AmbujaNeotia

THE NEOTIA UNIVERSITY

ज्ञानम् आत्म प्रदीपाय

BML- 292: BASICS OF HEMATOLOGY LAB

LAB MANUAL

DEPARTMENT OF

MEDICAL LABORATORY TECHNOLOGY

SCHOOL OF HEALTH SCIENCES

COMPILED BY

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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

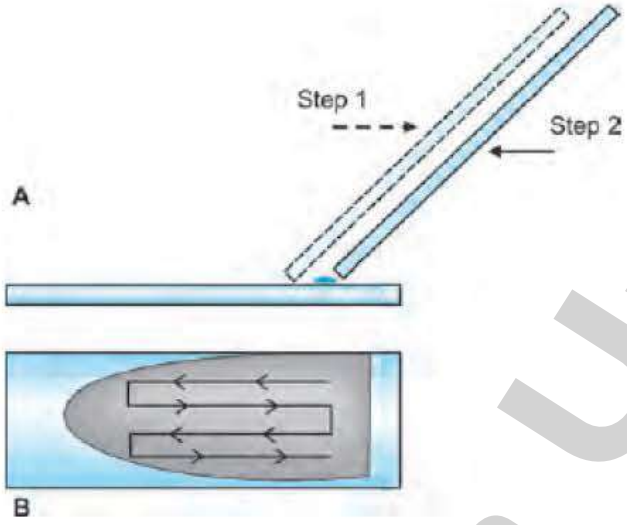

Figure 1-9: (A) Method of spreading a blood film. Step 1: The spreader is placed in front of the blood drop and pulled back till it touches the blood. Step 2: Spreader is pushed forwards to spread the film. (B) The appearance of a well-prepared film, showing the movement of the objective over it

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

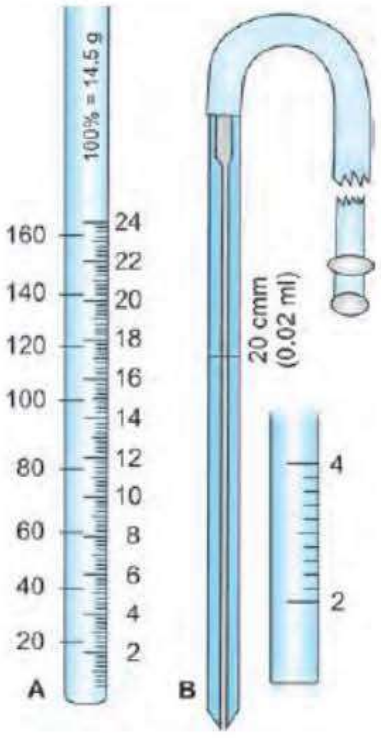

Figure 1-10: (A) Sahli-Adam's hemoglobin tube: It has graduations in g% on one side, and in percentage on the other. In this tube, 100% is equal to 14.5 g Hb per 100 ml blood. (B) Hemoglobin pipette: It has only one marking, indicating 20 cmm (0.02 ml, or 20 µl). There is no bulb (Inset: Each division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

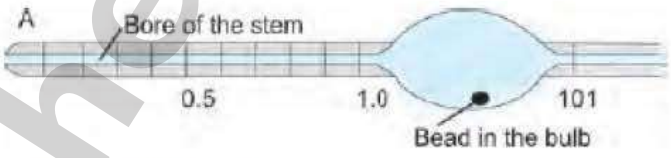


Figure 1-11: The RBC pipette. It has 3 markings—0.5, 1.0, and 101

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

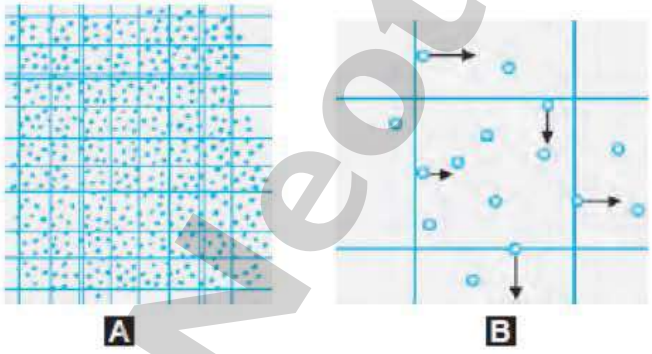
are moving and changing their positions due to currents in the fluid. During this time draw a diagram once again showing the RBC square. Then draw 5 groups of 16 squares each, showing their relative positions—the 4 corner groups and one central group for entering your counts.

