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DIAGNOSTIC VIROLOGY USING ELECTRON MICROSCOPIC TECHNIQUES

Anne M. Field

Virus Reference Laboratory
Central Public Health Laboratory
London, England

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I. Introduction

Diagnosis of viral infections by observation of virus particles in thin sections of infected tissues has been a continuing but perhaps rather underused technique for the last 30 years. Observation of virus particles in suspension in metal-shadowed preparations had some diagnostic applications, but when the negative staining technique was introduced in 1959 and, as a result, virus particles were more readily recognizable, diagnostic use of the electron microscope became extremely practical. The presence in the world of smallpox infection and the consequent necessity for rapid differentiation between the smallpox virus and other viruses established the electron microscope as an essential tool in a few selected laboratories. Naturally these instruments were utilized for other virus diagnostic problems and gradually experience accumulated. Confirmation of electron microscopy as a good diagnostic technique for samples direct from patients came in the late 1960s with hepatitis B serum testing. In the 1970s the essentially noncultivable fecal viruses of hepatitis A and various diarrheal conditions were discovered and in these studies electron microscopy played an indispensable role.

The purpose of this article is to review the development of the use of electron microscopy in viral diagnosis in the last 20 years and to place it in context with other laboratory techniques. The field covered is confined to medical viral diagnosis, but parallel developments have taken place in both veterinary and botanical fields and techniques derived from both these sources are included where relevant. Viral diagnostic electron microscopy in the medical field has been reviewed by Doane (1974), by Doane and Anderson (1977), by Donelli *et al.* (1979), and by Hsiung *et al.* (1979).

II. Viral Morphology

Virus particles have characteristic morphologies (i.e., shape, substructure, and size) which, because they are fundamental properties, are important in viral classification. Viral structure is thus the basis for the use of the electron microscope for diagnosis and viruses having the same structure constitute a morphologic group, which may be a family of viruses or a genus. Within a group individual viruses cannot be differentiated on appearance and more sophisticated methods of antigenic analysis must be used, but it is often sufficient for diagnostic purposes merely to place a virus within such a group. Many diagnoses

are made by recognition of the characteristic viral structure in the image displayed on the microscope screen and need not await confirmatory micrographs; for this an appreciation of the full range of viral morphology by the operator is essential.

The salient features of viral morphology as observed by negative staining and thin sectioning methods (Section III) are here briefly reviewed and illustrated diagrammatically in Fig. 1, and selected exam-

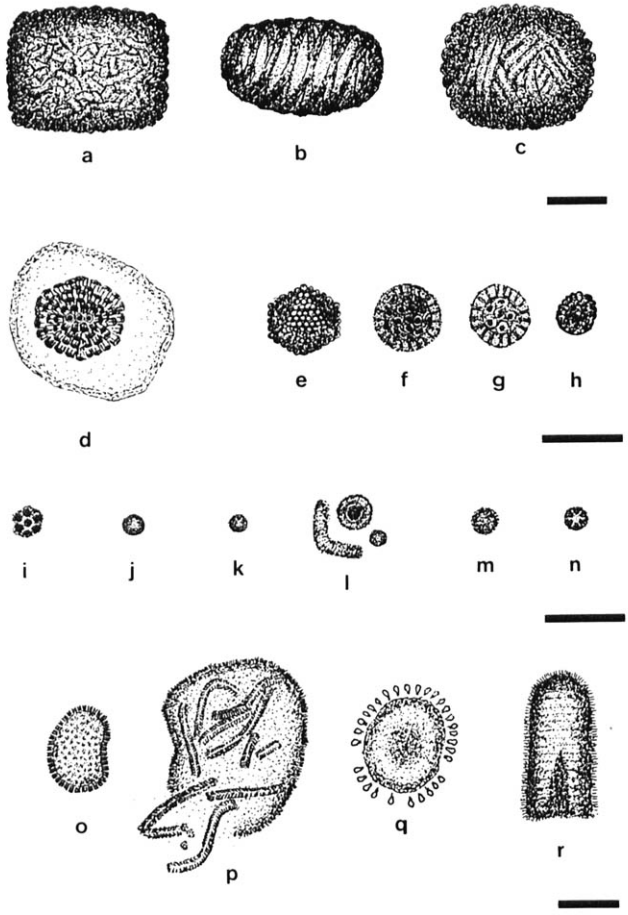


FIG. 1. Systematic morphology: the negatively stained morphology of virus particles represented diagrammatically. a, Orthopoxvirus; b, parapoxvirus; c, molluscum contagiosum virus; d, herpesvirus; e, adenovirus; f, reovirus; g, rotavirus; h, papovavirus; i, calicivirus; j, picornavirus; k, parvovirus; l, hepatitis B antigen; m, Norwalk agent; n, astrovirus; o, orthomyxovirus; p, paramyxovirus; q, coronavirus; r, rhabdovirus. Bars = 100 nm.

ples are illustrated photographically (Figs. 2 to 12). Reviews of viral structure have recently been published (Rabin and Jenson, 1967; Madeley, 1972; Dalton and Haguenau, 1973; Cheville, 1975). This review is not intended to be comprehensive but stresses the features with diagnostic significance and is largely based on personal observations and the reviews quoted above. Terminology is according to recent viral classification (REPORT, 1979).

A. Systematic Morphology

1. Poxviruses

Poxviruses are the largest and most complex virus particles. The *Orthopoxvirus* genus includes vaccinia, variola, cowpox, and monkeypox viruses, all of which can infect man. Negatively stained particles are brick-shaped, 220 to 270 nm long, and 180 to 220 nm wide. When penetrated by stain the particles appear larger than when unpenetrated. The latter display a random arrangement of 9-nm-wide surface filaments. Members of the *Parapoxvirus* genus causing human infections are orf virus and milker's nodule virus. The negatively stained particles are ovoid, 220 to 300 nm long, and 150 to 180 nm wide. The single surface filament is narrower than on *Orthopoxvirus* particles and is arranged in a regular spiral which usually gives a criss-cross appearance because both sides of the particle are imaged. Stain-penetrated particles are identical with orthopoxviruses except in the ovoid shape and generally greater length and lesser width. A probable member of the poxvirus family is molluscum contagiosum virus which has brick-shaped particles resembling orthopoxviruses but with more rounded corners. The slightly narrower surface filaments are arranged rather more regularly than on orthopoxviruses. Stain-penetrated particles closely resemble the orthopoxviruses except in the rounded corners. In thin sections of infected cells it has been shown that all poxviruses mature within a cytoplasmic matrix. In mature particles an outer coat encloses two lateral bodies which lie on each side of a dense core. Particles are released by cell lysis.

2. Large Icosahedral Viruses

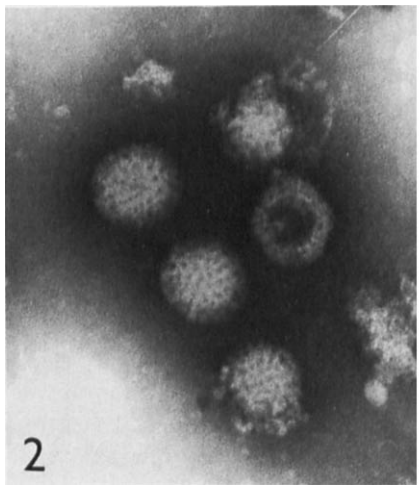
Because of their large size and complex structure the poxviruses cannot be confused with other viruses. Some of the larger icosahedral viruses, although of different families, can resemble each other under some circumstances, particularly if damaged. The families concerned are the Herpesviridae, Adenoviridae, Reoviridae (including reoviruses,

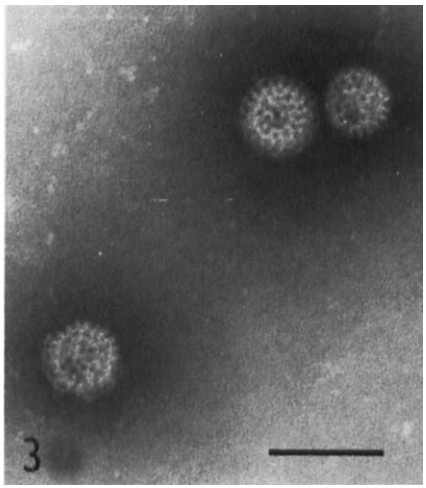
rotaviruses, and orbiviruses), and Papovaviridae, arranged in descending size order.

Negatively stained herpesvirus particles have icosahedral 100- to 110-nm-diameter capsids bearing 162 capsomeres which are hexagonal when seen end-on and tubular in profile. Capsids are often surrounded by a membranous envelope bearing irregular short projections. This gives the virus a total diameter of 120 to 150 nm. Varying degrees of stain penetration of both capsid and envelope have been observed (Watson and Wildy, 1963; Tyrrell and Almeida, 1967). In thin sections of infected cells it has been shown that capsids are assembled in the nucleus where they acquire cores, which have variable morphology, and where paracrystalline arrays of capsids may be seen. Envelopes are formed around capsids at several sites: the nuclear membrane, cytoplasmic membranes, and cell membrane. Members of the group infecting humans are herpes simplex virus, cytomegalovirus, Epstein–Barr (EB) virus, and varicella zoster virus.

Adenoviruses negatively stained have icosahedral 70- to 80-nm-diameter capsids with a rigid angular appearance, becoming spherical when damaged. There are 252 capsomeres which are 7 to 9 nm in diameter. Twelve vertex capsomeres bear fibers (Valentine and Pereira, 1965), but these are rarely observed in the conditions used to prepare diagnostic specimens. In thin sections of infected cells it can be seen that capsids assemble and mature in the nucleus where they often form paracrystalline arrays. At least 33 antigenic types of adenovirus can infect humans.

The Reoviridae have particles with icosahedral symmetry and two concentric protein coats both bearing capsomeres. Members of the *Reovirus* genus, which includes three types capable of infecting humans, have particles which are 70 to 80 nm in diameter when negatively stained. The outer layer of capsomeres is rarely spontaneously lost, but when it is an inner, 50- to 55-nm-diameter particle, it remains (Fig. 2). Rotaviruses occur in feces and negatively stained particles are 65 to 75 nm in diameter when complete and 55 to 60 nm without the outer coat which strips off readily in natural conditions. Complete Rotavirus particles have a smooth circular outline but removal of the outer layer leaves a particle coated with capsomeres having the appearance of spokes of a wheel (Fig. 3). Colorado tick fever virus is an *Orbivirus* genus member capable of infecting humans. The virus has a substructure resembling the Reoviruses but with less clearly defined capsomeres on the surface of a 65- to 75-nm-diameter capsid (Palmer *et al.*, 1977). Thin sections show that all the Reoviridae replicate in the cytoplasm and usually mature from a matrix of viroplasm. Reoviruses





FIGS. 2 AND 3. Reoviridae. Figure 2, reovirus particles from inoculated cell culture; Fig. 3, rotavirus particles from human feces. Negative stain. ×150,000. Bar = 100 nm.

frequently associate with the mitotic apparatus whereas rotaviruses replicate in close association with the endoplasmic reticulum. Viral release is by cell lysis except for orbiviruses which bud from cell membranes.

Papovaviruses when negatively stained show icosahedral symmetry, but with a skew arrangement, and the capsomeres are more prominent around one side of the particle than on the opposite side. Particles of the *Papillomavirus* genus are 50 to 55 nm in diameter. This genus includes human wart virus. Particles of the *Polyomavirus* genus have identical morphology but are smaller, 40 to 45 nm in diameter. This genus includes two human viruses, BK virus and JC virus. Filamentous forms and several isometric forms smaller than true virus particles can occur. In thin sections of infected cells capsids are seen to be assembled in the nucleus where they often form paracrystalline arrays. Particles may also be seen in cytoplasm, wrapped around by cellular membranes on entry to cells, while progeny virus particles are usually aligned on cytoplasmic membranes. Papillomaviruses tend to be more confined to the nucleus than polyomaviruses.

3. *Small Isometric Viruses*

Another collection of morphologically similar particles is the range of smaller sized (20- to 40-nm-diameter) isometric viruses, most of which do not have clearly recognizable surface subunits. This group includes

Caliciviridae, Picornaviridae, Parvoviridae, the hepatitis viruses, Norwalk agent, and astroviruses.

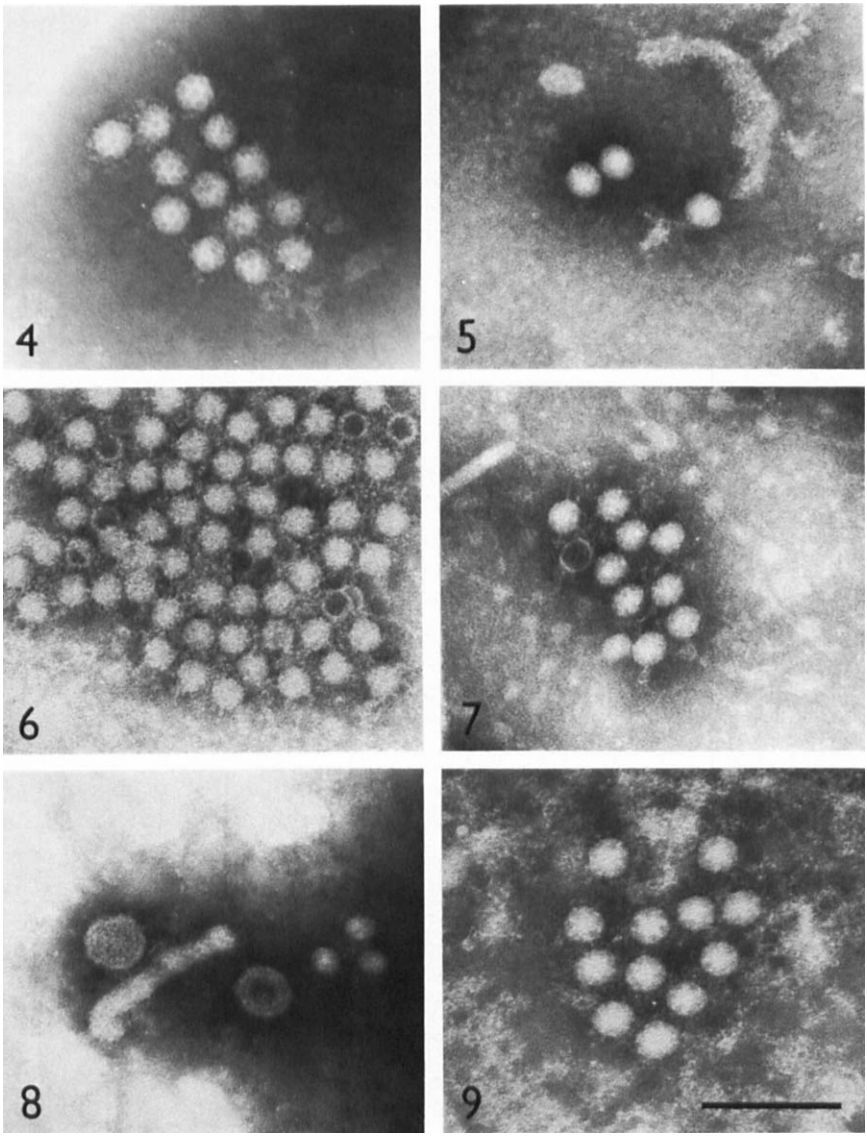
Members of the proposed family Caliciviridae negatively stained have roughly spherical particles, 29 to 40 nm in diameter, bearing 32 cup-shaped surface depressions arranged in icosahedral symmetry. In suitably oriented particles these give rise to a distinctive surface pattern of a hollow-centered star recently compared with the "Star of David" (Madeley, 1979). In thin sections the viruses are seen to mature in the cytoplasm in the cisternae of the endoplasmic reticulum and may form crystalline arrays (Love and Sabine, 1975). Human fecal calicivirus appears to be a member of this group (Fig. 4).

Negatively stained picornaviruses are spherical particles with a smooth surface and outline measuring 22 to 30 nm in diameter (Fig. 5). In infected cells they mature in the ground substance of the cytoplasm, sometimes forming paracrystalline arrays. Because they are only slightly larger than ribosomes individual particles may be difficult to recognize. The many antigenic types of poliovirus, Coxsackievirus, echovirus, and rhinovirus all infect humans. Rhinoviruses are more fragile than enteroviruses in negatively stained preparations and empty shells and partial shells are frequently observed. Enteroviruses have a peak buoyant density of 1.33 to 1.34 gm/ml in cesium chloride; the rhinovirus density is 1.38 to 1.41 gm/ml.

