HISTOCHEMICAL DETECTION OF CARBOHYDRATE

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M.Sc Semester II Cc 09

Histochemistry is an important technique that is used for the visualization of biological structures. As such, it is concerned with the identification and distribution of various chemical components of tissues through the use of stains, indicators as well as microscopy.

Essentially, identification and distribution of chemical constituents of tissues is achieved through the exploitation of unique chemical environments in cells, heterologous expression techniques as well as enzymatic activities.

Carbohydrates are the most abundant and one of the four essential macromolecules, required for the survival of living beings. Structurally, these are polyhydroxy aldehydes or ketones. Carbohydrates are divided into three classes depending upon the number of forming units (aldehyde or ketone), which are monosaccharides, oligosaccharides, and polysaccharides.

These carbohydrates have extensive roles to perform inside the living organism. Monosaccharides, the simplest unit of carbohydrates, including glucose, which acts as an energy source, and amino sugars are the structural part of oligosaccharides and polysaccharides. Disaccharides like sucrose and maltose are used as sweetener and sucrose also acts as a major source of energy in plants. Polysaccharides provide mechanical support to cells in different organisms and they also help in energy storage.

For the purpose of demonstration, the sample tissue should be prepared by freezing first, followed by sectioning and fixation of it.

Carbohydrate Fixation

For the best results, it is recommended to use different fixatives for different types of carbohydrates. This is elaborated in the table below:

Type of Carbohydrate	Fixative to Use
Glycogen	● Formaldehyde containing fixative (good if sample tissue source is liver, but no reasonable results will be obtained with muscle cell or placenta.) ● Bouin's fixative at 4 °C which contains picric acid, formaldehyde, and acetic acid. (streaming artifacts can be observed) ● For better results, instead of freezing, Lison's "Gendre fluid" at -73 °C which contains ethanol, formaldehyde, picric acid, and acetic acid can be used.
Glycoproteins	● It can be fixed with all protein fixatives. For example, Bouin's fixative or Formaldehyde containing fixative. ● Lead salts can be used as a fixative.
Proteoglycan	● Cetylpyridinium chloride (CPC) with an aqueous solution of formaldehyde. **Note:** Do not store or use any fixative at 0 °C that contains CPC. ● 5-amino-acridine chloride with 50% v/v ethanol.

Demonstration of Homopolysaccharide

1. Starch

Starch is a branched polymer of D-glucose units. It is a mixture of amylose and amylopectin. They are the storage form of polysaccharides in plants. The presence of starch in tissues can be determined by an iodine test.

- **Iodine Test**

Principle: Reaction of iodine with the amylose in starch results in the formation of a polyiodide chain which gives deep blue color.
Materials required: Sample tissue, Iodine-potassium solution (add 0.2g iodine in 2% potassium iodide solution), distilled water, glycerin jelly, and slides.
Procedure:

- Place the section of a sample tissue in iodine-potassium for 2 minutes.
- Rinse the section with distilled water.
- Mount in glycerine jelly.

Observation: You will observe deep blue or blue-black colored starch granules in the tissue section.

2. Glycogen

In animals, glycogen is the major storage form. It is a highly branched polymer of D-glucose units and is mostly found in the liver and the muscles.

- **Carmine Method**
  Principle: Carminic acid reacts with the hydroxyl group of glycogen (formation of hydrogen bonding) that results in red color glycogen.
  Materials required: Sample tissue, Hematoxylin crystal, Ferric chloride, concentrated HCl, Carmine, potassium carbonate, potassium chloride, ammonium hydroxide, absolute alcohol, methanol, distilled water, and slides.
- **Reagents preparation**
  Weigert's Iron Hematoxylin:
  Solution A**:**0 gm hematoxylin crystals in 100 ml of 90% alcohol;
  Solution B: Add 4 ml Ferric chloride in 95 ml distilled water followed by 1 ml of concentrated HC.

