Original Papers

National Institute of Public Health, Postuttak, Oslo, Norway

Application of Electron Microscopy in Viral Diagnosis

ELISABETH KJELDSBERG

Introduction

The development of the first electron microscope in the 1930's and the subsequent elaboration of preparation techniques for examining biological material in the 1940's made visualization of virus possible. Until then nothing was known about the morphology of virus particles, because of their extremely small size (20–500 nm in diameter). Most of them are below the detection limit of the light microscope, and their size was approximately determined through filtration experiments. It is the electron microscope with its high resolving power that has revealed the great variety of virus morphology.

The first report on use of the electron microscope in diagnostic virology came in 1948 when virus particles were demonstrated by shadow casting technique in clinical specimens from patients affected with smallpox, vaccinia and varicella (Nagler and Rake, 1948; van Rooyen and Scott, 1948). However, the preparation techniques at that time were cumbersome and time-consuming, and it was not until the introduction of the negative staining technique (Brenner and Horne, 1959), that the electron microscope became an instrument of practical value in virology.

Since then detection of a variety of viruses by EM in clinical specimens from infected humans has been reported. Poxvirus and herpesvirus were demonstrated in skin lesions (Peters et al., 1962; Nagington, 1964; Cruickshank et al., 1966), paramyxovirus in nasopharyngeal secretions (Doane et al., 1967, 1969; Joncas, 1969), hepatitis-B-antigen in serum (Bayer et al., 1968), rotavirus and adenovirus in faecal samples (Bishop et al., 1974; Flewett et al., 1973, 1974) and several more. But only quite recently, however, has the microscope been recognized as an important tool in rapid diagnosis of viral infections (Banatvala et al., 1975; Doane and Anderson, 1977).

The advantages of the electron microscopical techniques compared to other methods used in diagnostic virology are obvious. Speed is essential in cases of smallpox and herpes encephalitis, where the handling of the epidemiological situation and the treatment and prognosis depend on a correct and rapid diagnosis. And electron microscopy is a rapid method. By negative staining and electron microscopy preparation, examination and identification of a virus may be done in minutes or hours. By standard virological methods, primarily isolation of virus in cell cultures, eggs or animals, it may take days or weeks before the answer is available. But also in combination with inoculation of virus samples in cell cultures electron microscopy may be useful. If the cytopathic effect on the cells is not typical, a rapid demonstration of characteristic virus particles will save time and reagents (Doane et al., 1969; Pennington et al., 1975).

Another advantage of electron microscopy is its "lack" of specificity. Newer laboratory routines in diagnostic virology like immunofluorescence technique (FAT), radioimmunoassay (RIA) and enzymelinked immunoadsorbent assay (ELISA) are rapid and sensitive methods, but they require specific antibody and permit only demonstration of one particular virus or virus group at a time. The electron microscope visualizes any virus particle present, and there is no need to have any previous knowledge of what group it belongs to.

Finally the electron microscope is of decisive value when dealing with viruses that are difficult or impossible to culture, either because they do not grow in routine cell cultures at all or because they have lost their infectivity during transportation. The morphology is often stable even if the infectivity is lost. A number of such viruses have been demonstrated for the first time by EM, for example viruses associated with gastroenteritis (Flewett et al., 1973; Bishop et al., 1974; Kapikian et al., 1972; Paver et al., 1973; Madeley and Cosgrove, 1975), and hepatitis (Bayer et al., 1968; Feinstone et al., 1973).

Direct electron microscopy only places the virus in its morphological group, which, however, in some cases may be sufficient. Further serological typing of the virus requires immuno electron microscopy (IEM) or other serological methods.

This report reviews the most important electron microscopical techniques used in diagnostic virology to day and describes the main morphological characteristics of the various virus groups, which are most likely to be found in different clinical specimens.

Preparation Techniques

The first available preparatory technique was the shadow casting technique, which nowadays is practically never used in diagnostic virology. Ultrathin

sectioning was the second preparatory technique developed for electron microscopy. This technique is extensively used in cell biology, although rarely in viral diagnosis. Most work is done with the negative staining technique, which is far more simple and gives more information about virus morphology than the other two.

Negative Staining

Staining technique

This technique is mostly used for examination of particles in suspension, but it has been applied to thin sections, cells and cell-organells (Parsons, 1963). Any biological object, in this case a virus particle, is a poor electron scatterer. The negative stain surrounds the object with an amorphous, high electron scattering coating, which enhances the contrast. The virus particle appears white or "negative" against a dark background. The electron scattering material penetrates to a certain degree the particle and reveals details of the surface and in some cases the internal structure.

Suitable electron stains are solutions of heavy metal compounds such as phosphotungstic acid, sodiumsilicotungstate, ammoniummolybdate and uranyl salts. The appearance of the object may vary considerably with different stains and with the same stain at different pH-values (Müller, 1975). Ammoniummolybdate for example has a preserving effect on membranes, whereas uranylformate stabilizes lipid containing viruses by binding to phospholipids. As an acid solution reveals the finest surface detail, while an alkaline solution visualizes inner structures, an unknown object ought to be examined with different stains at different pH-values in order to find the optimal conditions for this particular particle. In virus diagnostic work, however, this approach would be much too time-consuming, and since most viruses are effectively stained with a 2–3% solution of phosphotungsticacid (PTA) adjusted with KOH to pH 7, this may be choosen as an allround stain.

The staining procedure itself may be performed in several ways (Nermuth, 1975; Johansen, 1978). One simple method is to mix equal volumes of the virus suspension and the stain. One drop of the mixture is placed on a formvar-carboncoated copper grid. After adsorbtion of the virus particles to the grid for one minute excess fluid is removed with a filterpaper, and the grid is airdried and examined in the microscope. In searching for unknown agents a magnification of 50,000 X is suitable, as this enlargement covers the whole size range of viruses.

Specimen grids with thin carbon support instead of the usual formvar-carbon film may be recommended in special cases, as the contrast in the virus structure is enhanced (Johansen, 1976). This may be important in distin-

guishing small spherical viruses with different surface structure (Kjeldsberg, 1977). Unfortunately it seems to be less stable than the formvar-carbon support and thus not quite suitable for routine use.

Preparation of specimens

If an EM examination should be successful, the specimen must fulfil certain conditions.

One of these conditions is that the concentration of virus particles is high. If a large and characteristic particle like the poxvirus is present, the concentration must be at least 10^7 particles pr ml. However, the minimum concentration depends on the size and shape of the virus. Small and anonymous particles like enteroviruses require as high concentrations as 10^9–10^{10} particles pr ml to be discovered.

