Ministry of Higher Education and Scientific Research
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Histological and Cytological Technique

Theoretical

For

Students of first class

Department of Medical Laboratory

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Syllabus for histological and cytological techniques

Definition of some terminology that deals with the subject.	1
Sample collection, fixation, effects of fixation on tissue.	2
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lecture (1+2)

Terminology:

Cell: The building unit of the living body. It consists of nucleus surrounded by cytoplasm & both are enclosed by a membrane.

Cytology: Is the study of cells, their origin. structure, function, and pathology

Histology: The study of the microscopic anatomy of cells and tissues of animals and plants. It is an essential tool of biology and medicine.

Pathology : the science which deals with diseases.

Cytopathology: Specialty of pathology on a cellular level with a focus on the diagnosis of diseases through specimens derived from fluids or smears.

Pathologist : A physician specializing in rendering medical diagnoses by examination of tissues and fluids removed from the body.

Histopathology: The microscopic study of diseased tissue, it is an important tool in anatomical pathology. since accurate diagnosis of cancer and other disease usually requires histopathological examination of samples.

Histotechnician: Trained scientists who perform the preparation of histological

Processing of tissue by paraffin method (paraffin method)

Steps of paraffin method:

1. Samples collection
2. Fixation
3. Washing
4. Dehydration
5. Clearing
6. Infiltration
7. Blocking and embedding (casting)
8. Trimming
9. Sectioning (microtomy)
10. Mounting
11. Staining
12. Mounting with Canada balsam and cover slide

1.Specimen collection:

Tissue specimen that collected for study is of two kinds:

a) Biopsy: Cells or tissues removed from the living body for examination.

b)Autopsy: Evaluation of a body to determine the cause of death.

2- Fixation :

This process is the most important step in the processing of tissue samples, it preserve cells and tissue components with minimal distortion .

It stabilizes proteins, rendering cells &its components resistance to further autolysis by Inactive lysosomal enzymes ,and changes the tissue response to further processing .

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Characteristics of good fixative :

1- The fixative must have the ability to prevent short –and long term destruction of the micro-architecture of the tissue by stopping the activity of catabolic enzymes and hence autolysis, minimizing the diffusion of soluble molecules from their original locations.

2-Agood fixative is characterized by the destruction of infectious agent which helps maintain tissue and cellular integrity.

3-it is useful for a wide variety of tissues including fatty lymphoid and neutral tissues.

4-it should preserve large and small specimens and support histochemical, Immunohistochemical.

5-it should penetrate &fix tissue rapidly and should be low cost.

How to fix tissue:-

1. Small blocks of tissue must be used (2 cm square) and not more than 4-5 mm thick.
2. Tissue specimen must be fixed immediately after removal, and if it is not possible they should be frozen to retard decomposition and autolysis.
3. Fixative volume should be 10-20 times volumes of tissues.
4. Fixation time depends on:
   a. Size and density of tissue
   b. Rate of penetration of fixative.
   c. Room temperature.
5. The tissue could be fixed in 2 or more fixatives for special studies.

Effects of fixation on tissues :

1- denatures proteins to make them in soluble .

2- The tissue become to the resistant the effects of the following steps.

3- The tissue will be more permeable to fluid (dead tissue).

4. It should preserve large & small specimens and support histochemical,

1. The fixative must have the ability to prevent short- and long- term destruction of

4-The tissue will be more acidic or basic , so staining will be strongly influenced.

5-Some fixative inhibitor interfere with dye reaction or acts as mordant to enhance staining result.

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Functions of fixation:

1-inhibit or stop autolysis and bacterial decomposition .

2-to coagulate and harden tissue.

3-To fortify tissue against the harmful effect of tissue processing e.g. dehydration, embedding…..

4-to improve the optical differentiation of tissues.

5-To make easily staining process of tissue.

Lecture (3-4)

Methods of fixation:

Fixation of tissues can be accomplished by physical or chemical methods.

I/ **Physical methods**

l. **Heating**: such as boiling egg, frozen section, or warming a slide with water to fix section

2. **Microwaving**: it accelerate fixation of gross specimen and tissue sections in a range of 20 min up to 12 hours.