Members of the Parvoviridae when negatively stained closely resemble picornaviruses. They are spherical with a smooth surface and outline and the diameter ranges from 18 to 26 nm. In both families some particles may appear to have hexagonal outlines. Empty shells and partial shells are, as with rhinoviruses, commonly seen in parvovirus preparations (Fig. 6). Thin sections of infected cells reveal empty and complete parvovirus capsids in the nucleus and at later stages progeny virus particles are found in the cytoplasm embedded in matrix material or in membrane-bounded spaces. The peak buoyant density in cesium chloride is 1.39 to 1.42 gm/ml. Possible human members of this family have been observed in feces and sera.

Negatively stained hepatitis A virus particles are 27 nm in diameter, spherical, smooth surfaced, and smooth outlined (Fig. 7). In thin sections they are found in the cytoplasm. Possession of ribonucleic acid and other properties indicate that this virus is a member of the Picornaviridae.

Hepatitis B virus is pleomorphic. In negatively stained preparations of serum three particle types are seen: small round particles, 19 to 23 nm in diameter with a smooth surface and edge; long filamentous forms, 19 to 23 nm wide and varying lengths with a smooth surface or



FIGS. 4–9. Small isometric viruses. Figure 4, calicivirus particles from human feces (courtesy of Mr. W. D. Cubitt); Fig. 5, picornavirus particles from inoculated cell culture; Fig. 6, parvovirus-like particles from human serum, aggregated by antibody; Fig. 7, hepatitis A virus particles from human feces, aggregated by antibody (courtesy of Dr. H. Appleton); Fig. 8, hepatitis B antigen particles from human serum; Fig. 9, astrovirus particles from human feces (courtesy of Mr. T. W. Lee). Negative stain. ×180,000. Bar = 100 nm.

with cross striations; Dane particles which are 42 nm in diameter and have a smooth outer layer enclosing a 27-nm spherical core (Fig. 8). The cores (HBcAg) can be extracted from the Dane particles and are antigenically different from the other pleomorphic particles (HBsAg) which are all antigenically similar (Almeida *et al.*, 1971). In thin sections of infected liver the HBcAg particles are seen in the nucleus and resemble parvoviruses. The three forms of HBsAg are seen in cisternae of endoplasmic reticulum in the cytoplasm (Huang and Neurath, 1979).

Norwalk agent and other morphologically similar viruses are at present unclassified. The particles bear some resemblance to caliciviruses but do not show the Star of David substructure. The major structural protein has properties similar to that of caliciviruses (Greenberg *et al.*, 1981). The outline of the particle is structured indicating the presence of some substructure. The particles are difficult to measure accurately because of the ragged outline but are approximately 27 to 34 nm in diameter. No thin section studies have been done. The agents are found in human feces (Kapikian *et al.*, 1972b).

Astroviruses are also unclassified at present. Negatively stained, they are 26- to 30-nm-diameter round particles with a smooth edge. A proportion of the particles bears a solid five- or six-pointed star on the surface (Madeley, 1979). No thin section studies of a full replicative cycle of the human virus are available but abortive cycles in cell culture show the virus located in cytoplasm (Kurtz *et al.*, 1979). Sheep astrovirus particles are cytoplasmic *in vivo* (E. Gray *et al.*, 1980). The particles are found in human feces (Fig. 9).

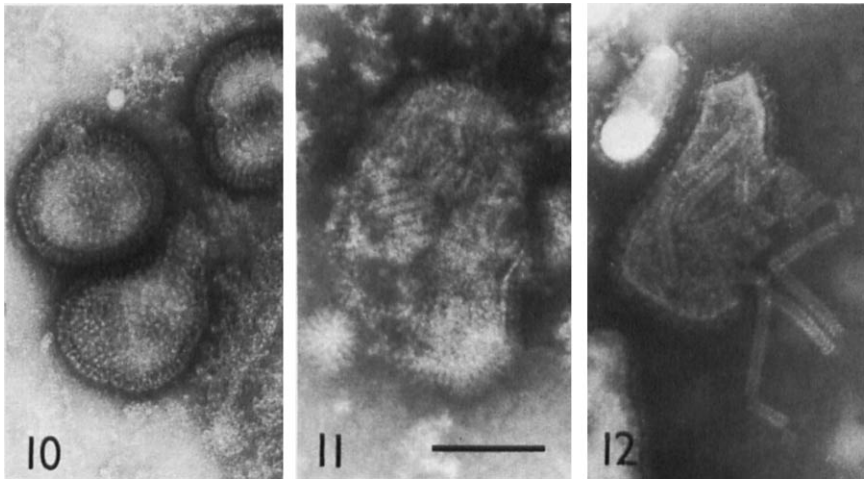
4. Viruses with a Fringe of Surface Projections

Viruses which are notable for bearing a fringed outer membrane include Orthomyxoviridae, Paramyxoviridae, Coronaviridae, Rhabdoviridae, and Marburg/Ebola viruses.

The orthomyxovirus particles are 80 to 120 nm in diameter and are pleomorphic in negatively stained preparations. Spherical and filamentous forms are the commonest. Spherical forms may show small blebs but these are probably preparation artifacts (Nermut, 1972). The surface of the limiting membrane is covered with 10-nm-long spike-like projections spaced 7 to 8 nm apart. This fringe has a regular appearance around the periphery of negatively stained particles (Fig. 10). End-on views of the projections give a regularly dotted appearance to the surface of particles. Some influenza C virus particles have a reticulate surface pattern (Apostolov and Flewett, 1969). The orthomyxovirus outer membrane is rarely penetrated by stain to reveal the internal component, which is a ribonucleoprotein helix, 9 nm in

width, arranged in a tight coil (Murti *et al.*, 1980) (Fig. 11). Nuclei of sectioned cells show various granular and fibrillar structures in the early stages of infection but virus particles are recognized only in the cytoplasm where they mature by budding at the plasma membrane where it covers the already assembled nucleocapsid. This group includes the influenza viruses.

Paramyxoviruses are also pleomorphic, membrane-bound particles, with diameters 120 to 450 nm. Surface projections are approximately 8 nm long and are spaced at 8- to 10-nm intervals. In contrast with orthomyxoviruses these particles frequently fracture revealing the enclosed ribonucleoprotein which is a loosely arranged helix with a pitch of 5 nm and a width of 17 to 18 nm in the *Paramyxovirus* genus (parainfluenza viruses and mumps virus) and in the *Morbillivirus* genus [measles and subacute sclerosing panencephalitis (SSPE) viruses] (Fig. 12). In the *Pneumovirus* genus [respiratory syncytial virus (RSV)] the helix width is only 12 to 15 nm (Joncas *et al.*, 1969a; Norrby *et al.*, 1970; Bächi and Howe, 1973; Berthiaume *et al.*, 1974). In sections the tubular nucleocapsid is seen to accumulate in the cytoplasm and to be aligned beneath the cellular membrane which acquires projections in those regions. Finally mature virus particles bud through the membrane. The morbilliviruses also accumulate nucleocapsid in the nu-



FIGS. 10–12. Orthomyxoviridae and paramyxoviridae. Figure 10, orthomyxovirus particles; Fig. 11, orthomyxovirus particle penetrated by stain and showing the helical nucleocapsid; Fig. 12, paramyxovirus particle and helical nucleocapsid. Negative stain. ×150,000. Bar = 100 nm.

cleus. SSPE virus has lost the budding maturation stage in the natural host.

Coronaviruses have pleomorphic membrane-bound particles with diameters ranging from 75 to 160 nm. They are surrounded by club-shaped surface projections approximately 20 nm long which however are easily lost. In thin sections of infected cells particles are seen to assemble in the cytoplasm and mature by budding into vesicles, accumulating there before being released by cell lysis. The particles in thin sections consist of a translucent core surrounded by a 20-nm-thick membrane bearing 15- to 20-nm-long projections. The human coronavirus affects the respiratory tract. Another possible member of the family is the human enteric coronavirus which, in common with some other animal enteric coronaviruses, has narrow surface spikes rather than club-shaped projections. These spikes are 20 nm long and frequently have knobs and extra T pieces at the distal ends (Caul *et al.*, 1977).

Rhabdoviruses are bullet-shaped particles 130 to 250 nm long and 70 to 80 nm wide. The group includes rabies and vesicular stomatitis virus. Rabies virus is very fragile and in negatively stained preparations most particles are pleomorphic and have variable lengths. Particles penetrated by the stain exhibit internal cross-striations at 4.5- to 5-nm intervals which is the coiled ribonucleoprotein helical component. Some particles exhibit a reticular surface structure but all have surface projections which are 8 to 10 nm long. In thin sections viral matrix material can be seen in the cytoplasm. The site of virus maturation depends on the virus and the cells used; rabies particles mostly bud from intracytoplasmic membranes but can bud in the viral matrix from *de novo* membranes.

Marburg and Ebola viruses, so far unclassified, have structural similarities to rhabdoviruses. In effect they are very long bullet-shaped particles which are frequently curved into hooked and circular forms and are 80 nm wide and 130 to 2500 nm long or even longer. Surface projections are 10 nm long and a helical inner structure with 40 to 50 nm diameter has 5 to 6 nm perodicity. In sections nucleocapsids are formed in the cytoplasm and particles mature by budding at the plasma membrane (Murphy *et al.*, 1978).

5. Viruses without Distinctive Morphology by Negative Staining

A collection of viruses which are not often encountered in diagnostic electron microscopy, largely because the negatively stained morphology is not distinctive, includes the Togaviridae, Arenaviridae, Bunyaviridae, and Retroviridae.

Togaviruses infecting man include Sindbis and Chikungunya in the *Alphavirus* genus, dengue, yellow fever, and Japanese B encephalitis in the *Flavivirus* genus, and rubella in the *Rubivirus* genus. With negative staining the viruses are difficult to recognize but they consist of a 40- to 70-nm-diameter envelope closely applied to a 25- to 35-nm nucleocapsid which probably has icosahedral symmetry. Most particles bear surface projections with little regularity. Viruses are more clearly seen in thin sections of infected cells where they multiply in the cytoplasm and mature by budding at the plasma membrane (alphaviruses), the endoplasmic reticulum and Golgi zone membranes (flaviviruses), and into vacuoles and at the plasma membrane (rubiviruses). Particles in thin sections exhibit an electron-dense core surrounded by a translucent zone bordered by the viral envelope (Holmes *et al.*, 1969; Matsumara *et al.*, 1971).

Arenaviruses are spherical or pleomorphic, 110- to 130-nm-diameter particles with club-shaped 10-nm-long surface projections. The most distinctive morphology is shown in thin sections where it can be seen that the limiting membrane encloses a varying number of 20- to 25-nm-diameter, ribosome-like particles. In the infected cells a mass of ribosomes accumulates in a dense cytoplasmic matrix and the virus particles mature by budding through the cell membrane (Murphy and Whitfield, 1975). Viruses of the Tacaribe complex, Lassa fever virus and lymphocytic choriomeningitis virus are human pathogens.

Bunyaviruses are spherical or oval, 90- to 100-nm-diameter particles with an envelope bearing surface projections. The envelope encloses helical ribonucleoprotein 2 to 2.5 nm wide. The particles assemble in the cytoplasm and mature by budding through smooth surfaced vesicles in the Golgi region. Human agents are Bunyamwera and California encephalitis virus.

The *Oncovirus* subgroup of the Retroviridae were originally described morphologically as A, B, C, and D type virus particles by Bernhard (1960). When negatively stained the particles are spherical, enveloped, 80 to 100 nm in diameter, and have surface projections which are seen best in the type B *Oncovirus* genus. Often particles have surface blebs or tails but these are probably preparation artifacts. Beneath the envelope the core is probably icosahedral and contains the ribonucleoprotein which may be helical. In thin sections the particle outer envelope encloses a nucleoid which resembles an A type particle and is eccentrically placed in mature type B oncoviruses and is central in type C oncoviruses. Mature type B particles are 90 to 200 nm in diameter and type C particles are 85 to 110 nm diameter (Dalton, 1972; de Harven, 1974). Although viruses of this family are found in mam-

mals, the morphological evidence for human oncoviruses is very slight. The other subfamily, the Spumaviridae, includes the foamy viruses and a possible human member. Negatively stained particles are enveloped, about 90 nm in diameter, with 12- to 15-nm-long surface projections. In thin sections of infected cells virus particles are observed in the cytoplasm budding into cytoplasmic vacuoles and through the plasma membrane. The moderately electron-dense core is 40 to 60 nm in diameter and is separated from the 70-nm envelope by empty space bridged by striations; spikes on the surface increase the total diameter to 90 nm (Clarke *et al.*, 1969).

B. Measuring Virus Particles

Although size is an important aid to identification it is unwise to rely on the accuracy of size estimates; the sizes quoted for virus particles in the preceding section are only approximate as measurement is subject to many variables. In practice it is useful to have a size marker in the instrument so that a rough estimate of virus particle size can be made using the image on the fluorescent screen. The microscope itself must be calibrated to ensure the accuracy of any given magnification as manufacturing tolerance is generally only within 5%. At high magnifications, such as those used for virus work, calibration based on cross-grating replicas is inaccurate and calibration using measurements of the lattice spacing in negatively stained catalase crystals is considered more reliable (Wrigley, 1968). Even small height changes of the specimen in the microscope affect magnification (Agar *et al.*, 1974) and this creates problems as it is almost impossible to ensure that specimen grids will stay perfectly flat and will be positioned at exactly the height of the calibration grid. Another cause of possible error is that lens currents vary over a period and this too can affect magnification.

In the photographic darkroom careless enlarger setting affects the magnification of prints and photographic paper dimensions can change. Thus measurements are best made on the negatives except when particle images are so small that this in itself induces errors. The thickness of stain surrounding particles is variable in negatively stained preparations, consequently particles may be flattened and so appear to be larger (Nermut, 1977); in addition excessive photographic contrast of the image can affect apparent particle size by obscuring the edge of the particle. In thin sections virus particles are usually smaller than in negatively stained preparations as a result of the processing used (Glauert, 1975).

A comparison of sizes obtained for bacteriophage particles by negative staining, thin sectioning, and freeze drying accompanied by metal shadowing, showed that negative staining gave the best correlation with the size derived from X-ray analysis (Earnshaw *et al.*, 1978).

C. Morphologic Variation

The preceding descriptions of negatively stained virus particles refer to viruses untreated as far as possible. The shape of the more delicate viruses can be altered by high-speed centrifugation (Polson and Stannard, 1970), and on the grid by stretching of the support film (Ronald *et al.*, 1977). Myxovirus pleomorphism is probably a preparation and storage artifact (Nermut, 1972). Chemicals used in purification and concentration of viruses may affect morphology (Almeida *et al.*, 1979). Even the negative stain can alter viral morphology: phosphotungstic acid is probably the least damaging in this respect but even this can disrupt the helical paramyxovirus ribonucleoprotein into short lengths (Hosaka, 1968) while uranyl acetate shrinks bacteriophage heads and causes the tails to swell (Ackermann *et al.*, 1974b) and potassium borotungstate splits myxovirus surface projections (Flewett and Apostolov, 1967).

Fixation of foot and mouth disease virus with glutaraldehyde before negative staining produced empty particles and increased their diameter by 25% (Sangar *et al.*, 1973). The internal details of enveloped herpesviruses were also obscured by either glutaraldehyde or osmium tetroxide fixation, probably because negative stain no longer penetrated the fixed envelope (Vernon *et al.*, 1976; Field, unpublished observations). Murphy *et al.* (1970) found that glutaraldehyde or osmium tetroxide fixation obscured the envelope detail of arenaviruses; glutaraldehyde-fixed coronaviruses lost the clarity of the surface projections in negatively stained preparations (Caul *et al.*, 1977). On the other hand rotavirus structure was stabilized by formaldehyde fixation (Woode *et al.*, 1976) and retroviruses were less pleomorphic if glutaraldehyde fixed and critical point dried than if unfixed and air dried (Gonda *et al.*, 1978). Influenza virus particles fixed in osmium tetroxide were less pleomorphic than when unfixed but the detailed surface structure was not so clear (Reuss *et al.*, 1967).

