VIRUS CULTIVATION, PURIFICATION AND ASSAYS

Techniques in cultivating and identifying animal viruses

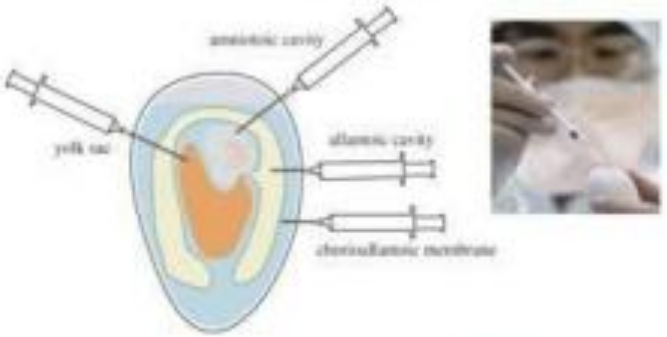
- viruses require living cells as their "medium".
  - In vivo – laboratory-bred animals and embryonic bird tissues.
  - In vitro - cell or tissue culture methods.

The primary purpose of virus cultivation is:

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

Methods for Cultivation of Virus

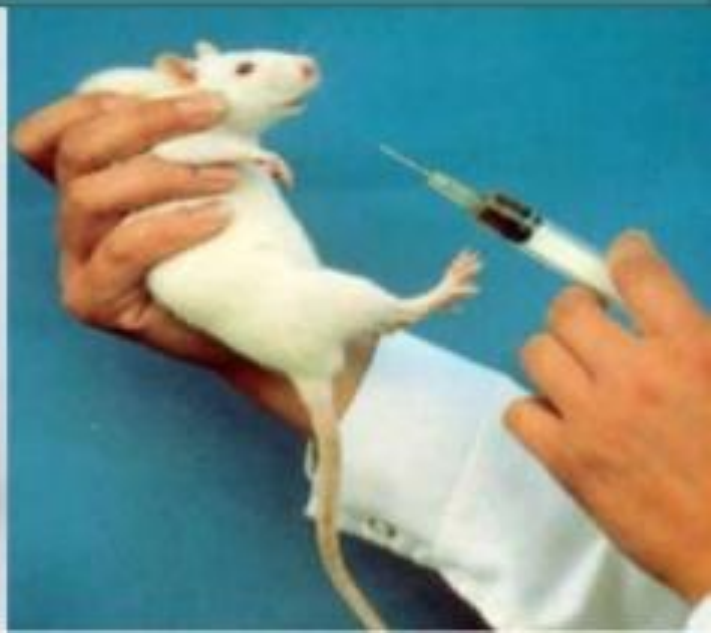
- Generally three methods are employed for the virus cultivation
  1. Inoculation of virus into animals
  2. Inoculation of virus into embryonated eggs
  3. Tissue culture



1. Animal Inoculation

- Viruses which are not cultivated in embryonated egg and tissue culture are cultivated in laboratory animals such as mice, guinea pig, hamster and rabbits are used.
- The selected animals should be healthy and free from any communicable diseases.
- Mice(less than 48 hours old) are most commonly used.
- Mice are susceptible to togavirus and coxsackie virues, which are inoculated by intracerebral and intranasal route.

- After inoculation, virus multiply in host and develops disease. The animals are observed for symptoms of disease and death.
- Then the virus is isolated and purified from the tissue of these animals.
- Live inoculation was first used on human volunteers for the study of yellow fever virus.



