Module 6: Nucleotide Sugar Analysis

Faculty Leader(s): *Natasha Zachara*

Background:

The enzymes that synthesize glycoconjugates utilize a variety of common high-energy activated nucleotide sugars. These sugars are derived from monosaccharides imported into the cell, are salvaged from degraded glycans, or are synthesized *de novo*. The interconversion of sugars and the activation of sugars occur primarily within the cytoplasm, and activated sugars can be transported into either the Golgi Apparatus (Golgi) or Endoplasmic Reticulum (ER) by a family of sugar nucleotide specific transporters.

Sugar transport into the cell is mediated by three types of sugar transporters: 1) Energy-independent transporters, such as the glucose transporters (GLUTs); 2) Energy-dependent transporters, such as the sodium-dependent glucose transporters (SGLTs), which are often found in the intestinal and kidney epithelial cells; and 3) transporters which couple ATP-dependent phosphorylation to sugar import, which are common in bacteria. Glucose is transported from the gut by SGLT1 and is recovered by SGLT2 in the kidney. The Km of these transporters is typically less than 1mM. GLUTs transport glucose into the cell, and the 14 GLUTs identified in mammals have different tissue expressions and Km's (typically, 2-20mM). In mammals, GLUTs predominantly transport glucose and fructose with variable efficiency. Whereas GLUT2, which is expressed in the liver, pancreas, intestine and kidney, also transports glucosamine. In mammals, there are also transporters for mannose and fucose. There are two types of mannose transporter: a) A SGLT, located on the brush border of enterocytes and on the surface of kidney epithelial cells; and b) a GLUT-like transporter found on most types of mammalian cell types with a Km for mannose of 50-100μM.

In addition to the uptake of sugars from the diet, mammals can synthesize sugar nucleotide *de novo* or salvage them from other pathways such as the degradation of glycoconjugates. The majority of glycoconjugates are degraded in the lysosome and released sugars are salvaged by lysosomal sugar carriers. There are distinct carriers for neutral hexoses (glucose, mannose, galactose, fucose, xylose; Km 50-75μM), N-acetylhexosamines (Km ~4μM) that interestingly do not transport hexosamines, and sialic acid and glucuronic acid (Km 330-550μM). While uronic acids are salvaged, they are not reused in the synthesis of sugar nucleotides. Instead, they are metabolized through the pentose phosphate pathway. Another internal source of glycans is Glycogen, which can contain up to 100,000 glucose units. The degradation of glycogen, by glycogen phosphorylase, releases glucose-1-phosphate.

The synthesis of activated sugar nucleotides requires nucleoside triphosphates and monosaccharides phosphorylated at the anomeric carbon (glycosyl-1-P). The *de novo* synthesis of sugar nucleotides proceeds via three reactions: 1) phosphorylation and activation of a monosaccharide by a kinase; and 2,3) the interconversion of an existing sugar nucleotide **(Figure 1)**. The majority of sugars can be synthesized from glucose and fructose (**Figure 2**), the sugars that make up sucrose (**Figure 3**), using glycan biosynthetic pathways. It should be noted that not all pathways are equally active in all cell types.

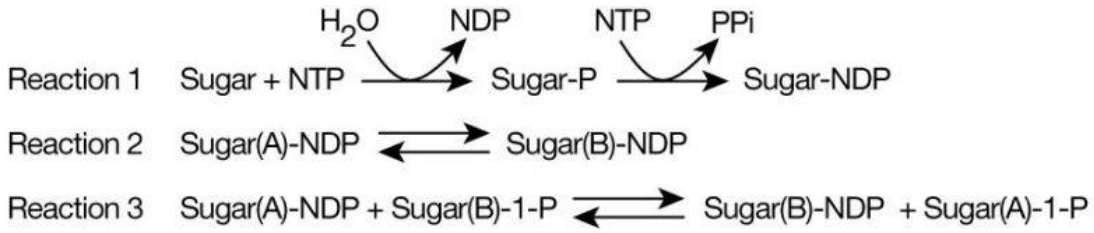

Figure 1: Reactions that generate sugar nucleotides

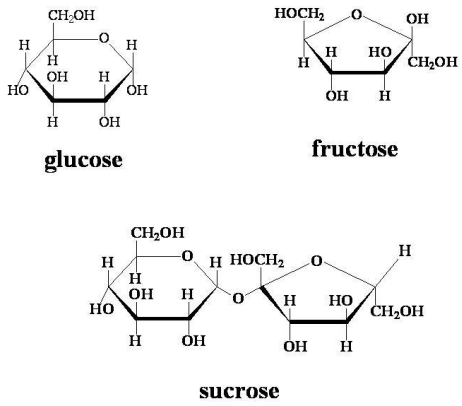

Figure 2: The structure of Glucose, Fructose and Sucrose. Sucrose is a disaccharide formed from one molecule of glucose and one molecule of fructose.

