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Topic: Principles and Methods of Staining

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PRINCIPLE AND METHODS OF STAINING

Staining of histological slides 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. Without the proper use of histochemistry techniques, it is hard and even, somewhere almost impossible to study structural details of cells, localization of subcellular structures and localization of different subcellular granules under microscope.

Only fixation of tissue specimen is not enough to visualize the structure and chemical characteristics of that tissue specimen, the process only fixes the tissue in their almost natural state with least deterioration. Normal, tissue section is colourless as the fixed protein has the same refractive index as that of glass. Dyes are used to impart colour to the different components of the tissue for the interpretation. A chromogenic dye absorbs the light of particular wavelength of the white light representing a specific colour and emits the light containing the rest of the colour. Therefore we see a coloured light from the dye that helps in imparting contrast to the substance which is a subject of study from their background.

The dye can be classified on the basis of electrical charge or chemical structure. The staining is the combination of a coloured dye with the tissue that retains the dye after washing. It is primarily a chemical reaction between the dye and the tissue, and the common chemical reactions are electrostatic bond, van der Waals attractions, hydrogen bond, covalent bond, hydrophobic bond and dye aggregations.

Dyes are roughly divided into acidic dyes and basic dyes. Basic dyes stain acidic components, and acidic dyes stain mainly basic components. The method used by P. Ehrlich in 1877 for using mixtures of acidic and basic dyes was an important milestone in the development of staining techniques. However, dyes can be classified as follows;

[A] On the basis of chemical structure, organic dyes can be divided into:

- **Azo dyes** (the greatest group), e.g. acid and basic azo dyes,
- **Nitro and nitroso dyes,**
- **Chinone dyes**, e.g. benzochinones, naphthachinones,
- **Di- and triphenylmethan dyes**,
- **Xanthene dyes** with the subdivisions of pyronines and phthaleines,
- **Acridine dyes,**
- **Azine dyes,**
- **Oxazine dyes,**
- **Thiazine dyes,**
- **Vat dyes.**

[B] The practical classification of dyes relies on their coloring effects:

- **Basic dyes**, e.g. methyl green, safranine and fuchsine,
- **Acidic dyes**, e.g. eosine, acid fuchsine and some anthrachinone dyes,
- **Substantive dyes**, e.g. benzopurpurine and Congo red,
- **Mordant dyes**, e.g. haematoxylin and carmine,
- **Developing dyes**, e.g. aniline black, naphthol red, Echtrot,
- **Vat dyes**, e.g. indigo and indanthrene dyes,
- **Sulfur dyes**, e.g. vidal black and pyrogene blue. Other dyes are mainly of industrial interest and not for microscopy.

Histological staining is usually done by staining of cut sections in asmuch as a dye in solution is offered to bind to defined tissue structures. Progressive and regressive techniques can be differentiated including direct and indirect procedures. One can operate with mixture of dyes simulateously or successively in order to discriminate different tissue textures by the respective dyes used. So, double, triple and multiple stainings can be achieved. Multiple staining in its proper sense is obtained when cell nuclei are stained in red color by carmine with concurrent staining of elastic material in dark violet by resorchin fuchsin. Effects of multiple tissue staining can be also obtained by a diffusely staining dye which is superimposed by staining of certain areas by a second dye. Many dyes have a priori only poor affinity to tissues, but this can be overcome by the use of metal salts. Those enhancing compounds are then called “mordants”. Their mechanism of action is not yet clear, but it seems that modants have a role in coordination bonding between the metal and the dye as well as in further coordination between that dye complex and tissue structures.

Dyes have some difference with stains. Dye is used to stain in single colour, whereas a stain is a combination of dyes used to stain a slide simultaneously with many colour. The difference between both of these is following;

Difference between dyes and stain	
Dyes	**Stain**
A single chemical reagent contained in a stain	A mixture of selecte dyes to colour a particular biological specimen
A chemical reagent	A mixture of dyes
Only highlights a specific components of the tissue in one colour	Highlights different components in different colours
To highlight a specific component within a tissue	To give a contrast to the tissue
Examples are methyl green, Pyronin G, Aninline blue, orange G	Examples are haematoxylin & eocin, toluidine blue, Masson’s trichrome stain, Wright’s stain

These dyes or stain have several affinities with the tissue as described above. Moreover, dye-to-dye interaction is also taking place in case of stain and multiple staining that can be understand in table given below.

