Examination of Fresh Blood: A. Drop Preparation, B. Preparing a Peripheral Blood Film

A. DROP PRESENTATION

The first and obvious way to study the cells of the blood is to examine fresh blood under the microscope in the form of a drop preparation and in a thin blood film or smear made on a glass slide.

In this experiment, the students will examine some features of blood cells and record their observations. They will also practice making (preparing) blood films and examine them without staining. In later experiments, they will prepare and stain blood smears and identify and count various blood cells, reticulocytes, and platelets.

- Anticoagulated blood obtained from a student volunteer, or spare blood obtained from the clinical laboratory may be provided to the students to avoid skin pricks at this time (a drop of blood can be put on the slide without touching it). Students may also use their own blood from skin pricks.

APPARATUS AND MATERIALS

1. Disposable, sterile blood lancet/pricking needle.
   - Sterile cotton/gauze swabs
   - 70% alcohol/methylated spirit.
2. 8–10 thin, absolutely transparent, grease-free standard glass slides (75 mm × 25 mm).
   - Vaseline
   - Toothpicks.

PROCEDURES

While you prepare the drop preparation, your work-partner can make blood films from the same finger-prick blood.

1. Get a finger-prick under aseptic conditions. Discard the first 2 drops and allow a good drop to form. Holding a coverslip by its edges between your thumb and finger, touch its center to the blood drop, thus forming a bead.

2. Invert and carefully drop the coverslip (along with the blood drop under it) in the center of a glass slide. Do not press. The blood drop will spread into a thick film by the weight of the coverslip.
3. Using a toothpick, apply a little vaseline all around the edges of the coverslip to seal the capillary space under it. This will prevent evaporation of water and drying up of the preparation.
4. Examine the preparation under low and high magnifications and record your observations.

B. PREPARATION OF A BLOOD FILM (BLOOD SMEAR)

Blood films can be made from anticoagulated, or finger-prick blood. (See Expt 1-12)

PROCEDURES

1. Place 3 or 4 slides on a white sheet of paper on your work-table, the surface of which should be even and smooth.
2. Allow a medium-sized drop of blood to form on the finger-tip.
3. Steady the pricked finger of your partner with your left hand. Lift a slide from the table, holding it along its long edges. Then touch its center, about 1 cm from the narrow end, to the blood drop. (If anticoagulated blood is being used place a drop of blood in a similar position with a dropper).

   Do not apply the blood drop at the finger to the slide placed on the table. One cannot see the amount of blood placed on the slide.
4. Place the slide flat on the table, with the blood drop to the right side (neither your fingers, nor the skin of the subject's finger should touch the surface of the slide).
5. Support the left end of the slide with your thumb and fingers of your left hand. Now grasp the long edges of a second slide, the "spreader", between thumb and fingers of your right hand, so that its free left end extends downwards and to the left at an angle of about 40° to the horizontal.

6. Place the narrow edge of the spreader on the first slide, at an angle of 40°, just in front of the blood drop (step 1, **Figure 1-9**). Pull the spreader back gently so that it touches the front of the blood drop. Hold it there, (or move it a little from side to side) till the blood, moving along the junction of the two slides by capillarity, almost reaches the ends of the spreader, except the last 2 mm on each side, thus distributing the blood evenly across its width. (If the blood drop is too big, you

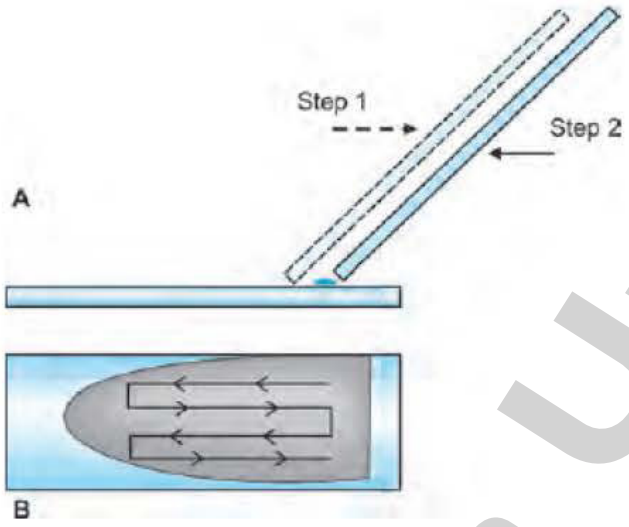

Figure 1-9: (A) Method of spreading a blood film. Step 1: The spreader is placed in front of the blood drop and pulled back till it touches the blood. Step 2: Spreader is pushed forwards to spread the film. (B) The appearance of a well-prepared film, showing the movement of the objective over it

may start to spread the smear before the whole of the blood spreads along the slide).

7. Steady the first slide with your left hand, and maintaining a light but even pressure and 40° angle (step 2, **Figure 1-9**), move the spreader forwards to the left in a single, smooth, fairly fast gliding motion, pulling the blood behind it in the form of a thin smear.

   The smear should be spread in about half a second. Any hesitation will result in striations in the film.

8. Make as many trials as possible to get acceptable films, keeping in mind the features of an ideal blood smear, as described below. Dry the film by waving the slide in the air (Do not try to blot-dry the film).

Staining a Peripheral Blood Film

APPARATUS AND MATERIALS

1. Microscope. •5–6 Clean glass slides. •Sterile lancet. •Cotton and gauze swabs. •70% alcohol. •Glass dropper.
2. A drop bottle containing Leishman's stain.
3. A wash bottle of distilled water (or buffered water, if available). • Fluff-free blotting paper.

Leishman's stain. This stain is a simplification of Romanowsky group of stains. It is probably one of the simplest and most precise method of staining blood for diagnostic purposes. It contains a compound dye—**eosinate of methylene-blue** dissolved in acetone-free methyl alcohol.

i. **Eosin.** It is an acidic dye (negatively charged) and stains basic (positive) particles—granules of eosinophils, and RBCs a pink color.

ii. **Methylene-blue.** It is a basic dye (positively charged) and stains acidic (negatively charged) granules in the cytoplasm, nuclei of leukocytes, especially the granules of basophils, a blue-violet color.

iii. **Acetone-free and water-free absolute methyl alcohol.** The methyl alcohol is a fixative and must be free from acetone and water. It serves two functions:

   a. It fixes the blood smear to the glass slide. The alcohol precipitates the plasma proteins, which then act as a 'glue' which attaches (fixes) the blood cells to the slide so that they are not washed away during staining.
   b. The alcohol preserves the morphology and chemical status of the cells.

- The alcohol must be free from acetone because acetone being a very strong lipid solvent, will, if present, cause crenation, shrinkage, or even destruction of cell membranes. This will make the identification of the cells difficult. (If acetone is present, the stain deteriorates quickly).
- The alcohol must be free from water since the latter may result in rouleaux formation and even hemolysis. The water may even wash away the blood film from the slide.

Estimation of Hemoglobin

PRINCIPLE

The Hb present in a measured amount of blood is converted by dilute hydrochloric acid into acid hematin, which in dilution is golden brown in color. The intensity of color depends on the concentration of acid hematin which, in turn, depends on the concentration of Hb. The color of the solution (i.e. its hue and depth), after dilution with water, is matched against golden-brown tinted glass rods by direct vision. The readings are obtained in g%.

APPARATUS AND MATERIALS

A. Sahli (Sahli-Adams) Hemoglobinometer (Hemometer). The set consists of:

1. **Comparator.** It is a rectangular plastic box with a slot in the middle which accommodates the calibrated Hb tube. Non-fading, standardized, golden-brown glass rods are fitted on each side of the slot for matching the color. An opaque white glass (or plastic) is fitted behind the slot to provide uniform illumination during direct visual color matching.
2. **Hemoglobin tube.** The square or round glass tube is calibrated in g Hb % (2–24 g%) in yellow color on one side, and in percentage Hb (20–160%) in red color on the other side. There is a brush to clean the tube **(Figure 1-10A)**.
3. **Hemoglobin pipette.** It is a glass capillary pipette with only a single calibration mark-0.02 ml (20 cmm, cubic millimeters; or 20 µl, micro liters). There is no bulb in this pipette (as compared to cell pipettes) as no dilution of blood is done. **Figure 1-10B** shows the Hb pipette.

   Note The calibration mark 20 cmm indicates a definite, measured volume and not an arbitrary volume, as is the case with diluting pipettes.
4. **Stirrer.** It is a thin glass rod with a flattened end which is used for stirring and mixing the blood and dilute acid.
5. **Pasteur pipette.** It is a 8–10 inch glass tube drawn to a long thin nozzle, and has a rubber

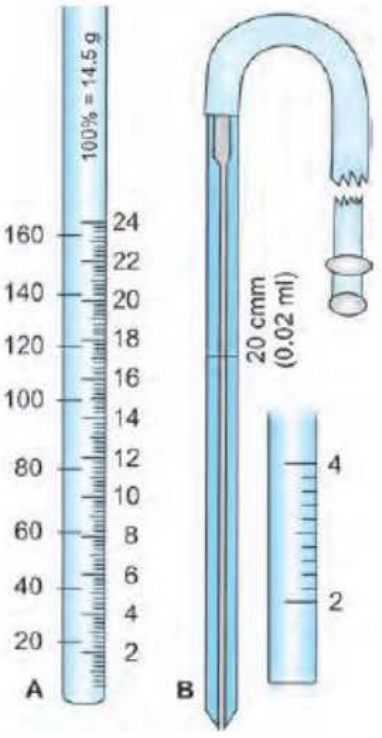

Figure 1-10: (A) Sahli-Adam's hemoglobin tube: It has graduations in g% on one side, and in percentage on the other. In this tube, 100% is equal to 14.5 g Hb per 100 ml blood. (B) Hemoglobin pipette: It has only one marking, indicating 20 cmm (0.02 ml, or 20 µl). There is no bulb (Inset: Each division represents 0.2 g of Hb)

teat. Ordinary glass dropper with a rubber teat also serves the purpose.

6. **Distilled water.**

B. Decinormal (N/10) hydrochloric acid (0.1 N HCl) solution. Mixing 36 g HCl in distilled water to 1 liter gives 'Normal' HCl; and diluting it 10 times will give N/10 HCl solution.

C. Materials for skin prick.

- Sterile lancet/needle
- Sterile gauze and cotton swabs
- Methylated spirit/70% alcohol.

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- Methylated spirit/70% alcohol.

PROCEDURES

Review the instructions for obtaining skin-prick blood, and filling a pipette as described in Expts 1-3 and 1-4.

1. Using a dropper, place 8–10 drops of N/10 HCl in the Hb tube, or up to the mark 20% or 3 g, or a little more till the tip of the pipette will submerge, and set it aside.
2. Get a finger prick under aseptic conditions, wipe away the first 2 drops of blood. When a large drop of free-flowing blood has formed again, draw blood up to the 20 cmm mark (0.02 ml). Carefully wipe the blood sticking to the tip of the pipette with a cotton swab, but avoid touching the bore or else blood will be drawn out by capillarity.
3. Without any waiting, immerse the tip of the pipette to the bottom of the acid solution and expel the blood gently. Rinse the pipette 3–4 times by drawing up and blowing out the clear upper part of the acid solution till all the blood has been washed out from it. Avoid frothing of the mixture. Note the time.
4. Withdraw the pipette from the tube, touching it to the side of the tube, thus ensuring that no mixture is carried out of the tube. Mix the blood with the acid solution with the flat end of the stirrer by rotating and gently moving it up and down.
5. Put the Hb tube back in the comparator and let it stand for 6–8 minutes (or as advised by the manufacturer). During this time, the acid ruptures the red cells, releasing their Hb into the solution (hemolysis). The acid acts on the Hb and converts it into acid hematin which is deep golden brown in color.

- The color of acid hematin does not develop fully immediately, but its intensity increases with time, reaching a maximum, after which it starts to decrease. An adequate time, usually 6–8 minutes, must be allowed before its dilution is started. Too little time and all Hb may not be converted into acid hematin. And, waiting too long, may result in fading of color. In either case, the result will be falsely low.

6. **Diluting and matching the color.** The next step is to dilute the acid hematin solution with distilled water (preferably buffered water, if available) till its color matches the color of the standard tinted glass rods in the comparator.
7. Take the Hb tube out of the comparator and add distilled water drop by drop (or larger amounts depending on the experience), stirring the mixture each time and comparing the color with the standard.
8. Hold the comparator at eye level, away from your face, against bright but diffused light. Read the lower meniscus (lower meniscus is read in colored transparent solutions).

OBSERVATIONS AND RESULTS

Compare your color matching with that of your work-partner and record the observations in your workbook. Take the average of 3 readings as shown below, and report your result as: Hb = ...g/dl.

1st reading, when the color is slightly darker than the standard:.....g/dl.

2nd reading, when, after adding a few drops of distilled water, the color exactly matches the standard: ..... g/dl.

3rd reading, when, after adding some more drops, the color becomes a little lighter than the standard:..... g/dl.

For report. Express your result as: Hb= .....g/dl.