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

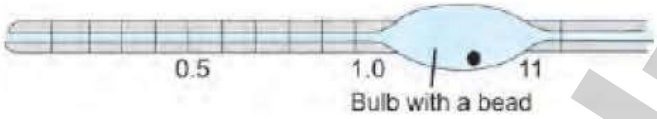


Figure 1-14: The WBC pipette. It has 3 markings—0.5, 1.0, and 11

   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.

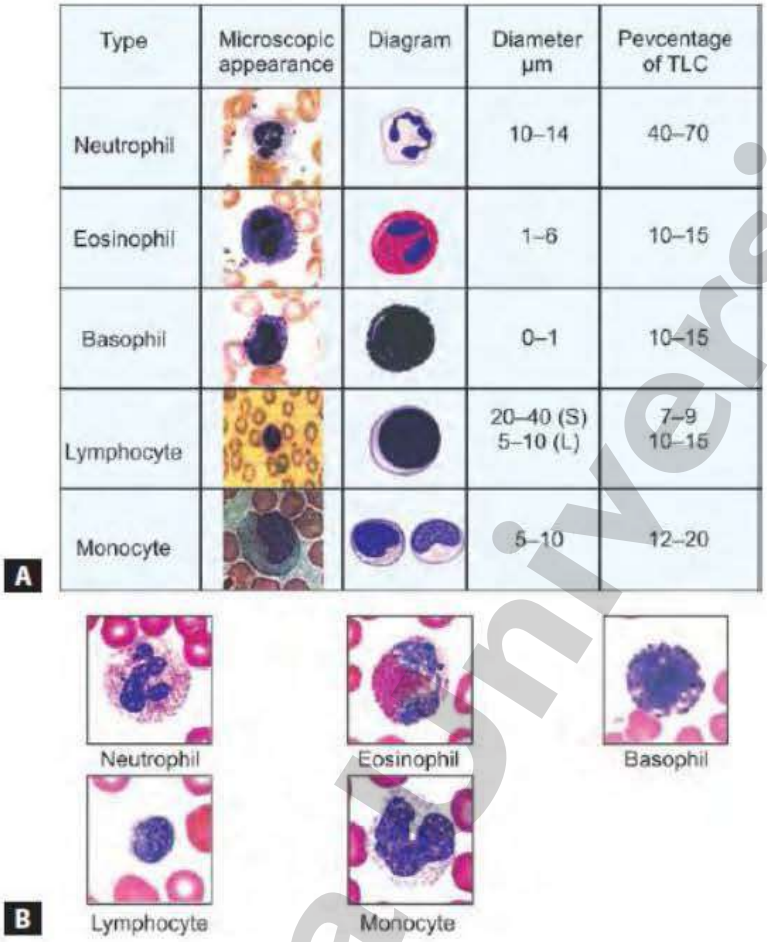
b. Identifying a Leukocyte. A leukocyte is identified from its size, its nucleus, and the cytoplasm—its color, whether vesicles (granules) are visible or not, their color and size if visible, and the cytoplasm/nucleus ratio **(Figure 1-15)**.

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

Absolute Eosinophil Count

Counting Methods

The absolute count of eosinophils can be done by two methods:

1. ***Direct method.*** The cells are counted directly by employing hemocytometry.
2. ***Indirect method.*** As mentioned in Expt 1-12 (DLC), the percentage of eosinophils is determined from a blood smear counting of leukocytes. If TLC is done simultaneously, the absolute count can be calculated.

