UNIT II

STERILIZATION

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IMPORTANCE OF STERILIZATION

- To prevent contamination in sterile products
- To prevent transmission of pathogenic microorganisms which are responsible for causing disease in plants, animals and human beings
- To prevent decomposition and spoilage of food and food products
- To prevent the contamination of unwanted microbes in pure cultures and other microbiology experiments performed for research studies
- To prevent unwanted microbial contamination in antibiotic, enzyme, vitamins, fermentation and other industries process
- To prevent contamination in aseptic areas/instruments which are used for the preparation of sterile dosage forms and sterility testing.

DEFINITION OF IMPORTANT TERMS

- **Sterilization :** It is a process by which an article, surface or medium is made free of all microorganisms either in vegetative or spore form.
- **Disinfection :** It is a process of destruction of all pathogens or organisms capable of producing infections in living cells but not necessarily spores. All organisms may not be killed but the number is reduced to a level that is no longer harmful to health.
- **Disinfectants:** these are antimicrobial agents that are applied to the surface of non-living objects to destroy microorganisms that are living on the objects.
- **Antiseptics :** Chemicals which can safely be applied to living tissues and are used to prevent infection by inhibiting the growth of microorganisms.
- **Asepsis :** Technique by which the occurrence of infection into an uninfected tissue is prevented.
- **Bactericidal agents/germicides:** These are the chemical substances which able to kill bacteria/germs.

DIFFERENTIATE BETWEEN ANTISEPTICS AND DISINFECTANTS

Antiseptic	Disinfectant
Used for humans and animals	Used for non-living things like furniture and other household items
Commonly found in healthcare centers or hospitals	Commonly found in homes or public places
Cleanses wounds and surgical sites to prevent infection and other complications	Kills microorganisms on the surface of non-living things
Includes mouthwash and cold sore and yeast infection treatment creams	Includes cleaning products for houses and public places
Transports through the lymphatic system and destroys bacteria within the human body	Destroys the cell wall of microorganisms or interferes with the metabolism of microbes thriving on the surface of tangible objects
Not harmful to humans and animals	Harmful to humans and animals

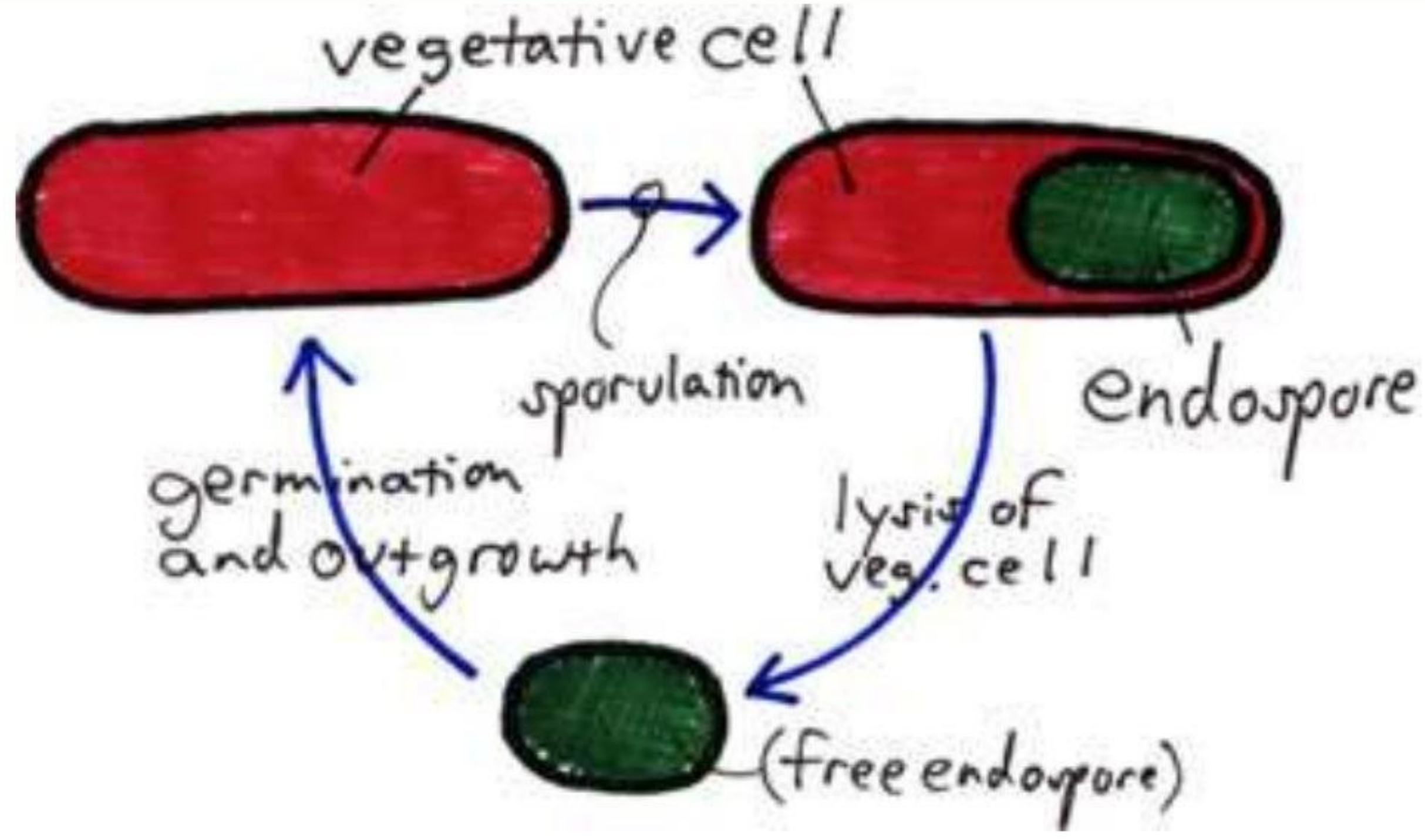
Difference between Bacterial Endospores and Vegetative Cells

Characteristics	Vegetative Cells	Bacterial Endospores
Appearance under Microscope	Non refractive	Refractive
Structure	Typical Gram Positive or Negative bacterial Cell	Thick spore like structure with exosporium, spore coat, cortex and core wall
Level of Calcium	Low level in vegetative cells	High calcium level in endospores
Dipicolinic acid	Absent in vegetative cells	Present in endospores
Activity of Enzymes	High enzymatic activity	Very low enzymatic activity

Staining properties	Stainable with common bacterial dyes	Un-stainable with common dyes, requires special stains
Effect of lysozyme enzyme	Vegetative cells are sensitive to the activity of lysozyme	Endospores are resistant to the action of lysozyme
Level of water content	High (80 – 90% of the cell)	Very low (10 – 20%)
pH of Cytoplasm	Always maintained above pH 7.0	About 5.5 to 6 in the core of the spore
Small Acid Soluble Proteins (SASPs)	SASPs absent in vegetative cells	SASPs Present in vegetative cells
Conformation of DNA	Usually B-form of DNA	Usually A-form of DNA

Respiration rate	Respiration rate high	Usually respiration absent, very less if present
Macromolecule Synthesis	Occurs	Not Occurs
Presence of mRNA	mRNA present in the vegetative cells	mRNA absent in the endospores
Heat resistance capacity	Very low heat resistance capacity	Very high heat resistance capacity
Resistance to Radiations	Very low	Very high resistance
Resistance to chemicals such as H_2O_2 or Acids	Very low	Very high resistance

Vegetative Vs spore

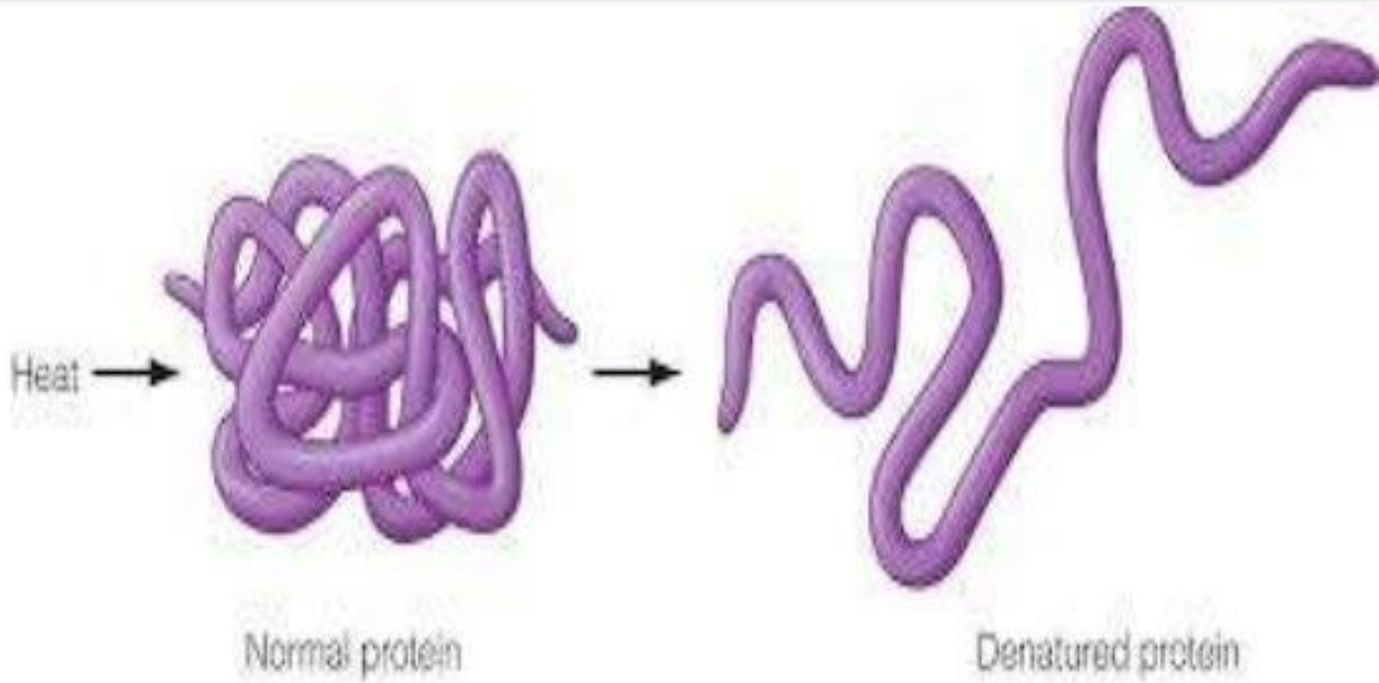


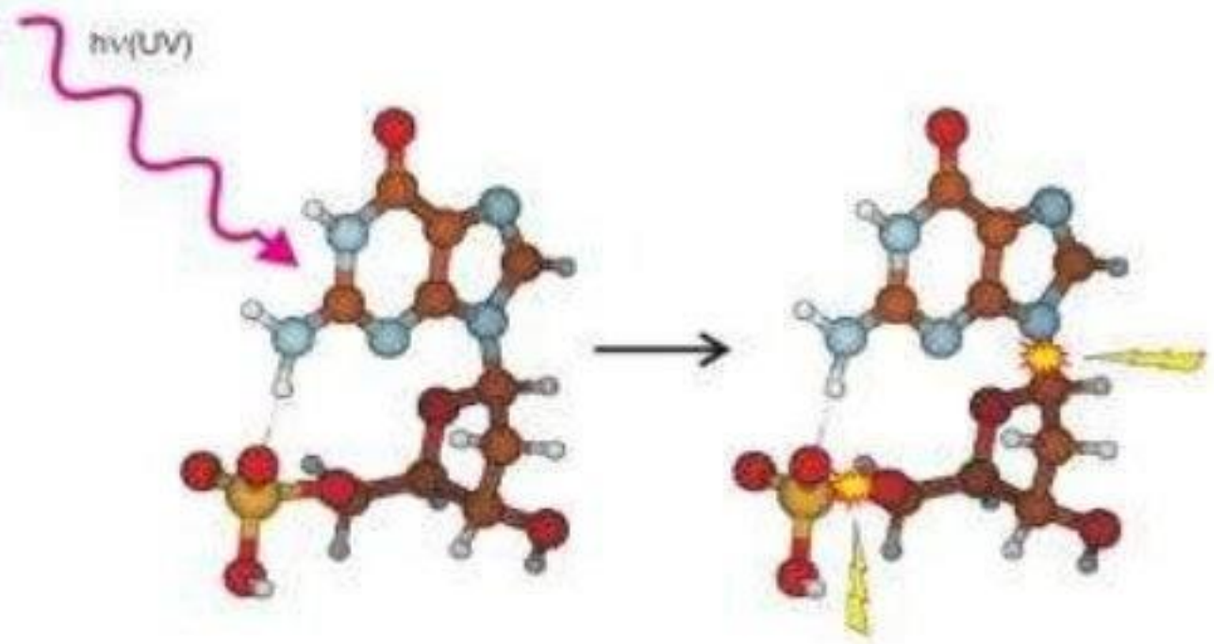
Why we need Sterilization

- Microorganisms capable of causing infection are constantly present in the external environment and on the human body.
- Microorganisms are responsible for contamination and infection.
- The aim of sterilization is to remove or destroy the microorganisms from materials or from surfaces.

How can microorganisms be killed?

- ✓ Denaturation of proteins
- ✓ Interference with protein synthesis
- ✓ Interruption of DNA synthesis/repair
- ✓ Oxidative damage of cell
- ✓ Disruption of cell membranes





Factors that influence efficacy of disinfection/sterilization

- Contact time
- Physico-chemical environment (e.g. pH)
- Presence of organic material
- Temperature
- Type of microorganism
- Number of microorganisms
- Material composition

What to sterilize?

- All instruments that penetrate soft tissues and bone.
- Instruments that are not intended to penetrate the tissues, but that may come into contact with oral tissues.
- If the sterilization procedure may damage the instruments, then sterilization can be replaced by Disinfection procedure.

METHOD OF STERILIZATION

TWO METHODS:-

1. Physical method

a)Dry heat sterilization:

Eg: Incineration, Direct flame, Red heat, Hot air.

b)Moist heat sterilization:

Eg: Pasteurization, Tyndallisation, Autoclave.

c)Sterilization by radiation:

Eg: Use of Ultra-violet rays: UV light(Non-ionising), Ionising radiations: X-rays, Gamma rays, beta rays.

d) Filtration/mechanical method:

Eg: Abestos filter(seitz), sintered glass filter(morton), filter candles(ceramic), membrane filter(millipore)

2. Chemical method

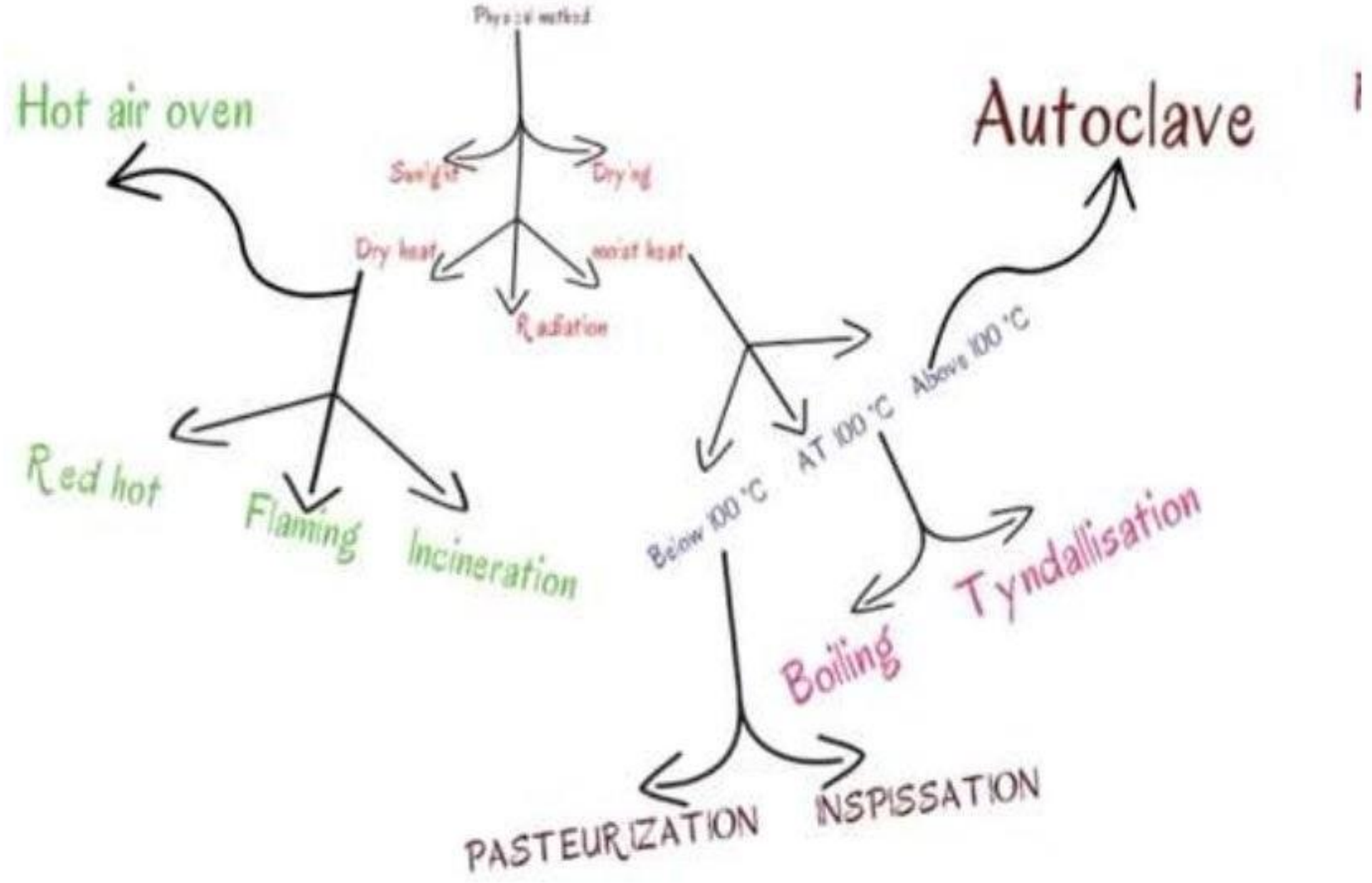
a) Gaseous sterilization

- **Eg: Ethylene oxide gas, Formaldehyde, Beta propiolactone**

b) Sterilization by disinfectant

Eg: Alcohols and Aldehydes, Phenols and Halogens, Oxidizing agents and Salts

METHOD OF STERILIZATION



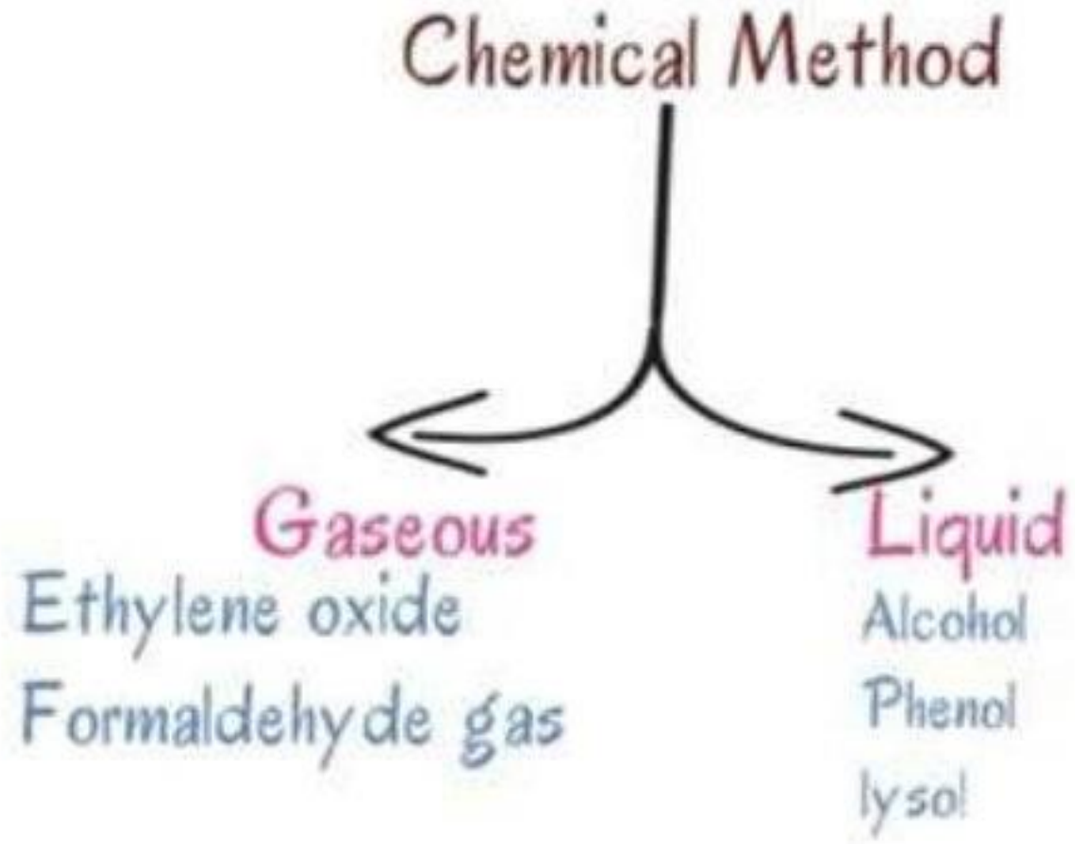
Radiation

Cold Sterlization
x-ray . gamma rays

hot sterlization
u.v & infrared

Nonionising

Ionising


Chemical Method
Gaseous
Ethylene oxide
Formaldehyde gas
Liquid
Alcohol
Phenol
lysol

Filteration Method

heat labile liquid

Candle Filter- purification of water for drinking

Asbestos Filter single use disc
Carcinogenic

Sintered Filter- Expensive
easily brittle

Membrane Filter- made up of cellulose ester
mainly used in microbiology
Average pore diameter-0.22mm

Sl. No.	Physical Method of Sterilization	Instruments used
1	Dry Heat	Oven
2	Moist Heat	Autoclave
3	Radiation	Gamma-ray Chamber

1. Physical method

- Involves processes by the use of physical means
- Utilisation of heat in the presence or in the absence of heat, moisture, radiation or membrane filtration methods.

