

## THE ELECTRON MICROSCOPE

The EM is similar in principle to the light microscope except that electrons are used as radiation. The high resolution of the EM derives from the very short wavelength of electrons compared to the much longer wavelength of visible light. Like the light microscope the EM consists of a radiation source, a condenser lens system, a specimen stage, and objective and projector (eyepiece) lenses. The electron source is a white-hot tungsten filament and the emitted electrons are accelerated to form a beam across a potential difference of about 100,000 volts. The lenses are electro-magnetic and are therefore continuously variable, and the system is maintained in a vacuum ( $10^{-5}$  atmospheres) to prevent deflection of electrons by gas molecules. The specimen must be electron opaque and for biological specimens this usually involves staining with a heavy metal. For viruses, negative staining has proved particularly useful; a stain is used (usually phosphotungstic acid) which does not bind to virus particles, so that the particles are seen as electron transparent objects on an electron-dense background.

The EM is an invaluable tool in the study of viruses. Its major uses are :

1. Examination of the structure of virus particles.
2. Examination of virus-infected cells. This allows the intracellular sites of virus assembly to be determined.
3. *Virus Assay*. The EM can be used to count the number of particles per unit volume of liquid. This is achieved simply by mixing an unknown concentration of virus with an equal volume of a known concentration of small particles (usually latex beads). A specimen of the mixture is examined with the EM and the ratio of the virus particles to latex particles is found by counting. The concentration of virus particles can then be calculated.
4. *Identification*. Although examination of a virus with the EM will not usually allow specific identification, the morphology of the particle should allow the virus to be placed within a group, and this may be of great diagnostic importance. A striking example was the use of the EM for distinguishing between smallpox (a poxvirus) and chicken pox (varicella-zoster, a herpesvirus). Clinical differentiation between these diseases was difficult in some instances, but the identification of poxvirus particles or herpes virus particles in the vesicular fluid was diagnostic. The speed of specimen preparation allows diagnosis in minutes rather than days and this was formerly of great importance in the rapid identification and isolation of smallpox cases.
5. *Immune Electronmicroscopy*. The precise identification of a virus often requires the use of serological techniques, and the EM is of use for this purpose. Unknown virus is mixed with antisera of known specificity and the mixture is examined with the EM for the presence of virus-antibody complexes. Secondary antibodies conjugated to electron-dense gold particles are also useful.

## ASSAY OF VIRUSES

### A *As infectious agents*

The plaque assay on a layer of susceptible cells is the most reliable and simple assay method, and it is used whenever possible. The methods discussed in the practical class for assaying T4 and HSV are typical. These methods rely on a suitable host cell for the virus, and growth of the virus in these cells must result in observable cytopathic effects which allow discrete foci of infection to be counted and an infectivity titre calculated in terms of plaque forming units (pfu) per ml. These conditions have not been met for all viruses, and it is sometimes necessary to use a quantal assay in which dilutions of virus are inoculated into animals or eggs and the dilution end point found. The results of this kind of assay are usually expressed in terms of the dose required to infect 50% of the

targets. Thus a preparation of influenza A virus might have a titre of 100 EID<sub>50</sub> per ml (egg infectious dose 50%). This means that if a large number of eggs were inoculated with 0.01 ml., half of these would become infected.

### **B As particles**

Viruses can also be assayed as physical entities rather than infectious agents : they may be counted directly using the EM; in some instances they can be assayed by their ability to agglutinate cells; provided specific sera are available they can be assayed by quantitative serological techniques such as complement fixation.

The relationship between the measurement of particles and the measurement of infectivity will depend on the efficiency of the infectivity assay. The assay of T4 is very efficient, and we find that a preparation which contains 100 particles/ml will have an infectivity titre of about 100 p.f.u./ml when assayed on E. coli by the technique described in the practical class. The infectivity assays for animal viruses are less efficient and we find for fowl plague virus, for example, that very approximately : 1 HA unit = 10<sup>7</sup> particles = 10<sup>6</sup> EID<sub>50</sub> = 10<sup>5</sup> p.f.u.

