Chapter 2

Anticoagulants

Chapter Outline

- Anticoagulants
- Preparation and Staining of Peripheral Blood Smears
- Haemoglobin Estimation
- Total Red Blood Cell (TRBC) Count
- Determination of Packed Red Cell Volume (PCV) and Absolute Indices
- Erythrocyte Sedimentation Rate (ESR)
- Differential Leucocyte Count (DLC)
- Absolute Eosinophil Count
- Platelet Count
- Reticulocyte Count

ANTICOAGULANTS

For various purposes a number of different anticoagulants available. The anticoagulants in use are:

1. Disodium or dipotassium ethylenediaminetetraacetic acid (EDTA; Sequestrene).
2. Ammonium and potassium oxalate mixture
3. Tri-sodium citrate
4. Heparin and
5. Acid-citrate-dextrose (ACD) solution

1. Ethylenediaminetetraacetic acid (EDTA)

The sodium and potassium salts of EDTA are powerful anticoagulants and they are the anticoagulants of choice for routine haematological work. EDTA acts by its chelating effect on the calcium molecules in blood. To achieve this requires a concentration of 1.2 mg (approximately 4 µmol) of the anhydrous salt per ml of blood. The recommended concentration of the dipotassium salt is 1.50 ± 0.25 mg/ml of blood.

The dilithium salt of EDTA is equally effective as an anticoagulant, and its use has the advantage that the same sample of blood can be used for chemical investigations. However, it is less soluble than the dipotassium salt (160 g/L).

EDTA is not suitable for use in the investigation of coagulation problems and should not be used in the estimation of prothrombin time.

2. Ammonium and potassium oxalate mixture

This consists of a mixture of 2 parts of potassium oxalate and 3 parts of ammonium oxalate. Take 1% solution of potassium oxalate 0.4 ml and 1% solution of ammonium oxalate 0.6 ml in a test tube. Evaporate to dryness in the incubator. This amount of oxalates is sufficient to prevent coagulation of 5 ml blood.

3. Trisodium citrate

0.109 mol/L trisodium citrate (32 g/L $Na_3C_6H_5O_7 2H_2O$) is the anticoagulant of choice in coagulation studies. Nine volumes of blood are added to 1 volume of the sodium citrate solution and immediately well mixed with it. Sodium citrate is also the anticoagulant most widely used in the estimation of the sedimentation rate (ESR); for 4 volumes of venous blood are diluted with 1 volume of the sodium citrate solution.

4. Heparin

This may be used at a concentration of 15 ± 2.5 IU per ml of blood. Heparin is an effective anticoagulant and does not alter the size of the red cells and a good dry anticoagulant when it is important to reduce to a minimum the chance of lysis occurring after blood, has been withdrawn. However, heparinized blood should not be used for making blood films as it gives a faint blue colouration to the background when the films are stained by Romanowsky dyes. This is especially marked in the presence of abnormal proteins. Heparin is the best anticoagulant to use for osmotic fragility tests; otherwise it is inferior to EDTA for general use and should not be used for leucocyte count as it tends to cause the leucocytes to clump.

5. Acid-citrate-dextrose (ACD) solution

This is preferred for blood transfusions, for preserving red cells, for enzyme studies, and for the study of haemolytic processes. A standard preparation consists of sodium citrate 1.32 gm, citric acid 0.48 gm, dextrose 1.40 gm, and distilled water to 100 ml. One ml of ACD solution is sufficient to prevent coagulation of 4 ml of blood.

Mode of Action of Anticoagulants

EDTA and sodium citrate remove calcium which is essential for coagulation. Calcium is either precipitated as insoluble oxalate (crystals of which may be seen in oxalated blood) or bound in a non-ionized form. Heparin works in a different way; it neutralizes thrombin by inhibiting the interaction of several clotting factors in the presence of a plasma co-factor, antithrombih Ill. Sodium citrate or heparin can be used to render blood incoagulable before transfusion.

For better long-term preservation of red cell for certain tests and for transfusion purposes citrate is used in combination with dextrose in the form of acid-citrate-dextrose (ACD), citrate-phosphate-dextrose (CPD) or Alsever's solution.

Effects of Anticoagulants of Blood Cell Morphology

If blood is allowed to stand in the laboratory before films are made, degenerative changes occur. The'changes are not solely due to the presence of an anticoagulant for they also occur in defibrinated blood.

Irrespective of anticoagulant, films made from blood which has been standing for not more than 1 hr at room temperature (18–25°C), are not easily distinguished from films made immediately after collection of the blood. By 3 hours changes may be discernible and by 12–18 hours these become striking. Some but not all neutrophils are affected: their nuclei may stain more homogeneously than in fresh blood, the nuclear lobes may become separated and the cytoplasmic margin may appear ragged or less well defined; small vacuoles appear in the cytoplasm. Some or many of the large mononuclears develop marked changes; small vacuoles appear in the cytoplasm and the nucleus undergoes irregular lobulation which may almost amount to disintegration. Some of the lymphocytes, too, undergo a similar type of change; a few vacuoles may be seen in the cytoplasm and the nucleus may undergo major budding so as to give rise to nuclei with two or three lobes. Other lymphocyte nuclei may stain more homogeneously than usual.

The red cells (of normal blood at least) are little affected by standing for up to 6 hours at room temperature (18–25°C). Longer periods lead to progressive crenation and sphering.

The cells in defibrinated blood undergo degenerative changes at about the same rate as those in EDTA blood.

All the above changes are retarded but not abolished in blood kept at 4°C. Their occurrence underlines the importance of making films as soon as possible after withdrawal. But delay of up to 1–3 hours or so is certainly permissible.

The practice of making films of blood before it is added to the antfcoagulant (e.g. at the bedside) is to be commended, especially when screening for lead toxicity, as the granules of punctate basophilia may not stain in anticoagulated blood. In fresh blood films, however, the platelets usually clump and it is less easy to estimate the platelet count from inspection of the film. Such films are nevertheless of particular value in investigating patients suspected of suffering from purpura, as in certain rare conditions, the absence of platelet clumping is a useful pointer to the diagnosis.

PREPARATION AND STAINING OF PERIPHERAL BLOOD SMEARS

Peripheral smear provides information about red cells—their number, shape, size and variation in morphology to diagnose different types of anaemia and other haematologic disorders. It also provides information for differential leucocyte count, platelets, abnormal cells and haemoparasites.

Procedure

1. Take a drop of blood from a finger prick and put it on a clean slide 1 cm from the end.
2. Take another slide with a smooth edgespreader and place the edge over the drop so that blood spreads along the edge.
3. Keep the spreader slide at approximately 30–40° angle and make a smear with a forward movement of the spreader. Smear should be 2.5–3.5 cm in length and spreader should preferably have smaller edge than the slide on which smear is being made.
4. Air dry the smear.
5. Make at least 2 smears of each sample.

Fixation of the Smear

Smears should be fixed within 4 hours to get good staining of cells and avoid background staining of plasma.

Take methanol in a Coplin jar. Give 4–6 dips to the smear in methanol. Let it get air dry.

Staining of the Smear

Staining of the smears is carried out using Romanowsky's stains which stain red cells, white cells, platelets and parasites. Romanowsky's stains consist of mixture of methylene blue, eosin and methylene blue oxidation products—azures which are formed by interaction and chemical treatment of the stain.These products are responsible for different shades of staining, e.g. red cells—pink, eosinophilic granules—red—orange; basophilic granules—bluish black; neutrophilic granules—lilac (azurophilic granules combine with azures of the stain); nuclear chromatin—purple black; white cell cytoplasm—faint purple.

Following are the commonly used stains:

- Leishman stain
- Giemsa stain
- Wright's stain

- Jenner's stain
- Jenner-Giemsa stain

Most of the routine laboratories use

- Giemsa stain
- Leishman stain

The details are given as follows.

Leishman stain

Leishman stain powder	1 gm
Methanol (acetone free)	500 ml

Mix the two. Warm the mixture to 50°C for 15 minutes in a conical flask. Filter the stain and the stain is ready for use after 2 days.

Giemsa stain

Giemsa stain powder	1 gm
Glycerol	66 ml
Methanol	66 ml

Dissolve Giemsa stain in glycerol at 60°C for 2 hours. Cool it. Add 66 ml of methanol (acetone free) and mix. Keep it at 37°C for 1 week to ripen. Stain is filtered before use.

Buffer water (Sorensen's phosphate buffer)

Solution A

Potassium dihydrogen phosphate	9.08 gm/L

Solution B

Dibasic sodium phosphate	9.47 gm/L

For pH 6.8

Solution A	50.8 ml
Solution B	49.2 ml

Mix the two solutions to get 100 ml of buffer water.

Leishman's Staining

- Place the PS (peripheral smear) on the staining rack.
- Pour the stain on the slide with a dropper and count the drops and cover the whole slide with the stain.
- After 2 minutes, pour double the number of drops of buffer water or distilled water on the slide and mix buffer with stain using a dropper.
- Let the slide be stained by this mixture for 15–30 minutes. Exact time to be decided for each batch of stain.
- Pour running water from one side of the slide so that the stain scum is drained off the slide and no more stain is left on the slide.
- Pick up the slide and keep it slanting for it to dry up.
- The slide is ready for examination.

Sources of Error

- If the pH of the buffer is too acidic, then red cells stain pink, and WBCs stain very light.
- If the pH of the buffer is too basic, red cells stain blue and WBCs take a bluish black hue. Hence for proper staining buffer with correct pH 6.8 should be used.
- Sometimes red cells demonstrate holes in them. These are due to air bubbles because of air drying in humid weather. To overcome this, smear should be dried up quickly by waving it vigorously in air.

Giemsa Staining

- Fixation of the smears 8–10 dips in methanol in a Coplin jar.
- Dry the slides and then pour the diluted (1 in 10) stain for 20–30 minutes. Exact dilution and time of staining to be determined for each batch of stain.
- Wash the slide (as in Leishman staining) and dry it.