Factors contributing to dye tissue affinities	
Interactions	**Example**
Solvent – solvent interaction	
Hydrophobic bonding	Staining systems using aq. Solution of dyes or other organic reagent, e.g. enzyme substrate
Stain – stain interaction	Metachromatic staining with basic dyes
Reagents – tissue interaction	
Coulombic attraction	Acid & basic dyes
Van der Waal's forces	Elastic fibre stain
Hydrogen bonding	Staining of polysaccharide
Covalent bonding	PAS staining
Coulombic attraction – force of attraction between positive and negative charges. *Van der Waal's force – weak electrostatic attraction between uncharged molecules*	

Similar to the dyes, stains can also be classified, which are as follows;

[A] According to their chemical composition;

- Organic stains: Examples are haematoxylin stains, carmine stains, aninlin stains
- Inorganic Stains

[B] According to pH value;

- Basic stains
- Acidic stains
- Neutral

[C] Histological stains are;

- Nuclear stain (Chromatic stains)
- Plasma or general stains
- Special stains
- Impregnations

General theory of staining of histological specimen states that as there are no methods to demonstrate all tissue material and as sometimes material may need a number of staining methods. Primary purpose of staining is the rendering of outlines and structures more distinct by giving them a colour contrast with their surrounding or background as discussed above. One more important use of staining is for the differentiation of particular structures or substances which by their selective staining facilitate the histological as well as histochemical analysis. Up

to the date, there are about hundreds of staining methods to be practiced in histological study and histochemistry, and this number is continuously growing. Staining method is followed after sectioning and spreading of paraffinized tissue block which has been previously. Some of the important staining methods are;

Perl's Reaction

This method is particularly important for the detection of ion levels (ferric ions). Because it can help detect the presence of ferric ions, this technique is used to determine the level of these ions in such organs as the spleen and bone marrow. It can be used to tell whether there are excessive amounts of the ion as observed in hemochromatosis (excessive levels of ferric ions with deposits in the liver and pancreas) or hemosiderosis where deposits can be found in the liver, spleen and the lymph nodes. In plants, this technique has also been shown to help understand the homeostasis of ferric ions.

Principle

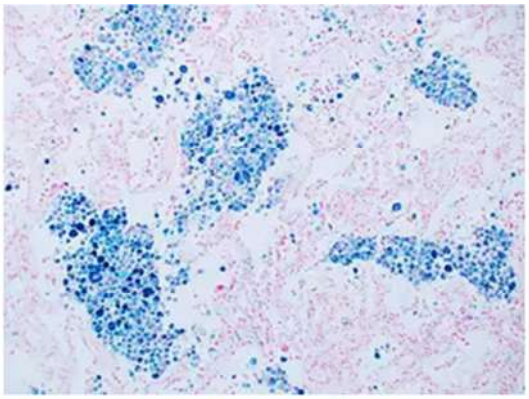
With this technique, ferric ions present in the tissue will combine with ferrocyanide resulting in the formation of a pigment called Prussian blue (ferric ferrocyanide). In plants, the technique is also based on the conversion of ferrocyanide into insoluble crystals (Prussian blue) in the presence of Ferric ions under acidic conditions. Prussian blue (resulting from the reaction) is bright blue in color, which indicates the presence of ferric ions.

Staining procedure

In Perl's reaction technique, a known positive control tissue is used as control while 10 percent formalin is used as a fixative.

Requirements

- Microwave oven
- Acid cleaned glassware
- Non-metallic forceps
- 5 percent potassium ferrocyanide
- 5 percent hydrochloric acid
- Sample section (cut to about 4µ)



Prussian blue staining of pulmonary section

Procedure

- Deparaffinize the section(s) and hydrate with distilled water
- Microwave for about 30 seconds and allow the specimen to stand in the working solution for about 5 minutes in the fume hood. The section can be treated with the working solution (acid ferrocyanide) for between 10 and 30 minutes.
- Rinse/wash the section using distilled water

- Stain (lightly) the section with 0.5% aqueous neutral red or 0.1% nuclear fast red. This part of the procedure is used to stain the nuclei.
- Rapidly wash the section using distilled water.
- Dehydrate the section, clear and mount on the microscope stage for viewing.