- **Oxygen carrying capacity:** Knowing your Hb concentration, and that 1.0 g of Hb can carry 1.34 ml of O_2, calculate its oxygen-carrying capacity as .....ml O_2/dl.
- **100 % Saturation.** When blood is equilibrated with pure (100 %) oxygen at a PO_2 of 120 mm Hg, the Hb gets 100 % saturated, i.e. it picks up as much O_2 as it possibly can.

For report:

- Oxygen carrying capacity
- 100% saturation.

Normal Values

The levels of Hb in normal Indian adults, especially in the economically deprived population, are on the lower side of those reported from affluent countries. The reason may be the poor intake of grade 1 proteins and other nutrients. The average levels and their ranges are as follows:
Males: 14.5 g/dl (13.5–18 g/dl).
Females: 12.5 g/dl (11.5–16 g/dl).

Advantages of Sahli Method

The method is simple, fairly quick, and accurate. It does not require any costly apparatus, since it needs only direct color matching. Its running cost is minimal and can, therefore, be used in mass surveys.

Disadvantages of Sahli Method

Since the acid hematin is not in true solution, some turbidity may occur. The method estimates only the oxyHb and reduced Hb, other forms, such as carboxyHb and metHb are not estimated. Also the degree of error may be high if proper precautions are not taken.

The Red Cell Count

PRINCIPLE

The blood is diluted 200 times in a red cell pipette and the cells are counted in the counting chamber.

Knowing the dilution employed, their number in undiluted blood can easily be calculated.

APPARATUS AND MATERIALS

1. **RBC pipette:** Consult Expt 1-4 and **Figures 1-6 and 1-11** for its discription. It should be clean and dry and the bead should roll freely.
2. **Improved Neubauer chamber with coverslip.** These should be clean and dust free.
3. **Microscope with LP and HP objectives and 10 x eyepiece.**
4. **Disposable blood lancet/pricking needle.**
   - Sterile cotton/gauze swabs
   - 70% alcohol/methylated spirit.
5. **Hayem's fluid (RBC diluting fluid):** The ideal fluid for diluting the blood should be isotonic and neither cause hemolysis nor crenation of red cells. It should have a fixative to preserve the shape of RBCs and also prevent their autolysis so that they could be counted even several hours after diluting the blood if necessary. It should prevent agglutination and not get spoiled on keeping. All these properties are found in Hayem's fluid.

   Composition of Hayem's fluid.

Sodium chloride (NaCl)	0.50 g
Sodium sulfate (Na_2SO_4)	2.50 g
Mercuric chloride ($Hg\ Cl_2$)	0.25 g
Distilled water	100 ml

Dissolve all these chemical in distilled water and filter several times through the same filter paper. Discard the solution if a precipitate forms.

- Sodium chloride and sodium sulfate provide isotonicity so that the red cells remain suspended in diluted blood without changing their shape and

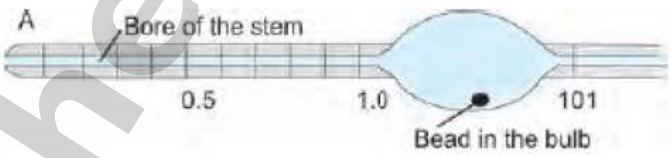


Figure 1-11: The RBC pipette. It has 3 markings—0.5, 1.0, and 101

size. Sodium sulphate also acts as an anticoagulant, and as a fixative to preserve their shape and to prevent rouleaux formation (piling together of red cells)

- Mercuric chloride acts as an antifungal and antimicrobial agent and prevents contamination and growth of microorganisms.

PROCEDURES

- Consult **Expt 1-3** for obtaining a sample of capillary blood, **Expt 1-4** on filling a pipette with blood and diluting it, and **Expt 1-5** on charging a counting chamber.

1. Place about 2 ml of Hayem's fluid in a watch glass.
2. Examine the chamber, with the coverslip 'centred' on it, under low magnification. Adjust the illumination and focus the central 1 mm square (RBC square on the counting grid) containing 25 groups of 16 smallest squares each. All these squares will be visible in one field. Do not change the focus or the field.

- Admitting too much light is a common cause of the inability to see the grid lines and squares clearly.

3. Move the chamber to your work-table for charging it with diluted blood. (It can be charged while on the stage, but it is more convenient to charge it on the table).
4. **Filling the pipette with blood and diluting it:** Get a finger-prick. Wipe the first 2 drops of blood and fill the pipette from a fresh drop of blood up to the mark 0.5. Suck Hayem's fluid to the mark 101 and mix the contents of the bulb for 3–4 minutes as described earlier.
5. **Charging the chamber:** Observing all the precautions, fill the chamber with diluted blood.

- Since the RBC pipette is a slow-speed pipette, it will need to be kept at an angle of 70–80° while charging the chamber.

6. Move the chamber to the microscope and focus the grid once again to see the central 1 mm square with the red cells distributed all over.

- Wait for 3–4 minutes for the cells to settle down because they cannot be counted when they

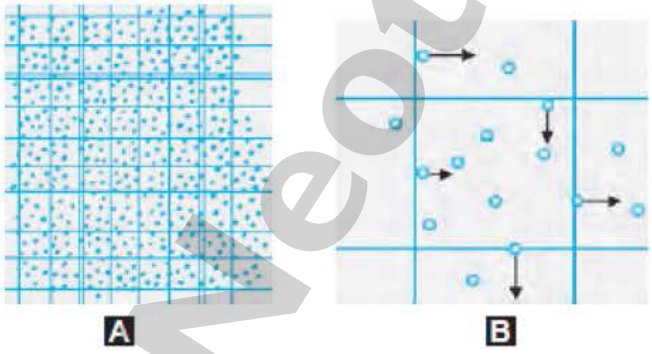
are moving and changing their positions due to currents in the fluid. During this time draw a diagram once again showing the RBC square. Then draw 5 groups of 16 squares each, showing their relative positions—the 4 corner groups and one central group for entering your counts.

7. **Counting the cells:** Switch over to high magnification (HP lens) and check the distribution of cells. If they are unevenly distributed, i.e., bunched at some places and scanty at others, the chamber has to be washed, dried, and recharged.
8. Move the chamber carefully and bring the left upper corner block of 16 smallest squares in the field of view. (There are no smallest squares above and to its left).

Rules for Counting

Note that the immediate boundary of each smallest square is formed by the 4 lines forming the square (side: 1/20 mm; area: 1/400 mm^2) the other lines of the tram or triple lines do not form part of the boundary of that square **(Figure 1-12)**.

i. Cells lying within a square are to be counted with that square.
ii. Cells lying on or touching its upper horizontal and left vertical lines are to be counted with that particular square.



Figures 1-12 A and B: (A) Microscopic view of a charged chamber showing even distribution of red cells. A group of 16 smallest squares is shown in the middle. (B) Rules of counting: Count the cells lying within a square and those lying on or touching its upper horizontal and left vertical line cells lying or touching its lower horizontal and right vertical lines are to be omitted as they will be counted in the adjacent squares. Arrows indicate the squares to which the red cells belong

iii. Cells lying on or touching its lower horizontal and right vertical lines are to be omitted from that square because they will be counted with the adjacent squares. In this way you will avoid counting a cell twice. (You may omit cells lying on the upper horizontal and left vertical lines and count those lying on its lower and right lines. But whichever method is chosen, it is best to follow it for all cell counts).

- While counting the cells, continuously "rack", the fine adjustment up and down so that cells sticking to the underside of the coverslip are not missed
- An occasional WBC (may be 1 in 600-700 RBCs) may be seen—appearing greyish and granular but it is not to be counted with the red cells.

9. We have already focused the upper left block of 16 smallest squares in the high power field. First count the cells in the upper 4 horizontal squares from left to right, then come down to the next row and count the cells in each square from right to left. Then count the cells in the 3rd row from left to right, and in the 4th row, from right to left. As the counts are made, enter your results in the appropriate squares drawn in your workbook, showing the count in each square.

- Count once more in these 16 squares and note the result in your work-book. The difference between the two counts should not be more than 10.

10. Move the chamber carefully till you reach the right upper corner block of 16 smallest squares (there are no smallest squares above and to the right of this group), and count the cells as before. Then move on to the right lower corner and then left lower corner groups, and finally count the cells in the central block of 16 smallest squares.

Thus, the counting will have been done in 80 smallest squares, i.e., in 5 blocks of 16 squares each.

OBSERVATIONS AND RESULTS

Add up the number of cells in each of the 5 blocks of 16 smallest squares. A difference of more than 20 between any 2 blocks indicates uneven distribution.

A. Calculation of dilution obtained (dilution factor).

Consult **Expt 1-4** once again. Recall that the dilution with this pipette can be 1 in 100 or 1 in 200 depending on whether blood is taken to mark 1.0 or 0.5.

Thus, the dilution factor is $= \frac{\text{Final volume attained (100 parts)}}{\text{Volume of blood taken (0.5 part)}}$

B. Calculation of volume of fluid examined. We know the count in 80 smallest squares which have a volume (space) of 1/50 mm^3. We can also know the cell count in 1 smallest square, which has a volume (space) of 1/4000 mm^3. We can now calculate the number of red cells in two ways as shown below:

C. Calculation of red cell count

i. Let x be the number of cells in 1/50 mm^3 of diluted blood.

Cells in 1 mm^3 of diluted blood = x × 50
Dilution employed was = 1 in 200
∴ Number of cells in 1 mm^3 of undiluted blood will be = x × 50 × 200
= x × 10000

Thus, adding, 4 zeros in front of x will give the RBC count per 1 mm^3 of undiluted blood.

Example

Number of cells in 80 smallest squares = 480
These cells are present in 1/50 mm^3 of diluted blood.
Dilution employed is = 1 in 200
∴ Number of cells in 1 mm^3 of undiluted blood will be
= 480 × 50 × 200
= 480 × 10,000
= 4800000, i.e., 4.8 million/mm^3.

ii. The other way to calculate is

Number of cells in 80 smallest squares = x
Number of cells in 1 smallest square = $\frac{x}{80}$
$\frac{x}{80}$ cells are present in 1/4000 mm^3 of diluted blood.

Dilution employed = 1 in 200
∴ Number of cells in 1 mm^3 of undiluted blood
$\frac{x}{80}$ × 4000 × 200 = x × 50 × 200 = x × 10,000

Normal Red Cell Count

Express your result as ..... million/mm^3
The average cell counts and their ranges are:
Males = 5.0 million/mm^3 (4.75 – 6.0 million/mm^3)
Females = 4.5 million/mm^3 (4.0 – 5.5 million/mm^3).

The Total Leukocyte Count (TLC) White Cell Count (WCC)

PRINCIPLE

A sample of blood is diluted with a diluting fluid which destroys the red cells and stains the nuclei of the leukocytes. The cells are then counted in a counting chamber and their number in undiluted blood reported as leukocytes/mm^3.

PROCEDURES

1. Take 1 ml of Turk's fluid in a watch glass. Place the counting chamber on the microscope stage. Adjust

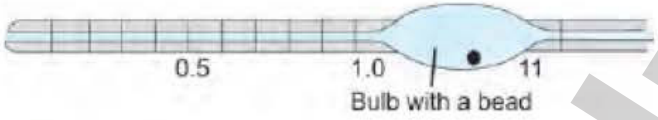


Figure 1-14: The WBC pipette. It has 3 markings—0.5, 1.0, and 11

   the illumination, and focus the right upper group of 16 WBC squares. You will see all the squares in one field.
2. Observing all the aseptic precautions, get a finger-prick, discard the first 2 drops of blood, and let a good-sized drop to form.
3. **Filling the pipette:** Dip the tip of the pipette in the edge of the drop, draw blood to the mark 0.5 and suck Turk's fluid to the mark 11. Mix the contents of the bulb thoroughly for 3–4 minutes.
   - Your partner can draw blood up to the mark 1.0 in the second pipette, followed by Turk's fluid to mark 11. This will give a dilution of 1 in 10.
4. **Charging the chamber:** Discard the first 2 drops of fluid from the pipette and charge the chamber on both sides, 1 in 10 dilution on one side and 1 in 20 dilution on the other. The chamber should neither be over-charged nor under-charged.
5. Allow the cells to settle for 3–4 minutes, then carefully transfer the chamber to the microscope. Use the fine adjustment again and try to identify the WBCs.

   Under low magnification: The leukocytes appear as round, shiny (refractile), darkish dots, with a halo around them. These 'dots' represent the nuclei, which have been stained by gential violet. The cytoplasm is not stained.

6. **Switch to high magnification** and study the leukocytes. By racking the microscope, you should be able to make out the morphology, of these cells—their round shapes, the clear unstained cytoplasm, and the deep blue-violet nuclei which appear lobed in some cells and single in others. You will also see the remnants of the red cell membranes; these are called 'ghost' cells since they are faintly visible.
7. **Counting the cells:** The procedure for counting the WBCs is similar to that employed for red cells.
   - Count the cells under high power lens; once some practice is gained they can be counted under low power
   - You may count the WBCs in 16 squares under low power and then under high power and compare the results
   - Count the cells in the 4 groups of 16 squares each, i.e., in a total of 64 squares
   - Draw appropriate squares in your work-book for entering the counts.

