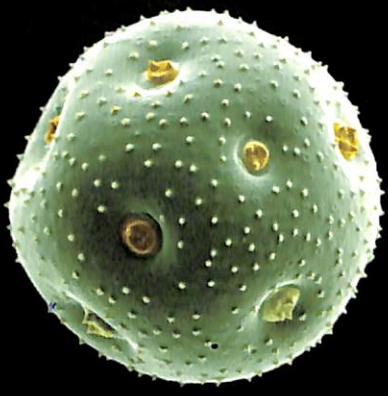
Agricultural Microbiology



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Preface

The science of Agricultural Microbiology envisages the utilization of modern biological and biochemical tools towards amelioration of the degrading eco-health and conservation of natural resources related to the field of agriculture. A student being exposed for the first time to this interesting subject needs to be given an idea about it. The present volume has tried to do this precisely. All kinds of micro-organisms from viruses to nematodes through bacteria, fungi, cyanobacteria have been touched to create an impression about the diversity. The scope and practices of using different micro-organisms have been shown which may attract future generations. The enormous prospect of application of microbiology in profitably using agricultural wastes and produces have been indicated. It also delineates strategies and protocols for utilization of microbes in solving problems of environment.

I have greatly aided by the advice and assistance given to me by my colleagues, friends and authorities on the subject and without their cooperation and contribution, it would not have been possible for me to complete this work.

As it is my firm conviction that any form of presentation of any matter can always be improved upon. I will gratefully accept all suggestions, comments or criticism, which can be effectively used to improve the book.

—P. K. Biswas

CONTENTS

1

VIRUSES

A *virus* is a genetic element containing either DNA or RNA that can alternate between two distinct states, intracellular and extracellular. In the extracellular state, a virus is a submicroscopic particle containing nucleic acid surrounded by protein and occasionally containing other components. In this extracellular state, the *virus particle*, also called the virion, is metabolically inert and does not carry out respiratory or biosynthetic functions. The virion is the structure by which the virus genome is carried from the cell in which the virion has been produced to another cell where the viral nucleic acid can be introduced and the intracellular state initiated. In the intracellular state, *virus reproduction* occurs: the virus genome is produced and the components which make up the virus coat are synthesized. When a virus genome is introduced into a cell and reproduces, the process is called infection. The cell that a virus can infect and in which it can replicate is called a host. The virus redirects preexisting host machinery and metabolic functions necessary for virus replication.

Viruses may thus be considered in two ways: as agents of *disease* and as agents of *heredity*. As agents of disease, viruses can enter cells and cause harmful changes in these cells, leading to disrupted function or death. As agents of heredity, viruses can enter cells and initiate permanent, genetic changes that are usually not harmful and may even be beneficial. In many cases, whether a virus causes disease or hereditary change depends upon the host cell and on the environmental conditions.

Viruses are smaller than cells, ranging in size from 0.02 μm to 0.3 μm. A common unit of measure for viruses is the nanometer (abbreviated nm), which is 1000 times smaller than a μm and one million times smaller than a millimeter.

Viruses are classified initially on the basis of the hosts they infect. Thus we have animal viruses, plant viruses, and bacterial viruses. Bacterial viruses, sometimes called *bacteriophages* (or *phage* for short, from the Greek *phago* meaning *to eat),* have been studied primarily as convenient model systems for research on the molecular biology and genetics of virus reproduction. Many of the basic concepts of

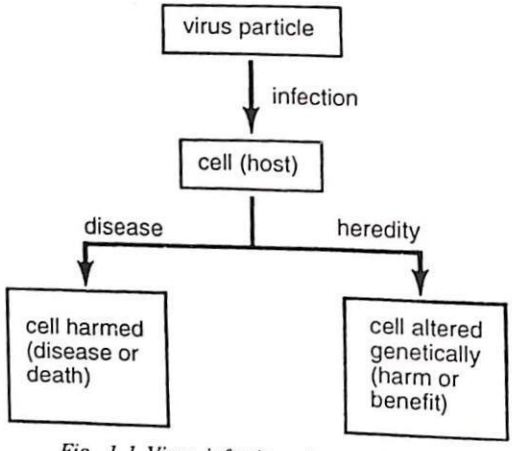

Fig. 1.1 Virus infection: the two-fold path.

virology were first worked out with bacterial viruses and subsequently applied to viruses of higher organisms. Because of their frequent medical importance, *animal viruses* have been extensively studied. The two groups of animal viruses most studied are those infecting insects and those infecting warm-blooded animals. *Plant viruses* are often important in agriculture but have been less studied than animal viruses. Although viruses are known which infect eucaryotic microorganisms, they have been little studied. In the present chapter, we discuss the structure, replication, and genetics of viruses infecting bacteria and warm-blooded animals. In a nutshell:

1. The virus genome consists of either RNA or DNA. The genome is surrounded by a *coat* of *protein* (and occasionally other material). When the virus genome is inside the coat it is called a *virus particle* or *virion.*

2. Viruses lack independent metabolism. They multiply only inside living cells, using the host cell metabolic machinery. Some virus

particles do contain enzymes, however, that are under the genetic control of the virus genome. Such enzymes are only produced during the infection cycle.

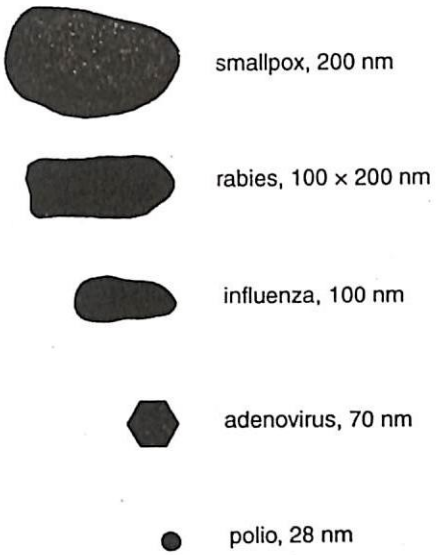

Fig. 1.2 Relative sizes of some common viruses infecting humans. DNA viruses are green, RNA viruses are red.

3. When a virus multiplies, the genome becomes released from the coat. This process occurs during the infection process. The present chapter is divided into three parts. The first part deals with basic concepts of virus structure and function. The second part deals with the nature and manner of multiplication of the bacterial viruses (bacteriophages). In this part we introduce the basic molecular biology of virus multiplication. The third part deals with important groups of animal viruses, with emphasis on molecular aspects of animal virus multiplication.

The Nature of the Virus Particle

Virus particles vary widely in size and shape. As we have stated, some viruses contain RNA, others DNA. We have discussed nucleic acids in previous chapters and have noted that the DNA of the cell genome is in the double-stranded form. Some viruses have double-stranded DNA whereas others have single-stranded DNA (Figure 6.3).

We have also noted in Section 5.8 that the RNA of the cell is generally in the single-stranded configuration. Interestingly, although single-stranded RNA viruses are more common, viruses are known in which the RNA is in the double-stranded form.

The structures of virions (virus particles) are quite diverse. Viruses vary widely in size, shape, and chemical composition. The nucleic acid of the virion is always located within the particle, surrounded by a protein coat called the *capsid*. The terms *coat, shell,* and *capsid* are often used interchangeably to refer to this outer layer. The protein coat is always formed of a number of individual protein molecules, called *protein subunits,* (sometimes called *capsomeres)* which are arranged in a precise and highly repetitive pattern around the nucleic acid. A few viruses have only a single kind of protein subunit, but most viruses have several chemically distinct kinds of protein subunits which are themselves associated in specific ways to form larger assemblies called *morphological units*. It is the morphological unit which is seen with the electron microscope. Genetic economydictates that the variety of virus proteins be kept small, since virus genomes do not have sufficient genetic information to code for a large number of different kinds of proteins.

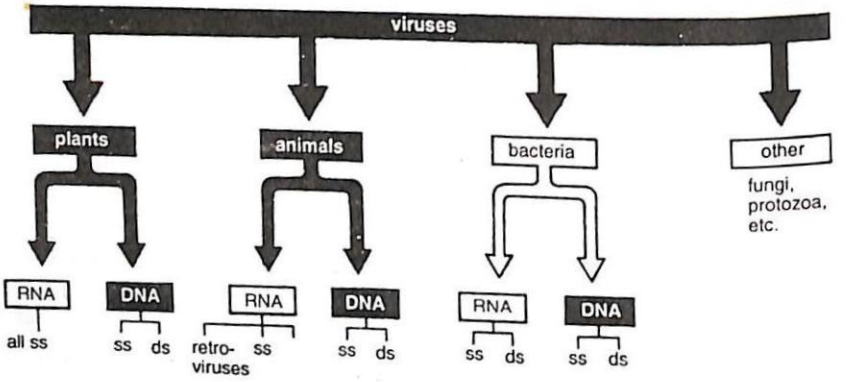

Fig. 1.3 Diversity of viruses. ss: single stranded; ds: double stranded.

The information for proper aggregation of the protein subunits into the morphological units is contained within the structure of the subunits themselves, and the overall process of assembly is thus called self-assembly. A single virion generally has a large number of morphological units.

