The Isolation and Synthesis of Guanosine Diphosphate Glucose

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Cabib and Leloir (2) isolated and characterized guanosine diphosphate (GDP) mannose from bakers' yeast and postulated that this nucleotide may serve as a donor of mannose residues for the synthesis of polymers which are present in the yeast cell wall. The occurrence of GDP-mannose may be ubiquitous since it has been reported to be present in hen's oviduct (3), the molds Penicillium chrysogenum (4) and Eremothecium ashbyii (5), the red alga Porphyra perforata (6), guinea pig mammary gland (7), sheep mammary gland (8), and the milk of the sheep, goat, and sow (9). The biosynthesis of GDP-mannose, first reported by Munch-Peterson (10), was shown to be catalyzed by a pyrophosphorylase from brewers' yeast (Reaction 1).

\[ \text{GTP} + \text{mannose-1-P} \rightleftharpoons \text{GDP-mannose} + \text{PP}_1 \] (1)

Pontis, James, and Baddiley (11) have reported the presence of small quantities of GDP-glucose and GDP-fructose in association with GDP-mannose isolated from a mold. After acid hydrolysis, the sugars were identified by chromatography and ionophoresis. The resorcinol-HCl absorption spectrum and the indole-H_2SO_4 colorimetric test were used specifically to identify fructose. From quantitative estimations of the sugars, it was reported that glucose and fructose together accounted for approximately 6% of the total hexose present in the GDP-mannose fraction.

The object of this report is to establish the presence of GDP-glucose in mammary tissue and to report for the first time both the biological and chemical synthesis of GDP-glucose.

MATERIALS AND METHODS

Nucleotides were purchased from Sigma Chemical Company, St. Louis, and Pabst Brewing Company, Milwaukee. Glucose 1-P-C^{14} was prepared from C^{14}-labeled starch (12) by the action of phosphorylase (13), and mannose-1-P was prepared from glucose to glucose-1-P as measured by the disappearance of UDP-glucose and by the formation of glucose-1-P.

UDP-glucose dehydrogenase was purified from calf liver (16); crystalline phosphoglucomutase, from rabbit muscle (17); and glucose-6-P dehydrogenase, from dried brewers' yeast (18). The Crotalus atrox venom (obtained from Ross Allen's Reptile Institute, Silver Springs, Florida) that was used as a source of nucleotide pyrophosphatase gave quantitative cleavage of UDP-glucose to glucose-1-P as measured by the disappearance of UDP-glucose and by the formation of glucose-1-P.

The Eremothecium ashbyii culture was purchased from the American Type Culture Collection (6747) and was cultivated according to the method of MacLaren (19). Hansenula holitii, grown according to the procedure of Anderson et al. (20), was provided by R. K. Brethauer.

Nucleotides were separated on ion exchange columns (21) and were identified by descending paper chromatography on Whatman No. 1 filter paper with the following solvent systems: ethanol-ammonium acetate, pH 7.5 (22), ethanol-ammonium acetate, pH 3.8 (22), and isobutyric acid-ammonium hydroxide-water, pH 4.3 (23). An ultraviolet lamp (Mineralight SL 2557) was used for the detection of nucleotides on paper, and photographs of the ultraviolet-absorbing spots were made by contact prints prepared by a modification of the procedure of Markham and Smith (24) as outlined by Wilken (25). The paper electrophoretic conditions of Wade and Morgan (26), with the use of 0.05 M citrate buffer, pH 4.4, were used. Solvents used in paper chromatography for the identification of sugars were butanol-pyridine-water (27), phenol-water (28), and ethyl acetate-pyridine-water (29); the sugars were detected by the ammoniacal silver nitrate method (30).

Total phosphorus determinations were performed by the method of King (31), and acid-labile and inorganic phosphorus, by the method of Fiske and SubbaRow (32). Ribose was assayed by the Meijbaum pentose determination procedure (33) and reducing sugars, by the method of Park and Johnson (34). Protein was measured by the method of Lowry et al. (35).

