B.Sc. NUTRITION LAB MANUAL

2nd Semester

Prepared By

Biological Science Dept.

Nutrition

MIDNAPORE CITY COLLEGE

To study the general properties of urease and salivary amylase

a. Principle:

Urease hydrolases urea to ammonia and CO2. The ammonia further reacts with a phenolic chromogen and hypochlorite to form green coloured complex. Intensity of the colour formed is directly proportional to the amount of urea present in the sample.

Urease

Urea+ H2O ----------Ammonia +CO2

Ammonia + phenolic chromogen ----— Green coloured complex+hypochloritre

b. Normal reference values

Serum/plasma: 14-40mg/dl

Urine: upto 20g/l

c. Requirement:

i. Semiautoanalyser/ Colorimeter
ii. Eppendorf
iii. Plasma or serum sample
iv. 1000ml and 100ml micropipette with tips
v. Distilled water
vi. Uric acid kit

d. Procedure:

i.

Addition sequence	**Blank (ml)**	**Standard (ml)**	**Test (ml)**
Buffer reagent (L1)	1	1	1
Enzyme reagent (L2)	0.1	0.1	0.1
Distilled water	0.01	-	-
Urea standard	-	0.01	-
Sample	-	-	0.01
Mix the above contents well and incubate at 370c for 5 minutes or at RT(25°c) for 10 min			
Chromogen reagent (L3)	0.2	0.2	0.2
Mix the above contents well and incubate at 37°c for 5 minutes or at RT(25°c) for 10 min. Measure the absorbance of the standard (Abs.s) and test sample (Abs.T) against the blank within 60 min at 570nm wavelength.			

e. Result:

Absorbance can be measured in colorimeter or by using semiautoanalyser. If the absorbance is measured in colorimeter the total volume of standard and sample should be at least 3 ml.

Uric acid concentration (mg/dl) = $\frac{\text{Absorbance of testx40}}{\text{Absorbance of standard}}$

f. Interpretation:

Uric acid is the end product of purine metabolism and that are catalyzed by urease enzyme to form ammonia that are excreted through urine. The sample have uric acid value of …….mg/dl of uric acid. A serum uric test is done to detect arthritis, gout and kidney disorder patients.

To study the properties of salivary amylase at different temperature

The presence of a specific enzyme actually begins the chemical digestion. The enzyme that makes this possible, called **salivary amylase**, is found in the mouth and begins the process of chemical digestion by aiding in the breakdown of starch molecules, complex carbohydrates found in food.

Salivary amylase is just one of several types of amylase enzymes. Amylases are categorized into three classes called Alpha, Beta, and Gamma amylase.

- Alpha amylase is found in many living things including animals like humans, also plants, and even microbes like bacteria.
- Beta amylases are only found in plants and animals. It is beta amylase that facilitates the ripening of fruits, making them taste sweet. In addition, beta amylase is used by brewers and distillers in the making fermented beverages.
- Gamma amylases are made only by animals and microbes.

Salivary amylase is an alpha amylase. In addition to the mouth, this type of amylase is found in some other organs and tissues. These vary from reproductive organs, the ovaries and fallopian tubes, to the lungs, gastrointestinal tract, and muscle tissue. Another type of alpha amylase is made in the pancreas and aids in the chemical digestion of food. This amylase can be detected in the blood and is sampled by health care providers to measure the function of the pancreas.

Aim

To study the effects of variation in temperature on the activity of salivary amylase on starch.

Necessary Materials & Apparatus

- Water.
- Ice cubes.

- Test tubes.
- Droppers.
- Wire gauze.
- Thermometer.
- Bunsen burner.
- Saliva solution.
- Iodine solution.
- pH tablets of 5, 6.7, 8.
- Beaker with water and a thermometer.
- 15 ml 1% starch solution + 3 ml 1% NaCl.
- 3 series of test tubes, each containing iodine solution.

Effect of Various Temperatures on the activity of salivary amylase on starch

- Divide and pour the 15 ml 1% starch solution + 3 ml 1% NaCl solution into three test tubes and name them as A, B and C.
- Pour a few ice cubes in a beaker and ensure that they stay at 5 °C.
- Transfer tube- A to the beaker with ice.
- Take two more beakers and fill them with water.
- Heat the two beakers, one up to 37 °C and the other at 50 °C.
- Ensure that the temperatures for the two beakers are constant.
- Transfer test tube B into the beaker which is set at 37 °C.
- Similarly, transfer test tube C into the beaker set at 50 °C.
- Draw 1 ml of saliva solution and add it into test tube A. Do the same for test tube B and C.
- Quickly draw a few drops using a dropper from test tube A and transfer the same to the first series of test tubes having iodine solution.

- Repeat the same: transfer a few drops from test tube B and C into the second and third series of test tubes having iodine solutions.
- Note the time as “0-minute reading” and wait 2 minutes before proceeding to the next step.
- Draw a few drops from each tube and add it to the tubes with the iodine solution. Note the change in colour.
- Repeat the experiment in intervals of 2 minutes until the colour of iodine does not change.

Observation

Effect of Various Temperatures on the activity of salivary amylase on starch:

The test tube at 37 °C reaches the achromic point quickest compared to the other two. At high temperatures, the enzyme gets denatured and at low temperatures, the enzyme is deactivated. Hence, it takes more time for starch to be digested at temperatures outside 37° C.

Phosphate Buffer (pH 5.8 to 7.4) Preparation and Recipe

A simple phosphate buffer is used ubiquitously in biological experiments, as it can be adapted to a variety of pH levels, including isotonic. This wide range is due to phosphoric acid having 3 dissociation constants, (known in chemistry as a triprotic acid) allowing for formulation of buffers near each of the pH levels of 2.15, 6.86, or 12.32. Phosphate buffer is highly water soluble and has a high buffering capacity, but will inhibit enzymatic activity and precipitates in ethanol. The buffer is one of the most popular currently used, and is commonly employed in molecular and cell biology, chemistry, and material science.

- **1 L of Phosphate Buffer (pH 5.8 to 7.4):**

Current molarity: 0.1 M Current pH: 7.4

Table 1. Required components

Component	**Amount**	**Concentration**
$Na_2HPO_4•7H_2O$ (mw: 268.07 g/mol)	20.214 g	0.0754 M
$NaH_2PO_4•H_2O$ (mw: 137.99 g/mol)	3.394 g	0.0246 M

1. Prepare 800 mL of distilled water in a suitable container.
2. Add 20.214 g of $Na_2HPO_4•7H_2O$ to the solution.
3. Add 3.394 g of $NaH_2PO_4•H_2O$ to the solution.
4. Adjust solution to final desired pH using HCl or NaOH.
5. Add distilled water until volume is 1 L.

Tris Buffer (1 M, pH 7.2) Preparation and Recipe

Tricine is derived from the amino acids tris and glycine. It is a dipolar ion (Zwitterion) and hydroxyl radical scavenger, and is used extensively for SDS-PAGE applications for small proteins. Tricine buffer is also commonly used for electrophoresis procedures.

To prepare - 1 L of Tris Buffer (1 M, pH 7.2):

Table 1. Required components:

Component	**Amount**	**Concentration**
Tris base (mw: 121.14 g/mol)	121.14 g	1 M

1. Prepare 800 mL of distilled water in a suitable container.
2. Add 121.14 g of Tris base to the solution.
3. Adjust solution to desired pH using HCl (typically pH ≈ 7.0).
4. Add distilled water until volume is 1 L.

Determination of Strength of $KMNO_4$ Using Primary Standard (Oxalic Acid)

Principle: Potassium Permanganate $KMNO_4$ is a strong oxidising agent permanganate (Mno_4) is a deep dark purple colour. Reduction of purple permanganate ion to the colourless Mn^{2+} ion. The solution will turn from dark purple colour to fluent pink colour at the equivalence point. No additional indicator is needed for this titration. The reduction of permanganate requires strong acidic conditions.

In this experiment permanganate will be reduced by oxalate (c2o4-2) in acidic condition. Oxalate reacts very slowly at the room temperature. So, solution are titrated hot to make the procedure practical. The unbalance redox reaction is shown below-

$$MnO_4 + C2O4\text{-}2 \longrightarrow Mn^{2+} + Co_2 \text{ (Acidic condition)}$$

Materials: 1. Oxalic acid solution 0.1n

2. dilute sulphuric acid 2n

3. potassium permanganate solution of unknown normality

Procedure: 1. Transfer 10ml of oxalic acid solution to the conical flask and add equivalent amount (10ml) of dilute sulphuric acid (2n).

