FIXATION

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INTRODUCTION

Preparation of tissues for microscopic examination, through a series of processes namely **fixation, dehydration, clearing, embedding, cutting** and **staining**.

Tissues decompose when removed from the body or it is cut off from the blood supply – **Autolysis** and **Putrefaction**

DEFINITION OF FIXATION

- Process by which the constituents of the cells and therefore of the tissues are fixed in a physical, partly also in a chemical state so that they will withstand the subsequent treatment with various reagents with **minimum of loss , distortion or decomposition**.

Fixation is the foundation for the subsequent stages in the preparation of the sections

- Complex series of chemical events which differs for different groups of substances found in tissues.

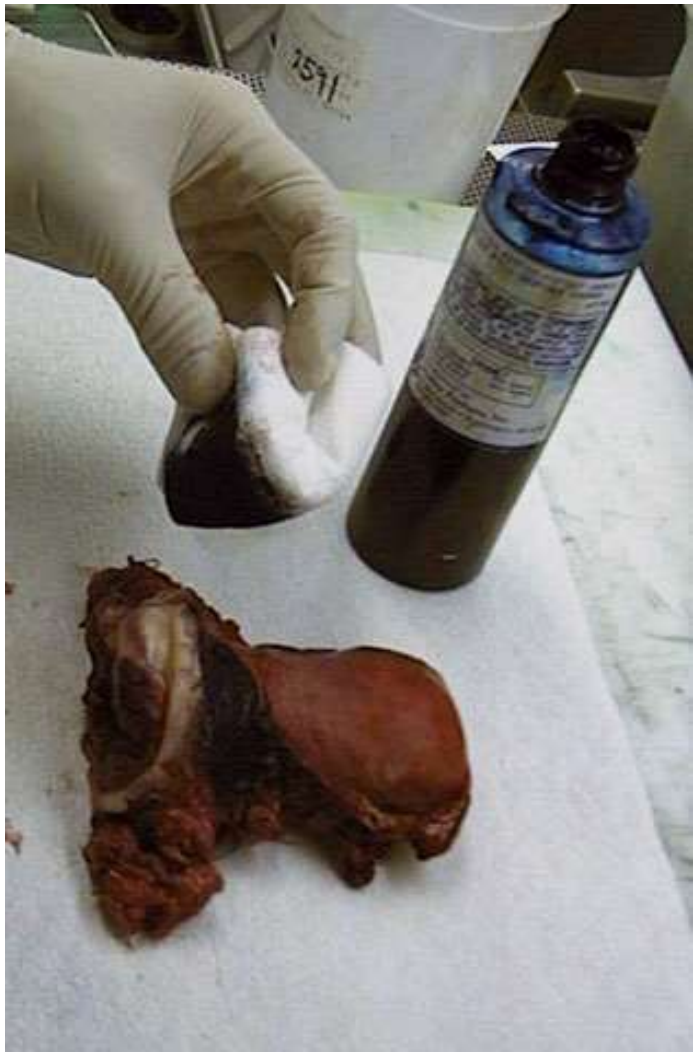
PRE-FIXATION PROCEDURES & PRECAUTION:

- Dissecting table made up of hard wood with a working surface area of 45  35 cm
- Wood- actual cutting surface
- Rubber pads of various dimensions- suitable
- Instrument- thin bladed knives 20-30 cm in length, scissors, probes, scalpels, stainless-steel rule, forceps, sponges
- First-aid box & eye-wash bottle
- Cutting board & instruments should be cleaned before procedures
- Powdering of the gloves & washing of the gloved hands before dissection

- Fixation of tissue- as soon as possible after death or removal from the body
- Screw capped specimen jars containing appropriate fixatives – in OT, post-mortem room, animal house
- Amount of fluid in the jars- 15-20 times
- Early dispatch of the specimens to histology laboratory
- Tissues should be washed in physiological saline
- Excessive blood & mucous should not be there
- Tissues selected for sectioning should be sufficiently thin to be adequately fixed throughout a reasonable time
- Best thickness- **3-5mm**

AIM

- **Preservation** of cells & tissue constituents in a condition identical to that existing during life.
- To do this in a manner that will allow preparation of **thin, stained sections**.

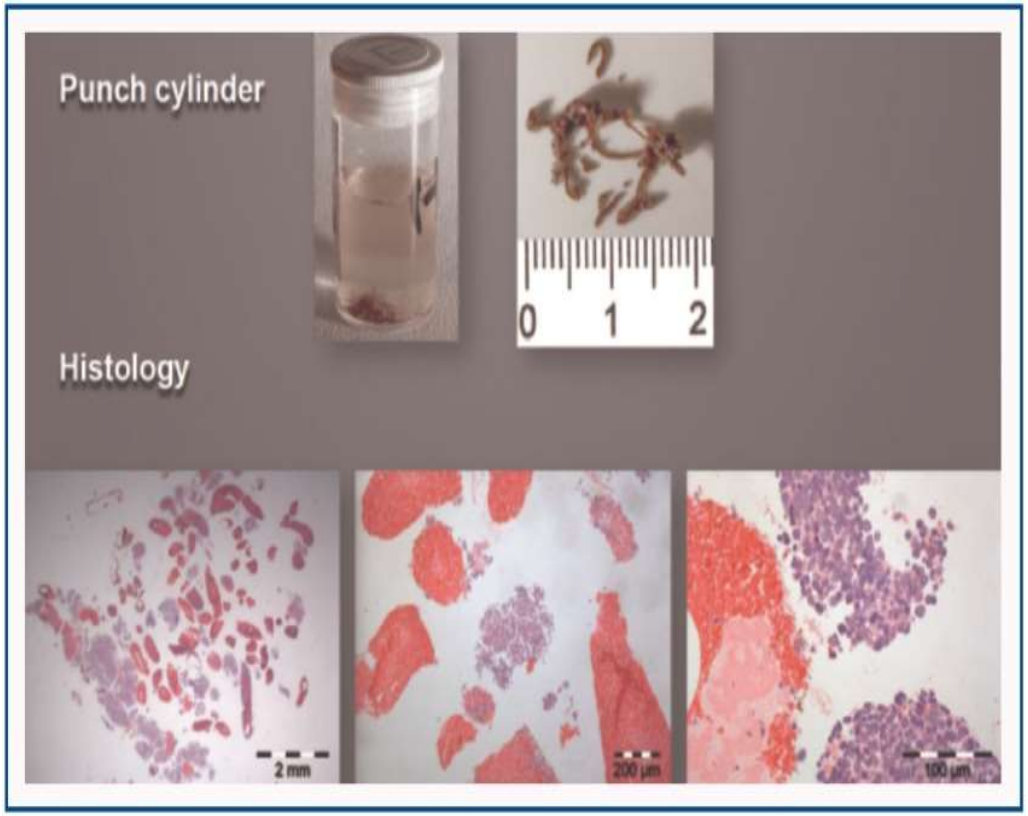


PURPOSE

1. To **prevent** autolysis, bacterial decomposition & putrefaction.
2. To **coagulate** the tissue as to prevent loss of easily diffusible substances.
3. To **fortify** the tissue against deleterious effects of further stages.
4. To **facilitate** differential staining with dyes & other reagents.