OBSERVATIONS AND RESULTS

Calculations

The leukocytes were counted in 64 squares, the volume of one square being 1/160 mm^3.

Volume of 64 squares = 1/160 × 64 = 4/10 mm^3.

Thus, the total volume of diluted blood in which WBCs were counted = 4/10 mm^3.

Let the count in 4/10 mm^3 be = x

Then 1 mm^3 of diluted blood will contain

= x × 10/4 white cells.

Since the dilution employed is 20 times (10 times in the 2nd pipette)

1 mm^3 of undiluted blood

will contain = x × 10/4 × 20

= x × 200/4

= x × 50

(x × 10/4 × 10 in the 2nd pipette)

This means that multiplying the number of cells in 64 squares with 50 will give the total leukocyte count (multiply the number of cells in 64 squares with 25 in the 2nd pipette).

Compare the two counts. The difference between the two should not be more than 10%. It will confirm the accuracy of your procedures in the two countings.

PLATELET COUNT

Platelet counting. There are two methods for this count: *direct method* and the *indirect method.* Automated counters are also available.

A. DIRECT METHODS

You will require: • Microscope • RBC pipette • Counting chamber with cover slip • Equipment for fingerpick • Rees-Ecker diluting fluid—OR—Freshly prepared 1.0% ammonium oxalate solution.

PROCEDURES

I. Ammonium Oxalate Method. This fluid destroys red cells but preserves platelets; it also acts as an anticoagulant.

1. Get a finger- prick and draw blood up to the mark 1.0. Suck the diluting fluid to the mark 101.
2. Mix the contents thoroughly and wait for 20 minutes. The red cells will be hemolyzed, leaving only the platelets. Mix the contents once again and charge the chamber on both sides.

   Place the charged chamber on wet filter paper and cover it with a petri dish to avoid evaporation.
3. Focus the RBC square under HP; adjust the diaphragm and position of condenser till you see the platelets – which appear as small, round or oval structures lying separately, highly refractile bodies with a silvery appearance. Rack the microscope continuously and count the platelets in 5 groups of 16 squares each, as was done for red cell count.

Knowing the dilution (1 in 100) employed and the dimensions of the squares, calculate the number of platelets in 1 mm^3 of undiluted blood.

II. Rees-Ecker Method. The Rees-Ecker fluid contains the following:

• Brilliant cresyl blue = 0.1 g • Sodium citrate = 3.8 g • Formalin 40% formaldehyde) = 0.2ml • Distilled water = 100 ml	(The dye stains platelets formalin prevents fungal growth and lyses red cells, citrate prevents clotting and makes the fluid isotonic

1. Draw freshly filtered diluent to the mark 0.5 in the RBC pipette. Get a finger-prick and draw blood in the pipette so that the diluent reaches the mark 1.0. Wipe the tip and fill the pipette with diluent once again to the mark 101. This gives a dilution of 1 in 200.
2. Roll the pipette gently between your palms for 3–4 minutes. (Taking the diluent first in the pipette prevents clumping and disintegration of platelets which occurs if blood is taken directly into the pipette).
3. Discard the first two drops and charge both sides of the chamber in the usual manner. Place it on a wet filter paper and cover with a petri dish, and wait for 10 minutes to allow the platelets to settle.
4. Count the platelets (which appear as bluish, round or oval bodies, highly refractile on racking the microscope) in 5 groups of 16 squares each, as was done for red cells. Calculate their number in 1 mm^3 of undiluted blood.

B. INDIRECT METHOD

1. Place a drop of 14% magnesium sulfate solution on your finger tip, and get a prick through this drop. Blood oozes directly into the solution which prevents clumping, and disintegration of platelets.
2. Spread a blood film with the diluted blood, dry it, and stain it with Leishman's stain.
3. Examine the stained film under oil immersion lens. Count the platelets and red cells in every 5th field until 1000 red cells have been counted. Determine the "platelet ratio", i.e. the ratio of platelets to red cells (usually, there is 1 platelet to 16–18 red cells).
4. Do the RBC count from a fresh finger-prick in a counting chamber, and calculate the count in 1 mm^3 of undiluted blood.

Calculation of platelet count. With the knowledge of platelet count, and the RBC count, the actual number of platelets per mm^3 blood can now be calculated.

(While doing DLC in a stained blood film, the platelets appear in groups of 3–15, and most of them show different degrees of disintegration. In the present case, however, the platelets lie separately from each other and their morphology can also be studied).

Normal platelet count = 250,000 - 500,000/mm^3.

Differential Leukocyte Count (DLC)

Steps in Differential Leukocyte Counting

1. Getting a blood sample from a finger-prick and making blood smears. If blood is obtained from a vein, place a drop of blood (through the needle) on each of the 4–5 slides and spread blood films.
2. Examining the blood smears under LP and HP and choosing the ideal films for staining.
3. Fixing and staining the blood films.
4. Identification and counting of various leukocytes.

PROCEDURES

A. Preparing the Blood Films

1. Prepare 4 or 5 blood films as described in Expt 1–6.
2. Air dry the slides immediately by waving them in the air.
3. Examine them under low and then under high magnifications, and choose the best for staining.

B. Fixing and Staining of Blood Films

While supravital staining is employed for living cells, the staining of blood films involves dead cells. **Fixation** is the process that makes the blood film and its cells adhere to the glass slide. It also preserves the shape and chemistry of blood cells as near living cells as possible. (See Q/A 8). **Staining** is the process that stains (colors) the nuclei and cytoplasm of the cells. Both these purposes are achieved by the Leishman's stain.

1. **Fixing the Blood Films.** Place the slides, smear side up, on a 'staining rack' assembled over a sink (two glass rods placed across the sink, with the ends fitted into short pieces of rubber tubing). Ensure that they are horizontal.
2. Pour 8–10 drops of the stain on each unfixed slide by dripping it from a drop bottle, or use a dropper. This amount of stain usually covers the entire surface and "stands up" from the edges of the slides without running off. Note the time.

3. Allow the stain to remain undisturbed for 1–2 minutes, as advised.

- During this time, watch the stain carefully, especially during hot weather, and see that it does not become syrupy (thick) due to evaporation of alcohol. If the stain dries, it will precipitate on the blood film and appear as round, blue granules. This can be prevented by pouring more stain on the slides as required.

4. **Staining the blood film.** After the fixing time is over, add an equal number of drops of distilled water (or buffered water, if available) to the stain. If the water is carefully dripped from a drop bottle or a dropper, the entire mixture will stand up from the edges of the slides (due to surface tension) without spilling over.
5. Mix the stain and water by gently blowing at different places on the slides through a dropper, without scratching the smear. A glossy greenish layer (scum) soon appears on the surface of the diluted stain. Allow the diluted stain to remain on the slide for 6–8 minutes, or as advised.
6. Flush off the diluted stain in a gentle stream of distilled water for about 30 seconds and leave the slides on the rack for about a minute with the last wash of water covering them. Drain the slides and put them in an inclined position against a support, stained sides facing downwards (to prevent dust particles settling on them) to drain and dry. The under sides of the slides may be blotted with filter paper.

OBSERVATIONS AND RESULTS

C. Assessment of Stained Blood Smears

Before starting the actual counting of WBCs, you should—

i. Take an assessment of all the blood films. Examine with naked eye first, and then under low and high magnifications. Choose the best stained films for cell counting.
ii. Make sure that you can identify all the leukocytes with certainty.

Identification of Leukocytes Under Oil-immersion

a. **Cells seen in a Blood Film.** The following cells can be identified:

1. **The red cells.** Stained orange-pink, the red cells appear as numerous, evenly spread out, non-nucleated, biconcave discs of uniform size of 7.2–7.8 µm. Normally, the central paleness occupies the middle third of the cells but is wider in anemias. There may be some overcrowding and overlapping, or even rouleaux formation in the head end of the blood film.
2. **The leukocytes.** Five main types of WBCs are commonly seen in blood films. They are all larger than the red cells, nucleated, and unevenly distributed here and there among the red cells. They include 3 types of granulocytes (polymorphonuclear leukocytes, PMNs; neutrophils being the most numerous), and 2 types of agranulocytes (monocytes and lymphocytes). A sixth type of leukocyte, the plasma cell, is occasionally seen in the blood films. The plasma cells are found in abundance in the lymphoid tissues. It is a specialized lymphocyte (B lymphocyte) that secretes antibodies. The chromatin of this cell gives a typical "cartwheel" appearance.

Population-wise, neutrophils are the most numerous leukocytes, then come the lymphocytes, monocytes, eosinophils, and basophils, in that order.

3. **The Platelets.** They are membrane-bound round or oval bodies, with a diameter of 2–4 µm. They lie here and there in groups of 2–12, which is an *in vitro* effect, i.e. they do not form clumps in the circulating blood. They stain pink-purple, and being fragments of megakaryocytes, they do not possess nuclei.

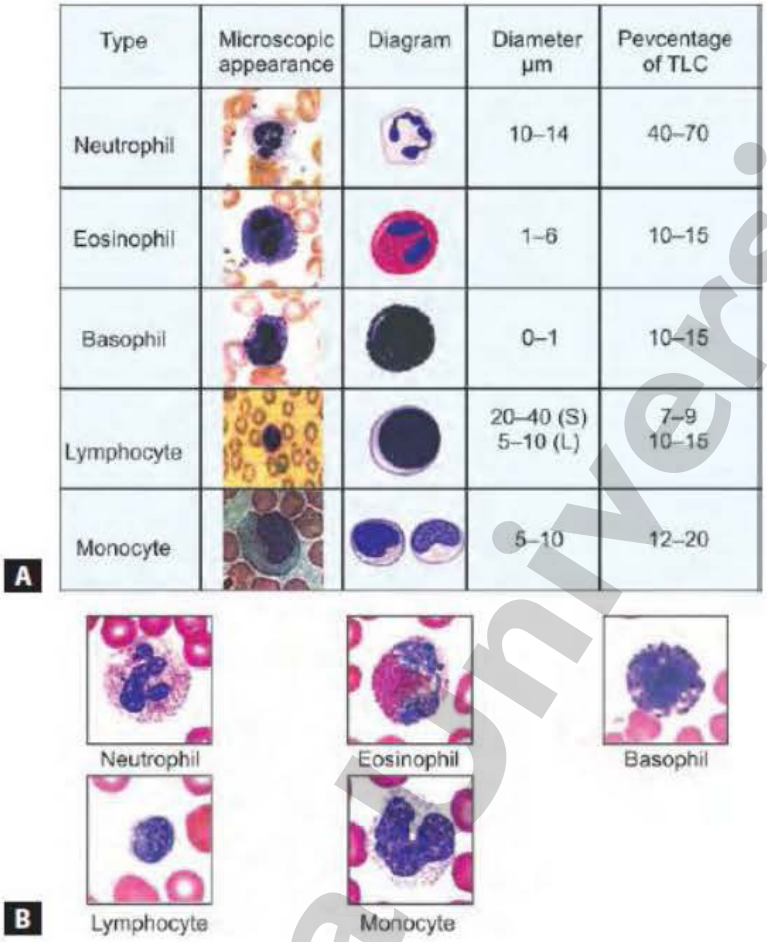
b. Identifying a Leukocyte. A leukocyte is identified from its size, its nucleus, and the cytoplasm—its color, whether vesicles (granules) are visible or not, their color and size if visible, and the cytoplasm/nucleus ratio **(Figure 1-15)**.

1. **Size.** The size of a WBC is assessed by comparing it with that of the surrounding red cells which have a uniform size of 7.2–7.8 µm. Try to assess whether 1½, 2, or 3 red cells will span across a leukocyte under study.

2. **The nucleus.** Note if the nucleus can be clearly seen through the cytoplasm and whether it is single or lobed. If single, note its location—central or eccentric, its shape—round, oval, or horse-shoe or kidney-shaped. Study its chromatin and whether condensed and lumpy or open and reticular. If lobed, count their number. Also note whether the lobes are connected by chromatin filaments or wider bands (**Figure 1-15**).
3. **The cytoplasm and cytoplasmic granules.** The cytoplasm may or may not show "visible" granules, or they may be very fine and not visible separately. Note the color of the cytoplasm and the granules, whether neutral color (light violet-pink taking up both acid and basic stains), or large and coarse—brick-red or red-orange (eosinophils) or deep blue-violet (basophils).
4. **Cytoplasm nucleus ratio.** Note the amount of cytoplasm in relation to the size of the cell and the nucleus.

Differentiation Between Various Leukocytes

Neutrophils, Eosinophils, and Basophils. All these cells are about the same size (10–14 µm) and have lobed nuclei. Neutrophils (polymorphonuclear leukocytes, PMNs, nuclei of many shapes) are the predominant WBCs. Their usual 3–5 lobed nucleus, and the fine, sand-like, sky-blue cytoplasmic granules identify them easily. The granules are not visible clearly and separately even with Leishman's stain, but give the cytoplasm a ground-glass (translucent) appearance. Some difficulty may arise when its nucleus has 2 lobes (which may appear spectacle-



Type	Microscopic appearance	Diagram	Diameter µm	Pevcentage of TLC
Neutrophil			10–14	40–70
Eosinophil			1–6	10–15
Basophil			0–1	10–15
Lymphocyte			20–40 (S) 5–10 (L)	7–9 10–15
Monocyte			5–10	12–20

FIgure 1-15: Different types of blood cells in a blood film stained with Leishman's stain. The size, shape of the nucleus, and staining features of the cytoplasmic granules distinguish them from one another. (A) Whiteblood cells, their microscopic appearance, diagrams, diameter, and their percentage of TLC is shown. (400 X) (B) White blood cells. (1000 X)

shaped) and there appear to be visible granules in the cytoplasm. Such a cell is likely to be mistaken for an eosinophil or a basophil.

