

Detection and Identification of Viruses by Electron Microscopy

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ABSTRACT Electron microscopy can aid in the rapid diagnosis of viral diseases, as it can be performed in a matter of hours, but on a routine basis it should be used in conjunction with other techniques. Initially, the specimen source and patient symptoms should be ascertained, as these will lend suggestions of possible agents while eliminating others; however, this information should not be allowed to prejudice observation in such a way as to cause oversight of an unlikely pathogen. Second, selection of the method of preparation should be based on sample consistency; extraction, debris clarification, concentration, tissue culture amplification, or embedment may be necessary. Finally, false-positive results must be avoided by differentiating viruses from cell organelles or debris, mycoplasmal or bacterial contamination, and bacteriophages.

INTRODUCTION

The detection and identification of viral agents in clinical specimens is becoming increasingly important. In addition to previous rationales for viral diagnosis—including quarantine of infected patients, immunoglobulin and vaccination prophylaxis, and avoidance of unnecessary antibacterial therapy—the advent of new and better antiviral agents has prompted an increasing demand for laboratory analysis of clinical viral pathogens. Many hospitals have viral diagnostic laboratories that make use of tissue culture and light microscope examination of the cytopathological effect (CPE) of inocula on cells. Obtaining the results from a viral culture takes several days or longer. Additionally, some viruses are nonculturable. For these reasons, direct examination of the specimen by electron microscopy (EM) within minutes to a few hours can be of value. Few hospitals are equipped yet to handle routine EM of viral specimens; thus, frequently, an existing research laboratory, proficient in EM but not necessarily involved in virus research, is asked to examine clinical samples. This paper is designed as an aid to microscopists in this situation, and it is organized from the point of view of determining an unknown in

a given material rather than describing viruses in taxonomic families. Preparation of specimens likely to be received will be discussed along with a description of potential agents in each specimen. Micrographs of representative viruses are presented here. Several good atlases, organized by virus family, are available as aids in virus identification (Dalton and Haguenuau, 1973; Hsiung, 1982; Hsiung and Green, 1978; Madeley, 1972; McLean and Wong, 1984; Palmer and Martin, 1982).

BACKGROUND

Many techniques have been described for identifying viruses or their components. Detection can be performed by four broad categories.

Serological

Serological techniques include the following: a) enzyme-linked immunosorbent assay (ELISA); b) solid-phase radioimmunoassay (SPRIA); c) reverse passive hemagglutina-

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¹Mention herein of products by trade name does not imply endorsement.

tion (RPHA); d) single radial hemolysis (SRH); e) thin-layer immunoassay (TLA); f) immune diffusion (ID); g) immunoelectrophoresis (IEOP), also known as counter-immunoelectrophoresis (CIE); h) gas chromatography (GC); (techniques a-h are described in WHO, 1981); i) latex agglutination (LA); j) passive hemagglutination (PHA); k) heterophil agglutination; l) hemagglutination inhibition (HI); m) complement fixation (CF) (Giunta and Groppa, 1983); n) neutralization; (techniques i-n are described by Dembert and Kaiser, 1983; Cajal et al., 1983; Russell et al., 1976); o) monoclonal antibodies (Yolken, 1983); p) immune complex detection (Casali and Oldstone, 1983); q) use of Staphylococcal protein A (Richman, 1983).

Biochemical (Brown, 1981)

Biochemical techniques include the following: a) viral nucleic acid detection (Herring et al., 1982; Bishop, 1983; Bornkamm et al., 1983; Ranki et al., 1983; Roizman and Tognon, 1983; Stålhandske et al., 1983; Specter et al., 1984; Whitton et al., 1983); b) viral protein identification (Anderson, 1983; Gronowitz and Källander, 1983; King et al., 1983); c) reverse transcriptase assay (Gallo and Reitz, 1976; Kacian, 1977); d) interferon (IFN) detection (Negreanu et al., 1983); e) virus physical properties (e.g., density, pH stability, solvent stability) (Brown, 1981).

Cultural

Among the cultural techniques are a) tissue culture (Knudson and Buckley, 1977; Hsiung and Green, 1978; Hsiung, 1982; Microbiological Associates, 1978; Miller and Lang, 1982; Swack, 1978); and b) animal inoculation (Field, 1982; Hsiung, 1973).

Microscopy

Microscopy includes the following techniques: a) light microscopy (Fong and Hsiung, 1978; Hsiung, 1973, 1982; Malherbe and Strickland-Cholmley, 1980), usually of inoculated tissue cultures (see above for culture references), which may be immunoperoxidase-stained (Smith, 1983) [patient material can sometimes be stained and viewed directly as in the Tzanck procedure (Rawls, 1979)]; b) fluorescence microscopy of infected cultures (Emmons and Riggs, 1977; Hsiung, 1973; WHO, 1981) and of patient material (Athanasu et al., 1983; McIntosh, 1983); c) fluorimetry (Halonen et al., 1983); and d) electron microscopy (EM) and immune elec-

tron microscopy (IEM) (Almeida, 1980; Field, 1982; Flewett, 1978; Fong and Hsiung, 1978; Hsiung et al., 1979; Kapikian et al., 1976; McLean and Wong, 1984; Miller, 1984).

Diagnostic tests for specific viruses are listed by Almeida et al. (1979) and Evans (1984). Serological tests for viral antibodies are hampered by the fact that in early infection, titers may not have risen and may be totally absent in immunodeficient patients. Serological tests for the virus itself may not detect low amounts of antigens in early infection; some viruses do not have the hemagglutinating antigens used in LA, PHA, and HI tests. The advantages of serological tests are the relatively simple and inexpensive procedures, as well as rapid results. Culturing techniques have disadvantages in that several cell lines must be maintained continuously for inoculation at appropriate cell confluency, in that some viruses do not grow (easily or at all) in culture, and in that light microscope CPE may require several days before becoming evident. However, if virus numbers are low owing to early infection or inadequate sampling (as in biopsies), amplification in tissue culture can increase the chances of visualization. In tests designed to identify viral components, the disadvantage is that one must have a wide variety of reagents (e.g., antisera, nucleic acid probes, protein standards) on hand, as well as have some idea of possible infectious agents so that the number of tests is not prohibitive. The advantages are that they are highly sensitive and specific.

Electron microscope examinations have the advantages that they can be performed in a few minutes to a few hours and are capable of detecting some viruses that cannot be grown in tissue culture. The disadvantages are the relatively large viral numbers required for visualization, the inability to distinguish different viruses of the same family, and the expensive instrumentation, which small hospitals may lack. Clearly EM can be advantageous when diagnostic time is of the essence, and it can be a valuable tool when used in conjunction with other techniques.

MATERIALS AND METHODS

Techniques

Biohazard precautions

All specimens should be treated as potentially pathogenic. The best working conditions include a biohazard hood with filtered

air and UV light; in the absence of one, a work bench area in a quiet corner away from traffic should be covered with diaper paper, underpads, or other absorbent, plastic-backed paper. Neither drying nor negative staining inactivates virus. There should be a container for contaminated disposable articles such as filter paper, parafilm, pipettes, etc., which will be autoclaved before discarding. All materials that have come into contact with specimen should be sterilized before reuse or discarding. Take care not to create an aerosol of the specimen when mixing it. Rubber gloves may be worn, especially if hepatitis is suspected; however, remove them when moving away from the preparation area; do not handle other articles such as doorknobs, microscope parts, etc., with contaminated gloves. If gloves are not worn, thoroughly clean hands with 70% ethanol. If grids are unfixed, there should be a grid depository for autoclaving and a container of 70% ethanol near the microscope for the specimen holder and forceps. Other reusable articles can be sterilized with 10% formalin, hypochlorite (a 10% Clorox solution will do), Lysol, or other suitable disinfectant.

Negative staining (Brenner and Horne, 1959; Haschmeyer and Meyers, 1972)

Direct examination of fluid specimens is fast and simple; however, a relatively high concentration (10^5 – 10^6 particles per 1 ml) is necessary for visualization. (Concentration methods are described below.) The sample should be cleared of cell debris by low-speed centrifugation at 1,500g for 1–2 min. If the supernatant is still cloudy, the speed and/or time can be increased. Do not exceed about 2,200g for 10 min, since it is possible for virus to be present in large clumps or attached to membrane fragments that would be pelleted and discarded (Narang and Codd, 1981). Put a drop of supernatant onto a waxed surface such as parafilm; place a formvar or colloidion and carbon-coated grid, membrane side down, on the drop and cover with a petri dish lid for 5–10 min. If the specimen drop appears thick or darkly colored, wash the grid briefly before draining by touching it to a drop of water. If not, drain with filter paper directly upon removal from the drop. If a suspected virus is in a salt solution (buffer or gradient material) after attempted concentration or purification, or if a serious health hazard such as hepatitis is suspected, place the grid onto the specimen drop for 5–10 min;

drain briefly, but do not dry; then place it onto a drop of 3% glutaraldehyde in buffer (phosphate or cacodylate, 0.1M, pH 7.4) for 5–10 min. Wash the grid gently with water, and drain. Negatively stain by placing the grid, membrane side down, onto a drop of 2% aqueous uranyl acetate (UA) or phosphotungstic acid (PTA). If no virions are evident, try one of the concentration methods. Other methods that may be useful for viruses in salt solution are the pseudoreplica technique and the agar-diffusion-filtration method (see below). Bear in mind that negative stains are heavy metals and should not be left to dry on pipettes and in places where the dust can become airborne and breathed. Also, UA is radioactive.

Ultracentrifugation

Liquid samples may be too dilute to demonstrate virus without concentration. If the sample is a large volume of fluid (2–10 ml), (e.g., cerebrospinal fluid (CSF), serum, lavages), a large ultracentrifuge can be used. First, clarify the sample of cells and debris (see above) and ultracentrifuge it for a minimum of 1 hr at 100,000g. The exact speed and time will depend on the rotor radius. Suggestions are Beckman SW 41 or SW 40, 38,000 rpm, 90 min; or SW 27, 24,000 rpm, 3 hr.¹ Decant the supernatant, blot the mouth of the inverted tube on filter paper or paper towel to remove additional remaining liquid, and resuspend the pellet in one drop (10–20 μ l) of distilled water. If no pellet is visible, remove the supernatant carefully with a Pasteur pipette, leaving a small drop in the tube; triturate the remaining drop several times against the bottom of the tube. Negatively stain (see above).

Airfuge ultracentrifugation

After debris clarification, small samples (0.2 ml) can be pelleted by spinning at a pressure of 30 pounds per square inch (psi) (100,000g) for 30 min in an airfuge (Beckman Instruments, Fullerton, CA). The pellet should be resuspended (as above) and negatively stained. A special rotor is also available that accepts EM grids so that material can be spun directly onto the membrane for negative staining (Hammond et al., 1981). The latter procedure collects everything in the suspension onto the grid and thus is less likely to lose virions; however, this often results in thick preparations that are difficult

to see through; therefore, a couple of different specimen dilutions should be included.

Ultrafiltration

Dilute suspensions can be concentrated by a number of ultrafiltration methods. The basic principle is that liquid is absorbed through a membrane with a small pore size that retains viruses in the original vessel. The pore size selected should retain particles that are greater than or equal to 20 nm (e.g., microfilter 55009 and membrane E142, pore size 0.015–0.010 μm ; Schleicher and Schuell, Keene, NH). It is imperative that debris be removed by centrifugation prior to ultrafiltration, even at the expense of pelleting viral clumps; otherwise the membrane will become clogged. The filtration unit with clarified suspension is then centrifuged at 3,500 rpm for a few minutes (3–5 min). The material on the filter is rinsed off with a drop (20 μl) of water, placed on a membrane-coated grid, and negatively stained. Another unit, called a miniultrafiltration cell, model 3 is available from Amicon (Amicon Corp., Danver, MA) and can be used with a membrane of 100,000 MW cutoff, which should retain particles of 20 nm. The unit costs several hundred dollars, requires a stirrer and an N_2 source, and may require up to 2 hr to achieve concentration. As above, clogging by debris may be a problem. These methods can be used in the absence of access to an ultracentrifuge, but then may lose clumped virions in the clarification step.

Pseudoreplica technique (Sharp, 1960)

Urine from infants with cytomegalovirus (CMV) infection is the main type of specimen prepared by this technique (Lee et al., 1978), though other specimens with a reasonably high virus concentration ($10^3/\text{ml}$) can be used (e.g., bands of virus purified on gradients and stool suspensions). This method allows excess liquid and salts to be absorbed into agar, leaving virions on the surface. Clarify the suspension of debris by low-speed centrifugation and place a drop onto each of several agar blocks previously prepared (see below). When the liquid has been absorbed into the agar, drop 0.5% formvar from a Pasteur pipette onto the agar. With a new, cleaned razor blade, trim the block sides so that the edges are smooth. Float the membrane off the agar onto a water surface in the same manner as floating it off a glass slide for the preparation of membrane-coated grids

(Hyatt, 1970). If trouble is encountered in detaching the film from the agar, try turning the block on its side on the slide and trimming the edges perpendicular to the membrane surface so that a 1-mm slice is removed from each edge. This prevents the blade from pushing the membrane into the agar from the top surface. Place a grid(s) on the membrane surface. (Virus, if present, is underneath.) To pick up the grids, place a small piece (1–2 cm^2) of filter paper over the grids and membrane on the surface of the water, and when it becomes wet, lift it out by the edge with a pair of forceps. This filter paper technique is easier than removing the grids with a peg, screen, or glass slide. Virus, if present, will now be on top of the membrane. Negatively stain. Sharp (1960) originally described the pseudoreplica technique with instructions to "float the membrane off onto 2% PTA." This method requires a large volume of stain and may retain virions in the stain, preventing its use for other specimens.

Agar preparation

Dissolve 2 g purified agar (Difco Laboratories, Detroit, MI) in 100 ml of water by heating and stirring. Autoclave, and aseptically pour a 0.5-cm-thick layer into sterile Petri plates. Agar plates may be stored in the refrigerator in a moist container for several weeks; discard when the agar dries out or if mold grows. When needed, cut out 1-cm² blocks with a spatula and transfer them to a glass microscope slide for easier handling.

Agar-diffusion-filtration method (Anderson and Doane, 1972)

Clarify the suspension of debris at low speed, and place a drop of supernatant onto a 2% agar block. Place a membraned grid onto the drop and allow diffusion of liquid and salts into the agar. When dry, pick up the grid and negatively stain.

Serum in agar diffusion (Anderson and Doane, 1973)

Prepare microtiter plates by adding pooled or specific serum to molten agar, cooled to 56°C, at a final dilution of 1:50, and fill wells three-fourths full. Label, seal with tape, and store the plates at 4°C. If moisture collects, place the plate at 37°C before use until the agar surface appears dry. Add a drop (20 μl) of clarified specimen per well and place a grid onto the drop(s). Allow the liquid to diffuse into the agar. When the drop has disap-

peared, remove the grid and negatively stain. If virus is recognized by antibody, it will be clumped and concentrated up to 100 times.

Immunolectron microscopy (Almeida, 1980; Kapikian et al., 1976)

Chances of visualizing viruses in dilute solutions can be increased by clumping them with antisera; however, the antibody must be specific for the virus. If there is already a hint of viral identity from other studies or symptoms, specific antisera can be used, but if the agent is unknown, pooled gamma-globulin may contain the right antibody. We have found serum from migrant farm workers to be particularly useful, since they have been exposed to many pathogens. Commercial gamma-globulin is also available. After clarification of large particles, add pooled serum to the sample at a final dilution of 1:100 or 1:200; specific antiserum can be diluted up to 1:500 to 1:1,000 depending on the serum. Complex formation will be diminished or lost if there is antibody or antigen excess (Almeida and Waterson, 1969), but when both specimen and antibody concentrations are unknown, these dilutions are a range within which to start. The volume of antiserum added should be kept low to prevent dilution of the virus; thus, antiserum stored frozen at a 1:10 dilution can be added to the virus suspension at a 1:10, 1:20, 1:50, or 1:100 dilution without substantially increasing the volume. Incubate 0.5 hr at 37°C, 1 hr at 25°C, or overnight at 4°C, inverting periodically to mix. Centrifuge at 15,000g for 1 hr (e.g., 17,000 rpm in a Sorvall RC2-B with SS 34 rotor, 23,000 rpm in a Beckman ultracentrifuge with 40.2 rotor, or equivalent instrument). We use an airfuge at 100,000g (30 psi) for 30 min, which also pellets single virions in the event no recognition has occurred. Decant the supernatant, drain well with filter paper, and resuspend the pellet in 1 drop (10–20 μ l) of distilled water. Negatively stain.

Mincing tissue

Because of the need for an urgent diagnosis, biopsy material is occasionally sent with a request that it be negatively stained. First, select a few small pieces of tissue and begin embedment for thin sections (see Embedment and Rapid Processing sections below). Mince the remaining tissue with a clean razor blade and grind it with a tissue homogenizer or mortar and pestle. If grinding instruments are unavailable, mince the tis-

sue as finely as possible with the razor blade; mash it with a glass rod; place it into a sturdy (polypropylene or pyrex) tube with phosphate-buffered saline (PBS) to cover, and freeze and thaw three times. This can be accomplished by lowering the bottom of the tube into liquid nitrogen, an acetone-dry ice bath, or acetone prechilled in a -20° C freezer. Thaw in a 37°C water bath. Clarify the suspension at low speed and examine the supernatant by negatively staining (see above). One of the concentration methods described above may also be useful. This method is faster than embedment but is generally of low yield. If negative, proceed with embedment.