2. Warm the solution gently until the temperature of the solution reaches 60-80℃ then add the permanganate solution slowly from the burette till the solution acquires a light rose colour. Keep the solution hot during the titration. A brown ppt is formed during the titration, this may be due to one of the following reasons.

a. the temperature of the solution may be due to below 60℃

b. the addition of the permanganate solution was carried out rapidly.

c. the amount of the sulphuric acid is in sufficient

3. Repeat experiment 3 times and take the mean value of your reading:

No. of observation	Burette reading volume of 0.1M oxalic acid solution (ml)		Mean volume (ml)
	Initial reading(ml)	Final reading(ml)	
1	4.9	5.9	5.0

Calculation: Molarity of the $KMNO_4$ solution=

$a_1 v_1 s_1 = a_2 v_2 s_2$

a1= 2 (number of electrons lost per formula unit of oxalic acid in a balanced equation of half cell reaction which is 2)

a2=5 (number of electrons gained per formula unit of potassium permanganate in a balanced equation of half cell reaction which is 5)

from the balanced chemical equation it is clear that 2 mols of $KMNO_4$ reacts with 5 mols of oxalic acid

v1=volume of oxalic acid

s1=molarity of oxalic acid

v2=volume of $KMNO_4$

s2=molarity of $KMNO_4$

so from $a_1\ v_1\ s_1 = a_2\ v_2\ s_2$

2 × volume of oxalic acid × molarity of oxalic acid = 5 × volume of $KMNO_4$ × molarity of $KMNO_4$

So, $2\times10\times0.1=5\times5.0\times$molarity of $KMNO_4$

So, molarity of $KMNO_4 = \frac{2\times10\times0.1}{5\times5.0}$

$=0.08M$

Strength of $KMNO_4$ solution:

Molarity mass of $KMNO_4$ is 158

Strength (g/lit)= Molarity × molecular mass

$= 0.08\times158=12.64$g/lit

Interpretation: Titration is common laboratory method of equalization of chemical analysis that can be used to determine the unknown concentration of solution. The basis of this process is the reaction between the analyte and a solution of unknown concentration (standard solution). The analyte taken in a conical flask using a pipette and a solution of unknown concentration is taken in a burette. The strength of $KMNO_4$ is 12.64g/lit.

Gel Electrophoresis

Gel electrophoresis is a procedure used to separate biological molecules by size. The separation of these molecules is achieved by placing them in a gel with small pores and creating an electric field across the gel. The molecules will move faster or slower based on their size and electric charge.

Purpose of Gel Electrophoresis

The purpose of gel electrophoresis is to **visualize, identify and distinguish molecules** that have been processed by a previous method such as PCR, enzymatic digestion or an experimental condition. Often, mixtures of nucleic acids or proteins that are collected from a previous experiment/method are run through gel electrophoresis to determine the identity or differentiate between molecules.

Gel Electrophoresis Steps

The broad steps involved in a common DNA gel electrophoresis protocol:

1. Preparing the samples for running

The DNA is isolated and preprocessed **(e.g. PCR, enzymatic digestion)** and made up in solution with some basic blue dye to help visualize the movement of the sample through the gel.

2. An agarose TAE gel solution is prepared

TAE buffer provides a source of ions for setting up the electric field during electrophoresis. The weight-to-volume concentration of agarose in TAE buffer is used to prepare the solution. For example, if a 1% agarose gel is required, 1g of agarose is added to 100mL of TAE. **The agarose percentage used is determined by how big or small the DNA is expected to be.** If one is looking at separating a pool of smaller size DNA bands (<500bp), a higher percentage agarose gel (>1%) is prepared. The higher percentage of agarose creates a denser sieve to increase the separation of small DNA length differences. The agarose-TAE solution is heated to dissolve the agarose.

3. Casting the gel

The agarose TAE solution is **poured into a casting tray** that, once the gel solution has cooled down and solidified, creates a gel slab with a row of wells at the top.

4. Setting up the electrophoresis chamber

The solid gel is placed into a chamber filled with **TAE buffer.** The gel is positioned so that the chamber wells are closest to the negative electrode of the chamber.

5. Loading the gel

The **gel chamber wells** are loaded with the DNA samples and usually, a DNA ladder is also loaded as reference for sizes.

6. Electrophoresis

The negative and positive leads are connected to the chamber and to a power supply where the voltage is set. **Turning on the power supply sets up the electric field** and the negatively charged DNA samples will start to migrate through the gel and away from the negative electrode towards the positive.

7. Stopping electrophoresis and visualizing the DNA

Once the blue dye in the DNA samples has migrated through the gel far enough, the power supply is turned off and the gel is removed and placed into an ethidium bromide solution. Ethidium bromide intercalates between DNA and is visible in UV light. Sometimes ethidium bromide is added directly to the agarose gel solution in step 2. **The ethidium bromide stained gel is then exposed to UV light and a picture is taken.** DNA bands are visualized in from each lane corresponding to a chamber well. The DNA ladder that was loaded is also visualized and the length of the DNA bands can be estimated. An example is given in the figure below.

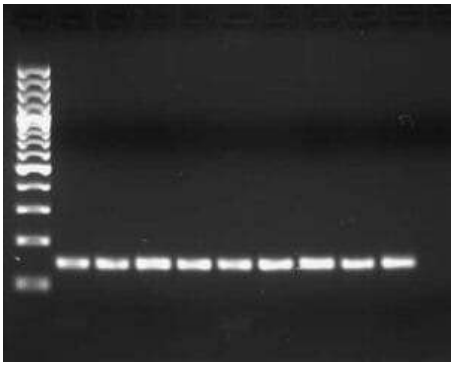


Fig: Agarose Gel electrophoresis of GAPDH

SDS PAGE/ Protein gel electrophoresis

The principle of SDS PAGE states that a charges molecule migrates to the electrode with the opposite side when placed in an electric field. The separation of the charged molecule depends upon the relative mobility of charged species.

The smaller molecule migrate faster due to less resistance during electrophoresis. The structure and the charge of the proteins also influence the rate of migration. Sodium dodecyl sulphate and polyacrylamide eliminate the influence of structure and charge of the proteins, the proteins are separateds based on the length of the polypeptide chain.

Role of SDS in SDS PAGE: SDS is a detergent present in the SDS PAGE sample buffer. SDS along with some reducing agent function to break the disulphide bond of proteins, disrupting the tertiary structure of proteins.

Procedure of SDS PAGE:

Preparation of protein sample then quantify the sample.

↓

SDS gel preparation

↓

Stacking gel (5%) and separating gel (12%) will be loaded

↓

After the completion of SDS gel electrophoresis gel will be placed to a petriplate

↓

Stain the gel with staining solution for over night

↓

Distain the gel with distaning solution. Repeat the solution for several time until the background of the gel is fully distained.

↓

Then detect the particular protein band.

Preparation of Staining solution: 50% methanol

10% glacial acetic acid

0-1% coomassie brilliant blue dye.

Preparation of Distaining solution: 40% methanol

10% glacial acetic acid

12% separating gel preparation:

Distilled water 1.7ml

Acrylamide 2 ml

8.8 tris buffer 1.25ml

10% SDS 0.05ml

10% APS 25µl

TEMED 5µl

5% stacking gel procedure:

Distilled water 2.9ml

Acrylamide 0.85 ml

6.8 tris buffer 1.25ml

10% SDS 0.05ml

10% APS 25µl

TEMED 3µl

Application of SDS PAGE:

- It is used to measure to the molecular weight of the molecules
- It is used to estimate the size of the protein
- It is used in peptide mapping
- It is used to estimate the purify of the protein

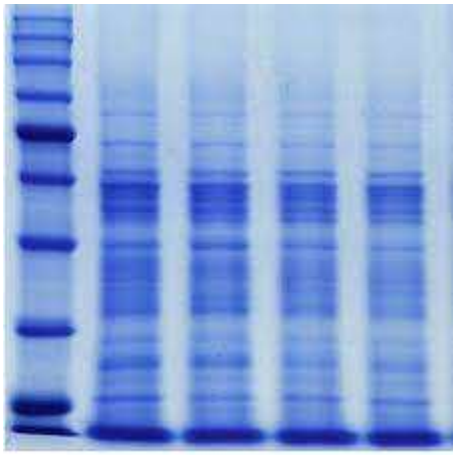


Fig: SDS PAGE electrophoresis of β-actin

Dialysis

(Dialysis Method for Macromolecule)

Introduction: In working with protein which often necessary to eliminate small molecular weight substances such as reducing agent DTT (Dithiothreitol), BME2-Marcapto Ethanol, biotin, preservatives that might interfere with subsequent state in different experiments.

Dialysis is one method for accomplishing both contentment removal and buffer exchange for macromolecular sample such as protein.

Principle: Dialysis is a separation technique that facilitates the removal of small unwanted compounds for macromolecule in solution by selective and passive diffusion to a semipermeable membrane. A sample add a buffer solution called dialysis, then it was placed on opposite site on the membrane, the membrane contain various size pores, molecule larger than the pore cannot pass through the membrane but small molecule and buffer salt pass freely to the membrane.

Sample preparation procedure:

500mg sample was collected and mixed with 1ml RIPA with protease inhibitor in an eppendrof.

