

Veterinary Clinical pathology- II

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Rumen Function Tests

1. Introduction

- The specific function & structure of the rumen makes ruminants different from other animals.
- Understanding the workings of the rumen, & the nature of rumen microbes, is key to maximising feed efficiency & productivity in ruminants.

- Digestion of feed in the rumen is primarily carried out by micro flora (bacteria, protozoa & fungi).

✓ *They secrete enzymes capable of digesting feed constituents*

- ✓ They are obligate or facultative anaerobes
- ✓ Bacteria are the predominant, make up about half, of the total microbial biomass in the normal rumen

➤ Functional groups rumen microbes:

- 1) *Fibrolytic*** - digest structural carbohydrates (Hemicellulose, Cellulose, Lignin....)
- 2) *Amylolytic*** - non-structural carbohydrates and
- 3) *Proteolytic*** – protein/NPN

- ❖ Carbohydrates/sugars → ¹ ***volatile fatty acids*** (acetate, propionate & butyrate), ² Co_2 & ³ ***methane***
- ✓ ***Propionate*** is a more energy-efficient fuel source for cattle
- ❖ Protein/NPN → into ammonia (NH_3)
- Rumen diseases: **Lactic acidosis, bloat ...**

Assessment of the rumen fluid is essential:

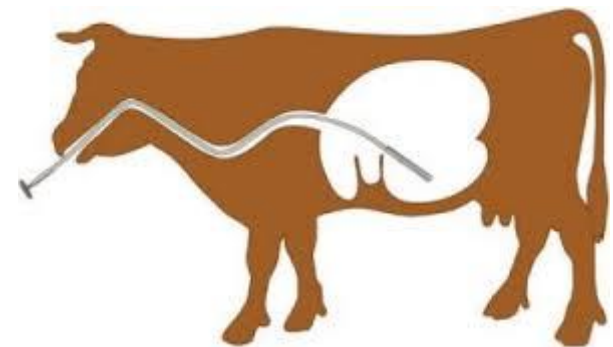
- To assist with the diagnosis of disorders of the rumen e.g. **indigestion, abomasal reflux, Lactic acidosis...**
- To check the rumen health at an individual (e.g. selection of a suitable candidate as a donor for ***rumen transfaunation***)

2. Rumen Fluid Analysis

2.1 Methods of rumen fluid collection

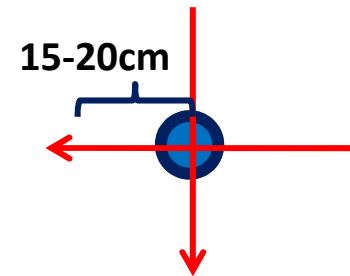
1. Stomach tube/ rumen tube

- ✓ Rumen fluid collected via rumen tube is often mixed with bicarbonate-rich saliva.
- ✓ Discard the first **50-100mL** of the collected sample

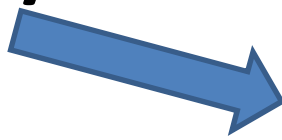


2. Rumenocentesis: needle size of 1.2-2.1mm x 8-12cm attached to a 10 - 20mL syringe

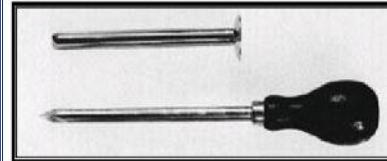
➤ *Intersection of a horizontal line from the point of the patella and a vertical line 15-20cm caudal to the last rib on the left side.*



3. Rumen Cannula/fistulae



4. Trocarization



2.2 Assessment/Examination of Rumen Fluid Sample

- The volume of rumen fluid sample to be collected is **20 -100 ml.**
- Analysis must be performed **immediately** after collection.
- If not; keep the sample for a maximum of:
 - ✓ 9 hrs in air-tight containers at room T° or
 - ✓ 24 hours at refrigerator

- ❖ Delayed assessment of rumen fluid has the most significant effect on, **misinterpretation of**, the physical, chemical & microbial properties of rumen fluid sample.

Examination of rumen fluid

1. Physical properties

- Color
- Odor
- Consistency
- Sedimentation activity test

2. Chemical tests

- pH
- Cellulose digestion &
- Methylene blue reduction tests

3. Microbial properties

- Bacteriological &
- Protozoan examinations

Examination of rumen fluid

Other tests:

- glucose fermentation, nitrate reduction, rumen osmolarity, etc. require specialised equipment, prolonged times to complete or yield little additional practical information.

