FORMATION OF GDP-L-GALACTOSE FROM GDP-D-MANNOSE

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L-Galactose is an uncommon sugar found thus far only in polysaccharides of one group of marine algae (Mori, 1953; Su and Hassid, 1962a), one genus of sea urchins (Vasseur, 1950) and one genus of snails (Bell and Baldwin, 1941). Interest in the biosynthesis of polymers containing L-galactose led to the isolation of GDP-L-galactose from the red alga, Porphyra perforata (Su and Hassid, 1962b) and from the albumen gland of the land snail, Helix pomatia (Goudsmit and Neufeld, 1966). This sugar nucleotide was postulated to be an intermediate in the formation of L-galactose and its eventual incorporation into polysaccharide.

The present report gives evidence for the conversion of GDP-D-mannose to GDP-L-galactose in the presence of cell-free extracts of H. pomatia albumen glands. D-Mannose and L-galactose differ from each other in the configuration about the third and fifth carbon atoms (Fig. 1). This is the

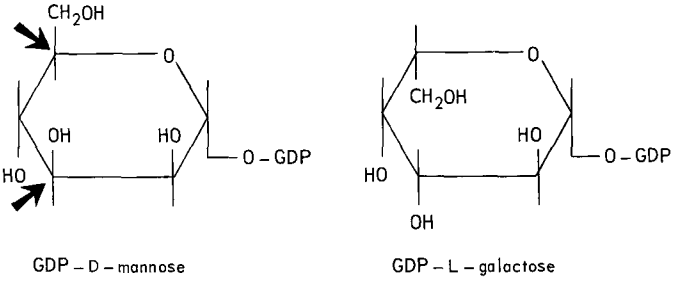

Figure 1. Structure of GDP-D-mannose and GDP-L-galactose.

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first instance of a double epimerization which does not involve the formation of a deoxysugar.

METHODS

GDP-D-Mannose-^{14}C was prepared by the method of Rosen and Zeleznick (1966) with three modifications: mannose 6-phosphate was isolated by chromatography in solvent II (see below), its conversion to GDP-mannose was carried out in the presence of 0.01M cysteine, and the GDP-mannose was recovered by paper chromatography in solvents II and I. From 100 μC of D-mannose-^{14}C (Schwarz BioResearch, Inc.) the yield was 13 μC of GDP-D-mannose-^{14}C, presumably of the same specific activity as the starting material (91 μC/μmole).

GDP-D-Glucose-^{14}C (0.68μC/μmole) was synthesized by the procedure of Roseman et al (1961).

An extract of secreting albumen glands was prepared by suspending one gland (1 gm wet weight) in 1.5 ml cold water and gently homogenizing in a Kontes glass vessel with a motor-driven Teflon pestle. The homogenate was centrifuged at 10,000 x g for 20 min and the supernatant solution used immediately since all activity was lost upon freezing and thawing or upon storage at 4°.

The following solvents were used for paper chromatography: (I) ethanol: 1M ammonium acetate, pH 3.8 (75:30); (II) ethanol:1M ammonium acetate, pH 7.5 (75:30); (III) isobutyric acid: 1N NH_4OH (10:6); (IV) n-butanol:pyridine:water (6:4:3); (V) 80% phenol; (VI) n-propanol:ethyl acetate:water (7:1:2).

RESULTS

The rate of L-galactose formation is described in Figure 2. Conversion as high as reported in that experiment was not always obtained, a yield of 30 to 50% L-galactose being more usual. DPN, though routinely used, did not significantly stimulate the reaction.

GDP-D-Glucose-^{14}C was compared with GDP-D-mannose-^{14}C as a possible substrate for the reaction. The nucleotides (0.02 μmole), the specific activities of which were equalized to 0.68 μC/μmole, were incubated in the assay

