oxidized in the presence of NAD; d-arabitol, and to a lesser extent xylitol, were oxidized in the presence of nicotinamide adenine dinucleotide phosphate (NADP).

Purification and properties of xylitol (→ d-xylulose) dehydrogenase. Since d-arabitol is the product formed by the reduction of d-xylulose and NADH\textsubscript{2} in extracts of A. aerogenes (Wood et al., 1962), the d-xylulose-reducing activity from S. rouxii was purified to facilitate identification of the reduction product. A 33-fold purification was obtained by: (i) precipitation of the nucleic acids with 0.4% protamine sulfate in the presence of 0.1 M ammonium sulfate; (ii) precipitation of the activity between 50 and 70% saturation of ammonium sulfate; (iii) adsorption on calcium phosphate gel (3.4 mg of gel per mg of protein) and elution with 0.2 M phosphate buffer,
Table 4. Identification of reduction and oxidation products of purified xylitol (→ D-xylulose) dehydrogenase from Saccharomyces rouxii

<table>
<thead>
<tr>
<th>Test</th>
<th>Pentitol from D-xylulose reduction</th>
<th>Authentic xylitol</th>
<th>Ketose from xylitol oxidation</th>
<th>Authentic D-xylulose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periodate-formaldehyde test</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cysteine-carbazole test (time for maximal color)</td>
<td>Negative</td>
<td>Negative</td>
<td>&lt;60 min</td>
<td>&lt;60 min</td>
</tr>
<tr>
<td>Paper chromatography R_p, butanol-acetic acid-water (4:1:5)</td>
<td>0.39</td>
<td>0.39</td>
<td>Rose-gray</td>
<td>Rose-gray</td>
</tr>
<tr>
<td>p-Dimethylphenaline spray†</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Dowex-1-borate chromatography</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Enzyme tests</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Arabitol (→ D-xylulose) dehydrogenase</td>
<td>Negative</td>
<td>Negative</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Xylitol (→ D-xylulose) dehydrogenase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Xylitol (→ L-xylulose) dehydrogenase</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ribitol (→ D-ribulose) dehydrogenase</td>
<td>—</td>
<td>—</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-Xylulokinase</td>
<td>—</td>
<td>—</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

* A dash indicates that the test was not run.
† Koch, Geddes, and Smith (1951).

pH 7.0; (iv) precipitation with ammonium sulfate between 60 and 75% saturation.

Reaction characteristics of the dehydrogenase were examined with the partially purified preparation. The enzyme exhibited a sharp pH optimum of pH 6.0 for D-xylulose reduction. The Michaelis constant (K_m) for D-xylulose in cacodylate buffer (pH 6.0) was 1.2 × 10^{-2} M.

For identification of the pentitol and ketopentose involved, the reactions were performed at pH 6 in the presence of D-xylulose and NADH, or at pH 9.2 with xylitol, NAD, sodium pyruvate, and lactic dehydrogenase. The reactions were terminated by the addition of perchloric acid, and the perchlorate was removed by neutralization with KOH. The supernatant liquid was deionized by passage through IR-120 (H^+) and IR-45 (OH⁻). The ketopentose or pentitol was purified by chromatography on Dowex-1-borate, and the eluates containing the peak were treated with methanol to remove the borate, as described by Khym and Zill (1952).

The products of ketopentose reduction and pentitol oxidation were examined after purification. Paper chromatography, Dowex-1-borate chromatography, and specific enzymes were used for positive identification of the pentitol. The cysteine-carbazole test, as well as paper chromatography and specific enzyme tests, was used to identify the ketopentose formed on pentitol oxidation. The product formed by reduction of D-xylulose gave a positive periodate-formaldehyde test and a negative cysteine-carbazole test (Table 4). The R_p value on paper chromatography (detected with silver nitrate) was identical to that of authentic xylitol and the product co-chromatographed with xylitol on Dowex-1-borate. When tested as a substrate for specific enzymes, it again behaved as did xylitol. Thus, it was evident that D-xylulose is reduced to xylitol.

In a similar manner, the compound isolated after oxidation of xylitol behaved identically to D-xylulose in the same series of tests. Based upon these identifications, the enzyme is a xylitol (→ D-xylulose) dehydrogenase.

Purification and properties of D-arabitol (→ D-ribulose) dehydrogenase. The dehydrogenase reducing D-ribulose with NADPH, was purified 12-fold by the following steps: (i) precipitation of nucleic acids with 0.4% protamine sulfate in the presence of 0.1 M ammonium sulfate; (ii) precipitation of the activity between 50 and 75% saturation of ammonium sulfate; and (iii) adsorption to calcium phosphate gel and elution with 0.2 M phosphate buffer, pH 7.0.

