E-content for Programme: M.Sc. Zoology (Semester - II)
Core Course (CC- 7): Biochemistry
Unit V: Principles of Histology and Histochemistry
5.1 General principles of fixation and types of fixatives

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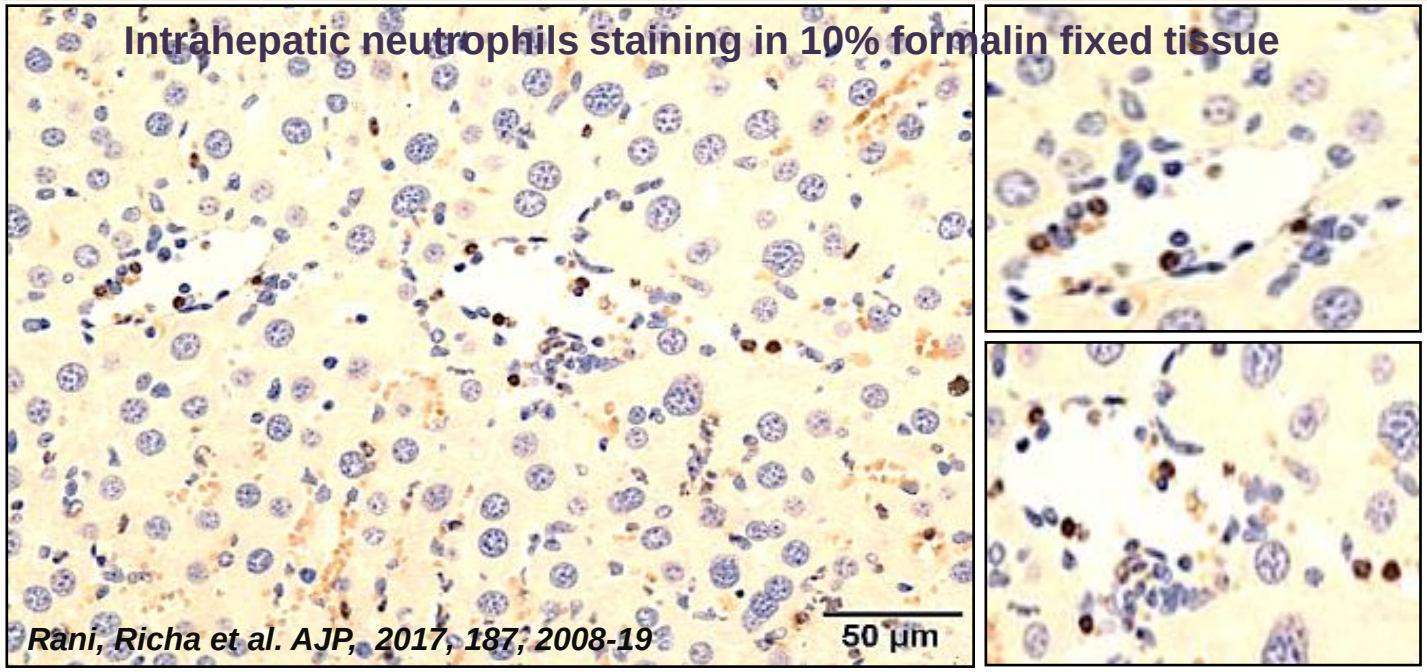


FIXATION

- A process used to preserve the constituents of cells and tissue so that they can withstand the subsequent treatment with various reagents with minimum loss of architecture.

- It is the most crucial step in the preparation of cells or tissues for microscopy analysis.

- It consists of two steps:

  - ✓ Termination of ongoing biochemical reactions i.e. killing

FIXATION (Cont'd)

- ✓ Stabilization of the cellular structure and composition i.e. preservation

- ❖ The process results in denaturation and coagulation of proteins in the tissues.

- ❖ The speed of fixation depends on the rate of diffusion of fixative into the tissue and the rate of chemical reactions with various components.

