Studies on the Biosynthesis of Guanosine Diphosphate L-Fucose*

VICTOR GINSBURG

From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service, Bethesda 14, Maryland

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An enzyme preparation from Aerobacter aerogenes is capable of carrying out the multistep conversion of guanosine 5'-diphosphate-D-mannose to guanosine 5'-diphosphate-L-fucose as shown in Equation 1 (1, 2).

\[
\text{GDP-D-mannose} + \text{TPNH} + \text{H}^+ \rightarrow \text{GDP-L-fucose} + \text{TPN} + \text{H}_2\text{O} \tag{1}
\]

The kinetics of reduced triphosphopyridine nucleotide oxidation suggested that guanosine 5'-diphosphate-D-mannose was converted to intermediates before reduction by reduced triphosphopyridine nucleotide (2). Subsequent studies revealed that the intermediates could be chemically reduced to guanosine 5'-diphosphate-6-deoxyhexoses (3). The present paper is concerned with the nature of the intermediates and the formation and identification of the reduction products.

EXPERIMENTAL PROCEDURE

Chemicals—GDP-mannose was a commercial preparation (Sigma Chemical Company) isolated from bakers' yeast and containing approximately 8% of a GDP-aldohexose (4). The GDP-aldohexose, however, was not metabolized by the enzyme preparation and did not appear to interfere with the experiments described in this paper.

The rare 6-deoxyhexoses used as chromatographic standards were gifts from Professor T. Reichstein.

TPNH was kindly supplied by Dr. G. Popják and Dr. D. S. Goodman, who prepared it by the chemical reduction of TPN in the presence of H_2O (5). It contained 270,000 c.p.m. per pmole of H which was assumed to be equally distributed between the α and β positions. The figure given in Table V is therefore 135,000 c.p.m. per pmole.

Enzyme Preparation—The enzyme preparation was an ammonium sulfate fraction prepared from A. aerogenes (ATCC 12637) as described in a previous publication (2). The amounts of enzyme used in the individual experiments are expressed as units of enzymic activity as measured by a spectrophotometric assay. A unit is defined as that amount of activity which will catalyze the GDP-D-mannose-induced oxidation of 1 pmole of TPNH per hour under specified conditions (2).

Analysis of GDP-linked Sugars—The GDP-sugars were adsorbed on charcoal which was then washed with water. The washed charcoal was suspended in 0.01 N HCl and heated at 100° for 10 minutes, thereby releasing the GDP-linked sugars. The charcoal was removed by centrifugation and the supernant solution was deionized with Amberlite MB-3 resin. The sugars in the deionized solution were identified by paper chromatography and specific chemical tests as described. AgNO₃ reagent (6) was used to visualize the sugars after chromatography. Quantitative determinations were carried out by eluting appropriate areas of the chromatograms with water and estimating the sugar content of the eluate by reducing value (7) or by the specific colorimetric assay for 6-deoxyhexoses (8).

Chromatography of Nucleotides—The nucleotides were separated by paper chromatography with ethanol-neutral ammonium acetate solution (9) as a solvent. The nucleotides from incubation mixtures were partially purified before chromatography by adsorption on charcoal followed by elution with 50% ethanol containing 0.1% concentrated NH₄OH. Omission of the charcoal step resulted in poorer resolution of the nucleotides without altering the qualitative or quantitative aspects of the resulting ultraviolet light-absorbing bands.

