VIROLOGY & MICROBIAL BIOCHEMISTRY AND ANALYTICAL TECHNIQUES

M.Sc. MICROBIOLOGY

SEMESTER-I

PRACTICAL PAPER-I (105MB24)

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Virology & Microbial Biochemistry and Analytical Techniques

First Edition : 2025

No. of Copies :

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Published by:

Prof. V. VENKATESWARLU
Director, I/c
Centre for Distance Education,
Acharya Nagarjuna University

Printed at:

FOREWORD

Since its establishment in 1976, Acharya Nagarjuna University has been forging ahead in the path of progress and dynamism, offering a variety of courses and research contributions. I am extremely happy that by gaining 'A+' grade from the NAAC in the year 2024, Acharya Nagarjuna University is offering educational opportunities at the UG, PG levels apart from research degrees to students from over 221 affiliated colleges spread over the two districts of Guntur and Prakasam.

The University has also started the Centre for Distance Education in 2003-04 with the aim of taking higher education to the door step of all the sectors of the society. The centre will be a great help to those who cannot join in colleges, those who cannot afford the exorbitant fees as regular students, and even to housewives desirous of pursuing higher studies. Acharya Nagarjuna University has started offering B.Sc., B.A., B.B.A., and B.Com courses at the Degree level and M.A., M.Com., M.Sc., M.B.A., and L.L.M., courses at the PG level from the academic year 2003-2004 onwards.

To facilitate easier understanding by students studying through the distance mode, these self-instruction materials have been prepared by eminent and experienced teachers. The lessons have been drafted with great care and expertise in the stipulated time by these teachers. Constructive ideas and scholarly suggestions are welcome from students and teachers involved respectively. Such ideas will be incorporated for the greater efficacy of this distance mode of education. For clarification of doubts and feedback, weekly classes and contact classes will be arranged at the UG and PG levels respectively.

It is my aim that students getting higher education through the Centre for Distance Education should improve their qualification, have better employment opportunities and in turn be part of country's progress. It is my fond desire that in the years to come, the Centre for Distance Education will go from strength to strength in the form of new courses and by catering to larger number of people. My congratulations to all the Directors, Academic Coordinators, Editors and Lesson-writers of the Centre who have helped in these endeavors.

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M.SC. MICROBIOLOGY
SEMESTER-I

105MB24 - Practical-I Virology & Microbial Biochemistry and Analytical Techniques

Virology

1. Study of morphological characters of plant and animal viruses (photographs/ diagrams)
2. Study of symptoms caused by plant viruses
3. Chlorophyll estimation in healthy and viral infected leaves
4. Mechanical/Sap transmission of plant viruses
5. Seed transmission of plant viral disease
6. Isolation of bacterial viruses from sewage water
7. Routes of inoculation and cultivation of viruses in embryonated chicken eggs

Microbial Biochemistry and Analytical Techniques

1. Quantitative estimation of carbohydrates by DNS method
2. Quantitative estimation of proteins by Lowry's method
3. Separation of Aminoacids by paper chromatography
4. Determination of saponification number of fats
5. SDS PAGE separation of soluble proteins
6. PAGE separation of DNA and RNA

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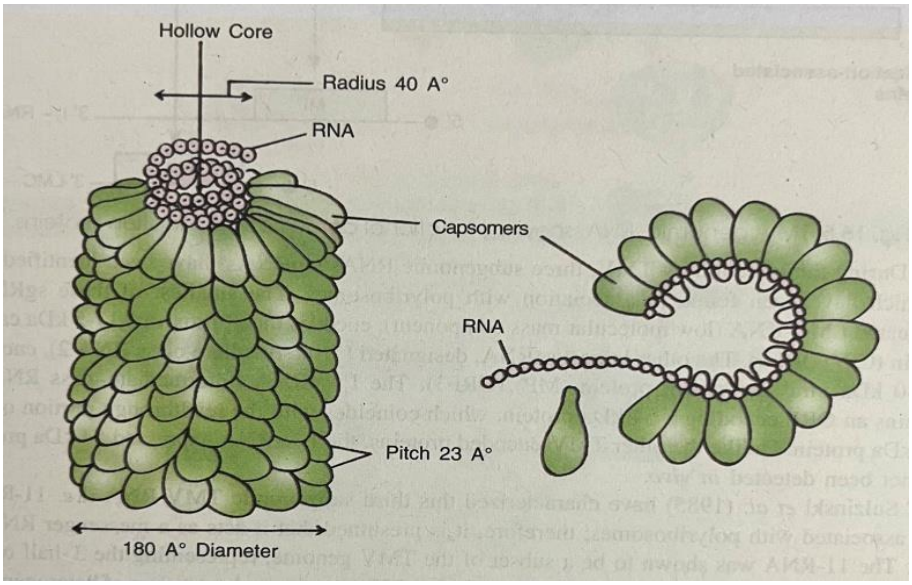
STUDY OF MORPHOLOGICAL CHARACTERS OF PLANT AND ANIMAL VIRUSES

PLANT VIRUSES

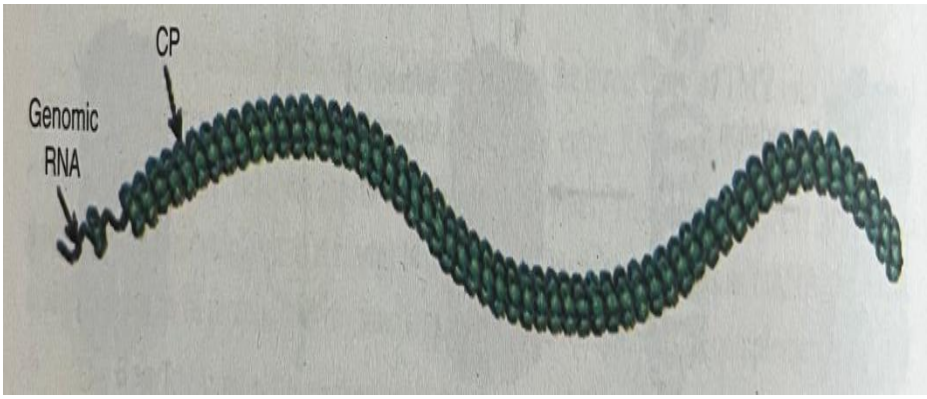
1. Tobacco Mosaic Virus



Tobacco Mosaic Virus

- TMV is a rod-shaped helical virus measuring about 280 x 150 µm with a molecular weight of 39 x 10^6 Daltons.
- It is made up of 2,130 protein subunits (capsomers) of identical size and are arranged around a central hollow core of 4 nm.
- Each protein subunit is made up of a single polypeptide chain which possess 158 amino acids and 17, 500 Daltons of molecular weight.
- Inside the protein capsid, a single stranded RNA molecule is spirally coiled to form helix.
- Virus RNA consists of 6,500 nucleotides.
- In one turn, the RNA contains 49 nucleotides and single protein subunit is linked with 3 nucleotides of RNA.
- The genome of TMV is a monopartite, linear, (+) sense mRNA.
- The mRNA is of 6.3 – 6.5 kb and produce five proteins during virus infection.
- TMV is the most serious pathogen that causes mosaic disease on tobacco leaves.
- The virus reduces the yield of the affected plant as well as the quality of the product i.e., nicotine content.

