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CHAPTER 3

# Cultivation and Assay of Viruses



Viruses replicate only within living cells. Some viruses are restricted n the kinds of cells in which they replicate, and a few have not yet been :ultivated at all under laboratory conditions. Fortunately, however, nost viruses can be grown in cultured cells, embryonated hen's eggs, or aboratory animals.

In veterinary virology, the natural host animal can be used for the :ultivation of viruses; indeed the earliest viral assay was carried out by Loeffler and Frosch with foot-and-mouth disease virus in cattle. The natural host is still useful for studies of pathogenesis and immunology, vaccine trials, experiments in chemotherapy, and occasionally for diagnostic purposes. However, *in vitro* cultivation of viruses in cell cultures s essential for the study of their mode of replication (Chapter 4) and for diagnostic virology (Chapter 13).

## CELL CULTURE

Although over 70 years have elapsed since mammalian cells were first grown *in vitro*, it is only since the advent of antibiotics that cell culture (also referred to as tissue culture) became a matter of simple routine.

Aseptic precautions are still essential, but the problems of contamination with bacteria, mycoplasmas, fungi, and yeasts are no longer insurmountable. Today, most kinds of animal cells can be cultivated *in vitro* for at least a few generations, and numerous immortal cell lines have been derived. Since 1949, when Enders, Weller, and Robbins reported that poliovirus could be grown in cultured nonneural cells with the production of recognizable cytopathic changes, hundreds of previously unknown viruses have been isolated and identified in cell cultures. The discovery of the adenoviruses, rhinoviruses, and many others during the 1950s and 1960s was directly attributable to their use, as were the consequent revolution in the diagnosis of viral diseases, the development of vaccines, and the dramatic advances in knowledge of the molecular biology of animal viruses.

## Methods of Cell Culture

Cells may be grown *in vitro* as explants of tissue, such as respiratory or intestinal epithelium, or as cell cultures. Explant cultures are occasionally used for research purposes or for the cultivation of certain viruses, but almost all diagnostic and research work involving viral cultivation is carried out in cell cultures—usually in monolayers, occasionally as suspension cultures.

To produce cell monolayers, tissue is cut into small pieces and placed in a medium containing a proteolytic enzyme such as trypsin. After the cells have dispersed into a single-cell suspension, they are washed, counted, diluted in a growth medium, and permitted to settle on the flat surface of a glass or plastic container. Most types of cells adhere quickly, and under optimal conditions they divide about once a day until the surface is covered with a confluent monolayer.

## Media

Cell culture has been greatly aided by the development of chemically defined media containing almost all the nutrients required for cell growth. The best known of these media, developed by Eagle, is an isotonic salt solution with added glucose, vitamins, and amino acids, buffered at pH 7.4, and containing antibiotics to inhibit the growth of bacteria and fungi. Serum must be added to Eagle's medium, and to most others, to supply additional growth factors, without which most cells will not multiply satisfactorily. In recent years several growth factors have been identified, and certain cell lines can now be grown in media that are totally defined chemically. For instance, the Madin–Darby canine kidney cell line has been grown on fibronectin or in poly-

lysine- or collagen-coated dishes, in serum-free medium supplemented with hormones including insulin, binding proteins (e.g., transferrin and albumin), and attachment factors. Such serum-free media are particularly useful for the cultivation of "hybridoma" cells used for the production of monoclonal antibodies, where there is a need to ensure that all the immunoglobulin in the medium is antibody of a single specificity, produced by the hybridoma cells. Defined media also present advantages for the isolation of viruses that are likely to be neutralized by antibody present in "normal" animal serum, but this is generally not a problem if fetal calf serum is employed. Fetal calf serum (5–10%) is therefore incorporated in the media used for the initial growth of cells in culture. Once the monolayer is established, the "growth medium" is removed, virus is inoculated, and "maintenance medium" containing little or no serum is added to the culture.

