TROPICAL COLLEGE OF MEDICINE

DEPARTMENT OF MEDICAL LABORATORY TECHNOLOGY

MODULE

ON

Performing Hematological Tests

MODULE CODE: HLT MLS4 M07 10 11

FOR

Medical Laboratory Services Level IV

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Preface

This Learning Module on Performing Haematological tests has been designed for level III Medical Laboratory Technicians based on the current curriculum.

The Learning Module comprises the following chapters: Introduction, Receive samples and process associated request forms, Performing Haematological tests, Overview of blood disorders.

This module aims to provide the learners with the knowledge, skills and right attitudes to exercise good laboratory practice and effective participation in Performing Haematological tests.

The Learning Module comprises of four Learning Outcomes. Assessment method and criteria, as well the list of References books are included in the module.

TABLE OF CONTENT

Module contents **Page**

LEARNING MODULE	Logo of TVET Provider

TVET-PROGRAMME TITLE: **Medical Laboratory Service Level IV**

MODULE TITLE: **perfoming hematological test**

MODULE CODE: **HLT MLS4 M07 10 11**

NOMINAL DURATION: **200 Hours**

MODULE DESCRIPTION: This module aims to provide the learners with the knowledge, skills and right attitudes to determine levels, function, activity and interactions of cellular and plasma components of blood using tests and procedures identified with the discipline of hematology laboratory.

LEARNING OUTCOMES

At the end of the module the learner will be able to:

2. Describe the function, production and interactions of blood components.
3. Perform hematological tests according to documented methodologist for diagnostic purpose.
4. Apply OHS and safe working practices to maintain own health, health of others & environment following OHS guidelines.
5. Exercise good laboratory practice with effective participation in quality improvement using pre-set procedures.

LEARNING METHODS

- Lecture
- Group discussion
- Demonstration
- Practical exercise
- Project work
- Co-operative training

MODULE ASSESSMENT

- **Assessment Methods**
- Practical assessment by direct observation of tasks
- Written exam/test on underpinning knowledge
- questioning or interview on underpinning knowledge
- project-related conditions (real or simulated) and require evidence of process

- **Assessment Criteria**
  - Able to perform hematological test
  - Able to follow OHS
  - Able to achieve passing mark in written exam

REFERENCE BOOKS

1. Robinson, Stephen H. and Paul Reich. Haematology: Patho physiologic bases for clinical practice. 3rd edition, 1983 Little Brown and co.
2. A.V. Hoffbrand and J. E. Petties .Essential Haematology, 2nd edition 1980
3. GeyA.Mc Donald et al .Atlas of Haematology, 4th edition 1978
4. Arther and Simons .Technical Haematology .3rd edition 1980
5. Richard A. Rifkind Fundamentals of Haematology, 1986
6. Williams, Beutler, Erslev, Lichtman .Haematology. 3rd ed.1983
7. Seiverd. Haematology for medical technologists. 5th ed.1983
8. Sahile Mariam Z. Hematology for Medical Laboratory Technology students Lecture note series ,Jimma University.

CHAPTER ONE

INTRODUCTION TO HAEMATOLOGY

The word haematology comes from Greek, ‘’hemat’’ meaning blood, ‘’ology’’ meaning study. Thus; **Haematology** is the study of blood.

Include in its concerns are analysis of:

- The concentration
    - structure (morphology)
    - function of blood cells
- precursors of blood cells in the bone marrow
- Chemical constituents of plasma, serum and whole blood intimately linked with blood cell structure and function.
- Function of platelets and proteins involved in coagulation

Changes in one or more of these characteristics may produce haematologic disease or may be haematologic manifestations of other disease processes.

History of hematology dates back to primitive man.
Loss of large amount of blood was associated with death.
Excess blood, plethora, was also considered as the cause of all ills by the ancient Greeks
In support of this belief they introduced the practice of bloodletting supported by starvation to induce anemia.
Introduction of the microscope in the 17th century by Antoni van Leeuwenhoek and others:

- allowed the study of circulating blood cells, and interest arose in their possible function
- Theory and dogma were replaced by scientific understanding

In the 19th century, studies were made on the causes of anemia and its deleterious effects on the patient; and haematology becomes one of the fields of medical science.

1. COMPOSITION, FORMATION AND FUNCTION OF BLOOD:

- Blood is a tissue fluid that baths in the vascular system accounts about 7 – 8% of the body weight.
- Blood is a complex liquid tissue composed of a pale yellow fluid called **plasma** in which are suspended **formed elements** comprising red cells (erythrocytes), white cells (leukocytes), and platelets (thrombocytes). These formed elements constitute about 45% of the blood; the remaining portion which accounts 55% is called Blood Plasma.
- Plasma contains water (95%) while the remaining parts are different nutrients like glucose, proteins (fibrinogen, albumin, globulin), amino acids, vitamins like vitamin B6, B12, K, minerals like Iron, Copper, Magnesium, Potassium, Sodium, Hormones (Erythropoiten), salts, enzymes, products of digestion, and waste products for excretion.
- The total blood volume in an adult is 5 – 6L or 7 – 8% of the body weight.

When you centrifuge whole blood or called anticoagulated blood, it will form 3 layers:

1. The packed one called red blood cells which is the largest portion
2. In between the red blood cells and the plasma layer there is Buffy coat which includes white blood cells and platelets; it is the smallest in composition
3. The supernatant portion called Plasma

1.2. FUNCTION OF BLOOD

Blood has important transport, distribution, regulatory, and protective functions in the body.

Transportation and distribution:

- Blood transport oxygen from the lungs to the cells of the body and carbon dioxide from the cells of the lungs.
- Nutrients absorbed from the digestive tract, e.g. monosaccharides (especially glucose), amino acids, fatty acids, glycerol, and vitamins, are transported to the cells of the body for use or storage.
- Waste products of metabolism are transported from the tissues to site of excretion, e.g. carbon dioxide produced from cellular activity is carried to the lungs for excretion, and the waste products of protein metabolism (urea, creatinine, uric acid) are transported to the kidneys for excretion.
- Hormones are carried from endocrine glands to the organs where they are needed.

Regulatory:

- Buffer systems in the plasma maintain the pH of the blood between pH 7.35–7.45 and the pH in body tissues within the physiological limits required for normal cellular activity
- Regulate the amount of water & electrolytes in the body fluids
- Blood assists in regulating the temperature of the body by absorbing and distributing heat throughout the body and to the skin surface where heat which is not required is dissipated.

Protective:

- When a blood vessel is damaged, platelets and blood coagulation factors interact to control blood loss. Platelets adhere to the damaged tissue and to one another and activated coagulation factors lead to the formation of fibrin and a thrombus clot which reinforce the platelet plug
- Leukocytes are involved in the body's immune defenses, producing antibodies in response to infection, and protecting the body from damage by viruses, bacteria, parasites, toxins and tumor cells.

FORMED ELEMENTS OF THE BLOOD:

The formed elements of blood are generally classified into three parts, namely Red blood cells, White blood cells and Platelets. The composition of each is constant for healthy individuals unless there are pathological situations.

In general there are **500 Red cells** for **30 Platelets** and **1 White blood cell**.

A. Red blood cells (erythrocytes)

- Are the most numerous cells in the blood which form the main cellular component of blood, i.e. about 45% of total blood volume in an adult, giving blood its red color.
- Each litre of blood contains about 5×10^{12} red cells, the exact number varying with age, gender, and state of health.

- Red blood cells are non nucleated cells shaped like biconcave disks size approximately 7 to 8μm in diameter with a thickness of 1.7-2.4μm.
- The **biconcave disk shape** gives red blood cells (RBCs) the flexibility to squeeze their way through capillaries and other small blood vessels.
- Its shape also provides a large surface area for the exchange of respiratory gases.
- A large proportion of their cytoplasm consists of the iron containing oxygen transport molecule called **hemoglobin.**
- In stained smears, RBCs appeared as circular (spherical) with a central hole, or **central pallor area** (free from hemoglobin), which is approximately one-third the diameter of the cell; while the peripheral area is fully hemoglobinated unless there is anemic condition.
- The main function of RBCs is gas exchange (cellular respiration):
  - carry **oxygen** from the lungs to the tissues
  - return **carbon dioxide (CO_2)**, a waste product of metabolism, from the tissues to the lungs to be exhaled
- Red blood cells are formed and matured in the bone marrow.
- The life span of an erythrocyte is only about 120 days. After finishing its life span, it is removed by the phagocytic cells of the reticuloendothelial system, broken down and some of its constituents (iron and amino acids) are reutilized for formation of new cells.

B. White Blood Cells (Leukocytes):

- Leukocytes are :
  - a heterogeneous group of nucleated cells
  - responsible for the body's defenses and are transported by the blood to the various tissues where they exert their physiologic role, e.g. phagocytosis.
- The normal WBC count is ~4,000 to 10,000/μL ($4.0–10.0 \times 10^9$ /L)
- There site of production (formation) is in the bone marrow but developed and differentiated in the lymphoid organs like Thymus, Lymph nodes, and Spleen.

- Leukocytes are usually divided into:

☞ Granulocytes:

- have specific granules (have a number of lobes and nucleus).
- Also called **Polymorpholeukocytes**
  - Neutrophils
  - Eosinophils
  - Basophils

☞ Agranulocytes:

- lack specific granules (have one nucleus and do not form a lobe/branch).
- Also called **Mononuclear leukocytes**
  - Lymphocytes
  - Monocytes

A. Neutrophils (55 – 75%)

- are the most common type of WBCs in adults
- The segmented neutrophils "segs," also called polymorphonuclear neutrophil leukocytes [PMNs or "polys"]
- are the primary defense against bacterial infection
- Their size ranges from 10-12μm in diameter.
- They are capable of amoeboid movement.
- There are 2-5 lobes to their nucleus that stain purple violet.
- The cytoplasm stains light pink with pinkish dust like granules.
- Normal range: 2.0-7.5 x 10^3/μl.
  Increased in acute bacterial infections.

Function

- Destroys microorganisms and other foreign particles by phagocytosis.

B. Eosinophils (1 – 3%)

- Slightly larger than a neutrophil, measuring 12–14 μm in diameter.
- Usually contains a bi-lobed nucleus in a typical "spectacle arrangement"
- The nucleus of most eosinophils has two lobes (occasionally three).
- The cytoplasm contains many large round orange-red granules and occasionally vacuoles.
- The underlying cytoplasm, which is usually obscured by the granules, is pale blue
- Eosinophils are important in allergic reaction and parasitic helminth immune responses in which IgG and IgE antibodies are produced.
- Normal range: 40-400/μl.

C. Basophils (0 – 1%)

- Are the rarest (<1%) of the circulating leucocytes.
- Measures 10–12 μm in diameter.
- Have a kidney shaped nucleus often obscured by a mass of large deep purple/dark blue staining (basophilic) granules.
- The granules contain:
  - heparin (an anticoagulant),
  - histamine (a fast vasodilator),
  - the slow-reacting substance of anaphylaxis (a slow vasodilator), and other compounds
- They are rich in histamine, serotonin, and heparin substances and Involved in allergic reaction & inflammation
- involved in immediate hypersensitivity reactions related to immunoglobulin class E (IgE)
- Normal range: 20-200/μl.

D. Monocytes(2 – 6%)

- Largest of the circulating white cells, measuring 15–20μm in diameter, often with an irregular shape.
- After 8 to 14 hours in the blood, they enter tissue to become tissue macrophages (also called histiocytes)
- Cytoplasm:
  - abundant staining light gray to light blue
  - finely granular
- Nucleus has very finely granular chromatin and is often folded, bean shaped, oval, or irregular
- Monocytes have two functions:
  - Phagocytosis of microorganisms (particularly fungi and mycobacteria) and cellular debris including malarial pigment
  - Antigen processing and presentation. In this role, they are critical in initiation of immune reactions
- Normal range: 700-1500/μl.
- Monocytosis is seen in bacterial infections (e.g., tuberculosis) and protozoan infections.

E. Lymphocytes (20 – 35%)

- are the second most common type of leukocytes in adults (~20–35% of WBC)
- The average number of lymphocytes in the peripheral blood is 2500/μl.
- The lymphocyte number is higher in children and also increases with viral infections
- Morphologically, lymphocytes appear as small lymphocytes and large lymphocytes.

Small Lymphocytes/Resting lymphocytes:

- are usually small (7-10μm in diameter)
- has a dark round to oval nucleus, and
- only a rim of pale blue staining cytoplasm
- nucleus is about the same diameter as a normal erythrocyte & occupies most of the cell
- are the predominant forms found in the blood.

Large Lymphocyte:

- A small number of lymphocytes in the blood
- Slightly larger than resting lymphocytes, with reddish purple (azurophilic) granules. This appearance generally corresponds to **natural killer (NK) cells**
- Size: 12-14μm in diameter
- Nucleus:
  - a little paler than small lymphocytes
  - is usually eccentrically placed in the cell
- Cytoplasm:
  - Is more plentiful
  - stains pale blue and may contain a few reddish (azurophilic) granules.
- Lymphocytes are involved in immune response (intracellular infection) and synthesis of antibodies

C. THE PLATELETS (Thrombocytes):

- are small, non nucleated (anucleated), round/oval cells/cell fragments
- Their size ranges 1-4μm in diameter
- They are produced in the bone marrow by simple fragmentation from Megakaryocytes, hence they may not be considered as true cells like red cells and white blood cells.
- Megakaryocytes are multinuclear and giant cells that are precursor cells for platelets.
- Their primary function is preventing blood loss from hemorrhage by forming a **platelet plug**
- Platelets have a life span of approximately 10 days.
- Senescent platelets are removed by the spleen
- Normal range: 150-400 x 10^3 /μl

Characteristics of Blood:

1. **Temperature**
   - Roughly 38°C (100.4 °F)
2. **Viscosity**
   - Five times that of H_2O due to interactions among dissolved proteins, formed elements, & surrounding H_2O molecules
   - Sticky, cohesive, and resistant to flow
3. **pH**
   - Ranges from 7.35- 7.45, averaging 7.4
4. **Volume**
   a. 5-6 liters in adult male
   b. 4-5 liters in adult female
   c. differences between genders reflect differences in body size
   d. Blood volume (BV) can be estimated by calculating 7% of the body wt in Kg
      E.g. 75 Kg individual would have a BV of approximately 5.25 liters (~1.4 gallons)
      i. Hypovolemic = below normal
      ii. Normovolemic = normal
      iii. Hypervolemic = above normal

   Abnormally high BV can place severe stress on the heart.

1.3 HAEMATOPOIESIS

- The process by which blood cells are formed is called haematopoiesis, or haemopoiesis.
- It refers to the formation and development (maturation) of all types of blood cells from their parental precursors.

There have been two theories about the origin of blood cells:

Monophyletic theory – all blood cells originate from a single mother cell (Pluripotent stem cells)

Polyphyletic theory – several mother cells give rise to the different cell lineages

- Monophyletic theory is accepted by many hematologists.

The Haemopoitic Tissues:

- In the embryo, blood cell production begins in the **yolk sack**.
- yolk sack probably drived from embryonic connective tissue mesenchymal cells
- These primitive mesenchymal cells of the embryo differentiated to produce hemocytoblasts.
- The primitive mesoblastic erythropoitic activity is recognized 14th day of development lasts for approximately 10 weeks.
- In the third months of embryonic development this mesoblstic stage fades & liver becomes the main focus of blood production.
- The liver activity (hepatic phase) begins between 6th & 9th weeks of embryonic life, the chief site of red cell and granulocyte production, and it is added by spleen, thymus and lymph-nodes.
- By the 4th month of embryonic life the bone marrow starts to play an increased role in the production of granulocyte and erythroid cells.
- At birth this organ is the principal source of both granulocyte, and erythrode cells as well as thrombocytes.
- B – Lymphocytes continue to be produced in marrow as well as lymphnodes.
- T – Lymphocytes produced in the thymus and secondary lymphoid organs.
- At birth particularly the whole bone skeleton contains active marrow (hematopoitic active marrow)
- By 18 years of age and for the remainder of the adult life, the red marrow is normally confined to the flat bones only (skull, clavicle, sternum, ribs, vertebrae, pelvis, & the proximal ends of the long bones (femurous & humerus).
- The remaining marrow space is occupied by fat cells which can be replaced by hematopoietic cells under certain situations of intensive stimulation.

Medullary hemopoiesis: Blood cell production within the bone marrow (medulla)**.**

Extramedulary hemopoiesis:

- Formation of apparently normal blood cells outside the confines of the bone marrow mainly in the **liver** and **spleen** in post fetal life is known as Extramedullary Hemopoiesis.
- Occurs when the bone marrow becomes dysfunctional e.g., aplastic anemia, infiltration by malignant cells, or over proliferation of a certain cell (e.g. leukemia)
- When the bone marrow is unable to meet increased demand for cells, e.g., hemolytic anemia

- If extramedulary hemopoiesis develops, the liver and spleen are enlarged (hepatospleenomegaly).

The Hematopoietic microenvironment:

The Haematopoietic microenvironment comprises stromal cells, fat cells, fibroblasts, macrophages and epithelial cells. Fibronectin, collagen and laminin are also present that support for cell proliferation. This is mainly occur in the bone marrow. In fact cell activation, differentiation and maturation happens not only in the bone marrow but also in Secondary Lymphoid Organs like Lymphnodes, Mucosal associated lymphoid organs and Gut associated lymphoid organs.

- The common ancestral cell of all mature blood cells in humans is the **Pluropotent hematopoietic stem cells (HSCs).**
- These stem cells are the first in a sequence of regular and orderly steps of cell growth and maturation.
- As stem cells, they are defined by their ability to form multiple cell types (multipotency) & their ability to self renew.
- HSCs can differentiate to form either a :
  I. Lymphoid stem cell (CFU - L, colony forming unit lymphoid)
  II. A non lymphoid stem cell(CFU - GEMM, colony forming unit granulocyte, erythroid, macrophage, megakaryocyte)
- Multipotential (pluripotential) stem cells are capable of differentiating in to a number of different unipotential stem cells. These are committed to differentiation along a single cell line.

Eg. BFU – E (Brust forming unit erythroid) can only differentiate in to mature red cells.

- In general the development of matured blood cells from multipotential takes place in three phases.
  I. Differentiation phase
  II. Proliferation phase
  III. Maturation phase

ERYTROPOIESIS:

Erytropoiesis is the formation of erythrocytes from committed progenitor cells through a process of mitotic growth & maturation.

The first recognizable erythroid cell in the bone marrow is the pronormoblast.
Red cells are produced in the bone marrow. Tissue hypoxia (lack of oxygen) leads to the release of the hormone erythropoietin which stimulates progenitor cells to develop into pronormoblasts (proerythroblasts).

BFU-E
↓
CFU-E
↓
Pronormoblast
↓
Basophilic normoblast
↓
Polychromatic normoblast
↓
Orthochromatic normoblast
↓
Reticulocyte
↓
Erythrocyte

A. PRONORMOBLAST (RUBRIBLAST)

- Measures 20 – 25µm in diameter
- Has large to oval nucleus & contains 0-2 light bluish, indistinct nucleoli.
- Has a narrow rim of dark blue cytoplasm

B. BASOPHILIC NORMOBLAST (PRORBRICYTE)

- Measures 16 – 18µm in diameter
- Has slightly wider ring of deep blue cytoplasm than in the pronormoblast
- The nucleus is round or oval & smaller than in the previous stage

C. POLYCHROMATIC NORMOBLST (RUBRICYTE)

- Measures 12 – 14µm in diameter
- Has blue gray to pink gray cytoplasm (shows a large range in color) due to the start of Hgb production in the cell
- The cytoplasm slightly increases in relative amount
- The nucleus is round, smaller & more condensed than the previous stage
- The chromatin pattern is more coarse & clumped stains a deeper blue-purple.

D. ORTHOCHROMATIC NORMOBLAST (METARUBRICYTE)

- Measures 10 – 12µm in diameter
- The cytoplasm is pinker & increased in amount than previous stage
- Has a pyknotic nucleus (a homogenous blue black mass with no structure). This is the primary difference between the rubricyte & metarubricyte.

E. RETICULOCYTE

- Measures 8 – 10µm in diameter
- Have no nucleus and the cytoplasm stain pink to slight pinkish gray.

- They contain large amounts of Hgb and only remnants of ribosomal RNA, which only stains with supravital stain.
- Reticulocytes enter the peripheral blood circulation & within 48hours develop into fully haemoglobinized pink-red staining erythrocytes.

F. MATURE ERYTHROCYTES

- Measures 7 – 8µm in diameter
- Are biconcave in shape with orange-pink cytoplasm with a pale staining center occupying one-third of the cell area.
- They are mainly composed of Hgb surrounded by a flexible protein membrane and outer lipid bilayer.
- The biconcave form of the red cell and membrane which is made of specialized deformable protein fibres, enable the cell to pass through capillaries of small diameter.
- Its shape also provides a large surface area for the exchange of respiratory gases.

