PRINCIPLES OF ANIMAL VIROLOGY

W. K. Joklik

Principles of Animal Virology

edited by

Wolfgang K. Joklik, D. Phil.

James B. Duke Distinguished Professor of Microbiology and Immunology
Chairman, Department of Microbiology and Immunology
Duke University Medical Center

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PREFACE

Virology occupies a unique position in contemporary biology. It is the discipline *par excellence* for studies at the molecular level: the opportunity to observe the functioning of small genomes encouraged the development of sophisticated genetic and biochemical techniques the exploitation of which has yielded a rich harvest of fascinating fundamental discoveries ranging from the very existence of messenger RNA in 1959 to recently acquired totally unexpected and surprising insight into the arrangement of genetic material. Viruses also represent the last major challenge in infectious disease: although many human viral pathogens were isolated and characterized during the first half of this century culminating in a golden period during the early 1950's, viruses that cause important human diseases are still being discovered nowadays—such as rotavirus and the virus that causes African hemorrhagic fever. Further, our gradually developing knowledge concerning the nature of latent and persistent viral infections may soon provide clues regarding the causes of chronic debilitating conditions such as diabetes, lupus, multiple sclerosis and Creutzfeld-Jakob disease, and the involvement of viruses in cancer.

In view of its central role in contemporary biology and medicine, virology provides an important conceptual framework that must not only be mastered by graduate and medical students, but is also becoming increasingly important in undergraduate curricula. This textbook is designed to fill several clearly defined needs. It is intended to be a text for advanced undergraduates who intend to proceed to graduate and medical school, as well as for graduate students in cell and molecular biology, genetics, biochemistry, microbiology and immunology, and related disciplines. It is also written for medical students experiencing their first exposure to medical microbiology, as well as for advanced medical students, house officers and practicing physicians. Finally, the book is designed as a reference source for instructors. To achieve these aims, the book comprises two sections, one that discusses the biochemistry, molecular biology and genetics of animal viruses (and also includes a chapter on the molecular virology of bacteriophages), and another that describes the viruses that are pathogenic for man, the nature and symptoms of the diseases that they cause, and how to treat and prevent such diseases.

In reference to the bibliography, we have elected not to reference specific statements in the text, but have appended to each chapter a list of recent reviews and important original papers. The former will quickly guide the reader to any specific aspect of virology that he wishes to pursue; the latter makes available the detailed considerations and circumstances that generated key discoveries. Many of the papers that are cited already are, or no doubt will soon become, "classics."

The list of individuals who have helped produce this volume is long and we are profoundly indebted to them. We would especially like to thank our many colleagues who permitted us to use illustrative

material and who almost invariably supplied us with original photographs, and the many publishers who allowed us to reproduce previously published material. We would also like to thank Lynda Frejlach, who did a superb job in drawing the numerous charts and diagrams, and all the secretaries who cheerfully typed and retyped the manuscript. Finally, we wish to express our appreciation to the staff of Appleton-Century-Crofts for their efficient cooperation in producing this book.

Wolfgang K. Joklik
David W. Barry
Thomas E. Frothingham
Samuel L. Katz
David J. Lang
Catherine M. Wilfert

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SECTION I

BASIC VIROLOGY

CHAPTER 1

The Nature, Isolation, and Measurement of Animal Viruses

Many important infectious diseases that afflict mankind are caused by viruses. Some are important because they are frequently fatal; among such are rabies, smallpox, poliomyelitis, hepatitis, yellow fever, and various encephalitic diseases. Others are important because they are very contagious and create acute discomfort; among such are influenza, the common cold, measles, mumps, and chickenpox, as well as respiratory-gastrointestinal disorders. Still other viruses, such as rubella and cytomegalovirus, can cause congenital abnormalities; and finally there are viruses that can cause tumors and cancer in animals and perhaps also in humans.

There is little that can be done to interfere with the growth of viruses, since they multiply within cells, using the cells' synthetic capabilities. Only a limited number of highly specialized reactions are under their own control. It is hoped that their selective inhibition will form the basis of a rational system of antiviral chemotherapy, thereby permitting virus diseases to be brought under effective control, just as antibiotics have brought most bacterial diseases under control.

