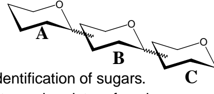
BCMB8020
April 11, 2006

Requirements for Structural Determination of a Carbohydrate



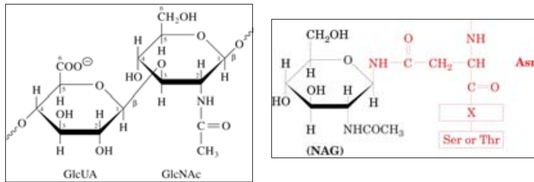
- -Identification of sugars.
- -Stereochemistry of each sugar.
- -Types of linkages.
- -Types of ring structures.
- -Anomeric configuration of each sugar.
- -Sequence of the different sugar residues.

Methods to Determine Carbohydrate Structure

The structural characterization of carbohydrates & glycoconjugates is challenging due to the complexity & diversity of carbohydrate structure. To completely characterize an oligo- or polysaccharide the following must be done/determined:

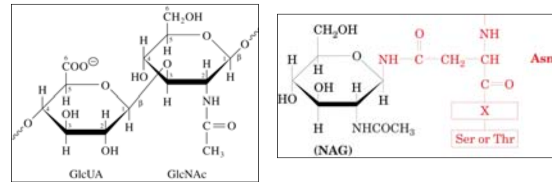
Methods to Determine Carbohydrate Structure

1. **Isolate "pure" oligo-, polysaacharide, glycoconjugate**
2. **Cleave the carbohydrate(s) from the aglycone**
3. **Determine glycosyl residue composition**



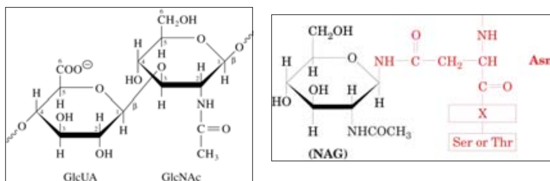
Methods to Determine Carbohydrate Structure

4. Determine absolute configuration (i.e. D or L) of each glycosyl residue (specific rotation; c.d. absorption; enzymatic susceptibility; derivatization and M.S.; NMR)



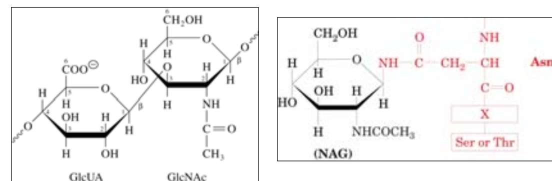
Methods to Determine Carbohydrate Structure

5. Determine glycosyl linkage(s) (methylation analysis; GC-MS; GLC-MS)



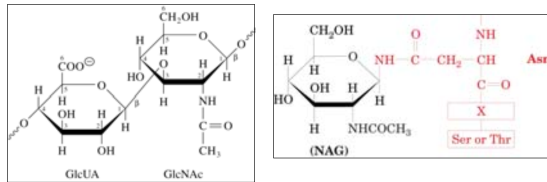
Methods to Determine Carbohydrate Structure

6. Establish ring form (i.e. furanose or pyranose) of each glycosyl residue (methylation analysis ± ethylation)



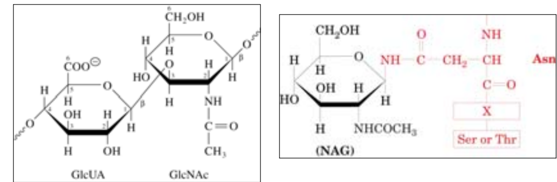
Methods to Determine Carbohydrate Structure

7. Determine sequence of the glycosyl residues (multiple approaches)



Methods to Determine Carbohydrate Structure

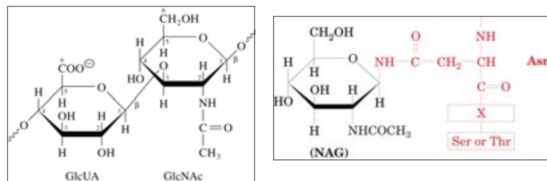
8. Determine the anomeric configuration (i.e. α or β) of each glycosyl residue (NMR; enzyme susceptibility)



