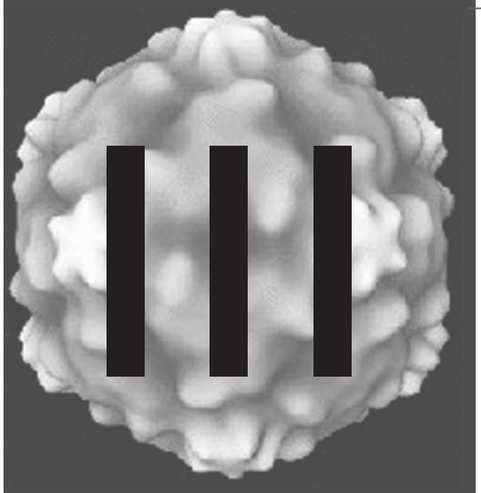
Working With Virus



PART III

- VISUALIZATION AND ENUMERATION OF VIRUS PARTICLES
- REPLICATING AND MEASURING BIOLOGICAL ACTIVITY OF VIRUSES
- THE OUTCOME OF VIRUS INFECTION IN CELLS
- MEASUREMENT OF THE BIOLOGICAL ACTIVITY OF VIRUSES
- PHYSICAL AND CHEMICAL MANIPULATION OF THE STRUCTURAL COMPONENTS OF VIRUSES
- VIRAL STRUCTURAL PROTEINS
- CHARACTERIZING VIRAL GENOMES
- CHARACTERIZATION OF VIRAL PRODUCTS EXPRESSED IN THE INFECTED CELL
- CHARACTERIZATION OF VIRAL PROTEINS IN THE INFECTED CELL
- DETECTING AND CHARACTERIZING VIRAL NUCLEIC ACIDS IN INFECTED CELLS
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- DELIBERATE AND ACCIDENTAL ALTERATIONS IN VIRAL GENOMES AS A RESULT OF LABORATORY REPLICATION
- PROBLEMS FOR PART III
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CHAPTER 9

Visualization and Enumeration of Virus Particles

- Using the electron microscope to study and count viruses
- Atomic force microscopy – a rapid and sensitive method for visualization of viruses and infected cells, potentially in real time
- Indirect methods for "counting" virus particles
- QUESTIONS FOR CHAPTER 9

Most viruses are submicroscopic physical particles, and while the largest can be discerned in an ultraviolet (UV)-light microscope, detailed visualization requires other methods. The development of physical and chemical methods for the study of viral properties and their unique shapes and sizes provides an important impetus for applying these techniques to the study of biological processes in general.

An investigator must know how many virus particles are in a sample, and what the sample's relationship is to the biological properties of the virus (measured in other ways) in order to carry out a meaningful physical study of virus particles.

The ability to count viruses ultimately depends on the ability to see them, and this requires special techniques that were not available until just prior to World War II. Notable among these is the **electron microscope (EM)**, whose design required a sophisticated knowledge of modern particle physics and modern electrical and mechanical engineering. The electron microscope has allowed scientists to see into the cell and biological processes, and much of the progress taken for granted in molecular biology and medicine would have been impossible without it.

Using the electron microscope to study and count viruses

The dimensions of viruses are below the resolving power of visible light, so their visualization requires the shorter wavelengths available with the EM. The EM (schematically shown in Fig. 9.1) accelerates electrons to high energy and magnetically focuses them. High energy gives the electrons a short wavelength, one that is much "smaller" than the virus particles. In fact, the EM can visualize DNA, RNA, and large proteins.

Despite the value of the EM's high resolving power, the energy needed to attain short wavelengths poses a problem. High-speed (short-wavelength) electrons are quite penetrating, and most biological subjects are transparent to them. Thus, in order to visualize viruses, they are generally either *stained* or *coated* with a heavy metal such as platinum or osmium. This coating or staining is done in such a way that the basic arrangements of the proteins and structure of the virus are not destroyed. The particles then are visualized by passing electrons through the specimen and observing it on a fluorescent screen. Areas where electrons do not pass because of the heavy