### Types of Cultured Cells

Many types of cells undergo only a few divisions *in vitro* before dying out, whereas others will survive for up to a hundred cell generations and some can be propagated indefinitely. These differences, the nature of which are not fully understood, give us three main types of cultured cells (Plate 3-1).

**Primary Cell Cultures.** When cultures are established initially from tissue taken directly from animals (often from fetal organs or tissues), they contain several cell types, most of which are capable of only limited growth *in vitro*—perhaps 5 or 10 divisions at most. This restricts their value, whether for routine diagnostic work or vaccine production, because of the high cost and inconvenience of having to obtain fresh tissue each time, as well as lack of consistency from batch to batch. Furthermore, the donor animals often harbor latent viruses which can confuse diagnosis or contaminate vaccines. Nevertheless, the presence of a diverse range of differentiated cell types in such primary cultures means that they tend to be very sensitive to many animal viruses. In veterinary diagnostic virology, it is common practice to inoculate samples suspected to contain virus into primary cultures derived from the same species of animal as that providing the samples.

**Diploid Cell Strains.** These are cells that are capable of undergoing a number of divisions in culture that is roughly related to the life span of the species of animal—about 50 for fetal human cells and about 10 for fetal cells from horses and cows. They retain their original diploid chromosome number throughout. Diploid strains of fibroblasts established from human fetuses or embryos are widely used in human diagnostic

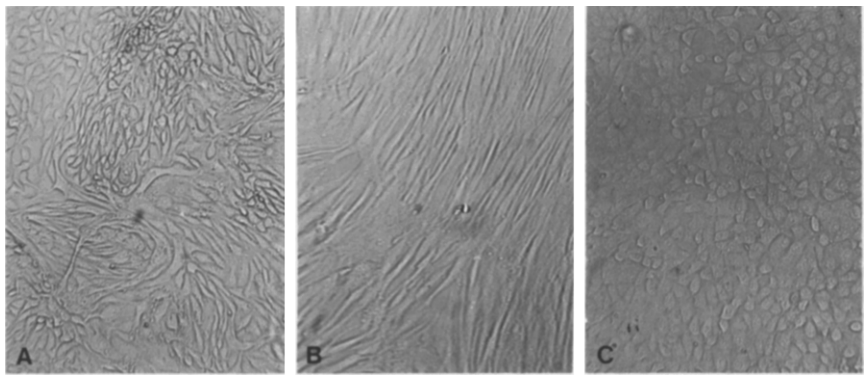


PLATE 3-1. *Unstained confluent monolayers of the three main types of cultured cells, as they appear by conventional low-power light microscopy, through the wall of the tissue culture vessel (×45). (A) Primary monkey kidney epithelium. (B) Diploid strain of fetal fibroblasts. (C) Continuous line of epithelial cells. (Courtesy I. Jack.)*

virology and vaccine production, but diploid strains have not been much used in veterinary vaccine production.

**Continuous Cell Lines.** These are cells of a single type that are capable of indefinite propagation *in vitro*. Such immortal cell lines originate from cancers, or by spontaneous transformation of a diploid cell strain. Often they no longer bear close resemblance to their cell of origin, as they undergo many mutations during their prolonged culture. The usual indication of these changes is that the cells have lost the specialized morphology and biochemical abilities that they possessed as differentiated cells *in vivo*. For example, it is no longer possible to distinguish microscopically between the epithelial cell lines arising from various cells of ectodermal or endodermal origin, or between the fibroblastic cell lines arising from cells of mesodermal origin. Cells of continuous cell lines are often aneuploid in chromosome number, especially if of malignant origin.

Continuous cell lines derived from monkey (e.g., the Vero cell line), dog (MDCK), cattle (MDBK), pig (PK15), cat (CFK), mouse (L929, 3T3), hamster (BHK-21), rabbit (RK-13), and others are widely used in experimental and diagnostic virology (see Table 13-3).