EFFECTS

1. Inhibition of autolysis & putrefaction
2. Preservation
3. Hardening
4. Solidification of colloid material
5. Optical differentiation
6. Effect on staining



IDEAL FIXATIVES:

- Produces immediate death of cells (life like appearances)
- Prevents autolysis & putrefaction
- Reacts rapidly & completely with the tissue
- Fixes all the constituents of the tissue
- Neither shrinks nor swells
- Makes the specimen hard enough
- Raises refractive indices
- Provides full range of staining methods with great selectivity

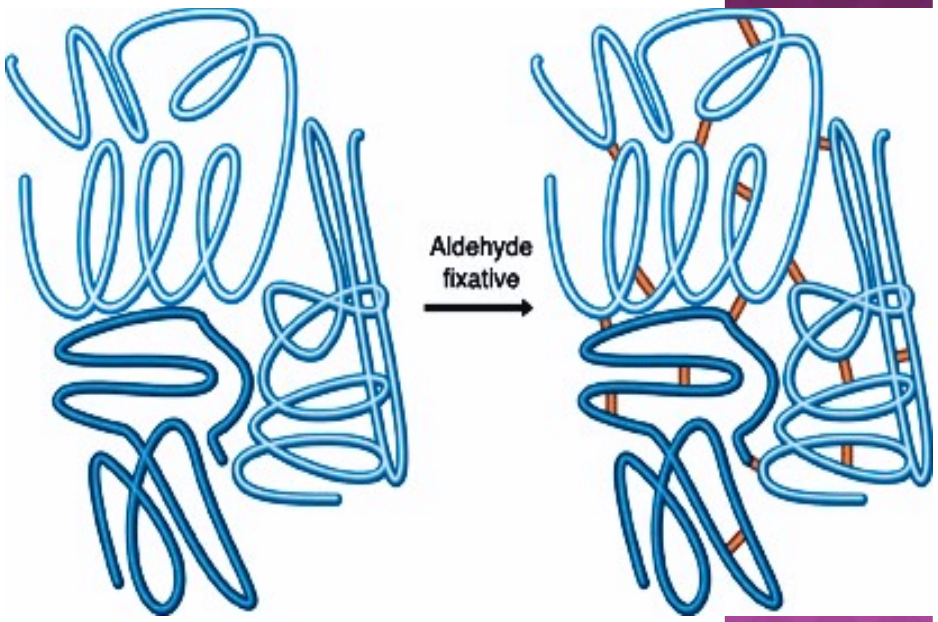
- No rigid upper limit of fixing time
- No tendency to deteriorate
- Easily prepared
- Cheap, safe to handle
- Non-toxic, stable
- Non-inflammable & non-irritant
- Should penetrate a tissue quickly
- Cause minimum physical and chemical alteration of the tissue and its components.
- **As such no ideal fixative.**

- Choice depends on the cell or tissue constituent to be demonstrated

REACTION OF FIXATIVES

1. **Proteins:**

- **Cross links** are formed between proteins.
- Soluble proteins are fixed to structural proteins- insoluble- mechanical strength-allowing subsequent maneuvers.
- Formaldehyde -reversible.
- Gluteraldehyde -rapid & irreversible.
- React with basic amino-acid residues



2. **Nucleic acid:**

- Fixation brings a change in the physical & chemical state of RNA & DNA.
- Uncoiling of DNA & RNA occurs with formalin when heated to 45˚C & 65˚C respectively.

3. Lipids:

- Phospholipids are fixed by aldehydes.
- Formaldehyde reacts with unsaturated fatty acids hence less lipid can be demonstrated in tissue stored in it for a long time.
- Mercuric chloride reacts with lipids to form complexes.
- Ultrastructural demonstration of lipids – post fixing in imidazole-osmium tetroxide.

CARBOHYDRATES:

- Carbohydrates are more water soluble- difficulty in total preservation
- They bind with fixed protein
- So the fixatives which are used for proteins, can be used for carbohydrate preservation.
- Fixed protein traps carbohydrates.
- **Glycogen not bound to protein-** fixed protein form lattice around glycogen to preserve it
- **Glycogen are more demonstrable in liver cells**

FACTORS INVOLVED

1. Buffers & hydrogen ion concentration:

- Best fixation occurs between pH 6-8
- Buffers used – phosphate, s-collidine, bicarbonate,

2. Temperature:

- Most tissues fixed– room temp
- Electron microscopy & histochemistry – 0-4˚C

3. Penetration of tissues:

- Blocks should be small or thin to ensure adequate penetration.

4. **Volume changes:**

- Due to inhibition of respiration, membrane permeability changes, changes in ion transport through membrane.
- Tissues fixed in formaldehyde & embedded in paraffin shrink by 33%

5. **Osmolality of fixative:**

- Hypertonic solutions – cell shrinkage
- hypotonic solutions – swelling of cells & poor fixation.
- **Best** – slightly hypertonic solutions.

6. **Substances to vehicle:**

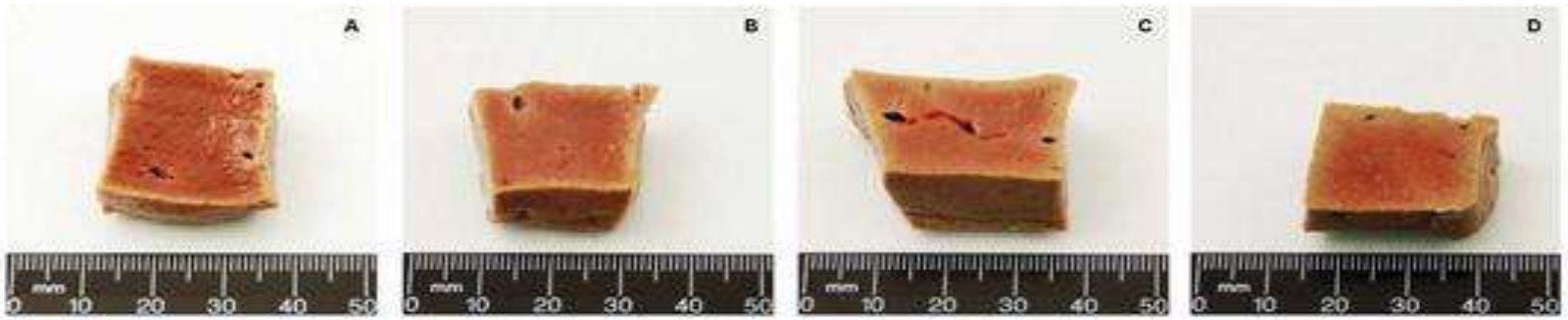
- Adding substances to fulfill certain functions.
- Denaturing effects, some stabilize proteins.
- Eg. Sodium chloride & sodium sulphate used with mercuric chloride.
- **Tannic acid enhances fixation of lipids & proteins in EM**