↓

Then sample was homogenized at 4°c and incubate for 30 min at 4°c

↓

It was centrifuged at 12000rpm for 1 min at 4°c

↓

Sample was collected by collecting the supernatant into a new test tube

Activation of dialysis membrane:

Cut the membrane at desire length.

↓

Immerse the tube into 1 lit of 2% sodium bicarbonate

↓

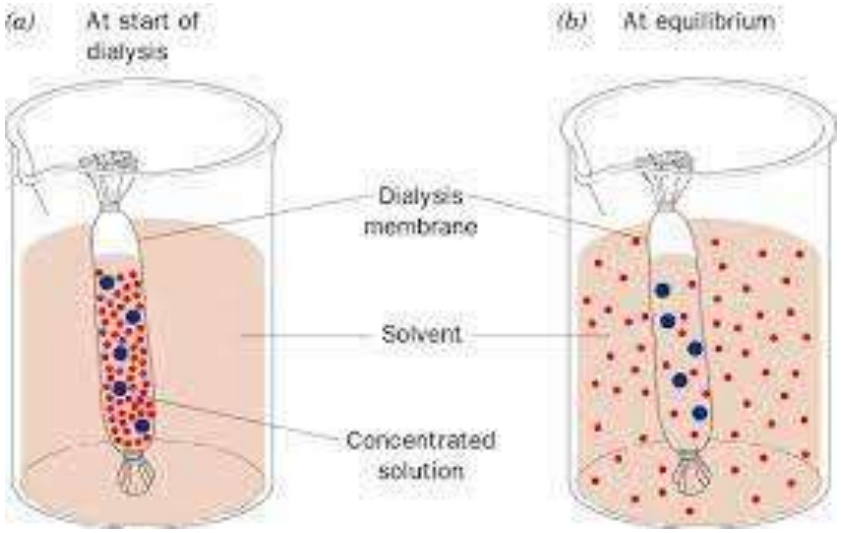
Boil tubing for 10 min

↓

Rinse the tubing thoroughly in distilled water in 10min

Discard the distilled water

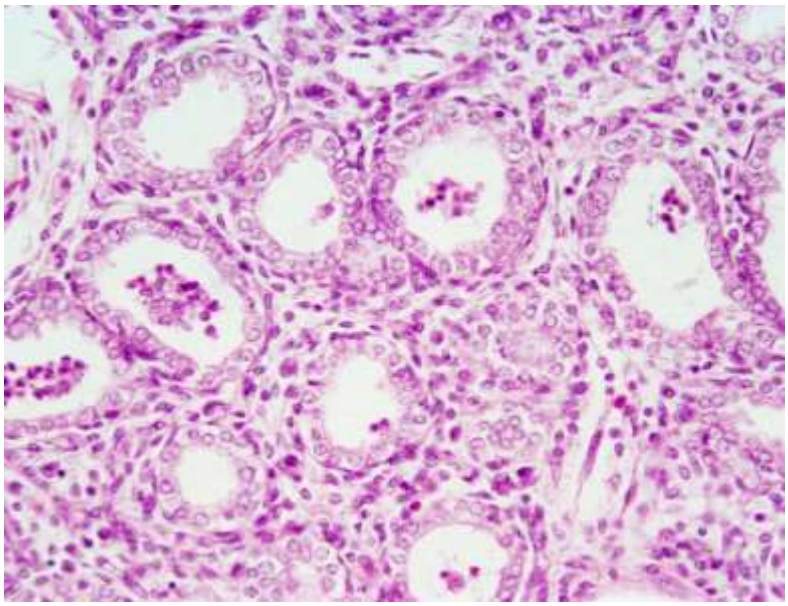
Then pour the sample in the dialysis membrane and tie two sides of membrane with a thread supporting by a glass rod. Start the dialysis process against 0.05 molar Tris HCL buffer.



HUMAN PHYSIOLOGY (Practical)

1. Identification of prepared Slides:

(a) Lungs:

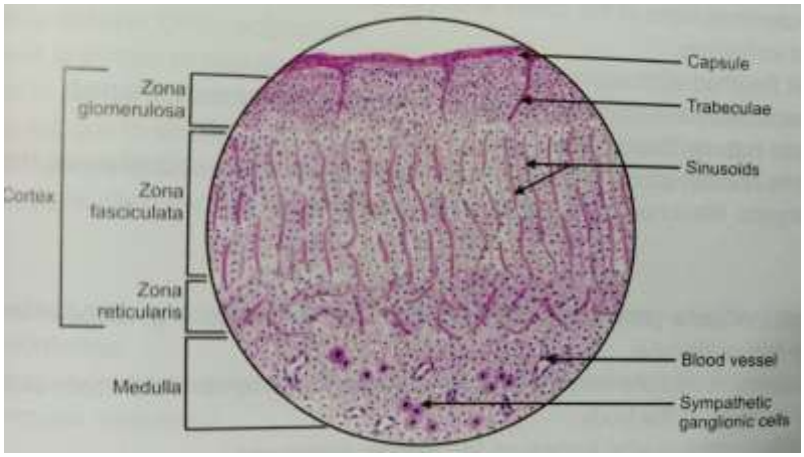


Identifying characteristics:

- Presence of Alveolar Sacs.
- Presence of Terminal Bronchiole.
- Presence of Respiratory Bronchiole.
- Presence of Pulmonary Artery.

Hence, it is the section of Lungs.

(b)Supra Renal Gland:

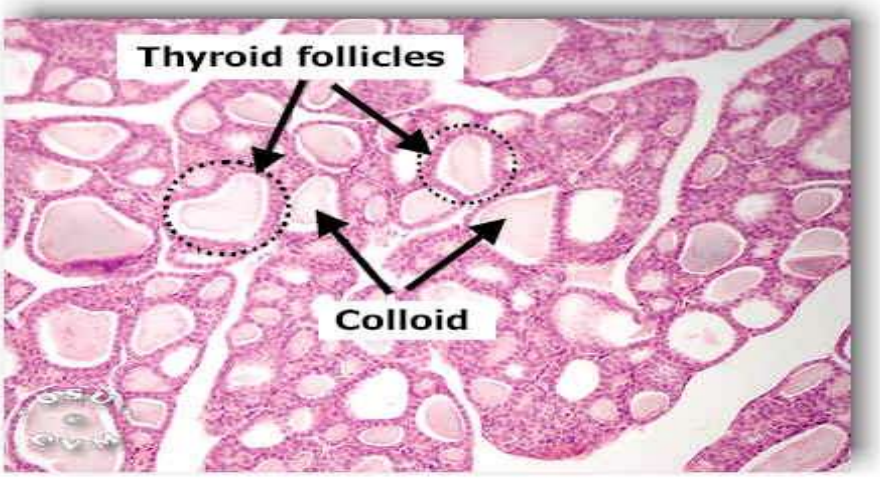


Identifying Characteristics:

- Presence of Capsule.
- Presence of Zona glomerulosa.
- Presence of Zona fasciculate.
- Zona reticularis is present.

Hence, it is the section of Supra Renal Gland.

(c)Thyroid Gland:

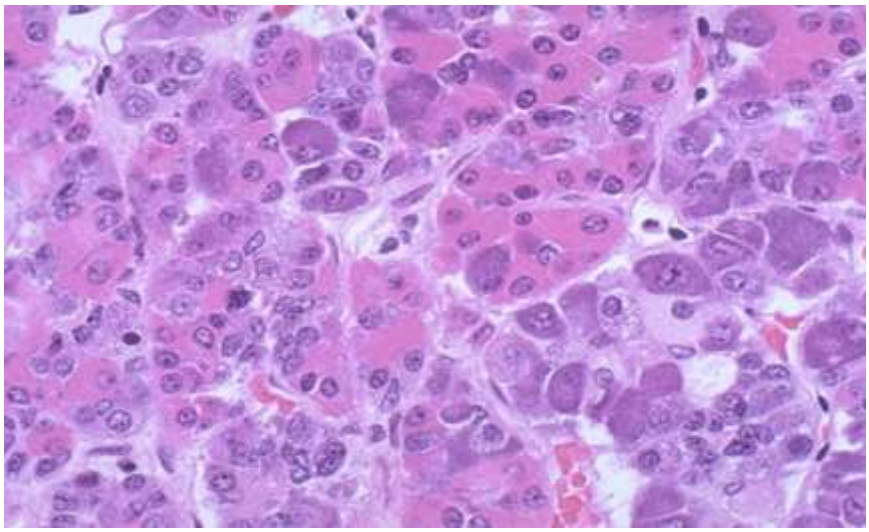


Identifying Characteristics:

- Presence of follicle filled with colloid.
- Presence of parafollicular cells.

Hence, it is the section of thyroid gland.

