APPENDIX
MEASUREMENT OF BLOOD PARAMETERS USING CLINICAL PROCEDURES

The measurement of blood parameters using clinical lab procedure is described in this section.

1. COLLECTION OF BLOOD

There are two ways of obtaining the blood samples:

1. From the Vein
2. From the Capillary

Venous blood is preferred because a number of investigations can be done and repeated on the same sample and because the examination can be done at leisure.

Venous Blood

Venous blood is obtained venepuncture. Good light and a comfortable position for both the patient and the operator are necessary. Ideally, the patient should be lying down. In adults, one of the veins in the antecubital fossa is chosen. Make the veins prominent by applying a tourniquet or a blood pressure cuff kept at the diastolic pressure; further the patient is asked to open and choose the hand several times vigorously and then to keep the first clinched on a roll of bondage. Select the vein that is both visible and palpable and well fixed to the surrounding tissue. Check all the equipment, the needle should be sharp patient and tight fitted; the syringe should be dry and with all the air expelled from it; and the bottle or the tube to collect the sample must be ready in hands. The patient is reassured and the part is cleaned with clean gauge or cotton moistened with rectified spirit. The patient’s forearm is grasped with left hand and to steady the vein, the thumb retracts...
downwards soft tissues below the site of the puncture. The needle is brought to the skin over the vein, and the bevel of the needle turned up. If the vein is large and well fixed, the skin and the vein may be punctured with a single short thrust. If the vein is small or slippery the skin is punctured first and the vein next, the plunger is pulled back gently. When the blood starts flowing in the syringe, the tourniquet is released and after sufficient blood has been collected, the needle is withdrawn.

The punctured site is gently pressed with a swab of cotton or gauge moistened with spirit for two minutes, the patient should elevate the arm and is asked to maintain the pressure for a few minutes more to prevent the haematoma. Before evacuating the syringe into the bottle, and then the needle must be removed. The contents are delivered slowly and mixed with the anticoagulant by gentle shaking for a minute.

**Capillary Blood**

Capillary blood is obtained by pricking the skin. Only a few drops of blood can be collected in this manner. Blood is collected directly into appropriate pipettes and has to be manipulated immediately. Hemoglobin estimation, red cell count, leukocyte count, platelet count and reticulocyte count can be done with capillary blood. For preparing smears for a differential count, capillary blood without an anticoagulant is preferred. In adults, capillary blood is obtained from the ball of the finger or the lobe of the ear; but in infants, the sites are the ball of the thumb, the great toe or the heel.
**Technique**

Examine the site of the puncture to make sure that there is no oedema or congestion or that the site is not too cold or blood less. If the last, place part in warm water to promote congestion. Both the patient and the operator should be seated comfortably and in good light. All the apparatus must be ready at hand before the site is punctured. The site chosen is cleaned with spirit and the part must be allowed to dry completely before the puncture is made. The point of the needle is touched to the skin and a bold quick prick is made. The prick must be deep enough (2 – 4 mm) to ensure a free flow of blood. The needle must be sharp and sterilized. Blood must flow spontaneously from the stab. The first drop is wiped off and the second one used for filling the pipette. Squeezing of the finger is not permissible as this will dilute the blood with the tissue fluid.

**2. ESTIMATION OF BLOOD UREA**

The wastes in the blood come from the normal breakdown of active tissues and from the food we eat. The body uses the food for energy and self-repair. After the body has taken what it needs from the food, waste is sent to the blood. If the kidneys do not remove these wastes, the wastes would build up in the blood and damage our body. The actual filtering occurs in tiny units inside the kidneys called nephrons. Every kidney has about a million nephrons [120-122]. In the nephron, a glomerulus—which is a tiny blood vessel or capillary, intertwines with a tiny urine-collecting tube called a tubule. A complicated chemical exchange takes place, as waste material and water leaves the blood and enter the urinary system.
Urea is one of the main nitrogenous waste products excreted in the urine. It is the major excretion product of protein catabolism. Liver is the sole site of urea formation. Urea is formed from CO₂ and NH₃, passed on to the blood and filtered at glomeruli and partly reabsorbed by the tubes.

Techniques for blood Urea estimation

There are two major techniques. The initial step in both the techniques is same, liberation of ammonia from urea by urease.

1. Nesselerization: It involves reaction of NH₃ with Nessler’s reagent.
2. Bertha lot’s reaction: In this method the liberated ammonia reacts with phenol and hypochlorite to give blue colored Indophenols.
3. Diacetyl monoxime method. In this method Urea directly reacts with diacetyl Compounds to give colored compounds.

The method of Nesselerization is explained as follows

**Principle**

Blood sample is digested with urease and urea is converted into ammonia. After precipitating proteins, ammonia is made to react with Nessler’ reagent, producing a colored compound which can be compared with any of the following.

