Observations on the Mechanism of the Reversible Epimerization of GDP-\(d\)-mannose to GDP-L-galactose by an Enzyme from Chlorella pyrenoidosa*

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An enzyme fraction from the green alga Chlorella pyrenoidosa that catalyzes the reversible epimerization of guanosine 5'-diphosphate \(d\)-mannose to guanosine 5'-diphosphate \(L\)-galactose brings about the incorporation of tritium from tritium-labeled water into the hexosyl moieties of those sugar nucleotides. The hexoses were degraded by periodate oxidation whereby the tritium was found to be equally distributed between carbon atoms 3 and 5. That observation was taken to imply that the epimerizations proceed via ene-diol intermediates.

An enzyme extract of the green alga Chlorella pyrenoidosa has been shown to catalyze the reversible epimerization of the sugar nucleotide guanosine 5’-diphosphate \(d\)-mannose to guanosine 5’-diphosphate \(L\)-galactose (1, 2). That process brings about inversion of configuration at carbon atoms 3 and 5 of the hexosyl moieties. As far as I know, double epimerizations of that type have heretofore been observed only in those enzymic reactions in which a 6-deoxy sugar nucleotide is produced.

The mechanism of the double epimerization of \(d\)-mannose to \(L\)-galactose is therefore of considerable interest, at least in the comparative sense. If epimerization proceeds as it does in the enzymic conversion of UDP-\(d\)-glucose to UDP-\(L\)-galactose, oxidation and subsequent reduction with inversion of the configuration at the epimerization site would be expected (3, 4). On the other hand, if the mechanism by which \(d\)-TDP-\(L\)-rhamnose is formed enzymically were followed (3, 5), there would first be oxidation at carbon 4, epimerization through the formation and rehydration of ene-diol intermediates, and subsequent stereospecific reduction of the keto group at carbon 4. When inversion occurs through introduction of hydroxyl across the ene-diol double bond, hydrogen from water in the medium would be expected to be incorporated into the molecule. This does not occur in the interconversion of UDP-\(d\)-glucose and UDP-\(L\)-galactose (4).

In this report I present evidence that \(^3\)\(^t\)H from \([^{3}H]H_{2}O\) is taken up by GDP-\(d\)-mannose and GDP-\(L\)-galactose in the presence of the Chlorella epimerase and that those \(^3\)\(^t\)H atoms are equally distributed between carbon atoms 3 and 5 of the hexosyl moieties.

MATERIALS AND METHODS

C. pyrenoidosa was cultured and enzyme extracts were prepared as described previously (1, 2). An additional 18-fold purification of the enzyme was achieved by chromatography on a column of hydroxylapatite (2), and that preparation was used for these experiments.

GDP \(d\)-mannose, GDP \(d\)-glucose, and other standard biochemicals were obtained from the Sigma Chemical Co. GDP-\(L\)-galactose was synthesized chemically in this laboratory (2). \(^3\)\(^t\)H-labeled water (about 50 Ci/\(\mu\)mol) and GDP-\(d\)-mannose labeled uniformly with \(^13\)C in the mannose moiety (150 \(\mu\)Ci/\(\mu\)mol) were purchased from the Amersham Corp.

Radioactivity of labeled compounds was measured in a Beckman model LS-230 scintillation spectrometer in a mixture consisting of 1 ml of water containing the sample and 10 ml of 33% Triton, 0.01% 1,4-bis[2-(5-phenyloxazolyl)]benzene and 0.4% 2,5-diphenyloxazole in toluene.

Electrophoresis was carried out on Schleicher and Schuell acid-washed paper (No. 589, Orange Ribbon) in an apparatus constructed after the design of Crestfield and Allen (6). For the electrophoretic isolation of GDP sugars from the reaction mixtures, a buffer of 0.1 M ammonium formate made to pH 2.7 with formic acid was used at about 40 V/cm. At that pH GDP sugars are cleanly separated from GDP, GMP, and hexose phosphates.