I. Gross Evaluation/Physical **Characteristics:**

A. Color: the colour of rumen fluid is assessed by visual inspection of rumen fluid samples in a transparent plastic/glass tube of a smaller diameter (<1.5cm).

➤ The **colour** of normal rumen fluid depends on the nature of feed:

- brownish green (Hay ration)
- green (Green ration)
- Yellowish brown (Grain or silage ration)

• **Abnormalities in Rumen Fluid Colour**

✓ **Darker greenish** – rumen stasis and decomposition of rumen contents.

✓ **Milky gray** - Grain overfeeding

- ✓ **Gray with** clots of milk and smell of abomasal contents - pyloric outflow obstruction and/or backflow of abomasal content.

The colour of the rumen fluid may also change due to:

- ✓ **diet:** feeding beetroot results in reddish **tinge color**
- ✓ administration of activated charcoal results in black coloration
- ✓ bleeding due to rumenocentesis - reddish coloration

B. Odor: The **odour** of rumen fluid is assessed by **smell**

- For efficient detection of the odours, the sample should be closed in an air-tight container for a minimum of five minutes and then opened to assess the odour.
- **Normal:** a sweet and fermentative smell (often described as aromatic and non-repellent).

➤ **Abnormal odor**

- ✓ **Ammonia smell** --- Urea poisoning
- ✓ **Moldy & foul/foetid smell** ---
putrefaction of rumen ingesta or due to
prolonged ruminal stasis (e.g. vagal
indigestion).
- ✓ **Intensely sour odor**---- lactic acidosis

C. Consistency: the consistency of rumen fluid in 'normal', healthy cattle is **slightly viscous**.

➤ **Abnormalities in rumen fluid consistency** - the consistency of rumen fluid changes from slightly viscous in normal, healthy cattle to:

✓ **Watery** - rumen dysfunction and inactivity (Inactive bacteria/protozoa and starvation)

- ✓ **Frothy /bubbles** - frothy bloat
- ✓ Sample of rumen fluid with **pasty consistency** that contain a **large number of small bubbles** are indicative of vagus indigestion.
- ✓ ***An excessively viscous sample - composed of saliva***

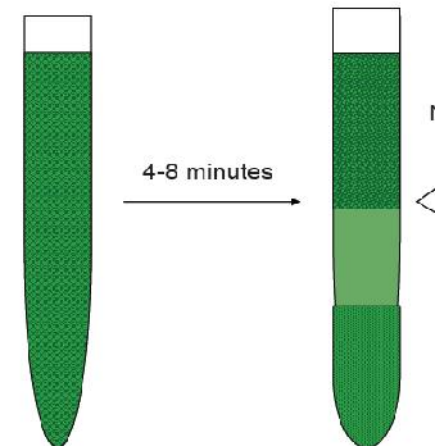
D. Rumen Fluid Sedimentation activity

- This test is highly underutilised in practice but it is often a very rewarding **ancillary technique**
- It is a qualitative measurement of microbial activity
- The test should be carried out on a fresh sample (< 2 hours old) in a plain test tube.

✓ *For easier assessment the sample can be filtered through a gauze swab.*

➤ The normal time needed for sedimentation of fine **particles and floatation of** larger particles or bubbles due to microbial gas production is **4-10 minutes**

-Sedimentation activity
(Evaluation of microfl)