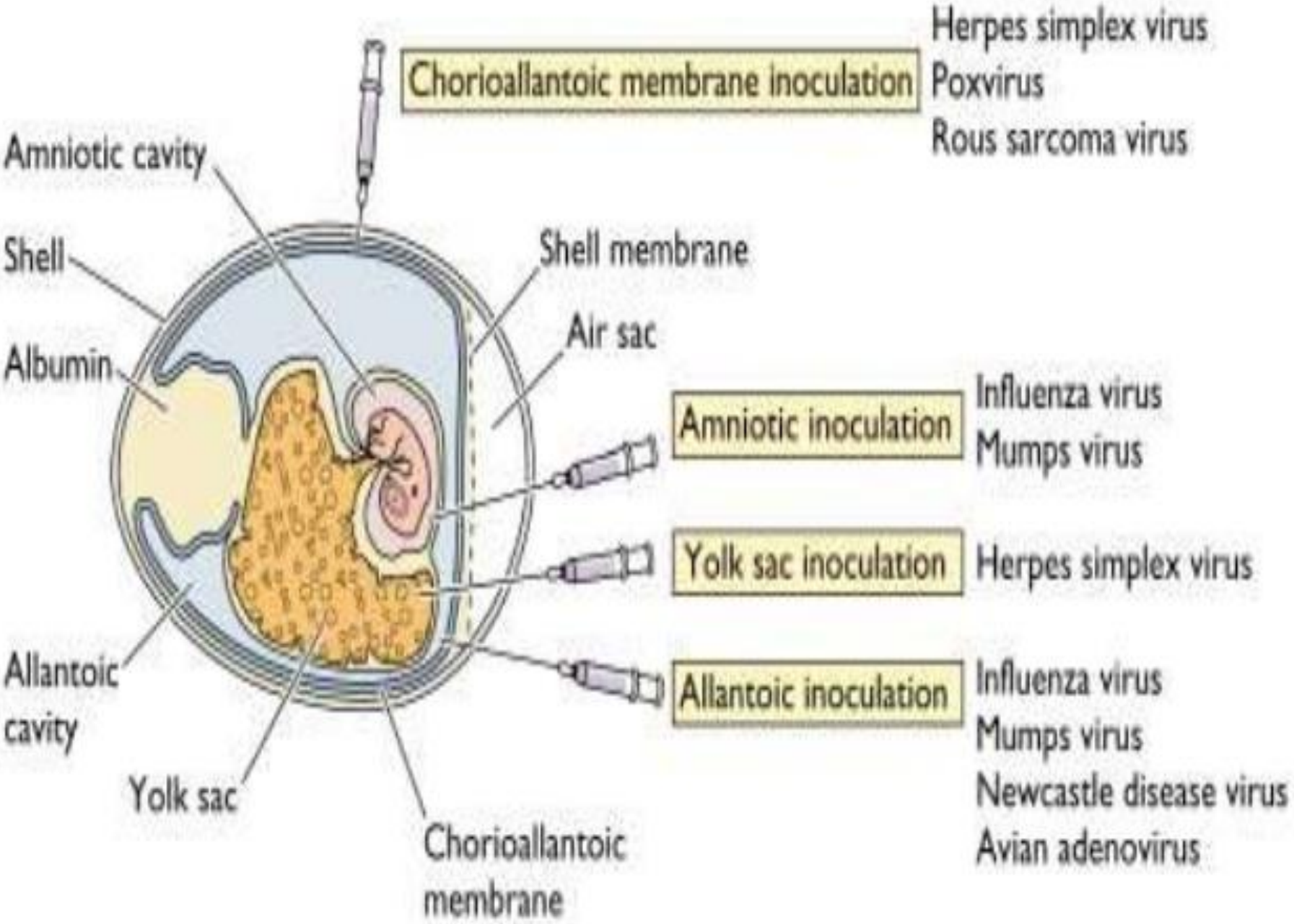
Disadvantages of Animal Inoculation

- Expensive and difficulties in maintenance of 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 disease.
- Mice do not provide models for vaccine development.
- Issues related to animal welfare systems.

2. Inoculation into embryonated egg

- Good pasture in 1931 first used the embryonated hen's egg for the cultivation of virus.
- The process of cultivation of viruses in embryonated eggs depends on the type of egg which is used.
- Viruses are inoculated into chick embryo of 7-12 days old.
- For inoculation, eggs are first prepared for cultivation, the shell surface is first disinfected with iodine and penetrated with a small sterile drill.

- After inoculation, the opening is sealed with gelatin or paraffin and incubated at 36°c for 2-3 days.
- After incubation, the egg is broken and virus is isolated from tissue of egg.
- Viral 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 membranes
- Viruses can be cultivated in various parts of egg like chorioallantoic membrane, allantoic cavity, amniotic sac and yolk sac.


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

Chorioallantoic Membrane (CAM):

- Inoculation is mainly for growing poxvirus.
- After incubation and , 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.

Allantoic cavity:

- Inoculation is mainly done for production of vaccine of influenza virus, yellow fever, rabies.
- Most of avian viruses can be isolated using this method.

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.

Yolk sac inoculation

- It is also a simplest method for growth and multiplication of virus.
- It is inoculated for cultivation of some viruses and some bacteria (Chlamydia, Rickettsiae)
- Immune interference mechanism can be detected in most of avian viruses.

Advantages of Inoculation into embryonated egg

- Widely used method for the isolation of virus and growth.
- Ideal substrate for the viral growth and replication.
- Isolation and cultivation of many avian and few mammalian viruses.
- Cost effective and maintenance is much easier.
- Less labor is needed.
- The embryonated eggs are readily available.
- They are free from contaminating bacteria and many viruses.
- Widely used method to grow virus for some vaccine production.

Disadvantages of Inoculation into embryonated egg

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

Animal viruses can also be grown in tissue (cell) culture on monolayers of animal cells. This technique is made possible by the development of growth media for animal cells and by the use of antimicrobial agents that prevent bacterial and fungal contamination.
Viruses are added to a layer of animal cells in a specially prepared petri dish and allowed time to attach to the cells. The cells are then covered with a thin layer of agar to limit virion spread so that only adjacent cells are infected by newly produced virions.

As a result, localized areas of cellular destruction and lysis called **plaques** often are formed and may be detected if stained with dyes, such as neutral red or trypan blue, that can distinguish living from dead cells

Animal viruses, in particular, can cause microscopic or macroscopic degenerative changes or abnormalities in host cells and in tissues

Detection of virus growth in cell culture

- **Cytopathic effects** – Many viruses causes morphological changes in cultured cells in which they grow. These changes can be observed microscopically and are known as cytopathic effects.

  The cytopathic effect are characteristic for a particular group of viruses and helps in the presumptive identification of viruses.

