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Note

High-performance liquid chromatographic study of GDP-mannose and GDP-fucose metabolism

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Glycoprotein biosynthesis involves specific glycosyltransferases utilizing sugar-nucleotides as glycosyl residue donors. The regulation of the glycoprotein biosynthetic pathway is a complex phenomenon, which includes sugar-nucleotide availability. The fucosylation process in the rat intestine was found to be very sensitive to regulation by nutritional [1] or developmental [2] factors; thus, it was important to study GDP-fucose availability in this organ. This availability principally depends on two opposing systems: GDP-fucose degradation by glycosylnucleotide pyrophosphatases (EC 3.6.1.21) [3] and GDP-fucose formation from GDP-mannose by GDP-mannose 4,6-dehydratase (EC 4.2.1.47) [4–6]. The study of these systems with radioactive sugar-nucleotides implies the separation of GDP-fucose breakdown products (fucose, fucose phosphate) from GDP-fucose and the separation of GDP-mannose from GDP-fucose. Some published reports have dealt with the high-performance liquid chromatographic (HPLC) separation of UDP-sugars and degradation products [7,8], using perchloric acid deproteinization of the samples. However, some problems were encountered with the correct neutralization of perchloric acid in the small incubation volumes used in enzymic studies, which were particularly crucial for the study of GDP-fucose metabolism, owing to the sensitivity of this sugar-nucleotide to spontaneous hydrolysis in acid solutions.

This paper describes an approach to this problem and reports the separation

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of GDP-mannose, GDP-fucose and intermediate metabolic products by isocratic reversed-phase HPLC.

EXPERIMENTAL

Chemicals

Chemicals used for buffers and precipitation techniques were of reagent grade. The following radioactive sugar-nucleotides were purchased from New England Nuclear (Dupont de Nemours, Paris, France): GDP-[^{14}C]fucose (8.8 GBq/mmol) and GDP-[^{14}C]mannose (9.4 GBq/mmol).

Samples

Rat intestinal cell sap, used for enzymic studies, was prepared as described elsewhere [2]. Enzymic reactions in 0.2 ml of incubation media were stopped either by addition of 0.3 ml of cold 1 *M* perchloric acid (0.6 *M* final concentration) (procedure A) or by heating for 20 s at 100°C (procedure B). All subsequent steps were performed at 4°C. In procedure A, after centrifugation, perchloric acid in the supernatant was partially neutralized with 50 μl of 6 *M* potassium hydroxide, then the mixture was buffered with 0.2 ml of 1 *M* K_2HPO_4 to ensure a correct neutralization. In procedure B, after heat denaturation of proteins, 0.1 ml of 1 *M* sodium perchlorate (pH 4.1) was added and kept for 15 min, followed by the addition of 0.2 ml of 1 *M* potassium acetate. In both procedures the samples were subjected to a freeze–thaw cycle to achieve complete precipitation of potassium perchlorate salts [9], then centrifuged. After filtration of the clear supernatant on 0.45-μm filter tips (Millipore, St. Quentin Yvelines, France), 0.1-ml aliquots were directly analysed by HPLC.

Chromatography

Sugar-nucleotide and sugar-nucleotide breakdown products were separated on a SAX Partisil column (10 cm × 0.8 cm I.D., 10 μm) in a radial compression module (Waters Division of Millipore) by an adaptation of the method of Holstege et al. [8]. A linear gradient from 100% buffer A (20 m*M* KH_2PO_4, pH 4.1) to 100% buffer B (0.5 *M* KH_2PO_4, pH 4.6) was applied from the start to 12 min at a flow-rate of 2.0 ml/min. Then the column was equilibrated for 5 min with buffer A at a flow-rate of 4.0 ml/min before the next injection. The separation of GDP-fucose and GDP-mannose was achieved isocratically in a C_{18} μBondapak column (10 cm × 0.8 cm I.D., 10 μm average particle size) in a radial compression module with buffer B at a flow-rate of 1.0 ml/min. Radioactivity was continuously monitored using a solid cell counter (Trace II, Packard Instruments).