Embedment (Hyatt, 1981)

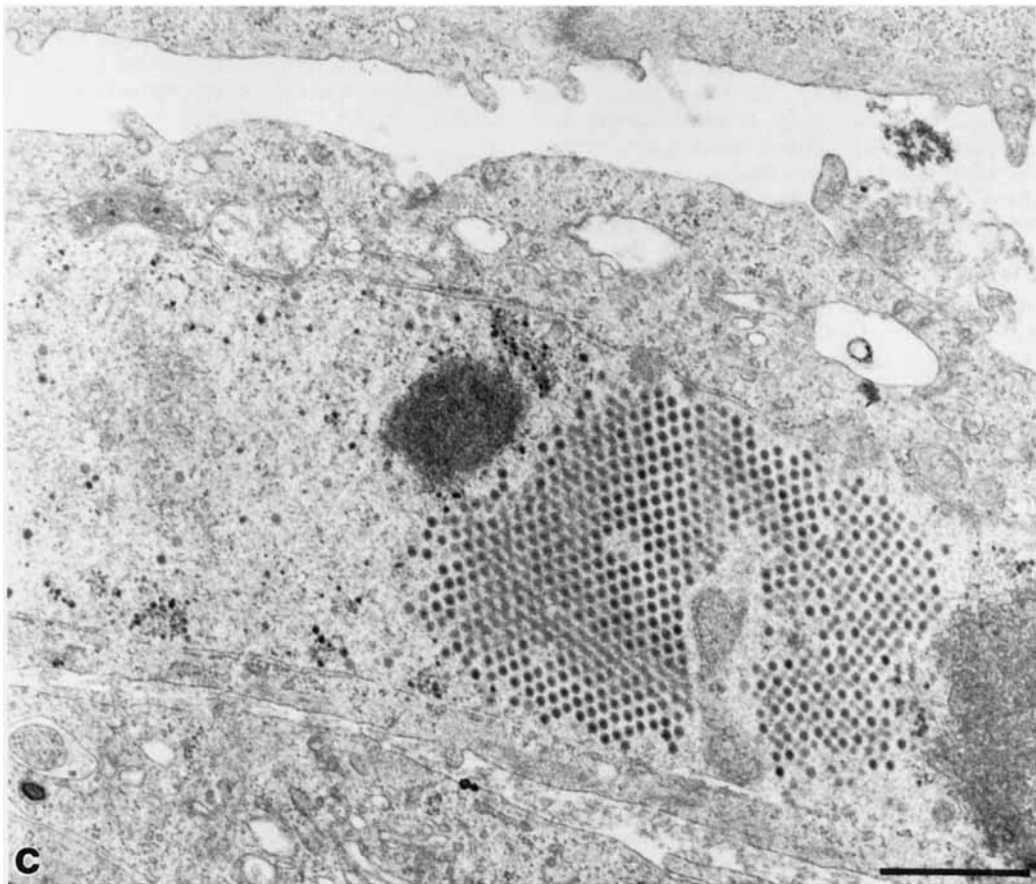
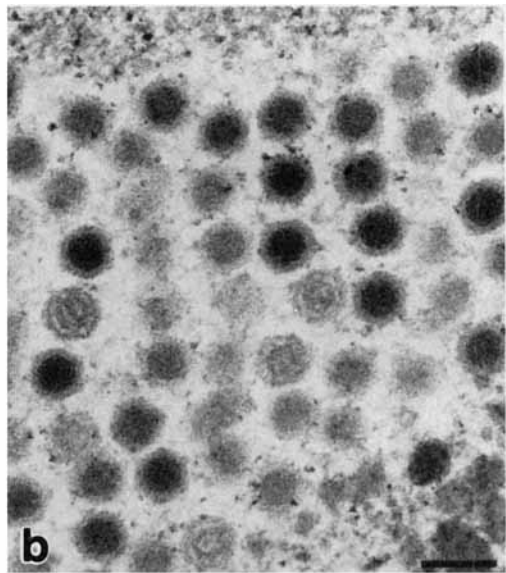
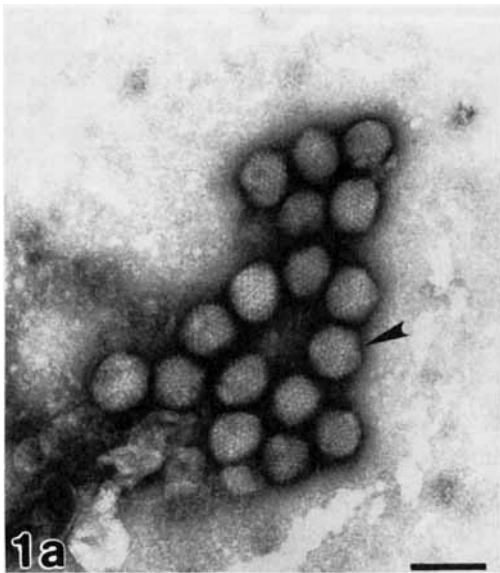
Each electron microscopist surely has a preferred method. Presuming that most readers are familiar with this technique, we will not elaborate here. For routine sectioning, we prefer one that includes 2% glutaraldehyde, 1% osmium tetroxide, and 1% uranyl acetate, followed by Spurr resin because of its short (8-hr) curing time.

Rapid processing

We have previously described a procedure for the EM examination of thin sections 2 hr from receipt of specimen; this time includes cutting (Miller and Nielsen, 1979; Miller and Lang, 1982; Miller, 1983b). Briefly, with a clean, sharp razor blade or scalpel, cut the tissue into very small slivers approximately 0.25 \times 0.25 \times 0.5 mm. If the situation requires prompt results, such as in herpesvirus encephalitis, the schedule in Table 1 will give satisfactory sections in about 2 hr. It requires constant attention and necessitates that the solutions be prepared early and that the Drierite be prechilled. Note that the times are total minutes, during which several solution changes may be necessary; all reagents are at room temperature. The specimens should be continuously agitated on a shaker.

This procedure, of course, can be lengthened to a comfortable working pace, but it proves that rapid embedment can be successful when time is of the essence. Others have also described rapid processing procedures (Bencosme and Tsutsumi, 1970; Doane et al., 1974; Hyatt and Giaquinta, 1970). The keys to success are as follows:

1. Keep the tissue slivers *very* small.
2. Use continual agitation.



Negative stains are all printed at the same size ($\times 100,000$) for easy direct comparison; bars are $0.1 \mu\text{m}$. Thin sections are at $\times 20,000$ to show virus-cell relationships; bars represent $1.0 \mu\text{m}$. Some thin sections are enlarged to $\times 100,000$, where bars are $0.1 \mu\text{m}$.

Fig. 1. a. Negatively stained adenovirus, a nonenveloped virus of $75\text{--}80 \text{ nm}$. The overall shape is a geodesic sphere, with the surface made up of triangular facets.

The capsomers are round, with holes in the centers, like beads. Depending upon its orientation on the grid, the virion may appear hexagonal (arrowhead). b. In thin sections of infected cells, adenoviruses appear in nuclei as round or hexagonal bodies with electron-dense cores and light shells or less dense structures with sharply defined shells. c. Owing to the flat facets, virions often line up in a crystalline array. Bars in a,b represent $0.1 \mu\text{m}$; c, $1 \mu\text{m}$.

TABLE 1. Procedures for rapid processing of specimens for thin sectioning

Procedure	Total time (min)
5% glutaraldehyde, phosphate (P) or cacodylate (C) buffered ¹	10
Buffer wash, 3 changes	5
4% OsO ₄ , P- or C-buffered	10
Wash, 2 changes P, 2 changes veronal acetate (VA) buffer	5
2% uranyl acetate (UA), VA-buffered	4
H ₂ O rinse, 2 changes	1
70% ethanol	4
95% ethanol	4
100% ethanol, 5 changes	10
100% ethanol/Spurr resin (3 × catalyst) 1:1, 4 changes	8
100% Spurr resin (3 × catalyst), 5 changes	15
Bake at 95° C	25
Bury in chilled (-20° C) Drierite or silica gel pellets	5
Cut thin sections	15
View without poststain at 60 kV or poststained at 80 kV	

¹Buffers. Any buffer system that maintains physiological conditions and in which fixatives and stains are soluble is sufficient. Cell ultrastructure is best preserved when the buffer itself is at physiological osmolarity (300 mOsm) and pH (7.4). The osmotic pressure added (above 300 mOsm) by glutaraldehyde does not alter morphology. Attempts at maintaining the final osmolarity at 300 mOsm by reducing that of the buffer vehicle result in cell blebbing (Brunk et al., 1981). (Physiological conditions are those in which cells are accustomed to living—obviously, some cells, e.g., those of marine animals, have different requirements.) Phosphate-buffered saline (PBS), 0.135 M PO₄ buffer, 0.1 M PO₄ buffer plus 0.053 M (1.8%) sucrose, and 0.1 M cacodylate plus 0.1 M (3.4%) sucrose are examples of vehicles in which glutaraldehyde and osmium tetroxide are soluble. However, UA is not soluble in any of these and must be dissolved in VA (0.11 M). Some researchers omit UA entirely, but it does fix nucleic acids and lipids. Others avoid the insolubility problem by using aqueous UA, assuming prior tissue stabilization by glutaraldehyde and OsO₄. Before UA is used, the tissue should be washed with the solvent in which UA is dissolved.

3. Exchange dehydrating agents, particularly the absolute (100%) ethanol, several times, draining well but using care not to let specimen dry *at all* on the surface.

4. Exchange the 100% resin several times, also draining well before each new addition.

5. Triple the catalyst concentration, but be sure to add it slowly and dropwise while stirring on a magnetic stirrer; otherwise a clump of glue is liable to form.

6. Do not allow the oven temperature to go above 95°C or the BEEM capsules will melt, and the resin will drip down onto the oven floor.

Tissue culture amplification (Miller et al., 1980; Miller and Lang, 1982)

Direct examination of biopsy specimens by fluorescence or electron microscopy may be fruitless owing to low virus concentration. Viral numbers can be amplified by allowing a round or two of replication in tissue culture. Evidence of CPE by light microscopy may require several days, but even immature virus particles can be identified by EM in 24–48 hr. Certain viruses grow best in specific cell lines, and if an educated guess leads to suspicion of a particular one, these lines should be used. Rhinoviruses (respiratory picorna) and myxoviruses usually grow

best in monkey kidney cells; herpes in rabbit kidney cells; CMV and varicella zoster virus (VZV) in human diploid fibroblasts; respiratory syncytial, adeno-, and measles viruses in HEp-2 cells; and Coxsackie A in rhabdomyosarcoma cells, and toga- and rubella viruses in Vero cells. Otherwise, for general unknowns, primary human kidney and human fibroblast cells can be used. Other possibilities are primary monkey kidney cells or HeLa or HEp-2 lines. For a detailed list of viruses and the cell cultures that can support them, see Microbiological Associates (1978). Some viruses, such as rotavirus and hepatitis B, are for the most part noncultivable under normal conditions. Some viruses may require special cells or culture conditions not routinely available in a viral diagnostic lab. Thus, a negative culture may not be informative.

Amplification requires maintenance of tissue cultures (or contact with a lab that does). It also has the disadvantage that if only a few cells become infected, one may have to search several sections before viruses are seen. But the technique has proven to be of use in cases where inocula are low and time is of the essence, such as in herpes virus encephalitis. Basic cell culture preparation and light and electron microscopy CPE are elaborated by Hsiung (1982). Many other detailed books on cell culture are available

(Barnes et al., 1984; Colowick and Kaplan, 1979; Freshney, 1983; Harris et al., 1980; Swack, 1978).

Aseptically mince biopsy material, and grind it in a sterile tissue homogenizer. Add culture medium and mix well in the homogenizer. Filter out the tissue pieces with a sterile large-pore filter or cheesecloth. Divide the filtrate into two aliquots. Refilter one portion through a 0.45- or 0.22- μm filter and add to several containers (3–4) of 90% confluent cells in a volume that will cover the cells. Add the second portion to several other cultures without microfiltration. Allow adsorption for 2 hr; remove the inoculum and add medium. Cultures without sterile filtration should receive medium containing antibiotics to reduce bacterial contamination. Penicillin, 100 units/ml or oxacillin, 20 $\mu\text{g}/\text{ml}$ and/or gentamicin, 50 $\mu\text{g}/\text{ml}$ are commonly used; tetracycline, 10 $\mu\text{g}/\text{ml}$ and amphotericin B, 3 $\mu\text{g}/\text{ml}$ give broad coverage. Other antibiotics and final concentrations are listed by Armstrong (1973) and Hsiung (1982). Incubate at 37°C. The cultures will not show any light microscope CPE at the early times unless something in the inoculum is toxic to

the cells. Harvest at 24, 36, 48, 60, and 72 hr and process by the rapid embedding method (Miller, 1983a,b).

Specimens

Stool

The specimen most likely to be sent for electron microscopy is fecal material from children with gastroenteritis. The reasons are multiple: a) Some of the viral agents that cause this problem either cannot be grown in tissue culture or are culturable only with special techniques not routinely used in a viral diagnostic lab; b) a rapid diagnosis is necessary so that clinicians can differentiate bacterial from viral infections for proper treatment; c) biochemical and serological assays (see Background) are so specific that one must have some hint as to which agent to seek; the Rotazyme assay (Abbott Laboratories, North Chicago, IL) detects rotavirus but would miss the many other enteric viruses. To prepare the specimen, suspend fecal material in water at approximately 20%; e.g., using an applicator stick, scrape about 2–3 mm^3 of sample and transfer it to a microfuge tube; stir to a smooth consistency; add 2–3

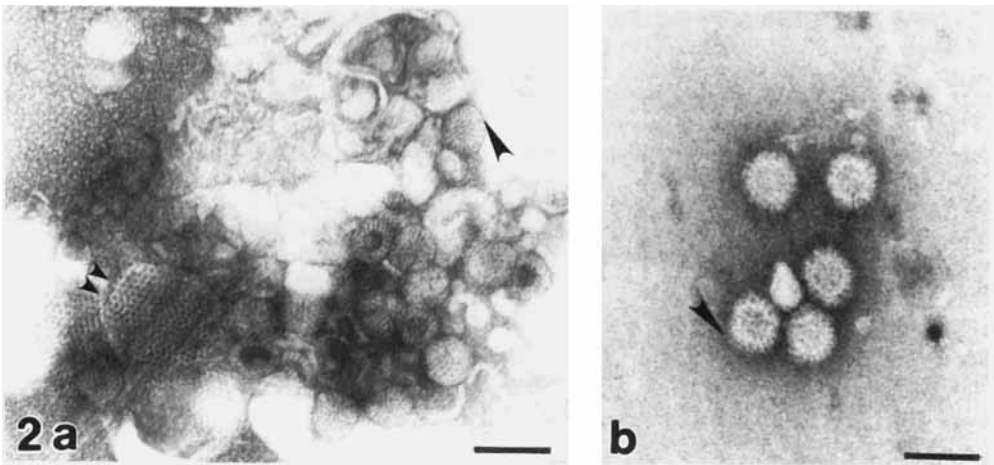
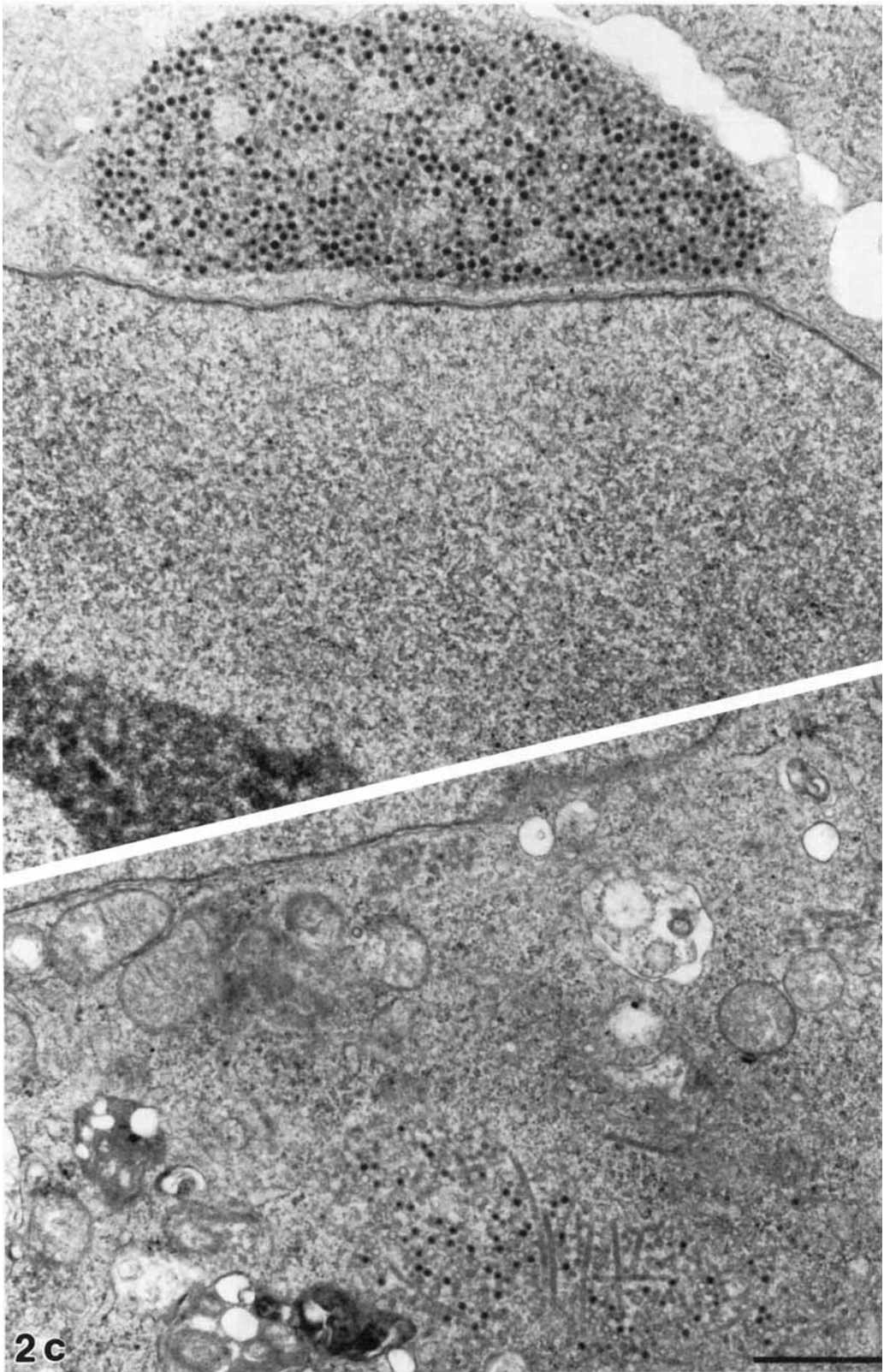
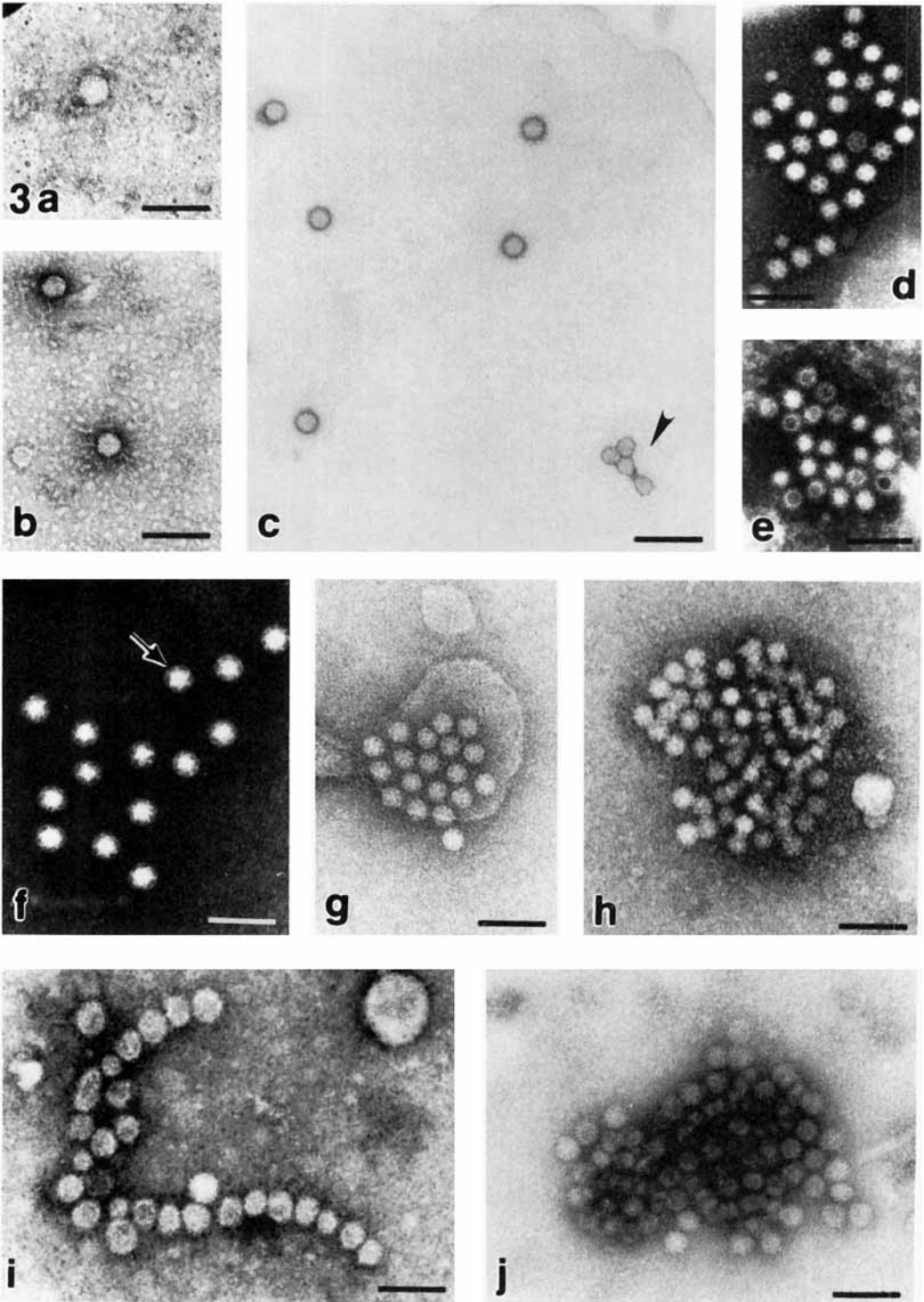