For Weigert's Iron Hematoxylin solution, mix solution A and B in equal parts for use.
Carmine solution (stock): Add 2.0 gm carmine, 1.0 gm potassium carbonate, 5.0 gm potassium chloride in 60 ml distilled water. Boil solution for 5 minutes; cool it down; add 20 ml of 28% ammonium hydroxide; you can store it in the refrigerator.
Carmine working solution: Mix 10 ml carmine solution, 15 ml 28% ammonium hydroxide, and methyl alcohol.
Differential solution: mix 20 ml absolute alcohol, 10 ml methanol, and 25 distilled water.
Procedure:

- Deparaffinize the section containing slide and hydrate it by using distilled water.
- Put the slide in Weigert's iron Hematoxylin for 1 minute.

- Wash the slide under running water.
- Rinse the slide with 0.5 % HCl followed by 70% alcohol for 10 seconds.
- Wash the slide under running water for 5 minutes.
- Rinse the slide with distilled water.
- Put the slide in a working carmine solution for 30 minutes.
- Transfer the slide in differentiating solution for 3 seconds.
- Rinse the slide in 70% alcohol.
- Dehydrate the slides in graded alcohol.
- Clear the slide in xylene and mount in synthetic resin.

Observation: You will observe glycogen granules in pink to red color.

3. Cellulose and Chitin

Cellulose is a linear polysaccharide of glucose monomer having B-1, 4 glycosidic linkages. This is insoluble in various organic solvents, as well as in water. This can be obtained from the by-product of various plants such as sorghum, sugarcane, wheat, and rice.

Chitin is a structurally long polymer of N-acetylglucosamine, which is an amino sugar and a derivative of glucose. It is present in the cell wall of plants and in some fungi, where it plays a role in structural support and protection under harsh conditions.

Calcofluor white staining method

Principle: Calcofluor white is a fluorochrome stain. It is non-specific in nature and stains cellulose and chitin by binding with it in the tissue environment.

Materials required: Sample tissue, calcofluor white, distilled water, fluorescence microscope, and slide.

Procedure:

- Deparaffinize the tissue section containing the slide.
- Put the slide in 1 % calcofluor white for 20 seconds.
- Wash the slide twice with distilled water.
- Mount the slide.
- Observe the slide under a fluorescence microscope.

Observation: You will observe blue-colored fluorescent cellulose under the microscope.

Note: Calcofluor white can also bind to callose, chitin and other polysaccharides.

Demonstration of Heteropolysaccharides

Heteropolysaccharides are also called as heteroglycans. They are composed of two or more different units of monosaccharides. Mainly it includes glycosaminoglycans (example- hyaluronate, chondroitin sulfate, and keratin sulfate) and peptidoglycan.

1. Glycosaminoglycans

These are also called mucopolysaccharides or proteoglycans, which are linear molecules containing uronic acid and sulfated groups (presence of uronic acid and sulfated group at free ends make it highly acidic). It mainly includes four groups, Hyaluronate, heparin sulfate, keratin sulfate, and chondroitin sulfate. Hyaluronic acid is a non-sulfated group of glycosaminoglycans (GAG). These are components of the extracellular matrix which also give mechanical support to the cell.

These mucosubstances (acidic and non-sulfated/sulfated) can be demonstrated by various methods like Hale's colloidal iron method, Periodic-acid-Schiff's reaction (PAS), Alcian blue, and Metachromatic dyes.

1. **Hale's colloidal iron method**
   Principle: At very low pH, carboxyl and sulfate-containing substances absorb the colloidal ferric ions. Prussian blue staining reaction then stains the absorbed ferric substance in blue.
   Materials required: Tissue section, 12% acetic acid, 2% aqueous potassium ferrocyanide, colloidal iron suspension (make a working colloidal solution by adding colloidal iron suspension in acetic acid, in equal volumes), and Perl's solution (mix 2% ferrocyanide and 2% HCl in equal volumes).

2. **Procedure:**

- Deparaffinize the section and rinse it with distilled water.
- Again, rinse the slides well in the 12% acetic acid.
- Put the section in a working colloidal solution for 15-20 minutes.
- Rinse the section three times with a 12 % acetic acid solution.
- Put the section in Perl's solution for 20 minutes.

- Wash the section with distilled water to remove the extra solution.
- Counterstain the section with nuclear-fast-red for 1 minute.
- Dehydrate the section, and mount it in DPX.

Observation: You will observe the acid mucopolysaccharides stained in deep blue color, which shows the presence of glycoproteins (example GAGs).