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Practical Note

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Biochemistry of Blood
(BCH 471)

TABLE OF CONTENTS

EXPERIMENT (1)

1. Separation of Plasma and Serum from Whole Blood

1.1 Objectives:

1- To know how to separate plasma and serum from whole blood.
2- To know what is haemolysis and the nature of haemolysing agents.
3- To detect the presence of Blood in a sample.

1.2 Introduction

The average person circulates about 5L of blood (1/13 of body weight), of which 3L is plasma and 2L is cells. Plasma fluid derives from the intestines and organs, and provides a vehicle for cell measurement. The cells are produced primarily by bone marrow and account for blood "solids". Blood cells are classified as red blood cells (erythrocytes), white cells (leukocytes) and platelets. The size of cells differs: white cells are the largest, red cells fall into the middle, and platelets are the smallest.

1.2.1 The General Functions of Blood

The general functions of blood are in metabolism and its regulation, transport, osmotic balance and defense. The metabolic and transport roles of blood overlap to some extent, for instance in the carriage of oxygen and carbon dioxide. Blood plays an important part in the body's defense mechanism. The immune response system is able to recognize foreign material within the body and a sequence of events is triggered that neutralizes and destroys the foreign material. The complex composition of blood is not constant, but changes during stress, starvation, exercise and as the result of injury or disease.

1.2.2 Whole Blood

It is living tissue that circulates through the heart, arteries, veins, and capillaries carrying nourishment, electrolytes, hormones, vitamins, antibodies, heat, and oxygen to the body's tissues. Whole blood contains

red blood cells, white blood cells, and platelets suspended in fluid called plasma. If blood is treated to prevent clotting and permitted to stand in a container, the red blood cells, which weigh more than the other components, will settle to the bottom; the plasma will stay on top; and the white blood cells and platelets will remain suspended between the plasma and the red blood cells. A centrifuge may be used to fasten this separation process. The platelet-rich plasma is then removed and placed into a sterile bag, and it can be used to prepare platelets and plasma.

1.2.3 Blood Cells

1.2.4 Red Blood Cells

They are perhaps the most recognizable component of whole blood. Red blood cells contain hemoglobin, a complex iron-containing protein that carries oxygen throughout the body and gives blood its red color. The percentage of blood volume composed of red blood cells is called the "hematocrit". The average hematocrit in an adult male is 47 percent. There are about one billion red blood cells in two to three drops of blood, and, for every 600 red blood cells, there are about 40 platelets and one white cell. Manufactured in the bone marrow, red blood cells are continuously being produced and broken down. They live for approximately 120 days in the circulatory system and are eventually removed by the spleen.

1.2.5 White Blood Cells

They are responsible for protecting the body from invasion by foreign substances such as bacteria, fungi, and viruses. The majority of white blood cells are produced in the bone marrow, where they outnumber red blood cells by two to one. However, in the blood stream, there are about 600 red blood cells for every white blood cell. There are several types of white blood cells; Granulocyes and macrophages protect against infection by surrounding and destroying invading bacteria and viruses, and lymphocytes aid in immune defense.

1.2.6 Platelets (or thrombocytes)

They are very small cellular components of blood that help the clotting process by sticking to the lining of blood vessels. Platelets are made in the bone marrow and survive in the circulatory system for an average of 9-10 days before being removed from the body by the spleen. The platelet is vital to life, because it helps prevent massive blood loss resulting from trauma, as well as blood vessel leakage that would otherwise occur in the course of normal, day-to-day activity.

1.2.7 B. The Liquid Phase of Blood

1.2.7.1 Plasma

It is the liquid portion of the blood in which red and white blood cells and platelets are suspended. Plasma, which is 90 percent water, constitutes about 55 percent of blood volume. Plasma contains albumin (the chief protein constituent), fibrinogen (responsible, in part, for the clotting of blood), globulins (including antibodies), and other clotting proteins.

1.2.7.2 Serum

Serum resembles plasma in composition but lacks the coagulation factors. It is obtained by letting a blood specimen clot prior to centrifugation or by centrifugation of plasma to precipitate Fibrinogen and the liquid phase will be the serum. Serum is preferred for many tests as the anticoagulants in plasma can sometimes interfere with the results.

1.2.8 Collection of Blood Specimens

1.2.8.1 Capillary Blood

It is most frequently obtained from a finger or thumb. The most convenient place is one the thumb about 5 mm from the side of the nail. The tip of a finger is also used.

1.2.8.2 Venous Blood

It is most often used, while the blood may be taken from any prominent vein, a vein on the front of the elbow or forearm is almost universally employed.

1.2.9 Anticoagulants

If whole blood or plasma is desired, an anticoagulant must be added to the specimen immediately after it is drawn or placed into the tube into which the blood is collected.

1.2.10 Types of Anticoagulants

1.2.10.1 Heparin (20 mg / 10ml of blood)

It is the most satisfactory anticoagulant since it does not produce a change in red cell volume or interfere with subsequent determinations. It inhibits the formation of thrombin from prothrombin and thus preventing the formation of fibrin from fibrinogen.

1.2.10.2 EDTA. disod.salt (20 mg / 10ml of blood)

It is a chelating agent, drives its anticoagulant activity from the fact that it binds calcium, which is essential for the clotting mechanism.

1.2.10.3 Potassium Oxalate (20 mg / 10ml of blood)

Oxalates act by precipitating the calcium, and we use potassium oxalate since it is the most soluble. It inhibits blood coagulation by forming rather insoluble complexes with calcium ions, which is necessary for coagulation.

1.2.10.4 Sodium Citrate (30 mg / 10ml of blood)

It does not precipitate the calcium, but converts it into a non-ionized form, and hence prevent clotting of blood.

1.2.10.5 Sodium Fluoride (10 mg / 10ml of blood)

It acts as a weak anticoagulant, therefore larger amounts are required than of either oxalates or citrates. It has been used chiefly as a preservative since it inhibits red cell metabolism and bacterial action.

1.2.10.6 Laboratory Use of Plasma and Serum

Different anticoagulants interfere with different tests; using serum means the same sample can be used for many tests. Some tests require serum, others plasma, while some can be carried out on either or whole blood.

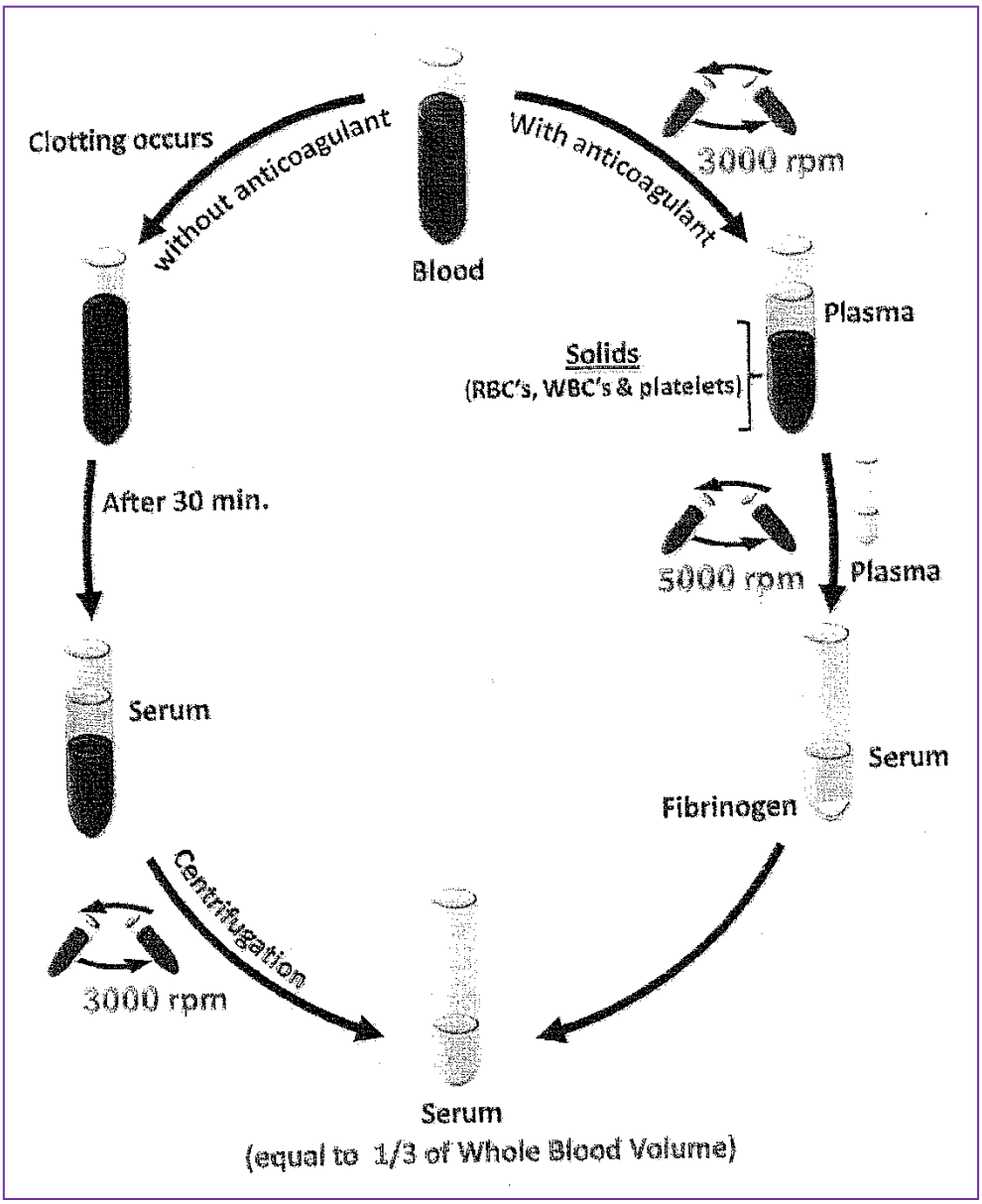
1.2.11 Changes in Blood on Keeping

1- Loss of carbon dioxide.
2- Conversion of glucose to lactic acid (glycolysis).
3- Increase in plasma inorganic phosphate.
4- Formation of ammonia from nitrogenous substances.
5- Passage of substances through the red cell envelope.
6- Conversion of pyruvate into lactate.

1.2.12 Normal Values

Total blood volume	55 – 80 ml / kg
RBC's volume	20 – 35 ml / kg
Plasma volume	30 – 45 ml / kg
Serum volume	18 – 27 ml / kg

How to Separate Blood Components



Part 1

Method of Separation of Plasma and Serum from Whole Blood

1.3 Materials

1- Whole blood
2- Centrifuge (up to 5000 rpm)
3- Centrifuge tubes suitable for the rotor of the centrifuge (preferably plastic and capped).
4- Disposable gloves
5- Disposable Pasteur pipette.
6- Measuring cylinder 10 ml.

1.4 Principle

1- If the blood is placed in a plain container and allowed to clot, the clot shrinks and expresses serum which can be obtained by centrifugation at 5000 rpm.
(Volume of serum obtained = 1/3 of the volume of whole blood)
2- Clotting can be prevented by placing the blood into a container containing an anticoagulant.
3- For hematological, biochemical, blood bank-related, immunological and other kinds of tests, plasma is obtained from whole blood. To prevent clotting, an anticoagulant is added to the blood specimen immediately after it is obtained and then we get (**Whole Blood**). The specimen is then centrifuged to separate plasma from blood cells. **Further centrifugation of plasma at 5000 rpm will precipitate the fibrinogen and we get SERUM (Plasma – Fibrinogen = Serum).**
4- To obtain platelets, units of platelets are prepared by using a centrifuge to separate the platelet-rich plasma from the donated unit of whole blood. The platelet-rich plasma is then centrifuged again to concentrate the platelets further.

1.5 Procedure

1- Into dry clean Centrifuge tube, pipette 15 ml of whole blood (V_1).

2- Place the centrifuge tube in the centrifuge machine and run it at 3000 rpm for 10 minutes. Centrifugation of whole blood separates the solid from the supernatant plasma.

3- Remove the tube, withdraw the liquid layer (plasma) by pasture pipette and measure its volume using small measuring cylinder (V_2). Determine the volume of blood cells too V_3 (equal to $V_1 - V_2$). Red blood cells which prepared from whole blood by removing the plasma, are kept to be used in part 2.

4- **Transfer** the supernatant (plasma) in another centrifuge tube and make further centrifugation at 3000 rpm. This will precipitate fibrinogen and the supernatant will be **SERUM**. Measure its Volume (V_4).

1.6.**RESULTS**

-Record your results in the following table:

	Component	**Total Volume**	**Percentage**	
1	Whole Blood	V_1 =		
2	RBC's	V_3 =		
3	Plasma	V_2 =		
4	Serum	V_4 =		

1.7.DISCUSSION

1.8.REFERANCES

Dacie.J.V. and Lewis. S.M .2001.Practical hematology .Longman group UK Limited. Ninth edition

EXPERIMENT (2)

2. Haemolysing Agents& Detection of blood

2.1 Introduction

2.1.1 Haemolysis (from the Greek Hemo: meaning blood, - lysis, meaning to break open):

It is the breaking open of red blood cells and the release of hemoglobin and the red cell contents into the surrounding fluid (plasma). The concentration of potassium inside red blood cells is much higher than in the plasma and so elevated potassium is usually found in biochemistry tests of hemolysed blood. Conditions that can cause hemolysis include: Immune reactions, Infections, Medications. Toxins and poisons.

2.1.2 Osmotic Pressure

Diffusion of water across a membrane – osmosis – generates a pressure called osmotic pressure.

If the pressure in the compartment into which water is flowing is raised to the equivalent of the osmotic pressure, movement of water will stop.

2.1.3 Isotonic Solution

A solution that has the same salt concentration as the normal cells of the body and the blood, having equal osmotic pressure.As opposed to a hypertonic solution or a hypotonic solution. Solutions which are isotonic with blood, such as sodium chloride 0.9%, have the same osmotic pressure as serum and they do not affect the membranes of the red blood cells. In hospitals, intravenous fluids are isotonic (iso = equal or even, and tonic = tonicity). Since the cell membranes of red blood cells are selectively permeable (allowing for diffusion of solvent, when the concentration of

solvent is greater on one side), equilibrium allows the red blood cells to retain their shape.

2.1.4 Hypotonic Solution

It has less than normal tension, the concentration of solute. In a hypotonic solution, there is a lower concentration of solute outside a cell, creating an environment with lower osmotic pressure than what is contained within the cell. For example, a hypotonic sodium chloride solution is less concentrated that isotonic or hypertonic solutions. If an IV solution was hypotonic (less solvent = more dilute), there would be less pressure on the red blood cells. The red blood cell would actually swell, in an attempt to equalize the concentration or tension (known as osmotic pressure) of solutes and solvents. As a result, the red blood cells would hemolyze or burst.

2.1.5 Examples of Hypotonic Solutions

1- 0.45% NaCl (half – normal saline solution); since normal saline is 0.9% NaCl, any solution less than 0.9% is hypotonic.
2- Dextrose 2.5% in water.
3- Dextrose 2% in water.

2.1.6 Hypertonic Solution

A solution that has a lower water potential and a correspondingly higher osmotic pressure than another solution. In a hypertonic solution, the plasma membrane of a red blood cell would separate and pull away from the cell membrane.

2.1.7 Examples of Hypertonic Solutions

1.2% NaCl (more than concentration in normal saline solution); since normal saline is 0.9% NaCl, any solution higher than 0.9% is hypertonic.

The next table indicates the changes on the nature of the red blood cells.

No.	Type of Solution	Changes on Red Blood Cell
1	**Isotonic Solution**	the cells were diluted in serum: Note the beautiful biconcave shape of the cells as they circulate in blood.
2	**Hypotonic Solution**	Most have swollen so much that they have ruptured, leaving what are called red blood cell ghosts. In a hypotonic solution, water rushes into cells.
3	**Hypertonic Solution**	A concentrated solution of NaCl was mixed with the cells and serum to increase osmolarity; water has flowed out of the cells, causing them to collapse.

2.2 Procedure

2.2.1 Materials

The packed Red Blood cells prepared from part 1 suspended in saline solution.

1- Saline Solution (0.9% sodium chloride NaCl) as an isotonic solution.
2- Sodium Chloride Solution 0.45%, as a hypotonic solution.
3- Sodium Chloride Solution 1.2% as a hypertonic solution.
4- Sucrose Solution 6%.
5- Sodium Hydroxide Solution 0.1 M.
6- Hydrochloric Acid Solution 0.1 M.
7- Water bath (variable temperature).
8- Dry clean test tubes.
9- Centrifuge.