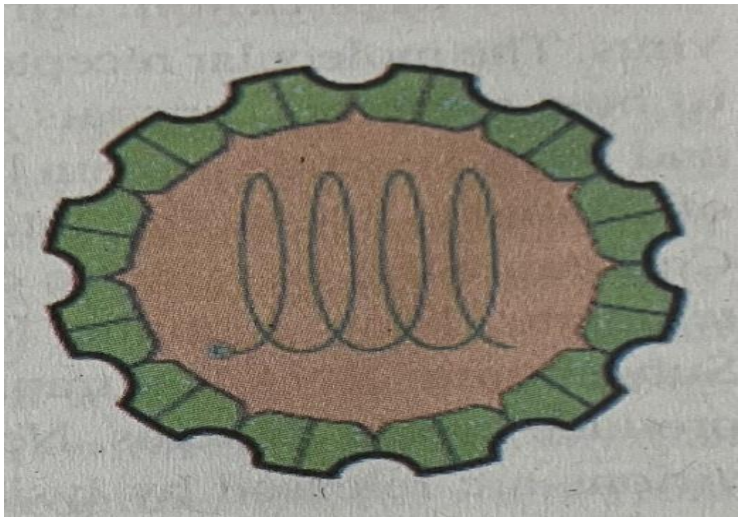
2. Potato Virus X



Potato Virus X

- Potato virus X is also called as *Solanum* virus and Potato latent mottle virus.
- It was first isolated by Vasudeva and Lal in 1945.
- The virion is non-enveloped, flexuous, and filamentous in structure.
- It measures 470 – 1000 nm or more long and 12 – 13 nm in diameter.
- Capsid is made up of identical protein subunits forming a helix of 3.3 nm pitch with a hole of 3 nm diameter.
- Possibly, a single subunit is associated with 3 or 4 nucleotides.
- The RNA of virus is infectious and serves as both the genome and viral mRNA.
- It consists of a single linear genome of (+) sense ssRNA having 5.8 – 9 kb of nucleotides.
- It encodes for 3 to 6 proteins and the 3’ terminus is polyadenylated.
- The virus infects several solanaceous plants and the infected plants become dwarf and deformity in foliage occurs.

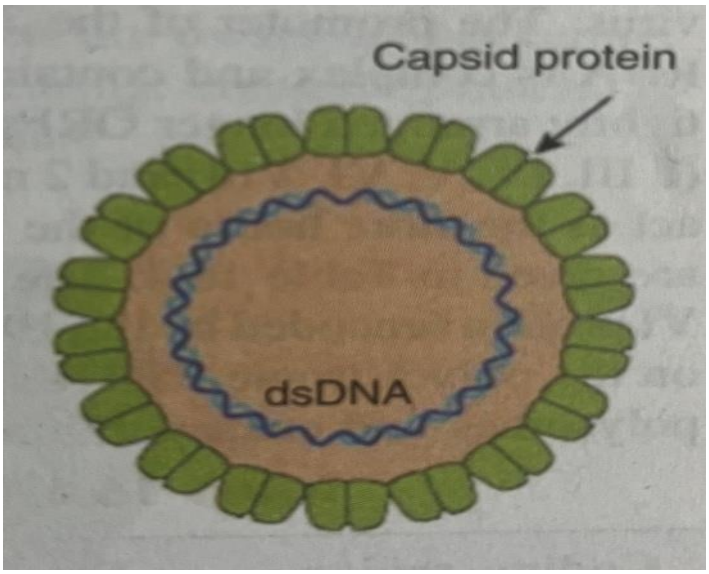
3. Cucumber Mosaic Virus



Structural organization of Cucumber Mosaic Virus

- Cucumber Mosaic Virus (CMV) belongs to the genus *Cucumovirus* in the family Bromoviridae.
- Virion consists of a capsid which is non-enveloped, icosahedral or bacilliform and measures 26 – 35 nm in diameter.
- The three genomic and one sub-genomic segments are encapsidated in distinct particles, resulting in several different types of virion.
- The capsid consists of 32 capsomers.
- Genome is segmented, tripartite linear (+) sense ssRNA which is composed of RNA1, RNA2, and RNA3.
- The viral genomes codes for both structural proteins and non-structural proteins.
- Genomic nucleic acid is infectious and it does not require coat protein or sub-genomic mRNA4 for infection.
- The complete genome is 8,621 nucleotides long.
- The symptoms caused by this virus include leaf mottling, crinkling and curling of edges on cucumber. Petioles and internodes are shortened resulting in stunted and compact appearance of the plant.
- The fruits are mottled with light yellow and white patches.

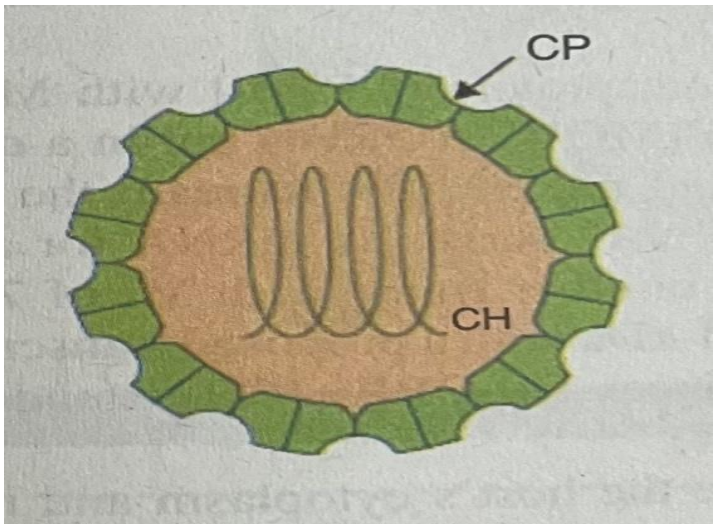
4. Cauliflower Mosaic Virus (CaMV)



Cauliflower Mosaic virus

- Cauliflower Mosaic Virus is a type member of the caulimoviruses which belongs to the family Caulimovididae.
- CaMV are the only plant viruses that contain dsDNA genome.
- The virion shows an icosahedral symmetry with a diameter of 52 nm.
- It is made up of 420 capsid protein subunits arranged with a triangulation T=7.
- Each virion consists of a circular dsDNA molecule of about 8.0 kb.
- Virions occur in the cytoplasm and in some cases in the nucleus.
- In the cytoplasm of infected cauliflower leaves, CaMV forms characteristic X bodies which are rounded structures.

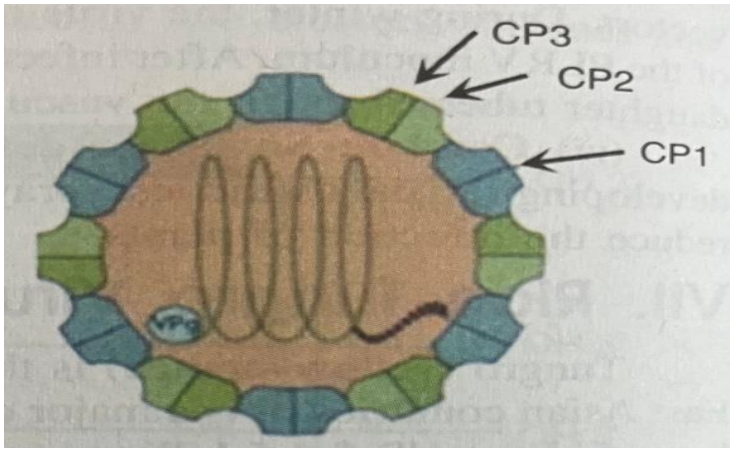
5. Potato Leaf Roll virus



Potato Leaf Roll virus

- Potato leaf roll virus (PLRV) belongs to the genus *Polerovirus* and family Luteoviridae.
- PLRV particles are isometric icosahedral, non-enveloped virion of 25 – 30 nm diameter with 180 copies of capsid protein.
- It contains 30% of single stranded, positive-sense RNA with a molecular weight of about 5.9 kb.
- The viral genome is covalently linked to a 7.2 kDa protein (VPg) at its 5' start without any poly (A) tail at 3' end.
- The viral RNA codes for at least 8 open reading frames (ORFs) that are located in two clusters of genes and separated by 197 nucleotides of non-coding sequences.
- The viral host range is restricted to Solanaceae family members and is transmitted via green peach aphids.
- Symptoms caused by PLRV in plants include dwarfing, puckering, mottling, vein clearing, excessive branching etc.