Fig. 2. a. Negatively stained rotavirus, a nonenveloped virus of 70–75 nm, found only in stool. The overall shape is spherical (round in negative stain); compare to the hexagonal (or geodesic) shape of adenovirus. The capsomers are tubular and are oriented in the shape of "wheel spokes." A thin rim surrounds the spokes (single arrowhead); this outer layer sometimes is lost if the virus is degraded. The presence of lattice structures (double arrowheads), the subunits of which are of the size and hexagonal shape of capsomers, is diagnostic; they are sometimes seen late in infection in the absence of complete virions. This material should not be confused with bacteriophage tail and neck proteins (see text and Fig. 14). b. Another isolate from stool. It is most likely degraded rotavirus, as the capsomers seem to have re-

laxed, and the size has increased; also, some areas have patches of material resembling the outer rim (arrowhead). Other researchers might argue that it is reovirus, which can be seen in the respiratory tract and occasionally in stool. Reovirus does not have the outer rim, but otherwise resembles rotavirus. c. Reovirus in tissue culture; the micrograph is of two areas of the same cell. Human rotavirus, which does not grow in tissue culture except under very special conditions, resembles reovirus when seen in biopsy tissue. Cytoplasmic factories contain virions with an electron-dense center and occasionally empty capsid shells. The viruses are usually in a granular matrix, associated with microtubules, and do not form crystals as adenoviruses do. Bars in a,b represent 0.1 μm ; c, 1 μm .





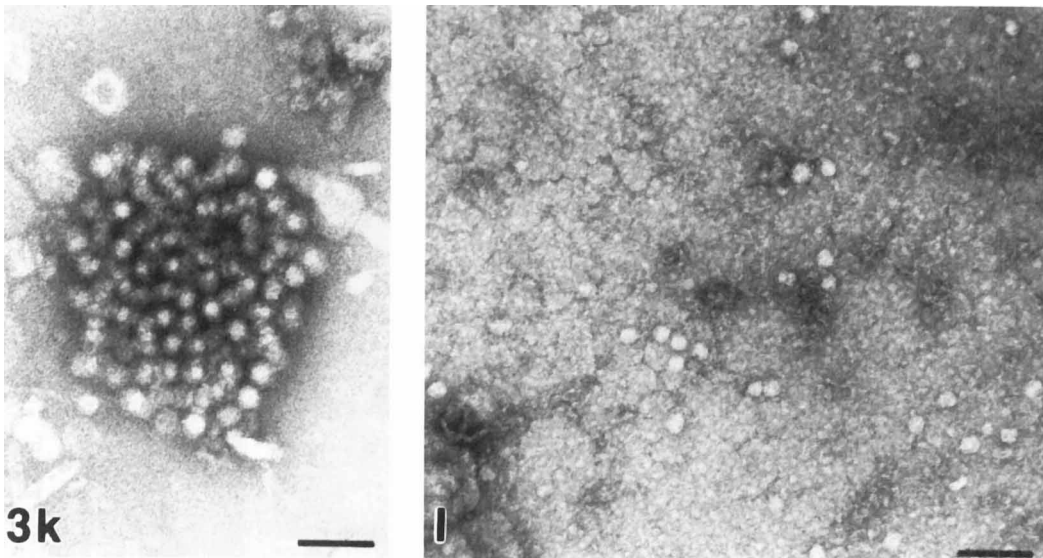


Fig. 3. There are many small round viruses (picorna- and parvoviruses) around 20–50 nm, many of which are nondescript and cannot be classified in an unknown by negative staining. a. Poliovirus. b. Echovirus. c. Coxsackie B virus. All are 30 nm or less; occasionally, as in polio, there may be a slight hexagonal appearance. (Viruses in a–c were provided by Joan Zeller, Duke Medical Center, Durham, NC.) d. Calicivirus from stool; spherical virions are 35–40 nm, and capsomers are cup-shaped. e. Minireovirus from stool; 35- to 40-nm virions with distinct capsomers resembling the reovirus family, of which rotavirus is a member. f. Astrovirus from stool;

spherical virions are 35–40 nm, and the surface structure has a six-pointed star appearance across the whole face (arrow). (Micrographs d–f were supplied by courtesy of Dr. M. T. Szymanski, Hospital for Sick Children, Toronto; Canada; reprinted from *Infectious Diseases of Children ed. 8*, with permission of Krugman, S., Katz, S.L., Gershon, A.A., and Wilfert, C.M. (1985). The C.V. Mosby Co., St. Louis.) g–l. Unknown viruses from stool; note the differing appearances and compare to confusing elements in Figure 15. The arrow in c denotes uneven staining (see Confusing Elements). All bars represent 0.1 μ m.

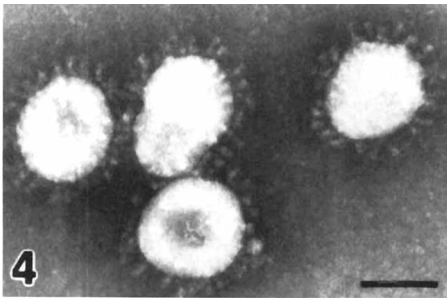


Fig. 4. Negatively stained coronavirus, 80–130 nm in diameter with long club-shaped spikes. (Micrograph courtesy of Dr. D.W. Bradley, Centers for Disease Control, Atlanta, GA.; reprinted from *Infectious Diseases of Children*, with permission of C.V. Mosby Co., St. Louis.) Bar represents 0.1 μ m.

drops of water and mix well. Add water up to about 0.5 ml, and stir again. If mixing is difficult, close the cap and hold the tube in a water bath sonicator for 30–60 sec. Remove debris by low-speed centrifugation (see above,

Negative Staining; note comment on speed). A microfuge is handy because only a small amount of sample is needed, but if it is not available, use a desk-top centrifuge or other instrument. Place a drop of supernatant on a membrane-covered grid and negatively stain (see above). Agents found in stool are rota-, adeno-, entero-, parvo-, minireo-, calici-, astro-, and coronaviruses and Norwalk agent. In cases of hepatitis, hepatitis A virus may be seen, though hepatitis is diagnosed routinely by serology. Most of the small viruses cannot be differentiated owing to lack of capsomeric detail, but some do have definitive morphology (e.g., calici-, astro-, and minireovirus). In reporting results, “small, round, fuzzy viruses” may be described as 20-, 30-, 40-, or 50-nm particles, singly or in clumps, with an indication of numbers, (e.g., many, few, number per grid hole, etc.), but not by name. Viruses with a definitive morphology can be diagnosed by name, e.g., adeno- or rotavirus; if the morphology is definite, one

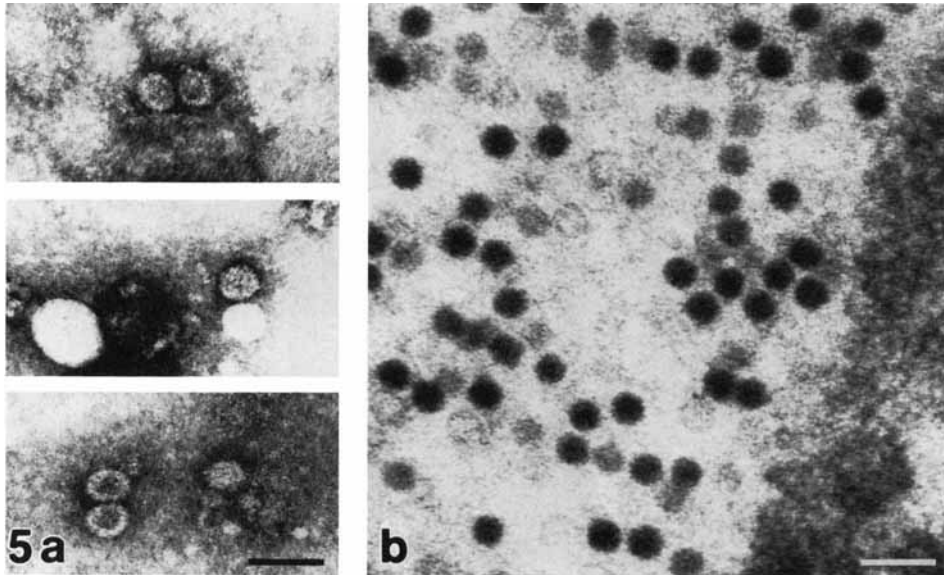


Fig. 5. Another group of small, round particles is the papovaviruses (papilloma, polyoma); they are not found in stool, but rather in skin and other tissues. a. Negatively stained papilloma virus, 45–55 nm. b. Thin section of papilloma virus in biopsied tissue. (Virus and tissue were provided by Dr. Susan Watts, UNC School of Medicine, Chapel Hill, NC) All bars represent 0.1 μm .

particle is sufficient for a diagnosis. The smaller viruses, however, must be differentiated from bacteriophages; see the section on Confusing Elements. Clues in the detection of an etiological agent are the presence of many particles or clumps of particles. Also look for uniformity of size and apparent “hardness” of the protein coat. Bacteriophages sometimes have membranous coatings that become distorted during preparation and then appear to have wrinkles. The membrane may become broken so that stain penetrates easily, creating a “bull’s eye” appearance. Except for adenovirus, particles with a tail is a phage and though interesting, is of no significance in the disease. See the Agents section in Results and Discussion and the figures for a detailed description of the virions. Intestinal viruses are also covered in detail by Tyrell and Kapikian (1982).

Urine

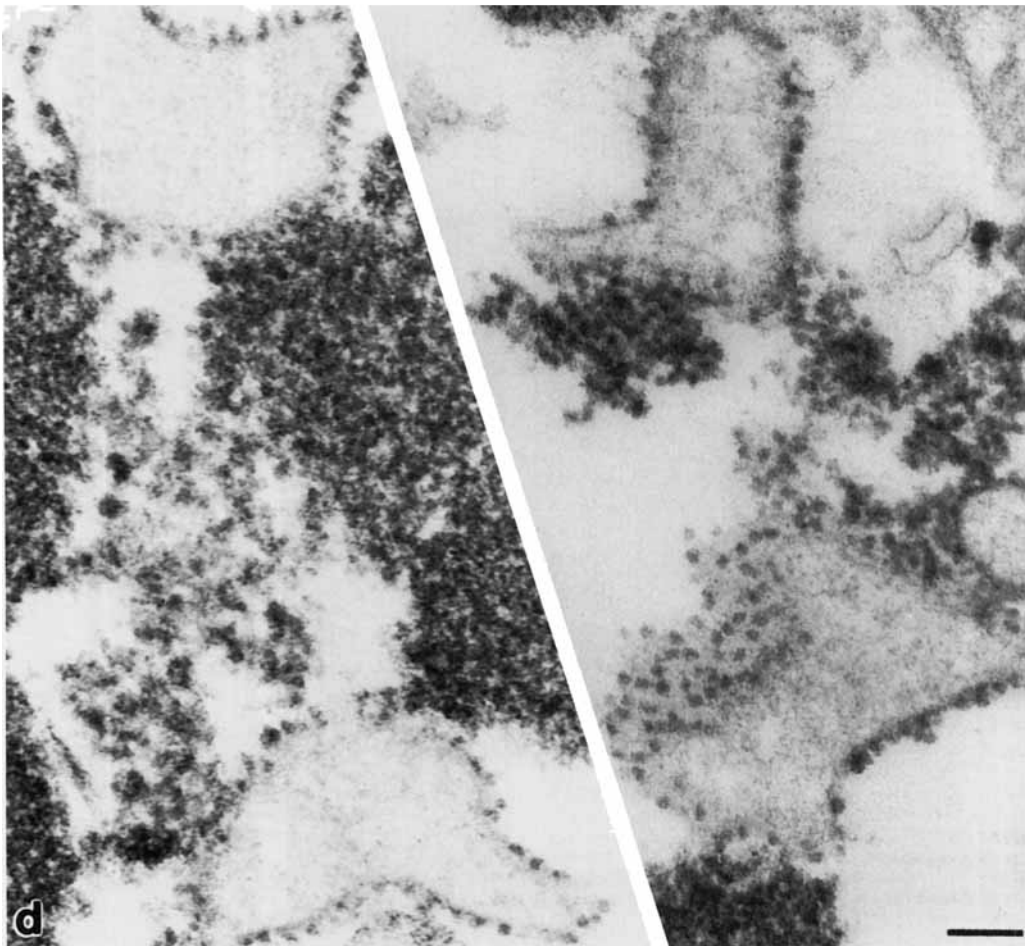
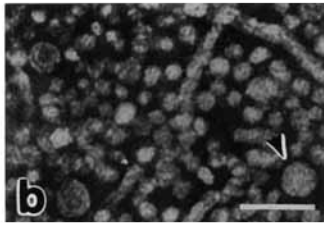
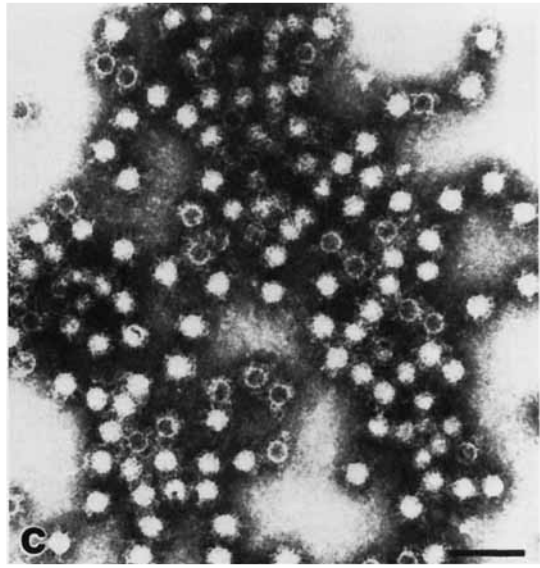
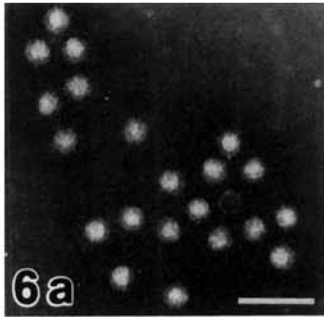
Large numbers of CMV can be found in the urine of infected infants. In transplant patients, BK virus can be seen in urine, and mumps virus can be seen in urine of patients with parotitis or orchitis. Direct examination

of unconcentrated urine can often demonstrate the virus, but if not, concentrate the sample by the pseudoreplica or the agar-diffusion-filtration technique or by ultracentrifuging (see above). Negatively stain.

