Biosynthesis of Guanosine Diphosphate D-Rhamnose and Guanosine Diphosphate D-Talomethylose from Guanosine Diphosphate α-D-Mannose*

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Strain GS, a gram-negative motile bacterium isolated from soil, synthesizes two polysaccharides. The synthesis of a uronic acid-containing polysaccharide is derepressed by high concentrations of NaCl, while the synthesis of the polysaccharide containing equal quantities of D-rhamnose and D-talomethylose is repressed by NaCl (1, 2). L-Fucose and L-rhamnose are the methylpentoses commonly found in nature. Recently, the biosynthesis of both methylpentoses has been shown to occur from GDP-n-mannose by extracts of strain GS and provides evidence that at least two enzymes are involved in these conversions. In addition, data are presented which demonstrate that at least two enzymes are involved in these conversions. In addition, data are presented which demonstrate that at least two enzymes are involved in these conversions.

EXPERIMENTAL PROCEDURE

All nucleotides and glucose-6-P dehydrogenase (type V) were purchased from Sigma Chemical Company, unless otherwise indicated. GDP-mannose (90%) contained small amounts of GDP-n-glycero-n-mannoheptose (6). DPNH (Pabst Laboratories and Sigma Chemical Company) was stored at room temperature in desiccators in the dark. DPNH solutions were prepared on the day of use, but solutions of TPNH were stored in the frozen state.

Methylpentose was determined by the cysteine-sulfuric acid method (7) with L-rhamnose or D-talomethylose as standards (10-minute boiling period). Since D-talomethylose has a molar absorption 1.5 times that of L- or D-rhamnose in this method, the use of L-rhamnose as a standard necessitated appropriate correction in the presence of D-talomethylose. Reducing sugar was determined by the method of Park and Johnson (8) with appropriate standards of either n-glucose, n-rhamnose, or n-mannose isomerase prepared from Pseudomonas saccharophila (9). Keto sugars produced were estimated by the method of Dische and Borenfreund (10) with appropriate standards of D-fructose and L-rhamnulose.

Fucose was determined by the orcinol method (11) with ribose as a standard, and total phosphate and acid-labile phosphate were determined by the method of Fiske and SubbaRow (12). Guanosine in nucleotides was calculated from ultraviolet absorption data by assuming an E_{280} of 11.7 (13). Protein and nucleic acid were estimated on the basis of ultraviolet absorption according to the method of Warburg and Christian, as described by Layne (14).

The following solvents were used for chromatography: Solvent 1, neutral 1 M ammonium acetate-ethanol (3:7) (15); Solvent 2, isobutyric acid-0.5 N NH₄OH (5:3); Solvent 3, acetic acid-1-butanol-water (1:4:1); Solvent 4, 2-butanol saturated with water (10); Solvent 5, tert-amyl alcohol-isopropyl alcohol-water (8:2:3) (17); Solvent 6, pyridine-1-butanol-water (4:6:3); Solvent 7, pyridine-ethyl acetate-water (2:7:1) (18).

Preparation of Nucleotide Intermediate from GDP-mannose
Nucleotide intermediate preparations were obtained by incubation of enzyme Preparation A (see below) with GDP-mannose or enzyme Preparation C with GDP-mannose. When Preparation C was utilized, sufficient TPNH was added so that absorption at 340 nm was evident for 15 minutes. For preparation of nucleotide intermediate, the mixture was heated at 100° for 1 minute, since longer heating destroyed nucleotide intermediate in certain experiments. Nucleotide intermediate was partially purified by the following methods.

Method 1—The reaction mixture was passed over a Sephadex G-50 column of sufficient bed volume to permit separation of large from small molecular components (10). The nucleotides were retarded.

Method 2—Proteins and polysaccharide contaminants could be removed by ethanol precipitation, provided that ammonium acetate was present to insure complete precipitation of polysaccharides. The resultant supernatant fluid contained the nucleotides.

Isolation of GDP-rhamnose and GDP-talomethylose
After inactivation and removal of proteins by boiling and centrifugation, the nucleotides were adsorbed on acid-washed Norit A (10 to 20 mg of Norit A per µmole of nucleotide) at

* This investigation was aided by a grant from The National Foundation and by Grant AM-05996 from The National Institute of Arthritis and Metabolic Diseases of the United States Public Health Service.
room temperature for 15 minutes. The Norit A was washed by suspension in cold distilled water and sedimentation. Nucleotides were eluted from Norit A by repeated suspension in 50% aqueous ethanol solution containing 0.1% NH$_4$OH. After concentration at 30-40°C in a vacuum, the nucleotides were separated by descending paper chromatography in Solvent 1 or 2. GDP-rhamnose and GDP-talomethylose were not separated from one another in those solvents.

Hydrolysis of Nucleotide-bound Carbohydrates

Procedure 1—Nucleotides in solution were hydrolyzed in 0.03 N HCl for 10 to 30 minutes at 100°C. The acid was neutralized and the nucleotides were removed with Dowex 1-HCO$_3^-$ resin. The supernatant and water washes of the resin were concentrated at 45-55°C in a vacuum.

