BACHELOR OF MEDICAL LABORATORY TECHNOLOGY LAB MANUAL

2nd Semester

Prepared By

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BMLT

MIDNAPORE CITY COLLEGE

Physiology Lab Manual

Human Physiology: BMLT paper – IV, Unit – 8

1.Staining of Squamous epithelium.

Aim: Preparation of temporary slide of animal tissues and their study.

Principle:

Group of similar type of cells that perform a specific function, is called a tissue. Tissues are organised in a specific proportion and pattern to form different organs. There are four basic types of tissues: (i) Epithelial, (ii) Connective, (iii) Muscular and (iv) Nervous. The epithelium or epithelial tissue provides a covering or lining for some parts of the body. Connective tissues have special function of linking and supporting other tissues or organs of the body. Muscular tissue plays an active role in all movements of the body. Nervous tissue controls the body's responsiveness to changing conditions within and outside the body.

Requirement:

Live material/concerned tissue, beakers, glass slides, coverslips, watch glasses, dropping bottle, dropper, methylene blue stain, glycerine, NaCl solution (0.9% w/v), brush, toothpick, water, wash-bottle, microscope.

Procedure:

• Rinse your mouth well with water.

• Gently scrap the inside of your cheek with the broad end of a clean toothpick. Discard this material.

• Scrap again, and spread these cells gently on a clean slide. Add a drop of 0.9% NaCl solution or physiological saline and a drop of methylene blue with the help of a dropper.

• After two minutes, remove the excess stain and saline using the edge of a filter paper. Now, put a drop of glycerine on the cells.

• Place a coverslip over the tissue and gently press it with the back of a pencil to spread the cells.

• Examine the slide under the low power of microscope.

Observation:

Record your observations in the tabular form given below:

Features	Observations
1. No. of cells in a focus	
2. Shape of cells	
3. Nature of cell boundary	
4. Nucleus: present/absent Shape Location	

2.Measurement of Pulse rate

Principle:

Pulse/heart rate is the wave of blood in the artery created by contraction of the left ventricle during a cardiac cycle. The strength or amplitude of the pulse reflects the amount of blood ejected with myocardial contraction (stroke volume). Normal pulse rate range for an adult is between **60-100 beats per minute**. A well-trained athlete may have a resting heart rate of 40 to 60 beats per minute, according to the American Heart Association.

Procedure:

Ask whether the patient has walked, climbed stairs, or otherwise exerted themself in the last 20 minutes. If not, you can proceed. If the answer is yes, wait 20 minutes before taking the reading. This will help to prevent false readings.

Make sure the patient is relaxed and comfortable.

Place the tips of your first and second finger on the inside of the patient's wrist.

Press gently against the pulse. Take your time to note any irregularities in strength or rhythm.

If the pulse is regular and strong, measure the pulse for 30 seconds. Double the number to give the beats per minute (e.g.: 32 beats in 30 seconds means the pulse is 64 beats per minute). If you noticed changes in rhythm or strength, you must measure the pulse for a full minute.

Record the pulse rate (the number of beats per minute) in the patient's notes and describe its strength and rhythm. Compare the pulse rate with the values in the Table land record whether the pulse is normal, slow or fast. Any abnormalities should be recorded and reported to the senior nurse and doctor.

Strength of the pulse is a very subjective measurement, but an experienced nurse will compare it with what has been felt previously in other patients. Describe the puse as ‘weak’, ‘faint’, ‘strong' or' bounding’.

Think about the rhythm of the pulse. Is it regular? If irregular, in what way? Cardiac problems may present as a regular missed beat, for example, so is the irregularity regular (described as regularly irregular) or is there no pattern (described as irregularly irregular)?

Discuss with your patient the result of the pulse measurement and if any further investigations are required.

Wash and dry your hands.


Elmien Wolvaardt Ellison

3.Measurement of blood pressure

The term blood pressure refers to the force exerted by the blood as it presses against and attempts to stretch the walls of blood vessels. Although blood exerts this outward force throughout the CVS, the term blood pressure, used unqualified, refers to systemic arterial blood pressure (others are: venous, capillary pressure, etc). The blood pressure (BP) is not steady (unchanging) throughout the cardiac cycle but fluctuating, i.e. it is pulsatile. It rises and falls; reaching its maximum during systole of the heart, when it is called the systolic blood pressure (SBP); and falling to its minimum during diastole of the heart when it is called diastolic blood pressure (DBP) Measurement of blood pressure is an important clinical procedure as it provides valuable information about the cardiovascular system (CVS) under normal and disease conditions.

PRINCIPLE:

A sufficient length of a single artery is selected in the arm (brachial artery), or in the thigh (femoral artery). The artery is first compressed by inflating a rubber bag (connected to a manometer) placed around the arm (or thigh) to stop the blood flow through the occluded section of the artery. The pressure is then slowly released and the flow of blood through the obstructed segment of the artery is studied by:

i. Feeling the pulse—the palpatory method.

ii. Observing the oscillations of the mercury column—the oscillometric method, and

iii. Listening to the sounds produced in the part of the artery just below the obstructed segment— the auscultatory method.

PROCEDURES:

The subject may be lying down (supine) or sitting, but should be mentally and physically relaxed and free from excitation and anticipation.

Lay the arm bare up to the shoulder and record the blood pressure first with the palpatory method, followed by auscultatory method. The upper arm on which the BP cuff is to be tied must be at the level of the heart. (In the supine position, the arm resting on the bed will be

nearly at the heart level. In the sitting position the arm resting on the table of a suitable height will be at the correct level).

In obese subjects, the cuff may be applied on the forearm with the stethoscope placed over the radial artery for auscultatory method. (If no sounds are heard a reasonably reliable determination can be obtained by palpation at the wrist).

A. Palpatory Method (Riva Rocci 1896)

1. Make the subject sit or lie supine and allow 5 minutes for mental and physical relaxation.
2. Open the lid of the apparatus until you hear the "click". Release the lock on the mercury reservoir and check that the mercury is at the zero level. If it is above zero, subtract the difference from the final reading. If it is below zero, add the required amount of mercury to bring it to zero level.
3. Place the cuff around the upper arm, with the centre of the bag lying over the brachial artery, keeping its lower edge about 3 cm above the elbow. Wrap the cloth covering around the arm so as to cover the rubber bag completely, and to prevent it bulging out from under the wrapping on inflation. The cuff should neither be too tight nor very loose.
4. Palpate the radial artery at the wrist and feel its pulsations with the tips of your fingers. Keeping your fingers on the pulse, hold the air bulb in the palm of your other hand and tighten the leak valve screw with your thumb and fingers.
5. Inflate the cuff slowly until the pulsations disappear; note the reading then raise the pressure another 30–40 mm Hg.
6. Open the leak valve and control it so that the pressure gradually falls in steps of 2–3 mm. Note the reading when the pulse just reappears. **The pressure at which the pulse is first felt is the systolic pressure**. (It corresponds to the time when, at the peak of each systole, small amounts of blood start to flow through the compressed segment of the brachial artery). Deflate the bag quickly to bring the mercury to the zero level.

7. Record the pressure in the other arm. Take 3 readings in each arm, deflating the cuff for a few minutes between each determination.

Advantages of palpatory method.

This method avoids the pitfall of the auscultatory method in missing the auscultatory gap.

Disadvantages of palpatory method:
This method measures only the systolic pressure, the diastolic pressure cannot be measured.
This method lacks accuracy because the systolic pressure measured by it is lower than the actual by 4–6 mm Hg. It assumes that the first escape of blood under the cuff will cause pulsations in the peripheral artery (radial in this case). However, there is no evidence that the amount of blood that escapes when the artery first opens is enough to produce a pulse wave detectable by the fingers. Thus, definite pulsation may not occur until the cuff pressure has been reduced by 6–8 mm Hg.

C. Auscultatory Method (Korotkoff, 1905)

1. Place the cuff over the upper arm as described above, and record the BP by the palpatory method.
2. Locate the bifurcation of brachial artery (it divides into radial and ulnar branches) in the cubital space just medial to the tendon of the biceps which can be easily palpated in a semi-flexed elbow as a thick, hard, elongated structure. Mark the point of arterial pulsation with a sketch pen.
3. Place the chest-piece of the stethoscope on this point and keep it in position with your fingers and thumb of the left hand (if you are right-handed).
4. Inflate the cuff rapidly, by compressing and releasing the air pump alternately (sounds may be heard as the mercury column goes up). Raise the pressure to 40 to 50 mm Hg above the systolic level as determined by the palpatory method.
5. Lower the pressure gradually until a clear, sharp, tapping sound is heard. Continue to lower the pressure and try to note a change in the character of the sounds.

<u>These sounds are called Korotkoff sounds and show the following phases:</u>

Phase I This phase starts with a clear, sharp tap when a jet of blood is able to cross the previously obstructed artery. (Sometimes this phase may start with a faint tap, especially when the systolic pressure is very high). As the pressure is lowered, the sounds continue as sharp and clear taps. This phase lasts for 10–12 mm Hg fall in pressure.

Phase II The sounds become murmurish and remain so during the next 10–15 mm Hg fall in pressure when they again become clear and banging.

Phase III It starts with clear, knocking, or banging sounds that continue for the next 12 to 14 mm Hg pressure, when they suddenly become muffled.

Phase IV The transition from phase III to phase IV is usually very sudden. The sounds remain muffled, dull, faint and indistinct (as if coming from a distance) until they disappear. The muffling of sounds and their disappearance occurs nearly at the same time, there being a difference of 4–5 mm Hg (i.e. phase IV lasts for 4–5 mm Hg).

Phase V This phase begins when the Korotkoff sounds disappear completely. If you reduce the pressure slowly, you will note that total silence continues right up to the zero level.

6. Take 3 readings with the auscultatory method and repeat 3 readings on the other arm.

7. Effects of posture, gravity, and muscular exercise on blood pressure are discussed in the next experiment.

EFFECT OF CHANGE IN POSTURE

The effect of changes in posture depends on whether these are recorded immediately after standing from supine position, or after prolonged standing. They also depend on whether a person stands against a support (e.g. a wall), or is standing ‘free’ and still.

Immediate Effect. As the person assumes erect position, blood tends to pool in the lower parts of the body (especially in the veins) due to gravity. This decreases the venous return, and hence CO and BP. A pooling of 250–300 ml of blood can decrease the systolic pressure by 10–15

mm Hg. However, within some 8–10 seconds the sino-aortic baroreceptor mechanism restores BP to normal level.

Effect of Prolonged Standing. If a person stands still, especially against a support, more than 500 ml of blood may pool in the lower body. Also, increased capillary hydrostatic pressure causes fluid to be filtered out into the tissues, which further reduces venous return. The CO and BP fall, resulting in cerebral ischemia that causes the person to fall down unconscious. The fainting is actually a homeostatic mechanism, as it restores venous return, CO and BP, thus relieving cerebral ischemia.

PROCEDURES

1. Allow the subject to rest and relax for a few minutes in the supine position. Record the heart rate (pulse rate) and BP by the palpatory method and auscultatory method (later on by auscultatory method alone). Disconnect the cuff from the BP apparatus.

2. Ask the subject to sit up and immediately record the BP and heart rate (HR). Repeat the determinations after 1 minute, 2 and 5 minutes.

3. Make the subject lie down again and rest for a few minutes. Then record the BP and HR. Now ask him to suddenly stand up, and record the BP and HR.

4. Record your observations in your workbook.

Results:

Pulse rate	***Systolic pressure***	***Diastolic pressure***	***Mean arterial pressure***	***pressure***
Effect of Posture				
Supine position				
Sitting from supine				
Standing from sitting				
Standing from supine				

4.Measurement of physical fitness index

Harvard step test

The **Harvard step test** is a type of cardiac stress test for detecting and diagnosing cardiovascular disease. It is also a good measurement of fitness and a person's ability to recover after a strenuous exercise by checking the recovery rate.

Procedure:

Participants were asked to be clothed loosely. They were asked to sit quietly for 5 min. Thereafter, they were asked to perform the stepping up and down on 20-inch high step for men and 18-inch high step for women for 5 min at the frequency of 30 times per min. The step used was a heavy wooden step so that it remained steady during the test. The participant performed this exercise as long as he/she could, but not in excess of 5 min. Time for which the participant can perform the test was noted. The time at which the participant felt that he/she cannot perform the test any more was taken into consideration. This time is known as the time of exhaustion. After the completion of the test, pulse rate was counted from 1 to 1½ min (pulse rate 1), 2–2½ min (pulse rate 2), and 3–3½ min (pulse rate 3).
PFI was then calculated using the following formula:
PFI = Duration of exercise (in seconds) ×100/2 (Pulse rate 1 + 2 + 3).
The fitness of the participant was graded on the basis of score of PFI as poor, low average, high average, good, and excellent.

Rating physical fitness index score
Poor <55
Low average 55–64
High average 65–79
Good 80–89
Excellent >90.

5.Determination of Blood Group and Rh factor

Principle:

The surfaces of red cell membrane contain a variety of genetically determined antigens, called **isoantigens** or **agglutinogens,** while the plasma contains antibodies **(agglutinins).** To determine the blood group of a person, his/her red cells are made to react with commercially available antisera containing known agglutinins. The slide is then examined under the microscope to detect the presence or absence of clumping and hemolysis (agglutination) of red cells which occurs as a result of antigen-antibody reaction.

APPARATUS AND MATERIALS

1. Microscope. •Glass dropper with a long nozzle. •Sterile blood lancet or needle. •Sterile cotton/ gauze swabs. •Alcohol. •5 ml test tube. •Toothpicks.
2. Clean, dry microscope slides. (A special porcelain tile with 12 depressions is available for this purpose and may be used in place of glass slides.)
3. 1% sodium citrate in normal saline (or normal saline alone).
4. **Anti-A serum:** [contains monoclonal anti-A antibodies (against human); these antibodies are also called anti-A or alpha (α) agglutinins]. The anti-A serum can also be obtained from a person with blood group B.

5. **Anti-B serum:** [contains monoclonal anti-B antibodies (against human); these antibodies are also called anti-B or beta (β) agglutinins]. The anti-B serum can also be obtained from a person with blood group A.
6. Anti-D (anti-Rh) serum: [Contains monoclonal anti-Rh (D) antibodies (against human). These antibodies are also called anti-D agglutinins.

PROCEDURES

1. Using a glass-marking pencil, divide 3 slides, each into two halves by a line drawn down the middle (the left sides will act as "test sides" and right sides as the "control sides"). Mark the left corner of 1st slide anti-A, left corner of 2nd slide "anti-B, and the left corner of 3rd slide "anti-D". Mark the right corners of these 3 slides 'C' (for control).
2. Mark another slide (4th) 'S' (for only red cell suspension in saline, i.e. no antiserum will be added on this slide).
3. Place 8–10 drops of saline in the center of slide 'S'.
4. **Preparation of red cell suspension.** A suspension of red cells in saline should preferably be prepared and used instead of adding blood drops directly from the fingerpick to the antisera for the following reasons:
    a. Dilution of blood permits easy detection of agglutination and hemolysis, if present. (Red cells in undiluted blood tend to form large rouleaux and masses. These may be difficult to disperse and may be mistaken for agglutination).
    b. Plasma factors likely to interfere with agglutination are eliminated.
5. Get a finger-prick under aseptic conditions, and add 2 drops of blood to the saline on the slide marked 'S'. Mix the saline and blood with a clean glass dropper to get a suspension of red cells. You may use a toothpick for this purpose.
        i. A better method is to place 2 ml of saline in a small (5 ml) test tube. Then get a finger pricked and allow a blood drop to form. Now place the pricked fingertip on top of the test tube and invert it. Mix the blood and saline by inverting the tube 2 or 3 times. A suspension of red cells is now ready.
        ii. Washed red cell suspension gives the best results. The red cells are "washed" in saline by centrifuging the diluted blood, removing the supernatant, and adding fresh saline to get a suspension of "washed" red cells.