Methods to Determine Carbohydrate Structure

9. Determine points of attachment of any non-carbohydrate substituents

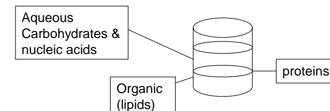
10. Determine higher levels of structure, when applicable



Purification of Carbohydrates

The preparation of a "purified" carbohydrate or glycoconjugate may be challenging.

Carbohydrates can be separated from proteins & lipids by aqueous:organic extraction



Glycolipids & glycoproteins are more difficult to purify. Specific enzymes are often used to cleave the carbohydrate from the aglycone.

Oligo- and polysaccharides can be further purified by standard chromatography (i.e. ion exchange, size exclusion, HPLC, preparative electrophoresis, electrofocusing, etc.

Detection of Carbohydrates

Colorimetric Assays for Carbohydrates. Often done in microtitre plates & compared to standard curves. Sensitivity in the nanomole range.

General Carbohydrate Assay

Phenol-Sulfuric Acid Assay

Sample + 5% phenol + H_2SO_4. Read OD at 490 nm. Not good for amino and acetylated sugars. (Dubois,M. et al, 1956, Anal. Chem. 28:350-356)

Ferricyanide Assay

Hydrolyze samples in 2N HCl for 2 hr at 100°C. Treat with reducing agents. Read OD at 690 nm. (Park & Johnson, 1949, J.Biol. Chem. 181:149)

Reducing sugar assays

Para-Hydroxybenzoic Acid Hydrazide (PAHBAH) Assay

Add reagent, boil 10 min. Read OD 410 nm. (Lever, 1972, Anal. Biochem. 47:248)

Nelson-Somogyi Assay

Heat samples, add reducing agent, heat. Read absorbance at 500 nm. (Nelson, 1944, J.Biol. Chem 153:375-380; Somogyi, 1952, J. Biol. Chem 195:19-23;Spiro, 1966, Methods Enzymol. 7:3-26; Green et al., 1989, Anal. Biochem. 182:197-199)

Hexose Assays

Anthrone Assay

Samples + 0.2% anthrone in H_2SO_4, Boil. Read OD at 620 nm. Color may vary with type of sugar. (Dische, A., 1962, In Methods Carbohydr. Chem. 1:478-512)

Uronic Acids

Meta-hydroxybiphenyl Assay for uronic acids

Hydrolyze in boiling H_2SO_4 5 min, add colorimetric reagent. Read at 520 nm. (Blumenkrantz & Asboe-Hansen, 1973, Anal. Biochem. 54:484; York *et al*., 1985, Methods in Enzymology 118:3-40, see pg. 26)

Orcinol Assay for pentoses & uronic acids

Add colorimetric reagent & HCL, boil 20 min. Read OD 665 nm. (Dische, 1953, J.Bio.Chem 204:983)

KDO Assay

Thiobarbituric Acid (TBA) Assay for KDO (and Sialic Acids)

Treat with periodate followed by colorimetric reagent. Read OD 549 nm. (Aminoff, 1961, Biochem. J. 81:384; Uchida, 1977, J. Biochem, 82:1425)

Sialic Acid Assays

Resorcinol Assay for Sialic Acid

Add colorimetric reagent in HCl, boil 15 min, add methylbutanol, centrifuge. Read upper phase at OD 450nm & 580 nm. Correct for hexose (OD 450). (Svennerholm, 1959, Biochim. Biophys. Acta 24:604-611; Beeley, 1985, In Laboaratroy techniuqes in biochm. & Mol. Biol., Vol 16, Elsevier)

Other types of carbohydrate detection

*Electrochemical Detection (DIONEX) (good sensitivity)

*Refractive Index (poor sensitivity)

