Biological Mechanisms Involved in the Formation of Deoxy Sugars

VII BIOSYNTHESIS OF 6-DEOXY-L-TALOSE*

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SUMMARY

Several microorganisms have been reported to contain the unusual sugar 6-deoxy-L-talose as a cell wall component. It is the objective of this paper to present experimental evidence for the biosynthesis of this sugar in a cell-free extract of *Escherichia coli* 045 according to the following reaction sequence:

$$\begin{align*}
\text{TDP-}D-\text{glucose} & \xrightarrow{\text{TDP-glucose oxido-reductase}} \text{TDP-6-deoxy-D-xylo-4-hexosulose} \\
\text{TDP-6-deoxy-D-xylo-4-hexosulose} & \xrightarrow{3,5-\text{epimerase}} \text{[TDP-6-deoxy-L-lyxo-4-hexosulose]} \\
\text{[TDP-6-deoxy-L-lyxo-4-hexosulose]} & \xrightarrow{4-\text{reductase}} \text{TDP-6-deoxy-L-talose}
\end{align*}$$

The over-all reaction yielding thymidine diphospho (TDP)-6-deoxy-L-talose was first established using crude enzyme preparations catalyzing Reactions 1, 2, and 3. Unambiguous proof for the formation of the L isomer of 6-deoxytalose was provided by radioisotope dilution experiments. Purification of sonic extracts of *E. coli* 045 into fractions catalyzing individual steps of the above scheme revealed intermediary formation of TDP-6-deoxy-D-xylo-4-hexosulose. This intermediate was used as substrate to study Reactions 2 and 3. As indicated by brackets, TDP-6-deoxy-L-lyxo-4-hexosulose is a postulated enzyme-bound intermediate. TDP-4-Keto-L-rhamhose-3,5-epimerase (3,5-epimerase) and TDP-6-deoxy-L-talose dehydrogenase (4-reductase) were separated by conventional methods. Neither fraction alone was capable of 6-deoxyhexose biosynthesis, only when combined TDP-6-deoxy-L-talose was formed. When 3,5-epimerase and 4-reductase isolated from *Pseudomonas aeruginosa* were examined, the reaction sequence proceeded as outlined above except that TDP-6-deoxy-L-mannose (L-rhamnose) was the end product. Mixing of 3,5-epimerase from *E. coli* 045 and 4-reductase from *P. aeruginosa* resulted in net 6-deoxyhexose biosynthesis with TDP-L-rhamnose as the only product. Conversely, when 3,5-epimerase from *P. aerugi- nosa* was mixed with 4-reductase of *E. coli* 045, TDP-6-deoxy-L-talose was the sole product of the reaction sequence. Thus, it appears that 3,5-epimerase and 4-reductase isolated from different sources can work together, but the product of the reaction sequence is determined by the stereospecificity of 4-reductase.

Unique properties concerning the stability of TDP-6-deoxy-L-talose which are quite different from other sugar nucleotides are reported. Exposure to mild alkaline conditions results in quantitative degradation to thymidine monophosphate and 6-deoxy-L-talose-1,2-monophosphate. A specific degradation procedure to establish the structure of the cyclic sugar monophosphate is presented.

Some of the structural entities of bacterial cell walls responsible for antigenic specificity are 6-deoxyhexoses (3, 4). The role of 6-deoxyhexoses as immunological determinants has stimulated interest in their biosynthesis and has led to the elucidation of 6-deoxyhexose pathways in bacterial (5) and plant (6) as well as in mammalian systems (7).

Recently, several reports have documented the occurrence of a rare 6-deoxy sugar, 6-deoxytalose, as a cell wall component in several different microorganisms. 6-Deoxy-D-talose was found in the capsular polysaccharide of a gram-negative soil bacterium (8). The isolation and identification of the L isomer of 6-deoxytalose was first accomplished by MacLennan from the cell wall polysaccharide of *Actinomyces bovis* (9). The same sugar has been found in at least one strain of *Escherichia coli* (3) and several...
strains of *Mycobacterium* (10, 11). Similarly, analysis of the lipopolysaccharides of several strains of the genus *Citrobacter* (12) revealed the presence of 6-deoxytalose. Recently, 6-deoxy-L-talose was reported to be a constituent of an antigenic heteroglycan isolated from *Streptococcus bovis* (13).