It is apparent from this that one plaque results from plating about 100 fowl plague virus particles on chick embryo fibroblasts. We should not interpret this to mean that 100 particles are required to form a plaque, or that only 1% of virus particles are infectious. Rather, we suppose that nearly every virus particles has only one chance in 100 of entering a cell and causing an infection. Infectivity measurements, therefore, are not absolute in that they do not detect every infectious particle. Nevertheless, the results of plaque assays are highly reproducible, provided that a standard assay technique is used, and are therefore a meaningful measure of the infectivity.

The concept of the plaque forming unit can be usefully used in the laboratory for experimental purposes:

When infecting tissue culture cells, a researcher can decide whether to infect at a high or low multiplicity of infection (moi); that is, add a relatively large amount of virus for the number of cells or a relatively small amount. This is expressed as "plaque forming units per cell" eg 5pfu/cell - this particular infection would be termed as a relatively high moi infection, whereas 0.01pfu/cell would be considered a low moi infection.

Different species of viruses may produce different numbers of progeny virus from one infected cell. With a correctly designed experiment, it is possible to measure an average of this from a bulk population of infected cells. This is sometimes referred to as "burst size".

## **IDENTIFICATION OF VIRUSES & DIAGNOSIS OF VIRUS DISEASE**

This is a complex area which can be approximately divided into four categories: identification by structural properties, by biological properties, by serological properties and by genome sequence.

**A Structural properties.** Examination of the virus with the EM will serve to place the virus in a particular group, and in combination with clinical symptoms this may be sufficient to make a confident diagnosis (e.g., chicken pox).

**B Biological properties.** This involves the examination of virus growth in animals, eggs or tissue culture cells. The host-range, type of cytopathic effect (CPE) caused, temperature for optimal growth, and the presence of inclusion bodies may all be of importance.

**C Serological properties.** Serological methods provide the most important approach to the identification of viruses. These methods, of course, require a catalogue of specific antisera, and the specificity of these sera determine the accuracy of the tests. A number of serological tests may be used for identification of a virus :

### **Immunofluorescence of infected cells**

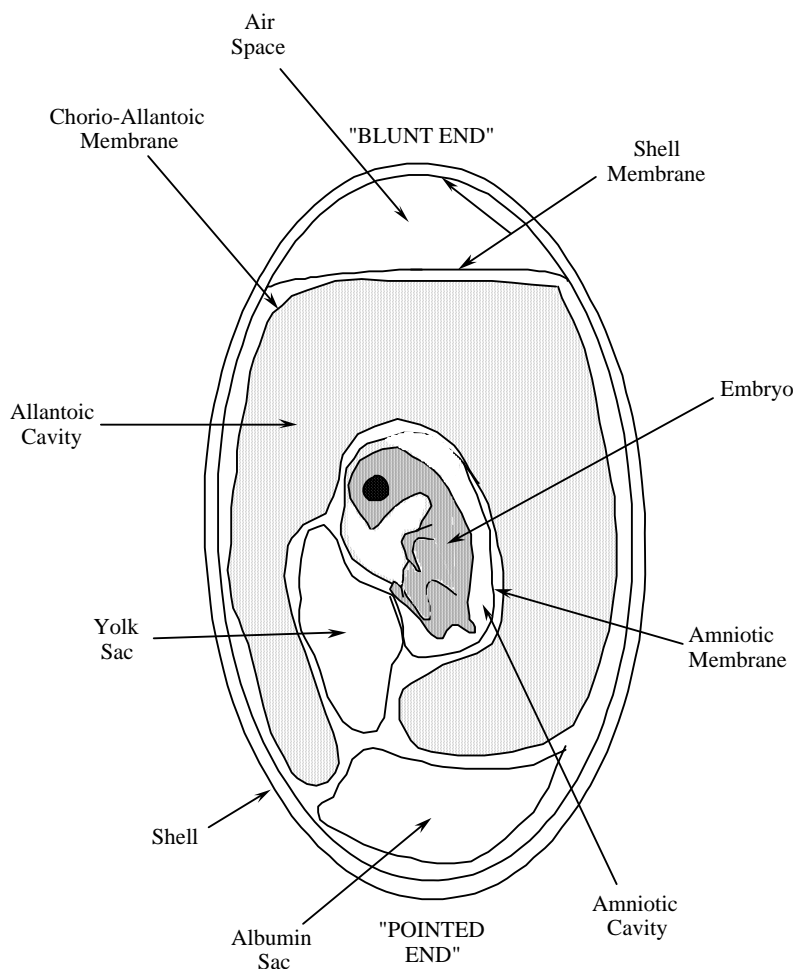
Serological neutralisation of virus infectivity  
 Inhibition of virus-mediated cell agglutination  
 Immuno-electron microscopy  
 ELISA/western blotting