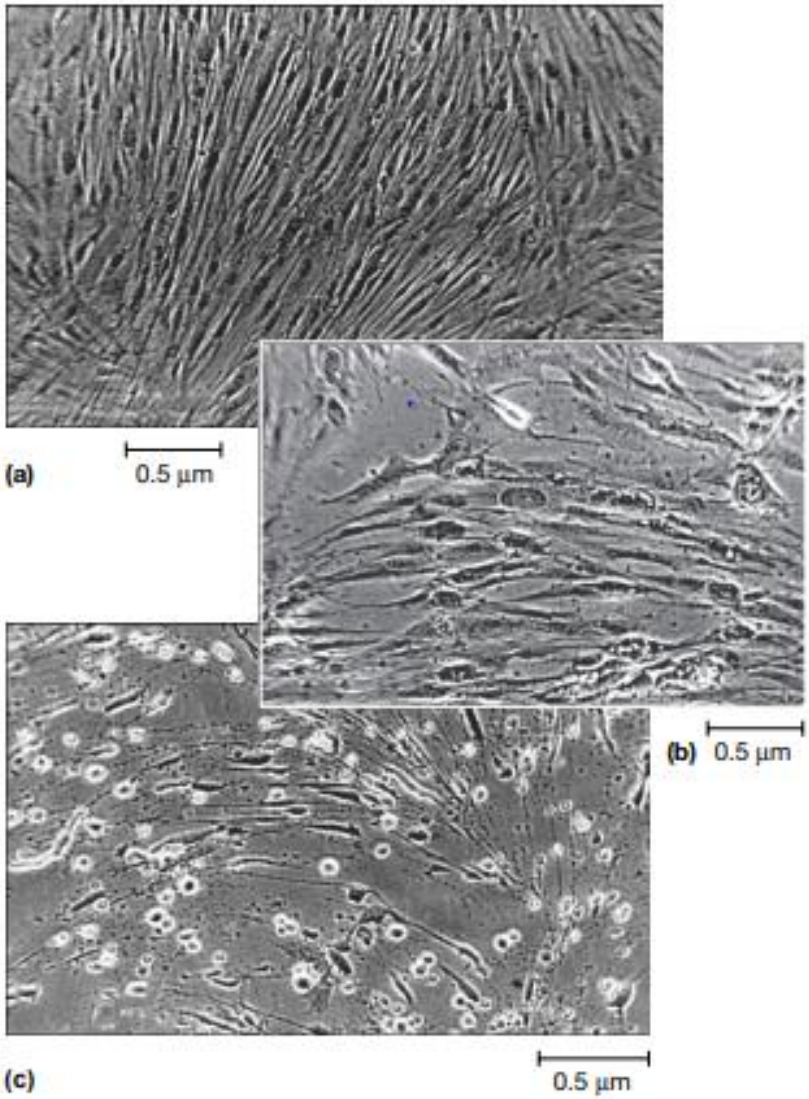


Figure 16.15 Cytopathic Effects of Viruses. **(a)** A monolayer of normal fibroblast cells from fetal tonsils. **(b)** Cytopathic effects caused by infection of fetal tonsil fibroblasts with adenovirus. **(c)** Cytopathic effects caused by infection of fetal tonsil fibroblasts with herpes simplex virus.

- **Metabollic inhibition** – In normal cell cultures the medium turns acidic due to cellular metabolism. Growth of viruses inhibits metabolism and hence no acidic production. This can be made out by the colour of the indicator (phenol red).

- **Haemadsorption** - When Haemogglutinating viruses (influenza and Para influenza) grow in cell culture. They adsorb Guinea pig RBCs onto the surface of cell cultures.