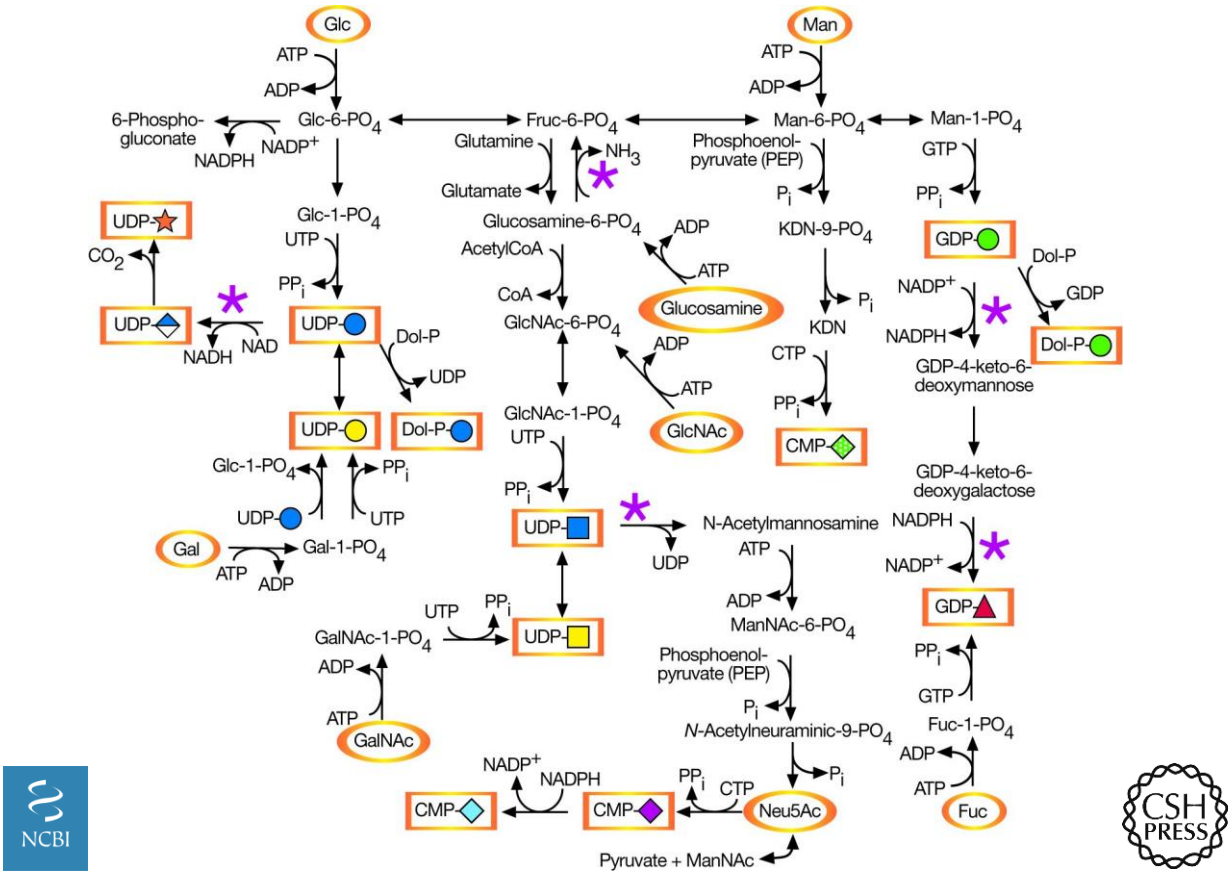

Figure 3: Mammalian sugar nucleotide biosynthesis (Essentials in Glycobiology, Figure 4.1). Biosynthesis and interconversion of monosaccharides. The relative contributions of each pathway under physiological conditions are unknown. (*Rectangles*) Donors; (*ovals*) monosaccharides; (*asterisks*) control points; (6PG) 6-phosphogluconate; (PEP) phosphoenolpyruvate; (KDN) 2-keto-3-deoxy-D-glycero-D-galactonononic acid; (Dol) dolichol.

Table 1: Common mammalian sugar nucleotides

Sugar	Activated Form	Anomeric Configuration
Glucose	UDP	α
Galactose	UDP	α
GlcNAc	UDP	α
GalNAc	UDP	α
GlcA	UDP	α
Xylose	UDP	α
Mannose	GDP	α
Fucose	GDP	α
Sialic Acid	CMP	

Note: There is no UDP-iduronic acid as iduronic acid is created by epimerization of glucuronic

acid, after the incorporation of this sugar into glycosaminoglycan chains.