The complete complex of nucleic acid and protein, packaged in the virus particle, is called the virus *nucleocapsid*. Although the virus structure just described is frequently the total structure of a virus particle, a number of animal viruses (and a few bacterial viruses) have more complex structures. These viruses are *enveloped* viruses, in which the nucleocapsid is enclosed in a membrane. *Virus membranes* are generally lipid bilayer membranes, but associated with these

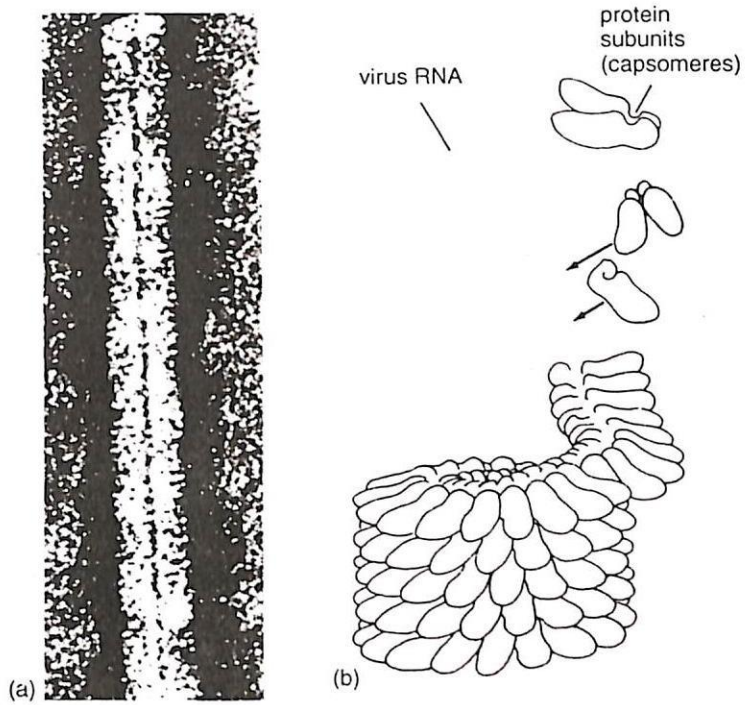

Fig. 1.4 Structure and manner of assembly of a simple virus, tobacco mosaic virus. (a) Electron micrograph at high resolution of a portion of the virus particle. (b) Assembly of the tobacco mosaic virion. The RNA assumes a helical configuration surrounded by the protein capsomeres. The center of the particle is hollow.

membranes are often virus-specific proteins. Inside the virion are often one or more *virus-specific enzymes*. Such enzymes usually play roles during the infection and replication process.

Virus symmetry The nucleocapsids of viruses are constructed in highly symmetrical ways. Symmetry refers to the way in which the protein morphological units are arranged in the virus shell. When a symmetrical structure is rotated around an axis, the same form is seen again after a certain number of degrees of rotation. Two kinds of symmetry are recognized in viruses which correspond to the two primary shapes, rod and spherical. Rod-shaped viruses have helical symmetry and spherical viruses have icosahedral symmetry.

A typical virus with **helical symmetry** is the tobacco mosaic virus (TMV). This is an RNA virus in which the 2130 identical protein subunits (each 158 amino acids in length) are arranged in a helix. In TMV, the helix has 16 1/2 subunits per turn and the overall dimensions of the virus particle are 18 × 300 nm. The lengths of helical viruses

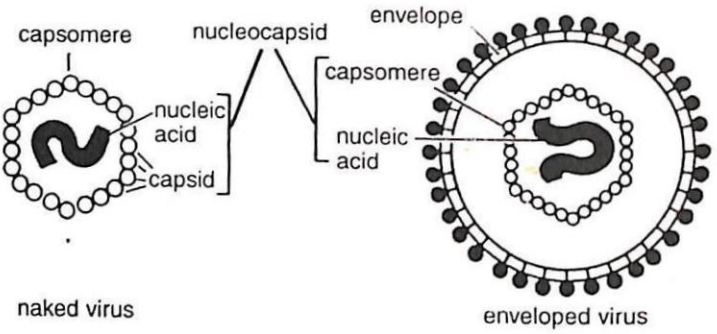

Fig. 1.5 Comparison of naked and enveloped virus, two basic types of virus particles.

are determined by the length of the nucleic acid, but the width of the helical virus particle is determined by the size and packing of the protein subunits.

An **icosahedron** is a symmetrical structure roughly spherical in shape which has 20 faces. Icosahedral symmetry is the most efficient arrangement for subunits in a closed shell because it uses the smallest number of units to build a shell. The simplest arrangement of morphological units is 3 per face, for a total of 60 units per virus particle. The three units at each face can be either identical or different. Most viruses have more nucleic acid than can be packed into a shell made of just 60 morphological units. The next possible structure which permits close packing contains 180 units and many viruses have shells with this configuration. Other known configurations involve 240 units and 420 units.

When discussing symmetry, one speaks *of axes of rotation.* A flat triangle shape, for instance, has one three-fold axis of symmetry, since there are three possible rotations that will lead to the exact configuration seen originally. Three dimensional objects such as viruses can have more than one axis of symmetry. An icosahedron, for instance, has three different axes of symmetry, two-fold, three-fold, and fivefold. When a rod is placed through the two-fold axis of symmetry (one of the edges) in the model, the model can be turned once around this axis (1/2 way or 180°) to obtain the same configuration again. When the rod is placed through one of the three-fold axes of symmetry (one of the faces), the model can be turned three times, and if the rod is placed through one of the five-fold axes of symmetry (one of the vertices) the model can be turned five times.

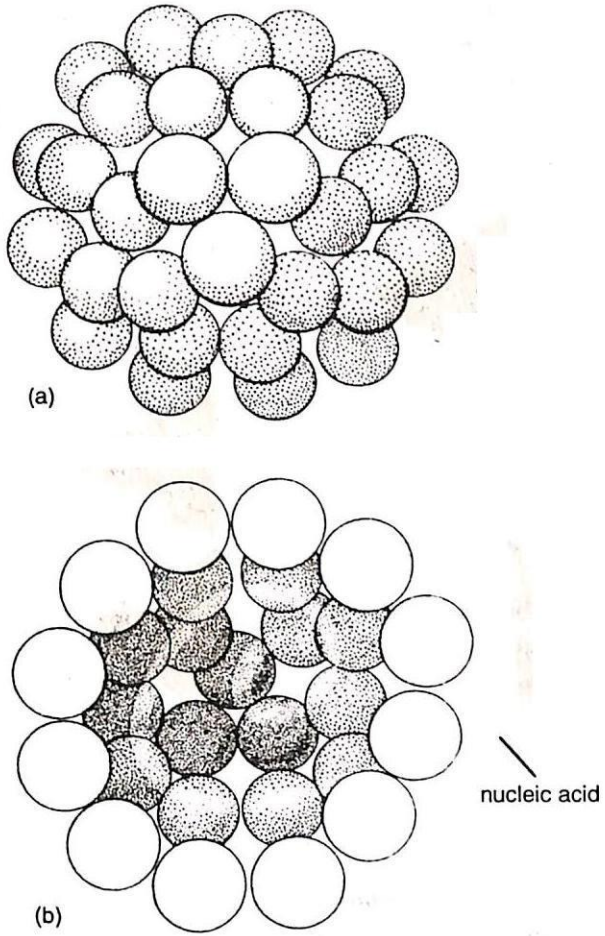

Fig. 1.6 A simple icosahedral virus. Each face has three subunits. A single subunit consists of one or more proteins. (a) Whole virus particle. (b) Virus particle opened up; nucleic acid released.

In all cases, the characteristic structure of the virus is determined by the structure of the protein subunits of which it is constructed. Self-assembly leads to the final virus particle.

Enveloped viruses Many viruses have complex membranous structures surrounding the nucleocapsid. Enveloped viruses are common in the animal world (for example, influenza virus), but some enveloped bacterial viruses are also known. The virus envelope consists of a lipid bilayer with proteins, usually glycoproteins, embedded in it. Although the glycoproteins of the virus membrane are encoded by the virus, the lipids are derived from the membranes of the host cell.

The symmetry of enveloped viruses is expressed not in terms of the virion as a whole but in terms of the nucleocapsid present inside the virus membrane.

