E-content for Programme: M.Sc. Zoology (Semester-II)

Core Course (CC-7): Biochemistry

Unit V: Principles of Histology and Histochemistry
5.3 General principles of histochemistry:
Carbohydrate, Protein, Lipid, Nucleic acids, and Enzymes

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Carbohydrate Histochemistry

A. Demonstration of Homopolysaccharide

1. Starch

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.

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 that results in red color glycogen.

3. Cellulose and Chitin

The presence of these can be determined by

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.

Carbohydrate Histochemistry (Cont'd….)

B. Demonstration of Heteropolysaccharides

Heteropolysaccharides are also called as heteroglycans. Mainly, it includes glycosaminoglycans (example- hyaluronate, chondroitin sulfate, and keratin sulfate) and peptidoglycan.

1. Glycosaminoglycans

Also known as mucopolysaccharides or proteoglycans, are linear molecules containing uronic acid and sulfated groups that make it highly acidic. These mucosubstances (acidic and non-sulfated/sulfated) can be demonstrated by various methods:

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.

Periodic-acid-Schiff Reaction

Principle: The free hydroxyl group is oxidized by periodic acid to aldehyde. which after reaction with Schiff's reagent shows carbohydrates of tissue in purple color. Carbohydrates that are stained by this method are Sulphomucins, Proteoglycan, Glycogen, Glycolipids, etc.

Carbohydrate Histochemistry (Cont'd….)

Alcian blue

Principle: Alcian blue is a basic dye which mainly stains acidic mucosubstances that are carboxylated and sulfated by forming a salt bridge with them.

Iron diamine method

Principle: Diamine stain the O-sulfate esters by oxidizing itself in the reaction with ferric chloride.

C. Demonstration of Glycoproteins

Glycoproteins are branched molecules containing sialic acid and fucose groups. The presence of sialic acid at the free end of the glycoproteins makes it a negatively charged compound. Most of the glycoproteins compose the integral membrane protein, where they have an essential role in cell-cell interaction.

Methods of demonstration: All the method which are involved in the demonstration of Glycosaminoglycans (GAGs) can also be used to demonstrate Glycoproteins; such as PAS (Periodic acid Schiff) reaction, alcian blue, and Cuprolinic blue staining method.

Protein Histochemistry

- Illustration of proteins histochemically is only done when one or more amino acids are very high in their composition in the protein structure. But when the protein to be demonstrated is mixed with other proteins and is in less concentration, their demonstration is done by enzyme activity. Some proteins are identified either by their tissue location or physio-chemical properties.

- There are mainly two principles involved in the illustration of protein:
  - ✓ Reaction with amino acids, which after covalent bonding with a dye, highlights the protein.
  - ✓ Reaction of charged dyes with a net charge on the protein (either positive or negative)

- Basic proteins like histones, myoglobin, and ribosomal basic proteins are also stained and localized by staining with Anionic dyes.

- Depending upon the type of amino acid to be demonstrated in the protein, there are various methods involved, for ex- Tryptophan can be demonstrated by dimethylaminobenzaldehyde (DMAB) which is followed by coupling to diazonium salt. In this test, the blue color of the solution specifically shows the presence of tryptophan in the sample.

Protein Histochemistry (Cont'd….)

Test for proteins

1. Biuret test

The presence of protein in the test sample is often determined by the Biuret test. This test specifically checks the presence of the peptide bond. Two or more peptide bonds presence gives a positive test result.

Principle: Alkaline $CuSO_4$ reacts with the protein present in the sample, forming a complex that gives a violet-colored product.

2. Ninhydrin test

This method is useful to detect amino acids, peptides, and proteins in the test sample. Ninhydrin reacts with a α-amino group of amino acids and proteins.

Principle: Ninhydrin is a very strong oxidative agent. Reaction with a α-amino group causes oxidative deamination of the amino acid, which gives a reduced form of ninhydrin, CO2, and ammonia. Then, the reduced ninhydrin reacts with liberated ammonia and other ninhydrin, which results in the formation of a blue-colored complex

Protein Histochemistry (Cont'd….)

Tests for amino acids and lower peptides:

1. Xanthoproteic Reaction

This test is used to check the presence of protein having aromatic amino acid.

Principle: The Reaction of aromatic amino acids of the protein with the nitric acid on heating gives a yellow color because of the nitration of benzene ring. The colored product changes to orange when alkali is added to the solution.

2. Sakaguchi test (Arginine reaction)

This method tests the presence of arginine in the test sample.

Principle: The reagent used in this method is comprised of α-naphthol and one drop of sodium hypochlorite (sometimes also called as Sakaguchi reagent). α-naphthol reacts with the guanidyl group of arginine and the obtained product is oxidized by sodium hypochlorite. The oxidation results in the red color of the compound.

3. Hopkin's Cole test (Tryptophan reaction)

This method tests the presence of tryptophan in the sample.

Principle: This method is based on the reaction of tryptophan present in the sample with glyoxylic acid in the presence of conc. H_2So_4, that gives purple color to the solution.