Critical roles of Fixation

- To preserve and stabilize the cell morphology and tissue architecture in a “life-like state”.
- To prevent or arrest the degenerative processes.
- To prevent autolysis (tissue digestion by intracellular enzymes released when organelle membranes rupture) and bacterial decomposition (or putrefaction).

Critical roles of Fixation (Cont'd)

- To enhance the refractive index of tissue constituents.
- To harden or strengthen the samples so that they can withstand the downstream staining procedures.
- To improve the optical differentiation of cells and tissues.
- To retain the structure of the tissue and avoid excessive shrinkage or swelling of the cells.

FIXATION: Principle and Methods

❑Principle:

- ➢ 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.

❑Methods of Fixation:

- ➢ Fixation of tissues can be achieved by chemical or physical means.

❖ Physical methods:

- ➢ It includes heating, microwaving and cryopreservation (freeze drying).

FIXATION: Methods (Cont'd)

❖ Chemical methods:

- Usually achieved by immersing the specimen in the fixative (immersion fixation) or, in the case of small animals or some whole organs such as a heart, by perfusing the vascular system with fixative (perfusion fixation).

- For some specialized histochemical procedures fixatives have occasionally been applied in the vapor form. For example paraformaldehyde and osmium tetroxide can be used to vapour-fix freeze-dried tissues.

Common methods of Fixation

❑Drying:

- ➢In this method of fixation, specimen is passed through the flame of a Bunsen burner to adhere onto the slide. Method preserves overall morphology, but denatures the proteolytic enzymes.

❑Freezing:

- ➢Samples with antigens that are too labile for chemical fixation used for de-paraffinization can be embedded in a cryoprotective embedding medium, such as optimal cutting temperature (OCT) compound, and then snap-frozen and stored in liquid nitrogen.

Common methods of Fixation (Cont'd)

❑Perfusion:

- ➢Fixative is pumped into the organ and fixes the tissue through the outside and inside of the organ.

❑Immersion:

- ➢It is a method, wherein the samples are dissected out and immersed in fixative of volume about 20 times greater than the volume of tissue.
- ➢Immersion fixation fixes from the outside and leaves the insides rotting until the fixative diffuses into the tissue or cell sample.
- ➢Immersion is often combined with perfusion to ensure thorough fixation throughout the tissue.

Mechanisms of Fixation

The mechanism of fixation is dependent on the reagents used.

Two major mechanisms of chemical fixation which are important in fixation of proteins and protein complexes are:

- denaturation, and
- addition and cross-link formation.

Mechanisms of Fixation (Cont'd)

❑ Denaturation:

- Generally induced by dehydrants such as the alcohols or acetone.

- These reagents remove and replace free water in cells and tissues, leading to a change in the tertiary structure of proteins by destabilizing hydrophobic bonding.

- The conformational changes in the protein molecules cause a change in the solubility of the protein, rendering water soluble proteins insoluble.

Mechanisms of Fixation (Cont'd)

❑ Addition and cross-link formation:

➢ The non-coagulant fixatives bind with proteins and other cell and tissue components by addition and forming inter- and intra-molecular cross-links.

➢ **Examples:**

- The mercuric-containing fixatives, for example B-5 and Zenker's, act through binding to sulfhydryl and amino groups in an additive reaction.

- Bouin's, like Carnoy's, consisting of picric acid, acetic acid and formaldehyde, has both a coagulative and cross-linking effect on proteins.

Mechanisms of Fixation (Cont'd)

- The most commonly used fixative for histopathology is a 4% aqueous solution of formaldehyde, often called 10% formalin because it is made by tenfold dilution of formalin.

The mechanism of action of formaldehyde occurs through the formation of intra- and inter-molecular cross-links. The principal cross-links occur between the amino group of the N-terminal amino acid residue and the side-chains of arginine, cysteine, histidine, and lysine residues.