(d) Pituitary :

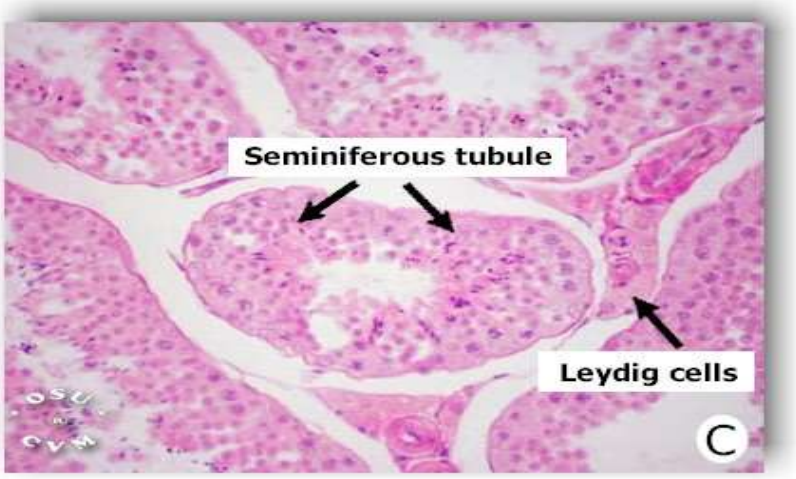


Identifying Characteristics:

- Presence of cortex.
- Presence of Renal pelvis.
- Presence of Renal papilla.

Hence, it is the section of pituitary.

(e)Testis:

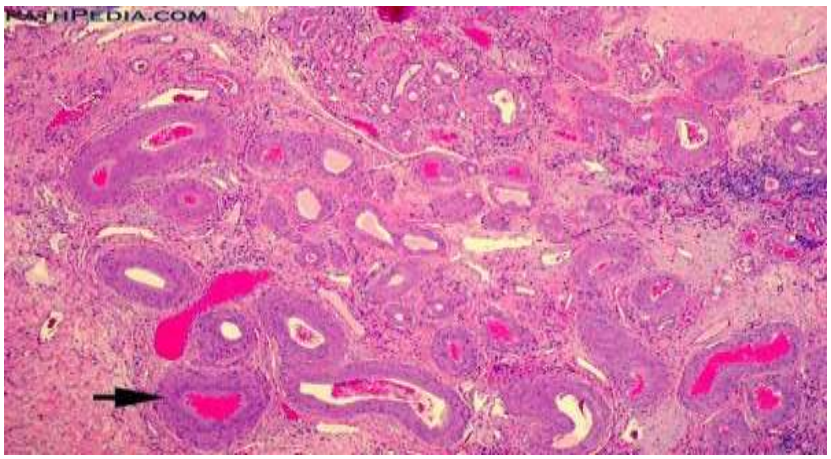


Identifying Characteristics:

- Presence of Leydig cell.
- Presence of Seminiferous tubule.

Hence, it is the section of Testis.

(f)Ovary:

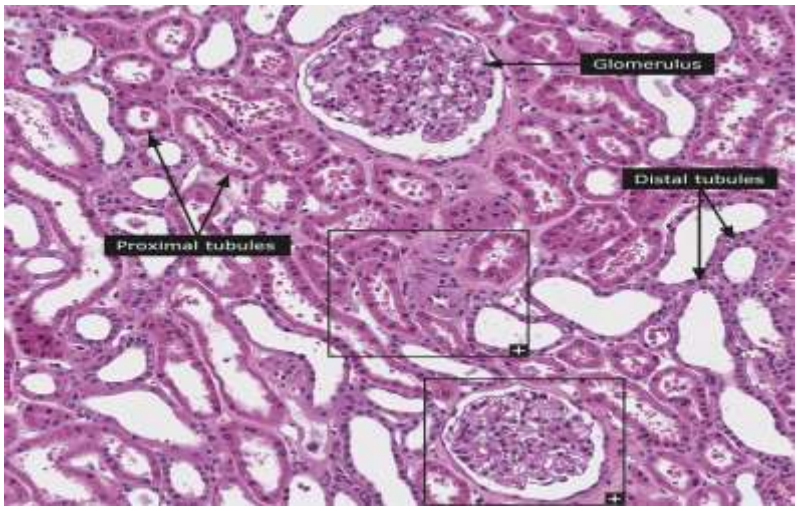


Identifying Characteristics:

- Presence of graffian follicle.
- Presence of corpus leuteum.

Hence, the section is ovary.

(g) Kidney:

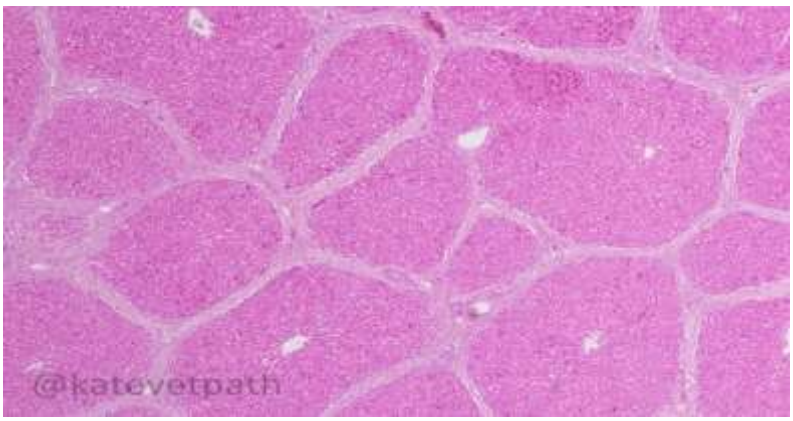


Identifying Characteristics:

- Presence of glomerulus.
- Presence of renal tubule.

Hence, it is the section of Kidney.

(h)Liver:

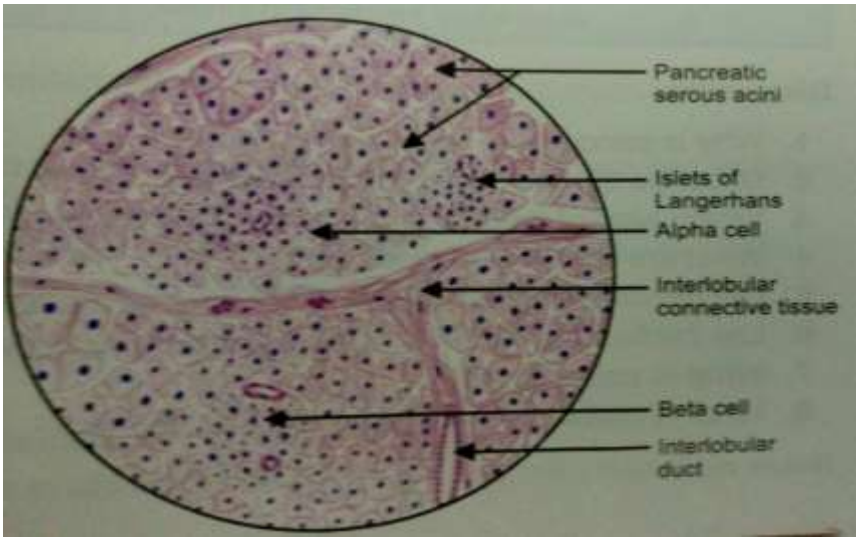


Identifying Characteristics:

- Presence of hepatic lobule arrange as spoke.
- Central vein is present.

Hence, it is the section of liver.

(i)Pancreas:



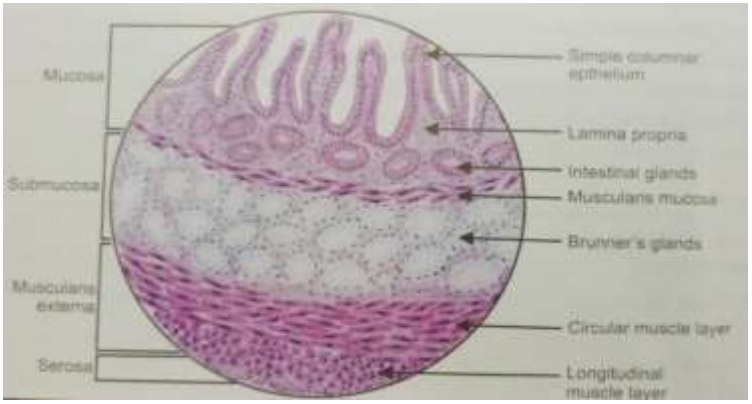
Identifying Characteristics:

- Presence of Islets of Langerhance.
- Presence of Pancreatic acini.
- Presence of connective tissue.

Hence, it is the section of Pancreas.