In addition to their medical importance viruses provide the simplest model systems for many basic problems in biology. The reason is that viruses are essentially small segments of genetic material encased in protective shells. Since the information encoded in viral genomes differs from that in host cell genomes, viruses afford unrivaled opportunities for the study of the mechanisms that control the replication, transcription, and translation of genetic information. Knowledge of these mechanisms is fundamental to an understanding of the development and operation of differentiated functions in higher organisms and is therefore directly applicable to the practice of medicine and the improvement of human welfare.

Historical background

There are three major classes of viruses: animal viruses, plant viruses, and bacterial viruses. Since knowledge concerning each of these classes has accumulated along distinctive lines, extensive specialization has developed. Bacterial viruses are, therefore, dealt with only briefly in this book, and plant viruses are not considered at all. Yet discoveries made concerning each of these classes of viruses have influenced profoundly our understanding of the nature of each of the others.

The existence of viruses became evident during the closing years of the nineteenth century when, as the result of newly acquired expertise in the handling of bacteria, the infectious agents of numerous diseases were being isolated. For some infectious diseases this proved to be an elusive task until it was realized that the agents causing them were smaller than bacteria. Iwanowski in 1892 was probably the first to record the transmission of an infection (tobacco mosaic disease) by a suspension filtered through a bacteria-proof filter. This was followed in 1898 by a similar report by Loeffler and Frosch concerning foot-and-mouth disease of cattle. Beijerinck (1898) considered the infectious agents in bacteria-free filtrates to be living but fluid—that is, nonparticulate—and introduced the term "virus" (Latin, poison) to describe them. It quickly became clear, however, that viruses were particulate, and the term "virus" became the operational definition of infectious agents smaller than bacteria and unable to multiply outside living cells. In 1911 Rous discovered a virus that produced malignant tumors in chickens, and during World War I Twort and d'Herelle independently discovered the viruses growing in bacteria, the bacteriophages.

During the next 25 years the experimental approaches in the three areas of virology diverged. Plant viruses proved easy to obtain in large amounts, thus permitting extensive chemical and physical studies. This work first led to the demonstration that plant viruses consisted only of nucleic acid and protein, and culminated in the crystallization of tobacco mosaic virus by Stanley in 1935. This feat evoked great astonishment, since it cut across preconceived ideas concerning the attributes of living organisms and demonstrated that agents able to reproduce in living cells behaved under certain conditions as typical macromolecules.

Work with bacteriophages concentrated on their clinical application. It was hoped that bacteria could be destroyed inside the body by injecting appropriate bacteriophages. Their activ-

ity in vivo, however, never matched their activity in vitro, most probably because they are eliminated efficiently from the bloodstream.

Work with animal viruses concentrated on the pathogenesis of viral infections and on epidemiology. Throughout this period, fundamental studies on animal cell-virus interactions were severely hampered by the absence of rapid and efficient techniques for quantitating viruses. The only method then available was the expensive and time-consuming serial end point dilution method, using animals (p. 11).

Around the year 1940 came several breakthroughs. First, the advent of electron microscopy permitted visualization of viruses for the first time. As will become evident, not only is morphology an important criterion of virus classification, but the study of the morphology of viruses has also had a profound impact on our understanding of their behavior and function. Second, techniques for purifying certain animal viruses were being perfected, and a group of workers at the Rockefeller Institute headed by Rivers carried out some excellent chemical studies on vaccinia virus. Third, Hirst discovered that influenza virus agglutinates chicken red cells. This phenomenon, hemagglutination, was rapidly developed into an accurate method for quantitating myxoviruses, as a result of which this group of viruses became in the 1940's the most intensively investigated group of animal viruses. Finally, this period marked the beginning of the modern era of bacterial virology. Until then the interaction of bacteriophages with bacteria had been analyzed principally in terms of populations, rather than at the level of a single virus particle interacting with a single cell. This conceptual block was removed by Ellis and Delbrück's study of the one-step growth cycle, as a result of which the bacteriophage-bacterium system became extraordinarily amenable to experimentation. Indeed, during the last three decades, many of the major advances in molecular biology have resulted from work in the bacteriophage field. Among these are the demonstration that initiation of virus infection involves the separation of virus nucleic acid and protein, the demonstration that the virus genome can become integrated into the genome of the host cell, the discovery of messenger RNA, and elucidation of the factors that control initiation and termination of both transcription and translation of genetic information.

In animal virology, rapid advances followed the development, in the late 1940's, of techniques for growing animal cells in vitro. Strains of many types of mammalian cells can now be grown in media of defined composition. As a result, animal cell-virus interactions can now be analyzed with the same techniques that have proved so powerful in the case of bacteriophages.