Evaluation of a Peripheral Smear

Apply very thin layer of oil all over the smear and examine it under low power. Area for DLC-tail and edges of the smear should be avoided while doing DLC, since monocytes and neutrophils predominate in these areas. This area is better suited for parasites like malarial parasite, microfilaria, since these are heavier than red cells carried to the tail by the spreader. DLC should be carried out in thin part of the smear next to the tail. For red cell morphology, the best area is between tail and body of the smear. Tail, head and edges should be avoided.

HAEMOGLOBIN ESTIMATION

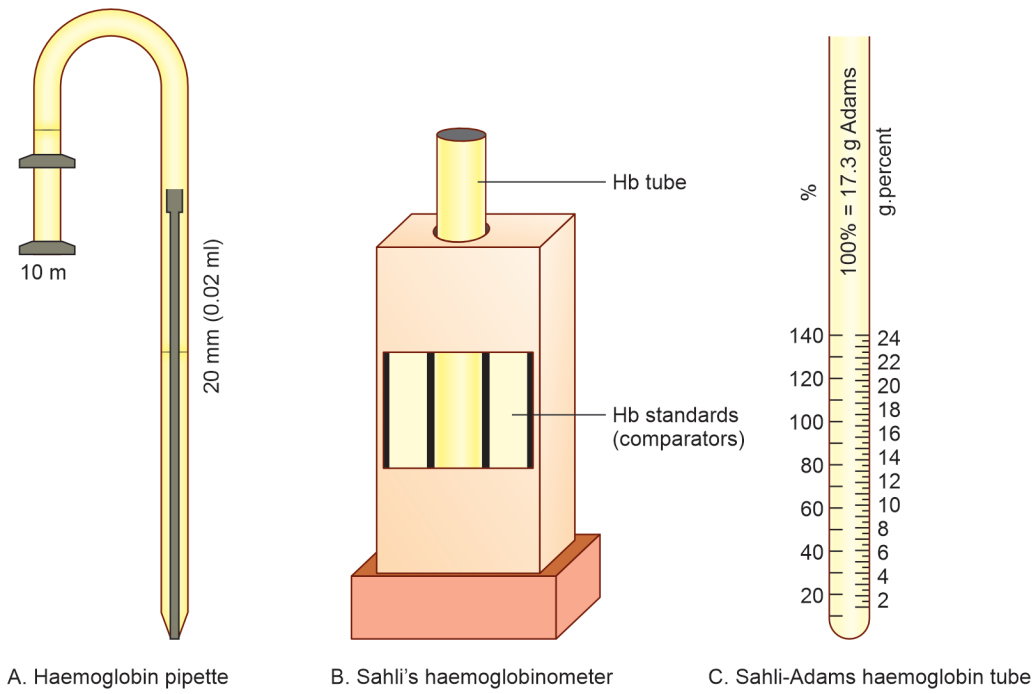
The haemoglobin (Hb) content in a blood sample may be determined by measurement of its colour, its power of combining with oxygen or carbon monoxide or by its iron content. The clinical methods for routine purpose are all based on colour or light intensity matching techniques:

A. i. Sahli or acid-haematin method (Fig. 2.1)
   ii. Alkaline-haematin method
   iii. Haldanes carboxyhaemoglobin method
   iv. Oxyhaemoglobin method
   v. Cyanmethaemoglobin method

B. Copper sulphate specific gravity method

Haemoglobin is a chromoprotein consisting of the colourless globin molecule attached to four red coloured haem molecules. The globin molecule consists of two alpha polypeptide chains and two beta polypeptide chains. Haem is a metal complex containing an iron atom in the centre of a porphyrin structure. Haemoglobin is formed in developing erythrocytes (normoblasts) in the marrow.

The biosynthesis of haemoglobin involves the triad of manufacture of haem, manufacture of globin and iron metabolism. Methods for estimation of haemoglobin are as follows:



A. Haemoglobin pipette B. Sahli's haemoglobinometer C. Sahli-Adams haemoglobin tube

Fig. 2.1: Sahli's acid haematin method

A. i. Sahli's Acid Haematin Method

Principle: Hb is converted into acid haematin by hydrochloric acid. The brown colour of the compound is matched against a brown glass standard in a comparator.

Apparatus: Sahli-type haemoglobinometer consisting of the comparator with glass standards, a square Hb tube marked both grams and percentage figures and Hb pipette marked at 20 cu mm, 0.1 N HCl and distilled water.

Technique

1. Fill the Hb calibrated tube up to the mark 20 (not less) with 0.1 N HCl by means of a dropper.
2. Fill the Hb pipette exactly up to 20 cu mm mark by gently controlled sucking; the pipette is held horizontal while taking the blood. If a slight excess is drawn in, it may be removed by touching the point of the pipette with the finger or gauge. If a great excess has been drawn in, inaccuracy will result, in this case the pipette must be cleaned, dried and refilled. Wipe off with gauge the blood on the outside of the pipette.
3. Empty the pipette into acid in the tube by keeping the point of the pipette to the bottom of the tube and gently blowing off the blood without causing bubbles. Rinse the pipette at least three times by drawing in and discharging the blood acid mixture. Now withdraw the pipette half way up the tube and rinse the outside of pipette with a few drops of the acid.
4. Mix the acid haematin solution in the tube with the glass rod and allow the tube to stand for 10 minutes. In this interval at least 95% of the colour of acid haematin is developed.
5. Now dilute the solution of acid haematin by adding distilled water, drop by drop, stirring the mixture all the time wtih glass rod. The comparator is held against good daylight and the additon of water continued till the colour of solution matches perfectly with that of the standards. Take the reading in grams per cent. The bottom of the meniscus is read.

Normal Range

Males	13–18 gm
Females	11–16 gm
Newborns	14–19 gm%

Significance

Low Hb values are seen in various types of anaemias.

High Hb values are seen in polycythaemia rubra vera, secondary erythrocytosis.

ii. Alkaline-Haematin Method

Haemoglobin, methaemoglobin, carboxyhaemoglobin and sulphaemoglobin are converted to alkaline haematin by addition of sodium hydroxide, a strong alkali. It forms a true solution and the brown colour can be read against comparable standards or in a colourimeter. Foetal haemoglobin and Hb-Barts are alkali-resistant, but can be converted by heating in a boiling water bath for 4 minutes or by collecting the blood first into acid and then adding alkali (acid-alkali method).

iii. Haldane's Carboxyhaemoglobin Method

Haemoglobin is converted to carboxyhaemoglobin (which is bright red in colour), by exposing it to carbon monoxide (CO). It is a relatively accurate method but CO is dangerous.

iv. Oxyhaemoglobin Method

Haemoglobin is converted to oxyhaemoglobin by mixing blood with a dilute solution of sodium carbonate or ammonium hydroxide. The intensity of the colour obtained is measured colourimetrically. It is a fast and accurate method, but traces of copper can give errors.

v. Cyanmethaemoglobin Method

Principle: Potassium ferricyanide converts Hb from ferrous to ferric state to form meth-Hb. The resulting meth-Hb combines with potassium cyanide to produce the stable pigment cyanmeth-Hb. Cyanmeth-Hb represents the sum of oxy-Hb, carboxy-Hb and meth-Hb.

Apparatus: Photoelectric colourimeter with filter 540 nm, test tubes, 5 ml pipettes and 20 µl micropipette (an accurately calibrated Sahli's pipette may be used).

Precaution: Do not pipette reagent by mouth. This reagent is poisonous. The concentration of KCN in Hb diluting reagent is 50 mg/L. The lethal dose of Hb diluting reagent is about 4,000 mg/L.

Reagent Drabkin's solution: Dissolve 5 gm of sodium bicabonate in distilled water first. Add 0.250 gm of potassium cyanide KCN in the solution, add 1 litre of distilled water. Then add 1.0 gm of potassium ferricyanide $K_4Fe(CN)_6$ in the solution. Shake well, make final volume with distilled water to 5 litres.

Interfering substance: Gross lipemia may result in a positive error of up to 3 g/dl.

Calibration curve: Hb standard for the cyanmeth-Hb method is used for calibrating. Into three clean and dry test tubes add solutions as described below (Fig. 2.2):

1. Pipette 5 ml of Hb standard in the first tube (60 mg/100 ml)
2. Into the second test tube pipette exactly 2.5 ml of Hb standard and add in the same tube exactly 2.5 ml of Hb diluting reagent. Stopper the tube and mix by repeated inversion, this is 1:1 dilution of standards (30 mg cyanmeth-Hb per 100 ml).
3. In the third test tube add 5.0 ml of Hb diluting reagent which serves as a blank.

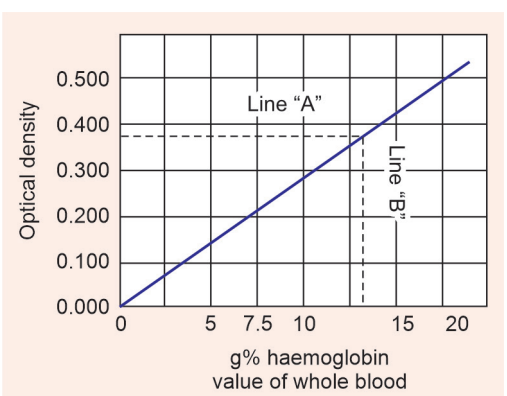


Fig. 2.2: Calibration curve

Measurement of Optical Density/Transmittance

1. Set the wavelength of photoelectric colourimeter at 540 nm, pour the blank (test tube no. 3) into the cuvette. Set the optical density to zero or transmission at 100%.
2. Pour the diluted Hb standard (test tube no. 2) into the cuvette and record the optical density or per cent transmission (cyanmeth-Hb standard).
3. Pour the undiluted Hb standard into the cuvette and record the optical density or per cent transmission.
4. If per cent transmission is determined, convert the reading to optical density.
5. Plot a graph Hb in mg/dl on horizontal axis and optical density on the vertical axis.
6. The value of cyanmeth-Hb in mg/dl is given on each vial. Half of this value will give the concentration of cyanmeth-Hb in the tube containing 1 : 1 dilution of Hb standard.
7. The equivalent g/dl Hb conc. in the undiluted and diluted standard can be calculated as shown below:
   Equivalent g/dl Hb value of undiluted standard.
   - g/dl Hb value of undiluted standard × dilution factor.
   - 0.06 × 251 (if the conc. of Hb standard is 60 mg/dl).
   - 15.06 equivalent g/dl Hb value of 1:1 diluted standard.
   - g/dl Hb value of diluted standard × dilution factor.
   - 0.03 × 251 (Half conc. of the Hb standard 60 mg/dl).
   - 7.53 = 7.5

> **Note:** The mg/dl Hb value (printed on the label divided by 1000 gives the g/dl Hb.