(j)Small Intestine:

Duodenum:

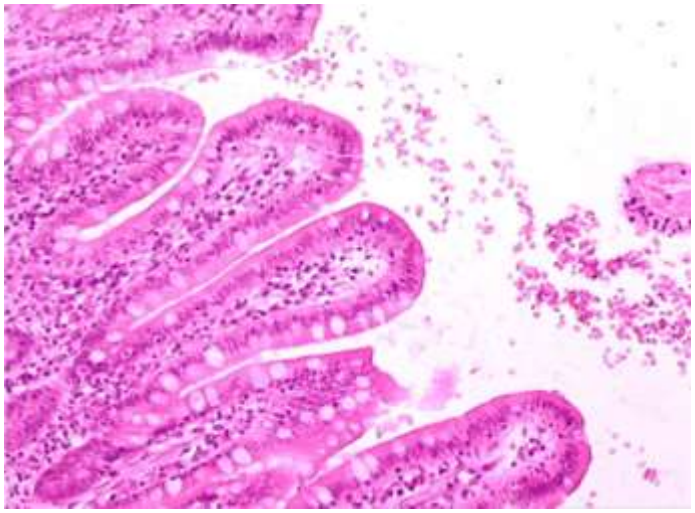


Identifying Characteristics:

- Presence of intravillous spaces.
- Presence of Brunner's gland.
- Presence of Lamina propria.

Hence, it is the section of <u>Duodenum.</u>

Jejunum:

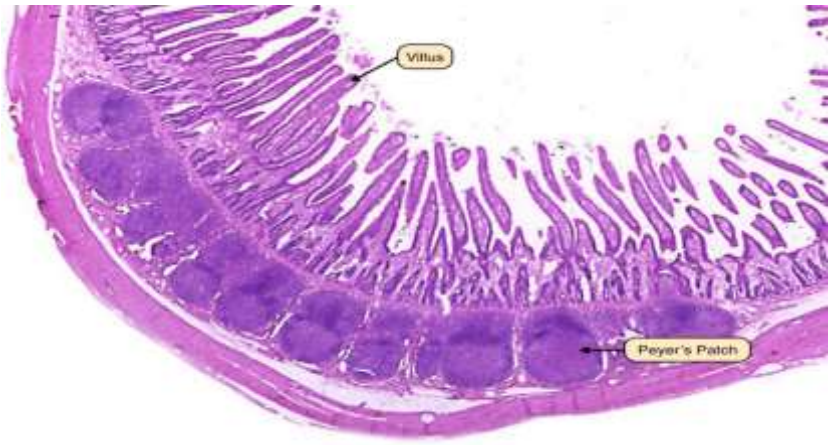


Identifying Characteristics:

- Presence of villi (leaf shaped).
- Presence of pyramidal lymphatic nodule.

Hence, it is the section of Jejunum.

Ileum:

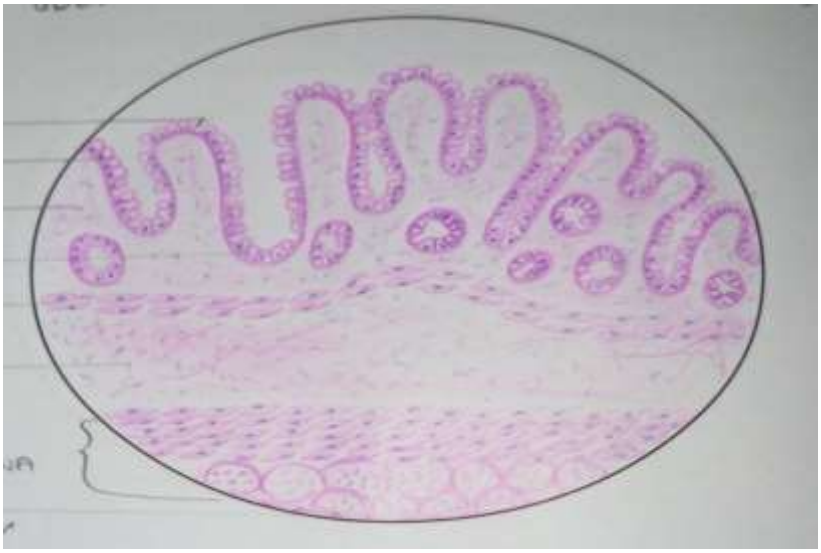


Identification Characteristics:

- Presence of villi (Cluv shaped).
- Presence of peyer's patch.

Hence, it is the section of Ileum.

(k)Large Intestine:

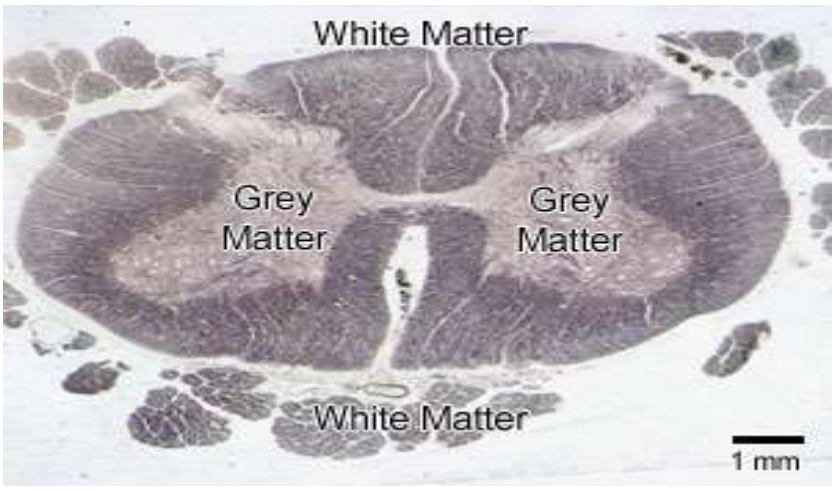


Identifying Characteristics:

- Presence of villi (Flower shaped).
- Presence of goblet cell.

Hence, it is the section of large intestine.

(l)Spinal Cord:

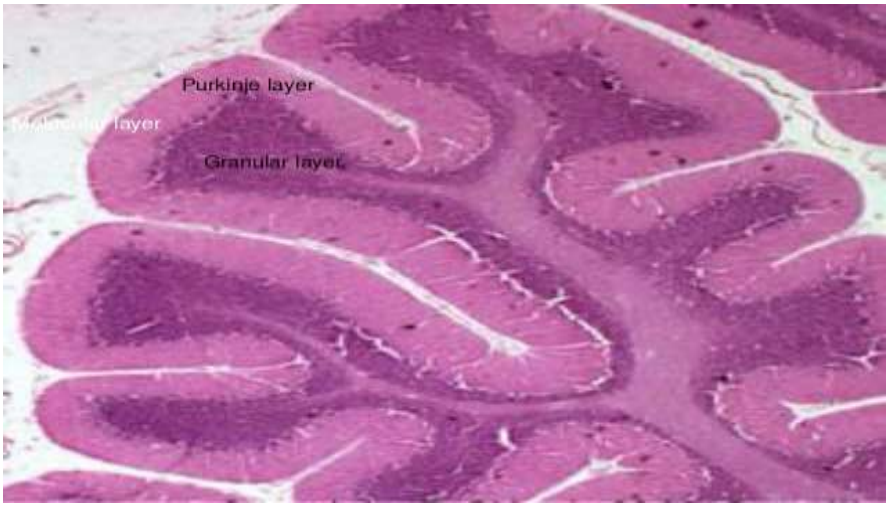


Identifying Characteristics:

- Presence of outer white matter and 'H' shaped inner gray matter.
- Presence of central canal.

Hence, the section is Spinal cord.

(j) Cerebellum:



Identifying Characteristics:

- Presence of outer molecular layer, inner granular layer.
- Presence of purkinje layer in between two layer.
- Hence, it is the section of cerebellum.

2. Preparation of blood film and identification of white blood cells, Differential count.

Methods of preparation of smear:-

The blood smear is prepared in two stages :- 1. Making a blood smear.

2. Fixing and staining the blood.

Making a blood smear:-

A blood smear is made by three methods:- 1. Cover glass method

2. Glass slide (wedge) method and

3. Centrifugal method

The glass slide method is commonly practiced.

Principle:-

Spreading a drop of blood on the glass slide makes a thin film.

Requirements:-

1. Glass Slides.
2. Spreader: These are specially designed glass slides with a smooth edge, the breadth is slightly less than that of usual glass slides. If spreaders are not available, a glass slide with a smooth edge can be used.
3. Equipment of sterile finger puncture.

Procedure:-

1. Take four clean and grease-free glass slides and select one slide as a spreader.
2. Clean the tip of the middle finger with alcohol, allow the finger to air dry and prick the finger tip with a sterile lancet.
3. Discard the first drop of blood.
4. Place a drop of blood on one end (Say the right end) of a slide on the middle, about 1 cm from the end, by touching the slide to the top of the blood drop. Take care about not to touch the skin of the tip of the finger with the slide.
5. Place the specimen slide on the flat surface of the table, and hold it in position at the left end of the slide (the end opposite to the blood drop) with the middle or index finger and thumb of the left hand.
6. Place the smooth clean edge of the spreader slide on the specimen slide just in front of the drop of the blood. There should be an approximate angle of 30º- 45º between the two slides.
7. Using the right hand, draw the speaker back until it touches the drop of blood. Let the blood run along the edge of the spreaders.
8. When the blood has spread evenly across the edge of the spreader slide, push the spreader to the other end of the slide with a smooth, quick and controlled movement.
9. Turn the spreader slide over (this gives another clean edge) and prepare a second blood film using the same procedure.
10. Dry the blood smears quickly by waving them in the air , or if the moisture is too high, as occurs in the rainy season, dry the films by waving them rapidly about 5 cm above the flame of a spirit lamp. Never bring the smear close to the flame as excess heat may damage it.