A liquid scintillation counter was used for the determination of radioactivity. The sample was dissolved in 0.01 ml of water and 1 ml of absolute ethanol to which were added 14 ml of a toluene solution containing, per liter, 4.0 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis-2-(5-phenyloxazole)benzene (36). Radioactive spots were detected by exposing paper chromatograms to "no screen" x-ray film, and their radioactivity was quantitated with a gas flow counter equipped with a thin Mylar window Geiger-Müller tube. Absorption spectra were obtained with a Beckman model DK-2 recording spectrophotometer.

Enzyme assays involving the oxidation or reduction of pyridine nucleotides were performed on a Beckman D2 spectrophotometer equipped with a Gilford cuvette changer and...
Isolation of GDP-Hexoses—Lactating bovine mammary gland obtained at a local abattoir was excised and frozen in dry ice for transport to the laboratory. Portions of the frozen tissue (500 g each) were then pulverized and extracted in a Waring Blender with 0.6 N perchloric acid 2 ml of acid per g of tissue), and the extract was centrifuged. The supernatant fluid was brought to pH 6 with 5 N KOH, the precipitate was removed by filtration, and the filtrate was placed directly on a Dowex 1-formate column for separation of the nucleotides (21), which were detected by absorbancy at 260 μm. GDP-hexoses were eluted immediately after ADP and were contaminated by this fraction (Fig. 1). To remove electrolytes, the principal GDP-hexose peak was adsorbed on a minimal amount of Norit, and the nucleotides were eluted from the Norit with 50% ethanol containing 1% ammonia. This nucleotide fraction was further purified by paper chromatography by in ammonium acetate-ethanol, pH 7.5. The GDP-hexose fraction was eluted from the paper with water, and the eluate was lyophilized. Characterization of some of the components of this peak is described below under "Characterization of GDP-glucose."

Biosynthesis of GDP-Glucose—For the demonstration of the biosynthesis of GDP-glucose from GTP and glucose-1-P, bovine mammary gland extracts were prepared from the same gland used for the isolation of GDP-hexose. A portion of the frozen tissue was pulverized, and an acetone powder was prepared by two extractions with 10 volumes of cold acetone (−10 °C) followed by extraction with 5 volumes of cold ether before drying. Rat mammary gland acetone powder was prepared from the posterior glands of animals that had been lactating for 7 to 10 days. Unless otherwise indicated, all operations were conducted between 0 and 5 °C, and centrifugations were for 10 minutes at 10,000 X g.

The acetone powder was extracted with 10 volumes of a compound buffer (0.1 M Tris, 0.01 M MgCl2, 0.001 M EDTA, and 0.001 M mercaptoethanol, brought to pH 7.5 with 5 N acetic acid) in a Servall Omni-Mixer for 10 minutes, and the resulting homogenate was centrifuged. The precipitate was discarded, and 0.05 volume of 1 M MnCl2 was added to the supernatant fluid with stirring. The addition of MnCl2 lowered the pH to approximately 6, at which point proteins as well as nucleic acids were precipitated. After centrifugation, the usually heavy precipitate was discarded and the supernatant fluid was brought to 60% ammonium sulfate saturation (37 g per 100 ml of supernatant), stirred for 20 minutes, and centrifuged. The supernatant fluid was discarded, and the precipitate was dissolved in 0.1 ml of 50% ethanol and then rechromatographed in isobutyric acid-ammonium hydroxide-water. This procedure gave excellent recovery of GDP-glucose, but no attempts were made to quantitate the assay at this point.

In the enzymic preparation of GDP-glucose for characterization, twelve identical tubes were incubated as described for the chromatographic assay, and the reaction mixtures were heated and centrifuged. The supernatant liquids were combined, and the nucleotides were separated by column chromatography as described earlier. The omission of ATP from the incubation mixtures resulted in approximately a 50% decrease in GDP-glucose formation.

Chemical Synthesis of GDP-Glucose—GDP-glucose and GDP-glucose-1-C14 were synthesized by the procedure given for the synthesis of GDP-mannose (39). Bis-(tri-n-octylammonium)-p-glucose 1-phosphate was incubated with 4-morpholine-N,N'-dicyclohexylcarbodiimide guanosine 5'-phosphoromorpholide (40) in anhydrous pyridine for 6 days. Electrophoresis of an aliquot of the reaction mixture in 0.05 M phosphate buffer, pH 7.5, was used to demonstrate completion of reaction. GDP-glucose was isolated by absorbing the reaction mixture on a column of Dowex 1-chloride and eluting the nucleotides with LiCl (39). The major peak was isolated by precipitation from methanol with acetone and ether in the standard way.