*If carbohydrate is derivatized: UV or fluorescence

*Radioactive

Glycosyl Residue Composition Analysis

Determination of the molar ratio of the constituent monosaccharides

Basic steps:

1. **Cleave glycosidic bonds (e.g. hydrolysis or methanolysis or solvolysis by hydrogen fluoride).** ***Note:*** **glycosidic bonds differ in their susceptibility to acid hydrolysis: depends on monosaccharide and on position and anomeric configuration of glycosic linkage.**

2. **Separate monosaccharides. Four common methods are:**

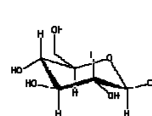
   A. Preparation of Alditol acetates & GC (can not distinguish uronic acids)

   B. Preparation of Trimethylsily (TMS) methylglycosides & GC Useful to detect uronic and amino sugars. Spectrum more complex than alditol acetate: get α & β anomer and maybe pyranose & furanose)

   C. Separate by high pH anion exchange chromatography (HPAEC)

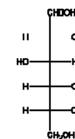
   D. Monosaccharide composition analysis by Carbohydrate Electrophoresis (FACE™ , Fluorophore-Assisted Carbohydrate Electrophoresis)

Preparation of alditol acetates & GC (composition analysis)



Glucose

Hydrolyzed monosaccharide



Glucitol

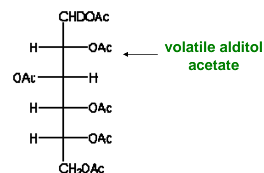
Reduced monosaccharide

($NaBD_4$ + NH_4OH)

Remove $NaBD_4$ & borate with methanol acetic acid

Add acetic anhydride + base

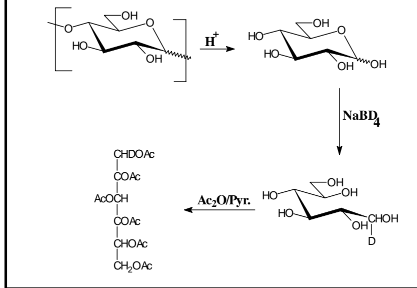
Separate by GC



volatile alditol acetate

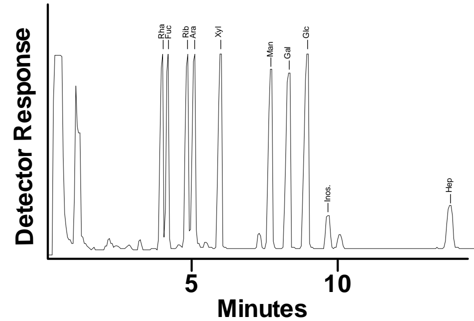
NOTE: can not specifically detect acidic sugars with this protocol!

Preparation of Alditol Acetates



GLC Profile of Alditol Acetates

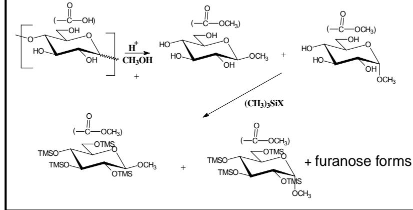
(Supelco SP2330 Column)



Preparation of Trimethylsilyl (TMS) Methyl Glycosides

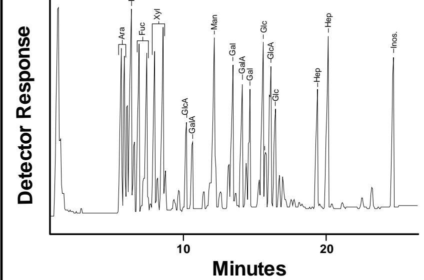
This methods does allow detection of acidic sugars

Preparation of Trimethylsilyl (TMS) Methyl Glycosides



GLC of TMS Methyl Glycosides

(J&W Scientific DB1 column)



Separate by high pH anion exchange chromatography (HPAEC)

Chromatography often done on a DIONEX HPLC system using a anion exchange column (e.g. CarboPacPA1) with Pulsed amperometric (PAD) or electrochemical (ED) detection.