2.2.2 Method

Into seven dry clean test tubes (A, B, C, D, E, F, G), pipette 3 drops of the suspended RBC's in Saline solution, and add to each tube as indicated the following table:

	Tube A	**Tube B**	**Tube C**	**Tube D**	**Tube E**	**Tube F**	**Tube G**
NaCl 0.45%	5 ml						
NaCl 1.2%		5 ml					
Sucrose 6%			5 ml				
NaOH 0.1 M				3 Drops			
HCl 0.1 M					3 Drops		
Dis. Water						5 ml	
NaCl 0.9%				5 ml	5 ml		5ml Heat slowly in the water bath and note the temperature at which haemolysis started.
Wait 30 minutes. Observe whether Haemolysis has taken place, i.e. whether the colour of the solution is changed or Centrifugation may be of help.							
Observation							
Conclusion							

2.3 Part 3: Detection of Blood by BENZIDINE TST

2.3.1 Principle

It is often necessary to detect the presence of small quantities of blood in urine, stomach contents etc. Minute amounts of blood in presence of peroxide catalyze the oxidation of benzidine giving a blue colour. However, the test is not specific for blood as peroxidases present in milk, potatoes and pus, as well as the ions of Fe^{+3}, Cu^{+2} and K^{+1} will give false positive results.

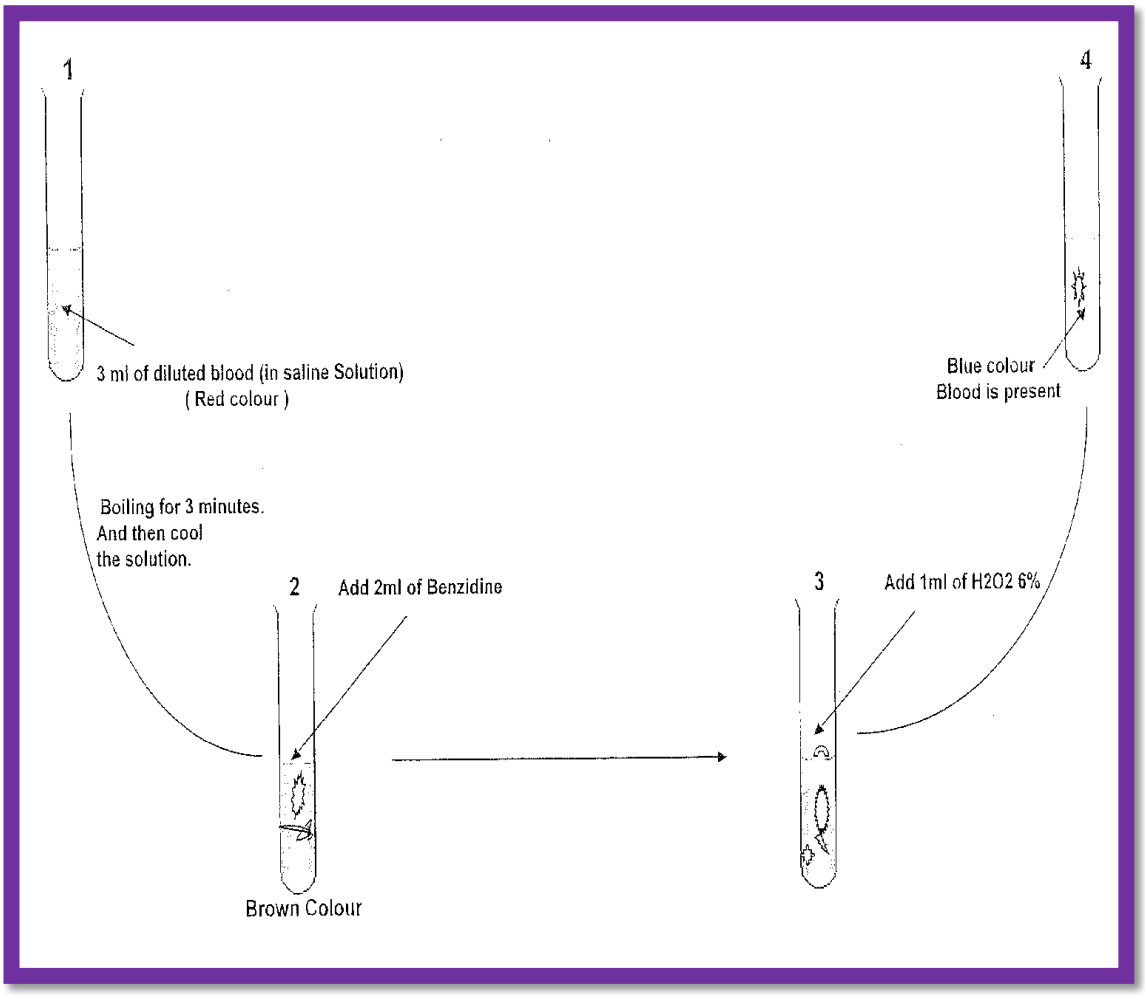
2.3.2 Reagents

1- Red Blood cells suspended in saline solution.
2- Benzidine solution 3% in glacial acetic acid (freshly prepared).
3- Hydrogen peroxide solution 6% (freshly prepared).
4- Boiling water bath.

2.3.3 Procedure

1- 3ml of suspended blood cells solution is boiled in water bath for 3 minutes and then cool it under tap water.
2- Add 2 ml of benzidine solution, followed by 1 ml of hydrogen peroxide solution. **A blue color is obtained.**

Detection of Blood by Benzidine Test



2.4.References

1- Varley.H; Gowenlock.A.H and Bell.M.1980. Practical Clinical Biochemistry, Vol. 1. 5th edition William. Heinemann Medical Books. Prince Henry's Hospital, Melbourne

2- Dacie .J.V.and Lewis.S.M.2001. Practical Haematology, 9th Ed. Churchill-Livingston

EXPERIMENT (3)

3. Separation of main protein in plasma and serum

3.1 Objectives:

To separate the principle proteins in serum and plasma and determination albumin/globulin ratio .

3.2 Introduction:

The main plasma protein are albumin,globulins and fibrinogen .fibrinogen may be salted out from plasma and identified by the biuret test and by the fact that clotting occurs on addition to serum which still contains active thrombin.
Proteins , which contain peptide linkages form a complex with copper in alkaline medium giving a violet color (Biuret reaction).
The intensity of the color is proportional to the number of peptide bonds and thus is a measure of the concentrations of proteins.

Normal value – Total protein 6.0 to 8.0 g/100 ml. albumin, 3.5 to 5.0 g/100 ml, globulin 2.3 to 3.6 g/ 100ml, fibrinogen, 0.3 to 0.6 g/ 100 ml.

Total serum protein consists of two main fractions, albumin and globulin
In normal people the A / G ratio is from 1.2 to 1.5 .
Generally the decrease in total protein is due to decrease in albumin fraction and increase is due to increase in globulin components. Dehydration is one conditionin which the increase in total protein is due to increase in both albumin and globulin fractions because of haemoconcentration. In this case the A / G ratio remains unaltered. Except dehydration, an increase in albumin very rarely occurs.

A low serum albumin may be due to:

i. A heavy loss of albumin in urine
ii. Loss of protein into alimentary tract.
iii. Malabsorption of protein from the alimentary tract
iv. Decreased formation by the liver due to defective liver and

v. Increase catabolism of protein or due to insufficient intake of protein in diet.

Total serum protein is appreciably reduced with low albumin in severe haemorrhage both acute and chronic, shock whether post operative following extensive burns or traumatic as in crush injuries, malignant disease of stomach, intestine and pancreas, peptic ulcer, sprue and steatorrheas etc.

In liver disease, particularly severe ones, albumin is reduced and A/G ratio altered. Total protein may be reduced but more commonly it is found within normal limits or even may be increased because globulin is increased in liver disease. Increase in globulin occurs most commonly in advanced liver disease, multiple myeloma and a number of chronic infections.

3.3 Materials

3.3.1.Chemicals

-blood serum.

-blood plasma.

-0.9 saline solution.

-2 N acetic acid.

-Biuret reagent: dissolve 9 g of sodium potassium tartrate in 500 ml of 0.2 N sodium hydroxide solution .add of 3 g of cupric sulphate and dissolve by stirring add 5 g of potassium iodide , make up the volume 1 liter with 0.2 N sodium hydroxide solution.

-saturated sodium chloride solution.

-5% calcium chloride solution.

-28% sodium sulphateor sulphit Na_2SO_3

-OR Saturated ammonium sulphate solution

3.3.2.Equipments

1-Water path at 37C.

Glassware

10 ml graduated pipettes.

Test tubes

3.4.Method:

3.4.1.Part I

A-fibrinogen:

Add an equal volume of saturate sodium chloride solution to 5 ml of plasma. fibrinogen precipitates.

To fibrinogen precipitate:

Redissolve in normal saline and divide into 3portion and carry out the following tests.

1-Biuret test: add an equal volume of biuret reagent , mix and allow to stand in a water path at 37^0c.

The development of a blue colour confirms the presence of protein (fibrinogen).

2-Clotting test :Add an equal volume of serum and a few drops of calcium chloride solution, incubate at 37^0c for 10 minutes.

Clotting occurs because serum contains active thrombin which converts fibrinogen to insoluble fibrin.

3-Heat coagulation test:add diluted acetic acid drop until the pH is between 5 and 6. Heat the contents of the tube. A cloudiness confirm the presence of protein

B- serum proteins

Two dry test tubes labeled P (total protein) and G (globulin)

	P	G
Saline	3.8 ml	-
28% sodium sulphate or saturated ammonium sulphate	-	3.8 ml
Serum	0.2 ml	0.2 ml

-Mix G by inverting the tube a couple of times, then filter immediately to separate globulins (a centrifuge can be used)

To globulin precipitate

Redissolve globulin in normal saline and divide into3portion and carry out the following tests.

1-Biuret test

2-Clotting test

3-Heat coagulation test.

3.3.2.Part I1

Label 7 test tubes as T (test), A (albumin), B (blank), and S (standard)

	T_1& T_2	A_1& A_2	B	S_1&S_2
Saline	-	-	1 ml	-
BSA (standard)	-	-	-	1 ml
Solution from P	1 ml	-	-	-
Filtrate of G	-	1 ml	-	-
Biuret reagent	5 ml	5 ml	5 ml	5 ml

Mix and keep standing for 10 minutes, read the absorbance using a spectrophotometer at 540 nm

3.5.RESULTS:

Part I

Test	fibrinogen	globulin
Biuret		
Clotting		
Heat coagulation		

Part I1

1. Concentration of proteins in serum:

$$\frac{\text{Absorbance of T}}{\text{Absorbance of S}} \times \text{Conc of standard}$$

2. Concentration of albumin in serum:

$$\frac{\text{Absorbance of A}}{\text{Absorbance of S}} \times \text{Conc of std}$$

3. Concentration of globulin = Total protein – Albumin

4. Calculate the A/G ratio

3.6.DISCUSSION:

3.7Questions:

1- What is different between plasma and serum?

2- give the formula of biuret?

3- What is the normal A/G ratio? And what does a higher ratio indicates?

4- Name two other methods for protein estimation.

5- Explain the need to test for the level of proteins.

3.8.References:

Singh.S.P.(2007).Practical manual of biochemistry. 6th edition

Tietz, N. W. (1987) .Fundamentals of Clinical Chemistry . W.B. Saunders. Philadelphia. third Edition .

EXPERIMENT (4)

4. Electrophoretic Separation of Serum Proteins

4.1 Introduction

4.1.1 Serum:

Is the name given to the supernatant fluid obtained after blood has clotted, i.e. after the fibrinogen has been converted into insoluble fibrin and this fibrin together with the cells have been removed by centrifugation.

4.1.2 Electrophoresis

Is a technique used for separation of ionic substances. Any substance which exists in an ionized form will migrate in an electric field, as in the electrolysis of NaCl, when Na^+ ions move to the cathode and Cl^- ions to anode. Amino acids and proteins are examples on substances which carry a net negative or a net positive charge depending o the pH or their environment.

The rate of migration for an ionized substance in an electrical field depends on:

a. Ionic charge (the bigger the net charge, the faster the movement).
b. Ionic size – (the larger the ion, the slower it moves).
c. The potential gradient across the medium.

In zone electrophoresis, a strip of filter paper or cellulose acetate (a modified paper) is moistened with buffer so that it will conduct a current, and the mixture to be investigated is placed as a narrow band or spot on this supporting strip. A potential is then applied and the ions begin to migrate, to the suitable pole and at the suitable rate. Coloured dyes are used to stain colourless substances so that their positions can be localized.

Serum is used rather than plasma as this avoids the additional peak produced by fibrinogen and should be fresh or otherwise at stored at $4^{o}C$ as short time as possible. Thus for simple separation into the usual five bands, cellulose acetate membrane has many advantage including the virtual elimination of trailing because of the very small amount of adsorption. Well-defined bands are obtained on an almost colourless background making accurate quantitation possible. Noteworthy is the good separation of albumin and $\alpha_1$bands. The volume of serum required is very small and with the small scale technique only a half to two hours is required for the separation. Subsequent staining, washing and drying is rapid so that the entire procedure usually takes no more 1-2 hours.

4.2 Materials and Methods

1- Cellulose acetate membrane.
2- Electrophoresis tank.
3- Power supply unit.
4- Staining solution.
5- A suitable buffer.
6- Plastic forceps.
7- Serum sample.

4.3 Method:

1- A cellulose acetate strip 12 x 5 cm is labeled.
2- Impregnate with buffer by floating the strip on the surface of the buffer solution contained in a flat dish, so that the liquid soaks up from underneath (this avoids entrapping of air in the pores), leave for 15 minutes.
3- Blot lightly with clean filter paper and place across the pieces of the tank which should be 6 – 8 cm apart.
4- Connect to a suitable D.C source, which is a capable of delivering either constant voltage or constant current.

5- Allow either 0.4 mA per cm width of strip (i.e. 4 x 5 strips require 8mA) or 15-25 V per cem.

6- Constant current is preferable but as this changes with the number of strips, a constant voltage of 200 is less liable to error.

7- The polarity should be reversed on alternate runs. Run for 60 minutes; this should give 4 to 5 cm separation.

8- Turn off the current, carefully place the strips in the dye which contains trichloroacetic acid to fix the protein.

9- Leave for 10 minutes. Remove excess dye by agitating the strips in wash solution for 5 minutes. This is repeated until the background is white.

10- Complete the washing in pure methanol for 5 to 10 minutes and dry between blotting paper under pressure.

11- To evaluate the electrophoresis strips, 4 different methods are available:

Inspection, Elution, Solution and Scanning.

We combine visual examination with scanning. The exact details of the scanning procedure will depend on the scanning procedure will depend on the instrument available.

4.4 Results

Count the bands you obtain and define them.

Compare the results you obtain with those in the attached table and figure.

4.5 Referance :

- Tietz, N. W. (1987) .Fundamentals of Clinical Chemistry . W.B.Saunders. Philadelphia. third Edition .
- .Dacie. J.V and.Lewis .S.M .2001. Practical haematology , 9th edition, Churchill-Livingston.

EXPERIMENT (5)

5. Determination of plasma enzymes using the clinical analyzer

Determination of LDH (Lactate Dehydrogenase) in Serum

5.1 Introduction

Lactic acid dehydrogenase (LDH) is an enzyme that helps produce energy. It is present in almost all of the tissues in the body and becomes elevated in response to cell damage. Normal LDH levels range from 45 units per liter (U/L) to 90 U/L. LDH is most often measured to evaluate the presence of tissue damage. The enzyme LDH is in many body tissues, especially the heart, liver, kidney, skeletal muscle, brain, blood cells, and lungs. Lactate is released into the blood and is eventually taken up by the liver. The liver converts lactate back to glucose and releases glucose into the blood. This glucose is then taken up by resting muscles, red blood cells, and other tissue.

LDH exists in 5 forms (isoenzymes), which differ slightly in structure.

The LDH content in various tissues is seen in the following table:

LDH Isoenzyme	**Tissues or o**	**Enzyme Level U/G**
LDH-1	is found primarily in heart muscle and red blood cells.	160,000
LDH-2	is concentrated in while blood cells.	16,000 – 62,000
LDH-3	is highest in the lung.	
LDH-4	is highest in the kidney, placenta, and pancreas	250,000-300,000
LDH-5	is highest in the liver	260,000
	and in skeletal muscle	133,000

All of these isoenzymes can be measured in the blood, and can be separated by electrophoresis.

Exercising muscles convert (and red blood cells metabolize) glucose to lactate. The optimum pH for lactate pyruvate (L→ P) reaction is 8.8 – 9.8, while for pyruvate to lactate (P→ L) is 7.7 – 7.8. The enzyme is inhibited by sulfhydryl reagents such as P-chloromercuribenzoate and mercuric ions.