A) DRY HEAT STERILIZATION

- **Heat is the most reliable and rapid method of sterilization**
- **Mechanism: Protein denaturation, oxidative damage and toxic effect of elevated levels of electrolytes.**
- **Time required for sterilization is inversely proportional to the temperature of exposure. This can be expressed as thermal death time, which is the minimum time required to kill a suspension of microorganisms at a temperature and specific conditions.**

1. Sunlight and drying:

- Action primarily due to UV rays however, effects vary due to places

Eg: Natural method for sterilization of water in tanks, reservoir, lakes, etc

2. Heat:

- Most reliable method of sterilization and should be the method of choice.

Eg: Inoculating wire, needles, forceps, etc

3. Flaming:

- Passed over flame without allowing it to become red hot

Eg: Culture tube, glass slides, scalpels, needles, cover slips, etc.

4. Incineration:

- Excellent method for rapid destroying materials

Eg: Pathological material, contaminated cloth, animals carcasses

5. HOT AIR OVEN

- Hot air ovens are electrical devices used in sterilization.
- The oven uses dry heat to sterilize articles.
- Generally, they can be operated from 50 to 300 C (122 to 572 F) .
- There is a thermostat controlling the temperature.
- This is the most widely used method of sterilization by dry heat.
- Items: glassware, forceps, scissors, scalpels, all-glass syringes, swabs, liquid paraffin, dusting powder, fats, grease.
- (Materials should be properly arranged to allow free circulation of air)
- IT IS NOT SUITABLE FOR SURGICAL DRESSING, RUBBER, PLASTIC, VOLATILE AND HEAT LIABLE SUBSTANCES.

INSTRUMENT IMAGE

Precautions:

- Glass wares should be dry.
- Oven should not be over loaded.
- Glass materials after drying are allow to cold down before use
- Articles are to be arranged in a manner to allow free circular of air.
- Door of the Oven should be opened after it cools down (2Hours).

Temperature (c)	Time(in minutes)
170	60
160	120
150	150
140	180

NORMALY THE SPORES AS WELL AS THE VEGETATIVE FORMS OF ALL MICROORGANISMS ARE KILLED IN TWO HOURS AT A TEMPERATURE OF 160°C

Advantages & Disadvantages:

Advantages:

- It is suitable method for sterilization of substances destroyed by moisture.
- They do not require water and there is not much pressure build up within the oven, unlike an autoclave, making them safer to work with.
- Suitable and easy to be use in a laboratory environment.
- They are much smaller than autoclaves but can still be as effective.

Disadvantages:

- long heating time, high temperature.
- As they use dry heat instead of moist heat, some organisms like prions, may not be killed by them every time.

B) MOIST HEAT STERILIZATION

- Killing of microorganisms with hot water or steam
- Mechanism: **Denaturation and coagulation of proteins**
- Divided into **three forms in terms of temperature:**

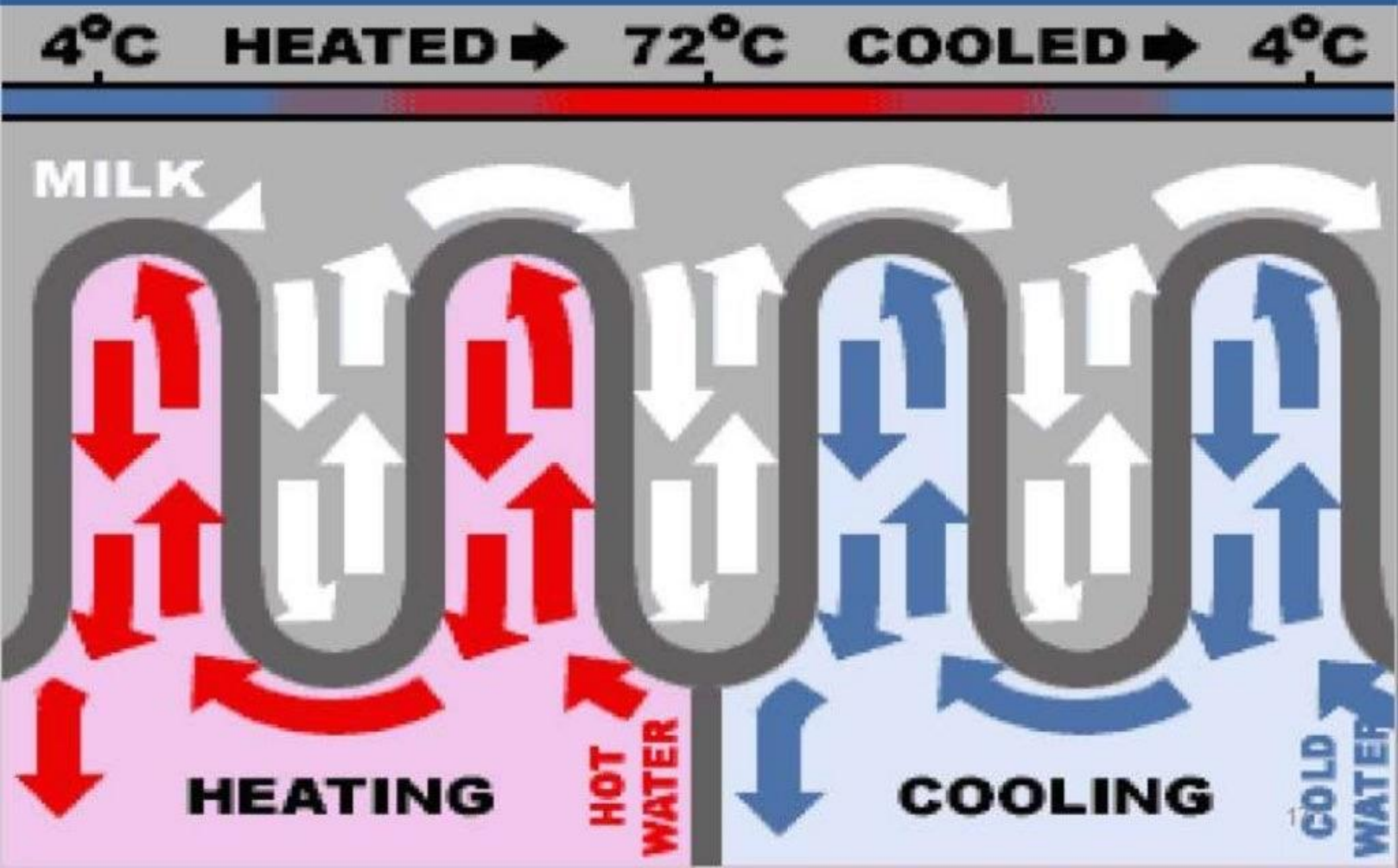
1. Temperature below 100°C (Pasteurization method)
2. Temperature at 100°C (Tyndallization method)
3. Temperature above 100°C (Autoclaving method)

Temperature below 100°C

I : Pasteurization: below100°C

- Used for milk, ice cream, yogurt, and fruit juices
- Heat-tolerant microbes survive
- Batch method
- Temperature below 100° **Pasteurization of milk**
- Developed by Louis Pasteur to prevent the spoilage of beverages.
- Used to reduce microbes responsible for spoilage of beer, milk, wine, juices, etc.
- Milk was exposed to 65°C for 30 minutes.
- **Inspissation** is the process used when heating high-protein containing media; for example to enable recovery of bacteria for testing.(by making THICKINING /DENSE)
- **High Temperature Short Time Pasteurization (HTST): Used** today. Milk is exposed to 72°C for 15 seconds.
- **Target: all non-sporing pathogens**
- **Eg: Mycobacteria, Brucellae, Salmonella, relatively heat resistant, may survive the holder method.**

Principle of Pasteurization



II: A temperature at 100°C

1. Boiling : Boiling for 10 – 30 minutes may kill most of vegetative forms but spores with stand boiling.
2. Tyndallisation: Steam at 100°C for 20 minutes on three successive days. Used for egg , serum and sugar containing media.
3. Steam sterilizer : Steam at 100°C for 90 minutes. Used for media which are decomposed at high temperature.

III. A temperature above 100°C

Autoclave :

- Steam above 100°C has a better killing power than dry heat.
- Bacteria are more susceptible to moist heat.
- TARGETS BOTH VEGETAIVE AND SPORES

Components of autoclave:

- Consists of vertical or horizontal cylinder of gunmetal or stainless steel.
- Lid is fastened by screw clamps and rendered air tight by an asbestos washer.
- Lid bears a discharge tap for air and steam, a pressure gauge and a safety valve.

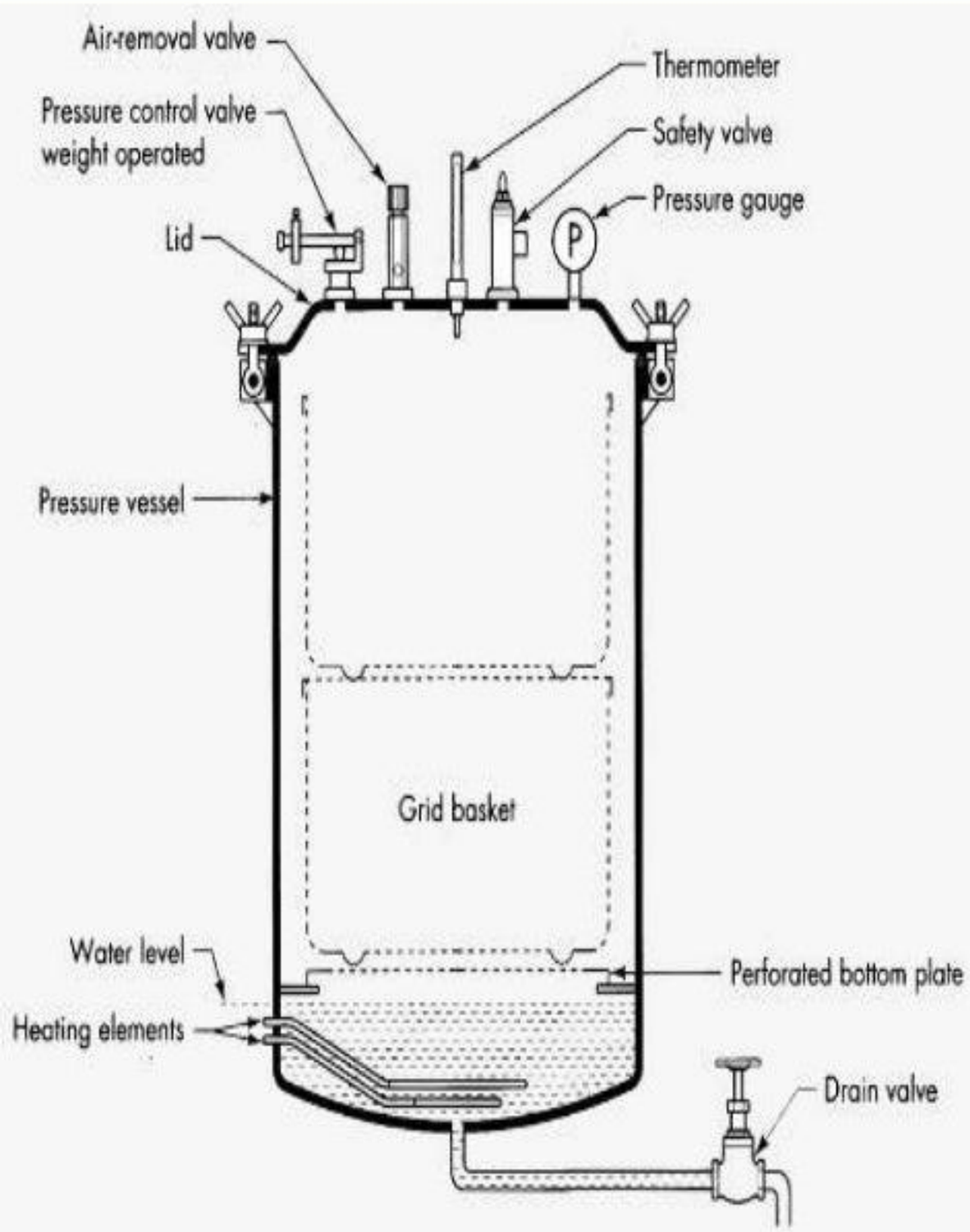
Precautions :

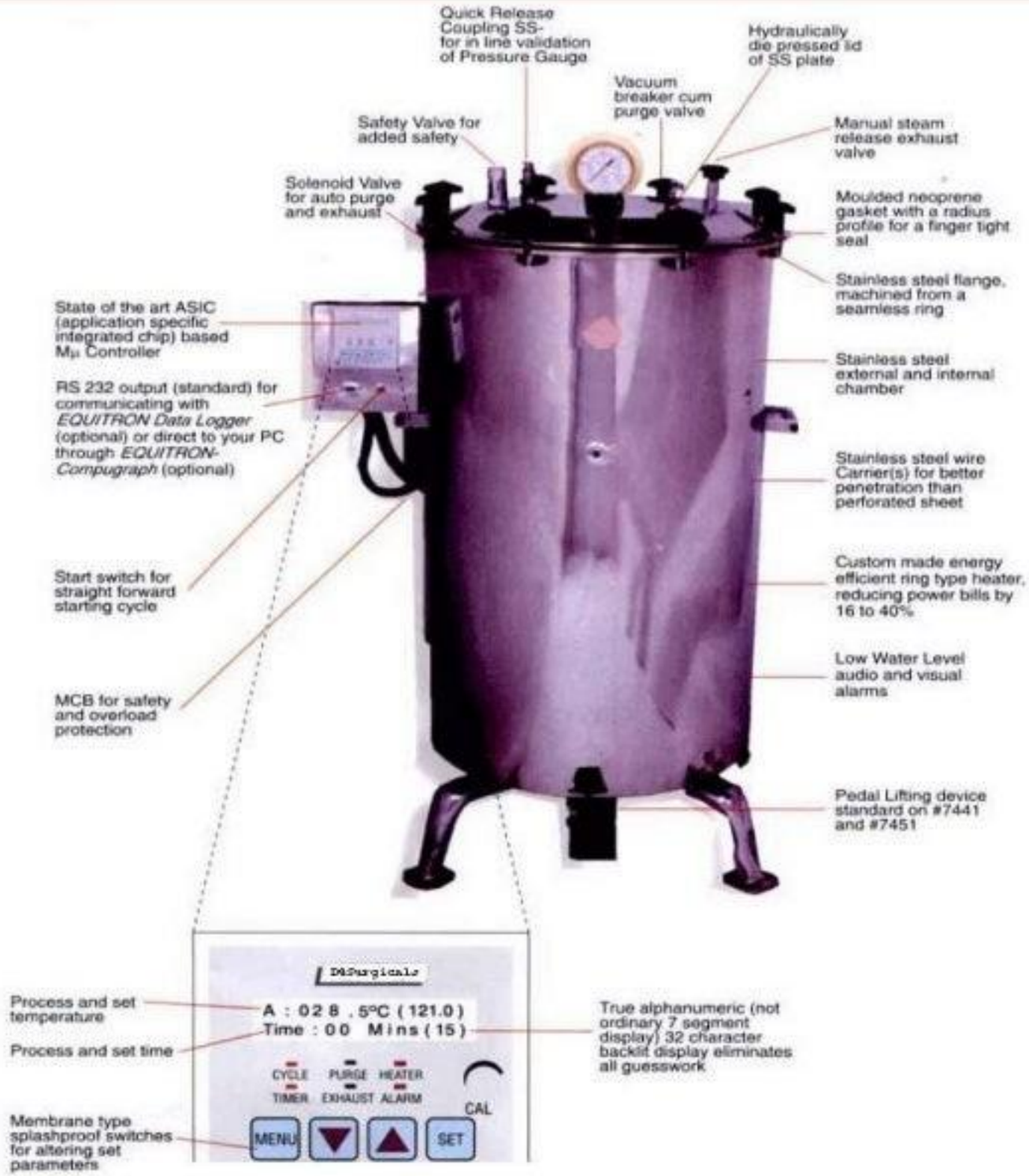
1. Should not be overloaded
2. Arranged in a manner which allows free circulation of air
3. Material to be sterilized should be perfectly dry.
4. Test tubes, flasks etc. should be fitted with cotton plugs.
5. petridishes and pipetts should be wrapped in paper.
6. Rubber materials and inflammable materials should not be kept inside.
7. The oven must be allowed to cool for two hours before opening, since glass ware may crack by sudden cooling.

REQUIRED TEMPERTURE AND TIME

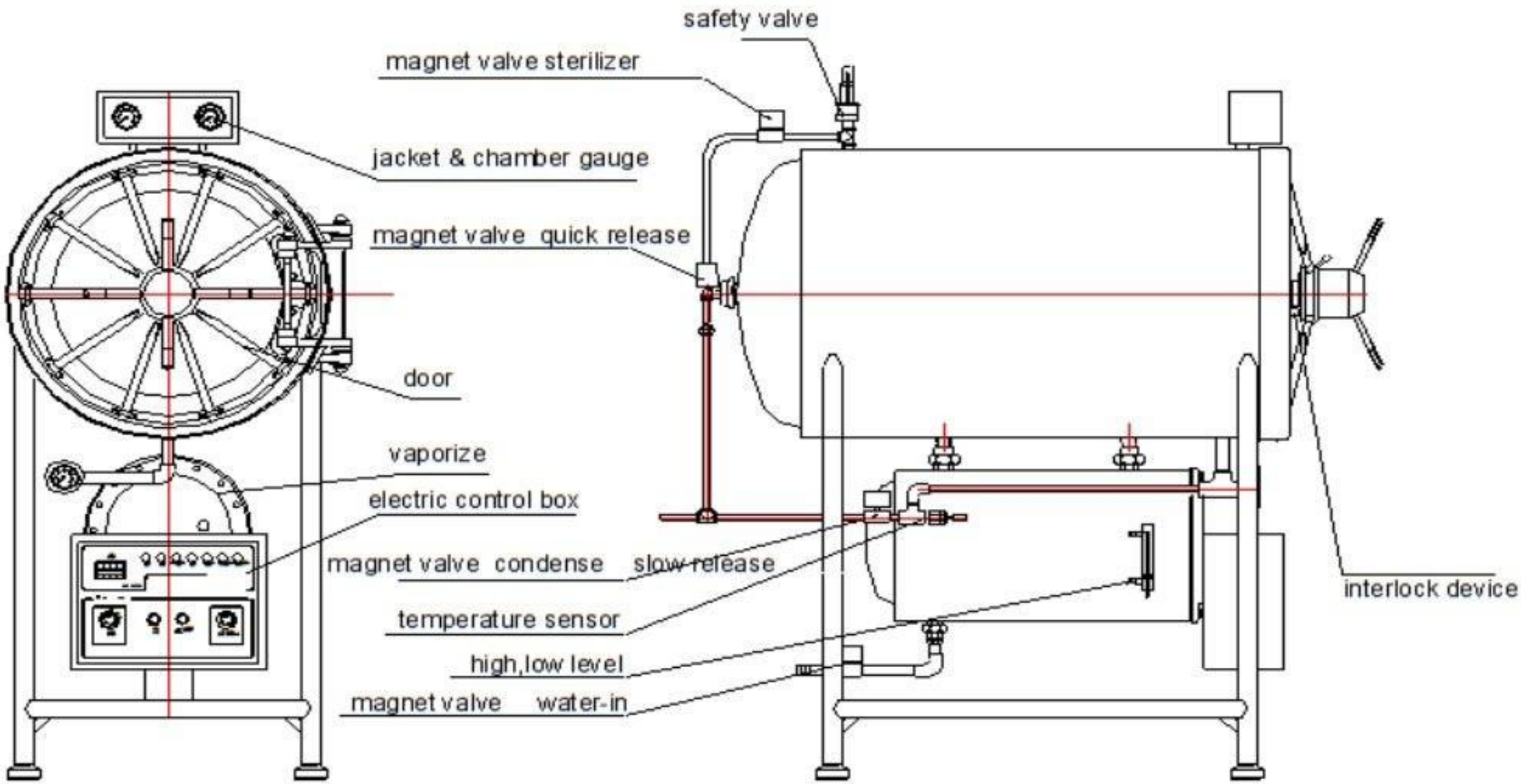
Temperature(°C)	Duration(min)
121	15
126	10
134	3