6. Rice Tungro Spherical virus



Rice Tungro Spherical virus

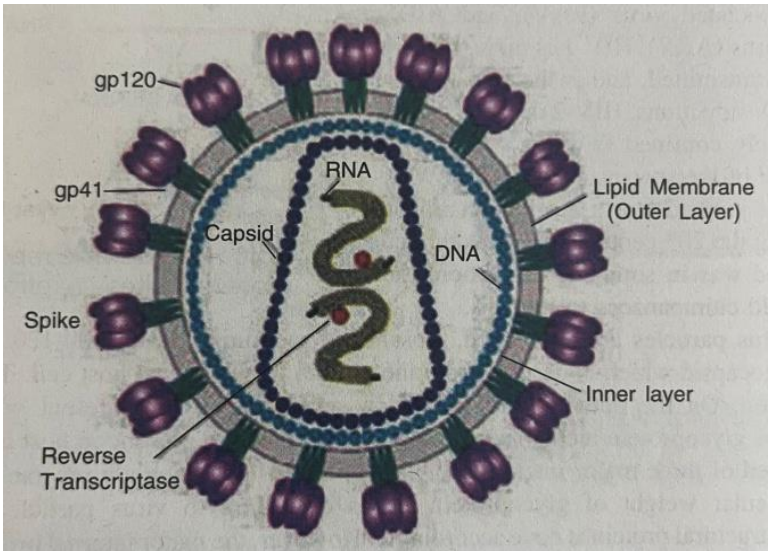
- The Rice tungro spherical virus (RTSV) is non-enveloped, isometric, and icosahedral

particles of about 30 nm in diameter.

- It consists of three capsid proteins namely CP1, CP2 and CP3.
- Virion genome is 9 – 12 kb long, linear positive strand ssRNA.
- The 5' terminus has a genome linked protein (VPg) and a poly (A) tail is present at 3' end.
- RTSV is transmitted by aphids and green leafhoppers.

ANIMAL VIRUSES

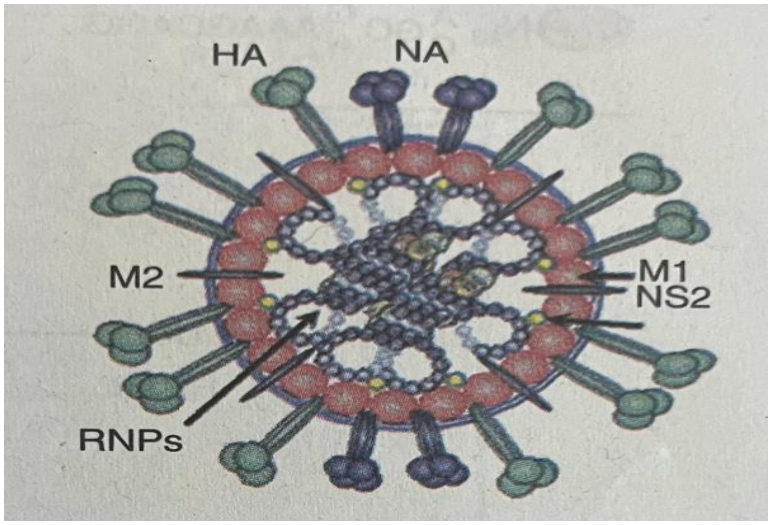
1. Human Immunodeficiency Virus



HIV structure

- HIV is different in structure from other retroviruses.
- It causes Acquired Immuno-deficiency syndrome (AIDS) in humans.
- HIV is of 100-200 nm diameter containing a protein envelope to which spicules of glycoprotein are attached.
- Three dimensional structure of the viral envelope appears like a sphere made up of from an assembly of 12 pentamers and 20 hexamers.
- The icosahedral capsid is conical and composed of 2,000 copies of the viral protein p24.
- Two copies of positive sense ssRNA molecules are tightly bound with nucleocapsid proteins, p7 and enzymes such as reverse transcriptase, proteases, ribonuclease and integrase.
- Matrix is composed of viral protein p17 which surrounds the capsid.
- The viral envelope is composed of two layers of phospholipids.
- The RNA genome consists nine genes and encode 19 proteins.
- Three of nine genes (gag, pol and env genes) contain the information which is required to synthesize the structural proteins. The other six genes are regulatory genes.

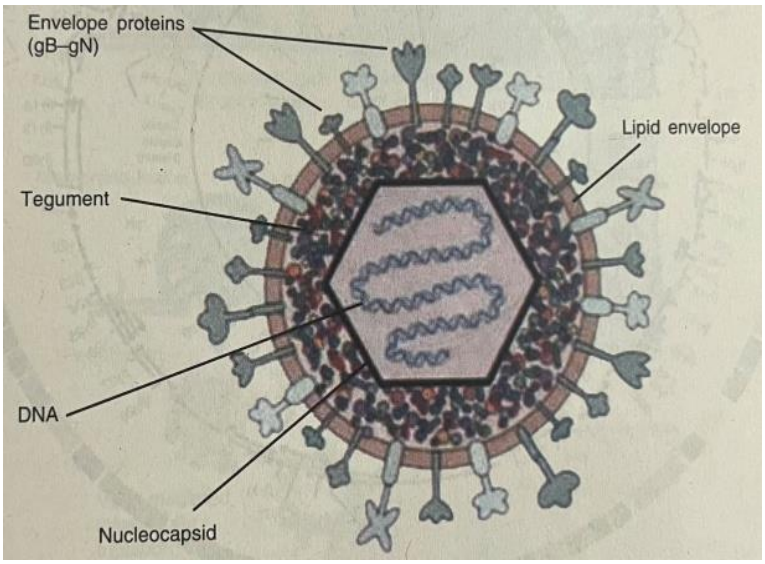
2. Influenza Virus



Influenza Virus

- Particles of influenza virus are highly pleomorphic, mostly spherical having 80-120 nm diameter.
- The outer surface of the viral particle consists of a lipid envelope.
- From lipid envelope, prominently two types of about 500 glycoprotein spikes are projected, Haemagglutinin (HA) and Neuraminidase (NA). Each of the spikes projects 10 to 14 nm from the surface.
- The number of N spikes per virion is about 100.
- The nucleocapsid is enclosed by lipoprotein membrane.
- The inner side of the envelope is lined by two types of the matrix proteins, M1 and M2 as well as non-structural proteins at some places.
- The genome consists of 7 to 8 segments of linear negative sense ssRNA.
- The total length of the genome is 12,000 – 15,000 nucleotides.

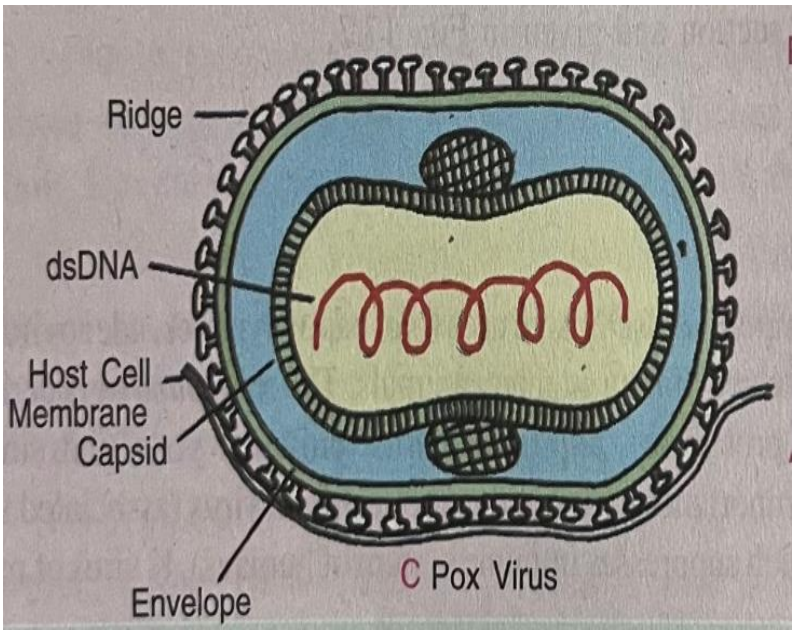
3. Herpes Virus



Herpes Virus

- The nucleocapsid is spherical icosahedral and of about 100 nm diameter which is enclosed in a 30 nm thick glycoprotein lipid envelope.
- The nucleocapsid contains the spikes.
- The structure of the herpes virus is very complex.
- Capsid of the herpes virus is an icosahedron of triangulation number T = 16.
- The capsid measures about 100 nm and made up of hollow columnar capsomers (162) forming icosahedral symmetry.
- There are 150 hexamers and 12 pentamers.
- Outside the capsid is the tegument (matrix), a protein filled region.
- The envelope contains numerous viruses-encoded glycoproteins that are visible as spikes which project from surface.
- Genome is large and the nucleic acid is a linear dsDNA of about 235 kb.
- The DNA consists of two fragments – one long and other short DNA molecule- which are covalently linked at the identical region.