Mechanisms of Fixation (Cont'd)

- Cross-linking involves covalent bond formation both within proteins and between them, which causes tissue to stiffen and therefore resist degradation.

- This can have an effect on the subsequent staining characteristics of a particular protein as well as altering its molecular conformation and thus its solubility.

- For example, paraformaldehyde causes covalent cross-links between molecules, effectively gluing them together into an insoluble meshwork.

Mechanisms of Fixation (Cont'd)

- Tissue fixed with formaldehyde stains poorly with eosin, as it reacts extensively with amino groups to form methylene bridges and thus these groups are no longer available to bind negatively charged dye molecules such as those of eosin.

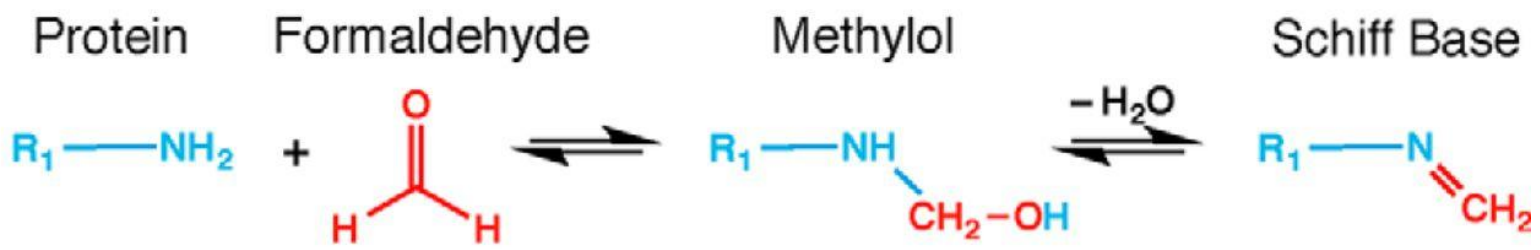
- Glutaraldehyde is more effective at forming cross-links than formaldehyde. That is why it so effectively preserves the ultrastructure of cells and is the fixative of choice for electron microscopy. But, glutaraldehyde-fixed tissues stain poorly with conventional dye-staining methods.

Mechanisms of Fixation (Cont'd)

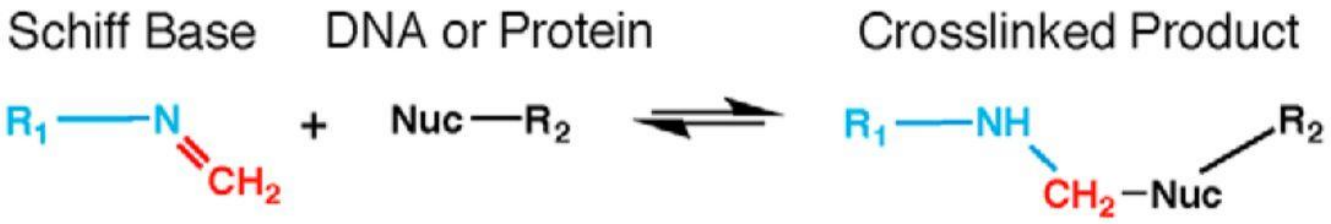
- **Fixation reaction of Formaldehyde:**