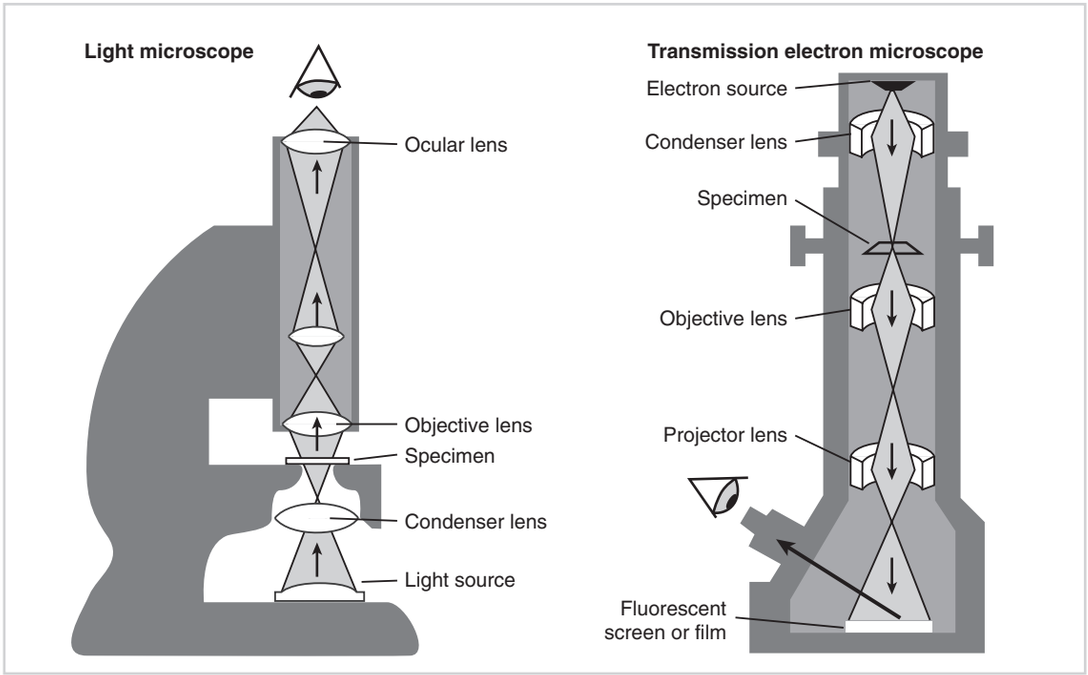

Fig. 9.1 A schematic comparison of light and electron microscopes. The principles behind the focusing of the image are similar except that magnetic fields must be used to focus electrons. The higher energy of the electrons accelerated through high voltage produces very short wavelengths with resulting high resolving power.

metal appear dark on the screen, but appear white (light) in prints because they are photographed in negative.

The physics of electron acceleration and focusing mean that specimens must be observed in a vacuum; therefore, the sample must be completely dry and fixed. For this reason, the EM picture is only a representation of structure because subtle effects of protein hydration on the arrangement of the polypeptide chains, for example, may be altered or lost by preparation for the EM. Further, sample preparation means that the EM cannot visualize objects in motion but only "frozen" in time. The "snapshots" of virus entry, egress, or alterations to the infected cell therefore must be interpreted with caution. One never knows whether the observed virus is biologically functional (able to replicate) or whether the process seen is exactly the one leading to biological effects. This point is important to remember when interpreting the EM views of virus entry into and egress from cells such as those presented in Chapter 6.

The process of "shadowing" a virus particle with heavy metal is shown in Fig. 9.2. Such a shadow-cast can provide exquisite detail of the geometry of the virus. Much early development of shadowing and visualization methods of viruses was carried out by Robley C. Williams at the University of California, Berkeley.

Even richer detail can be obtained with the use of subtle staining procedures where the heavy metal is linked to protein molecules. Other types of shadowing, such as carbon shadowing, can also increase detail. Application of computer image enhancement can provide further striking increases in apparent resolution and resolve features that are obscured in conventional EM. Many examples of such detail can be seen in references cited in the introduction to this book and elsewhere.

