



HISTOPATHOLOGIC & CYTOLOGIC TECHNIQUES LEC

Module 8: Bone Preparation: Decalcification

MTHC31-24

Prof. Neil Allen Elquinto

TOPIC OUTLINE

- I. Bone
- II. Decalcification
 - A. Preparation of Bone Specimens for Decalcification
 - B. Types of Specimens
 - C. Principle of Decalcification
 - D. Factors Affecting the Process of Decalcification
 - E. Measuring the Extent of Decalcification
 - F. Post-Decalcification
 - G. Tissue Softeners

BONE

Bones are the main object of decalcification in a surgical pathology laboratory, but other specimens, such as teeth, calcified heart valves are also required in this procedure. It is necessary for the calcified tissues to adjust the hard substances of bones to the softness of paraffin embedding medium.

There are two types of adult bones. The **compact bone**, known as the strong bone, supports the shaft of long bones like femur, tibia, and external surface of flat bones ex. the skull. The **trabecular or spongy bone** is composed of a mesh of bone strands which forms the weight-bearing structure of the diaphysis, epiphysis, or vertebrae.

The three major components of the bone are the mineral, cells, and organic extracellular matrix.

Component	Description
Mineral	➤ During the initial mineralization phase, the mineral in the bone is approximately 38% calcium deposited as amorphous calcium phosphate. ➤ Later on, it is transformed into hydroxyapatite with addition of calcium, it forms a crystal lattice of needle-like crystals in which carbonate, citrate, and fluoride ions as well as magnesium, potassium, and strontium can be substituted or included.
Cells	➤ The three types of bone cells are the osteoblasts, osteocytes and osteoclasts. ➤ Some of the osteoblasts are fully differentiated cells and form an osteoid. ➤ On the surface of actively forming bone, they appear as plump cells with eccentric nuclei distal to the bone surface and supported by a basophilic cytoplasm.
Organic Extracellular Matrix	➤ Composed of collagen fibers and ground substance.

DECALFICATION

Decalcification is the process of removing Calcium or Lime Salts from tissues. It is performed on bones, teeth, calcified tissues (ex. Tuberculous lungs, arteriosclerotic vessels). I adjust the hard substance of bones to the softness of paraffin embedding medium.

Decalcification enables the histotechnologist to cut soft sections of the bone using the microtome, so that they can be processed like any other soft tissue of the body. It prevents poor cutting of hard tissues/knife damage.

Decalcification is a lengthy procedure, as bone pieces have to be left in the decalcifying agents for several days or even weeks depending on the size of the tissue. Many of the grossing and cutting techniques for bone require the use of a high-speed saw and/or long periods in decalcifying solution, prior reducing the specimen to a size that can be easily processed, embedded and sectioned.

Decalcification should be done after fixation and before impregnation, to ensure and facilitate the normal cutting of sections and to prevent obscuring the microanatomic detail of such sections by dust and other cellular debris.

If the tissue size is very large, use a fine-fret saw. Decalcifying agents should be changed regularly.

- **“GRATING”** sensation during cutting = place block in 10 % HCl for 1 hour.
- **Rapid decalcification** produces an effect on nuclear staining – (failure of nuclear chromatin to take up hematoxylin).

Inadequate decalcification may result in poor cutting of hard tissues and hard tissue and damage to the knife edge during sectioning. Poorly-fixed specimens become macerated during decalcification and stain poorly afterwards. Extending the fixation time for bone specimens before commencing decalcification is



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important. It is important to provide ready access for the fixative to penetrate the bone, so skin and soft tissue should be removed from large specimens if practicable.

Cartilage does not require any softening, except if some calcified areas are present. After fixation, depending on the amount of adjusted soft tissue, the toenail should be rinsed off with soapy water once it becomes pliable.

Preparation of Bone Specimens for Decalcification

Bone biopsy is similar to soft tissue biopsy except that bone needs decalcification before processing. An X-ray of the specimen may also select samples for processing. Interpretation of biopsy requires assessment of the relationship between mineralized and non-mineralized bone or osteoid. The methods used are:

1. Mineralized bone sections embedded in methyl methacrylate (MMA) plastic. It is the preferred method for metabolic bone disease work-up.
2. Frozen section forms an undecalcified bone biopsy.
3. Silver impregnation method of Tripp and McKay (1972) demonstrating bone and osteoid in a decalcified, paraffin embedded bone section.

Types of Specimens

Specimen	Description
Amputated Limbs	➤ These are often sent to the laboratory immediately after surgery secondary to tumor, inflammation and gangrene. ➤ They are often delivered without fixative, and must be taken care of as soon as possible.
Resected Specimens	➤ These include bones with benign or low-grade malignant tumors and arthritic femoral heads. ➤ They are usually with established diagnosis and considered diagnosis and are considered less urgent.