However, the presence of 15–20 round or ovoid, coarse, closely-packed, refractive (shining), even-sized, red-orange or brick-red acidophilic (= acid or eosin loving) granules; and the clearly visible 2 lobes of the nucleus connected with a thick band of chromatin (spectacle-shaped nucleus) are typical of an eosinophil (the nucleus may sometimes be obscured by the granules).

The basophil (a rare cell of the blood) may be of the same size but is commonly smaller. The nucleus, which is bilobed or S-shaped, is usually obscured (covered, making it indistinct) by the deep blue-violet, basophilic (= basic loving) granules. These granules are round, variable-sized, coarse and closely packed **(Figure 1-15)**.

Table 1-2: Appearance of white blood corpuscles in a stained blood film

Cell type	Diameter (μm)	Nucleus	Cytoplasm	Cytoplasmic granules
Granulocytes Neutrophils (40–70%)	10–14 (1.5-2X a RBC)	• Blue-violet • 2–6 lobes, connected by chromatin threads Seen clearly through cytoplasm	• Slate-blue in color	• Fine, closely-packed violet pink • Not seen separately • Give ground-glass appearance • Do not cover nucleus
Eosinophils (1–6%)	10–15	• Blue-violet • 2–3 lobes, often bi-lobed, lobes connected by thick or thin chromatin band • Seen clearly through cytoplasm	• Eosinophilic • Light pink-red • Granular	• Large, coarse • Uniform-sized • Brick-red to orange • Seen separately • Do not cover nucleus
Basophils (0–1%)	10–15	• Blue-violet • Irregular shape, may be S-shaped, rarely bilobed • Not clearly seen, because overlaid with granules	• Basophilic • Bluish • Granular	• Large, very coarse • Variable-sized • Deep purple • Seen separately • Completely fill the cell, and cover the nucleus
Agranulocytes				
Monocytes (5–10%)	12–20 (1.5-3 X a RBC)	• Pale blue-violet • Large single • May be indented horse-shoe, or kidney shaped (can appear oval or round, if seen from the side)	• Abundant • 'Frosty' • Slate-blue • Amount may be larger than that of nucleus	• No visible granules
Small Lymphocytes				
(20–40%)	7–9	• Deep blue-violet • Single, large, round, almost fills cell. • Condensed, lumpy chromatin, gives 'ink-spot' appearance	• Hardly visible • Thin crescent of clear, light blue cytoplasm	• No visible granules
Large lymphocytes (5–10½)	10–15	• Deep blue-violet • Single, large, round or oval, almost fills cell • May be central or eccentric	• Large, crescent of clear, light blue cytoplasm • Amount larger than in small lymphocyte	• No visible granules

Small and large lymphocytes. Though small (7–9 µm) and large (10–15 µm) lymphocytes are commonly seen, a few intermediate cells also occur. The small lymphocyte, filled with a round or slightly indented and intensely blue-violet nucleus, and hardly containing any cytoplasm, is easy to differentiate from a large lymphocyte. It is about the size of a red cell, and its condensed chromatin gives it an ink-spot appearance. The bigger size of the large lymphocyte, with a round, oval, or indented nucleus, and larger amount of cytoplasm, which forms a small crescent on one side, help to identify it. However, the cytoplasm may form a thin rim around the nucleus in both cells. (The small lymphocyte is the more mature of the two cells).

- A plasma cell shows the typical "cartwheel" appearance of the chromatin of its nucleus.

Monocyte and large lymphocyte. The monocyte, which is the largest of the blood cells (12–20 µm) and 2–2½ times bigger than a red cell, can be identified by its pale-staining, oval, kidney or horseshoe-shaped nucleus, which is usually eccentric. The difficulty in differentiating it from a large lymphocyte may arise when the cell is seen from the side when it will appear oval or round. (The kidney shape of the nucleus cannot appear so from all directions). Then the much larger amount of pale-blue cytoplasm (about 1–2 times the size of the nucleus), as compared to a large lymphocyte (where it forms only a rim or crescent), and its frosty nature helps to identify it.

Differential Counting of Leukocytes

1. Draw 200 squares in your workbook for recording various WBCs as they are encountered and identified one after another. Enter these cells by using the letters 'N' for neutrophils, 'M' for monocytes, 'LL' for large lymphocytes, 'SL' for small lymphocytes, 'E' for eosinophils, and 'B' for basophils.
   - You can indicate these cells in a column (instead of the 200 squares), and as you identify a cell, put a short vertical stroke against that cell. In this way, you can place different types of cells in groups of 5, a horizontal stroke representing the 5th cell (e.g. Neutrophils = ~~IIII~~ III, etc.)

2. Place a drop of cedar wood oil on the right upper corner of the film, a few mm away from the head end. Bring the oil immersion lens into position till it enters the oil drop. Adjust the focus.
   - Do not flood the entire surface of the slide with oil; as you move the slide, the oil will move with the objective lens.
3. Move the slide slowly to the right (the image will move to the left) and as you encounter a leukocyte, identify it, and enter it in your workbook. As you approach the end of the smear, move 2 fields down and scan the film in the opposite direction. As you near the head, again move 2 fields down and scan the film towards the tail. Traverse the film in this to and fro fashion till you have examined 200 cells (count 400 cells for good results). This "battlement" procedure, as shown in Figure 1-9, ensures that you do not count a leukocyte more than once.
   - The possibility of WBCs sticking to the edge of the spreader should be kept in mind.
4. **Recount.** After you have, counted 200 (or 400) cells, count the leukocytes once more, starting from the lower left corner of the film, and going up in the "battlement" procedure.

Differential leukocyte count. When counting has been done, calculate the percentage of each type of cell in your count of 200 (or 400) white cells. The neutrophils are the prominent cells of the blood and constitute about 50–60% of the WBCs. The next predominant cells are lymphocytes (20–40%), which may be small or large. The third cell in the order of population is the monocyte which constitutes 8–10% of the WBCs.

Absolute leukocyte count. Use your value of TLC (though it should be done at the same time as DLC) obtained in the last experiment to express these percentages in terms of absolute values of each type of leukocyte per mm^3 of blood.

The absolute values are more significant than the DLC values alone. The reason is that the DLC may show only a relative increase or decrease of a particular type of cell with a corresponding change in the other cell types. For example, a neutrophil count of 85% may suggest neutrophilia, but if the TLC is, say, 8000/mm^3, then the absolute neutrophil count of 6800/mm^3 (8000 × 85/100 = 6800) would be within the normal range.

Normal values. The normal values for differential and absolute counts are given below:

Differential count	*(percent)*	*Absolute count (per mm^3)*
Neutrophils	40–75	2000–7500
Eosinophils	1–6	4–440
Basophils	0–1	0–100
Monocytes	2–10	500–800
Lymphocytes (both)	20–45	1300–3500

Determination of Hematocrit (Hct) (Packed Cell Volume; PCV)

APPARATUS AND MATERIALS

1. **Equipment for venepuncture.**
- Sterile swabs, alcohol, syringe and needle
- Container (penicillin vial or bulb) with anticoagulant (double oxalate or sequestrene).

2. **Wintrobe tube (hematocrit tube): Figure 1-17** shows a Wintrobe tube. It is 11 cm long, heavy, cylindrical glass tube, with a uniform bore diameter of 2 mm. Its lower end is closed and flat. The tube is calibrated in cm and mm from 0 to 10 cm from above downwards on one side of the scale (for ESR), and 10 to 0 cm on the other side (for PCV). The mouth of the tube can be covered with a rubber cap to prevent loss of fluid by evaporation. A Wintrobe stand is provided for holding the tube upright when doing ESR.
3. **Pasteur pipette:** It is a glass tubing drawn to a long thin nozzle about 14 cm long. A rubber teat is provided to suck blood into the pipette by a slight pressure. It is used for filling the Wintrobe tube.
4. **Centrifuge machine:** It packs the red cells in the Hct tube by centrifugal force. The magnitude of force produced by rotation of the tube depends on:
   a. The radius, i.e., the distance between the center of the shaft and the bottom of the centrifuge tube when laid horizontally.
   b. The number of revolutions per minute (rpm).

   In terms of gravitational force (G), the value of this force should be 2260 units. This much force is created when the radius is 9 inches and the speed is 3000 rpm.

PROCEDURES

1. Draw 5 ml of venous blood and transfer it to a container (penicillin vial or bulb) of anticoagulant. Rotate the bulb between your palms.
   i. To ensure proper mixing of cells and plasma (inaccurate results are likely if this precaution is not taken).
   ii. To oxygenate blood cells to remove CO_2 (red cells are larger when CO_2 is high, in venous blood).

2. Fill the pasteur pipette with blood and take its nozzle to the bottom of the Wintrobe tube. Expel the blood gently by pressing the rubber teat, and fill the tube from below upwards while withdrawing the pipette but always keeping its tip below the level of blood. Ensure that there is no air bubble trapped in the blood.
   - Do not try to fill the tube from its top as blood will not flow down to its bottom because of air present in the tube.
3. Bring the blood column exactly to the mark 10 (or the mark 0 on the other side of the scale) at the top. There should not be any bubbles at the top of blood.
   - If less blood is available, note the level.

   For ESR. Before centrifuging the tube, ESR can be determined by placing the tube in its stand and taking the reading of clear plasma above the red cells, as described in Expt 1-17.
4. Close the mouth of the tube with its rubber cap and centrifuge it at 3000 rpm for 30 minutes (slower speed will not pack the red cells fully). Balance this tube with another tube filled with water, or another sample of blood placed in the opposite tube holder.
5. At the end of 30 minutes, take the reading of upper level of packed red cells on the side of the scale where zero is at the bottom. Replace the tube in the machine and centrifuge it again for 15 minutes. Read the packed cell height again; it should be the same as before. If the height is reduced, centrifuge it again for 5 minutes. To be reliable, at least 3 successive readings, at intervals of 5 minutes, should be the same.

OBSERVATIONS AND RESULTS

Note that the blood has been separated into 3 layers:

i. A tall upper layer of clear plasma—amber or straw-colored. It should not be pink or red which would indicate hemolysis of red cells in the sample or within the body (i.e., before withdrawal of venous blood) in hemolytic diseases. If there is hemolysis, the test must be repeated on a fresh sample.

ii. A greyish-white, thin layer (about 1 mm thick) the so-called "buffy layer", consisting of platelets above and leukocytes below it.

iii. A tall bottom layer of red cells which have been closely packed together. A greyish red line separates red cell layer from the layer of leukocytes above it. This line is due to the presence of reduced Hb in the red cells lying next to the leukocytes which reduce the oxyHb of the cells. The line marks the upper limit of the red cell layer.

- The percentage of the volume of blood occupied by the red cells constitutes hematocrit or packed cell volume, i.e., the percentage of whole blood that is red cells

$$\text{Hematocrit (Hct)} = \frac{\text{Height of packed red cells (mm)}}{\text{Height of packed RBCs and plasma (ie., height of blood column)}} \times 100$$

For example, if the height of packed red cells is 45 mm, then

$$= \frac{45}{100} \times 100 = 45 \text{ percent.}$$

- It also means that out of 100 volumes (or parts) of blood 45 volumes (or parts) are red cells and 55 volumes (or parts) are plasma. Thus, out of 1 liter of blood, 450 ml are red cells and 550 ml are plasma.

Normal values. The average value of PCV is 42% when the RBC count is 5 million/mm^3 and their size and shape are normal.

Males: 44 percent (38–50 percent)

Females: 42 percent (36–45 percent)

The PCV for newborns is about 50 percent.

'True' hematocrit [true cell volume (TCV)]: Even under optimum conditions it is impossible to completely pack the red cells together, and about 2% plasma remains trapped in between the red cells. This percentage is more (i.e., more plasma) if the red cells are abnormal in shape (e.g. spherocytosis, sickle cells). To compensate for the trapped plasma, the 'true' cell volume (true hematocrit) can be obtained by multiplying the observed Hct value with 0.98.

The microhematocrit: Two heparinised glass capillary tubes, each about 6 cm long, are filled with blood and centrifuged at 12,000 rpm for 3 minutes. Readings of packed cells are taken from the scale on the tube holder. This method is very accurate and can be used on free-flowing capillary blood from a skin puncture in infants, or when the amount of blood available is very small, or in mass surveys for anemia.