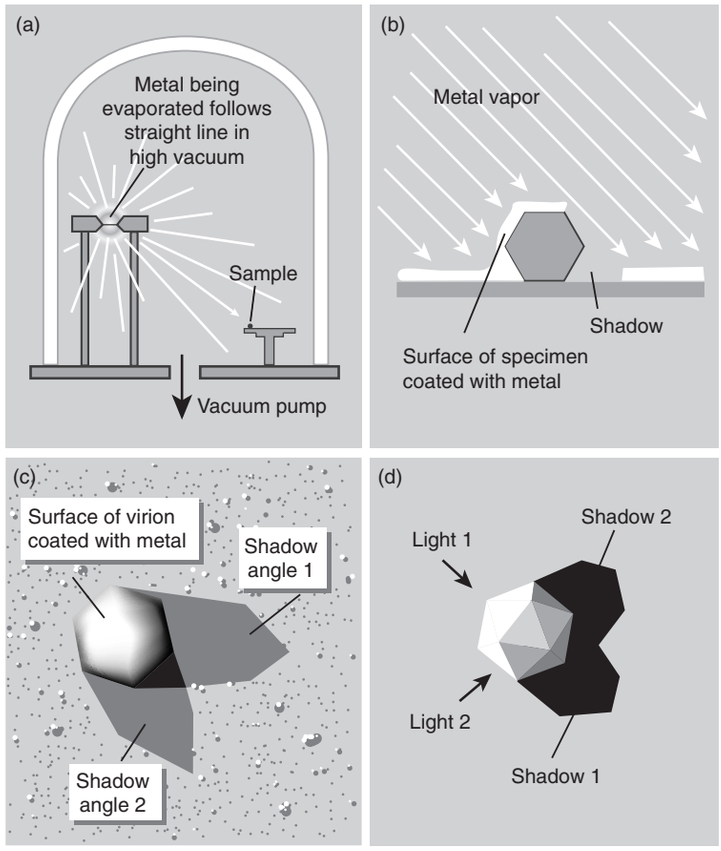

Fig. 9.2 Shadowing specimens for viewing in the electron microscope. *a.* A sample of heavy metal is vaporized in a vacuum chamber. This vapor travels in straight lines from the source and forms a layer on all surfaces in its path. *b.* Any object in the path will cast a shadow on the grid on which it is supported. *c.* A double-shadowed virus in the electron microscope. *d.* An icosahedral model is placed in two light beams to show the equivalence of the shadows. This equivalence occurs because metal particles in vapor travel in straight lines, as does light. (*c* and *d* are drawn from photographs originally made by Robley C. Williams.)

To avoid the problems of structural deformation of particles that result during preparation for conventional EM, especially with enveloped viruses, a technique called **cryo-electron microscopy** was developed. This method employs no stains or heavy-metal shadowing and therefore results in greater preservation of the particles. Instead, the virus particles are rapidly frozen on the EM grid such that they are captured in a thin film of vitreous ice (ice in which large crystals cannot form). Within this glasslike matrix, the particles are hydrated in what may be more like a normal state, as opposed to metal ion—stained and dried specimens of a conventional method. Since no stains are used, the frozen-hydrated particles are imaged by taking advantage of the difference between the electron density of protein or lipid and that of the surrounding water matrix. To prevent unwanted changes, the specimens are viewed in a microscope equipped with a cold stage to maintain the ice structure under vacuum and data are collected at a very low dose of electrons to reduce damage from the intensity of the beam. The images observed can be enhanced by computer methods similar to those applied to the resolution of x-ray diffraction information. The HSV capsid image shown in Fig. 9.3 was produced by these techniques.