Determination of Rh factor.

Put one drop of anti-A serum on the left half ("test side") of 1st slide (marked anti-A), one drop of anti-B serum on the left half of 2nd slide (marked anti-B), and one drop of anti-D serum on the left half of 3rd slide (marked anti-D).

7. Put one drop each of normal saline on the "control" sides (right halves) of the 3 slides (i.e. areas marked 'C').

8. Add a drop each of red cell suspension (from the slide 'S', or from the test tube of red cell suspension) on anti-A, one drop on anti-B and one drop on anti-D sera, and one drop each on the normal saline taken on the "control" sides of the 3 slides. In this way, the red cells-saline mixture on the "control" sides of each slide will act as a control to confirm agglutination or no agglutination on the corresponding test side.
9. Mix the anti-sera and red cells, and saline and red cells on each slide by gently tilting it first one way.
10. Wait for 8–10 minutes, then inspect the 3 antisera-red cell mixtures ("test" mixtures) and "control" mixtures, first with the naked eye to see whether agglutination (clumping and hemolysis of red cells) has taken place or not. Then confirm under low magnification microscope, comparing each "test mixture" with its corresponding "control mixture".

OBSERVATIONS AND RESULTS

It is essential that you should be able to distinguish between ***"agglutination"*** and ***"no agglutination"***. The features of each are:

Agglutination.

i. If agglutination occurs, it is usually visible to the naked eye. The hemolysed red cells appear as isolated (separate), dark-red masses (clumps) of different sizes and shapes.
ii. There is brick-red coloring of the serum by the hemoglobin released from ruptured red cells.
iii. Tilting or rocking the slide a few times, or blowing on it does not break or disperse the clumps.
iv. Under LP objective, the clumps are visible as dark masses and the outline of the red cells cannot be seen.

No Agglutination

i. In the "control" mixtures, the red cells may form a bunch, or rouleaux. These sedimented red cells give an orange tinge of a suspension of red cells rather than "isolated dark red masses" of ruptured red cells.
ii. The red cells will disperse if you gently blow on the slides, or tilt them a few times. Confirm all these features of "no agglutination" under the microscope.

PRECAUTIONS

1. The slides should be dry, dust-free and grease-free.
2. Identify and mark all slides, containers, and test tubes clearly and legibly. Double-check every step of the procedure.
3. The droppers supplied with the antisera bottles should not be interchanged.
4. Examine the slides with the naked eye and then under the microscope after 8–10 minutes but before the sera-blood mixtures dry up.
5. Do not add undiluted blood from the finger-prick directly on to the antisera for 2 reasons, one, the sera may get intermixed, and two, false positive reaction may develop. Sometimes

it is not possible to say with certainty whether agglutination has occurred or not. In such cases the grouping must be repeated with diluted blood.

6. A control should always be used to exclude false positive result.

6. Measurement of total count of RBC.

PRINCIPLE:

The blood is diluted 200 times in a red cell pipette and the cells are counted in the counting chamber. Knowing the dilution employed, their number in undiluted blood can easily be calculated.

APPARATUS AND MATERIALS

1. **RBC pipette:** It should be clean and dry and the bead should roll freely.
2. **Improved Neubauer chamber with coverslip.** These should be clean and dust free.
3. **Microscope with LP and HP objectives and 10 x eyepiece.**
4. **Disposable blood lancet/pricking needle.**
   - Sterile cotton/gauze swabs
   - 70% alcohol/methylated spirit.
5. **Hayem's fluid (RBC diluting fluid):** The ideal fluid for diluting the blood should be isotonic and neither cause hemolysis nor crenation of red cells. It should have a fixative to preserve the shape of RBCs and also prevent their autolysis so that they could be counted even several hours after diluting the blood if necessary. It should prevent agglutination and not get spoiled on keeping. All these properties are found in Hayem's fluid.

Composition of Hayem's fluid.

Sodium chloride (NaCl) 0.50 g

Sodium sulfate (Na2SO4) 2.50 g

Mercuric chloride (Hg Cl2) 0.25 g

Distilled water 100 ml

Dissolve all these chemical in distilled water and filter several times through the same filter paper. Discard the solution if a precipitate forms.

- Sodium chloride and sodium sulfate provide isotonicity so that the red cells remain suspended in diluted blood without changing their shape and size. Sodium sulphate also acts as an anticoagulant, and as a fixative to preserve their shape and to prevent rouleaux formation (piling together of red cells)
- Mercuric chloride acts as an antifungal and antimicrobial agent and prevents contamination and growth of microorganisms.

PROCEDURES

1. Place about 2 ml of Hayem's fluid in a watch glass.
2. Examine the chamber, with the coverslip 'centred' on it, under low magnification. Adjust the illumination and focus the central 1 mm square (RBC square on the counting grid) containing 25 groups of 16 smallest squares each. All these squares will be visible in one field. Do not change the focus or the field. • Admitting too much light is a common cause of the inability to see the grid lines and squares clearly.
3. Move the chamber to your work-table for charging it with diluted blood. (It can be charged while on the stage, but it is more convenient to charge it on the table).
4. **Filling the pipette with blood and diluting it:** Get a finger-prick. Wipe the first 2 drops of blood and fill the pipette from a fresh drop of blood up to the mark 0.5. Suck Hayem's fluid to the mark 101 and mix the contents of the bulb for 3–4 minutes as described earlier.
5. **Charging the chamber:** Observing all the precautions, fill the chamber with diluted blood.
   - Since the RBC pipette is a slow-speed pipette, it will need to be kept at an angle of 70–80° while charging the chamber.
6. Move the chamber to the microscope and focus the grid once again to see the central 1 mm square with the red cells distributed all over.
   - Wait for 3–4 minutes for the cells to settle down because they cannot be counted when they are moving and changing their positions due to currents in the fluid. During this time draw a diagram once again showing the RBC square. Then draw 5 groups of 16 squares each, showing their relative positions—the 4 corner groups and one central group for entering your counts.
7. **Counting the cells:** Switch over to high magnification (HP lens) and check the distribution of cells. If they are unevenly distributed, i.e., bunched at some places and scanty at others, the chamber has to be washed, dried, and recharged.
8. Move the chamber carefully and bring the left upper corner block of 16 smallest squares in the field of view. (There are no smallest squares above and to its left).

Rules for Counting

Note that the immediate boundary of each smallest square is formed by the 4 lines forming the square (side: 1/20 mm; area: 1/400 mm2) the other lines of the tram or triple lines do not form part of the boundary of that square.

i. Cells lying within a square are to be counted with that square.

ii. Cells lying on or touching its upper horizontal and left vertical lines are to be counted with that particular square.

iii. Cells lying on or touching its lower horizontal and right vertical lines are to be omitted from that square because they will be counted with the adjacent squares. In this way you will avoid counting a cell twice. (You may omit cells lying on the upper horizontal and left vertical lines and count those lying on its lower and right lines. But whichever method is chosen, it is best to follow it for all cell counts).

• While counting the cells, continuously "rack", the fine adjustment up and down so that cells sticking to the underside of the coverslip are not missed

• An occasional WBC (may be 1 in 600-700 RBCs) may be seen—appearing greyish and granular but it is not to be counted with the red cells.

9. We have already focused the upper left block of 16 smallest squares in the high power field. First count the cells in the upper 4 horizontal squares from left to right, then come down to the next row and count the cells in each square from right to left. Then count the cells in the 3rd row from left to right, and in the 4th row, from right to left. As the counts are made, enter your results in the appropriate squares drawn in your workbook, showing the count in each square.

• Count once more in these 16 squares and note the result in your work-book. The difference between the two counts should not be more than 10.

10. Move the chamber carefully till you reach the right upper corner block of 16 smallest squares (there are no smallest squares above and to the right of this group), and count the cells as before. Then move on to the right lower corner and then left lower corner groups, and finally count the cells in the central block of 16 smallest squares.

Thus, the counting will have been done in 80 smallest squares, i.e., in 5 blocks of 16 squares each.

OBSERVATIONS AND RESULTS

Add up the number of cells in each of the 5 blocks of 16 smallest squares. A difference of more than 20 between any 2 blocks indicates uneven distribution.

A. Calculation of dilution obtained (dilution factor).

Recall that the dilution with this pipette can be 1 in 100 or 1 in 200 depending on whether blood is taken to mark 1.0 or 0.5.

Thus, the dilution factor is = Final volume attained (100 parts) Volume of blood taken (0.5 part)

B. Calculation of volume of fluid examined. We know the count in 80 smallest squares which have a volume (space) of 1/50 mm3. We can also know the cell count in 1 smallest square, which has a volume (space) of 1/4000 mm3. We can now calculate the number of red cells in two ways as shown below:

C. Calculation of red cell count

i. Let x be the number of cells in 1/50 mm^3 of diluted blood.

Cells in 1 mm3 of diluted blood = x × 50

Dilution employed was = 1 in 200

∴ Number of cells in 1 mm^3 of

undiluted blood will be = x × 50 × 200

= x × 10000

Thus, adding, 4 zeros in front of x will give the RBC count per 1 cubic mm of undiluted blood.

7.Determination of Packed cell volume.

PRINCIPLE:

Measurement of hematocrit (Hct) or packed cell volume (PCV) is the most accurate and simplest of all tests in clinical hematology for detecting the presence and degree of anemia or polycythemia. In comparison, hemoglobin estimation is less accurate, and RBC count far less accurate. Also, if Hb, RBC count, and PCV are determined at the same time, various absolute corpuscular values (e.g., volume and Hb content of a single red cell) of a person can be determined. These values help in the laboratory diagnosis of the type of anemia in a person.

APPARATUS AND MATERIALS

1. Equipment for venepuncture.

• Sterile swabs, alcohol, syringe and needle

• Container (penicillin vial or bulb) with anticoagulant (double oxalate or sequestrene).

2. Wintrobe tube (hematocrit tube): It is 11 cm long, heavy, cylindrical glass tube, with a uniform bore diameter of 2 mm. Its lower end is closed and flat. The tube is calibrated in cm and mm from 0 to 10 cm from above downwards on one side of the scale (for ESR), and 10 to 0 cm on the other side (for PCV). The mouth of the tube can be covered with a rubber cap to prevent loss of fluid by evaporation.

3. Pasteur pipette: It is a glass tubing drawn to a long thin nozzle about 14 cm long. A rubber teat is provided to suck blood into the pipette by a slight pressure. It is used for filling the Wintrobe tube.

4. Centrifuge machine: lt packs the red cells in the Hct tube by centrifugal force. The magnitude of force produced by rotation of the tube depends on:

a. The radius, i.e., the distance between the center of the shaft and the bottom of the centrifuge tube when laid horizontally.

b. The number of revolutions per minute (rpm).

In terms of gravitational force (G), the value of this force should be 2260 units. This much force is created when the radius is 9 inches and the speed is 3000 rpm.

PROCEDURES:

1. Draw 5 ml of venous blood and transfer it to a container (penicillin vial or bulb) of anticoagulant. Rotate the bulb between your palms.

i. To ensure proper mixing of cells and plasma (inaccurate results are likely if this precaution is not taken).

ii. To oxygenate blood cells to remove CO2 (red cells are larger when CO2 is high, in venous blood).

2. Fill the pasteur pipette with blood and take its nozzle to the bottom of the Wintrobe tube. Expel the blood gently by pressing the rubber teat, and fill the tube from below upwards while withdrawing the pipette but always keeping its tip below the level of blood. Ensure that there is no air bubble trapped in the blood.

• Do not try to fill the tube from its top as blood will not flow down to its bottom because of air present in the tube.

3. Bring the blood column exactly to the mark 10 (or the mark 0 on the other side of the scale) at the top. There should not be any bubbles at the top of blood.
• lf less blood is available, note the level.
4. Close the mouth of the tube with its rubber cap and centrifuge it at 3000 rpm for 30 minutes (slower speed will not pack the red cells fully). Balance this tube with another tube filled with water, or another sample of blood placed in the opposite tube holder.
5. At the end of 30 minutes, take the reading of upper level of packed red cells on the side of the scale where zero is at the bottom. Replace the tube in the machine and centrifuge it again for 15 minutes. Read the packed cell height again; it should be the same as before. If the height is reduced, centrifuge it again for 5 minutes. To be reliable, at least 3 successive readings, at intervals of 5 minutes, should be the same.
• Note that unnecessarily prolonged centrifugation may cause mechanical hemolysis of red cells which must be avoided.

OBSERVATIONS AND RESULTS

Note that the blood has been separated into 3 layers:
i. A tall upper layer of clear plasma—amber or straw-colored. It should not be pink or red which would indicate hemolysis of red cells in the sample or within the body (i.e., before withdrawal of venous blood) in hemolytic diseases. If there is hemolysis, the test must be repeated on a fresh sample.
ii. A greyish-white, thin layer (about 1 mm thick) the so-called “buffy layer”, consisting of platelets above and leukocytes below it.
iii. A tall bottom layer of red cells which have been closely packed together. A greyish red line separates red cell layer from the layer of leukocytes above it. This line is due to the presence of reduced Hb in the red cells lying next to the leukocytes which reduce the oxyHb of the cells. The line marks the upper limit of the red cell layer.
The percentage of the volume of blood occupied by the red cells constitutes hematocrit or packed cell volume, i.e., the percentage of whole blood that is red cells
Hematocrit (Hct) = Height of packed red cells (mm) / Height of packed RBCs and plasma × 100.

Normal values. The average value of PCV is 42% when the RBC count is 5 million/mm3 and their size and shape are normal.

Males: 44 percent (38–50 percent)

Females: 42 percent (36–45 percent)

The PCV for new borns is about 50 percent.

8. Measurement of total count of WBC.

PRINCIPLE

A sample of blood is diluted with a diluting fluid which destroys the red cells and stains the nuclei of the leukocytes. The cells are then counted in a counting chamber and their number in undiluted blood reported as leukocytes/mm3.

APPARATUS AND MATERIALS

1. Microscope •Counting chamber with a heavy coverslip. •Blood lancet/pricking needle. •Sterile cotton/gauze swabs. •70% alcohol.
2. **WBC pipettes:** white bead in bulb, and markings 0.5, 1.0, and 11. Two such, clean and dry pipettes, with free-rolling beads are required.
3. **Turk's fluid.** This fluid is used for diluting the blood.
Glacial acetic acid = 1.5 ml (hemolyzes RBCs without affecting WBCs).
Gentian violet (1% solution) =1.5 ml (it stains the nuclei of leukocytes). Distilled water to 100 ml.