**D Sequence properties.** The advent of PCR-based methodologies has superseded many older diagnostic methods. The presence of a particular virus suspected from clinical symptoms can be tested for by PCR (or reverse transcription-PCR for RNA viruses) amplification of a segment of a virus genome using validated specific primer sets. If an amplicon of the expected size is produced, further useful information (virus strain for instance) can be gained from determining its nucleotide sequence.

The diagnosis of virus disease by the isolation and identification of the virus is frequently impossible because of the difficulty of virus isolation. In this instance we must rely on antibody titre as indication of recent virus infection. Technically the approach to this problem is similar to the serological methods used for virus identification, except that a catalogue of known viruses is required to test unknown sera, and the tests must be quantitative. High antibody titres are indicative of recent infection, but greater confidence may be placed in the diagnosis if increases in antibody titre occur during the course of the disease.

### USE OF THE FERTILE EGG

Although the cultivation and assay of viruses is now usually achieved by the use of tissue culture cells, the membranes of the fertile egg have been widely used for this purpose in the past, and the egg remains a convenient system for the detection and cultivation of some viruses. Indeed, hundreds of millions of influenza vaccine doses are prepared every year by this route. Three parts of the egg are of use (see diagram) :



**A The amniotic cavity:** This cavity surrounds the embryo and is lined by a single layer of epithelial cells. The amniotic fluid bathes the external surface of the embryo and comes into contact with the respiratory and alimentary tracts.

**B The allantoic cavity:** The cavity comprises an outgrowth of the hind-gut of the embryo and is lined with endoderm.

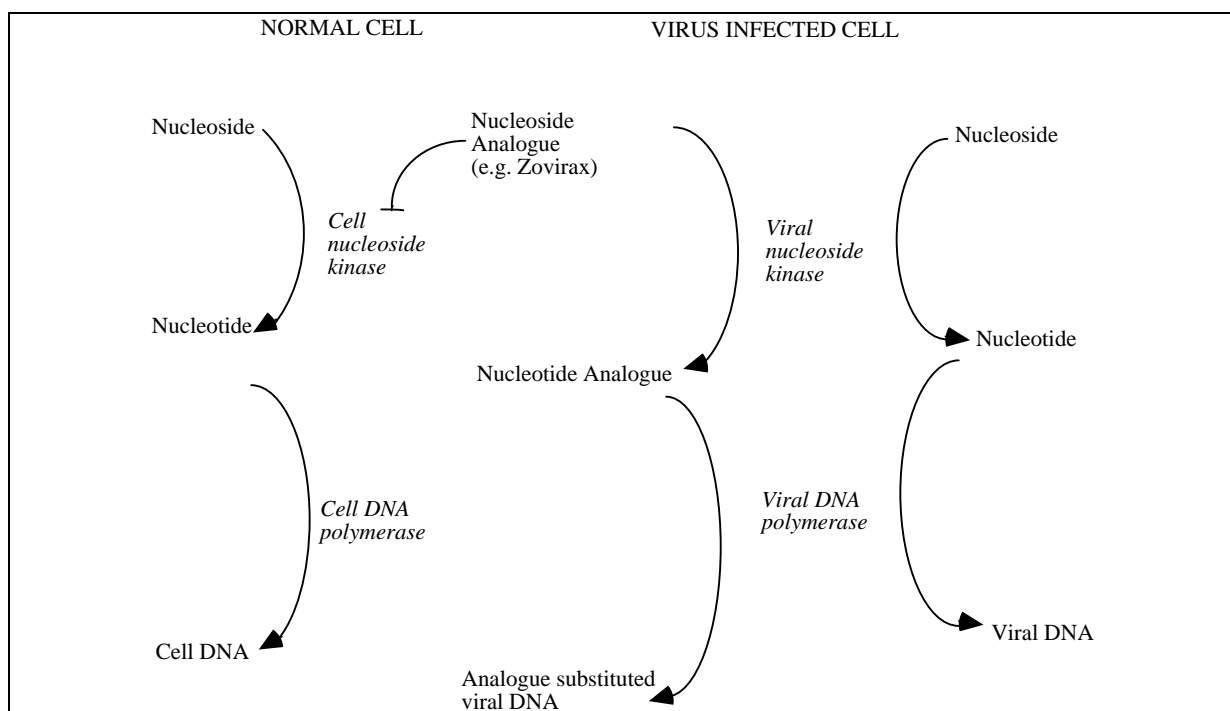
Both of these cavities are useful for the cultivation of viruses, particularly for orthomyxoviruses (e.g., influenza) and paramyxoviruses (e.g., mumps). The membranes which line the cavities are the major source of cells in which virus growth occurs, but the embryo may also become infected. The allantoic cavity is routinely used because it is technically much simpler to inoculate. However, some viruses (e.g., human influenza isolates) may need to be egg-adapted by growth in the amniotic cavity before they will grow efficiently in the allantoic cavity.