7. **Concentration of fixatives:**

- Different concentrations have different effects on morphology.
- Effects subsequent staining

8. **Duration of fixation:**

- Formalin – 2-6hours
- Electron microscopy – 3 hours
- Formaldehyde – prolonged fixation – shrinkage & hardening of tissue.
- Gluteraldehyde – prolonged fixation – advantageous.
- **Long fixation in aldehydes** - inhibits enzyme activity.
- **Long fixation in oxidizing fixatives** – degrade the tissue.



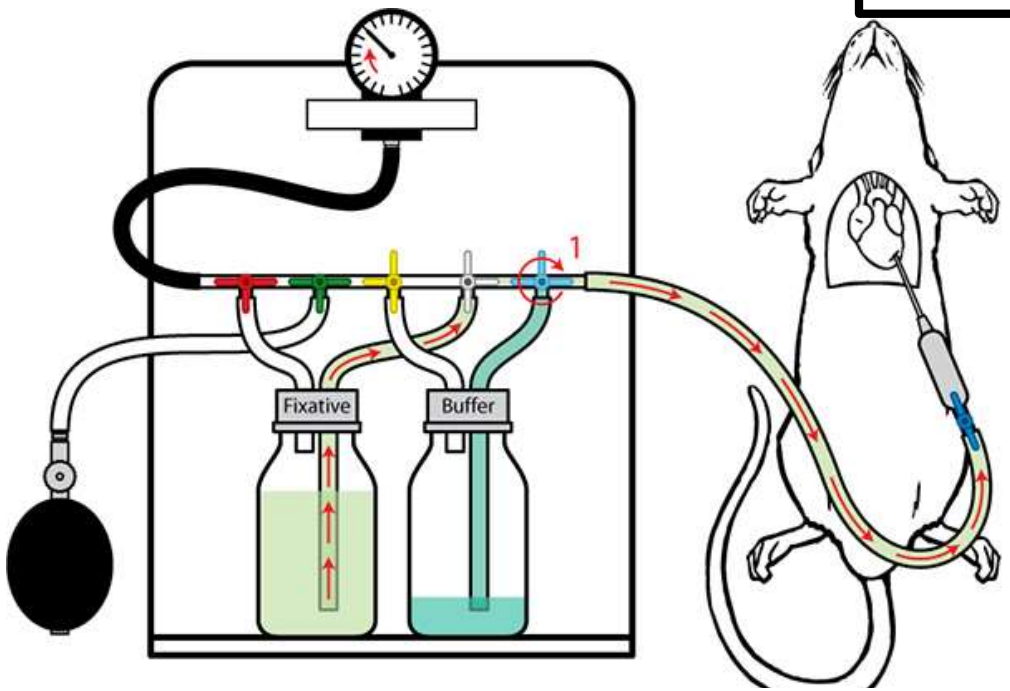
METHODS OF FIXATION:

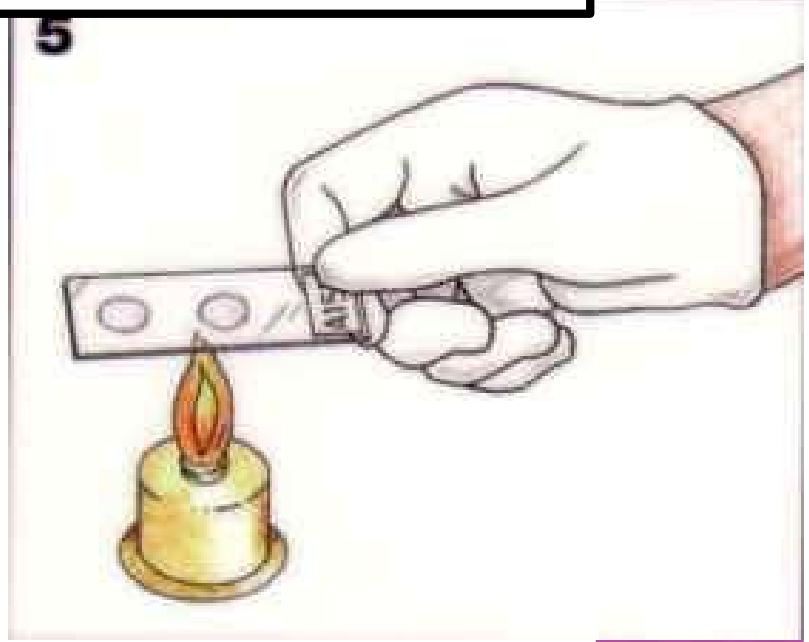
CHEMICAL METHODS

- Immersion/ in vitro fixation
- Perfusion/ in vivo fixation
- Vapour fixation
- Coating/Spray fixation

PHYSICAL METHODS

- Heat fixation
- Freeze drying and freeze substitution
- Microwave fixation/Stabilization





TYPES OF FIXATION

- Three types of fixation

Heat fixation

Perfusion

Immersion

PHYSICAL METHODS

- Heating, microwaving, and freeze-drying
- Independent processes
- Not used commonly in the routine practice of medical or veterinary pathology, anatomy, and histology, except for the use of dry heat fixation of microorganisms prior to Gram staining.

PHYSICAL METHODS: HEAT FIXATION:

- After a smear has dried at room temperature, the slide is gripped by tongs or a clothespin and passed through the flame of a Bunsen burner several times to heat-kill and adhere the organism to the slide.
- Routinely used with bacteria and archaea.
- generally preserves overall morphology but not internal structures.
- Heat denatures the proteolytic enzyme and prevents autolysis.

PHYSICAL METHODS: MICROWAVE FIXATION

- Microwave heating speeds fixation
- Reduce times for fixation of some gross specimens and histological sections from more than 12 hours to less than 20 minutes
- Microwaving tissue in formalin results in the production of large amounts of dangerous vapors, so in the absence of a hood for fixation, or a microwave processing system designed to handle these vapors, this may cause safety problems..