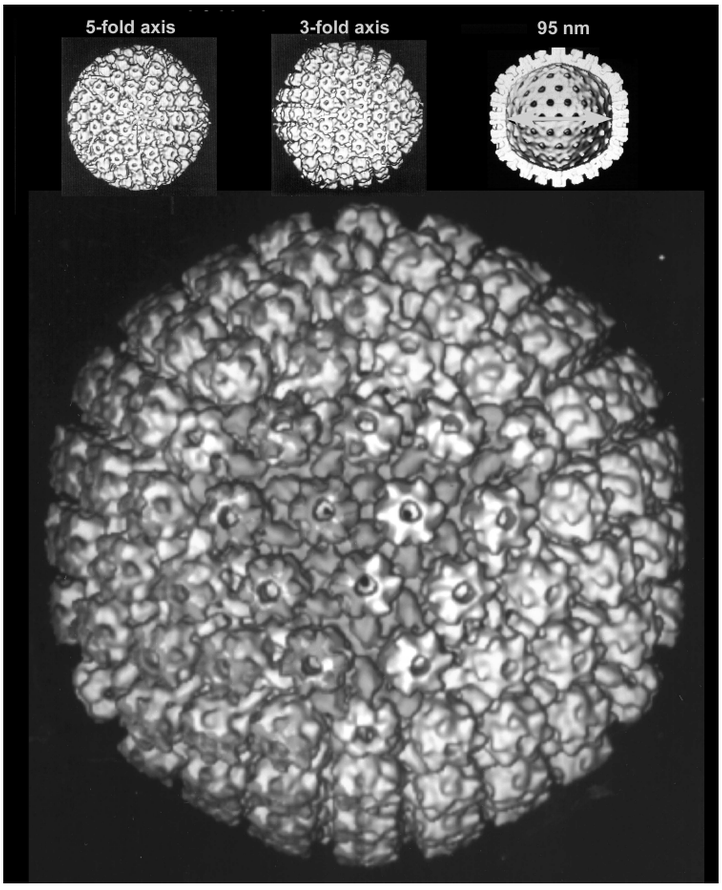

Fig. 9.3 Computer-enhanced three-dimensional reconstruction of the HSV-1 capsid. The reconstruction is computed from electron micrographs of capsids preserved by freezing. For this type of electron microscopy, the samples are frozen and irradiated at liquid N_2 temperature with a very low flux of electrons to minimize damage. Information from many individual micrographs of particles is then combined in the computer to produce a reconstruction with a resolution higher than that of any single micrograph. The reconstructions shown here were from 60 to 80 images, which provides a resolution on the order of 2.5 nm, but as many as 200 or more can be combined. Views showing the three major axes of symmetry and a cross section are shown at top. The bottom figure is a false color rendering of the information. One triangular face of the icosahedral capsid is shown in color. Pentons are orange, hexons red, and triplexes green. VP26, a small protein (molecular weight 12,000) associated with the hexons, is coded in blue. One VP26 molecule is bound to each VP5 molecule in each hexon. No VP26 is present in pentons. More detail concerning herpesvirus capsid structure can be found in Chapter 18. See Plate 4 for color image. (Photographs courtesy of J. C. Brown and James Conway.)

Counting (enumeration) of virions with the electron microscope

Since virus particles can be purified and visualized, they can be counted. Such counting does not tell how many of the particles are infectious (biologically active), but a count of particles in a solution free of contaminating cellular material is very useful. Once the total number of particles is known in a solution, the measurement of total nucleic acid (genomes) allows calculation of the amount of genome per particle, and thus a measure of genome size. Again, particle number can be used to tell the absolute amount of protein per capsid, and this (along with knowing the molar ratios of different capsid proteins determined by methods discussed in Chapter 11) allows one to work out details of the virus structure. Finally, the ability to count virus particles can be very useful for diagnostic and other medical purposes.

All counts require visualization, but once it is known that a certain number of virus particles contains a given amount of enzyme (i.e., reverse transcriptase for a retrovirus), or interacts with a certain number of test red blood cells (hemagglutination), or contains a given amount of DNA or RNA, then measure of these latter parameters can be related to particle number.

Counting of particles is simple in theory. For example, if one could be sure that each EM field contained virus from a specific volume of solution, one could readily calculate particle number. All

that is required is knowledge of the fraction of the original sample being utilized for visualization. This fraction is a function of the volume of the observed sample as well as any dilution steps used in preparing the sample.

For example, if there were 30 particles in an average microscopic field and the volume of solution visualized corresponded to 10^{-4} ml of the original virus suspension, then that original suspension could be estimated to contain 3×10^5 ($30/10^{-4}$) particles. However, this is not a particularly accurate way of measuring particle concentrations. The problem comes from the fact that despite the basic simplicity of the approach, it is difficult to achieve careful dilution and even spread of virus in the field of view of the EM, and many artifacts can arise.

Some uncertainties can be minimized by addition of a known amount of some standard in the original suspension, such as latex beads of uniform size. Then the number of both beads and particles can be counted in the EM field. The ratio of these, and knowledge of the number of beads used to make the solution, allow calculation of the number of particles in the original suspension. Since it is easy to add a known number of beads from a standard solution, the process can be applied to a series of different virus preparations.