Isolation, cultivation, identification

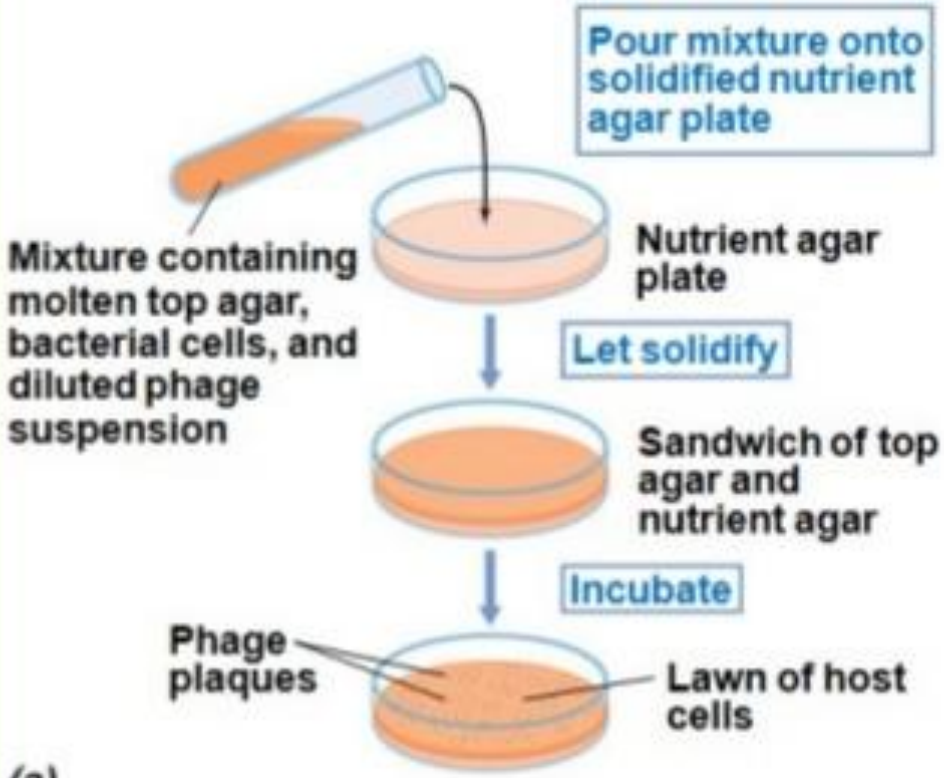
- **Bacteriophages can be grown**

1- In suspension of bacteria in liquid media

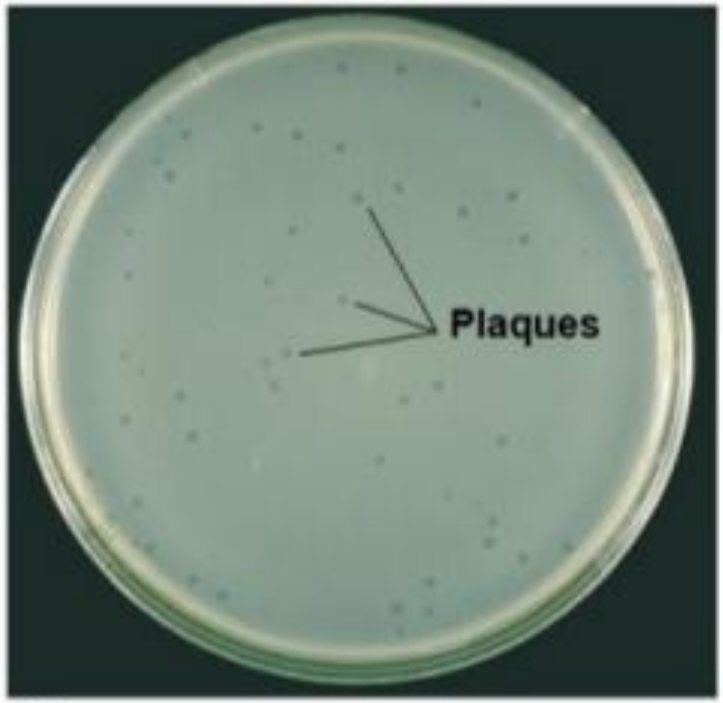
2- In bacterial cultures on solid media.

- On solid media the phage infection produces plaques that can be counted and theoretically correspond to one virus per plaque. The count is given as **pfu** (plaque forming units).

Quantification of bacterial virus by plaque assay using the agar overlay technique

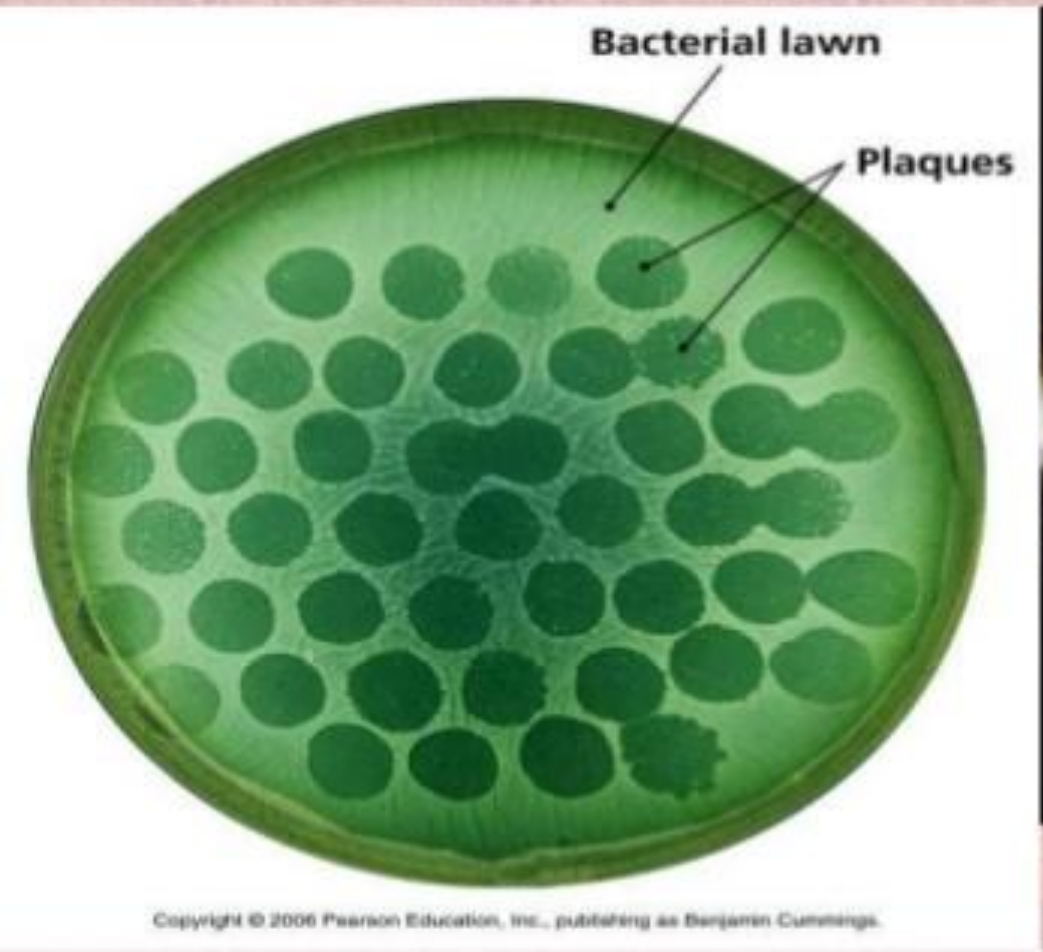


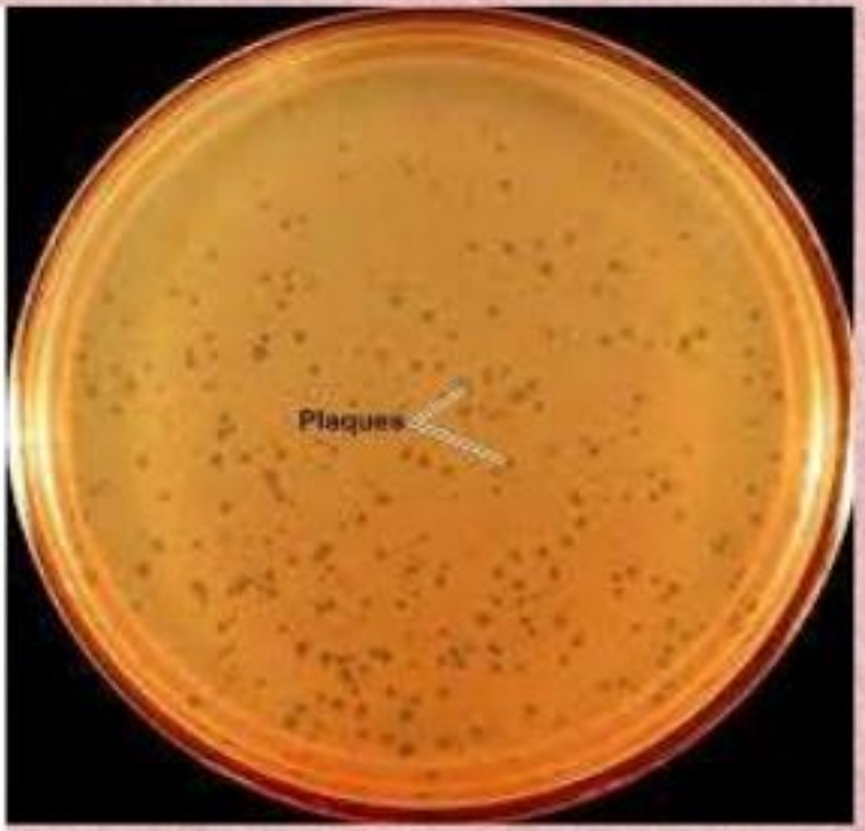
(a)