PHYSICAL METHODS:FREEZE-DRYING AND FREEZE SUBSTITUTION

- Useful technique for studying soluble materials and small molecules;
- tissues are cut into thin sections, immersed in liquid nitrogen, and the water is removed in a vacuum chamber at −40°C.
- The tissue can be post-fixed with formaldehyde vapor.
- In substitution, specimens are immersed in fixatives at −40°C, such as acetone or alcohol, which slowly remove water through dissolution of ice crystals, and the proteins are not denatured; bringing the temperature gradually to 4°C will complete the fixation process .
- These methods of fixation are used primarily in the research environment and are rarely used in the clinical laboratory setting

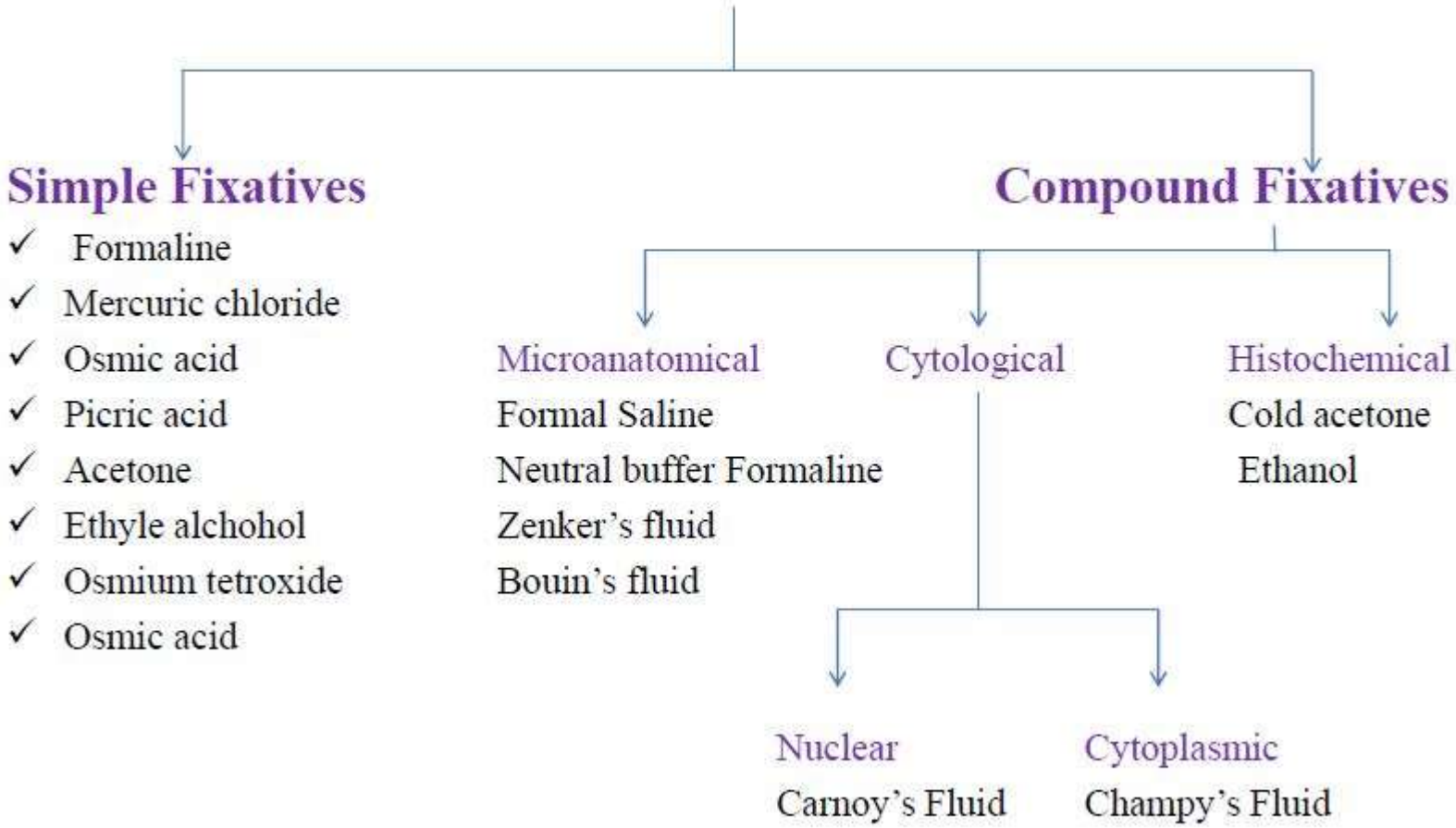
CHEMICAL METHODS: IMMERSION

- The sample of tissue is immersed in fixative solution of volume at a minimum of 20 times greater than the volume of the tissue to be fixed.
- The fixative must diffuse through the tissue to fix, so tissue size and density, as well as type of fixative must be considered.
- This is a common technique for cellular applications.
- Using a larger sample means it takes longer for the fixative to reach the deeper tissue.

CHEMICAL METHODS: PERFUSION

- Fixation via blood flow.
- The fixative is injected into the heart with the injection volume matching cardiac output.
- The fixative spreads through the entire body, and the tissue doesn't die until it is fixed.
- This has the advantage of preserving perfect morphology
- Disadvantages are that the subject dies and the cost of the volume of fixative needed for larger organisms is high.

Chemical Fixatives



Based on chemical agents

1. cross- linking fixatives / aldehydes

-formaldehyde, glutaraldehyde

2. protein denaturing fixatives/ coagulants

- acetic acid, methyl alcohol, ethyl alcohol

3. oxidizing agents

- osmium tetroxide, potassium permanganate, potassium dichromate

4.Other cross linking agents

- carbodiimides

5.Miscellaneous

- mercuric chloride, picric acid, non-aldehyde –containing fixatives, dye stuffs

General classification

1. **Aldehydes:** formaldehyde, gluteraldehyde, acrolein
2. **Oxidizing agents:** osmium tetroxide, potassium permanganate, potassium dichromate
3. **Protein denaturing agents:** acetic acid, methyl alcohol, ethyl alcohol
4. **Other cross linking agents:** carbodiimides
5. **Physical:** heat, microwave
6. **Unknown mechanism:** mercuric chloride, picric acid
7. **HOPE fixatives:** formalin like morphology, good protein antigenicity for enzyme histochemistry, good for RNA & DNA yeilds

Baker classification

- **Coagulant fixatives includes:**

1. Formaldehyde
2. Gluteraldehyde
3. Osmium Tetroxide
4. Potassium Dichromate
5. Acetic Acid

- **Non-Coagulant fixatives includes:**

1. Alcohol
2. Zinc salts
3. Mercuric chloride
4. Chromium trioxide
5. Picric Acid

CLASSIFICATION-BASED ON STRUCTURE

1. **Micro-anatomical fixatives:**

   When anatomy of tissues with correct relationship of tissue layers & large aggregate of cells is to be preserved.