1 During purification by paper chromatography, a band was detected which had both ultraviolet adsorption and ninhydrin-positive material. After the elution and lyophilization of this material, Dr. E. Okuhara of our laboratory hydrolyzed it for 20 hours in 6 N HCl and identified glutamic acid, cysteic acid, glycine, and taurine by using two-dimensional paper chromatography with water-saturated phenol in the first direction and butanol-propanol-acetic acid-water in the second direction. The amino acids were detected and identified with the polychromatic ninhydrin spray of Moffat and Lytle (38).

2 The authors wish to thank Dr. S. Roseman and J. J. Distler, University of Michigan, for their assistance in the chemical preparation of GDP-glucose.
bating GTP and glucose-1-P with mammary gland preparations, hydrate, was 61.6%.

The yield of GDP glucose, based on the spectrophotometric determination of its guanosine content and isolated as a tetrahydrate, was 61.6%.

\[
\text{Guanosine concentration (\mu moles) = } \frac{(A_{260} - A_{2})}{(a_{260} - a_{M}A)} \times 100
\]

where \(A_{260}\) is the absorbancy at 260 nm and \(A_{2}\) is the absorbancy at wave length \(\lambda; a_{260}\) is the extinction coefficient at 250 nm and \(a_{M}A\) is the extinction coefficient at wave length \(\lambda\). The same results were obtained with \(\lambda = 230\) nm or \(\lambda = 280\) nm.

† Results based on total phosphorus value of 2.00.

\[
\text{Guanosine concentration (pmoles) = } \frac{(A_{260} - A_{1})}{a_{M}X} \times 100
\]

where \(A_{260}\) is the absorbancy at 260 nm and \(A_{1}\) is the absorbancy at wave length \(\lambda; a_{260}\) is the extinction coefficient at 250 nm and \(a_{M}X\) is the extinction coefficient at wave length \(\lambda\). The characteristic spectral shifts of guanosine compounds (23).

\[\text{Acid-labile phosphorus.} \]

\[\text{Found: C 27.1\%, H 4.42\%, N 10.10\%, P 9.01\%} \]

\[\text{C_{16}H_{23}N_{6}O_{16}P_{2}Li_{2.4}H_{2}O} \text{ (mol. wt., 689)} \]

\[\text{Calculated: C 27.97\%, H 4.51\%, N 10.16\%, P 9.01\%} \]

\[\text{Reducing value} \text{ (pmoles glucose X 100 = per cent hydrolysis)} \]

\[\text{Chemical analyses for phosphorus, ribose, and hexose agreed with guanosine values obtained by quantitative ultraviolet absorption as measured at 260 nm, assuming an } E_{260} \text{ of 11.7 (21).} \]

\[\text{The analytical data for the three GDP-hexose preparations compared favorably with the theoretical values (Table I).} \]

\[\text{4. Chromatographic and Electrophoretic Characterization of Hexose Nucleotides and Their Components—The isolated GDP-hexose and the chemically and enzymically formed GDP-glycoses behave in an identical manner. These hydrolysis curves also are similar to that obtained by Cabib and Leloir for GDP-mannose (2).} \]

\[\text{The yield of GDP glucose, based on the spectrophotometric determination of its guanosine content and isolated as a tetrahyd} \]

\[\text{Chromatographic and electrophoretic mobilities of hexose nucleotides} \]

\[\text{Chemical analyses of GDP-hexoses} \]

\[\text{Acid-hydrolysis of enzymically and chemically prepared GDP-glucose and isolated GDP-hexose in 0.01 n HCl for 15 minutes and for 3 hours gave rise to GDP and GMP, respectively. The quantitative appearance of reducing sugar during the course of the hydrolysis (Fig. 2) indicates that the chemically and enzymically prepared GDP-glucoses behave in an identical manner. These hydrolysis curves also are similar to that obtained by Cabib and Leloir for GDP-mannose (2).} \]

\[\text{Results based on total phosphorus value of 2.00.} \]

\[\text{TABLE I} \]