   The procedure using 0.02 ml of whole blood in 5 ml Hb diluting reagent, the dilution factor is 251. Alternatively, the procedure using 0.02 ml of whole blood in 6 ml of Hb diluting reagent, the dilution factor is 301.
8. Draw a straight line connecting the two plotted points on the graph which should pass through origin. Reagents should be room temperature 25 ± 5°C.

Method

	Test	*Blar*
Hb diluting reagent	5.0 ml	5.0 ml
Fresh whole blood	0.02 ml	–

Mix well, allow to stand at room temperature for 3 minutes and measure optical density/transmittance of the test against blank 540 nm.

Record the readings: The colour is stable for more than 24 hours in well-stoppered tubes, kept in dark.

From the optical density reading of the test specimen determine the concentration of Hb in g/dl from the calibration curve.

If the optical density reading of test specimen is 0.385, then from the optical density reading of 0.385

draw a straight line parallel to the horizontal axis until it intersects the calibration curve. From this intersection draw a line parallel to the vertical axis down horizontal axis. Alternatively if a single standard reading is taken, the following calculation method may be used.

Calculation

$$\text{Hb} = \frac{\text{OD of unknown}}{\text{OD of standard}} \times \frac{\text{Conc. of standard in mg/dl} \times \text{dilution factor}}{1000} \text{ g/dl}$$

$$\text{Example: } \frac{0.385 \times 60 \times 251}{0.418 \times 1000} = 13.8 \text{ g/dl}$$

Normal range of Hb

Men	15.5 ± 2.5 g/dl
Women	14.0 ± 2.5 g/dl
Infants (full-term cord blood)	16.5 ± 3.0 g/dl
Children 3 months	11.0 ± 1.5 g/dl
Children 3–6 years	12.0 ± 1.0 g/dl
Children 10–12 years	13.0 ± 1.5 g/dl

B. Copper Sulphate Specific Gravity Method

The method is based on specific gravity and is a reasonably reliable method for determining the haemoglobin of the blood donor. It is indirect measure of the Hb value.

Preparation of Copper Sulphate Solution

1. Preparation of copper sulphate solution (sp. gravity 1100).

   Dissolve 159.63 g of pure air dried crystals of copper sulphate ($CuSO_4 \cdot 5H_2O$) in water and make up to exactly 1000 ml at 25°C. The specific gravity of the solution must be 1100.
2. Prepare standard copper sulphate solution of specific gravity 1052 to 1055 is given in Table 2.1.

The solution should be stored at room temperature in tightly capped containers to prevent evaporation.

Check specific gravity of $CuSO_4$ solution which must be within ±.0003 of required value. Specific gravity is determined using a 50 cc capacity specific gravity bottle and weighing in the 4th decimal place:

1. Weigh specific gravity bottle, clean and dry.
2. Weigh specific gravity bottle + distilled H_2O.
3. Weigh specific gravity bottle + $CuSO_4$ solution. Bottle is rinsed out three times with solution to be weighed before filling with that solution and weighing.

$$\frac{3-1}{2-1} = \frac{\text{Weight of } CuSO_4 \text{ solution}}{\text{Weight of water}} = \text{Sp. Gr. of } CuSO_4$$

Adjust specific gravity if necessary (a rough guide is 10 ml saturated $CuSO_4$ solution added to 1 litre of $CuSO_4$ solution increase that specific gravity by approx 0.001) and recheck specific gravity.

Table 2.1: Preparation of copper sulphate solution of 1052–1055 from stock solution

Sp. gravity	*Stock solution*	*Distilled water to make*	*Hb equivalent*
1052	51 ml	100 ml	12.0 g
1053	52 ml	100 ml	12.5 g
1054	53 ml	l00 ml	13.0 g
1055	54 ml	100 ml	13.4 g

Method

A solution of specific gravity 1053 is used for determining haemoglobin level of 12.5 g/dl:

1. Dispense 30 ml of copper sulphate solution (specific gravity 1053) into appropriately labelled clean, dry, tubes or bottles. Change the solution daily of after 25 tests and be sure that the solution is properly mixed before performing tests daily.
2. The site of the skin puncture, i.e. fingertip is cleaned with antiseptic solution and allowed to dry.
3. Disposable lancet is used for puncture and there should be free flow of blood.
4. One drop of blood is collected either in capillary tube or pipette and allowed to fall generaly from a height of 1 cm above the surface of the copper sulphate solution.
5. The drop gets encased in a sac of copper proteinate, which prevents any change in specific gravity for about 12 seconds. If the drop of blood remains at the surface, or rises from the bottom of the solution, the drop is lighter than the $CuSO_4$ solution and the haemoglobin content of the blood is below normal levels. However, if the drop of blood has the specific gravity same or higher than the solution, it will sink within 15 seconds, i.e. haemoglobin will be equal to or higher than 12.5 ± 0.1 g%.

☞ **Note:** If plasma protein level of donors is on lower limit of normal, it is possible a donor may be rejected through he/she may be having required Hb.

Remediable source of errors in HB estimation

i. Taking first drop of blood from finger prick
ii. Squeezing the finger because blood not free flowing.
iii. Inside of blood pipette not flushed out completely.
iv. Dirty pipette.
v. Chip of delivering end of blood pipette.

TOTAL RED BLOOD CELL (TRBC) COUNT

Specimen: Erythrocyte count can be done on oxalated blood or on capillary blood directly collected into the pipette. In the former case, the sample, unless refrigerated must not be more than 6 hours old.

Apparatus

Red cell pipette	
Diluting fluid: 40% formaldehyde	10 ml
Trisodium citrate (3% w/v)	990 ml
or	
Trisodium citrate	3.8 gm
Formalin	1 ml
Distilled water	1 ml
Neubauer's chamber with coverslip	99 ml

Technique

1. If oxalated blood is to be used, first mix it thoroughly by gentle shaking.
2. Fill the red cell pipette exactly up to 0.5 mark by holding the pipette almost horizontally. The pipette must be clean and dry.
3. Now draw in the diluting fluid up to the mark 101 (dilution 1 in 200). While filling the bulb the pipette should be gently rotated to obtain good mixing (Fig. 2.3)

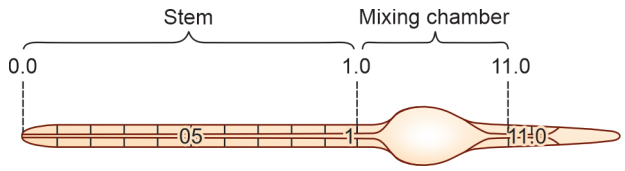


Fig. 2.3: RBC pipette

4. The coverslip is placed over the Neubauer's chamber so as to cover both the ruled platforms evenly.
5. Now load the chamber. This is done in three steps:
   a. Mix the contents of pipette for 3 minutes.
   b. Expel 6 drops from the pipette to remove the fluid in the stem which has not been mixed with blood.
   c. By holding the pipette at an angle of 45° and touching the space between the coverslip and the chamber by the point of the pipette, an appropriate drop of the mixture is allowed to run under the coverglass by capillary action. It must be large enough to cover the whole ruled area of platform of Neubauer chamber.
6. Allow two minutes for setting of the cells and then count.
7. The count is done as follows.

In the erythrocytic count, the central double ruled square is used. Red cells lying in 80 very small squares have to be counted. These 80 small squares comprise 5 medium-sized squares, each of which is bound by a triple line (Fig. 2.4). It is recommended that the five medium-sized squares chosen for counting cells should consist of four corners and one central; this is to secure an even distribution of cells. In counting, cells which touch the left hand lines or the upper lines of the square are taken to be within that square and those which touch the lower or right hand lines are omitted as outside the square.

To obtain accuracy at least 400 to 600 cells must be counted. If the count of the 80 small squares is less than this figure, more squares must be counted.

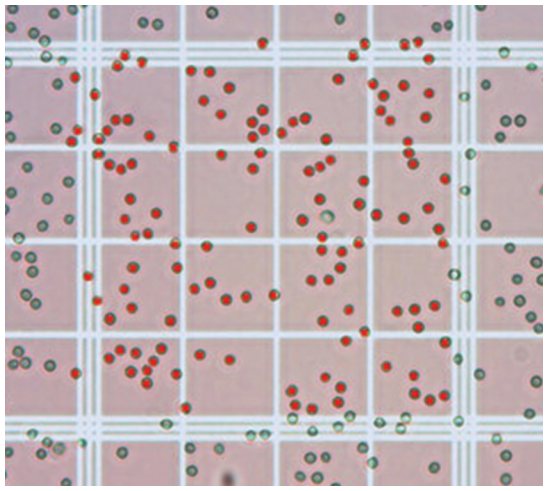


Fig. 2.4: Microphotograph showing RBC in Neubauer chamber

Calculation: The total area of the whole large central square is 1 sq mm. The smallest square has side of 1/20 mm so that its area is 1/400 sq mm and since the depth is 1/10 mm, its volume is 1/4000 cu mm. Total volume of 80 small squares is therefore 80/4,000 cu mm = 1/50 cu mm.

Dilution is 1 in 200

$$\text{Total RBC count} = \frac{\text{Dilution} \times \text{No. of cells counted}}{\text{Volume}}$$

$$= 200 \times N \times 50$$

$$= N \times 10{,}000 \text{ cells/cu mm}$$

Sources of Error

1. Sampling error in collection of blood.
2. Equipment error in the pipette and haemocytometer.
3. Technical errors involved in the exercise from the filling of the pipette to the final count.