The great advantage of continuous cell lines over primary cell cultures is that they can be propagated indefinitely by subculturing the cells at regular intervals. Like other cells, they retain viability for many years when frozen in serum-containing medium with added dimethylsulfoxide and stored at very low temperature, e.g., in liquid nitrogen (−196°C) or

an electric deep-freezer at −70°C or lower. Good laboratories follow the general microbiological precept that the surest way of faithfully maintaining the characteristics of a cultured cell line is to replace it periodically from frozen stocks.

Some continuous cell lines have been adapted to grow as suspensions of single cells. Such suspension cultures are particularly useful for biochemical studies of viral replication, because large numbers of identical cells are continuously available for regular sampling and processing.

Various methods have been devised to maximize the surface area to which cells can attach, while keeping the overall size of the vessel and the volume of medium within reasonable bounds. Round bottles can be continuously rolled, or may be filled with glass tubes, glass beads, or spiral plastic film. Perhaps the most useful method for growing cells on a large scale for vaccine production is on plastic or Sephadex beads (microcarriers) maintained in suspension in large fermentation tanks.

### Applications of Cell Culture

Of paramount inportance for virologists is the selection of cell lines that will allow optimal growth of the virus under study. Some viruses replicate in almost any cell line and some cell lines support the replication of many different types of viruses. On the other hand, many viruses are quite restricted in the kinds of cells in which they can be isolated from an infected animal. On adaptation by serial passage, however, mutants with somewhat greater growth potential for a given cell line can be selected. A useful general rule is to use a cell strain of low passage number from the same animal species for primary isolation of a virus. Once the virus has been isolated, alternative more convenient cell substrates may be sought.

Cultured cells serve three main purposes: (1) isolation of viruses from clinical specimens (see Chapter 13), for which purpose a type of cell culture should be selected which is known for its high sensitivity and in which cell abnormality is readily recognized, (2) production of vaccines and antigens for serological diagnosis, for which the principal requirement is for a cell line giving a high yield of virus and free from contaminating agents (see Chapter 14), and (3) biochemical studies of viral replication (see Chapter 4), for which continuous cell lines, preferably growing as suspension cultures, are usually chosen.

### Recognition of Viral Growth in Cell Culture

The growth of viruses in cell culture can be monitored by a number of biochemical procedures indicative of the intracellular increase in viral macromolecules and virions (see Chapter 4). In addition, there are sim-