Neutral sugars were isolated by partition chromatography on Schleicher and Schuell No. 589 White Ribbon paper developed with 1-propyl alcohol/ethyl acetate/water (7:1:2 by volume). They were made visible with the alkaline silver nitrate or \(p\)-anisidine-HCl spray reagents (7). \(^13\)C-labeled compounds were located on paper by exposure to x-ray film (Kodak No. SB-3).

RESULTS AND DISCUSSION

A typical enzymic reaction mixture used to bring about the incorporation of tritium into the hexosyl moiety of GDP-\(d\)-mannose and GDP-\(L\)-galactose was composed of the following: 0.1 \(\mu\)mol of GDP-\(L\)-galactose or GDP-\(d\)-mannose, 0.5 \(\mu\)mol of Tris-HCl, pH 8.0, 0.1 mg of Chlorella epimerase (lyophilized hydroxylapatite protein fraction), and 50 \(\mu\)Ci of \([^{3}H]H_{2}O\) in a total volume of 30 \(\mu\)l. It was incubated for 2 h at 23°C. At the end of the reaction period, 0.10 ml of 95% ethanol was added to the mixture on a plastic planchet. It was dried down in a vacuum desiccator over silica gel adsorbent. The residue was taken up in 0.1 ml of water and again evaporated to dryness in vacuo. The material remaining was then electrophoresed on paper in ammonium formate buffer, pH 2.7. GDP-Hexose was located by its UV absorption and was eluted with water. The aqueous eluate was weighed to determine its volume, and a suitable aliquot was counted in the scintillation spectrometer.

In order to learn how radioactive label was distributed between the GDP-hexoses, a portion of the \(^3\)\(^t\)H-labeled material was hydrolyzed in acid (1 M trifluoroacetic, 10 min at 100°C) and chromatographed on paper against authentic mannose and galactose. The appropriate areas of the paper were cut out, chopped into small pieces, and extracted by shaking with 1 ml of water in a scintillation vial for 20 min. The Triton scintillation mixture (10 ml) was added to each vial, and radioactivity was measured in the counter. An old enzyme preparation that was found to have no active epimer-
ase inadvertently served as a control for this experiment. It brought about no incorporation of \(^{3}\text{H}\) into GDP-hexose when incubated in the standard reaction. Results of this experiment are shown in Table I. The variation in the proportion of the total label recovered in L-galactose and D-mannose after hydrolysis of each sugar nucleotide preparation is thought to be a result of the difficulty in precisely locating \(^{3}\text{H}\)-labeled sugars on paper chromatograms. Nonetheless, I think it has been satisfactorily established that most of the \(^{3}\text{H}\) incorporated into the sugar nucleotides is in the hexosyl moieties. It should be noted that when similar mixtures were incubated with this enzyme preparation in the absence of tritiated water (2), equilibrium was reached in 15 min from GDP-d-mannose and in 30 min from GDP-L-galactose.

From these data the theoretical maximum incorporation of \(^{3}\text{H}\) into GDP-hexose was calculated as follows. In the incubation mixture \([^{3}\text{H}]\text{H}_{2}\text{O}\) was diluted to a specific activity of 30 \(\mu\text{Ci}/\mu\text{mol}\). Assuming that one hydrogen from \(\text{H}_{2}\text{O}\) would be incorporated for each of the two epimerizations, there would be a stoichiometry of 1 mol of \(\text{H}_{2}\text{O}/\text{mol of hexose epimerized}\). Hence, for 0.1 \(\mu\text{mol}\) of substrate there could be a maximum of 3 \(\mu\text{Ci}\) of \(^{3}\text{H}\) incorporated or about 3.4 \(\times\) 10^3 cpm in this counting system. The incorporation of 2.12 \(\times\) 10^5 cpm into GDP-hexose therefore represents about 6% of the theoretical.