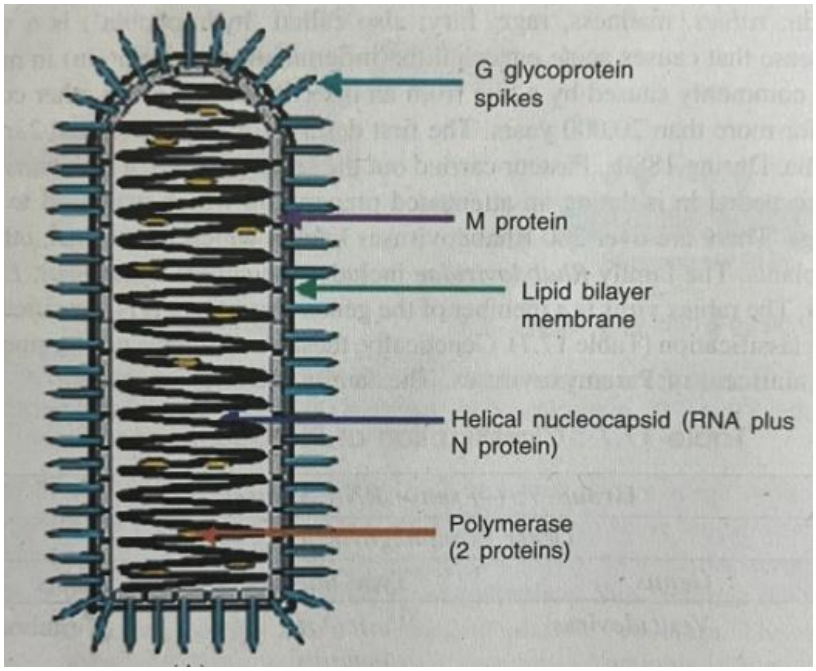
4. Pox Virus



Pox Virus

- Oval or brick-shaped virus with 200 nm diameter and 300 nm length.
- The external surface is ridged in parallel rows.
- Virion is enveloped externally and internally.
- The genome is a single, linear, ds DNA.
- The particles are extremely complex and contain more than 100 different proteins.
- The genome has about 250 genes.
- Most of the essential genes are located in the central part of the genome, whereas non-essential genomes are located at the ends.
- Antigenically, pox virus is very complex inducing both specific and cross-reacting antibodies.

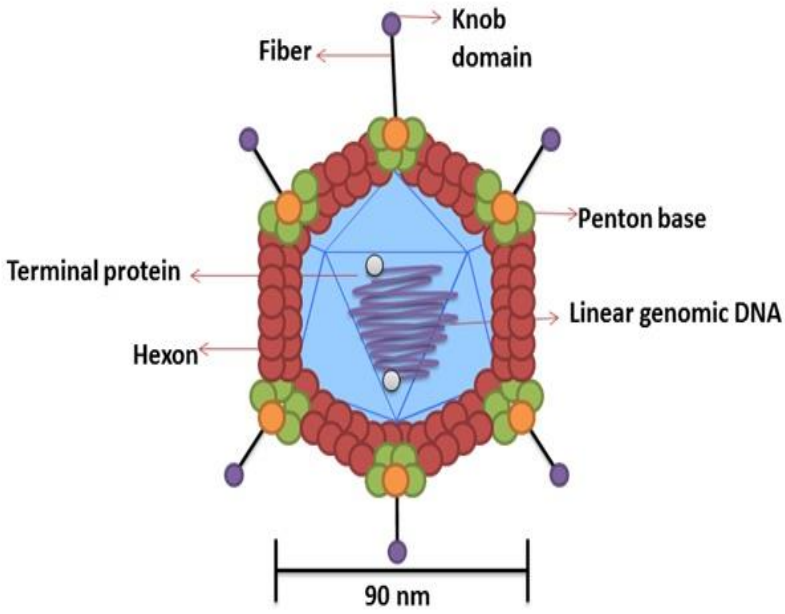
5. Rabies Virus



Rabies virus

- Rabies virus or Rhaddo virus is a member of the genus *Lyssavirus*.
- It has a unique bullet-shaped appearance and measures about 180 x 75 mm.
- It has an envelope with prominent spikes on surface.
- Spikes are made up of G protein that haemagglutinate RBCs.
- The envelope is lined by matrix protein and contains nucleocapsid which wound helically inside the core.
- Rabies virus genome encodes five structural proteins and two non-structural proteins.
- The glycoprotein forms approximately 400 trimeric spikes which are tightly arranged on the surface of the virus.
- The M protein is associated both with the envelope and the RNP.
- The genome is monopartite, linear and ssRNA and consists 11,000 – 15,000 nucleotides.

6. Adeno Virus



Adeno virus

- Adeno virus is non-enveloped having 90 – 100 nm diameter with icosahedral symmetry.
- It is composed of 252 capsomers – 240 hexons forming the faces and 12 pentons at vertices of icosahedron.
- The penton bears a slender fiber with a slender shaft and globular head.
- The penton fibers involve in the process of attachment of virus particles to host.
- The globular domain of the fiber is responsible for recognition of the cellular receptor.
- Genome of adeno virus is linear, non-segmented, dsDNA of 30 -38 kb size.
- The dsDNA is associated with two major core proteins – terminal protein and VII.
- The terminal protein is covalently attached to the 5’ end of the genome strand.
- Protein VII is arginine-rich basic protein which is covalently associated with the genome.

Experiment No. 2

STUDY OF SYMPTOMS CAUSED BY PLANT VIRUSES

Aim: To observe and note the important symptoms of plants infected with disease causing viruses.

Tomato leaf curl:

- Characterized by severe stunting of the plants with downward rolling and crinkling of the leaves together with mottling.
- Newly formed leaves show chlorosis.
- Older, curled leaves become leathery and brittle.
- Stunting of plant is due to shortening of the internodes.
- The diseased plants look pale and produce more lateral branches resulting in bushy growth.
- Causes complete sterility of plant.
- Virus is transmitted by whitefly and by grafting.

Spotted wilt of tomato:

- Characterized by bronze markings on the upper surface of young leaves, which extend from leaf blade to the petiole and stem.
- The upward rolling of leaves occurs.
- Yellow spotting of fruits and finally wilting of stem occurs.
- Disease is transmitted by vectors.

Leaf curl of papaya:

- Prevalent in several parts of India.
- Characterized by severe curling, crinkling and deformation of leaves. Mostly young leaves are affected.
- Other symptoms like vein-clearing, reduced size, inward rolling of leaves and thickening of veins are also common.
- Twisting of petioles may occur.
- Diseased leaves become leathery and brittle, plants become stunted, fruit yield reduced, defoliation often results.
- Caused by virus which is transmitted readily by grafting and by means of whitefly.

Papaya mosaic:

- Most devastating viral disease prevalent in the central belt of India.
- Most serious in young plants.
- The top young leaves of diseased plant are much reduced in size and show blister- like patches of dark green tissue, alternating with yellowish-green lamina.
- The leaf petiole is reduced in length and the top leaves assume an upright position.
- The infected plants show degeneration and marked reduction in growth.
- Fruits develop circular water-soaked lesions with a central solid spot.
- Fruits are elongated and reduced in size.
- Virus is mechanically transmitted.

Yellow vein mosaic of Bhendi:

- Characterized by yellowing of entire network of veins in leaf blade.
- In severe cases young leaves turn yellow, reduced in size, plant is stunted.
- Infection may start at any stage of plant growth.
- Vein clearing is followed by veinal chlorosis of leaves.
- Veins and veinlets may appear thickened.
- Due to infection, flowering of the plant is restricted; fruits if formed are small, rough and harder.
- Caused by bhendi vein-clearing virus transmitted by vectors.