RESULTS AND DISCUSSION

Formation of Intermediates The transformation of GDP-D-mannose to GDP-L-fucose by enzyme preparations from A. aerogenes requires TPNH. In the absence of TPNH, the GDP-D-mannose is converted to intermediates on the pathway of GDP-L-fucose formation. The disappearance of GDP-D-mannose can be followed either by chromatography of the nucleotides present in the incubation mixture at various times or by estimating the mannose liberated on mild acid hydrolysis after adsorption of the nucleotides on charcoal. Neither of these methods, however, reveals the nature of the intermediates in the conversion process. Chromatography of the sugars liberated by mild acid hydrolysis showed only decreasing amounts of mannose without the concomitant appearance of any well defined product. Chromatography of the nucleotides at various times during the incubation likewise showed a decreasing amount of ultraviolet-absorbing material with the chromatographic properties of GDP-D-mannose. However, the appearance of a new ultraviolet-absorbing compound in the GDP area of the chromatogram could be seen. This compound was indistinguishable from GDP by chromatography or electrophoresis. As shown by the following experiment, it is most likely GDP and is an artifact arising from the breakdown of the intermediates during the isolation procedure. GDP-D-mannose, 1.4 μmoles, was incubated with 5 units of enzyme in 10 ml of 0.02 μ Tris buffer,
pH 8.0, at 22° for 1 hour. The incubation mixture was then placed in a boiling water bath for 20 seconds and the denatured protein discarded by centrifugation. Chromatography and analysis of the nucleotides in an aliquot of the supernatant solution revealed a virtual disappearance of GDP-d-mannose and the appearance of GDP. However, when the remainder of the supernatant solution was reincubated with fresh enzyme and excess TPNH, oxidation of TPNH occurred without the usual lag that is given with GDP-d-mannose as substrate (Z), and analysis of the nucleotides in the incubation mixture revealed mainly GDP-L-fucose and only a small amount of GDP. As neither authentic GDP nor the GDP isolated from the incubation mixtures gives rise to GDP-L-fucose when incubated with TPNH and the enzyme, it would appear that the actual intermediates are labile and are degraded to GDP during isolation.

Formation of GDP-6-Deoxyhexoses by Chemical Reduction of Intermediates—In attempts to make stable derivatives from which the nature of the intermediates could be deduced, an incubation mixture of GDP-d-mannose and enzyme was treated with H2 and rhodium catalyst. It was found that this treatment could replace TPNH in that a GDP-6-deoxyhexose was produced. However, as previously reported (3), the GDP-6-deoxyhexose formed was not GDP-L-fucose, as it liberated 6-deoxymannose and an unidentified 6-deoxyhexose on mild acid hydrolysis. This 6-deoxyhexose, which is the predominant reduction product, has now been identified as 6-deoxytalose. In some experiments, trace amounts of two additional sugars were detected.

In a typical preparation, 1.7 μmoles of GDP-d-mannose were incubated for 2 hours with 4 units of enzyme at 22° in 10 ml of 0.02 M Tris buffer, pH 8.0. The incubation mixture was then placed in boiling water for 20 seconds, and the denatured protein was removed by centrifugation. Rhodium catalyst (5% Rh on alumina powder, Baker and Company, Inc., Newark, New Jersey), 30 mg, was added, and H2 was bubbled through the solution for 30 minutes. Charcoal, 15 mg, was then added, and the mixture of charcoal and catalyst was collected by centrifugation. After being washed with two 10 ml aliquots of water, the nucleotides were eluted from the charcoal and catalyst with 50% ethanol containing 0.1% concentrated NH4OH. The GDP-6-deoxyhexose was then separated by chromatography from the remaining GDP-d-mannose, the GDP-leptose, and the GDP that is formed during the incubation. The yield of GDP-6-deoxyhexose was 0.6 μmole calculated spectrophotometrically as guanosine (10).

Characterization of GDP-6-Deoxyhexoses—The isolated nucleotide was identified as a mixture of GDP-6-deoxyhexoses by the following evidence.

(a) During paper chromatography in ethanol-neutral ammonium acetate the nucleotide migrated faster than GDP-d-mannose and had the same mobility as GDP-L-fucose (2). The isolated material exhibited an ultraviolet absorption spectrum typical of a guanosine derivative. It reacted in the specific colorimetric test for 6-deoxyhexoses (8) to produce a chromophore with an absorption spectrum identical to that given by authentic fucose. A chemical analysis is given in Table II. The reducing value exhibited before hydrolysis presumably reflects the lability of the nucleotide under alkaline conditions.

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**Table I**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>6-Deoxyhexose formed (μmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>25</td>
</tr>
<tr>
<td>Incubation omitted</td>
<td>0</td>
</tr>
<tr>
<td>Catalyst omitted</td>
<td>0</td>
</tr>
<tr>
<td>H2 omitted</td>
<td>1</td>
</tr>
</tbody>
</table>

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**Table II**

Chemical analysis of GDP-6-deoxyhexose

<table>
<thead>
<tr>
<th>Test</th>
<th>Results (μmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid-labile phosphorus</td>
<td>1.1</td>
</tr>
<tr>
<td>Total phosphorus</td>
<td>2.2</td>
</tr>
<tr>
<td>6-Deoxyhexose (fucose)</td>
<td>1.2</td>
</tr>
<tr>
<td>Pentose (L), as ribose</td>
<td>1.1</td>
</tr>
<tr>
<td>Reducing value (7), as fucose</td>
<td>0.6</td>
</tr>
</tbody>
</table>