The reaction involved in the biosynthesis of 6-deoxyhexoses have been studied in some detail for a variety of deoxy sugars (14) with the exception of 6-deoxy-L-talose. It is the purpose of this paper to present data for the enzymatic conversion of thymidine diphosphoglucose to thymidine diphospho-6-deoxy-L-talose and to describe some properties of the enzymes involved in these conversions. 6-Deoxy-L-talose biosynthesis proceeds in the following way.

\[
\text{TDP-}\text{d-glucose} \rightarrow \text{TDPG}^+ \rightarrow \text{TDP-6-deoxy-\text{n-xylo-4-hexosulose}}
\]

\[
\text{TDP-6-deoxy-\text{d-xylo-4-hexosulose}} \rightarrow \text{3,5-epimerase} \rightarrow \text{TDP-6-deoxy-\text{d-xylo-4-hexosulose}}
\]

\[
\text{TDP-6-deoxy-\text{d-xylo-4-hexosulose}} \rightarrow \text{4-reductase} \rightarrow \text{TDP-6-deoxy-L-talose}
\]

\[
\text{NADPH} + \text{H}^+ \rightarrow \text{NADP}^+
\]

In addition we wish to report unusual lability of TDP-6-deoxy-L-talose under alkaline conditions which results in formation of TMP and 6-deoxy-L-talose-1,2-monophosphate.

**MATERIALS AND METHODS**

L-Fucose and D-fucose were purchased from Pfannstiel Chemicals. TDP-glucose, NADH and NADPH were commercial preparations from Calbiochem and Boehringer. TDP(4,4'-methylenebis)[d-glucose] and TDP-[3-3H]glucose were prepared as described earlier (15, 16).

**Chromatography and Electrophoresis**—Descending paper chromatography was carried out on Whatman No. 1 paper at room temperature in the following solvent systems: I, pyridine-ethanol-acetic acid-H2O (1:3.6:1.15, v/v); II, ethanol-1 m ammonium acetate, pH 7.0 (7:3, v/v); III, ethanol-1 m ammonium acetate, pH 3.8 (7:3, v/v). High voltage electrophoresis was performed in a Gilson model D Electrophorator in 0.05 M citrate buffer, pH 4.0, for 30 min at 80 volts per cm using Whatman No. 1 paper.

**Thin Layer Chromatography**—PEI Chromatography (V)—PEI plates (Brinkmann) were used in the following solvent systems: A, 10% aqueous ethyleneglycol containing 1% Na2BO3, 10H2O, 2% H3BO3, and 0.75% LiCl; B, 10% aqueous ethyleneglycol containing 2% NaH2SO4; C, 1 m acetic acid and 0.6 m LiCl. The plates were developed at room temperature. Elution of compounds from PEI chromatograms was carried out with 1.5 m LiCl in 0.05 m acetic acid.

**TLC-Silica Gel Chromatography**—TLC-silica gel was carried out on Silica Gel G (Merck) in the following solvent systems: a, ethylacetate-pentane (3:1, v/v); b, benzene methanol (99:1, v/v); c, benzene-methanol (95:5, v/v); d, benzene-methanol (90:10, v/v). Solvent systems a to d were used with plates spread with a suspension of 25 g of silica gel in 62 ml of water and activated for 30 min at 110°. The time for development was about 1 hour. Micoscopic slides (75 x 25 mm) coated with Silica Gel G were used for analytical purposes. The following detection methods on Silica Gel G plates were used: (a) examination with ultraviolet light; (b) spraying with 10% sulfuric acid in methanol followed by heating on a hot plate; most organic compounds will form brown to black spots; (c) spraying with 2,4-dinitrophenylhydrazine (0.1 g of 2,4-dinitrophenylhydrazine in 5 ml of concentrated HCl and 95 ml of ethanol); (d) detection of hydrophobic sugar derivatives by spraying with water.

**Location of Compounds on Paper and PEI Plates**—Compounds with ultraviolet absorption were located on paper as well as on thin layer plates with an ultraviolet lamp (Universalight). Reducing sugars were detected on paper by the silver nitrate dip reagent (19). Location of phosphorylated intermediates on PEI plates was carried out as follows. The plate was sprayed with a mixture containing 4.3 ml of 70% perchloric acid, 10 ml of 1 n HCl, and 25 ml of 4% (w/v) ammonium molybdate, and was diluted with water to a total volume of 100 ml of reagent. After spraying, the plates were heated for a few minutes in a 100° oven and exposed to ultraviolet light until the blue color appeared. Exposure to moist air may accelerate the color development. Scanning of chromatograms with radioactive compounds was carried out with a Packard model 7201 radiochromatogram scanner with thin layer attachment.

**Analytical Procedures**—Phosphorus was determined by the method of Ames and Dubin (20). 6-Deoxyhexoses were measured by the cysteine-sulfuric acid method according to Dische (21). When fucose is used as a standard in the 3-min cysteine-sulfuric acid determination, 6-deoxytalose gave 1.5 times the color value given by fucose. Reducing sugar was determined by the method of Nelson (22). For radioactivity measurement a liquid scintillation system Mark I, Nuclear-Chicago was used. To 1 ml of aqueous sample 10 ml of Triton X-100 mixture were added (23). Melting points were determined using a Kofler hot stage.