The nature of viruses

Viruses are a heterogeneous class of agents. They vary in size and morphology; they vary in chemical composition; they vary in host range and in the effect that they have on their hosts. There are certain characteristics, however, that are shared by all viruses:

1. Viruses consist of a genome, either RNA or DNA, that is surrounded by a protective protein shell. Frequently this shell is itself enclosed within an envelope that contains both protein and lipid.
2. Viruses multiply only inside cells. They are absolutely dependent on the host cells' synthetic and energy-yielding apparatus. They are parasites at the genetic level.
3. The multiplication of viruses involves as an initial step the separation of either their genomes or their nucleocapsids from their protective shells (see below).

In essence, therefore, viruses are nucleic acid molecules that can enter cells, replicate in them, and code for proteins capable of forming protective shells around them.

Given this definition of viruses, are they to be regarded as living organisms or as lifeless arrangements of molecules? The answer to this question depends on whether one is concerned with viruses as extracellular suspensions of particles or as infectious agents. Isolated virus particles are arrangements of nucleic and protein molecules with no metabolism of their own; they are no more active than isolated chromo-

somes. Within cells, however, virus particles are capable of reproducing their own kind manyfold by virtue of precisely regulated sequences of reactions. Considered in this light, viruses may indeed be said to possess at least some of the attributes of life. Such terms as "organism" and "living," however, are not really applicable to viruses; it is preferable to refer to viruses as being functionally active or inactive, rather than living or dead.

The origin of viruses

The question of the origin of viruses poses a fascinating problem. The two likeliest hypotheses are (1) viruses are the products of regressive evolution of free-living cells. An evolutionary pathway of this type has been suggested for mitochondria, which still retain vestiges of cellular organization, as well as a mechanism for replicating, transcribing, and translating genetic information. The largest animal viruses, the poxviruses, are so complex that one could imagine them also to be derived from a cellular ancestor. (2) Viruses are derived from cellular genetic material that has acquired the capacity to exist and function independently. Nowadays the latter hypothesis is considered much more likely for all viruses (with the possible exception of poxviruses).

The characteristics of cultured animal cells

The medical practitioner should understand not only how viruses affect the patient as a whole but also how viruses interact with cells. This understanding can be acquired far more readily by studying isolated infected cells than by examining infected cells in the intact organism. Animal virology provided the main impetus for the development of tissue culture—the technique of growing cells in vitro. Tissue culture is now used extensively for fundamental studies in areas ranging from growth, differentiation, and aging to molecular biology and genetics. Since knowledge concerning the normal cell is crucial to an understanding of virus-cell interaction, we will first examine briefly the characteristics of animal cells cultured in vitro.

The Establishment of Animal Cell Strains

Cells of many organs can be grown in vitro. As a rule, small pieces of the tissue in question are dissociated into single cells by treatment with a dilute solution of trypsin, and a suspension of the cells is then placed into a flask, bottle, or petri dish. There the cells attach to the flat surface, and provided that they are supplied with a growth medium, they multiply. The essential constituents of a growth medium are physiologic amounts of 13 essential amino acids and 9 vitamins, salts, glucose, and a buffering system that generally consists of bicarbonate in equilibrium with an atmosphere containing about 5 percent carbon dioxide. This medium is supplemented to the extent of about 5 percent with serum, the source of which is not predicated by the species from which the cells were derived; calf and fetal calf serum are the two most commonly employed. Antibiotics, such as penicillin and streptomycin, are also usually added in order to minimize the growth of bacterial contaminants, and a dye, such as phenol red, is generally included as a pH indicator. This medium, or more complex versions of it, will permit most cell types to multiply with a division time of 24 to 48 hours.

When cells are brought into contact with a surface, they generally attach firmly and flatten so as to occupy the maximum surface area. The only time when they are not maximally extended is during mitosis, when they become round and are therefore easily dislodged from the substratum. Cells multiply until they occupy all available surface area—that is, until they are confluent, but no further. The reason for this is the cells cease dividing when they make contact with neighboring cells, a phenomenon known as "contact inhibition" (see Chap. 9).

Animal cells can be cloned just like bacterial

cells, although the efficiency of cloning is frequently less than 100 percent. Numerous genetically pure cell strains are now available. They fall into two morphologic categories, epithelial cells with a polygonal outline, and fibroblasts with a narrow spindlelike shape (Fig. 1-1).