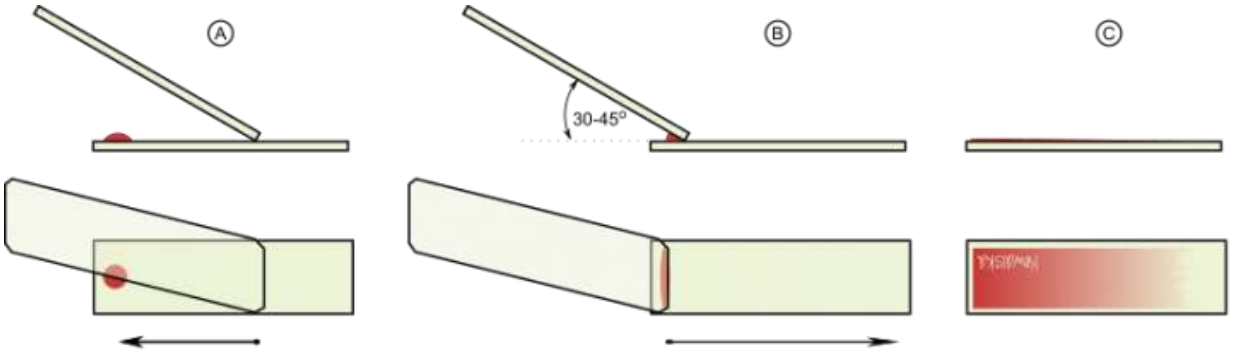


Fig- Blood smear preparation

Precautions:-

1. The glass slides must be scrupulously cleaned. Dirty slides do not give an even smear.
2. Discard the first drop of blood as it contains tissue fluid and is rich in neutrophils.
3. Do not squeeze to make a drop of blood.
4. Use a medium size blood drop.
5. Make the smear immediately after placing the drop of blood on the slide. A delay will cause uneven distribution of which cells on the film.
6. The spreader slide must be moved steadily and confidently with a single, quick and smooth movement.
7. Before the smear is made (before the spreader slide is moved) ensure that the blood has spread uniformly along the edge of the spreader; otherwise a thick narrow smear results.
8. The angle between the spreader slide and the glass slide should be about 45º.
9. The smear should be completely dry. A wet smear if stained will not stick to the slide. Then cells are distorted if the smear takes a long time to dry.

Fixing and staining a smear:

Principle:-

Fixation is the process by which blood cells are made to adhere to the slide, and staining is the process by which the cells (cytoplasm and nuclei) are stained. The blood cells are fixes by methanol. It is advisable to stain the smear soon after making it. If it cannot be stained within few hours, it should be fixed be immersion in absolute methyl alcohol (methanol) for 2-3 seconds, and then air dried.

Requirement:-

1. **Leishman stain: -** This is a mixture of methylene blue and eosin in acetone free methyl alcohol.
    - Methylene Blue (basic dye):- Stains the acidic part of the cell, i.e, the nuclei (DNA) and cytoplasm (RNA) of WBCs and granules of basophils.
    - Eosin (acidic dye):- Stains the basic part of the cell eosinophilic granules and Hb of red cells.
    - Methyl alcohol:- Fixes the smear to the slide. It is acetone free because acetone causes lysis of the cells (breaks cell membrane).
2. **Staining rack :-** This consist of two glass rods placed parallel about 5 cm apart on a tray. The glass rods hold the slides and the tray holds the stain and water poured on the slides.
3. Distilled Water.
4. Pasteur pipette.
5. Blood smears.

Procedure:-

1. Place the slides on the staining rack with the blood smear (dull side of the slide) facing up. If the staining rack is not available, place two glass rod over a tray to hold the slide.
2. Pour 8-12 drops of Leishman stain on the slide. The stain should just cover the smear.
3. Note the time and leave it for ½- 2 minutes.
4. Add double the amount of distilled water on the smear, with the help of dropper , taking care that the water does not spill.
5. Mix the stain and water evenly by blowing gently or by blowing air
6. Note the time and leave it for 7-10 minutes.
7. Pour off the stain and wash the slide gently and thoroughly under tap water.
8. Shake off all water adhering to the slide and set the slide in an upright position in a drying rack.

Precautions:-

1. The smear should dry completely before staining.
2. Adequate quantity (10-12 drops) of stain should cover the entire smear. Do not pour excess stain (it should just cover the smear).
3. Allow adequate fixing time.
4. The distilled Water should just cover the slide. Care should be taken to prevent spillover of the mixture of water and stain.
5. It is important that the staining rack be level so that the stain is uniform through out the film and the mixture does not spill over.

6. Water and stain should be mixed by gentle blowing. Mixing should be done immediately after pouring the distilled water.
7. Exact timing for staining should be followed.
8. When the mixture of stained water is poured off the slide, take care to prevent deposition of scum of the smear.
9. While washing ensure that the smear is not directly under the stream of water.

Identification of cells

Identify various types of cells on the basis of the following characteristics as a result of staining with leishman's stain. Even if the smear is not properly stained, the shape and size of the cells provide sufficient cells for their identification.

(a) Red blood cells:

Appearance:- Red cells appear as round bodies containing no nucleus, granules, or discrete materials.

Staining:- Stain orange red. The red color is darker at the edge of the cell than in the centre. This variation is caused by the biconcare shape of the cells which contain less hemoglobin in its (thinner) center.

Size:- The diameter of the cells is about 7.2 µm.

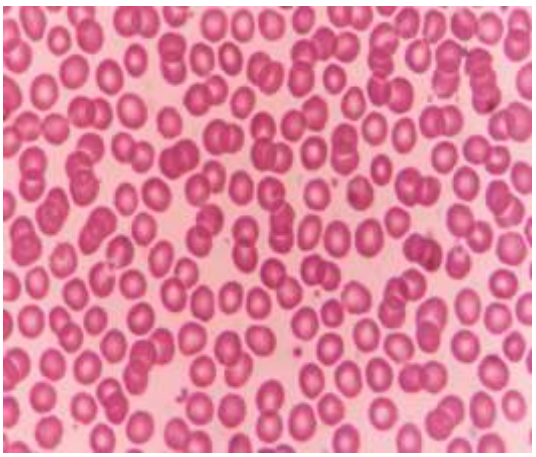


Fig- Red blood cell

(b) Platelets:

Appearance:- Under high power, they look like dirt and stain deposits. Under oil immersion, they appear like pin heads. They contain no nuclei. On fine adjustment, platelets look refractive, this is a characteristic feature that distinguishes them from deposited stained particles.

Stain :- Stain massive pink.

Distribution:- Usually they are present in groups or aggregates (many platelets lying close to each other) but presence of a single (isolated) platelet is not unusual.

Size :- They are the smallest cells in the peripheral smear. The diameter of the platelet is 2-4µm.

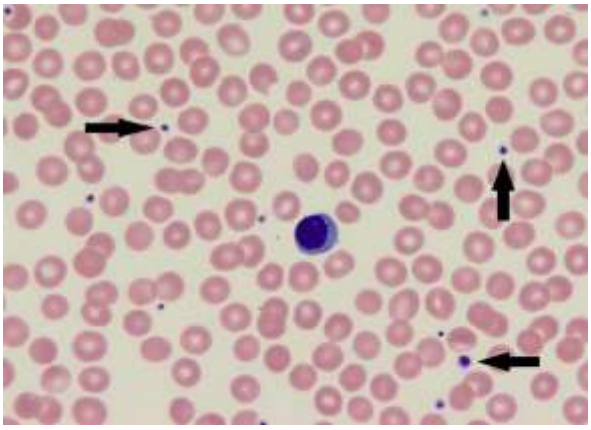


Fig- Platelet

(c) Leucocytes:-

Neutrophils:-

Size:- 10-14µm

Nucleus:- Multilobed (2-6 lobes), lobes are connected by thin strands. In band form, the nucleus is sausage shaped(also called stab form).

Cytoplasm:- Looks pale pink, containing fine pink granules.

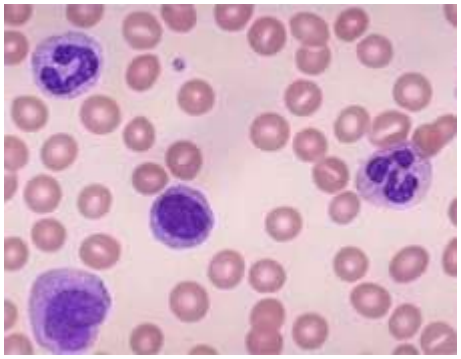


Fig- Neutrophil

Eosinophil:-

Size:- 10-14µm

Nucleus:- Usually bio lobed, lobes are connected by a thick strand, giving the appearance of spectacles (spectacle shaped nucleus).