Another requirement for a successful result is that the salt content is low. When drying on the specimen grid the salts crystallize, and if there is too much salt present, this will obliterate the virus particles during examination.

Finally the specimen should contain as little extraneous material as possible. It may be very hard to spot an occasional virus particle among other circular or oval objects of virus size but of non-viral nature. In highly crude samples therefore, the requirement of a high concentration of virus is of importance.

Vesicular- and pustular fluids, scabs, nasopharyngeal secretions, biopsy- and autopsy tissue are clinical specimens that often contain a large amount of morphologically recognizable virus particles and may be examined directly by EM without prior concentration. Smears of vesicular- and pustular fluids are resuspended in a few drops of distilled water before staining. Scabs are homogenized in a Dounce homogenizer in a small volume of distilled water and the homogenate stained. Nasopharyngeal secretions are stained directly. Specimens collected on cotton swabs may be resuspended in distilled water and stained without prior treatment. Biopsy- and autopsy tissue are cut into small pieces (1 mm^3) and frozen and thawn a few times on a metal planchet on dry ice in order to rupture the tissue and release the virus particles. A few drops of distilled water are added and the mixture is suspended and stained.

Most fluid specimens, however, contain a considerable amount of salt and the result may be improved by using the "water-drop" method to avoid this problem (Doane et al., 1969). A drop of the specimen is placed on a drop of distilled water on a wax plate, and a grid touched quickly to the surface of the drop before negative staining.

Other types of clinical specimens usually contain too small a number of viruses to be examined directly and have to be concentrated by centrifugation prior to negative staining. These include faeces, spinal fluid, urine, serum and

throat- and eyewashings. Faecal samples are suspended in phosphate buffered saline (PBS) 10–20% v/v and then concentrated. All specimens are clarified by light centrifugation 30 minutes at 3000 g to remove bacteria and cell debris. The supernatant is recentrifuged for 90 minutes at 50,000 g to deposit virus. The pellet is drained off, resuspended in a few drops of distilled water and negatively stained. By this treatment the virus suspension is concentrated, partly purified and freed from disturbing content of salt.

Treatment of cell culture specimens for EM, whether it concerns cells inoculated with virus or control cultures, is often actual and may be done as follows. Cells from culture tubes are scraped off the tube into the medium, and the cell suspension is centrifuged for 90 minutes at 50,000 g. The pellet is transferred to a metal planchet and freeze-thawed a few times to rupture all cells and release virus. A drop of distilled water is added and the suspension is negatively stained and examined in the microscope.

Thin Sectioning

Ultrathin sectioning technique combined with positive staining may be used for demonstration of intracellular virus particles in biopsy- and autopsy tissue and in cell cultures. The technique involves ultramicrotomy, and to be able to be cut into ultrathin sections (approx. 50 nm thick) 1 mm³ pieces of tissue must be fixed and embedded in a polymerizable resin. Glutaraldehyde or glutaraldehyde-formaldehyde mixtures followed by osmiumtetroxide are the most commonly used fixatives. Epoxy-type resins polymerizable at a high temperature are often used for embedding. Ultrathin sections are cut on an ultramicrotome, collected on grids and positively stained. Heavy metal compounds as uranylacetate and lead citrate, usually in combination, are suitable stains in positive staining, where the salt solution directly interacts with molecules in the tissue; and the object appears dark on a light background (Sevéus and Johannessen, 1978).

Biopsy tissue, which has been formalin fixed and paraffin embedded for light microscopy, may be used for thin section EM (Johannessen, 1977; Johannessen and Bøhler, 1978). In diagnostic virology, where the aim is demonstration of the presens of virus particles, this technique may give valuable information, as the virus particles are amazingly well preserved even under less ideal conditions.

However, in spite of recently published rapid embedding methods (Johannessen, 1973; Doane et al., 1974), this technique is complicated and fairly lengthy compaired to negative staining. As it gives less information of the ultrastructure of the virus, it is not routinely used in viral diagnosis.

Immune Electron Microscopy (IEM)

In IEM specific antibody is added and allowed to react with virus antigens in an immunological reaction, and the product is visualized in the electron microscope (Almeida and Waterson, 1969). The technique may be applied to either virus particles in suspension using negative staining, or to intracellular virus in tissues or cell cultures by thin sectioning.

When specific antibody is added to a specimen containing virus particles, the antibody molecules will adhere to and aggregate the particles. The appearance depends on the relative amounts of antigen and antibody. Virus and antibody, present in appropriate proportions, form large aggregates of virus particles linked by antibody molecules. Excess antigen or antibody cause a low degree of aggregation. When the aggregation is prevented by too much antibody the virus particles are covered with a thick fuzz of antibody molecules. With unknown concentrations of antigen and antibody, therefore, series of dilutions of the one must be put up against series of dilutions of the other to get satisfactory results.

IEM is a useful method by examination of crude specimens with a low concentration of virus, especially when the virus particles are small and without a distinctive morphology. Aggregates are easier to detect among other disturbing material than single particles. Some of the recently detected gastroenterit viruses like Norwalk agent and astrovirus were demonstrated in faeces by IEM (Kapikian et al., 1972; Madeley and Cosgrove, 1975). Hepatitis A virus was found in faeces from patients with infectious hepatitis also by using the patients reconvalescent serum in the IEM method (Kapikian et al., 1976).

Utilizing the specificity of immunoreactions, further typing of virus within a morphological group is possible by IEM directly on the specimen grid. This has been reported for enteroviruses (Doane, 1974; Anderson and Doane, 1973) and myxoviruses (Kelen and McLoed, 1974), but the method may of course be applied to any system.

In practice one volume of the specimen may be mixed with one volume of antiserum and the mixture incubated 1 hour at room temperature or overnight at + 4 °C to allow the antigen/antibody reaction to take place. The antiserum used in the reaction should be inactivated for 30 min at 56 °C to avoid complications arising from presence of complement, and ultracentrifuged for 1 hour at 100,000 g. Furthermore, the antiserum ought to be used in a dilution of at least $\frac{1}{10}$ (rather $\frac{1}{50}$) to avoid cross reaction. After an appropriate time of incubation the aggregates may be concentrated either by centrifugation for 1 hour at 50,000 g, or by an agar-diffusion technique (Doane, 1974) and finally negatively stained.

IEM on thin sections should be mentioned as a possibility in special cases, not as a routine technique. Virus antigens may be localized intracellular or on cell surfaces by use of antibody coupled to electron dense marker systems like ferritin or peroxidase (Howe et al., 1974; Dmochowski et al., 1974).