3. **Freeze-drying:** lt is useful technique in studying soluble materials and small molecules ,tissues are cut in sections immersed in liquid nitrogen and water is removed in a vacuum chamber at 40c.

Ill / Chemical methods

Chemical fixation utilizes organic and non-organic solutions to maintain adequate morphological preservation. These are:

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A/ Simple fixative:

1. Coagulant fixatives:

Both organic and non-organic fixative s may coagulate protein s making them insoluble . Coagulating lipoprotein and fibrous proteins such as glycogen will maintain tissue . histomorphology at light microscope level. The most commonly used coagulating fixatives are alcohol (e.g. ethanol, methanol) and acetone.

2. Non-coagulant cross-linking fixatives:

Chemicals used as fixatives have potential action of forming cross-links within and between proteins and nucleic acids as well as between nucleic acids and proteins. Formaldehyde is one ofthese chemicals(aldehyde).

3. Cross-linking fixatives:

Cell organelles such as cytoplasmic and nuclear membranes, mitochondria, membrane-bound secretory granules, and smooth & rough endoplasmic reticulum need to be preserved carefully for electron microscopy. In these structures fixative used must not solubilize lipids. The preferred fixatives are a strong cross-linking fixatives such as glutaraldehyde, a combination of glutaraldehyde & formaldehyde. These fixatives slowly so specimen must be thin.

B/ Compound fixatives :

Pathologists use formaldehyde-based fixatives and other agents may be added to formaldehyde to produce specific effects .Ethanol can be added to formaldehyde to produce alcoholic formalin which preserve molecules such glycogen , produces less shrinkage and hardening.

Compound fixatives are useful for specific tissues:

l. Alcoholic formalin is good fixative for fatty tissue.

2_ = = may aid identifying lymph node embedded in fat.

3. Alcoholic formalin & some compound fixatives are good in preserving antigen immunorecognition.

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4. Some compound fixatives such as glutaraldehyde- formaldehyde fixation may increase background staining

Example of compound fixatives:

a) Neutral buffered 10% formalin (NBF)

Tap water 900ml
Formalin ……. 100ml
Sodium phosphate, mono basic, monohydrate ..4gm
Diabasic,anhydrous……. 6.5gm

b) Formal (10% formalin) saline

Tap water 900ml

Formaldehyde (37%) 100ml

Sodium chloride …….. 9 gm

c) Formalin, buffered saline

Tap water 900ml

Formaldehyde (37%) 100ml

Sodium chloride 9gm

Sodium phosphate, dibasic. l2gm

d)Formal (10% formalin), calcium acetate

Tap water 900ml

Formaldehyde (37%) 100ml

Calcium acetate 20gm

- Good fixative for preservation of lipids.

e) Formal (10% formalin), zinc un-buffered

Tap water 900ml

Formaldehyde (37%) 100ml

Sodium chloride 4.5gm

Zinc chloride or (zinc sulfate).....l.6gm or (3.6gm)

- An excellent fixative, for immunohistochemistry studies.

Lecture(5+6)

(2)Mercuric fixatives :

A problem with fixatives containing mercury, is that several types of pigments may combine with the mercury and precipitate as black pigments which could be removed by using iodine treatment followed by sodium thiosulfate.

a) Zinker ‘s solution

Distilled water 250ml

Vlercuric chloride l2.5gm

Potassium dichromate 6.3gm

Sodium sulfate 2.5gm

- Just before use add 5ml of glacial acetic acid to 95ml of the above solution. It is good fixative for bloody (congested) specimens and trichrome stains.

b)Helly's solution

Distilled water 250ml

Potassium dichromate 6.3gm

Mercuric chloride l2.5gm

Sodium sulfate 2.5gm

- Just before use add 5ml of 37% formaldehyde to 95ml of the above solution. It is excellent for bone marrow

C) B5 fixative

Stock solution:

Mercuric chloride	12gm
Sodium acetate	2.5gm
Distilled water	200ml

- Add 2ml of formaldehyde (37%) to 20ml of the above solution just before use.

It is used for bone marrow, lymph nodes, spleen and other hematopoietic tissue.

