Notes

5

FIXATION OF TISSUES

5.1 INTRODUCTION

It is a process by which the cells or tissues are fixed in chemical and partly physical state so that they can withstand subsequent treatment with various reagents, with minimal distortion of morphology and no decomposition.

OBJECTIVES

After reading this lesson, you will be able to:

- state the aims of fixation
- explain the principle of fixation
- describe the properties and factors affecting fixation
- explain types of fixation.

5.2 AIMS OF FIXATION

(a) To preserve the tissues as close to their living state as possible

(b) To prevent autolysis and bacterial attack

(c) To prevent tissues from changing their shape and size during processing

(d) To harden the tissues

(e) To allow clear staining of sections subsequently

(f) To improve the optical differentiation of cells & tissues

5.3 PRINCIPLE OF FIXATION

Fixation results in denaturation and coagulation of protein in the tissues. The fixatives have a property of forming cross links between proteins, thereby forming a gel, keeping everything in their in vivo relation to each other.

5.4 PROPERTIES OF FIXATIVES AND FACTORS AFFECTING FIXATION

1. Coagulation and precipitation of proteins in tissues.
2. Penetration rate differs with different fixatives depending on the molecular weight of the fixative
3. pH of fixatives – Satisfactory fixation occurs between pH 6 and 8. Outside this range, alteration in structure of cell may take place.
4. Temperature – Room temperature is alright for fixation. At high temperature there may be distortion of tissues.
5. Volume changes – Cell volume changes because of the membrane permeability and inhibition of respiration.
6. An ideal fixative should be cheap, nontoxic and non-inflammable. The tissues may be kept in the fixative for a long time.

5.5 TYPE OF FIXATION

- Immersion fixation
- Perfusion fixation
- Vapour fixation
- Coating/Spray fixation
- Freeze drying
- Microwave fixation/Stabilization

The most commonly used technique is simple immersion of tissues/smears in an excess of fixative. For all practical purposes immersion fixatives are most useful. These may be divided into routine and special.

5.6 SIMPLE FIXATIVES

1. **Formaldehyde**: Commercially available solution contains 35%-40% gas by weight, called as formalin. Formaldehyde is commonly used as 4% solution, giving 10% formalin for tissue fixation. Formalin is most commonly used fixative. It is cheap, penetrates rapidly and does not over- harden the tissues. The primary action of formalin is to form additive compounds with proteins without precipitation. Formalin brings about fixation by converting the free amine groups to methylene derivatives.

   If formalin is kept standing for a long time, a large amount of formic acid is formed due to oxidation of formaldehyde and this tends to form artefact which is seen as brown pigment in the tissues. To avoid this buffered formalin is used.

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2. **Absolute alcohol** – it may be used as a fixative as it coagulates protein. Due to its dehydrating property it removes water too fast from the tissues and produces shrinkage of cells and distortion of morphology. It penetrates slowly and over-hardens the tissues.
3. **Acetone** – Sometimes it is used for the study of enzymes especially phosphatases and lipases. Disadvantages are the same as of alcohol.
4. **Mercuric chloride** – It is a protein precipitant. However it causes great shrinkage of tissues hence seldom used alone. It gives brown colour to the tissues which needs to be removed by treatment with Iodine during dehydration.
5. **Potassium dichromate** – It has a binding effect on protein similar to that of formalin. Following fixation with Potassium dichromate tissue must be well washed in running water before dehydration.
6. **Osmic acid** – It is used for fixation of fatty tissues and nerves.
7. **Chromic acid** – It precipitates all proteins and preserves carbohydrates. Tissues fixed in chromic acid also require thorough washing with water before dehydration.
8. **Osmium tetraoxide** – It gives excellent preservation of cellular details, hence used for electron-microscopy.
9. **Picric acid** – It precipitates proteins and combines with them to form picrates. Owing to its explosive nature when dry; it must be kept under a layer of water. Tissue fixed in picric acid also require thorough washing with water to remove colour. Tissue can not be kept in picric acid more than 24 hrs.