PROCEDURE:

1. Take 1 ml of Turk's fluid in a watch glass. Place the counting chamber on the microscope stage. Adjust the illumination, and focus the right upper group of 16 WBC squares. You will see all the squares in one field.
2. Observing all the aseptic precautions, get a finger-prick, discard the first 2 drops of blood, and let a good-sized drop to form.
3. **Filling the pipette:** Dip the tip of the pipette in the edge of the drop, draw blood to the mark 0.5 and suck Turk's fluid to the mark 11. Mix the contents of the bulb thoroughly for 3–4 minutes. • Your partner can draw blood up to the mark 1.0 in the second pipette, followed by Turk's fluid to mark 11. This will give a dilution of 1 in 10.
4. **Charging the chamber:** Discard the first 2 drops of fluid from the pipette and charge the chamber on both sides, 1 in 10 dilution on one side and 1 in 20 dilution on the other. The chamber should neither be over-charged nor under-charged.
5. Allow the cells to settle for 3–4 minutes, then carefully transfer the chamber to the microscope. Use the fine adjustment again and try to identify the WBCs.
Under low magnification: The leukocytes appear as round, shiny (refractile), darkish dots, with a halo around them. These 'dots' represent the nuclei, which have been stained by gential violet. The cytoplasm is not stained.
• Do not confuse with dust particles which have varying sizes and shapes, often angular. They are usually opaque, with no 'halo' around them. They may be brown, black or yellow in color.
6. **Switch to high magnification** and study the leukocytes. By racking the microscope, you should be able to make out the morphology, of these cells—their round shapes, the clear unstained cytoplasm, and the deep blue-violet nuclei which appear lobed in some cells and single in others. You will also see the remnants of the red cell membranes; these are called 'ghost' cells since they are faintly visible.

7. **Counting the cells:** The procedure for counting the WBCs is similar to that employed for red cells.

• Count the cells under high power lens; once some practice is gained they can be counted under low power.

• You may count the WBCs in 16 squares under low power and then under high power and compare the results.

• Count the cells in the 4 groups of 16 squares each, i.e., in a total of 64 squares.

• Draw appropriate squares in your work-book for entering the counts.

OBSERVATIONS AND RESULTS

Note that the deep brown color of the diluted blood is due to the formation of acid hematin by the action of acetic acid on the Hb released from the ruptured red cells. However, hemolysis and formation of acid hematin (the principle used for estimation of Hb by the Sahli method) does not interfere with the counting of leukocytes.

Calculations

The leukocytes were counted in 64 squares, the volume of one square being 1/160 mm^3.

Volume of 64 squares = 1/160 × 64 = 4/10 mm^3.

Thus, the total volume of diluted blood in which WBCs were counted = 4/10 mm^3.

Let the count in 4/10 mm^3 be = x

Then 1 mm^3 of diluted blood will contain

= x × 10/4 white cells.

Since the dilution employed is 20 times (10 times in the 2nd pipette)

1 mm^3 of undiluted blood

will contain = x × 10/4 × 20

= x × 200/4

= x × 50

(x × 10/4 × 10 in the 2nd pipette)

This means that multiplying the number of cells in 64 squares with 50 will give the total leukocyte count (multiply the number of cells in 64 squares with 25 in the 2nd pipette).

Compare the two counts. The difference between the two should not be more than 10%. It will confirm the accuracy of your procedures in the two counting's.

9. Measurement of differential count of WBC.

PRINCIPLE:

A blood film is stained with Leishman's stain and scanned under oil immersion, from one end to the other. As each WBC is encountered, it is identified until 200 leukocytes have been examined. The percentage distribution of each type of WBC is then calculated. Knowing the TLC and the differential count, it is easy to determine the number of each type of cell per mm3.

Special Importance of a Blood Smear

The special importance of a stained blood smear is that, unlike any other routine blood test, the smear can be retained and preserved as a permanent original record. The slide can be taken out and re-assessed whenever required after days, weeks, months or even years. The slide can also be conveniently sent to specialists for their opinion in doubtful cases.
The stained smears can also provide information about the morphology and count of red cells and platelets, and Hb status, besides detecting the presence of various parasites (e.g. malaria).

APPARATUS AND MATERIALS

1. Microscope. •5–6 Clean glass slides. •Sterile lancet. •Cotton and gauze swabs. •70% alcohol. •Glass dropper.
2. A drop bottle containing Leishman's stain.
3. A wash bottle of distilled water (or buffered water, if available). • Fluff-free blotting paper.

Leishman's stain. This stain is a simplification of Romanowsky group of stains. It is probably one of the simplest and most precise method of staining blood for diagnostic purposes. It contains a compound dye—**eosinate of methylene-blue** dissolved in acetone-free methyl alcohol.
i. Eosin. It is an acidic dye (negatively charged) and stains basic (positive) particles—granules of eosinophils, and RBCs a pink color.
ii. Methylene-blue. It is a basic dye (positively charged) and stains acidic (negatively charged) granules in the cytoplasm, nuclei of leukocytes, especially the granules of basophils, a blue-violet color.
iii. Acetone-free and water-free absolute methyl alcohol. The methyl alcohol is a fixative and must be free from acetone and water. It serves two functions:
a. It fixes the blood smear to the glass slide. The alcohol precipitates the plasma proteins, which then act as a 'glue' which attaches (fixes) the blood cells to the slide so that they are not washed away during staining.
b. The alcohol preserves the morphology and chemical status of the cells.
• The alcohol must be free from acetone because acetone being a very strong lipid solvent, will, if present, cause crenation, shrinkage, or even destruction of cell membranes. This will make the identification of the cells difficult. (If acetone is present, the stain deteriorates quickly).
• The alcohol must be free from water since the latter may result in rouleaux formation and even hemolysis. The water may even wash away the blood film from the slide.

Steps in Differential Leukocyte Counting

1. Getting a blood sample from a finger-prick and making blood smears. If blood is obtained from a vein, place a drop of blood (through the needle) on each of the 4–5 slides and spread blood films.
2. Examining the blood smears under LP and HP and choosing the ideal films for staining.
3. Fixing and staining the blood films.
4. Identification and counting of various leukocytes.

PROCEDURES:

A. Preparing the Blood Films

1. Prepare 4 or 5 blood films.
2. Air dry the slides immediately by waving them in the air.
3. Examine them under low and then under high magnifications, and choose the best for staining.

B. Fixing and Staining of Blood Films

While supravital staining is employed for living cells, the staining of blood films involves dead cells. **Fixation** is the process that makes the blood film and its cells adhere to the glass slide. It also preserves the shape and chemistry of blood cells as near living cells as possible. (See Q/A 8). **Staining** is the process that stains (colors) the nuclei and cytoplasm of the cells. Both these purposes are achieved by the Leishman's stain.

• Since the timings for fixing and staining of the films with the Leishman's stain vary with different batches of the stain, check the timings with the laboratory assistant.

1. Fixing the Blood Films. Place the slides, smear side up, on a 'staining rack' assembled over a sink (two glass rods placed across the sink, with the ends fitted into short pieces of rubber tubing). Ensure that they are horizontal.

2. Pour 8–10 drops of the stain on each unfixed slide by dripping it from a drop bottle, or use a dropper. This amount of stain usually covers the entire surface and "stands up" from the edges of the slides without running off. Note the time.

3. Allow the stain to remain undisturbed for 1–2 minutes, as advised.

• During this time, watch the stain carefully, especially during hot weather, and see that it does not become syrupy (thick) due to evaporation of alcohol. If the stain dries, it will precipitate on the blood film and appear as round, blue granules.

This can be prevented by pouring more stain on the slides as required.

4. Staining the blood film. After the fixing time is over, add an equal number of drops of distilled water (or buffered water, if available) to the stain. If the water is carefully dripped from a drop bottle or a dropper, the entire mixture will stand up from the edges of the slides (due to surface tension) without spilling over.

5. Mix the stain and water by gently blowing at different places on the slides through a dropper, without scratching the smear. A glossy greenish layer (scum) soon appears on the surface of the diluted stain. Allow the diluted stain to remain on the slide for 6–8 minutes, or as advised.

6. Flush off the diluted stain in a gentle stream of distilled water for about 30 seconds and leave the slides on the rack for about a minute with the last wash of water covering them. Drain the slides and put them in an inclined position against a support, stained sides facing downwards (to prevent dust particles settling on them) to drain and dry. The under sides of the slides may be blotted with filter paper.

OBSERVATIONS AND RESULTS

C. Assessment of Stained Blood Smears

Before starting the actual counting of WBCs, you should—

i. Take an assessment of all the blood films. Examine with naked eye first, and then under low and high magnifications. Choose the best stained films for cell counting.

ii. Make sure that you can identify all the leukocytes with certainty.

Ensure that you are examining the blood smear side of the slide. Hold the slide in bright light and tilt it this way and that to see if there are any reflections. The clean side shows reflections, while the side which has the blood smear appears dull and does not show any reflections.

1. Draw 200 squares in your workbook for recording various WBCs as they are encountered and identified one after another. Enter these cells by using the letters 'N' for neutrophils, 'M' for monocytes, 'LL' for large lymphocytes, 'SL' for small lymphocytes, 'E' for eosinophils, and 'B' for basophils.

• You can indicate these cells in a column (instead of the 200 squares), and as you identify a cell, put a short vertical stroke against that cell. In this way, you can place different types of cells in groups of 5, a horizontal stroke representing the 5th cell (e.g. Neutrophils = ~~IIII~~ III, etc.)

2. Place a drop of cedar wood oil on the right upper corner of the film, a few mm away from the head end. Bring the oil immersion lens into position till it enters the oil drop. Adjust the focus.

• Do not flood the entire surface of the slide with oil; as you move the slide, the oil will move with the objective lens.

3. Move the slide slowly to the right (the image will move to the left) and as you encounter a leukocyte, identify it, and enter it in your workbook. As you approach the end of the smear, move 2 fields down and scan the film in the opposite direction. As you near the head, again move 2 fields down and scan the film towards the tail. Traverse the film in this to and fro fashion till you have examined 200 cells (count 400 cells for good results). This "battlement" procedure, as shown in Figure 1-9, ensures that you do not count a leukocyte more than once.

• The possibility of WBCs sticking to the edge of the spreader should be kept in mind.

4. **Recount.** After you have, counted 200 (or 400) cells, count the leukocytes once more, starting from the lower left corner of the film, and going up in the "battlement" procedure.

Differential leukocyte count. When counting has been done, calculate the percentage of each type of cell in your count of 200 (or 400) white cells. The neutrophils are the prominent cells of the blood and constitute about 50–60% of the WBCs. The next predominant cells are lymphocytes (20–40%), which may be small or large. The third cell in the order of population is the monocyte which constitutes 8–10% of the WBCs.

10. Haemoglobin estimation by Sahli's of Drabkin's method.

PRINCIPLE:

The Hb present in a measured amount of blood is converted by dilute hydrochloric acid into acid hematin, which in dilution is golden brown in color. The intensity of color depends on the concentration of acid hematin which, in turn, depends on the concentration of Hb. The color of the solution (i.e. its hue and depth), after dilution with water, is matched against golden-brown tinted glass rods by direct vision. The readings are obtained in g%.

APPARATUS AND MATERIALS:

A. Sahli (Sahli-Adams) Hemoglobinometer (Hemometer). The set consists of:

1. Comparator. It is a rectangular plastic box with a slot in the middle which accommodates the calibrated Hb tube. Non-fading, standardized, golden-brown glass rods are fitted on each side of the slot for matching the color. An opaque white glass (or plastic) is fitted behind the slot to provide uniform illumination during direct visual color matching.

2. Hemoglobin tube. The square or round glass tube is calibrated in g Hb % (2–24 g%) in yellow color on one side, and in percentage Hb (20–160%) in red color on the other side. There is a brush to clean the tube.

3. Hemoglobin pipette. It is a glass capillary pipette with only a single calibration mark-0.02 ml (20 cmm, cubic millimeters; or 20 ml, micro liters). There is no bulb in this pipette (as compared to cell pipettes) as no dilution of blood is done. **Figure 1-10B** shows the Hb pipette. Note The calibration mark 20 cmm indicates a definite, measured volume and not an arbitrary volume, as is the case with diluting pipettes.

4. Stirrer. It is a thin glass rod with a flattened end which is used for stirring and mixing the blood and dilute acid.

5. Pasteur pipette. It is a 8–10 inch glass tube drawn to a long thin nozzle, and has a rubber teat. Ordinary glass dropper with a rubber teat also serves the purpose.

6. Distilled water.

B. Decinormal (N/10) hydrochloric acid (0.1 N HCl) solution. Mixing 36 g HCl in distilled water to 1 liter gives 'Normal' HCl; and diluting it 10 times will give N/10 HCl solution.

C. Materials for skin prick.

- Sterile lancet/needle
- Sterile gauze and cotton swabs
- Methylated spirit/70% alcohol.

PROCEDURES:

1. Using a dropper, place 8–10 drops of N/10 HCl in the Hb tube, or up to the mark 20% or 3 g, or a little more till the tip of the pipette will submerge, and set it aside.
2. Get a finger prick under aseptic conditions, wipe away the first 2 drops of blood. When a large drop of free-flowing blood has formed again, draw blood up to the 20 cmm mark (0.02 ml). Carefully wipe the blood sticking to the tip of the pipette with a cotton swab, but avoid touching the bore or else blood will be drawn out by capillarity.

3. Without any waiting, immerse the tip of the pipette to the bottom of the acid solution and expel the blood gently. Rinse the pipette 3–4 times by drawing up and blowing out the clear upper part of the acid solution till all the blood has been washed out from it. Avoid frothing of the mixture. Note the time.

4. Withdraw the pipette from the tube, touching it to the side of the tube, thus ensuring that no mixture is carried out of the tube. Mix the blood with the acid solution with the flat end of the stirrer by rotating and gently moving it up and down.

5. Put the Hb tube back in the comparator and let it stand for 6–8 minutes (or as advised by the manufacturer). During this time, the acid ruptures the red cells, releasing their Hb into the solution (hemolysis). The acid acts on the Hb and converts it into acid hematin which is deep golden brown in color.

• The color of acid hematin does not develop fully immediately, but its intensity increases with time, reaching a maximum, after which it starts to decrease. An adequate time, usually 6–8 minutes, must be allowed before its dilution is started. Too little time and all Hb may not be converted into acid hematin. And, waiting too long, may result in fading of color. In either case, the result will be falsely low.

6. **Diluting and matching the color.** The next step is to dilute the acid hematin solution with distilled water (preferably buffered water, if available) till its color matches the color of the standard tinted glass rods in the comparator.

7. Take the Hb tube out of the comparator and add distilled water drop by drop (or larger amounts depending on the experience), stirring the mixture each time and comparing the color with the standard.

8. Hold the comparator at eye level, away from your face, against bright but diffused light. Read the lower meniscus (lower meniscus is read in colored transparent solutions).

OBSERVATIONS AND RESULTS

Compare your colour matching with that of your work-partner and record the observations in your workbook. Take the average of 3 readings as shown below, and report your result as: Hb = …….g/dl.

1st reading, when the colour is slightly darker than the standard:……..…...g/dl.