(3) Dichromate fixatives :

1. It is good for making proteins insoluble without coagulation at pH of 3.5
2. It makes unsaturated lipids insoluble upon prolonged fixation (more than 48hr).
3. It preserves mitochondria well.
4. It is used in fixing endocrine tissues which will be stained, especially normal adrenal medulla and its tumors.
5. Time of fixation is 24hr.; washing is done with water then specimens transferred to 70% ethanol

(a)Miller's solution

Potassium dichromate	2.5gm
Sodium sulfate	l gm
Distilled water	100ml

(b)Moller's solution

Potassium dichromate	3gm
Distilled water	80ml

- At time of use add 20ml of 37% formaldehyde.

(4)Dehydrant fixatives :

100% ethanol, 95%ethanol, 70%ethanol, these solutions may cause Excessive shrinkage of tissue components after more than 3-4hr of fixation. These fixatives could be modified by adding some chemicals to produce specific effects. Methanol is useful for smears especially blood smears. Acetone fixation should be short (lhr) at 4C only on small specimens. It causes shrinkage and hardening; it is useful for immunuhistochemistry, enzyme studies, and in the detection of rabies.

(a) Carnoy's fixative

Useful in:

1. Cytology to clear bloody specimen.
2. Staining of RNA
3. Preservation ofglycogen.

(b) Clarke's solution

Absolute ethanol	60m]
Glacial acetic acid	29ml

(5) Dehydration gross-linkage fixatives :

Compound fixatives with both dehydrant and cross-linking include alcohol-formalin mixture. These produce excellent results the immunohistochemical identification of specific antigen, also alcohol-formalin fixation or post fixation is advantageous in large specimen fat (breast). Lymph nodes can be detected much more easily in specimens with alcohol-formalin fixation due to the extraction of lipids texture differences compared with tissue fixed in NBF.

For post-fixation, Carson (1990) recommends the following formula:

Absolute ethanol	650 ml
Distilled water	250 ml
Formaldehyde	100 ml

Other formulas are used for better fixation, these are:

(a)Alcoholic formalin

Ethanol (95%)	895ml
Formaldehyde (37%)	105ml

(b)Alcoholic-formalin-acetic acid fixative

Ethanol (95%)	85ml
Formaldehyde (37%)	10ml
Glacial acetic acid	5ml

(c)Alcoholic Bouin's (Gendre's solution)

95%ethanol saturated with picric acid	
(5gm per 100ml)	800ml
Formaldehyde (37%)	150ml
Glacial acetic acid	50ml

1. This solution is less aqueous than Bouin's fixative.
2. It is good for some carbohydrate retention (glycogen)
3. Fixation should be between 4hr to overnight.
4. Washing with 70% ethanol followed by 95% ethanol (sevsral changes).
5. It is the only fixative that improves with aging.

Fixation and decalcification

(a) Bouin's decalcifying solution:

Saturated aqueous solution of picric acid

(l0.5gm per 100ml)	500ml
Formaldehyde(37%)	167ml
Formic acid	33ml

(b)Fixation for fatty acids

Bouin's solution	75 ml
95°/oethanol	35 ml

Fixation may be up to 48hr for good sections of lipomas or well differentiated liposarcoma.

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Lecture(7+8)

3.Washing Tissues after fixation

It is the removal of fixative by using different solutions, and it depends on the type of the used fixative.

Examples of washing tissue after fixation:

1. Tissues fixed in Helly's or Zinker's fluids should be washed by running tap water (1 -24 hrs) to get rid of the yellow potassium dichromate color.
2. Tissue fixed in fixatives containing formalin should be washed in running tap water for' hr. then transferred to 70% ethanol (formalin moves out of tissue faster).
3. If tissues fixed in Bouin's fluid, it should be washed in several changes of 50% or 70% ethanol to remove the yellow color of the fixative; if some of the yellow color remains after sectioning and mounting, tissue sections must be left for a period of time in 70% ethanol (Hydration) during staining procedure.
4. Tissues fixed in Carnoy's or alcoholic fluid, it should be transferred into absolute alcohol for dehydration.

4.-Dehydration

lt is the removal of unbounded water & aqueous fixatives from tissue. Many dehydrating agents are hydrophilic (water loving), possessing strong polar groups that interact with water molecules in the tissue.

Dehydration should be accomplished slowly through a graded series of reagents of increasing concentrations.