Procedure 2—Nucleotides, while adsorbed to Norit A, were hydrolyzed as in Procedure 1, releasing the free carbohydrates into the supernatant solution. Acid was removed from the solution by repeated lyophilization.

Enzyme Preparations from Strain GS

Enzymes were prepared at 0-4°C from cultures of strain GS grown as described previously (1, 2).

Preparation A—Extracts were prepared from washed cells suspended in 50 mM Na$_2$HPO$_4$-KH$_2$PO$_4$, pH 7.5, by sonic disruption in a Raytheon 9-kc sonic disintegrator for 15 or 30 minutes followed by centrifugation at 34,000 × g for 10 to 20 minutes.

Preparation B—Preparation A was mixed with streptomycin sulfate (1.5 mg per mg of protein) and after 15 minutes the precipitate was removed by centrifugation. After dilution of the supernatant solution to contain 22 mg of protein in 7.5 ml of 25 mM Na$_2$HPO$_4$-KH$_2$PO$_4$, pH 7.5, it was placed on a DEAE-cellulose column (20 × 11 mm) that had previously been equilibrated with 10 mM Na$_2$HPO$_4$-KH$_2$PO$_4$, pH 7.5. Under these conditions, the enzymes which catalyze GDP-mannose-dependent TPNH oxidation were retained on the column. The column was eluted with 5 ml of 50 mM Na$_2$HPO$_4$-KH$_2$PO$_4$, pH 7.5, followed by 5 ml of the same buffer containing 300 mM Na$_2$SO$_4$. The latter fraction (34% of the protein applied to the column) contained enzymes which convert GDP-mannose to GDP-methylpentoses.

Preparation C—EDTA was added to Preparation A to a final concentration of 10 mM. Part of the nucleic acids were removed with streptomycin sulfate as in enzyme Preparation B. Solid ammonium sulfate was added slowly to the streptomycin sulfate supernatant fraction to a concentration of 40% (0.28 g per ml at 0°C). After removal of the inactive precipitated protein, additional ammonium sulfate was added until 80% saturation was reached. This second precipitate was collected by centrifugation, dissolved in 50 mM Na$_2$HPO$_4$-KH$_2$PO$_4$, 10 mM EDTA, pH 7.5, and dialyzed against 100 volumes of the same buffer for 18 hours. The 40 to 80% ammonium sulfate fraction contained enzymes which convert GDP-mannose to GDP-methylpentoses.

Preparation D—Streptomycin sulfate was used (as above) to remove part of the nucleic acid from 3.8 g of protein of Preparation A. EDTA and MgCl$_2$ were added to the protein solution (and to all subsequent eluents) to final concentrations of 0.2 and 0.4 mM, respectively, and the volume was adjusted to 380 ml, resulting in a Na$_2$HPO$_4$-KH$_2$PO$_4$ concentration of 25 mM. A DEAE-cellulose column (5 × 35 em) equilibrated with 25 mM K$_2$HPO$_4$, pH 7.5, was used to fractionate the proteins at constant pH as follows. The protein solution was passed through the column at a rate of about 40 ml per hour. To the column were then added 900 ml of 25 mM K$_2$HPO$_4$, followed by 1300 ml of 50 mM K$_2$HPO$_4$, and 20-ml fractions were collected. A concave upward gradient was used (20) as follows. The mixing flask connected to the column initially contained 2900 ml of 50 mM K$_2$HPO$_4$, while the feeding flask contained 2000 ml of 50 mM K$_2$HPO$_4$-400 mM NaCl. Under these conditions, protein was eluted from the column after 2200 ml of the gradient mixture, and the first protein fraction contained an enzyme or enzymes that reduced GDP-4-ketorhamnose to GDP-rhamnose and GDP-talomethylose in the presence of either DPNH or TPNH. This fraction, Preparation D, does not catalyze the reaction of GDP-mannose with either DPNH or TPNH. Subsequent fractions eluted from the column were inactive when assayed for enzymes involved in conversion of GDP-mannose to GDP-methylpentoses.

RESULTS

Fig. 1 illustrates the GDP-mannose-stimulated oxidation of TPNH with enzyme Preparation A, as well as the effect of preincubation of GDP-mannose with enzyme on TPNH oxidation. Simultaneous addition of GDP-mannose and TPNH to the enzyme resulted in GDP-mannose-stimulated TPNH oxidation without lag. However, preincubation of Preparation A with GDP-mannose for varying periods results in a more rapid initial rate of TPNH oxidation (Fig. 1). Preincubation of Preparation A with GDP-mannose for 11 minutes leads to an additional TPNH oxidation equal to 0.9 mole per mole of GDP-mannose. Hydrolysis of GDP-mannose in 0.01 N HCl at 100°C...
for 30 minutes destroyed the ability of the nucleotide to stimulate TPNH oxidation.

