ENZYMATIC SYNTHESIS OF GALACTOGEN IN THE SNAIL, HELIX POMATIA

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The galactose polymer, galactogen, has been reported in numerous pulmonate snails (Ghose, 1963; Horstmann, 1956; May, 1931; McMahon et al., 1957; Meenakshi, 1954; Rigby, 1963). Whereas this polysaccharide is found exclusively in the reproductive system (albumen gland) and freshly laid eggs, glycogen is present in the rest of the body. As isolated from the albumen gland of the land snail, Helix pomatia, the polymer has been reported to contain both D- and L-galactose in the approximate ratio of 6:1 (Bell and Baldwin, 1941). The methylation studies of Bell and Baldwin (1938), yielding essentially equimolar proportions of 2,3,4,6-tetramethyl- and 2,4-dimethyl-D-galactose, are the basis for a proposed structure of galactogen consisting of either a β-1→3 or a β-1→6 linear chain wherein each galactopyranose unit bears a terminal galactose substituent on C-6 or C-3, respectively. L-Galactose is thought to be part of the side chain (Bell and Baldwin, 1941; May and Weinland, 1956). Heretofore, however, no information relating to the mode of biosynthesis of this polysaccharide has been available.

The present report describes the preparation of an extract from the albumen gland of H. pomatia which utilizes UDP-D-galactose-C^{14} for the formation of a radioactive galactose polymer, tentatively identified as galactogen.

Materials and Methods--UDP-D-Galactose-C^{14}, GDP-D-glucose-C^{14} and TDP-D-glucose-C^{14} were synthesized by the procedure of Roseman et al.

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(1961). UDP-D-Glucose-C^{14} (Anderson et al., 1959) and glucose-1-phosphate-UC^{14} (Doudoroff et al., 1955) were prepared enzymatically. TDP-D-Galactose-C^{14} was a gift from Dr. Elizabeth Neufeld of this Institute; galactose-1-phosphate-UC^{14} and ADP-glucose-C^{14} were obtained from the International Chemical and Nuclear Corporation, California. Lymnaea stagnalis albumen gland galactogen, which consists only of D-galactose, was a gift from Dr. Theodor von Brand.

Galactose was measured colorimetrically by the phenol-sulfuric acid method (Dubois et al., 1956). Protein was determined according to Lowry (1951). The paper chromatographic systems employed were (I) pyridine:ethylacetate:water (1:3.6:1.15) and (II) ethanol:1 M ammonium acetate, pH 3.8, (7:3).

Galactogen was prepared from freshly excised albumen glands by extraction with 5 N KOH at 100° (50 mg tissue/ml). Polysaccharide was recovered from the supernatant fluid by the addition of 1.5 volumes of 95% ethanol. This material, after resolution and precipitation with ethanol several times, was dialyzed against distilled water for 48 hours and again precipitated with ethanol. After washing with absolute ethanol and ether, the polysaccharide was dried in a vacuum desiccator over $CaCl_2$. This preparation exhibited an $[\alpha]_D$ of -12.3° in agreement with earlier reported values of -13.5° (May, 1931) and -16.1° (Bell and Baldwin, 1938). Acid hydrolysis resulted in the formation of galactose; no other sugar was detectable on chromatography.

Preparation and Assay of the Enzyme--An albumen gland was suspended in cold 0.25 M sucrose containing 0.001 M EDTA (800-900 mg/ml wet wgt. tissue) and homogenized in a glass vessel with a Teflon pestle. The crude extract was centrifuged at 18,000 x g for 10 minutes and the pellet discarded. Cold, saturated ammonium sulfate, pH 7.0, was added to the turbid solution and the fraction precipitating between 20-50% of saturation was collected. The precipitate was suspended in 0.5 ml of

0.05 M Tris buffer, pH 7.1, and dialyzed overnight at 2° against 1,000 volumes of 0.05 M Tris buffer, pH 7.1. Upon storage at 2°, this preparation retained activity for at least 2 weeks and was used without further purification.

The standard assay mixture contained, in a final volume of 40 µl, 0.04 µmoles of UDP-D-galactose (39,400 cpm), 0.50 mg of galactogen and 20 µl of enzyme (0.4 mg protein) suspended in 0.05 M Tris buffer, pH 7.1. The samples were incubated for 2 hours at room temperature and the reaction stopped by the addition of 0.10 ml of 5 N KOH. The reaction tubes were placed in a boiling water bath for 15 minutes, cooled, and 0.2 ml of 95% ethanol added. The precipitated material was redissolved in distilled water and precipitated repeatedly with ethanol until the radioactivity remained constant. After washing with ethanol and ether, the polysaccharide was dissolved in 0.2 ml of distilled water and aliquots were plated on copper planchets. Radioactivity was determined