Excessive dehydration may cause the tissue to become hard, brittle, and shrunken .while incomplete dehydration will prohibit the penetration of the clearing agent into the tissue leaving the specimen soft and non-receptive to infiltration.

Types of Dehydration fluids:

I/Alcoholic solutions:

a)Ethanol:

1. It is clear, colorless, inflammable liquid.
2. Miscible with water and other organic solvents.
3. Hydrophilic.
4. Fast acting.
5. It ensures total dehydration by graded concentrations and it may be used for electron microscopy.

b)industrial methylated spirit (denatured ethanol)

lt is used in the same manner as ethanol in dehydration.

c) Methanol

1- Clear, colorless, flammable fluid.
2- high toxic.
3- miscible with water & organic solvents.
4-it can be used instead of ethanol

d) lsopropyl alcohol

1. Miscible with water 8- organic solvents.
2. Does not cause over-hardening or shrinkage of the tissue.

e) Butyl alcohol (butanol)

1. It is used primarily for plant and animal histology

2. Slow dehydrate.

3.causes less shrinkage and hardening of the tissue.

II/ Acetone

1. Clear, colorless, flammable liquid.

2. Miscible with water, alcohol, and organic solvent.

3. Rapid in action, poor penetration. It is used in two ways, either 4 changes of fresh acetone 20min for each change.

4 changes of fresh acetone 30min for each change.

5. Prolonged use causes brittleness in tissue.

6. lt removes lipids from tissue during tissue processing.

lll/ Dioxane (diethylene dioxide)

1. It is an excellent reagent since it could be used as dehydrant and clearing agent.
2. It is readily miscible with water, alcohol, xylene and paraffin.
3. lt produces less shrinkage than alcohol.
4. Tissue can be left in dioxane for long period of time without affecting tissue.
5. After dehydration tissue transferred to melted paraffin for infiltration.
6. It should be used in a well-ventilated room.
7. It is recommended for processing soft tissues due to their hardening properties.

Lecture(9-10)

5.Clearing

This term is related to the appearance of the tissues after they have been treated by special fluids chosen to remove the dehydrating agent; these fluids are called (clearing agents). Time for clearing tissue is proportional to the thickness of tissue.

Clearing agents:

1-They act as an intermediary between the dehydration & infiltration solutions.

2- Most of these agents are hydrocarbons with refractive indices similar to protein

- At the end of this process the tissue will have a translucent appearance.

Selection of a suitable clearing agent must be based on:

1. Speed of removal of alcohol.
2. Ease of removal by molten embedding medium.
3. Gentleness towards tissue.
4. Flammability.
5. Toxicity.
6. Cost.

- Prolonged treatment with clearing agents causes the tissue to become brittle.

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Some of the clearing agents that used:

1**. Xylene:**

a. The most commonly reagent used for clearing and it is recyclable.

b. Colorless liquid with petroleum odor.

c. Relatively rapid in its displacement of alcohol.

d. Readily miscible with paraffin.

O. Over exposure during processing will cause over hardening

2. Toluene:

a. it is similar to xylene.

b. Does not harden tissue with prolonged treatment.

c. A good clearing agent in which to leave tissue overnight.

3. **chloroform**:

a. slower action than xylene and toluene.

b. It does not make tissue brittle as xylene. liver.

c. lts vapor is dangerous since it affect the liver.

d. It is used in processing specimens of the central nervous system.

4. **Benzene**:

ar a. it is similar to xylene.

b. It is not recommended for use due to its possible

c. It is very rapid in clearing.

5. Cedar wood oil:

a. lt is very penetrating.

b. cause no shrinkage of tissue.

c. For clearing it must be of thin low viscosity quality.

d. tissue may be left immersed in it identify without harm.

e-After clearing tissue must be immersed in xylene ,followed by changes of paraffin to insure removal of oil

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6. Citrus fruit oil-limonene reagents:

a. Limonene reagents are extracted from orange and lemon rids.
b. Non-toxic.
c. Miscible with water.
d. Disadvantages are: 1.The strong pungent odor. 2. Copper or calcium may dissolve.

Notice:

We speak of the clearing in two procedures:
1. In the embedding process, we clear tissue after dehydration.
2. In the mounting procedure, we clear tissue after staining and dehydration.