Virus Morphology

In spite of the great variety in sizes and shapes viruses may be divided into three main geometrical groups and each main group further into several subgroups on the basis of their distinctive morphology and symmetry (Madeley, 1972; Horne, 1974). By direct EM a virus may be placed in one of these morphological groups. The three main groups comprise virions with a) cubic (icosahedral) symmetry, b) helical symmetry and c) complex structure. The symmetry relates to the construction of the nucleocapsid, which is the combined nucleicacid and protein covering of the virus. Nucleocapsids may be enclosed in a cell derived envelope or they may be naked.

The group *icosahedral* viruses comprises a large number of spherical-shaped viruses with a diameter ranging from 20 to 100 nm. The central core of nucleicacid is surrounded by a protein shell possessing icosahedral symmetry and assembled from aggregates of identical proteins (capsomeres). These capsomeres may appear as hollow tubes, holes or spherical units, and each morphological subgroup has a distinct number of capsomeres. Adenovirus (Fig. 6), for example has 252. Other examples of cubic viruses are herpesvirus (Fig. 3a, b and c), papovavirus (Fig. 4), and enterovirus (Fig. 7).

Viruses with *helical* symmetry consist of a helical nucleocapsid, more or less randomly coiled, and by animal and human viruses always enclosed in an envelope. By some of these viruses the envelope is easily ruptured, releasing the inner helical structure. They are highly pleomorphic structures varying in both size and shape (80–500 nm) and the envelope is covered with spike-like projections placed at regular spacings and easily distinguished. The most important subgroups are orthomyxovirus and paramyxovirus, the former including influenzavirus (Fig. 11), the latter mumpsvirus (Fig. 12a and b), parainfluenza- and measlesvirus. Quite recently coronaviruses (Fig. 16) have been demonstrated to contain ribonucleoprotein helices, which probably place them in this same group (Davies et al., 1978).

The third geometrical main group includes viruses with a complex structure. Their protein shell is assembled according to a mixture of symmetrical plans. Typical examples are the vaccinia- and paravaccinia subgroups, considered under the heading of poxviruses. They are large particles approximately 250 nm long, and both subgroups appear in two different morphological forms (Fig. 1 and 2).

Because of the distinct morphological differences between the subgroups, herpesvirus, for example, may easily be distinguished from vacciniavirus or enterovirus. On the other hand, differentiation between viruses within a morphological group is impossible by direct EM. All members of the herpesvirus group are morphologically indistinguishable from each other.

Viruses in Clinical Specimens and Cell Cultures

Skin lesions

Clinical specimens from skin lesions often provide excellent sources for direct examination by EM because of their high content of virus. Poxvirus and herpesvirus are easily detected in vesicle fluid and crusts from patients with smallpox and chickenpox, and the differentiation between these two virus groups has been of great importance in diagnostic virology (Peters et al., 1962; Nagington, 1964; Cruickshank et al., 1966). Orfvirus, the etiological agent of ectyma contagiosum, and molluscum contagiosum are without any difficulties demonstrated in skin tumors (Macrae et al., 1969; Johannessen et al., 1975), and papovavirus in common warts and genital warts (Williams et al., 1961; Oriel and Almeida, 1970). Figures 1–4 show some examples of the virus groups, one may expect to find in specimens from the skin.


Fig. 1–4. Negatively stained (phosphotungstic acid) virus particles usually found in skin lesions. Horizontal bar, 200 nm.

Fig. 1. Vacciniavirus in a secondary pustel on the cheek of a vaccinia vaccinated person. Two morphological forms, both brickshaped.

Fig. 2. Orfvirus in a tumor-like growth on a finger. Note the bacillus shape and the quite regular spiral of surface threads on one of the two morphological forms.

Fig. 3. Herpesviruses from vesicles. a) Two virus particles with intact envelopes (E). b) Particle with ruptured envelope (E) and the nucleocapsid partly penetrated with stain showing the capsomeres (C). c) Completely released nucleocapsids with clearly visible capsomeres on the surface. Note the castellated edge on the apparently empty particle (C).

Fig. 4. Papovaviruses. Examples of the morphological group, to which wartvirus belongs.
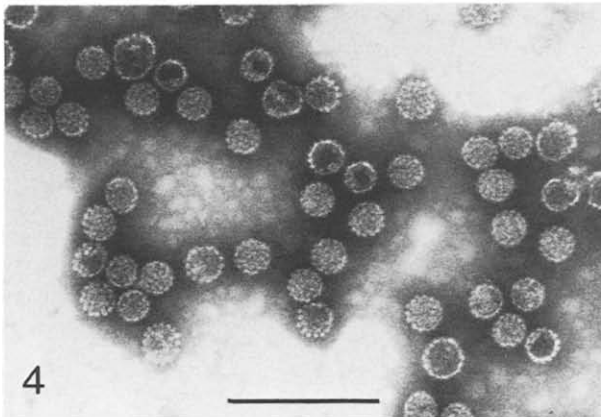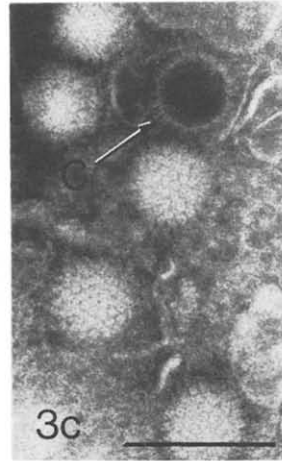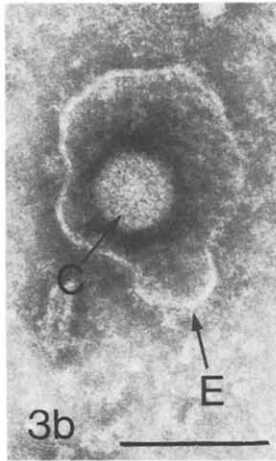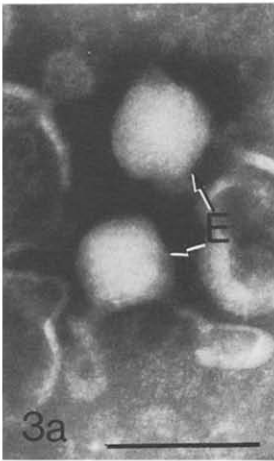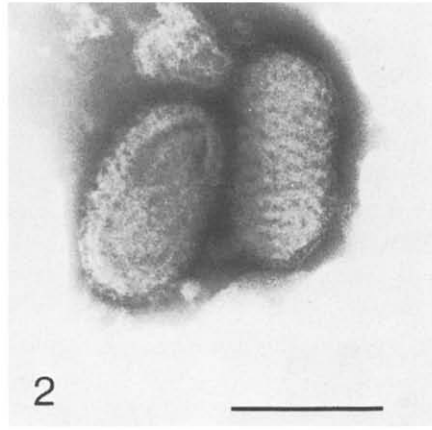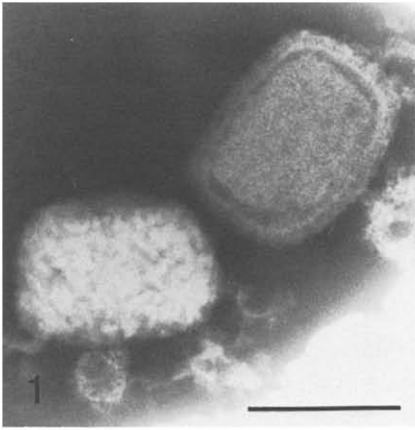
Faeces