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**Table III**

Chromatographic properties of 6-deoxyhexoses formed by chemical reduction

<table>
<thead>
<tr>
<th>Solvent</th>
<th>6-Deoxyhexose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>6-Deoxygalactose (fucose)</td>
<td>0.6</td>
</tr>
<tr>
<td>6-Deoxyglucose</td>
<td>0.8</td>
</tr>
<tr>
<td>6-Deoxyriboside</td>
<td>0.9</td>
</tr>
<tr>
<td>6-Deoxymannose</td>
<td>1.0</td>
</tr>
<tr>
<td>6-Deoxyallose</td>
<td>(1.4)</td>
</tr>
<tr>
<td>6-Deoxysialtose</td>
<td>1.4</td>
</tr>
<tr>
<td>6-Deoxylactose</td>
<td>1.6</td>
</tr>
<tr>
<td>6-Deoxydose</td>
<td>1.7</td>
</tr>
<tr>
<td>Unknown A</td>
<td>1.6</td>
</tr>
<tr>
<td>Unknown B</td>
<td>1.0</td>
</tr>
</tbody>
</table>

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(b) Treatment of the isolated nucleotide with 0.01 N HCl for 10 minutes at 100° liberated free sugars and changed the chromatographic properties of the guanosine derivative from that of a fast-running compound to one that cochromatographed with GDP. Longer hydrolysis led to the formation of a second guanosine derivative that cochromatographed with GMP.

c) Chromatographic analysis of the sugars liberated by hydrolysis usually revealed only two sugars, both of which exhibited the characteristic 6-deoxyhexose reaction (8). These were identified as 6-deoxytalose and 6-deoxymannose from the table given in Table III. Additional evidence for the identity of 6-deoxytalose was supplied by demonstrating chromatographically the formation of a spot corresponding to fucose after epimerization of the isolated sugar with 0.05 M phosphate buffer, pH 7.4, at 100° for 1 hour. The ratio of 6-deoxytalose to 6-deoxymannose was approximately 12:1. This ratio was not affected by varying the time of incubation before chemical reduction. In some experiments, trace amounts of two additional sugars were detected in the hydrolysate. Their chromatographic properties with Solvent 1 (Table III) were similar to fucose and 6-deoxyglucose, but their low concentrations precluded further characterization. The amounts of these trace sugars were estimated to be generally less than 5% of the total 6-deoxyhexose liberated from the nucleotide by hydrolysis.

Nature of Intermediates—The constant ratio of GDP-6-deoxytalose to GDP-6-deoxymannose and the fact that they are C-4 epimers suggest that these compounds arise from the reduction of a single intermediate, GDP-4-keto-6-deoxy-α-mannose (Compound I, Fig. 1). Because of the trace reduction products mentioned above, it is tempting to postulate the presence of a second intermediate, GDP-4-keto-6-deoxy-α-galactose (Compound II). However, the existence of Compound II in the incubation mixture is by no means certain, and if it does exist, it must be present in low concentration.

Evidence that GDP-6-mannose is converted into a keto derivative is furnished by the o-phenylenediamine test of Lanning and Cohen (17). This test for α-keto acids was found by Bernaerts and de Ley (18) to be valuable for 3-keto sugars which gave absorption peaks in the neighborhood of 350 μ. Although compounds analogous to Compound I are not available for com-
Biosynthesis

The L-fucose was then treated with periodate, and the acetaldehyde arising from C-5 and C-6 was isolated as its crystalline dimer dimeric derivative (19). The acetaldehyde dimer was then assayed for H3-activity. A correction was applied for the dilution by unlabeled L-fucose.