2. **Cytological fixatives:**

   To preserve constituent elements of cells.

   Elements being preserved at the expense of penetration, ease of cutting & loss of other cell structures.

3. **Histochemical fixatives:**

   When histochemical tests are to be applied.

MICROANATOMICAL FIXATIVES

I. **Routine formalin fixatives:**

1. 10% Formol-Saline:

- 10% formalin in 0.9% sodium chloride.
- Layer of marble chips/calcium carbonate added to neutralize formic acid production.

2. Buffered Formalin:

- Formalin – 10ml
- Acid sodium phosphate monohydrate 0.4g
- Anhydrous disodium phosphate 0.65g
- Water to 100ml

3. Formol Calcium(Baker)

- Formalin – 10ml
- Calcium chloride – 2g
- Water – 100ml
- Chloride preserves phospholipids

4. Formol Calcium(Lillie)

- Acetate instead of chloride
- Easily prepared
- Widely used for routine fixation.

II. **FORMALIN FIXATIVES FOR CARBOHYDRATES:**

1. Buffered Gluteraldehyde
2. Heidenhain's Susa:

- Mercuric chloride – 4.5g
- Sodium chloride – 0.5g
- Trichloroacetic acid – 2g
- Acetic acid – 4ml
- Formalin – 20ml
- Distilled water to 100ml
- Adv – **Excellent fixative for routine biopsy work**.
- Brilliant staining with **good cytological detail**.
- Rapid penetration, minimum shrinkage
- **Disadv** – intolerant fixative
- Transfer to absolute alcohol is required

3. Zenker's fluid:

- Mercuric chloride – 5g
- Potassium dichromate – 2.5g
- Sodium sulphate – 1g
- Distilled water to 100ml
- Glacial acetic acid immediately before use – 5ml
- Adv – rapid & even penetration

beneficial effect on **cytologic, nuclear chromatin & fibre stain**

- Wash in running water.
- Fixation complete in 12hours

4. HELLY'S FLUID (ZENKER FORMOL)

- Distilled water 100 ml
- Potassium dichromate 2.5g
- Sodium sulphate 1g
- Mercuric chloride 5g

- 5ml of 40% formaldehyde before use
- Formalin instead of acetic acid.
- Excellent fixative for **bone marrow, spleen**
- Aka Spuler's or Maximow's fluid

7. Bouin's fluid:

- Picric acid – 75ml
- Formalin – 25ml
- Glacial acetic acid – 5ml
- Rapid & even peneration
- Fixed tissue gives brilliant staining with trichome methods
- Used to demonstrate **glycogen.**
- Good for **GIT biopsies**

5. Gendre's Fluid – good glycogen fixation

6. Rossman's Fluid – carbohydrate fixation

CYTOLOGICAL FIXATIVES

I. **NUCLEAR FIXATIVES:**

1. Carnoy's Fluid:

- Absolute alcohol – 60ml
- Chloroform – 30ml
- Glacial acetic acid – 10ml
- Penetrates very rapidly
- Excellent nuclear fixation
- Preserves **Nissl substance& glycogen**
- chromosomes
- Destroys cytoplasmic elements.
- Rapid fixative – urgent diagnosis.

2. Flemming fluid:

- Only 5-10 times of tissue bulk is required.
- Used as secondary fixative for **myelin** following primary formalin fixation.

3. Newcomer's fluid:

- Fixation of **chromosomes**
- Preserves chromatin better than Carnoy's.

II. CYTOPLASMIC FIXATIVES:

1. Champy's fluid:

- Should be freshly prepared.
- Poor & uneven penetration
- Preserves **mitochondria, fat, yolk, lipids**
- Preferred for mitochondria

2. Regaud's fluid – **mitochondria & chromaffin tissue**
3. Muller's fluid – bone specimens
4. Zenker Formol
5. Schaudinn's fluid – For wet smears

HISTOCHEMICAL FIXATIVES

- Preserve the constituent to be demonstrated & its morphological relationships.
- Without affecting the reactive groups & reagent to be used in its visualization.
- **Best** – cryostat cut sections of rapidly frozen tissue.
- Formol saline
- **Cold acetone** – where enzymes are to be studied especially **phosphatases**
- Absolute alcohol
- Vapour fixatives like formaldehyde, acetaldehyde, gluteraldehyde, acrolein to fix cryostat cut sections of fresh tissue & blocks of frozen dried tissue

FIXATION FOR SPECIALIZED TECHNIQUES

- **Urgent paraffin section**

  Thin slices are fixed in alcohol containing fixatives Carnoy's , formol alcohol

- **Enzyme Histochemistry-**

  Fix in cold formol calcium

- **Transmission Electron Microscopy**

  Osmium tetroxide, glutaraldehyde.

- **Electron microscopy** - 2% glutaraldehyde

- **Immunoflourescence** – unfixed cryostat

MECHANISM OF ACTION:

- **Aldehydes (cross linking agents)-** act by creating covalent chemical bonds between proteins of tissues- anchor the insoluble compound to cytoskeleton-protect secondary as well as tertiary structure of protein- provide mechanical strength/ additional rigidity to tissue structure.

- **Oxidizing agents-** joins with various side chains of protein molecules & other biomolecules- allow formation of cross link- stabilizes tissue structure

- **Protein denaturing agents**: reduce the solubility of protein without combining with it & disrupts the hydrophobic bonds which is needed for its tertiary structure to form.
- **Mercurials (B5 fixatives**): it increases the staining brightness & give good nuclear detail. Good for reticulo-endothelial tissue & haemo-poetic tissue.

- **Picrates**:binds with histone & basic proteins to form crystalline picrates with amino acid & precipitates protein.

FORMALDEHYDE

- Gas soluble in water up to 40% by wt.
- Available as 40% formaldehyde or formalin.
- **Stabilizer** – 10-14% **methanol**
- **10% formalin**
- Acidic solution.
- On storage becomes acidic by formation of formic acid.
- Colourless.
- **Turbid** on keeping - **paraformaldehyde.**