Faecal samples, too, are suitable for EM, but usually not until after centrifugation. Recently a wide variety of viruses have been found in negatively stained faecal specimens and most of them in association with nonbacterial gastroenteritis. Rotavirus (Flewett et al., 1973), Norwalk agent (Kapikian et al., 1972), adenovirus and enterovirus (Flewett et al., 1974), hepatitis A virus (Feinstone et al., 1973), astrovirus and calicivirus (Madeley and Cosgrove, 1975, 1976), coronavirus (Caul et al., 1975) and some "viruslike" particles (Paver et al., 1973; Mathan et al., 1975) have been demonstrated by negative staining and EM of faecal samples. In case of gastroenteritis rotavirus is far the most common followed be adenovirus, while the others are more or less rare. Examples of gastroenterit viruses are shown in figures 5–10.

Respiratory tract specimens

From patients with respiratory illness samples drawn from the nasopharynx are usable in demonstration of viruses. Orthomyxo- and paramyxoviruses are the most common (Doane et al., 1967; Joncas et al., 1969). But coronavirus are found to be responsible for respiratory infections (Almeida and Tyrrell, 1967) and the virus particles have been demonstrated directly by EM in nasal secretions from acute upper respiratory tract infections as referred by Doane and Anderson (1977). Orthomyxo-, paramyxo- and coronavirus are illustrated in figures 11, 12a, 12b and 16.

Figures 5–10. Virus particles in faecal samples from patients with gastroenteritis. Negatively stained with PTA. Horizontal bar, 100 nm.

Fig. 5. Rotaviruses usually appearing in two morphological forms. Double shelled particles with a sharply defined outline (D) and single shelled particles lacking the outer layer (S).

Fig. 6. Adenoviruses. The triangular sides on the icosahedron may be discerned on some of the particles (arrow).

Fig. 7. Enteroviruses appearing as featureless spheres but with a very constant size and a sharp outline (arrows). One "empty" stain penetrated particle is visible (arrowhead).

Fig. 8. Caliciviruses showing holes on the surface and a characteristic "star of David" configuration in a certain orientation of the particle.

Fig. 9. Astroviruses with a distinct outline and a 5-pointed star visible on the surface of some of the particles.