Some Levels of LDH in Different Diseases

	Disease		LDH Level
1	Myocardial infarction		↑ LDH level 6-10 times normal
2	Liver Disease	Toxic jaundice	↑LDL level 10 times
		Viral hepatitis	↑LDH level is lower than 10 times
		Obstructive jaundice	↑LDL level 2 times
3	Anemia	Pernicious anemia	↑LDH level 50 times
		Megaloblastic anemia	↑LDH level 20 times
4	Renal Diseases	Tubular necrosis	↑LDH
		Pyelonephritis	↑LDH
5	Malignant Disease	Lung Cancer	↑LDH
		Hodgkin's disease	↑LDH

In heart attack is suspected, the LDH levels will be reached at 24 and 48 hours to monitor for changes. They also help determine how well chemotherapy is working during treatment for lymphoma.

5.2.Principle

LDH is a hydrogen transfer enzyme which catalyzes the interconversion of pyruvate and lactate.

In the liver, it catalyzes the oxidation of L-lactate to pyruvate (L→P) with the mediation of NAD as hydrogen acceptor. The reaction is reversible and the reaction equilibrium strongly favors the reverse reaction, namely the reduction of pyruvate to lactate (P→L). The formation of NADH produces an increase in

absorbance at 340 nm. The rate of absorbance change is directly proportional to the activity of LDH in the specimen.

5.3.Reagents

1. Tris buffer (pH 8.9, 57.5 mmol)

Dissolve 7 gm of tris in 700 ml of distilled water, warm if necessary and make up to 1000 ml with distilled water.

2. LDH Reagent

[Lactate solution (51.6 mmol/l) + NAD (8.26 mmol/l) in tris buffer)

a. Dissolve 2.9 gm of sodium lactate in 200 ml tris buffer (pH 8.9, 57.5 mmol).
b. Dissolve 2.75 gm of NAD in 200 of tris buffer (pH 8.9, 57.5mmol).

Mix a and b, make up to 500 ml with the buffer.

5.4.Procedure

Into 2 dry clean test tubes, pipette the following:

	Blank	**Unknown**
Sample	---	100 µl
LDH Reagent	3 ml	3 ml
Mix thoroughly, incubate at 37^{o}C in water bath for 1 min. Read the absorption at 34 nm, A_1, and read it again after 2, 3 minutes (A_2, A_3).		

Then calculate

ΔA_1, = A2 - A1

$$\rightarrow \Delta A/min = (\Delta A_1, \Delta A_2) / 2$$

ΔA_2 = A3 – A2

Calculations

LDH (U/L) =ΔA x 4921

Expected Values

109 to 245 U/L

EXPERIMENT (6)

6. Prothrombin time and coagulation time

6.1 Objectives:

The test can be used as an important screening test for deficiencies specific factors occurring spontaneously in disease or produced as the result of the administration of anticoagulant drugs such as coumarin and indanedione.

In the screening tests ,a method based on the original technique of Quick and will be described. Although an inaccurate title, the term 'prothrombin time' is so firmly established that it seems pointless to advocate the use of a more accurately descriptive term such as 'brain-thromboplastin time.'

6.2 Introduction:

The introduction by Quick of the measurement of the clotting time of plasma after the addition of brain extract can be seen in retrospect to be landmark in the study of blood coagulation and the haemorrhagic disorders. Introduced originally as a test for prothrombin activity – hence the name – the test is now known to measure in addition, and more importantly, factors, V, VII and X. It is also relatively sensitive to the presence of heparin in the blood and hypofibrinogenaemia.

6.3 Principle.

A potent preparation of human or rabbit brain emulsion is added to citrated plasma. The mixture is then recalcified and the clotting time estimated.

The main plasma protein are albumin(36-50 g/l) , globulins (18-32 g/l) and fibrinogen (2-4 g/l). fibrinogen may be salted out from plasma and identified by the biurt test and by the fact that clotting occurs on addition to serum which still contains active thrombin.

The biuret reaction is given by proteins , which is then reacted under strongly alkaline conditions with copper sulphate to give a blue color.

6.4 Materials:

Preparation of Solutions

control plasma samples.

Centrifuge citrated blood without delay at 1200-1500 g for 15 min. Then remove the supernatant plasma and place it in a clean glass tube. If the test is not carried out at once, the sample should be placed at 4oC where it may remain for several hours. A delay of more than 6 h is undesirable and specimens so kept usually show a shortening of their prothrombin time by several seconds. This phenomenon is due to activation of kallikrein in the cold and conversion of the factor VII to the more active form. If the specimen is visibly haemolyzed it should be discarded. Human brain emulsion. An extract of acetone-dried brain is widely used (for preparation).

Suspend 0.03 g of the dry brain powder in 5 ml of 9g/l NaCl (saline) and warm at 37^{o}C for 15-30 min, with occasional shaking. The coarse particles are allowed to sediment and the opalescent supernatant is used for the test.

Ideally, a new tube for brain powder should be used every time a batch of tests is performed, but the saline suspension may be kept for a day or two at 4oC without deterioration, or longer if frozen at – 20oC. It is convenient o make up large batches of brain powder; if suitably stored, human brain powder retains its potency for many months. Many laboratories in Britain use a phenol-saline suspension of human brain known as the British Comparative Thromboplastin as a working reagent for prothrombin time tests. The material is produced in large volumes without significant variation in sensitivity between batches. Rabbit brain emulsion. A number of commercial rabbit brain preparations, as well as preparations containing rabbit brain and calcium chloride, are available. Rabbit brain thromboplastin is not as sensitive as human brain to factor-VII deficiency, and these preparations usually give faster clotting times with deficient plasma samples. However, the commercial preparation are usually satisfactory for the investigation of patients with bleeding disorders. In all instances, the manufacturer's instructions should be followed. Calcium chloride. 0.025 mol/l calcium chloride may be prepared conveniently from a commercial molar solution.

6.5 Method

6.5.1. Prothrombin time **.**

Deliver 0.1 ml of plasma into the bottom of a 75 x 10 mm glass test-tube placed in a water-bath at 37oC and add 0.1 ml of brain suspension to it. After a delay of about 1 min, add 0.1 ml of warmed 0.025 mol/l calcium chloride and mix the contents of the tube carefully. Start a stop-watch and hold the tube with its lower end submerged. Tilt the tube continuously but gently from the vertical to just short of the horizontal so that its contents can be observed for the first signs of clotting. A fibrin clot developing within a second marks the end-point. A shielded horizontal source of light should be arranged to provide effective lighting of the sample being tested. Repeat the test at least once for each specimen and record the mean time. Include at least one normal control plasma sample in each batch of tests.

Normal range:- (Quick method): 10 – 14 s. It has been repeatedly demonstrated that the normal values obtained in any laboratory depend upon exactly how the test is carried out. In particular, the values observed with normal and pathological plasma samples depend greatly on the source and type of brain thromboplastin used. Each laboratory has to establish its own normal range.

Significance of an abnormal prothrombin time:- The prothrombin time test is non-specific indicator of the extrinsic blood, coagulation mechanism. As already mentioned, deficiencies of prothrombin and factors, V, VII and X give rise to a prolonged time, as well as the presence of heparin in the blood and hypofibrinogenaemia.

The common causes of a long one-stage time are:

1- Therapy with coumarin or indanedione drugs.
2- obstructive jaundice.
3- Haemorrhagic disease of the newborn.
4- Liver disease.

Less common causes are:

5- Heparin therapy.
6- Loss of clotting proteins from the blood via the kidneys in renal disease, e.g. in nephritic syndromes.
7- Congenital deficiency of one or more of factors II, V, VII or X.
8- Fibrinogen deficiency.
9- Malabsorption states (vitamin K deficiency).

There are, too, a n\umber of common artefactual causes which should be considered if a long time is not expected. These include the following:

1- Faulty collection of the specimen, resulting in partial clotting and serum being tested instead of plasma.

2- An excess of citrate or insufficient blood so that there is an incorrect volume of citrate in relation to the blood.

3- An unsuitable anticoagulant, such as EDTA, used in collecting the sample.

4- An unduly high PCV, so that there is less plasma than normal per unit volume of blood and consequently an excess of anticoagulant.

5- Technical errors, such as a hole in the tube containing calcium chloride, the water-bath set at an incorrect temperature or a faulty thromboplastin reagent.

If an artefactual cause of an increased time is suspected, a normal control plasma should be tested, the patient's blood sample should be checked for small clots and a further sample obtained, if necessary. If the PCV of the blood is high, the effect

of adding 0.05 mol/l calcium chloride in place of 0.025 mol/l calcium chloride should be tried.

6.5.2 Coagulation time:

1- Put the four glass tubes of 10 mm external bore into a 37 ˚C water bath.

2- 5 ml of venous blood taken from the subject. Timing is started.

3- 1 ml of blood is put into each of the tubes. The tubes remain in the water bath.

4- Every minute, the tubes in rotation are gently tilted until one can be titled through an angle greater than 90˚without spilling the blood.

5- The tilting must be done in the same way each time end in the same tube order.

6- When the contents of one tube coagulates, the time is recorded.

7- The process is continued for the remaining tubes, the time being recorded when the contents of each coagulate.

8- The mean of the results is the coagulation time.

9- If the coagulation is greatly prolonged, as in haemophilia, (e.g. it may take one hour or more) then the mean is taken of the time taken for coagulation in at least two of the tubes.

6.6 Results:

Part 1

-Prothrombin time

Part 11: Time for coagulation to occur in each of the tubes;

- (a) min.
- (b) min.
- (c) min.
- (d) min.
- coagulation time min. (Normal range 4-9 min. at 37 ˚ C).

6.7.Discussion

6.8 Questions:

1- Giving reasons, would you expect the coagulation time to be greater or less in tubes of narrow bore than in tubes of larger bore?

6.9 References:

Dacie, S. J.V. and Lewis, S.M., Practical Haematology, 6th edition, Longman Group Limited, 1984, Chp.13.

EXPERIMENT (7)

7. ABO BLOOD GROUPNG& Rh GROUPS

7.1 Objectives:

1- to determine the blood group and therefore the type of antigen carried on the surface of erythrocytes in the ABO system.

2- to test for the availability of the Rh factor (D antigen) on the surface of erythrocytes

7.2 Introduction:

ABO blood group system is one of30 genetically independent human blood –group system. Each of these system contain a group of structurally related antigens. All are inherited according to mendelian laws of genetics. blood group antigens are not found only as part of erythrocyte membrane but also found in a wide variety of tissues and biological fluids such as saliva, milk , seminal fluid, urine , and gastric juice. blood group antigens must be determined to secure a safe practice of blood transfusion. They are also useful in determining familial relationships in forensic medicine. the chemical nature of only a few blood –group systems is known.

The ABO system is associated with three blood group substances (antigens) on erythrocytes designated as the A,B and H antigens. These antigens have the following antigenic determinants at the non-reducing termini of oligosaccharides

7.3 Materials:

Citrate saline

Glassware

Slides.

Tooth pick

7.4 Method:

Pick the finger and draw up blood with the W.b.c. pipette up to mark 0.5 .such citrated saline up to mark 11.this gives a 1:20 suspension of

erythrocytes (red blood cells). Divide the slides into three compartments with grease pencil line. Label them A, B and x . On no account should the dropper of one serum core in contact with the other serum. Add one fair sized drop or erythrocytes suspension to each compartment .mix the suspension and serum in each compartment by racking the slide gently to and one or two minutes and then look for agglutination.

7.5 Results:

Agglutination looks like red pepper grains. it is easily visible in most cases. If there is a beaker, racking it occasionally, and noting for agglutiation from time to time. In case of doubt compare the appearance of mixture in compartment A and B and that X which acts as a control. If no agglutination occurs for 10 minutes, it may be examined under low power of microscope to make certain that agglutination has occurred. Rouleaux formation is no agglutination .Record your findings in a tabular form and draws a diagram of the appearance on the slide. Record the name of your blood group. Repeat the procedure using anti-D sera to find the Rh group using undiluted blood and after mixing the sera and the blood , the slide is incubated for 1/2 -1 hour at 37c.

7.6 Discussion and conclusion:

7.7 Questions:

1- for what group can you act as a donor and recipient?
2- what is the importance of blood groups in transfusion of blood?
3- what other grouping factors do you keep in mind in addition to the classical groups?
4- what is the importance of Rh factor?
5- what is percent distribution of blood groups among the world population?
6- what is the importance of blood groups in the medical jurisprudence?

7.8 References:

Henery.J.B .Clinical Diagnosis and Management by Laboratory Methods, 17th Edition, ,1984.

EXPERIMENT (8)

8 Haemoglobin ,Anaemia ,HCT and ESR

8.1 Haemoglobin

8.1.1 Introduction

8.1.1.1 Haemoglobin Synthesis

The circulating blood of a normal adult man contains about 750 gm of **haemoglobin**, and of this about 1/12o or 7 – 8 or 7 – 8 gm are degraded daily. This amount has to be newly synthesized each day because:

- The globin part of haemoglobin can be reutilized only after catabolism into its constituent amino acids,
- the haem moiety is broken down into bile pigment, which is excreted,
- iron alone is reutilized in the synthesis of Haemoglobin.

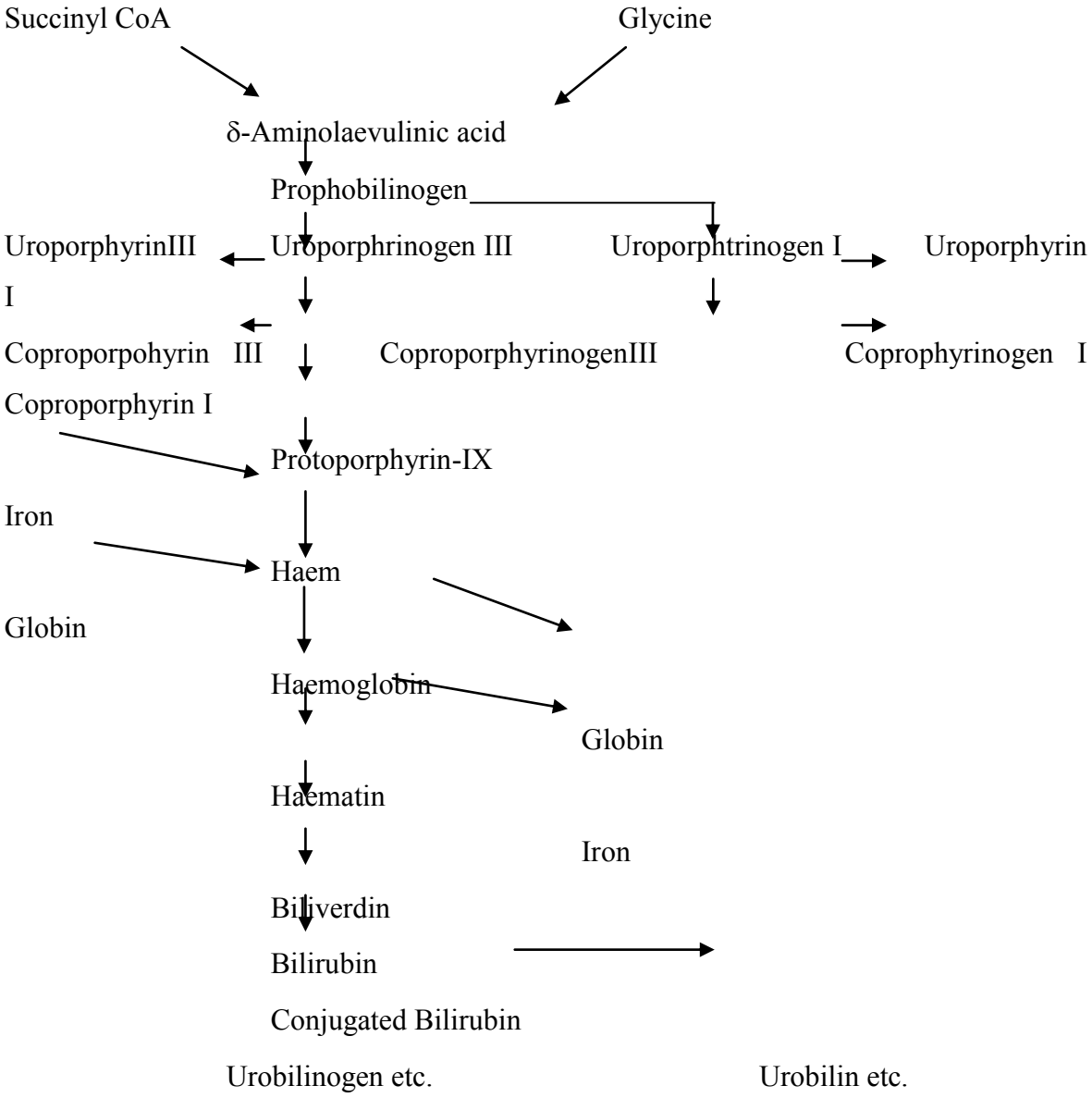
The rates at which haemoglobin is synthesized and at which red cells are formed, are related to the oxygen content of the blood. And therefore depend not only upon the amount of oxygen reaching the blood but also upon the capacity of the blood to carry oxygen, which in turn depends on the amount of circulating haemoglobin. Therefore, haemoglobin synthesis is stimulated by anoxia, whether due to oxygen deficiency or due to anaemia. The erythrocytes are derived from primitive nucleated cells in the bone marrow by successive processes of mitosis and maturation.