Cerebrospinal fluid

Viruses in CSF, when present, are not numerous and are difficult to detect. They may

Fig. 6. Hepatitis is routinely diagnosed serologically, but particles may be seen by EM. a. Hepatitis A can be seen in stool as a 27-nm fuzzy particle. b. Hepatitis B can be seen in serum after concentration by centrifugation or aggregation by antiserum. Infectious Dane particles, 42 nm (arrowhead), and noninfectious 22-nm Australia antigen and filaments may be present. c. Hepatitis may also be caused by an agent(s) serologically unrelated to A or B; such infections are referred to as Hepatitis non-A, non-B. One such agent is shown here as a 27-nm fuzzy particle. (Micrographs a–c by courtesy of Dr. D. W. Bradley, Centers for Disease Control, Atlanta, GA.) d. Sections of hepatitis A-infected cells show dilated endoplasmic reticulum and small, round electron-dense particles as seen here in different areas of the same cell. Note the lack of distinguishable characteristics; most small RNA viruses in cells display the same characteristics. Sometimes virions collect into a group or crystalline array, but still do not show characteristic identifiable shape. (Cells were provided Dr. Ross McKinney, Duke Medical Center, Durham, NC.) Bars represent 0.1 μm .



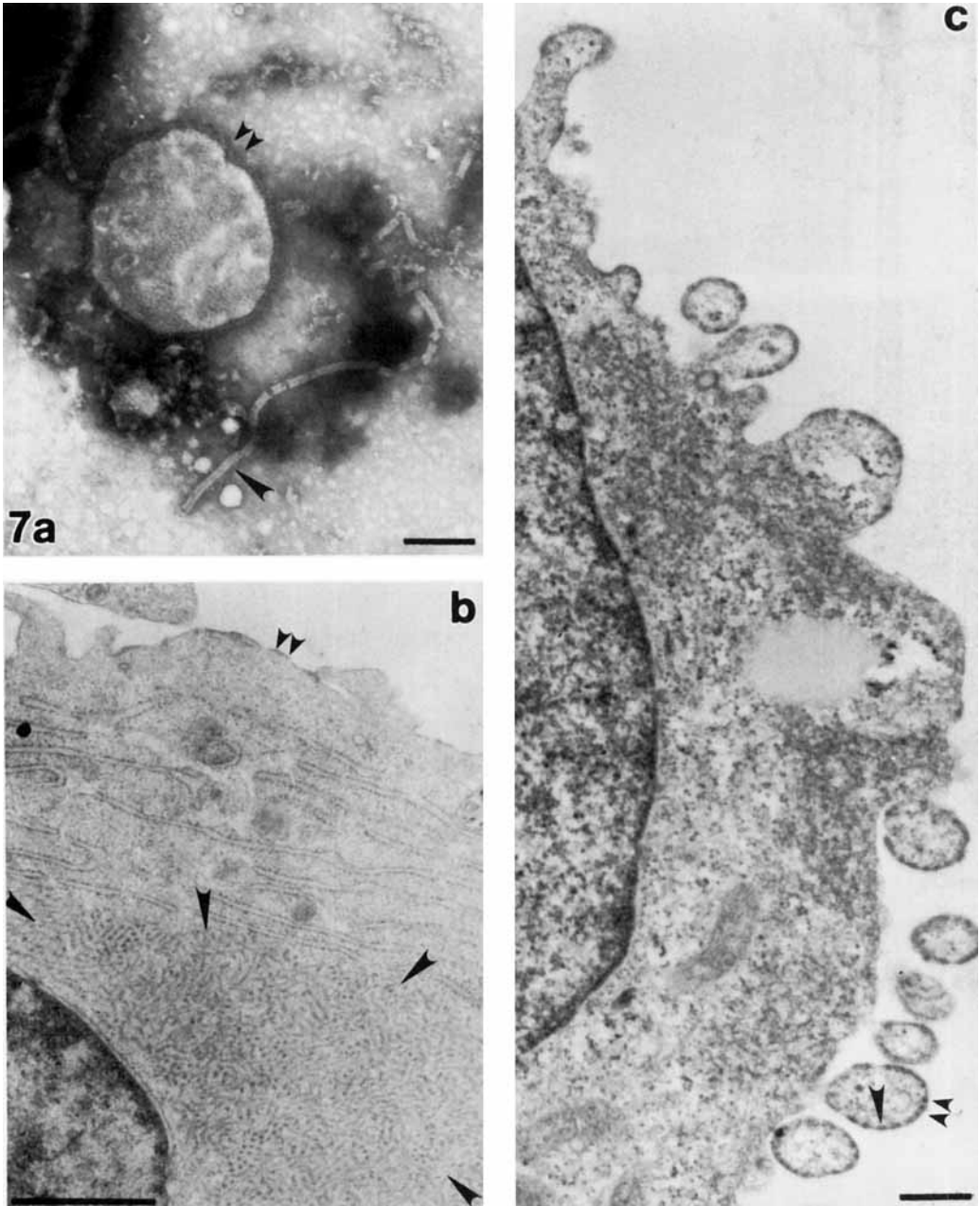


Fig. 7. a. A negative stain of measles virus shows a 200- to 300-nm pleomorphic-membraned virion with spikes on the surface (double arrowhead). Nucleocapsids, 18 nm in diameter, are helical and demonstrate a herringbone pattern (single arrowhead). b. In tissue sections, nucleocapsids appear as a "tangle of worms" (single arrowheads). They migrate to areas of thickened cell membrane (double arrowhead) containing virus-en-

coded material and bud out of the cell. c. Dots (single arrowhead) inside virions (double arrowhead) are nucleocapsids in cross section. Measles virions with fuzz around the outside of the membrane should not be confused with microvilli (Fig. 17b), caveolae (Fig. 18a,b) or coated pits (Fig. 18c,d). Other myxoviruses (influenza, parainfluenza, and mumps) resemble measles. Bars in a,c, represent 0.1 μm ; b, 1 μm .

be demonstrated after concentration of a substantial amount of fluid (2–5 ml) by ultracentrifugation. If only a small amount is available, use an airfuge. Immunoelectron microscopy with pooled serum may be helpful. Since there is no guarantee that the serum will recognize the virus that is present, we employ both immunoagglutination and ultracentrifugation. If virus is recognized, it will be in clumps, and more easily visualized; if not, the high-speed spin should pellet the individual particles. Negatively stain. In warm weather, enterovirus may be found. Herpes virus (simplex and zoster), mumps, and rubella may be present. Because of the dilute nature of the sample, tissue cultures should be also inoculated for amplification of viral numbers (see above for amplification methods, below for virus description).

Tears

Preparation of tears is the same as for CSF. Possible agents are adeno-, herpes-, echo-, entero-, and Coxsackie viruses.

Brain biopsy/autopsy material

Slice some tissue into slivers for embedding, and begin processing. Grind the remaining piece in a tissue homogenizer, and add buffer. Spin out the debris and negatively stain the supernatant (see Negative Staining); if negative, try concentration. Herpes, picorna-, mumps, JC, and rabies viruses may be seen.

Eye biopsy

Treat as above for brain. Herpes, CMV, rubella, and adenovirus are possible agents.

Respiratory system lavages or aspirations, pleural or pericardial fluids

If the specimen is thick with mucus, add a small amount of water (10%) and mix well; ultrasonicate the tube if necessary. Clear of debris by low-speed centrifugation (see above, Negative Staining) and examine the supernatant. If negative, concentrate by immunoaggregation and ultracentrifugation or airfuging. Rhino-, entero-, adeno-, respiratory syncytial, influenza, parainfluenza, and Coxsackie B virus are likely agents; measles, mumps, corona-, and rabiesvirus, if present, may be seen. In immunocompromised patients, almost any agent can be present in the lungs.

Blister fluids

Aspirate the fluid with a needle and place it directly onto a grid. If little fluid is available, inject a drop of sterile saline or water into the blister and remove. In some cases, there has been success in placing a grid directly onto the underside of the blister roof. Negatively stain. Agents to be differentiated are herpes and pox viruses. If negative, inoculate cultures.

Serum

Clarify at low speed. Negatively stain. Concentrate if necessary. Rubella and hepatitis B may be seen.

Liver or kidney biopsy

Treat as above for brain. Herpes, CMV, and adenovirus are likely agents. In kidney, antibody-antigen complexes in glomerulonephritis may resemble myxovirus nucleocapsids.

See Joklik (1984, pp. 835–846) for a detailed list of viruses by family and the diseases they cause. Dembert and Kaiser (1983) list specimens and the likely associated agents.

RESULTS AND DISCUSSION

General morphological characteristics

Figures are printed with the negative stains at the same magnification ($\times 100,000$) for direct size comparison. Some thin sections are at $\times 100,000$; others are at lower magnifications ($\times 20,000$) to show the relationship of viruses to the infected cell. The micrographs are grouped with respect to the agents that often must be differentiated from each other, e.g., enteric viruses all together. They are representative of the major variations in morphology, but are not all-inclusive. For classification by family, the reader is referred to the atlases listed in the Introduction and to Fraenkel-Conrat (1985).

Viruses are discussed here in morphologically similar groups rather than in the usual genetic/biochemical classification, because the differentiation by EM is visual. With this in mind, there are two major groups, naked and membraned virions. Naked viruses have a rigid protein coat encasing the genetic material. The coat is made up of subunits called capsomers, which are often resolvable by EM, as in the case of adenovirus. Most naked viruses are spherical (icosahedral) and range in size from 20 nm to 75 nm. Generally, naked virions get out of cells by lysing them.

Membraned viruses, on the other hand, have a more or less nonuniform shape. The virion is contained by a membrane that it obtains as it buds from the cell surface (except in the case of poxvirus); the membrane contains some cell-encoded and some virus-encoded proteins. The nucleocapsid containing the nucleic acid may (e.g., herpes) or may not (e.g., retrovirus) be surrounded by a rigid protein coat; and the capsid may be spherical (e.g., herpes), helical (e.g., measles), or complex (e.g., pox). Because of the pliable nature of the membrane, these viruses are pleomorphic, and some (e.g., rubella) may be difficult to distinguish by negative stain from cell membrane debris. If the stain penetrates the outer membrane so that the inner structure is visible (Fig. 8a), or if the surface has spikes (Fig. 7a), identification is easier.

A few membraned virions have a definite shape: Rhabdoviruses are bullet-shaped, and poxviruses are brick-shaped. In the case of pox, the membrane is synthesized *de novo* in the cytoplasm, rather than during budding; this membrane tightly surrounds the nucleocapsid so that the virion is not pleomorphic. In thin sections, the cellular location of virus can be a hint as to the type of nucleic acid, and thus to identification. DNA viruses are generally produced in cell nuclei (e.g., adenovirus), whereas RNA viruses are generally produced in the cytoplasm (e.g., picornavirus), but there are exceptions. Poxviruses (DNA) are constructed in the cytoplasm. Herpes viruses replicate in the nucleus and bud through the nuclear membrane; although only nucleocapsids are found in the nucleus, nucleocapsids and whole virions can be seen in the cytoplasm. Some viruses have a preference for certain organ systems; thus, if the sample origin is considered, some agents can be ruled out. However, in immune deficient or suppressed patients, almost any agent can be found almost anywhere; such individuals are particularly prone to disseminated infections with herpes, CMV, VZV, and enteroviruses.

Agents

Naked viruses

Adenovirus (Fig. 1) is a large (75–80 nm) nonenveloped icosahedral virus that can be found in stool and most other tissues. The capsomers are arranged to form triangular facets, and in negative stains, depending upon how the virion lands on the grid membrane, the circumference may appear hexag-

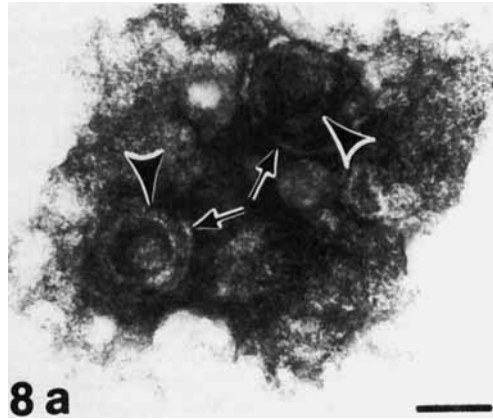
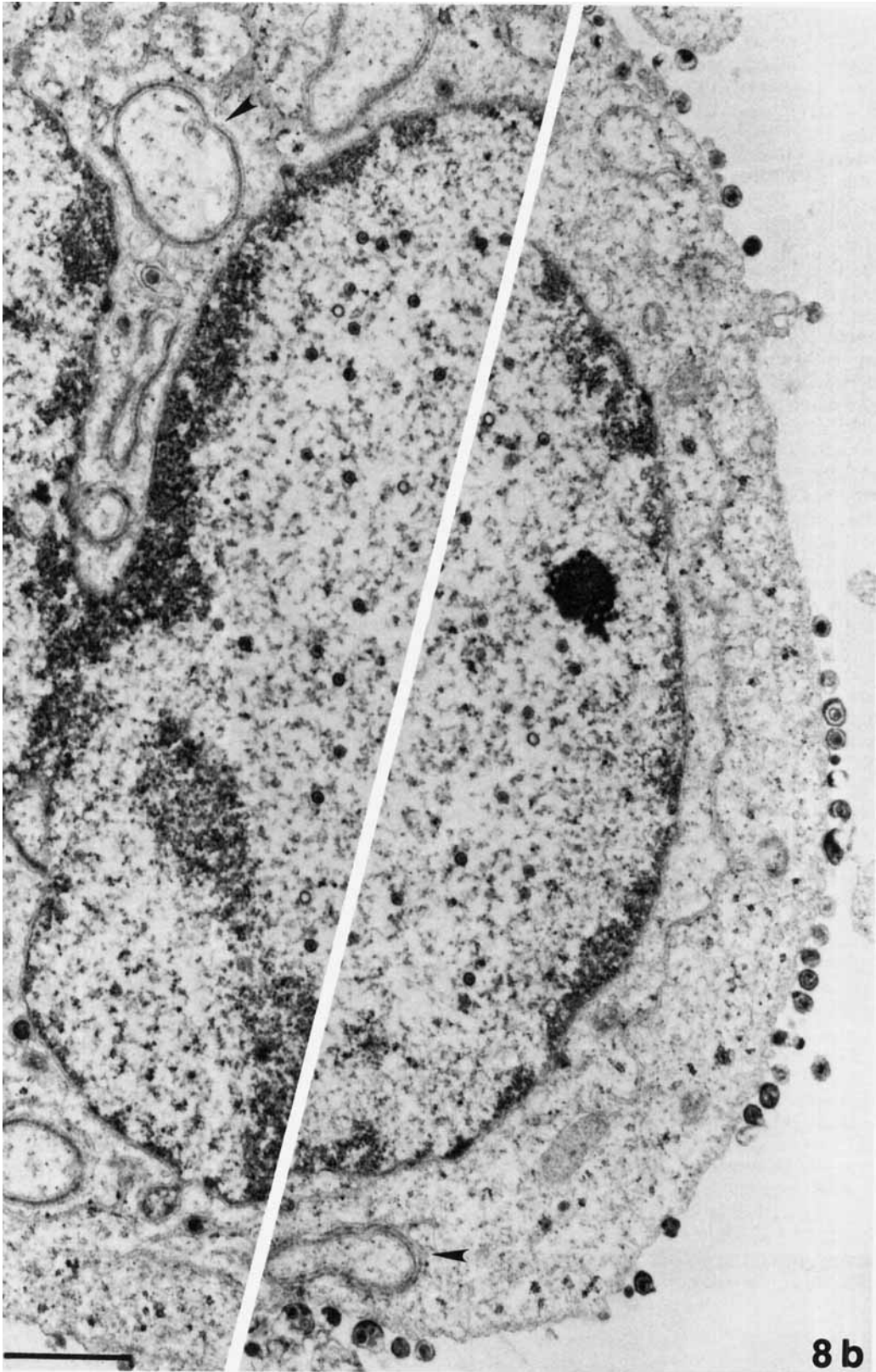


Fig. 8. a. Herpes viruses are membraned 200- to 300-nm particles, and unless the stain penetrates the membrane (arrowheads) so that the 100-nm spherical nucleocapsid is visible (arrows), particles cannot be differentiated from cell membrane debris (see Fig. 15a). In purified preparations where the membrane has been removed, the capsomers resemble those of rotavirus (tubular, Fig. 2a). b. Thin section of an infected cell showing nucleocapsids in the nucleus, and whole virions both in the cytoplasm and at the cell periphery. Note the overproduction of cell membranes (arrowheads). Herpesvirus in tissue sections should be differentiated from adenovirus, which is not seen in the cytoplasm. Herpes usually does not form crystalline lattices as does adenovirus. Bar in a represents 0.1 μm ; b, 1 μm .

onal (Fig. 1a). Fibers that are attached to the vertices (penton antigens) are rarely seen in patient material. In thin sections of infected cells, adenovirus (a DNA virus) is found in the nucleus. It has an electron-dense core with a lighter shell; some virions are less dense, with a crisp shell definition (Fig. 1b). Virions may appear hexagonal or round, depending on the section plane. Many times they line up with flat sides together to form a crystalline array (Fig. 1c). Adenovirus should be differentiated from herpesvirus in the nucleus. Some adenoviruses are fastidious and are not easily grown in culture, further emphasizing the importance of direct EM identification. They may be seen in patients with respiratory illnesses, conjunctivitis, gastroenteritis, renal transplants, and immune deficiencies.