When viewed under the microscope, blue parts are indicative of iron while the red and pink parts indicate the nuclei and background respectively. For this staining method, it is very important to use gloves, goggles and a lab coat due to hazardous nature of some chemicals that may cause irritation.

Von Kossa Techniques

This is also an ion based technique that is used in histochemistry. It is a more sensitive technique that can be used to identify the presence of calcium deposits on cyst fluids, ductal ectasia and papillomatosis. However, excessive amount of calcium may be found in any given part of the body and can be demonstrated using the Von Kossa technique.

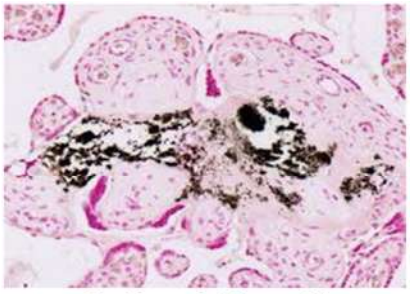
Although this technique is used to demonstrate the presence of calcium, it demonstrates an anion rather than the calcium ion itself.

Principle

For this technique, the sample section is treated with the solution of silver nitrate and is reduced and the calcium (if present in the sample) is reduced by the strong light and replaced with deposits of silver. As a result, it is visualized as metallic silver.

Requirements for Von Kossa technique

- A control sample (a tissue that contains known calcium deposits or undecalcified bone)
- 10 percent formalin (fixative)
- Glassware (clean)
- 60 watt lamp
- A mirror or a foil
- 5 percent of silver nitrate solution
- 5 percent hypo (sodium thiosulfate)


Von Kossa Calcium staining

Procedure

- Deparaffinize and hydrate the section using distilled water.
- Place the section in the silver solution (in a glass jar) and place it in bright light (or in front of the 60 watt lamp). Place a mirror or a paper foil behind the jar so as to reflect the light. Leave it standing for about one hour or until the calcium turns black.
- Rinse the section in distilled water.
- Stain using 5 percent hypo solution for about 5 minutes.
- Wash the section using tap water or rinse in distilled water.
- Introduce the sample to nuclear-fast Red for about 5 minutes.
- Wash using distilled water.
- Dehydrate and mount for viewing.

Observation

A black color indicates the presence of calcium (calcium salts), red indicates the nuclei while the cytoplasm will appear pink.

Lipids Staining

This technique is dependent on dyes that are soluble in lipids. Some of the most common lipid soluble dyes used in lipid staining include:

- Sudan VI
- Sudan black
- Oil Red O
- Nile blue

Lipid staining is a useful technique that is used for demonstrating intracellular lipids in various tissue sections.

Principle

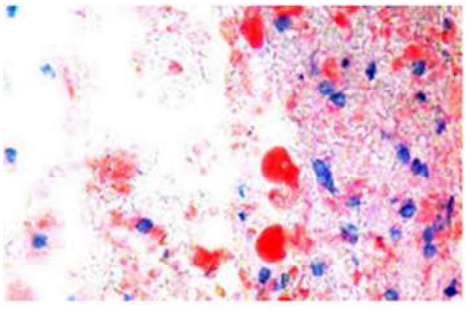
For this technique, the dye is more soluble in the lipid, which allows it to be more demonstrated than in the vehicular solvent. The dyes used in this technique are all interchangeable, which means that they can be substituted for each other for the staining process.

Requirements/reagents

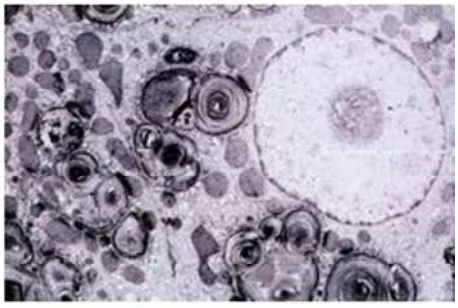
- ORO (Oil Red O) solution
- Glycerine jelly mounting medium

Procedure

- Cut the sample to obtain sections of between 8 and 10 microns and air dry.
- Rinse the section with 60 percent isopropanol.
- Stain the section with the Oil Red O working solution for about 15 minutes.
- Rinse the specimen with 60 percent isopropanol.
- Dip the section in Alum hematoxylin a few times in order to stain the nuclei.
- Rinse in distilled water.
- Mount the specimen in water or in glycerin jelly.