A primitive stem cell divides to form two cells, one of which retains its behavior as a stem cell while the other successively divides to form two basophile normoblasts, four polychromatic normablasts and eight ortho-chromatic normoblasts, after which maturation through late normoblast and reticulocyte stages to the mature non-nucleated fully haemoglobionized erythrocyte involves

no further mitotic division. These processes must involve the biosynthesis not only of haemoglobin, but also of large quantities of purine bases, nucleic acids and protein. The ability of the haemopoietic tissues to manufacture erythrocytes depends on a variety of hormones, trace metals, enzymes, coenzymes and an adequate provision of essential amino acids, glycine, acetyl coenzyme A and iron. There is a strong evidence that the marrow response to the stimulus of hypoxia is dependent upon glycoprotein hormone, erythropoietin, which in response to hypoxia may act on differentiation of the stem cells rather than upon any particular step in haemoglobin synthesis. Erythropoietin is formed in the kidney from a prohormone (erythrogenin) by the action of a plasma factor synthesized in the liver.

8.1.1.2 HaemoglobinCatabolism

1- In the reticuloendothelial system, erythrocytes are destroyed and the haemoglobin is released.
2- Some haem is released in the narrow during erythroblast maturation or from the dead cells of ineffective erythropoiesis.
3- Globin is separated from haem, and haematin is formed in which the iron of the harm is oxidized to [ferric] iron (III).
4- The porphyrin ring is then opened and the iron is removed with the formation of the straight chain compound biliverdin, which is converted to bilirubin by reduction. A minor pathway first opens the ring to form choleglobin and then removes the iron and globin to produce biliverdin globin and then biliverdin.
5- The iron and the amino acids of the globin, are retained, the pyrrole rings are eventually excreted as bilirubin.



The principal steps in the synthesis and degradation of Haemoglobin

8.1.1.3.Role of Vitamins, Trace Metals and Cofactors Deficiencies in Producing HumanDiseases

1- Biotin, pantothenic acid, pyridoxal phosphate and coenzyme A; are essential coenzymes required for the synthesis of haem. The deficiency of pyridix I phosphate plays a role in human disease while the deficiency of folic acid can cause megaloblastic anaemia.

2- Of the trace metals, only copper and cobalt are known to play a role. Copper is playing a role in the absorption of ion, while Cobalt is an essential constituent of vitamin B_{12}.

3- Deficiency of intrinsic factor can cause vitamin B_{12} deficiency, with abnormal maturation of red cells leading to a megaloblastic stage and consequent failure to liberate sufficient red cells to maintain a normal amount of circulating haemoglobin.

I) Iron-Deficiency Anaemia

Deficiency of iron is essentially due to blood loss with failure to replace the iron stores because of dietary deficiency, increased requirement or defective absorption; plasma iron is low, the iron-binding capacity normal but percentage saturation is low. Microcytes containing a subnormal quantity of haemoglobin may be released into the circulation, and be ineffective in raising the haemoglobin level to normal. Accompanying changes include brittleness of the nail and atropy of mucous membranes.

II) Megaloblastic Anaemia

This may be due to deficiency of folic acid or cobalamin (vitamin B_{12}) both of which acts as coenzymes and exist in various forms within the body. In megaloblastic erythropoiesis, there is general disturbance of metabolism which leads not only to the characteristic megaloblastic marrow but also to lesions of the

oral, gastrointestinal and vaginal epithelium. Deficiency of both cobalamins and folate has aroused much interest with regard to CNS function, which is affected in deficiencies of two other members of the vitamin B group, nicotinamide and thiamine. Thus psychoses, confusion and depression as well as sub acute combined degeneratin occur in cobalamin deficiency while there is an association between anticonvulsant drug therapy for epilepsy and later development of the folate deficiency with megaloblastosis. The effectiveness of anticonvulsant therapy is interfered with by folate leading to an increase in fit frequency.

III) Membrane Defects

In these conditions there is a defect of the erythrocyte membrane and an abnormally in the sodium pumps: the fundamental causes have not been elucidated. The best-known disorders are hereditary spherocytosis and hereditary elliptocytosis.

8.1.1.4 Glucose-6-phosphate dehydrogenate (G6PD) deficiency [non-spherocytic]

This is relatively common, especially in negroes, south Chinese, and Mediterranean people, and may protect against malaria. G6PD is the enzyme responsible for the initial deviation of glucose into pentose phosphate pathway to form 6-phosphogluconate. This pathway provides $NADPH_2$ in the erythrocyte for the conversion of oxidized reduced glutathione and for other reactions such as reduction of methaemoglobin.

Deficiency of G6PD

- The enzyme deficiency may cause haemolysis, but chiefly occurs after sensitization of the erythrocyte by a wide variety of agents e.g. primaquine, broad beans (favism) or in infections.

- The cells accumulate methaemoglobin and are deficient in reduced glutathione which is necessary for cell integrity. Haemolysis, dark urine and jaundice are present.
- In homozygote, enzyme activity is reduced to less than 15% of normal.
- The deficiency of G6PD may also produce neonatal jaundice.

Normal Range

Men	14 – 18 g/dl
Women	12 – 16 g/dl

8.1.2 Principle

The Fe(II) atom in each of the haem in the red haemoglobin is oxidized by ferricyanide to Fe(III)-methaemoglobin. A cyanide group is then attached to the iron atom (which is positively charged) by reaction with KCN to give the brown cyanmethaemoglobin. Qyanmethaemoglobin can be estimated quantitatively.

Haemoglobin + Cyanide + Ferricyanide → Cyanmethaemogloibn

8.1.3 Material

- Potassium hexacyanoferrate (III) solution

Potassium hexacyanoferrate (III)0.6 mmol/l

Potassium phosphate buffer 0.5 mmol/l, pH 7.20

-Potassium cyanide solution

Potassium cyanide 0.75 mmol/l

-Potassium phosphate buffer2.50 mmol/l, pH 7.20

-Working Solution

Mix equal volumes of both reagents 1 and 2.

-Sample Preparation

Use whole blood immediately. Heparinized blood and EDTA blood can be stored up to 4 days at +4 to 25°C.

-Caution

All solutions are poisonous. Use safety pipettes.

-Requirements

Wavelength	546 nm
Cuvette	1 cm light path
Incubation temp.	20-25°C
Measure against	redist. Water

8.1.4 Procedure

Pipette into two dry clean test tubes

	Blank	**Test**
Working Solution	---	5.00 ml
Blood Sample	---	0.02 ml
Redist. Water	5.00 ml	---

Flush pipette thoroughly with the working solution.

Mix solution well, and incubate at 20-25°C for 5 min.

Measure the absorbance of the sample (A_{HGB}).

8.1.5 Calculation

$C_{HGB} = 36.77 \times A_{HGB}$ [g/dl]

$= 22.82 \times A_{HGB}$ [mmol/l]

- **Note:**

  You can obtain the concentration of HGB in your sample from the attached table of values.

Table of values for measurement at Hg 546 nm

Absorbance A	Haemoglobin			Absorbance A	Haemoglobin		
	g/100 ml	Hb/4 mmol/ l	%		g/100 ml	Hb/4 mmol/ l	%
0.100	3.7	2.3	23.1	0.400	14.7	9.1	91.8
105	3.9	2.4	24.1	405	14.9	9.2	93.2
110	4.0	2.5	25.2	410	15.1	9.4	94.3
115	4.2	2.6	26.4	415	15.3	9.5	95.4
120	4.4	2.7	27.6	420	15.5	9.6	96.6
125	4.6	2.9	28.7	425	15.6	9.7	97.7
130	4.8	3.0	29.9	430	15.8	9.8	98.7
135	5.0	3.1	31.1	435	16.0	9.9	99.9
140	5.1	3.2	31.7	440	16.2	10.0	101.1
145	5.3	3.3	33.2	445	16.4	10.2	102.3
150	5.5	3.4	34.5	450	16.6	10.3	103.4
155	5.7	3.5	35.6	455	16.7	10.4	104.3
160	5.9	3.7	36.7	460	16.9	10.5	105.6
165	6.1	3.8	37.9	465	17.1	10.6	107.8
170	6.3	3.9	38.9	470	17.3	10.7	108.0
175	6.4	4.0	40.1	475	17.5	10.8	109.1
180	6.6	4.1	41.1	480	17.7	11.0	110.4
185	6.8	4.2	42.5	485	17.8	11.1	111.5
190	7.0	4.3	43.6	490	18.0	11.2	112.5
195	7.2	4.5	44.8	495	18.2	11.3	113.7
0.200	7.4	4.6	45.9	0.500	18.4	11.4	114.8
205	7.5	4.7	47.1	505	18.6	11.5	116.0
210	7.7	4.8	48.2	510	18.8	11.6	117.1

215	7.9	4.9	49.4	515	18.9	11.8	118.3
220	8.1	5.0	50.6	520	19.1	11.9	119.5
225	8.3	5.1	51.7	525	19.3	12.0	120.6
230	8.5	5.2	52.8	530	19.5	12.1	121.9
235	8.6	5.4	54.0	535	19.7	12.2	123.0
240	8.8	5.5	55.1	540	19.9	12.3	124.0
245	9.0	5.6	56.2	545	20.0	12.4	125.3
250	9.2	5.7	57.4	550	20.2	12.6	126.4
255	9.4	5.8	58.6	555	20.4	12.7	127.6
260	9.6	5.9	59.8	560	20.6	12.8	128.7
265	9.7	6.0	60.9	565	20.8	12.9	129.7
270	9.9	6.2	62.0	570	21.0	13.0	131.0
275	10.1	6.3	63.2	575	21.1	13.1	132.1
280	10.3	6.4	64.3	580	21.3	13.2	133.3
285	10.5	6.5	65.5	585	21.5	13.4	134.5
290	10.7	6.6	66.6	590	21.7	13.5	135.6
295	10.9	6.7	67.8	595	21.9	13.6	136.6
0.300	11.0	6.8	68.9	0.600	22.1	13.7	138.3
305	11.2	7.0	70.1	605	22.2	13.8	139.0
310	11.4	7.1	71.3	610	22.4	13.9	140.3
315	11.6	7.2	72.3	615	22.6	14.0	141.4
320	11.8	7.3	73.5	620	22.8	14.2	142.5
325	12.0	7.4	74.7	625	23.0	14.3	143.6
330	12.1	7.5	75.8	630	23.2	14.4	144.6
335	12.3	7.6	77.0	635	23.3	14.5	145.8
340	12.5	7.8	78.2	640	23.5	14.6	147.8
345	12.7	7.9	79.2	645	23.7	14.7	148.2
350	12.9	8.0	80.5	650	23.9	14.8	149.3
355	13.1	8.1	81.6	655	24.1	15.0	150.5
360	13.2	8.2	82.7	660	24.3	15.1	151.5
365	13.4	8.3	83.9	665	24.5	15.2	142.8
370	13.6	8.4	85.0	670	24.6	15.3	154.0
375	13.8	8.6	86.2	675	24.8	15.4	155.3

380	14.0	8.7	87.4	0.680	25.0	15.5	156.3
385	14.2	8.8	88.4				
390	14.3	8.9	89.6				
0.395	14.5	9.0	90.8				

8.2 Determination of Hematocrit (HCT)

8.2.1 Introduction

Hematocrit, or packed cell volume (PCV), determination is part of the daily routine in hematological laboratories. It is used as a simple screening test for anemia and is used in conjunction with the mean cell volume (MCV) and mean cell haemoglobin concentration (MCHC). Blood is collected in heparinised capillary tube, which is then sealed, centrifuged and the red cell volume expressed as a percentage of the whole blood.

8.2.2 Procedure

1- The blood of a colleague may be taken in a heparinised capillary tube or a blood sample in such a tube may be used which has been taken not more than 6 hours before and stored at $4^{o}C$.

2- Seal the dry end of the tube, and centrifuge for 5 min.

3- The column of red cells will be seen, topped by the grayish-red layer of leukocytes and above this a thin creamy layer of platelets, the "Buffy coat".

4- Measure the length of the column of red blood cells (A), and the total length of blood components (B).

$$\text{Haematocrit} = \frac{A}{B}$$

Normal Range Men 0.40 – 0.54

Women 0.37 – 0.47

8.3 C. Determination of Erythrocyte Sedimentation Rate [ESR]

8.3.1 Principle

- ESR is the rate at which erythrocytes settle out of anticoagulated blood in 1 hour. This test is based on the fact that inflammatory and necrotic processes cause an alteration in blood proteins, resulting in an aggregation of red cells, which make them heavier and more likely to fall rapidly when placed in a special vertical tube.
- ESR is dependent on the plasma concentration of asymmetric macromolecules such as globulins, fibrinogen, besides the concentration of red cells.
- ESR is used clinically as a non-specific screening test to detect the presence of infection in the body in general. It is used as well as a means of monitoring the status of chronic inflammatory disease such as rheumatoid arthritis.
- ESR is not diagnostic of any particular disease, but rather is an indication that a disease process is ongoing and must be investigated.

8.3.2 Procedure

In this technique, cells are allowed to sediment under the effect of gravity, using a Westergren's tube. 106 cc of blood is drawn out from the vein of a subject by a syringe. Transfer it into an EDTA tube, and thend raw the blood up into a Westergren's tube exactly to the zero mark. The tube is placed upright in the rack and left undisturbed. The length of the column of clear plasma at the top is noted at the end of 1 hour and again at he end of 2 hours.

Normal Range

	After 1 hour	**After 2 hours**
Men	0 – 5 mm	7 – 15 mm
Women	0 – 10 mm	10 – 20 mm

8.3.3 References Ranges

	Male	**Male/Female**	**Female**	**Units**
RBC	4.2 – 5.5		3.7 – 5.0	10^{12}/l
HCT	38 – 48		36 – 46	%
MCV		80 – 100		Fl
RDW		11.5 – 15		%
HGB	135 – 165		115 – 140	g/l
MCH		28 – 35		Pg
MCHC		330 – 360		g/l
PLT		150 – 380		10^9/l
MPV		7.5 – 10.5		Fl
WBC		4.0 – 9.0		10^9/l
LYM		1.2 – 3.5		10^9/l
MID		0.1 – 0.6		10^9/l
GRA		1.4 – 7.0		10^9/l
LYM		20 – 48		%
MID		2 – 10		%
GRA		42 – 80		%
Bands		0 – 5		%
Neutrophils		55 – 65		%

Eosinophils	1 – 5	%
Basophils	0 – 1	%
Lymphocytes	22 – 35	%
Monocytes	3 – 8	%
Reticulocytes	0.2 – 2.0	%

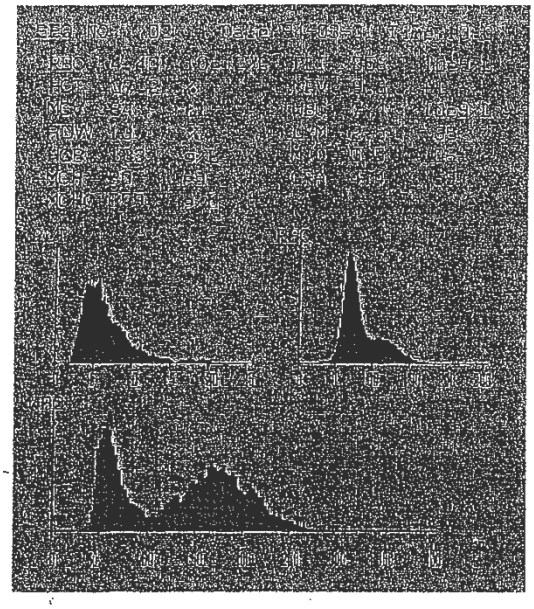
Abbreviations

RBC	Red Blood Cells
HCT	Haematocrit
MCV	Mean Cell Volume
RDW	Red Cell Distribution Width
HGB	Haemoglobin
MCH	Mean Cell Haemoglobin Concentration
MCHC	Mean Cell Haemoglobin Concentration
PLT	Platelets
MPV	Mean Platelet Volume
WBC	White Blood Cells
LYM	Lymphocytes
MID	Midcells
GRA	Granulocytes

fl	femtolitre	10^{-15} litre
pg	picogram	10^{-12} gram

8.3.4 Normal Sample

Haematologically healthy individual. The blood sample was taken with K_3EDTA as the anti-coagulant and analysed 30 min after venepuncture. It is important to allow the blood sample to adapt to the EDTA-environment. The sample cannot be kept too long before analysis otherwise the leukocyte differential count will be erroneous. The analysis should take place 15 min – 8 hours after drawing. If the particle concentration alone is requested then analysis can be performed up to 24 hours after drawing. It is essential that the sample is not older than 8 hours if a leukocyte differential count is requested.