Potato mosaic:

- Caused by potato virus Y.
- Chief symptom is a blotching mottle, which becomes apparent about 3 weeks after planting.
- The mottled mosaic symptoms are more pronounced on young and newly formed leaves.
- Later necrosis appears on the veins on the lower surface of leaf followed by similar lesions on the upper surface.
- Necrotic lesions spread along the veins to the petiole and stem.
- The affected leaves droop and wither.
- The entire plant becomes stunted and is easily identified even from a distance.

Potato streak:

- Caused by virus namely Solanum virus 4.
- Causes chlorophyll mottling, which appears as streaks on the leaf.
- Often necrotic spots are produced on the young shoots, and the tender shoots and buds may die.

Rosette disease of Ground nut:

- Virus is transmitted through grafting and by *Aphis craccivora*.
- Characterized by a clumping together of the foliage.
- Reduction in the size of leaf blade occurs.
- Occurrence of severe mottling.
- Bunching and erect appearance of the plant occur.

Little leaf of apple:

- Characterized by reduction in the size of leaves.
- Reduction of internode length.
- Mottling and deformation of the leaf blade occur.
- The leaf buds either open late or not open at all and eventually die.

Sugarcane mosaic disease:

- The causative virus of sugarcane mosaic disease belongs to the Potato Virus Y group.
- More commonly, elongated yellowish stripes alternate with the normal green portions of the leaf to give a mosaic appearance.
- In severe infections, the chlorotic area may be greater than the healthy.
- The mosaic symptoms are more clearly apparent on the younger basal portions

than on older leaves.

- Similar symptoms are seen on the leaf sheaths and stalks, and in highly susceptible varieties yellow stripes are seen even on the canes.
- Sometimes, elongated necrotic lesions are produced on stalks, in which case stem splitting is common.
- The entire plant is stunted and becomes chlorotic, and easily identified from a distance.

Tobacco mosaic disease:

- Disease is caused by Nicotiana Virus I.
- It is the first recognized viral disease of plants.
- First symptoms are light discoloration along the veins of the youngest leaves.
- Soon after the infection, leaves develop a characteristic light and dark green pattern, the dark green areas usually associated with the veins.
- Sometimes, the disease is accompanied by a blistered appearance of the leaf due to more rapid growth of the dark green tissues.
- Plants that are infected early in the season are very much stunted while those infected late in the season show little reduction in size.

Leaf curl of Tobacco:

- Disease appears in fields usually about 4-6 weeks after transplanting.
- Characterized by downward curling of young leaves i.e., leaf margin turn downwards and come together at bottom, exposing the middle upper surface of the leaf blade.
- Usually thickened leaf blade exhibits vein clearing symptoms.
- As disease advances, plant becomes dwarf and most of the leaves are curled.
- The inflorescence is greatly condensed and the veins of the calyx thickened and green enation and leaf-like outgrowths along the veins are also common.

Tungro disease of Rice:

- Disease is characterized by stunting of plant and discoloration of leaves, ranging from various shades of yellow to orange and rusty blotches spreading downwards from the leaf tip.
- Young leaves how a mottled appearance and slightly twisted, whereas older leaves appear rusty colored.
- In less susceptible varieties infection delays flowering.
- In highly susceptible varieties, if infection takes place at early stages the plants may die before flowering.
- The virus is transmitted by green leaf hoppers.

Orange leaf disease of tobacco:

- The disease is characterized by an intense orange color which may later change to a golden or brownish yellow.
- Reduced tillering occurs in diseased plants.
- As the disease advances, there may be longitudinal rolling of leaves and rapid death of plant.
- Transmission occurs by leaf hopper.

Experiment No. 3

CHLOROPHYLL ESTIMATION IN HEALTHY AND VIRAL INFECTED LEAVES

Aim: To estimate and compare the total chlorophyll content in healthy and virus infected leaves.

Requirements: Healthy and virus infected leaf samples, Pestle and mortar, Centrifuge, Centrifuge tubes, Test tubes, Measuring cylinder, Spectrophotometer, Acetone.

Principle: Chlorophyll is extracted in 80% acetone and the absorption at 663 nm and 645 nm are read in spectrophotometer. Using the absorption coefficients, the amount of chlorophyll is calculated.

Procedure:

1. Weigh 1.0 g of finely cut and well mixed representative sample of healthy leaves into a clean mortar.
2. Grind the leaf material to a fine pulp with addition of 20 ml of 80% acetone.
3. Centrifuge the ground contents for 5 minutes at 5,000 rpm and transfer the supernatant to a 100 ml volumetric flask.
4. Grind the residue with 20 ml of 80% acetone, centrifuge and transfer the supernatant to the same volumetric flask.
5. Repeat the same procedure until the residue become colorless. Wash the mortar and pestle thoroughly with 80% acetone and collect the clear washings in the volumetric flask.
6. Make up the volume to 100 ml with 80% acetone.
7. Read the absorbance of the solution at 645 and 663 nm wavelength in spectrophotometer against the solvent blank (80% acetone).
8. Repeat the steps from 1-7 with virus infected leaf sample.
9. Calculate the total chlorophyll amount present in healthy leaves and virus infected leaves per gram tissue using the following equation

$$\text{mg total chlorophyll / gram tissue} = 20.2\,(A_{645}) + 8.02\,(A_{663}) \times \frac{V}{1000 \times W}$$

where A = absorbance at specific wavelengths
V = Final volume of chlorophyll extract in 80% acetone
W = Fresh weight of tissue taken

Result: Relatively less amount of total chlorophyll is seen in virus infected leaves when compared to that of healthy leaves.

Experiment No. 4

MECHANICAL / SAP TRANSMISSION OF PLANT VIRUSES

Aim: To transmit the Peanut green mosaic virus (PGMV) of groundnut and *Tobacco streak Ilarvirus* (TSV) of Pumpkin to healthy groundnut and Pumpkin plants by mechanical inoculation.

Requirements:
Pestle and Mortar, Monobasic and Dibasic Potassium Phosphate salts, 2-Mercaptoethenol, 600 mesh Carborundum power, Muslin cloth, Paper tags.

Virus source: Peanut green mosaic virus (PGMV) infected groundnut young fresh leaves.
Test source: Healthy groundnut plant at two to three leaf stage.

Preparation of inoculation buffer, pH 7.0:

K_2HPO_4 - 1.201 g.
KH_2PO_4 - 0.421 g.

Dissolve in 1000 ml of dist. H_2O and added 2 ml of 2-mercptoethanol.

Procedure:

- The required number of groundnut healthy potted test plants was selected and these plants were labeled with paper tags.
- Few young PGMV infected groundnut leaves were collected and ground separately in mortars using pestle by adding chilled inoculation buffer at the rate of 2 ml/g of leaf tissue.
- After thorough grinding, the extracts were filtered separately through muslin cloth.
- Carborundum (600 mesh) was uniformly dusted on the leaves of test plants.
- The prepared inocula were gently rubbed on the surface of abrasive dusted leaves of test plants using muslin cloth pad in one direction only i.e. from the petiole to the apex of the leaf.
- After 30 min. of inoculation the inoculated plants were sprinkled with tap water to remove the excessive inoculum and source tissue debris.
- The inoculated pots were kept in insect proof wire mesh house for development of symptoms.
- The symptoms were observed and after 2-3 weeks.

No. of plants inoculated	No. of plants infected	No. of leaves infected	% of Transmission	Average leaves infected/plants.

Results: Symptoms development indicates the sap transmission of viruses.

Experiment No. 5

SEED TRANSMISSION OF PLANT VIRUSES

Aim: To demonstrate the transmission of plant viruses through seeds.

Principle: Seed transmission of plant viruses, also known as vertical transmission, occurs when a virus infects the seed embryo, leading to infected seedlings upon germination. This process can happen through direct invasion of the embryo from the parental plant or indirectly through infected pollen or ovules.

Procedure:

1. Collect Seeds from Symptomatic Plants:
Gather seeds from plants exhibiting viral symptoms. Seeds from non-symptomatic plants can serve as a control.

2. Seed Treatment:
Extract seeds and wash them with a 4% hydrochloric acid solution followed by a 10% bleach solution to ensure that any surface contamination is removed.