Inherent or field errors of the distribution of cells in the counting chamber.

Normal Values

Men	$5.5 \pm 1.0 \times 10^{12}$/L
Women	$4.8 \pm 1.0 \times 10^{12}$/L
Infants (full term cord blood)	$5.0 \pm 1.0 \times 10^{12}$/L
Children 3 months	$4.0 \pm 1.0 \times 10^{12}$/L
Children 1 year	$4.4 \pm 0.8 \times 10^{12}$/L
Children 3–6 years	$4.8 \pm 0.7 \times 10^{12}$/L
Children 10–12 years	$4.7 \pm 0.7 \times 10^{12}$/L

DETERMINATION OF PACKED RED CELL VOLUME (PCV) AND ABSOLUTE INDICES

Principle: An oxalated sample of the blood is centrifuged to pack red cells to the maximum. The volume of packed red cells is determined. The procedure is reliable because of its reproducibility.

Specimen: Though haematocrit determination (PCV) can be done on capillary blood (with the use of special haematocrit) venous blood is preferred. The blood must be oxalated with the special mixture of potassium and ammonium oxalate. The sample must be processed within two hours of collection.

Apparatus: The Wintrobe haematocrit tubes, pasteur pipettes and a centrifuge with a speed of 3000 rpm.

Procedure: The haematocrit tube must be clean and dry. Mix the oxalated sample of blood thoroughly by gently shaking for 3 minutes. With the pasteur pipette fill the haematocrit tube to the 10 mark. This is done by passing the pipette to the bottom of the haematocrit. There must be no air bubbles.

Fill a second haematocrit with either another sample of blood or with water. This tube is to counterbalance the first one during centrifugation. Put the tubes in the centrifuge. Centrifuge at 3,000 rpm for 30 minutes, preferably for 60 minutes. Take the reading of packed red cells. Recentrifuge for 5 minutes more at the same speed and take the reading again. There should be no difference between the two readings if the packing has been complete. If there is a difference, further centrifugation is indicated.

At the uppermost portion of packed red cells, a narrow dark band (due to reduced haemoglobin) is seen. The reading is made at the uppermost level of the band. Above the band will be seen a reddish grey layer of white cells. The volume of packed red cells read is for 10 ml. Multiply by 10 to get the value for 100. This value is the PCV. Normal range for adult males 43 to 54 per 100 ml of blood for adult females 37 to 47 per 100 ml of blood.

Calculation of MCV, MCH and MCHC (absolute indices):

$$\text{MCV (in cubic microns)} = \frac{\text{PCV}}{\text{RBCs in millions per cu mm}} \times 10$$

$$\text{MCHC (in percent)} = \frac{\text{Hb in g per 100 ml}}{\text{PCV}} \times 100$$

$$\text{MCH (in picograms)} = \frac{\text{Hb in gm per 100 ml}}{\text{RBCs in million per cu mm}} \times 10$$

Clinical Usefulness of Red Blood Cell Indices

Now with the Coulter counter type of automated equipment, a suspension of RBCs in a metered volume of electrolyte solution simply passes through a small orifice which is electrically charged. Since each RBC is a relative nonconductor, a change in the electrical charge occurs, which is then expressed as an RBC count. The magnitude of the charge change is proportional to the cell volume, and thus an MCV is derived as well. Finally, a haemoglobin is determined by an automated colourimetric method. Thus, only the Hb, MCV and RBC count are actually measured, while the Hct, MCH, and MCHC are computed internally using the above equations.

Normal Values for MCV, MCH, MCHC

The normal MCV in newborns is higher than at any other time of life, but beginning at a few months of age the MCV becomes lower than adult values. An MCV of 70, for example, may be normal for a 1-year-old infant. This development change in MCV is paralleled by changes in the MCH. The MCHC is fairly constant throughout the life.

The Significance of the MCV

The MCV may be low, normal or high in various disorders, and specific diagnostic tests for anaemia should be based on clues provided by the MCV.

A. The Low MCV

Iron deficiency: The earliest changes in iron deficiency are a fall in bone marrow iron and a concomitant fall in serum ferritin. This is followed by a decrease in serum iron and rise in the quantity of RBC free erythrocyte porphyrin (FEP). The MCV then falls and finally the Hb drops. Currently, the MCV and the FEP are the most widely used screening tests for iron deficiency, but the serum ferritin may soon be both then screening and the diagnostic test.

Lead (Pb) poisoning: Microcytosis may be seen in lead poisoning but is not uniformly present, while a rise in FEP is almost invariably found. The FEP is now the standard screening test for Pl poisoning. The FEP tends to be much higher in Pb poisoning than in iron deficiency, although some overlap may exist.

Thalassaemia minor: A low MCV is the hallmark of thalassaemia minor, and the MCV may be used as a screening test of high sensitivity in the detection of beta thalassaemia minor. In alpha thalassaemia, the MCV on the average is slightly higher than in beta thalassaemia. A Hb electrophoresis is best considered a confirmatory test for thalassaemia.

A very simple calculation may be used to distinguish iron deficiency and lead poisoning from thalassaemia minor based on the MCV and RBC count. The RBC count falls in iron deficiency in Pb poisoning but is normal in thalassaemia minor.

If the MCV divided by the RBC count, a discriminate index is obtained where

$$\frac{MCV}{RBC} < 12 = \text{Thalassemia minor}$$

$$\frac{MCV}{RBC} > 14 = \text{Iron deficiency or Pb poisoning}$$

Values between 12 and 14 are indiscriminate. Since these formulae are correct 95% of the time, a more rational selection of confirmatory tests is possible.

Miscellaneous: Other causes of microcytosis are much less common but include the anaemia of chronic disorders (this is usually normocytic, but 10% are microcytic), severe protein malnutrition, sideroblastic anaemia, and copper deficiency.

B. Normocytic Anaemia

Anaemia with a normal MCV is usually caused by bone marrow failure, recent blood loss, or haemolysis.

C. The High MCV

Unlike in adults, macrocytosis in a child should not call B_{12} deficiency to mind first. The causes of a high MCV include:

1. **Normal newborn:** All normal newborns are macrocytic. In fact, an MCV of 94 or less should suggest a diagnosis of alpha thalassaemia minor, the most common cause of microcytosis in newborns.
2. **Reticulocytosis:** Reticulocytes are very large and, when averaged with more mature RBCs, may produce a high MCV. A review of the peripheral blood smear will show that an elevated MCV is merely a reflection of young RBCs.
3. **Down's syndrome:** In Down's syndrome, the MCV averages 10 fl higher than normal.
4. **Liver disease:** This will deposit excessive lipids on the RBC, producing enlargement.
5. **Hypothyroidism:** A low T_4 commonly produces large speculated and targeted RBC.
6. **Drugs:** Any drug such as methotrexate which alters the DNA synthesis causes macrocytosis.
7. **Folate deficiency**
8. **B_{12} deficiency:** B_{12} and folate deficiency, in addition to producing macrocytosis, will cause hypersegmentation of neutrophils, distinguishing them from other causes of macrocytosis.
9. **Miscellaneous:** Other causes include preleukemic states and congenital pure RBC aplasia (Blackfan-Diamond syndrome).

Alterations of MCH and MCHC

Since the MCH falls as the Hb falls, relatively a little information is obtained from the MCH itself. The same is somewhat true of a low MCHC, which adds nothing to what is already known from the MCV. A high MCHC is important to recognize, since it indicates that the haemoglobin content of the RBC is being very tightly packed. A high MCHC almost invariably means spherocytes are present and suggests such diagnosis as hereditary spherocytosis, AHU incompatibility, autoimmune haemolytic anaemia, and occasionally microangiopathic haemolytic anaemias. Normal values of MCV, MCH and MCHC at different ages are given in Table 2.2.

Table 2.2: Normal values of MCV, MCH and MCHC at different ages

	MCV (fl)	*MCH (pg)*	*MCHC*
Normal adult	90 ± 10	31 ± 4	34 ± 3
Normal newborn	119 ± 9.0	36 ± 2	32 ± 2
Ages 10–17 months	77 ± 7	26.1 ± 2.8	34.2 ± 1.6
18–48 months	80 ± 6	27.0 ± 3.0	33.6 ± 1.4
4–7 years	81 ± 5	27.6 ± 2.4	33.6 ± 1.6

ERYTHROCYTE SEDIMENTATION RATE (ESR)

If an anticoagulant is added to the blood and the specimen allowed to stand in a tube, red cells slowly sediment to the bottom of the tube leaving clear plasma as the supernatant. The rate of sedimentation estimated under standard conditions is known as the erythrocyte sedimentation rate (ESR). Sedimentation takes place in three stages:

1. Formation of rouleaux.
2. Sinking of rouleaux.
3. Packing of the rouleaux.

Influencing Factors

1. Difference in specific gravity between red cells and plasma.
2. The extent to which the red cells form rouleaux, which sediment more rapidly than single cells.
3. The ratio of red cells to plasma, i.e. the PCV.
4. Plasma viscosity.
5. Verticality or otherwise of the sedimentation tube.
6. The bore of the tube .
7. Dilution, if any, of the blood.

Methods of Estimation

1. Wintrobe Method

Wintrobe Tube

Length 110 mm
Diameter 3. 0 mm
Graduation of lower 100 mm from 0–100

Anticoagulant used: Double oxalate

Method: The tube is filled up to the 100 mm mark, allowed to stand in a vertical position at the room temperature, read the fall of red cells at the end of one hour (Fig. 2.5).

Advantages: Can be used for
1. Packed cell volume.
2. Buffy coat preparation.

2. Westergren Method

Westergren tube

Length	300 mm
Diameter	2.5 mm

Graduation in mm from 0 to 200

Anticoagulant used: 3.13% sodium citrate solution (0.106 M). 0.5 ml of anticoagulant is used for 2 ml blood.