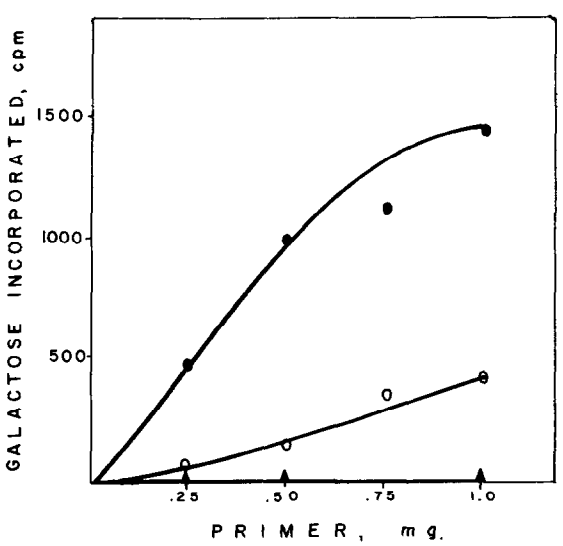

Fig. 1. Primer requirement for polysaccharide synthesis. The complete incubation mixture contained the following in a total volume of 0.065 ml: 4 x 10^{-2} µmoles of UDP-D-galactose-C^{14} (39,400 cpm); 0.04 ml of enzyme (0.5 mg protein) suspended in 0.05 M Tris buffer pH 7.15; and the indicated primer. ●——●, H. pomatia galactogen; o——o, L. stagnalis galactogen; ▲——▲, glycogen.

with an end-window gas flow counter. Control samples contained KOH at zero time. Under the above conditions, the assay was proportional to protein concentration, from 0 to 0.5 mg, and to time for at least 4 hrs.

The enzyme preparation exhibited an absolute requirement for galactogen (Fig. 1). Addition of galactogen, as carrier, after termination of the reaction was without effect. When H. pomatia galactogen was replaced by galactogen from L. stagnalis, incorporation was markedly reduced. Glycogen was unable to replace galactogen in the formation of radioactive polymer. Data on the substrate specificity of the active extract are summarized in Table I.

TABLE I. Substrate specificity for polysaccharide synthesis

C^{14} Donor	Product-% incorporation	C^{14} Donor	Product-% incorporation
UDP-D-Galactose	10	GDP-D-Glucose	0
UDP-D-Glucose	3.7*	α-D-Galactose-1-P	0
TDP-D-Galactose	4.3	α-D-Glucose-1-P	0
TDP-D-Glucose	<0.5	D-Galactose	0
ADP-D-Glucose	0.6	D-Glucose	0

The complete incubation mixture contained the following in a total volume of 0.045 ml: 1×10^{-2} μmoles of the indicated C^{14} donor, 0.5 mg of galactogen; and 0.02 ml of enzyme (0.60 mg protein) suspended in 0.05 M Tris buffer, pH 7.15. Procedures for incubation and for isolation of the product are described in the text.

* Acid hydrolysis of this product yielded only galactose.

Identification of the Reaction Product--The 3-fold reprecipitated products from a number of small-scale incubations were pooled and dialyzed for 24 hours against 200 volumes of cold, distilled water. The specific activity of the radioactive polymer averaged 400 cpm per μmole of galactose. Paper chromatographic examination of the dialyzed product in solvents I and II revealed a single radioactive peak remaining at the origin; mild acid hydrolysis (0.15 N HCl, 100°, 15 minutes) produced no change in mobility. Comparable control mixtures containing boiled enzyme, carrier galactogen and UDP-galactose-C^{14} were completely re-

solved in solvent II; no radioactivity was detected at the origin. Upon subjection to high-voltage electrophoresis at pH 4.0, the radioactive polymer did not migrate.

Complete hydrolysis of the product was accomplished by refluxing for 2 hours with 2 N H_2SO_4. Upon rechromatography of the neutralized product in solvent I, all of the radioactive and silver nitrate-positive material (Anet, 1953) was found to co-chromatograph with known galactose. The specific rotation of the hydrolysate, $[\alpha]_D$ + 54.8°, was in good agreement with the figures of +53.6° (May, 1931) and +56.5° (Bell and Baldwin, 1941) reported for galactogen.

Further identification of the hydrolyzed reaction product as galactose was obtained by preparation of the crystalline flavazole derivative. Thirty milligrams of enzymatically prepared radioactive polysaccharide (2,600 cpm/mg galactose) was completely hydrolyzed as described above. To the neutralized hydrolysate was added 150 mg of D-galactose and the derivative, D-threo-trihydroxypropyl-1-phenyl-flavazole, was prepared, m.p. 196.5-198.5°, (Ohle and Liebig, 1942). The specific activity remained constant after 4 recrystallizations from ethanol (--, 182, 184, 194 cpm/mg) (theoretical 220 cpm/mg).

DISCUSSION

The radioactive product formed has been tentatively identified as galactogen. Evidence in support of this identification is based upon the following: (1) The synthesizing system has been isolated from the albumen gland, which is the exclusive site of galactogen accumulation in the adult snail. (2) The formation of radioactive polymer has been shown to be absolutely dependent upon the presence of primer galactogen; glycogen was inert. (3) Galactose was identified as the sole radioactive component of the enzymatically formed polysaccharide. A more rigorous identification of this polymer as galactogen, must, of necessity, await the complete structural determination of authentic

galactogen.

The present study provides no information on the identity of the optical isomer of the incorporated galactose. However, it is of interest that, in addition to UDP-D-galactose-C^{14}, TDP-D-galactose-C^{14} also serves as a galactosyl donor in this system. Whether it, or yet another sugar nucleotide, may function in the formation and transfer of L-galactose is a problem requiring further study.

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