The intentional disruption of virus particles on the grid with detergent has been used to study their internal structure (Almeida and Brand, 1975).

III. Techniques for Electron Microscopy of Viruses

A. Negative Stain

The basis of negative staining is that the electron-dense stain surrounds the virus particles and flows into surface crevices, giving a clear image of the outside surface of the particles. Sometimes stain penetrates to the interior of the capsids and an image of the outer shell in profile results (Horne, 1975). The technique was first described in 1959 (Brenner and Horne, 1959) and was soon applied successfully to viral diagnostic specimens. The usual method for viral diagnosis is to mix virus suspension and negative stain in equal volumes, place a drop of mixture onto a Formvar–carbon-coated grid, remove excess fluid by touching the edge of the grid to filter paper, and allow the grid to air-dry. Alternatively virus suspension can be applied to the grid and dried before stain is added. The stain used most commonly is phosphotungstic acid.

Samples must be rich in virus to overcome the limitations of the technique. Some samples, even diagnostic specimens from patients, contain sufficient virus to be examined without concentration; however, others need concentration and a simple method is to pellet virus by ultracentrifugation of a clarified sample (Almeida *et al.*, 1967b). A further refinement is purification by density gradient centrifugation. Virus may be concentrated from samples by adding Lyphogel, which is a polyacrylamide hydrogel capable of absorbing water, salts, and small molecules to leave virus particles in a greatly reduced volume (Ashcavai and Peters, 1971; Whitby and Rodgers, 1980). Virus can be concentrated from very large volumes by membrane filtration (Torrella and Morita, 1979) or can be adsorbed to a polyelectrolyte and eluted to a smaller volume (Chaudhary and Westwood, 1972). Virus particles can be released from infected cells of solid tissues, cell cultures, and organ cultures by lysing the concentrated cells in a small volume of distilled water (Almeida *et al.*, 1967c; Almeida and Tyrrell, 1967) and, if necessary, further concentration of virus in such cell lysates can be effected by one of the techniques mentioned above.

Various methods have been used to remove contaminating salts from samples. An agar diffusion technique devised by Kellenberger and Arber (1957) was developed by Kelen *et al.* (1971) and by Anderson and Doane (1972a). It utilizes an agar substrate and either the grid with a microdrop (approximately 0.01 ml) of virus suspension on it is placed

on the agar or the microdrop is applied to the agar surface and the grid is floated on the microdrop. When the drop has dried the fluid, salts, and low-molecular-weight substances have diffused into the agar leaving virus particles and larger debris on the grid ready for negative staining. Salts may also be removed by careful washing of grids after air-drying the viral sample onto them (Cartwright *et al.*, 1969; Bond and Hall, 1972). Sucrose can be dialyzed from viral samples already applied to grids by using a wick of filter paper to run a buffer solution continuously across the grid (Webb, 1973).

The pseudoreplica method concentrates virus from samples and removes salts simultaneously (Smith and Melnick, 1962). The viral sample is applied to the surface of a small block of agar and allowed to dry. The agar surface is covered with Formvar solution, and the resultant virus-coated film is floated onto negative stain and mounted on a grid. The technique has been used successfully with diagnostic samples (Burtonboy *et al.*, 1978; Lee *et al.*, 1978) but is perhaps unnecessarily complicated.

Although excellent viral morphology results from spraying virus particles onto grids, it is not to be recommended for diagnostic samples since the technique is relatively insensitive, needing very high concentrations of virus, and the resultant aerosol could be dangerous (Horne, 1967; England and Reed, 1980).

It is generally accepted that the threshold concentration of virus necessary for detection in negatively stained preparations is 10^5 to 10^6 particles per milliliter (Galasso, 1967; Monroe and Brandt, 1970; Ball and Harris, 1972; Chaudhary and Westwood, 1972).

If viral samples are too well purified it may be difficult to obtain adherence to grids, but adding a wetting agent such as bovine serum albumin or bacitracin may solve this problem (Horne, 1967; Gregory and Pirie, 1972).

Of the many different negative stains available phosphotungstic acid at pH 6.4 has been found to be the most reliable for general diagnostic work. Silicotungstate gives a less granular background and preserves paramyxovirus structure better than phosphotungstate (Bloth and Norrby, 1967). Phosphotungstate often gave better definition of the viruses encountered in veterinary diagnosis when used at pH 6 as opposed to pH 7 (Spadbrow and Francis, 1969). Caul *et al.* (1977) found phosphotungstate superior to ammonium molybdate or uranyl acetate for examination of fecal coronaviruses. Flewett and Apostolov (1967) found potassium borotungstate damaged myxovirus surface projections.

B. Negative Stain Immune Electron Microscopy

The morphology of negatively stained virus particles is sufficient for grouping purposes but it is necessary to use immune electron microscopy (IEM) to differentiate morphologically identical but antigenically distinct viruses. When specific antiserum is added to a suspension of virus particles molecules of antibody attach to the particles and can be seen after negative staining. When optimal proportions of virus and antiserum are used the virus particles are agglutinated by the antibody into immune complexes. Methods and applications have been reviewed by Almeida and Waterson (1969a), Doane (1974), and Doane and Anderson (1977).

The simplest method for negative stain IEM is to mix small volumes (i.e., 0.1 ml) of viral suspension and antiserum, incubate at 37°C or room temperature for 1 hour, dilute to a reasonable volume, and ultracentrifuge to pellet the immune complexes for negative staining (Almeida and Waterson, 1969a). When reagents are scarce smaller volumes (0.01 to 0.02 ml) can be mixed and incubated, then microdrops are removed to an agar surface and grids are floated on the drops. When the fluid has dried the grids are treated with negative stain (Kelen *et al.*, 1971). Alternatively, microdrops of virus–serum mixture may be applied directly to the grids, but before negative staining repeated washing is necessary to remove salts and low-molecular-weight substances which would otherwise obscure the immune reaction (Milne and Luisoni, 1977). Unfortunately it is necessary to use salt-containing fluids for immune electron microscopy to ensure optimal combination of virus with antibody (Ball and Brakke, 1968). For routine virus identification specific antibodies can be incorporated into agar in microtiter plate wells and the plates can be stored with a grid on top of the agar in each well. When required, microdrop samples of the virus to be identified are added on top of the grids in the wells and allowed to dry into the agar. Grids are removed and treated with negative stain (Doane, 1974). Gel immunodiffusion test precipitin lines can be cut out, homogenized, and negatively stained to show virus–antibody complexes (Beale and Mason, 1968). A method of specific attraction of virus particles to antiserum-treated grids was developed for plant viruses (Derrick, 1973) and further refined by Shukla and Gough (1979) who used staphylococcal Protein A to enhance antibody coating of the grid. The technique has recently been used successfully in rotavirus diagnosis (Nicolaieff *et al.*, 1980) but it was noted that coronaviruses and small round viruses also present in the samples were not seen on the

rotavirus–antiserum-treated grids. Virus particles specifically adsorbed onto antiserum-coated grids may be treated further with antisera to investigate their antigenic nature (Milne and Luisoni, 1977).

Complement causes immune lysis of some viruses and may change the appearance of immune complexes so sera are best heat inactivated before use to eliminate these effects (Almeida and Waterson, 1969a). Addition of a second antiserum specific for the immunoglobulin in the original immune complex increases the total antibody layer around the virus particles making detection of complexes easier (Saif *et al.*, 1977). Antibody may be conjugated to ferritin molecules which makes identification of antibody easier (Brzosko *et al.*, 1970; Patterson, 1975), although antibody molecules can be seen readily without a marker when negatively stained. Ferritin labeling has been found useful when a second layer of antibody is used to identify the species of immunoglobulin involved in immune complexes (Locarnini *et al.*, 1977).

The basic necessities for satisfactory negative stain IEM are virus particles in large numbers, free from cell debris and free of antibody, and an antibody preparation which is also free of immune complexes. If the viral antigen is a sample from a patient some antibody may be present. Although it is possible to use such antigens by careful grading of the amount of antibody seen in immune complexes interpretation is more difficult. False clumping of virus particles occurs particularly during abrupt pH changes (Floyd, 1979). Rheumatoid factor can induce mixed clumping of nonidentical viruses to give misleading results in typing tests by IEM (Stannard *et al.*, 1980). It is best to use viruses unfixed because some lose antigenicity when fixed (Narayan *et al.*, 1973), although others retain activity (Woode *et al.*, 1976; Chaudhary *et al.*, 1979).

The application of negative stain IEM has been particularly useful for the study of the antigenic nature of some of the newly discovered noncultivable viruses. For example human parvovirus-like particles have been compared antigenically by this technique (Paver *et al.*, 1975) as have rotaviruses from humans and from veterinary samples (Woode *et al.*, 1976). The system can be reversed and, using known viral antigens, specific viral antibodies can be detected in human sera. Again the technique is most useful when the antigen is a noncultivable virus such as hepatitis A virus (Dienstag *et al.*, 1976a) or Norwalk agent (Parrino *et al.*, 1977).

Negative stain IEM can be used to detect viruses in clinical samples or after culture *in vitro* since with certain viruses it increases the sensitivity of negative stain visualization some 100 times (Doane,

1974). Such enhanced sensitivity depends upon the titer of the antiserum used (Lamontagne *et al.*, 1980) and Zissis *et al.* (1978) found negative stain IEM did not in fact increase sensitivity of rotavirus detection. When single virus particles are readily recognizable, such as rotaviruses, it is probably more sensitive to have many scattered single particles in an ordinary negative stain rather than a much smaller number of virus aggregates in immune preparations. Perhaps the main advantage of IEM in virus detection is the specific aggregation of virus particles of unremarkable morphology so that their viral nature can be appreciated. This was the method used to identify rubella virus (Best *et al.*, 1967) and the small round virus particles such as hepatitis B antigen (Almeida *et al.*, 1969b), rhinoviruses (Kapikian *et al.*, 1972a), and hepatitis A virus (Feinstone *et al.*, 1973).

Incorporation of atypical forms along with typical virus particles in the same specific immune complex demonstrates their common viral antigenicity. This was shown for the tubular forms of human polyomaviruses (Albert and Zu Rhein, 1974) and for filamentous forms of rotaviruses (Holmes *et al.*, 1975). Conversely negative stain IEM demonstrated that HBcAg particles differed antigenically from the three forms of HBsAg because they were not associated in the same immune complexes (Almeida *et al.*, 1970). Amorphous material possessing viral antigenicity can also be identified using negative stain IEM methods (Almeida *et al.*, 1981).

C. Thin Sections

While tissues can be homogenized to extract virus particles for negative staining it is often preferable to search for viruses *in situ* in thin sections. When particles are scanty the thin section technique may be more sensitive than negative staining. Some viruses have a more distinctive morphology in thin sections than when negatively stained. Thin sectioning allows observation of the pathogenesis of the infection as well as identification of the viral cause.

There are many standard schedules for tissue fixation and embedding and methods will not be reviewed here. Examination of thin sections for viruses entails the use of relatively high magnifications in the electron microscope and methods should be chosen which will satisfy this condition. For samples where results are required urgently there are rapid embedding methods (Doane and Anderson, 1977). Examination of thick sections of resin-embedded material stained with toluidine blue for light microscopy may reduce sampling error and so reduce time spent examining thin sections. Even with such selection the thin

sectioning technique has very limited potential in rapid viral diagnosis.

Confirmation of viral infection by electron microscopy on tissues originally processed for light microscopy is frequently useful. For example, virus particles have been seen in thin sections of tissues which had been stored in formalin over a long period (Zu Rhein and Chou, 1965; Hashida and Yunis, 1970). Paraffin sections can be marked to indicate cells with inclusions and the appropriate areas of the section reembedded for electron microscopic examination of the same cells (Blank *et al.*, 1970; Rossi *et al.*, 1970; Pinkerton and Carroll, 1971; Bhatnagar *et al.*, 1977). Cytologic preparations have been reembedded in a similar fashion and virus particles seen in cells (Takeda, 1969; Coleman *et al.*, 1977b). Cells positive for viral antigens by immunofluorescence have also been reembedded and the same cells have been found to contain the expected virus particles (Epstein and Achong, 1968). Tissue and cell structure is generally adversely affected in reprocessed samples, because the original processing for light microscopy is not suitable for electron microscopy, but viral structures rarely disintegrate so far as to be unrecognizable. However, initial use of Bouin's fixative did destroy herpesvirus structure (Cockson and Holmes, 1977).

D. Thin Section Immune Electron Microscopy

Viral antigens can be detected in thin sections of infected cells by IEM with suitably labeled specific antibodies. Certain factors limit the technique for diagnostic virology: prolonged fixation and the use of standard concentrations of fixatives reduce antigenicity and also limit the penetration of reagents into fixed cells, thus making it difficult to investigate intracellular antigens (Smit *et al.*, 1974; Brown and Thormar, 1976). The average diagnostic schedule is not oriented toward light fixation of specimens immediately followed by intricate processing. Various methods are available to process already heavily fixed tissues but the preservation of fine structure is generally poor (Miyamoto *et al.*, 1971; Hadler and Dourmashkin, 1975; Mohanty, 1975; Wendelschafer-Crabb *et al.*, 1976; Bohn, 1980; Sisson and Vernier, 1980). Reactions can be attempted on already thin-sectioned material but nonspecific staining is a problem (Thomson *et al.*, 1967; Hansen *et al.*, 1979; Takamiya *et al.*, 1979). Trypsin digestion has restored viral antigenicity to formalin-fixed material for immunofluorescence and might be useful for thin section IEM (Huang *et al.*, 1976; Swoveland and Johnson, 1979; Johnson *et al.*, 1980).

The antibody markers used in thin section IEM are usually ferritin or peroxidase and the methods have been reviewed by Howe *et al.* (1974) and by Kurstak and Kurstak (1974). Cytochrome c has also been used to label antibodies (Singer, 1974) and peroxidase–antiperoxidase methods based on those used in light microscopy have recently been developed (Hsu and Ree, 1980).

Reprocessing immune light microscopy preparations for thin sections or thin section IEM has confirmed reaction specificities (Epstein and Achong, 1968; Chapman, 1970; Kumanishi and Hirano, 1978).

IV. Application of the Techniques to Samples from Patients

Virus particles are sometimes present in such large numbers in clinical specimens that they can be detected directly by electron microscopy and negative staining methods in particular can be used to provide a rapid diagnosis. There are of course limitations in sensitivity and in the fact that the technique gives only a morphological grouping in the first instance. However, IEM can be used in certain circumstances to give further identification.

A. Lesions of Skin and Mucous Membranes

Viral skin lesions may contain a large number of virus particles and samples are thus highly suitable for electron microscopy. Before the advent of negative staining techniques viruses extracted from such samples were examined, with variable success, after metal shadowing (Nagler and Rake, 1948; Van Rooyen and Scott, 1948; Melnick *et al.*, 1952) and tissue biopsies have been thin sectioned to demonstrate virus particles (cf. Sutton and Burnett, 1969; Kimura *et al.*, 1972). However with negative staining a rapid diagnosis can be made. Vesicular fluid is a suitable starting material for this technique; equally good are dried smears of lesion scrapings, rehydrated in a minimal quantity of distilled water. Crude extracts of solid tissue in distilled water may also be utilized (Macrae *et al.*, 1969). Because of the high viral content of the lesions it is usually unnecessary to concentrate virus before negative staining. Samples are best unfixed as this facilitates extraction of virus from the cells and fixation before negative staining may hinder virus recognition, especially of herpesviruses. Because of variation of viral content in different samples it is advisable to prepare specimens from more than one lesion (Cruickshank *et al.*, 1966; Harkness *et al.*, 1977).

Application of negative staining techniques to diagnosis of viral skin

lesions, particularly to the differential diagnosis of smallpox (a poxvirus as opposed to the herpesvirus of varicella zoster), was a major factor in establishing electron microscopy in diagnostic virology (Peters *et al.*, 1962; Williams *et al.*, 1962). Although smallpox has been eradicated other diagnostic problems remain for solution by electron microscopy. Human monkeypox infections were diagnosed by electron microscopy and in some cases other laboratory tests were negative (Breman *et al.*, 1980). Orf virus grows only with difficulty and electron microscopy is the only practical diagnostic method; similarly, laboratory diagnosis of molluscum contagiosum is possible only by electron microscopy. Compared with virus isolation and gel precipitin tests electron microscopy was conspicuously effective in diagnosis of varicella zoster virus infections (Cruickshank *et al.*, 1966; Macrae *et al.*, 1969). Although poxviruses and herpesviruses are morphologically distinguishable, individual orthopoxviruses (vaccinia, variola, and cowpox) cannot be differentiated morphologically nor can the herpesviruses (herpes simplex and varicella zoster). Negative stain IEM is not useful in this diagnostic situation because of the small sample size and because other laboratory tests can be more simply used to give precise identification (Macrae *et al.*, 1969).