1. A std \((\text{NH}_4)_2\text{SO}_4\) solution
2. A urea solution
Reagents

1. 10% Sod tungstate solution
2. 2/3 NH₂SO₄
3. Urease solution
   5mg soya bean powder is shaken with 100ml of water. Prepare fresh one daily. Alternatively urease tablet may be dissolved in 3ml of 30% alcohol or, urease powder can be used.
4. Urea std 0.5mg/dl
5. Nesseler's reagent

Procedure

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<tr>
<th></th>
<th>Blank</th>
<th>Test</th>
<th>Standard</th>
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</thead>
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<tr>
<td>Distilled water</td>
<td>3.4ml</td>
<td>3.2ml</td>
<td>3.2ml</td>
</tr>
<tr>
<td>Blood</td>
<td>---</td>
<td>0.2ml</td>
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</tr>
<tr>
<td>Urea std solution</td>
<td>---</td>
<td>---</td>
<td>0.2ml</td>
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<tr>
<td>(0.5 mg/ml)</td>
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Add 20 mgm of urease powder to each tube and incubate all of them at 37°C for 20 mins. Then add:

10% Sod. Tungstate 0.3ml 0.3ml 0.3ml
2/3 NH₂SO₄ 0.3ml 0.3ml 0.3ml

Mix well. Let it stand for few minutes, and centrifuge

Clear supernatant from above 2.0ml 2.0ml 2.0ml
Distilled water 5.0ml 5.0ml 5.0ml
Cool for 2 mins.

Add 1.0ml of Nessle’s reagent to each tube and read at 480nm.

Calculations: \[
\text{Blood Urea} = \frac{O.D(T)}{O.D(s)} \times 100 \text{mg/dl}
\]

Interpretation

Normal blood urea level in adults is 10-20mg/dl. Increase in blood urea occurs with

2. Renal failure
3. Renal stasis

Only condition known to decrease blood urea is normal pregnancy.

3 ESTIMATION OF BLOOD SUGAR

Body and brain cells need different types of nourishment, one of which is sugar. The circulatory system carries sugar and transfers it to the cells with the aid of a chemical substance called insulin. The pancreas, located in the abdominal cavity, manufactures insulin. When the insulin production and sugar are balanced the body functions normally. An individual suffering from a reduction in the production of insulin is said to have diabetes mellitus. Diabetes mellitus is a condition in which the body is not able to satisfactorily process ingested sugar. As a result of this unbalance, the body is adversely affected. However, many diabetics lead healthy, normal lives through a program of balanced diet and medication. When the diabetic’s condition is not controlled, certain disorders may occur.
Determination of blood sugar is one of the first biochemical estimations to be applied clinically. The abnormalities of blood glucose levels reflect disturbances in carbohydrate metabolism. At room temperature, glycolysis in the RBCs reduces the blood glucose at a rate of 5% per hour. To prevent this loss, either the estimation should be performed within half an hour after collection or an inhibitor of glycolysis such as NaF is added. More often, the sample is collected in the tube containing a fiprode-oxalate mixture. Blood glucose values are 10-15% lower than plasma values due to lower water content within RBCs. Also, normal range for glucose in whole blood obviously varies with hematocrit.

**Techniques**

1. Glucose oxidase technique-specific and represents true glucose.

2. Techniques are dependent on the reducing property of glucose. These may over-estimate the blood glucose levels by as much as 5-20%.

**Principle**

A protein free blood filtrate is treated with alkaline copper solution. The cuprous oxide formed is treated with a phosphomolybdic acid solution, blue color being obtained which is compared with that of a standard.

**Reagents**

1. Alkaline CuSO₄ solution

2. Phosphomolybdic acid solution

3. Standard glucose solution (0.1 mg/ml)

4. 10% sodium tungstate solution

5. 2/3 NH₂ SO₄
Procedure

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<td>3.2ml</td>
</tr>
<tr>
<td>Blood</td>
<td></td>
<td>0.2ml</td>
<td>--</td>
</tr>
<tr>
<td>Standard</td>
<td></td>
<td></td>
<td>0.2ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.2ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% sod. Tungstate</td>
<td>0.3ml</td>
<td>0.3ml</td>
<td>0.3ml</td>
</tr>
<tr>
<td>2/3 $\text{NH}_2\text{SO}_4$</td>
<td>0.3ml</td>
<td>0.3ml</td>
<td>0.3ml</td>
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</tbody>
</table>

Mix well and centrifuge at 3000 rpm for 5 mins.