**Chemical Synthesis of 6-Deoxy-L-talose**—In order to facilitate identification of the enzymatically formed sugar, authentic 6-deoxy-L-talose was prepared according to Collins and Overend (24) and was isolated by paper chromatography in Solvent I. The product was characterized as 1-methyl-2,3,4-tri-O-acetyl-6-deoxy-L-talopyranose; m.p. 93.5° [α]D = −76.7° (c = 0.65 in methanol).

**Growth and Harvesting of Cells**—A culture of *E. coli* 045 (kindly provided by Dr. B. Jann and Dr. K. Jann, Max Planck Institute for Immunobiology, Freiburg, Germany), kept on nutrient agar slants (Difeo), was used for inoculation of growth medium containing 17.5 g per liter of Antibiotic Medium No. 3 (Difeo). Four 1-liter Erlenmeyer flasks, each containing 250 ml of medium, were placed in a gyratory shaker (New Brunswick) at 37°. The optical density was followed at 750 nm and the cells were grown...
up to early logarithmic growth phase (O.D. at 750 nm about 0.800). The cells were harvested by centrifugation and washed with ice-cold water. The total yield of cells was about 2 g wet weight. The cells were suspended in ice-cold 0.025 M Tris-chloride buffer, pH 8.0, containing 0.001 M MgCl₂, 0.01 M β-mercaptoethanol, and 0.01 M sodium EDTA. About 5 ml of buffer were used per g wet weight of cells. The cells were disrupted by sonication (Heat Systems, Ultrasound, Inc.) for a total of 60 s in 10-s pulses. The temperature of the solution remained below 10° at all times. The cell debris was removed by centrifugation at 15,000 x g for 20 min and the supernatant solution (about 15 to 20 mg of protein per ml) was used for the experiments.

Preparation of TDP-6-deoxy-d-xylo-4-hexosulose—Incubation mixtures contained, in a total volume of 200 μl: TDP-glucose, 0.1 μmole; Tris-chloride buffer, pH 8.0, 2.5 μmoles; β-mercaptoethanol, 0.1 μmole; and 25 to 50 μl of the above sonic extract. The reaction mixture was kept for 1 hour at 37°. Termination of the incubation was accomplished by the addition of 900 μl (3 volumes) of ethanol and heating of the sample in a boiling water bath for 1 min. For preparative purposes, it is suggested that up to 10 individual incubation mixtures be prepared simultaneously, each of them containing 0.1 μmole of sugar nucleotide. The samples were combined, and protein was precipitated and removed by centrifugation. The supernatant liquid was evaporated under reduced pressure to a small volume (200 μl). The solution was diluted to 5 ml with water, the pH was adjusted to 1.0 with 1 N acetic acid, and 10 mg of Norit were added. The charcoal was washed with water and the nucleotide was eluted with 0.5% ammonia in 50% ethanol. The ethanol was removed by evaporation under reduced pressure. An aliquot was tested for completeness of the reaction by the addition of NaOH to a final concentration of 0.1 m. A spectrophotometric reading at 318 nm was taken after 15 min at 37°. (E₃₁₈ = 4.8 x 10⁹) and between 70 and 75% of the expected 4-keto intermediate was recovered. In each instance the purity of the product was established by reduction of an aliquot with sodium borohydride followed by acid hydrolysis and chromatography in Solvent I. The only sugars released from the nucleotide were found to be 6-deoxy-d-glucose and 6-deoxy-d-galactose.

Preparation of TDP-6-deoxy-d-β-[3-3H]xylo-4-hexosulose—The identical procedure was followed as outlined above except that the starting material used was TDP-[β-3H]glucose (16). It is advantageous to use purified preparations of TDPG oxidoreductase (25) for preparation of tritiated intermediates to avoid exchange with the medium.

RESULTS

Identification of TDP-6-deoxy-l-talose—The first objective of our investigation was to provide experimental evidence that the end product of Reactions 1, 2, and 3 is TDP-6-deoxy-l-talose. This was accomplished by using TDP-[U-14C]glucose as precursor followed by isolation and identification of the nucleotide-linked sugar by isotope dilution.

TDP-[U-14C]glucose, in an incubation mixture containing NADPH and the sonic extract, was converted to a 6-deoxyhexose as determined in an aliquot by the cysteine sulfuric acid method. The sugar nucleotide was isolated by adsorption on charcoal, followed by paper electrophoresis and paper chromatography in Solvent III. After acid hydrolysis of the sugar nucleotide (0.01 N HCl for 10 min at 100°) the sample was subjected to paper chromatography in Solvent I. A single radioactive component was located with a mobility identical with authentic 6-deoxy-l-talose. The precursor relationship between TDP-glucose and the product of the enzymatic reaction was established by measurement of specific activities. The results of these experiments are shown in Table I and establish constant specific activity for precursor and end product.