The viral etiology of skin warts has been repeatedly demonstrated by electron microscopy. In thin sections paracrystalline arrays of papillomavirus particles have been observed (Strauss *et al.*, 1949; Bunting, 1953) and these thin section appearances have been correlated with intranuclear inclusions seen by light microscopy (Almeida *et al.*, 1962). The virus particles reacted with wart virus antiserum in thin section IEM preparations (Viac *et al.*, 1978). Negative staining was successfully applied to homogenates of skin warts (Williams *et al.*, 1962) and negative stain IEM was used to study antigenicity of extracted virus (Almeida *et al.*, 1969a). Some skin warts contain only a small number of virus particles in thin sections (Maciejewski *et al.*, 1973) and genital warts usually have a low viral content. Oriel and Almeida (1970) suggested that the best method to extract virus from biopsies of genital warts was by light grinding to disrupt only the surface layers rather than complete homogenization of the tissue. A recent study of the wart virus lesions of epidermodysplasia verruciformis showed that negative staining was slightly more sensitive than thin sections to detect virus in the early malignant lesions where viral content is very low (Yabe and Sadakane, 1975). Thin sections have shown typical papillomavirus particles in lesions of focal epithelial hyperplasia, a rare condition of the oral mucosa (Praetorius-Clausen and Willis, 1971; Hanks *et al.*,

1972; Van Wyk *et al.*, 1977), but as yet there has only been one report of a successful negative stain diagnosis (Goodfellow and Calvert, 1979). Laryngeal wart thin sections have shown scanty papillomavirus-like particles some of which were not entirely convincing in the published micrographs (Dmochowski *et al.*, 1964; Boyle *et al.*, 1971; Spoendlin and Kistler, 1978), but these findings were confirmed when papillomavirus antigen was found in laryngeal papillomas by light microscopy peroxidase–antiperoxidase techniques (Lack *et al.*, 1980). Papillomavirus particles have also been observed in thin sections of atypical genital warts resembling early dysplasia in the cervix and vagina (Laverty *et al.*, 1978; Morin and Meisels, 1980).

Particles resembling paramyxovirus nucleocapsid have been reported in thin sections of cells in measles skin rash biopsies (Kimura *et al.*, 1975), in skin lesions of discoid lupus (Hashimoto and Thompson, 1970), in Behçet skin lesion biopsies (Tawara *et al.*, 1976), and in warts, Bowen tumours, and basal cell carcinomas (Maciejewski *et al.*, 1973). These findings must be viewed with caution as artifacts which resemble nucleocapsid are not uncommon. This will be discussed in Section VI,B.

B. Nasopharyngeal Secretions

Nasopharyngeal secretions have been examined for virus particles after dilution in distilled water and negative staining. In samples revealing paramyxovirus particles studied by Doane *et al.* (1967) all yielded parainfluenza virus type 1 in cell culture. Joncas *et al.* (1969b) examined lysates of cells in nasopharyngeal secretions by negative staining and also used Doane's method and found that several samples contained paramyxoviruses. These were differentiated into respiratory syncytial virus (RSV) and other paramyxoviruses according to the width of the nucleocapsid. Isolation studies confirmed all the RSV identifications. However adenoviruses and picornaviruses isolated from these samples were never detected by electron microscopy. In a large survey of routine specimens examined by electron microscopy and virus isolation it was shown that electron microscopy was comparatively insensitive for the detection of myxoviruses and paramyxoviruses (Pavilanis *et al.*, 1971) although Valters *et al.* (1975) found that negative stain IEM increased the sensitivity of electron microscopy for detection of viruses in throat swabs. The difficulty of differentiating the paramyxoviruses seen has made it necessary to continue either routine virus isolation or immunofluorescence, which has

proved the most useful rapid and specific diagnostic method providing suitable samples are available (Gardner and McQuillin, 1980; Minnich and Ray, 1980).

Partially enveloped herpesvirus particles observed in negatively stained preparations of concentrated throat washings from a patient excreting virus were identified as EB virus by culture methods (Lipman *et al.*, 1975) and Lee *et al.* (1978) detected herpesvirus particles by the pseudoreplica method in throat swabs of five infants which were identified as cytomegalovirus in cultures from all samples.

Many patients excreting rotavirus in their feces have respiratory as well as gastrointestinal symptoms, but a study of nine such patients using negative staining methods failed to detect rotaviruses in throat swabs or nasopharyngeal secretions (Lewis *et al.*, 1979).

Saliva has often been subjected to negative staining and negative stain IEM studies for hepatitis B particles. Occasionally the search has been successful (Kistler *et al.*, 1973; Bancroft *et al.*, 1977) though the photographic evidence is not always completely convincing, probably because so few particles are present. A recent report correlated the presence of Dane particles in negatively stained density gradient fractions of saliva with the presence of DNA polymerase. Small round particles resembling those of HBsAg were also present but negative stain IEM was not done to prove their identity (Macaya *et al.*, 1979).

Norwalk agent particles have been found in a sample of vomit concentrated 100-fold by ultracentrifugation before negative staining. However, three more vomit samples positive for Norwalk agent by radioimmunoassay were negative by electron microscopy (Greenberg *et al.*, 1979).

C. Serum

The transmission of hepatitis B in blood was known for many years before the first electron micrographs of negatively stained small round and long particles of HBsAg in serum were published (Bayer *et al.*, 1968). Shortly after this negative stain IEM was used to aggregate the particles so rendering them more recognizable (Almeida *et al.*, 1969b; Hirschman *et al.*, 1969). This was immediately useful in diagnosis and sera began to be widely examined by negative stain IEM after concentration. Circulating immune complexes were observed in some sera which were examined without adding antibody (Almeida and Waterson, 1969b). Sera with such complexes frequently gave false-negative results in the other relatively crude detection tests then available (Krohn *et al.*, 1970; Cossart *et al.*, 1971). It has been suggested that

electron microscopy is more sensitive than the modern radioimmune and hemagglutination assays for detection of immune complexes (Trepo *et al.*, 1974). Dane described the larger particles bearing his name in 1970 (Dane *et al.*, 1970) and Almeida *et al.* (1971) showed that the cores of the Dane particles could be extracted and, by negative stain IEM, were antigenically distinct from the other particles. Positive sera always contain the small round particles; long particles are next in frequency and Dane particles are the least common (Dane *et al.*, 1970; Stannard *et al.*, 1973). The extra sensitivity of modern tests has rendered electron microscopy less useful in routine detection of hepatitis B antigens but it is a relatively easy way to identify sera containing Dane particles and their presence can be correlated with high risk of hepatitis transmission. Gel precipitin techniques are still used in routine testing and confirmation of the specificity of these tests by negative staining of extracted gel lines is useful.

Hepatitis A infection is not blood transmitted but there is one report of virus-like particles of variable size in human sera in the acute stages (Zanen-Lim, 1976). The samples examined were gel precipitin lines formed between patients' sera and animal sera raised against them, a system susceptible to nonspecific reactions. Some particles illustrated resembled common artifacts found in human sera which will be discussed in Section VI,A.

It is uncertain if non-A, non-B hepatitis has one etiologic agent or many. Studies are underway to examine sera by negative staining for possible particles but the results which have been obtained are inconsistent with each other. In one study seemingly specific gel precipitin lines between sera were examined by an unusual method: the gel containing the line was fixed in osmium and embedded in araldite blocks before negative staining. No significant particles were seen (Tabor *et al.*, 1979). In another study particles 60 nm in diameter with 40-nm-diameter cores were seen in some sera (Coursaget *et al.*, 1979). Hantz *et al.* (1980) reported the presence of particles closely resembling those of HBsAg in size and shape; however these were antigenically distinct from HBsAg, were present in very low concentration, and were not reactive in negative stain IEM with antisera which gave gel precipitin lines with the same sera. The gel lines were not examined. Yoshizawa *et al.* (1980) reported the presence in very low concentrations of virus-like particles approximately 27 nm in size detectable only by negative stain IEM using large volumes of sera from patients with non-A, non-B hepatitis. Similar particles were observed in chimpanzee sera after experimental infection. Mori *et al.* (1980), in a negative staining study of density gradient fractions of non-A, non-B sera, showed hexagonal

32-nm enveloped particles with 22-nm hexagonal cores as well as free 22- to 24-nm particles, but no IEM was done.

Theoretically, any virus causing massive viremia could be detected in serum by electron microscopy. Ebola virus was seen in human serum in the course of one infection (Bowen *et al.*, 1978).

When testing sera by negative stain IEM for HBsAg we occasionally observed parvovirus-like particles with a diameter of approximately 23 nm. The human HBsAg detector serum routinely used had antibodies to the parvovirus-like particles and so formed them into immune complexes closely resembling those of HBsAg small round particles (Cossart *et al.*, 1975). These parvovirus-like agents are antigenically distinct from those found in feces (Paver *et al.*, 1975) and no diseases, except possibly a short febrile illness (Shneerson *et al.*, 1980) and onset of hypoplastic crisis in sickle cell anemia (Pattison *et al.*, 1981), have yet been associated with them. Particles resembling coronaviruses have also been seen in sera during routine HBsAg testing by negative staining techniques (Zuckerman *et al.*, 1970; Stannard *et al.*, 1973), but these particles are probably forms of serum lipoproteins (Ackermann *et al.*, 1974a).

D. Urine

Negative staining examination of urine after suitable concentration has revealed herpesvirus particles in patients excreting cytomegalovirus (Paradis *et al.*, 1969). Montplaisir *et al.* (1972) found electron microscopy was more successful if large volumes, 30 to 50 ml, were used. They also noted that cytomegalovirus isolation in suitable cell cultures, although slower than electron microscopy, was much more sensitive. This was confirmed, comparing isolation with pseudoreplica methods, by Lee *et al.* (1978), who observed however that electron microscopy was most sensitive in tests on children younger than 6 months; presumably in these congenital infections large numbers of cytomegalovirus particles are excreted. In contrast Henry *et al.* (1978) found that negative staining of ultracentrifugation pellets from 5-ml samples of urine was more sensitive than virus isolation, however, they were using a detection level of only four or five virus particles to a grid indicating prolonged examination in the electron microscope. The same authors examined thin sections of cells excreted in urine but saw no herpesvirus particles. Immune electron microscopy has not been used to differentiate between herpesviruses seen in urine. We have seen herpesvirus particles in urine from which herpes simplex virus has been grown; without the culture results the particles might have been assumed to be cytomegalovirus (Field and Gardner, unpub-

lished observations). Our experience with urine of immunosuppressed adults has been that cytomegalovirus is frequently cultured but rarely seen (Coleman *et al.*, 1973).

We first observed papovaviruses, of the polyomavirus subgroup, in concentrated urine samples from an immunosuppressed renal transplant recipient in 1971 (Gardner *et al.*, 1971). Since then we and other groups of workers have seen similar particles in urine of various categories of patients: renal transplant recipients (Coleman *et al.*, 1973; Lecatsas *et al.*, 1973; Dougherty and Di Stefano, 1974; Hogan *et al.*, 1980a), patients under treatment for malignancy (Reese *et al.*, 1975; Gardner, 1977), and pregnant women (Coleman *et al.*, 1977a, 1980; Lecatsas *et al.*, 1978). Sometimes the virus particles are coated with an antibody-like substance and specific antiviral antibodies can be detected in urine by negative stain IEM and other tests (Gardner *et al.*, 1971; Reese *et al.*, 1975). When sufficient virus is present in urine it is possible to identify the precise type of virus by negative stain IEM provided the particles are not already coated with antibody (Gardner, 1977).

All our attempts to detect polyomavirus particles in urine by negative stain electron microscopy have been accompanied by virus culture and cytologic studies. To confirm cytologic detection of virus the urine cells with viral inclusions were embedded for thin sectioning. Although ultrastructural preservation of the cells was poor, polyomavirus particles were clearly identified (Coleman *et al.*, 1977b). Cytology is often more sensitive than either virus isolation or negative stain electron microscopy and reprocessing in this way has increased the diagnostic potential of the electron microscope (Coleman *et al.*, 1980).

As well as typical polyomavirus particles Lecatsas and Prozesky (1975) observed filamentous and minispherical forms in one urine. Recently papillomavirus particles were seen in urine from pregnant women (Lecatsas and Boes, 1979).

Urine samples can be contaminated by feces and this may be the origin of rotavirus particles which were seen in one sample of urine of nine examined from babies excreting rotaviruses in feces (Chrystie *et cl.*, 1975).

Urine of two patients in the acute phase of non-A, non-B hepatitis contained virus-like particles 60 nm in diameter with 40-nm cores (Coursaget *et al.*, 1979).

E. Feces

In 1972 Anderson and Doane described an agar filtration technique to rid samples of undesirable salts and to concentrate virus particles

onto the grids. A sample chosen to illustrate the technique was a fecal extract and the micrograph showed particles resembling rotaviruses though the identification made was reovirus. This may be the first picture published of human rotavirus (Anderson and Doane, 1972a) though it is not clearly stated in the text that the feces were human. In the same year the detection by negative stain IEM of Norwalk virus particles in fecal extracts from volunteers in gastroenteritis experiments was reported (Kapikian *et al.*, 1972b). The observation in 1973 of rotaviruses in thin sections of duodenal biopsies of infants with diarrhea (Bishop *et al.*, 1973) was closely followed by the observation of rotaviruses in fecal extracts by negative staining methods (Flewett *et al.*, 1973; Bishop *et al.*, 1974; Middleton *et al.*, 1974). All these illustrated the suitability of electron microscopy for the diagnosis of viral agents in diarrhea and opened up a new field of fecal virology which continues to expand (Flewett, 1979; Holmes, 1979).

Fecal extracts may be examined by negative staining without concentration but this is successful only if virus content is very high. It is more usual to employ some method, usually ultracentrifugation, to concentrate virus (Flewett *et al.*, 1973). Portnoy *et al.* (1977) compared three methods: direct examination of uncentrifuged fecal extracts; a pseudoreplica method using clarified fecal extracts; and virus concentration by ultracentrifugation. They found that the last two techniques were more sensitive than direct examination and that the small viruses were more readily detected by ultracentrifugation than by the pseudoreplica method. Concentration of enteroviruses from fecal extracts by adsorption onto a polyelectrolyte followed by elution into a smaller volume for negative staining was successful for detection of 10^6 poliovirus particles per milliliter (Chaudhary and Westwood, 1972). Concentration of virus in fecal extracts by selective removal of water, salts, and low-molecular-weight substances into Lyphogel had a sensitivity equal to or greater than ultracentrifugation and the viral morphology was not affected (Whitby and Rodgers, 1980). Ammonium sulfate precipitation of viruses from fecal extracts has been found useful and the morphology of coronaviruses was particularly well preserved (Caul *et al.*, 1978). Narang and Codd (1980a) found that low-speed centrifugation of lightly clarified fecal extracts onto grids placed at the base of specially shaped tubes was sufficient for most fecal diagnostic work although smaller virus particles (20 to 35 nm) did not appear on the grids unless present in large aggregates (Narang and Codd, 1980b). Comparing Narang and Codd's method with other techniques, Roberts *et al.* (1980) found that leaving the prepared centrifuge tubes on the bench gave the same results as the low-speed centrifugation and they

suggested the aggregates of virus seen were released from infected cells which had settled on the grid to be lysed in the subsequent staining. Narang and Codd's experiments show that the comparatively high centrifugal forces used in most laboratories to clarify fecal extracts might rid the preparation of much of the desired virus particularly if it is aggregated. Juneau (1979) suggested incorporating normal human immunoglobulin into agar used in the agar-filtration techniques thus making virus particles in fecal extracts more easily recognized by coating them with antibody. However, if there is too much antibody on the particles viral structure may be obscured and identification becomes difficult. It has also been shown that human antibodies are capable of indiscriminate coating of viruses, virus-like particles, and bacteriophages (Almeida *et al.*, 1974; Locarnini *et al.*, 1974).