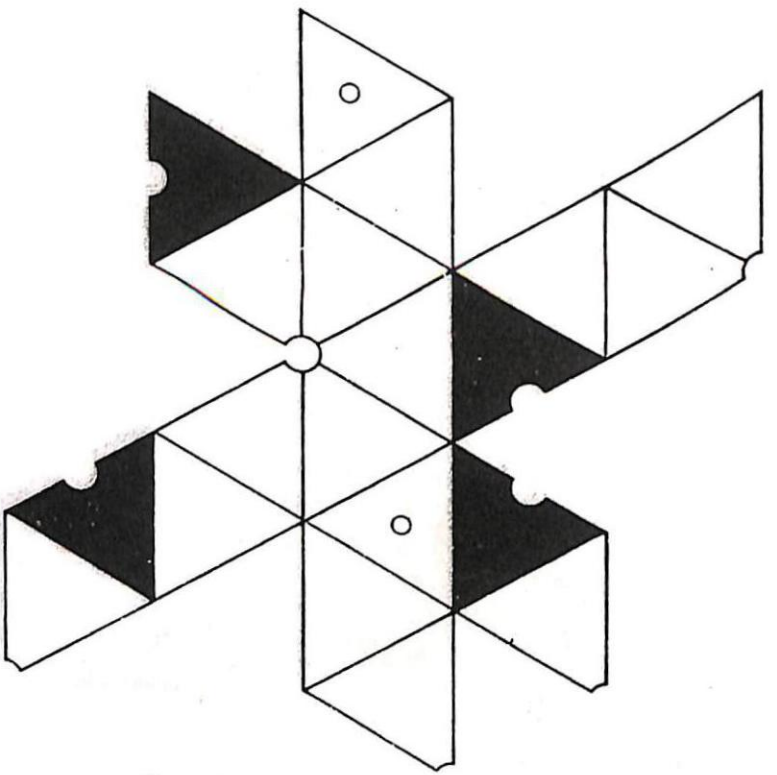

Fig. 1.7 Demonstration of icosahedral symmetry.

What is the function of the membrane in a virus particle? We will discuss this in detail later but note that because of its location in the virion, the membrane is the structural component of the virus particle that interacts first with the cell. The specificity of virus infection, and some aspects of virus penetration, are controlled in part by characteristics of virus membranes.

Complex viruses Some virions are even more complex, being composed of several separate parts, with separate shapes and symmetries. The most complicated viruses in terms of structure are some of the bacterial viruses, which possess not only icosahedral heads but helical tails. In some bacterial viruses, such as the T4 virus of *Escherichia coli*, the tail itself is a complex structure. For instance, T4 has almost 20 separate proteins in the tail, and the T4 head has

several more proteins. In such complex viruses, assembly is also complex. For instance, in T4 the complete tail is formed as a subassembly, and then the tail is added to the DNA-containing head. Finally, tail fibers formed from another protein are added to make the mature, infectious virus particle.

The virus genome We have stated that the virus genome consists of either DNA or RNA, never both. Viruses differ in size, amount, and character of their nucleic acid. Both single-stranded and doublestranded nucleic acid is found in viruses, and the amount of nucleic acid per virion may vary greatly from one virus type to another. In general, in enveloped viruses the nucleic acid constitutes only a small part of the mass of the virus particle (1-2 percent), whereas in nonenveloped viruses the percent of the particle which is nucleic acid is much larger, often 25-50 percent.

Interestingly, the nucleic acid in some viruses is not present in a single molecule, the genome being segmented into several or many molecules. For instance, *retroviruses-causal* agents of some cancers and AIDS, among other diseases-have two identical RNA molecules, influenza virus has 8 RNA molecules of sizes varying over about three-fold, and some other animal viruses have even more RNA molecules. The manner in which these various pieces of nucleic acid are replicated in the cell and then assembled into mature virions is of considerable interest-how do all these nucleic acid pieces end up together in one particle?

Enzymes in viruses We have stated that virus particles do not carry out metabolic processes. Outside of a host cell, a virus particle is metabolically inert. However, some viruses do contain enzymes which play roles in the infectious process. For instance, many viruses contain their own nucleic acid polymerases which transcribe the viral nucleic acid into messenger RNA once the infection process has begun. The retroviruses are RNA viruses which replicate inside the cell as DNA intermediates. These viruses possess an enzyme, an RNA-dependent DNA popo called *reverse transcriptase,* which transcribes the information in the incoming RNA into a DNA intermediate. It should be noted that reverse transcriptase is unique to the retroviruses and is not found in any other viruses or in cells.

A number of viruses contain enzymes which aid in release of the virus from the host cells in the final stages of the infectious process. One group of such enzymes, called *neuraminadases,* break down glycosidic bonds of glycoproteins and glycolipids of the

connective tissue of animal cells, thus aiding in the liberation of the virus. Virions infecting some bacteria possess an enzyme, *lysozyme*, which hydrolyzes the cell wall, causing lysis of the host cell and release of the virus particles. We will discuss some of these enzymes in more detail later.

The Classification of Viruses

As we have noted, viruses can be classified into broad groups depending on their hosts. For instance, there are plant viruses, animal viruses, and bacterial viruses. A number of viruses infecting insects are also known and although viruses are known for fungi, protozoa, and algae, these viruses have been so little studied that no classification has been developed. In the present chapter, we discuss only the animal (primarily mammalian) and bacterial viruses, and we discuss here briefly how these two groups of viruses are classified.

Classification of bacterial viruses In the bacterial viruses, a formal classification scheme is rarely used. Rather, each bacterial virus is designated in terms of its principal bacterial host, followed by an arbitrary alphanumeric. Thus, we speak of T4 virus of *Escherichia coli* or P22 virus of *Salmonella typhimurium*. An overview of some of the major types of bacterial viruses is given later. We should note, however, that although a bacterial virus may be designated in reference to its principal host, the actual host range of the virus may be broader. Thus, bacteriophage Mu, generally studied with *Escherichia coli*, also infects *Citrobacter* and *Salmonella*.

Classification of animal viruses We should note first that classification of animal viruses presents some major differences from the classification of organisms. The conventional approach to classification of organisms, involving hierarchical categories such as species, genera, families, etc., has been applied only to animal viruses. Even here, the higher levels of classification are not used. The highest level of animal virus classification is the virus family. Virus families are designated by terms ending in *-viridae*. Thus, the group of poxviruses is called the *Poxviridae* and the herpesviruses are called the *Herpesviridae*. Note that the major criteria used in classifying animal viruses are the type of nucleic acid, the presence or absence of an envelope, and, for certain families, the manner of replication.

Virus genera are designated by terms ending in - *virus*. Thus, among the *Poxviridae* those poxviruses which infect fowl are called by the genus name *Avipoxvirus*. Note that frequently in the animal viruses, the genus is defined based on the host which the virus infects.

Except in a few cases, virus species have not been formally designated, but would refer to specific virus entities that have been recognized. At present, virus species are only designated by common names, such as mumps virus, poliovirus 1, and smallpox virus. For instance, in the virus genus *Orthopoxvirus* two virus species currently recognized are vaccinia and smallpox, but are not given Latin names. At present, it does not appear useful to use Latin names for virus species.

When contemplating the problem of virus classification, we can be truly impressed with the enormous diversity of viruses. Undoubtedly, many new viruses are awaiting discovery, although most undiscovered viruses will probably be considered members of existing virus families.

The Virus Host

Because viruses only replicate inside living cells, research on viruses requires use of appropriate hosts. For the study of bacterial viruses, pure cultures are used either in liquid or on semi-solid (agar) medium. Because bacteria are so easy to culture, it is quite easy to study bacterial viruses and this is why such detailed knowledge of bacterial virus reproduction is available.

With animal viruses, the initial host may be a whole animal which is susceptible to the virus, but for research purposes it is desirable to have a more convenient host. Many animal viruses can be cultivated in *tissue* or *cell cultures*, and the use of such cultures has enormously facilitated research on animal viruses.

Cell cultures A cell culture is obtained by enabling growth of cells taken from an organ of the experimental animal. Cell cultures are generally obtained by aseptically removing pieces of the tissue in question, dissociating the cells by treatment with an enzyme which breaks apart the intercellular cement, and spreading the resulting suspension out on the bottom of a flat surface, such as a bottle or petri dish. The cells generally produce glycoprotein-like materials that permit them to adhere to glass surfaces. The thin layer of cells adhering to the glass or plastic dish, called a *monolayer, is* then overlayed with a suitable culture medium and the culture incubated. The culture media used for cell cultures are generally quite complex, employing a number of amino acids and vitamins, salts, glucose, and a bicarbonate buffer system. To obtain best growth, addition of a small amount of blood serum is usually necessary, and several antibiotics are generally added to prevent bacterial contamination.

Some cell cultures prepared in this way will grow indefinitely, and can be established as *permanent cell lines*. Such cell cultures are most convenient for virus research because continuously available cell material can be available for research purposes. In other cases, indefinite growth does not occur but the culture may remain alive for a number of days. Such cultures, called *primary cell cultures*, may still be useful for virus research, although of course new cultures will have to be prepared from fresh sources from time to time.

Cancer Cancer is a cellular phenomenon of uncontrolled growth that is sometimes induced by virus infection. Most cells in a mature animal, although alive, do not divide extensively, apparently because of the presence of growth-inhibiting factors which prevent them from initiating cell division. Under a variety of pathological conditions, among which is included infection by certain viruses, growth inhibition is overcome and the cells begin to divide uncontrollably. Under some conditions, this extensive cellular growth is so excessive that the animal body is virtually consumed by cancer cells: the animal dies. Cancerous growth is thus due to a derangement in the control of cellular growth, and is of great medical as well as theoretical interest.