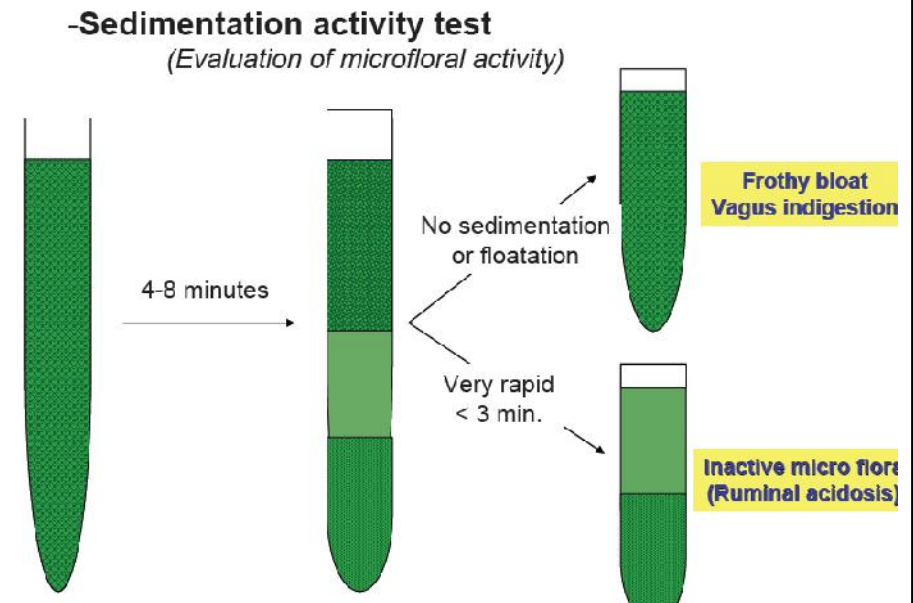


Abnormalities in rumen fluid sedimentation

- ✓ Rapid sedimentation (often < 2 minutes) with no or delayed floatation

➤ *Inactive microflora*

- ✓ Rumen acidosis
- ✓ Prolonged anorexia
- ✓ Indigestible roughages



➤ *No appreciable sedimentation or floatation:*

- ✓ **Frothy bloat** will have particles suspended within the fluid for extended periods of time and may not show any flotation.

II. Chemical tests

A. pH of the rumen fluid

- Should be measured as soon as possible after collection of the sample (within minutes)-
- It can be measured using narrow range ***pH indicator papers*** or, ideally, **by a pH meter.**
- The normal pH of the rumen fluid varies significantly depending on the type of diet:
Normal pH = ranges from **5.5 – 7.2**

- ✓ Cattle on an exclusively pasture diet = **6.0 - 7.2**
- ✓ Feedlot cattle = **5.0 - 6.0**
- ✓ *The pH of the rumen sample collected by stomach tube is usually **0.2-1.0 point** higher compared to samples collected by ruminocentesis.*

Abnormalities of the rumen fluid pH

✓ *pH < 5.5 (rumen acidosis)*

- Acute, severe carbohydrate overload
- over-feeding of rapidly fermented carbohydrates

✓ *pH > 7.0 (Rumen alkalosis)*

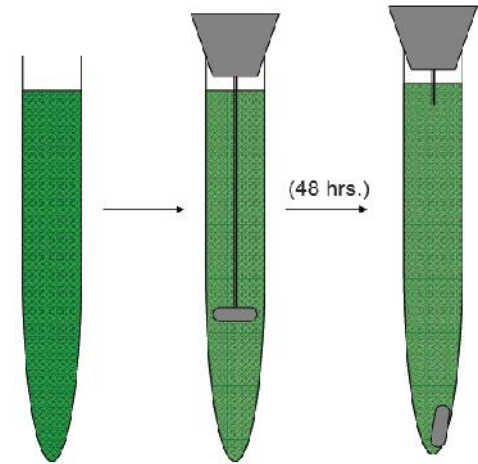
- Decreased fermentation (microflora is inactivated)
- Excess ammonia formation (Protein overload or Urea toxicosis)
- dilution with saliva
- withdrawal of feed for 24 hours or longer.

B. Cellulose Digestion Test

- It is used to evaluate the cellulose digesting ability of rumen micro flora.
- **Procedure**
 - Take 10ml of rumen content in the test tube
 - Add about 0.3ml of 10% glucose solution
 - Suspend **cellulose fiber/cotton thread** to the solution and incubate in 37°C for 48 hrs