**C The chorio-allantoic membrane:** The membrane consists of an outer layer of stratified epithelium which constitutes the respiratory surface of the egg, and an inner layer of endoderm (the lining of the allantoic cavity). The membrane may be used as a cell sheet provided it is first dropped away from the shell membrane. Dermotropic viruses (poxviruses and some herpes viruses) will grow on this membrane, and at low concentrations, will give discrete foci of infection which consist of centres of cell proliferation and necrosis (pocks).

The membrane may therefore be used to assay these viruses. In addition, different viruses cause pocks of different colour and morphology, and this is of diagnostic value for distinguishing between different poxviruses.

## CHEMOTHERAPY

Anti-virus chemotherapy is an area where increasing progress is being made. The difficulty of achieving successful chemotherapy of virus disease reflects the nature of virus replication : the virus is an intracellular parasite which uses the machinery of the host for its replication. Most drugs which interfere with normal functions of the host cell will also interfere with virus growth, but such drugs are, of course, cytotoxic. However, one function, the replication of virus nucleic acid, is often performed by a virus-specified polymerase and not by the polymerases of the host cells. In addition, some viruses specify nucleoside kinases, enzymes that phosphorylate nucleosides to nucleotides, thus increasing the supply of nucleotides for virus nucleic acid synthesis. With this in mind, a rational approach to anti-virus chemotherapy has been to examine a wide range of nucleoside analogues in the hope that some of these analogues will interact with virus enzymes (kinases and polymerases) but not with the equivalent enzyme of the uninfected cell. The rationale of this approach is outlined below.

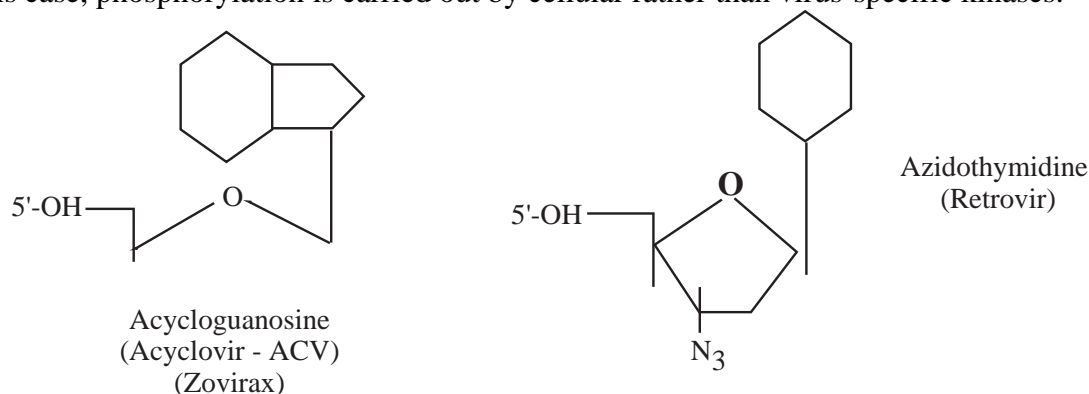


The nucleoside analogue is phosphorylated by the virus kinase to a nucleotide analogue which interacts with the virus polymerase to prevent the production of normal virus nucleic acid. In the uninfected cell