Atomic force microscopy – a rapid and sensitive method for visualization of viruses and infected cells, potentially in real time

While the electron microscope can provide three-dimensional structural information, it is merely an averaging technique — that is, highly detailed structures are based upon the entire population of particles observed. This is an inherent limitation of even the highest resolution techniques available for studying molecular structures — **X-ray crystallography**. Further, these methods require extensive sample preparation and fixing, and subtle information regarding the characteristics of the individual particles and structures in a population of viruses as well as dynamic changes can only be inferred by painstaking statistical analysis, and then only with caution. Thus grandly symmetrical, and apparently perfect models of larger viruses derived from X-ray crystallography and cryo-EM may be somewhat deceptive, and not entirely representative of the entire population. Further, as has been discussed in Chapter 6, details about virus — cell interactions are often open to multiple interpretations.

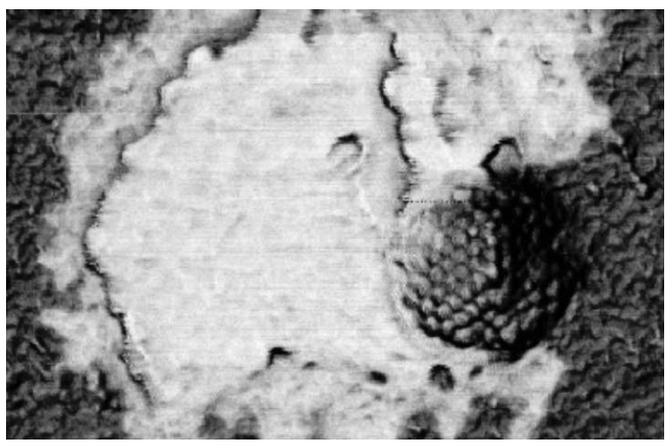

Fig. 9.4 Atomic Force microscopy of a herpes simplex virus capsid.

A rather bizarre feature of molecules interacting at extremely close (quantum scale) distances is that electrons can "tunnel" between atoms producing a small but measurable force between them. This quantum force has been utilized in the technique of **atomic force microscopy (AFM)** where a molecule-scale probe is held at a constant tunneling force over the surface of a cell, sub cellular component, or virus so that as the probe is moved over the sample a "contour map" of the surface can be generated. This method requires little or no sample preparation, and, in theory at least, could be done on living cells to provide animated real time analyses of changes in cellular surface structure as virus infection proceeds. It introduces an effective complement to the techniques above. Most importantly, it can be used to examine the architecture of a single virus particle, or a collection of distinct individuals, and this may be carried out at a resolution very near that of cryo-electron microscopy. This method has been used for imaging capsid structures of viruses in crystals as well as viruses interacting with cells. An example of an atomic force microscopic view of herpes simplex virus capsids is shown in Fig. 9.4 and Plate 5.