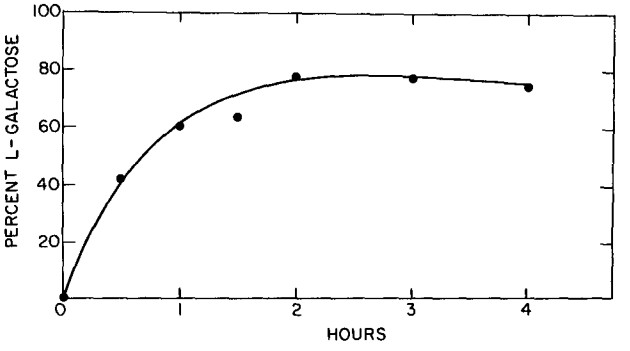

Figure 2. Rate of L-galactose synthesis. The standard mixture contained, in a volume of 0.2 ml: 1.3×10^{-4} μmole GDP-mannose-^{14}C (0.012 μC), 0.03 μmole DPN, 0.30 μmole $MgCl_2$, 2.5 μmoles Tris buffer, pH 8.0, and 0.15 ml enzyme. After incubation at 30°, the samples were acidified to pH 4 by the addition of 0.3 ml cold water followed by 0.015 ml of 0.2N acetic acid. Precipitated protein was removed by centrifugation, and 30 mg of acid-washed Norit A added to the supernatant solution. The charcoal was collected by centrifugation, washed thoroughly with water and heated at 100° for 15 min in 0.3 ml of 0.01N HCl in order to hydrolyze nucleotide-linked sugars which had been adsorbed. After the charcoal was removed by centrifugation, the supernatant solution containing the sugars was deionized on Amberlite MB-3 and subjected to chromatography in solvent IV for 15 hr. Radioactive areas, located with a strip scanner, were cut out, placed in toluene phosphor, and counted in a liquid scintillation spectrometer.

mixture similar to that described in Fig. 2, but scaled up ten-fold. After 2 hr at 30° there was no detectable conversion of GDP-D-glucose, while 55% of the GDP-D-mannose had been converted to GDP-L-galactose.

Isolation of GDP-L-galactose-^{14}C - To obtain enough product for characterization, ten reaction mixtures as described in Fig. 2 were each supplemented with 0.006 μmole of GDP-D-mannose-^{14}C (20 μC/μmole) and incubated 2 hr at 30°. They were then pooled and applied to Whatman 3MM paper for chromatography in solvent I. All the radioactivity was located in the GDP-hexose area. It was eluted and subjected to further chromatography in solvents II and III. GDP-L-Galactose, which separates from GDP-D-mannose in the latter solvent (Goudsmit and Neufeld, 1966), was further purified by adsorption to charcoal (5 mg of acid-washed Norit A) and elution with 50% ethanol containing 0.1% concentrated NH_4OH. Its specific activity was the same as that of the starting material; the yield, 10%.

Another preparation of GDP-L-galactose of higher specific activity (91 μC/μmole), made in similar fashion, was used for experiment 2 below.

Characterization of GDP-L-galactose-^{14}C - Identification of the isolated reaction product as GDP-L-galactose is based on the following evidence:

1) Its spectrum at pH 7 and pH 1 was similar to that of a guanosine derivative (Fig. 3).

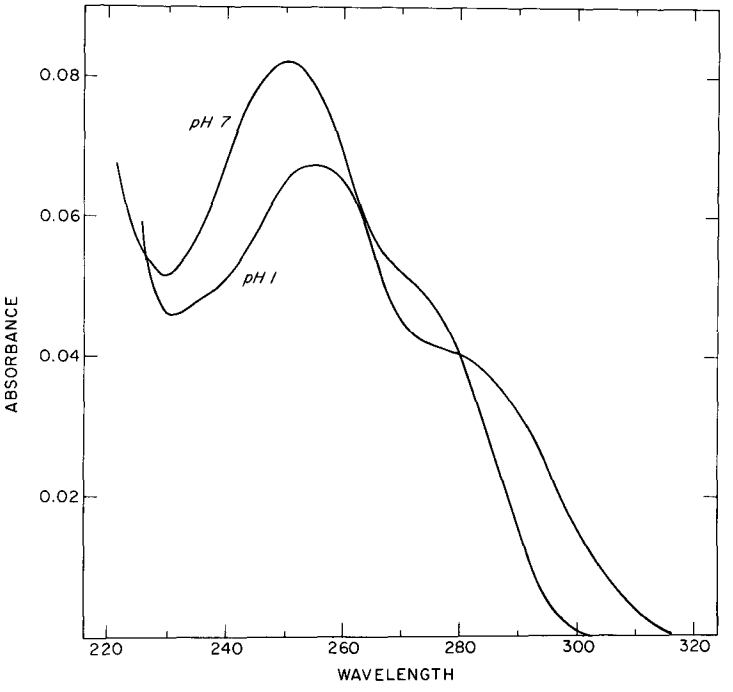

Figure 3. Spectrum of GDP-L-galactose at pH 7 and 1. These were recorded on a Cary spectrophotometer, and corrected for the absorbance obtained from a filter paper blank treated in the same way.