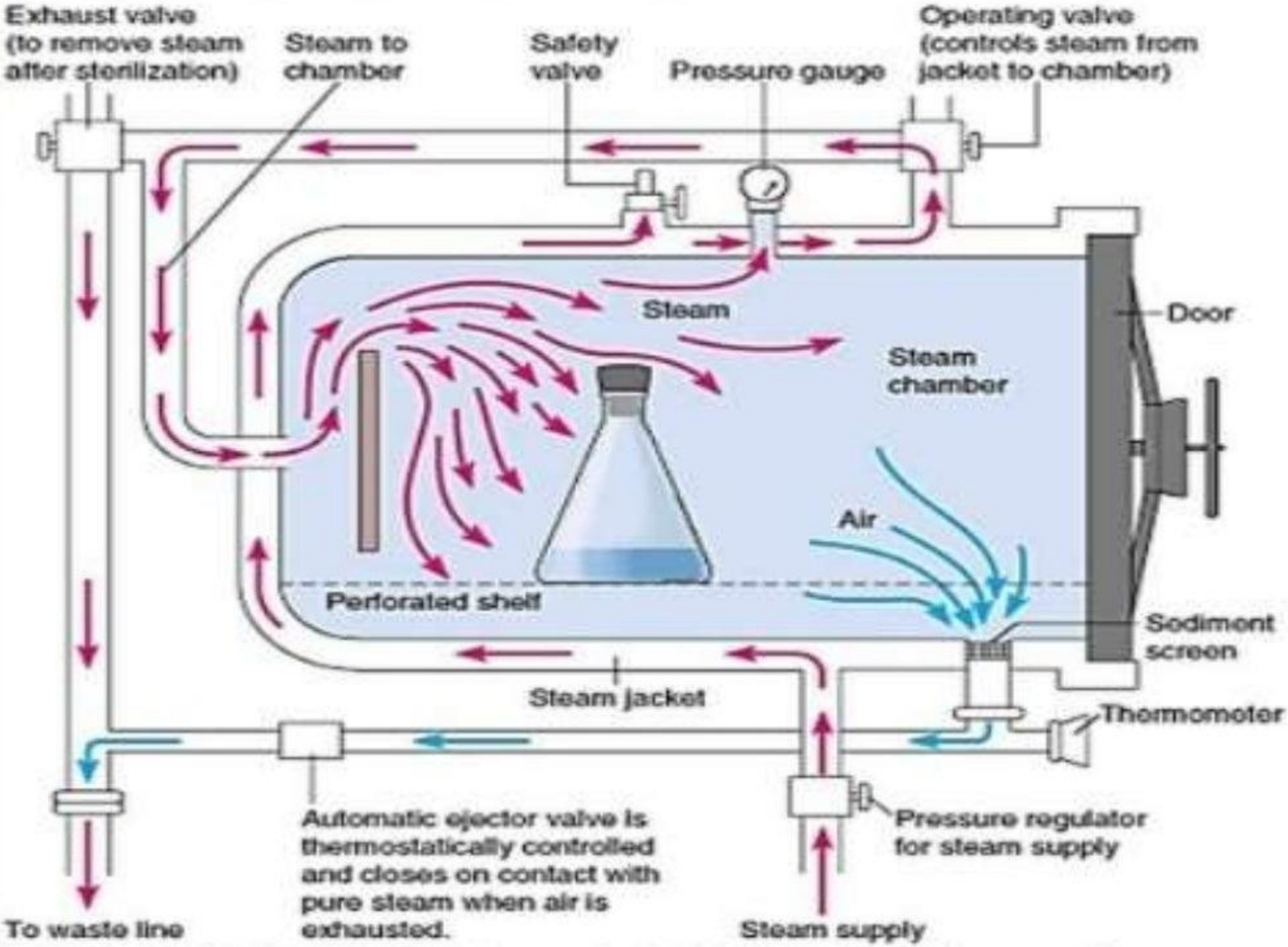
Vertical autoclave





Horizontal autoclave





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Transformation in design

BEFORE

AFTER

Procedure

- Water is added on the bottom of the autoclave and articles to be sterilized are placed in a perforated shelf.
- The lid is closed, discharge tap is opened and safety valve is adjusted to the required pressure.
- When the air bubbles stop emitting from the discharge tap it indicates all the air from inside the autoclave has been removed.
- At this stage, the discharge tap is closed.
- Steam pressure rises inside and when it reaches the desire set level(15p.s.i) the safety valve opens and excess steam escapes.
- From this point the holding time(15mins) is counted
- When the holding time is over, the heating is stopped and autoclave is allowed to cool till pressure gauze indicates that the inside pressure has reached to the atmospheric pressure
- The discharge tap is opened slowly and air is allowed to removed from the autoclave.
- The lid is opened and the sterilized articles are removed.

Uses of Autoclaves:

- Useful for materials which can not withstand high temp.
- To sterilize culture media, heat stable liquids, saline solutions, heat resistant equipments and instruments, glasswares, ampoules, syringes, rubber material, gowns, surgical dressings, gloves etc.

❖ Unsuitable for anhydrous material such as powders, oils, fats, ointments.

More efficient than Dry heat sterilization

i) It provides greater lethal action of moist heat

ii) It is quicker in heat up the exposed articles

iii) It can penetrate easily porous material such as cotton wool stoppers, paper and cloths wrappers.

C) RADIATION

- **Two types of radiation: Ionising radiation & Non-ionising radiation**

1. Non-ionising radiation(HOT STERILIZATION)

- **Infrared- Used for rapid mass sterilization of pre-packed items such as Syringe, Cathaters**
- **UV**
- **Used for disinfecting enclosed area such as entryways, operation theatres and labs.**

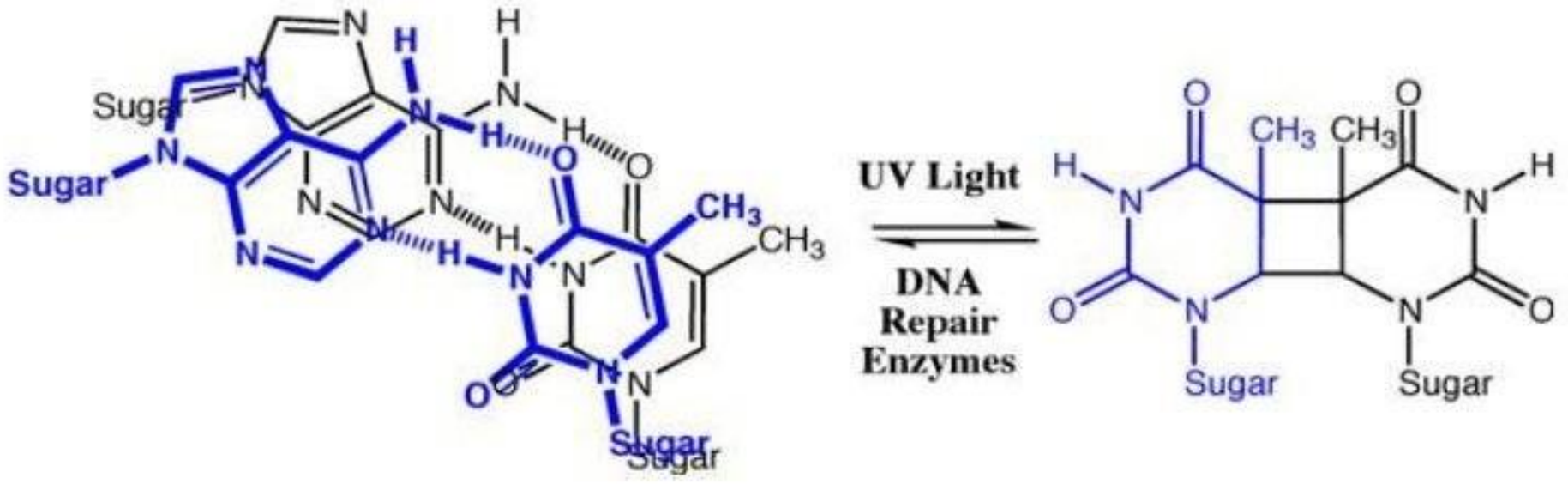
2. Ionising radiation(Cold sterilization)

- **Gamma rays: X-rays:**
- **Used for sterilising plastics, syringes, swabs, catheters, animal feeds, cardboard, oils, greases, fabric and metal foils.**

MECHANISM

1. Non-ionizing radiations(UV light)

- Induce the production of abnormal nucleotides such as thymine dimers in the bacterial cell.



2.Ionizing radiation(X-rays, gamma rays,cathode rays)

- Produces microbial mutant
- Causes ionization resulting in the death of cell

D) Filtration (mechanical) method

- Helps to remove bacteria from heat labile liquids
- Items: sera and solutions of sugars or antibiotics.
- Principle: as viruses pass through the ordinary filters, filtration can be used to obtain bacteria-free filtrates of clinical samples for virus isolation.

Types of filters:

- Candle filters
- Asbestos filters
- Sintered glass filters
- Membrane filters.

Candle filter

- Manufactured in different grades of porosity and widely used for purification of water for industrial and drinking purposes.
- Made up of porous procelain or kieselguhr
- Inexpensive and available in different sizes

Asbestos filter(Seitz filter)

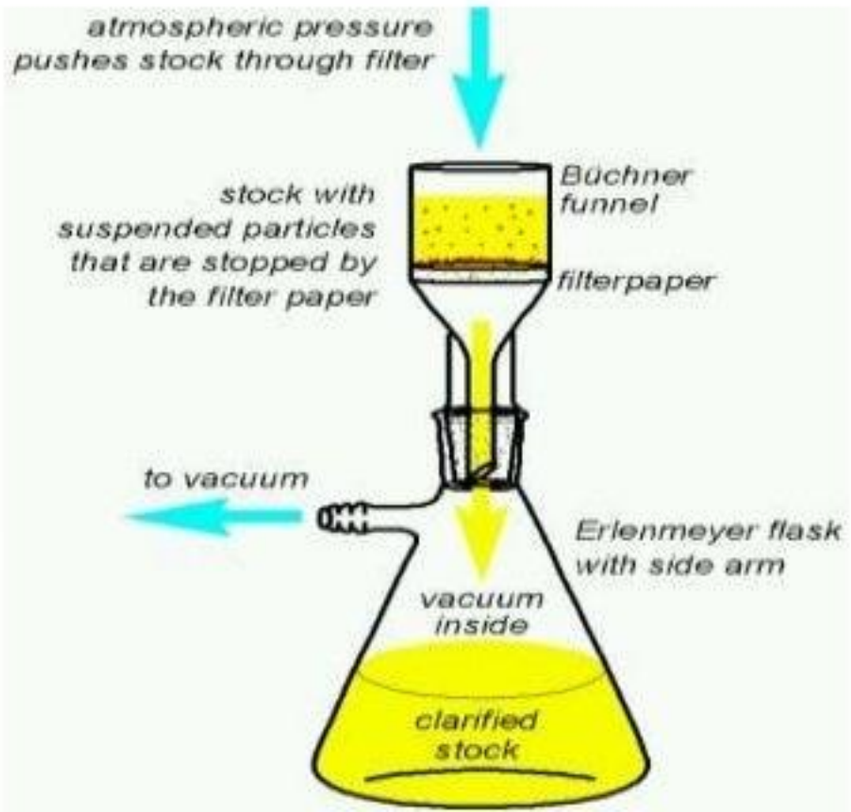
- Disposable
- Single use disc made up of asbestos(magnesium trisilicate)
- **Tend to alkalinise filtered liquids.**
- The pore size of filter ranges from 0.01 to 5 microns.
- **Usage is discouraged because of its carcinogenic property.**

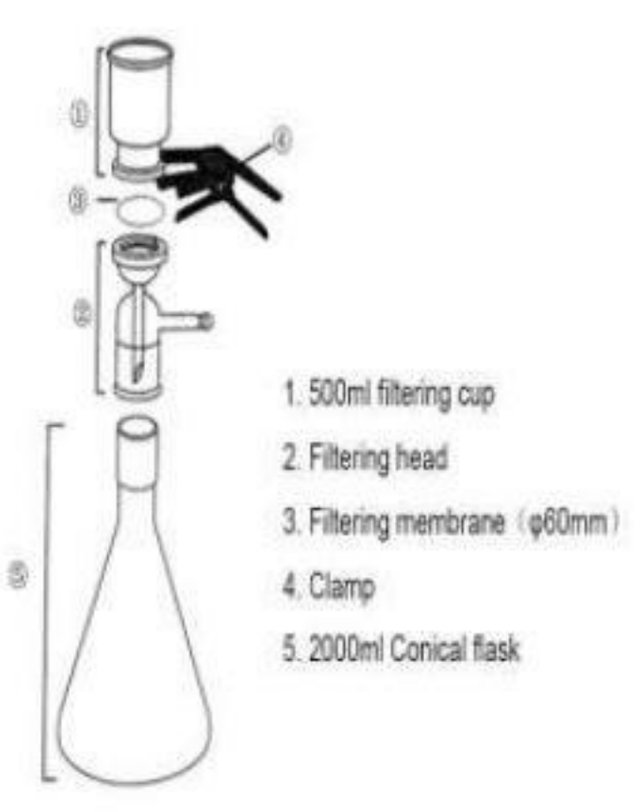
Sintered glass filter(morton filter)

- Has low absorptive properties
- Borosilicate glass is finely powdered in a ball mill and packed into disc mould and heated until suitable adhesion take place between the granules.
- Brittle and expensive.

Membrane filter(millipore/ultra filter)

- Made of cellulose esters or other polymers
- Usually used for water purification and analysis, sterilization and sterility testing and preparation of solutions for parenteral use.
- The are 150µm thick and contain millions of microscopic pores ranging from 0.01 to 10µm in diameter.





Advantages and disadvantages

Adv:

1. All microorganisms are separated by process of sieving
2. Membranes have a high and uniform porosity permitting a rapid rate of filtration
3. Membranes are disposable. Hence, there is no cross contamination between filtered products
4. Adsorption is very less

Disadv:

1. Prefilter is used before the membrane filter to avoid clogging and breaking
2. They have less chemical resistance to certain organic solvents such as chloroform, ketone and esters

CHEMICAL METHODS OF STERILIZATION

Action of chemical agents

Mechanism:

- Protein coagulation
- Disruption of cell membrane resulting in exposure, damage/loss of contents
- Removal of sulfhydryl group essential for normal functioning Of enzyme
- Substrate competition.

Commonly used chemical

1. Reagents:

Alcohol

- Frequently used are Ethyl alcohol ,Isopropyl alcohol
- These must be used at concentration 60-90%.
- Isopropyl alcohol used in disinfection of clinical thermometer.
- Methyl alcohol is effective against fungal spores, treating cabinets and incubators.
- Methyl alcohol is also toxic and inflammable.

Aldehyde

- Formaldehyde:
- Having Bactericidal, sporicidal and has lethal effect on viruses.
- Used to preserve anatomical specimens, destroying anthrax
- spores on hair and wool.

Glutaldehyde:

- Effective against tubercle bacilli, fungi, viruses.
- Less toxic and irritant to eyes, skin
- Used to treat anaesthetic rubber, face masks, plastic endotracheal tubes, metal instruments and polythene tubing.

2. Dyes:

Two groups of dyes:

1.Aniline dye

2.Acridine dye

- Both are bacteriostatic in high dilution but are of low bactericidal activity.
- Aniline dye is more active against gram +ve than gram-ve organisms.

Some important dyes:

- Proflavine
- Acriflavine
- Euflavine
- Aminacrine
- These Impair the DNA complexes of the organisms and thus kill or destroy the reproductive capacity of the cell.

Halogens

- **Iodine**
- **Used as Skin disinfectant**
- **Having Active bactericidal activity &moderate action on spores.**
- **Chlorine**
- **Used to disinfect Water supplies, swimming pools and food and dairy industries.**
- **Along with hypochlorides are bactericidal. Also act on viruses.**

Phenols

- These are obtained from distillation of coal tar between 170- 270 C.
- Lethal effects are:
- Capacity to cause cell membrane damage, releasing cell contents and causing lysis.
- Low concentration will precipitate proteins.

3. Gases:

- Types of gases used for sterilization:
- Ethylene oxide
- Formaldehyde gas
- Beta propiolactone (BPL).

Ethylene oxide:

- Action is due to its alkylating the amino, carboxyl, hydroxyl and sulphydryl groups in protein molecules.
- Also on DNA and RNA.
- Items: heart-lung machines, respirators, sutures, dental equipment, books, clothing.

Formaldehyde gas:

- This is widely employed for fumigation of Operation Theatre and other rooms.
- Formaldehyde is produced by adding 150g of KMnO4 to 280ml of formalin for every 1000cu.ft of roomvolume, after closing the windows and other outlets.
- After fumigation, the doors should be sealed and left unopened for 48 hours.

Beta propiolactone:

- Product of ketane and formaldehyde with a boiling point of 163 C.
- Having rapid bactericidal activity but carcinogenic.
- Capable of killing all microorganisms and is very active against viruses.

Table 7.4: Methods of sterilization used to control microbial growth

Sterilization method	Mechanism of action	Instrument/chemical	Temperature/ Dose	Applications
Dry heat sterilization				
(a) Red heat	Burning contaminants	Bunsen burner	Till red hot	Inoculating loops, forceps
(b) Incineration	Burning to ashes	Bunsen burner or any other	Suitable temp. to burn	Contaminated dressings, animal carcasses, bags.
(c) Hot air sterilization	Oxidation	Hot air oven	160°C for 2 hrs.	Instruments, needles, glasswares, sealed materials like oils, dry powder etc.
Moist heat sterilization				
(a) Below 100°C	Protein denaturation	Water bath	65 to 75°C for 10 min	Serum, body fluids and vaccines
(b) At 100°C	Protein denaturation	Water bath	100°C for 10-20 min	Glass, metal and rubber items
(c) Above 100°C	Protein denaturation	Autoclave	121°C for 15 min	Culture media, surgical dressings, equipments, solution
Radiation sterilization				
(a) Ionizing	Destruction of DNA	Cobalt 60 (x-rays, gamma rays)	2.5 Mrad	Packaged food, antibiotics, hormones sutures, plastic syringes, canulas etc.
(b) Non-ionizing	Damage to DNA	UV lamps, UV units	250-260 nm wavelength for 30 min.	Hospital operating rooms, laboratory, wards.
Gaseous sterilization				
(a) Formaldehyde	Denaturation of protein and nucleic acid	Formaldehyde	150 gm $KMnO_4$ in 280 ml formalin for 1,000 cu. ft of room	Hospital rooms, aseptic area
(b) Ethylene oxide	Denaturation of protein and enzyme	Ethylene oxide	44 mg/lit for 24 hrs OR 88 mg/lit for 2 hrs.	Medical and biological preparations, catgut, plastic equipments, antibiotics
Filtration sterilization	Separation of microbes from liquids	Membrane Filter and assembly	Pore size of filter 0.45 µm (HA) or 0.22 µm (GS)	Sterilizing liquids, enzymes, vaccines

Sterility criteria

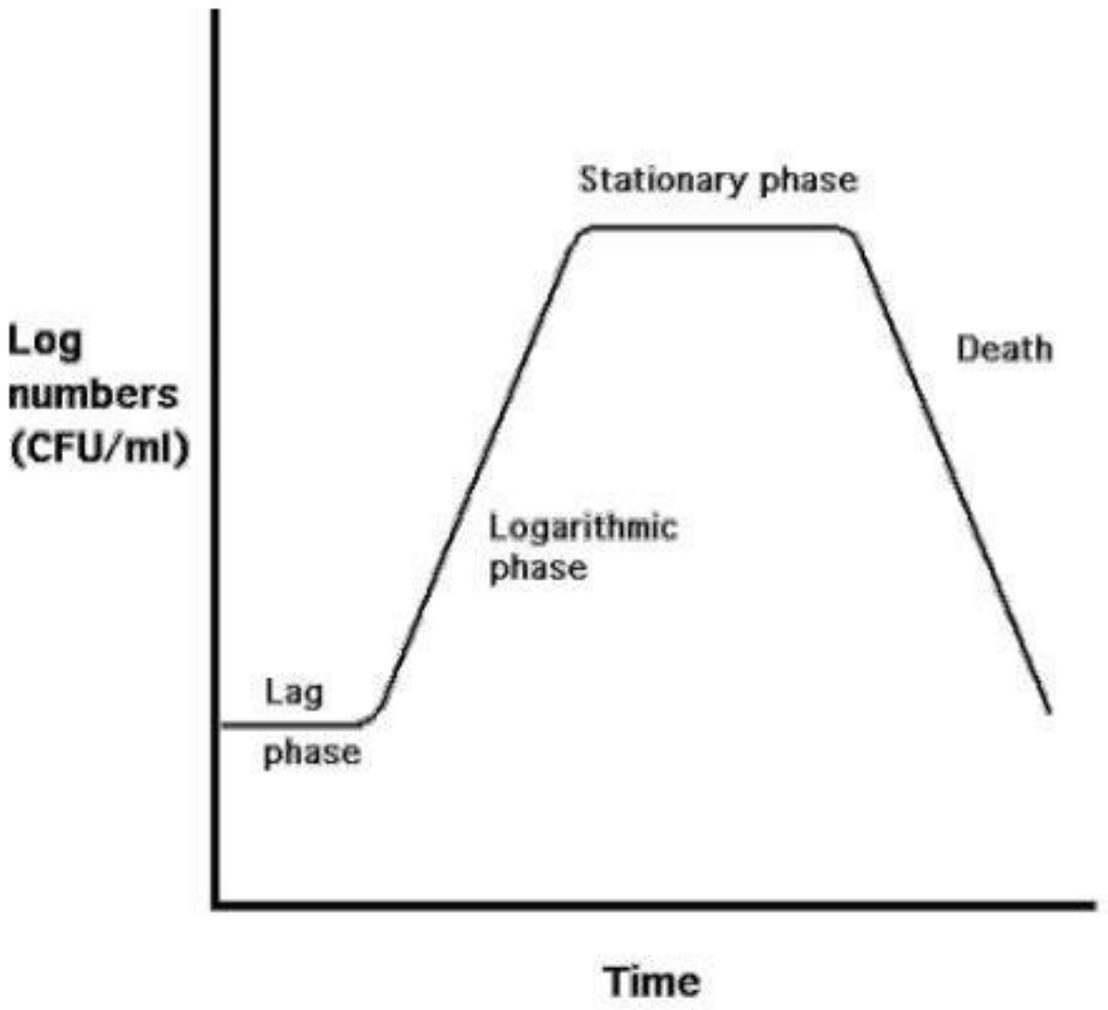
- **Bioburden** is normally defined as the number of bacteria living on a surface that has not been sterilized.
- The term is most often used in the context of **bioburden testing,** also known as microbial limit testing, which is performed on pharmaceutical products and medical products for quality control purposes.
- Time and temperature relationship for steam sterilization to ensure that a large number of the most resistant pathogens would be killed.