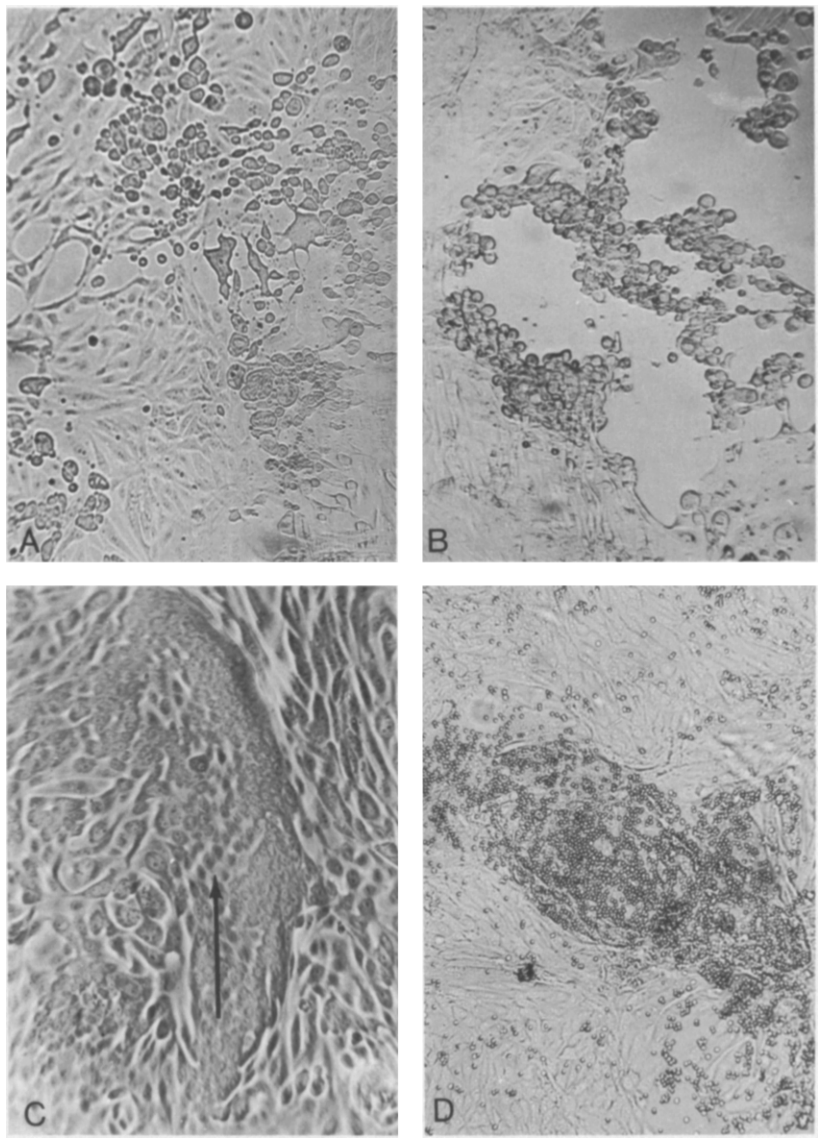


PLATE 3-2. *Cytopathic effects produced by different viruses. The cell monolayers are shown as they would normally be viewed in the laboratory, unfixed and unstained (×60). (A) Enterovirus—rapid rounding of cells, progressing to complete cell destruction. (B) Herpesvirus—focal areas of enlarged, rounded cells. (C) Paramyxovirus—focal areas of cells are fused to form syncytia or giant cells. (D) Hemadsorption. Erythrocytes adsorb to those cells in the monolayer that are infected. The technique is applicable to any virus that incorporated hemagglutinin into the plasma membrane. Most of the enveloped viruses that mature by budding from the cell membranes produce hemadsorption. (Courtesy I. Jack.)*

TABLE 3-1
*Cytopathic Effects of Some Viruses in Cell Culture*

| Cytopathic effect | Virus |
|---|---|
| Cell lysis[a] | Adenoviruses[b] |
| | Alphaherpesviruses |
| | Poxviruses |
| | Enteroviruses |
| | Rhinoviruses[c] |
| | Togaviruses[c] |
| | Reoviruses |
| Cell fusion (syncytium formation) | Herpesviruses |
| | Paramyxoviruses |
| Minimal | Parvoviruses |
| | Orthomyxoviruses[d] |
| | Morbilliviruses |
| | Coronaviruses |
| | Retroviruses |
| | Arenaviruses |

[a]Rounding, pyknosis, then detachment.
[b]Some types cause aggregation of cells, and some cause foci of CPE.
[c]Often produce incomplete cytopathic effect.
[d]In most cell types.

pler methods that are more commonly used for diagnostic work (see Chapter 13), as outlined below.

**Cytopathic Effects.** Many viruses kill the cells in which they replicate, so that infected cell monolayers gradually develop visible evidence of cell damage, as newly formed virions spread to involve more and more cells in the culture. These changes are known as *cytopathic effects* (CPE), and the responsible virus is said to be *cytopathogenic*. Most cytopathic effects can be readily observed in unfixed, unstained cell cultures, under low power of the light microscope with the condenser racked down and the iris diaphragm partly closed to obtain the required contrast.