Rotavirus (Fig. 2) is also a large (75-nm) nonenveloped spherical particle, but it is seen only in stool; its capsomers are short, hollow tubes arranged to resemble the spokes of a wheel (hence, "rota"). There are two shells with a thin coating outside (Fig. 2a, single arrowhead), which often is missing in patient



material (Fig. 2b). Many times lattices are visible (Fig. 2a, double arrowhead), the subunits of which are the size of capsomers. These structures may be seen in late stages of infection and are diagnostic, even in the absence of whole rotavirions, but must be differentiated from other crystalline structures such as bacteriophage neck and head proteins. (See section on Confusing Elements.) Some rotaviruses can be grown in culture in the research laboratory under special conditions, but human rotavirus is not culturable in the diagnostic laboratory. Tissue samples containing rotavirus (e.g., segments of necrotic bowel removed surgically) are unusual, but may occasionally be received. In such specimens, the virus resembles reovirus, which is produced in a granular matrix in the cytoplasm (Fig. 2c). Reovirus in negative staining resembles rotavirus without the thin outer rim (Fig. 2b). It has been seen in the respiratory tract of patients and normal individuals and has been reported in stool, but its role as an etiological agent is equivocal.

Medium-sized entero- and rhinoviruses range from 40 nm to 45 nm, are spherical, and lack envelopes. Most do not have a distinctive capsomeric morphology that allows them to be identified in an unknown. One can only report the presence of a 40- to 50-nm particle. These, however, must be distinguished from bacteriophages when the sample is a stool. (See Confusing Elements.) Papova viruses are spherical 40- to 55-nm particles with a rough surface. Examples are papilloma (wart) virus, which measures 50–55 nm (Fig. 5); BK virus, which is found in urine of transplant patients; and JC virus, which has been seen in brains of patients who have died of progressive multifocal leukoencephalopathy (PML). Most are not commonly grown in tissue culture, but in infected tissue nondescript dense virions are found in clusters (Fig. 5b) and sometimes in crystalline arrays in the nucleus.

Most small viruses (20–40 nm) also do not have a distinctive morphology by negative staining. Examples are the picornaviruses, including poliovirus (Fig. 3a), echovirus (Fig. 3b), Coxsackie B virus (Fig. 3c); and Norwalk agent (a parvovirus). Some can be identified morphologically. Calicivirus (Fig. 3d) is 35–40 nm and has capsomers that are cup-shaped. Minireovirus (Fig. 3e) resembles members of the family Reoviridae, such as rotavirus and reovirus, but does not belong

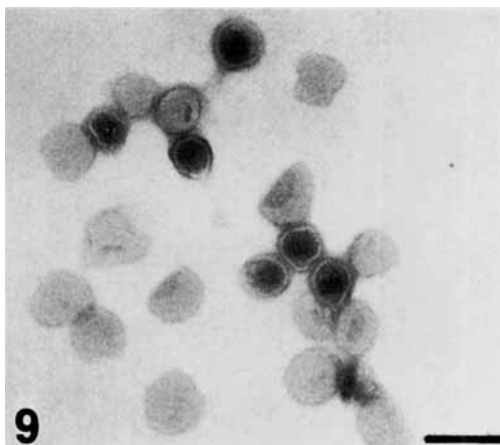


Fig. 9. A purified preparation of rubella virus, negatively stained; virions are about 40–60 nm. Membraned viruses without spikes are pleomorphic, fuzzy, and impossible to differentiate from cell debris by negative stain. (Micrograph courtesy of Robert Simmons, Atlanta University, Atlanta, GA.) In thin sections of infected tissue, rubella resembles retrovirus, but depending on the plane of section, some virions have a more dense core. Bar represents 0.1 μ m.

to the same family; it is about 35 nm. Astrovirus sometimes has a six-sided star appearance (Fig. 3f) and is about 35 nm. These agents, too, must be distinguished from bacteriophages in stool; clues to the presence of a pathogen are large numbers, large clumps, uniform size, and the apparent rigidity of the shell. Unidentifiable viruses from stool are shown in Figure 3, panels g–l; bacteriophages and confusing elements are shown in Figures 13–15.

In tissue, DNA viruses are found in the nucleus, RNA viruses in the cytoplasm. In thin section, the small and medium-sized icosahedral DNA viruses can be seen as uniform small, dense bodies in the nucleus without characteristic structure (Fig. 5b), which must be distinguished from nuclear granules and nuclear bodies (Fig. 23); sometimes they may be in crystalline arrays. Icosahedral RNA viruses may cause dilated endoplasmic reticulum and collections of protein around ribosomes resulting in an appearance of large beads on a string (Fig. 6d). Sometimes factories of viral protein form a granular deposit or crystalline arrays of nucleocapsids in the cytoplasm.

Membraned viruses

Coronavirus (Fig. 4) is the only membraned virus that is occasionally seen in stools; it

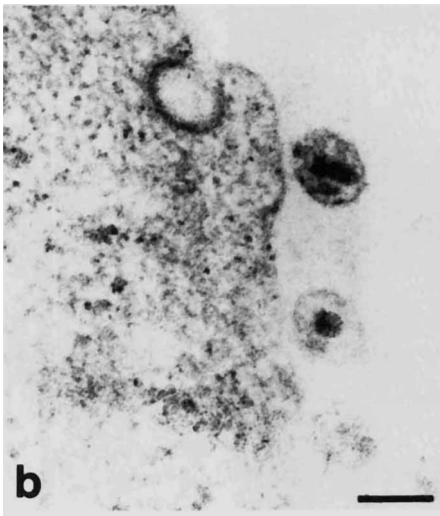
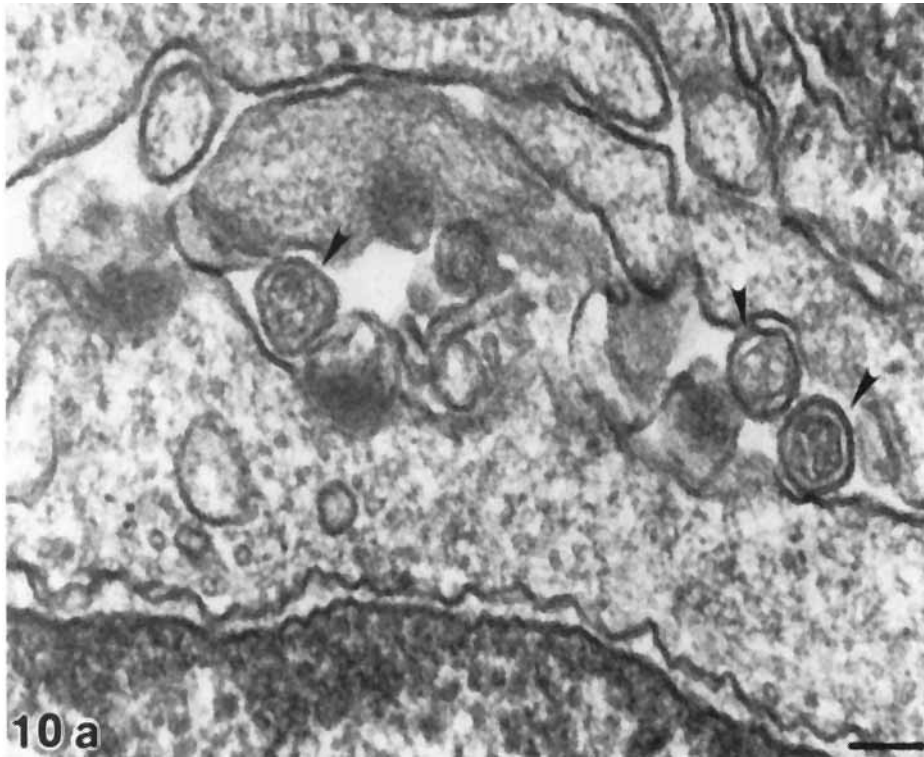


Fig. 10. Retroviruses by negative stain also resemble cell debris. a. HTLV-1 (arrowheads) in tissue is found budding from the cell membrane, often in groups or patches on one side of the cell; it has a nucleoid less dense than herpes and is never seen in the nucleus; b. HTLV-3 in thin section, depending on the cutting plane, may show a rod-shaped core. Bar represents $0.1 \mu\text{m}$.

has been reported in respiratory tract infections. Pleomorphic and measuring 80–130 nm in diameter, it differs from the myxoviruses in that its spikes are long and club-shaped. Arenaviruses are pleomorphic, 50–300 nm in diameter, and closely resemble

coronaviruses. They cause hemorrhagic fevers, but human infections in the United States are rare.

Other membraned viruses are shown in Figures 7–11. Measles, a paramyxovirus, is a large (200–300 nm) pleomorphic virion with

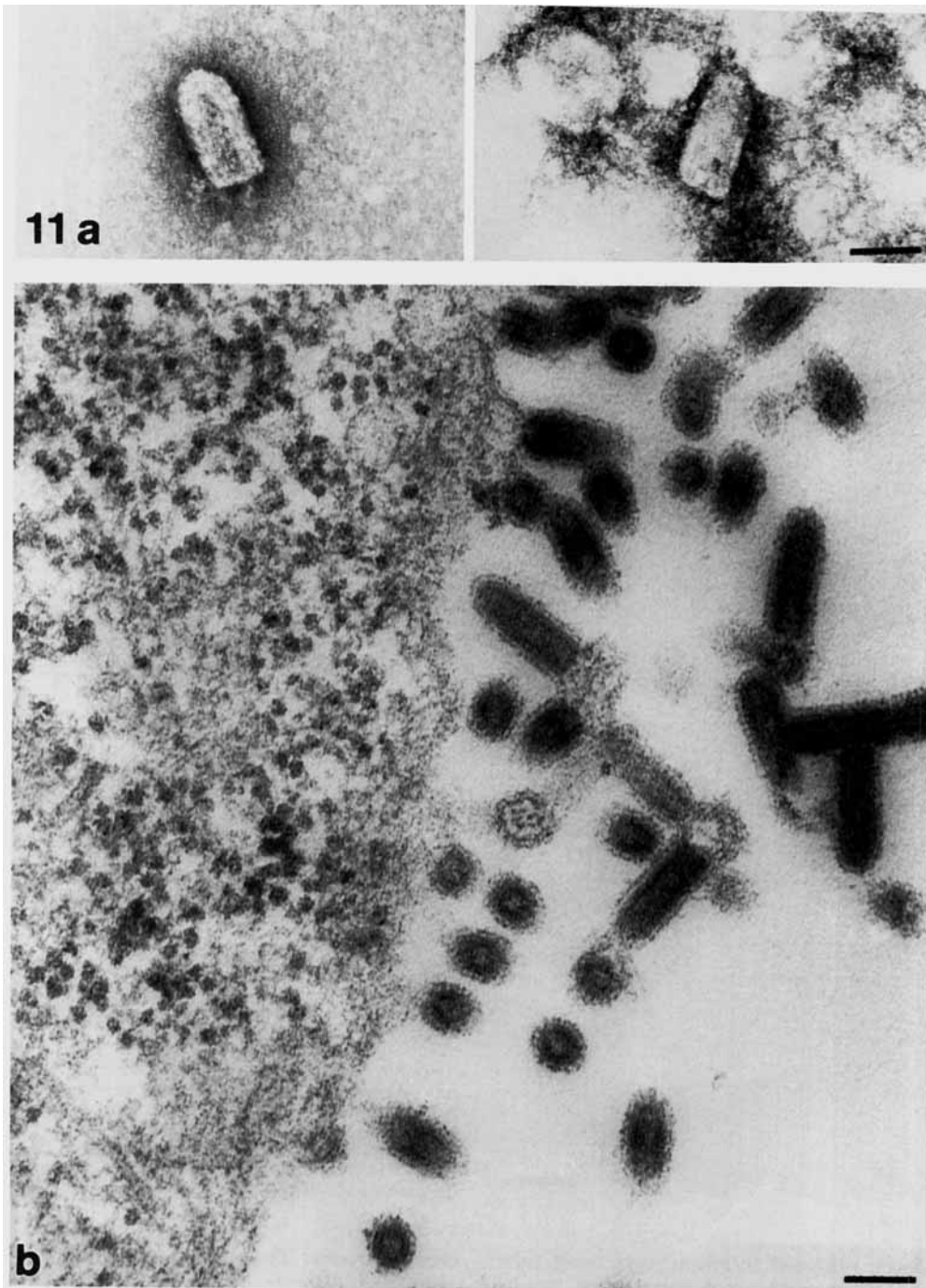


Fig. 11. Rabies virus is routinely diagnosed by fluorescence microscopy of tissue culture. By EM, it is bullet-shaped and resembles closely vesicular stomatitis virus, shown here. a. Negative stain of VSV virions, 60×180

nm; projections are 6–8 nm. b. Thin section of virions budding from the cell membrane. (Infected cells and virus were provided by Lucille Fresco, Duke University Medical Center, Durham, NC.) Bars represent $0.1 \mu\text{m}$.

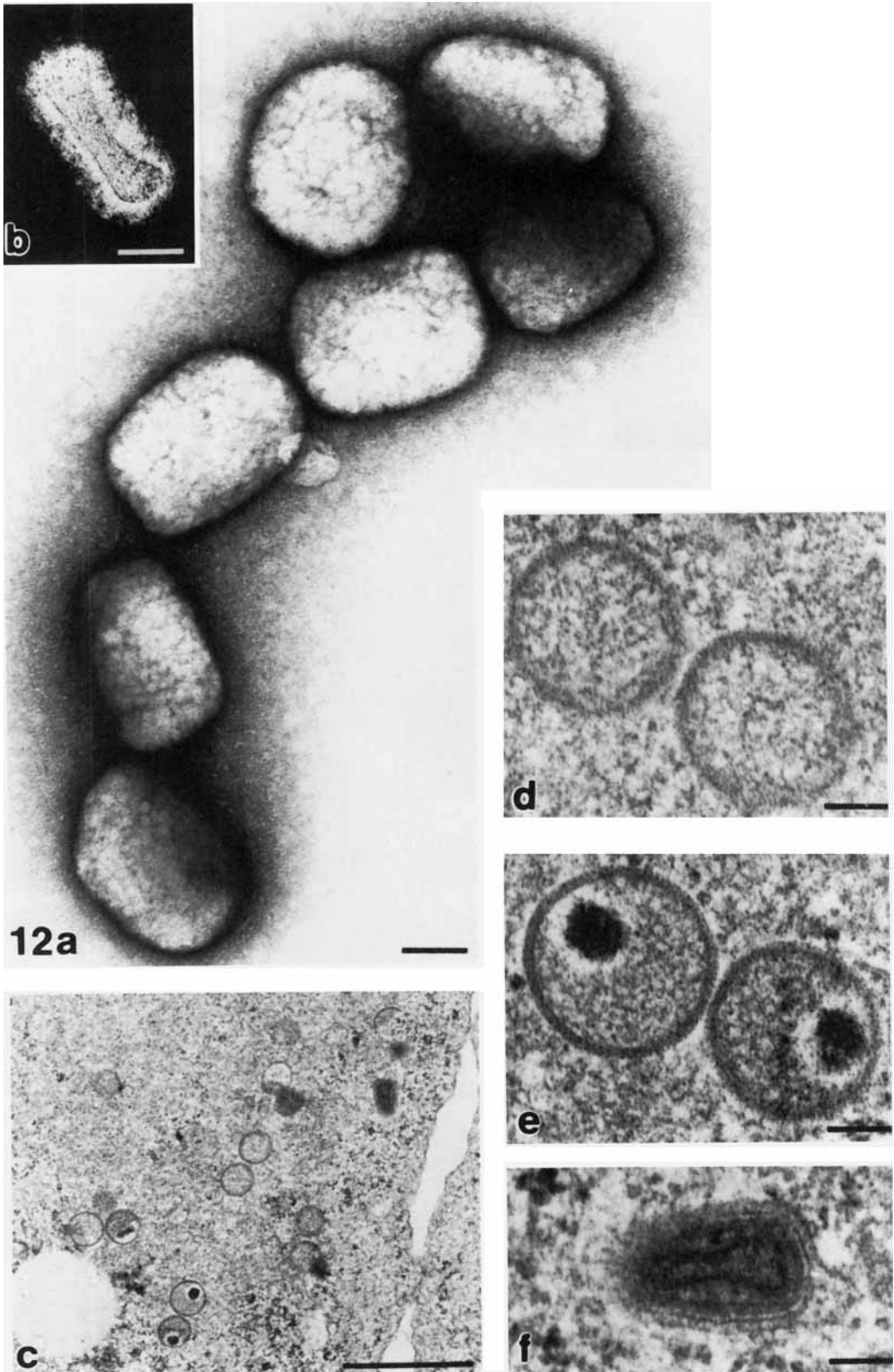


Fig. 12. Pox viruses are large (150–250 × 200–400 nm), brick-shaped particles. a. Cowpox, negative stain showing the rough exterior. b. Thin stain has penetrated the capsid showing the bone-shaped core. c. Thin section of cowpox in the cytoplasm of an infected cell. d–f. Pro-

gressively more mature particles. In skin lesions, a differential diagnosis between pox and herpes virus would be significant. (Infected cells and virus were provided by Dr. David Pickup, Duke University Medical Center, Durham, NC.) Bars in a,b,d,e,f represent 0.1 μm; c, 1 μm.