Sensitivity of microorganisms

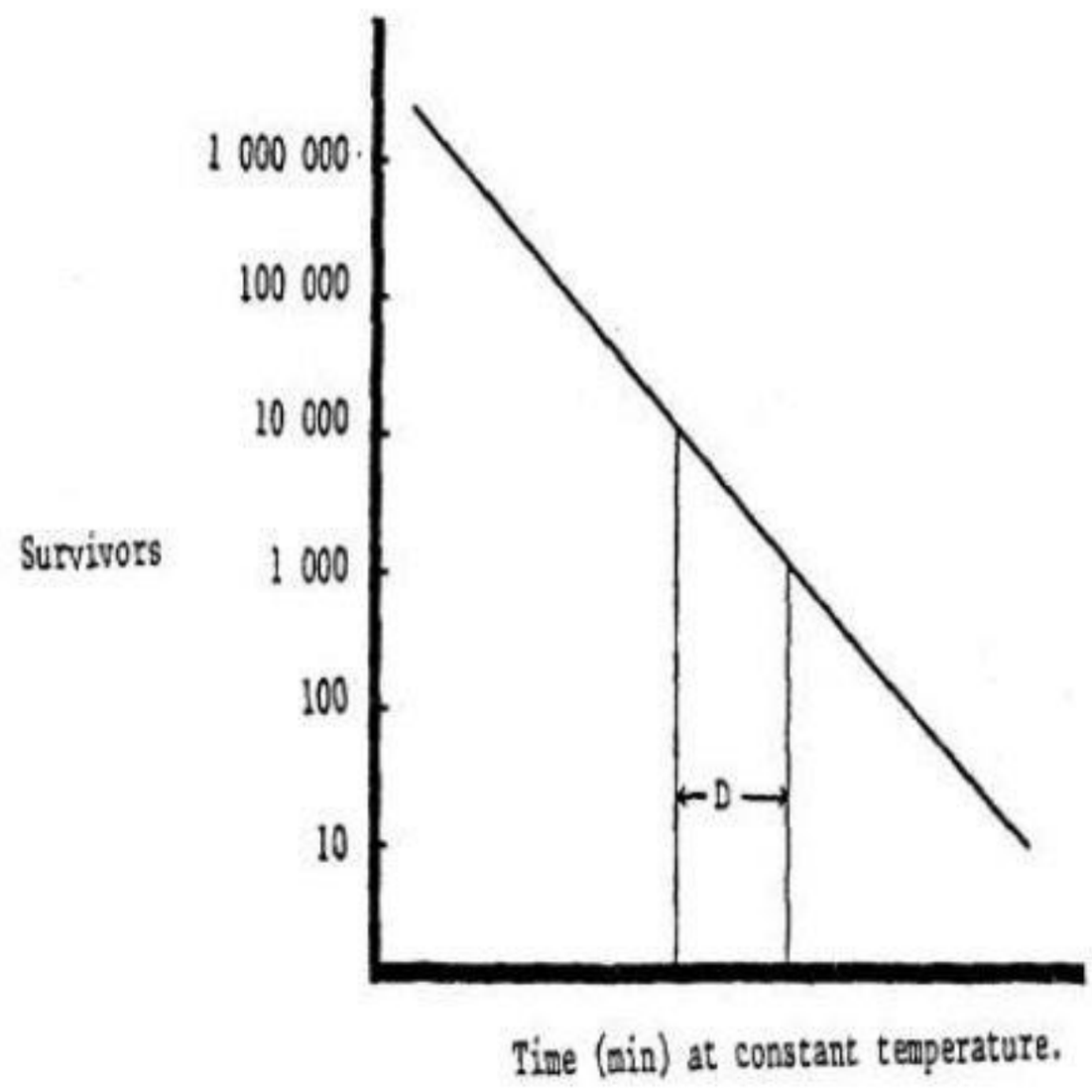
- Microorganisms shows resistance to heat, radiation and chemicals.
- The vegetative forms of bacteria and fungi are most sensitive.
- The thermophilic bacteria, smaller viruses and mould spores are killed at temperature between 70 to 90°C, while bacteria spores may be destroyed at 90 to 120°C temperatures.

Death rates or survivor curve

- It is determined by assessing the reduction in the number of viable microorganisms resulting from contact with a given destructive force.



Hypothetical bacterial growth curve.



Sterility indicators

- Changing appearances in colour or pattern, the sterilization indicators visually show if cleaning conditions are passing or procedures have been completed.
- Eliminating any confusion or possibility instruments will not be sterile, indicators are used routinely in clinical and research environments where contamination elimination is crucial.
- With the temperature resistance required to endure the purification, the sterilization indicators are available in different forms such as tapes, ampoules, and sticks.
- Monitoring of sterilization process can be achieved by the use of **physical, chemical or biological indicators.**

1. Physical indicators

i) Moist heat Indicator:

- ✓ A **Master Process Record (MPR)** is prepared as part of the validation procedure for a particular autoclave
- ✓ The MPR should be checked at annual intervals and whenever significant changes occur in the BPR(Batch Production **Records**)when compared with the MPR.
- ✓ Microprocessor-controlled sterilization cycles are now a part of modern autoclaves.

ii) Dry heat:

- ✓ in dry sterilization processes, a temperature record chart is made of each sterilization cycle and is compared against a master temperature record.

iii) Radio sterilization:

- ✓ A plastic dosimeter gives an accurate measure of the radiation does absorbed and is considered to be the best technique currently available for the radio sterilization process.

iv) Gaseous methods:

- ✓ For gaseous sterilization procedures, elevated temperatures are monitored for each sterilization cycle by temperature probes and routine leak test are performed to ensure gas-tight seals. Gas concentration is measured independently of pressure rise, often by reference to the weight of gas used. Pressure and humidity measurements are record.

v) Filtration:

- ✓ Bubble point pressure test is a technique employed for determining the pore size of filters and may also be used to check the integrity of certain types of filter devices immediately after use. The principle of the is that the filter is soaked in an appropriate fluid and pressure is applied to the filter. The pressure difference when the first bubble of air breaks away from the filter is equivalent to the maximum pore size. When the air pressure is further increased slowly, there is general eruption of bubbles over the entire surface. The pressure difffrence is equivalent to the mean pore size.

2.Chemical indicators

- Chemical monitoring of a sterilization process is based on the ability of heat, steam, sterility gases and ionizing radiation to alter the chemical or physical characteristics of a variety of chemical substances.

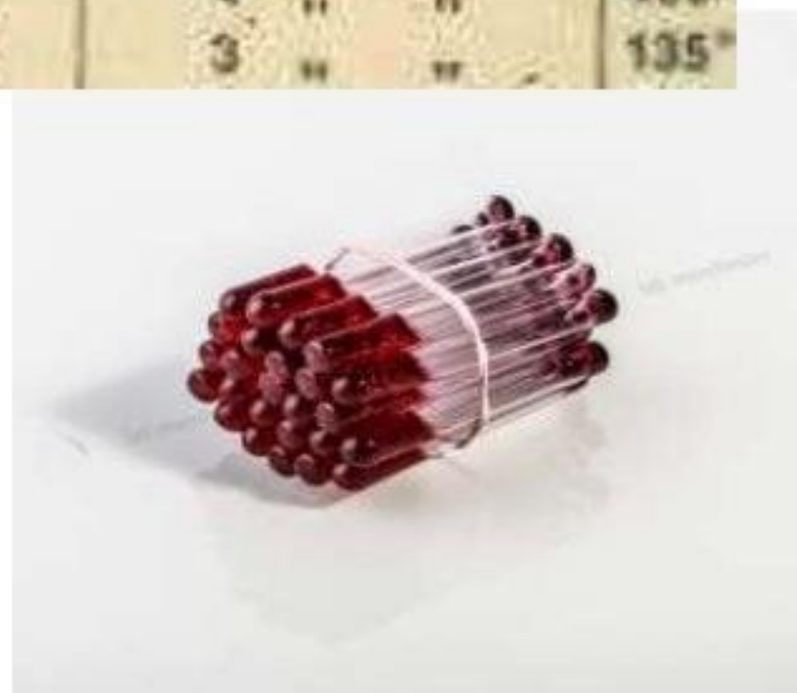
i) Browne's tubes:

- ➢ Most commonly used chemical indicator for heat process
- ➢ Contains small sealed coloured tubes having a reaction mixture and an indicator
- ➢ Expose to high temperature resulting in the change of colour of the indicators.(Red to green)

TYPES OF Browne's tubes:

FOR DIRECTIONS SEE LEAFLET

		UNUSED	UNSAFE		TURNING POINT	EFFECTIVE TREATMENT	
		APPROX TIMES IN MINUTES TO PRODUCE THESE COLOURS AT:					
Tubes Type 1	O	12	20	23	25 and over		115°
(Black Spot)	O	8	13	15	16 " "		120°
	O	5	9	10	11 " "		125°
Tubes Type 2	O	2	3	3½	4 " "		130°
(Yellow Spot)	O	1½	2½	2½-3	3 " "		135°



II) WITTNESS TUBES

- Consist of single crystalline substances of known melting point contained in glass tubes
- Ex: Sulphur(115°C), Succinic anhydride(120°C), Benzoic acid(121°C), etc.
- A dye may be included to show more clearly that the crystals have melted.
- Indicates that a certain temperature has been reached.

WITTNESS TUBES

3. Biological indicator

- Consist of a suitable organism deposited on a carrier and are distributed throughout the sterilizer load
- At the end of the sterilization process, the units are recovered and cultured to determine the presence or absence of survivors.
- Confirm the ability of the sterilization process to kill microbial spores
- Can check large number of spore
- Includes all the parameters of the sterilization process
- Most critical test of sterilization process

Table 7.6: Biological indicators for monitoring sterilization processes

Sterilization process	Species	D-value
Autoclave at 121°C	*Bacillus stearothermophilus*	1.5 min
	Clostridium sporogenes	0.8 min
Dry heat at 160°C	*Bacillus subtilis* var. niger	5 – 10 min
Ethylene oxide at 600 mg/lit. (Temperature - 54°C, 60% - relative humidity)	*Bacillus subtilis* var. niger	2.5 min
Ionizing radiation	*Bacillus pumilus*	3 kGy (0.3 M rad)
Membrane filter (0.45 µm pore size)	*Serratia marcescens*	–
Membrane filter (0.22 µm pore size)	*Pseudomonas diminuta*	–

Thank you

UNIT-III

The Virus

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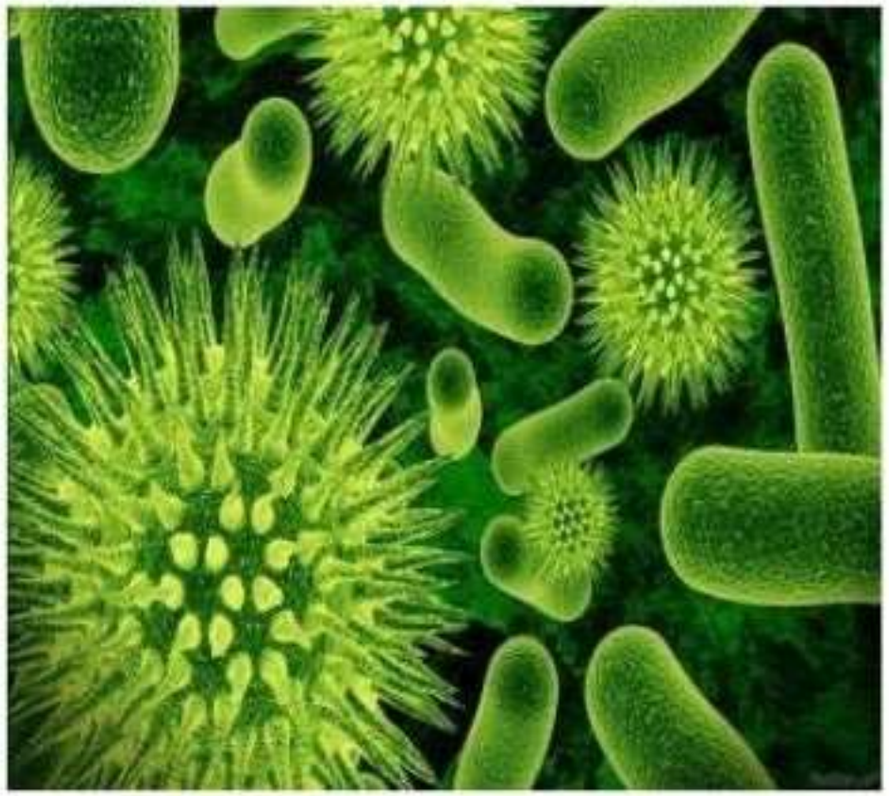
CONTENT

- Morphology
- Classification
- Reproduction/ Replication
- Cultivation

An infective agent that typically consists of a nucleic acid molecule in a protein coat, is too small to be seen by light microscopy, and is able to multiply only within the living cells of a host.



Viruses:

- Viruses do not have cells that divide; new viruses are assembled in the infected host cell
- But unlike still simpler infectious agents, viruses contain genes, which gives them the ability to mutate and evolve.
- Evolved from plasmids: pieces of DNA that can move between cells
- while others may have evolved from bacteria.
- Over 5,000 species of viruses have been discovered.

Introduction to viruses

- A virus consists of two or three parts:
- genes, made from either DNA or RNA, long molecules that carry genetic information
- protein coat that protects the genes; and in some viruses, an envelope of fat
- Viruses vary in shape from the simple helical and icosahedral to more complex structures.
- Viruses range in size from 20 to 300 nanometres; it would take 30,000 to 750,000 of them, side by side, to stretch to 1 centimeter.

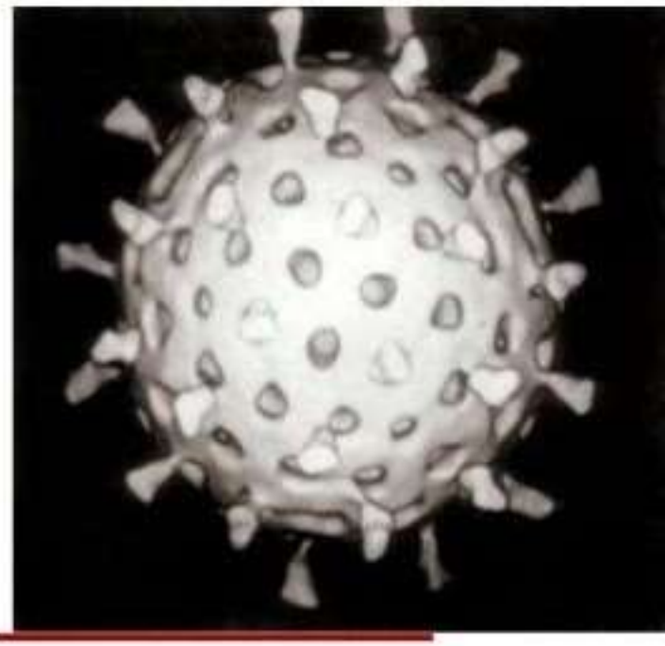
Introduction to viruses

- Viruses spread in many ways. Just as many viruses are very specific as to which host species or tissue they attack,
- Plant viruses are often spread from plant to plant by insects and other organisms, known as *vectors*.
- Some viruses of animals, including humans, are spread by exposure to infected bodily fluids
- Viruses such as influenza are spread through the air by droplets of moisture when people cough or sneeze.
- Viruses such as norovirus are transmitted by the faecal–oral route, which involves the contamination of hands, food and water.

Spreading , Vectors:

- The human immunodeficiency virus, HIV, is transmitted by bodily fluids transferred during sex.
- Dengue virus, are spread by blood-sucking insects.
- Rotavirus is often spread by direct contact with infected children.
- Antibiotics have no effect on viruses,

but antiviral drugs have been developed to treat life-threatening
infections. Vaccines that produce lifelong immunity can prevent some viral infections.



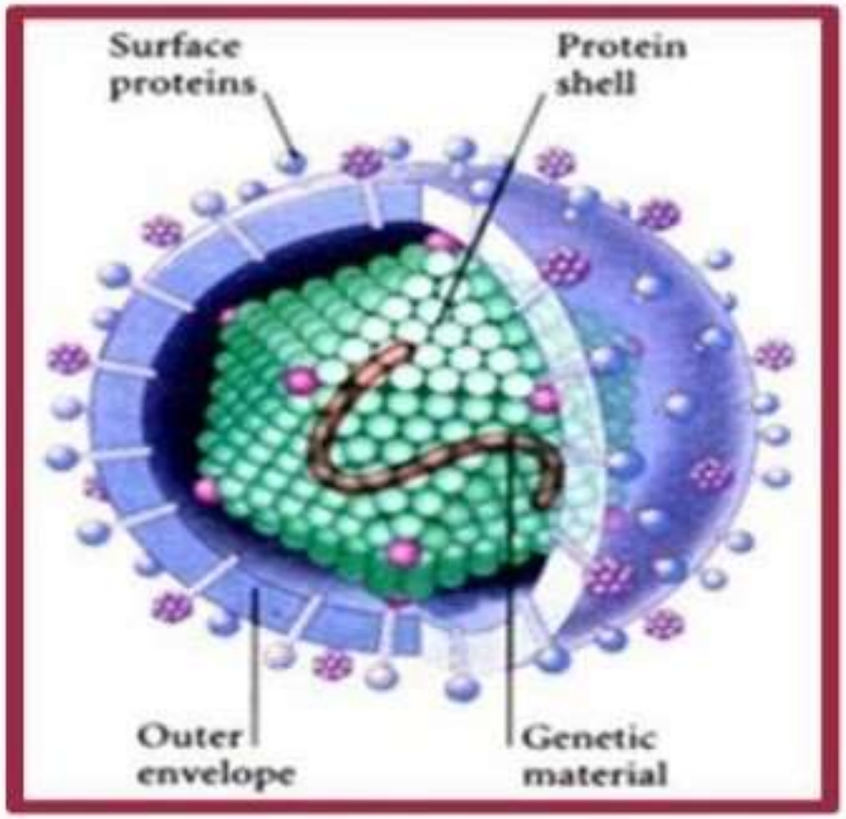
- Obligate intracellular parasites of bacteria, protozoa, fungi, algae, plants, and animals.
- Ultramicroscopic size, ranging from 20 nm up to 450 nm (diameter).
- Not cellular in nature; structure is very compact and economical.
- Do not independently fulfill the characteristics of life.
- Inactive macromolecules outside the host cell and active only inside host cells.
- Basic structure consists of protein shell (capsid) surrounding nucleic acid core.
- Nucleic acid can be either DNA or RNA but not both

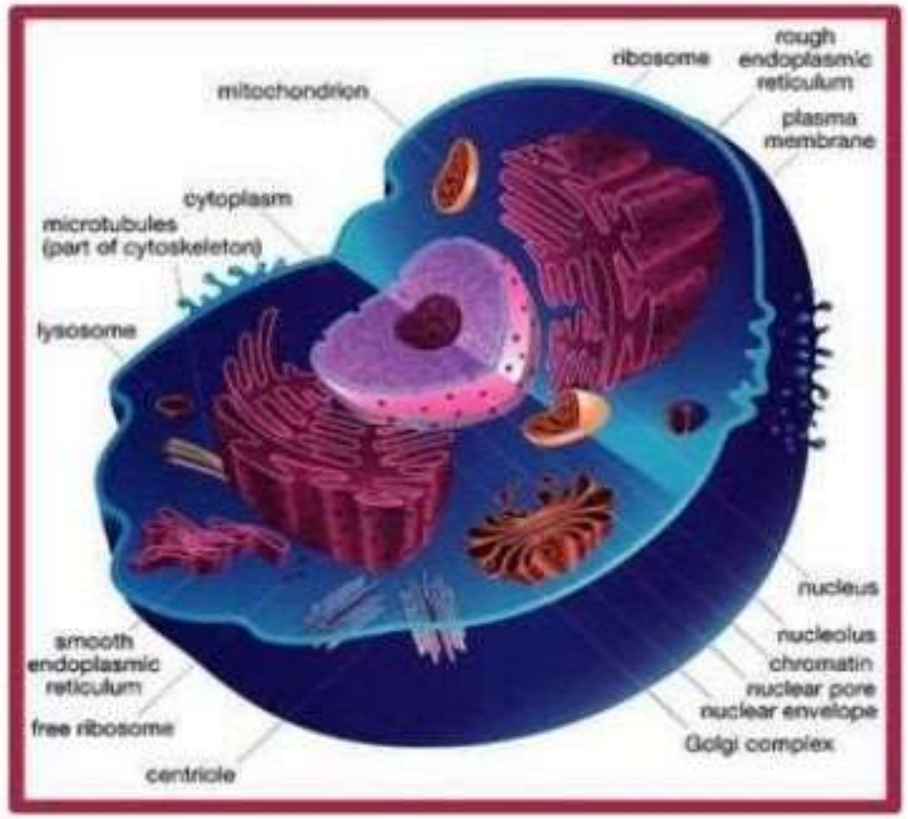
Characteristics

- Nucleic acid can be double-stranded DNA, single- stranded DNA single-stranded RNA, or double-stranded RNA.
- Molecules on virus surface impart high specificity for
- attachment to host cell.
- Multiply by taking control of host cell's genetic material and regulating the synthesis and assembly of new viruses.
- Lack enzymes for most metabolic processes.
- Lack machinery for synthesizing proteins.
- Most RNA viruses multiply in & are released from the cytoplasm.
- Viral infections range from very mild to life threatening.