- Normal

- If the rumen is active, there will be break down of the thread

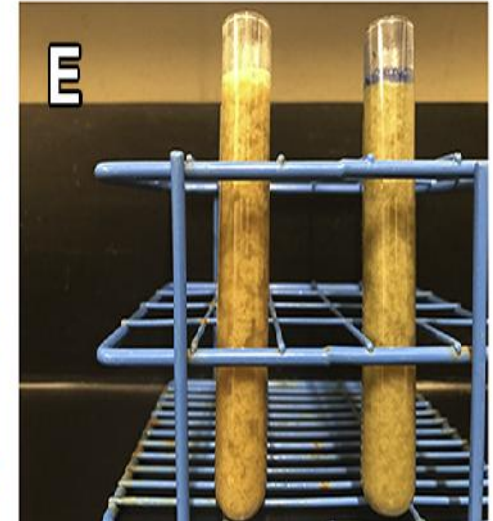
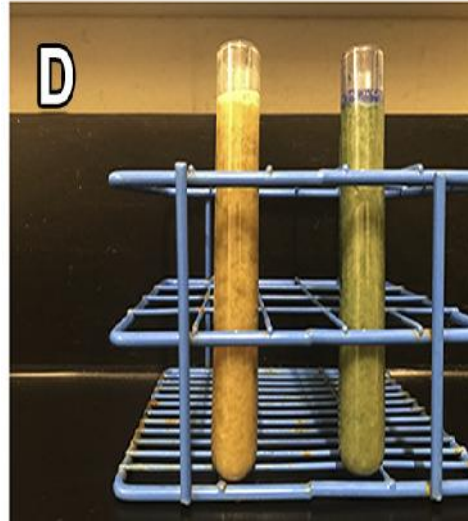


C. Methylene blue (MB) reduction test

- The redox potential of the rumen is primarily maintained by the microbial population of the rumen and can be measured by using a methylene blue reduction test.
- It reflects the anaerobic fermentative metabolism of the bacterial population.

- It is one of the most reliable tests to assess the microbial status of rumen fluid.
- ✓ To perform the test, 0.5 ml of 0.03% methylene blue should be mixed with 10 mL of fresh rumen fluid.
- ✓ The time for the sample to clear the blue color should be measured
- ❖ In health animal, active microflora decolourises (reduces) the methylene blue within 3-6 minutes.

(MB) reduction test



- Prolonged decolourisation time (10 minutes) indicates decreased microbial activity, and occurs in acidosis, rumen atony, indigestible roughage, and anorexia of several days.

III. Microbial Properties: Microscopic Examination

1. Microscopic Examination of Rumen Protozoan's:

- ❖ Rumen protozoa are highly reactive to changes in their environment. The presence, diversity & activity of the rumen protozoa are the most sensitive measure/ indicators of rumen health and function.

III. Microbial Properties: Microscopic Examination

- Protozoa can be evaluated by placing a drop of fresh/warm rumen fluid on a slide (40x).
- ✓ *Lugols iodine can be used to stop motility and allow counting of protozoa*
- There are two form of actively-motile protozoa (Ciliates and Flagellates) in rumen fluid.

- a. **Normal' rumen fluid:** It should contain variety of species, sizes and forms of actively motile protozoa
- I. **Motility:** The protozoa should be active:
 - >10 protozoa entering a single microscopic field over a period of 30 seconds.

Motility is judged & graded as follows:

- +++ = Highly motile and very crowded (> 15)
- ++ = Motile and crowded (8 - 15)
- + = Sluggish motility and low number (< 5)
- 0 = No or sporadic alive protozoa

Interpretation: Normal fluid Fauna are motile

- Abnormal fluid Sluggish or no movement at all

II. Forms of protozoa: Both ciliated & flagellated should be detected

- ✓ Cattle on a high grain diet usually have predominantly flagellated protozoa.
- ✓ Cattle on a high roughage diet usually have predominantly ciliated protozoa.

III. Size of protozoa : three sizes of protozoa should be present: small, medium and large.

IV. Numbers of protozoa: ≥ 40 protozoa per microscopic visual field.

Interpretation:

- Low numbers of protozoa (<8 per field)
 - Inactive protozoa (<5 entering the field)
 - Loss of diversity
 - or any combination of the above are indicative of a 'non-healthy' rumen environment and associated with most rumen disease
- ❖ ***Large & medium protozoa are most susceptible to ruminal environmental change and will die first***

2. Bacteria Examination : The rumen bacteria microflora is comprised of a large number of species with a variety of morphological forms.