RESULTS AND DISCUSSION

Chemical and enzymic degradation of sugar-nucleotides

The chromatographic conditions described in Experimental lead to the rapid separation of glycosyl-nucleotide pyrophosphatase products on the SAX column (retention times: free fucose, 1.7 min; fucose phosphate, 6.2 min; GDP-fucose, 10.4 min) (Fig. 1).

Preliminary experiments indicated that problems arose from the spontaneous hydrolysis of GDP-fucose to free fucose in 0.6 *M* perchloric acid, which at 4°C occurs at a rate of 0.5% per min, with a calculated half-life of 100 min. (Under the same conditions, GDP-mannose is not hydrolysed upon incubation up to 30 min.) This hydrolysis of GDP-fucose increases drastically with temperature: it is almost complete at room temperature after 5 min (only 7% of GDP-mannose is hydrolysed under these conditions). Moreover, the use of even dilute solutions of strong acid and base makes it difficult to adjust the pH in small volumes, so that the buffering capacity of a concentrated salt is needed (procedure A).

Since correct results could not be obtained for GDP-fucose using perchloric acid precipitation, ethanol precipitation or heat denaturation was tested and also found unsuitable. Ethanol must be carefully eliminated under nitrogen in

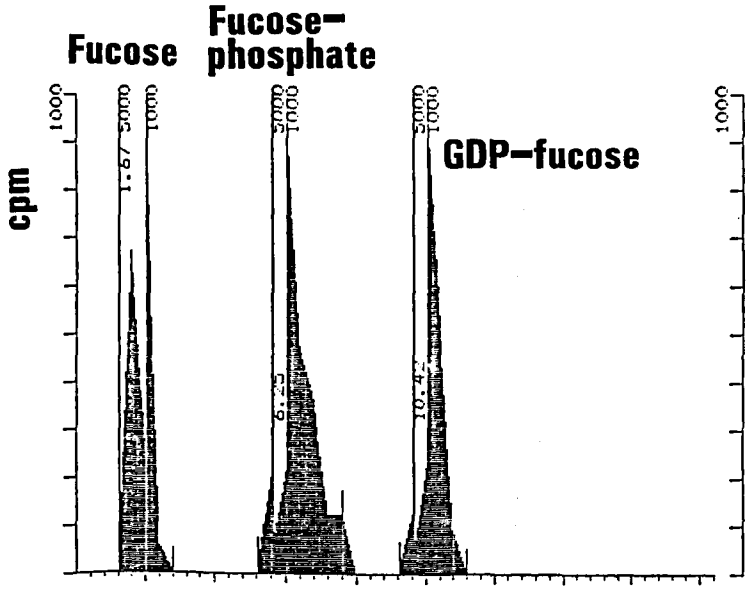

Fig. 1. Radioactivity monitoring (with automatic ranging), after HPLC separation on SAX Partisil, of GDP-fucose and GDP-fucose breakdown products. *y*-axis: radioactivity (cpm); *x*-axis: time (25-s integration periods).

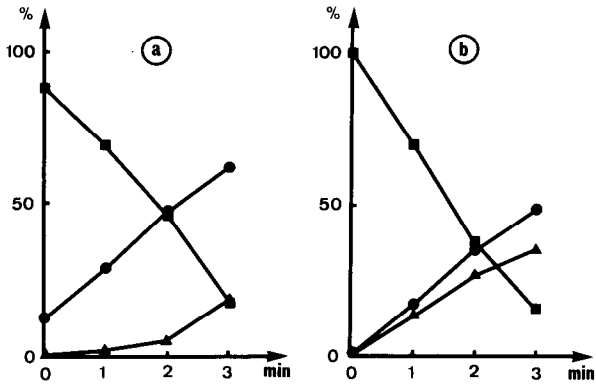

Fig. 2. Kinetic determinations at 30°C of GDP-fucose pyrophosphatase activity in rat intestinal cell sap [50 µl of cell sap, 150 µl of 10 m*M* Tris-HCl (pH 7.4), 1 kBq radioactive GDP-fucose]. (a) Reaction stopped with procedure A (0.6 *M* perchloric acid, final concentration). (b) reaction stopped with procedure B. (■) GDP-fucose; (▲) fucose phosphate (●) fucose.