2nd reading, when, after adding a few drops of distilled water, the colour exactly matches the standard: ……..…… g/dl.

3rd reading, when, after adding some more drops, the colour becomes a little lighter than the standard:……..…….. g/dl.

For report. Express your result as: Hb= ...….g/dl.

• **Oxygen carrying capacity:** Knowing your Hb concentration, and that 1.0 g of Hb can carry 1.34 ml of O2, calculate its oxygen-carrying capacity as …….……ml O2/dl.

• **100 % Saturation.** When blood is equilibrated with pure (100 %) oxygen at a PO2 of 120 mm Hg, the Hb gets 100 % saturated, i.e. it picks up as much O2 as it possibly can.

For report:

• Oxygen carrying capacity

• 100% saturation.

Normal Values

The levels of Hb in normal Indian adults, especially in the economically deprived population, are on the lower side of those reported from affluent countries. The reason may be the poor intake of grade 1 proteins and other nutrients. The average levels and their ranges are as follows:
Males: 14.5 g/dl (13.5–18 g/dl).
Females: 12.5 g/dl (11.5–16 g/dl).

Advantages of Sahli Method

The method is simple, fairly quick, and accurate. It does not require any costly apparatus, since it needs only direct color matching. Its running cost is minimal and can, therefore, be used in mass surveys.

Disadvantages of Sahli Method

Since the acid hematin is not in true solution, some turbidity may occur. The method estimates only the oxyHb and reduced Hb, other forms, such as carboxyHb and metHb are not estimated. Also the degree of error may be high if proper precautions are not taken.

11. Determination of ESR

PRINCIPLE:

In the circulating blood the red cells remain uniformly suspended in the plasma. However, when a sample of blood, to which an anticoagulant has been added, is allowed to stand in a narrow vertical tube, the red cells (specific gravity = 1.095) being heavier (denser) than the colloid plasma (specific gravity = 1.032), settle or sediment gradually towards the bottom of the tube. The rate, in mm, at which the red cells sediment, called ESR, is recorded at the end of one hour.

Sedimentation of red cells

The settling or sedimentation of red cells in a sample of anticoagulated blood occurs in 3 stages:
i. In the ***first stage,*** the RBCs pile up (like a stack of coins), and form rouleaux that become heavier during the first 10-15 minutes.
ii. During the ***second stage,*** the rouleau (pleural of rouleaux) being heavier (see below) sink to the bottom. This stage lasts for 40–45 minutes.
iii. In the **third stage,** there is packing of massed bunches of red cells at the bottom of the blood column. This stage lasts for about 10–12 minutes.
Thus, most of the settling of the red cells occurs in the first hour or so.

WESTERGREN'S METHOD:

APPARATUS AND MATERIALS:

1. 2 ml disposable syringe with needle •Sterile cotton/gauze swabs moist with alcohol •Container (discarded penicillin bottle).

2. Sterile solution of 3.8 percent sodium citrate as the anticoagulant.

3. Westergren pipette (tube) and stand. It is 300 mm long and has a bore diameter of 2.5 mm. It is calibrated in cm and mm from 0 to 200, from above downwards in its lower two-thirds. The Westergren stand can accommodate up to 4 tubes at a time. For each pipette, there is a screw cap that slips over its top, and, at its lower end, the pipette presses into a rubber pad or cushion. When the pipette is fixed in position, there is enough pressure of the screw cap to prevent leakage of blood from its lower end. There is a spirit level to ensure vertical position of the pipette.

PROCEDURES:

1. Draw 2.0 ml of venous blood and transfer it into a vial containing 0.5 ml of 3.8% sodium citrate solution. This will give a blood: citrate ratio of 4:1. Mix the contents by inverting or swirling the vial. Do not shake, as it will cause frothing.
2. Fill the Westergren's pipette with blood-citrate mixture by sucking, after placing the tip of your finger over the top of the pipette to control the flow of blood into and out of it, or with a rubber bulb. Bring the blood column to exact zero mark. (If there is a difference of 1–2 mm, it should be noted and taken into account before giving the final report at the end of one hour).
3. Keeping your finger (or the rubber bulb) over the pipette, transfer it to the Westergren stand by firmly pressing its lower end into the rubber cushion. Now slip the upper end of the pipette under the screw cap. Confirm that there is no leakage of blood and that the pipette will remain vertical.
4. Leave the pipette undisturbed for one hour at the end of which read the mm of clear plasma above the red cells.

Express your results as:………mm 1st hour (Westergren).

Normal values

Males : 3–9 mm 1st hour

Females : 5–12 mm 1st hour.

12. Identification of superficial reflex.

These include the plantar response; the epigastric and abdominal reflexes; cremasteric, gluteal, and anal reflexes; the ciliospinal reflex; and the various mucous membrane reflexes described earlier with cranial nerves.

Response. In all the skin reflexes, there is contraction of the underlying muscles when a particular area of the skin is stimulated by scratching, stroking, or pinching.

Reflex Arcs. The reflex arcs for the skin reflexes appear to be long and complex, and include a number of interneurons between the sensory and the motor neurons of the reflex arc. The afferent impulses appear to be carried up by dorsal columns and spinothalamic tracts and end somewhere in the midbrain, thalamus or cerebral cortex. From here, impulses are carried by corticospinal and extrapyramidal tracts to the anterior horn cells innervating the muscles involved in the reflex. This is the reason why the skin reflexes are absent in the upper motor neuron lesions.

Flexor Plantar Reflex (Plantar Flexor Reflex).

The subject is asked to relax the muscles of the legs. A light scratch is given with a thumbnail (it should always be tried first), a key, or the blunt point of the patellar hammer, along the **outer edge of the sole of the foot, from the heel toward the little toe, and then medially along the base of the toes up to the 2nd toe.** The response to this stimulation of the skin in healthy adults is: plantar flexion and drawing together of the toes, often including the big toe, dorsiflexion and inversion of the ankle, and sometimes, contraction of the tensor fascia lata. With stronger stimuli, the limb may be withdrawn (flexed at the knee and hip) and adducted at the hip. This is the normal response in the adults, and is called the flexor plantar reflex (or the plantar flexor reflex). It is never completely absent in healthy individuals. Afferent (tibial nerve): L-5, S-1, 2; Center: S-1, 2; Efferent (tibial nerve): L-4,5 segments of the spinal cord.

Extensor Plantar Reflex (Plantar Extensor Reflex).

In infants, the response is a dorsiflexion of the big toe and retraction of the foot and occasionally dorsiflexion and fan-like spreading of the other toes. In adults, such a response (first described by Babinski in 1896) is seen in lesions of corticospinal system. This abnormal response is called the **extensor plantar reflex** or the **Babinski sign (Babinski toe sign; positive Babinski;** or **"upgoing toe")**. In this response the dorsiflexion of the toes (the big toe dorsiflexes first) is followed by dorsiflexion of the ankle and flexion of the knee and the hip. (The stimulus must be applied over the lateral region of the sole because the medial region may give a normal response).

In some cases of positive Babinski, the reflexogenic area (i.e. the region from which it is obtained), spreads out over a large area so that the same response is obtained by squeezing the calf muscles, by a firm downward movement over anterior tibia (Oppenheim's sign), by pinching the Achilles tendon (Gordon's reflex), or by stroking the lateral malleolus (Chaddok's sign). [The clawing movement of the fingers and the thumb upon flicking the terminal phalanx of the index finger is called the **Hoffmann's sign** (the equivalent of Babinski in the upper limb)].

The Babinski sign is perhaps the most important single physical sign in clinical neurology. It has great significance in differentiating between an organic lesion and a functional disorder (e.g. psychoneurosis) because it never occurs in the latter conditions.

13. Identification deep reflex's.

The deep reflexes are also called the **tendon reflexes**, or **tendon jerks** or simply **"jerks"** because when the tendon of a lightly stretched muscle is given a single, sharp blow with a rubber hammer (patellar or percussion hammer), the muscle contracts briefly and then relaxes, i.e. it gives a "jerky" response.

Patellar (knee) Hammer. It is a simple device employed for eliciting deep or tendon reflexes. It has a long metallic handle that bears a triangular rubber piece. The rubber is employed for delivering a sharp blow on the tendon of a slightly stretched muscle under study. The sudden stretch of the muscle causes a reflex contraction of the muscle.

When using it, the hammer should be held between the thumb and fingers and the swing should be at the wrist and not at the elbow or shoulder. The upper part of the hammer which can be unscrewed has a sharp point for eliciting superficial reflexes.

Stimulus

The **stimulus** that initiates a deep reflex is the sudden **stretching of the muscle spindles**, which sends a synchronous volley of impulses from the primary sensory endings into the spinal cord. In the cord, these impulses directly (monosynaptically) stimulate the anterior horn cells which innervate the stretched muscle. Thus, these reflexes are monosynaptic stretch reflexes.

The knee jerk reflex.

Supine position. The subject is asked to relax his legs, and is reassured that the patellar hammer will not cause injury. His legs are semiflexed, and the observer supports both knees by placing a hand behind them. The patellar tendon is then struck midway between the patella and the insertion of the tendon on the tibial tuberosity. (The tendon is located by palpation before striking it. The response is extension of the knee due to contraction of the quadriceps femoris muscle. Afferent and efferent paths: Femoral nerve; Center: Lumbar 3,4 segments.

Sitting position. The subject is seated in a chair and is asked to cross one leg over the other, and then the reflex is elicited. The leg can be seen to kick forwards; the muscle can also be felt to contract if the observer places his hand on the lower front of the thigh.

A better way to elicit this reflex is to ask the subject to sit with both legs dangling loosely over the edge of the chair. It permits a more rapid comparison of the two knee jerks.

The knee jerk may be pendular in acute cerebellar disease and present on the side of the lesion. It may be sustained in chorea. In hypothyroidism, there may be delayed return of the leg to the resting position. In hyperthyroidism, the jerks are brisk.

Elicit the ankle jerk.

The subject lies supine, the knee is semiflexed, and the hip externally rotated. Then with one hand, the examiner slightly dorsiflexes the foot so as to stretch the Achilles tendon (tendo-calcaneus), and with the other hand, the tendon is struck on its posterior surface. The response is plantar flexion of the foot due to contraction of the calf muscles.

Another method is to ask the subject to kneel over a chair so that he faces the back of the chair and his ankles lie, over its edge. The ankle jerks are then tested as described above. Afferent and efferent: Tibial nerve; Center: Sacral 1,2 segments.

Test the biceps jerk in the subject provided.

The subject's elbow is flexed to a right angle and the forearm semipronated and supported on the examiner's arm. The examiner then places his thumb on the biceps tendon and strikes it with the hammer. The response is contraction of the biceps causing flexion and slight pronation of the forearm (If the patient is in bed, his forearm may rest across his chest). The afferent and efferent paths are musculocutaneous nerve and the center is in 5th and 6th cervical segments.

14. Muscle striation study by Methelene blue.

Procedure:

(a) Taking out the tissue • Place a preserved cockroach/frog or other available animal in a dissecting tray containing water. • Cut open the animal to expose its thigh region. (As an alternate preserved sample of striated muscle can be provided) • Take a small piece of muscle from this region and tease it on a slide with the help of needles to get a few thinnest possible fibres. • Wash it in water in a petridish, changing the water 2-3 times to remove the preservative, as it may interfere with staining. (b) Staining and mounting • Add a few drops of methylene blue to stain the muscle fibres. • After staining, put the muscle fibres on a slide and tease it further, if necessary, with needles so that the muscle fibres are well separated. • Blot out the excess of water and stain. • Add a drop of glycerine on the slide and with the help of a needle gently put the coverslip and avoid the entry of air bubbles. • Press the coverslip gently with a needle to spread the glycerine and the muscles properly. • Examine the slide under the microscope.

Observation:

Look for the following features in the muscle fibre • Muscle fibres are elongated, cylindrical and multinucleated (syncytium). • These fibres are enclosed in a membrane called sarcolemma. • Several dark and light bands are alternately arranged perpendicularly to the long axis of the fibre. Presence of these bands alternately produce striations; hence these muscles are called striated muscles. Draw a labelled diagram of your preparation.

16. Study of nodes of Ranvier by Silver chloride method.

15. Bielschowsky's Silver Staining Protocol for Nerve Fibers, Axons

Principle:

Bielschowsky's silver stain a very useful tool to detect nerve fibers. It can be used to stain axons, neurofibrils and senile plaques in the central nervous system. This method is easy to perform and is routinely used in the study of Alzheimer's disease together with antibody staining.

Fixation: 10% formalin

Section: Paraffin sections at 10 um thick.

Solutions and Reagents:

10% Silver Nitrate Stock Solution:

Silver nitrate ---------- 5 g
Distilled water ---------- 50 ml

1% Ammonium Hydroxide Solution:
Ammonium hydroxide, concentrated ---------- 1 ml
Distilled water ---------- 100 ml

Developer Stock Solution (make fresh):

37-40% Formaldehyde ---------- 20 ml
Citric acid (trisodium dihydrate, Sigma) ----- 0.5 g
Nitric acid, concentrated ---------- 2 drops
Distilled water ---------- 100 ml

Developer Working Solution (make fresh immediately before use, discard after use):
Developer Stock Solution ---------- 8 drops
Ammonium hydroxyide, concentrated --------- 8 drops
Distilled water ---------- 50 ml

5% Sodium Thiosolfate (HYPO):

Sodium Thiosolfate ---------- 5 g
Distilled water ---------- 100 ml

Procedure:

1. Deparaffinize sections to distilled water and wash three times.
2. Place slides in pre-warmed (40 ºC) 10% silver nitrate solution and stain for 15 minutes and sections become light brown color (thicker sections may look dark brown).
3. Place slides in distilled water and wash for 3 times.
4. To the silver nitrate solution, add concentrated ammonium hydroxide drop by drop until the precipitate formed is JUST clear. Excess ammonia may cause a precipitate or result in a poor impregnation of the fibers. If ammonia is added too much, add few more drops of 10% silver nitrate solutions to make solution JUST becomes cloudy.
5. Place slides back in this ammonium silver solution and stain in 40 ºC oven for 30 minutes or until sections become dark brown (may not look very dark brown if the sections are only 10 um thick and may look very dark brown if the sections are 30 um thick).

6. Place slides DIRECTLY (do not wash slides) in developer working solution for about 1 minute or less (reaction may be very fast, so test a slide, check microscopically to determine exact incubation time).
7. Dip slides for 1 minute in 1% ammonium hydroxide solution to stop the silver reaction.
8. Wash slides in 3 changes of distilled water.
9. Place slides in 5% sodium thiosulfate solution for 5 minutes.
10. Wash in 3 changes of distilled water
11. Dehydrate and clear through 95% ethyl alcohol, absolute alcohol and xylene.
12. Mount with resinous medium.

Results:

Axons, neurofibrillary tangles and senile plaques ---------- black

Positive Controls:

Brain tissue.

Notes:

1) Use glass stainers/containers for all procedure. All the glass containers must be completely cleaned with distilled water.
2) Developing time is critical so test a few slides and check microscopically to determine exact developing time.

