**Fixation**

**The object of fixation:**

**1-** The cells and extracellular materials must be preserved so that there has been as little alteration as possible to the structure and chemical composition of the living tissue.

**2-** the tissue's structural proteins and other constituents must be rendered insoluble in all the reagents to which they will subsequently be exposed.

**Physical methods of fixation:**

**1- Heat:** The simplest physical method is the application of heat. This results in the coagulation of proteins and the melting of lipids. The resemblance to the living state is not very close after such treatment,

but the method is often used in diagnostic microbiology. Larger specimens are usually heated in a microwave oven.

**2- Freezing:** Animal tissues are sometimes processed by the techniques known as freeze-drying. A specimen must be frozen in such a way as to minimize the formation of ice crystals, which can replace the architecture of any tissue with a meaningless array of cell-sized holes. This artifact is usually seen in large specimens that have been frozen slowly. Suitable quick freezing techniques include immersion in isopentane cooled to its freezing point (–170°C) by liquid nitrogen.

**Chemical methods of fixation:** Liquid fixatives are used for most histological and histochemical purposes. These substances affect

the tissues both physically and chemically. The principal physical changes produced are shrinkage or swelling, and many of the fixatives in common use are mixtures of different agents, formulated to balance these two undesirable effects. Most fixatives harden tissues.

**General properties of fixatives:**

The structure of an animal tissue is determined largely by the configuration of its contained **proteins**. The main contributors to the structure are the **lipoproteins**, which are major components of the plasmalemmae and membranous organelles of cells, **cytoskeletal fibrous proteins**, the **fibrous glycoproteins** of such extracellular elements as **collagen** and **basement membranes**, and the **globular proteins**, which are dissolved in the cytoplasm and extracellular fluid. **All these substances must be stabilized by fixation**. The **nucleic acids and nucleoproteins s**hould also be preserved, as should the **mucosubstances** and, if their histochemical demonstration is required, the **lipids**. Many fixatives do not directly affect lipids, whose preservation depends largely on the avoidance of agents that dissolve them.

**Physical considerations:** The rates of penetration of fixatives have been studied with solid organs, especially the liver. These experiments the distance penetrated was visible as a change in appearance or a colour change in an indicator. Penetration follows a simple law of diffusion: D = K√t where **D** is the distance (mm) penetrated in **time t (hours)**. The coefficient **K varies with the medium being penetrated**, is **different** for each **chemical fixative**, and **increases with concentration or temperature.**

**Rapidly penetrating fixatives** will usually fix in **24 h** a specimen whose smallest dimension is **5 mm**. For **slowly penetrating fixatives** the thickness of the block should not exceed **2 mm.** The duration of fixation should **not exceed 24 h** except in the case of **formaldehyde**, which **penetrates quickly** but takes a week to cause full stabilization of histological structure.

**Coagulants and non-coagulants:**

Fixatives that coagulate proteins can destroy or distort cytoplasmic organelles such as mitochondria, lysosomes and secretory granules, but they do not seriously disturb the supporting extracellular materials, which are already partly solid before being fixed. It is thought that coagulant fixatives produce a **sponge-like proteinaceous** **reticulum** that is easily permeated. **Coagulant fixation** usually **allows easy sectioning** of **wax-embedded animal** and plant tissue.

**Non-coagulant fixatives** act by **cross-linking** the structural macromolecules of a tissue. **Crosslinking of proteins converts** the cytoplasm into an **insoluble gel** in which the organelles are **well preserved.** Such a gel may be less easily penetrated by paraffin than coagulated cytoplasm.

Whether coagulant or non-coagulant, a fixative may also be designated as **additive** or **nonadditive**. An additive fixative has molecules or ions that combine chemically with proteins, cellulose or other structural macromolecules. All cross-linking fixatives are additive. **A non-additive fixative** such as **alcohol or acetic acid** causes changes in the tissue but is **removed by washing** or other later procedures. **Many fixative mixtures** contain both coagulant and non-coagulant compounds and combine the advantages of both. For **light microscopy**, it is necessary to settle for either a mixture that gives adequate **cytoplasmic fixation** (due mainly to non-coagulant insolubilization of protein) or one that provides superior structural **preservation on a larger scale** (for which coagulation is necessary).