Analytical examination of the isolated sugar nucleotide revealed the data shown in Table II, consistent with identification of the sole end product of the enzymatic conversion to l-6-deoxytalose. In order to provide unambiguous proof for the configuration of the product, the following experiment was carried out. The sugar nucleotide containing the hexose moiety uniformly labeled with 14C was hydrolyzed, and the radioactive sugar was isolated by paper chromatography in Solvent I. The radioactive sample was divided into two equal parts and authentic unlabeled d- or l-6-deoxygalactose, respectively, was added as carrier. Following a procedure used previously by Ashwell and Volk (26), the individual samples were converted to their corresponding 1-phenylthioflavose derivatives (Fig. 1). It should be noted that this procedure retains only the centers of asymmetry at carbons 4 and 5. Measurement of optical rotation of this derivative permits differentiation between the d and l isomers.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Specific activityебмом/μмоль</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDP-[U-14C]glucose</td>
<td>2.09 x 10⁴</td>
</tr>
<tr>
<td>TDP-[U-14C]deoxyhexose isolated after:</td>
<td></td>
</tr>
<tr>
<td>1. Elution from Norit</td>
<td>1.97 x 10⁴</td>
</tr>
<tr>
<td>2. High voltage electrophoresis</td>
<td>1.39 x 10⁴</td>
</tr>
<tr>
<td>3. Paper chromatography in Solvent III</td>
<td>1.89 x 10⁴</td>
</tr>
</tbody>
</table>
Upon repeated recrystallization of the 1-phenylflavazole derivatives, specific activity of the D isomer decreased sharply while the specific activity of the L isomer remained constant (Fig. 2). The specific rotation of ([α]_D^20 = +38.1°) the compound with constant specific activity establishes it clearly as the L isomer. Further corroboration was obtained by conversion of each of the 1-phenylflavazole derivatives to their corresponding di-O-acetyl-1-phenylflavazoles. This conversion resulted in complete loss of radioactivity for the D isomer and retention of constant specific activity for the L isomer.

**Table II**

*Analysis of thymidine diphospho-6-deoxytalose*

The material analyzed was obtained by the procedures outlined in Table I after chromatography in Solvent III.

<table>
<thead>
<tr>
<th>Analytical determination</th>
<th>Found</th>
<th>Ratio of component analyzed to 6-deoxyhexose</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-Deoxyhexose^a^</td>
<td>0.052</td>
<td>1.00</td>
</tr>
<tr>
<td>Thymidine^b^</td>
<td>0.054</td>
<td>1.04</td>
</tr>
<tr>
<td>Total phosphate^c^</td>
<td>0.110</td>
<td>2.12</td>
</tr>
<tr>
<td>Acid-labile phosphate^d^</td>
<td>0.048</td>
<td>0.92</td>
</tr>
<tr>
<td>Reducing sugar before hydrolysis^e^</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Reducing sugar after hydrolysis^f^</td>
<td>0.041</td>
<td>0.78</td>
</tr>
</tbody>
</table>

^a^ Cysteine sulfuric acid reaction (21) using 1.5 times the value of fucose standard.

^b^ $E_{270} = 9.6 \times 10^4$.

^c^ Measured according to Ames and Dubin (20).

^d^ Hydrolysis in 1 N HCl for 7 min at 100°.

^e^ Method of Nelson (22).

^f^ Hydrolysis in 0.1 N HCl for 30 min at 100°.

*Intermediary Formation of TDP-6-deoxy-D-xylo-4-hexosulose—*When sonic extracts of E. coli 045 were incubated with TDPG in the absence of NADPH, as indicated under "Materials and Methods," quantitative conversion to TDP-6-deoxy-D-xylo-4-hexosulose was observed. The 4-keto intermediate was characterized using criteria reported previously by Okazaki et al. (27). (a) Addition of NaOH to a final concentration of 0.1 M resulted in the characteristic ultraviolet absorption of 318 nm. (b) Reduction of the product with sodium borohydride yielded TDP-6-deoxy-D-glucose and TDP-6-deoxy-D-galactose as the sole products.

*Conversion of TDP-6-deoxy-D-xylo-4-hexosulose to TDP-6-deoxy-L-talose— Evidence for further conversion of this 4-keto intermediate to TDP-6-deoxy-L-talose in the presence of NADPH was provided in several independent ways. (a) Some extracts were incubated with TDP-6-deoxy-D-xylo-4-hexosulose and NADPH as described under "Materials and Methods." A TDP-6-deoxy-D-xylo-4-hexosulose-dependent disappearance of NADPH was observed. Appearance of 6-deoxyhexose was measured simultaneously by the cysteine sulfuric acid method (21). Estimates for the stoichiometry of this reaction using crude extracts were complicated by high endogenous NADPH oxidation (20%) caused by oxidases. When purified enzymes were used (assay procedure described below), substrate-unrelated NADPH oxidation was eliminated. Synthesis of TDP-6-deoxy-L-talose, was found to require a stoichiometric amount of NADPH and a molar ratio of one to one was observed in this reaction. (b) TDP-6-deoxy-D-[U-14C]xylo-4-hexosulose (1.25 μ mole, 138,000 cpm) was incubated with sonic extracts in the presence of NADPH. The reaction conditions and isolation of the nucleotides by Norit were as described under "Materials and Methods." Essentially all of the radioactivity (111,300