Virus	Bacteria
1) Virus is ultra microscopic.	1) Bacteria is microscopic.
2)Virus is acellular	2)Bacteria is unicellular
3) Either DNA or RNA is present in Virus body.	3) Both DNA and RNA is present in Bacteria body.
4)Virus is true Parasite	4) Bacteria is parasite or saprophytic or photosynthetic.
5) They cannot multiply outside host body.	5)They can multiply inside and outside host body.
6)Virus causes Meales, Pox, Sneezing, Coughing , AIDS, Influenza etc	6)Bacteria causes Pneumonia, Dysentery, Cholera, Tetanus etc

A comparison with bacterial cell





- Viruses have no nucleus, no organelles, no cytoplasm or cell membrane—Non-cellular

- Smallest infectious agents
- Most are so small, they can only be seen with an electron microscope
- Proviruses- around 20 nm in diameter
- Mimi viruses- up to 450 nm in length
- Special stains and an electron microscope
- Negative staining outlines the shape
- Positive staining shows internal details

Size of virus ?

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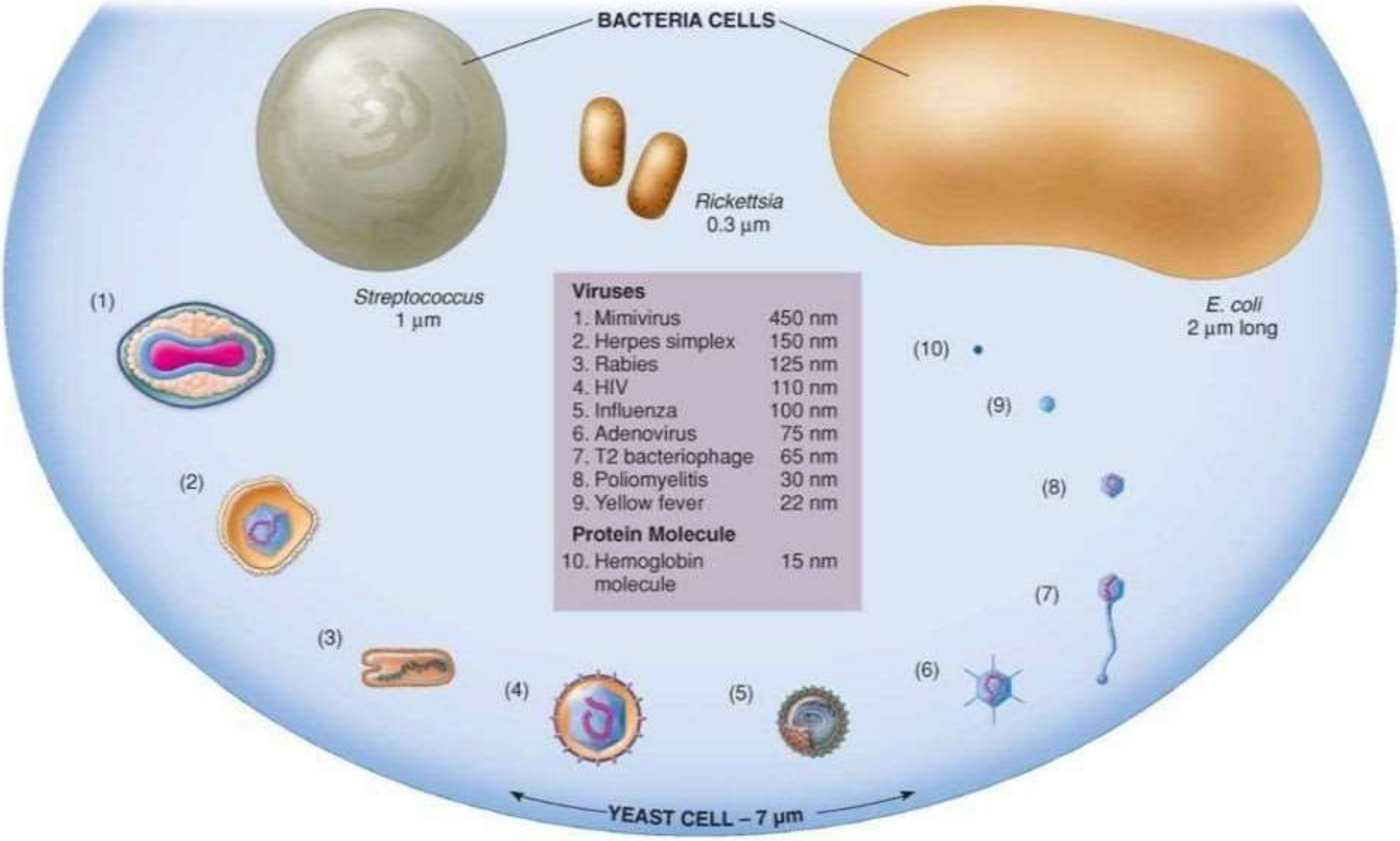


Classification

- Viruses are classified on the basis of habitat (host).which is trivial system beside this Viruses are classified on following criteria.
- Structure
- Chemical composition
- Similarities in genetic makeup
- International Committee on the Taxonomy of Viruses, which includes
- 3 orders
- 63 families "-viridae"
- 263 genera "-virus"

- 3 Types of systems were proposed to classify the viruses:
- Baltimore Classification.
- Classical System Classification.
- Genetic Classification.

Types of Classification:

7 groups were made.

Its principles are fundamental to an understanding of virus classification and genome replication.

The Baltimore classification has + RNA as its central point.

I: **dsDNA viruses** (e.g. Adenoviruses, Herpesviruses, Poxviruses)

II: **ssDNA viruses** (+ strand or "sense") DNA (e.g. Parvoviruses)

III: **dsRNA viruses** (e.g. Reoviruses)

IV: **(+)ssRNA viruses** (+ strand or sense) RNA (e.g. Picornaviruses, Togaviruses)

V: **(−)ssRNA viruses** (− strand or antisense) RNA (e.g. Orthomyxoviruses, Rhabdoviruses)

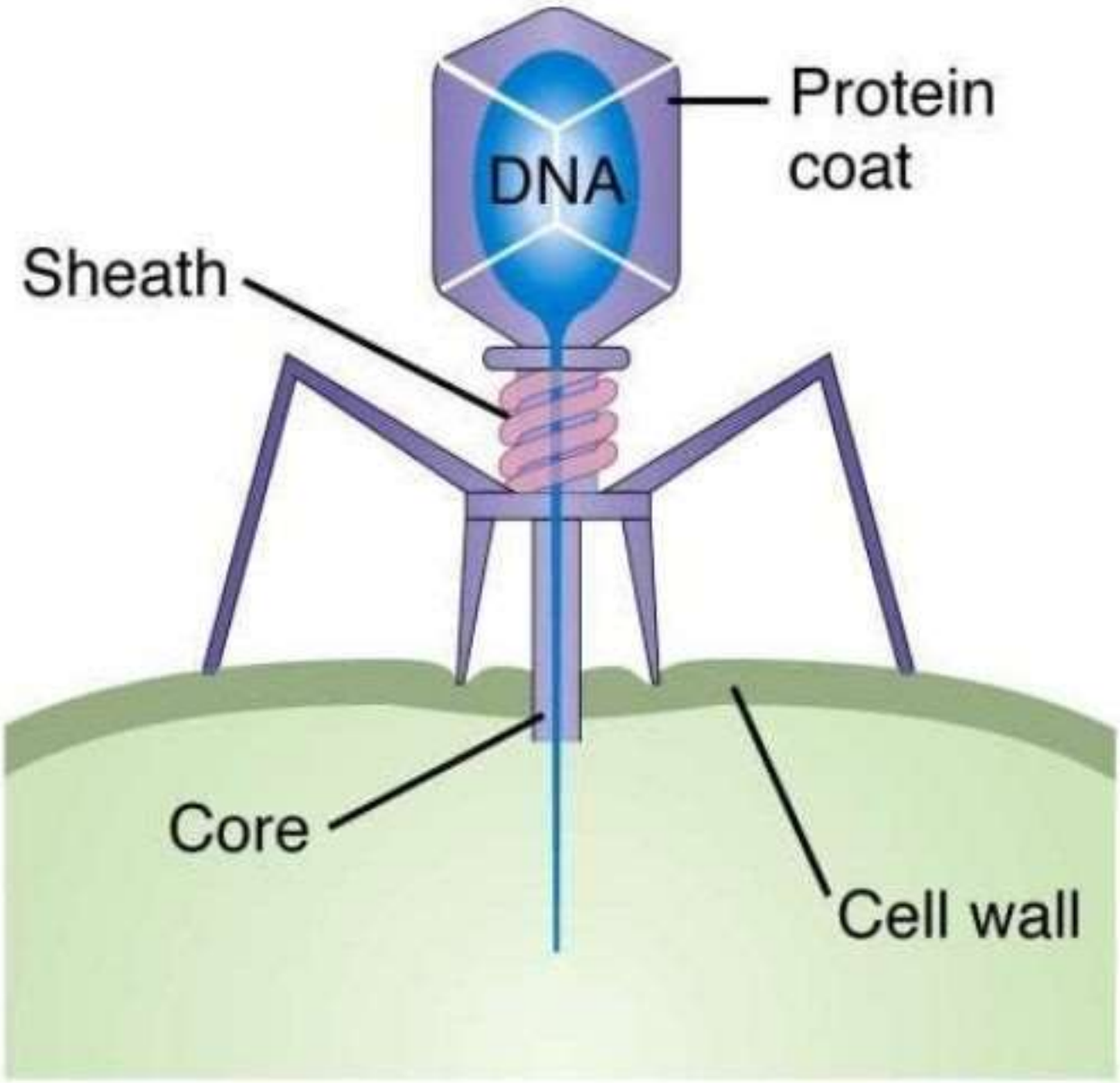
VI: **ssRNA viruses** (+ strand or sense) RNA with DNA intermediate in life-cycle (e.g. Retroviruses)

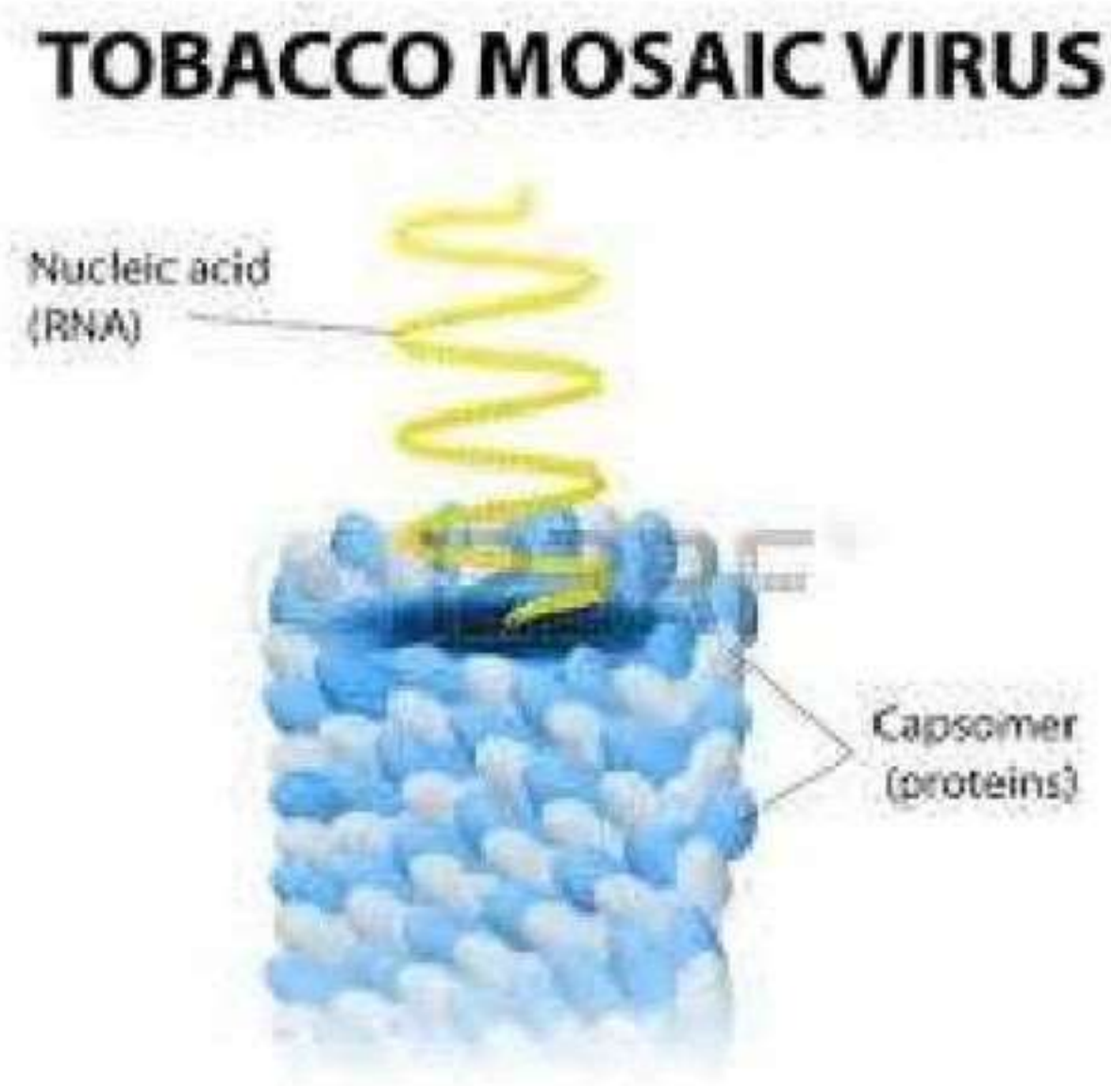
VII: **dsDNA viruses** (e.g. Heptadnaviruses)

Baltimore Classification:

- **Animal viruses**:
- Viruses of animal host
- Rabies, Polio, Mumps, Chicken pox, Small pox, and Influenza.
- **Plant Viruses**:
- viruses which show their live characteristics when attached to plants.
- Tobacco mosaic virus (TMV), Banana streak virus, Carrot thin leaf virus
- **Bacterial Virus**: Bacteriophages (T1, T2, T3, and T4.)

Classification on basis of host


DNA
Protein coat
Sheath
Core
Cell wall


TOBACCO MOSAIC VIRUS
Nucleic acid (RNA)
Capsomer (proteins)

- According to genetic consequences viruses are classified as. DNA Viruses and RNA Viruses
- Genes may be linear or circular
- The smallest have only 4 genes and largest have several hundred.
- **DNA Viruses**
- DNA Viruses are the viruses which consist of DNA genome . They complete their activities by transcription and most of them attack on organisms of similar genome.
- **RNA Viruses**
- RNA Viruses are the viruses which consist of RNA genome. They complete their activities by reverse transcription.

Classification on Genetics basis

- With relevant to morphology of viral structure viruses are organized as Enveloped and Nonenveloped viruses.
- However they are also arranges subclasses of DNA and RNA viruses

Classification on structural basis

- **Questions Relating to Structure**
  - Is it rigid?
  - How big is it?
  - Is it flexible?
- **Structure Must Serve Virus**
  - It should provide protection for genome
  - It should allow virus to move from one host to next
  - It should allow for attachment of virus on to new host

Structure of virus

225 nm

Bacteriophage T4

Rabies virus
170 × 70 nm

Human red blood cell
10,000 nm in diameter

Adenovirus
90 nm

Rhinovirus
30 nm

Bacteriophage M13
800 × 10 nm

Chlamydia elementary body
300 nm

Bacteriophages
f2, MS2
24 nm

Tobacco mosaic virus
250 × 18 nm

Viroid
300 × 10 nm

Prion
200 × 20 nm

Poliovirus
30 nm

Vaccinia virus
300 × 200 × 100 nm

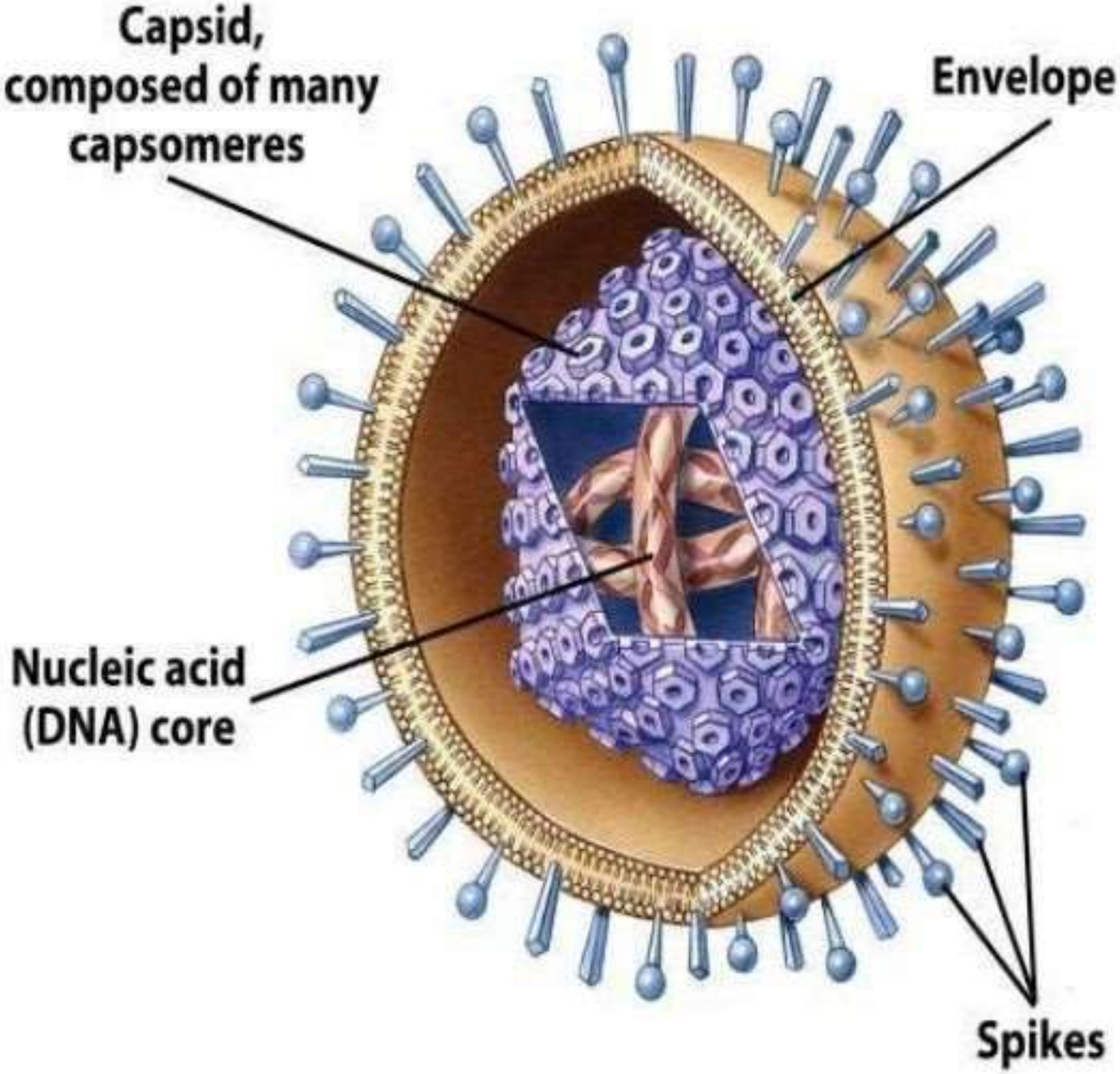
Ebola virus
970 nm

Plasma membrane of red blood cell
10 nm thick

E. coli
(a bacterium)
3000 × 1000 nm

Viral components

- Nucleic acids
- Capsid
- Envelope



Generalized Structure:

- Viruses consists of nucleic acid core surrounded by a protein called capsid.
- Capsid is composed of large number of capsomer which is made up of polypeptide molecules.
- The capsid with the enclosed nucleic acid is known as nucleocapsid.
- Fully formed virus that is able to establish an infection in a host cell is known as virion.

Capsids:

Capsomere

Nucleic acid

Capsid

(a) A polyhedral virus

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Functions of Capsid

- It protects the viral genome from physical destruction and enzymatic inactivation by nucleases in biological material.
- It provides the binding site which enable the virus to attach to specific site on the host cell.
- It facilitates the assembly and packaging of viral genetic information.
- It serves as a vehicle of transmission from host to another.
- It is antigenic and specific for each viruses
- It provides the structural symmetry to the virus particle.