  It occurs in two steps

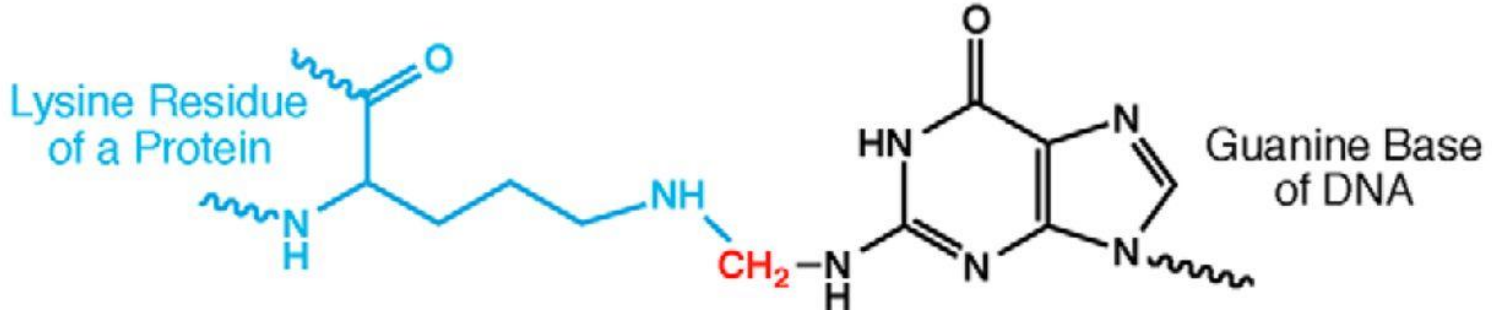
Step 1



Step 2



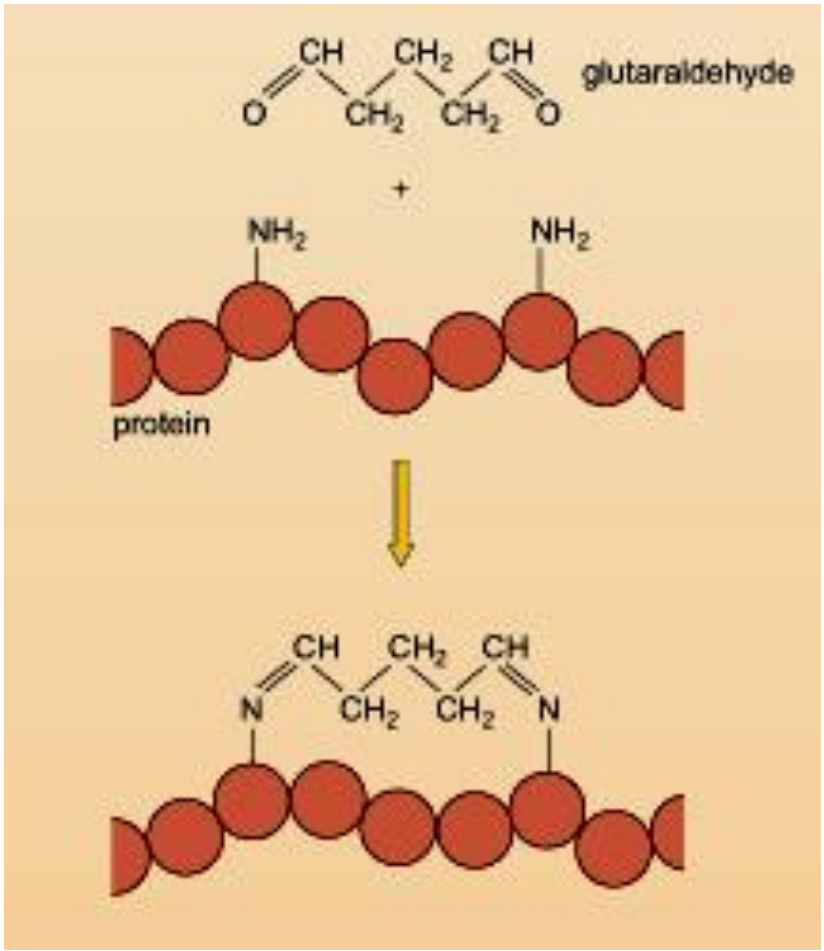
Example Protein-DNA Crosslink



JBC. 2015. 290, 26404-26411.

Mechanisms of Fixation (Cont'd)

- **Fixation reaction of glutaraldehyde:**
  - The amine groups ($-NH_2$) in the protein react with aldehyde groups (-CHO) in the fixative.