The diagnosis of rotavirus infection has considerable clinical value. Tests other than electron microscopy to detect rotaviruses are available (WHO Scientific Working Group, 1980) but are all limited by a requirement for high quality viral antisera. Rotaviruses have a type-specific antigen on the outer layer of capsomeres and a group-specific antigen on the inner layer. Loss of the outer layer is common and immune detection methods are imprecise as a result. Rotaviruses may already be coated with antibody when excreted (Watanabe and Holmes, 1977) which also renders detection by immune methods more difficult. This has ensured a continued role for electron microscopy in rotavirus diagnosis. In addition electron microscopy is a nonselective method and in the search for rotaviruses other agents may be revealed.

Aberrant rotavirus capsids in tubular form are sometimes observed. Negative stain IEM studies showed these were antigenically identical to the spherical capsids (Holmes *et al.*, 1975). Human rotavirus subtypes have been identified by various techniques including negative stain IEM (Zissis and Lambert, 1978).

Adenovirus particles have been observed by negative staining in feces of patients with gastroenteritis and frequently are noncultivable (Bruce White and Stancliffe, 1975; Bryden *et al.*, 1975). It has recently been demonstrated that they belong to a new adenovirus serotype (Johansson *et al.*, 1980).

Coronavirus-like particles have been seen in human feces but their presence often cannot be correlated with illness (Caul *et al.*, 1975; Mathan *et al.*, 1975; Schnagl *et al.*, 1978; Clarke *et al.*, 1979). Morphologically they differ from the classical coronavirus by having greater pleomorphism and narrower surface projections (Caul and Egglestone, 1977; Caul *et al.*, 1977). The viral nature of these particles

has not yet been established conclusively. Although coronavirus-like particles were seen in the abortive replication cycle in cell culture the thin sections failed to show typical coronavirus maturation stages (Caul and Egglestone, 1977). The viral nature of the fecal particles has been questioned by Dourmashkin *et al.* (1980) who sectioned a fecal extract pellet which by negative staining contained typical pleomorphic coronavirus-like particles. No typical coronaviruses were seen in the thin sections although large numbers of fringed membrane-bound objects were present. However, it is possible that the pleomorphism of the negatively stained particles reflects some abnormality, such as lack of core material, which would give rise to this appearance in thin sections.

Norwalk agent was first described in feces from volunteers with experimental gastroenteritis (Kapikian *et al.*, 1972b). Unlike the smooth-surfaced parvoviruses and picornaviruses, negatively stained Norwalk particles appear to have a structured surface and edge. Particles are 27 to 34 nm in diameter with a density of 1.36 to 1.41 gm/ml. Particles similar in appearance and antigenically related were observed in feces in a gastroenteritis outbreak in Montgomery County and other morphologically similar but antigenically unrelated particles were reported from Hawaii (Thornhill *et al.*, 1977). Particles resembling Norwalk morphologically and antigenically, 27 to 30 nm in diameter and with density 1.38 gm/ml, were excreted by patients with gastroenteritis following the consumption of oysters in Australia (Cross *et al.*, 1979; Murphy *et al.*, 1979). Two further agents morphologically identical to Norwalk, with densities 1.35 to 1.37 and 1.37 to 1.4 mg/ml, but both antigenically distinct from Norwalk were found in feces of gastroenteritis patients in Japan (Taniguchi *et al.*, 1979; Kogasaka *et al.*, 1980). Morphologically similar particles have also been seen in the United Kingdom and, based on the examination of these particles and of Norwalk agent, it has been suggested that there are morphological similarities with caliciviruses (Caul *et al.*, 1979).

Viruses morphologically indistinguishable from classical caliciviruses were observed in human feces (Madeley and Cosgrove, 1976) but were not associated with illness until more recently when they were implicated in winter vomiting disease and gastroenteritis (McSwiggan *et al.*, 1978; Chiba *et al.*, 1979, 1980; Cubitt *et al.*, 1979, 1980; Suzuki *et al.*, 1979). Negative stain IEM has been used in these studies to detect the appearance of antibodies to the agents seen and to investigate the antigenic nature of the caliciviruses.

Particles measuring 26 to 30 nm with a solid star-shaped pattern on their surface have been termed astroviruses. The surface pattern dif-

fers from the hollow Star of David pattern on the 29- to 33-nm-diameter calicivirus. Madeley (1979) has described the differential morphology in detail. Astroviruses were first observed in negative staining studies of feces of babies in maternity ward outbreaks of gastroenteritis and in other infants with gastroenteritis (Madeley and Cosgrove, 1975).

Parvovirus-like particles with a diameter of 22 nm were first observed in human feces by Paver *et al.* (1973). For their detection it was found necessary to use negative stain IEM with postinfection human sera to agglutinate the particles. Identification of parvoviruses depends upon the smooth-surfaced morphology; the size, which is slightly smaller than the morphologically similar enteroviruses; and the density, which is higher than enterovirus density. Because of overlap in both size and density ranges between these two groups precise identification is often impossible. Fecal parvovirus-like agents are noncultivable whereas enteroviruses can usually be grown in cell cultures. Appleton *et al.* (1977) described 26-nm parvovirus-like particles with density 1.38 to 1.40 gm/ml in feces from a school outbreak of winter vomiting disease (the Ditchling agent) and other 25- to 26-nm parvovirus-like particles with density 1.40 gm/ml (Appleton and Pereira, 1977) in feces of patients with gastroenteritis after eating cockles (cockle agent). Negative stain IEM showed that these two agents differed antigenically, both were unrelated to Norwalk but Ditchling was related to the W agent, an earlier reported parvovirus-like particle (Paver *et al.*, 1973). Another school outbreak of gastroenteritis was associated with a 23- to 26-nm parvovirus-like particle but no density estimations or cross-reactions with other parvovirus-like agents were described for the Paramatta agent (Christopher *et al.*, 1978). Although Norwalk was assumed to be the cause of the Australian oyster-associated gastroenteritis, parvovirus-like particles, 22 to 25 nm in diameter, were also seen in many of the fecal samples and similar particles were seen in one oyster sample (Murphy *et al.*, 1979).

Negative staining examination of feces of hepatitis patients, originally in a search for HBsAg and latterly for hepatitis A virus, has revealed interesting particles. Cross *et al.* (1971) found 15- to 25-nm-diameter small round particles and 35- to 45-nm-diameter particles resembling Dane particles. By gel diffusion and negative stain IEM there was no antigenic similarity between the smaller particles and the small round particles of serum HBsAg but slight cross-reactivity between the larger particles and the Dane particles of HBsAg was observed. Moodie *et al.* (1974) observed that gut digestive enzymes would degrade all hepatitis B antigen with the exception of the Dane particle

cores and in fact there have been no convincing electron microscopy reports of hepatitis B particles in feces.

The causative agent of hepatitis A was revealed as a 27-nm-diameter small, round, smooth-surfaced virus particle by Feinstone *et al.* (1973) who used volunteers' fecal samples and negative stain IEM with convalescent sera to detect the virus. Excretion of the particles was time related to symptoms. These findings have been confirmed by others (Locarnini *et al.*, 1974; Gravelle *et al.*, 1975) and particles seen in different outbreaks have been found to be antigenically identical. Coulepis *et al.* (1980) showed that maximal virus excretion occurred just before the onset of symptoms, fell slightly in the 5 days after onset while patients had dark urine, and then reduced steadily until by 2 weeks after onset virus was only just detectable by electron microscopy.

F. Breast Milk

Breast milk has been tested for HBsAg by negative stain IEM after concentration of the samples by ultracentrifugation and small round particles have been seen (Boxall *et al.*, 1974), but no strict tests were done to exclude the presence of occult blood.

Following the analogy of the mouse mammary tumor virus human breast milk has been surveyed for retroviruses. Particles resembling type B oncoviruses were observed by Moore *et al.* (1969) using thin sections and negative staining in parallel with biochemical studies on fractionated milk. Some samples of human breast milk degraded the structure of true type B oncovirus particles added to them (Sarkar and Moore, 1972) rendering the particles unrecognizable. Chopra *et al.* (1973) found type D oncovirus-like particles in breast milk using negative staining but could not correlate electron microscopy and biochemical findings.

G. Cerebrospinal Fluid

It is possible to demonstrate viral antigens by immunofluorescence in cells in cerebrospinal fluid (CSF) in viral encephalitis or meningitis (Dayan and Stokes, 1973; Taber *et al.*, 1973). A paramyxovirus has been seen in CSF by negative staining and mumps virus was isolated from the sample (Doane *et al.*, 1967). Thin sections of CSF cells in presumed mumps virus meningitis showed cytoplasmic collections of tubules resembling paramyxovirus nucleocapsid (Herndon *et al.*, 1974).

H. Tissues

Most human tissues have been examined by electron microscopic methods for virus particles at some time. However, it is comparatively rare for such investigations to be included in routine viral diagnosis. Sample selection is a considerable problem for both thin-sectioned and negatively stained preparations of tissues. Immune electron microscopy on sectioned material presents considerable technical difficulties and viral content of tissue homogenates may be too low for negative stain IEM so a virus seen in the tissue cannot always be sufficiently well identified for diagnostic purposes. Immunofluorescence and other light microscopy immune methods have greater diagnostic potential because they are simpler, larger samples are used, and precise virus identification is possible. Nevertheless, electron microscopy has been important in revealing viral etiology, often for the first time, and it has frequently been the impetus for development of the more convenient light microscopy techniques. Of recent years examination of tissues for viruses by electron microscopy has concentrated upon three major areas: the brain, the liver, and tumors. These applications illustrate well the techniques, problems, and achievements in viral diagnosis by electron microscopy of tissues.

1. Brain

Ultrastructural studies of virus infections of the human brain have been reviewed recently (Mirra and Takei, 1976).

The light microscopy neuropathology of brain affected by the rare condition progressive multifocal leukoencephalopathy (PML) was first described by Åström *et al.* (1958). Lesions were later shown by thin section electron microscopy to contain papovavirus particles by Zu Rhein and Chou (1965). Extracts of formalin-fixed brain were negatively stained and typical papovavirus particles were observed which were 41 nm in diameter and were clearly members of the polyomavirus subgroup (Howatson *et al.*, 1965). Virus particles were also demonstrated in unfixed PML brain homogenates by negative staining (Schwerdt *et al.*, 1966). It was not until 1971 that a new polyomavirus, JC virus, was cultivated from PML brain (Padgett *et al.*, 1971). The following year two isolates of a polyomavirus antigenically similar to simian virus 40 (SV40) were reported from PML brains (Weiner *et al.*, 1972) but all subsequent strains isolated from such material have been identified as JC virus (Padgett *et al.*, 1976). In thin sections of brain typical spherical polyomavirus particles are found in the nuclei and cytoplasm of oligodendrocytes and filamentous forms of the virus are

also frequently present (Fig. 13). Brain homogenates may be so rich in virus that concentration by ultracentrifugation before negative staining is unnecessary. Filamentous particles have not been seen in negatively stained brain extracts even when they were plentiful in the thin sections (Field, unpublished observations). When JC virus had been grown *in vitro* and specific antisera were prepared, virus particles extracted from infected brains were identified by negative stain IEM and parallel immunofluorescence studies were performed on brain sections (Narayan *et al.*, 1973). For successful negative stain IEM virus content of the brain sample must be high and virus particles must be free of cell debris. Because JC virus is relatively difficult to cultivate this technique has great potential. Although diagnosis of the polyomavirus infection by recognition of the typical particles in thin sections and negative stains of formalin-fixed brain is straightforward, it has proved impossible to identify the virus antigenically by negative stain IEM after formalin fixation (Narayan *et al.*, 1973; Padgett *et al.*, 1976; Field and Gardner, unpublished observations).

Laboratory diagnosis of infection with the measles-like virus of subacute sclerosing panencephalitis is generally based upon detection of measles antibodies in serum and in cerebrospinal fluid. In rare

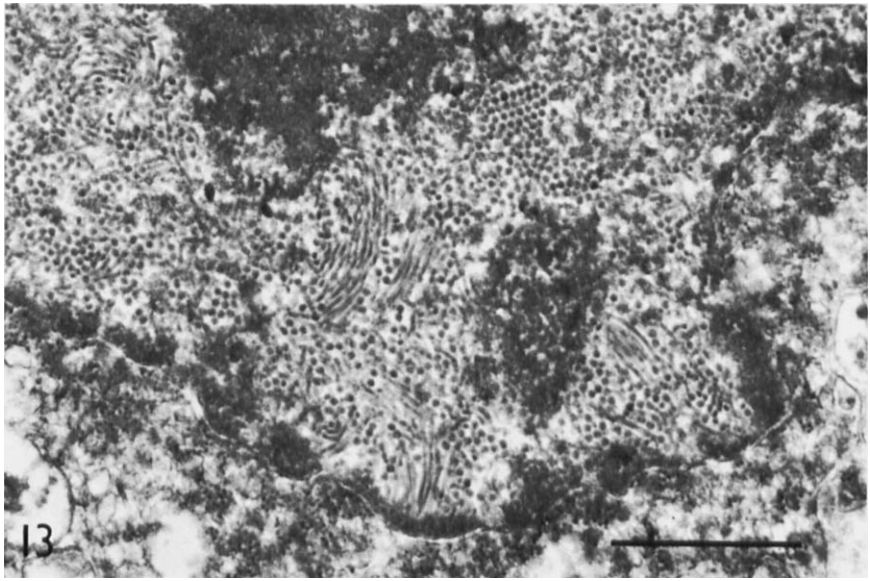


FIG. 13. Thin section of PML brain showing both spherical and filamentous polyomavirus particles within the nucleus. ×25,000. Bar = 1 μm.

cases when brain biopsy is performed the usual laboratory diagnosis is by immunofluorescence with specific measles virus antiserum or, less desirably, measles convalescent human serum. Confirmation by electron microscopy may be sought because virus culture, although possible (Chen *et al.,* 1969; Horta-Barbosa *et al.,* 1969), is technically difficult. Thin sections of SSPE brain have been found to contain collections of 17- to 19-nm-diameter tubular paramyxovirus nucleocapsids in nuclei and cytoplasm of oligodendrocytes and neurons (Herndon and Rubinstein, 1968) (Fig. 14). The nuclear particles are clearly tubular and have no surface coating but cytoplasmic nucleocapsid is usually coated with a granular substance. There have been only two reports of clearly recognizable paramyxovirus nucleocapsid in negatively stained SSPE brain homogenates (Dayan and Cumings, 1969; Dayan and Almeida, 1975). We have examined concentrated homogenates from nine SSPE brains, of which four contained typical SSPE viral tubules in thin sections and two contained measles antigen by immunofluorescence, but in none have we seen any paramyxovirus nucleocapsid by negative staining (Richmond and Field, unpublished observations). Antigenic identification of the virus seen in brain is thus dependent

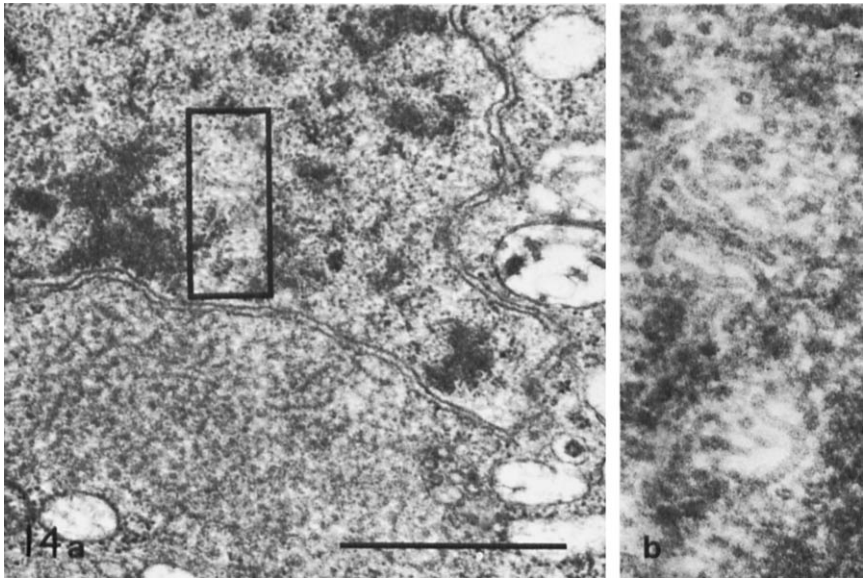


FIG. 14. Thin section of SSPE brain showing (a) intranuclear and cytoplasmic paramyxovirus nucleocapsids, ×30,000, bar = 1 μm; (b) enlarged boxed area from (a) of intranuclear nucleocapsids, ×100,000. (Courtesy of Mrs. J. E. Richmond.)

upon thin section IEM with all its attendant technical difficulties (Jenis *et al.*, 1973). SSPE brain cells cultured *in vitro* have been shown to contain paramyxovirus nucleocapsids in nuclei and cytoplasm (Chen *et al.*, 1969; Katz *et al.*, 1969) and typical negatively stained paramyxovirus helix has been seen in extracts of these cultured cells (Iwasaki and Koprowski, 1974).

