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Practical Note

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Ministry of Higher Education
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Biochemistry of Blood
(BCH 220)

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.

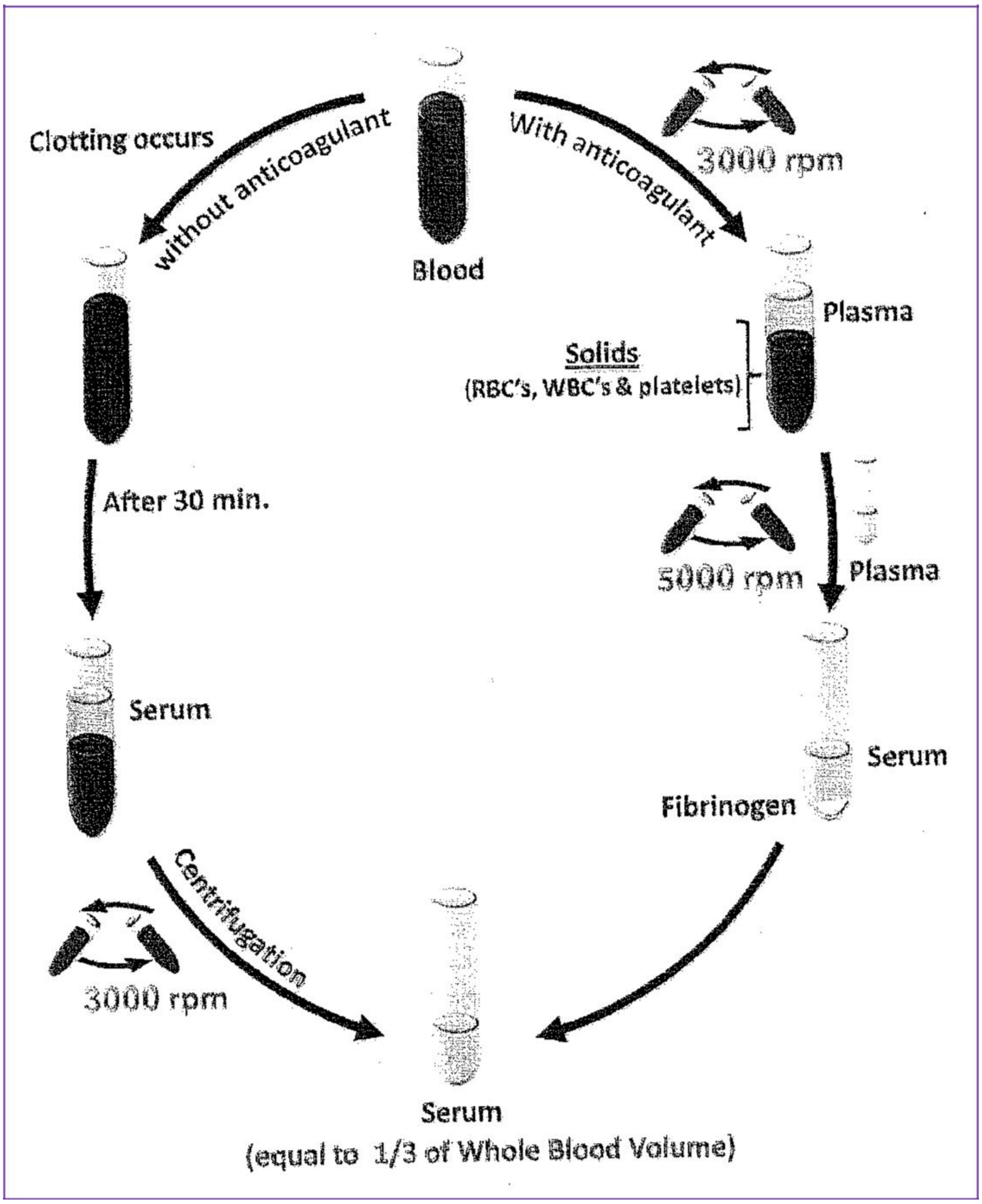
1.2.11 Changes in Blood on Keeping

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							

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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:

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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.