Clear filtrate from above

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<tbody>
<tr>
<td>2.0ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkaline Copper regent</td>
<td>2.0ml</td>
<td>2.0ml</td>
<td>2.0ml</td>
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</table>

Mix and keep in boiling water baths for 6 minutes. Cool for 2-3 mins. Add 2ml of phosphomolybdic acid to each one and dilute to 25 ml with distilled water. Read in colorimeter

$$\frac{T - B}{S - B} \times 100 = \text{Sugar} \text{ in mg/dl}$$
Interpretation

The normal range of fasting venous blood sugar values by this method is 90-120 mg/dl of whole blood. Of this non-glucose reducing substances constitute 20-30 mg/dl. However this saccharoid fraction remains remarkably constant. Fasting hyperglycemia is a highly suggestive of diabetes. On the other hand, diabetes can never be ruled out by a normal fasting blood sugar. A blood glucose below 40-60 mg/dl occurs most frequently as a result of over dosage of insulin in treatment of diabetes mellitus.

Modifications of Folin Wu method

1. SOMOGYI—SHAFFER HARTMAN method:

   Hemolyzed blood is deprotenized with Zn(OH)₂, giving a filtrate containing practically no reducing substances other than sugar.

NELSON SOMOGYI METHOD

   Blood is deprotenized by Zn(OH)₂, BaSO₄: This procedure gives a filtrate containing practically no reducing substance other than glucose.

   Results of these methods are considerably lower than the classical Folin Wu method.

Glucose oxidase method

It is based on the following reactions.

\[
\text{Glucose} + \text{O}_2 \xrightarrow{\text{Oxidase}} \text{Glucic acid} + \text{H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + \text{Chromogen Peroxidase Chromogen}
\]
The color of this chromogen is estimated colorimetrically. This method is highly specific for glucose.

4. TOTAL COUNT OF BLOOD CELLS

Leukemia is a form of cancer that affects the body blood-making system, including the lymph and bone marrow. Leukemia is either acute (coming on suddenly) or chronic (lasting a long time). Leukemia rarely affects children while acute leukemia affects both adults and children.

Leukemia accounts for about two percent of all cancers. It strikes nine out of every 100,000 people in every year. Men are more likely to develop leukaemia than women. Adults are 10 times more likely to develop leukaemia than children, and it is most often in the elders. When leukaemia occurs in children, it happens most often before age 4. In acute leukemia, immature blood cells reproduce quickly in the bone marrow, where they eventually eat out healthy cells. These immature, abnormal cells can also spread to other organs, leading to damage of the organs. The types of acute leukemia involve different types of blood cells:

(a) Haemocytometer:

The Haemocytometer Neubauer counting chamber has the total ruled area 9 sq mm. It consists of a centrally heavy ruled area of 1 sq mm in size of four others of the same size in each corner. The central area is divided into 25 squares and each square is subdivided into 16 squares. For total RBC counts one each at the corner and the
centre (i.e. 80 small squares). The four outer 1 sq mm areas are divided into 16 squares. The squares are used for total WBC counts.

(b) Red Blood Cells (Erythrocytes):

The Mature erythrocytes are biconcave disks, circular in shape, centrally unstained and periphery stained, pink in color. Size: 7.2 microns in average diameter. It contains hemoglobin.

(a) Formal Citrate Solutions:

Trisodium citrate ............... 3g
Distilled water ............... 99ml
Formalin, commercial ...........1ml

(b) Hayem’s Fluid:

Sodium chloride .............0.5g
Sodium sulphate ............2.5g
Mercuric chloride ............0.25g
Distilled Water ..............to 100ml

Collect the blood from the finger directly into RBC pipette or from a sample of oxalate blood exactly to the 0.5 mark (Thoma pipette mark101) and immediately draw up the diluting fluid (formal citrate solution or Hayem’s fluid) to the mark 101, and rotate the pipette between the thumb and forefinger. This will give a dilution of 1:200.

Clean the counting chamber and cover glass thoroughly. Place the cover glass in position over the ruled area, using gentle pressure. Mix the suspension
thoroughly by rotating the pipette for about a minute, holding it in horizontal position, and finally shake sidewise. Expel the fluid from the stem of the pipette and without loss of time, fill the chamber by holding the pipette at an angle of 45 degrees and lightly touching the tip against the edge of the cover glass. Care should not be taken to ensure that the suspension does not flow into the moats on either side, nor should any bubble from under cover glass. Allow two or three min for the red corpuscles to settle.

Count the number of RBC's in 80 small squares (4 squares of 16 at the four corners and one of at centre). Do not count the cells touching the lower and right hand lines, but count the cells touching the upper and left hand lines.

**Bulk dilution method:**

In a clean test tube take 3.98 ml of RBC diluting fluid. Add 0.02 ml of blood with help of a hemoglobin Sahli pipette. Mix well. Now the dilution is 1 in 200. Fill the counting chamber as usual, count and calculate.