Fig. 10. Norwalk-agent-like particles with a capsomere-like surface structure.
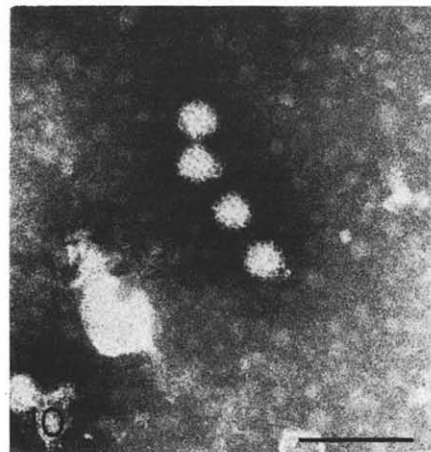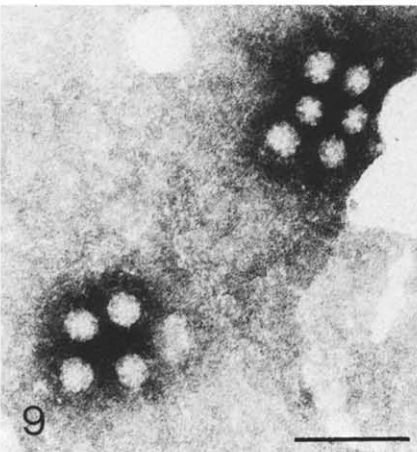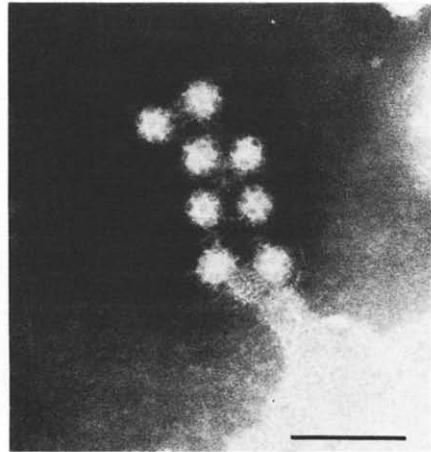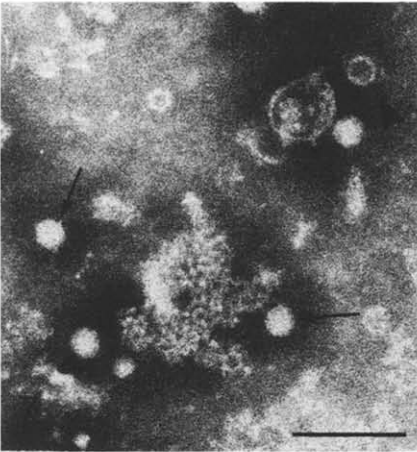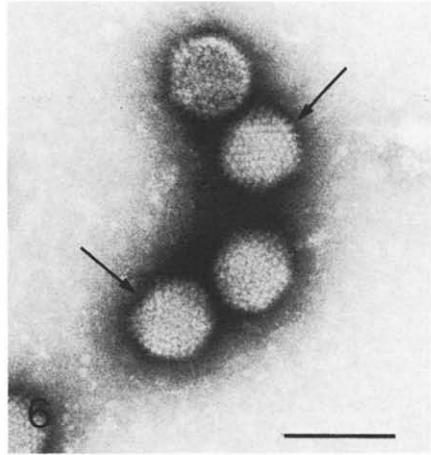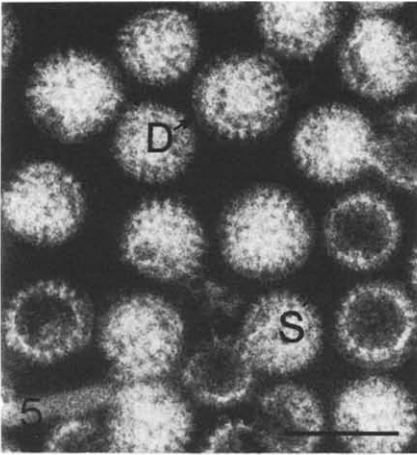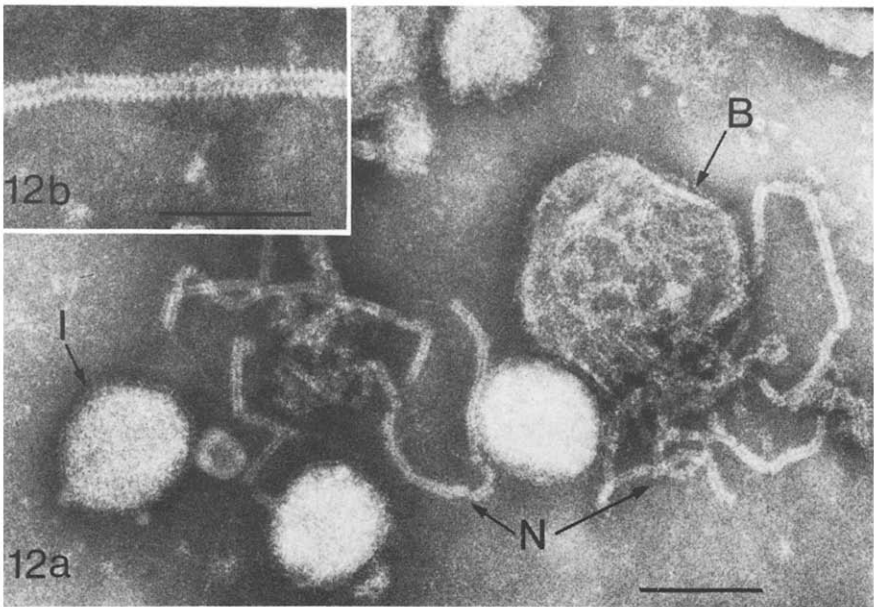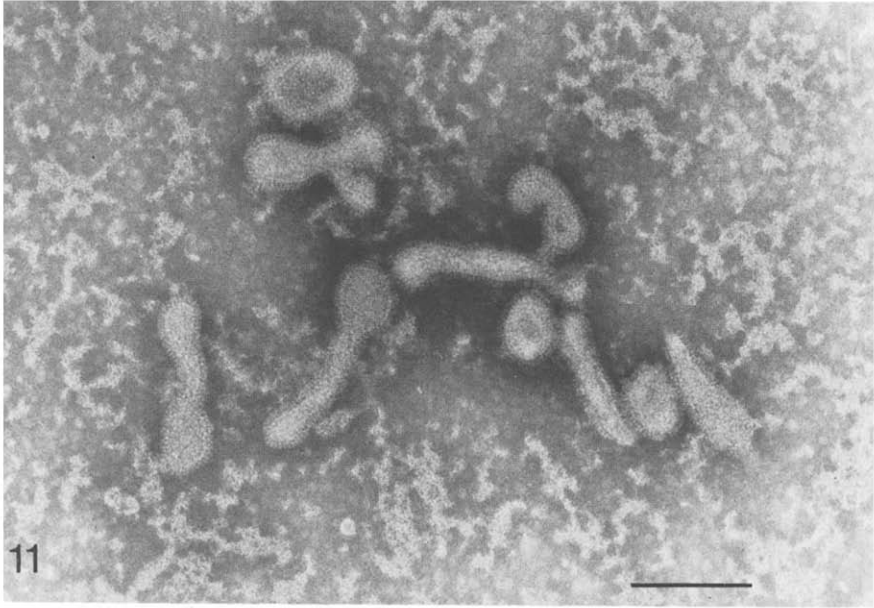
Fig. 11.   Orthomyxo viruses (influenza). Pleomorphic forms with clearly visible surface spikes. Negative staining with PTA. Bar, 200 nm.

Fig. 12a.   Paramyxo viruses (mumps). Virus particles with intact envelope covered with surface spikes (I). Broken particle (B) penetrated by stain and releasing the inner helical nucleocapsid (N). Negative staining with PTA. Bar, 200 nm.

Fig. 12b.   Inset. Helical nucleocapsid at a higher magnification showing the heerıng bone pattern. Bar, 100 nm.
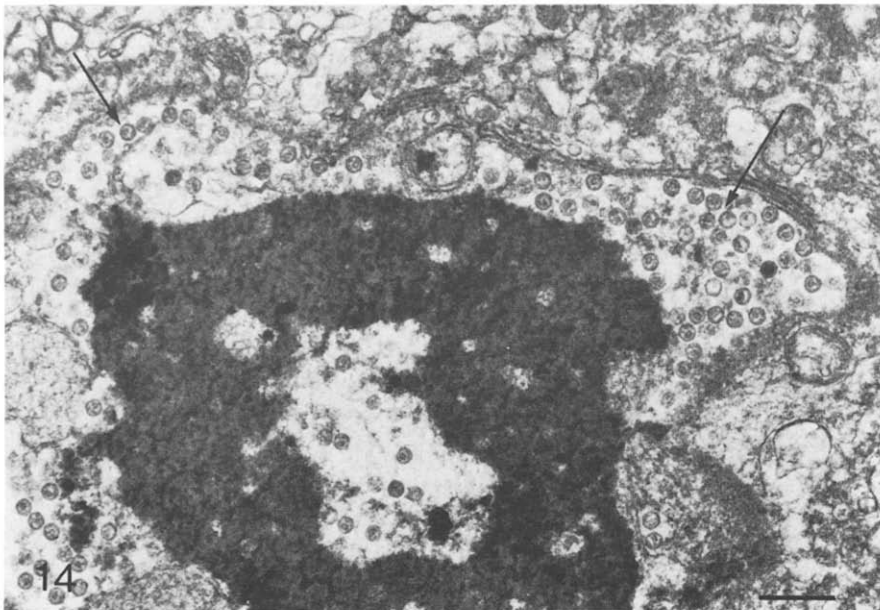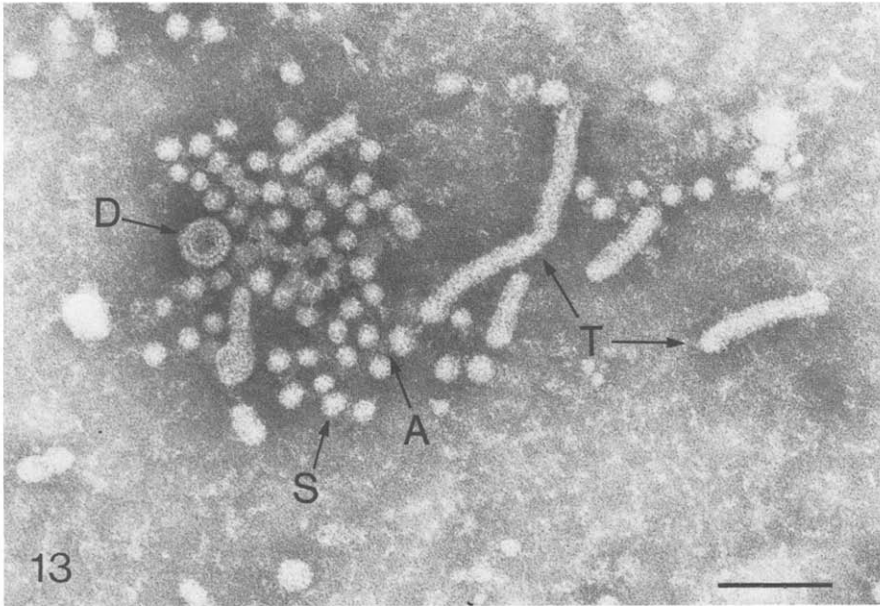
Fig. 13.   Hepatitis B antigens from a patient with serumhepatitis. The antigens are aggregated by antibody. 20 nm spherical particles (S), tubular forms (T) and a Dane particle (D) are seen. Antibody molecules (A) may be discerned between the antigen particles. Negatively stained with PTA. Bar, 100 nm.