order to avoid precipitation of phosphate ions in the chromatographic system. Moreover, the use of ethanol leads to the extraction of components (especially in membrane-bound enzyme determinations), which are retained on the column and rapidly decrease both the retention times and the radioactivity recovery. The same problem was also found after heat denaturation, because of incomplete precipitation. However, in neither case was spontaneous breakdown of GDP-fucose observed. Therefore, a method was developed (procedure B), utilizing heat treatment (20 s at 100°C) for enzyme denaturation and the precipitating properties of perchlorate ions without acidification of the incubation mixture. Sodium perchlorate (pH 4.1) was added, then, after incubation for 15 min at 4°C, potassium acetate was added. The mixture was left for 15 min at 4°C for ion exchange, subjected to a freeze–thaw cycle [9] and centrifuged for the elimination of proteins and potassium perchlorate. This technique provides a simple, rapid and efficient method for preparing many samples from small-volume incubation mixtures, without chemical hydrolysis of GDP-fucose, and gives reproducible retention times and complete radioactivity recovery.

As an example, Fig. 2 shows the results of two similar kinetic determinations of GDP-fucose pyrophosphatase in intestinal cell sap. It is clear that perchloric acid (procedure A, Fig. 2a) to stop enzymic reactions utilizing GDP-fucose leads to results different from those obtained by procedure B (Fig. 2b): the amount of sugar phosphate is greatly underestimated with perchloric acid precipitation, whereas that of free fucose is overestimated.

Enzymic conversion of GDP-mannose into GDP-fucose

The incubation of intestinal cell sap with radioactive GDP-mannose, in the presence of AMP as an inhibitor of glycosyl-nucleotide pyrophosphatase ac-

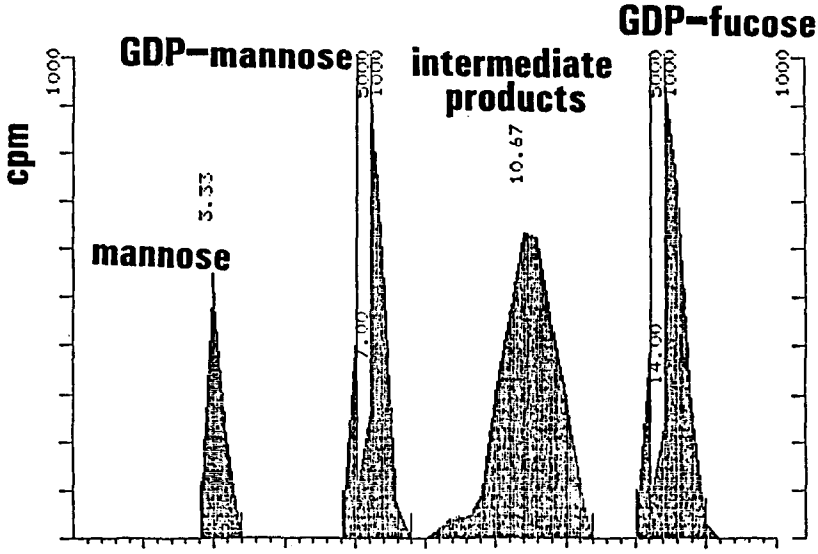

Fig. 3. Radioactivity monitoring (with automatic ranging), after HPLC separation on a C_{18} column, of GDP-mannose, GDP-fucose and intermediate products. Incubation of intestinal cell sap was performed as in Fig. 2, in the presence of 1 m*M* AMP as a glycosyl-nucleotide pyrophosphatase inhibitor and 2 kBq radioactive GDP-mannose. *y*-axis: radioactivity (cpm); *x*-axis: time (25-s integration periods).

tivity, produces GDP-fucose. Fig. 3 shows the separation of GDP-fucose from GDP-mannose and intermediate metabolic products on a C_{18} column (retention times: free sugars, 3.3 min; GDP-mannose, 7.0 min; intermediate products, 10.7 min; GDP-fucose, 14.0 min). The use of 0.5 *M* potassium phosphate is important: a decrease in concentration drastically decreases retention times and alters the separation of peaks.