3. Sow Seeds in Sterile Medium:
Sow the seeds in sterile soil under controlled conditions, such as an insect-proof glasshouse, to prevent further infection.

4. Germination and Observation:
Provide optimal conditions for germination and growth. Observe for the appearance of typical viral symptoms on the seedlings.

5. Virus Detection:
Use the methods like Immunosorbent Electron Microscopy (ISEM) to detect and identify the virus within the seeds.

Result: Appearance of viral symptoms on the seedlings confirms the seed transmission of viruses.

Experiment No. 6

ISOLATION OF BACTERIOPHAGES FROM SEWAGE WATER

Aim: To isolate the bacteriophages (coliphages) from sewage water.

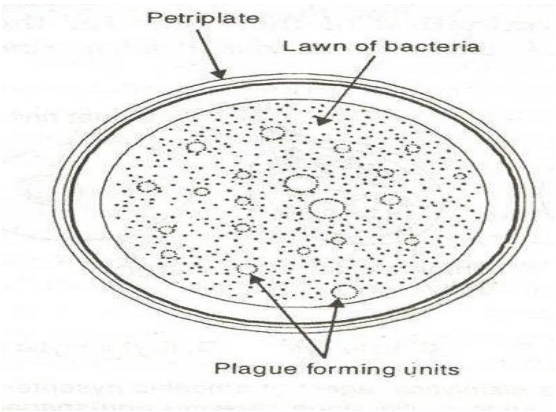
Requirements: Sewage sample, phage broth, *Escherichia coli* culture, Centrifuge, Incubator, Tryptone agar plates, Tryptone soft agar, Membrane filters, Pipettes.

Principle: The phages of the sewage sample when added to molten soft agar medium containing the host bacterial culture and allowed for incubation result in the formation of plaques amidst the lawn of bacterial growth. Each plaque represents a large number of phages that are formed from a single phage particle on multiplication in their host and release to the outside.

Procedure:

1. Add 5 ml of bacteriophage broth, 5 ml of *E. coli* culture and 45 ml of raw sewage sample to 250 ml conical flask in aseptic conditions.
2. Incubate the culture for 24 hours at 37^{o}C and centrifuge the culture at 2,500 rpm for 20 minutes.
3. Decant the supernatant into a 125 ml flask.
4. Filter the supernatant through sterile membrane filter to collect the bacteria-free but phage containing filtrate.
5. Prepare the tryptone agar plates by pouring sterilized tryptone agar into the plates at molten state and allow the medium to solidify.
6. Prepare tryptone soft agar tubes and sterilize the medium.
7. To the molten tryptone soft agar tubes, add 1 ml of *E. coli* culture and few drops of filtrate, mix thoroughly and pour into the tryptone agar plates.
8. Allow the plates for the solidification of soft agar.
9. Incubate the plates in inverted position at 37^{o}C for 24 hours.
10. Observe the plates after incubation for plaques formation.

Result: Formation of plaques indicate the presence of coliphages in the raw sewage sample.



Plaque formation

Experiment No. 7

ROUTES OF INOCULATION AND CULTIVATION OF VIRUSES IN EMBRYONATED CHICKEN EGGS

Aim: To inoculate and cultivate the animal viruses in different locations of an embryonated chicken egg.

Principle: Embryonated egg inoculation of animal viruses involves injecting a viral suspension into the fluid of a developing chicken egg to cultivate and study the virus. This method is commonly used for isolating, propagating, and producing vaccines against various viruses.

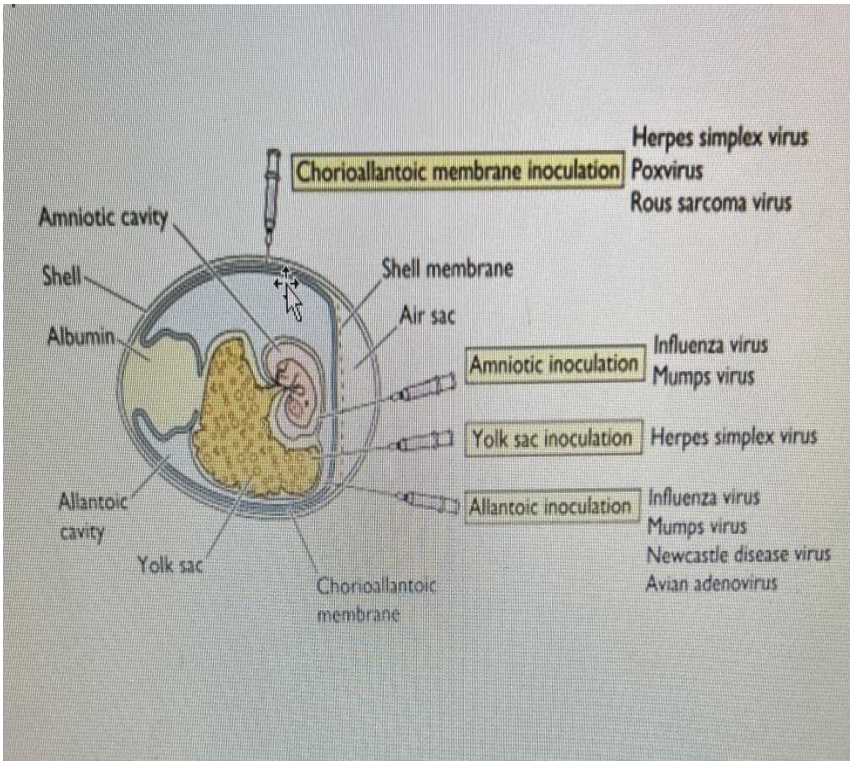
Procedure:

- Select and use 5-12 day old fertile eggs, ensuring the shell is healthy and sterile.
- Place the egg before some light source and locate the different sites of an embryonated egg i.e, allantoic cavity, amniotic cavity, chorioallantoic membrane (CAM), yolk sac, embryo etc., to guide the inoculation site. This is called as candling process.
- Then sterilize the eggshell with alcohol (70% ethanol).
- Make a small hole in the shell using a sterile eggshell puncher or carborundum disc.
- Inject the viral suspension (0.1 ml) into appropriate inoculation site of the egg using a syringe and needle.
- The needle should penetrate approximately 16 mm into the egg to reach the specific inoculation site namely amniotic cavity, allantoic cavity etc.
- Then seal the hole with a tape or melted wax material.
- Incubate the inoculated egg at 37^{o} C for 36-72 hours.
- Observe the egg for signs of viral growth, such as embryo death, pathological changes, cell damage, or the formation of pocks or lesions on the egg membranes.
- If necessary, perform a hemagglutination test to confirm viral growth.
- For harvesting and storage, cool the eggs to 4^{o} C after incubation to stop viral replication.
- Then carefully open the eggshell and membrane, avoiding contamination.
- Collect the fluid, from the inoculated site, containing the virus. Clear the fluid of debris by centrifugation and store at -80o C for long-term preservation.

Result: Appearance of different signs specific to viruses and their locations indicate the presence of cultivated viruses.

Precautions:

- Maintaining sterility throughout the process is crucial to prevent contamination.
- Different viruses have different growth requirements, so it's important to optimize the protocol accordingly.
- Handle embryonated chicken eggs and viral samples with appropriate safety precautions.



Inoculation of viruses into different sites/locations of an embryonated egg

MICROBIAL BIOCHEMISTRY AND ANALYTICAL TECHNIQUES EXPERIMENTS

Experiment No.1

QUANTITATIVE ESTIMATION OF CARBOHYDRATES BY DNS METHOD

Aim: To quantitatively estimate the amount of reducing sugars in a sample using the DNS (3,5-dinitrosalicylic acid) method.

Principle:

The DNS method is based on the reduction of 3, 5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid by the free aldehyde or ketone groups of reducing sugars under alkaline conditions, producing an orange-red color. The intensity of this color is proportional to the concentration of reducing sugars in the sample. This reduction causes a shift in absorbance that can be measured spectrophotometrically at 540 nm. A standard curve is generated using known concentrations of glucose (or another reducing sugar), which is then used to determine the sugar concentration in unknown samples.