In certain experiments with Preparation A, GDP-mannose-stimulated TPNH oxidation did not occur unless the enzyme preparation was preincubated with GDP-mannose. This anomalous behavior could be eliminated by the addition of EDTA (10 mM) to the reaction mixtures. The interaction of TPNH, GDP-mannose, enzyme, and EDTA is not clear at the present time. Preparation of the enzyme in the presence of 3 mM cysteine or addition of lipoic acid to the reaction mixture did not affect the reaction.

Net Synthesis of Methylpentoses from GDP-mannose—Fig. 2 shows the synthesis of methylpentoses as a function of time when GDP-mannose, TPNH, and a TPNH-generating system are incubated with either Preparation A or B. Omission of GDP-mannose in other experiments with Preparation A prevented the formation of methylpentoses (7), while omission of TPNH and a TPNH-generating system resulted in the formation of methylpentoses equal to only 18% of that obtained with the complete system. For each mole of GDP-mannose added, 0.83 and 0.75 mole of methylpentose were formed by Preparations A and B, respectively.

Analyses of GDP-methylpentoses—GDP-methylpentoses were isolated from the reaction mixtures described in the legend of Fig. 2 with Solvent 2. The GDP-methylpentoses are not separable from each other in either Solvents 1 or 2, but they are separable from GDP-mannose. Analyses of the isolated GDP-methylpentoses are shown in Table I. With Preparation A and B, approximately equal quantities of rhamnose and talomethylose varied in the analyses shown in Table I suggested the presence of unidentified carbohydrates which yield a color with aniline-oxalate on paper chromatography in Solvent 4. Neither mannose nor other carbohydrates which yield a color with aniline-oxalate on paper chromatograms were detected in these preparations. However, the analyses shown in Table I exhibited ultraviolet absorption spectra typical of a guanosine derivative. They reacted in the specific test for methylpentoses (7) to give absorption spectra identical with that obtained from authentic n-rhamnose and n-talomethylose.

Hydrolysis of GDP-bound carbohydrates was performed by Procedure 1 as described in “Experimental Procedure.” Approximately equal quantities of rhamnose and talomethylose were found by colorimetric analyses (7) of eluates following paper chromatography in Solvent 4. Neither mannose nor other carbohydrates which yield a color with aniline-oxalate on paper chromatograms were detected in these preparations. However, the analyses shown in Table I suggested the presence of unidentified carbohydrate in addition to GDP-rhamnose and GDP-talomethylose. Therefore, the GDP-methylpentose fraction was examined spectrophotometrically for the presence of GDP-4-ketorhamnose by the use of GDP-4-ketorhamnose reductase (enzyme Preparation D) and TPNH. The results indicated that intact GDP-4-ketorhamnose was not present. Okazaki et al. (22) showed that TDP-4-keto-6-deoxyglucose does not react in the Dische test for methylpentose (7), but gives a reducing test (8) before and after acid hydrolysis. On the basis of their findings, it is possible that the low yield of GDP-methylpentoses was investigated in other experiments described below.

Of interest is the fact that these nucleotides exhibited approximately 75% of their maximum reducing sugar value (8) without prior acid hydrolysis (Table I). This result confirms those obtained previously with GDP-rhamnose and GDP-talomethylose prepared by chemical reduction of GDP-4-ketorhamnose (21). The nucleotides isolated in the experiments presented in Table I exhibited ultraviolet absorption spectra typical of a guanosine derivative. They reacted in the specific test for methylpentoses (7) to give an absorption spectrum identical with that obtained from authentic n-rhamnose and n-talomethylose.

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>GDP-mannose</th>
<th>GDP-talomethylose</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>B</td>
<td>0.74</td>
<td>0.73</td>
</tr>
<tr>
<td>Methylpentose (7)^b</td>
<td>0.67</td>
<td>0.59</td>
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<tr>
<td>Pentose (11)</td>
<td>0.89</td>
<td>0.91</td>
</tr>
<tr>
<td>Reducing sugar (8)^c</td>
<td>0.80</td>
<td>0.81</td>
</tr>
<tr>
<td>Reducing sugar (8)^d</td>
<td>0.98</td>
<td>1.1</td>
</tr>
</tbody>
</table>

^a Calculated from ultraviolet absorption data by assuming an E\_\text{1\_\text{μm}} of 11.7 (13).
^b Calculated as an equimolar mixture of talomethylose and rhamnose.
^c After hydrolysis for 15 minutes at 100° in 0.01 N HCl.
^d The low yield of GDP-methylpentoses was investigated in other experiments described below.