Fig. 1. Conversion of 6-deoxy-L-talose to its 1-phenylflavazole. Note that only the centers of asymmetry at carbon atoms 4 and 5 are retained.
TDP-6-deoxy-n-talose was found. 

of the charcoal eluate obtained in identical mobility to 6-deoxy-n-talose. (c) The second aliquot deoxy-n-talose (RF = 0.40). When the chromatogram was chromatography on PEI. Solvent B will differentiate between scanned for radioactivity, a single peak with the mobility of 6-deoxy-r-talose were observed when routine procedures used for hydrolyzed (0.01 cpmp per mole) was recovered as nucleotide-bound intermediate. The radioactive sample was treated with sodium borohydride, and divided into two equal portions. One aliquot of the sample was hydrolyzed (0.01 × HCl, 100°, 10 min) and subjected to chromatography in Solvent I. The only radioactive sugar found had identical mobility to 6-deoxy-n-talose. (c) The second aliquot of the charcoal eluate obtained in δ was subjected to thin layer chromatography on PEI. Solvent B will differentiate between TDP-6-deoxy-n-xylol-4-xylosulose (Rf = 0.22) and TDP-6-deoxy-l-talose (Rf = 0.40). When the chromatogram was scanned for radioactivity, a single peak with the mobility of TDP-6-deoxy-l-talose was found.

Stability of TDP 6-deoxy l-talose—Consistent losses of TDP 6-deoxy-l-talose were observed when routine procedures used for sugar nucleotide isolation were employed. A systematic study was undertaken to examine the properties of the new sugar nucleotide, and the results obtained are shown in Table III. It is apparent that the nucleotide is sensitive to alkali treatment, resulting in degradation to 5’-TMP and sugar monophosphate. The alkaline degradation of sugar nucleotides leading to formation of sugar-1,2-phosphates is well known (28) and we have confirmed the nature of the reaction in our case by characterization of the 6-deoxy-l-talose-1,2-monophosphate obtained from TDP-6-deoxy-l-talose (Fig. 3). TDP-6-deoxy-l-[U-14C]talose (Compound I), 1.14 μmoles (2.12 × 10^6 cpm per pmole) in a total volume of 500 μl, was treated with 1 N NaOH at 100°. The reaction mixture was evaporated under reduced pressure to dryness. The sample was subjected to paper chromatography in Solvent II. Examination of the paper in ultraviolet light revealed one component with a mobility (Rf TDPG = 0.91) identical with authentic 5’-TMP. No radioactivity was associated with this component. A second component (Rf TDPG = 1.52) was located by scanning for radioactivity. Upon elution from the paper, this component was radioactive, but had no ultraviolet absorption (6-deoxy-l-talopyranose, 1,2-monophosphate (Compound II)). Treatment of Compound II with 0.01 × HCl for 1/2 hour at 100° yielded 6-deoxy-l-talose-2-phosphate (Compound III). When either Compounds II or III were subjected to vigorous acid hydrolysis (1 N HCl at 100° for 1 hour), the sole products were free 6-deoxy-l-talose and inorganic phosphate. Each of the sugar phosphates (Compounds II and III) was distinguished by paper chromatography in Solvent Systems II and III from 6-deoxy-l-talose-1-phosphate, formed by the action of snake venom phosphodiesterase on the intact nucleotide (Compound I). A summary of some properties of the different 6-deoxy-l-talose monophosphates is shown in Table IV.

Table III

<table>
<thead>
<tr>
<th>Reaction conditions</th>
<th>Intact nucleotide</th>
<th>5’-TMP + sugar monophosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 M sodium citrate buffer, pH 4.0, 2 hr at 37°</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0.05 M sodium phosphate buffer, pH 7.6, 2 hr at 37°</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0.05 M Tris-chloride buffer, pH 8.0, 2 hr at 37°</td>
<td>77</td>
<td>23</td>
</tr>
<tr>
<td>0.05 M Tris-chloride buffer, pH 8.0 + 3 volumes ethanol, 1 min at 100°</td>
<td>78</td>
<td>22</td>
</tr>
<tr>
<td>1 N NaOH, 2 hr at 37°</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1 N NaOH, 10 min at 100°</td>
<td>11</td>
<td>89</td>
</tr>
<tr>
<td>16-hr chromatography in Solvent II (pH 7.0)</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>16-hr chromatography in Solvent III (pH 8.8)</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>
Fig. 3. Identification of 6-deoxy-L-talopyranose 1,2-monophosphate as degradation product of TDP-6-deoxy-L-talose. The Roman numerals refer to the following compounds: I, TDP-6-deoxy-L-[U-14C]talose; II, 6-deoxy-L-[U-14C]talopyranose 1,2-monophosphate; III, 6-deoxy-L-[U-14C]talose-2-phosphate; IV, 6-deoxy-L-[U-14C, 1-3H]talitol-2-phosphate; V, L-[U-14C, 3-3H]glycerol 2-phosphate; VI, n-[U-14C, 1-3H]glycerol 2-phosphate; VII, n-[U-14C, 1-3H]glycerol.