Materials Required:

- DNS reagent
- Standard glucose solution (1 mg/mL)
- Unknown carbohydrate sample
- Distilled water
- Test tubes
- Pipettes and micropipettes
- Water bath
- Spectrophotometer
- Cuvettes
- Beaker, measuring cylinder

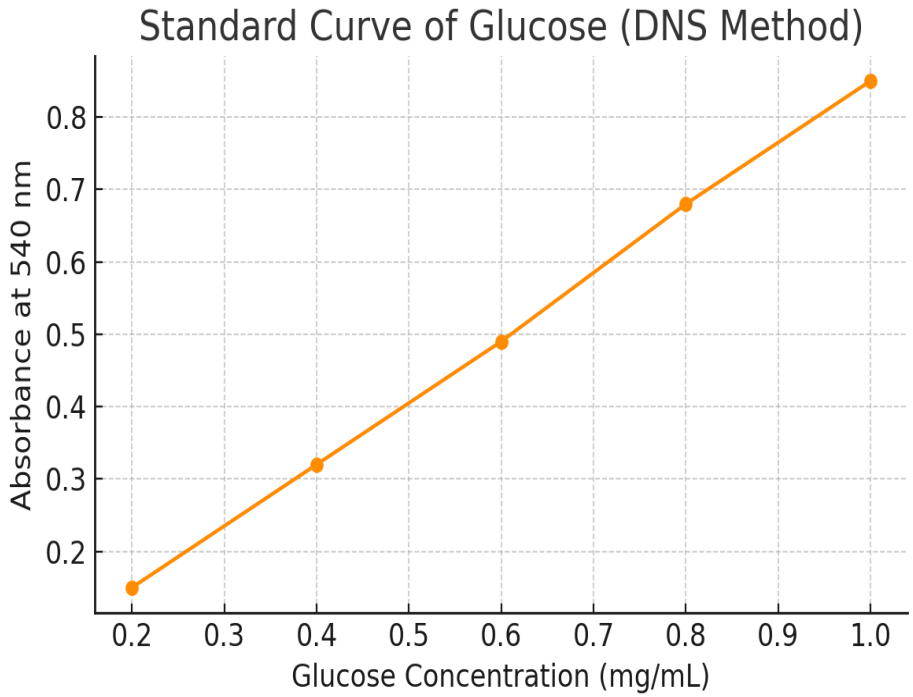
Procedure:

1. Prepare a series of glucose standard solutions: 0.2, 0.4, 0.6, 0.8, and 1.0 mL of glucose solution, making up each to 1 mL with distilled water.
2. Add 1 mL of DNS reagent to each test tube.
3. Prepare a blank with 1 mL distilled water + 1 mL DNS.
4. Add 1 mL of unknown sample to another tube with 1 mL DNS reagent.
5. Heat all the tubes in a boiling water bath for 5–10 minutes.
6. Cool the tubes to room temperature.
7. Dilute each tube with 10 mL distilled water.
8. Measure the absorbance at 540 nm using a spectrophotometer.

9. Plot a standard graph of absorbance vs. glucose concentration.

Results:

Glucose Concentration (mg/mL)	Absorbance at 540 nm
0.2	0.15
0.4	0.32
0.6	0.49
0.8	0.68
1.0	0.85



Discussion / Interpretation of Results:

The linear relationship observed between glucose concentration and absorbance confirms the reliability of the DNS method for estimating reducing sugars. The orange-red color intensity increases with sugar concentration, as expected. Any deviation or non-linearity in the graph could be due to sample impurities, reagent degradation, or incorrect boiling time. The concentration of reducing sugar in the unknown sample can be accurately determined from the graph. This technique is simple and widely used for carbohydrate quantification in microbiological, food, and fermentation studies.

Experiment No. 2

QUANTITATIVE ESTIMATION OF PROTEINS BY LOWRY'S METHOD

Aim:

To quantitatively estimate the protein concentration in a given sample using the Lowry's method.

Principle:

Lowry's method relies on two chemical reactions: first, the peptide bonds in proteins react with copper under alkaline conditions (Biuret reaction) to form a complex; second, the aromatic amino acids in the protein reduce the Folin-Ciocalteu reagent, resulting in a blue-colored complex. The color intensity is directly proportional to the protein concentration and can be measured at 660 nm. A standard curve is prepared using bovine serum albumin (BSA) as a reference protein to determine the concentration of unknown samples.

Materials Required:

- Standard BSA solution (1 mg/mL)
- Unknown protein sample
- Alkaline copper sulfate reagent
- Folin-Ciocalteu reagent (diluted)
- Distilled water
- Pipettes and micropipettes
- Test tubes
- Water bath
- Spectrophotometer
- Cuvettes

Procedure:

1. Prepare standard BSA solutions: 0.2, 0.4, 0.6, 0.8, and 1.0 mL, making up to 1 mL with distilled water.
2. Add 5 mL of alkaline copper sulfate reagent to each tube.
3. Incubate at room temperature for 10 minutes.
4. Add 0.5 mL of diluted Folin-Ciocalteu reagent to each tube.
5. Mix well and incubate for 30 minutes at room temperature.
6. After incubation, measure the absorbance at 660 nm.
7. Plot a standard curve of absorbance vs. protein concentration.

Results:

BSA Concentration (mg/mL)	Absorbance at 660 nm
0.2	0.12
0.4	0.26
0.6	0.41
0.8	0.57
1.0	0.72

Discussion / Interpretation of Results:

A standard curve was plotted based on the absorbance values of known BSA concentrations. The curve was linear, confirming the accuracy of the Lowry's method within this concentration range. From the absorbance of the unknown sample, its protein concentration was interpolated from the standard curve. Lowry's method is sensitive and suitable for protein concentrations in the range of 10–100 µg/mL. However, it is susceptible to interference by reducing agents, detergents, and buffer components.

Experiment No. 3

SEPARATION OF AMINO ACIDS BY PAPER CHROMATOGRAPHY

Aim:
To separate and identify the amino acids in a mixture using paper chromatography based on their differential solubility and adsorption.

Principle:
Paper chromatography is a simple analytical technique used to separate compounds based on their differential partition between a stationary phase (the chromatography paper) and a mobile phase (solvent system). Amino acids travel at different speeds with the solvent front depending on their solubility in the solvent and their interaction with the paper. When the chromatogram is developed, the separated amino acids are colourless and invisible. Spraying the chromatogram with **ninhydrin reagent** and heating it causes the amino acids to react and form coloured spots (usually purple), allowing visualization. Each amino acid produces a unique **Rf value**, calculated as the ratio of the distance travelled by the substance to the distance travelled by the solvent.

Materials Required:
- Whatman No. 1 filter paper
- Amino acid mixture
- Standard amino acids (e.g., glycine, alanine)
- Solvent system (butanol:acetic acid:water = 4:1:5)
- Ninhydrin solution
- Chromatography jar
- Capillary tubes
- Pencil and ruler
- Hot air oven

Procedure:
1. Cut a strip of chromatography paper and draw a light pencil line 2 cm from the bottom.
2. Using capillary tubes, apply small spots of standard amino acids and the unknown mixture on the pencil line. Allow to dry.
3. Place the strip vertically in a chromatography jar with the prepared solvent. Ensure the spots are above the solvent level.
4. Close the jar and allow the solvent to rise until it’s about 2 cm from the top.
5. Remove the strip, immediately mark the solvent front, and dry the paper.
6. Spray the paper with **ninhydrin** and heat in a hot air oven at 60°C for 10 minutes to visualize the spots.
7. Measure the distance from the origin to each spot and to the solvent front. Calculate the **Rf values**.

Results:

Amino Acid	Distance moved by Spot (cm)	Rf Value
Glycine	**4.0**	**0.67**
Alanine	**3.5**	**0.58**
Unknown	**4.1**	**0.68**

Discussion / Interpretation of Results:

The Rf values for glycine and alanine were consistent with known standards. The unknown sample showed an Rf value of 0.68, which closely matched that of glycine (0.67), suggesting its presence in the mixture. This technique is effective for qualitative analysis of amino acids. However, slight variations in temperature, humidity, and solvent composition can influence Rf values. For higher accuracy and quantification, complementary techniques like HPLC may be employed.