Fig. 14.   Thin section of a brain biopsy from a patient with herpes encephalitis. Positively stained with uranyl acetate and lead citrate. Herpes nucleocapsids are seen in the nucleus of the cell (arrows). Bar, 500 nm.

Cerebrospinal fluid

Cerebrospinal fluid (CSF) rarely contains sufficient quantities of virus to be examined by direct EM. However, herpesviruses have been detected in CSF from a patient with herpes zoster (Evans and Melnick, 1949) and mumpsvirus from a patient with encephalitis (Doane et al., 1967).

Urine

Direct EM of urine is usually of no use. However, cytomegalovirus has been found by centrifugation of a large volume, 30–50 ml, of urine and negative staining of the concentrated virus (Montplaisir et al., 1972), and papovavirus has been demonstrated in urine from renal-transplanted patients (Gardner et al., 1971).

Blood

Demonstration of hepatitis B antigens in serum was earlier to a great extent done by immune electron microscopy. This technique is still used in hepatitis diagnosis, as a supplement to RIA, for detection of Dane particles, the infectious agent of serumhepatitis (Dane et al., 1970) Fig. 13. Demonstration of small spherical parvovirus-like particles in serum from healthy blooddonors and from patients was reported by Cossart et al. (1975), but their pathogenicity is unknown. However, it appears that serum is not very suitable for direct EM detection of viruses.

Biopsy- and autopsy tissue

Biopsy- or autopsy tissue may be treated either by negative staining technique or by thin sectioning as mentioned above. Thin sectioning is usually the most reliable especially with brain biopsy because of the great content of lipid. Herpesviruses are demonstrated in brain tissue from herpetic encephalitis (Harland et al., 1967), papovavirus from progressive multifocal leukoencephalopathy (zu Rhein and Chou, 1965) and measlesvirus-like structures from subacute sclerosing panencephalitis (Tellez-Nagel and Harter, 1966). Rotavirus was demonstrated in duodenal mucosa (Bishop et al., 1973) and hepatitis B virus in liver biopsy (Huang, 1971). Herpesviruses in a brain biopsy are seen in figure 14.

Cell cultures

Isolation of virus in cell cultures may at times be rather troublesome. If the infection produces an atypical cytopathic effect the virus isolate may however be identified by electron microscopy (Doane et al., 1969; Pennington et al.,
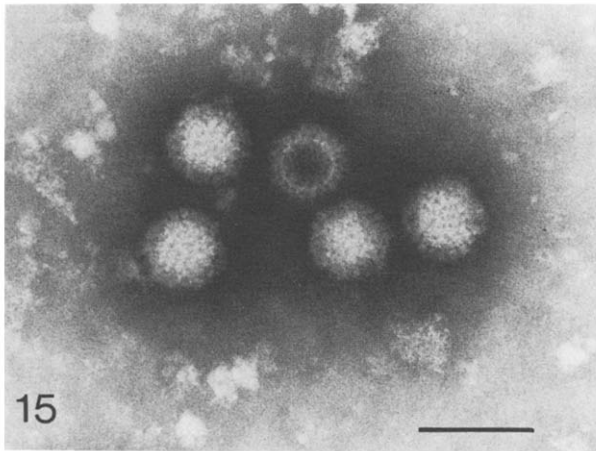
Fig. 15. Reoviruses demonstrated in a cell culture inoculated with faeces from a patient with periodically fever. The virus particles are morphologically similar to rotavirus but without a clear outline. Negatively stained with PTA. Bar, 100 nm.
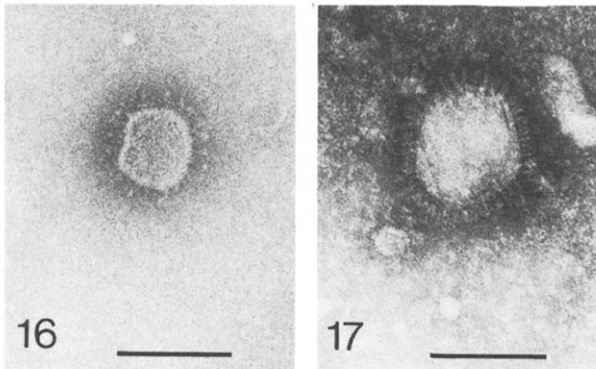
Fig. 16. Coronavirus-like particle isolated in a cell culture inoculated with cerebrospinal fluid from a patient with meningitis. The particle is surrounded by a "corona" of wide spaced, club shaped projections. Negative staining with PTA. Bar, 100 nm.

Fig. 17. Foamy agent, a contaminant virus, demonstrated in green monkey kidney (GMK) cells. The particle has long well developed surface projections. Negatively stained with PTA. Bar, 100 nm.