Biochemistry & Biophysics: BMLT paper – V, Unit – 10

Qualitative Tests for Carbohydrates

Potato consists of different carbohydrates like starch, reducing sugars etc. Difficulties are encountered in the qualitative and quantitative analysis of samples containing mixtures of carbohydrates, particularly the sugars, because of their structural and chemical similarity and also with respect to their stereoisomers. During biochemical investigations it may because necessary to establish whether a given sample, particularly of a purified preparation, consist carbohydrates or not. Several rapid tests are available the presence or absence of a sugar or a carbohydrate in a sample. These tests are based on specific colour reactions typical for their group and are described below. For laboratory practical, it may be advised to perform these tests with the individual rather than mixture of sugars. Use of sugar solutions of different concentrations (0.1-1%) during these experiments would also provide valuable information about the sensitivity of these tests. The types of carbohydrates detected by these tests are:

Name of the test Application

1. Molisch's Test General test for carbohydrates
2. Anthone Test General test for carbohydrates
3. Iodine Test For glycans (starch, glycogen)
4. Barfoed's Test To distinguish between mono-saccharides from reducing diasaccharides
5. Seliwanoff's Test For Ketones
6. Fehling's Test For reducing sugars
7. Bendict's Test For reducing sugars
8. Picric acid Test For reducing sugars
9. Bial's Test For pentoses

MOLISCH'S TEST

Principle

This is a general test for all carbohydrates. Conc. H_2SO_4 hydrates glycosidic bonds to yield monosaccharides which in the presence of an acid get dehydrated to form furfural and its derivatives. These products react with sulphonated α-naphthol to give a purple complex. Polysaccharides and glycoproteins also give a positive reaction.

Reagents

1. Conc. H_2SO_4

2. α-naphthol: 5% (w/v) in ethanol (prepare **Procedure and**

observations

Add 2-3 drops of α-naphthol solution to 2 ml of the test solution. Very gently pipette 1ml conc. H_2SO_4 along the side if the test tube so that the two distinct layers are formed. Carefully observe any color change at the junction two layers. Appearance of purpose color indicates the presence of carbohydrates in the sample preparation or the test solution.

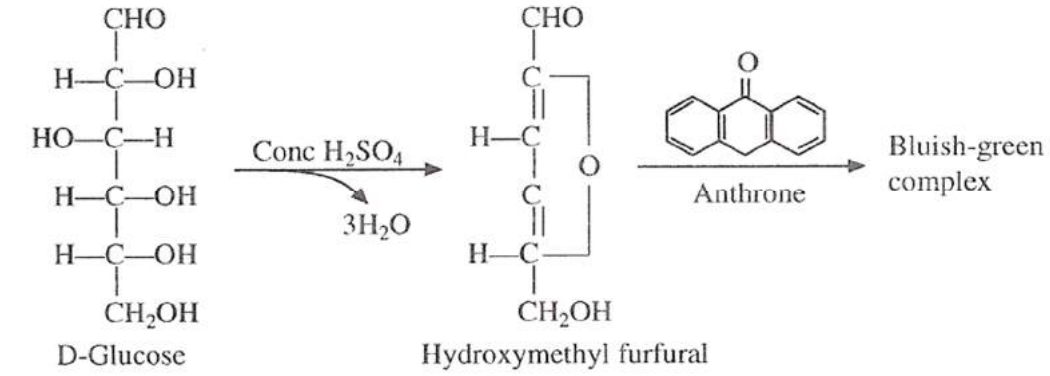
Precautions

1. α-naphthol solution is unstable and should be prepared fresh.

2. Conc. H_2SO_4 should be along the sides of the test tubes causing minimal disturbance to the contents in the tube.

ANTHRONE TEST
Principle:

Reaction



Materials and Reagents

Anthrone reaction is another general test for carbohydrates. In this the furfural produced reacts with anthrone to give bluish green colored complex.

Materials and Reagents

1. Boiling water bath.
2. Conc. H_2SO_4
3. 0.2% (w/v) anthrone solution **Procedure and observations**

Add 0.5 - 1 ml of the test solution to about 2 ml of anthrone reagent and mix thoroughly. Observe whether the color changes to bluish green. If not, examine the tubes again keeping them in boiling water bath for 10 min.

IODINE TEST

Principle

Iodine forms colored adsorption complexes with polysacchaides. Starch gives blue color with iodine, while glycogen reacts to form reddish brown complex. Hence it is useful, convenient and rapid test for detection of amylase, amylopectin and glycogen.

Reagents

Iodine solution: Prepare 0.005N iodine solution in 3% (w/v) potassium iodine solution.

1% Test solutions of glucose, sucrose, starch, glycogen, cellulose etc.

Procedure and observations

Take 1 ml of the sample extract or test solution in a test tube. Add 4 - 5 drops of iodine solution to it and mix the contents gently. Observe if any coloured product is formed

Barfoed's Test

Principle:

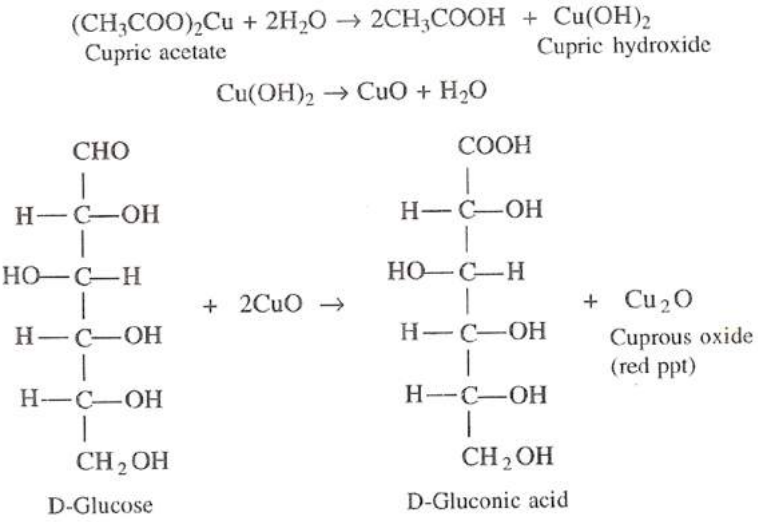
This test is used for distinguishing monosaccharides from reducing disaccharides. Monosaccharides usually react in about 1 - 2 min while the reducing disaccharides take much longer time between 7 - 12 min to get hydrolysed and then react with the reagent. Brick red color is obtained in this test which is due to the formation of cuprous oxide.

Reaction

$(CH_3COO)_2Cu_2 + H_2O$ ® $2CH_3COOH + Cu(OH)_2$

Cupric acetate Cupric hydroxide $Cu(OH)_2$

® $CuO+H_2O$



Materials and Reagents

1. Boiling water bath
2. Barfoed's reagents: Dissolve 13.3 g of copper acetate in 200 ml water and add 1.8 ml of glacial acetic acid to it.

Procedure and observations

Take 2 ml of Barfoed's solution in a test tube and add 1ml of sample solution to it. Keep the test tubes in a boiling water bath. A briskly boiling water bath should be used for obtaining reliable results. Look for the formation of brick red color and also note the time taken for its appearance.

SELIWANOFF'S TEST

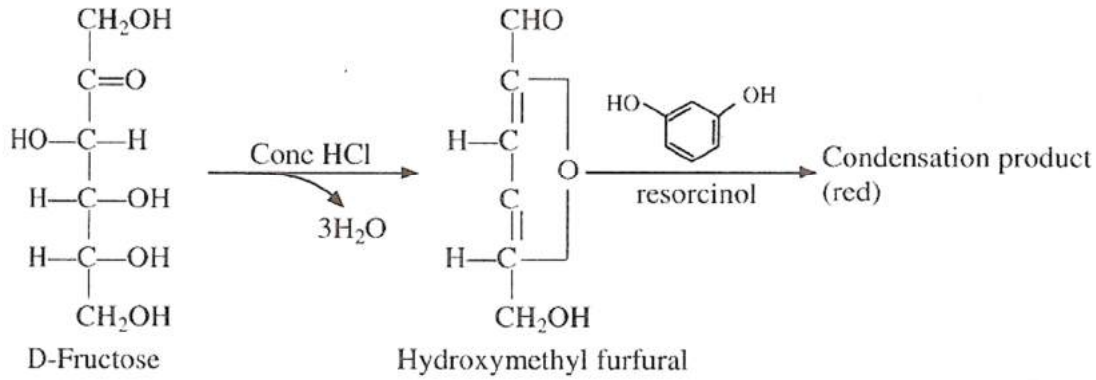
Principle

This test is used to distinguish aldoses from ketoses. Ketoses undergo dehydration to give furfural derivatives, which then condense with resorcinol to form a red complex. Prolonged heating will hydrolyze disaccharides and other monosaccharides will also eventually give color.

Reaction

Materials and Reagents

Reaction



3. Boiling water bath

4. Seliwanoff's reagent: 0.05% (w/v) resorcinol in 3 HCl **Procedure and**

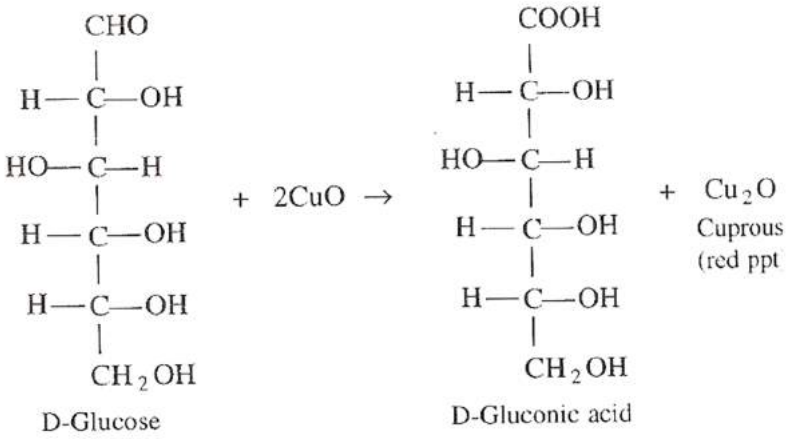
Observations

Add 1ml of the test solution to 2 ml of Seliwanoff's reagent and warm in a boiling water bath for 1min. Note for the appearance of a deep red color. This would indicate that the sample solution contains a keto sugar.

Fehling's
Principle

Fehling's test is a specific and highly sensitive for detection of reducing sugars. Formation of yellow or red cuprous oxide denotes the presence of reducing sugars. Rochelle salt acts as the chelating agent in this reaction.

Reaction



Materials and Reagents

5. Boiling water bath.
6. Fehling's solution A: Dissolve 35 g of $CuSO_4.5H_2O$ in water and make the volume to 500 ml.
7. Fehling's solution B: Dissolve 120 g of KOH and 173 g Na-K tartrate (Rochelle salt) in water and make the volume to 500 ml.
8. Fehling's reagent: Mix equal volumes of Fehling's solution A and B. These solutions must be mixed immediately prior to use.

Procedure and observations

Add 1 ml of Fehling's reagent (Reagent No. 4) to 1 ml of aliquot of the test solution. Mix thoroughly and place the test tubes in vigorously boiling water bath. Look out for the formation of red ppt of cuprous oxide which would indicate the presence of reducing sugars in the solution.

Benedict's Test

Benedict's test is more convenient and this reagent in more stable. In this method sodium citrate functions as a chelating agent. Presence of reducing sugars results in the formation of red ppt of cuprous oxide.

Reaction

$$Na_2CO_3 + 2H_2O \rightarrow 2NaOH + H_2CO_3$$
$$2NaOH + CuSO_4 \rightarrow Cu(OH)_2 + Na_2SO_4$$
$$Cu(OH)_2 \rightarrow CuO + H_2O$$
$$\text{D-Glucose} + 2\ CuO \rightarrow \text{D-gluconic acid} + Cu_2O\ (\text{Red ppt})$$

Materials and Reagents

- Boiling water bath.
- Benedict's reagents: Dissolve 173 g of sodium citrate and 100 g of anhydrous Na_2CO_3 in 600 ml of hot H_2O. Dilute to 800 ml with water.
- Dissolve 17.3 g of $CuSO_4.H_2O$ in 100 ml hot water. Cool and dilute to 100 ml.
- Add Reagent No.2 to Reagent No.3 slowly with constant stirring. Make the final volume to 1 L.

Procedure and observations

Add 0.5 - 1 ml of the test solution or sample extract to 2 ml of Benedict's reagent (Reagent No. 4). Keep the test tubes in a vigorously boiling water bath. Observe for the formation of red precipitates whose appearance would suggest the presence of reducing sugars in the given or sample extract.

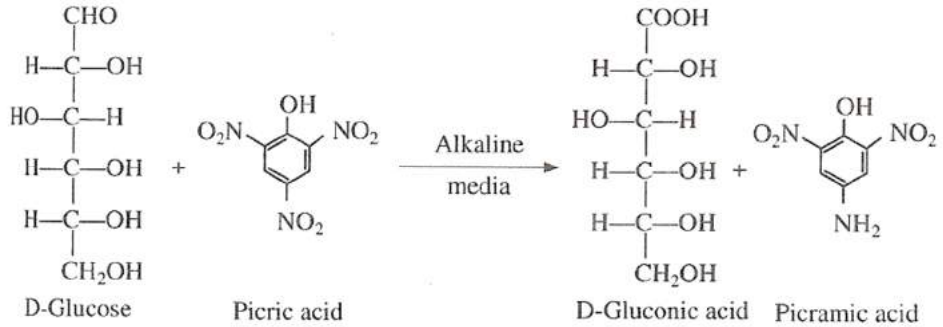
Picric acid Test

Principle:

It is another test for detection of reducing sugars. The reducing sugars react with picric acid to form a red colored picramic acid.

Reaction

Reaction



Materials and Reagents

Materials and Reagents

- Boiling water bath.
- Saturated picric acid: Dissolve 13 g picric acid in distilled water, boil and cool.
- 10% Na_2CO_3.

Procedure and observations

Add 1 ml saturated picric acid to 1 ml of sample solution followed by 0.5 ml 10% Na_2CO_3. Heat the test tubes in a boiling water bath. Appearance of red color would indicate the presence of reducing sugars in the sample solution.

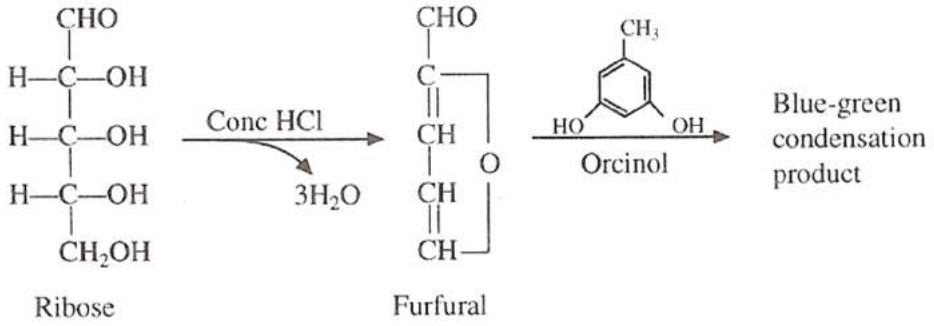
Bial's test

Principle

This test is useful in the determination of pentose sugars. Reaction is due to formation of furfural in the acid medium which condenses with orcinol in presence of ferric ions to give a blue-green colored complex which is soluble in butyl alcohol.