All radioactivity counting was done by means of a liquid scintillation counter. The samples were dissolved in a solution with the following composition: H2O, 0.2 ml; ethanol, 1.8 ml; and 8.0 ml of toluene containing 400 mg of 2,5-diphenyloxazole and 5 mg of 1,4-di[2-(5-phenyloxazolyl)]benzene per 100 ml (20).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Radioactivity (cpm/mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPNH†</td>
<td>135,000</td>
</tr>
<tr>
<td>L-Fucose</td>
<td>27,500</td>
</tr>
<tr>
<td>Acetaldehyde dimered</td>
<td>&lt;800</td>
</tr>
</tbody>
</table>

Comparison, it is reasonable to assume that 4-keto sugars would exhibit a similar reaction. When GDP-δ-mannose was incubated with the enzyme, the accumulation of a compound exhibiting this reaction was found. The spectrum given by an incubation mixture in this test has an absorption maximum at 345 mp as shown in Fig. 2. A correlation between the accumulation of the α-phenylendiamine-reacting compound and the amount of 6-deoxyhexose that can be produced by chemical reduction is given in Table IV. From the observed correlation it is probable that the compound responsible for the α-phenylendiamine reaction is indeed the intermediate formed from GDP-α-mannose.

Experiments with Tritium—Incubation of GDP-δ-mannose and TPNH with the enzyme preparation resulted in the formation of tritium-labeled GDP-L-fucose. The L-fucose was isolated and its specific activity determined. The L-fucose was then treated with periodate and the acetaldehyde arising from C-5 and C-6 was isolated as its crystalline dimeric derivative (18). The acetaldehyde dimer was then assayed for H3-activity. A correction was applied for the dilution by unlabeled L-fucose.

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When the experiment described in Table V was repeated with H2O in place of TPNH as a source of H3, the L-fucose formed also became labeled. However, in this case 20% of the H3 of the L-fucose was recovered in the dimeric derivative (H2O, 71,000 c.p.m. per μmole; L-fucose, 9,100 c.p.m. per μmole; acetaldehyde dimered, 1,800 c.p.m. per μmole).

Effect of DPN on Conversion of GDP-δ-Mannose to GDP-L-Fucose—DPN stimulates the formation of GDP-L-fucose from GDP-δ-mannose as shown in Fig. 3. It can be seen that DPN increases the maximal rate attained by the reaction without decreasing the lag period. This would indicate that DPN exerts its effect at an early step in the conversion process. It is probable that this early step is involved in the formation of GDP-4-keto-6-deoxy-δ-mannose by analogy to the finding of Kornfeld and Glaser (22) that DPN stimulates the formation of TDP-4-keto-6-deoxy-glucose, which they find to be an intermediate in the conversion of TDP-glucose to TDP-L-rhamnose (23-25). However, direct evidence for this is lacking.

Pathway for Biosynthesis of GDP-L-Fucose—A provisional pathway is presented in Fig. 1 that is consistent with the data obtained although it is evident that many uncertainties remain. It is possible, for example, that the actual intermediates are tautomers of the compounds depicted in Fig. 1. Even if it be assumed that this is the correct pathway, the mechanism of formation of Compound I from GDP-δ-mannose is still to be determined. This conversion involves a reduction at C-6 and an oxidation at C-4 of GDP-δ-mannose. The experiment with H2O makes it probable that this oxidation-reduction is intramolecular as one might expect to find much more H3 in C-5 and C-6 than was actually found if the hydrogens on C-6 were supplied by water. This would suggest a possible function for DPN as a mediator for intramolecular hydrogen transfer.

The conversion of I to II involves an inversion of configuration at C-3 and C-5. This transformation could be carried out by one-deoxymannose in which case H3 from H2O might be expected to be incorporated into C-3 and C-5.

Compound II could be stereospecifically reduced by TPNH directly to GDP-L-fucose. This would be consistent with the fact that no sugars other than δ-mannose or L-fucose were detected in the incubation mixture in the absence of DPN.
detected in reaction mixtures (2). Presumably, the 4-keto-6-deoxyhexose is unstable when released from the nucleotide by acid hydrolysis and escapes chromatographic detection. The H3-labeled L-fucose that was formed with the use of TPNH did not contain H3 in either the C-5 or C-6 position. This result is also consistent with a reduction of II, which would introduce H3 into the C-4 position.

It is hoped that work now in progress on the purification of the enzymes involved will provide more information on the validity of the proposed pathway.

SUMMARY

Evidence is presented that guanosine 5'-diphosphate 4-keto-6-deoxy-d-mannose is an intermediate in the conversion of guanosine 5'-diphosphate d-mannose to guanosine 5'-diphosphate L-fucose by an enzyme preparation from Aerobacter aerogenes. A pathway for the biosynthesis of guanosine 5'-diphosphate L-fucose is proposed that is consistent with the available data.

REFERENCES

Studies on the Biosynthesis of Guanosine Diphosphate l-Fucose
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