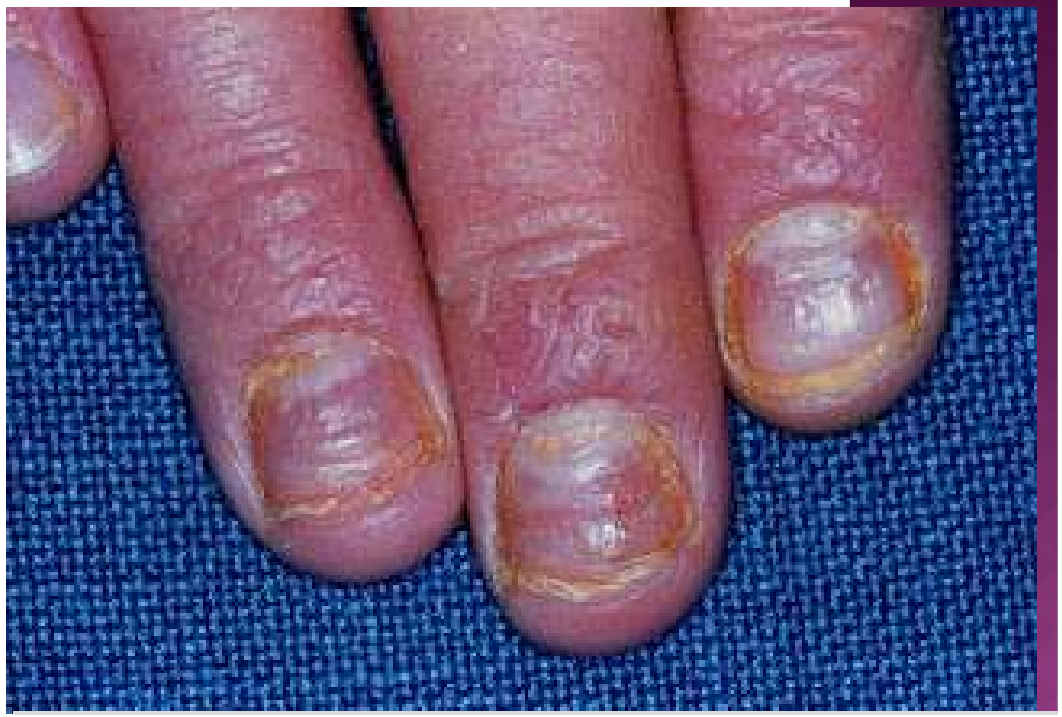
- **Yellow** – contaminated with **ferric iron** from metal containers. **Positive prussian blue** reaction.
- fixes protein, lipids well preserved.
- **Favors** staining of acidic structures like **nuclei** with basic dyes
- **Diminishes** effect of acid dyes on basic structures like **cytoplasm**.

ADVANTAGES:

- Formalin is cheap
- Easy to prepare
- Relatively stable
- Frozen sections can be prepared with ease.
- Staining of fat and tissue enzymes.
- Penetrates tissues well.
- Beneficial hardening with little shrinkage

- Natural tissue colors are retained.
- Does not require washing before processing.
- **Best fixative for nervous system**

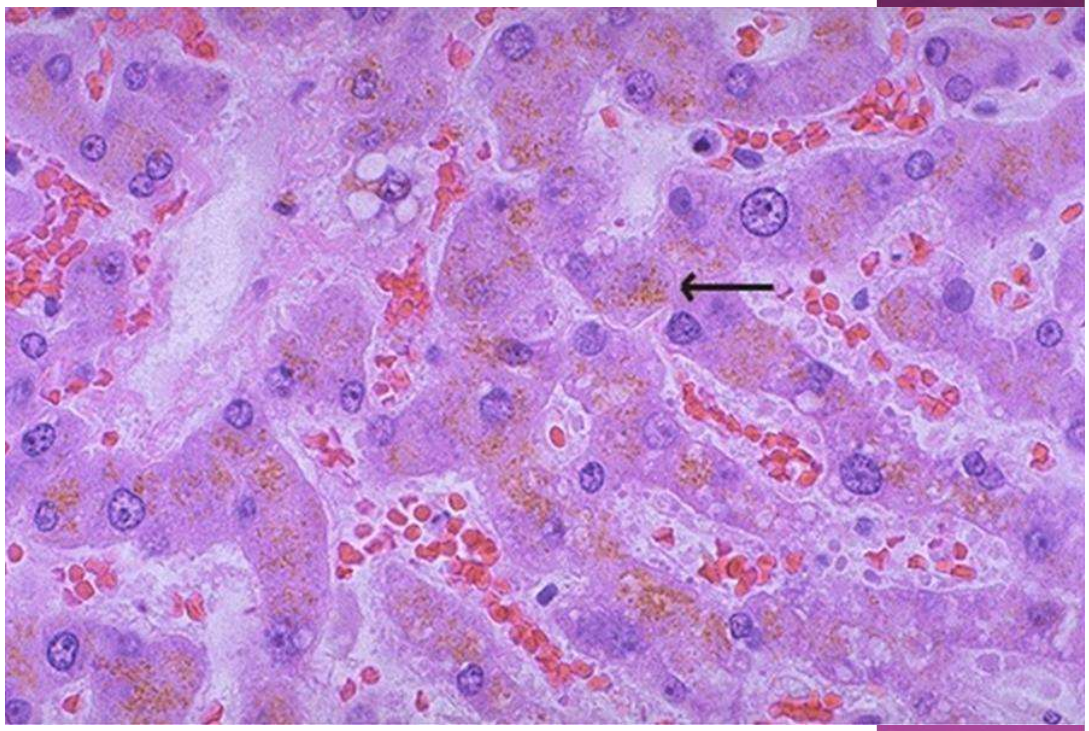
- **Disadvantage:**
- unpleasant vapour irritation to eyes & respiratory epithelium
- Formalin dermatitis



CHEMICAL BASIS OF FIXATION

FORMALIN PIGMENT

- Brown, granular material, **extracellular**, birefringent
- Progressive in deposition
- Often seen after several days
- Action of acid formalin on blood
- **Avoided** by using **buffered** **formalin**.
- **Removed** – treatment with saturated alcoholic solution of **picric acid** for 20mins

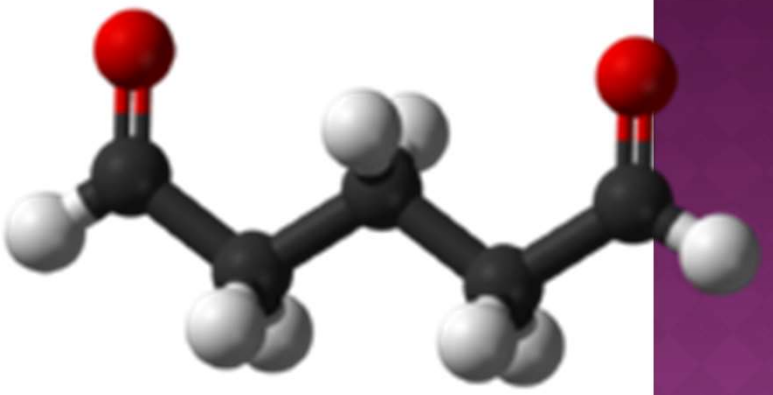


PINK DISEASE

- Peculiar **artifact** seen in sections fixed in formal saline & stained with **H&E**.
- Complete or partial failure of nuclei to stain with haematoxylin – **take up eosin – loss of nuclear margin distinction.**
- Lymphoid & epithelial tissue – most distinct
- Patchy distribution
- **Avoided** by using - **2% acetic acid** in 10% formalin
- **When present** – treat with **1% hydrochloric acid** in **absolute alcohol** for 1 hour before staining with H&E.