The molar ratio of talomethylose to rhamnose formed from GDP-mannose was determined in several experiments (Table II), and both methylpentoses were identified in Solvents 3 through 7. When Preparation A was used, the ratio of talomethylose to rhamnose that was adsorbed to Norit A varied in Experiments P-48 and P-49. In Experiment P-115 of Table II,
but no talomethylose. After elution of the rhamnose and GDP-rhamnose fraction (Table II, Experiment P-115) isolated after paper chromatography in Solvent 1. A fraction that migrates somewhat faster than GDP-mannose was eluted. Hydrolysis was performed as in Procedure 1. A fraction that migrates somewhat faster than GDP-rhamnose and either DPN or TPN produced no detectable DPNH or TPNH under incubation conditions similar to those of Fig. 3, the GDP-4-ketorhamnose reductase reaction does not appear to be reversible. The purified GDP-4-ketorhamnose reductase was used to determine whether or not other fractions from the same column of TPNH or DPNH oxidized per hour per mg of protein), the GDP-4-ketorhamnose reductase (Preparation D) was purified 25-fold when compared with Preparation A. Since endogenous TPNH oxidation was negligible, it was a suitable preparation for reduction of large quantities of nucleotide intermediate in the presence of TPNH or DPNH. The purified GDP-4-keto-

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Enzyme preparation</th>
<th>Reducing system</th>
<th>Isolation method</th>
<th>Talomethylose to rhamnose ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-48</td>
<td>A</td>
<td>TPNH + TPNH generator</td>
<td>N</td>
<td>0.54</td>
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<tr>
<td>P-49</td>
<td>A</td>
<td>TPNH</td>
<td>N</td>
<td>1.2</td>
</tr>
<tr>
<td>P-115</td>
<td>C</td>
<td>TPNH generator</td>
<td>F</td>
<td>0.50*d</td>
</tr>
<tr>
<td>P-115</td>
<td>C</td>
<td>TPNH generator</td>
<td>F</td>
<td>0.0*</td>
</tr>
</tbody>
</table>

* The TPNH generator contained glucose-6-P, glucose-6-P dehydrogenase, and TPN.

Table II

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Enzyme preparation</th>
<th>Reducing system</th>
<th>Isolation method</th>
<th>Talomethylose to rhamnose ratio</th>
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</thead>
<tbody>
<tr>
<td>P-48</td>
<td>A</td>
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<tr>
<td>P-115</td>
<td>C</td>
<td>TPNH generator</td>
<td>F</td>
<td>0.0*</td>
</tr>
</tbody>
</table>

The ratio of free talomethylose to free rhamnose was 0.59. In contrast, the GDP-linked methylpentose that was retained on Norit A and isolated by paper chromatography from the same experiment (Table II) was free of talomethylose and contained rhamnose as the only methylpentose. The free methylpentoses in the reaction mixture are probably an indication of breakdown of the GDP-rhamnose and GDP-talomethylose, either during the incubation period or during the treatment with Norit A. It is therefore quite clear that if the total quantities of talomethylose and rhamnose synthesized are to be determined, both the free methylpentose and that bound to nucleotide should be included. The variations in ratio of talomethylose to rhamnose observed in Experiments P-48 and P-49 (Table II) may be due to the fact that free methylpentoses were not measured.

Identification of D-rhamnose Obtained from GDP-rhamnose Preparation—The GDP-rhamnose fraction (Table II, Experiment P-115) isolated after paper chromatography in Solvent 1 showed a molar ratio of rhamnose to guanosine of 0.74. The nucleotide-bound carbohydrates were hydrolyzed as indicated in Table II, and the free carbohydrate fraction was chromatographed in Solvent 3. Staining with aniline-oxalate indicated the presence of rhamnose, a faint spot with an Rf-glucose of 1.05, but no talomethylose. After elution of the rhamnose and talomethylose areas, 1.1 micromoles of rhamnose, but no talomethylose, were found. The rhamnose reacted with d-mannose isomerase of P. saccharophila (9) to yield a compound with an absorption spectrum identical with that of rhamnulose (2) in the cysteine-carbazole reaction (10). The enzymatic test has been used previously to identify d-rhamnose present in the polysaccharide of strain GS (2). From these results, it is concluded that the rhamnose is the d isomer.