Cytoplasm:- Stains faint pink containing coarse brick red or red orange granules. The cytoplasm is usually not visible as it is observed by granules.

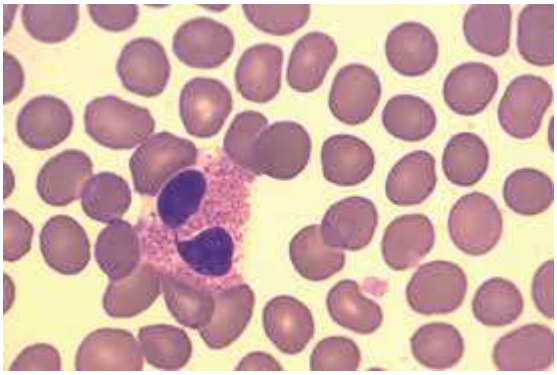


Fig- Eosinophil

Basophils:-

Size: -10-14 µm

Nucleus :- usually boiled, but lobes are usually not distinctly visible because the cell is stubbed with granules.

Cytoplasm:- Contain numerous coarse granules. Granules are blue black and fill the cells and obscure the nucleus.

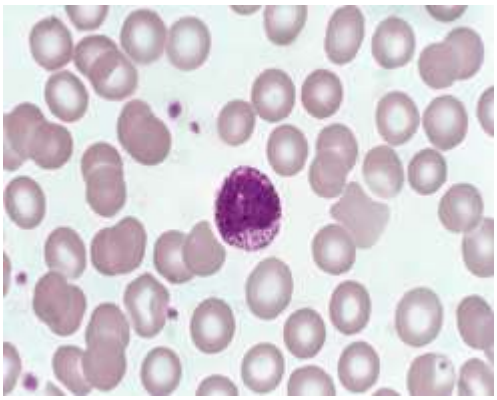


Fig- Basophile with red blood cell

Large lymphocytes:-

Size:-10-14 µm.

Nucleus: - occupies 80-90% of the cell. It is eccentrically placed and oval or round with or without a dent. It is homogenous (compact) and violet in colour.

Cytoplasm:- Clear blue cytoplasm , usually contains no granules.

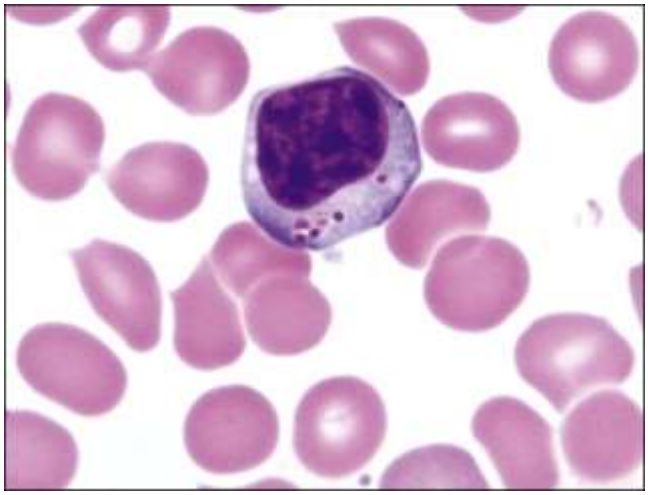


Fig- Large lymphocyte

Small lymphocytes :-

Size :- 7-8 µm. (same as or slightly larger than red cells).

Nucleus:- occupies almost the whole cell. It is homogenous (compact) and deep violet in color.

Cytoplasm:- May not be present or sometimes there may be a thin rim of clear blue cytoplasm. Present at the periphery. There are no granules.

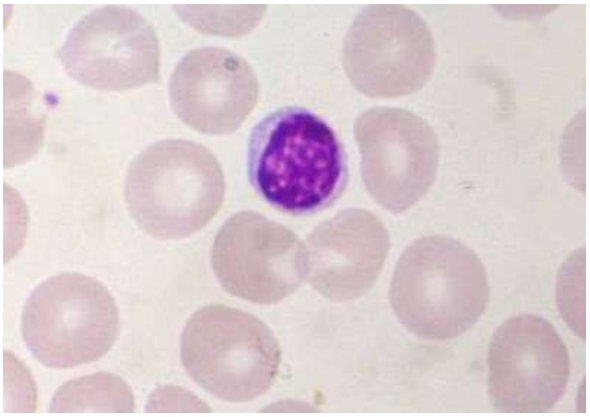


Fig- Small lymphocyte

Monocytes:

Size: 12-24 µm the largest leucocytes.

Nucleus: Occupies 50 percent of the cell, centrally or slightly eccentrically placed. It is usually kidney shaped or horse shoe-shaped, but may be round, oval, dump-bell shaped, or irregular.

Non homogenous (spongy) in appearance and pinkish violet in color.

Cytoplasm:-

- Ground glass (hazy, turbid) appearance.
- Grey blue in color.
- Usually contains no granules, but sometimes (15-30% of monocytes) fine granules may be present.

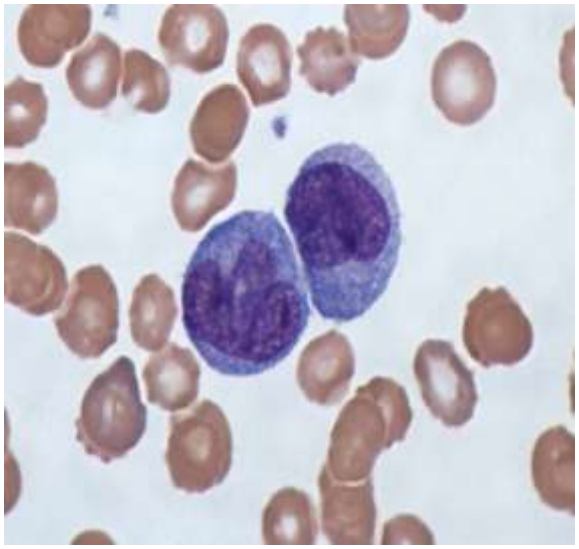


Fig- Monocyte

Discussion

A bad smear results from lack of sincerity and interest in making the smear. If all the steps of preparing and staining the smear are properly followed the smear will definitely be a good one. The smear may be prepared well but improper staining may spoil it totally.

Differential Leucocytes Count

Introduction:

The types and number of each type of leucocytes counted are traditionally reported as percentages. Determination of percentage distribution of leucocytes in the peripheral blood is known as differential leucocytes count (DLC). Five types of leucocytes are encountered in normal peripheral blood. They are divide into granulocytes and agranulocytes. Neutrophils, eosinophils, and basophils are granulocytes and lymphocytes and monocytes are agranulocyte.

Method of counting:

Principle:- A blood film stained with leishman stain is examined under an oil immersion objective and the different white blood cells are identified. The percentage distribution of these cells is then determined.

Requirement: -

1. Glass slides
2. Leishman stain
3. Microscope
4. Distilled water
5. Pasteur pipette

Procedure:-

1. Prepare blood smear and stain the smear with leishman stain.
2. First, examine the smear under low power objective for general scanning and assessing the quality of the smear, and for studying the distribution pattern of the cells.

Subject Name-
Age-
Sex-

Observation and result:

Field status	Neutrophils	Eosinophil	Basophile	Monocyte	Large lymphocyte	Small lymphocyte	Total	Grand total

3.Estimation of Haemoglobin by cyanametric Method

Principle: Potassium fericyanide converts the Hb in the sample to methaemoglobin. The methaemoglobin further reacts with potassium cyanide to form a cyanmeth haemoglobin complex. Intensity of the complex formed is directly proportional to the amount of Hb present in the sample.

Requirements:

1. Haemocor-D solution.
2. Fresh blood sample collected in anticoagulant.
3. Colorimeter.
4. 20 microliter (µl) micropipette with tips.
5. Test tube
6. Hb standard.

Procedure: 5 ml haemocor D solution is added to test tube lebelled as 'B' (Blank) 'T' (Test) and 'S' (Standard).

↓

20 µl of sample (whole blood sample0 is added to the test tube marked as 'T'.

↓

Mixed well and incubate at room temperature atleast 3 mins.

↓

Measure the absorbance of test sample(T) and standard against blank(B).