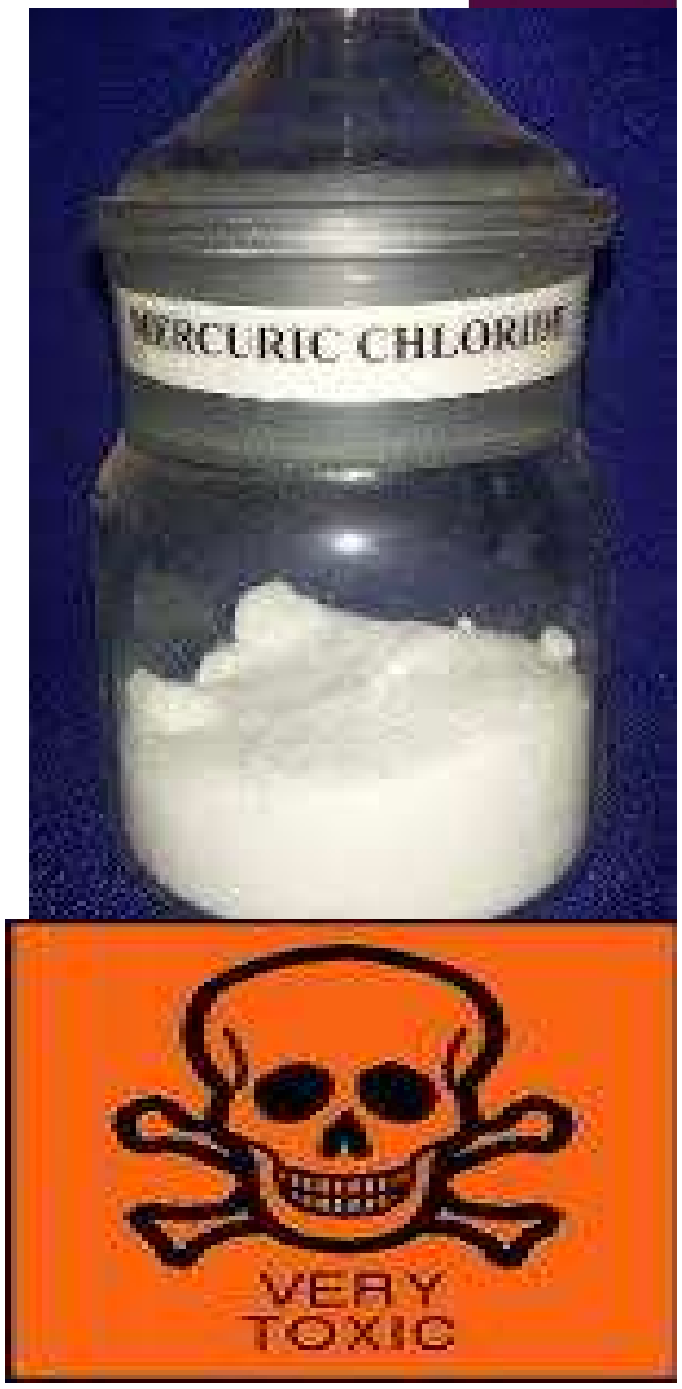
GLUTERALDEHYDE

- Used for **Electron Microscopy** with osmium tetroxide
- **Advantage:**
- Most efficient cross linking agent for **collagen**
- More rapid fixation than formalin.
- **Disadvantage**:
- Poorer penetration
- False positivity with **PAS**
- More costly



MERCURIC CHLORIDE

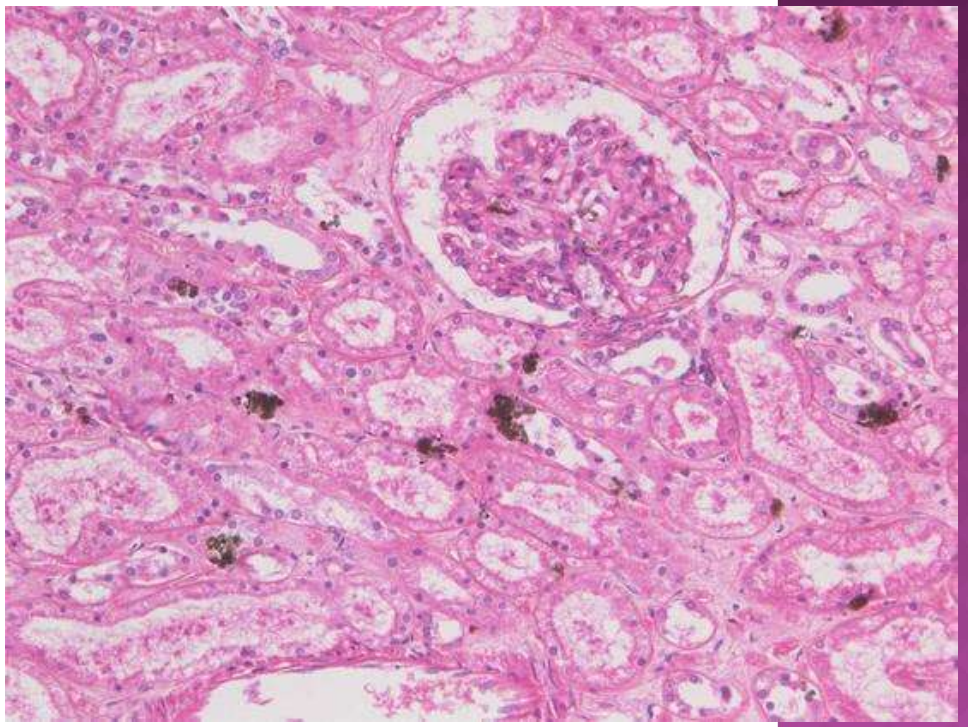
- White crystalline substance.
- Powerful **protein precipitant**, fixes both nucleus & **cytoplasm** well favoring its staining.
- **Conjunction with other fixatives**.
- **Adv**: rapidly penetrates & hardens tissue, radio-opaque
- **Disadv**: Extremely **poisonous & corrosive to metals**.
- Intolerant fixative.
- Pollution to environment.



MERCURIC PIGMENT

- Mercury pigment – brown to black granular deposit.
- **Treatment-**

1. Place section in 0.5% iodine in 80% alcohol for 3mins
2. Rinse in water
3. Place in 3% aqueous sodium thiosulphate for 3mins
4. Wash in running water for 1-2mins



PICRIC ACID

- Bright yellow crystalline substance.
- Damped with water because of **explosive properties**.
- Enhances **cytoplasmic staining**.
- Acts as **mordant**
- Much shrinkage but little hardening.