Since monosaccharides area weak acids (pKa's ~ 12.2-13.8), an alkaline eluent (usually NaOH) is used to transform the hydroxyl groups on the monosaccharides into oxyanions.

Separation is due to the small differences in the pKa's of the monosaccharides and differences in their interactions with the strong anion resin in the column.

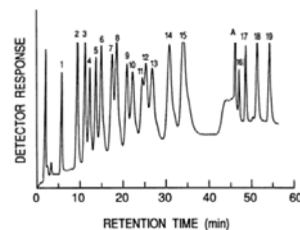


FIG. 1. HPLC chromatogram showing separation of a standard solution of 13 neutral and 2 amino sugars together with 3 uronic acids and muramic acid. Isocratic elution with 4.6 mM NaOH at 1.0 ml/min for 39 min eluted the neutral and amino sugars and then a step gradient to 6.0 mM NaOH–100 mM sodium acetate eluted acidic sugars. Injected amounts of each sugar were 1.5 nmol, except Fuc and MurA (0.75 nmol), and Fru and Rib (4.5 nmol). The column and detector settings used are described in the text. 1, fucose (Fuc); 2, 6-deoxyglucose (6-dGlc; internal standard); 3, 2-deoxyglucose (2-dGlc; internal standard); 4, rhamnose (Rha); 5, arabinose (Ara); 6, mannosamine (ManN; internal standard); 7, glucosamine (GlcN); 8, galactose (Gal); 9, glucose (Glc); 10, lyxose (Lyx); 11, xylose (Xyl); 12, mannose (Man); 13, sucrose (Suc); 14, fructose (Fru); 15, ribose (Rib); 16, muramic acid (MurA); 17, galacturonic acid (GalA); 18, glucuronic acid (GlcA); 19, mannuronic acid (ManA); A, peak due to the acetate step gradient. Note baseline drift for 39 min despite isocratic elution.

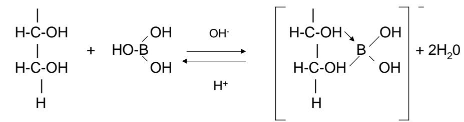
Separation by high pH anion exchange chromatography (HPAEC)

from *Wicks et al., 1991, Applied & Environ. Microbiol. 57:3135-3143*

Monosaccharide composition analysis by Carbohydrate Electrophoresis (FACETM , Fluorophore-Assisted Carbohydrate Electrophoresis)

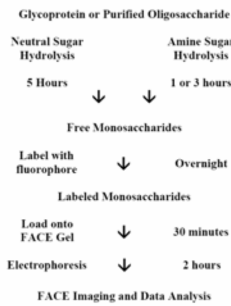
Monosaccharides are reducing end-derivatized with a fluorescent tag (e.g. 2-aminoacridone) and the derivatized monosaccharides are separated by PAGE in a buffer containing borate ions.

The monosaccharides form a complex with borate as shown below and, thus, being charged, move by PAGE.



Novotny, M. V. and Sudor (1993) Electrophoresis 14: 373-389

Summary of Monosaccharide Composition Prot



GLYCO FACE® Monosaccharide Composition Kit

Results:

The results can be interpreted using two different methods, a U.V. light box or the FACE Imaging system

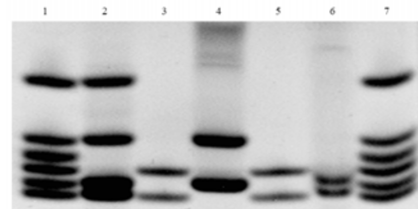
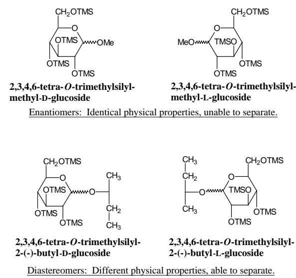