The first cultures after tissue dispersion are known as "primary cultures." When such cultures are confluent they are passaged by dislodgment from the surface by means of trypsin or the chelating agent ethylene diamine tetraacetate (EDTA) and reseeded into several new containers, in which they form secondary cultures. Passaging can then be continued in this manner, provided that an adequate supply of growth medium is supplied at regular intervals.

The overall properties of cell strains are generally stable on continuous culturing. However, mutations occur constantly, so that one particular mutant, or variant, usually emerges as the dominant population component under any given set of conditions. As a result, the same cell strain cultured in two different laboratories may exhibit detectable phenotypic differences.

The Multiplication Cycle

The multiplication of each individual cell conforms to a regular pattern which can be thought of as a cycle (Fig. 1-2). According to this scheme, the interval between successive mitoses is divided into three periods: the G1 period that precedes DNA replication, the S period during which DNA replicates, and the G2 period during which the cell prepares for the next mitosis. RNA and protein are not synthesized while mitosis proceeds—that is, during metaphase—but are otherwise synthesized throughout the multiplication cycle. Nongrowing cells are usually arrested in the G1 period; the resting state is often referred to as G0 (G zero). The relative durations of the periods are quite variable, but metaphase rarely occupies more than one hour.

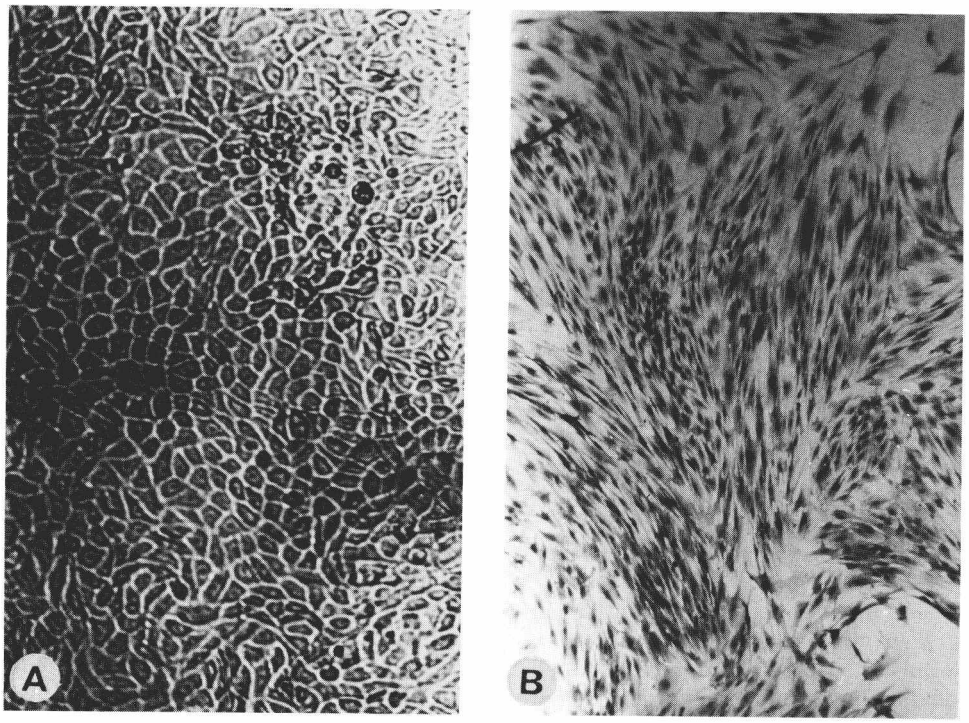

Fig. 1-1 Cultured mammalian cells. **A.** Unstained monkey kidney cells, which exhibit a typical epithelioid morphology. **B.** Chick embryo fibroblasts (Giemsa stain). Note characteristic spindle shape and orderly alignment. (A, from Eagle and Foley, Cancer Res 18:1017, 1958. B, courtesy of Dr. R. E. Smith.)