Characteristics of the partially purified enzyme preparations were examined as previously described. The enzyme for D-ribulose reduction had a broad pH optimum at pH 6.0. The K_m for D-ribulose in cacodylate buffer (pH 6.0) was 2.0 × 10^{-2} M. Products of D-ribulose reduction and D-arabitol oxidation were prepared and purified as described above for D-xylulose and xylitol, except that NADP was regenerated in a system containing glutamic dehydrogenase, which was present in the crude extract, α-ketoglutarate, and ammonium hydroxide-ammonium chloride buffer, pH 9.4 (Weimberg, 1962). In a combination of chemical, chromatographic, and
enzymatic tests (Table 5), the reduction product corresponded closely to authentic d-arabitol, and the oxidation product to d-ribulose. These identifications strongly suggested that the enzyme is a d-arabitol (→ d-ribulose) dehydrogenase.

**Formation of d-ribulose by phosphatase action.** Since d-ribulose, rather than d-xylulose or a ketopentose phosphate, appeared to be the immediate precursor of d-arabitol, the path of free d-ribulose formation in *S. rouxii* was investigated. Both alkaline and acid phosphatases were found in crude extracts, and both were purified and examined for activity on d-ribulose-5-phosphate. All attempts to demonstrate the existence of a specific phosphatase for d-ribulose-5-phosphate have failed.

Alkaline phosphatase was purified by: (i) treatment of the crude extract in 0.1 M ammonium sulfate with 0.2% protamine sulfate, (ii) chromatography on DEAE cellulose previously equilibrated with 0.02 M Tris buffer (pH 8.0). One major and two minor peaks were batched with 0.05 to 0.2 M NaCl.0.02 M Tris buffer (pH 8.0). The major peak was diluted to 0.02 M and rechromatographed on DEAE cellulose. One peak was obtained when the Tris concentrations varied (0.1 to 0.3 M, pH 8), and the NaCl concentration was constant at 0.1 M. This procedure yielded a 50-fold purified alkaline phosphatase. The phosphatase was active on a large number of phosphate esters, especially nucleotides, but no activity was observed with d-ribulose-5-phosphate (Table 6).

The acid phosphatase was purified 40-fold by: (i) adjusting the pH of the crude extract to 3.5 with HCl and removing foreign protein by centrifugation; (ii) precipitation of the majority of remaining protein by ammonium sulfate, 90% saturation; (iii) exhaustive dialysis against 0.02 M Tris buffer (pH 7.0); (iv) lyophilization to dryness and solution of the residue in a small volume of water. The specificity towards various phosphate esters is shown in Table 6. A series of carbohydrate phosphate esters, including d-ribulose-5-phosphate and d-xylulose-5-phosphate, were hydrolyzed; however, the activity was considerably higher on hexose phosphates.

**Coupled phosphatase-pentitol dehydrogenase reactions.** In exploratory experiments, it was found that the phosphatase could be assayed spectrophotometrically in the presence of d-ribulose-5-phosphate, NADH₂, and excess ribitol (→ d-ribulose) dehydrogenase purified from *A. aerogenes*. The reaction was dependent upon d-ribulose-5-phosphate, ribitol (→ d-ribulose) dehydrogenase, and phosphatase (Fig. 2).

In a large scale reaction, ribitol was identified enzymatically as the final end product with ribitol (→ d-ribulose) dehydrogenase. The product did not react with d-arabitol (→ d-ribulose) dehydrogenase from *A. aerogenes*. The *Kₘ* for d-ribulose-5-phosphate, as determined in this assay, was 4.0 × 10⁻⁴ M. The phosphatase was inactivated by heating at 70°C for 10 min, and inhibited 96% by fluoride ion (6.7 × 10⁻² M) and 90% by phosphate ion (6.7 × 10⁻² M).

In a similar manner, the phosphatase was coupled to the d-arabitol (→ d-ribulose) dehydrogenase purified from *S. rouxii*. Figure 2 shows the dependence of the reaction upon substrate, dehydrogenase, and phosphatase. The product of the coupled reaction was identified as d-arabitol with d-arabitol (→ d-xylulose) dehydrogenase. The product gave no reaction in the presence of ribitol (→ d-ribulose) dehydrogenase.