Whole body hematocrit: Determination of total red cell mass by using chromium $^{51}(Cr)$ has shown that the Hct of venous blood is higher than that of the blood in microcirculation vessels (metarterioles, arterioles, capillaries). In these small vessels, the red cells tend to move in the centre of the blood stream (axial flow). As a result, the blood along the sides of the vessels has a low Hct, and branches leaving at right angles may receive cell-poor blood. In capillaries, the effect is marked because the RBCs move in a single file in the middle of the stream. This phenomenon, called plasma skimming, may be responsible for the capillary blood hematocrit being about 20% lower than the whole body hematocrit.

Venous blood hematocrit: The Hct of venous blood is slightly higher than that of arterial blood, because as the pH changes from the arterial value of 7.41 to 7.37 in the venous blood, the red cells gain a little water.

Normal Blood Standards (Absolute Corpuscular Values and Indices)

APPARATUS AND MATERIALS

The apparatus and materials required are those used for Hb, RBC count, and PCV.

PROCEDURES

1. Use your own values of Hb, RBC count and the value of PCV obtained during the demonstration experiment on a volunteer. This value of PCV, however, will not be strictly applicable to any person other than the volunteer.
   Your teacher may also provide each one of you with an arbitrary value of PCV from your Hb and RBC counts.
2. Calculate your absolute values for MCV, MCH, MCHC, and color index as shown below:

I. Mean Corpuscular Volume (MCV)

The MCV is the average or mean volume of a single red blood cell expressed in cubic micrometers (μm^3 or femtoliters). It is calculated from the following two basic values:

i. Red cell count in million/mm^3
ii. Packed cell volume (PCV) in 100 ml blood.

Formula

$$\frac{PCV \times 10}{\text{RBC count in million/mm}^3} \text{ or } \frac{\text{PCV per liter}}{\text{RBC } (10^3/\text{mm})}$$

For example

PCV = 45% RBC count = 5.0 million/mm^3

$$MCV = \frac{45 \times 10}{5} \; 90\mu m^3$$

Normal range = 74 – 95 μm^3

Derivation of the formula: In order to know the volume of one red cell from this formula, we have to determine either of the following two:

A. The packed volume of RBCs in 1 mm^3 of blood, since we know their number (i.e., 5 million). or
B. The number of RBCs in 100 ml blood, since we know their packed volume (e.g., 45 ml) in the same volume of blood.

The calculations according to both the methods are described below:

[A] Since PCV is 45%, the volume of packed cells in 1 mm^3 of blood = 0.45 mm^3

Number of red cells in the same volume of blood (i.e. 1 mm^3) = 5 million

Thus, there are 5,000,000 red cells in 0.45 mm^3 of blood.

Therefore, the volume of 1 red cell = $\frac{0.45\ mm^3}{5,000,000}$

Because 1 mm = 1000 micrometers, the MCV is:

$$MCV = \frac{0.45 \times 1000 \times 1000 \times 1000}{5,000,000}$$

$$= \frac{0.45 \times 1000}{5} = 90\ \mu m^3$$

[B] The PCV of RBCs in 100 ml blood = 45 ml

Because 1 meter (100 cm) = 10^6 micrometers

1 cm = 10^4 micrometers

For volume, 1 cubic cm = $10^4 \times 10^4 \times 10^4 = 10^{12}$ µm [Since the density of water at 4°C is taken as 1, for practical purposes, the density at room temperature may also be taken as 1]

So, 1 cubic cm = 1 ml, and 1 ml = 10^{12} cubic micrometers (μm^3)

∴ 45 ml = 45×10^{12} cubic micrometers (μm^3)

[We now have converted the volume of red cells (i.e., 45 ml) that are present in 100 ml blood into cubic microns (45×10^{12}), we now want to find out the number of RBCs in 100 ml blood, i.e., in 45 ml of packed red cells].

Because 1 cm = 10 mm

1 cubic cm = $10 \times 10 \times 10 = 10^3\ mm^3$ (cmm)

∴ 1 ml = $10^3\ mm^3$

And, 100 ml = $10^3 \times 10^2 = 10^5\ mm^3$

Since 1 mm^3 blood contains = 5×10^6 red cells

100 ml will contain = $5 \times 10^6 \times 10^5$

= 5×10^{11} red cells

Thus, the volume of 5×10^{11} RBCs in 100 ml blood = 45×10^{12} cubic micrometers (μm^3).

The volume of 1 red cell will be = $\frac{45 \times 10^{12}}{5 \times 10^{11}} = \frac{45 \times 10}{5} = 90\ \mu m^3$

So, $MCV = \frac{PCV \times 10}{\text{RBC count in million/mm}^3}$

II. Mean Corpuscular Hemoglobin (MCH)

The MCH, which is also determined indirectly, is the average hemoglobin content (weight of Hb) in a single red blood cell expressed in picograms (micro-microgram, μμg). It is calculated from the following basic values:

i. RBC count in million/mm^3.
ii. Hb in g percent.

Formula

$$\frac{\text{Hb in g\%} \times 10}{\text{RBC count in million/mm}^3}$$

For example

$$\text{Hb} = 15 \text{ g\%}$$
$$\text{RBC count} = 5 \text{ million/mm}^3$$
$$\text{MCH} = \frac{15 \times 10}{5} = 30 \text{ pg}$$

Normal range = 27–32 pg

Derivation of the formula. The derivation of the formula is on the same lines as that for MCV. We want to convert the g Hb into picograms (pg).

Since 1 g = 10^{12} pg

15 g = 15 × 10^{12} pg

Thus, the Hb content of 5 × 10^{11} RBCs in 100 ml blood = 15 × 10^{12} pg

The Hb content of 1 red cell will be

$$= \frac{15 \times 10^{12}}{5 \times 10^{11}} = \frac{15 \times 10}{5} = 30 \text{ pg}$$

(5 × 10^{11} red cells are present in 100 ml blood, as described earlier)

The formula can also be expressed as $= \frac{\text{Hb in g per liter}}{\text{RBC count in million/mm}^3}$

In macrocytic (large red cells) anemia, the MCH may be as high as 39 pg, because the cells are larger, but MCHC (see below) would be within normal range.

III. Mean Corpuscular Hemoglobin Concentration (MCHC)

The MCHC represents the relationship between the red cell volume and its degree or percentage saturation with hemoglobin, that is, how many parts or volumes of a red cell are occupied by Hb. The MCHC does not take into consideration the RBC count, but represents the actual Hb concentration in red cells only, (i.e., not in whole blood)—expressed as saturation of these cells with Hb.

The Hb synthesizing machinery of red cells does not have the Hb concentrating capacity beyond a

certain limit, i.e., RBCs cannot be, say 70% "filled" with Hb; this upper limit is only 36%. MCHC is calculated from the following formula:

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

$$\left[\frac{\text{Hb g\%}}{\text{PCV\%}} \times 100\right]$$

For example

Hb = 15 g%, PCV = 45%

$$\text{MCHC} = \frac{15}{45} \times 100 = 33.3 \text{ percent}$$

Normal range = 30–36%

If the MCHC is within the normal range, the cell is normochromic, if it is below the range, the cell is hypochromic. However, it cannot be hyperchromic for the reason mentioned above. A large cell may contain more Hb, but its percentage saturation will not be more than 36%.

Derivation of the formula. The derivation of the formula is as under:

45 volumes of red cells contain = 15 g of Hb

1 volume of red cells contain $= \frac{15}{45}$ g

100 volumes of red cells will contain $= \frac{15}{45} \times 100 = 33.3\%$

Another way of expressing MCHC is as follows:

$$\text{MCHC} = \frac{\text{MCH}}{\text{MCV}} \times 100$$

(Because MCH is Hb concentration in 1 red cell, and MCV is the volume of one red cell)

Taking MCH as 30 pg, and MCV as 90 cubic microns, MCHC

$$= \frac{30}{90} \times 100 = 33.3\%$$

IV. Mean Corpuscular Diameter (MCD)

The MCD is determined by direct micrometric measurements of the red cells in a stained film. The range is 6.9 to 8 micrometers, with an average of 7.5 µm. MCD can be used for measuring the mean corpuscular average thickness (MCAT).

V. Color Index (CI)

For the determination of CI, the results obtained in a particular case are compared with arbitrarily set "normal" values. The three traditional indices are: color index, volume index, and saturation index, which are the relative measures of Hb, cell size, and Hb concentration of red cells as compared to "normal" values. Only the color index is mentioned below.

To establish a relation between Hb concentration and the RBC count, they are expressed in the same unit, i.e., "percentage of normal", it being assumed that a normal person has 100% Hb, and 100% RBC count. Traditionally, the normal 100% RBC count is fixed at 5 million/mm^3, and the normal Hb at 15 g%, irrespective of age and sex.

For CI, we require the Hb concentration and the RBC count determined in an individual.

$$\text{Color index} = \frac{\text{Hb concentration (percentage of normal)}}{\text{RBC count (percentage of normal)}}$$

$$= \frac{100}{100} = 1.0$$

Normal range = 0.85 – 1.15

$$\text{Color index} = \frac{\dfrac{\text{g\% Hb found}}{\text{Normal Hb (15 g\%)}}}{\dfrac{\text{RBC count found}}{\text{Normal RBC count (5 mill./mm}^3\text{)}}}$$

The color index is low in iron deficiency anemia and high in macrocytic anemias. But since both RBC count and Hb may decrease simultaneously in a way that the CI remains normal, the CI does not have much clinical value.

Erythrocyte Sedimentation Rate (ESR)

PRINCIPLE

In the circulating blood the red cells remain uniformly suspended in the plasma. However, when a sample of blood, to which an anticoagulant has been added, is allowed to stand in a narrow vertical tube, the red cells (specific gravity = 1.095) being heavier (denser) than the colloid plasma (specific gravity = 1.032), settle or sediment gradually towards the bottom of the tube. The rate, in mm, at which the red cells sediment, called ESR, is recorded at the end of one hour.

Sedimentation of red cells

The settling or sedimentation of red cells in a sample of anticoagulated blood occurs in 3 stages:

i. In the ***first stage,*** the RBCs pile up (like a stack of coins), and form rouleaux that become heavier during the first 10-15 minutes.
ii. During the ***second stage,*** the rouleau (pleural of rouleaux) being heavier (see below) sink to the bottom. This stage lasts for 40–45 minutes.
iii. In the **third stage,** there is packing of massed bunches of red cells at the bottom of the blood column. This stage lasts for about 10–12 minutes.

Thus, most of the settling of the red cells occurs in the first hour or so.

WINTROBE'S METHOD

APPARATUS AND MATERIALS

1. Disposable syringe and needle •Sterile swabs moist with 70% alcohol •Container (discarded penicillin bottle, etc.) with double oxalate mixture, or sequestrene.
2. Pasteur pipette with a long thin nozzle.
3. Wintrobe tube and stand. **Figure 1-17** shows a Wintrobe tube (recall that it is also used for hematocrit or PCV; as was done in Expt1-9). Check out its dimensions and markings once again. It is graduated 0 to 10 cm from above downwards on one side (for ESR) and 10 to 0 cm on the other side (for Hct). The Wintrobe stand can hold up to 3 (or 6) tubes at a time. It is provided with a spirit level to ensure that the tubes are held vertical throughout the test.

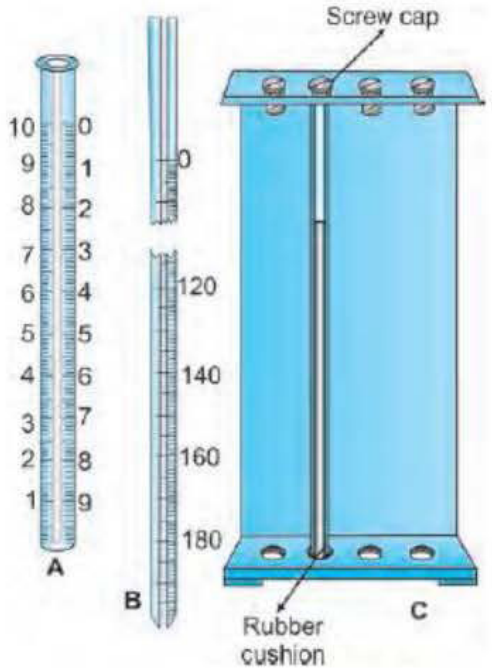


Figure 1-17: Determination of ESR. (A) Wintrobe tube; (B) Westergren tube; (C) Westergren stand with the Westergren tube in position

PROCEDURES

1. Draw 2.0 ml of venous blood and transfer it to a container of anticoagulant. Mix the contents gently but well by inverting the vial a few times, or by swirling it. Do not shake, as it will cause frothing.
2. Using the Pasteur pipette, fill the Wintrobe tube from below upwards as was done in Expt 1-9. Ensure that there are no air bubbles.
3. Transfer the tube to its stand and adjust the screws so that it will remain vertical. Leave the tube undisturbed in this position for one hour, at the end of which read the mm of clear plasma above the red cells.

   Express your result as: .....mm 1st hour (Wintrobe).

Normal values

Males	:	2–8 mm 1st hour
Females	:	4–10 mm 1st hour.