Reaction

Reaction



Materials and Reagents

- Boiling water bath
- Dissolve 1.5 g of orcinol in 100 ml of conc. HCl and add 20-30 drops of 10 % ferric chloride solution to it.

Procedure and observations

To 2 ml of Bial's reagent add 4-5 drops of test solution and heat in a boiling water bath. Observe for the formation of blue-green colored complex.

Known Test for Glucose

Sl.No	Experiment	Observation	Inference
1.	**Molisch's Test:** A carbohydrate solution of 2-3 ml is taken in a test tube. Molish's reagent (2-3 drops) is added to carbohydreate solution and mix it well. Then 1-2 ml of concentrated H2SO4 solution is added slowly in the test tube without stirring or mixing	Development of a purple-coloured ring between the junction of two liquids in the test tube.	This is general test for all Carbohydrate due to the formation of hydroxymithyle furfural. Glucose being a monosaccharide it reacts with the solution
2.	**Benedict's Test:** Given carbohydrate solution (2-3ml) is taken in a dry and clean test tube. Half Benedict reagent is added to an amount of half of the carbohydrate solution and mixed. Then boil the solution on the reducing flame of a Bunsen burner.	Greenish yellow colour is developed initially after that it turns to brick red colour after vigorous boiling.	This is the positive test for all reducing sugar. Glucose being a reducing monosaccharide so, it gives red colour due to formation of Cu2O (Cuprous oxide)
3.	**Barfoed's Test:** About 2-3 ml of supplied carbohydrate solution is taken in a dry test tube. Then just 2-3 drops of Barfoed's reagent is added to it and mix well. Then heat vigorously and finally cooled at room temperature	Red precipitate is developed at the bottom of the test tube	This is a positive test for only reducing monosaccharide it reacts with the solution due to the formation of Cu2O.
4.	**Fehling's Test:** About 2-3 ml of supplied carbohydrate solution is taken in a dry test tube Then just 2-3 drops of Fehling's reagent are added to it and mix well. Then heat vigorously.	Initially yellow colour developed and then red precipitate is formed.	for reducing sugar due to formation of Cu2O. Glucose being a reducing monosaccharide, it reacts with the solution
5.	**Iodine Test:** Given carbohydrate solution is taken in a dry and clean test tube. Then 2-3 drops of Iodine solution is added to it and mix well and wait for 3 mins	No change was observed	This is the positive test for the polysaccharide. As glucose is a reducing monosaccharide so, it does not react with iodine.

Known test for Fructose

Sl.No.	Experiment	Observation	Inference
1.	**Molisch's Test:** A carbohydrate solution of 2-3 ml is taken in a test tube. Molish's reagent (2-3 drops) is added to carbohydreate solution and mix it well. Then 1-2 ml of concentrated H_2SO_4 solution is added slowly in the test tube without stirring or mixing	Development of a purple coloured ring between the junction of two liquids in the test tube.	This is general test for all Carbohydrate due to the formation of hydroxymithyle furfural. As fructose is a monosaccharide it reacts with the solution.
2.	**Barfoed's Test:** About 2-3 ml of supplied carbohydrate solution is taken in a dry test tube. Then just 2-3 drops of Barfoed's reagent is added to it and mix well. Then heat vigorously and finally cooled at room temperature	Red precipitate is developed at the bottom of the test tube	This is a positive test for only reducing monosaccharide. As fructose is a reducing monosaccharide so it gives positive result. It reacts with the solution due to the formation of Cu_2O.
3.	**Fehling's Test:** About 2-3 ml of supplied carbohydrate solution is taken in a dry test tube Then just 2-3 drops of Fehling's reagent is added to it and mix well. Then heat vigorously.	At first yellow colour developed and then red precipitate is formed.	This is the positive test for reducing sugar due to formation of Cu_2O. As fructose is a reducing monosaccharide, it reacts with the solution.
4.	**Iodine Test:** Given carbohydrate solution is taken in a dry and clean test tube. Then 2-3 drops of Iodine solution is added to it and mix well and wait for 3 mins	No change was observed	Positive test for the polysaccharide. Fructose is a reducing monosaccharide so, it does not react with iodine.
5.	**Benedict's Test:** Given carbohydrate solution (2-3ml) is taken in a dry and clean test tube. Half Benedict reagent is added to an amount of half of the carbohydrate solution and mixed. Then boil the solution on the reducing flame of a Bunsen burner.	Greenish yellow colour is developed nitially after that it turns to brick red olour after vigorous boiling.	Positive test for all reducing sugar. Fructose is a reducing monosaccharide so, it gives red colour due to formation of Cu_2O (Cuprous oxide).

Known Test for Lactose

Sl. No.	Experiment	Observation	Inference
1.	**Molisch's Test:** A carbohydrate solution of 2-3 ml is taken in a test tube. Molish's reagent (2-3 drops) is added to carbohydreate solution and mix it well. Then 1-2 ml of concentrated H2SO4 solution is added slowly in the test tube without stirring or mixing	Development of a purple-coloured ring between the junction of two liquids in the test tube.	This is general test for all Carbohydrate so, lactose (reducing disaccharide) react with molisch's test due to the formation of hydroximethayl furfural.
2.	**Benedict's Test:** Given carbohydrate solution (2-3ml) is taken in a dry and clean test tube. Half Benedict reagent is added to a amount of half of the carbohydrate solution and mixed. Then boil the solution on the reducing flame of a Bunsen burner.	Greenish yellow colour is developed initially after that it turns to brick red colour after vigorous boiling.	Positive test for all reducing sugar. As lactose is a reducing monosaccharide so, it gives red colour due to formation of Cu2O (Cuprous oxide)
3.	**Iodine Test:** Given carbohydrate solution is taken in a dry and clean test tube. Then 2-3 drops of Iodine solution are added to it and mix well and wait for 3 mins	No change was observed	positive test for the polysaccharide
4.	**Fehling's Test:** Given carbohydrate solution is taken in a dry and clean test tube. 2-3 drops of Fehling's reagent are added to it and mix well. After that solution was heated vigorously	Yellow colour Developed initially and then red precipitate is observed.	It is the positive test for reducing sugar due to formation of Cu2O. As lactose is a reducing monosaccharide, it reacts with the solution.

5.	**Barfoed's Test:** About 2-3 ml of supplied carbohydrate solution is taken in a dry test tube. Then just 2-3 drops of Barfoed's reagent is added to it and mix well. Then heat vigorously and finally cooled at room temperature	Red precipitate is developed at the bottom of the test tube	This is a positive test for only reducing sugar. As lactose is reducing sugar. It react in benedict test due to the formation of cuprous oxide (cu2O)

Known test for Maltose

Sl. No.	Experiment	Observation	Inference
1.	**Molisch's Test:** A carbohydrate solution of 2-3 ml is taken in a test tube. Molish's reagent (2-3 drops) is added to carbohydreate solution and mix it well. Then 1-2 ml of concentrated H2SO4 solution is added slowly in the test tube without stirring or mixing.	Development of a purple-coloured ring between the junction of two liquids in the test tube.	It is general test for all Carbohydrate so, maltose (reducing disaccharide) react with molisch's test due to the formation of hydroximethayl furfural.
2.	**Fehling's Test:** Given carbohydrate solution is taken in a dry and clean test tube. 2-3 drops of Fehling's reagent is added to it and mix well. After that solution was heated vigorously	Yellow colour Developed initially and then red precipitate is observed.	This is the positive test for reducing sugar due to formation of Cu2O. As maltose is a reducing monosaccharide, it reacts with the solution.
3.	**Iodine Test:** Given carbohydrate solution is taken in a dry and clean test tube. Then 2-3 drops of Iodine solution are added to it and mix well and wait for 3 mins	No change was observed.	This is the positive test for the polysaccharide. Maltose is a disaccharide so; it does not react with iodine reagent.

4.	**Barfoed's Test:** Given carbohydrate solution is taken in a dry and clean test tube. 2-3 drops of Barfoed's reagent is added to it and mix well. Then heat the solution vigorously and finally cooled at room temperature.	At the bottom of the test tube Red precipitate is Observed.	Positive test for only reducing sugar. Maltose is reducing sugar. So, it reacts in benedict test due to the formation of cuprous oxide (cu2o).
5.	**Benedict's Test:** Given carbohydrate solution (2-3ml) is taken in a dry and clean test tube. Half Benedict reagent is added to an amount of half of the carbohydrate solution and mixed. Then boil the solution on the reducing flame of a Bunsen burner.	At first greenish yellow colour is developed just after heating then it turns to brick red colour after vigorous boiling.	This is the positive test for all reducing sugar. Maltose is a reducing monosaccharide so, it gives red colour due to formation of Cu2O (Cuprous oxide).

Known test for Sucrose

Sl.No.	Experiment	Observation	Inference
1.	**Molisch's Test:** A carbohydrate solution of 2-3 ml is taken in a test tube. Molish's reagent (2-3 drops) is added to carbohydreate solution and mix it well. Then 1-2 ml of concentrated H2SO4 solution is added slowly in the test tube without stirring or mixing.	Development of a purple-coloured ring between the junction of two liquids in the test tube.	Sucrose is present in the sample. hydroximethayl furfural is formed in the molisch's test due to reaction of carbohydrate with concentrated H2SO4.
2.	**Benedict's Test:** Given carbohydrate solution (2-3ml) is taken in a dry and clean test tube. Half Benedict reagent is added to a amount of half of the carbohydrate solution and mixed. Then boil the solution on the reducing flame of a Bunsen burner.	No change was observed.	Sucrose is a nonreducing sugar so, it does not react with this solution.

3.	**Barfoed's Test:** Given carbohydrate solution is taken in a dry and clean test tube. 2-3 drops of Barfoed's reagent is added to it and mix well. Then heat the solution vigorously and finally cooled at room temperature.	No change was observed.	Sucrose is a nonreducing sugar so, it does not react with this solution.
4.	**Hydrolysis Test:** 2-3ml of given carbohydrate solution is taken in a dry and clean test tube. Then few drops of conc. H2SO4, con. HCl is added to it and then boiled. After boiling cooled the solution under tap water and then neutralize the solution by adding Na2CO3 until no further bubbles occur. Then perform Benedict's Test	Developed greenish yellow colour after heating then it turns to brick red colour after vigorous boiling.	This is the positive test for all carbohydrate. Sucrose is a nonreducing sugar and converted to glucose after acid hydrolysis. Reducing sugar test is perform by using Benedict's Test and it gives the brick red colour due to formation of Cu2O
5.	**Iodine Test:** Given carbohydrate solution is taken in a dry and clean test tube. Then 2-3 drops of Iodine solution is added to it and mix well and wait for 3 mins.	No change was observed	This is the positive test for the polysaccharide. as sucrose is a disaccharide so, it does not react with iodine reagent.
6.	**Fehling's Test:** Given carbohydrate solution is taken in a dry and clean test tube. 2-3 drops of Fehling's reagent are added to it and mix well. After that solution was heated vigorously.	No change was observed	This is the positive test for all reducing sugar. Sucrose is a nonreducing sugar so, it does not react with Fehling's solution.

Known test for Starch

Sl. No.	Experiment	Observation	Inference
1.	**Molisch's Test:** A carbohydrate solution of 2-3 ml is taken in a test tube. Molish's reagent (2-3 drops) is added to carbohydreate solution and mix it well. Then 1-2 ml of concentrated H2SO4 solution is added slowly in the test tube without stirring or mixing.	Development of a purple-coloured ring between the junction of two liquids in the test tube.	Sucrose is present in the sample. hydroximethayl furfural is formed in the molisch's test due to reaction of carbohydrate with concentrated H2SO4
2.	**Benedict's Test:** Given carbohydrate solution (2-3ml) is taken in a dry and clean test tube. Half Benedict reagent is added to a amount of half of the carbohydrate solution and mixed. Then boil the solution on the reducing flame of a Bunsen burner.	No change was observed	Starch is a nonreducing sugar so, it does not react with this solution.
3.	**Barfoed's Test:** Given carbohydrate solution is taken in a dry and clean test tube. 2-3 drops of Barfoed's reagent are added to it and mix well. Then heat the solution vigorously and finally cooled at room temperature.	No change was observed	Starch is a nonreducing sugar so, it does not react with this solution.
4.	**Iodine Test:** Given carbohydrate solution is taken in a dry and clean test tube. Then 2-3 drops of Iodine solution is added to it and mix well and wait for 3 mins.	Blue color was observed	This is the positive test for the polysaccharide. Starch is a polysaccharide; it reacts with iodine reagent.
5.	**Fehling's Test:** Given carbohydrate solution is taken in a dry and clean test tube. 2-3 drops of Fehling's reagent are added to it and mix well. After that solution was heated vigorously.	No change was observed	This is the positive test for all reducing sugar. Starch is a nonreducing sugar so, it does not react with Fehling's solution.

6.	**Hydrolysis Test:** 2-3ml of given carbohydrate solution is taken in a dry and clean test tube. Then few drops of conc. H_2SO_4, con. HCl is added to it and then boiled. After boiling solution was cooled under tap water and then neutralize the solution by adding Na_2CO_3 until no further bubbles occur. Then perform Benedict's Test.	Developed greenish yellow colour after heating then it turns to brick red colour after vigorous boiling.	This is the positive test for all carbohydrate. Starch is a nonreducing sugar and converted to glucose after acid hydrolysis. Reducing sugar test is perform by using Benedict's Test and it gives the brick red colour due to formation of Cu_2O

Known test for Peptone

Sl. No.	Experiment	Observation	Inference
1.	**Biuret test:** 1% copper sulphate solution, one drop taken in a dry clean test tube. Then 3ml of 10% NaOH solution added to it. Then equal volume of supplied protein solution is taken and mixed well.	rose pink (Violet) colour observed.	Positive test for peptone
2.	**Million's Test:** Given protein solution (2-3 ml) taken in a dry and clean test tube. Then 3-4 drops of Million's reagent added to it and heated slightly heated with stirring.	At first very little precipitate was observed and after heating it was dissolved	Positive test for peptone.
3.	**Xanthoprotein Test:** About 2-3ml of supplied protein solution is taken in a dry test tube then HNO_3 is added to it. Then heated and boiled and cooled	Yellow colour is developed without precipitate.	positive test for peptone

4.	**Adamkiewicz Test:** Abo Given protein solution (2-3 ml) is taken in a dry test tube. 2ml of glacial CH3COOH added to it and mixed well. Then con. H2SO4 mixed into the test tube.	A purple colour is developed at the junction of two liquid	positive test for peptone
5.	**Esbach test:** Few ml of supplied protein sample is taken in a dry test tube. Then few ml of Esbach solution is added to it.	No precipitate is formed.	Presence of peptone
6.	**Heat coagulation:** Little amount of given protein solution is taken in a dry and clean test tube. Then few drops of glacial CH3COOH is added to it and heated.	No coagulation Observed after heating	It is the negative test for peptone.