**In electron microscopy** the **cytoplasmic disruption** due to coagulant fixation is unacceptable, and **non-coagulant agents must be used**. Fortunately the plastics used as embedding media for electron microscopy cause much less distortion of the delicate architecture of tissues than does paraffin wax.

**The nuclei** of cells are deliberately stained in most histological preparations, especially of animal tissue. A f**ixative mixture should therefore contain** a substance that either coagulates the chromatin or renders it resistant to extraction by water and other solvents.

**Most fixatives do not react chemically with macromolecular carbohydrates and lipids**, though these substances are often protected from extraction as a consequence of the insolubilization of associated proteins.

**Effects on staining:** Another important consideration is the **effect of fixation** on the subsequent reactivity of the tissues with dyes or histochemical reagents.

**Individual fixative agents:**

**1- Simple organic coagulants**

This group includes liquids such as **acetone, ethanol, and methanol** that **displace water from proteinaceous materials**, thereby **breaking hydrogen bonds** and **disturbing the tertiary structure of proteins** and other macromolecules. **Simple coagulants distort protein molecules** without changing the sequences of their amino acids.

**2- Mercuric chloride**

 It is a very poisonous substance, and for this reason, its use in laboratories is often avoided. The chemistry of fixation by mercuric chloride is **poorly understood**. Similar **mercury–nitrogen bonds** are formed with amides and amino acids. **Cross-linking of two nitrogens by mercury can also occur**. Thus, addition of mercury and cross-linking may account for the **insolubilization of proteins by mercuric chloride**. A **crystalline precipitate** (of uncertain chemical composition but probably mostly mercurous chloride, Hg2 Cl2 ) forms within mercury-fixed tissues and must be **removed**, before the **sections are stained**, by treatment with a solution of **iodine followed by sodium thiosulphate.**

**3- Zinc salts**

Zinc chloride was first used in a fixative more than a **century ago.** The fixative action of the zinc ion is due to protein coagulation.

4- **Picric acid**

A near-saturated aqueous solution of picric acid (pH 1.5–2.0) causes coagulation by forming salts (picrates) with the basic groups of proteins. The low pH of a picric acid solution brings about hydrolysis of nucleic acids. Fixatives containing picric acid are avoided for quantitative histochemical studies of DNA and RNA. Blocks fixed in picric acid are passed through several changes of 70% alcohol to remove as much as possible of the yellow colour. According to Luna (1968), prolonged contact with picric acid, even in solid paraffin wax, may cause structural deterioration and poor staining.

**Acetic acid**

Acetic acid does not fix proteins, but it coagulates nucleic acids and may partly extract their associated basic proteins. The mechanism by which this change is brought about is obscure. **Acetic acid is included in fixative mixtures to preserve chromosomes of dividing cells** to precipitate the chromatin of interphase nuclei, and to oppose the shrinking actions of other agents such as ethanol and picric acid.

**Osmium tetroxide**

It is a non-ionic solid, OsO4 , which is volatile at room temperature. OsO4 is soluble (without ionization) in water but much more soluble in non-polar organic solvents. The best-understood fixative action is with the unsaturated linkages of lipids. Osmium tetroxide penetrates tissue to a depth of only 0.5–1.0 mm, so its use as a fixative is limited to small blocks. By stabilizing membranes and gelating dissolved proteins, OsO4 provides lifelike fixation of the internal structures of cells. Unfortunately, however, pieces of osmium-fixed tissue have a crumbly consistency which is made worse by embedding in wax, and leads to the formation of cracks and shrinkage spaces. Osmium tetroxide may be used as a secondary fixative (post-fixation) after formaldehyde or glutaraldehyde, as in electron microscopy, and it may be used to stain frozen sections for unsaturated lipids. The vapor above an aqueous solution of OsO4 is as effective as the solution itself, both as a fixative and as a stain.