WESTERGREN'S METHOD

APPARATUS AND MATERIALS

1. 2 ml disposable syringe with needle •Sterile cotton/gauze swabs moist with alcohol •Container (discarded penicillin bottle).
2. Sterile solution of 3.8 percent sodium citrate as the anticoagulant.
3. **Westergren pipette (tube) and stand. Figure 1-17** shows a Westergren pipette. It is 300 mm long and has a bore diameter of 2.5 mm. It is calibrated in cm and mm from 0 to 200, from above downwards in its lower two-thirds. The Westergren stand can accommodate up to 4 tubes at a time. For each pipette, there is a screw cap that slips over its top, and, at its lower end, the pipette presses into a rubber pad or cushion. When the pipette is fixed in position, there is enough pressure of the screw cap to prevent leakage of blood from its lower end. There is a spirit level to ensure vertical position of the pipette.

PROCEDURES

1. Draw 2.0 ml of venous blood and transfer it into a vial containing 0.5 ml of 3.8% sodium citrate solution. This will give a blood: citrate ratio of 4:1. Mix the contents by inverting or swirling the vial. Do not shake, as it will cause frothing.
2. Fill the Westergren's pipette with blood-citrate mixture by sucking, after placing the tip of your finger over the top of the pipette to control the flow of blood into and out of it, or with a rubber bulb. Bring the blood column to exact zero mark. (If there is a difference of 1–2 mm, it should be noted and taken into account before giving the final report at the end of one hour).
3. Keeping your finger (or the rubber bulb) over the pipette, transfer it to the Westergren stand by firmly pressing its lower end into the rubber cushion. Now slip the upper end of the pipette under the screw cap. Confirm that there is no leakage of blood and that the pipette will remain vertical.
4. Leave the pipette undisturbed for one hour at the end of which read the mm of clear plasma above the red cells.

   Express your results as:.....mm 1st hour (Westergren).

Normal values

Males	:	3–9 mm 1st hour
Females	:	5–12 mm 1st hour.

Modified experiment. The above procedure is standardized for clinical use. However, you can modify it as an extension of the standard procedure. Instead of taking just one reading at the end of one hour, take ESR readings (to the nearest mm) every 15 minutes from the zero time for 3–4 hours. Using these figures, plot a graph of the distance of fall of red cells against time. Note if the rate of settling of RBCs is constant or not.

For comparison. Using this method, you can compare the ESR of **"normal"** blood with that of "abnormal" blood. Place 0.5 ml citrate in each of 2 dry containers. Draw 5 ml blood from a suitable vein and gently expel 2 ml blood into each container (citrate: blood ratio = 1:4). Add enough glucose to one container to make its blood "abnormal". Set up 2 separate Westergren pipettes containing "normal" and "abnormal" blood samples and take the readings at the end of one hour.

OBSERVATIONS AND RESULTS

Note that if there is no hemolysis, there is a sharp line of demarcation between the red cells and the clear, cell-free, and straw-colored plasma. The bore of the pipette if not less than 2 mm has no effect on ESR, but inclination from the vertical gives false high values. Higher values are also obtained at extremes of temperature, in anemia, and after ingesting food.

Tests for Hemostasis (Bleeding time; Coagulation time)

BLEEDING TIME (BT)

[I] "Duke" Bleeding time (finger-tip; ear-lobe)

- Since the skin of the fingertip is quite thick in some persons, a small cut in the skin of the earlobe with the corner edge of a sterile blade gives better results. The earlobe method is the original "Duke" method for BT.
- Ask your partner to fill the capillary tube with blood from the same skin puncture from where you are doing the BT (see below for CT).
- **Materials** •Equipment for sterile finger-prick. • Clean filter papers. • Chemically clean, 10–12 cm long, glass capillary tubes with a uniform bore diameter of 1–2 mm. • Stopwatch.

PROCEDURES

1. Get a deep finger-prick under aseptic conditions to get free-flowing blood. Start the stop watch and note the time.
2. Absorb/remove the blood drops every 30 seconds by touching the puncture site with the filter paper along its edges, without pressing or squeezing the wound. Number the blood spots 1 onwards.
3. Note the time when bleeding stops, i.e. when there is no trace of blood spot on the filter paper. Encircle this spot and number it as well. This is the end point. (Do not keep the filter paper on the table and then press your wound on it).
4. Count the number of blood spots and express your result in minutes and seconds.

Normal bleeding time = 1–5 minutes.

- The test is simple and quite reliable in spite of the fact that the depth of the wound cannot be controlled.
- The BT is prolonged in purpura (platelet deficiency, or vessel wall defects) while it is usually normal in hemophilia.
- Lack of several clotting factors may prolong BT, though it is especially prolonged by lack of platelets.

[II] Another method is to get a finger-prick and dip the finger in a beaker containing normal saline at 37°C. The blood drops will be seen falling to the bottom in a continuous stream. Note the time when bleeding stops.

[III] "Ivy" Bleeding Time (Hemostasis Bleeding Time).

This method is more reliable than the "Duke" method. However, it requires some practice to apply the BP cuff and maintain the pressure.

Procedure

1. Clean the skin over the front of the forearm with 70% alcohol.
2. Apply a blood pressure cuff on the upper arm, raise the pressure to 40 mm Hg and maintain it there till the end of the experiment.
3. Clean the skin area once again. Grasp the underside of the forearm tightly, make a 1–3 mm deep skin puncture, about 5–6 cm below the cubital fossa. Note the time.
4. Remove the blood every 30 seconds by absorbing it along the edges of a clean filter paper by gently touching the wound with it, till the bleeding stops. This is the end-point.

Normal bleeding time with this method is upto 9 minutes.

CLOTTING TIME (CT) COAGULATION TIME

[I] CAPILLARY BLOOD CLOTTING TIME (WRIGHT'S CAPILLARY GLASS TUBE METHOD)

(While your partner is doing BT on your finger prick, you can proceed with your CT.

1. Absorb the first 2 drops of blood on a separate filter paper and allow a large drop to form. Now dip one end of the capillary tube in the blood; the blood rises into the tube by capillary action. This can be enhanced by keeping its open end at a lower level.
2. Note the time when blood starts to enter the tube. This is the zero time.
3. Hold the capillary tube between the palms of your hands to keep the blood near body temperature (in winter, you may blow on it).
4. Gently break off 1 cm bits of glass tube from one end, at intervals of 30 seconds, and look for the formation of fibrin threads between the broken ends. The end-point is reached when fibrin threads span a gap of 5 mm between the broken ends ("rope formation"). Note the time.

Normal clotting time = 3–6 minutes.

Comments

(i) The clotting of blood with this method involves both the intrinsic and the extrinsic systems of clotting. There is injury to the blood (coming in contact with glass, intrinsic pathway), and the injury to the tissues (extrinsic pathway).

(ii) The CT is prolonged in hemophilia and other clotting disorders, because thrombin cannot normally be generated. Yet, the BT, which reflects platelet plug formation and vasoconstriction, independently of clot formation, is normal.

[II] Drop Method

This method is less accurate than the above method. Place a large drop of blood from a skin puncture on a clean and dry glass slide. Draw a pin through the drop every 30 seconds, and note the time when fibrin threads adhere to the pin and move with it out of the blood drop. The time elapse between placing the blood drop on the slide and the formation of fibrin threads is the clotting time.

Normal clotting time = 2–4 minutes.

- In the original Duke's Drop Method for CT, two drops of 4–5 mm diameter are placed on a glass slide. The slide is tilted at 30 second intervals. The end-point is absence of change in the previous shape when the slide is held vertical.

[III] *VENOUS BLOOD CLOTTING TIME* (LEE AND WHITE TEST-TUBE METHOD)

A. Single test-tube method. This is the most widely used method for the determination of clotting time.

1. Draw 5 ml venous blood by a clean, non-traumatic venepuncture. Note the time when blood starts to enter the syringe. This is the zero time. Transfer the blood to a chemically clean and dry test tube.
2. Holding the test tube in a water bath at 37°C, take it out at 30 second intervals and tilt it. The end-point is when the tube can be tilted without spilling the blood.

Normal clotting time with this method = 5–10 minutes.

B. Multiple test-tube method. The CT can be determined more accurately by using 3 test tubes rather than one only.

1. Rinse 3 test tubes of 8 mm diameter with normal saline, drain them and place them in a metal rack kept in water at 37°C. Transfer 1.5 ml blood into each test tube.
2. Take out the first tube after 1 minute, tilt it to 45° and return it to the rack. Repeat every 30 seconds until clotting occurs, i.e. where the test tube can be tilted without spilling the blood. Note the time.
3. Repeat the tilting on the second test tube and note the time when clotting occurs, (this happens a few seconds later because tilting the tube hastens clotting). The third tube acts as a control and a check on the end-point in the 2nd test tube.

If a siliconized test tube is used at the same time, a delayed clotting time (40–70 minutes) can be shown.

Normal clotting time with this method = 5–10 minutes.

- The CT depends on the condition of the glass itself, and even on the size of the test tube. Therefore, a high-degree of standardization is needed.

Blood Grouping

PRINCIPLE

The surfaces of red cell membrane contain a variety of genetically determined antigens, called **isoantigens** or **agglutinogens**, while the plasma contains antibodies **(agglutinins)**. To determine the blood group of a person, his/her red cells are made to react with commercially available antisera containing known agglutinins. The slide is then examined under the microscope to detect the presence or absence of clumping and hemolysis (agglutination) of red cells which occurs as a result of antigen-antibody reaction.

APPARATUS AND MATERIALS

1. Microscope. •Glass dropper with a long nozzle. •Sterile blood lancet or needle. •Sterile cotton/ gauze swabs. •Alcohol. •5 ml test tube. •Toothpicks.
2. Clean, dry microscope slides. (A special porcelain tile with 12 depressions is available for this purpose and may be used in place of glass slides.)
3. 1% sodium citrate in normal saline (or normal saline alone).
4. **Anti-A serum:** [contains monoclonal anti-A antibodies (against human); these antibodies are also called anti-A or alpha (α) agglutinins]. The anti-A serum can also be obtained from a person with blood group B. (see Q/A 6).
5. **Anti-B serum:** [contains monoclonal anti-B antibodies (against human); these antibodies are also called anti-B or beta (β) agglutinins]. The anti-B serum can also be obtained from a person with blood group A. (see Q/A 6).
6. Anti-D (anti-Rh) serum: [Contains monoclonal anti-Rh (D) antibodies (against human). These antibodies are also called anti-D agglutinins.

Note
The antibodies against Rh factor do not occur naturally (see below).

Note
These antisera are available commercially. For a quick identification, the anti-A serum is tinted blue, anti-B serum yellow, while the anti-D serum is colorless.

Caution
Do not interchange the droppers provided with antisera bottles.

PROCEDURES

1. Using a glass-marking pencil, divide 3 slides, each into two halves by a line drawn down the middle (the left sides will act as "test sides" and right sides as the "control sides"). Mark the left corner of 1st slide anti-A, left corner of 2nd slide "anti-B, and the left corner of 3rd slide "anti-D". Mark the right corners of these 3 slides 'C' (for control).
2. Mark another slide (4th) 'S' (for only red cell suspension in saline, i.e. no antiserum will be added on this slide).
3. Place 8–10 drops of saline in the center of slide 'S'.
4. **Preparation of red cell suspension.** A suspension of red cells in saline should preferably be prepared and used instead of adding blood drops directly from the fingerpick to the antisera for the following reasons:
   a. Dilution of blood permits easy detection of agglutination and hemolysis, if present. (Red cells in undiluted blood tend to form large rouleaux and masses. These may be difficult to disperse and may be mistaken for agglutination).
   b. Plasma factors likely to interfere with agglutination are eliminated.
5. Get a finger-prick under aseptic conditions, and add 2 drops of blood to the saline on the slide marked 'S'. Mix the saline and blood with a clean glass dropper to get a suspension of red cells. You may use a toothpick for this purpose.
   - A better method is to place 2 ml of saline in a small (5 ml) test tube. Then get a finger pricked and allow a blood drop to form. Now place the pricked fingertip on top of the test tube and invert it. Mix the blood and saline by inverting the tube 2 or 3 times. A suspension of red cells is now ready.
   - Washed red cell suspension gives the best results. The red cells are "washed" in saline by centrifuging the diluted blood, removing the

supernatant, and adding fresh saline to get a suspension of "washed" red cells.

6. **Determination of Blood group.**
   Put one drop of anti-A serum on the left half ("test side") of 1st slide (marked anti-A), one drop of anti-B serum on the left half of 2nd slide (marked anti-B), and one drop of anti-D serum on the left half of 3rd slide (marked anti-D).
7. Put one drop each of normal saline on the "control" sides (right halves) of the 3 slides (i.e. areas marked 'C').
8. Add a drop each of red cell suspension (from the slide 'S', or from the test tube of red cell suspension) on anti-A, one drop on anti-B and one drop on anti-D sera, and one drop each on the normal saline taken on the "control" sides of the 3 slides.
   In this way, the red cells-saline mixture on the "control" sides of each slide will act as a control to confirm agglutination or no agglutination on the corresponding test side **(Figure 1-18)**.
9. Mix the anti-sera and red cells, and saline and red cells on each slide by gently tilting it first one way and then the other a few times. Take utmost care that the "test" mixtures and "control" mixtures do not flow into each other and get mixed up.
   - The red cells and sera can also be mixed by gently blowing on them. You may use 3 separate toothpicks to transfer red cell suspension to the three anti-sera, and for mixing them, and 3 toothpicks to transfer red cells to saline drops taken on the "control" sides of the 3 slides.