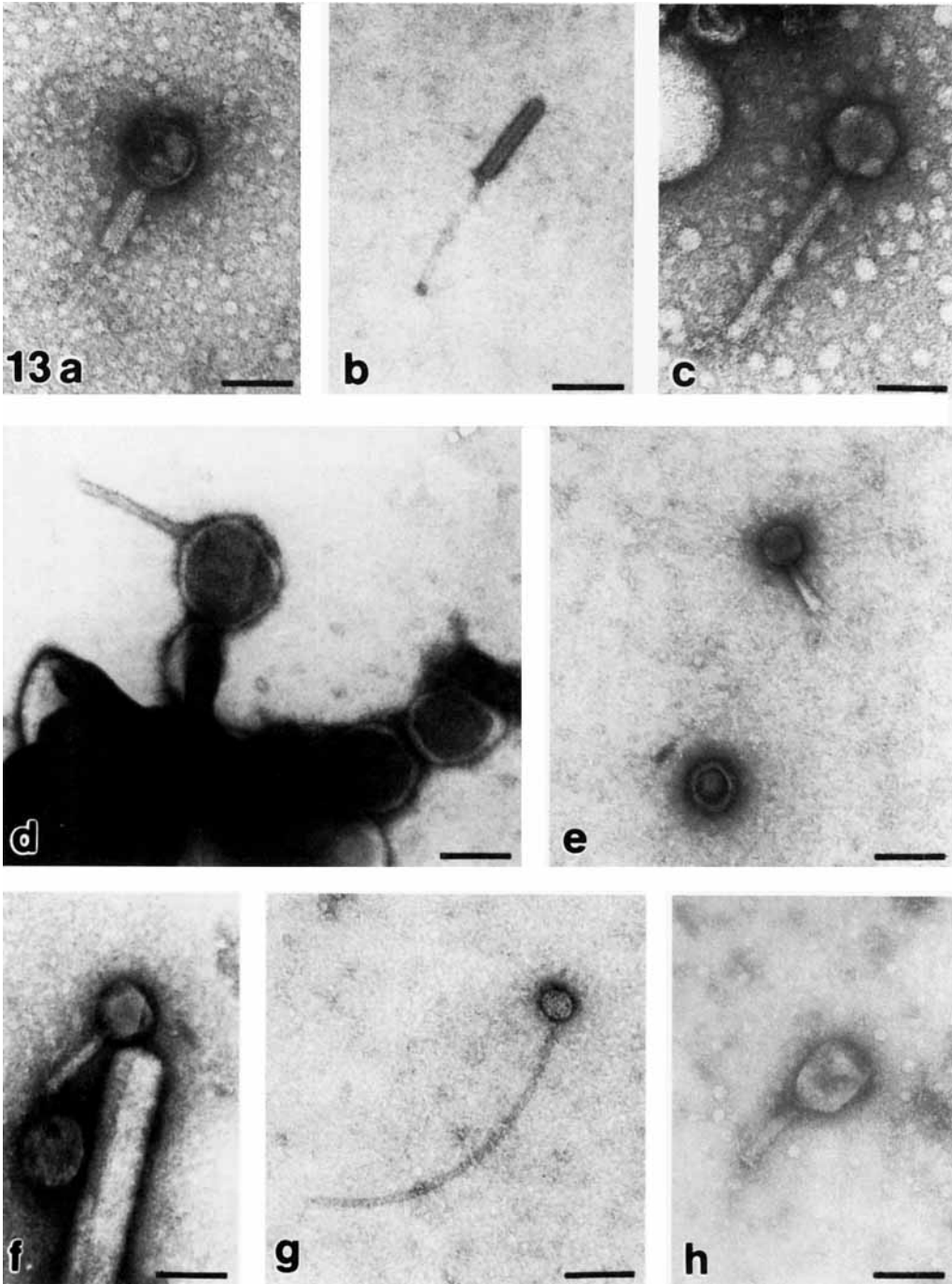
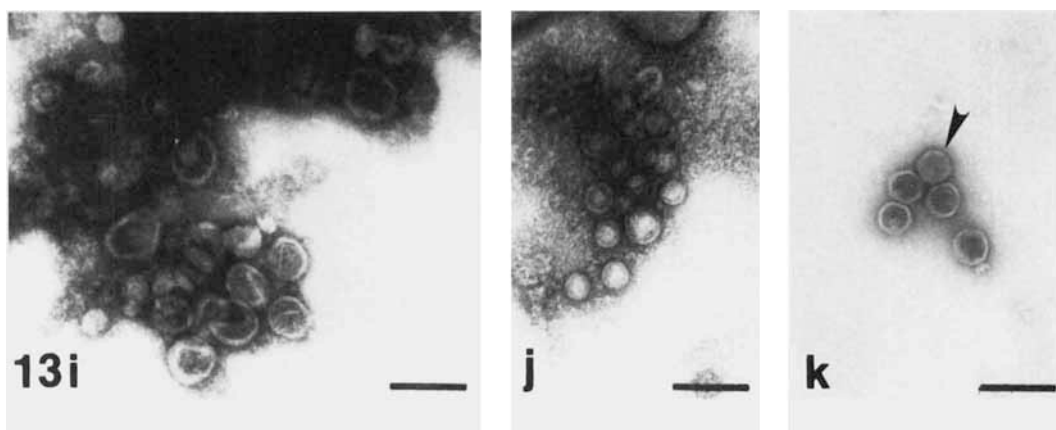


Fig. 13. Bacteriophages with tails (a–h) are easy to discern, but tailless varieties may be confused with enteroviruses in negative stains. Note the “bullseye” appearance (e,i,j) and the wrinkled appearance (i); some are angular (k, arrowhead). Sizes may range from 20 nm to over 100 nm. All bars represent 0.1 μm .



spikes on the surface (Fig. 7a, double arrowhead). Inside the virion are long filamentous nucleocapsids, about 18 nm in diameter, which in negative stain have a herring bone appearance (Fig. 7a, arrowhead). In thin sections of infected cells, nucleocapsids appear as a "tangle of worms" in the cytoplasm (Fig. 7b, arrowheads). They emerge from the cell by budding through areas in the cytoplasmic membrane that bear viral-encoded and cell-encoded proteins. These areas have a thickened or fuzzy appearance (Fig. 7b, double arrowhead) that corresponds to the spikes in negative stain. A high magnification shows mature virions at the cell surface (Fig. 7c, double arrowhead); the dots inside the membrane (single arrowhead) are nucleocapsids cut in cross section. Measles can be seen in nasopharyngeal secretions and occasionally in skin scrapings. A measles-like agent has been isolated from brains of children with subacute sclerosing panencephalitis (SSPE). Influenza is an orthomyxovirus with a spiked membrane and helical nucleocapsid about 9 nm in diameter, similar to that of measles. In general, virion size is somewhat smaller than measles; some strains are more rounded, but others are elongated or filamentous. Virions of respiratory syncytial virus, like influenza, may be round or filamentous; nucleocapsids are 13–14 nm, similar to those of measles. Bunyaviruses morphologically resemble measles. They cause fevers and encephalitis, but are not a problem in the United States.

Nonspiked membraned viruses are difficult to discern by negative staining unless their nucleocapsid is distinctive and unless the stain penetrates the membrane so that

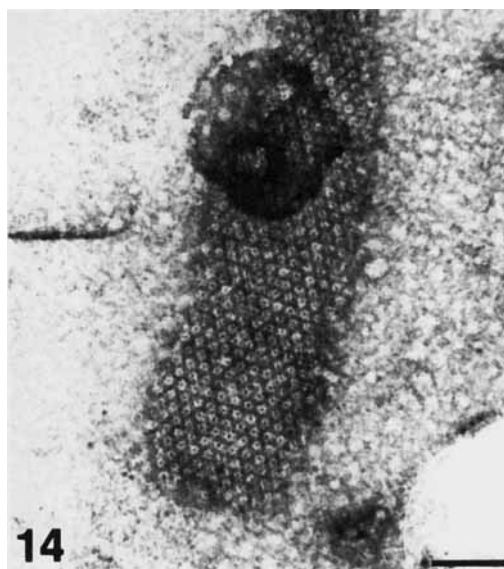


Fig. 14. Bacteriophage neck proteins can aggregate and resemble rotavirus lattice material (Fig. 2a). In rotavirus lattices, the sides of one hexagon are integral parts of other hexagons, whereas entire hexagons of phage proteins are separated by granular material. Bar represents 0.1 μm .

the core is visible. In Figure 8a, the stain has penetrated so that the 100-nm icosahedral herpesvirus nucleocapsid is recognizable within the 200–300-nm virion. A sample of cell debris is pictured (Fig. 15a) to demonstrate possible confusion.

Herpesviridae can be found in a number of sites. Herpes and VZV may be demonstrated in blisters by negative staining. CMV can

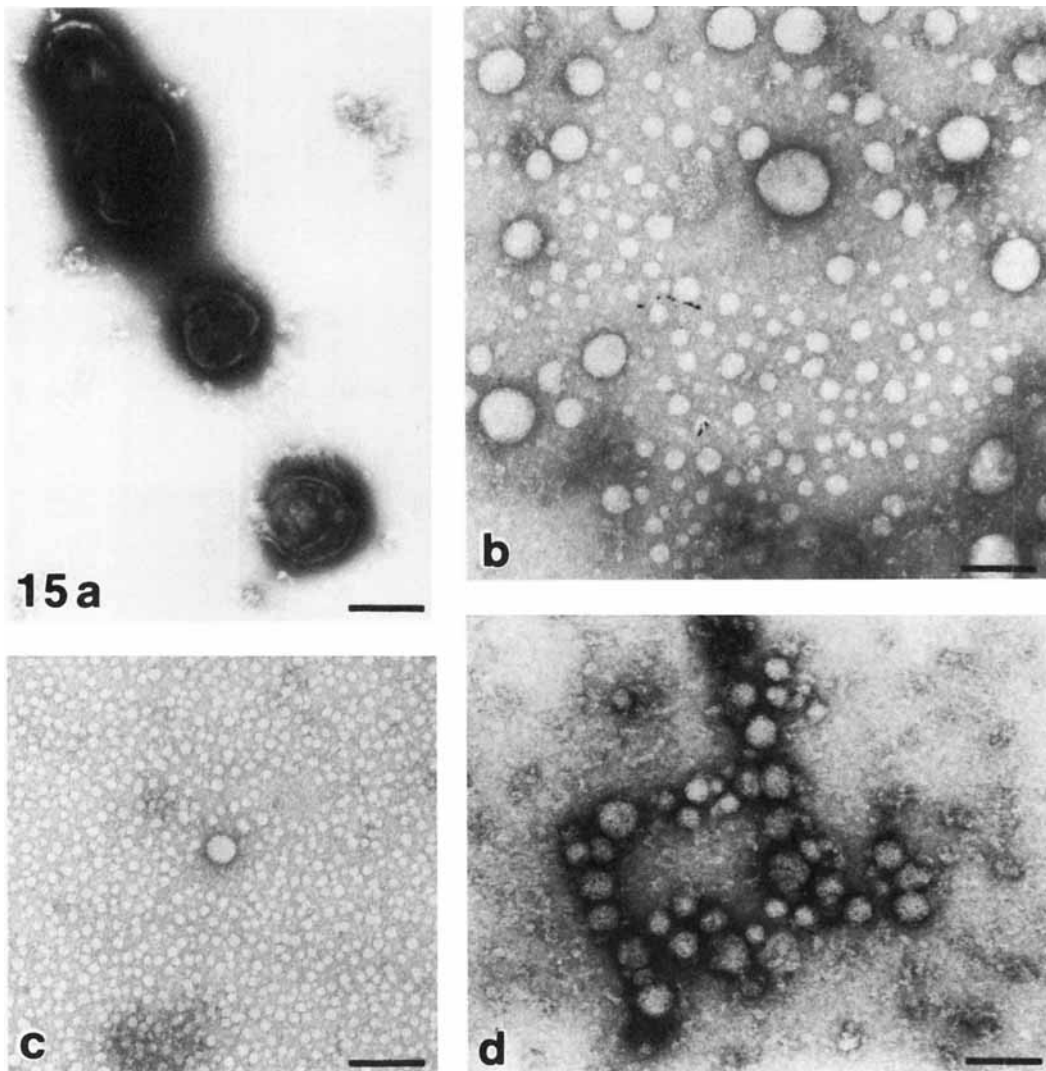


Fig. 15. a. In negative stains, membranous cell debris can be confused with enveloped viruses; compare this material, especially the structure at the bottom right, to Figure 8a. b. Nonviral lipid-like droplets from stool. c. An unknown enterovirus in a field of droplets or bubbles

in the stain; had this virion been in the field in panel b, it would have been unrecognizable. d. Clumped material from stool; note the nonuniformity of droplets. All bars represent $0.1 \mu\text{m}$.

cause hepatitis in young people and pneumonitis. It can be isolated from urine, saliva, milk, cervix, and fetal tissues. Epstein-Barr virus can be seen in cases of mononucleosis and pneumonitis in the United States, where it is found in blood, leukocytes, and pharyngeal secretions. It is associated with Burkitt's lymphoma in African patients and nasopharyngeal carcinoma in Oriental patients. Herpes, CMV, and VZV can also cause

severe, disseminated infections in immunocompromised patients. In infected tissue, nucleocapsids with a dense core are seen randomly in the nucleus (Fig. 8b); compare these to the crystalline array of adenovirus (Fig. 1c). The outer membrane is added as the nucleocapsid buds through the nuclear membrane, into cellular vesicles, or through the cytoplasmic membrane. Infected cells have many whorls of extra membrane (Fig.

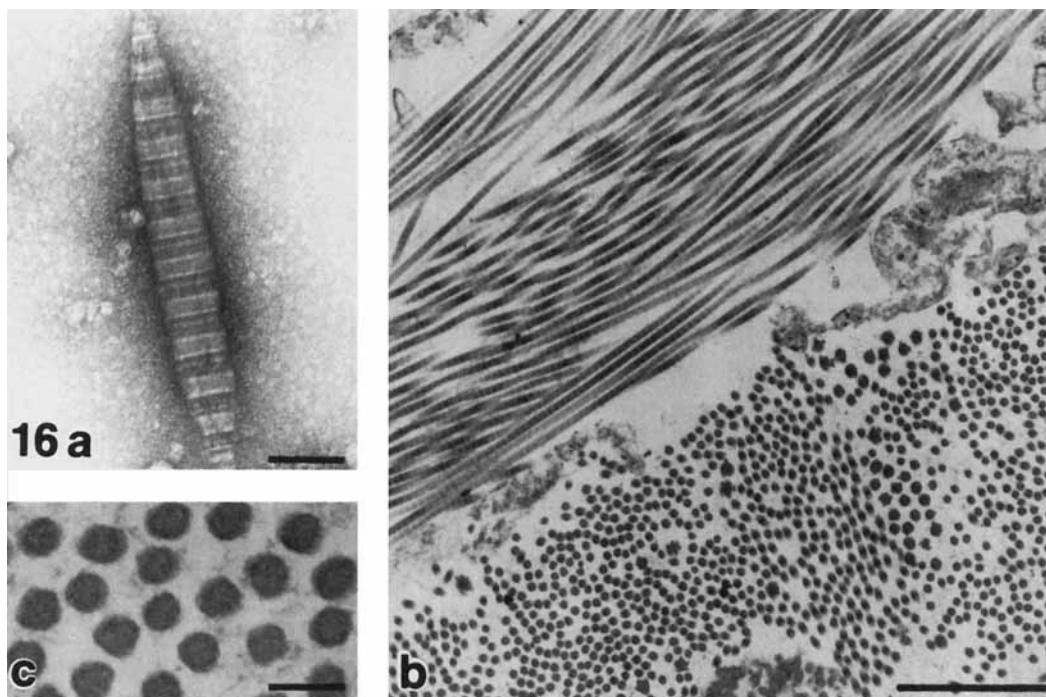


Fig. 16. a. Banded filaments of collagen are often seen in negative stains of stool preparations, but are insignificant in the diagnosis. b, c. In cross sections, their uniform density and lack of internal structure distinguishes them from viruses. (Micrographs b,c taken by Lynne Bartling in this lab.) Bars in a,c represent $0.1 \mu\text{m}$; b, $1 \mu\text{m}$.

8b, arrowhead). Members of the Herpesviridae family can be differentiated from other families, but not usually from each other, though herpes type 2 produces 20-nm intranuclear filaments, which are not seen in type 1 infections.

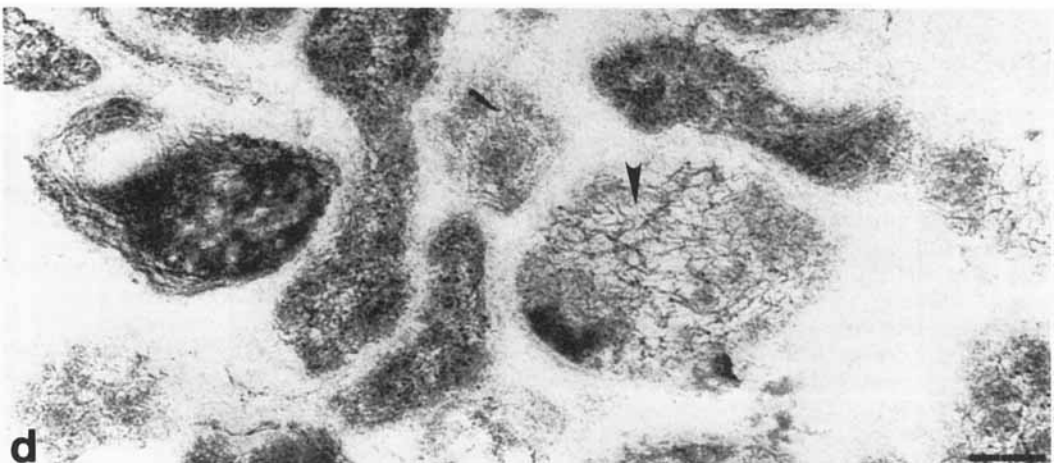
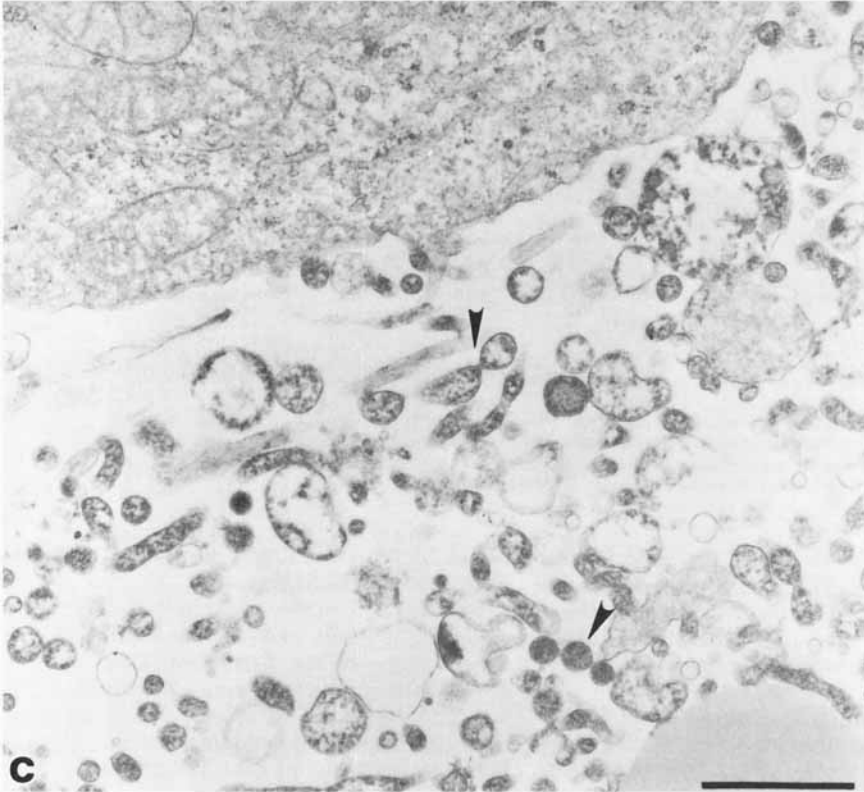
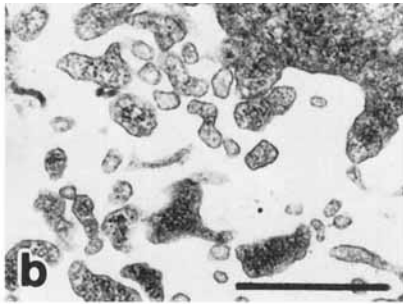
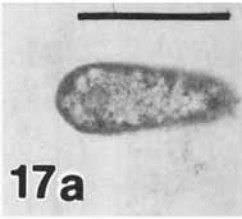
Other nonspiked membraned viruses are members of the Rubiviridae (Fig. 9), Togaviridae, and Retroviridae (Fig. 10); some are rough with short projections, but lack myxovirus-like spikes. In thin section, intracellular varieties of these agents must be differentiated from secretory products (Fig. 20a).