Observation: Absorption of (Abs) T (Test sample)- 0.23
Absorption of S (Standard sample)- 0.31

Normal Reference value of Haemoglobin:

New born- 16.25 gm/dl
Infant- 11-14 gm/dl
Children (upto 10 yrs)- 12-16 gm/dl
Male- 12-18 gm/dl
Female- 12-16 gm/dl

Calculation & Result:

$$\text{Hb gm/dl} = \frac{\text{Abs-T}}{\text{Abs-S}} \times \frac{251}{1000} \times 60$$

$$= \frac{0.23}{0.31} \times \frac{251}{1000} \times 60$$

$$= 11.15 \text{ gm/dl}$$

- 251 is the dilution factor i.e

$$= \frac{\text{Total reagent volume (5.02 ml)}}{\text{Sample volume (0.02 ml)}}$$

- 1000 is the multiplication factor to convert mg to gm.
- 60 is the concentration of the Hb standard in mg %.

4. Determination of Bleeding Time and Clotting Time of Blood, Blood grouping

Determination of Bleeding Time

Principle: Bleeding time is the interval between the moments when bleeding stop. Normal bleeding time (Duke's method) is about 4 min. Bleeding time is prolonged in purpose but normal in coagulation disorder like haemophilia. Normal bleeding time is about 2-5 min.

Requirement:

1. Sterile lancet
2. Cotton
3. Rectified Spirit
4. Stop watch
5. Filter paper

Procedure: (Duke's Method)

1. Sterilize the finger tip using rectified spirit and allow to dry.
2. Make a sufficiently deep prick, using a sterile lancet so that blood come out freely without squeezing.
3. Note the time (start the stopwatch) when bleeding start.
4. After 30 sec wipe the blood by touching the finger tip with a filter paper.
5. This is repeated every 30 sec. Each time using a fresh portion of filter paper till bleeding stops.
6. Note the time (stop the watch) when it is seen then the bloos stain on the filter paper gets smaller to disappear finally when bleeding stops.

Fig: Bleeding Time

Result:

- **Name of the subject-**
- **Age-**
- **Sex-**
- **Body weight-**

Normal bleeding time: Normal bleeding time is 2-5 min.

Determination of Clotting Time

It is the time required for blood to clot without the presence of any substances. Clotting time is the interval between the moments when fibrin thread is past seen. Normal value is 3-10min. Bleeding time and clotting time are not the same. Bleeding time depends on the integrity of platelets and vessel walls where as clotting time depends on the availability of coagulation factor. In coagulation disorder like haemophilia, clotting time is prolonged but bleeding time is remained normal. Clotting time is also prolonged is depending upon some conditions like vit-k deficiency liver diseases, overdose of anti coagulants etc.

Requirement:

1. Fine capillary glass tubes about 10mm length.
2. Rectified Spirit
3. Cotton
4. Stop watch
5. Filter paper
6. Niddle

Procedure:

1. Sterilize the finger tip using rectified spirit and allow to dry.
2. Prick the finger by niddle and remove the first drop of blood.
3. Squeeze the finger to obtain a large drop of blood and fill the capillary tube with blood.
4. After 1 min start breaking small pieces of the capillary tube in every 30 sec. Until a fibrin thread is seen between the two broken ends.

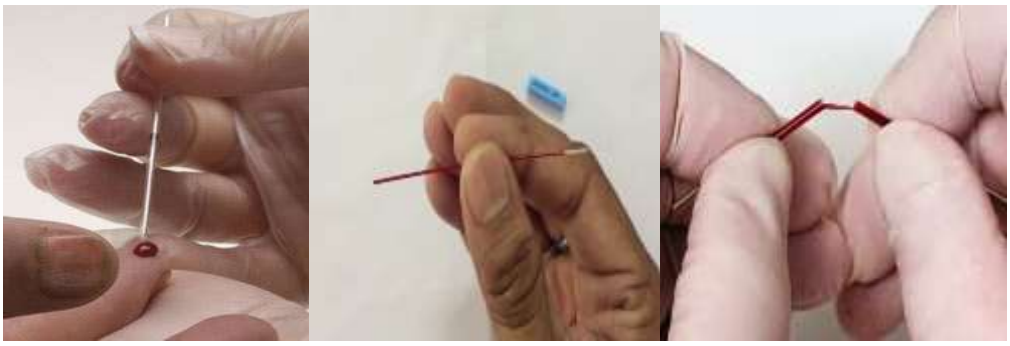


Fig: Procedure of Clotting Time

Result:

- **Name of the subject-**
- **Age-**
- **Sex-**
- **Body weight-**

Normal clotting time: Normal clotting time is 4-5 min.

Determination of Blood Group and Rh-factor

The blood grouping test is based on the haem agglutination reaction. Human RBCs contain A and B or AB antigen agglutinase with the corresponding antibody. Agglutination of RBCs with anti A monoclonal and anti B monoclonal and or anti AB monoclonal is a positive test result and indicates the presence of the corresponding antigen. Absence of agglutination of RBCs with anti-A, anti-B and or anti-AB monoclonal is a negative test result that indicates the absence of corresponding antigen. Mixing of RBCs with anti-D antiserum will result in agglutination reaction. If corresponding D antigen is present in the RBCs. Visible agglutination of RBCs with anti-D antiserum indicates positive reaction where no agglutination with anti-D negative reaction and absence of antigen.

Materials require:

1. Glass slide
2. Disposable sticks
3. Anti A, B, D monoclonal kits
4. Niddle (Disposable)

Procedure:

1. Take a clean and dry glass slide and placed it on a horizontal surface.

↓

2. Labelled it with anti-A, anti-B and anti-D respectively.

↓

3. Distance one drop of anti-A, anti-B and anti-D monoclonal serum respectively on the glass slide.

↓

4. One drop of whole blood will be added to anti-A, anti-B and anti-D monoclonal respectively, collecting by niddle, pricking in finger.

↓

5. Mixed well by disposable stick and wait of up to 1 min.

↓

6. Observed it for the evidence of agglutination.

Reaction with anti-A monoclonal	Reaction with anti-B monoclonal	Reaction with anti-D (AB) monoclonal	Blood Group
+	-	+	**A+**
+	-	-	**A-**
-	+	+	**B+**
-	+	-	**B-**
+	+	+	**AB+**
+	+	-	**AB-**
-	-	-	**O-**
-	-	+	**O+**

Result:

- **Name of the subject-**
- **Age-**
- **Sex-**
- **Blood Group-**

Anti-A monoclonal	Anti-B monoclonal	Anti-D (AB) monoclonal	Blood Group

5. Measurement of Blood Pressure and Pulse Rate

Determination of Blood Pressure

Introduction:

During flow of blood through the blood vessels a pressure excreted on the wall of vessels perpendicularly called blood pressure (BP). There are 4 types BP. They are-

Systolic BP	Diastolic BP	Pulse Bp	Mean BP
Pressure due to Ventricular systolic pressure	Pressure due to ventricular diastolic 120±10 (mmHg) 80±10 (mmHg)	Systolic Pressure- Diastolic Pressure	Diastolic Pressure- 1/3 pulse

Principle:

The cuff of the sphygmomanometer is wrapped around the arm of the subject. The bag is then inflated until the air pressure in the cuff overcomes the arterial pressure and obliterates the arterial lumen. This is confirmed by palpating the radial pulse that disappears when the cuff pressure is raised above the arterial pressure.

When the pressure is the cuff reaches just below the arterial pressure, blood escape beyond the occlusion into the periplural past of the artery and the pulse starts reappearing. That is detected by appearance of sound is the stethoscope and is taken as the systolic pressure. Subsequently, the quality of the sound changes and finally disappears. The level where sound disappears is taken as the diastolic pressure. The sound disappears because the flow in the blood vessels became laminar.

Requirement:

1. Sphygmomanometer (Doctor)
2. Stethoscope (Doctor)
3. A sitting chair (height 1.6 feet from floor)

Procedure:

1. **Blood pressure measurement during sitting posture:**

i) Subject ask to sit down on the chair and allowed to take rest for 5 min.

ii) Exposed of the left arm up to shoulder.

iii) Wrapped the Rina-Rocci-Cuff (which consist of inflatable rubber bag attaching to two tubes, 1 tubes attached to mercury manometer and other tube is connective to air pump.

iv) Raised blood pressure of the manometer by compressing the rubber bulb to disappear the radical point.

v) Reduced the pressure gradually and noted the level of indications, where pulse reappear. This is systolic pressure.

vi) Gradually fall the pressure and again pulse disappearance and noted the level of the indication this is the diastolic pressure.

2. **Blood pressure measurement during standing posture:**
   Ask the subject to stand erect and blood pressure was measured as sitting posture.

3. **Blood pressure measurement during supine posture:**
   Ask the subject lying out the floor and measure the blood pressure by previous manner.

Result:

- **Name of the subject-**
- **Age-**
- **Sex-**
- **Body weight-**

Different posture	No. of observation	Systolic BP (mmHg)		Diastolic BP (mmHg)		Pulse BP (SBP-DBP mmHg)	Mean pressure (DBP+1/3PP mmHg)
		Avg		Avg			
Sitting posture	1						
	2						
	3						
Standing posture	1						
	2						
	3						
Supine posture	1						
	2						
	3						

Normal Range: