POST GRADUATE AND RESEARCH
DEPARTMENT OF ZOOLOGY
SREE NARAYANA COLLEGE, KOLLAM

LABORATORY MANUAL

IN ZOOLOGY CBCSS
UNIVERSITY OF KERALA
VOLUME I

POST GRADUATE AND RESEARCH
DEPARTMENT OF ZOOLOGY
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Funded by
DBT Star College Scheme

ISBN : 978-81-965347-2-1

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1. Saponification Value of Oil or Fat

Aim: To determine the saponification value of a given oil or fat sample.

Principle: The saponification value is the number of milligrams of potassium hydroxide (KOH) required to saponify 1 gram of oil or fat. It is an important parameter indicating the average molecular weight of the fatty acids present. The use of a solvent mixture (ethanol-ether) ensures the complete dissolution of the fat or oil, allowing for efficient saponification.

Materials Required:

1. Oil or fat sample
2. Ethanol-ether mixture (1:1 by volume)
3. Potassium hydroxide (KOH) solution (0.5 N in ethanol)
4. Phenolphthalein indicator
5. Hydrochloric acid (HCl) solution (0.5 N)
6. Distilled water
7. Conical flask, burette, pipette
8. Reflux condenser
9. Hot plate
10. Weighing balance

Procedure:

1. Weigh accurately about 1 g of the oil or fat sample and transfer it into a 250 ml conical flask.
2. Add 10 ml of the ethanol-ether mixture (1:1) to dissolve the oil or fat sample completely.
3. Add 25 ml of 0.5 N alcoholic KOH solution to the flask.
4. Attach a reflux condenser and heat the mixture on a hot plate for 30 minutes, ensuring complete saponification.
5. Allow the flask to cool, then add a few drops of phenolphthalein indicator.
6. Titrate the mixture with 0.5 N HCl until the pink color disappears.
7. Conduct a blank titration following the same procedure without the oil or fat sample.

Result:

The saponification value of the given oil or fat sample is ……………………………..
,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,, mg KOH/g of oil or fat.

2. Estimation of DNA From Tissue Extract Using Diphenylamine (DPA) Method

To estimate the amount of DNA present in a tissue extract using the Diphenylamine (DPA) method**,** which is based on the reaction between deoxyribose sugar in DNA and diphenylamine under acidic conditions, forming a blue-colored complex that can be measured spectrophotometrically at 595 nm**.**

Principle:

- The DPA method is a colorimetric assay used for the quantitative estimation of deoxyribonucleic acid (DNA).
- Under acidic conditions, deoxyribose in DNA undergoes hydrolysis to form hydroxymethylfurfural, which reacts with diphenylamine reagent to produce a blue-colored complex.
- The intensity of the blue color is proportional to the DNA concentration and is measured using a spectrophotometer at 595 nm.
- A standard curve is prepared using known concentrations of calf thymus DNA, and the unknown DNA concentration is determined by extrapolation.

Materials Required:

Reagents:

1. Tissue sample (e.g., liver, kidney, or plant leaf)
2. Lysis buffer (Tris-HCl, EDTA, NaCl, SDS)
3. Chloroform: Isoamyl alcohol (24:1)
4. Cold ethanol (for DNA precipitation)
5. Diphenylamine reagent:
6. Calf thymus DNA standard solution (100 µg/ml)

Equipment:

- UV-Vis spectrophotometer
- Centrifuge
- Water bath
- Test tubes
- Micropipettes
- Cuvettes

Procedure:

Step 1: DNA Extraction from Tissue Sample

1. Take 100 mg of fresh tissue and homogenize it in 1 ml of lysis buffer using a mortar and pestle.
2. Incubate the homogenized mixture at 55°C for 30 minutes to allow complete lysis.
3. Add an equal volume of chloroform: isoamyl alcohol (24:1) and mix well by gentle inversion.
4. Centrifuge the mixture at 10000 rpm for 10 minutes at 4°C.
5. Transfer the upper aqueous phase to a fresh tube without disturbing the interphase.
6. Add two volumes of cold ethanol to precipitate the DNA.
7. Incubate at -20°C for 30 minutes and then centrifuge at 12000 rpm for 10 minutes.
8. Discard the supernatant and wash the pellet with 70% ethanol.
9. Air-dry the pellet and dissolve the extracted DNA in TE buffer or distilled water for further estimation.

Step 2: DNA Estimation using Diphenylamine (DPA) Method

1. Prepare a series of standard DNA solutions (0, 20, 40, 60, 80, and 100 µg/ml) from the calf thymus DNA stock solution**.**
2. Take **0.5 ml** of each standard and the unknown DNA sample in separate test tubes.
3. Add 2 ml of diphenylamine reagent to each tube.
4. Mix well and incubate the tubes in a boiling water bath (100°C) for 10 minutes.
5. Allow the tubes to cool to room temperature.
6. Measure the absorbance at 595 nm using a UV-Vis spectrophotometer.
7. Plot a standard curve of absorbance vs. DNA concentration.
8. Determine the DNA concentration in the unknown sample by comparing its absorbance with the standard curve.

Results:

1. The DNA concentration of the given tissue extract was determined to be ____ µg/ml, based on the standard calibration curve.
2. The intensity of the blue color obtained confirmed the presence of DNA.

3. Quantitative Estimation of Protein by Lowry Method

Aim:

To estimate the amount of protein present in the given sample by Lowry method.

Principle:

The principle of this method is based on two reactions leading to color complex formation. First, the Biuret reaction, in which Cu^{2+} from the reaction mixture reacts with the peptide bonds of proteins under alkaline conditions, results in their reduction to cuprous ions (Cu^{+}). Second, Lowry's reaction involves the Folin-Ciocalteu reagent. The phenolic groups of the amino acid residues (tyrosine and tryptophan) will produce a blue-purple color due to the reduction of phosphomolybdotungstate to heteropolymolybdenum blue by the copper-catalyzed oxidation of the amino acids. The intensity of this color depends on the amount of these aromatic amino acids present. Thus, the blue-purple color formed will differ from protein to protein.

Protein (Peptide bonds) + Cu^{2} ----------Protein-Cu1+ Complex (1)

Protein-Cu1+ Complex + Folin-Ciocalteu……>Blue colour (2)

Reagents:

Lowry Reagent, Folin-Ciocalteau reagent, BSÁ

Procedure:

Seven test tubes are taken and labelled as 1,2,3,4,5,6,7. 1 ml of distilled water is pipetted out to the test tube labelled as blank. Pipette out 0.2, 0.4, 0.6, 0.8, and 1.0 ml of the protein solution into a series of tubes. Make up the total volume to 1 ml with the addition of water. To each tube, pipette out 5 ml of the alkaline-copper sulfate solution, mix well, and allow it to stand at room temperature for 10 to 15 minutes Pipette out 0.5 ml of the Folin-Ciocalteau reagent into each tube, mixing rapidly after each addition. Leave the tubes for 30 minutes, and measure the blue color formed at 700 nm (or a red filter). Prepare a blank with 1 ml of distilled water instead of the protein solution and with 1 ml of the unknown solution, and proceed as per standards. Prepare a calibration curve with mg of protein on the X-axis and O.D. on the Y-axis. Prepare a standard curve by plotting the absorbance values of the standard glucose solutions (y-axis) against the concentration of glucose (x-axis). Calculate the concentration of the unknown protein sample using the standard curve equation:

Concentration of unknown = (Absorbance of unknown (y)-intercept)/slope.

The y-intercept and slope values can be obtained from the standard curve equation. The result should be expressed in mg/mL or µg/mL, depending on the initial sample volume and dilution factor.

Result:

Amount of protein present in the given sample=……. µg/ml

Amount of protein present in the given sample (Graph) =…….µg/ml

Amount of protein present in the given sample (Standard Curve) =…….µg/ml

Observation and calculation

Calculations:

Volume of test = ….ml

OD of test=

Amount of protein present in test solution=

$$\frac{\text{Concentration of standard x OD of test}}{\text{OD of standard X Volume of test}}$$

= …….µg/ml

4.Quantitative Estimation of Glucose by Anthrone Method

Aim:

To estimate the amount of glucose present in the given sample by Anthrone method.

Principle:

Carbohydrates are dehydrated by conc. H_2SO_4 to form furfural. The active form of the reagent is anthranol, the enol tautomer of anthrone, which reacts by condensing with the carbohydrate furfural derivative to give a green colour in dilute solutions and a blue colour in concentrated solutions, which is determined colorimetrically. The blue-green solution shows an absorption maximum of 620 nm.

(i) Hydrolysis to monosaccharides

Disaccharide → Monosaccharide

(ii) Dehydration—product is a furfural

Monosaccharide → Furfural

(iii) Reaction of furfural with Anthrone

Furfural + Anthrone reagent → Blue-green complex.

Reagents

Anthrone reagent, Con H_2SO_4, Glucose solution

Procedure:

Seven test tubes are taken and labelled as 1,2,3,4,5,6,7. 1 ml of distilled water is pipetted out to the test tube labelled as blank. Pipette out 0.2, 0.4, 0.6, 0.8, and 1.0 ml of the glucose solution from the stock solution into a series of tubes. 0.5 ml of unknown sample is taken as test solution. Then make up the total volume to 1 ml with the addition of distilled water. Add 4 ml of the anthrone reagent to each tube and mix well. Cool the tubes. Cover the tubes with caps on top and incubate at 80°C in boiling water bath for 10 minutes. Cool the tubes to room temperature and measure the optical density of the solutions at 620 nm against a blank. A graph is plotted by taking concentration of standards along X axis and their corresponding OD value along Y axis. The

concentration of the unknown sample is read directly from the graph. Prepare a standard curve by plotting the absorbance values of the standard glucose solutions (y-axis) against the concentration of glucose (x-axis). Calculate the concentration of the unknown carbohydrate sample using the standard curve equation:

Concentration of unknown = (Absorbance of unknown (y)-intercept)/slope.

The y-intercept and slope values can be obtained from the standard curve equation. The result should be expressed in mg/mL or µg/mL, depending on the initial sample volume and dilution factor.

Result

Amount of glucose present in the given sample=…….µg/ml

Amount of glucose present in the given sample (Graph) =…….µg/ml

Amount of glucose present in the given sample (Standard Curve) =…….µg/ml

5. Quantitative Estimation of Cholesterol

Aim:

To estimate the amount of cholesterol present in the given sample by Zak method.

Principle:

Serum proteins are precipitated using a ferric chloride-acetic acid reagent. The resulting protein-free filtrate, containing cholesterol, is then treated with concentrated sulfuric acid. The 3-Hydroxy -5 ene part of Cholestrol is dehydrated to form Cholestrol-3,5-diene in the presence of glacial acetic acid. This compound is then oxidised to form Bis cholesta 3,5-diene. Upon sulphonation by H_2SO_4 it gives Cholestra diene disulphonate which is pinkish red colour. The intensity of the pinkish red color is measured using a spectrophotometer, usually at a wavelength of 570 nm. The measured absorbance is then compared to a standard curve to determine the cholesterol concentration in the original sample

Cholestrol + Dehydration ---------- Cholesta -3-5 diene

Cholesta -3-5 diene + Oxidation ---------- Bischolesta -3-5 Diene

Bischolesta -3-5 Diene-+ Sulphonation --------- Cholestra diene disulphonate (Pinkish red Colour)

Reagents

Ferric chloride, Glacial acetic acid, Cholesterol powder

Procedure:

Seven test tubes are taken and labelled as 1,2,3,4,5,6,7. 1 ml of distilled water is pipetted out to the test tube labelled as blank. Pipette out 0.2, 0.4, 0.6, 0.8, and 1.0 ml of the cholesterol solution into a series of tubes. Make up the total volume to 1 ml with the addition of glacial acetic acid. To each tube, pipette out 3 ml of the ferric chloride solution, mix well, and allow it to stand at room temperature for 5 minutes Pipette out 2 ml of the H_2SO_4 into each tube, mixing rapidly after each addition. Leave the tubes for 20 minutes, and measure the pinkish red formed at 570nm (or a red filter). Prepare a blank with 1 ml of glacial acetic acid instead of the Cholesterol solution and with 1 ml of the unknown solution, and proceed as per standards. Prepare a calibration curve with µg of cholesterol on the X-axis and O.D. on the Y-axis. Prepare a standard curve by plotting the

absorbance values of the standard cholesterol solutions (y-axis) against the concentration of cholesterol (x-axis). Calculate the concentration of the unknown cholesterol sample using the standard curve equation:

Concentration of unknown = (Absorbance of unknown (y)-intercept)/slope.

The y-intercept and slope values can be obtained from the standard curve equation. The result should be expressed in mg/mL or µg/mL, depending on the initial sample volume and dilution factor.

Result

1. Amount of cholesterol present in the given sample=……. µg/ml

2. Amount of cholesterol present in the given sample (Graph) =……. µg/ml

3. Amount of cholesterol present in the given sample (Standard Curve) =…….

6. Test For Abnormal Constituents of Urine (Glucose and Albumin)

Biuret Test

Aim:

To detect the presence of albumin in the given sample of urine

Principle:

The reaction in the biuret test is a colorimetric reaction where the result is indicated by a colour change from blue to purple or violet. In an alkaline environment, the cupric (Cu^{+2}) ions in the biuret reagent bind to the nitrogen atoms in the peptide bonds of proteins forming a violet-coloured copper coordination complex. The formation of purple color indicates the presence of peptide bonds in the sample. The intensity of the developed purple color is directly proportional to the concentration of peptide bonds present in the solution.

Materials Required:

Alkaline copper sulphate (0.5 % $CuSO_4$) solution, test tubes, dropper, test tube stand.

Procedure:

In a test tube, take 2 ml of the sample and add equal volume of Biuret reagent (0.5 % $CuSO_4$). Shake well and let it stand at room temperature for 5 minutes. Observe the tubes for the development of violet colour in the suspension.

Observation and Conclusion

Formation of purple colour after the addition of Biuret reagent. (tube with albumin solution will turn purple.) indicates the presence of albumin in the sample.

Nitric acid ring test (Heller's Test)

Aim:

To detect the presence of albumin in the given sample.

Principle:

Nitric acid causes the precipitation of albumin.

Materials Required:

Test tubes, dropper, concentrated nitric acid.

Procedure:

Take 5 ml of concentrated nitric acid in a test tube and incline the tube to add the sample, so that the latter flows down slowly along the side of the test tube to form a separate layer. A white ring develops at the junction of the two liquids which indicates the presence of albumin in the urine sample.

Observation and conclusion

The given sample appears as a cloudy turbid solution or whitish which indicates that albumin is present in the sample.

Fehlings Test

Aim:

To detect the presence of glucose in the given sample.

Principle:

Fehling's test is based on the principle that the aldehyde functional group of sugar gets oxidised by the complexed copper ions to form acid. Then copper (I) oxide then precipitates as red precipitates which is the indicator of redox reaction.

Materials Required:

Fehling's solution A & B, test tubes, dropper, spirit lamp.

Procedure:

Take 2 ml of a given sample in a clean and dry test tube. Add equal volume of Fehling's reagent A & B. Heat the test tube over a spirit lamp for almost 2 minutes with gentle shaking. The colour change is observed in the test tube.

Observation and conclusion

A brick red precipitate appears, indicating the presence of glucose in the sample.

Benedict's Test

Aim:

To detect the presence of glucose in the given sample.

Principle:

When Benedict's solution and simple carbohydrates are heated, the solution changes to orange red/ brick red. This reaction is caused by the reducing property of simple carbohydrates. The copper (II) ions in the Benedict's solution are reduced to Copper (I) ions, which causes the color change.

Materials Required:

Benedict's reagent, test tubes, dropper, spirit lamp.

Procedure:

Take 2 ml of the given sample in a test tube, using a dropper, take a small quantity of Benedict's reagent. Heat the sample over a burner for 2 minutes holding the test tube firmly with a test tube holder. Keep shaking the test tube as it is being heated. The colour change is observed in the test tube.

Observation and Conclusion

A brick red precipitate appears, indicating the presence of glucose.

7.Measurement of Microorganisms Under the Microscope Using Micrometry Technique

Aim:

To measure the size of microorganisms using a micrometer.

Requirements:

Ocular micrometer, stage micrometer, compound microscope, and specimen.

Introduction:

Micrometry is the measurement of microscopic objects (microorganisms). Since microorganisms can be seen only under a microscope, a suitable scale for their measurements should be somewhere in the microscope itself. For this, an ocular micrometer (eye piece micrometer) and a stage micrometer are used.

An ocular micrometer is simply a disc of glass upon which etched lines. There are usually 100 equally spaced divisions, marked 0 to 100, upon an ocular micrometer. However, the scale on the ocular micrometer does not have any standard value.

A stage micrometer is simply a microscope glass slide having in its centre a known (one millimeter) distance etched into 100 equally spaced divisions. Thus, each division of the stage micrometer equals 0.01 mm or 10 μm.

Principle:

The Ocular micrometer is calibrated under different objective lens systems of the microscope by superimposing the graduations of the stage micrometer. By determining how many divisions of the ocular micrometer superimpose a known distance on the stage micrometer, we may find out the exact value of one division of the ocular micrometer in the microscope field. Once calibrated, the ocular micrometer can be used to measure the size of various microbes in terms of length, breadth, and diameter.

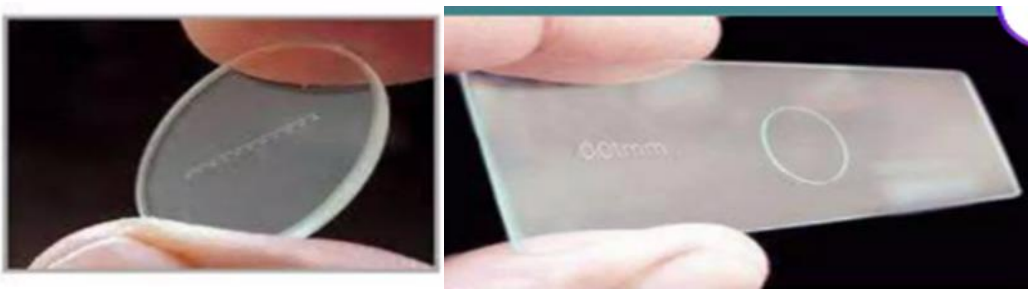
Procedure:

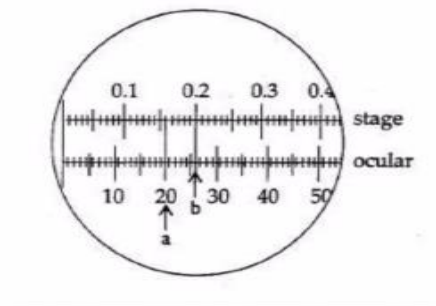
1. Replace the regular ocular lens with an ocular eye piece lens with an ocular micrometer and observe. The scale lines of the ocular micrometer are in sharp focus. These lines and distances will remain unchanged under different objectives.

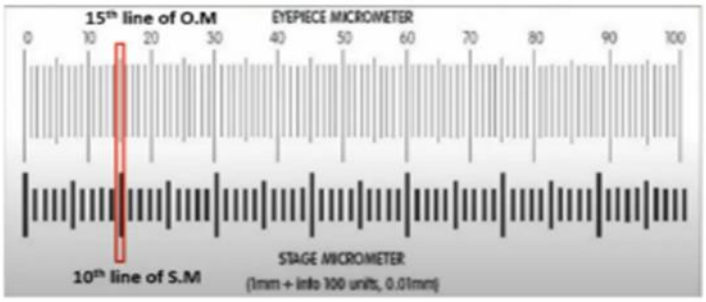
2. Mount the stage micrometer on the microscope stage and bring its scale to the center of the microscope field under a sharp focus. This is done first with a low-power objective and thereafter also with high-power objectives.

3. Adjust the scales of the ocular micrometer and stage micrometer in such a way that the lines of the former superimpose upon those of the latter. If necessary, the ocular micrometer may be rotated or the stage micrometer may be moved on the stage so that lines of the ocular micrometer superimpose upon the stage micrometer. The scales of both micrometers are to be adjusted in such a way that the lines of the two coincide at one end of the microscope field. Now count the spaces of each micrometer to a point where the lines of the two micrometers coincide again. In this way, we can find out how many divisions of ocular micrometer (unknown scale) are equal to how many divisions of stage micrometer (known scale).

4. Replace the stage micrometer with the slide bearing the microorganisms whose measurements are to be taken.







$$\text{One division of ocular micrometer (C)} = \frac{\text{No. of dividions of stage micrometer (A)}}{\text{No. of divisions of ocular micrometer (B)}} \times 100$$

= ------- µm

Calibration of ocular micrometer for 10 x objective

Trial No.	A	B	C $\left(C = \frac{A}{B} \times 10\right)$
1.			C_1 =
2.			C_2 =
3.			C_3 =
		Average (C)=	

One division of ocular micrometer (C) =-------- µm.

For calibration of 40/65/100 x eye pieces follow the above procedure.
Dimension of the given specimen = Length (L) x Breadth (B) µm.

Trial No.	L	B	Dimension (L x B)
1.			D_1 =
2.			D_2 =
3.			D_3 =
		Average (D)=	

Average size of the given specimen (D) = ------ µm.

8.Study of Mitosis in Onion (*Allium cepa*) Root Tip Cells

Aim

1. To observe and identify the different stages of mitosis in onion root tip cells.

2. To estimate the relative duration of each stage of mitosis.

Principle

Somatic growth requires an increase in the number of cells. This occurs through mitosis, a type of equational cell division that produces two genetically identical daughter cells with the same chromosome number as the parent cell.

- Plants show active mitosis in meristematic tissues (root and shoot apices).

- Onion (*Allium cepa*) root tips are ideal because they are easy to grow and have large chromosomes, making the mitotic stages distinct and easily visible under a compound microscope.

Introduction

The term mitosis (Greek mitos = thread) was introduced by Walter Flemming (1882).

Mitosis proceeds through four main stages:

1. Prophase (including prometaphase)

2. Metaphase

3. Anaphase

4. Telophase, followed by cytokinesis.

Mitosis ensures tissue growth, repair, and replacement of worn-out cells.

Requirements

- Freshly sprouted onion bulbs with young root tips.

Equipment

- Compound microscope
- Glass jar or beaker
- Spirit lamp or hot water bath (60 °C)
- Clean glass slides
- Cover slips
- Razor blade
- Blotting paper
- Forceps and dropper

Chemicals & Reagents

- Fixative: Aceto-alcohol (1:3 mixture of glacial acetic acid and 95 % ethanol)
- Stain: Acetocarmine (1 % carmine in 45 % acetic acid) or Aceto-orcein (1 % orcein in 45 % acetic acid)
- Hydrochloric acid: 1 N and 0.1 N (for maceration)
- Distilled water
- 70 % ethanol (for storage)

Procedure

For growing onion root: Take freshly grown onion root (dried or old roots are not good for the experiment). For this, take an onion bulb, carefully remove dried roots, and place it in a glass jar filled with water for 3 to 6 days to grow. Cut 1 cm long freshly grown roots and transfer them to freshly prepared aceto-alcohol fixative. Keep it for 24 hrs. or transfer root tips to 70% ethanol for use (root tip is preserved).

Preparation of root tips: Soften the root tips so that they are easily spread on the slide. ─ For this, fill the tube about 2/3 full with 1N HCl. ─ Place the tube in a 60 °C water bath, and incubate for 12 minutes. ─ Remove the tube from the water bath. o Rinse the root tips in water 3 times

Preparation of slide:

o Take rinsed root tips (1 or 2) on a clean slide.

o Place one drop of 1/1 N HCl on the root tip, followed by 2-3 drops of acetocarmine or aceto-orecein stain on it.

o Leave the slides for 12 minutes.

o Blot the excess stain carefully using blotting paper and rinse the root tips thrice.

o Cut 2-3 mm tip portion of the root tip using a razor blade and discard the rest.

o Put a drop of water and mount a cover slip carefully by avoiding any air

bubbles.

o Squash with thumb using slight pressure.

o Observe the slide under the compound microscope.

Observation

Under the microscope, the following stages are visible:

Interphase - Nucleus distinct; chromatin appears as a dense granular network; nucleolus visible; nuclear membrane intact.

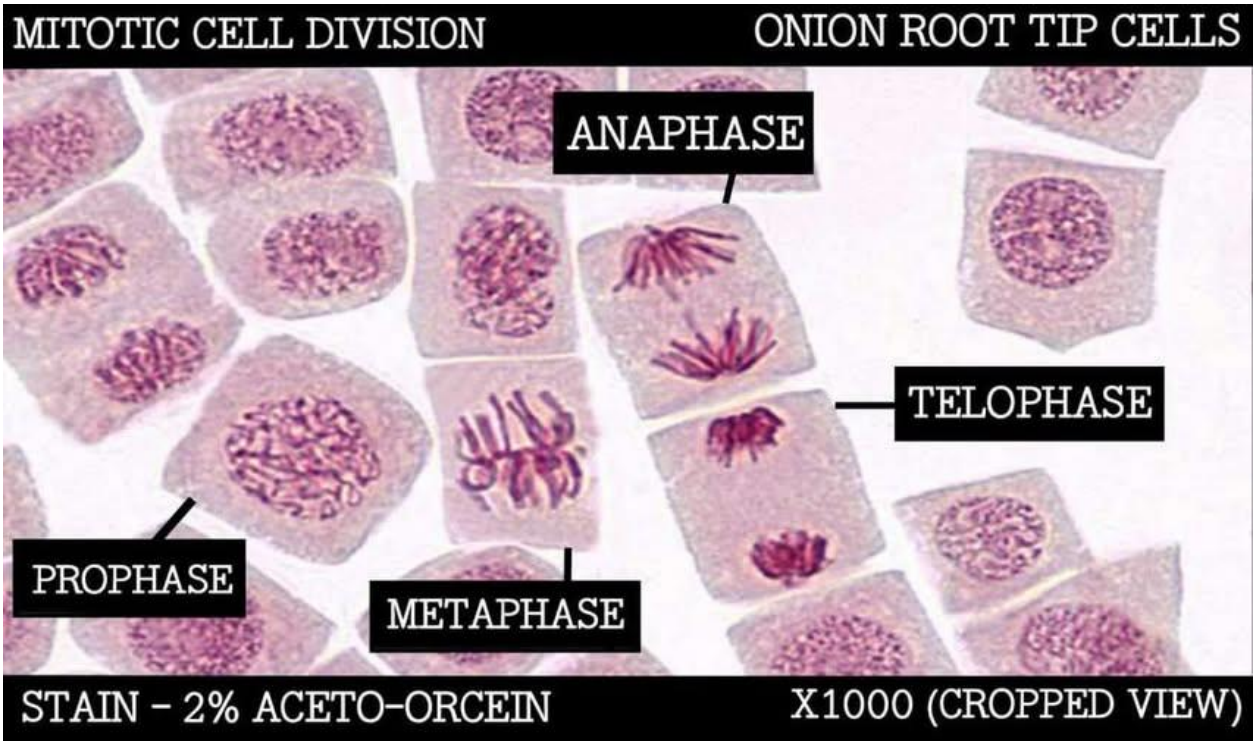
Prophase - Chromosomes condense into fine threads; nucleolus disappears; nuclear membrane still visible initially.

Metaphase - Chromosomes thick, distinct, and aligned on the equatorial plate; nuclear membrane absent; sister chromatids clearly attached at centromeres.

Anaphase- Sister chromatids separate and migrate to opposite poles; appear V-, J-, or I-shaped depending on centromere position.

Telophase - Chromosomes reach poles and decondense; nuclear membrane and nucleolus reappear forming two daughter nuclei.

Cytokinesis - Cell plate forms at the equator, expanding outward to divide the cytoplasm into two identical daughter cells.


MITOTIC CELL DIVISION
ONION ROOT TIP CELLS
ANAPHASE
TELOPHASE
PROPHASE
METAPHASE
STAIN – 2% ACETO-ORCEIN
X1000 (CROPPED VIEW)

9. Study of Meiosis in Grasshopper (*Orthoptera*) Testis Using the Squash Technique

Aim

To prepare a squash preparation of chromosomes from the testis of a grasshopper and to observe, identify, and study the various stages of Meiosis I and Meiosis II under a compound microscope.

Introduction

Meiosis is a specialized type of cell division in which the chromosome number is reduced to half, producing haploid gametes (sperm or egg) from diploid germ cells.

- The process occurs in two successive divisions:
    - Meiosis I (Reductional Division): Homologous chromosomes pair (synapsis), exchange segments (crossing over), and separate, reducing the chromosome number by half.
    - Meiosis II (Equational Division): Sister chromatids separate without further reduction, similar to mitosis.
- Grasshoppers are ideal for studying meiosis because their testes contain numerous primary spermatocytes and their chromosomes are relatively large and few, making the stages easy to observe.

Principle

The squash technique is used to spread cells from the grasshopper testis on a slide. Fixation preserves chromosome structure, and acetocarmine or aceto-orcein stain highlights chromatin, enabling the observation of different stages of meiosis under a compound microscope.

Materials Required

Specimens & Equipment	Reagents
Live grasshopper	Chloroform (for anesthesia)
Dissecting tray & dissection box	70 % ethanol
Compound microscope	Glacial acetic acid

Specimens & Equipment	Reagents
Spirit lamp / gentle heat source	Aceto-alcohol fixative (1:3 glacial acetic acid: ethanol)
Petri dishes, forceps, scalpel, needles	1 % Acetocarmine stain (or 1 % Aceto-orcein stain)
Clean slides & cover slips	0.1 N HCl
Blotting paper, glass rod	Distilled water

Preparation of Stains

- **Acetocarmine:** 1 % carmine in 45 % glacial acetic acid. Dissolve 10 g carmine in 1 L of 45 % glacial acetic acid, reflux for 24 h, filter, and store in a dark bottle at 4 °C.
- **Aceto-orcein:** Pour 55 mL of boiling 45 % glacial acetic acid over 1 g orcein powder, cool, add distilled water to 100 mL, filter, and store.

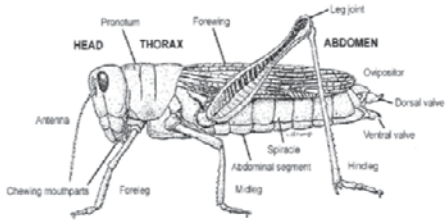
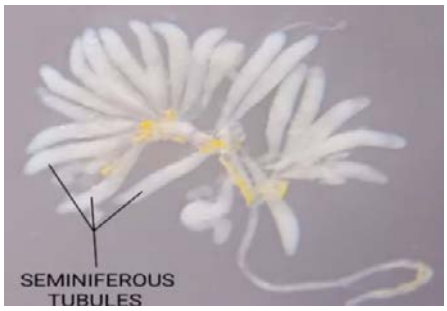


Fig. Morphology of Grasshopper



Procedure

1. **Anesthetize the Sspecimen**
    - Place a live grasshopper in a small container with a few drops of chloroform until it is immobilized.

2. **Dissection**
    - Place the insect dorsal side up in a dissecting tray with a few drops of saline.
    - Make a longitudinal cut along the dorsal abdominal wall.
    - Locate the testes—orange-yellow, lobed structures in the anterior half of the abdomen—beneath fatty tissue.
3. **Fixation**
    - Gently remove the testes and transfer them to aceto-alcohol fixative (1:3) for about 5 minutes.
4. **Staining**
    - Place a drop of acetocarmine or aceto-orcein stain in the center of a clean slide.
    - With a scalpel or needle, cut the testis lobes into tiny pieces in the stain. Leave for 10 minutes to allow penetration.
5. **Squash Preparation**
    - Remove 3–4-stained follicles, blot excess liquid with blotting paper.
    - Add a fresh drop of stain if needed, place a cover slip, and gently tap with the flat end of a glass rod to spread the cells.
6. **Gentle Heating**
    - Warm the slide briefly over a spirit lamp or hot plate (do **not boil**) to intensify staining.
7. **Final Squash**
    - Place the slide between sheets of blotting paper and press gently with your thumb or a pencil eraser to flatten the tissue and remove excess stain.
8. **Microscopic Observation**
    - Observe under low power to locate the germinal tissue, then under high power to identify the meiotic stages.

Observation

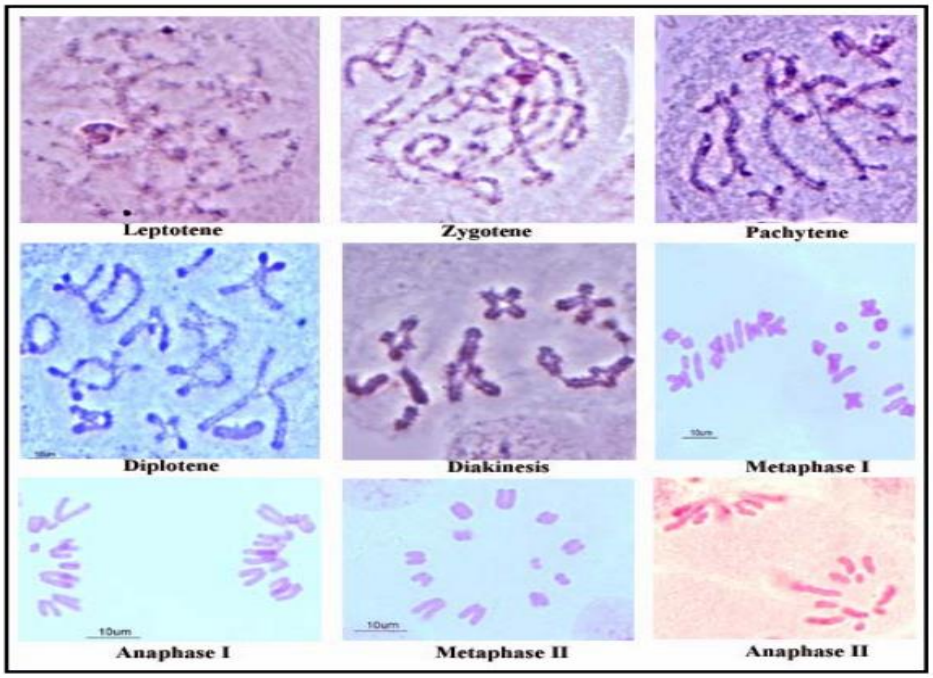
Under the microscope, the following **meiotic** stages can be identified:

Stage	Key Features
Prophase I	Substages: leptotene (thin thread-like chromosomes), zygotene (synapsis of homologous chromosomes), pachytene (crossing over; thick chromosomes), diplotene (chiasmata visible), diakinesis (chromosomes condensed, spindle formation begins).
Metaphase I	Bivalents (paired homologous chromosomes) align on the equatorial plate.
Anaphase I	Homologous chromosomes separate and move to opposite poles (sister chromatids remain attached).
Telophase I	Two haploid nuclei form; brief interkinesis follows without DNA replication.
Prophase II	Chromosomes condense again without further replication.
Metaphase II	Chromosomes align individually at the equator.
Anaphase II	Sister chromatids separate and move to opposite poles.
Telophase II & Cytokinesis	Four haploid nuclei form; cytokinesis produces four spermatids.

Result: All major stages of **Meiosis I and Meiosis II** can be successfully observed in squash preparations of grasshopper testis.

Precautions

- Handle chloroform carefully and use minimal quantity.
- Do not overheat the slide while staining—excess heat may distort chromosomes.
- Apply gentle, even pressure when squashing to avoid breaking the cover slip or displacing cells.
- Use fresh, actively dividing testes for best results.


Leptotene
Zygotene
Pachytene
Diplotene
Diakinesis
Metaphase I
10um
Anaphase I
10um
Metaphase II
Anaphase II

10. D**emonstration of Polytene (Giant) Chromosomes in the Salivary Glands of *Drosophila melanogaster* Larvae**

Aim

To dissect the salivary glands of *Drosophila melanogaster* (fruit fly) larvae, prepare a squash slide, and observe the polytene (giant) chromosomes under the microscope.

Introduction

The salivary glands of Dipteran insect larvae were first described by Balbiani (1881). Their chromosomes are exceptionally large—about 100 times longer than normal metaphase chromosomes—and are called giant or polytene chromosomes.

• During larval development, the DNA undergoes repeated replication without cell division (endoreduplication).

• As a result, many sister chromatids remain aligned side by side, forming a single thick structure called a polytene chromosome.

• These chromosomes, measuring 0.25–0.55 mm when fully extended, exhibit alternating dark and light bands, each representing specific genes or chromosomal regions.

• They are ideal for cytogenetic studies because the banding pattern is reproducible and can be mapped to specific gene loci.

Principle

The squash preparation technique is used to spread and stain the salivary gland cells.

• Aceto-orcein stain binds strongly to chromatin, allowing the banding pattern of the polytene chromosomes to be clearly visible under a compound microscope.

Materials Required

- Third-instar larvae of Drosophila melanogaster
- Equipment:
  - Dissecting microscope
  - Compound microscope
  - Dissection kit (scissors, fine forceps, needles)
  - Clean glass slides and cover slips
  - Pasteur pipette
  - Filter/blotting paper
  - 1 % (w/v) Aceto-orcein stain

Procedure

1. Collection of Larvae
   - Collect healthy third-instar larvae from a Drosophila culture bottle.
   - Place them in a drop of distilled water on a clean slide to prevent drying.

2. Dissection of Salivary Glands

o Transfer a larva to a drop of 1 % aceto-orcein stain on a clean slide.

o Under a dissecting microscope, hold the posterior end of the larva with forceps.

o Gently pull the anterior end with another pair of forceps until the salivary glands (paired, transparent, and tubular) emerge.

3. Fixation and Staining

o Keep the dissected salivary glands immersed in the drop of 1 % aceto-orcein for 5–10 minutes to ensure proper staining.

4. Slide Preparation

o Carefully remove any excess tissue surrounding the salivary glands.

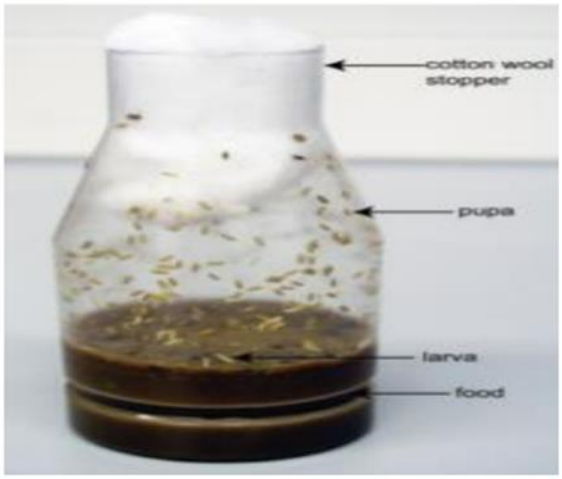
o Place a clean cover slip over the stained glands.

o Gently press (squash) with the blunt end of a pencil or the thumb, using filter paper to absorb excess stain and avoid air bubbles.

5. Microscopic Observation

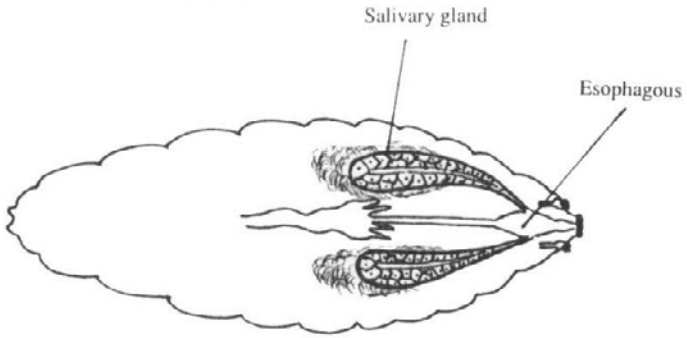
o Examine the preparation first under low power to locate the salivary gland nuclei.

o Switch to high power (40X or oil immersion if needed) to observe the giant polytene chromosomes with their characteristic banding pattern.



Adult Fruit fly (*Drosophila melanogaster*)

Drosophila melanogaster larva

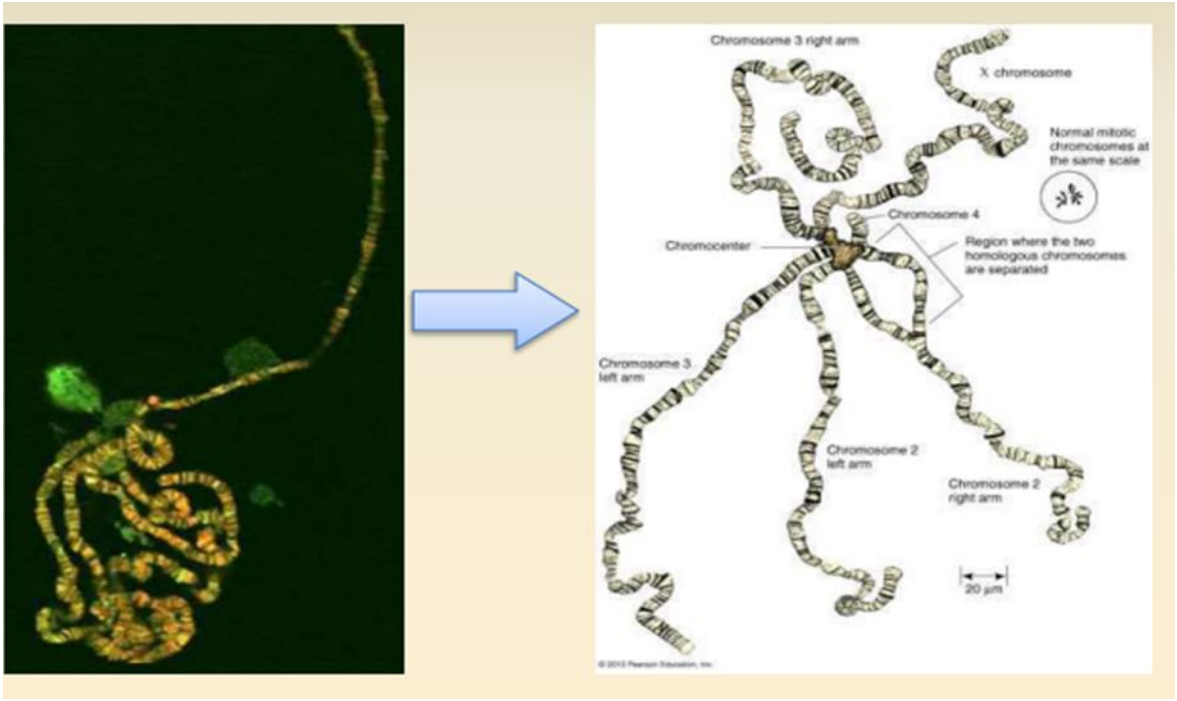


Observation

• Thick, thread-like polytene chromosomes with distinct dark and light transverse bands.

• Chromosome arms may show puffs or Balbiani rings, which indicate regions of active transcription (RNA synthesis).

• The chromosomes are significantly larger and more prominent than typical mitotic chromosomes.



11.Study of Developmental Stages of Chick Embryo Using Window Method and Vital Staining

Aim:

- To observe and identify the developmental stages of chick embryo in an incubated egg using the window method.
- To demonstrate embryonic structures by vital staining.

Principle:

- Chick embryo development is a classic model for studying vertebrate embryology.
- The window method allows direct observation of the embryo through a small opening in the eggshell without killing the embryo.
- Vital staining involves using non-toxic dyes (e.g., neutral red) that selectively stain living embryonic tissues, allowing visualization of structures such as blood vessels, heart, and somites.
- The chick embryo develops from a fertilized egg into a fully formed chick through recognizable stages: zygote → blastoderm → primitive streak → somites → organogenesis → hatching.

Materials Required:

- Fertilized chicken eggs (2–3 days incubation for early stages, up to 5–6 days for mid-stages)
- Incubator (37–38°C, 50–60% humidity)
- Fine forceps and scissors
- Sterile needle / pins
- Filter paper rings
- Vital stain: Neutral red or methylene blue (0.1–0.5% solution)
- Small Petri dish

- Pipette / dropper
- Watch glass or cover slip
- Cotton

Procedure:

A. Incubation of Eggs

1. Place fertilized eggs in the incubator at 37–38°C with moderate humidity.
2. Rotate eggs twice daily to prevent adhesion of the yolk to the shell membrane.
3. Incubate for desired developmental stage:
    - 24 hours → early blastoderm
    - 48 hours → primitive streak formation
    - 72 hours → somites formation
    - 96–120 hours → heart, limb buds, and circulatory system visible

B. Window Method

1. Clean the eggshell surface with a cotton swab soaked in spirit.
2. Make a small hole (1–2 cm^2) on the broad end of the egg using fine scissors or forceps.
3. Remove shell fragments carefully, keeping the inner shell membrane intact.
4. Use a small piece of filter paper to gently hold and stabilize the embryo if needed.
5. Examine the embryo under a stereomicroscope.

C. Vital Staining

1. Prepare 0.1–0.5% neutral red solution in physiological saline.
2. Using a pipette, drop a small amount of dye on the exposed yolk over the embryo.
3. Incubate the egg for 2–5 minutes to allow staining of living embryonic cells.
4. Remove excess stain by gently rinsing with saline.
5. Observe stained embryo under a stereomicroscope to identify:

- Head fold and tail fold
- Primitive streak
- Somites
- Heart and blood vessels
- Limb buds (in later stages)

Observations

Incubation Time	Developmental Stage	Visible Structures (after vital staining)
24 hours	Early blastoderm	Blastodisc, area pellucida and area opaca
48 hours	Primitive streak	Head fold, tail fold, primitive streak
72 hours	Somite formation	Somites, notochord, neural folds
96–120 hours	Organogenesis	Heart, blood vessels, limb buds

- Record sketches of each stage with labeled structures.
- Note the size, shape, and position of embryonic features.

Precautions

1. Handle eggs gently to avoid rupturing the yolk or embryo.
2. Maintain sterile conditions to prevent contamination.
3. Use non-toxic vital stains; avoid overdosing as it can kill the embryo.
4. Keep the egg covered during observation to maintain temperature and humidity.
5. Dispose of used eggs properly after observation.

12. Estimation of Primary Productivity Using Light and Dark Bottle - Winkler Method

Introduction

Primary productivity refers to the rate at which energy is captured by photosynthetic organisms (mainly plants and algae) and converted into organic matter. In aquatic ecosystems, this is typically measured by assessing the rate of oxygen production (photosynthesis) and consumption (respiration). The light and dark bottle method, often combined with the Winkler titration method, is a technique used to estimate primary productivity by determining changes in oxygen concentration in light and dark conditions.

- **Light Bottle**: Measures oxygen produced through photosynthesis (net primary productivity).
- **Dark Bottle**: Measures oxygen consumed by respiration in the absence of light.

Principle

1. **Light Bottle**: In the light bottle, photosynthesis occurs, producing oxygen, and respiration also occurs, consuming some oxygen.
2. **Dark Bottle**: In the dark bottle, photosynthesis is inhibited, and only respiration occurs, consuming oxygen.

By comparing the oxygen changes in the light and dark bottles, we can calculate the primary productivity.

Materials Required

1. Glass Bottles (1000 ml) - Two bottles: one for light and one for dark.
2. Dissolved Oxygen (DO) Meter or Winkler Titration Kit
3. Oxygen-absorbing stirring bar
4. Burette
5. Pipettes
6. Sodium Thiosulfate Solution (for Winkler titration)
7. Manganese Sulfate Solution ($MnSO_4$)
8. Alkaline Iodide-Azide Reagent
9. Sulfuric Acid (H_2SO_4, concentrated)
10. Sodium Hydroxide (NaOH)
11. Wash bottle (distilled water)
12. Incubator or Water bath (optional, for temperature control)
13. Water sample from the aquatic environment (lake, pond, river, etc.)

Preparation of Reagents

1. **Manganese Sulfate Solution ($MnSO_4$)**
    - Dissolve 4.0 g of Manganese Sulfate in 100 ml distilled water.
    - Store in a clean, dark container.
2. **Alkaline Iodide-Azide Reagent**
    - Dissolve 35 g of potassium iodide (KI) in 100 ml distilled water.
    - Add 1.0 g of sodium azide (NaN_3) and mix.
    - Dissolve the mixture in 100 ml of 1.0 N sodium hydroxide (NaOH).
    - Store in a tightly sealed bottle.
3. **Sulfuric Acid Solution (H_2SO_4)**
    - Concentrated sulfuric acid is used in the Winkler titration method to release iodine from the iodine-starch complex.
4. **Sodium Thiosulfate Solution (0.025 N)**
    - Dissolve 6.25 g of sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) in distilled water to prepare 1 liter of 0.025 N solution.
    - Standardize the solution if necessary, using a known volume of iodine solution.

Procedure

1. **Collection of water sample**
    - Collect a water sample from the desired aquatic environment. Use a clean, non-reactive container (preferably glass) to avoid contamination.
    - If measuring primary productivity in situ, collect the water sample in a way that avoids introducing air bubbles and contamination.
2. **Preparation of Bottles**
    - Label two bottles: one for light and one for dark.
    - Fill both bottles with the collected water sample, making sure there are no air bubbles inside.
    - Seal the bottles tightly with stoppers or caps.
3. **Incubation**
    - **Light Bottle**: Place the light bottle in a location where it will be exposed to light, such as underwater or in a transparent container under natural sunlight.
    - **Dark Bottle**: Place the dark bottle in a location where no light reaches, such as in a covered container or wrapped with aluminum foil.
    - **Incubation Time**: Allow both bottles to incubate for a period (usually 2–4 hours, depending on environmental conditions).
4. **Measurement of Dissolved Oxygen (DO)**
    - After incubation, measure the dissolved oxygen (DO) concentration in both the light and dark bottles.

Winkler Titration Method for DO Measurement

1. **Add Manganese Sulfate Solution**: Add 1 ml of manganese sulfate ($MnSO_4$) solution to each sample. This reacts with dissolved oxygen to form manganese oxide (MnO_2) in the solution.
2. **Add Alkaline Iodide-Azide Reagent**: Add 1 ml of the alkaline iodide-azide reagent. The MnO_2 formed reacts with iodide ions to produce iodine (I_2).
3. **Add Sulfuric Acid**: Add 1 ml of concentrated sulfuric acid (H_2SO_4) to the solution. The iodine formed will now be in the form of free Iodine (I_2).
4. **Titrate the Iodine Solution**: Titrate the liberated iodine with 0.025 N Sodium Thiosulfate ($Na_2S_2O_3$) solution. The iodine will react with Sodium Thiosulfate, turning the solution from brown to colorless.
    - **End Point**: The endpoint is reached when the yellow-brown color disappears completely, indicating all iodine has been neutralized.
5. **Record the Volume of Sodium Thiosulfate Used**: Note the volume of Sodium Thiosulfate used in the titration.

Calculations

1. **Net Primary Productivity (NPP)**:
    - NPP=Oxygen produced in light bottle−Oxygen consumed by respiration in dark b ottle NPP = text (Oxygen produced in light bottle) - text (Oxygen consumed by respiration in dark bottle) NPP = Oxygen produced in light bottle − Oxygen consumed by respiration in dark bottle.
    - The difference between the oxygen concentration in the light and dark bottles gives the rate of net primary productivity (NPP) in the system.
2. **Gross Primary Productivity (GPP)**:
    - GPP=Oxygen produced in light bottle+Oxygen consumed by respiration in dark b ottle GPP = text (Oxygen produced in light bottle) + text (Oxygen consumed by respiration in dark bottle) GPP = Oxygen produced in light bottle + Oxygen consumed by respiration in dark bottle
    - GPP represents the total oxygen produced by photosynthesis before respiration takes place.
3. **Respiration Rate**:
    - R = Oxygen consumed in dark bottle This is the rate of oxygen consumption due to respiration by organisms in the water.

13. Estimation of Dissolved Oxygen from Water sample

Reagents:

1. Manganous sulphate solution: Dissolved 100 g of Manganous Sulphate in 200 ml of distilled water filter it.

2. Alkaline potassium iodide solution: Dissolved 100 g of KOH and 50 g of KI in 200 ml of preboiled distilled water.

3. Sodium thiosulphate ($Na_2S_203.5H_2O$) 0.025 N: Dissolved 24.82 g of sodium thiosulphate in preboiled distilled water and made up to the volume of 1 liter. Added a pellet of sodium hydroxide as a stabilizer. The concentration of the solution was 0.1 N. Diluted to 4 times to prepare 0.025 N standard solution (1000 ml).

4. Starch indicator solution: Dissolved 1 g of starch in 100 ml of distilled water and warmed for complete dissolution.

5. Concentrated Sulphuric acid.

Procedure:

Fill the water samples in 300 ml BOD bottle carefully without allowing air bubbles. Added 2 ml of manganous sulphate and 2ml of alkaline Potassium Iodide reagent. A brown precipitate formed was allowed to settle. Added 2 ml of concentrated Sulphuric acid and mixed well until the precipitate dissolved. About 50 ml of the solution was taken and titrated against sodium thiosulphate until a straw yellow colour appeared. Few drops of starch indicator is added and titrated again until the blue colour disappeared.

Calculation:

Dissolved oxygen, mg/l= ml of Sodium Thiosulphate×0.025×8×1000

V2 [(V1-V)/V1)]

Were,

V1 = Volume of sample bottle

V2 = Volume of contents titrated

V = Volume of MnSO4 and KI added (2 ml)

14. Estimation of Free Carbon dioxide from the water sample

Free carbon dioxide in the waters accumulates due to microbial activity and respiration of organisms. This imparts the acidity to the waters because of the formation of carbonic acid. Free carbon dioxide reacts with sodium carbonate or sodium hydroxide to form sodium bicarbonate. Completion of the reaction is indicated titrimetric ally by the development of pink colour with phenolphthalein as the indicator.

Reagents:

1. Sodium hydroxide, 0.05 N: Dissolved 40 g of sodium hydroxide in boiled CO_2 free distilled water and made up the volume to 1 liter. The solution was filtered to remove Na_2CO_3. This gave 0.1 N NaOH solution. Diluted the solution 20 times to prepare 0.05 N solution.

2. Phenolphthalein indicator solution: Dissolved 0.5 g of phenolphthalein in 50 ml of distilled water. 0.05 N Sodium hydroxide solution was added dropwise until the solution just turned pink.

Procedure:

About 50 ml of the sample was taken in a conical flask and added a few drops of phenolphthalein indicator. If colour turns pink, free Carbon dioxide absent. The solution was titrated against 0.05 N sodium hydroxide. The endpoint is the appearance of pink colour.

Calculation:

Free CO2, mg/l= ml of sodium hydroxide×0.05×44×1000 ml of sample taken

15.Estimation of Alkalinity of the Water Sample

Aim:

To determine the alkalinity of the given water sample

Reagents:

1. Hydrochloric Acid 0.1 N: Diluted 12 N concentrated HCl (sp, gr.1.18) to 12 times (8.34à100 ml) to get 1.0 N HCl. Further dilute it to 10 times to prepare 0.1 N HCL (100à1000 ml). Standardize it against Sodium Carbonate solution

2. Sodium carbonate, 0.1N: Dissolve 5.300g of Na2CO3 (predried at 250ºC for 4 Hours) in distilled water to prepare 1 liter of solution.

3. Methyl Orange indicator: Dissolve 0.5 g of methyl orange in 100 ml of distilled water.

4.Phenolphthalein indicator: Dissolved 0.5 g of phenolphthalein in 50 ml of 95% ethyl alcohol. Added 50 ml of distilled water and 0.05 N sodium hydroxide dropwise, till a faint pink colour appeared.

Procedure:

Take 100 ml of sample in conical flask and add drops of Phenolphthalein indicator. If the solution remains colour less, Phenolphthalein alkalinity (PA) is zero (indicating absence of carbonate) and total alkalinity with methyl orange is only determined. If the colour changes to pink after addition of Phenolphthalein indicator, titrate it with 0.1N HCl until the colour disappears at end point. This is phenolphthalein alkalinity (PA). Now add 2-3 drops of methyl orange to the same sample and continue the titration further, until the yellow colour changes to pink at the end point to obtain total alkalinity.

PA as CaCO3 mg/l= (A × N) of HCl × 1000 × 50 mL of sample

TA as CaCO3 mg/l= (B × N) of Hcl × 1000 × 50 mL of sample

Where,

A= ml of HCl used only with Phenolphthalein

B= ml of HCl used with Phenolphthalein and methyl orange i.e. total HCl used with both the indicators.

16. Estimation of Total Hardness of the Water Sample

Aim:

To determine the total hardness of the water sample

Reagents:

Buffer solution:

(a) Dissolve 16.9 g Ammonium Chloride (NH4Cl) in 143ml of Ammonium Hydroxide (NH_4OH) solution.

(b) Dissolve 1.179 g of EDTA and 0.780 g of $MnSO_4.7H_2O$ in 50 ml distilled water

Mix both (a) and (b) solutions and dilute to 250 ml with distilled water

EDTA (Ethylene Diamine Tetra Acetic acid)

Dissolve 3.723 g of disodium salt of EDTA in distilled water to prepare 1 L of solution store in polythene or Pyrex bottle.

Eriochrome Black T:

Mixed 0.40 g of Eriochrome Black T with 100 g Sodium Chloride and grind.

Sodium Sulphide solution

Dissolve 5.0 g of $Na_2S.9H_2O$ or 3.7 g $Na_2S.5H_2O$ in 100 ml of distilled water and store in a tightly closed bottle.

Procedure:

Take 50 ml of sample in a conical flask. In case of sample with high hardness a smaller aliquot may also be taken. Add 1 ml of buffer to this. If sample is having higher concentration of heavy metals, add 1 ml of Na_2S solution. Add approximately 100 g of Eriochrome Black T indicator, the solution will turn wine-red. Titrate the contents with EDTA solution; the colour changes to blue at end point.

Hardness, mg/l as CaCo3 = ml of EDTA used ×100 ml of sample

17. **Determination of Turbidity of a Water Sample**

Aim

To determine the turbidity of a given water sample.

Principle

The Nephelometry method is based upon a comparison of the intensity of light scattered by the sample under defined conditions with the intensity of light scattered by a standard reference suspension. The higher the intensity of scattered light, the higher is the turbidity. Readings of the Nephelometer are given in Nephelometric Turbidity Unit (NTU). A primary standard suspension is used to calibrate the instrument. A secondary standard suspension is used as a daily calibration check and is monitored periodically for deterioration using one of the primary standards.

Apparatus

Nephelometer

Reagents

• Dissolve 1.000 g hydrazine sulfate [$(NH_2)2\ H_2SO_4$] in filtered water and dilute to 100 mL in a volumetric flask.

• Dissolve 10.00 g hexamethylenetetramine [$(CH_2)6N_4$] in filtered water and dilute to 100 mL in a volumetric flask.

• Mix 5.0 mL of Hydrazine Sulfate and 5.0 mL of hexamethylenetetramine solutions in a 100-mL volumetric flask and let stand 24 hours at 25 ± 3°C; dilute to the mark and mix. To prepare 500 mL of 400 NTU standard, mix 25 mL of the reagent solutions in a 500- mL flask, dilute to the mark, and mix.

• For a 40 NTU standard, dilute 10.00 mL of the 400 NTU stock suspension to 100 mL with turbidity-free water (sample or deionized water passed through a filter media of 0.2 mm)

Procedure

Calibration of Nephelometer

The calibration of Nephelometer can be done using the following steps:

• Switch the turbidimeter on and allow it to warm up.

• Check instrument focus: insert template in the cell holder. The lamp image should just fill the inside circle.

• Draw out the sample cell from the Nephelometer and hold it by the rim not beneath the lip.

• Pour the deionized water into the sample cell and wipe out the exterior with a soft dry tissue paper.

• Using Set Zero dial calibrate the instrument to Zero.

• Next, pour the standard solution into the sample cell and wipe out the exterior with a soft dry tissue paper. Place the cell in its holder with correct orientation.

• Select the desired NTU range dial of the Nephelometer and rotate the calibrate dial of the Nephelometer to match the digital reading exactly with the preset range.

• The instrument is now calibrated.

• The Sample cell can now be filled with unknown sample to measure the NTU.

Calculation

The NTU of diluted sample can be calculated using the following formula:

$NTU = A \times (B+C) / C$

Where,

A = NTU found in diluted sample,

B = Volume of dilution water, in milliliters, and

C = Sample volume taken for dilution, in milliliters.

18. Temperature Preference of Fishes Using Thermometers

Aim:

To determine the preferred temperature zones of fishes using a metal box with thermometers. To record the number of fishes in each temperature zone and establish a temperature reference for fish behavior and physiology.

Principle:

Fish are ectothermic; their body temperature and activity are influenced by surrounding water temperature. When provided with multiple temperature zones, fish move to regions of optimal comfort. By dividing a metal box into five zones and measuring temperature with five thermometers, we can quantitatively observe fish preference.

Materials Required:

Metal box (long or rectangular, thermometers (one for each zone), water, ice cubes or warm water for adjusting temperatures, Live fish (sufficient number for observation), Divider markers (to mark 5 zones in the box), Stopwatch or timer and observation sheet.

Procedure:

A. Preparation of Temperature Zones

1. Clean the metal box thoroughly.
2. Divide the box into 5 equal zones using markers.
3. Place one thermometer in each zone to monitor temperature.
4. Adjust water in each zone using ice or warm water to achieve a temperature gradient (e.g., Zone 1: coldest, Zone 5: warmest).
5. Ensure temperatures are stable before adding fish.

B. Introduction of Fish

1. Gently place a group of fish in the metal box.
2. Allow 2–3 minutes for the fish to acclimate.
3. Observe the number of fish in each zone over a fixed period (e.g., 10 minutes).
4. Repeat the observation 3–5 times to ensure accuracy.

C. Recording Observations

1. Count the number of fish in each zone at regular intervals.

2. Note behavioral activity, such as swimming speed or clustering.

3. Record temperature in each zone simultaneously. After recording, plot a bar graph of fish numbers vs. temperature zones to visualize preference.

19. pH Preference of Fishes Using pH Gradient

Aim:

To determine the preferred pH range of fishes using a controlled pH gradient. To record the distribution of fishes across different pH zones and establish a pH preference reference.

Principle:

Fishes are sensitive to changes in water pH, which can affect physiology, behavior, and survival. When exposed to a gradient of pH, fishes tend to aggregate in zones with optimal pH, reflecting their preference. By observing the distribution of fishes across different pH zones, their preferred pH range can be determined. This method provides a standard reference for ecological and physiological studies.

Materials Required:

Live fishes (sufficient number for observation), Rectangular tank or metal box, pH meter or pH strips, buffer solutions (acidic, neutral, basic), divider markers (to create multiple pH zones), Stopwatch / timer and observation sheet.

Procedure:

A. Preparation of pH Gradient

1. Divide the tank or box into 5 equal zones.

2. Prepare water in each zone with different pH values (e.g., Zone 1: pH 5, Zone 2: pH 6, Zone 3: pH 7, Zone 4: pH 8, Zone 5: pH 9).

3. Measure and adjust pH precisely using a pH meter or strips.

4. Allow the gradient to stabilize before introducing fishes.

B. Observation of Fish Distribution

1. Introduce fishes gently into the tank.

2. Allow 2–3 minutes for acclimation.

3. Observe and record the number of fishes in each pH zone at regular intervals (e.g., every 2–5 minutes).

4. Note any behavioral responses such as swimming, clustering, or avoidance.

5. Repeat the observation 3–5 times for accuracy.

5. Plot a graph of fish number vs. pH zone to visualize pH preference.

20. **Determination of Total Ammonia Nitrogen (TAN) in water.**

Aim:

To determine the total ammonia nitrogen (TAN) in a water sample.

Principle:

Nessler's reagent consists of potassium tetraiodomercurate (II), K2HgI4. Under alkaline conditions it reacts with ammonia, producing a yellow to brownish-yellow color whose intensity is directly proportional to the ammonia concentration.

$$2K_2HgI_4 + NH_3 + 3KOH \rightarrow HgO \cdot Hg(NH_2)I + 7KI + 2H_2O$$

Materials:

Ammonium chloride (NH_4Cl), Distilled water, Nessler's reagent, 100 mL Volumetric flask, Pipettes, Test tubes, Colorimeter, Cuvettes.

Procedure:

1. Preparation of Ammonia Stock Solution

Accurately weigh 0.1 g of ammonium chloride (NH_4Cl). Transfer the weighed ammonium chloride to a 100 mL volumetric flask. Add distilled water up to the 100 mL mark on the volumetric flask. Ensure thorough mixing.

2. Preparation of Standard Solution

Using a 10 mL measuring cylinder, accurately transfer 10 mL of the ammonia stock solution into a 100 mL volumetric flask. Add distilled water until the bottom of the meniscus aligns with the 100 mL mark. Ensure thorough mixing.

Take 5 mL of the standard solution into a clean test tube. Add one drop of Nessler's reagent and allow it to rest for 15 minutes. This allows for color development.

3. Preparation of Test Solution

Take 5 mL of the test solution into a clean test tube. Add one drop of Nessler's reagent and allow the test solution to rest for 15 minutes. This allows for color development.

4. Prepare the Blank Solution

Take 5 mL of distilled water into a clean beaker or test tube.

5. Colorimetric Measurement

Turn on the colorimeter and set the wavelength to 420 nm and calibrate it by setting the blank to 0 absorbance. Pour the blank solution into a clean cuvette. Place the cuvette into the colorimeter and read the Optical Density (OD) at 420 nm. Similarly record the OD of the standard and test solution.

Observations:

The Nessler's reagent reacts with ammonia to produce a yellow-brown color, the intensity of which is proportional to the concentration of ammonia. By comparing the OD of the test solution to the OD of the standard solution, the ammonia concentration of the test sample is determined. A higher OD indicates a higher ammonia concentration.

Calculation:

$$\text{OD of the sample} = \frac{\text{Concentration of the standard x OD of test}}{\text{OD of standard x Volume of test}} \times 1000$$

Where,
Concentration of standard = 0.1 mg
Volume of test = 5 ml

Result: Total Ammonia Nitrogen (TAN) the test sample is ……..µg ml^{-1}

Table

Sl.No	Sample	Volume of Sample (ml)	OD at 420 nm
1	Blank	5 ml	
2	Standard	5 ml	
3	Test	5 ml	

21. Preparation and Microscopic Examination of a Human Blood Smear

Aim:

To prepare a thin smear of human blood, stain it using **Leishman's stain**, and observe the different types of blood cells—erythrocytes, leukocytes (granulocytes and agranulocytes), and platelets—under the microscope for studying their morphology and relative proportions.

Preparation of Materials

- **Slides and spreader:** Clean glass slides thoroughly to ensure a grease-free surface. Keep a smooth-edged spreader slide ready.
- **Stain:** Prepare Leishman's stain and distilled water.
- **Other materials:** Sterilized lancet/prick needle, cotton swabs, spirit, and antiseptic solution are kept ready for use.

Collection of Blood Sample

- Clean the fingertip (middle or ring finger) using a cotton swab dipped in spirit and allow it to dry.
- Prick the fingertip gently with a sterile lancet.
- Wipe away the first drop of blood with sterile cotton to remove any tissue fluid contamination.

Preparation of Blood Smear

- Place a small drop of fresh blood near one end of the clean slide.
- Hold the spreader slide at an angle of about 45° C in front of the drop.
- Pull the spreader backward until it touches the drop, allowing the blood to spread by capillary action along the edge.
- Push the spreader smoothly and quickly forward in a single stroke to create a thin, even, tongue-shaped smear about 3–4 cm long.
- Air-dry the smear thoroughly.

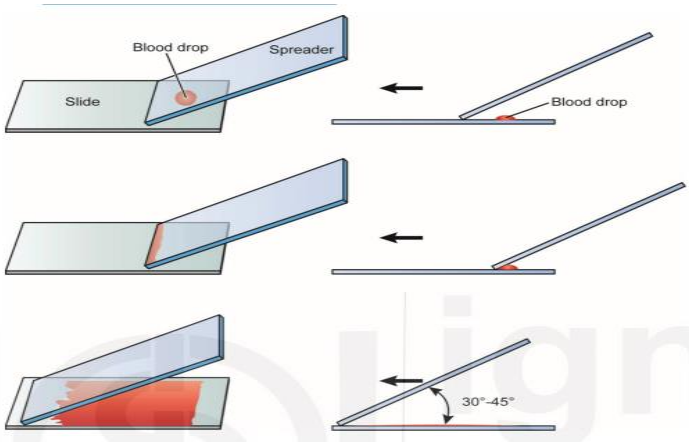
Fixation and Staining

- Flood the air-dried smear with Leishman's stain and leave it for 2 minutes to allow fixation.
- Add an equal amount of distilled water to dilute the stain and mix gently.

- Allow the diluted stain to act for 2–3 minutes, then gently rinse the slide under running tap water to remove excess stain.
- Drain the slide vertically and air-dry completely.

Mounting and Microscopic Observation

- Mount the smear with a clean cover slip using a drop of distilled water if necessary to avoid drying artifacts.
- Examine the slide first under low power to locate the ideal area (between the body and tail of the smear) where cells are evenly distributed and not overlapping.
- Switch to high power (40× objective) to study cell morphology in detail.



Identification of Cells in Human Blood Smear

When the stained blood smear is observed under the microscope (preferably with a 40× or 100× oil-immersion objective), the following cells can be identified based on their shape, size, nucleus, and staining characteristics:

Erythrocytes (Red Blood Cells – RBCs)

- **Shape & Size:** Small, round, biconcave discs; ~7–8 µm in diameter.
- **Color/Staining:** Pale pink or light reddish because of hemoglobin; no nucleus.
- **Special Features:** Central pale area due to biconcavity; the most numerous cells in the smear.
- **Function:** Transport oxygen and carbon dioxide.

Leukocytes (White Blood Cells – WBCs)

Divided into granulocytes and agranulocytes. All WBCs are larger than RBCs and contain a distinct nucleus.

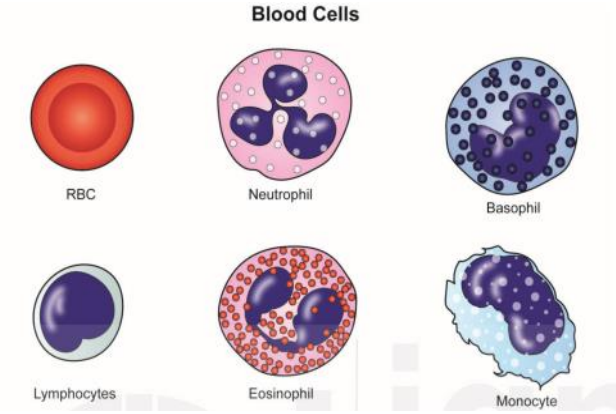
A. Granulocytes (Cytoplasm contains visible granules)

1. **Neutrophils**
    - **Size:** 10–12 μm
    - **Nucleus:** Multi-lobed (3–5 lobes) connected by thin strands.
    - **Cytoplasm:** Pale pink with fine, faint granules.
    - **Function:** Phagocytosis of bacteria and debris; first line of defense.
2. **Eosinophils**
    - **Size:** 10–12 μm
    - **Nucleus:** Bilobed (two lobes).
    - **Cytoplasm:** Large, coarse, red–orange granules.
    - **Function:** Defense against parasitic infections; modulate allergic reactions.
3. **Basophils**
    - **Size:** 8–10 μm
    - **Nucleus:** Irregular or S-shaped, often obscured by granules.
    - **Cytoplasm:** Large, deep blue–black granules.
    - **Function:** Release histamine and heparin during inflammatory and allergic responses.

B. Agranulocytes (No visible cytoplasmic granules)

1. **Lymphocytes**
    - **Size:** 6–10 μm (small) or up to 12 μm (large).
    - **Nucleus:** Large, dark-staining, round nucleus occupying most of the cell.
    - **Cytoplasm:** Thin rim of pale blue cytoplasm.
    - **Function:** Adaptive immunity—T cells (cell-mediated immunity) and B cells (antibody production).
2. **Monocytes**

- **Size:** 12–20 µm (largest WBC).
- **Nucleus:** Kidney-shaped or horseshoe-shaped.
- **Cytoplasm:** Abundant grayish-blue cytoplasm without prominent granules.
- **Function:** Differentiate into macrophages for phagocytosis and antigen presentation.



PRECAUTIONS

1. Blood drop should be of proper size.
2. The edges of the spreaders should be smooth and it should be smaller than the slide on which smear is being made.
3. Apply the pressure properly and uniformly.
4. Pull the drop of blood with spreader, but do not push.
5. Prepare the smear with a single stroke.
6. Mount the cover slips to prevent damage to smear during handling and examination.
7. Never let the stain dry on the slide; otherwise, it will deposit and we will not be able to observe the cells clearly.

22. Determination of Human Blood Group using Antisera Aim

Aim:

To understand the basic concept of the ABO blood group system and to know our blood group and type.

Materials Required

- Toothpicks
- Blood sample
- Alcohol Swabs
- Lancet
- Clean glass slide
- Sterile cotton balls
- Biohazard disposal container
- Monoclonal Antibodies (Anti-A, B, and D)

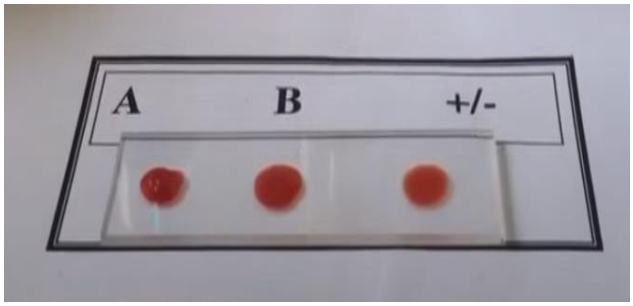
Procedure

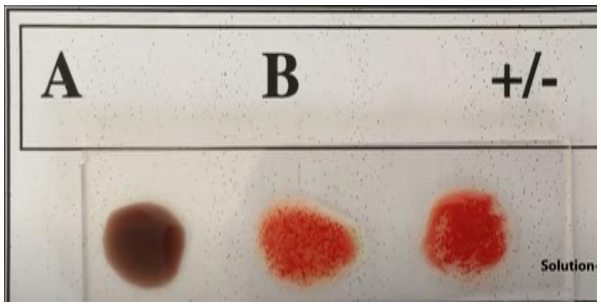
- Take a clean glass slide and draw three circles on it.
- Unpack the Monoclonal Antibodies (MAB) kit. In the first circle add Anti-A, to the second circle add Anti-B and to the third circle add Anti-D with the help of a dropper.
- Keep the slide aside safely without disturbing.
- Now wipe the ring finger with the alcohol swabs and rub gently near the fingertip, where the blood sample will be collected.
- Prick the ring fingertip with the lancet and wipe off the first drop of the blood.
- As blood starts oozing out, allow it to fall on the three circles of the glass slide by gently pressing the fingertip.
- Apply pressure on the site where it was pricked and to stop blood flow. Use the cotton ball if required.
- Mix the blood sample gently with the help of a toothpick and wait for a minute to observe the result.

Conclusion

The chart helps to predicts the different types of blood groups along with its Rh factor.

Blood Type	A	B	O	AB
Rh-positive	A+	B+	O+	AB+
Rh-negative	A-	B-	O-	AB-





23. Gram Staining (+\-) of bacteria

Danish microbiologist and physician Hans Christian Gram in 1884, developed a staining procedure by which bacteria can be classified in two groups as Gram positive and Gram-negative bacteria. Most of the bacteria is encased by a strong cell wall in which a carbohydrate matrix is cross-linked by short polypeptide chains. Gram positive bacteria (encased by thick layer of peptidoglycan- 90% of cell wall) retain the color of the stain and the Gram-negative bacteria (thin layer of peptidoglycan- 10% of cell wall and high lipid content) don't retain the color of the stain, when the bacteria is subjected to it. After the staining, Gram-positive bacteria will appear as violet or blue-black and the Gram-negative bacteria will appear as red/pink.

A Gram stain is a test used to identify Gram positive and negative bacteria.

Materials required:

Crystal violet, Gram's iodine, 95% ethanol, safranin. glass slide, Bunsen burner, wire loop, forceps, distilled water in wash bottle, blotting paper, bacterial culture on plate and compound microscope.

Procedure

1. Add a few drops of crystal violet (primary stain) to the smear and let it sit for 60 seconds.
2. Rinse the slide with water.
3. Add a few drops of Gram's iodine (mordant) to the smear and let it sit for 60 seconds.
4. Rinse the slide with water.
5. Decolorize with 95% ethanol: let the alcohol run over surface of slide until no more crystal violet colour comes out of the smear (time varies—no more than 5-10 seconds).
6. Rinse with water.
7. Add a few drops of safranin (counterstain) and let it sit for 60 seconds.
8. Rinse with water. Blot dry with filter paper. Be careful not to wipe off the bacteria.
9. Observe the slide under the microscope.

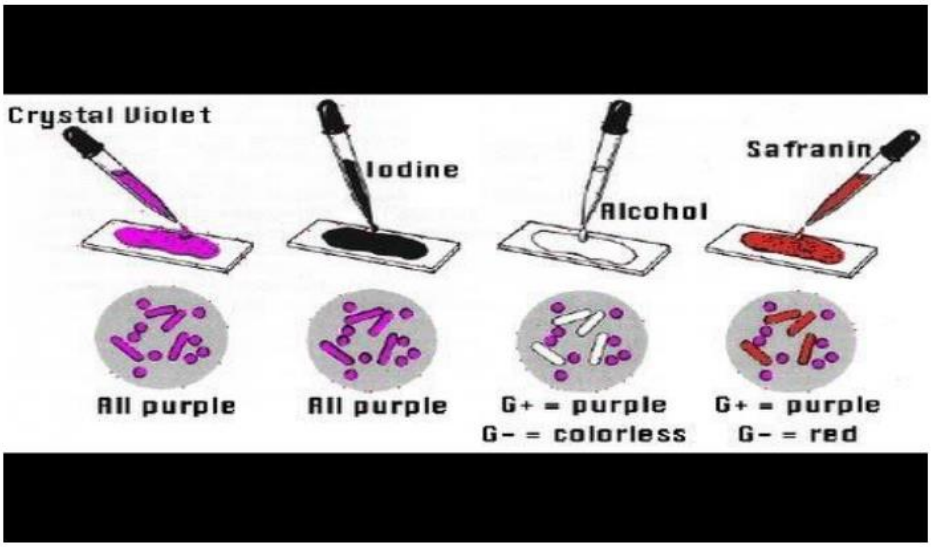


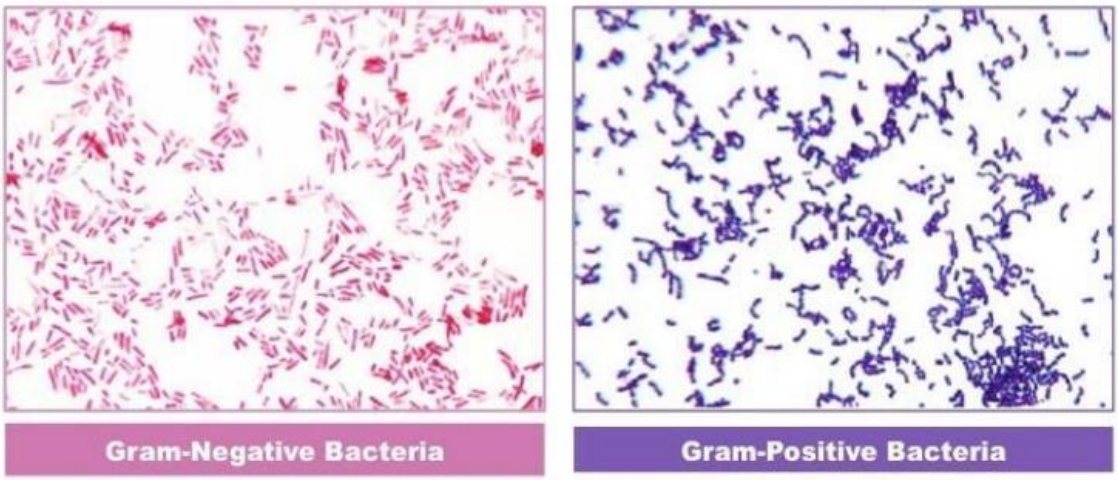
Fig 2: use of different chemicals (photo courtesy- Uploaded on YouTube by: Amrita Vlaboratory; Link- http://www.amrita.edu/create)

Observation:

Observations under compound microscope - the Gram-positive bacteria will appear in violet or blue-black and the Gram-negative bacteria will appear in red or pinkish color.

Precautions to be taken:

- Don't over-stain the bacterial smear either by crystal-violet or safranin stain.
- Don't delay in collecting the sample on glass slide from the bacterial colony.
- Glass slide needs to be properly cleaned.
- Correct thickness of bacterial smear is very important.
- Don't wash the slide by acetone or alcohol for longer time unnecessarily.



(Image Credit: BioNinja; Link- https://ib.bioninja.com.au/options/untitled/b1-microbiology-organisms/gram-staining.html)

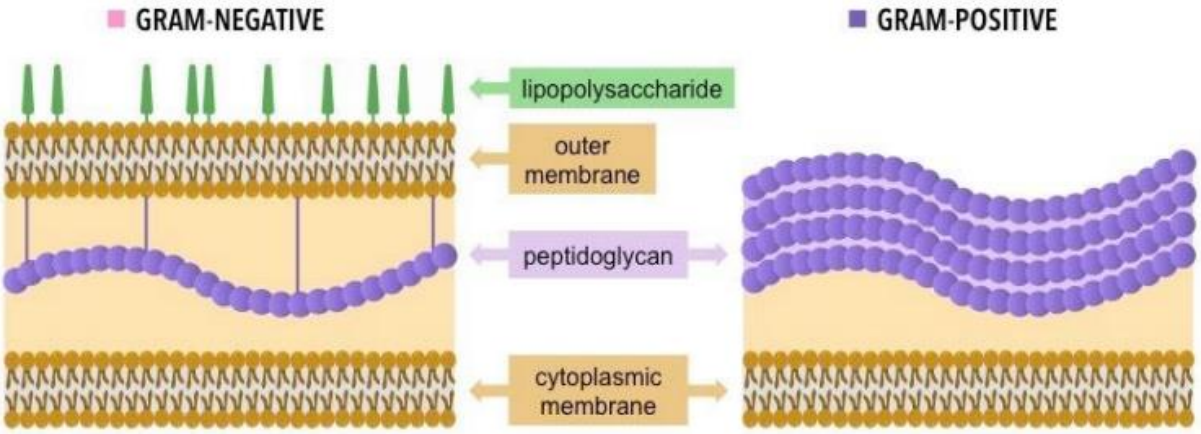


Fig. 1: chemical components of cell wall in Bacteria (Image Credit: BioNinja; Link- https://ib.bioninja.com.au/options/untitled/b1-microbiology-organisms/gram-staining.html)

24. Determination of Quality of Milk by Methylene-Blue Reductase Test

Milk is the white fresh lacteal secretion from the female of the cattle through the udder to feed young ones. Milk is a liquid food for human infants and adults. It is an excellent culture medium for microorganisms. The chemical composition of milk is 1.3% water, 5% lactose, 3.8% fat, 2.5%casein and 14% proteins. The pH of milk varies from 6.5-6.7. It contains body building proteins, bone forming minerals, energy giving lactose, milk fat and fatty acids.

Various types of microorganisms are found in the milk. The presence or absence of microorganisms depends upon the quality of milk. The important microorganisms in milk are bacteria, yeast, molds, bacteriophages, viruses and protozoans. They may produce desirable and considerable changes in the milk and milk products. The cow's milk always contains 500 several millions of bacteria per milliliters. The number and kind of microorganisms fluctuated with production of particular milk and the degree of contamination. The different sources of contamination of the milk are from the udder of cow, skin of cow, utensils and equipment, foul air of cow's shed, water and hands of human beings etc.

AIM:

To determine the quality of given milk samples A, B and C.

PRINCIPLE:

Large number of bacteria are present in cow's milk especially E. coli and Streptococcus lactis. The bacteria present in milk utilizes oxygen which is present in small amounts producing or reducing the environment. The reductase test is made upon oxidation-reduction activities of bacteria present in milk. The methylene blue which is colour sensitive to oxygen, is added to the milk as indicator. It is blue in the oxidized state and white in the reduced state. The speed of colour disappearance of methylene blue or methylene blue retention time is proportional to the number of bacteria present in the sample. It is taken as an indicator of bacterial load. The more the number of bacteria present, the faster are reduction rate.

Milk may be classified according to the following type of decolorization relationships.

MBRT	Classification of Milk Samples	Approx no: of bacteria per ml
0 – 30 min	Very poor quality	>2,00,00,000
31 – 120 min	Poor quality	40,00,000 to 2,00,00,000
121 – 360 min	Fair quality	5,00,000 to 40,00,000
361 – 480 min	Good quality	<5,00,000

REQUIREMENTS:

1. 3 milk samples A, B and C.
2. Methylene blue solution (1:25,000)
3. Sterile test tubes.
4. Sterile 10ml measuring cylinder.
5. Sterile 1ml pipette.
6. Water bath at 31℃.
7. Sterile distilled water.

PROCEDURE:

Thoroughly mix each sample. Transfer 10 ml of each sample of milk into the labelled test tubes A, B and C using separate sterile measuring cylinder. Add 1ml of methylene blue into each test tube. Close the test tube in a water bath at 37 ℃ for 2 hours. One control test tube containing 10 ml of boiled milk and 1ml of methylene blue is also incubated.

RESULT:

According to the standard table given;

Sample A = Poor quality

Sample B = Poor quality

Sample C = Fair quality

COMMENT:

Milk is a complete food. It contains proteins, fats, carbohydrates. It is also rich in Vitamin A, B, B2, C and D. Milk is susceptible to degradation by one or another species of microorganisms. This impact abnormal taste, flavour or appearance to milk. This alteration in the physical and chemical properties of milk is not due to mere presence of these alternative organisms. This results from the individual cells during the period of their development or reproduction or from substances produced during such activity. It is quite understood that milk and dairy products will transmit these disease-causing microorganisms to the consumers of these products. It is important to know the microbial content in order to prevent the spoilage of milk to produce desirable products and to prevent spread of disease. Among the diseases, which are most commonly carried through milk and milk products are tuberculosis, typhoid, fever, diphtheria, scarlet fever, sore throat and various intestinal disturbances. In order to prevent this, it is important to know the channels through which microorganisms gain access to milk or products of milk.

25. Detection of Barr Bodies in Human Buccal Smear

Aim:

The buccal smear barr body test is performed to identify the inactive X-chromosome in the female cells.

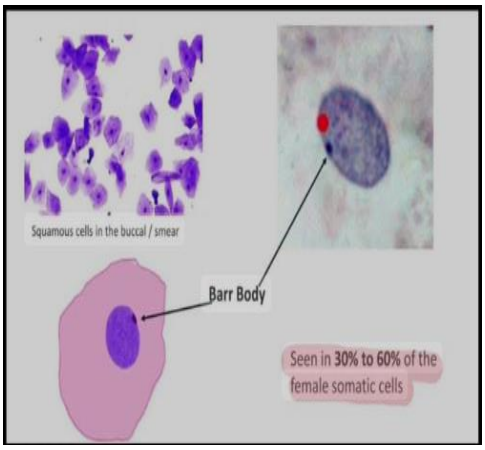
Materials required:

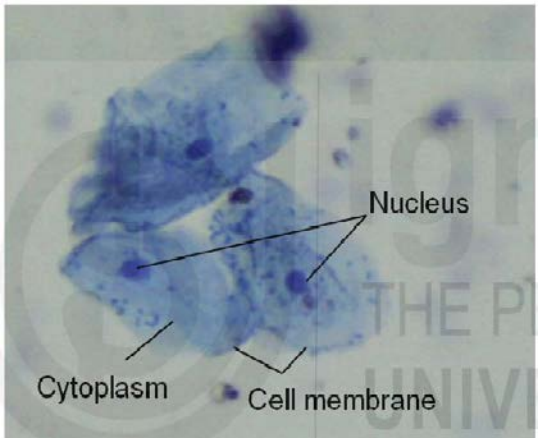
Clean glass slides, toothpicks, methylene blue stain.

Procedure:

Scrape off a specimen of tissue from the inner lining of the mouth with a clean toothpick. This tissue is then laid on a clean and dry glass slide and a thin smear is prepared. It is stained with methylene blue for 6-8 minutes. Excess stain is removed by rinsing in distilled water. The slide is air dried/water removed with a blotting paper and observed under a microscope.

Observation: One can observe a darkly stained body just below the nuclear membrane in about half the cells. This is the Barr body is not present in all cells but in





26. Preparation of bar diagrams, histogram, frequency polygon and pie diagram using suitable software (MS Excel)

Aim: To create bar charts, pie charts, histograms, and frequency polygons in Microsoft Excel

Procedure

1. Bar Chart:

- **Prepare Data:** Enter your data into columns, with labels for categories and values in adjacent columns.
- **Select Data:** Highlight the data you want to chart, including labels.
- **Insert Chart:** Go to the "Insert" tab and choose "Recommended Charts" or "Column Chart" for a bar chart.
- **Customize:** Adjust chart title, axis labels, and chart layout

2. Pie Chart:

- **Prepare Data:** Similar to bar charts, ensure your data is organized with categories and values.
- **Select Data:** Highlight the data, including categories and values.
- **Insert Chart:** Go to the "Insert" tab and choose "Pie Chart."
- **Customize:** Adjust chart title, labels, and chart layout as needed.

3. Histogram:

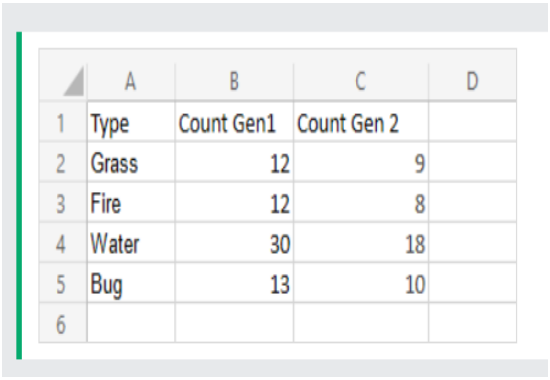
- **Prepare Data:** Enter your data into a single column and optionally create "bin ranges" (class intervals) in another column.
- **Select Data:** Highlight the data and the bin ranges (if using).
- **Insert Histogram:** Go to the "Insert" tab and select "Histogram" under "Statistic Charts" (or use the "Data Analysis" tool).
- **Customize:** Adjust bin size, axis labels, and chart layout.

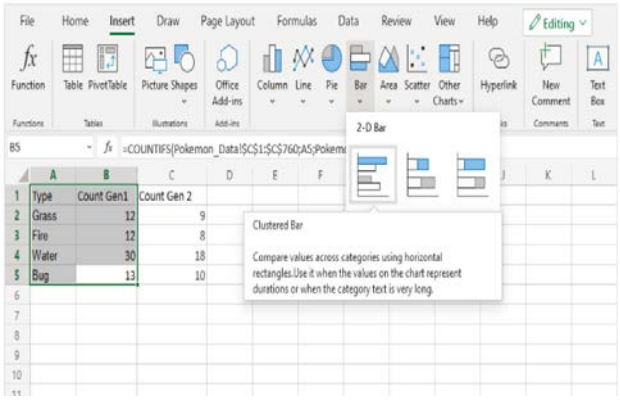
4. Frequency Polygon:

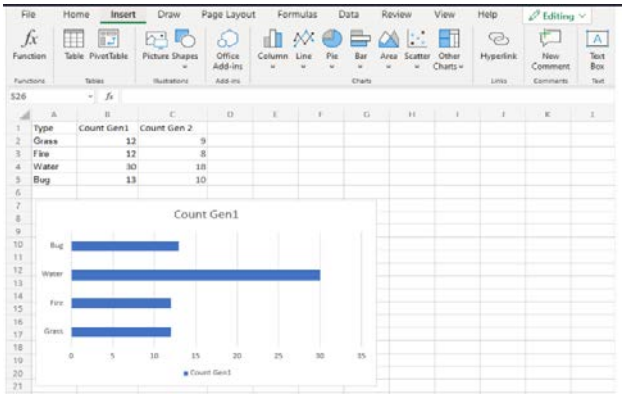
- **Start with a Histogram:** Create a histogram as described above.

- **Convert to Line Graph:** Right-click on the histogram bars and select "Change Chart Type."
- **Choose Line Graph:** Select "Line" as the chart type.
- **Customize:** Adjust the line style, remove gaps between the lines if desired, and add labels. You may also need to adjust the bin size to match the class intervals for a more accurate frequency polygon.

	A	B	C	D
1	Type	Count Gen1	Count Gen 2	
2	Grass	12	9	
3	Fire	12	8	
4	Water	30	18	
5	Bug	13	10	
6				







27. Study of following biodiversity indices and estimation using PAST (PAleontological STatistics) programme: Shannon-Wiener index, Dominance index, Margalef index, Simson index, Evenness index.

PAST (PAleontological STatistics), is a powerful software for scientific data analysis, with functions for data manipulation, plotting, univariate and multivariate statistics, ecological analysis, time series and spatial analysis, morphometries and stratigraphy. Many of the functions are specific to paleontology and ecology, and these functions are not found in standard, more extensive, statistical packages. Data may be entered via a spreadsheet-type interface, or pasted from the clipboard.

Diversity index is a quantitative measure that reflects how many different types (such as species) are there in a dataset (a community). These indices are statistical representations of biodiversity in different aspects (richness, evenness, and dominance).

1. **Dominance index (D):** Measures the probability that two randomly selected individuals from a community belong to the same species. A higher dominance index indicates a greater likelihood of selecting the same species, suggesting a less diverse community where one or a few species are dominant. Dominance index value ranges from 0 (all taxa are equally present) to 1 (one taxon dominates the community completely). Simpson's Dominance Index is the inverse of the Simpson's Index.

2. **Simpson index (**1-dominance): The Simpson index, also known as Simpson's Diversity Index, measures 'evenness' of the community from 0 to 1. It is an index of diversity which is a measure of probability--the less diversity, the greater the probability that two randomly selected individuals will be the same species. With this index, 0 represents infinite diversity and 1, no diversity.

3. **Shannon wiener index:** The index considers both the number of species (richness) and the evenness of their distribution. It is denoted as *H*, the higher the value of *H*, the higher the diversity of species in a particular community. The lower the value of *H*, the lower the diversity. A value of $H = 0$ indicates a community that only has one species.

4. **Margalef index:** The Margalef species richness index (d) is a measure of species richness in a sample. It provides a measure of species diversity that takes into account the total number of individuals and the overall sample size. The higher index value suggests greater species richness.

5. **Evenness index.** The equitability (J) index, also known as Pielou's evenness index, is a measure of how evenly distributed individuals are among different species in a community. The value of J ranges from 0 to 1. Higher values indicate higher levels of evenness.

Step 1: Download and install PAST

Step 2: Enter or import your data

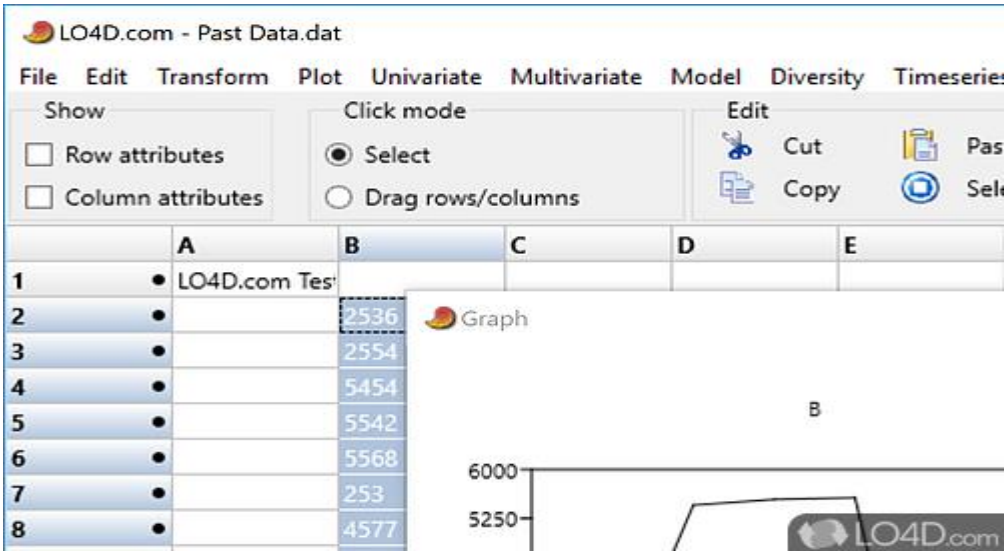
PAST has a spreadsheet-like interface for data entry.

1. Enable Edit mode: Click the "Edit" checkbox above the spreadsheet area to enable editing.
2. Input data manually: Click on any cell and type your data. The convention is that items or samples occupy rows, and variables or taxa occupy columns.
3. Name rows and columns: Give your rows and columns descriptive labels for easier reference. Go to the Edit menu and select Rename rows or Rename columns.
4. Paste from another program: You can copy data from programs like Excel and paste it directly into the PAST spreadsheet.
5. Load a data file: Use the File > Open function to load a pre-existing data file. PAST can read ASCII, Excel, and other formats.

Step 3: Perform a statistical analysis

PAST offers a wide array of statistical functions accessible from the main menu bar.

1. **Select your data**: Most operations are performed on the data that is selected (highlighted) in the spreadsheet. A single column, multiple columns, or the entire data set can be selected.
2. **Choose analysis**:
    - For diversity statistics, go to Diversity to calculate diversity indices like Shannon-Wiener index, Dominance index, Margalef index, Simson index, Evenness index.



28. Estimation of RBCs and WBCs in Vertebrate Blood

Aim

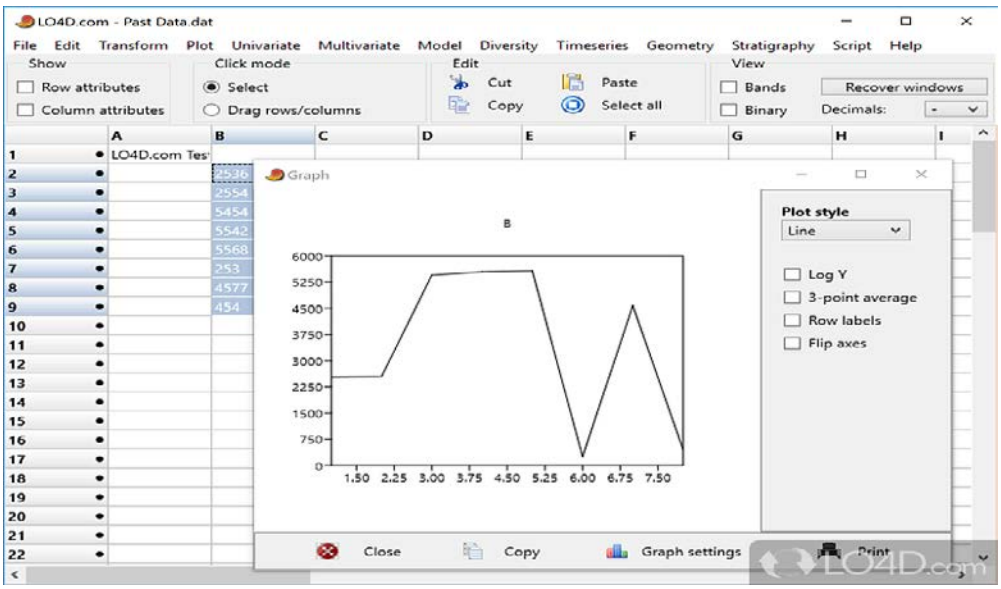
To estimate the number of Red Blood Cells (RBCs) and White Blood Cells (WBCs) in vertebrate blood using a hemocytometer.

I. Estimation of RBCs

Principle

RBC count is determined using a hemocytometer, where a known volume of blood is diluted with a diluting fluid and counted under a microscope. The dilution helps in spreading the cells evenly for accurate counting.

Materials Required



- Fresh vertebrate blood sample
- Hemocytometer (Neubauer's counting chamber)
- RBC diluting fluid (Hayem's or Formal-Citrate solution)
- Micropipette
- Coverslip
- Microscope

Procedure

1. Dilution of Blood Sample:

o Take 0.02 ml (20 µl) of blood in a pipette.

o Mix it with 4 ml of RBC diluting fluid (dilution ratio 1:200).

2. Filling the Hemocytometer:

o Place a coverslip over the hemocytometer grid.

o Using a micropipette, fill the counting chamber with the diluted blood.

o Allow the cells to settle for 2-3 minutes before counting.

3. Counting RBCs:

o Place the hemocytometer under a microscope at high power (40X objective).

o Count the RBCs in the five small squares of the central large square.

4. Calculation of RBC Count: Use the formula:

$$\text{Total RBC count} = \frac{\text{Number of cells counted} \times \text{Dilution factor} \times 10^6}{\text{Number of squares counted}}$$

o Dilution factor = 20

o Depth of chamber = 0.1 mm

o Area of one small square = 1/25 mm^2

II. Estimation of WBCs

Principle

The WBC count is estimated similarly using a different diluting fluid (Turk's fluid), which lyses RBCs and stains WBCs for easier counting.

Materials Required

- Fresh vertebrate blood sample
- Hemocytometer
- WBC diluting fluid (Turk's solution: Glacial acetic acid + Gentian violet)
- Micropipette

- Coverslip
- Microscope

Procedure

1. Dilution of Blood Sample:

o Take 0.02 ml (20 µl) of blood in a pipette.

o Mix it with 0.38 ml of WBC diluting fluid (dilution ratio 1:20).

2. Filling the Hemocytometer:

o Place a coverslip on the hemocytometer.

o Load the diluted blood sample carefully without overflowing.

3. Counting WBCs:

o Observe under low power (10X objective).

o Count the WBCs in the four large corner squares of the grid.

4. Calculation of WBC Count:

Total WBC count=Number of cells counted×Dilution factor×106

Number of squares counted

Dilution factor = 20

o Depth of chamber = 0.1 mm

o Area of one large square = 1 mm^2

29. Histochemical localization of Protein in Paraffin Section

INTRODUCTION

Proteins are chains of amino acids they play an important role in structural and functional organization of cells. Amino acids contains both carbonyl and amino group. proteins may be acidic, basic, neutral. If it contains great number of amino acids that possess high proportions of carbonyl radicals such as aspartic acid, glutamic acid, it will be an acidic protein. If it contains predominant amount of basic amino acids such as arginine and lysine it will be a basic protein. Proteins that contain amino acids with one amino group and one carbonyl group or balanced amount of acidic and basic amino acids it is said to be neutral. However amino acids are amphoteric that is they have the capacity to ionize as acid or base according to the medium in which they are. Generally, proteins are histochemically detected by mercuric bromophenol blue introduced by Durrum (1950) and later modified by Kunkel (1955)

Mercuric Bromophenol blue method

Principle

Proteins may be acidic, basic or neutral depending upon the proportion of carboxyl(acidic) and amino (basic) group. Bromophenol blue is an acidic dye and can normally bind with basic groups (NH2) of protein. But in the presence of mercuric salt, it can also bind with acid group of amino acid through the salt. Therefore, mercuric bromophenol reagent (a combination of mercuric chloride and bromophenol blue) combines with both basic and acid group of amino acids present in proteins. Hence it is a general test for all proteins. The mercuric chloride coagulates with cytoplasmic as well as nucleo protein.

AIM

To detect the presence of proteins in the given tissue.

Materials required

Tissues, stains, grades of alcohol, bromophenol blue stain, DPX (diphthalate xylene), microscope, coverslip.

Procedure

1. Deparafinize the slides in xylene in for 10 mints.
2. Pass through down grade absolute alcohol,90% alcohol and finally to distilled water each for 5 mints.
3. Dehydrate the slides again by passing it through clean 30% alcohol,50% and 70% alcohol.

4. Stain the slides by transferring it into the bromophenol blue (rinse the stain) for about 10-15 minutes.
5. Rinse the slides in tap water for 5 mints.
6. Transferr the slides to tertiary butyl alcohol for 5 mints.
7. Dry well and immerse in xylene for 5 mints.
8. Mount the slides using DPX
9. Observe the slide under microscope

RESULT

Proteins are stained blue in colour

COMMENT

Mercuric bromophenol blue is an indicative of protein. So all the region which contain protein are stained blue.it is a general technique for staining protein. All proteins get stained according to Beer lambert's law. That is whatever proteins are present in greater quantities they get more stained.

Preparation of test slide

1) Deparaffinize the slide in Xylene for 10 mints.
2) Bring the slides to water by passing it through absolute alcohol,90%,70%,50% and 30% alcohol and finally to distilled water each for 5 mints.
3) Treat the slides with 5% trypsin solution.
4) Again, upgrade to 70% alcohol.
5) Stain the slide by transferring it into bromophenol blue stain (since the stain is alcoholic) for 10-15 mints.
6) Rinse the slide in tap water for 5 mints.
7) Blot well and transfer the slides to tertiary butyl alcohol for 5 mints
8) Dry the slide well
9) Immerse the slide in xylene again for 5 mints
10) Mount by DPX
11) Observe the slide under microscope

Observation

When the slide is observed under microscope, the section is not stained.

CommentThe section is not stained because it is treated with trypsin. Trypsin is a proteolytic enzyme. When the section is treated with trypsin all the protein in the tissue is digested. Thus no blue colour is retained when treated with bromophenol blue.

30. Histochemical localization of Glycogen in Paraffin Section

PAS Technique

The histochemistry of carbohydrate centres around the periodic acid Schiff's reaction, a procedure used routinely in histology. It permits localization of carbohydrate such as glycogen, microproteins, and glycoproteins, glycogens the principal storage form of carbohydrates in animals and its identification and localization is often important. The specificity of PAS reaction is derived from sequential views of two selective reagents periodic acids and the Schiff's reagent periodic acid oxidise the free OH groups on two carbon atoms such as 1-4 glycol linkage in hexoses for the adjacent OH and aminogroups, hexoamines the OH groups are converted to aldehyde. The C-C Bond is cleaved and under the condition of PAS procedure and oxidation does not further proceed. The resulting aldehyde reacts with Schiff's reagent to produce a stable colored complex. The usual Schiff's reagent is fuschin sulphorous acid achromogenic bisulphide which when combined it forms an additional product with aldehyde and produce insoluble majenta product. Majenta colouration demonstrating the tissue to which the carbohydrate is attached.The Carbohydrate involved are 1,2 glycols. Carbohydrates which stain with this method include polysaccharides, monosaccharides, glycosaccharides.

AIM

To detect the presence of glycogen in the given tissue

MATERIALS REQUIRED

Tissue, stain, grades of alcohol, DPX, microscope, Schiff's reagent, periodic acid and coverslip

Procedure

1. Deparaffinized the slides by dipping in Xylene.
2. Hydrated the slide with water
3. Treated the slides with periodic acid for 10 mints.
4. After that wash the slide in running water for 5 mints.
5. Treated the slides with Schiff's reagent in darkness for 10 mints.
6. The slide is then passed through two changes of sulphuric acid solution about 1-2 mints
7. Washed again in running water for 5 mints.
8. Dehydration of the slide in upgrading series of starting from 30%,50% ,70%,90% and absolute alcohol
9. Cleaned the slide in xylene and mounted in DPX
10. Observed under microscope
11.

RESULT

Glycogen is stained in red colour.

COMMENT

PAS is a general staining technique for all carbohydrate. Periodic acid oxidises the free hydroxyl group of two adjacent carbon atom and converts into aldehyde. This resulting aldehyde reacts readily with the Schiff's reagent to produce stable colour complex

PREPARATION OF CONTROL SLIDE

PROCEDURE

1) Deparaffinized the slide by dipping in Xylene
2) Hydrated the slide with water
3) Treat the slide with saliva for half an hour to avoid oxidation with periodic acid
4) After that they are washed in running water for 5 mints
5) Treated the slides with Schiff;s reagent for 10 mints
6) The slide is then passed through two changes of sulphide solution for 1-2 mints each
7) Washed again in running water for 5 mints.
8) Dehydrated the slides in upgrading series of alcohol (since staining from 30%,50%,70%,90% alcohol)
9) Cleaned the slide in xylene and mounted by DPX
10) Observed under microscope

Result

The section was not stained

Comment: The section was not stained because it was treated with saliva solution. Saliva contains carbohydrate digesting enzymes" diesterase" which digest all carbohydrate present in section. Hence when they are treated with Schiff's reagent, no colour is developed.

31. Study of the Effect of Different Tonicities (Isotonic, Hypotonic, and Hypertonic Solutions) on Human Blood Cells Using Micrometry.

Aim:
To examine the effect of hypertonic, isotonic, and hypotonic solutions on the size and shape of human red blood cells (RBCs) and to measure the changes in cell diameter using an ocular micrometer.

Principle:
Blood is a liquid connective tissue composed of RBCs, WBCs, and platelets suspended in plasma. RBCs are surrounded by a semi-permeable membrane, which allows osmosis—the movement of water across the membrane in response to solute concentration differences.

• Isotonic solution (0.9% NaCl): Same solute concentration as cytoplasm; no net water movement → RBCs retain normal size and shape.
• Hypotonic solution (distilled water): Lower solute concentration than cytoplasm; water enters the cells → RBCs swell and may burst (hemolysis).
• Hypertonic solution (5% NaCl): Higher solute concentration than cytoplasm; water leaves the cells → RBCs shrink (crenation).

Micrometry allows precise measurement of RBC diameters under these conditions using a calibrated ocular micrometer.

Materials Required
- Fresh human blood (finger prick)
- Solutions:
  - 0.9% NaCl (isotonic)
  - 5% NaCl (hypertonic)
  - Distilled water (hypotonic)
- Compound microscope with ocular micrometer and stage micrometer
- Clean glass slides and cover slips
- Sterile lancet or needle
- Dropper or Pasteur pipette
- Cotton and spirit for sterilization

Procedure
A. Calibration of Ocular Micrometer
1. Place the stage micrometer on the microscope stage and focus it with the desired objective (e.g., 40×).

2. Superimpose the ocular micrometer scale (in the eyepiece) on the stage micrometer divisions.
3. Count how many ocular divisions coincide with a known length on the stage micrometer.
Example: 10 ocular divisions = 80 µm → 1 ocular division = 8 µm.

3. Record the calibration for each objective (10×, 40×).
4.

B. Preparation of Slides
1. Finger Prick: Clean the fingertip with spirit, prick with a sterile lancet, and discard the first drop of blood.
2. Place three drops of fresh blood on three clean slides.
3. To each slide, add one of the following:
O A drop of 0.9% NaCl (isotonic)
O A drop of distilled water (hypotonic)
O A drop of 5% NaCl (hypertonic)
4. Gently mix and cover each drop with a clean cover slip.

Observations

Solution Type	Appearance of RBCs	Ocular Divisions	Diameter (µm)
Isotonic	Normal biconcave discs	… …	
Hypotonic	Swollen, spherical, some lysed	… …	
Hypertonic	Shrunken, spiky (crenated)	… …	

Result
• Isotonic (0.9% NaCl): RBCs retain normal size (~7–8 µm) and shape.
• Hypotonic (distilled water): RBCs swell, diameter increases, some cells hemolyze.
• Hypertonic (5% NaCl): RBCs shrink, diameter decreases, cells become crenated.
This confirms that osmotic movement of water occurs depending on the tonicity of the surrounding solution.

Precautions
1. Use fresh blood to prevent clotting and ensure accurate results.
2. Slides and cover slips must be clean and dry.
3. Avoid air bubbles while mounting.
4. Measure several cells to obtain a reliable average.
5. Do not use excessive pressure when placing the cover slip.

32. Study of Mouthparts of Insects – Mosquito, Honey Bee and Housefly

Aim:

To dissect and display the mouth parts of Mosquito/ Honey bee/ Mosquito.

Materials required:

Glass slide, cover slip, Glycerine, dissection needle, blade.

Procedure:

1. Take a Mosquito/ Honey bee/ House fly, hold its head firmly and separate the head.

2. Transfer the mouthpart into a watch glass to 10 % KOH for 10 minutes for mosquito or 30% KOH for 10 minutes for house fly and honey bee.

3. Remove the KOH by repeated washing with distilled water and mount in glycerine

The mouthparts of a female mosquito are highly modified to form a proboscis that is adapted for piercing skin and sucking blood. Males have similar mouthparts, but they feed only on nectar. The proboscis is similar to a sword within a scabbard. A mosquito proboscis consists of a labium, labrum, hypopharynx, two mandibles and two maxillae. Mouthparts of a mosquito that goes into the skin are actually not rigid, but very flexible. The mouth parts of mosquito include:

- Labium – Labium is an outer sheath that

covers other mouthparts, it is not used to puncture the skin and does not go into the skin at all. When a mosquito punctures the skin, labium slides upwards its head and uncovers mandibles and maxillae, which are inserted into the skin.

- Mandibles – A female mosquito has a pair

of mandibles, which similar to maxillae are very thin filaments used to pierce the skin. Mandibles have sharp ends to help them go deeper into the flesh.

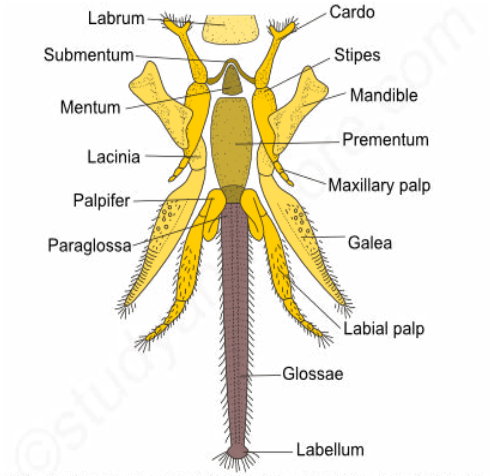
- Maxillae – A female mosquito has a pair of

maxillae, which also are used to pierce the skin, but instead of mandibles, maxillae have toothed blades to better grip the flesh, so other mouthparts can be moved deeper under the skin.

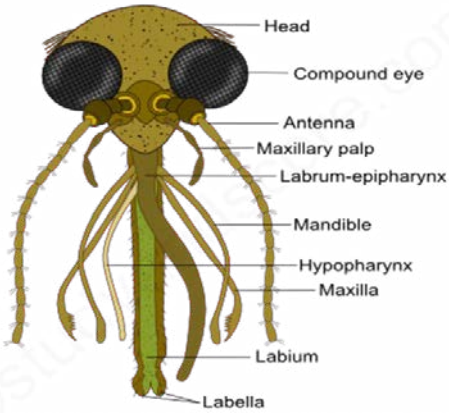
- Hypopharynx – A tube like organ with empty middle, through which a mosquito sends saliva down to a blood vessel.

- Labrum – A tube like organ with empty middle through which a mosquito pumps blood

middle, through which a mosquito pumps blood back up form a blood vessel

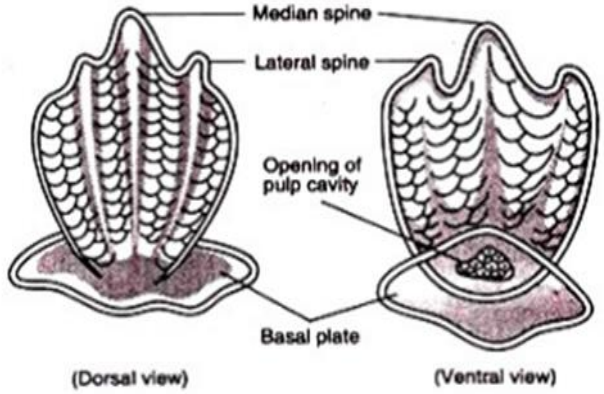


MOUTHPARTS OF HONEY BEE - CHEWING & LAPPING TYPE

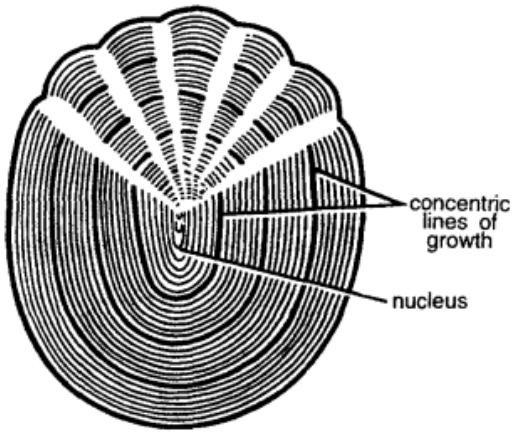


MOUTHPARTS OF MOSQUITO - PIERCING & CHEWING TYPE

PLACOID SCALES



Cycloid Scales



Ctenoid Scales