(b)

Plaque : A clear area in a lawn of host cells that results from the lysis of host cells by viruses.





Plant viruses are cultivated in a variety of ways. Plant tissue cultures, cultures of separated cells, or cultures of protoplasts (cells lacking cell walls) may be used. Viruses also can be grown in whole plants. Leaves are mechanically inoculated when rubbed with a mixture of viruses and an abrasive. When the cell walls are broken by the abrasive, the viruses directly contact the plasma membrane and infect the exposed host cells. (In nature, the role of the abrasive is frequently filled by insects that suck or crush plant leaves and thus transmit viruses.) **A localized necrotic lesion** often develops due to the rapid death of cells in the infected area

(a)

(b)

Figure 16.17 Necrotic Lesions on Plant Leaves.
(a) Tobacco mosaic virus on *Nicotiana glutinosa*. **(b)** Tobacco mosaic virus infection of an orchid showing leaf color changes.

Virus Purification

- four commonly used methods
  - differential centrifugation and density gradient centrifugation
  - precipitation of viruses
  - denaturation of contaminants
  - enzymatic digestion of cell constituents

Differential centrifugation

- separates based on size

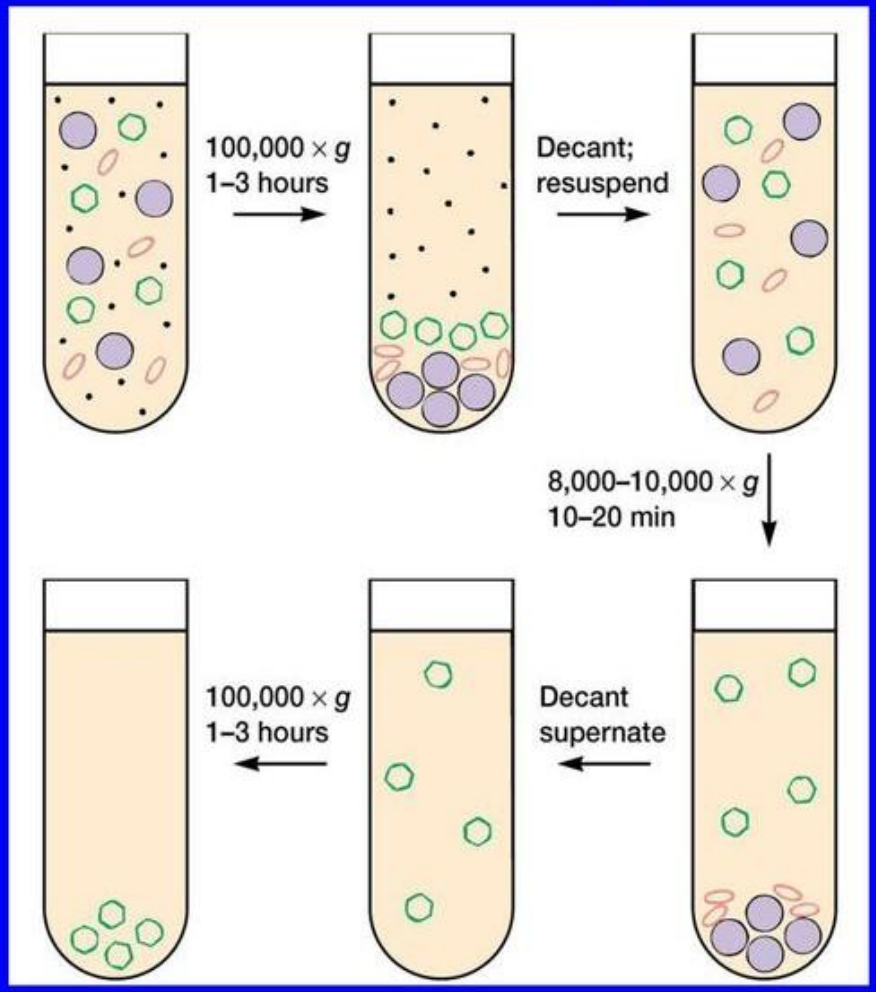


Figure 16.6

Density gradient centrifugation

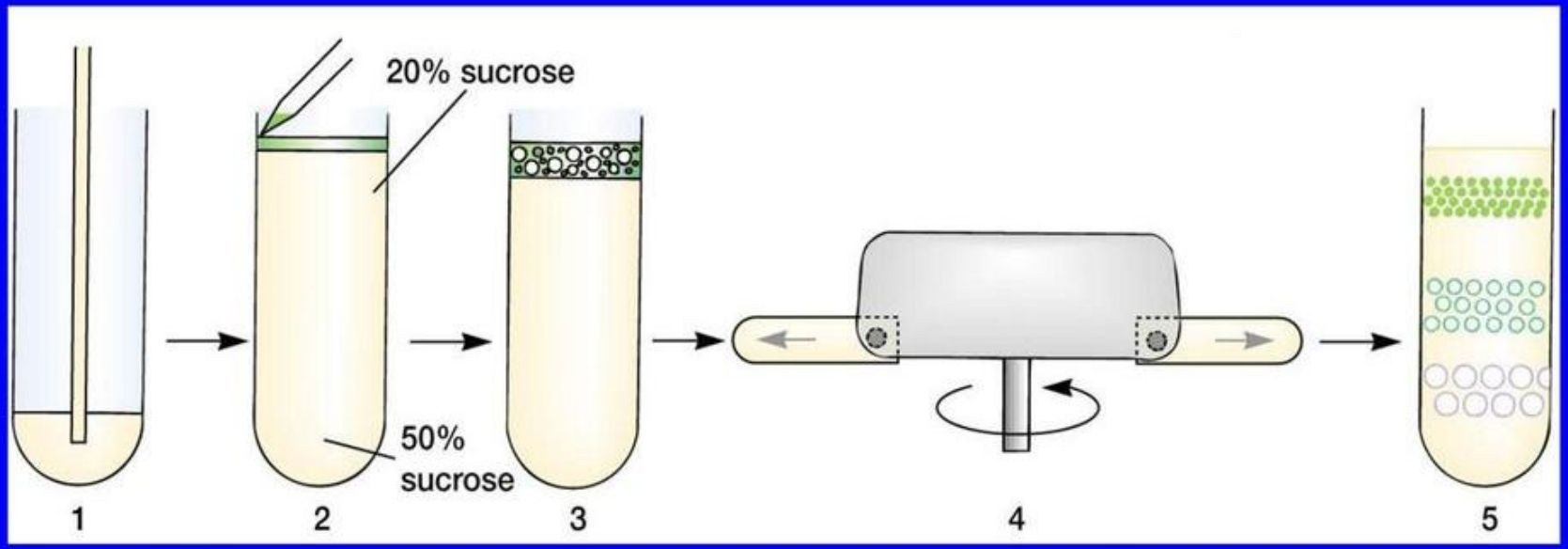


Figure 16.7a

separates based on size and density

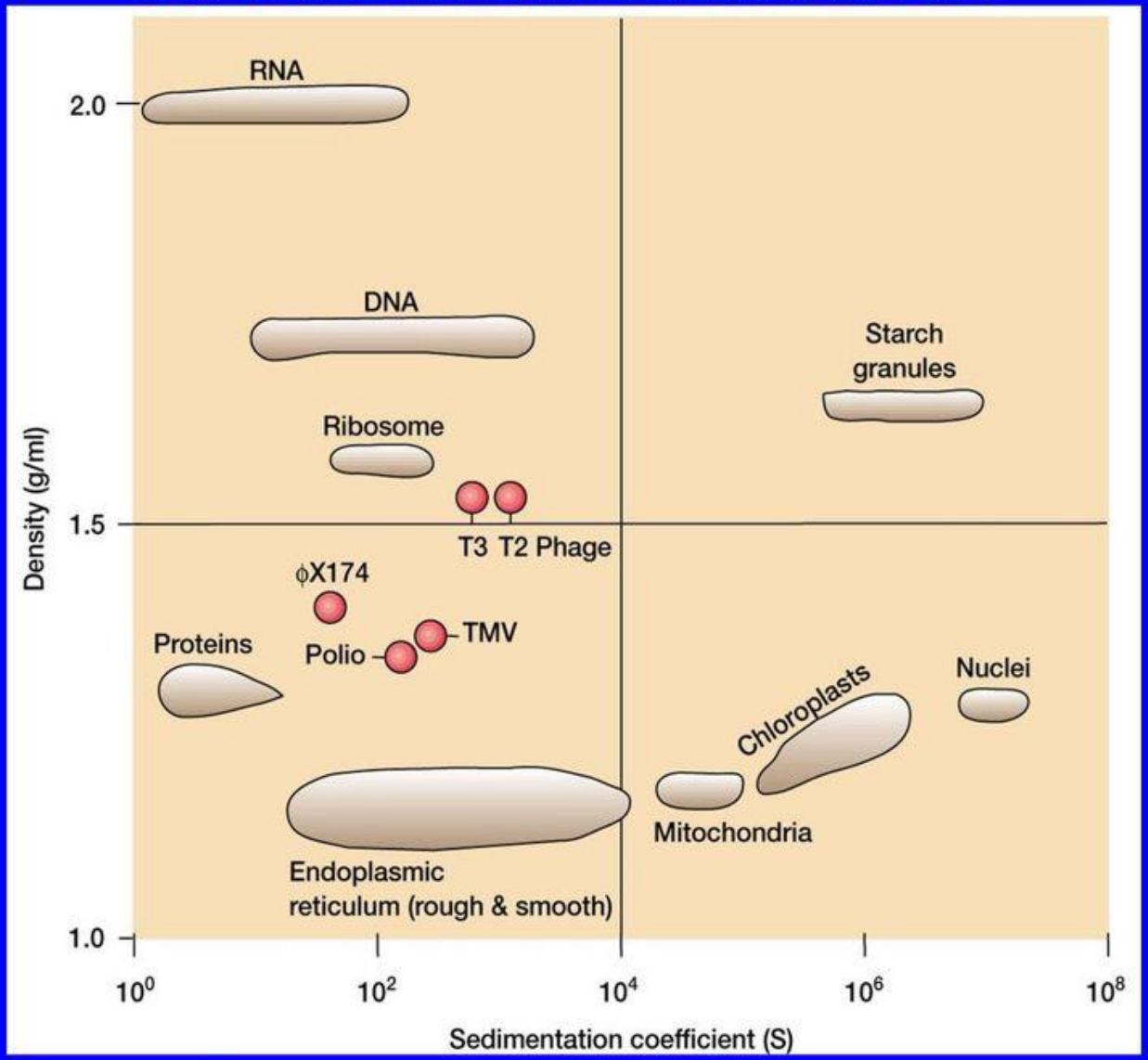


Figure 16.7b

Differential precipitation

- commonly uses ammonium sulfate or polyethylene glycol
- separates viruses from other components in a virus preparation mixture