In cases of suspected herpes encephalitis laboratory confirmation of herpes simplex is sometimes urgently required and the most sensitive and rapid method for this is immunofluorescence on brain biopsy material. Herpesvirus particles are found in thin sections (Fig. 15) in nuclei and cytoplasm of neurons and glial cells (Harland *et al.*, 1967; Roy and Wolman, 1969; Baringer and Swoveland, 1972; Viloria and Garcia, 1976). The thin section technique is too slow for rapid diagnosis and as virus-infected cells are distributed unevenly sample selection is a major problem. Virus can be seen in negatively stained brain homogenates but this procedure is comparatively insensitive (Flewett, 1973; Ross, 1973; Joncas *et al.*, 1975). In our experience virus particles are extremely sparse even after ultracentrifugation of brain homogenates (Field, Porter, and Richmond, unpublished observations). Other herpesviruses besides herpes simplex virus can infect brain: McCormick

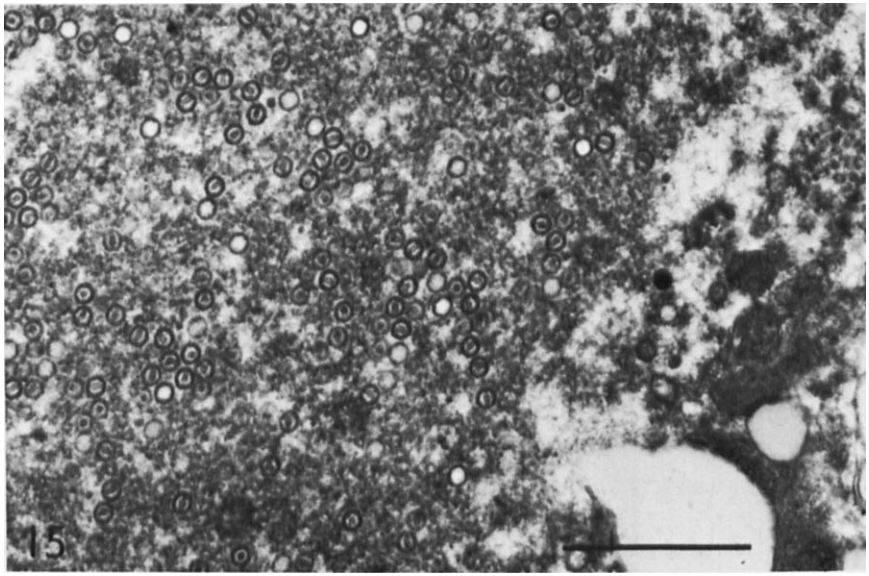


FIG. 15. Thin section of herpes encephalitis brain showing herpesvirus particles. ×25,000. Bar = 1 μm.

et al. (1969) saw herpesvirus particles in glial cells in thin sections of the brain from a patient with encephalomyelitis and cultured varicella zoster virus. In the absence of virus culture complete identification of an observed herpesvirus is difficult. Thin section IEM is technically difficult although possible (Kumanishi and Hirano, 1978); negative stain IEM is unsatisfactory because there is insufficient virus and even by immunofluorescence there are problems with antigenic cross-reactions with other herpesviruses (Emmons and Riggs, 1977).

Electron microscopy has not on the whole proved useful for the laboratory diagnosis of rabies infection of human brain. The methods more generally used are histology, immunofluorescence, and virus isolation. The Negri bodies in histological sections are sometimes but not always identical with the cytoplasmic viral factory areas of thin sections (Morecki and Zimmerman, 1969; Lemercier *et al.*, 1970; Vallat *et al.*, 1977) and rabies virus particles may bud from factory sites or from diverse cytoplasmic membranes of neurons. Factory sites and virus particles are easily recognized in thin sections. Cell culture-derived rabies virus is difficult to identify in negatively stained preparations because of its fragility, indicating that attempts at rapid diagnosis by this technique on brain samples would be unsatisfactory.

Adenovirus encephalitis was investigated by Chou *et al.* (1973) who found inclusion bodies in histological sections and typical, mostly intranuclear, adenovirus particles in neurons and glial cells in thin sections. Adenovirus particles were also seen in negatively stained brain homogenate, from which adenovirus type 32 was later isolated.

Investigating eastern equine encephalomyelitis, Bastian *et al.* (1975) isolated the virus, found typical histology, and for the first time demonstrated typical togavirus particles in thin sections of human brain.

Particles resembling paramyxovirus nucleocapsids have been observed in thin sections of multiple sclerosis brain lesions, mostly sited in nuclei with probable leakage to the cytoplasm (Prineas, 1972; Lhermitte *et al.*, 1973; Watanabe and Okazaki, 1973) and sometimes exclusively in the cytoplasm (Narang and Field, 1973; Pathak and Webb, 1976). However, similar particles have been seen in unrelated conditions of the brain, in normal brain, and in other organs; immunofluorescence and IEM studies have failed to identify the particles as viral. Suggestions have been made that abnormal condensation of nuclear chromatin and multiple invaginations of cytoplasmic membranes might be the cause of these virus-like structures (Baringer and Swoveland, 1972; Dubois-Dalcq *et al.*, 1973; Tanaka *et al.*, 1974, 1975b; Hayano *et al.*, 1976; Kirk and Hutchinson, 1978; Lehrich and Arnason, 1978). Although a paramyxovirus (parainfluenza virus type 1) was re-

trieved from multiple sclerosis brains by cell fusion techniques, no virus particles were seen in thin sections of the original brains, although some cytoplasmic nucleocapsid was seen at the eleventh pass of brain cells in culture (Ter Meulen *et al.*, 1972). Not surprisingly, in view of the experience with SSPE brain, no paramyxovirus particles have been reported in negatively stained extracts of multiple sclerosis brain.

Particles identified by Bastian (1971) as papovaviruses were seen in cytoplasm and extracellularly in a human choroid papilloma, but the particles were not morphologically characteristic and the lack of intranuclear particles was unusual. Similar particles in other human choroid papillomas were shown to be glycogen (Carter *et al.*, 1972). Early reports claimed ultrastructural evidence for association of a papovavirus with the SSPE paramyxovirus (Koprowski *et al.*, 1970; Oyanagi *et al.*, 1970) but none of the published micrographs has convincingly demonstrated papovavirus and the particles were never intranuclear and were seen only in cultured brain cells and not in the original tissue. A large number of 45-nm-diameter granules with irregular edges were observed in astrocyte cell processes in a Creutzfeldt–Jakob diseased brain and were identified as papovaviruses although the morphology was not convincing (De Reuck *et al.*, 1976). Kirk and Hutchinson (1978) believe that these cytoplasmic papovavirus-like structures are probably reticulosomes and related structures normally present in cells.

Creutzfeldt–Jakob disease has some characteristics of a virus infection and brain tissue has been examined by electron microscopy for a possible etiologic agent. The histology, characteristic of a spongioform encephalopathy, is the usual means of laboratory diagnosis. In thin sections tubular particles and variably sized round and hexagonal particles with and without cores have been reported (Vernon *et al.*, 1970; Bots *et al.*, 1971; Narang, 1975), but none has been demonstrated to be viral. Recently objects resembling spiroplasmas have been observed in thin sections of Creutzfeldt–Jakob brains (Bastian, 1979; A. Gray *et al.*, 1980).

2. Liver

Infection of the liver with hepatitis B virus is accompanied by ultrastructural changes. Parvovirus-like particles were first described in hepatocyte nuclei in thin sections by Nowosławski *et al.* (1970) and these observations were soon confirmed (Nelson *et al.*, 1970; Scotto *et al.*, 1970) (Fig. 16). For some time it was assumed that these particles were identical to the small round particles of HBsAg in negatively stained serum and immunofluorescence and thin section IEM using

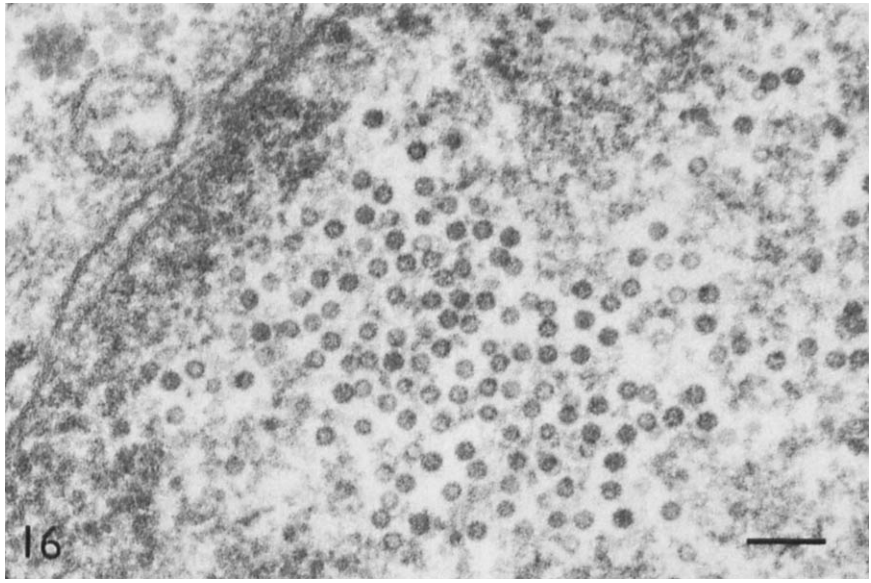


FIG. 16. Thin section of human liver showing intranuclear HBcAg particles. ×100,000. Bar = 100 nm. (Courtesy of Mrs. J. E. Richmond.)

human antisera tended to confirm this impression (Gerber *et al.*, 1972; Huang *et al.*, 1972). Meanwhile cytoplasmic particles resembling the pleomorphic forms of serum HBsAg were seen in liver cells in thin sections (Huang, 1971; Stein *et al.*, 1971). By immunofluorescence, sera specific for HBsAg revealed antigen in cytoplasm and sera specific for HBcAg traced a nuclear antigen and so it was suggested that the nuclear, parvovirus-like particles must be HBcAg and the pleomorphic cytoplasmic particles HBsAg (Gerber *et al.*, 1974; Gyorkey *et al.*, 1974). Thin section IEM has confirmed this and has also shown HBcAg in cytoplasmic maturing Dane particles (Huang and Neurath, 1979). Examination of liver homogenates with negative staining had already shown the three morphologic forms of HBsAg and when 25- to 27-nm round particles were also seen it was suggested that these might be the intranuclear, parvovirus-like particles since they were certainly antigenically different from the particles of HBsAg (Almeida *et al.*, 1970; Huang and Groh, 1973a). Extracts of heavily infected human liver are a good source of HBcAg for detection of antibodies by negative stain IEM (Cohen and Cossart, 1977; Cohen, 1978).

Recent reports have described intranuclear, roughly spherical, 27-nm-diameter particles in human liver thin sections in non-A, non-B

hepatitis (Gmelin *et al.*, 1980; Grimaud *et al.*, 1980). These particles are however somewhat unconvincing as virus because of their size range and irregular outlines. Particles with similar morphology to the HBcAg have been extracted from non-A, non-B liver but they are antigenically distinct (Hantz *et al.*, 1980).

Herpesvirus particles have been seen in the nuclei and cytoplasm of liver cells of a patient who died with infectious mononucleosis and extensive hepatic necrosis (Chang and Campbell, 1975). Arenavirus particles were described in liver thin sections of a patient with Lassa fever (Winn *et al.*, 1975) and of another patient with Argentine hemorrhagic fever (Junin virus) (Maiztegui *et al.*, 1975). Ebola virus particles have been observed in large numbers in hepatic cells and bile canaliculi in human infection (Ellis *et al.*, 1978).

3. Tumors

The benign human tumors molluscum contagiosum and warts have been shown by electron microscopy to have poxvirus and papovavirus etiology, respectively, and have already been described in Section IV,A. Papovavirus-like particles measuring 45–50 nm were seen in a cell line derived from a nephroblastoma (Wilms' tumor) at the fortieth and subsequent pass levels. The particles were recognized in nuclei in thin sections but were less convincing in negatively stained preparations. Attempts to confirm the findings on other Wilms' tumor cell lines were fruitless and no virus particles were seen in the original tumor (Smith *et al.*, 1969).

Although herpesvirus particles of EB virus have frequently been observed in cultured Burkitt lymphoma cells by thin section electron microscopy since they were first described by Epstein *et al.* (1964), there has been no report of such particles in the original tumor. EB virus particles have also been seen in lymphoblastoid cell lines derived from nasopharyngeal carcinoma (Vuillaume and de Thé, 1973), in leukemic buffy coat cell cultures (Zeve *et al.*, 1966), and in lymphoid cell lines derived from lymph nodes from patients with various cancers (Jensen *et al.*, 1967), and in one of these cases herpesvirus particles were seen in a few cells of the original lymph node biopsy. Epstein–Barr virus particles have also been seen in lymphoid cell lines from patients with infectious mononucleosis (Moses *et al.*, 1968; Steel and Edmond, 1971), with hepatitis (Douglas *et al.*, 1969), and from an apparently normal patient (Moore *et al.*, 1967). Generally thin section methods have been used to detect the virus. Negative staining has been used (Hummeler *et al.*, 1966) but appears to be less sensitive (Moses *et al.*, 1968; Hillman *et al.*, 1977). The sophisticated immunofluorescence

tests for the various EB virus antigens have supplanted electron microscopy in diagnosis particularly as even uncultured Burkitt tumor cells carry one of these antigens. Katayama *et al.* (1974) suggested the morphology of Burkitt tumor cells in thin sections was so characteristic, even though no virus was seen, that electron microscopic diagnosis was superior to histology. Full identification of particles seen in cultured lymphoid cell lines is not always attempted, but herpesvirus particles seen in cell lines from patients with Kaposi's sarcomas were associated with cytomegalovirus in some instances (Giraldo *et al.*, 1972).

The search for retroviruses in human tumors, cell cultures of tumors, and in placentas by electron microscopy was reviewed by De Harven (1974) who reluctantly concluded that the morphologic evidence for human types A, B, and C oncoviruses at that time was extremely slight. Since 1974 the situation has not changed markedly. Tests for biochemical markers for the presence of a retrovirus are now frequently performed in parallel with electron microscopy. Such parallel studies have utilized thin sectioning (Birkmayer *et al.*, 1974; Warnaar *et al.*, 1976) and negative staining (Mak *et al.*, 1974) of fractionated cell extracts.

Retrovirus-like particles were seen in cultured myeloid cells from a patient with acute myelogenous leukemia but not in the original tumor, although there was biochemical evidence for retrovirus in the uncultured cells (Gallagher and Gallo, 1975). This virus was later shown to be closely related to simian sarcoma-associated virus isolated from a woolly monkey fibrosarcoma.

The placenta has provided somewhat better morphological evidence for human retroviruses (Dalton *et al.*, 1974; Imamura *et al.*, 1976) but the viral morphology was not absolutely comparable with other mammalian C-type particles (Dalton *et al.*, 1974; Dirksen and Levy, 1977). Similar particles were detected in cultured testicular tumor cells but parallel biochemical studies were negative (Bronson *et al.*, 1979). Human embryonic cells in culture have been studied and particles resembling type C oncoviruses have been observed (Chandra *et al.*, 1970; Panem *et al.*, 1975).