**Formaldehyde**

Formaldehyde is a gas (BP –21°C). It is sold as a solution (formalin) containing 37–40% by weight of the gas in water, and as a solid

polymer, paraformaldehyde, Paraformaldehyde is also seen as the white precipitate that forms in old bottles of formalin. It act to inhibits many enzymes, thereby preventing autolysis, but does not structurally stabilize the tissue. Formaldehyde fixes tissue by cross-linking the proteins, primarily the residues of the basic amino acid lysine. Its effects are reversible by excess water and it avoids formalin pigmentation. Paraformaldehyde is also commonly used and will depolymerize back to formalin when heated, also making it an effective fixative.

**Glutaraldehyde**

Glutaraldehyde cross-links proteins in a manner analogous to formaldehyde, but targeting different components, such as amino groups. During this process, it stabilizes the protein mass and preserves the morphology. It fixes somewhat faster than formaldehyde, but its penetration rate is poorer.

**Choice of fixative**

A fixative is chosen according to the structural or chemical components of the tissue that are to be demonstrated. Often a mixture of different agents is employed in order to offset undesirable effects of individual substances and to obtain more than one type of chemical fixation. Most frequently, the routine fixative will be neutral buffered formalin with other agents used for bone marrow trephines (perhaps a zinc formalin), renal biopsies, frozen sections, etc. Buffered formalin is widely used because it is probably the most flexible of agents.

**Non-aqueous fixatives**

The following mixtures are used for general histology and for preserving nucleic acids and macromolecular carbohydrates.

**Methanol alone**, for 1–2 minutes, is an adequate fixative for thin preparations such as blood smears and cell cultures.

**Clarke’s fluid:**

Absolute ethanol: 3 volumes plus Glacial acetic acid: 1 volume. This is one of the oldest fixatives and is notable for excellent results with subsequent paraffin embedding.

**Carnoy’s fluid**

Mix of Absolute ethanol: 60 ml, Chloroform: 30 ml, and Glacial acetic acid: 10 ml. Fix for up to 15 h in at least 20 times the volume of the specimen. Like Clarke’s, this is a rapidly penetrating fixative that coagulates protein and nucleic acids and extracts lipids. Blocks of tissue up to 5 mm thick are fixed for 6–8 h and then moved into 100% alcohol.

**Aqueous solutions fixations**

**Bouin’s fluid:** Saturated aqueous picric acid: 750 ml, Formalin (37–40% HCHO): 250 ml, Glacial acetic acid: 50 ml Keeps indefinitely. Bouin’s fluid preserves morphological features, especially of nuclei and connective tissue well. This fixative is valuable for general histological work because the physical distortion of tissues is minimal, paraffin sections are easy to cut, and staining methods using multiple dyes usually give bright, well-separated colors. usually fixed for 24 hours, then transferred to 70% or 95% alcohol.

**Neutral buffered formalin**

Formaldehyde, 37–40%: 100 ml, Water: 900 ml, Sodium phosphate, monobasic (NaH2 PO4 .H2O): 18.6 g, Sodium hydroxide (NaOH): 4.2 g. Can be kept for several months.

**Formaldehyde with glutaraldehyde:** The addition of 10 ml of 25% aqueous glutaraldehyde to 90 ml of neutral buffered formaldehyde gives a solution that preserves many intracytoplasmic structures such as mitochondria.

**Gendre’s fluid** (alcoholic Bouin):

Saturated alcoholic picric acid: 800 ml Formalin, (40% HCHO): 150 ml, and Glacial acetic acid: 50 ml. This mixture can be kept indefinitely.

**Zenker’s fluids:** Stock Zenker solution: Mercuric chloride (HgCl2 ): 50 g Potassium dichromate (K2 Cr2 O7 ): 25 g Sodium sulphate (Na2 SO4 .10H2 O): 10 g Water: Zenker contain mercuric chloride and potassium dichromate. These fixatives are excellent for morphological work, but are not compatible with many histochemical techniques.