Rubella virus causes German measles; in the first trimester of pregnancy, it causes congenital defects in the infant. A purified preparation shows a roughly spherical particle of 40–60 nm; the surface appears rough or fuzzy, but measles-like spikes are not evident (Fig. 9). Thin sections would show virions budding from the cytoplasmic membrane like retrovirus, but with a somewhat denser center. Rubella virus can be isolated from pharyngeal secretions, acute-

phase blood, CSF, urine, amniotic fluid, and placental and fetal tissues.

Togaviruses cause encephalitis (e.g., eastern and western equine encephalitis), and fevers (e.g., yellow fever and Powassan fever). By negative staining, they are 40–70 nm and resemble rubella. In thin sections, they can be seen in endoplasmic reticulum or budding from the plasma membrane or into vesicles. Virions are nondescript, with a dark center surrounded by a membrane.

Retroviruses are RNA viruses that contain an enzyme, reverse transcriptase, that makes DNA from an RNA template. Research laboratories have used assays for reverse transcriptase to detect the presence of these viruses (Gallo and Reitz, 1976; Kacian, 1977). Complete retroviruses are rarely seen in patient material unless it is cultured for several days or cocultivated with susceptible cells (Poesz et al., 1980). In thin sections, they have a moderately dense core surrounded by a membrane that is obtained as they bud through the cytoplasmic membrane. They are roughly spherical and about



100 nm in diameter. Human T-cell leukemia (leukotropic) virus (HTLV), type 1 is shown in Figure 10a. The AIDS-associated agent HTLV-3, depending on the plane of section, may show a rod-shaped core (Fig. 10b). There are no exterior distinguishing characteristics, making these viruses unrecognizable from cell debris in negative stain.

Rabies is rare in humans, but because of the serious consequences, identification of infected animals is important. The disease is usually diagnosed by immunofluorescence microscopy in a state laboratory. By EM, the virus is bullet-shaped, 60×180 nm with 6–8 nm spikes, and arises from the cell by budding. It resembles vesicular stomatitis virus (VSV) (Fig. 11), which is not important in human disease.

Two agents that one might be asked to differentiate are herpes and poxvirus, both of which can cause illnesses with blisters. Poxviruses differ somewhat among themselves, but all are large, brick-shaped structures ($150\text{--}250 \times 200\text{--}400$ nm (Fig. 12). The membrane is lipoprotein, but is synthesized de novo in the cytoplasm of cells, rather than being added during budding. In negative stains, the outer surface has ridges that, depending on the species, may resemble coils of rope wound around a brick or the gyri of a brain (Fig. 12a). If stain penetrates the shell, a "dumbbell-shaped" core can be seen (Fig. 12b). In thin sections, pox, a DNA virus, is seen in the cytoplasm of cells (Fig. 12c), rather than in the nucleus as other DNA viruses. Depending on the maturity of the virion and the plane of section, the core may be less dense and oval (Fig. 12 c,d), contain a dense nucleoid (Fig. 12c,e), or have a "dumbbell-shaped" nucleoid (Fig. 12c,f). Virions may be associated with lipid-like or finely granular inclusions in the cytoplasm.

Several different agents can cause inflammation of the liver, or hepatitis, which is usually diagnosed serologically. Most often,

the agent is hepatitis A, a food- and water-borne virus that can be seen in stool as a 27-nm icosahedral particle (Fig. 6a). In infected cells (Fig. 6d), swelled endoplasmic reticulum with ribosomes and viral protein resembles large beads on a string. Virions are dense, nondescript bodies. Hepatitis B is transmitted by blood products or intravenous drug use, and it can be seen in serum and extracts of liver biopsies as 42-nm icosahedra called Dane particles (Fig. 6b, arrowhead). The Dane particles are infectious, but the smaller, accompanying 22-nm particles (Australia antigen) and 22-nm \times 50- to 200-nm filaments, made up of coat proteins, are not. Hepatitis B cannot be grown in tissue culture. Other viruses, which do not crossreact serologically with either of these, have been isolated from blood of hepatitis patients; these are called non-A, non-B hepatitis. There are probably two or more different agents in this group. One example is shown in Fig. 6c; another has been reported to resemble the AIDS virus.

Confusing elements

Many structures in negatively stained preparations can be mistaken for viral pathogens, e.g., cell organelles such as ribosomes and vesicles, lipid droplets, and most notably in stool specimens, bacteriophages. Figures 13–25 illustrate some of these structures. It will be obvious to most observers that anything between 20 nm and 70 nm with a tail is a bacteriophage (Fig. 13a–h) (Ackerman and Nguyen, 1983; Kawaoka et al., 1982; Teuber and Lembke, 1983; Walker and Walker, 1983), is not pathogenic to humans, and thus is insignificant in viral gastroenteritis. However, many phages lack tails and can easily be confused with enteroviruses. Clues to the relevance of small particles are a) large numbers (10 to several hundred per grid hole), b) clumps of 3 or 4 to 100, and c) uniformity of size and shape. Enteroviruses range from 20 to 50 nm (Fig. 3), but within a given type, are all one size; they have rigid protein coats that retain their spherical shape (are not deformable), rarely show facets, and never show wrinkles. Phages may be produced as proheads, complete heads, or head and tailed varieties; thus, one kind may come in several different sizes. Some phages are oval (Driedonks and Caldentey, 1983). Many have soft membrane-like heads, which, after drying, appear to have wrinkles (Fig. 13i) (Driedonks and Caldentey, 1983; Fuller

Fig. 17. a. Bacteria are larger than viruses, have a thick cell wall, and may show internal strands of nucleic acid. b. Cell microvilli and ruffles contain ribosomes, but no DNA strands. c. Mycoplasma are larger than most viruses, and are very irregular in shape, since they do not have a rigid cell wall. Some may resemble microvilli, others may be more dense; budding forms are usually present (arrowheads). d. At high magnification nucleic acid strands (arrowhead), not present in microvilli, are visible. (Micrograph d taken by Owen McMillan in this lab.) Bars in a-c represent 1 μ m; d, 0.1 μ m.

and King, 1982; Walker and Walker, 1983). Phages may be seen attached to bacteria, flagella, and pili (Beibricher and Düker, 1984; Coetzee et al., 1983). They also may clump together in a manner similar to human virus clumped by antibody, but usually the stain, especially uranyl acetate, penetrates many of the particles, making the nucleic acid stand out and producing a "bull's eye" pattern (Fig. 13e,i,j) (Fuller and King, 1982: Figs. 8, 10). If a particle is less than 75 nm, has triangular facets, or appears strongly hexagonal or pentagonal (Fig. 13k, arrowhead), it is probably a phage (Kwiatkowski et al., 1982; Ramsay and Ritchie, 1984; Walker and Walker, 1983), though picornaviruses are icosahedral, and some may demonstrate facets, e.g., polio (Fig. 3a). Adenovirus may also appear hexagonal, but it is large (75 nm), with clearly distinguishable capsomers (Fig. 1a), and is not easily confused with any other virus.

Sometimes present in rotavirus infections are lattice structures, presumably composed of viral capsid material (Fig. 2a, double arrowheads), which can be diagnostic. However, these must be clearly differentiated from other crystalline structures such as ribosomes (Haschemeyer and Meyers, 1972; Kuhlbrandt, 1982: Figs. 3-6; Palmer and Martin, 1982: Fig. 21-1) and lattices of bacteriophage neck (Carrascosa et al., 1983) or head (Parker et al., 1983) proteins (Fig. 14). Note that in rotaviral lattices, one side of a hexagon is an integral part of an adjacent hexagon, like "chicken wire"; whereas in phage material, each hexagon is entire and separated from other hexagons by dense material.

In negative stains, membranous cell debris can be confused with nonspiked, enveloped viruses; compare Figure 15 with Figures 8 and 9. Differentiation can be made only if the nucleocapsid is recognizable inside. Globular material can resemble the 20- to 50-nm naked viruses; clues to the recognition of viruses are uniform size and shape, and the existence of clumps; compare Figure 3a-k to Figure 15b-d. In Figure 15c, a 40-nm virus particle stands out among smaller droplets; if it had been in the field of Figure 15b, it would be unrecognizable. The small irregular droplets should not be confused with picornaviruses. Another factor in virus identification, particularly in sizing, is to select areas where the negative stain has spread evenly and without bubbles. In Fig-

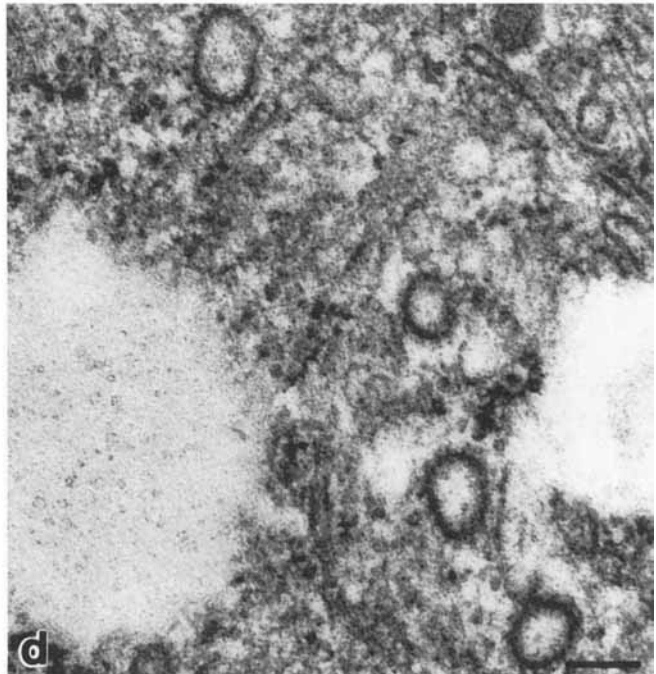
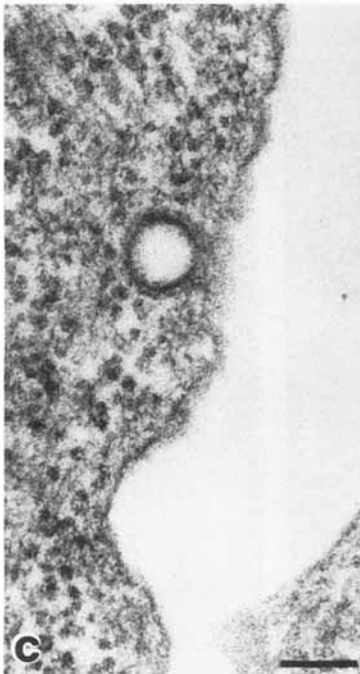
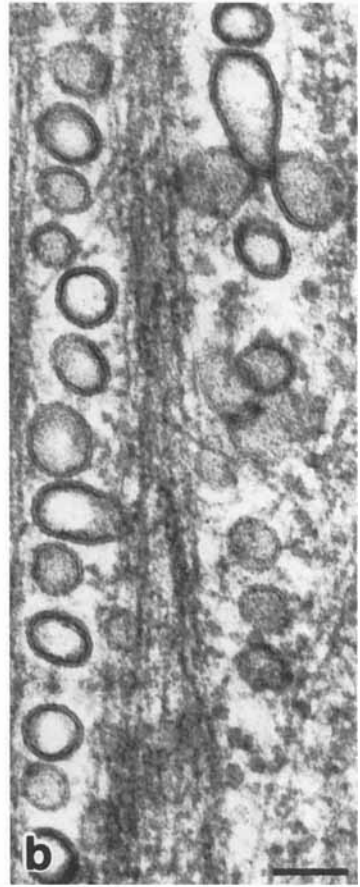
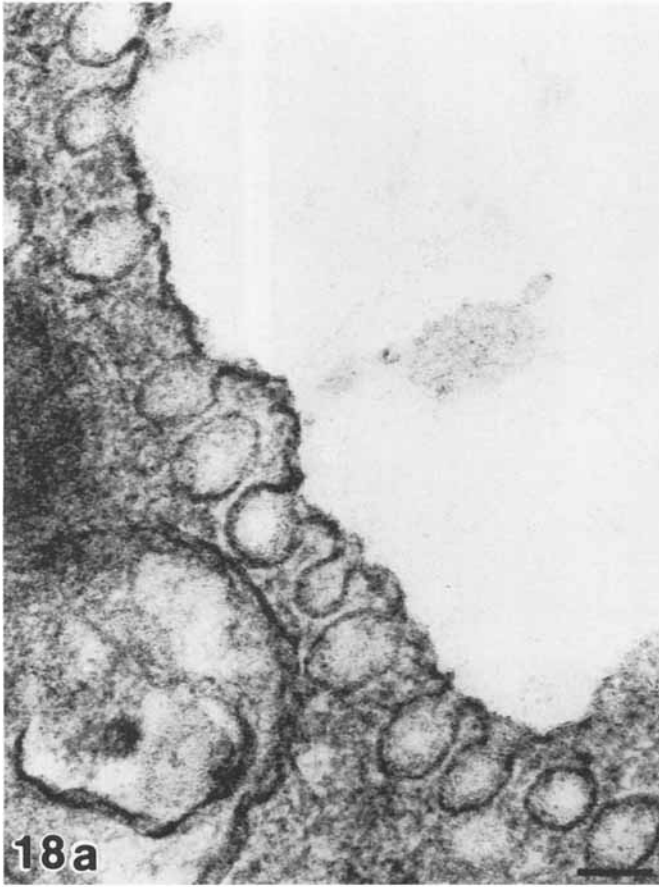
ure 3c, some virions are of a uniform spherical shape, whereas others appear to have excess stain around them (arrowhead). This drying artifact results in distorted shape and size.

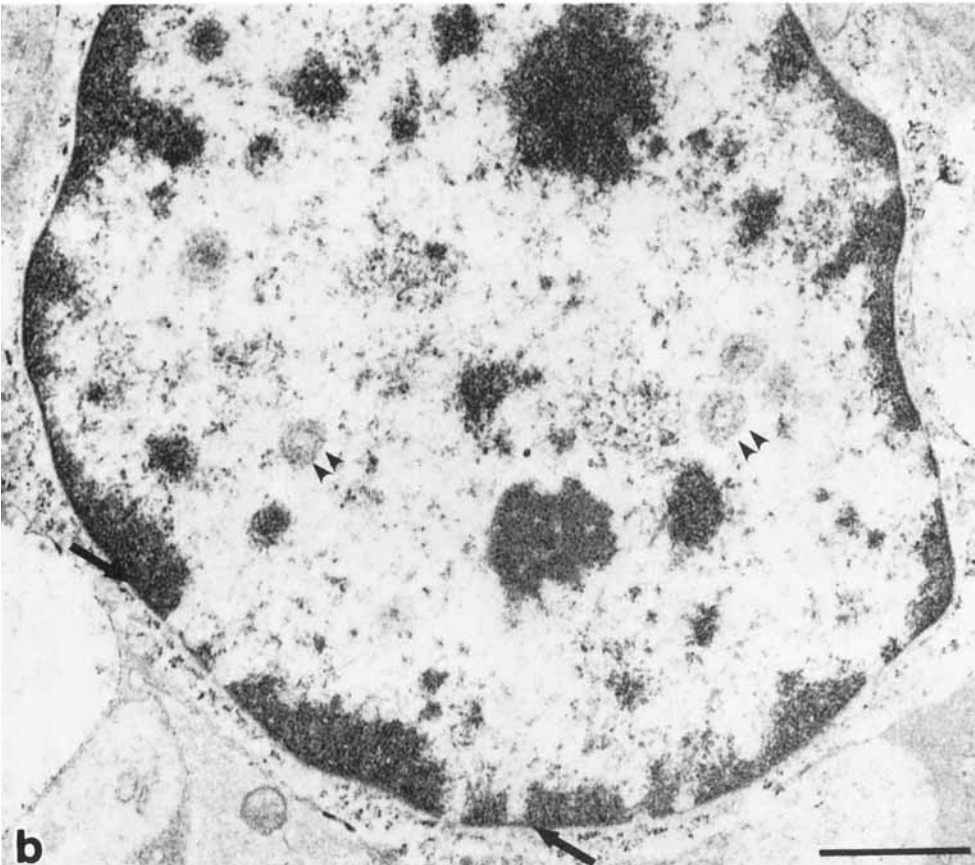
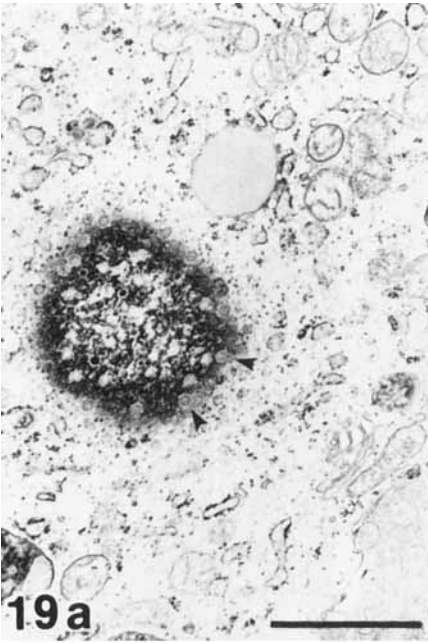
Collagen is often seen in stool preparations (Fig. 16a) but is insignificant in the diagnosis. In cross section (Fig. 16b,c), it is extracellular and fairly uniform in size and density, and it may be confused with budded viruses; it does not have a dense core.