2) Its chromatographic mobility in solvents I, II and III was identical with that of GDP-L-galactose which has been isolated from albumen glands (Goudsmit and Neufeld, 1966). Treatment with Crotalus adamanteus venom, which has nucleotide pyrophosphatase activity, released a radioactive product which migrated with authentic galactose 1-phosphate in solvent I. Further reaction of this product with alkaline phosphatase of E. coli (Worthington) resulted in its complete hydrolysis to a free sugar, chromatographically identical to galactose in solvent IV.

3) The radioactive sugar residue obtained by hydrolysis of the sugar nucleotide (100°, 15 min, pH 1) behaved as galactose upon co-chromatography in solvents IV, V and VI, and co-electrophoresis in sodium tetraborate (pH 9.2, 80 V/cm, 25 min).

The L-configuration of the radioactive galactose was indicated by its reactivity with L-fucose isomerase (Green and Cohen, 1956). Authentic L-galactose (0.02 μmole) was mixed with the radioactive sugar (4,300 cpm), 0.1 ml L-fucose isomerase and 1 μmole $Na_2B_4O_7$, pH 8.0, as previously described (Goudsmit and Neufeld, 1966). After 5 hr at 37°, the mixtures were passed through Rexyn RG 50-H^+ and freed of borate by repeated drying in methanol. Tagatose, 0.01 μmole (Dische and Borenfreund, 1951), was recovered by chromatography in solvent IV. The specific activity of this product was essentially identical to that of the L-galactose substrate (1.8×10^5 cpm/μmole).

Definitive identification of the radioactive sugar as L-galactose was obtained by preparation of the crystalline 1-methyl-1-phenylhydrazone (Fletcher, 1948). The radioactive sugar (32,520 cpm) was mixed with 50 mg of authentic L-galactose in a total volume of 1.0 ml water. Seventy-five mg of solid 1-methyl-1-phenylhydrazine sulfate (Eastman Kodak) was added to the solution and stirred until completely dissolved. After 4 hr at room temperature, crystals were collected, washed with ice-cold absolute ethanol, and recrystallized 3 times from warm 30% ethanol.[2] During the 3 recrystallizations, the specific activity of the derivative remained constant (400, 407, 400 cpm/mg; theoretical, 410 cpm/mg). By contrast, when the same amount of the radioactive sugar was mixed with 50 mg of D-galactose, the specific activity of the methyl-phenylhydrazone was less than 10 cpm/mg.

Other Products - In the experiments described above, GDP-L-galactose-^{14}C was the only product formed. In earlier experiments, using a different batch of snails, there were two, and occasionally three, other radioactive products

[2] After the third recrystallization, the colorless, rectangular platelets melted at 189.5° - 191°, compared to 191° reported by Fletcher (1948).

in the acid hydrolysate of the material adsorbed on charcoal. One of these was tentatively identified as L-fucose on the basis of co-chromatography in solvents IV and V, co-electrophoresis in 0.05 M sodium tetraborate, and by reaction with L-fucose isomerase to yield a radioactive material behaving chromatographically as fuculose. The other, minor, products of incubation of snail extract with GDP-mannose have not been identified. Since the synthesis of albumen is a seasonal activity of the gland, related to egg-laying, the difference between the two batches of snails may possibly be due to the fact that they were used at different times of the year.

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REFERENCES

Bell, D. J., and Baldwin, E., J. Chem. Soc. 125 (1941).
Dische, Z., and Borenfreund, E., J. Biol. Chem. 192, 583 (1951).
Fletcher, H. G., J. Am. Chem. Soc. 71, 3679 (1948).
Goudsmit, E. M., and Neufeld, E. F., Biochim. Biophys. Acta 121, 192 (1966).
Green, M., and Cohen, S. S., J. Biol. Chem. 219, 557 (1956).
Mori, T., Adv. Carbohydrate Chem. 8, 316 (1953).
Roseman, S., Distler, J. J., Moffatt, J. G., and Khorana, H. G., J. Am. Chem. Soc. 83, 659 (1961).
Rosen, S. M., and Zeleznick, L. D., in E. F. Neufeld and V. Ginsburg (Eds.), Methods in Enzymology, Vol. 8, Academic Press, New York, 1966, p. 145.
Su, J. C., and Hassid, W. Z., Biochemistry 1, 468 (1962a).
Su, J. C., and Hassid, W. Z., Biochemistry 1, 474 (1962b).
Vasseur, E., Acta Chem. Scand. 4, 1144 (1950).