The tumorigenic or cancer-causing ability of viruses can often be detected by observing the induction in cell cultures of uncontrolled growth. In cell cultures, the general arrangement of the cells is as a *monolayer*, arising because growth generally ceases when the cells, as a result of growth, come in contact with each other. Cancer cells have altered growth requirements and continue to grow, piling up to form a small *focus of growth*. By observing for the induction of such foci of growth from virus infection, it is possible to observe the tumorigenic properties of viruses.

In some cases, cell culture monolayers can not be obtained but whole organs, or pieces of organs, can be cultured. Such *organ cultures* may still be useful in virus research, since they permit growth of viruses under more or less controlled laboratory conditions.

Quantification of Viruses

In order to obtain any significant understanding of the nature of viruses and virus replication, it is necessary to be able to *quantify* the number of virus particles. Virus particles are almost always too small to be seen under the light microscope. Although virus particles can be observed under the electron microscope, the use of this instrument is cumbersome for routine study. In general, viruses are quantified by measuring their effects on the host cells which they

infect. It is common to speak of a *virus infectious unit,* which is the smallest unit that causes a detectable effect when placed with a susceptible host. By determining the number of infectious units per volume of fluid, a measure of virus quantity can be obtained. We discuss here several approaches to assessment of the virus infectious unit.

Plaque assay When a virus particle initiates an infection upon a layer or lawn of host cells which is growing spread out on a flat surface, a zone of *lysis* or *growth inhibition* may occur which results in a clearing of the cell growth. This clearing is called a *plaque*, and it is assumed that each plaque has originated from one virus particle.

Plaques are essentially "windows" in the lawn of confluent cell growth. With bacterial viruses, plaques may be obtained when virus particles are mixed into a thin layer of host bacteria which is spread out as an agar overlay on the surface of an agar medium. During incubation of the culture, the bacteria grow and form a turbid layer which is visible

to the naked eye. However, wherever a successful virus infection has been initiated, lysis of the cells occurs, resulting in the formation of a clear zone, called *a plaque.*

The plaque procedure also permits the isolation of pure virus strains, since if a plaque has arisen from one virus particle, all the virus particles in this plaque are probably genetically identical. Some of the particles from this plaque can be picked and inoculated into a fresh bacterial culture to establish a pure virus line. The development of the plaque assay technique was as important for the advance of virology as was Koch's development of solid media for bacteriology.

Plaques may be obtained for animal viruses by using animal cell-culture systems as hosts. A monolayer of cultured animals cells is prepared on a plate or flat bottle and the virus suspension overlayed. Plaques are revealed by zones of destruction of the animal cells.

In some cases, the virus may not actually destroy the cells, but cause changes in morphology or growth rate which can be recognized. For instance, tumor viruses may not destroy cells but cause the cells to grow faster than uninfected cells, a phenomenon called *transformation.* As we have noted, in a tissue culture monolayer, these transformed cells gradually develop into a recognizable cluster of cells called a *focus of infection.* By counting foci of infection, a quantitative measure of virus may be obtained.

Efficiency of plating One important concept in quantitative

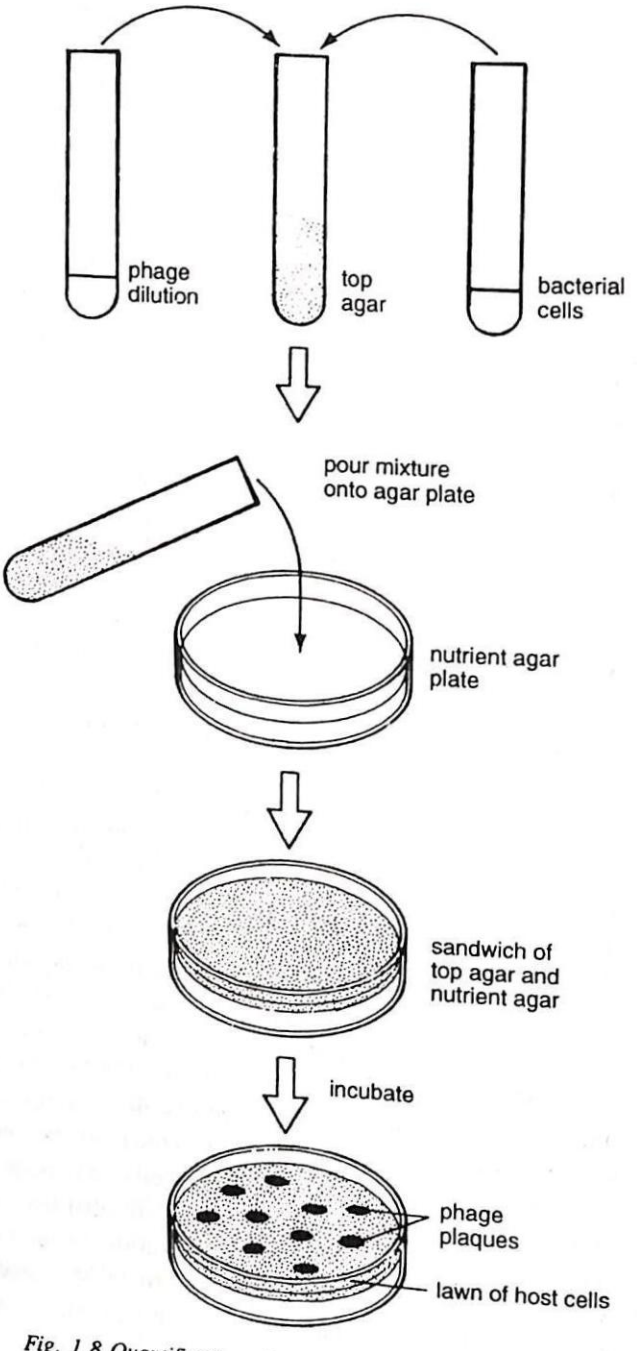

Fig. 1.8 Quantification of bacterial virus by plaque assay using the agar overlay technique.

virology involves the idea of *efficiency of plating*. Counts made by plaque assay are always lower than counts made with the electron microscope. The efficiency with which virus particles infect host cells is almost never 100 percent and may often be considerably less. This does not mean that the virus particles which have not caused infection are inactive. It may merely mean that under the conditions used, successful infection with these particles has not occurred. Although with bacterial viruses, efficiency of plating is often higher than 50 percent, with many animal viruses it may be very low, 0.1 or 1 percent. Why virus particles vary in infectivity is not well understood. It is possible that the conditions used for quantification are not optimal. Because it is technically difficult to count virus particles with the electron microscope, it is difficult to assess the actual efficiency of plating, but the concept is important in both research and medical practice. Because the efficiency of plating is rarely close to 100 percent, when the plaque method is used to quantify virus, it is accurate to express the titer of the virus suspension not as the number of virion units, but as the number of *plaque forming units*.

Animal infectivity methods Some viruses do not cause recognizable effects in cell cultures but cause death in the whole animal. In such cases, quantification can only be done by some sort of titration in infected animals. The general procedure is to carry out a serial dilution of the unknown sample, generally at ten-fold dilutions, and samples of each dilution are injected into numbers of sensitive animals. After a suitable incubation period, the fraction of dead and live animals at each dilution is tabulated and an *end point dilution is* calculated. This is the dilution at which, for example, *half* of the injected animals die. Although such serial dilution methods are much more cumbersome and much less accurate than cell culture methods, they may be essential for the study of certain types of viruses.

General Features of Virus Reproduction

The basic problem of virus replication can be simply put; the virus must somehow induce a living host cell to synthesize all of the essential components needed to make more virus particles. These components must then be assembled into the proper structure and the new virus particles must escape from the cell and infect other cells. The various phases of this replication process in a bacteriophage can be categorized in seven steps:

1. **Attachment** (adsorption) of the virion to a susceptible host cell;