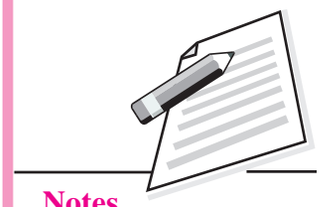
5.7 COMPOUND FIXATIVES

1. **Formal saline** - It is most widely used fixative. Tissue can be left in this for long period without excessive hardening or damage. Tissues fixed for a long time occasionally contain a pigment (formalin pigment). This may be removed in sections before staining by treatment with picric alcohol or 10% alcoholic solution of sodium hydroxide. The formation of this pigment can be prevented by neutralizing or buffering the formal saline.

   Fixation time – 24 hours at room temprature
2. **Formal calcium** – Useful for demonstration of phospholipids.

   Fixation time-24 hours at room temperature
3. **Zenker's fluid** – It contains mercuric chloride, potassium-di-chromate, sodium sulphate and glacial acetic acid.

   Advantages – even penetration, rapid fixation



Disadvantages – After fixation the tissue must be washed in running water to remove excess dichromate. Mercury pigment must be removed with Lugol's iodine.

4. **Zenker's formal (Helly's fluid)** – In stock Zenker's fluid, formalin is added instead of acetic acid.

   Advantages – excellent microanatomical fixative especially for bone marrow, spleen & kidney.

5. **Bouins fluid** – It contains picric acid, glacial acetic acid and 40% formaldehyde.

   Advantages – (a) Rapid and even penetration without any shrinkage. (b) Brilliant staining by trichrome method. It is routinely used for preservation of testicular biopsies.

Points to Remember

1. 10% buffered formalin is the commonest fixative.
2. Tissues may be kept in 10% buffered formalin for long duration.
3. Volume of the fixative should be atleast ten times of the volume of the specimen. The specimen should be completely submerged.
4. Special fixatives are used for preserving particular tissues.
5. Formalin vapours cause throat/ eye irritation hence mask/ eye glasses and gloves should be used.
6. Tissues should be well fixed before dehydration.
7. Penetration of fixatives takes some time. It is necessary that the bigger specimen should be given cuts so that the central part does not remain unfixed.
8. Mercury pigment must be removed with Lugol's iodine.
9. Biopsies cannot be kept for more than 24 hours in bouin's fluid without changing the alcohol.
10. Glutaraldehyde and osmion tetraoxide are used as fixatives for electron microscopy.

Most Commonly used Fixatives in the Laboratory are

10% Formalin

Formaldehyde (40%)	-	10 ml
Distilled water	-	90 ml

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Formal Saline

Formaldehyde (40%)	-	100 ml
Sodium Chloride	-	9 gm
Distilled Water	-	900 ml

10% Buffered Formalin

Formaldehyde (40%)	-	10 ml
Sodium dihydrogen phosphate	-	0.4 gm
Disodium hydrogen phosphate (anhydrous)	-	0.65 gm
Distilled water	-	90 ml

The advantage of this fixative is that it prevents the formation of formalin pigment

Bouin's solution

Saturated picric acid (1.2 gm/ 100 ml)	-	750 ml
Formaldehyde (40%)	-	250 ml
Glacial acetic acid	-	50 ml

Alcoholic formaldehyde

40% formaldehyde	-	100 ml
95% alcohol	-	900 ml

0.5 g calcium acetate may be added to this mixture to ensure neutrality

Alcohol containing fixatives

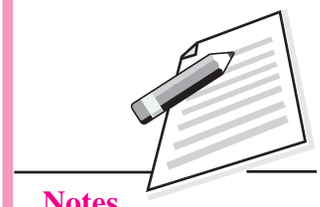
Carnoy's fixatives -

Absolute ethanol	-	60 ml
Chloroform	-	30 ml
Glacial acetic acid	-	10 ml

Mercury salt containing fixatives

Zenker's fluid

Distilled water	-	950 ml
Potassium dichromate	-	25 gm
Mercuric Chloride	-	50 gm
Glacial acetic acid	-	50 gm