PRINCIPLE

Blood is diluted 10 times in a WBC pipette using Pilot's diluting fluid that lyses RBCs and leukocytes other than eosinophils. The stained cells are then counted in a counting chamber.

APPARATUS AND MATERIALS

1. Microscope •Counting chamber •WBC pipette •Coverslips •Equipment for finger prick.
2. ***Pilot's diluting fluid*** for eosinophil counting:
   Stock solutions:
   a. Propylene glycol
   b. Phloxine: 1 % solution in water (0.5% eosin may be used but phloxine is superior).
   c. Sodium carbonate: 10 % solution in water.

Working solution: It is made by mixing and filtering the following:

Propylene glycol	= 50 ml
Phloxine (1 %)	= 10 ml
Sodium carbonate (10 %)	= 1 ml
Heparin	= 100 units
Distilled water	= 40 ml

PROCEDURES

1. Get a finger prick under asepsis, discard the first 2 drops, and then fill 2 WBC pipettes with blood to the mark 1.0 (EDTA anticoagulated blood can be used).
2. Suck the diluting fluid to the mark 11 in both pipettes. Mix the contents of each for 2 minutes.

3. Place the pipettes on 2–3 layers of moistened filter papers and cover them with a petri dish. Allow to stand for 15 minutes for proper lysis and staining. (The purpose of moist filter papers is to prevent evaporation of water from the pipettes).
4. Take out the pipettes and mix the contents once again for 30 seconds. Discard the fluid in the stems and charge each side of the chamber from each pipette and bring the chamber into focus in the usual manner.
5. Using HP objective, count the eosinophils in the 4 corner groups of 16 squares each, i.e. in a total of 64 squares that were used for TLC. When the counting has been done, calculate the number of cells in 1 mm^3 of undiluted blood. Enter your observations in appropriate squares drawn in your notebooks.

OBSERVATIONS AND RESULTS

Calculations

Volume of 64 squares
(each = 1/160 mm^3)
= 1/160 × 64 = 4/10 mm^3

Let the eosinophil count in 4/10 mm^3
(i.e. 64 squares) be = x

Then, 1 mm^3 of diluted blood will contain
= x × 10/4 cells

Since the dilution is 10 times, 1 mm^3
of undiluted blood will contain
= x × 10/4 × 10
= x × 25 cells.

Compare the count from the two pipettes. The difference should not be more than 10%.

Normal absolute eosinophil count = 10–400/mm^3 (Eosinophil count of capillary blood is usually 10–15% higher).

Indirect Method of Counting Eosinophil

This method was mentioned in Expt 1-11. For this both TLC and DLC are required. For example, if the TLC is 8000/mm^3 and eosinophils are 2% in DLC, then the absolute count would be = 2/100 × 8000 = 160/mm^3 of undiluted blood. This method can act as a check on the result of direct method.

Reticulocyte Count

PRINCIPLE

A mixture of blood and a dye (stain) is spread in the form of a thin smear on a glass slide and suitably counterstained to bring out their reticulum. They are then counted per 1000 red cells and their percentage calculated.

APPARATUS AND MATERIALS

1. Microscope. • Glass slides. • Equipment for finger prick. • Petri dish. • Blotting paper.
2. Reticulocyte stains (supravital stains). These stains are used for staining unfixed, "living" cells and tissues *in vitro* (outside the body).
   - i. ***Brilliant cresyl blue.*** 1.0 g of this dye dissolved in 100 ml of citrated saline (1.0 volume of 3.8% sodium citrate and 4 volumes of normal saline). The dye stains the RNA of reticulocytes, citrate prevents clotting of blood, and normal saline provides tonicity (1.0% solution of the dye in methyl alcohol can also be used).
   - ii. ***New methylene blue.*** While methylene blue does not stain the reticulum, new methylene blue (which is chemically different) stains this material more deeply and uniformly. 1.0 g of the dye is dissolved in 100 ml of citrate saline.