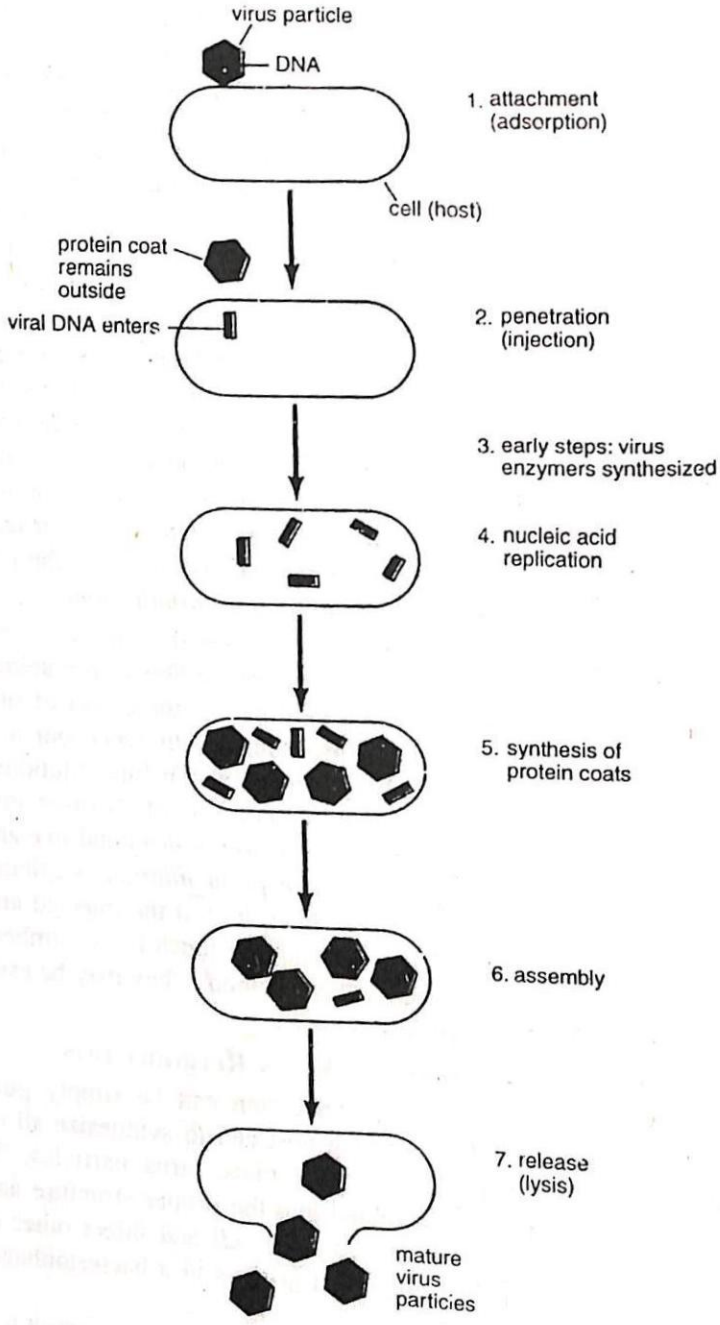

Fig. 1.9 The replication cycle of a bacterial virus. The general stages of virus replication are indicated.

2. **Penetration** (injection) into the cell by the virion or by its nucleic acid;
3. **Early steps in replication** of the virus nucleic acid, in which the host cell biosynthetic machinery is altered as a prelude to virus nucleic acid synthesis. Virus-specific enzymes may be made;
4. **Replication** of the virus nucleic acid;
5. **Synthesis of protein subunits** of the virus coat;
6. **Assembly** of nucleic acid and protein subunits (and membrane components in enveloped viruses) into new virus particles;
7. **Release** of mature virus particles from the cell (lysis).

These stages in virus replication are recognized when virus particles infect cells in culture and are illustrated in Figure 6.13, which exhibits what is called a **one-step growth curve.** In the first few minutes after infection, a period called the *eclipse* occurs, in which the virus nucleic acid has become separated from its protein coat so that the virus particle no longer exists as an infectious entity. Although virus nucleic acid may be infectious, the infectivity of virus nucleic acid is many times lower than that of whole virus particles because the machinery for bringing the virus genome into the cell is lacking. Also, outside the virion the nucleic acid is no longer protected from deleterious activities of the environment as it was when it was inside the protein coat.

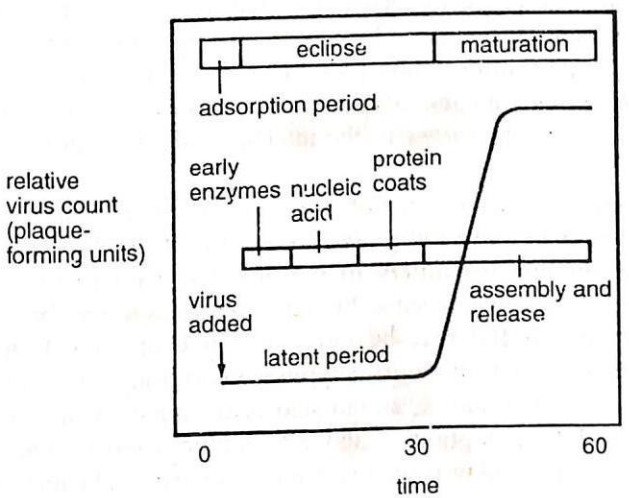

Fig. 1.10 The one-step growth curve of virus replication. This graph displays the results of a single round of viral multiplication in a population of cells.

The eclipse is the period during which the stages of virus multiplication occur. This is called the *latent period,* because no infectious virus particles are evident. Finally, maturation begins as the newly synthesized nucleic acid molecules become assembled inside protein coats. During the *maturation* phase, the titer of active virus particles inside the cell rises dramatically. At the end of maturation, *release* of mature virus particles occurs, either as a result of cell lysis or because of some budding or excretion process. The number of virus particles released, called the *burst size, will* vary with the particular virus and the particular host cell, and can range from a few to a few thousand. The timing of this overall virus replication cycle varies from 20-30 minutes in many bacterial viruses to 8-40 hours in most animal viruses. We now consider each of the steps of the virus multiplication cycle in more detail.

Early Events of Virus Multiplication

In order to discuss the stages of virus multiplication, we must return briefly to a consideration of the virus genome. As we have noted, virus genomes consist of either DNA or RNA, and both single-stranded and double-stranded forms of each of these nucleic acids is known to occur in different viruses. In the case of DNA viruses, the nucleic acid may be in either a linear or a circular form. The nucleic acid of RNA viruses is always in a linear form. Some virus nucleic acids also contain covalently linked polypeptides or amino acids which play roles in replication. In addition, in some RNA viruses the genome is not present in a single molecule, but may be divided over two or many nucleic acid molecules. Even more complicated, once inside the cell, the genetic information present in the virus genome may be transfered to another nucleic acid molecule. To avoid confusion, we restrict the term *virus genome* to the nucleic acid found in the virion (virus particle).

As we have noted, the outcome of a virus infection is the synthesis of viral nucleic acid and viral protein coats. In effect, the virus takes over the biosynthetic machinery of the host and uses it for its own synthesis. A few enzymes needed for virus replication may be present in the virus particle and may be introduced into the cell during the infection process, but the host supplies everything else: energy-generating system, ribosomes, amino-acid activating enzymes, transfer RNA (with a few exceptions), and all soluble factors. The virus genome codes for all new proteins. Such proteins would include the coat protein subunits (of which there are generally more than one kind) plus any new virus-specific enzymes.

Attachment There is a high specificity in the interaction between virus and host. The most common basis for host specificity involves the attachment process. The virus particle itself has one or more proteins on the outside which interact with specific cell surface components called *receptors*. The receptors on the cell surface are normal surface components of the host, such as proteins, polysaccharides, or lipoprotein-polysaccharide complexes, to which the virus particle attaches. In the absence of the receptor site, the virus cannot adsorb, and hence cannot infect. If the receptor site is altered, the host may become resistant to virus infection. However, mutants of the virus can also arise which are able to adsorb to resistant hosts.

In general, virus receptors carry out normal functions in the cell. For example, in bacteria some phage receptors are pili or flagella, others are cell-envelope components, and others are transport binding proteins. The receptor for influenza virus is a glycoprotein found on red blood cells and on cells of the mucous membrane of susceptible animals, whereas the receptor site of poliovirus is a lipoprotein. However, many animal and plant viruses do not have specific attachment sites at all and the virus enters passively as a result of phagocytosis or some other endocytotic process.

Penetration The means by which the virus penetrates into the cell depends on the nature of the host cell, especially on its surface structures. Cells with cell walls, such as bacteria, are infected in a different manner from animal cells, which lack a cell wall. The most complicated penetration mechanisms have been found in viruses that infect bacteria. The bacteriophage T4, which infects *E. coli,* can be used as an example.

The particle has a head, within which the viral DNA is folded, and a long, fairly complex tail, at the end of which is a series of tail fibers. During the attachment process, the virus particles first attach to the cells by means of the tail fibers. These tail fibers then contract, and the core of the tail makes contact with the cell envelope of the bacterium. The action of a lysozyme-like enzyme results in the formation of a small hole. The tail sheath contracts and the DNA of the virus passes into the cell through a hole in the tip of the tail, the majority of the coat protein remaining outside. The DNA of T4 has a total length of about 50 μm, whereas the dimensions of the head of the T4 particle are 0.095 Am by 0.065 μm. This means that the DNA must be highly folded and packed very tightly within the head.

With animal cells, the whole virus particle penetrates the cell,

being carried inside by endocytosis (phagocytosis or pinocytosis), an active cellular process. We describe some of these processes in detail later in this chapter.

Virus restriction and modification by the host We have already seen that one form of host resistance to virus arises when there is no receptor site on the cell surface to which the virus can attach. Another and more specific kind of host resistance involves destruction of the viral nucleic acid after it has been injected. This destruction is brought about by host enzymes that cleave the viral DNA at one or several places, thus preventing its replication. This phenomenon is called *restriction*, and is part of a general host mechanism to prevent the invasion of foreign nucleic acid.