**DISCUSSION**

The specificity of the pentitol dehydrogenases and identification of the products formed in both the oxidation of pentitols and the reduction of
Table 6. Substrate specificity for purified alkaline and acid phosphatases from Saccharomyces rouxii.

<table>
<thead>
<tr>
<th>Substrate*</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alkaline</td>
</tr>
<tr>
<td></td>
<td>phosphatase</td>
</tr>
<tr>
<td>Adenosine monophosphate</td>
<td>1.22</td>
</tr>
<tr>
<td>Adenosine diphosphate</td>
<td>0.87</td>
</tr>
<tr>
<td>Adenosine triphosphate</td>
<td>0.03</td>
</tr>
<tr>
<td>Cytosine monophosphate</td>
<td>0.85</td>
</tr>
<tr>
<td>Guanosine monophosphate</td>
<td>1.35</td>
</tr>
<tr>
<td>Uracil monophosphate</td>
<td>1.00</td>
</tr>
<tr>
<td>D-Ribulose-5-phosphate</td>
<td>2.00</td>
</tr>
<tr>
<td>D-Xylose-5-phosphate</td>
<td>1.00</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>0.01</td>
</tr>
<tr>
<td>6-Phosphogluconate</td>
<td>0.01</td>
</tr>
<tr>
<td>Fructose-6-phosphate</td>
<td>—</td>
</tr>
<tr>
<td>Fructose-1,6-diphosphate</td>
<td>—</td>
</tr>
<tr>
<td>D-Ribose-5-phosphate</td>
<td>0.03</td>
</tr>
<tr>
<td>a-Glycerophosphate</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* Reaction mixture contained for alkaline phosphatase: 5 μmoles of substrate; 5 μmoles of MgCl2; 80 μmoles of Tris buffer, pH 9.0; enzyme and water to 1.0 ml. For acid phosphatase, the reaction mixture contained: 2.5 μmoles of substrate; 2.5 μmoles of MgCl2; 80 μmoles of acetate buffer, pH 5.5; enzyme and water to 1.0 ml. The same amount of alkaline phosphatase, eluted from DEAE cellulose, was used for each substrate. Similarly, a constant amount of lyophilized acid phosphatase was used for the assay of each substrate.

† Activity of alkaline phosphatase expressed as micromoles of phosphate released per 30 min.

‡ Acid phosphatase activity expressed as that amount of enzyme which caused an absorbancy change of 1.0 at 600 μm in a reaction volume of 1.0 ml in 5 min.

ketopentoses by crude extracts or purified preparations demonstrate the presence of only one route of d-arabitol production; this route is the reduction of d-ribose in the presence of NADPH2. No reduction of d-ribose-5-phosphate or d-xylulose-5-phosphate with NADH2 or NADPH2 has ever been observed. Contrary to the specificity of the d-arabitol (→ d-ribose) dehydrogenase of A. aerogenes, the reduction of d-ribose by the dehydrogenase of S. rouxii yields xylitol, as is the case in mammalian liver (Hickman and Ashwell, 1959).

That d-arabitol (→ d-ribose) dehydrogenase functions in vivo in d-arabitol formation is strongly supported by isotope data from the fermentation of d-glucose-C14. C-6 of glucose gives rise to C-5 of both d-ribose of RNA and d-arabitol, each with similar specific activities. Thus, it is likely that the precursors of each are in equilibrium with, or identical to, d-ribose-, d-ribose-, and d-xylulose-5-phosphates. In addition, the labeling patterns have unique value in determining which of the possible ketopentoses is directly responsible for d-arabitol synthesis. In the reduction of d-ribose, C-5 would become C-5 of d-arabitol, whereas, in the reduction of d-xylulose to d-arabitol, C-5 would become C-1 of d-arabitol. Clearly, the latter possibility is not supported by the isotope data.

d-Ribulose can arise by the action of a phosphatase on d-ribose-5-phosphate, but a specific d-ribose-5-phosphate phosphatase has not been found. Furthermore, the probability of the existence of a specific d-ribose-5-phosphate phosphatase seems to be diminished by the fact that mannitol, erythritol, and glycerol are also often produced, presumably from the ketoses derived from phosphate esters. This suggests that a number of phosphate esters are attacked by a nonspecific phosphatase and then reduced to a polyol.