1975). Figures 15 and 16 show examples of viruses, which were identified in cell cultures by EM after months with confusing results by other methods. Control of cell cultures used for propagation of virus is important, as primary cell cultures from monkey kidney tissue are widely used, but unfortunately harbour simian viruses. These viruses do not always produce a recognizable cytopathic effect, but may however interfere with or inhibit isolation of human virus (Anderson and Doane, 1970). Periodic checks by EM of primary cell cultures and virus pools prepared in simian cell cultures help to avoid this problem. Figure 17 shows a Foamy agent particle, one of the contaminant viruses of monkey cells.

Conclusion

Summarizing the advantages and disadvantages of EM one must come to the conclusion, that it is an invaluable help as a supplement to other virological methods, although it may not be able to replace standard techniques in daily routine work at a great laboratory with a large number of specimens.

The technique is an expensive one due to the high price of both purchase and maintenance of the microscope. Furthermore, it has a low capacity. Few laboratories have more than, at best, one microscope, and only a very limited number of samples can be examined per day. Examination of a specimen in the microscope takes on the average half an hour.

The most important application of electron microscopy is probably in situations where speed is really essential. But also when dealing with viruses, which can not yet be propagated in any *in vivo* or *in vitro* system, or grow with difficulty and atypical or no cytopathic effect, and in periodic checks of cell cultures and virus pools, the microscope may be very useful to virologists.

In the last few years the number of viruses, known to be responsible for human diseases, has grown, largely due to electron microscopy. The future importance of both direct and immune electron microscopy in diagnostic virology as well as in the search for new agents might therefore be obvious.

References

Almeida, J. D. and Tyrrell, D. A. J.: The morphology of three previous uncharacterized human respiratory viruses that grow in organ culture. J. gen. Virol. *1*, 175–178 (1967)

Almeida, J. D. and Waterson, A. P.: The morphology of virus-antibody reaction. Advanc. Virus Res. *15*, 307–338 (1969)

Anderson, N. and Doane, F. W.: Electron microscopy in diagnostic virology. Seventh international congress for electron microscopy. In: Microscopie electronique. Ed. Favard, P. Société française de microscopie electronique. Vol III, 303 (1970) (Abstract)

Anderson, N. and Doane F. W.: Specific identification of enteroviruses by immune electron microscopy using serum-in-agar diffusion method. Canad. J. Microbiol. *19*, 585–589 (1973)

Banatvala, J. E., Chrystie, I. J., and Flower, A. J. E.: Rapid diagnosis of viral infections. Lancet II, 79–80 (1975)

Bayer, M. E., Blumberg, B. S., and Werner, B.: Particles associated with australia antigen in the sera of patients with leukaemia, Down's syndrome and hepatitis. Nature, *218*, 1057–1059 (1968)

Bishop, R. F., Davidson, G. P., Holmes, I. H., and Ruck, B. J.: Virus particles in epithelial cells of duodenal mucosa from children with acute non-bacterial gastroenteritis. Lancet II, 1281–1283 (1973)

Bishop, R. F., Davidson, G. P., Holmes, J. H., and Ruck, B. J.: Detection of a new virus by electron microscopy of faecal extracts from children with gastroenteritis. Lancet I, 149–151 (1974)

Brenner, S. and Horne, R. W.: A negative staining method for high resolution electron microscopy of viruses. Biochim. biophys. Acta *34*, 103–110 (1959)

Cossart, Y. E., Cant, B., Field, A. M. and Widdows, D.: Parvovirus like particles in human sera. Lancet I, 72–73 (1975)

Cruickshank, J. G., Bedson, H. S. and Watson, D. H.: Electron microscopy in rapid diagnosis of smallpox. Lancet II, 527–530 (1966)

Dane, D. S., Cameron, C. H. and Briggs, M.: Virus-like particles in serum of patients with australia-antigen-associated hepatitis. Lancet I, 695–700 (1970)

Davies, H. A. and Macnaughton, M. R.: Comparison of the morphology of three strains of coronavirus. Abstract of the Fourth International Congress for Virology, p. 453. Centre for Agricultural Publishing and Documentation, Wageningen (1978)

Dmochowski, L., Hoshino, M., Shigematsu, T., Hiraki, S., and Priori, E. S.: Studies of oncornaviruses by immunoferritin and immunoperoxidase electron microscopy. In: Viral immunodiagnosis. Ed. Kurstak, E. and Morisset, R. Acad. Press. New York, 183–213 (1974)

Doane, F. W., Anderson, N., Chadiyanonda, K., Banatyne, R. M., McLean, D. M., and Rhodes, A. J.: Rapid laboratory diagnosis of paramyxovirus infections by electron microscopy. Lancet II, 751–753 (1967)

Doane, F. W., Anderson, N., Zbitnew, A., and Rhodes, A. J.: Application of electron microscopy to the diagnosis of virus infections. Canad. med. Ass. J. *100*, 1043–1049 (1969)

Doane, F. W.: Identification of viruses by immune electron microscopy. In: Viral immunodiagnosis. Eds. Kurstak, E. and Morisset, R. Acad. Press. New York, 237–255 (1974)

Doane, F. W., Anderson, N., Chao, J., and Noonan, A.: Two-hour embedding procedure for intracellular detection of viruses by electron microscopy. Appl. Microbiol. *27*, 407–410 (1974)

Doane, F. W. and Anderson, N.: Electron and immunoelectron microscopic procedures for diagnosis of viral infections. In: Comparative diagnosis of viral diseases. Eds. Kurstak, E. and Kurstak, C. Acad. Press. New York. II, part B, 505–539 (1977)

Evans, A. S. and Melnick, J. L.: Electron microscope studies of the vesicle and spinal fluids from a case of herpes zoster. Proc. Soc. exp. Biol. Med. *71*, 283–286 (1949)

Feinstone, S. M., Kapikian, A. Z., and Purcell, R. H.: Hepatitis A: Detection by immuno electron microscopy of a viruslike antigen associated with acute illness. Science *182*, 1026–1028 (1973)

Flewett, T. H., Bryden, A. S., and Davies, H.: Virus particles in gastroenteritis. Lancet II, 1497 (1973)

Flewett, T. H., Bryden, A. S., and Davies, H.: Diagnostic electron microscopy of faeces. J. Clin. Path. *27*, 603–614 (1974)

Gardner, S. D., Coleman, D. V., Field, A. M., and Hulme, B.: New human papovavirus (BK) isolated from urine after renal transplantation. Lancet I, 1253–1257 (1971)

Harland, W. A., Adams, J. H., and McSeveney, D.: Herpes simplex particles in acute necrotising encephalitis. Lancet II, 581–582 (1967)

Horne, R. W. (ed): Virus structure. Acad. Press. New York (1974)