Denaturation and precipitation of contaminants

- can be achieved with heat, pH, and organic solvents

Enzymatic degradation of cellular constituents

- proteases and nucleases used to remove cellular proteins and nucleic acids

Virus Assays

- used to determine quantity of viruses in a sample
- two types of approaches
  - count particles
  - measure concentration of infectious units

Particle counts

- direct counts
  - made with an electron microscope

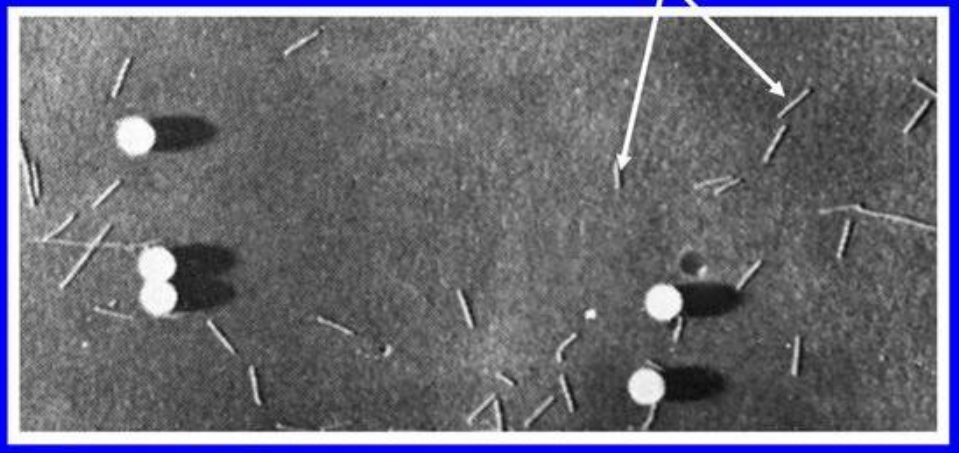
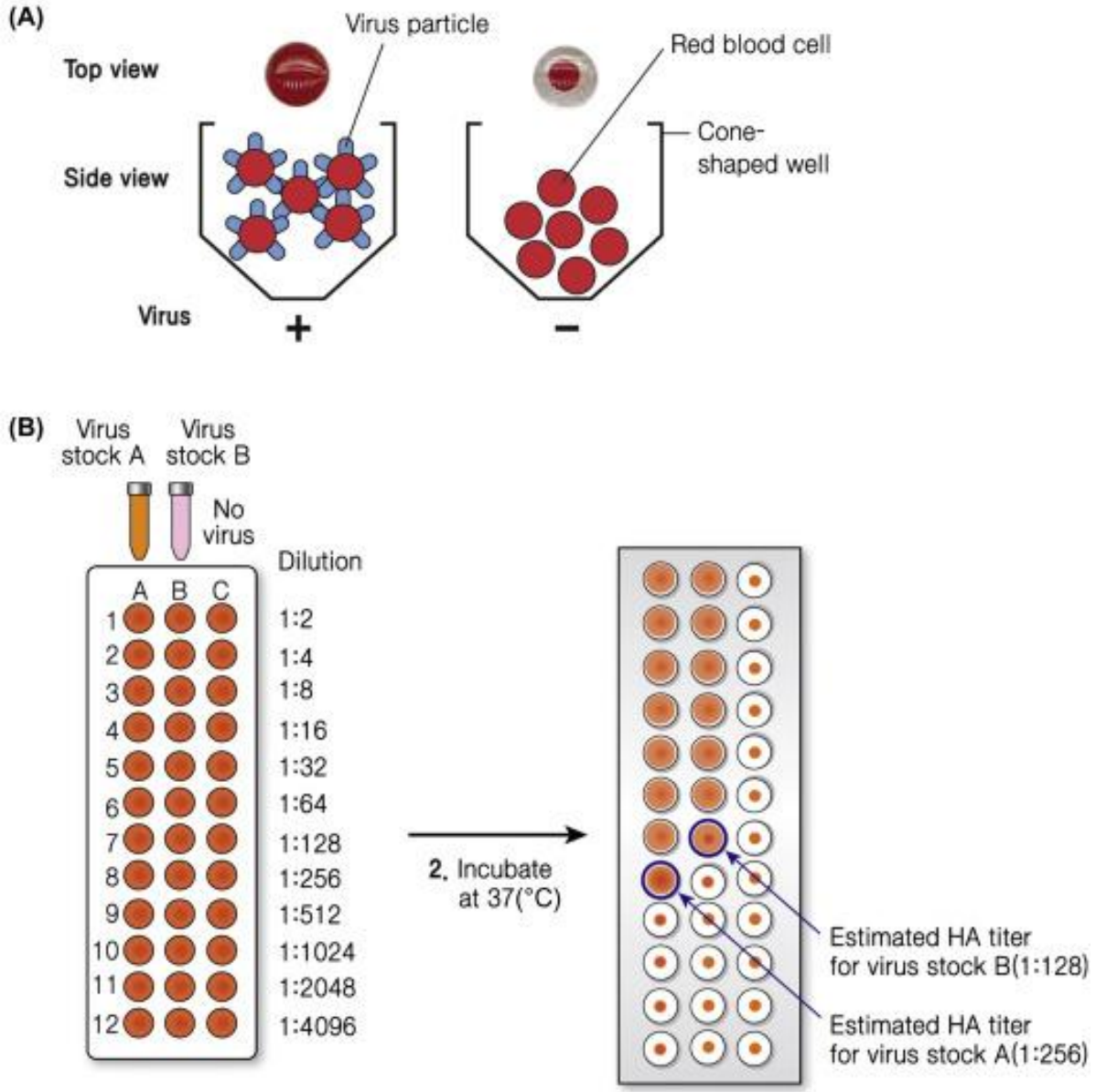