**Calculation:**

Count the number of cells in 80 small squares and add four ciphers, e.g. Total number of cells in 80 small squares =435. Therefore total RBC's per c.mm is 4350000 in other words 4.35 million per c.mm.

The area of the small square is 1/400 sq.mm.

The depth of the counting chamber is 1/10 mm.
Therefore the volume of a small square is \( \frac{1}{400} \times \frac{1}{10} = \frac{1}{4000} \) c.mm

The dilution of the blood is 1/200.

Total RBC's \( \frac{435}{80} \times \frac{4000}{1} \times \frac{200}{1} = 43,500 \text{ per c.mm.} \)

Normal: Men 4.5 to 6.5 million per c.mm. Women 3.9 to 5.6 million per c.mm.

(c) White Blood Cell (Leucocytes) Counts:

WBC Dilution Fluid, Truck's:

Acetic acid, glacial 3ml

Distilled water 97ml

Add gentian violet to give a pale violet color.

Draw the blood up to 0.5 marks in WBC pipette marked 11 and dilute up to the 11 mark with WBC fluid as described in RBC counting. Then fill the counting chamber in the same manner. Allow 3 mins for the cells to settle. If the Neubauer counting chamber is used. Count the cells in the four corner blocks. Each of these 4 square mm area is subdivided into 16 squares, by using the low power objective and a medium ocular. In counting the cells include those touching on the inner lines on the right and top., but do not count the cells touching the lines left and bottom. The difference between the two square mm area should not be more than 10 WBC's.
**Bulk dilution method:**

In a clean test tube, take 0.38 ml of WBC diluting fluid. ADD 0.02 ml of blood with the help of a haemoglobin Shali pipette. Mix well. Now the dilution is 1 in 20. Fill the counting chamber as usual, count and calculate.

**Calculation:**

Total number of cells counted in 4 squares/2 and adds two zeros. e.g No of cells counted = 120/2 = 60 and 2 zeros, that is =6000 per c.mm.

To get WBC’s per c.mm:

No WBC’s counted in four squares : 120 cells

The volume of a square is 1/10 c.mm

The blood was diluted to 1/20

Therefore the number of cells per c.mm of undiluted blood =

\[
\frac{120 \times 10 \times 20}{4} = 6000 \text{ Per c.mm}
\]

Normal : WBC’s per c.mm of blood 4000 to 10,000 per c.mm.

**5. TYPHOID**

Typhoid fever is a bacterial infection of the intestinal tract and occasionally of the blood stream. It is an uncommon disease with only 30 – 50 cases occurring in each year. Most of the cases are acquired during foreign travel to under developed countries. The germ that causes typhoid is a unique human strain of salmonella called Salmonella typhi. Any one can get typhoid fever but the greatest risk exists to travellers visiting countries where the disease is common.
Occasionally, local cases can be traced to exposure to a person has the chronic carrier. Typhoid germs are passed in the feces and, to some extent, the urine of infected people. The germs are spread by eating or drinking water or foods.

**WIDAL REACTION**

Collect 5 ml of blood in sterile tube by venous puncture using sterile dry syringe. Allow the blood to clot and separate the serum. It should be free from haemolysis. The serum is diluted in a series of dilutions in normal saline (NaCl) 0.85% and equal quantities of antigen is added to it. Then tubes are incubated. The Highest dilution at which the agglutination occurs is noted. Make a 1 in 10 dilution of the patient’s serum in 0.85% normal saline (NaCl). In a rack place 8 small test tubes (3* ½ inch) in a row. Like this four rows are to be arranged for Salmonella Typhi ‘H’ (TH), Salmonella typhi ‘O’( TO), Salmonella paratyphi (AO) and Salmonella paratyphi (BO). Take 0.5 ml of normal saline 0.85% in test tubes No.2 to No.8 in all four rows. Then add 0.5 ml of patient’s diluted serum in No.1 and No.2 tubes. Mix and withdraw 0.5 ml from No. 1 tube into No.3 tube. Mix and withdraw 0.5 ml from No.3 tube into No.4 tube and so on till No.7 tube. Withdraw and discard 0.5 ml from tube No 7. Tube No.8 contains normal saline only. The same procedure is adopted for all the four rows, 0.5 ml of bacterial suspension is added to each tube. Shake the rack and keep it in the incubator for one and half an hour at 37°C or in water bath at 50°C to 55°C for two hours. Keep in room temperature for half an hour for ‘H’ types and for ‘O’ types, readings should be taken after four hours and 24 hours.
Normal: Normal Sera may agglutinate H antigen at 1:20 and O antigen in final dilution up to 1:80.

In the same way put up TO, AO and BO and add antigen respectively. Incubate and keep in room temperature as described above. Clumping or agglutination can easily be seen with naked eye in a satisfactory light. This deposit is seen at the bottom of the tube.