The cytogram presents 16 parameters as numbers and 3 histograms.

RBC	Red Blood Cells
HCT	Haematocrit
MCV	Mean Cell Volume
RDW	Red Cell Distribution Width
HGB	Haemoglobin
MCH	Mean Cell Haemoglobin
MCHC	Mean Cell Haemoglobin Concentration
PLT	Platelets
MPV	Mean Platelet Volume
WBC	White Blood Cells
LYM	Lymphocytes
MID	Midcells (monocytes)
GRA	Granulocytes

Red Blood Cell Histogram shows the distribution of erythrocytes between 35 and 250 fl. The low peak to the right shows erythrocytes which have passed the measuring point at the same time, usually two or three together (doublets and triplets respectively) These large "cells" are not included in the calculation of mean cell volume but are nevertheless presented in the histogram. *Further information is to be found in the instruction manual.*

Platelet Histogram shows the size distribution of the platelets. In normal cases this lies between 3 and 20 fl and has a positive skew shape. *Further information is to be found in the instruction manual.*

White Blood Cell Histogram shows three different populations within the 35 – 420 fl area.

LYM	35 – 85 fl	lymphocytes	Nucleated red blood cells, clumped platelets, macrocytic platelets, atypical lymphocytes, blasts)
MID	85 – 115 fl	monocytes	(atypical lymphocytes, blasts, immature granulocytes, plasma cells, eosinophils, basophils, precursor cells, agranular neutrophils, hyposegmented granulocytes)
GRA	115 – 420 fl	granulocytes	(eosinophils, bands, immature granulocytes, hypersegmented granulocytes)

Index calculation

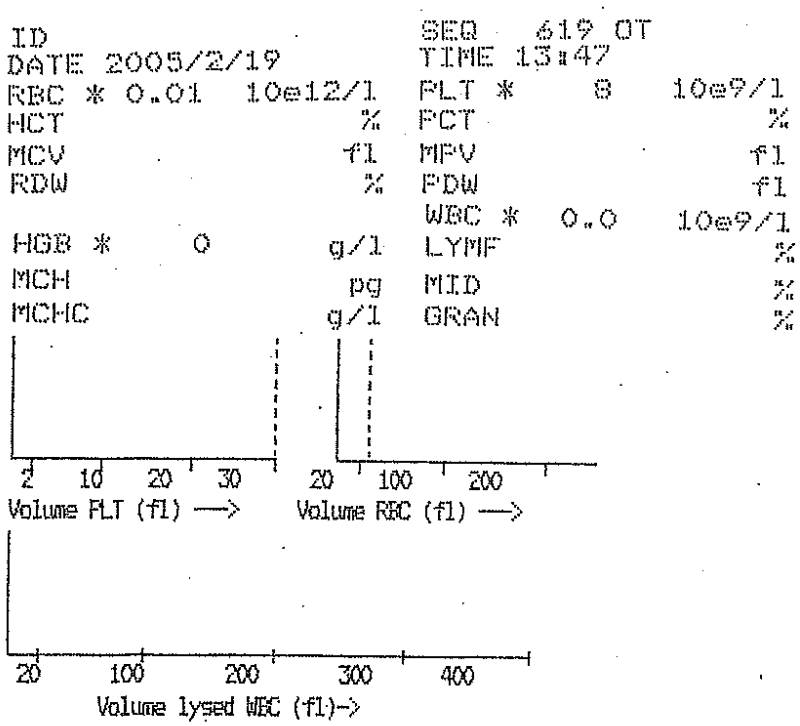
HCT	=	RBC x MCV	%
MCH	=	HGB / RBC	pg
MCHC	=	HGB / HCT	g/l
RDW	=	SD / MCV	%

	Male	**Male/Female**	**Female**	**Units**
RBC	4.2 – 5.5		3.7 – 5.0	10^{12}/l
HCT	38 – 48		36 – 46	%
MCV		80 – 100		Fl
RDW		11.5 – 15		%
HGB	135 – 165		115 – 140	g/l
MCH		28 – 35		Pg
MCHC		330 – 360		g/l
PLT		150 – 380		10^9/l
MPV		7.5 – 10.5		Fl
WBC		4.0 – 9.0		10^9/l
LYM		1.2 – 3.5		10^9/l
MID		0.1 – 0.6		10^9/l
GRA		1.4 – 7.0		10^9/l
LYM		20 – 48		%
MID		2 – 10		%
GRA		42 – 80		%
Bands		0 – 5		%
Neutrophils		55 – 65		%
Eosinophils		1 – 5		%
Basophils		0 – 1		%
Lymphocytes		22 – 35		%
Monocytes		3 – 8		%
Reticulocytes		0.2 – 2.0		%

Abbreviations

RBC	Red Blood Cells
HCT	Haematocrit
MCV	Mean Cell Volume
RDW	Red Cell Distribution Width
HGB	Haemoglobin
MCH	Mean Cell Haemoglobin Concentration
MCHC	Mean Cell Haemoglobin Concentration
PLT	Platelets
MPV	Mean Platelet Volume
WBC	White Blood Cells
LYM	Lymphocytes
MID	Midcells
GRA	Granulocytes

fl	femtolitre	10^{-15} litre
pg	picogram	10^{-12} gram



8.4 References

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- Russell. N J; Powell. G. M; Jones J .G., Winterburn P J and Basford J M. 1982. Blood Chemistry. Croom Helm, London and Canberra.

EXPERIMENT (9)

9.1 Estimation of Serum Bilirubin [Total and Direct]

9.1 Introduction

9.1.1 Bilirubin **:**

Is a breakdown product of hemoglobin. Bilirubin metabolism begins on the death of the red blood cell and its breakdown. Red blood cells contain hemoglobin, which is broken down to heme and globin. Heme is converted to bilirubin, which is then carried by albumin in the blood to the liver. The haem is broken down into bilverdin and then to unconjugated bilirubin. In the liver, most of the bilirubin is chemically attached to a glucuronide before it is excreted in the bile. This "conjugated" bilirubin is called direct bilirubin; unconjugated bilirubin is called indirect bilirubin. Total serum bilirubin equals direct bilirubin plus indirect bilirubin.

Conjugated bilirubin is excreted into the bile by the liver and stored in the gall bladder or transferred directly to the small intestines. Bilirubin is further broken down by bacteria in the intestines to urobilins, which contribute to the color of the feces. A small percentage of these compounds are reabsorbed and eventually appear in the urine, where they are referred to as urobilinogen.

- **Unconjugated bilirubin** is only springly soluble in water but is bound to **albumin** for transportation purposes.
- The healthy liver conjugates **glucuronic acid** to the **biliburin** by means of **Bilirubin-UDP Glucuronosyl Transferase** rendering in water soluble.
  The water soluble conjugated bilirubin is then excreted into the bile. About 200 mg per day of unconjugated bilirubin are transported to the liver and disturbances in the powers of conjugated and/or excretion of the liver of this yellow compound will lead to raised levels in serum. Above about 2 mg/dl in the blood, we get the condition known as **Jaundice.**

9.1.2 Jaundice

is the discoloration of skin and sclera of the eye, which occurs when bilirubin accumulates in the blood at a level greater than approximately 2.5 mg/dl. Jaundice occurs because red blood cells are being broken down too fast for the liver to process, because of disease in the liver, or because of bile duct blockage.

The causes of jaundice may be classified as:

Pre-Hepatidc Jaundice, Hepatic Jaundice and Post-Hepatic Jaundice.

The liver function test results expected for examples of each are as follows:

1- Pre-Hepatic Jaundice

E.g. haemolytic disease

The productin of unconjugated bilirubin may exceed the conjugating capacity of the liver and hence the serum levels of indirect (and of total) bilirubin will be raised and that of direct in the upper normal range or just a little elevated. The other liver function tests will usually give normal results.

2- Hepatic Jaundice

***A*. Cirrhosis of the liver** (in the absence of infection)

Destruction of liver cells will lead to a reduced conjugating capacity with a raised serum level of indirect (and of total) bilirubin but with a low level of direct bilirubin and an abnormally high release, into the blood, of the enzymes: AST, ALT and ALP. Synthesizing power will be diminished and hence low levels of total protein, albumin and cholesterol. The insoluble unconjugated bilirubin will not be excreted in the urine, and bilirubin will be absent in severe cases.

***B*. Infective Hepatitis**

The conjugative capacity of the liver is approximately normal, but there is the inability to transport the conjugated bilirubin from the liver cells to the caniculi of the biliary system, and it will be

regurgitated back into the blood. Hence the serum level of unconjugated bilirubin will be normal, and that of conjugated (and total) bilirubin will be raised. Synthesizing power is diminished leading to low serum levels of proteins which are made in the liver and of cholesterol, but the raising of antibodies to infection usually leads to raised total proteins level.

C. **Neonatal Jaundice**

Conjugating enzymes in the liver are often absent at birth. Hence raised serum level of indirect (and total) bilirubin is to be expected, with a low level of direct bilirubin. The other liver functions are normal. The indirect bilirubin level will rise for the first few days after birth until the conjugating enzymes begin to synthesize. If the latter process is delayed and the serum level of indirect bilirubin rises towards 20 mg/dl, an ultraviolet therapy or an exchange blood transfusion should be carried out owing to the danger of deposition of the insoluble unconjugated bilirubin in the basal ganglia of the brain leading to the condition known as **Kernicterus**, and permanent **Brain Damage**.

3- Post-Hepatic Jaundice

E.g. Cholecystitis

Here, the bile duct is blocked. The indirect bilirubin level is normal but conjugated bilirubin is regurgitated into the blood and excreted into the urine (raised conjugated and total bilirubin). Enzymes will be regurgitated into the blood giving raised levels. The other liver function tests azre normal. If the bile ducts are obstructed, direct bilirubin will build up, escape from the liver, and end up in the blood. If the levels are high enough, some of it will appear in the urine. Only direct bilirubin appears in the urine. Increased direct bilirubin usually means that the biliary (liver secretion) ducts are obstructed. This test is useful in determining if a patient has liver disease or a blocked bile duct.

9.1.3 Role of Drugs

1- **Drugs that can increase bilirubin measurements** include anabolic steroids, some antibiotics, antimalarials, codeine, diuretics, epinephrine, morphine, nicotinic acid, oral contraceptives, salicylates, steroids, sulfonamides, and theophylline.

2- **Drugs that can decrease bilirubin measurements** include barbiturates, caffeine, penicillin, and high-dose salicylates.

9.1.4 Normal Range

- Direct bilirubin: 0 to 0.3 mg/dl
- Total bilirubin: 0.3 to 1.9 mg/dl

9.2 Principle

Total and direct bilirubin are usually measured to screen for or to monitor liver or gall bladder dysfunction. The estimation of unconjugated aznd conjugated bilirubin are two important tests of liver function. The water soluble conjugated bilirubin reacts easily with reagents such as diazotized sulphanilic acid (as used in this experiment) and is thus known often as direct bilirubin while the water insoluble unconjugated bilirubin requires a solubilising reagent, such as **Caffeine**, in order to react with the diazotized sulphanilic acid. For this reason, it is often called indirect bilirubin. In this experiment, the direct bilirubin is estimated in the absence of the solubilising agent and then further bilirubin estimatin in thepresence of the solubilising agent will give the total bilirubin level (conjugated + unconjugated). The indirect or unconjugated bilirubin is then found by difference.

9.3 Material

Solution 1:

Sulpohanilic acid	29 mmol/l
HCl	0.17 M

Solution 2:

Sodium nitrite	25 mmol/l

Solution 3:

Caffeine	0.26 mmol/l
Sodium benzoate	0.52 mmol/l

Solution 4:

Tartarate	0.93 mmol/l
NaOH	9 M
Sodium chloride solution	0.9%

Sample Preparation

1. Do not expose sample to sunlight or other light.
2. Carry out assay immediately.
3. Hemolysis interferes with the test.

A) Total Bilirubin

Requirements

Wavelength	578 nm
Cuvette	1 cm light path
Incubation temp.	20-25^{o}C

Measure against sample blank

9.4 Procedure and calculation

Part 1

Pipette into two dry clean test tubes:

	Sample Blank	Sample
Solution 1	0.20 ml	0.20 ml
Solution 2	--	1 drop (0.05 ml)
Solution 3	1.00 ml	1.00 ml
Solution	0.20 ml	0.20 ml

Mix, let stand for 30 min. at 20 – 25^{o}C.

Solution 4	1.00 ml	1.00 ml

Mix, let stand for 15 min. at 20-25^{o}C.

Read absorbance of sample against sample blank (A_{TB}).

-Calculation

$$\mathbf{C_{TB} = 10.8\ A_{TB}} \quad \mathbf{[mg/dl]}$$

$$\mathbf{= 185 \times A_{TB}} \quad \mathbf{[\mu mol/l]}$$

Normal Values

Up to: 1 mg/dl OR 17μmol/l

Part 11

Direct Bilirubin

-Requirements

Wavelength	546 nm
Cuvette	1 cm light path
Incubation temp.	20-25^{o}C

Measure against sample blank

-Procedure and calculation

Pipette into two dry clean test tubes:

	Sample Blank	**Sample**
Solution 1	0.20 ml	0.20 ml
Solution 2	--	1 drop (0.05 ml)
NaCl solution 0.9%	2.00 ml	2.00 ml
Sample	0.20 ml	0.20 ml

Mix, let stand for 5 min. at 20-25^{o}C.

Read absorbance of sample against sample blank (A_{DB}).

-Calculation

$$\mathbf{C_{DB} = 14.4\ A_{DB}} \quad \textbf{[mg/dl]}$$

$$\mathbf{= 246 \times A_{DB}} \quad \textbf{[µmol/l]}$$

Normal Values

Up to: 0.25 mg/dl OR 4.3 µmol/l

N.B

Indirect bilirubin = Total bilirubin – Direct bilirubin

9.5 QUESTIONS:

1. What are the differences between direct and indirect serum bilirubin? give an abnormality associated with each.
2. How can serum bilirubin be lowered?

9.6.REFERANCES

- Tietz, N. W. (1987) .Fundamentals of Clinical Chemistry . W.B. Saunders. Philadelphia. third Edition .
- Singh.S.P.(2007).Practical manual of biochemistry. 6th edition
- Toro.g and Ackermann .P.G. 1973.Practical clinical chemistry. Pub. Little, Brown and company Boston, p.320.

EXPERIMENT (10)

10. Glucose-6-phosphate dehydrogenase deficiency

- Quantitative determination of glucose 6-phosphate dehydrogenase (G6P-DH) activity in erythrocytes (hemolysate).

10.1 Introduction

G6PD is the enzyme responsible for the initial deviation of glucose into pentose phosphate pathway to form 6-phosphogluconate. .

Most of the interest of G6P-DH focuses on its role in the erythrocyte. Here, it functions to maintain NADPH in its reduced form. An adequate concentration of NADPH is required to regenerate sulfhydryl-containing proteins ,such as glutathione , form the oxidized to the reduced state. Glutathione in the reduced form, in turn, protects hemoglobin from oxidation by agents that may be present in the cell.

A deficiency of G6P-DH consequently results in an inadequate supply of NADPH and, ultimately, in the inability to maintain reduced glutathione levels. When erythrocytes are exposed to oxidizing agents, hemolysis occurs because of oxidation of hemoglobin and to damage of the cell membrane.

G6P-DH deficiency is an inherited Sex-linked recessive trait. The disorder can results in several different clinical manifestations, one of which is drug-induced hemolytic anemia. When exposed to an oxidant drug such as primaquine, an antimalarial drug, affected individuals experience a hemolytic episode. G6P-DH deficiency is most common in African Americans , but has been reported in virtually every ethnic group.

A red cell hemolysate is used to assay for deficiency of the enzyme, while serum is used for evaluation of enzyme elevations.

Expected values

Serum : 0-0.18 U/L or (0-0. 18 mU/mL).

Erythrocytes : 0.131 (0.118-0.144) U/10^9 cells or 131 (118-144) mU/10^9 cells.

10.2 Principle

The enzyme G6PD is catalyses the dehydrogenation of glucose 6-phosphate as the first step in pentose phosphate pathway. $NADP^+$, the electron acceptor, is reduced to NADPH in the reaction. The pH optimum for the G6P-DH reaction is 8.3 for the enzyme from yeast or blood cells. The rate of formation of NADPH is a measure of the G6P-DH activity and it can be followed by means of the increase in extinction at 340, 334 or 365 nm . The overall reaction is outlined as follows:

$$\text{Glucose-6-phosphate} + NADP^+ \xrightarrow{\text{G6P-DH}} \text{6-Phosphogluconate} + NADPH + H^+$$

10.3 Material and Method :

As indicate by the kit

Precautions:

G6P-DH lysing reagent contains saponin which is harmful if taken internally. Avoid contact with eyes and skin. In case of contact flush with copious amount of water and seek medical attention. Exercise the normal precautions required for the handling of other reagents.