B5 fixative

Stock reagent A

Mercuric chloride	-	60 g
Sodium acetate	-	12.5 g
Distilled water	-	1000 ml

Stock Reagent B

10% buffered neutral formalin

Working Solution

Stock reagent A	-	90 ml
Stock reagent B	-	10 ml
Fixation time	-	5-8 hrs

Adequate time should be given for fixation. Formalin fixation should ideally be given for at least 8 hours before processing. (Not the whole specimen but the cut sections).

INTEXT QUESTIONS 5.1

1. Fixation results in ..... & ..... of protein in the tissues.
2. Most commonly used fixation technique is .....
3. ..... is used as fixation for fatty tissues and nerves
4. Most widely used fixative is .....
5. Volume of fixatives shuld be atleast ..... of the volume of the specimen
6. Mercury pigment should be removed with .....
7. ..... prevents the formation of formalin pigment
8. Which is the commonly used fixative for tissues

   (a) Buffered formalin (b) Saline

   (c) Glutaraldehyde (d) Bouin's fluid

9. Which of the following is the best fixative for testicular biopsies?

   (a) Buffered formalin (b) Zenker's solution

   (c) Saline (d) Bouin's fluid

Notes

10. What is used to remove colour from tissues fixed in Zenker's solution?

(a) Alcohol (b) Lugol's iodine

(c) Tap water (d) Acetone

11. Which of the following is used for fixation of tissues for electron microscopy?

(a) Glutaraldehyde (b) Saline

(c) Osmic acid (d) Picric acid

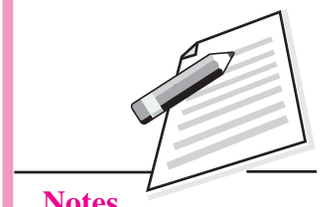
12. What should be the optimum pH of fixative to preserve good morphology?

(a) 5 (b) 6

(c) 7 (d) 8

WHAT HAVE YOU LEARNT

- Fixation of tissues is a process by which the cells of tissue are fixed in chemical and partly physical state so that they can withstand subsequent treatment with various reagants
- Fixation results in denaturation and coagulation of protein in the tissues
- Penetration rate differs with molecular weight of the fixative
- Saturation fixation occurs between pH of 6 & 8 and optimally at 7
- An ideal fixative should be cheap, nontoxic and non inflammable
- Immersion, perfusion, vapour, coating/spray, freeze drying, micro waved fixation are the different types of fixatives used
- The most commonly used technique is simple immersion of tissues/smears in an excess of fixation
- Buffered formalin is the most commonly used fixative and prevents brown pigment formation on tissues
- Following fixation with potassium dichromate tissue must be well washed in running water
- Osmic acid is used for fixation of fatty tissues and nerves
- Osmium tetraoxide and glutaraldehyde are used for electron microscopy
- Formal saline is the most widely used fixative
- Formal Calcium is useful for demonstration of phospholipids
- Bouins fluid is routinely used for preservation of testicular biopsies



- Mercury pigment must be removed with lugol's iodine
- Formalin fixation should ideally be given for atleast 8 hours before processing. Whole specimens should not be fixed without giving cuts.

TERMINAL QUESTIONS

1. What is a fixative?
2. What is the commonest fixative?
3. Write the properties of an ideal fixative.
4. What precautions should be observed when using formalin as fixative?
5. Write names of two special fixatives and their use.

ANSWERS TO INTEXT QUESTIONS

5.1

1. Denaturation and Coagulation
2. Simple immersion
3. Osmic acid
4. Formal Saline
5. Ten times
6. Lugol's Iodine
7. Buffered Formalin
8. (a) Buffered formalin
9. (d) Bouin's fluid
10. (b) Lugol's iodine
11. (a) Glutaraldehyde
12. (c) 7