Succinyl CoA Glycine

δ-Aminolaevulinic acid

Prophobilinogen

UroporphyrinIII Uroporphrinogen III Uroporphtrinogen I Uroporphyrin

I

Coproporpohyrin III CoproporphyrinogenIII Coprophyrinogen I

Coproporphyrin I

Protoporphyrin-IX

Iron

Haem

Globin

Haemoglobin

Globin

Haematin

Iron

Biliverdin

Bilirubin

Conjugated Bilirubin

Urobilinogen etc. Urobilin etc.

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^{o}C.

-Caution

All solutions are poisonous. Use safety pipettes.

-Requirements

Wavelength	546 nm
Cuvette	1 cm light path
Incubation temp.	20-25$_{o}$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^{o}C for 5 min.

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

8.1.5 Calculation

C_{HGB} = 36.77 x A_{HGB} [g/dl]

= 22.82 x 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).

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

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	%

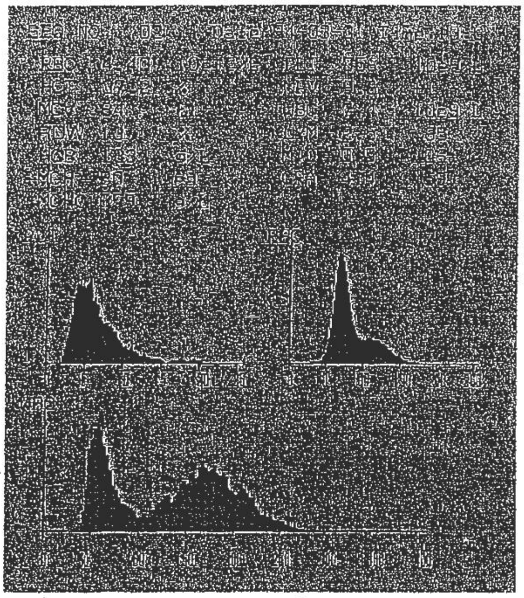
Abbreviations

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 K3EDTA 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

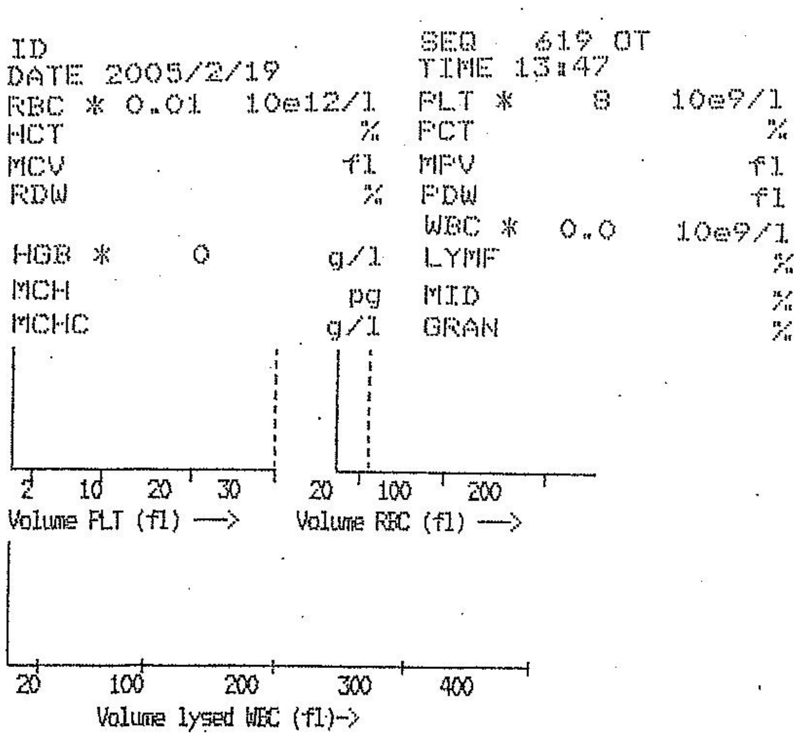
HCT	=	RBC x MCV	%
MCH	=	HGB / RBC	pg
MCHC	=	HGB / HCT	g/l
RDW	=	SD / MCV	%