A number of diseases have been associated with either reduced or elevated levels of sugar nucleotides, we will offer a few examples here. GDP-mannose, which is required for the synthesis of numerous glycoconjugates, is synthesized from mannose-1-phosphate which its self is derived from mannose-6-phosphate. Mannose-6-phosphate is generated by phosphorylation of mannose by hexokinase or conversion of fructose-6-phosphate to mannose-6 phosphate, a reaction catalyzed by phosphomannose isomerase. Mutations in phosphomannose isomerase leads to a congenital disorder of glycosylation (type II), this effect of which is exacerbated as mannose is not a common constituent of mammalian diets. In both yeast and humans, many of the effects of mutations in phosphomannose isomerase can be overcome by mannose feeding. Notably, feeding exogenous mannose can lead to the honeybee effect, in which ATP levels are depleted.

Deficiencies in one of the pathways utilized for the synthesis of UDP-Gal leads to a condition called galactosemina, which if left uncontrolled can lead to mental retardation, liver damages, and death. Galactose-1-phosphate can be converted to UDP-Galactose through a UDP-exchange reaction with UDP-Glc. In an effort to reduce the buildup of galactose-1-phosphate, cells convert it to galactitol and galactonate that are toxic. Mutations in three enzymes can lead to galactosemia, with mutations in the galactose-1-phosphate uridyltransferase being the most common. Some of the effects of galactosemia can be ameliorated by reducing galactose in the diet, which includes lactose.

Due to the negative charge, sugar-nucleotides cannot diffuse across the ER and Golgi membrane. In Eukaryotes, sugar-nucleotides are exchanged for nucleoside-monophosphates by a set of energy-independent nucleotide sugar antiporters (Km 1-10μM), and results in concentrations of sugars 10- to 50- higher that of the cytoplasm.

Techniques for sugar nucleotide analysis

The assessment of sugar nucleotides starts with the efficient extraction of sugar nucleotides from cells and tissues in a manner that facilitates quantification. Traditionally, this has been achieved using perchloric acid (which precipitates proteins); and more recently using chloroform/methanol followed by solid-phase clean up. To quantify sugar-nucleotides and other intermediates effectively, two steps must be taken: a) there must be some method for assessing the amount of protein/tissue from which the nucleotides were extracted; and b) an internal standard must be included which can be used to normalize between different samples.

Sugar nucleotides have been successfully separated using a number of methods (as described below) and are easily detected due to the nucleotide. Sugar phosphate intermediates are more difficult to detect, and

GC-MS or HPAEC with pulsed ampometric detection is more effective.

Separation and Detection:

1) **HPLC** using porous graphitized carbon (PGC), ultra-high-pressure chromatography, and mass spectrometry (2,3). High throughput. Poor Separation of UDP-GlcNAc and UDP-GalNAc. Significant regeneration of the column is required.
2) **Reversed Phase:** Quick, but poor separation of highly charged sugar nucleotides.
3) **Ion Exchange (HPAEC):** One advantage of the HPAEC with PAD detection is the ability to detect hydroxyl residues that facilitates the detection of sugar phosphates.
4) **High resolution reversed phase HPLC with Ion-pairing:** While the ion-pairing reagents are not compatible with mass spectrometry (4), they improve the separation of highly charged sugar nucleotides.
5) **CE:** Capillary electrophoresis in borate buffer has been used and can be effectively coupled to mass spectrometry.
6) **NMR:** Best used for studying metabolite flux through sugar-metabolite pathways and requires the use of labeled sugars.

Citations

1. Hudson H. Freeze and Alan D. Elbein. Glycoylation Precursors (Chapter 4), Essentials in Glycobiology (2009) 2nd edition. Edited by Ajit Varki, Richard D Cummings, Jeffrey D Esko, Hudson H Freeze, Pamela Stanley, Carolyn R Bertozzi, Gerald W Hart, and Marilynn E Etzler. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2009.
2. Garcia AD, Chavez JL, Mechref Y. Sugar nucleotide quantification using multiple reaction monitoring liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom. 2013 Aug 15;27(15):1794-800. doi: 10.1002/rcm.6631.
3. Pabst M, Grass J, Fischl R, Léonard R, Jin C, Hinterkörner G, Borth N, Altmann F. Nucleotide and nucleotide sugar analysis by liquid chromatography-electrospray ionization-mass spectrometry on surface-conditioned porous graphitic carbon. Anal Chem. 2010 Dec 1;82(23):9782-8. doi: 10.1021/ac101975k. Epub 2010 Nov 2.
4. Kochanowski N, Blanchard F, Cacan R, Chirat F, Guedon E, Marc A, Goergen JL. Intracellular nucleotide and nucleotide sugar contents of cultured CHO cells determined by a fast, sensitive, and high-resolution ion-pair RP-HPLC. Anal Biochem. 2006 Jan 15;348(2):243-51. Epub 2005 Nov 15.