Oil Red O Lipid Staining



Sudan black Lipid Staining

Observation

Red color indicates the lipid while blue coloration indicates the nuclei. Lipid staining technique is useful for showing the normal distribution of lipids as well as disease-related lipid accumulation. Preferably, Oil Red O and Sudan stain are used to stain lipid in tissue specimen for histochemical analysis.

For Proteins and Amino Acids, some of specialized methods are used which are as follows;

- ***Millon's reaction***
- ***Sakaguchi reaction***
- ***Tetrazotized benzidine reaction***

Millon's reaction

This reaction is not widely used for histochemical localization of protein molecules especially those which contains amino acid tyrosine or histochemical analysis of tyrosine in the protein. Because, it is not specific for proteins; it also gives a positive test for other compounds containing the phenol functional group. Therefore, the biuret test or the ninhydrin reaction is used along with it to confirm the presence of proteins.

Principle

In this technique, the mercurous and mercuric nitrate (components of the reagent) reacts with hydroxybenzene radicals to form a compound that is red in color. Tyrosine contains the phenolic group, which forms the red coloration in the presence of Millon's reagent. The compound formed through this reaction is called mercuric fumarate.

Millon's reagent

It is prepared by dissolving mercuric nitrate in nitric acid, and then adding water to dilute it. The reagent is used in Millon reaction test wherein few drops of the reagent are added to the test solution as is described in procedure.

Procedure

- Add about 2ml of the protein solution into a test-tube.
- Add a few drops of the reagent (Millon's reagent) in to the test-tube that contains the original solution.
- Hold the test-tube over boiling water or flame using a tube holder and boil for about half a minute.

Observation

If the solution turns reddish in color after boiling, then tyrosine is present in the solution.

Sakaguchi Test

The Sakaguchi reaction test involves the use of the Sakaguchi reagent. This reagent is composed of 1-naphthol and sodium hypobromite and forms a reddish compound when mixed with the sample containing arginine.

The test is positive for any amino acid that contains the guanidine group in Arginine. Therefore, the Guanidine group in an amino acid will react with the α-Naphthol and alkaline hypobromite in the reagent to give of a red-colored complex indicating the presence of such amino-acids.