Important
Do this one at a time, and then discard each toothpick.

10. Wait for 8–10 minutes, then inspect the 3 antisera-red cell mixtures ("test" mixtures) and "control" mixtures, first with the naked eye to see whether agglutination (clumping and hemolysis of red cells) has taken place or not. Then confirm under low magnification microscope, comparing each "test mixture" with its corresponding "control mixture".

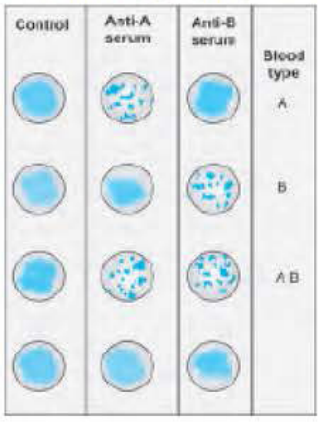


Figure 1-18: Determination of blood groups (types) Showing agglutionation (hemolysis and clumping of red cells) and no-agglutionation (cells remain unformly distributed). The reaction between anti-D serum and red cells is not shown

OBSERVATIONS AND RESULTS

It is essential that you should be able to distinguish between ***"agglutination"*** and ***"no agglutination"***. The features of each are:

Agglutination.

i. If agglutination occurs, it is usually visible to the naked eye. The hemolysed red cells appear as isolated (separate), dark-red masses (clumps) of different sizes and shapes.
ii. There is brick-red coloring of the serum by the hemoglobin released from ruptured red cells.
iii. Tilting or rocking the slide a few times, or blowing on it does not break or disperse the clumps.
iv. Under LP objective, the clumps are visible as dark masses and the outline of the red cells cannot be seen.

Grading of Agglutination Reaction. The agglutination reaction may be graded according to whether there is a single large agglutinate (4+), or a number of large (3+), medium or small masses with no free red cells (2+), or many small aggregates in the background of free red cells (1+).

Table 1-3: Determination of blood groups

(+) denotes agglutination		*(–) denotes no agglutination*			
Agglutination		***Your blood group is***	***Your RBCs contain agglutinogens***	***Your plasma contains agglutinins***	***Your plasma will agglutinate RBCs of group***
Anti-A serum	***Anti-B serum***				
+	–	A	A	Anti-B	B, AB
–	+	B	B	Anti-A	A, AB
+	+	AB	A, B	None	None
–	–	O	O	Both anti-A and anti-B	A, B, AB
Similarly, for Rh(D) blood group:					
Agglutination	+ Your RBCs contain Rh(D) antigen. You are Rh(D) +ve				
No agglutination	– No Rh(D) antigen in your red cells. You are Rh(D) –ve				
Note					
Agglutinin against agglutinogen not present in the red cells is present in the plasma of each type. There are no naturally occurring antibodies against Rh(D) antigen. It is obvious from the above that if a known type of 'A' or 'B' blood is available, it is possible to determine the blood group of any unknown person.					

No Agglutination

i. In the "control" mixtures, the red cells may form a bunch, or rouleaux. These sedimented red cells give an orange tinge of a suspension of red cells rather than "isolated dark red masses" of ruptured red cells.
ii. The red cells will disperse if you gently blow on the slides, or tilt them a few times.

Confirm all these features of "no agglutination" under the microscope.

Spirometry (Determination of Vital Capacity, Peak Expiratory Flow Rate, and Lung Volumes and Capacities)

Measurement

Vital capacity may be measured either on a simple spirometer or a recording spirometer (spiro = breathe; meter = measuring device):

A. **Simple spirometer (student spirometer;** also called a **Vitalograph**). It is a common low-cost instrument, either a metallic or a bellows type, used in colleges, hospitals, sports facilities, and gymnasia.

B. **Recording spirometer.** It is a sophisticated, electrically-driven, recording system used in respiratory physiology laboratories, hospitals, etc. It provides a graphic record of various lung volumes and capacities.

C. **Wright's peak flow meter.** It is a small portable instrument which can give information about the state of respiratory passages (it can be carried in one's pocket for bed-side use by the physician).

Note

In this experiment, the students will use a simple spirometer and the peak flow meter to measure some of the ventilatory functions of the lungs under normal conditions. The working of a recording spirometer will then be briefly described.

DEFINITIONS AND TERMINOLOGY

The rate. The rate of respiration is somewhat variable even at rest, varying from 12–16/min. However, when one becomes conscious of one's breathing, it may speed up, slow down, or show a temporary disturbance in its rate, depth, and rhythm.

The respiratory cycle. There is a cycle of inspiration, expiration, and a pause. The duration of the pause varies inversely with the rate. (The pause may not be obvious in some cases).

LUNG VOLUMES AND CAPACITIES

The volume of air in the lungs changes considerably during a respiratory cycle. However, for convenience, 4 lung volumes and 4 lung capacities are distinguished, as shown in **Figure 2-3**.

The term **lung volumes** refers to the non-overlapping subdivisions, or fractions of the total lung air, while the term **capacities** refers to combination of two or more lung volumes.

Lung Volumes

1. **Tidal Volume (TV).** It is the amount of air that moves into the lungs with each inspiration (or the amount that moves out with each expiration) during normal quiet breathing (tidal respiration). It amounts to about 500 ml. (It varies greatly in different persons and in the same person at different times).
2. **Inspiratory Reserve Volume (IRV).** It is the extra volume of air that can be inspired over and above the normal (resting, quiet) tidal volume (i.e. from the spontaneous end-inspiratory point), with maximum effort. It amounts to about 2500 ml.
3. **Expiratory Reserve Volume (ERV).** It is the extra amount of air that can be expelled (expired) by forceful expiration from the spontaneous end-expiratory point, i.e. over and above the normal tidal expiration. It amounts to about 1100 ml.
   - (The term 'reserve' in these 2 volumes means this; during quiet breathing (i.e. without effort, spontaneous), we take in 500 ml air and breathe out 500 ml air. In addition to this 500,

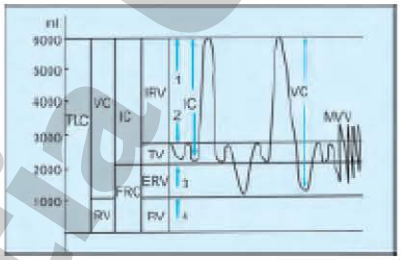


Figure 2-3: Lung volumes and capacities. The volumes do not overlap; the capacities are made up of two or more volumes. Arrows: (1) Maximum inspiratory level; (2) Resting inspiratory level; (3) Resting expiratory level; and (4) Maximum expiratory level. Standard abbreviations used

one can, with maximum effort, take in more air (IRV), or expel more air (ERV).

4. **Residual Volume (RV).** It is the amount of air that remains behind in the lungs after a maximum voluntary expiration. It amounts to 1100 ml. The lungs cannot be emptied out completely of air even with maximum effort because as the pressure outside small air passages increases (i.e. the high intrathoracic pressure due to maximum expiratory effort) they are compressed and thus block the flow of air out of the lungs.

Lung Capacities

1. **Vital Capacity, VC; forced vital capacity, FVC; forced expired (or expiratory) volume, FEV.** It is the largest volume of air a person can expel from the lungs with maximum effort after first filling the lungs fully by a deepest possible inspiration. It amounts to 3.5 to 5.5 liters.
2. **Inspiratory Capacity (IC).** It is the maximum amount of air that a person can breathe in with maximum effort, starting from the normal end-expiratory point. It amounts to about 3000 ml. (IRV, 2500 ml + TV, 500 ml = 3000 ml).
3. **Functional Residual Capacity (FRC).** This is the amount of air remaining in the lungs at the end of a normal (quiet) expiration. It amounts to about 2500 ml. (ERV, 1100 ml + RV, 1100 ml = 2200 ml).
4. **Total Lung Capacity (TLC).** It is the volume of air that is present in the lungs at the end of a deepest possible inspiration. It is a measure of VC + RV, and amounts to 4500–6000 ml.

Minute Ventilation (minute volume, MV). This is the amount of air that is breathed in or breathed out per minute when the person is at rest. It amounts to 6–8 liters/min. (TV × Rate of respiration, i.e. 500 ×12 to 16 = 6–8 liters per minute).

Alveolar Ventilation. Out of a tidal volume of 500 ml, 150 ml air remains in the upper respiratory passages up to respiratory bronchioles (anatomical dead space), while only 350 ml reaches the respiratory zone (respiratory bronchioles, alveolar ducts and alveoli) for exchange of gases. Thus, alveolar ventilation would be = 350 × 12 to 16 = 4.2 —5.6 liters per minute.

Maximum Voluntary Ventilation (Maximum ventilation volume, MVV). It is the amount of air which can be moved into or out of the lungs with maximum effort during one minute. Formerly called **Maximum Breathing Capacity (MBC)**, the MVV amounts to 100–140 liters/min. The subject breathes quickly and deeply for 15 seconds, and MVV is calculated for one minute. (This means that pulmonary ventilation of 6–8 l/min can be increased by 15–20 times with maximum effort, though for short periods).

STATIC AND DYNAMIC LUNG VOLUMES AND CAPACITIES

The lung volumes and capacities may either be **static** or **dynamic,** depending on whether or not time factor has been taken into consideration.

A. Static volumes and capacities. These measurements are those where time factor is not taken into consideration. They are expressed in milliliters or liters, and include:
Static volumes: TV, IRV, ERV, and RV.
Static capacities: IC, VC (FEV, without timing), FRC, and TLC.

B. Dynamic volumes and capacities. These measurements are those where time factor is taken into account, that is, they are time-dependent. They are expressed in milliliters or liters per second or per minute and include:
Dynamic volumes: MV, MVV.
Dynamic capacities: VC (timed vital capacity, FEV_1) maximum mid-expiratory flow rate (MMFR).

A. SPIROMETRY (VITALOMETRY) EFFECT OF POSTURE ON VITAL CAPACITY

Apparatus and Materials. Spirometer; potassium permanganate solution.

Spirometer (Vitalograph; 'student' (or simple) spirometer). It consists of a double-walled metal cylindrical chamber, having an outer container filled with water in which a light-metal gas bell of 6-liter capacity floats. The bell (or float) is attached on

its upper surface to a chain which passes over a graduated frictionless pulley. The pulley bears a spring-mounted indicator needle that moves with the pulley and indicates the volume of air present in the bell. The gas bell is counterpoised by a weight (counter-weight) attached to the other end of the chain (**Figure 2-4**). This weight allows a smooth up and down movement of the bell).

The inlet tube, through which air moves into or out of the bell, is corrugated canvas-rubber tubing bearing a mouthpiece. (This tube is attached to a metal pipe fitted at the bottom of the apparatus, the upper end of which lies above the level of water in the outer container). When air is blown into the inlet tube, it raises the bell, the water acting as an airtight seal.

PROCEDURES

Note
One can record not only vital capacity with this apparatus but also a few other lung volumes and capacities, though only approximately. Their accurate recording is done on recording spirometer, as described later.

Important
Explain the procedure in detail to the subject and even give her a pilot rehearsal to ensure that the procedure has been fully understood. Also, the subject should be encouraged to exert full expiratory effort during the test. Record the age, height, and body weight before starting the test.

1. Bring the bell to its lowest position by gently pushing it down. Adjust the pointer needle at zero, which indicates that the bell is completely empty.
2. **Measuring VC by collecting expired air in the spirometer (Standard Method).** Ask the subject to stand comfortably, facing the spirometer so that she/he can see the movement of the bell. Tell her to breathe normally (quietly) for a minute or so. Now direct her to inspire as deeply and as fully as possible to fill the lungs. Then, while keeping the nostrils closed with a thumb and fingers, and the mouthpiece held firmly between the lips, tell him to expel all the air that he can with maximum effort into the spirometer. The bell moves up and the pointer on the pulley indicates the volume of expired air. The forced expiration should be deep and quick but without haste. (Normally, this procedure takes about 3 seconds.) Take 2 more readings at intervals of 5 minutes in the standing position as before.
3. **Effect of posture.** Ask the subject to sit comfortably on a stool and record the VC 3 times as before, at intervals of 2 minutes. Then ask her to lie down on the couch in supine position (face up) and record the VC 3 times. (See Q/A 7 for the effect of posture on VC).
4. **Measuring VC by breathing in from the full spirometer.** This method is a variation of the above method and there is no special name given to it. However, it is an instructive exercise.
   i. Raise and lower the bell a few times, finally raising it to its highest position so that it gets filled with fresh room air. Note the reading.
   ii. Tell the subject to breathe deeply a few times, then to expel air, with maximum effort, from

Figure 2-4: Simple spirometer. 1: Outer container; 2: Gas float; 3: Calibrated pulley with spring-mounted indicator needle; 4: Tap for draining water; 5: Wide-bore inlet tube; 6: Counterpoise system

the lungs into the atmosphere. This leaves only the RV in the lungs.

iii. Now ask the subject to hold the mouthpiece firmly between the lips and then to breathe in maximally from the spirometer (thus breathing in ERV, TV, and IRV in that order, as shown in **Figure 2-3**). Note the reading. The difference between the two readings is the vital capacity. Compare this value with that obtained with the standard method, and explain the difference, if any.