REGULATION OF ERYTHROPOIESIS:

- Maintenance of the circulating red cell mass within the narrow limits seen in health is achieved by a feedback mechanism, which senses body oxygen demands & adjusts the rate of erythropoiesis accordingly.
- The feedback mechanism is mediated by the glycoprotein hormone erythropoietin (secrated mainly 90% by kidney cells and rarely in liver and lung) as follows:
    - A fall in the circulating red cell mass leads to decreased delivery of oxygen to the tissues & hypoxia develops.
    - Tissue hypoxia is sensed by an enzyme linked mechanism in the kidney & synthesis of erythropoietin (EPO) by the peritubular endothelial cells of the kidney is stimulated.
    - EPO binds to specific receptors on BFU – E & CFU – E in the bone marrow, resulting in a shortening of cell cycle time, an increased rate of formation & release of red cells from the bone marrow.
    - The increased red cell count improves oxygen delivery to the tissues, the hypoxia is corrected & EPO synthesis is switched off.

Whenever there is:

- Low Red Blood Cells/haemoglobin in the peripherial circulation
- Hemoglobinopathy (a defect in haemoglobin function)
- Poor blood flow in the circulation
- Altitude of the place (high altitude may result in low oxygen tension)

All the above factors may favor tissue hypoxia that is recognized by the kidney cells, which in turn produce erythropoiten. The secreted erythropoiten hormone will sensitize the bone marrow for rapid production of Red Blood Cells.

The actions of erythropoiten in the bone marrow include:

- Rapid division and differentiation of stem cells
- It facilitates the assembly of heme part with globulin part in the stem cells
- It will shorten the time required for cell maturation

- It also facilitate urgent entrance of juvenile red cells to the circulation

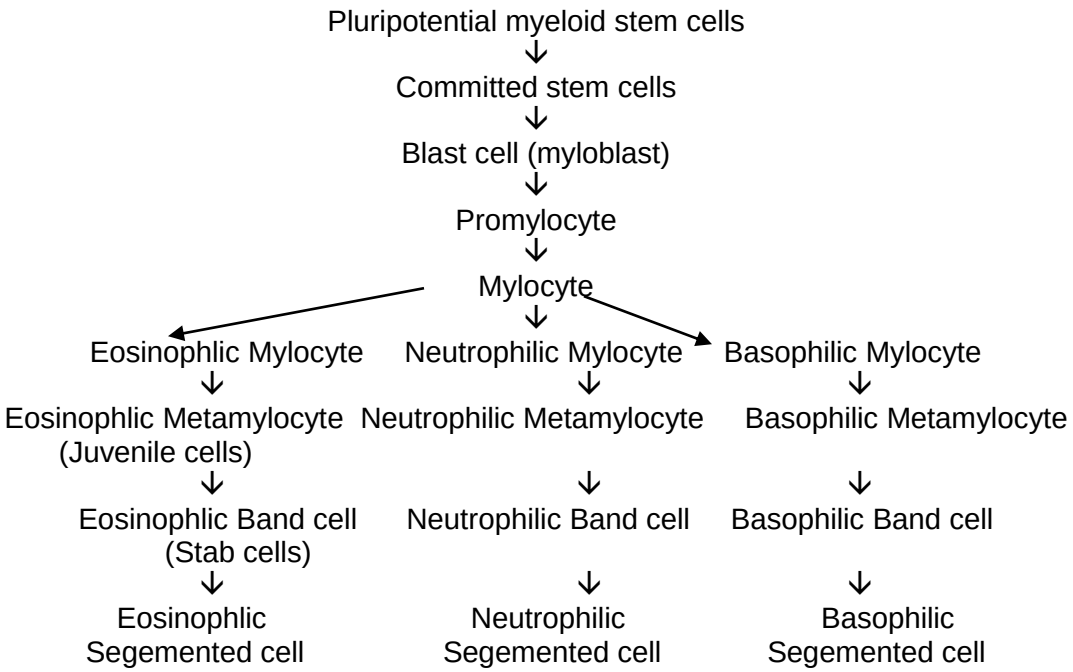
Ineffective erythropoiesis/Intramedullary hemolysis:

Erythropoiesis is not entirely efficient since 10-15% of eryhtropoiesis in a normal bone marrow is ineffective, i.e., the developing erythroblasts die within the marrow without producing mature cells. Together with their hemoglobin, they are ingested by macrophages. This Hematology process is substantially increased in a number of anemias.

Megaloblastic Erythropoiesis:

Megaloblasts are pathologic cells that are not present in the normal adult bone marrow, their appearance being caused by a deficiency in vitamin B_{12} or folic acid or both leading to defective DNA synthesis. In megaloblastic erythropoiesis, the nucleus and cytoplasm do not mature at the same rate so that nuclear maturation lags behind cytoplasmic hemoglobinization. This nuclear lag appears to be caused by interference with DNA synthesis while RNA and protein synthesis continue at a normal rate. The end stage of megaloblastic maturation is the megalocyte which is abnormally large in size (9-12µm in diameter).

LEUKOCYTE FORMATION & DEVELOPMENT:



CFU-GM → CFU-M → Monoblast → Promonocyte → Monocyte

PSC→Lymphoid progenitor→Lymphoblast→Prolymphocyte → B cells → B lymphocytes
T cells→ T lymphocyte

Hemopoietic stemcell→Megakaryoblast→Promegakaryocyte→Megakaryocyte→Platelets

CHAPTER TWO

RECEIVE SAMPLES AND PROCESS ASSOCIATED REQUEST FORMS

Blood must be collected with care and adequate safety precautions to ensure:

- test results are reliable
- contamination of the test sample is avoided and
- infection from the blood transmissible pathogens is prevented.

Proper collection and reliable processing of blood specimens is a vital part of the laboratory diagnostic process in hematology as well as other laboratory disciplines.

ANTISEPTICS: are disinfectants that are applied on human bodies or other animals used to clean/ make the site sterile so that microorganisms cannot enteed to the specimen as well as to the patient. These include:

70% Alcohol (denatured alcohol):

- most common antiseptic
- sometimes might cause allergy for some patients

0.5 – 2% Iodine:

- is a good antiseptic and it is sporocidal (destroy spore forming microorganisms) but it will stain the skin (leave a mark on the skin).

Diluted savlon:

- can serve as a good antiseptic if there is shortage or lack of alcohol solution.

TYPES OF BLOOD SAMPLE FOR HEMATOLOGY LABORATORY:

- Capillary blood
- Venous blood
- Arterial blood – used for blood gas analysis

Capillary Blood:

- Also referred to as microblood samples collection/dermal puncture
- Is collecting blood after an incision is made to the skin with a lancet
- The amount of blood obtained is small; not more than 100-200µl
- Preferred for tests like total WBC count, Blood Film, RBC count, Differential WBC count, Platelet count, Haemoglobin determination.
- also used when venipuncture is impractical, e.g.,
    - In infants
    - In cases of severe burns
    - In extreme obesity where locating the veins could be a problem
    - In patient whose arm veins are being used for intravenous medication

- **Sites of Puncture**
  - **Adults and children**: Palmer surface of the tip of the ring or middle finger or free margin of the ear lobe
  - **Infants:** plantar surface of the big toe or the heel.
- **Advantages of Capillary Blood**
  - Easy to collect
  - The preferred specimen for making peripheral blood films since no anticoagulant is added that may affect cell morphology.
- **Disadvantages of Capillary Blood**
  - Only small amounts of blood can be obtained and repeated examinations require a new specimen.
  - Platelet count cannot be performed on capillary blood since some platelets are unavoidably lost by adherence onto the wound.
  - Precision is poorer in capillary than venous blood because of variation in blood flow and dilution with interstitial fluid.
  - Blood in microtubes frequently hemolyse that interferes with most laboratory tests.

Venous Blood:

- Collection of blood from the vein (venipuncture)
- Also referred to as phlebotomy
- Necessary for most tests that require anticoagulation or larger quantities of blood, plasma or serum.
- **Sites of Puncture**
  - Veins of the forearm are preferred; wrist or ankle can also be used
  - veins in the **Antecubital fossa** of the arm are the preferred sites
    - they are larger than those in the wrist or ankle regions
    - hence are easily located and palpated in most people.
    - Median cubital vein is the first choice as it is well anchored and easy to penetrate than Cephalic and Basilic veins.
- **Advantages of Venous Blood:**
  - various tests to be repeated in case of accident or breakage
  - checking of a doubtful result
  - performance of additional tests
  - aliquots of the specimen (plasma and serum) may be frozen for future reference.
  - reduces the possibility of errors resulting from
    - tissue dilution with interstitial fluid
    - constriction of skin vessels by cold that may occur in taking blood by skin puncture.
- **Disadvantages of Venous Blood:**
  - lengthy procedure
  - technically difficult in children, obese individuals and in patients in shock.

- more likelihood of occurrence of Hemolysis
    - hemolysis must be prevented because it leads to lowered red cell counts and interferes with many chemical tests.
- Hematoma (or blood clot formation inside or outside the veins) may also occur

NB: The peripheral blood (capillary blood) is diluted with tissue fluid but the hematocrit value, hemoglobin value, WBC count and red cell count is slightly higher than venous blood; in contrast the platelet count could be smaller in peripheral blood due to adherence of platelet in the damaged tissue.

Venous Blood Collection using Vacutainer:

Sometimes we can use a vacutainer for venous blood collection.

The following are the advantages of vacutainer method of venous blood collection:

- Multiple samples can be collected by exchanging tubes
- Eliminates many of the factors that cause hemolysis.
- No preparation of anticoagulants and containers needed (ready made special tubes coated with anticoagulant are used for blood collection)
- Because the evacuated tubes are sterile, avoids possible bacterial contamination. Thus, an ideal blood sample for microbiological analysis

PREPARATION OF PLASMA AND SERUM:

Preparation of blood plasma:

PLASMA: is the fluid portion of anticoagulated whole blood that is obtained afte centrifugation. In plasma all proteins are present.

To obtain plasma proceed as follows:

i. Make a vein-puncture & withdraw about 5cc of blood sample
ii. Transfer the blood to a test tube containing the specified type & amount of anticoagulant
iii. Mix the blood & anticoagulant by inverting the test tube 10 -12 times. Do not shake!
iv. Centrifuge the blood at high speed for about 5 minutes
v. Remove the blood from the centrifuge
vi. Using a dropper or pipette, remove the plasma & transfer in to another tube.

Preparation of blood serum:

SERUM: is the fluid portion of blood that is formed after coagulation of whole blood. In serum all proteins are present except Fibrinogen. The Fibrinogen is converted into Fibrin and the remaining fluid known as serum.

To obtain serum:

i. Make a vein-puncture & withdraw about 5cc of blood sample
ii. Transfer the blood to a glass test tube
iii. Let it stand at least 30 minutes
iv. Get a toothpick or applicator stick & gently loosen the clot so it does not stick to the walls of the test tube
v. Centrifuge the blood at high speed for about 5 minutes
vi. Remove the blood from the centrifuge

Using a medicine dropper or pipette, remove the serum & transfer it in to another tube

HEMOLYSIS: is the disruption of red cells and they may release their content to the whole blood. Haemolysed blood may interfere with haematological tests and other tests. Haemolysis may result from improper collection, processing and handling of blood and sometimes during pathological conditions.

Prevention of Hemolysis:

- Use syringe, needle and test tubes that are dry and free from detergent
  - traces of water or detergent cause hemolysis
- Use smooth and good quality sharp needles
- Avoid rough handling of blood at any stage
  - Do NOT eject the blood from the syringe through the needle as this may cause mechanical destruction of the cells
  - Transfer the blood from the syringe by gently pouring down the side of the tube
  - Mix blood with anticoagulant by gentle inversion not by shaking
- Tourniquet should NOT be too tight and should be released before blood is aspirated
- If examination is to be delayed beyond 1-3 hrs, do not allow the sample to stand unplugged or at room temperature.
  - Apply stopper and store in a refrigerator at 4^0C overnight
- Blood should NOT be stored in a freezer because the red cells will hemolyse on thawing
- Make sure that all solutions with which blood is to be mixed or diluted are correctly prepared and are isotonic
  - Hypotonic solutions will lead to hemolysis.
- When obtaining blood by skin puncture:
  - Make sure the skin is dry before pricking
  - Use sharp, 2-3mm lancets that produce clean puncture wounds
  - Allow the blood to flow freely

CHAPTER 3

PERFORMING HAEMATOLOGICAL TESTS:

HAEMOCYTOMETERY:

Introduction:

The enumeration of blood cells is a fundamental examination in the clinical laboratory.
Cell counts are nowadays performed by automated procedures/instruments.
The classical manual procedures are still used in many laboratories also used as a back up & QC for automated methods
The manual counting involves:

- ☞ Diluting the blood specimen in an appropriate diluting fluid
- ☞ Loading it into a special counting chamber known as a Hemocytometer
- ☞ Counting the cells

The cells counted in routine practice are white cells, red cells, and platelets.

Cell counts are expressed as the number of cells or formed elements (e.g. Plts, WBCs, RBCs) per liter of blood (ICSH (International Committee in Standardization in Hematology) recommendation).

The traditional unit of reporting was cubic millimeters (mm^3 or mm^2).

- 1 mm^3 = 1.00003 µL is felt to be insignificant, 1 µL is considered equivalent to 1 mm^3. Thus,**1 mm^3 = 1 µL = 10^{-6} liters**

HAEMOCYTOMETER:

Haemocytometer is a special glass that will present the cells to the observer in such a way that the number of cells per unit volume of fluid can be counted. The haemocytometer is a thick glass slide with inscribed platforms of known area & precisely controlled depth under the cover glass. In the center of the upper surface there are ruled areas separated by moats / channels/ from the rest of the slide and two raised transverse bars one of which is present on each side of the ruled area.

When an optically plane cover glass is rested on the raised bars there is a predetermined gap or chamber formed between its lower surface and the ruled area. This is called the depth of the chamber and it varies with the type of the chamber. The ruled area itself is divided by microscopic lines in to a pattern that varies again with the type of the chamber.

A haemocytometer consists of a counting chamber, a cover glass for the counting chamber & the diluting pipettes. Many types of counting chambers are available. Improved Neubauer & Fuchs Rosenthal are the two most commonly used chambers in laboratories.

The Improved Neubauer ruled chamber is recommended for cell counts (WBC, RBC, PLT) where as Fusch Rosenthal is recommended for Eosinophil count.

The Improved Neubauer counting chamber

- Each hemocytometer side has a counting chamber (two)
- Each counting chamber is 3mm x 3mm (area of 9mm^2).
- The space between the cover glass & surface of the counting area (i.e. the depth) is exactly 0.1mm if the cover glass is correctly applied.
- Each ruled area is a square of 9mm divided in to nine large squares each of 1mm side. The central square of these nine is divided by engraved lines in to 400 tiny squares of arranged in 25 groups of 16 by triple boundary lines. Each large square is 1mm^2, each of the 25 medium squares is of 0.004 mm^2 area & each of the 400 tiny squares has an area of 0.0025 mm^2.

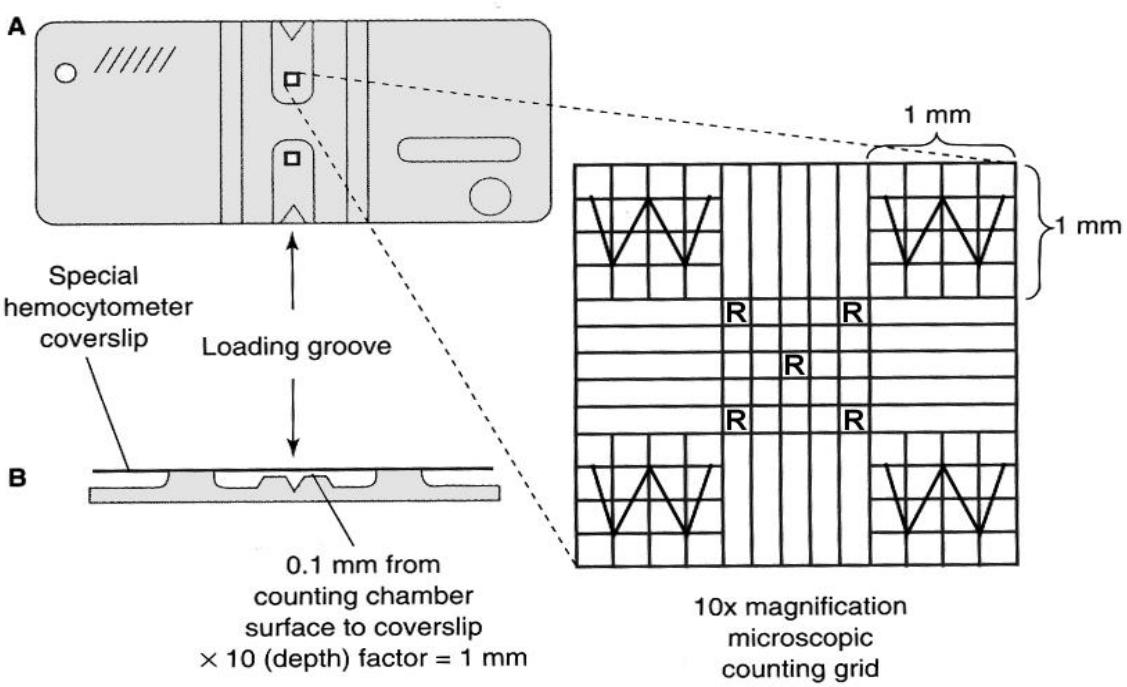


Fig.1. Improved Neubauer counting chamber

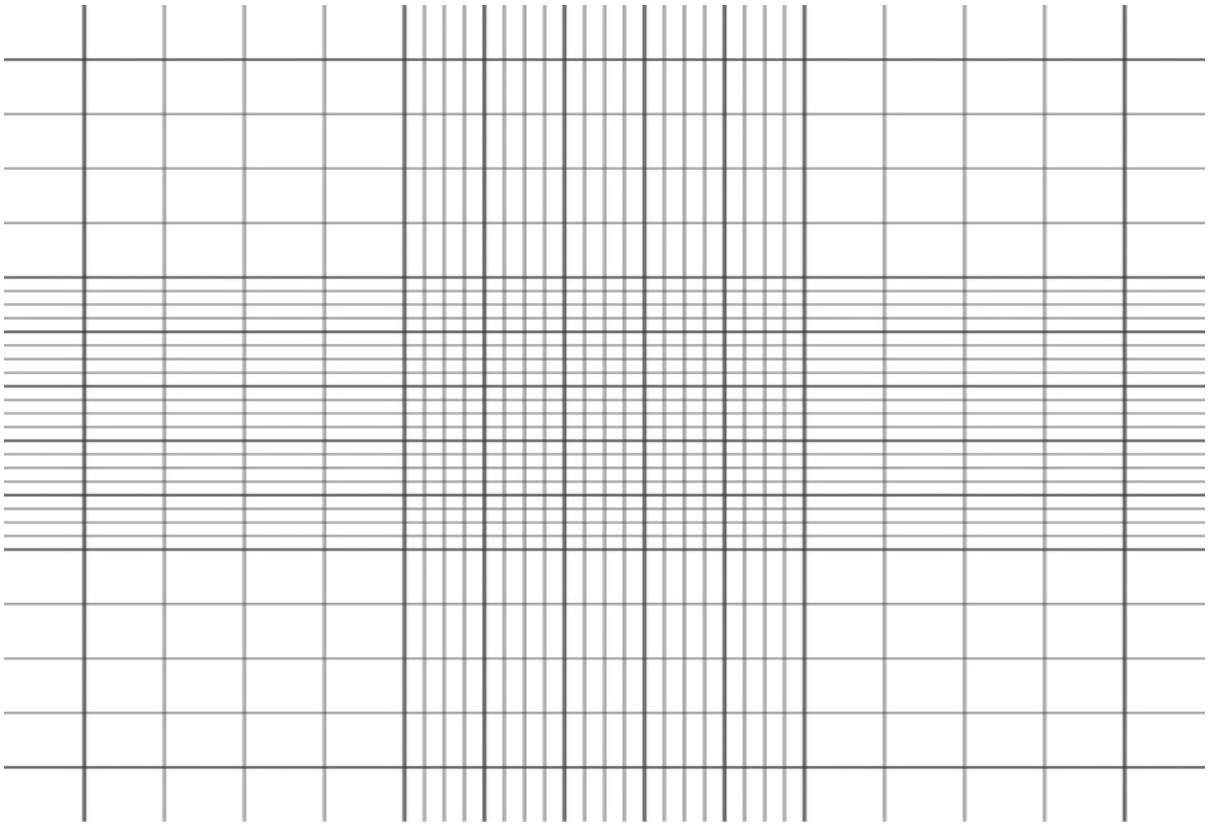


Figure 2. Improved Neubauer counting chamber. The central area consists of 25 groups of 16 small squares separated by closely ruled triple lines (which appear as thick black lines in the photograph).

DILUTION OF BLOOD SAMPLE FOR CELL COUNTS:

Dilution of the Sample is accomplished by using:
1.Thomma pipette: - a small calibrated diluting pipettes designed for WBC or RBC count
The WBC pipette has an upper numerical value of 11 while that of the RBC pipette marked by 101.
2.Tube dilution method:
- larger volumes of blood and diluting fluid are used
- greater accuracy compared with the smaller volumes used in the thomma pipette techniques.