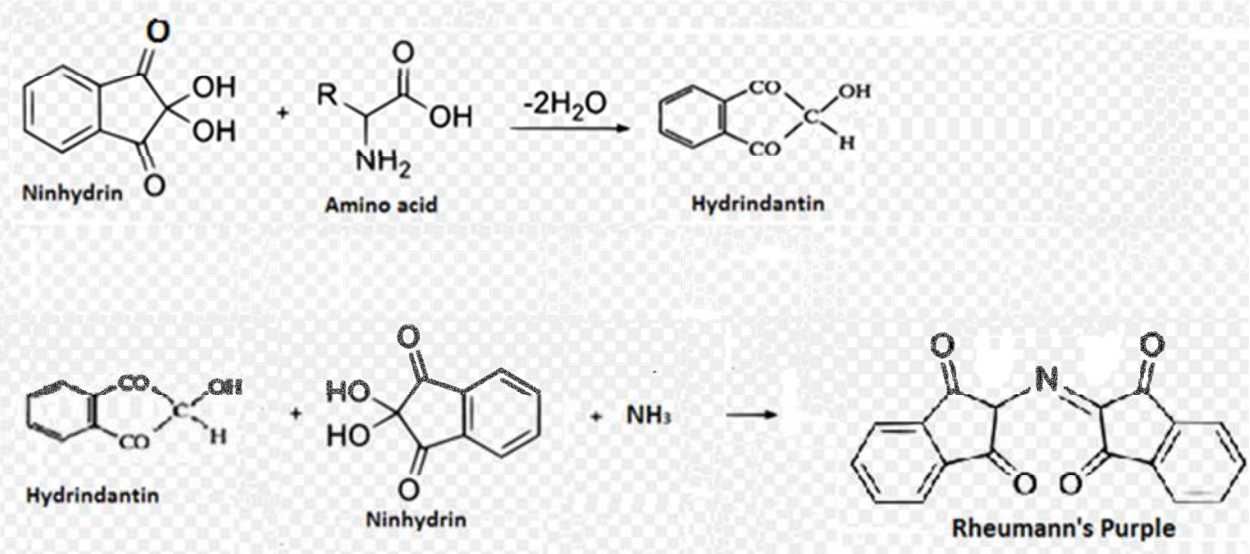
Procedure

- Add 1 ml of the protein solution in to a test-tube.
- Add 2 or 3 drops of 40 percent of sodium hydroxide, 2 drops of ethanolic a-Naphthol and 5 drops of bromide water.
- Mix the contents in the test-tube well.
- If a red-color complex forms when shaking the contents, then it is positive that the solution contains Arginine or a protein containing Arginine.

Ninhydrin reaction

Principle

This test is a general test and thus given by all amino acids. This test is due to a reaction between a amino group of free amino acid and ninhydrin. Ninhydrin is a powerful oxidizing agent and its presence, amino acid undergo oxidative deamination liberating ammonia, CO2, a corresponding aldehyde and reduced form of ninhydrin (hydrindantin). The NH3 formed from a amino group reacts with another molecule of ninhydrin and is reduced product (hydrindatin) to give a blue substance diketohydrin (Ruhemanns complex). However, in case of amino acid like proline and hydroxyproline, a different product having a bright yellow color is formed. Asparagine, which has a free amide group, reacts to give a brown colored product.



Requirements

- Protein solution test solution
- 2 % ninhydrin in acetone
- Water bath
- Dry test tubes
- Pipettes

i. Preparation of 2% ninhydrin solution:

- weigh 0.2 gm of Ninhydrin and dissolve in 10ml of acetone or ethanol

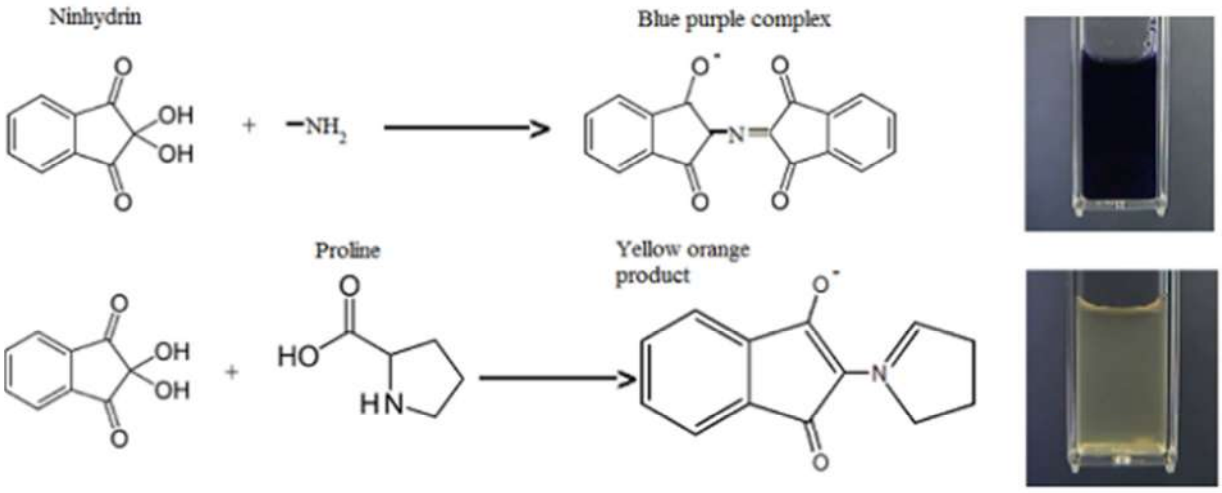
ii. Preparation of test solution:

- Prepare 1% amino acid solution in distilled water.

Procedure

- Take 1 ml test solution in dry test tube and 1 ml distilled water in another tube as a control.
- Pour few drops of 2% ninhydrin in both the test tubes.
- Keep the test tubes in water bath for 5 minutes.
- Look for the development of blue or violet color.

**But in the case of proline and hydroxyproline, yellow color will develop instead of blue color. Similarly, asparagine will give brown color as shown in figure below.