Principle of Decalcification

Strong mineral acids such as 10% HCl, or weak organic acids, such as 5-10% HCOOH, form soluble calcium salts in an ion exchange that moves calcium into the decalcifying solution. The same final effect makes 14% EDTA an ideal chelating agent that sequesters metallic ions, including calcium, in aqueous solution. It is also possible to prepare bone specimens by infiltrating them with acrylic or epoxy resins which, when polymerized, have a hardness equivalent to that of mineralized bone and hence do not require decalcification at all.

Buffered HCHO is a satisfactory fixative for bone but where the preservation of bone marrow is important, some laboratories use alternatives such as zinc formalin mixtures, B-5, formol-acetic alcohol (Davidson's fixatives), or Bouin's solution.

Factors Affecting the Process of Decalcification

Factor	Description
Concentration and Volume of Decalcifying Agent	➤ The more concentrated the decalcifying agent is, the decalcification time becomes more rapidly, however, it is more harmful to the tissue. Rapid depletion of an acid or chelator by their reaction with calcium can be avoided by using large volumes of fluids compared with the volume of tissue. ➤ Recommended fluid volume to tissue ratio is 20:1.
Fluid Access	➤ A decalcifier should have ready access to all surfaces of the specimen. This will enhance diffusion and penetration to the specimen and will facilitate solution, ionization, and removal of calcium. Decalcification can be hastened by suspending the tissue in a decalcifying solution for greater fluid access.
Size and Consistency	➤ Increase in size and consistency of tissues will require longer periods for complete decalcification. Dense bone tissues usually require up to 14 days or longer in order to complete the process.
Agitation	➤ Gentle agitation may increase the rate of decalcification. Mechanical agitation and moving of the tissue in solution usually influences fluid exchange, accelerates the rate of diffusion and speeds up the decalcification process.
Temperature	➤ Increased temperature will hasten decalcification, but it will also increase the damaging effects of acids on tissue. Standard temperature +25°C but in practice a room temperature (RT) range of +18°C to +30°C is acceptable. At +60°C, bones tissues and cells may become completely macerated almost as soon as they are decalcified.

Measuring the Extent of Decalcification

Method	Description
Physical or Mechanical Test	➤ Bending, probing, needling / pricking or inserting a pin, slicing the tissues, razor, touching, squeezing of tissues or scalper directly into the tissues. Prone to produce artifacts and destroy cellular details.



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X-ray method	➤ Very expensive but most ideal. It is not for mercuric chloride fixed tissues.
Chemical method (CaOx)	➤ It is simple, reliable and convenient. Detection of calcium in acid solutions by precipitation of Calcium Hydroxide or Calcium Oxalates. ➤ Solutions: ○ concentrated NH_4OH ○ saturated $(\text{NH}_4)_2\text{C}_2\text{O}_4$ performed on the discarded fluid. ○ Litmus paper: blue to red ○ Clear solution à complete decalcification ○ Cloudiness or Precipitation à incomplete

Post-Decalcification

- Decalcified tissues are neutralized by:
 - Immersing in saturated Li_2CO_3 , or 5-10% NaHCO_3
 - Rinsing in running tap water.
 - Storing in formol saline containing 15% sucrose or PBS with 15-20% sucrose at $+4^\circ\text{C}$

Tissue Softeners

- Perenyi's
- Lendrum's :4% phenol
- Molliflex
- 2%HCl
- 1% HCl in 70% Alcohol



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Module 8: Bone Preparation: Decalcification (Decalcifying Agents / Methods)

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TOPIC OUTLINE

- I. Decalcifying Agents / Methods of Decalcification
- II. Acids
 - A. Nitric Acid
 1. Formol Nitric Acid
 2. Perenyi's Fluid
 3. Phloroglucin–Nitric Acid
 - B. Hydrochloric Acid (HCl)
 1. Von Ebner's Fluid
 - C. Formic Acid
 1. Formic Acid- Sodium Citrate Solution
 - D. Trichloroacetic Acid
 - E. Sulfurous Acid
 - F. Chromic Acid (Flemming's Fluid)
 - G. Citric Acid – Citrate Buffer
- III. Chelating Agents
- IV. Ion Exchange Resins
- V. Electrophoresis (Electrical Ionization)

DECALCIFYING AGENTS / METHODS OF DECALCIFICATION

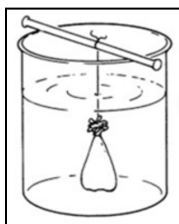
- **Acids** – HNO₃, HCl, Formic, TCA, Sulfurous, Chromic, Citric
- **Chelating Agents** – EDTA (Versene)
- **Ion Exchange Resins (Ammonium form of polystyrene resin)** – 1-14 days – spread on the bottom of the container.
- **Electrical Ionization (Electrophoresis)** – attraction of Ca to negative electrode

ACIDS

- It is the most widely used for routine decalcification.
- It is also stable, easily available and cheap.

A. Nitric Acid

- The most commonly used, rapid decalcifying agent, can be removed by 70% alcohol.
- Inhibits nuclear stains – prevented by combining with formaldehyde or alcohol.



1. Formol Nitric Acid

- Rapid acting, good nuclear staining, less tissue discoloration by 5% sodium sulfate or 0.1% urea.
- Decalcification time: 1-3 days

Composition	
Conc. Nitric Acid	10mL
Strong Formaldehyde 40%	5mL
Distilled water	85mL

2. Perenyi's Fluid

- Decalcified and softens tissues at the same time. Good nuclear and cytoplasmic staining. Maceration avoided by chromic acid and ethyl alcohol.
- Disadvantages: slow, difficult to assess complete decalcification by chemical means.
- Decalcification time: 2-7 days

Composition	
Nitric Acid 10%	40mL
Chromic Acid 0.5%	30mL
Absolute Ethyl Alcohol	30mL

Mix shortly before use.
Chromic acid must be collected for proper disposal.

3. Phloroglucin-Nitric Acid

- Most rapid decalcifying agent. Poor nuclear staining.
- Decalcification time: 12-24 hours
- When decalcification is complete, acid must be removed by 3 changes of 70 to 90% ethanol.

Composition	
Conc. Nitric Acid	10mL
Phloroglucin	1gm
Nitric Acid 10%	100mL

To be added after disappearance of dense white fumes formed by combining the first two ingredients.

B. Hydrochloric Acid (HCl)

- Inferior compared to Nitric Acid as a decalcifying agent.
- Slower action, greater tissue distortion. Good nuclear staining.
- Recommended for surface decalcification.

1. Von Ebner's Fluid

- Good cytologic staining.
- Recommended for teeth and small pieces of bones.

Composition	
Saturated aqueous solution of NaCl 36%	50mL
Conc. Hydrochloric acid	8mL
Distilled water	50mL

C. Formic Acid



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- Better nuclear staining with less tissue distortion. Safer to handle than Nitric and HCl.
- Recommended for postmortem research tissues upon the addition of sodium citrate. It is both fixative and decalcifying agent. For small pieces of bone and teeth.
- Decalcification time: 2-7 days

Composition	
Formic acid (sp.grav 1.20)	10mL
Formol Saline	90mL

1. Formic Acid-Sodium Citrate Solution

- Slow, not recommended for routine purposes. Permits better nuclear staining than Nitric Acid. Requires neutralization with 5% Na sulphate. Recommended for autopsy materials, Bone Marrow, Cartilage and tissues for research.

Composition	
45% Formic Acid	50mL
20% Na Citrate	50mL

D. Trichloroacetic Acid

- Weak decalcifying agent. Permits good nuclear staining. Slow decalcifying agent. Suitable only for small bone spicules.
- Decalcification time: 4-8 days.

Composition	
Trichloroacetic acid	5gm
10% Formol Saline	95mL

E. Sulfurous Acid

- Very weak decalcifying agent. Suitable only for minute pieces of bone.

F. Chromic ACid (Flemming's Fluid)

- Fixative and decalcifying agent. Nuclear staining with hematoxylin is inhibited. Forms precipitate at the bottom. Carcinogenic, corrosive to skin.

Composition	
Chromic acid	15mL
Osmium tetroxide	4mL
Glacial Hac	1mL

G. Citric Acid-Citrate Buffer

- Permits good nuclear and cytoplasmic staining.
- Decalcification time: 6 days.

Composition	
7% Citric Acid	5mL

4% Ammonium Citrate	95mL
1% Zinc sulfate	0.2mL
Chloroform (preservative) few drops	

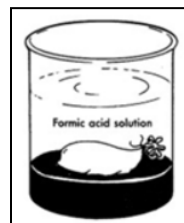
CHELATING AGENTS

- Substances which combine with Calcium ions and other salts (Fe, Mg).
- EDTA (Versene, Sequestrene) – most common chelating agent, will not bind Ca at pH below 3.0. Permits excellent staining. Very slow – 1-3 weeks. Slight tissue hardening produced EDTA inactivates alkaline phosphatase – add magnesium chloride.

Composition	
EDTA disodium salt	5.5mL
Distilled Water	90mL
Formaldehyde (37-40% stock)	10mL

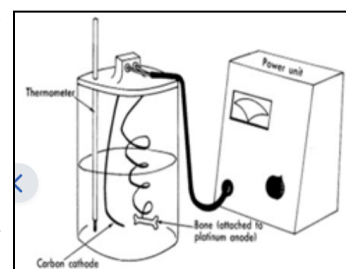
ION EXCHANGE RESINS

- Ammonia form of Polystyrene Resin. Hastens decalcification by removing Ca ions from Formic acid- containing decalcifying solutions. Artifacts produced, usually caused by CO2 bubbles. Slow decalcification time– 1-14 days. Degree of calcification cannot be measured by chemical means.



ELECTROPHORESIS (ELECTRICAL IONIZATION)

- Process whereby positively charged Ca ions are attracted to a negative electrode and subsequently removed from the decalcifying agent, satisfactory for small bone fragments. Uses electricity:



Solution used for Electrolytic Decalcification	
Formic Acid 88%	100mL
Conc. Hydrochloric Acid	80mL
Distilled water	1000mL