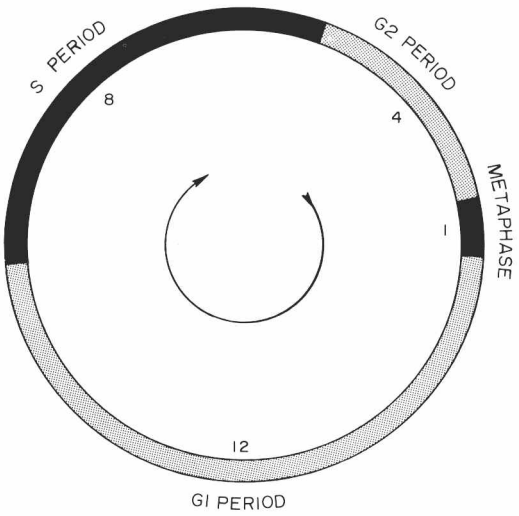

FIG. 1-2. The multiplication cycle of mammalian cells. The duration of the cycle illustrated here is 25 hours; the average lengths (in hours) of the individual periods is indicated by the numbers inside the cycle.

Under conditions of normal growth, the individual cells of a growing culture pass through this multiplication cycle in an unsynchronized fashion, so that cells at all stages of the cycle are always present. It is, however, possible to synchronize cells so that they multiply in step for several generations. Synchronized cell cultures are useful in studies of the reaction that are essential for progression through the multiplication cycle.

The Aging of Cell Strains

Cells derived from normal tissues cannot be passaged indefinitely. Instead, after about 50 passages, which generally occupy about one year, their growth rate inevitably begins to slow. The amount of time that they spend in G0 following each mitosis gradually increases, fewer and fewer cells enter the S period, and the cells' karyotype, that is, their chromosomal complement, changes from the euploid (diploid) pattern characteristic of normal cells to an aneuploid one, characterized by the appearance of supernumerary chromosomes, chromosome fragments and chromosomal aberrations, that is, changes in the structure of individual chromosomes. Finally, the cell strain dies out. Loss of cell strains in this manner is generally guarded against by growing large numbers of cells during the early passages and storing them at −196C, the boiling point of liquid nitrogen.

Continuous Cell Lines

While cells derived from normal tissues have the properties described thus far, malignant tissues give rise to aneuploid cell lines that have an infinite life span and are referred to as "established cell lines." Infrequently such cell lines seem to arise from euploid cell strains, but the possibility that malignant or premalignant cells were not present originally is difficult to rule out. In addition to being aneuploid and immortal, such cell lines usually have two other significant properties: they form tumors when transplanted into animals, and they can grow in suspension culture like bacteria. Cells growing in suspension are used extensively for studies of virus multiplication, since they are easier to handle experimentally than cells growing as monolayers.

Patterns of Macromolecular Biosynthesis

Since virus multiplication consists essentially of nucleic acid and protein synthesis, a brief description of the patterns of macromolecular synthesis in animal cells is relevant. The essential feature of the animal cell is its compartmentalization. The DNA of the animal cell is restricted to the nucleus at all stages of the cell cycle except during metaphase, when no nucleus exists. All RNA is synthesized in the nucleus. Most of it remains there, but messenger RNA and transfer RNA migrate to the cytoplasm. Ribosomal RNA is synthesized in the nucleolus; the two ribosomal subunits are assembled partly in the nucleolus and partly in the nucleus, and then they also migrate to the cytoplasm. All protein synthesis proceeds in the cytoplasm. The only exception to this brief summary concerns the mitochondria, which contain DNA-, RNA-, and protein-synthesizing systems of their own and which are located only in the cytoplasm.

The detection of animal viruses

The presence of viruses is recognized by the manifestation of some abnormality in host organisms or host cells. In the organism, symptoms of virus infection vary widely, from unapparent infections (detectable only by the formation of antibody), the development of local lesions, or mild disease characterized by light febrile response, to progressively more severe disease culminating in death. In cells, the symptoms of viral infection vary from changes in morphology and growth patterns to cytopathic effects, such as rounding, breakdown of cell organelles, the development of inclusion bodies, and general necrotic reactions, finally resulting in complete disintegration.

The isolation of animal viruses

Many techniques have been developed for isolating viruses. The source of virus may be excreted or secreted material, the bloodstream, or some tissue. Samples are collected and, unless processed immediately, sheltered from heat, preferably by storage at −70C, the temperature of dry ice. If necessary, a suspension is then prepared by grinding or sonicating in the presence of cold buffer solution, and this is then centrifuged in order to remove large debris and contaminating microorganisms.