DILUTING FLUIDS:

Diluting fluids are solutions that are useful to dilute blood and other body fluids for enumeration purpose. In order to count cells in smaller volume and manageable level, blood cells should be diluted.

Properties of diluting fluids:

- It has to keep the structure of the cells that we are interested to innumerate.
- It has to destroy unwanted cells other than our interest.

- It has to facilitate identification of cells
- Diluting fluids must also avoid aggregation of cells so that one can easily observe cellular structures

In haematology laboratory there are different types of diluting fluids. These are:

White blood cell diluting fluids:

Are diluting fluids useful for counting of white blood cells. They have the following features:

- It must hemolyse red cells so that white cells may be counted more readily (not masked by red cells)
- The fluid should keep the structures of white blood cells
- If possible the diluting fluid stain the nuclear part of white blood cells so that differentiation is easier

The commonly used WBC diluting fluids include:

- **Turk's solution**: 2% aqueous solution of glacial acetic acid colored pale violet with gentian violet/ methylene blue. The glacial acetic acid causes erythrocyte lysis while the gentian violet lightly stains the leucocytes permitting easier enumeration.
  Advantage: since Methylene blue can stain the nucleus of WBCs, it will facilitate enumeration and identification of cells.
- **1% HCL solution**
- **2% Glacial acetic acid**

Red blood cell diluting fluids:

The commonly used red cell diluting fluids are:

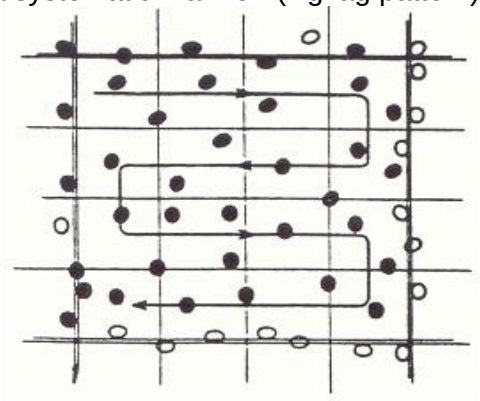
- **1% Formol citrate solution:** the main purpose of red cell diluting fluid is**:**
  - to keep the red cells as intact as possible without any distortion
  - has a proper specific gravity, so that the cells will settle as evenly as possible
- **Gower's solution**
- **Hayme's solution**
- **0.85% Sodium chloride solution**

Platelets diluting fluids:

- **1% ammonium oxalate**
- **Resker solution:** more preferable since it stains platelets

COUNTING OF CELLS & CALCULATION:

- The diluted cells are introduced in to the counting chamber & allowed to settle.
- Then the counting chamber is surveyed with low power objective (10x) to ascertain whether the cells are evenly distributed. The cells are then counted in the designated area (areas).
- While counting the cells in the squares include those that touch the lines on the left side or on top of the squares, & exclude those that touch the lines on the right side and at the bottom of the squares.
- Count cells in a systematic manner (zigzag pattern)



- Calculation:

No of cells/mm^3 = **N × DF × VF**

No of cells/L = **N × DF × VF × 10^6**

Where:

- ✓ N – No of cells counted in a given area.
- ✓ DF – Dilution Correction factor
- ✓ VF – Volume Correction Factor

COUNTING CELLS:

TOTAL WHITE BLOOD CELLS COUNT:

- ☞ Is the number of white cells in 1 liter (L) of whole blood.

Significance of WBC count:

- ✓ to investigate infections and unexplained fever
- ✓ to follow prognosis and
- ✓ to monitor treatments, which can cause leukopenia

Principle of test: Whole blood is diluted 1 in 20 in an acid reagent which haemolyzes the red cells (not the nucleus of nucleated red cells), leaving the white cells to be counted. White cells are counted microscopically using an Improved Neubauer counting chamber and the number of WBCs per litre of blood is calculated.

***Note*:** When after examining a stained blood film, many nucleated red cells are present (more than 10%), the WBC count should be corrected.

SPECIMEN: Whole blood anticoagulated with EDTA. Capillary blood can be used if it is diluted immediately after collection.

Required materials:

- Counting chamber (haemocytometer)
- Counting chamber cover glasses
- Thomma WBC Pipette
- WBC thoma pipettes
- Hand counter
- WBC diluting fluid
- Cotton roll (non absorbent)
- Gauze

Procedure:

1. After blood collection, take blood sample using WBC thoma pipette upto 0.5 mark.

2. Rinse excess blood from the pipette and suck diluting fluid upto 11 mark of the thoma pipette.

3. Mix very well or rack it on the rotator.

4. after 3-5 minutes discard the fluid at the stem part of the thoma pipette where there is no dilution with blood. (just discard 3-4 drops of the mixture)

5. fill (charge) the chamber gently and carefully.

6. wait for 3-5 minutes until the cells are uniformly distributed and settled.

7. count using 10x objective in the WBC counting areas (in 4 large corner squares of the chamber)

8. Report the number of white cells per mm^3 of blood using the following simple calculation:

No. of WBCs/mm^3 = No. of WBCs counted in 4 counting areas X Volume correction factor X Dilution correction factor

Area for WBC counting = 4 areas each has $1mm^3$ and a volume of 0.1 mm^3

Total WBCcounting area volume = 4 x 0.1 mm^3 = 0.4 mm^3

Volume correction should be done; i.e:

Volume correction factor = $\frac{\text{Volume desired}}{\text{Volume used}} = \frac{1mm^3}{0.4mm^3} = 2.5\ mm^3$

The dilution factor will be 1:20

No of WBCs/mm^3 **= No. of WBC × 2.5 x 20**

Some considerations in dilution and counting of WBCs:

- If the number of leukocytes is suspected small, one can dilute blood as 1:10

- In case of Leukemia (high WBC) we can dilute the blood 1:100 or 1:200 using red cell thoma pipette or tube method
- The distribution of WBCs in each counting area should not vary significantly; arbitrarily the difference should not be more than 10 cells. If so, repeat the procedure to have uniform distribution.
- As much as possible we have to count the two sides of the haemocytometer and take the average of the two counts. This gives a better result than a single count.

Correction for nucleated red cells:

The WBC diluting fluids normally lyse only matured red cells. Small number of nucleated red cells could be released into the peripheral circulation from the marrow and can be considered as white blood cells. The presence of nucleated red cells is usually observed from the stained blood film. If these cells are observed in significant numbers, a correction for the total white cell count must be carried out using the following formula:

$$\text{Corrected WBC count} = \frac{\text{Uncorrected WBC count /mm}^3 \times 100}{\text{No. of nucleated RBCs/100 WBCS} + 100}$$

SOURCE OF ERRORS IN MANUAL WBCs COUNT:

- Incorrect measurement of blood due to poor technique or using a wet or chipped pipette.
- When using anticoagulated blood, not mixing the blood sufficiently or not checking the sample for clots.
- Inadequate mixing of blood with diluting fluid.
- Not checking whether the chamber and cover glass are completely clean.
- Not using a haemocytometer cover glass.
- Over-filling of a counting chamber or counting cells when the sample contains air-bubbles.
- Not allowing sufficient time for the cells to settle in the chamber.
- Using too intense a light source or not reducing the iris diaphragm sufficiently to give good contrast.
- Counting too few cells. Precision increases with the number of cells counted.
- Not correcting a count when the sample contains many nucleated RBCs.

CLINICAL SIGNIFICANCE OF WBC COUNT:

An increase in total white blood cells is known as **Leukocytosis.**
A decrease in total WBC count is called **Leukopenia.**
Reference ranges for WBC counts vary with age, with higher counts being found in children. There are also gender differences with higher total WBC & neutrophil counts being found in women of child-bearing age and during pregnancy.

WBC reference range:
Children at 1 y . . . . . 6.0–18.0 × 10^9/l
Children 4–7 y . . . . . 5.0–15.0 × 10^9/l
Adults . . . . . 4.0–10.0 × 10^9/l

Pregnant women . . . . . Up to 15 × 10^9/l

Leukocytosis:

The main causes of a raised WBC count are:

- Acute bacterial infections
- Pregnancy
- Inflammation and tissue necrosis
- HDN
- Metabolic disorders
- Poisoning
- Leukemia and myeloproliferative disorders
- Stress, menstruation, strenuous exercise.

Leukopenia:

The main causes of a reduced WBC count are:

- Viral, parasitic infections
- Drugs (e.g. cytotoxic) and reactions to chemicals
- Hypersplenism
- Rheumatoid arthritis
- Cirrhosis of liver
- Aplastic anaemia
- Folate and vitamin B_{12} deficiencies (megaloblastic anaemia)
- Bone marrow infiltration (e.g. lymphomas, myelofibrosis, myelomatosis)
- Ionizing radiation

RED BLOOD CELLS COUNT:

The red cell count is one of the outdated manual haematological procedure due to:

- The procedure is very tiresome/ boring
- The automated method give a more precise and accurate tests
- Other haematological tests like haemoglobin, hematocrit and ESR gives good estimation of the red cell count and function
- The manual method is so time consuming and not suitable for routine activities

Principle of the test: A sample of blood is diluted with an isotonic diluent that maintains the disc-like shape of the red cells & prevents agglutination of the red cells. The number of red cells in a known volume & of known dilution is counted using a counting chamber.

Procedure:

Required materials:

All the materials used for WBCs count are required for RBCs count except that we use Thoma RBCs pipette for the red cell count in place of Thoma WBCs pipette.

1. Take blood sample up to 0.5 mark of the red cell thoma pipette

2. Rinse excess blood from the pipette using cotton carefully.

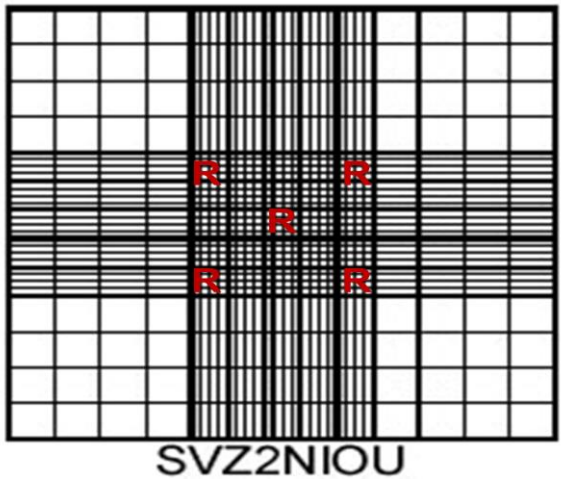
3. Rinse the blood using red cell diluting fluid up to 101 mark.

4. Mix very well or rack it on the rotator.

5. After 3-5 minutes discard the fluid at the stem part of the thoma pipette where there is no dilution with blood. (just discard 3-4 drops of the mixture)

5. Fill (charge) the chamber gently and carefully and wait for 3-5 minutes until the red cells cells are uniformly distributed and settled.

7. Count in the 5 small squares of RBC counting areas using 40x objective.



No. of Red cells/ mm^3 = No. of red cells counted in 5 small squares x Volume correction factor x dilution correction factor

Each volume of the five small squares = 0.004 mm^3

Total volume = 5 x 0.004 = 0.02 mm^3

So, Volume correction factor = $\frac{\text{Volume desired}}{\text{Volume used}} = \frac{1\ mm^3}{0.02\ mm^3} = 50$

Dilution correction factor = 200

RBCs /mm3 = No. of RBCs X 50 X 200

= No. of RBCs X 10,000

N.B. If the patient is very anemic or if less than 400 cells have been counted, the dilution of blood should be decreased to 1:100 by filling the pipettes with blood to the 1.0 mark instead of the 0.5 mark.

RBC reference range:

Adult Men. . . . . 4.5–6.2 × $10^6/mm^3$
Adult Women ……. . . . . 4.0– 5.5 × $10^6/mm^3$
new borns……..5.0- 6.5 x $10^6/mm^3$

Polycythemia – an increase in red blood cell count beyond the normal ranges.
-occures in Polycythemia vera
Anemia – a decreased red cell count.

-occurs in bleeding disorders, intestinal parasitosis like hook worm infection, abnormal production of red cells in the bonemarrow, hemolysis due to inherited or acquired disease.

THE PLATELET COUNT:

Value of test: A platelet count may be requested to investigate abnormal skin and mucosal bleeding which can occur when the platelet count is very low. Platelet counts are also performed when patients are being treated with cytotoxic drugs or other drugs which may cause thrombocytopenia.

Principle of test: Blood is diluted 1 in 20 in a filtered solution of 1% ammonium oxalate reagent which lyzes the red cells. Platelets are counted microscopically using a counting chamber and the number of platelets per litre of blood is calculated.

Platelets are difficult to count because:

- They agglutinate, fragment & break down readily and quickly
- Because of their small size they are hard to differentiate from debris
- They adhere to glass
- Platelets are not evenly distributed throughout the blood & have a tendency to clump together

SPECIMEN: Use EDTA anticoagulated venous blood. Capillary blood should not be used because platelets clump when the blood is being collected.

PROCEDURE:

Required materials:

Bright-line counting chamber and other equipment as described previously for WBC & RBC counting are required for counting platelets.
Ammonium oxalate 10 g/l (1% w/v) diluting fluid (Should be kept at **2-8ºC**)

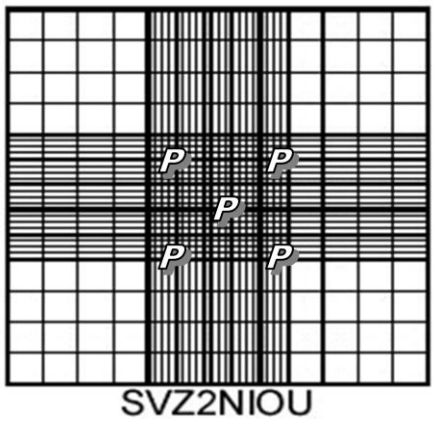
1. Mix the anticoagulated blood well; here we have to use EDTA venous blood as platelet count become more reliable.
2. Take blood sample using Red cell thoma pipette upto 1 mark
3. Rinse the excess blood using cotton
4. Take platelet diluting fluid by rinsing up the blood to the bulb of the pipette up to 101 mark
5. Mix or rack on a rotator for 10 minutes so that red cells could be hemolysed
6. Discard the fluid in the stem part of the pipette and the dilution becomes 1:100
7. Fill (charge) carefully the hemocytometer chamber and wait for 30 minutes (allows time for platelets to settle). Place the chamber in a petridish with wet cotton and cover with a lid (to prevent drying)
8. Count in the 10 small square areas of the red cell counting area using 40x objective.
9. They will be seen as small bright fragments (refractile) against a dark background Dirt and debris are distinguishable because of their high refractility
10. Report the number of platelets per mm^3 of blood using the following simple

No. of Platelets/ mm^3 = No. of Platelets in 10 small areas x Volume correction factor x Dilution correction factor

= No. of Platelets X 1/(10 x 0.004 mm^3) X 100

= No. of Platelets X 25 X 100

= No. of Platelets X 2500



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SOME CONSIDERATIONS IN COUNTING PLATELETS:

- If the platelet count in 10 small squares is less than 80, repeat the procedure by counting in all 25 small squares.
- If the platelet count in 10 small squares is less than 50, use WBC thoma pipette (apply 1:20 dilution)
- Phase contrast microscopy is more appropriate than bright field microscopy.

Interpretation of platelets counts:

In health there are about **150–400 ×10^9 platelets/litre** of blood. Platelet counts are lower in Africans.

Thrombocytopenia:

The main causes for a reduction in platelet numbers are:

I. REDUCED PRODUCTION OF PLATELETS

- ✓ Infections, e.g. typhoid, brucellosis
- ✓ Deficiency of folate or vitamin B12
- ✓ Aplastic anaemia
- ✓ Drugs (e.g. cytotoxic, aspirin), chemicals (e.g. benzene), alcoholism ---etc.
- ✓ Leukemia, lymphoma, myeloma, myelofibrosis, carcinoma
- ✓ Hereditary thrombocytopenia (rare condition).

II. INCREASED DESTRUCTION OR CONSUMPTION OF PLATELETS

- ✓ Infections, e.g. acute F.malaria, trypanosomiasis, visceral leishmaniasis
- ✓ Disseminated intravascular coagulation (DIC)
- ✓ Hypersplenism

- ✓ Immune destruction of platelets, e.g. idiopathic thrombocytopenic purpura (ITP), systemic lupus erythematosus (SLE), chronic lymphatic leukaemia, lymphomas and HIV/AIDS.

Thrombocytosis

Causes of an increase in platelet numbers include:

- ✓ Chronic myeloproliferative diseases, e.g. essential thrombocythaemia, polycythaemia vera, chronic myeloid leukaemia, myelofibrosis.
- ✓ Carcinoma (disseminated)
- ✓ Chronic inflammatory disease, e.g. tuberculosis
- ✓ Haemorrhage
- ✓ Sickle cell disease associated with a nonfunctioning spleen or after splenectomy.
- ✓ Iron deficiency anemia, associated with active bleeding

EOSINOPHIL COUNT:

Although total eosinophil Count can be roughly calculated from the total & differential WBC count, the staining property of eosinophils make it possible to count them directly and accurately in a counting chamber.

Principle: Whole blood is diluted appropriately using a diluents (**Hinkleman's solution**) which lysis the red cells & stains granules in the eosinophils, but does not stain the other leukocytes. The total numbers of eosinophils in a known volume of known dilution are counted using a counting chamber using low power objective.

SPECIMEN: Whole blood anticoagulated with EDTA. Capillary blood can be used if it is diluted immediately after collection.

TEST PROCEDURE:
Required materials:
Counting chamber (**Fuchs Rosenthal counting chamber** has a depth of 0.2 mm) and other equipment as described previously for WBC & RBC counting are required for counting eosinophils.

1. Take blood sample (anticoagulated with EDTA/ Heparin) and mix it very well.
2. Take blood up to 1 mark of WBC thoma pipette. Mostly a Fuchs-Rosenthal chamber (with a total area of $16mm^2$ and depth of 0.2mm) is used for counting.
3. Rinse excess blood and take Eosinophil diluting fluid up to 11 mark.
4. Mix very well and discard the fluid in the stem part. All the cells in the ruled area are counted (i.e., in 3.2μl volume).
5. Fill the chamber and wait for 10-15 minutes in a moisture petridish. Counting is carried out as soon as the cells are settled.
6. Count cells in all rulled areas of Neubaour or Fuchs-Rosenthal chamber using 10x objective.

Calculation: If E is the number of eosinophils in 16 large squares (in 3.2μl volume), then the absolute eosinophil count per μl of blood is:

Eosinophil count/mm^3 = No. of Eosinophil counted in all rulled areas X Volume correction factor X Dilution correction factor

When using Improved Neubour chamber,

Eosinophil count/mm^3 = 3 x 3 = 9 mm^2 and a base of 0.1

Total Eosinophil counting area volume = = 9 mm^2 x 0.1mm = 0.9 mm^3

Since we use all the rulled areas (2 ruled ares),

Total Eosinophil counting area volume = 0.9 mm^3 x 2 = 1.8 mm^3

Volume correction should be done; i.e:

Volume correction factor = $\frac{\text{Volume desired}}{\text{Volume used}} = \frac{1mm3}{1.8mm3}$ = 0.555 mm3

The dilution factor will be 1:10

No of Eosinophils/mm^3 = No. of Eosinophils × 0.555 x 10

If using Fuchs Rosenthal counting chamber

Eosinophil/mm3 = No. of Eosinophil counted x 1/6.4 mm3 x 10

Eosinophil/mm3 = No. of Eosinophil counted x 0.15 x 10

***Normal range*: 50 – 450 /mm^3 in adults.**

Eosinophlia: increased Eosinophils common in:

- Intestinal helminthes
- Allergic condition (hypersensitivity reaction)
- Asthmatic patients

Eosinopenia: a decrease in Eosinophil count observed in:

- Bonemarrow depression
- Radiotherapy

RETICULOCYTE COUNT

- Reticulocytes are immature red cells normally present in small numbers in the blood (up to 2%).
- Are young red cells that have lost their nuclei but not the cytoplasmic RNA.
- They contain remnants of the ribosomal ribonucleic acid (RNA) that was present in larger amounts in the cytoplasm of the nucleated precursors from which they were derived.
- Reticulocyte numbers increase when there is an increase in erythropoietic activity.
- A reticulocyte count assesses bone marrow activity, e.g. whether there is an effective erythropoietic response when there is a reduction in the number of red cells due to haemolysis or haemorrhage.

- ❖ Reticulocytes are demonstrated with a **supravital stain**, when the RNA appears as a network of strands or granules.

Principle of test: An isotonic solution of a supravital stain is incubated with a few drops of blood. To detect ribosomal RNA in reticulocytes, the red cells must be stained while they are still living (not fixed). A thin preparation is made and the reticulocytes counted microscopically. Reticulocytes are recognized by the violet-blue stained granules of ribosomal RNA (reticulin) they contain.