Factors affecting Fixation

❑Temperature:

- An increase in temperature increases the rate of fixation but also increases the rate of autolysis and diffusion of cellular components.

- For light microscopy, initial fixation is usually carried out at room temperature and may be followed by further fixation at temperatures up to 45°C during tissue processing.

- For electron microscopy and some histochemical and cytological procedures, 0 to 4°C has been considered the ideal temperature or the fixation.

Factors affecting Fixation (Cont'd)

- **Specimen dimensions:**
  - The tissue is typically cut into thin sections (5-10 µm) or smaller pieces (for whole mount studies) to facilitate further study.

- **Volume ratio:**
  - Volume of fixative should be at least 20 times greater than the volume of tissue to be fixed.

- **Time:**
  - Time of fixation ranges from 4-24 hr. Prolonged fixation can cause shrinking and hardening of the tissue and can inhibit enzyme activity.

Factors affecting Fixation (Cont'd)

- **pH and buffers:**
  - Should be in between pH 4-9 or in physiological range.

- **Selection of fixatives:**
  - For ex- for electron microscopy glutaraldehyde is preferred as fixative.

- **Penetration rate:**
  - The penetration rate of a fixing agent depends on its diffusion characteristics and varies from agent to agent.

Factors affecting Fixation (Cont'd)

❑Tissue storage:

- ➢Non-fixed tissues are generally stored in 70% methanol and tissue fixed in neutral buffered saline are safe to use. Delicate tissues are first embedded in cryoprotective embedding medium, such as OCT compound, and then snap-frozen and stored in liquid nitrogen until they are sectioned.

❑Osmolality:

- ➢Hypotonic and isotonic fixatives cause cell swelling and poor fixation, whereas hypertonic fixative can result in cell shrinking.

Fixative or Fixing agent

- Any substance that aims to fix the cells or tissues to preserve their morphological and chemical characteristics.

Properties of ideal fixatives:

- Should be cheap, non-toxic, non-allergic for user, and non-inflammable.
- Should be able to make the cellular components insoluble to reagent used in tissue processing.
- Should prevent the excessive hardness of tissue.
- Should enhance the staining of the tissues.

Classification of Fixatives

- **Physical fixatives:**
  - It is an alternate approach to prepare samples for staining, and the specific method depends on the sample source and the stability of the target antigen. For ex- blood smears are air-dried to heat-fix the cells to the slide.
  - Example- Heat, Microwave, Cryopreservation
- **Chemical fixatives:**
  - Chemical fixatives crosslink or precipitate sample proteins.

Classification of Fixatives (Cont'd)

Chemical fixatives may be:

- **Aldehydes:**
  - Formaldehyde, Glutaraldehyde, Acrolein
- **Oxidizing agents:**
  - Osmium tetroxide, Potassium permanganate, Potassium dichromate
- **Protein denaturing agents:**
  - Acetic acid, Methanol, Ethanol
- **Others:**
  - Mercuric chloride, Picric acid, non aldehyde containing fixatives.

Classification of Fixatives (Cont'd)

❖ **Based on the types of fixation, fixatives are classified as follows:**

❑ **Microanatomical fixatives:**

➢ These are used to preserve the anatomy of the tissue. For ex- Neutral buffered formalin

Classification of Fixatives (Cont'd)

❑Cytological fixatives:

➢These are used to fix intracellular structures.

- **Nuclear fixatives:** Ex- Carnoy's Fluid, Clarke's Fluid, Newcomer's Fluid, Flemming's Fluid etc.
- **Cytoplasmic Fixatives:** Ex- Champy's Fluid, Regaud's Fluid etc.

❑Histochemical fixatives:

➢These are used to demonstrate the chemical constituents of the cell. For ex- Formal saline, Cold acetone, Absolute alcohol etc.