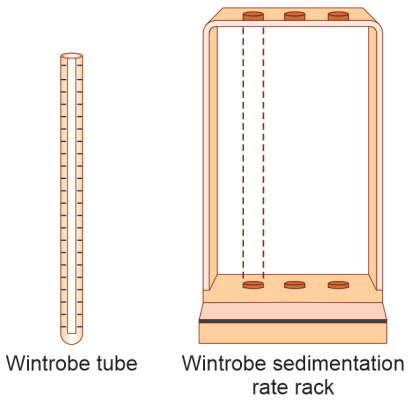


Fig. 2.5: Wintrobe tube and rack for ESR

Method: The mixture is drawn into a Westergren tube up to the zero mark and the tube set upright in a stand with a spring clip on top and rubber at bottom.

The level of the top of the red cell column is read at the end of 1 hour.

Precautions

1. The tubes must be scrupulously clean.
2. They must be filled properly without air bubbles.
3. They must be kept vertically.
4. Anaemia, if present, will raise the ESR, irrespective of the primary condition.

Normal Values

Wintrobe method:	Men—0 to 9 mm in 1st hour
	Women—0 to 20 mm in 1st hour
Westergren method:	Men—0 to 5 mm in 1st hour
	Women—0 to 7 mm in 1st hour

Corrected ESR

The corrected ESR is needed to eliminate the influence of anaemia on sedimentation rate. The rate is then corrected according to the volume of cells by referring to chart for correction (Fig. 2.6).

Find the horizontal line which represents the sedimentation in mm for 1st hour. Follow this across the chart until it intersects the vertical line which represents the blood cell volume per cent.

Follow the nearest curved line until it intersects the heavy line at 42% per 100 ml, if the patient is female, or

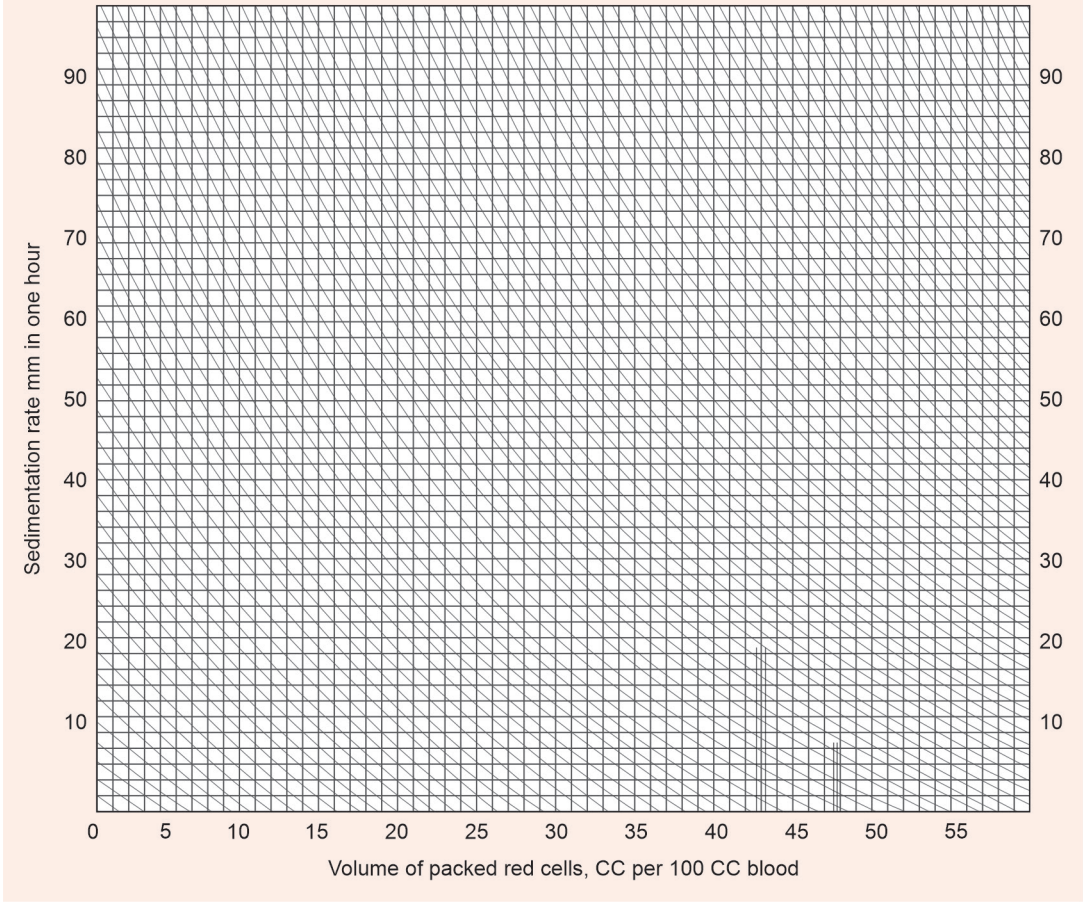


Fig. 2.6: Graph 1: Wintrobe and Landsberg chart for correcting the sedimentation rate for variations resulting from differences in the concentration of erythrocytes as measured by the volume of packed cells

the line at 47% if the patient is male. Then, at the point of intersection, read the value on the horizontal line for the corrected sedimentation rate. The normal average sedimentation in one hour by this method is 9.6 mm for healthy female (0–20 mm) and 0–9 mm for healthy male.

Total Leucocyte Count (TLC)

Specimen: The leucocyte count may be done on oxalated blood or capillary blood. In the former case the sample must be examined within 6 hours of collection.

Apparatus

- A white cell pipette (white lead).
- Diluting fluid—4% acetic acid with two drops of gentian violet per litre.
- Neubauer's chamber with coverslip.
- Microscope.

Procedure

1. Draw blood in a clean dry pipette up to the mark 0.5 (Fig. 2.7) with all possible accuracy. The oxalated sample must be thoroughly mixed before use.
2. Wipe off the outside of the pipette with gauge.
3. Now draw the diluting fluid up to mark 11 (dilution 1 in 20). While drawing the fluid rotate the pipette.
4. Mix the contents of the pipette for 3 minutes.
5. Dispel the first 4 drops of the contents.
6. Adjust the Neubauer's chamber. It must be clean and dry. By holding the coverglass between the fingers at the edges, place it in such a manner that both the ruled platforms are evenly covered by it. Load it with the mixture, by holding the pipette at angle of 45° and touching the space between the coverglass an the chamber by the point of the pipette, and appropriate drop of the mixture is allowed to run under the coverglass by capillary action. It must be sufficiently large to cover the whole ruled platform, yet not large enough to fill the moat. Also there must be no air bubbles.
7. Allow 2 minutes for setting of cells, then count.
8. The count is done as follows: The improved Neubauer chamber has two central platforms each of which is ruled. When the coverglass is in place, there is a space of 0.1 mm depth over the ruled area. The surface of the ruled area is 3 × 3 mm (9 sq mm) (Fig. 2.8). Nine large squares can be recognised in the ruled area; the four corner squares are single ruled and the five central squares are double ruled. Each of the corner single ruled square is divided into 16 smaller squares (Fig. 2.9). The central double ruled square is divided into 400 very small squares. Each of the four large corner squares (with 16 small squares) has an area of 1 sq mm; each of the very small squares (400 in all) in the central square has an area of 1/400 sq mm. The central square is meant for the erythrocyte count; the four large corner squares are used for the leucocytic count. Figure 2.10 shows pattern of good and poor distribution of white blood cells and Fig. 2.11 shows procedure and score of counting. In counting, cells which touch the left hand lines or the upper lines of the square are taken to be within that square and those which touch the lower and right lines are omitted as outside the square. WBC as viewed under low power magnification are shown in Fig. 2.12.
9. **Calculation:** The area of each large square is 1 sq mm, the depth of the chamber being 0.1 mm, the volume of the square is 0.1 cu mm.

   Volume of four corner squares = 0.1 × 4 = 0.4 cu mm

   No. of cells in four corner squares = N

   0.4 cu mm contains = N cells.

$$1 \text{ cu mm contains} = \frac{N \times 20\,(\text{dilution factor})}{0.4}$$

$$= N \times 50$$

Sources of Error

1. Sampling error in collection of blood.
2. Equipment error in the pipette and chamber.
3. Technical errors involved in the exercise from the filling of the pipette to the final count.
4. Inherent or field errors of the distribution of cells in the counting chamber. This can be minimised by counting a large number of cells.

