Determination of Carbohydrate Structure

Introduction:

The mention of carbohydrate likely evokes thoughts of common simple sugars such as glucose, sucrose, fructose and their associated calories. A bit more thought may bring to mind the polysaccharides, glycogen and starch. The importance of carbohydrates extends beyond these relatively simple molecules and there are journals, such as “Glycoconjugate Journal” that are devoted to the study of the function of the carbohydrate groups of glycolipids and glycoproteins. There is considerable diversity in the composition and structure of the carbohydrate portion and many important biochemical and biological functions are facilitated and regulated by these groups. This is illustrated in the following titles and abstracts from recent journal publications.

1) Galactosylation of IgG from rheumatoid arthritis patients
2) UDP-Gal:GlcNAc-R β 1,4-galtosyltransferase – a target enzyme for drug design
3) Ectopic expression of N-acetylglucosamine 6-O-sulfotransferase 2 in chemotherapy-resistant ovarian adenocarcinomas
4) Role of sialic acid-containing molecules in paramyxovirus entry into the host cell: A minireview
5) Utilization of lectin-histochemistry in forensic neuropathology: lectin staining provides useful information for postmortem diagnosis in forensic neuropathology.
6) Detection of ABO blood group-active glycolipids extracted from red cell membrane and heat hematomata using TLC-immunostaining.

Glycolipids extracted from groups A, B, and O erythrocytes were developed on thin-layer plates; their ABO blood group antigenicities were detected by immunostaining method using avidin-biotin-complex (ABC). Among series of glycolipids of different flow rates, antigen-specific staining was observed in five bands from group A1 erythrocytes, four bands from group B, and two bands from group O. Monoclonal anti-A, -B, and -H antibodies specifically stained glycolipids from A1, B, and O erythrocytes, respectively. ABO blood grouping was possible from 5 g of epidural heat hematomata of a charred body by this method. ABC immunostaining on thin-layer chromatography is a useful and reliable method for ABO blood grouping in forensic practice.

In this laboratory exercise, you will be given a carbohydrate, which is a mono, di, or trisaccharide. Your goal will be to determine the composition and structure through a series of tests and analysis. The text Biochemical Techniques: Theory and Practice, J.F. Robyt and B.J. White, pages 322-338 is a good reference for this laboratory. Also refer to pertinent pages in chapter 4 (107-108) and chapter 7 (213-227) of Lehninger, Principles of Biochemistry, fourth edition.

Methods:

Solution Preparation:

Pre-weighed samples of an unknown sugar (about 600 mg) will be distributed. Prepare 2.0 mL of a solution in water that is approximately 20 mg/mL. The exact volume and concentration of this sample need not be known accurately. This sample can be employed for sections (A – D). A second sample is required for section (E), optical rotation. The concentration of this solution should be known as accurately as possible and should be near 20.0 mg/mL. This solution should be prepared in a 25 volumetric flask.

A. Test for a reducing sugar:

Add 0.2 mL of your carbohydrate solution to 2 mL of Benedict’s reagent and heat in a boiling water bath for 3 min. Control solutions, a reducing sugar and a non-reducing sugar, will be available. Treat controls like unknowns and compare color changes in sample and controls. Benedict’s alkaline copper reagent will be available. The composition of the reagent employed in this procedure is slightly different than described in “Robyt and White”, but the chemical reduction of Cu²⁺ to Cu₂O is the same.
B. Determination of composition/glycosidic linkage by acid hydrolysis:

The composition of glycoconjugates can be determined by hydrolysis of all glycosidic links followed by separation and detection of components. Glycosidic links of saccharides are susceptible to acid hydrolysis. Although they are not equally susceptible to hydrolysis, rigorous conditions will hydrolyze common glycosidic linkages to completion.

Place 100 μL of unknown carbohydrate solution in a tube and add 10 μL trifluoroacetic acid. Tightly stopper the tube and heat in a boiling water bath for 30 min. Remove from heat and allow sample to cool.

C. Determination of composition/glycosidic linkage by enzymatic hydrolysis:

Enzymatic hydrolysis, unlike acidic hydrolysis, is stereospecific and should yield information on the specific linkages present in the unknown compound. Three different enzymes, α-glucosidase, β-glucosidase, and invertase, will be employed in this section. These enzymes are available as solutions of 50 units/mL in the following buffers respectively, 50 mM imidazole chloride pH 6, 50 mM pyridine acetate pH 5.0 and 50 mM pyridine acetate pH 4.5.