- An assessment of the bacterial population can be made by Gram staining the fluid
- ✓ In the normal rumen, there is a predominance of **Gram-negative bacteria**.
- ✓ Cattle fed predominantly on roughage should have a high proportion of **large bacterial forms**.
- ✓ Cattle fed on high-grain diets should have a high proportion of **small bacterial forms**.
- ✓ Cattle fed on mixed rations should have mixed-size bacterial forms

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➤ **Interpretation:** In cases of acute carbohydrate overload the bacterial population changes and *Gram positive bacteria* predominantly present

Other tests: The presence of anaerobic bacteria can be confirmed using the methylene blue reduction test. It must be carried out shortly after collection.

- **Fungi:** comprises 8% of ruminal microbial biomass

Pancreas

➤ Exocrine Pancreas

1. Proteases

- ✓ Trypsinogen – activated to trypsin by Enterokinase
- ✓ Chymotrypsinogen -activated by trypsin

2. Lipase - secreted in the active form

- ✓ bile and alkaline pH enhanced the activity of lipase

3. Amylase - secreted in the active form

- **Abnormalities** -Pancreatic Inflammation and Necrosis, Exocrine Pancreatic Insufficiency (EPI) and Tumor

Pancreatic Inflammation and Necrosis

- Systemic signs (anorexia, vomiting, pyrexia, abdominal pain etc.) in dogs & cats
- Pancreatic enzymes gain access into blood

➤ **Laboratory tests:**

A) Serum amylase & lipase level

B) Trypsin-like Immunoreactivity (TLI)

A) Serum amylase and lipase: Increased

- Enzyme leakage from the pancreatic cells directly into the peripheral blood or into the blood via lymphatics (altered cell membrane permeability)

Non specific (increased in renal failure, salivary lesions, prostatitis, and other intraabdominal diseases)

Pancreatic Inflammation and Necrosis

B) TLI: *it is more specific than amylase and lipase for pancreatic inflammation.*

- ✓ Peak in TLI can occur 1-2 days before peak of amylase and lipase activities
- ✓ Species specific (canine = cTLI, feline = fTLI)
- **Increased in:** Pancreatic inflammation & decreased renal clearance

Pancreatic Exocrine Insufficiency

- Clinical signs associated with maldigestion = weight loss, polyphagia, diarrhoea (fatty faeces)
- Decrease of exocrine pancreatic enzymes
 - 1. TLI** is the test of choice: Low TLI = EPI
 - ✓ Highly sensitive and specific
 - 2. Microscopic fecal examination**
 - a. Sudan III/IV stained smears** - to detect steatorrhea
 - ✓ Fats appear as large orange or red droplets
 - ✓ Deficiency of pancreatic lipase

b. Lugol's stained smears – to detect starch granules (amylorrhea)= blue-black or blue-green structures

✓ Increased numbers indicate amylase deficiency.

c. Muscle fibers may be observed in unstained or Lugol's stained smears === blunt ends and cross striations are visible (creatorrhea).

- Their presence in feces from animals on a meat diet indicates deficiency in protein digestion.

3. Total fecal fat: it is performed on a 24hr stool sample.

- Normal amount of fat excreted is < 7 grams/24 hrs.
- Greater amounts indicate steatorrhea

4. Gelatin digestion tests - to detect protease action in feces, i.e., trypsin.

- Film test- A strip of x-ray film
- Tube test: **more sensitive**
- Mix faecal sample with melted 7.5% gelatin
- Digestion of the emulsion (liquification of the gelatin) indicates the presence of proteases.
- Failure to digest the emulsion or solidification of gelatin indicates protease deficiency.

4. Fat absorption test (plasma turbidity test).

a. Hyperlipemia

- 1) Postprandial hyperlipemia occurs in a normal animal 1-3 hours following a high fat meal.
- 2) Fasting hyperlipemia -mobilization of body fat

Fat absorption test: Fast the animal 12 hours and determine if the plasma is turbid (fasting hyperlipemia). If plasma is clear, proceed with the test. Orally administer corn oil (3 ml/kg) and examine the plasma for turbidity 2-3 hours later.

- The presence of plasma turbidity indicates fat is being absorbed.
- The absence of plasma turbidity indicates a lack of absorption of fat.
- ✓ Exocrine pancreatic insufficiency/lipase deficiency
- ✓ Bile insufficiency
- ✓ Intestinal malabsorption

PREPARATION OF HISTOLOGICAL SPECIMENS

- Living cells and tissues are difficult to examine because they are relatively transparent and thick.
- To reduce this problem, tissues/cells are usually killed & processed in such a manner that it is sufficiently thin/thick to be examined microscopically and all the structures in a tissue may be differentiated.