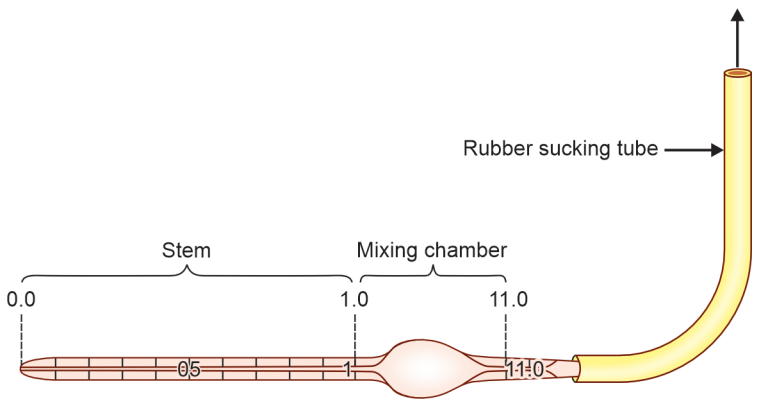


Fig. 2.7: White cell pipette with markings

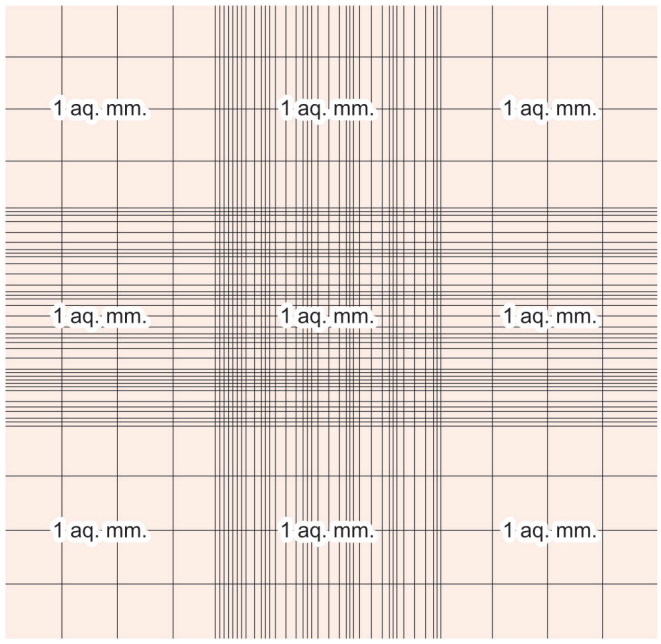


Fig. 2.8: Ruled area of Neubauer chamber

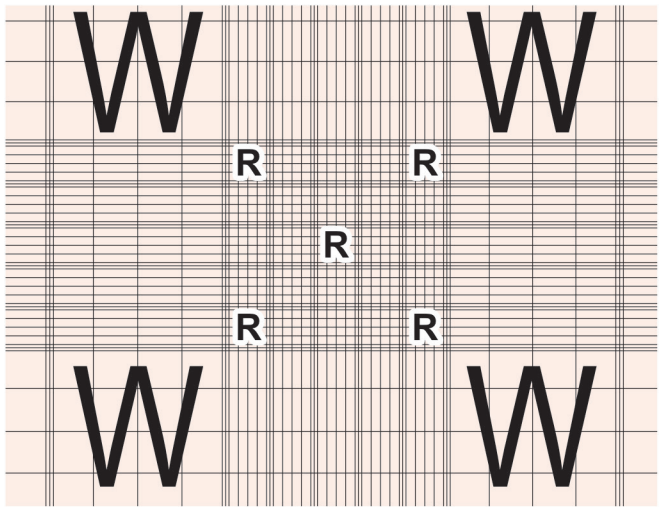


Fig. 2.9: 'W' areas for white cell count

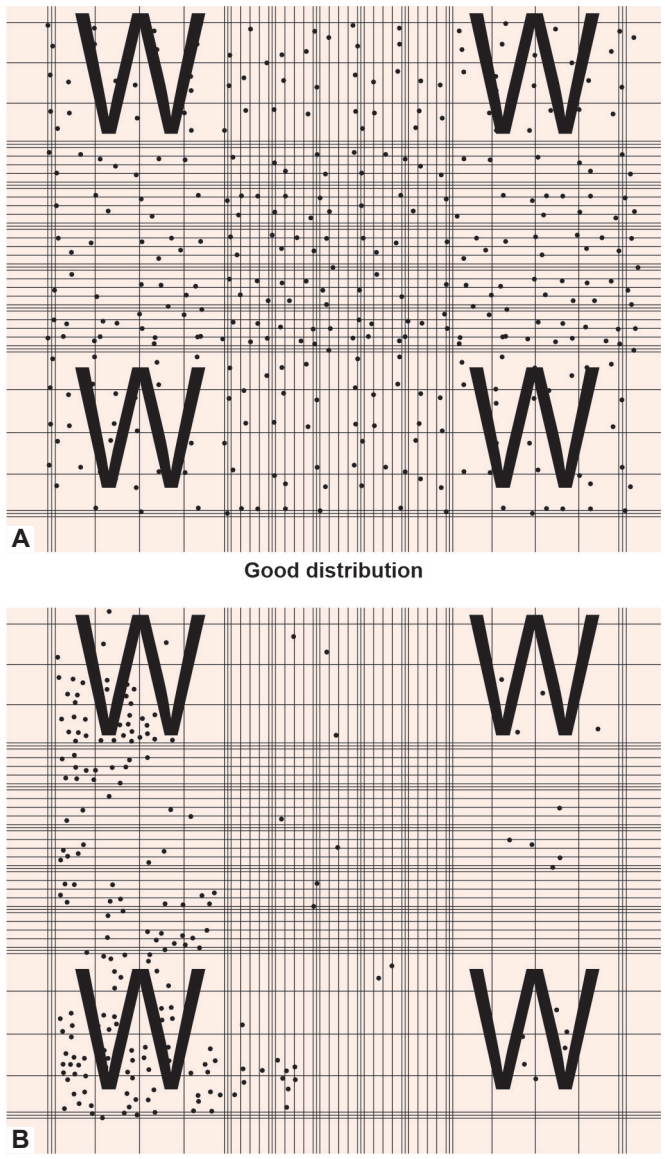


Fig. 2.10A and B: Pattern of good and poor distribution of WBC

Range of TLC

Adults	$7.5 \pm 3.5 \times 10^3$/cu mm
Infants (full term 1st day)	$18 \pm 8 \times 10^3$/cu mm
Infants, 1 year	$12 \pm 6 \times 10^3$ cumm
Children 4–7 years	$10 \pm 5 \times 10^3$/cu mm
Children 8–12 years	$9 \pm 4.5 \times 10^3$/cu mm

The spreader

1. Should have a smooth edge.
2. Should be narrower in breadth than the slide on which film is to be made so that the edges of the film may be readily examined.
3. Should not be used for making more than six slides in succession without being cleaned.
4. Should be washed in running water and dried immediately after being used.

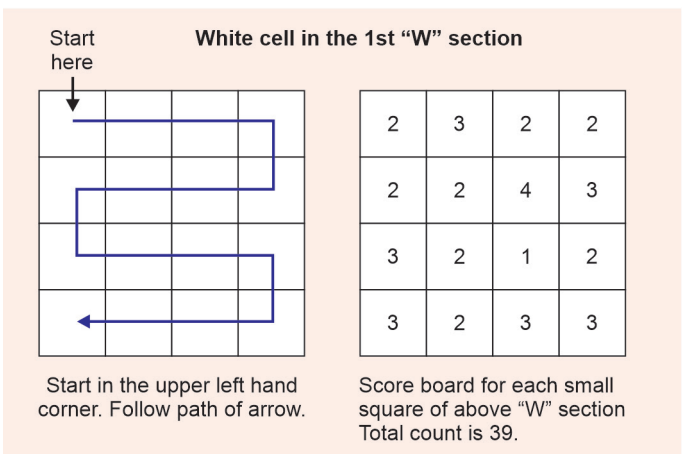


Fig. 2.11: Procedure and score of counting

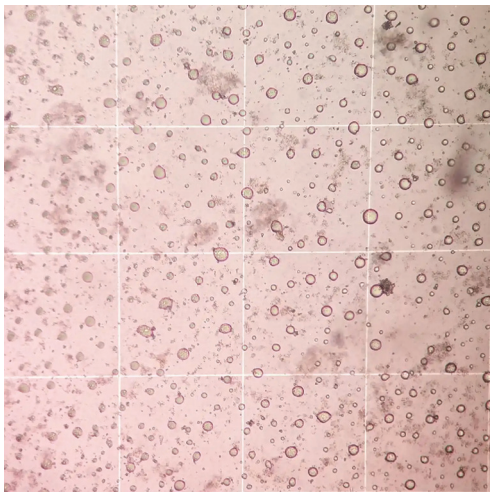


Fig. 2.12: WBC as viewed under low power magnification (× 10)

Table 2.3: Conditions accompanied by abnormal white cell counts

High white cell count (leucocytosis)	*Low white cell count (leucopenia)*
Appendicitis	Measles
Pneumonia	Influenza
Leukaemia	Brucellosis
Tonsillitis	Typhoid fever
Meningitis	Agranulocytosis
Abscesses	Infectious hepatitis
Rheumatic fever	Lupus erythematosus
Diphtheria	Cirrhosis of liver
Smallpox	Paratyphoid fever
Chickenpox	Protein therapy
Peritonitis	Radiation
Erythroblastosis fetalis	Myxedema
Uremia	Psittacosis
Ulcers	Sandfly fever
Newborn	Scrub typhus
Pregnancy	Dengue
Menstruation	Rheumatoid arthritis

Preparation of Film

1. A small drop of blood is placed in the central line of a slide about 1–2 cm. from one end.
2. The spreader is placed at an angle of 45° to the slide and then moved back to make contact with the drop.
3. The drop should spread out quickly alone the line of contact of the spreader with the slide (Fig. 2.13).

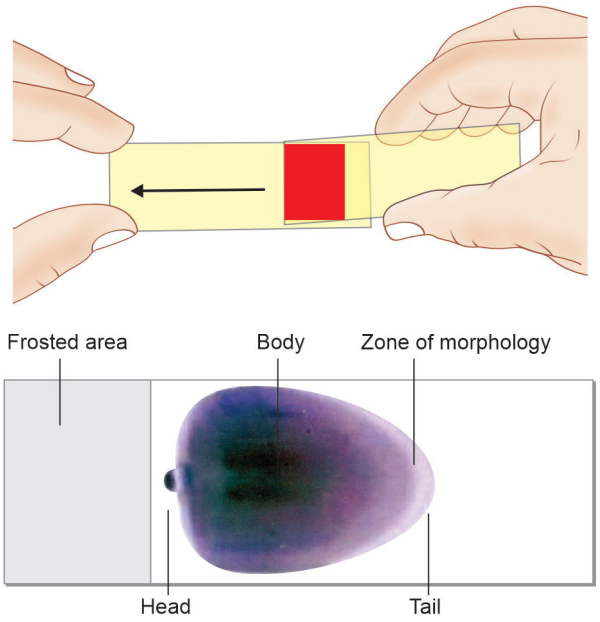


Fig. 2.13: Method of preparation of blood smear

4. The moment this occurs the film should be spread by a rapid smooth, forward movement of the spreader.
5. The drop should be of such size that the film is 3–4 cm in length.

Sources of error: If the film is made too thin or if a rough edged spreader is used, many of the leucocytes, perhaps even 50% of them, accumulate at the edges and in the tail. Moreover, a gross qualitative irregularity in distribution is the rule. Polymorphonuclear neutrophils and monocytes predominate at the margins and the tail and lymphocytes in the middle of the film. This separation probably depends upon differences in stickiness, size and specific gravity among the different classes of cells (Fig. 2.14).

