4 Decalcification of Bony and Hard Tissue for Histopathology Processing

4.1 Introduction

The presence of calcium salt in the tissues makes them very firm to hard and this may damage the knife. Therefore, it is often necessary to remove calcium salt from the tissue and to make it soft for cutting in a microtome. The process of removal calcium salt from the tissue is known as decalcification (Box 4.1).

Aim: The basic aims of decalcification are:

1. Removal of calcium salt from tissue
2. No damage to tissue morphology
3. No significant effect in staining

Calcium-containing tissue: The tissue containing heavy amount of calcium salts are (1) the bone, (2) tooth, (3) pathological tissue such as in tuberculous lymph node, dystrophic calcification, and certain tumours such as teratomas, etc.

Requisites for successful decalcification: The following measures are helpful for successful decalcification:

- *Consistency*: Exact assessment of the consistency of the tissue is required for successful decalcification.
- *Small pieces*: The tissue should be cut in 2–6 mm thick sections because thicker tissue may take longer time to be decalcified.
- *Fixation*: Adequate fixation of the tissue is necessary for proper decalcification.

Box 4.1 Decalcification

Aim: Removal of calcium salt from tissue without damaging the morphology of the tissue

Calcium-containing tissue: (1) bone, (2) tooth, and (3) pathological calcification in tissue

Requisites for successful decalcification:

- Small tissue
- Adequate fixation
- Consistency
- Adequate volume of decalcifying agent
- Suitable choice of the decalcifying agent
- Exact end point detection

Factors controlling the rate of decalcification:

- Facilitates decalcification
- Higher concentration of the decalcifying agent
- Higher temperature
- Agitation of the solution
- Thin tissue
- Low density

Methods of decalcification

- Acid decalcification
- Ion-exchange resin

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Box 4.1 (continued)

- Electrical ionization
- Chelating solution
- Surface decalcification

End point determination of decalcification

- Radiographic examination
- Chemical test
- Physical test

- *Washing*: The fixed tissue should be washed thoroughly before decalcification.
- *Choice of decalcifying agent*: Suitable choice of the decalcifying agent is required.
- *Volume*: Optimum volume of the decalcifying agent is a prerequisite for proper decalcification.
- *End point detection*: The end point of the decalcification should be determined correctly.

4.1.1 Factors Controlling the Rate of Decalcification

- *Concentration*: The increased concentration of the decalcifying agent increases the rate of decalcification.
- *Temperature*: Increased temperature fastens the decalcification rate.
- *Density of bone*: Hard bone takes longer time to be decalcified.
- *Agitation*: Mild agitation of the decalcifying solution increases the rate.
- *Thickness of tissue*: Thinner tissue is quickly decalcified.

4.2 The Methods of Decalcification [1]

1. Acid decalcification
2. Ion-exchange resin
3. Electrical ionization
4. Chelating solution
5. Surface decalcification

Acid Decalcification This is the commonest method of decalcification in routine laboratory process. Acid makes the soluble calcium salt, and thereby calcium is removed from the tissue.

The strong acids:

- Hydrochloric acid
- Nitric acid

Weak acids:

- Formic acid
- Trichloroacetic acid

Strong Acid The strong acids are used in 5–10% concentration. They are rapid in action. However, careful attention is needed to prevent tissue damage. Neutralizer is also used to prevent any tissue distortion.

Aqueous Nitric Acid This is rapid in action. It does not impair staining if the end point is not crossed.

Preparation:

Nitric acid	5 ml
Distilled water	100 ml

Advantages:

1. Rapid in action
2. Good nuclear stain

Precaution: Nitric acid may give yellow colour to the tissue that can be removed by urea.

Nitric acid formaldehyde (10%)

Nitric acid	10 ml
Formalin	10 ml
Distilled water	80 ml

Advantages:

1. Rapid action.
2. Good nuclear stain.
3. Less chance of tissue damage and swelling.
4. Long-time washing by water is not needed.

Von Ebner's fluid

Saturated solution of sodium chloride: 175 g
Hydrochloric acid (concentrated): 15 ml
Distilled water: make it up to 1000 ml

Advantages:

1. Rapid action
2. Ideal decalcifying agent for the tooth

Disadvantage:

1. Nuclear staining is not good.

Perenyi's fluid

Nitric acid (10%)	40 ml
Chromic acid (0.5%)	30 ml
Absolute alcohol	30 ml

Advantages:

1. Provides excellent result
2. Softens the fibrous tissue
3. Cellular morphology well-preserved

Disadvantages:

1. Slower in action.
2. End point detection is difficult.

Weak acids

Gooding and Stewart solution

Formic acid	5 ml
Formalin (40% formaldehyde)	5 ml
Distilled water	90 ml

Advantage:

1. Decalcifying agent of choice in routine laboratory process

Disadvantages:

1. Slow acting and takes many days for decalcification.
2. Increased concentration of formic acid may enhance its capacity.

Trichloroacetic acid

10% Formal saline	95 ml
Trichloroacetic acid	5 g

Advantages:

1. Good for small biopsies
2. Good nuclear stain

Disadvantages:

1. Not good for hard bony tissue
2. Slower in action and takes 4–5 days

Table 4.1 highlights the advantages and disadvantages of various acid decalcifies.