Lipid Histochemistry

➢Lipid staining technique is used for demonstrating intracellular lipids in various tissue sections. It involves the use of disparate Lysochromes (Lipid soluble dyes) like Sudan Black B, Nile red, and Oil Red O, etc. The dyes are selected depending on the type of lipid to be studied.

Principle

For this technique, the dye is more soluble in the lipid, which allows it to be more demonstrated than in the vehicular solvent. The dyes used in this technique are all interchangeable, which means that they can be substituted for each other for the staining process.

Demonstration of all types of Lipids (including hydrocarbons and higher alcohols)

Oil Red O Method

Principle: Staining with Oil Red O involves the principle of solubility of these dyes in lipids than in the usual hydroalcoholic solvent.

Osmium Tetroxide method

Principle: Osmium tetroxide is fat-soluble and when it reacts with fats, it gets associated with the lipid head and forms a black colored reduction compound.

Lipid Histochemistry (Cont'd….)

Demonstration of Hydrophobic or Hydrophilic Lipids

Bromine-Sudan Black method

Principle: It is a basic dye and it reacts with the acidic group of lipids. This reaction results in the formation of a black colored product.

Marchi Method

Principle: The oxidation-reduction reaction of osmium tetroxide with lipid droplets results in the formation of a black colored product.

Demonstration of Hydrophobic Lipids (storage lipids)

Hydrophobic lipids include waxes, lipofuscin, free fatty acids, cholesterols, and triglycerides.

Nile Blue Method

Principle: Nile blue is the composition of two dyes that is blue oxazine and red oxazone (an oxidation product of oxazine). Oxazone is a lysochrome which reacts with the lipids to give a red to pink color.

Lipid Histochemistry (Cont'd….)

Demonstration of Heterophasic Lipids

Heterophasic lipids are also called amphipathic lipids. This group includes phospholipids, glycosphingolipids, and cerebrosides. Histochemical techniques to demonstrate these lipids are: **Nile Blue Method and PAS Method.**

Demonstration of Phospholipids

OTAN Method
Principle: Osmium-α-naphthylamine chelates with the hydrophilic lipid present in the tissue that gives it orange to red color.

Chromatin-acid hematin Method
Principle: Reaction of divalent chromate with the phospholipids form a chelated product. This product, when it reacts with acid Hematin solution, forms a dark blue to black colored product.

Nucleic Acid Histochemistry

Various methods that are involved in staining of Nucleic acids are as follows:

Feulgen's nuclear reaction: Most widely used method involves the principle of hydrolysis of DNA by HCl which exposes the deoxyriboses. Then Fuchsine reacts with the aldehyde group which colors the DNA in red.

S-Bromo-2′ -Deoxyuridine Method: BrdU is incorporated in DNA and for its visualization, BrdU specific monoclonal antibodies are used. Mainly used for the visualization of DNA in cultured cells, smears, and chromosomal spreads.

In-situ Hybridization: This method involves the melting of double-stranded nucleic acid and then hybridizing them with DNA or RNA probes having radioactive elements like ^{125}I or ^{3}H or non-radioactive elements like biotin for visualization of nucleic acid.

➢RNA is mainly stained by using basic dyes such as Toluidine Blue and methylene blue.Dyes that are used to stain both DNA and RNA include, Methyl green pyronin stain which is just used to observe the presence of nucleic acid; and Acridine orange which stains DNA in yellow-green and RNA in red-orange color.

Enzyme Histochemistry

A morphological technique that serves to demonstrate the activity of enzymes present on tissues.

Principle:

The visualization is based on the action of the enzyme on a specific substrate. Following this reaction, an insoluble product develops providing the location of enzyme. If the product is not stained, a metal precipitation technique or coupling with azoic dye and tetrazolium salt can be performed.

Methods to study the enzyme histochemistry depend upon the class of enzyme.

General principles of enzyme histochemical techniques

➢Enzyme histochemistry combines the biochemical analysis of enzyme activity with information on its topographical localization.

Enzyme Histochemistry (Cont'd….)

- In a dehydrogenase reaction, enzyme substrates like sodium succinate or sodium L-lactate are oxidized and a stoichiometric color indicator tetranitrotetrazolium chloride blue (TNBT) is reduced to black or blue formazan. The formazan immediately binds to local protein and permits the precise localization of the enzyme dehydrogenase in a particular tissue compartment.

- The enzyme histochemical reaction follows the stoichiometric principles of biochemistry.

- Whereas biochemistry is applied to tissue homogenates or extracts, expressing enzyme activity in turnover rates, enzyme histochemistry indicates the locus of an enzyme in the tissue section.

- A second group of enzyme reactions use diazonium salt instead of tetrazolium chloride as color indicator. Enzymes stained with this kind of reaction are mainly esterases and phosphatases. The ester group or phosphate group of a naphthyl salt is split off by the enzyme reaction and the naphthyl rest couples to a diazo-salt and stains the esterase- or phosphatase-containing compartment like in a formazan color reaction.

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