This suspension is then tested for the presence of virus in several ways. First, it is injected back into the original host species in order to determine whether the first noted abnormality is produced. Second, the suspension is injected into other animals in order to establish whether there exist more susceptible hosts in which the disease develops more rapidly, more severely, or in a more easily recognizable manner. Newborn or suckling animals (often mice or hamsters) or developing chick embryos are hosts which permit many viruses to multiply more extensively than do adult animals and are accordingly widely used for virus isolation. Third, a search is conducted for an optimal cultured animal cell strain or line in which the virus will multiply and in which it may actually be isolated and also assayed. The cells which will eventually be chosen will usually be ones in which the virus rapidly elicits readily observable cytopathic effects.

The final stage of the isolation procedure is passage at limiting dilution in order to ensure that only a single unique virus is being isolated. This may be accomplished either by limiting serial dilution, when the virus suspension is diluted to such an extent that only one out of several aliquots inoculated gives a positive response, or by plaque isolation (p. 9). The latter is preferable wherever possible, since plaques originate from single virus particles, just as bacterial colonies originate from single bacterial cells.

While virus isolation from severely diseased hosts may present no difficulty, it may be a formidable task if the original source is merely suspected of containing a small amount of virus. As a result, no symptoms may result when the initial virus suspension is inoculated into the various test systems. In such cases one generally resorts to so-called blind passaging, in the hope that gradual enrichment of virus will occur. In this procedure, cells are disrupted several days after inoculation even if they appear healthy and unaltered, and an extract of them is inoculated into fresh cells. This is repeated several times until symptoms appear. It is important that this procedure be adequately controlled by passaging extracts of uninfected cells under the same conditions, since animal cells are known to harbor latent viruses which may be induced to multiply and which may then be mistaken for the etiologic agent of whatever condition is under study.

Adaptation and Virulence

During the isolation of viruses, there may emerge variants capable of multiplying more efficiently in the host cells used for this purpose than the original wild-type virus. This phenomenon, which is known as "adaptation," has as its basis the selection of spontaneous mutants,

which constantly arise during virus multiplication. These mutants multiply more efficiently in the cells used for isolating the virus than in the cells of the original infected tissue. Such variants damage the original host less severely than the wild-type virus and are therefore said to be less "virulent." Viruses are often purposely adapted in order to alter growth and virulence characterisitics. An example is provided by the attenuated vaccine virus strains, which are obtained by repeated passaging of virus virulent for one host in some different host, until virus strains with decreased virulence for the original host are selected.

The measurement of animal viruses

Viruses are measured by several methods that can be divided into two categories. First, viruses may be measured as infectious units—that is, in terms of their ability to infect, multiply, and produce progeny. Second, viruses may be measured in terms of the total number of virus particles, irrespective of their function as infectious agents.

MEASUREMENT OF VIRUSES AS INFECTIOUS UNITS

Measurement of the amount of virus in terms of the number of infectious units per unit volume is known as titration. There are several ways of determining the titer of a virus suspension, all of them involving infection of host or target cells in such a way that each particle that causes productive infection elicits a recognizable reaction.

Plaque Formation

In this method a series of monolayers of susceptible cells are inoculated with small aliquots of serial dilutions of the virus suspension to be titrated. Wherever virus particles infect cells, progeny virus particles are produced and released and then immediately infect adjoining cells. This process is repeated until, after a period ranging from 2 to 12 days or more, there develop areas of infected cells which can be seen with the naked eye. These are called plaques. In order to ensure that progeny virus particles liberated into the medium do not diffuse away and initiate separate (or secondary) plaques, agar is frequently incorporated into the medium.

The fundamental prerequisite for this method of enumerating infectious units is that the infected cells must differ in some way from noninfected cells: for example, they must either be completely destroyed, become detached from the surface on which they grow, or possess staining properties different from those of normal cells. In practice, the most common method of visualizing plaques is to apply the vital stain neutral red to infected cell monolayers after a certain number of days and to count the number of areas that do not stain (Fig. 1-3). Titers are expressed in terms of numbers of plaque-forming units (PFU) per ml.

There is a linear relationship between the amount of virus and the number of plaques produced; that is, the dose-response curve is linear. This indicates that each plaque is formed by a single virus particle. The virus progeny in each plaque therefore are clones, and virus stocks derived from single plaques are said to be plaque purified. Plaque purification is an important technique for the isolation of genetically pure virus strains.

Plaque formation is often the most desirable method of titrating viruses. It is economical of cells and virus, as well as technically simple. However, not all viruses can be measured in this way, because there may be no cells that develop the desired cytopathic effects. For these viruses, alternate titration methods must be used.