Partial Purification of GDP-4-ketorhamnose Reductase—The results presented in Fig. 1 suggested that at least two enzymes are involved in the conversion of GDP-mannose to GDP-rhamnose and GDP-talomethylose. It was apparent that both enzymes were absorbed and eluted from a DEAE-cellulose column (Fig. 2 and Table I). Therefore, experiments were performed to separate the enzymes on large DEAE-cellulose columns (“Experimental Procedure,” “Preparation D”). Protein fractions obtained from DEAE-cellulose columns were assayed for TPNH oxidation stimulated by nucleotide intermediate or GDP-rhamnose (see Figs. 1 and 3 for conditions). An enzyme fraction that catalyzed TPNH or DPNH oxidation in the presence of nucleotide intermediate, but not in the presence of GDP-rhamnose, was obtained. A typical spectrophotometric assay of such an enzyme (Preparation D) is shown in Fig. 3. Only one asymmetric protein peak (Preparation D) from the DEAE-cellulose column catalyzed nucleotide intermediate-stimulated TPNH (and DPNH) oxidation. On the basis of specific enzymatic activity measurements (micromoles of TPNH or DPNH oxidized per hour per mg of protein), the GDP-4-ketorhamnose reductase (Preparation D) was purified 25-fold when compared with Preparation A. Since endogenous TPNH oxidation was negligible, it was a suitable preparation for reduction of large quantities of nucleotide intermediate in the presence of TPNH or DPNH. The purified GDP-4-ketorhamnose reductase showed the same specific enzymatic activity, when assayed spectrophotometrically, whether TPNH or DPNH was the substrate oxidized. Therefore, it was of interest to determine whether or not the purified GDP-4-ketorhamnose reductase activity with TPNH as well as with DPNH resulted from the presence of a transhydrogenase. In certain preparations of GDP-4-ketorhamnose reductase, a TPNH-specific glucose-6-P dehydrogenase activity was present. Addition of glucose-6-P, excess DPN, and limiting amounts of TPN to such preparations resulted in reduction of only the added TPN. Such results indicated that a transhydrogenase, which would have catalyzed a reaction between TPNH and DPNH, was not present. Since incubation of the enzyme with GDP-rhamnose, GDP-talomethylose, and either DPN or TPN produced no detectable DPNH or TPNH under incubation conditions similar to those of Fig. 3, the GDP-4-ketorhamnose reductase reaction does not appear to be reversible.

The purified GDP-4-ketorhamnose reductase was used to determine whether or not other fractions from the same column

![Fig. 3. TPNH oxidation stimulated by nucleotide intermediate in the presence of purified GDP-4-ketorhamnose reductase.](http://www.jbc.org/)

Fig. 3. TPNH oxidation stimulated by nucleotide intermediate in the presence of purified GDP-4-ketorhamnose reductase. The reaction mixture contained the following components in a total volume of 0.5 ml: 50 mM Na2HPO4-KH2PO4, pH 8.0; 2 mM MgCl2; 2 mM EDTA; 1 mM DPN; 0.12 mM TPNH; 4.4 

units of protein from Preparation D; and 0.028 mM acid-hydrolyzed nucleotide intermediate (added at zero time). At the time indicated by the arrow, nucleotide intermediate, derived from 0.024 
mole of GDP-rhamnose, was added.
were able to form the nucleotide intermediate from GDP-mannose. No such separate activity was detected in several experiments.

Characterisation of Nucleotide Intermediate as GDP-4-keto-d-talose (GDP-4-keto-d-talomethylene)—The products formed by the reaction of nucleotide intermediate with o-phenylenediamine exhibited an absorption spectrum similar to that formed by GDP-4-ketorhamnose previously identified (21).

Okazaki et al. (22) have found that TDP-4-keto-6-deoxy-d-glucose reacts in 0.1 N NaOH to give an absorption peak at 320 μμ, while the sugar phosphate released by the action of pyrophosphatase on the nucleotide was found to have an absorption maximum at 334 μμ in NaOH. The nucleotide intermediate produced from GDP-mannose gave an absorption peak at 325 μμ in 0.1 N NaOH (Fig. 4).

The following experiments were carried out to characterize further the nucleotide intermediate and, at the same time, investigate the enzymatic reaction or reactions involved in its reduction. The nucleotide intermediate was reduced chemically with NaBH₄ or enzymatically with purified GDP-4-ketorhamnose reductase with TPNH or DPNH as the reductant (Table III). It is to be noted that either chemical or enzymatic reduction results in the formation of substantial quantities of both bound rhamnose and talomethylene. The ratio of the methylpentoses varied in the two experiments in which NaBH₄ was used as reductant. Reduction of the nucleotide intermediate by GDP-4-ketorhamnose previously identified (21).

In Experiment P-59, the quantities of talomethylene and rhamnose and their ratio were determined on eluates from paper after chromatography in Solvent 7. In P-117 the ratio of talomethylene to rhamnose was determined as in P-59 with Solvent 4; the total quantities were calculated from methylpentose determinations on incubation mixtures after correcting for methylpentose in controls without NaBH₄ or without TPNH or DPNH. These controls contained less than 10% of the amounts synthesized. Between 90 and 95% of the methylpentoses adhered to Norit A in P-117. However, approximately half of the initially adhering methylpentoses were recovered with six water washings as free talomethylene and free rhamnose; the rest was eluted (footnote a). The eluates from Norit A contained methylpentose-bound rhamnose and talomethylene in different ratios than the water washings of Norit A. Methylpentoses were separated in Solvent 4. The GDP-4-ketorhamnose converted to rhamnose by incubation mixtures in the four experiments was 89, 88, 81, and 47%, respectively.