The transformation of GDP-mannose into GDP-fucose is catalysed by GDP-mannose 4,6-dehydratase, which produces two intermediate products, the reduction of which by NADPH produces GDP-fucose [4,5]. The radioactive peak observed between GDP-mannose and GDP-fucose was characterized as these intermediate products in two ways: demonstration of a precursor–product relationship and chemical characterization. First, intestinal cell sap was dialysed against 10 m*M* Tris buffer (pH 7.4), to eliminate endogenous NADPH, and incubated with labelled GDP-mannose; under these conditions, only intermediate products were formed. After addition of 1 μM NADPH, this peak progressively disappeared with a concomitant increase in the GDP-fucose peak. Secondly, after isolation of this intermediate peak, the products were reduced by an excess of sodium borohydrate, concentrated on a small Dowex 1 column, eluted by 1 *M* trifluoroacetic acid and hydrolysed for 2 h at 100°C. After neutralization, the sugars were separated by paper chromatography in pyridine–ethyl acetate–water (1:3.6:1.15, v/v). The chromatographic pattern is essen-

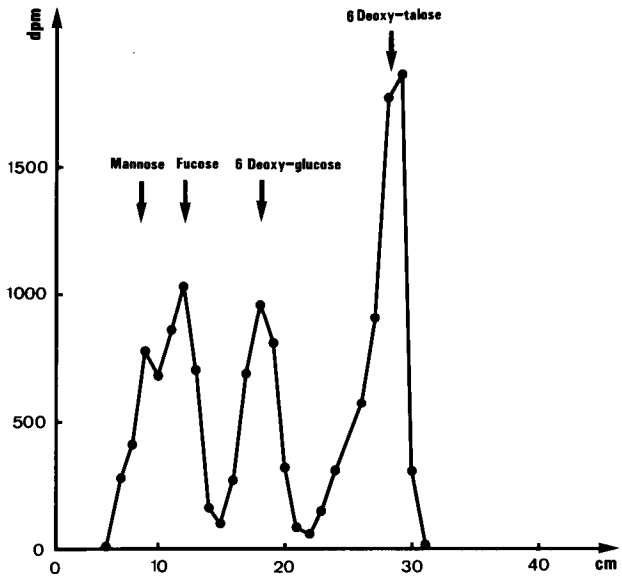

Fig. 4. Paper chromatography in pyridine-ethyl acetate-water (1:3.6:1.15, v/v) of the sugars obtained by reduction and hydrolysis of the intermediate products separated between GDP-mannose and GDP-fucose on a C_{18} column. (Mannose, fucose and deoxyglucose were determined with authentic standards; deoxytalose was determined from R_F).

tially the same as reported by Bulet et al. [5] and shows, in addition to fucose and mannose, the presence of 6-deoxy-L-glucose and 6-deoxy-D-talose (Fig. 4). Therefore, the peak observed between GDP-mannose and GDP-fucose is a mixture of GDP-4-oxo-6-deoxy-D-mannose (giving deoxytalose or deoxymannose after reduction and hydrolysis) and GDP-4-oxo-6-deoxy-L-galactose (giving essentially deoxyglucose and fucose after reduction and hydrolysis). Unfortunately, these intermediates could not be separated by HPLC in the same run, even by varying the chromatographic conditions.

Thus, HPLC appears convenient for the study of GDP-fucose-metabolizing enzymes, provided some care is taken to avoid chemical hydrolysis during sample handling (using procedure B reported here). This technique is less time-consuming than paper chromatography. It avoids the use of isobutyric acid in a paper chromatographic separation of GDP-fucose and GDP-mannose [4,5] and provides a better separation of intermediate products, allowing the study of the different steps of the enzymic production of GDP-fucose from GDP-mannose [6].

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