Pock Formation

Many viruses cause macroscopically recognizable foci of infection or lesions on the chorioallantoic membrane of the developing chick embryo; these lesions may be used in a manner similar to the cell monolayers employed for plaque assay. The main advantage is ready availability, wide virus susceptibility, and ease of handling. The main disadvantage is variation in virus susceptibility among different eggs of

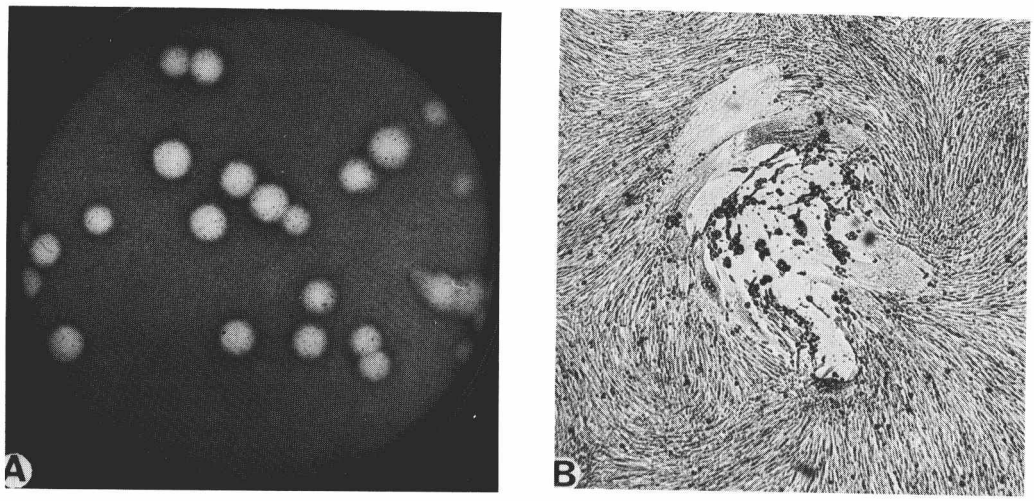

FIG. 1-3. Virus plaques. **A.** Plaques of influenza virus on monolayers of chick embryo cells, 4 days after inoculation. The monolayers were stained with neutral red on day 3. **B.** Photograph showing the microanatomy of a herpesvirus plaque on BHK 21 cells. (A, courtesy of Dr. G. Appleyard. B, courtesy of Dr. S. Moira Brown.)

even the same hatch, so that larger numbers of eggs than cell tissue culture monolayers are necessary in order to attain the same level of statistical significance. The lesions caused by viruses are known as pocks and are generally recognizable as opaque white or red areas caused by cell disintegration, migration, and proliferation, as well as edema and hemorrhage (in the case of red pocks) (Fig. 1-4). The actual titration is carried out as described for plaques, with enumeration of pocks taking the place of plaque counting.

Focus Formation

Certain tumor viruses do not destroy the cells in which they multiply and therefore produce

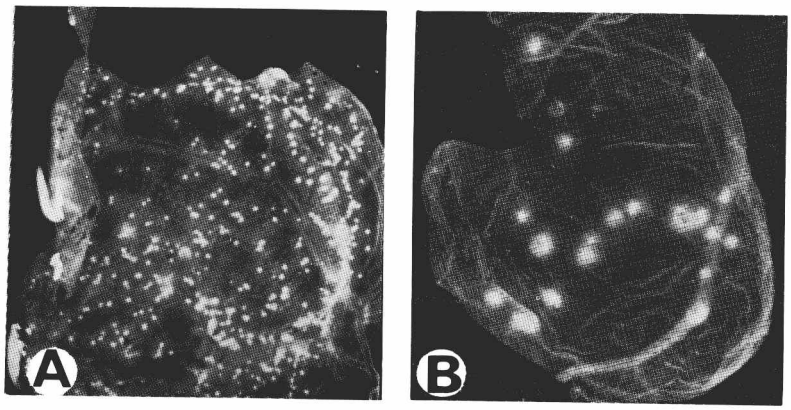

FIG. 1-4. Pocks on the chorioallantoic membrane of the developing chick embryo. The membrane is cut out two or three days after inoculation, washed and spread on a flat surface. **A.** Variola. **B.** Vaccinia. (From Kempe: Fed Proc 14:468, 1955.)