6-infiltration

After tissues have been thoroughly cleared with a clearing agent, it is necessary to infiltrate the tissue with a supporting medium that holds the cells and intercellular structure in proper relation to each other ,so that they may be cut into thin sections.

infiltrating media also used to support and enclose specimens which are to be subsequently cut into thin sections.

Paraffin wax continues to be the most popular infiltration and embedding medium in the histology laboratories.

Paraffin is solid at normal temperature. The hardness of the paraffin wax used for infiltration is matched to the hardness of the tissue.

Paraffin wax has a wide range of melting points because:

a. it is inexpensive
b.provide quality sections
c.it can be used most routine & special stains.
d. Easily adaptable to a variety of uses.

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Types of paraffin wax:-

A. Soft paraffin wax (low melting point of about 45° - 50° C). It can be used only in cool temperature.

B. Medium (semi-solid) paraffin wax (melting point of 50° "58° C) which is adopted for routine work sometimes.

C. Hard paraffin wax (melting point of 58° ' 62° C) this allow thinner sections to be obtained, provides better sectioning of hard objects.

M4”

The tissue is submerged in melted paraffin wax which replace the clearing agent in the tissue, two or three changes of molten paraffin wax with time not more than 3 hours. Prolonged immersion of tissue in molten paraffin wax causes hardness. Brain and spinal cord need longer time for infiltration due to their compact nature.

Method of heating paraffin wax:

There should be available a thermostatically controlled oven, set at 2 or 3 degrees above the melting point of the wax. lf wax clipping from embedded tissues is to be re-used it is recommended that this wax be filtered. A funnel with No. 1 filter paper may be permanently kept in the wax oven.

7.Embedding (Casting ,Blocking)

During this process the tissue samples are placed into molds along with liquid embedding material which is then hardened. This is achieved by cooling in case of paraffin wax and heating in case of epoxy resins.

Certain precautions should be taken when we embed tissue in paraffin wax:

1. The wax must contain no trace of clearing agent.
2. No dust particles must be present.
3. Immediately after tissue casting, the wax must be rapid cooled to reduce the wax crystal size.

Orientation of tissues

Specimen orientation during embedding is important for the demonstration of proper morphology. lV lost tissues are embedded flat; the margin of embedding medium around the tissue will assure support of the tissue.

Tissues require special orientation are:

1. Tubular structures: arteries, veins, fallopian tubes, and vas deferens; cut in cross-section of the lumen.
2. Skin, intestine, gallbladder, and other epithelial biopsies; cut in a plane at right angles to the surface.
3. Muscle biopsies are cut in both transverse and longitudinal sections.
4. Multiple pieces of tissue oriented side by side with epithelial surface facing in the same direction.

There are instances where different medium is instead of paraffin wax if: the

1. The impregnating medium is not sufficiently hard to support tissue.
2. The tissue may be affected by heat.
3. The use of dehydrate and clearing agents may destroy or distort the tissue or tissue components.
4. The adhesion between the paraffin wax and the tissue is inadequate so the tissue will break away from the wax during sectioning.
5. Large crystals in the paraffin wax.
6. The sections cannot be out thin enough.

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Alternative embedding media are:

a. Water soluble: Easter wax, polyester and microcrystalline wax.
b. Alternative resins: acrylic , Epoxy and urea-formaldehyde
c. Other media: agar. Gelatin and Celloidin.

8-Trimming

lt is the process of removing the excess of paraffin wax around the embedded tissue, to get a block with regular geometrical figure that can be sectioned to have straight paraffin stripe with good tissue sections which will ease mounting and staining.

Lecture (11+12)

9-Microtomy

Microtome is the means by which tissue can be sectioned and attached to a surface for further microscopic examination. The basic instrument used in microtome, is the microtome. Most microtome is performed on paraffin-embedded tissue blocks.

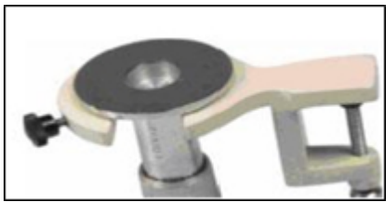
The microtome is nothing more than a knife with a mechanism for advancing a paraffin block standard distances across it.

Vertical sectioning perpendicular to the surface of the tissue is the usual method. Horizontal sectioning is often done in the evaluation of hair follicles and pilosebaceous units.