SUPRAVITAL STAINING:

- ✓ Is the process of staining of a cell after its somatic death but before its molecular death
- ✓ Is a stain used to stain cells or tissues while they are still living
- ✓ When blood cells drawn from the body it is said to be somatically dead
- ✓ When a reticulocyte is stained with vital dyes, the dye reacts with the RNA & ribosomes of the cells and an identifiable precipitate of RNA and ribosomes is formed.

Staining solutions:

- New methylene blue(1%)
- Brilliant cresyl blue

SPECIMEN: Capillary blood or anticoagulated venous blood taken in to EDTA or sodium citrate.

TEST PROCEDURE:

Required materials

- ✓ Staining solution
- ✓ Pasture pipette
- ✓ Microscope
- ✓ Microscope slides
- ✓ 37^{o}c incubator

Test method:

1. Filter 2–3 drops of the stain into a small tube or vial.
2. Add about 4 drops of blood sample and mix well.
3. Incubate at room temperature for 20 minutes or 10–15 minutes at 35–37 ^{o}C.
4. Mix gently to re-suspend the red cells and using a capillary or plastic bulb pipette, transfer a drop of the stained blood to a slide. Spread to make an evenly spread *thin* film. Wave the slide back and forth to air-dry the films.
5. Count the reticulocytes microscopically. Use the 10× objective (with reduced condenser iris diaphragm) to check the distribution of the red cells. Select an area where the red cells can be seen individually, add a drop of immersion oil, and examine using the oil immersion objective.

6. Count systematically 1000 RBCs in consecutive oil immersion fields & record the number of reticulocytes encountered along the 1000 RBCs.

Appearance of reticulocytes:

Reticulocytes appear as greenish-blue with deep blue intracellular precipitate. Matured red cells appear greenish blue or pale-blue.

***Counting reticulocytes*:** A convenient method of counting reticulocytes is to reduce the size of the microscope field by inserting in each eyepiece a circular piece of black (opaque) paper which has a punched out hole of about 5 mm.

7. Calculate the percentage of reticulocytes as follows & report the result:

$$\text{Reticulocyte \%} = \frac{\text{Reti. Counted in 1000 RBCs} \times 100}{1000}$$

Absolute Reti. Count = Reti. % × RBCs count/mm3

Sources of errors in reticulocyte count:

- Not mixing adequately the stained blood prior to making the blood films.
- Not counting the cells accurately or counting too few cells.
- Using stain which has become contaminated and has not been filtered before use.
- Confusing Heinz bodies or precipitated stain for the reticulin of reticulocytes.

Interpretation of reticulocyte counts

Infants at birth 2–5%

Children and adults 0.5–2.5%

***Note*:** Whenever an RBC count is available, express the reticulocyte in absolute numbers.

Raised reticulocyte counts: Found when there is an increase in red blood cell production as occurs in:

- Haemolytic anaemias (with effective erythropoiesis).
- Following acute blood loss
- After iron therapy for iron deficiency anaemia or specific therapy for megaloblastic anaemia

***Decreased reticulocyte count*:** Associated with ineffective erythropoiesis or decreased production of red cells.

CELL COUNTING IN BODY FLUIDS:

Blood is the commonest specimen that is sent to haematology laboratory for cell counting. However, it is possible to count cells in different body fluids. The following body fluids could be possible specimen for cell counting:

- Celrebrospinal fluid (CSF)
- Synovial fluid (joint fluid)
- Peritoneal fluid (abdominal fluid)

- Pericardial fluid (cardiac fluid)
- Pleural fluid (Lung fluid)

The distribution of cells in the above body fluids differs significantly one from the other. Normally the number of WBCs and RBCs could be very small per milliliters as compared to blood. In case of pathological conditions there might be infiltration of blood cells to the aforementioned body fluids and the cell count could be elevated.

CSF:

- **WBC---- 0-5 cells/ mm^3**
- **RBC---- 0-2 cells/ mm^3**

Synovial fluid
Peritoneal fluid } **WBC----- 100-200 cells/ mm^3**
Pericardial fluid } **RBC----- 10-100 cells/ mm^3**
Pleural fluid

When we perform cell counting in body fluids, we have to consider the following:

- The nature of the specimen (body fluids), haematogenous or not
- The turbidity of specimen (due to protein)
- The presence of pus cells (pyuria)
- Most body fluids are precious and their collection procedure should be done by well-trained physicians
- This observation may guide us to dilute or avoid any dilution of the body fluids using cell diluting fluids.

CSF is the most frequently used body fluid that is sent to the haematology laboratory for cell count. If the CSF is turbid and haematogeous, dilute as 1:10 or 1:20 using **10% acetic acid** diluting fluid and proceed as the usual cell counting procedure. If it is clear, directly fill the chamber (commonly Fuchs-Rosental) and count in the whole areas.

TOTAL WHITE BLOOD CELL COUNT IN CSF:

CSF should be collected by experienced physicians using the lumbar puncture procedure. The lumbar vertebrae between 4th and 5th are the preferred site for collection of CSF.

CSF is useful to nourish brain cells, it maintains the intracranial pressure, it acts a cushion for meninges and gives protection from damage. It is also useful to administer drugs.

The amount of CSF in the body is small and care has to be given during collection. Only 1-2ml of CSF is adequate for cell counting. The collection tube has to be free from any chemical and detergent especially the tube must be sterile for bacteriological analysis.

Cell count in CSF is very vital to rule out septic and aseptic meningitis. Usually in septic meningitis the total WBC count will be elevated mostly Neutrophils. While in aseptic meningitis the predominating cells are Lymphocytes.

Principle:

Depending on the CSF physical appearance, one can dilute or directly fill the haemocytometer usually the Fuchs Rosental chamber and count in the whole ruled areas.

Procedure:

1. Carefully handle your CSF, and take up to 0.5 mark of the thoma pipette if the CSF is turbid and haematogeous.
2. Take Turk's solution up to 11 mark.
3. Or one can take CSF up to 1 mark of the thoma pipette and Turk's solution up to 11 mark (in the first case the dilution will be 1:20 while in the later case it will be 1:10)
4. Allow lyzing all RBCs and after filling the chamber count the cells.
5. Report cell count per milliliters of CSF or per liters of CSF.
   WBC/mm3 CSF = No. of WBCs X Volume correction X Dilution correction
   = No. of WBCs X 0.31 X 20 (for Fuchs Rosental counting chamber)
   = No. of WBCs X 0.9 X 20 (for Improved Neubour chamber)

NORMAL VALUES:

WBC---- 1-5 /ml of CSF

Increased WBC during bacterial aseptic meningitis, Niesseria meningitis, Hemophilus influenza, Streptococcus pneumonia. Mainly Polymorphonuclear leukocytes are involved.

CHAPTER 4

DIFFERENTIAL WHITE BLOOD CELL COUNT

- It is the determination of the percentage distribution of the different leukocytes & their quantitative study on a stained blood film.
- It includes the identification & counting of various types of WBCs and expressing the number of each type per 100 white cells.
- The count is usually performed by visual examination of blood films that are prepared on slides by the spread or "wedge" technique.
- For a reliable differential count on films spread on slides, the film must not be too thin and the tail of the film should be smooth. To achieve this, the film should be made with a rapid movement using a smooth glass spreader.

Purpose of differential cell count:

- ♦ To evaluate the different leukocyte distribution in the circulation in normal and pathological state
- ♦ A differential white cell count provides information on the different white cells present in the circulating blood, i.e. neutrophils, lymphocytes, monocytes, eosinophils, basophils.
- ♦ Providing the total WBC count is known, the absolute number of each white cell type, i.e. number of each cell per litre of blood, can be calculated and an assessment made of whether the number of a particular cell type is increased or decreased (compared with the accepted reference range).

Techniques in differential counting:

Various systems for performing the differential count have been advocated, but none can compensate for the gross irregularities in distribution in a badly made film. Of the three methods indicated underneath for doing the differential count, the **lateral strip method** appears to be the method of choice.

I. The longitudinal strip method

- ✓ Counting of cells longitudinally. It starts from the thick end to the feather end encompassing the cells from one part of the original drop of blood.
- ✓ The cells are counted using the 100× objective in a strip running the whole length of the film until 100 cells are counted.
- ✓ Inspect the film from the head to the tail, and if fewer than 100 cells are encountered in a single narrow strip, examine one or more additional strips until at least 100 cells have been counted.
- ✓ If all the cells are counted in such a strip, the differential totals will closely approximate the true differential count.

Disadvantage

- This technique is liable to error if cells in the thick part of the film cannot be identified
- It does not allow for any excess of neutrophils and monocytes at the edges of the film, but this preponderance is slight in a well-made film and in practice makes little difference to the result.

II. The Exaggerated battlement method

The film is examined systematically, by being traversed, three – fields along the edge, two – fields up, two – fields along & two fields down. The sequence is continued until a minimum of 100 cells are counted.

Disadvantage:

- ➢ It is difficult to make demarcation point between two microscopic fields.

III. The lateral strip ('Crenellation') Technique

The field of view is moved from side to side across the width of the slide in the counting area just behind the feather edge where the cells are separated from one another & are free from artifacts.

Two types of counting are available:

A. Relative Count:

- ☞ The relative differential count is the simple percentage distribution of white blood cells.

B. Absolute Count:

- ☞ An absolute differential count is the exact count of leukocytes in the peripheral blood. The absolute count can be given as the product of the relative count with the total leukocyte count preferably the corrected leukocyte count.
- ❖ It is preferable to count the absolute differential count as we obtain relatively the exact number of leukocytes in the peripheral blood.

- ❖ For proper interpretation the relative count is of little use by itself.
  For example, the fact that a sample may have 60% polymorphs is of little use
  A patient sample may have 60% neutrophil and a total leukocyte count of 8.0 x 10^9/L giving 4.8 x 10^9/L neutrophils, which is quite normal
  but if the patient has 60% neutrophils in a total leucocyte count of 3.0 x 10^9/L, then the patient's neutrophil count is1.8 x 10^9/L neutrophils. In this case the patient has granulocytopenia.

Principle:

After taking blood sample, blood film is prepared, and after staining with Romanowsky stains, 100, 200, 300, etc., cells will be counted; then the percent distribution of each cell is calculated.

Type of specimen:

EDTA anticoagulated venous or capillary blood

Material and equipment:

- Slides
- Microscope
- Diff counter
- Oil immersion
- Wright stain
- Timer
- Ethanol/methanol for fixation

Procedure:

1. Collect the blood sample in an aseptic condition
2. Prepare thin blood film
3. Dry the film and fix it with absolute methanol or methanol
4. Stain the fixed film with Wright stain
5. Let it dry
6. Count the relative leukocyte distribution in the selected area of the film using 100x objective

HOW COULD YOU IDENTIFY EACH TYPE OF LEUKOCYTES:

Leukocytes are identified & classified based on:

- ✓ Size of the cell
- ✓ Nuclear pattern (shape, staining depth of nucleus, cytoplasm ----etc.)
- ✓ Presence or absence of granules in cytoplasm or nucleus
- ✓ Staining characteristics of cytoplasm (color)

Leukocytes normally present in the blood:

There are five types of leukocytes observed in a normal peripheral blood smear.

These are:

- ✓ Neutrophils
- ✓ Eosinophils
- ✓ Basophils
- ✓ Lymphocytes
- ✓ Monocytes

1. Neutrophils

- Measures 10–12 µm in diameter.
- Smaller than monocytes & eosinophils but slightly larger than basophils.
- The nucleus has 2 - 5 clear lobes, separated by chromatin threads and stains deep reddish purple.
- Has an irregular shape like letters of **E, Z, S**
- The cytoplasm stains light pink with pinkish dust like granule

Band Neutrophils:

- A normal blood smear may contain a few (up to 3%) **band cells** which are neutrophils in a slightly premature stage in which the band shaped long nucleus is not yet divided in to lobes.
- These cells have a strand of nuclear material which is thicker than a filament connecting the lobes. It usually has **U** or **C** shaped nucleus with uniform thickness.

Segmented Neutrophils:

- Segmented neutrophils has at least two lobes which is separated by filament.
- Segmented neutrophils has the following percentage distribution:
- Two lobes …… 10 – 30%
- Three lobes ……. 40 – 50%
- Four lobes ……... 10 – 20%
- Five lobes …….. 5%
- Six or more lobes ……is called **Hypersegmentation.**

2. **Eosinophils**

- Slightly larger than a neutrophil, measuring 12–14 µm in diameter.
- Usually contains a bi-lobed nucleus in a typical “spectacle arrangement”
- The cytoplasm contains many large round bright orange-red granules and occasionally vacuoles.
- The underlying cytoplasm, which is usually obscured by the granules, is pale blue or color less.
- Their structure is similar to Polymorphonuclear neutrophils (PMN) but the cytoplasm of eosinophil contains large round or oval granules having a strong affinity for acidic stains.

3. Basophils

- Measures 10–12 µm in diameter.
- The nucleus is usually bi-lobed but is obscured (covered) by large, irregular in size basophilic granules which have strong affinity for basic dyes.
- The cytoplasm is slightly basophlic having pale blue or colorless color and contains large granules which stain purple or black dye to their strongly basophilic nature.

4. Monocytes

- Largest of circulating white cells, measuring 15–20µm in diameter containing a single nucleus.
- The nucleus is large, often appearing indented, convoluted or folded, brainy looking nucleus, mauve staining with a delicate lacy looking chromatin pattern.
- The nucleus is large and curved, often in the shape of a horseshoe, but it may be folded or curled. It never undergoes segmentation.
- Monocyte nucleus can sometimes take many shapes from a band shape, to an oblong shape, to a convolutes brainy shape.
- The cytoplasm is a clear grey-blue and contains fine granules.

5. Lymphocytes

- Are mononuclear cells like monocytes and have no specific granules.

Small lymphocytes:

- Measures 7–12µm in diameter.
- They have a round or irregular shaped dark mauve staining nucleus surrounded by a thin rim of blue cytoplasm.
- cytoplasm stains pale blue

Large lymphocytes:

- measures 12–16µm in diameter
- Less densely stained nuclei and more abundant cytoplasm
- Cytoplasm stains sky blue
- The cytoplasm is pale and more abundant than the small lymphocyte

★ While performing the deferential count, all elements of the blood film must be observed. For example:

- ✓ Erythrocytes
- ✓ Platelets
- ✓ Leukocytes
- ✓ Hemoparasites

REPORTING THE DIFF. LEUKOCYTE COUNT

The differential count, expressed as the percentage of each type of cell, should be related to the total leukocyte count and the results should be reported in absolute numbers ($\times 10^9$/l).

Differential WBC reference range:

ADULTS	**Absolute number**	**Percentage**
Neutrophils	1.5–7.5 × 10^9/L	40–75%
Lymphocytes	1.2–4.0 × 10^9/L	21–40%
Monocytes	0.2–1.0 × 10^9/L	2–10%
Eosinophils	0.02–0.6 × 10^9/L	1–6%
Basophils	0.01–0.1 × 10^9/L	0–1%
CHILDREN (2–6 y)		
Neutrophils	1.5–6.5 × 10^9/L	20–45%
Lymphocytes	6.0–8.5 × 10^9/L	45–70%
Monocytes	0.1–1.0 × 10^9/L	2–10%
Eosinophils	0.3–1.0 × 10^9/L	1–6%
Basophils	0.01–0.1 × 10^9/L	0.1–1%

ABNORMALITIES IN LEUKOCYTES:

I. Neutrophils

Neutrophilia:

- Is an increase in the number of circulating neutrophils above normal
- **Also called neutrophilic leucocytosis**
- The conditions associated with this include:
  - ✓ Overwhelming infections
  - ✓ Metabolic disorders
  - ✓ Haematological disoreders (Eg. Myelogenous leukemia)
  - ✓ Physical & emotional stress
  - ✓ Drugs & chemicals

Neutropenia:

- Is a reduction of the absolute neutrophil count below normal
- The conditions associated with this include:
  - ✓ Myeloid hypoplasia
  - ✓ Ionizing radiation

Hypergranular neutrophils (neutrophils with toxic granules):

- Are neutrophils with coarse blue black or purple granules
- Such granules are indicative of severe infection or other toxic conditions

Vacuolation:

- Multiple clear vacuoles in the cytoplasm of neutrophils may be seen in progressive muscular dystrophy

Hypersegementation:

- Neutrophil with more than six lobes to their nucleus is an important diagnostic observation indicative of megaloblastic erythropoiesis (vitamin B_{12} and/or folic acid deficiency), iron deficiency anemia.

Agranular nutrophils:

- Neutrophils devoid of granules & having a pale blue cytoplasm are features of leukemia

Left shift of neutrophils:

- Immature neutrophils and precursor cells seen, e.g. band cells, metamyelocytes and sometimes myelocytes.
- Cells may contain darkly staining coarse granules (toxic granulation) and sometimes vacuoles

Shift to the right of Neutrophils:

- When there are many segmented neutrophils or hypersegmented neutrophils in the peripheral blood film.

II. Eosinophils

Eosinophilia:

- Is an increase in the number of circulating eosinophils above normal
- The conditions associated with this include:
  - ✓ Allergic diseases like bronchial asthma, seasonal rhinitis
  - ✓ Parasitic infections
  - ✓ Skin disorders
  - ✓ Chronic myelogenous leukemia

Eosinopenia:

- Is a reduction of the absolute eosinophil count below normal
- The conditions associated with this include:
  - ✓ Acute stress due to secretion of adrenal glucocorticoid & epinephrine
  - ✓ Acute inflammatory stress

III. Basophils

Basophilia:

- Is an increase in the number of circulating basophil above normal
- It is rare condition associated with:
  - ✓ Allergic reactions
  - ✓ Chronic myelogenous leukemia
  - ✓ Polycythemia vera

IV. Monocytes

Monocytosis:

- Is an increase in the number of circulating monocytes above normal
- The conditions associated with this include:
  - ✓ Recovery from acute infections
  - ✓ Tuberculosis
  - ✓ Monocytic leukemia

Monocytopenia:

- Is a reduction of the absolute monocyte count below normal
- The conditions associated with this include:
  - ✓ Treatment with prednisone
  - ✓ Hairy cell leukemia

V. Lymphocytes

Lymphocytosis:

- Is an increase in the number of circulating lymphocytes above normal
- The conditions associated with this include:
  - ✓ Infectious lymphocytosis associated with some viral infections
  - ✓ Acute & chronic lymphocytic leukemia

- ✓ Toxoplasmosis

Lymphocytopenia:

- ♦ Is a reduction of the absolute lymphocyte count below normal
- ♦ The conditions associated with this include:
  - ✓ Immune deficiency disorders (HIV/AIDS)
  - ✓ Drugs & radiation therapy

Atypical lymphocytes (Reactive lymphocytes):

- ✓ Irregularly shaped larger than normal lymphocytes with a less dense nucleus (may contain nucleoli) and abundant blue cytoplasm often appearing dark blue and folded at periphery of cell
- ✓ Indicate transformation of lymphoid cells as a response to antigenic stimulation
- ✓ Are primarily seen in infectious mononucleosis which is an acute, self-limiting infectious disease of the reticuloendothelial tissues, especially the lymphatic tissues

ABNORMAL CELLS IN THE PERIPHERAL BLOOD:

1. **Nucleated Red Cells:**
   - Also called Normoblasts
   - Their nucleus is round and usually in an eccentric position.
   - Chromatin materials are dense and clumped which stained darkly.
   - The cytoplasm is pink and grayish blue without granules

2. **Blast cells:**
   - Youngest form of blood cells.
   - N**:**C ratio increased, have nucleoli, the cytoplasm is blue and there is granules.

3. **Immature granulocytes:**
   - Present in leukemia and other conditions. Includes the following:

Band cells:

- Also called stab cells.
- Are precursors of segmented neutrophils
- Can present in normal conditions (2-6%)

Metamyelocyte:

- Also called a juvenile
- Rarely seen normally in the peripheral blood
- Nucleus characteristically indented or kidney-shaped
- The nuclear chromatin is clumped and condensed
- Specific granules appear the same as in the band

Myelocyte:

- Should never be seen in the peripheral blood
- The nucleus is round or oval and often eccentrically located in the cell
- The nuclear chromatin is beginning to clump; no nucleoli are visible

- A moderate amount of patchy blue cytoplasm can be seen
- The cell is characterized by the presence of light pink or tan (neutrophilic) specific granules

Promyelocyte:

- Should never be seen in the peripheral blood
- Are arge cells
- The nucleus may be eccentric and slightly indented with nucleoli still visible
- The nuclear chromatin is loose and open
- The cell is characterized by the presence of few to many, prominent, dark red or purple cytoplasmic granules

Myeloblast:

- Should never be seen in the peripheral blood
- Large cell (size may vary)
- The nucleus is large and round, with one or more visible nucleoli
- The nuclear chromatin is loose and open
- The cytoplasm is scanty to moderate and deeply basophilic

4. **Megakaryocytes:**
   - Are the biggest cells in the marrow & are mother cells (precursors) for platelets.
   - Normally they are present in marrow and rarely in the peripheral circulation.
   - Their nucleus irregular in shape, highly lobulated.
   - Cytoplasm is highly lobulated, full of granules and some platelets.