A trained virologist can distinguish several types of cytopathic effects, even in unstained, living cultures (Plate 3-2, Table 3-1). Fixation and staining of the cell monolayer reveals further diagnostic details, notably *inclusion bodies* (Plate 6-1, Fig. 6-1) and *syncytia* (Plate 3-2C). These morphological consequences of viral infection are discussed in Chapter 6.

**Hemadsorption and Hemagglutination.** Cultured cells infected with orthomyxoviruses, paramyxoviruses, or togaviruses, all of which bud

from cytoplasmic membranes, acquire the ability to adsorb erythrocytes. This phenomenon, know as *hemadsorption,* is due to the incorporation into the plasma membrane of newly synthesized viral protein that binds red blood cells (Plate 3-2D). Hemadsorption can be used to demonstrate infection with noncytopathogenic as well as cytocidal viruses, and can be demonstrated very early, e.g., after 24 hours, when only a small number of cells in the culture are infected. *Hemagglutination* is a different, though related phenomenon, in which erythrocytes are agglutinated by free virus (see below). Virions or hemagglutinin may thus be demonstrated in the supernatant fluid of an infected culture.

**Immunofluorescence.** Newly synthesized intracellular viral antigen can be detected by staining the fixed cell monolayer with specific antiviral antibody which has been labeled with a fluorescent dye, or with an enzyme such as peroxidase. Full details of these techniques are given in Chapter 13.

**Interference.** The replication of one virus in a cell usually inhibits the replication of another virus (see Chapters 6 and 8). The viruses of rubella and of the common cold were first discovered by showing that infected cell cultures, which showed no cytopathic effect, were nevertheless resistant to challenge with an unrelated enterovirus. Cell lines have now become available in which these viruses produce cytopathic effects, but interference is still used for the diagnosis of bovine virus diarrhea virus.

## EMBRYONATED EGGS

Prior to the 1950s, when cell culture began to be widely adopted for the cultivation of viruses, the standard host for the cultivation of many viruses was the embryonated hen's egg (developing chick embryo). The technique was devised by Goodpasture in 1930 and was extensively developed by Burnet over the ensuing years. Nearly all of the viruses that were known at that time could be grown in the cells of one or another of the embryonic membranes, namely the amnion (plus the lung of the chick within the amniotic sac), allantois, chorion, or yolk sac.

Although now largely supplanted by cultured cells, embryonated hen's eggs are still used for the isolation and cultivation of many avian viruses (Table 3-2). The chorioallantoic membrane and the amniotic sac are convenient and sensitive substrates for the growth of many poxviruses and influenza viruses, respectively, and intravenous inoculation is used for the isolation of bluetongue viruses. Furthermore, the allantois produces such high yields of certain viruses, notably influenza virus

TABLE 3-2
*Some Examples of Viruses Grown in Embryonated Eggs*

| Route of inoculation | Viruses | Signs of growth |
|---|---|---|
| Yolk sac | Avian infectious bronchitits virus | Dwarfing of embryo |
| | Avian encephalomyelitis virus | Encephalitis |
| Chorioallantoic membrane | Orthopoxviruses | Pocks |
| Amniotic | Influenza virus | Hemagglutination |
| Allantoic | Influenza virus<br>Newcastle disease virus | Hemagglutination |
| Intravenous | Bluetongue viruses | Death of embryo |

and Newcastle disease virus, that this system is still used by research laboratories and for vaccine production.

## LABORATORY ANIMALS

Like embryonated eggs, laboratory animals have almost disappeared now from diagnostic laboratories, since cell cultures are so much simpler to handle and much more versatile. Nevertheless, infant mice are still used for the isolation of arboviruses, and the natural host animal is still used for agents that do not grow in cell culture, or when a positive isolation is of critical importance.

Animals are still essential for many kinds of virological research. Experiments on pathogenic mechanisms and the immune response are commonly carried out in the natural animal host, or in inbred strains of mice. Hamsters and other rodents are widely used in tumor virology, because they are highly susceptible to tumor production by a number of tumorigenic viruses. Finally, since serology looms large in much virological research, laboratory animals, usually rabbits, are extensively used for producing antisera. Mice are also commonly used for intraperitoneal implantation of hybridomas which secrete monoclonal antibody as an ascitic fluid.