![Fig. 4. Spectral changes occurring during synthesis of nucleotide intermediate from GDP-mannose and a requirement for catalytic amounts of TPNH to produce the spectral changes. Reaction mixtures A, B, and C contained the following components in a total volume of 0.5 ml: 50 mM NaHPO₄-KH₂PO₄, pH 8.0; 10 mM EDTA; 2 mM MgCl₂; 1.7 mg of protein from enzyme Preparation C; 0.96 mM GDP-mannose where indicated; and 0.12 mM TPNH where indicated. Incubation was performed at room temperature. Aliquots (0.1 ml) were withdrawn at 0, 15, 60, and 120 minutes and mixed with 1.0 ml of 0.1 N NaOH, and the spectra were read between 1 and 11 hours after addition of alkali.](http://www.jbc.org/)

**Table III**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Reductant</th>
<th>Isolation method</th>
<th>Talo-</th>
<th>Rhamnose</th>
<th>Talomethylene to rhamnose ratio (μmoles)</th>
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<tr>
<td>P 50</td>
<td>NaBH₄</td>
<td>F</td>
<td>5.6</td>
<td>0.50</td>
<td>3.5</td>
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<tr>
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<td>NaBH₄</td>
<td>W + E</td>
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<td>P-117b</td>
<td>TPNH</td>
<td>W + E</td>
<td>1.9</td>
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<tr>
<td>P-117c</td>
<td>DPNH</td>
<td>W + E</td>
<td>0.84</td>
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</table>

*Method F, free methylpentoses that did not adsorb to Norit A. All of the methylpentose was in this form in Experiment P-59. W, methylpentoses released by washing Norit A-adsorbed nucleotides with water. E, methylpentoses released by elution of Norit A-adsorbed nucleotides with 50% ethanol containing 0.1% of concentrated NH₄OH.*

*GDP-4-ketorhamnose, prepared from 7 μmoles of GDP-mannose by the action of enzyme Preparation A, was separated from enzymes and polysaccharide by Method 2 (see “Experimental Procedure”). The GDP-4-ketorhamnose, in a total volume of 2 ml of 4 mM NaHPO₄-KH₂PO₄, pH 7.5, was adjusted to pH 8.9 with NaOH. NaBH₄ (130 μmoles) was added and the mixture was incubated at 0° for 3 hours. The pH was then adjusted to 5 with HCl; nitrogen was bubbled through the solution for 2 hours at 0°, and the pH was adjusted to 8 with NaOH.*

*GDP-4-ketorhamnose prepared from 10.6 μmoles of GDP-mannose by the action of enzyme Preparation C (with a catalytic quality of TPNH) was separated from enzymes and polysaccharide by Method 1 (see “Experimental Procedure”). Spectrophotometric assay of this preparation of GDP-4-ketorhamnose with GDP-4-ketorhamnose reductase and TPNH indicated the presence of 3.8 μmoles of GDP-4-ketorhamnose. The GDP-4-ketorhamnose, in a total volume of 20 ml of 45 mM EDTA-500 mM Tris-5 mM NaHPO₄, pH 8, was incubated at 0° for 3 hours (the pH remained constant). The reaction mixture was then treated as in the last sentence of footnote b.*

*a Method F, free methylpentoses that did not adsorb to Norit A. All of the methylpentose was in this form in Experiment P-59. W, methylpentoses released by washing Norit A-adsorbed nucleotides with water. E, methylpentoses released by elution of Norit A-adsorbed nucleotides with 50% ethanol containing 0.1% of concentrated NH₄OH.*

*b GDP-4-ketorhamnose, prepared from 7 μmoles of GDP-mannose by the action of enzyme Preparation A, was separated from enzymes and polysaccharide by Method 2 (see “Experimental Procedure”). The GDP-4-ketorhamnose, in a total volume of 2 ml of 4 mM NaHPO₄-KH₂PO₄, pH 8.0; 10 mM EDTA; 2 mM MgCl₂; 1.7 mg of protein from enzyme Preparation C; 0.96 mM GDP-mannose where indicated; and 0.12 mM TPNH where indicated. Incubation was performed at room temperature. Aliquots (0.1 ml) were withdrawn at 0, 15, 60, and 120 minutes and mixed with 1.0 ml of 0.1 N NaOH, and the spectra were read between 1 and 11 hours after addition of alkali.
with DPNH or TPNH and purified GDP-4-ketorhamnose reductase resulted in talomethylose to rhamnose ratios of 1.0 and 1.9, respectively.

Properties of Enzyme Preparation C—Preparation C behaved quite differently from the crude extracts in the spectrophotometric assay for GDP-mannose-stimulated TPNH oxidation and in the assay that measured net synthesis of methylpentoses. Figs. 5 and 6 show a lag in TPNH oxidation stimulated by GDP-mannose and a lag in net methylpentose synthesis with Preparation C. However, Preparation C contained large quantities of GDP-4-ketorhamnose reductase, since GDP-4-ketorhamnose-stimulated TPNH oxidation occurred immediately on addition of GDP-4-ketorhamnose. Therefore, the assays shown in Figs. 5 and 6 measured the rate of formation of GDP-4-ketorhamnose when TPNH was present. The time lag varied from 5 to 12 minutes. The addition of DPN had no detectable effect on the reaction as measured by TPNH oxidation (assay similar to that shown in Fig. 5). However, addition of DPN in the absence of TPNH did result in the formation of methylpentose, but at a greatly reduced rate and with a lag of 45 minutes (Fig. 6).