Add 100 μL of your unknown carbohydrate solution to three separate test tubes. Add 20 μL of one of the three enzymes (α-glucosidase, β-glucosidase, and invertase) to each test tube. Cover the tubes and allow them to react at room temperature for 12-48 hours.

D. Thin-layer chromatography:

Monosaccharides released by acid and enzymatic hydrolysis will be separated by thin layer chromatography and detected by acid/heat charring of individual spots. The R_f values of unknown spots will be compared to the R_f values of standards to determine monosaccharide identity.

Prepare a Whatman K5 TLC plate by drawing a line about one inch from the top. Prepare the plate such that there is at least 10 lanes for spotting.

Using a capillary tube, spot about 2 μL of the original unknown solution and about 2 μL of the acid hydrolyzed unknown. This sample can be spotted as soon as it is ready. Store the plate in a safe spot until the enzymatically hydrolyzed samples are ready.

Spot about 2 μL of each enzyme reaction onto the same TLC plate used above.

Spot about 2 μL of standard solutions, S1, S2, and S3, each in a separate lane. Spot, also in a single lane, one simple carbohydrate to help in identifying your TLC plate. Depending on lane availability, you may have one or two sets of standards per plate.

Each plate should have the following samples.

1) sample with no hydrolysis  
2) sample after acid hydrolysis
3) sample after invertase treatment  
4) sample after β-glucosidase treatment
5) sample after α-glucosidase treatment  
5) S1, S2, S3, monosaccharide

Components will be separated with a solvent system of acetonitrile:water (85:15) (v/v). Due to low solubility in the mobile phase, the separation must by performed 4 times, drying the plate thoroughly between each ascent of the solvent. Develop the plate by spraying with methanol:sulfuric acid (4:1) (v/v) or water:sulfuric acid (1:1) and heating at 110 °C - 120 °C for 5 min. Care must be used when removing the plates from the oven or they will crack. Do not place them immediately on the cold lab bench but allow them to cool in the air first. Cover plates tightly with Saran wrap to make a copy on the copier machine.
E. Optical Rotation:

Most saccharides are optically active and can be identified with relatively high accuracy by their rotation of plane polarized light.

A solution of at least 10 mg/mL should be prepared. The angle of rotation is proportional to the concentration so a more concentrated solution will result in greater rotation. To improve the signal to noise relationship, a solution of 20 mg/mL is recommended. The solution should be prepared carefully to allow accurate determination of the concentration. The polarimeter is quite precise and rotation of as little as 0.001 degree can be observed.

Determine the optical rotation of the carbohydrate solution according to the following steps.

Use of the polarimeter:

1) Do not prepare your sample until immediately before you are ready to determine its optical rotation.
2) Turn the polarimeter on with the black switch on the end. Allow the polarimeter to warm up for at least 15-20 min. Adequate warm up of the lamp provides for a more stable light source.
3) After an appropriate warm up, zero the instrument by pressing the red button on the end (digital).
4) Carefully weigh your carbohydrate (to within 0.001 g) and dissolve in water.
5) Fill the polarimeter tube. Avoid any air bubbles. A very high reading (indicated by the reading value flashing on the digital polarimeter) is indicative of an air bubble.
6) Determine the rotation of this solution (α\text{observed}). Monitor the optical rotation over time, collecting data at several times. Note the time of each measurement. If there is a significant trend in one direction, mutarotation may be occurring and the system is approaching equilibrium. Extrapolate data points to determine the optical rotation of the original conformer.

Optional

7) Prepare a second sample that contains a drop of dilute HCl. This will catalyze the approach to equilibrium.
8) Determine the optical rotation at 5 min. intervals until an equilibrium value is reached.
9) Calculate the specific rotation.

\[ [\alpha]_D^{20} = (\alpha_{\text{observed}} \times 100)/(l \times c) \]

where \([\alpha]_D^{20}\) is the specific rotation of the D line of sodium at 20°C
\(\alpha_{\text{observed}}\) is the observed rotation of the solution in degrees
\(l\) is the path length in decimeters
\(c\) is the concentration in grams per 100 mL

Calculate the specific optical rotation and compare it with literature values. Note that \(\alpha\) linkages give large positive rotations and \(\beta\) linkages give low or negative rotations of the polarized light. Optical rotation can be determined with a manual or a digital, more automatic polarimeter. The same sample and sample tube can be used for both.
### Specific Optical Rotation of Some Carbohydrates, $[\alpha]_D^{20}$