The acid phosphatase of baker's yeast is reported to be associated primarily with the external cell surface (Schmidt et al., 1963). If a similar situation existed in S. rouxii, d-ribose-5-phosphate would be unavailable for dephosphorylation. However, Suomalainen, Linko, and Ours (1960) reported that approximately 30% of baker's yeast acid phosphatase was located intracellularly. In a similar study, Tonino and Steyn-Parvé (1963) concluded that the presence and location of nonspecific phosphatases depended upon the type of yeast, the age of the
culture, and the composition of the growth medium. Preliminary experiments with whole cells of *S. rouxii* indicated an inability to dephosphorylate D-ribose-5-phosphate, as measured by the liberation of inorganic phosphate. Secondly, the optimal pH of whole baker's yeast acid phosphatase is between 3 and 4 (Schmidt et al., 1963). The activity at pH 6.0 is negligible, whereas the optimum of the acid phosphatase of *S. rouxii* is between 5.5 and 6.5. Apparently, the acid phosphatase of *S. rouxii* does not resemble that of baker's yeast and, hence, may not be located entirely at the cell surface. Although not tested directly, it is considered likely that D-ribose-5-phosphate is available for attack by acid phosphatase.

The demonstrated activity of an acid phosphatase on D-ribose-5-phosphate provides a means for D-ribose formation. However, this phosphatase has high activity in vitro on a number of other sugar phosphates, and an indiscriminate dephosphorylation of all phosphate esters might be expected. Indeed, the production of mannitol, erythritol, and glycerol may result from this lack of specificity, as well as from the presence of the necessary dehydrogenases. The extent of indiscriminate phosphatase action may be regulated by the steady-state levels, the *Km* values of the phosphatase, and the turnover velocity of the various esters. For example, it is doubtful that D-xylulose-5-phosphate is hydrolyzed, perhaps due to one of the above parameters, because neither D-xylulose nor xylitol has been found in fermentation liquor or cell-free extracts. On the other hand, D-fructose, rather than D-fructose-6-phosphate, is reduced with NADH, presumably to mannitol. Further studies of the relative quantities of the phosphate esters present during the fermentation will be necessary to answer this question.

The stimulatory effect of a low concentration of phosphate ion and a high oxygen tension on the conversion of glucose to D-arabitol, reported by Spencer and Shu (1957), may now be partially understood. Weinberg and Orton (1963) reported that the synthesis of acid phosphatase by *S. mellis* is inhibited by a high extracellular inorganic phosphate concentration. This in vivo effect was not investigated in *S. rouxii*, but the activity of the purified acid phosphatase was found to be markedly inhibited by inorganic phosphate. Thus, it appears that both the synthesis and activity of the acid phosphatase may be under regulation of the intracellular inorganic phosphate concentration. The stimulation of D-arabitol synthesis by a high oxygen tension is considered to result from an increased glucose oxidation through the hexose monophosphate pathway, accompanied by an accumulation of NADPH. This would be accompanied by an accumulation of phosphate esters and a decrease in the level of inorganic phosphate. The acid phosphatase, now more active at low phosphate concentration, is able to dephosphorylate the first ketopentose formed, D-ribose-5-phosphate, to D-arabitol, with regeneration of inorganic phosphate. D-Arabitol (→ D-ribose) dehydrogenase then regenerates NADP by the reduction of D-arabitol to D-arabitol. Since the oxidation of 1 mole of glucose to 1 mole of D-arabitol-5-phosphate generates 2 moles of NADPH, sufficient NADPH is available for both biosynthetic and maintenance reactions and for production of D-arabitol, as described. The stoichiometry of D-ribose-5-phosphate formation vs. NADPH formation may be balanced through an independent route to D-ribose-5-phosphate from D-fructose-6-phosphate via transaldolase and transketolase reactions.

An increased yield of D-arabitol has been reported to be coupled to a decreased yield of ethanol under aerobic conditions (Spencer and Shu, 1957). This would be expected for increased participation of the hexose monophosphate pathway and the regeneration of NADP via D-arabitol formation. These considerations indicate that NADPH is not readily oxidized by molecular oxygen, but that NADP is regenerated by a system analogous to the anaerobic produc-

![Fig. 3. Metabolic reactions leading to synthesis of D-arabitol from D-glucose by *Saccharomyces rouxii*.](image-url)
tion of ethanol and regeneration of NAD by alcohol dehydrogenase.

The overall sequence of reactions for D-arabitol synthesis is illustrated in Fig. 3. The plausibility of this scheme is further supported by the observed synthesis of D-arabitol from a mixture of purified acid phosphatase, D-arabitol (→ d-ribulose) dehydrogenase, NADPH, and d-ribulose-5-phosphate.

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Literature Cited


Weimberg, R., and W. L. Ortong. 1963. Repres-