Types of microtome:-

1. Hand microtome

a. May be used successfully for botanical sections.
b. Its use for animal tissues is limited.



2. **Rocking microtome :** lt is used for soft tissue

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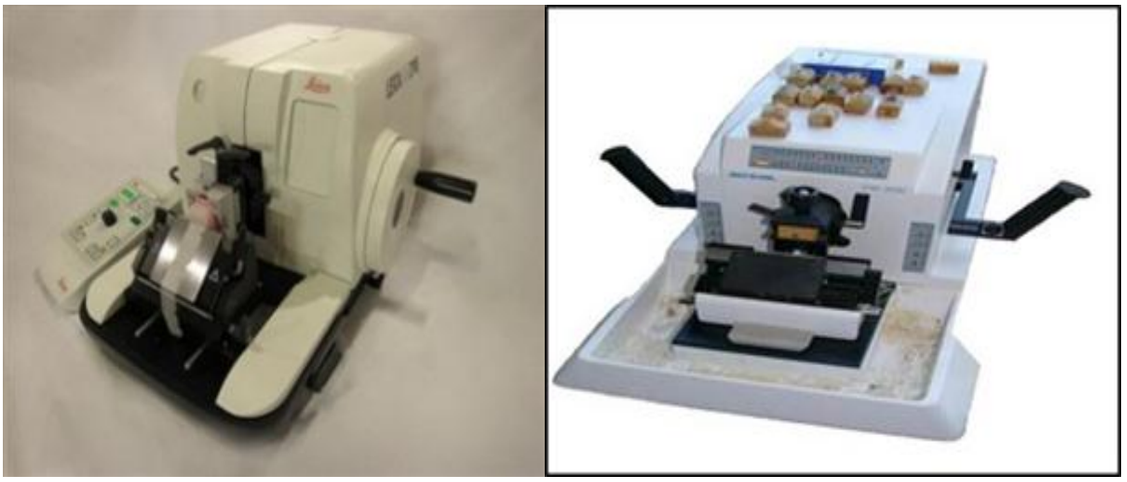
3. Rotary microtome:

a. It is easy adaptable for all types of tissue which are embedded in paraffin wax (hard, fragile , or fatty).

b. Its ability to cut thin (2-3p) sections.



4. Base slede microtome

a. The specimen is held stationary and the knife slide across the top of the specimen during sectioning.

b. lt is used for large blocks.

c. = = = = hard tissues.

d. = = = = whole mounts.

e. It is ideal for the production of celloidin-embedded sections.

f. Especially useful in neuropathology and ophthalmic pathology.

g. Also used in sectioning of un-decalcified bones.

5. Sliding microtome

a. The knife or blade stationary and the specimen slides under it.

b. It is used with celloidin-embedded tissue blocks.

10.Mounting & Adhesives

Once sections are cut, they are floated on a warm water bath that helps remove wrinkles. Then they are picked up on a glass microscopic slide, which is covered by a thin film of adhesive that provides good adhering of the sections.

Types of adhesives:-

1. Albumin:

a. Mostly used in histopathological labs.

b. Used for more adhering of sections.

c. Source of albumin is egg, bovine, or human albumin

d. Preservative should be used to prevent putrefaction.

e. Glycerol is added to albumin to increase viscosity & prevent complete drying.

2. **Gelatin:**

a. Gelatin adhesives provide affirmer attachment of sections than albumin.

b. 0.5% in distilled water with preservative is satisfactory before use it should be heated gently to melt the gelatin and then used.

3. Starch:

a. Starch adhesives provide greater adhesion than gelatin.

b. lts disadvantage is of interfering with many dyes.

c. Since it is carbohydrate, its use isn't recommend when detecting for these substances.

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4. **Cellulose:**

a. 1% methyl cellulose solution found to be a good adhesive.

b. It does not interfere with commonly used dyes.

5. Sodium silicate:

a. Used by diluting the commercial syrup up to 10 (11100)

b. Good adhesive.

c. Tend to stain with most dyes.

6. Resins:

a. A greater adhesion is made by using epoxy resins(araldite).

b. This adhesive, diluted 1in 10 with acetone, it should be painted onto clean slide immediately before use.