- **Paraffin technique:** This is achieved by passing the tissue through a series of process/steps;
 1. Acquisition and Fixation
 2. Dehydration, clearing and Embedding = Tissue processing
 3. Sectioning and Staining
 4. Examination and Interpretation

1. Acquisition

❖ Points to be considered

- Knowledge of gross anatomy and identification of desired sample
- Collect and handle tissue sample must atraumatically
- ✓ ***Avoid crushing/squeezing/traction of the tissue with forceps***
- ✓ ***Avoid dull and dirty scalpels or scissors***

- Tissue sample should be taken from the periphery that includes normal and diseased tissue.
- Tissue sample should be removed rapidly and placed immediately into container (glass, plastic or metal container or plastic bag) with adequate volume of fixative and transported to laboratory

2. Fixation

- This is the process by which the constituents of cells and tissue are fixed/preserved in a physical and a chemical state so that they will withstand subsequent treatment with various reagents with minimum loss of architecture.
- This is achieved by exposing the tissue to chemical compounds, called **Fixative**.

Classification of Fixatives

- 1. Tissue fixatives** - Buffered formalin, Buffered gluteraldehyde, Zenker's formal saline, Bowen's fluid....
 - *10% neutral- buffered formalin commonly used*
- 2. Cytological fixatives** – Ethanol, Methanol, Ether...
- 3. Histochemical fixatives**- Formal saline, Cold acetone...

Amount of fixative fluid

- This should be approximately **10-20** times the volume of the specimen. Fixative should surround the specimen on all sides.
- No fixative will penetrate a piece of tissue **thicker than 1 cm**. Therefore;
 1. **Solid organ** should be cut not thicker than 5mm
 2. **Hollow organ** should be opened or filled with fixative or pack lightly with wool soaked in fixative.
 3. **Large specimen** - Inject fixative along the vessels or bronchi as in case of lung so that it reaches.

If fixative is not available at hand, place the specimen in refrigerator at 4oC to slow down autolysis.

Properties of an Ideal Fixative:

1. Prevents autolysis and bacterial decomposition.
2. Preserves tissue volume and structure in their natural state and fix all components.
3. Make the cellular components insoluble to reagent used in tissue processing.
4. Avoid excessive hardness of tissue.
5. Allows enhanced staining of tissue
6. Should be non-toxic and non-allergic for user.
7. Should not be very expensive.

2. Tissue processing

- It is a long procedure, require 24 hrs, & can be done by manually or mechanically. It is done in stages and subdivided into; **dehydration, clearing, impregnating** and **embedding**.
- It is important that all specimens are properly ***labeled*** before processing is started.
- ✓ Printed, or graphite pencil written should be used



➤ *To permit sectioning **75% of water** must be removed and replaced/infiltrated with paraffin*

A. Dehydration - to remove fixative and water from the tissue and replace them with **dehydrating fluid/Dehydrants**: *Ethyl alcohol, Methyl alcohol, Butyl alcohol and Isopropyl alcohol.*

➤ To minimize tissue distortion from diffusion currents, tissues are dehydrated by using increasing strength of alcohol; e.g. 50%, 70%, 90% and 100%.

✓ *The volume of alcohol should be 50- 100X that of tissue.*

B. Clearing - The next step alcohol should be replaced by paraffin wax/embedding medium.

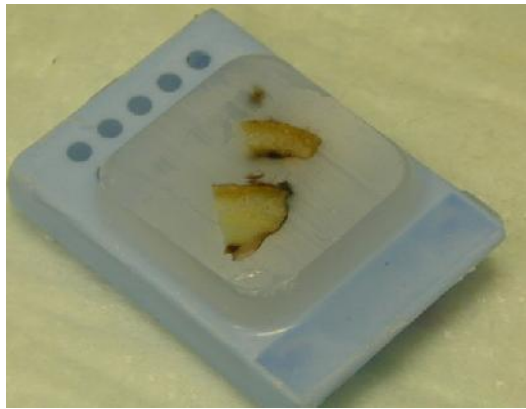
- As paraffin wax is not alcohol soluble, alcohol is replaced with ***a substance/ fluid*** that is totally miscible with both the dehydrating fluid and the embedding medium. This step is call ***clearing***.
- It is achieved by any of the following reagents: Xylene, Chloroform, Benzene, Carbon tetrachloride, Toluene... ***Xylene is commonly used.***

C. Impregnation with Wax

- This is allowed to occur at **melting point T₀ of paraffin wax, which is 54-60°C---**
(melted wax)
- *Types of Wax employed for Impregnation:*
 - ✓ Paraffin wax, Water soluble wax....
 - ✓ Paraffin wax is used routinely.