the analogue is not recognised by the cell kinase, does not become phosphorylated and does not, therefore, interfere with cell nucleic acid synthesis. Alternatively, the analogue may become phosphorylated, but will not be recognised by the cell polymerase. An analogue with these properties would fulfil the requirements of a chemotherapeutic agent. It would inhibit virus synthesis but not be cytotoxic.

The drug used in the class practicals, acycloguanosine (Zovirax) acts in this way. Acycloguanosine is an analogue which is licensed for clinical use. It is highly specific : it is not phosphorylated in uninfected cells and is therefore not cytotoxic, and the drug effectively inhibits the growth of a number of herpes viruses both *in vitro* and *in vivo*.

A more recent example of a nucleoside analogue in clinical use against virus infection is the drug azidothymidine (AZT) (Retrovir) which has activity against the human immunodeficiency virus (HIV), the agent of AIDS. The specificity of the drug stems from its inhibitory effect on the virus-encoded reverse transcriptase enzyme. Once again, the molecule has to be phosphorylated before it is active, but in this case, phosphorylation is carried out by cellular rather than virus-specific kinases.



Both compounds lack a 3' hydroxyl group. After addition to the growing DNA chain, a further nucleotide cannot therefore be added. These compounds are **chain terminating**.

This kind of approach to the problems of virus chemotherapy offers considerable hope for the future treatment of virus disease. For instance, given adequate financial resources, HIV infection can now be controlled for many years using a cocktail of specific antivirals directed against different targets within the virus. Incidentally, the success of this approach highlights the importance of fundamental research into the mechanisms of virus replication. Only through such research can we recognise suitable target enzymes for chemotherapeutic attack.

## PRACTICAL CLASS EXERCISES

**DURING THE COURSE OF THE PRACTICALS**, you should have a look at the following questions and satisfy yourself that you understand them. If you have any difficulties, the Demonstrators will happily go over them with you.

1. What is a plaque?
2. Do you understand the term plaque forming unit (pfu)?
3. Do you know how pfu's are related to virus particle numbers? How could the EM be used to examine this?
4. Do you understand the infectious centre assay? What is the eclipse phase?
5. Do you understand the terms cell monolayer and cytopathic effect (cpe)?
6. In the first practical, assay and serological identification of animal viruses, the anti-virus antibodies were added to the viruses prior to infection of the cell monolayers. However, in the acyclovir (ACV) experiment, the monolayers were infected with the viruses prior to exposure of the cells to ACV. Why was this?

7. Why is ACV such a "good" drug?
8. Do you understand the influenza haemagglutination (HA) and influenza haemagglutination inhibition (HAI) assays?  
**Remember**, in these assays, it is the **influenza virus haemagglutinin molecule** on the virion surface which is responsible for agglutinating the red blood cells.
9. A virus was isolated from a nasal swab of a patient with 'flu like symptoms and cultured in a 12-day old embryonated hen's egg for 48 hours. After this time, the allantoic fluid was harvested, and a series of two-fold dilutions made in phosphate-buffered saline. The dilutions were then tested for their ability to agglutinate red blood cells. The highest dilution at which haemagglutination was seen was 1:128.

What was the titre of the virus (in HA units per ml) recovered in the allantoic fluid (assuming that each well contained 0.2ml before addition of red cells)?

In a separate experiment, the allantoic fluid was diluted 1:100 and 1ml of this dilution mixed with 1ml of a suspension of latex beads (concentration  $10^7$  beads/ml). The mixture was examined in the EM. The ratio of influenza particles to latex particles was found to be 6:1. Calculate how many influenza virus particles are present in one HA unit.