Control experiments with \(^{14}\text{C}\)-labeled GDP-d-mannose (Table II) indicated that the recovery of sugar nucleotides by these techniques is almost complete, hence the low incorporation of \(^{3}\text{H}\) from \([^{3}\text{H}]\text{H}_{2}\text{O}\) by this system probably represents isotope discrimination (8). What level of discrimination should be expected is not readily apparent, however. There are at least two separate events in which \(^{3}\text{H}\) is incorporated into and released from the molecule, and the product of the first incorporation might well affect the rate of the second reaction. It has also been observed with the \(\text{Chlorella}\) epimerase that the apparent equilibrium (GDP-L-galactose)/(GDP-d-mannose) reached when the reaction is initiated with GDP-d-mannose is different from the equilibrium arrived at from GDP-L-galactose, 0.8 and 0.2, respectively (9). I have been unable to explain that anomaly, but it may indeed have some bearing on these results.

A periodate degradation was used to determine the position of the \(^{3}\text{H}\) atoms incorporated into d-mannose and L-galactose. It was expected to proceed as shown in Fig. 1. If d-mannose and L-galactose are labeled at positions 3 and 5, the two formic acids produced per mol of hexose by this sequence of reactions should contain all the radioactivity.

### Table I

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(^{3}\text{H}) incorporated into sugar nucleotide</th>
<th>cpm recovered in L-Galactose</th>
<th>cpm recovered in D-Mannose</th>
<th>Par cent. of total (^{3}\text{H}) incorporated recovered in L-Galactose and D-Mannose</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDP-d-mannose</td>
<td>(1.7 \times 10^{-3} \text{ M})</td>
<td>213,100</td>
<td>35,700</td>
<td>110,700</td>
</tr>
<tr>
<td></td>
<td>(3.4 \times 10^{-3} \text{ M})</td>
<td>174,000</td>
<td>65,300</td>
<td>134,900</td>
</tr>
<tr>
<td>GDP-L-galactose</td>
<td>(1.7 \times 10^{-3} \text{ M})</td>
<td>168,100</td>
<td>54,200</td>
<td>71,500</td>
</tr>
<tr>
<td></td>
<td>(3.4 \times 10^{-3} \text{ M})</td>
<td>201,300</td>
<td>39,100</td>
<td>43,600</td>
</tr>
</tbody>
</table>

The periodate oxidation mixture contained about 0.07 \(\mu\text{Ci}\) (47,000 cpm) of GDP-d-mannose/GDP-L-galactose labeled with \(^{3}\text{H}\) in the hexosyl moieties and 0.5 \(\mu\text{mol}\) of GDP-D-glucose in a total volume of 0.110 ml of 0.03 M NaIO\(_4\), pH 4.9. It was let stand overnight (15 h) at room temperature in the dark. At the end of the reaction period excess periodate was removed by the addition of 6 \(\mu\text{mol}\) of ethylene glycol. The mixture was let stand for 30 min at room temperature.

To measure the amount of formic acid produced in the first oxidation, half of the above reaction mixture was acidified with 0.2 ml of 0.1 M H\(_2\)SO\(_4\), and formic acid was distilled over in \(\text{vacuo}\) by the low temperature method of Grant (10).

Material in the other half of the periodate oxidation mixture was reduced with NaBH\(_4\), by adding 6 \(\mu\text{mol}\) of NaBH\(_4\), as a freshly prepared solution (1 m) and allowing the mixture to stand at room temperature for 90 min. Excess NaBH\(_4\) was oxidized by the addition of about 20 \(\mu\text{mol}\) of formaldehyde. After bubbling had ceased, the mixture was evaporated to dryness on a plastic planchet in \(\text{vacuo}\) over concentrated H\(_2\)SO\(_4\), and pellets of KOH. The residue was dissolved in a small volume of water and electrophoresed on paper at pH 2.7.