Figure 16.8

- indirect counts
  - e.g., **hemagglutination assay**
    - determines highest dilution of virus that causes red blood cells to clump together


(A)
Top view
Side view
Virus
Virus particle
Red blood cell
Cone-shaped well
+
−
(B)
Virus stock A
Virus stock B
No virus
Dilution
A B C
1 1:2
2 1:4
3 1:8
4 1:16
5 1:32
6 1:64
7 1:128
8 1:256
9 1:512
10 1:1024
11 1:2048
12 1:4096
2. Incubate at 37(°C)
Estimated HA titer for virus stock B(1:128)
Estimated HA titer for virus stock A(1:256)
1. Add twofold serial dilutions of virus to red blood cells in a 96-well plate
3. Examine the cells in each well for hemagglutination

Measuring concentration of infectious units

- plaque assays
  - dilutions of virus preparation made and plated on lawn of host cells
  - number of plaques counted
  - results expressed as plaque-forming units (PFU)

Measuring concentration of infectious units…

- infectious dose and lethal dose assays
  - determine smallest amount of virus needed to cause infection or death of 50% of exposed host cells or organisms
  - results expressed as ID_{50} or LD_{50}

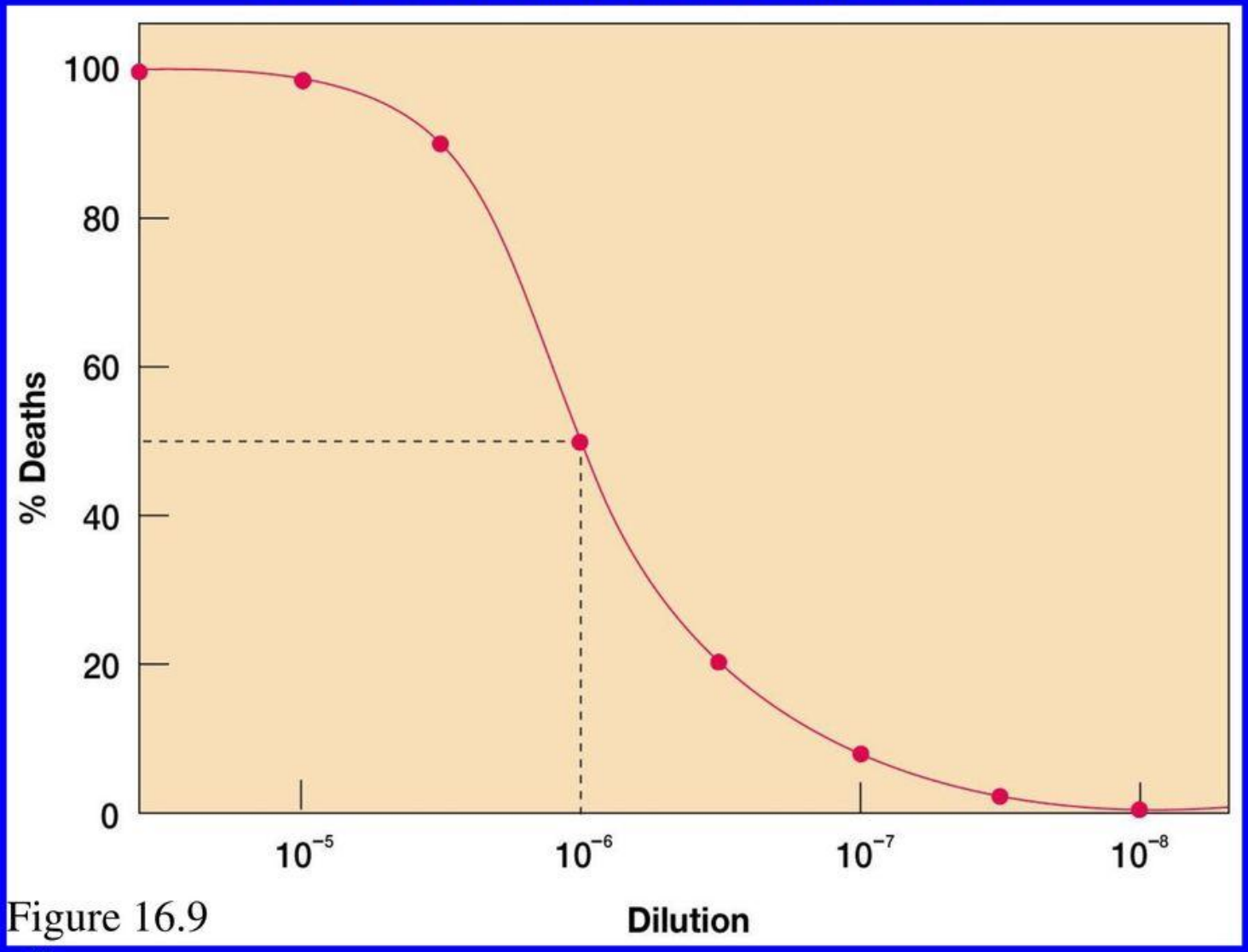


Figure 16.9