Restriction enzymes are highly specific, attacking only certain sequences (generally four or six base pairs). The host protects its own DNA from the action of restriction enzymes by *modifying* its

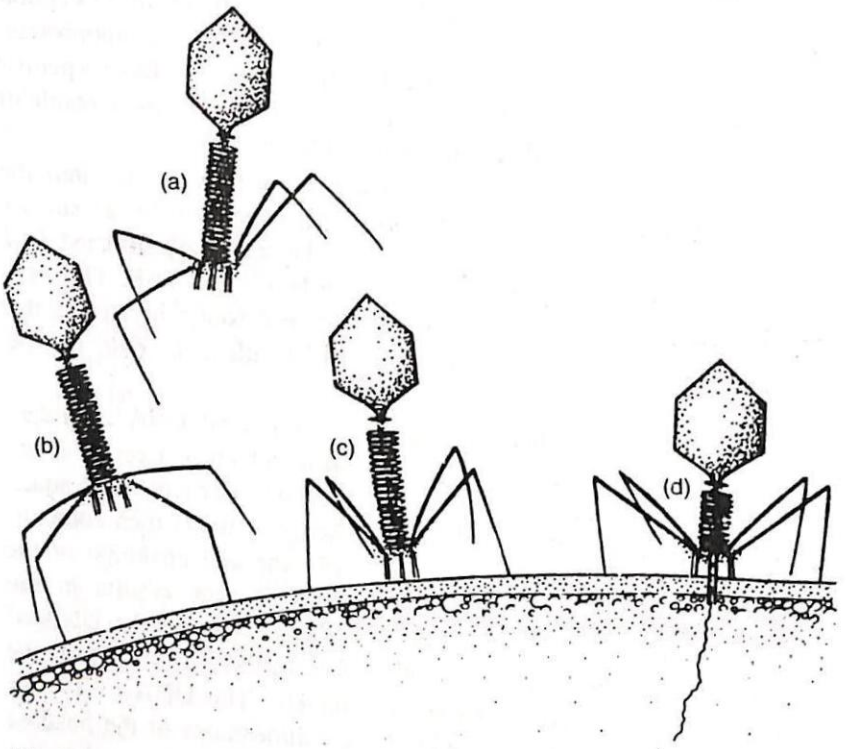

Fig. 1.11 Attachment of T4 bacteriophage particle to the cell wall of E. coli *and injection of DNA: (a) Unattached particle. (b) Attachment to the wall by the long tail fibers. (c) Contact of cell wall by the tail pin. (d) Contraction of the tail sheath and injection of the DNA.*

DNA at the sites where the restriction enzymes will act. Modification of host DNA is brought about by methylation of purine or pyrimidine bases.

Viruses can overcome host restriction mechanisms by modifications of their nucleic acids so that they are no longer subject to enzymatic attack. Two kinds of chemical modifications of viral DNA have been recognized, glucosylation and methylation. The bacteriophages T2, T4, and T6 have their DNA glucosylated to varying degrees, and the glucosylation prevents or greatly reduces nuclease attack. In bacteriophage *lambda* the amino groups of adenine and cytosine bases are methylated by an enzyme that uses S-adenosylmethionine as methyl donor. Many other viral nucleic acids have been found to be modified by methylation but glucosylation has been found only in the T-even bacteriophages (bacteriophages T2, T4, and T6). It should be emphasized that modification of viral nucleic acid occurs after replication has occurred and the modified bases are not copied directly. The enzymes for methylation are actually present in the host before infection, and hence are not virusinduced functions. These host modification enzymes probably have as their main role the modification of host DNA so that it can be transferred without inactivation into other cells during genetic recombination.

The ability to modify nucleic acid is not found in all strains that support the growth of a given virus. Thus, when bacteriophage *lambda is* grown on *E. coii* strain C it is not modified *(E. coli* strain C lacks both the *lambda* modification and restriction enzymes), and nucleic acid of virus grown on strain C is destroyed when it enters *E. coli* strain K- 12, which does have the restriction enzyme. However, strain K12 also has the modification enzyme, and, *if lambda* is grown on K- 12, its nucleic acid is modified and it will infect both strains K-12 and C equally well. However, if *lambda is* grown on a K-12 strain made methionine deficient, methylation cannot occur and the phage particles released are unable to replicate in K12. In the case of the T-even phages, glucosylation requires uridine diphosphoglucose (UDPG), and if a T-even phage is grown on a host deficient in UDPG its nucleic acid is not glucosylated and it is unable to replicate in susceptible cells.

A knowledge of modification and restriction systems is of considerable practical utility in studying DNA chemistry. So far, no evidence exists that either modification or restriction occurs in eucaryotic organisms.

Virus messenger RNA In order for the new virus-specific proteins to be made from the virus genome, it is necessary for new virus-specific RNA molecules to be made. Exactly how the virus brings about new mRNA synthesis depends upon the type of virus, and especially upon whether its genetic material is RNA or DNA, and whether it is single-stranded or double-stranded. Which copy is read into mRNA depends upon the location of the appropriate promoter, since the promoter points the direction that the RNA polymerase will follow. In cells (uninfected with virus) all mRNA is made on the DNA template, but with RNA viruses the situation is obviously different.

A virus-specific RNA RNA polymerase is needed, since the cell RNA polymerase will generally not copy double-stranded RNA (and ribosomes are not able to translate double-stranded RNA either). A wide variety of modes of viral mRNA synthesis are outlined in Figure By convention, the chemical sense of the mRNA is considered to be of the *plus (+)* configuration. The sense of the viral genome nucleic acid is then indicated by *a plus* if it is the same as the mRNA and a ***minus*** if it is of oppposite sense. If the virus has double-stranded DNA (ds DNA), then mRNA synthesis can proceed directly as in uninfected cells. However, if the virus has a singlestranded DNA (ss DNA), then it is first converted to ds DNA and the latter serves as the template for mRNA synthesis with the cell RNA polymerase.

If the virus has double-stranded RNA (ds RNA), this RNA serves as a template in a manner analogous to DNA. There are three classes of viruses with ss RNA and they differ in the mechanism by which mRNA is synthesized. In the simplest case, the incoming viral RNA is the *plus* sense and hence serves directly as mRNA, and copies of this viral RNA are also copied to make further mRNA molecules. In another class, the viral RNA has a *minus (-)* sense. In such viruses a copy is made (*plus* sense) and this copy becomes the mRNA. In the case of the retroviruses (causal agents of certain kinds of cancers and AIDS), a new phenomenon called **reverse transcription** is seen, in which virion ss RNA is copied to a double-stranded DNA (through a ss DNA intermediate) and the ds DNA then serves as the template for mRNA synthesis (thus: ss RNA ss DNA ds DNA). Retrovirus replication is of unusual interest and complexity.

Viral proteins Once mRNA is made, viral proteins (for example, enzymes, capsomers) can be synthesized. The proteins synthesized as a result of virus infection can be grouped into two broad categories,

the enzymes synthesized soon after infection, called the early enzymes, which are necessary for the replication of virus nucleic acid, and the proteins synthesized later, called the late proteins, which include the proteins of the virus coat. Generally, both the time of appearance and the amount of these two groups of virus proteins are regulated. The early proteins are enzymes which, because they act catalytically, are synthesized in smaller amounts and the late proteins, often structural, are made in much larger amounts.

Virus infection obviously upsets the regulatory mechanisms of the host, since there is a marked overproduction of nucleic acid and protein in the infected cell. In some cases, virus infection causes a complete shutdown of host macromolecular synthesis while in other cases host synthesis proceeds concurrently with virus synthesis. In either case, the regulation of virus synthesis is under the control of the virus rather than the host. There are several elements of this control which are similar to the host regulatory mechanisms, but there are also some uniquely viral regulatory mechanisms. We discuss various regulatory mechanisms when we consider the individual viruses later in this chapter.

Viral Genetics

Viruses exhibit genetic phenomena similar to those of cells. Studies of viral genetics have played a significant role in understanding many aspects of genetics at the molecular level. In addition, knowledge of the basic phenomena of viral genetics has increased our understanding of processes involved in virus replication. Understanding these processes has also led to some practical developments, especially in the isolation of viruses which are of use in immunization procedures. Most of the detailed work on viral genetics has been carried out with bacteriophages, because of the convenience of working with these viruses. We mention here briefly some of the types of genetic phenomena of viruses.

Mutations Much of our knowledge of viral reproduction and how it is regulated has depended on the isolation and characterization of virus mutants. Several kinds of mutants have been studied in viruses: host-range mutants, plaque-type mutants, temperature-sensitive mutants, nonsense mutants, transposons, and inversions.

Host-range mutations are those that change the range of hosts that the virus can infect. Host resistance to phage infection can be due to an alteration in receptor sites on the surface of the host cell, so that the virus can no longer attach, and host-range mutations of

the virus can then be recognized as virus strains able to attach to and infect these virus-resistant hosts. Other host-range mutants may involve changes in the viral and host enzymes involved in replication, or in the restriction and modification systems.

Plaque-morphology mutations are recognized as changes in the characteristics of the plaques formed when a phage infects cells in the conventional agarplate technique. Characteristics of the plaque, such as whether it is clear or turbid, and its size, are under genetic control. The underlying basis of plaque morphology lies in processes taking place during the virus multiplication cycle, such as the rate of replication and the rate of lysis. Under appropriate experimental conditions plaque morphology can be a highly reproducible characteristic of the virus. The advantage of plaque mutants for genetic studies is that they can be easily recognized on the agar plate, but a disadvantage is that there is no convenient way of selecting for them among the large background of normal particles.