Biuret Test

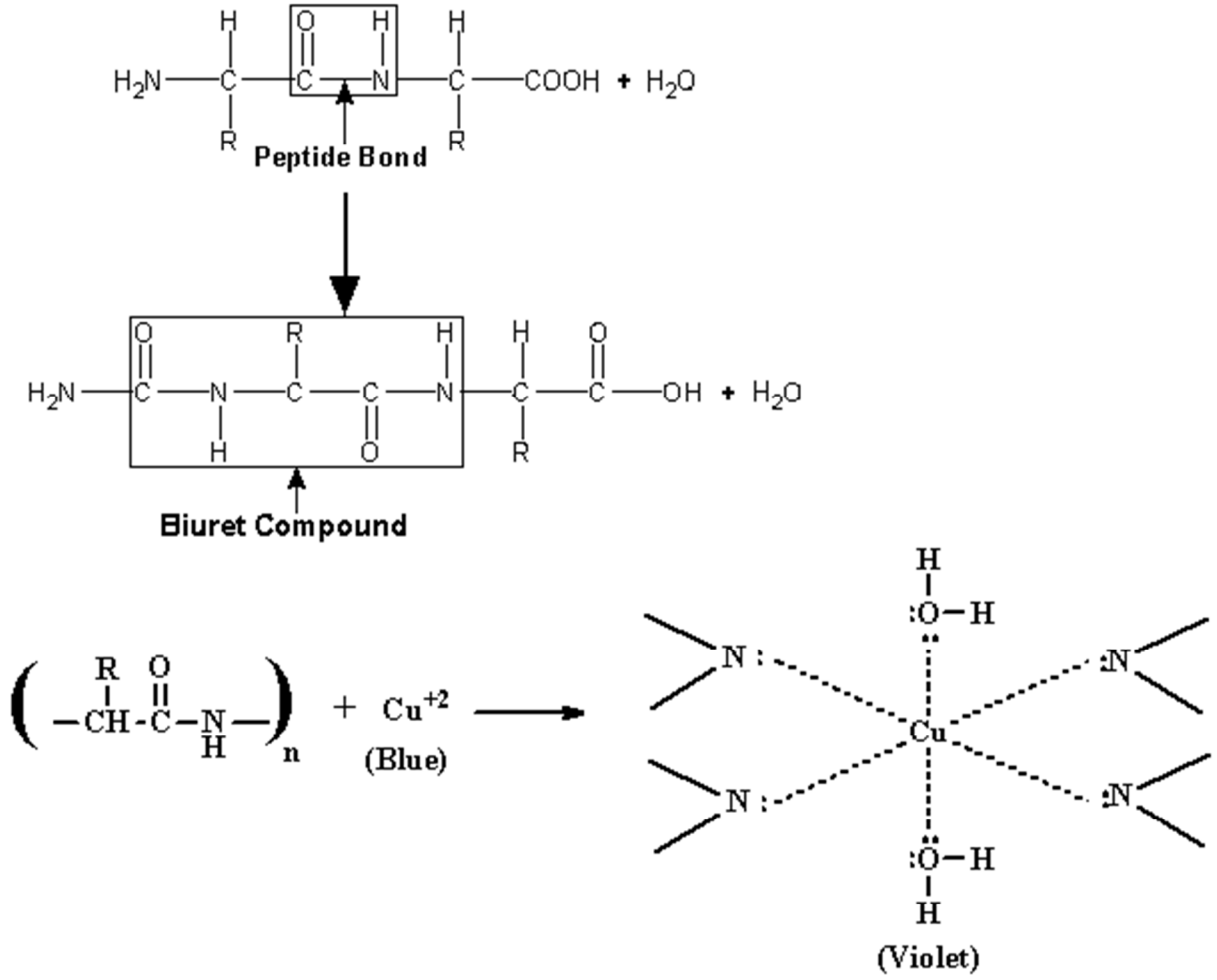
Principle

Biuret test is a general test for compounds having a **peptide** bond. Biuret is a compound formed by heating urea to 180° C. When biuret is treated with dilute copper sulfate in alkaline condition, a purple colored compound is formed. This is the basis of biuret test widely used for identification of proteins and amino acids.

This test is given by compounds containing two or more peptide bond (CO-NH group). Since all proteins and peptides possessing at least two peptide linkage i.e. tripeptide, it gives positive biuret test.