- **Genome**:

- the sum total of the genetic information carried by an organism
- Number of viral genes compared with a cell- quite small
- They only have the genes necessary to invade host cells and redirect their activity

Nucleic Acids:

DNA Viruses :

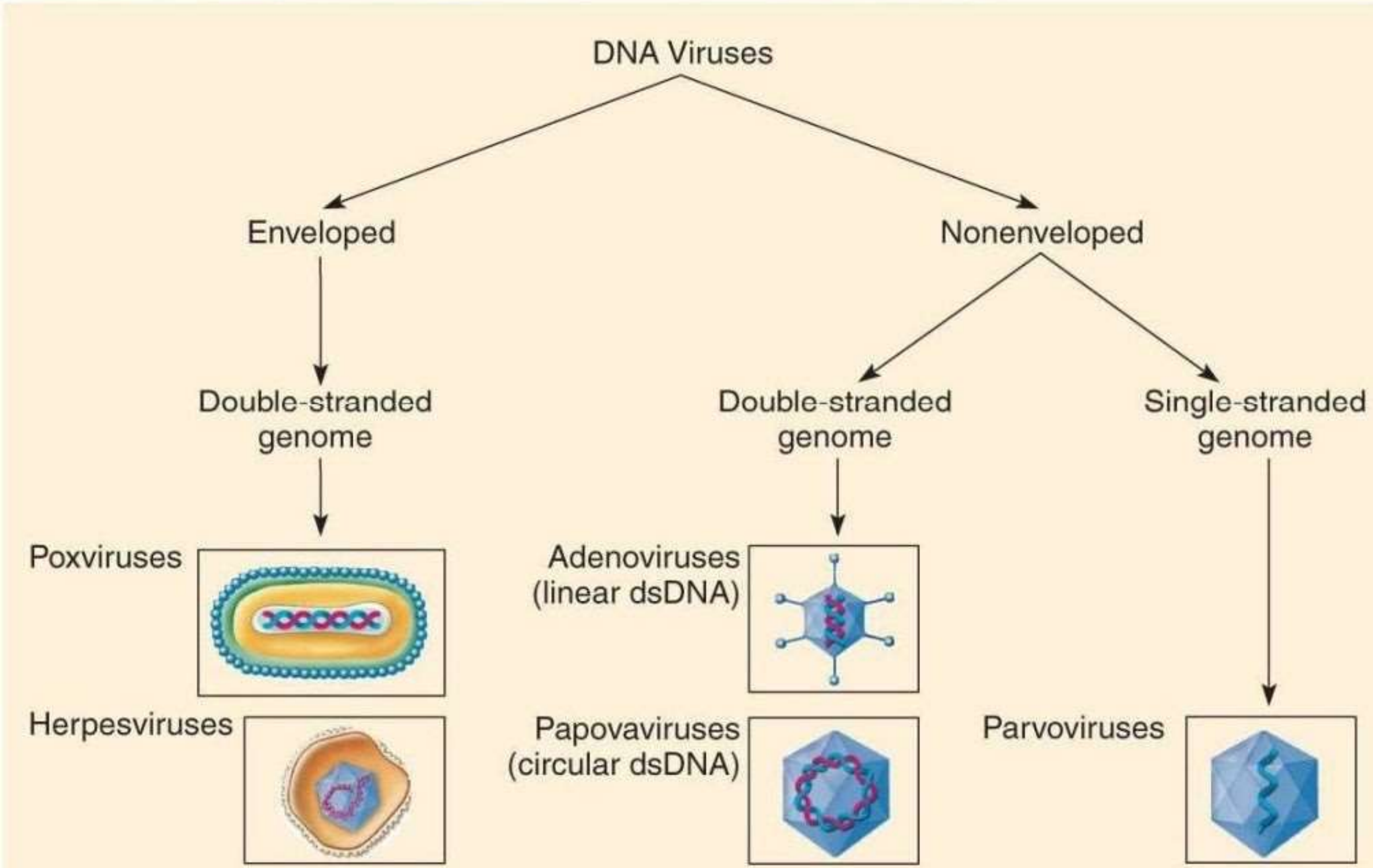
- **ssDND**

  (single stranded DNA)

- **dsDNA**

  (double stranded DNA)

TABLE 6.2 Medically Relevant DNA Virus Groups



Source: Adapted from: *Poxviridae* from Buller et al., National Institute of Allergy & Infectious Disease, Department of Health & Human Services.

- **Mostly single-stranded**
  - Positive-sense RNA: genomes that are ready for immediate translation into proteins
  - Negative-sense RNA: genomes have to be converted into the proper form to be made into protein

RNA Viruses:

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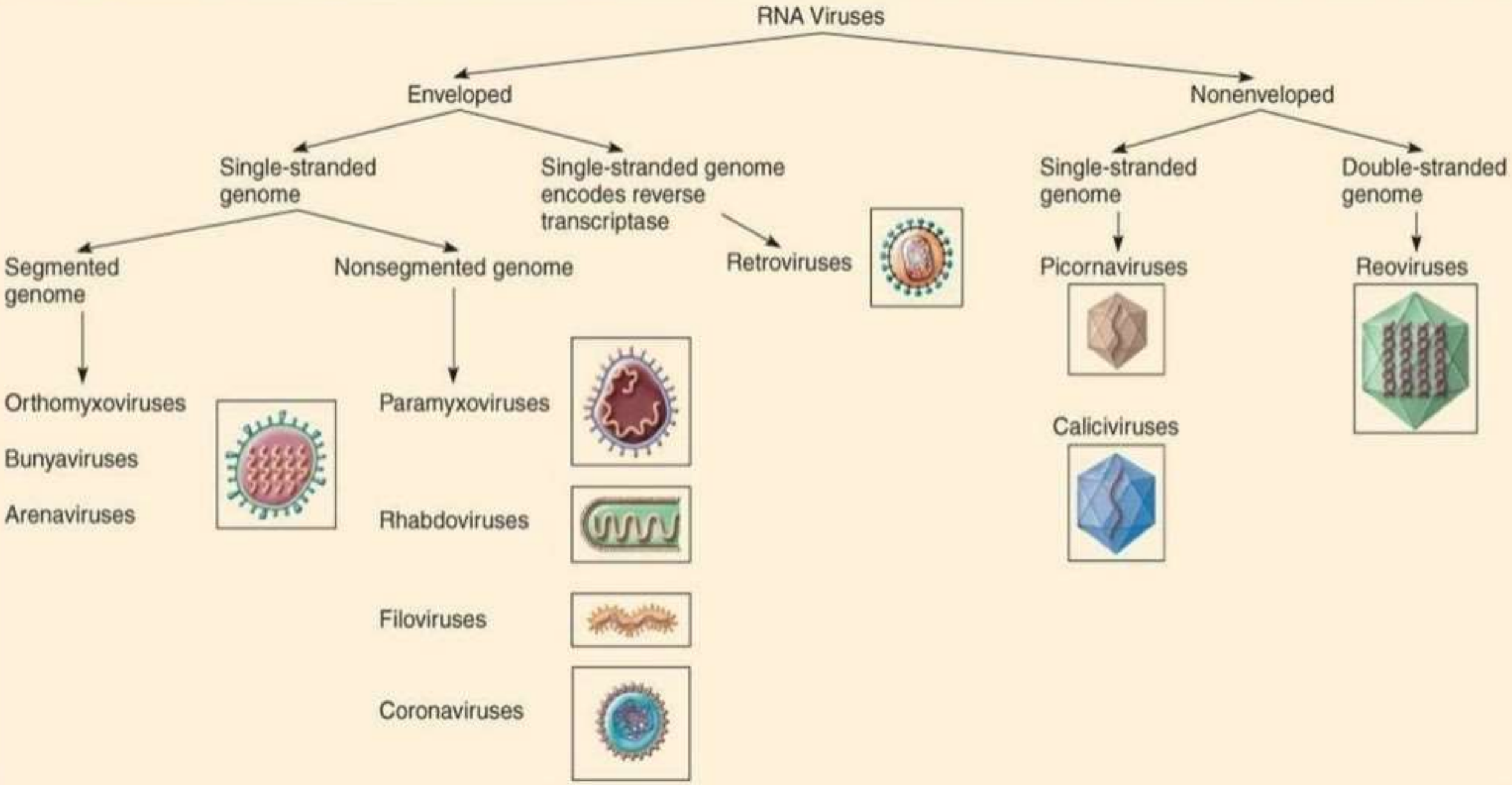
TABLE 6.3 **Medically Relevant RNA Viruses**



RNA viruses

Viral Envelop

Enveloped Virus

- The envelop or outer covering of virus containing lipid is derived from the plasma membrane of the host cell during the release by budding from the cell surface.
- The envelop is glycoprotein in nature.
- Enveloped viruses are susceptible to the action of lipid solvent such as ether, chloroform and detergent.
- Eg. Herpes virus, Hepatitis B virus, HIV virus

Non Enveloped Virus

- Viruses which does not have outer covering.
- Naked viruses are more likely to be resistant to lipid solvents like ether, chloroform and detergent.

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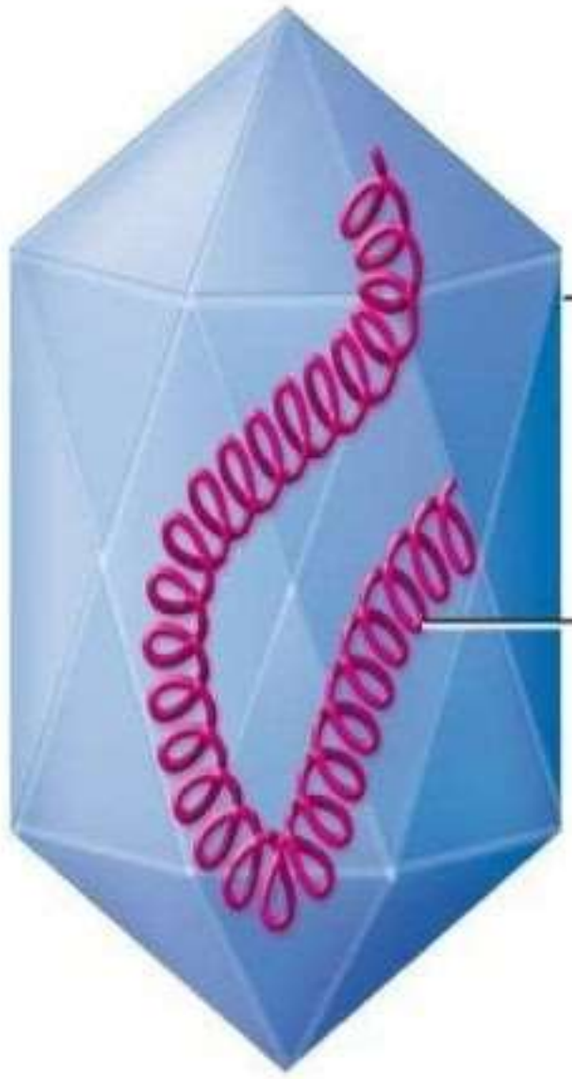
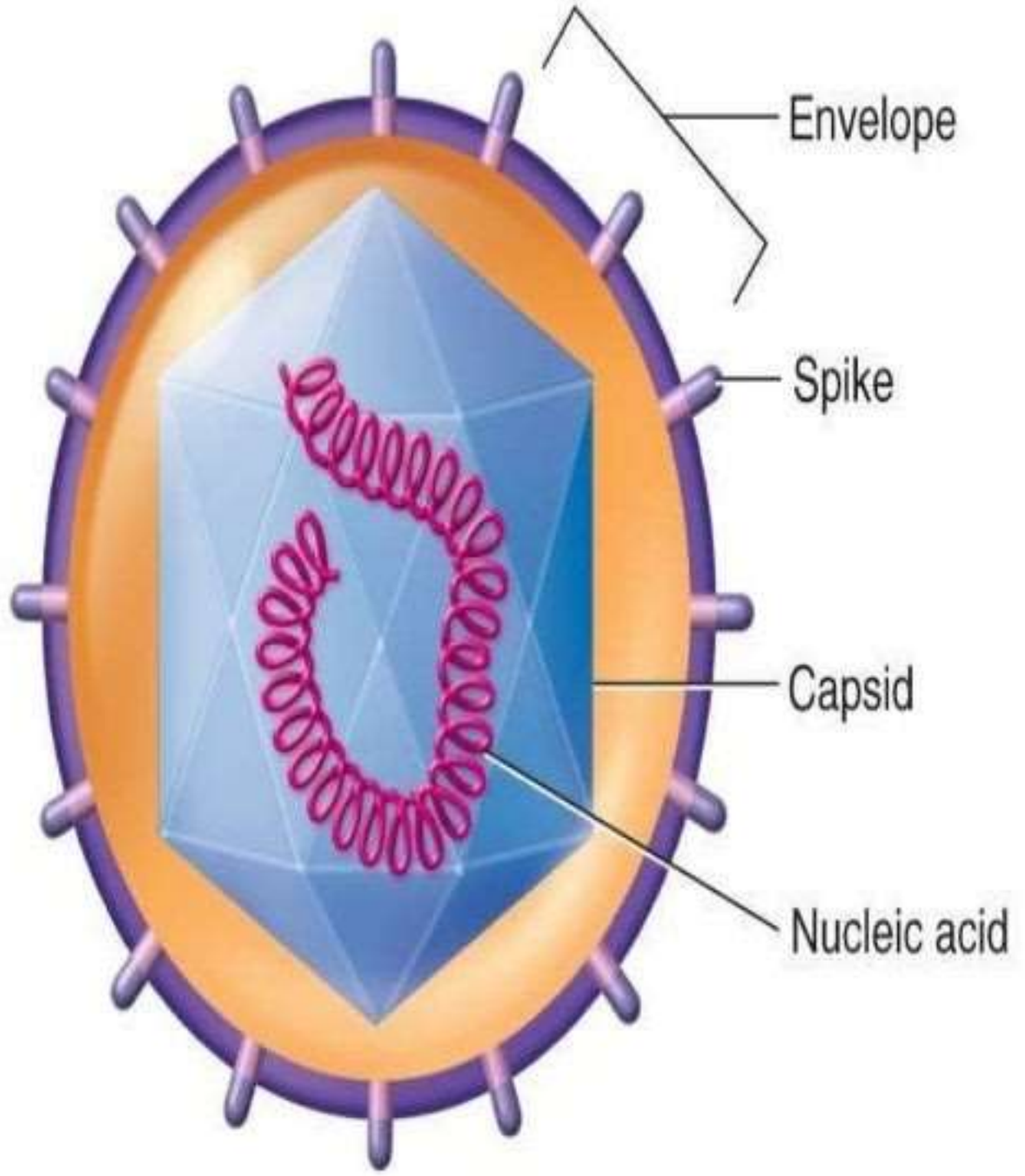


(a) **Naked Nucleocapsid Virus**



(b) **Enveloped Virus**

Peplomers

- In mature virus particle, the glycoproteins often appear as projecting spikes on the outer surface of the envelop which are known as peplomers.
- A virus may have more than one type of peplomers. E.g the influenza virus carries two types of peplomers, the hemagglutinin and neuraminidase

Functions of Peplomers

- It helps for attachment of virus to the host cell receptors to initiate the entrance of the virion into the cell.
- It attach to receptors on red blood cells, causing these cell to agglutinate.
- It has enzymatic activity like neuraminidase which cleave neuraminic acid from host cell glycoproteins.
- It has antigenic properties.

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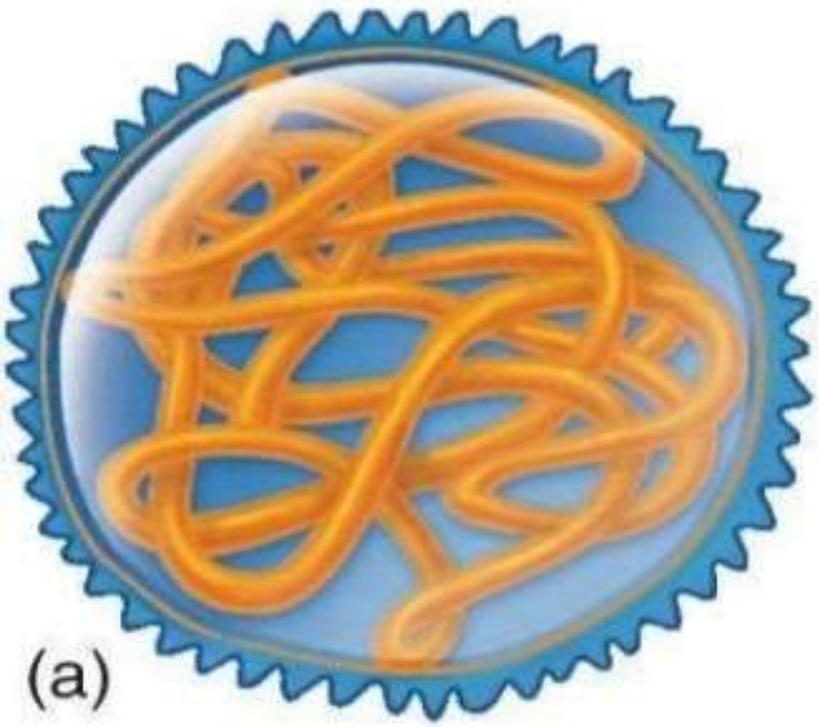
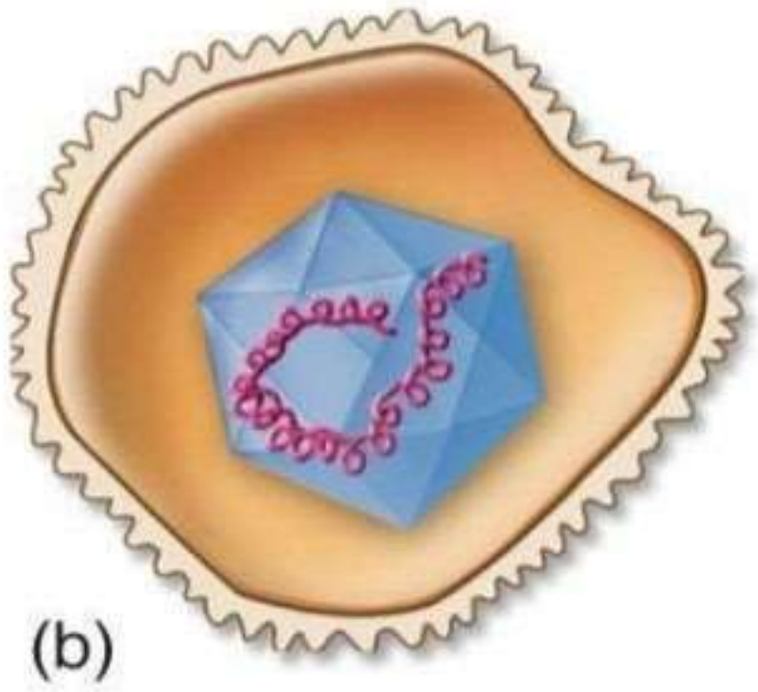
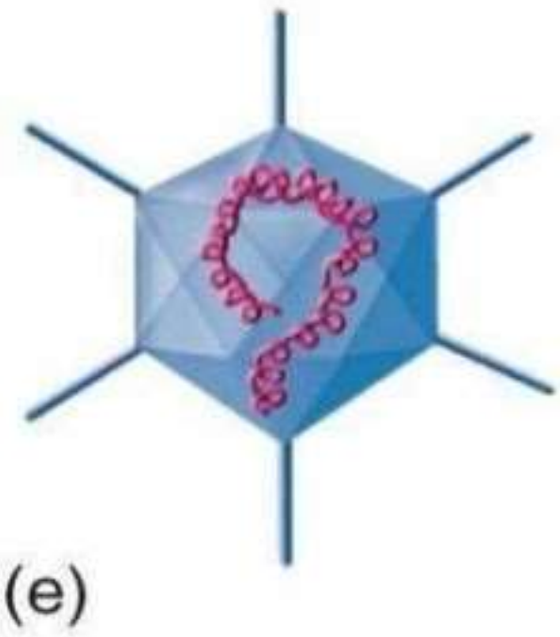
Enveloped Viruses

Naked Viruses

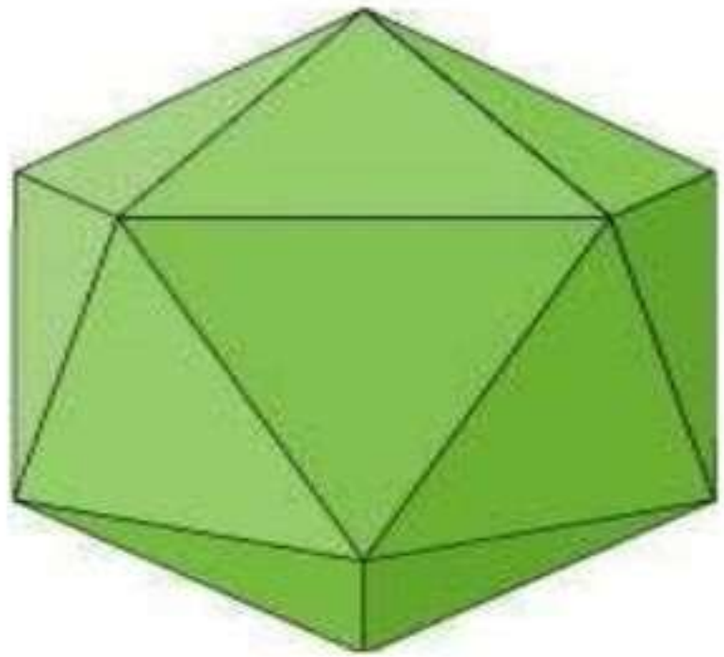






Icosahedral Symmetry

- An icosahedral (icosa, meaning 20 in greek) is a polgon with 12 vertices or corners and 20 facets or sides.
- Each facet is in the shape of an equilateral triangle.
- Pentagonal capsomers at the vertices (pentons) and hexagonal capsomers making up the facets (hexons)
- Eg. Adeno viruses



Structural Symmetries:

Helical Symmetry

- The nucleic acid and capsomers are wound together in the form of helix or spiral.
- Eg. Influenza virus, parainfluenz virus, rabies virus.