Selection of Fixatives

- The selection of the types of fixatives is influenced by the target antigen as well as the desired detection technique (fluorescent or chromogenic).

- The most widely used fixatives are:

- **Formaldehyde:**
  - Formaldehyde (CH_2O) is the only gaseous aldehyde and is dissolved in water to saturation at 37–40% (w/v). Also known as “formalin” or “concentrated formaldehyde solution”.
  - For fixation, one part formalin is usually diluted with nine parts of water or buffer. This produces a 10% formalin solution which contains about 4% formaldehyde (w/v).

Selection of Fixatives (Cont'd)

- **Glutaraldehyde:**
  - Glutaraldehyde or glutaric dialdehyde ($CHO(CH_2)_3CHO$) posses aldehyde groups at either end of the molecule and react with the same chemical groups as formaldehyde.

  - For electron microscopy, glutaraldehyde primary fixation is commonly followed by secondary fixation in osmium tetroxide. Glutaraldehyde is not normally used for routine histopathology.

- Other chemical fixatives used are acrolein, glyoxal, osmium tetroxide, picric acid, ethanol, methanol, acetone, acetic acid, etc.

Selection of Fixatives (Cont'd)

- **Critical differences between aldehyde- and alcohol-based fixatives:**

- While aldehyde-based fixatives destroy amine groups, but tend to maintain tissue structure well; alcohol-based fixatives usually result in poorer preservation of structure as they dehydrate cells through the removal of water from the free carboxyl, hydroxyl, amino, amido and imino groups of the proteins but do not destroy amine groups and they can preserve some secondary structure in proteins.

- Alcohol-based fixatives result in protein coagulation and tissue shrinkage.

Function of Fixatives

- ✓ Help in maintaining a proper relationship between cells and extracellular substances such as connective tissue fibres (collagen reticulin, elastin) and amorphous ground substances.
- ✓ Confer differences in refractive indexes and increases the visibility or contrast between different tissue components.
- ✓ Render cell constituent's insoluble, with proteins as the primary target for stabilization.

Guidelines for choosing a fixative

Antigen	Fixative
Most proteins, peptides and enzymes of low molecular weight	Cells / cytological preparations: 4% formaldehyde Tissue sections: 10% Neutral-Buffered Formalin (NBF)
Delicate tissue	Bouin's fixative
Small molecules such as amino acids	4% formaldehyde
Blood-forming organs (liver, spleen, bone marrow)	Zenker’s solution
Connective tissue	Helly's solution
Nucleic acids	Carnoy’s solution
Large protein antigens (e.g., immunoglobulin)	Ice-cold acetone or methanol (100%)
Nuclear morphology	Zinc formalin
For electron microscopy	4% formaldehyde - 1% glutaraldehyde

https://www.abcam.com/kits/tissue-fixation-embedding-and-sectioning

Different fixatives and their composition

Fixative	Method of fixation	Composition
B-5	Denaturing	5.4% Mercuric Chloride (w/v), 1.1% Sodium Acetate (w/v), 4% Formaldehyde (v/v), Water
Bouin's	Denaturing, cross-linking	25% of 37% formaldehyde solution, 70% picric acid, 5% acetic acid
Carnoy's	Denaturing	60% ethanol, 30% chloroform, 10% Glacial acetic acid
Glutaraldehyde	Cross-linking	Generally, 2% v/v of glutaraldehyde to water/PBS
Methacarn	Denaturing	60% methanol, 30% chloroform, 10% Glacial acetic acid
Neutral buffered formalin (NBF)	Cross-linking	10% of 37% formaldehyde solution, in a neutral pH
Paraformaldehyde (PFA)	Cross-linking	Generally, 4% w/v of paraformaldehyde to Water/PBS
Zenker's	Denaturing	5% Mercuric Chloride (w/v), 2.5% Potassium Dichromate (w/v), 5% Glacial acetic acid (v/v), Water

Howat and Wilson, 2014

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Thank You!