In order to follow this portion of the reaction conveniently, a mixture containing GDP-d-mannose labeled uniformly with \(^{14}\text{C}\) in the mannosyl moiety was similarly oxidized with periodate and reduced with NaBH\(_4\). It was discovered thereby that the conditions of the reduction apparently brought about partial hydrolysis of the degraded sugar nucleotide. Thus, after electrophoresis, UV-absorbing materials in the positions of GDP and GMP were observed on the paper, and \(^{14}\text{C}\) was associated with neutral compounds and hexose phosphate. No UV absorption or radioactivity was detected in the GDP-hexose area. Therefore, after this stage in the isolation procedure, material in the hexose phosphate and neutral areas of the electrophoreogram was eluted with water and the hexose phosphates were hydrolyzed (1 m trifuloroacetic acid, 15 min at 100°C). The hydrolysate was combined with the neutral sugars, and the mixture was chromatographed on paper. \(^{14}\text{C}\)-labeled compound (depending upon the substrate) with the mobility of glycerol was the only radioactive compound detected by this method. Glyceroldehyde, which should be derived from C1 and C2 of the degraded hexose, was never recovered either by paper chromatography or by electrophoresis of the bisulfite addition product (11). Indeed, when
Epimerization of GDP-\(\alpha\)-mannose

![Diagram of reactions](image)

**FIG. 1.** The sequence of reactions expected to occur in the periodate degradation of GDP-\(\alpha\)-hexose (GDP-\(\alpha\)-mannose is depicted here). Details of the procedures used to degrade \(^3\)H-labeled substrate are given in the text.

![Diagram of reactions](image)

**TABLE III**

| Oxidation of GDP-\(\alpha\)-mannose and GDP-\(\alpha\)-\(^3\)Hgalactose
<table>
<thead>
<tr>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Total cpm in GDP-hexose</td>
<td>cpm recovered in formic acid</td>
<td>Per cent total in formic acid</td>
<td>cpm in glycerol</td>
<td>Per cent total recovered in glycerol</td>
<td>cpm recovered in glycerol</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>23,400</td>
<td>11,500</td>
<td>49</td>
<td>8,900</td>
<td>38</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>50,900</td>
<td>23,100</td>
<td>45</td>
<td>32,800</td>
<td>65</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>25,000</td>
<td>12,600</td>
<td>50</td>
<td>12,400</td>
<td>49</td>
</tr>
</tbody>
</table>

authentic glycolaldehyde was chromatographed or simply left to dry on a paper in the hood for several hours, it could no longer be made visible with the alkaline AgNO\(_3\) reagent. It may be sufficiently volatile under those conditions to be lost into the atmosphere.

\(^3\)H-Glycerol from C4, 5, 6 of the hexose was degraded further as follows: It was eluted from the chromatography paper with water and made 0.03 M with NaIO\(_4\). The mixture was held for 4 h at room temperature and then left overnight in the refrigerator. The mixture was made alkaline with 2 M NaOH (pH 9 to 10) and distilled over at low temperature. The residue was acidified with 0.5 ml of 2 M \(\text{H}_2\text{SO}_4\) and distilled over again. Radioactivity was measured in the alkaline and acid distillates. The alkaline distillate should contain formaldehyde derived from the end carbon atoms of glycerol (C4 and C6 of the hexose), and the acid distillate should contain formic acid from the center carbon (C5 of the hexose). These results are shown in Table III.

Essentially half the radioactivity from \(^3\)H\(\text{H}_2\text{O}\) incorporated into \(\alpha\)-mannose and L-galactose was found in carbon 3 and the other half in carbon 5. It seems likely therefore that epimerization in this enzymic system proceeds by oxidation of the hexosyl moiety at carbon 4 to a keto intermediate, enediol formation, and inversion of the configurations at C3 and C5 upon rehydration of the double bonds and stereospecific reduction of the keto group (Fig. 2). This is analogous to the
Epimerization of GDP-\(\alpha\)-mannose

Epimerization of dTDP-\(\alpha\)-glucose to dTDP-\(\alpha\)-rhamnose (3, 5).

Since the epimerization envisaged here requires an oxidation and reduction of the substrate, it should be possible to detect a keto intermediate and an electron carrier. I have as yet been unable to demonstrate any requirement by the system for a pyridine nucleotide or to demonstrate the presence of one on the enzyme surface.

Acknowledgments—I should like to thank Doctors E. J. Behrman and G. S. Serif for much helpful advice, and Dr. P. A. Hebda for further purification of the enzyme preparation.

REFERENCES

Observations on the mechanism of the reversible epimerization of GDP-D-mannose to GDP-L-galactose by an enzyme from Chlorella pyrenoidosa.
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