Qualities of a Good Film

1. Should not be too thin and the tail of the film should be smooth (diagrammatic representation Fig. 2.14).
2. There should be some overlap of the red cells. diminishing to separate near the tail.
3. The leucocytes in the body of the film should not be badly shrunken.

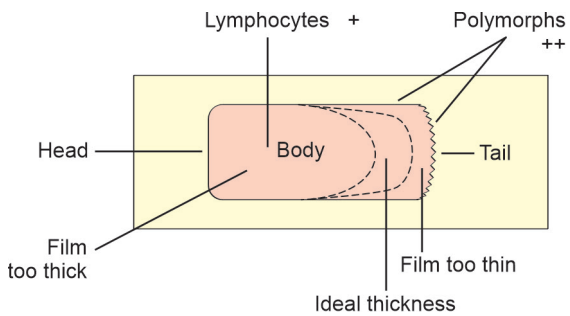
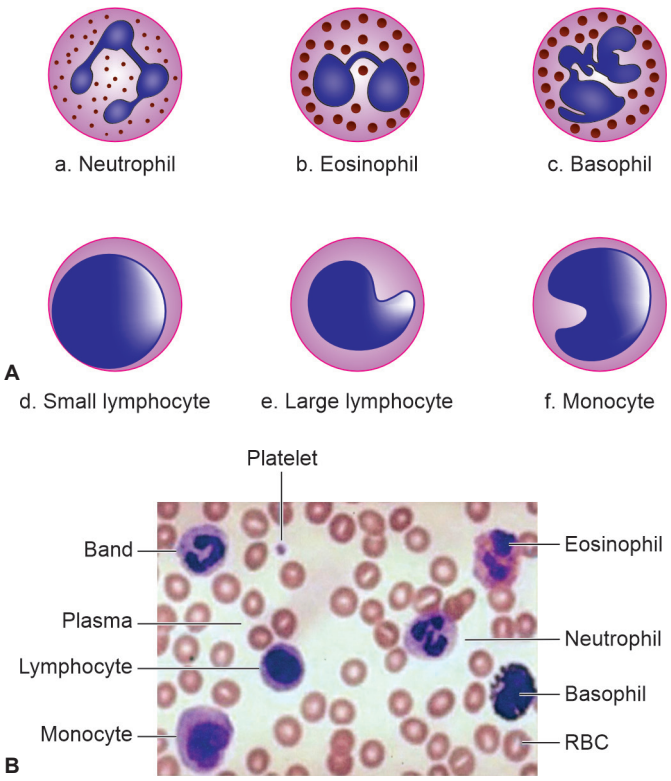


Fig. 2.14: Diagrammatic representation of distribution of cells and parts of the smear

DIFFERENTIAL LEUCOCYTE COUNT (DLC)

Gives an information about the type of the cells responsible for the rise or fall in the total leucocyte count. Properly spread and stained films are essential for accurate evalution. Smears made from capillary finger blood (direct smear) are preferred to smears made from anticoagulated blood (indirect smear) (Fig. 2.15).



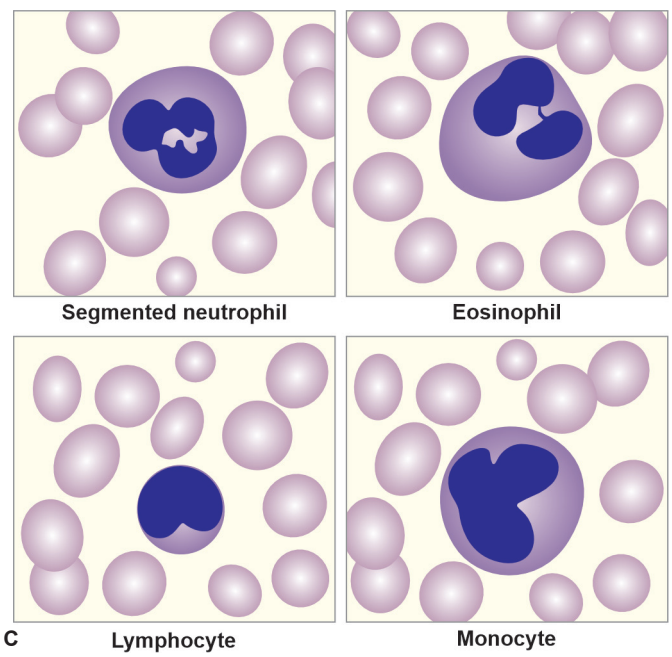


Fig. 2.15: (A) Labelled DIC (a–f) diagram; (B) Labelled photograph DLC; (C) Diagram labelled—segmented neutrophil, eosinophil, lymphocyte and monocyte

Method: A small drop of blood is placed on a clean glass slide, 1–2 cm from one end. The spreader (having a smooth edge, narrower breadth) is placed at an angle of 45° in front of the drop and moved backwards to touch the drop. The drop will spread along the line of contact. The smear is made by a rapid, smooth, forward movement of the spreader. The angle is important to avoid aggregation of leukocytes at the periphery. The smear should be dried rapidly in the air and never heated.

Characteristics of a good smear

- Good film has a thick and thin portion and gradual transition.
- The film should have smooth, even appearance and free from ridges, waves or holes.
- The edge of spreader must be smooth, if it is rough, the film has ragged tails containing many leukocytes.
- The film has some overlap of red cells in much of the film but even distribution and separation of red cells towards the thin tail.
- The faster the film is air dried, the better is the spreading of the individual cells and there is no shrinkage of cells. The slide is labeled by writing with a lead pencil on thick end of the film.

Fixation of smears: Most Romanowsky stains are dissolved in methyl alcohol and combine fixation with staining. Wright's and Leishman stains are alcoholic stains that fix the smears during staining. For Giemsa stain, a prior fixation in methyl alcohol (2–3 minutes) is necessary. If staining is delayed, all the smears must be fixed in methyl alcohol.

Staining of the smears: Romanowsky stains are used in routine practice. Romanowsky stains include Wright's stain, Leishman stain, Giemsa stain (common ones) May-Grunwald stain and Jenner stain.

Staining the smear with Wright's stain/Leishman stain

1. Cover the air dried smear with Wright's stain.
2. Add double the volume of buffered water or distilled water after 1 minute. The water should not run of the slide.
3. Mix the stain and the water by blowing gently on the surface. Keep the slide for 5–8 minutes, till a scum with metallic sheen is formed on the surface.
4. Wash the slide directly under running water (to avoid deposition of the scum on the stained film).
5. Clean the back of the slide, and air dry the smear. Examination of smear is done under oil immersion.

Procedure of Count

1. The cells should be counted using high power coil immersion lens in a strip running the whole length of the film.
2. The lateral edges of the film are avoided.
3. The film should be inspected from the head to the tail.

4. If less than 100 cells are encountered in a single narrow strip, one or more additional strips should be examined until at least 100 cells have been counted.
5. In patients with very high counts (as in leukaemia) the cells should be counted in any well spread area where the cell types are easy to identify.

Cell Identification

Leucocytes include segmented cell of myeloid series and mononuclear cells of lymphoid and monocytic cells. *Mature cells of myeloid series include*:

1. Neutrophil
2. Eosinophil
3. Basophil

These are segmented cells of 10–12 μ size. The nucleus shows lobes connected by filaments, cytoplasm contains granules.

- **Neutrophil or polymorph:** Nucleus may show 2–5 lobes. Cytoplasmic granules are fine and pale pink violet.
- **Eosinophil:** Nucleus usually bilobed like a spectacle. Cytoplasmic granules are coarse and orange.
- **Basophil obscure:** Cytoplasmic granules are large deep purple and nucleus.

Mature Cells of Lymphoid Series

Small Lymphocytes

- Size same as that of RBC
- Round, dark staining nucleus fills the cell
- Scanty pale blue cytoplasm.

Size 10–14 μm

- Pale nucleus large, rounded or indented
- Cytoplasm abundant, pale blue, may show a few reddish blue granules.

Monocytes

- Large cell, 14–20 μm
- Nucleus round, oval kidney-shaped, convoluted
- Cytoplasm abundant, grayish blue.

Normal Range of DLC

- Neutrophils, 40–60%
- Lymphocytes, 20–45%
- Monocyte, 2–10%
- Eosinophil, 1–6%
- Basophils, 0–1%.

Abnormalities of Leucocytes

Increase in neutrophils with toxic granules	Pyogenic infections
Increase in lymphocytes	Viral infections
Increase in eosinophils	Allergies parasitic infestation
Increase in monocytes	Chronic infections
Presence of immature leucocytes	Infections, leukaemia

ABSOLUTE EOSINOPHIL COUNT

Apparatus

- WBC pipette
- Eosinophil diluting fluid (Dunger's solution)

Stock Solution

- Eosin yellow: 0.5 gm
- Formaldehyde (40%): 0.5 ml
- Phenol (95%) (aqueous): 0.5 ml
- Distilled water up to: 100 ml

Working solution: 6 ml of stock solution diluted with distilled water up to 100 ml.

- Neubauer's chamber with coverslip
- Microscope.

Procedure: Suck blood up to 0.5 mark in a WBC pipette and dilute up to 11 mark with the diluting fluid. Gently rotate the pipette. Discard first 3 or 4 drops of fluid. Charge the Neubauer counting chamber and allow it to stand for 3 minutes, so that the cells settle down (Fig. 2.16).

Count all the eosinophils in the whole of the ruled area (i.e. 9 squares).