Known test for Glycerol

Sl. No.	**Experiment**	**Observation**	**Inference**
1.	**Smell test:** Smell taken from the given sample.	No smell	Presence of glycerol
2.	**Solubility Test:** 1-2ml of Given sample taken in a dry and clean test tube and add water in the test tube. In a test tube few ml of given sample is taken and mixed with chloroform.	Soluble in water. Insoluble in present. chloroform	Water soluble lipid glycerol may be present.
3.	**Acrolin Test:** About 2-3 ml of given sample is taken in a test tube and then add potassium bisulphate (KHSO4)	pungent odour is developed	Glycerol may be present

4.	**Dunston's Test:** About 4-5ml of 0.5% borax s solution is taken in a dry test tube. 1-2 drops of 1% alcoholic phenoptheline is added to it drop wise and 1-2ml of given sample is added.	At first red colour of Borax was observed and phenoptheline mixture discharge by lipid solution.	Glycerol may be present

Known test for Cholesterol

Sl. No.	Experiment	Observation	Inference
1.	**Smell test:** Smell taken from the given sample.	Smell of chloroform	Presence of Cholesterol
2.	**Solubility Test:** 1-2ml of Given sample taken in a dry test tube and add water in the test tube. In a test tube few ml of given sample is taken and add chloroform.	Insoluble in water. Soluble in chloroform	Cholesterol may be present
3.	**Libermann-burchared Test:** About 2-3ml of given fat sample is taken in a dry test tube. Then 10-15 drops of acidic anhydrous and 3 drops of conc. H2SO4 added to it.	At first cherry red colour appear and then it turns to blue.	Presence of Cholesterol
4.	**Salkowski's Test:** About 2-3ml of given fat is taken in a test tube. Then equal volume of concentrated H2SO4 is added to it.	Upper layer turns in to red to purple colour but lower layer exhibited green fluorescence colour.	Presence of Cholesterol

PREPARATION OF BUFFER

AIM:

To prepare the buffer at required pH.

PRINCIPLE:

The pH meter measures at electrical potential developed by pair of electrode pins in a solution. For measurement of pH, an electrode system sensitive to change in H+ ion concentration of solution is taken. The electrode system consists of sequence of electrode whose potential raise with pH (H+ concentration of the solution).

PROCEDURE:

1. **ACETIC ACID- SODIUM ACETATE BUFFER:**

REAGENTS REQUIRED:

Acetic Acid 0.2M: 1.5 ml of glacial acetic acid is made upto 100ml with distilled water.

Sodium Acetate Solution**:** 0.64 gm of sodium acetate or 2.72gm of sodium acetate trihydrate is dissolved in 100ml Distilled water.

PROCEDURE:

Pipette out exactly 36.2ml of sodium acetate solution into 100ml of standard flask and add 14.8ml of glacial acetic acid, make the volume 100ml using distilled water using distilled water. This gives 0.2 M of acetic acid and sodium acetate buffer. The pH is measured with pH meter.

The pH meter is first standararised with pH buffer. Wash electrode with distilled water and introduced into 0.2M acetic acid-sodium acetate buffer prepared, the pH of solution is 4.6.

RESULT:

36.2ml Sodium acetate and 14.8 ml glacial acetic acid were mixed and buffer was prepared. pH was measured initial reading observed was 4 which made upto 4.6 with 5N NaOH.

2. BARBITONE BUFFER:

REAGENTS REQUIRED:

- Diethyl barbituric acid.
- Sodium diethyl barbititrate

PROCEDURE:

Dissolve 2.85gm of diethyl barbituric acid and 14.2gm of sodium diethyl barbititrate in distilled water and upto 1 liter. This gives the barbitone buffer. The pH meter is first standararised with pH buffer. Wash electrode with distilled water and introduced into barbitone buffer prepared, the pH of solution is 6.8.

3. CITRATE BUFFER:

REAGENT S REQUIRED:

- Citric acid: Dissolve 2.101 gm of citric acid in 100ml distilled water.
- Sodium citrate solution 0.1 M: Dissolved 2.941gm of sodium citrate in 100ml distilled water.

PROCEDURE:

46.5ml of citric acid with 3.5ml of sodium citrate solution and upto 100ml with distilled water. It corresponds to 0.1 M citrate buffer and standardised with pH meter and measures the pH of the prepared solution. This gives citrate buffer at pH 2.5.

RESULT:

Citrate buffer was prepared and the pH observed was 4.8 which was adjusted to 2.5 using 1N Hcl and 5N NaoH.

CARBONATE- BICARBONATE BUFFER:

REAGENTS REQUIRED:

- Sodium carbonate solution 0.2M: Dissolve 2.12gm of anhydrous sodium carbonate in 100ml Distilled water.
- Sodium bicarbonate solution: Dissolve 1.68gm of sodium bicarbonate in 100ml of distilled water.

PROCEDURE:

Pipette out exactly 27.5ml of sodium carbonate (Na_2Co_3) solution. To this add 22.5ml of sodium bicarbonate solution and made upto 100ml with distilled water which corresponds to 0.2 M sodium carbonate and bicarbonate buffer.

Standardise pH meter and measure the pH of required buffer. This gives the Carbonate-bicarbonate buffer pH 10.2.

RESULT:

Carbonate bicarbonate buffer was prepared and pH observed was 7.5 which was adjusted to 10.2 using 1N Hcl and 5N NaoH.

4. PHOSPHATE BUFFER:

REAGENTS REQUIRED:

- Monobasic: Dissolve 2.78gm of sodium dihydrogen phosphate in 100ml of distilled water.
- Dibasic sodium phosphate (0.2M): Dissolve 5.3gm of disodium hydrogen phosphate or 7.17 gm sodium hydrogen phosphate in 100ml distilled water.

PROCEDURE:

39 ml of dihydrogen sodium phosphate is mixed with 61 ml of disodium hydrogen phosphate This made up to 200ml with distilled water. This gives phosphate $(Po_4)_2$ buffer of 0.2M.

Standardized pH meter with standard buffer. Washed electrode with distilled water and introduced it into phosphate buffer prepared. The pH of the solution is 6.8.

RESULT:

Phosphate buffer was prepared and pH was observed 8.5 which was made upto 6.8 using 1N Hcl and 5N NaoH.

5. POTASSIUM PHOSPHATE BUFFER:

REAGENTS REQUIRED:

- Dipotassium hydrogen phosphate
- Potassium dihydrogen phosphate

PROCEDURE:

174.18 g/mol dipotassium hydrogen phosphate and 136.09 g/mol potassium dihydrogen phosphate was taken and made up to 200ml using distilled water. This gives the potassium buffer.

Standardised pH meter with standard buffer. Washed electrode with distilled water and introduced it into potassium buffer prepared. The pH of the solution is 6.5.

RESULT:

Dipotassium hydrogen phosphate (K2HPO4) and potassium dihydrogen phosphate (KH2PO4) solution were prepared and the pH was measured to be 9.87 and 4.23 respectively, the solution were made using 1N Hcl and 5N NaoH respectively and the pH was found to be 6.5.

Determination of Glucose, Sucrose and Fructose in a specific sample

Application

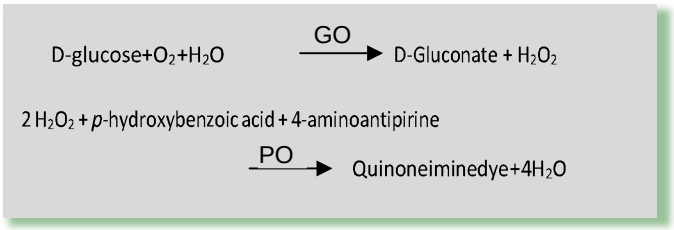
This rapid and simple specific enzymatic method is used for the simultaneous determination of lactose, sucrose and D- glucose in flour mixtures as well as other foodstuffs, pharmaceuticals, cosmetics and biological samples.

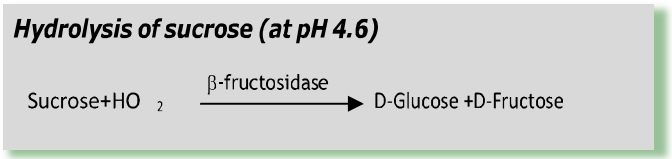
Introduction

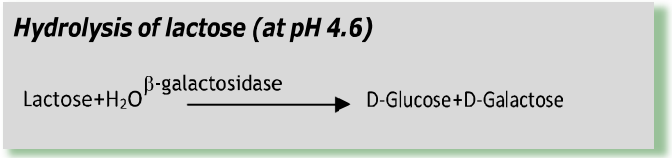
Sucrose and D-glucose occur widely in plant organisms. In foods, they occur mainly in honey, wine and beer, and a range of solid foodstuffs such as bread and pastries, chocolate and candies. Lactose, or milk sugar, is formed in the mammary glands of all lactating animals and is present in milk and milk products.

Sucrose and milk solids (containing lactose) are major constituents of many flour mixtures used in the production of cakes, biscuits, breads and confectionary goods.

Principles







Free D-glucose in the sample is determined directly with GOPOD Reagent by conversion to a red coloured quinoneimine dye compound through the combined action of glucose oxidase and peroxidase. Sucrose is hydrolysed to D-glucose and D-fructose with □-fructosidase, and measured as D-glucose. Lactose is hydrolysed to D-glucose and D-galactose using β-galactosidase (lactase) and measured as D- glucose.

Specificity

Using high purity of glucose oxidase, peroxidase, β- fructosidase and β-galactosidase, this colorimetric method is specific for lactose, sucrose and D-glucose measurement in plant and food extracts, namely in flour mixtures. The colour formed through the reaction is stable at room temperature for at least 2 hours after development.

Linearity and precision

Linearity of the determination exists from 10 to 100 µg D- glucose lactose or sucrose per assay (see Figure 1). Standard errors below 5% are reached routinely.

Kit composition

Solution 1. Buffer (20 mL, pH 4.6). Stable for 2 years at 4 °C.

Dilute the content of bottle 1 to 100 mL with distilled water before use. Stable for > 1 year at 4ºC.

Suspension 2. □-Fructosidase (2.2 mL). Stable for 2 years at 4

°C. Swirl bottle before use.

Add 0.02 ml of Suspension 2 plus 0.180 ml of Solution 1, per assay, to a test tube and homogenise (**Solution 1+2**). This solution should be prepared for each assay day.

Suspension 3. □-Galactosidase (2.2 mL). Stable for 2 years at 4 °C. Swirl bottle before use.

Add 0.02 ml of Suspension 3 plus 0.180 ml of Solution 1, per assay, to a test tube and homogenise (**Solution 1+3**). This solution should be prepared for each assay day.

Solution 4. GOD-POD reagent buffer (30 mL, pH 7.4), *p*- hydroxybenzoic acid and sodium azide (0.64% w/v) as a preservative. Stable for 3 years at 4 °C.

Dilute the contents of the bottle to 1.0 L with distilled water and use immediately.

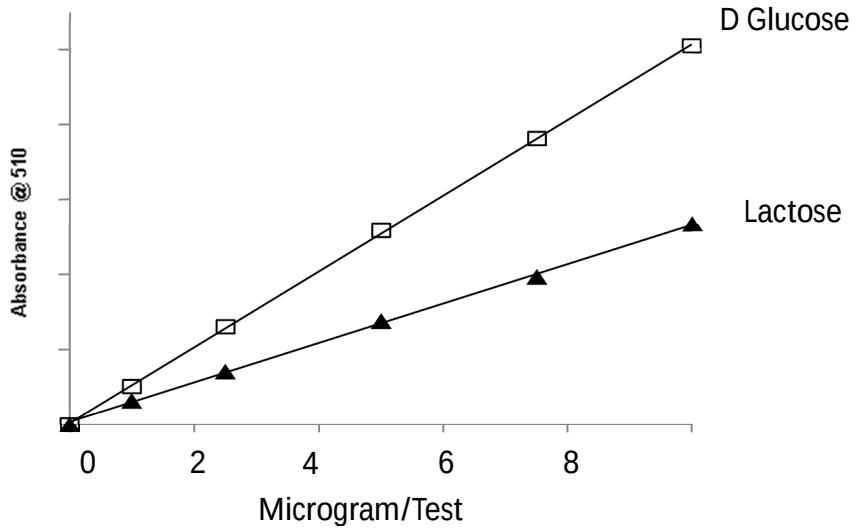
Mixture 5. GOD-POD reagent enzymes. Freeze-dried powder of glucose oxidase (GOD), peroxidase (POD) and 4- aminoantipyrine. Stable for 5 years at -20 °C.

Dissolve the contents of one bottle 5 in approx. 20 mL of solution 4 and quantitatively transfer this to the bottle containing the remainder of solution 4. Cover this bottle with aluminum foil to protect the enclosed reagent from light. Stable for 3 months at 2-5 °C or 12 months at -20 °C.

Solution 6. D-Glucose standard solution (5 mL, 1.0 mg/mL) in 0.2% (w/v) benzoic acid. Stable for 5 years at room temperature. Use as supplied.

Powder 7. Control flour sample. Lactose, sucrose and D- glucose contents shown on vial label.

Use as supplied. Prepare as a sample (see Examples of sample preparation)



Safety

The general safety measures that apply to all chemical substances should be followed.

Precautions and controls

Include reagent blanks and D-glucose controls (quadruplicate) with each set of assays.

Analyse an extract from the control powder with each set of assays.

The time of incubation with GOPOD reagent is not critical, but should be at least 20 min.

However, the time for maximum colour formation with 100 µg of D-glucose standard should be checked, for each new GOPOD reagent.

Procedure (endpoint analysis)

Wavelength: 510 nm

Cuvette: 1 cm light path (glass or plastic)

Temperature: · 50 °C

Final volume: 3.3 mL

Sample solution: 10-100 µg of total lactose, sucrose and D-glucose per cuvette

Read against re

For each sample

Pipette into cuvettes (mL)	**Blank**	**D-Glucose Standard**	**D-Glucose assay (A)**	**Sucrose + D-Glucose assay (B)**	**Lactose + D-Glucose assay (C)**
Solution 1	-	-	0.20	-	-
Solution 1+2*	-	-	-	0.20	-
Solution 1+3*	-	-	-	-	0.20
Sample	-	0.10	0.10	0.10	0.10
H2O	0.30	0.20	-	-	
Mix. Incubate for 20 min at 50 ºC. Then add:					
GOD-POD reagent (3+4)	3.00	3.00	3.00	3.00	3.00

Mixtures can be obtained with a plastic spatula or by gentle inversion after sealing with a cuvette cap. Pipette both Solution 1+2/3 and sample into the bottom of the cuvette and mix by gentle swirling

Calculation

The concentration of D-glucose, sucrose and lactose (g/L) are calculated as follows:

$$[\text{D-Glucose}] = \frac{\Delta A_{\text{Sample}}\,(A)}{\Delta A_{\text{D-Glucose Standard}}} \quad [\text{g/L}]$$

$$[\text{Sucrose}] = \frac{\Delta A_{\text{Sample}}\,(B) - \Delta A_{\text{Sample}}\,(A)}{\Delta A_{\text{D-Glucose Standard}}} \times \frac{342}{180} \quad [\text{g/L}]$$

$$[\text{Lactose}] = \frac{\Delta A_{\text{Sample}}\,(C) - \Delta A_{\text{Sample}}\,(A)}{\Delta A_{\text{D-Glucose Standard}}} \times \frac{342}{180}$$

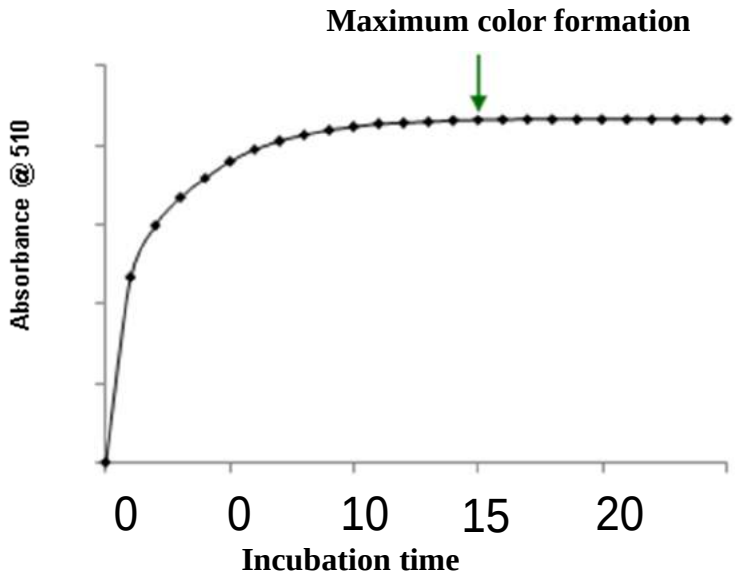
Sucrose and sucrose calculation takes in account the conversion of µg of D-Glucose (as measured) to µg of sucrose and lactose, respectively.