### Table IV

Properties of 6-deoxy-L-talose monophosphates

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent II</th>
<th>Solvent III</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-Deoxy-L-talose-1,2-monophosphate (Compound II)</td>
<td>1.52</td>
<td>1.94</td>
</tr>
<tr>
<td>6-Deoxy-L-talose-2-phosphate (Compound III)</td>
<td>0.94</td>
<td>1.66</td>
</tr>
<tr>
<td>6-Deoxy-L-talose-1-phosphate</td>
<td>0.82</td>
<td>1.48</td>
</tr>
</tbody>
</table>

Analytical determination:

<table>
<thead>
<tr>
<th>Component</th>
<th>Solvent II</th>
<th>Solvent III</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-Deoxytalose</td>
<td>1.32</td>
<td>0.107</td>
</tr>
<tr>
<td>Total phosphate</td>
<td>1.29</td>
<td>0.108</td>
</tr>
<tr>
<td>Acid-labile phosphate</td>
<td>0.84</td>
<td>0.063</td>
</tr>
<tr>
<td>Inorganic phosphate</td>
<td>0.35</td>
<td>0.036</td>
</tr>
<tr>
<td>Reducing sugar</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

a All analytical determinations were carried out as described in Table II.

b Elevated values were found consistently; condition of assay causes release of inorganic phosphate.

c Hexose 2-phosphates have been reported previously (28) to give a negative reaction for reducing sugar.

### Table V

Degradation of 6-deoxy-L-[U-14C, 1-3H]talitol-2-phosphate (Compound IV)

<table>
<thead>
<tr>
<th>Compound</th>
<th>dH</th>
<th>dC</th>
<th>Ratio of dH/dC</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-Deoxy-L-[U-14C, 1-3H]talitol-2-phosphate (IV)</td>
<td>5670</td>
<td>655</td>
<td>8.6</td>
</tr>
<tr>
<td>n-[U-14C, 1-3H]glycerol (VII)</td>
<td>6980</td>
<td>358</td>
<td>16.7</td>
</tr>
</tbody>
</table>

The Roman numerals refer to structures in Fig. 3.

pH 7.6, and Compound V was reduced with sodium borohydride to β-glycerophosphate (Compound VI). Isolation of Compound VI was carried out by chromatography on Dowex 1 (0.5 × 4.5 cm) using a linear gradient of 0 to 0.1 N HCl in a total volume of 100 ml. Collection of 1-ml fractions revealed the presence of a compound containing organic-bound phosphate and tritium at Fractions 51 to 60. Authentic β-glycerophosphate is eluted at the same position. Treatment of the isolated β-glycerophosphate with alkaline phosphatase resulted in liberation of inorganic phosphate and formation of glycerol (Compound VII). The glycerol (Compound VII) was isolated by thin layer chromatography on Silica Gel G impregnated with boric acid. The results obtained are shown in Table V.
Separation of 3,5-Epimerase (Reaction 2) and 4-Reductase (Reaction 3)—Separation of the crude enzyme mixture into individual fractions catalyzing Reactions 2 and 3, respectively, was achieved by employing conventional methods of enzyme purification.

For the assay of 3,5-epimerase (Reaction 2) incubation with TDP-6-deoxy-D-[3-3H]xylo-4-hexosulose as substrate was used. Transfer of tritium label into the medium is a result of 3,5-epimerase activity.

4-Reductase activity (Reaction 3) can be measured by direct spectrophotometric determination of NADPH oxidation at 340 nm. The progress of the over-all reaction is best followed in the crude system by colorimetric determination of 6-deoxyhexose synthesis according to Dische (21).