	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

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

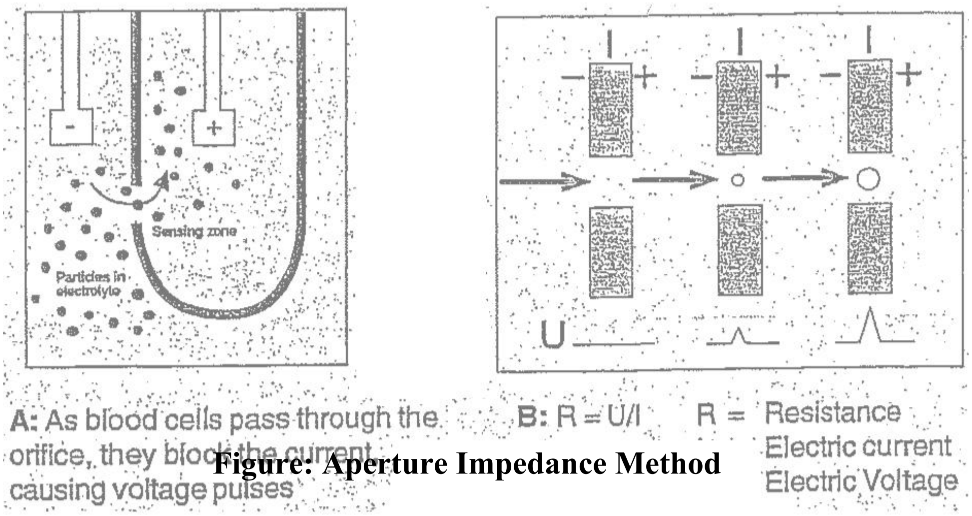


Figure: Aperture Impedance Method

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.

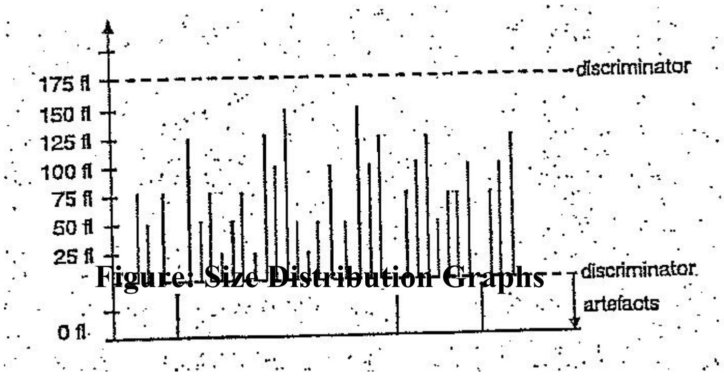


Figure: Size Distribution Graphs

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.

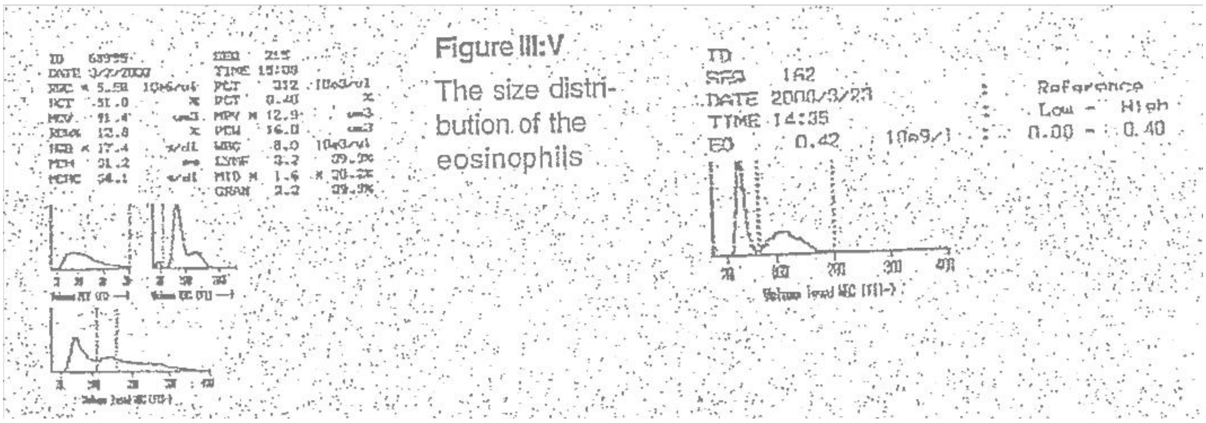


Figure: WBC Histogram with a MID Cell Fraction

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.