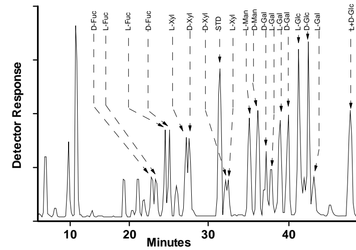
Figure 1. *Lane 1 Monosaccharide Standards*
Lane 2 Fetuin – amine hydrolysis
Lane 3 Glucose and GlcNAc
Lane 4 Fetuin – neutral hydrolysis
Lane 5 Glucose and GlcNAc
Lane 6 N-acetyllactosamine monosaccharides
Lane 7 Monosaccharide Standards

GLYCO FACE® Monosaccharide Composition Kit

Determination of the Stereochemical Configuration of Glycosyl Residues

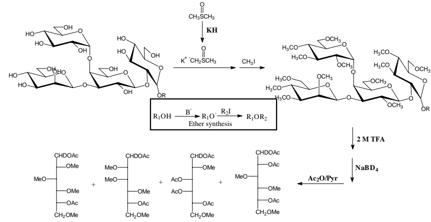


GC Separation of Trimethylsilyl (-)-2-Butyl Glycosides



Gerwig, G.J., Kamerling,J.P., and Vliegenthart, J.F.G. 1978. *Carbohydr. Res.* **62**:349-357

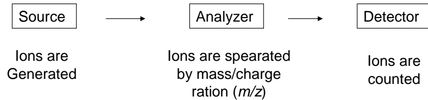
Glycosyl Linkages by Preparing Partially Methylated Alditol Acetates (PMAAs)



Mass Spectroscopy of PMAAs

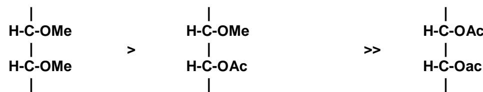
A mass spectrum (spectrum of masses of intact and fragmented original species) of Parially Methylated Alditol Acetates is used to identify glycosyl residue linkage.

In brief: a beam of electrons breaks the PMAAs apart (generates fragment ions in the gas phase), the ions are accelerated using an electric field, the ions are projected into a mass analyzer, and the mass of the fragments is counted.

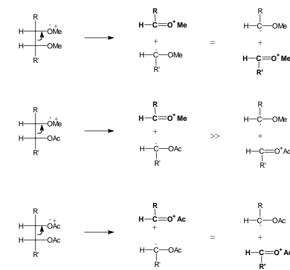


There are preferences for which –C-C- bonds will be broken; so diagnostic fragmentation patterns can be interpreted.

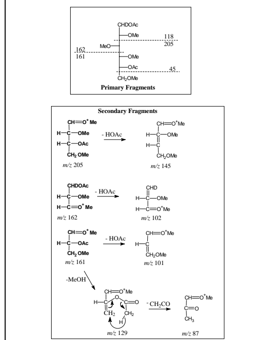
The preference of fragmentation for specific –C-C- bonds in PMAA is:



Primary Fragments for PMAAs



Primary and Secondary Fragments of the PMAA of Terminal Glucose



High-resolution **NMR (nuclear magnetic resonance) spectroscopy** is a powerful technique to study carbohydrate structure.

All structural information (except D vs L configuration) can be obtained by NMR. NMR is particularly useful for determining **glycosidic linkage (α vs β)** and for determining secondary structure.

In brief: magnetically active nuclei in carbohydrates (1H, 13C, 31P, most common) are placed in a strong magnetic field.

The field generated by the spinning charged magnetically active nuclei tend to lie parallel (i.e. low energy state) to the external magnetic field.

A short pulse of electromagnetic radiation of (wavelength 1.5 – 60 m; generally 200-500 MHz) is applied and the nuclei absorb energy (resonance).

This causes the magnetically active nuclei to line up parallel to the magnetic field (high energy state).

As the nuclei try to get back to their lower energy, signals result that represent the absorption of energy as a function of the structural environment of the protons in the carbohydrate. These signals are portrayed as a series of peaks called an NMR spectrum.