5. **Plasma cells:**
   - Are big cells, round in shape and an eccentric nucleus with clumped chromatin.
   - Have dark blue cytoplasm with a pale staining area round the nucleus.

Quality control of Differential count:

- ☞ Well prepared well stained blood film which has 3 zones (Head, body, tail)
- ☞ WBC should contain blue nucleus along with a lighter staining cytoplasm
- ☞ RBC should have good quality of color ranging from buff pink to orange
- ☞ Platelet should be blue with granules and no nucleus

Sources of errors:

- Use of unclean slide and improper smear preparation and staining technique
- Counting cells in an area not suitable for counting
- Misidentification of white cells
- Interpersonal skill

CHAPTER 5

RED CELL MORPHOLOGY STUDY

- Is the study of morphological appearance or characteristics of RBCs on a stained blood film preparation on different disease conditions.
- The morphology of blood cells in stained films is the basis of laboratory diagnosis of hematological disorders.

Microscopic examination of blood film:

If the blood film has been properly stained, it should appear light purple or pink to the naked eye. Then thoroughly examine the blood film as follows:

I. Examine the blood film under low power objective

The purpose of this examination is:

- To get an idea of the quality of the preparation, i.e., whether red cell agglutination or excessive roulex is present.
- To get an idea of the number, distribution & staining of the leukocytes.
- To find an area where the red cells are evenly distributed and are not distorted.

II. Examine the blood film under oil immersion (100X) objective

- Having selected a suitable area, the oil immersion objective is used to appreciate variation in red cell size, shape & staining and fine details such as cytoplasmic granules & other red cell inclusions.

During the examination of the peripheral blood film, the following characteristics in the red cell morphology should be observed & reported:

- ✓ Color (Hgb content)
- ✓ Shape
- ✓ Distribution pattern
- ✓ Artifacts
- ✓ Size
- ✓ Inclusions
- ✓ Nucleated red cells

Normal mature red blood cells (Discocytes):

- ✓ In health, red cells are said to be normocytic & normochromic.
- ✓ In well spread & stained films the great majority of cells have round smooth contours & have diameters within the comparatively narrow range of 6 - 8µm.
- ✓ It stained pink or orange red to the periphery & the intensity of the stain decreased towards the central area.

But in many disease conditions, there is an alteration in size, shape, & staining characteristics of the red cells.

A. variation in color (Change in RBCs haemoglobinization)

The depth of staining (color) may give rough estimation of Hgb content.

1. Normochromic erythrocytes

- Are erythrocytes that contain normal amount of Hgb.
- On stained blood film it shows deep orange red color to the periphery, which gradually diminishes towards the center of the cell.
- It has normal MCV, MCH, and MCHC.

2. Hypochromic erythrocytes (Hypochromasia)

- A red cell with low Hgb content as a result the central pallor area is increased to more than one – third of the cell diameter.
- Have low MCH & MCHC.
- The central pale area is increased to more than one-third of the cell diameter
- Poor drying of the film may cause a **'false hypochromia'**
    - This can be distinguished from a true one in that the change in the central pale area is sudden while in true hypochromia it is gradual
- It is a consistent finding in iron deficiency anemia, thalassemia & sidroblastic anemia.

3. Hyperchromic erythrocytes (Hyperchromasia)

- The Hgb content in the individual cells appears to be increased, since the cells are thicker than normal, but the Hgb content is actually higher than the normal limits.
- The entire cells stained deep pink lacking the central pallor.
- Have increased MCH & MCHC.

4. Polychromatophilia (polychromasia)

- Are cells with diffuse light blue or bluish – gray coloration
- On Wright's stained film appear as large young RBCs that represent marrow reticulocytes "shifted" out of the bone marrow prematurely in response to accelerated erythropoietic activity.
- They are larger than normal cells
- Therefore it is an indicator of an increased reticulocytes
- Polychromasia occur because of the affinity of RNA to basic dyes & the affinity of Hgb for acidic dyes.
- It occurs in blood loss anemia & marked hemolysis.

5. Dimorphism (Anisochromasia)

- It is the presence of two populations of erythrocytes namely hypochromic & normochromic cells on the same stained blood film in approximately equal proportions.
- It is a finding in treated iron deficiency anemia where there is the new normochromic red cell population & the original hypochromic population and in patients with hypochromic anemia who have been transfused.

B. variation in size (Anisocytosis)

- Is the variation in size of erythrocytes

On the basis of their diameter the cells are divided in to:

I. Normocytic RBCs: Erythrocytes with normal size.

II. Microcytes

- ✓ Abnormally small erythrocytes usually less than 6µm in diameter
- ✓ Its MCV is decreased
- ✓ It is associated with hypochromasia
- ✓ Area of central pallor usually increases because of the coexistent hypochromia
- ✓ Microcytosis is common in iron deficiency anemia & hepatic disease.

III. Macrocytes

- ✓ The cells are greater than 8 µm in size
- ✓ There may be young red cells that can be shown by supravital staining to be reticulocytes.
- ✓ Macrocytosis is seen in stress erythropoiesis as seen in hemolytic anemia & also during recovery from acute blood loss.

IV. Megalocytes

- ✓ Large (9 – 12 µm in diameter), often oval shaped cells with increased Hgb content.
- ✓ Megalocytes are seen in Vitamin B_{12} & folic acid deficiency.

C. variation in shape (Poikilocytosis)

- Poikilocytosis is the term which describes the change in shape of erythrocytes.

I. Shistocytes (fragmented cells)

- Schistocytes are red cells fragmented by trauma & found in many blood diseases.
- They are smaller than normal red cells and of varying shape.
- Sometimes they have sharp angles or spines (spurs), & sometimes they are round in contour, usually staining deeply but occasionally palely as the result of loss of Hgb at the time of fragmentation.
- Shistocytes occur in the following situations:
  - ✓ In certain genetically determined disorders (e.g. thalassaemias & congenital dyserythropoietic anaemia).
  - ✓ In acquired disorders of red cell formation when erythropoiesis is megaloblastic or dyserythropoietic.
  - ✓ As the consequence of mechanical stresses

II. Acanthocytes (Spiny cells)

- The term *acanthocytosis* was introduced to describe an abnormality of the red cell in which there are a small number of spicules (spines) of inconstant length, thickness, and shape, irregularly disposed over the surface of the cell.
- They are often associated with abnormal phospholipid metabolism or with inherited abnormalities of red cell membrane proteins.
- They are present in varying numbers following splenectomy & in hyposplenism. A similar cell occurs in severe liver disease ("spur cell" anaemia).

III. Echinocytes (Crenated cells)

- Echinocytosis or crenation describes the process by which red cells develop many or numerous short, evenly distributed regular projections (spines) from their surface.
- Crenation has many causes. A few crenated cells may be seen in many blood films, even in those from healthy subjects.
- Crenation regularly develops if blood is allowed to stand overnight at 20°C before films are made.
- Consistently found in blood samples that have been stored for some time at room temperature and because of diffusion of alkaline substances from the slide into the cells resulting in an increase in pH and thus crenation of the cells
- Ehinocytes are seen in uremia, pyruvate kinase deficiency, & neonatal liver disease.

IV. Target cells (Leptocytes/'Mexican hat cells')

- The term *target cell* refers to a cell in which there is a central round stained area & a peripheral rim of haemoglobinized cytoplasm separated by non- staining or more lightly staining cytoplasm.
- Target cells result from cells having a surface that is disproportionately large compared with their volume.
- They may be normal in size, microcytic, or macrocytic.
- They are seen in films in chronic liver diseases in which the cell membrane may be loaded with cholesterol, in hereditary hypobetalipoproteinaemia, and in varying numbers in iron deficiency anaemia and in thalassaemia.
- They are often conspicuous in certain haemoglobinopathies, Hgb C disease, Hgb H disease, sickle cell anaemia.

V. Stomatocytes

- Stomatocytes are red cells in which the central biconcave area appears slit-like in dried films. These are cells with a narrow slit like area of central pallor.
- In "wet" preparations, the stomatocyte is a cup-shaped red cell.
- The slit-like appearance of the cell's concavity, as seen in dried films, is thus to some extent an artifact.
- The term was first used to describe the appearance of some of the cells in a rare type of haemolytic anaemia, hereditary stomatocytosis.

- They are observed in liver disease, in alcoholism, and occasionally in the myelodysplastic syndromes.
- There is a suspicion that in some films the occurrence of stomatocytosis is an *in vitro* artifact because it is known that the change can be produced by decreased pH and as the result of exposure to cationic detergent-like compounds.

VI. Dacrocytes ('Tear drop cells')

- These are tear drop or pear shaped red cells
- Could be considered to be discocytes with a single drawn out spicule.
- It is thought that stretching of the cell membrane beyond a certain limit results in loss of deformability and ability to revert to normal discoid shape.
- Seen in Myelofibrosis, Myeloid metaplasia, Tumour metastases to the bone marrow, Tuberculosis, Drug-induced Heinz body formation

VII. Drepanocytes ('sickle cells')

- These are crescent shaped red cells because of the formation of rod-like polymers of Hb S or some other rare hemoglobins
- Have an increased surface area and increased mechanical fragility which leads to hemolysis and hence severe anemia
- They are primarily seen in sickle cell anemia where there is substitution of valine for glutamic acid at position 6 of the beta chain in the hemoglobin molecule

VIII. Elliptocytes/ovalocytes

- Are elliptical or oval shaped red cells.
- Normally less than 1% of the red cells are elliptical/oval shaped.
- Found in almost all anemias where approximately 10% of the red cells may assume elliptical/oval shape and in hereditary elliptocytosis where almost all the red cells are elliptical.

IX. Spherocytes/Microspherocytes

- Dense staining spherical cells with smaller diameter and greater thickness than normal
- They are formed as a result of loss of membrane due to: Genetic lack of structural proteins in the red cell membrane, Chemicals, Bacterial toxins, Antibody-mediated hemolytic anemias, Burn injury.

X. Rouleaux formation

- This phenomenon is the adherence of red cells resembling stacks of coins
- May be seen as artifacts in the thick areas of the blood film
- They are often associated with: Hyperproteinemia, chronic inflammatory disorders, multiple myeloma, macroglobulinemia.

D. Red cell inclusions

A normal red cell does not contain any inclusion. Various types of inclusions may be seen in RBCs which may indicate disorders or disease conditions.

1. **Basophilic stippling**
   - The red cells contain small irregularly shaped granules which stain blue in Wright's stain & which are found distributed throughout the cell surface.
   - It is a common finding in lead poisoning, anemias associated with disorders of hemoglobin synthesis.

2. **Howell – Jolly bodies**
   - Small, round inclusions that contains DNA & is usually eccentrically located in the cell.
   - They stain deep purple.
   - Found in megaloblastic anemia, some hemolytic anemias & after splenoctomy.

3. **Cabot's rings**
   - These are incomplete or complete rings, even figures of "8", that appears as reddish - violet fine filamentous configuration in Wright stained films.
   - They are remnants of the microtubules of the microtubules of the mitotic spindle.

4. **Heinz bodies**
   - Heinz bodies are oxidized denatured Hgb.
   - They can be demonstrated only by supravital staining with brilliant cresyl blue.
   - Heinz bodies may appear in the peripheral blood smear after removal of the spleen, or in hemolytic anemias such as that due to G_6PD.

Red cell abnormalities

Red cell abnormalities	Causes
 Normal	
 Macrocyte	Liver disease, alcoholism. Oval in megaloblastic anaemia
 Target cell	Iron deficiency, liver disease, haemoglobinopathies, post-splenectomy
 Stomatocyte	Liver disease, alcoholism
 Pencil cell	Iron deficiency
 Ecchinocyte	Liver disease, post-splenectomy
 Acanthocyte	Liver disease, abetalipoprotein-aemia, renal failure
 Sickle cell	Sickle cell anaemia
 Microcyte	Iron deficiency, haemoglobinopathy

Red cell abnormalities	Causes
 Spherocyte	Hereditary spherocytosis autoimmune haemolytic anaemia, septicaemia
 Fragments	DIC, microangiopathy, HUS, TTP, burns, cardiac valves
 Elliptocyte	Hereditary elliptocytosis
 Tear drop poikilocyte	Myelofibrosis, extramedullary haemopoiesis
 Basket cell	Oxidant damage–e.g. G6PD deficiency, unstable haemoglobin
 Howell–Jolly body	Hyposplenism, post-splenectomy
 Basophilic stippling	Haemoglobinopathy, lead poisoning, myelodysplasia, haemolytic anaemia
 Malarial parasite	Malaria. Other intra-erythrocytic parasites include *Bartonella bacilliformis*, babesiosis
 Siderotic granules (Pappenheimer bodies)	Disordered iron metabolism e.g. sideroblastic anaemia, post-splenectomy

CHAPTER 6

DETERMINATION OF HAEMOGLOBIN

STRUCTURE OF HAEMOGLOBIN

- Haemoglobin (Hgb), the main component of RBC, is a conjugated protein that serves as the vehicle for the transportation of oxygen and carbon dioxide.
- When fully saturated, each gram of Hgb holds 1.34ml of oxygen. The red cell mass of the adult contains approximately 600g of Hgb, capable of carrying 800ml of oxygen.
- A molecule of Hgb consists of two pairs of polypeptide (globin) chains and four prosthetic haem groups, each containing one atom of ferrous iron.
- Each haem group is precisely located in a pocket or fold of one of polypeptide chains.
- The globin molecule consists of two alpha (α) & two beta (β) polypeptide chains.
- Heam is a metal complex containing an iron atom in the center of a porphyrin structure.
- Hgb is formed in the developing erythrocyte in the bone marrow.

SYNTHSIS OF Hgb

- Haem synthesis occurs in most cells of the body, except the mature erythrocytes, but most abundantly in the erythroid precursors.
- This synthesis occurs in the mitochondria by a series of biochemical reaction.

I. Glycine + succinyl coenzyme A condensed by delta amino laevulinic acid (ALA) synthetase in the presence of pyridoxal phosphate (Vit. B_6). This leads to the formation of protoporyphyrin.

Glycine + Vit. B_6 succinyl co-A __ALA_Synthetase -------⇨ protoporyphyrin

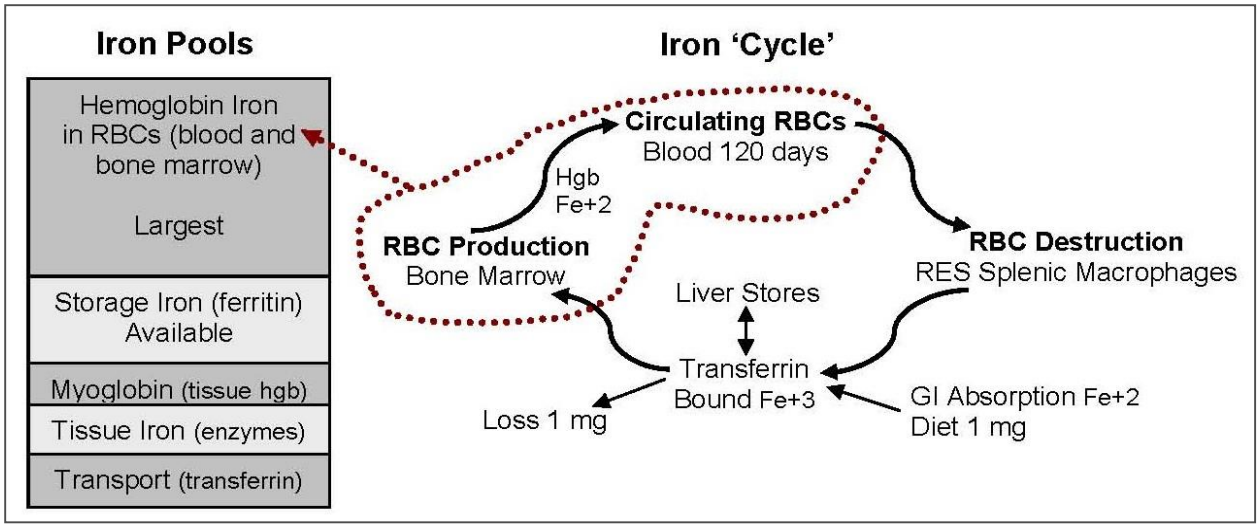
II. Protoporyphyrin combines with iron to form heam.

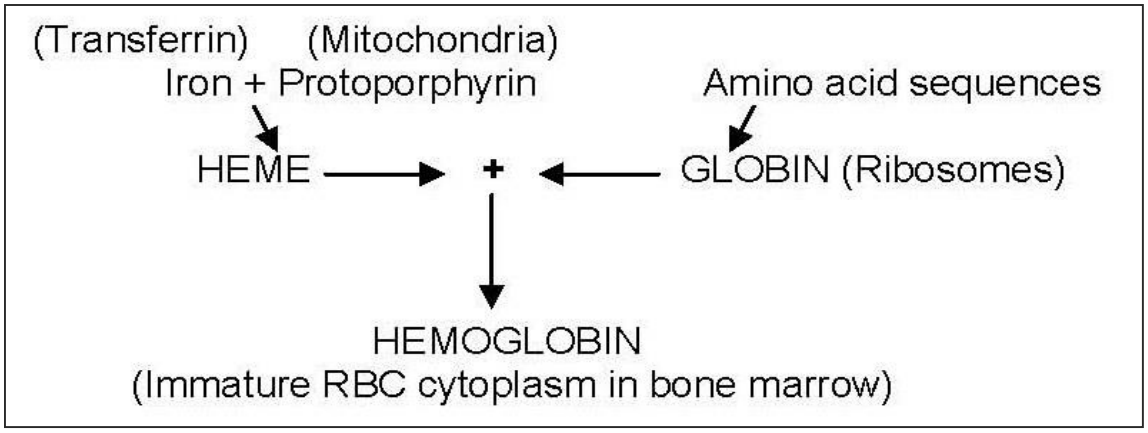
Fe + protoporyphyrin -------⇨ Heam

- Globin synthesis occurs in the cytoplasm of the normoblast & reticulocyte.

III, Each molecule of heam combines with the globin chains on polyribosomes.

IV. Tetramer of 4 – globin chains, each with its own heam group, make up haemoglobin molecule.





Function of Hgb

- Transport of oxygen from the lungs to the tissues and of carbon dioxide in the reverse direction
  - ✓ At an oxygen tension of 100mmHg in the pulmonary capillaries, 95 – 98% of Hgb is combined with oxygen.
  - ✓ In the tissues, where oxygen tension is as low as 20mmHg, the oxygen readily dissociates from Hgb; in this instance, less than 30% of oxygen would remain combined with Hgb.
- Assisting in acid base regulation by eliminating carbon dioxide in the lungs and by the buffering action of Hgb.

Hemoglobin breakdown (building blocks):

Protoporphyrin ring of heme is cleaved
↓ + CO ↑
biliverdin is reduced to bilirubin (unconjugated)
↓ transported by albumin to parenchymal cells in liver where it is conjugated by glucuronyl transferase
bilirubin diglucuronide (conjugated) → excreted into bile
↓ reduced by anaerobic bacteria in colon
urobilinogens → stool or urine

Iron is released, bound to transferrin and recycled for hemoglobin production or goes to storage.

Globin - polypeptides are hydrolyzed to amino acids and returned to amino acid pool for protein synthesis.

Forms of Hgb

Depending on the content & sequence of amino-acids in the globin chains, different forms of Hgb may occur in the red cells.

At least three distinct Hgb forms are found postnatally in normal individuals, & the structure of each has been determined. These are HbA, HbA_2 & HbF.

- **HbA** is the major (96 – 98%) normal adult Hgb. The polypeptide chains of the globin part of the molecules are of two types: two identical α – chains and two identical β – chains (α_2 β_2).
- **Hb A_2** account for 1.5 – 3.5% of normal adult Hgb. It has two α chains and two δ chains (α_2 δ_2).
- **HbF** is the major Hgb of the fetus & the new born infant. Hb F has two α chains and two γ chains (α_2 γ_2).
  - ✓ **HbF** is slowly replaced by **HbA** & at the end of the first year, the **HbF** concentration is reduced to 1 – 2% from 70 – 90% at birth.
  - ✓ A normal adult may have 1 – 2% HbF in blood.
  - ✓ **HbF** combines more readily with oxygen than **HbA**, thus helping the foetus in acquiring oxygen in placental circulation.

- ✓ It also differs from **HbA** in that; it resists denaturation with alkali, unlike **HbA** which is easily denatured.

- Reduced haemoglobin (Hgb) is Hgb with iron unassociated with oxygen. When each haem group is associated with one molecule of oxygen, the Hgb is referred to as oxyhaemoglobin (HbO_2). In both Hb & HbO_2 iron remains in the ferrous state.
- When iron oxidizes to ferric state, metheamoglobin (haemoglobin, Hi) is formed, & the molecule loses its capacity to carry oxygen or carbon dioxide.

MEASUREMENT OF HAEMOGLOBIN CONCENTRATION

- Hgb is measured to detect anemia and its severity and to monitor an anemic patient's response to treatment.
- The test is also performed to check the Hgb level of a blood donor prior to donating blood.
- Haemoglobinometery is the measurement of the concentration of Hgb in the blood.