Assay System for 3,5-Epimerase (Reaction 2)—TDP-6-deoxy-D-[3-3H]xylo-4-hexosulose, 2 nmole, was incubated with enzyme in a total volume of 60 μl containing 0.025 M Tris-chloride buffer, pH 8.0. After 30 min at 37° the reaction was terminated by the addition of 10 μmoles of sodium borohydride. The mixture was kept for 30 min at room temperature and finally the reaction mixture was diluted with water to a total volume of 500 μl. An aliquot was taken and the tritium activity was determined. Another aliquot was taken and evaporated to dryness. The dry sample was dissolved in water and again evaporated to dryness. This process was repeated once more. The sample was dissolved in a volume identical with that of the aliquot taken originally. The difference in radioactivity determined before and after repeated evaporation represents the amount of tritium exchanged by the enzymatic reaction. A control experiment, containing the same components but heat-inactivated enzyme, was carried out simultaneously with each determination.

Assay for 4-Reductase (Reaction 3)—Disappearance of NADPH was measured at 340 nm in a microcuvette in a total volume of 300 μl. The incubation mixture contained 0.03 μmole of TDP-6-deoxy-D-xylo-4-hexosulose and 0.04 μmole of NADPH in 0.025 M Tris-chloride buffer, pH 8.0. It is important to establish first endogenous oxidation of NADPH by a control experiment omitting the substrate. Endogenous oxidation of NADPH in crude extracts amounts to about 20% of the rate found for substrate-dependent oxidation. When purified 3,5-epimerase and 4-reductase were used, endogenous NADPH oxidation was less than 5% under the conditions of this assay. Since the rate of NADPH oxidation is proportional to the concentration of TDP-6-deoxy-D-xylo-4-hexosulose, increase of substrate concentration led to values of endogenous NADPH oxidation of less than 1%.

Enzyme Purification—A large scale preparation for the isolation of 3,5-epimerase and reductase to be published elsewhere (29) was carried out using 950 g of cell wet weight of E. coli 045. The sequential use of protamine sulfate precipitation, ammonium sulfate fractionation, and DEAE chromatography led to two enzyme preparations catalyzing either Reaction 2 or Reaction 3 (Table VI). Only after mixing of the two enzyme fractions was 6-deoxyhexose biosynthesis observed, while either fraction alone did not yield any TDP-6-deoxy-L-talose.

Similar observations were made previously by Melo and Glaser (30) but in a system isolated from Pseudomonas aeruginosa. The reaction sequence is identical with the one described in this paper, except that the end product of deoxyhexose biosynthesis is TDP-L-rhamnose (TDP-6-deoxy-L-rhamnose). Since the common postulated intermediate for both pathways, TDP-6-deoxy-L-lyxo-4-hexosulose, appears to remain enzyme-bound, we decided to investigate the possibility that analogous enzymes isolated from different sources can be interchanged. Following the procedures described by Melo and Glaser (30) we purified 3,5-epimerase and the 4-reductase from P. aeruginosa. Both enzyme preparations were obtained free of cross-contamination. Experiments carried out by mixing 3,5-epimerase and 4-reductase isolated from E. coli 045 and P. aeruginosa are summarized in Table VII.

TABLE VI

Examination of cross-contamination of 3,5-epimerase and 4-reductase isolated from Escherichia coli 045

<table>
<thead>
<tr>
<th>Incubation mixture containing:</th>
<th>Exchange of</th>
<th>Synthesis of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>epm</td>
<td>μmoles</td>
</tr>
<tr>
<td>Ten microliters of 3,5-epimerase</td>
<td>12,000</td>
<td>0</td>
</tr>
<tr>
<td>Fifty microliters of 4-reductase</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ten microliters of 3,5-epimerase + 50 m</td>
<td>Not deter-</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Discussion

In the lipopolysaccharide of E. coli 045, 6-deoxy-L-talose was reported to occur (3) instead of the commonly found 6-deoxy-L-rhamnose (L-rhamnose). A study of the biosynthesis of 6-deoxy-L-talose in this microorganism provided experimental evidence for its formation according to Reactions 1, 2, and 3 and led to identification of TDP-6-deoxy-L-talose as the end product of the reaction sequence.

The pathways for L-rhamnose and 6-deoxy-L-talose formation are identical with the exception of the last step. After 3,5-epimerization of TDP-6-deoxy-D-xylo-4-hexosulose to yield the postulated intermediate TDP-6-deoxy-L-lyxo-4-hexosulose, stereospecific reduction with 4-reductase leads to TDP-6-deoxy-L-talose as the only product (Reaction 3). The occurrence of a single product excludes the possibility considered by us earlier, that 6-deoxy-L-talose may be the result of 4-epimerization of TDP-6-deoxy-L-rhamnose (TDP-L-rhamnose). Specific attempts made by us to demonstrate a 4-epimerase activity for this conversion failed.