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Source</th>
<th>Ethanol-ammonium acetate</th>
<th>Isoamyl-alcoholic acid-alcoholic ammonium hydroxide-buffer*</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDP glucose</td>
<td>Chemical synthesis</td>
<td>1.34</td>
<td>0.65</td>
</tr>
<tr>
<td>GDP glucose</td>
<td>Enzymic synthesis</td>
<td>1.50</td>
<td>0.65</td>
</tr>
<tr>
<td>GDP mannose</td>
<td>Chemical synthesis</td>
<td>1.30</td>
<td>0.67</td>
</tr>
<tr>
<td>GDP hexose</td>
<td>Isolated</td>
<td>1.30</td>
<td>0.66</td>
</tr>
<tr>
<td>UDP-glucose</td>
<td></td>
<td>2.74</td>
<td>0.64</td>
</tr>
</tbody>
</table>

* Mobility relative to GMP.
Isolated GDP-hexose gave compounds corresponding to mannose and glucose plus faint spots with mobilities similar to fructose and fucose. Although fructose and mannose have the same mobility in butanol-pyridine-water, they can be separated with phenol-water. The faint fucose spot was not detectable when phenol-water was used because of the darker background encountered with ammoniacal silver nitrate as the developing reagent.

5. Hydrolysis with Snake Venom Nuclease Pyrophosphatase—Treatment of both the chemically and enzymically formed GDP-glucose with snake venom gave quantitative liberation of glucose-1-P as determined by the reduction of TPN in the presence of phosphoglucomutase and glucose-6-P dehydrogenase. As phosphoglucomutase is specific for the α form of glucose-1-P (41), the compounds exist in the α configuration as does UDP-glucose. Incubation of β-glucose-1-P with snake venom did not yield a product capable of TPN reduction, whereas incubation of α-glucose-1-P together with β-glucose-1-P produced an immediate reaction under the assay conditions.

6. Incorporation of Glucose-1-P-C14—Incubation of glucose-1-P C14 and GTP with the mammary gland enzyme extract yielded a radioactive nucleotide which was identical to GDP-glucose as demonstrated by paper chromatography in ethanol-ammonium acetate, pH 7.5, and also in isobutylc acid-ammonium hydroxide-water. Acid hydrolysis of the isolated nucleotide and subsequent electrophromatography of the hexose portion in three solvents yielded only glucose.

Formation of Hexose Nucleotides Catalyzed by Pyrophosphorylase from Different Sources—Pyrophosphorylase activity of nine preparations with respect to the synthesis of UDP-glucose, GDP-mannose, and GDP-glucose is recorded in Table IV. Enzyme extracts were prepared as previously described from desiccated Saccharomyces cerevisiae and acetone powders of the mammary glands, calf liver, hen oviduct, Hansenula holstii, and Eremothecium ashbyii. With the assay conditions described, only mammary gland contained an enzyme that formed detectable amounts of GDP-glucose, whereas UDP-glucose pyrophosphorylase was found in all nine preparations. GDP-mannose pyrophosphorylase activity was not found in calf liver, hen oviduct, or Hansenula holstii, and Eremothecium ashbyii. With the assay conditions described, only mammary gland contained an enzyme that formed detectable amounts of GDP-glucose, whereas UDP-glucose pyrophosphorylase was found in all nine preparations. GDP-mannose pyrophosphorylase activity was not found in calf liver, hen oviduct, or Hansenula holstii, and Eremothecium ashbyii. The absence of PPi. However, no difficulties arose when rat mammary gland acetone powder was used as the enzyme source. The final reaction mixture contained 0.2 μmole of GDP-glucose, 1.5 μmoles of Na₂HPO₄; 10 μmole of TPN and, phosphoglucomutase and glucose 6-P dehydrogenase in nonlimiting amounts, all made to a total volume of 0.48 ml with the compound buffer. The reaction was initiated by the addition of 0.02 ml of the pyrophosphorylase preparation. Controls lacked -