Pipetting by mouth is not recommended for any laboratory reagent.

10.4 Result:

10.5 References:

1- Bishop .M; Fody. E and Schoeff .L.(2005) .Clinical chemistry .Principle , procedures and correlations .Fifth edition. Lippincott Williams &Wilkins.

2. Tietz, N. W. (1987) .Fundamentals of Clinical Chemistry . W.B. Saunders. Philadelphia. third Edition .

EXPERIMENT (11)

11. SICKLE CELL TEST

- Qualitative determination of hemoglobin S (HbS) in blood using a phosphate solubility method.

11.1 Introduction:

This test is a simple and rapid method for detection of the presence of hemoglobin S in blood.

In normal adults, 95% or more of the hemoglobin is present as hemoglobin A (HbA). Hemoglobin S can be inherited in the homozygous state (S/S) produce sickle cell anemia , or in heterozygous ,also called sickle cell trait (A/S)), usually don't exhibit symptoms of the sickle cell anaemia disease (unless under extreme hypoxia).

A point mutation in the **Hb β gene** is responsible for the sickling of RBCs seen in sickle cell anemia .The abnormality is due to Substitution of non polar valine for a charged Glutamic acid in position 6 in the **β** chain .

HbS can also occur in the presence of other abnormal hemoglobin i.e., HbC (S/C) and thalassemia (S-Thal) .These are all sickle cell variants and can produce symptoms of varying severity.

Individuals with Hb-S will be at high risk when exposed to conditions of low oxygen tension such as surgery, high altitude or athletics which may results in serious and fatal clinical complications. In order to avoid or minimise these clinical complications, it is important to screen the individuals for the presence of Hb-S.

11.2 PRINCIPLE

sickling solution contains saponin and dithionite in a phosphate buffer .

Erythrocytes are lysed by saponin and the released hemoglobin is reduced by dithionite in a phosphate buffer. Reduced HbS is characterized by its very low

solubility and by the formation of nematic liquid crystals (tactoids). So that in the presence of HbS or non-S sickling hemoglobins the system becomes turbid.

11.3 MATERIALS and METHOD

As shown in the provided kit.

11.4 CHEMICAL PRECAUTIONS

Dithionite reagent is highly Toxic. .

Do not pipette by mouth.

For other reagents, exercise the normal precautions required for the handling of laboratory reagents. Pipetting by mouth is not recommended for any laboratory reagent.

11.5. INTERPRETATION OF RESULTS

- POSITIVE :

If HbS or any other sickling hemoglobin is present, the solution is turbid and the lines behind the test tubes will not be visible.

- NEGATIVE:

If no sickling hemoglobin is present, the clear solution will permit the lines to be seen through the test tubes.

All doubtful tests along with all positive tests should be submitted for electrophoretic confirmation.

IF THE TEST IS POSITIVE, THE FOLLOWING PERFORMED:

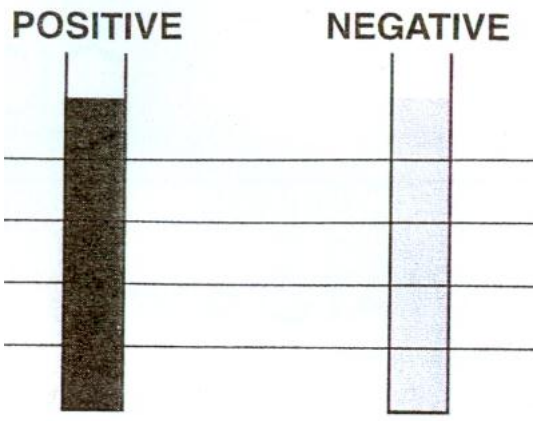
1.Repeat the test and add 4-8 drops urea reagent before the positive whole blood specimen add (and mix by inversion).

2. Allow to stand at room temperature for 5 minutes.

3-. Read the test by holding the test Tubes approximately 3cm in front of a lined scale on the card provided with the kit.

An originally positive HbS test will be confirmed if the solution becomes clear upon addition of the Sickle Cell Urea Reagent. The only known variant that will also clarify is HbC (Harlem).

If the originally positive HbS is still turbid after addition of Urea reagent, a non S-Sickling hemoglobin is indicated.



SCALE FOR SICKLE CELL TUBE TEST

11.5 REFERENCES:

- Tietz, N. W. (1987) .Fundamentals of Clinical Chemistry . W.B. Saunders. Philadelphia. third Edition .
- Bishop .M; Fody. E and Schoeff .L.(2005) .Clinical chemistry .Principle , procedures and correlations .Fifth edition. Lippincott Williams &Wilkins.
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EXPERIMENT(12)

12. Determination of serum iron

- Quantitative determination of iron, unsaturated iron binding capacity and total iron binding capacity in serum .

12.1 OBJECTIVES:

1. To determine the normal level of serum iron.
2. To determine the use of this test in diagnosis of anemias.

12.2 INTRODUCTION & PRINCIPLE:

Iron is themetal component of haemoglobin, myoglobin cytochromes and some proteins of the electron transport chain. The total iron of an adult male is 4-5g and of a female 3-4g. of this 65% is as haemoglobin, 25% as stored iron (ferritin and haemosiderin),10% as other forms (myoglobin, cytochromes etc.) and only 0.1 %as serum iron.

Iron is carried in Fe^{3+} state bound to a specific iron transport protein known as transferrin.

Individuals who lack transferrin show severe hypochromic anaemia and are also susceptible to bacterial and viral infections.

Transferrin distributes iron to those tissues which have a demand for its utilization.

Serum iron is low in iron deficiency anaemia whether due to insufficient intake, malabsorbtion or blood loss.

Low values are also found with anaemias of most chronic infections.

Serum iron concentration is high when marrow cannot utilize iron as in pernicious anaemia, in thalassemia and hemolysis. High values are also found in severe hepatitis due to release from liver cells. In patients with anaemia, serum iron value is usually confirmed by measurement of total iron binding capacity (TIBC).

This value is indicative of the binding capacity of transferrin for iron.

It is of value in differentiation of iron deficiency anaemia from that due to infection. In iron deficiency TIBC is high, and normal or decreased in anaemia due to infection.

TABLE : LABORATORY MARKERS OF IRON STATUS IN SEVERAL DISEASE STATES

CONDITION	**SERUM IRON (50-160μg/dL)**	**TRANSFERRIN (200-400 mg/dL)**	**% SATURATION (20-55)**	**FERRITIN (20-250 μg/L)**
Iron deficiency	Decreased	Increased	Decreased	Decreased
Iron poisoning/overdose	Increased	Decreased	Increased	Increased
Hematochromatosis	Increased	Decreased	Increased	Increased
Malnutrition	Decreased	Decreased	Variable	Decreased
Malignancy	Decreased	Decreased	Decreased	Increased
Chronic infection	Decreased	Decreased	Decreased	Increased
Viral hepatitis	Increased	Increased	Normal/increased	Increased
Anemia of chronic disease	Decreased	Normal/decreased	Decreased	Normal/increased
Sideroblastic anemia	Increased	Normal/decreased	Increased	Increased

Spectrophotometric determinations have the following steps: Fe^{3+} is released from binding proteins by acidification, reduced to Fe^{2+} by ascorbate or a similar reducing agent, and complexed with a color reagent such as ferrozine, ferene, or bathophenanthroline.

Normal range:

Serum iron

CHILD : 50 - 120 μg/dL

ADULT : MALE : 50- 160 μg/dL

FEMALE : 45- 150μg/dL

Serum TIBC

250-425 μ**g**/dL

12.3 Principle

A). The iron in serum is dissociated from its Fe (III)-transferrin complex by the addition of an acidic buffer containing hydroxylamine which reduces the Fe (III) to Fe (II). The chormogenic agent, PDTS*, forms a highly colored Fe (II)-complex that is measured, photometrically at 565 ± 3 nm.

B). The unsaturated iron binding capacity (UIBC) is determined by adding Fe (II) ions to serum, so that they bind to the unsaturated iron binding sites on transferrin . The excess Fe (II) ions are reacted with PDTS to form the color complex which is measured photometrically.

The difference between the amount of Fe (II) added and the amount of Fe (II) measured represents the unsaturated iron binding capacity

.

C). The total iron binding capacity (TIBC) is determined by adding the serum iron value to the UIBC value.

(SERUM IRON + SERUM UIBC = SERUM TIBC)
(μg/dL) (μg/dL) (μg/dL)

D)- transferring **saturation,%=** serum iron concentration **. 100**
TIBC

*PDTS :3-(2-Pyridyl)5, 6-Diphenyl-1, 2, 4-Triazine-p, p'-Disulfonic Acid Disodium Salt Trihydrate.

12.4.Material and method

As shown in the provided kit.

12.5 Result:

12.6 Discussion

12.7 References:

- Aendy.W and Brickell.J. 2007. Clinical chemistry. F.A.Davis company. Philadelphia
- Bishop .M; Fody. E and Schoeff .L.(2005) .Clinical chemistry .Principle , procedures and correlations .Fifth edition. Lippincott Williams &Wilkins.
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EXPERIMENT (13)

13 Red & White Blood Cell Count Differential Count& Blood cell autocounter

13.1 Red Blood Cell Count

13.1.1 Introduction:

Healthy male people have between 4.5 and 6.5 million red cell in each ml of their blood, while healthy female people have between 4 and 5.5 million per ml of their blood. It is often useful to know if patients have more or less red cells than they should have. In sickle cell anaemia, for example, the number of red cells is usually less than normal due to the fact that sickle cell anaemia is a haemolytic disease. Red cell count determination is very useful as a diagnostic tool especially when used in conjunction with indices such as mean cell volume (MCV), mean cellular haemoglobin (MCH) and mean cellular haemoglobin concentration (MCHC).

The red blood cell possesses a most unusual cell shape. The discoid form, together with a distinctive red colouration, makes it one of the most easily recognized cell types in the body. RBC's are made in bone marrow, where they arise from stem cells which differentiate to become reticulocytes. All these precursors are nucleated. Reticulocytes specialize in the production of two polypeptides, α-and β–globin, and protoporphyrin which, together with an iron atom, constitute normal adult haemoglobin. Inherited disorders in any of these syntheses can lead to anaemia. The major function of the red cell is the transport of oxygen from the lungs to the tissues. The amount of haemoglobin increases by seventy-fold the oxygen carrying capacity of the blood over that which can be carried in free solution.

RBC's play a significant role in the control of blood pH: the CO_2 from tissues into the plasma and is taken up by red cells where it is converted

into bicarbonate by the enzyme carbonic anhydrase. The bicarbonate, on passing back into the plasma, forms a major part of the buffering capacity of blood. As an erythrocyte ages, it begins to lose its characteristic discocyte shape, the cell membrane loses lipid, becomes leaky and the cell density increases. After approximately 120 days in the circulation, the red cells are removed by the reticuloendothelial system of the spleen and liver.

13.1.2.Principle

Suspension of RBC's in an isotonic solution, should be used in this experiment, to avoid the destruction of the blood cells (haemolysis). If we suspend the red blood cells in either hypotonic or hypertonic solution, this will lead to the absorption of water by the cells and their rupture. Certain agents will also cause this e.g. detergents and surfactants. Isotonic solutions of substances normally present in plasma inhibit haemolysis.

13.1.3.Reagent and Equipments

1- Hayem's solution

NaCl	0.5%
Na_2SO_4	0.5%
$HgCl_2$	0.25%

2- Haemocytometer

3- Microscope

4- Blood lancet and alcohol swab

13.1.4.Procedure

1- Prick the finger, wipe away first drop of blood and allow collecting another drop without milking the finger (it dilutes the blood with tissue fluid).

2- Insert the end of the pipette into the blood and draw the blood up to 0.5 mark, adjust the column of the blood and clean the adherent blood from the tip of the pipette with a tissue paper.
3- Dip the end of the pipette quickly into the Hayem's fluid and suck until the mixture reaches 101 mark.
4- Rotate the pipette continuously while the diluting fluid is being drawn to facilitate mixing and to prevent coagulation.
5- Remove the mouthpiece, shake the pipette vigorously for one minute to obtain an equal distribution of erythrocytes in the fluid. Close the ends of the pipette with parafilm and place it at a safe place.
6- Take a clean haemocytometer slide, and place over it a clean cover-slip; focus the ruling of the haemocytometer for the erythrocytes under low power of the microscope.
7- Attach the mouthpiece to the pipette and shaking it vigorously discarding the first 4 or 5 drops of the fluid and then wipe the tip of the pipette, and let a small drop forms at the tip.
8- Touch the drop to the rules slide at the margin of the cover slip, the counting chamber will be filled by the capillary action.
9- Whenever counting chamber is filed, the pipette should be shaken thoroughly before putting the drop onto the counting slide.
10- Focus the chamber first under the low power and then under the high power, and count the cells **in 80 smallest squares (5 'R' squares).**

N.B. Cells which touch the upper and the left borders should be included in the count, but those which touch the lower borders must not be included.

13.1.5.Results

1- The surface of the middle platform is 0.1mm lower than those of the other two. It follows that, when the special cover-glass is placed upon the

counting area, a depth of 0.1mm is established between the lower surface of the cover glass and the surface of the ruled central platform.

2- Find the total cells in 80- small squares, divide it with 80 to find the average in one square, multiply it by 200 (dilution factor) and then by 4000 to obtain the number per cubic millimeter.

Thus

If the number of the cells in 80 small squares is N

Then

$$\text{RBC's count} = \frac{N}{80} \times 200 \times 4000 = N \times 10000/ml$$

13.2 White Blood Cells Count

13.2.1.Introduction

Healthy people have between 4000 and 10,000 white cells per ml of their blood. White blood cells originate from precursor cells known as pluripotent stem cells, so-named because they also give rise to red cells and platelets. Unlike the cells and platelets, white cells are not confined to blood, but are present in the tissues also, where they fulfill many of their important functions.

There are two broad classes of white cell:

1- The non-granular leucocytes
2- The granulocyes which contain densely staining granules in their cytoplasm.

White cells are concerned with the defense of the body. The different white cell types do not function independently, but form a part of co-coordinated recognition and defense system that also involves other cells,

such as platelets, or the complement system. This defense system consists of two types of immune response, the production of antibodies and

phagocytosis. **Lymphnodes** in particular contain large number of lymphocytes.

Lymphocytes are the cells responsible for both short and long immunity. They are divided into two broad classes:

1- B lymphocytes, which produce antibodies.

2- T lymphocytes, which participate in various cellular immune reactions.

The phagocytic response is performed mainly by granulocytes which form a 'line of defense' removing unwanted or harmful materials.

13.2.2 Principle

In this experiment, it is necessary to obtain RBC's free preparation of WBC's from blood. Suspension of the red blood in a very hypotonic solution will lead to the destruction of RBC's. We will discuss TURK'S FLUID [0.5% Gentian violet in 1.0% acetic acid] for this purpose. Acetic acid lyses the red cells without harming the white cells while Gentian violet will stain leucocytes.

13.2.3 Materials

- Haemocytometer
- Turk's fluid
- Blood lancet
- Alcohol swab

13.2.4 Procedure

1- Prick the finger, wipe away first drop of blood and allow collecting another drop without milking the finger (it dilutes the blood with tissue fluid).

2- Insert the end of the pipette into the blood and draw the blood up to **0.5**mark, adjust the column of the blood and clean the adherent blood from the tip of the pipette with a tissue paper.

3- Dip the end of the pipette quickly into the Turk's fluid and suck until the mixture reaches **11**mark.

4- Rotate the pipette continuously while the diluting fluid is being drawn to facilitate mixing and to prevent coagulation.

5- Remove the mouthpiece, shake the pipette for one minute to obtain an equal distribution of WBC's in the fluid. Close the ends of the pipette with parafilm and leave it for 30 min.

6- Take a clean haemocytometer slide, and place over it a clean cover-slip; focus the ruling of the haemocytometer fr the erythrocytes under low power of the microscope.

7- Attach the mouthpiece to the pipette and shaking it vigorously discarding the first 4 or 5 drops of the fluid and then wipe the tip of the pipette, and let a small drop forms at the tip.

8- Touch the drop to the rules slide at the margin of the cover slip, the counting chamber will be filled by the capillary action.

9- Whenever counting chamber is filled, the pipette should be shaken thoroughly before putting the drop onto the counting slide.

10- Focus the chamber first under the low power and then under the high power, and count the cells **in four large squares each of which is formed by the sixteen squares.**

N.B. Cells which touch the upper and the left borders should be included in the count, but those which touch the lower borders must not be included.

13.2.5 Results

Find the total cells in four large squares, divide it by 64 (No. of small squares in 4 large square), multiply it by 20 (dilution factor) and then by 160 to obtain the number per cubic millimeter.