4.3 Chelating Agents

Chelating agents are organic substances that adsorb metals. *Ethylenediaminetetraacetic acid (EDTA)*: EDTA is the most commonly chelating

Table 4.1 Acid decalcifying agents

Chemicals	Concentration	Advantages	Disadvantages
Aqueous nitric acid	5%	1. Rapid in action 2. Good nuclear stain	Gives yellow colour to the tissue
Nitric acid formaldehyde	10%	1. Rapid action: 1–3 days 2. Less chance of tissue damage and swelling 3. Long-time washing by water is not needed	Not a very good nuclear stain
Hydrochloric acid	8%	1. Rapid action 2. Ideal decalcifying agent for the tooth	Nuclear staining is not very good
Trichloroacetic acid	5 g in 95 ml formal saline	1. Good for small biopsies 2. Good nuclear stain	1. Not good for hard bony tissue 2. Slower in action and takes 4–5 days
Formic acid	5%	Decalcifying agent of choice in routine laboratory process	1. Slow acting and takes many days for decalcification

agents in the routine laboratory decalcification (Box 4.2). It binds with calcium of the hydroxyapatite crystals and forms a non-ionized soluble complex. The action of EDTA is slow and gentle, and it may take several weeks to remove calcium from the tissue. Therefore EDTA is not a suitable decalcification agent for dense bone or urgent removal of calcium. The main advantage of EDTA is the preservation of morphology and to maintain the tissue for various other techniques for research purpose. The action of EDTA is pH dependant and it works best in pH 7–7.6.

EDTA solution

EDTA	5.5 g
Formalin	100 ml
Distilled water	900 ml

Advantages:

1. It gives best morphological preservation of tissue.
2. Various other laboratory tests can be done on the tissue such as immunohistochemistry, fluorescent in situ hybridization technique, etc.
3. It is very good for bone marrow trephine biopsy as glycogen is preserved in the tissue.

Disadvantages:

1. Very slow process.
2. Maintenance of pH around 7 is necessary.
3. Thin tissue is needed.

Box 4.2 Ethylenediaminetetraacetic Acid (EDTA)

- Most commonly used
- Chelating agents
- Mode of action: Binds with calcium of the hydroxyapatite crystals to form a non-ionized-soluble complex
- Slow and gentle in action

Advantages:

- Morphological preservation of tissue
- Suitable to do various other laboratory tests
- Very good for bone marrow trephine biopsy

Disadvantages:

- Very slow process.
- Maintenance of pH around 7 is necessary.
- Thin tissue is needed.

4.3.1 Other Procedures of Decalcification

The other uncommon procedures for decalcification include:

- *Ion-exchange resin method*: In this technique an ion-exchange resin (sulfonated polystyrene resin) is used along with an organic acid as decalcifying fluid. It also produces faster decalcification with preservation of morphological details of the tissue.
- *Electrolysis method*: In this process electrolysis of the tissue is done in a solution of hydrochloric acid and formic acid. Calcium from the tissue moves to the cathode plate. This is a very rapid method of decalcification and takes only a few hours to decalcify the bone. However there is a risk of tissue damage in this technique.

4.4 Surface Decalcification

In case of surface decalcification, the surface layer of paraffin blocks is inverted in 1% hydrochloric acid (HCl) for 1 h. The exposed top 30 µm tissue of the paraffin block is decalcified. The block should be washed thoroughly before cutting. Only the first few paraffin sections are expected to be free from calcium.

4.5 End Point Determination of Decalcification

The end point of decalcification can be detected by:

1. Radiographic examination
2. Chemical test
3. Physical test

1. *Radiographic examination*: X-ray examination of the tissue is the most accurate technique to detect the end point of decalcification. However, this is a costly procedure, and the pre-decalcification radiograph is also needed to assess the extent of decalcification.
2. *Chemical test*: This test is done to assess the presence of calcium in the decalcifying solution in two successive times. The chemical test is applied when weak acid solution (e.g. formic acid) is used.

Chemical solution

Stock solution	
Ammonium hydroxide stock solution	
Ammonium hydroxide (28%)	5 ml
Distilled water	95 ml
Ammonium oxalate stock solution	
Ammonium oxalate	5 ml
Distilled water	95 ml

Before use equal volume of both the stock solution is mixed.

Method

- Now for the chemical test, 5 ml of the decalcifying agent from the container containing the tissue is withdrawn.
- Mix the decalcifying agent with 5 ml of ammonium hydroxide and ammonium oxalate mixture solution.
- The mixture is kept overnight.
- Any precipitation is noted.
- The presence of precipitation (calcium oxalate) indicates that the decalcifying agent contains calcium and decalcification is not completed.

3. *Physical test*: This is a crude test and it does not accurately detect the end point of decalcification. The tissue is bent or a pin is introduced within the tissue. In case of adequate decalcification, it is expected that the tissue will be soft and could be bent easily. Pin also should penetrate easily within the tissue. The major disadvantage of physical test is the tissue damage by making a hole or by bending it.

Reference

1. Skinner RA, Hickmon SG, Lumpkin CK, et al. Decalcified bone: twenty years of successful specimen management. J Histotechnol. 1997;20:267–77.