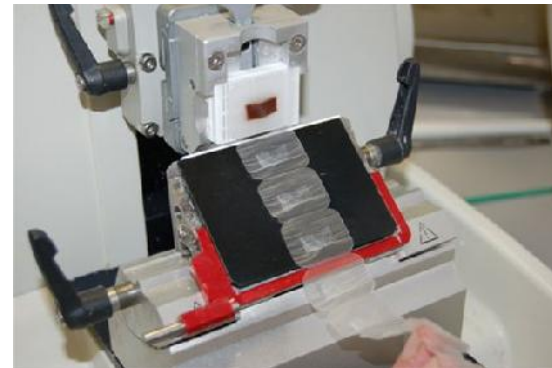
D. Embedding or Blocking - is the process by which tissues are surrounded by a medium such as agar, gelatin, or **Paraffin wax**

- ✓ Permits the specimen to be sectioned sufficiently thin
- ✓ Impregnated tissues are placed in a mould with their labels and then fresh melted wax is poured in it and allowed to settle and solidify.



E. Sectioning/Cutting - tissue blocks are mounted on a rotary microtome and sectioned at 5-7 μ m thickness
(*These sections form a ribbon*)

- Ribbons may be floated on warm water bath, the specimen will be subsequently stretched, and picked up on slides for staining



3. Staining is a process by which we give colour to a section.

➤ There are hundreds of stains available.
Generally the stains are classified as:

A. Acid Dyes: Acid dyes stain basic components.

✓ In an acid dye the basic component is coloured and the acid component is colourless.

e.g. *Eosin* stains cytoplasm red.

B. Basic Dyes: Basic dyes stain acidic components

✓ In a basic dye the acid component is coloured and the basic component is colourless.

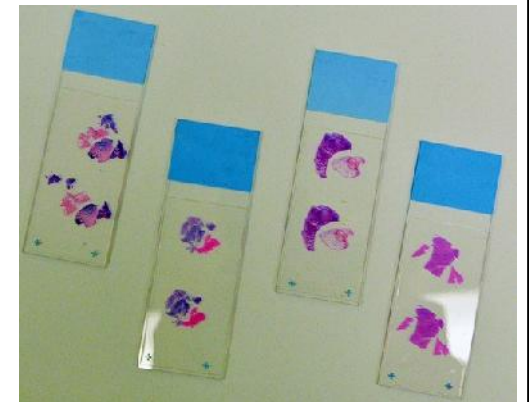
e.g. basic fuchsin stains nucleus blue.

C. Neutral Dyes: When an acid dye is combined with a basic dye a neutral dye is formed. As it contains both coloured radicals, it gives different colours to cytoplasm and nucleus simultaneously

This is the basis of Leishman stain.

Procedure of staining - either manually or in an automatic stainer. Use stain according to a specified method.

- **Haematoxylin & Eosin (HE) staining:** is the most common used routine stain in histopathology laboratory
- ✓ Tissue sections (paraffin- embedded) fixed to glass slides
- ✓ Paraffin must be removed before staining.
- ✓ The reverse order of the previous procedure is used
- ✓ Then slides should be stained and again dehydrated, cleared, and covered with a mounting medium
- ✓ A cover – slip is applied to produce **permanent preparation**



Special Stains

1. PAS (Periodic Acid Schiff) stain: This stain demonstrates glycogen
2. Stains for micro-organism:
 - ✓ Gram-stain:
 - ✓ Ziehl_Neelsen stain: This stain detect acid fast bacilli.
 - ✓ PAS stain: It is used for fungi, amoeba and Tricomonas.
 - ✓ Modified Giemsa (2% in water): For Helicobacter pylori.
3. Congo-red: It is used for identification of amyloid.
4. Sudan-Black: It is used for fat staining.
5. Masson's Trichrome: It is used for differentiation of connective tissue