Theory of Reticulocyte Staining

The basophilic remnants of RNA and ribosomes in the cytoplasm of reticulocytes cannot be stained by the basic dye methylene blue, which is a component of Leishman's stain. The material can only be stained with certain dyes such as brilliant cresyl blue. The dye enters the cells and stains the basophilic material to form bluish precipitates of dots, short strands, and filaments. This reaction can occur only in **supravitally (or vitally)** stained cells, i.e. in "unfixed" and "living" cells. The more the immature cells, greater is the amount of precipitable ribosomal material present in them.

PROCEDURES

1. Take 2–3 clean, grease-free glass slides and place a drop of reticulocyte stain in the center of each slide about 1 cm from its end.
2. Get a finger prick under aseptic precautions and add an equal-sized drop of blood to each drop of stain. Stir with a pin and put the slides on moist

filter paper and cover with a petri dish. Allow the mixture to remain on the slides for 1 minute. (The slides may be incubated at body temperature for 15 minutes to simulate the living conditions so that the stain may better penetrate the reticulocytes).

3. Spread a smear of the blood-dye mixture on each slide, then counterstain with Leishman's stain in the usual manner. (This will stain all cells).

OBSERVATIONS AND RESULTS

1. Using oil-immersion objective, bring the blood cells into focus and identify reticulocytes. They stain lighter than the red cells and also contain dots, strands, and filaments, etc. of bluish-stained material.

   Identification of Reticulocytes. These non-nucleated cells are slightly larger (diameter about 8 µm) than the red cells (average diameter = 7.5 µm). They also stain lighter than the red cells, and contain dots, strands, and filaments of bluish-stained material.
2. Count the reticulocytes in 100 alternate fields, i.e., move one uncounted field before counting them again. (In some fields you may not see any such cells.)
3. Count the red cells in every 5th field, for a total of 10 fields. (When counting red cells in a field, divide the field into 4 imaginary quadrants. This will make the counting easier).

Calculations

If, say, the number of reticulocytes in 100 fields is = 72
and the number of red cells in 10 fields is = 450
The number of red cells in 100 fields = 450 × 10 = 4500
Percentage of reticulocytes = 72/4500 × 100 = 1.6%

Normal Values

1. Newborns = 30–40%. Their number decreases to 1–2% during the first week of life.
2. Infants = 2–6 %
3. Children and adults
   a. 0.2–2.0% (average = 1%)
   b. Absolute count = 20,000–90,000/mm^3.

Absolute Reticulocyte Count

A direct reticulocyte counting by hemocytometry is not possible. An indirect absolute count can be obtained from the relative percentage by doing a total red cell count.

= Reticulocyte percent × red cell count/100

Normal value = 25,000 – 100,000 / mm^3

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

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

For example

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

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

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.

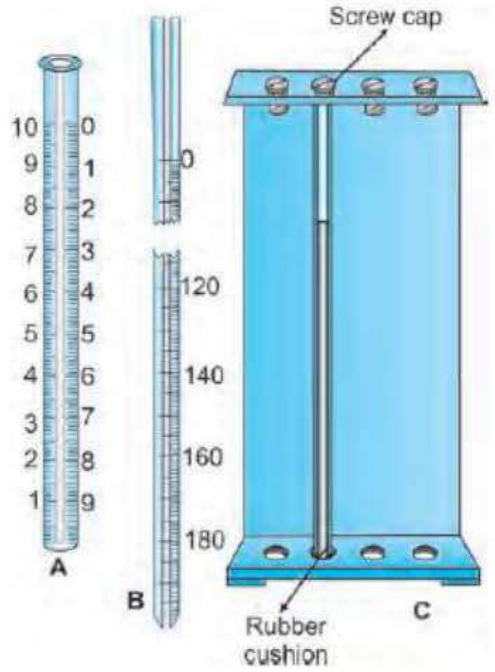


Figure 1-17: Determination of ESR. (A) Wintrobe tube; (B) Westergren tube; (C) Westergren stand with the Westergren tube in position

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.