Howe, C., Bächi, T. and Hsu, K. C.: Application of immunoferritin techniques for detection of viral and cellular antigens. In: Viral immunodiagnosis. Eds. Kurstak, E. and Morisset, R. Acad. Press. New York, 215–234 (1974)

Huang, S.: Hepatitis-associated antigen hepatitis. An electron microscopic study of virus-like particles in liver cells. Amer. J. Path. *64*, 483–500 (1971)

Johannessen, J. V.: Rapid processing of kidney biopsies for electron microscopy. Kidney Int. *3*, 46–50 (1973)

Johannessen, J. V., Krogh, H.-K., Solberg, I., Dalen, A., Wijngaarden, H. van, and Johansen, B.: Human orf. J. cutan. Path. *2*, 265–283 (1975)

Johannessen, J. V.: Electron microscopy of paraffin material. In Pathology Annual vol 12, part 2., pp 189–224 Eds. S. C. Sommers and P. P. Rosen, Appleton-Century-Crofts, New York (1977)

Johannessen, J. V. and Bøhler, P. J.: Paraffin material. In: Electron microscopy in human medicine. Vol I., pp 280–284 Ed. J. V. Johannessen, McGrawHill International Book Company. New York – London – Toronto, (1978)

Johansen, B. V.: Bright field electron microscopy of biological specimens. VI. Signal-to-noise ratio in specimens prepared on amorphous carbon and graphite crystal supports. Micron 7, 157–170 (1976)

Johansen, B. V.: Negative staining. In: Electron microscopy in human medicine. Vol. I., Ed. J. V. Johannessen, McGraw-Hill International Book Company, New York – London – Tornoto, 84–98 (1978)

Joncas, J. H., Berthiaume, L., Williams, R., and Beaudry, P.: Diagnosis of viral respiratory infections by electron microscopy. Lancet I, 956–959 (1969)

Kapikian, A. Z., Wyatt, R. G., Dolin, R., Thornhill, T. S., Kalica, A. R., and Chanock, R. M.: Visualization by immune electron microscopy of a 27 nm particle associated with acute infectious non-bacterial gastroenteritis. J. Virol. 10, 1075–1081 (1972)

Kapikian, A. Z., Feinstone, S. M., Purcell, R. H., Wyatt, R. G., Thornhill, T. S., Kalica, A. R., and Chanock, R. M.: Detection and identification by immuno electron microscopy of fastidious agents associated with respiratory illnes, acute non-bacterial gastroenteritis and hepatitis A. In: Perspectives in Virology. Ed. M. Pollard, Acad. Press. New York, Vol. IX, 9–47 (1976)

Kelen, A. H. and McLoed, D. A.: Differentiation of myxoviruses by electron microscopy and immuno electron microscopy. In: Viral immunodiagnosis. Eds. E. Kurstak and R. Morisset, Acad. Press. New York, 257–275 (1974)

Kjeldsberg, E.: Small spherical viruses in faeces from gastroenteritis patients. Acta path. microbiol. scand. Sect. B. 85, 351–354 (1977)

Macrae, A. D., Field, A. M., McDonald, J. R., Meurisse, E. V., and Porter, A. A.: Laboratory differential diagnosis of vesicular skin rashes. Lancet II, 313–316 (1969)

Madeley, C. R. (ed.): Virus morphology. Churchill Livingstone, Edinburgh (1972)

Madeley, C. R. and Cosgrove, B. P.: 28 nm particles in faeces in infantile gastroenteritis. Lancet II, 451–452 (1975)

Madeley, C. R. and Cosgrove, B. P.: Calicivirus in man. Lancet I, 199 (1976)

Mathan, M., Swaminathan, S. P., Mathan, V. I., Yesudoss, S., and Baker, S. J.: Pleomorphic virus-like particles in human faeces. Lancet I, 1068–1069 (1975)

Montplaisir, S., Belloncik, S., Leduc, N. P., Onji, P. A., Martineau, B., and Kurstak, E.: Electron microscopy in the rapid diagnosis of cytomegalovirus: Ultrastructural observations and comparison of methods. J. infect. Dis. 125, 533–538 (1972)

Müller, G.: Negativkontrastierung von Viren. In: Methodensammlung der Elektronenmikroskopie. Eds. G. Schimmel and W. Vogell, Wissenschaftliche Verlagsgesellschaft mbH. Stuttgart (1975)

Nagington, J.: Electron microscopy in differential diagnosis of poxvirus infections. Brit. Med. J. 2, 1499–1500 (1964)

Nagler, F. P. O. and Rake, G.: The use of the electron microscope in diagnosis of variola, vaccinia and varicella. J. Bacteriol. 55, 45–51 (1948)

Nermuth, M. V.: Methoden der Negativkontrastierung. In: Methodensammlung der Elektronenmikroskopie. Eds. G. Schimmel and W. Vogell, Wissenschaftliche Verlagsgesellschaft mbH. Stuttgart (1975)

Oriel, J. D. and Almeida, J. D.: Demonstration of virus particles in human genital warts. Brit. J. vener. Dis. 46, 37–42 (1970)

Parsons, D. F.: Negative staining of thinly spread cells and associated virus. J. Cell Biol. *16*, 620–626 (1963)

Paver, W. K., Ashley, C. R., Caul, E. O., and Clarke, S. K. R.: A small virus in human faeces. Lancet I, 237–240 (1973)

Pennington, T. H., Follet, E. A. C., and Timbury, M. C.: Rapid diagnosis of virus infections. Lancet II, 182 (1975)

Peters, D., Nielsen, G., and Bayer, M. E.: Variola. Dtsch. med. Wschr. *87*, 2240–2246 (1962)

Sevéus, L. and Johannessen, J. V.: Embedding, sectioning and staining. In: Electron microscopy in human medicine. PP. 116–186, Vol I., Ed. J. V. Johannessen, McGraw-Hill International Book Company, New York – London – Toronto (1978)

Tellez-Nagel, J. and Harter, D. H.: Subacute sclerosing leucoencephalitis: Ultrastructure of intranuclear intracytoplasmic inclusions. Science *154*, 899–901 (1966)

van Rooyen, C. E. and Scott, G. D.: Smallpox diagnosis with special reference to electron microscopy. Canad. J. publ. Hlth *39*, 467–477 (1948)

Williams, M. G., Howatson, A. F., and Almeida, J. D.: Morphological characterization of the virus of the human common wart. Nature *189*, 895–897 (1961)

zu Rhein, G. M. and Chou, S. M.: Particles resembling papovaviruses in human cerebral demyelinating disease. Science *148*, 1477–1479 (1965)

Dr. Elisabeth Kjeldsberg, National Institute of Public Health, Postuttak, Oslo 1, Norway