Structural Symmetries:

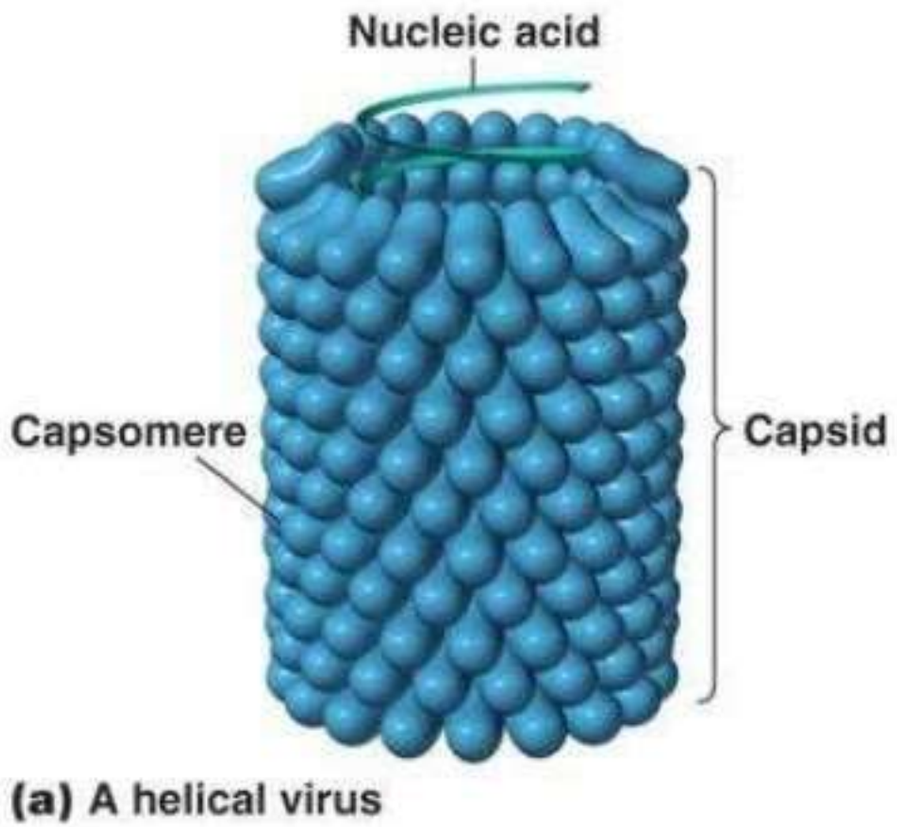
Complex Symmetry

- Viruses which don not show either icoshedral or helical symmetry

  due to complexity of their

  structure are referred to have

  complex symmetry.
- Eg. Pox viruses



(a) A helical virus

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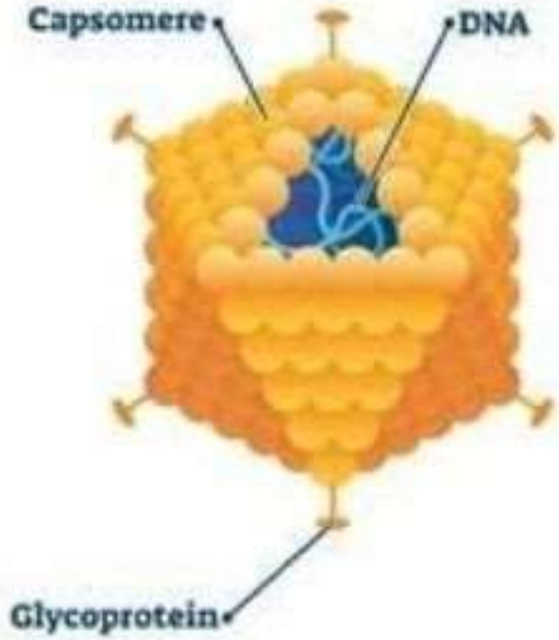
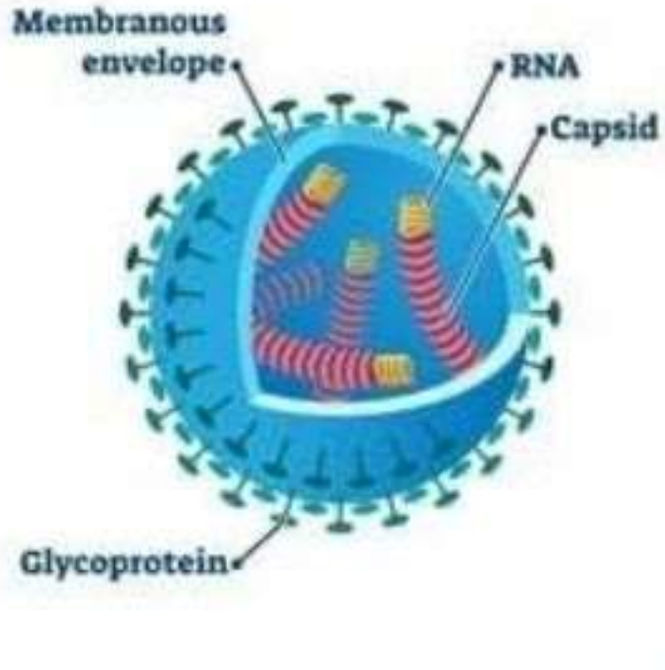
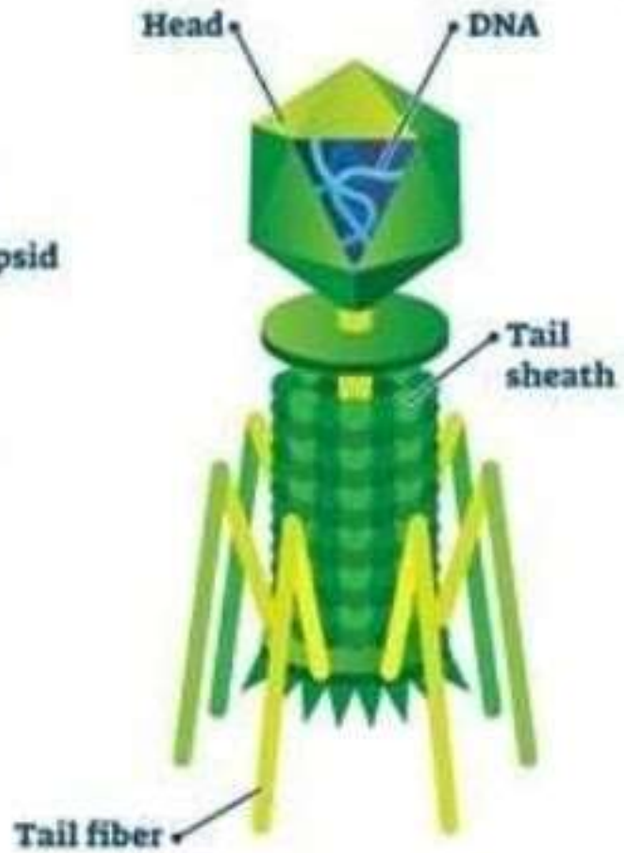
Structural Symmetries:







HELICAL

Tobacco Mosaic Virus

POLYHEDRAL

Adenovirus

SPHERICAL

Influenza Virus

COMPLEX

Bacteriophage

The genomic information necessary for viral replication is contained in the viral nucleic acid but lacking biosynthetic enzymes.

The virus depend on the synthetic machinery of the host cell for replication.

Viral multiplication proceeds as following manner.

- **Adsorption,**
- **Penetration,**
- **Uncoating,**
- **Synthesis,**
- **Assembly and Release**
- **Adsorption.**

Viral Replication

Adsorption/Attachment

- Virus encounters susceptible host cells
- Adsorbs specifically to receptor sites on the cell membrane
- Because of the exact fit required, viruses have a limited host range

- Flexible cell membrane of the host is penetrated by the whole virus or its nucleic acid
- Endocytosis: entire virus engulfed by the cell and enclosed in a vacuole or vesicle
- The viral envelope can also directly fuse with the host cell membrane

Penetration

- Enzymes in the vacuole dissolve the envelope and capsid
- The virus is now uncoated

Uncoating

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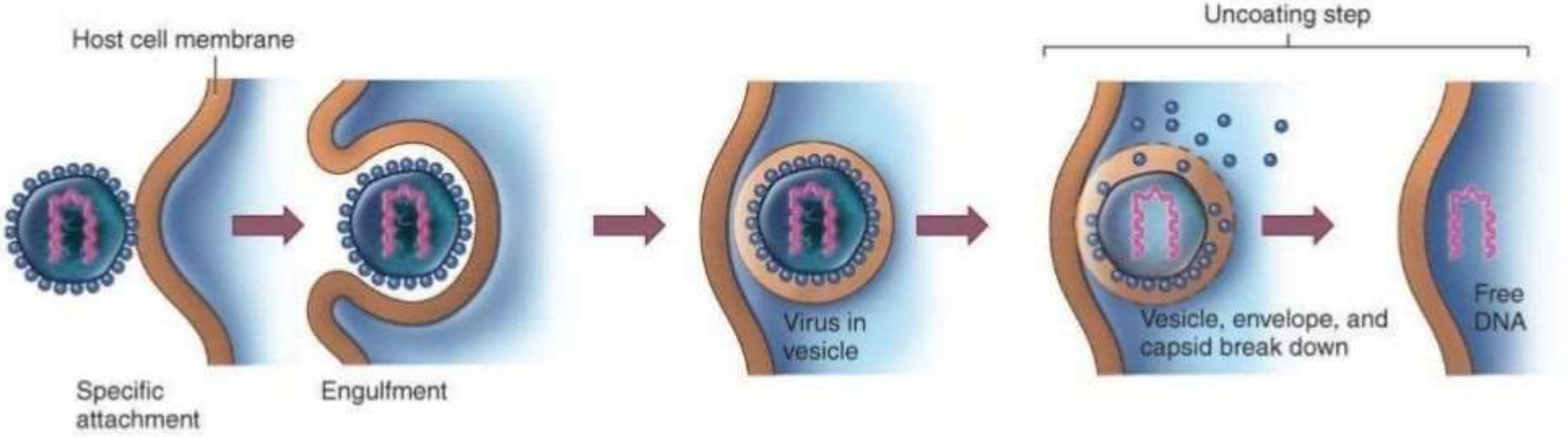
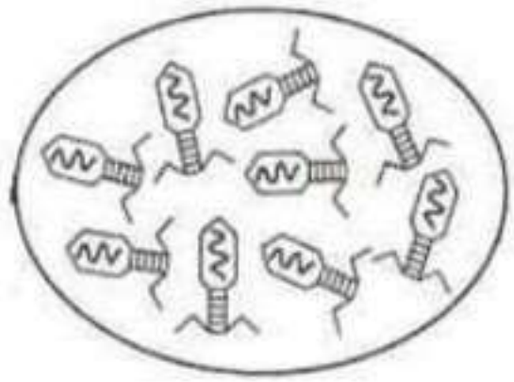


- Free viral nucleic acid exerts control over the host's synthetic and metabolic machinery
- DNA viruses- enter host cell's nucleus where they are replicated and assembled
- DNA enters the nucleus and is transcribed into RNA
- The RNA becomes a message for synthesizing viral proteins (translation)
- New DNA is synthesized using host nucleotides
- RNA viruses- replicated and assembled in the cytoplasm

Synthesis

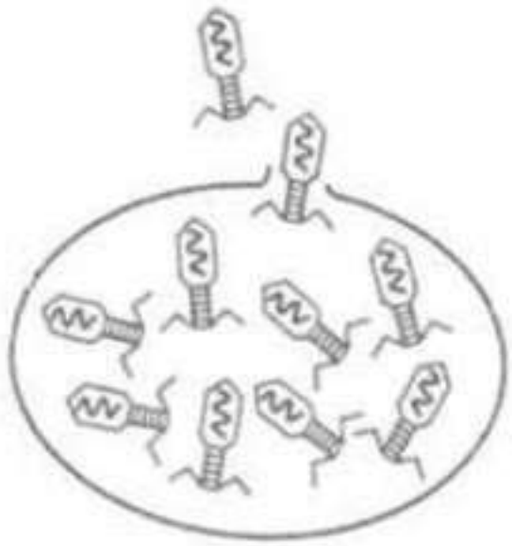
- Mature virus particles are constructed from the growing pool of parts

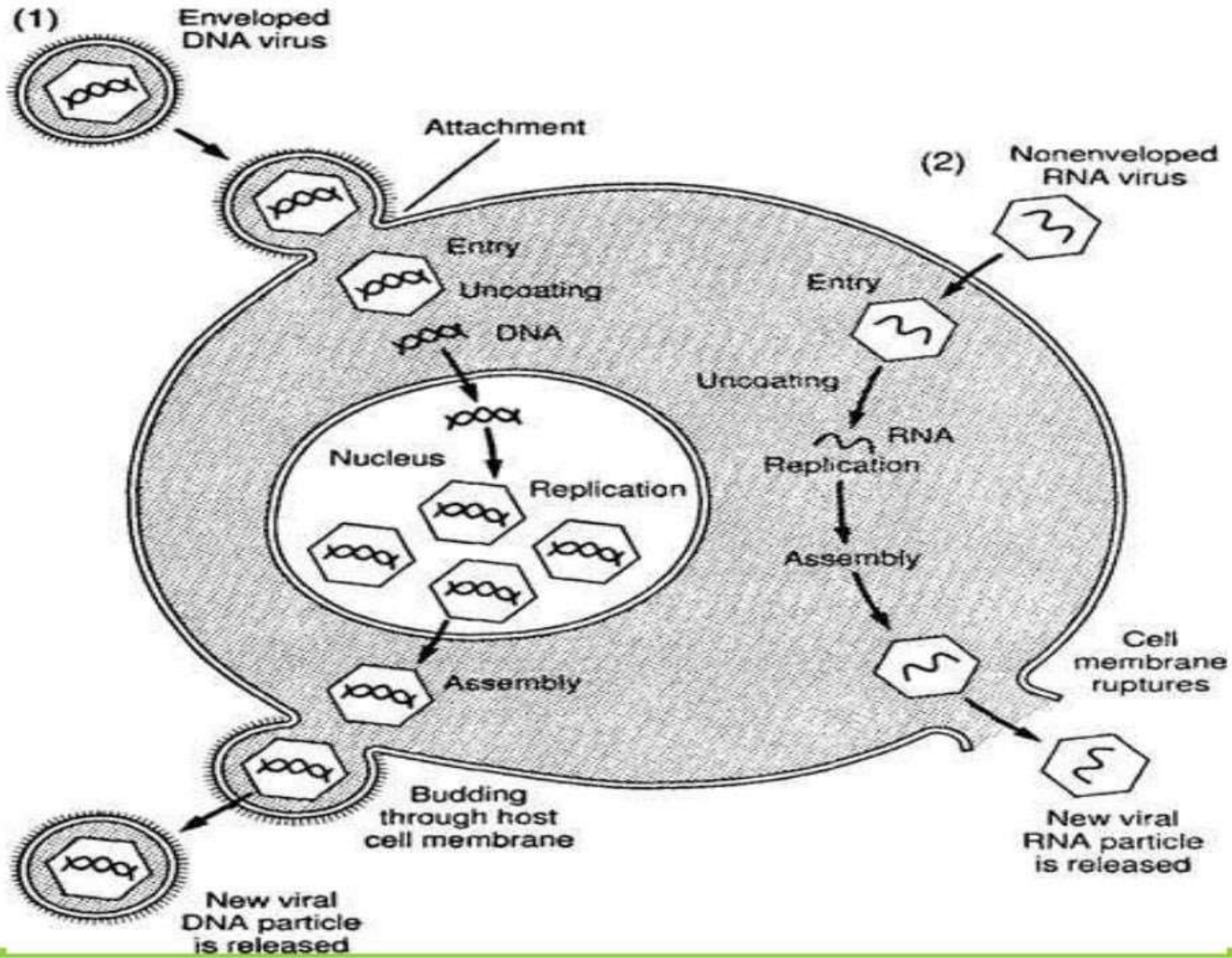


Assembly

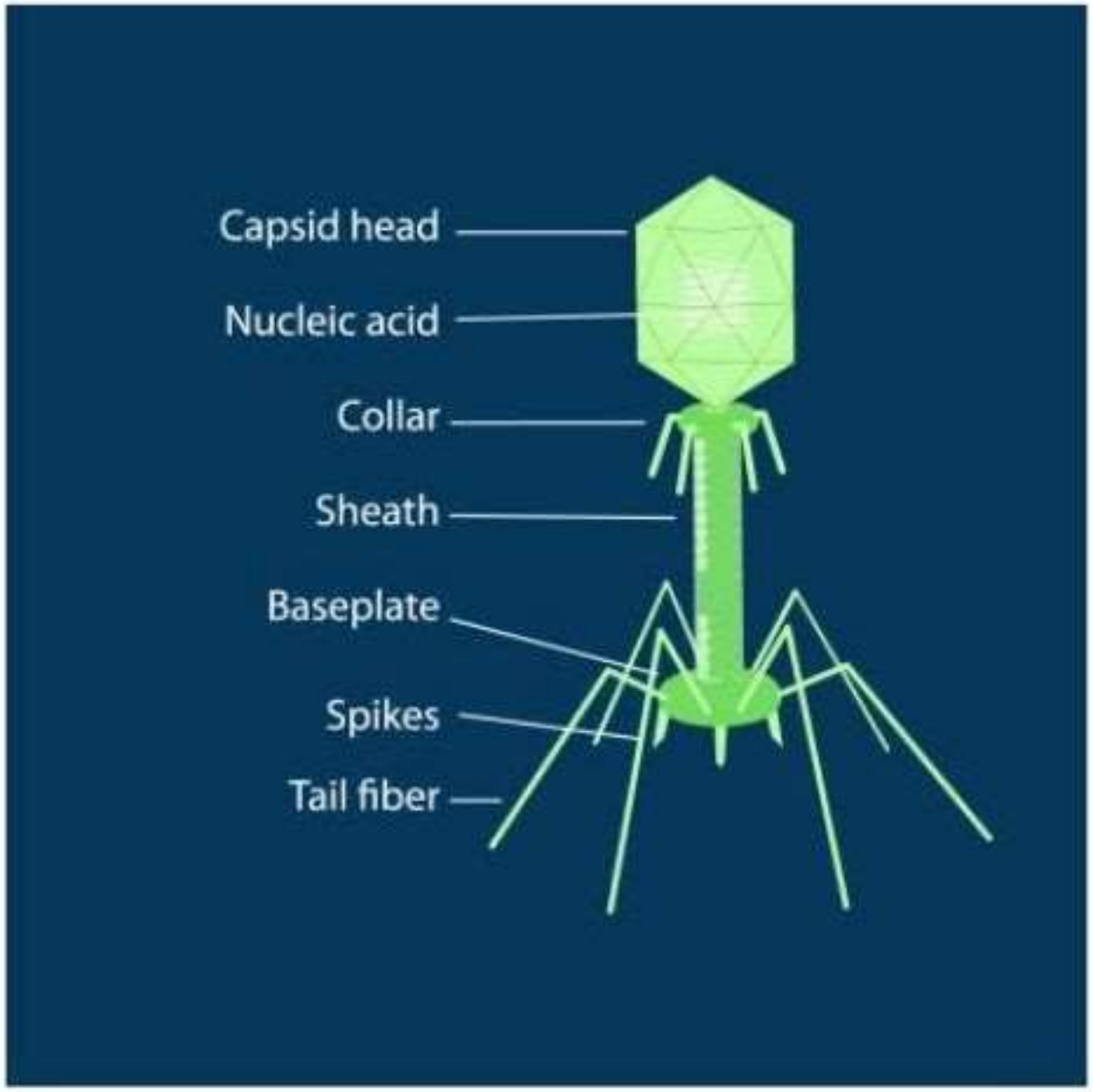
Release

- Nonenveloped and complex viruses are released when the cell lyses or ruptures
- Enveloped viruses are liberated by budding or exocytosis
- Anywhere from 3,000 to 100,000 virions may be released, depending on the virus
- Entire length of cycle- anywhere from 8 to 36 hours




(1)
Enveloped
DNA virus
Attachment
(2)
Nonenveloped
RNA virus
Entry
Uncoating
DNA
Nucleus
Replication
Assembly
Budding
through host
cell membrane
New viral
DNA particle
is released
Entry
Uncoating
RNA
Replication
Assembly
Cell
membrane
ruptures
New viral
RNA particle
is released

LYTIC CYCLE & LYSOGENIC CYCLE



Bacteriophage life cycle

BACTERIOPHAGE

-Bactria Eater

- Virus that infect the bacteria

-T4 Bacteriophage is one of the phage from total number of Bacteriophage of *E. Coli*

- Total Number – T1-T7

-Which replicate only by Lytic Cycle.