Methods of measuring haemoglobin

The Hgb content of a solution may be estimated by several methods:

- By measurement of its color
- Its power of combining with oxygen or carbon monoxide
- By its iron content
- Oxyhaemoglobin
- cyanmaethemoglobin

In district laboratories, depending on available facilities and resources, Hgb is measured photometrically or estimated using a visual comparative technique.

- ✓ In photometric techniques the absorbance of Hgb in a blood sample is measured electronically using a filter colorimeter or a direct read out Hgb meter.
- ✓ When it is not possible to measure Hgb accurately using a photometric technique a visual comparative technique can help to detect anemia.
- ✓ Hgb values are expressed in grams per liter (g/l) or grams per deciliter (g/dl).

Photometric techniques

I. Cyanmethaemoglobin (Haemiglobincyanide)

- ✓ Is the recommended method because stable haemiglobincyanide (HiCN) standards are available to calibrate instruments.
- ✓ Is also used as a reference method against which all other color comparison methods should be calibrated.

Principle: The Hgb is treated with drabkin's reagent containing potassium ferricyanide, potassium cyanide & potassium dihydrogen phosphate. The ferricyanide forms methaemoglobin which is converted to cyanmethaemoglobin by the cyanide.

Reagents

A. Drabkin's solution
   Potassium cyanide ---------- 50mg
   Potassium ferricyanide ---------- 200mg
   Potassium dihydrogen phosphate ---------- 140mg
   Non – ionic detergent ---------- 1ml
   Distilled water ---------- to 1L
B. Cyanmethaemoglobin standard
   - ✓ Is needed to calibrate a filter colorimeter.
   - ✓ It is available commercially in sealed ampoules.
   - ✓ A calibration curve can be prepared by diluting the standard 1:2, 1:3, 1:4 -----etc & the concentration plotted against the absorbance.

Test procedure

Required materials

- ✓ Photometer with green filters (540nm)
- ✓ Cuvette
- ✓ Cyanmethemoglobin standard solution
- ✓ Drabkin's reagent
- ✓ Test tubes & test tube rack
- ✓ Measuring pipette
- ✓ Gauze pads
- ✓ Blood with anticoagulant

Method

1. Measure carefully 20µl of blood & dispense it in to 3.98ml Drabkin's diluting fluid.

2. Stopper the tube, mix, & leave the diluted blood at room temperature, protected from sunlight, for 4 – 5 minutes.

3. Place a yellow – green filter in the colorimeter or set the wavelength at 540nm.

4. Zero the colorimeter with Drabkin's fluid & read the absorbance of the patient's sample.

5. using the table prepared from the calibration graph, read off the patient's value.

Advantages of Cyanmethaemoglobin method

- Is convenient
- Readily available & stable standard solution
- All forms of Hgb except sulfhemoglobin are readily converted to HiCN.

Source of errors

- ✓ Using of chipped and/or dirt pipettes.
- ✓ Inaccurate measuring of blood sample.
- ✓ Excessive squeezing of finger prick to obtain blood.
- ✓ When using anticoagulated venous blood, not mixing the sample sufficiently.
- ✓ Note insuring that the optical surfaces of a cuvette are clean dry and there are no air bubbles in the solution.

II. Non-dilution photometric techniques

- Blood is collected directly into a specially designed single-use micro-cuvette or other sampling device which is internally coated with reagent to lyze the blood and converts the haemoglobin to a form which can be read in a direct read-out meter.
- Eg. HemoCue non – dilution photometric technique

III. Visual comparative technique

When it is not possible to measure haemoglobin accurately using a photometric technique, e.g. in a health centre or antenatal unit, a visual comparative technique such as the *Haemoglobin Colour Scale*, can help to detect anemia and assess its severity.

Acid haematin method (Sahli – Hellige)

This visual comparative method of estimating Hgb although still used in some health centers and hospitals is not recommended because of its unacceptable imprecision and inaccuracy.

Most of the problems associated with the Sahli method are:

- ✓ Due to the instability of acid haematin,
- ✓ Fading of the colour glass standard & difficulty in matching it to the acid haematin solution.
- ✓ Conversion to acid haematin is slow.
- ✓ HbF is not converted to acid haematin & therefore the Sahli method is not suitable for measuring Hgb levels in infants up to 3 months.

Principle: Hgb in a sample of blood is converted to a brown colored acid hematin by treatment with 0.1N HCL & after allowing the diluted sample to stand for 5 minutes to ensure complete conversion to acid hematin, then it is diluted with distilled water until its color match as with the color of an artificial standard (tinted glass reference).

Test Procedure

Required materials

- Sahli haemoglobinometer
- Sahli pipette graduated
- Small glass rod
- Dropping pipette
- Non absorbent cotton
- 0.1N HCL
- Distilled water

Method

1. Fill haemoglobinometer graduated tube to 20 mark with 0.1N HCL.

2. Draw venous blood or capillary blood to the 0.02ml mark of Sahli pipette.

3. Wipe the outside of the pipette with non-absorbent cotton. Check that the blood is still on mark.

4. Blow the blood from the pipette in to the graduated tube of acid solution. Rinse the pipette by drawing & blowing out the acid solution three times. The mixture of the blood & acid gives a brownish color. Allow to stand for 5 minutes.

5. Place the graduated tube in the haemoglobinometer stand facing a window. Compare the color of the tube containing diluted blood with the color of the reference tube. If the color of the diluted sample is darker than that of the reference, continue to dilute by adding 0.1 N HCL or distilled water drop by drop. Stir with glass rod after adding each drop.

6. Remove the rod and compare the color of the diluted blood with the reference glass or tube.

7. Note the mark and read, depending on the type of haemoglobinometer.

Source of errors

- Failure to maintain normality of HCL (0.1N)
- Difference in person's inability to compare colors.
- Improper light source
- Contaminated diluting fluid
- Failure to make proper dilution

Interpretation of haemoglobin test results

Normal haemoglobin levels vary according to age and gender, and the altitude at which a person lives.

Children at birth . . . . . 13.5–19.5 g/dl

Children 2 y–5 y . . . . . 11.0–14.0 g/dl

Children 6 y–12 y . . . . . 11.5–15.5 g/dl

Adult men. . . . . .13.0–18.0 g/dl

Adult women . . . . . 12.0–15.0 g/dl

(Pregnant women) . . . . . .11.0–13.8 g/dl

RBC count, HGB & HCT values <u>parallel</u> each other

RBC Count, Hemoglobin, Hematocrit Reference Ranges Vary with sex, age, altitude and testing procedure			
	RBC Count	**HGB**	**HCT**
Males	4.40-5.80 million/cmm	13.0-17.0 g/dL	37.0-51.0 %
Females	3.80-5.20 million/cmm	11.5-15.5 g/dL	35.0-46.0 %
Newborns	5.00-6.50 million/cmm	14.0-25.0 g/dL	44.0-64.0 %
Children (1-10 yo)	3.70-5.50 million/cmm	11.0-14.0 g/dL	34.0-42.0 %

- **Significance**
  - **Decreased RBC, HGB and HCT values….Anemia**
    - **Decreased production, increased loss/destruction**
  - **Increased RBC, HGB and HCT values….Polycythemia**
    - **Increased production**
- **Critical values:**
  - **HGB <7.0 or >18.5 g/dL**

RBC Count, HGB, HCT Correlation

- **Relationship: Hb x 3 = HCT <u>±</u> 3%**

RBC x 3 = Hb or RBC x 9 = HCT

- **Used to estimate values or check data correlation**
- **'Rules' only apply *if red cells are <u>normal</u> in size and hgb content***

CHAPTER 7

HAEMATOCRIT (PACKED CELL VOLUME)

When anticoagulated whole blood is centrifuged, the space occupied by the packed red blood cells is termed as the haematocrit reading & is expressed as the percentage of RBCs in a volume of whole blood.

Purpose

- The hematocrit is a test to determine the ratio of cells to fluid. This test is rapidly replacing the red cell count for general purpose; since it is easier, quicker and more accurate.
- It is used to screen for anaemia when it is not possible to measure Hgb accurately and mains electricity is available to operate a microhaematocrit centrifuge.
- Is also used in the investigation of dehydration, burns, dengue haemorrhagic fever and polycythaemia.
- In the investigation of anaemia, the PCV with Hgb value is used to calculate the mean cell haemoglobin concentration (MCHC).

Methods of haematocrit determination

There are two methods to determine PCV:

- ✓ Microhematocrit method
- ✓ Macrohematocrit method

I. Microhematocrit method

Principle: The packed cell volume is that proportion of whole blood occupied by red cells, expressed as a ratio (L/L). Anticoagulated blood in a glass capillary of specified length bore size, & wall-thickness is centrifuged in a microhaematocrit centrifuge for 3–5 minutes to obtain constant packing of the red cells. A small amount of plasma remains trapped between the packed red cells. The PCV value is read from the scale of a microhaematocrit reader or calculated by dividing the height of the red cell column by the height of the total column of blood.

Equipment

- ✓ Microhaematocrit centrifuge
- ✓ Capillary tubes for measuring PCV
- ✓ Sealant
- ✓ Microhaematocrit reader
- ✓ Blood collection materials

Test method

1. About three quarters fill the capillary tube with well mixed blood.

2. Seal the capillary tubes by vertically placing the dry end in to a tray of sealing compound. Rotate the capillary tube slightly & remove it fro the tray.

3. Place the filled sealed capillary tube in the grooves (slots) of the centrifuge with the sealed end toward the periphery. Write the number of the slot on the patient's form. Position the inner lid carefully to avoid dislodging the tubes.

4. Set the timer of the centrifuge at 5 minutes & spin at 10,000 – 15,000g.

5. Immediately after centrifuging, read the PCV. First check that there has been no leakage of blood from the capillary or breakage.

- To read the PCV in a hand-held microhaematocrit reader, align the base of the red cell column (above the sealant) on the **0** line and the top of the plasma column on the **100** line. Read off the PCV from scale. The reading point is the top of the red cell column, just below the buffy coat layer (consisting of WBCs and platelets).
- **When no reader is available:** Use a ruler to measure the length of the total column of blood (top of plasma to bottom of red cell column) in mm and the length of the red cell column (base to below buffy coat layer). Calculate the PCV as follows:

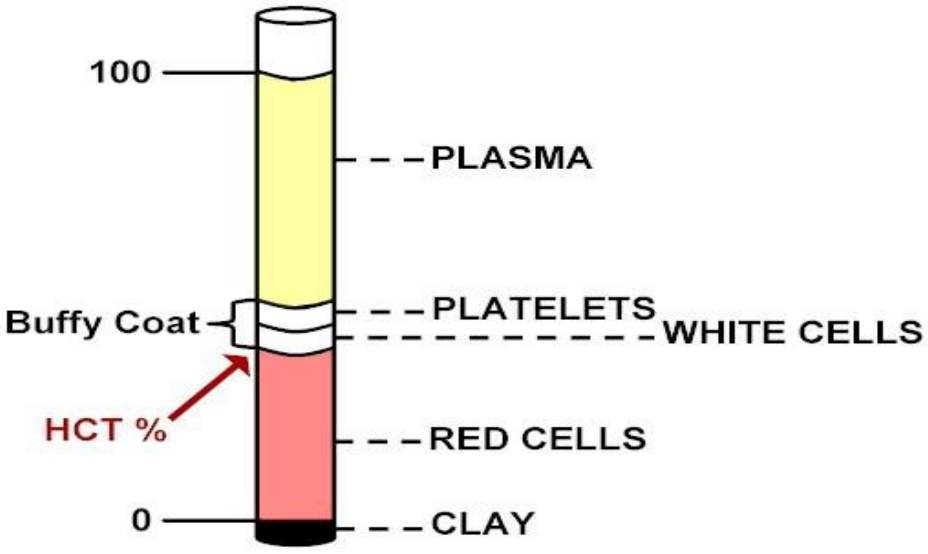
$$PCV = \frac{\text{Length of red cell column (mm)}}{\text{Length of total column (mm)}}$$

Example: height of red cell column = 19mm

height of total blood column = 49mm

PCV = 19mm/49mm = 0.388 (l/l) or 38.8%

6 Report any abnormal appearance of the plasma.



Interpretation of PCV

In a similar way to haemoglobin levels, PCV values vary according to age, gender, and altitude.

Children at birth 0.44–0.54

Children 2–5 y 0.34–0.40

Children 6–12 y 0.35–0.45

Adult men. 0.40–0.54

Adult women 0.36–0.46

- PCV values are reduced in anaemia.
- Increased PCV values are found when there is loss of plasma as in severe burns, dehydration and in dengue haemorrhagic fever.
- It also increases in all forms of polycythaemia.

Sources of error in measuring PCV

- ✓ Specimen collection errors
- ✓ Inadequate mixing of specimen (for EDTA blood)
- ✓ Improper use of the hematocrit reader or including the buffy coat layer
- ✓ Improper centrifugation
- ✓ Wrong RPMs (speed) of the centrifuge
- ✓ Incorrect centrifuge time
- ✓ Manually stopping the centrifuge
- ✓ wrong sealing method used (e.g use of soap, heating)
- ✓ Incorrect reading due to uneven clay plug
- ✓ Excess anticoagulant
- ✓ Centrifuging at too low speed or for an insufficient length of time resulting in a PCV value being higher than it should be.
- ✓ Delay in reading the PCV after centrifugation, allowing plasma to evaporate.
- ✓ Using an anticoagulated blood sample containing excess EDTA
- ✓ Clots in an anticoagulated blood sample can result in a falsely low PCV value.
- ✓ Incomplete sealing of the haematocrit tube gives falsely low results.
- ✓ Rises in PCV (up to 6% error) can occur when there is an increase in trapped plasma due to changes in red cell size or shape, e.g. in spherocytosis, microcytosis …etc.
- ✓ Using capillary tubes that are not designed for measuring PCV.
- ✓ Not cleaning and maintaining the microhaematocrit centrifuge as recommended by the manufacturer.

II. Macrohematocrit method

- Procedure is no longer in routine use
- Wintrobe tube is filled with well mixed EDTA anticoagulated venous blood (0.1ml EDTA/1ml blood) to the mark "0" on top using a long stem Pasteur pipette making sure that no air bubbles are trapped.
- The preparation is then spun at 2300g for 30 minutes.
- The hematocrit is read from the scale on the right hand side of the tube taking the top of the black band of reduced erythrocytes immediately beneath the reddish gray leukocyte layer.

Advantages of the Microhematocrit Method

- ☐ It enables higher centrifugation speeds with consequent shorter centrifugation times and superior packing.
- ☐ The amount of trapped plasma is less than that in the Wintrobe method by virtue of the higher centrifugation speed employed.

CHAPTER 8

ERYTROCYTE SEDIMENTATION RATE (ESR)

When well mixed venous blood is placed in a vertical tube, erythrocytes will tend to fall toward the bottom. The length of fall of the top of the column of erythrocytes in a given interval of time is called the ESR. The rate is expressed in mm/hr.

The ESR is a non-specific test. It is raised in a wide range of infectious, inflammatory, degenerative, and malignant conditions associated with changes in plasma proteins, particularly increases in fibrinogen, immunoglobulins, & C-reactive protein.

Principle:

If blood, to which an anticoagulant has been added, is placed in a narrow tube in a vertical position for a set time, the red cells settle out of the suspension, leaving plasma above them. In erythrocyte sedimentation test, the distance from the top of the column to the interface between the plasma and the sedimented red cells is recorded and expressed in **mm/hr**.

STAGES IN ESR

Three stages can be observed

- ✓ The initial period of ten minutes, during which rouleaux formation occurs.
- ✓ The period of fast settling during this period the settling rate is constant. It lasts about 40 minutes.
- ✓ A slower rate of fall during which packing of the sedimented red cell column occurs. It lasts about 10 minutes.

Methods of ESR determination

Two basic methods have been recommended & have gained wide acceptance. These are:

- Westergren method
- Wintrobe method

I. Westergren method

This is ICSH (International council for standardization in hematology) reference method for ESR determination.

Equipment

- ✓ Westergren ESR pipette
- ✓ Westergren ESR stand with labeling device
- ✓ Timer capable of timing accurately 1 hour

- ✓ Tri-Sodium citrate (3.2% W/v) anticoagulant
- ✓ Pipette filling device

Test method 8cstle 2georg 4walya 1H2O

1. Venous blood is diluted accurately in the proportion of one volume of citrate to four volumes of blood.

2. A clean dry Westergren pipette is carefully filled & adjusted to the “0”mark on top.

3. The tube is placed in a strictly vertical position in the Westergren stand under room temperature conditions not exposed to direct sunlight & away from vibrations & draughts. Allow it to stand for exactly 1hour.

4. After one hour read the level at which the plasma meets the red cells in mm.

5. After reading the ESR, return the blood to its container, remove carefully the pipette & soak it in sodium hypochlorite (0.25%) disinfectant. Dispose of the blood safely & decontaminate the container.

Advantage of Westergren method

- ✓ It more reliably reflects the clinical state & is the most sensitive method for serial study of chronic disease.

Disadvantage of Westergren method

- ✓ It requires a large amount of blood & involves dilution which may be one source of error.

Interpretation of result

Reference range

Men . . . . . 0 to 15mm/hour

Women . . . . . 0 to 20 mm/hour

***Note*:** Higher values are obtained during menstruation, pregnancy, puerperium.

II. Wintrobe method

It uses a tube closed at one end, 11cm long with a bore of 2.5mm & having a graduating scale from 0 – 100mm and a special Wintrobe rack.

Equipment

- ✓ Wintrobe tube
- ✓ Wintrobe rack
- ✓ Pasture pipette
- ✓ Timer capable of timing accurately 1 hour
- ✓ Tri-Sodium citrate (3.2% W/v) anticoagulant

Test method

2. Blood is collected with EDTA in the right proportion.
3. Enough blood to fill the Wintrobe tube (about 1ml) is drawn in to a pasture pipette having a long steam.
4. The Wintrobe tube is then filled from the bottom up to the "0" mark.
5. The tube is placed in the wintrobe rack in exactly vertical position & the time is noted.
6. At the end of 1hour the ESR is read as the length of the plasma column above the cells & is expressed as mm/hr.

Advantage of Wintrobe method

- ✓ The method is simple, requires a small amount of blood & there is no dilution.
- ✓ With the same preparation, once the ESR has been read, the hematocrit value can be determined.
- ✓ Smears of buffy coat can be made.

Disadvantage of Wintrobe method

- ✓ Because of the short column, it is only sensitive when the ESR is low & when the disease is in the acute stage.

Interpretation of result

Reference range

Men . . . . . 0 to 7mm/hour

Women . . . . . 0 to 15 mm/hour

Factors affecting ESR

Some of the factors that affect ESR include:

- ♦ Plasma proteins
- ♦ Plasma viscosity
- ♦ Red cell factors
- ♦ Mechanical influences
- ♦ Temperature

Sources of error in ESR determination

- ✓ Using the wrong volume of blood to anticoagulant.
- ✓ Blood not sufficiently mixed with anticoagulant.
- ✓ Clots in the blood. Even the smallest fibrin clot in the sample will invalidate the test result.
- ✓ Air bubbles at the top of the column.
- ✓ Testing blood samples at the hottest time of the day, or leaving tests in direct sunlight. Temperatures over 25^{o}C increase sedimentation.
- ✓ Using a pipette which is not clean or not dry.
- ✓ Pipette not positioned vertically. Even slight variations from the upright increase sedimentation. A 3^{o} inclination can increase the ESR by 30%.
- ✓ Not checking whether the ESR stand is level on the bench.
- ✓ Placing an ESR stand on the same bench as a centrifuge where vibration will interfere with sedimentation.

CHAPTER 9

RED CELL INDICES

- Red cell indices are measurements that describe the size & oxygen carrying protein (Hgb) content of red blood cells.
- Wintrobe introduced calculations for determining the size, content and Hgb concentration of red cells.
- Red cell indices are useful for morphological characteristics & classification of anemia.
- To calculate red cell indices, an accurate red blood cell count is required. To perform an accurate RBC count, an electronic cell analyzer is needed. Most district laboratories will not therefore be able to calculate these indices; however, examining a well-stained blood film can help to detect macrocytosis or microcytosis.

Red cell indices most frequently used in the investigation of anemia are:

- ✓ Mean cell volume (MCV)
- ✓ Mean cell haemoglobin (MCH)
- ✓ Mean cell haemoglobin concentration (MCHC)
- ✓ Red cell distribution width (RDW)

Red cell indices are calculated from:

- The red cell count
- Hgb concentration
- Haematocrit (PCV)

I. Mean cell volume (MCV)

- ♦ Is the average volume of red cell and is calculated from the hematocrit value & the red cell count.
- ♦ MCV provides information on red cell size. It is measured in femtolitres (fl) and is determined from the PCV and electronically obtained RBC count.
- ♦ It can be calculated as follows:

$$\text{MCV (fl)} = \frac{\text{PCV (L/L)}}{\text{No of RBCs/L}}$$

- ♦ MCV is expressed in femtoliters (fl) or cubic micrometer.