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>$\alpha$-form</th>
<th>Equilibrium mixture</th>
<th>$\beta$-form</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-ribose</td>
<td>-23.1</td>
<td>-23.7</td>
<td></td>
</tr>
<tr>
<td>L-arabinose</td>
<td>+54.0</td>
<td>+104.5</td>
<td>+175.0</td>
</tr>
<tr>
<td>D-xylose</td>
<td>+92.0</td>
<td>+19.0</td>
<td></td>
</tr>
<tr>
<td>D-glucose</td>
<td>+113.4</td>
<td>+52.2</td>
<td>+19.0</td>
</tr>
<tr>
<td>D-galactose</td>
<td>+144</td>
<td>+80.5</td>
<td>+52.0</td>
</tr>
<tr>
<td>D-fructose</td>
<td></td>
<td>-92.0</td>
<td>-133.5</td>
</tr>
<tr>
<td>D-mannose</td>
<td>+34.0</td>
<td>+14.6</td>
<td>-17.0</td>
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<tr>
<td>lactose</td>
<td>+90.0</td>
<td>+55.3</td>
<td>+35.0</td>
</tr>
<tr>
<td>sucrose</td>
<td></td>
<td>+66.5</td>
<td></td>
</tr>
<tr>
<td>maltose</td>
<td></td>
<td>+130.4</td>
<td></td>
</tr>
<tr>
<td>cellobiose</td>
<td></td>
<td>+35</td>
<td></td>
</tr>
<tr>
<td>melibiose</td>
<td></td>
<td>+129.5</td>
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</tr>
<tr>
<td>turanose</td>
<td></td>
<td>+75.8</td>
<td></td>
</tr>
<tr>
<td>$\alpha,\alpha'$-trehalose</td>
<td></td>
<td>+178</td>
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</tr>
<tr>
<td>raffinose</td>
<td></td>
<td>+105.2</td>
<td></td>
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<tr>
<td>melezitose</td>
<td></td>
<td>+88</td>
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</tbody>
</table>
The potential carbohydrate unknowns are as follows. Structures should be available from textbooks or the web.

- sucrose
- lactose
- melezitose
- melibiose
- raffinose
- turanose
- cellobiose
- maltose
- trehalose

Standard mixtures for TLC

<table>
<thead>
<tr>
<th>S1</th>
<th>S2</th>
<th>S3</th>
</tr>
</thead>
<tbody>
<tr>
<td>xylose</td>
<td>xylose</td>
<td>xylose</td>
</tr>
<tr>
<td>fructose</td>
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<td>fructose</td>
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<td>glucose</td>
<td>glucose</td>
<td>glucose</td>
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<td>galactose</td>
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<tr>
<td>sucrose</td>
<td>turanose</td>
<td>sucrose</td>
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<tr>
<td>lactose</td>
<td>cellobiose</td>
<td>maltose</td>
</tr>
<tr>
<td>melizitose</td>
<td>melizitose</td>
<td>melizitose</td>
</tr>
<tr>
<td>melibiose</td>
<td>melibiose</td>
<td>melibiose</td>
</tr>
<tr>
<td>raffinose</td>
<td>raffinose</td>
<td>raffinose</td>
</tr>
</tbody>
</table>

Enzymes: Each enzyme employed has a different glycosidic link specificity.

- **α-glucosidase** specifically attacks α-glycosidic links. It will hydrolyze the \(\alpha-1,4\) linkage in maltose, the \(\alpha-1,2\) linkage of sucrose and the \(\alpha-1,3\) linkage in melezitose. It will not hydrolyze the \(\alpha-1,6\) galactosyl linkage in melibiose or raffinose. It may slowly attack \(\alpha-1,6\) glycosyl linkages.

- **β-glucosidase** specifically attacks β-glycosidic links. It will hydrolyze the \(\beta-1,4\) glucosidic linkage of cellobiose, the \(\beta-1,4\) galactosidic linkage of lactose and the \(\beta\)-fructosidic linkage of sucrose.

Invertase is also called fructosidase and will cleave sucrose and related linkages such as that in raffinose. It does not cleave the sucrose linkage of melezitose.

**Lab Report Write Up:**

Include your data, observations, identification of your unknown, and the conclusions/reasoning you reached in solving the identity of your unknown. The identification of your unknown should include the trivial name of the carbohydrate as well as the component sugars and the glycosidic links present. In your report, provide information on a known composition of a glycolipid or glycoprotein and its role.