Types A, B, and C oncoviruses have somewhat variable structure in both thin sections and negatively stained preparations (Sarkar and Moore, 1972; Sarkar *et al.*, 1975) which makes morphological diagnosis difficult. The budding stage of maturation, which is perhaps the most convincing evidence of the viral nature of these particles, has rarely been observed in human tissues. The negatively stained morphology, particularly of type C oncoviruses, is so variable that this method is

unsuitable for diagnosis although Lo and Howatson (1978) have suggested detergent treatment before negative staining to standardize particle shape.

The other retrovirus subgroup, the Spumaviridae, have more distinctive morphology. Although not seen in the original tumor a spumavirus was identified in thin sections of cultured nasopharyngeal carcinoma cells (Achong *et al.*, 1971).

V. Application of the Techniques to Laboratory Samples

Electron microscopy alone rarely gives a sufficiently specific diagnosis of viral infection on samples taken directly from the patient and other techniques are generally employed to reach a more conclusive result. In the course of laboratory tests on such samples electron microscopy can be used to detect the presence of virus in inoculated cell cultures, in the precipitin lines of gel immunodiffusion tests, and following passage of an agent in laboratory animals. Morphology can be very helpful in the preliminary identification of the virus, and, provided suitable controls are examined, electron microscopy can also detect contamination with endogenous viruses from cell cultures. Density gradient studies to characterize a virus are often monitored by electron microscopy, particularly if the virus is noncultivable. Simple negative staining techniques are most useful and negative stain IEM can be used for more specific identification of viruses seen. Thin sections are useful to detect viruses which do not have clear negatively stained morphology, such as rubella virus, and thin sections are also useful in examination of tissues from inoculated laboratory animals.

A. Cell Cultures

Viruses inoculated into cell cultures usually take some days to grow and produce typical cytopathic effects. Negative staining can give early confirmation of the presence of a virus and preliminary identification by its morphology. This is a useful diagnostic aid in most circumstances and particularly so when the diagnosis is urgent as in dangerous infections such as Ebola (Bowen *et al.*, 1977; Johnson *et al.*, 1977). But early electron microscopic examination of cultures, before cytopathic effects are well advanced, is often fruitless (Field, unpublished observations). However, the use of negative stain IEM in-

creased the sensitivity of virus detection so that cultures examined only 24 hours after inoculation showed virus particles (Edwards *et al.*, 1975). Rhinoviruses are particularly difficult to detect in cell cultures by negative staining (Tyrrell and Almeida, 1967) and Kapikian *et al.* (1972a) found negative stain IEM was better able to detect these viruses in crude cell culture extracts. In practice diagnostic use of IEM is seldom feasible because of the large number of possible serotypes.

We have found that examination of inoculated cell cultures by negative staining is useful for rapid differentiation of viruses when initial biological observations cannot group them. Examples are differentiation of myxoviruses from paramyxoviruses, adenoviruses from herpesviruses, and vaccinia virus (an orthopoxvirus) from enteroviruses.

Electron microscopy of viruses which grew in cell cultures without clearly discernible cytopathic effects was used in initial work with human coronaviruses (Almeida and Tyrrell, 1967; McIntosh *et al.*, 1967; Tyrrell and Almeida, 1967) and human polyomaviruses (Gardner *et al.*, 1971). This procedure has remained useful in our laboratory to monitor growth of the human polyomaviruses both on isolation and further passage in cell cultures.

Viruses which are endogenous in cell cultures are a particular hazard in diagnostic laboratories and electron microscopy by negative staining and thin sectioning is useful for the detection of these agents (Anderson and Doane, 1972b). Frequently endogenous viruses cause no cytopathic effects to arouse suspicion of their presence. For those who work with monkey kidney cell cultures the simian paramyxovirus SV5 and the simian polyomavirus SV40 have been particular problems, but these agents can be easily detected in negatively stained preparations. On the other hand simian foamy virus, a retrovirus, can be easily detected by its cytopathic effect but by electron microscopy is more difficult, thin sections being more sensitive than negative staining (Anderson and Doane, 1972b). Polyomaviruses have been detected by electron microscopy in Vero cell lines (Waldeck and Sauer, 1977; Gardner and Field, unpublished observations) and in pig kidney cell lines (Newman and Smith, 1972; Tischer *et al.*, 1974). BHK-21 hamster cell lines carry the hamster R virus detectable in thin sections (Shipman *et al.*, 1969). Parvoviruses were detected in many continuous cell lines by techniques which included electron microscopy (Hallauer *et al.*, 1971). It was assumed that many of these originated from trypsin used when subculturing, but recently parvoviruses detected in cultured cells had as their source the calf serum used in culture media (Nettleton and Rweyemamu, 1980).

B. Gel Diffusions

Virus particles may be purified by gel electrophoresis. The particles are concentrated into certain regions of the gel which can then be extracted for electron microscopy. Weintraub *et al.* (1962) purified plant viruses from plant sap by this technique and examined the gel fractions by metal shadowing to prove the purity of the harvests. Ahmad-Zadeh *et al.* (1968) used negative staining techniques on gel electrophoresis eluates to prove separation of adenovirus soluble antigens into hexons, fibers, and pentons.

Gel immunoprecipitin lines formed between viral antigens and homologous antibodies can be treated in the same way and the specificity of the lines becomes evident when the expected virus particle–antibody complexes are seen. Beale and Mason (1968) investigated the antigenic nature of full and empty poliovirus particles this way. Huang and Groh (1973b) applied the technique to HBsAg–antibody and HBcAg–antibody reactions. However, Almeida *et al.* (1974) found that immunoprecipitin lines between fecal extracts and human sera were frequently devoid of recognizable virus particles. When gel precipitin lines were examined for herpesviruses in thin sections by Konn *et al.* (1969), of the three lines formed between EB virus and a rabbit antiserum, herpes particles, which were surrounded by antibody, were seen in only one line.

C. Density Gradients

Fractions from density gradient centrifugation can be examined in the electron microscope for virus particles to determine their buoyant density. The cesium or sucrose must be removed before examination by application of the methods for eliminating salts described in Section III,A. Use of the technique depends upon the presence in the fractions of enough virus for particles to be detectable either by negative staining or by negative stain IEM. A typical study was that of Torikai *et al.* (1970) on a parvovirus using negative stain on the gradient fractions: particles banding at a density of 1.43 gm/ml were complete and were not penetrated by stain; particles in the 1.34 gm/ml fraction were partially penetrated by stain but were intact; at 1.30 gm/ml only shells completely penetrated by stain were seen. Parallel studies showed the particles with 1.43 gm/ml density contained normal nucleic acid while those at 1.34 gm/ml had little if any nucleic acid.

Cultivable virus can be located in density gradient harvests by its infectivity but for noncultivable viruses electron microscopy is a prac-

tical detection method which has been widely applied in density determination of agents such as the fecal parvovirus-like particles (Paver *et al.*, 1974; Appleton *et al.*, 1977; Appleton and Pereira, 1977), Norwalk agent (Kapikian *et al.*, 1973), other fecal agents resembling Norwalk (Kogasaka *et al.*, 1980), and HBsAg (Bond and Hall, 1972). The interpretation of results in the absence of infectivity experiments can be difficult as shown by the discrepancy in original estimates of the density of hepatitis A virus. Some estimates gave a peak density around 1.4 gm/ml, consistent with parvoviruses (Feinstone *et al.*, 1974), while other estimates were 1.34 gm/ml, consistent with enterovirus density (Provost *et al.*, 1975b; Maynard *et al.*, 1975). Eventually, general agreement was reached that the latter estimates were correct (Moritsugu *et al.*, 1976; Schulman *et al.*, 1976). The confusion arose because both enteroviruses and parvoviruses display multiple peaks in density gradients and the ranges overlap significantly.

D. Laboratory Animals

Laboratory animals are sometimes inoculated with known viruses in order to develop model systems for the study of human disease. Examples are picornavirus-induced hepatitis in mice (Burch *et al.*, 1973), adenovirus infection of mouse adrenal glands as a model for Allison's disease (Hoenig *et al.*, 1974), and parainfluenza type 1 infection of mouse brain as a model for multiple sclerosis (Tanaka *et al.*, 1975a). Electron microscopy of affected tissues in these experimental infections can ultimately be useful in providing examples of what might be expected in the human diagnostic situation.

Laboratory animals infected with viruses which do not grow in cell cultures may generate sufficient virus for use as reagents in diagnostic tests. Production of HBcAg from chimpanzee livers for use in detecting antibodies by complement fixation (Hoofnagle *et al.*, 1973) was suggested by thin sectioning electron microscopy studies which demonstrated intranuclear HBcAg particles in liver cells, the antigenicity being confirmed by immunofluorescence (Barker *et al.*, 1973). Similarly, thin section studies on livers of marmosets infected with hepatitis A virus showed cytoplasmic picornavirus-like particles which could be extracted for use as antigen to detect antibodies by negative stain IEM (Provost *et al.*, 1975b) and by complement fixation (Provost *et al.*, 1975a).

Specimens from animals infected with virus-containing material may be examined by electron microscopy, using all the same techniques as for human samples. The GB hepatitis agent passaged in

marmosets has been examined using serum, liver, and feces in negative staining and negative stain IEM studies (Almeida *et al.*, 1976; Dienstag *et al.*, 1976b; Appleton, 1977), but no firm conclusions resulted. Current descriptions of non-A, non-B hepatitis agents in chimpanzees are similarly conflicting (Bradley *et al.*, 1979; Shimizu *et al.*, 1979; Tsiquaye *et al.*, 1980; Yoshizawa *et al.*, 1980).

VI. Artifacts

A. Negative Stains

Since any sample examined by negative staining in diagnostic virology is likely to contain some cellular debris it is important to appreciate that this can give rise to artifacts which can be confused with virus particles. Cellular membranes may bear projections comparable in size with the surface projections of orthomyxoviruses, paramyxoviruses, and rhabdoviruses (Cunningham and Crane, 1966; Berg *et al.*, 1969). Most cellular membrane projections tend to be globular rather than spike-like, particularly those on mitochondrial internal membranes, and with practice it is easy to differentiate such artifacts from virus particles. Particles with 5- to 10-nm projections are occasionally seen in sera and have been tentatively identified as coronaviruses, but Ackermann *et al.* (1974a), by enzyme digestion experiments, proved they were composed of lipoprotein. It is worth noting that negatively stained cell debris is indistinguishable from mycoplasmas (Wolanski and Maramorosch, 1970), thus the negative staining technique should not be used for the detection of mycoplasma contamination of cell cultures and thin sectioning and even scanning electron microscopy are the methods of choice (Boatman *et al.*, 1976).

Small lipoprotein particles in some sera have diameters about 20 to 23 nm and closely resemble the small round particles of HBsAg (Solaas, 1978). While individual particles are difficult to distinguish from HBsAg they tend to cluster together into a palisade which differs from HBsAg immune complexes because the edges of adjacent particles tend to flatten against one another (Fig. 17).

Virus-like artifacts in feces are common and are a serious problem since confirmation of the viral nature of the particles seen by culture is rarely possible. Bacterial cell walls often display substructure similar to arrays of small virus particles (Dalen, 1978). The smaller isometric bacteriophages and viruses from edible plants which might be expected to make an occasional appearance in feces are often comparable in size,

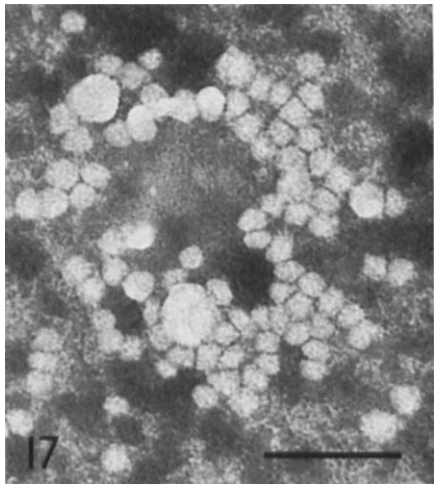


FIG. 17. Artifact in human serum resembling hepatitis B antigen. Negative stain. ×180,000. Bar = 100 nm.

appearance, and buoyant density with the small fecal viruses (Tikhonenko, 1970; Brown and Hull, 1973). Even the development of antibodies to an agent is not proof that it is a human virus as human convalescent sera do contain antibodies to fecal bacteriophage particles (Almeida *et al.*, 1974; Locarnini *et al.*, 1974).

B. Thin Sections

Brief consideration has already been given to papovavirus-like and paramyxovirus-like particles in thin sections resulting from artifacts (Section IV,H,1). Kirk and Hutchinson (1978) have explained cytoplasmic particles in both these categories as normal cellular components. Cytoplasmic paramyxovirus-like tubules in dilated cisternae of endoplasmic reticulum have often been described. In high-quality micrographs Schürch and Fukuda (1974) demonstrated continuity between the tubules and the cisternal membranes, proving that the tubules arose as invaginations of the membrane and were cellular rather than viral. Eady and Odland (1975) confirmed this in wounded tissue and suggested the phenomenon occurred in regenerating cells. Intranuclear filaments which sometimes appear to be tubular have also been identified as paramyxovirus nucleocapsid, but the interpretation generally favored is that this is a postmortem chromatin change (Blin-

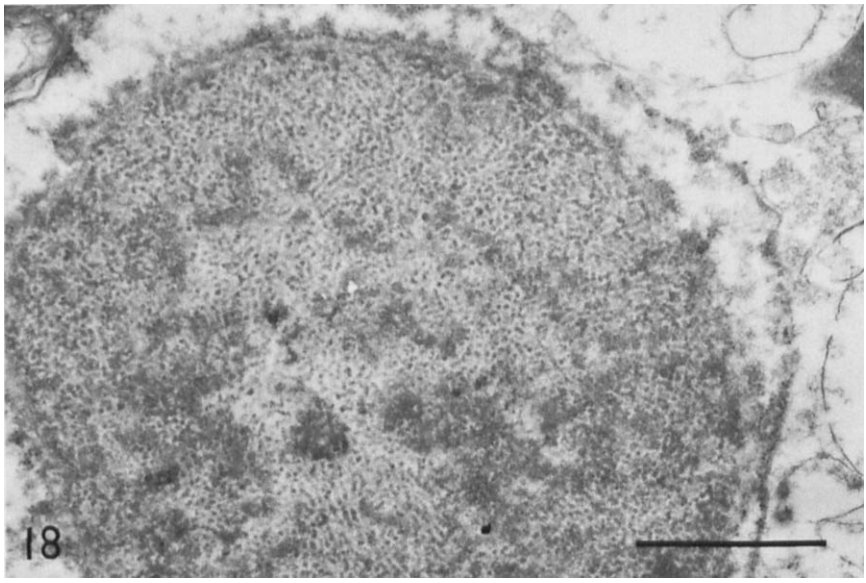


FIG. 18. Thin section of human brain showing intranuclear artifacts resembling paramyxovirus nucleocapsids. ×25,000. Bar = 1 μm.

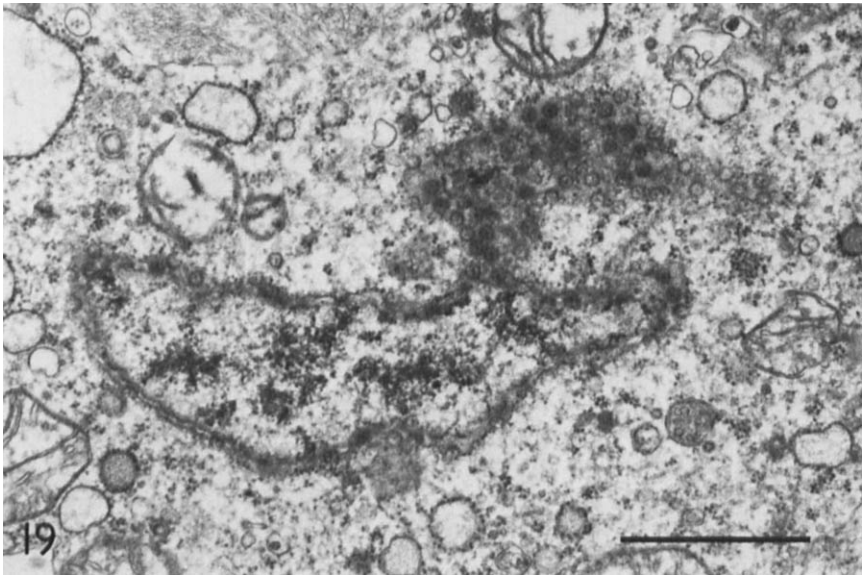


FIG. 19. Thin section of human brain showing tangentially sectioned nuclear pores which resemble herpesvirus particles. ×25,000. Bar = 1 μm. (Courtesy of Mrs. J. E. Richmond.)

zinger *et al.*, 1974; Hayano *et al.*, 1976) (Fig. 18). In addition, tangentially sectioned nuclear pores can resemble herpesviruses (Fig. 19).