  1 femtoliter (fl) = 10^{-15}L = 1 cubic micrometer
- ♦ The MCV categorize red cells by size. Cells of normal size are called normocytic, smaller cells are microcytic, and larger cells are macropcytic.

Interpretation of MCV results

Normal value: Men & women 92 $\pm$ 9fl.

- Low MCV values are found in microcytic anaemias particularly iron deficiency, anaemia of chronic disease and thalassaemia.
- Raised MCV values are found in macrocytic anaemias, marked reticulocytosis, and chronic alcoholism.

II. Mean cell haemoglobin (MCH)

- ♦ The average weight of Hgb in a red blood cell is measured by the MCH.
- ♦ It is calculated from the haemoglobin and electronically obtained RBC count.

$$\text{MCH (pg)} = \frac{\text{Hgb (g/l)}}{\text{No of RBCs/L}}$$

- MCH is expressed in picograms (pg) or cubic micrometer.

  1 picograms (pg) = 10^{-12}g = 1 micro-microgram (µµg)

Interpretation of MCH result

- ✓ The reference range for MCH in health is 29.5 ± 2.5pg.
- ✓ Low MCH values are found in microcytic hypochromic anaemias and also when red cells are microcytic & normochromic.
- ✓ Raised MCH values are found in macrocytic normochromic anaemias.

III. Mean cell haemoglobin concentration (MCHC)

- MCHC measures the average concentration of Hgb in a red blood cell.
- It is calculated from Hgb and PCV as follows:

  $$MCHC\ (g/l) = \frac{Hgb\ (g/l)}{PVC\ (l/l)}$$

  ✓ MCHC is expressed in g/l.
- MCHC categorizes RBCs according to their concentration of Hgb.
  - ✓ Cells with a normal concentration of Hgb are called normochromic.
  - ✓ Cells with a lower than normal concentrations of Hgb are called hypochromic.
  - ✓ Because there is a physical limit to the amount of Hgb that can fit in a cell, there is no hyperchromic category.
- Just as MCV relates to the size of the cells, MCHC relates to the color of the cells. When examined under a microscope,
  - ✓ Normal red blood cells that contain a normal amount of Hgb stain pinkish red with a paler area in the center. These normochromic cells have a normal MCHC.
  - ✓ Cells with too little Hgb are lighter in color with a large pale area in the center. These hypochromic cells have a low MCHC.
- According to the MCHC index, anemia can be categorized as hypochromic & normochromic.

Interpretation of MCHC result

- The reference range for MCHC in health is 330 ± 15g/l.
- Low MCHC values are found in iron deficiency anaemia and other conditions in which the red cells are microcytic and hypochromic.
- An increased MCHC can occur in marked spherocytosis but this is a rare condition.

	MCHC	MCV	MCH
Reference range	315 -345 g/l	92 ± 9fl	29.5 ± 2.5pg.
Anemias			
I. Normocytic normochromic	Normal	Normal	Normal
II. Microcytic hypochromic	Reduced	Reduced	Reduced
III. Macrocytic normochromic	Normal	Increased	Increased

Table -1: Summary of red cell indices in common anemias.

CHAPTER 10

SCREENING BLEEDING DISORDERS

3.7.1. HEMOSTASIS

Hemostasis (Haema ⇨ Blood & Stasis ⇨ Arrest) is a complex process which continually ensures prevention of spontaneous blood loss & stops hemorrhage caused by damage of vascular system.

- Hemostasis is the arrest of blood flow & control of hemorrhage from an injured blood vessel.
- It is the process by which the blood is retained with in the vascular system. Hemostasis is initiated when a blood vessel is injured.
- The mechanism of haemostasis is very complex & involves platelets, the coagulation factors & fibrinolytic system.

Hemostasis has the following **functions**:

- Arrests bleeding
- Keeps blood in fluid state
- Localize reactions involved in the bleeding site
- Repair & reestablish the blood flow through the injured vessels
- Remove hemostatic plug

- If any of the above functions is exaggerated or impaired it will cause either thrombosis or hemorrhage respectively; so hemostasis is a balance between thrombosis & hemorrhage.

Components of normal hemostasis

The normal hemostatic process requires the following major components:

- ✓ Blood vessels
- ✓ Platelets
- ✓ Plasma coagulation factors
- ✓ Coagulation inhibitors
- ✓ Fibrinolytic system

1. Blood vessel

The blood vessel wall has three layers: intima, media, and adventitia. The intima consists of endothelium and subendothelial connective tissue and is separated from the media by the elastic lamina interna. Endothelial cells form a continuous monolayer lining all blood vessels.

Function of endothelial cells:

- Endothelial cells contain heparan sulphate that can activate antithrombin which is an important inhibitor of coagulation enzymes.
- It can also produce thrombomodulin to which thrombin can be attached to it & activate protein C. The activation of protein C results in the degradation & inhibition of factors Va & VIIIa and a carboxy peptidase which inhibits fibrinolysis.
- Endothelium produces von Willebrand factor (VWF), essential for platelet adhesion to the sub-endothelium.
- Angeotension II which is a local vasoconstrictor is produced by the endothelium.
- Under appropriate stimulation the endothelial cell can produce vasodilators such as nitric oxide (NO) and prostacyclin or vasoconstrictors such as endothelin and thromboxane.
- The sub-endothelium consists of connective tissues composed of collagen, elastic tissues, proteoglycans, and non-collagenous glycoproteins, including fibronectin and

VWF. After vessel wall damage has occurred, these components are exposed and are then responsible for platelet adherence.

2. Platelets

- The main function of platelets is the formation of mechanical plug during the normal hemostasis.
- The main steps in platelet functions are adhesion, activation with shape change, and aggregation.
- Following blood vessel injury platelets adhere to the exposed sub-endothelial connective tissue & up on adhesion platelets become spherical and extrude long pseudopods which enhance interaction between adjacent platelets.
- Platelet activation is then achieved by glycoprotein binding fibrinogen to produce platelet aggregation.
- Platelet aggregation may occur by at least two independent but closely linked pathways. The first one is through the metabolism of arachidonic acid & the second is through different platelet agonists.
- Thus platelets have at least three roles in haemostasis:
    - ✓ Adhesion and aggregation forming the primary haemostatic plug.
    - ✓ Release of platelet activating and procoagulant molecules.
    - ✓ Provision of a procoagulant surface for the reactions of the coagulation system.

3. Coagulation factors

Blood coagulation involves a biological amplification system in which relatively few initiation substances subsequently activate by proteolysis a cascade of circulating proteins (coagulation factors) which culminates the generation of thrombin & subsequently fibrin.

- Coagulation factors are zymogens, protein cofactors, membrane phospholipids & ions like calcium ion that play an active role in the development of the fibrin clot.
- By international agreement & common usage, the coagulation proteins are designed by Roman numerals: Factor I (fibrinogen) through XIII. Numeral VI is not used.
- The numeral order does not reflect reaction sequence.
- Roman numerals are not used for prekallikrein & high molecular weight kininogen (HMWK).
- The activated form of a coagulation factor is indicated by the appropriate Roman numeral followed by the suffix **"a"**.
- Coagulation factors can be divided in to three groups based on their properties.

Thrombin sensitive groups:

- ✓ Consists of factors I, V, VIII, XIII
- ✓ They are consumed during the coagulation process
- ✓ They are absent in serum & present in plasma
- ✓ Factors V & VIII are susceptible to denaturation & are reduced in quantity in stored plasma.

Vitamin K dependent group

- ✓ Consists of factors II, VII, IX & X
- ✓ Vitamin K is necessary for their synthesis
- ✓ Factors VII, IX and X are not consumed during coagulation process.
- ✓ All these factors are stable & reserved in stored plasma.

Contact group

- ✓ Composed of factors XI, XII, prekallikrein & HMWK

- ✓ They are not consumed during coagulation
- ✓ They are relatively stable

Coagulation factors Number and/or name	**Function**
I (Fibrinogen)	Forms clot (Fibrin)
II (Prothrombin)	Its active form IIa activates I, V, VIII, XI, XIII, Protein C , platelets
III (Tissue factor or Thromboplastin)	Co-factor of VIIa
IV (Calcium ion)	Required for coagulation factors to bind to phospholipid
V (Proaccelerin, Liable factor)	Co-factor of X with which it forms the prothrombinase complex
VII (Stable factor or proconvertin)	Activates IX, X
VIII (Antihemophilic factor)	Co-factor of IX with which it forms the tenase complex
IX (Christmas factor)	Activates X: forms tenase complex with factor VIII
X (Stuart – power factor)	Activates II: Forms prothrombinase complex with factor V
XI (Plasma thromboplastin antecedent)	Activates IX
XII (Hageman factor)	Activates factor XI & prekallikrein
XII (Fibrin stabilizing factor)	Cross links fibrin
Prekallikrein (Fletcher factor)	Activates XII & cleaves HMWK
HMWK (Fitzgerald factor)	Supports reciprocal activation of XII, XI & prekallikrein

3.7.2. BLOOD COAGULATION

The central event in the coagulation pathways is the production of thrombin, which acts upon fibrinogen to produce fibrin and thus the fibrin clot. This clot is further strengthened by the cross-linking action of factor XIII, which itself is activated by thrombin.

PHASES OF HEMOSTASIS

Hemostasis can occur in two phases.

A. Primary hemostasis

- ♦ It is defined as the formation of primary plug
- ♦ It involves platelets, the blood vessel wall & von Willbrand factor (vWF)
- ♦ Vasoconstriction is the initial event in hemostasis; it retards vascular blood loss & slows local blood flow enhancing the adherence of platelets to the exposed sub-endothelial surface & activation of the coagulation process.
- ♦ The formation of primary platelet plug involves platelet adhesion, platelet activation & platelet aggregation.

Platelet adhesion

- ✓ Is the adhesion of platelets to the exposed sub-endothelium.
- ✓ This is mediated by vWF & fibrinogen which bind to the glycoprotein membrane of platelets.

Platelet activation

Adhesion of platelets causes platelets:

- To change their shape from dic shapes to spherical shape with the extrusion of many pseudopods.
- To activate the collagen receptors on their surface
- To undergo release reaction

Platelet aggregation

- Up on activation platelets synthesize & release thromboxine A_2 (TXA_2) and platelet activation factor (PAF).
- TXA_2 & PAF together with ADP and serotonin, which are secreted from dense granules, cause activation and recruitment of additional platelets which bind to the adhered platelets resulting in platelet aggregation.
- This aggregation leads to the formation of primary platelet plug. This primary platelet plug should be stabilized by the fibrin clot.

B. Secondary hemostasis

- Is the process of formation of fibrin through the coagulation cascade.
- This involves circulating coagulation factors & cofactors, calcium and platelets.
- Platelets provide a source of phospholipid (PF3) and a binding surface up on which the coagulation cascade proceeds.

Pathways for the coagulation process

The coagulation cascade of secondary hemostasis has two pathways:

1. The contact activation pathway (formerly known as the Intrinsic pathway), and
2. The tissue factor pathway (formerly known as the Extrinsic pathway)

Both the above pathways lead to a common pathway. The various factors, their precursors and other reacting substances respond in an orderly controlled process called the coagulation cascade.

Contact activation pathway (Intrinsic pathway)

In this pathway, all the necessary components are found with in the circulating blood. When blood comes in contact with a foreign surface; for example exposed collagen fibres in the wall of the blood vessel or a glass surface; a series of reactions mediated by enzymes start.

On contact with a foreign surface, prekallikrein & HMWK participate in the activation of factor XII to XIIa. Factor XIIa in turn activates factor XI to form XIa. This process continues further involving factors IX, VIII, & X. Factor X is converted to Xa by the action of calcium ions and phospholipid on the platelets.

Tissue factor pathway (Extrinsic pathway)

The extrinsic system is initiated when there is tissue damage together with an injured blood vessel. The damaged tissue releases thromboplastin which is not normally present in the blood. This thromboplastin along with factor VII in the presence of calcium activates factor X to produce Xa. The extrinsic pathway is much shorter and quicker than the intrinsic pathway.

Common pathway

The common pathway involves the activation of factor X to Xa via the extrinsic or intrinsic pathway. Factor Xa, in the presence of calcium ions, platelet factor (PF3) and factor V, converts factor II (prothrombin) to the active enzyme thrombin. Thrombin acts on factor I (fibrinogen) to convert it to fibrin. Factor XIII helps in the formation of stabilized, stronger clot.

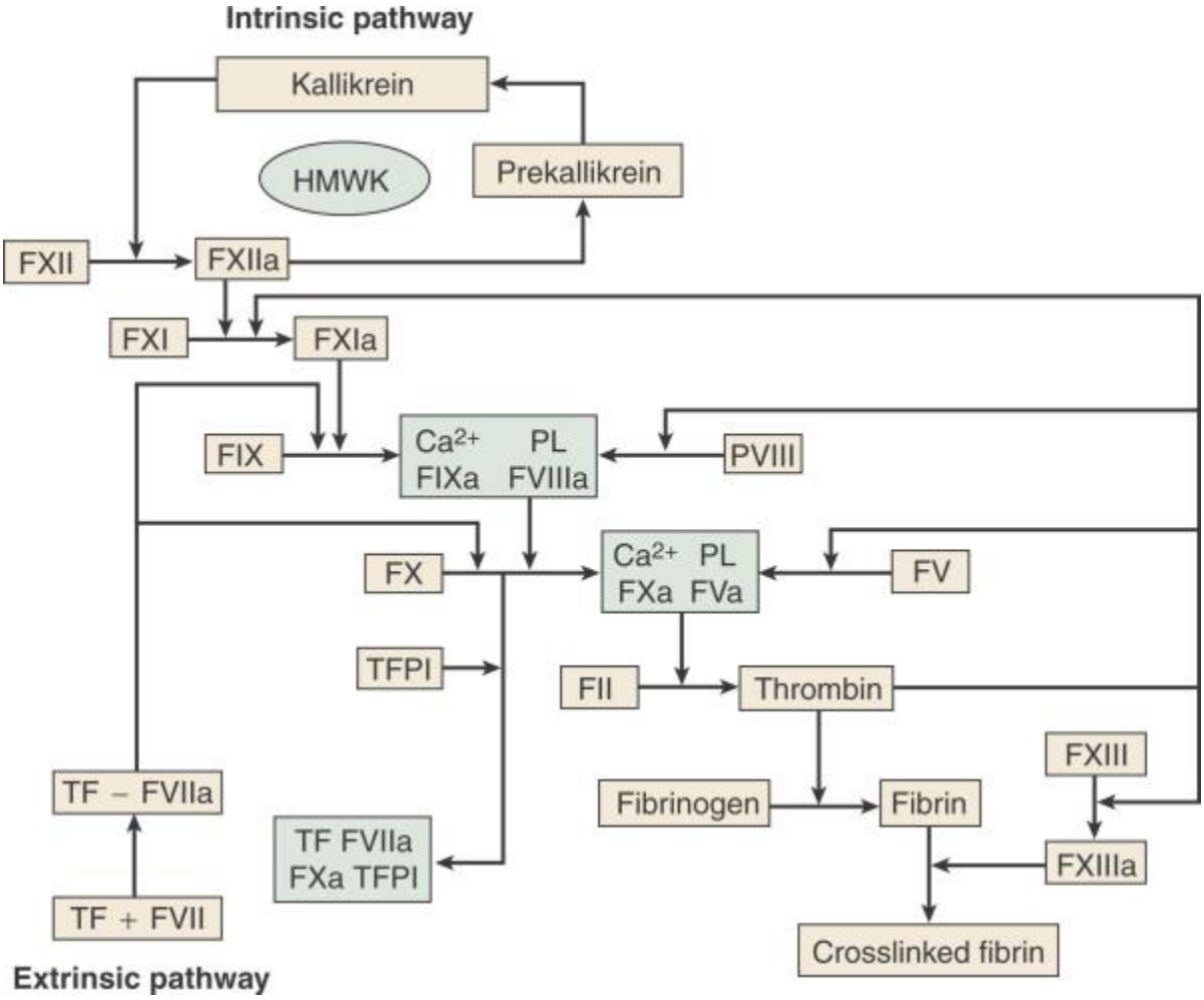


Figure 3.7.1: Schematic representation of the coagulation network.
The major interactions are shown by the bold arrows. HMWK, high molecular weight kininogen; PL, phospholipid; TFPI, tissue factor pathway inhibitor

4. Coagulation inhibitors

A number of mechanisms exist to ensure that the production of the fibrin clot is limited to the site of injury and is not allowed to propagate indefinitely. First there are a number of proteins that bind to and inactivate the enzymes of the coagulation cascade.

Antithrombin III

- ✓ Is the principal physiological inactivator of thrombin & factor Xa.
- ✓ It binds to factor IIa forming an inactive thrombin–antithrombin complex, which is subsequently cleared from the circulation by the liver.
- ✓ It also capable of inactivating factors IXa, XIa, and XIIa but to lesser degrees than thrombin.
- ✓ These inhibiting action are progressive but become immediate in the presence of heparin.

Protein C

- ✓ Is a vitamin K dependent plasma enzyme synthesized in the liver.
- ✓ It inactivates major proteins factor Va & VIIIa.

Protein S

- ✓ Is also a vitamin K dependent plasma enzyme synthesized in the liver.
- ✓ Circulating protein S is active as cofactor of protein C.

Heparin cofactor II

- ✓ Is a glycoprotein of hepatic origin
- ✓ Is a very specific inhibitor & only neutralizes thrombin effectively.

5. Fibrinolytic system

- ♦ Fibrinolysis is the process of removing unwanted, insoluble deposits form as a result of coagulation.
- ♦ It is a physiological process in which a fibrin clot is broken down by enzymes in to soluble fragements.

The fibrinolytic system involves conversion of a plasma proenzyme plasminogen in to plasmin. Plasmin digests fibrin and breaks it in to soluble fragments.

Plasminogen $\xrightarrow{\text{Activators}}$ plasmin

Fibrin polymer $\xrightarrow{\text{plasmin}}$ soluble fragments

3.7.3. DISORDERS OF BLOOD COAGULATION

The common causes of coagulation disorders include deficiencies of coagulation factors, inhibitors of coagulation factors and defects in platelet function. Disorders of blood coagulation can result in uncontrolled hemorrhaging into joints, muscles and deep tissues with the formation of haematomas. Coagulation disorders may be:

A. Hereditary, as in haemophilia A due to a deficiency of clotting factor VIII (commonest hereditary coagulation disorder), haemophilia B (Christmas disease) due to a deficiency of factor IX, and Von Willebrand's disease caused by a deficiency or abnormality of the von Willebrand factor resulting in a defect in platelet adhesion.

B. Acquired coagulation disorders as in vitamin K deficiency, severe liver disease, and disseminated intravascular haemolysis (DIC) associated with infections, obstetric complications (septic abortion, eclampsia, fetal retention, & ruptured uterus), haemorrhagic disease of the newborn, snake envenomation, and malignancies.

3.7.4. LABORATORY ASPECTS OF THE BLEEDING DISORDER

Sample collection and preparation

- ✓ Venous blood samples are commonly recommended.
- ✓ All blood samples must be collected by personnel who are trained and experienced in the technique.
- ✓ Patients requiring venipuncture should be relaxed and in warm surroundings.
- ✓ Excessive stress & vigorous exercise cause changes in blood clotting & fibrinolysis. Stress and exercise will increase factor VIII, VWF antigen, and fibrinolysis.
- ✓ Whenever possible, venous samples should be collected without applying tourniquet, allowing the blood to enter the syringe by continuous free flow or by the negative pressure from an evacuated tube.
- ✓ Venous occlusion causes haemoconcentration, increase of fibrinolytic activity, platelet release, and activation of some clotting factors.
- ✓ The most commonly used anticoagulant for coagulation samples is trisodium citrate. A 32 g/l solution is recommended. For routine blood coagulation testing, 9 volumes of blood are added to 1 volume of anticoagulant.

TESTS OF HEMOSTASIS & COAGULATION

A. BLEEDING TIME

- The bleeding time is a measure of vascular and platelet integrity.
- It is measured by determining the time required for bleeding to stop from small subcutaneous vessels that have been severed by a standard incision.
- It is a screening test for detecting disorders of platelet function, vW disease, & vascular problems.

- It is directly affected by platelet number and the ability of platelets to form a plug.

➢ Three generation of tests have been developed each with increasing standardization of a wound of uniform depth and length.

I. Duke method

The test is performed using a sterile blood lancet to make a cut either on the finger or ear lobe, recording the time, touching a piece of filter paper the cut every 30 seconds until bleeding ceases and record the final time.

The normal value is 1 – 3 minutes.

The method is no more recommended today owing to the following drawbacks:

- ✓ It is not possible to standardize the depth of the wound
- ✓ If the patient has a significant bleeding disorder, bleeding in to the soft subcutaneous tissue in the ear lobe could lead to a large hematoma.

II. The Ivy method

Principle: Three incisions are made on the volar surface of the forearm using a lancet known as a Stylet that has a shoulder to limit the depth of the cut. The bleeding times of the three wounds are averaged.