## ASSAY OF VIRAL INFECTIVITY

All scientific research depends on reliable methods of measurement, and with viruses the property we are most obviously concerned with

measuring is infectivity. The content of infectious virions in a given suspension can be titrated by infecting cell cultures (or, more rarely, chick embryos, laboratory animals, or the natural host) with dilutions of viral suspensions, then observing for evidence of viral replication. Two types of infectivity assays should be distinguished: quantitative and quantal.

### Quantitative Assays

A familiar example of this type of assay is the bacterial colony count on an agar plate. Each viable organism multiplies to produce a discrete clone, recognized as a colony, and the colony count therefore represents a direct estimate of the number of viable bacteria originally plated. The parallel in virology is the *plaque assay*, using monolayers of cultured cells.

**Plaque Assays.** In 1952 Dulbecco introduced a modification of the bacteriophage plaque assay into animal virology, and this is now the standard procedure for the quantitation of most animal viruses. A series of 10-fold dilutions of a viral suspension is inoculated onto monolayers of cultured cells for an hour or so to allow the virions to adsorb to the cells. The infected cells are then overlaid with medium in an agar or methylcellulose gel, to ensure that the spread of viral progeny is restricted to the immediate vicinity of the originally infected cell. Hence, each infective particle gives rise to a localized focus of infected cells that becomes, after a few days, large enough to see with the naked eye as an area of cytopathology. To render the plaques more readily visible, the cell monolayers are usually stained with neutral red or crystal violet; the living (uninfected) cells take up the stain and the plaques appear as clear areas against a red or purple background (Plate 3-3). Some viruses, e.g., herpesviruses and poxviruses, will produce plaques even in cell monolayers maintained in liquid medium, because most of the newly formed virions remain cell-associated and plaques form by direct spread of virus to adjacent cells. Infection with a single virus particle is sufficient to form a plaque; the infectivity titer of the original viral suspension is expressed in terms of plaque-forming units (PFU) per milliliter.

**Transformation Assays.** Some oncogenic viruses do not kill cells but "transform" them (see Plate 6-3), so that they display reduced contact inhibition, and grow in an unrestrained fashion to produce a heaped-up "microtumor" that stands out conspicuously against the background of normal cells in the monolayer. Like malignant cells excised from a tumor, the transformed cells have also acquired the ability to grow in medium in semisolid agar or methylcellulose. Both properties have been exploited by tumor virologists to assay tumorigenic viruses.

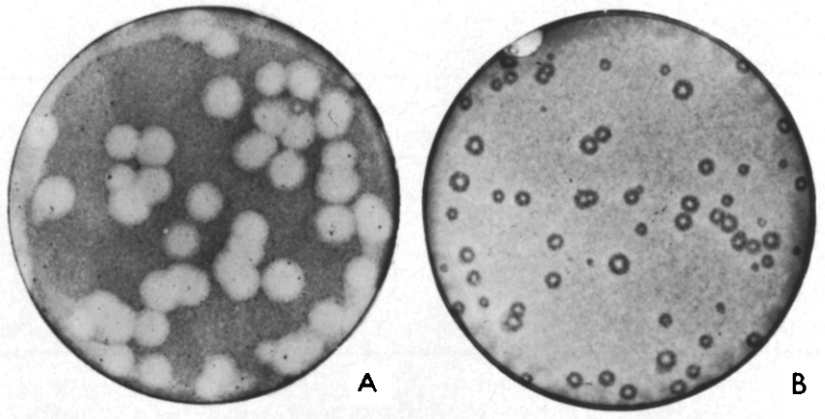


PLATE 3-3. *Plaques produced by influenza virus in monolayers of a continuous cell line derived from human conjunctival cells (Chang). Each plaque is initiated by a single infectious virus particle and yields a clone. (A) Normal plaques, seen as clear areas in monolayer stained with neutral red. (B) "Red" plaques, characteristic of certain strains of influenza virus, and some other viruses. [From E. D. Kilbourne,* In *"Fundamental Techniques in Virology" (K. Habel, ed.), pp. 154, 155. Academic Press, New York; courtesy Dr. E. D. Kilbourne.]*