Addition of ATP, ADP, GTP, or GDP did not prevent the lag observed with Preparation C. Preincubation of this preparation for 30 minutes either with GDP-mannose or with TPNH alone did not remove the lag observed when the omitted component (GDP-mannose or TPNH) was subsequently added. Other experiments indicated that a limiting amount of GDP-4-ketorhamnose was completely reduced by an appropriate amount of Preparation C within 5 minutes. When this amount of GDP-4-ketorhamnose and GDP-mannose was preincubated with the same amount of Preparation C, the subsequent addition of TPNH resulted in difference curves characteristic of immediate reduction of the added GDP-4-ketorhamnose, followed by formation of more GDP-4-ketorhamnose and its reduction 5 minutes later. When small amounts of GDP-mannose were used with Preparation C and the reaction was allowed to go to completion, introduction of additional GDP-mannose resulted in immediate GDP-mannose-stimulated TPNH oxidation. This result indicates that the enzyme that converts GDP-mannose to GDP-4-ketorhamnose had been activated. If, following such activation, the enzyme is precipitated with ammonium sulfate and dissolved, the characteristic lag period is again evident. These results suggest that the lag period is due to a reversible activation of the enzyme which occurs in the presence of GDP-mannose and TPNH.

The requirements for formation of GDP-4-ketorhamnose with Preparation C have also been tested with a two-step reaction: Step 1, incubation with GDP-mannose and other components followed by inactivation of enzyme by 1 minute of heating at 100°, and Step 2, spectrophotometric assay for GDP-4-ketorhamnose with purified GDP-4-ketorhamnose reductase (Preparation D) and spectrophotometric assay for residual GDP-mannose with Preparation C. The results of such experiments are reported in Fig. 7. It is apparent that catalytic quantities of TPNH are necessary for accumulation of GDP-4-ketorhamnose. Similar requirements for the formation of GDP-4-ketorhamnose were found when its absorption spectrum in alkaline solution was used for the determination of the nucleotide intermediate (Fig. 4).

The data illustrated in Fig. 7 indicate that DPN increased the rate of GDP-4-ketorhamnose accumulation in the presence of TPNH. DPN (in the absence of TPNH) led to no accumulation of GDP-4-ketorhamnose. In similar experiments with this two-step reaction, DPNH, TPN, or both together could not replace the TPNH requirement for the accumulation of GDP-4-ketorhamnose with Preparation C. In contrast, it is apparent from the data of Fig. 6 that the addition of DPN (in the absence of TPNH) resulted in some methylpentose formation. It is possible that small amounts of GDP-4-ketorhamnose, formed with DPN present, were rapidly reduced to GDP-rhamnose and GDP-talomethylose utilizing some endogenous component of
minute incubations. The amount of GDP-mannose in zero time
latter instances, GDP-mannose was assayed only in the 150-
samples was taken as the amount added. The results of Fig. 5
indicate that GDP-mannose assays yield only 62% of theoretical
corrected accordingly.
the system to reduce DPN. This would explain the limited
methylpentose synthesis observed with DPN and no TPNH
(Fig. 6) and the absence of accumulation of GDP-4-ketorhamnose
under similar conditions (Fig. 7).
the nature of the effect of DPN on the conversion of GDP-
mannose to GDP-4-ketorhamnose by Preparation C is not clear
from the results presented and requires further study with
purified enzyme preparations free of GDP-4-ketorhamnose
reductase. Such preparations have not been obtained.

**Fig. 7. Requirements for synthesis of GDP-4-ketorhamnose**
with enzyme Preparation C. The complete reaction mixture
contained the following in a total volume of 0.5 ml; 50 mM Na2-
HPO4-KH2P04, pH 8.0; 2 mM MgCl2; 10 mM EDTA; 0.1 mM DPN;
0.12 mM TPNH; 0.96 mM GDP-mannose; and 1.7 mg of protein
from enzyme Preparation C. Aliquots (0.1 ml) were removed
after incubation at room temperature for the indicated time,
diluted to 0.4 ml, and placed in a boiling water bath for 1 minute;
0.1 ml of this mixture was analyzed spectrophotometrically for
GDP 4 ketorhamnose with GDP 4-ketorhamnose reductase
(Preparation D) and for GDP-mannose with Preparation C.
GDP-4-ketorhamnose was not detected at any of the indicated
times when TPNH or TPNH and DPN were omitted. In these
latter instances, GDP-mannose was assayed only in the 150-
minute incubations. The amount of GDP-mannose in zero time
samples was taken as the amount added. The results of Fig. 5
indicate that GDP-mannose assays yield only 62% of theoretical
with Preparation C. The GDP-mannose analyses have not been