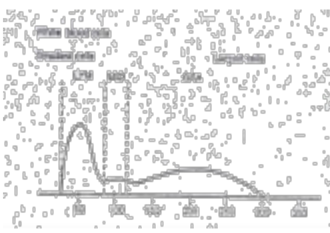


Figure: Normal Distribution Curve

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 = 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

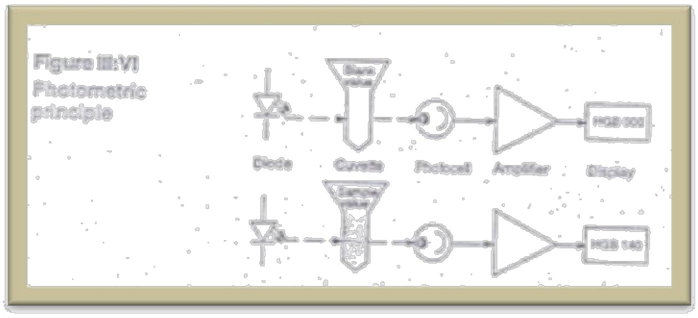
The PCT is presented in percent or litre per litre. The PCT is the volume of packed platelets in relation to the total blood volume. PCT = PLT x MPV

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

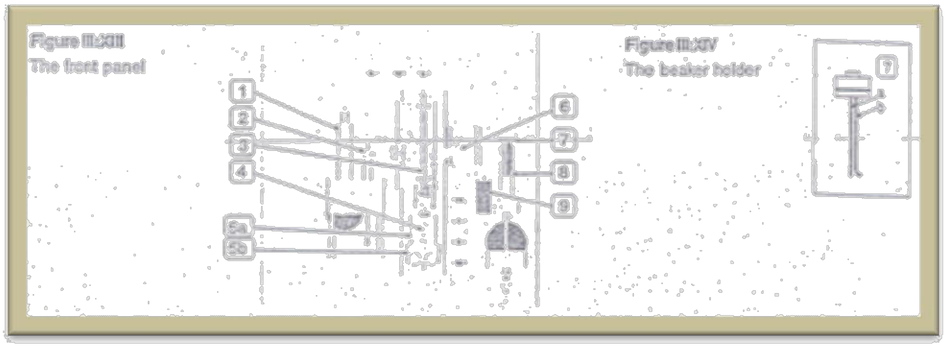
The quantitative determination of the prepared sample is obtained by measuring the light absorption. Light from a diode is passing through the cuvette. First only with the reagents as a zero reference known as a blank value. The zero reference value for each sample is obtained from the RBC/PLT dilution 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:

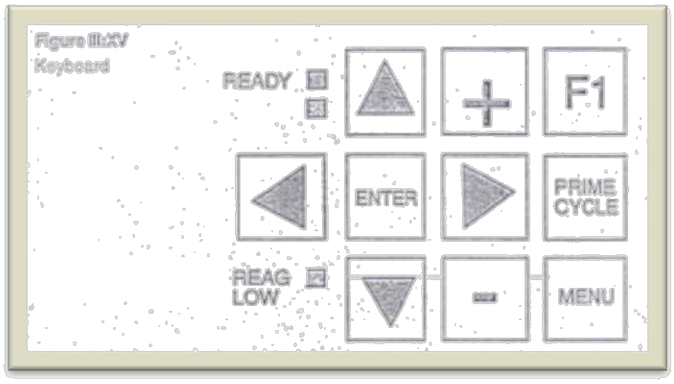
   a. Tube for transfer the dilution to the counting beaker.

   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

<u>**Measurement EO**</u>

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.

<u>Note:</u> 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.