The principle of biuret test is conveniently used to detect the presence of proteins in biological fluids.

Alkaline CuSO4 reacts with compounds containing two or more peptide bonds to give a violet colored product which is due to formation of co-ordination complex of cupric ions with un-shared electron pairs of peptide nitrogen and O2 of water.



Requirements

- 1 % alanine, 5 % egg white (albumin)
- Biuret reagent
- Water bath
- Dry test tubes

- Pipettes

Biuret reagents

- Copper sulfate (CuSO40
- Sodium hydroxide (NaOH)
- Sodium potassium tartarate (commonly known as Rochelle salt)

Preparation Biuret reagent

Biuret reagent is prepared by adding NaOH in CuSO4 solution, making it alkaline.

- **To prepare 1000ml of Biuret reagent**
  - Take 1.5 gram of pentavalent copper sulphate (CuSO4) and 6 gram of Sodium
  - Potassium tartarate and dissolve them in 500 ml of distilled water
  - Sodium potassium tartarate is a chelating agent and it stabilize the copper ion
  - Take 375 ml of 2 molar Sodium hydroxide
  - Mix both the solution in volumetric flask and make it final volume to 1000 ml by adding distilled water.

Procedure

- Take 1 ml of test solutions in dry test tubes and in another tube take 1 ml distilled water as control.
- Add 1 ml of biuret reagent to all test tubes, mix well.
- Look for the development of blue colors

Result

Colour of solution changes to purple. All peptides and proteins give the test positive. Histidine is the only amino acid that gives positive test. However, magnesium and ammonium ions present in the solution interfere in reaction which can be overcome by adding little excess alkali.

Tetrazotized Benzidine Reaction

Although it has been shown to be less effective, Tetrazotized benzidine is used in histochemistry to detect non-collagen proteins. The procedure involves the coupling of Tetrazotized benzidine with beta naphthol or Hyaluronic acid.

The method, commonly referred to as Tetrazotized benzidine helps in the detection of such non-collagen proteins as tyrosine, histidine as well as tryptophan.

Staining of nuceic acid such as DNA is achieved by doing Feulgon's reaction, which is mentioned below.

Feulgon's Reaction

This is a relatively new technique that is used for demonstrating DNA in tissue sections. It is a sensitive means of detecting aldehydes, which makes it the ideal method for detecting the presence of DNA. Here, the section is treated with dilute hydrochloric acid in order to remove the bases.

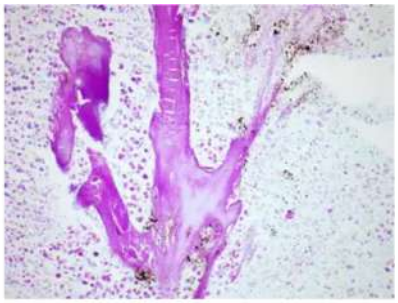
The sugar part that remains reacts as an aldehyde ultimately forming a visible color. Therefore, this method can be said to be divided in to two main parts:

1 – The first part of the procedure is the hydrolysis phase that involves the use of 5N HCl, ambient temperature for 40 minutes. This step is aimed at separately selecting 2 purine bases (adenine and guanine) which are removed from the DNA molecule.

2 – The second step is the staining phase. The reagent used is preferred because it is highly selective for DNA rather than RNA. Here, RNA does not react because of the presence of hydroxyl on carbon 2 of ribose, which prevents the acid (HCl) from hydrolyzing sugar. The reaction is also precise for the localization of DNA given that deoxyribose radicals are bound to phosphoric acid of the apurinic acid molecule following the removal of purine bases.

Some of the stains used for both DNA and RNA include:

- Methyl green pyronin stain
- Acridine orange



Staining of Saccharides is achieved by doing Periodic Acid Schiff Reaction, which is mentioned below.

Periodic Acid Schiff's Reaction (PAS Reaction)

This is one of the most popular histochemical techniques for the detection of glycogen. It has been shown to be one of the best techniques for demonstrating carbohydrates in tissue. In this technique, the periodic acid oxidizes tissue carbohydrates to produce aldehyde groups. This group then condenses with the reagent to form a bright red coloration to demonstrate the tissue component with carbohydrate attachments.