POTASSIUM DICHROMATE

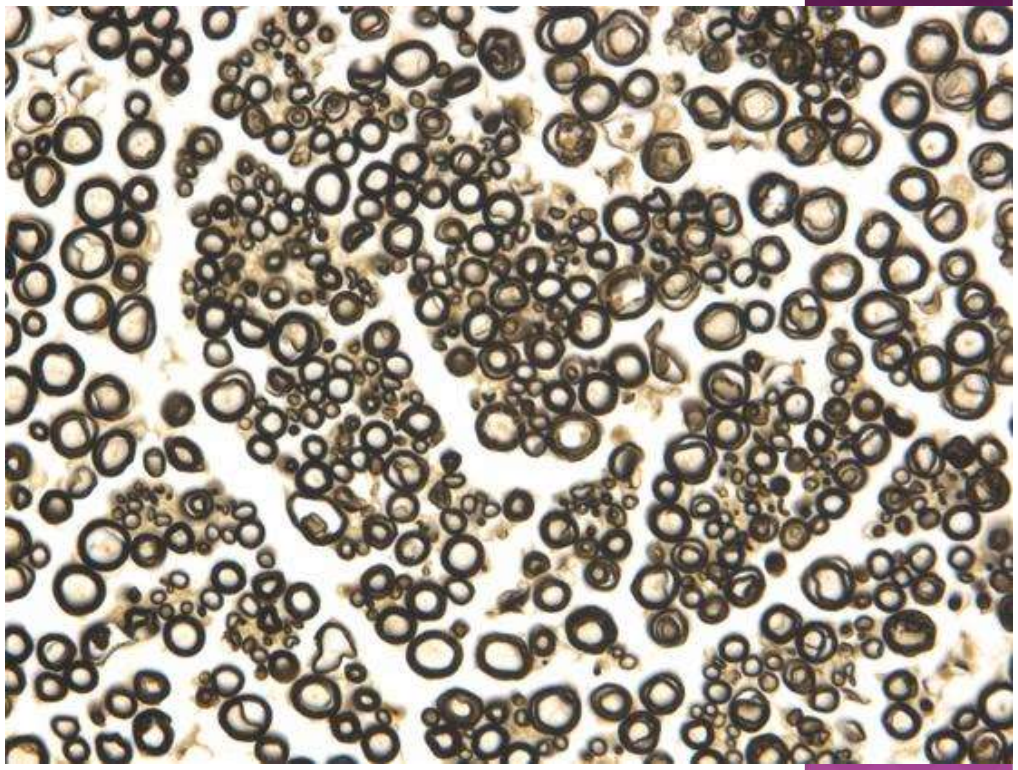
- Orange crystalline substance
- **Less acidic pH** – fixes cytoplasm & **mitochondria**
- **More acidic pH** – fixes nucleus & cytoplasm
- Mordant
- Wash in running water after to prevent formation of insoluble precipitate.
- Prolonged exposure causes tissue to become brittle.

CHROMIC ACID

- Dark red crystals of anhydride.
- Powerful oxidizing agent,
- Requires washing with running water.

OSMIUM TETROXIDE

- Pale yellow.
- Demonstrates lipid like **myelin.**
- Excellent preservation of detail of single cells hence used for **EM.**
- Uneven penetration for pieces more than 2-3mm
- Storage in dark, cool place
- Vapour is irritating, causes conjunctivitis
- Uneven fixation



ACETIC ACID

- Colour-less liquid with pungent odour.
- Used in compound fixatives
- Swells collagen fibres
- Precipitates nucleoproteins
- Solvent action on cytoplasmic granules.

ETHYL ALCOHOL

- Colour-less.
- Powerful dehydrating agent.
- Causes shrinkage & hardening
- Coagulates protein but not nucleoprotein.
- Precipitates glycogen.
- Used in histochemical method for enzymes.

FIXATION FOR INDIVIDUAL TISSUES

tissue	Time interval	fixative
Adrenal gland	1 or 2 hr	Formaldehyde
eye	Immediately after death or within 2 hrs	Formol saline
Alimentary tract	Immediately after death	Susa fixative
Blood forming organ	-do-	Zenker's fluid
Testis/ovary	-do-	Susa fixative
Lung/kidney/bone	Fairly resistant to postmortem changes	Susa fixative
Renal biopsy		Neutral buffer formalin

Target	Fixative of Choice
Proteins	Neutral Buffered Formalin
Enzymes	Frozen Sections
Lipids	Frozen Sections*, Glutaraldehyde/Osmium Tetroxide
Nucleic Acids	Alcoholic fixatives, HOPE
Mucopolysaccharides	Frozen Sections
Biogenic Amines	Bouin Solution, Neutral Buffered Formalin
Glycogen	Alcoholic based fixatives

SECONDARY FIXATION

- Tissues fixed with 2 fixatives in succession.
- Improved preservation & staining.
- Tissues fixed in buffered formalin – fixed with mercuric chloride.
- Tissues fixed with gluteraldehyde is post fixed with osmium tetroxide for electron microscopy.

POST CHROMATIZATION

- Treatment of tissues with 3% potassium dichromate following normal fixation.
- Before processing – tissue in dichromate solution for 6-8days
- After processing – for 12- 24 hrs
- Both followed by washing in running water
- Employed to mordant tissues.
- Mitochondria & myelin demonstrated.
- Improved preservation and staining of elements.

Is fixation always necessary????

WASHING AFTER FIXATION

↓

Good staining

SUMMARY

- **Formalin** is used for all **routine surgical pathology and autopsy tissues** when an H & E slide is to be produced.
- **Formalin** is the most forgiving of all fixatives when conditions are not ideal.
- **B5 fixatives** are recommended for **reticulo-endothelial tissues including lymph nodes, spleen, thymus, and bone marrow.**

- **Bouin's solution** is recommended for fixation of **testis**, general fixative for **connective tissue stains.**

- **Glutaraldehyde** is recommended for fixation of tissues for **electron microscopy**.

- **Alcohols,** specifically ethanol, are used primarily for **cytologic smears**.

IMPORTANT POINTS

- Bouin's fluid- embryo fixation
- Commercially 40% by volume
- 37% by weight
- Dessication- to study inorganic components
- Post fixation- softening by 4% phenol for 3-4 days- for better cut sections of tumours
- Secondary fixation- to prevent hardening effect
- Phenoxitol- ideal fixative, but very costly

REFERENCES:

- Carleton's histological technique
- Histology, Microscopy & Photomicrography by Dr D.R. Singh
- Histopathological & histochemical techniques- C.F.A. CULLING


Thank You