**Pock Assays.** A much older assay, still occasionally used for the poxviruses, is the titration of viruses on the chorioallantoic membrane of the chick embryo. Newly synthesized virus escaping from infected cells spreads mainly to adjacent cells, so that each infecting particle eventually gives rise to a localized lesion, known as pock. The morphology and color of the pock is often characteristic of a particular group of viruses or even a particular mutant.

### Quantal Assays

The second type of infectivity assay is not quantitative but quantal, i.e., it does not measure the exact number of infectious virus particles in the inoculum, but only whether there are any at all. Being an all-or-none assay, it is not as precise as a quantitative assay. Serial (e.g., 10-fold) dilutions of virus are inoculated into several replicate cell cultures, eggs, or animals. Adequate time is allowed for virus to replicate and spread to destroy the whole cell culture, or kill the embryo or animal, as the case may be. Hence, each host yields only a single piece of information, namely, whether or not it was infected by that particular dilution of virus.

The results of typical quantal and quantitative infectivity titrations are

TABLE 3-3
*Comparison of Quantitative and Quantal Infectivity Titrations*

| Virus dilution[a] | Quantitative assay (plaque count) | Quantal assay[b] (cpe or death of host) |
|---|---|---|
| $10^{-2}$ | C, C, C, C, C[c] | +++++ |
| $10^{-3}$ | 50, 42, 54, 59, 45 | +++++ |
| $10^{-4}$ | 5, 7, 3, 6, 4 | ++−++ |
| $10^{-5}$ | 0, 0, 1, 0, 1 | −+−−− |
| $10^{-6}$ | 0, 0, 0, 0, 0 | −−−−− |
| Titer | $10^{5.4}$ PFU per ml | $10^{5.2}$ $TCID_{50}$ per ml |

[a]Inoculum: 0.2 ml.
[b]Each symbol (+ or −) represents the result in one tissue culture tube or animal.
[c]C, Confluent (uncountable).

given in Table 3-3. The end point of a quantal titration is taken to be that dilution of virus which infects (or kills) 50% of the inoculated hosts; the titer of the original virus suspension is then expressed in terms of 50% infectious doses ($ID_{50}$ or $TCID_{50}$ in tissue cultures) or 50% lethal doses ($LD_{50}$) per milliliter. Statistical procedures must be used to calculate the end point of quantal titrations. Commonly used procedures are those introduced by Reed and Muench, and Karber, details of which can be found in laboratory manuals.

## ASSAYS BASED ON OTHER PROPERTIES OF VIRIONS

### Hemagglutination

Many viruses contain, in their outer coat, virus-coded proteins capable of binding to erythrocytes (Table 3-4). Such virions can, therefore, bridge red blood cells to form a lattice. This phenomenon, known as *hemagglutination* (HA), was first described in 1941 by Hirst, who then went on to analyze the mechanism of hemagglutination by influenza virus. The *hemagglutinin* of influenza is a glycoprotein, which projects from the envelope of the virion (see Plate 26-1). The virus will attach to any species of erythrocyte carrying complementary receptors, which are glycoproteins of a different sort. Hemagglutination by influenza virus and the paramyxoviruses is complicated by the fact that the virions also carry an enzyme, neuraminidase, which destroys the glycoprotein receptors on the erythrocyte surface (by removing terminal neuraminic acid) and allows the virus to elute, unless the test is carried out at