If the sample has been diluted or a different sample volume was used during the reaction, the result must be multiplied by the corresponding dilution/concentration factor.

Interferences

An internal standard should be included during sample analysis if the presence of interfering substances is suspected. A quantitative recovery of this standard should be expected.

With each new batch of GOD-POD Reagent, the time of maximum colour formation with 100 µg of D-Glucose standard should be checked. This is approximately 15 min. (See Figure 2).



General information on sample preparation

The total amount of lactose, sucrose and D-glucose present in the cuvette should range between 10 and 100 µg. Thus, if a sample volume of 0.10 mL is used the sample solution must be diluted to yield a sugar concentration between 100 and 1000 mg/L.

To implement this assay use clear, colourless and practically neutral liquid samples directly, or after dilution; filter turbid solutions; degas samples containing carbon dioxide (*e.g.* by filtration; measure "coloured" samples (against a sample blank; treat "strongly coloured" samples that are used undiluted or with a higher sample volume with PVPP (ad 0.2 g of PVPP/10 mL sample, shake vigorously for 5 min and filter through Whatman nº1 filter paper); crush or homogenize solid or semi-solid samples, extract with water or dissolve; extract samples containing fat with hot water.

Sample dilution buffer (Sodium acetate buffer (50 mM, pH 4.5) Add 2.9 mL of glacial acetic acid (1.05 g/mL) to 900 mL of distilled water. Adjust the pH to 4.5 by careful addition of 1 M (4 g/100 mL) sodium hydroxide solution. Adjust the volume to 1 litre and store the buffer at 4°C. Sodium azide (0.2 g) can be added as a preservative. Stable for > 6 months at 4°C.

Examples of sample preparation

Determination of lactose, sucrose and D-glucose in flour mixtures (Enzyme Inactivation and Sugar Extraction)

Accurately weigh 0.5 g of milled sample into a glass test-tube and add 5 mL of ethanol (95% v/v). Incubate the tube in a water bath at 85-90°C and allow to reflux for 5 min (This inactivates endogenous enzymes).

Quantitatively transfer the tube contents to a 50 mL volumetric flask using Sample dilution buffer from a wash bottle to ensure complete transfer. Adjust to volume (50 mL) with Sample dilution buffer and thoroughly mix the solution. Filter an aliquot of this solution through filter through Whatman nº1 filter paper. Add 1.0 mL of the filtrate to 3 mL of distilled water and mix thoroughly (Sample will be at 2.5 mg solid matter/ml).

Determination of lactose in milk, cream or yogurt

Accurately weigh approx. 1 g of milk, cream, yogurt or condensed milk into a 100 mL volumetric flask, add approx. 60 mL of distilled water, mix and store at 50°C for 15 min with occasional swirling. Add 2 mL of Carrez I solution (3.60 g of potassium hexacyanoferrate (II) in 100 mL of distilled water), and mix. Add 2 mL of Carrez II solution (7.20 g of zinc sulphate in 100 mL of distilled water) and mix. Add 4 mL of 100 mM NaOH solution and mix vigorously. Dilute to volume with distilled water and mix thoroughly. Filter an aliquot of the solution through Whatman No. 1 filter paper. Discard the first few mL of filtrate. Use the clear filtrate (sample solution) in the assay. Typically, for milk, cream and yogurt, no extra dilution is required; for condensed milk, an additional dilution of 1:3 is satisfactory.

Determination of lactose in cheese and chocolate

Add 10 g of grated cheese or 0.5 g of grated chocolate to a 200 mL beaker. Add approx. 60 mL of distilled water and mix on a magnetic stirrer at 50°C for approx. 15 min. Add 2 mL of Carrez I solution (3.60 g of potassium hexacyanoferrate (II) in 100 mL of distilled water) and mix. Add 2 mL of Carrez II solution (7.20 g of zinc sulphate in 100 mL of distilled water) and mix. Add 4 mL of 100 mM NaOH solution and mix vigorously. Quantitatively transfer the solution to a 100 mL volumetric flask and dilute to volume with distilled water. Mix thoroughly and filter an aliquot of the solution through Whatman No.1 filter paper. Discard the first few mL of filtrate. Use the clear filtrate (sample solution) in the assay. Typically, for chocolate and most cheeses, no extra dilution is required, however for some cheeses, a dilution of 1:10 might be necessary.

Potassium test kit is for the quantitative determination of Potassium concentration in human serum.

INTRODUCTION

Potassium is present in all body tissues and is required for normal cell function because of its role in maintaining intracellular fluid volume and transmembrane electrochemical gradients ·

Elevated potassium levels (hyperkalemia) are often associated with renal failure, dehydra- tion shock or adrenal insufficiency.Decreased potassium levels (hypokalemia) are associat- ed with malnutrition, negative nitrogen balance, gastrointestinal fluid losses and hyperactivi- ty of the adrenal cortex.

Principle:

The amount of Potassium is determined by using sodium tetraphenylboron in a specifically prepared mixture to produce a colloidal suspension. The turbidity of which is proportional to potassium concentration in the range of 2-7 mEq/L.

Tetra phenyl Boron + K^+ ⟶ White Turbidity

The extent of turbidity is proportional to the potassium concentration and is measured photometrically at 620 nm (610-620)

Pack Size

Kit Size	**1 x 50 ml**	**25 T**	**50 T**
Cat. No.	**ADX361**	**ADX362**	**ADX363**
Kit Contents			
1) Potassium Reagent	1 x 50 ml	25 x 1 ml	50 x 1 ml
2) Potassium Standard (5 mmol /L)	1 x 2 ml	1 x 2 ml	1 x 2 ml

Storage and Stability

The components of the kit, stored at 2 - 8 ºC, will remain stable until the expiry date stated on the label.

Reagent Preparation:

Ready to use reagents.

Sample and specimen storage

Serum is the preferred specimen.

Do not use lipemic / turbid/ icteric samples.

Warning and precautions

1. For in vitro diagnostic use.
2. Specimens should be considered infectious and handled appropriately.
3. Avoid ingestion. DO NOT PIPETTE BY MOUTH.
4. The reagent contains sodium hydroxide that is corrosive. In case of contact with skin, flush with water. For eyes, seek medical attention.
5. The disposal of the residues has to be done as per local legal regulations

Materials required but not provided

1. Pipettes to accurately measure required volumes.
2. Test tubes/rack
3. Timer
4. 37 °C heating block or water bath
5. Photometer capable of accurately measuring absorbance at 620 nm

Test Procedure

Primary wavelength 620 nm

Temperature 37 °C

Prewarm the Reagent to reaction temperature

	Blank (ml)	Standard (ml)	Sample (ml)
Potassium Reagent	1.000	1.000	1.000
Potassium Standard	--	0.050	--
Sample	--	--	0.050

Mix well and incubate for 5 min at room temperature. After incubation, zero the Photometer with the reagent blank. Read and record the incubated Standard and samples.

$$\text{Calculation: } \frac{Sample\ OD}{Standard\ OD} \times 5 = \text{mmol Potassium / L}$$

Note: All glassware and cuvettes should be washed with quality distilled water before use.

Ouality Controls

Control Sera are recommended to monitor the performance of manual and automated assay procedures. Each laboratory should establish its own Quality Control scheme and corrective actions if controls do not meet the acceptable tolerances.

Normal values

Serum, plasma: 3.4 – 5.3 mmol/L

It is strongly recommended that each laboratory establish its own normal range

Automated Procedure

Appropriate program sheet is available for different analyzers upon request.

Calibration

The procedures are calibrated with the standard solution which is included with each series of tests. The absorbance is used to calculate the results.

Limitations of Test

Linearity : 8.5 mmol/L Potassium.

Sensitivity: 0.140 mmol/L Potassium.

Samples that have Potassium values greater than 8.5 mmol/L should be diluted with saline water (NaCl 0.9 %) 1:1, re-assayed and the results multiplied by 2.

Interferences

Turbid or icteric samples produce falsely elevated results. Bilirubin above 40 mg/dl and Urea Nitrogen above 80 mg/dl will produce elevated results. Hemolyzed sera produce elevated results. Sera containing high levels of ammonia should be avoided.

System Parameters

Mode	:	End point
Std. Conc.	:	5
Wave length	:	620 nm
Units	:	mmol/L
Flow cell Temp.	:	37 °C
Blank	:	Reagent
Reagent volume	:	1000 µL
Sample volume	:	50 µL
Incubation	:	5 min. at R.T
Reaction Direction	:	Increasing
Low Normal	:	3.4
High Normal	:	5.3

Quantitative, Colorimetric Determination of Sodium in Serum, Plasma or Urine

Summary and Principle

Prior to flame photometry and ion-selective electrodes, the most popular method of determining sodium in body fluids involved its precipitation as the triple salt, sodium uranyl zinc acetate. This technique was introduced by Kolthoff in 1927, with subsequent utilization of the precipitate in several ways. One approach was the colorimetric measurement of the solubilized residue itself, either directly, as reported by Albanese and Lein, or by monitoring the color fade of the yellow supernate after precipitation, as described by Bradbury. The method presented is essentially an adaptation of the latter scheme, 3 wherein sodium is precipitated from a protein-free supernate as the triple salt. The resulting decrease in absorbance of the supernate-color reagent mixture is proportional to sodium content of the specimen.

Reagents

Sodium Color Reagent

Solution of uranyl acetate, 5.3 g/dL, and zinc acetate, 15.4 g/dL, in aqueous acetic acid-ethanol mixture.

Precipitating Reagent

Aqueous solution of trichloroacetic acid (TCA), 10 g/dL.

Sodium Standard - (140 mmol/L),

Sodium chloride, 4.091 g/L, in aqueous TCA. Equivalent to sodium value of 140 mmol/L when used as directed in method presented.

Precautions:

For In Vitro Diagnostic Use. Use care in handling Precipitating Reagent and Sodium Standard since they are mildly caustic.

Materials Required

Spectrophotometer capable of absorbance readings at 420 nm Centrifuge with high speed capacity (>1500 rpm) Pipets capable of accurately delivering 0.5 and 2.5 mL Test tubes & Cuvets Vortex mixer (optional) Interval timer

Specimen Collection and Preparation

Serum: Remove from clot promptly and carefully to prevent hemolysis.

Plasma: Use lithium heparinate, ammonium heparinate, or lithium oxalate as anticoagulant.

Urine: Dilute portion of a well-mixed and measured 24-hour collection 1:10 (1+9) with distilled water. Depending on sodium content, a dilution of 1:5 (1+4) or 1:2 (1+1) may be required.

Sample Stability:

Sodium levels remain stable for at least 14 days at 15-25^{o}C.

Interfering Substances:

Contaminated glassware is the greatest source of error. All glassware should be washed with 10-20% nitric acid, rinsed thoroughly with distilled water, dried and stored in dust free area.

Procedure

Preparation of Protein-Free Supernate

1. To properly labeled test tubes, add 0.5 mL serum plasma or diluted urine. (Do not use Standard in this step!)

2. Add 0.5 mL Precipitating Reagent dropwise to each tube with vigorous mixing (vortexing suggested). 3. Allow to stand for 5 minutes, then centrifuge at high speed for 5-10minutes.

Test Procedure

1. Pipet into marked tubes the following volumes (mL), mixing promptly after each addition of the color reagent: REAGENT BLANK (RB) STANDARD (S) SAMPLE (U) Distilled Water 0.5 Standard 0.5Supernate 0.5 Color Reagent .

	Reagent Blank (RB)	Standard (S)	Sample (U)
Distilled water	0.5		
Standard		0.5	
Supernate			0.5
Color reagent	2.5	2.5	2.5

2. Again re-mix contents of all tubes.
3. Incubate tubes for 10 minutes at room temperature (15-30o C).
4. After incubation period, mix thoroughly and centrifuge at high speed for 5 minutes.
5. Carefully transfer supernate of each tube to appropriate cuvet.
6. With spectrophotometer set at 420 nm, zero the instrument with water. Read and record the absorbance of the Reagent Blank (RB), Standard (S) and Unknowns (U) within 30 minutes.

Quality Control:

Control sera and/or urines, assayed for sodium content by this method, flame photometry or ion-selective electrode methods, should be included with each set of unknowns. Stanbio SerT-Fy I, Normal Control, Cat. No. G427-86 and Stanbio Ser-T-Fy II, Abnormal Control, Cat. No. G428-86 are recommended for each test run.

Results

Values are derived by the following calculation:

$$\text{Serum, Plasma or Urine Sodium (mmol/L)} = \frac{\text{Abs (RB)} - \text{Abs (U)} \times 140}{\text{Abs (RB)} - \text{Abs (S)}}$$

Where Abs (RB), Abs (U) and Abs (S) represent the absorbance of the Reagent Blank, Unknowns, and Standard, respectively, and 140 the equivalent value of the sodium standard in mmol/L.

Example: A serum sample assayed by the method described gave an absorbance reading of 0.936, with the Reagent Blank reading 1.385 and the standard reading 0.967. Therefore:

Sodium mmol/Lit = 1.385 - 0.967/1.385 - 0.936 x 140 = 150

NOTE: Urine values must be multiplied by the appropriate dilution factor.

$$\text{Urine Sodium (mmol/24h)} = \frac{\text{Urine Sodium (mmol/L)} \times \text{24h volume (mL)}}{1000}$$

Precision: Multiple assays (n=20) on a serum pool (mean = 100.7 mmol/L) over an 11 day period revealed a standard deviation (SD) of 1.5 mmol/L and a coefficient of variation (CV) of 1.4%. Correlation: Each of six serum pools were assayed for sodium by flame photometry (range: 126-149 mmol/L). Replicate analyses (n = 6) using the method presented revealed an average deviation from the reference method of - 1.5 mmol/L.

Linearity: When performed as directed, the method is linear from 0 - 160 mmol/L.