Calculation

$$\text{Absolute eosinophil count (AEC)} = \frac{N \times \text{Dilution}}{\text{Volume}}$$

Where N = No. of cells counted

$$\text{Volume} = \text{length} \times \text{breadth} \times \text{depth}$$
$$= 3 \times 3 \times 0.1$$
$$= 0.9 \text{ cu mm}$$

$$\text{Dilution is 1 in 20} = \frac{N \times 20}{0.9}$$

$$\therefore \text{AEC} = N \times 22.2 \text{ cu mm}$$

$$\text{Normal range of AEC} = 40\text{–}440 \text{ cell/cu mm}$$

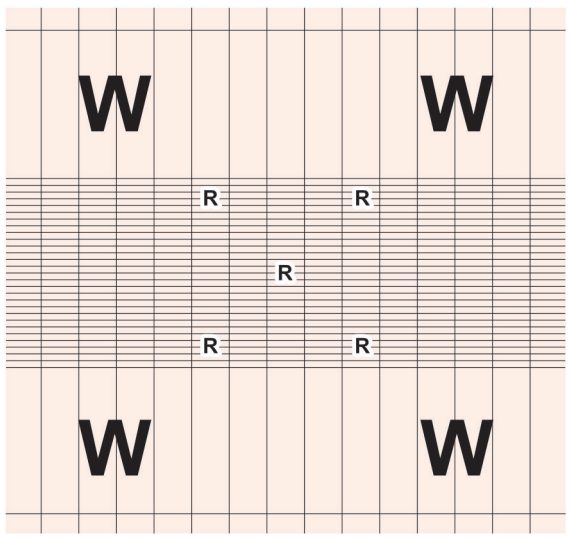


Fig. 2.16: Ruled area of Neubauer's chamber showing counting areas

Interpretation: An increase in eosinophil count or eosinophilia is seen in:

- Allergic diseases such as bronchial asthma.
- Skin disorders, e.g. eczema.
- Parasitic infestation.

PLATELET COUNT

Direct Method (1)

Specimen: Venous blood is collected by a clean puncture and delivered without frothing into a bottle or a tube containing the anticoagulant dipotassium EDTA. EDTA prevents the clumping of platelet or it may be collected finger prick. Venous blood is preferred.

Procedure

1. Draw the blood into a clean dry RBC pipette up to the mark 0.5. The accuracy in filling the pipette has to be of high order.
2. Wipe off the outside of the pipette.
3. Draw the diluting fluid up to the mark 101.

   The diluting fluid (Rees-Ecker) has the following formula:

Sodium citrate	3.8 g
Brilliant cresyl blue	0.05 g
Neutral formalin	0.65 ml
Distilled water	100 ml

   Filter and centrifuge at 2,800 rpm for 30 minutes. Stock the solution in a refrigerator in a well-stoppered bottle and filter before use every time.

   Another good diluent is the formol citrate solution used for red cell count.
4. Shake the pipette for 5 minutes.
5. Discard the first 4 drops. Load the chamber; both the haemocytometer and the coverslip must be scrupulously clean.
6. Allow the preparation to stand for 15 minutes in a moist chamber. An inverted Petri dish with a piece of wet filter paper in the top makes a good moist chamber.
7. Count under the high power with the light partially cut off. Platelets are lilac-coloured. Count at least 300 platelets and calculate by the formula.

PLATELET COUNT (by visual impression)

A peripheral smear prepared from EDTA anticoagulated blood helps to judge roughly whether adequate platelets are present and usually if 2 to 10 platelets per 100 red cells or if clumps of platelets are present; it suggests that platelets are adequate. The size and morphology of the platelets can also be studied by peripheral smear examination. They are bluish, spherical or oval structures, 2–4 microns in diameter. With Romanowsky's stain azure granules are seen in the hyaline, light blue cytoplasm, no nucleus is present.

Platelet counts can be made by visual counting or by electronic counters. Accuracy in visual counting (direct or indirect method) is only achieved by scrupulous cleanliness during blood preparation and by experience in differentiating platelets from extraneous matter. Phase contrast microscopy helps considerably in recognising and counting platelets. 1% ammonium oxalate can be used as a diluent in which red cells are lysed.

Count: Calculation

$$\frac{\text{No. of platelets counted} \times 10 \times \text{dilution}}{\text{No. of 1 mm squares counted}} = \text{Platelets per cu mm}$$

Normal values—1,50,000 to 4,00,000 cells/cu mm

Direct Method (2)

The blood is diluted in 1% ammonium oxalate stored refrigerated at 4°C which haemolyzes the red blood cells. (Prepared by dissolving 1 gm of ammonium oxalate in 100 ml of distilled water.)

Fill blood and diluent (in this case 1% ammonium oxalate) as described for the RBC count and using the RBC pipette. If platelet count is low, a WBC pipette can be used instead:

- Charge the chamber with the help of the pipette employed.
- Using 40 x objective with reduced condenser aperture count the platelets in the same squares as indicated for RBC counting.
- Calculate as (if RBC pipette used) =

$$\frac{\text{Cells counted} \times \text{blood dilution} \times \text{chamber depth factor}}{\text{Area of chamber counted}}$$

$= N \times 200 \times 5 \times 10$

$= N \times 10000$

(However, if a WBC pipette is employed the appropriate formula and method should be used. Platelet counts are made in the small 5 RBC squares only. Platelet count = $N \times 20 \times 5 \times 10$ or $N \times 1000$).

Indirect method

1. A drop of 14% magnesium sulfate is placed on the fingertip and the finger is pricked through it to dilute the blood at once in order to prevent the clumping or disintegration of platelets.
2. With a drop, a thin smear is prepared and stained with Leishman's or Wright's stain.
3. With another drop of blood, the RBC count is done simultaneously.
4. On the smear 1000 RBCs are counted and the number of platelets seen whilst counting is noted.

Platelet count (per litre).

$$\frac{\text{Number of platelets counted} \times \text{Red cell count}}{1000}$$

Normal platelet counts = 1.5–4.0 Lakhs/cu mm.
Raised platelet count (thrombocytosis).
Reduced platelet count (thrombocytopenia).

Causes of Thrombocytosis

1. During infections
2. Immediately after surgery and following bleeding
3. Iron deficiency anaemia
4. Chronic myeloid leukaemia
5. Polycythaemia vera
6. Myelofibrosis
7. Trauma
8. Acute haemolytic states
9. Hemorrhagic thrombocythaemia
10. Hereditary spherocytosis
11. Spleenectomy
12. Thalasemia

Causes of Thrombocytopenia

a. Causes of platelet production failure
   i. Selective megakaryocyte depression:
   - *Drugs:* Antazoline, benzene compounds and chemicals
     - Chloramphenicol
     - Chlorothiazide
     - Chlorpropamide
     - Digitoxin
     - Phenobarbitone
     - Phenylbutazone
     - Quinidine
     - Quinine
     - Salicylates
     - Sulphonamides
   - *Viral infections:* Measles, infectious mononucleosis, rubella, scarlet fever, septicemia, tuberculosis

   ii. Part of general bone marrow failure:
   - Aplastic anaemia
   - Leukaemia
   - Myelosclerosis
   - Marrow infiltration, e.g. in carcinoma, lymphoma
   - Multiple myeloma
   - Megaloblastic anaemia.

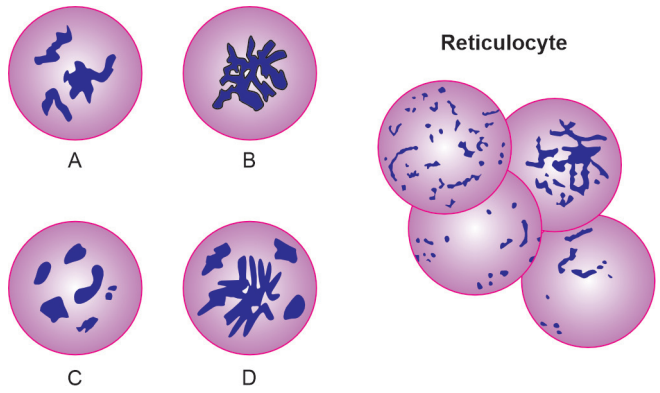
b. Increased destruction of platelets
   - Acute or chronic (ITP) idiopathic thrombocytopenic purpura
   - Secondary immune thrombocytopenia (post-infection, SLE, CLL, and lymphomas)

c. Abnormal distribution of platelets
   - Splenomegaly.

d. Dilutional loss
   - Massive transfusion of old blood to bleeding patients

RETICULOCYTE COUNT

Reticulocytes are young red cells which contain basophilic ribonucleoprotein but no nucleus. With Romanowsky stains they may shown in Fig. 2.17.



A. Basophilic multiple granules
B. Confluent basophilic granular, branching pattern
C. Confluent ganular rod type
D. Basophilic, branching bush appearance

Fig. 2.17: Reticulocytes stained with methylene blue

1. Diffuse basophilia (polychromasia)
2. Punctate basophilia
3. Filaments in the cells (Skein cells)

Basophilic ribonucleoprotein (RNP) reacts with brilliant cresyl blue to form blue precipitate of granules or filaments. They take 1–2 days to ripe to mature RBC in spleen or peripheral blood. ln smear they may be confused with:

1. Papenheimer bodies which usually contain single dot like granular material and stain darker blue shade.
2. Heinz bodies stain lighter blue as compared to reticulocytes.

Staining Solution

Brilliant cresyl blue	1.0 gm
Sodium carbonate (anhydrous)	400 gm
Nomal saline	100 ml

First dissolve dye in normal saline, then add sodium carbonate. Mix and filter the stain and keep at room temperature.

Procedure: Put 3–4 drops of staining solution in a small test tube. Add equal amount of oxalated or EDTA blood. Mix and keep in incubator at 37°C for 15–20 minutes. After that, gently mix the solution and prepare smears. If patient

is anaemic, add more blood. lf patient is polycythaemic, add less blood. Films are examined without fixing or counter staining, under oil immersion.

Calculation

Count 100 reticulocytes

For example: No. of reticulocyte in 150 fields = 100

Total no. of red cells in 150 fields = 3,000

Therefore,

$$\text{Reticulocyte\%} = \frac{100}{3000} \times 100 = 3.3\%$$

Normal Range

Adults and children	0.2–2.0%
Newborn infants	2.0–6.0%

Decreased Reticulocyte Count

In aplastic anaemia and in conditions where the bone marrow is not producing red blood cells.

Increased Reticulocyte Count

In haemolytic anaemias, iron deficiency anaemias receiving iron therapy, thalassaemia, sideroblastic anaemias, and acute and chronic blood loss.