Thus, if the number of the cells in 4 large squares is Y

Then

$$\text{WBC's count} = \frac{Y}{64} \times 200 \times 160 = Y \times 50/ml$$

13.3 Differential Count of WBC's

13.3.1 Introduction:

The differential count is the relative number of each cell type compared to the total cells. There are two broad classes of white cell

The non-granular leucocytes: which are classified as two types:

a. Lymphocytes: which are formed mainly in the thymus and lymphoid tissue. They recycle many times between the blood, tissues and lymph.

b. Plasma cells: which are rarely seen in blood, but present in the tissues.

The granulocytes: which are formed mainly in the bone marrow, and contain densely staining granules in their cytoplasm. They consists of two types:

1- Polymorpohonuclear granulocytes (PMN)

which are distinguished by the appearance of their nuclei of multiple lobes and are classified as: **Neutrophils – Basophiles – Eosinophils.**

PMN granulocytes may spend only a few hours in blood before entering the tissues. Their overall lifespan is probably a few days and they do not return to the blood.

2- Monocytes

which conain fine granules in their cytoplasm and have a round nucleus. Monocytes arise in the spleen and lymphoid tissues. They are present in the blood for a few days only. They migrates to the tissues, where they differentiate into macrophages which survive for months or years without re-entering the blood stream.

Class of White Cells	**White Cell Type**		**% of total white cell population**
Granulocytes	Polymorphonuclear Granulocytes PMN	Neutrophils	40 – 75
		Bashophiles	Approx. 1
		Eosinophils	1 – 6
	Monocytes		2 – 10
Non-granular Leucocytes	Lymphocytes		20 – 45
	Plasma cells		Ŏ

Ŏ: Rarely seen in blood, but present in the tissues.

13.3.2 Principle:

The differential count has a great diagnostic significance such as in certain disease conditions, one type of cell will increase relative to the other. For example, in case of parasitic infestation, eosinophils count might exceed (10%). Classification of PMN granulocytes is based on the size, shape, number, and staining characteristics of their granules. We will use **Leishman's** stain to differentiate between **WBC's** as indicated in the following table:

	Type of Cell	**Colour of the Stain**
1	Neutrophils nuclei	Purple
2	Eosinophil granules	Orange – Red
3	Basophiles granules	Dark Blue
4	Lymphocytes nuclei	Dark Purple
5	Platelets granules	Violet
6	RBC	Pink

13.3.3.Materials

1- Lishman's stain: Dissolve 0.13 gm of Leishman powder in 100 ml of methanol.
2- Phosphate buffer: 0.5M, pH 7.00
3- Prepared blood fillm.
4- Microscope with oil immersion lens.

13.3.4.Procedure

On staining the blood film, cover the smears with 10 – 15 drops of stain for 1 – 2 minutes. Add about 30 drops of buffer, mix well with blood. Let stain for 7 – 10 minutes, wash off with distilled water, dry and see under oil immersion lens.

13.3.5.Counting and Results

- A total number of 400 – 800 cells are counted and each type is recorded separately.
- Results are recorded for each type of cells as a percentage of the total.

- Differential Count of WBC's

	Type of Cell	Colour	Number you found
1	Neutrophils nuclei		
2	Eosinophil granules		
3	Basophiles granules		
4	Lymphocytes nuclei		
5	Platelet granules		
6	RBC		

13.3.6 Reference Ranges

	Male	Male/Female	Female	Units
RBC	4.2 – 5.5		3.7 – 5.0	10^{12}/l
HCT	38 – 48		36 – 46	%
MCV		80 – 100		fl
RDW		11.5 – 15		%
HGB	135 – 165		115 – 140	g/l
MCH		28 – 35		pg
MCHC		330 – 360		g/l
PLT		150 – 380		10^9/l
MPV		7.5 – 10.5		fl
WBC		4.0 – 9.0		10^9/l
LYM		1.2 – 3.5		10^9/l
MID		0.1 – 0.6		10^9/l
GRA		1.4 – 7.0		10^9/l
LYM		20 – 48		%
MID		2 – 10		%
GRA		42 – 80		%
Bands		0 – 5		%
Neutrophils		55 – 65		%
Eosinophils		1 – 5		%
Basophils		0 – 1		%
Lymphocytes		22 – 35		%
Monocytes		3 – 8		%

Reticulocytes	0.2 – 2.0	%

Abbreviations

RBC	Red Blood Cells
HCT	Haematocrit
MCV	Mean Cell Volume
RDW	Red Cell Distribution Width
HGB	Haemoglobin
MCH	Mean Cell Haemoglobin Concentration
MCHC	Mean Cell Haemoglobin Concentration
PLT	Platelets
MPV	Mean Platelet Volume
WBC	White Blood Cells
LYM	Lymphocytes
MID	Midcells
GRA	Granulocytes

fl	femtolitre	10^{-15} litre
pg	picogram	10^{-12} gram

13.4 References:

- Dacie .J.V and Lewis.S.M. Practical Haematology, Ninth Edition, 2001. Churchill-Livingston

EXPERIMENT (14)

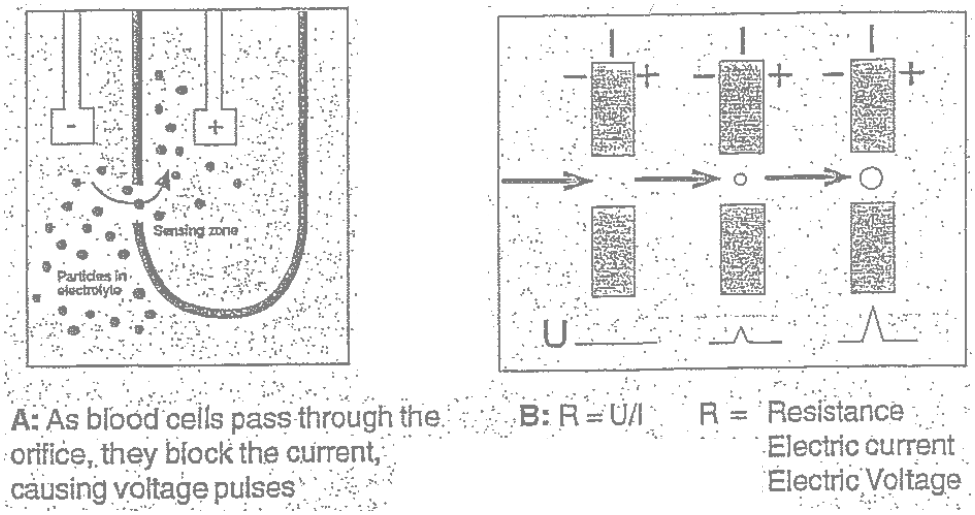
14 Blood Cells Autocounter

14.1 Description of the Autocounter

14.1.1 Description of the Parameters

14.1.1.1 Aperture Impedance Method

Detection of RBC, PLT and WBC is accomplished by measuring the impedance in the orifice of the transducer. The transducer is mounted in a conductive solution. Electrodes with opposite charges establish a weak current. As blood cells pass through the orifice, they block the current, causing voltage pulses. The amplitude of the pulse is directly related to the size of the represented cell. The number of pulses is equivalent to the number of cells passing through the orifice during the counting period.

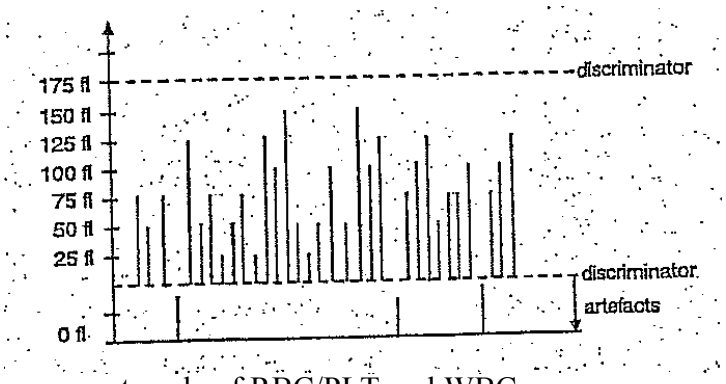


With this technique, thousands of particles can be counted in a few seconds. To be able to count blood cells they must be diluted in an isotonic solution. Thereby the RBC/PLT can be counted and the volume determined. In order to count WBC, the red blood cells must first be destroyed i.e. hemolysed. Otherwise the red blood cells interfere with the white cell counting, both due to their size and the fact that the number of th red blood cells are approximately 10^3 more per litre blood compared to the white blood cells.

The amplitude of each pulse, that directly corresponds to the cell volume, is measured and accumulated. The AutoCounter has LOW and HIGH discriminators to filter any amplitudes not within the required range.

The size distribution graphs show the size of the counted cells in femtolitre along the x-axis and the relative number of cells along the y-axis. The x-axis is divided in 4096 different channels in varying width depending on the cell type. The AutoCounter reports the number of cells which have been registered in the respective channels. The findings are then presented in a histogram in relation to the number of cells in each channel.

Each RBC, PLT and WBC count is measured on a precise volume of the dilution. The amount measured is determined by the distance between two optical sensors, which are mounted on a precision column called the measuring tube.



During each measurement cycle of RBC/PLT and WBC a vacuum pump pulls the dilute through the measuring tube. When the liquid meniscus passes the optical path of the start sensor, the counting is activated. Detected pulses within the discriminators are accepted and accumulated only when the cycle is in counting mode.

When the liquid meniscus reaches the optical path of the stoop sensor, the counting stops. During each measurement, two or more cells can enter the orifice simultaneously. The corresponding change in impedance is detected as a single pulse with a high amplitude, resulting in the loss of one or more pulses (counts). The reduction, referred to as coincidence passage loss, is statistically predictable,

and is related to the effective volume of the orifice and to the concentration of the dilution. The AutoCounter automatically corrects each RBC, PLT and WBC count for coincidence passage loss.

In order for the method to work properly the following is required:

- A correct cell dilution.
- A sufficient and repeated mixing of the cell dilution.
- A constant flow rate through the orifice.
- A constant radius of the orifice.
- A constant measuring volume.

(The orifice radius is influenced by proteins which are concentrated in the transducer, thereby reducing the radius. This results in an imprecise determination of the cell size. Frequent cleaning of the transducer and its orifice is thus important in order to eliminate the proteins).

14.2 RBC – Red Blood Cell Count

RBC is presented in number of cells per litre or microlitre. For human blood the RBC discrimintors are set to minimum 30 and maximum 250 femtolitre.

14.3 MCV – Mean Cell Volume

MCV is presented in femtolitre or cubic micrometer. Determination is based on statistical methods from size distribution span of counted red blood cells.

MID region (mid size cells): Ranges from 95 to 120 femtolitre. Cells in this area typically correlate to monocytes, eosinophils and basophils and also degranulated neutrophils, precursor cells, blasts and plasmacytes. GRA region (large size cells): Ranges from 120 to 420 femtolitre. Cells in this area typically correlate to neutrophils. In approximately 20% of the samples eosinophils can also locate in this region. Precursor granulocytic

cells, especially bands, have a tendency to locate close to the mid cell region.

14.4 EO-Eosinophils

In the models AC910EO-0, AC920EO+2, AC920EO+0 and AC970EO-0 it is impossible to determine the eosinophils using the Swelab EO kit. EO is presented in number of cells per litre or microlitre. The eosinophils belong to the granulocytes and in normal samples the total amount is low and can not be detected in a 3-part differential. The semi automatic EO measurement is a quantitative method that is performed when a significant high MID cell count is obtained or when a high EO content can be suspected. Detection of DO is accomplished by lysing all cells except the eosinophils using an alkaline non-ionic based surfactant. The remains, activated and non-activated eosinophils, are counted In the AutoCounter.

The EO apper in an intermediate position overlapping the MID and GRA areas in the WBC histogram. After treatment with the lyse reagent the eosinophil nuclei is similar in size to nuclies of monocytes, some abnormal cells and occasionally granulocyees. The presence of elevated MID cells can therefore be an indication of high eosinophils level. The discriminators set in the "6 Set up menu", determine the minimum and maximum size of the eosinophils. The EO discriminators are set to 70 and 200 femtoliter.

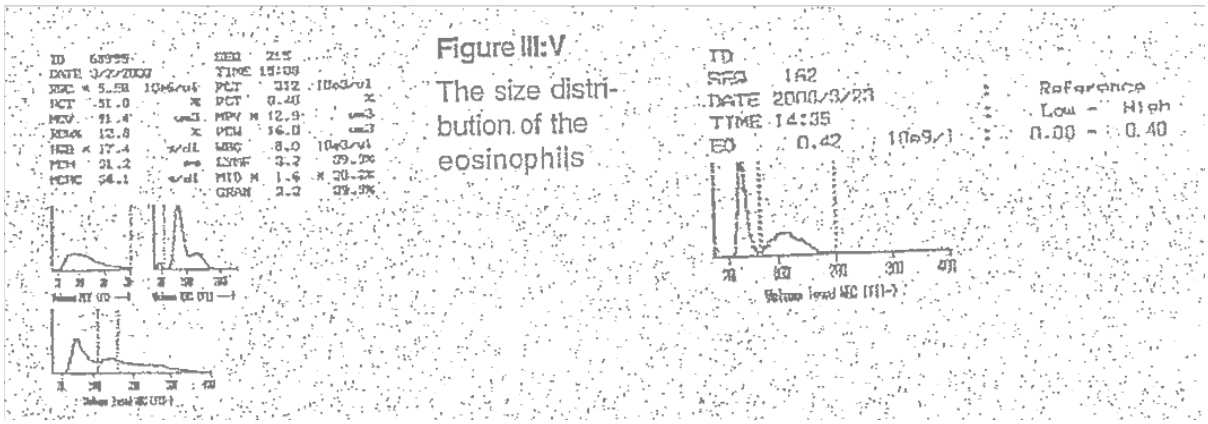


Figure: WBC Histogram with a MID Cell Fraction

14.5 PLT – Platelet Cell Count

PLT is presented in number of cells per litre or microlitre. The AutoCounter uses floating discriminators for PLT counting. Within the defined limits the software automatically find the minimum concentration of cells and set the discriminator to this point. The range for human samples is from 2 and the upper limit is floating between 15 and 30 fl. This means that the AutoCounter will search for a distinct discrimination point between 15 and 30 fl.

14.6 MPV – Mean Platelet Volume

MPV is presented in femtolitre or cubic micrometer determined on the total number of PLT counted. The histograms describes the size distribution span of the counted cells. When the PLT count is less than 40 x 10^9/l MPV is not reported.

14.7 WBC – White Blood cell Count

The differentiation of the WBC cells into lymphocytes, mid-cells and granulocytes is presented in number of cells per litre or microlitre and in percentage of total number of WBC cells. The MID discriminator of WBC WBC is set to 95 and 120 fl. The WBC histogram is automatically adjusted depending on number of cells, i.e. expanded for low values and compressed for high values. The size distribution of non-differential WBC should be seen as a check of the hemolysing process only. A too low concentration of hemolyzer gives a too high number of cells due to presence of only partially hemolyzed red blood cells at 30 femtolitre or just above. A too high concentration of hemolyzer gives a too low number of WBC. The cells will decrease in size to below 30 femtolitre. The WBC differentiation as in the AutoCounter, is a screening method. Les common normal and abnormal cells and cell distribution must be visually investigated in a microscope.

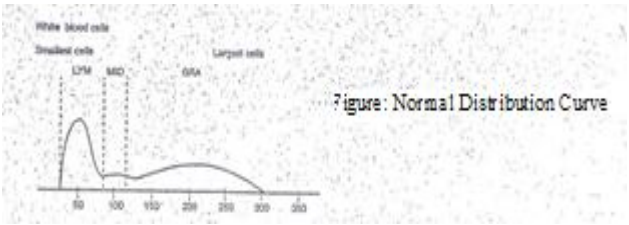


Figure: Normal Distribution Curve

LYM region (small size cells): Ranges from 30 to 95 femtolitre. Cells in this area typically to lymphocytes. Other cell type that could locate in this region arenucleated red blood cells, clumped platelets, macrocyte platelets, variant (atypical) lymphocytes or blasts.

14.8 Calculated Parameters

HCT-Hematocrit

The HCT is presented in percent or litre per litre. The HCT is the volume of packed erythrocytes in relation to the total blood volume.

HCT = RBC x MCV

14.9 RDW-Red Cell Distribution Width

The RDW is presented in percentage of the red cell volume distribution. The RDW is an index of the variation in red cell size (anisocytosis). The RDW is calculated directly from the RBC histogram. Not all cells are included in the RDW calculation thus RDW is only measured on a portion of the RBC histogram.

14.10 MCH and MCHC, Indices Calculation

MCH – Mean Cell Hemoglobin –s presented in pictogram or femtomol.