In thin sections, bacteria (Fig. 17a), microvilli (Fig. 17b), and mycoplasma (Fig. 17c) are all larger than most viruses. Bacteria have strands of nucleic acid visible inside, and a thick cell wall. Microvilli and ruffles are bound by the cytoplasmic membrane, which is thinner than bacterial cell walls. The membrane may or may not be contiguous with the membrane around the cell due to the plane of section. Microvilli contain ribosomes and granular material identical to that in the cytoplasm. Mycoplasma (Fig. 17c), on the other hand, do not have cell walls and are bound only by a membrane. Mycoplasma cell contents are usually less granular than cell cytoplasm, and the density varies more than that of cell projections. Mycoplasma are pleomorphic and may show budding forms (Fig. 17c, arrowheads). Often, strands of nucleic acid can be seen at high magnification (Fig. 17d, arrowhead).

Cell structures such as caveolae (pinocytotic vesicles) and clathrin-coated pits and vesicles should not be confused with enveloped viruses. Though some viruses mature by budding through the plasma membrane to the outside of the cell (e.g., herpes, HTLV), others may bud into intracytoplasmic vesicles (e.g., herpes, Fig. 8b). Caveolae cut perpendicular to the cell membrane can be seen invaginating (Fig. 18a); cut parallel to the plane of the membrane, they may show more or less regular circles (Fig. 18b). They do not contain dense cores; see Figures 8 and 10. Coated pits and vesicles (Fig. 18c,d) are studded with clathrin projections on the outside of the structure, but internal to the cell. Most

Fig. 18. a,b. Caveolae are invaginations of cell membrane and do not contain dense centers as do viruses. They are somewhat irregular in size. Compare them to coated vesicles (c,d) which have spikes on the cytoplasmic side of the membrane. Coated pits and vesicles should not be confused with myxoviruses, where spikes occur on the extracellular side (Fig. 7). Bars represent 0.1 μ m.





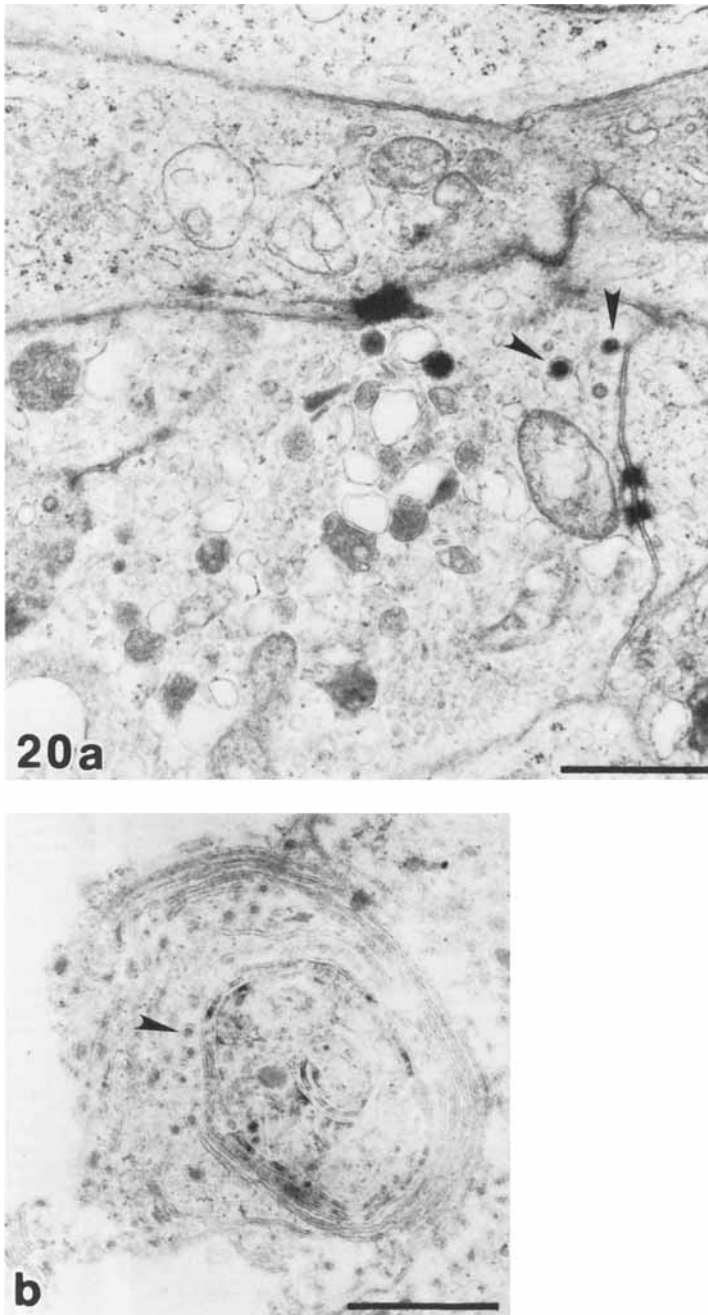


Fig. 20. Secretory products (a, arrowheads), and dilated endoplasmic reticulum and Golgi vesicles cut in cross section (b, arrowhead) may resemble viruses. (Fig. 20a courtesy of Ben Spurlock, Medical College of Georgia, Augusta, GA.) Bars represent 1 μ m.

Fig. 19. a. A section tangential to the nucleus may cut nuclear pores (arrowheads) in such a way that they resemble a factory of RNA viruses in the cytoplasm. b. If the plane transects the two nuclear membranes, pores (double arrowheads) may resemble DNA viruses in the nucleus. c. If the section plane is just inside the inner

membrane, as is a section along the plane of the long arrows in b, the nucleus may appear "holey" where the heterochromatin does not approach the pores. (Micrographs courtesy of Ben Spurlock, Medical College of Georgia, Augusta, GA.) Bars represent 1 μ m.

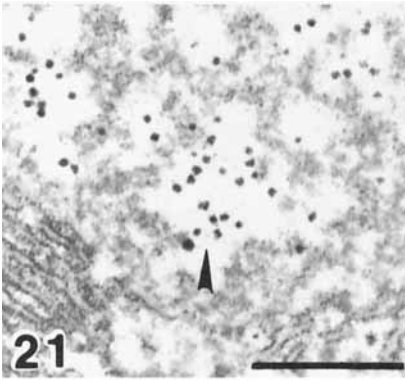


Fig. 21. Glycogen (arrowhead), a normal storage product, should not be confused with the early stages of picornavirus infection. (Micrograph was taken by Lynne Bartling in this lab.) Bar represents 1 μm .

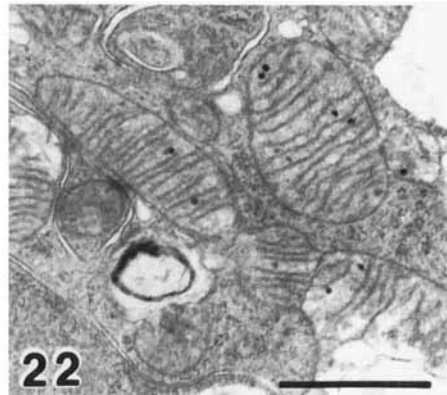


Fig. 22. Mitochondria may contain dense granules, which are normal. Bar represents 1 μm .

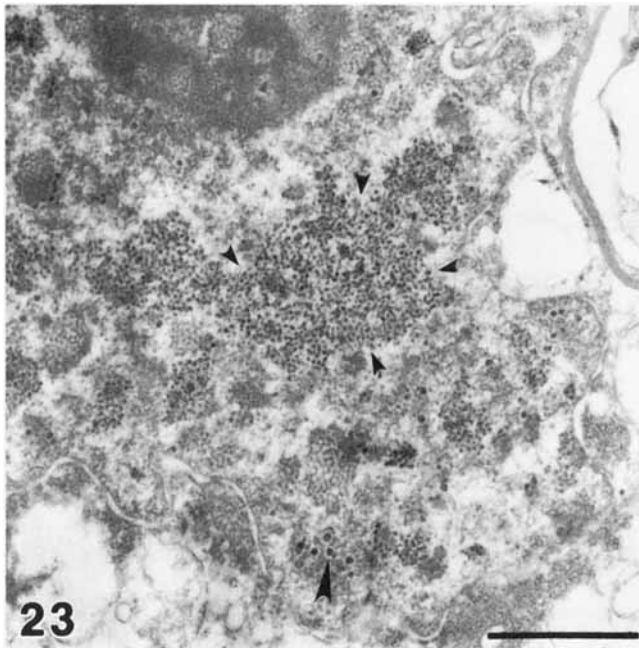


Fig. 23. Nuclear granules (small arrowheads) and nuclear bodies (large arrowheads) are cell reactions to injury and should not be confused with viruses. Bar represents 1 μm .

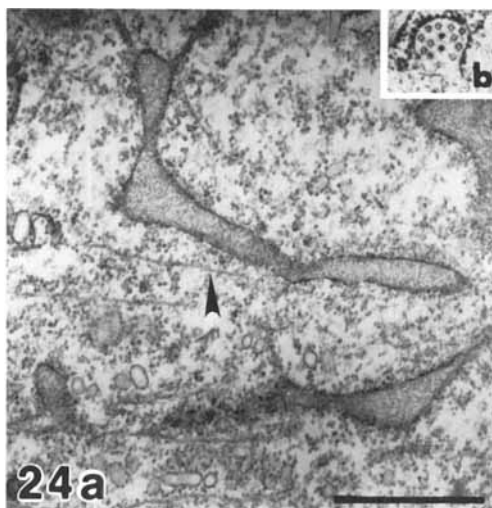


Fig. 24. a. Microtubules (arrowhead) are straighter than filamentous myxovirus nucleocapsids (Fig. 7b). b. In cross section, they are 22–24 nm, about the size of some picornaviruses (20–30 nm), but usually they are hollow, with side arms radiating from the tube. (Micrograph b courtesy of Dr. B. Mackay, University of Texas M.D. Anderson Hospital and Tumor Institute, Houston, TX; reprinted from *Introduction to Diagnostic Electron Microscopy*, with permission of Appleton-Century-Crofts, New York.) Bar represents 1 μ m.

spiked viruses (e.g., measles [Fig. 7] and influenza) obtain their membranes as they bud out of the cell with the projections exterior. Thus, complete virions are found extracellularly. An exception to this is coronavirus, which buds into cell vesicles. However, this agent is rarely seen in tissue sections; it is usually diagnosed by negative staining.

A grazing section of the nucleus may show nuclear pores that resemble cytoplasmic virus (Fig. 19a). If the section bisects the two nuclear membranes, pores may resemble nuclear viruses (Fig. 19b). If the section plane is just inside the inner membrane, the nucleus will appear to have holes where the heterochromatin does not approach the pore (Fig. 19c); this can be seen in cross section in Figure 19b (see the plane between the long arrows).

Secretory granules (Fig. 20a) in endocrine cells and fusion and fragmentation of endoplasmic reticulum (Fig. 20b) in some tumor cells can resemble membraned viruses; endoplasmic reticulum can sometimes be distinguished by examining the cell for tubular forms of the inclusion where the cisternae may have been cut in transverse section.

Some cells, especially hepatic cells, contain deposits of glycogen (Fig. 21), which should not be confused with collections of ribonucleoprotein in the early stage of picornavirus infection.

Often, normal mitochondria contain dense granules that are not viral (Fig. 22).

Nuclei in cells that are unhealthy for any reason (e.g., infection, toxins, nutrient deficit) may have nuclear granules (Fig. 23, small arrowheads) and/or nuclear bodies (large arrowhead), which are not diagnostic for anything specific.

Microtubules are approximately the same size as myxovirus nucleocapsids (22–24 nm vs. 18–20 nm for measles). In thin section, microtubules are straighter; filamentous nucleocapsids are curly and look like “worms.” Compare Figure 24a with Figure 7b. In cross section, microtubules are the size of some picorna- and parvoviruses; they differ in having an electron-translucent center (Fig. 24a), whereas small viruses are dense throughout (Fig. 6d).

Many cells contain storage and secretory granules, microbodies, lysosomal inclusions, multivesicular bodies, smooth endoplasmic reticulum, golgi vesicles, or lipid (Fig. 25). These structures are normal components of cells and should not be confused with viruses or virus-induced structures. Cell components are not uniform in size, whereas internal viral nucleocapsids and naked virions are. Membraned viruses vary in size, but are usually found external to the cell.

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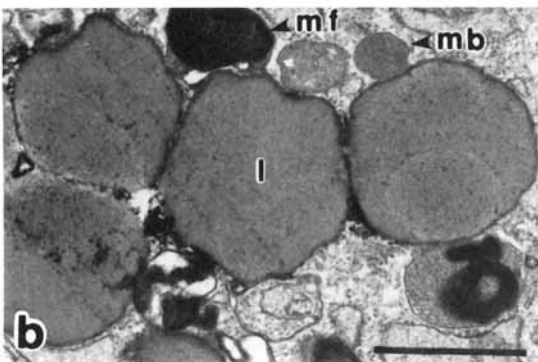
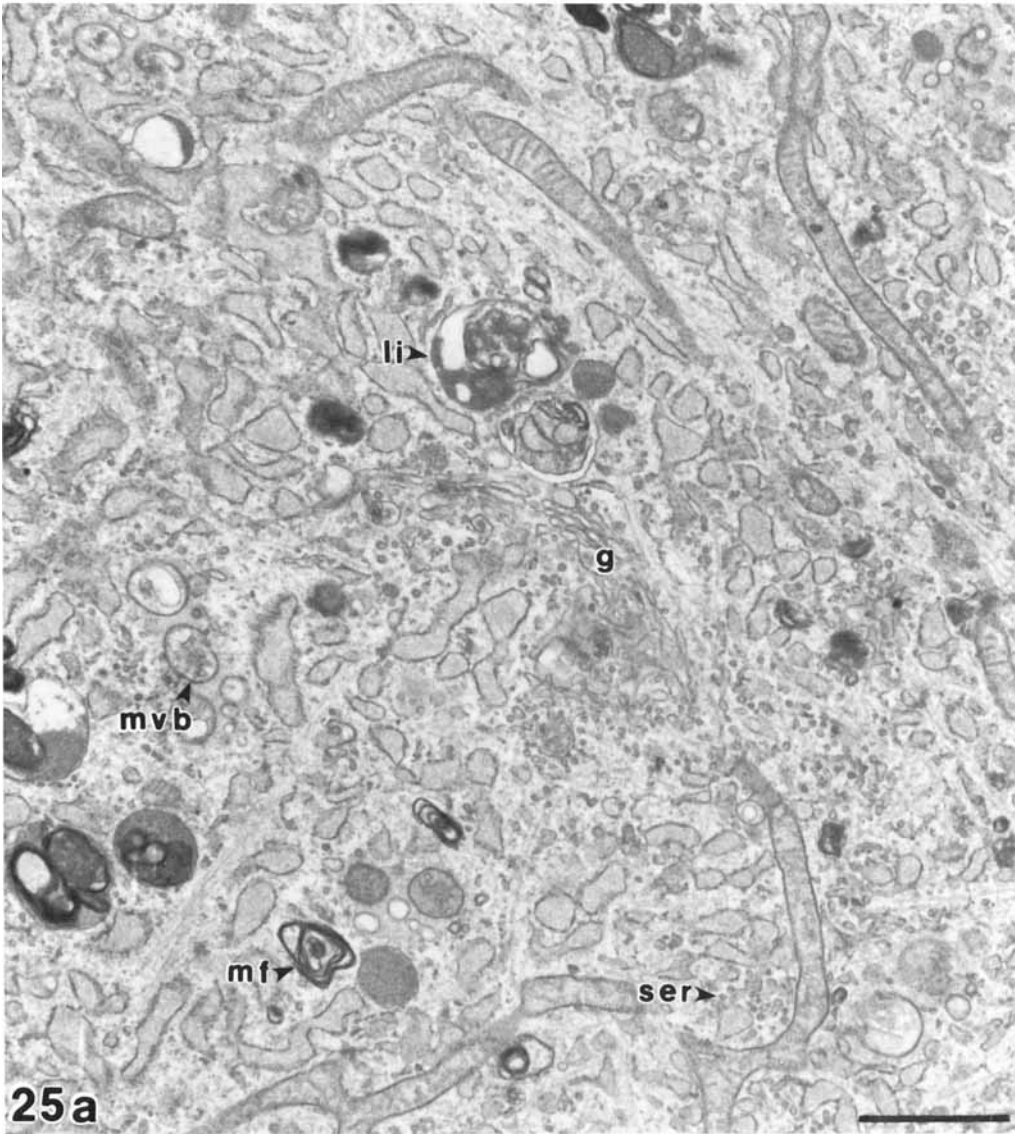


Fig. 25. Other normal cell organelles that could be confusing to the novice "virus hunter." a. lysosomal inclusions (li), myelin figures (mf), smooth endoplasmic reticulum (ser), Golgi vesicles (g), multivesicular bodies (mvb). b. Microbodies (mb), myelin figures (mf), and lipid (l). Bars represent 1 μ m.

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