discussion

Scheme 1 summarizes the enzymatic steps required for the
conversion of GDP D-mannose to GDP D-rhamnose and GDP-
d-talomethyllose. On the basis of the spectrum of the inter-
mediate in the o-phenylenediamine reaction and in the reaction
with alkali, it is likely that the nucleotide intermediate is a keto
sugar compound. The enzymatic and chemical reduction to
GDP-rhamnose and GDP-talomethyllose indicate that a keto
group is present at position 4 of the methylpentose moiety.
When reduction was carried out with NaBH4, the talomethyllose
to rhamnose ratio varied in two different experiments. Wide
variations in ratios of the two possible products of reduction
were observed by Moses, Ferrier, and Calvin (23) when a keto
sugar was reduced with NaBH4. Although the absolute configu-
raration of the talomethyllose derived from GDP-talomethyllose
was not directly determined, the demonstration that rhamnose
obtained from GDP-rhamnose was the D isomer is evidence that
the GDP-4-ketorhamnose and GDP-talomethyllose are the D
isomers. These results are consistent with the fact that poly-
saccharide from strain GS contains D-rhamnose and D-talomethyllose (2).

Enzyme Preparation C exhibits a requirement for the simultan-
eous presence of TPNH and GDP-mannose to "activate"
the enzyme fraction that converts GDP-mannose to GDP-4-
ketorhamnose (tentatively designated GDP-mannose dehydrase).
The activation and inactivation were reversible, since represen-
tation with ammonium sulfate of the activated GDP-mannose
dehydrase yielded inactive enzyme that was reactivated by
GDP-mannose and TPNH. Such a reversible activation system
could provide a convenient control mechanism in vivo, apart
from protein synthesis. It is possible that the reversible steps
are an indication of an aggregation reaction, but no evidence
has been obtained to support this view. In this connection,
Fincham has isolated mutants of Neurospora crassa, in which
the glutamate dehydrogenase is inactive but can be activated
by TPN and high concentrations of glutamate (24) and in which
the enzyme appears to be composed of subunits (25).

GDP-4-ketorhamnose is an intermediate in the synthesis of
GDP-L-fucose in Aerobacter aerogenes ATCC 12658 (21), in
mucoi'd mutants of Escherichia coli K12 (26), and in the syn-
thesis of GDP-colitose (GDP-3,6-dideoxy-L-galactose) in E.
coli 0111-B4 (27). The final products in the above reactions
(L-fucose and GDP-colitose) each are one of two possible
C4 epimers. Epimerization of GDP-4-keto-rhamnose at C-3
and C-5 is required for GDP-L-fucose synthesis, and epimeriza-
tion at C-5 and reduction at C-3 are required for GDP-colitose
synthesis in addition to a specific reduction at C-4 in both.
An analogous situation is apparent in the conversion of TDP-p-
glucoce to TDP-L-rhamnose, in which TDP-4-keto-6-deoxy-p-
glucoce has been identified as an intermediate (22, 28). No
other intermediates have been found in the above reactions.
In view of the requirements for GDP-mannose dehydrase activa-
tion, it would be of interest to determine whether or not inter-

**Scheme I**
mediates other than GDP-4-ketorhamnose or TDP-4-keto-0-deoxy-0-glucose form in the appropriate systems when catalytic quantities of TPNH (or DPNH) are added. A mechanism for the formation of GDP-4-ketorhamnose from GDP-mannose has been proposed in which the DPN is considered "as a mediator in intramolecular hydrogen transfer" (21). An analogous mechanism has been proposed for conversion of TDP-D-glucose to TDP-4-keto-6-deoxy-0-glucose (3). The name mechanism may apply to the enzyme from strain GS, since DPN increased the rate of accumulation of GDP-4-ketorhamnose.

The isolation of an enzyme fraction that reduces GDP-4-keto-D-rhamnose to GDP-D-rhamnose and GDP-D-talomehylose with either TPNH or DPNH as a reductant is of considerable interest. Further fractionation is needed to determine whether one or two reductases are present in the preparation, but the identical rate of reaction with TPNH or DPNH as reductant, before and after 25-fold purification, provides no evidence for the presence of two different enzymes. The name GDP-4-keto-D-rhamnose reductase is tentatively suggested for this enzyme fraction. The direct epimerization of GDP-rhamnose to GDP-talomehylose either with reduced di- or triphosphopyridine nucleotide as a reductant. GDP-D-mannose is inactive with this enzyme fraction, for which the name guanosine diphosphate 4-keto-D-rhamnose reductase is tentatively suggested.

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Biosynthesis of Guanosine Diphosphate d-Rhamnose and Guanosine Diphosphate d-Talomethylose from Guanosine Diphosphate α-d-Mannose
Alvin Markovitz


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