Temperature-sensitive mutations are those which allow a virus to replicate at one temperature and not at another, due to a mutational alteration in a virus protein that renders the protein unstable at moderately high temperatures. For instance, temperature-sensitive mutants are known in which the phage will not be replicated in the host at 43°C but will at 25°C, although the host functions at both temperatures. Such mutations are called *conditionally lethal*, since the virus is unable to reproduce at the higher temperature, but replicates at the lower temperature.

Nonsense mutations change normal codons into nonsense codons In viruses, nonsense mutations are recognized because hosts are available that contain suppressors able to read nonsense codons. The virus mutant will be able to grow in the host containing the suppressor, but not in the normal host.

Transpositions several viruses are known which act essentially as transposons and transposition events involving viruses can lead to their genetic change.

Genetic recombination in viruses The availability of virus mutants makes possible the investigation of genetic recombination. If two virus particles infect the same cell, there is a possibility for genetic exchange between the two virus genomes during the replication process. If recombination does occur, the progeny of such a mixed infection should include not only the parental types, but recombinant types as well. With appropriate mutants, it is possible to recognize

both the parental types and the recombinants and to study the events involved in the recombination process. Genetic recombination in viruses is an extremely complex process to analyze because recombination does not occur as a single discrete event during mixed infection, but may occur over and over again during the replication cycle. It has been calculated that the T-even bacteriophages undergo, on the average, four or five rounds of recombination during a single infection cycle. By detailed and careful analysis of a wide variety of virus crosses, it has been possible to construct genetic maps of a number of bacterial viruses. Such maps have provided important information about the genetic structure of viruses. We present a few genetic maps when discussing specific viruses later in this chapter.

Genetic recombination arises by exchange of homologous segments of DNA between viral genomes, most often during the replication process. The enzymes involved in recombination are DNA polymerases, endonucleases, and ligases, which also play a role in DNA repair and synthesis processes.

Phenotypic mixing During studies on genetic recombination between viruses, another phenomenon was discovered which superficially resembles recombination but has a quite different basis. Phenotypic mixing occurs when the DNA of one virus is incorporated inside the protein coat of a different virus. For phenotypic mixing to occur, the two viruses must be closely related, so that the protein coat is of proper construction of the packaging of either viral DNA. As an example of phenotypic mixing, in phage *T2* of *E. coli* there is a gene called the *h* gene which controls host specificity through modification of the tail fibers of the phage. If a mixed infection is set up with two *T2* phages, mutant *T2h* and wild-type $T2h^+$, tail fibers of *b* specificity may be incorporated onto the particles containing DNA of h^+ specificity. Since it is the *h* function of the tail fibers that affects attachment, these mixed particles will show *b* specificity during the next round of infection, even though they contain h^+ DNA, but the particles resulting from this second round of infection will phenotypically become b^+, because the DNA has been unchanged.

General Overview of Bacterial Viruses

Most of the bacterial viruses which have been studied in any detail infect bacteria of the enteric group, such as *Escherichia coli* and *Salmonella typhimurium*. However, viruses are known that infect a variety of procaryotes, both eubacteria and archaebacteria. A few bacterial viruses have lipid envelopes but most do not. However, many

bacterial viruses are structurally complex, with head and complex tail structures. The tail is involved in the injection of the nucleic acid into the cell.

We now discuss some of the bacterial viruses for which molecular details of the multiplication process are known. Although these bacterial viruses were first studied as *model systems* for understanding general features of virus multiplication many of them now serve as convenient tools for *genetic engineering*. Thus, the information on bacterial viruses is not only valuable as background for the discussion of animal viruses, but is essential for the material presented in the next two chapters on microbial genetics and genetic engineering.

It should already be clear from what has been stated that a great diversity of viruses exist. It should therefore not be surprising that there is also a great diversity in the manner by which virus multiplication occurs. Interestingly, many viruses have special features of their nucleic acid and protein synthesis processes that are not found in cells. In the present chapter, we are only able to present some of the major types of virus replication patterns, and must skip some of the interesting exceptional cases.

RNA Bacteriophages

A number of bacterial viruses have RNA genomes. The best-known bacterial RNA viruses have single-stranded RNA. Interestingly, the bacterial RNA viruses known in the enteric bacteria group infect only bacterial cells which behave as gene donors (males) in genetic recombination. This restriction to male bacterial cells arises because these viruses infect bacteria by attaching to *male-specific pili*. Since such pili are absent on female cells, these RNA viruses are unable to attach to the females, and hence do not initiate infection in females.

The bacterial RNA viruses are all of quite small size, about 26 nm in size, and they are all icosahedral, with 180 copies of coat protein per virus particle. The complete nucleotide sequence of several RNA phages are known. In the RNA phage *MS2*, which infects *Escherichia coli*, the viral RNA is 3,569 nucleotides long. The virus RNA, although single stranded, has extensive regions of secondary and tertiary structure. The RNA strand in the virion has the plus (+) sense, acting directly as mRNA upon entry into the cell.

The genetic map is shown and the flow of events of *MS2* multiplication. The infecting RNA goes to the host ribosome, where it is translated into four (or more) proteins. The four proteins that have been recognized are *maturation protein* (A-protein; present in

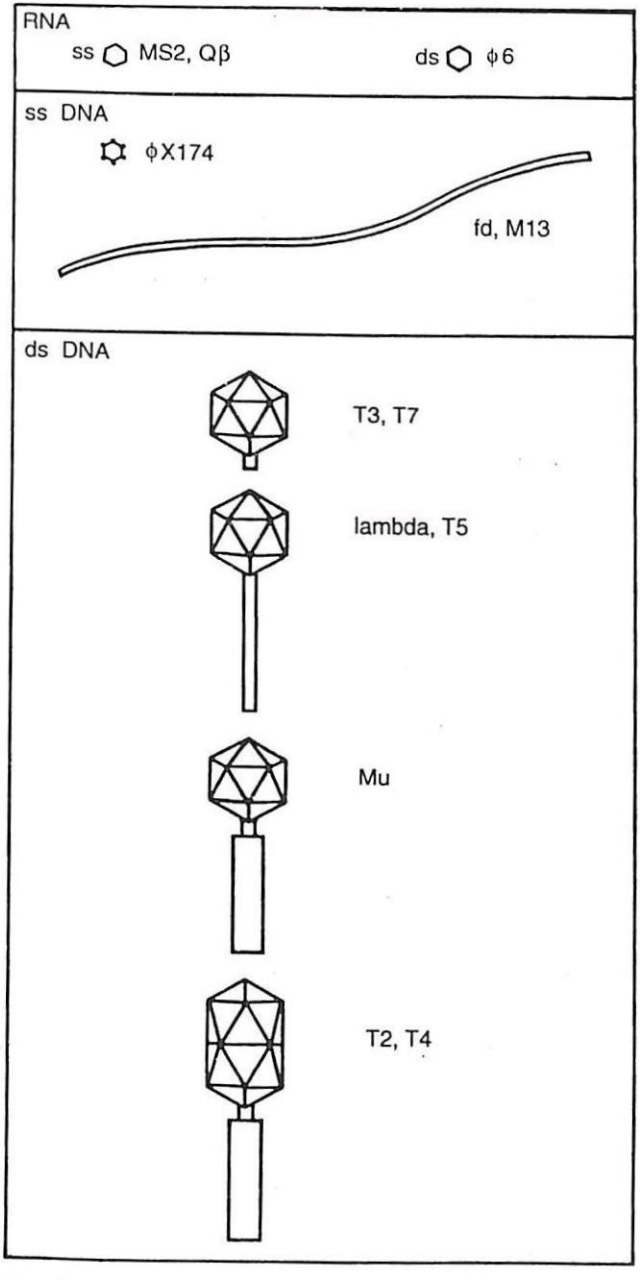

Fig. 1.12 Schematic representations of the main types of bacterial viruses. Those discussed in detail are fd, M13, ϕX174, MS2, T4, lambda, T 7, and Mu. Sizes are to approximate scale.