5. **Two-stage Vital Capacity Determination.**
   Stage I: Fill the spirometer with fresh room air, and note the reading. Ask the subject to breathe normally a few times. Then, starting from resting end-expiratory position, to take a maximum inspiration from the spirometer bell. Note the reading. The difference will give you inspiratory capacity (IC).
   Stage II: Bring the bell to its lowest position (zero level). Tell the subject to breathe normally a few times. And then, starting from resting end-expiratory position, to expel all the air from the lungs with maximum effort into the spirometer. This will give ERV.
   The sum of the above, i.e. IC and ERV will give you vital capacity. Compare it with the value obtained with the standard method and explain the difference, if any.
6. **Measurement of inspiratory capacity.** This can be done in two ways:
   a. **By breathing from the spirometer.** Fill the bell with fresh air as before and note the reading. Carry out the procedure described above for stage I employed for vital capacity. This give IC.
   b. **By breathing out into the spirometer.** Bring the bell down to zero level. Then tell the subject to take a deep breath and to expire forcefully into the spirometer up to the point of resting end-expiratory position. This will give IC, i.e. TV + IRV.
7. **Measurement of expiratory reserve volume.** Carry out the procedure described above for stage II for vital capacity.
8. **Tidal volume.** Only an approximate idea can be obtained by breathing 2–3 times in and out of spirometer and taking the readings. This step cannot be repeated for more than 2–3 times because this spirometer is a closed system and there is no provision for absorbing CO_2 from the expired air.

OBSERVATIONS AND RESULTS

Record your observations as indicated below:

Name.... Age.... Sex.... Date of experiment....
Weight.... kg.... Height..... cm

1. Rate of respiration ...../min
2. Tidal volume ...../ml

Minute ventilation (at rest): ....liters/min

	Readings (ml) 1st	2nd	3rd	Max value
3. Vital capacity				
a. Standard method	....	....	....	....
b. Two-stage method	....	....	....	....
4. Inspiratory capacity				
a. Breathing from spirometer	....	....	....	....
b. Breathing out into spirometer				
5. Expiratory reserve volume	....	....	....	....
6. Effect of posture on VC:				
a. Standing	....	....	....	....
b. Sitting	....	....	....	....
c. Supine				

Calculate the vital capacity:
per kg body weight ....
per cm height ....
per m^2 BSA (vital index) ....
(Consult nomogram for body surface area calculation at the end of the book).

Recording of Systemic Arterial Blood Pressure

Indirect Methods

Obviously, the direct method is not suitable as a routine clinical procedure. Indirect methods were, therefore, introduced; methods that are variations of a procedure called sphygmomanometry.

PRINCIPLE

A sufficient length of a single artery is selected in the arm (brachial artery), or in the thigh (femoral artery). The artery is first compressed by inflating a rubber bag (connected to a manometer) placed around the arm (or thigh) to stop the blood flow through the occluded section of the artery. The pressure is then slowly released and the flow of blood through the obstructed segment of the artery is studied by:

i. Feeling the pulse—the palpatory method.
ii. Observing the oscillations of the mercury column—the oscillometric method, and
iii. Listening to the sounds produced in the part of the artery just below the obstructed segment—the auscultatory method.

APPARATUSES

Stethoscope
(Steth = chest, scope = to inspect)

Though introduced in its present form by Laennec in 1819, it was not until 1905 that Korotkoff used it for recording the blood pressure. The sounds produced in the chest and elsewhere in the body are heard with a stethoscope. The instrument has the following 3 parts:

a. **The chest-piece.** The chest-piece has two end pieces—a bell and a flat diaphragm, though some have only the diaphragm.
b. **The rubber tubing.** In the commonly used stethoscope, a single soft-rubber pressure tube (inner diameter 3 mm) leads from the chest-

piece to a metal Y-shaped connector. The plastic diaphragm causes magnification of low-pitched sounds though it distorts them a little. The bell-shaped chest-piece conducts sounds without distortion but with little magnification. Murmurs which precede, accompany, or follow the heart sounds are better heard with the bell.

c. **The ear-frame.** It consists of two curved metallic tubes joined together with a flat U-shaped spring which keeps them pulled together. The upper ends of the tubes are curved so that they correspond to the curve of the external auditory meatus, i.e. they are directed forwards and downwards. Two plastic knobs threaded over the ends of the tubes fit snugly in the ear. Two rubber tubes connect the Y-shaped connector to the metal tubes.

Sphygmomanometer (Commonly called the "BP apparatus")

The sphygmomanometer is the instrument routinely used for recording arterial blood pressure in humans. The term "sphygmomanometer" is derived from three Greek roots with Latin equivalents "sphygmo" means pulse, "manos" means thin, and "metron" refers to measure. In early procedures, when physicians used to feel the pulse during measurement of BP, they described its first appearance as "thin", hence the term. Different types of BP instruments are in use, but the one in common use is the mercury sphygmomanometer. It consists of the following parts:

a. **Mercury manometer.** The manometer is fitted in the lid of the instrument. One arm of the manometer is the reservoir for mercury—a broad and short well that contains enough mercury to be driven up in the other limb—the graduated glass tube **(Figure 2-13)**.

b. **Graduated tube.** The manometer glass tube is graduated in mm from 0 to 300, each division representing 2 mm, though actually slightly less than 2 mm. The reason for this is the greater diameter of the mercury reservoir than that of the glass tube. For example, when mercury is driven up the tube for, say, 20 mm Hg, the meniscus in the reservoir falls less so that the actual pressure on its mercury is slightly greater than 20 mm Hg.

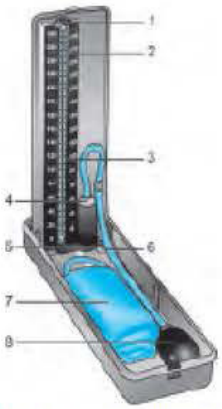


Figure 2-13: Sphygmomanometer (the " BP apparatus"). 1: Spring-loaded clip; 2: Lid of the apparatus; 3: Graduated glass tube; 4 One-way valve; 5: Mercury reservoir; 6: Stop cock; 7: Armlet; 8: Air pump (rubber bulb, with leak valve.)

And, to compensate for this, the tube is calibrated with divisions that are slightly less than 2 mm apart.

A stopcock between the two limbs, when closed, prevents the mercury from entering the glass tube. The one-way valve fitted at the top of the mercury well prevents spilling of mercury when the lid is closed, while allowing pressure to be transmitted from the rubber bag to the mercury reservoir. A spring-loaded clip at the top of the tube keeps it firmly pressed into a rubber washer at its lower end to prevent leakage of mercury.

c. **The armlet (rubber bag; Riva Rocci cuff).** The "cuff" as it is usually called, consist of an inflatable rubber bag, 24 cm × 12 cm, which is fitted with 2 rubber tubes—one connecting it to the mercury reservoir and the other to a rubber bulb (air pump). The bag is enclosed in a long strip of inelastic cloth with a long tapering free end. The cloth covering keeps the rubber bag in position around the arm when pressure is being measured. In some cuffs, 2 velcron strips are provided in appropriate locations for the same purpose.

The rubber bag is 12 cm wide which is enough to form a pressure cone that reaches the underlying artery even in a thick arm. As a general rule, the width of the bag should be 20% more than the diameter of the arm, though it should be wider in an obese person. The recommended width of the bag in different age groups is as under:

Infants (below 1 year)	:	2.5 cm
Below 4 years	:	5 cm
Below 8 years	:	8 cm
Adults	:	12 cm

d. Air pump (rubber bulb). It is an oval-shaped rubber bulb of a size that conveniently fits into one's fist. It has a one-way valve at its free end, and a leak-valve with a knurled screw, at the other where the rubber tube leading to the cuff is attached. The cuff can be inflated by turning the leak valve screw clockwise, and alternately compressing and releasing the bulb. Deflation of the bag is achieved by turning this screw anti-clockwise.

Aneroid manometer. In this manometer, in which metal bellows, mechanical links, and a calibrated dial replace the mercury manometer, is also in common use. However, it should be calibrated against a mercury manometer from time to time.

PROCEDURES

The subject may be lying down (supine) or sitting, but should be mentally and physically relaxed and free from excitation and anticipation.

Lay the arm bare up to the shoulder and record the blood pressure first with the palpatory method, followed by auscultatory method. The upper arm on which the BP cuff is to be tied must be at the level of the heart. (In the supine position, the arm resting on the bed will be nearly at the heart level. In the sitting position the arm resting on the table of a suitable height will be at the correct level).

In obese subjects, the cuff may be applied on the forearm with the stethoscope placed over the radial artery for auscultatory method. (If no sounds are heard a reasonably reliable determination can be obtained by palpation at the wrist).

:. Auscultatory Method (Korotkoff, 1905)

1. Place the cuff over the upper arm as described above, and record the BP by the palpatory method.
2. Locate the bifurcation of brachial artery (it divides into radial and ulnar branches) in the cubital space just medial to the tendon of the biceps which can be easily palpated in a semiflexed elbow as a thick, hard, elongated structure. Mark the point of arterial pulsation with a sketch pen.
3. Place the chest-piece of the stethoscope on this point and keep it in position with your fingers and thumb of the left hand (if you are right-handed).

> **Note**
> The chest-piece should not rub against the cuff, rubber tubes, or the skin in this area because these disturbing noises will interfere with auscultation of sounds.

4. Inflate the cuff rapidly, by compressing and releasing the air pump alternately (sounds may be heard as the mercury column goes up). Raise the pressure to 40 to 50 mm Hg above the systolic level as determined by the palpatory method.
5. Lower the pressure gradually until a clear, sharp, tapping sound is heard. Continue to lower the pressure and try to note a change in the character of the sounds.

These sounds are called Korotkoff sounds and show the following phases:

Phase I This phase starts with a clear, sharp tap when a jet of blood is able to cross the previously obstructed artery. (Sometimes this phase may start with a faint tap, especially when the systolic pressure is very high). As the pressure is lowered, the sounds continue as sharp and clear taps. This phase lasts for 10–12 mm Hg fall in pressure (**Figure 2-14**).

> **Note**
> **Criterion of systolic pressure** The level at which the first sound (clear, sharp, or faint) is heard, is taken as the systolic pressure.

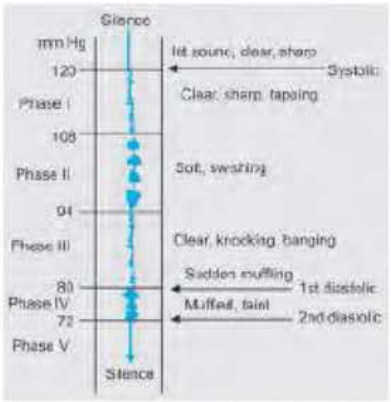


Figure 2-14: Phases of Korotkoff sounds, showing the changes in their character during each phase as the mercury column is gradually lowered. Systolic pressure: 1st appearance of sounds. Diastolic pressure: sudden muffling of sounds

Phase II The sounds become murmurish and remain so during the next 10–15 mm Hg fall in pressure when they again become clear and banging.

Phase III It starts with clear, knocking, or banging sounds that continue for the next 12 to 14 mm Hg pressure, when they suddenly become muffled.

Phase IV The transition from phase III to phase IV is usually very sudden. The sounds remain muffled, dull, faint and indistinct (as if coming from a distance) until they disappear. The muffling of sounds and their disappearance occurs nearly at the same time, there being a difference of 4–5 mm Hg (i.e. phase IV lasts for 4–5 mm Hg).

Important
Note the reading at muffling and another at disappearance of sounds, after which deflate the cuff quickly.

Phase V This phase begins when the Korotkoff sounds disappear completely. If you reduce the pressure slowly, you will note that total silence continues right up to the zero level.

6. Take 3 readings with the auscultatory method and repeat 3 readings on the other arm.

7. Effects of posture, gravity, and muscular exercise on blood pressure are discussed in the next experiment.

Table 2-1: Record of systemic arterial blood pressure

A. PALPATORY METHOD (mm Hg)				
		Right arm		**Left arm**
1st reading				
2nd reading				
3rd reading				
Maximum value = ... mm Hg **Heart rate =** ..... mm Hg /min				
B. OSCILLATORY METHOD				
C. AUSCULTATORY METHOD (mm Hg)				
	Systolic pressure	*Diastolic pressure*	*Mean arterial pressure*	*Pulse pressure*
Right arm				
1st reading	.....	.....	.....	.....
2nd reading	.....	.....	.....	.....
3rd reading	.....	.....	.....	.....
Left arm				
1st reading	.....	.....	.....	.....
2nd reading	.....	.....	.....	.....
3rd reading	.....	.....	.....	.....
Maximum values:				
Right arm =				
Left arm =				
Digital BP Monitor				