LYTIC CYCLE

VIRULANT VIRUS → VIRAL NUCLEIC ACID REPLICATE BY RUPTURING HOST CELL → VIRAL DNA REPLICATE SEPRATELY WITHOUT FUSING WITH HOST DNA

LYSOGENIC CYCLE

NON VIRULANT VIRUS / TEMPERATE VIRUS → VIRAL NUCLEIC ACID REPLICATE BY FUSION WITH HOST CELL → VIRAL DNA FUSED AND CONVERTED TO PROPHAGE

PURPOSE OF VIRUS CULTIVATION

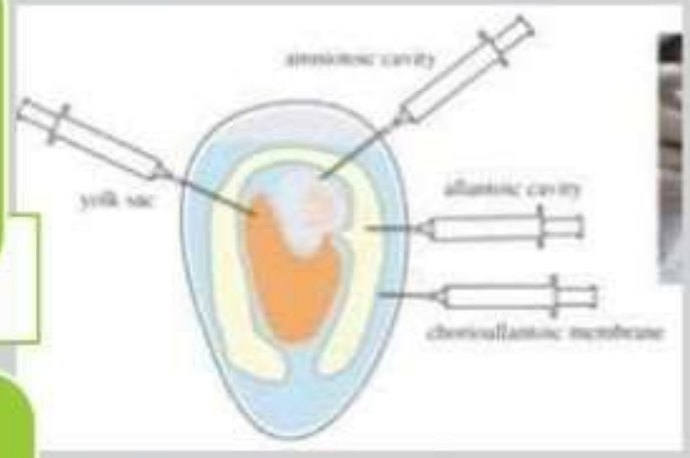
- To isolate and identify viruses in clinical samples.
- To prepare viruses for vaccine production.
- To do research on viral structure, replication , genetics and effects on host cells.

CULTIVATION OF VIRUS

METHODS FOR CULTIVATION OF VIRUSES

- Inoculation of Virus into Animal

- Inoculation of Virus into Embryonated Eggs.



- Tissue culture

Inoculation of Virus in Animals

1. Viruses which are not cultivated in embryonated egg and tissue culture are cultivated in laboratory animals. e.g: mice, guinea pig , hamester , rabbits and primates are used.
2. The selected animals should be healthy and free from any communicable diseases.
3. Suckling mice (less than 48 hours old) are most commonly used.
4. Route for Inoculation-

Intracerebral.

Subcutaneous.

Intraperitoneal.

Intranasal.

It is administered into the cerebrum. It means when a diseased blood vessel within the brain bursts allowing blood to leak inside the brain.

A subcutaneous injection is an injection in which a needle is inserted just under the skin.

Intraperitoneal injection is given to peritoneal cavity.

It lying within or administered by way of the nasal structure.

ADVANTAGES

- Production of antibodies can be identified.
- Diagnosis, pathogenesis and clinical symptoms are determined.
- Primary isolation of certain viruses.
- Mice provide a reliable model for studying viral replication.
- Used for the study of immune responses, epidemiology and oncogenesis

DISADVANTAGES

- Expensive and difficulties to maintained animals.
- Difficulty in choosing of animals for particular virus.
- Some human viruses cannot be grown in animals or can be grown but do not cause diseases.
- Mice do not provide models for vaccine development.

Inoculation of Virus in Egg Embryo

1. The process of cultivation of viruses in embryonated eggs depend upon the type of egg being used.
2. Egg provide a suitable means for :

-The primary isolation and identification of viruses.

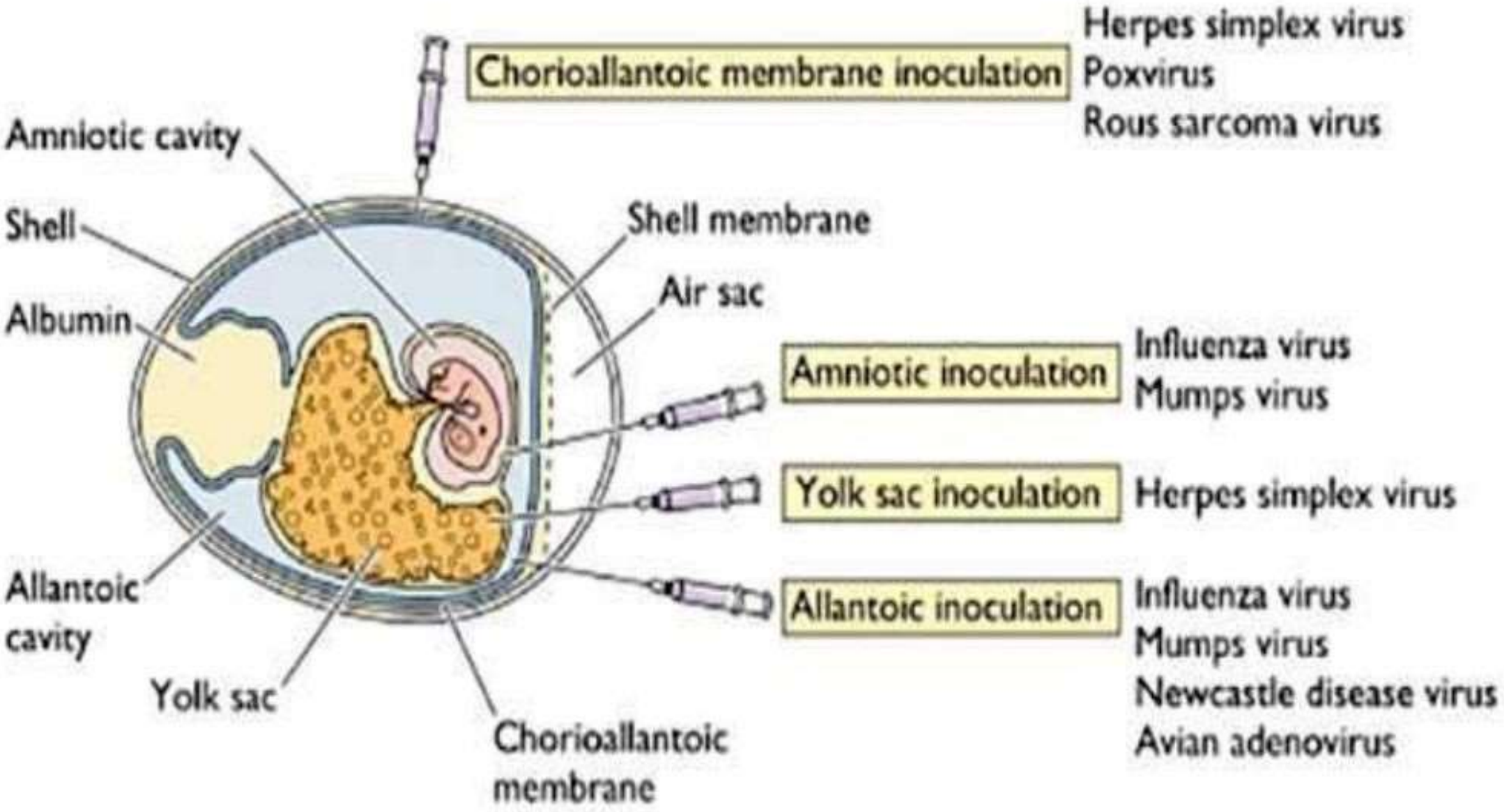
-The production of vaccines.

-The maintained of stock culture.

Viruses are inoculated into chick embryo of 7-12 days old.

For inoculation , eggs are first prepared for cultivation , the sheel surface are first prepared for cultivation , the shell surface is first disinfected with iodine and penetrated with a small sterile drill.

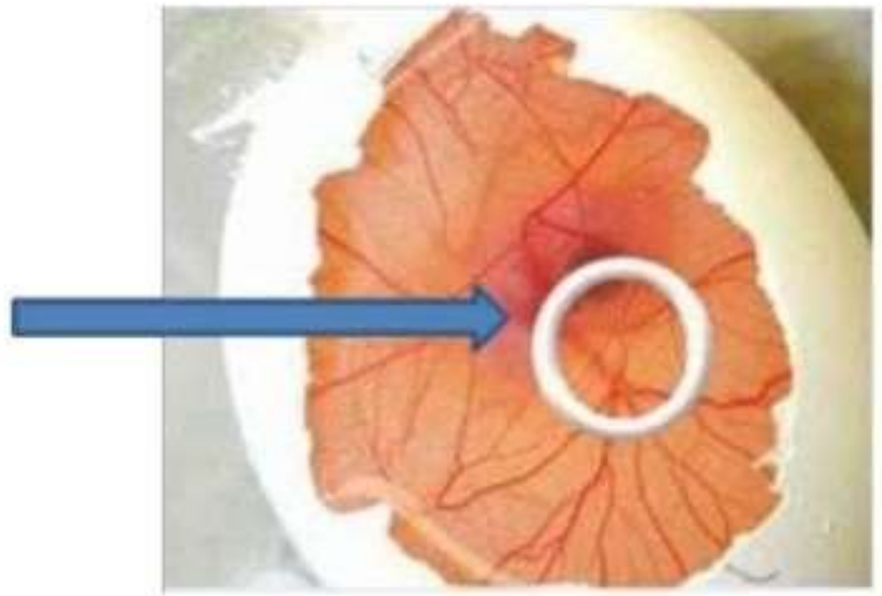
After incubation , the egg is broken and virus is isolated from tissue of egg.


Chorioallantoic membrane inoculation
Herpes simplex virus
Poxvirus
Rous sarcoma virus
Amniotic cavity
Shell
Albumin
Shell membrane
Air sac
Amniotic inoculation
Influenza virus
Mumps virus
Yolk sac inoculation
Herpes simplex virus
Allantoic cavity
Allantoic inoculation
Influenza virus
Mumps virus
Newcastle disease virus
Avian adenovirus
Yolk sac
Chorioallantoic membrane

- Virus growth and multiplication in the egg embryo is indicated by the death of the embryo , by embryo cell damage , or by the formation of typical pocks or lesions on the egg membrane.

Viruses can be cultivated in various pats of egg like :

1. Chorioallantoic membrane (CAM)
2. Allantoic cavity
3. Amniotic sac
4. Yolk sac



1. Chorioallantoic Membrane (CAM)

- Inoculation is mainly for growing provirus.
- After incubation visible lesions called pocks are observed , which is grey white area in transparent CAM.
- Herpes simplex virus is also grown.
- Single virus gives single pocks.
- This method is suitable for plaque studies.

2. Allantoic Cavity

- Inoculation is mainly done for production of vaccine of influenza virus , yellow fever , rabies.
- Most of avaian viruses can be isolated using this method.
- Allantoic inoculation is a quick and easy method that yields large amounts (8-15ml) of virus-infected egg fluids.

3. Amniotic Sac

- Inoculation is mainly done for primary isolation of influenza virus and the mumps virus.
- Growth and replication of virus in egg embryo can be detected by haemagglutination assay.
- The virus is introduced directly into the amniotic fluid that bathes the developing embryo.

4. Yolk Sac

- Inoculation is done for some bacteria and some viruses.
- This method is simple for cultivation and multiplication of virus.

ADVANTAGES

- Widely used method for the isolation of virus and growth.
- Cost effective and maintenance is much easier.
- The embryonated eggs are readily available.
- They are free from contaminating bacteria and many latent viruses.
- Ideal substrate for the viral growth and replication.
- less labor is needed.
- Widely used method to grow virus for some vaccine production.
- Defense mechanisms are not involved in embryonated eggs.

DISADVANTAGES

- The site of inoculation for varies with different virus . That is , each virus have different sites for growth and replication.

Inoculation of Virus Using Tissue Culture

There are three types of tissue culture:

- **Organ culture.**
- **Explant culture.**
- **Cell culture.**

Inoculation of Virus Using Organ Culture

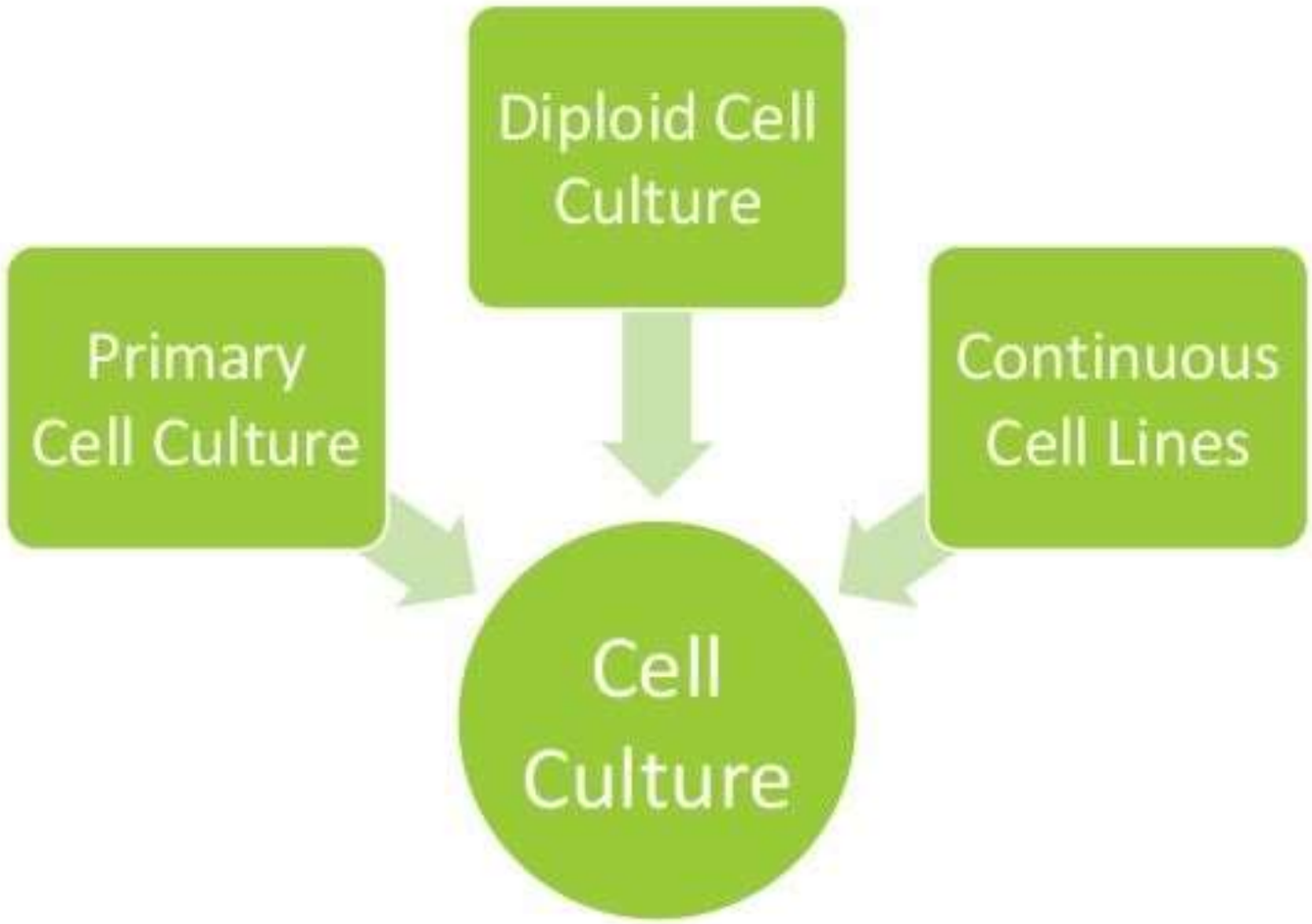
1. Small bits of organs can be maintained in vitro for days and weeks, preserving their original architecture and function.
2. Organs culture are useful for the isolation of some viruses which appear to be highly specialised parasite of certain organs.
3. For example, the tracheal ring organ culture is employed for the isolation of coronavirus, a respiratory pathogen.

Inoculation of Virus Using Explant Culture

1. Fragments of minced tissue can be grown as 'explants' embedded in plasma clots.
2. They may also be cultivated in suspension.
3. This method is now seldom employed in virology.
4. Adenoid tissue explant culture were used for the isolation of adenoviruses.

Inoculation of Virus Using Cell Culture

This is the type of culture routinely employed for growing



- Tissue are dissociated into the component cell by the action of proteolytic enzyme.

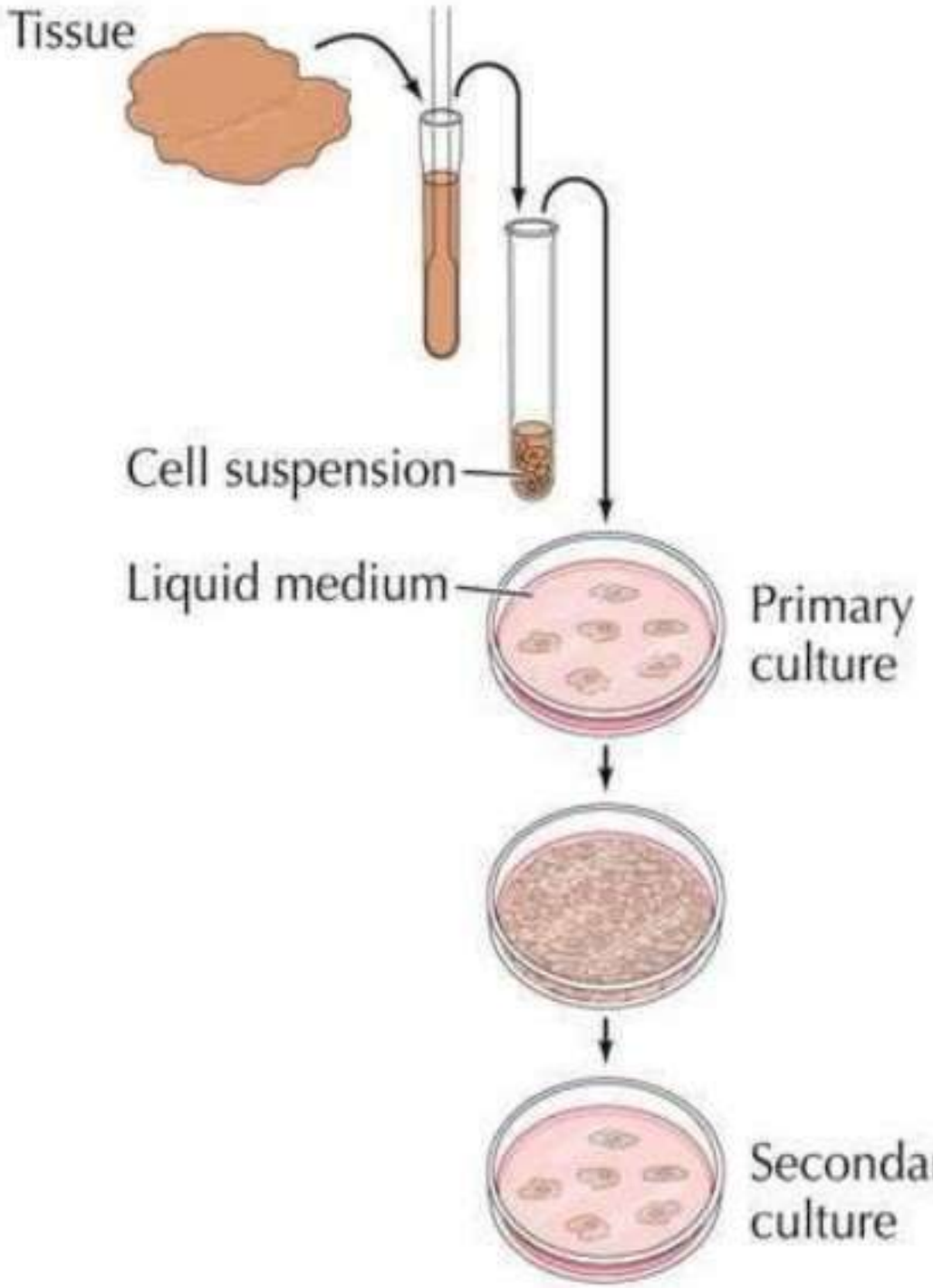
-The cells are washed , counted and suspended in a growth medium.

-Such media will enable most cell types to multiply with a division time of 24-48 hrs.

- The cell suspension is dispensed in bottles, tubes or petridishes

- The cell adhere to the glass surface and on incubation, divide to form a confluent monolayer sheet of cells covering the surface within about a week.

1.41 Culture of animal cells



THE CELL, Fourth Edition, Figure 1.41 © 2006 ASM Press and Sinauer Associates, Inc.

Primary Cell Culture

1. These are normal cells obtained from fresh organs of animals or human being and cultured.
2. They are capable of only limited growth in culture and cannot be maintained in serial culture.e.g. monkey kidney cell culture.human embryonic kidney. chick embryo cell culture.
3. They are commonly employed for primary isolation of viruses and in preparation of vaccine.

Diploid Cell Culture

1. These are cells of single type, contain the same number of chromosome as the parent cells and are diploid.
2. The diploid cell strains can be subculture for limited number of times.
3. After about 50 serial passage they undergo senescence.
4. They are also employed for the production of viral vaccine. Eg. human embryonic lung cell strain WI-38

Continuous Cell Line

1. Animal cells capable of indefinite growth are called continuous cell lines or cell lines.
2. These are the cells of a single type , usually derived from cancer cells , that are capable of continuous serial cultivation indefinitely.
3. Standard cell lines derived from human cancers , such as HeLa , HEp – 2 and KB cell lines have been used in laboratories throughout the world for many years.
4. These cell lines may be maintained by serial subcultivation or stored in the cold (
5. -70^0C) for use when necessary.
6. Some cell lines are now permitted to be used for vaccine manufacture, for example: Vero cells for rabies vaccine.

Advantages

- Relative ease, broad spectrum, cheaper and sensitivity

Disadvantages

- The process requires trained technicians with experience in working on a full time basis.
- Tissue or serum for analysis is sent to central laboratories to identify virus.
- State health laboratory and hospital Laboratory do not isolate and identify virus for clinical work.

ThankYou

For Your Precious Time