It is of interest to provide some insight into relationships between structure and stability when comparing TDP-6-deoxy-L-rhamnose and TDP-6-deoxy-L-talose. The C-1 conformation of 6-deoxy-L-talose places the substituents at carbons 2 and 4 into a cis-diaxial arrangement. The difference in the stabilities of TDP-6-deoxy-L-talose places the substituents at carbons 2 and 4 into a cis-diaxial arrangement. The difference in the stabilities of TDP-6-deoxy-L-talose places the substituents at carbons 2 and 4 into a cis-diaxial arrangement.
always requires the presence of 3,5-epimerase. Consequently, but remain enzyme-bound and, therefore, assay of 4-reductase. The reaction products of the 3,5-epimerase are never found free from the medium to the 4-keto intermediate was measured after termination of the reaction by the addition of sodium borohydride. Determination of tritium activity in the isolated TDP-6-deoxy-6-L-talose in the complete system (4-reductase present in excess) the ability to transfer protons to the medium appeared lower. Several reasons could be responsible for this disparity. Ketol-enediol transformations involving proton shifts in this reaction is compared with the rate of net biosynthesis of 6-deoxy-L-lyxo-4-hexosulose by 4-reductase. To study this interaction in more detail, 3,5-epimerase and 4-reductase were isolated from different sources. The enzymes obtained from E. coli 045 yielded TDP-6-deoxy-L-talose, whereas analogous enzymes isolated from P. aeruginosa led to formation of TDP-L-rhamnose. Individual preparations of 3,5-epimerase and 4-reductase free of cross-contamination were mixed and used interchangeably and led in each instance to formation of 6-deoxyhexoses. The nature of the end product was determined by the stereospecificity of 4-reductase (Table VII). Thus, 4-reductase from E. coli 045 yielded TDP-6-deoxy-L-talose, while the analogous enzyme from P. aeruginosa resulted in formation of TDP-L-rhamnose.

Kinetic analysis of the enzyme system isolated from E. coli 045 revealed inhibition of the over-all reaction by 5'-TMP. By varying the relative amounts of 3,5-epimerase and 4-reductase, only at conditions when 3,5-epimerase is rate-limiting, 5'-TMP was found to be a competitive inhibitor.

The biosynthesis of the D isomer, 6-deoxy-D-talose, reported by Winkler and Markovits (35, 36) has quite different properties when compared to all other systems known for 6-deoxyhexose formation. Most notably, in this system a nucleotide-4-keto intermediate is reduced enzymatically to both 4 epimers, 6-deoxy-D-mannose (D-rhamnose) and 6-deoxy-D-talose, without prior epimerization.

### REFERENCES


### Table VII

**End products of 3,5-epimerase and 4-reductase, isolated from different sources**

Incubations were carried out for 3 hours at 35° containing TDP-6-deoxy-d-[U-14C]-xylo-4-hexosulose, 0.1 μ mole, 11,500 cpm; NADPH, 0.3 μ mole; 0.05 M Tris-chloride, pH 8.0; 0.001 M EDTA; and 0.01 M 3-mercaptoethanol, in a total volume of 300 μl. After completion of incubation, residual 4-keto intermediate was reduced with sodium borohydride. The radioactive sugars were released from the nucleotide by mild acid hydrolysis, separated by paper chromatography in Solvent I, and located by scanning for radioactivity.

<table>
<thead>
<tr>
<th>Enzyme (sources)</th>
<th>TDP-linked sugars identified as:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control experiments</td>
<td>6-Deoxy-D-glucose, 6-deoxy-D-galactose</td>
</tr>
<tr>
<td>10 μl of 3,5-epimerase (E. coli 045)</td>
<td>6-Deoxy-D-glucose, 6-deoxy-D-galactose</td>
</tr>
<tr>
<td>30 μl of 4-reductase (E. coli 045)</td>
<td>6-Deoxy-D-glucose, 6-deoxy-D-galactose</td>
</tr>
<tr>
<td>10 μl of 3,5-epimerase (P. aeruginosa)</td>
<td>6-Deoxy-D-glucose, 6-deoxy-D-galactose</td>
</tr>
<tr>
<td>25 μl of 4-reductase (P. aeruginosa)</td>
<td>6-Deoxy-D-glucose, 6-deoxy-D-galactose</td>
</tr>
</tbody>
</table>

**Mixing experiments**

| 10 μl of 3,5-epimerase (E. coli 045) + 30 μl of 4-reductase (E. coli 045) | 6-Deoxy-D-glucose, 6-deoxy-D-galactose |
| 10 μl of 3,5-epimerase (P. aeruginosa) + 25 μl of 4-reductase (P. aeruginosa) | 6-Deoxy-D-glucose, 6-deoxy-D-galactose |
| 10 μl of 3,5-epimerase (P. aeruginosa) + 25 μl of 4-reductase (P. aeruginosa) | 6-Deoxy-D-glucose, 6-deoxy-D-galactose |
| 10 μl of 3,5-epimerase (P. aeruginosa) + 30 μl of 4-reductase (E. coli 045) | 6-Deoxy-D-glucose, 6-deoxy-D-galactose |
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