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>UDP-glucose</th>
<th>GDP-mannose</th>
<th>GDP-glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine mammary gland</td>
<td>+*</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rat mammary gland</td>
<td>+</td>
<td>N.T.</td>
<td>+</td>
</tr>
<tr>
<td>Rabbit mammary gland</td>
<td>+</td>
<td>N.T.</td>
<td>-</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Hansenula holstii</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Calf liver</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hen oviduct</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Eremothecium ashbyii</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Enzymes present in detectable amounts are indicated by +; not detectable, by -, and not tested, by N.T.
ing PPi and TPN were run simultaneously, and all assays were corrected for TPNH formation caused by nucleotide pyrophosphatase activity. There was no reduction of TPN until rat mammary gland enzyme was added. A unit of enzyme is defined as the amount of activity that will form 1 μmole of glucose-1-P per minute under the conditions of the assay.

For the enzyme fractionation, 1 g of rat mammary gland acetone powder was extracted with 15 ml of the compound buffer. The extract was centrifuged, the precipitate was discarded, and 10 ml of the supernatant fluid were used for subsequent purification. Three ammonium sulfate fractions were prepared by the successive addition of 4.7, 3.2, and 4.8 ml of saturated ammonium sulfate at pH 8.3 to the respective supernatant liquids (Table V). A 4-fold purification was obtained with 75% of the original activity in Fraction 2. The change in the ratio of UDP-glucose pyrophosphorylase to GDP-glucose pyrophosphorylase is evidence that the formation of GDP-glucose is due to a different enzyme.

Properties of Partially Purified Enzyme—The suitability of the assay for further purification studies is demonstrated in Fig. 3. The reaction is linear with respect to time and over at least a 4-fold change in enzyme concentration. Fig. 4 demonstrates the requirement for PPi and shows the effect of different levels of PPi on the rate of reaction. The reaction rate increased up to 1.5 μmoles of PPi, whereas above 2.5 μmoles, the rate decreased. There was no loss of activity after centrifugation of the crude homogenate for 1 hour at 100,000 × g, which demonstrates that the enzyme is located in the soluble fraction.

Incorporation of P32 into GTP—Attempts to demonstrate the reversibility of GDP-glucose synthesis by incorporation into GTP of P32 from labeled PPi (prepared by pyrolysis of P32i) were not successful with the crude bovine enzyme extracts. However, when the partially purified enzyme preparation fraction 2 (see Table V) was used, such incorporation was readily demonstrated. Two incubation mixtures were prepared as described for the spectrophotometric assay, except that the amount of GDP-glucose was doubled; the first was devoid of PPi, and the second contained 2.15 μmoles of PPi (150,000 c.p.m. per μmole). After 30 minutes at room temperature, the mixtures were heated at 100° for 1 minute, and the nucleotides were adsorbed on Norit and separated from contaminants by washing the Norit exhaustively with water. The nucleotides were then eluted with 10% aqueous pyridine, and portions of the wash water and the pyridine eluent were counted with a gas flow counter. The wash water contained a total of 276,000 c.p.m. and the pyridine eluent contained 31,200 c.p.m. Thus, the nucleotide fraction accounted for approximately one-tenth of the P32 recovered. The labeled GTP was cochromatographed with authentic GTP in ethanol-ammonium acetate, pH 7.5, and was identical with GTP on electrophoresis in 0.05 M citrate buffer, pH 4.4.

**Summary**

The biological and chemical synthesis of guanosine diphosphate glucose has been reported for the first time. The characterization of guanosine diphosphate hexoses obtained from lactating bovine mammary gland has revealed the presence of guanosine diphosphate mannose and guanosine diphosphate glucose. Tentative evidence for the presence of guanosine diphosphate fucose and guanosine diphosphate fructose is presented. The enzyme responsible for catalyzing the synthesis of guanosine diphosphate glucose has been shown to be separate and distinct from uridine diphosphate glucose pyrophosphorylase. Guanosine 5'-diphosphate-α-D-glucose has been identified as the product of the reaction.

**References**

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