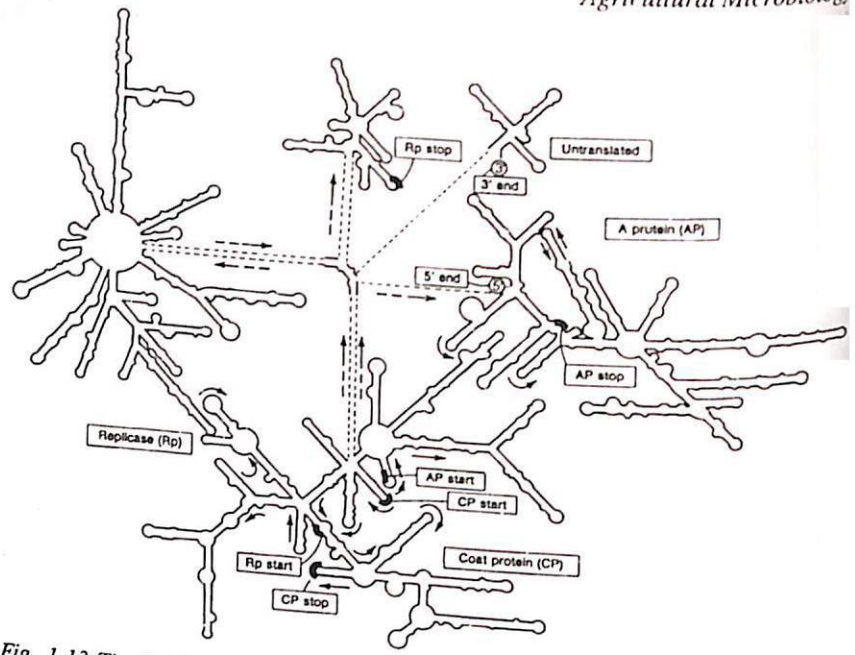

Fig. 1.13 The RNA of bacteriophage MS2. The molecule is single stranded but there are extensive regions of complementary bases, so that pairing within the strand leads to the secondary structure shown. Note that the start sites for three coding regions are in the same part of the folded molecule.

the mature virus particle as a single copy), *coat protein*, *lysis protein* (involved in the lysis process which results in release of mature virus particles), and RNA replicase, the enzyme which brings about the replication of the viral RNA. Interestingly, the RNA replicase is a composite protein, composed partly of a virus-encoded polypeptide and partly of host polypeptides. The host proteins involved in the formation of active viral replicase are *ribosomal protein SI* (one of the subunits of the 30S ribosome), and elongation factors Tu and Ts, involved in the translation process. Thus, the virus appears to co-opt host proteins that normally have entirely distinct functions and make them become part of active viral replicase.

As noted, the viral RNA is of the *plus (+)* sense. Replicase synthesizes RNA of *minus (-)* sense using the infecting RNA as template. After *minus* RNA has been synthesized, *plus* RNA is made from this *minus* RNA. The newly made *plus* RNA strands now serve as messengers for virus protein synthesis. The gene for the maturation protein is at the 5' end of the RNA. Translation of the gene coding for the maturation protein (needed in only one copy per virus particle), occurs only from the newly formed plus-strand RNA as the replication

process occurs. In this way, the amount of maturation protein needed is limited. As the virus RNA is made, it folds into a complex form with extensive secondary and tertiary structure. Of the four AUG start sites, the most accessible to the translation process is that for the coat protein. As coat protein molecules increase in number in the cell, they combine with the RNA around the AUG start site for the replicase protein, effectively turning off synthesis of replicase. Thus, the major virus protein synthesized is coat protein, which is needed in 180 copies per RNA molecule.

Another interesting feature of *MS2* RNA virus is that the fourth virus protein, the *lysis* protein, is coded by a gene which overlaps with both the coat protein gene and the replicase gene. The start of this lysis gene is not directly accessible to ribosomes. As the ribosome passes over the coat protein gene, a frame shift occasionally occurs, resulting in reading of the lysis gene. By restricting the efficiency of translation in this way, premature lysis of the cell is probably avoided. Only after sufficient coat protein is available for the assembly of mature virus particles, does lysis commence. (In another RNA phage, Qβ, the maturation protein itself also functions as a lysis protein, and a separate lysis gene as such is not present.)

Ultimately, assembly occurs and release of virions from the cell occurs as a result of cell lysis. The features of replication of these simple RNA viruses are themselves fairly simple. The viral RNA itself functions as an mRNA and regulation occurs primarily by way of controlling access of ribosomes to the appropriate start sites on the viral RNA.

Single-Stranded Icosahedral DNA Bacteriophages

A number of small bacterial viruses have genomes consisting of single-stranded DNA in circular configuration. These viruses are very small, about 25 nm in diameter, and the principle building block of the protein coat is a single protein present in 60 copies (the minimum number of protein subunits possible in an icosahedral virus), to which are attached at the vertices of the icosahedron several other proteins which make up spike-like structures. In contrast to the RNA viruses, much of the enzymatic machinery for the replication of DNA already exists in the cell. These small DNA viruses possess only a limited amount of genetic information in their genomes, and the host cell DNA replication machinery is used in the replication of virus DNA.

The most extensively studied virus of this group is the phage designated ϕX174, which infects *Escherichia coli*. ϕX174 is of special interest because it was the first genetic element shown to have overlapping genes. The genomes of cells are organized in linear fashion, with the gene coding for each protein separate from that for all other genes. In very small viruses such as ϕX 174 there is insufficient DNA to code for all virus-specific proteins. ϕX174 has solved this problem by the use of overlapping genes. Thus, parts of certain nucleotide sequences are read twice, in different directions and in different reading frames. It should be noted that although the use of overlapping genes makes possible more efficient use of genetic information, it seriously complicates the evolution process, since a mutation in a region of gene overlap may affect two genes simultaneously.

As seen in the genetic map, the sequences of genes D and E overlap each other, gene E being contained completely *within* gene D. In addition, the termination codon of gene D overlaps the initiation codon of gene J by one nucleotide. The reading frame of gene E is therefore in a different phase (starting point) from that of gene D. Obviously, any mutation in gene E will also lead to an alteration in the sequence of gene D, but whether a given mutation affects one or both proteins will depend on the exact nature of the alteration (because the genetic code is degenerate). Other instances of gene overlap through use of overlapping reading frames in ϕX 174 DNA are genes A/B, K/B, K/C, K/A, A/C, and D/E. Additionally, a small gene A protein, called A* protein, is formed by *reinitiation of translation* (not transcription) within gene A mRNA, with A' protein being read and terminated from the same mRNA reading frame as A protein.

The DNA of OX174 consists of a circular singlestranded molecule of 5386 nucleotide residues. The DNA of ϕX174 was the first DNA to be completely sequenced, a remarkable achievement when it was accomplished by Sanger and colleagues in 1977. Now, DNA sequencing is a routine procedure. The replication process of such a circular singlestranded DNA molecule is of considerable general interest, since cellular DNA replicates always in the double-stranded configuration. The DNA strand in the virion is referred to as the plus (+) strand and the complementary strand the *minus* (-) strand. Upon infection, the viral *plus* strand becomes separated from the protein coat; entrance into the cell is accompanied by the conversion of this singlestranded DNA into a double-stranded form called the *replicative form* (RF) DNA. Cell-coded proteins involved in the

conversion of viral DNA into RF consist of the enzyme *RNA primase* and *DNA polymerase, ligase,* and *gyrase.* No virus-coded proteins are involved in the conversion of single-stranded DNA to RF. The RF is a closed, double-stranded, circular DNA which has extensive supercoiling.

DNA replication differs between the leading strand and the lagging strand of the DNA double helix. In cells, replication of the lagging strand involves the formation of short *RNA primers* by action of an enzyme called *RNA primase* (or *primase* for short). Such RNA primers are made at intervals on the lagging strand and are then removed and replaced with DNA by DNA polymerase.

In ϕX174, however, replication begins with a single stranded closed circle, a rather atypical situation. First, primase brings about the synthesis of a short RNA primer, beginning at one or more specific initiation sites on the DNA.

Once priming of DNA synthesis has been carried out, the RNA primer is replaced with DNA through action of *DNA polymerase.* Continuation of DNA replication around the closed circle leads to the formation of the complete double-stranded RF. Once the complete second strand has been formed, its circle is closed with *DNA ligase* and a *DNA gyrase* introduces twists that result in supercoiling. DNA gyrase introduces supercoils by cutting one of the two strands of the DNA double helix, holding the two ends apart without rotation, passing a distant region of the circle through the cut, and resealing the ends. The degree of supercoiling is determined by the number of twists that have been introduced into the DNA. One result of supercoiling is that it converts the DNA into a more compact form where it takes up less room in the cell or virion.

Once the RF is formed, nucleic acid replication occurs by conventional semiconservative replication, resulting in the formation of new RF molecules. As in general DNA synthesis, *initiation* of the formation of a new strand begins at a unique site on the DNA, the *origin of replication.* In ϕX174, the origin of replication is at residue 4395. Formation of single-stranded viral progeny begins with a single-stranded cleavage of the viral *(plus)* strand of the RF at the origin of replication. Cleavage is brought about by a protein called *gene A protein*; this protein also makes a covalent bond to the *5′ P* of the viral strand. Asymmetric replication by the rolling circle mechanism results in the formation of single-stranded molecules that will become the virus progeny. When the growing viral strand reaches unit length

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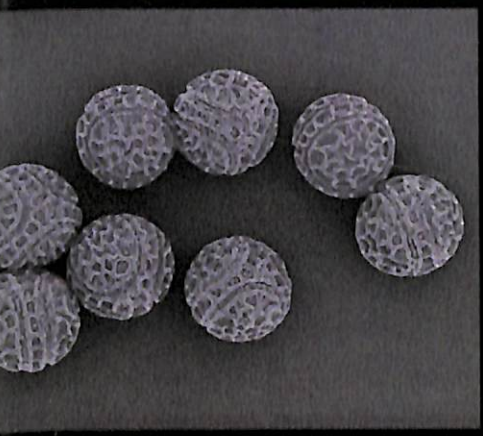

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