MCH = HGB / RBC

MCHC – Mean Cell Hemoglobin Concentration – is presented in grams per litre, grams per deciliter or millimol per litre.

$$MCHC = \frac{HGB}{HCT}$$

The red cell indices provide an indication of red cell morphology and can also be used to indicate instrument calibration and stability. The indices are very stable parameters. They do not significantly change from day to day or year to year even though the parameters which are used to calculate them dramatically increase or decrease. The indices are calculated automatically.

14.11 PCT – Plateletcrit

The PCT is presented in percent or litre per litre. The PCT is the volume of packed platelets in relation to the total blood volume.

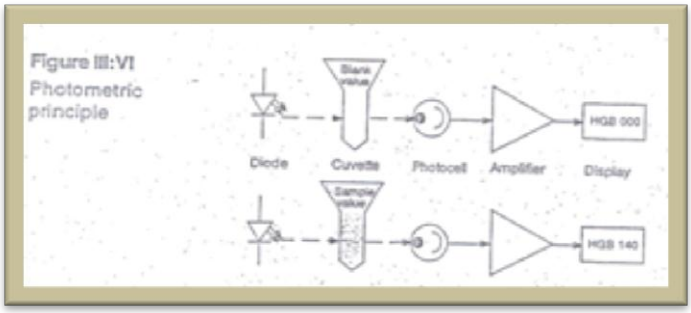
PCT = PLT x MPV

14.12 PDW – Platelet Distribution Width

The PDW is presented in percentage of the platelet cell volume distribution. The PDW is an index of the variation in platelet cell size. The PDW is calculated directly from the histogram. Not all cells are included in the PDW calculation thus PDW is only measured on a portion of the PLT histogram.

Note: PCT and PDW are for laboratory use only.

14.13 Photometric Method



14.14 HGB – Hemoglobin

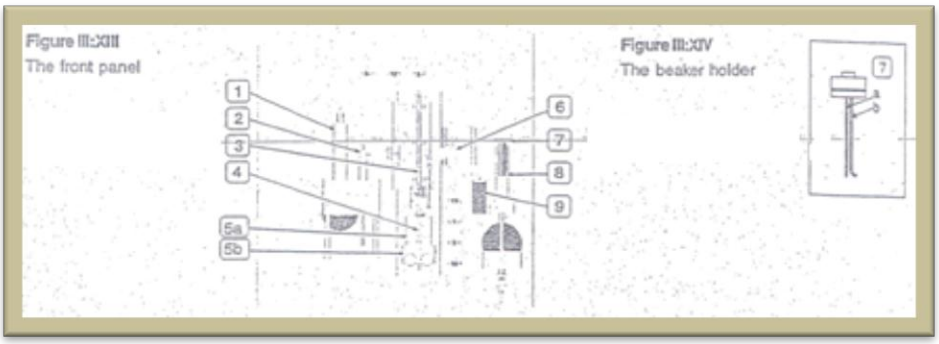
The quantitative determination of the prepared sample is obtained by measuring the light absorption. Light from a diode is passing through the cuvette. First only with the reagents as a zero reference known as a blank value. The zero reference value for each sample is obtained from the RBC/PLT dilution immediately before this dilution is drained from the counting beaker. The light transmission is measured by a photocell.

The light transmission is measured once again the WBC.HGB dilutin to absorb light at 555 nm and is converted to a digital value. HGB – the hemoglobin concentration in blood is measured by the photometer and is presented in grams per litre, grams per deciliter or millimol per litre. The hemolysing reagent is lysing the RBC-membranes and the hemoglobin molecule are released. The Fe^{2+} is oxidated to Fe^{3+} and a stable hemoglobin complex is formed. The photometer measures the absorption and calculate the concentration of hemoglobin.

AC910EO+

14.15 Components

The Front Panel



1- **Isotonic Diluent Syringe**. The syringe is set to approx. 4 ml.
2- The blood volume is determined by the Blood Volume Syringe and is 20 µl.
3- The dilution is pulled into the **Measuring Tube** by vacuum pump.
4- The cells are counted when passing the orifice in the **Transducer**.
5- The 5a **Counting Beaker** has nozzles for delivery of the RBC/PLT dilution and the hemolyzed WBC/HGB dilution. The air used for mixing the secondary RBC/PLT dilution in the counting beaker enters via the bottom nozzle which also is the drain. The lower part of the counting beaker is the HGB cuvette, fitted into the **5b HGB Photometer**.

6- After aspiration of a sample the **Pipette Arm** moves down to the counting beaker and the AutoCounter dilutes the sample with diluent.

7- The **Beaker Holder** consists of two tubes: Figure:

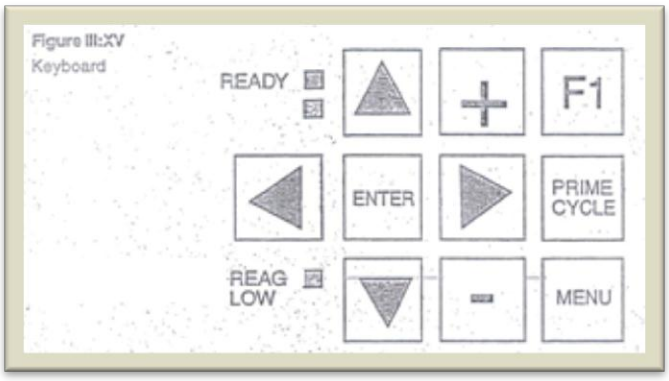
   a. Tube for transfer the dilution to the counting beaker.

   b. Tube for delivery of the hemolysing reagent

8- **Hemolysing Reagent Syringe**. The syringe is set to approx. 3 ml.

9- The drain from the measuring is transferred into the **Drain Cup**.

Keyboard



The **ENTER** key is used to:

Enter into a selected menu

Enter options within a menu

◄, ▲, ▼, ► The arrow keys are used to:

Step forwards or backwards within a menu

Step sideways within a menu.

Change digital position.

The + (Plus) and – (minus) keys are used to:

Switch on or off a function

Increase (+) or decrease (-) a numerical value.

The **F1** key is not in use

The **PRIME CYCLE** key is used to:

Flush or fill up the AutoCounter with reagent in the "1 Measurement"

The **MENU** key is used to return to the previous menu.

14.15.1READY Lamp:

Green light = home position, ready to start next analysis.

Red light = sample aspiration.

Red flashing light = aspirating competed, waiting for next move.

No light = the time between aspiration and home position

14.15.2 REAG LOW Lamp:

Is flashing red when the reagent level is low in any of the reagent containers.

The reagent which is too low is indicated on the display when entering the "1 Measurement".

14.16 Sample Collection

14.16.1 Venous Blood

Collect the blood by venepuncture in a tube containing tripotassium ethylenediaminetetra-acetic acid (K_3EDTA) as anticoagulant (0.07 mol/ml blood). After blood collection the test tubes should immediate be gently mixed by reversing them approx. 10 times and there after rest of 15 minutes prior to analysis in order for the cells to stabilize. If the sample is analysed immediately, the MVC and WBC differential can be affected.

14.16.2 Stability

For whole blood cell counts which include WBC differential, the best results are obtained when the samples are analysed within 8 hours after drawing. These samples shall be kept at room temperature.

Note: For good quality results it is recommended that hematology samples are analysed as quick as possible after 15 minutes rest.

The blood count, except WBC differential can be analysed up to 24 hours after drawing if the specimens are stored in refrigerator. Make sure that the samples are brought to room temperature and well mixed before analyzing.

14.16.3 Capillary Blood

Use the Swelab dispenser calibrated for the AutoCounter. Dispense isotonic diluent into a sample beaker. In the C910EO+ Dispense-function is used. Collect 20 µl capillary blood using a micro capillary tube and immediately transfer the blood into the sample beaker with 4 ml diluent. Rinse the capillary tube carefully with the isotonic diluent. Seal the sample beaker and ix gently.

14.16.4 Stability

The analysis of the prediluted sample should be performed as soon as possible but no later than within 60 minutes after collection and the sample dilution shall be kept at room temperature.

Note: The MVC value in prediluted control blood may decrease up to 5 femtolitre if not measured immediately, due to the dilution effect.

14.17 Analysis Process

14.17.1 Whole Blood

A. At green READY light, 20 µl blood is aspirated from the whole blood sample via the pipette when the ▲-key is pressed. The aspiration is indicated by red light and when the aspiration is finished a flashing red light is shown. An unused beaker is placed under the pipette and the ▼-key is pressed, the primary dilution is performed.

B. 20 µl of the primary dilution is aspirated via the pipette when the ▲-key is pressed. The remaining of the primary dilution is placed in the WBC/HGB position and when the start lever is pulled the analysis process starts.

The 20 µl of the primary dilution is mixed with 4ml isotonic diluent delivered from the diluid syringe directly into the counting beaker. The RBC/PLT dilution is mixed using air. The dilution is pulled into the measuring tube by the vacuum pump and the RBC/PLT count starts.

While RBC and PLT are counted the primary dilution in WBC/HGB position is hemolysed. When the RBC/PLT counting is ready the HGB blank is measured, the orifice is cleaned and the dilution is drained. The WBC/HGB dilution is transferred to the counting beaker and the WBC is counted. When the WBC counting is ready HGB is measured aznd the orifice is cleaned. The dilution is drained and the counting beaker is rinsed twice with isolation diluent.

The results are displayed. The READY lamp shows green light when the analysis process is ready and a new sample can be aspirated.

14.17.2 Prediluted Blood

The pre-diluted blood sample is prepared by adding 20 µl of blood to 4 ml diluent. The 4 ml diluent is dispensed using the Dispense-function of AC910EO+. The 20 µl blood is added to the 4 ml diluent using e.g. micro capillary tube. The description of the analysis process of the prediluted sample is the same as above from step B.

14.17.3 Dispense (in AC910EO+)

The menu is only available in the AC910EO+ software and is used to dispense 4 ml diluent for preparation of prediluted samples.

1- From the MAIN MENU step to "3 Dispense" with the ▼ key and press ENTER.
2- Place an unused beaker under the pipette and press the ▼ key to dispense 4 ml diluent into the beaker.
3- Exit with the MENU key.

14.17.4 Prediluted Blood

The pre-diluted blood sample is prepared by adding 20 µl of blood to 4 ml diluent. The 4 ml diluent is dispensed using the Dispense-function AC910EO+. The 20 µl is added to the 4 ml diluent using e.g. micro capillary tube. The description of the analysis process of the prediluted sample is the same as above from step B.

B 20 µl of the primary dilution is aspirated via the pipette when the ▼-key is pressed. The remaining of the primary dilution is placed in the WBC/HGB position and when the start lever is pulled the analysis process starts.

The 20 µl of the primary dilution is mixed with 4ml isotonic diluent delivered from the diluid syringe directly into the counting beaker. The RBC/PLT dilution is mixed using air. The dilution is pulled into the measuring tube by the vacuum pump and the RBC/PLT count starts.

While RBC and PLT are counted the primary dilution in WBC/HGB position is hemolysed. When the RBC/PLT counting is ready the HGB blank is measured, the orifice is cleaned and the dilution is drained. The WBC/HGB dilution is transferred to the counting beaker and the WBC is counted. When the WBC counting is ready HGB is measured aznd the orifice is cleaned. The dilution is drained and the counting beaker is rinsed twice with isolation diluent.

The results are displayed. The READY lamp shows green light when the analysis process is ready and a new sample can be aspirated.

14.18 Measurement in AC910EO+

14.18.1Background Count

1- In the Main Menu step to "1 Measurement" and press ENTER.
2- Aspirate with the ▲–key.
3- Dispense the blank dilution into an unused beaker with the ▼-key.
4- Aspirate diluent from the beaker with the ▲-key.
5- Place the beaker in the WBC/HGB position. Pull the start lever towards the beaker. The analysis starts.
6- Repeat the background count until the values do not exceed the recommended level.

RBC	£0.02 X 10^{12}/l	PLT	£10 x 10^{9}/l
HGB	00 g/l	WBC	£0.2 x 10^{9}/l

Note: Always start and finish a measurement serie with a background count.

14.18.2 Blood Count of Whole Blood

2- In the Main Menu step to "1 Measurement" and press ENTER.
3- Mix the blood sample carefully and aspirate it through the pipette with the ▲–key. When the READY lamp shows red flashing light, the aspiration is completed. Wipe the pipette carefully.

4- Dispense the primary dilution into an unused beaker with the ▼-key and continue with step 2 in the below section "Blood Count of Prediluted Samples".

14.18.3 Blood Count of Prediluted Blood

1- In the Main Menu step to "1 Measurement" and press ENTER.
2- Mix the prediluted sample by gentle swirling and aspirate via the pipette with the ▲–key. When the READY lamp shows red flashing light, the aspiration is completed. Wipe the outside of the pipette carefully.
3- Place the beaker in the WBC/HGB position. Pull the start lever towards the beaker. The analysis starts.
4- Press ENTER to enter the ID-number with the + (plus) or – (minus) keys. It is possible to enter the ID-number during the total counting time. The AutoCounter measures the prediluted blood sample. The measurement is completed when the READY lamp shows green light. The measurement results remain on the display until start of next analysis. To view the histogram pres the ▶–key. Press the ◀–key to return to the results of the analysis.
5- Repeat from step 2 for all prediluted samples.

Set Next Seq. No.

If the sequence number has to be changed see "6.3 Set next sequence number."

14.19 EO Menu

Measurement EO

Sample Separation

1- Switch on the AutoHeater. The red light diode marked with POWER is switched ON during warm up of the AutoHeater. When the AutoHeater has reached the right temperature, after approx. 10 minutes, the green light diode marked TEMP is switched ON.

2- Dispense 4.5 ml of the EO reagent with SWELAB's EO-dispenser into a sample beaker.

3- Preheat the EO reagent in the position 1-5, approx. 10 minutes. If more than 5 EO samples shall be analysed, load the AutoHeater during the measurement process.

4- Prepare the AutoCounter for an EO measurement:

- In the Main Menu step to "2 EO menu" and press ENTER. Step to "2.1 Measurement EO" and press ENTER.
- Start to measure a background count with pre-heated EO-reagent.

14.19.1In AC 970EO+/Ac920EO+:

b. Take one beaker of preheated EO reagent and place it in the prediluted postion.

c. Press "start PreDilute". The READY lamp switches from green to red light and at the same time the dilution is aspirated.

14.19.2In AC910EO+:

Take one beaker of preheated EO reagent and place it in the WBC/HGB position and pull the start lever towards the beaker.

14.20 Measurement of EO Dilution in the AutoCounter

1- Remove the beaker from position 1 in the AutoHeater and turn the beaker wheel clockwise one step.

2- Add 20 µl of blood using the micro capillary tubes and mix the dilution gently by swirling the beaker. Put the beaker lid on.

Note: Do not turn the beaker upside down. The reagent can leak out due to the surface active ingredient in the EO reagent.

3- Place the EO dilution in the position marked green and press the beaker to the bottom. An alarm sounds and the timer starts.

4- Fill position 5 in the beaker wheel with a new beaker if necessary.

5- After 90 seconds the lysing of all cells, except EO, is completed and an alarm sounds. Press once again the beaker to the bottom to switch off the alarm. Measure the sample within 30 seconds.

6- In AC970EO+/AC920EO+:

a. Swirl the EO dilution carefully and place it in the prediluted position.

b. Press "START PreDilute". The READY lamp switches from green to red light and at the same time the sample is aspirated.

c. Press ENTER to enter ID-number with the + (plus) or – (minus) keys. It is possible to enter the ID-number during the total counting time.

.1 In AC910EO+:

a. Swirl the EO dilution carefully and place the dilution in the WBC/HGB position.

b. Pull the start lever towards the beaker. The READY lamp fo the AutoCounter switches from green to red light and start he analysis.

c. Press ENTER to enter the ID-number with the + (plus) or – (minus) keys. It is possible to enter the ID-number during the total counting time.

7- The AutoCounter measures the EO sample and presents the results on the display. When measurement is completed the READY lamp shows green light. The results and the histogram remain on the display until start of next analysis.

Note: Results below 0.10 should be reported as $< 0.10 \times 10^9/l$.

8- Repeat from step 1 for all EO samples.

9- Clean and restore the system when all EO samples are measured. Run a background count in an unused beaker with 4 ml diluent.

10- After the background count the instrument is ready for measurement of routine blood samples.

14.21 EO Memory

The "2.2 EO Memory" is designed in the same way as for the '4 Sample memory" but the EO memory only contains the eosinophil results including the histograms.

When the memory is "full" the first sample entered is automatically deleted. In the EO memory a search of ID-number, DATE or SEQ - number can be performed.

2. Select the different ways of search conditions ID, DATE and/or SEQ-number.
3. Select one of the different options below, and press ENTER.
    a. View selected EO samples.
    b. Statistical calculation
    c. Print selected samples
    d. Send selected samples
    e. Delete selected samples
4. Exit with the MENU key.