Densely stained spherical cytoplasmic particles approximately 20 nm in diameter have been observed in muscle cells and variously interpreted as picornaviruses (Gyorkey *et al.*, 1978) or, with histochemical proof, as glycogen (Collins and Gilbert, 1977; Green *et al.*, 1979) or, when histochemistry disproved glycogen, as arrays of ribosomes (Oshiro *et al.*, 1976).

Dalton (1975) described 30- to 60-nm-diameter structures which resembled viruses and could be observed in thin sections of cultured cells, both intra- and extracellularly. He demonstrated that these originated from the fetal bovine serum used in the media and showed that none of the particles was viral.

VII. Electron Microscopy and Other Diagnostic Methods Compared

A. Virus Detection

Electron microscopy is a relatively good test in virus diagnostic work because positive results are convincing, there is photographic evidence, and results obtained from samples direct from the patient are not complicated by possibilities of cross-contamination. But it does suffer from a low sensitivity and from a lack of automation for large-scale studies compared with some of the other available tests. It is not the method of choice in the diagnosis of herpes simplex encephalitis because, although rapid, electron microscopy is insensitive compared with immunofluorescence on brain tissue (Flewett, 1973; Ross, 1973). It is, however, the method of choice for varicella zoster diagnosis because of the difficulty of culturing virus from the generally unsuitable specimens provided and the lack of sensitivity of other available tests (Macrae *et al.*, 1969). Electron microscopy is useful in cytomegalovirus detection only in very young children and the need for parallel virus isolation procedures is apparent, for even cytology is less sensitive than virus culture (Montplaisir *et al.*, 1972; Henry *et al.*, 1978; Lee *et al.*, 1978).

When virus particles are antibody coated and unable to multiply in cell culture, electron microscopy has the advantage as a detection method. This can be seen in studying polyomaviruses in urine where virus isolation and negative stain electron microscopy otherwise have roughly equivalent sensitivities (Gardner *et al.*, 1971; Coleman *et al.*,

1973). Cytology is more sensitive in polyomavirus detection than either of the other two techniques and reprocessed cytologically positive cells can be thin-sectioned to confirm the presence of polyomavirus (Coleman *et al.*, 1980). A new immunofluorescence test promises greater sensitivity for the detection of urine polyomavirus (Hogan *et al.*, 1980b) and a peroxidase–antiperoxidase light microscopy technique for polyomaviruses in tissues should also prove useful (Gerber *et al.*, 1980).

Electron microscopy was initially sufficiently sensitive to confirm positive results by complement fixation and gel immunodiffusion tests for HBsAg (Cossart *et al.*, 1971). When the more sensitive radioimmunoassay (RIA) and hemagglutination techniques were developed as routine tests for hepatitis B, the use of electron microscopy was largely discontinued except for specialized applications such as the assessment of Dane particle content of positive sera. Immunofluorescence and immunoperoxidase light microscopic detection of hepatitis B antigens in liver are more sensitive than thin section electron microscopy (Roos *et al.*, 1976), but false-positive reactions can be a problem (Omata *et al.*, 1980).

For the detection of hepatitis A virus RIA and enzyme-linked immunosorbent assay (ELISA) seem to be equally as good as negative stain IEM (Hollinger *et al.*, 1975; Purcell *et al.*, 1976; Locarnini *et al.*, 1978; Mathiesen *et al.*, 1978).

Electron microscopy has been of major diagnostic importance in the detection of noncultivable fecal viruses, but for large-scale investigations of rotavirus-associated nonbacterial gastroenteritis more suitable tests have been developed. An early report indicated that gel immunoelectrophoresis was not very sensitive for detecting rotaviruses but complement fixation was almost as sensitive as electron microscopy (Spence *et al.*, 1975). The immunofluorescence test on cells after centrifugation with rotavirus-containing fecal extracts was not as sensitive as electron microscopy, perhaps because particles lacking the outer capsomere layer were not taken into the cells (Banatvala *et al.*, 1975; Bryden *et al.*, 1977). Direct immunofluorescence on the fecal extract, however, was as specific as electron microscopy and slightly more sensitive (Yolken *et al.*, 1977). RIA and ELISA tests have greater sensitivity than electron microscopy (Middleton *et al.*, 1977; Birch *et al.*, 1979; Sarkkinen *et al.*, 1979; Seigneurin *et al.*, 1979) and usually specificity is good, though ELISA false positives have been observed (Yolken and Stopa, 1979).

Similarly, the RIA for Norwalk is specific, at least as sensitive as negative stain IEM, and more sensitive than immune adherence assay (IAHA) (Greenberg *et al.*, 1978, 1979). But in a recent series of tests

negative stain IEM proved more sensitive than RIA in detecting Australian strains of Norwalk agent (Grohmann *et al.*, 1980).

An ELISA to detect noncultivable adenoviruses has recently been described and is almost as sensitive as electron microscopy but might not detect antibody-coated particles (Johansson *et al.*, 1980). Adenovirus in tonsils was detected by the presence of viral nucleic acid in the tissue with greater sensitivity than by virus isolation (Lord *et al.*, 1980), and similar studies with wart virus have been reported (Krzyzek *et al.*, 1980).

The great advantage of electron microscopy not possessed by RIA, ELISA, and other such immune tests is that detection of a range of agents is possible in a single test. This advantage is inevitably lost if specific IEM methods are used to concentrate virus from specimens.

B. Seroidentification of Viruses

Negative stain IEM can be used to serotype viruses but it is scarcely justifiable to use this difficult technique for viruses which can be cultivated and thus serotyped more easily by conventional means. Despite this, IEM typing has been described for adenoviruses (Luton, 1973), picornaviruses (Chaudhary *et al.*, 1971; Hughes *et al.*, 1977), and ortho- and paramyxoviruses (Kelen and McLeod, 1974). Papovaviruses were also serotyped by negative stain IEM because, although some grew well in cell culture and were easy to type by conventional means, others were not as amenable (Almeida *et al.*, 1969a; Field *et al.*, 1974).

Negative stain IEM has been a standard method of comparing strains of the noncultivable fecal viruses such as hepatitis A viruses (Locarnini *et al.*, 1974; Gravelle *et al.*, 1975), rotaviruses (Woode *et al.*, 1976; Zissis and Lambert, 1978), parvovirus-like particles (Paver *et al.*, 1975; Appleton and Pereira, 1977), and Norwalk group agents (Thornhill *et al.*, 1977; Kogasaka *et al.*, 1980). The development of alternative tests for the detection of these agents has also facilitated serotyping and, for example, Norwalk-like agents found in an Australian outbreak of oyster-associated gastroenteritis were confirmed as Norwalk by RIA (Murphy *et al.*, 1979).

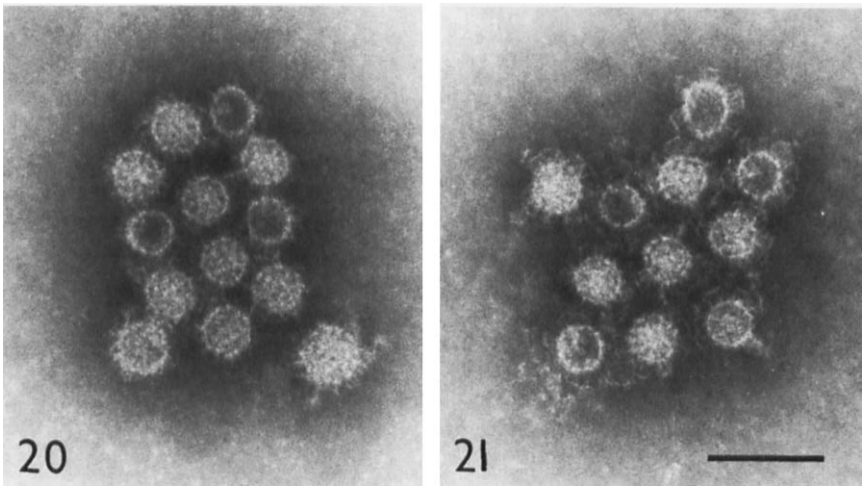
C. Antibody Detection

Negative stain IEM utilizing known viral particle antigens has been used in recent years to assess the antibody response. Certain precautions are necessary and ideally the antigen should consist of virus particles which are well separated and clear of all attached cell debris.

If particles are already antibody coated interpretation of results becomes difficult. These tests have been used to detect antibody response to EB virus (Henle *et al.*, 1966), rabies virus (Chaudhary *et al.*, 1979), papovaviruses (Ogilvie, 1970; Gardner *et al.*, 1971), hepatitis A virus (Feinstone *et al.*, 1973; Gravelle *et al.*, 1975; Locarnini *et al.*, 1977; Coulepis *et al.*, 1980), hepatitis B virus (Almeida *et al.*, 1971; Cohen, 1978), rotavirus (Kapikian *et al.*, 1974), Norwalk agent (Kapikian *et al.*, 1972b; Parrino *et al.*, 1977; Thornhill *et al.*, 1977; Murphy *et al.*, 1979), fecal calicivirus (Chiba *et al.*, 1979; Cubitt *et al.*, 1979; Suzuki *et al.*, 1979), and astrovirus (Kurtz *et al.*, 1977). The interpretation of the Norwalk negative stain IEM for antibody detection has been most difficult because sera taken in both the acute and the convalescent phases of infection contain antibody and careful grading of the amount of antibody coating the particles has been necessary to demonstrate rising titers.

Other tests originally developed to detect these agents, such as RIA and ELISA, can be reversed if suitable amounts of antigen are available to detect antibodies as, for example, in hepatitis B (Cohen, 1978), in hepatitis A (Purcell *et al.*, 1976; Mathiesen *et al.*, 1978), in Norwalk (Greenberg *et al.*, 1978) and, by immunofluorescence, in astrovirus infections (Kurtz and Lee, 1978). Generally these tests are at least as sensitive for antibody detection as negative stain IEM and have advantages for mass screening. Because of the limited excretion period of hepatitis A virus the detection of antibody, particularly immunoglobulin M, is more likely to be used to establish the diagnosis. Specificity of some of these tests may be a problem and negative stain IEM can be used to monitor this aspect.

Immunoglobulin M (IgM) molecules have a distinctive shape and can be differentiated from immunoglobulin G (IgG) in negatively stained preparations of immune complexes between virus particles and serum immunoglobulin fractions (Almeida *et al.*, 1967a; Svehag and Bloth, 1967; Green, 1969). It is necessary to use fractionated serum; otherwise, the IgM structure would be obscured by any IgG present. Thus negative stain IEM can be used to detect specific antiviral IgM response, a useful criterion for recent infection. This test has been used successfully with papillomavirus (Goffe *et al.*, 1966), polyomavirus (Flower *et al.*, 1977), and hepatitis A virus (Locarnini *et al.*, 1977; Coulepis *et al.*, 1980). Used in conjunction with other tests for IgM directed toward HBcAg, Cohen (1978) noted that IgM was undetectable in IAHA and complement fixation tests. Gibson *et al.* (1981) used a variety of tests for polyomavirus IgM and found that negative stain IEM, although less sensitive, was the only completely reliable test (Figs. 20 and 21).



FIGS. 20 and 21. Figure 20, polyomavirus particles coated with immunoglobulin G. Figure 21, polyomavirus particles coated with immunoglobulin M. Negative stain. ×150,000. Bar = 100 nm.

D. Electron Microscopy in Surveillance of Other Diagnostic Methods

The emergence of diagnostic tests which may be more sensitive than electron microscopy and more suitable for large-scale screening of specimens has still demanded the continuing use of electron microscopy to monitor the performance of these tests by confirmation of results on selected specimens. Electron microscopy also plays a role in quality control of the viral reagents utilized in these tests.

VIII. SAFETY

There is now a general awareness of the need for greater safety in the laboratory, not only for the laboratory worker but also for engineers servicing equipment, and all potentially infectious material must be inactivated before examination by electron microscopy. The electron beam may kill viruses, but only in the area of the specimen grid irradiated, and the high vacuum conditions will not inactivate virus. Chemical fixation prior to embedding and thin sectioning does kill viruses but some agents are not inactivated. Negative staining procedures do not inactivate viruses (Horne and Wildy, 1963). The whole aim of electron microscopy in viral diagnosis is to examine recognizable virus particles and some of the most effective ways of sterilizing the

starting material have an adverse effect on negatively stained viral morphology, as discussed in Section II,C.

Exposure to ultraviolet irradiation inactivates most viruses (Cameron *et al.*, 1979; Hughes *et al.*, 1979), but papovaviruses are comparatively resistant (Shah *et al.*, 1976; Cameron *et al.*, 1979). Negatively stained viral morphology is usually unaffected by ultraviolet irradiation although poliovirus particles were penetrated by stain after prolonged exposure (Katagiri *et al.*, 1967). Our standard virus inactivation procedure is to expose negatively stained grids to a high intensity, short wavelength, ultraviolet lamp at a distance of 6.5 cm from the source where the emission registers 700 to 800 $\mu W/cm^2$. The grid is irradiated for 5 minutes on each side. The lamp has been tested for its capacity to kill vaccinia virus and human polyomaviruses under these conditions (unpublished observations).

Inactivating viruses in tissues for embedding and thin sectioning must be done with a fixative which also preserves viral and cellular structure. Glutaraldehyde and formalin are most commonly used and are quite effective virucidal agents at the usual fixative concentrations, although the markedly lower concentrations needed for thin section IEM studies may not be active (Borick, 1968; Graham and Jaeger, 1968; Bowen *et al.*, 1969; Sabel *et al.*, 1969; Saitanu and Lund, 1975). Papovaviruses tend to be more resistant (Tevethia and Tevethia, 1976) and viruses in tissues may resist fixative action longer than when tested in suspension (Cunliffe *et al.*, 1979).

The agents of slow virus CNS diseases such as Creutzfeldt–Jakob disease are highly resistant to formalin (Gajdusek and Gibbs, 1976) and special care must be exercised when handling any material from such cases.

Care must also be taken when handling the various toxic chemicals which are routinely used in electron microscopy (Drury, 1980).

IX. Summary and Conclusions

The supreme advantage of electron microscopy in virus diagnosis is that any virus, if present in the sample in sufficient quantity, will be recognized. The technique is flexible because it is nonselective and this applies to both negative staining and thin sectioning. The disadvantages are the high cost of the electron microscope, the need for highly trained operators, the comparative lack of sensitivity for virus detection, and the relatively small number of specimens which can be examined in a given time. As a means of opening up new fields of diagnostic

virology electron microscopy has been preeminent, but after this initial stage it tends to be replaced by techniques based on newer more biochemical concepts, especially for large-scale diagnostic work.

The slowly developing techniques of electron microscopy itself seem at present to have little to offer diagnostic virology. Even high-resolution scanning electron microscopy is incapable of revealing virus particle–cell interactions in a way which can be utilized in diagnostic work. The scanning transmission mode of operation, which can induce image contrast changes electronically, may enhance studies with unstained sections and perhaps facilitate thin section IEM. It might even alter ways of examining virus particles in suspension, but early results are not particularly encouraging.

The immense contribution of electron microscopy to diagnostic virology in the last 10 years should not be underestimated. The whole concept of a virus diagnostic laboratory has changed from one in which most diagnoses are serological but with an occasional virus being isolated from a large number of samples tested, to a laboratory in which some virus isolation work continues together with a great deal of useful diagnostic work on infections with viruses which are never cultured in the laboratory. This change of outlook has been largely brought about by discoveries made with the electron microscope.

For the future, electron microscopes already heavily used in virus diagnostic work will continue to be used in this field and new discoveries will be made from time to time as in the past. The newly established laboratory, especially in financially less well endowed parts of the world, will probably utilize diagnostic tests in kit form rather than electron microscopy to search for clinically important noncultivable viruses as a first priority. However, the electron microscope has now become an established and essential part of any large virus diagnostic laboratory.

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