The diastase and a-amylase in the reagent act on the glycogen and depolymerize it into smaller sugar units (maltose and glucose) which are then washed out of the section.

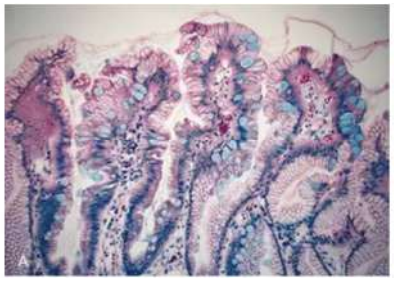
Procedure

- Deparaffinize and hydrate the slide using distilled water.

- Place the section in preheated diastate solution (at 37 degrees centigrade) for about an hour.
- Wash the sample in running water for about 5 minutes.
- Place the sections in 0.5 percent periodic acid solution for about 5 minutes.
- Wash the section in distilled water.
- Place the section in Schiff reagent for about 15 minutes.
- Wash the section for about a minute in 0.55 percent potassium metabisulfite in order to remove excess stains.
- Wash in running tap water for about 10 minutes.
- Counterstain using Harris's hematoxylin with acetic acid for half a minute.
- Wash with running water.
- Dehydrate with two changes using absolute alcohol, clear with xylene and mount to view.

Some of the other stains used for staining sacchrides include:

- Lectins
- Ruthenium red
- Alcian blue



New staining techniques continue to be introduced, and older ones continue to be used and improved. Several factors control specificity, selectivity and visibility of the end product in any procedure using dyes, fluorochromes, inorganic reagents or histochemical reactions applied to sections or similar preparations. Local concentration of the tissue target often determines the intensity of the observed color, as does the fine structure within the object being stained, which may facilitate or impede diffusion of dyes and other reagents. Several contributions to affinity control the specificity of staining. These include electrical forces, which result in accumulation of dye ions in regions of oppositely charged tissue polyions. Weaker short-range attractions (hydrogen bonding, van der Waals forces or hydrophobic bonding, depending on the solvent) hold dyes ions and histochemical end products in contact with their macromolecular substrates. Nonionic forces can also increase visibility of stained sites by causing aggregation of dye molecules. Covalent bonds between dye and tissue result in the strongest binding, such as in methods using Schiff's reagent and possibly also some mordant dyes. The rate at which a reagent gains access to or is removed from targets in a section or other specimen affect what is stained, especially when more than one dye is used, together or sequentially. Rate-controlled staining is greatly influenced by the presence and type of embedding medium, such as a resin, that infiltrates the tissue. The rates of chemical reactions are major determinants of outcome in many histochemical techniques. Selective staining of different organelles within living cells is accomplished mainly with fluorochromes and is controlled by mechanisms different from those that apply to fixed tissues. Quantitative structure-activity relations (QSAR) of such reagents can be derived from such molecular properties as hydrophilic-hydrophobic balance, extent of conjugated bond systems, acid-base properties and ionic charge. The QSAR correlates with

staining of endoplasmic reticulum, lysosomes, mitochondria, DNA, or the plasma membranes of living cells.

Despite of utmost care take in performing histological study and/or histochemical analysis, many errors has been observed. Common errors during the histology laboratory procedure are listed below.

Common errors in histology laboratory practices

Receipt of	Data entry	Tissue	Tissue	Embedding	Microtomy	Staining	Mounting
No tissue received in specimen container	Sample and request form allocated wrong number	Request form and specimen match not performed	Tissue not sufficiently fixed prior to processing	Tissue contaminated during embedding	Tissue lost during section cutting	Oven drying time of slides inadequate	Loss of tissue from slide during mounting
Specimen received	Sample and request form	Specimen containers	Tissue processing	Samples mixed up	Identification mix-up of	Staining reagents in	Slides incorrectly labelled
Number of samples not consistent with request form	Data entered incorrectly into LIMS	Slide numbering sequence incorrect	Tissue processing incomplete	Tissue lost during embedding	Paraffin sections floated onto wrong side or slide	Sections detached from slide during staining	Quality control not performed on stained sections
Incomplete or incorrectly labelled		Tissue lost or contaminated during	Incorrect programme selected		Paraffin section mounted	Sections contaminated during	Slides and request forms wrongly

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