Experiment No. 4

DETERMINATION OF SAPONIFICATION NUMBER OF FATS

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

Principle: Saponification number is defined as the number of milligrams of potassium hydroxide (KOH) required to saponify one gram of fat. Fats and oils are triglycerides that, under alkaline hydrolysis, break down into glycerol and soap (fatty acid salts). This reaction allows quantification of the total ester content present in a fat. In this experiment, a known weight of fat or oil is refluxed with an excess of alcoholic KOH. After the reaction, the remaining (unreacted) KOH is back-titrated with standard hydrochloric acid (HCl). The difference between the blank and sample titration values gives the amount of KOH consumed in saponification, from which the saponification number is calculated.

Materials Required:

- Fat/oil sample
- 0.5 N alcoholic KOH
- 0.5 N HCl
- Phenolphthalein indicator
- Round-bottom flask
- Reflux condenser
- Water bath
- Burette and pipette
- Analytical balance

Procedure:

1. Accurately weigh 1.5 grams of the fat/oil sample into a round-bottom flask.
2. Add 25 mL of 0.5 N alcoholic KOH to the flask.
3. Attach a reflux condenser and heat the flask in a boiling water bath for 30 minutes.
4. Cool the flask and add a few drops of phenolphthalein.
5. Titrate the excess KOH with 0.5 N HCl until the pink colour disappears.
6. Perform a blank titration using the same volume of alcoholic KOH but without the fat/oil.
7. Calculate the saponification number using the formula:

Saponification Number=(B−S)×N×56.1W\text{Saponification Number} = \frac{(B - S) \times N \times 56.1}{W}Saponification Number=W(B−S)×N×56.1
Where:

- **B** = Volume of HCl used in blank (mL)
- **S** = Volume of HCl used in sample (mL)
- **N** = Normality of HCl
- **W** = Weight of fat/oil sample (g)

Results:

Sample	Volume of HCl (Blank) (mL)	Volume of HCl (Sample) (mL)	Saponification Number
Coconut oil	**25.0**	**15.5**	**179.08**

(Calculation: (25.0 – 15.5) × 0.5 × 56.1 / 1.5 = 179.08)

Discussion / Interpretation of Results:

The saponification number provides an estimate of the average molecular weight (chain length) of fatty acids in the fat. A higher value indicates shorter chain fatty acids. The observed saponification number for coconut oil (≈179) is lower than typical literature values (~250), which might be due to incomplete saponification, inaccurate weighing, or procedural deviations. In practice, this method is valuable for fat and oil characterization in quality control and biodiesel feedstock evaluation.

Experiment No. 5

SDS-PAGE SEPARATION OF SOLUBLE PROTEINS

Aim:
To separate and analyse soluble proteins based on their molecular weight using SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis).

Principle:
SDS-PAGE is an electrophoretic technique that separates proteins primarily by molecular weight. Proteins are denatured with **Sodium Dodecyl Sulphate (SDS)**, which imparts a uniform negative charge to them, eliminating differences in native shape and charge. When subjected to an electric field in a polyacrylamide gel matrix, proteins migrate toward the positive electrode at rates inversely proportional to their size. The gel consists of two parts: the **stacking gel**, which concentrates proteins into tight bands, and the **resolving gel**, where actual separation occurs. A **protein ladder (marker)** is run alongside samples to estimate the molecular weights of separated bands.

Materials Required:
- SDS-PAGE apparatus
- Acrylamide/Bis-acrylamide solution
- Tris-HCl buffer
- SDS, TEMED, Ammonium persulfate (APS)
- Protein sample
- Sample buffer (Laemmli buffer with β-mercaptoethanol)
- Protein molecular weight marker
- Coomassie Brilliant Blue staining solution
- Destaining solution
- Micropipettes, Gel caster and combs

Procedure:
1. **Preparation of Gel:**
    - Cast the resolving gel (e.g., 10–12% acrylamide), allow to polymerize.
    - Overlay with stacking gel (4–5%) and insert comb to form wells. Allow to set.
2. **Sample Preparation:**
    - Mix protein samples with sample buffer and heat at 95°C for 5 minutes.
3. **Loading and Running the Gel:**
    - Mount the gel in the electrophoresis unit and fill with running buffer.
    - Load protein samples and marker into wells.
    - Run at 80 V through the stacking gel, and then increase to 120 V for the resolving gel.
4. **Staining and Destaining:**
    - After run completion, remove gel and stain with Coomassie Brilliant Blue for 30–60 min.

- Destain using destaining solution (methanol:acetic acid:water) until clear background appears.

Results:

Lane Sample Loaded	Observed Bands (kDa)
Protein markers	**100,70,50,35,25,15**
Crude enzyme extract	**70,35,25**
Purified enzyme	**35**

(Approximate molecular weights based on marker band comparison)

Discussion / Interpretation of Results:

The SDS-PAGE profile allows qualitative and semi-quantitative assessment of protein composition. The presence of multiple bands in the crude extract indicates a mixture of proteins. The single prominent band in the purified sample suggests successful isolation of a protein with an approximate molecular weight of 35 kDa. This technique is widely used for protein purity analysis, subunit characterization, and molecular weight determination. Precision depends on gel concentration, buffer system, and proper sample preparation.

Experiment No. 6

SEPARATION OF DNA AND RNA USING AGAROSE GEL ELECTROPHORESIS

Aim:
To separate and visualize the DNA and RNA molecules using Agarose Gel Electrophoresis.

Principle:
Nucleic acids like DNA and RNA are negatively charged molecules due to their phosphate backbone. In an electric field, they migrate towards the positive electrode (anode) through a porous agarose matrix. The rate of migration is inversely proportional to their molecular size; smaller fragments migrate faster and farther than larger ones. Agarose gel electrophoresis effectively separates DNA and RNA based on size, enabling the distinction between genomic DNA, plasmid DNA, and various RNA species (e.g., 28S, 18S rRNA). Ethidium bromide or safe dyes intercalate into nucleic acids, allowing visualization under UV light.

Materials Required:

- Agarose powder
- 1X TAE or TBE buffer
- DNA and RNA samples
- DNA ladder/marker
- Gel casting tray and comb
- Electrophoresis apparatus
- Ethidium bromide or SYBR Safe (or alternative stain)
- Micropipettes and tips
- Gel documentation system
- Loading dye

Procedure:

Gel Preparation:

- Weigh 1% agarose (1g in 100 mL) and dissolve it in 1X TAE buffer by heating.
- Cool to ~60° C, add ethidium bromide (0.5 µg/mL) or SYBR Safe.
- Pour into gel tray with comb and allow to solidify (~30 minutes).

Sample Preparation:

- Mix each DNA/RNA sample with loading dye (6X).
- Prepare a DNA ladder as molecular weight marker.

Loading and Running the Gel:

- Place the gel in the electrophoresis chamber, submerge in 1X TAE.
- Load DNA ladder and samples into respective wells.
- Run the gel at 90–100 V for ~45 minutes.

Visualization:

- Visualize gel under UV light using a gel documentation system.
- Document band patterns and migration distances.

Results:

Sample Band Observations

Lane	Sample	Band(s) Observed	Interpretation
1	DNA Ladder	1000, 800, 600, 400, 200 bp	Reference for size estimation
2	Genomic DNA	Single band – 800 bp_	High molecular weight DNA
3	Total RNA	3 bands- 1000,700,400 bp	Distinct ribosomal RNA bands
4	Plasmid DNA	2 bands – 600,200 bp	Supercoiled and open circular forms

Discussion / Interpretation of Results:

The DNA ladder provides a size reference. Migration distances correlate with known fragment lengths. Genomic DNA appears as a single high-molecular-weight band, indicating intact, non-degraded DNA. Total RNA shows characteristic rRNA bands—28S and 18S rRNA are typically sharp and well-separated in high-quality RNA preparations. Plasmid DNA shows two bands: supercoiled DNA migrates faster (appears lower), while open circular forms migrate slower. The quality and integrity of nucleic acids can be assessed: smeared bands suggest degradation, whereas sharp bands indicate good sample quality.