TABLE 3-4
*Hemagglutination by Viruses*

| Virus | | Erythrocytes |
|---|---|---|
| Family | Genera or species | |
| *Adenoviridae* | Most species | Monkey and/or rat, 37°C |
| *Poxviridae* | *Orthopoxvirus* | Chicken, 37°C |
| *Parvoviridae* | Most species | Guinea pig, hamster, pig, human, monkey, 4°C |
| *Togaviridae* | *Alphavirus* | Goose, pigeon, chick; pH and temperature critical |
| *Flaviviridae* | *Flavivirus* | |
| *Orthomyxoviridae* | Influenza A | Chick, human, guinea pig, 4°C |
| *Paramyxoviridae* | Parainfluenza<br>Newcastle disease | Chick, human, guinea pig, 4°C |
| *Coronaviridae* | Several species | Rat, mouse, chick |
| *Bunyaviridae* | Several species | Goose; pH critical |
| *Rhabdoviridae* | Rabies | Goose, 4°C |
| *Reoviridae* | *Reovirus*<br>*Rotavirus* | Human |

ambient or lower temperature. About $10^7$ influenza virions are needed to cause agglutination of sufficient erythrocytes to permit the test to be read with the naked eye. Thus, hemagglutination is not a sensitive indicator of the presence of small numbers of virions, but because of its simplicity it provides a very convenient assay if large amounts of virus are available (see Plate 13-3).

## Counting Virions by Electron Microscopy

*Negative staining* with potassium phosphotungstate makes it possible to count the number of particles in viral suspensions by electron microscopy. There are three main approaches. The viral suspension can be mixed with a known concentration of polystyrene latex particles (or with a morphologically distinct virus of known particle count), to provide an easily recognizable marker; the ratio of "unknown" virus particles to latex beads enables its concentration to be determined. Alternatively, a known volume of viral suspension is deposited on the grid by ultracentrifugation. Another method is to remove water and salts from a drop of viral suspension hanging from the underside of an ultrathin carbon-coated plastic ("Formvar") film mounted on a copper grid, by diffusion downwards into agar; thus the number of particles in a known volume may be counted.

TABLE 3-5
*Comparison of Assays for Influenza Virus*

| Method | Titer (per ml) |
|---|---|
| Electron microscope count | $10^{10}$ virions |
| Quantal infectivity assay in eggs | $10^{9}$ egg $ID_{50}$ |
| Quantal infectivity assay in cultured cells[a] | $10^{7.8}$ $TCID_{50}$ |
| Quantitative infectivity assay by plaque formation[a] | $10^{8}$ PFU |
| Hemagglutination assay | $10^{3}$ HA units |

[a]In the same cell line.

## Comparison of Different Assays

If a given preparation of viral particles were to be assayed by all of the methods described above, the "titer" would be different in every case. For example, an influenza virus suspension may provide the data set out in Table 3-5. The difference between the electron microscope and hemagglutination titers reflects merely the difference in sensitivity between the two assays. On the other hand, the ratio between the infectivity titer and the particle count, known as the infectivity to particle ratio, requires deeper analysis. In part, it is explained by the fact that most of the virions visible by electron microscopy are noninfectious, having being inactivated by heat (even at 37°C) or by other mechanisms during extraction and purification, or having been assembled unsatisfactorily in the first place (typically having a defective genome, hence known as "defective" or "incomplete" virus—see Chapter 5). However, the situation is complicated by another factor, namely, the susceptibility of the cell system in which viral infectivity is assayed; here one speaks of the *plating efficiency* of the virus. Even a fully infectious virion has only a certain chance of successfully negotiating all the barriers it may encounter in the course of entering and infecting a cell. The susceptibility of one cell system may be much lower (i.e., resistance much higher) than that of another, hence a given viral preparation may have a lower efficiency of plating in one system than in the other; for example, there is a 1.2 $\log_{10}$ difference between egg $ID_{50}$ and $TCID_{50}$ in Table 3-5.

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