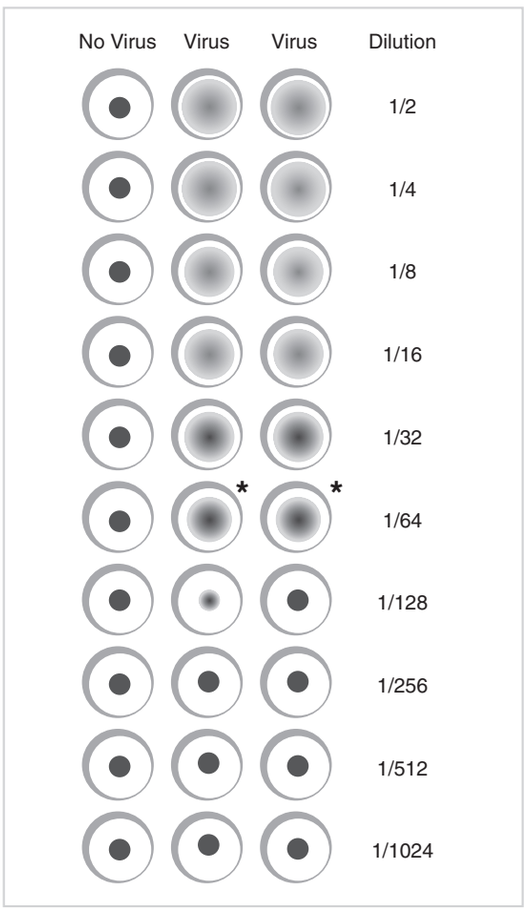

Fig. 9.5 Assay of influenza virus by hemagglutination. The same number of red blood cells was added to each well, and duplicate samples of a virus stock were added to the wells at the indicated dilutions. Two-fold dilution steps are very convenient to handle and require only a small amount of virus sample. The wells in which there is enough virus present to agglutinate red blood cells have a gelatinous suspension of the colored cells. In wells with no virus, or an amount too low to agglutinate the cells, the red blood cells can be pelleted at the well's bottom with low-speed centrifugation. If more virus particles were in the original suspension, more dilution would be required to lower the concentration below the critical level for the hemagglutination measured. This would result in a *higher* HA titer, which is just the dilution factor required to dilute the agglutination. (Based on fig. 2.5 in Dimmock, N.J., and Primrose, S.B. *Introduction to modern virology*, 4th edn. Boston: Blackwell Science, 1994.)

Indirect methods for "counting" virus particles

Once the number of virus particles in standard solution is known, this information can be correlated with other readily measurable properties of the virion. For example, the amount of virus causing agglutination can be related to particle number. As discussed in Chapter 7, many enveloped viruses can agglutinate red blood cells, and this property can be used as a measure of virus particles because it takes a certain number to coat the red blood cells to cause agglutination. Under standard conditions for the assay, the number of influenza virions is about 10^4 virus particles per hemagglutination unit (*HA unit*). An HA unit is just enough virus to cause agglutination of the standard sample. (Actual details of an HA unit definition can be found in many medical laboratory protocols.)

An HA titration of influenza virus is shown in Fig. 9.5. The basic procedure is as follows: Standard samples of red blood cells (guinea pig or chicken red blood cells for influenza virus) are mixed with different dilutions of unknown virus stock, which could be from a patient's serum. After a suitable period of time, the solution is gently shaken and subjected to low-speed centrifugation. If the red blood cells agglutinate, the cells make a jelly-like clump and cannot sediment. Remember from the discussion of hemagglutination inhibition titrations in Chapter 7, that agglutination is characterized by a diffuse red or salmon-pink solution. If the red blood cells do not agglutinate, the cells pellet to form a red "button" at the bottom of the tube. The beauty of hemagglutination is not accuracy; it is speed and ease of operation. This is very important in small clinical laboratories, especially in developing countries.

Similar tests using enzymes can be used to estimate particle number, *but only after one knows how much enzyme is contained in a single particle*. For example, the enzyme reverse transcriptase is found in retrovirus virions, and can be rather readily assayed in the laboratory. The number of enzyme units of reverse transcriptase per virus particle, which is a constant, can be determined just once, and the amount of enzyme recovered in an unknown sample can be used to estimate the number of virus particles using simple arithmetic. Remember, however, all these indirect methods require the ability to count the particles in the first place.

QUESTIONS FOR CHAPTER 9

1 The data in the table below show the results of attempting to infect three different cell lines with La Crosse encephalitis virus (LAC). With electron microscopy, observations were made to detect virus particles on the surface of the cells and virus particles present in endocytotic vesicles (endosomes) inside of the cell. A "+" indicates that the virus was present in the majority (>80%) of the cells observed, whereas "+/–" indicates that the virus was present in only a few (<5%) of the cells observed. In addition, the *average* yield of virus per cell was measured. Using these data, answer the following questions about these cell lines.

	Data for La Crosse Encephalitis Virus		
Cell line	Virus on surface	Virus in endosomes	Virus yield per cell
HeLa	+	+/–	5
CEF	–	–	0
BHK-21	+	+	200

Continued

a Which cell lines are susceptible to infection by LAC? Why?
b Which cell lines appear to be permissive for LAC infection? Why?
c Propose a hypothesis to explain the data for HeLa cells compared to BHK-21 cells. How can you explain the difference in average yield per cell? How would you test your hypothesis?

2 You isolate virus particles and resuspend them in 2 ml of a buffered solution containing a total of 6×10^9 latex beads. After doing laborious and careful dilutions, shadowing, and other things necessary for electron microscopic examination, you view a number of equal fields and determine that you have 3 beads for every 9 virions. What is the approximate number of virions present in each milliliter of your beginning stock solution?

3 What features of the electron microscope make it an excellent tool for examining virus particles?