Advantage

- ✓ Standardization of incision
- ✓ Improved standardization of pressure

Test procedure

Required materials

- ➢ Sphygmomanometer
- ➢ Cleansing swabs
- ➢ Stopwatch
- ➢ Circular filter paper
- ➢ 70% alcohol
- ➢ Cotton wool pads or gauze
- ➢ Disposable stylets
- ➢ Sterile bandages

Method

1. Apply the sphygmomanometer cuff around the patient's arm above the elbow, inflate to 40 mm Hg, and keep it at this pressure throughout the test.
2. Clean the area with 70% ethanol and allow to air dry.
3. Choose an area of skin on the volar surface of the forearm that is devoid of visible superficial veins.
4. Using a Stylet make three incisions. Start one stopwatch for each puncture wound when bleeding begins.
5. With the edge of a circular filter paper, at 15 second intervals blot off the blood exuding from the cut. Avoid contact with the wound during this procedure because this may disturb the formation of the platelet plug.
6. The end point is reached when blood no longer stains the filter paper. Record the time at this point for each puncture wound. Average the bleeding time of the three wound.
7. Clean the puncture sites and apply a sterile bandage.

Normal value

- Children **<** 8 Minutes
- Adult **<** 6 minutes

III. The template method

Principle: The same as Ivy's

Test procedure

Required materials

- ➢ Sphygmomanometer
- ➢ Cleansing swabs
- ➢ Stopwatch
- ➢ 70% alcohol
- ➢ Cotton wool pads or gauze
- ➢ Template, blade handle & gauge

- Circular filter paper
- Surgical blade
- Sterile bandages

Method

1. Mount the surgical blade on the handle. Standardize the depth of the blade by placing the handle on the gauge & arrange that the tip of the blade protrudes 1 mm through the template slit.
2. Apply the pressure cuff on the upper arm; gently cleans the forearm with an alcohol pad and allow to dry.
3. Inflate the cuff to 40mmHg. Maintain this pressure through out the test.
4. Place the template on the forearm about 5cm from the antecubital fossa.
5. Apply firm pressure to the template while introducing the blade at a right angle on the upper portion of the template slot. This guides the blade to make an incision that is 1mm deep and 9mm long. Make the incision smoothly & rapidly. Start the stop watch immediately. Make a second or third incision parallel to the first & start separate stop watches.
6. Gently the bloo d with a circular filter paper at 30 second intervals.
7. The end point is reached when blood no longer stains the filter paper. Record the time at this point for each wound. Average the bleeding times of the two or three incisions.
8. Clean the wounds & apply a bandage or adhesive strip.

Normal value: 2 – 7 minutes with 9mm length incision.

Source of errors in bleeding time

I. Mistakes that will decrease the bleeding time
- ✓ Failure to thoroughly cleans the area to be punctured
- ✓ Choosing and puncturing a cold bloodless area
- ✓ Making only superficial puncture

II. Mistakes that will result prolonged bleeding time
- ✓ Choosing and puncturing red fleshed area
- ✓ Making too deep puncture
- ✓ higher pressure
- ✓ touching the filter paper to the wound and removing the platelet plug

Interpretation of results

A prolonged bleeding time may result from the following:
- ✓ *Thrombocytopenia*. It is advisable to check the platelet count before carrying out the bleeding time test. Patients with a platelet count below 50 × 10^9/l may have a very long bleeding time and the bleeding may be difficult to arrest.
- ✓ *Disorders of platelet function*.
- ✓ *VWD*. This may occur as a result of defective platelet adherence to the subendothelium in the absence of a normal amount or of normally functioning VWF.
- ✓ Occasionally, severe deficiency of factor V or XI.
- ✓ Vascular abnormalities.

B. COAGULATION TIME (Clotting time)

- Is the time required for blood to coagulate.
- It may be used in the diagnosis and treatment of hemorrhagic disease.
- It is an important test before operation.

Methods

I. Capillary blood method
- ✓ Slide method
- ✓ Capillary tube method

II. Venous blood method
- ✓ Lee & White method

- ✓ Howell method

Lee & White method

Principle: Whole blood is delivered using carefully controlled venipuncture & collection process in to a standardized glass tube. The clotting time of blood is recorded & expressed in minutes.

Procedure

1. Draw the venous blood, recording the time at which blood appears in the syringe.
2. Deliver the blood inn three test tubes that have been incubated at 37^{o}C.
3. Tilt the tube every 30 seconds until coagulation takes place.
4. Record the time & report the average.

Interpretation

Normal range: 4 – 10 minutes.

The clotting time is prolonged in defects of intrinsic & extrinsic coagulation.

C. Prothrombin Time

Principle: The prothrombin time is the time required for plasma to clot after tissue thromboplastin and optimal amount of calcium chloride have been added.

- ✓ It is used to asses the overall efficiency of the extrinsic clotting system.
- ✓ It is used to screen factors VII, V, and X deficiencies.
- ✓ The test depends on the activity of factors V, VII, X, II and I.

Test procedure

Required materials

- ➢ Patient and Control Plasma Samples
- ➢ $CaCl_2$ (0.025 mol/l)
- ➢ Round bottom glass test tubes
- ➢ Thromboplastin
- ➢ Water bath
- ➢ Stop watch

Method

Deliver 0.1 ml of plasma into a glass tube placed in a water-bath and add 0.1 ml of thromboplastin. Wait 1–3 min to allow the mixture to warm. Then add 0.1 ml of warmed $CaCl_2$ and start the stopwatch. Mix the contents of the tube and record the end-point. Carry out the test in duplicate on the patient's plasma and the control plasma.

Some thromboplastins contain calcium chloride, in which case 0.2 ml of thromboplastin is added to 0.1 ml plasma and timing is started immediately.

Interpretation

Normal value: 11 – 16 seconds

The common causes of prolonged prothrombin time are as follows:

- ✓ Administration of oral anticoagulant drugs (vitamin K antagonists)
- ✓ Liver disease, particularly obstructive
- ✓ Vitamin K deficiency
- ✓ Disseminated intravascular coagulation

D. Activated Partial Thromboplastin Time (APTT) Test

- The APTT is a screening test of the intrinsic clotting system.
- It will detect the inhibition or deficiency of one or more of the following factors: prothrombin, V, VIII, IX, X, XI, XII and fibrinogen.

Test Principle: Kaolin (surface activator) and platelet substitute (phospholipid) are incubated with citrated plasma at 37^{o}C for the time specified in the test method. Calcium chloride (CaCl2) is added and the time taken for the mixture to clot is measured.

Test procedure

Required materials

- Patient and Control Plasma Samples
- $CaCl_2$ (0.025 mol/l)
- Round bottom glass test tubes
- Platelet poor plasma
- Phospholipid
- Thromboplastin
- Water bath
- Stop watch
- Kaolin (5g/l) platelet substitute mixture

Method

1. Mix equal volumes of the phospholipid reagent and the kaolin suspension and leave in a glass tube in the water bath at 37°C.
2. Place 0.1 ml of plasma into a new glass tube. Add 0.2 ml of the kaolin–phospholipid solution, mix the contents, and start the stopwatch simultaneously.
3. Leave at 37°C for 10 min with occasional shaking. At exactly 10 min add 0.1 ml of prewarmed $CaCl_2$ and start a second stopwatch.
4. Record the time taken for the mixture to clot. Repeat the test at least once on both the patient's plasma and the control plasma.

Interpretation

The normal range is typically within 30–40 seconds. The actual times depend on the reagents used and the duration of the pre-incubation period, which varies in manufacturer's recommendations for different reagents.

The common causes of a prolonged APTT are as follows:

- ✓ Disseminated intravascular coagulation
- ✓ Liver disease
- ✓ Massive transfusion with plasma-depleted red blood cells
- ✓ Deficiency of a coagulation factor other than factor VII

E. Thrombin Time (TT)

Principle: Thrombin is added to plasma and the clotting time is measured.

- ♦ The TT determines the rate of thrombin induced, cleavage of fibrinogen to fibrin monomers and subsequent polymerization to form fibrin clot.
- ♦ The TT is affected by the concentration and reaction of fibrinogen.
- ♦ The procedure is particularly useful when the other parameters such as APTT & PT are prolonged.

Test procedure

1. Incubate 0.2ml of plasma at 37oC for 3min.
2. Add 0.1ml of pre-warmed thrombin - calcium solution and record the time.
3. Observe the clot formation and record the time.

Interpretation

The normal value is less than 20 seconds.

The common causes of prolonged TT are:

- ✓ Fibrinogen deficiency (Fibrinogen is less than 100mg/dl)
- ✓ Presence of thrombin inhibitors
- ✓ High concentration of immunoglobulins

Chapter 10

SYTEMIC LUPUS ERYTHEMATOSUS (SLE)

Systemic lupus erythematosus (SLE) is an autoimmune relapsing disease in which auto-antibodies to nuclear proteins are produced, including anti-DNA antibodies. It is SLE a chronic rheumatic disease which affects joints, muscles and other parts of the body.

Immune complexes cause tissue damage. The features of the disease include arthritis, arthralgia, fever, malaise, typical facial 'butterfly' skin rash, cytopenias.

Genetic, viral, and environmental factors are thought to be involved in the development of SLE.

Laboratory features

- ❖ Leukopenia (neutropenia and lymphopenia), thrombocytopenia, and usually anaemia.
- ❖ Occasionally circulating lupus anticoagulant is present which can cause prolonged prothrombin time and activated partial thromboplastin time (APTT).
- ❖ Presence of anti-deoxyribonuclear protein (anti-DNP) antibodies in active SLE which can be detected by a slide agglutination test using latex particles coated with deoxynucleoprotein.
- ❖ Presence of Lupus erythematosus cells in buffy coat smears prepared from defibrinated blood.

Lupus erythematosus (LE) cell

- ✓ Neutrophils that contain inclusions that are reddish-purple & structure less are called LE cells.
- ✓ The LE cell is usually a neutrophil polymorph (occasionally a monocyte or eosinophil) that has ingested the altered nucleus of another polymorph.
- ✓ The bulk of the cell is occupied by a spherical, homogenous mass that stains purplish brown. The lobes of the ingesting polymorph appear wrapped around the ingested material.

Demonstration of LE cells

Many methods of demonstrating LE cells have been described. It seems clear that some degree of trauma to leukocytes is necessary for a successful preparation for the LE factor does not appear to be capable of acting up on healthy living leucocytes.

A good method of achieving the necessary degree of trauma is to rotate the whole blood sample to which glass beads have been before concentrating the leucocytes by centrifugation.

Method using patient's blood

The rotary method of Zinkham and Conley

1. 1ml of patient's blood collected in heparin is transferred in to a glass test tube.

2. Four glass beads are added & the tube is sealed with a tightly fitting rubber bung.

3. The preparation is rotated at 33rpm at room temperature for 30 minutes & placed at 37oC for 10 – 15 minutes.

4. The contents of the tube are transferred to a Wintrobe tube & centrifuge at 200g for 10 minutes.

5. Buffy coat smears are prepared, dried in the air, fixed in methanol and are stained with Romanowsky stains in the usual manner.

Examination of Films

The films especially their edges and tails are searched for a minimum of 10 minutes (a minimum of 500 polymorphs should be counted) before a negative report is given.

Appearance of LE cells

An LE cell is a neutrophil that has ingested nuclear material that has been denatured by antibody to nucleoprotein. The lyzed nuclear inclusion fills the neutrophil & stains an even pale mauve color. It is surrounded by the lobes of the neutrophils.

LE cells must be differentiated from "tart cells" which are usually monocytes that have phagocytosed the nucleus of a lymphocyte. The ingested nuclear material is well preserved in tart cells in contrast to the LE cell inclusion body.

Interpretation

A positive LE cell test is very suggestive of SLE and the test is a very useful diagnostic test. The test is positive in 75% of patients with SLE.

CHAPTER 12

OSMOTIC FRAGILITY OF THE RED CELL

The red cell envelope is a semi-permeable membrane. When red cells are placed in hypotonic solution they imbibe fluid & thereby swell. It follows then that there is a limit to the hypotonicity of a solution that normal red cells can stand.

The normal biconcave disc shape of the red blood cells are linked to excess surface area to volume ratio. In addition RBCs has a fluidy nature, these allow the normal deformability of the red cells. If the red cell area decreased in relation to its volume spherical shape cell will be formed which impart some rigidity, this interfere with the passage in the microcirculation.

Osmotic fragility Test (OFT)

- OFT is the measure of the rate of hemolysis of RBCs when exposed to different hypotonic solution of saline solution (NaCl).
- The OFT roughly evaluate the relation ship of surface area to volume of normal & abnormal cells.

Example: Sperocytes show a decrease resistance to lysins in hypotonic solution (Eg. 0.65%) that do not deform normal red cells.

- Increased OFT is indicative of red cell sphere rformation. i.e, a decrease surface area to volume ratio.
- Increased resistance to hypotonic solution ⇨ a decrease OFT ⇨ an increased surface area, volume, or biochemical content of red cells.

Example: Target cells fail to lyse completely in low saline solution (0.3%) which completely lyse normal red cells.

- The greatest usefulness of OFT is in the diagnosis of hereditary spherocytosis. The test may also be used in screening for thalassaemia.

Parpart and coworkers method of OFT determination

Principle: Small volumes of blood are mixed with a large excess of buffered saline solutions of varying concentration. The fraction of red cells lysed at each saline concentration is determined photometrically. The test is normally carried out at room temperature (15-25°C).

Reagents: Prepare a stock solution of buffered sodium chloride, osmotically equivalent to 100 g/l (1.71mol/l) NaCl.

In preparing hypotonic solutions for use, it is convenient to make first a 10 g/l solution from the 100 g/l NaCl stock solution by dilution with water. Dilutions equivalent to 9.0, 7.5, 6.5, 6.0, 5.5, 5.0, 4.0, 3.5, 3.0, 2.0, and 1.0 g/l are convenient concentrations. Intermediate concentrations such as 4.75 and 5.25 g/l are useful in critical work, and an additional 12.0 g/l dilution should be used for incubated samples.

It is convenient to make up 50 ml of each dilution. The solutions keep well at 4°C if sterile, but should be inspected for moulds before use and discarded if moulds develop.

Specimen: Heparinized venous blood or defibrinated blood may be used; oxalated or citrated blood is not suitable because of the additional salts added to it. The test should be carried out within 2 hours of collection with blood stored at room temperature or within 6 hours if the blood has been kept at 4°C.

Procedure

1. Deliver 5ml of each of the 11 saline solutions into test tubes. Add 5ml of water to the 12th tube.

2. Add to each tube 50 µl of well-mixed blood, and mix immediately by inverting the tubes several times, avoiding foam.

3. Leave the suspensions for 30 minutes at room temperature. Mix again, and then centrifuge for 5 minutes at 1200 rpm.

4. Remove the supernatants and estimate the amount of lysis in each using a spectrometer at a wavelength setting of 540 nm. Use as a blank the supernatant from tube 1 (osmotically equivalent to 9 g/l NaCl).

5. Assign a value of 100% lysis to the reading with the supernatant of tube 12 (water), and express the readings from the other tubes as a percentage of the value of tube 12. Plot the results against the NaCl concentration (Fig. 3.9.1).

Note

A. The measurement of osmotic fragility is a simple procedure that requires a minimum of equipment. It will yield gratifying results if carried out carefully.
B. The blood must be delivered into the 12 tubes with great care. The critical point is not that the amount be exactly 50 µl, but rather that the amount added to each tube must be the same.

The sigmoid shape of the normal osmotic fragility curve indicates that normal red cells vary in their resistance to hypotonic solutions. Indeed, this resistance varies gradually (osmotically) as a function of red cell age, with the youngest cells being the most resistant and the oldest cells

are being the most fragile. The reason for this is that old cells have higher sodium content and a decreased capacity to pump out sodium.

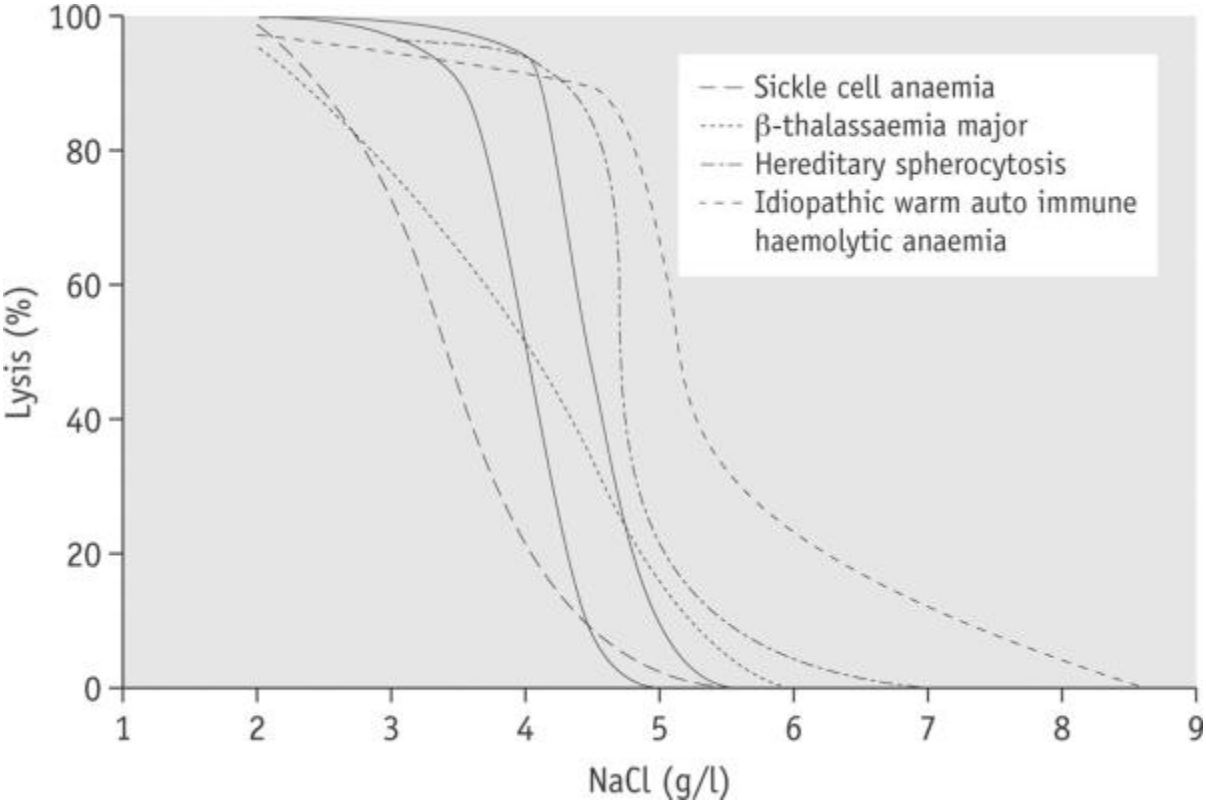


Figure 3.9.1 Osmotic fragility curves. Osmotic fragility curves of patients suffering from the following: sickle cell anaemia, β-thalassaemia major, hereditary spherocytosis, and "idiopathic" warm autoimmune haemolytic anaemia. The normal range is indicated by the unbroken lines.

Reporting the Results of Osmotic Fragility Tests

Osmotic fragility most often has been expressed in terms of the highest concentration of saline at which lysis is just detectable (initial lysis or minimum resistance) and the highest concentration of saline in which lysis appears to be complete (complete lysis or maximum resistance). It is, however, useful also to record the concentration of saline causing 50% lysis (i.e., the median corpuscular fragility [MCF]) and to inspect the entire fragility curve (Fig. 3.9.1).

The findings in health are summarized the following table:

Amount of hemolysis	**Fresh blood (g/l NaCl)**
Initial lysis	5.0
Complete lysis	3.0
median corpuscular fragility (MCF) (50% lysis)	

	4.0–4.45

Interpretation of Results

- The osmotic fragility of freshly taken red cells reflects their ability to take up a certain amount of water before lysing. This is determined by their volume-to-surface area ratio.
- The ability of the normal red cell to withstand hypotonicity results from its biconcave shape, which allows the cell to increase its volume by about 70% before the surface membrane is stretched; once this limit is reached lysis occurs.
- Spherocytes have an increased volume-to-surface area ratio; their ability to take in water before stretching the surface membrane is thus more limited than normal and they are therefore particularly susceptible to osmotic lysis.
- The increase in osmotic fragility is a property of the spheroidal shape of the cell and is independent of the cause of the spherocytosis.
- Decreased osmotic fragility indicates the presence of unusually flattened red cells (leptocytes) in which the volume-to-surface area ratio is decreased. Such a change occurs in iron deficiency anaemia and thalassaemia in which the red cells with a low MCH and MCV are unusually resistant to osmotic lysis.

Factors Affecting Osmotic Fragility Tests

In carrying out OFT by any method, three variables capable of markedly affecting the results must be controlled, quite apart from the accuracy with which the saline solutions have been made up. These are as follows:

- ✓ The relative volumes of blood and saline
- ✓ The final pH of the blood in saline suspension
- ✓ The temperature at which the tests are carried out